Introduction of the *MDM2* T309G Mutation in Primary Human Retinal Epithelial Cells Enhances Experimental Proliferative Vitreoretinopathy

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PURPOSE. The murine double minute (MDM)2 is a critical negative regulator of the p53 tumor suppressor, and *MDM2* SNP309G is associated with a higher risk of proliferative vitreoretinopathy (PVR); in addition, the *MDM2* T309G created using clustered regularly interspaced short palindromic repeats (CRISPR)/associated endonuclease (Cas)9 enhances normal rabbit vitreous-induced expression of MDM2 and survival of primary human retinal pigment epithelial (hRPE) cells in vitro. The goal of this study was to determine whether this *MDM2* T309G contributes to the development of experimental PVR.

METHODS. hRPE cells expressing *MDM2* T309G or T309T only were treated with vitreous from human PVR donors (HV). The expression of MDM2 and p53 in the treated cells was examined by Western blot. The in vitro vitreous-induced cellular responses, such as contraction were assessed, and PVR was induced by intravitreal injection of the hRPE cells with *MDM2* T309G or T309T only into rabbit eyes.

RESULTS. Western blot analyses indicated that treatment of hRPE cells with HV led to a significant increase (1.7 ± 0.2-fold) in the expression of MDM2 and a significant decrease in p53 in the cells expressing *MDM2* T309G compared with those with *MDM2* T309T. In addition, HV promoted contraction of the hRPE cells expressing *MDM2* T309G significantly more than those with *MDM2* T309T only. Furthermore, *MDM2* T309G in the hRPE cells enhanced the development of PVR in a rabbit model.

CONCLUSIONS. The *MDM2* SNP309 in RPE cells enhances their potential of PVR pathogenesis.

Keywords: *MDM2*, CRISPR/Cas9, SNP309, RPE, PVR

The major cause of failure in surgery to correct rhegmatogenous retinal detachment (RRD) is proliferative vitreoretinopathy (PVR),[1–3] which is characterized with formation of fibrotic-cellular epiretinal membranes (ERMs) composed of extracellular matrix proteins and cells, including RPE cells, retinal glial cells, fibroblasts, and macrophages. Indeed, RPE and glial cells in these vitreal membranes appear to play an especially critical role in the pathogenesis of PVR.[4]

PVR occurs in 5% to 10% of patients who undergo the surgical repair of RRD, and accounts for approximately 75% of all primary failures after the surgery.[1,5–10] Currently, repeat surgery is the only option for PVR[2,3]; however, the visual outcome of the surgery is poor because of the retinal damage resulting from recurrent detachment and the PVR process itself.[11] Adjuvant pharmacotherapies that have been evaluated thus far have neither blocked the formation of PVR nor reduced the rates of retinal redetachment secondary to PVR consistently.[11] Incomplete understanding of the PVR pathogenesis is a major obstacle for developing new therapeutic options for individuals who are afflicted by this potentially blinding disease.

The oncogenic protein murine double minute 2 (MDM2), an E3 ubiquitin-protein ligase, with a human homologue called Hdm2, is an important negative regulator of the p53 tumor suppressor.[12–14] We have previously shown that vitreous from experimental rabbits or PVR patients can trigger the signaling pathway of phosphoinositide 3 kinase (PI3K)/Akt, which phosphorylates MDM2. The phosphorylated MDM2 enhances p53 degradation[15]; in addition, blocking MDM2 binding to p53 with a small molecule nutlin-3 protects rabbits from retinal detachment in a PVR rabbit model.[5]

Importantly, the G allele of single nucleotide polymorphisms (SNPs) (rs2279744) in the *MDM2* intron promoter locus between exons 1 and 2 attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans.[16] Intriguingly, this SNP is also associated with a higher risk of

PVR for RRD patients,[1] but whether this *MDM2* G309 SNP contributes to the development of PVR is still unknown.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (Cas) in bacteria and archaea form their adaptive immune system, in which CRISPR RNAs (crRNAs) can guide the Cas to cleave the foreign nucleic acids.[17–19] In *Streptococcus pyogenes* (Sp) there are two nuclease domains in the SpCas9, RuvC and HNH, each of which can cleave one strand of the double-stranded target DNA when directed by the crRNA and trans-activating crRNA (tracrRNA).[19,20] Importantly, this SpCas9 can be engineered to target specific genomic loci in mammalian cells together with the processed single guide RNAs (sgRNAs), which consist of the crRNA and tracrRNA, at a prior protospacer adjacent motif.[19,21,22] With this CRISPR/Cas9 technology, we have created the *MDM2* T309G in the genomic DNA of human primary RPE (hRPE) cells and found that the *MDM2* T309G mutation enhances rabbit vitreous (RV)-induced expression of MDM2 and cell proliferation,[21] but whether this mutation contributes to the pathogenesis of PVR is still unclear. The goal of the studies presented in this article aims to resolve this question.

MATERIALS AND METHODS

Major Reagents and Cell Culture

The antibodies against p53 and MDM2 were purchased from Cell Signaling Technology (Danvers, MA, USA) and from Abgent (San Diego, CA, USA), respectively. The primary antibody against β-actin and the secondary antibodies of the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescent substrate for detection of HRP was from Thermo Scientific (Waltham, MA, USA).

hRPE cells were purchased from Lonza (Walkersville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 medium (F12) (Thermo Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The hRPE cells with *MDM2* T309G or T309T only were created using a system of AAV-SpCas9 D10A and AAV-SpGuide (MDM2 or lacZ) and a homology direct recombinant DNA template with *MDM2* G309 as described previously.[21] All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Quantitative PCR

The hRPE cells with *MDM2* T309G were plated into six-well plates at a density of 1×10^5 cells per well in DMEM/F12 supplemented with 10% FBS overnight and then serum-starved for 24 hours. Subsequently, the cells were treated with or without vitreous from patients with PVR (HV) (diluted 1:3 in DMEM/F12) for 0.5, 2, 4, and 16 hours. Then total RNA was respectively isolated with a RNeasy mini kit (Qiagen, Germantown, MD, USA), and cDNA was synthesized with an ISCRIPT cDNA synthesis kit (BioRAD, Hercules, CA, USA) in a program (25°C, 5 minutes; 42°C, 30 minutes; 85°C, 5 minutes; 4°C, forever). The cDNA was subjected to quantitative PCR using a Fast Start universal SYBR green Master mix (Roche, Basel, Switzerland) in a Light Cycler 480 II machine (Roche). Primers of quantitative PCR synthesized by Integrated DNA Technology (Coralville, IA, USA) were (forward: 5′-AGAAGGACAAGAACTCTCAGATG-3′, reverse: 5′-GTGCATTTCCAATAGTCAGCTAA-3′) for *MDM2* and (forward:

5′-CCTGGCGTCGTGATTAGTGAT-3′, reverse: 5′-AGACGTT CAGTCCTGTCCATAA-3′) for a housekeeping gene hHPRT1.

Western Blot

hRPE cells with *MDM2* T309G or T309T cultured to 90% confluence in 24-well plates were switched to serum-free medium for 24 hours and then were treated with or without HV (diluted 1:3 in DMEM/F12) for 16 hours. After rinsing twice with ice-cold PBS, cells were lysed in $1 \times$ sample buffer, which was diluted with extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/ mL aprotinin, 2 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride) from the $5 \times$ protein sample buffer (25 mM EDTA, pH 7.0, 10% SDS, 500 mM dithiothreitol, 50% sucrose, 500 mM Tris-HCl, pH 6.8, and 0.5% bromophenol blue). The samples were heated at 95°C for 5 minutes and then centrifuged for 5 minutes at 13,000g. Proteins in the samples were then separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to Western blot analysis. Signal intensity was determined by densitometry using NIH Image J (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).[21,22]

Cell Proliferation Assay

The hRPE cells with *MDM2* T309G or T309T were plated into 24-well plates at a density of 3.0×10^5 cells per well in DMEM/ F12 supplemented with 10% FBS. After the cells had attached to the plates, the medium was replaced with DMEM/F12 supplemented with HV (1:3 dilution in DMEM/F12). Three days later, the cells were counted in a hemocytometer; at least three independent experiments were performed.[21,23]

Apoptosis Assay

The hRPE cells with *MDM2* T309G or T309T were seeded into 6-cm dishes (1×10^5 cells/dish) in DMEM/F12 with 10% FBS. After cell attachment, the medium was replaced with DMEM/ F12 supplemented with HV (1:3 dilution in DMEM/F12). On day 3, the cells were harvested and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) following the instructions provided with the apoptosis kit (BD Biosciences, Palo Alto, CA, USA). The cells were analyzed using fluorescence-activated cell sorting (FACS) in a Coulter Beckman XL instrument. At least three independent experiments were performed.[21,23]

Collagen Contraction Assay

The hRPE cells with *MDM2* T309G or T309T only were resuspended in 1.5 mg/mL of neutralized collagen I (INAMED, Fremont, CA, USA) (pH 7.2) on ice at a density (1×10^6 cells/ mL).[24,25] The mixture was transferred into wells of 24-well plates that had been preincubated overnight with 5 mg/mL BSA/PBS. Ninety minutes later at 37°C, 0.5 mL DMEM/F12 or HV (1:3 dilution in DMEM/F12) was added. On day 3 after being photographed, the gel diameter was measured, and the gel area was calculated using a formula $3.14 \times r^2$, where r is the radius of the gel. At least three independent experiments were performed.[25–27]

PVR Patient Vitreous

As previously described,[25,27] human vitreous from patients with PVR (HV) (1.0 to 1.5 mL) was obtained during pars plana vitrectomy before initiating the pars plana infusion at the
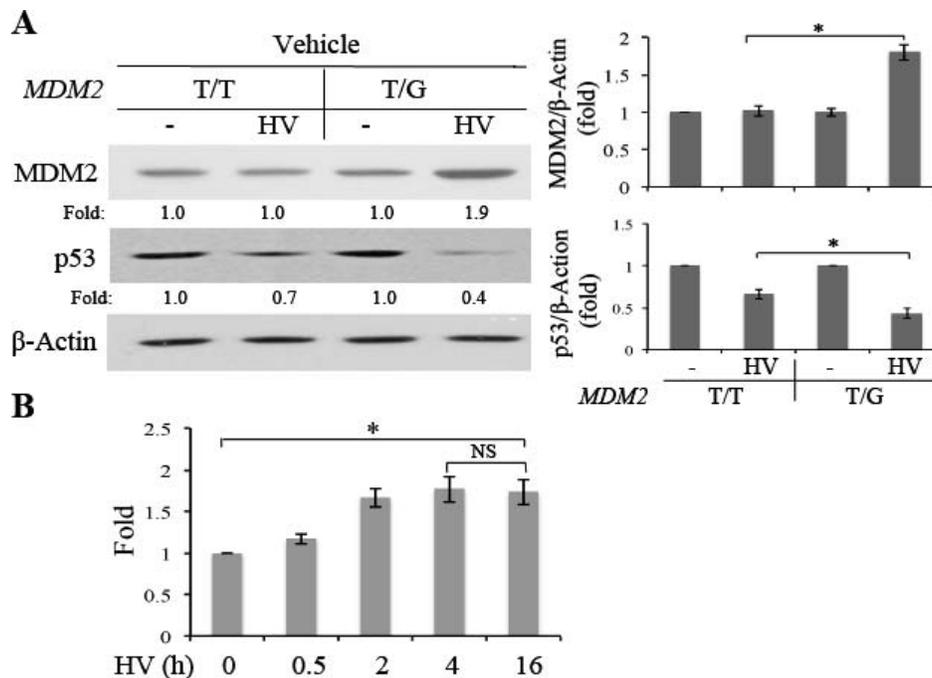
FIGURE 1. Patient PVR vitreous enhanced expression of MDM2 in the hRPE cells with *MDM2* T309G. (A) hRPE cells with *MDM2* T309 only (T/T) or *MDM2* T309 plus G309 (T/G) were serum-deprived 24 hours and treated with or without patient PVR vitreous (HV) for 16 hours, and the cell lysates were subjected to Western blot analysis with indicted antibodies. "Fold" was calculated by first normalizing to the level of β-actin and then calculating the ratio of the stimulated over the basal (i.e., unstimulated). The data were from three independent experiments, and the *asterisk* indicates significant difference between the two compared groups. (B) The hRPE cells with *MDM2* T309G were serum-starved for 24 hours and then treated with or without HV for 0.5, 2, 4, and 16 hours. Subsequently, the total RNA was isolated and the synthesized cDNA was subjected to quantitative PCR for HV-induced expression of *MDM2* mRNA. "Fold" for expression of *MDM2* mRNA was calculated by first normalizing to the level of a housekeeping gene hHPRT1 and then calculating the ratio of the stimulated over the basal (i.e., unstimulated). The data normalized by a housekeeping gene were from three independent experiments. The *asterisk* indicates significant difference between the two compared groups, and NS denotes no significant difference.

Vancouver Hospital. An ethical approval was obtained before the initiation of this project from the Vancouver Hospital and University of British Columbia Clinical Research Ethics Board and from Nova Scotia Health Research Ethics Board, Dalhousie University. The University of British Columbia Clinical Research Ethics Board and the Nova Scotia Health Research Ethics Board policies comply with the Tri Council Policy and the Good Clinical Practice Guidelines, which have their origins in the ethical principles in the Declaration of Helsinki. Written informed consent was obtained from patients.

Experimental PVR in Rabbits

As described previously,[26-28] PVR was induced in right eyes of Dutch Belted rabbits (6 months old, 2–3 kg, male and female) (Covance, Denver, PA, USA). Briefly, a gas vitrectomy was performed by injecting 0.1 mL perfluoropropane (C_3F_8) (Alcon, Fort Worth, TX, USA) into the vitreous cavity 4 mm posterior to the corneal limbus. One week later, all rabbits were injected with 0.1 mL of platelet-rich plasma (PRP) from rabbits and 0.1 mL DMEM/F12 containing either 3.0×10^5 cells of hRPE cells with *MDM2* T309G or T309T only (10 rabbits per group) under an operative microscope. The retinal status was examined with an indirect ophthalmoscope through a +30 diopter (D) fundus lens on days 1, 3, 5, 7, 14, 21, and 28 by two ophthalmologists without knowing the injected cell types. PVR was graded using a standard of the Fastenberg classification from 0 through 5.[27,29] On day 28, the rabbits were killed. All surgeries adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and a protocol for

the use of animals was approved by the Schepens Animal Care and Use Committee (Boston, MA, USA).

Statistics

The data were analyzed using an unpaired *t*-test or a Mann-Whitney test. A power (*P*) value less than 0.05 was considered statistically significant.

RESULTS

HV-Induced Expression of MDM2 in the hRPE Cells With *MDM2* T309G

MDM2 SNP309 is associated with a higher risk of PVR[1] and the CRISPR/Cas9-created *MDM2* T309G in hRPE cells enhanced RV-induced expression of MDM2 and a reduction in p53.[21] Thus, we investigated whether this mutation in hRPE cells rendered the cells responsive to HV from patients with PVR. hRPE cells containing *MDM2* T309G or T309T were treated with HV for 16 hours, and the cell lysates were subjected to Western blot analyses. As shown in Figure 1, HV treatment led to a 1.7 ± 0.2-fold increase in the protein expression and a 1.73 ± 0.15-fold increase in the mRNA of *MDM2* in the hRPE cells with *MDM2* T309G, compared with the cells with *MDM2* T309T only. In addition, HV treatment suppressed expression of p53 by 1.75-fold more in the hRPE cells with *MDM2* T309G than in the cells with *MDM2* T309T only.
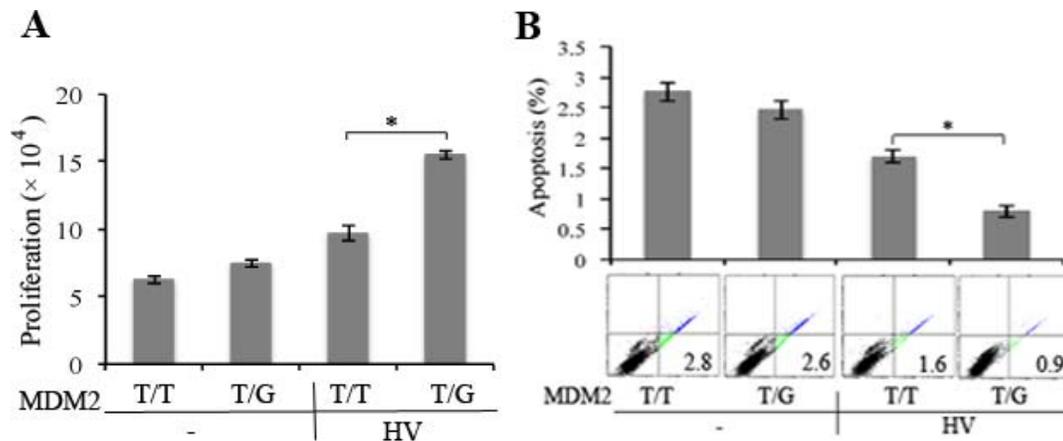
FIGURE 2. HV enhanced proliferation and survival of hRPE cells with *MDM2* T309G compared with those with *MDM2* T309T only. (**A**) The hRPE cells with *MDM2* T309 only (T/T) or *MDM2* T309 plus G309 (T/G) were plated into a 24-well plate at a density of 3×10^4 cells per well. After the cells had attached the plates, the medium was switched to either DMEM/F12 or HV (diluted 1:3 in DMEM/F12). The media were replaced every day. On day 3, cells were counted with a hemocytometer under a light microscope. Mean ± SD of three independent experiments is shown. *$P < 0.05$, unpaired *t*-test. (**B**) Serum-starved hRPE cells with *MDM2* T309G or 309T only were plated into 60-mm dishes at a density of 100,000 cells per dish. After the cells attached to the dishes, the medium was switched to either DMEM/F12 or HV (diluted to 1:3 in DMEM/F12). The media were replaced every day. On day 3, the cells were stained with FITC-conjugated annexin V and PI in an apoptosis assay kit by following the manufacturer's instructions. Cells that were stained with annexin V and/or PI were detected and quantified by flow cytometry in a Beckman Coulter (Brea, CA, USA) XL instrument. The mean ± SD of three independent experiments is shown, and one of the experimental raw data is shown *below the bar graphs*.

MDM2 T309G Enhanced HV-Induced Cell Proliferation and Survival Against Apoptosis

We speculated that an increase in MDM2 and decrease in p53 might enhance cell proliferation and survival.[15,16] Thus, we treated the hRPE cells expressing *MDM2* T309G or T309T only with HV and assessed their proliferation and survival. As shown in Figure 2A, whereas HV-induced proliferation of hRPE cells with *MDM2* T309T only, the proliferative response was greater in hRPE cells with *MDM2* T309G than those with *MDM2* T309T. Moreover, the two types of hRPE cells were starved for serum for 3 days to induce apoptosis, and the cells were treated with or without HV, and the apoptosis was analyzed using a FITC-conjugated annexin V-based apoptosis kit by FACS. The results (Fig. 2B) showed that HV promoted survival of hRPE cells with *MDM2* T309G better than those with *MDM2* T309T only.

MDM2 T309G Promoted HV-Induced Contraction of hRPE Cells and Enhanced Experimental PVR

One of the major characteristics of PVR is the formation of epiretinal membranes,[27] which can contract and cause the retinal redetachment. Thus, we assessed the possible contribution of *MDM2* T309G to the development of PVR. In a contraction assay, cells were mixed with collagen to mimic the conditions of an epiretinal membrane. As shown in Figure 3, hRPE cells expressing *MDM2* T309G contracted more in response to HV than cells with *MDM2* T309T only, indicating that *MDM2* T309G in the hRPE cells might contribute to the pathogenesis of PVR.

Thus, we investigated whether the *MDM2* T309G polymorphism influenced the pathogenesis of PVR. hRPE cells expressing *MDM2* T309T only or the cells with *MDM2* T309G were injected intravitreally into rabbit eyes. On the seventh day after cell injection, the cells with *MDM2* T309G had begun to form fibrotic membranes, whereas all those with *MDM2* T309T remained as cell aggregates in the vitreous (Fig. 4). Observation on weeks 2, 3, and 4 indicated significant differences in the development of PVR in a rabbit model

between the two groups of hRPE cells with *MDM2* T309T only and those with *MDM2* T309G based on the standard of the Fastenberg classification from 0 through 5,[27,29] as shown in Figure 4A. On week 4, for instance, the retina detached in 3 of 10 rabbits that were intravitreally injected with the *MDM2* T309G hRPE cells, but none of the 10 rabbits containing *MDM2* T309T only developed retinal detachment. Histologic examination indicated that there were more cells migrating into the retinas in the vitreous injected with hRPE cells expressing *MDM2* T309G than those expressing *MDM2* T309T (Fig. 4B). Taken together, these experiments demonstrated that *MDM2* T309G polymorphism in the hRPE cells contributes to the pathogenesis of experimental PVR.
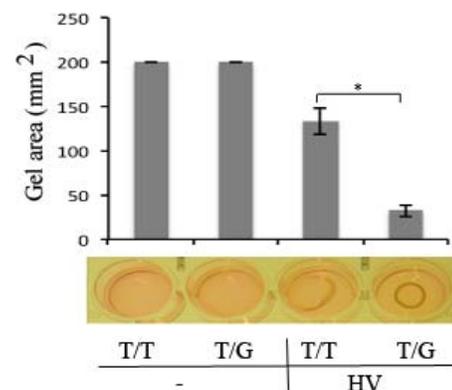

FIGURE 3. HV promoted more contraction of hRPE cells with *MDM2* T309G than those with *MDM2* T309T only. The hRPE cells with *MDM2* T309G or T309T only were resuspended in 1.5 mg/mL of neutralized collagen (pH 7.2) at a density of 1×10^6 cells/mL and seeded into wells of a 24-well plate that had been preincubated overnight with 5 mg/mL BSA/PBS. The mixture of the collagen and cells was incubated at 37°C for 90 minutes. The collagen gels were overlaid with DMEM/F12 alone (−) or HV. On day 3, the gel diameter was measured and calculated using the formula $(3.14 \times [diameter/2]^2)$. The mean ± SD of the three independent experiments is shown. *Denotes $P < 0.05$ using an unpaired *t*-test. A photograph of the representative experiment is shown at the bottom of the bar graphs.
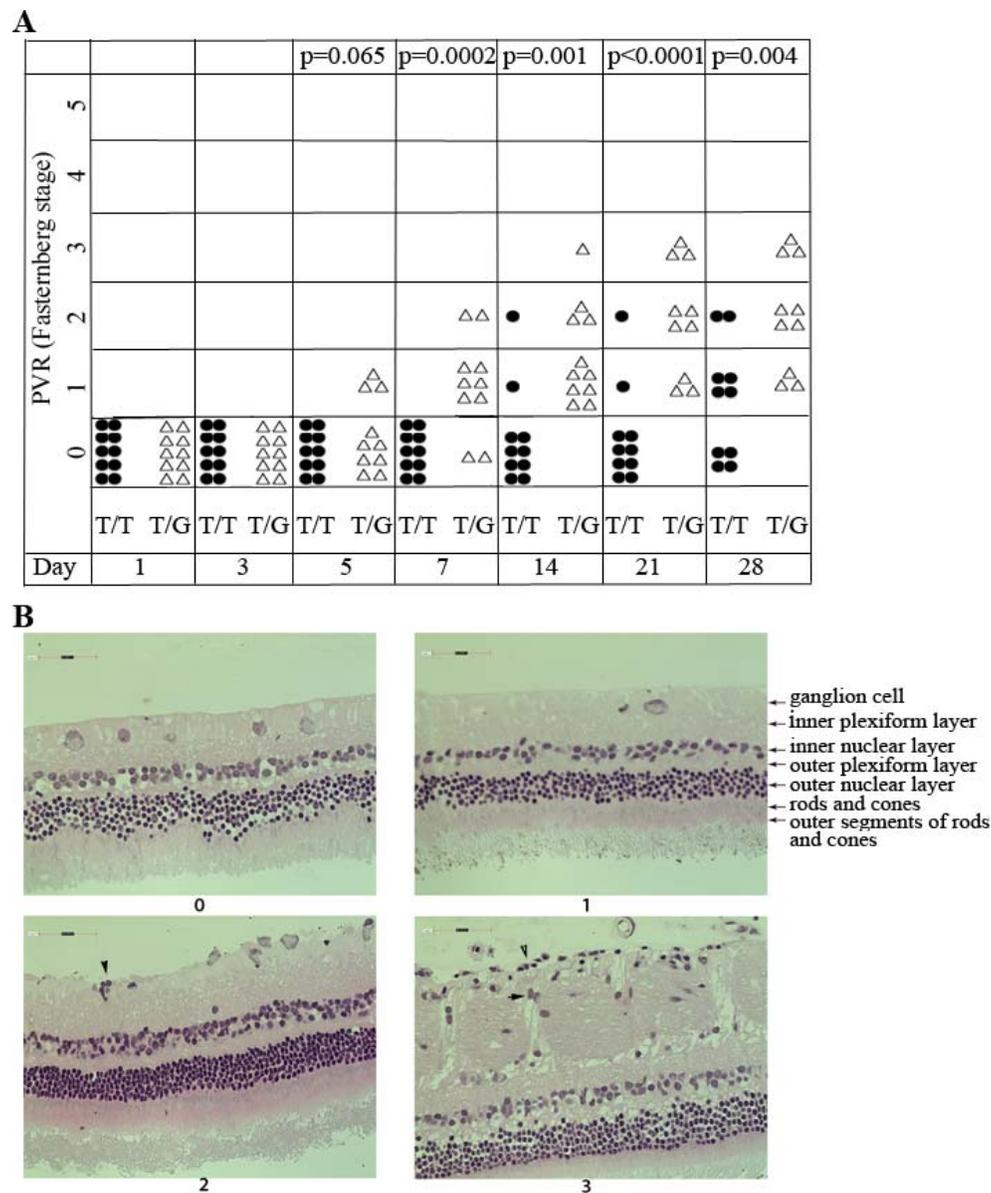
FIGURE 4. The hRPE cells with *MDM2* T309G had more potential to induce PVR in a rabbit model. (**A**) PVR was induced in the right eyes of Dutch Belted rabbits (10 rabbits per group) as previously described.[25,27,28] Briefly, 1 week after gas vitrectomy, rabbits were intravitreally injected with 0.1 ml PRP and hRPE cells (3×10^5) with *MDM2* T309 only (T/T) or *MDM2* T309 plus G309 (T/G) in 0.1 mL DMEM/F12. The rabbits were examined with an indirect ophthalmoscope and the PVR status for each rabbit was plotted on days 1, 3, 5, 7, 14, 21, and 28. The data were subjected to Mann-Whitney analysis. A *P* value less than 0.05 is considered a significant difference between the two groups (T/T and T/G). (**B**) The representative eyeballs from rabbits with PVR stages 0 and 1 (hRPE T309T), and 2 and 3 (hRPE T309G) were fixed, sectioned and stained with hematoxylin-eosin. In the eyes with PVR stages 2 and 3 injected with hRPE expressing *MDM2* T309G, there were some cells (*arrowheads*) attaching to the retina.

Discussion

MDM2 T309G, which was generated in the hRPE cells using the CRISPR/Cas9,[21] led to enhancement of HV-stimulated expression of MDM2 and cell proliferation, survival, and contraction in response to HV, as well as development of PVR in a rabbit model. Although the cells used in the in vivo PVR model were a mixed population of *MDM2* genotypes (20% T309T, 70% T309G, and 10% G309G),[21] they demonstrated more significant membrane formation than those expressing only *MDM2* T309T.

We chose rabbits for the experimental PVR because of the ease of working in these animals' eyes. The smaller size of the rabbit lens compared with the eyeball permits manipulations to be performed within the eye, without causing any damage to the lens/retina. Rabbits lack a distinct macula; however, the medullary wing detachment simulates a retinal detachment in humans and shows PVR-like features.[2] Retinas from rabbits are only partially vascularized, in contrast to those in humans, which are completely vascularized. However, the use of other common laboratory animals such as rats and mice, which have higher retinal vascularization, can be difficult to work with due to the difficulties in isolating and purifying the small volume of the vitreous. Indeed, the mouse lens occupies nearly 75% of the eye.[30] It is also important to note that the cell injection into rabbit eyes to induce experimental PVR does not involve any break in the retina. Indeed, RRD models leading to the

development of PVR[31] may be considered in future studies in an effort to more closely resemble the clinical condition.

Vitreous can induce the transcription factor specific protein (Sp)1[32,33] to bind the intron promoter of *MDM2* in the hRPE cells with *MDM* T309G and thus increase its expression,[21] resulting in a reduction in p53, and enhanced cell proliferation, viability, and contraction, leading to an increased potential of PVR. These findings might be relevant to other eye diseases, such as proliferative diabetic retinopathy (PDR), in which there are similar fibrotic-cellular membranes. Thus, it will be intriguing to determine whether this *MDM2* T309G polymorphism also contributes to the development of PDR.

Both normal RV[21] and HV (Fig. 1) induce expression of MDM2 as well as proliferation and survival of hRPE cells. Vitreous contains numerous growth factors and cytokines, including epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor (TGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), connective tissue growth factor and interleukins (IL)-6 and -8, which are implicated in PVR.[34] Some growth factors have been reported to be increased during the development of PVR, such as platelet-derived growth factor (PDGF)[35] and TGF-β[8] and these vitreal growth factors and cytokines may be essential drivers of the PVR pathogenesis.[34] Moreover, the exposure of cells, such as RPE cells, to vitreous containing rich growth factors and cytokines may stimulate them to secrete growth factors and/or cytokines, increasing the local concentrations of these agents.[34] A cocktail of agents neutralizing EGF, TGF-α, FGF-2, IL-8, TGF-βs, HGF, IGF-1, and PDGFs blocks experimental PVR induced by fibroblasts,[36] but whether the same approach would prevent PVR induced by RPE cells that harbor the *MDM2* 309SNP is unknown.

Adeno-associated viruses (AAVs) are small viruses that are not at present known to cause any disease, and thus their derived vectors have great potential for human gene therapy.[37,38] The eye is an especially ideal target organ for gene therapy, as it is easily accessible and immune privileged.[39] In this study, we showed that the AAV-CRISPR/Cas9-created *MDM2* T309G contributed to the development of experimental PVR in a rabbit model, further demonstrating that this CRISPR/Cas9 system is a novel powerful tool for the targeted introduction of mutations into eukaryotic genomes for the generation of reproducible disease models.[40,41]

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