Inhibition of Cell Proliferation of Tenon’s Capsule Fibroblast by S-Phase Kinase-Interacting Protein 2 Targeting SiRNA through Increasing p27 Protein Level

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PURPOSE. Although antiproliferative drugs have been used to prevent scarring after filtration surgery in patients with glaucoma, there are complications associated with their use. In the present study, the authors investigated whether small interfering RNA (siRNA)-mediated gene silencing of Skp2 can be used to increase p27kip1 level and inhibit cell proliferation in rabbit Tenon’s capsule fibroblast (rTF).

METHODS. A plasmid containing Skp2 siRNA was used to decrease the high constitutive level of Skp2 protein in rTF, which can lead to consequent degradation of p27kip1. Cell proliferation was assayed by immunocytochemistry using antibodies against S-phase nuclear antigen (PCNA). Skp2 siRNA was delivered to a trabeculectomy animal model to study the effect on rTF proliferation in vivo.

RESULTS. Immunocytochemistry and Western blot analysis showed a decreased level of Skp2 and an increased level of p27kip1 in cells transfected with pSkp2 siRNA but not in vehicle transfection and uninfected cells in vitro and in vivo. MTT assay showed that cell viability significantly declined in rTF transfected with Skp2 siRNA. Skp2 siRNA-transfected cells showed significantly less BrdU and PCNA-positive staining than control cells in vitro and in vivo. Infiltration bleb was detected in the Skp2 siRNA group 14 days after trabeculectomy.

CONCLUSIONS. Skp2 siRNA inhibited cell proliferation and decreased cell viability of rTF in vitro and in vivo. These findings suggest that siRNA-mediated gene silencing of Skp2 can be a novel gene therapy to treat scarring after glaucoma surgery by the suppression of p27kip1 downregulation. (Invest Ophtalmol Vis Sci. 2010;51:1475-1482) DOI:10.1167/iovs.09-4363

In glaucoma surgery, an additional drainage pathway for the aqueous humor is normally created to reduce intraocular pressure. The postoperative healing response can render the desired outcome of such surgery ineffective if scar formation increases outflow resistance of the artificially created drainage pathway. Antimetabolites such as 5-fluorouracil and mitomycin C have been shown to be clinically effective in preventing bleb failure after filtration surgery and recently were used in non-penetrating glaucoma surgery.1 The antiblific effect of those antimetabolites has been shown to derive mostly from the inhibition of Tenon’s capsule fibroblast (rTF) proliferation2 and from the promotion of rTF apoptotic cell death.3-4 However, alternative treatments are needed because of the complications associated with the use of antimetabolites, such as bleb leaks that might lead to endophthalmitis and chronic hypotony with maculopathy. Gene therapy targeting cell cycle genes might provide an alternative long-term antiscarring adjuvant for glaucoma surgery without the complications associated with antimetabolites.5-7

S-phase-kinase-interacting protein 2 (Skp2) is identified as an E3 ubiquitin ligase that targets p27kip1 for ubiquitination and plays a very important role in cell cycle regulation.8-10 Skp2-cullin-F-box (SCF) complexes represent an evolutionarily conserved class of E3 enzymes that contain four subunits: Skp1, Cul1, F-box proteins, and Roc1/Rbx1.11 Skp2 is a rate-limiting component of the machinery that is specifically required for p27kip1 ubiquitination and degradation.12 Skp2 is frequently overexpressed in tumor cell lines, and forced expression of Skp2 in quiescent fibroblasts induces DNA synthesis. However, no previous studies have demonstrated a role for Skp2 in rTF proliferation or scarring after glaucoma surgery.

RNA interference (RNAi) can easily and effectively inhibit the expression of a specific gene.13 The RNAi process is mediated through small, double-stranded RNA molecules called small interfering RNAs (siRNAs), which specifically trigger the cleavage and subsequent degradation of their target mRNA in a sequence-dependent manner. Therefore, RNAi can prevent synthesis of a protein encoded by the target mRNA.14 Recently, RNAi-mediated gene silencing has been shown to be efficient in mammalian cells, and this has led to the increasing feasibility of RNAi technology for the therapy of certain human diseases.15 IKKβ-targeting siRNA was reported to inhibit the proliferation of in vitro human rTF.16 However, this is the only gene whose inhibition by siRNA has been studied in rTF.

In this study, we examined whether siRNA-mediated gene silencing of Skp2 could inhibit p27kip1 downregulation in rTF and the effects of downregulation of p27kip1 on rTF proliferation in vivo and in vitro.

MATERIALS AND METHODS

Animals

Sixty male rabbits (weight range, 2–3 kg) were supplied by the Experimental Animal Center of Harbin Medical University. The rabbits were randomly divided into three groups in vivo: Skp2 siRNA-transfection group (n = 20), vehicle control group (n = 20), and blank control group (n = 20). rTF cells were extracted from nine rabbits. All animal care and experimental procedures were conducted in accordance with our institutional guidelines for animal care, the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of
Health (NIH publication no. 85–23, revised 1996), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents
Pentobarbital sodium was purchased from Shanghai Biological Company (Shanghai, China). Suppression agents (pSuppressor containing Skp2 siRNA and control pSuppressor) were purchased from Imgenex (San Diego, CA). Synthetic oligonucleotide primers were purchased from Qiagen (San Diego, CA). Vimentin antibody, keratin antibody, Skp2 antibody, and streptavidin-alkaline phosphatase complex (SABC) kit were purchased from Boster Company (Wuhan, China). p27kip1 antibody and PCNA antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein-conjugated anti-sheep IgG was purchased from Zhongshan Biotechnology (Beijing, China). Mounting medium (Vectashield) was purchased from Vector Laboratories (Burlington, Canada). Dulbecco’s modified Eagle’s medium and F12 medium (DMEM F12) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal calf serum and reagent (Trizol) were purchased from Invitrogen (Carlsbad, CA). Hanks balanced salt solution was purchased from Hyclone (Logan, UT). Poly-lysine was purchased from Sigma (St. Louis, MO). Culture plate was purchased from BD Biosciences (San Jose, CA). Transfection reagent (Hiperfect) was purchased from Qiagen. Polyvinylidene difluoride (PVDF; Hybrid-B) membrane was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Methylthiazolyldiazotetrazolium (MTT) was purchased from Sigma. MITT cell proliferation kit was purchased from ATCC (Manassas, VA). Cell counter was purchased from Coulter Z1 (Hialeah, FL).

Equipment
Fluorescence (IX70) and optical microscopes were purchased from Olympus (Tokyo, Japan). CO2 incubator (BB16HF) was purchased from Heal Force (Hong Kong, China). Ultraclean work table (D8C-010) was purchased from Boster Company (Wuhan, China). Ultraclean work table (D8C-010) was purchased from Vector Laboratories (Burlington, Canada). Dulbecco’s modified Eagle’s medium and F12 medium (DMEM F12) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal calf serum and reagent (Trizol) were purchased from Invitrogen (Carlsbad, CA). Hanks balanced salt solution was purchased from Hyclone (Logan, UT). Poly-lysine was purchased from Sigma (St. Louis, MO). Culture plate was purchased from BD Biosciences (San Jose, CA). Transfection reagent (Hiperfect) was purchased from Qiagen. Polyvinylidene difluoride (PVDF; Hybrid-B) membrane was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Methylthiazolyldiazotetrazolium (MTT) was purchased from Sigma. MITT cell proliferation kit was purchased from ATCC (Manassas, VA). Cell counter was purchased from Coulter Z1 (Hialeah, FL).

Cell Culture of rTF
Rabbits were anesthetized with ketamine xylazine (60 mg/kg, 80 mg/kg). rTFs were propagated from subconjunctival Tenon’s capsule. The planted tissue was attached to the bottom of a six-well plate with a sterile coverslip and overlaid with DMEM F12 medium. All culture media were supplemented with streptomycin 50 mg and penicillin 500,000 U/L (Invitrogen/Gibco, Uxbridge, UK). To ensure fibroblast cell proliferation, the media were also supplemented with fetal calf serum (FCS; 10% of final volume; Invitrogen-Gibco). Cells from three to five passages were used for all experiments. All cells were grown at 37°C with 5% CO2 ventilation.

Plasmids and Transfection
Vector (pSuppressor[β]-Neo; Imgenex, San Diego, CA) was used to generate biologically active siRNAs from the 16 promoter. Synthetic oligonucleotide primers (5’-tcgaGGGAGGACAAAGGAGACUGCAAAACCUCUUU-3’) containing XhoI and XbaI overhangs were annealed and then were introduced into vector (pSuppressor[β]-Neo; Imgenex). Oligonucleotide sequences corresponded to a 19-nucleotide sequence from Skp2 (nucleotides 114–133), which was separated by an eight-nucleotide linker from the reverse complement of the same 19-nucleotide sequence. A transcriptional termination (UUUUU) was added to the end of the oligonucleotide. We used a circular plasmid containing a scrambled sequence as the control. rTF transfected with pSuppressor containing Skp2 siRNA, pSuppressor only, and medium served as the experimental group, the vehicle control group, and the blank control group, respectively. Transfection was performed in 60-mm plates using 2 μg (1 μg/μL) vector in 10 μL reagent (Metafectene Pro; Biontex, Martinstried, Germany). After 48 hours of transfection, cells were treated with G418 (Gibco Life Technologies) for 2 weeks for positive clone selection. After G418 treatment, several stable transfected cells were cloned. Each clone was screened for expression of Skp2 by Western blot analysis.

Immunocytochemistry
Cells cultured on coverslips were fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized, and blocked with 0.1% Triton X-100 and 5% goat serum for 30 minutes. Cells were incubated with rabbit anti-vimentin antibody (1:500) at 4°C overnight, followed by goat-anti-rabbit IgG (1:500) for 1 hour at room temperature. Then cells were incubated with SABC at 37°C for 30 minutes and colored with 3,3’-diaminobenzidine (DAB). After coloration with DAB, dehydration, and dimethyl benzene treatment, slides were mounted. Controls were stained by omitting the primary antibody.

Skp2 monoclonal antibody (sheep, 1:500 dilution) and anti-sheep IgG conjugated to distinct fluorescence (1:500 fluorescein-conjugated) were used for immunofluorescence-staining experiments.

Western Blot Analysis
The protein expressions of Skp2, p27kip1, and PCNA from three different samples of rTF in each treatment were examined by Western blot analysis. Cells (6 × 103) or 3 × 3-mm Tenon’s capsule fibroblast treated with pSuppressor containing Skp2 siRNA, pSuppressor only, and medium were prepared in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-100, 0.1% SDS, 1 mM EDTA, 1 mM AEBSF, 20 μg/mL aprotinin, and 20 μg/mL leupeptin. Equal amounts of total protein (10 μg) were separated by 10% SDS-PAGE and transferred to PVDF membrane (Hybrid-B; Amersham Pharmacia Biotech). After blocking with 5% nonfat dry milk in PBS containing 0.1% Tween-20, membranes were probed with anti-Skp2 (1:500) or anti-p27kip1 (1:500) mouse monoclonal antibody, followed by incubation with the appropriate secondary antibody, respectively. Visualization of the protein bands was performed by the enhanced chemiluminescence kit (Santa Cruz Biotechnology). A parallel Western blot was probed with an anti-GAPDH antibody (Abcam Company, Cambridge, MA) as a loading control. Band intensity was quantified using appropriate software (Quantity One 4.4.1; Bio-Rad).

MTT Assay
Cell viability was examined with the MTT cell proliferation kit (ATCC, Manassas, VA) in accordance with the manufacturer’s instructions. The assay is based on measuring the reduction of yellow tetrazolium to purple formazan, as facilitated by dehydrogenases of metabolically active cells. Intracellular formazan can be solubilized and quantified by spectrophotometric means. Quadruple samples of rTF from the same animal were grown on 96-well plates and were infected with 2 μg (1 μg/μL) of either of the two vectors in 10 μL reagent (Metafectene Pro; Biontex) or were not infected. After 2, 4, 6, 8, and 12 days, wells were incubated for 20 hours in a medium containing yellow tetrazolium.

Cell Proliferation and BrdU Incorporation
In Vitro
Cells (5.0 × 103) were plated onto a 24-well multilwell plate (3047; Falcon; Becton Dickinson, Franklin Lakes, NJ) and allowed to attach for 24 hours. The culture medium was then replaced with fresh medium. Cells were trypsinized and counted using a cell counter (Coulter Z1; Coulter) at 0, 2, 4, and 6 days. For bromodeoxyuridine (BrdU) incorporation, cells growing on coverslips were incubated with 10 μM BrdU (Sigma) for 3 hours. After fixing in cold methanol/acetone 1:1 for 10 minutes, the cells were sequentially incubated in 1.5 M HCl for 10 minutes. Then cells were washed with PBS and incubated with the mouse anti-BrdU primary antibody (Roche) for 1 hour. The cells were washed four times with PBS. The nuclei were simultaneously incubated with 10 μg/mL of 4V, 6-diamidino-2-phenylindole. Cells with different BrdU incorporation patterns were analyzed and counted with a conventional microscope.
Trabeculectomy Animal Model, pSkp2 siRNA Delivery, and BrdU Incorporation In Vivo

To study the effect of pSkp2 siRNA on rTF proliferation in vivo, the trabeculectomy was performed as follows. Briefly, the rabbits were anesthetized with ketamine xylazine (60 mg/kg, 80 mg/kg). The eyeball was exposed with a wire lid speculum. A fornix-based flap of the conjunctiva and Tenon’s capsule were raised in the superior nasal quadrant of either the left or the right eye, selected randomly. A limbus-based triangular scleral flap was outlined with a steel blade and carefully dissected. A stab knife was then used to create the entry into the anterior chamber at the surgical limbus. Consequently, the tissue blocking the entrance was excised, and a peripheral iridectomy was performed through the fistula. The flaps of sclera and conjunctiva were closed with a 10–0 nylon suture.

Two micrograms (1 μg/μL) vector in 10 μL reagent (Metafectene Pro; Biontext) was delivered to the conjunctiva by subconjunctival injection at the superonasal quadrant during trabeculectomy. After surgery, neomycin sulfate and dexamethasone were applied to the eye.

The Tenon’s capsule fibroblast was cut 14 days after transfection. The protein expression of Skp2, p27kip1, and PCNA in Tenon’s capsule fibroblast cells in vivo was examined by Western blot analysis. The Western blot procedure in vivo was the same as that in vitro. After 14 days of transfection, 0.5 mL BrdU (10 μM) was injected into the Tenon’s capsule fibroblast.

Pathologic and Histochemistry Examinations

The animals were perfused through the heart with heparin saline, followed by 4% paraformaldehyde after 14-day transfection. Eyes were fixed overnight and were transferred to a 30% sucrose solution overnight (4°C). Frozen sections (5 μm) were cut longitudinally on a cryostat, thaw mounted onto coated glass slides, and stained with hematoxylin and eosin,3 skp2 antibody, p27kip1 antibody, PCNA antibody, and anti–BrdU-fluorescein primary antibody. The sections of vehicle control were stained with vimentin antibody to identify the body, and anti–BrdU-fluorescein primary antibody. The sections of Tenon’s capsule fibroblast. The histochemistry procedure in vivo was the same as that in vitro.

Statistical Analysis

Data were analyzed by the two-tailed Student’s t-test with data analysis software (Origin, version 6.0; OriginLab Corp., Northampton, MA). P < 0.05 was considered significant.

RESULTS

Identification of rTF Cells

Immunocytochemistry assay of vimentin, a special cell marker of rTF, was used in our study to identify rTF. As shown in Figures 1A and 1B, most of the fibroblast cells isolated from subconjunctival Tenon’s capsule expressed vimentin protein in the cytoplasm, which indicated rTF in vitro and in vivo.

Downregulation of Skp2 Protein by siRNA in rTF

After 48 hours of transfection and 2 weeks of treatment with G418, we cloned several stable transfectant cells. Immunofluorescence staining demonstrated high constitutive expression of Skp2 protein in the nucleolus of rTF transfected with pSuppressor vehicle (Fig. 2A) or without transfection (Fig. 2C). Transfection with Skp2 siRNA decreased the expression of Skp2 protein in cells of rTF (Fig. 2B). Each clone was also screened for Skp2 expression by Western blot analysis. Skp2 expression could be detected in rTF from the vehicle control group and the blank control group (Fig. 2D, lanes 1, 3). However, there was little expression of Skp2 in the experimental group, which indicated that transfection with Skp2 siRNA could inhibit the expression of Skp2 in rTF in vitro (Fig. 2D, lane 3).

The rabbits underwent trabeculectomy, and 2 μg (1 μg/μL) vector in 10 μL reagent (Metafectene Pro; Biontext) was delivered to the conjunctiva by subconjunctival injection at the superonasal quadrant. After 2-week transfection in vivo, immunofluorescence staining demonstrated high constitutive expression of Skp2 protein in the rTF transfected with pSuppressor vehicle (Fig. 3A) or without transfection (Fig. 3C). Transfection with Skp2 siRNA during trabeculectomy decreased the expression of Skp2 protein in rTF cells (Fig. 3B). The rTF was also screened for Skp2 expression by Western blot analysis. Skp2 expression could be detected in rTF from the vehicle and blank control groups (Fig. 3D, lanes 1, 3). However, there was little expression of Skp2 in the experimental group, which indicated that transfection with Skp2 siRNA could inhibit the expression of Skp2 in rTF in vitro (Fig. 3D, lane 3).
it possible for us to investigate the effect of p27kip1 inhibition on the proliferation of rTF.

Western blot analysis demonstrated that p27kip1 expression of rTF in the experimental group increased the expression of p27kip1 protein (Figs. 4B, 4E) in vitro and in vivo. Transfection with Skp2 siRNA increased p27kip1 protein expression in rTF cells (Figs. 4C, 4F) in vitro and in vivo. There was a decline in p27kip1 expression of Skp2 siRNA transfected rTF compared with control cells (Fig. 5, lane 2). GAPDH served as a loading control.

FIGURE 2. Downregulation of Skp2 protein by siRNA in rTF in vitro. Immunofluorescence staining (1 of 3 representative experiments) indicated high constitutive levels of Skp2 protein (green fluorescence, arrow) in the nucleus of rTF transfected with pSuppressor vehicle (A) or without transfection (C). Transfection with Skp2 siRNA dramatically decreased the expression of Skp2 protein in rTF cells (B). Scale bar, 40 μm. After 48 hours of transfection and 2 weeks of treatment with G418, several stable transfectant cells were cloned. Western blot analysis (1 of 3 representative experiments) showed that high constitutive Skp2 expression can be detected in rTF of the vehicle control group and the blank control group, respectively (D, lanes 1, 2). However, little expression of Skp2 was detected in the experimental group, indicating transfection with Skp2 siRNA can inhibit the expression of Skp2 in rTF in vitro (D, lane 2). GAPDH served as a loading control.

Skp2 siRNA–Induced p27kip1 Accumulation in rTF

Skp2 is required for the ubiquitination and consequent degradation of p27kip1. Western blot analysis in our study demonstrated that p27kip1 expression of rTF in the experimental group increased (Figs. 4G, 4H) when the expression of Skp2 decreased (Figs. 2, 3). Immunofluorescence staining demonstrated little p27 protein expression in the rTF transfected with pSuppressor vehicle (Figs. 4A, 4D) or without transfection (Figs. 4B, 4E) in vitro and in vivo. Transfection with Skp2 siRNA increased the expression of p27kip1 protein in rTF cells (Figs. 4C, 4F) in vitro and in vivo. Upregulation of p27kip1 made it possible for us to investigate the effect of p27kip1 inhibition on the proliferation of rTF.

Skp2 siRNA–Decreased rTF Cell Viability In Vitro

MTT. As shown in Figure 5, cell viability in the vehicle control and blank control did not exhibit significant differences, indicating that the plasmid did not influence cell metabolism. However, cell viability in Skp2 siRNA-transfected rTF (experimental group) significantly decreased at 6 and 12 days after transfection when compared with vehicle control and blank control.

Incorporation of BrdU. For the BrdU incorporation assay, cells growing on coverslips were incubated with BrdU. Incorporation of BrdU was detected using antibodies as described in Materials and Methods. High levels of BrdU staining could be detected in rTF from the vehicle control (Figs. 6A, 7A) and the blank control (Figs. 6B, 7B), but staining was decreased in Skp2 siRNA-transfection cells (Figs. 6C, 7C). Statistical analysis (Figs. 6D, 7D) after cell counting showed that BrdU-positive cells in rTF transfected with Skp2 siRNA was significantly decreased compared with control cells (P < 0.01 vs. vehicle and blank control).

Expression of PCNA. PCNA is a marker that indicates the proliferation potential of cells. Immunofluorescence staining demonstrated little expression of PCNA protein in the rTF transfected with pSuppressor vehicle (Figs. 8A, 8D) or without transfection (Figs. 8B, 8E) in vitro and in vivo. Transfection with Skp2 siRNA increased PCNA protein expression in rTF cells (Figs. 8C, 8F) in vitro and in vivo. There was a decline in PCNA expression after transfection with Skp2 siRNA (Fig. 8G, lanes 1, 2; Fig. 8H, lane 3) compared with the other two groups (Fig. 8G, lanes 1, 2; Fig. 8H, lanes 1, 2).

Histologic Observation for Inhibition of rTF Proliferation

Trabeculectomy was performed to further study the effect of pSkp2 siRNA on rTF proliferation. At 14 days after trabeculectomy, high levels of rTF proliferation could be detected in vehicle control (Fig. 9A) and blank control (Fig. 9C). However, rTF proliferation dramatically decreased, and the infiltration bleb could be detected in the Skp2 siRNA-transfection group (Fig. 9B).
DISCUSSION

Glaucoma is a disease resulting from increased intraocular pressure that leads to irreversible functional impairment of the optic nerve. Filtration surgery to enhance the drainage of aqueous humor is one of the most effective therapies for glaucoma, but the success rate is not perfect because of blockage of the surgically created drainage channel by subconjunctival scarring that may occur as a result of wound healing. Fibroblasts located in the subconjunctival area play a major role in scar formation after filtration surgery through the proliferation, migration, and synthesis of the extracellular matrix. Thus, regulating the biological activities, particularly cell proliferation, of subconjunctival Tenon’s capsule fibroblasts during the wound healing process is a major antiscarring strategy that would be beneficial in glaucoma filtration surgery.

Cellular proliferation is regulated primarily by cell cycle regulation. Cell cycle progression is regulated by a combination of positive and negative regulators. Cyclin-dependent kinase (CDKs) inhibitors (CKI) negatively regulate progression of the cell cycle by inhibiting the activity of cyclin-CDK complexes. p27kip1, a member of the CKI family, plays a pivotal role in the control of cell proliferation. The p27kip1 level is high during the G0 phase but decreases rapidly on reentry of the cells into the G1 phase. Rapid removal of p27kip1 at the G0/G1 transition is required for effective progression of the cell cycle to the S phase. It means a negative correlation between the expression of Skp2 and p27kip1 in fibroblasts from pathologic scar tissue. However, there are no previous studies involving the role of Skp2 in rTF proliferation or scarring after glaucoma surgery. Our results demonstrated that Skp2 was highly expressed and that p27kip1 displayed very little expression in rTF. Furthermore, for the first time, we showed that the inhibition of p27kip1 expression by Skp2 siRNA was correlated with high proliferative and migratory capacity, whereas nuclear accumulation of CKI was associated with a quiescent and static phenotype. In previous studies, Yoshida et al. suggested that the disappearance of p27kip1 was correlated with cell proliferation in the corneal epithelium after injury. Yang et al. showed that adenovirus-mediated p27kip1 overexpression significantly improved wound healing after filtration surgery by inhibiting postoperative proliferation in rabbit eyes.

The level of p27kip1 is regulated by Skp2. Skp2 is specifically required for p27kip1 ubiquitination and is a rate-limiting component of the machinery that degrades phosphorylated p27kip1. Skp2 is constitutively expressed in normal skin tissue and scar tissue. High expression of Skp2 and decreased expression of p27kip1 were observed in fibroblasts from pathologic scar tissue. It means a negative correlation between the expression of Skp2 and p27kip1 in fibroblasts from pathologic scar tissue. However, there are no previous studies involving the role of Skp2 in rTF proliferation or scarring after glaucoma surgery. Our results demonstrated that Skp2 was highly expressed and that p27kip1 displayed very little expression in rTF.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933247/)

**Figure 4.** Skp2 siRNA induced p27kip1 accumulation in rTF in vitro and in vivo. Immunofluorescence staining indicated the expression of p27kip1 dramatically increased in rTF transfection with Skp2 siRNA (C, F) when compared with rTF transfection with pSuppressor vehicle (A, D) or without transfection (B, E). Scale bar, 40 μm. Western blot analysis (1 of 3 representative experiments) showed that the expression of p27kip1 dramatically increased in rTF transfection with Skp2 siRNA (G, H; lane 3) when compared with rTF transfection with pSuppressor vehicle (G, H; lane 1) or without transfection (G, H; lane 2). GAPDH served as a loading control.

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933247/)

**Figure 5.** Cell viability assay by MTT. After 6 to 12 days of transfection with Skp2 siRNA, cell viability was significantly decreased in cultured rTF cells compared with vehicle control and blank control.

*P < 0.01 vs. vehicle and blank control; **P < 0.05 vs. vehicle and blank control.
FIGURE 6. Skp2 siRNA inhibited the cell proliferation of rTF in vitro (BrdU). For BrdU incorporation, cells growing on coverslips were incubated with BrdU. Incorporated BrdU was detected with antibodies, as described in Materials and Methods. High levels of BrdU with dense brown staining can be detected in rTF of vehicle control (A) and blank control (B) but were decreased in Skp2 siRNA-transfection cells (C). Statistical analysis (D) after cell counting showed that BrdU-positive cells decreased after rTF transfection with Skp2 siRNA compared with control cells. *P < 0.01 vs vehicle and blank control.

FIGURE 7. Skp2 siRNA inhibited the cell proliferation of rTF in vivo (BrdU). High levels of BrdU staining were detected in the rTF of vehicle control (A) and blank control (B) but were dramatically decreased in Skp2 siRNA-transfection cells. (C). Scale bar, 40 μm.
of Skp2 expression by siRNA enhanced p27kip1 protein levels and prevented rTF proliferation. Thus, it may be used to prevent excess scarring after glaucoma surgery.

Approaches to modify the wound healing response after filtering surgery by gene transfer have been reported by different research groups. Perkins et al. delivered the recombinant adenovirus-containing p21 gene to conjunctiva and found that it can prevent rTF cell proliferation after glaucoma surgery. Akimoto et al. blocked the transcription factor E2F in hTF by transferring double-stranded phosphorothioate oligonucleo-

**FIGURE 8.** Upregulation of PCNA protein by siRNA in rTF in vitro and in vivo. Immunofluorescence staining indicated the expression of PCNA increased in rTF transfection with Skp2 siRNA (C, F) compared with rTF transfection with pSuppressor vehicle (A, D) or without transfection (B, E). Scale bar, 40 μm. Western blot (1 of 3 representative experiments) showed that the expression of PCNA dramatically decreased after rTF transfection with Skp2 siRNA (G, H, lane 3) when compared with rTF transfection with pSuppressor vehicle (G, H, lane 1) or without transfection (G, H, lane 2). GAPDH served as a loading control.

**FIGURE 9.** Skp2 siRNA inhibited the proliferation of rTF in vivo (hematoxylin and eosin staining). Hematoxylin and eosin staining was performed to examine histologic changes 14 days after trabeculectomy and transfection. Obvious rTF proliferation was detected in pSuppressor vehicle group or without transfection after trabeculectomy (A, C). Infiltration bleb in the Skp2 siRNA-transfection group (B). Scale bar, 20 μm.
tides containing E2F transcription factor cis element with the hemagglutinating virus of Japan cationic liposomes and found growth inhibition of rTF. Johnson et al.12 constructed a recombinant adenoviral vector to overexpress p53 in hTF and found that adenoviral p53 gene transfer leads to significant proliferation inhibition of hTF.

Finally, in view of the growing interest in Skp2 and p27kip1 as a target for drug development in the inhibition of fibroblast cell proliferation to regulate wound healing after glaucoma surgery, it is our hope that the data presented here will help to enhance the success ratio of glaucoma surgery.

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References