Optimized Protocol for Regeneration of the Conjunctival Epithelium Using the Cell Suspension Technique

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Purpose: To develop autologous tissue-engineered conjunctival epithelial sheets to be used as advanced therapy medicinal products for severe ocular surface disorders involving the conjunctiva.

Methods: Methods used aimed at 1) mapping the conjunctiva for identification of the stem cell location, 2) establishing proper cell culturing conditions, 3) identifying the proper scaffold, and 4) characterizing the conjunctival grafts better. For these purposes, immunostaining and PAS staining, serial cultivation of cells, and quantitative polymerase chain reaction (ΔNp63α and MUC5AC) were performed.

Results: The inferior fornix represents the ideal area where to take the conjunctival biopsies from, with at least +3.58% of clonogenic colonies and higher percentages of stem cells compared with other areas, as confirmed by ΔNp63α expression levels (6.79% ± 1.18%). The standard culture conditions are necessary when cells are cultured on bare plastic, while animal-free media can be used for conjunctival cell culture on the scaffold. Fibrin glue represents the ideal scaffold for production of epithelial conjunctival grafts because it allows physiological expression of the main conjunctival cell markers, with K19 as the ideal one (98.5% ± 0.5% positive cells). The presence of goblet cells (6.3% ± 1.3%) and expression of the stem cell marker ΔNp63α (1.65% ± 0.35% positive cells) were also assessed.

Conclusions: Our findings pave the way for ex vivo cultivation of conjunctival epithelial cells onto a scaffold using the cell suspension technique by means of animal-free media. This would allow us to obtain conjunctival grafts for clinical purposes, thus giving a therapeutic option to patients with conjunctival diseases refractory to current therapies.

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Every year more than a 1000 patients in Italy and more than 100,000 worldwide develop severe disorders of the ocular surface, and in particular of the conjunctiva, such as ocular cicatricial pemphigoid, Stevens–Johnson syndrome, toxic epidermal necrolysis, recurrent pterygia, and chemical/thermal burns. Given that the ocular surface has a conjunctival-to-cornea area ratio of 12.8[1] and that a normal functioning conjunctiva is crucial to maintain a healthy ocular surface,[2] it is evident that even minor conjunctival involvement can have a great impact. The conjunctiva protects the ocular surface from various environmental insults through production of gel-forming and membrane-associated mucins,[3] formation of a solid barrier composed of an extended tight junctional network,[4] and production of antimicrobial β-defensins.[5] When the protective role of the conjunctiva diminishes or even ceases, the ocular surface becomes vulnerable to ulcer formation, secondary bacterial infections, severe dry eye disease, and even visual impairment in the case of secondary corneal involvement.[6] The former can manifest itself as fornix shortening, symblepharon and eyelid distortions,[7] which in turn can further damage the ocular surface epithelia and reduce eye movement.[8]

Conventional treatments include surgical excision of the lesion, followed by amniotic membrane (AM) transplantation over the exposed bare sclera.[9] AM transplantation is, however, limited to treatment of patients with mild conjunctival defects, as reepithelialization relies on migration of the surrounding healthy conjunctival epithelium.[10] Hence, autologous cellularized grafts have been proposed as an alternative, including conjunctival autografts,[11] oral mucous membrane grafts,[12] and nasal mucosa grafts.[13] Although these autografts may restore the epithelial barrier, disadvantages have been reported and include limited donor tissue availability, comorbidity at the donor site, nonfavorable cosmetic appearance, and risk of recurrence.[14,15] These drawbacks have led to development of tissue-engineered conjunctival equivalents.

Over the past 3 decades, stem cell therapy has been shown to be a potent and valuable therapeutic tool in clinical practice. It seems plausible that even transplants of autologous conjunctival epithelial sheets, generated by ex vivo cultured

conjunctival stem cells, could represent an appropriate therapeutic option for conjunctival diseases refractory to current therapies.[14] Many groups are working on development of cell grafts for treatment of conjunctival diseases, but so far results are limited,[16] and few clinical studies have been reported.[17,18] All these studies differ in the choice of the scaffold, the culture medium, and the cell culturing strategy.[19] With regard to the *in vitro* conjunctival cell culture (CCC), all studies make use of the AM as a scaffold, media containing animal components and the explant technique (cells outgrowing from a biopsy plated onto a scaffold).[17,18,20] On the basis of our previous experience with patients with limbal stem cell deficiency,[21] in this article, we evaluate whether conjunctival epithelial grafts can be regenerated through the "cell suspension technique." For this purpose, we have tested 2 different scaffolds: the amniotic membrane[17,18] and the fibrin glue gel (GEL), which represents our gold standard.[21] Although the AM presents proven biologically variability,[22] it is currently the most used scaffold in CCC, thanks to several beneficial biological effects (eg hypoimmunogenicity, antiangiogenic properties, etc.) and easy surgical manipulation.

Currently, most of the reports based on the epithelial cell suspension technique still make use of animal-derived products, such as fetal bovine serum (FBS), and feeder-layers of murine fibroblasts 3T3-J2 (FL) to support optimal cell growth.[17,20,23–25] One of the aims of this study was, therefore, to standardize a protocol for CCC using the cell suspension technique, animal-free media,[26] and no presence of FL and to compare the results with the conventional approach. This is crucial for development of a new stem cell–based advanced therapy medicinal product because the presence of animal-derived products might carry the theoretical health risk of pathogen transmission (eg, prions, viruses, etc.).[27]

Finally, to identify the location of epithelial conjunctival stem cells and thus define the optimal site where the conjunctival biopsies should be harvested from, we analyzed different areas of the conjunctival epithelium and defined the clonogenic potential of the corresponding cell population. The location of human conjunctival stem cells is controversial,[16] with 2 studies reported. Pellegrini et al suggested uniform localization of the human conjunctival stem cells in the bulbar and forniceal regions,[28] even though only 1 donor was evaluated. Stewart et al[29] detected human conjunctival stem cells across all areas of the conjunctival basal layer, although a significantly higher concentration was found in the medial canthal and inferior forniceal areas.

In this study, we show the possibility to obtain conjunctival epithelial grafts generated by culturing conjunctival stem cells in animal-free conditions for clinical purposes. Our strategy might become a therapeutic option for those conjunctival diseases that are refractory to current therapies.

MATERIALS AND METHODS

Cell Culture Media

The standard control medium (K) consists of Dulbecco Modified Eagle Medium (DMEM) and Ham F12 (F12) (Dulbecco Modified Eagle Medium/F12 2:1) supplemented with 10% FBS (all from Gibco, Life Technology, Italy), 50 μg/mL penicillin–streptomycin (Euroclone, Italy), 4 mM glutamine (Euroclone, Italy), 5 μg/mL insulin (Humulin R, Lilly, Canada), 0.4 μg/mL hydrocortisone (Flebocortid Richter, Sanofi, Italy), 0.18 mM adenine (Adenine grade I, Pharma Waldhof GMBH, Germany), 8.1 μg/mL cholera toxin (Cholera Toxin QD; List Biological Laboratories), 2 nM triiodothyronine (Liotir; IBSA, Italy), and 10 ng/mL epidermal growth factor (EGF, GMP Cellgro; Cell Genix GmbH, Germany). The K medium was compared with two serum-free culture media, of which the first mentioned is xeno-free as well:

1. Control medium supplemented with 10% XerumFree XF205 (TNCBIO, Italy) as a replacement for FBS (XF);
2. Keratinocyte-Serum Free Medium (Thermo Fisher Scientific, Italy) (KSFM) completed with Human Recombinant EGF and Bovine Pituitary Extract, according to the manufacturer's recommendations.

EGF was added to the K and XF media from the first day of culture, and the KSFM medium was always tested without FL. The XF medium was investigated with FL during cell cultivation in cell culture plates, and with or without FL during cell cultivation on scaffolds.

Conjunctival Cell Culture

Human conjunctival cells were cultured as previously described.[30–32] Briefly, the cells were isolated from human conjunctival biopsies treated with 0.05% trypsin/0.01% EDTA (Life Technologies, Italy) at 37°C and plated on cell culture plates with or without a FL of lethally irradiated 3T3-J2 fibroblasts (40,000 cells/cm^2).

Several in vitro Life Span Tests (LSTs, serial cultivation of the cells until senescence) were performed to compare the ability of the different media to support conjunctival cell growth. The LST allows us to analyze a range of parameters including morphology, number of passages, cumulative cell doubling (CCD) rate before senescence, and percentages of total and clonogenic colonies. The following conditions were tested:

1. K medium with FL
2. XF medium with FL
3. SFM medium without FL

LSTs (n = 3) at standard seeding concentrations (15,000 cells/cm^2) were performed for all the serum-free media; LSTs (n = 3) at inferior seeding concentrations (5,000 cells/cm^2) were also performed for the XF condition.

CCC on scaffolds, AM or GEL (Tisseel, Baxter, Italy, diluted to 3 UI/mL thrombin and to 44 mg/mL fibrin), was performed using the cell suspension protocol that we previously described.[21,26] Primary conjunctival cells were seeded on the 2 different scaffolds (17,000 cells/cm^2 and 35,000 cells/cm^2 for the GELs and AMs, respectively) both in control and serum-free culture media (XF and SFM) To test the AM as a scaffold, secondary cultures were performed using the following conditions: control medium + FL (n = 3) and −FL (n = 3), XF medium − FL (n = 5), and KSFM medium − FL (n = 3). To test the GEL scaffold, secondary cultures were

evaluated using the following conditions: control media + FL (n = 16) and XF − FL (n = 16).

Once confluent (~4–7 days both for AM and GEL), the grafts were punched and the central part (AM: Ø 0.4 cm, 0.13 cm^2; GEL: Ø 2.2 cm, 3.8 cm^2) was used for subsequent histological evaluation. The external rings of the grafts (AM: 2.17 cm^2, GEL: 4.7 cm^2) were enzymatically treated (trypsin/EDTA digestion for AM and dispase II and trypsin/EDTA digestions for GEL) to dissociate the cells. The collected cells were analyzed by means of the LST (n = 3–5 for AM and n = 5 for GEL), cytology (n = 5 for GEL), and quantitative polymerase chain reaction (qPCR) for detection of the stem cell marker ΔNp63α (n = 5 for GEL) and the goblet cell marker MUC5AC (n = 11 for GEL).

All cultures were incubated at 37°C and 5% CO_2 in humidified atmosphere, and media were changed every 2 to 3 days. A colony forming efficiency (CFE) assay was performed at each passage. To reduce the variability in CFE performances[26] and make the results comparable, CFEs were cultivated in the control medium with FL for all the tested conditions.

Location of Conjunctival Stem Cells (Mapping Analysis)

Thirty-six conjunctival biopsies were collectively harvested from both living subjects or cornea donors. Biopsies from living subjects (n = 8) were obtained following surgical interventions for pterygium removal or retinal detachment, and approval for the study was granted by the Ethical Committee of Venice December 11, 2009 (Protocol 2009/75,666). Biopsies from corneoscleral buttons of cadaveric donors (n = 5) with ages ranging from 4 to 79 years were isolated after signed informed consent forms were obtained from the donor's next of kin. Donor corneas were harvested within 12 hours after death.

Biopsies were harvested from 6 different areas (Fig. 1A): inferior fornix (IF), superior fornix (SF), inferior bulbar (IB), superior bulbar (SB), nasal bulbar (NB), and temporal bulbar (TB). The analysis of each area includes two biopsies from patients and 4 from cadaveric donors The series of experiments performed to localize the optimal source of conjunctival stem cells included CCC and cell biology–based quality control assays (LSTs). CCC was performed only in the K medium.

Scaffold Preparation

The cryopreserved human AMs (3 × 3 cm) were obtained from the Treviso Tissue Bank (Treviso, Italy—signed informed consent forms were obtained from the Treviso Tissue Bank) and were prepared as previously described.[20,26] Briefly, the epithelial layer was enzymatically detached, whereas the spongy layer was gently removed using a cell scraper. The denuded AMs, with the basement membrane facing up, were secured in an interlockable ring (2.3 cm^2) system as previously described.[26] The GELs were prepared as previously described.[33]

Immunohistochemistry

Parts of the AMs, GELs, and cells dissociated from the grafts were fixed with 4% paraformaldehyde (PFA; CremCruz, Santa Cruz Biotechnology, DBA, Italy) overnight. Samples for histology were embedded in the OCT compound (Cryobloc, Diapath, Italy), frozen, and sectioned (5 μm sections). Samples for cytology were prepared using a Cytospin4 machine (Thermo Shandon), by splitting 10,000 to 15,000 cells/slide. Both samples for histology and cytology were analyzed through indirect immunofluorescence using the following primary antibodies (overnight at +4°C): p63 (mouse monoclonal, 1:100; Dako Denmark, Italy), MUC5AC (mouse monoclonal, 1:100; M5293 Sigma-Aldrich, Italy), Mucin 1 (mouse monoclonal, 1:100 MA5-13168 Invitrogen; Life technology, Italy), Keratin 19 (rabbit polyclonal, 1:200, RB-9021; NeoMarkers, Freemont, CA), Keratin 13 (mouse monoclonal, 1:100, sc-101460; Santa Cruz Biotechnology, DBA, Italy), and Keratin 14 (rabbit polyclonal, 1:800, 905,301; BioLegend, Italy). Fluorescein-conjugated secondary antibodies were purchased from Invitrogen Life Technology (Alexa Fluor 488) and ThermoFisher Scientific (Rhodamine-conjugated). Histochemical periodic acid–Schiff staining (PAS; Sigma-Aldrich 3958-1KT, Italy) was performed to the same samples. A Nikon microscope (Eclipse Ti) was used for taking pictures. The same immunohistochemistry protocol was used to stain the conjunctival biopsies (IF and IB areas) and XF cytological samples (n = 5).

Real-Time qPCR

Total RNA was extracted from cells to analyze expression of ΔNp63α and MUC5AC. For determination of the proper CCC condition on plates, cells collected at the end of the first and third passages of LSTs were used. For determination of the proper CCC conditions on scaffolds, cells collected at the end of second passage (after dissociation from the scaffolds) and third/fourth passages were examined. RNAs were extracted and purified using the RNeasy Micro kit (Qiagen, Italy) according to the manufacturer's instructions. ΔNp63α analysis was performed according to the study by Barbaro et al,[34] 2016. MUC5AC analysis was performed as follows: both intron-spanning MUC5AC primers and gene-specific synthetic DNA template of MUC5AC were purchased from Bio-Rad Laboratories (Hercules, CA). Samples were run in SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad CFX96 Real-Time System. Gyceraldehyde 3-phosphate dehydrogenase levels were used for normalization purposes. The PrimePCR cycling protocol consisted of an activation step (95°C for 30 seconds), followed by 40 amplification cycles of denaturation (95°C for 5 seconds) and annealing/extension (60°C for 30 seconds).

Statistical Analysis

The results were expressed as mean ± standard error of the mean. The parametric ANOVA and Student *t* test (*t* test) were used in several experiments to compare differences between groups. The level of significance (*P*) was set at <0.05 for all experiments.

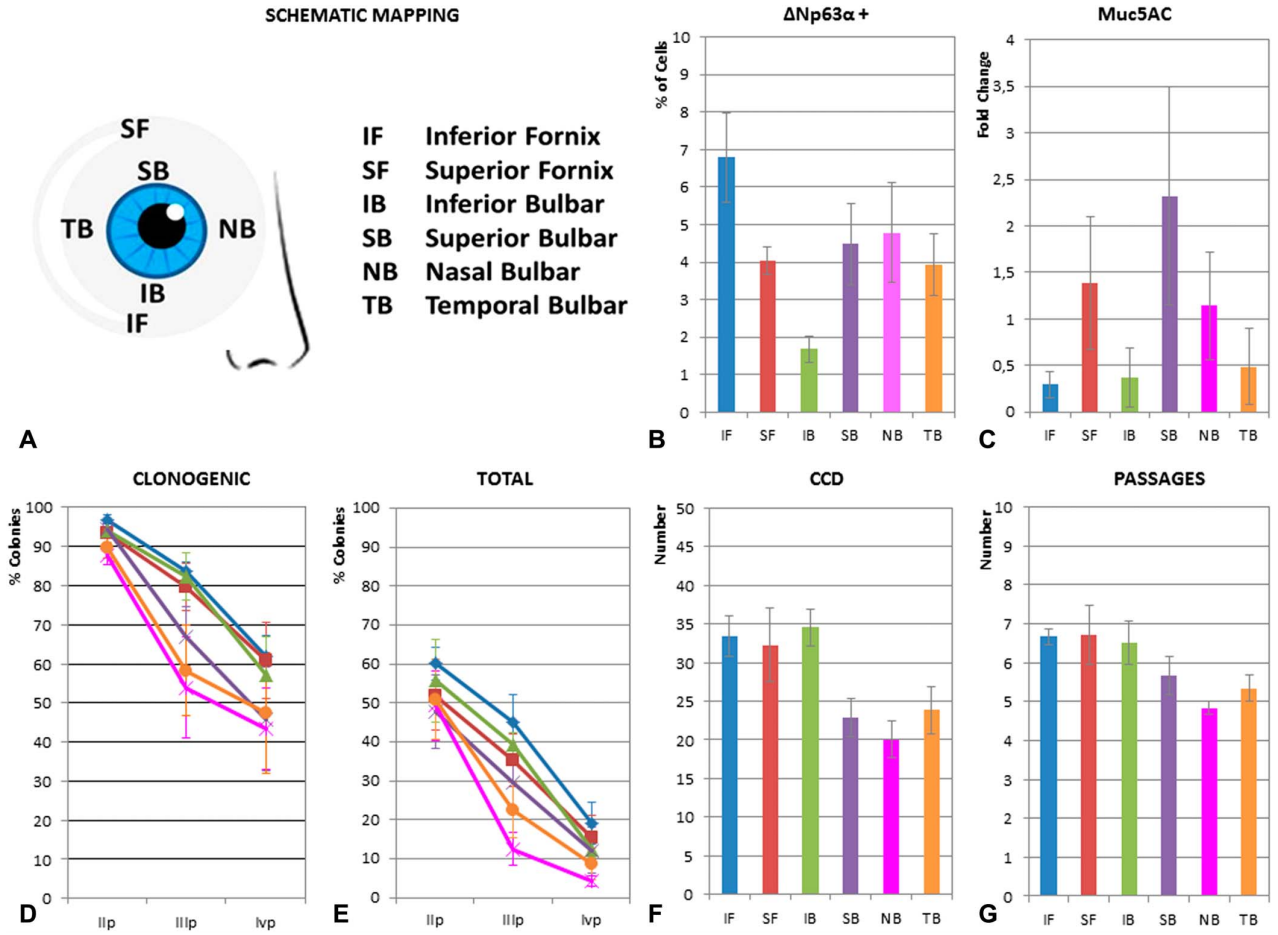

FIGURE 1. Localization of the conjunctival stem cells. A, Mapping of the conjunctival epithelium. B, Real-time qPCR of ΔNp63α showing a higher expression in the IF area ($P < 0.05$). C, Real-time qPCR of Muc5AC showing 3 groups with a similar fold change: 1) SB (fold change > 2), 2) SF and NB (2< fold change > 1), and 3) IF, IB, and TB (fold change < 2) (Kruskal–Wallis test $P < 0.05$). D–G, LSTs with cells obtained from different areas of the conjunctival epithelium. D, Percentage of clonogenic colonies at passages (p) II, III, and IV. E, Percentage of total colonies grown (both clonogenic and abortive) at passages (p) II, III, and IV. F, Total number of CCDs. G, Number of passages in culture.

RESULTS

Mapping of the Conjunctival Epithelium

Figure 1A shows the areas of the conjunctival epithelium, which have been analyzed. As shown in Figures 1D–G, the results of the LSTs allow us to divide the areas into 2 main groups: 1) NB-TB and 2) IF-SF-IB, with the SB area having intermediate performances. For all the parameters analyzed, no statistically significant differences were observed between conditions inside the NB-TB group (see Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/ICO/A685). Several differences were instead observed between the two groups, with a better in vitro performance of the IF-SF-IB group ($P < 0.05$). The IF area was considered the best performing one. In addition, IF differed also from SB ($P < 0.05$) when CCDs were analyzed (Fig. 1E). The SB area showed no statistically significant differences compared with the areas of both groups (see Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/ICO/A685), except for CCD, in which IF and IB showed better outcomes than BS ($P < 0.05$) (Fig. 1E).

In conclusion, the LSTs seem to suggest that the IF is the ideal area where to harvest the conjunctival biopsy from.

ΔNp63α expression data further confirmed these results (Fig. 1B): IF is the area with the most abundant percentage of conjunctival stem cells. More precisely, the ΔNp63α analysis showed a statistically significant higher expression ($P < 0.05$) in the IF area (6.79% ± 1.18% of ΔNp63α-positive cells) compared with IB, SB, and TB areas (1.67% ± 0.36%, 4.47% ± 1.08%, and 3.92% ± 0.81%, respectively) (Fig. 1B).

The expression analysis of Muc5AC by means of real-time PCR allowed us to identify 3 groups with similar fold changes: 1) SB (fold change > 2), 2) SF and NB (2< fold change > 1), and 3) IF, IB, and TB (fold change <2) (Fig. 1C). P values for the 3 groups were 0.0249, 0.0249, and 0.0373, respectively.

Evaluation of the Proper CCC Conditions on the Plate

To identify the best culturing conditions, 3 media (K, XF, and SFM) were tested at both standard and low-seeding density conditions, and cells were analyzed after LSTs.

The LSTs performed on cells plated at standard seeding concentrations did not reveal any statistically significant difference between the XF and control conditions (K) for all the parameters analyzed (see Supplemental Table 2a, Supplemental Digital Content 1, http://links.lww.com/ICO/A685) (Figs. 2A, B).

The performances of the KSFM medium were found to be comparable with those obtained with the K medium (see Supplemental Table 2b, Supplemental Digital Content 1, http://links.lww.com/ICO/A685) (Figs. 2C, D).

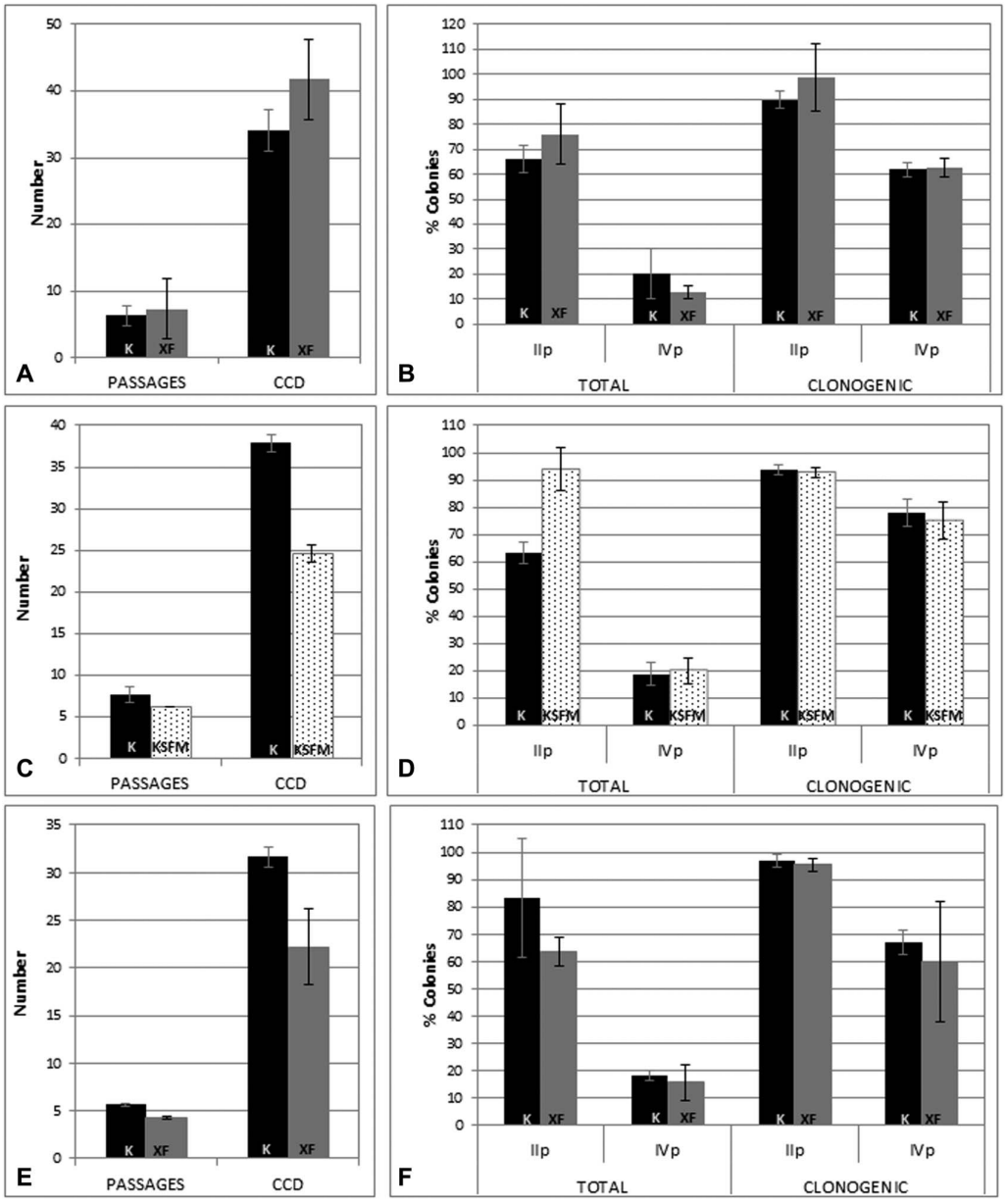

FIGURE 2. LSTs in XF and KSFM. A and B, XF medium versus K medium at standard seeding concentrations (15,000/cm^2): no appreciable increased performances of the XF + FL condition were observed compared with the control condition (K + FL). C and D, XF medium versus K medium at lower seeding concentrations (5,000/cm^2): a higher performance of K when compared with the XF condition. E and F, KSFM medium versus K medium at standard seeding concentrations (15,000/cm^2): no statistically appreciable increased performances of the control (K + FL) condition were observed compared with the XF condition.

On the contrary, the LSTs performed in conditions of low-seeding density led to statistically significant differences ($P < 0.05$) between K and XF (Figs. 2E, F): the number of passages was different (K = 5.67 ±0.16, XF = 4.33 ± 0.16), whereas for the other analyzed parameters, no differences were detected (see Supplemental Table 2c, Supplemental Digital Content 1, http://links.lww.com/ICO/A685). This is a crucial point because the initial number of cells collected from a biopsy is highly variable and normally represents the critical point for subsequent expansion and proliferation of cells in vitro.

Assessment of the Optimal CCC Conditions on the Amniotic Membrane

When cells cultured on the AM were dissociated from the scaffold and analyzed for their morphology, we found that both K and XF conditions (with or without FL) were characterized by squamous and homogeneous epithelia, with small cuboidal-shaped and well-organized and stratified cells (see Supplemental Figure 1A–C, Supplemental Digital Content 1, http://links.lww.com/ICO/A686). On the contrary, cells cultured in KSFM maintained a rounded shape, could not bind to the surface properly, and therefore were unable to become confluent, even when the duration of the cell culture process was increased (see Supplemental Figure 1D, Supplemental Digital Content 1, http://links.lww.com/ICO/A686). For this reason, the KSFM condition was discontinued and no longer investigated.

LSTs did not reveal any differences between the K conditions, with or without FL for all the parameters that we analyzed (see Supplemental Figure 2, Supplemental Digital Content 1, http://links.lww.com/ICO/A687). Statistically significant differences ($P < 0.05$) were instead observed between K and XF without FL, thus confirming that the XF condition was the best performing one (see Supplemental Table 3, Supplemental Digital Content 1, http://links.lww.com/ICO/A685).

The immunostaining analyses performed on biopsies (Fig. 3) and grafts (Fig. 4) showed that both types of grafts (K and XF) and biopsies were positive for the epithelial stem cell marker ΔNp63α, which was found expressed in the basal layers. Moreover, the grafts properly expressed the putative conjunctival epithelial markers: K13 (Figs. 4G, H), K14 (Figs. 4I, J), K19 (Figs. 4K, L), and MUC1 (Figs. 4C, D) but did not show specific expression of the MUC5AC goblet cell marker (Figs. 4 E, F). More precisely, MUC1 was detected in the upper layers of both graft samples and biopsies (Figs. 3, 4C, D), whereas K14 showed specific basal expression in biopsies but not in the graft samples (Figs. 3G, H, K, Land Figs. 4I, J, M, N). PAS staining performed to detect goblet cells showed a weak and nonspecific positivity on the grafts (Figs. 4O, P) but was able to detect goblet cells in biopsies (Figs. 3M, N).

Assessment of the Optimal CCC Conditions on GEL

With regard to the morphological analyses of cells cultured on GEL, both K and XF grafts showed a similar morphology at confluence, that is, small cuboidal-shaped cells forming a squamous and homogeneous epithelium. In the XF condition, cells often grew faster and showed a higher capacity to digest the scaffold compared with the control ones (data not shown).

As shown in Figure 5, no statistically significant differences were observed between the K and XF conditions after the analysis of the LSTs (Figs. 5A, B) for all the parameters investigated (see Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/ICO/A685), including ΔNp63α expression after real-time qPCR (ΔNp63α: K = 0.079% ± 0.092%, XF = 0.023% ± 0.011%) (Fig. 5C).

The immunostaining analysis was performed on the K and XF GEL grafts (Fig. 6), IF and IB biopsies (Fig. 3). The stem cell content of the grafts was confirmed by expression of p63 and K14 (Figs. 6A, B, I, J). The conjunctival markers K13, K14, K19, and MUC1 were expressed both on K and XF grafts (Figs. 6G–L, respectively). Apparently, similar distribution of the markers in the conjunctival epithelium could be observed, but low stratification of the grafts did not allow us to appreciate it: in vivo K13 and MUC1 are generally expressed in the upper layers (for K13 Figs. 3E, F and Figs. 6G, H; for MUC1 Figs. 3C, D, K, L and Figs. 6C, D, M, N) and K14 in the basal layers (Figs. 3I, J and Figs. 6I, J, M, N). No expression of the goblet cell marker MUC5AC was detected in the GEL (Figs. 6E, F). On the contrary, K19 was expressed throughout the thickness of the grafts (Figs. 3I, J and Figs. 6K, L), thus suggesting that K19 could be considered the ideal identity marker of the conjunctival GEL grafts. The PAS staining for goblet cells led to a weak and nonspecific positivity on the graft samples (Figs. 6O, P).

To support these data further, immunocytochemistry was performed and the following results were obtained: K19 (98.5% ± 0.5%), K14 (97.2% ± 0.7%), K13 (9.2% ± 3%), and Muc1 (16.0% ± 2.4%), thus confirming K19 as the ideal identity marker of the conjunctival grafts.

The presence of goblet cells was detected with 3 different methods:

1. Immunocytochemistry (MUC5AC expression <1%) (Fig. 5D);
2. PAS staining performed on XF cytological samples (6.3% ± 1.3% of bright cells) (Fig. 5E);
3. Real-time qPCR.

Of these 3 tests, the latter was performed on cells from both K and XF conditions. Muc5AC expression was detected only in a few samples of the total (N = 2/11 for XF and N = 3/11 for K), thus not allowing any statistical comparison between the two conditions.

DISCUSSION

In recent years, several approaches to reconstruct the conjunctival epithelium have been attempted, and in vitro regeneration of the conjunctival epithelium seems to be a promising strategy.[15] The aim of our study was, therefore, to establish a standardized protocol for the manufacture of

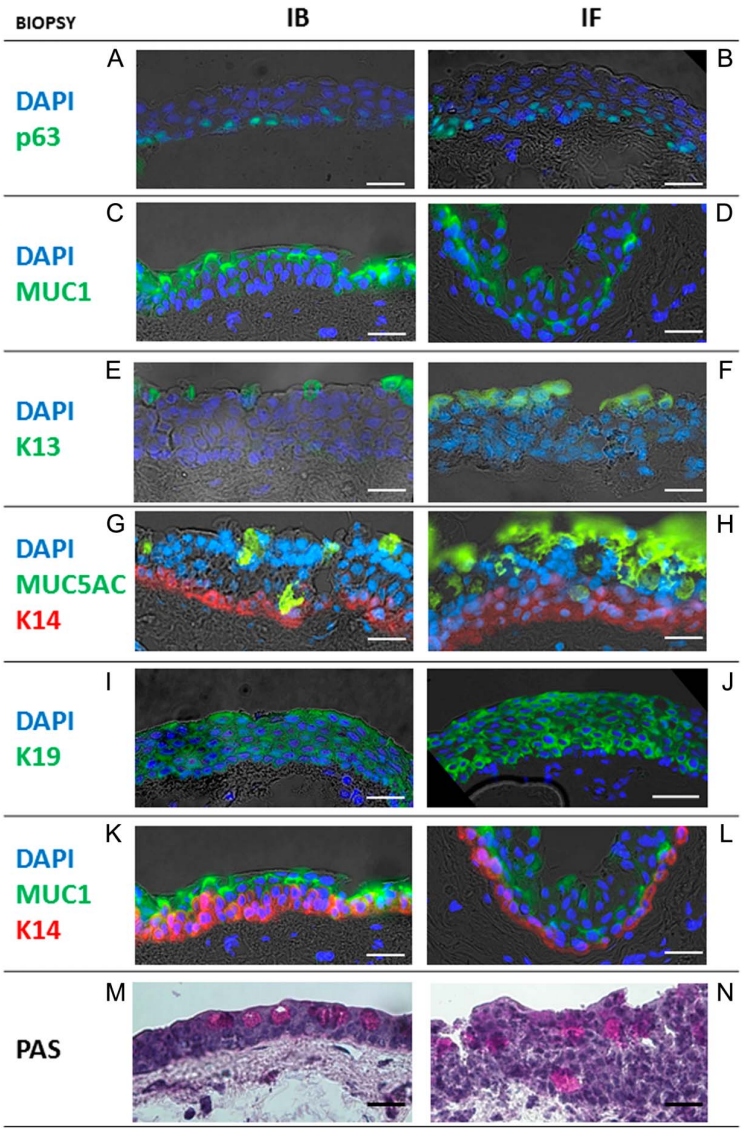

FIGURE 3. Immunohistochemical analysis of human conjunctival biopsies. Immunostaining for p63, MUC1, K13, MUC5AC, K14 (A–L), and periodic acid–Schiff staining (PAS, M and N) on IB (A, C, E, G, I, K, and M) and IF (B, D, F, H, J, L, and N) biopsies. K19 was the only marker expressed throughout the thickness of the samples (I, J). Scale bar = 100 μm.

conjunctival epithelial stem cell grafts. To achieve this, we investigated several parameters including the starting material, the proper scaffold, and the culturing conditions.

We demonstrated that the best area for isolating higher percentages of stem cells was the inferior forniceal area. Stem cells isolated from the IF were able to lead to significantly better regeneration of the tissue, both on culture plates and when grown onto a scaffold.

As for the scaffold, although a significant body of literature on this topic is available, none of the existing materials met all the criteria for an optimal conjunctival repair.[35] The AM seemed to be the most suitable carrier for in vitro culturing of conjunctival explants, although availability, costs, and processing are serious drawbacks to standardization of this application.[15] When the cell suspension technique was evaluated on the AM, 3 main hurdles were identified. The first one is caused by the intrinsic AM variability because each batch differs, in terms of thickness, transparency, and fragility. It was therefore difficult to monitor cell adhesion and proliferating activity and to have daily control of cell morphology. The second main issue was the presence of small holes in the AMs that could interfere with homogenous growth of the cells and eventually with the integrity of the AM surface. The third obstacle is that we will not be able to adopt the "no touch technique"[20] because this would not allow us to perform the quality control analyses required to release the final product, compulsory information needed by the GMP guidelines. To conclude, the batch-to-batch variability, the presence of holes while cells are growing, and the difficulties to perform quality control tests before releasing the graft make impossible to standardize a product made of conjunctival cells grown onto an AM scaffold.

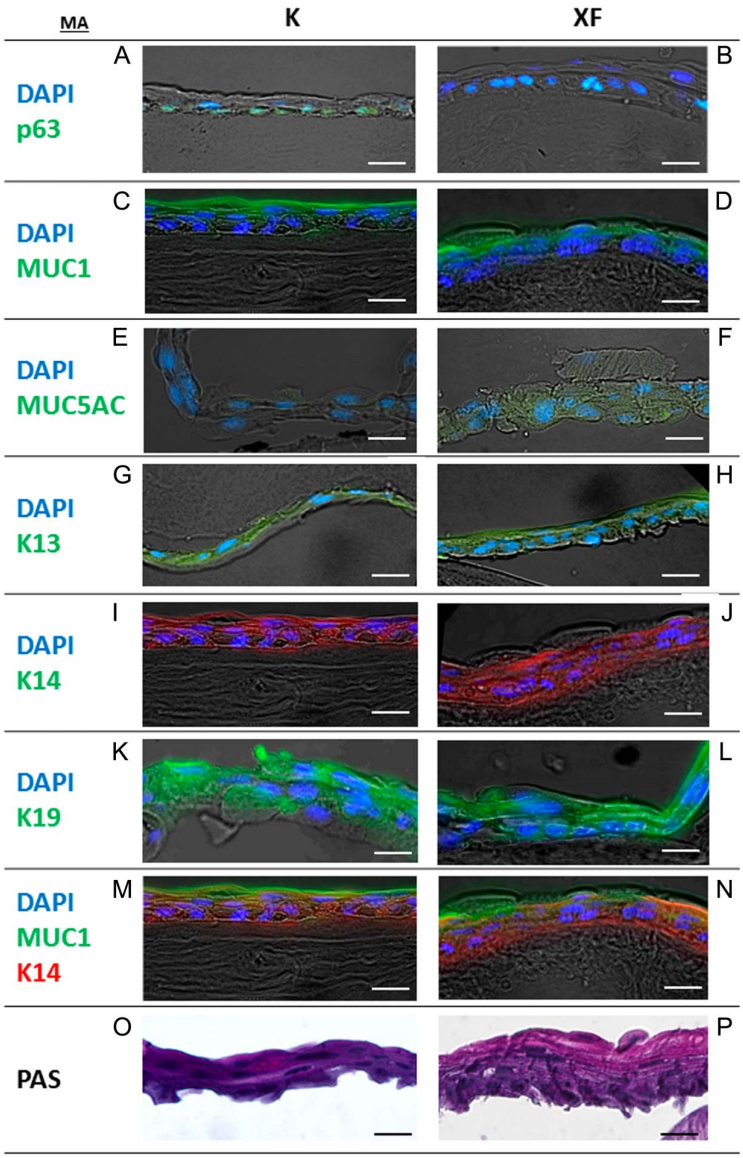

FIGURE 4. Immunohistochemical analysis of conjunctival epithelial grafts on the amniotic membrane. Immunostaining for p63, MUC1, MUC5AC, K13, K14, K19 (A–N), and periodic acid–Schiff staining (PAS, O–P) on K and XF conjunctival grafts on the amniotic membrane. The proliferative markers p63 and K14 (A, B, I, and J) and the putative conjunctival epithelial markers MUC1, K13, K14, and K19 (C, D, G, H, I, J, K, and L) were positive in both types of grafts. As for goblet cell detection, Muc5AC showed nonspecific expression (E and F). A weak positivity on the grafts samples was also observed after PAS staining (O and P). Scale bar = 100 μm.

Because of these issues, we decided to move forward and test the GEL as a scaffold. Previous clinical applications, in fact, showed that the batch-to-batch variability is reduced to a minimum (as these are pharmaceutical products), transparency is not an issue and the quality control tests before transplantation can be easily performed, with no damage to the final product.[21,31] The GEL is therefore the ideal scaffold for growing epithelial conjunctival grafts using the cell suspension technique. Both standard (K) and animal-free (XF) conditions can be carried out and allow proper characterization (morphology, identity, etc.) of the final product.

Regarding identification of a specific marker of conjunctival cells, despite our continuous search for a more specific one, K19 still resulted to be the keratin that was more expressed in the cultured conjunctival epithelial grafts.

We also investigated the presence of goblet cells. We found that immunohistology was not the ideal assay to detect the goblet cells in the conjunctival grafts. Negative results were obtained on cryosections, and a percentage of goblet cells inferior to 1% was found on cytological samples. Only a few studies do report immunostaining assays performed on human conjunctival cultures, with a very low number of cells expressing MUC5AC. Furthermore, in those studies, expression was not detectable through Western blotting.[36] We also showed that real-time qPCR is not always useful to detect Muc5AC expression in conjunctival cultures. These data agree with those reported by Gipson and colleagues[37] showing that the level of MUC5AC in conjunctival culture was approximately 4.3×10^4-fold lower compared with that of the native tissue, thus suggesting that only a very small

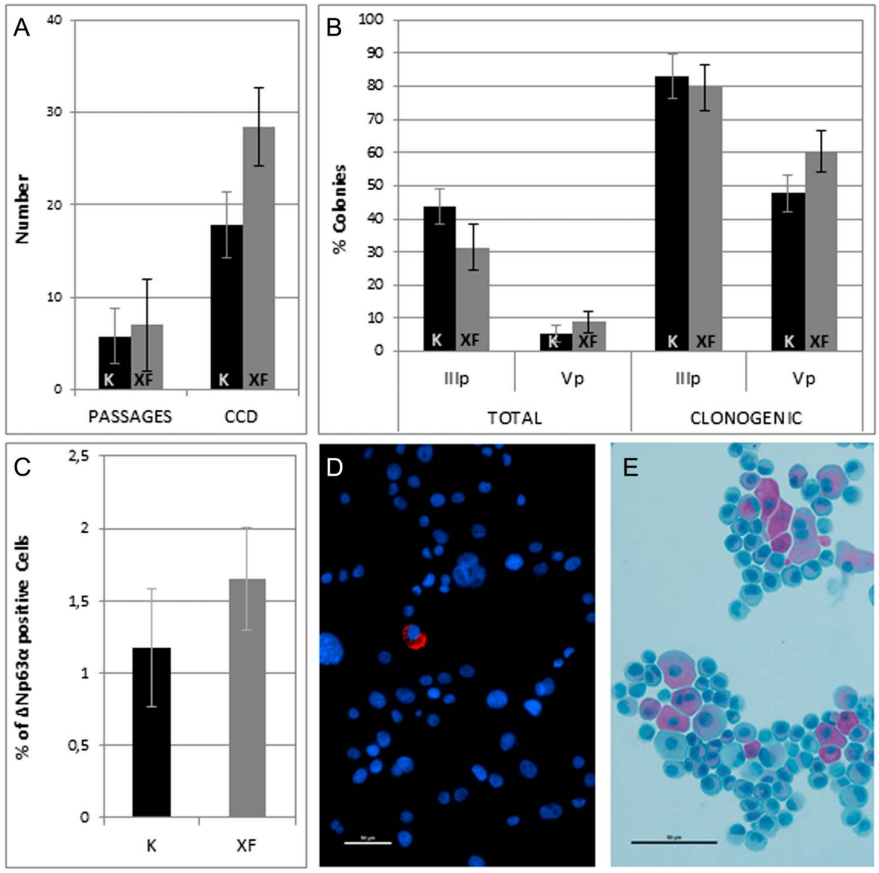

FIGURE 5. Analysis of CCC dissociated from the GEL scaffold. LST (A, B), real-time qPCR (C), immunocytochemistry (D), and PAS staining (E) were performed on the K and XF conditions. No statistically significant differences were observed between the K and XF conditions after the analysis of the LSTs for all the parameters investigated: (A) number of passages and number of total CCDs, (B) percentage of total colonies at passage III and at passage V and percentage of clonogenic colonies at passage III and at passage V, and (C) ΔNp63α expression after real-time qPCR. Scale bars for D and E = 100 μm.

population of cells in culture expressed MUC5AC transcripts (as verified through immunohistology) and that a sensitive real-time PCR protocol should be used instead. It is generally accepted that messenger RNA levels are often insufficient to predict protein expression levels. A clear dose–response correlation between MUC5AC messenger RNA and protein can be observed only in human conjunctival epithelial cell cultures that were previously stimulated (through nerve growth factor addition, feeder layer and air-lifting, etc.), and MUC5AC production diminishes or halts unless a certain stimulus is introduced.[38] The absence of these stimuli in our culture conditions could therefore explain downregulated MUC5AC transcription. The presence of MUC5AC packed vesicles in our cultures could be hypothesized as a result of previous translation and a delay in MUC5AC secretion.

In 2 recent clinical trials with conjunctival cells, 1 reported positive staining for MUC5AC on paraffin sections, without quantifying the number of goblet cells,[18] whereas the other showed positive immunocytochemistry for MUC5AC on in vitro cultures and a few PAS+ cells on histological sections.[17] No real-time qPCR analyses were performed in either study. We must point out that we obtained conjunctival grafts starting from cells of the IF biopsy, that is the area with the highest amount of stem cells, but it is at the same time one of the areas of the ocular surface with the lowest concentration of goblet cells.

In most of the reports published so far, the presence of goblet cells was assessed by PAS staining on impression cytology samples. The concentration of goblet cells is conventionally reported as the number of cells/mm^2, and these values have been notably variable, ranging from 24 to 2,226 cells/mm^2.[38] According to Doughty and colleagues,[38] the Nelson grading scheme considered the goblet cell density across the bulbar surface >500 cells/mm^2, even if goblet cell density values of 100 to 300 cells/mm^2 can still be considered acceptable. PAS staining that we performed on cells dissociated from the grafts showed a percentage of goblet cells of 6.3%. We therefore went on comparing our data with those obtained on impression cytology samples through PAS staining[39]: being the standard cell density of bulbar conjunctiva around 2,000 total cells/mm^2 ±250,[40–43] 6.3% of goblet cells should correspond to a cell density of 126 cells/mm^2, which is acceptable. Moreover, our data also agree with those of Lambiase and colleagues[44] showing a 5% of goblet cells in in vitro cultured conjunctival cells when PAS staining was performed.

In conclusion, our study could pave the way for ex vivo cultivation of conjunctival epithelial cells onto a GEL scaffold by means of animal-free media and reagents, thus replacing the standard conditions based on the use of FBS and FL. If confirmed, our results would allow us to obtain

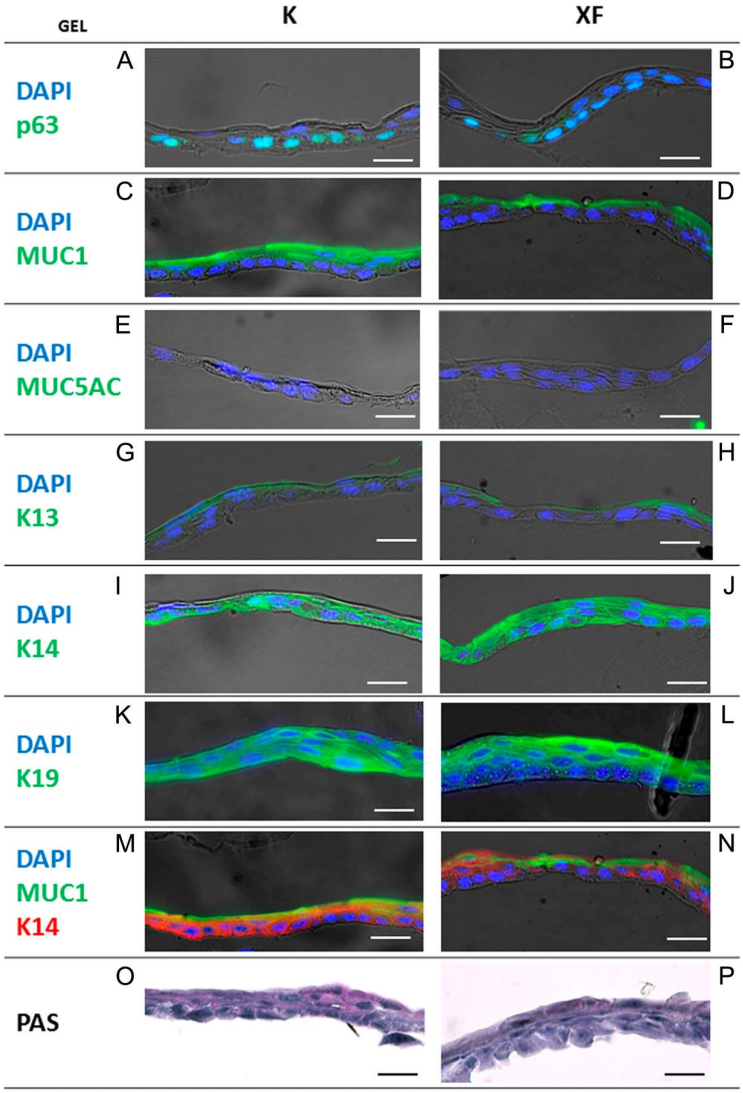

FIGURE 6. Immunohistochemical analysis of conjunctival epithelial grafts on GEL. Immunostaining for p63, MUC1, MUC5AC, K13, K14, K19 (A–N), and periodic acid–Schiff staining (PAS, O–P) on K and XF conjunctival grafts on GEL. The stem cell content of the grafts was confirmed by expression of p63 and K14 (A, B, I, J). The putative conjunctival markers MUC1, K13, K14, and K19 were expressed both on K and on XF grafts (C, D, and G–L, respectively). Distribution of the markers in the conjunctival epithelium was similar to that observed in Figure 5, but low stratification of the GEL grafts did not allow us to appreciate it. PAS staining performed to detect the presence of goblet cells highlighted a weak and nonspecific positivity (O–P). Scale bar = 100 μm.

epithelial conjunctival grafts for clinical purposes, thus giving a therapeutic option to patients who have conjunctival diseases that are refractory to current therapies.

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