

# Silk Fibroin Films for Corneal Endothelial Regeneration: Transplant in a Rabbit Descemet Membrane Endothelial Keratoplasty

Natalia Vázquez,<sup>1</sup> Carlos A. Rodríguez-Barrientos,<sup>1</sup> Salvador D. Aznar-Cervantes,<sup>2</sup> Manuel Chacón,<sup>1</sup> José L. Cenis,<sup>2</sup> Ana C. Riestra,<sup>1</sup> Ronald M. Sánchez-Avila,<sup>1</sup> Mairobi Persinal,<sup>1</sup> Agustín Brea-Pastor,<sup>3</sup> Luis Fernández-Vega Cueto,<sup>1</sup> Álvaro Meana,<sup>1</sup> and Jesús Merayo-Llves<sup>1</sup>

<sup>1</sup>Instituto Universitario Fernández-Vega, Fundación de Investigación Oftalmológica, Universidad de Oviedo, Asturias, Spain

<sup>2</sup>Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, Departamento de Biotecnología de La Alberca, Murcia, Spain

<sup>3</sup>Unidad de Bioterio e Imagen Preclínica, Universidad de Oviedo, Asturias, Spain

Correspondence: Natalia Vázquez, Instituto Universitario Fernández-Vega, Avda. Dres. Fernández-Vega 34, Oviedo 33012, Asturias, Spain; natalia.vazquez@fio.as.

NV and CAR-B contributed equally to the work presented here and therefore should be regarded as equivalent authors.

Submitted: March 2, 2017

Accepted: May 10, 2017

Citation: Vázquez N, Rodríguez-Barrientos CA, Aznar-Cervantes SD, et al. Silk fibroin films for corneal endothelial regeneration: transplant in a rabbit Descemet membrane endothelial keratoplasty. *Invest Ophthalmol Vis Sci.* 2017;58:3357–3365. DOI:10.1167/iov.17-21797

**PURPOSE.** Develop a silk fibroin (SF)-based artificial endothelial graft for its use in a rabbit Descemet membrane endothelial keratoplasty (DMEK).

**METHODS.** Human and rabbit artificial corneal endothelial grafts were developed through the culture of human and rabbit corneal endothelial cells (CECs) on SF films. Rabbit artificial SF endothelial grafts were transplanted in a DMEK surgery into a rabbit in vivo model.

**RESULTS.** SF artificial endothelial grafts showed the characteristic endothelial markers: zonula occludens (ZO-1) and Na<sup>+</sup>/K<sup>+</sup> ATPase. In a rabbit model of DMEK surgery, SF artificial endothelial graft restored the corneal transparency and thickness at 6 week of follow-up. Anterior segment optical coherence tomography revealed the SF graft as a fully integrated component in the corneal tissue, displaying a similar corneal thickness and endothelial cell count when compared with its healthy contralateral cornea. Histologic analysis showed that the SF artificial endothelial graft was attached and integrated on the surface of the corneal stroma without a significant inflammatory reaction, and rabbit CECs consisted in a monolayer that showed their characteristic markers ZO-1 and Na<sup>+</sup>/K<sup>+</sup> ATPase, suggesting proper intercellular junctions and cellular pump function.

**CONCLUSIONS.** We have developed SF films with biological properties that supported the growth of rabbit and human CECs, which showed normal morphology and characteristic markers; and with mechanical properties that allowed its use in a DMEK surgery, proving its in vivo functionality in a rabbit model of endothelial dysfunction.

**Keywords:** silk fibroin films, biomaterials, corneal endothelial cells, DMEK

Descemet membrane endothelial keratoplasty (DMEK) and Descemet stripping automated endothelial keratoplasty (DSAEK) are two of the most used methods in the treatment of corneal endothelial dysfunction.<sup>1</sup> In these techniques, Descemet's membrane and the endothelial monolayer, or Descemet's membrane with its endothelium and a thin layer of stroma, are transplanted. These procedures have inherent advantages<sup>1,2</sup> in terms of visual recovery, final visual acuity, and rejection risk compared with penetrant keratoplasty (PK), which involves the replacement of the full thickness of the cornea. Moreover, these types of surgeries are minimally invasive compared with the current PK technique, in which the anterior chamber is exposed to the environment, avoiding the risk of ocular surface complications,<sup>3–5</sup> suture-related infections,<sup>6,7</sup> and devastating expulsive haemorrhage intraoperatively or postoperatively.<sup>8,9</sup>

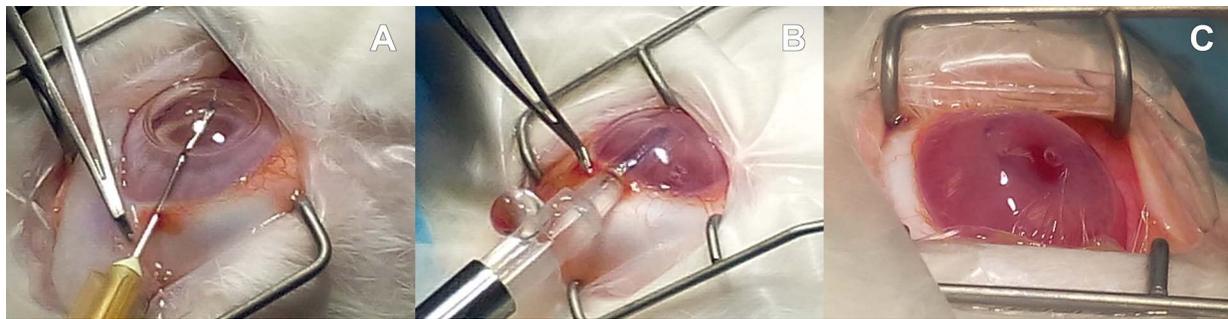
Despite that DMEK surgery is more complex in graft preparation and handling than other posterior lamellar keratoplasties,<sup>10</sup> it is the most in-demand surgical technique because it does not have the disadvantages of transferring another cell type and offers faster and more complete visual

rehabilitation than DSAEK.<sup>11–17</sup> However, as in other surgical techniques, it still requires of human donors.

The treatment of corneal pathologies using artificial endothelial grafts<sup>18</sup> or isolated endothelial cells, associated<sup>19,20</sup> or not<sup>21</sup> with the use of ROCK kinase, represents a promising future in which there would be less need of human donors when performing surgical interventions. However, cell therapy with injected corneal endothelial cells (CECs) into the anterior chamber may lead to systemic cell dispersion via aqueous flow<sup>20</sup> or could lead to secondary glaucoma if the cells obstruct the trabecular meshwork.<sup>22</sup> This inherent risk could be avoided by grafting CECs already attached to an artificial Descemet membrane.

In recent years, tissue engineering techniques have been aimed at developing new artificial endothelial grafts, which could supplant the natural endothelium, through optimization of CEC culture techniques<sup>23–25</sup> and the development of adequate scaffolds that support their growth.<sup>18,26–40</sup> These scaffolds not only have to offer an adequate environment for CEC growth, moreover, they must be able to be used in the





**FIGURE 1.** DMEK surgical procedure for SF film transplantation. Descemetorhexis from the posterior stroma (A), injecting SF films with or without rabbit CECs into the anterior chamber (B) and unfolded SF films inside the anterior chamber (C).

current minimally invasive surgical techniques while maintaining endothelial function when they are grafted.

Silk fibroin (SF), the structural protein obtained from the cocoon of the silk worm *Bombyx mori*, has been used in tissue engineering due to optical properties, nonimmunogenic response, controllable degradation rates, and tuneable and robust mechanical properties.<sup>41–48</sup> Several studies have reported SF films as an optimal scaffold for different types of cell cultures.<sup>49,50</sup> On the ocular surface, SF films also have been shown to be an optimal substrate for the culture of corneal cells.<sup>42,51,52</sup> All these facts make SF films a good candidate for their use in current surgical techniques.

In the present study, we used SF films for the development of artificial endothelial grafts. These artificial endothelial grafts were transplanted into rabbit corneas using a minimally invasive surgical technique. The tissue-engineered SF endothelial graft restored endothelial function, leading to complete recovery of the corneal transparency and thickness at 6 weeks. Finally, we show that human CECs also can be cultured on SF films, so that SF films may be an alternative for the development of corneal endothelial artificial grafts for its use in a human DMEK surgery.

## MATERIALS AND METHODS

### Preparation of the SF Films

*Bombyx mori* cocoons were obtained from silkworms reared in the sericulture facilities of the IMIDA (Murcia, Spain). Cocoons were chopped into 4 or 5 pieces and boiled in 0.02M Na<sub>2</sub>CO<sub>3</sub> for 30 minutes to remove the glue-like sericin proteins. Then raw SF was rinsed thoroughly with water and dried at room temperature for 3 days. The extracted SF was dissolved in 9.3M LiBr (Acros Organics, Geel, Belgium) for 3 hours at 60°C to generate a 20% wt/vol solution that was dialyzed against distilled water for 3 days (Snakeskin Dialysis Tubing 3.5 kDa molecular weight cutoff; Thermo Scientific, Waltham, MA, USA) with eight total water changes. The resultant 5% wt/vol SF dissolution was

recovered, filtered, and stored at 4°C for no longer than 30 days. SF films were obtained by casting 580 μL 5% wt/vol SF aqueous dissolution on a plastic Petri dish, 5.8 cm in diameter, to give a 10-μm-thick film. Once dried at room temperature, the water-annealing was performed by placing the SF films in a water-filled desiccator in vacuum conditions for 24 hours.

SF films were sterilized in 70% ethanol for 15 minutes and rinsed in a sterile solution of PBS pH 7.4 before cell seeding.

### Characterization of Mechanical and Optical Properties of the SF Films

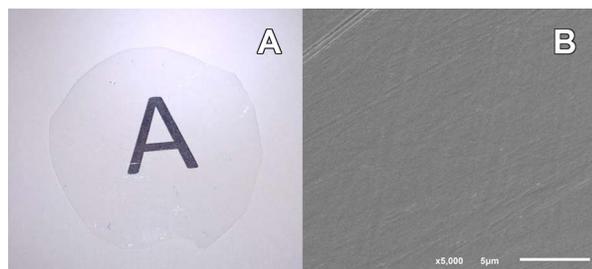
Tensile tests were performed using a universal test frame machine (Qtest; MTS Systems, Eden Prairie, MN, USA). The mechanical properties of specimens (10 × 30 mm) were recorded with a crosshead speed of 0.1 mm/s and a load cell of 5N, under ambient conditions. The thickness of each piece of film was determined with an electronic digital micrometer, with an accuracy of 1 μm. Young's modulus (MPa), ultimate strength (MPa), and elongation at rupture (%) were determined using the stress-strain curves. This studio was performed using either dry films or prewet films (in PBS 1X, for 24 hours at room temperature). Each test was performed at least three times per condition.

Light transmission measurements were made using a narrow spectral region between 400 and 700 nm using a SPELEC Spectroelectrochemical Instrument (Dropsens, Asturias, Spain) equipped with a Deuterium 215 to 400 nm and Tungsten Halogen 360 to 2500 nm light source and a linear silicon charge-coupled device array with a detection range 200 to 900 nm detector. The entire tests were carried out in duplicate at 20°C ± 0.1°C, using prewet (1X PBS for 24 hours at room temperature) 10-mm-diameter SF films.

For the statistical analyses, IBM SPSS Statistics v.22 software (IBM Corp., New York, NY, USA) was used. Data followed the normality and homogeneity of variance requirements and they were compared by means of the parametric ANOVA test ( $P < 0.05$ ).

### Cell Isolation and Culture of CECs on SF Films

CECs were cultured in Optimum I (Life Technologies, Carlsbad, CA, USA) supplemented with 8% vol/vol fetal bovine serum,



**FIGURE 2.** Macroscopic view (A) and SEM micrographs (B) of SF films.

**TABLE.** Mechanical Properties of Dry and Prewet SF Films

	Dry Films	Prewet Films
Young's modulus, MPa	346 ± 33	22 ± 2
Elongation, %	8.3 ± 1.2	23.5 ± 9.1
Ultimate strength, MPa	21.5 ± 3.3	2.1 ± 1.0

Values are shown as mean ± SD.

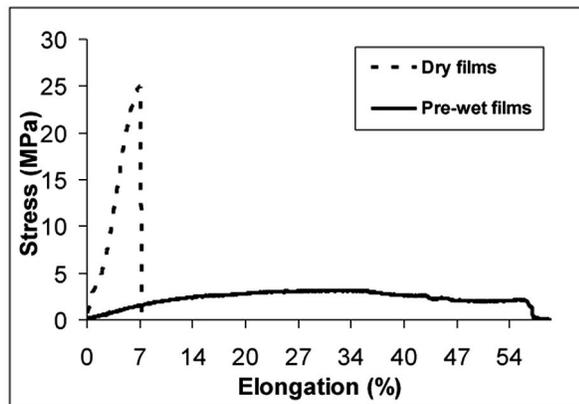


FIGURE 3. Stress-strain curves of dry and prewet SF films.

0.3 mM ascorbic acid 2-phosphate, 200 mg/L calcium chloride, 0.04% chondroitin sulfate, 10 U/mL penicillin, 10  $\mu$ g/mL streptomycin, 20 ng/mL nerve growth factor (Sigma-Aldrich Corp., St. Louis, MO, USA), and 5 ng/mL epidermal growth factor (Austral Biologicals, San Ramon, CA, USA).

**Culture of Human CECs.** Human tissue was handled according to the Declaration of Helsinki. Corneoscleral rings from corneas previously used for PK were obtained from the local Eye Bank (Centro Comunitario de Sangre y Tejidos, Oviedo, Asturias, Spain) and the Instituto Oftalmológico Fernández-Vega (Oviedo, Asturias, Spain). All tissues were maintained at 4°C in Eusol-C storage medium (Alchimia, Ponte S. Nicolò, Italy) for fewer than 10 days before use.

Corneoscleral rings were placed endothelial side up in a Petri dish containing endothelial culture medium. Descemet's membrane, along with endothelial cells, was carefully dissected under a dissecting stereomicroscope following the Schwalbe line and the peripheral endothelial ring was maintained overnight at 37°C in a culture plate (2 cm<sup>2</sup>), previously treated with FNC Coating Mix (Athena Environmental Sciences, Baltimore, MD, USA), with 1 mL culture medium. The following day, the excess medium was removed to a volume of 150  $\mu$ L and then, the peripheral endothelial ring was placed as an explant in the culture plate.

**Culture of Rabbit CECs.** Rabbit CECs were isolated from healthy male New Zealand white rabbits (2 months of age and body weight of 2.0–2.5 kg) obtained from the Animal Housing Facility of the University of Oviedo (Oviedo, Asturias, Spain). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved (PROAE 14/2014) by the Committee on the Ethics of Animal Experiments of the University of Oviedo and the Animal Production and Health

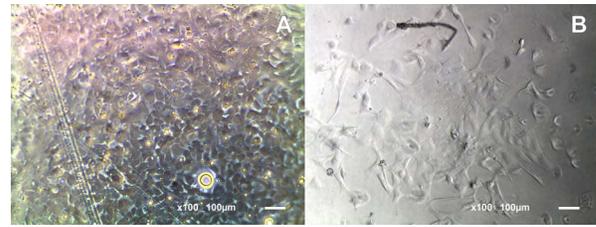


FIGURE 5. Micrographs of human CECs cultured on SF films with (A) or without (B) pretreatment with FNC Coating Mix.

Service of Asturias. Rabbits were kept under a 12/12 day/night light cycle with food and water ad libitum and were monitored on a daily basis.

Descemet's membrane along with endothelial cells were carefully peeled and digested with trypsin/EDTA 0.25% (Sigma-Aldrich Corp.) for 30 minutes at 37°C. After that, the trypsin was neutralized with culture medium. The loosened cells were centrifuged using an Eppendorf 5702R centrifuge (Eppendorf, Hamburg, Germany) at 0.4g for 10 minutes, and the supernatant was removed. Fresh medium was added and the cells were seeded on a culture plate (10 cm<sup>2</sup>) previously treated with FNC Coating Mix.

**Culture of CECs on SF Films.** When human or rabbit cultures were confluent, cells were digested with Accutase (Sigma-Aldrich). The loosened cells were centrifuged using an Eppendorf 5702R centrifuge at 0.4g for 10 minutes and the supernatant was removed. Fresh medium was added and the cells were seeded (100,000 cells/cm<sup>2</sup>; passage 1) on one of the sides of a SF film, previously treated with FNC Coating Mix, being cultured for a week in an 11-mm-diameter culture device.<sup>53</sup>

All cells were cultured under the same conditions (humidified atmosphere at 37°C, 5% CO<sub>2</sub>, medium changed three times per week).

## Examination of Cell Cultures

Cellular growth was assessed using a Leica DMIL LED phase-contrast microscope (Leica, Wetzlar, Germany); photos were taken with an attached EC3 camera (Leica).

Confluent cultures on SF films were fixed using ice-cold methanol for 10 minutes, an 8.5-mm-diameter punch was performed and used for phase-contrast microscopy, immunocytochemistry, and scanning electron microscopy (SEM).

Methanol fixed cultures were rinsed with PBS solution twice for 10 minutes and permeabilized in a PBS solution containing 0.3% Triton-X 100 for another 10 minutes. Next, the samples were incubated with primary antibody containing 10% normal goat serum (Abcam, Cambridge, UK) at 4°C overnight. Mouse Na<sup>+</sup>/K<sup>+</sup> ATPase (Millipore, Billerica, MA, USA) (1:100)

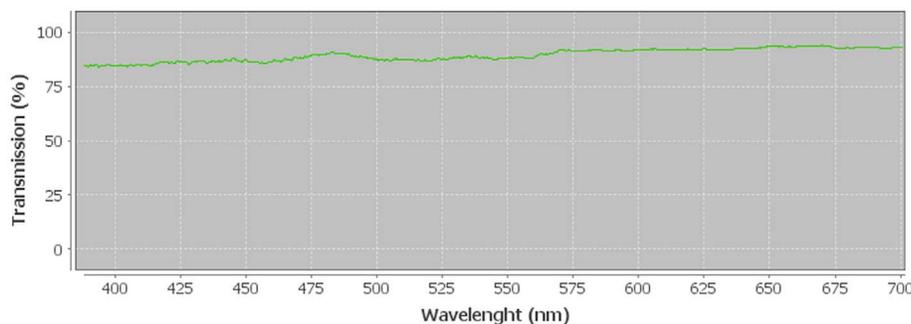


FIGURE 4. Optical properties (% light transmission) of SF films.

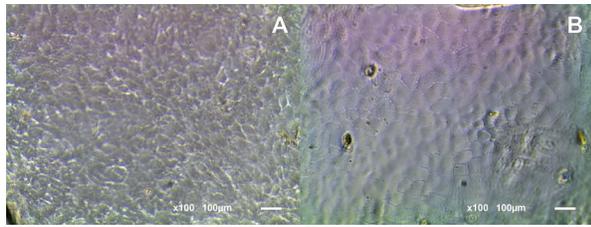


FIGURE 6. Micrographs of rabbit CECs cultured on SF films with (A) or without (B) pretreatment with FNC Coating Mix.

and rabbit zonula occludens-1 (ZO-1) (Life Technologies) (1:100) double immunostains were performed to confirm their phenotype. Subsequently, the samples were incubated with corresponding secondary antibodies (Life Technologies) (1:500) for 2 hours at room temperature. Between incubations, samples were washed three times with PBS for 10 minutes. Immunolabeled cells were stained with 4',6-diamidino-2-phenylindole to allow nuclei visualization. All the samples were examined using a Leica DM6000B fluorescence microscope.

Surface morphology of cultured and noncultured SF films was examined by SEM. Cultured and noncultured SF films fixed in ice-cold methanol were rinsed in PBS, followed by dehydration through a graded series of acetone (30%, 50%, 70%, 90%, and 100%) for 10 minutes, respectively, and dried by the critical-point method. Afterward, they were coated with gold under vacuum and observed with a JEOL 6610LV scanning electron microscope (JEOL Co., Tokyo, Japan) at 20 kV accelerated voltage.

### Transplantation of SF Films in a Rabbit Model

DMEK surgery was performed unilaterally on six New Zealand white rabbits. Rabbits were divided into three groups: rabbits transplanted with SF films with ( $n = 2$ ) or without ( $n = 2$ ) cultured rabbit CECs and rabbits with only peeled off Descemet's membranes ( $n = 2$ ).

Animals were anesthetized with buprenorphine and meloxicam, and then intubated and ventilated with isoflurane 2%. After topical administration of double anesthetic Colicursi (tetracaine 0.1% and oxybuprocaine 0.4%), a 4-mm wound incision was made at the limit of the corneoscleral tissue of each right eye with a slit knife, and the corneal endothelium was removed from the anterior chamber with a 30-gauge needle. SF films with or without rabbit CECs were cut using an 8.5-mm-diameter trephine and stained with trypan blue solution. Grafts of SF films, as a half-rolled taco with CECs facing inward, were placed in a disposable inserting set for Descemet lenticle with a small volume of viscoelastic agent (E. Janach, Como, Italy), injected and unfolded into the anterior chamber and fixed to the posterior stroma stripped of Descemet's membrane (Fig. 1). The corneoscleral wound was closed with nylon sutures and a subconjunctival dose of Trigon Depot (triamcinolone acetonide 40 mg/mL) was administered.

After transplantation, rabbits were treated with Tobradex (dexamethasone 1 mg/mL + tobramycin 3 mg/mL) and Timabak (timolol 2.5 mg/mL) eye drops twice a day during the follow-up period. The exterior appearance of rabbit eyes was monitored by taking photographs on the day of surgery, 24 hours after surgery, and once a week for the duration of the experiment. Six weeks after transplantation, corneal thickness was measured by anterior segment optical coherence tomography (AS-OCT) using an OCT CASIA SS-100 (Tomey, Erlangen, Germany) and a corneal endothelial count was performed using a specular microscope SP-2000P (TOPCON, Tokyo,

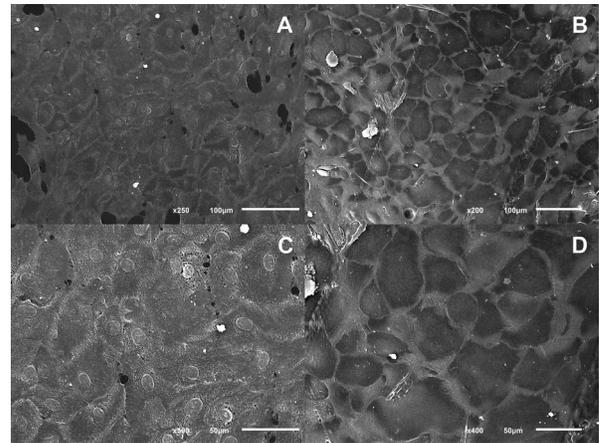


FIGURE 7. SEM micrographs of human CECs (A, C) or rabbit CECs (B, D) cultured on SF films pretreated with FNC Coating Mix.

Japan). Finally, rabbits were euthanized by an intravenous overdose of pentobarbital sodium.

Corneas were excised, rinsed with PBS solution, and fixed using ice-cold methanol for 4 hours. Corneal tissues were embedded in paraffin and then, hematoxylin-eosin stain and immunostaining against ZO-1 and  $\text{Na}^+/\text{K}^+$  ATPase was performed as described in the previous section.

## RESULTS

### Preparation of the SF Films

SF films were obtained by evaporating the SF dissolution extracted from cocoons of *Bombyx mori* after removing the glue-like sericin proteins. Fabricated films had an average area of  $3.46 \text{ cm}^2$  and thickness of  $10 \mu\text{m}$  showing a smooth surface when observed under SEM (Fig. 2).

### Characterization of Mechanical and Optical Properties of the SF Films

Average values of Young's modulus, the elongation at break, and the ultimate strength were calculated using stress-strain curves generated from tensile tests and are shown in the Table. All were found to be statistically different depending on the hydration of the film (ANOVA,  $P < 0.05$ ).

As can be observed in the Table, the average value of Young's modulus was much higher in dry films (346 MPa) than in prewet films (22 MPa), with the latter being less rigid. The elongation was improved with the hydration of the films, with average values of 8.3% and 23.5% in dry and prewet films, respectively (Fig. 3). Otherwise, the estimated ultimate

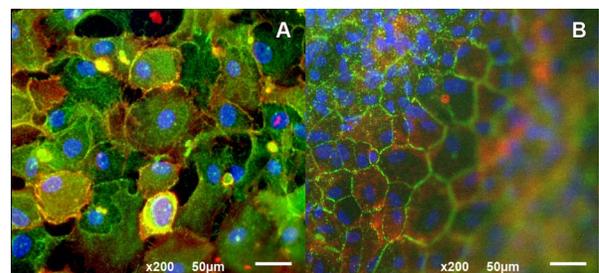


FIGURE 8. Micrographs of human (A) and rabbit (B) CECs cultured on SF films. ZO-1 (green) and  $\text{Na}^+/\text{K}^+$  ATPase (red).

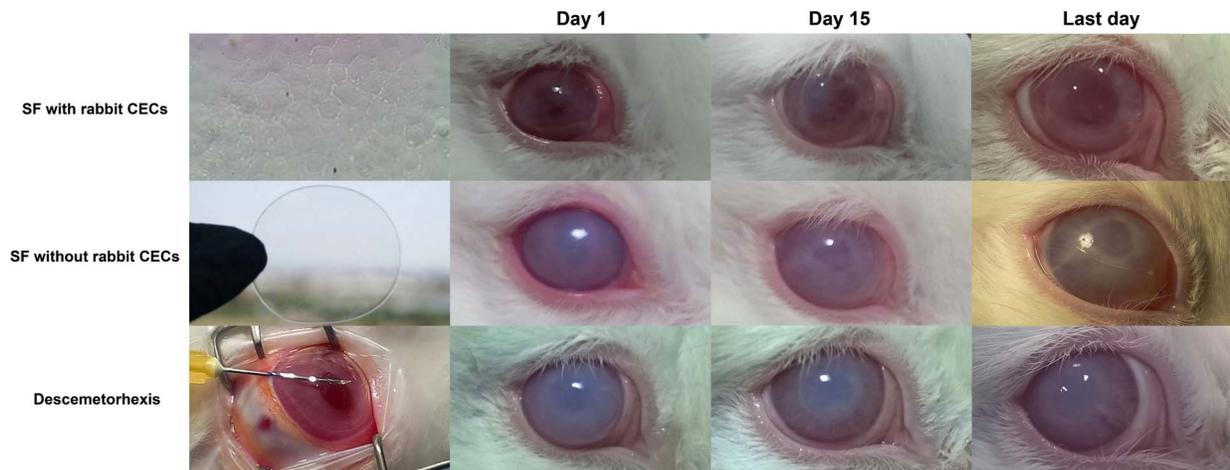


FIGURE 9. Photographs of the exterior appearance of transplanted rabbit eyes during the follow-up period. From *top to bottom*: rabbits transplanted with or without rabbit CECs cultured on SF films and rabbits with only peeled Descemet's membrane.

strength decreased from 21.5 MPa to 2.1 MPa after prewetting the films.

Optical testing revealed (Fig. 4) that SF films were optically transparent at all wavelengths of the visible light electromagnetic spectrum with a mean value of light transmission of  $89.447\% \pm 0.84\%$ .

#### Cell Isolation, Culture, and Examination of CECs on SF Films

Human CECs were observed to have migrated from the explant onto the plate after 3 to 4 days in culture, and by day 30 a monolayer of compact cells had formed on the plate immediately adjacent to the explant. These cells displayed their typical hexagonal endothelial morphology. After 30 days, confluent ( $80,000 \text{ cell/cm}^2$ ) hexagonal human CECs were detached by Accutase digestion, and subcultured for a week on SF films previously treated with FNC Coating Mix. Without this

treatment, human CECs showed signs of endothelial-mesenchymal transition with an elongated and fibroblast-like abnormal phenotype (Fig. 5).

In the same way, rabbit CECs were able to attach and proliferate until confluence ( $150,000 \text{ cells/cm}^2$ ) when isolated by trypsin/EDTA digestion. In this case, when rabbit CECs were subcultured for a week on SF films, treatment with FNC improved morphology of rabbit CECs only slightly (Fig. 6).

These results were confirmed under SEM, whereby human and rabbit CECs displayed their polygonal morphology when they were cultured on SF films previously treated with FNC Coating Mix (Fig. 7).

Immunohistochemical analysis revealed positive staining for ZO-1 and  $\text{Na}^+/\text{K}^+$  ATPase, a tight junction-associated protein and an integral membrane protein, respectively, characteristic markers of CECs and responsible for endothelial barrier and pump functions (Fig. 8).

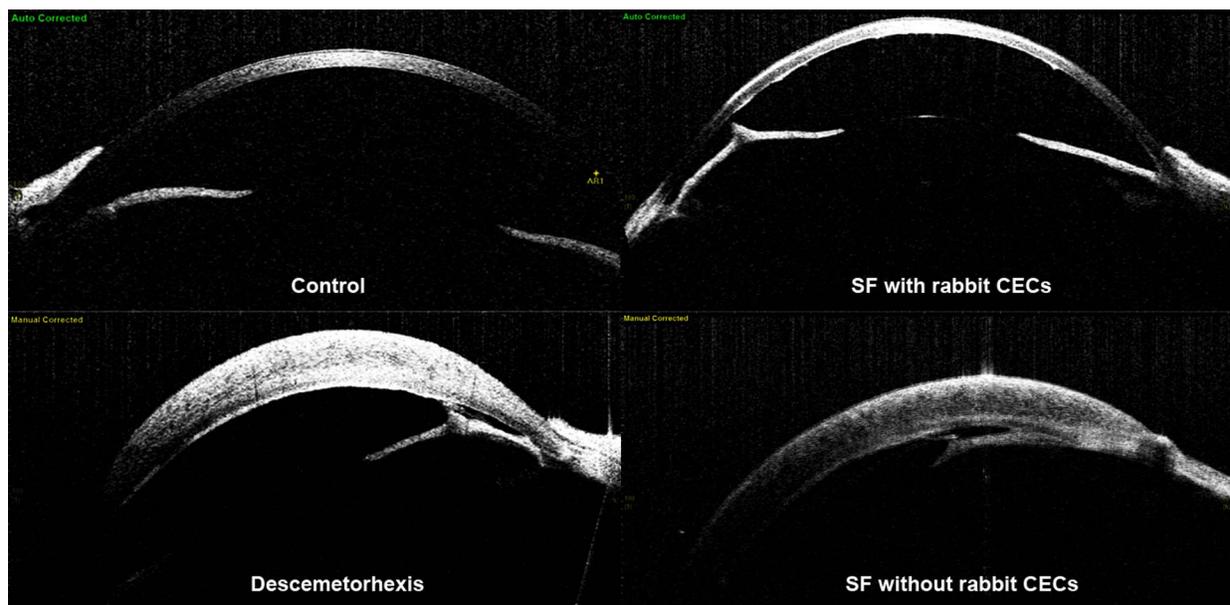


FIGURE 10. AS-OCT of control, descemetorhexis, and transplanted rabbit corneas displaying corneal thickness at 6 weeks.

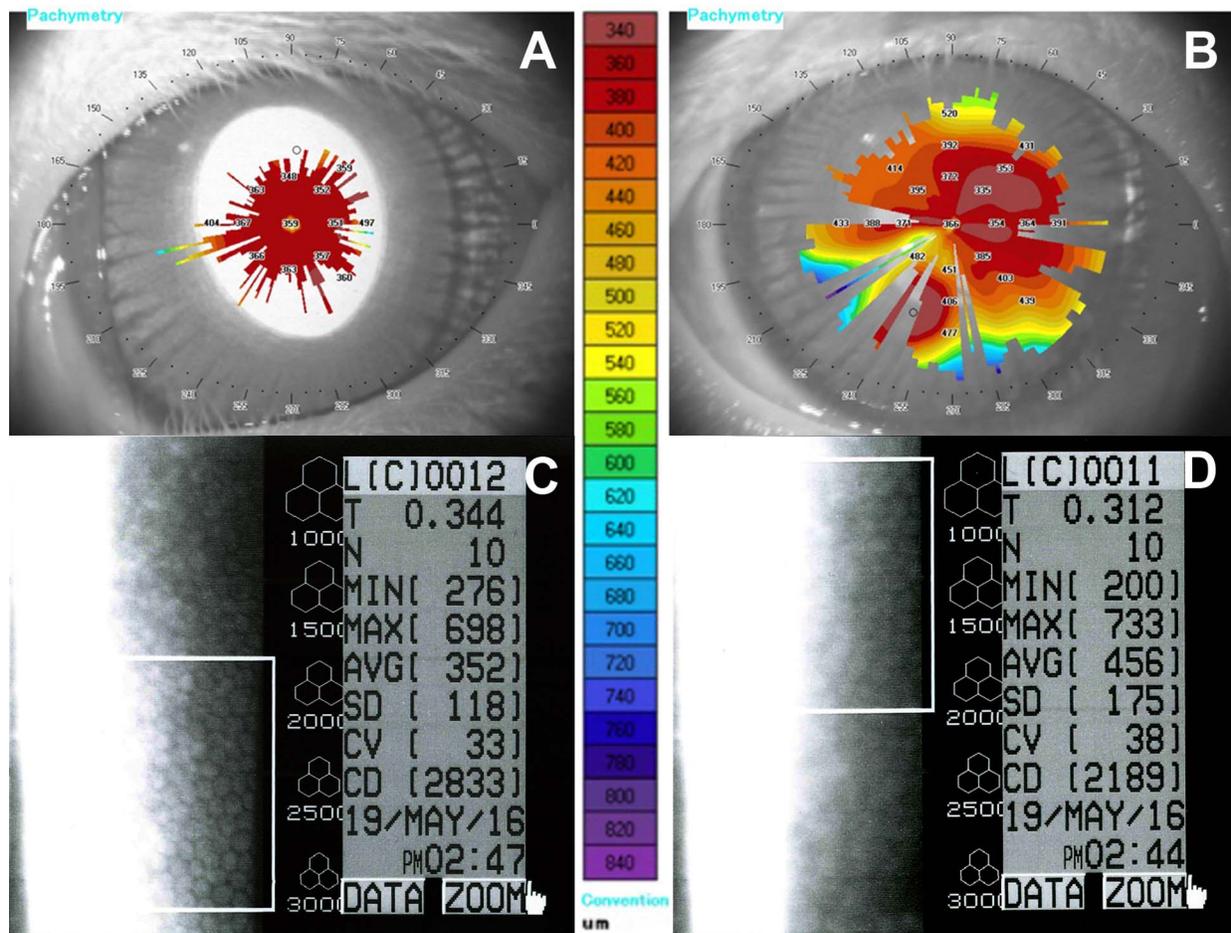


FIGURE 11. Corneal pachymetry (A, B) and CEC count (C, D) of contralateral control rabbit eye (A, C) and transplanted rabbit eye with rabbit CECs cultured on SF films (B, D).

### Transplantation of SF Films in a Rabbit Model

Corneal edema and white turbidity appeared a few days after surgery in the three groups: rabbits transplanted with or without rabbit CECs cultured on SF films and rabbits with only peeled Descemet's membrane. Corneas transplanted with cultured rabbit CECs on SF films began to become transparent by day 15, and corneal transparency was maintained up to 6 weeks. However, the eyes in the group without rabbit CECs cultured on SF films and the eyes in the group of rabbits with peeled off Descemet's membrane did not restore corneal transparency and retained the corneal edema during the follow-up period. No obvious signs of immune rejection were found in any group (Fig. 9).

Results of AS-OCT revealed SF films with cultured rabbit CECs as a fully integrated component in the corneal tissue, displaying a similar corneal thickness when compared with its healthy contralateral cornea. SF films without cultured rabbit CECs and rabbits with peeled off Descemet's membrane revealed only an enhancement in corneal thickness reflecting a loss of corneal endothelial functionality (Fig. 10).

Topography mapping of transplanted corneas revealed irregular values of peripheral corneal thickness (392–477  $\mu\text{m}$ ), whereas central cornea appeared homogeneous (366  $\mu\text{m}$ ) in corneas with cultured rabbit CECs, displaying a similar distribution when compared with its healthy contralateral cornea (359  $\mu\text{m}$ ) (Fig. 11). Moreover, the CECs count in eyes transplanted with cultured rabbit CECs on SF films had a

number of cells with a size and shape similar to the ones found in the contralateral control eye (Fig. 11).

The histologic analysis showed a slightly marked fibrotic tissue in the posterior segment of the stroma in rabbits transplanted with SF films without cultured rabbit CECs and an apparent corneal edema and fibrotic tissue in the rabbits with peeled off Descemet's membrane. SF films, in rabbits transplanted with cultured cells, was attached tightly to the corneal stroma, and rabbit CECs formed a continuous monolayer with the same morphology and phenotypical markers, ZO-1 and  $\text{Na}^+/\text{K}^+$  ATPase, as a healthy control eye (Fig. 12). No sign of endothelia remains were found in any of the transplanted corneas.

### DISCUSSION

Currently, corneal transplantation from cadaveric donors is the main alternative for the treatment of endothelial dysfunctions.<sup>2</sup> However, this procedure is limited by the shortage of high-quality donor corneas.<sup>10</sup> Due to recent advances in the field of biomaterials and the culture of CECs, the development of artificial endothelial grafts could be an alternative to traditional surgeries.

In this study, we have isolated SF from cocoons of *Bombyx mori* and manufactured it into 10- $\mu\text{m}$ -thick transparent films that support human and rabbit CEC growth. Madden et al.<sup>52</sup> were the first to prepare 5- $\mu\text{m}$  thick SF transparent films, modified with coatings of collagen IV, FNC Coating Mix, and

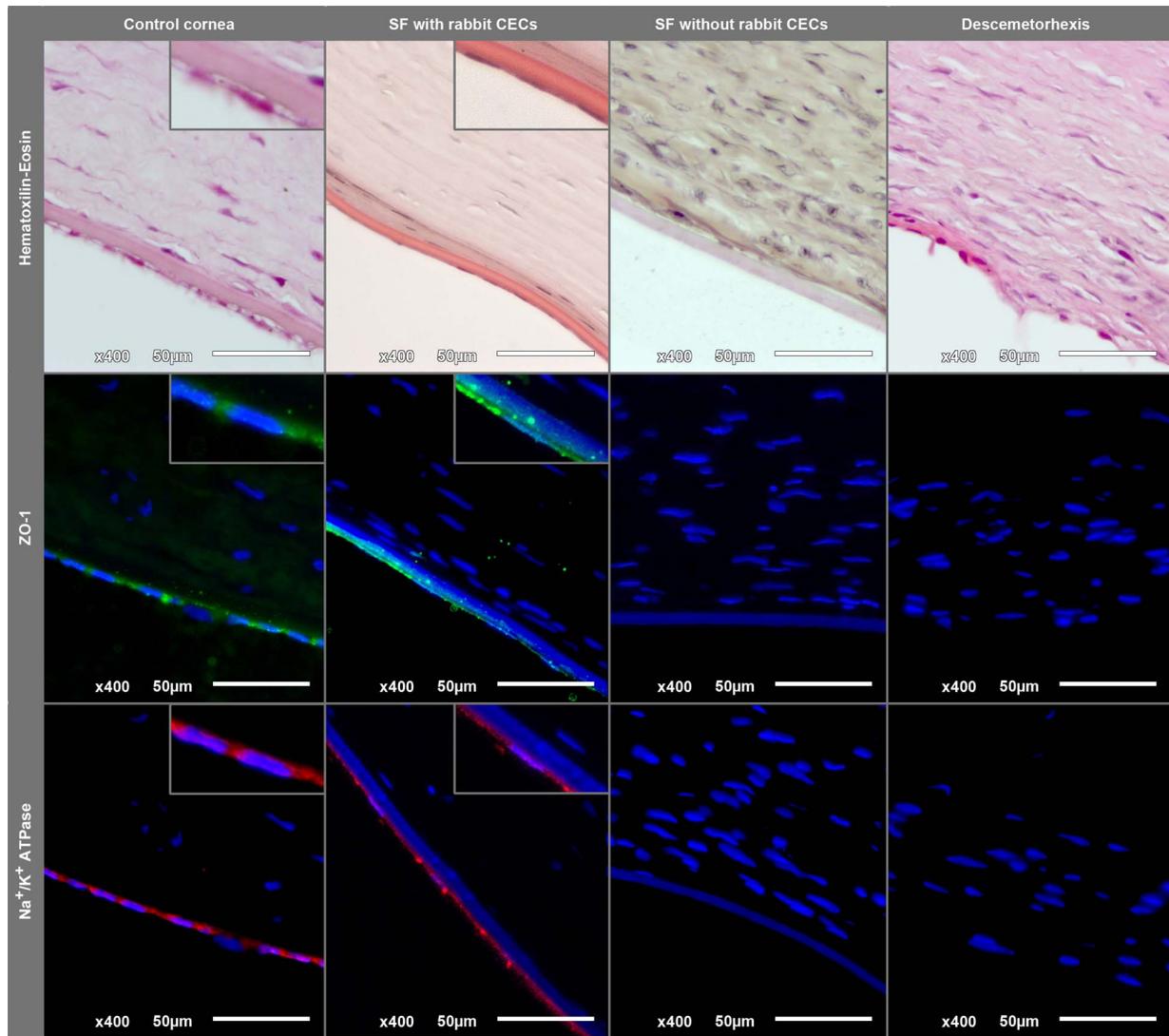


FIGURE 12. Histologic and immunofluorescent analysis after SF film transplantation.

chondroitin sulfate laminin mixture to assess cell attachment and proliferation of the B4G12 cell line and primary culture of human CECs. The group achieved successful cell confluence and proliferation with the collagen coating, concluding that SF films could be a potential substratum for the transplantation of artificial endothelial grafts for keratoplasty. In our case, the treatment of SF films with FNC Coating Mix allowed the growth of both rabbit and human CECs, maintaining the hexagonal morphology and the expression of its characteristic markers ZO-1 and  $\text{Na}^+/\text{K}^+$  ATPase.

The second outcome of this study was that SF films have mechanical properties that permit their use in a disposable inserting set for Descemet lenticle. Our SF prewet films showed a Young's modulus value of 22 MPa and an ultimate strength of 2.1 MPa. These values are similar to the values of posterior corneal stroma described in the literature<sup>54</sup> and allowed that SF films were easily handled for transplantation into rabbit eyes in a DMEK model. Our SF artificial graft was able to fold in the interior of a disposable inserting set for Descemet lenticle, and was easily introduced and unfolded in the anterior chamber in a DMEK surgical procedure, indicating that it could be used in this type of surgery. This point is important in corneal regenerative medicine because a tissue-

engineered endothelium should be able to adapt to the current surgical procedures, avoiding the need for the development of new grafting techniques, and, therefore, making it easier to implement in the clinical practice.

In our study, we established a 6-week follow-up period. Throughout this follow-up period, it was observed that transplanted corneas with cultured rabbit CECs on SF films began to become transparent by day 15 and corneal transparency was maintained up to 6 weeks. Results of AS-OCT revealed SF films with rabbit CECs as a fully integrated component in the corneal tissue, displaying a similar average corneal thickness when compared with their healthy contralateral cornea. Although CEC density was found to be lower in the rabbits transplanted with SF films with cultured CECs, the density of CECs seems adequate because it restored corneal transparency.

It is known that rabbit CECs have the ability to proliferate both *in vivo* and *in vitro*.<sup>55</sup> However, in rabbits transplanted with SF films without rabbit CECs and in rabbits with peeled off Descemet's membrane only, the cornea remained edematous during the entire follow-up period and the corneal thickness was greater than that of the healthy contralateral cornea. These findings indicate that corneal transparency, at

the 6-week follow-up period, is due to SF artificial endothelial graft and not due to spontaneous regeneration of autologous rabbit endothelium.

Histologic staining showed that the SF artificial endothelial graft was attached and integrated in the corneal stroma without a significant inflammatory reaction, and rabbit CECs consisted of a monolayer that showed their characteristic markers ZO-1 and Na<sup>+</sup>/K<sup>+</sup> ATPase, suggesting proper intercellular junctions and cellular pump function. These results confirmed that the SF artificial endothelial graft performs as expected on in vivo implantation and contributes to the maintenance of corneal clarity.

Several groups, including our own,<sup>56</sup> have already developed different artificial endothelial grafts that allow the recovery of endothelial functionality in different in vivo models.<sup>18,27,29,57,58</sup> From our point of view, SF films present several advantages, such as having better mechanical properties than other biomaterials, being a biomaterial that has been used for centuries in medicine and that could be easily accessible and have a lower cost of production than other biomaterials in some countries. However, future studies with more animals and a longer follow-up period will be necessary to elucidate the ideal biomaterial for this purpose.

Our current results still have some issues to resolve. On the one hand, the number of animals was limited, and on the other hand the differences in the size of anterior chamber and the IOP between rabbits and humans make rabbit DMEK surgery even a more complex procedure, limiting the correct placing and unfolding of the graft. In our case, pachymetry and AS-OCT revealed some irregular values of corneal thickness in the peripheral cornea. Several studies<sup>59-61</sup> have demonstrated that the presence of anomalies, such as folds or wrinkles in the graft, secondary to posterior keratoplasties, could compromise visual acuity. However, this limitation could be overcome with an appropriate handling of the graft.

In conclusion, we developed SF films with biological properties that supported the growth of rabbit CECs, which expressed its normal morphology and characteristic markers; and with mechanical properties that allowed its use in a DMEK surgery, proving its in vivo functionality in a rabbit model of endothelial dysfunction. Furthermore, clinical translation to human therapies might be feasible because, as shown, human CECs also can be cultured on SF films.

### Acknowledgments

The authors thank the diagnostic team from the Instituto Oftalmológico Fernández-Vega for their skillful technical assistance.

Supported by Programa INNPACTO (IPT-2012-1029-010000).

Disclosure: N. Vázquez, None; C.A. Rodríguez-Barrientos, None; S.D. Aznar-Cervantes, None; M. Chacón, None; J.L. Cenis, None; A.C. Riestra, None; R.M. Sánchez-Avila, None; M. Persinal, None; A. Brea-Pastor, None; L. Fernández-Vega Cueto, None; Á. Meana, None; J. Merayo-Llodes, None

### References

- Price MO, Price FW Jr. Endothelial keratoplasty - a review. *Clin Exp Ophthalmol*. 2010;38:128-140.
- Tan DTH, Dart JKG, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet*. 2012;379:1749-1761.
- Mannis MJ, Zadnik K, Miller MR, Marquez M. Preoperative risk factors for surface disease after penetrating keratoplasty. *Cornea*. 1997;16:7-11.
- Meyer RE, Bobb KC. Corneal epithelium in penetrating keratoplasty. *Am J Ophthalmol*. 1980;90:142-147.
- Thompson RW, Price MO, Bowers PJ, Price FW. Long-term graft survival after penetrating keratoplasty. *Ophthalmology*. 2003;110:1396-1402.
- Christo CG, van Rooij J, Geerards AJ, Remeijer L, Beekhuis WH. Suture-related complications following keratoplasty: a 5-year retrospective study. *Cornea*. 2001;20:816-819.
- Forstot SL, Abel R, Binder PS. Bacterial endophthalmitis following suture removal after penetrating keratoplasty. *Am J Ophthalmol*. 1975;80:509-512.
- Price FW, Whitson WE, Ahad KA, Tavakkoli H. Suprachoroidal hemorrhage in penetrating keratoplasty. *Ophthalmic Surg*. 1994;25:521-525.
- Purcell JJ Jr, Krachmer JH, Doughman DJ, Bourne WM. Expulsive hemorrhage in penetrating keratoplasty. *Ophthalmology*. 1982;89:41-43.
- Rajan MS. Surgical strategies to improve visual outcomes in corneal transplantation. *Eye (Lond)*. 2014;28:196-201.
- Guerra FP, Anshu A, Price MO, Giebel AW, Price FW. Descemet's membrane endothelial keratoplasty: prospective study of 1-year visual outcomes, graft survival, and endothelial cell loss. *Ophthalmology*. 2011;118:2368-2373.
- Guerra FP, Anshu A, Price MO, Price FW. Endothelial keratoplasty: fellow eyes comparison of descemet stripping automated endothelial keratoplasty and Descemet membrane endothelial keratoplasty. *Cornea*. 2011;30:1382-1386.
- Kruse FE, Schrehardt US, Tourtas T. Optimizing outcomes with Descemet's membrane endothelial keratoplasty. *Curr Opin Ophthalmol*. 2014;25:325-334.
- Melles GRJ, Lander F, Rietveld FJR. Transplantation of Descemet's membrane carrying viable endothelium through a small scleral incision. *Cornea*. 2002;21:415-418.
- Melles GRJ, Ong TS, Ververs B, van der Wees J. Descemet membrane endothelial keratoplasty (DMEK). *Cornea*. 2006;25:987-990.
- Price MO, Price FW. Descemet's membrane endothelial keratoplasty surgery: update on the evidence and hurdles to acceptance. *Curr Opin Ophthalmol*. 2013;24:329-335.
- Tourtas T, Laaser K, Bachmann BO, Cursiefen C, Kruse FE. Descemet membrane endothelial keratoplasty versus descemet stripping automated endothelial keratoplasty. *Am J Ophthalmol*. 2012;153:1082-1090.
- Mimura T, Yamagami S, Yokoo S, et al. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. *Invest Ophthalmol Vis Sci*. 2004;45:2992-2997.
- Okumura N, Koizumi N, Ueno M, et al. ROCK inhibitor converts corneal endothelial cells into a phenotype capable of regenerating in vivo endothelial tissue. *Am J Pathol*. 2012;181:268-277.
- Okumura N, Sakamoto Y, Fujii K, et al. Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction. *Sci Rep*. 2016;6:26113.
- Mimura T, Shimomura N, Usui T, et al. Magnetic attraction of iron-endocytosed corneal endothelial cells to Descemet's membrane. *Exp Eye Res*. 2003;76:745-751.
- Mimura T, Yamagami S, Usui T, et al. Long-term outcome of iron-endocytosing cultured corneal endothelial cell transplantation with magnetic attraction. *Exp Eye Res*. 2005;80:149-157.
- Engelmann K, Bednarz J, Valtink M. Prospects for endothelial transplantation. *Exp Eye Res*. 2004;78:573-578.
- Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci*. 2000;41:660-667.
- Yokoo S, Yamagami S, Yanagi Y, et al. Human corneal endothelial cell precursors isolated by sphere-forming assay. *Invest Ophthalmol Vis Sci*. 2005;46:1626-1631.

26. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea*. 2001;20:731-737.
27. Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci*. 2004;45:800-806.
28. Islam MM, Cepla V, He C, et al. Functional fabrication of recombinant human collagen-phosphorylcholine hydrogels for regenerative medicine applications. *Acta Biomater*. 2015;12:70-80.
29. Jumblatt MM, Maurice DM, Schwartz BD. A gelatin membrane substrate for the transplantation of tissue cultured cells. *Transplantation*. 1980;29:498-499.
30. Koizumi N, Sakamoto Y, Okumura N, et al. Cultivated corneal endothelial cell sheet transplantation in a primate model. *Invest Ophthalmol Vis Sci*. 2007;48:4519-4526.
31. Koizumi N, Sakamoto Y, Okumura N, et al. Cultivated corneal endothelial transplantation in a primate: possible future clinical application in corneal endothelial regenerative medicine. *Cornea*. 2008;27:S48-S55.
32. Lai J-Y, Chen K-H, Hsiue G-H. Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. *Transplantation*. 2007;84:1222-1232.
33. Lange TM, Wood TO, McLaughlin BJ. Corneal endothelial cell transplantation using Descemet's membrane as a carrier. *J Cataract Refract Surg*. 1993;19:232-235.
34. McCulley JP, Maurice DM, Schwartz BD. Corneal endothelial transplantation. *Ophthalmology*. 1980;87:194-201.
35. Orwin EJ, Hubel A. In vitro culture characteristics of corneal epithelial, endothelial, and keratocyte cells in a native collagen matrix. *Tissue Eng*. 2000;6:307-319.
36. Ozcelik B, Brown KD, Blencowe A, Daniell M, Stevens GW, Qiao GG. Ultrathin chitosan-poly(ethylene glycol) hydrogel films for corneal tissue engineering. *Acta Biomater*. 2013;9:6594-6605.
37. Schwartz BD, McCulley JP. Morphology of transplanted corneal endothelium derived from tissue culture. *Invest Ophthalmol Vis Sci*. 1981;20:467-480.
38. Yoeruek E, Saygili O, Spitzer MS, Tatar O, Bartz-Schmidt KU, Szurman P. Human anterior lens capsule as carrier matrix for cultivated human corneal endothelial cells. *Cornea*. 2009;28:416-420.
39. Yoshida J, Oshikata-Miyazaki A, Yokoo S, Yamagami S, Takezawa T, Amano S. Development and evaluation of porcine atelocollagen vitrigel membrane with a spherical curve and transplantable artificial corneal endothelial grafts. *Invest Ophthalmol Vis Sci*. 2014;55:4975-4981.
40. Zhang J, Sisley AMG, Anderson AJ, Taberner AJ, McGhee CNJ, Patel DV. Characterization of a novel collagen scaffold for corneal tissue engineering. *Tissue Eng Part C Methods*. 2015;22:1-28.
41. Arai T, Freddi G, Innocenti R, Tsukada M. Biodegradation of *Bombyx mori* silk fibroin fibers and films. *J Appl Polym Sci*. 2004;91:2383-2390.
42. Chirila T, Barnard Z, Zainuddin, Harkin DG, Schwab IR, Hirst L. *Bombyx mori* silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders. *Tissue Eng A*. 2008;14:1203-1211.
43. Jin HJ, Park J, Karageorgiou V, et al. Water-stable silk films with reduced beta-sheet content. *Adv Funct Mater*. 2005;15:1241-1247.
44. Meinel L, Hofmann S, Karageorgiou V, et al. The inflammatory responses to silk films in vitro and in vivo. *Biomaterials*. 2005;26:147-155.
45. Motta A, Fambri L, Migliaresi C. Regenerated silk fibroin films: thermal and dynamic mechanical analysis. *Macromol Chem Phys*. 2002;203:1658-1665.
46. Panilaitis B, Altman GH, Chen J, Jin HJ, Karageorgiou V, Kaplan DL. Macrophage responses to silk. *Biomaterials*. 2003;24:3079-3085.
47. Shao Z, Vollrath F. Materials: surprising strength of silkworm silk. *Nature*. 2002;418:741-741.
48. Vepari C, Kaplan DL. Silk as a biomaterial. *Prog Polym Sci*. 2007;32:991-1007.
49. Ghassemifar R, Redmond S, Zainuddin, Chirila TV. Advancing towards a tissue-engineered tympanic membrane: silk fibroin as a substratum for growing human eardrum keratinocytes. *J Biomater Appl*. 2010;24:591-606.
50. Wang Y, Kim HJ, Vunjak-Novakovic G, Kaplan DL. Stem cell-based tissue engineering with silk biomaterials. *Biomaterials*. 2006;27:6064-6082.
51. Chirila T, Barnard Z, Zainuddin, Harkin D. Silk as substratum for cell attachment and proliferation. *Materials Science Forum*. 2007;561-565:1549-1552.
52. Madden PW, Lai JN, George KA, Giovenco T, Harkin DG, Chirila TV. Human corneal endothelial cell growth on a silk fibroin membrane. *Biomaterials*. 2011;32:4076-4084.
53. Vazquez N, Chacón M, Meana A, et al. Keratin-chitosan membranes as scaffold for tissue engineering of human cornea. *Histol Histopathol*. 2015;30:813-821.
54. Lombardo M, Lombardo G, Carbone G, de Santo MP, Barberi R, Serrao S. Biomechanics of the anterior human corneal tissue investigated with atomic force microscopy. *Invest Ophthalmol Vis Sci*. 2012;53:1050-1057.
55. Proulx S, Brunette I. Methods being developed for preparation, delivery and transplantation of a tissue-engineered corneal endothelium. *Exp Eye Res*. 2012;95:68-75.
56. Vázquez N, Chacón M, Rodríguez-Barrientos CA, et al. Human bone derived collagen for the development of an artificial corneal endothelial graft. In vivo results in a rabbit model. *PLoS One*. 2016;11:e0167578.
57. Fan T, Ma X, Zhao J, et al. Transplantation of tissue-engineered human corneal endothelium in cat models. *Mol Vis*. 2013;19:400-407.
58. Mimura T, Amano S, Usui T, et al. Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats. *Exp Eye Res*. 2004;79:231-237.
59. Muftuoglu O, Prasher P, Bowman RW, McCulley JP, Mootha VV. Corneal higher-order aberrations after Descemet's stripping automated. *Ophthalmology*. 2010;117:878-884.e6.
60. Pantanelli SM, Sabesan R, Ching SST, Yoon G, Hindman HB. Visual performance with wave aberration correction after penetrating, deep anterior lamellar, or endothelial keratoplasty. *Invest Ophthalmol Vis Sci*. 2012;53:4797-4804.
61. Seery LS, Nau CB, McLaren JW, Baratz KH, Patel SV. Graft thickness, graft folds, and aberrations after descemet stripping endothelial keratoplasty for fuchs dystrophy. *Am J Ophthalmol*. 2011;152:910-916.