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Research article

Human corneal epithelial cells harvested from advanced surface ablation (ASA): An optimized *in vitro* culture protocol

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ABSTRACT

Purpose: Corneal epithelial cells obtained from advanced surface ablation (ASA) surgery provide a valuable resource for *in vitro* models of ocular surface diseases. The aim of this study is to enhance culture conditions and characterize the functionality of EpiASA cells in culture, focusing on their ability to keep the distinctive properties of corneal epithelial cells.

Methods: EpiASA samples from 51 patients were included in the study. Two different collection media were tested, and their effect on sample preservation and initial viability was evaluated. Then, cells were disaggregated and cultured using different strategies to increase cell viability, which was measured by AlamarBlue assay. Once the optimized conditions were established, cells were cultured and passaged, and structural and functional characterization of native tissue, primary cultures, and first-passage cultures was performed using atomic force microscopy (AFM), qPCR, and immunofluorescence stainings.

Results: The addition of trehalose to the basal collection medium increased EpiASA initial viability. Culture surface coating with type I collagen, along with the supplementation of culture medium with hydrocortisone, significantly increased cell viability. On the contrary, co-cultures with different ocular cell lines, or the use of human serum, did not provide a sustained benefit. Further low-concentration trehalose supplementation of EpiASA cultures enhanced monolayer formation and allowed subculturing. AFM and immunofluorescence confirmed that passage 1 EpiASA cells retained corneal epithelial characteristics, including well-organized microvilli and uniform expression of barrier and epithelial markers.

Conclusion: This research provides an optimized protocol (EpiKeraMAX) for using EpiASA samples for *in vitro* studies of human corneal cells.

1. Introduction

Ocular surface diseases (OSDs) encompass a range of disorders that affect the normal structure and function of the ocular surface, with some leading to visual impairment and significantly affecting the quality of life for millions of people (Craig et al., 2023; Papas, 2021; Qi et al., 2024). Within OSDs, we find a variety of diverse pathologies such as dry eye syndrome (Siregar and Ferreri, 2022), keratitis (Anwar et al., 2024), corneal opacities (Wilson et al., 2022), and degenerative keratopathies (Vera-Duarte et al., 2024), among others (Fung et al., 2024; Stapleton et al., 2024).

Preclinical OSD research, necessary for the advance of the knowledge in these pathologies and the development of new treatments, is performed in animal and *in vitro* models (Somayajulu et al., 2024; Chen et al., 2024). However, existing *in vitro* models are based on immortalized cell lines, which do not completely resemble the physiological and functional characteristics of primary corneal epithelial cells due to the immortalization process. Unlike what happens in other tissues, such as skin (Sierra-Sánchez et al., 2021) or adipose tissue (Rogal et al., 2022), obtaining primary cells from the cornea is challenging due to ethical considerations. Acknowledging this limitation, we propose that advanced surface ablation (ASA) refractive surgery represents an undervalued source of corneal epithelial cells for research. During this procedure, an epithelial cell sheet is removed from the central corneal

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tissue, often after ethanol-enhanced debridement, and then discarded. However, the viability and functionality of ASA-derived cells (referred to as EpiASA tissue or cells) is affected by the ethanol exposure, thus limiting their potential uses (Sobas et al., 2015). Furthermore, corneal epithelial cells are organized in several layers where only some cells in the basal layers maintain the ability to divide (Swamynathan and Swamynathan, 2023; Medeiros et al., 2018), affecting the proliferative activity and apoptosis rate of this cell population (Chen et al., 2002).

Despite these limitations, we previously showed that EpiASA cells can be an appropriate source of material for *in vitro* research and that these cells could be maintained in culture (García-Posadas et al., 2022). However, to ensure optimal preservation and superior performance of this cell culture it is necessary to implement different improvements in culture conditions. Several survival and growth enhancers have been reported for primary cultures, such as hydrocortisone and trehalose. Hydrocortisone is a natural glucocorticoid, with anti-inflammatory, immunomodulatory and cell environment stabilizing effects (Zerillo et al., 2024),. In *in vivo* and *in vitro* rabbit models of dry eye disease, hydrocortisone exhibited potent anti-inflammatory and protective effects, improving corneal health (Bucolo et al., 2019). Also, in cultures of human keratinocytes from normal and keratoconic corneas, hydrocortisone downregulated inflammatory and growth factors (Song et al., 2016). Trehalose is a disaccharide known for its cytoprotective, oxidative, chemical and osmotic stress reducing properties (Matsuo, 2001; Gao et al., 2024; Bertl et al., 2019), and has been used as the sole cryoprotective agent to enhance post-cryopreservation viability in human kidney cell cultures (Hara et al., 2017). In addition, the use of different coatings or scaffolds has been reported to enhance adhesion and viability of eye-derived cell cultures (Bhardwaj and Sridhar, 2022; Viheriälä et al., 2021). Collagen is one of the most used materials for this end, and it has been shown that coating the culture substrate with collagen promotes the growth and differentiation of corneal epithelial cells in culture (Toropainen et al., 2001). Finally, another strategy that can be employed to improve cell culture of highly specialized cells is the use of co-culture systems. Different studies have shown that the factors secreted by the supporting cells favor epithelial adhesion, proliferation and viability (Jung et al., 2020; Zeng et al., 2024).

In this study, we analyzed the potential of different strategies to enhance the viability of EpiASA cells while maintaining uniform expression of barrier and epithelial markers. The final goal is to develop a protocol to use human primary corneal cells obtained from surgical discarded tissue as an *in vitro* model suitable for cornea research. For that purpose, we tested different protocols to collect and culture EpiASA cells and managed to establish an optimized protocol that allows for extended culture of EpiASA cells and their subculturing.

2. Methods

2.1. Human sample collection

This study was conducted in accordance with the Declaration of Helsinki and was approved by the "Ethics Committee for Research with Medicines in the Valladolid Health Area" (Project code: PI-23-3076). Experiments with human tissue complied with the guidelines of ARVO Best Practices for Using Human Eye Tissue in Research (Nov2021). Written informed consent was obtained from each patient previous to their participation in the study. Corneal epithelial cells were collected from 51 patients (30 male, 21 female, mean age 33,3 ± 8,9 years) undergoing ASA refractive surgery, all performed by the same surgeon (author MJM). Human cadaveric corneas from ocular-healthy donors, not exposed to ethanol, were provided by the Barraquer Eye Bank (Barcelona, Spain) and used as controls in immunofluorescence characterization experiments.

The ASA surgery was performed following a standardized protocol at the Institute of Applied Ophthalmobiology (IOBA). A 20 % ethanol solution was applied to the central 9-milimiter in diameter cornea for 30 s. Excess ethanol was removed using a Merocel® sponge (Medtronic Xomed Ophthalmics, Inc., Jacksonville, FL, USA), the corneal surface was then irrigated with balanced salt solution (BSS, Alcon Laboratories, Inc., Fort Worth, TX, USA) for 30 s, and the epithelial sheet was debrided with a blunt spatula. The detached corneal epithelium (EpiASA tissue) was passed to a BSS-soaked Merocel® sponge and then was transferred to and collected in Eppendorf® tubes containing 500 µl of collection medium under two experimental conditions DMEM/F12 (1:1) supplemented with GlutaMAX™ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) without additives (Basal Collection Medium), and DMEM/F12 supplemented with 100 nM Trehalose (D-(+)-Trehalose dihydrate, ≥99 % HPLC, Sigma-Aldrich, St. Louis, MO, USA), (Trehalose Collection Medium) (Table 1).

Samples were transported to the laboratory within 20 min of collection. For immunofluorescence and other immunological assays, EpiASA tissue specimens were directly embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and stored at −20 °C until further processing.

All experiments were performed using at least three independent biological replicates. Each biological replicate corresponds to a unique donor sample, and no pooling of biological material from different patients was carried out at any stage of the study.

2.2. Cell viability assessment using the LIVE/DEAD™ cytotoxicity kit

The initial viability of the EpiASA tissue was evaluated using the LIVE/DEAD™ Cytotoxicity/Viability Kit for Mammalian Cells (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). After a 20-min incubation in either basal medium or Trehalose collection medium (Table 1), the tissues were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (1X Gibco™) and incubated in 100 µl of staining solution prepared with 5 µl of Calcein AM, 20 µl of Ethidium homodimer-1, and 10 ml of DPBS for 30 min at room temperature. Subsequently, the

Table 1
Composition of cell culture media used for EpiASA cells.

Culture Medium	Composition
Basal Collection Medium	DMEM/F12 GlutaMAX™ (Gibco, Thermo Fisher Scientific)
Trehalose Collection Medium	DMEM/F12 GlutaMAX™ Trehalose 100 nM (Sigma-Aldrich)
Control Culture Medium (CM)	DMEM/F12 GlutaMAX™ 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen-GIBCO) 15 % FBS (Sigma-Aldrich) 10 ng/mL epidermal growth factor (EGF) (Invitrogen) 5 µg/mL insulin (Invitrogen)
Human Serum Medium (HS)	DMEM/F12 GlutaMAX™ 100 U/mL penicillin and 0.1 mg/mL streptomycin 15 % heat-inactivated human serum (Sigma-Aldrich) 10 ng/mL epidermal growth factor (EGF) 5 µg/mL insulin
Enhanced Growth Corneal Medium (EGCM)	DMEM/F12 GlutaMAX™ 100 U/mL penicillin and 0.1 mg/mL streptomycin 15 % FBS 10 ng/mL epidermal growth factor (EGF) 5 µg/mL insulin 1 µM hydrocortisone (MP Biomedicals™)
EGCM with Trehalose (EGCM-Tre)	EGCM 2 mM Trehalose (Sigma-Aldrich)
EGCM with Ascorbic Acid (EGCM-AA)	EGCM 100 µM L-(+)-Ascorbic Acid (Gibco, Thermo Fisher Scientific)
EGCM with Trehalose + Ascorbic Acid (EGCM-Tre-AA)	EGCM 2 mM Trehalose (Sigma-Aldrich) 100 µM L-(+)-Ascorbic Acid

samples were washed twice with DPBS and stained with Hoechst 33342 (1:1000 dilution, Thermo Fisher Scientific). Images were captured using a Leica CTR6000 epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Histological processing and hematoxylin-eosin staining

EpiASA tissues collected with either basal medium or Trehalose collection medium were washed three times with DPBS and fixed in 4 % paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA), for 24 h. The samples were then processed for paraffin embedding. Sections of 5 μm were cut and mounted on poly-L-lysine-coated slides, stained with hematoxylin-eosin (H/E), and analyzed using a Leica DM4000 B inverted microscope (Leica Microsystems).

2.4. Immunofluorescence

EpiASA tissue sections were characterized by immunofluorescence. Specifically, the corneal epithelial markers cytokeratin (CK) 3 and CK12, the barrier and junctional markers E-cadherin, Claudin-1 and ZO-1, the proliferation marker Ki67, and the fibroblast marker fibroblast specific protein 1 (FSP-1), were analyzed. All procedures were conducted in a humid chamber, and each wash step was performed three times for 5 min with 1X DPBS. Samples were permeabilized with 0.3 % Triton X-100 (Sigma-Aldrich) for 10 min (except those stained with antibodies against E-Cadherin) and blocked with 5 % donkey serum (Sigma-Aldrich) for 1 h at room temperature. The primary antibodies (Table 2) were incubated overnight at 4 °C, except Ki-67, incubated for 1 h at 37 °C. The next day, samples were washed, incubated with 10 μg/mL AlexaFluor® 488 secondary antibodies for 1 h at room temperature, and cell nuclei counterstained with Hoechst. Finally, samples were mounted with coverslips using Fluoromount™ (Sigma-Aldrich) and visualized with a Leica CTR6000 epifluorescence microscope (Leica Microsystems). All experiments included negative controls (with omission of primary antibody) and positive controls using healthy human cadaveric corneal tissue not exposed to ethanol or corneal fibroblasts (for FSP-1 marker).

2.5. Disaggregation of EpiASA samples and cell culture

EpiASA samples were divided in two and enzymatically disaggregated using 0.05 % trypsin-EDTA (Gibco, Thermo Fisher Scientific). Two trypsin exposure times were evaluated: 10 and 15 min. After trypsinization, the number of cells and cell viability percentage were measured using an automated cell counter (Countess™, Thermo Fisher Scientific).

Dissociated cells were seeded at a density of 50,000 cells/cm^2 in the different culture media (Table 1). Also, when indicated, a pre-coating of the cell culture surface with 66 μg/mL type I collagen (10 mg/mL, Corning® Collagen I from rat tail, Corning, NY, USA) was used. Cultures were maintained under standard conditions (37 °C, 5 % CO_2), and cell viability was measured at days 2, 4 and 6 using the AlamarBlue™ assay (Sigma-Aldrich).

2.6. Co-culture of EpiASA cells with epithelial and fibroblast cell lines

EpiASA cells were co-cultured with the following cell lines: immortalized human corneal epithelial cells (IM-HCEpiC; catalog number P10871-IM; Innoprot, Derio, Spain), immortalized human conjunctival epithelial cell line (IM-HConEpiC; catalog number P10870-IM; Innoprot), and immortalized human keratocytes (IM-HK; catalog number P10872-IM; Innoprot). EpiASA cells were grown in Transwell inserts designed for 24-well plates, equipped with a 0.4 μm transparent PET membrane (Corning Incorporated, Corning, NY, USA), at a density of 50,000 cells/cm^2 using control culture media (Table 1). Cell viability was evaluated with the AlamarBlue assay at days 2, 4 and 6.

2.7. Passage of primary cultures of EpiASA cells

After seven days, cells cultured with Enhanced Growth Corneal Medium with Trehalose (EGCM-Tre) were trypsinized for 15 min and subcultured establishing passage 1 cell cultures.

Subcultures were maintained under the same conditions as those used during the primary culture phase and were evaluated daily by microscopy for 7 days to visually assess adhesion to the culture plate, cell confluence, and cell structure. Passage 1 cells were then characterized by immunofluorescence as previously described for primary cultures. CK3 was used to specifically identify corneal epithelial cells, confirming their phenotypic identity. ZO-1 and E-Cadherin were analyzed to assess barrier integrity and adhesion properties, respectively. Nuclear morphology was visualized by staining with propidium iodide (PI, 1:12,000 dilution, Sigma-Aldrich). A negative control for FSP-1 was also included to rule out mesenchymal or stromal cell contamination.

2.8. Gene expression analysis in corneal epithelium and EpiASA cultured cells by qPCR

To assess the expression of genes associated with corneal epithelial identity (CK3 and CK12), barrier integrity (E-Cadherin and ZO-1), and proliferative capacity (Ki67), total RNA was extracted from native EpiASA tissues (control), primary, and first-passage cultures. RNA extraction was performed using the PureLink RNA Mini Kit (Invitrogen). cDNA synthesis was conducted with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and the resulting cDNA was quantified, diluted to 20 ng/μl, and used for qPCR. Reactions included Fast SYBR Green Master Mix and primers (Table 3), with amplification conducted on a 7500 Real-Time PCR System (Applied Biosystems). The protocol consisted of 40 cycles (95 °C for 15 s, 60 °C for 1 min) and a melting curve analysis. GAPDH served as the reference gene, and expression levels were determined using the $2^{-\Delta\Delta CT}$ method. All assays were conducted in triplicate.

2.9. Atomic force microscopy

Native EpiASA tissue, EpiASA primary cultures, and passage 1 cultures were evaluated by atomic force microscopy (AFM). AFM imaging was performed with an MFP-3D Bio Asylum AFM (Oxford Instruments, Scotts Valley, CA, USA). For image acquisition, liquid contact mode and

Table 2
Antibodies used for protein detection in EpiASA tissue and in primary and first-passage cell cultures.

Target Protein	Specificity	Antibody Type (Clone)	Supplier	Final Concentration
Cytokeratin 3	Intermediate filaments in corneal epithelium	Mouse monoclonal (AE5)	MP Biomedicals	4 μg/mL
Cytokeratin 12	Intermediate filaments in corneal epithelium	Mouse monoclonal (E8)	Santa Cruz Biotechnology	4 μg/mL
E-cadherin	Cell adhesion molecule	Mouse monoclonal (36/E-cadherin)	BD Transduction Laboratories	2.5 μg/mL
Zonula Occludens-1 (ZO-1)	Tight junction protein	Rabbit polyclonal	Invitrogen	2.5 μg/mL
Ki-67	Proliferation marker	Mouse monoclonal (MIB-1)	Dako	0.7 μg/mL
Claudin-1	Tight junction protein	Mouse monoclonal (XX7)	Santa Cruz Biotechnology	2 μg/mL
FSP-1	Fibroblast-Specific Protein 1	Rabbit polyclonal (S100A4)	Abcam	7.52 μg/mL.

Table 3
Primers Sequences and Target Genes for PCR amplification.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Amplicon Size (bp)	Citation
CK3	ATTTGTGACTCTGAAGAAGG	TCCTTAAGAAGTCGATCTCATC	[101]	Sikora et al. 2019
CK12	TCTAAAGACCCAACCAAAAC	TCATGGGGCAGATCTTGTGA	[168]	Sikora et al. 2019
Ki67	TGACCCTGATGAGAAAGCTCAA	CCCTGAGCAACACTGTCTTTT	[141]	Sobecki et al. 2017
E-Cadherin	CGACCCAACCCAAGAATCTA	AGGCTGTGCCTTCCTACAGA	[172]	Zhu et al. 2009
ZO-1	TCTTCGGCCCAGCATGAAAT	CTTGGCTGCAGGGCTATCTT	[124]	This study
GAPDH	TGTCGCCATCAATGACCCC	TGACAAGCTTCCCATTCTC	[116]	Sikora et al. 2019

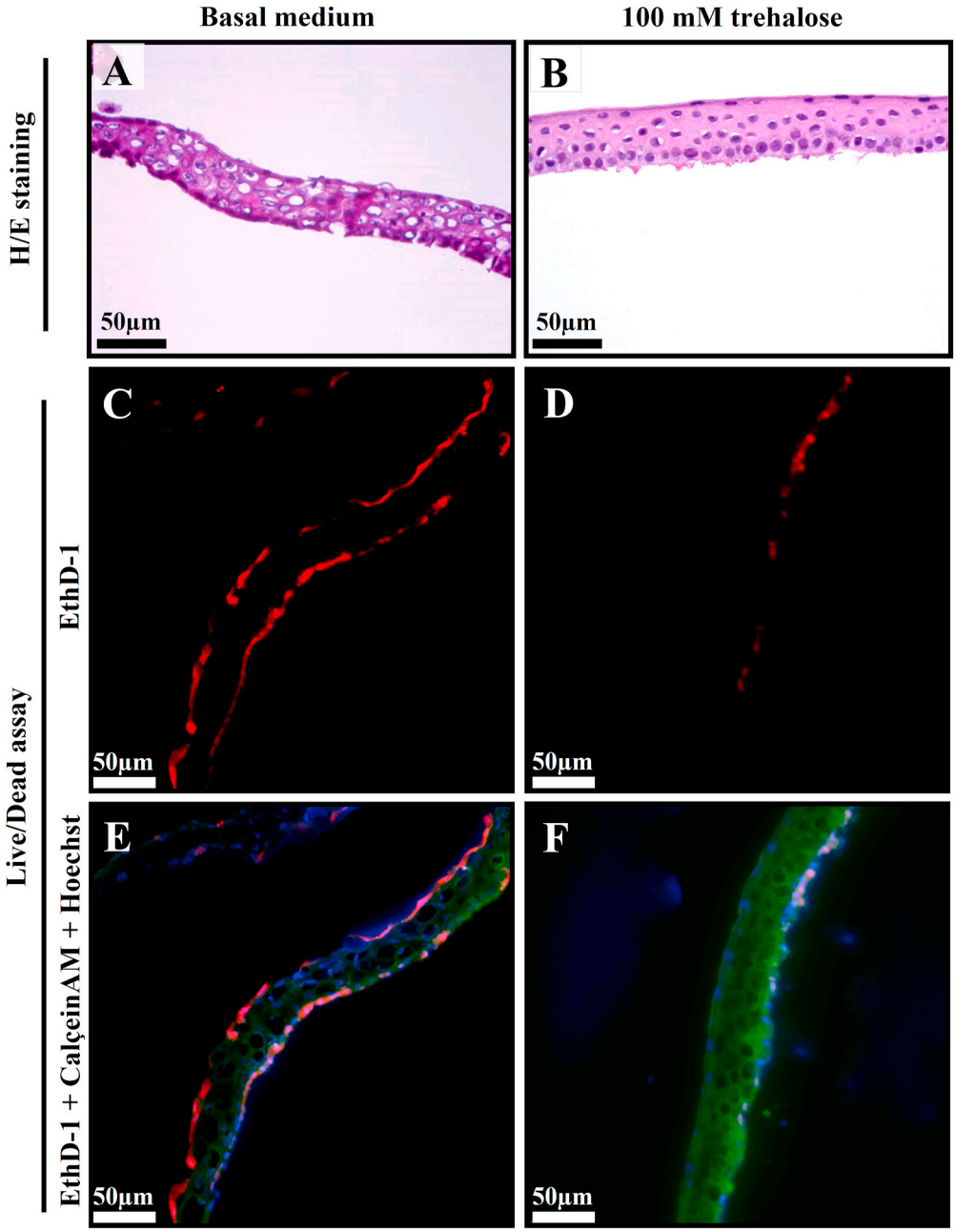

Fig. 1. Histological And Cell Viability Analyses of EpiASA Samples. EpiASA samples were obtained with Basal Collection Medium and with medium enriched with 100 mM Trehalose (Trehalose Collection Medium). Hematoxylin and eosin staining (A–B) revealed less vacuolization in the samples collected with Trehalose Collection Medium (B), compared to the Basal Collection Medium (A). Panels C–F show representative images of the LIVE/DEAD cell viability assay, where dead cells are stained in red by ethidium homodimer-1 (EthD-1), live cells are stained in green with calcein AM (Cal-AM), and nuclei are stained in blue with Hoechst. Increased cell viability is observed in the samples collected with Trehalose (D, F). Scale bars = 50 µm. A total of 12 biological samples were analyzed: 6 for hematoxylin and eosin staining and 6 for the LIVE/DEAD assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

air-tapping mode were used. For liquid contact mode, gold-coated silicon nitride probes (MikroMash, XNC12/Cr-Au BS, k = 0.08N/m, 17 kHz) were used. Gold-coated probes (MikroMash, AC 240 NG, k = 2N/m, 70Hz) were used for imaging in air-tapping mode. Areas of 80 × 80 μm and 20 × 20 μm were scanned at 512x512 lines in both AFM modes. The images were processed with Gwyddion 2.60 data analysis software. AFM images were obtained from three independent samples, with multiple areas scanned per sample to ensure data consistency.

2.10. Statistical analysis

Data are presented as mean ± standard deviation (SD). Experiments were performed using at least three biological samples from different patients, each one analyzed in triplicates. Data normality was checked with the Shapiro-Wilk test. For comparisons between two groups a Student's *t*-test was used. For pairwise comparisons between more than two groups a one-way ANOVA was performed followed by Dunnett's post-hoc test to define differences between conditions. For comparisons between groups with proportion data, such as percentage of viability, a Chi-squared test was used to test difference in distribution of live and death cells on each condition. Quantitative differences in Ki67-positive nuclei between samples were tested with a Z-test. The significance threshold was set for two-tailed p value ≤ 0.05.

3. Results

3.1. Characterization of the EpiASA tissue

Histological analysis revealed clear differences in the structural integrity of EpiASA tissue according to the collection medium used (Fig. 1A and B). Hematoxylin/eosin staining of samples obtained in Basal Collection Medium showed abundant intracellular vacuoles (Fig. 1A), indicative of structural damage, and extensive EthD-1 labeling reflecting cell death (Fig. 1C). In contrast, hematoxylin/eosin staining of samples obtained in Trehalose Collection Medium (Fig. 1B) showed better preserved cell structure with fewer vacuoles, together with a limited EthD-1 labeling and more widespread Cal-AM labeling, indicative of an overall higher cell viability (Fig. 1D and F). Quantitatively, we found a higher cell viability in samples collected in Trehalose Collection Medium (86.3 ± 6 %) compared to those obtained in Basal Collection Medium (60.7 ± 5 %). These results suggest a trend supporting the contribution of trehalose to the preservation of cellular integrity of the corneal epithelium during the collection process.

Immunofluorescence analysis of EpiASA tissue samples confirmed the presence of the corneal epithelial differentiation markers CK3 and CK12 (Fig. 2). In addition, expression of the cell adhesion protein E-Cadherin, and the tight junction proteins ZO-1 and claudin-1 was observed throughout the tissue, indicating that the epithelial barrier remained largely intact after ethanol treatment. However, areas of irregular ZO-1 expression were detected, which may correspond to intracellular vacuoles previously identified in the histopathological analyses. These localized alterations suggest possible disturbances in cell-cell adhesion. The EpiASA samples showed no FSP-1 staining, confirming that only epithelial cells were recovered from the surgery.

Proliferative capacity, assessed by Ki67 staining, was detected mainly in the basal epithelial layers. A quantitative comparison of Ki67-positive cells between EpiASA tissue samples and healthy donor corneal tissue revealed no significant differences ($p = 0.874$, Z-test), suggesting normal proliferative activity of EpiASA tissue samples.

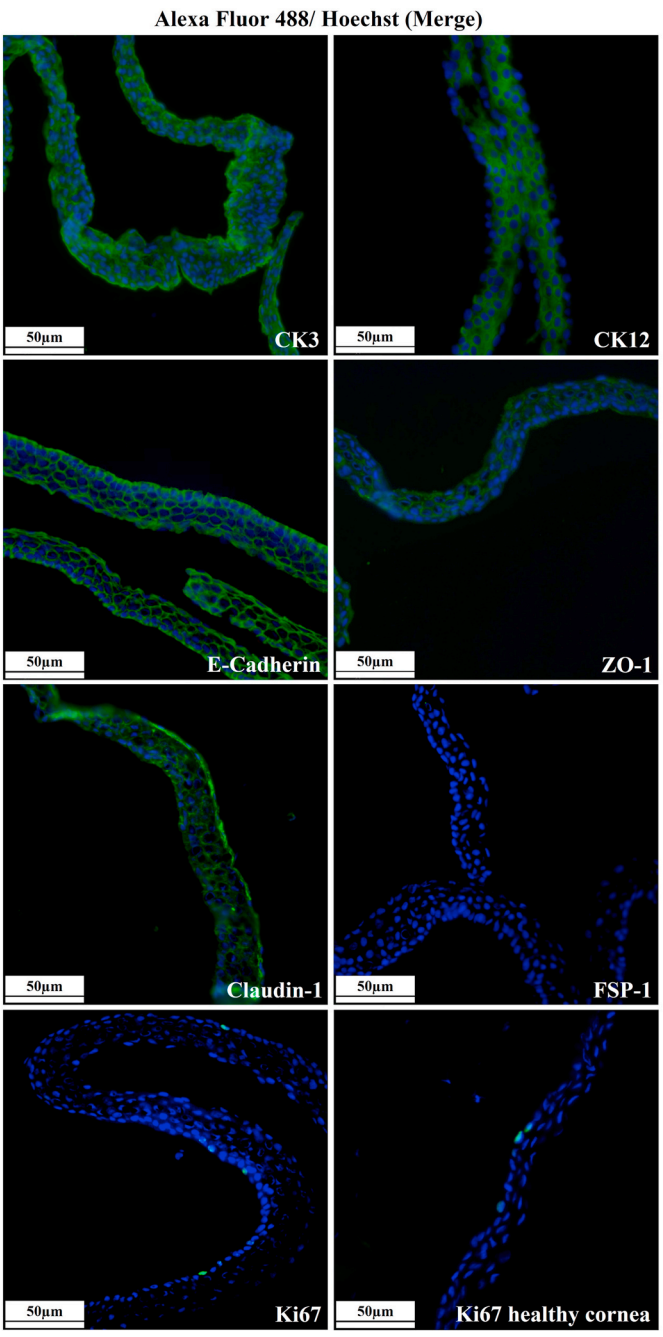

Fig. 2. Expression of Corneal Epithelial Markers in EpiASA Tissue Samples. Representative images of immunodetection of corneal epithelial markers CK3 and CK12, barrier markers E-Cadherin, ZO-1, and claudin-1, and proliferation marker Ki67 (all in green). FSP-1, a fibroblast marker, was employed as a negative control to ensure the specificity of epithelial staining. Nuclei were counterstained in blue with Hoechst dye. The Ki67-positive nuclei of EpiASA samples were compared with those of a healthy donor corneal tissue, showing no significant differences ($p = 0.874$, Z-test). Scale bars = 50 μm. Three biological samples of EpiASA tissue and three human cadaveric corneas were used for these experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Impact of the trypsin exposure time on EpiASA cell viability

Trypsin-EDTA mixture is used as a standardized solution to detach cells from the culture surface and subculture them. We used it in this work to disaggregate EpiASA tissue samples. The time of trypsin exposure significantly impacted the cell number and viability of the disaggregated cells, with the 15-min condition displaying both higher cell number (574,667 ± 136,906 cells vs 276,833 ± 91,125 cells, $p = 0.0023$) and higher cell viability (52.71 ± 4.70 % vs 31.71 ± 8.10 %, $p = 0.0005$) compared to 10-min trypsin exposure time (Fig. 3A). It should be noted that a shorter trypsin exposure time produced higher cell clustering (Fig. 3B).

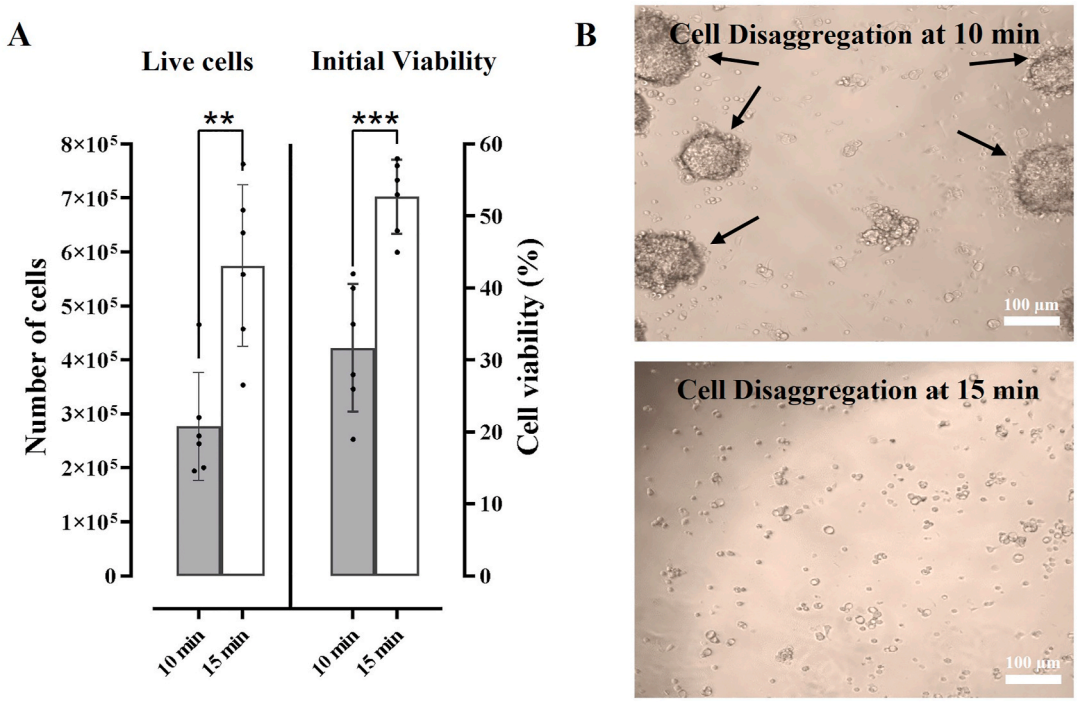

Fig. 3. Optimization of Trypsin Exposure Time for EpiASA Primary Culture Establishment. A) Cell counts and viability after trypsin disaggregation of EpiASA samples. Trypsinization for 15 min recovered more cells and with higher cell viability than trypsinization for 10 min (** = $p \leq 0.01$, *** = $p \leq 0.001$). B) Phase-contrast microscopy images showing the level of cell disaggregation obtained after 10 (top) and 15 (bottom) minutes of trypsin exposure. Cell clumps are indicated with black arrows. Six biological samples of EpiASA tissue were used.

3.3. Co-culture of EpiASA cells with ocular epithelial and stromal cell lines

We found a positive and significant effect of the co-culture with human corneal fibroblasts (IM-HK) on the viability of EpiASA cultured cells at day 2 (2.6-fold increase, which was statistically significant ($p = 0.0001$, Fig. 4A). At days 4 and 6, an overall reduction in EpiASA cells' viability with no effect of co-culture was observed.

Interestingly, on day 6, although in a low viability state, EpiASA cells under the different co-culture conditions showed remarkable differences in cell morphology and cell-cell interactions (Fig. 4B). Here, slight aggregation and morphological changes were observed in the cells co-cultured with IM-HK, suggesting a response to the modified culture medium.

In co-cultures with the human corneal epithelial cell line IM-HCEpiC, a slight aggregation of EpiASA cells was observed. Still, no significant changes were observed with respect to the control.

Finally, cells co-cultured with the human conjunctival epithelial cell line IM-HConEpiC showed a statistically significant 1.7-fold increase in IM-HConEpiC cells compared to the control medium ($p < 0.0001$). Although there was a slightly substantial trend toward preserved EpiASA cell viability on day 4 ($p = 0.0482$), we observed that viability was not maintained on day 6. Microscopically, we observed cell-cell aggregation of EpiASA at day 6 (Fig. 4B). This microscopic analysis suggests a marked beneficial effect of co-culture of corneal fibroblasts and conjunctival epithelial cells on EpiASA cell viability at day 2. Although overall viability remained low at day 6, cell-cell interactions were observed, suggesting a role for the co-culture in stimulating epithelial organization or stability under prolonged culture conditions.

3.4. Effect of human serum on EpiASA cell viability

Alternative medium supplementation with human serum instead of FBS (Control Culture Medium), revealed that FBS rendered significantly higher cell viability than human serum at days 2 and 4 (Fig. 5). The consistent decrease in cell viability observed from day 4 may be attributed to cellular senescence, affecting all culture conditions similarly.

3.5. Culture media optimization for enhanced EpiASA cell viability and functionality

To optimize our culture conditions, we tested the effect of hydrocortisone addition to the culture medium, the coating of wells with type I collagen and a combination of both, as a stress-protective and growth promoting strategy, respectively.

The results indicate that cells cultured in media supplemented with hydrocortisone and in wells with type I collagen coating exhibited significantly higher viability compared to the other conditions at all time points analyzed (Fig. 6A). This effect was maintained despite an overall reduction in viability observed from day 4 onwards. Also, microscopy images reveal a positive effect of this condition over cell morphology and culture organization at day 6 (Fig. 6B). The improvement observed in cell viability shows that culture surface coating with type I collagen and the addition of hydrocortisone benefit the stability and function of EpiASA cells, what lead us to define this new culture medium composition as Enhanced Growth Corneal Medium (EGCM) (Table 1).

3.6. Optimization of the EGCM for increased EpiASA cell viability and functionality

Next, we tested the addition of 2 mM trehalose (EGCM-Tre), 100 µM ascorbic acid (EGCM-AA), or both (EGCM-Tre-AA) to our defined EGCM (Fig. 7A and B). We observed a general trend toward enhanced cell viability under all three conditions at every time point analyzed, and a significant effect on day 2 with the addition of 2 mM trehalose ($p = 0.0202$) (Fig. 7A). These results suggest a beneficial impact of the addition of trehalose on the viability of primary EpiASA cell cultures. In addition to the viability measurement, microscopy analysis revealed remarkable differences in cell adhesion and cell-cell interactions in

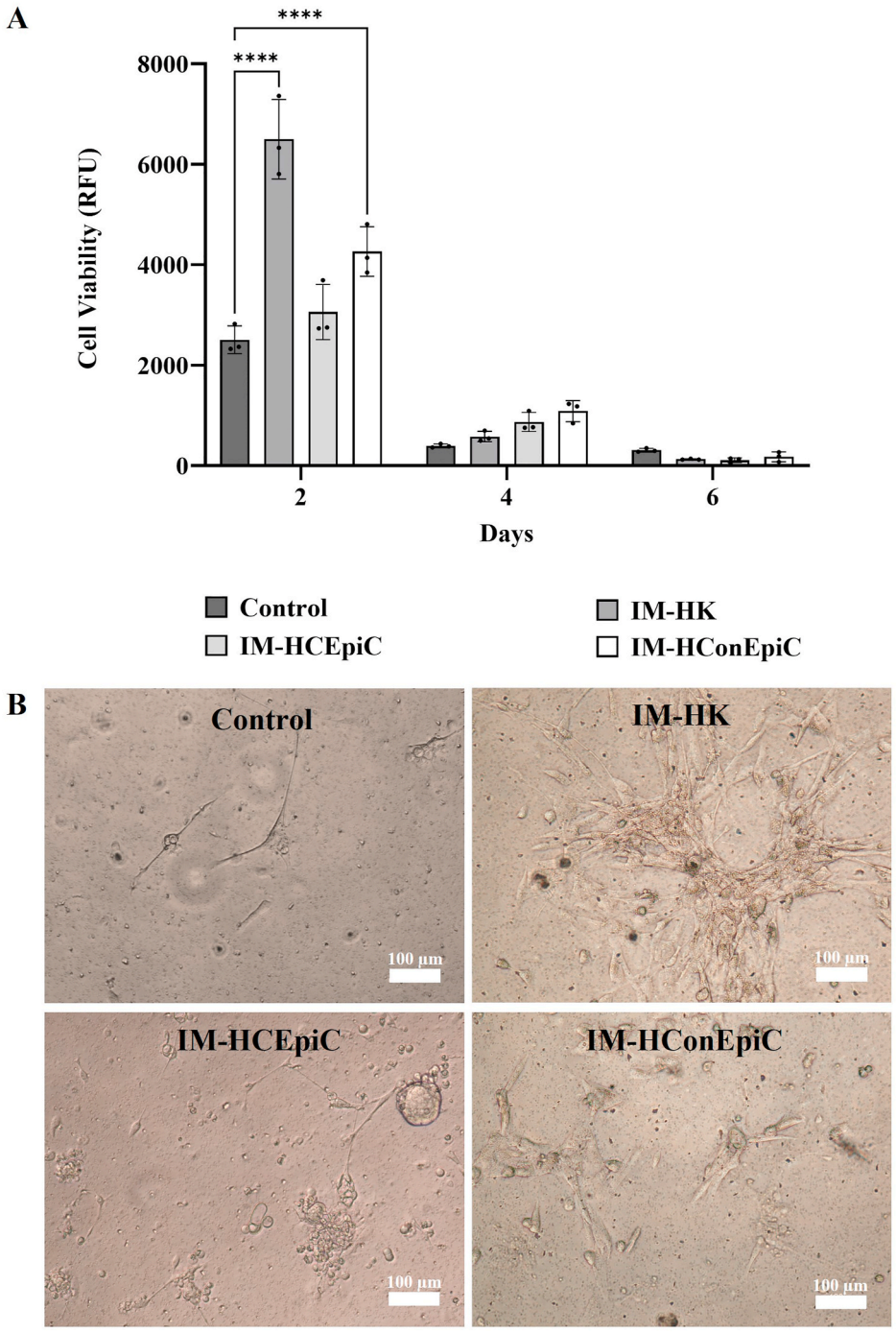

Fig. 4. Effect of the Co-culture of EpiASA Cells with Different Ocular Surface *Cell Lines*. A) Cell viability of EpiASA cells without supporting cells (control) or co-cultured with corneal fibroblasts (IM-HK: immortalized human keratocytes), corneal epithelial cells (IM-HCEpiC: immortalized human corneal epithelial cells) or conjunctival epithelial cells (IM-HConEpiC: immortalized human conjunctival epithelial cell line), at days 2, 4 and 6 as determined by the AlamarBlue assay. Bars represent cell viability (mean +SD) measured in relative fluorescence units (RFU). Significant differences in each co-culture compared with the control were determined at each time point using a one-way ANOVA followed by Dunnett's multiple comparisons post hoc test. On day 2, significant increases in cell viability were observed in the co-culture with IM-HK and IM-HConEpiC (**** $= p \leq 0.0001$) compared to the control medium. No statistically significant differences were observed on days 4 and 6. B) Phase-contrast microscopy images of EpiASA cells showing their morphology and cell-cell interactions after 6 days of co-culture with each cell type (scale bar = 100 µm). Three biological samples were used in triplicate.

EpiASA cultures (Fig. 7B). Compared to EGCM alone and EGCM-AA, or EGCM-Tre-AA conditions, EGCM-Tre markedly improved cell morphology, with more adherent cells and more defined cell structures. Thus, the addition of trehalose helped to form organized monolayer structures with higher cell-cell interactions.

Taking all these results together, we established the EpiKeraMAX protocol, an optimized procedure to maximize the potential of EpiASA cells, collecting the samples in Trehalose collection medium, dis-aggregating cells in 0.05 % Trypsin/EDTA solution for 15 min, and culturing the cells on collagen-I coated surfaces with EGCM-Tre (Fig. 7).

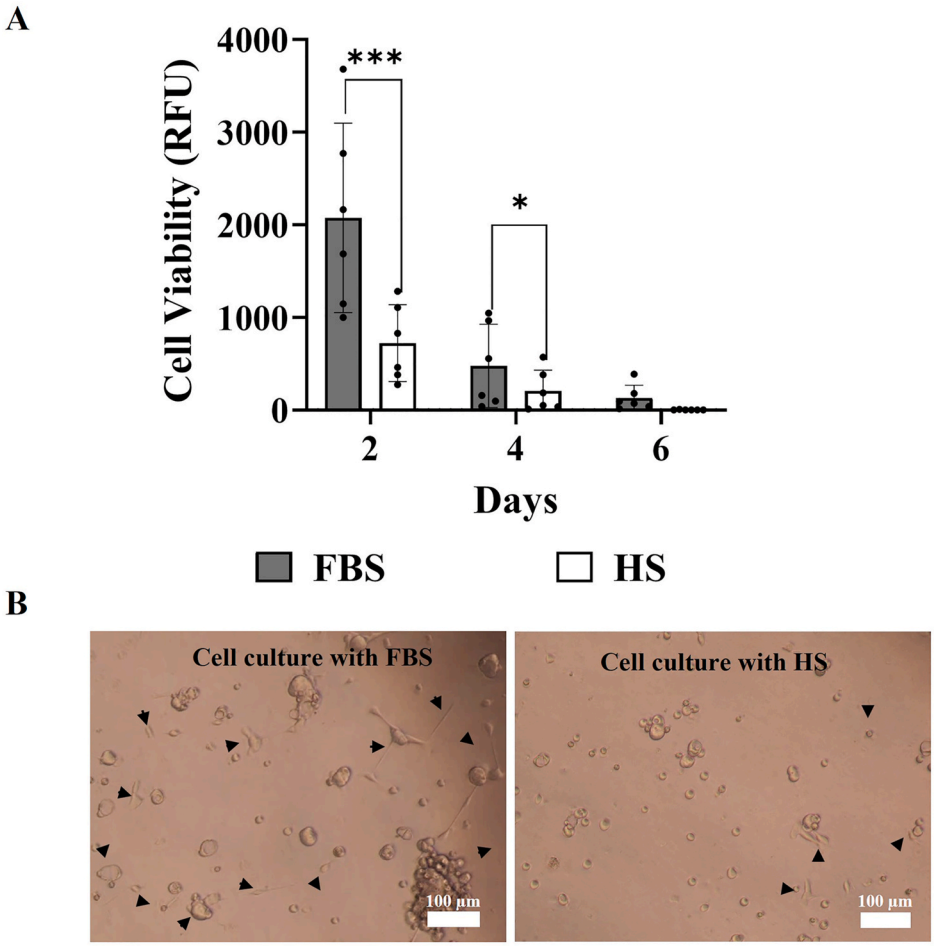

Fig. 5. Optimization of Control Medium (FB*S vs.* HS*)*. A) Graph showing the cell viability (mean ± SD) of EpiASA cells cultured with Control Culture Medium (CM) or Human Serum (HS) Medium. Cell viability was significantly higher with the Control Culture Medium at days 2 and 4. ($p = 0.0001$, $p = 0.0050$, respectively, unpaired Student's *t*-test). B) Phase-contrast microscopy images showing adherent cells (black arrow) with control culture medium with 15 % FBS (top) or HS medium (bottom) at day 6. Three biological samples were used, with triplicate analyses performed for each condition.

3.7. Molecular and structural characterization of first-passage EpiASA cultures

Using our stablished EpiKeraMAX protocol (Fig. 8), we successfully passaged primary EpiASA cultures using 15 min exposure to trypsin-EDTA.

The evaluation of gene expression in EpiASA-derived samples revealed an overall preservation of epithelial markers when comparing primary cultures (P0) and first-passage cells (P1) to the original tissue. Although no statistically significant differences were observed, the expression levels of CK3, CK12, and Ki67 remain detectable across P0 and P1, indicating that cultured cells retain epithelial characteristics and proliferative capacity. The expression of E-cadherin and ZO-1 -key markers of cell–cell junctions - remains stable or even shows a slight increase in cultured samples, particularly at passage 1, compared to the original tissue (Fig. 9).

We also demonstrated that first-passage EpiASA cultures expressed CK3 and the epithelial barrier markers ZO-1 and E-Cadherin proteins, as demonstrated by immunofluorescent staining (Fig. 10). CK3 expression confirmed the corneal epithelial identity of the cultured cells. ZO-1 was localized at cell junctions, indicating well-formed tight junctions. E-Cadherin expression demonstrated well-defined cell-cell adhesion, supporting the integrity of the epithelial barrier. These findings demonstrate the ability of EpiASA cells to maintain their epithelial identity and form tight and adherent junctions after subculturing under optimized conditions.

Immunofluorescence analysis showed no detectable FSP-1 expression in the cultured cells, indicating the maintenance of the epithelial phenotype and the absence of epithelial-to-mesenchymal transition under our optimized cultured conditions. As expected, positive FSP-1 staining was observed in corneal fibroblasts used as positive control.

3.8. Atomic force microscopy (AFM) analysis

AFM revealed some differences in surface organization between native EpiASA tissue, primary cultured cells, and first-passage cells (Fig. 11). The native EpiASA tissue displayed a smooth, compact surface with uniform structures in 3D topography images, and in Z-sensor topography, it showed surface irregularities similar to microvilli, reflecting an apparently intact epithelial integrity, just as the tight barrier junctions appeared intact in altitude and HR topography images. In contrast, the primary cultures presented asymmetrical surfaces with HR topography and a distribution of microvilli in sensor topography, indicating structural changes derived from *in vitro* conditions but with characteristics typical of epithelial cells. However, the first-passage cultures showed improved organization, with a more organized surface, structures similar to microvilli, and strongly integrated barrier junctions, suggesting a partial recovery of the structural organization of the native EpiASA tissue. These findings highlight the impact of culturing and passaging on the morphology of epithelial cells and their resemblance to native tissue.

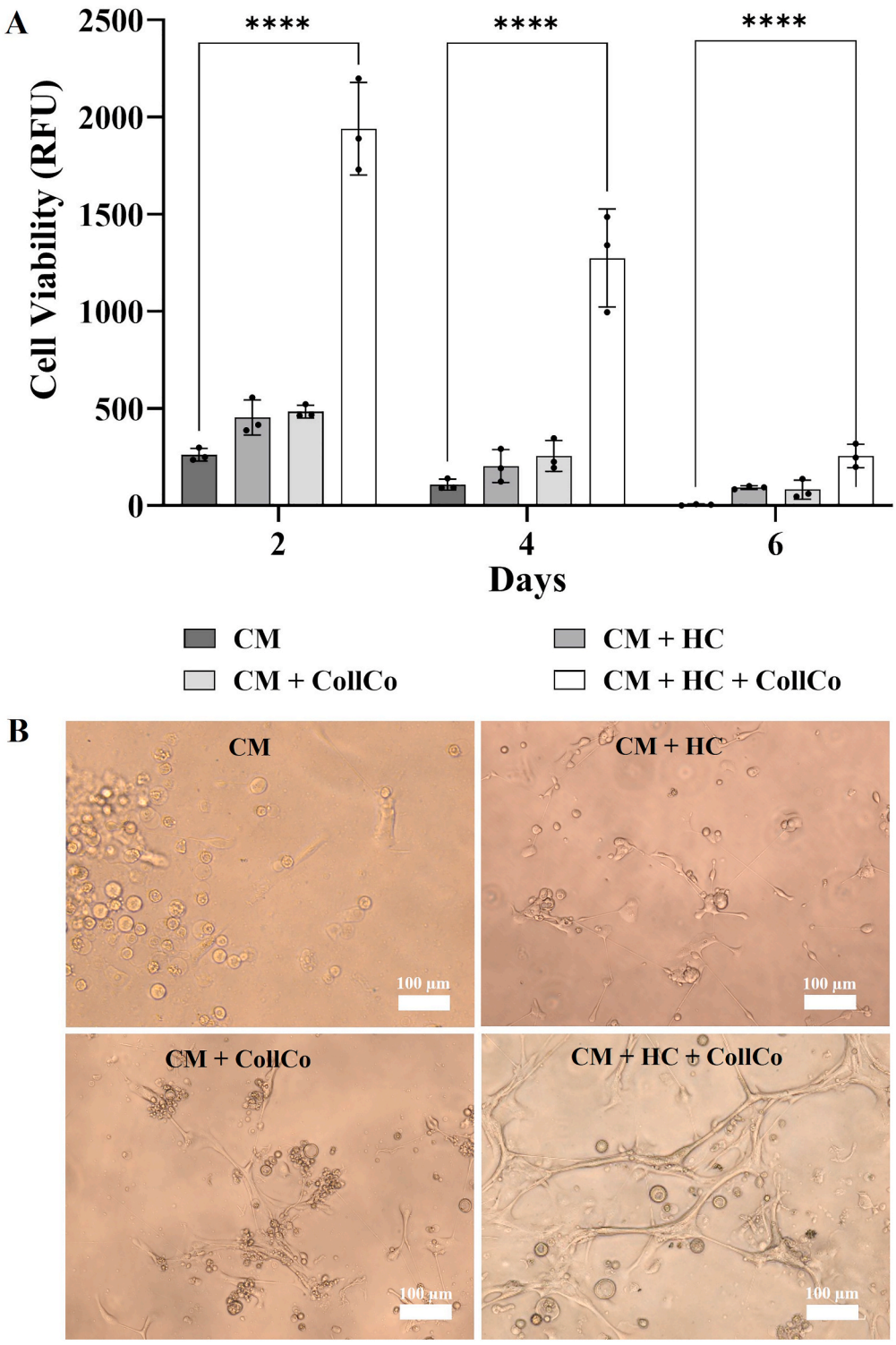

Fig. 6. Establishment of a Specialized Culture Media. (CM: control medium; CM + HC: control medium + hydrocortisone; CM + CollCo: control medium + type I collagen substrate coating; CM + HC + CollCo: control medium + hydrocortisone + type I collagen substrate coating). A) Graph showing the cell viability (mean ± SD) over time of EpiASA cells cultured with control medium (CM) supplemented with 1 µM hydrocortisone (HC), with type I collagen substrate coating (CollCo), or with both. At each time point, Cell viability was significantly higher in the 1 µM hydrocortisone + CollCo condition compared to the control at all time points (day 2: $p < 0.0001$; day 4: $p < 0.0001$; day 6: $p = 0.0301$; Dunnett's multiple comparisons test, n = 3). Significance levels: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$. B) Phase-contrast microscopy images showing the effect of the different conditions tested on cell morphology and culture organization. EpiASA cells cultured with hydrocortisone (1 µM) added to the culture medium and type I collagen coating displayed a more organized cell layer and higher cell-to-cell interactions; this culture condition is hereafter referred to as Enhanced Growth Corneal Medium (EGCM). Three biological samples were used in triplicate.

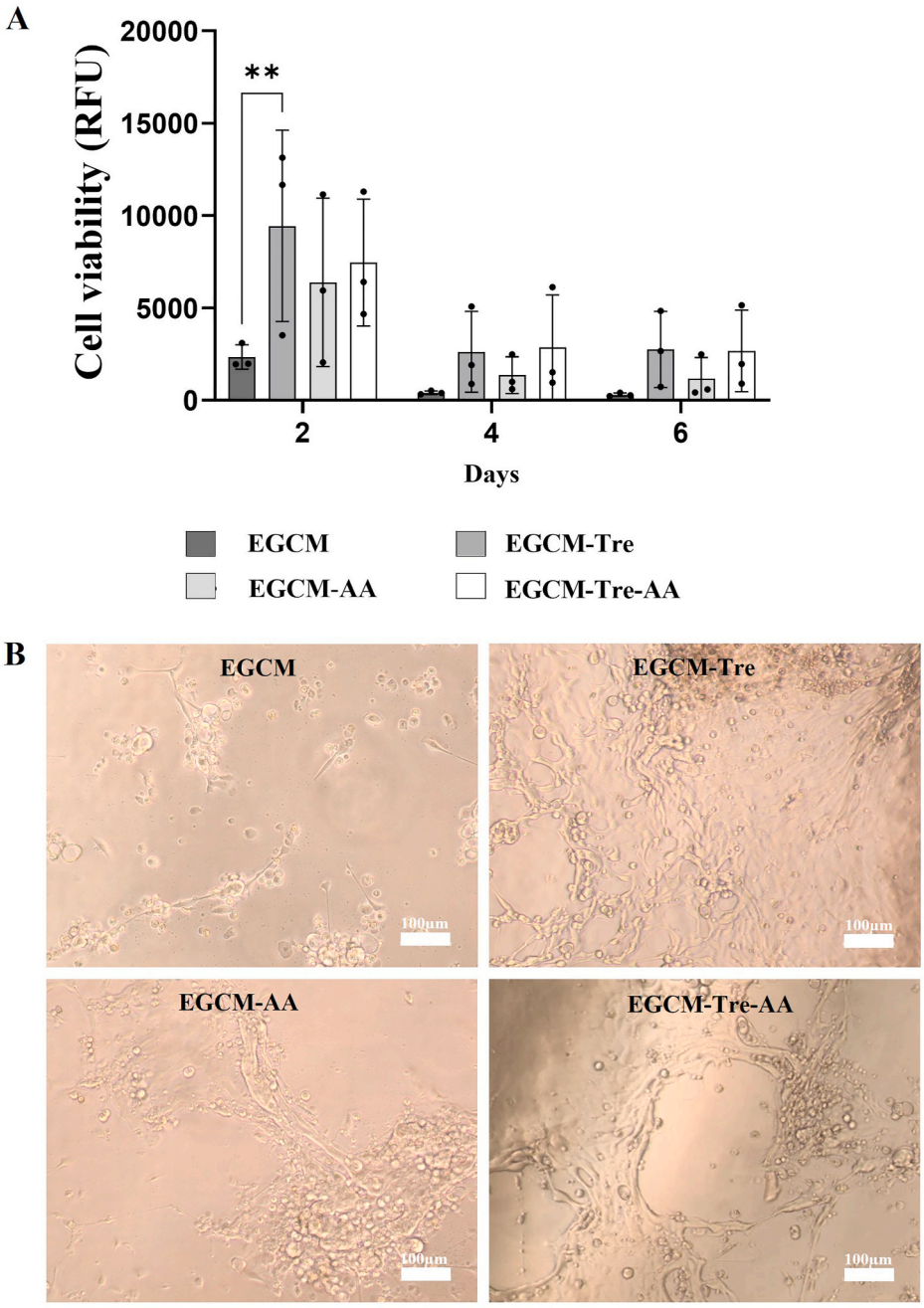

Fig. 7. Effect of Enhanced Growth Corneal Medium (EGCM) supplementation with Trehalose (Tre) and/or Ascorbic *Acid (AA)*. A) Cell viability (mean ± SD) of EpiASA cells cultured in enhanced growth control medium (EGCM), or EGCM supplemented with 2 mM trehalose and/or 100 µM ascorbic acid (AA) after 2, 4, and 6 days. A significant increase in viability was observed with EGCM + trehalose on day 2 compared to EGCM alone ($p = 0.0085$, Dunnett's post hoc test, n = 3). No statistically significant differences were found between conditions at days 4 and 6. B) Representative phase-contrast microscopy images showing EpiASA cell morphology and distribution under each condition at day 6. Three biological samples were used in triplicate.

4. Discussion

In this study, we first characterized the EpiASA tissues and enhanced their harvesting and culture conditions. After that, we were able to establish a specific optimized cell culture protocol and to design a specialized culture medium that allowed us, not only to maintain EpiASA cells in culture for 14 days, but to achieve subculturing. Moreover, we demonstrated that our primary and first-passage cultures preserve the key structural and functional characteristics of the human corneal epithelium (Oh et al., 2013), showing that these cultures can be used for robust and relevant *in vitro* research.

In the EpiASA tissue sample collection phase, the addition of 100 nM trehalose to the collection medium significantly mitigated the ethanol-induced cell damage, what was observed as a reduced number of intracellular vacuoles, and higher numbers of recovered viable cells. The use of 20 % ethanol for 30 s is a common step in ASA procedures, and several studies have analyzed its effects on the cornea in both patients and animal models. These studies have reported that such exposure can induce vacuolization, transiently compromise cell viability, and alter the expression of junctional proteins (Chen et al., 2002; Liao, 2012). Oh et al. showed that ethanol exposure compromises corneal cell viability in a concentration-dependent manner (Oh et al., 2013), with a high cytotoxicity observed at concentrations exceeding 20 %. Gablet et al., analyzed the effect of 20 % ethanol at different exposure times in human

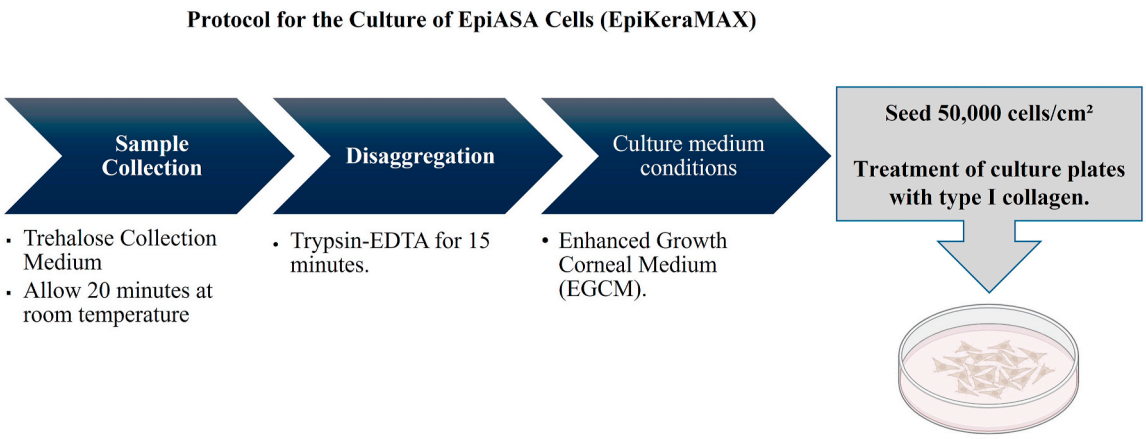

Fig. 8. EpiKeraMAX Protocol. Schematic summarizing the main characteristics of our optimized protocol to culture EpiASA cells.

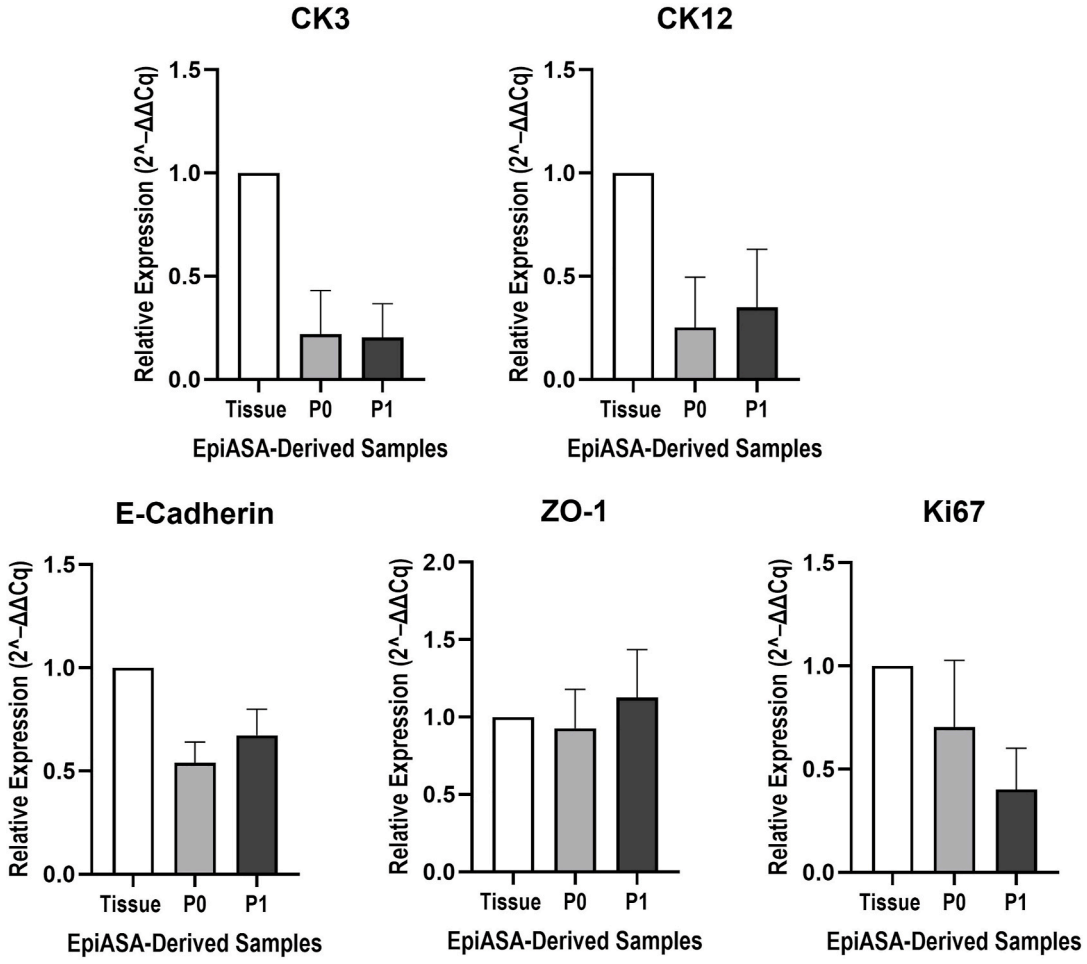

Fig. 9. Expression of Epithelial and Junctional Markers in EpiASA-derived *Samples*.
qPCR analysis of CK3, CK12, E-cadherin, ZO-1, and Ki67 in native EpiASA epithelial tissue (Tissue), primary cultures (P0), and first-passage cultures (P1). CK3 and CK12, markers of corneal epithelial identity, remain detectable in cultured samples. E-cadherin and ZO-1 expression levels are stable or slightly increased in P1, suggesting the retention and potential reinforcement of cell–cell adhesion and barrier function during early subculture. Ki67 expression indicates maintained proliferative activity across passages. Data show the mean ± SD of 12 biological samples (n = 12), each analyzed in technical triplicates.

cadaveric corneas, showing that most epithelial cells remained viable when exposure time was up to 30 s, whereas at 45 s the proportion of viable and dead cells was approximately equal, and longer exposure times resulted in predominantly non-viable cells (Gabler et al., 2002). Nevertheless, the clinical use of ethanol in procedures such as LASEK shows that the damage induced by short ethanol exposure is at least partially reversible(Liao, 2012). Our study is in accordance with these observations, since cultured EpiASA cells show a healthy morphology, no signs of vacuolization, and recovered the expression of key epithelial barrier markers (Miyake et al., 2020).

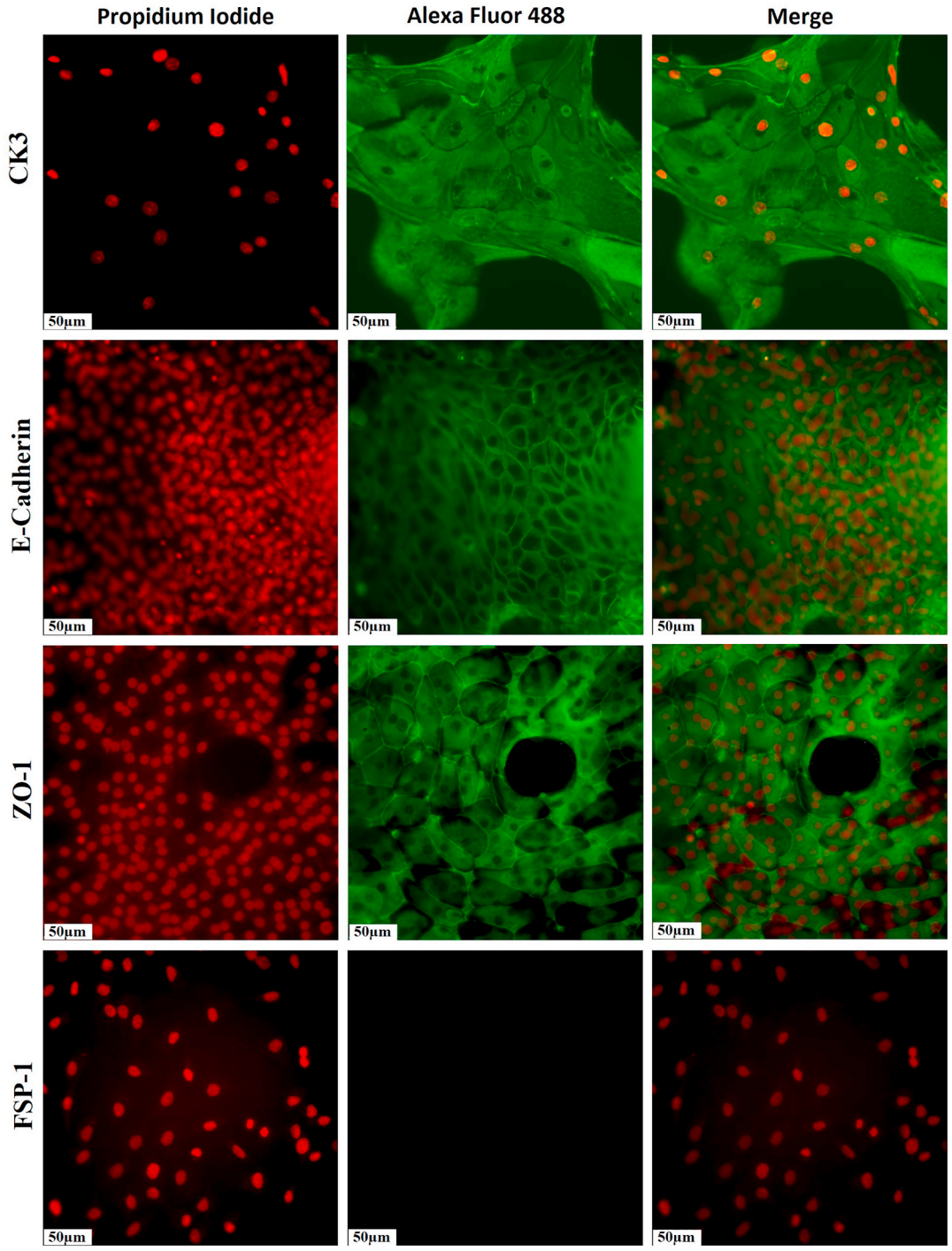

Fig. 10. Immunofluorescence Staining of Passage 1 EpiASA Cells. Representative fluorescence microscopy images of First-passage EpiASA cells expressing CK3, the tight junction protein E-Cadherin, ZO-1, and FSP-1 (all in green). Nuclei were counterstained with propidium iodide (PI, in red). Scale bars: 50 µm. Three biological samples were evaluated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The cytoprotective effects of trehalose has been previously documented in tissue cryopreservation, the reduction of oxidative stress, and the improvement of osmotic balance (Matsuo, 2001; Gao et al., 2024; Bertl et al., 2019; Miyake et al., 2020; Liu et al., 2020; Panigrahi et al., 2019). In our hands, this cytoprotective effect was particularly valuable for the post-surgical culture of the discarded corneal tissue, for which the maintenance of the tissue structural integrity and mitigation of the damage associated to ethanol exposure is crucial. Another demonstration that the tissue retains its intrinsic structural and functional characteristics such as adhesion, barrier function, and proliferative capacity is the positive expression of specific markers. CK3 and CK12 confirmed epithelial identity, E-cadherin, Claudin-1 and ZO-1 reflected the presence of intercellular junctions, and Ki67 indicated proliferative activity. Although ZO-1 staining showed some discontinuities, likely associated with ethanol exposure, it is noteworthy that this protein was re-expressed during primary culture, and its expression was maintained through subsequent subculturing. This finding highlights the tissue's ability to recover key junctional features *in vitro*.

In order to obtain reproducible cell cultures, appropriate cell disaggregation is necessary. We analyzed the influence of two trypsin-EDTA

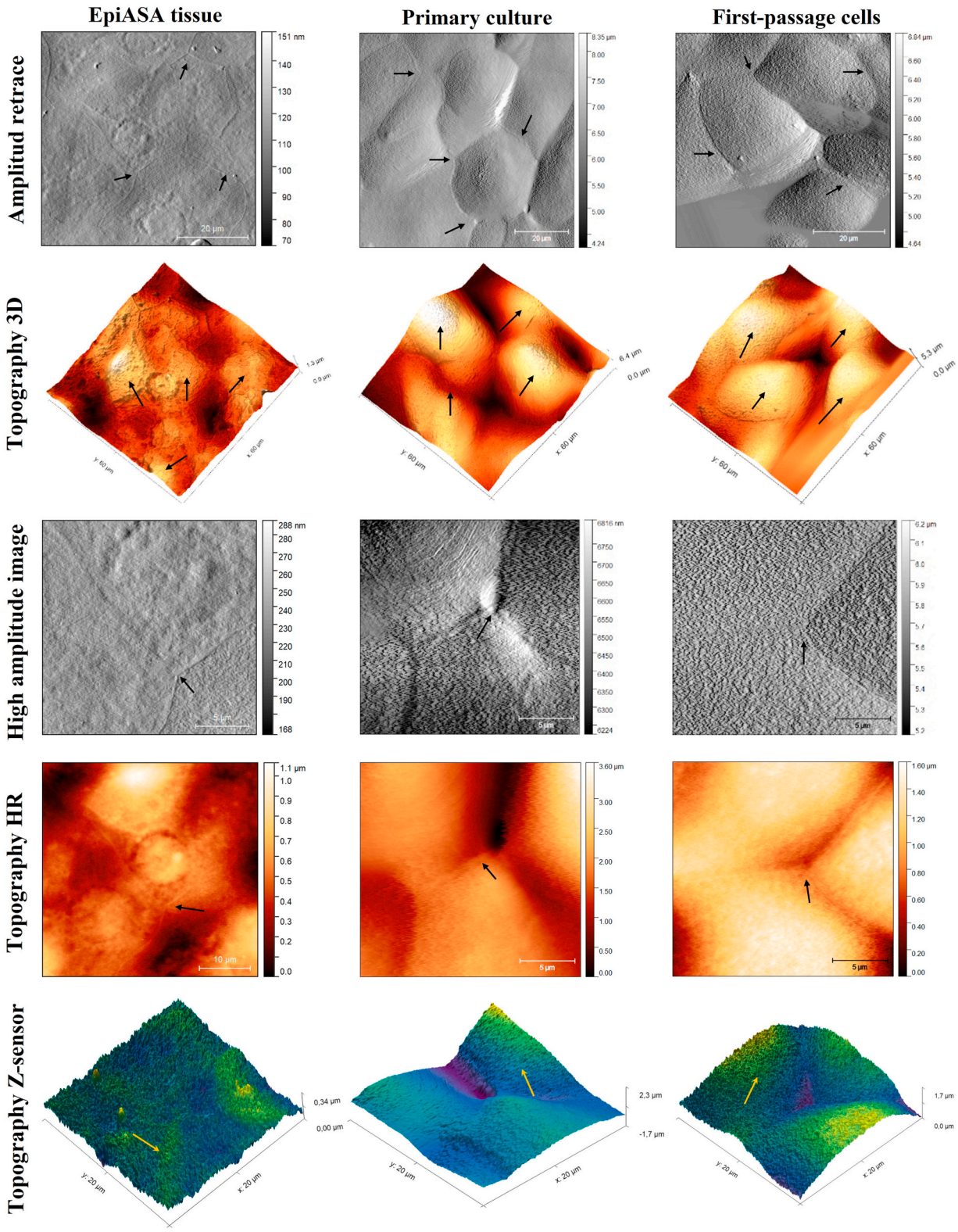

Fig. 11. Analysis of Surface Topography and Cellular Organization of EpiASA Samples by Atomic Force Microsc*opy (AFM)*. In the amplitude retardation images, native EpiASA tissue, primary culture, and first-passage cells display cellular junctions (black arrows), confluent monolayers, and intact, well-defined junctions without interruption in high amplitude images. The 3D topographic images reveal uniform structures of lower elevation in native EpiASA tissue compared to the other two cultures, marked by black arrows. In the high amplitude images, the junctions in EpiASA tissue are barely distinguishable due to their neat arrangement, similar to those in the First-passage culture, whereas in primary culture, the junctions appear thicker (black arrows). The HR topography shows that while high magnification images focus on cellular junctions within the same plane in EpiASA tissue and are well-defined in the first-passage culture, primary cultures display varying elevations, marked by black arrows. In the Z-sensor topography images, elevated irregularities similar to microvilli are observed across the entire surface (black arrows), indicative of the detailed textural differences between the samples. This highlights the dynamic changes in cellular architecture from native tissue to subculture processes. Scale bars: 20 μm for general views and 2 μm for high magnification frames. A total of six biological samples were used.

exposure times on EpiASA tissue disaggregation. The analysis revealed that a 15-min incubation with trypsin-EDTA resulted in better cell disaggregation and higher cell viability compared to that resulting from the 10-min incubation. Nevertheless, it should be noted that a high proportion of cellular aggregates was obtained after 10 min of incubation, which likely influenced the lower cell count and cell viability rate observed in this condition. In contrast, 15 min of trypsin treatment produced a higher proportion of viable dispersed cells, which will facilitate the production of more reproducible primary cultures in terms of cell density and distribution. Further optimization using alternative disaggregation techniques could provide cells with higher viability rates.

Once the EpiASA-derived cell suspension was obtained, we explored different conditions to improve cell culture. Co-culture experiments demonstrated that both corneal fibroblasts and the conjunctival epithelial cell line enhanced the viability and organization of primary epithelial cells, presumably through paracrine signaling mechanisms. These results align with prior studies that demonstrated the critical role of stromal cells in epithelial cell growth (Toropainen et al., 2001). However, we observed that these benefits were short-termed and found a decline in cell viability around day 4. For that reason, and bearing in mind that co-cultures systems increase the complexity of the models and can incorporate higher variability, different approaches to improve EpiASA cell cultures were evaluated.

We used a systematic approach to medium supplementation to define an optimized culture condition specifically for EpiASA cells. This strategy allowed us to adjust the culture environment directly for the cells in the primary culture, improving their viability and stability without the complexities and variable factors inherent in a co-culture with cell lines (Jung et al., 2020).

We first compared the effect of FBS included in our control medium with the effect of HS and found that FBS consistently outperformed HS in supporting EpiASA primary cell viability and structural integrity at all evaluated time points. It is widely known that both FBS and HS can promote cellular growth in a cell type–dependent manner (Bieback et al., 2009; Liu et al., 2023; Heger et al., 2018). FBS is commonly used in cell culture due to its high content in growth factors and its low complement activity (Triglia and Linscott, 1980). It also has a high amount of fibronectin, that can also promote the adhesion and better growth of EpiASA cells (Hayman and Ruoslahti, 1979). These two factors may contribute to the superior performance of FBS over HS in supporting EpiASA cultures. Additionally, it should be noted that human serum contains active human immunoglobulins, which may influence immunoregulatory gene expression, thereby increasing the variability of the results. (Guo et al., 2016). Our results support the use of FBS to promote EpiASA primary cell proliferation.

When we tested, either alone or in combination, the effect of hydrocortisone-supplemented medium and type I collagen culture substrate coating, we found that the combination of these two actions strikingly increased primary cell viability compared to the non-supplemented control medium. Hydrocortisone has been reported to strongly promote the development and maintenance of barrier properties in cerebral capillary endothelial cells cultured in a serum-free system without the need for co-culture with astrocytes (Hoheisel et al., 1998; Yang et al., 2017; Yu et al., 2016) (Somaiah et al., 2015). This approach significantly improves these properties in different cell types, with optimal effects observed at concentrations above 5.8×10^{-7} mg/cm^2, as reflected in reduced adhesion time and doubling time (Yashiki et al., 2001). In our EpiASA culture system, both supplements marginally increase cell viability when used separately, although it is possible that the enhanced cell attachment derived from the collagen coating consequently potentiates the barrier formation effect of hydrocortisone when used in combination. From these results, we defined our Enhanced Growth Corneal Medium (EGCM) by supplementing the control medium with hydrocortisone and established the need for coating the culture wells with type I collagen.

Additional supplementation of EGCM with trehalose (EGCM-Tre) significantly increased cell viability on day 2; however, this increase was not sustained in subsequent measurements on days 4 and 6. Interestingly, an improvement in the morphology of the primary EpiASA culture was observed starting from day 2. By days 4 and 6, the EpiASA cells, which had formed a monolayer, remained attached to the plate, indicating that they were still viable.

The improvements observed in the primary cultures of EpiASA cells with EGCM-Tre over 7 days allowed us to subculture the cells, and then we analyzed these first-passage cells after another 7 days in culture. Both qPCR and immunofluorescence analysis confirmed the expression of key corneal epithelial markers in the first-passage cells, including CK3, ZO-1, and E-cadherin, which are essential for validating the differentiation and structural integrity of the corneal epithelium (Polisetti et al., 2023; Li et al., 2024).

One of the main concerns that arise when culturing epithelial cells, and sometimes related to the use of FBS-containing media, is the risk of epithelial-to-mesenchymal transition (EMT). Several studies have shown that prolonged exposure to FBS can induce the loss of epithelial markers, cytoskeletal reorganization, and the expression of mesenchymal-associated proteins (Malm et al., 2018). Nevertheless, we did not observe EMT in EpiASA cultures, as shown by the absence of FSP-1 expression, and by the maintenance of epithelial markers such as CK and E-cadherin. E-cadherin is strongly associated with suppression of EMT (Wei et al., 2022; Bardag-Gorce et al., 2016; Shome et al., 2023). The risk of EMT is also higher at higher cell passages. Different studies have demonstrated that prolonged culture or higher passage numbers can lead to altered morphology and reduced marker expression in ocular surface epithelia, even under optimized conditions(Harper, 2024; Martinovich et al., 2017). On the contrary, primary cultures and first-passage cells have been shown to reliably preserve epithelial identity and ensure greater translational relevance(Miserocchi et al., 2017). For those reasons, it is highly recommended to use cells in low passages.

We used AFM to perform the structural characterization of the primary and first-passage cultures due to its capability to obtain non-destructive live-cell imaging (Berquand et al., 2010). AFM allowed us to observe the positive progression in structural organization from primary to first-passage culture (Li et al., 2021). The application of AFM enables the observation of key structural features that validate the advancements in the functionality of EpiASA cultures under our optimized conditions. This analysis demonstrated the effectiveness of our optimized EpiKeraMAX protocol (Fig. 8) in supporting cellular reorganization that resembles the structure of native tissue, which is partially lost in primary cultures following tissue disaggregation.

5. Conclusion

In conclusion, this research provides an optimized protocol (EpiKeraMAX) for using EpiASA samples for *in vitro* studies on OSDs. The use of different strategies, including culture medium supplementation and culture surface coating, helped us to address biological and technical challenges in the establishment of reliable primary cultures derived from EpiASA cells. Further investigations should focus on long-term stability and functional characterization, to maximize the potential of EpiASA cells for disease modeling and therapeutic applications.

CRediT authorship contribution statement

Helen Gutiérrez: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Antonio López-García:** Writing – review & editing, Methodology, Investigation. **Miguel J. Maldonado:** Writing – review & editing, Resources, Conceptualization. **Laura García-Posadas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Commercial relationships disclosure

H. Gutiérrez, None; A. López-García, None; M.J. Maldonado, None; L. García-Posadas, None.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI, San Francisco, CA, USA) to assist in improving the English language. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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