

Effects of resveratrol on ARPE-19 cell proliferation and migration *via* regulating the expression of proliferating cell nuclear antigen, P21, P27 and p38MAPK/MMP-9

Xiao-Ning Hao¹, Wen-Jie Wang², Jian Chen¹, Qing Zhou¹, Yi-Xin Qu¹, Xiao-Yong Liu¹, Wei Xu¹

¹Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China

²Department of Ophthalmology, Shenzhen Children's Hospital, Shenzhen 518026, Guangdong Province, China

Co-first authors: Xiao-Ning Hao and Wen-Jie Wang

Correspondence to: Jian Chen and Qing Zhou. Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China. drchenj@163.com; kerryzh@163.com

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INTRODUCTION

Retinal pigment epithelial (RPE) cells have proved to be a major cell type on the process of proliferative vitreoretinopathy (PVR), which remains one of the major causes of failure to repair rhegmatogenous retinal detachment [1]. Briefly, after the formation of retinal tears, retina cells (mainly RPE cells, also including fibroblasts, glial cells and macrophages) are exposed to vitreous humor which is rich in a series of growth factors and cytokines, thus resulting in cell migration, proliferation, epithelial-to-mesenchymal transition (EMT) and synthesis of extracellular matrix, and then producing a contractile non-vascular cellular membrane, and consequently, this contractile proliferative membrane causes subsequent tractional retinal detachment [2-3]. RPE cells have been widely confirmed to be the major component of this contractile membrane [2,4-6].

Currently, surgeries aimed at removing proliferative membranes, achieving retinal reattachment and restoring visual function are standard treatments, mainly including scleral buckling, vitrectomy, peeling the proliferative membranes, silicone oil or long-active gas tamponade and retinal-release or retinectomy if necessary [3]. But the outcomes are always disappointing, and few of the patients could gain satisfied vision improvement [7-8]. Researches exploring pharmacotherapy as an assistant treatment have been ongoing for decades, such as anti-inflammatory agents (corticosteroids) [9-10], anti-metabolites agents (daunomycin and 5-fluorouracil), anti-coagulant drugs which could combine with multiple growth factors in vitreous (low molecular weight heparin) [11-13], all of them have controversial efficacy and none has been applied to clinical practice as a regular method [7,14].

Resveratrol (Res), 3, 4', 5-Trihydroxy-trans-stilbene, is a nature polyphenolic compound which possesses a variety of biological activities, *i.e.* anti-proliferation, anti-tumor, anti-oxidation, anti-inflammation and cardiovascular

Abstract

• **AIM:** To explore whether resveratrol (Res) can inhibit human retinal pigment epithelial cell (ARPE-19 cell) proliferation and migration, and to research the molecular mechanisms.

• **METHODS:** ARPE-19 cells were pretreated with various concentrations at 0, 50, 100, 150, 200 and 300 $\mu\text{mol/L}$ of Res, and with 0 $\mu\text{mol/L}$ Res as the control for 24, 48 and 72h. The cell proliferation, apoptosis and migration were measured with cell counting kit -8 (CCK-8), flow cytometry, and wound-healing and Transwell assays, respectively. The expression of proliferating cell nuclear antigen (PCNA), P21 and P27, as well as matrix metalloproteinase-9 (MMP-9) and p38 mitogen-activated protein kinases (p38MAPK) was identified by Western blot.

• **RESULTS:** Cell proliferation was effectively inhibited by Res ($P < 0.05$). When pretreated with Res, cells arrested in S-phase increased remarkably ($P < 0.05$), but the apoptosis ratios showed no significant difference between the treatment and control groups ($P > 0.05$). Cell migration was suppressed by Res both in wound-healing assay and Transwell migration assay ($P < 0.05$). Decreases of PCNA, MMP-9 and p38MAPK, as well as increases of P21 and P27 were detected by Western blot ($P < 0.05$).

• **CONCLUSION:** Res can inhibit ARPE-19 cell proliferation and migration in a concentration-dependent manner with up-regulation of the expression of P21 and P27, and down-regulation of PCNA, MMP-9 and p38MAPK.

• **KEYWORDS:** retinal pigment epithelium; resveratrol; cell proliferation; cell migration inhibition

protection [15-16]. The effect of anti-proliferation has been proved *in vitro* on animal and in a few clinical studies, which makes Res a promising medication for many diseases. Proliferating cell nuclear antigen (PCNA), P21 (P21^{Cip1}) and P27 (P27^{Kip1}) were highly demonstrated as important cell cycle regulatory factors. PCNA, an auxiliary factor of DNA polymerases- δ/ϵ (forming the DNA pol- δ/ϵ -PCNA complex), acts as a positive regulatory factor of DNA synthesis in S-phase [17-18]; P21 and P27, which belong to the cyclin dependent kinase inhibitory proteins (CKIs), exert negative regulatory activities in cell cycle mainly by interacting with cyclins/Cdk complex [19]. In addition, P21 is consistently recognized as a cofactor interacting with PCNA to play its part in cell cycle regulation [17,19-20]. Matrix metalloproteinase-9 (MMP-9) plays an important role in degrading extracellular matrix (ECM), and thus regulates cell migration as well as tumor metastasis [21]. Mitogen-activated protein kinases (MAPKs) are tightly involved in cell growth, cell apoptosis, cellular stress and metastasis, mainly through three members targeting different down-streams, that is, p38MAPK, extracellular signal regulated kinase 1 and 2 (ERK1/2) and c-Jun N-terminal kinase 1 and 2 (JNK1/2) [22]. p38MAPK has been confirmed to participate in cell migration by regulating MMP-9 [23-24].

Whether Res can inhibit the proliferation and migration of human RPE cells and its potential molecular mechanisms have not been clarified. So we carried out this study aimed at answering these questions, and exploring whether Res could be a promising drug therapy for the treatment of PVR.

MATERIALS AND METHODS

Materials Res and dimethyl sulfoxide (DMSO) were bought from Sigma (USA); cell counting kit-8 (CCK-8) was from Dojindo (Japan); mouse anti-human PCNA, rabbit anti-human P21 were from Proteintech (USA); rabbit anti-human P27 was from Abcam (UK); rabbit anti-human MMP-9 and p38MAPK were from Cell Signaling Technology (CST, USA); 24-well Transwell chambers (8.0- μ m pore size) were from BD Biosciences (USA).

Methods

Cell culture ARPE-19 cells were given by Zhongshan Ophthalmic Center, Sun Yat-Sen University. Cells were incubated at 37°C in a 5% CO₂ and sufficiently humid incubator, and maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham's (DMEM/F12; Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA).

Preparation of resveratrol and experimental design Powder of Res was dissolved in DMSO as stock solution at a concentration of 100 mmol/L, sub-packaged and then stored at -20°C. Except Transwell migration assay, the stock solution was diluted with serum-containing medium previously mentioned to gain the intended concentrations.

Res for Transwell migration assay was diluted in serum-free medium. Each test was independently repeated for 3 times with 0 μ mol/L Res group as the control group.

Cell proliferation assay CCK-8 test was performed to determine the proliferation of ARPE-19 cells treated with Res. Cells were harvested and seeded in 96-well plates at a density of 1×10^5 cells/mL with the volume of 100 μ L/well. After 24h, they were treated with Res (at the final concentrations of 0, 50, 100, 150, 200 and 300 μ mol/L), for 24, 48 and 72h respectively. After the incubation for intended periods, 100 μ L medium containing 10% CCK-8 was added to each well, and after continued incubation for another 2h, OD values were measured at 450 nm using a microplate Reader (Safire, Tecan, Swiss). Each well was made in quintuplicate with 100 μ L medium containing 10% CCK-8 as the blank control group.

Flow cytometric analysis To investigate cell cycle distribution and apoptosis, flow cytometric analysis was conducted. General procedures are the same as mentioned in other articles. Briefly, cells treated with Res (0, 100, 150 and 200 μ mol/L; for 48h) were digested and washed (in duplicate) by PBS, transferred into 1.5 mL Eppendorf tubes, and then fixed in 70% cold ethanol at 4°C overnight. The next day, cells were washed twice, and stained with 200 μ L propidium iodide (PI, BD, USA) at 37°C for 15min in the dark. Following filtration, a flow cytometer (FACSAria, BD, USA) was used to analyze cell cycle. For apoptosis analysis, a kit of Annexin V-FITC/PI (BD, USA) was used. Similar steps were repeated until cells were transferred into 1.5 mL Eppendorf tubes. The re-suspended cells were centrifuged at 1000 rpm for 5min, dyed with Annexin V-FITC (5 μ L, in 200 μ L Binding Buffer) at 37°C for 10min in the dark, centrifuged again and dyed with PI (5 μ L, in 200 μ L Binding Buffer) followed by analyzing using a flow cytometer (FACSAria, BD, USA).

Wound – healing assay Cell density was adjusted to 1×10^7 cells/mL, and 2 mL cell suspension was added to each well of a pre-marked 6-well plate. The plate was shaken slightly and cells were distributed evenly. When cells were grown to 95% confluence, 2 parallel straight lines were made using a sterile 100-1000 μ L tip for each wall. Thereafter, cellular debris was washed away by PBS, medium containing Res (0, 100 and 200 μ mol/L) was added (each in duplicate) and photographs were taken at selected regions using $\times 100$ magnification of an inverted phase contrast microscope (Eclipse TE2000-u, Leica, Swiss). After incubation for another 24h, pictures were taken again at the same regions.

Transwell migration assay For this part, cells were re-suspended with serum-free medium in the presence of Res (0, 100 and 200 μ mol/L) to the density of 4×10^4 cells/mL. The 200 μ L cell suspension was loaded on the upper

chamber of each transwell insert (each in duplicate), and 600 μ L serum-containing medium on the lower chamber. Cultured for 16 and 24h, cells on the upper side of the filter were removed with a cotton swab, and cells migrated to the lower side were fixed with 4% paraformaldehyde (PAF) at room temperature for 10min, stained with crystal violet solution (Beyotime Biotechnology, China) for 5min. Ten random un-overlapping fields were photographed under $\times 100$ magnification (Eclipse TE2000-u, Leica, Swiss) for each membrane.

Western blot Cell suspension was loaded on a 6-well plate (1×10^5 cells/mL, 2mL per well; each in duplicate), cultured for 24h before the treatment with Res in distinct concentrations (0, 100, 150 and 200 μ mol/L). After continued incubation for 48h, total protein in duplicate was extracted with newly-prepared lysis buffer (500 μ L per well) containing 100 mmol/L phenylmethanesulfonyl fluoride (PMSF; Sigma, USA) for 30min on ice, homogenates were cleared by centrifugation at 12 000 rpm for 15min at 4 $^{\circ}$ C, and the supernatants were collected. The protein content was qualified by a Pierce BCA protein assay kit. Equal protein in each sample was loaded and run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF; Milipore, USA) membrane. After blocked by 5% nonfat milk dissolved in phosphate buffer solution with Tween-20 (PBST) at room temperature for 1h, PVDF membranes were probed by primary antibodies [anti-PCNA, -P21 and -P27; glyceraldehyde-3-phosphate dehydrogenase (GADPH) as internal control] diluted (1:300) in 5% nonfat milk dissolved in PBST overnight at 4 $^{\circ}$ C on a rotator, and then bound by secondary antibodies (anti-IgG-Cy3; 1:1000) at 4 $^{\circ}$ C on a rotator for 1h. Immunoblots were detected by enhanced chemiluminescence (ECL) for 2min, followed by film exposure (1-5min). For the detection of MMP-9 and p38MAPK, experimental design was distinct from the previous as cells were treated with Res of 0, 100 and 200 μ mol/L for 24h and α -tublin was designed as the internal control.

Statistical Analysis Data were shown as mean \pm SD, analyzed using SPSS 16.0 for windows. The statistical difference was assessed by One-way analysis of variance (ANOVA). $P < 0.05$ was considered to be significant difference.

RESULTS

The Effect of Resveratrol on ARPE -19 Cell Proliferation CCK-8 test revealed that Res with different concentrations (ranging from 50 to 300 μ mol/L) reduced the proliferation index of ARPE-19 cells in a concentration- and time-dependent manner (Figure 1). The 100-200 μ mol/L Res effectively decreased the proliferation index (%) to around 50% under the treatment for 48h, and the difference was significant compared with the control group ($P < 0.01$).

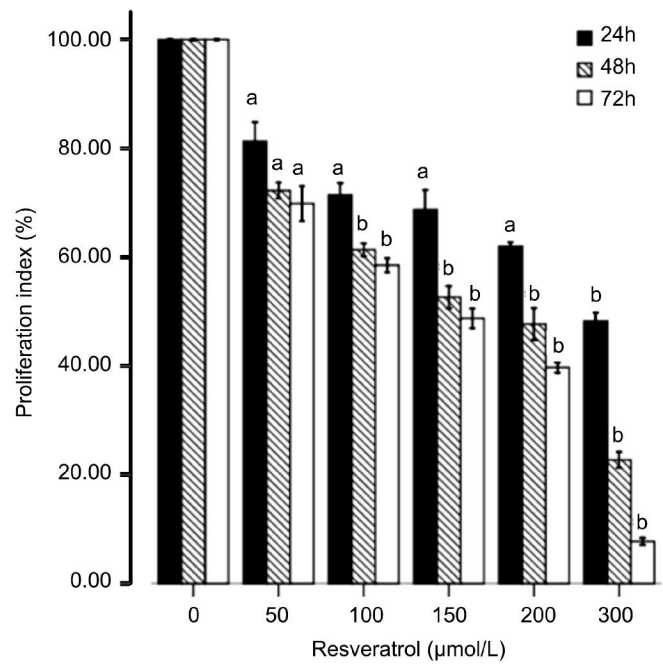


Figure 1 The inhibitory effect of Res on ARPE -19 cell proliferation Cells were treated with Res of 0, 50, 100, 150, 200 and 300 μ mol/L for 24, 48, 72h. CCK-8 test was performed to gain the OD values at 450 nm. The proliferation index was calculated according to the formula that proliferation index (%) = $(A - A_{\text{blank}}) / (A_0 - A_{\text{blank}})$, where A is the OD value in the treatment group; A_0 and A_{blank} are the OD values of the control and blank control groups. The difference was significant compared with the control group. ^a $P < 0.05$, ^b $P < 0.01$ vs the control group ($n = 15$).

Cell Cycle and Apoptosis Analysis According to Flow cytometric technology, cells arrested in S-phase increased in a concentration-dependent manner when treated with Res (0, 100, 150 and 200 μ mol/L; for 48h) (Figure 2A). The percentages of cells in S-phase of 100, 150 and 200 μ mol/L groups were $(38.80 \pm 0.28)\%$, $(41.28 \pm 0.07)\%$ and $(49.57 \pm 0.41)\%$, and compared with the control ($11.33 \pm 0.03\%$), the statistical differences were significant ($P < 0.05$) (Figure 2B). The apoptosis analysis revealed the apoptosis ratio of the early and late stage (zone B_4 and B_2 in Figure 3) in each treatment group was of no significant difference compared with the control ($P > 0.05$).

The Effect of Resveratrol on ARPE-19 Cell Migration

Wound-healing assay and Transwell migration assay were performed to testify migration ability of ARPE-19 cells when cells treated with Res. In wound-healing assay, under the treatment of 100 and 200 μ mol/L Res for 24h, the wound-healing was obviously suppressed compared with the control group, and 200 μ mol/L Res provided the best inhibitory effect (Figure 4). This was verified by a quantitative analysis of Transwell migration assay (Figure 5A), by which the migration ratio could be obtained. The migration ratios in 100 and 200 μ mol/L groups were $(0.6479 \pm 0.075)\%$ and $(0.4313 \pm 0.040)\%$ at 24h, $(0.6049 \pm 0.076)\%$ and $(0.3598 \pm 0.062)\%$ at 16h, respectively (Figure 5B). The statistical difference

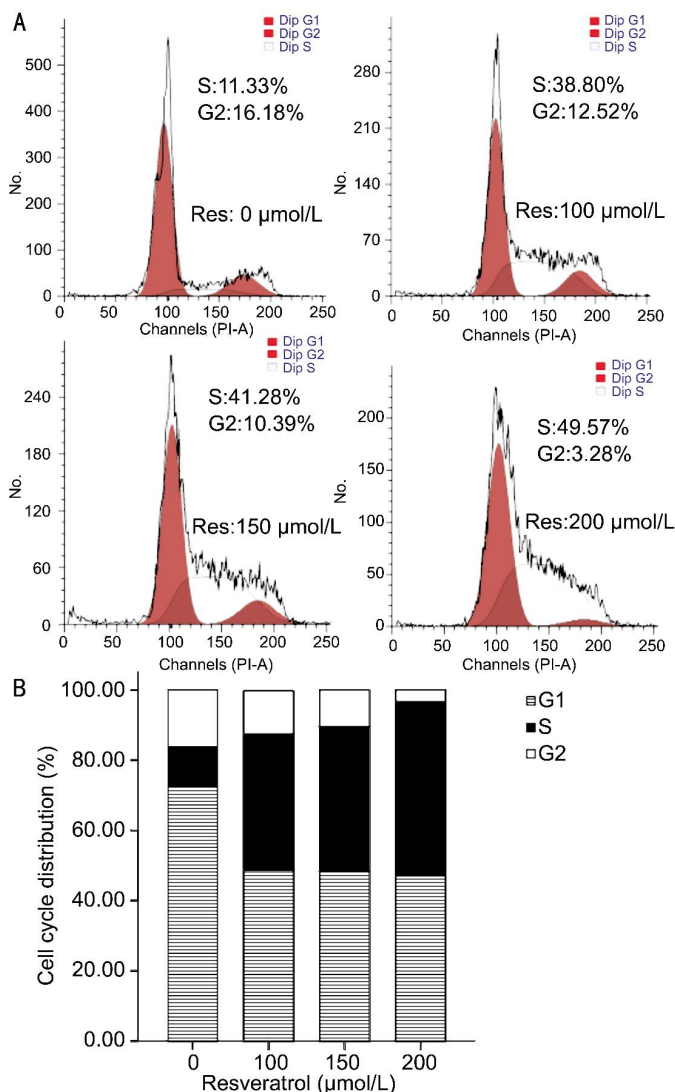


Figure 2 The analysis of cell cycle arrest of Res on ARPE-19 cells. Cells were exposed to selected concentrations of Res (0, 100, 150 and 200 µmol/L) for 48h, stained by propidium iodide (PI, 50 µg/mL), and analyzed by a flow cytometer. A: A representative experiment; B: Cell cycle distribution in each phase (G1, S and G2). Cells in S-phase increased with the concentration of Res increasing, and the difference was significant compared with the control group. $P < 0.05$ vs the control group ($n = 6$).

was significant between each treatment group and the control ($P < 0.05$).

The Expression of Relevant Proteins Protein expression was quantified by Western blot. The relative protein content was computed based on gray level analysis. A reduction of PCNA and increases of P21 and P27 were detected along with the increase of experimental concentrations (100, 150 and 200 µmol/L, for 48h) (Figure 6A, 6B). Meanwhile, descending trends of MMP-9 and p38MAPK were observed under the pre-treatment with Res at 100 and 200 µmol/L for 24h (Figure 7A, 7B). MMP-9 in 100 and 200 µmol/L groups to the control were (0.74±0.20)% and (0.54±0.28)%, and p38MAPK were (0.78±0.16)% and (0.48±0.13)%, respectively. The statistical difference was significant between each treatment group and the control ($P < 0.05$).

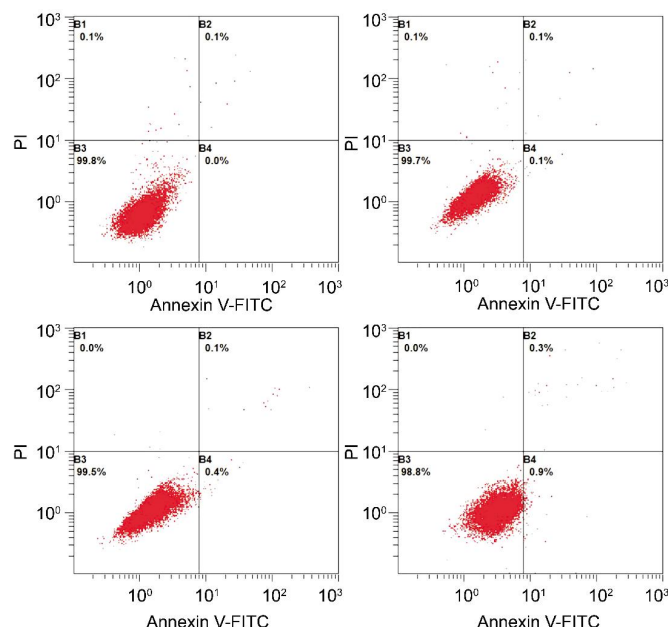


Figure 3 The apoptosis effect of Res on APRE-19 cell. Pretreated with Res in the same way as cell cycle analysis, cells were dyed with a method of Annexin V-FITC/PI double stain, and read using a flow cytometer. A typical experiment is shown above, where zone B₄ is the early apoptosis and zone B₂ represents the late apoptosis. $P > 0.05$ vs the control group ($n = 6$).

DISCUSSION

RPE cell proliferation and migration plays a critical role in the development of PVR, which raises a speculation whether a pharmacotherapy intending to obstruct these pathological behaviors could prevent PVR. Res, as a retarding factor to cell proliferation and migration, has been widely researched on disorders due to excessive cell growth like neoplasms and atherosclerosis [25-26]. In our study, CCK-8 test indicated that APRE-19 cell proliferation was inhibited by each selected dosage of Res (0, 50, 100, 150, 200 and 300 µmol/L). As shown, under the treatment with 150 µmol/L Res for 48h, cell proliferation index was reduced by 50%. Cell Cycle Analysis showed that Res (0, 100, 150 and 200 µmol/L; for 48h) could arrest cell cycle in S-phase, and meanwhile, Apoptosis Analysis revealed that under these conditions, Res was non-toxic to ARPE-19 cell. So we selected safety dosages and acting times within these ranges for subsequent experiments. As expected, in wound-healing assay and Transwell migration assay, Res with chosen dosages (100 and 200 µmol/L) could inhibit ARPE-19 cell migration. Cell proliferation is a complicated process closely related to cell cycle progress. PCNA is a well-conserved nucleoprotein constantly expressed in cells with vigorously proliferative capacities, functions as an activator for DNA pol-δ/ε and other regulatory proteins relative with DNA replication [17]. Among its multiple functions, i.e. chromatin re-modelling, DNA repair, sister-chromatid, cohesion and cell cycle control, cell cycle control has been elucidated as an important part. Besides, cell cycle is regulated by the sequential activation of

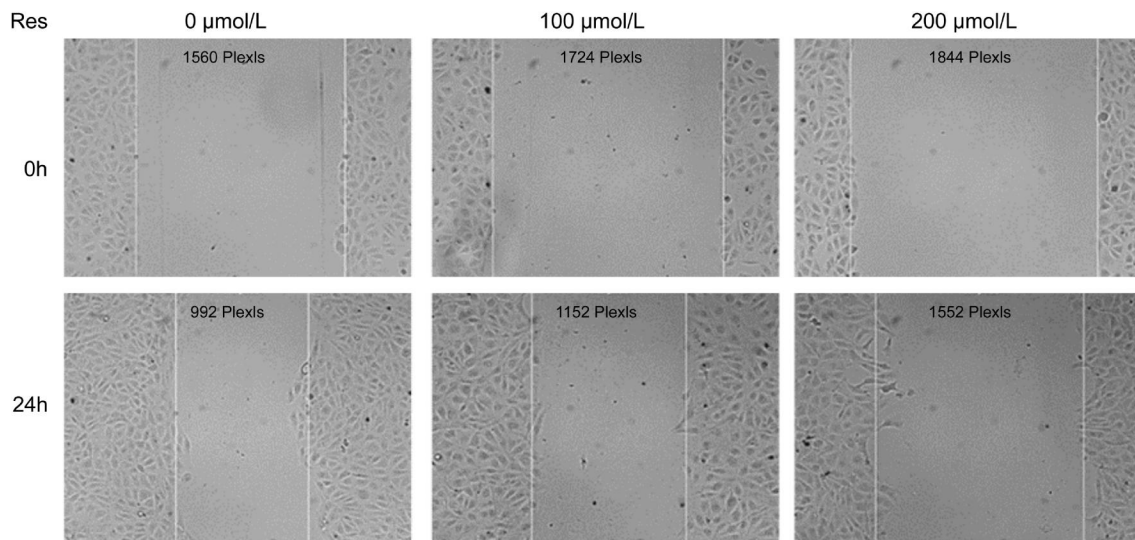


Figure 4 The inhibitory effect of Res on ARPE-19 cell migration A scratch was made on the monolayer cells, and Res at concentrations of 0, 100 and 200 $\mu\text{mol/L}$ was added for further culture of 24h. The wound-healing extent was observed and photographed ($\times 100$ magnification) at 0 and 24h. Pictures were disposed by Image J (National Institutes of Health, USA). A representative outcome is displayed as above.

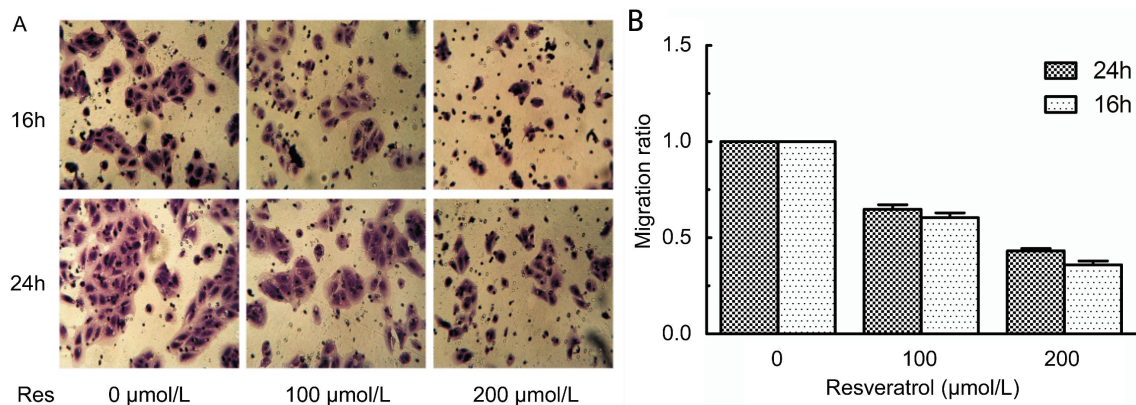


Figure 5 The inhibitory effect of Res on ARPE-19 cell migration Cells were allowed to migrate at a Transwell migration device under the treatment with Res (0, 100 and 200 $\mu\text{mol/L}$; for 16 and 24h) as stated. Thereafter, migrated cells were fixed, stained (crystal violet staining) and photographed ($\times 100$ magnification). Cells in each field were counted and the migration ratio was computed. A: The migrated cells; B: The histogram of the migration ratio. The statistical difference was significant between each treatment group and the control. $P < 0.05$ vs the control group ($n = 60$).

various cyclin/Cdk complexes, *i.e.* cyclin-D/Cdk4 (6), cyclin-E/Cdk2, cyclin-B/Cdk1/ and cyclin-A/Cdk1 [19]. P21 (P21^{Cip1}) and P27 (P27^{Kip1}), which belong to the Cip/Kip family of CKIs, are nonspecific inhibitive factors to cell cycle progress, functioning by associating with cyclin/Cdk complexes and inhibiting activities of kinases. What's more, it has been illustrated that the C terminal domain of P21 has the unique affinity with PCNA, thus facilitates the inhibitory effect of P21 [20]. Hsieh *et al* [27] have demonstrated that Res exhibits an inhibitive effect on bovine pulmonary artery endothelial cell proliferation accompanying with an elevating level of P21, and an effect of arresting cell progression in S and G2 phases. Similarly, Hseu *et al* [28] have proved that PCNA is up-regulated, and P21 together with P27 are down-regulated when human breast cancer cell (MDA-MB-231) proliferation is inhibited by Antrodia camphorate. In our study, firstly, we conducted the Flow cytometric analysis. Cell cycle and apoptosis analysis demonstrated that Res,

under selected dosages (100, 150 and 200 $\mu\text{mol/L}$), showed the effect of cell cycle arrest in S-phase and was non-toxic on ARPE-19 cells. Studies have reported that Res arrests different cells at different cell cycle stages (G1-, S- and G2-phase) and its effects on cell cycle and apoptosis of different cell types are highly variable [15]. What's obvious in our study, S-phase cells in treatment groups increased and G₂-phase cells decreased along with the increase of Res. Thus we could draw a conclusion that Res inhibits ARPE-19 cell proliferation via arresting cells in S-phase rather than promoting cell apoptosis or arresting cells in G1- or G2-phase. Then we performed Western blot to learn whether Res functions on ARPE-19 cell proliferation via regulating PCNA, P21 and P27. As expected, a decrease of PCNA, and sharp increases of P21 and P27 were detected when cells were treated with 100, 150 and 200 $\mu\text{mol/L}$ Res for 48h. Cell migration is a complex activity involving multi-steps including the degrading of ECM. MMPs are a family of

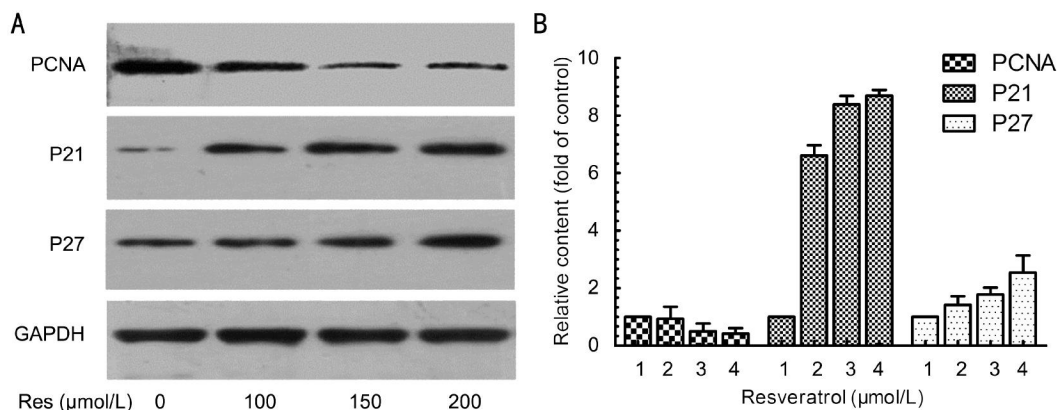


Figure 6 The expression of PCNA, P21 and P27 Cells were pre-treated with Res of 100, 150 and 200 μmol/L for 48h, followed by Western blot. Gel imaging analysis system and Image J were used to dispose images. Relative content of each protein in treatment groups to the control was gained by the grey value, and the bargraph was obtained using GraphPad Prism 5. A: Gel images of PCNA, P21 and P27; B: The bargraph of the relative content of PCNA, P21 and P27, where 1, 2, 3 and 4 in X-axis refer to different experimental concentrations of 0, 100, 150 and 200 μmol/L. The statistical difference was significant between each treatment group and the control. $P < 0.05$ vs the control group ($n = 3$).

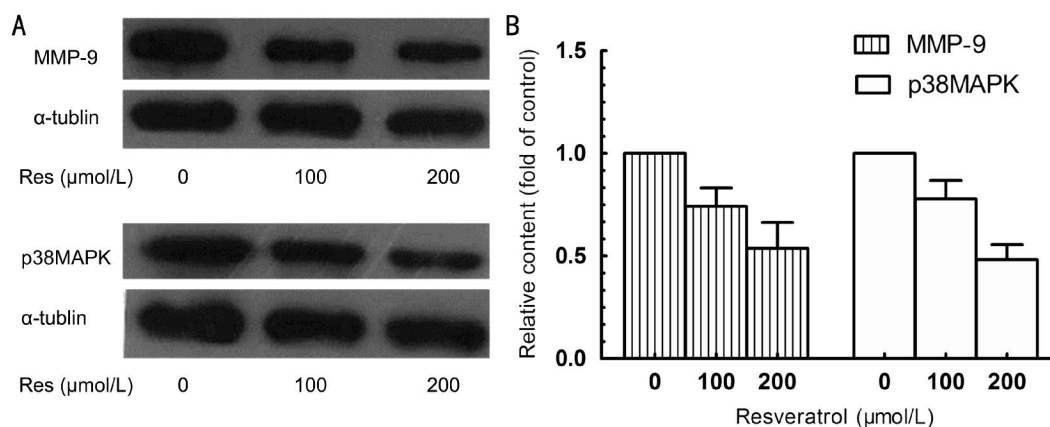


Figure 7 The expression of MMP-9 and p38MAPK Cells were treated with Res (0, 100 and 200 μmol/L) as the same way mentioned previously for 24h. A: Gel images of MMP-9 and p38MAPK; B: The bargraph of the relative content of MMP-9 and p38MAPK. The statistical difference was significant between each treatment group and the control. $P < 0.05$ vs the control group ($n = 3$).

zinc-dependent endopeptidases known as proteolytic enzymes mainly targeting at ECM^[29]. MMP-9 (also known as gelatinase B) is reported one of the most important subclasses of MMPs, mainly degrading type IV collagen of the ECM^[21]. MAPK pathway is important in regulating multi-processes like cell migration, invasion, proliferation, survival. At the same time, MAPKs are intricately involved in MMPs expression^[30]. Existing studies indicated that MMP-9 and p38MAPK decrease when cell migration is restrained^[30-32]. In our study, MMP-9 expression decreased under the treatment with 100 and 200 μmol/L Res, which went with a parallel reduction of p38MAPK.

It's worth noting that PCNA and MMPs have been investigated in some experiments as the therapeutic targets of PVR. Mandava *et al*^[33] conducted an experiment where they used a chimeric ribozyme targeted rabbit PCNA and successfully prevented PVR in dispase-induced rabbit models, and in vitro, they verified the inhibitory effect on cell proliferation by delivering the PCNA ribozyme into RPE cells and fibroblast cells. Ozerdem *et al*^[34] proved MMP-9 was an available therapeutic target by injecting 0.5 mg

Prinomastat, a synthetic inhibitor of matrix metalloproteinases, into vitreous cavity of dispase-induced rabbit models. These reports, together with the present study support the point that PVR might be prevented by interfering relevant proteins, and obstructing ARPE-19 cell proliferation and migration.

In conclusion, our study verified that ARPE-19 cell proliferation and migration could be inhibited by Res, and the expression of PCNA, P21, P27 as well as MMP-9 and p38MAPK could be simultaneously regulated. Given our previous results on ARPE-19 cell, further studies observing morphological changes of retina, testing electrophysiological function of retina and measuring protein expression in vitreous humor and retina of PVR models on animals when they are administrated with Res are expected to be the next step.

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Conflicts of Interest: Hao XN, None; Wang WJ, None; Chen J, None; Zhou Q, None; Qu YX, None; Liu XY, None; Xu W, None.

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