Inhibition of proliferation of rabbit lens epithelial cells by S-phase kinase-interacting protein 2 targeting small interfering RNA

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Purpose: Improper proliferation of lens epithelial cells is causally related to posterior capsule opacification. In the present study, we investigated whether small interfering RNA (siRNA)-mediated gene silencing of S-phase kinase-interacting protein 2 (Skp2) can be employed to inhibit rabbit lens epithelial cell (rLEC) proliferation by increasing the p27kip1 level.

Methods: A plasmid containing Skp2 siRNA was used to decrease the high constitutive level of Skp2 protein in rLECs, which can lead to consequent degradation of p27kip1. Protein expression of Skp2 and p27kip1 was detected by immunocytochemistry and western blot. Cell viability was measured using the tetrazolium reduction (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide [MTT]) assay. Cell proliferation was assayed by cell counts, immunocytochemistry, and western blot by using antibodies against proliferating cell nuclear antigen.

Results: Immunocytochemistry and western blot showed a decreased level of Skp2 and increased level of p27kip1 in cells transfected with pSkp2 siRNA but not in vehicle transfection and uninfected cells. MTT assay showed that cell viability significantly declined in rLECs transfected with Skp2 siRNA. Skp2 siRNA transfected cells showed significantly less 5-bromodeoxyuridine- and proliferating cell nuclear antigen-positive staining compared with control cells.

Conclusions: Skp2 siRNA inhibits cell proliferation and decreases cell viability of rLECs in vitro by suppression of p27kip1 downregulation. Our findings suggest that siRNA-mediated gene silencing of Skp2 can be a novel gene therapy for posterior capsule opacification induced by LEC abnormal proliferation.

RNA interference (RNAi) can easily and effectively inhibit the expression of a specific gene [10]. 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 [11]. 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 [12]. IkkapaB kinase subunit beta (IKKβ) targeting siRNA was reported to inhibit the proliferation of in vitro human Tenon’s capsule fibroblast [13]. Our recent study showed that transfection of Skp2 siRNA can effectively inhibit the proliferation of rabbit tenon’s fibroblast cells after glaucoma surgery [14].

In this study, we examined the expression of Skp2 in rabbit LEC (rLEC) and investigated if siRNA-mediated gene silencing of Skp2 could inhibit p27kip1 downregulation and repress rLEC proliferation in vitro.

METHODS

All experimental procedures were carried out in accordance with Harbin Medical University guidelines for animal care and the Guide for Care and Use of Laboratory Animals.
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Source of reagents: Keratin antibody, skp2 antibody, and streptavidin biotin complex (SABC) kit were purchased from Boster company (Wuhan, China). P27 kip1 antibody, PCNA antibody and Enhanced chemiluminescence kit were from Santa cruz biotechnology Inc (Santa Cruz, CA). Fluorescein-conjugated anti-sheep IgG was purchased from Zhongshan biotechnology (Beijing, China). Vectashield mounting medium was from Vector laboratories (Burlington, Canada). Modified Eagle's medium (MEM) was from Gibco (Burlington, VT). Fetal calf serum, Trizol TM were from Invitrogen (Carlsbad, CA). Hanks solution was from Hyclone (Logan, UT). Poly-lysine and Phosphate buffer solution (PBS) were purchased from Sigma (St. Louis, MO). Culture plate was from BD Biosciences (San Jose, CA). Hipercell transfection reagent was purchased from Qiagen gene technology company (Carlsbad, CA). Anti- GAPDH antibody was purchased from Abcam company (Cambridge, MA). Mouse anti-BrdU-fluorescein primary antibody was purchased from Roche (Madison, WI).

Equipment used: Fluorescence microscope (IX70) and optic microscope was purchased from Olympus company (Tokyo, Japan). CO₂ incubator (BB16HF) and Irriclean work table (D8C-010) were purchased from Heal Force (Hong Kong,China). Incubation plate was from Coster corporation (Cambridge, MA). Cell Counter (Coulter Z1) was purchased from Coulter company (Hialeah, FL).

Cell culture of rabbit lens epithelial cells: Adult albino rabbits weighing between 2 and 3 kg, purchased from experimental animal center of Harbin Medical University, were used for experiment. The rabbits used in this investigation were handled in accordance with the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve-week-old rabbits weighing 1–1.5 kg were killed by CO₂ inhalation. The entire eye was removed and then slides were mounted. Controls were stained by omitting the primary antibody. Skp2 monoclonal antibody (1:500 dilution), p27kip1 antibody (1:500 dilution), and PCNA antibody (1:1,000 dilution) were also used for immunofluorescence staining experiments.

Western blot analysis: The protein expression of Skp2, p27kip1, and PCNA from three different rLEC samples in each
treatment was examined by western blot analysis. rLEC cells \((6 \times 10^5)\) 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 X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM 4-(2-Aminoethyl)-benzenesulfonl (AEBSF), 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Equal amounts of total protein (10 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond-P polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in PBS with 0.1% Tween-20, membranes were probed with anti-Skp2 (1:500), anti-p27kip1 (1:500), or anti-PCNA mouse monoclonal antibodies (1:1,000), followed by incubation with the appropriate secondary antibody. Visualization of the protein bands was performed by the enhanced chemiluminescence kit. A parallel western blot was probed with an anti-GAPDH antibody as a loading control. Band intensity was quantified using Quantity One 4.4.1 software (Bio-Rad, Hercules, CA).

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide assay: Cell viability was examined by the MTT cell proliferation kit following the instructions of the manufacturer. The assay is based on measuring the reduction of yellow tetrazolium to purple formazan as facilitated by dehydrogenases of metabolically active cells. The intracellular formazan can be solubilized and quantified by spectrophotometric means. Quadruple samples of rLEC were grown on 96-well plates and were infected with 2 μg (1 μg/µl) of either of the two vectors in 10 µl of Metafectene proreagent or were not infected. After 2, 4, 6, 8, and 14 days, wells were incubated in a medium containing yellow tetrazolium for 20 h.

Cell proliferation and bromodeoxyuridine incorporation: Cells \((5.0 \times 10^3)\) were plated onto a 24-well multiwell plate (Falcon; Becton Dickinson, Franklin Lakes, NJ) and allowed to attach for 24 h. The culture medium was then replaced with fresh medium. Cells were trypsinized and counted with a cell counter at 0, 2, 4, and 6 days. For bromodeoxyuridine (BrdU) incorporation, cells growing on coverslips were incubated with 10 μM Brdu (Sigma) for 3 h. After fixing in cold methanol/acetic acid 1:1 for 10 min, the cells were sequentially incubated in 1.5 mol/l HCl for 10 min. Cells were then washed with PBS and incubated with mouse anti-
BrdUrd-fluorescein primary antibody for 1 h. The cells were washed four times with PBS. The nuclei were simultaneously stained with 10 µg/ml of 4V, 6-diamidino-2-phenylindole. Cells with different BrdUrd incorporation patterns were analyzed and counted with a conventional fluorescence microscope.

Statistical analysis: The data were analyzed by the two-tailed Student t test using SPSS 10.0; p<0.05 was considered significant.

RESULTS
The identification of rabbit lens epithelial cells: An immunocytochemistry assay of keratin, a special cell marker of rLECs, was used in our study to identify rLECs. As shown in Figure 1, cultured cells expressed keratin protein (brown staining) in the nucleus, which indicated rLECs.

Downregulation of Skp2 protein by small interfering ribonucleic acid in rabbit lens epithelial cells: After 48 h 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 rLECs transfected with pSuppressor vehicle (B) or without transfection (C). Transfection with Skp2 siRNA dramatically decreased the expression of Skp2 protein in rLECs (A). Scale bar is equal to 40 μm. After 48 h of transfection and 2 weeks of treatment with G418, several stable transfectant cells were cloned. Western blot analysis (one representative of three experiments) showed that high constitutive Skp2 expression can be detected in rLECs of the vehicle control group and blank control group (D). However, little expression of Skp2 was detected in the experimental group, indicating transfection with Skp2 siRNA can inhibit expression of Skp2 in rLECs in vitro (Figure 2D, lane 1).

Upregulation of p27kip1 protein by Skp2 small interfering ribonucleic acid in rabbit lens epithelial cells: Skp2 is required for the ubiquitination and consequent degradation of p27kip1 [2]. Immunofluorescence staining demonstrated little expression of p27 protein in the rLECs transfected with pSuppressor vehicle (Figure 3A) or without transfection (Figure 3B). Transfection with Skp2 siRNA increased the expression of p27kip1 protein in rLECs (Figure 3C) in vitro. Western blot analysis in our study demonstrated that p27kip1 expression of rLECs in the experimental group increased (Figure 3D, lane 3) when expression of Skp2 decreased (Figure 2). Upregulation of p27kip1 made it possible for us to investigate the effect of p27kip1 inhibition on the proliferation of rLECs.

Skp2 small interfering ribonucleic acid decreased the rabbit lens epithelial cells viability and proliferation in vitro:

3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyltetrazoliumbromide (MTT)—As shown in Figure 4, cell viability did not show any significant differences after vehicle transfection compared with the blank control group, indicating that the plasmid did not influence cell metabolism.
However, cell viability in Skp2 siRNA transfected rLECs (experimental group) significantly decreased at 6 and 10 days after transfection when compared with the vehicle control and blank control.

Incorporation of bromodeoxyuridine—High-level staining of BrdU was detected in the rLECs of the vehicle control (Figure 5A) and blank control (Figure 5B) but dramatically decreased in Skp2 siRNA transfectant cells (Figure 5C). Statistical analysis (Figure 5D) after cell counting showed that BrdU-positive cells in rLECs transfected with Skp2 siRNA significantly decreased compared with control cells (p<0.01 versus vehicle and blank controls).

PCNA protein expression in rLECs after transfection with Skp2 siRNA: PCNA is a marker that indicates the proliferation potential of cells. Immunofluorescence staining demonstrated obvious expression of PCNA protein in the rLECs transfected with the pSuppressor vehicle (Figure 6A) or without transfection (Figure 6B) in vitro. Transfection with Skp2 siRNA decreased the expression of PCNA protein in rLECs (Figure 6C) in vitro. Western blot further confirmed that the expression of PCNA dramatically decreased after rLEC transfection with Skp2 siRNA.
transfection with Skp2 siRNA (Figure 6D, lane 3) when compared with rLEC transfection with pSuppressor vehicle (Figure 6D, lane 1) or without transfection (Figure 6D, lane 2).

**DISCUSSION**

Cataract surgery can induce a wound-healing response in the lens that leads to PCO. PCO incidence is approximately 50% in adults and 100% in children after cataract surgery [15] and is the most common postoperative complication of cataract surgery that causes visual loss [16]. PCO arises from residual LECs at the equator and under the anterior lens capsule after cataract surgery. These cells proliferate and migrate onto the posterior capsule underlying the intraocular lens and into the light path. Many of these cells undergo epithelial-to-mesenchymal transition (EMT), resulting in the formation of fibroblasts and spindle-like myofibroblasts, which lead to capsular opacity [16]. Clinically, there are two morphological types of PCO: fibrosis type and pearl type. The fibrosis-type PCO is caused by the proliferation and migration of LECs, which undergo EMT [15-18]. In the pearl-type PCO, LECs located at the equatorial lens region can cause regeneration of crystallin-expressing lenticular fibers and form Elschnig pearls and a Soemmering ring.

The histological features of PCO are now well established, but to date the molecular mechanisms influencing leftover LEC behavior after cataract surgery are not completely clear. LECs left behind in the capsular bag after any type of extracapsular cataract surgery are mainly responsible for PCO development [1], which involves LEC abnormal proliferation, migration, EMT, collagen deposition, and lens fiber regeneration [19-21].

Studies show that levels of several cytokines and growth factors increase in the aqueous humor and influence the behavior of the remaining LECs after cataract surgery. These factors include transforming growth factor [22-24], fibroblast growth factor 2, hepatocyte growth factor, and interleukins 1 and 6 [25,26].

Cellular proliferation is regulated primarily by cell-cycle regulation. Cell-cycle progression is regulated by a combination of positive and negative regulators. Cycin-dependent kinase (CDKs) inhibitors (CKI) negatively regulate progression of the cell cycle by inhibiting the activity of cyclin-CDK complexes. p27\(^{kip1}\), a member of the CKI family, plays a pivotal role in the control of cell proliferation [27-29]. The level of p27\(^{kip1}\) is high during the G0 phase but decreases rapidly upon re-entry of the cells into the G1 phase. Rapid removal of p27\(^{kip1}\) at the G0/G1 transition is required for effective progression of the cell cycle to S phase [30-32]. In previous studies, Yoshida et al. [33] suggested that the disappearance of p27\(^{kip1}\) was correlated with cell proliferation in the corneal epithelium after injury. According to our study, a low level of p27\(^{kip1}\) expression was correlated with high proliferative and migratory capacity, whereas nuclear accumulation of the CKI was associated with a quiescent and static phenotype.
The level of p27<sup>kip1</sup> is regulated by Skp2. Skp2 is specifically required for p27<sup>kip1</sup> ubiquitination and is a rate-limiting component of the machinery that degrades phosphorylated p27<sup>kip1</sup> [22]. Skp2 is constitutively expressed in normal skin tissue and scar tissue. High expression of Skp2 and decreased expression of p27<sup>kip1</sup> are observed in fibroblasts from pathological scar tissue [30], which indicates a negative correlation between expression of Skp2 and p27<sup>kip1</sup> in fibroblasts from pathological scar tissue. However, there have been no previous studies on the role of Skp2 in LEC proliferation. Our study demonstrated that Skp2 was highly expressed and p27<sup>kip1</sup> displayed little expression in rLECs. Furthermore, we showed that inhibition of Skp2 expression by siRNA enhanced the p27<sup>kip1</sup> protein level and prevented rLEC proliferation.

At present, the only effective treatment of PCO is neodymium-doped yttrium aluminium garnet (Nd:YAG) laser capsulotomy, which involves clearing the visual axis by creating a central opening in the opacified posterior capsule. Although this procedure is easy and rapid, there are complications, including retinal detachment, damage to the intraocular lens (IOL), cystoid macular edema, an increase in intraocular pressure, iris hemorrhage, corneal edema, IOL subluxation, and exacerbation of localized endophthalmitis. Changes induced by Nd:YAG capsulotomy have been shown to be affected by IOL material and design. In addition, this treatment represents a considerable cost burden to national health care systems, and such laser treatment is not readily available in developing countries. Therefore, a better understanding of the pathogenic mechanism of PCO is highly desirable as a basis for improving the outcome of cataract surgery and eradicating PCO [34-36].

Approaches to inhibiting LEC proliferation after cataract surgery by gene transfer have been reported by different research groups. Malecaze et al. [37] and Coundrec et al. [38] delivered adenoviral vector-mediated transfer systems, including herpes simplex virus-thymidine kinase/ganciclovir (HSV-tk/GCV) into rabbit LECs in vitro, which can inhibit the proliferation of rLECs. Malecaze et al. [39] demonstrated that adenoviral-mediated Bax or procaspase-3 overexpression is capable of inducing therapeutic programmed cell death in vitro and in vivo in resident lens cells and preventing PCO in a rabbit model. Kampmeier et al. [40] showed that transfection of antisense cyclin G1 retroviral vectors can inhibit proliferation of LECs. In this study we showed that siRNA targeting Skp2 prevented rLEC proliferation in vitro. Thus, the method might be used to prevent PCO after cataract surgery.

Finally, in view of the growing interest in Skp2 and p27<sup>kip1</sup> as a target for drug development in inhibiting LEC proliferation following cataract surgery, it is our hope that the data presented here will help to decrease the PCO incidence from cataract surgery.

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