Modulation of Contact Inhibition by ZO-1/ZONAB Gene Transfer—A New Strategy to Increase the Endothelial Cell Density of Corneal Grafts

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Submitted: November 28, 2018
Accepted: June 24, 2019

Citation: Kampik D, Basche M, Georgiadis A, et al. Modulation of contact inhibition by ZO-1/ZONAB gene transfer—a new strategy to increase the endothelial cell density of corneal grafts. *Invest Ophthalmol Vis Sci*. 2019;60:3170–3177. https://doi.org/10.1167/iovs.18-26260

Purpose. Endothelial cell density (ECD) is the principal factor determining the success of corneal transplants. Here we explored a strategy to increase corneal ECD in human explants via modulation of the ZO-1/ZONAB pathway. In multiple cell types, ZO-1 maintains G1 cell cycle arrest via cytoplasmic sequestration of the mitosis-inducing transcription factor ZONAB. In this study, we assessed the effects of lentiviral vector-mediated downregulation of ZO-1 or overexpression of ZONAB upon ECD and the integrity of the endothelial monolayer.

Methods. HIV-based lentiviral vectors were used to deliver either constitutively expressed ZONAB (LNT-ZONAB), or a small hairpin RNA targeting ZO-1 (LNT-shZO1). Human corneal specimens were bisected and each half was exposed to either treatment or control vector. After 1 week in ex vivo culture, effects were assessed by quantitative RT-PCR, immunohistochemistry, and ECD assessment.

Results. LNT-shZO1 achieved an ∼45% knockdown of ZO-1 mRNA in corneal endothelial cells cultured ex vivo, reduced ZO-1 staining, and did not affect morphologic endothelial monolayer integrity. The proliferative effect of LNT-shZO1 correlated with control ECD but not with donor age. Within a low-ECD cohort an ∼30% increase in ECD was observed. LNT-ZONAB achieved a >200-fold overexpression of ZONAB mRNA, which led to an ∼25% increase in ECD.

Conclusions. ZO-1 downregulation or ZONAB upregulation increases corneal ECD via interference with contact inhibition and cell cycle control. With further development, such approaches might provide a means for improving ECD in donor corneas before transplantation.

Keywords: corneal endothelial cells, ZO-1/ZONAB, gene therapy, cell proliferation

Corneal transplantation is the most common form of transplantation surgery undertaken with approximately 3500 grafts performed annually in the United Kingdom,[1] 75,000 in the United States,[2] and 185,000 globally.[3] Endothelial disease that results in cellular decompensation and an associated loss of corneal transparency is a major indication for corneal transplantation,[2,4] and posttransplantation endothelial decompensation represents a major cause of eventual graft failure.[5,6] This argues for strict selection of donor corneas on the basis of a sufficiently high endothelial cell density (ECD). However, in many countries such selection has led to a shortage of donor corneas deemed suitable for transplantation. The increasing average age of tissue donors contributes further to lowering average ECD[7] in donated corneas. A further decline in the ECD of donor material is associated with extended storage in organ culture.[8,9] In UK eye banks the number of corneas deemed unsuitable for transplantation increased from 17% to 33% after 28 days of storage.[8] Finally, transplantation surgery itself can result in endothelial cells losses of up to 23%.[10]

In humans, corneal endothelial cells do not divide at a sufficient rate to replace lost cells. After complete formation of the endothelial monolayer at birth, a constant cell loss can be observed throughout life. Histologic and in vivo confocal microscopy studies indicate that the average rate of cell loss is 0.3% to 0.6% per year in normal subjects throughout adult life, and is 10-fold higher after a corneal transplant.[11–14] Several mechanisms of active repression of corneal endothelial cell reentry to the cell cycle have been identified: (1) a lack of response to positive growth factors, (2) the antiproliferative effect of TGF-β2 in the aqueous humor, and (3) contact inhibition though mature cell-cell junctions.[15–17] Contact inhibition, that is, the presence of mature cell–cell junctions, is considered the most important factor inhibiting endothelial proliferation in vivo.[18] Release of cells from their confluent monolayer to create a single cell suspension is the principal requirement for culturing primary corneal endothelial cells in vitro. Mitotic inhibition in vivo has been investigated in rats, where corneal endothelial cells continue to mature after birth.[19] Proliferation of endothelial cells does not cease until stable cell-

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cell contacts, represented by connexin-43 and ZO-1 expression, and cell-substrate contacts, represented by an organized pattern of fibronectin and collagen IV, are established. Senoo et al.[20] have attempted to disrupt the endothelial monolayer of human corneal samples ex vivo by using EDTA, and assessed proliferation by Ki67 immunostaining. EDTA disruption alone is not sufficient to induce proliferation, nor is incubation in mitogen-containing medium. A combination of EDTA-mediated contact disruption and mitogenic factors do promote proliferation, but this method does not increase total ECD.[21]

The ZO-1/ZONAB pathway has been identified as a key molecular pathway controlling contact inhibition.[22,23] ZO-1, a membrane protein located at the intracellular side of tight junctions (but that is not essential to their assembly[24]), binds a Y-box transcription factor, *ZO-1*–associated nucleic acid binding protein (ZONAB). Subcellular localization of ZONAB determines its activity as a transcription factor. In confluent cells containing many tight junctions, ZONAB is bound to the SH3 domain of ZO-1. If, however, low cell density prevents the establishment of tight junctions, ZONAB is released from its binding partner and is free to accumulate within the nucleus. Here it acts as a repressor of the *erb-2* gene, a tyrosine kinase coreceptor regulating epithelial differentiation.[23] Furthermore, free ZONAB associates with cell division kinase 4 (CDK4), a central regulator of the cell cycle. By facilitating nuclear accumulation of CDK4, ZONAB promotes S phase entry.[22] ZONAB also acts as a transcription factor directly regulating the expression of proliferating cell nuclear antigen (*PCNA*), encoding a crucial component of the DNA replication machinery, and C*yclin D1*, a key regulator of G1/S-phase transition.[25] These mechanisms lead to proliferation and eventually increased cell density with formation of tight junctions. By sequestering ZONAB at the tight junctions, ZO-1 reduces cell proliferation in a cell density–dependent manner (Supplementary Fig. S1). This concept has been investigated in vitro in MDCK and MCF-10A cells, mammalian epithelial cells derived from the kidney and mammary gland, respectively. Overexpression of *ZO-1* reduces proliferation, while overexpression of *ZONAB* increases cell density even in mature monolayers.[22,25] We have also investigated this signalling pathway in vivo, in the retinal pigment epithelium of mice.[26] Both overexpression of *ZONAB* or knockdown of *ZO-1* promotes RPE proliferation. Modulation of the ZO-1/ZONAB pathway may therefore provide an effective strategy to overcome contact inhibition and induce cell proliferation in corneal endothelial cells. In this study, we assessed the effects of lentiviral vector-mediated knockdown of *ZO-1* or overexpression of *ZONAB* on the proliferation of corneal endothelial cells in excised human corneas, analyzing the change in ECD as the primary outcome measure.

Methods

Recombinant Lentiviral Vectors

Second-generation, self-inactivating HIV-based lentiviral vectors pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) were produced by transient transfection of three plasmids into HEK-293T cells as previously described.[27,28] The pHR'SIN plasmid backbone was used as transfer vector delivering either *ZONAB* or *hrGFP* under the ubiquitous active spleen focus-forming virus (SFFV) promoter, as described previously.[25] For knockdown, a small hairpin targeting ZO-1 was first cloned into the mU6pro plasmid and then transferred into the pHR'SIN plasmid. The sense strand of the targeting hairpins[26] was 5′-AAGATAGTTTGGCAGCAAGAG-3′. Vector in cell culture supernatant was purified by filtration (0.45-µm filter), concentrated by ultracentrifugation (65,000*g* for 105 minutes), and resuspended in OptiMEM (Invitrogen, Paisley, UK), yielding vector concentrated to 10^8 to 10^9 infectious particles per milliliter.

Corneal Samples

Two sources of human corneal samples were used.

1. Whole human corneas from deceased multiorgan donors, unsuitable for transplantation owing to reasons unrelated to endothelial function, were obtained from Moorfields Eye Bank, London, United Kingdom, after consent for use in research was obtained from relatives. These will be referred to as "whole corneas." Whole corneas were maintained in culture no longer than 10 days before the start of experiments; during this time they were stored either in Cornea Medium I without Dextran T500 (Biochrom, Berlin, Germany) supplemented with 2% fetal calf serum or in Cornea Medium II with 5% Dextran T500 (Biochrom) supplemented with 2% fetal calf serum. In total this study used three samples of this type.
2. Most samples were full-thickness cornea specimens excised at corneal transplantation, obtained from Moorfields Eye Hospital, London, United Kingdom, with informed consent. These will be referred to as "corneal buttons." In most cases corneal buttons derived from patients undergoing penetrating keratoplasty for keratoconus. Specimen collection and arrangements for consent were managed in accordance with the Declaration of Helsinki and approved by the local research ethics committee (ref. 09/H0721/6). Only specimens with an apparently healthy endothelium were collected. In total this study used 24 samples of this type.

Corneal samples were usually bisected with a diamond blade before use. Whole corneas were bisected upon receipt into the laboratory, whereas corneal buttons were bisected by the surgeon immediately after excision. In all cases care was taken when bisecting to minimize damage to the endothelium. Upon receipt into the laboratory all corneas were maintained at 37°C in Cornea Medium I (no dextran, serum free). Whole corneas were kept in 25 mL medium in T25 tissue culture flasks, secured to float in the middle of the flask by a suture. Corneal buttons were kept in 8 mL medium in six-well plates with care taken to keep corneas lying endothelium-side up. In both cases the medium was replaced weekly.

Transduction of Corneal Samples With Lentiviral Vectors

For viral transduction of endothelium, human corneal specimens were incubated in OptiMEM with concentrated virus preparation for 4 hours at 37°C. To achieve the highest possible vector concentration, the volume of medium was minimized to 250 µL. In the case of larger samples (bisected whole corneas) this was achieved by tilting the container at 45° to increase the depth of the media so as to cover the sample. Multiplicity of infection (MOI) was calculated as the number of infectious particles per target cell during transduction. The total number of endothelial cells per whole cornea was estimated to be 3×10^5. Using 50 µL of a typical lentiviral vector preparation (titer: 2–3 $\times$ 10^8 infectious particles/mL) for one whole cornea resulted in an MOI of ~40. For an excised corneal specimen, endothelial cell number was estimated at 1.5×10^5 cells. To maximize the potential effect size, the MOI in each experiment was the maximum permitted by the viral titer available. After transduction corneal samples were cultured in Corneal Medium I for a further 5 to 7 days before assessment.

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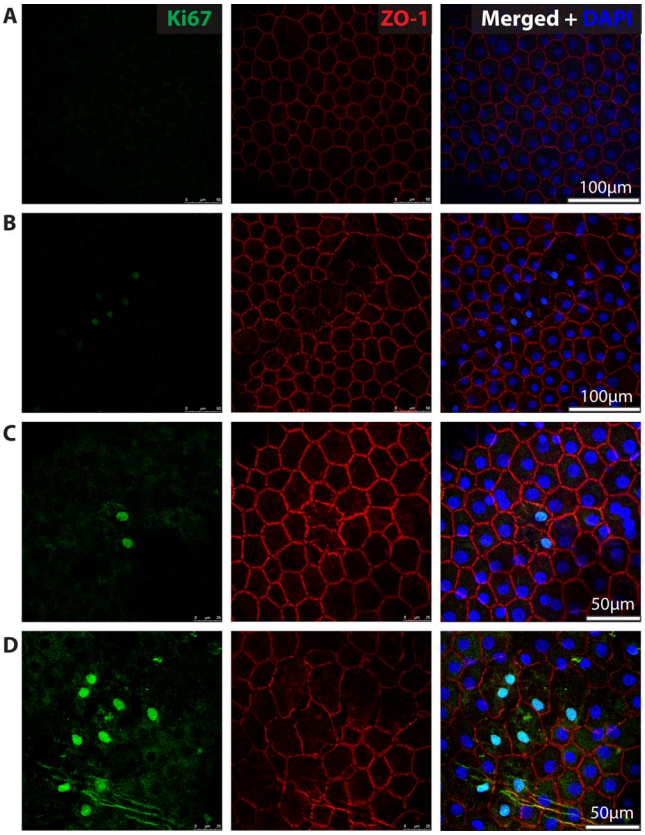

FIGURE 1. Correlation between breakdown of tight junctions and proliferation in human corneal endothelium. Flat-mount of full-thickness cornea fixed within 1 hour of excision. Ki67-positive nuclei can only be found in cells with reduced ZO-1 expression. (**A**) Midperipheral endothelium, intact cell-cell contacts (ZO-1), no Ki67 staining. (**B**) Midperipheral endothelium, disrupted cell-cell boundaries along Descemet's membrane fold with Ki67-positive nuclei. (**C**, **D**) Possible wounds to the endothelium associated with loose cell-cell contacts and Ki67-positive nuclei.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Descemet's membrane with corneal endothelium was peeled off under an operating microscope and immediately lysed for total RNA extraction by using a silica membrane/salt buffer system (RNeasy Mini Kit, Qiagen, Hilden, Germany). For each experiment, equal amounts of RNA were immediately reverse transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's directions. Primers listed in Supplementary Table S1 were used for standard qPCR reactions with a dark quencher dye (Roche, Basel, Switzerland). The PCR was run on an ABI Prism 7900HT Fast Real-time Sequence Detection System (Applied Biosystems, Waltham, MA, USA) using 10 to 70 ng of cDNA. Three identical replicates were run for each sample, and the averaged C_T value was used for relative quantification using the comparative C_T method against 18s rRNA as reference gene ($\Delta\Delta C_T$ method). REST software was used for qPCR analysis (Relative Expression Software Tool, REST 2009, version 2.0.13).

Immunohistochemistry

Corneal samples were washed in PBS; fixed in 2% paraformaldehyde in PBS, pH 7.4, for 15 minutes; permeabilized with 0.2% Triton X-100 for 8 minutes; and blocked in 2% normal goat serum, 2% fetal calf serum in PBS for 1 hour, all at room temperature. Primary antibody was diluted in blocking solution and incubated with the sample for 2 hours at room temperature or overnight at 4°C (anti–ZO-1 and anti-ZONAB, Invitrogen; anti-Ki67, Sigma-Aldrich, Gillingham, UK). After washing twice in PBS, sample was incubated with secondary antibody (Alexa Fluor Secondary Antibodies; Invitrogen) diluted 1:500 in block solution (1 hour at room temperature). For flat-mounting, four radial cuts were made to flatten corneal samples. They were attached to a glass microscopic slide endothelium-side up, using cyanoacrylate glue; submerged in fluorescent mounting medium (Agilent, Santa Clara, CA, USA); and covered with a glass coverslip, using pressure-sensitive adhesive as a spacer. Pressure was applied to flatten Descemet's membrane folds. Specimens were imaged by using an upright confocal laser-scanning microscope (Leica TCS SPE DM5500 Q; Leica Mirosystems, Wetzlar, Germany) with the manufacturer's software (Leica LAS AF, version 2.4.1).

ECD Assessment

Corneal ECD was quantified in flat-mounts, with the endothelium en face, stained with 4′,6-diamidino-2-phenylindole (DAPI) by counting nuclei in micrographs obtained on the confocal laser scanning microscope, with a defined image size of 275 × 275 µm (~0.076 mm^2). The focus plane was chosen so as to only image endothelial nuclei by adjusting aperture and laser power. In some highly irregular endothelial surfaces, a z-stack of approximately five images was overlaid. At least six images were taken per sample, scanning through the flat-mount in a standardized method. A distance of at least one microscope field of view was left between micrographs counted. An area of 0.5 mm from the cut edges of the cornea was excluded. If the area of interest revealed gross damage to the endothelium, it was also excluded. DAPI-positive nuclei were counted manually by using ImageJ (version 1.46a; http://rsbweb.nih.gov/ij/, provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Counts of the six or more images were averaged, comprising a minimum total of 1000 to 1400 nuclei, and cell density was expressed as cells/mm^2. To obtain standardized and unbiased counts, treatment and control samples were masked at the beginning of the experiment by an independent observer. Equal numbers of micrographs were counted for treatment and control sample. Treatment and control sample of one cornea were analyzed as pairs. If any cornea had apparent extensive endothelial damage that prevented standardized imaging and counting, both the treatment and the corresponding control sample were excluded from further analysis.

Statistical Analysis

In all cases where statistics were applied, no more than two groups or variables were compared and the threshold applied for statistical significance was $P \leq 0.05$. All tests used to compare two groups were parametric two-tailed *t*-tests. All error bars represent the standard error of the mean unless otherwise stated. In cases where bisected corneas were exposed to two independent treatments and compared as a group, the test applied was a paired two-tailed *t*-test, with the two halves of the same corneal sample being treated as pairs. In cases where multiple measurements were performed in a single bisected pair, the test applied was an unpaired two-tailed *t*-test. In cases where correlation between two variables was assessed, a linear regression analysis was performed and both the P value and Pearson's R were presented as measures of correlation.

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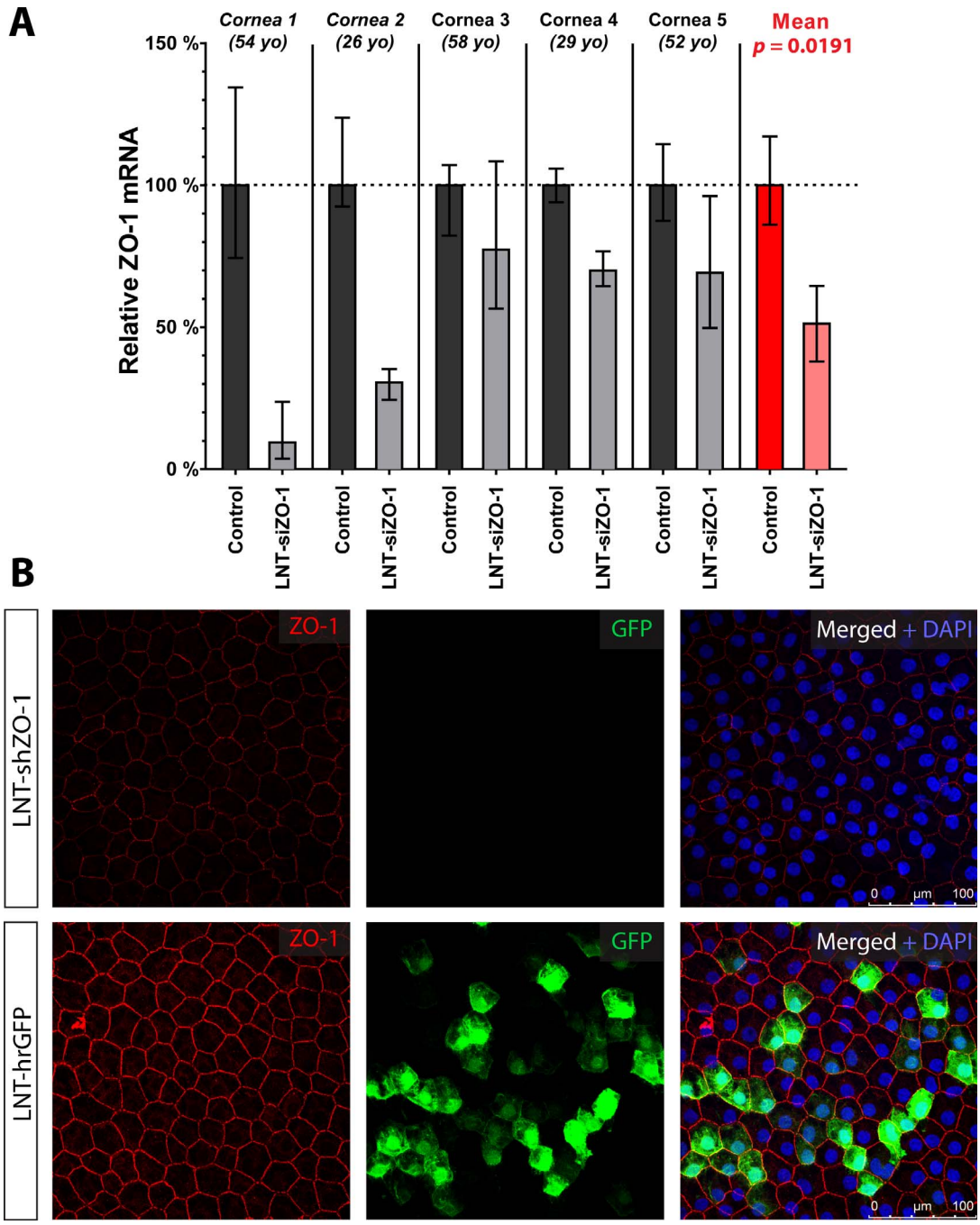

Figure 2. Downregulation of ZO-1 by LNT-shZO1. (**A**) qRT-PCR was performed in five different bisected corneal specimens (donors: 26–58 years of age) after endothelial cell isolation 6 days post transduction. LNT-shZO1–transduced samples show a mean 51% ± 30% reduction in ZO-1 mRNA compared to LNT-hrGFP–transduced control hemicorneas ($P = 0.0191$, paired *t*-test, error bars = 95% CI). (**B**) Example of a cornea excised from a keratoconus patient 1 week after transduction. Reduced ZO-1 immunostaining (*red*) is seen in the LNT-shZO1–transduced half, while the endothelial layer remains intact. Normal ZO-1 staining is seen in the control half transduced with LNT-hrGFP.

Results

Correlation Between Loss of Contact Inhibition and Proliferation in Endothelium

In normal human corneal endothelium, occasional signs of cell proliferation can be observed in the form of cells that are positive for Ki67 or bromodeoxyuridine (BrdU) staining, or in the form of an occasional mitotic figure. To analyze any correlation between these endogenous proliferating cells and contact inhibition, we first examined normal human corneal endothelium not maintained in long-term ex vivo culture. Areas of obvious iatrogenic endothelial damage, such as cut edges, were excluded from analysis. In total three samples (two buttons and one whole cornea) were examined.

Immunostaining for ZO-1 in these samples revealed an almost ubiquitously intact endothelial monolayer with Ki67-negative nuclei, as shown in Figure 1A. However, very rarely, small isolated areas displaying both an impaired/interrupted pattern of ZO-1 staining and Ki67-positive nuclei were found

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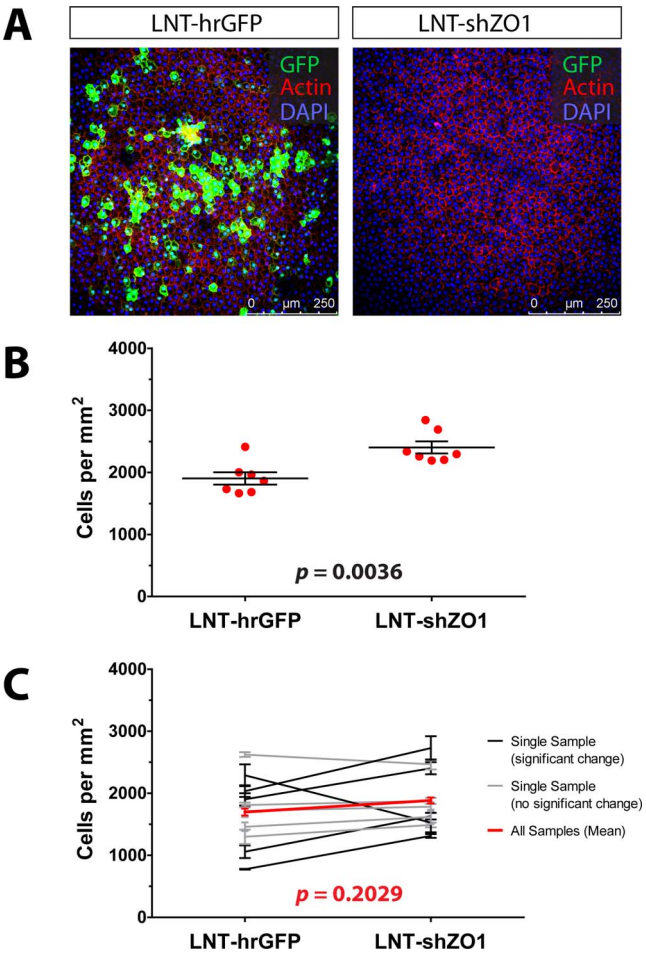

Figure 3. Effect of downregulation of ZO-1 in human corneal endothelium ex vivo. (**A**) Integrity of the endothelial cell monolayer assessed 7 days post vector transduction by f-actin and DAPI staining of corneal flat mounts. Transgene expression (GFP) is visible in the control sample. *Scale bar*: 250 µm. (**B**) Effect of LNT-shZO1 upon ECD in a single corneal sample. ECD quantified in seven micrographs (area of each: 0.076 mm^2) per hemicornea and calculated as cells/mm^2. Sample shown derives from a 20-year-old keratoconus patient and shows a 30% increase in ECD ($P = 0.0036$, unpaired *t*-test). (**C**) Effect of LNT-shZO1 upon ECD across all 10 corneal samples (donor age range, 17 to 81 years). No significant change in ECD was induced by LNT-shZO1 ($P = 0.2029$, paired *t*-test). *Red line*: Mean data. *Black lines*: Individual samples with significant ($P \leq 0.05$) changes in ECD. *Gray lines*: Samples in which no such difference was found. Unpaired *t*-tests.

(Figs. 1B, 1C), indicating a disruption of tight junctions and cell-cell contact. These regions of loosened contact inhibition appeared to correspond to folds in Descemet's membrane or areas of possible damage and appeared more common in the periphery of the sample (Fig. 1D) than in the central or midperipheral regions.

Effect of Knockdown of ZO-1 Upon ECD

To disrupt tight junctions, we first sought to downregulate *ZO-1* mRNA by using a lentiviral vector (LNT-shZO1) expressing an shRNA against ZO-1 that has been previously shown to effectively reduce ZO-1 protein levels in the human non-transformed mammary epithelial cell line MCF-10A.[25] The effect of lentivirus-mediated downregulation of *ZO-1* mRNA on human corneal endothelium was assessed by quantitative RT-PCR. Five corneal samples, whole corneas (×2), or corneal buttons (×3) were bisected and transduced with either LNT-shZO1 or LNT-hrGFP as control, at an MOI of ∼40 for 4 hours. After 6 days there was a 51% ± 30% (mean ± SD) reduction of *ZO-1* mRNA in LNT-shZO1–transduced cells in comparison with LNT-hrGFP–transduced controls (two-tailed paired *t*-test, $P = 0.0191$) (Fig. 2A). Endothelium transduced with LNT-shZO1 also revealed no obvious changes in the integrity of the monolayer, as visualized by ZO-1 immunohistochemistry on flat-mounts ($n = 2$). A representative example area of a keratoconus specimen (28-year-old male patient) is shown in Figure 2B. Five days after transduction, the half transduced with LNT-shZO1 displayed uniformly reduced immunostaining for ZO-1 in comparison with an LNT-hrGFP–transduced control.

ECD was the principal outcome measure for ZO-1 downregulation in situ. In total, 10 corneal buttons (age range, 17–81 years) were analyzed for ECD. Each was bisected and half was transduced with either LNT-shZO1 or LNT-hrGFP at an MOI of ∼50 to 100 for 4 hours, the latter acting as a control for both transduction of and variation in initial ECD. Ex vivo culture time was 7 days. Visualization of GFP in the LNT-hrGFP–transduced samples allowed an approximate assessment of transduction efficacy, estimated as ranging between 30% and 60%. The monolayer remained intact at the end of the experiment, as verified by f-actin (phalloidin) staining with DAPI nuclear counterstain. Representative immunofluorescence micrographs are shown in Figure 3A. Assessment of ECD (six 0.076-mm^2 areas per sample, Fig. 3B) revealed that when analyzed individually, transduction by LNT-shZO1 resulted in a significant increase in ECD in 5 of 10 samples. When analyzed as a whole there was an average ECD increase of 18% within the group, but this did not reach significance ($P = 0.2029$, two-tailed paired *t*-test) (Fig. 3C).

Proliferative Effect of LNT-shZO1 Correlates With ECD but Not With Donor Age

We next assessed whether the observed increases in ECD induced by LNT-shZO1 correlated with either the age or the cell density of the control sample. Analyzing the same cohort of 10 samples (age range, 17–81 years) we found no significant correlation between donor age and ECD (Fig. 4A) and no significant correlation between donor age and the change in ECD induced by LNT-shZO1 (Fig. 4B). However, a significant negative correlation ($P = 0.01$) was found between the change in ECD induced by LNT-shZO1 and the ECD in LNT-hrGFP–treated controls (Fig. 4C). From these findings we split our cohort of 10 into two equal groups on the basis of control ECD and reanalyzed for the proliferative effect of LNT-shZO1. The high ECD group was not responsive to LNT-shZO1 ($P = 0.803$) (Fig. 4D), but in the low ECD group there was a significant average increase of 31% ($P = 0.042$, two-tailed paired *t*-test) (Fig. 4E). These results suggest that the initial ECD of the donor cornea is the critical factor determining the proliferating effect of LNT-shZO1, and that this factor may be more important than age.

Effect of Overexpression of ZONAB Upon ECD

ZO-1 regulates proliferation by sequestering and thereby inhibiting the activity of ZONAB, a transcription factor. Thus, a complementary approach to downregulating ZO-1 would be overexpression of ZONAB. We therefore examined the effect of transducing the endothelium with a lentiviral vector expressing ZONAB (LNT-ZONAB).[26] Corneal buttons from nine patients aged between 21 and 33 years were bisected and each half transduced with either LNT-ZONAB or LNT-hrGFP at an MOI of ∼50 to 100 for 4 hours. Specimens were maintained

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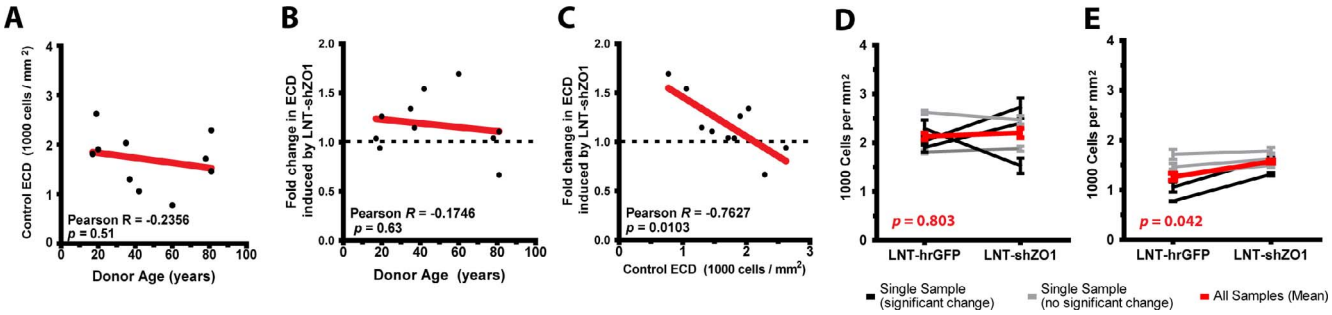

Figure 4. Effect of LNT-shZO1 in relation to donor age and control ECD. (**A**) CEC density in control condition (LNT-hrGFP) correlated with age of donor for all samples. No significant correlation. Pearson's $R = -0.24$, $P = 0.51$. (**B**) Responsiveness to LNT-shZO1, measured as ratio between ECD in LNT-shZO1– and LNT-hrGFP–treated halves, correlated with age of donor for all samples. No significant correlation. Pearson's $R = -0.17$, $P = 0.63$. (**C**) Responsiveness to LNT-shZO1, measured as ratio between ECD in LNT-shZO1– and LNT-hrGFP–treated halves, correlated with ECD of control half. Significant negative correlation. Pearson's $R = -0.76$, $P = 0.01$. (**D**) Effect of LNT-shZO1 upon ECD across the five corneal samples with the highest control ECD. No significant change in ECD was induced by LNT-shZO1 ($P = 0.803$, paired *t*-test). (**E**) Effect of LNT-shZO1 upon ECD across the five corneal samples with the lowest control ECD. A significant mean increase in ECD of 31% was induced by LNT-shZO1 ($P = 0.042$, paired *t*-test). *Red line*: Mean data. *Black lines*: Individual samples with significant ($P \leq 0.05$) changes in ECD. *Gray lines*: Samples in which no such difference was found. Unpaired *t*-tests.

in ex vivo culture for 7 days. Four of the samples were analyzed by RT-PCR and confirmed overexpression of ZONAB (Fig. 5B). The remaining five samples were immunostained for ZONAB to verify transgene overexpression, and f-actin to assess cell monolayer integrity (Fig. 5A). Vector-mediated ZONAB was also shown to correlate and colocalize with Ki67 activation (Supplementary Fig. S2). These samples were also analyzed for changes in ECD resulting from transduction with LNT-ZONAB over at least six areas per sample (Fig. 5C). Following LNT-ZONAB transduction, all five samples displayed a significant increase in ECD when compared with controls (Fig. 5D). The overall mean increase in ECD was 26% ($P = 0.0047$, two-tailed paired *t*-test), with a maximum increase of 33%.

Discussion

Adult human corneal endothelium is subject to strong inhibition of cell proliferation at a molecular level. Contact inhibition, one molecular axis of which being the ZO-1/ZONAB pathway, is recognized to have the strongest inhibitory effect.[18] Mechanical release from contact inhibition is the basic principle used to generate a primary cell culture of nearly all cells and can be applied to the culture of endothelial cells, which then proliferate, albeit slowly, in vitro. By confocal microscopy of healthy corneal endothelium, we have shown disruption in ZO-1 staining to be associated with Ki67 positivity, further confirming the close correlation between the breakdown of cell-cell contacts and proliferation. The observation of such a loss of intercellular contact in a normal cornea so shortly after excision suggests that in human corneal endothelium, breakdown of tight junctions can occur naturally to allow cell cycle progression, and indeed there is some evidence for such limited endothelial renewal in vivo.[29] In endothelium of eyes cultivated in the eye bank over the longer term, we also observed BrdU uptake after endothelial wounding, that is, mechanically disrupting cell-cell contacts (Supplementary Fig. S3). Despite these observations, it is clear that any inherent capability of the corneal endothelium to regenerate by mitosis is insufficient to compensate natural age-related endothelial cell loss, let alone the increased loss occurring after corneal transplantation, surgery, or in disease states. One possible reason for this might be that contact inhibition continues to be maintained even during cell loss, as even in endothelium with very low cell density cell-cell contacts remain intact over many years, resulting in enlarged cells.[30–32] By overcoming contact inhibition at a molecular level, we were able to induce endothelial cell proliferation and thereby increase cell density.

One of the first reported studies involving disruption of contact inhibition in eye bank corneas was undertaken by Senoo et al.[20] By depleting calcium with EDTA in the presence of mitogens, they could induce Ki67-positive mitotic figures. However, this approach does not increase overall cell density.[21] Nakano et al.[33] have achieved downregulation of connexin 43, a component of gap junctions that is required for contact inhibition, by using antisense oligodeoxynucleotides or small interfering RNA, in an in vivo rat corneal scrape injury model. This induced Ki67 upregulation even in unwounded rat epithelium, but overall cell density was not assessed and human samples were not tested.

This study is the first to show an increase in corneal ECD after molecular breakdown of contact inhibition in human corneal endothelium ex vivo. Both vectors used in this study could generate significant increases in ECD of similar magnitudes, although the effect of LNT-shZO1 only became significant within a low ECD cohort. By contrast the ECD increases mediated by LNT-ZONAB were significant across a broader range of densities. This may imply that directly increasing the levels of ZONAB is more effective than indirect modulation of ZONAB levels through ZO-1. Indeed, our qPCR analysis revealed ZO-1 knockdown to be variable, and it is possible that both this and any inefficiencies in translating the effect downstream via ZONAB translocation and CDK4 activation may be responsible for the more limited effect of ZO-1 knockdown. It is also important to note that control ECD is not directly equivalent to initial, untreated ECD. Control samples were transduced by control vector (LNT-hrGFP) and spent a week in organ culture and we cannot rule out any deleterious effects of these interventions. However, any resulting change in ECD is controlled for by sham treatment and we would expect any such effect to be both mild and relatively consistent. Control density can therefore be considered reasonably representative of the untreated density. These observations validate our hypothesis, which was based upon several studies that have established a correlation between either ZO-1 downregulation or ZONAB upregulation and proliferation.[22,25,26] Our results confirm the role of the ZO-1/ZONAB pathway to be a significant molecular basis of contact inhibition in the corneal endothelium. This study focused upon the most clinically relevant endpoint, ECD, as its outcome measure and did not further examine the downstream effects

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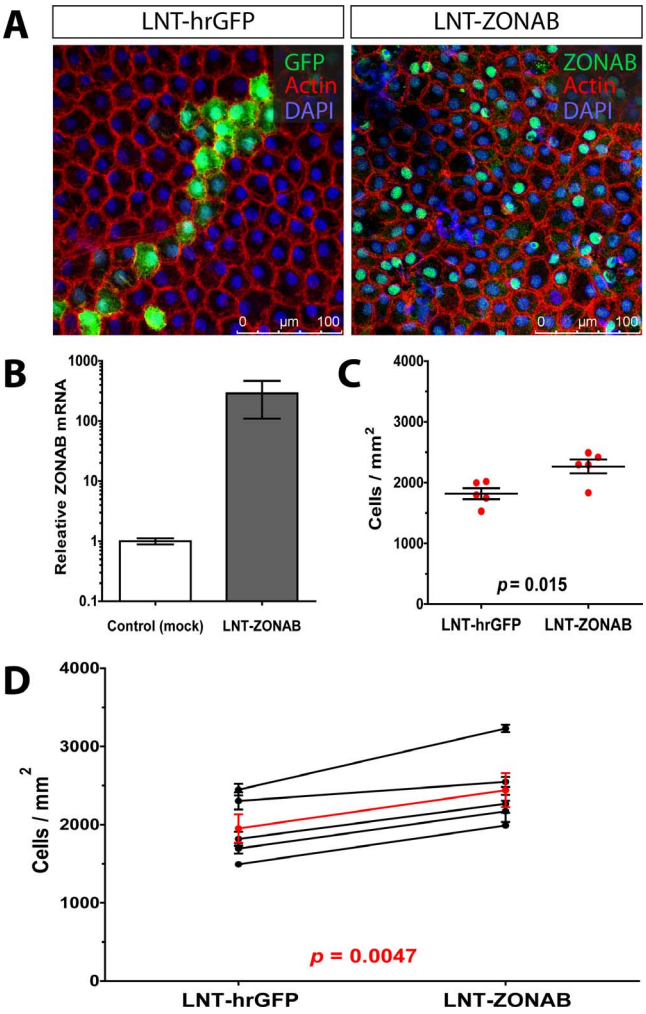

FIGURE 5. Effect of overexpression of *ZONAB* in human corneal endothelium ex vivo. (**A**) Representative immunofluorescence micrographs showing the integrity of the endothelial cell monolayer as assessed by f-actin and DAPI staining of corneal flat mounts, and transgene expression of *ZONAB* or GFP after 7 days. (**B**) qRT-PCR was performed in four bisected corneal specimens after endothelial cell isolation 6 days post transduction. LNT-ZONAB–transduced samples show a more than 200-fold overexpression of ZONAB mRNA compared to untransduced controls. (**C**) Effect of LNT-ZONAB upon ECD in a single corneal sample. ECD quantified in seven micrographs (area of each: 0.076 mm^2) per hemicornea, calculated as cells/mm^2. Sample shown derives from a 31-year-old keratoconus patient and shows a 25% increase in ECD ($P = 0.015$, unpaired *t*-test). (**D**) Effect of LNT-shZO1 upon ECD across five corneal samples (donor age range, 21 to 32 years). A significant mean increase in ECD of 26% was induced by LNT-ZONAB ($P = 0.0047$, paired *t*-test). Mean data are represented by the *red line*. All individual samples (*black lines*) also show significant increases in ECD ($P \leq 0.05$, unpaired *t*-tests).

of ZO-1/ZONAB modulation. However, Sourisseau et al.[25] have shown that within the mammary epithelial cell line MCF-10A, transcription of Cyclin D1 (a key regulator of G_1/S-phase transition) and PCNA (a crucial component of the DNA replication machinery) are both directly regulated by ZONAB at the transcriptional level.

Previous studies attempting to mediate cell cycle modulation within corneal endothelial cells have observed that the proliferative response of the corneal endothelium is dependent on the donor age.[34] We did not observe such an effect in our LNT-shZO1 cohort, nor did we observe any correlation between donor age and ECD. This is probably because our cohort was relatively small. Irrespective of age, however, we did observe a strong negative correlation between responsiveness to LNT-shZO1 and ECD. This finding underpins the importance of contact inhibition in corneal endothelium. It would appear that in low-density endothelium, where contact inhibition is reduced, there is space for the cells to divide and they are thus able to replicate in response to ZO-1 modulation. In dense endothelium, stronger contact inhibition prevents this response. It should also be noted that in low-ECD samples the vector MOI would be proportionally increased and it is possible that this may drive a stronger, dosage-driven response. However, given that the MOI in all cases was set relatively high we would not expect this to be the primary factor determining the proliferative response.

Unfortunately, owing to the sample size, we were unable to draw any conclusions with regard to the effect of age on responsiveness to treatment. It is possible that the lower ECD more generally associated with older donors might render such a cornea more responsive to ZO-1 modulation. However, it is possible that senescence of the endothelial cell population might counteract this. The ability of endothelial cells to be established in culture is reduced with donor age and the kinetics of cell cycle entry is different in young and old cells.[35] However, any age-related decrease in proliferative capacity is not caused by replicative senescence, that is, the shortening of telomeres with age. Telomere length remains constant over the lifetime of the endothelium.[36,37] Instead it has been proposed that nuclear DNA damage from oxidative stress is the main reason why some older cells are incapable of undergoing a full cell cycle.[38] Further studies are required to establish the capacity of older endothelial cells to respond to the modulation of contact inhibition.

In conclusion, we demonstrated that knockdown of ZO-1 expression and overexpression of ZONAB is possible in human corneal endothelium ex vivo. Harmful effects on the endothelium were not evident in the samples tested. The modulation in expression of ZO-1 and ZONAB was associated with approximately a 30% increase in cell density and we found some evidence to suggest that this approach was less effective in corneas from donors older than 70 years. Although the increase seems relatively modest, this would be sufficient to rescue a substantial number of donor corneas that would otherwise be discarded owing to ECD below 2000 cells/mm^2, the standard applied in eye banks. Changes in ECD might be increased further if cell transduction were more efficient than the ~30% to 60% observed in this study, due to changes in vector properties or design. Moreover, we have not investigated a dual approach in which both ZO-1 and ZONAB expression are modulated in the same specimens by exposure to both vectors. The observation that none of the treated corneas reached an ECD > 3000 cells/mm^2 (the density found in humans at birth) might indicate that intrinsic mechanisms of contact inhibition are strong enough to prevent any uncontrolled proliferation above this density level.

Further work to investigate the safety of ZO-1 and ZONAB modulation and testing of endothelium function in corneas modified ex vivo will be required before any potential clinical application. However, with appropriate safety measures in place, such as transient viral gene transfer mediated by a nonintegrating lentiviral vector,[39] this approach may become a useful method to improve the quality of corneas for transplantation.

Acknowledgments

The authors thank Selina Azam for providing technical support in relation to viral vector production.

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Supported by Fight for Sight and the National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital National Health Service Foundation Trust and University College London Institute of Ophthalmology.

Disclosure: **D. Kampik**, None; **M. Basche**, None; **A. Georgiadis**, None; **U.F.O. Luhmann**, None; **D.F. Larkin**, None; **A.J. Smith**, None; **R.R. Ali**, None

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