$p27^{kip1}$ Antisense-Induced Proliferative Activity of Rat Corneal Endothelial Cells

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Purpose. To determine whether antisense downregulation of $p27^{kip1}$ will overcome G_1-phase arrest and promote cell cycle progression in rat corneal endothelial cells (CECs).

Methods. Confluent cultures of rat CECs were incubated for 24 hours in the presence of $p27^{kip1}$ antisense (AS) oligonucleotides (oligoS) using nonliposomal lipid transfection. Control cultures were incubated under one of the following conditions: no oligos or lipid-containing buffer, lipid-containing buffer alone, or lipid-containing buffer plus missense (MS) $p27^{kip1}$ oligo. Viability was tested by a cell-viability assay after 0, 24, 48, and 72 hours. After postincubation for 0, 24, 48, or 72 hours, cultures were fixed and immunostained for $p27^{kip1}$, to test for downregulation, or for Ki67 or BrdU, to detect actively cycling cells. Western blot and immunocytochemistry (ICC) studies were conducted to determine the effect of $p27^{kip1}$ antisense treatment on the relative protein level and subcellular localization of several cell cycle proteins, including cyclin-D1, -E, -A, and -B1; CDK2 and -4; $p21^{cip1}$; and $p15^{INK4b}$. Proliferation was determined by direct counting of propidium iodide (PI) or 4′,6′-diamino-2-phenylindole (DAPI)-stained cells.

Results. Viability was not significantly affected by lipid-based oligo transfection for up to 48 hours, after which a decline was noted. The protein level of $p27^{kip1}$ was reduced after AS transfection in a time-dependent manner. Nuclear staining for $p27^{kip1}$ was greatly reduced in CECs incubated with AS oligo. No change in $p27^{kip1}$ levels was observed in controls at any time point tested. $p27^{kip1}$ AS oligo transfection increased cyclin-D1, -E, -A, and -B1 protein levels, and all cyclins were localized to the nucleus. No changes in protein level were observed for CDK2, CDK4, $p21^{cip1}$, or $p15^{INK4B}$. A time-dependent increase in the relative number of Ki67- and BrdU-positive cells was noted in CECs incubated with AS oligo. In contrast, no to few Ki67- or BrdU-positive cells were observed in CECs incubated with MS oligo or the buffer-treated control cells. The percentage increase in the number of cells transfected with AS oligo increased with time, compared with that of cells transfected with MS oligo.

Conclusions. Treatment with $p27^{kip1}$ antisense oligonucleotides followed by postincubation in 10% FBS lowers endogenous $p27^{kip1}$ protein levels and promotes proliferation in confluent cultures of rat CECs. (*Invest Ophthalmol Vis Sci.* 2004; 45:1763–1770) DOI:10.1167/iovs.03-0885

Corneal endothelium is the single layer of cells located at the posterior of the cornea and is responsible for maintaining corneal transparency.[1] The density of human corneal endothelial cells (CECs) decreases with age,[2,3] and this cell loss can be accelerated by disease,[4,5] intraocular surgery,[6,7] or laser procedures.[8] Maintenance of corneal clarity requires an intact endothelial monolayer, and transparency can be lost when endothelial cell density is reduced below a critical level. Cell density decreases over time, indicating that the rate of cell division does not keep pace with the rate of cell loss. Although corneal endothelial cells in vivo exhibit limited mitotic activity,[1,9] they possess proliferative capacity. Corneal endothelial cells in vivo are arrested in the G_1-phase of the cell cycle[10,11] and are actively maintained in a nonproliferative state.

Proteins that positively regulate the transition between G_1- and S-phase include E2F, cyclin-D1, cyclin-E, cyclin-dependent kinase (CDK)-2, and CDK4. Cyclin-D1 is a regulatory factor that binds to and activates CDK4,[12] and these complexes mediate phosphorylation of the retinoblastoma gene product pRb.[13] In quiescent cells, pRb binds and inactivates E2F, a transcription factor that must be activated for S-phase entry.[14] Hyperphosphorylation of pRb by the cyclin-D–CDK4 kinase complex promotes E2F activation and the subsequent transcription of S-phase genes.[15] Cyclin-E is a regulatory protein synthesized in late G1-phase. Association of cyclin-E with CDK2 activates its kinase activity, which is also important for S-phase entry.[12] Cyclin-A is synthesized just before the S-phase and helps mediate the activation of DNA synthesis in association with CDK2.[16] Cyclin-B is synthesized late in the S-phase and reaches its peak cellular concentration late in the G_2-phase. Cyclin-B1 helps mediate chromosome condensation, reorganization of microtubules, and disassembly of the nuclear lamina and Golgi apparatus.[17]

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 cyclin-dependent kinase-interacting protein/cyclin-dependent kinase inhibitory protein (Cip/Kip) and inhibitor of cyclin-dependent kinase (INK) families, and that of cyclin–CDK complexes, such as cyclin-D1–CDK4 and cyclin-E–CDK2. On mitogenic stimulation, cyclin-D and -E are synthesized, increasing the concentration of positive stimulators relative to that of G_1-phase inhibitors. 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. The Cip/Kip and INK families of proteins are CKIs and induce G_1-phase arrest. The INK family, including $p16^{INK4a}$,[18] $p15^{INK4b}$,[19] $p18^{INK4c}$,[20] and $p19^{INK4d}$[21] bind to CDK4 and prevent its binding to and activation by cyclin-D.[22] $p15^{INK4b}$ induces release of $p27^{kip1}$ from cyclin-D–CDK4 and increases the binding of $p27^{kip1}$ to cyclin-E–CDK2 complexes, leading to the inactivation of CDK2 kinase

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activity.[23] The Cip/Kip family, including $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$, bind to cyclin–CDK complexes and prevent kinase activation.[24–26] $p21^{cip1}$ inhibits both the G_1- and G_2-phase cyclin–kinase complexes.[27]

In many cell types, $p27^{kip1}$, a member of the Cip/Kip family, helps mediate cell cycle arrest induced by cell–cell contact and TGF-β.[23,28,29] The level of $p27^{kip1}$ protein expression is high in G_0/G_1 resting cells and declines as cells progress toward the S-phase. Overexpression of $p27^{kip1}$ inhibits entry into The S-phase in normal and malignant cells,[30,31] whereas, downregulation of $p27^{kip1}$ in (−/−) knockout mice results in unregulated cell growth and tumor formation.[32] A recent study[33] demonstrated a correlation between high levels of $p27^{kip1}$ protein and inhibition of proliferation in contact inhibited rat corneal endothelial cells, providing evidence that $p27^{kip1}$ is an important mediator of contact inhibition in corneal endothelium. Studies[34–36] have also demonstrated that fibroblast growth factor (FGF)-2 causes a decrease in $p27^{kip1}$ levels, induces phosphorylation of $p27^{kip1}$, and plays an important role in antiproliferation in rabbit CECs.

In other cell types, downregulation of $p27^{kip1}$ protein levels by antisense (AS) methods leads to increased proliferative activity.[37] In previous studies from this laboratory,[38] rat corneal endothelial cells were transfected with AS oligonucleotides (AS oligos) for PKC-α at more than 90% efficiency. AS oligo binding to target mRNA may interfere with the intermediary metabolism of the mRNA and lead to its inactivation.[39] Although several AS mechanisms have been considered, little is known about the precise mechanisms that lead to inhibition of protein expression. Possible mechanisms include prevention of mRNA transport,[40] transcriptional[41] or translational arrest,[40] and RNase-H–mediated cleavage.[40] AS technology represents a useful method for reducing cellular levels of functional proteins. However, there are some problems associated with this technology. Apart from nonspecific and occasional toxic effects, AS oligos may fail to produce complete inhibition of gene expression. In contrast, an important advantage of AS technology is the high specificity and the ability to inhibit the expression of biologically active proteins selectively.

A major goal of this laboratory is to develop therapies to increase cell density in stressed corneal endothelium. The proposed approach is to increase cell density by transiently stimulating corneal endothelial proliferation. We hypothesize that corneal endothelial cell proliferation will be promoted by decreasing the cellular concentration of $p27^{kip1}$, by using AS methods.

Materials and Methods

Corneal Tissue

Corneas were obtained from adult male Sprague-Dawley rats maintained in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Endothelial cell explant cultures were prepared according to Chen et al.[42] Primary cultures were grown to confluence in Medium-199 (M199; Invitrogen-Gibco, Grand Island, NY) supplemented with 50 μg/mL gentamicin (Invitrogen-Gibco), and 10% fetal bovine serum (FBS; HyClone, Logan, UT). Confluent cells were subcultured onto sterile four-chamber slides (Laboratory Tek, Napierville, IL) and T25 flasks, grown to confluence and maintained in culture medium containing 10% FBS for another week to ensure formation of a fully confluent monolayer. All incubations were performed in a 5% CO_2 and 95% air, humidified atmosphere at 37°C.

$p27^{kip1}$ AS Transfection

AS and missense (MS) oligos for $p27^{kip1}$ were obtained from Midland Certified Reagent Company (Midland, TX) (AS sequence: 5′ (PS) GCGTCTGCTCCACAG3′; mismatch sequence: 5′(PS)GCATCCCCTGTGCAG3′).[43] Cells were transfected with $p27^{kip1}$ AS or MS oligo during a 6-, 12-, 18-, 24-, or 48-hour period using a nonliposomal lipid transfection reagents kit (Qiagen Effectene; Qiagen, Inc., Valencia, CA). A DNA-to-transfection reagent ratio of 1:26 was used. Cells were fixed immediately or washed in PBS, incubated in 10% FBS for 24, 48, 72, or 96 hours, and either fixed for immunocytochemistry (ICC) or extracted for Western blot analysis. Control cultures were incubated under one of the following conditions: (1) no oligo or lipid-containing buffer, (2) lipid-containing buffer alone, or (3) lipid-containing buffer plus MS $p27^{kip1}$ oligo.

Viability of CECs

Viability was assessed using a cell viability assay kit (Molecular Probes, Eugene, OR) after 0, 24, 48, 72, or 96 hours of AS incubation, and 24, 48, or 72 hours after incubation. Staining was visualized by fluorescence microscopy (Eclipse YS100; Nikon, Melville, NY) equipped with a digital camera (Coolpix 995; Nikon).

Antibodies

Polyclonal rabbit antibodies to $p27^{kip1}$, $p21^{cip1}$, and cyclin-D1 -A, and CDK4; polyclonal mouse antibodies to cyclin-E and -B1 and CDK2; and polyclonal goat antibody to $p15^{INK4b}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)– and horseradish peroxidase (HRP)–conjugated anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Bromodeoxyuridine (BrdU) reagents, including mouse monoclonal anti-BrdU, were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). BrdU-labeling reagents were used according to the manufacturer's protocol. The mouse monoclonal antibody to Ki67 was purchased from Novocastra (Newcastle-upon-Tyne, UK). Nonmuscle myosin antibody was obtained from Biomedical Technologies (Stoughton, MA).

Immunocytochemistry

Endothelial cells subcultured on four-chamber slides were rinsed with phosphate-buffered saline (PBS; Invitrogen-Gibco) and fixed with 99.9% methanol for 10 minutes at −20°C. All further incubations were at room temperature. Slides were rinsed with PBS and cells permeabilized for 10 minutes with 0.1% Triton X-100 in PBS. Nonspecific sites were blocked for 10 minutes with 4% bovine serum albumin in PBS. Cells were incubated for 2 hours with primary antibody diluted in PBS (Ki67, 1:100; $p27^{kip1}$, 1:200; $p21^{cip1}$, 1:500; $p15^{INK4b}$, 1:100; cyclin-D1, 1:200; cyclin-E, 1:100; cyclin-A, 1:100; cyclin-B1, 1:200; CDK2, 1:100; and CDK4, 1:50). Slides were rinsed with PBS and incubated for 2 hours with secondary antibody (1:100 in PBS). Controls were incubated with secondary antibody alone. For BrdU staining, BrdU labeling reagent (1:1000 dilution) was added to the cultures for 24 hours before immunostaining according to the supplier's protocol. Coverslips were mounted in antifade medium (Vectashield; Vector Laboratories, Inc., Burlingame, CA) containing propidium iodide (PI) or DAPI (4′6 diamidino-2-phenylindole), which stain cell nuclei (Vector Laboratories, Inc.).

Western Blot Analysis

Cultured cells were trypsinized and pelleted, and proteins were extracted by incubating cells for 30 minutes at 4°C in buffer containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), 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 primary antibody diluted in blocking buffer. Antibody dilutions, prepared in

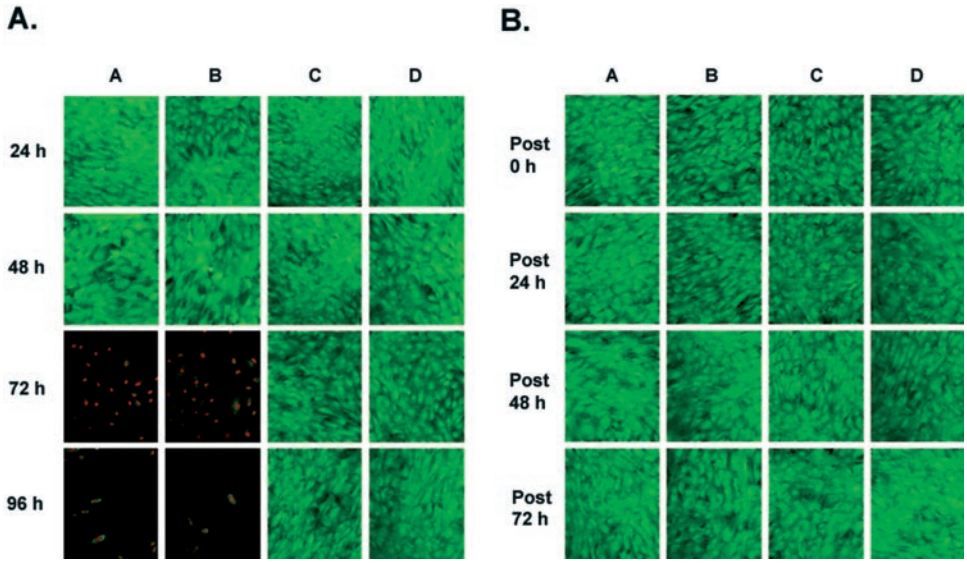

FIGURE 1. (**A**) Viability assay of rat CEC cultures incubated with AS oligo (*column A*), MS oligo (*column B*), lipid-containing buffer (*column C*), or no oligo or lipid-containing buffer (*column D*) for 24, 48, 72, or 96 hours. (**B**) Viability assay of rat CEC cultures incubated for 24 hours with AS oligo, washed in PBS, then postincubated in 10% FBS for 0, 24, 48, or 72 hours. Live cells are stained *green*. Dead cells are stained *red*. *Black* areas indicate regions of cell loss. Original magnification, ×10.

blocking buffer, were as follows: p27^{kip1}, 1:200; p21^{cip1}, 1:100; p15^{INK4b}, 1:100; cyclin-D1, 1:200; cyclin-E , 1:100; cyclin-A, 1:100; cyclin-B1, 1:200; CDK2, 1:100; CDK4, 1:50; and nonmuscle myosin, 1:200. Blots were rinsed for 10 minutes three times with 0.1% Triton X-100, then reblocked and exposed for 1 hour to HRP-conjugated donkey anti-rabbit, -mouse, or -goat IgG, diluted 1:10,000 in blocking solution. The same blots were probed with rabbit anti-nonmuscle myosin to control for protein load. After a thorough wash, peptides were detected using chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL). For quantification, films were digitally scanned (BDS-Image; Biological Detection Systems, Pittsburgh, PA), scans were analyzed by computer (NIH Image ver. 1.61; W. Rasband, National Institutes of Health, Bethesda, MD; available by FTP from zippy.nimh.nih.gov or on floppy disc from NTIS, Springfield, VA), and protein content was normalized according to nonmuscle myosin protein content.

Quantification of Proliferating Cells

For proliferation studies, cells were immunostained for Ki67 or BrdU, mounted in DAPI, and counted in a masked fashion, with a microscope (Eclipse E800; Nikon). At least five random 20× fields were examined per specimen. The total number of nuclei (PI- or DAPI-positive) was counted by using the rhodamine or ultraviolet channel, respectively. BrdU- or Ki67-positive cells were counted using the FITC channel. The percentage increase in cell number was calculated by dividing the number of BrdU- or Ki67-positive cells by the total number of cell nuclei ×100, and results were compared with untreated controls. All experiments were conducted in duplicate and repeated at least three times for statistical evaluation. Statistical analysis was performed on computer (Sigma Stat version 2.0; SPSS, Chicago, IL), to calculate significance according to the paired *t*-test.

RESULTS

Effect of AS Oligo Transfection on Viability

In a previous study,[38] AS oligo incubation conditions were optimized and yielded around 90% transfection efficiency. Because AS treatment has had occasional toxic effects,[39] we first performed viability assays to determine the optimal incubation conditions for AS oligo transfection. Confluent rat CEC were incubated for 24, 48, 74, or 96 hours with AS oligo, MS oligo, lipid-containing buffer, or buffer containing no oligo or lipid followed by viability assay. Figure 1A shows that viability was not significantly affected by lipid-based oligo transfection for up to 48 hours, after which a decline in viability was noted. To test viability after transfection, cultures were incubated under the same conditions as above for 24 or 48 hours; washed in PBS, incubated with 10% FBS for 0, 24, 48, or 72 hours; and then assessed for viability. Figure 1B shows that, in cultures incubated with oligo for 24 hours, viability was not affected up to 72 hours after transfection. In contrast, in cultures incubated in oligo for 48 hours, a decline in viability was noted (data not shown). As a result of these studies, all subsequent incubations with AS oligo were performed for 24 hours.

Effect of AS Transfection on p27^{kip1} Protein Levels

The effect of AS oligo transfection on p27^{kip1} levels was determined by Western blot analysis and ICC. After transfection for 24 hours with p27^{kip1} AS oligo, cells were washed and postincubated for 24 or 48 hours in 10% FBS before preparation of cells for Western blot analysis or ICC, as indicated in the Materials and Methods section. Figure 2A demonstrates that transfection with p27^{kip1} AS oligo reduced p27^{kip1} protein

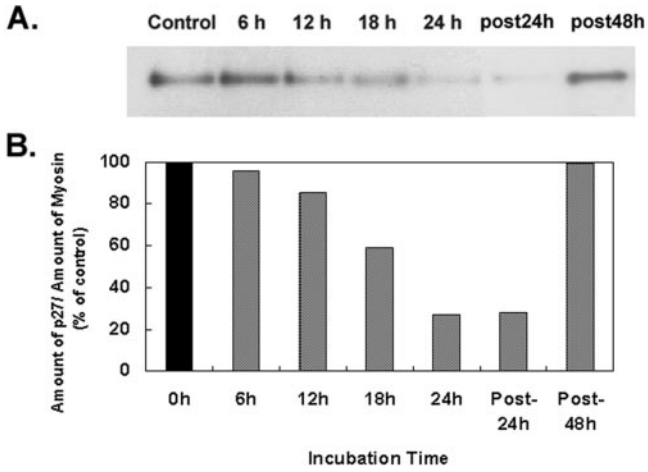

FIGURE 2. (**A**) p27^{kip1} protein level of rat CECs transfected with p27^{kip1} AS oligo for 6, 12, 18, or 24 hours or transfected for 24 hours in AS oligo, washed, and postincubated in 10% FBS for 24 or 48 hours. Control cells were not transfected. (**B**) The p27^{kip1} protein level standardized to the amount of nonmuscle myosin present in the same samples.

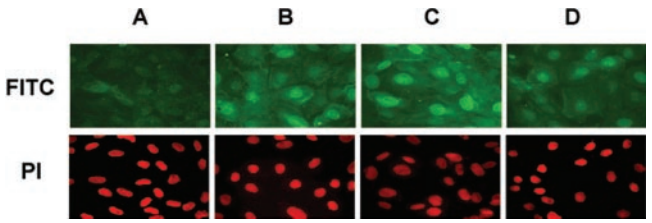

FIGURE 3. p27^{kip1}staining of rat CECs incubated for 24 hours with AS oligo (**A**), MS oligo (**B**), lipid-containing buffer (**C**), or buffer containing no oligo or lipid (**D**). *Green* fluorescence (FITC) localized p27^{kip1}. *Red* fluorescence (PI) stained all nuclei. Original magnification, ×60.

levels in a time-dependent manner. Semiquantitative analysis of the blots (Fig. 2B) indicate that the protein level of p27^{kip1} began to reduce within 6 to 12 hours after initiation of transfection and was suppressed to approximately 30% of control levels by 24 hours. Protein levels returned to normal after 48 hours after incubation. In contrast, p27^{kip1} MS oligo transfection did not reduce p27^{kip1} protein levels (data not shown).

For ICC evaluation of the effect of p27^{kip1} AS oligo transfection on p27^{kip1} levels, cultures were incubated for 24 hours with AS oligo, MS oligo, lipid-containing buffer alone, or buffer containing no oligo or lipid, and then processed for ICC. Images of control samples in Figure 3 show a strong nuclear staining for p27^{kip1} in the nuclei of rat CECs. This pattern is very similar to that observed by Lee and Kay[34,35] and Kim et al.[36] in rabbit CECs. Nuclear staining for p27^{kip1} was greatly reduced in CECs incubated with AS oligo.

Effect of p27^{kip1} AS Treatment on the Localization and Expression of Cell Cycle Regulatory Proteins

We next studied the effects of p27^{kip1} AS oligo treatment on the relative expression of cell cycle regulatory proteins (Fig. 4). Confluent rat CECs were transfected for 24 hours with p27^{kip1} AS oligo or with buffer containing no oligo or lipid as a control, and postincubated for 24 or 48 hours in 10% FBS. Cells were fixed for ICC or extracted for Western blot analysis. Localization patterns and relative staining intensity of the cell cycle regulatory proteins were compared. In cells incubated with no

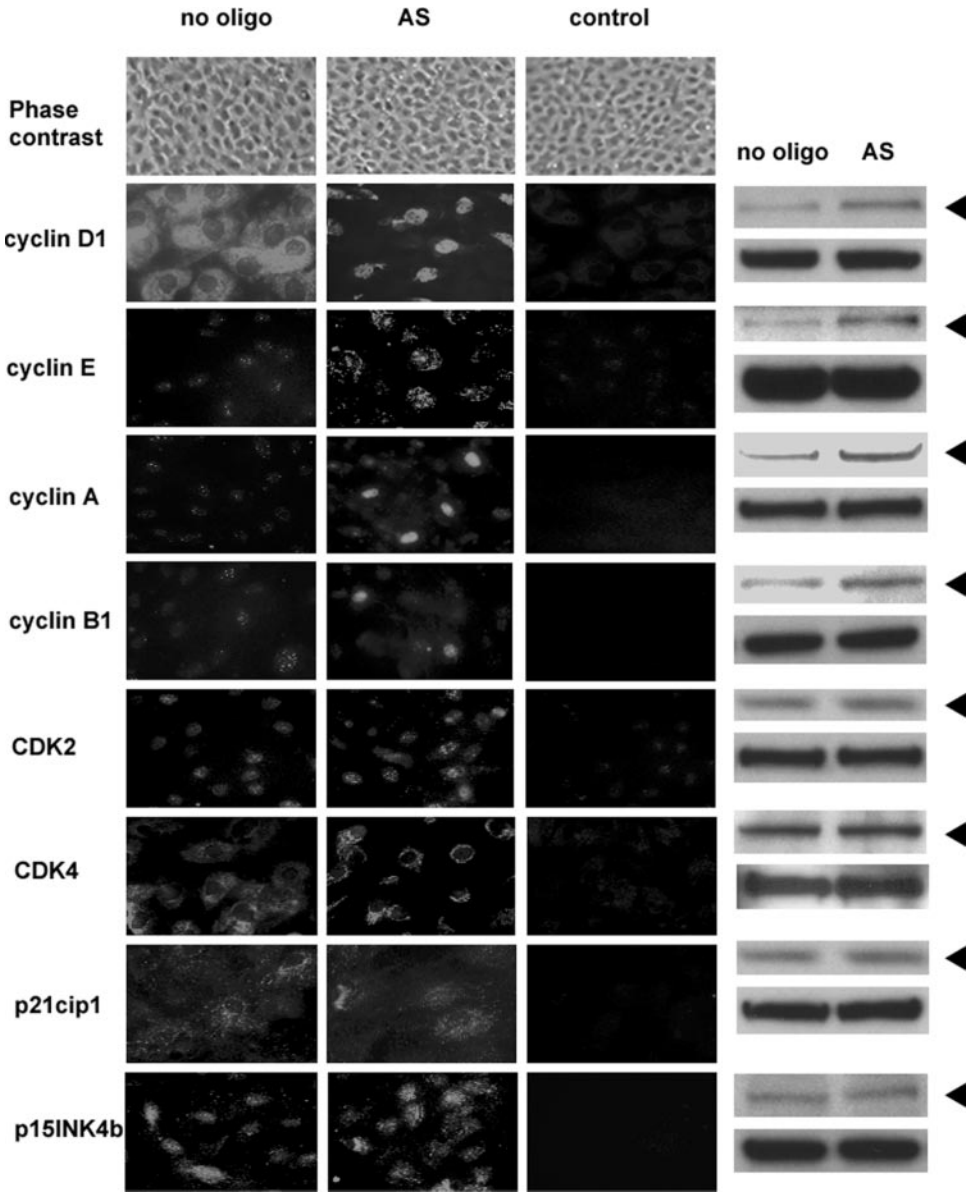

FIGURE 4. Phase-contrast images, immunolocalization, and Western blot studies of cell cycle regulatory proteins in confluent rat CECs incubated for 24 hours in buffer containing no oligo or lipid (no oligo) or with p27^{kip1} AS oligo (AS). Both cultures were postincubated in 10% FBS for 24 hours (except cyclin-A and -B1) or 48 hours (cyclin-A and -B1) and then fixed for ICC or extracted for Western blot analysis. Representative phase-contrast images were also taken. Control column shows results of the ICC using secondary antibody alone. For Western blot analysis, the *top panel* of each pair (*arrowhead*) shows the result for each cell cycle protein; *bottom lanes* show the corresponding nonmuscle myosin bands used for normalization of the data. Original magnification: (ICC) ×60; (phase-contrast) ×4.

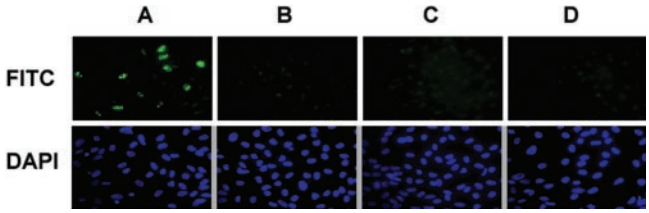

FIGURE 5. Ki67 immunostaining of confluent rat CECs incubated for 24 hours with AS oligo (**A**), MS oligo (**B**), lipid-containing buffer (**C**), or buffer containing no oligo or lipid (**D**). *Green* fluorescence (FITC) localized Ki67, while *blue* fluorescence (DAPI) stained all nuclei. Original magnification, ×40.

oligo or lipid, positive staining for cyclin-D1 was detected in the cytoplasm, but not in the nucleus; however, strong nuclear staining was evident in AS-treated cells. Western blot analysis demonstrated a 2.1-fold increase in cyclin-D1 protein in AS-treated cells compared with the no-oligo control. The apparent difference in localization of cyclin-D1 appears to be significant, because cyclin-D1 plays an important role in activating CDK4 during the G_1-/S-phase transition. Faint nuclear staining for cyclin-E was noted in control cultures, whereas, an intense, punctate nuclear pattern was present in AS-treated cells. The increased staining intensity correlated with a 1.9-fold increase in the cyclin-E protein level in AS-treated cells. Both cyclin-A and -B1 showed faint nuclear staining in control cells, but more intense nuclear staining in some cells of the AS-treated cultures. Western blot analysis indicated that cyclin-A protein level increased by 1.3-fold, whereas cyclin-B1 increased 1.4-fold compared with the control. Nuclear staining for CDK2 was similar under both experimental conditions. CDK4 appeared to be more strongly perinuclear in AS-treated cells compared with control cells, in which CDK4 appeared diffusely distributed within the cytoplasm as well as perinuclearly. Little change in total CDK2 or -4 protein was noted on Western blot analysis. In both AS-treated and control cells, faint cytoplasmic staining was noted for p21^{cip1}, whereas p15^{INK4b} was localized to the nucleus. Similar levels of p21^{cip1} and p15^{INK4b} protein were found on Western blot analysis, indicating no change in the protein levels of these two CKIs.

Effect of p27^{kip1} AS Treatment on Proliferation of CECs

We questioned whether p27^{kip1} inhibition could overcome G_1-phase arrest in a confluent monolayer of cells and thereby promote proliferation. To study the effects of p27^{kip1} AS treatment on the corneal endothelial cell cycle, confluent cells were transfected for 24 hours with p27^{kip1} AS oligo, MS oligo, lipid-containing buffer, or buffer containing no oligo or lipid, and then processed for ICC of Ki67, a marker of actively cycling cells.[44,45] As shown in Figure 5, Ki67-positive cells were noted in CECs incubated with AS oligo. In contrast, no to few Ki67-positive cells were observed in CECs incubated with MS oligo or the buffer control.

Immunostaining in Figure 6A demonstrates the presence of Ki67-positive cells immediately after the 24-hour incubation with p27^{kip1} AS oligo, as well as in AS oligo-treated cells postincubated in 10% FBS for 24, 48, or 72 hours. To quantify Ki67-positive staining, confluent cultures were transfected with p27^{kip1} AS oligo or incubated in buffer containing no oligo or lipid as a negative control. Cells were then washed in PBS and postincubated in 10% FBS for 24, 48, or 72 hours before immunostaining for Ki67. At all time points (Fig. 6B), the number of Ki67-positive cells was significantly increased ($P < 0.05$) in AS oligo-treated cultures above that of the respective control. A significant increase ($P < 0.001$) was observed in the number of Ki67-positive cells between the time the AS oligo incubation was completed (24 hours) and the 24-hour postincubation time point (post-24 hours). The total number of Ki67-positive cells peaked at 48 hours after incubation and represented 38.7% of the total cell population; however, the increase in positive cells between the 24- and 48-hour postincubation time points was not statistically significant ($P < 0.090$). By 72 hours after incubation, the number of positive cells had begun to decrease.

We next determined whether lowering of p27^{kip1} protein levels alone was sufficient to promote cell cycle progression. Preliminary studies had determined that subconfluent rat corneal endothelial cells will not proliferate in the presence of 0.1% FBS, but will readily proliferate in 10% FBS. Under normal conditions, confluent, contact-inhibited cells will not proliferate even in the presence of 10% FBS, as demonstrated earlier. To test whether lowering p27^{kip1} protein levels was sufficient to induce cell cycle progression, we transfected confluent cultures with p27^{kip1} AS oligo and then postincubated them for 24, 48, or 72 hours in medium containing 0.1%, rather than 10%, FBS. Compared with the results shown in Figure 6B, Figure 6C shows that there was no difference in the relative number of Ki67-positive cells between AS oligo-transfected cultures incubated under low-serum conditions and control cultures. These results indicate that lowering of p27^{kip1} protein levels by treatment with p27^{kip1} AS oligo was necessary, but not sufficient, to induce cell cycle progression.

Staining for BrdU was used to detect S-phase cells as a second indicator of cell cycle progression (Fig. 6D). The same transfection and incubation conditions were used as in Figure 6B. BrdU was added to the cultures 24 hours before preparation for staining. Positive BrdU staining demonstrated a significant increase ($P < 0.05$) in the relative number of S-phase cells in AS oligo-treated cultures compared with untransfected control cells. The relative number of BrdU-positive cells was significantly higher ($P < 0.003$) after 24 hours postincubation in 10% FBS than immediately after AS oligo treatment. There was no significant difference in the relative number of BrdU-positive cells between the 24- and 48-hour postincubation time points ($P < 0.204$); however, a maximum number of BrdU-positive cells (22.8%) was observed at 48 hours. The relative percent of BrdU-positive cells was lower at each time point than that of Ki67 staining.

Immunostaining with Ki67 permits semiquantitative analysis of the relative number of cells in the late G_1-phase through mitosis.[46,47] Senoo and Joyce[48] and Senoo et al.,[49] have demonstrated that Ki67 staining patterns in human CECs are useful for classifying cells in the late G_1-phase through M-phase of the cell cycle. Figure 7 shows endothelial cell cycle kinetics in confluent cultures of rat CECs transfected with p27^{kip1} AS oligo for 24 hours and postincubated in 10% FBS for 0, 24, 48, or 72 hours. Ki67 patterns changed with time. Mitotic figures were observed after 24 hours postincubation. A maximum of approximately 13% of total Ki67-positive cells exhibited mitotic figures at 24 hours postincubation. Transfection with p27^{kip1} AS oligo followed by 24 hours of postincubation promoted cell cycle progression in the endothelium, revealed by the presence of Ki67-stained nuclei and mitotic figures. The relative percent of the Ki67-positive population showing mitotic figures declined by 72 hours after infection, even in the presence of 10% FBS.

To confirm the proliferative effect of p27^{kip1} AS oligo transfection, confluent cultures were transfected with p27^{kip1} AS oligo, MS oligo, or buffer containing no oligo or lipid as a control, and postincubated for 24, 48, 72, or 96 hours in 10% FBS. Cells were then stained with DAPI to visualize nuclei. Nuclei were counted in a masked fashion in at least five random 20× fields per specimen. Percentage increase in the

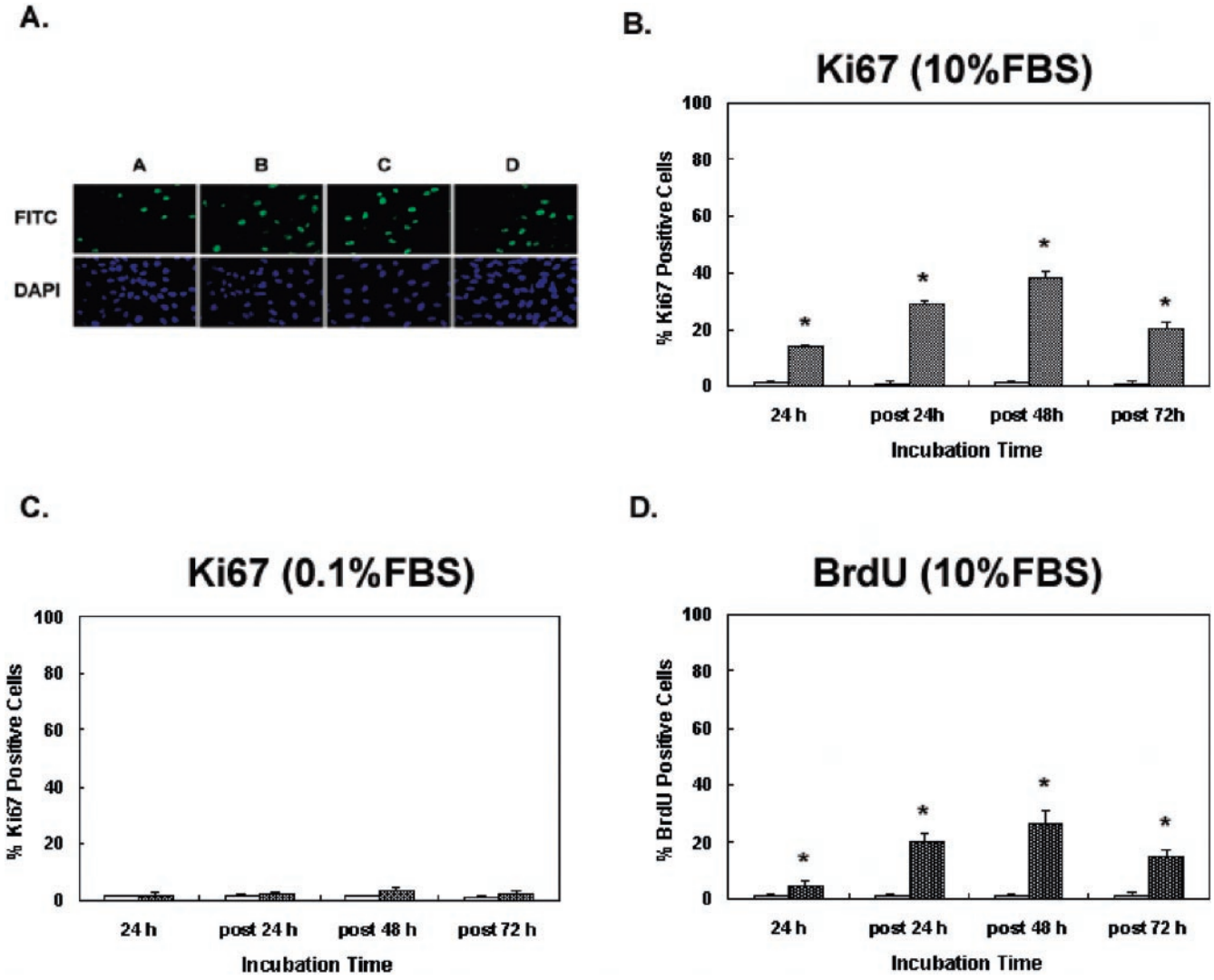

FIGURE 6. (**A**) Ki67 ICC of rat CEC cultures incubated with AS oligo for 24 hours, washed in PBS, and incubated with 10% FBS for 0 (**A**), 24 (**B**), 48 (**C**), or 72 (**D**) hours. *Green* fluorescence (FITC) localized Ki67, whereas *blue* fluorescence (DAPI) stained all nuclei. Original magnification, ×40. (**B**) Mean percentage of Ki67-positive cells in cultures incubated for 24 hours with AS oligo (▨) or in buffer containing no oligo or lipid (□), then postincubated with 10% FBS for 24, 48, or 72 hours (**C**). Mean percentage of Ki67-positive cells in cultures transfected under the same conditions as in (**B**), but postincubated in only 0.1% FBS. (**D**) Mean percentage of BrdU-positive cells in cultures transfected under the same conditions as in (**B**). Results in (**B**), (**C**), and (**D**) are expressed as mean ± SEM. $^*P < 0.05$.

number of cells in oligo-treated cultures was calculated compared with the total number of cells in control cultures. Figure 8 shows that, in cultures transfected with $p27^{kip1}$ AS oligo, the number of cells increased with time to approximately 122% of control at 72 hours after incubation, compared with that of cells transfected with MS oligo (approximately 102% of control). After this time, percentages appeared to plateau. From these data, we concluded that $p27^{kip1}$ AS oligo treatment promotes corneal endothelial cell proliferation in the presence of 10% FBS by decreasing the inhibitory barrier to G_1-phase progression.

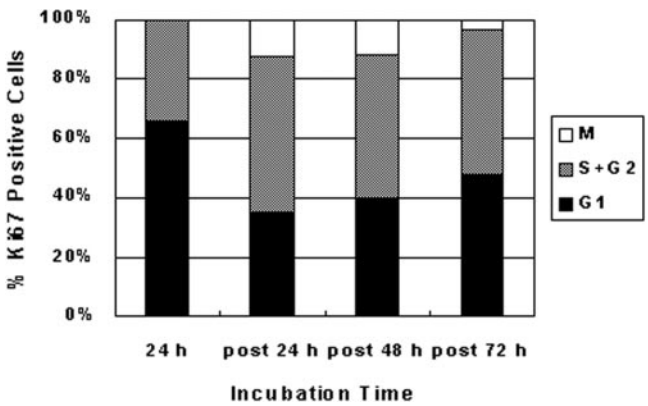

FIGURE 7. Effects of $p27^{kip1}$ AS oligo on cell cycle kinetics. Confluent rat CEC were transfected with AS oligo for 24 hours and postincubated in medium containing 10% FBS for 0, 24, 48, or 72 hours. Results are expressed as the percentage of Ki67-positive cells exhibiting staining patterns for the G_1-, S/G_2-, or M-phase.

DISCUSSION

There have been few reported studies in which AS methods were used in corneal endothelium to alter the synthesis of cell cycle regulatory proteins. Kay et al.[50] successfully used this technique to reduce synthesis of bFGF and thereby block by 50% the enhanced growth potential mediated by bFGF in cultured rabbit CECs. In other cells, AS techniques have been

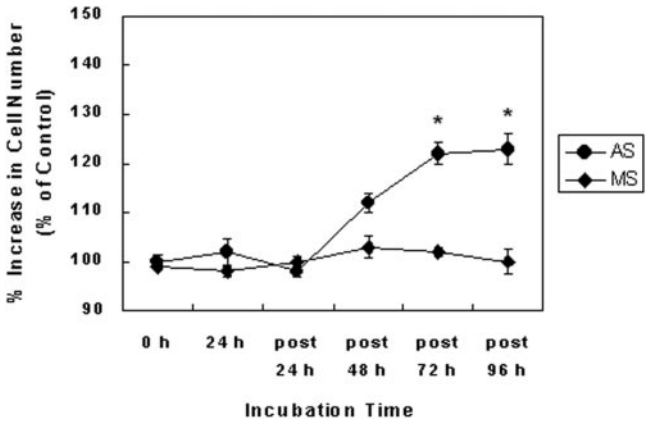

FIGURE 8. Relative percent increase in cell numbers in rat CEC treated with p27^{kip1}AS oligo. Confluent rat CEC were transfected for 0 or 24 hours with p27^{kip1} AS or MS oligos or with buffer containing no oligo or lipid and postincubated for 24, 48, 72, or 96 hours. DAPI-stained nuclei were counted in a masked fashion. Results are expressed as the mean ± SEM. *$P < 0.05$.

widely used for studying biological functions and developing new therapeutic strategies.[51–53] For the current studies, p27^{kip1} was chosen as the target CDK inhibitor, because it is capable of inhibiting both cyclin-D–CDK4 and cyclin-E–CDK2 activity. In contrast, p15^{INK4b}, a member of the INK family of inhibitors that is expressed in rat CECs,[33] binds only CDK4.[54] Our studies demonstrate that p27^{kip1} AS oligo treatment can successfully decrease the cellular concentration of p27^{kip1} in a time-dependent manner. In contrast, the protein levels of p21^{cip1} and p15^{INK4b} did not change after AS transfection, indicating that the AS treatment specifically altered only p27^{kip1} levels and did not result in a compensatory increase in other cell cycle inhibitors.

Treatment of confluent cells with p27^{kip1} AS oligo and subsequent incubation in 10% FBS altered both the protein level and subcellular localization of cell cycle regulatory proteins, such as cyclin-D1, -E, -A, and -B1, suggesting that this treatment can influence the expression and activity of positive cell cycle regulators. Cyclin-D1 and -E regulate the activity of CDK4 and -2, and active cyclin-D1–CDK4 and cyclin-E–CDK2 complexes are required for S-phase entry.[55] After p27^{kip1} AS treatment, the protein level of both cyclin-D1 and cyclin-E greatly increased. Cyclin-D1 was translocated from the cytoplasm to the nucleus. Cyclin-E also showed a nuclear localization. These changes suggest that cyclin-D1- and -E–containing complexes are active in p27^{kip1} AS oligo-treated cells. Protein levels of CDK2 and CDK4 did not appear to be significantly affected by p27^{kip1} AS oligo transfection. However, the translocation of CDK4 from the cytoplasm to the perinuclear region suggests the activation of this kinase. Lee and Kay[35] have reported that the protein level of CDK4 is upregulated and the localization of CDK4 is changed from the cytoplasm into nuclei by the stimulation of FGF-2 in rabbit CECs. These differences may be due to the difference of species (rat and rabbit) used in the two studies and the stimulators of proliferation (p27^{kip1} AS oligo with 10% FBS versus FGF-2 alone). In addition, different mechanisms may exist in the relative intracellular signaling pathways induced by these two methods. The levels of cyclins-A and -B1 also increased by 1.3-fold (cyclin-A) and 1.4-fold (cyclin-B1), and strong nuclear staining of these proteins was observed in response to p27^{kip1} AS oligo treatment. Cyclin-A is normally synthesized later in G1-phase than cyclin-E, whereas cyclin-B1 is synthesized in late S-phase, and its protein levels peak in G_2-phase. Both proteins are required for transition from G_2- to M-phase. Together, these ICC and Western blot results indicate that p27^{kip1} AS treatment followed by incubation in 10% FBS induce cell cycle progression.

AS treatment to decrease p27^{kip1} levels promoted proliferation in confluent cultures of rat CECs without apparent negative effects on viability. Counts of Ki67-positive cells indicate that approximately 38% of the cell population entered the cell cycle after transfection of rat CECs with p27^{kip1} AS oligo and after incubation in 10% FBS. Similar findings were obtained using BrdU to quantify the S-phase population. As expected, the relative percent of BrdU-positive cells was lower at each time point than that of Ki67-positive cells, because Ki67 is highly expressed in all actively cycling cells from the late G_1-phase through the M-phase, whereas BrdU shows only cells that have entered the S-phase of the cycle. Of importance, was the fact that total endothelial cell numbers increased in AS-treated cells compared with either MS-treated or untreated cells.

In this study, transfection of confluent rat CECs with p27^{kip1} AS oligo alone was not sufficient to induce proliferation, as indicated by the lack of Ki67 staining in AS-treated cultures postincubated in 0.1% FBS. These results differ from those in a study[37] using cultured fibroblasts transfected with p27^{kip1} AS oligo, which reported increased proliferation, even under low-serum conditions. This indicates that lowering of p27^{kip1} protein levels in those cells reduces the requirement for growth factor stimulation. The proliferative response of endothelial cells incubated in 10% FBS may be because reduction of p27^{kip1} levels in confluent rat CECs lowers an inhibitory barrier and because the cells are then able to enter the cell cycle on appropriate mitogenic stimulation. Further study is needed to elucidate the specific mechanism by which the lowering of p27^{kip1} promotes proliferation under these conditions.

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