

Original Article

RKIP inhibits the proliferation and migration of human retinal pigment epithelial cells by suppressing the MAPK pathway

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Received December 17, 2015; Accepted March 5, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Retinal pigment epithelial (RPE) cells play important roles in diabetic retinopathy. Raf-1 kinase inhibitory protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, has been involved in regulating cell proliferation and migration. However, the role of RKIP in RPE cells is still unclear. Therefore, in the present study, we investigated the effects of RKIP on the proliferation and migration in RPE cells, and the signaling mechanism of RKIP involved in these processes. Our results showed that the expression of RKIP was markedly decreased in the vitreous fluid of diabetic retinopathy patients. Overexpression of RKIP significantly inhibited the proliferation and migration in ARPE-19 cells. In addition, overexpression of RKIP markedly inhibited the expression of p-p38 and p-ERK1/2 in ARPE-19 cells. In conclusion, our findings suggest that overexpression of RKIP inhibited the proliferation and migration of RPE cells by suppressing the MAPK pathway. Therefore, RKIP may serve as a potential molecular target for the treatment of diabetic retinopathy.

Keywords: Raf-1 kinase inhibitory protein (RKIP), diabetic retinopathy, retinal pigment epithelial (RPE)

Introduction

Diabetic retinopathy is one of the leading causes of visual impairment among aged adults in the world [1]. It is a chronic, progressive, sight-threatening disease associated with prolonged hyperglycemia. Nevertheless, there is no effective treatment, because the mechanism of diabetic retinopathy remains largely enigmatic. It is, therefore, necessary to develop new therapeutic strategies for this disease. The retinal pigment epithelial (RPE), a monolayer of cells located between choroid and the photoreceptors, plays a crucial role in the pathogenesis of diabetic retinopathy. A number of studies have shown that the proliferative and migratory phenotype of RPE cells occurs in diabetic retinopathy [2-4]. Therefore, inhibiting the proliferation and migration of RPE cells may be a therapeutic approach for the treatment of diabetic retinopathy.

The Raf kinase inhibitor protein (RKIP) belongs to an evolutionarily conserved family of phosphatidylethanolamine-binding proteins (PEBPs), which have important functions as inhibitors of

kinase signaling pathways [5, 6]. It plays important roles in tumorigenesis, neural development, cardiac function and spermatogenesis [7-9]. For example, Li *et al.* reported that overexpression of RKIP in breast cancer cells impaired invasiveness and metastasis, whereas down-regulation of RKIP expression promoted invasiveness and metastasis [10]. In addition, dysregulated RKIP expression has the potential to contribute to diabetic nephropathy [11]. However, the function and impact of RKIP in diabetic retinopathy is not well understood. In this study, we investigated the effect of RKIP on the proliferation and migration of RPE cells, and the signaling mechanism of RKIP involved in these processes. We found that overexpression of RKIP inhibited the proliferation and migration of RPE cells by suppressing the MAPK signaling pathway.

Materials and methods

Collection of vitreous fluid samples

Undiluted vitreous fluid samples (~0.5 ml) from 10 patients with diabetic retinopathy were col-

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lected through the vitreous cutter using a syringe attached to an automated vitrector before opening the infusion line at the start of a standard three-port pars planovitrectomy. Control vitreous samples were obtained from 10 patients without diabetic retinopathy. Samples were transferred to a tube, placed immediately on ice and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was separated and stored at -80°C.

Cell culture

The human ARPE-19 cell line were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium, Ham's F-12 nutrient mixture (DMEM/F12) supplemented with 10% fetal bovine serum (FBS, Gibco/Invitrogen, NY), and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin, Sigma, MO) at 37°C in 5% CO₂ and 95% humidity.

Plasmid construction and transfection

FLAG-tagged RKIP expression vector was constructed by inserting PCR amplified RKIP fragment into a pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) linked with FLAG tag at the amino terminus. The RKIP expression vector and empty pcDNA3 were transfected into human ARPE-19 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were selected with G418 at the concentration 800 µg/ml, and the resistant clones were further confirmed by Western blot.

Cell proliferation assay

ARPE-19 cells transfected with empty vector or Flag-RKIP were seeded into 96-well plates (5×10³ cells/well) and incubated for 24 h, 48 h and 72 h, respectively. Then, cells were stained with an equal volume (100 µl) of fresh medium containing 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and incubated for 4 h at 37°C in the dark, followed by the addition of 150 µl dimethyl sulfoxide (Sigma) to dissolve the formed formazan crystals. For colorimetric analysis, the absorbance at 490 nm was recorded using a microplate reader.

Cell migration assay

Cell migration ability was analyzed by the Transwell chamber assay. In brief, ARPE-19

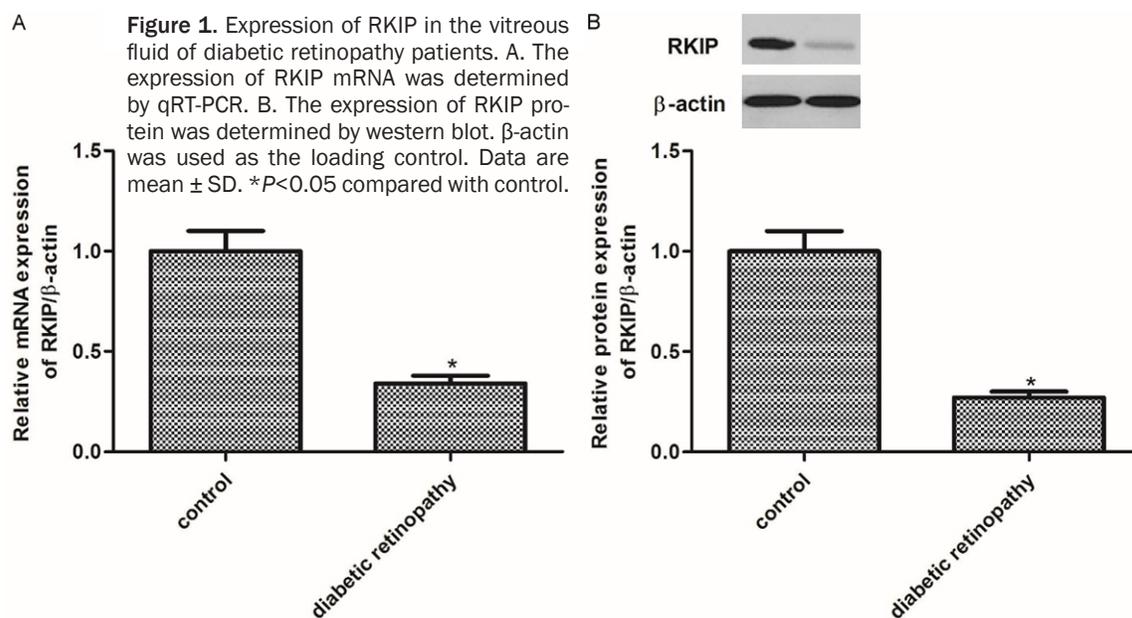
cells transfected with empty vector or Flag-RKIP (1×10⁵ cells per well) suspended in 0.1% FBS medium were seeded in the upper compartment, and the lower chamber was filled with DMEM containing 10% FBS. After 24 h of incubation, cells were fixed in methanol for 15 minutes and stained with 0.05% crystal violet in PBS for 15 min. The remaining cells on the upper surface of the filter were removed by wiping with a cotton swab. Cells on the underside of the filters were viewed and counted under a microscope (Olympus, Tokyo, Japan).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from ARPE-19 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized with 2 µg total RNA using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany). The RT-PCR reactions were performed based on the thermal cycler dice real-time system. PCR primers were as follows: human RKIP: forward 5'-AGCAGTGGCACAGTCCTC-3', reverse 5'-TGG-TCTCCAGATCGGTTG-3'; human β-actin: forward 5'-TGT TCG ACA GTC AGC CG AT-3'; reverse 5'-ACT CCG ACC TTC ACC TTC CC-3'. These primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR procedure was as follows: 94°C for 4 min; 94°C for 20 s, 55°C for 30 s, and 72°C for 20 s; 2 s for plate reading for 35 cycles; and melt curve from 65 to 95°C. For relative quantification, the levels of individual gene mRNA transcripts were first normalized to the control β-actin. Subsequently, the differential expression of these genes was analyzed by the 2^{-ΔΔCT} method and expressed as fold changes.

Western blot

The proteins were extracted from ARPE-19 cells using RIPA lysis buffer (Beyotime, Nantong, China). The protein concentration in the lysates was determined using a BCA protein assay kit (Beyotime). Forty micrograms of protein per lane was separated on 10% SDS-PAGE gel and transferred to the nitrocellulose membranes. Then, nonspecific binding was blocked by incubating with 5% nonfat milk in TBST buffer at room temperature for 1 h. The target proteins were probed with primary antibody (anti-RKIP, anti-p38, anti-p-p38, anti-EKR1/2, anti-p-ERK1/2 or anti-β-actin) (Abcam, Cambridge,



MA) overnight at 4°C. Subsequently, the membrane was washed and incubated with peroxidase-conjugated secondary antibody goat anti-rabbit IgG (Boster Corporation, Wuhan, Hubei, China; diluted 1:3,000 in the blocking buffer) for 1 h. Expression was visualized by using ECL Western blotting detection reagent (GE Healthcare, UK). BandScan 5.0 software was used for the quantification of all the proteins after western blot analysis.

Statistical analysis

Data are expressed as the means \pm SD. Statistical significance was analyzed with the one-way factorial ANOVA or the Student's two-tailed t-test. A p value less than 0.05 was considered statistically significant.

Results

Expression of RKIP in the vitreous fluid of diabetic retinopathy patients

To investigate the role of RKIP in diabetic retinopathy, we detected the expression of RKIP in the vitreous fluid of diabetic retinopathy patients using qRT-PCR and Western blot. As indicated in **Figure 1A**, the expression of RKIP mRNA was significantly lower in the vitreous fluid of diabetic retinopathy patients than in controls with non-diabetic ocular diseases. Similarly, Western blot analysis demonstrated

that the expression of RKIP protein was also obviously lower in the vitreous fluid of diabetic retinopathy patients (**Figure 1B**).

Effect of RKIP on human RPE cell proliferation

To further examine the role of RKIP in RPE cell proliferation, ARPE-19 cells were transduced with empty vector or Flag-RKIP. The transfection efficiency was evaluated by RT-qPCR and Western blot at 24 h after transfection. The results of qRT-PCR analysis demonstrated that RKIP overexpression significantly increased the expression of RKIP mRNA in ARPE-19 (**Figure 2A**). Similarly, Western blot analysis also indicated that the expression of RKIP protein was greatly increased in RKIP overexpression-transfected cells (**Figure 2B**). Then, the effect of RKIP on RPE cell proliferation was determined by the MTT assay. The results demonstrated that RKIP overexpression significantly inhibited ARPE-19 cell proliferation, exhibiting a time-dependent manner, as compared with the vector group (**Figure 2C**).

Effect of RKIP on human RPE cell migration

To explore the effect of RKIP on RPE cell migration, ARPE-19 cells were transfected with empty vector or Flag-RKIP and were placed in a Transwell chamber. As shown in **Figure 3**, as compared with the vector group, restoration of

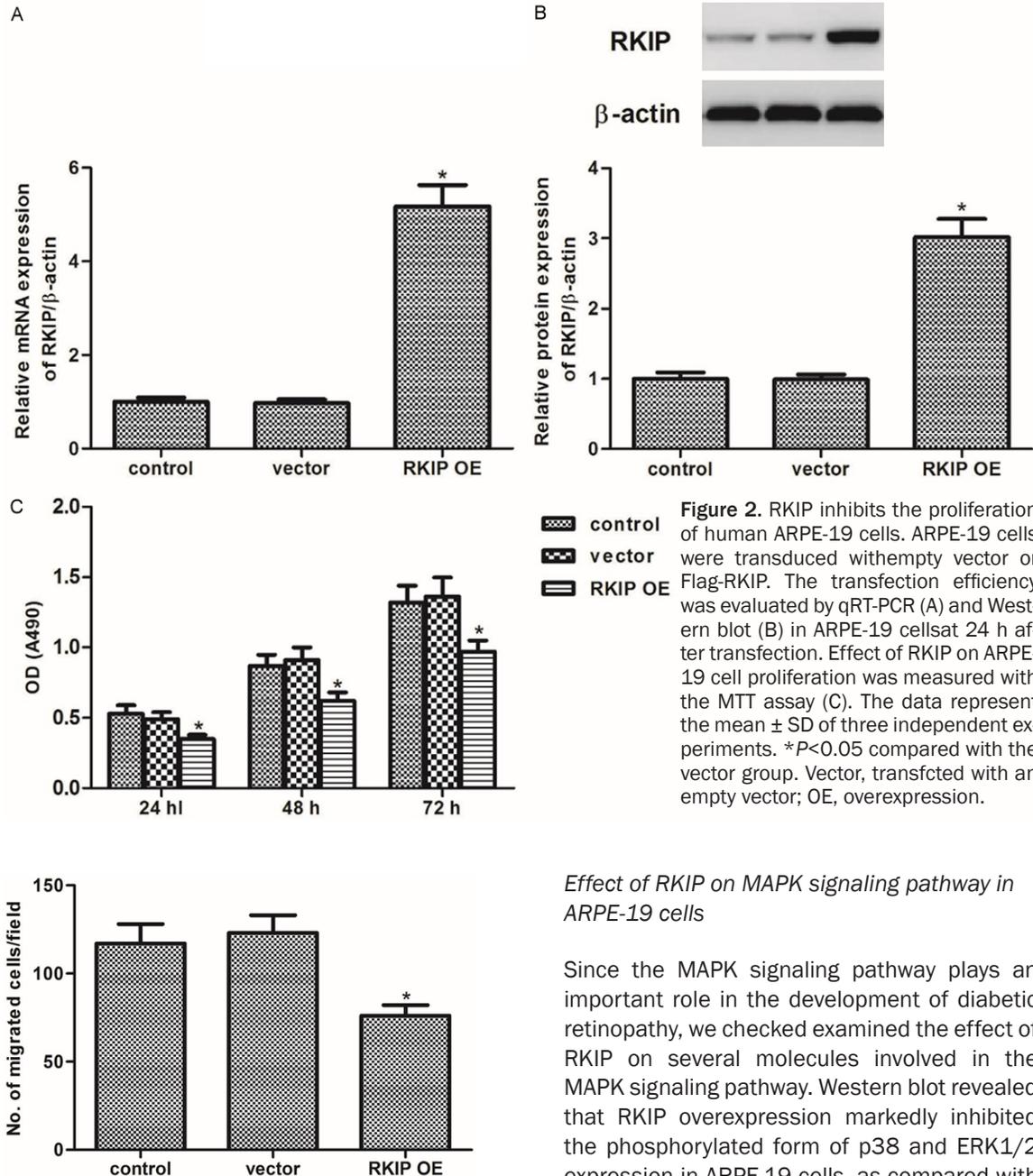


Figure 2. RKIP inhibits the proliferation of human ARPE-19 cells. ARPE-19 cells were transduced with empty vector or Flag-RKIP. The transfection efficiency was evaluated by qRT-PCR (A) and Western blot (B) in ARPE-19 cells at 24 h after transfection. Effect of RKIP on ARPE-19 cell proliferation was measured with the MTT assay (C). The data represent the mean \pm SD of three independent experiments. * $P < 0.05$ compared with the vector group. Vector, transfected with an empty vector; OE, overexpression.

Effect of RKIP on MAPK signaling pathway in ARPE-19 cells

Since the MAPK signaling pathway plays an important role in the development of diabetic retinopathy, we checked examined the effect of RKIP on several molecules involved in the MAPK signaling pathway. Western blot revealed that RKIP overexpression markedly inhibited the phosphorylated form of p38 and ERK1/2 expression in ARPE-19 cells, as compared with the vector group (Figure 4).

Discussion

In this study, we found that expression of RKIP was markedly decreased in the vitreous fluid of diabetic retinopathy patients. Overexpression of RKIP significantly inhibited the proliferation and migration in ARPE-19 cells. In addition, overexpression of RKIP markedly inhibited the expression of p-p38 and p-ERK1/2 in ARPE-19 cells.

Figure 3. RKIP inhibits the migration of human ARPE-19 cells. ARPE-19 cells were transduced with empty vector or Flag-RKIP for 24 h. Transwell assay was performed to evaluate the migration potential of ARPE-19 cells. The data represent the mean \pm SD of three independent experiments. * $P < 0.05$ compared with the vector group. Vector, transfected with an empty vector; OE, overexpression.

RKIP expression leads to significant reduction in migration of ARPE-19 cells. There was no significant difference between the control group and the vector group.

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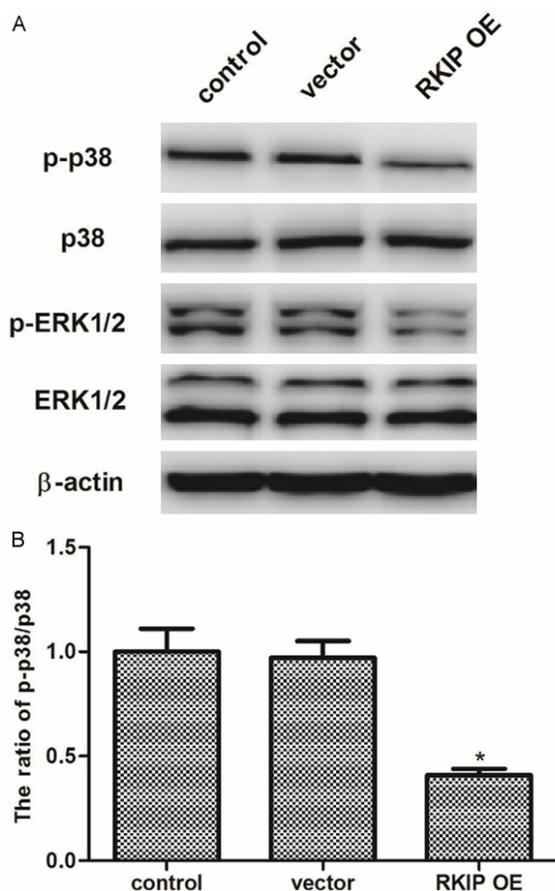


Figure 4. RKIP inhibits the activation of MAPK signaling pathway in ARPE-19 cells. ARPE-19 cells were transfected with empty vector or Flag-RKIP for 30 min. A. The levels of phosphorylated and total p38 and ERK1/2 were determined with western blot analysis. β -actin was used as the loading control. B, C. p-p38 and p-ERK levels were measured densitometrically and normalized to the levels of p38 and ERK, respectively. The data represent the mean \pm SD of three independent experiments. * $P < 0.05$ compared with the vector group. Vector, transfected with an empty vector; OE, overexpression.

RPE cells play a key role in diabetic retinopathy. Proliferation of these cells is an essential step in the pathogenesis of retinal diseases that lead to vision loss [12]. And, RPE cell migration is a multiple-step process with interactions between RPE cells and their surrounding micro-environment that begins with RPE cells leaving the basement membrane site and relocating in the vitreous cavities [13]. Without migration, RPE cells would not gain access to the vitreous and form vitreoretinal membranes. In addition, emerging evidence is increasingly showing that aberrant expression of RKIP may contribute to the development and progression of various cancers and many other diseases [14, 15]. It has been reported that RKIP significantly suppressed the proliferation and migration of human breast cancer cells, as well as the expression of matrix metalloproteinase (MMP-1) and MMP-2 expression in MDA-MB-231 cells [16]. Another study demonstrated that RKIP also inhibits gastric cancer cell proliferation and invasion [17]. In this study, we found that the expression of RKIP was significantly lower

in the vitreous fluid of diabetic retinopathy patients, and RKIP overexpression inhibited the proliferation migration of RPE cells, which is consistent with its effects on the proliferation and migration of cancer cells. These results suggest that RKIP may play a role in the pathogenesis of diabetic retinopathy.

The mitogen-activated protein kinase (MAPK) signaling pathway has been shown to play a central role in cell proliferation, migration and invasion [18-20]. There are three major MAPK pathways: the p38 MAPK, the extracellular-signal-regulated kinases (ERKs) and the c-Jun N-terminal kinase (JNK) [21]. In RPE cells, p38MAPK and ERK1/2 signaling pathways were activated by transforming growth factor- β 2 (TGF- β 2) [22]. Several studies have demonstrated that the proliferation and migration of RPE cells depended on the MAPK signaling pathway. ERK1/2 has been shown to be involved in the RPE cell proliferation stimulated by fibroblast growth factors (FGFs) [23]. Furthermore, endothelial growth factor recep-

tor (EGFR) ligand activation promotes RPE cell proliferation and survival, signaling through the ERK/MAPK and PI3K pathways [24]. In addition, RKIP has been identified as a suppressor of the MAPK signaling pathway. RKIP inhibits the activation phosphorylation of MEK by Raf-1 by competitively inhibiting the binding of MEK to Raf-1 and thus exerting an inhibitory effect on the Raf-MEK-ERK pathway [6]. In the current study, we found that overexpression of RKIP obviously decreased levels of p38 and ERK1/2 phosphorylation in ARPE-19 cells. These results suggest that RKIP overexpression inhibits the proliferation and migration in human RPE cells through suppressing the MAPK signaling pathway.

In summary, this study highlights the important role of RKIP in the pathogenesis of diabetic retinopathy. Our results suggest that RKIP inhibits proliferation and migration in human RPE cells by suppressing the MAPK signaling pathway. Thus, RKIP may serve as a potential molecular target for the treatment of diabetic retinopathy.

Disclosure of conflict of interest

None.

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