Using HSV-TK/GCV suicide gene therapy to inhibit lens epithelial cell proliferation for treatment of posterior capsular opacification

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Purpose: To establish a novel, targeted lentivirus-based HSV-tk (herpes simplex virus thymidine kinase)/GCV (ganciclovir) gene therapy system to inhibit lens epithelial cell proliferation for treatment of posterior capsular opacification (PCO) after cataract surgery.

Methods: An enhanced Cre recombinase (Cre/loxP) system with a lentiviral vector expressing Cre under the control of the lens-specific promoter LEP503 (Lenti-LEP503-HSVtk-Cre [LTKCRE]) was constructed, as well as another lentiviral vector containing a switching unit. The latter vector contains a stuffer sequence encoding EGFP (Lenti-hPGK-Loxp-EGFP-pA-LoxP-HSVtk [PGFPTK]) with a functional polyadenylation signal between two loxP sites, followed by the herpes simplex virus thymidine kinase (HSV-tk) gene, both under the control of the human phosphoglycerate kinase (hPGK) promoter. Expression of the downstream gene (HSV-tk) is activated by co-expression of Cre. Human lens epithelial cells (HLECs) or retinal pigment epithelial cells (RPECs) were co-infected with LTKCRE and PGFPTK. The inhibitory effects on HLECs and RPECs infected by the enhanced specific lentiviral vector combination at the concentration of 20 µg/ml GCV were assayed and compared.

Results: The specific gene expression of Cre and HSV-tk in HLECs is activated by the LEP503 promoter. LTKCRE and PGFPTK co-infected HLECs, but not RPECs, expressed high levels of the HSV-tk protein. After 96 h of GCV treatment, the percentage of apoptotic HLECs infected by the enhanced specific lentiviral vector combination was 87.23%, whereas that of apoptotic RPECs was only 10.12%. Electron microscopy showed that GCV induced apoptosis and necrosis of the infected HLECs.

Conclusions: The enhanced specific lentiviral vector combination selectively and effectively expressed HSV-tk in HLECs. A concentration of 20 µg/ml GCV is effective against the proliferation of HLECs in vitro. This cell-type-specific gene therapy using a Cre/loxP lentivirus system may be a feasible treatment strategy to prevent PCO.

Posterior capsular opacification (PCO) caused by proliferation of residual epithelial cells over the lens equator and onto the posterior lens capsule [1] is the leading cause of visual impairment and blindness after cataract surgery [2-4]. There are currently no effective means by which to eradicate the residual lens epithelial cells during the operation [5,6]. In spite of improvements in the basic research on development of cataracts, surgical techniques, and the material or the design of the intraocular lens, the incidence of PCO is still 8~34.3% in adults, and nearly 100% in children [7-10]. One new promising approach for treatment of PCO is a gene therapy system uses a so-called suicide gene, the herpes simplex virus type 1 thymidine kinase (HSV-tk) gene, and the anti-herpes nucleoside analog drug, ganciclovir (GCV) [11-13]. In this system, GCV is phosphorylated by HSV-tk into a deoxynucleotide analog that becomes incorporated into DNA during strand replacement in proliferating cells, where it acts as a chain terminator of DNA synthesis and kills the dividing cells [14-16].

However, the standard HSV-tk/GCV system is driven by a constitutive promoter [11,12], and was shown to not only cause the death of the lens epithelial cells, but also of the corneal endothelial cells and the iris pigmental epithelial cells [11]. This cytotoxic side effect is the main obstacle for further clinical application of this system. However, the use of a lens-specific promoter would greatly benefit the application of this system for treatment of PCO. LEP503 (lens epithelium gene product 503), which is a highly conserved gene involved in lens epithelial cell differentiation in different vertebrate species, is localized in the epithelial cells along the entire anterior surface of the lens. LEP503 may be an important lens epithelial cell gene involved in the processes of epithelial cell differentiation [17]. The expression of LEP503 is highly restricted to lens epithelial cells in vivo, and 2.5-kb flanking sequence-directed high-level promoter activity in lens epithelial cells, but not in other cell types [18]. Maleczae et al. [19] found that LEP503,MIP (major intrinsic protein), and Filensin promoters induced strong lens-specific expression of a reporter gene in human lens cells. The efficacy of LEP503 promoters for a reporter gene expression is restricted to the...
residual lens cells post-PCO. We have found that human cytomegalovirus (CMV) promoter driven HSV-TK can inhibited the HLEC proliferation, though this system has no cell specification [20,21]. To avoid the toxic effects of the constitutive promoter on the surrounding normal cells, we constructed the HSV-tk/GCV vector with the lens-specific promoter LEP503 (Lenti-LEP503-EGFP-HSVtk [LGFTPTK]) and found that it can specifically express the HSV-tk protein in lens epithelial cells. However, the promoter inserted in this vector cannot provide high levels of expression. Indeed, the transduction efficiency of this vector was only 17.32%. Because the expression of HSV-tk induced by the lens-specific promoter LEP503 was lower than that of the CMV promoter, we reasoned that it would not effectively inhibit the proliferation of lens epithelial cells.

It was recently reported that gene therapy using the Cre/loxP system greatly enhances the expression of the HSV-tk gene [15,22-24], especially that transduced by adenoviruses under the control of tissue-specific promoters such as the carcinoembryonic antigen (CEA) promoter and thyroglobulin promoter [24,25]. In these studies, the sensitivity of tumor cells to GCV was increased up to 5 to 10-fold compared with sensitivity in the presence of the promoter alone [26].

In the present study, to enhance the expression of the lens-specific promoter LEP503, we employed the HSV-tk/Cre/loxP system for gene therapy, targeting human lens epithelial cells (HLECs). Cre-loxP system-mediated lentiviruses, bearing an ON/OFF switching unit for activation by Cre recombinase, were used. We constructed an enhanced specific lentiviral vector combining two vectors: one is a regulatory vector (Lenti-LEP503-HSVtk-Cre [LTKCRE]) that expresses the Cre recombinase gene under the control of the LEP503 promoter, while the other is a target vector (Lenti-hPGK-Loxp-EGFP-pA-Loxp-HSVtk [PGFPTK]). The switching unit in the lentiviral vector contains a stuffer sequence encoding enhanced green fluorescent protein (EGFP) with a functional polyA sequence between the strong human phosphoglycerate kinase (hPGK) promoter and the inserted HSV-tk fragment, thereby inducing EGFP gene expression without HSV-tk expression. A pair of loxP sites flanking the stuffer sequence allows its excision by the Cre recombinase, leading to expression of the HSV-tk sequence instead of EGFP.

Thus, we used the regulatory vector (LTKCRE) to express the HSV-tk protein and Cre recombinase after infection of HLECs by the two lentiviral vectors. The induced Cre recombinase should excise the functional polyA sequence interposed between the two loxP sites in the target vector (PGFPTK). Consequently, HSV-tk gene expression would be driven by the stronger hPGK promoter after activation by Cre recombinase. The amount of HSV-tk expressed by the Cre/loxP system-mediated lentiviruses should be greater than that expressed by the lentiviruses driven only by lens-specific promoter LEP503 (Figure 1). We then evaluated the efficacy of gene therapy against proliferation of HLECs using these vectors with the Cre/loxP system and GCV treatment. Our findings provide experimental evidence for further development of this potential therapy for clinical treatment of PCO.

METHODS
Construction of the enhanced specific lentiviral vector combination: The enhanced specific lentiviral vector combination includes two lentiviral vectors. One is a regulatory vector (LTKCRE), while the other is a target vector (PGFPTK). Cre recombinase was cloned from the Lenti-Cre
plasmid by PCR, and sub-cloned into a T vector, which was double-digested by EcoRI and Sall enzymes. At the same time, the LGFPTK plasmid (constructed in our laboratory, unpublished data) was also digested by EcoRI and Sall enzymes. The two digested sections were ligated together to construct the regulatory vector LTKCRE. Loxp-polyA-Loxp was artificially synthesized with a Pmel enzyme site at the 5′ end of the polyA, and was inserted into a T vector along with EGFP, which had Pmel enzyme-digested sites at both terminal ends, resulting in the Loxp-EGFP-pA-Loxp vector. The Loxp-EGFP-pA-Loxp vector and plasmid PRRL were both double-digested by BamHI and Sall enzymes and then directionally combined. IRES-HSV/tk was cloned by PCR with Sall restriction sites at both ends and inserted into the Sall-digested construction vector to produce the target vector PGFPTK.

**Production of lentiviral vectors:** Replication-defective lentiviral particles pseudotyped with a VSV-G envelope were produced by three plasmid transient transfections of 293T cells, as previously described [20], with 20 μg of one of the gene transfer constructs (LTKCRE, PGFPTK), 12 μg of psPAX2, and 5 μg of pMD2.G using a calcium phosphate transfection kit (Gibco-BRL, Gaithersburg, MD). The transfection medium was replaced with fresh culture medium after 14 to 16 h. The conditioned medium was collected for 4 h, cleared by low-speed centrifugation, and filtered through 0.45-mm filters (Nalgene, Rochester, NY). The virus was collected by ultracentrifugation for 90 min at 80,000× g at 4 °C. The pellet was resuspended in 1 ml phosphate buffered saline (PBS).

**FACS and PCR analysis of the infection efficiency of the lentiviral infection:** To determine the infection efficiency of the enhanced specific lentiviral vector combination (PGFPTK and LTKCRE), EGFP expression was visualized by fluorescence microscopy and analyzed by FACS. Because the regulatory vector LTKCRE does not express EGFP, we detected the infection efficiency of LGFPTK. The HLEC line (SRA 01/04, cell line transformed by large T antigen) and retinal pigment epithelial cells (RPECs, A PRE-19) were obtained from the ATCC (Manassas, VA ) and cultured in DMEM with 10% FBS. The HLECs and RPECs were plated separately at 2×10^5 cells/well in 6-well plates for 24 h, and then LGFPTK and PGFPTK at a multiplicity of infection (MOI) of 20 were added. Polybrene (8 μg/ml) was added to the two sets of cultures, and the infected HLECs and RPECs were cultured for 72 h. EGFP expression in the HLEC group was visualized by fluorescence microscopy and analyzed by FACS. The primer sequences were as follows: **EGFP** (upstream 5′-cga gct gga cgg cga cgt aaa c-3′; downstream 5′-ggc ctt ctc gtt ggg ttt g-3′) and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**; upstream 5′-aac gag ggg ttc cga tgc cct ggg-3′; downstream 5′-tct gtc ctc cgt tcc ctt cgt aag c-3′). Cycling conditions for amplification were: 94 °C for 5 min; 28 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and finally, 72 °C for 10 min. The expected length of the EGFP PCR products was 597 bp. Differences in expression were normalized to the *GAPDH* signal (590 bp). Ten microliters from each RT-PCR product was electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide.

**The expression of EGFP in HLECs and RPECs infected by the enhanced specific lentiviral vector combination:** To analyze the expression of EGF, the HLECs and RPECs were plated separately at 2×10^5 cells/well in 6-well plates, and LTKCRE (MOI=100), PGFPTK (MOI=20), or the enhanced specific lentiviral vector combination (PGFPTK [MOI=20] and LTKCRE [MOI=100]) were added. Polybrene (8 μg/ml) was added to all cultures, and the infected HLECs and RPECs were cultured for 72 h. EGFP expression in the HLEC group and RPEC group were visualized by fluorescence microscopy and analyzed by FACS.

**Western blotting analysis:** To analyze the protein expression of Cre, EGFP, and HSV-tk from the lentiviral vectors, 1×10^6 HLECs were seeded in 25 mm^2 cell culture flasks for 24 h and then LTKCRE (MOI=100), PGFPTK (MOI=20), or the enhanced specific lentiviral vector combination (PGFPTK [MOI=20] and LTKCRE [MOI=100]), was added. At 72 h after the infection, the cells were boiled with 6× loading buffer for 10 min, and the cell proteins (20 μg) were separated in SDS–PAGE gels, followed by blotting onto polyvinylidene difluoride (PVDF) membranes. The anti-β-actin antibody from Santa Cruz Biotechnology (1:5000, Heidelberg, Germany) and anti-Cre, anti-EGFP, and anti-HSV-tk antibodies from Sigma-Aldrich (1:2,000; St. Louis, MO) were used to detect the lentivirally expressed Cre, EGFP, and HSV-tk proteins. The membranes were then incubated with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Amersham, Arlington Heights, IL) and visualized using a chemiluminescence system (Amersham™ ECL).

**Cytotoxicity of GCV:** To evaluate the cytotoxicity of GCV (InvivoGen, SanDiego, CA) treatment, apoptotic levels of HLECs and RPECs were evaluated. HLECs and RPECs were plated separately in 96-well plates at a density of 5×10^3 cells/well and incubated for 24 h. Cells infected with Lenti-hPGK-EGFP-HSV/tk (MOI=20), LTKCRE (MOI=100), or the enhanced specific lentiviral vector combination (PGFPTK [MOI=20] and LTKCRE [MOI=100]) served as the control group. After 24 h, the four groups of cells were treated with GCV at increasing concentrations (0, 10, 20, 30, 40, and 50 μg/ml) and incubated at 37 °C in 5% CO_2_ for 4 days. Each group was assayed in triplicate for each concentration. Apoptosis of infected cells after the 4-day incubation was evaluated quantitatively by measuring phosphatidylserine externalization, an early apoptosis-related event. This was performed by fluorescence
staining using the annexin V(blue)-PI staining kit (Roche, Basel, Switzerland) as per the manufacturer’s recommendations.

To further evaluate the cytotoxicity of GCV treatment, HLECs were plated in 96-well plates at a density of 5×10^4 cells/well and incubated for 24 h. Cells infected with Lenti-hPGK-EGFP-HSVtk (MOI=20), LTKCRE (MOI=100), or the enhanced specific lentiviral vector combination [(PGFPTK (MOI=20) and LTKCRE (MOI=100)] served as therapeutic groups, and cells infected with Lenti-IRES-EGFP (MOI=20) served as a control group. After 24 h, the four groups were treated with GCV at increasing concentrations (0, 10, 20, 30, 40, and 50 μg/ml) and incubated at 37 °C in 5% CO₂ for 1 to 4 days. Cell viability of the four groups was determined using the Cell Counting kit-8 Cell Proliferation Assay (Dojindo, Kumamoto, Japan) after 24 or 96 h. At each time point, cells were assayed in triplicate for each concentration. The cytotoxic effect was indicated as the percentage of surviving cells (ratio of surviving cells after treatment and without treatment) using the following formula: cell viability=(absorption of lenti-HSV-tk-EGFP−absorption of background)/(absorption of lenti-EGFP absorption of background) × 100%. A p value of <0.05 was considered statistically significant.

Electron microscopy: HLECs (1×10^6/well, 6-well plates) infected with the enhanced specific lentivector combination (PGFPTK [MOI=20] and LTKCRE [MOI=100]) served as a therapeutic group; HLECs infected with Lenti-IRES-EGFP (MOI=20) served as a control group. After treatment with GCV (20 μg/ml) for 96 h, cells were collected with a cell scraper, followed by three 10-min washes in PBS at room temperature. The cell pellet was fixed with 5% glutaraldehyde for at least 30 min, fixed in PBS containing 1% osmium tetroxide for 1 h, dehydrated in grading ethanol, and embedded in resin. Sections (100 nm thick) were counterstained with uranyl acetate and lead citrate and examined by transmission electron microscopy (H-7650; Hitachi, Tokyo, Japan). The characteristic morphologic changes of infected HLECs were recorded, including chromatin condensation, plasma membrane blebbing, cell shrinkage, and fragmentation into membranebound bodies.

Statistical analysis: Treatments were examined and analyzed statistically using the SPSS statistical package, version 11.0 (SPSS Inc., Chicago, IL) for Windows. Data are expressed as mean±standard deviation of separate experiments. ANOVA and t tests were performed to assess the statistical significance between different groups. A p value of <0.05 was considered statistically significant.

RESULTS

The LEP503 promoter drives EGFP expression specifically but relative weakly in HLECs: In comparing HLECs at 3 days post-infection by two lentiviral vectors (MOI=20), a stronger fluorescent signal was detected after PGFPTK infection (Figure 2A). However, a low level of EGFP expression was detected in the HLECs infected by LGFPTK under a fluorescence microscope (Figure 2B). FACS detections of positive EGFP were 98.64% (Figure 2C) and 25.58% (Figure 2D) by PGFPTK and LGFPTK infection, respectively. This demonstrated that the constitutive promoter hPGK was stronger than the LEP503 promoter in HLECs. To detect the specificity of the LEP503 promoter in HLECs, we analyzed the expression of EGFP in HLECs and RPECs, which are both important cells related to visual function in eye. RT–PCR results showed that PGFPTK expressed well in both HLECs and RPECs. However, LGFPTK was only expressed in HLECs, as there was no detectable EGFP in RPECs (Figure 2E). This result demonstrated that the LEP503 promoter can specifically induce HSVtk-EGFP expression in HLECs with lower efficiency than the constitutive promoter hPGK.

The enhanced specific lentiviral vector combination drives high HSVtk protein expression in HLECs: The enhanced specific lentiviral vector combination was composed of LTKCRE and PGFPTK. The mechanism of this expression system is shown in Figure 1. LTKCRE specifically expressed the HSV-tk protein and Cre recombinase in HLECs. PGFPTK could express EGFP well, but not HSV-tk with the polyA behind EGFP. When both of them were expressed in HLECs, LEP503 specifically induced the Cre recombinase (LTKCRE) to excise the functional polyA sequence interposed between the two loxP sites in the target vector (PGFPTK). Then, HSV-tk gene expression could be driven by the stronger hPGK promoter after activation by the Cre recombinase. The HSV-tk expressed by this vector combination was greater than that expressed in the presence of the promoter alone (LTKCRE). To estimate the transduction efficiency and specificity of the lentiviral vectors, we infected HLECs (Figure 3A-C) and RPECs (Figure 3D-F). The regulatory vector LTKCRE did not express EGFP (Figure 3A,D). The transduction efficiencies of PGFPTK in HLECs and RPECs measured by FACS analysis were 97.43% and 92.15%, respectively (Figure 3B,E). Meanwhile, the expression of EGFP was drastically reduced to 6.3% by this enhanced specific lentiviral vector combination in HLECs (Figure 3C), and there was no significant change in expression between the lentiviral vector combination applied to RPECs (86.46%; Figure 3F) and that from the infection of PGFPTK in RPECs (Figure 3E). We analyzed the expressions of Cre, EGFP, and HSV-tk by western blot analysis (Figure 3G). As expected, Cre was expressed only in the HLECs infected by LTKCRE and the enhanced specific lentiviral vector combination. No EGFP expression was observed in the HLECs infected by LTKCRE, whereas EGFP expression in HLECs infected by PGFPTK was stronger than infection by the enhanced specific lentiviral vector combination. For HSV-tk, HLECs infected by the enhanced specific lentiviral vector combination showed
A stronger expression than by LTKCRE, whereas there was very weak expression in the HLECs infected by PGFPTK.

Apoptosis of HLECs treated with GCV in different lentiviral vector groups: The cell morphologic changes of the therapeutic group (the enhanced specific lentiviral vector combination) and the control group (Lenti-IRES-EGFP) were studied using transmission electron microscopy. Compared with the normal shapes and sizes of the cells in the control group (Figure 4A), the typical morphologic changes of apoptosis were seen in the enhanced specific lentiviral vector combination infected cells treated with 20 μg/ml GCV for 96 h (Figure 4B). The characteristics of apoptosis included cell shrinkage, chromatin condensation, nuclear fragmentation, and cytoplasm degradation. The nuclei were irregular and bulbous; at the same time, necrosis was seen in a few cells of the therapeutic group (Figure 4C). The cells were swollen with a collapsed membrane, loss of structural integrity, and rounded mitochondria. Intracellular contents were released, which indicated a typical pattern of cell necrosis. The electron microscopy results showed that GCV at a concentration of 20 μg/ml can induce apoptosis and necrosis of cells infected with the enhanced specific lentiviral vector combination.

Apoptotic levels of HLECs and RPECs were analyzed by FACS 96 h after infection in different lentiviral vector groups treated with GCV at the concentration of 20 μg/ml (Figure 4D). The percentage of apoptosis in the HLECs infected with the enhanced specific lentiviral vector combination (PGFPTK and LTKCRE) was 87.23%, and the percentage of apoptosis in the positive control vector (Lenti-hPGK-EGFP-HSVtk) group was 90.12%. The apoptotic level was somewhat lower, at 53.2%, in the cells infected individually with the vectors carrying the lens epithelial cell specific promoter (LTKCRE), while that of the negative control vector Lenti-IRES-EGFP was 0.96%. For the RPECs, the percentages of apoptosis in cells infected with different vectors were 10.12% (PGFPTK and LTKCRE), 89.68% (Lenti-hPGK-EGFP-HSVtk), 8.62% (LTKCRE), and 0.75% (Lenti-IRES-EGFP).

The levels of apoptosis in HLECs infected with the enhanced specific lentiviral vector combination were obviously higher than those in the LTKCRE-infected groups, and a specificity for HLECs over RPECs was also shown.

Cytotoxicity of the HSV-tk/GCV system: To determine the effect of GCV on HLECs, the drug was added at increasing concentrations (from 0 to 50 μg/ml) to four groups of cells [infected with Lenti-hPGK-EGFP-HSVtk, LTKCRE, the enhanced specific lentiviral vector combination (PGFPTK and LTKCRE), and Lenti-IRES-EGFP], which were then incubated for 24 or 96 h. At each time point, the cytotoxicities of the cells were assayed in triplicate for each concentration using the Cell Counting Kit-8 (CCK-8).

The cell cytotoxicity gradually became more obvious with increasing GCV concentrations (Figure 5). The results indicated a dose-dependent effect on survival of the infected HLECs treated with GCV. At a concentration of 20 μg/ml after 96 h, there was no significant difference (p>0.05) in the cell viability between the enhanced specific lentiviral vector combination and the Lenti-hPGK-EGFP-HSVtk group. However, the cell viability with the enhanced specific
lentiviral combination infection was significantly lower (p<0.05) than that of LTKCRE-infected cells. The cell viability of the enhanced specific lentiviral vector combination at 24 h was significantly higher (p<0.05) than that at 96 h.

DISCUSSION

Clinically, PCO is treated by Nd:YAG capsulotomy or secondary capsulotomy, which carries a risk of sight-threatening complications such as cystoid macular edema, retinal detachment, and increased intraocular pressure [3,4]. With the development of molecular biology, gene therapy for PCO in vitro has made further advances. Several studies have experimentally demonstrated the cytotoxic effects of the HSV-tk/GCV system on lens epithelial cells and showed that it effectively inhibits the proliferation of lens epithelial cells in vitro [11,13,19,25,26]. However, further application of HSV-tk gene therapy for PCO treatment in vivo is dependent on eliminating the toxic side effects on the cells surrounding the lens epithelial cells. The use of the lens-specific promoter LEP503 may be a desirable strategy for targeted gene therapy for PCO. To increase the low expression activity of this tissue-specific promoter [27], we used the Cre/loxP system involving double lentivirus vectors in the present in vitro study.

In comparing HLECs infected by various vectors, no EGFP-positive cells could be seen in the regulatory vector LTKCRE-infected group, and very few were infected by the enhanced specific lentiviral vector combination expressed-EGFP; however, almost all of the HLECs in the targeted vector PGFPTK group expressed EGFP, as observed by fluorescent microscopy. Western blot analysis confirmed these EGFP expressions. For HSV-tk, there was weak expression in the HLECs infected by the targeted vector PGFPTK, while those infected by the regulatory vector...
LTKCRE showed stronger expression of HSV-tk. Finally, the strongest expression of HSV-tk was observed in HLECs infected by the enhanced specific lentiviral vector combination. Cre was only expressed in the HLECs infected by the regulatory vector LTKCRE and the enhanced specific lentiviral vector combination. These data demonstrated that the use of the constructed double lentiviral vector combination was successful in terms of the high level of the promoter expression. However, there was weak expression of the HSV-tk protein in HLECs infected by the targeted vector PGFPPTK, suggesting that the efficacy of the functional polyA sequence needs further improvement.

This expression of HSV-tk in HLECs resulted in marked specific enhancement of cytotoxicity after exposure to GCV. Similar dose-dependent cytotoxic effects of GCV on infected HLECs, as well as in other virus-mediated systems, have been previously observed [11,20]. In our previous experiments, we found that 20 to 25 μg/ml of GCV was the best range of concentrations of GCV for inhibiting HSV-tk-positive cells without cytotoxicity to normal cells [20]. We evaluated the inhibitory effect of the HSV-tk/GCV system on HLECs through analysis of apoptosis and viability using CCK-8.

After treatment of the cells with GCV at a concentration of 20 μg/ml for 96 h, the apoptosis rate detected by flow cytometry in the HLECs transduced by the enhanced specific lentiviral vector combination was high, at 87.23%. Additionally, the cell viability of the group treated with the enhanced specific lentiviral vector combination was significantly suppressed compared with that treated with LTKCRE.

The double lentiviral vectors combination of the HSV-tk/GCV system effectively inhibited the proliferation of HLECs in our study. To verify our observations, electron microscopy and EGFP detection were also applied, which showed that the cytotoxic mechanism of the HSV-tk/GCV system includes apoptosis and necrosis of infected cells. The fact that the apoptosis rate was relatively high at 87.23% but not 100% is...
perhaps an advantage. That is, killing all residual lens epithelial cells may ultimately result in capsular defects since growth and/or maintenance of the capsule requires living lens cells, especially in children [28,29].

In addition to the high HSV-tk expression in the lens epithelial cells, the specific HSV-tk expression is another important index in the evaluation of the enhanced specific lentiviral vector combination. After the RPECs were infected with LGFPPTK, RT–PCR analysis showed there was no detectable EGFP in the cells, while the infection of the targeted vector PGFPPTK or the enhanced specific lentiviral vector combination resulted in nearly the same high level of EGFP positive cells in the two groups. These data indicated that there was no HSV-tk and Cre expression in RPECs under the control of the lens-specific promoter LEP503. After treatment of the cells with GCV (20 µg/ml), even for 96 h, there was little apoptotic effect on RPECs infected by the enhanced specific lentiviral vector combination. Therefore, the double lentiviral vectors combination as constructed would have restricted expression of HSV-tk in HLECs, thereby avoiding toxic effects to the surrounding normal cells.

In conclusion, the lentiviral vector combination using the Cre/loxP system can improve the expression of the HSV-tk gene driven by the lens-specific promoter LEP503. At a concentration of 20 µg/ml, GCV was effective against the proliferation of HLECs in vitro, but had little killing effect on RPECs. In future experiments, we will verify the inhibitory effects of this lentiviral vector combination on the lens capsule or PCO models.

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