

# Effect of Overexpressing the Transcription Factor E2F2 on Cell Cycle Progression in Rabbit Corneal Endothelial Cells

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**PURPOSE.** To test the hypothesis that overexpression of the transcription factor E2F2 promotes cell cycle progression in nonproliferating corneal endothelial cells.

**METHODS.** Ex vivo rabbit corneas were transfected with a lipid transfection reagent and either a control plasmid containing full-length cDNA for enhanced green fluorescent protein (pIRES2-EGFP) or a plasmid containing full-length cDNA for both E2F2 and EGFP (pIRES2-E2F2/EGFP). Transfection control experiments consisted of corneas incubated in buffer without transfection reagent or plasmid or incubated in reagent without plasmid. After transfection, corneas were incubated for various periods in 0.1% FBS (a concentration that maintains cell health, but does not promote proliferation). Immunocytochemical (ICC) localization tested for overexpression of E2F2 in transfected corneal endothelial cells and permitted calculation of transfection efficiency. Endothelial cell viability was tested in transfected ex vivo corneas and confluent cultures by using a cell-viability assay. Apoptosis was detected in confluent cultures by TUNEL assay. RT-PCR tested for mRNA expression of Ki67 (a marker of actively cycling cells) and cyclin B1 (a marker for the G<sub>2</sub>-phase of the cell cycle). Semiquantitative densitometric analysis compared the relative amounts of PCR reaction products.

**RESULTS.** ICC demonstrated the colocalization of E2F2 and EGFP in corneal endothelium with a transfection efficiency of 10% to 12%, using the pIRES2-based plasmid and transfection reagent. The cell-viability assay revealed very few dead cells in ex vivo corneal endothelium that overexpressed E2F2. Cell viability and TUNEL assays of confluent cultures revealed that approximately 27% of cells died in all cultures incubated with transfection reagent, but death appeared not to be due to apoptosis. No additional cell death was noted by either assay in cells that overexpressed E2F2. RT-PCR of endothelial samples obtained 48 hours after transfection showed the presence of higher levels of reaction product for Ki67 (a 5.1-fold increase) and cyclin B1 (a 2.3-fold increase) in cells that overexpressed E2F2 than in control samples.

**CONCLUSIONS.** Overexpression of the transcription factor E2F2 in nonproliferating rabbit corneal endothelial cells induces cell cycle progression without inducing significant apoptosis. (*Invest Ophthalmol Vis Sci.* 2004;45:1340-1348) DOI:10.1167/iov.03-0335

Corneal endothelium is considered to be a nonreplicative tissue, because throughout life proliferation does not keep pace with cell loss. As a result, endothelial cell density decreases with age at a rate of approximately 0.6% per year.<sup>1-3</sup> Endothelial cell density is usually sufficient to maintain corneal transparency, even in older individuals.<sup>4</sup> In some cases, however, accidental or surgical trauma,<sup>5,6</sup> corneal transplantation,<sup>7,8</sup> diseases such as diabetes,<sup>9</sup> treatments for glaucoma,<sup>10</sup> and endothelial dystrophies<sup>11,12</sup> can increase the rate of endothelial loss. When density decreases to approximately 300 to 500 cells/mm<sup>2</sup>, monolayer integrity can be compromised, resulting in stromal edema, corneal clouding, and loss of visual acuity. Currently, there are no medical treatments for loss of endothelial integrity, and normal vision can be restored only by corneal transplantation.

Despite ample evidence for lack of mitotic activity in vivo, there is sufficient evidence to indicate that human corneal endothelial cells possess proliferative capacity. For example, Wilson et al.<sup>13,14</sup> have shown that cultured human corneal endothelial cells undergo multiple rounds of division when transfected with viral oncogenes, such as simian virus (SV)40 large-T antigen or the E6/E7 proteins from human papilloma virus. In addition, human corneal endothelial cells have long telomeres throughout life, indicating that their limited replicative ability does not result from critically short telomere lengths.<sup>15</sup> Our studies indicate that corneal endothelial cells in vivo have not exited the cell cycle but are arrested in the G<sub>1</sub>-phase.<sup>16,17</sup> These findings suggest that endothelial cells must be actively maintained in a nonreplicative state. Several mechanisms appear to contribute to maintenance of G<sub>1</sub>-phase arrest in vivo. These include the formation and maintenance of stable cell-cell and cell-substrate contacts,<sup>18-20</sup> the apparent lack of positive growth factor stimulation,<sup>21</sup> the antiproliferative activity of TGF- $\beta$ 2 in aqueous humor,<sup>22</sup> and an age-related decrease in sensitivity to mitogens.<sup>23</sup>

Within the cell cycle, the transition between the G<sub>1</sub>- and S-phase is highly regulated to assure accurate transmission of genetic information and controlled cell replication. Central to the temporal control of the G<sub>1</sub> to S-phase transition is regulation of the activity of the E2F transcription factor family. The E2F family consists of at least six distinct isoforms. E2F1, E2F2, and E2F3 mediate the G<sub>1</sub> to S-phase transition,<sup>24-26</sup> while E2F4 and -5 prevent entry of cells from the quiescent, nonreplicating state (G<sub>0</sub>-phase) into the G<sub>1</sub>-phase.<sup>27</sup> E2F6 negatively regulates the transcription of E2F-responsive genes.<sup>28</sup> E2Fs regulate expression of target genes by binding to specific sequences within their promoter regions and either activating or repressing their transcription in a cell-cycle-dependent manner. The transcriptional activity of E2F1, -2, and -3 is tightly regulated by

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the retinoblastoma tumor suppressor, pRb.<sup>29</sup> In quiescent cells, pRb is hypophosphorylated and tightly binds E2F, preventing its activation. On mitogenic stimulation, cells enter the G<sub>1</sub>-phase of the cell cycle. Early in this phase, cyclin D is synthesized and associates with the cyclin-dependent kinase, CDK4.<sup>30</sup> Formation of the cyclin D/CDK4 complex activates the kinase activity of CDK4. pRb is a specific substrate for this complex.<sup>31</sup> Phosphorylation of pRb by this complex alters the pRb-E2F interaction, promoting release and activation of E2F.<sup>32</sup> E2F isoforms form heterodimeric complexes with either DP-1 or -2.<sup>33,34</sup> These E2F-binding proteins are expressed constitutively and are present in relative abundance throughout the cell cycle. Formation of E2F-DP complexes promotes efficient DNA-binding, resulting in optimal E2F activity.

Of the three E2F isoforms that mediate the G<sub>1</sub> to S-phase transition, E2F1 has been the most thoroughly studied. Dominant negative mutants of E2F1 decrease the number of cells entering the S-phase and significantly decrease expression of E2F1-induced S-phase genes, indicating the importance of E2F1 in S-phase induction.<sup>35</sup> Overexpression of E2F1 stimulates division in quiescent REF52 cells,<sup>24</sup> overcomes G<sub>1</sub>-phase arrest induced by TGF- $\beta$ ,<sup>36</sup> and inhibits *in vitro* myogenic differentiation while inducing proliferation.<sup>37</sup> Unregulated overexpression of E2F1 can result in cellular transformation.<sup>38</sup> Overexpression of E2F1 has also been shown to induce significant apoptosis in a p53-dependent manner.<sup>24,39,40</sup> E2F2 was first cloned by Ivey-Hoyle et al.<sup>41</sup> and exhibits 46% amino acid identity with E2F1. Both the sequence and function of the DNA- and pRb-binding domains of E2F1 are conserved in E2F2. E2F2 also induces S-phase entry, but does not appear to have as strong an effect on apoptosis as E2F1. This is evidenced by the fact that the ability of the transcription factor, Myc, to induce S-phase in primary mouse embryo fibroblasts is impaired by the absence of either E2F2 or -3, but not by E2F1. In contrast, Myc-induced apoptosis is reduced in cells in which E2F1 is deleted, rather than E2F2 or -3.<sup>42</sup> Studies have been conducted in cultured primary sensory neurons from adult rats to determine the effect on cell cycle progression of overexpressed E2F2.<sup>43</sup> These neuronal cells are considered "terminally" post-mitotic and do not replicate *in vivo* or in culture, even in the presence of serum. E2F2 overexpression in these cells induced true DNA replication (as indicated by fluorescent *in situ* hybridization [FISH] analysis of doublet signals) in the absence of serum and laminin. The importance of E2F3 in regulation of proliferation is shown by the fact that mutation of this E2F isoform dramatically impairs mitogen-induced transcriptional activation of numerous E2F-responsive genes.<sup>44</sup> E2F3 is proposed to play an essential role during the cell cycle by controlling the expression of genes whose products are rate-limiting for initiation of DNA replication<sup>45</sup>; however, it appears to have a function somewhat different from that of E2F1 or -2. Although overall E2F DNA-binding activity accumulates during the initial G<sub>1</sub>-phase after growth stimulation, only E2F3-binding activity reaccumulates at subsequent G<sub>1</sub> to S-phase transitions.

Human and rabbit corneal endothelial cells *in vivo* express both pRb and E2F.<sup>17</sup> A crucial role for both pRb and E2F in regulating corneal endothelial proliferation is suggested from studies of viral oncoproteins. When expressed in quiescent cells, viral oncoproteins, such as the SV40 large-T antigen,<sup>46,47</sup> human papilloma virus E7,<sup>47,48</sup> and adenovirus E1A,<sup>47,49</sup> strongly compete with E2F for binding to pRb, causing the release and subsequent activation of E2F. Viral oncoproteins and pRb phosphorylation appear to induce S-phase entry through a similar mechanism (i.e., by releasing bound E2F). The major difference between these two mechanisms is that viral oncoprotein induction of proliferation is usually unregulated, resulting in transformation, whereas stimulation of division through phosphorylation of pRb is regulated by a complex



FIGURE 1. pIRES2-E2F2/EGFP plasmid construct.

feedback mechanism that prevents uncontrolled division. As mentioned earlier, expression of viral oncoproteins in cultured human corneal endothelial cells was capable of inducing proliferation for several generations,<sup>13,14</sup> suggesting that free E2F released by viral oncoprotein binding to pRb is sufficient to induce proliferation in human corneal endothelial cells and that, once G<sub>1</sub> arrest is overcome, division proceeds.

In the present study, we tested the hypothesis that overexpression of the transcription factor E2F2 promotes cell cycle progression in nonproliferating rabbit corneal endothelial cells. The E2F2 isoform was chosen because it was expected to induce S-phase entry in a manner similar to that of E2F1, but to induce less apoptosis. Although it is well-known in the field that corneal endothelial cells from rabbits have greater proliferative capacity than their human counterparts, we chose to use *ex vivo* rabbit corneas as a model system for several reasons. A similar model has been used successfully for both viral- and non-viral-based gene delivery to corneal endothelium.<sup>50-53</sup> In preliminary studies, we tested both *ex vivo* corneas and confluent monolayers of cultured rabbit corneal endothelial cells for their ability to maintain contact-dependent inhibition of proliferation under all experimental conditions. Results indicated that mitotic quiescence could be optimally maintained in the endothelium of *ex vivo* corneas. In addition, RT-PCR analysis to detect mRNA expression for cell cycle proteins was aided by the fact that, in rabbit corneas, Descemet's membrane and the underlying endothelium can be easily dissected from the overlying stroma. Thus, a sufficient number of pure isolates of endothelial cells were obtained for these studies.

## MATERIALS AND METHODS

### Plasmid Construct

Full length E2F2 cDNA (1313 bp) was originally cloned from a human HeLa S3 cDNA  $\lambda$ gt11 library.<sup>41</sup> A pcDNA3 plasmid containing a CMV promoter and full-length E2F2 cDNA was kindly provided by Joseph Nevins, PhD (Duke University Medical Center, Durham, NC). In our laboratory, E2F2 cDNA was amplified by PCR with primers containing *EcoRI* and *BamHI* restriction enzyme sites, blunt-end ligated into a pCRBluntII-TOPO plasmid (Invitrogen, Carlsbad, CA), excised by double restriction enzyme digestion, and then ligated into a pIRES2-EGFP plasmid (BD Biosciences-Clontech, Palo Alto, CA). As shown in Figure 1, E2F2 cDNA was inserted after the CMV promoter within the multiple cloning site (MCS). This is followed by the internal ribosome entry site (IRES) of the encephalomyocarditis virus, followed by the (enhanced green fluorescent protein) EGFP reporter cDNA. This pIRES2-E2F2/EGFP plasmid construct permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA, but the resultant proteins are separate gene products, not fusion proteins. This plasmid construct was sequenced at Tufts University Medical Center (Boston, MA). Sequence results indicate that the E2F2 cDNA was inserted in the proper orientation and that the DNA sequence is correct and does not contain mutations (data not shown).

### Transfection Method

Male New Zealand White rabbits (2.5–3 kg) were used as a source of corneal tissue. All rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Whole corneas were excised, washed in Medium 199 (M199, Invitro-

gen) supplemented with 50  $\mu\text{g}/\text{mL}$  gentamicin (Invitrogen) and 1 $\times$  antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO), and incubated for 24 hours at 37°C in M199, 10  $\mu\text{g}/\text{mL}$  gentamicin, and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). This treatment stabilized the endothelium and permitted completion of any rounds of cell division that might have been initiated to repair the monolayer after corneal dissection. Corneas were then washed in M199 and transfected for 3 hours at 37°C in M199 supplemented with transfection reagent (LipofectAMINE-Plus; Invitrogen) and 1% FBS. Experimental conditions included (1) no transfection reagent or plasmid, (2) transfection reagent alone (no plasmid), (3) reagent plus the pIRES2-EGFP plasmid, and (4) reagent plus the pIRES2-E2F2/EGFP plasmid. The amount of plasmid was optimized at 4  $\mu\text{g}$  per intact cornea. The reagent-to-plasmid ratio was 8:1. Corneas were then washed in M199 and postincubated at 37°C in a 5% CO<sub>2</sub> incubator for various periods in M199 and 0.1% FBS to maintain cell viability without promoting serum-stimulated proliferation.

### Calculation of Transfection Efficiency

Rabbit corneas were cut into quarters, and cells were transfected for 3 hours with the pIRES2-EGFP plasmid in the presence of reagent (LipofectAMINE-Plus; Invitrogen), as just described. After transfection, corneas were washed and incubated for 48 hours in M199 and 0.1% FBS. Corneas were then fixed and mounted in medium containing propidium iodide (PI; Vectashield; Vector Laboratories, Inc., Burlingame, CA) to stain all nuclei. EGFP and PI were visualized by fluorescence confocal microscopy (Model TCS 4D, equipped with a DMRBE laser; Leitz, Wetzlar, Germany; and SCANware ver. 4.2 software; Leica Laser-technik, Heidelberg, Germany). For calculation of transfection efficiency, regions were chosen in the middle of each corneal quarter. Micrographs of this area were taken at 20 $\times$  magnification. Five separate areas were photographed per corneal quarter, and the images were recorded for further analysis using NIH Image version 1.62 (W. Rasband, National Institutes of Health, Bethesda, MD; available by FTP from [http://zippy.nimh.nih.gov] or on floppy disc from NTIS, Springfield, VA; and Adobe Photoshop 4.0 software; Adobe Systems, Inc., Mountain View, CA). EGFP-expressing cells were counted in each of the five areas and then all PI-stained nuclei were counted within the same areas. The relative percentage of EGFP-expressing cells per 20 $\times$  area was determined by dividing the number of EGFP-positive cells by the total number of PI-stained nuclei times 100. The average percentage of transfected cells was then calculated using the data from the five areas analyzed. The experiment was conducted twice.

### Immunodetection of E2F2-Overexpressing Cells

All incubations for immunocytochemistry (ICC) were conducted at room temperature. Corneas were washed in phosphate-buffered saline (PBS), fixed for 30 minutes in 4% formaldehyde (Polysciences, Inc., Warrington, PA), washed, and permeabilized for 10 minutes with 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Nonspecific binding was blocked by incubating the corneas for 10 minutes in 4% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA) in PBS after permeabilization. Corneas were then incubated for 2 hours with rabbit anti-human E2F2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This antibody was diluted in 4% BSA in PBS at 1:800, because preliminary studies indicated that this dilution permitted visualization of overexpressed E2F2, but not of lower levels of endogenous E2F2. Corneas were washed and incubated for 1 hour in Rhodamine Red-X-conjugated anti-rabbit IgG (1:200 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After they were washed, corneas were mounted endothelial side up and viewed using fluorescence confocal microscopy.

### Viability and Apoptosis Testing

To determine the effect of overexpressed E2F2 on endothelial cell viability, cell-viability assays were conducted on ex vivo corneas. Samples for assay included untransfected corneas and corneas transfected for 3 hours with either the pIRES2-EGFP or pIRES2-E2F2/EGFP plasmid

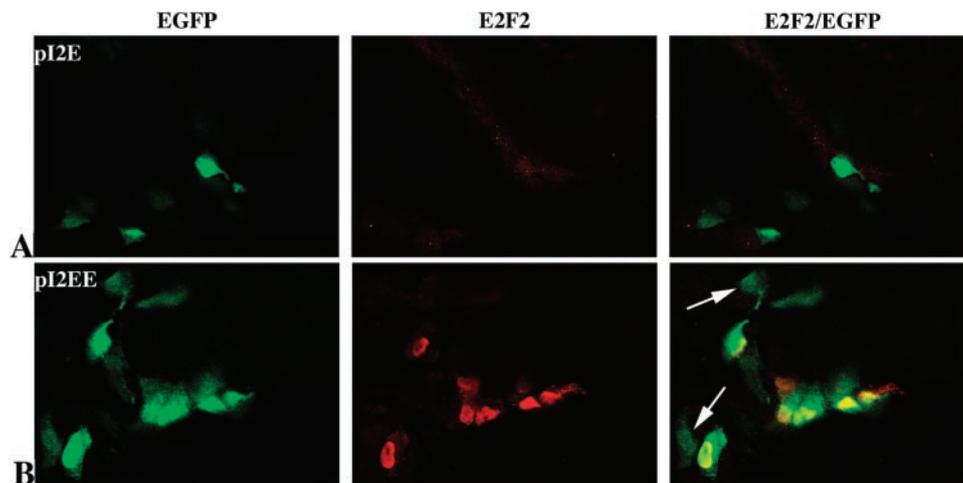
and then postincubated for 48 hours in M199 and 0.1% FBS. Unfixed corneas were washed in PBS and stained with a cell-viability assay kit (Live/Dead Viability/Cytotoxicity Kit; Molecular Probes, Eugene, OR). Staining conditions were as follows: After the 48 hours of incubation, cells were washed in PBS, incubated in 5  $\mu\text{M}$  each of calcein AM and ethidium D for 45 minutes, and washed in PBS. Duplicate samples for each condition were mounted endothelial side up for fluorescence microscopy (Eclipse E800; with a VFM Epi-Fluorescence Attachment; Nikon Inc., Melville, NY; equipped with a Spot digital camera and Spot ver. 1.1 CE software; Diagnostic Instruments, Sterling Heights, MD). To further test the effect of E2F2 overexpression on endothelial cell viability, studies were conducted using confluent rabbit corneal endothelial cells cultured according to established protocols.<sup>54</sup> Cells were subcultured onto two-well tissue culture chamber slides and grown to confluence. Confluent conditions included the following: (1) incubation of confluent cells without lipid reagent (LipofectAMINE-Plus; Invitrogen) or plasmid; (2) incubation of confluent cells with lipid reagent, but without plasmid; (3) transfection with lipid reagent and the pIRES2-EGFP control plasmid, or (4) transfection with lipid reagent and the pIRES2-E2F2/EGFP plasmid. For transfection, cultures were incubated with plasmid for 3 hours under the conditions described earlier, washed, and then postincubated for 48 hours in M199 and 0.1% FBS. The cell-viability assay was performed to determine overall viability. Controls for cell death using the assay included cultures fixed in absolute methanol for 10 minutes at -20°C before staining. TUNEL assays were conducted according to the supplier's directions (ApoptTag Apoptosis Detection Systems; Chemicon International, Temecula, CA) to detect apoptotic cells. The apoptotic control for the TUNEL assay included confluent cultures incubated for 24 hours in 0.1 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in M199 and 0.1% FBS before staining. All experiments were repeated at least twice and included duplicates for each condition. Staining was evaluated by fluorescence microscopy with the microscope described earlier. For quantification of the relative percentage of dead or apoptotic cells, at least five 20 $\times$  areas were photographed per tissue culture well. The relative number of total nuclei and dead or apoptotic nuclei was determined with Image-J software (http://rsb.info.nih.gov/ij/, W. Rasband, National Institutes of Health, Bethesda, MD), and the percentage of dead and apoptotic cells was calculated.

### Reverse Transcription-Polymerase Chain Reaction

Descemet's membrane with attached endothelium was excised from rabbit corneas. Tissue was processed for total RNA extraction as recommended by the manufacturer (TRIzol Reagent; Invitrogen). cDNA was prepared from 1  $\mu\text{g}$  of total RNA by reverse transcription in a volume of 20  $\mu\text{L}$ , using a kit from Promega (Madison, WI). PCR was performed, electrophoresis was run, and RNA samples were tested for genomic DNA contamination, as previously described.<sup>20</sup> Annealing temperatures and number of cycles were as follows: EGFP: 65°C, 30 cycles; E2F2: 50°C, 30 cycles; Ki67: 45°C, 30 cycles; and cyclin B1: 50°C, 35 cycles. Each RT-PCR experiment was conducted in duplicate. Glyceroldehyde-3-phosphate dehydrogenase (G3PDH; BD Biosciences-Clontech) acted as a positive control for the PCR and yielded a 452-bp product. EGFP acted as a control for relative transfection in each sample. The primer sequences were obtained from BD Biosciences-Clontech. Negative controls consisted of the PCR reaction mixture, including primers, but without cDNA.

### Primers

Sequences were EGFP: 5'-GCAAGCTGACCCTGAAGTTCATC-3', 5'-TTGTAGTTGCCGTCGTCCTTG-3', 202 bp; E2F2<sup>55</sup>: 5'-AAGAAGTTCATTACCTCCTGA-3', 5'-AATCACTGTCTGCTCCTTAAA-3', 386 bp; Ki67<sup>17</sup>: 5'-ACTTGCTCCTAATACGCC-3', 5'-TTACTACATCTGCCATGA-3', 437 bp; and cyclin B<sup>36</sup>: 5'-GGAGGAGAGCAGTCAGTTA-3', 5'-GTCAAAAAGCGAAGTACC-3', 275 bp.



**FIGURE 2.** Localization of EGFP and E2F2 in the endothelium of ex vivo rabbit corneas transfected with the pIRES2-EGFP control plasmid (pI2E in **A**) or the pIRES-E2F2/EGFP plasmid (pI2EE in **B**). After transfection, corneas were washed, incubated for 24 hours in 0.1% FBS, and then immunostained for high levels of E2F2. EGFP marker fluorescence (green) was visible in both specimens, indicating successful transfection. E2F2 overexpression (red) was visible only in cells transfected with pIRES-E2F2/EGFP. Superimposition of the two images indicated that E2F2 was localized to the nucleus, whereas EGFP was both nuclear and cytoplasmic. It should be noted that, at this 24-hour time-point, some cells in (**B**) showed light EGFP fluorescence, but were negative for E2F2 (arrows). Original magnification,  $\times 40$ .

### Semiquantitative Densitometric Analysis

Images of PCR gels were obtained using an image-analysis system (BDS Image, ver. 1.3; Biological Detection Systems Inc., Pittsburgh, PA). Semiquantitative analysis of the images was made using NIH-Image version 1.62. Within a single experiment, the density measurement of each band was first adjusted by using G3PDH to normalize for gel sample load. Density was then adjusted using EGFP to normalize for relative differences in transfection efficiency in each sample. All results represent the average density of positive bands obtained from two to three separate experiments.

## RESULTS

### Overexpression of E2F2 in Corneal Endothelium

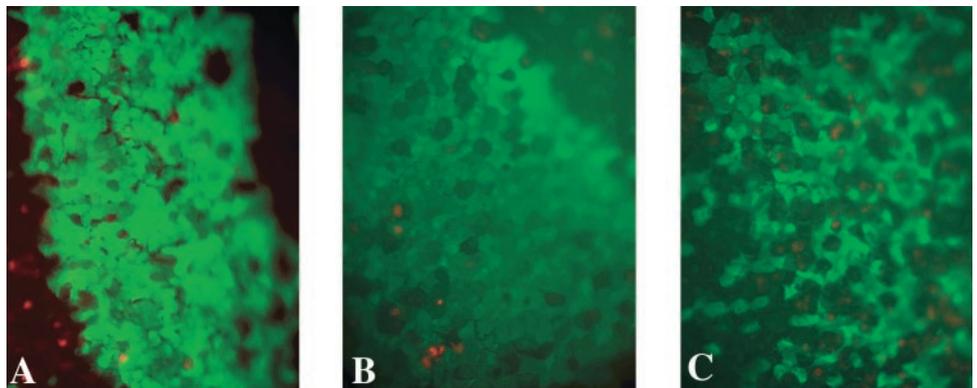
Wholemounts of rabbit corneal endothelium were examined by fluorescence microscopy after transfection with pIRES2-E2F2/EGFP or the control plasmid. As shown in Figure 2, cells coexpressing the EGFP reporter gene and E2F2 were present within the endothelial monolayer, indicating the presence of transfected cells that overexpressed E2F2. As expected, E2F2 localized to the nucleus, whereas EGFP was both nuclear and cytoplasmic. In control experiments, EGFP fluorescence was

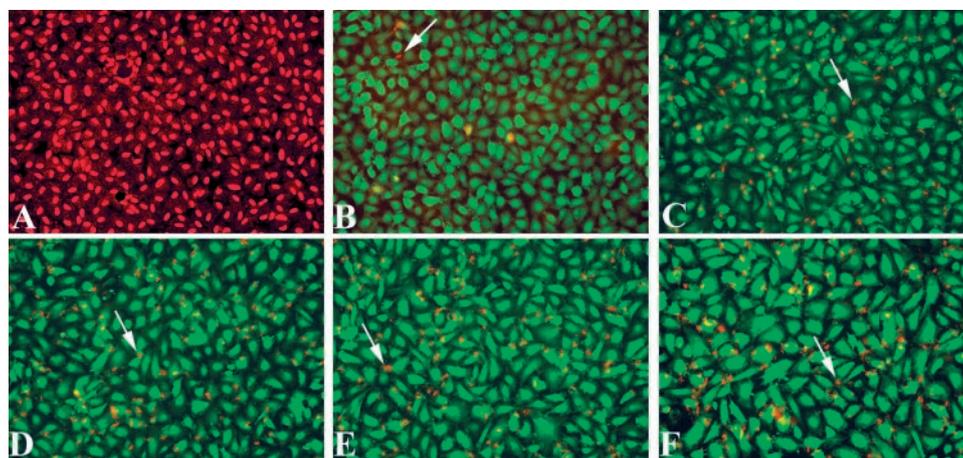
visible; however, no staining for high levels of E2F2 was observed, indicating lack of E2F2 overexpression. It should be noted that a low level of endogenous E2F2 is normally detectable in rabbit corneal endothelium by ICC; however, the antibody concentration used in this study (1/800 dilution) permitted visualization of overexpressed E2F2 only. Examination of micrographs from corneas incubated for 24, 48, or 72 hours after transfection indicated the presence of E2F2-overexpressing cells at all three time points. Relative fluorescence intensity and the number of E2F2-positive cells reached its maximum 48 hours after transfection and decreased thereafter (data not shown). Transfection efficiency was calculated in the endothelium of corneas incubated for 48 hours after transfection. Results showed good agreement in duplicate samples and averaged 10% to 12%.

### Effect of E2F2 Overexpression on Cell Viability

A fluorescence-based cell-viability assay was used to test endothelial cell viability in ex vivo corneas. Control, untransfected corneas and corneas incubated for 3 hours with either the pIRES2-EGFP or pIRES2-E2F2/EGFP plasmid were incubated for 48 hours in M199 and 0.1% FBS before assay. The 48-hour time point was chosen, since this was the time determined to yield

**FIGURE 3.** En face view of the endothelium of ex vivo untransfected rabbit corneas (**A**) and corneas transfected with either the pIRES2-EGFP (**B**) or pIRES2-E2F2/EGFP plasmid (**C**), incubated for 48 hours in M199 and 0.1% FBS, and stained for viability using a cell-viability assay. Stromal swelling of all corneas prevented clear visualization of the endothelium within the entire microscopic field. Green: viable cells; red: dead cells. Original magnification,  $\times 20$ .





**FIGURE 4.** Representative images from the cell-viability assay to test the effect of E2F2 overexpression on viability of confluent rabbit corneal endothelial cells. Fluorescein (*green*: live) and rhodamine (*red*: dead) images have been overlaid. Methanol fixation of confluent cultures (**A**) acted as a positive control to demonstrate staining of dead cells. Confluent cultures were incubated without lipid reagent or plasmid (**B**); with lipid reagent, but without plasmid (**C**); transfected with lipid reagent and the pIRES2-EGFP control plasmid (**D**); or transfected with lipid reagent and the pIRES2-E2F2/EGFP plasmid (**E**, **F**). In (**B**-**F**), dead cells were interspersed between live cells. *Arrows*: representative dead cells. Original magnification,  $\times 20$ .

the greatest number of E2F2-positive cells. Figure 3 presents representative fluorescence micrographs of the results. The endothelium of untransfected corneas (Fig. 3A) shows mainly green nuclear fluorescence (calcein-AM), indicating live cells and only a few red nuclei (ethidium-D), indicating dead cells. Similar staining patterns were observed in the endothelium of ex vivo corneas transfected with either the pIRES2-EGFP (Fig. 3B) or pIRES2-E2F2/EGFP plasmid (Fig. 3C). Cell-viability and TUNEL assays were conducted on fully confluent cultures of rabbit corneal endothelial cells to evaluate further the effect of lipid-based transfection and E2F2 overexpression on viability. As indicated in the Materials and Methods section, culture conditions included the following: (1) incubation of confluent cells without lipid reagent (LipofectAMINE-Plus; Invitrogen) or plasmid, (2) incubation of confluent cells with lipid reagent, but without plasmid, (3) transfection with the pIRES2-EGFP control plasmid, or (4) transfection with pIRES2-E2F2/EGFP plasmid. As with the ex vivo corneas, assays were performed 48 hours after transfection. Representative micrographs of the cell-viability assay are presented in Figure 4. Table 1 presents the quantitative data. For this assay, methanol-fixed cultures acted as positive controls for cell death. Fluorescence microscopy of methanol-fixed cultures revealed red-stained nuclei throughout the culture (Fig. 4A). A small number of dead cells were observed in control cultures incubated without transfection

reagent or plasmid (Fig. 4B). These cells were interspersed between the live cells of the endothelial monolayer. Cultures incubated in the reagent but without plasmid (Fig. 4C) contained a larger number of red-stained nuclei. Of note was the presence of cellular debris associated with red-stained nuclei in the lipid-treated cultures. This debris was not as evident in control cultures incubated in the absence of the reagent, but was found in cultures transfected with either the pIRES2-EGFP (Fig. 4D) or pIRES2-E2F2/EGFP plasmid (Figs. 4E, 4F), using a lipid-based transfection reagent. As shown in Table 1, cell death increased from 0.657% to 27.16% when cultures were incubated in lipid-based reagent alone. Little or no additional cell death was observed in cultures incubated in this reagent plus either pIRES2-E2F2/EGFP or the control plasmid. The same incubation conditions were used to test for apoptosis using the TUNEL assay. Representative micrographs are presented in Figure 5 and quantification of the results is given in Table 2. For this assay, hydrogen peroxide ( $H_2O_2$ ) treatment was used as a positive control for apoptosis. As shown in Figure 5A, 24 hours after  $H_2O_2$  treatment most of the cells had died and lifted from the chamber slide. All remaining attached cells were TUNEL positive. No TUNEL-positive apoptotic cells were observed in cultures incubated in the absence of transfection reagent and plasmid (Fig. 5B). In cultures incubated with reagent but without plasmid (Fig. 5C), very few cells

**TABLE 1.** Quantification of Dead Cells in a Cell Viability Assay of Confluent Rabbit Corneal Endothelial Cultures

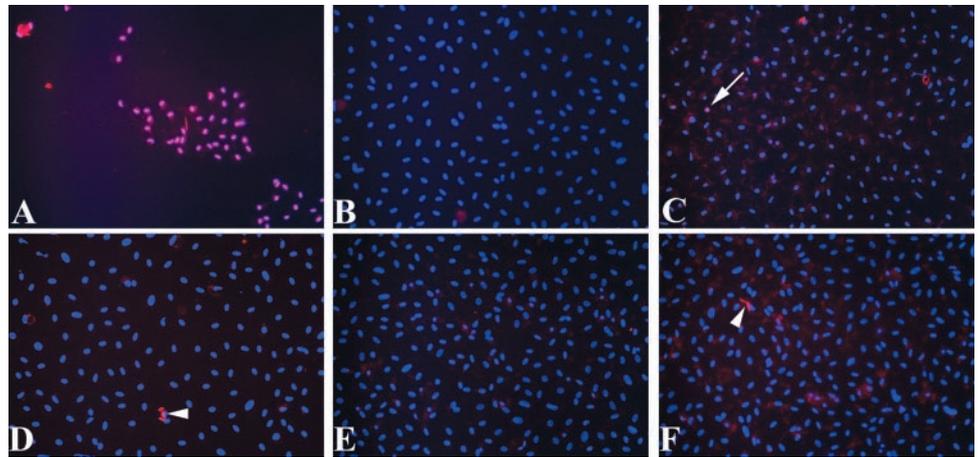
Treatment	Total Nuclei*	Total Ethidium D-Positive Nuclei†	Ethidium D-Positive Nuclei (%)
No lipid, no plasmid	3,197	21	0.657
Methanol-fixed	2,120	2,120	100.00
Lipid, no plasmid	5,126	1,392	27.16
pIRES2-EGFP	5,334	1,472	27.60
pIRES2-E2F2/EGFP	5,063	1,448	28.60

*n* = 4.

\* Total nuclei in 5–20 $\times$  microscopic fields.

† Ethidium D indicates dead cells.

**FIGURE 5.** Representative images from the TUNEL assay to test the effect of E2F2 overexpression on the induction of apoptosis in confluent rabbit corneal endothelial cells. DAPI (blue: stains all nuclei) and rhodamine (red: stains apoptotic cells) images have been overlaid. Hydrogen peroxide treatment of confluent cultures (A) acted as a positive control to demonstrate staining of dead cells. Only a portion of the cells remained attached to the culture dish and the DAPI and TUNEL-positive staining overlaps, producing a purple-pink color. Confluent cultures were incubated without lipid reagent or plasmid (B); with lipid reagent, but without plasmid (C); transfected with lipid reagent and the pIRES2-EGFP control plasmid (D); or transfected with lipid reagent and the pIRES2-E2F2/EGFP plasmid (E, F). (C, arrow) indicates a dead cell. (D, F, arrowheads) indicate rhodamine-positive debris, not dead cells. Original magnification,  $\times 20$ .



(0.324%) were TUNEL positive. No TUNEL-positive cells were observed in cultures transfected with pIRES2-EGFP (Fig. 5D) and only 0.059% of cells transfected with the pIRES2-E2F2/EGFP plasmid were TUNEL positive (Figs. 5E, 5F).

### RT-PCR Detection of E2F2-Induced Cell Cycle Progression

E2F2 is expected to be active at the  $G_1$  to S-phase transition, resulting in movement of cells into the S-phase of the cell cycle. Initial studies used RT-PCR to detect mRNA for Ki67, a marker of actively cycling cells,<sup>57</sup> in endothelial cells transfected with the control plasmid and cells transfected with pIRES2-E2F2/EGFP. As shown in Figure 6, cells transfected with pIRES2-E2F2/EGFP yielded a positive band for Ki67 by 48 hours after transfection, whereas, this product was barely detectable in controls. Semiquantitative densitometric analysis normalized for both gel sample load and transfection efficiency indicated that, at 48 hours after transfection, the relative amount of Ki67 PCR reaction product in cells incubated with pIRES2-EGFP/E2F2 averaged 5.1 times greater than that in cells transfected with the control plasmid. This finding demonstrates a correlation between E2F2 overexpression and proliferative activity.

RT-PCR studies were also conducted to compare the relative levels of cyclin B1 mRNA in cells transfected with the control or pIRES2-E2F2/EGFP plasmid. The rationale for using cyclin B1 as a marker of cell cycle progression is based on the fact that the cyclin B1 promoter does not become activated until the end of the S-phase,<sup>58</sup> and the corresponding protein level does not peak until late in the  $G_2$ -phase of the cell cycle.<sup>59</sup> In addition, accumulation of cyclin B1 requires the upstream E2F-dependent expression and regulatory activity of cyclin A.<sup>60</sup>

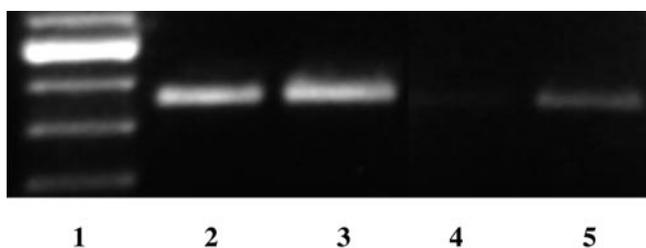
Thus, detection of increased cyclin B1 mRNA levels indicate that the cell cycle has progressed through the S-phase into the  $G_2$ -phase. Preliminary studies were conducted to determine whether cyclin B1 mRNA expression could be used as a marker of cell cycle progression in rabbit corneal endothelial cells. For these studies, subconfluent cultures of rabbit corneal endothelial cells (prepared according to established protocols<sup>54</sup>) were incubated for 24 or 48 hours in either 0.1% or 10% FBS. Previous studies indicated that, at a concentration of 0.1% FBS, cells remained healthy, but did not proliferate, whereas, 10% FBS supported proliferation (data not shown). Total RNA was extracted, and RT-PCR was conducted with primers specific for cyclin B1. Figure 7A shows cyclin B1 reaction product only in cells incubated in 10% FBS, indicating that cyclin B1 mRNA is detectable only in proliferating cells. To test the effect of E2F2 overexpression on cyclin B1 levels, ex vivo rabbit corneas were transfected with pIRES2-E2F2/EGFP or the control plasmid and incubated in M199 and 0.1% FBS for 24 or 48 hours. Other controls included cells incubated with no plasmid or transfection reagent (LipofectAMINE-Plus; Invitrogen), and cells incubated with reagent but without plasmid. Figure 7B shows a low level of cyclin B1 PCR reaction product in cells transfected with the control plasmid, but detectably higher levels in cells overexpressing E2F2. Semiquantitative densitometric analysis normalizing for both gel load and relative transfection efficiency indicated that, at 24 hours after transfection, the relative amount of cyclin B1 PCR product averaged 2.2 times greater in E2F2-transfected cells than in plasmid controls. After 48 hours, cyclin B1 reaction product averaged to 2.3 times higher in E2F2 overexpressing cells. Little-to-no detectable cyclin B1 reaction product was obtained from cells incubated under the other control conditions.

**TABLE 2** Quantification of TUNEL-Positive Nuclei in Confluent Rabbit Corneal Endothelial Cultures

Treatment	Total Nuclei*	Total TUNEL-Positive Nuclei	TUNEL-Positive Nuclei (%)
No lipid, no plasmid	847	0	0
H <sub>2</sub> O <sub>2</sub>	175	175	100
Lipid, no plasmid	1,853	6	0.324
pIRES2-EGFP	1,429	0	0
pIRES2-E2F2/EGFP	3,373	2	0.059

*n* = 2.

\* Total nuclei in 5–20 $\times$  microscopic fields.



**FIGURE 6.** Representative RT-PCR results demonstrate the effect of E2F2 overexpression on the relative levels of Ki67 mRNA in ex vivo rabbit corneal endothelium. PCR products are shown for G3PDH (lanes 2 and 3) and Ki67 (lanes 4 and 5) in cells transfected with the pIRES2-EGFP (lanes 2 and 4) or the pIRES2-E2F2/EGFP plasmid (lanes 3 and 5). At 48 hours after transfection, PCR product for Ki67 was clearly detectable at higher levels in tissue transfected with the pIRES2-E2F2/EGFP plasmid than with the control plasmid. G3PDH results indicate similar sample loads. Portion of 100-bp ladder (lane 1).

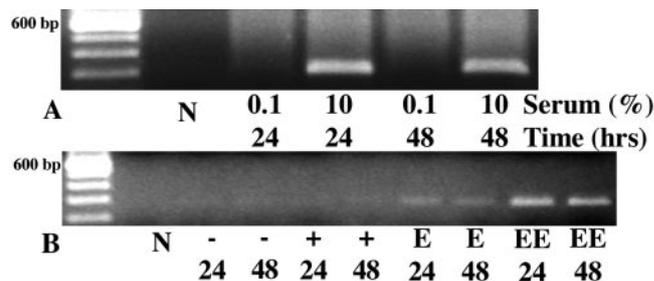
## DISCUSSION

Human corneal endothelial cells *in vivo* are arrested in G<sub>1</sub>-phase of the cell cycle and, as such, normally do not proliferate.<sup>16,17</sup> Previous studies by Wilson et al.<sup>13,14</sup> in which cultured human corneal endothelial cells proliferated in response to the overexpression of viral oncogenes have indicated that endothelial cells retain proliferative capacity despite their normally nonreplicative state. That these cells could be induced to proliferate on expression of SV40 large-T antigen or E6/E7 suggests a possible cellular mechanism that could be exploited to induce transient proliferation. Both the SV40 large-T antigen and E6/E7 papilloma virus proteins are known to compete strongly with E2F for binding to pRb, resulting in the subsequent release and activation of E2F.<sup>47</sup> This suggests that overexpression of E2F may act in a manner similar to that of viral oncogenes without the requirement for expressing “foreign” proteins in these cells. The current studies tested the hypothesis that it should be possible to bypass the G<sub>1</sub>-phase arrest in corneal endothelial cells by overexpressing E2F2. This E2F isoform was chosen because studies in other cell types indicated that it could promote cell cycle entry while inducing significantly lower levels of apoptosis compared with E2F1.<sup>24,42</sup> In preliminary RT-PCR studies, we determined that E2F2 is endogenously synthesized in both nonproliferating rabbit and human corneal endothelial cells. This established that E2F2 is already native to endothelial cells and that its overexpression would not introduce high levels of a foreign protein. In addition, expression of this isoform in both human and rabbit cells indicates that results obtained using the rabbit model would be relevant to human tissue. Using an ex vivo rabbit cornea model, we have now shown by ICC that lipid-based transfection with a cDNA plasmid for full-length E2F2 resulted in coexpression of high levels of E2F2 and marker EGFP in endothelial cells. Use of transfection reagent as a vehicle resulted in a transfection efficiency of at least 10% to 12%.

Assays were performed to determine the viability of the endothelium in ex vivo rabbit corneas and of confluent rabbit corneal endothelial cells after lipid-based transfection and E2F2 overexpression. Micrographs of the endothelium in ex vivo corneas showed little difference in the relative number of dead cells in control, untransfected corneas compared with those transfected with the E2F2-containing or control plasmid. Further evaluation using confluent cultures showed that incubation of cells in lipid reagent alone negatively affected cell viability. Transfection with pIRES2-E2F2/EGFP or the control plasmid did not further decrease viability. These results indi-

cate that cell viability was negatively affected by lipid treatment, but that overexpression of E2F2 did not have an additional negative effect. Results of the TUNEL assay suggest that, in rabbit corneal endothelial cells, lipid treatment did not induce cell death through apoptosis. The lack of a significant number of TUNEL-positive cells in cultures transfected with the E2F2 plasmid, at a time when a maximum number of cells express E2F2, suggests that E2F2 overexpression does not induce apoptosis in these cells. Since transfection efficiency using our lipid-based technique was 10% to 12%, it is possible that E2F2 overexpression could have induced apoptosis, but that the relative number of TUNEL-positive cells was too low to provide relevant quantitative data. In the cell-viability assay, the average percent of ethidium D-positive (dead) nuclei observed in cultures transfected with the E2F2 plasmid was only 1% higher than that of the control plasmid. Therefore, it appears that overexpression of E2F2 does not induce significant cell death. Future studies using adenoviral vectors will investigate the effect of E2F2 overexpression in a larger cell population and should provide important additional data regarding the potential induction of apoptosis.

RT-PCR was the method of choice to examine the effect of E2F2 overexpression in ex vivo rabbit corneal endothelium for several reasons. One method to observe cell cycle progression and obtain semiquantitative data would be to immunostain the endothelium for various cell cycle markers and then count the relative number of positive cells double-stained for the marker plus EGFP compared with total nuclei. This method was discarded, because it was difficult to obtain flat preparations of rabbit cornea consistently, even with incubation in high-molecular-weight dextran to reduce stromal swelling, thus making accurate observation and counting of positive cells very difficult. Western blot studies were ruled out because of the relatively large amount of protein required for gel electrophoresis. RT-PCR was chosen because reverse transcription made it possible to amplify the signal obtained from the relatively small amount of mRNA isolated from the endothelium of individual rabbit corneas and to obtain reliable semiquantitative data. These RT-PCR experiments demonstrated that overexpression of E2F2 mRNA correlated with increased levels of mRNA for



**FIGURE 7.** RT-PCR for cyclin B1 in subconfluent rabbit corneal endothelial cells (A) and in the endothelium of ex vivo corneas overexpressing E2F2 (B). In (A), subconfluent cells grown in 10% FBS (a concentration that supports proliferation) yielded a PCR product for cyclin B1 after incubation for either 24 or 48 hours after transfection. Subconfluent cells incubated in 0.1% FBS (a concentration that does not support proliferation) did not yield a cyclin B1 PCR product at either time point. RT-PCR in (B) is representative of results obtained from rabbit corneas incubated under one of the following conditions: no lipid/no plasmid (-); with lipid/no plasmid (+); with lipid and pIRES2-EGFP (E); with lipid and pIRES2-E2F2/EGFP (EE). A low level of PCR product for cyclin B1 was detected in endothelium transfected with the control plasmid, but a higher level of product was obtained in endothelium transfected with the plasmid containing full-length cDNA for E2F2 (EE). No positive cyclin B1 PCR product was detected in controls at either time-point. N, PCR control containing no cDNA. *Left:* 100-bp ladder.

both Ki67, a marker of actively cycling cells, and cyclin B1, the G<sub>2</sub>-regulatory protein whose gene promoter becomes activated at the end of the S-phase. The finding that mRNA levels of both Ki67 and cyclin B1 increased 5- and 2-fold, respectively, in the endothelium of corneas transfected with full-length cDNA for E2F2 provides strong evidence that E2F2 overexpression induces cell cycle progression from the normal G<sub>1</sub>-phase arrested state through at least the G<sub>2</sub>-phase of the cell cycle. Studies by Ishida et al.<sup>61</sup> support our finding that E2F2 overexpression results in increased mRNA expression of cyclin B1. Those studies used high-density DNA microarray analysis to identify genes induced by the activity of E2F isoforms. Results indicated that overexpression of either E2F1 or E2F2 increased the expression of a cluster of S-phase-related genes, as well as a cluster of genes whose products are active in the G<sub>2</sub>-phase, including cyclin B1. This finding adds strength to the argument that, in rabbit corneal endothelial cells, overexpressed E2F2 is active and that induction of G<sub>2</sub>-phase genes is an expected downstream effect of normal E2F activity.

In summary, in these studies, overexpression of the transcription factor E2F2 induced cell cycle progression in the endothelium of ex vivo rabbit corneas. Viability assays indicated that the lipid transfection reagent induced a nonapoptotic form of cell death, but overexpression of E2F2 did not negatively affect cell viability. RT-PCR analysis provided evidence that overexpressing E2F2 is sufficient to increase the expression of cell cycle markers, such as Ki67 and cyclin B1. Because Ki67 is a known marker of actively cycling cells and cyclin B1 levels only increase in the S to G<sub>2</sub>-phase of the cell cycle, the results suggest that E2F2 overexpression induces cell cycle progression from the normal state of G<sub>1</sub>-phase arrest at least through the G<sub>2</sub>-phase of the cycle. Of importance is the fact that this study did not demonstrate actual cell division. With the relatively low transfection efficiency obtained using lipid-based methods, it was not possible to demonstrate unequivocal cell division by in situ or in vitro cell counts or by ICC methods. Recently, we reported (Mc Alister JC, et al. *IOVS* 2003;44:ARVO E-Abstract 4334) that infection of ex vivo human donor corneas with an adenoviral vector containing full-length E2F2 cDNA induced a significant ( $P < 0.001$ ) increase in the density of endothelial cells, providing additional evidence that overexpression of this E2F isoform promotes cell cycle progression in corneal endothelium.

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