Phosphoinositide 3-kinase δ inactivation prevents vitreous-induced activation of AKT/MDM2/p53 and migration of retinal pigment epithelial cells

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that play a critical role in transmitting signals from cell-surface molecules to intracellular protein effectors. Key PI3Ks include PI3Kα, PI3Kβ, and PI3Kδ, which are regulated by receptors. The signaling pathway comprising the PI3Ks, along with a Ser/Thr kinase (AKT), a proto-oncogene product (mouse double minute (MDM)2), and a tumor suppressor protein (p53), plays an essential role in experimental proliferative vitreoretinopathy (PVR), which is a fibrotic blinding eye disorder. However, which PI3K isoforms are involved in PVR is unknown. A major characteristic of PVR is the formation of epi (or sub)-retinal membranes that consist of extracellular matrix and cells, including retinal pigment epithelium (RPE) cells, glial cells, and macrophages. RPE cells are considered key players in PVR pathogenesis. Using immunoblotting and immunofluorescence analyses, we herein provide the evidence that PI3Kδ is highly expressed in human RPEs when it is primarily expressed in leukocytes. We also found that PI3Kδ inactivation through two approaches, CRISPR/Cas9-mediated depletion and a PI3Kδ-specific inhibitor (idalisib), not only blocks vitreous-induced activation of AKT and MDM2 but also abrogates a vitreous-stimulated decrease in p53. Furthermore, we demonstrate that PI3Kδ inactivation prevents vitreous-induced proliferation, migration, and contraction of human RPEs. These results suggest that PI3Kδ may represent a potential therapeutic target for RPE-related eye diseases, including PVR.

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases, which phosphorylate the 3-hydroxy of the inositol ring of inositol lipids to generate phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-biphosphate, and phosphatidylinositol 3,4,5-trisphosphate at the inner leaflet of the plasma membrane (1, 2). Receptor-regulated PI3Ks include heterodimeric PI3Kα, PI3Kβ, and PI3Kδ (1, 2). Their regulatory subunit p85α contains Src homology 2 and 3 domains can bind to the phosphorylated tyrosine in the YYXM motif of receptor tyrosine kinases (e.g. platelet-derived growth factor receptor) so that their catalytic subunit (p110α, p110β, or p110δ) can phosphorylate phosphatidylinositol 4,5-bisphosphate to become phosphatidylinositol 3,4,5-trisphosphate, which in turn can be bound by pleckstrin homology domain-containing proteins such as AKT, an oncogene product also known as protein kinase B (1, 3–6). This binding facilitates AKT to be phosphorylated by phosphoinositide-dependent kinase 1 at threonine 308 and by the mammalian target of rapamycin complex 2 at serine 473 (1, 6, 7). Activation of AKT can stimulate multiple cellular processes such as cell survival, proliferation, growth, as well as migration (6, 8, 9). So PI3Ks play an essential role in transmitting signals from cell-surface molecules into the intracellular enzymes as well as in stimulating cellular responses (1, 2, 10). However, the contribution of most of the PI3K isoforms to organizational function and disease still remains obscure.

PI3Kα and PI3Kβ are ubiquitously expressed, and knockout of their catalytic subunit p110α or p110β is embryonic lethal, whereas mice without a catalytic subunit (p110δ) of PI3Kδ are viable (2, 10–12). In humans, high expression of PI3Kδ is seen in spleen and thymus (13). However, the expression pattern and function of PI3Kδ in retinal pigment epithelial cells (RPEs) have not been explored.

Deregulation of PI3Ks may initiate multiple diseases such as cancer and proliferative vitreoretinopathy (PVR) (14–16), a blinding eye disease with a major characteristic of epi (or sub)-retinal membrane; ERK, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; sgRNA, single-guide RNA; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated endonuclease 9.

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2 The abbreviations used are: PI3K, phosphoinositide 3-kinase; RCF, rabbit conjunctival fibroblast; HCF, human corneal fibroblast; PAEC, porcine aortic endothelial cell; ANOVA, analysis of variance; RV, rabbit vitreous; PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PVR, proliferative vitreoretinopathy; RPE, retinal pigment epithelium; PRPE, primary human fetal RPE; ERM, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; LTR, long-terminal repeat; FDA, Food and Drug Administration; MTU, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; sgRNA, single-guide RNA; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated endonuclease 9.
PI3KÎ± inactivation blocks AKT/MDM2/p53 in RPEs

**Results**

**PI3KÎ± is highly expressed in RPEs**

Previous studies have demonstrated that PI3Ks play a central role in experimental PVR (14, 16), but which PI3K isoform contributes to the pathogenesis of PVR is still a mystery. In addition, PI3KÎ± was originally reported to be restricted to hematopoietic cells (2, 23–25); however, surprisingly, Western blot analysis of the cell lysates showed that p110Î±, a catalytic isoform of PI3KÎ±, was highly expressed in cultured human RPEs, including ARPE-19, a spontaneously arising retinal pigment epithelial cell line, and primary human fetal RPE (PRPEs), whereas low levels of p110Î± expression were detected in other cell lines examined, including human corneal fibroblast (HCF), mouse cone cells (661W), a human retinoblastoma cell line (Y-79), rabbit conjunctival fibroblast (RCF), and porcine aortic vascular endothelial cells (PAECs) (Fig. 1A). In addition, p110Î± (Fig. 1, A and D) was expressed in all the examined cell lines at similar levels, and p110Î² was expressed approximately twice more and p110Î³ less in RPEs than those in 661W cells (Fig. 1, B, E, and F). Specifically, expression of p110Î± in RPEs was ~10 times higher than that in 661W cells and around 1-fold lower than that in a mouse macrophage RAW264.7 cell line, which served as a positive control (Fig. 1, C and G). Taken together, these results suggest that PI3KÎ± may play an essential role in vitreous-induced signaling events (e.g. AKT activation) in RPEs.

**Depletion of p110Î± with CRISPR/Cas9**

The technology of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) can be harnessed for depletion gene expression by genome editing (26–29). Next, we identified that one sgRNA (named PK2, AGAGCCGCTCATACTGGCCG) from exon 4 in the human genomic PIK3CD locus (Fig. S1A) was able to cleave the locus at its expected site (Fig. S1B), resulting in ~90% depletion of p110Î± in ARPE-19 cells analyzed by Western blotting (Fig. S1, C and D); in addition, p110Î± was hardly detectable by immunofluorescence in most of the ARPE-19 cells expressing PK2–sgRNA/Cas9 compared with those expressing lacZ–sgRNA/Cas9 (Fig. S1F). Furthermore, Western blotting and immunofluorescence analysis showed that this CRISPR/Cas9-mediated depletion of p110Î± did not have significant impact on expression of p110Î± (Fig. S1, C, E, and G), suggesting that this PK2–sgRNA is specific to PIK3CD, and the p110Î± antibody we used herein is specific to p110Î±.
PI3Kδ inactivation blocks AKT/MDM2/p53 in RPEs

Previously, we found that PI3K is critical for vitreous-mediated activation of AKT and MDM2 and decrease of p53 (16), and blockade of the interaction of MDM2 with p53 suppresses experimental PVR (30). Therefore, we investigated whether depletion of p110δ in ARPE-19 cells had any influence on the pathway of AKT/MDM2/p53. Compared with treatments with a single growth factor or cytokine or their combination, vitreous treatment is more relevant to an in vivo environment for PVR development (31–34). In addition, rabbits are usually used to induce PVR (15, 34–36) because the smaller size of the rabbit lens as compared with that of the eyeball permits manipulations to be performed within the eye, without causing any damage to the lens and retina; however, other common laboratory animals as such as rats and mice are difficult to work with because of the small volume of vitreous (20, 37, 38). Therefore, ARPE-19 cells were treated with normal vitreous from experimental rabbits (RV) for 2 h based on a previously established time course (16). As shown in Fig. 2, vitreous induced an increase (4.1 ± 0.3-fold) in phosphorylated AKT (Ser-473) and (3.3 ± 0.2-fold) in phosphorylated MDM2 (Ser-166), and an ~70% decrease in p53, but depletion of p110δ in these cells blocked these biochemical events, suggesting that PI3Kδ is a primary PI3K isoform mediating the vitreous-induced pathway of AKT/MDM2/p53.

Depletion of p110δ attenuates vitreous-induced activation of AKT and MDM2 and decrease of p53

Re-expression of p110δ restores vitreous-induced activation of AKT and MDM2 and decrease of p53

To exclude the off-target effects of CRISPR/Cas9, we re-expressed a mouse PI3Kδ cDNA in APR-19 cells whose endogenous one had been depleted by CRISPR/Cas9 because the mouse PI3Kδ would not be recognized by the human-specific sgRNA (PK2) of the CRISPR/Cas9. As shown in Fig. 3, transducing the mouse PI3Kδ into the p110δ-depleted ARPE-19 cells raised their p110δ levels slightly higher than their original ones; in addition, re-expression of mouse p110δ in the p110δ-depleted ARPE-19 cells recovered RV-induced AKT and MDM2 activation and p53 degradation (Fig. 3). These results further illustrate that PI3Kδ plays an essential role in the vitreous-induced signaling pathway of PI3K/AKT/MDM2/p53 in RPE cells.

Pharmacological inhibition of PI3Kδ effectively prevents vitreous-induced activation of AKT and MDM2 and reduction of p53

Next, a pharmacological approach was used to assess whether PI3Kδ was required for vitreous-induced activation of AKT and MDM2 and degradation of p53. Idelalisib, a specific inhibitor of PI3Kδ, is a Food and Drug Administration (FDA)-approved drug for treating certain cancers. To establish the maximum-tolerated dose of idelalisib in cultured RPEs, we treated ARPE-19 cells with serially increasing concentrations of idelalisib; 5, 10, 20, 40, 60, and 80 μM in serum-free media for 72 h (10). The results showed that 80 μM idelalisib became obviously toxic to ARPE-19 cells by morphology (e.g. cells shrinking) (Fig. S2A), and in these cells the IC50 of idelalisib was 61.5 μM, which was determined by MTT assay (Fig. S2B). However, 10 μM idelalisib did not show obvious toxicity to ARPE-19 cells except that cell number was less than those treated by its vehicle dimethyl sulfoxide (DMSO), which was assessed by morphology under a microscope and MTT assay (Fig. S2).

To examine the impact of idelalisib on the PI3K/AKT/MDM2/p53 pathway, we still monitored phosphorylation of AKT at serine 473 and phosphorylation of ERK at tyrosine 204 as indirect measures of activation of these kinases (39). In addition, it has been reported that the clinical trial dose of idelalisib for drinking is 150 mg twice per day (23), and there are a number of publications using 10 μM idelalisib to inactivate PI3Kδ in cultured cells (40–42) even though its IC50 to inhibit p110δ activity is 19 nM, which was evaluated by an in vitro biochemical assay (10). So we treated ARPE-19 cells with idelalisib (0.1, 1, and 10 μM) in addition to RV for 2 h. Western blot analysis of their lysates showed that idelalisib at 1 μM completely abrogated vitreous-induced activation of AKT, in contrast with ERK activation, which was not affected even if the cells were treated with idelalisib at 10 μM (Fig. 4, A and B). Importantly, idelalisib at 10 μM failed to inhibit PDGF-BB–induced AKT activation in 661W cells (Fig. 4, E and F), in which p110δ was expressed at very low levels compared with that in ARPE-19 cells (Fig. 1), indicative of idelalisib at 10 μM being selective. Furthermore, BYL719, whose IC50 to inhibit p110α activity is 4.0–4.8 nM (43), did not inhibit vitreous-induced AKT activation at 400 nM (100 times of its IC50) in ARPE-19 cells (Fig. 4, C and D), but at this dose BYL719 inhibited PDGF-BB–induced activation of AKT in 661W cells (Fig. 4, E and F). These results suggest that PI3Kδ is a predominantly functional receptor-regulated PI3K isoform for vitreous-induced activation of AKT in RPEs. Therefore, we next evaluated the impact of idelalisib on the AKT/MDM2/p53 pathway. As shown in Fig. 4, A, B, G, and H, idelalisib at 1 μM blocked vitreous-induced activation of AKT and MDM2 and suppressed vitreous-stimulated impact on p53 expression. This result is consistent with the depletion of p110δ.
Fig. 6. Illustration of the PI3K pathway and its involvement in ARPE-19 cell proliferation and migration. A, Western blot analysis showing the expression levels of p110α, β-Actin, p-AKT, AKT, Hsp90α, p-MDM2, MDM2, and p53 in ARPE-19 cells with or without PI3K inhibition using the two approaches. B, Graphs showing the fold change in p-AKT/AKT and p53/β-Actin expression in ARPE-19 cells treated with RV or idelalisib.

Inactivation of PI3Kα prevents vitreous-stimulated proliferation of RPEs

Activation of the PI3K/AKT/MDM2/p53 pathway can stimulate cell proliferation (2, 16), which is also a main characteristic for pathogenesis of PVR (16). Thus, we examined whether inactivation of PI3Kα using the two approaches could block vitreous-induced cell proliferation. As shown in Fig. 5, A and B, although vitreous stimulated (1.8 ± 0.2-fold) proliferation of ARPE-19 cells, either depletion of p110α with CRISPR/Cas9 or inhibition of PI3Kα with idelalisib significantly suppressed vitreous-induced proliferation.

Inactivation of PI3Kα blocks vitreous-induced migration of RPEs

Activation of the PI3K/AKT/MDM2/p53 signaling pathway also affects cell motility (2, 16). During PVR pathogenesis, RPE cells proliferate and migrate from the RPE monolayer to form sheets of dedifferentiated cells, a major cellular constituent of ERMs from patients with PVR (44). We next examined the impact of PI3Kα inactivation using the two approaches on vitreous-induced migration of ARPE-19 cells with a wound-healing assay, in which the wound was created by scratching the cells on culture plates with a 200-μl pipette tip (37, 45). As illustrated in Fig. 6, while vitreous stimulated cell migration into the wound areas, inactivation of PI3Kα by either p110α depletion with CRISPR/Cas9 or a specific pharmacological inhibitor idelalisib at 10 μM reversed vitreous-induced migration of ARPE-19 cells to levels that were even lower than in controls, indicating that both induced and constitutive PI3Kα activity contribute to cell migration.

Inactivation of PI3Kα abrogates vitreous-induced contraction of RPEs

A main characteristic of PVR is the formation of ERMs, whose contraction causes retinal detachment (17, 34, 46). To mimic the interaction of cells with extracellular matrix for contraction, an in vitro contraction assay was used even though this assay was far from the in vivo environment (37, 46). To investigate the impact of PI3Kα inactivation on the capability of the cell contraction, two approaches by either depletion of p110α with CRISPR/Cas9 or inhibiting its kinase activity with a specific inhibitor idelalisib were utilized. As shown in Fig. 7A, vitreous significantly induced contraction of cells expressing lacZ–sgRNA/Cas9, and the contraction capability was significantly attenuated in the RPEs expressing PK2–sgRNA/Cas9; in addition, idelalisib also blocked the vitreous-induced contraction of ARPE-19 cells in this collagen contraction assay (Fig. 7B).

Discussion

In this paper, we report that p110α is highly expressed in RPEs, and inactivation of PI3Kα using molecular and pharmacological approaches blocks vitreous-induced activation of AKT/MDM2/p53 as well as cellular responses intrinsic to the development of PVR. RPEs have several functions (light absorption, epithelial transport, spatial ion buffering, visual cycle, phagocytosis, secretion, and immune modulation) (47) and are involved in age-related macular degeneration (48), retinitis pigmentosa (49), diabetic retinopathy (50, 51), and PVR (35). p110α has been found to be mostly expressed in hematopoietic systems, including myeloid cells, B cells, and T cells, and plays key roles in leukocyte signaling, proliferation, differentiation, activation, and chemotaxis (2, 13, 52, 53). Our findings reported herein expand the research scope of PI3Kα, which at present is...
mostly focused on the hematopoietic system, indicating deregulation of PI3K\(\delta\) may involve in RPE-related pathology such as PVR.

PVR is a blinding complication that develops in 8–10% of patients with primary retinal detachment (19, 54–59) and in 40–60% of patients with open-globe injury (20, 54, 60–68). So far, repeat surgery is the only option to treat PVR, but this surgery has poor functional results (18, 20, 34). Even though tremendous efforts have been made to identify nonsurgical approaches for prevention of PVR, they have not been successful. The PI3K\(\delta\)/AKT-signaling pathway is hyperactive in the vitreal environment, suggesting PI3K\(\delta\) is a promising target for the therapy of PVR.

However, the mechanisms by which inactivation of PI3K\(\delta\) blocks vitreous-stimulated activation of AKT/MDM2/p53 warrants further investigation because RPE cells express multiple other isoforms of PI3Ks, including PI3K\(\alpha\), PI3K\(\beta\), and PI3K\(\gamma\). Based on that, idelalisib inhibits vitreous-induced activation of AKT in RPEs but not PDGF-BB–stimulated AKT activation in 661W cells (Fig. 4). Because RPEs express much more p110\(\delta\) than do 661W cells (Fig. 1), we propose the following three hypotheses: 1) in RPE cells PI3K\(\delta\) is one of the predominant isoforms of PI3Ks; 2) vitreous induces a specific p85 regulatory subunit to activate p110\(\delta\); and 3) vitreous stimulates a specific AKT isoform via PI3K\(\delta\) for activating MDM2. These hypotheses are actively being examined in my laboratory.

Idelalisib is a potent, small-molecule inhibitor of PI3K\(\delta\) that is highly selective for the \(\delta\) isoform of PI3K, as compared with the \(\alpha\), \(\beta\), and \(\gamma\) isoforms of PI3Ks. In lymphoid cell lines and primary samples from patients, idelalisib blocks PI3K\(\delta\)–AKT signaling (23, 42). Similarly, inactivation of PI3K\(\delta\) abrogated vitreous-stimulated activation of AKT and degradation of p53 (Figs. 2–4). AAV-based CRISPR/Cas9 has been in clinical trial for treating human diseases especially eye disease (29); in addi-
**Figure 5.** Inactivation of p110δ prevents vitreous-induced cell proliferation. ARPE-19 cells expressing lacZ-sgRNA/Cas9 or PK2-sgRNA/Cas9 (A) or plain ARPE-19 cells (B) were seeded into a 24-well plate at a density of $3 \times 10^5$ cells/well. After 8 h, the cells had attached to the plates, and the medium was switched to either 0.5 ml of DMEM (–) or RV (A) or RV supplemented with idelalisib (10 μM) or its vehicle (V) DMSO (B). After treating for 48 h, the cells were counted with a hemocytometer under a light microscope. The mean ± S.D. of three independent experiments is shown; *** denotes $p < 0.001$ using one-way ANOVA followed by the Tukey post test. ns, not significant.

**Figure 6.** Inactivation of p110δ abrogates vitreous-induced cell migration. After ARPE-19 cells expressing lacZ-sgRNA/Cas9 or PK2-sgRNA/Cas9 (A) or plain ARPE-19 cells (B) in a 24-well plate reached confluence, they were serum-starved for 24 h. Then, the wells with cells were scratched with a 200-μl pipette tip and treated with RV or RV in addition to idelalisib (10 μM) or its vehicle. 16–20 h later, pictures of the cells were taken and analyzed. Representative raw data of three experiments below the bar graphs is shown. Scale bar, 400 μm. The mean ± S.D. of three independent experiments is shown; *** indicates $p < 0.001$ using ANOVA followed by the Tukey post test. ns, not significant.

**Figure 7.** Inactivation of p110δ inhibits vitreous-induced contraction of RPEs. ARPE-19 cells expressing lacZ-sgRNA/Cas9 or PK2-sgRNA/Cas9 (A) or plain ARPE-19 cells (B) were resuspended in neutralized collagen and seeded into wells of a 24-well plate and treated with RV in addition to idelalisib (10 μM) for 2 days. The gel diameter was measured and calculated using the formula $(3.14 \times (\text{diameter}/2)^2)$. A, lacZ: ARPE-19 cells expressing lacZ-sgRNA/Cas9 as a control; PK2: ARPE-19 cells expressing P3Kδ-sgRNA/Cas9. B, V: vehicle, 0.1% DMSO; idelalisib 10 μM. The mean ± S.D. of the three independent experiments is shown, and the data were analyzed using one-way ANOVA followed by the Tukey’s post test. *** indicates $p < 0.001$. A photograph of the representative experiment is shown at the bottom of the bar graphs. ns, not significant.
**PI3Kδ inactivation blocks AKT/MDM2/p53 in RPEs**

...idelalisib has been approved by the FDA for the treatment of certain hematological malignancies (2, 53); therefore, these genome-editing and pharmacological (idelalisib) approaches to targeting PI3Kδ have great potential for therapeutic treatment of RPE-related diseases, including PVR.

**Materials and methods**

**Major reagents**

Primary antibodies against p-AKT (Ser-473), AKT, p-MDM2 (Ser-166), MDM2, p-ERK (Thr-202/Tyr-204), ERK, Hsp90α, p110α, p110β, p110δ, and p110γ were purchased from Cell Signaling Technology (Danvers, MA); antibodies against Hsp90, Hsp70, Hsp40, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, horseradish peroxidase–conjugated goat anti-rabbit IgG, and goat anti-mouse IgG, were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescent substrate for detection of horseradish peroxidase was from Thermo Fisher Scientific (Waltham, MA). Idenalisib was purchased from APExBIO (Houston, TX), and BYL719 was from Thermo Fisher Scientific (Waltham, MA).

**DNA constructs**

The 20-nt target DNA sequence preceding a 5′-NGG PAM sequence at exon 4 in the human genomic PIK3CD DNA constructs were purchased from Cayman Chemical (Ann Arbor, Michigan). PDGF-βB was purchased from Peprotech Inc. (Rocky Hill, NJ).

**Human primary fetal RPE cells** (Lonza, Walkersville, MD), ARPE-19 cells (American Type Culture Collection, Manassas, VA), and PAECs were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture (DMEM/F-12, Invitrogen) supplemented with 10% fetal bovine serum (FBS). RCFs (52) and PAECs (69) were gifts from the Kazlauskas’ lab at Schepens Eye Research Institute (Boston, MA), and HCFs were a gift of the Zieske lab (70) at Schepens Eye Research Institute. Mouse cone photoreceptor cells (661W) were obtained by material transfer agreement from University of Houston (71). Human retinoblastoma Y-79 cells and RAW264.7 mouse macrophages were purchased from American Type Culture Collection. Y-79, HCF, 661W, and RAW264.7 cells were cultured in DMEM supplemented with 10% FBS.

**Production of lentivirus**

Lentivirus was produced as described previously (72). Briefly, the lentICRISPR v2 vector inserted with sgRNA, the packaging plasmid psPAX2 (Addgene, 12260), and the envelope plasmid VSV-G (Addgene, 8454) were mixed together and then added to a mixture of Lipofectamine 3000 (Thermo Fisher Scientific) with Opti-MEM (Thermo Fisher Scientific). This transfection mix was kept at room temperature for 30 min and then carefully added into HEK 293T cells in a 60-mm cell culture dish. After 18 h (37 °C, 5% CO2), the medium was replaced with growth medium supplemented with 20% FBS, and lentiviruses were harvested at 24 h after changing the medium and then daily for 2 days. The virus-containing media were pooled and centrifuged at 800 × g for 5 min to remove the cell debris. The supernatant was used to infect APRE-19 cells supplemented with 8 μg/ml Polybrene (Sigma). The infected cells were selected in media using puromycin (Sigma) (4 μg/ml), and the resulting cells were examined by Western blotting (21, 31, 75).

**Re-expression of p110δ**

This experiment was performed as described previously (37). Briefly, the mouse PIK3CD cDNA in the vector pCMVSPORT6 (MMM1013-202765002, GE Dharmacon, Waltham, MA) was subcloned into a retroviral vector of pLNCX3™ (LTR—CMV-Sall-Hpal-SacI-NotI-BglII-Clai—LTR) derived from pLNCX (GenBank™ accession no. M28247; National Institutes of Health, Bethesda) by Sall/Notl (37) and confirmed by Sanger DNA sequencing. The sequence-confirmed retroviral vector was transfected into 293GPG cells for producing retroviruses, which were used for infecting ARPE-19 cells whose endogenous p110δ had been depleted by the CRISPR/Cas9. This mouse PIK3CD cDNA circumvented recognition by the human originated PK2–sgRNA of the CRISPR/Cas9, and its expression was confirmed by Western blotting.

**Western blotting**

When cells reached 90% confluence in 24-well plates, they were serum-starved for 24 h, and then treated with normal rabbit vitreous (RV, diluted 1:3 in DMEM/F-12) in addition to idelalisib or its vehicle (0.01% DMSO: DMSO) for 2 h. After being washed twice with ice-cold phosphate-buffered saline (PBS), the cells were lysed in 1× sample buffer diluted with protein 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 Na3VO4, 1 mM phenylmethylsulfonyl fluoride) from 5× sample buffer (25 mM EDTA, 10% SDS, 500 mM DTT, 50% sucrose, 500 mM Tris-HCl, pH 6.8, 0.5% bromphenol blue). The lysates were boiled for 5 min and then centrifuged for 5 min at 13,000 g × 4 °C. Proteins in samples were separated in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blot analysis using desired antibodies. Signal intensity was determined by densitometry using ImageJ software (National Institutes of Health) (21, 33).

**Immunofluorescence**

This experiment was performed as described previously (37). Briefly, cells were fixed in 3.7% formaldehyde/PBS for 10 min at...
room temperature. Then, the samples were blocked with 5% normal goat serum in 0.3% Triton X-100/PBS for 30 min and incubated with a primary antibody from rabbit against p110δ (Abcam) or p110α (1:100 dilution) in combination with mouse against pan-keratin or nonimmune mouse and rabbit IgG overnight at 4 °C. After thorough washes with PBS to get rid of the nonspecific binding, the samples were incubated with fluorescently-labeled secondary antibodies Dylight 549 (anti-rabbit IgG) and Dylight 488 (anti-mouse IgG) (Vector Laboratories, Inc., Burlingame, CA) (1:300 dilution in a blocking buffer) for 1 h. After thorough washes with PBS, the slides were mounted with a mount medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). These slides were observed and photographed under a fluorescent microscope (30, 37, 69).

**MTT assay**

The toxicity of Idealalisib on cell viability was assessed using the MTT assay (Solarbio). ARPE-19 cells were seeded into 96-well plates in their respective medium supplemented with 10% FBS and incubated for 24 h before exposure to a range of concentrations (0, 12.5, 25, 50, and 100 μM) of idealalisib dissolved in DMSO in 5% FBS-supplemented medium or serum-free medium for 24–72 h. Subsequently, the medium was removed and replaced with 200 μl of 0.5 mg/ml MTt in 5% FBS-containing medium or serum-free medium, and the cells were incubated in the CO2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the MTT dye was solubilized in 150 μl/well DMSO. Absorbance was measured at 570 nm on a plate reader. Each condition was tested with six replicates, and all assays were performed in triplicate (76, 77).

**Cell proliferation assay**

Cells were plated in 24-well plates at a density of 3 × 10⁴ cells/well in DMEM/F-12 supplemented with 10% FBS. Following attachment, the cells were treated with DMEM/F-12 or RV (1:3 dilution in DMEM/F-12) plus idelalisib (10 μM) or its vehicle DMSO. After treatment for 48 h, the cells were trypsin-detached from the plates and counted in a hemocytometer under a light microscope. At least three independent experiments were performed as described previously (31, 33, 75).

**Cell migration assay**

After cells in 24-well plates were grown to confluence, they were serum-starved overnight. Then, a wound was created by scratching the monolayer with a 200-μl pipette tip (37, 45). The cells were washed with PBS and treated with DMEM/F-12 or RV (1:3 dilution in DMEM/F-12) plus idelalisib (10 μM) or its vehicle DMSO. The scratched area was photographed to capture the initial width and photographed again 16–20 h later. Data were analyzed using Adobe Photoshop CS4 software. At least three independent experiments were performed.

**Collagen contraction assay**

Cells were resuspended in 1.5 mg/ml neutralized collagen I (INAMED, Fremont, CA), pH 7.2, on ice at a density of 1 × 10⁶/ml cells/ml (15, 75). The cell and gel mixture was transferred into wells of 24-well plates, which had been preincubated overnight with 5 mg/ml BSA/PBS. When the gel became a solid jelly-like soft material after incubation at 37 °C for 90 min, 0.5 ml of DMEM/F-12 or RV (1:3 dilution in DMEM/F-12) was added with or without idealalisib (10 μM). On day 3, 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 as described in previous reports (37, 75, 78).

**Statistics**

The experimental data were analyzed using an unpaired t test between two groups and one-way ANOVA among more than two groups followed by the Tukey post test using GraphPad Prism Version 5c. A p value less than 0.05 is considered statistically significant.

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