TAT-Mediated Protein Transduction into Human Corneal Epithelial Cells: p15^{INK4b} Inhibits Cell Proliferation and Stimulates Cell Migration

Xiaoqing Guo, Audrey E. K. Hutcheon, and James D. Zieske

Purpose. The cell cycle inhibitor p15^{INK4b} has been localized in migrating corneal epithelial cells. In this study, TAT-fusion protein technology was used to transduce p15^{INK4b} into human corneal epithelial cells to examine the effect on cell proliferation and migration.

Methods. Human p15^{INK4b}, obtained by RT-PCR, was cloned into a TAT-HA vector, and the fusion protein was purified from bacteria transformed with the TAT-HA-p15 construct. Various dilutions of TAT-HA-p15 were applied to primary human corneal epithelial cells to test potency. In addition, the effect of exposure time was examined. Cells were labeled with bromodeoxyuridine to detect proliferation, and indirect immunofluorescence was performed. Ki67 expression was also examined. To assay cell migration, human corneal epithelial cells were plated inside a cylinder and exposed to TAT-HA-p15. The cylinder was removed, the cells were allowed to spread for 2 days, and the area of cell coverage was calculated. TAT-HA-β-galactosidase served as the control in all experiments. Finally, the extent of retinoblastoma protein phosphorylation was assayed by Western blot in cells cultured with and without TAT-HA-p15.

Results. TAT-HA-p15 was successfully transduced into primary human corneal epithelial cells. TAT-HA-p15 decreased proliferation in a concentration- and time-dependent manner. The migration assay showed that TAT-HA-p15 stimulated cell migration 1.8-fold. TAT-HA-β-galactosidase had no effect on proliferation or migration. Finally, TAT-HA-p15 decreased the level of phosphorylated retinoblastoma protein by 4.9-fold.

Conclusions. Active p15^{INK4b} can be efficiently transduced into primary human corneal epithelial cells using TAT-fusion protein technology. p15^{INK4b} appears to be sufficient to inhibit corneal epithelial cell proliferation and to stimulate cell migration. (*Invest Ophthalmol Vis Sci.* 2004;45:1804–1811) DOI:10.1167/iovs.03-1164

Corneal epithelial wound repair is a complex process involving the flattening and elongation of cells during migration to cover the wound area and the proliferation of cells to repopulate the wound area.[1–5] We and others[2,6–10] have shown that the migratory and proliferative responses are compartmentalized, in that epithelial cells distal to the original wound exhibit an enhanced proliferative rate, while cells migrating to cover the wound do not proliferate. Over the years, many investigators have documented the fact that the levels of numerous epithelial proteins are dramatically altered during corneal wound repair.[1,4,11–16] One of these proteins is p15^{INK4b}, which is upregulated in the migrating epithelial cells.[15] p15^{INK4b} was first identified as being one of the proteins stimulated by transforming growth factor (TGF)-β in mink lung epithelial cells[17] and has subsequently been shown to be a member of the INK4 family (inhibitors of cyclin-dependent kinase 4).[18–22] It binds directly to isolated cyclin-dependent kinases (CDKs) and prevents the CDKs from binding to cyclins, thus, blocking the activation of the cyclins. This subsequently results in preventing phosphorylation of the retinoblastoma protein (pRb) and in blocking cells from proceeding through the cell cycle.[23–27]

Our observation that p15^{INK4b} is upregulated in migrating, nonproliferative epithelium has led us to hypothesize that TGF-β signaling is involved in inhibiting the proliferation of migrating cells.[15,28] To test this hypothesis, we investigated the use of protein transduction of fusion proteins into corneal epithelial cells.

With transduction, a fusion protein, which consists of a protein transduction domain (PTD) and the protein of interest, is simply added to the culture medium, and the PTD ferries the protein of interest across the cell membrane into the cell.[29] PTDs are generally a group of positively charged (at physiological pH), short peptides (consisting of 10–34 amino acid [aa] residues) that possess the ability to cross the biological membrane of a cell and carry a protein with them.[30–35] Transactivator protein (Tat, 86 aa) from the human immunodeficiency virus, type 1 (HIV-1), is the most widely studied protein with this domain.[36–39] In 1998, Nagahara et al.[29] developed a technology using a small portion of this Tat protein, an 11-aa transduction domain (TAT). This technology, TAT-fusion protein technology, involves a bacterial expression vector containing an N-terminal 6-histidine leader, followed by the 11-aa TAT protein transduction domain, a hemagglutinin (HA) tag, and a polylinker (TAT-HA). With this vector, a full-length protein can be introduced into a cell and expressed. Using this transduction method, more than 60 full-length proteins with functional domains ranging in size from 15 to 120 kDa have been made and transduced into ~100% of both primary and transformed cell types.[29,35,40,41] In the present study, we examined whether TAT-fusion proteins could be efficiently transduced into corneal epithelial cells in culture. In addition, the effect of p15^{INK4b} on human corneal epithelial (HCEC) proliferation and migration was investigated.

Methods

Generation of Full-Length TAT-HA-p15 Fusion Protein

The human sequence for p15^{INK4b} was obtained from GenBank (accession no. L36844; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Primers were designed for RT-PCR (5′ end:

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Supported by National Eye Institute Grant R01 EY05665 (JDZ).

Submitted for publication October 22, 2003; revised January 7, 2004; accepted January 26, 2004.

Disclosure: **X. Guo**, None; **A.E.K. Hutcheon**, None; **J.D. Zieske**, None

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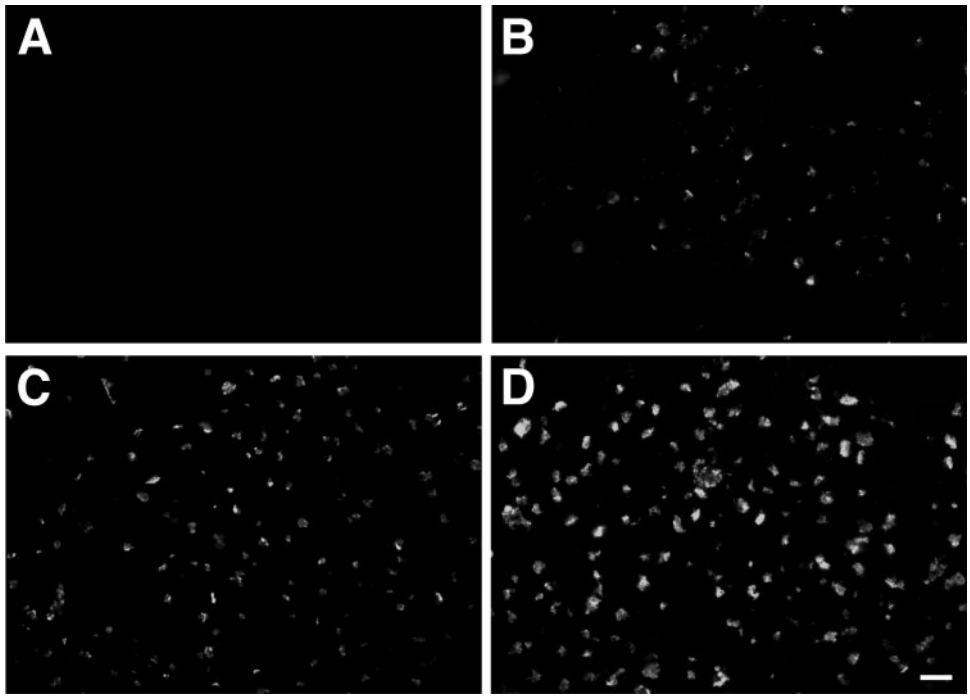

FIGURE 1. Immunolocalization of HA (a marker for TAT-HA-p15) in primary HCECs that have been either not transduced (**A**) or transduced with TAT-HA-p15 at 60 (**B**), 150 (**C**), or 300 (**D**) nM. The amount of HA appeared to increase with increasing concentration of TAT-HA-p15. Bar, 50 μm.

T*CTCGAG*ATGCGCGAGGAGAA [*Xho*I] and 3′: C*GAATTC*TCAGTCCCCCGTGGCTGT [*Eco*RI]), and cDNA was made of the entire human p15^{INK4b} sequence. Restriction enzyme sites (italic sequences) were added to the primers to enable the cloning of the p15^{INK4b} sequence into the TAT-HA vector (a gift from Steven Dowdy, PhD, University of California San Diego School of Medicine, La Jolla, CA), thus creating the TAT-HA-p15 construct. BL21 (DE3) pLysS bacteria (Novagen, Madison, WI) were then transformed with the TAT-HA-p15 construct, and bacteria colonies with high levels of TAT-HA-p15 expression were chosen for protein production.

TAT-HA-p15 Purification

TAT-HA-p15 was purified using a general protocol developed for isolating TAT-fusion proteins.[40] Transformed BL21 (DE3) pLysS bacteria were grown overnight in 1 L of Luria-Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO) plus 50 μg/mL ampicillin (Sigma-Aldrich). The next day, the bacteria were pelleted and resuspended in 20 mL of buffer Z (8 M urea, 100 mM NaCl, 20 mM HEPES [pH 8.0]) to denature the TAT-HA-p15 fusion protein. This suspension was sonicated three times for 15 seconds, with 30-second incubations on ice between sonications. The suspension was then clarified by centrifugation at 14,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a fresh tube and was adjusted to a final concentration of 15 mM imidazole (Sigma-Aldrich). The supernatant was then loaded onto a preequilibrated, 5 mL Ni-NTA column (Qiagen, Valencia, CA), at room temperature. After the supernatant flowed into the column, the column was washed with 50 mL of buffer Z/15 mM imidazole, and then 50 mL 15 mM imidazole/phosphate-buffered saline (PBS) plus protease inhibitors (PIs; 1 μg/mL aprotinin [Sigma-Aldrich] and leupeptin [Sigma-Aldrich]; 5 μg/mL phenylmethylsulfonyl fluoride [PMSF]; Roche Diagnostics, Indianapolis, IN). In a stepwise fashion, the TAT-HA-p15 was eluted with the addition of 5 mL of 100, 250, 500, and 1000 mM imidazole in PBS+PI. One-milliliter fractions were collected and checked by running a sample of each fraction on a 10% to 20% Tris-glycine gel (Invitrogen Corp., Carlsbad, CA) and staining the gel with Coomassie blue. After the fraction(s) with the TAT-HA-p15 was identified (the TAT-HA-p15 fusion protein, approximately 25 kDa, eluted with the last 1 mL of 100 mM imidazole), that fraction(s) was then applied to a separation column (PD-10; Amersham Pharmacia Biotech; Uppsala, Sweden) to remove salts. The relative concentration of TAT-HA-p15 was determined by protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Ten percent glycerol was added to the stock solution, and the stock solution was stored at −20°C.

As a control, TAT-HA-β-galactosidase (gal; bacteria containing the TAT-HA-β-gal construct—a gift from Steven Dowdy, PhD) was purified as described earlier. However, no urea was necessary for the denaturation of TAT-HA-β-gal fusion protein. TAT-HA-β-gal eluted with 250 mM imidazole in PBS.

Transduction of TAT Fusion Protein into Primary HCECs

Preparation of Primary Culture of HCECs. The methods for primary HCEC isolation and cultivation were outlined in Zieske et al.[28] Briefly, human limbal rims that otherwise would have been discarded after corneal transplantation (a gift from Kenneth Kenyon and Peter Repoza of University Eye and Ear Consultants of Boston, Boston, MA), were trimmed to remove conjunctival tissue, leaving the limbus and a small amount of cornea. The rims were then rinsed with Dulbecco's PBS (D-PBS; without calcium or magnesium; Invitrogen) containing 20 μg/mL of gentamicin (Invitrogen) for 2 to 3 minutes before each rim was cut into eight pieces of equal size. These small pieces of tissue were treated with dispase solution (25 caseinolytic U/mL; BD Biosciences, Franklin Lakes, NJ) in Hanks' balanced salt solution with 5 μg/mL of gentamicin for 18 to 24 hours at 4°C. After incubation in dispase, the epithelial layer was lifted from the stroma and digested in trypsin-EDTA solution (Invitrogen) for 5 minutes at 37°C. During this time, the mixture of tissue and trypsin was aspirated with a small pipette to dissociate the cells. After 5 minutes, an equal volume of 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA) in D-PBS was added to the tissue and trypsin mixture to neutralize the trypsin. Cells were pelleted at 1000 rpm for 5 minutes, resuspended in keratinocyte-serum-free medium (SFM; Invitrogen) and seeded onto a fibronectin-collagen–coated flask (FNC Coating Mix; AthenaES, Baltimore, MD). Medium was changed every other day until cells reached 70% to 80% confluence. Primary HCECs were isolated from at least 10 different corneal rims for these experiments and were used after one passage. Use of human tissue adhered to the tenets of the Declaration of Helsinki.

Effect of TAT-HA-p15 on Proliferation of HCECs. Primary HCECs were plated on two-well chamber slides (Nalge Nunc

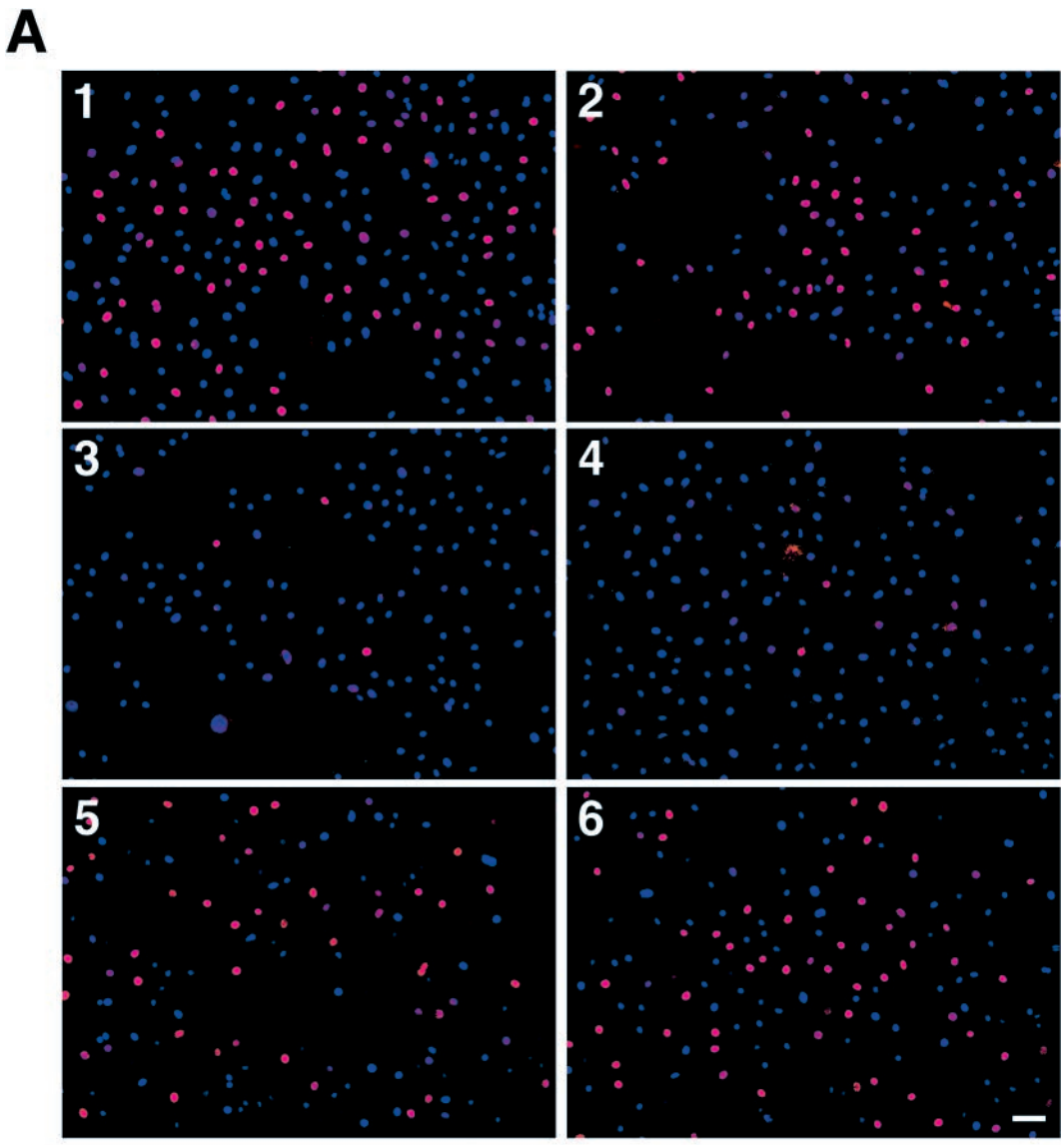

FIGURE 2. (**A**) Immunolocalization of BrdU (a marker of proliferating cells, *red*) in primary HCECs that were not transduced (**A1**, **A5**) or were transduced with TAT-HA-p15 (**A2**-**A4**) or TAT-HA-β-gal (**A6**) for 18 hours at 60 (**A2**), 150 (**A3**), or 300 (**A4**, **A6**) nM. DAPI (*blue*) was used as a counterstain to mark all cell nuclei. (**B**) Average percentage of BrdU-labeled cells under each of the above conditions, showing an inverse correlation between increasing concentrations of TAT-HA-p15 and cellular proliferation. Bar, 50 μm; $^*P < 0.01$.

International, Rochester, NY) with keratinocyte-SFM and cultured overnight at 37°C with 5% CO_2. The potency of TAT-HA-p15 was tested by applying various dilutions of TAT-HA-p15 to the HCECs and incubating them for 18 hours and by incubating HCECs in TAT-HA-p15-fusion protein for various times (2, 4, 6, 8, or 18 hours). Also, the longevity of TAT-HA-p15 activity was tested by incubating HCECs with TAT-HA-p15 at 150 nM for 18 hours, washing the cells with PBS, and incubating them for an additional 1, 2, 3, 4, 5, 6, or 7 days in fresh medium without TAT-HA-p15. Cells not transduced with TAT-HA-p15 and cells transduced with TAT-HA-β-gal served as control samples. At the appropriate time, the cells were washed three times with PBS and labeled with bromodeoxyuridine (BrdU) for 1 hour (10 μM; Roche Diagnostics). Indirect immunofluorescence was performed as previously described.[42] Both anti-BrdU (Roche Diagnostics) and anti-HA (Upstate Biotechnology, Lake Placid, NY) were applied to the slides, which were then incubated 1.5 hours, at room temperature at concentrations of 1:10 and 1:70, respectively. Secondary antibodies, rhodamine-conjugated donkey anti-mouse IgG and fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) were used at a concentration of 1:50, for 1 hour, at room temperature. Negative controls from which the primary antibody was omitted were performed with every experiment. After the secondary antibody incubation, the slides were washed and coverslipped with 4′,6′-diamino-2-phenylindole (DAPI) mounting media (Vectashield; Vector Laboratories, Inc., Burlingame, CA). Cells were viewed and photographed with a microscope (Eclipse E800; Nikon, Melville, NY) equipped with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). Cells labeled with BrdU (a marker of proliferating cells) and DAPI (a marker of all cell nuclei) were counted and analyzed statistically with a paired *t*-test. $P < 0.01$ was considered significant. Ten randomly selected areas were counted per sample. Each selected area was equal to that viewed within the 20× objective, for a final 200× field of view. To minimize bias, we viewed only the DAPI staining while selecting the areas to count. These areas had to fit the following criteria: (1) the epithelial cells within the 200× field of view had to have a confluence of 70% to 80%, and (2) the cells had to be a monolayer. Three wells were cultured per time point. Approximately 500 cells were counted per sample.

In addition, the effect of TAT-HA-p15 on Ki67 (a marker of actively proliferating cells)[43,44] was tested by Western blot and reverse transcription-polymerase chain reaction (RT-PCR). HCECs were plated in T75 flasks and grown to 80% confluence, after which the cells were incubated for 18 hours with fresh medium containing 150 nM of TAT-HA-p15. Cells not transduced with TAT-HA-p15 served as the control.

Western blot analysis was performed as previously described.[2] Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer,[45] and equal amounts of cell extracts were loaded, electrophoresed on a 3% to 8% Tris-acetate gel (NuPage; Invitrogen), and electrophoretically transferred to a membrane. Transfer of protein was confirmed by staining the membrane with 0.1% Ponceau S solution (Sigma-Aldrich). After a 1-hour incubation in blocking reagent (Blotto [5%]; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the membrane was incubated with anti-Ki67 (Vector Laboratories) diluted in the blocking reagent (1:100) overnight. The membrane was then washed briefly and incubated for 1 hour with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) diluted in blocking reagent (1:5000), soaked in substrate (Super Signal; Pierce Biotechnology, Rockford, IL) for 5 minutes, and exposed to x-ray film (Hyperfilm; Amersham Pharmacia Biotech). The film was then developed, and the band intensities quantified with NIH-Image 1.61/68K software (available by ftp at rsbweb.nih.gov/pub/nih-image or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

RT-PCR was performed as previously described,[46] with specific primers for human Ki67 (a gift from Nancy Joyce, PhD, Schepens Eye Research Institute, Boston, MA).[47] Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; BD BioSciences-Clontech, Palo Alto, CA) served as a control. PCR was performed for 25 (G3PDH) or 32 (Ki67) cycles. The

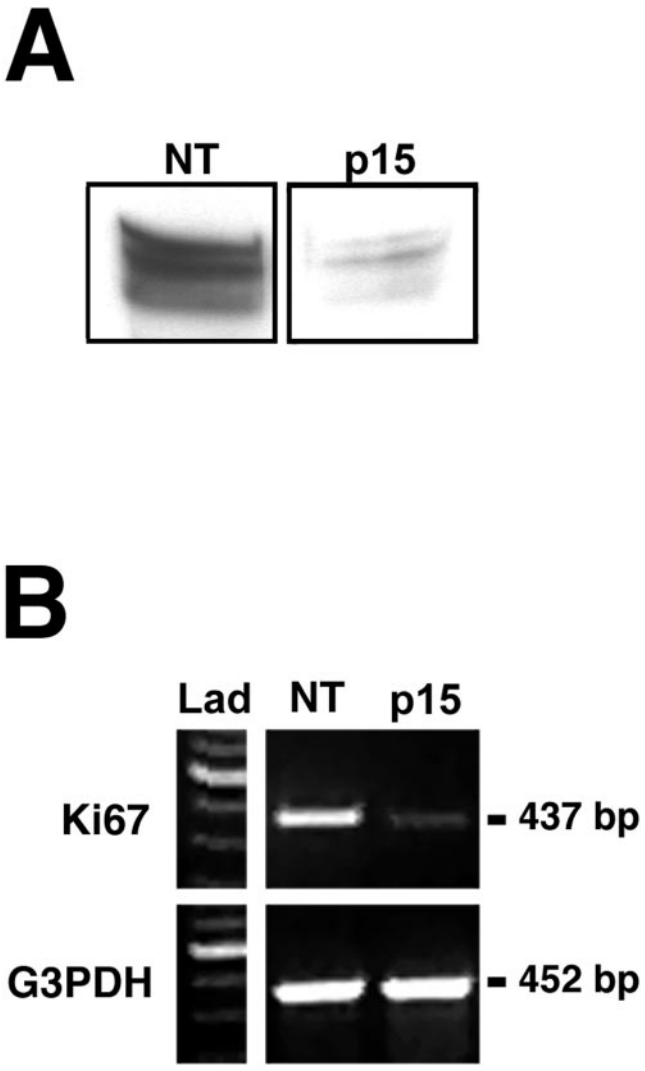

FIGURE 3. (**A**) Western blot analysis of primary HCECs that had been either not transduced (NT) or transduced with 150 nM TAT-HA-p15 (p15) for 18 hours. Equal amounts of protein were loaded into each well, and blots were reacted with anti-Ki67. Ki67 consists of at least two polypeptide bands of 345 and 385 kDa.[43,44] (**B**) RT-PCR analysis of primary HCECs that either were not transduced (NT) or were transduced with 150 nM TAT-HA-p15 (p15) for 18 hours.

PCR products were resolved on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide, and the gel was photographed (Digital Science DC40 camera; Eastman Kodak, Rochester, NY). G3PDH primers were used as the positive control for cDNA quality. Samples with no cDNA were also amplified and served as the negative control.

Effect of TAT-HA-p15 on Migration of HCECs. For the cell migration assay, 5.6×10^4 HCECs were seeded into an 8-mm cylinder in a six-well plate. Cells were allowed to attach for 3 hours at 37°C with 5% CO_2, after which the cylinders were removed. The wells were rinsed with PBS to remove any unattached cells, and medium with PBS, 200 nM TAT-HA-p15, or 200 nM TAT-HA-β-gal was added. Three wells were used for each condition. After 2 days, the migration assay was stopped by fixing cells with buffered formalin and staining the cells with hematoxylin. The new area covered by the cells was calculated by measuring the area with NIH-Image 1.61/68K software.

Effect of TAT-HA-p15 on Phosphorylation of Retinoblastoma Protein in Primary HCECs. Western blot analysis, as previously described, was used to quantify pRb and phospho(p)-pRb in normal and TAT-HA-p15–transduced HCECs. Cells in a T75 flask at 80%

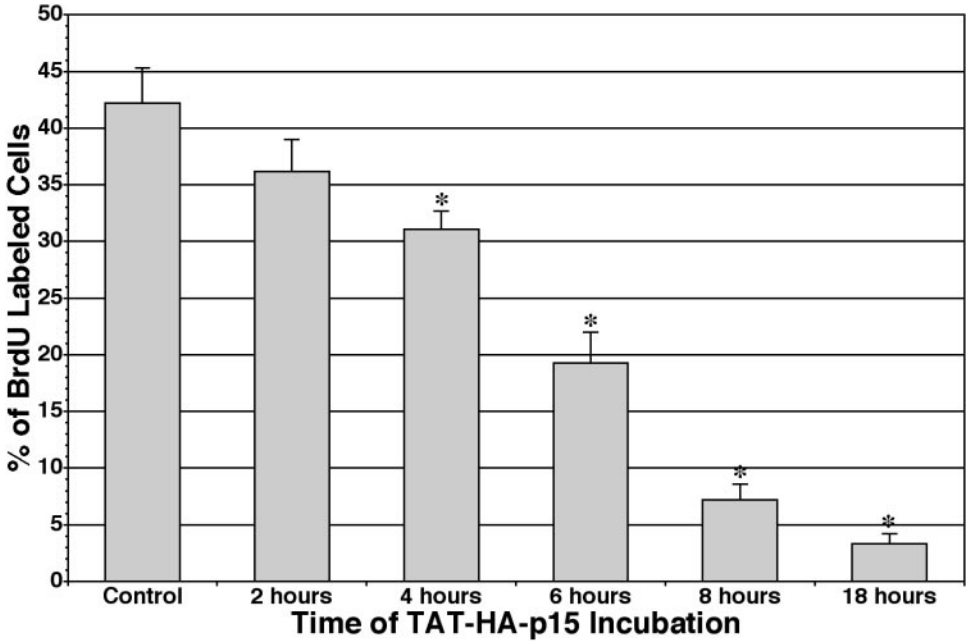

FIGURE 4. Average percentage of BrdU-labeled primary HCECs after incubation with 150 nM TAT-HA-p15 for various lengths of time. Note that the number of proliferating cells decreased by 2 hours; however, it was not until 4 hours that a significant decrease was detected. $^*P < 0.01$.

confluence were transduced with 150 nM of TAT-HA-p15 diluted in fresh keratinocyte-SFM for 18 hours, at 37°C, with 5% CO_2. Cells not transduced with TAT-HA-p15 served as the control. Cells were harvested and lysed with RIPA buffer.[45] An equal amount of cell extracts were loaded, electrophoresed on an 8% Tris-glycine gel (Invitrogen), and electrophoretically transferred to a membrane. After a 1-hour incubation in blocking reagent (Blotto; Santa Cruz Biotechnology), the membrane was incubated with either anti-p-pRb (807; Cell Signaling Technology, Beverly, MA) or anti-pRb (C-15; Santa Cruz Biotechnology) diluted in blocking reagent (1:2000) for 1 hour. The membrane was then washed briefly and incubated for one additional hour with peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) diluted in blocking reagent (1:3000). The membrane was then soaked in substrate (Super Signal; Pierce) for 5 minutes and exposed to x-ray film. The film was developed and the band intensities quantified using NIH-Image 1.61/68K software.

RESULTS

As seen in Figure 1, TAT-HA-p15 was successfully transduced into primary HCECs. Furthermore, the amount of TAT-HA-p15 taken into the cells appeared to be directly dependent on the concentration present in the culture medium.

To determine whether the transduced TAT-HA-p15 was functionally active, its effect on cell proliferation was examined (Fig. 2). After exposure for 18 hours to different concentrations of TAT-HA-p15, the number of proliferating cells decreased in a concentration-dependent manner (Figs. 2A1–4). As seen in Figure 2B, the percentage of BrdU-labeled cells was 45.7%, 21.4%, 3.3%, and 1.7% at 0, 60, 150, and 300 nM of TAT-HA-p15, respectively ($P < 0.01$). In contrast, TAT-HA-β-gal had no significant effect on cell proliferation (Figs. 2A5, 2A6) with 42.9%, 38.9%, 39.0%, and 40.2% BrdU-positive cells at 0, 60, 150, and 300 nM of TAT-HA-β-gal, respectively ($P > 0.3$; Fig. 2B).

To confirm that TAT-HA-p15 was inhibiting proliferation, the expression of Ki67 was examined. As seen in Figure 3, TAT-HA-p15 inhibited the expression of both Ki67 protein (7.6-fold; Fig. 3A) and mRNA (2.9-fold; Fig. 3B), in agreement with the data shown in Figure 2.

Next, we examined how long cells had to be exposed to TAT-HA-p15 to cause a decrease in their proliferation levels (or S-phase cells). As seen in Figure 4, the number of cells in S-phase decreased with time of exposure to TAT-HA-p15. After 0-, 2-, 4-, 6-, 8-, and 18-hour exposure to 150 nM of TAT-HA-p15, the percentage of BrdU-labeled cells were 41.4%, 36.5%, 29.9%, 18.6%, 7.0%, and 3.3%, respectively (Fig. 4). Although the percentage of BrdU-labeled cells had decreased from 41.4% to 36.5% by 2 hours of exposure, a significant difference was not seen until 4 hours ($P < 0.01$).

We then examined whether exposure to TAT-HA-p15 was toxic to the epithelial cells (Fig. 5). In this experiment, cells were treated with TAT-HA-p15, washed, and cultured with fresh medium for 1, 2, 3, 4, 5, 6, or 7 days. After an initial decrease in cell proliferation (Fig. 5A), the number of BrdU-positive cells returned to control levels by 2 days after removal of TAT-HA-p15 (Fig. 5B). These data indicate that TAT-HA-p15 does not kill the cells, but only inhibits cell proliferation and that TAT-HA-p15 activity in the cells is lost by 2 days.

Because $p15^{INK4b}$ is upregulated in cells migrating to cover a wound,[15] we wanted to determine whether TAT-HA-p15 expression had an effect on migration in corneal epithelial cells (Fig. 6). In these experiments, cells were plated in cylinders and allowed to spread in the presence of TAT-HA-p15, TAT-HA-β-gal or with no additions (PBS only). The results appeared to show that TAT-HA-p15 stimulated cell migration (Fig. 6). Compared with the area of the cylinder, the growth percentage of control cells (PBS only), TAT-HA-β-gal, and TAT-HA-p15 was 53.44%, 51.11%, and 96.44%, respectively (Fig. 6C). These data indicate that the stimulation of the cells to migrate was not caused by the TAT-HA portion of TAT-HA-p15, as the growth percentage of PBS only– and TAT-HA-β-gal–transduced cells was approximately equal. The distribution of cells at the edge of the outgrowths was distinct in the TAT-HA-p15–treated cells. In contrast to the control and TAT-HA-β-gal–treated cells, the TAT-HA-p15–treated cells at the edge of the culture appeared to migrate as single cells (Figs. 6A, 6B). This resulted in large gaps between cells compared with the other conditions, in which cells appeared to migrate more as a sheet.

Finally, to confirm that TAT-HA-p15 was involved in the regulation of cell-cycle progression, pRb and p-pRb were assayed by Western blot analysis. As seen in Figure 7A, the level of p-pRb decreased by 4.9-fold in the TAT-HA-p15–transduced HCECs. This finding was confirmed when total pRb was exam-

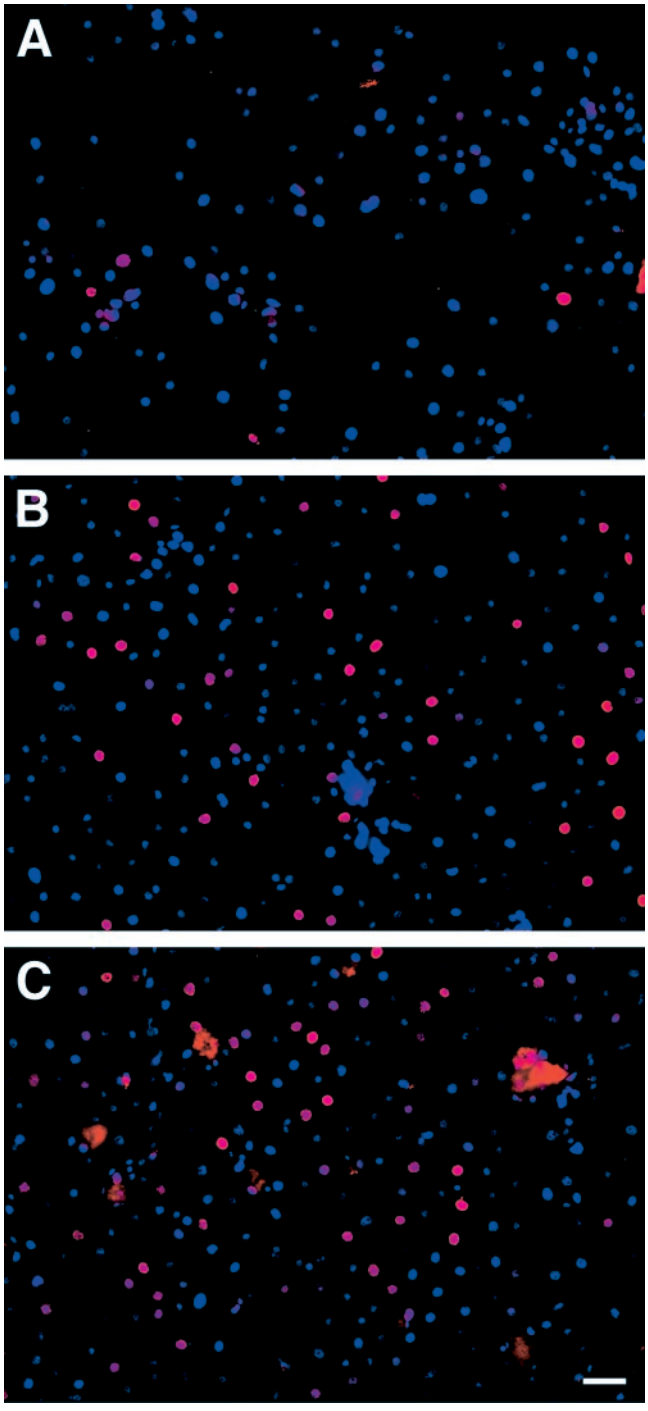

FIGURE 5. Immunolocalization of BrdU (*red*) in primary HCECs that have been transduced with 150 nM TAT-HA-p15 (**A–C**) for 18 hours, washed with PBS, and maintained in culture for an additional day (**A**), 2 days after washing (3 days total; **B**), or 6 days after washing (7 days total; **C**). Note that initially the number of BrdU-labeled cells decreased, but returned to control levels by 2 days after washing. DAPI (*blue*) was used as a counterstain to mark all cell nuclei. Bar, 50 μm.

ined (Fig. 7B). As seen in Figure 7B, the ratio of hyperphosphorylated to hypophosphorylated pRb in untransduced and transduced cells was 2.7 and 0.8, respectively. Thus, on treatment with TAT-HA-p15, there was a dramatic shift in the hyperphosphorylated versus hypophosphorylated pRb. It was also observed that the total amount of pRb was 1.7-fold lower in the TAT-HA-p15–transduced cells (low level of cell proliferation) compared with the actively proliferating untransduced cells. These results are in agreement with the findings in previous studies[18–22,48–50] that p15^{INK4b} negatively regulates the cell cycle by indirectly inhibiting the phosphorylation of pRb and that pRb levels are moderately enhanced in actively proliferating cells.

DISCUSSION

The regulation of epithelial cell proliferation during corneal wound repair presents an intriguing model, in that proliferation is enhanced in cells distal to the original wound but decreases to a level approaching zero in the cells migrating across the wound area.[2,9,10] One of the proteins that has been observed to be upregulated in the migratory corneal epithelial cells is the cell cycle inhibitor p15^{INK4b}.[15] This protein is a member of a family of cell cycle inhibitors termed the INK4 family. These proteins function by binding to CDK4, preventing its interaction with cyclin D.[18–22] Blocking this interaction prevents the activation of CDK4 and the subsequent phosphorylation of pRb, the key regulatory protein of the proliferative cell cycle.[23–27] In the current investigation, we have used

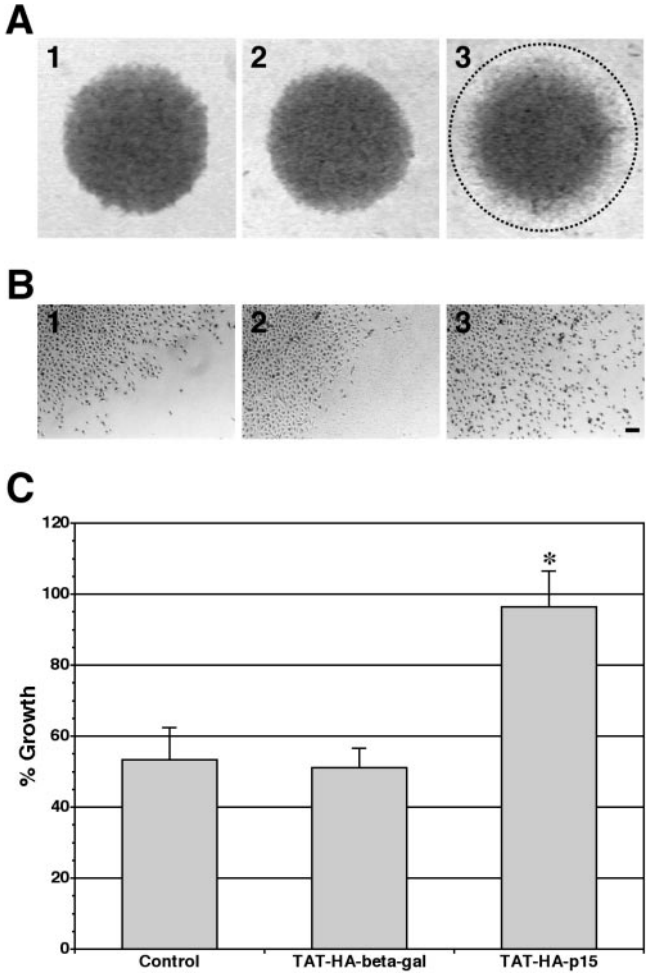

FIGURE 6. Micrographs of primary HCECs: (**A**) entire cell area and (**B**) edge of outgrowths. Cells were either not transduced (**A1**, **B1**) or transduced with either 200 nM TAT-β-gal (**A2** and **B2**) or 200 nM TAT-HA-p15 (**A3**, **B3**) for 2 days. (**C**) Percentage growth of the primary corneal epithelial cells. Cells transduced with TAT-HA-p15 appeared to spread more, thus giving a higher percentage growth than cells that were either not transduced or are transduced with TAT-HA-β-gal. (**A3**, *dotted circle*) Circumference of cell area. Bar, 50 μm; $^*P < 0.01$.

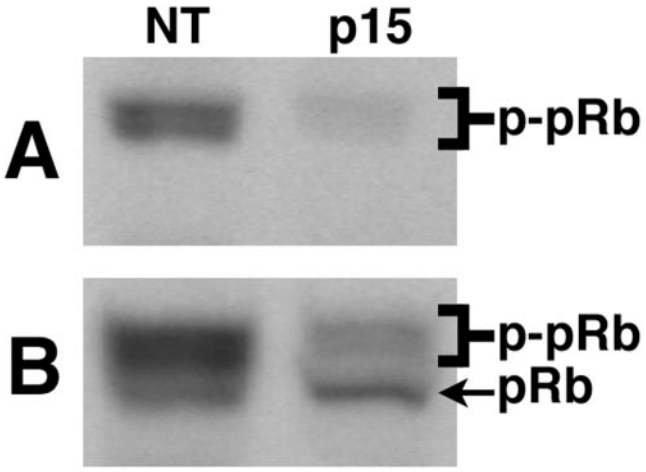

FIGURE 7. Western blot analysis of primary HCECs that either were not transduced (NT) or were transduced with 150 nM TAT-HA-p15 (p15) for 18 hours. Equal amounts of protein were loaded into each well, and blots were reacted with either anti-p-pRb (**A**) or anti-pRb (**B**). The phosphorylated pRb decreased with TAT-HA-p15 transduction, and p-pRb migrated at a higher apparent molecular mass than pRb.

TAT-fusion protein technology to demonstrate that $p15^{INK4b}$ blocks phosphorylation of pRb in HCECs and that the overexpression of $p15^{INK4b}$ is sufficient to inhibit proliferation in these cells. The inhibition of proliferation was confirmed by demonstrating that transduction of TAT-HA-p15 significantly reduced the incorporation of BrdU (Fig. 2) and the expression of Ki67, a marker of proliferating cells (Fig. 3).

Many reports have been made on the up- or downregulation of a variety of proteins during corneal wound repair.[1,4,5,11–14,16] Most of these studies have been correlative findings that the protein under study was spatially and/or temporally expressed in a manner suggesting that it plays a role in the healing process. In the current study, we investigated the use of TAT-fusion protein technology to examine more directly the effect of a protein on corneal epithelial cells. This technology allows the generation of TAT proteins that are rapidly ferried across the cell membrane. This transduction occurs within minutes and allows the manipulation of the concentration of transduced protein by altering the concentration of the TAT-fusion protein to which the cells are exposed.[29,34,35,40,51–54] This methodology has been used to generate more than 60 TAT-fusion proteins, and we have used it successfully to generate for the first time a $p15^{INK4b}$ fusion protein. This methodology holds great promise for examining the effect of various proteins in corneal epithelial cell biology. The methodology may also be adapted to use in organ culture or in vivo studies. The cell cycle inhibitor $p15^{INK4b}$ was originally discovered by investigators searching for the downstream proteins responsible for blocking cell proliferation after exposure to TGF-β.[17] The expression of $p15^{INK4b}$ in migrating corneal epithelial cells suggests that these cells have been stimulated by TGF-β during the healing process, resulting in the inhibition of cell proliferation. A TGF-β signaling pathway has also been postulated to be involved in inhibiting proliferation at the edge of skin wounds.[55] This is in agreement with previous findings that TGF-β receptors type I and II are both upregulated in response to a debridement wound in rat corneas.[28] In addition, we have seen that TGF-β stimulates the expression of $p15^{INK4b}$ in primary HCECs.[28] Results in the current investigation and previous results[15,28] suggest the hypothesis that wounding stimulates the release of active TGF-β and the upregulation of its receptors resulting in enhanced signaling through the TGF-β pathway. One of the downstream proteins upregulated by this stimulation is $p15^{INK4b}$, which is at least, in part, responsible for the inhibition of proliferation in the cells migrating to cover a wound. It is unclear how the signal that inhibits proliferation is confined to cells migrating to cover the wound, but one portion of the regulatory mechanism appears to be that TGF-β receptor II is upregulated only in the cells migrating across the wound.[28] It is unlikely that induction of $p15^{INK4b}$ is the sole mechanism involved in blocking cell proliferation during migration. For example, Sharma et al.[9] has recently discovered that the p38 pathway may also be involved. The downstream molecules directly responsible for the inhibition of proliferation in this pathway were not identified.

We anticipated that $p15^{INK4b}$ would inhibit corneal epithelial cell proliferation, since it has been shown to do so in other epithelial cells.[17–22] However, it was unclear what if any effect $p15^{INK4b}$ would have on migration of corneal epithelial cells. As seen in Figure 6, $p15^{INK4b}$ stimulated cell spreading in a cell culture model. This effect appeared to be the result of cells migrating as single cells rather than as intact sheets. We are currently investigating the effect of overexpression of $p15^{INK4b}$ in an organ culture model. It should also be noted that the level of cell proliferation was lower in the TAT-HA-p15–treated cells than under the other conditions tested. Thus, the effect of $p15^{INK4b}$ on migration may be even greater than measured in our assay. It is not clear whether the stimulation of spreading is a direct effect of $p15^{INK4b}$ or the stimulation is secondary to the inhibition of cell proliferation. Indeed, Nagahara et al.[29] have demonstrated that transducing the cell cycle inhibitor p27 into hepatocarcinoma cells also stimulates cell migration.

In summary, using TAT PTD as a "missile," $p15^{INK4b}$ has been efficiently introduced into primary HCECs in culture. These studies show that TAT-mediated protein transduction is an extremely useful tool in the studies of corneal wound healing and homeostasis. Our studies using TAT-HA-p15 demonstrate that $p15^{INK4b}$ is sufficient to inhibit corneal epithelial cell proliferation and that $p15^{INK4b}$ may promote migration. This may result in more efficient wound repair.

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