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Article

Epithelial Regeneration in Human Corneas Preserved in an Active Storage Machine

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Purpose: To characterize the corneal epithelium (CE) and limbal epithelium (LE) of human corneas stored in an innovative active storage machine (ASM) after a period of organ culture (OC).

Methods: Corneas unsuitable for graft and stored in a standard commercial OC medium for 2 to 5 weeks were transferred into our ASM for 14 days. The ASM actively maintained an overpressure on the endothelial side (20 mm Hg) while ensuring medium renewal. We compared three modalities of storage in the ASM's epithelial chamber: (1) alternating exposure to a supplemental hormonal epithelial medium (SHEM) and air (air-lifting), (2) continuous immersion in SHEM, and (3) continuous immersion in OC medium. Passive immersion of the whole cornea in OC medium or of the CE in SHEM with or without airlifting served as controls. Paired corneas were used for better comparability. Histology, differentiation (by immunolabeling), and ultrastructure were analyzed at the end.

Results: The ASM with air-lifting was most effective in regenerating a pluristratified and differentiated CE (apical ZO-1 and MUC16 staining and regeneration of the glycocalyx). In addition, the LE was stratified with preserved expression of ABCB5. The ASM with immersion in SHEM or OC medium gave a less stratified and differentiated CE. In the three control groups, the epithelia, when present, were paucistratified and less differentiated.

Conclusions: In human corneas previously stored in OC, the ASM regenerates a CE with differentiation characteristics close to normal.

Translational Relevance: Regeneration of the epithelium of human corneas discarded by eye banks will increase tissue availability for research.

Introduction

To test fundamental or applied hypotheses and to conduct preclinical research in the field of ocular surface diseases (e.g., drug testing, wound healing assays), ex vivo models using animal or human whole cornea have been developed to complement existing laboratory in vitro and in vivo models and circumvent their respective drawbacks.

Ex vivo models have been developed in particular for wound healing and pharmacologic[1–3] experiments. They allow the use of both animal corneas, used in a very short postmortem period, and human corneas from eye banks. Ex vivo storage of corneas aims to preserve the three main corneal cell types (epithelial

cells, stromal keratocytes, endothelial cells) to preserve cell interactions and tissue properties (transparency, wound healing). But corneal storage is challenging as culture conditions for the endothelial and epithelial layers differ. Corneas procured for eye banking or for ex vivo experiment quickly develop edema and epithelial alterations (desiccation, erosions, full abrasion, reduced stratification in most cases)[4] whether stored at 4°C[5] or in organ culture (OC).[6] To address this problem, we developed an active storage machine (ASM) that preserves epithelial integrity, endothelial viability, and stroma hydration control in porcine corneas to a degree unrivaled by conventional passive storage methods.[7] This was achieved by dissociating endothelial and epithelial culture conditions. The ASM restores intraocular pressure (IOP) and control-media flows and alternately exposes the corneal epithelium to air and to a medium intended specifically for corneal epithelium culture.

There would be great value in using human corneas as an ex vivo model to avoid physiologic interspecies discrepancies and the need for specific antibodies against animal-specific epitopes. Many corneas disqualified from transplantation by eye banks could become available. But having usually spent a short or long time in their storage medium, they cannot be deemed fresh and may show alterations listed above.

The purpose of the present study was to analyze the ASM's ability to regenerate the corneal epithelium of corneas disqualified by banks and already stored for weeks in OC. We assessed the influence of IOP restoration, of using a medium specifically for corneal epithelial cells, and of intermittent air exposure on regeneration of the corneal epithelium structure.

Material and Methods

Human Corneas

The study was conducted in accordance with the ARVO statement for the use of human subjects in ophthalmic and vision research. Handling of donor tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting donor confidentiality. Human corneas unsuitable for transplantation were obtained from the eye banks of Saint-Etienne and Besancon after checking with relatives that subjects expressed no opposition in their lifetime, as authorized by French bioethics laws. Corneas had been deemed unsuitable for transplantation because of a low endothelial cell count (<2000 cells/mm^2). As we needed a functional endothelium, we selected only corneas with an endothelial cell density (ECD) higher than 1000 cells/mm^2 and with OC less than or equal to 6 weeks.

Corneas were initially stored at 31°C in sealed glass flasks of 100 mL OC medium containing 2% fetal calf serum (CorneaMax; Eurobio, Les Ulis, France) in a dry incubator, without medium renewal. Only corneas with between 2 and 5 weeks' storage and ECD of between 1000 and 2000 cells/mm^2 were included, per the corneas that banks usually distribute for research.

To obtain reference images of fresh human corneal epithelium in transmission electron microscopy (TEM), we analyzed the corneal button of a patient with a penetrating keratoplasty for keratoconus. In this disease, even if the epithelium is potentially involved in the pathophysiology,[8] its architecture and histology are accepted as being very similar to those of a healthy epithelium. It is also one of the most easily accessible fresh controls. This corneal button was fixed within 10 minutes of trephination.

Comparison of Different Storage Conditions

We compared the corneal and limbal epithelia after storage in the ASM or with three static reference storage methods: conventional OC and a method used for an epithelial wound-healing assay using corneas mounted on an agar base ("Agar") with or without air-lifting. The specific features of each technique are detailed below and summarized in Figure 1 and Table 1.

Active Storage Machine

The ASM can be likened to the machine perfusions used for vascularized organs. We used the same model, machined in a biocompatible material (polyether ether ketone), that we recently used for studying the long-term eye banking of human corneas.[7,9] Briefly, it maintained a sterile closed environment, allowing long-term corneal storage and no-touch assessment through two optical-quality windows in front of the epithelial and endothelial sides of the tissue. Under a laminar flow hood, the cornea was tightly secured to the ASM base, using the scleral rim as a watertight seal to separate the epithelial and endothelial chambers, and then the lid was closed on the epithelial side. In the present model, a peristaltic pump controlled by a pressure sensor and a microcontroller continuously renewed the culture medium (CM) at a rate of 5 µL/min while creating a pressure in the endothelial chamber 20 mm Hg higher than atmospheric pressure. The machine, except its control panel, was placed at 31°C in a dry incubator with 5% CO_2, because some ASM

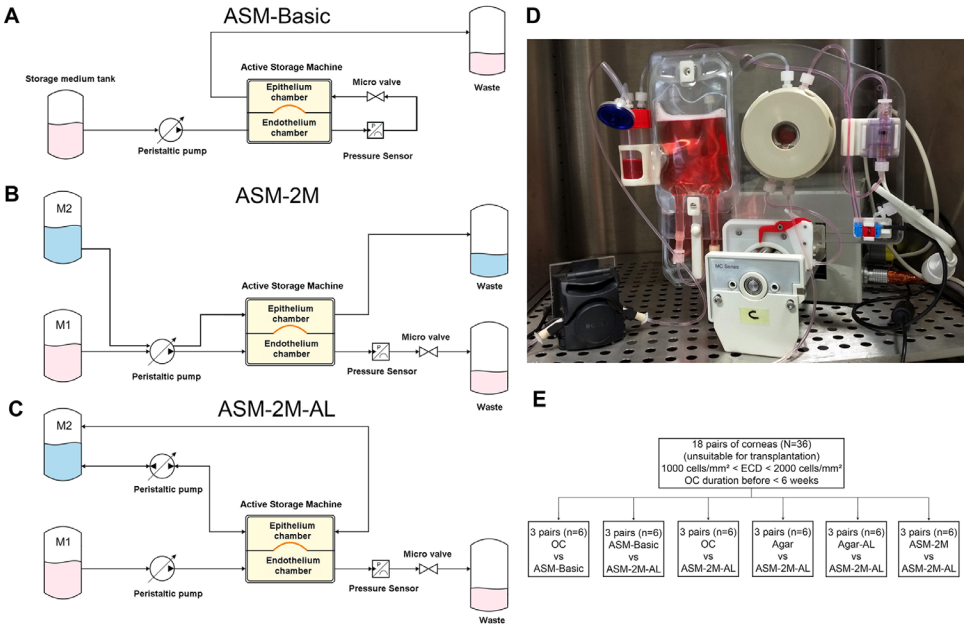

Figure 1. Functional diagram (**A**, **B**, **C**) of the three versions of the ASM. In all three, the cornea separated the endothelial and epithelial chambers. The endothelial chamber was filled with endothelial culture medium (CorneaMax) from a tank. Fluid was circulated by a peristaltic pump driven by a pressure sensor, a solenoid valve, and a microcontroller. (**A**) Functional diagram of the ASM-Basic, in which the culture medium first circulated in the endothelial chamber, then in the epithelial chamber, and was finally removed to a waste compartment. (**B**) Functional diagram of the ASM-2 media (ASM-2M), in which the two chambers were independent and an epithelial culture medium was circulated in the epithelial chamber. (**C**) Functional diagram. (**D**) Picture of the ASM-2 media and air-lifting (ASM-2M-AL), in which the epithelial chamber was exposed alternately to the epithelial medium for 1 second and to air for 30 seconds. (**E**) Flowchart of experiments.

Table 1. Characteristics of the Five Corneal Storage Methods

Denomination	Epithelial CM	Air-Lifting	Endothelial CM	Endothelial CM Flow and IOP, mm Hg	*n*
ASM-Basic	CMax	No	CMax	Yes/20	6
ASM-2M	SHEM	No	CMax	Yes/20	3
ASM-2M-AL	SHEM	Yes	CMax	Yes/20	15
OC	CMax	No	CMax	No	6
Agar	SHEM	No	None	No	3
Agar-AL	SHEM	Yes	None	No	3

The active storage machine was used with three conditions of increasing complexity and compared to two reference methods: organ culture and agar system. CMax, CorneaMax.

tubes were CO_2 permeable and the media used a mainly bicarbonate buffer requiring an atmosphere enriched to 5% CO_2. The ASM was used under three increasingly complex conditions: (1) in its simplest mode of operation (ASM-Basic), with one medium circulating first on the endothelial side and then on the epithelial side. This was a commercial OC medium based on Dulbecco's modified Eagle's medium (DMEM) and containing 2% fetal calf serum (Eurobio). This mode was previously used to validate 4-week storage in the ASM.[9] (2) In the ASM two-medium mode (ASM-2M), the endothelial chamber was filled with CorneaMax under the same circulation and pressure conditions as in ASM-Basic, but the epithelial chamber was filled with a CM usually dedicated to in vitro epithelial cell culture (hereafter called supplemental hormonal epithelial medium [SHEM]) that consisted of DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and Ham's F12 (Sigma, Saint-Quentin-Fallavier, France) (2:1) supplemented with 10% fetal bovine serum (Eurobio), 10 ng/mL epidermal growth factor (Sigma), 1% antibiotic antimycotic solution (Sigma), 0.1 nM cholera toxin (Sigma), 5 mg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 0.18 nM Adenin (Sigma), 0.4 µg/mL hydrocortisone (Sigma), 1 µg/mL bovine pituitary extract (Thermo Fisher Scientific),

and 2 nM 3,3′,5 Triiodo-L-thyronine (EMD Millipore, Burlington, MA, USA). The SHEM was renewed at a rate of 5 µL/min using the same peristaltic pump. (3) In the ASM-2M with air-lifting mode (ASM-2M-AL), the previous mode was supplemented by a system alternating, in the epithelial chamber, exposure to the SHEM medium for 1 second and exposure to air for 30 seconds by means of a peristaltic pump and a microcontroller independent of the main ASM system. The SHEM (25 mL) was changed every 2 days.

Organ Culture

Corneas were immersed in glass vials containing 100 mL CorneaMax and incubated at 31°C.

Agar System

Corneas were placed on a concave silicon support, epithelial side down. The endothelial side was filled with 2% agar (Sigma) in phosphate-buffered saline (PBS) at 35°C in sterile conditions. Once the agar solidified, the corneas were placed in a six-well plate (Corning Costar, Corning, NY, USA), epithelial side up, either fully immersed in SHEM or cultured with SHEM at an air–liquid interface and incubated at 31°C, 5% CO_2, and 90% relative humidity.

Organization and Aims of the Comparisons

In our previous studies using the ASM, storage time was extended to 4 weeks to match the long-term storage period in European eye banks[9] and to 3 months to develop extremely long storage.[10] Here, the aim was to rehabilitate epithelium-disqualified corneas to make them quickly available for research. Preliminary analysis in said studies after 7 days' storage showed results close to those found in this study, but stratification was improved by extending storage to 14 days. This compromise seemed sufficient to allow complete regeneration in the case of severely altered epithelium, without excessively extending storage time for corneas already organ cultured for weeks. To increase the power of the comparisons by minimizing the interindividual variability related to donors (age, genetics, pathology) and to preexperiment storage (time to death, OC duration), we only used pairs of corneas. Each cornea of the pair was preserved with a different method. The comparisons were organized as follows, each time in triplicate: (1) ASM-Basic versus OC to confirm the specific effect of active conservation after 14 days (the previous tests were performed over 28 days and with fresh corneas),[9] (2) ASM-Basic versus ASM-2M-AL to analyze if air-lifting with an epithelium-specific medium further improved regeneration and differentiation, (3) ASM-2M-AL versus ASM-2M to characterize the specific contribution of air-lifting, (4) ASM-2M-AL versus OC, (5) ASM-2M-AL versus agar, and (6) ASM-2M-AL versus Agar-AL to compare the most sophisticated method with the three conventional passive methods. Due to this, the overall number of ASM-2M-AL corneas was higher than in the other groups.

End-of-Storage Analysis

After 14 days' storage, corneas were cut into four pieces and processed as follows.

Histology

One-fourth of the cornea was fixed in 4% paraformaldehyde for 24 hours at room temperature (RT), dehydrated through ascending concentrations of ethanol, and embedded in paraffin. Cross sections 7 µm thick were cut, rehydrated, and stained with hematoxylin, eosin, and saffron. Bright-field tagged image format file (TIFF) images of the cross sections were acquired using an IX81 microscope (Olympus, Tokyo, Japan). The number of epithelial cell layers (semiquantitative) and the epithelial thickness were compared between the different experimental storage conditions on images taken with a 40× objective. For epithelial thickness, to avoid measurement bias due to local variations, cross-sectional epithelial surfaces were measured over a constant length for all corneas (125 µm), using ImageJ (US National Institutes of Health, Bethesda, MD, USA). Average epithelial thickness was calculated by dividing the measured epithelial area by 125 µm.

Immunohistochemistry

One-fourth of the cornea was immediately processed for flat-mounted immunostaining, as previously reported.[9,11] The sample was divided in two, and one piece was fixed in 0.5% paraformaldehyde for 1 hour at RT and then permeabilized in 1% Triton (Sigma) for 10 minutes at RT. The sample was rinsed twice in PBS for 5 minutes between each step; the other piece was fixed in methanol for 1 hour at RT and then rehydrated in three 5-minute PBS baths at RT. Nonspecific binding sites were blocked by incubation for 30 minutes at 37°C with blocking buffer, based on PBS supplemented with 2% heat-inactivated goat serum (Eurobio) and 2% bovine serum albumin (Thermo Fisher Scientific). Slides were incubated at 37°C for 1 hour or overnight at 4°C with the primary antibody diluted by 1:200 in blocking buffer. Primary antibodies were supplied as listed in Table 2. Samples were incubated for 2 hours at 37°C with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA) diluted by 1:500

Table 2. Primary Antibodies

Target Protein	Animal Source	Role (Expected Cell Compartment)	Reference and Manufacturer
ZO-1	Mouse	Tight junctions (apical plasmic membranes)	40-2200; Zymed, Carlsbad, CA, USA
ABCB5	Rabbit	Stemness (plasmic membranes)	NBP1-50547; Novus Biologicals, CO, USA
E-Cadherin	Mouse	Epithelial cells (all plasmic membranes)	33-4000; Thermo Fisher Scientific
Laminin 5	Rabbit	Basement membrane	AB14509; Abcam, Cambridge, UK
Cytokeratin 3 (K3)	Mouse	Differentiated corneal epithelial cells (cytoplasmic)	Sc-80000; Santa Cruz Biotechnology, Dallas, TX, USA
Cytokeratin 12 (K12)	Mouse	Differentiated corneal epithelial cells (cytoplasmic)	Sc-515882; Santa Cruz Biotechnology

in blocking buffer. Nuclei were counterstained with TO-PRO-3 Iodide (1:500) (Thermo Fisher Scientific) in blocking solution for 5 minutes at RT. Three rinses in PBS were performed between each step, except between blocking of nonspecific protein binding sites and incubation with the primary antibody. Last, the slides were mounted using Vectashield medium (Vector Laboratories, Burlingame, CA, USA).

One-fourth of the cornea was embedded in optimal cutting compound (Thermo Fisher Scientific) and snap-frozen using prechilled 2-methylbutane (Sigma) and liquid nitrogen. Samples were then stored at –80°C until further processing. Tissue sections (14 µm thick) were cut using Cryostat Microm HM550 (Thermo Fisher Scientific) and spread out on Surgipath X-tra Adhesive (Leica Biosystems, Nussloch, Germany). Cornea sections were immunolabeled as previously described[7]: nonspecific rabbit and mouse immunoglobulin G (IgG; Zymed, Carlsbad, CA, USA) were used as primary antibodies for negative controls; incubation with the secondary antibody was reduced to 1 hour.

Images were captured with a confocal microscope (IX83 Fluoview FV-1000; Olympus), equipped with the Olympus Fluoview software. The image acquisition parameters (laser power, 10%) were identical for both of each donor's corneas. For ZO-1 and MUC16 staining, maximum intensity projection was obtained from a Z stack of images using ImageJ.

Transmission Electron Microscopy

The corneal epithelium was examined by TEM as previously described.[12] One-fourth of the cornea was fixed in 1% glutaraldehyde (16220; Electron Microscopy Sciences, Hatfield, PA, USA) and 0.5% paraformaldehyde (P/0840/53; Thermo Fisher Scientific, Loughborough, UK) in 0.02 M Na_2HPO_4 (Prolabo, Paris, France)/0.08 M KH_2PO_4 (P3786; Sigma) buffer (pH 7.4), postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide (19180; Electron Microscopy Sciences) for 1 hour, dehydrated in a graded series of ethanol, and embedded in Epon resin (Oxford Instruments, Oxford, UK). Ultrathin 90-nm sections were cut with an ultramicrotome (MTXL; Leica Biosystems, Nussloch, Germany) and stained with 7% uranyl acetate (73943; Sigma) in methanol and Lead(II) citrate tribasic trihydrate (C6522; Sigma). Pictures were taken with a TEM (H-800; Hitachi, Tokyo, Japan) equipped with a charge-coupled device camera (XR41; AMT, Danvers, MA, USA). Three corneas from each storage condition were processed. Pictures were assembled using Adobe Photoshop CS6 (Adobe, San Jose, CA, USA).

Statistical Analysis

The nonparametric Kruskal-Wallis test was used to compare epithelial thickness between ASM-2M-AL and each of the other groups. Post hoc analysis was performed using Dunn's multiple-comparison tests. The null hypothesis was rejected by a type I error <0.05 ($\alpha < 0.05$). Statistical analyses were performed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA), and figures were produced using Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Thirty-six corneas were obtained from 18 donors aged 75 ± 35 (mean ± SD) years (range, 56–91 years; median, 77 years). Death to storage time and storage time were 13 ± 8 hours (range, 4–30 hours; median, 13 hours) and 22 ± 8 days (range, 13–40 days; median, 18 days), respectively. ECD, measured between days 2 and 5 by the eye bank using an advanced image analysis system,[13–17] was 1645 ± 234 cells/mm^2 (range, 1122–1992 cells/mm^2; median, 1660 cells/mm^2).

Corneal Epithelium

No cross section showed an epithelial defect. The corneal epithelium (CE) of corneas stored in ASM-Basic was more stratified compared to paired corneas stored in OC (Figs. 2–4 and Supplementary Fig. 1), confirming the beneficial effect of the ASM alone (IOP restoration, medium renewal). CE organization was limited to one or two layers in OC but was three or four layers thick in ASM-

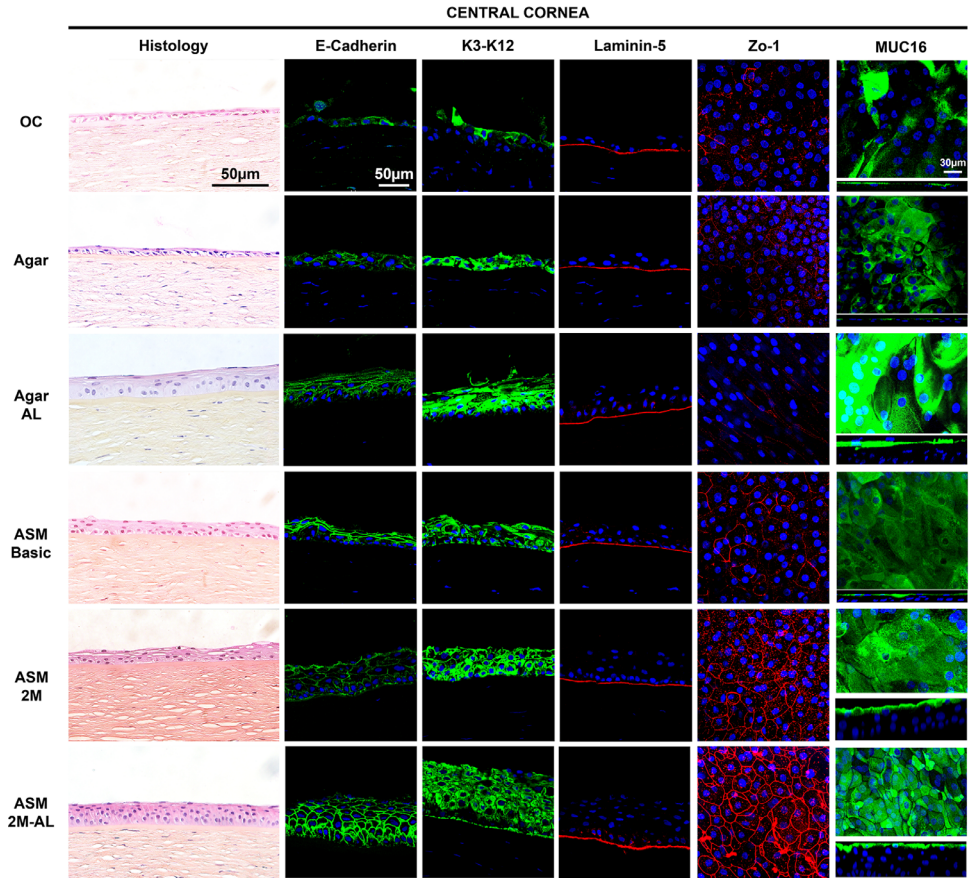

Figure 2. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining on corneal cross sections or on flat-mounted corneas after 14 days' storage in organ culture, on agar, on Agar-AL, or in the ASM. K3–K12 (*green*), E-cadherin (*green*), laminin 5 (*red*), ZO-1 (*red*), and MUC16 (*green*). Nuclei were stained with TO-PRO-3 Iodide (*blue*). MUC16 staining pictures presented are the en face (*upper image*) and the orthogonal (*lower image*) views. No staining was observed in negative controls (data not shown).

Basic. The CE of corneas stored in ASM-2M-AL was more stratified compared to paired corneas stored in ASM-Basic or ASM-2M, with five to seven layers in ASM-2M-AL, three to four layers in ASM-Basic (on a different triplicate from the one cited above), and three to five layers in ASM-2M, suggesting the additional role of the SHEM, specifically formulated for epithelial cell maintenance, and the effect of intermittent air-lifting, respectively. Increased stratification was observed in ASM-2M-AL compared to static condition (OC or Agar), which was limited to one or two layers. Last, corneas stored in Agar-AL seamed more stratified (two to four layers) than corneas stored in Agar alone, suggesting the beneficial effect of air-lifting on epithelial stratification independently of the culture medium (SHEM). Nevertheless, increased stratification was systematically observed in paired corneas stored in ASM-2M-AL compared to Agar-AL.

Interestingly, overall epithelial thickness was not fully correlated to stratification: the thickness values were similar in OC (one of the two sets), Agar, ASM-Basic, and ASM-2M despite intergroup differences in stratification. Two out of three corneas stored in Agar-AL had a thicker epithelium than other corneas stored in passive conditions. Nevertheless, only corneas stored in ASM-2M-AL systematically had a thicker epithelium than their matched corneas, irrespective of storage condition.

Besides the reduced stratification in the Agar, Agar-AL, and OC groups, CE architecture was abnormal with enlarged basal cells (Fig. 2). In the ASM (all conditions), CE basal cells were columnar, superficial cells were flattened (squamous), and in between, polyhedral cells (wing cells) were observed. Intercellular spaces were observed between the most superficial cells and those located underneath in the OC and Agar groups, when multilayered. Additionally, a reduced number of desmosomes was observed in these two static storage conditions compared to ASM stored corneas. At the apical surface of superficial cells, microvilli were observed in every storage condi-

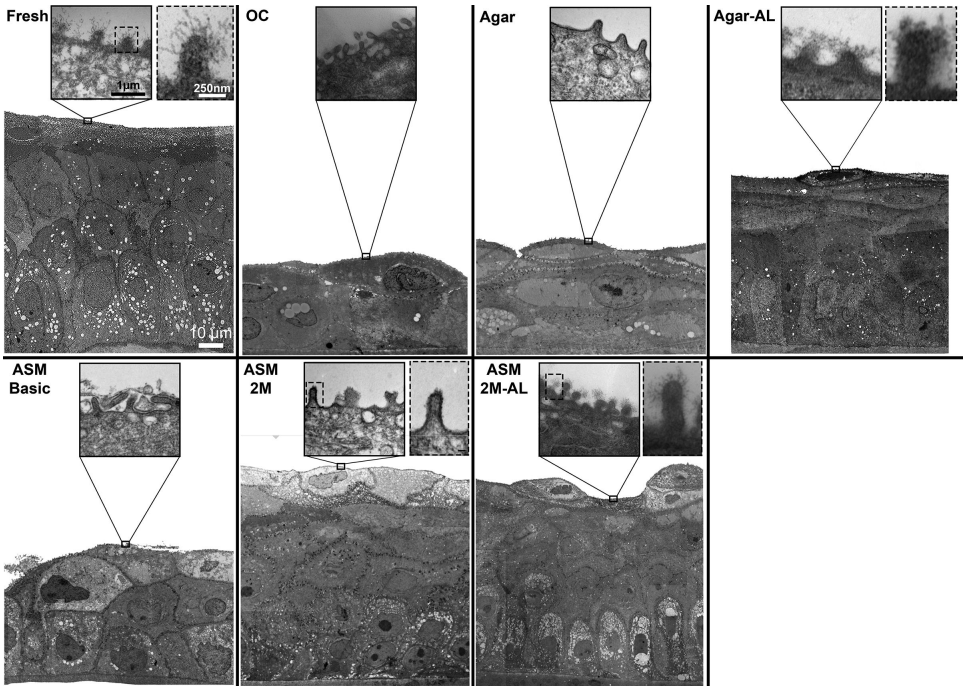

Figure 3. TEM on central epithelium of human corneas after 14 days' storage in organ culture, on agar, or in the ASM.

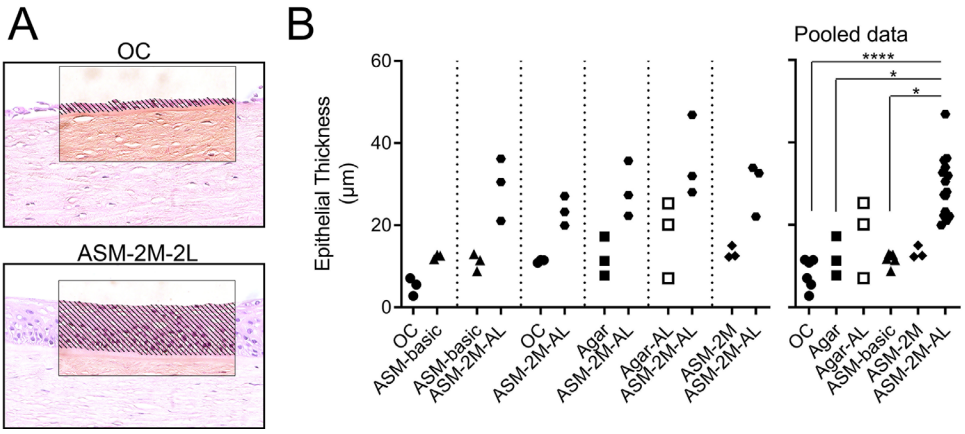

Figure 4. Assessment of epithelial thickness on histologic cross sections. (**A**) Epithelial area was measured in a fixed-width frame. It reflects the epithelial thickness (here on two examples). (**B**) Epithelial thickness of corneas stored in five different storage conditions. *Left*: the six comparisons are separated by *dotted lines*. *Right*: the data from each storage condition were pooled. *$P < 0.05$. ****$P < 0.0001$.

tion. Nevertheless, their density was reduced in OC, Agar, and Agar-AL stored corneas compared to ASM stored corneas (Fig. 3). The extrinsic cell surface coat prominent on the tips of the microvilli, the glycocalyx, was observed in corneas stored in ASM-2M-AL (two of three), ASM-2M (two of three), and ASM-Basic (one of three) but was not observed in any cornea stored in static conditions without air-lifting (OC, Agar). Corneas stored in Agar-AL displayed glycocalyx at the surface of microvilli. Vacuoles were observed mainly in basal cells of ASM stored corneas (all conditions) (Fig. 3). To a lesser extent, theses vacuoles were also observed in the fresh cornea analyzed by TEM.

Whatever the storage condition, the CE expressed corneal differentiation markers (E-cadherin and K3/K12+; Fig. 2) and rested on a continuous basement membrane (laminin 5; Fig. 2). The most superficial layer of the CE displayed ZO-1 staining (Fig. 2). However, ZO-1 staining was irregular and intermittent in corneas stored in a static condition (OC, Agar, and Agar-AL) but was enhanced in paired corneas stored

in ASM-Basic or ASM-2M-AL, showing the ASM's role in CE differentiation. Corneas stored in ASM-2M-AL also had enhanced ZO-1 staining compared to paired corneas stored in ASM-2M or ASM-Basic, suggesting the additional role of the culture media and of intermittent air exposure in corneal epithelial cell differentiation.

MUC16 staining was irregular in corneas stored in a static condition (OC and Agar [except for one]) but enhanced when corneas where exposed to air (Agar-AL); conversely, it was more even when corneas were stored in the ASM-Basic (five of six) or ASM with SHEM, with or without air-lifting (Fig. 2).

Limbal Epithelium

The limbal epithelium (LE) was also more stratified in ASM stored corneas compared to paired corneas stored in OC, Agar, or Agar-AL (Fig. 5). Similar to what was observed in the CE, the LE was more stratified when stored in ASM-2M-AL compared to the LE of corneas stored in ASM-Basic or ASM-2M. ABCB5 staining was observed in every storage condition (Fig. 5) and extended to the suprabasal layers.

Discussion

Having previously shown that the ASM keeps fresh porcine[7] and human[9,10] corneas in conditions unrivaled by conventional storage methods, we show in this article that the ASM promotes the epithelial regeneration of human corneas that had undergone prolonged storage in an eye bank. Cornea storage in the ASM, which combines IOP equivalent restoration with continuous CM renewal in the endothelial chamber, in conjunction with intermittent air-lifting and exposure to an epithelium-specific CM in the epithelial chamber, regenerated a more stratified and differentiated corneal epithelium than the other storage conditions. The systematic use of pairs of corneas increases the value of our observations.

At least three factors may explain why static (or passive, i.e., without physiologic pressure restoration) storage methods cannot preserve the integrity of the epithelium of animal[3] or human corneas[18–20] and why the ASM does: (1) the storage media, initially developed to preserve endothelial cells without proliferative capacity, may be suboptimal for epithelial cells. However, media formulation does not seem to be the most important factor, as we observed the same epithelial alterations despite using a SHEM medium used for in vitro culture of human corneal epithelium. (2)

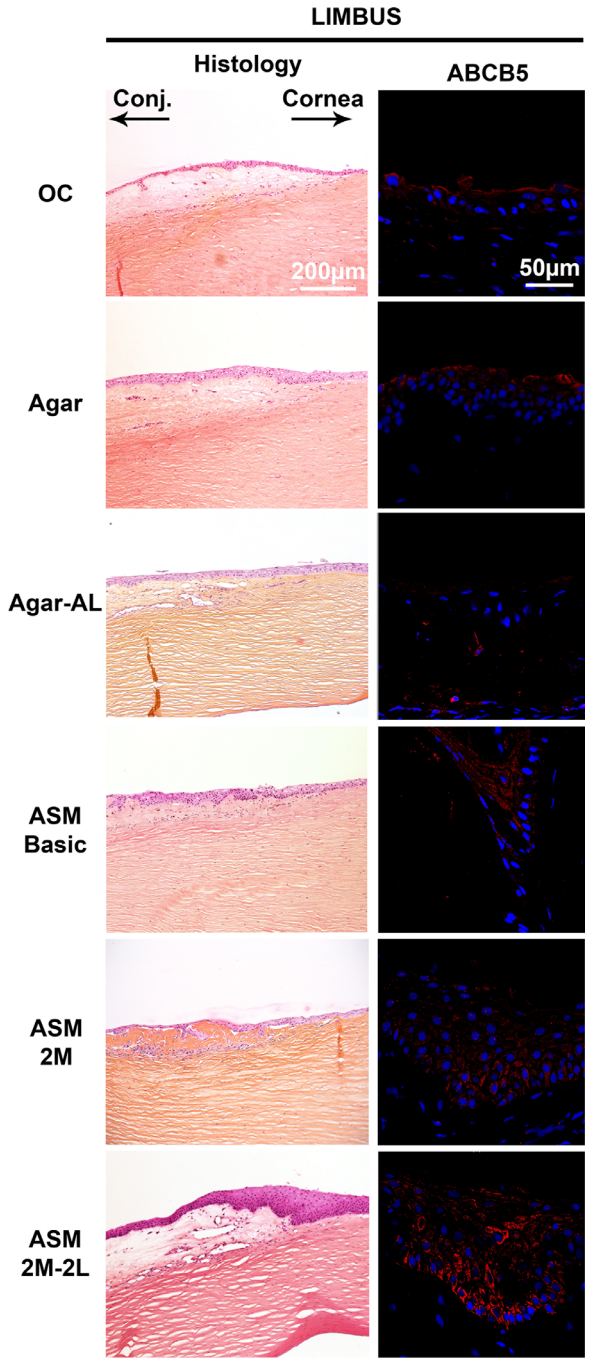

Figure 5. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining on sections of the limbal epithelium of human corneas after 14 days' storage in organ culture, on agar, on Agar-AL, and in the ASM. ABCB5 (*red*). Nuclei were stained with TO-PRO-3 Iodide (*blue*). No staining was observed in negative controls (data not shown).

Its static nature: the only difference between OC and ASM-Basic is IOP restoration and CM renewal, which is the same in both experimental groups. This confirms that dynamic storage, with enhanced endothelial viability/function and better control of stromal hydration, is involved in CE stratification. (3) The absence of

shear stress: shear force[21] and air exposure[22] on the ocular surface contribute to corneal epithelium preservation and differentiation. In vitro, shear stress was associated with stemness maintenance of limbal epithelial stem cells (LESCs),[23] with maturation of superficial epithelial cells (flattened, with enhanced expression of tight junction proteins)[21] and also with superficial cell shedding.[24] The air–liquid interface (ALI) condition has also been used to induce differentiation of multiple cell types, including primary epithelial cell cultures or airway,[25] skin,[26] and reproductive tract.[27] Under ALI, epithelial cells differentiate, regain baso-apical polarity and barrier function, and can generate in vivo–like apical fluid or mucus secretion.[3] The exposure to air of corneal epithelium is also a factor in its stratification and differentiation in vitro[22,26] and ex vivo.[3,7] Deshpande et al.[3] used a rocking system on rabbit cornea stored in a Petri dish to reproduce intermittent moisture and air exposure (air-lifting) of the corneal surface and found an improvement in corneal epithelium preservation. The ASM, initially developed for eye banking, was not focused on the survival of the corneal epithelium: in penetrating keratoplasty and anterior lamellar keratoplasty, the donor epithelium is gradually replaced by the recipient's.[28] However, in this first version (ASM-Basic), medium flow in the epithelial chamber may generate a shear stress at the surface of the most superficial epithelial cells but probably less efficiently than air-lifting. Air-lifting was therefore incorporated in a specific version (ASM-2M-AL), used in this study to enhance corneal epithelium maintenance. Nevertheless, air exposure is not the only factor involved, as shown in our study.

Other evidence in the literature suggests the role of the endothelium in CE and LE health. In vitro experiments reported that coculture of endothelial cells with CE plays a role in CE differentiation and epithelial basement membrane formation.[29] In clinical practice, corneal stratification is impaired in bullous keratopathy and could be associated with limbal stem cell deficiency.[30] Together, these results suggest the utility of corneal endothelial cell preservation when developing an ex vivo model to study corneal epithelium.

To favor CE preservation while reproducing the ex vivo corneal physiologic environment, perfusion chambers were developed for animal corneas (rabbit,[1] porcine,[7,31] and bovine[32]). The two advantages of animal corneas are their unlimited availability and the possibility of using them fresh, in the hours after the animal's death. However, extrapolation to humans may be of limited use. Brunette et al.[33] developed a perfusion chamber for human corneas, but their study was limited to CE analysis. Between 2012 and 2016, corneas not released for transplantation ranged from 25% to 39% of tissues procured by US eye banks, according to the Eye Bank Association of America.[34] These corneas are invaluable for research, but the constant deterioration in quality, particularly of the epithelium, which is observed with all storage methods, may limit some research.[5,6] We used corneas with a relatively well-preserved endothelium (i.e., with an ECD between 1000 and 2000 cells/mm^2). In situ, this ECD range is usually associated with a functional endothelium. This criterion may reinstate a large share of discarded corneas, making numerous corneas available for ocular surface research. In the ASM, the CE showed improved stratification and differentiation characteristics compared to corneas stored in static conditions. In particular, we noted enhanced staining of ZO-1, which belongs to the apical tight junction complex, as well as glycocalyx regeneration. These structures are observed in differentiated squamous corneal epithelial cells and are involved in ocular surface barrier function (OSBF).[35] OSBF is of paramount importance, with dysfunction being involved in the pathogenesis of several ocular diseases.[35] OSBF restoration in human cornea ex vivo could thus be useful for developing ocular surface disease models. Additionally, the bioavailability of topical drugs is influenced by permeability barriers[36] such as apical epithelial glycocalyx.[37] Further developments are ongoing to implement functional tests[35] for OSBF, because structural components of the OSBF may not correlate with the actual function. We also noticed extents of MUC16 staining in every storage condition. Taking into account the apical structures (glycocalyx) observed in TEM, this result suggests that MUC16 expression cannot be considered by itself to be a marker of OSBF restoration, highlighting the need for functional testing of OSBF.

In humans, the corneal epithelium is renewed by cells migrating from the corneal limbus,[38] which is the putative niche for LESCs. For corneal epithelium homeostasis, LESCs generate amplifying cells that proliferate, migrate, and differentiate according to the X, Y, Z hypothesis.[39] The absence of LESCs impairs CE renewal and leads to an invasion of the corneal surface by conjunctival epithelial cells (conjunctivalization).[40] Numerous LESC-specific markers were identified in an effort to characterize and isolate the most undifferentiated cells.[41] In homeostasis, these markers are usually expressed by the most basal limbal cells, but the expression of these markers may not be restricted to the basal cells when the corneal

epithelium is wounded.[42] In our study, ABCB5 staining in the limbus was not limited to the most basal layer, contrary to a previous report.[43] This marker was identified as an LESC marker[42,43] and also committed progenitor cells.[44] We hypothesize that the observed phenotype is related to an "activated limbus" in the context of CE regeneration, similar to what was reported for p63.[42] The observed phenotype could be associated with a healing phase, and it would be interesting to assess ABCB5 location after extended storage. Additionally, controversy persists over the role of limbal cells[45,46] in corneal epithelium homeostasis, so the importance of the preservation of limbal cells should be further assessed. Human corneas stored in ASM-Basic for 3 months had a stratified epithelium, which may suggest the niche was functional. We plan to study wound healing in the ASM, to test the ability of the limbus to fully regenerate CE. The present study has some limitations: (1) we did not assess regeneration at different time points (shorter or long term). It would be useful to further optimize the process, in order to analyze the minimum time allowing regeneration of a normal epithelium and the long-term survival time of the newly formed epithelium. This first study enabled selection of an effective method. In the future, we will determine the minimum time required for epithelial regeneration and whether normal epithelial structure can be maintained for several months. (2) Air-lifting can be optimized. No previous study determined an ideal air-exposure time, and this point requires further work. In humans, the blink rate is faster than the frequency we used. However, the closed ASM environment should offer increased humidity in the anterior chamber and limit desiccation. (3) We must test the ASM's utility for corneas stored at 4°C, the most common storage technique worldwide,[47] and in which the epithelium is also frequently altered.[5,34] (4) We did not assess functionally the epithelial barrier function; development of such assessment is ongoing in our laboratory. (5) The absence of corneal epithelial defects found on slices passing through the center suggests that the epithelium covers the whole cornea. However, the area covered by the epithelium should be assessed using either functional testing (e.g., fluorescein test) or en face imaging (e.g., optical coherence tomography).

Conclusion

CE stratification and differentiation of human corneas unsuitable for corneal graft can be enhanced after storage in an ASM that restores a near-physiologic environment (IOP, medium flow, epithelial air-lifting, specific CM for each side). The possible regeneration of epithelium from corneas disqualified from clinical use by eye banks could provide a new source of tissue for ocular surface research.

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References

1. Schrage N, Frentz M, Spoeler F. The Ex Vivo Eye Irritation Test (EVEIT) in evaluation of artificial tears: purite-preserved versus unpreserved eye drops. *Graefes Arch Clin Exp Ophthalmol*. 2012;250(9):1333–1340.
2. Tanelian DL, Bisla K. A new in vitro corneal preparation to study epithelial wound healing. *Invest Ophthalmol Vis Sci*. 1992;33(11):3024–3028.
3. Deshpande P, Ortega I, Sefat F, et al. Rocking media over ex vivo corneas improves this model and allows the study of the effect of proinflammatory cytokines on wound healing. *IOVS*. 2015;56(3):1553–1561.
4. Van Meter WS, Katz DG, White H, Gayheart R. Effect of death-to-preservation time on donor corneal epithelium. *Trans Am Ophthalmol Soc*. 2005;103:209–224.
5. Soni NG, Hoover CK, Silva HD, Jeng BH. Preservation of the corneal epithelium in different corneal storage media. *Cornea*. 2015;34(11):1400–1403.
6. Crewe JM, Armitage WJ. Integrity of epithelium and endothelium in organ-cultured

human corneas. *Invest Ophthalmol Vis Sci*. 2001;42(8):1757–1761.
7. Guindolet D, Crouzet E, He Z, et al. Storage of porcine cornea in an innovative bioreactor. *Invest Ophthalmol Vis Sci*. 2017;58(13):5907–5917.
8. Shetty R, Vunnava KP, Dhamodaran K, et al. Characterization of corneal epithelial cells in keratoconus. *Trans Vis Sci Tech*. 2019;8(1):2.
9. Garcin T, Gauthier A-S, Crouzet E, et al. Innovative corneal active storage machine for long-term eye banking. *Am J Transplant*. 2019;19(6):1641–1651.
10. Garcin T, Gauthier AS, Crouzet E, et al. Three-month storage of human corneas in an active storage machine. *Transplantation*. 2020;104(6):1159–1165.
11. Forest F, Thuret G, Gain P, et al. Optimization of immunostaining on flat-mounted human corneas. *Mol Vis*. 2015;21:1345–1356.
12. He Z, Forest F, Bernard A, et al. Cutting and decellularization of multiple corneal stromal lamellae for the bioengineering of endothelial grafts. *Invest Ophthalmol Vis Sci*. 2016;57(15):6639–6651.
13. Gain P, Thuret G, Kodjikian L, et al. Automated tri-image analysis of stored corneal endothelium. *Br J Ophthalmol*. 2002;86(7):801–808.
14. Deb-Joardar N, Thuret G, Racine G-A, et al. Standard microlithographic mosaics to assess endothelial cell counting methods by light microscopy in eye banks using organ culture. *Invest Ophthalmol Vis Sci*. 2006;47(10):4373–4377.
15. Deb-Joardar N, Thuret G, Zhao M, et al. Comparison of two semiautomated methods for evaluating endothelial cells of eye bank corneas. *Invest Ophthalmol Vis Sci*. 2007;48(7):3077–3082.
16. Acquart S, Gain P, Zhao M, et al. Endothelial morphometry by image analysis of corneas organ cultured at 31 degrees C. *Invest Ophthalmol Vis Sci*. 2010;51(3):1356–1364.
17. Deb-Joardar N, Thuret G, Gavet Y, et al. Reproducibility of endothelial assessment during corneal organ culture: comparison of a computer-assisted analyzer with manual methods. *Invest Ophthalmol Vis Sci*. 2007;48(5):2062–2067.
18. Haug K, Azqueta A, Johnsen-Soriano S, et al. Donor cornea transfer from Optisol GS to organ culture storage: a two-step procedure to increase donor tissue lifespan. *Acta Ophthalmol*. 2013;91(3):219–225.
19. Borderie VM, Kantelip BM, Delbosc BY, Oppermann MT, Laroche L. Morphology, histology, and ultrastructure of human C31 organ-cultured corneas. *Cornea*. 1995;14(3):300–310.
20. Crewe JM, Armitage WJ. Integrity of epithelium and endothelium in organ-cultured human corneas. *Invest Ophthalmol Vis Sci*. 2001;42(8):1757–1761.
21. Hampel U, Garreis F, Burgemeister F, Eßel N, Paulsen F. Effect of intermittent shear stress on corneal epithelial cells using an in vitro flow culture model. *Ocul Surf*. 2018;16(3):341–351.
22. Ban Y, Cooper LJ, Fullwood NJ, et al. Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting. *Exp Eye Res*. 2003;76(6):735–743.
23. Kang YG, Shin JW, Park SH, et al. Effects of flow-induced shear stress on limbal epithelial stem cell growth and enrichment. *PLoS ONE*. 2014;9(3):e93023.
24. Ren H, Wilson G. The effect of a shear force on the cell shedding rate of the corneal epithelium. *Acta Ophthalmologica Scandinavica*. 1997;75(4):383–387.
25. Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol*. 1995;268(3, pt 1):L347–L360.
26. Pruniéras M, Régnier M, Woodley D. Methods for cultivation of keratinocytes with an air-liquid interface. *J Invest Dermatol*. 1983;81(suppl 1):28s–33s.
27. Chen S, Schoen J. Air-liquid interface cell culture: from airway epithelium to the female reproductive tract. *Reprod Domest Anim*. 2019;54(suppl 3):38–45.
28. Stulting RD, Waring GO, Bridges WZ, Cavanagh HD. Effect of donor epithelium on corneal transplant survival. *Ophthalmology*. 1988;95(6):803–812.
29. Zieske JD, Mason VS, Wasson ME, et al. Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. *Exp Cell Res*. 1994;214(2):621–633.
30. Glasgow BJ, Gasymov OK, Casey RC. Exfoliative epitheliopathy of bullous keratopathy with breaches in the MUC16 Glycocalyx. *Invest Ophthalmol Vis Sci*. 2009;50(9):4060–4064.
31. Thiel M, Morlet N, Schulz D, et al. A simple corneal perfusion chamber for drug penetration and toxicity studies. *Br J Ophthalmol*. 2001;85(4):450–453.
32. Zhao B, Cooper LJ, Brahma A, MacNeil S, Rimmer S, Fullwood NJ. Development of a three-dimensional organ culture model for corneal

wound healing and corneal transplantation. *Invest Ophthalmol Vis Sci*. 2006;47(7):2840–2846.
33. Brunette I, Nelson LR, Bourne WM. A system for long-term corneal perfusion. *Invest Ophthalmol Vis Sci*. 1989;30(8):1813–1822.
34. 2016 EYE BANKING STATISTICAL REPORT. Eye Bank Association of America, DC, USA. Available at: http://restoresight.org/wp-content/uploads/2017/04/2016_Statistical_Report-Final-040717.pdf. Accessed April 7, 2019.
35. Leong Y-Y, Tong L. Barrier function in the ocular surface: from conventional paradigms to new opportunities. *Ocul Surf*. 2015;13(2):103–109.
36. Kompella UB, Kadam RS, Lee VH. Recent advances in ophthalmic drug delivery. *Ther Delivery*. 2010;1(3):435–456.
37. Mauris J, Mantelli F, Woodward AM, et al. Modulation of ocular surface glycocalyx barrier function by a galectin-3 N-terminal deletion mutant and membrane-anchored synthetic glycopolymers. *PLoS ONE*. 2013;8(8):e72304.
38. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57(2):201–209.
39. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci*. 1983;24(10):1442–1443.
40. Deng SX, Borderie V, Chan CC, et al. Global consensus on definition, classification, diagnosis, and staging of limbal stem cell deficiency. *Cornea*. 2019;38(3):364–375.
41. Schlötzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res*. 2005;81(3):247–264.
42. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA*. 2005;102(27):9523–9528.
43. Ksander BR, Kolovou PE, Wilson BJ, et al. ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature*. 2014;511(7509):353–357.
44. Liu L, Nielsen FM, Emmersen J, et al. Pigmentation is associated with stemness hierarchy of progenitor cells within cultured limbal epithelial cells. *Stem Cells*. 2018;36(9):1411–1420.
45. Dua HS, Miri A, Alomar T, Yeung AM, Said DG. The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. *Ophthalmology*. 2009;116(5):856–863.
46. Majo F, Rochat A, Nicolas M, Jaoudé GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature*. 2008;456(7219):250–254.
47. Gain P, Jullienne R, He Z, et al. Global survey of corneal transplantation and eye banking. *JAMA Ophthalmol*. 2016;134(2):167–173.