Mitochondria Transplantation Promotes Corneal Epithelial Wound Healing

Daniel Raz,[1,2] Keren Ben-Yaakov,[1] Michal Levi,[1,3] Marina Bertolin,[4] Stefano Ferrari,[4] Diego Ponzin,[4] Massimo Busin,[5–7] Hana Leiba,[1,3] Arie L. Marcovich,[1,3] Avital Eisenberg-Lerner,[1] and Ziv Rotfogel[1,3]

[1]Ophthalmology Research Laboratory, Department of Ophthalmology, Kaplan Medical Center, Israel
[2]Institute of Biochemistry, Food Science and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel
[3]Department of Ophthalmology, Kaplan Medical Center, Faculty of Medicine, Hebrew University of Jerusalem, Israel
[4]Fondazione Banca degli Occhi del Veneto, Venice, Italy
[5]Department of Translational Medicine, University of Ferrara, Ferrara, Italy
[6]Department of Ophthalmology, Ospedali Privati Forlì "Villa Igea," Forlì, Italy
[7]Istituto Internazionale per la Ricerca e Formazione in Oftalmologia, Forlì, Italy

Correspondence: Avital Eisenberg-Lerner, Ophthalmology Research Laboratory, Department of Ophthalmology, Kaplan Medical Center, Pasternak St. PO Box 1, Rehovot 76100, Israel; avital.eisenberg@mail.huji.ac.il.
Ziv Rotfogel, Department of Ophthalmology, Kaplan Medical Center, Faculty of Medicine, The Hebrew University of Jerusalem, International Office, Boyar Building, 5th Floor, Mount Scopus, Jerusalem 9190500, Israel; rotfogel@gmail.com.

AEL and ZR contributed equally to this work and should be considered co-senior authors.

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Purpose. The integrity of the corneal epithelium is essential in maintaining normal corneal function. Conditions disrupting the corneal epithelial layer range from chemical burns to dry eye disease and may result in impairment of both corneal transparency and sensation. Identifying factors that regulate corneal wound healing is key for the development of new treatment strategies. Here, we investigated a direct role of mitochondria in corneal wound healing via mitochondria transplantation.

Methods. Human corneal epithelial cells (hCECs) were isolated from human corneas and incubated with mitochondria which were isolated from human ARPE-19 cells. We determined the effect of mitochondria transplantation on wound healing and proliferation of hCECs. In vivo, we used a mouse model of corneal chemical injury. Mitochondria were isolated from mouse livers and topically applied to the ocular surface following injury. We evaluated the time of wound repair, corneal re-epithelization, and stromal abnormalities.

Results. Mitochondria transplantation induced the proliferation and wound healing of primary hCECs. Further, mitochondria transplantation promoted wound healing in vivo. Specifically, mice receiving mitochondria recovered twice as fast as control mice following corneal injury, presenting both enhanced and improved repair. Corneas treated with mitochondria demonstrated the re-epithelization of the wound area to a multi-layer appearance, compared to thinning and complete loss of the epithelium in control mice. Mitochondria transplantation also prevented the thickening and disorganization of the corneal stromal lamella, restoring normal corneal dehydration.

Conclusions. Mitochondria promote corneal re-epithelization and wound healing. Augmentation of mitochondria levels via mitochondria transplantation may serve as an effective treatment for inducing the rapid repair of corneal epithelial defects.

Keywords: corneal epithelium, corneal wound healing, mitochondria

Corneal epithelial irregularities and defects are among the most common ocular pathologies. Corneal re-epithelization is often a slow process and treatment of epithelial defects can be challenging. Incomplete re-epithelization may lead to persistent epithelial defects. Prolonged healing may lead to scarring and opacification requiring surgical interventions, such as corneal transplantation. Therefore, there is a great need for new therapeutic approaches to promote the rapid and effective healing of the corneal epithelium.

Wound healing is a complex process that involves cell proliferation, migration, and tissue remodeling. Accumulating evidence underscores the significant dependence of wound healing on mitochondria, which serve as the primary source of cellular energy production.[1–4] Mitochondria are increasingly recognized for their involvement in critical cellular processes, such as migration, proliferation, and metabolite regulation, all of which are essential for effective wound healing. Accordingly, mitochondria play a pivotal role in corneal wound healing, and various studies demonstrate that improving mitochondrial function enhances this crucial process. For instance, the administration of an ophthalmic solution containing Coenzyme Q10, an essential electron carrier in the mitochondrial respiratory chain, was shown to reduce corneal damage after UVB exposure by improving mitochondrial bioenerget-

ics and cell viability.[5] Additionally, administration of Coenzyme Q10 promoted corneal wound healing in vivo after epithelium removal.[5] Another example of enhancing mitochondrial function to promote corneal wound healing is through mitochondria-targeted antioxidant therapy using SkQ1. SkQ1 has been shown to enhance corneal wound healing under UV light and mechanical injury by promoting the survival of corneal epithelial cells through antioxidant defense and restoration of normal corneal metabolism.[6]

Moreover, mitochondria have been shown to play a key role in impaired corneal wound healing during diabetes. This effect was demonstrated to be mediated via the downregulation of PPARα, a transcription factor regulator of various cellular processes including mitochondrial biogenesis, energy metabolism, and oxidative stress.[7] Activation of PPARα improved mitochondrial function and promoted wound healing in the diabetic cornea.[8] Furthermore, in diabetic corneas, high glucose-induced hyperosmolarity was found to reduce mitochondrial respiration in corneal epithelial cells and negatively impact migration.[9]

Interestingly, lateral transfer of mitochondria between cells occurs in various cell types under stress conditions, and in some cases has been shown to facilitate wound healing.[10–17] In the context of corneal repair, topical application of mesenchymal stem cells (MSCs) to the corneal surface induced mitochondrial transfer to corneal epithelial cells (CECs) and facilitated wound healing in rabbits following chemical injury.[18] This observation underscores the potential of mitochondrial transfer as a therapeutic strategy for enhancing corneal wound healing.

Recently, a new therapeutic modality of mitochondria transplantation was developed, mimicking the endogenous process of mitochondrial transfer.[19] Mitochondria transplantation involves the augmentation of compromised or stressed cells or tissues with fresh mitochondria isolated from healthy tissues. Similar to the endogenous process, the efficiency of mitochondrial transplantation dramatically increases under stress conditions.[20] Recent studies have demonstrated the protective effect of mitochondria transplantation in various ischemic and stress conditions, including cardiac ischemia following infarction and ischemic damage,[21–24] neuronal damage,[25–30] renal ischemia,[31] and more. This emerging field holds promise for addressing mitochondrial dysfunction and promoting tissue repair in various pathological conditions.

In this work, we aimed to investigate the direct involvement of mitochondria in corneal wound healing. We used cultures of primary human corneal epithelial cells (hCECs) and a mouse model of corneal chemical burn, and demonstrated that mitochondria transplantation enhances corneal epithelial cell migration, wound healing, and repair. Our findings may contribute to future clinical investigation of mitochondria transplantation for the treatment for corneal epithelial defects.

Methods

Animals

Female C57BL/6 mice 7 to 8 weeks of age were purchased from Envigo, Israel. The mice were housed in the Kaplan Medical Center animal facility at 21°C to 22°C with 12/12-hour light-dark cycles. Prior to dissection of the livers for mitochondrial isolation, the mice were fasted overnight and euthanized. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All study protocols were approved by the Israeli National Council for Experimentation on Animals.

hCEC Isolation From Human Corneas

Human corneas, which were unsuitable for transplantation, were obtained from the Veneto Eye Bank Foundation (Venice, Italy) and used for research purposes, according to Italian legislation and guidelines set by the Italian Transplant Service (Centro Nazionale Trapianti, Rome, Italy) and the Regional Transplant Service (Centro Regionale Trapianti, Padua, Italy). To isolate hCECs from human corneas, limbal tissue was dissected from human corneas, cut into small pieces, and incubated with 10 mL trypsin (0.05% trypsin and 0.01% EDTA solution, Life Technologies, 25300-062) for 30 minutes in 37°C, after which the supernatants, containing the cells, were collected, and neutralized by addition of 10 mL KM media containing: Dulbecco's Modified Eagle Medium (21969035, Gibco) and F12 (21765029, Gibco) 2:1, fetal bovine serum (FBS) 10% (10099-141, Life Technologies), penicillin-streptomycin 50 mg/mL (15140122Life, Technologies), and Glutamine 4 mM (25030081, Life Technologies). Adenine grade I 0.18 mM, (4010-21-2, Pharma Waldhof GMBH), hydrocortisone 0.4 mg/mL (AIC013986029, Flebocortid Richter, Sanofi), insulin (HI0210, Humulin R, Lilly, Canada), Triiodothyronine 2 nM (AIC036906016, Liotir, IBSA), Cholera Toxin QD 8.1 mg/mL, 9100B, List Biological Laboratories), and EGF 10 ng/mL (1416-050, EGF, Cell Genix GmbH, Germany). This process was repeated three times on the limbus sections. Cells were pelleted by centrifugation for 5 minutes at 200 $\times$ g and resuspended in KM media. Cells were seeded in co-culture with a feeder layer of irradiated NIH-3T3 cells. Cultures were incubated at 37°C with 5% CO_2 and used after the first passage.

ARPE-19-GFP Cell Culture

ARPE-19 cells were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/Ham's F-12, Capricorn Scientific) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-Glutamine. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

The mitochondrial-targeting sequence of Steroidogenic Acute Regulatory Protein (StAR) cloned into pEGFP-N1 (StAR-GFP) was a kind gift from Joseph Orly, The Hebrew University of Jerusalem, Israel. The pEGFP-N1 plasmid backbone includes a Neomycin resistance gene which allows selection of cells which express the vector by exposure to G418, an analog of neomycin sulfate. Human retinal pigment epithelium ARPE-19 cells were transfected with StAR-GFP and stably expressing cells were selected with 1200 µg/mL G418.

Mitochondria Isolation From Mouse Liver

Mitochondria were isolated from mouse liver. The livers were washed with ice-cold isolation buffer (IB)-1 buffer (225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, 0.5% fatty acid free bovine serum albumin [FA-free BSA], 0.5 mM EGTA, pH 7.4) and homogenized by 10 up-and-down strokes

of a pestle rotating in 250 revolutions per minute (RPM). The homogenate was centrifuged twice at 750 × g for 10 minutes at 4°C, and the supernatant was separated and centrifuged again at 10,000 × g for 10 minutes at 4°C. The mitochondria pellet was re-suspended in IB-2 buffer (similar to IB-1 but lacking EGTA). Mitochondria were kept on ice until use.

Mitochondria Isolation From ARPE-19-GFP

Cells were grown to approximately 80% to 90% confluency in cell culture plates. Then, 20×10^7 cells were rinsed with cold phosphate-buffered saline (PBS) containing magnesium and calcium. Cells were detached using a scraper in cold PBS, and transferred to a centrifuge tube. Cells were pelleted by centrifugation at low speed (e.g. 300 × g) for 5 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in ice-cold homogenization buffer (containing 225 mM Mannitol, 75 mM sucrose, 30 mM Tris-HCl, and 7.4 PH) and homogenized to break cellular membranes. The homogenate was centrifuged at 750 × g for 10 minutes at 4°C to remove unbroken cells, nuclei, and large debris. The resulting supernatant containing the crude mitochondrial fraction was transferred to a fresh centrifuge tube and centrifuged at high speed (10,000 × g) for 10 minutes at 4°C. Following centrifugation, the pellet containing the mitochondrial fraction was re-suspended with IB-3 buffer (containing 225 mM Mannitol, 75 mM sucrose, 30 mM Tris-HCl, 0.1 mM EGTA, and 7.4 PH). Mitochondria were kept on ice until use.

ATP Production Measurement

ATP production was measured using ATPlite luminescence assay kit (PerkinElmer, USA) according to the manufacturer's instructions. Isolated mitochondria were diluted in respiration buffer (225 mM mannitol, 75 mM sucrose, 0.1% fatty acid free bovine serum albumin, 1 mM EGTA, 2 mM HEPES, 5 mM MgCl, 10 mM KH_2PO_4, and pH 7.4). Succinate (20 µM) was added as complex I substrate to all vials. ATP production was determined under basal conditions and after addition of 150 µM ADP. To validate mitochondrial-dependent ATP production, the uncoupler FCCP (12 µM) was added with succinate and ADP. Luminescence was measured by a microplate reader (Tecan, Switzerland).

Ki67 Proliferation Index Staining

hCECs were detached from human corneas as described above and cultured on glass coverslips in KM media. Cells were transplanted with either mitochondria, or vehicle (IB-3 buffer) as control, for 24 hours. Cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized for 30 minutes in permeabilization solution containing 0.1% Triton X-100, 2% bovine serum albumin (BSA), and 5% donkey serum (DS) in PBS. Subsequently, the cells were washed with PBS, and incubated overnight at 4°C with primary antibodies against Ki67 (#ab16667, Abcam) diluted in 2% BSA and 5% DS in PBS. Cells were extensively washed in PBS and incubated with CY3-conjugated secondary antibodies for 2 hours at room temperature. Finally, cells were stained with DAPI for nuclear counterstaining for 15 minutes and mounted on glass slides. Images were taken using EVOS (FL auto inverted) microscope in 10 × magnification. Multiple fields were imaged from each coverslip. The acquired images were analyzed using image analysis software (QuPath[32]) to obtain the total and Ki67 positive cell count ($n = > 3500$ cells/group). The number of Ki67 positive cells was calculated as the percentage from the total cell count.

Neutral Red Cell Viability Assay

hCEC cells were plated in 96-well plates (2000 cells/well) and cultured for 24 hours. Neutral Red solution (#N-2889, Sigma Aldrich) was filtered to avoid dye crystals, and incubated at a 1:50 dilution with hCECs for 2 hours at 37°C in a humidified 5% CO_2. The cultures were then carefully washed with PBS to eliminate extracellular Neutral Red. The incorporated dye was eluted from the cells by adding 150 µL elution medium (50% ethanol and 1% acetic acid) into each well, followed by gentle shaking of the plate for 10 minutes. The color intensity was measured in a plate reader at 540 nm.

In vitro Wound Healing Scratch Assay

hCECs were isolated from human corneas, as described above, and cultured in 24-well plates in KM media with irradiated 3T3 to reach confluent cell monolayers. A cross-shaped scratch was performed on the confluent monolayers using a 200 µL plastic pipette tip to create a cell-free area. Cultures were immediately washed three times with PBS to remove detached cells. The scratch area was imaged by Nikon eclipse Ts2 inverted microscope using 10 × magnification. Cells were then transplanted with either mitochondria, or vehicle (IB-3 buffer) as control, for 24 hours, and imaged again. The cell-free wound area was measured using image analysis software (Image J[19]). The wound area at 24 hours post transplantation of mitochondria/vehicle was calculated as percentage of the initial wound area per each well.

Alkali Corneal Injury

Female C57BL/6 mice were used for the corneal alkali burn studies. Previous studies demonstrate that there are no gender differences in corneal wound healing in experimental models.[33,34] Nevertheless, it has been suggested that female mice may have a higher susceptibility to corneal damage.[35] We therefore used female mice in these studies.

Mice were anesthetized by intraperitoneal injection of Ketamine 75 mg/Kg and medetomidine hydrochloride 0.75 mg/Kg. Localin (oxybuprocaine hydrochloride 0.4%) was topically applied to the eyes. Alkali burns were inflicted in the right eye of mice as follows: Whatman 3MM filter paper was cut into 5 mm diameter circles, soaked in 0.15M NaOH for 5 seconds, and placed on the eye for 30 seconds to produce an ocular surface alkali burn. Then, the eye was extensively rinsed by 5 mL sterile PBS.

Mitochondria Transplantation to Mouse Eyes

Isolated mitochondria were diluted in IB-1 buffer and a 25 µL drop was applied to the alkali-injured eyes of mice under anesthesia, as described above. The drop was replenished every 30 minutes to avoid drying for a total of 3 hours. At the end, the eyes were extensively irrigated by PBS. Finally, dethamycin (dexamethasone sodium phosphate 0.1% and neomycin sulfate 0.5%) were topically applied to the injured

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eyes. Celluspan (Hydroxyethylcellulose 1.4%) was applied to non-treated eye to avoid drying throughout the procedure. Anesthesia was reversed by subcutaneous injection of antipamezole hydrochloride 3.75 mg/Kg. Mitochondria transplantation was performed daily, with fresh mitochondria, from the day of injury and for 4 consecutive days. Mice in the control group underwent the same alkali injury procedure and were treated daily with vehicle (IB-1 buffer), followed by dethamycin, in the same manner described for mitochondria-transplanted mice. For demonstration of internalization, mitochondria were applied for 3 hours 1 day post alkali injury, followed by enucleation of the eyes for evaluation.

Transplantation of Inactive Mitochondria

After isolation, mitochondria were frozen in liquid nitrogen and thawed in 37°C. This process was repeated in five freeze-thaw cycles. Inactivation was verified by in vitro ATP production assays.

Slit Lamp Imaging

Mice were anesthetized by intraperitoneal injection of Ketamin 75 mg/g and medetomidine hydrochloride 0.75 mg/g. Corneas were stained by administration of 0.1% fluorescein and the epithelial damage was imaged by slit-lamp microscopy. The wound area was measured in ImageJ.

PCR Analysis

Mitochondria were isolated from livers of BALB/c mice and administered to the ocular surface of C57BL/6 mice following alkali burn as described in the section "Mitochondria transplantation to mouse eyes." Corneas from 3 to 5 C57BL/6 recipient mice were pooled and DNA was extracted using DNeasy Blood & Tissue kit (Qiagen). PCR analysis was performed using primers targeting a region containing differential nucleotides between BALB/c and C57BL/6 mtDNA, designed to match mtDNA from BALB/c. For normalization, primers targeting a shared sequence between BALB/c and C57BL/6 mtDNA were used. Primer sequences: BALB/c: F: CTG ACA TTT TGT AGA CGT AA, R: GAA GAT AAC AGT GTA CAG GTTG; shared mtDNA: F′: CCC AGC TAC ATC ATT CAAGT R′: GAT GGT TTG GGA GAT TGG TTG ATG T.

Confocal Microscopy

The eyes were enucleated, extensively washed in PBS, and then fixed for 1 hours in 4% PFA. Then, the corneas were isolated, stained with DAPI, and mounted on glass slides. Pictures were acquired using the Leica SP8 LIGHTING confocal microscope (Wetzlar, Germany) and analyzed by ImageJ software.

Histology

Murine eyes were enucleated after 4 days from the day of injury. Corneas were isolated from the enucleated eyes and fixed in 4% formaldehyde. Then, 4 µM sections of the specimens were stained with hematoxylin and eosin.

Statistical Analysis

Data were analyzed by Student's unpaired *t*-test using GraphPad Prism. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Results

Human Corneal Epithelial Cells Internalize Exogenous Mitochondria

To examine the effect of mitochondria transplantation on corneal epithelial wound healing, we first determined the ability of CECs to internalize exogenous mitochondria. We isolated fresh mitochondria from ARPE-19 cells expressing a mitochondrial GFP reporter protein (hereafter mito-GFP; Fig. 1A). To confirm that the isolated mitochondria were functional, we demonstrated that isolated mitochondria performed de novo ATP synthesis in vitro in response to succinate and ADP addition, in a manner which was sensitive to inhibition by the mitochondrial uncoupler FCCP (Fig. 1B). Then, the hCECs were isolated from fresh human corneas and cultured with isolated mito-GFP. To evaluate the internalization of exogenous mitochondria by hCEC, we performed confocal microscopy analysis which demonstrated the uptake of mito-GFP by hCECs (Fig. 2).

Mitochondria Transplantation Induced hCEC Proliferation and Wound Healing In Vitro

To examine whether mitochondria transplantation affects wound healing of the corneal epithelium, hCECs were isolated from human corneas and cultured. The cultures were scratched to create a wound and incubated with either isolated mitochondria or vehicle as control. hCEC wound healing was determined 24 hours post-wounding by measuring the acellular wound area at each time point and calculating the percentage of wound closure. Figure 3A shows representative images of the hCEC culture for each group, at the time of wounding and 24 hours later. Strikingly, whereas the mean percentage of wound closure was 60% in the control group, mitochondria-transplanted cells demonstrated complete closure of the wound (100%) 24 hours post-wounding (Fig. 3B). These results demonstrate that mitochondria transplantation induces wound healing in cultured primary hCECs.

We next wished to determine whether the observed wound healing effect of mitochondria transplantation in hCECs was dependent on enhancement of cell proliferation. To that end, we analyzed the expression of ki67, a marker of cell division, by immunostaining hCECs that received either mitochondria or vehicle for 24 hours. We found that the percentage of ki67-positive cells was approximately 12% higher in mitochondria transplanted cells, compared to control (Figs. 4A, 4B). To further evaluate the effect of mitochondria transplantation on cell proliferation, we quantified the uptake of Neutral Red, which is actively internalized by living cells and is therefore reflective of the live cell count, in hCECs that received either mitochondria or vehicle for 24 hours. In agreement with the ki67 results, Neutral Red uptake was approximately 17% higher, on average, in cells which received mitochondria compared to control cells (Fig. 4C). These results suggest that mitochondria transplantation

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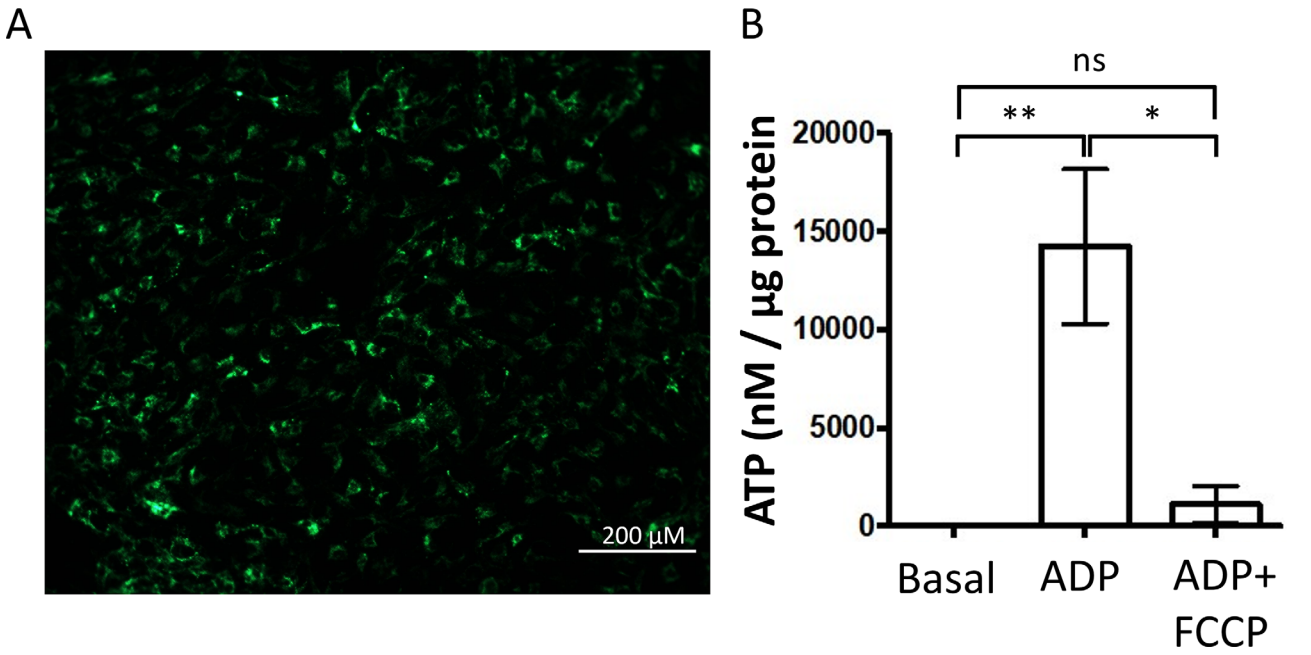

Figure 1. Mitochondria isolation from mito-GFP RPE cells. (**A**) Representative image of mito-GFP ARPE-19 cells. (**B**) Mitochondria was isolated from mito-GFP ARPE-19 cells and ATP production was measured by Clark electrode under basal conditions (20 µM succinate) or following addition of ADP (20 µM succinate + 150 µM ADP) or ADP + FCCP (20 µM succinate + 150 µM ADP + 12 µM FCCP). N = two individual experiments from independent mitochondria purifications; $^{*}P = 0.011$; $^{**}P < 0.009$.

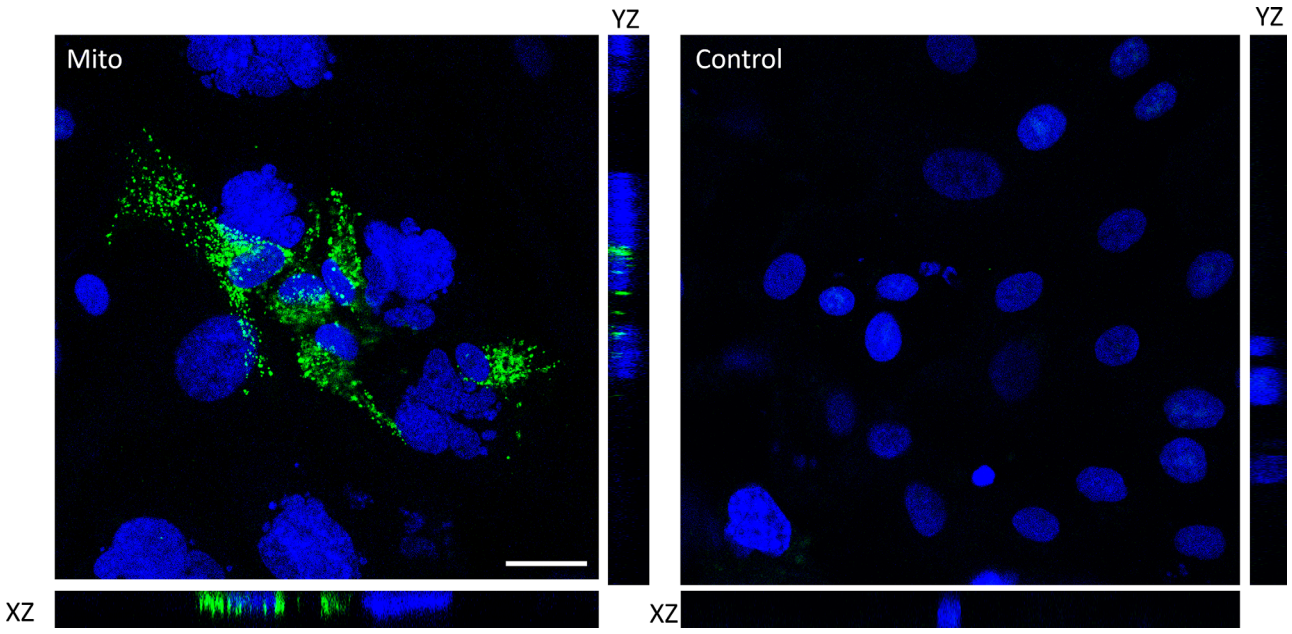

Figure 2. hCECs internalize exogenous mitochondria. Corneal epithelial cells were isolated from human corneas and cultured in the presence of GFP-tagged mitochondria, isolated from ARPE-19 cells, (mito) or vehicle as control (control). The cells were fixed 24 hours later, nuclei were stained with DAPI (*blue*), and imaged by confocal microscopy. Shown are representative images of cells that internalized exogenous mitochondria. Scale bar = 25 µM. Orthogonal XZ and YZ sections are shown on the *right* and *bottom* of each figure (Mito and Control), demonstrating a single focal plane containing exogenous mitochondria and nuclear staining.

induces a mild, yet significant, increase of hCEC proliferation. Together, these findings demonstrate that mitochondria transplantation enhances wound healing through the plausible combination of enhanced proliferation and cell migration.

Mitochondria Transplantation Induced Re-Epithelization After Alkali Burn In Vivo

Given the findings that mitochondria transplantation enhances hCEC wound healing in culture, we aimed to

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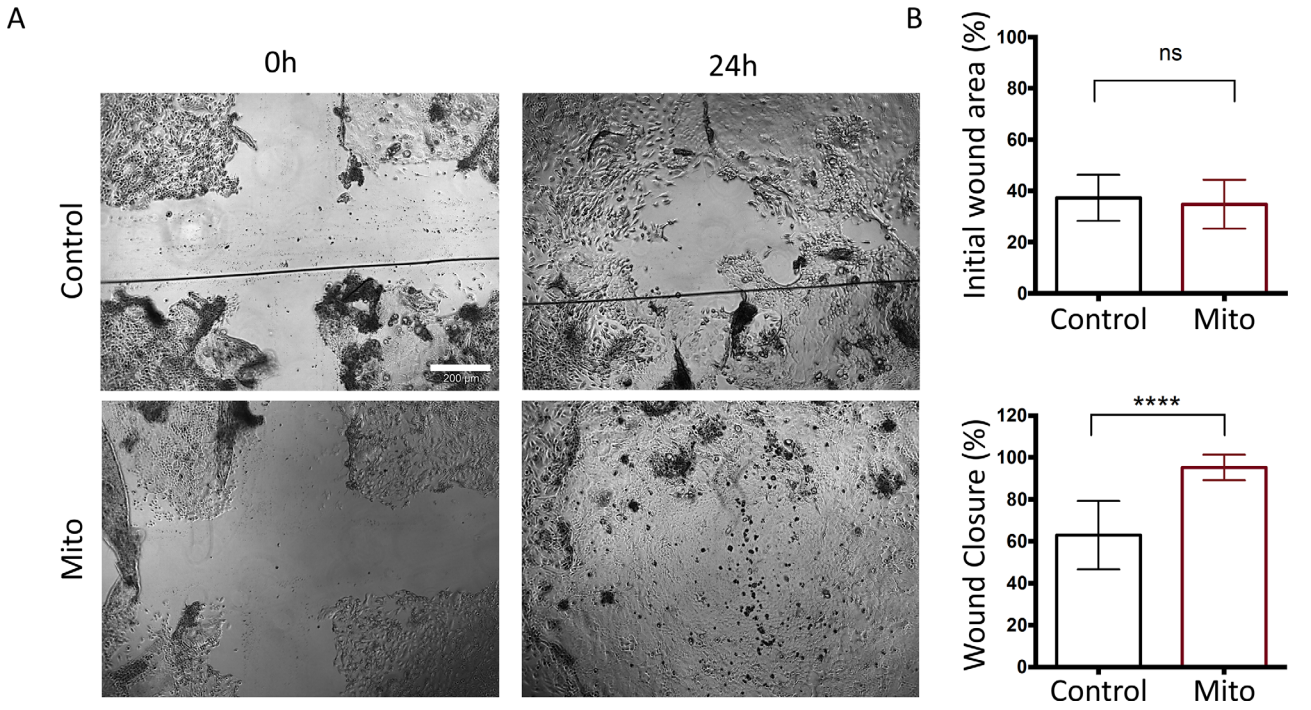

Figure 3. Mitochondria transplantation enhances CEC wound healing. hCECs were isolated from human corneas. Confluent cultures were wounded by a cross-shaped scratch. Cultures were incubated in the presence of either mitochondria (mito), or vehicle control (Control). (**A**) Representative images at the time of wounding (0 hours) and 24 hours post-wounding (24 hours). (**B**) Images were analyzed by ImageJ to determine the cell-free wound area immediately after wounding (*top*) and 24 hours later (*bottom*). Wound closure was calculated for each well as percentage of the initial wound area of that well; $^{****}P < 0.0001$. N = three individual experiments. In each experiment, hCECs were isolated from 6 human corneas, pooled, and seeded in 10 different wells (in total, 3 cell-isolate pools from 18 corneas were examined in 3 different experiments). Separate mitochondria isolations were prepared from ARPE-19 cells for each experiment. Scale bar = 200 µM.

examine whether mitochondria transplantation could induce the recovery of the corneal epithelium in vivo following injury. Chemical burns are a common cause of corneal injury, which can compromise corneal transparency and lead to visual impairment. Therefore, we sought to investigate the effect of mitochondria transplantation on corneal wound healing in a mouse model of corneal chemical burn. To induce corneal alkali burn, NaOH-soaked filter paper was placed on the ocular surface of mice for 30 seconds, as described.[36] We first evaluated the uptake of exogenous mitochondria by corneal cells in vivo. To that end, mitochondria were isolated from donor BALB/c mouse livers and administered to the ocular surface of recipient C57BL/6 mice following alkali injury. Corneas from recipient C57BL/6 mice were then harvested, subjected to DNA extraction, and analyzed by PCR targeting a mitochondrial DNA sequence specific to BALB/c mitochondrial DNA. Amplification using BALB/c-specific primers revealed the uptake of BALB/c mitochondria into C57BL/6 corneal epithelial cells after in vivo administration (Fig. 5A). Furthermore, we utilized transgenic mice expressing a mitochondrial matrix-targeted Dendra2 fluorescent protein, to isolate fluorescent Dendra2-labeled mitochondria from the livers of these mice. The fluorescent Dendra2-mitochondria were administered to the ocular surface of WT C57BL/6 mice following alkali burn, and the corneas of the recipient mice were harvested. Confocal microscopy analysis demonstrated the uptake of Dendra2-labeled mitochondria into corneal epithelial cells following in vivo administration (Figs. 5B–D). We estimate the ratio of corneal epithelial cells that demonstrated internalized mitochondria to be approximately 20%.

We next proceeded to examine the effect of mitochondria transplantation following corneal alkali burn on wound healing (Fig. 6A). Mice were initially examined using fluorescein staining under a slit-lamp microscope to assess corneal epithelial defects (Fig. 6B). Subsequently, the mice received daily treatment either with freshly isolated mitochondria from mouse livers or with a vehicle control, administered topically to the ocular surface. Mice were thereafter evaluated daily for epithelial damage by fluorescein staining under a slit-lamp microscope. The wound area was quantified using ImageJ software and expressed relative to each mouse's initial wound area.

Twenty-four hours after the injury, mice treated with mitochondria exhibited a 40% recovery of the epithelial damage, whereas only 10% recovery was observed in control mice (see Figs. 6B, 6C). This enhanced recovery persisted in mice receiving mitochondria, with 80% and 90% recovery on days 2 and 3, respectively, compared to 30% and 50% recovery in the control group (see Fig. 6C). By day 4 following the alkali burn, control mice still displayed fluorescein staining and epithelial damage close to 50% of the original wound. In contrast, mice treated with mitochondria demonstrated complete re-epithelization of the wound by day 4 (see Figs. 6B, 6C).

Consistent with these findings, hematoxylin and eosin (H&E) staining of corneas from mice receiving vehicle demonstrated broad damage including thinning and cell loss of the epithelial layer, thickening and disorganization

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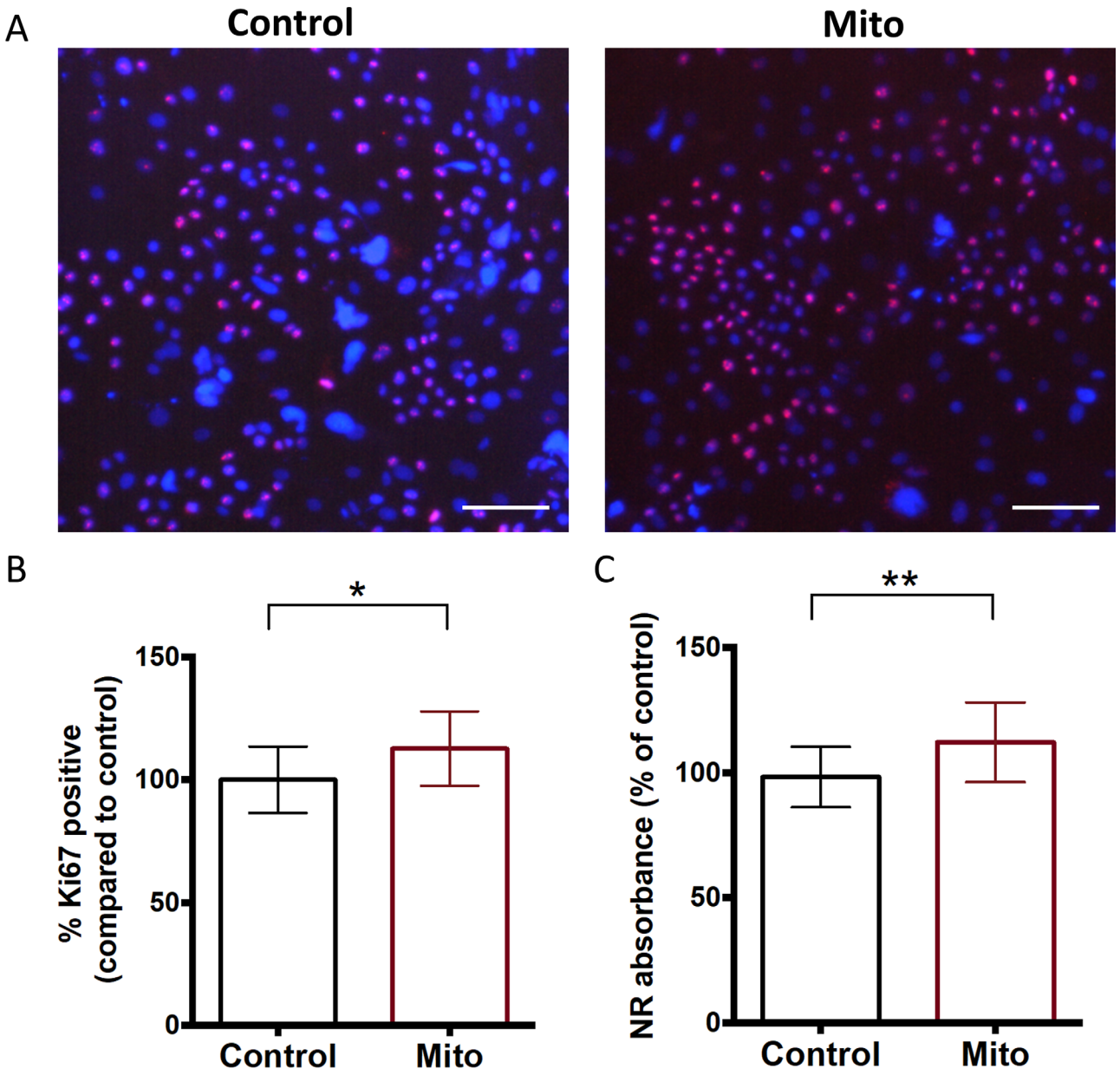

Figure 4. Mitochondria transplantation mildly induces hCEC proliferation. hCECs were transplanted with either mitochondria (mito), or vehicle as control (control). In (**A–C**): n = three individual experiments. In each experiment, hCECs were isolated from six human corneas, pooled, and seeded in different wells. In total, 18 corneas were analyzed. Separate mitochondria isolations were prepared from ARPE-19 cells for each experiment. (**A, B**) Cells were immunostained with antibodies against Ki67. (**A**) Representative images. Scale bar = 400 µM. (**B**) Quantification of % Ki67-positive cells (n = > 3500 cells, analyzed from 3 individual experiments). *P = 0.02. (**C**) Neutral Red uptake measurement (n = 3 individual experiments). **P =0.003.

of the stroma, and cell infiltrates. However, corneas from mice transplanted with mitochondria had a multi-layered epithelium, with normal stroma thickness and organization, and without increased infiltrates, supporting a phenotype of repaired epithelium (Fig. 7).

Finally, we asked whether transplanted mitochondria facilitate wound healing via ATP production. To disrupt ATP production, we subjected isolated mitochondria to repeated freeze-thaw cycles post-isolation. This process is expected to disturb mitochondrial membranes and impede ATP production. In vitro ATP production assays confirmed a substantial reduction in the ability of isolated mitochondria to produce ATP after freeze-thaw (Fig. 8A). Subsequently, we conducted an in vivo wound healing assay by applying mitochondria to the ocular surface after alkali burn, using either fresh or frozen mitochondria. Results revealed that wounds in mice treated with freeze-thawed mitochondria exhibited significantly slower healing compared to those treated with fresh mitochondria (Fig. 8B). These findings suggest that ATP synthesis contributes to the corneal wound healing effect of mitochondria transplantation.

Discussion

Mitochondria serve as the main source of cellular energy in the cornea and play a critical role in corneal wound

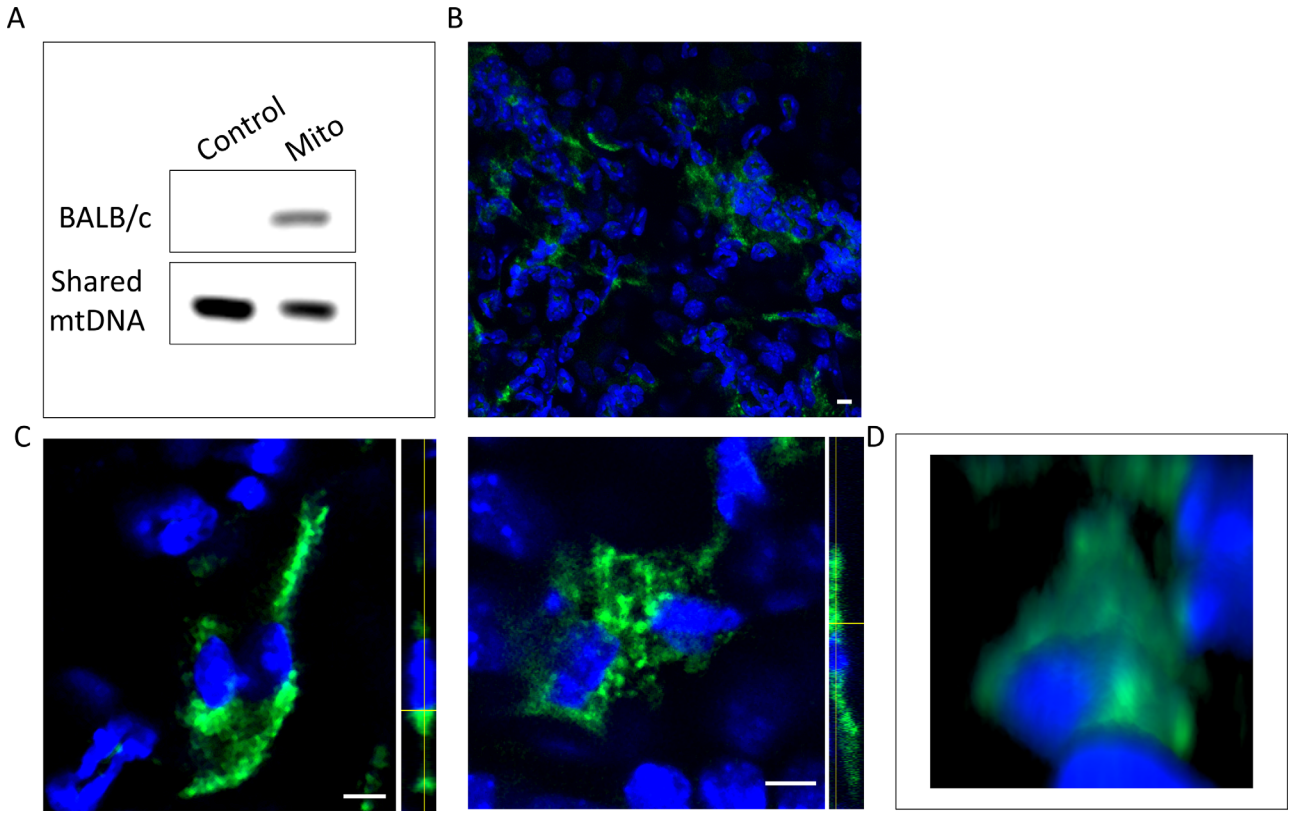

Figure 5. Corneal epithelial cells internalize Mitochondria after alkali-burn in vivo. (**A**) Mitochondria were isolated from donor BALB/c mice and administered to the ocular surface of recipient C57BL/6 mice following alkali burn. DNA was harvested from corneas of recipient C57BL/6 mice and analyzed by PCR with primers specific for BALB/c mtDNA and primers for a shared region of mtDNA as housekeeping control. Shown is a representative DNA gel of four experiments, each experiment was performed with separate mitochondria preparations and three to five mice per group. (**B, C**) Representative image of corneal epithelial cells which internalized Dendra2-labeled mitochondria after in vivo administration to the ocular surface following alkali burn. Scale bar = 5 µM. Orthogonal YZ sections demonstrating the presence of mitochondria and nuclear staining within a focal plane are shown on the right-hand side of images in **C** ($n = 5$ mice), from three individual experiments with separate mitochondria preparations from livers of transgenic mice expressing Dendra2-labeled mitochondria. (**D**) Confocal Z section images were combined into a 3D representation of a representative cell showing internalized Dendra2-labeled mitochondria.

healing.[4–6,8,9] Moreover, common causes of corneal insults, such as UV light exposure, oxidative stress, chemical injury, and epithelial debridement, compromise mitochondrial integrity, dynamics, and function.[4,6,37,38] Interestingly, restoration of corneal mitochondrial respiration has been linked to corneal wound healing.[4] In light of these findings, mitochondria-targeted therapies emerge as an attractive strategy for supporting wound healing.[3] For example, mitochondria-targeted antioxidant therapy was shown in a rabbit model to improve corneal epithelial healing following UV damage.[6]

Various studies have elegantly demonstrated that transfer of mitochondria between cells can promote cell survival, proliferation, and wound healing. Although the precise molecular mechanisms that underlie the effects of mitochondrial transfer are not fully defined, mitochondrial transfer was shown to restore cellular bioenergetics and alter the metabolic state of recipient cells.[10,13,14,17,39–49] In CECs, transfer of mitochondria from MSCs via tunneling nanotubes was shown to improve cell survival under conditions of oxidative stress. Mechanistically, the effect of the transferred mitochondria was correlated to increased respiration and reduced oxidative stress signaling such as NFkB.[18] Transplantation of MSC onto rabbit corneas was further shown to promote wound healing following alkali burn,[18] suggesting that mitochondrial augmentation may support corneal wound healing in vivo.

In the present study, we explored the ability to promote corneal epithelial wound healing via direct transplantation of isolated mitochondria. We show that mitochondria are spontaneously internalized by primary hCECs, and promote a wound healing effect by increasing cell migration in vitro. We further show that mitochondria transplantation increases the proliferation of hCECs by approximately 15%. These findings suggested that the fresh pool of internalized mitochondria promoted hCEC wound healing via a combination of enhanced proliferation and cell migration. Following demonstrating the effect of mitochondria transplantation in primary human cells, we set to establish the proficiency of mitochondria transplantation to support wound healing in vivo, in a mouse model of alkali burn, a common and serious corneal injury in humans. We demonstrated that topical administration of mitochondria to the ocular surface of mice accelerated and improved the repair of the cornea following alkali burn. Specifically, whereas the control mice still had substantial corneal damage 4 days post alkali burn, mice that received mitochondria had no apparent epithelial defect. This effect was evident both in fluorescein staining as well as in histology staining,

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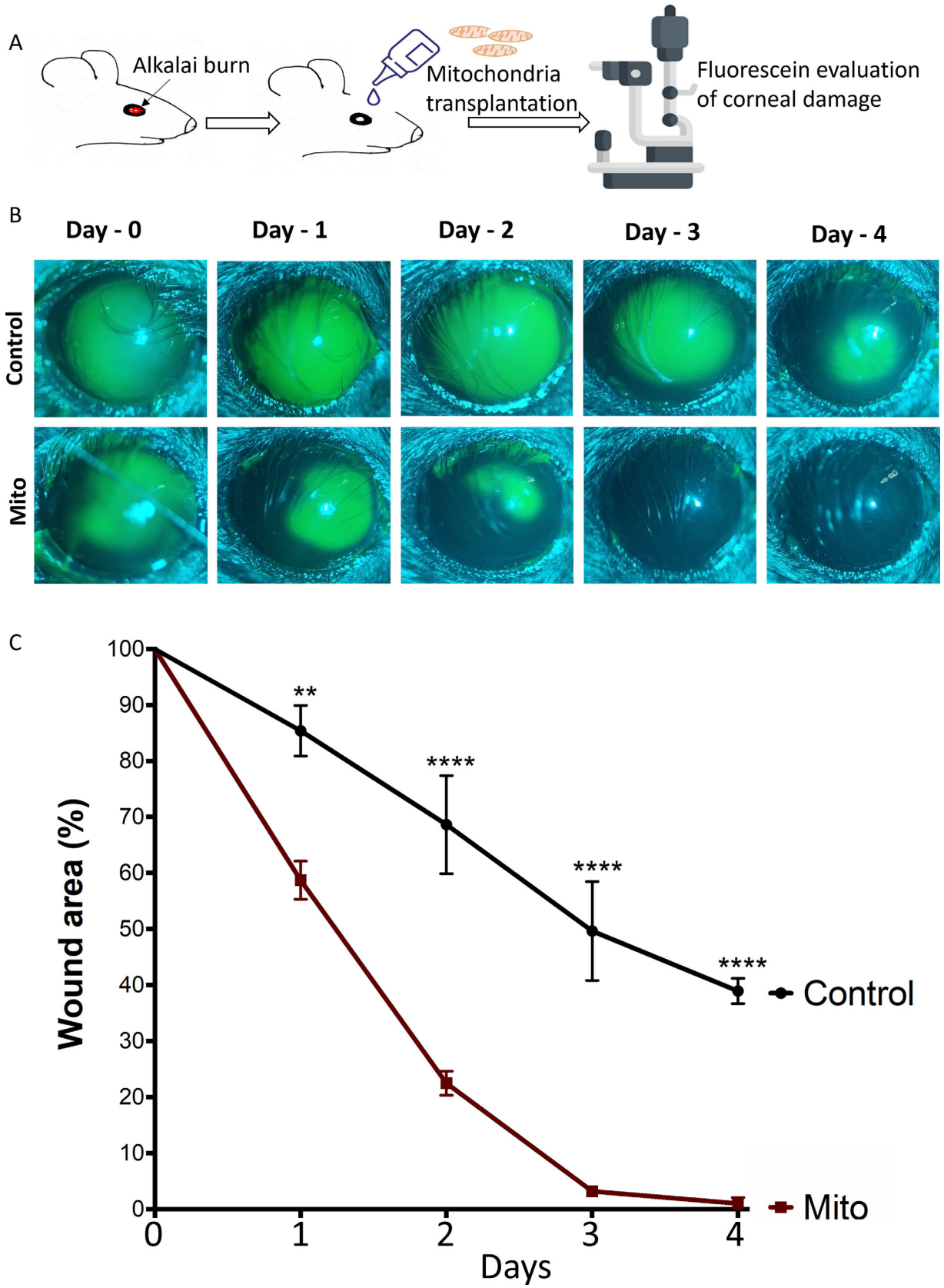

Figure 6. Mitochondria transplantation promotes corneal recovery after alkali burn. (**A**) Schematic representation of the experiment. Mitochondria were isolated from mouse livers and administered daily to the ocular surface of mice after corneal alkali burn. The wound area was measured daily by fluorescein staining and slit microscopy imaging. (**B**) Slit-lamp image of fluorescein staining from day 0 (day of alkali injury) and daily for 4 consecutive days. *Upper panels* show representative images of control mice, and the *lower panels* show representative images of mice receiving mitochondria transplantation. (**C**) The wound size was determined based on fluorescein staining, relative to the

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total cornea area. The graph shows the wound closure as the percentage from the original wound area, from day 1 to day 4 of control and mitochondria-transplanted mice ($n = 12$ mice per group, analyzed in 3 independent experiments, 4 alkali-injured eyes from 4 mice per group in each experiment). Separate mitochondria isolations were prepared for each experiment. $^{**}P = 0.002$; $^{****}P < 0.0001$.

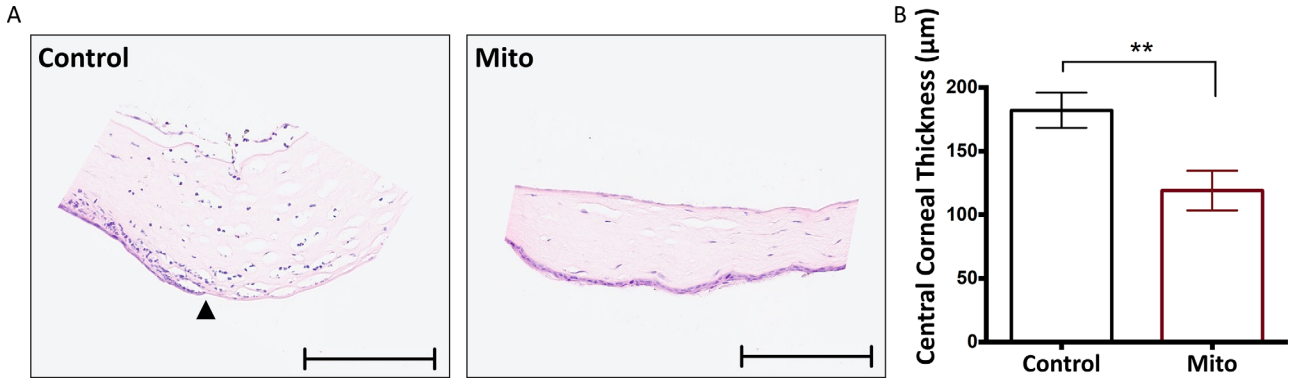

Figure 7. Mitochondria transplanted corneas have normal central thickness and stromal organization after alkali burn. (**A**) representative H&E images of corneas harvested 4 days post alkali damage from mice receiving mitochondria transplantation (Mito) or vehicle as control (Control). *Arrowhead* denotes epithelial thinning and loss. Scale bar = 200 µM. (**B**) Central corneal thickness was determined from the mean of three measurements at the central region of each cornea histology section ($n = 3$ different corneas from control mice and 3 different corneas from mitochondria-transplanted mice). $P = 0.002$.

which demonstrated full re-epithelization of corneas following mitochondria transplantation, compared to substantial epithelial thinning and cell loss in control mice. Moreover, although the corneas following alkali burn had a thickened and disorganized stromal lamella, the stroma of corneas from mitochondria-transplanted mice decreased to normal stromal thickness, suggestive of normal corneal hydration.

Our findings suggest that ATP synthesis by internalized mitochondria contributes to the wound healing effect of mitochondria transplantation. A subtle difference in response between mice that received frozen, inactive, mitochondria and the control group was insignificant. Nevertheless, residual ATP production activity after freeze-thaw cycles is evident in the ATP production assays and may

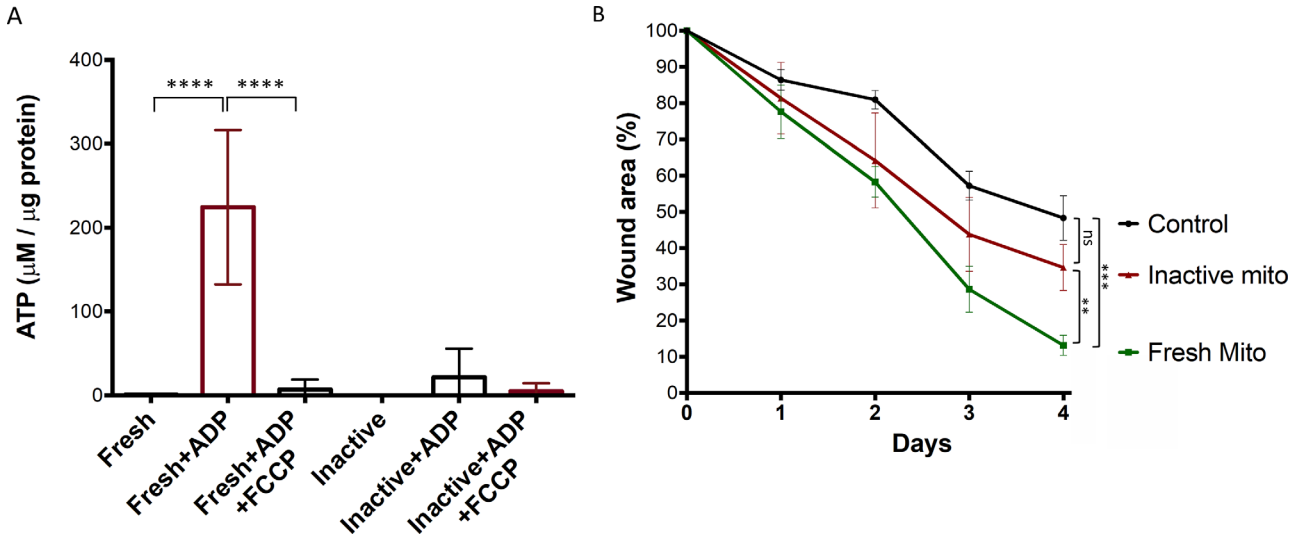

Figure 8. Transplanted mitochondria induce wound healing via ATP production. (**A**) Mitochondria were isolated from mouse livers and analyzed in in vitro ATP production assays either immediately after isolation (fresh) or following five repeated freeze-thaw cycles (inactive). ATP was measured in basal state (20 µM succinate) or with the addition of ADP (20 µM succinate + 150 µM ADP) or ADP + FCCP as an uncoupler control as indicated (20 µM succinate + 150 µM ADP + 12 µM FCCP, $n = 4$ individual mitochondria preparations). (**B**) Mice inflicted with corneal alkali burn were treated with either buffer (control), fresh mitochondria, or inactive mitochondria (prepared as described in **A**). The corneal wound size was determined daily based on fluorescein staining, relative to the total cornea area. The graph shows the wound closure as percentage from the original wound area, from day 1 to day 4. Fresh mitochondria versus control: $^{***}P = 0.0005$. Inactive mitochondria vs control: not significant (ns). Fresh mitochondria versus inactive mitochondria: $^{*}P = 0.02$ (n [control] = 9 mice; n [fresh mitochondria] = 9 mice; n [inactive mitochondria] = 9 mice). Analyzed in three independent experiments (3 mice per group in each) with separate mitochondria isolations for each experiment.

explain this difference. Alternatively, the transfer of mitochondrial components—such as substrates for ATP production, proteins, lipids, nucleic acids, etc.—could promote wound healing in recipient cells. This may occur either by indirectly enhancing ATP production in recipient cell mitochondria or through mechanisms independent of ATP production.

We estimated that approximately 20% of corneal cells internalized exogenous mitochondria during the in vivo incubation. It is interesting to discuss the relatively limited levels of mitochondria internalization percentages reported by previous studies, albeit the notable beneficial biological effects demonstrated in these investigations. For example, mitochondrial uptake was only 3% to 7% in ischemic cardiac myocytes/heart while producing a cell protective effect.[50,51] Likewise, internalization of 9% in brain cells of diabetic mice improved cognitive function,[52] and 7% to 14% internalization in neuronal cells was shown to confer neuroprotection under hypoxia.[53] These findings suggest that the estimation of incorporated mitochondria may be limited by factors such as the threshold of detection, turnover rates, and fusion with the endogenous mitochondrial network. However, despite these challenges, the consistent demonstration of beneficial biological effects resulting from the incorporation of exogenous mitochondria underscores their therapeutic potential, as evidenced by the work of others and our own research.

Improper or slow corneal epithelial repair may lead to extensive scarring and vison impairment, even in cases of low or medium severity injuries. Such cases may require surgical interventions, such as corneal transplantation. It is therefore of importance to develop treatments that support the efficient and rapid wound healing of the cornea, which may spare the need of transplantation. Immediate treatment of corneal chemical injuries aims mostly at evacuating and neutralizing the foreign material by irrigation with copious amounts of water or saline, administration of topical medications, such as antibiotics and corticosteroids to control infection and inflammation, and administration of lubricants. Traditional management of severe or refractory lesions that are unresponsive to standard care include pressure patching, amniotic membrane transplantation, therapeutic contact lens, and tarsorrhaphy. In recent years, biological based therapies have shown promising results in the treatment of corneal irregularities. For example, use of autologous serum eye drops, from peripheral blood or umbilical cord, was shown efficacious in several studies for treatment of corneal epithelial defects.[54–61] The beneficial effect was suggested to be mainly driven by the presence of growth factors, such as epidermal growth factor, neuronotrophic growth factor, and insulin-like growth factor, known to contribute to corneal epithelial maintenance and regeneration.[62,63] Studies examining cell-based therapy have also demonstrated promising results for treatment of corneal injuries. Preclinical studies have shown that stem cell therapy enhances corneal repair.[64–69] Although the mechanism of this effect was not fully elucidated, secreted factors were implicated in mediating the effect.[70–74] Interestingly, transfer of mitochondria from MSCs to corneal epithelial cells was shown to protect from oxidative stress and mitochondrial damage.[75] Therefore, transfer of mitochondria may contribute to the corneal recovery after burn following MSC therapy. Still, lack of response and persistent epithelial defects are commonly observed in clinical practice. Thus, additional treatment possibilities and new approaches are still needed.

Our findings highlight a direct role of mitochondria in promoting corneal wound healing, thus expanding the repertoire of known biological materials which contribute to corneal epithelial regeneration. We present for the first time the plausibility of administering topical mitochondria to the ocular surface as a treatment for ocular surface injuries and epithelial defects, which both accelerates and improves repair. In the last years, the mitochondria transplantation approach has shown positive results in preclinical and clinical trials like in pediatric patients with ischemia–reperfusion injury who were on extracorporeal membrane oxygenation (ECMO)[76] and in patients with Pearson syndrome.[77] Although still in its infancy, the potential to ameliorate mitochondrial function to mitigate stress and promote regeneration via mitochondria transplantation holds great therapeutic potential. Corneal epithelial defects are a very common problem, and are often hard to treat. Our finding suggests that mitochondrial transplantation may contribute in the management of corneal pathologies in the future.

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