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Acceleration of Regeneration of the Corneal Endothelial Layer After Descemet Stripping Induced by the Engineered FGF TTHX1114 in Human Corneas in Organ Culture

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Purpose: Descemet stripping only (DSO, descemetorhexis without endothelial keratoplasty) is increasing in clinical use but can impose long recovery times. The objective of this research was to determine whether TTHX1114, an engineered analog of FGF1, could accelerate healing in corneas after DSO.

Methods: Corneas obtained from eye banks were placed into suspension culture and subjected to DSO with a procedure comparable with that used clinically. The healing of the stripped area and the regeneration of the corneal endothelial cell (CEC) layer were evaluated intermittently for 14 days using trypan blue staining, alizarin red staining, and immunohistochemistry.

Results: Corneas subjected to DSO showed about 30% of the stripped area healed after 14 days in culture while those treated with TTHX1114 healed 81%. The healed area was similar in both normal corneas and corneas judged by the eye banks to be dystrophic. The regeneration of the endothelial layer in the stripped area was substantially more complete in TTHX1114-treated corneas, most of which demonstrated a contiguous monolayer of CECs expressing ZO-1 at the cell–cell junctions. In corneas not subject to DSO, incorporation of EdU, a marker of proliferation, was stimulated by TTHX1114 treatment.

Conclusions: The corneal organ culture model recapitulated clinical observations of DSO, only with much more rapid recovery. Within the immediate postsurgical time frame of 2 weeks, treatment with TTHX1114 stimulated near-total regeneration of the CEC layer, suggesting that TTHX1114 may be useful as an adjunct to DSO.

Corneal endothelial dystrophies, including Fuchs endothelial corneal dystrophy (FECD), are characterized by progressive loss of corneal endothelial cells (CECs).[1–3] Corneas that have lost sufficient CECs lose the ability to pump fluid out of the cornea leading to edema, corneal opacity, and loss of vision. The only therapeutic option for such patients is transplantation of healthy endothelial cells from a normal donor, and tens of thousands of such surgeries are performed every year.[4,5] More recently, stripping of only the central 4 mm of Descemet membrane without transplantation (Descemet stripping only or DSO) has been successful in selected patients. The success of DSO depends on the combined excision of a small central region of Descemet membrane, selection of patients with suitable peripheral CEC reserves, and regeneration of the central endothelium from the peripheral CECs. Suitable patients must have a healthy peripheral endothelial population for regeneration to be successful, and the failure rate of DSO is substantial compared with conventional Descemet membrane endothelial keratoplasty/Descemet stripping endothelial keratoplasty (DMEK/DSEK). In addition, DSO leaves patients with poor vision immediately after surgery, requiring weeks to months for a return to 20/40 or better vision.[6,7] A treatment that stimulates the regeneration of CECs, including both proliferation and migration to reconstruct the normal CEC barrier layer, has the potential to improve outcomes after DSO.

Although CECs do not seem to proliferate in vivo, there are several lines of evidence arguing that they retain the capacity to do so (reviewed in Ref. 8). When removed from patients and cultured as dissociated cells in vitro, CECs can proliferate[9–11] and retain function as shown by their ability to restore the normal endothelial pump mechanism when transplanted back into patients with FECD.[12] CECs in intact corneas express proliferative markers such as Ki-67 in a small subpopulation of cells.[13] CECs at the edge of the wounded area of a cornea in organ culture have increased Ki-67 expression,[14,15] suggesting that wounding of the endothelial layer can stimulate proliferation.

Pharmacological stimulation of CEC proliferation and migration while preserving normal CEC function and

phenotype would provide the equivalent of transplantation without the potential for rejection/surgical mishaps, the need for steroids with their concomitant adverse effects, or the high cost and production issues of transplantation or cell therapy.[16,17] Fibroblast growth factors (FGFs) are endogenous stimulators of CECs that are used as a standard component of CEC culture media and are both protective[18] and potently mitogenic for CECs.[19–24] FGFs stimulate CEC migration and corneal wound healing in vitro and can accelerate the recovery of the endothelial layer from wounding in vivo,[25,26] thus making them candidates for regenerative therapy.

Dissociated cell culture and animal models, although useful, are imperfect reflections of human responses in vivo. Primary or immortalized endothelial cell cultures may not accurately reflect the environment in the intact cornea, where cell–cell contact and the influence of the extracellular matrix, Descemet membrane, and stroma may be important regulators of CEC growth.[27,28] Culturing of CECs has been shown to change their protein expression profile including that of growth factors and their receptors.[29–31] CECs grow readily in dissociated cell culture[8] but do not proliferate meaningfully in organ culture,[32] demonstrating that the regulation of proliferation in these 2 environments is substantially different. Organ-cultured corneas retain function and can be successfully used as donor tissue for transplantation.[33] CECs in organ culture also retain functionality including intact barrier and pump functions as reflected by appropriate ZO-1 and Na^+,K^+-ATPase staining[34] and maintained corneal fluid balance when cultured in a bioreactor.[32] The differences between dissociated cell culture and organ culture make it important to investigate the regulation of proliferation and functional integrity of CECs in organ culture, which may be more representative of the in vivo condition.

Given the known effects of FGFs on wound healing and regeneration and the activity of FGFs in various corneal tissues, this study sought to explore whether an engineered FGF, TTHX1114, could accelerate endothelial wound healing in a corneal organ culture model. FGF1 and stabilized derivatives of it are known to accelerate dermal wound healing.[35] TTHX1114 is a derivative of FGF1 that has been modified to increase stability and biological half-life by the introduction of an internal disulfide bond (through the A66C mutation) and the elimination of unpaired cysteines (through the C16S and C117V mutations).[36] TTHX1114 has been shown to ameliorate chemical injury in rabbit corneal epithelial cells[37] and stimulate proliferation of rabbit and human CECs.[38] This study demonstrates that TTHX1114 accelerates the healing and regeneration of the CEC layer in human corneas including those that show signs of FECD. This accelerated healing was a function of both increased migration of CECs at the wound edge into the wound and the proliferation of CECs. Endothelial wound healing stimulated by TTHX1114 was not accompanied by expression of αSMA, indicating that endothelial–mesenchymal transition and scarring are not induced by drug treatment. A drug such as TTHX1114, if able to stimulate the proliferation of CECs in vivo, would have potential utility in the treatment of endothelial dystrophies including FECD.

MATERIALS AND METHODS

Ten (10) pairs of normal human research corneas and 17 pairs with guttae and other signs of dystrophy observed by specular microscopy were purchased from the Lions Eye Institute for Transplant and Research (Tampa, FL), Advancing Sight Network (Birmingham, AL), Eversight Eye Bank (Chicago, IL), Lions VisionGift (Portland, OR), Georgia Eye Bank (Atlanta, GA), San Diego Eye Bank (San Diego, CA), and OneLegacy Eye Bank (Los Angeles, CA). Donor information is listed in Table 1. Corneas were removed from Optisol, rinsed in 1× phosphate-buffered saline (PBS), and trimmed to leave a 1- to 2-mm scleral ring. Whole corneas were then incubated at 37°C with 6% CO_2 for 24 hours submerged in Opti-MEM (Invitrogen, Carlsbad, CA) supplemented with 1× insulin, transferrin and selenium (Corning, Ithaca, NY), 1× antibiotic/antimycotic (Thermo Scientific, Waltham, MA), 0.02 mg/mL $CaCl_2$ (Amresco, Solon, OH), and 0.2 mg/mL ascorbic acid (Sigma, St. Louis, MO) with 8% heat-inactivated fetal bovine serum (FBS, Thermo Scientific). After 24 hours, corneas were removed from the medium and rinsed in 1× PBS before lesioning. Lesions were created by pressing a 4-mm biopsy punch (Sklar Industries, West Chester, PA) onto the center of the cornea and using a sharp 30-gauge needle (BD, Franklin Lakes, NJ) to lift the edges of Descemet membrane along the perimeter of the punched area. A Sinskey hook and forceps were then used to strip the central 4 mm of Descemet, making sure not to peel any of the membrane outside of the lesion, similar to the methods of Soh et al.[17] Corneas were stained with trypan blue (Invitrogen) for 30 seconds, rinsed in 1× PBS containing calcium and magnesium, and imaged under a Nikon SMZ1270 dissecting microscope (Nikon, Tokyo, Japan). Excess fluid was removed before imaging by gently blotting corneas on the side of a petri dish, but a small amount of remaining liquid created a circular meniscus visible in most images. The left cornea from each pair was cultured in the above medium containing 0.8% FBS, and the right cornea cultured in the same medium supplemented with 100 ng/mL TTHX1114. Corneas were incubated at 37°C with 6% CO_2 for 14 days with daily media changes. 10 μM 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen) was added to the medium on day 12 for a 48-hour incubation. Trypan blue staining was repeated on days 3, 6, 9, 12, and 14 using forceps when necessary to maintain a centered and upright position that could be reproduced across all time points. On day 14 after trypan staining, corneas were stained with 5% alizarin red (Sigma) in 0.9% saline (pH unadjusted) for 2 minutes, then rinsed twice in PBS for 5 minutes before imaging.

After trypan and alizarin staining was complete, corneas were fixed for 30 minutes in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for immunohistochemistry. Cells were permeabilized for 5 minutes in 1× PBS containing 1% Triton X-100 (MP Biomedicals, Santa Ana, CA), then rinsed 3 times in 1× PBS. Corneas were blocked for 1 hour at 37°C in 1× PBS containing 2% bovine serum albumin (Fisher Scientific, Pittsburgh, PA) and 2% goat serum (Sigma), then incubated in the same blocking solution containing 2.5 μg/mL primary antibody mouse anti-

TABLE 1. Donor Information

Eye Bank Designation	Patient ID #	Eye	Age	Race	Sex	Days Stored in Optisol	Death to Collection Interval (h:min)	Cell Density (Cells/Mm²)	Cause of Death
Dystrophic	W411120201147	L	68	White	F	7	16:18	1859	Stroke
	W411120201147	R	68	White	F	7	16:18	1791	
	W403420063684	L	68	White	F	11	7:15	2506	Heart attack
	W403420063684	R	68	White	F	11	7:15	2463	
	W403420063585	L	65	Black	F	12	17:16	2347	Heart attack
	W403420063585	R	65	Black	F	12	17:16	2315	
	W411120201219[1]	L	46	White	F	13	14:48	2049	End-stage liver disease
	W411120201219	R	46	White	F	13	14:48	2083	
	W411120201255	L	67	White	M	7	8:00	1471	Multisystem organ failure
	W411120201255	R	67	White	M	7	8:00	1815	
	W404320000811[1]	L	63	White	F	12	4:36	2212	Ruptured aneurysm
	W404320000811	R	63	White	F	12	4:36	2294	
	W411120201506	L	77	White	M	7	11:54	1168	Cardiogenic shock
	W411120201506	R	77	White	M	7	11:54	2278	
	W406820000878	L	50	White	M	9	8:01	1292	Probable heart disease
	W406820000878	R	50	White	M	9	8:01	891	
	W407920036683	L	39	White	M	7	21:39	837	Not available
	W407920036683	R	39	White	M	7	21:39	1590	
	W411120201565	L	77	White	F	4	12:06	2404	CHF versus pleural effusion
	W411120201565	R	77	White	F	4	12:06	2545	
	W406820000904	L	73	White	F	13	4:30	1387	Probable heart attack
	W406820000904	R	73	White	F	13	4:30	1376	
	W407918065525[5]	L	54	Black	M	6	13:10	2146	Heart attack
	W407918065525[5]	R	54	Black	M	6	13:10	2315	
	W407918075831[5]	L	66	White	F	13	20:00	2045	Stroke
	W407918075863[5]	L	70	White	F	11	5:13	1287	Respiratory failure
	W407918075863[5]	R	70	White	F	11	5:13	1443	
	W407918076461[5]	L	59	White	F	8	5:56	NA	Lung cancer
	W407918076461[5]	R	59	White	F	8	5:56	NA	
	W407918098349[5]	L	31	Black	F	5	6:00	NA	Intracerebral hemorrhage
	W407918098349[5]	R	31	Black	F	5	6:00	NA	
	W407919004288[5]	L	66	White	M	7	15:11	NA	Subarachnoid hemorrhage
	W407919004288[5]	R	66	White	M	7	15:11	NA	
Normal	W407920001118	L	44	White	M	12	22:14	2723	Lung cancer
	W407920001118	R	44	White	M	12	22:14	3008	
	W407920001685	L	63	White	F	8	18:37	1832	Not available (NA)
	W407920001685	R	63	White	F	8	18:37	2008	
	W407920001364	L	71	White	M	11	16:15	2267	COPD exacerbation
	W407920001364	R	71	White	M	11	16:15	2002	
	W412920004905[1]	L	76	NA[2]	NA[3]	9	17:32	2375	Pneumonia
	W412920004905[1]	R	76	NA[2]	NA[3]	9	17:32	2747	
	W412920004908	L	60	NA[2]	NA[3]	7	11:16	2439	NA
	W412920004908	R	60	NA[2]	NA[3]	7	11:16	2890	
	W412920004914	L	34	NA[2]	NA[3]	4	6:18	3279	NA
	W412920004914	R	34	NA[2]	NA[3]	4	6:18	3289	
	W414220036516	L	49	NA[2]	F	10	2:20	3086	Stroke
	W414220036516	R	49	NA[2]	F	10	2:20	3322	
	W414220039270	L	18	NA[2]	M	6	5:25	3257	NA
	W414220039270	R	18	NA[2]	M	6	5:25	3215	
	W404220037435	L	54	NA[2]	M	14	9:17	2890	Metastatic colon cancer
	W404220037435	R	54	NA[2]	M	14	9:17	2933	
	W414220037949	L	57	NA[2]	F	11	11:57	2571	NA

TABLE 1. (*Continued*) Donor Information

Eye Bank Designation	Patient ID #	Eye	Age	Race	Sex	Days Stored in Optisol	Death to Collection Interval (h:min)	Cell Density (Cells/Mm²)	Cause of Death
	W414220037949	R	57	NA[2]	F	11	11:57	2481	
	W407921013965[4]	L	69	White	M	9	9.98	2838	Coronary artery disease
	W407921013965[4]	R	69	White	M	9	9.98	2936	
	W407921012676[4]	L	67	Black	M	3	8.33	3103	Overdose
	W407921012676[4]	R	67	Black	M	3	8.33	3385	

(1) not trimmed because the scleral ring is already too small; (2) eye bank does not report race data; (3) eye bank does not report sex data; (4) featured in confocal images only, data excluded from statistical analysis; and (5) used for EdU quantitation only.

ZO-1 (clone 1A12, Thermo Scientific) or 5 μg/mL primary antibody mouse anti-αSMA for 60 minutes at 37°C. Corneas were rinsed 3 times in 1× PBS containing 0.05% Tween-20 (Sigma). The EdU Click-iT reaction with Alexa Fluor 488 and subsequent staining with Hoechst 33342 (Invitrogen) was performed as described previously.[38] Corneas were rinsed in 1× PBS containing 0.05% Tween-20 for 30 minutes, incubated in blocking solution containing 2 μg/mL Alexa Fluor 555-labeled goat anti-mouse IgG (Thermo Scientific) and 1 unit/mL FITC-phalloidin (Invitrogen) for 60 minutes at 37°C, and then rinsed again 3 times in 1× PBS containing 0.05% Tween-20 for 30 minutes.

Corneas were flat mounted in VECTASHIELD H-1000 (Vector Laboratories, Burlingame, CA) and imaged using a Nikon A1 confocal microscope at the UCSD Nikon Imaging Center. The edges of the descemetorhexis and the border between the stripped and unlesioned areas were visualized using the low level of autofluorescence of Descemet visible under the UV channel; however, this feature is lost when removing background noise during image processing.

Trypan images at each time point were analyzed using ImageJ software (National Institutes of Health Bethesda, MD). Stained areas were measured using color thresholding, and the limbus was traced using the oval selection tool to determine the area of the total area of the cornea. The lesion area was quantified by calculating the percentage of the stained area in relation to the total area of the cornea. For statistical analyses, *t*-tests were performed using the data analysis package in Excel and nonparametric tests were performed using GraphPad Prism (version 9.0.0, GraphPad, San Diego, CA).

A subset of the corneas judged by the eye bank to be dystrophic were transverse sectioned and stained with H&E at the end of the study, and the presence of guttae in Descemet membrane confirmed in these samples. Experiments in quartered corneas were performed as described previously.[38] In brief, donor corneas separated from those used in the DSO experiments described above were cut into quarters using a scalpel. Quarters were incubated in the same culture media containing 0.4% FBS and 10 μM EdU with and without of 100 ng/mL TTHX1114 for 48 hours. Total cells and cells incorporating EdU were counted in images of corneal quarters taken on a Nikon E400 epifluorescence microscope. The mid and edge zones were evaluated separately by capturing 3 nonadjacent fields in the undisturbed center of the quarter at least 3 fields (~1–1.2 mm) away from the cut edges, corresponding to an area that would be just peripheral to the stripped area in the whole corneas above. The edge evaluation similarly captured 3 nonadjacent fields at or within 3 fields of the cut edge of the cornea. Two quarters were cultured in each condition for a total n of 6. Fields sampled typically contained between 100 and 400 cells in the mid zone, where the edge zone was on average less dense and more variable, counts ranging from 10 to 500 cells.

RESULTS

Enhanced Endothelial Wound Healing After DSO is Stimulated by TTHX1114

To test whether TTHX1114 could stimulate corneal endothelial wound healing, 10 pairs of normal research corneas were obtained from eye banks and placed into culture. The 4-mm central area of Descemet membrane was surgically removed, simulating the clinical DSO procedure, and healing of the descemetorhexis was followed by staining the corneas with trypan blue and quantitating the stained area. Over 14 days of culture, control corneas healed over 32% ± 11% of the wound area while corneas treated with 100 ng/mL TTHX1114 showed 81% ± 15% healing ($P < 0.001$, paired *t* test) (Fig. 1). Healing of the descemetorhexis generally proceeded from the edge of the wound toward the center, and in many corneas, small islands of trypan staining could be seen in the stripped area even after 14 days of recovery. All corneas developed progressive edema with folding of the endothelial layer.

This experiment was repeated using corneas that were judged to be dystrophic by the eye bank that supplied the corneas. The criteria for judging a cornea to be dystrophic included the presence of guttae and reduced CEC density by specular examination. Corneas judged to be dystrophic used in this study had a mean CEC density of 1864 versus a CEC
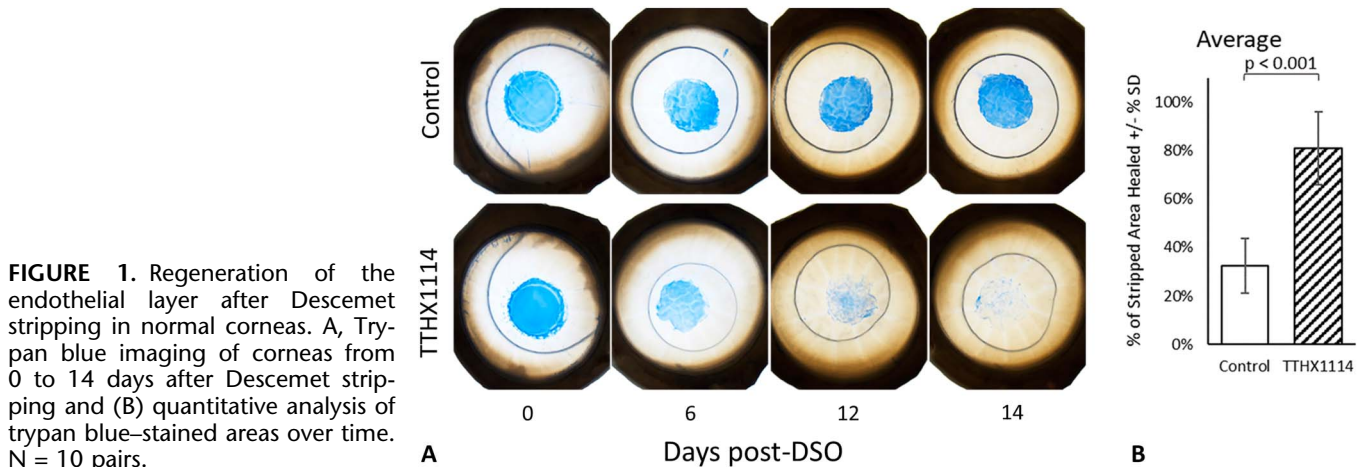
FIGURE 1. Regeneration of the endothelial layer after Descemet stripping in normal corneas. A, Trypan blue imaging of corneas from 0 to 14 days after Descemet stripping and (B) quantitative analysis of trypan blue–stained areas over time. N = 10 pairs.

density of 2731 in the normal cornea group ($P < 0.005$, t test). In all cases, both OD and OS corneas were judged dystrophic independently. In addition, several of these corneas were examined using H&E staining of transverse sections and the presence of guttae on Descemet membrane was confirmed (data not shown).

In 11 pairs of dystrophic corneas, healing of the descemetorhexis was remarkably similar to the normal corneas (Fig. 2), with control corneas healing over 38% ±23% of the wound area and TTHX1114-treated corneas healing 91% ±18% at 14 days ($P < 0.001$, paired t test).

Donor information characteristics (Table 1) of both normal and dystrophic corneas were analyzed to identify potential correlations between any of these variables and the percent healing on day 14. Age, days stored in Optisol, death to collection interval, and cell density were compared against healing using Pearson correlation coefficient (r), whereas sex was evaluated using an unpaired t test comparing male and female healing. The absolute value of r in all cases was below 0.25, suggesting that any correlations between these factors and healing are negligible. The t test showed no significant difference between healing in corneas from male and female

donors ($P = 0.53$). Race was not investigated because this information was not available for many of the corneas.

The accelerated healing of Descemet wounds in the presence of TTHX1114 is consistent with stimulation of migration of the CECs by FGFs previously reported in culture[39] and in vivo.[26] However, an important component of wound healing is the proliferation of CECs, especially in FECD where they may not have the same regenerative capacity as that in normal corneas. Therefore, using a previously established methodology,[38] we quantitated the incorporation of EdU in CECs both at the wound edge and in the intact CEC monolayer in quartered corneas judged by eye banks to be dystrophic. EdU incorporation in the undisturbed monolayer (mid zone) of dystrophic corneas is very low, with few or no EdU-incorporating cells in many fields (Fig. 3). EdU incorporation is seen in more cells near the wound edge. In quarters incubated with TTHX1114, more cells incorporated EdU both in the mid zone and near the wound edge (Figs. 3, 4). EdU-incorporating cells were found proximal to guttae, suggesting that guttae do not have a local inhibitory effect on CEC proliferation. Quantitation of EdU incorporation (Fig. 4) showed that the percentage of cells incorporating EdU was low and highly variable in the mid zone of the
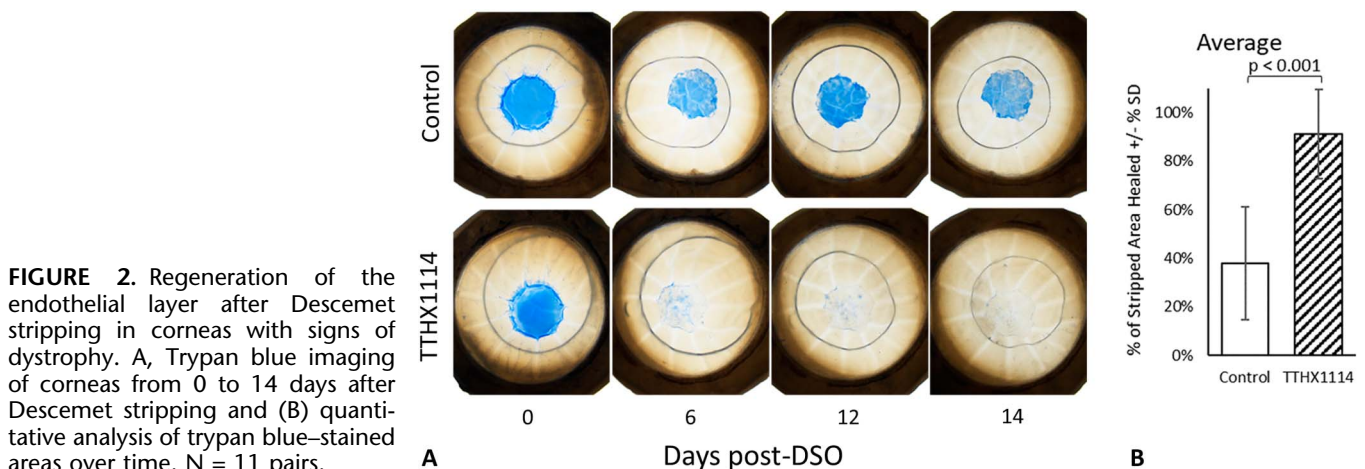

FIGURE 2. Regeneration of the endothelial layer after Descemet stripping in corneas with signs of dystrophy. A, Trypan blue imaging of corneas from 0 to 14 days after Descemet stripping and (B) quantitative analysis of trypan blue–stained areas over time. N = 11 pairs.
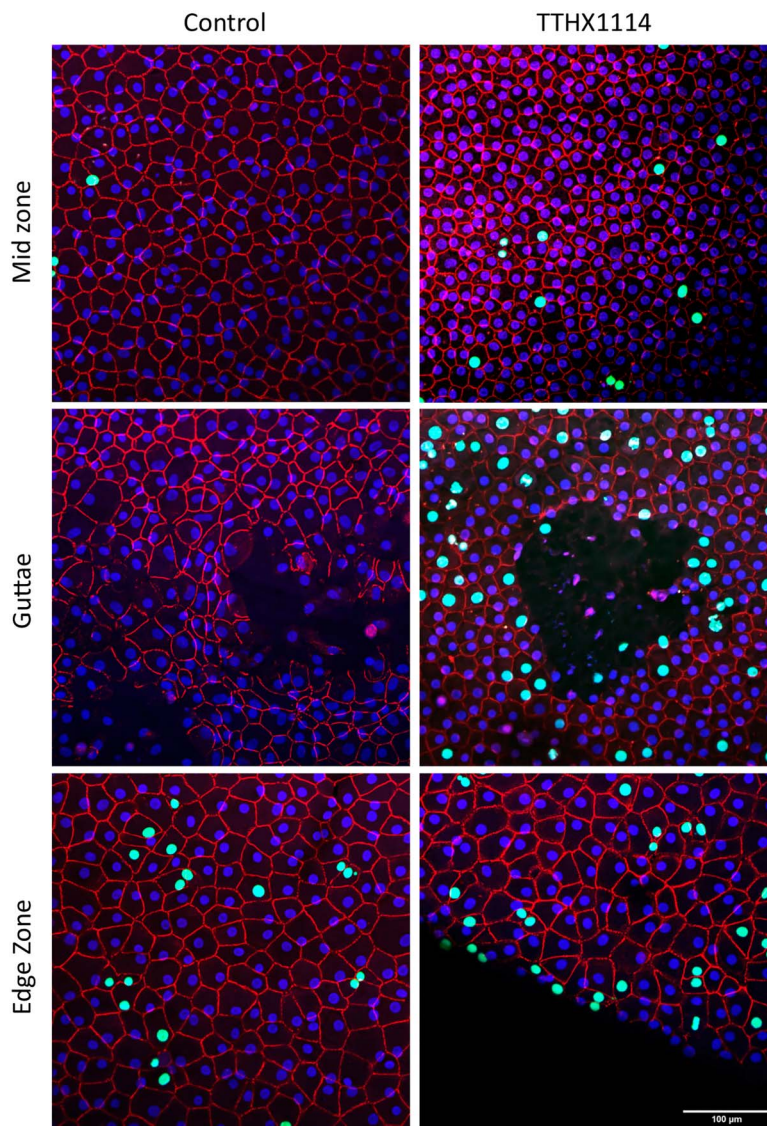
FIGURE 3. TTHX1114 stimulates EdU incorporation in CECs of corneas with signs of dystrophy. Confocal images of the endothelial layer in intact and wounded areas of corneas cultured for 48 hours with or without TTHX1114 in the presence of EdU. Blue, Hoechst; red, ZO-1; green, EdU.

cornea, with a percentage of EdU-incorporating cells of 0.9% ±1.3% (mean ± SD, range 0%–3.4%) in the absence of stimulation. EdU incorporation in the mid zone is increased to 4.4% ±6.9% (range 0%–22%) when treated with TTHX1114. Higher proliferation is observed at the wound edge, with 14% ±18% (range 0.3%–52%) in control quarters and 16% ±16% (range 0.1%–54%) in TTHX1114 treated (Fig. 4). Because of the high variability within each group, the differences between the control and TTHX1114 treatments were not statistically significant (paired *t* test *P*= 0.098 in the mid zone and *P* = 0.063 at the wound edge), although the difference between the mid zone and the edge was significant (*P* = 0.036) as was the comparison of control to TTHX1114-treated quarters when mid and edge were pooled (*P* = 0.012, paired *t* test). There were a large number of observations with zero proliferating cells, particularly in the mid zone of dystrophic corneas not exposed to TTHX1114. There were also some corneas with much larger numbers of proliferating cells, suggesting the possibility that the data are not normally distributed. A statistical analysis was performed using the nonparametric analog of the *t* test, the Wilcoxon matched-pairs signed-rank test. Statistical significance was demonstrated using this method; the *P* values for comparison of the control and TTHX1114-treated groups were 0.027 for the mid zone and 0.032 for the wound edge.

An important aspect of endothelial wound healing is the reformation of a contiguous sheet of CECs with tight junctions bound by ZO-1 such that the barrier and pump functions of the CECs can operate. To evaluate this, corneas subjected to descemetorhexis and allowed to recover were incubated with EdU for the last 2 days of incubation and then fixed and stained for ZO-1 and EdU as described previously. At the wound edge, control corneas showed limited to no EdU incorporation on the intact side and some EdU-incorporating cells in the stripped area (Fig. 5A); this observation is consistent for both normal corneas and corneas judged by the eye bank to be dystrophic. In corneas treated with TTHX1114, EdU-positive cells were observed on both
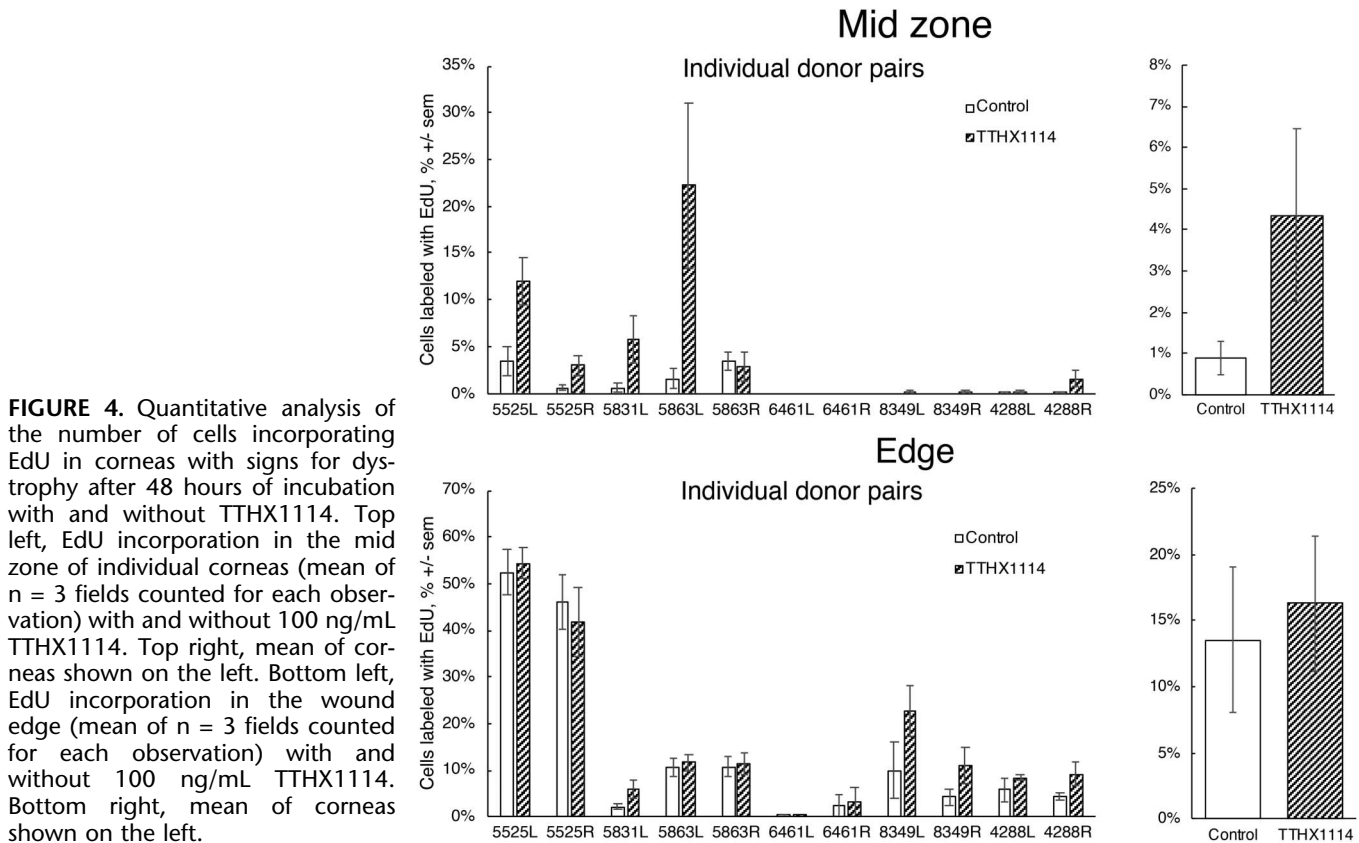
Mid zone

FIGURE 4. Quantitative analysis of the number of cells incorporating EdU in corneas with signs for dystrophy after 48 hours of incubation with and without TTHX1114. Top left, EdU incorporation in the mid zone of individual corneas (mean of n = 3 fields counted for each observation) with and without 100 ng/mL TTHX1114. Top right, mean of corneas shown on the left. Bottom left, EdU incorporation in the wound edge (mean of n = 3 fields counted for each observation) with and without 100 ng/mL TTHX1114. Bottom right, mean of corneas shown on the left.

Edge

sides of the wound edge. The CECs in the central wound area of treated corneas can be seen to have ZO-1-positive cell–cell contacts, although gaps in the endothelial layer are still visible.

A second method for evaluating the CEC layer is staining with alizarin red, which enables visualization of the cell–cell borders. Because this staining provides a stronger signal than ZO-1 fluorescence, it can be used to evaluate larger areas of the cornea at lower magnification. The alizarin red staining of control corneas and the contralateral TTHX1114-treated corneas from a normal pair and one judged by the eye bank to be dystrophic is shown in Figure 6. Areas that stained positive for trypan blue on day 14 corresponded well with negative staining by alizarin red (dark red areas). In the control corneas, very little of the stripped area was healed over, whereas in the TTHX1114-treated corneas, most of the wound area was covered with CECs and cell–cell borders were clearly visible. However, the CECs did not recreate the even hexagonal spacing of a normal endothelial layer.

A significant issue in wound healing in the cornea is scarring and the epithelial–mesenchymal transition of cells instead of appropriate regeneration. To examine the possibility that some migrating or proliferating CECs might undergo endothelial–mesenchymal transition (EnMT), corneas were stained with antibodies to αSMA, a marker of EnMT, and with phalloidin to evaluate actin stress fibers characteristic of myofibroblasts and/or cells that have undergone EnMT. In normal corneas (Fig. 7), αSMA staining was

absent in the mid zone, the wound edge, and the stripped area regardless of TTHX1114 treatment. αSMA staining was present in the vessels at the limbus, where αSMA is known to be located (data not shown). Phalloidin staining revealed an actin network at the cell periphery where cell–cell junctions were observed with ZO-1 staining, with no consistent difference in actin abundance observed between treated and untreated corneal endothelial cells. In corneas judged by the eye bank as dystrophic and treated with TTHX1114, a similar pattern was observed. αSMA staining in both regenerated endothelial cells and stromal fibroblasts was weak to absent.

DISCUSSION

This study sought to model the clinical DSO procedure using a corneal organ culture model and to explore the potential utility of the engineered FGF1 analog TTHX1114 to accelerate and strengthen regeneration of the CEC layer after DSO. Although the organ culture model was only taken out to 2 weeks, regeneration of the DSO lesion appeared consistent with what was observed in vivo. The control (untreated) corneas recovered approximately 30% of the CEC layer in 2 weeks, in contrast to the 4 to 12 weeks required for regeneration in human clinical studies.[6,7,40,41]

Treatment of the organ-cultured corneas with TTHX1114 dramatically accelerated recovery of the CEC layer and many treated corneas had nearly total regeneration of the CEC layer at 14 days as measured both by trypan blue staining and alizarin red staining. This accelerated regeneration
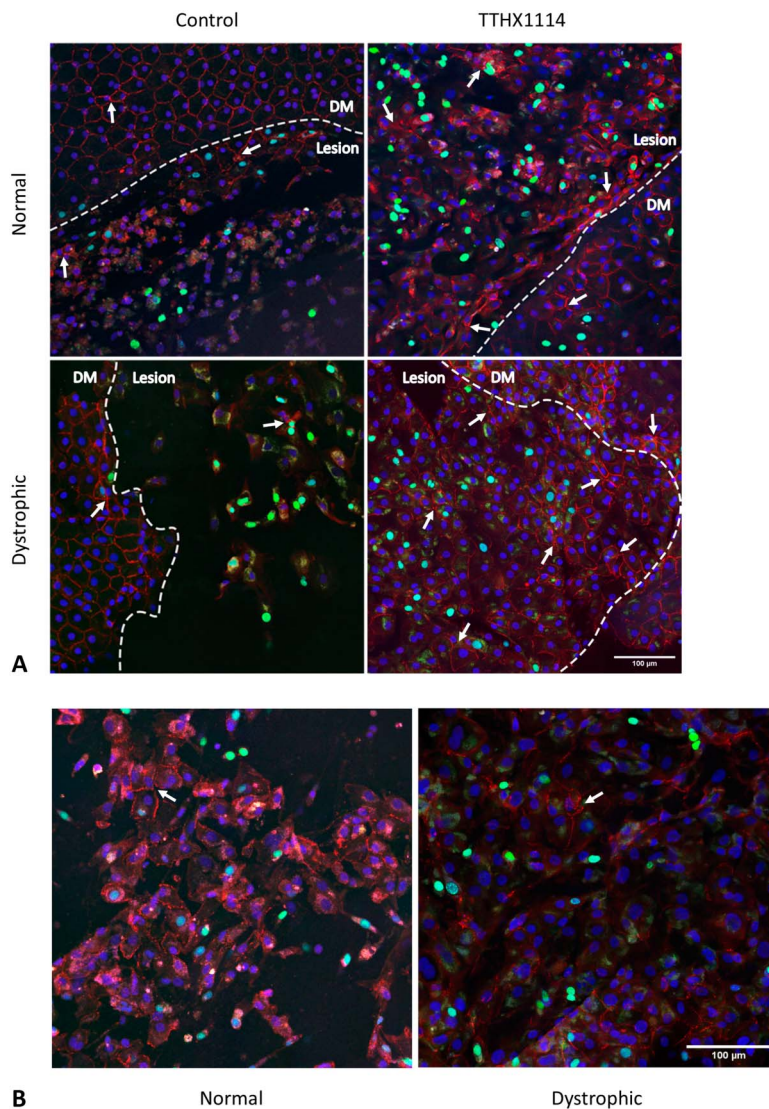
FIGURE 5. Regenerated area after TTHX1114 treatment is reforming the endothelial layer. A, Confocal images of the regenerated areas at 14 days (top row, normal corneas and bottom row, corneas with signs of dystrophy); the dotted line indicates the edge of the original descemetorhexis. Blue, Hoechst; red, ZO-1; green, EdU. B, Confocal images from the center of the stripped area in normal (left) and dystrophic (right) corneas treated with TTHX1114.

was similar in both normal corneas and in corneas judged to be dystrophic (with signs of FECD). The exclusion of trypan blue and the reformation of ZO-1-positive cell–cell junctions within the regenerated CEC layer argues that an intact barrier, part of the CEC's natural function, was restored. Because the corneas in this model were in suspension culture without an intact epithelial layer, swelling was expected and the regenerated CEC layer would not be expected to deturgescence the corneas even if fully functional. This is a challenge inherent to the corneal organ culture model, which prompted the limitation of the culture period to 14 days. Recent advances such as the active storage machine developed by Garcin et al[42] have the potential to extend the time in culture up to 3 months while minimizing corneal edema.

The dystrophic corneas used here came from donors who did not (to the eye bank's knowledge) have a premortem diagnosis of FECD; they all were judged dystrophic by the eye bank postmortem on the basis of specular microscopy (presence of guttae and reduced CEC density). They had

guttae visible after immunostaining (Fig. 3), and in some corneas, we confirmed the presence of guttae as thickening or buttons of Descemet membrane using transverse sectioning. However, the dystrophic corneas did not have large areas of confluent guttae and were not significantly edematous based on the central corneal thicknesses measured by specular microscopy. Therefore, although corneas were not graded for severity of dystrophy (eg, Krachmer scale), this likely represents an "early" FECD corneal phenotype. Although this is a limitation of this study, the differences between "early" and "late" FECD are of less relevance in the context of DSO where the central Descemet and guttae are surgically removed and a peripheral reserve of CECs are present as a substrate for regeneration.

Corneas of patients with FECD experience a progressive loss of CECs, and the associated loss of function drives corneal edema and vision loss associated with the disease. Therefore, a key component of the successful regeneration of the CEC layer after DSO is the proliferation of CECs.
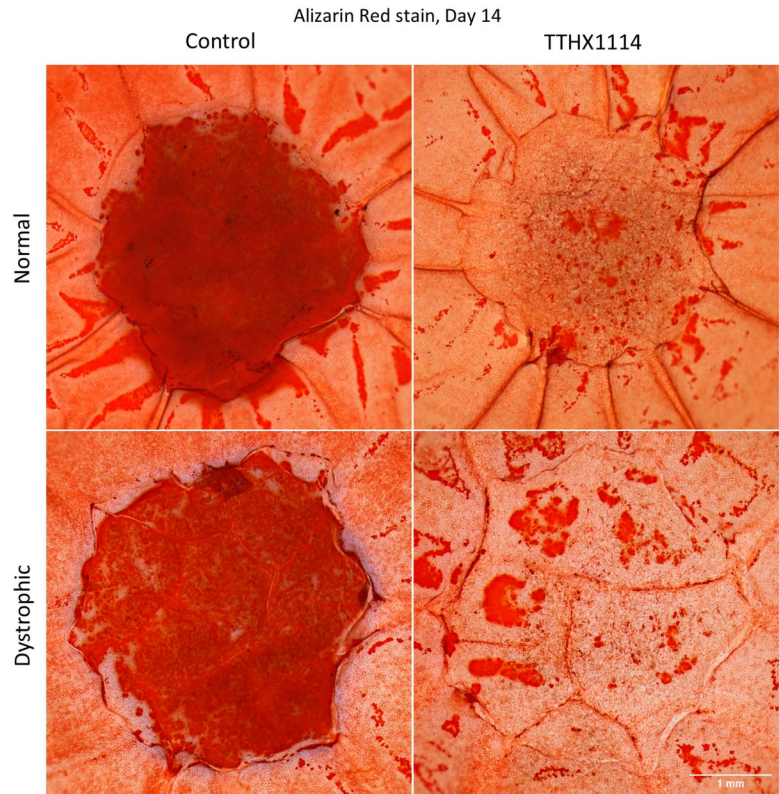
Alizarin Red stain, Day 14

FIGURE 6. Alizarin red images of the central region of normal corneas (top) and corneas with signs of dystrophy (bottom). Alizarin red staining was performed after 14 days of culture with (right) or without (left) 100 ng/mL TTHX1114.
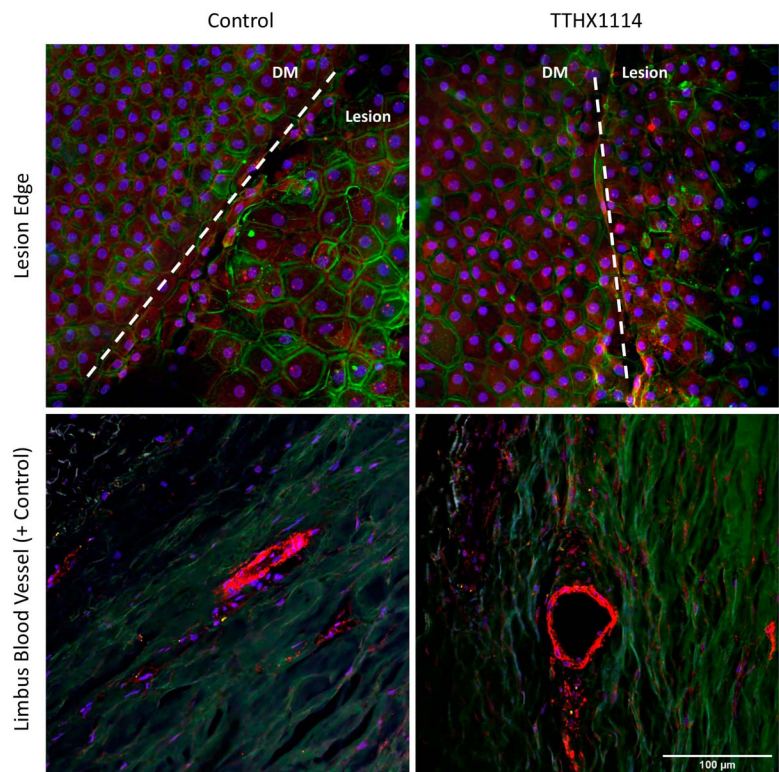

FIGURE 7. Regenerated regions of the endothelium do not express αSMA. Confocal imaging of filamentous actin and αSMA in the endothelium of normal corneas with and without TTHX1114. Whole corneas were subject to descemetorhexis and cultured in the presence (right) or absence (left) of TTHX1114 (100 ng/mL) for 14 days. Corneas were stained for filamentous actin (green) using phalloidin-Alexa Fluor 488 and for αSMA (red) using mouse anti-αSMA and Alexa Fluor 555-conjugated goat anti-mouse IgG2a. Nuclei (blue) were stained with Hoechst 33342. Top: The normal corneal endothelium with dotted lines indicating the edge of the descemetorhexis lesions with the stripped area to the right in both cases. Bottom: Positive αSMA staining of blood vessels of the limbus in transverse sections of each cornea.

There is substantial variability from cornea to cornea in proliferation, although less in healing of the DSO lesions. Although we measured proliferation in different sections of a single cornea, we have observed in pilot experiments that the proliferative capacity (both in the presence and absence of TTHX1114) of 2 corneas from a single individual tend to be similar [should we provide a figure on variability of the DSO closure response?]. Because the corneas became quite edematous over the 14 days of organ culture resulting in the CEC layer becoming irregular, we could not quantitate EdU staining in the recovered corneas and thus could not quantitate high proliferation with good DSO recovery. There was no correlation between proliferation or DSO recovery and the time from collection to organ culture (time in Optisol), death to collection interval, donor age, or cell density.

DSO should remove about 15% of the endothelial area, so if migration alone accounts for recovery, the expected total number of CECs postrecovery would be 15% less than prelesion. The presence of proliferating CECs was observed in both normal corneas and in corneas judged to be dystrophic by the incorporation of EdU into CECs in the DSO model. Proliferating CECs were observed proximal to guttae and within the lesion area. In addition, in the quartered cornea model (without DSO), stimulation of proliferation of the CECs by TTHX1114 was quantitatively demonstrated consistent with our previous observations,[38] although the high variability in the corneas judged to be dystrophic reduced the statistical significance of this observation. If TTHX1114 stimulates 4% of the cells in the mid zone to divide every 2 days consistent with this and our previous work[38] and this stimulation continues over 14 days to produce a 7-fold effect, then over 14 days it should increase the cell numbers by 28%, more than enough to replace the cells removed by the descemetorhexis and enabling the near complete regeneration of the CEC layer consistent with that shown here by trypan staining.

Quantitation of the CEC density in the central area postrecovery was not performed because the untreated corneas did not have a countable CEC layer in the center. These studies show that TTHX1114 stimulated EdU incorporation by the CECs both in the intact areas of the cornea and in the lesion area as the cells migrate to heal the lesion. This suggests that regeneration stimulated by TTHX1114 leads to a higher CEC density after wound healing. Failure of the CECs to regenerate and or relapse after recovery is well documented in clinical DSO series including in eyes treated with Rho kinase inhibitors.[6]

The level of EdU incorporation/proliferation seen in the corneas with signs of dystrophy is highly variable both with and without TTHX1114 stimulation, with a few corneas having zero cells incorporating EdU in the mid zone and some having very low levels of proliferation even at the wound edge with TTHX1114. This is consistent with observations in normal corneas[38] that the level of proliferation varies between individuals and can be very low in some but is correlated between corneas from the same individual. This contrasts with the healing of the corneas by trypan staining, where all corneas showed good recovery after TTHX1114 treatment and all corneas recovered to some extent even

without TTHX1114 treatment. Although proliferation and migration (trypan) were not measured in the same corneas, this suggests that TTHX1114 is able to increase migration of CECs even in corneas that have poor proliferation. The observation that all corneas recovered well with TTHX1114 stimulation argues that the poor proliferative response of some corneas is not driven by some aspect of the donation and storage process because that would likely affect both proliferation and migration.

Optimal regeneration of the endothelial layer would not only be rapid and robust but also be without aberrant scarring. FGFs are well known to oppose the epithelial–mesenchymal transition depending on context.[43–45] Corneal stromal cells can undergo EnMT and form myofibroblasts and contribute to fibrosis.[46] CECs can undergo endothelial–mesenchymal transition (EnMT) in response to injury or disease such as FECD, and these corneas have increased expression of αSMA.[47] In this study, no induction of αSMA staining was observed in either the control or TTHX1114-treated corneas including those with dystrophy. In addition, the actin cytoskeleton and ZO-1 staining were appropriate for differentiated CECs in all cases. This suggests that, consistent with clinical cases of DSO, the EnMT is not common and likely does not play a role in the failure or relapse of corneas. In addition, the EnMT was not stimulated by TTHX1114 treatment.

The effects of TTHX1114 shown in this organ culture model suggest that it may be useful as an adjunct to DSO to accelerate healing and increase the density of the CEC layer postrecovery. A clinical trial is currently underway to test this hypothesis.

REFERENCES

1. Vedana G, Villarreal G, Jr, Jun AS. Fuchs endothelial corneal dystrophy: current perspectives. *Clin Ophthalmol.* 2016;10:321–330.
2. Eghrari AO, Riazuddin SA, Gottsch JD. Fuchs corneal dystrophy. *Prog Mol Biol Transl Sci.* 2015;134:79–97.
3. Gipson IK. Age-related changes and diseases of the ocular surface and cornea. *Invest Ophthalmol Vis Sci.* 2013;54:ORSF48–53.
4. Afshari NA, Pittard AB, Siddiqui A, et al. Clinical study of Fuchs corneal endothelial dystrophy leading to penetrating keratoplasty: a 30-year experience. *Arch Ophthalmol.* 2006;124:777–780.
5. Van Meter W. *Eye Banking Statistical Report.* Washington, DC: Eye Bank Association of America; 2015.
6. Moloney G, Garcerant Congote D, Hirnschall N, et al. Descemet stripping only supplemented with topical ripasudil for Fuchs endothelial dystrophy 12-month outcomes of the sydney eye hospital study. *Cornea.* 2021;40:320–326.
7. Borkar DS, Veldman P, Colby KA. Treatment of Fuchs endothelial dystrophy by Descemet stripping without endothelial keratoplasty. *Cornea.* 2016;35:1267–1273.

8. Van den Bogerd B, Dhubhghaill SN, Koppen C, et al. A review of the evidence for in vivo corneal endothelial regeneration. *Surv Ophthalmol.* 2018;63:149–165.

9. Zaniolo K, Bostan C, Rochette Drouin O, et al. Culture of human corneal endothelial cells isolated from corneas with Fuchs endothelial corneal dystrophy. *Exp Eye Res.* 2012;94:22–31.

10. Joyce NC. Proliferative capacity of corneal endothelial cells. *Exp Eye Res.* 2012;95:16–23.

11. Zhu C, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. *Invest Ophthalmol Vis Sci.* 2004;45:1743–1751.

12. Kinoshita S, Koizumi N, Ueno M, et al. Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. *N Engl J Med.* 2018;378: 995–1003.

13. He Z, Campolmi N, Ha Thi BM, et al. Optimization of immunolocalization of cell cycle proteins in human corneal endothelial cells. *Mol Vis.* 2011;17:3494–3511.

14. Patel SP, Bourne WM. Corneal endothelial cell proliferation: a function of cell density. *Invest Ophthalmol Vis Sci.* 2009;50:2742–2746.

15. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci.* 2000;41:660–667.

16. Okumura N, Kinoshita S, Koizumi N. The role of rho kinase inhibitors in corneal endothelial dysfunction. *Curr Pharm Des.* 2017;23:660–666.

17. Soh YQ, Peh GS, Mehta JS. Evolving therapies for Fuchs' endothelial dystrophy. *Regen Med.* 2018;13:97–115.

18. Rieck PW, Gigon M, Jaroszewski J, et al. Increased endothelial survival of organ-cultured corneas stored in FGF-2-supplemented serum-free medium. *Invest Ophthalmol Vis Sci.* 2003;44:3826–3832.

19. Rieck P, OLiver L, Engelmann K, et al. The role of exogenous/endogenous basic fibroblast growth factor (FGF2) and transforming growth factor beta (TGF beta-1) on human corneal endothelial cells proliferation in vitro. *Exp Cell Res.* 1995;220:36–46.

20. Dannowski H, Bednarz J, Reszka R, et al. Lipid-mediated gene transfer of acidic fibroblast growth factor into human corneal endothelial cells. *Exp Eye Res.* 2005;80:93–101.

21. Lee JG, Jung E, Heur M. Fibroblast growth factor 2 induces proliferation and fibrosis via SNAI1-mediated activation of CDK2 and ZEB1 in corneal endothelium. *J Biol Chem.* 2018;293:3758–3769.

22. BAird A, BAird A, GospoDarowicz D, et al. Retina- and eye-derived endothelial cell growth factors: partial molecular characterization and identity with acidic and basic fibroblast growth factors. *Biochemistry.* 1985;24:7855–7860.

23. Gospodarowicz D, Mescher AL, Birdwell CR. Stimulation of corneal endothelial cell proliferations in vitro by fibroblast and epidermal growth factors. *Exp Eye Res.* 1977;25:75–89.

24. Savion N, Isaacs JD, Shuman MA, et al. Proliferation and differentiation of bovine corneal endothelial cells in culture. *Metab Pediatr Syst Ophthalmol.* 1982;6:305–320.

25. Schilling-Schön A, Schilling-Schon A, Pleyer U, et al. The role of endogenous growth factors to support corneal endothelial migration after wounding in vitro. *Exp Eye Res.* 2000;71:583–589.

26. Landshman N, BelkinM, Ben-Hanan I, et al. Regeneration of cat corneal endothelium induced in vivo by fibroblast growth factor. *Exp Eye Res.* 1987;45:805–811.

27. Kocaba V, Katikireddy KR, Gipson I, et al. Association of the gutta-induced microenvironment with corneal endothelial cell behavior and demise in Fuchs endothelial corneal dystrophy. *JAMA Ophthalmol.* 2018; 136:886–892.

28. Rizwan M, Peh GS, Adnan K, et al. In vitro topographical model of Fuchs dystrophy for evaluation of corneal endothelial cell monolayer formation. *Adv Healthc Mater.* 2016;5:2896–2910.

29. Chung DD, Zhang W, Jatavallabhula K, et al. Transcriptomic profiling of posterior polymorphous corneal dystrophy. *Invest Ophthalmol Vis Sci.* 2017;58:3202.

30. Frausto RF, Le DJ, Aldave AJ. Transcriptomic analysis of cultured corneal endothelial cells as a validation for their use in cell replacement therapy. *Cell Transpl.* 2016;25:1159–1176.

31. Wilson SE, Lloyd SA, He YG. Fibroblast growth factor-1 receptor messenger RNA expression in corneal cells. *Cornea.* 1993;12:249–254.

32. Guindolet D, Crouzet E, He Z, et al. Storage of porcine cornea in an innovative bioreactor. *Invest Ophthalmol Vis Sci.* 2017;58:5907–5917.

33. Thuret G, Chiquet C, Bernal F, et al. Prospective, randomized clinical and endothelial evaluation of 2 storage times for cornea donor tissue in organ culture at 31 degrees C. *Arch Ophthalmol.* 2003;121:442–450.

34. He Z, Gueudry J, Toubeau D, et al. Endothelial quality of eye bank-prestripped DMEK prepared form organ-cultured corneas with the Muraine technique. *Cell Tissue Bank.* 2018;19:705–716.

35. Blaber SI, Diaz J, Blaber M. Accelerated healing in NONcNZO10/LtJ type 2 diabetic mice by FGF-1. *Wound Repair Regen.* 2015;23:538–549.

36. Xia X, Kumru OS, Blaber SI, et al. Engineering a cysteine-free form of human fibroblast growth factor-1 for "second generation" therapeutic application. *J Pharm Sci.* 2016;105:1444–1453.

37. Eveleth DD, Eveleth JJ, Subramaniam A, et al. An engineered human fibroblast growth factor-1 derivative, TTHX1114, ameliorates short-term corneal nitrogen mustard injury in rabbit organ cultures. *Invest Ophthalmol Vis Sci.* 2018;59:4720–4730.

38. Eveleth D, Pizzuto S, Weant J, et al. Proliferation of human corneal endothelia in organ culture stimulated by wounding and the engineered human fibroblast growth factor 1 derivative TTHX1114. *J Ocul Pharmacol Ther.* 2020;36:686–696.

39. Lee JG, Kay EP. FGF-2-induced wound healing in corneal endothelial cells requires Cdc42 activation and Rho inactivation through the phosphatidylinositol 3-kinase pathway. *Invest Ophthalmol Vis Sci.* 2006;47:1376–1386.

40. Macsai MS, Shiloach M. Use of topical rho kinase inhibitors in the treatment of Fuchs dystrophy after descemet stripping only. *Cornea.* 2019;38:529–534.

41. Huang MJ, Kane S, Dhaliwal DK. Descemetorhexis without endothelial keratoplasty versus DMEK for treatment of fuchs endothelial corneal dystrophy. *Cornea.* 2018;37:1479–1483.

42. Garcin T, Gauthier AS, Crouzet E, et al. Innovative corneal active storage machine for long-term eye banking. *Am J Transpl.* 2019;19:1641–1651.

43. Becerril C, PArdo A, MontanoM, et al. Acidic fibroblast growth factor induces an antifibrogenic phenotype in human lung fibroblasts. *Am J Respir Cell Mol Biol.* 1999;20:1020–1027.

44. Ramos C, Becerril C, Montano M, et al. FGF-1 reverts epithelial-mesenchymal transition induced by TGF-{beta}1 through MAPK/ERK kinase pathway. *Am J Physiol Lung Cell Mol Physiol.* 2010;299:L222–L231.

45. Kubo E, Shibata S, Shibata T, et al. FGF2 antagonizes aberrant TGFβ regulation of tropomyosin: role for posterior capsule opacity. *J Cell Mol Med.* 2017;21:916–928.

46. Wilson SE. Corneal myofibroblasts and fibrosis. *Exp Eye Res.* 2020;201: 108272.

47. De Roo AK, Janssens T, Foets B, et al. Immunohistochemical profiling of corneas with fuchs endothelial corneal dystrophy. *Cornea.* 2017;36:866–874.