Original Article

Histological and Ultrastructural Features for Proliferation Inhibition by Delivery of Exogenous p27^{Kip1} to Rabbit Models after Glaucoma Filtration Surgery

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Abstract

Purpose: To investigate postoperative inhibition of cell proliferation in animals treated with exogenous p27^{Kip1} during an experimental glaucoma filtration surgery (GFS) by histological and ultrastructural examinations.

Methods: The primer was designed according to p27^{Kip1} gene sequence of GenBank. The p27^{Kip1}-expressing adenovirus-mediated Ad-p27 was constructed according to standard techniques. Gene therapy was performed by subconjunctival delivery of Ad-p27, mitomycin C(MMC) and PBS served as controls. Histological and ultastructural changes at surgical sites were observed for 28 days postoperatively.

Results: Histologically, evident cellular proliferation induced by Ad-p27 was observed on day 7, with appreciable filtering cavity. However, thin conjunctival layers were noted, and the number of fibroblasts decreased in the Tenon's capsule on day 14. On day 28, the filtering cavity partially disappeared. The histological features of the animals with MMC delivery were similar to those treated by Ad-p27. In contrast, in the PBS treatment group, thick conjunctival layers were observed, and the number of goblet cells and fibroblasts, increased markedly. The filtering cavity disappeared at postoperative day 21. Twenty eight day postoperatively, ultrastructural findings showed that most conjunctival epithelial cells in the Ad-p27 treatment group were under static state, and that organelles were inactive. The number of goblet cells, and the secretion of mucin, were decreased. The amount of fibroblasts also decreased with partial apoptotic cells. The ultrastructural features presented by rabbits with the MMC delivery resembled those treated with Ad-p27. In the PBS treatment group, the number of conjunctival epithelial cells increased; the amount of goblet cells increased as the secretion of mucin was strengthened, and a substantial amount of fibroblasts were observed with active and productive organelles.

Conclusion: Ad-p27 inhibits the proliferation of Tenon's capsule fibroblasts at surgical sites, and suppresses the formation of scars, thereby promoting surgical efficacy. (***Eye Science 2012; 27:127-133***)

Keywords: cyclin dependent kinase inhibitor; p27^{Kip1}; glaucoma; filtration surgery; proliferation

Fibroblasts proliferation and conjunctival fibrosis serve as the main reasons causing the failure of glaucoma filtration surgery (GFS), eventually leading to flap closure and failed filtering bleb[1]. Mito mycin C (MMC) delivery gains significant inhibitory effects, while has low specificity, likely resulting in several complications including ocular surface discomforts, corneal epithelial defects, chronic hypotony, filtering bleb fistula and endophthalmitis, etc[2,3]. Thus, seeking a proper inhibitory method which can prevent postoperative scar formation and prolong the survival of filtering blebs is highly required to enhance clinical efficacy and maintain uncontrolled intraocular pressure (IOP).

p27^{Kip1}, also known as CDKN1B, is a cyclin-dependent kianase inhibitor (CKI), belonging to Cip/Kip family. p27^{Kip1}, as the key negative cell cycle regulator, is able to suppress G1/S transition by the close bind between its two serine sites and cyclin-

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CDK complexes, including cyclin E-CDK2, cyclin D–CDK4/6, etc[4-6]. Our previous study found that $p27^{Kip1}$ can lower IOP by inhibiting wound healing after GFS in rabbits, but it fails to result in evident filtering bleb thinning[3]. In this study, we observed histological changes in surgical sites induced by $p27^{Kip1}$ and revealed the underlying mechanism of the inhibitory effect of $p27^{Kip1}$ upon scar formation.

Materials and methods

Animal model construction

Thirty six rabbits, weighed 2.5 to 4.0 kg, male and female, were provided by the experimental animal center of Xi'an Jiaotong University. All the rabbits were randomly allocated to three groups: p27 group (experimental group), MMC group as positive control and PBS group as negative control.

Primers were designed based on human p27 cDNA sequence GenBank (GenBank Accession: NM_004064.2): the forward primer, cgcgtcgacatgtcaaacgtgcgagtgtc, which contains SalI restriction site. The reverse primer, containing HindIII and Flag sites: ccaagctta tttatcgtcatcgtctttgtagtccgtttgacgtcttctgaggc. pShuttle-GFP-CMV plasmid and adenovirus shuttle plasmid pAd-p27 were constructed. The adenovirus vector carrying $p27^{Kip1}$ was transfected into 293 cell line and recombinant plasmid vector Ad-p27 was constructed. The cloned gene sequence was validated and the plaque of 293 cells was observed. Western blot analysis was performed to the expression of Ad-p27. The viral titer of Ad-p27 was 2.1×10^{15} pfu·L^{-1}.

Sclerectomy combined with a peripheral iridectomy was performed through thefistula.Ad-p27 of 8.0×10^{14} pfu·L^{-1} (1.0×10^{-4}L) was delivered to the conjunctiva by subconjunctival injection at the superonasal quadrant during surgery. Controls included animals dosed with equivalent PBS and MMC. All the procedures were performed by one single surgeon. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sample preparation

Two rabbit eyes were randomly collected 3, 7, 14, 21 and 28 d after surgery. Eyes were enucleated. Conjunctiva and cornea 5 mm from the surgical site were excised, rinsed in 10% paraformaldehyde for HE staining. For transmission electron microscope (TEM), animals were sacrificed 28 d after surgery. Conjunctival and corneal tissues were fixed with a mixture containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer at 4℃ after enucleation.

Histological findings

HE staining analysis was conducted to display the morphological changes of conjunctival, scleral and subconjunctival tissues, inflammatory reactions and openness of filtration path and fibroproliferation.

Ultrastructural findings

For TEM analysis, the tissue was fixed in 2% OsO4 for 2h, dehydrated in 30%, 50% and 70% acetone for 5 to 10 min, 90% for 10 to 15 min and 100% for 40 min, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and inspected with a transmission electron microscope.

Results

Histological observations at surgical sites

On day 3, filtration cavities were noted at surgical sites in the Ad-p27 group. Conjunctival epithelia consisted of 1 to 2 layers. Underlying connective tissues were loosely arranged with a relatively small number of fibroblasts. Collagen fiber was dispersely distributed. Few lymphocytes, monocytes and plasmocytes were infiltratrated. On day 7, partial filtration canal, filtration path remained unobstructed. Conjunctival epithelia consisted of 2 to 3 layers. Subconjunctival fibrous tissues were evidently increased. Lymphocyte, monocyte and plasmocyte infiltration were relatively obvious. Apparent capillary hyperplasia was seen. At day 14 after surgery, partial unobstructed filtration path was observed. Conjunctival epithelia contained 1 to 3 layers. The quantity of subconjunctival fibroblasts and infiltrated inflammatory cells decreased. On day 21, conjunctival epithelia had 1 to 3 layers. Subconjunctival tissues were loosely arranged, a low density of fibroblasts was seen and surrounding fibrous tissues were evenly distributed. On day 28, partial filtration path was blocked, conjunctival epithelia consisted of 2 to 3 layers, subconjunctival collagen fiber was densely arranged and a slight amount of fibroblasts was found.

In the MMC-treated animals, the filtration structures were similar to those treated with Ad-p27 on 3- and 7-d postoperatively. On day 14, evident filtration cavity could be observed, unobstructed filtration path, 1 to 3 layers of conjunctival cells. The epithelial cells were discontinuously arranged. The quantity of subconjunctival fibroblasts was small, collegen fiber was loosely distributed and a slight amount of infiltrated inflammatory cells was noted. At day 28 postoperatively, partial filtration path was blocked, conjunctival epithelia consisted of 1 to 3 layers, discontinuous conjunctival epithelia were seen. Subconjunctival collagen fiber was relatively densely arranged, but the number of fibroblasts was relatively small (Figure 1F-J).

In the PBS group, filtration cavity could be observed in filtration sites on day 3, conjunctival epithelia were intact, a small amount of goblet cells were found. Subconjunctival connective tissues were sparsely arranged with relatively plentiful fibroblasts and collegen fiber, and capillary formation was seen. Relatively evident lymphocyte, monocyte and plas-

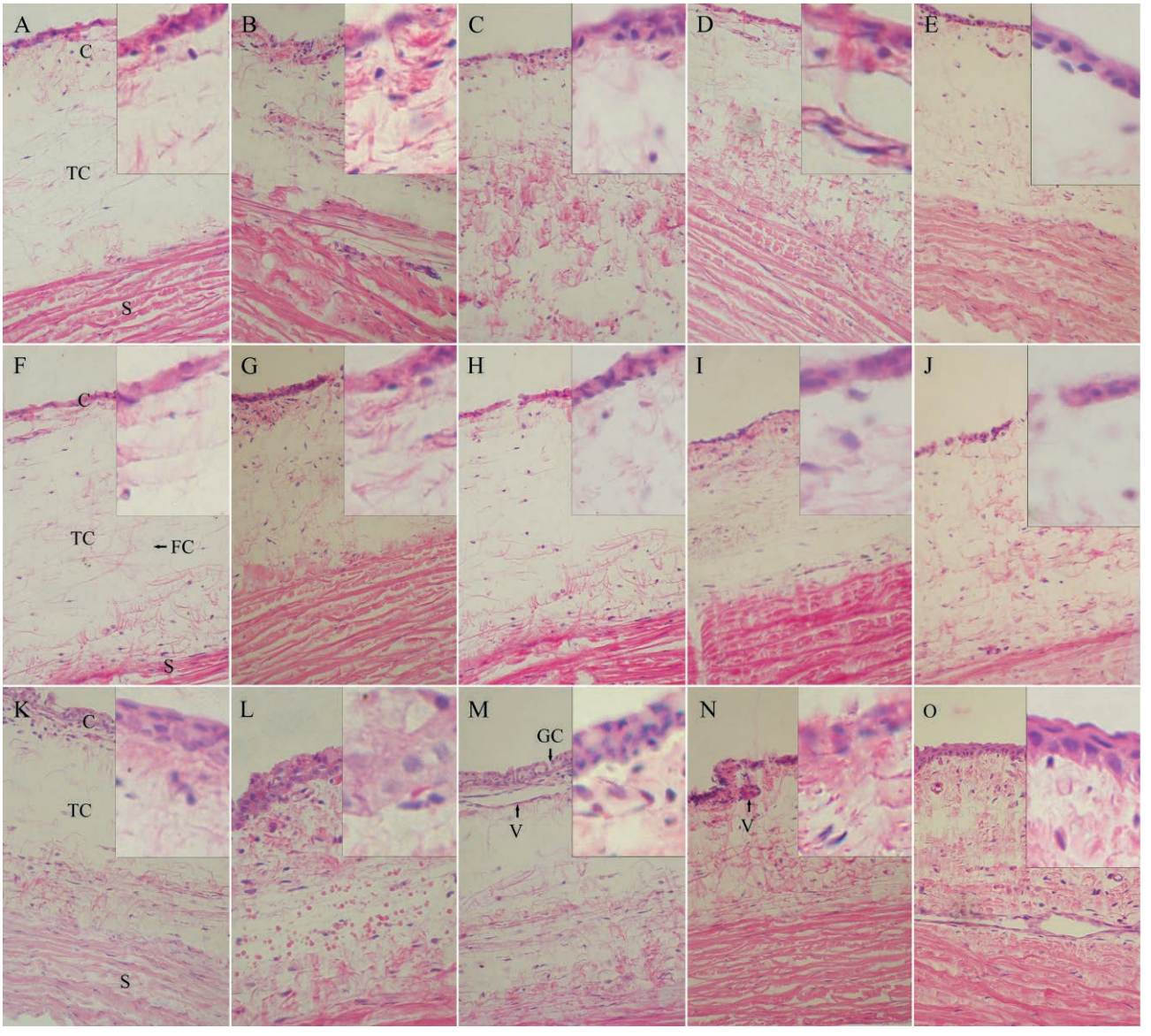

Figure 1 Postoperative histological features in conjunctiva and Tenon's capsule among three groups during a 28–day period

A–E: Histological alterations in conjunctiva and Tenon's capsule in the Ad–p27 group on days 3, 7, 14, 21 and 28. F–J: Histological changes in conjunctiva and Tenon's capsule in the MMC group on days 3, 7, 14, 21 and 28. K–O: Histological alterations in conjunctiva and Tenon's capsule in the PBS group on days 3, 7, 14, 21 and 28. Abbr. C: conjunctiva; TC: Tenon's capsule; S: sclera; GC: goblet cell. V: vessel; FC: filtrating cavity.(large panels, 100×; small panels, 400×).

mocyte infiltration were noted. On day 7, partial filtration path was unobstructed, conjunctival epithelia were made up of 3 to 6 layers, apparent subconjunctival inflammatory cell infiltration was observed, a large amount of capillaries formed and the quantity of inflammatory cells increased. At day 14, partial filtration cavity was found, conjunctival epithelia mainly had 3 to 4 layers, relatively plentiful goblet cells were noted, subconjunctival fibroblasts and fibrous tissue hyperplasia were frequently seen. However, the quantity of inflammatory cells and capillaries decreased. On day 21, filtration cavity disappeared, conjunctival epithelia had 3 to 4 layers, subconjunctival fibroblasts vigorously proliferated and

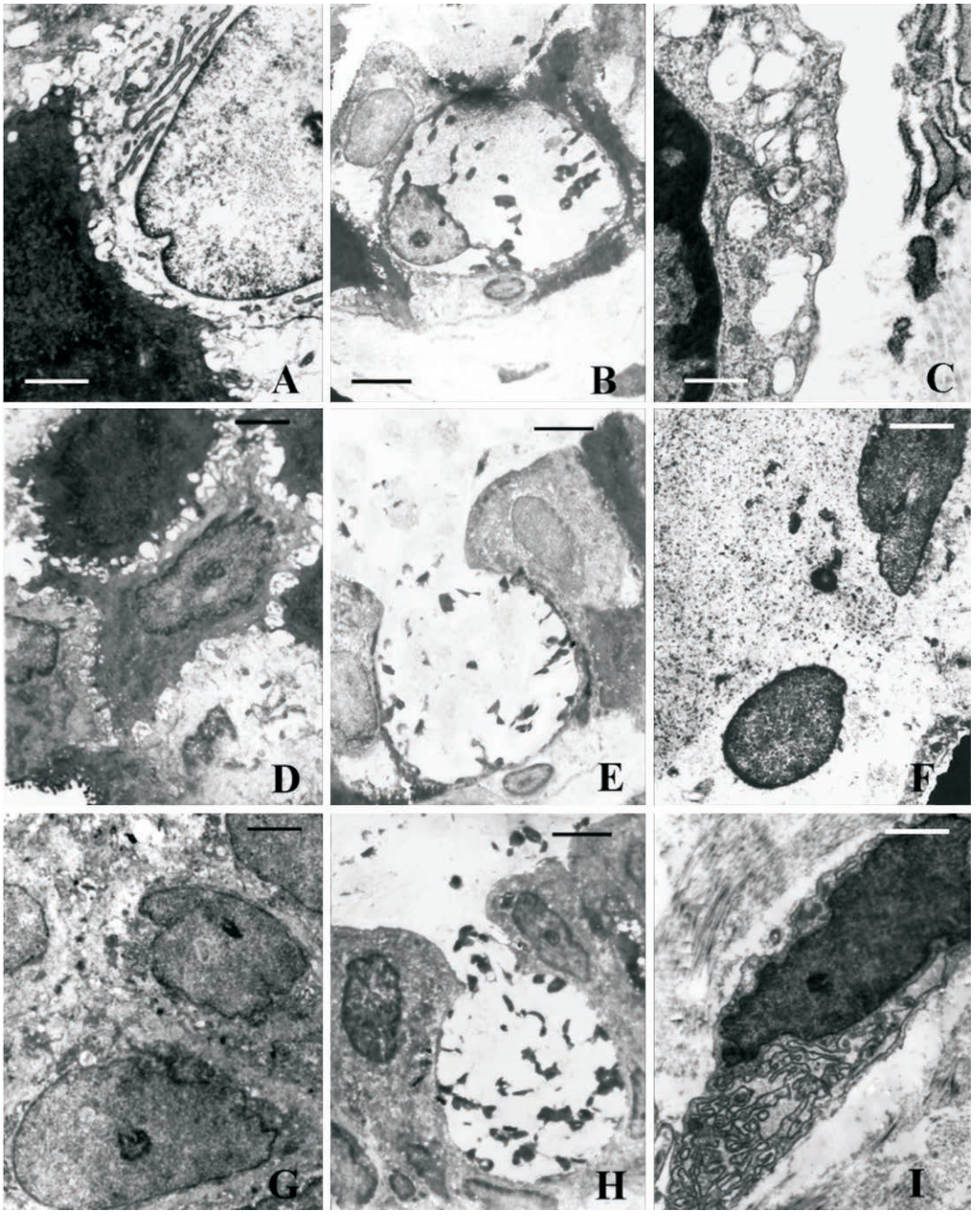

Figure 2 Postoperative ultrastructural observations at surgical sites among the three groups on day 28

A–C: Ultrastructural features in conjunctival epithelial cells, goblet cells and fibroblasts in the Ad-p27 group, respectively. D–F: Ultrastructural features in conjunctival epithelial cells, goblet cells and fibroblasts in the MMC group, respectively. G–I: Ultrastructural features in conjunctival epithelial cells, goblet cells and fibroblasts in the PBS group, respectively. Scale bar: A, D, F, G and I=1 μm; B, E and H=2 μm; C=0.5 μm.

thickened and dense collagen connective tissues were blocked and arranged in disorder. At 28 d postoperatively, conjunctival epithelia consisted of 3 to 4 layers, compact filtering blebs were observed, filtration cavity disappeared, completely displaced by fibroblasts and collagen connective tissues (Figure 1K-O).

Ultrastructural observations at surgical sites

In the Ad-p27 group, most conjunctival epithelia kept in a relatively static state and the amount of microvillus decreased; the number of organelle shrank and were damaged, mitochondrion presented with vacuolization and swollen RERs were found; perinuclear space was partially widened and nuclear membrane was rough. Conjunctival goblet cells secreted less amount of mucin. Fibroblasts had small nucleus, relatively dark-stained nucleolus, few nuclear membrane bulges and swollen cytoplasmic RERs were noted. Various sizes of cavities were noted in partial fibroblast cytoplasm, mitochondrion showed vacuolization alterations with a slight quantity of nucleoprotein bodies. Partial fibroblast apoptosis was noted (Figure 2A-C).

In the MMC group, a majority of epithelial cells were located in a relative static state, dark-stained nucleus and nucleous pycnosis, shrunk cytoplasm volume, organelle were reduced and disrupted; additionally, swollen fibroblasts were also found to have more heterochromatin, margination, enlarged perinucleus space, karyopyknosis and cytoplasm showed evidence of vacuolization (Figure 2 D-F).

In the PBS-treated group, a substantial amount of microvillus was found on the surface of conjunctival epithelia with oval nucleus and slight tortuosity in nuclear membrane. Plentiful mitochondrion, RER were seen to present with vigorous synthesis. A large quantity of desmosome was noted among cells. Abundant goblet cells and organelle were seen in conjunctiva and a substantial amount of mucin droplet was observed. Fibroblasts had large nucleus, less bulges on nuclear membrane, large densities of autosome, more cytoplasm, less signs of vacuolization and plentiful RER. Tenon's capsule was filled with protein substances. Plentiful Golgi body vesicles were noted to have a high level of secretion. Many polyribosomes while few lysosomes were found in cytoplasm (Figure 2 G-I).

Discussion

p27^{Kipl} is a key factor regulating cell cycle and its major function is to inhibit the cell division, referred as an independent prognostic factor and a potential therapeutic target of cancer[5,7]. p27^{Kipl} can suppress the activity of almost all the cyclin-CDK complexes via binding to these cyclin-CDK complexes during G1 phase, including cyclin A-CDK2, cyclin E-CDK2 and cyclin D-CDK4/6, etc. A stable triple-polymer complex was formed, which inhibited the activity of cyclin-CDK complexes and stop G1/S transition, eventually slowing down the cell cycle progression[6,8]. According to the studies related to p27^{Kipl} gene features, we found that p27^{Kipl}, as a target gene, is applicable in inhibiting cell proliferation. Exogenous p27^{Kipl} delivery can significantly lower IOP in rabbits following GSF and prevent the formation of filtration path scars[3]. After p27^{Kipl} treatment, epithelial cells were continuously arranged while filtering bleb fistula was not seen. MMC-treated animals did not have filtering bleb fistula. However, morphological findings revealed thin and discontinued layers of epithelial cells, as an evidence of the possible emergence of filtering bleb fistula in the future. It is safe to apply adenovirus-mediated p27^{Kipl} in rabbit filtration surgery without the incidence of common complications, such as thinning of filtering bleb and fistula.

On day 7 after GSF, scleratic limbus and subconjunctival fibroblasts achieved peak proliferation rate and entered active proliferation phase on day 11. The conjunctival epithelia vigorously proliferated and cell functions worked well accompanied by a substantial amount of goblet cells with active synthesis. The proliferation of fibroblasts returned to baseline level after day 14[9]. Thus, intervention and regulation during early stage of GFS, typically within 1 to 2 weeks, plays a vital role in constructing desirable filtration path and reducing the incidence of postoperative scarring.

Fibroblast is a type of effector cells of scar formation and dominates the postoperative scarring post GSF. The key underlying the formation of functional filtering bleb is to suppress the DNA replication of fibroblasts during the proliferation stage of filtering

bleb, block the division and proliferation of fibroblasts and reduce the synthesis of collagen fibers to alleviate or suppress the scar formation[10]. Following $p27^{Kip1}$ treatment, the quantity of fibroblasts and collagen fibers sharply decreased and fibroblast apoptosis was noted, suggesting that exogenous $p27^{Kip1}$ is able to prevent scar growth by inhibiting the proliferation of fibroblasts and suppressing the synthesis of novel collegen fibers, finally forming good filtering blebs. Over-expression of $p27^{Kip1}$ leads to fibroblast apoptosis, which is a main pattern for cell regression during wound healing process and also plays an extremely important role in wound healing and tissue remodeling.

Repairing conjunctival injuries is a dynamic and complicated process. After Ad-p27 delivery, conjunctival layer was thin, the number of resting epithelia increased and intercellular junction was expanded with wider cellular space. During inflammation period, platelets released less chemokines such as TGF-β and PDGF, etc. The proliferation of angiogenesis was inapparent. The ability of attracting neutrophil, monocyte and lymphocyte into wound sites was attenuated. The amount of inflammatory cells decreased at surgical sites. The secretion of cytokines, interferons and various growth factors, which functioned to stimulate cell proliferation and migration, was inhibited, thus, alleviating the effect of postoperative inflammatory reactions upon filtering blebs[11].

Conjunctival goblet cell is single cell mucous gland, located at conjunctival epithelial layer. The main functions of goblet cell are to synthesize, store and secrete mucin. Mucin is a major component of the innermost layer of tear film and plays a vital role in maintaining the ocular surface function. Changes in the quantity and secretion of goblet cells induced by a variety of endogenous and exogenous factors can cause dry eye and blurred vision, etc[12]. After the basilar epithelial cells gradually migrated to surface to secrete mucin during the development of goblet cells, some cells could re-started the synthesis of mucus after resting, and other cells could secrete mucin particles in the form of apocrine secretion, diminished in size and exfoliated from conjunctival epithelial layer[13]. Ad-p27 treatment reduced the number of conjunctival goblet cells, enhanced the quantity of surrounding conjunctival resting epithelial cells, enlarged cavities and decreased the secretion of mucin. Long-term observations probably indicated the tendency of dry eyes.

Exogenous $p27^{Kip1}$ delivery affects conjunctiva and Tenon's capsule in rabbit eyes after GFS and exerts a persistent and significant effect on inhibiting scar formation by suppressing cell cycle, which provides a novel adjuvant therapy post GFS.

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