Induction of Replication in Human Corneal Endothelial Cells by E2F2 Transcription Factor cDNA Transfer

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Purpose. Corneal endothelial cells in humans do not replicate to any meaningful extent. Diminishing density of the cell monolayer with age and in the disease states is a major cause of loss of corneal transparency. This study was conducted to test the hypothesis that overexpression of the transcription factor E2F2 results in replication in nonproliferating human corneal endothelial cells.

Methods. Whole human corneas were incubated for 2 hours in a solution of recombinant $E1^-/E3^-$ adenovirus incorporating cDNA encoding E2F2 and green fluorescent protein (GFP) under control of a bidirectional promoter and subsequently maintained in ex vivo culture. Control specimens were incubated with an identical virus bearing the GFP sequence only, or virus-free medium. Efficiency of gene transfer and localization was examined by fluorescence microscopy. En face confocal microscopy of the corneal endothelial surface was used to image recombinant E2F2 expression. 5-bromodeoxyuridine (BrdU) incorporation was used to examine progression to the S phase. Changes in density of the corneal endothelium were quantified by specular microscopy and counting of trypan-blue–stained cells. Apoptosis was tested with a TUNEL assay.

Results. Recombinant proteins were expressed predominantly in the endothelium and in a high proportion of endothelial cells in the first week after exposure to virus, diminishing thereafter. Compared with the control, transduction with E2F2 resulted in progression from the G_1 to the S phase in a significant number of cells and in increased cell density. Apoptosis was not found to any significant extent.

Conclusions. Overexpression of the transcription factor E2F2 in nonmitotic human corneal endothelial cells results in short-term expression, cell-cycle progression, and increased monolayer cell density. (*Invest Ophthalmol Vis Sci.* 2005;46:3597–3603) DOI:10.1167/iovs.04-0551

The monolayer of endothelial cells (ECs) on the internal surface of the cornea is critical to maintenance of corneal transparency and normal visual acuity. These cells have been shown to possess a metabolically coupled and osmotically active HCO_3^- transport system that regulates hydration of the corneal stroma.[1] Unlike other species in which endothelial cell replication readily occurs in vivo to maintain normal cell density,[2,3] human ECs do not undergo mitosis to any meaningful extent to replace lost or injured cells. Even in the absence of disease, there is a gradual decrease in EC density throughout life (~4000 cells/mm^2 in infancy to ~1500 in the elderly).[4,5] Death of ECs is also a major limiting factor in maintenance of scarce donor corneas ex vivo before transplantation, with falling EC density during storage accounting for approximately 30% of corneas being discarded, the endothelium being considered of insufficient quality for transplantation.[6] After surgical trauma, inflammation (such as corneal transplant rejection) or degenerative disease, human endothelium responds to cell loss by cell migration and spreading rather than by mitosis. If EC density is reduced below approximately 400 cells/mm^2, the monolayer decompensates and aqueous humor enters the stroma, resulting in corneal edema, the irreversible loss of transparency, and, ultimately, blindness.[7] The only treatment to restore vision in such eyes is replacement of whole-thickness cornea (i.e., epithelium, stroma, and endothelium) with a corneal transplant. The impact of endothelial decompensation is indicated by the fact that corneal disease restricted to that monolayer that is sufficient to cause loss of vision is the cause of more than half of all corneal transplantations[5,8,9] (~3000 per year of which are performed in the United Kingdom and ~40,000 in the United States).

The process of mitosis is controlled centrally within any one cell by the cell-division cycle. On mitogenic stimulation, resting cells enter the G_1 phase, which prepares the cell for DNA duplication. Advance from the G_1 into the S phase, in which DNA synthesis occurs, commits a cell-to-cell division, or if aborted, to apoptosis.[10] The subsequent G_2 and M phases allow a cell to complete DNA duplication and generate two daughter cells. In G_1 the retinoblastoma susceptibility gene product (pRb) is hypophosphorylated and in complex with a member of the early gene 2 factor (E2F) family of transcription factors. E2F/pRb complexes are transcriptional repressors and prevent cells from undergoing DNA replication. In preparation for DNA synthesis, pRb is phosphorylated by cyclin-dependent kinases (cdks), and E2F-pRb complexes dissociate. Free E2F acts as a transcriptional activator of genes that drive DNA synthesis.[11] After cell replication, this regulatory cycle is completed by dephosphorylation of pRb, which binds free E2F and prevents reentry into the cell cycle.

Analysis of isolated human corneal ECs by flow cytometric cell-cycle analysis and cyclin immunohistochemistry has demonstrated cell-cycle arrest. Staining for cell-cycle proteins, including Ki67, indicates that corneal ECs in vivo are arrested before the mid-G_1 phase.[12,13] Recent evidence from tissue culture studies indicates that human ECs from both young and older donors retain proliferative capacity.[14–16] Thus, it should be possible to take advantage of that capacity to stimulate proliferation and increase EC density. One possible strategy is transfection of human ECs with simian virus 40 large tumor antigen (SV40 T Ag), an oncoprotein that blocks exogenous TGF-β signaling while simultaneously phosphorylating pRB,[17] which has been demonstrated to induce proliferation by over-

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Supported by a training fellowship from T. F. C. Frost Charitable Trust (JCMcA) and National Eye Institute Grant R01 EY12700 (NCJ).

Submitted for publication May 18, 2004; revised October 21, 2004, and February 9, 2005; accepted February 9, 2005.

Disclosure: **J.C. McAlister**, None; **N.C. Joyce**, None; **D.L. Harris**, None; **R.R. Ali**, None; **D.F.P. Larkin**, None

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coming G_1- to S-phase arrest.[18,19] Although the resultant endothelium retained in vitro its characteristic morphology, a therapeutic strategy of even transient expression of a known proto-oncogene in human tissue would raise long-term safety concerns. However, proliferation after overexpression of E2F in postmitotic neuronal[20] and lens[21] tissue further supports modulation of this transcriptional network as a strategy to induce EC replication. As expression studies of the seven members of the E2F family have shown that the overexpression of E2F1 to -3 induce a high mitotic index (proportion of cells in a population entering the S phase) but that E2F2 induces significantly less apoptosis than E2F1 and -3,[22] we selected E2F2 as the most appropriate for overexpression in a direct molecular approach, to overcome cell-cycle arrest in the human corneal endothelium. We have demonstrated that overexpression of E2F2 leads to cell-cycle progression in endothelial cells in rabbit corneas in ex vivo culture.[23] In further studies described herein, we examined the effect of overexpression of this transcription factor on cell-cycle progression in human corneas.

Materials and Methods

Recombinant Adenovirus Production

An E1/E3-deleted recombinant adenovirus was amplified from a single plaque in transcomplementing 293A cells, a human embryonal kidney cell line stably transfected with adenovirus E1 and E3 region genes. After 48 hours, the infected cells were harvested, and virus was released by three freeze–thaw cycles. Viruses were purified by two cycles of cesium chloride density gradient centrifugation, passed through a 0.2-μm filter, and stored at −70°C. The infectious titer of viruses in each stock was determined by plaque assay.[24]

Shuttle Plasmid Preparation

Human E2F2 cDNA[25] (gift of Joseph Nevins, Duke University, Durham, NC) was subcloned into an adenoviral shuttle plasmid. The expression cassette of the shuttle plasmid was constructed to direct transcription from a bidirectional cytomegalovirus (CMV) promoter, using the human β-globin intervening sequence IVS II and a polyadenylation signal. The upstream gene was a second-generation membrane-bound form of green fluorescent protein (GFP), Us9GFP[26,27] (gift of Andrew Beavis, Princeton University, Princeton, NJ), whereas a downstream multiple cloning site was present after the human β-globin intron. Correct insertion was verified by sequencing. The cassette expressing Us9GFP alone (AdU; Fig. 1A) and the second cassette coexpressing human E2F2 in addition to Us9GFP (AdUE, Fig. 1B) were then subcloned into the recombinant adenovirus genome.

Adenovirus Infection of Human Corneas

Pairs of human corneas were used in experiments, consent for use in research having been obtained from Moorfields Eye Bank (London, UK), the CTS Eye Bank (Bristol, UK), and the National Disease Research Interchange (Philadelphia, PA). The corneas were managed in accordance with the guidelines of the Declaration of Helsinki. There were general contraindications to use in transplantation such as donor neurologic disease or unavailability of blood for virological testing. In these studies, we excluded corneas from donors with known corneal disease or who had undergone extended periods of chemotherapy. There was no age exclusion. Corneas were washed twice in warm serum-free medium (SFM; OptiMEM-I; Invitrogen-Gibco, Gaithersburg, MD) and placed in 1.0 mL of serum free-medium containing recombinant adenovirus for 2 hours at 37°C. One cornea of each pair was incubated with AdUE and the other with either AdU or virus-free medium. After a preliminary study titrating the virus concentration against the level of transgene expression at day 3 and which indicated a transduction efficiency of 60% to 75% at these concentrations (data not shown),

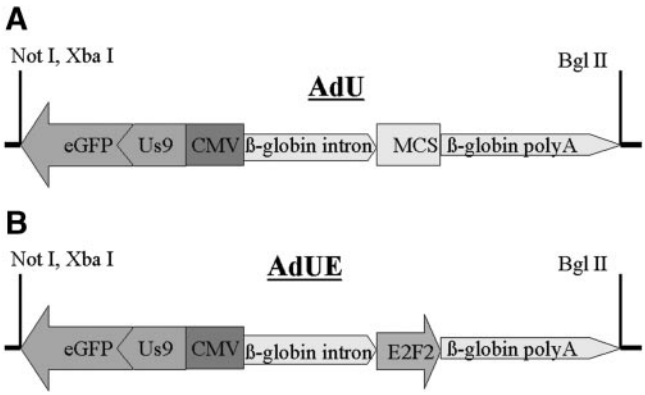

Figure 1. The expression cassette used in the recombinant adenovirus used a bidirectional CMV promoter. A control virus, AdU, was engineered incorporating only Us9-GFP cDNA (**A**). In AdUE, full-length cDNA encoding human E2F2 was inserted into the multiple cloning site (MCS) and expressed in one direction, and Us9-GFP cDNA was expressed in the opposite direction (**B**).

1.8×10^7 pfu/mL SFM of AdU and 1.5×10^7 pfu/mL of AdUE was used. Corneas were then washed three times before being placed in Eagle's minimal essential medium, 2% heat-inactivated fetal bovine serum (FBS), HEPES buffer, 26 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin and amphotericin B (Bristol Eye Bank Culture Medium). Corneas were maintained in culture in a volume of 80 mL medium at 37°C for up to 3 weeks and prepared for analysis as described in the following sections.

Detection of Gene Transfer to Cornea

Corneas from two different donors were washed in OptiMEM-I, incubated for 2 hours in AdU at 1.8×10^7 pfu/mL SFM and washed as indicated earlier. After incubation for 48 hours at 37°C, corneas were washed in phosphate-buffered saline (PBS), fixed for 10 minutes in ice-cold 100% methanol, and washed in PBS. Frozen 6-μm transverse sections were cut and mounted on glass slides with antifade medium containing propidium iodide (PI; Vectashield; Vector Laboratories, Burlingame, CA) to stain all nuclei. Fluorescence was visualized by microscope (Eclipse E800 microscope with a VFM Epifluorescence Attachment; Nikon Inc., Melville, NY) equipped with a digital camera and software (Spot camera with Ver. 1.1 CE software; Diagnostic Instruments, Sterling Heights, MI).

Quantification of Efficiency of Gene Transfer to Endothelium

Corneas were washed in SFM, incubated in AdU at 1.8×10^7 pfu/mL SFM, washed, and incubated for 48 hours. Corneas were then washed in PBS, fixed in ice-cold methanol for 10 minutes, washed, and incubated at 4°C overnight in 5% dextran (molecular mass, >144,000 kDa; Sigma-Aldrich). Small radial cuts were made to flatten the cornea. Tissue was mounted endothelium side up on glass slides in antifade medium with PI (Vectashield; Vector Laboratories) . Samples were visualized by fluorescence confocal microscopy (model TCS 4D microscope; Leica, Deerfield, IL, equipped with a model DMRBE laser and ScanWare ver. 4.2 software; Leitz Lasertechnik, Heidelberg, Germany). Five 40× images were taken of central corneal endothelium. Total PI-positive nuclei and GFP-positive cells were counted. The relative percent of Us9-GFP expressing cells per 20× area was determined by dividing the number of GFP-positive cells by the total number of PI-stained nuclei times 100. This experiment was repeated using corneas from at least two different donors. The mean percentage of transduced cells was then calculated.

Detection of E2F2 Protein Expression in Endothelial Cells

Donor corneas were washed and infected with the control vector (AdU) or vector containing full-length cDNA for E2F2 (AdUE) under the conditions described earlier for adenoviral infection of human corneas. After infection, corneas were washed in SFM and then incubated in Bristol Eye Bank Organ Culture Medium for 0 or 48 hours or 2 weeks. Corneas were then washed in PBS, fixed for 10 minutes in ice-cold methanol, washed in PBS, and immunostained for E2F2 as follows. All steps were conducted at room temperature. Corneas were incubated for 10 minutes in 1% Triton X-100 to permeabilize the cells and then were washed in PBS. Nonspecific antibody binding was blocked by incubation for 10 minutes in 4% bovine serum albumin (BSA: Fisher Scientific, Pittsburgh, PA) in PBS. Corneas were then incubated for 2 hours in a 1:800 dilution of polyclonal anti-E2F2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody concentration has been shown to detect mainly E2F2-overexpressing cells rather than the lower endogenous levels of E2F2.[23] After washing and reincubation in blocking buffer, corneas were incubated for 1 hour in 1:200 dilution of rhodamine-conjugated donkey anti-rabbit IgG (Rhodamine Red-X; Jackson ImmunoResearch, West Grove, PA), washed, incubated in 5% high-molecular-weight dextran as described earlier, and then mounted in antifade medium without PI, before visualization by fluorescence confocal microscopy.

Detection of S-Phase Entry by BrdU Incorporation

Corneal pairs from a 28- and a 70-year-old donor were used for these studies. One cornea from each donor was cut in quarters and infected with AdU; the other cornea was quartered and infected with AdUE. This permitted duplicate samples from each donor to be infected with either AdU or AdUE and examined 48 hours and 1 week after infection. After infection, half the tissue from each of the two donors was incubated for 48 hours in medium containing 5-bromodeoxyuridine (BrdU) labeling reagent (GE Healthcare, Piscataway, NJ) at a 1:1000 dilution. The remaining corneal quarters were incubated for 48 hours in the same medium, but without BrdU. At the 48-hour time point, BrdU was added and the tissue incubated for an additional 5 days (= 7 days postinfection). After incubation, the tissue was washed in PBS, fixed for 10 minutes in ice-cold methanol, washed, and then permeabilized for 10 minutes at room temperature with 1% Triton X-100 in PBS. Nonspecific binding was blocked by incubation for 10 minutes in 4% BSA. Corneal tissue was then incubated for 2 hours in undiluted monoclonal anti-BrdU (GE Healthcare). After washing and incubation for 10 minutes in blocking buffer, corneas were incubated for 1 hour in 1:200 rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) and 1 μM Cy5 nuclear stain (TO-PRO3; Molecular Probes, Eugene, OR), washed, and incubated in 5% dextran, as described earlier. Corneas were mounted endothelium side up in mounting medium without PI. Positive staining was viewed by fluorescence confocal microscopy.

Endothelial Specular Microscopy

To facilitate EC density quantification by specular microscopy, deswelling of the thickened corneal stroma was necessary. Corneas were first incubated overnight in SFM supplemented with 5% dextrose at 4°C. The endothelial surface of corneas was imaged en face by a specular microscope (Konan, Hyogo, Japan), and cell density quantified by analysis software (Kerato Analyzer EKA-98; Konan). One hundred cells per image field were used to generate each EC density measurement. The density and variance for each cornea was based on three to five specular images (typically four paracentral and one central) from the endothelial surface.

Trypan Blue Staining

The corneal specimens were divided into quadrants, placed in 5% dextrose for 3 minutes, and fixed in 100% ethanol for 90 seconds followed by 50% trypan blue (Sigma-Aldrich) staining for 3 minutes. The specimens were immediately mounted on glass slides endothelium side down and allowed to dehydrate partially for 2 minutes before cell density was assessed based on the intracellular staining pattern. En face preparations were examined by light microscopy at ×40 magnification in the central and peripheral cornea, peripheral being defined as the zone of endothelium adjacent to Schwalbe's line. A charge-coupled device (CCD) video camera coupled to image-capture software (LC200; Leica) was used to generate images to quantify EC density. Three to five representative fields in both the center and periphery of the corneal specimen were examined. A 0.1-mm^2 grid was used to measure cell density in each image. Mean EC density and variance were calculated.

TUNEL Assay

One cornea of a donor pair was washed and infected for 2 hours in AdU or AdUE, as described earlier. After a wash in SFM, the corneas were incubated for 48 hours. A TUNEL assay for detection of apoptotic cells was conducted (ApopTag Red in Situ Apoptosis Detection Kit; Chemicon/Serologicals Corp., Temecula, CA). After the TUNEL assay, corneas were stained with the nuclear stain (TO-PRO3; Molecular Probes), to permit visualization of all nuclei. Corneas were then incubated in dextran as described earlier and mounted endothelium side up in antifade medium. Staining was detected by fluorescence confocal microscopy. Rabbit corneal endothelial cells were cultured on glass slides according to published protocols,[28] grown to confluence, and used as a positive control for apoptosis. Cultures were incubated for 24 hours in 0.5 mM H_2O_2 (Sigma-Aldrich) diluted in Medium-199, 50 μM gentamicin (Invitrogen) and 10% FBS. The TUNEL assay was performed, and coverslips were mounted using antifade medium with 4′,6′-diamino-2-phenylindole (DAPI). Staining was visualized by conventional fluorescence microscopy.

Statistical Analysis

Differences in cell density and cell-cycle phase proportions in groups after incubation with recombinant viruses and virus-free medium were analyzed by Student's *t*-test, with $P < 0.05$ deemed significant.

Results

Efficient Adenovirus-Mediated GFP Gene Transfer to Human Corneas Ex Vivo

After infection with AdU and subsequent incubation, transverse corneal sections were then cut and analyzed by fluorescence microscopy to determine which cells within the cornea were infected by the adenovirus and expressed Us9-GFP. Multiple transverse sections of corneas from two different donors yielded the same results. No fluorescence was visible in central corneal epithelium or in stromal fibroblasts. Us9-GFP fluorescence was easily detected in the cytoplasm of the endothelium, indicating that the adenovirus primarily infects endothelial cells (Fig. 2). At 48 hours after infection with AdU, the mean efficiency of gene transfer to the endothelium calculated from two different donors was 75%.

Kinetics of E2F2 Overexpression in Endothelium of Corneas Ex Vivo

Anti-E2F2 antibody was used to detect and localize overexpressed E2F2 in corneal endothelial cells. Figure 3 demonstrates an expected lack of positive immunostaining for overexpressed E2F2 in human corneal endothelium immediately after incubation in AdUE (Fig. 3A). By 48 hours after infection (Fig. 3B), Us9-GFP protein was detected by cytoplasmic fluorescence in several cells. A low level of positive cytoplasmic staining for E2F2 was observed in some cells, although these cells usually did not show fluorescence. Of importance is the

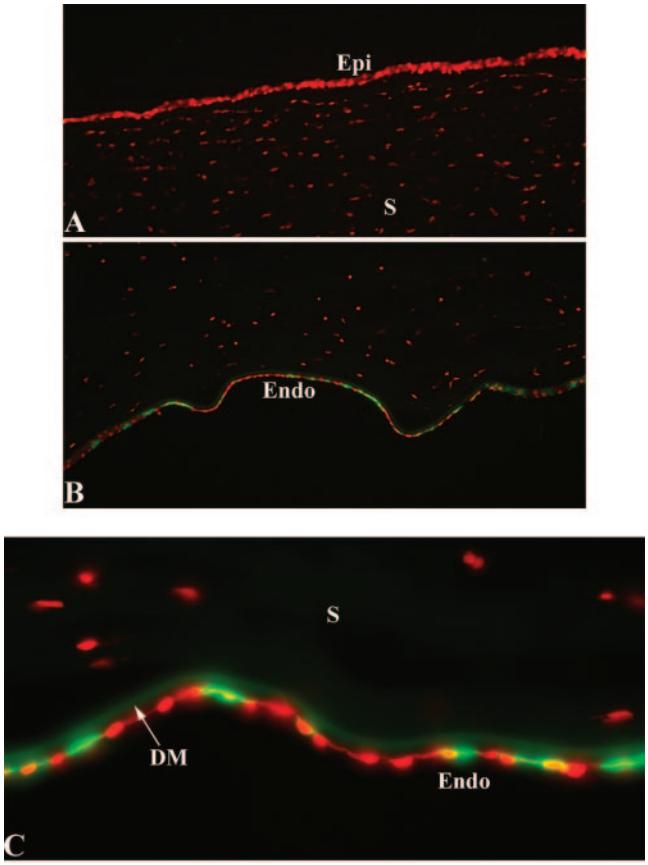

FIGURE 2. Location of Us9-GFP expression in transverse sections of cornea. Corneas from two donors were infected with the AdU vector at 1.8×10^7 pfu/mL for 2 hours at 37°C, washed, and incubated for 48 hours in medium containing 2% FBS. Representative transverse sections from the cornea of a 22-year-old donor (**A**) demonstrate a lack of Us9-GFP fluorescence (*green*) in corneal epithelial cells (Epi) and anterior stromal keratocytes (S). The epithelium was relatively thin, typical of epithelial morphology after corneal storage. Strong Us9-GFP expression was consistently observed in the cytoplasm of endothelial cells (Endo) (**B**, **C**). No Us9-GFP expression was observed in posterior stromal fibroblasts. (**C**) *Light green* autofluorescence in Descemet's membrane (DM) was visible at higher magnification. *Red*: PI. Final magnification: (**A**, **B**) ×100; (**C**) ×400.

fact that intense nuclear staining for E2F2 was present in cells coexpressing Us9-GFP, indicating that these cells were infected with the AdUE vector and overexpressing E2F2. The transcription factor E2F2 is known to be active only when it is localized to the nucleus,[29] suggesting that E2F2 was active in ECs within 48 hours after infection. By 2 weeks after infection (Fig. 3C), overexpression of E2F2 was found to persist in endothelial cell nuclei, as indicated by coexpression with Us9-GFP, but the relative number of cells coexpressing Us9-GFP and E2F2 appeared to be decreased.

Evidence for Endothelial Cell Cycle S-Phase Entry Determined by BrdU Incorporation

The E2F2 transcription factor is active during the G_1-to-S-phase transition in the cell cycle. As such, overexpression of E2F2 should facilitate progression of nonproliferating corneal ECs into the S phase. To determine the effect of E2F2 overexpression on S-phase entry, corneas from a 28- and a 70-year old donor were infected with AdUE or the control AdU vector and then incubated in the presence of BrdU. Figure 4 presents representative confocal images of the endothelium stained for BrdU. After incubation in BrdU for 48 hours, few-to-no (range 0%–4.5%) BrdU-positive nuclei were observed in AdU-treated corneas (Fig. 4A). It should be noted that a similar range of BrdU-positive nuclei was observed in mock-infected endothelium (data not shown), suggesting that adenoviral infection alone was not responsible for this staining. In contrast, BrdU-positive endothelial nuclei were easily detected in corneas treated with AdUE and postincubated for the same length of time (Figs. 4B, 4C). It should be noted that 48 hours after AdUE infection, there were more positive endothelial nuclei in the cornea obtained from the 28-year-old (Fig. 4B) than in that from the 70-year-old donor (Fig. 4C). To determine whether cells from the older donor would enter the S phase later than 48 hours after infection, AdUE-infected corneas were incubated for 48 hours in medium without BrdU, after which BrdU was added. At the 1-week time point (BrdU was present in the medium from 48 to 168 hours after infection), very few BrdU-positive cells were observed in the AdU-treated endothelium (Fig. 4D); however, positive staining was clearly observed in endothelial nuclei of the cornea infected with AdUE (Fig. 4E). Of particular interest is the fact that BrdU-positive mitotic figures were observed in the cornea from the 70-year-old donor 1 week after infection, indicating the ability of cells from older donors to undergo normal mitosis (Fig. 4F).

Effect of Recombinant E2F2 Expression on Corneal Endothelial Cell Density

EC density was examined in corneas maintained in culture for up to 3 weeks after exposure to the virus. In a preliminary study, cell density measurements were made after both specular microscopy and trypan blue staining at 1-, 2-, and 3-week intervals and were found to correlate closely (data not shown). In subsequent experiments, on account of the manipulation of corneas and incubation in dextran required for each examination, specular microscopy was used only for baseline and intermediate measurements. As tissue fixation was required for

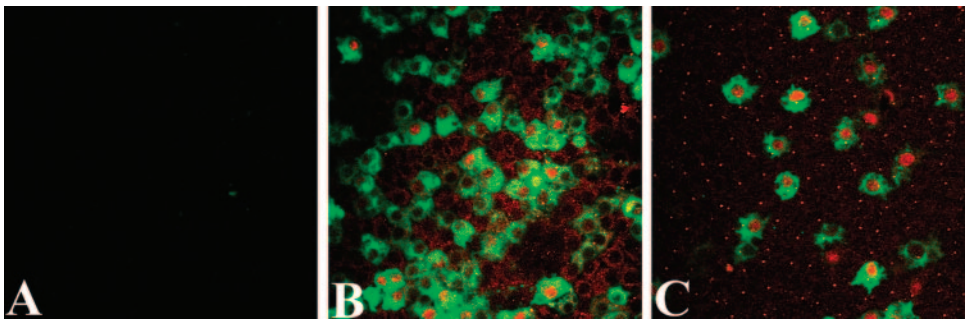

FIGURE 3. E2F2 expression in corneal endothelium. Representative images demonstrating E2F2 overexpression in endothelium of corneas infected ex vivo with AdUE. A cornea from a 22-year-old donor was cut in quarters and infected with AdUE at 1.5×10^7 pfu/mL for 2 hours at 37°C, washed, and incubated for 0 (**A**) or 48 hours (**B**), or 2 weeks (**C**) in medium containing 2% FCS. E2F2 overexpression is seen as bright *red* nuclear staining in cells coexpressing Us9-GFP (*green*) in the cytoplasm. (**B**) Background, endogenous levels of E2F2 were visible in the cytoplasm of noninfected cells. In some Us9-GFP-positive cells, nuclear E2F2 was not visible. Final magnification: ×400.

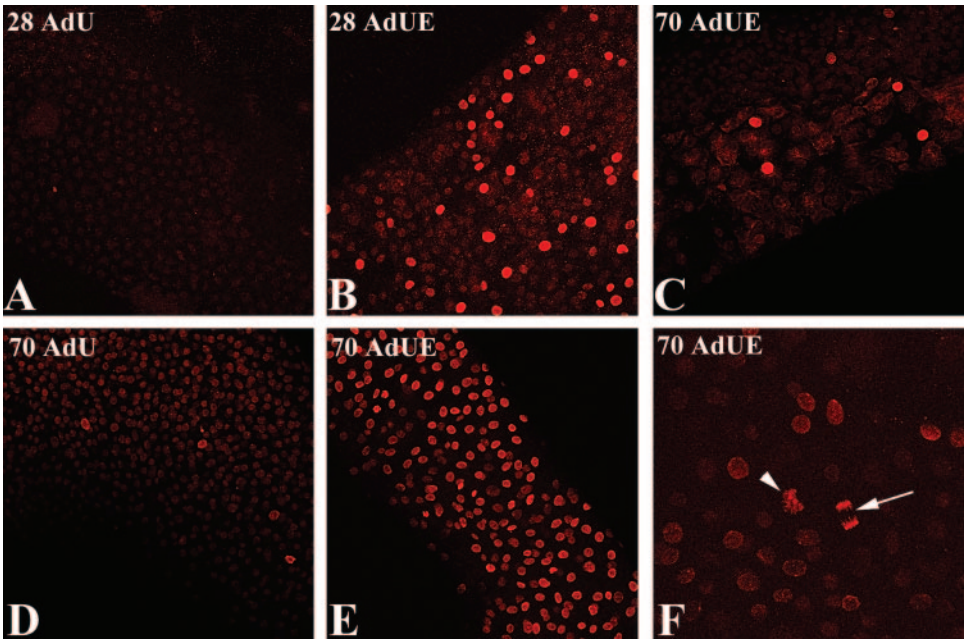

FIGURE 4. Effect of E2F2 cDNA transfer on BrdU incorporation. Representative images of BrdU staining in the endothelium of ex vivo corneas from (**A**, **B**) a 28- and (**C**–**F**) a 70-year-old donor. Corneal quarters were incubated for 2 hours in either the control vector (**A**, **D**) or the vector containing full-length cDNA for E2F2 (**B**, **C**, **E**, **F**). (**A**–**C**) Sections were infected and then incubated in the presence of BrdU for 48 hours. Corneal quarters from the 70-year-old donor (**D**–**F**) were postincubated for 48 hours in medium only, and then BrdU was added for a total of 5 days (= 7 days after infection). (**F**) *Arrow*: A late-telophase cell; *arrowhead*: a cell in prophase. Only the rhodamine (BrdU) staining is shown in these micrographs. Final magnification: (**A**–**E**) ×400, (**F**) ×1000.

assessments of density using trypan blue, this method was used only for final density assessments.

Over a period of 3 weeks in culture, corneal specimens in which human E2F2 was overexpressed demonstrated significant increases in cell density (assessed by trypan blue staining) compared with AdU-treated and mock-infected control subjects. Comparison of pre- and posttreatment EC density in E2F2-overexpression specimens indicated significant increase at 1, 2, and 3 weeks ($P = 0.003$, 0.04, and 0.013, respectively, paired *t*-test). The trend was for progressive decrease in cell density at all time points in AdU-treated and mock-infected control specimens, other than an insignificant increase in those corneal specimens treated with AdU after 1 week of culture (Fig. 5).

Effect of Recombinant E2F2 Expression on Apoptosis

Overexpression of the transcription factor E2F1 has been shown to induce significant apoptosis in various cell types.[22] Since E2F2 is a member of the same transcription factor family, we conducted a TUNEL assays to test for induction of apoptosis in response to E2F2 overexpression. Control studies were first conducted on cultured rabbit corneal endothelial cells to demonstrate our ability to detect TUNEL-positive cells. Confluent cultures were treated with H_2O_2, as described in the Materials and Methods section, and postincubated for 24 hours. Figure 6A shows that, on treatment with 0.5 mM H_2O_2, several cells disappeared from the culture dish, and the remaining attached cells were TUNEL-positive. Human corneas were then infected with AdU or AdUE as previously and maintained in culture for 48 hours, a time by which E2F2 is overexpressed. No TUNEL-positive cells were observed in the endothelium of either the control cornea (Fig. 6B) or cornea infected with AdUE (Fig. 6C), providing evidence that overexpression of E2F2 does not induce significant apoptosis.

DISCUSSION

Previous studies[23] have demonstrated that overexpression of E2F2 leads to cell-cycle progression in endothelial cells in rabbit corneas ex vivo. The studies described herein have expanded this information by demonstrating that E2F2 overexpression not only induces cell-cycle progression, but also increases the number of cells in the endothelium of human corneas ex vivo.

After infection of whole corneas ex vivo with AdU, fluorescence microscopy of transverse sections detected GFP expression in the endothelium only, indicating that adenovirus-mediated gene transfer is largely restricted to this cell layer. This finding is consistent with previous reports.[24,30] High levels of recombinant E2F2 were detectable in the nuclei of endothelial cells by 48 hours after AdUE infection. The relative number of cells overexpressing E2F2 appeared to decrease by 2 weeks after infection, although the results were not specifically quantified. Studies were not continued beyond this time point, but it is expected that E2F2 levels would decrease to normal endogenous levels by ~1 month after infection. This expectation is based on the documented kinetics of expression of cDNA encoding CTLA-4 Ig[30] and tumor necrosis factor receptor,[31] both of which were reduced to undetectable levels 28 days after adenoviral infection. Transient or short-term expression of recombinant E2F2 would be a requirement if it were to be used to increase endothelial cell density in humans, because constitutive overexpression would result in unregulated cell division.

The observation that E2F2 is overexpressed in corneal endothelial cells by 48 hours after infection with AdUE correlates with the presence of BrdU-positive cells at 48 hours, suggesting that E2F2 overexpression has induced S-phase entry. This conclusion is supported by the fact that few-to-no BrdU-positive cells in the endothelium were observed in corneas infected with the control vector. The low level of BrdU-positive, Ki67-negative nuclei in the negative controls may be due to BrdU incorporation as the result of DNA repair processes, rather than to the presence of actively cycling cells.[32,33] For immunocytochemistry studies, corneas are usually cut in quarters to increase the number of conditions for study. This treatment, plus the various incubations and washings needed to perform the immunocytochemistry, may have caused sublethal damage leading to DNA repair, a mechanism important in the survival of the corneal endothelium.[34] In control tissue, neither BrdU-positive mitotic figures nor paired nuclei were observed at any

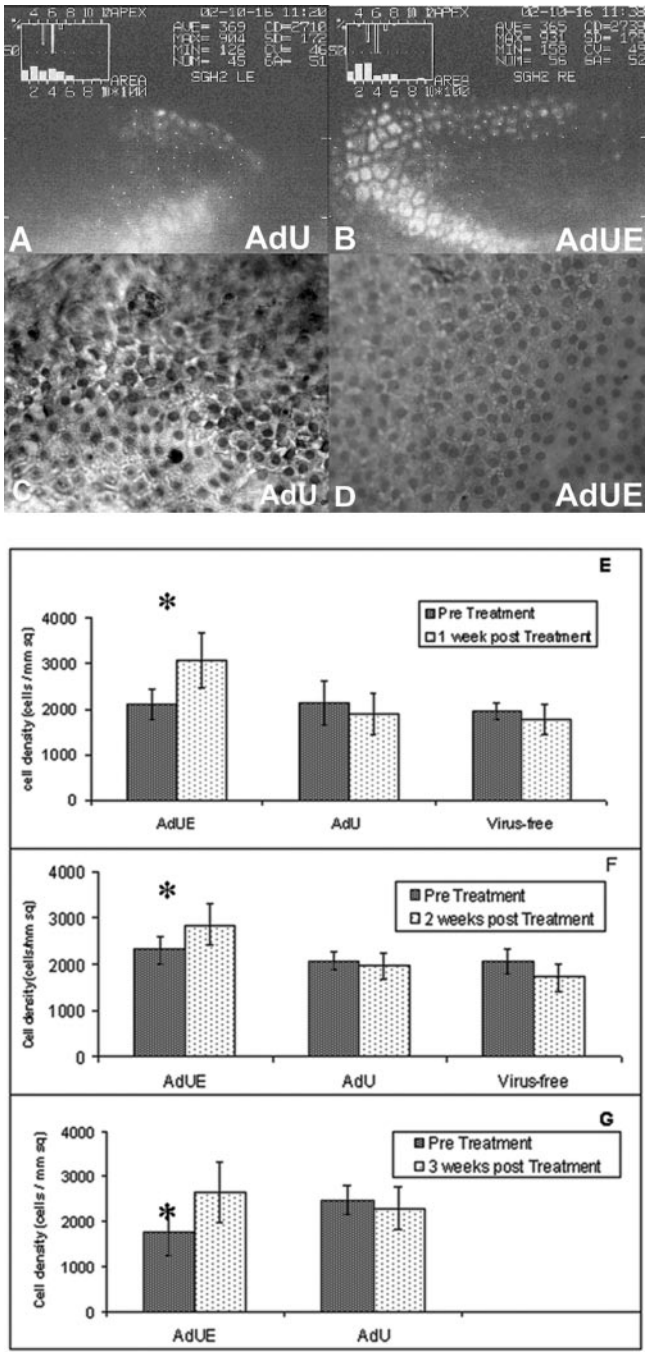

FIGURE 5. Effect of E2F2 cDNA transfer on endothelial cell density. Cell density of all corneas was measured before infection by specular microscopy with associated cell-counting software (**A**, **B**). After incubation with AdUE, AdU, or virus-free medium, cell density of corneas in culture was measured by trypan blue staining of fixed corneal specimens (**C**, **D**). Cell density in pairs of corneas before and 1 (**E**), 2 (**F**) and 3 weeks (**G**) after incubation with AdUE, AdU, or virus-free medium. Density in AdUE-treated corneas increased significantly at 1, 2, and 3 weeks compared with pretreatment. Error bars, SD. $^*P < 0.05$ on paired *t*-test analysis.

time points tested, suggesting that these cells most likely do not complete the cell cycle.

The finding at 48 hours after infection that more BrdU-positive cells were present in the endothelium of the younger donor is consistent with previous studies. Kinetic studies of growth factor effects on cell-cycle progression in endothelial cells from wounded ex vivo corneas[14] and in cell culture[15,16] indicate that cells from older donors respond more slowly to growth factor stimulation than those from younger donors. The population doubling time for cultured cells from older donors was calculated to be 90.25 hours compared with 46.25 hours for younger donors. The observation of an increased number of BrdU-positive cells in the endothelium from the older donor by 1 week after infection reflects this apparent slower response and indicates that these cells are responsive to E2F2, but enter the cell cycle at a slower rate. Although outside the scope of the current work, future studies will address the age-related differences in the response of ECs to AdUE treatment and E2F2 overexpression.

The observed decrease in cell density after 1 week is of interest. One possible reason is that transduced cells with limited proliferative potential may have completed several cell-division cycles. After the first completed round of division, some cells within the monolayer may undergo apoptosis as a result of further cycling induced by E2F2 overexpression. Another possibility is that after cell division, daughter cells with poor cell attachment to neighboring cells or Descemet's membrane may be lost in the culture medium spontaneously or with minor manipulations during extended culture. As previously demonstrated in rabbit corneas,[23] overexpression of E2F2 did not induce detectable levels of apoptosis in human corneal endothelium. The TUNEL assays in the current studies were conducted 48 hours after infection, a time when E2F2 was shown to be overexpressed. The results as presented suggest the relative lack of apoptosis; however, it is possible that the assay did not detect a cohort of cells that had either been lost from the endothelial monolayer or were in the early stages of apoptosis. It is also possible that it would take a longer time than 48 hours after infection for apoptosis to occur. This possibility cannot be ruled out, since the TUNEL assay was not conducted on corneas postincubated for longer periods. How-

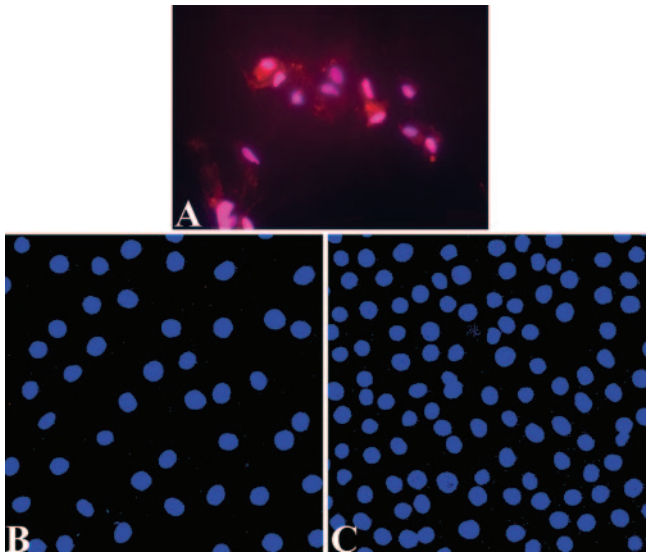

FIGURE 6. TUNEL assay to detect apoptotic corneal endothelial cells. Confluent rabbit corneal endothelial cells were used as a control for the TUNEL assay. Cells were treated for 24 hours with 0.5 mM H_2O_2 and then processed to detect TUNEL-positive cells. (**A**–**C**) Merger of the rhodamine (*red*: TUNEL positive) and nuclear stain (*blue*: nuclei) images. As seen in (**A**), after this treatment, most cells lifted from the culture dish and the remaining attached cells were TUNEL positive. In contrast, no TUNEL-positive cells were observed in corneal endothelium of ex vivo corneas infected with AdU (**B**) or AdUE (**C**) and postincubated for 48 hours. Final magnification: (**A**) ×400; (**B**, **C**) ×800.

ever, the finding of significantly increased cell density 1 week after infection with AdUE suggests that the net effect of E2F2 overexpression is positive and that if apoptosis or any form of virus-associated cytopathogenicity occurs, it does not adversely affect the number of cells. Future studies will further investigate these important problems.

In summary, adenovirus-mediated overexpression of E2F2 induces cell-cycle progression and increased endothelial cell density in human corneas in ex vivo culture. Effects on cell-cycle progression are evident within 2 to 3 days of exposure to recombinant virus for 2 hours. E2F2 expression diminishes after 1 week. Overexpression of this transcription factor has significant potential as a therapy in disorders of the human corneal endothelium.

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