p27kip1 siRNA Induces Proliferation in Corneal Endothelial Cells from Young but Not Older Donors

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Purpose. To determine whether small interfering (si)RNA downregulation of the cyclin-dependent kinase inhibitor p27kip1 overcomes G_1-phase arrest and promotes cell-cycle progression in human corneal endothelial cells (HCECs) from young (<30 years old) and older (>60 years old) donors.

Methods. Transfection of siRNA was confirmed by incubating confluent cultures of HCECs with FITC-labeled nonsilencing siRNA. Confluent cultures were transfected for 48 hours with p27kip1 siRNA (2.5, 5, 25, or 100 nM) or nonsilencing siRNA, with a lipid transfection reagent. As a comparison, cultures were also transfected for 48 hours with p27kip1 antisense (AS) or missense (MS) oligonucleotides (oligo). At various times after transfection, cells were fixed for immunocytochemical localization of p27kip1 or extracted for Western blot analysis to assess relative p27kip1 protein levels. Cultures were also prepared for ZO-1 immunolocalization, to assess the effect of transfection on the morphology of the monolayer. The number of cells was counted at 0, 48, 96, 144, and 192 hours after incubation, and a cell-viability assay was performed.

Results. A dose-dependent decrease in p27kip1 protein level was observed in Western blot analysis, and nuclear staining for p27kip1 was greatly reduced in HCECs incubated with p27kip1 siRNA. No change in p27kip1 levels or in nuclear staining was observed in the nonsilencing control. p27kip1 siRNA (25 nM) appeared to be quantitatively more efficient than antisense oligonucleotide (500 nM) in reducing p27kip1 protein levels. Viability was less affected by siRNA treatment than by AS oligo transfection. ZO-1 staining showed no effect on morphology of the monolayer. The number of HCECs from young donors (<30 years old) transfected with p27kip1 siRNA increased up to 144 hours after incubation, whereas no change in the number of cells was observed in HCECs transfected with nonsilencing siRNA. In contrast to the results from young donors, no change in the number of cells was observed at any time point tested in HCECs from older donors (>60 years old) after p27kip1 siRNA transfection.

Conclusions. Transfection of p27kip1 siRNA was sufficient to promote proliferation in confluent cultures of HCECs from younger, but not older donors. These results suggest that inhibition of proliferation in older donors is regulated by other mechanisms in addition to p27kip1. (*Invest Ophthalmol Vis Sci.* 2006;47:4803–4809) DOI:10.1167/iovs.06-0521

Corneal endothelium is the fragile monolayer of cells located at the posterior of the cornea that is responsible for maintaining corneal transparency through its barrier and ionic pump functions.[1] Human corneal endothelial cells (HCECs) are considered to be nonproliferative in vivo, since the rate of proliferation does not keep pace with the rate of cell loss. As a result, HCEC density decreases with age.[2,3] Maintenance of corneal clarity requires an intact endothelial monolayer, and transparency can be lost when endothelial cell density is reduced below a critical level. Although CECs in vivo exhibit limited mitotic activity,[4] they possess proliferative capacity.

Corneal endothelial cells in vivo are arrested in the G_1-phase of the cell cycle[5,6] and appear to be actively maintained in a nonproliferative state. The ability to progress through G_1-phase of the cell cycle is dependent on the relative balance between the activity of stimulatory and inhibitory factors. Cells progress through the G_1-phase when the balance favors the stimulatory factors.[7] The G_1-phase arrest occurs when the cellular concentration and activity of inhibitory factors exceeds that of stimulatory factors. Cell-cycle progression is negatively regulated by the relative balance between the cellular concentration of cyclin-dependent kinase inhibitors (CKIs), such as members of the Cip/Kip and INK families, and that of cyclin/cyclin-dependent kinase (CDK) complexes, such as cyclin D1-CDK4 and cyclin E-CDK2.[8] On mitogenic stimulation, cyclins D and E are synthesized, increasing the concentration of positive stimulators relative to that of G_1-phase inhibitors.[9] Once the overall concentration of cyclin/CDKs exceeds that of the inhibitors, active cyclin/CDK complexes will be formed, and the cell cycle will proceed.[10–12]

The Cip/Kip and INK families of proteins are cyclin-dependent kinase inhibitors and induce G_1-phase arrest.[13–15] In many cell types, p27kip1, a member of the Cip/Kip family, helps mediate cell cycle arrest induced by cell–cell contact and TGF-β.[14] The level of p27kip1 protein expression is high in G_0/G_1 resting cells and declines as cells progress toward the S-phase. Overexpression of p27kip1 inhibits entry into the S-phase in normal and malignant cells.[16,17] In a study by Joyce et al.[18], p27kip1 was shown to be important in maintaining cultured rat CECs in G_1-phase arrest. This study demonstrated a correlation between high levels of p27kip1 protein and inhibition of proliferation in contact inhibited cells, providing evidence that p27kip1 is an important mediator of contact inhibition in rat corneal endothelium. Lee and Kay[19] and Kim et al.[20] have demonstrated that p27kip1 plays an important role in the negative regulation of proliferation in rabbit CECs. FGF-2 stimulates proliferation in these cells, mainly through the activity of the PI 3-kinase pathway. FGF-2 induced activation of this pathway results in specific phosphorylation of nuclear p27kip1 on Thr^{187}, which in turn results in the proteolysis of p27kip1, reduction of p27kip1 protein levels, and subsequent reduction of inhibition of cell cycle progression. Downregulation of p27kip1 in knockout (−/−) mice results in

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unregulated cell growth and tumor formation.[21] In other cell types, downregulation of p27kip1 protein levels by antisense methods leads to increased proliferative activity.[22] In a previous study, we showed that lowering of p27kip1 protein levels using antisense methods promotes proliferation in confluent cultures of rat CECs.[23] Therefore, it would be expected that downregulation of p27kip1 protein expression using small interfering (si)RNA methods would overcome G_1-phase arrest in HCECs.

The long-term goal of our studies is to develop therapy that increases cell density in stressed corneal endothelium. Using the method of culturing HCECs that was established in this laboratory, we can consistently culture untransformed CECs from older donors that show normal polygonal morphology at confluence.[24,25] The present studies were designed to test the effect of lowering p27kip1 protein levels on induction of proliferation in HCECs cultured from young (<30 years old) and older (> 60 years old) donors. Viability studies comparing antisense and siRNA methods in cultured HCECs demonstrated that HCECs were much more sensitive to antisense treatment than rat corneal endothelium. As a result, siRNA technology was used to lower p27kip1 protein levels in HCECs.[26–29] siRNA has high specificity and the ability to inhibit the expression of biologically active proteins selectively and can produce complete inhibition of gene expression.[27] siRNA is also known to specifically block protein expression to a greater extent than antisense methods and it is possible to obtain the same blocking effect using smaller amounts of siRNA than of antisense oligonucleotides.[30,31] We hypothesized that HCEC proliferation would be stimulated by decreasing the cellular concentration of p27kip1 with siRNA methods.

Materials and Methods

Human Corneal Tissue

Donor corneas were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) and stored in corneal preserving medium at 4°C (Optisol-GS; Bausch & Lomb, Tampa, FL). Exclusion criteria were the same as described by Joyce and Zhu.[32] Handling of donor information by the NDRI and this laboratory adhered to the tenets of the Declaration of Helsinki in protecting donor confidentiality. HCEC cultures were prepared as previously described.[24,25] Primary cultures were grown to confluence in culture medium (OptiMEM-I; Invitrogen Life Technologies, Carlsbad, CA), containing 8% fetal bovine serum (FBS: Hyclone, Logan, UT), 5 ng/mL epidermal growth factor (EGF: Upstate Biotechnology, Lake Placid, NY), 20 ng/mL nerve growth factor (NGF: Biomedical Technologies, Stoughton, MA), 100 μg/mL bovine pituitary extract (Biomedical Technologies), 20 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 mg/L calcium chloride (Sigma-Aldrich), 0.08% chondroitin sulfate (Sigma-Aldrich), 50 μg/mL gentamicin (Invitrogen-Life Technologies), and antibiotic-antimycotic solution diluted 1:100 (Invitrogen-Life Technologies). Depending on the experiment, confluent cells were subcultured onto sterile four-chamber slides (Laboratory Tek, Naperville, IL) or into T25 flasks that had been precoated with undiluted coating mix (FNC; Biological Faculty and Facility, Inc., Ijamsville, MD) and grown to confluence. The medium was changed every other day. All incubations were performed in a 5% CO_2-95% air, humidified atmosphere at 37°C.

p27kip1 siRNA and Antisense Transfection

p27kip1 siRNA (r[GUACGAGUGGCAAGAGGUG]dTT), nonsilencing siRNA (r[UUCUCCGAACGUGUCACGU]dTT), FITC-labeled nonsilencing siRNA, and transfection reagent (RNAiFect) were purchased from Qiagen (Valencia, CA). In an initial experiment, confluent cells were incubated for 18 hours with 5 nM FITC-labeled nonsilencing siRNA, to confirm transfection into HCECs. For the p27kip1 siRNA studies, the siRNA-to-lipid ratio was 1 μg to 6 μL. This complex was diluted in MEM-I (Hyclone, Inc.) with 8% FBS. Confluent cells were then transfected for 48 hours with p27kip1 siRNA (2.5, 5, 25, or 100 nM). Control cultures were incubated in one of the following conditions: (1) no siRNA or reagent, (2) reagent alone, or (3) reagent plus 25 nM nonsilencing siRNA. At specific time points after transfection, cells were fixed immediately for immunocytochemistry, extracted for Western blot analysis, or prepared for viability assay.

Antisense (AS) and missense (MS) oligos for p27kip1 were obtained from Midland Certified Reagent Company (Midland, TX): antisense sequence, 5′ (PS)GCGTCTGCTCCACAG3′; mismatch sequence, 5′ (PS)GCATCCCCTGTGCAG3′.[33] Transfection of p27kip1 AS or MS oligonucleotide (500 nM) was performed for 48 hours using a nonliposomal lipid transfection kit (Effectene; Qiagen), according to the manufacturer's protocol. An oligo-to-transfection reagent ratio of 1:25 was used. The transfection complex was diluted in MEM-I with 8% FBS. Cells were incubated for 48 hours and either extracted for Western blot analysis or prepared for the cell-viability assay. Control cultures were incubated with no oligo or reagent.

Immunocytochemistry

Endothelial cells were subcultured on four-chamber slides, grown to confluence, and transfected for 48 hours with p27kip1 siRNA or nonsilencing siRNA. The cells were then rinsed with phosphate-buffered saline (PBS; Invitrogen-Life Technologies) and fixed with 99.9% methanol for 10 minutes at −20°C. All further incubations were at room temperature. The slides were rinsed with PBS, and the cells were permeabilized for 10 minutes with 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Nonspecific sites were blocked for 10 minutes using 4% bovine serum albumin (BSA) in PBS. The cells were incubated for 2 hours with rabbit polyclonal anti-p27kip1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 or with rabbit polyclonal anti-ZO-1 (Invitrogen) diluted 1:150. The slides were rinsed with PBS and incubated for 2 hours with fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 in PBS. Control cultures were incubated with secondary antibody alone. Coverslips were mounted with antifade medium containing DAPI (4′6 diamidino-2-phenylindole; Vectashield; Vector Laboratories, Inc., Burlingame, CA) to stain the cell nuclei. Positive staining of cultured cells was visualized on a microscope (Eclipse E800, with a VFM Epi-Fluorescence Attachment; Nikon Inc., Melville, NY), equipped with a digital camera (Spot and Spot version 1.1 CE software; Diagnostic Instruments, Sterling Heights, MI). These experiments were repeated at least three times.

Western Blot Analysis

After transfection for 48 hours with p27kip1 siRNA or nonsilencing siRNA, cultured cells were trypsinized and pelleted. Proteins were extracted by incubating cells for 30 minutes at 4°C in buffer containing 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich), followed by homogenization and centrifugation. Protein content was quantified by spectrophotometry. Equal protein was loaded on 4% to 12% Bis-Tris gels for SDS-PAGE. Peptides were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), and nonspecific binding was blocked by incubation overnight at 4°C in 5% nonfat milk diluted in PBS. Membranes were incubated for 2 hours with rabbit polyclonal anti-p27kip1 diluted 1:200 in blocking buffer. Blots were rinsed three times for 10 minutes with 0.1% Triton X-100, then reblocked and exposed for 1 hour to horseradish peroxidase (HRP)–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:10,000 in blocking solution. The same blots were probed with rabbit anti-nonmuscle myosin (Biomedical Technologies, Inc.) diluted 1:200 to control for protein load. After a thorough wash, peptides were detected with a chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL). For quantification, films were digitally scanned (BDS-Image; Biological Detection System, Pittsburgh, PA). Scans were ana-

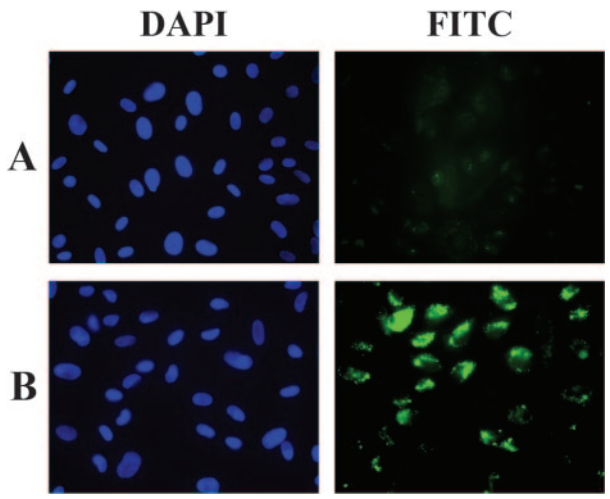

FIGURE 1. Confirmation of siRNA transfection. HCEC cultures were incubated for 18 hours with transfection reagent alone (**A**) or with reagent plus FITC-labeled nonsilencing siRNA (**B**). *Green* fluorescence (FITC): transfected cells; *blue* fluorescence (DAPI): all nuclei. Magnification, ×60.

lyzed with NIH Image software version 1.61 (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), and protein content was normalized according to nonmuscle myosin protein content. These experiments were repeated at least three times.

Quantification of Proliferating Cells

HCECs were cultured from five young donors (19, 22, 25, 27, and 28 years old) and from six older donors (60, 64, 67, 72, 72, and 75 years old). For all studies, passage 2 HCECs were used to test the effect of p27kip1 siRNA on cell proliferation. Cells were grown to confluence in 24-well culture plates, transfected for 48 hours with 25 nM p27kip1 siRNA or 25 nM nonsilencing siRNA, then postincubated for 0, 48, 96, 144, or 192 hours in the same culture medium described for primary culture. The medium was changed every other day. At each designated time point after transfection, cultures were trypsinized, and the number of cells was determined by cell counter (Coulter Electronics, Hialeah, FL). Cells from each well were counted three times, and at least three separate wells were counted per time point and condition. Results were averaged, and standard deviations were calculated. Statistical analysis using Student's paired *t*-test was performed using Sigma Stat version 2.0 (SPSS, Chicago, IL). $P < 0.05$ was considered to be significant.

Viability of HCECs

Viability was assessed (Live/Dead assay kit; Invitrogen-Molecular Probes, Eugene, OR) after 48 hours of incubation with 25 nM p27kip1 siRNA and 500 nM AS oligo, or incubation under control conditions. Staining was visualized by fluorescence microscopy using a microscope equipped with a digital camera (Eclipse YS100 with a Coolpix 995 camera; Nikon). The percentage of cell survival was calculated by dividing the number of healthy cells by the total number of cells. Results were compared with those of untreated control cultures in experiments repeated at least three times.

RESULTS

Confirmation of siRNA Transfection

Preliminary studies were conducted to confirm siRNA transfection using (RNAiFect Transfection Reagent; Qiagen). Confluent HCECs were incubated for 18 hours with transfection reagent alone (Fig. 1A) or with 5 nM FITC-labeled nonsilencing siRNA (Fig. 1B). Cultures were then washed, and siRNA was visualized by fluorescence microscopy. The presence of FITC-positive cells indicated successful siRNA transfection. Results of the cell-viability assay demonstrated that viability was not significantly affected by siRNA transfection under these conditions (data not shown).

Effect of siRNA Transfection on p27kip1 Protein Levels

The effect of siRNA transfection on p27kip1 levels was determined by Western blot analysis and immunocytochemistry (ICC). After transfection for 48 hours with increasing concentrations of p27kip1 siRNA or with transfection reagent without siRNA, cells were washed and either prepared for Western blot analysis or ICC. Figure 2A demonstrates that transfection of confluent HCECs with p27kip1 siRNA reduced p27kip1 protein levels in a dose-dependent manner. Semiquantitative analysis of the blots (Fig. 2B) indicates that the protein level of p27kip1 was reduced with 2.5 nM p27kip1 siRNA after transfection and was suppressed to approximately 17% of control levels by 25nM siRNA. As a result of these studies, 25 nM p27kip1 siRNA was used in all subsequent studies.

For ICC evaluation of the effect of p27kip1 siRNA transfection on p27kip1 levels, confluent cultures were incubated for 48 hours with 25 nM p27kip1 siRNA (Fig. 3A), 25 nM nonsilencing siRNA (Fig. 3B), transfection reagent alone (Fig. 3C), or no siRNA or reagent (Fig. 3D), and then processed for ICC of p27kip1. Staining for p27kip1 was greatly reduced in HCECs incubated with p27kip1 siRNA (Fig. 3A). Images in Figures 3B–D show strong staining for p27kip1 in the nuclei with a low level of staining in the cytoplasm of HCECs, indicating no change in p27kip1 protein levels in these control cells.

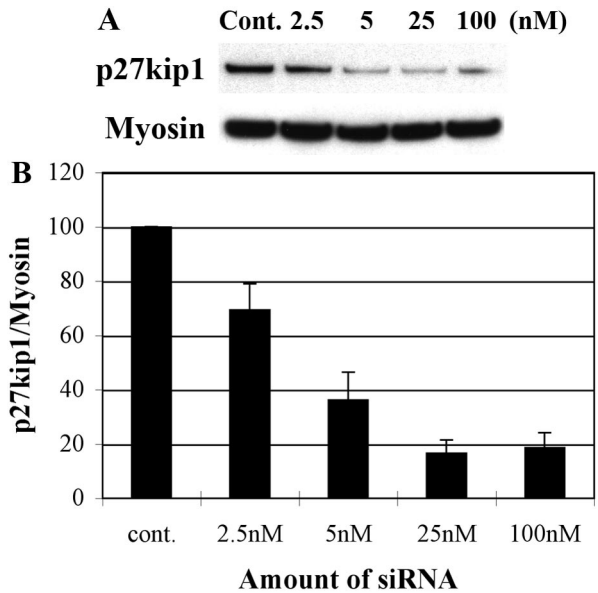

FIGURE 2. (**A**) Western blot analysis comparing p27kip1 protein expression after 48-hour treatment with increasing concentrations of p27kip1 siRNA or transfection reagent without siRNA (Cont.). Nonmuscle myosin was used for normalization of protein load. (**B**) p27kip1 protein level standardized to the amount of nonmuscle myosin present in the same samples. Bars, SD.

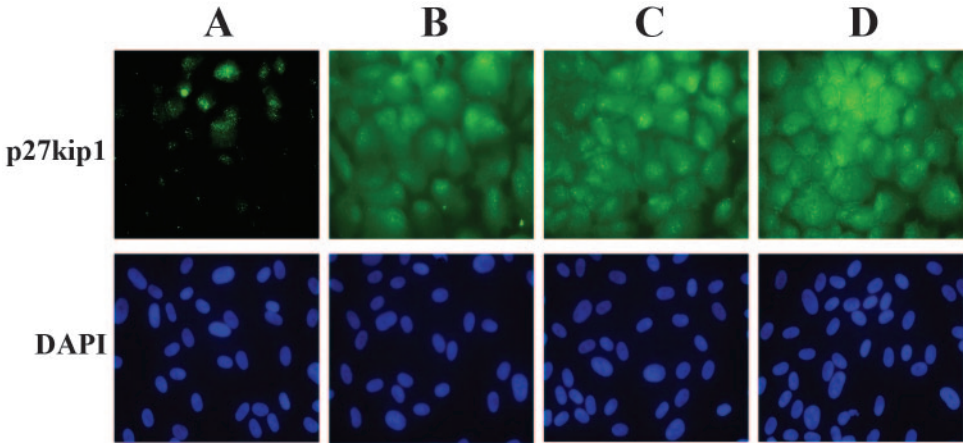

FIGURE 3. p27kip1 immunostaining of HCEC cultures incubated for 48 hours with 25 nM p27kip1 siRNA (**A**), 25nM nonsilencing siRNA (**B**), transfection reagent (**C**), or no siRNA or reagent (**D**). *Green* fluorescence (FITC) localizes p27kip1; *blue* fluorescence (DAPI) stains all nuclei. Magnification, ×60.

Comparison of the Effect of siRNA and Antisense Oligonucleotide Treatment on p27kip1 Protein Levels and Cell Viability

To compare the effect of siRNA and antisense oligonucleotide (oligo) treatment on p27kip1 protein levels and viability after transfection, HCEC cultures were incubated for 48 hours with 25 nM p27kip1 siRNA, 25 nM nonsilencing siRNA (Cont siRNA), no siRNA or reagent (Cont.), 500 nM p27kip1 antisense oligo (p27 AS), 500 nM missense oligo (p27 MS), or no oligo or reagent (Cont.) in the same conditions as just described and then processed for Western blot analysis (Figs. 4A, 4B) and viability assay (Fig. 4C). p27kip1 siRNA at 25 nM appeared to be quantitatively more efficient than antisense oligo at 500 nM (Figs. 4A, 4B). Viability was less affected by siRNA treatment than by antisense oligo (Fig. 4C).

Effect of p27kip1 siRNA on Monolayer Integrity

Confluent cultures of HCECs were immunostained for ZO-1, a tight junction-associated protein, to assess the effect of the transfection reagent and siRNA treatment on the morphology of the endothelial monolayer. Figure 5 shows a normal ZO-1 pattern in p27kip1 siRNA-treated and control cultures, indicating that siRNA transfection did not have a deleterious effect on cell shape or monolayer integrity.

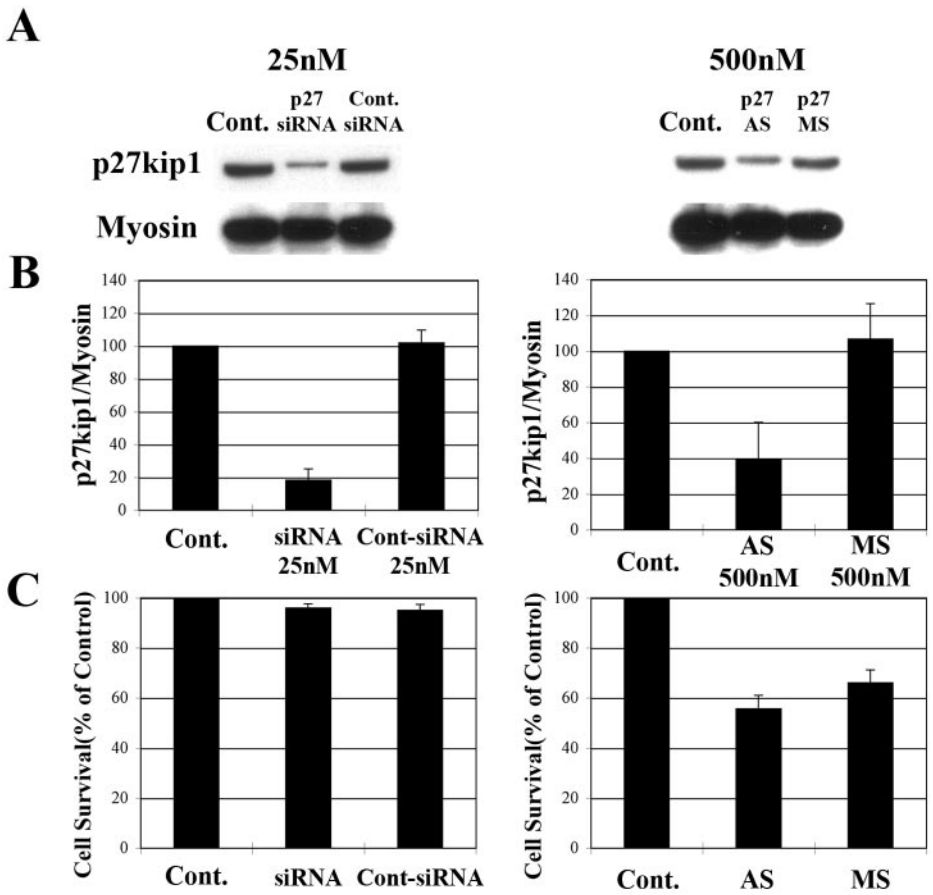

FIGURE 4. Comparison of the inhibition of p27kip1 protein expression (**A**, **B**) and of the effect on viability (**C**) induced by siRNA and antisense oligo treatment. Confluent HCECs were incubated for 48 hours with 25 nM p27kip1 siRNA, 25 nM nonsilencing siRNA (Cont siRNA), no siRNA or reagent (Cont.), 500 nM p27kip1 antisense oligo (p27 AS), 500 nM missense oligo (p27 MS), or no oligo or reagent (Cont.) and then extracted for Western blot analysis (**A**). (**B**) p27kip1 protein levels standardized to the amount of nonmuscle myosin present in the same samples. (**C**) Percentage of live cells.

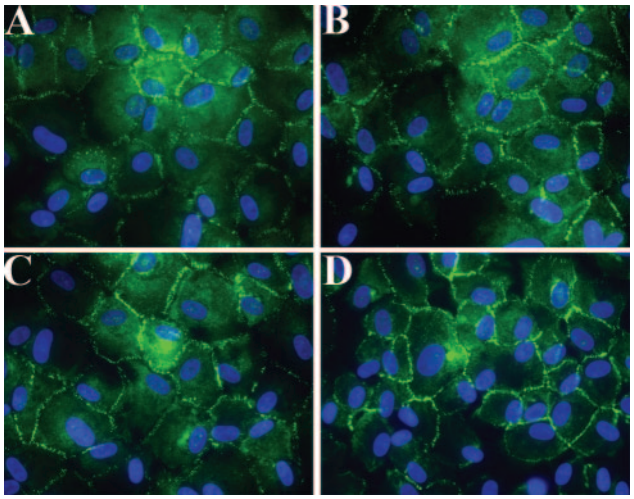

FIGURE 5. Immunolocalization of ZO-1 in confluent cultures of HCECs incubated without transfection reagent or siRNA (**A**), or incubated with transfection reagent only (**B**), control siRNA (**C**), or p27kip1 siRNA (**D**). The normal disrupted, linear ZO-1 pattern is observed under all treatment conditions. Magnification: ×100.

Effect of p27kip1 siRNA on Proliferation of HCECs from Young and Old Donors

HCECs do not normally replicate in vivo and are arrested in the G_1-phase, although they maintain proliferative capacity.[5,6] We questioned whether p27kip1 inhibition could overcome G_1-phase arrest in a confluent monolayer of cells and promote proliferation. To study the effects of p27kip1 siRNA treatment on HCECs, confluent HCEC cultures were transfected for 48 hours with 25 nM p27kip1 siRNA or 25 nM nonsilencing siRNA; postincubated for 0, 48, 96, 144, or 192 hours; and processed for cell counting. Six corneas from older donors (>60 years old) and five corneas from young donors (<30 years old) were used for the study. HCECs cultured from older donors and transfected with p27kip1 siRNA showed no change in the number of cells when compared with control cells at any time-point tested (Fig. 6A). In contrast, the relative number of HCECs from young donors that had been transfected with p27kip1 siRNA showed a statistically significant increase (to ~130% of control levels) by 144 hours after incubation. After this time, the number of cells appeared to plateau (Fig. 6B).

DISCUSSION

In many cell types, p27kip1 helps mediate inhibition of the cyclin-dependent kinase activity that is required for cell-cycle progression and is particularly active in mediating cell-cycle arrest induced by cell–cell contact and TGF-β. We have demonstrated that both cell–cell contact and TGF-β help maintain CECs in G_1-phase arrest[19,34] and suggested that p27kip1 must play a role in inhibiting proliferation in CECs. To test this idea, we demonstrated that p27kip1 antisense oligo treatment could successfully decrease the cellular concentration of p27kip1 in cultured rat CECs and promote proliferation, as evidenced by the increased number of cells.[23]

In the current studies, we extended our inquiry to determine whether decreasing p27kip1 protein levels would promote proliferation of HCECs. We found that HCECs are much more sensitive to antisense oligo treatment than are rat CECs, resulting in an increased loss of cell viability. As a result, we tested p27kip1 siRNA as an alternative method to decrease p27kip1 protein levels in HCECs. Although there have been no reported studies using siRNA methods in human corneal endothelium to alter the synthesis of cell cycle regulatory proteins, we successfully used this technique to reduce p27kip1 protein levels and to promote proliferation in cultured HCECs. In recent years, siRNA techniques have been used for studying biological function and developing new therapeutic strategies in other cell types.[35,36] Recent studies of the downregulation of gene expression indicate that siRNA is an effective method for functional gene analysis.[28,29] It has been reported that siRNA methods are more effective and less harmful to cells than are antisense methods and that siRNA is generally more efficient at reducing protein levels.[30,31] The current studies indicate the feasibility of using siRNA, not only for controlling p27kip1 protein levels, but also for reducing the cellular concentration of other proteins of interest.

Of particular interest, is the fact that p27kip1 siRNA treatment only promoted proliferation in cells cultured from young donors, whereas HCECs from older donors showed no change in the number of cells after similar treatment. These results strongly suggest that there is an age-related change in the molecular mechanisms that regulate proliferation in HCECs. Our laboratory has shown that HCECs from young donors are more efficient in responding to positive growth factors than are cells cultured from older donors.[25,32,37] We have also shown similar age-related differences in cell-cycle kinetics in an

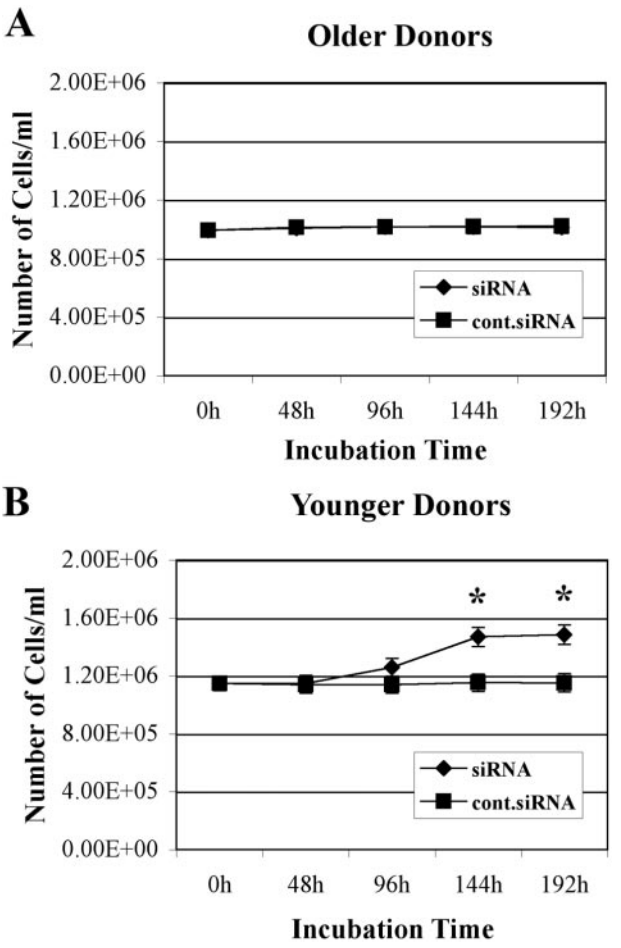

FIGURE 6. Confluent HCECs, cultured from five young (19, 22, 25, 27, and 28 years old) and six older donors (60, 64, 67, 72, 72, and 75 years old), were transfected for 48 hours with p27kip1 siRNA or nonsilencing siRNA (cont.), transferred to fresh culture medium, and counted at 0, 48, 96, 144, or 192 hours after incubation. No change from control levels was observed at any time point tested in HCECs of older donors. Results are expressed as the mean ± SD. $^*P < 0.05$.

ex vivo wound model, thus demonstrating that this is not a culture artifact.[38] In addition, recent studies using cultured HCECs[39] indicate that there is an age-related increase in the relative expression of the cyclin-dependent kinase inhibitors p21cip1 and p16INK4a, whereas, there was little change in the relative expression of p27kip1 with donor age. Together, evidence strongly suggests that HCECs from younger donors have a higher proliferative capacity than do older HCECs. Among the underlying reasons for this observed age-related difference may be increased inhibition of cyclin-dependent kinase activity by p21cip1 and/or p16INK4a. The results obtained in the present study clearly suggest that p27kip1 must be an important barrier to proliferation in HCECs from younger donors; however, it appears that there are additional inhibitory control mechanisms in HCECs from older donors.

Results of the current studies using HCECs appear to differ from those of previous studies from this laboratory that showed increased proliferation in rat CECs after p27kip1 antisense treatment. This discrepancy may be due to the relatively young rats used in those studies. Fitch et al.[40] demonstrated that there is a progressive decline in the number of cells and an increase in pleomorphism in rat corneal endothelium from age 6 months to 30 months, closely paralleling changes reported in human endothelium in individuals from 20 to 70 years old. Similar results were obtained by Meyer et al.,[41] who demonstrated an age-related decrease in the number of cells and an increase in polymorphism and polymegathism in rat corneal endothelium by 34 weeks of age. The rats used in our p27kip1 AS oligo studies were 6 weeks of age. Thus, our rat model and the cellular response appear to reflect more closely the behavior of young human donor endothelium.

In the past few years, studies have been conducted to determine the feasibility of transplanting the posterior cornea to replace endothelial cells[42] rather than transplanting full-thickness corneas. Another approach has been to culture HCECs on supports, such as amniotic membrane[43] and then transplant the construct to increase endothelial cell density. This second approach is hampered by the fact that HCECs are relatively difficult to culture. Cells from very young donors are most reliable for culture; however, these cells cannot be obtained in the quantities needed to make these new transplantation techniques widely available to patients. Our laboratory has successfully isolated and cultured HCECs from both young and older donors using reported techniques.[24,25] It is clear, however, that optimal culture of HCECs for transplantation purposes requires a deeper understanding of cell-cycle regulation.

From the current data, we conclude that p27kip1 plays an important role in G_1-phase arrest, at least in HCECs from young donors, and that decreasing its protein level using siRNA methods can promote proliferation in these cells. The induction of proliferation in this experiment occurred without apparent cell damage. Because this treatment might be applied to HCECs to increase cell density in individuals with abnormalities of endothelial cell density, it is important to understand the basis for the age-related difference observed in response to this treatment and to elucidate the specific mechanisms by which the lowering of p27kip1 promotes proliferation in HCECs.

References

1. Dikstein S, Maurice D. The metabolic basis to the fluid pump in the cornea. *J Physiol*. 1972;221:29–41.
2. Murphy C, Alvarado J, Juster P, Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium: a quantitative histologic study. *Invest Ophthalmol Vis Sci.* 1984;25:312–322.
3. Laing RA, Sandstrom MM, Berrospi AR, Leibowitz HM. Changes in the corneal endothelium as a function of age. *Exp Eye Res.* 1976; 22:587–594.
4. Laing RA, Neubauer L, Oak S, Kayne HL, Leibowitz HM. Evidence for mitosis in adult corneal endothelium. *Ophthalmology*. 1984; 91:1129–1134.
5. Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci*. 1996;37:645–755.
6. Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci*. 1996;37:1566–1575.
7. Sherr CJ. Mammalian G1 cyclins. *Cell*. 1993;73:1059–1065.
8. Kato J, Matsushima H, Hiebert SW, et al. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev*. 1993;7: 331–342.
9. Ewen ME, Sluss HK, Sherr CJ, et al. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*. 1993; 73:487–497.
10. Coverley D, Laman H, Laskey RA. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. *Nat Cell Biol*. 2002;4:523–528.
11. Draviam VM, Orrechia S, Lowe M, et al. The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. *J Cell Biol*. 2001;152:945–958.
12. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. 1993;366:704–707.
13. Jeffrey PD, Tong L, Pavletich NP. Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. *Genes Dev*. 2000;14: 3115–3125.
14. Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev*. 1995;9:1831–1845.
15. Harper JW, Adamin GF, Wei N, et al. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*. 1993;75:805–816.
16. Polyak K, Kato JY, Solomon MJ, et al. p27Kip1, a cyclin-cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes Dev*. 1994;8:9–22.
17. Levenberg S, Yarden A, Kam Z, Geiger B. p27 is involved in N-cadherin-mediated contact inhibition of cell growth and S-phase entry. *Oncogene*. 1999;18:869–876.
18. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: Contact inhibition and TGF-β2. *Invest Ophthalmol Vis Sci*. 2002;43:2152–2159.
19. Lee HT, Kay EDP. Regulatory role of PI 3-kinase on expression of Cdk4 and p27, nuclear localization of Cdk4, and phosphorylation of p27 in corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2003;44:1521–1528.
20. Kim TY, Kim WI, Smith RE, Kay EDP. Role of $p27^{Kip1}$ in cAMP- and TGF-β2–mediated antiproliferation in rabbit corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2001;42:3142–3149.
21. Nakayama K, Ishida N, Shirane M, et al. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*. 1996;85:707–720.
22. Rivard N, L'Allemain G, Bartek J, Pouyssegur J. Abrogation of p27Kip1 by cDNA antisense suppresses quiescence (G0 state) in fibroblasts. *J Biol Chem*. 1996;271:18337–18341.
23. Kikuchi M, Harris DL, Joyce NC, et al. p27kip1 antisense-induced proliferative activity of rat corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2004;45:1763–1770.
24. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea*. 2001; 20:731–737.
25. Zhu C, Joyce NC. Proliferative response of corneal endothelial cells from young and older donors. *Invest Ophthalmol Vis Sci*. 2004;45:1743–1751.
26. Fire A, Xu S, Montgomery MK, et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature.* 1998;19:391:806–811.
27. Scherr M, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem*. 2003;10: 245–256.

28. Shuey DJ, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today*. 2002;7:1040–1046.
29. Hannon GJ. RNA interference. *Nature*. 2002;418:244–251.
30. Bertrand JR, Pottier M, Vekris A. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem Biophys Res Commun*. 2002;296:1000–1004.
31. Miyagishi M, Hayashi M, Taira K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev*. 2004;2003;13:1–7.
32. Joyce NC, Zhu CC. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. *Cornea*. 2004;23(Suppl 1):S8–S19.
33. St Croix B, Florenes VA, Rak JW, et al. Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. *Nat Med*. 1996;2:1204–1210.
34. Chen KH, Harris DL, Joyce NC. TGF-β2 in aqueous humor suppresses S-phase entry in cultured corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1999;40:2513–2519.
35. Li K, Lin SY, Brunicardi FC, et al. Use of RNA interference to target cyclin E-overexpressing hepatocellular carcinoma. *Cancer Res*. 2003;63:3593–3597.
36. Reich SJ, Fosnot J, Kuroki A, et al. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol Vis*. 2003;9:210–216.
37. Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res*. 2003;22:359–389.
38. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci*. 2000;41:660–667.
39. Enomoto K, Mimura T, Harris DL, Joyce NC. Age differences in cyclin-dependent kinase inhibitor expression and Rb hyperphosphorylation in human corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2006;47:4330–4340.
40. Fitch KL, Nadakavukaren MJ, Richardson A. Age-related changes in the corneal endothelium of the rat. *Exp Gerontol*. 1982;17:179–183.
41. Meyer LA, Ubels JL, Edelhauser HF. Corneal endothelial morphology in the rat. Effects of aging, diabetes, and topical aldose reductase inhibitor treatment. *Invest Ophthalmol Vis Sci*. 1988;29:940–948.
42. Melles GRJ, Lander F,et al.Preliminary clinical results of posterior lamellar keratoplasty through a sclerocorneal pocket incision. *Ophthalmology.* 2000;107:1850–1857.
43. Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci*. 2004;45:800–806.