Fisetin inhibits epidermal growth factor–induced migration of ARPE-19 cells by suppression of AKT activation and Sp1-dependent MMP-9 expression

Hung-Yu Lin,[1,2,3,4] Yong-Syuan Chen,[5] Kai Wang,[1,6,7] Hsiang-Wen Chien,[1,6,7] Yi-Hsien Hsieh,[5,8,9] Shun-Fa Yang[1,10]

(The last two authors contributed equally to this work.)

[1]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan; [2]School of Medicine, Chung Shan Medical University, Taichung, Taiwan; [3]Department of Ophthalmology, Show Chwan Memorial Hospital, Changhua, Taiwan; [4]Department of Optometry, Yuan Pei University, Hsinchu, Taiwan; [5]Institute of Biochemistry, Microbiology and Immunology, Chung Shan Medical University, Taichung, Taiwan; [6]Departments of Ophthalmology, Cathay General Hospital Sijhih Branch, New Taipei City, Taiwan; [7]Department of Ophthalmology, Cathay General Hospital, Taipei, Taiwan; [8]Department of Biochemistry, School of Medicine, Chung Shan Medical University, Taichung, Taiwan; [9]Clinical laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan; [10]Department of Medical Research, Chung Shan Medical University, Taichung, Taiwan

Purpose: Proliferative vitreoretinopathy (PVR) can result in abnormal migration of RPE cells. Fisetin is a naturally occurring compound that has been reported to have antitumor effects, but its effects on epidermal growth factor (EGF)–induced cell migration and the underlying mechanisms remain unclear.
Methods: Effects of fisetin on EGF-induced cell viability and migration were examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and in vitro migration assays. Reverse transcription–PCR (RT–PCR) and immunoblotting were performed to evaluate matrix metallopeptidase-9 (MMP-9) expression and activation of specificity protein-1 (Sp1) and protein kinase B (AKT) in ARPE-19 cells treated with EGF and with or without fisetin. Luciferase and chromatin immunoprecipitation (ChIP) assays were performed to examine Sp1 transcription activity and MMP-9 binding activity.
Results: Fisetin did not affect ARPE-19 cell viability and significantly inhibited the EGF-induced migration capacity of ARPE-19 cells. Furthermore, fisetin exerted an antimigratory effect and suppressed MMP-9 mRNA and protein expression. Treatment with EGF induced phosphorylation of AKT and expression of MMP-9 and Sp1. Fisetin combined with LY294002 (an inhibitor of AKT) prevented the EGF-induced migration involved in downregulation of Sp1 and MMP-9 expression. Luciferase and ChIP assays suggested that fisetin remarkably decreased the EGF-induced transcription activity of MMP-9 and Sp1 and inhibited EGF-mediated Sp1 from directly binding to the MMP-9 promoter in ARPE-19 cells.
Conclusions: Fisetin inhibited EGF-induced cell migration via modulation of AKT/Sp1–dependent MMP-9 transcriptional activity. Therefore, fisetin may be a potential agent in the treatment of migratory PVR diseases.

Proliferative vitreoretinopathy (PVR) is a common complication of retinal detachment and open-globe injury in the posterior segment of the eye [1]. Pathologic changes in the RPE are considered to be a key element in the process of PVR [2]. The main cell not only forms and shrinks the proliferative membrane but also produces the driving factor to attract fibroblasts that participate in the formation of proliferative membranes [3]. These RPE cells can then proliferate, dedifferentiate, and undergo an epithelial-to-mesenchymal transformation to help create the preretinal membranes of

PVR [4-6]. The exact mechanism involved in the migration process of PVR remains to be elucidated.

Fisetin (3,7,3,4-tetrahydroxyflavone) is a flavonol, a structurally distinct chemical substance that belongs to the flavonoid group of polyphenols and has been isolated from many fruits and vegetables [7]. Previous studies have demonstrated that fisetin has antimicrobial, anti-inflammatory, antioxidant, antitumor, and antimigratory capacities against different cancers [8-11]. Hitt et al. reported that fisetin and luteolin inhibit the effects of oxidative stress-induced cell death in ARPE-19 cells [12]. Research has also shown that fisetin can protect ARPE-19 cells from DNA damage–induced cell death via decreased interleukin-6 (IL-6)/IL-8 expression, acetylation of p53, and promotion of the SIRT1 protein [13].

Correspondence to: Yi-Hsien Hsieh, Department of Biochemistry, School of Medicine, Chung Shan Medical University, Taichung, Taiwan; Shun-Fa Yang, Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan; Phone:+886-0424730022; FAX: +886-04-23248110; email: hyhsien@csmu.edu.tw; ysf@csmu.edu.tw.

The balance between production and degradation of the extracellular matrix (ECM) is tightly regulated, and matrix metalloproteinases (MMPs) are associated with the degradation of collagen and other ECM proteins [11]. The family of MMPs is thought to be involved in multiple pathways, including invasion and metastasis. Specifically, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) degrade collagen of the basement membrane and are involved in tumor progression and degenerative diseases [14,15]. In addition, other reports have shown that MMP-2 and MMP-9 activity correlates with PVR membrane formation [16] and facilitates cell migration in PVR [17]. Patients with PVR have higher levels of MMP-2 and MMP-9 expression [18]. However, the effects of fisetin on EGF-induced cell migration via MMP-9 expression in ARPE-19 cells remain unknown. During the PVR process, accumulating evidence indicates that tyrosine kinase growth factor receptors (RTK), such as epidermal growth factor receptor (EGFR), are activated, leading to cell proliferation and migration in retinal cells [19-21]. In the present study, we evaluated the molecular mechanism by which fisetin leads EGF-induced RPE cells to migrate. We found that fisetin inhibits EGF-induced cell migration by modulating the protein kinase B (AKT) regulation of MMP-9 proteins and reducing the expression of Sp1 transcription factors.

METHODS

Antibodies and reagents: Fisetin was purchased from Sigma (St. Louis, MO). EGF was purchased from R&D Systems, Inc (Minneapolis, MN). Antibodies against p-AKT (Ser 473; sc-7985-R), t-AKT (sc-56878), NF-κB (sc-372), c-fos (sc-52), Sp1, Lamin B (sc-6216), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Dallas, TX). MMP-2 (ab92536) and MMP-9 (ab137867) were purchased from Abcam (Cambridge, UK). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. LY294002 was purchased from Calbiochem (San Diego, CA).

Cell culture and treatments: The adult human RPE ARPE-19 cell line (BCRC No 60,383) was obtained from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). The ARPE-19 cell lines were analyzed to genotype with short tandem repeat (STR) analysis (Case Number: ECID20170003). Authentication Service (Mission Biotech, Taipei, Taiwan) using tandem repeat analysis plus the Amelogenin gender determining locus and was a perfect match for the ATCC human cell line CRL-2302 (ARPE-19). The STR analyses are presented in Appendix 1. Cells were cultured at 37 °C with 5% CO_2, in Dulbecco's modified Eagle's medium-F12 (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic. EGF with a 20 ng/ml final concentration was used for cell stimulation. For the general EGF treatment experiments, cells were starved in serum-free medium overnight followed by incubation with EGF (20 ng/ml) and fisetin (5 or 10 μM) for another 24 h. For the experiments with an inhibitor, LY294002 (30 μM) was added to the medium 2 h before EGF (20 ng/ml) or fisetin (5 or 10 μM) treatment.

Assessment of cell viability: Cell viability was determined with an MTT assay as previously described [22]. In the beginning, we plated the cells in 24-well culture plates (2×10^5 cells/well). Cells were treated for 24 h with different concentrations of fisetin or EGF (20 ng/ml) combined with fisetin. Then cells were incubated with MTT reagents (5 mg/mg) for 4 h. After incubation for 4 h, the cells were solubilized with isopropanol. Absorbance of the solution was measured using a Multiskan Ascent 96 Plate Reader (MTX Lab Systems, Bradenton, FL) at 570 nm.

In vitro scratch wound healing assay: The ARPE-19 cells were seeded at a density of 8×10^5 cells/well into a 24-well plate containing DMEM culture medium supplemented with 10% FBS. After incubation for 24 h, the DMEM media were completely removed, and the adherent cell layer was scratched with a sterile yellow pipette tip. The cells were treated with EGF or EGF combined different concentrations of fisetin in serum-free DMEM media. Percent migration was calculated for the left scratch and then the right scratch as previously reported [23].

In vitro migration assay: The ARPE-19 cells were incubated with different concentrations of fisetin or EGF (20 ng/ml) combined with fisetin for 24 h. Migration assays were performed in Boyden chambers with minor modifications [24]. The wells of the Boyden chambers (48-well, pore size 8 μm) were seeded with 1×10^5 cells in medium with 0.1% FBS. DMEM-F12 medium with 10% FBS was added to the lower chamber and served as a chemotactic agent. After 24 h incubation, nonmigrating cells were wiped from the upper side of the membrane. Cells that penetrated the membrane were fixed with methanol, the cells were stained with 0.1% Giemsa stain for 30 min, and then the migrated cells on the underside of the membrane were counted under a microscope at 100X magnification. Four random fields of each transwell membrane were counted and averaged.

Western blotting: Cells were lysed in NETN buffer, and protein concentrations were determined using a Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by sodium dodecyl
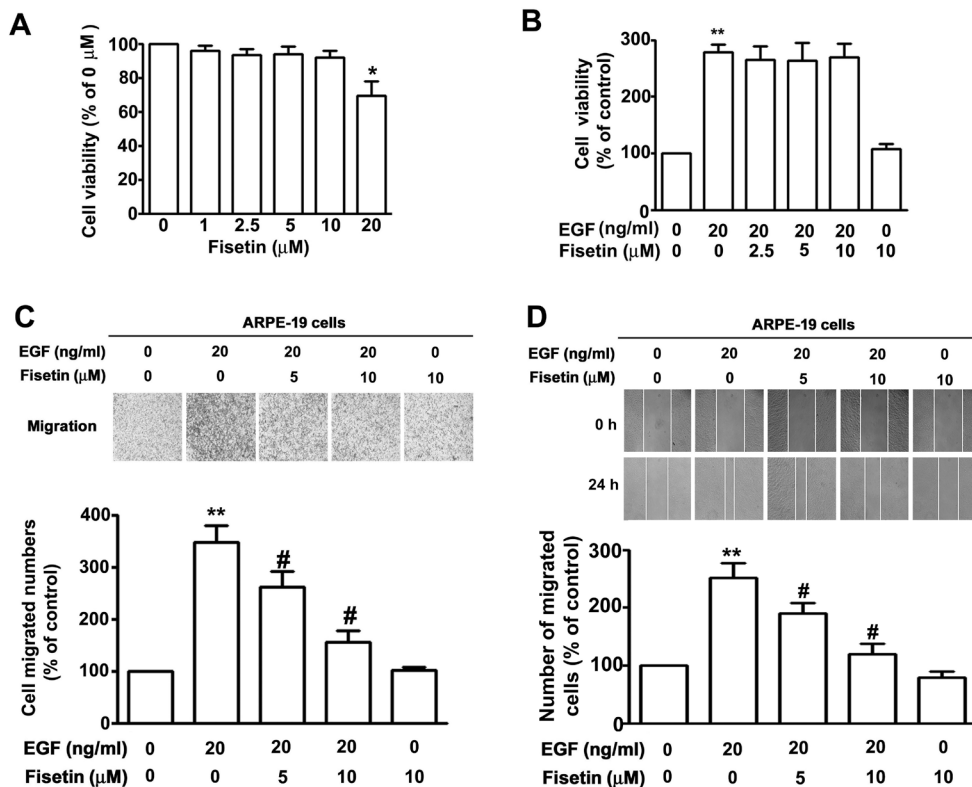
Figure 1. Effects of fisetin on EGF in terms of cell viability and migration of ARPE-19 cells. **A**: ARPE-19 cells were treated with different concentrations of fisetin for 24 h and were tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **B**: ARPE-19 cells were treated with epidermal growth factor (EGF; 20 ng/ml) or were cotreated with EGF (20 ng/ml) plus different concentrations of fisetin (5, 10, and 20 µM) for 24 h before analysis with the MTT assay. **C**: In vitro determination of cell migration. **D**: Microscopy images of ARPE-19 cell migration after scratch (time 0) and after 12 h of EGF, EGF (100 ng/ml) or EGF (100 ng/ml) plus different fisetin (5 and 10 µM) treatments. Quantification of migrating cells is shown as a histogram. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. **p<0.01 compared with control cells.

sulfate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking with Tris-buffered saline (TBS) containing 5% nonfat dry milk for 2 h, the membranes were incubated with primary antibodies overnight at 4 °C. After 24 h, the membranes were incubated with a horseradish peroxidase second antibody for 2 h at room temperature. Immunoreactive bands were detected with enhanced chemiluminescent substrate. Band intensities were quantified using Scion Image software (Frederick, MD) and were normalized to β-actin.

RNA extraction and reverse transcription–PCR (RT–PCR) analysis for mRNA expression of MMP-2 and MMP-9: Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with the PrimeScript RT Reagent Kit protocol (Takara Bio, Inc, CA). Semiquantitative PCR was performed using GoTaq®qPCR Master Mix reagents (Promega Corporation, Madison, WI). RT-PCR was performed using GoTaq® qPCR Master Mix reagents (Promega Corporation, Madison, WI). The PCR cycle was programmed for 30 s at 95 °C for initial denaturation, followed by 30 cycles of 30 s at 95 °C for denaturation, 30 s at 54°C for annealing, 60 s at 72 °C for extension, and 5 min

at 72 °C for the final extension. Gene-specific primer pairs used for amplification were as follows: for β-actin, 5′-CCA TCG TCC ACC GCA AAT-3′ (forward) and 5′-CAT GCC AAT CTC ATC TTG TTT-3′ (reverse); for MMP-2, 5′-CTC ATC GCA GAT GCC TGG AA-3′ (forward) and 5′-TTC AGG TAA TAG GCA CCC TTG AAG A-3′ (reverse); and for MMP-9, 5′-GTC CAC CCT TGT GCT CTT CC-3′ (forward) and 5′-GCC ACC CGA GTG TAA CCA T-3′ (reverse). β-actin was used as an endogenous control.

Extraction of Sp1 protein: The ARPE-19 cells (2 × 10⁶) were treated with fisetin in the presence or absence of EGF for 24 h. Cells were immediately washed twice and centrifuged at 1,500 ×g for 10 min. Cytoplasmic and nuclear extracts were prepared from the cells using the NE-PER nuclear and cytoplasmic extraction reagent (Rockford, IL).

Reporter assay analysis for Sp1, NF-κB, AP-1, and MMP-9 promoter activities: The ARPE-19 cells (5 × 10⁴ cells/well) were seeded in 24-well cell culture plates. The pGL3-MMP-9, mutant MMP-9 (Sp1 mutant), -Sp1, -NF-κB, and -AP1 promoter were kindly provided by Dr. Yang (Department of Medical Research, Chung Shan Medical University, Taichung, Taiwan). After 24 h of incubation, the pGL3-basic

(vector) and the MMP-9, -MMP-9 (Sp1 mutant), -Sp1, -NF-κB, and -AP1 promoter plasmid were cotransfected with a β-galactosidase expression vector (pCH110) into cells using TurboFect reagents (Fermentas, Carlsbad, CA). After 24 h of transfection, the cells were treated with EGF (20 ng/ml) in the presence or absence of fisetin for 24 h. Luciferase and β-galactosidase activities were reported as previously described [25]. Luminescence was measured using a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). The value of the luciferase activity was normalized for transfection efficiency and was monitored with β-galactosidase expression.

Chromatin immunoprecipitation (ChIP): Cells were grown to 70% confluence in 10-cm culture dishes. ARPE-19 cells (2×10^6/well) were treated with fisetin in the presence or absence of EGF for 24 h. Cells were cross-linked with 4% formaldehyde for 10 min, 125 mM glycine was added for 5 min at room temperature, and then the cells were washed and harvested in ice-cold PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4). The sonified chromatin was treated as described previously [26], using 2 μg of anti-Sp1 (Santa Cruz Biotechnology). Purified DNA was used for PCR analysis. The human MMP-9 promoter-specific primer pairs used for amplification were as follows: MMP-9, 5′-CCA TCG TCC ACC GCA AAT-3′ (forward) and 5′-CAT GCC AAT CTC ATC TTG TTT-3′ (reverse). Chromatin immunoprecipitates from the proteins were amplified with PCR, normalized to input, and calculated as percentages of inputs.

Statistical analysis: Each data set was presented as mean ± standard error of the mean (SEM) from at least three independent experiments. The significance of the differences between the sets was assessed with one-way ANOVA (ANOVA) followed by Tukey's multiple comparison test in GraphPad Prism version 4 (GraphPad Software, San Diego, CA). The differences were considered statistically significant at a p value of less than 0.05.

RESULTS

Effect of fisetin on EGF-treated ARPE-19 cell viability: In the present study, the results of the MTT assay suggested that limited cytotoxicity to ARPE-19 cells was noted after treatment with concentrations of fisetin lower than 20 μM (Figure 1A). Next, we examined the effect of fisetin on EGF-treated cell viability in ARPE-19 cells and found that fisetin presented no significant toxicity to the ARPE-19 cells that were treated with EGF at concentrations between 5 and 10 μM for 24 h or were not exposed to EGF (Figure 1B). These concentrations were studied further in subsequent experiments.

Effect of fisetin on EGF-treated ARPE-19 cell migration: In vitro migration assays suggested that EGF (20 ng/ml) treatment resulted in a significant increase in the cell migration (Figure 1C) of ARPE-19 cells. Conversely, pretreatment with fisetin (5 or 10 μM) markedly reduced cell migration in EGF-treated ARPE-19 cells (Figure 1C). Similar to the results with the wound-healing assay, we found that the rate of wound closure increased significantly in the EGF-treated cells and
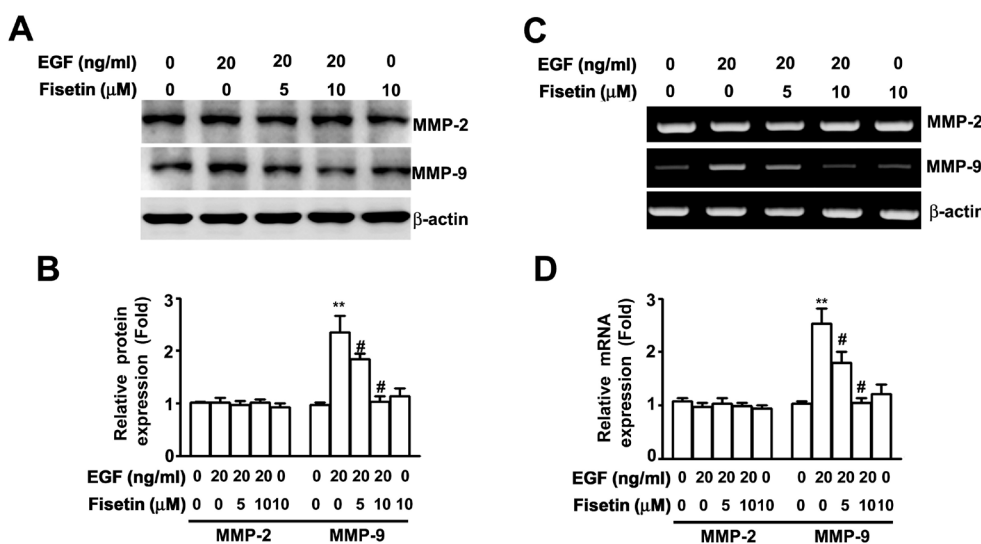

Figure 2. Effects of treatment with fisetin in EGF-induced MMP-2 and MMP-9 expression in ARPE-19 cells. **A**: Western blotting for the protein of matrix metallopeptidase-2 (MMP-2) and MMP-9 in epidermal growth factor (EGF; 20 ng/ml) or following treatment with EGF (20 ng/ml) plus fisetin (5 and 10 μM) for 24 h. **B**: Quantitative analysis of western blots. **C**: Reverse transcription–PCR (RT–PCR) analysis of MMP-2 and MMP-9 mRNA expression. **D**: Quantitative analysis of RT–PCR. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. β-actin was used as internal control. **p<0.01 compared with control cells; #p<0.01 compared with fisetin.
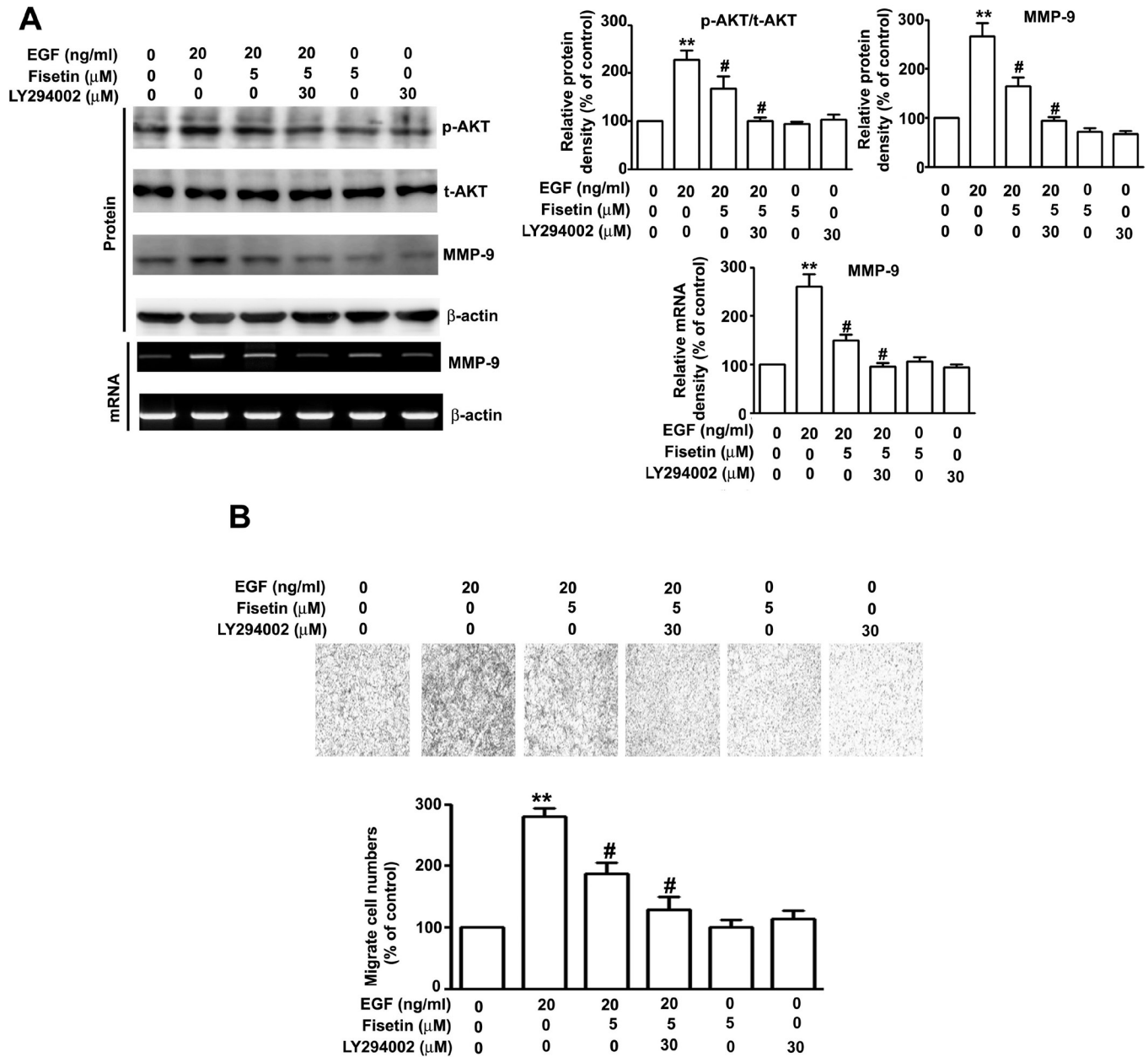
Figure 3. Effects of treatment with either fisetin or LY294002 on EGF-induced AKT activation in ARPE-19 cells. **A**: ARPE-19 cells were treated with epidermal growth factor (EGF; 20 ng/ml) or were cotreated with EGF (20 ng/ml) plus fisetin (5 μM) plus LY294002 (30 μM) for 24 h; western blots show p-AKT, t-AKT, and matrix metallopeptidase-9 (MMP-9) expression. The reverse transcription–PCR (RT–PCR) assay shows MMP-9 mRNA expression. **B**: The migratory abilities of cells were determined using an in vitro migration assay. Quantification of migrated cells is shown as a histogram. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. β-actin was used as an internal control. **p<0.01 compared with control cells; #p<0.01 compared with fisetin.

that treatment with EGF combined with fisetin significantly delayed wound closure (Figure 1D). These results indicated that fisetin significantly inhibits EGF-induced migration in ARPE-19 cells.

Fisetin suppressed EGF-induced MMP-9 expression, but not MMP-2 expression, in ARPE-19 cells: MMP-2 and

MMP-9 activation has been shown in the migration of ARPE-19 cells [27,28]. Therefore, the effects of fisetin on EGF-induced MMP-2/MMP-9 expression were examined. The results showed that treatment of ARPE-19 cells with fisetin significantly inhibited EGF-induced MMP-9 protein expression (Figure 2A,B). RT–PCR analysis showed that the
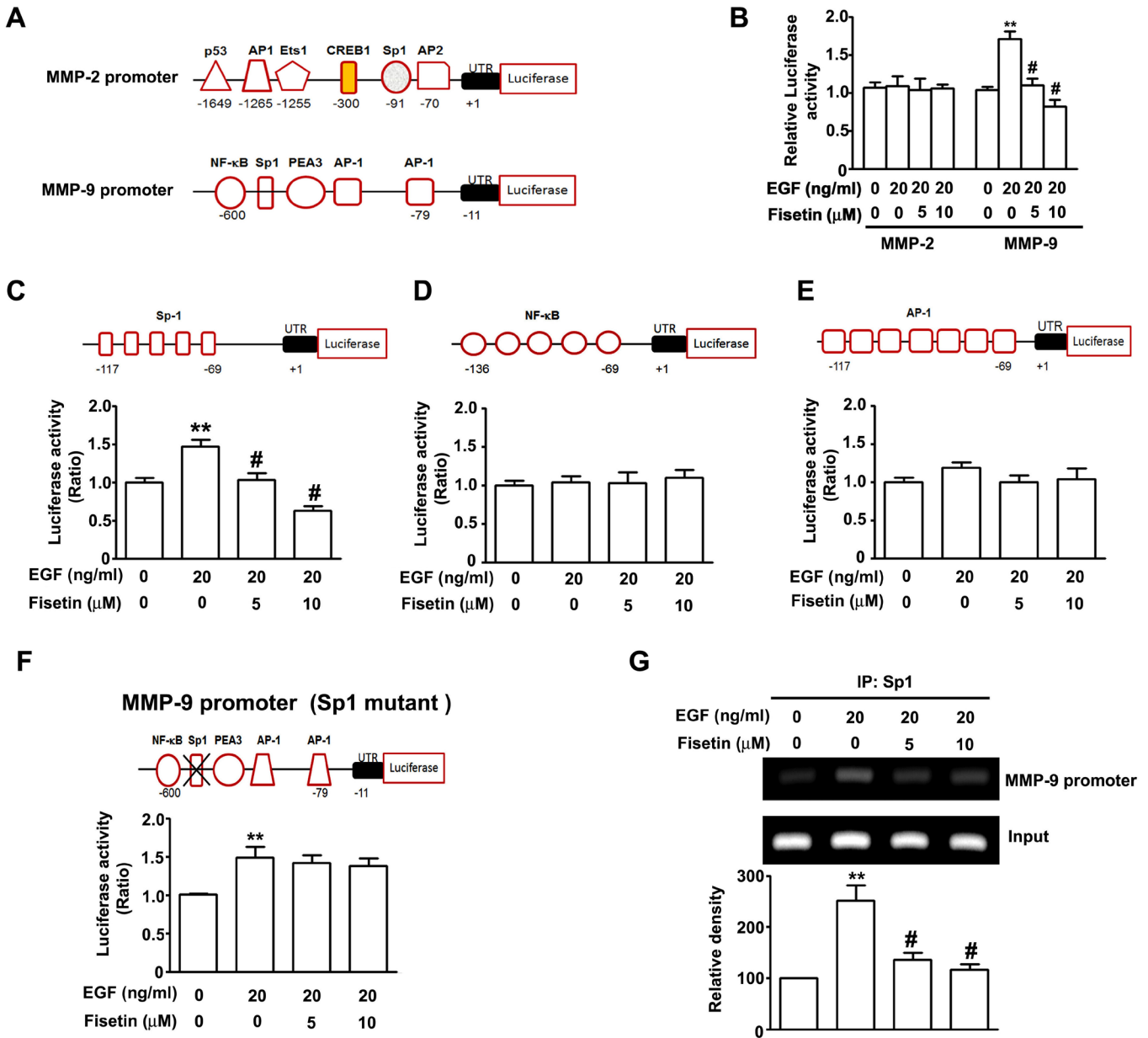
Figure 4. Fisetin inhibits the EGF-induced transcriptional activity of MMP-9 by decreasing the DNA-binding activity of Sp1 on the matrix metallopeptidase-9 (MMP-9) promoter in ARPE-19 cells. ARPE-19 cells were treated with epidermal growth factor (EGF; 20 ng/ml) or were treated with EGF (20 ng/ml) plus fisetin (5 and 10 μM) for 24 h. **A**: Schematic of the transcription-factor binding regions on the human MMP-2 and MMP-9 promoter. **B**: Luciferase assay analysis of MMP-2 and MMP-9 transcription activity. **C–E**: Schematic representation of reporter plasmids of Sp1, NF-κB, and AP1 promoters (left to right) and responsive elements (upper portion). ARPE-19 cells were transiently transfected with reporter plasmids of Sp1, NF-κB, and AP1 promoters and were treated with fisetin for 2 h with or without EGF for 24 h. **F**: Schematic presentation of the MMP-9 promoter Sp1 mutant plasmid (shown at the top) and the luciferase activity of MMP-9. **G**: Chromatin immunoprecipitation (ChIP) analysis of the Sp1 binding to the MMP-9 promoter region treated with fisetin for 2 h with or without EGF for 24 h in ARPE-19 cells. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. **p<0.01 compared with control cells; #p<0.01 compared with fisetin.
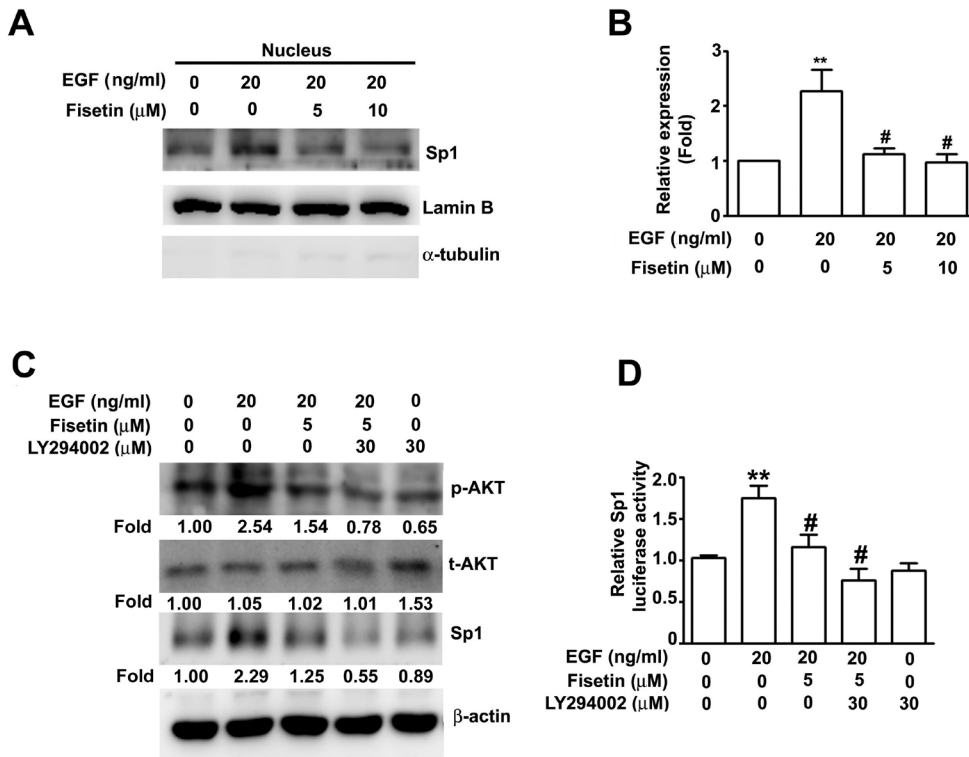
Figure 5. Effects of fisetin on EGF-induced the expression of AKT and Sp1 in ARPE-19 cells. **A**: Western blotting of the nuclear fraction of Sp1 in epidermal growth factor (EGF; 20 ng/ml) or treated with EGF (20 ng/ml) plus fisetin (5 and 10 μM) for 24 h. **B**: Quantitative analysis of western blots. **C**: Western blotting for the expression of p-AKT, AKT, and Sp1. **D**: Sp1 activity was detected with the luciferase assay. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. **p<0.01 compared with control cells; #p<0.01 compared with fisetin.

mRNA level of MMP-9 increased following EGF (20 ng/ml) treatment and then MMP-9 expression was reduced by treatment with fisetin (5 and 10 μM) in a dose-dependent manner (Figure 2C,D). However, we found no significant difference in the effects on MMP-2 protein or mRNA expression (Figure 2C,D).

Fisetin suppressed EGF-induced cell migration via inactivation of AKT in ARPE-19 cells: Next, we evaluated the effects of fisetin on EGF-regulated AKT signaling pathways, because AKT is the downstream effector kinase of EGFR and is an important mediator of ARPE-19 cell migration [29,30]. The results of the western blotting analysis showed that treatment of ARPE-19 cells with fisetin significantly inhibited EGF-induced AKT activation (Figure 3A, upper portion). To investigate whether the inhibition of MMP-9 by fisetin occurred mainly via the inhibition of AKT activation, fisetin was combined with EGF in the absence or presence of LY294002, an inhibitor of PI3K in ARPE-19 cells. We found that fisetin combined with LY294002 significantly reduced the EGF-induced protein expression of MMP-9 (Figure 3A, upper portion) and mRNA expression of MMP-9 (Figure 3A, lower portion). In addition, we showed that LY294002 combined with fisetin markedly inhibited EGF-mediated cell migration in ARPE-19 cells (Figure 3B).

Fisetin suppressed EGF-induced transcriptional activation of Sp1 and reduced Sp1 binding on the MMP-9 promoter in ARPE-19 cells: Based on the results of the RT–PCR assay, fisetin significantly decreased the EGF-induced MMP-9 mRNA expression of ARPE-19 cells. We further determined whether fisetin inhibited EGF-induced MMP-9 promoter activity and transcription-factor binding activity in ARPE-19 cells. The luciferase reporter assay demonstrated that EGF induced only the promoter activity of MMP-9 but did not affect MMP-2 promoter activity (Figure 4A,B). These results indicate that fisetin inhibited EGF-induced MMP-9 transcriptional activity. We assessed the effects of fisetin on EGF-mediated Sp1, NF-κB, and AP-1 luciferase promoter activities. In the results, EGF induced only Sp1 luciferase promoter activity (Figure 4C), and other luciferase promoters seemed not to be affected (Figure 4D,E). Treatment of ARPE-19 cells with fisetin markedly inhibited EGF-induced Sp1 luciferase activity in a dose-dependent manner (Figure 4C). Next, to determine the transcription factor Sp1, involved in regulation of MMP-9 transcription, we generated a promoter with a mutation in the Sp1-binding site. We observed that fisetin did not inhibit the EGF-induced MMP-9 promoter activity when the Sp1-binding site was mutated (Figure 4F). To explore whether fisetin-inhibited EGF induces the binding activity of Sp1, we performed the ChIP assay. We found that EGF induces Sp1 binding to MMP-9 promoters in ARPE-19

cells (Figure 4G). In contrast, fisetin treatment significantly decreased EGF-induced Sp1 binding to the MMP-9 promoter in a dose-dependent manner (Figure 4G). The overall results provide strong evidence that fisetin prevents the EGF-induced binding activity of Sp1 to reduce the transcription levels of MMP-9 in ARPE-19 cells.

Fisetin inhibits EGF-induced accumulation of nuclear Sp1 via AKT activation in ARPE-19 cells: To determine the role of the nuclear translocation of Sp1, we examined the expression of Sp1 transcription factors in the cells' nuclear fractions, and we evaluated the AKT-dependent pathway using western blotting. We found that fisetin significantly inhibited EGF-induced Sp1 translocation to the nucleus in ARPE-19 cells (Figure 5A,B). However, we also found that LY294002 combined with fisetin significantly inhibited the EGF-induced nuclear fraction of Sp1 (Figure 5C) and decreased the Sp1 luciferase promoter activity (Figure 5D) in ARPE-19 cells. These results showed that fisetin inhibits EGF-induced cell migration by suppression of AKT activation and decreased the accumulation of nuclear Sp1 expression.

DISCUSSION

This is the first report of the antimigration effects of fisetin on ARPE-19 cells. In this study, we found that the flavonoid compound fisetin was safe and nontoxic for ARPE-19 cells. Fisetin significantly decreased EGF-induced cell migration. We also found that the protein and mRNA expression of MMP-9 was inhibited by fisetin treatment in EGF-treated ARPE-19 cells. Fisetin decreased the EGF-mediated phosphorylation of AKT. Fisetin also decreased MMP-9 transcription activity and inhibited Sp1 direct binding to MMP-9 promoter in ARPE-19 cells. Based on these data, the present results provide evidence that fisetin prevented human RPE cells from EGF-induced migration with low toxicity and good efficacy. For these reasons, fisetin may be a useful supplement to slow the development of PVR in vitro.

Fisetin is isolated from the smoke tree (*Cotinus coggygria*) and is generally found in various fruits and vegetables. Some studies have suggested a suppressive effect of fisetin on various biologic and biomodulating activities, including antiproliferation, antioxidation, and antimigration in RPE cells [12,31,32]. Several studies have shown that
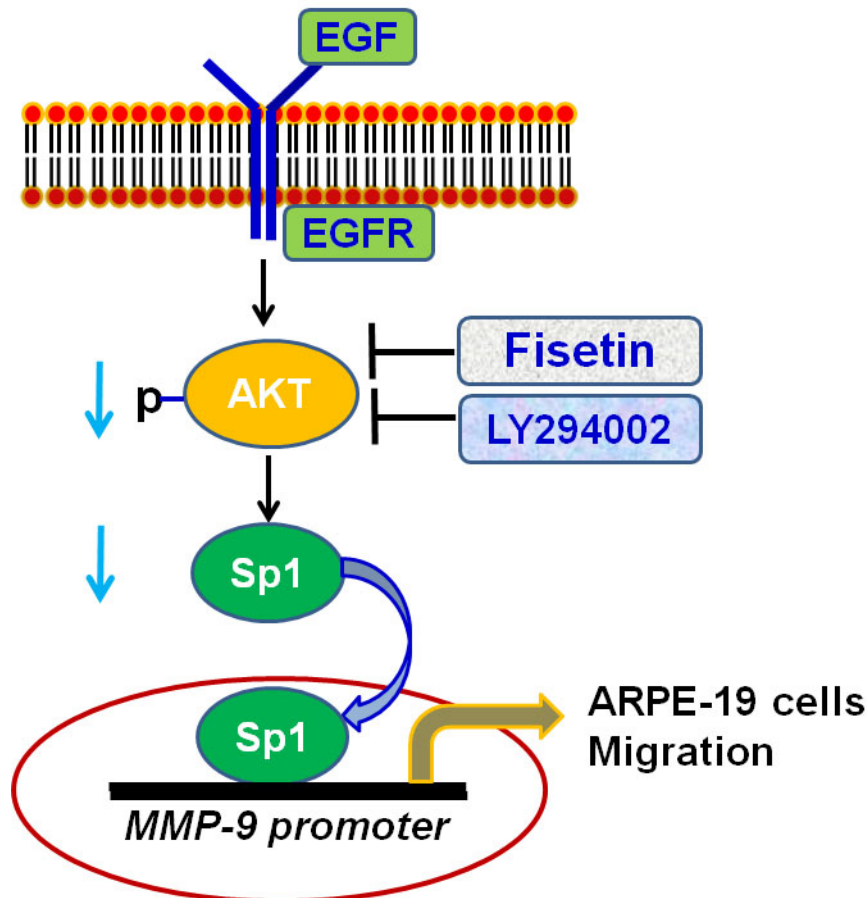

Figure 6. A model describing the molecular mechanisms by which fisetin suppresses EGF-induced ARPE-19 cell migration. Fisetin could inhibit EGF induced the phosphorylation of AKT, thereby inhibited the EGF-induced Sp1 translocation and MMP-9 transcription activity, and further reduced migration of ARPE-19 cells.

EGF induces the EGFR/mitogen-activated protein kinase (MAPK)/AKT signal transduction pathway and contributes to the proliferation and migration of RPE cells. For these reasons, EGF is considered a major factor in the development of PVR [33-36]. Previous reports suggested that hepatocyte growth factor (HGF) coupled with EGF or heparin-binding EGF-like growth factor (HB-EGF) mediates the PVR-related changes of RPE by increasing expression of the PKCδ and extracellular-signal-regulated kinase (ERK) pathways [37]. However, that fisetin with EGF induces PVR-related cellular changes in the RPE has not been reported. We found that AKT acts downstream of EGFR for cell migration of RPE induced by EGF (Figure 2). This finding is consistent with the findings of previous studies that demonstrated the role of ERK/AKT and PI3K/AKT in mediating cell migration of RPE cells induced by EGF [29,30]. Taking advantage of this finding, we showed that fisetin acts as an inhibitor of EGF signaling and suggested that fisetin could reduce the efficacy of EGF in the migration of RPE cells.

MMPs are a family of zinc-dependent endopeptidases and are known as proteolytic enzymes that mainly target ECM [38]. MMP-9 is a critical molecule and strongly correlates with cell migratory and invasive phenotypes, and MMP-9 activation reportedly has been correlated with RPE cell migration [39]. MMP-9-mediated cell signaling requires the presence of growth factor and receptors, such as the EGF [33], and EGF activates different intracellular signaling pathways in RPE cells, such as glycogen synthase kinase-3 beta (GSK-3β)/beta-catenin [40], PKCδ/ERK pathways [37], and PI3K/AKT [29]. In this study, we found that EGF/AKT-dependent migration in RPE cells was inhibited by fisetin. Interestingly, pretreatment with fisetin significantly reduced the EGF-induced phosphorylation of AKT and the MMP-9 expression (Figure 6). These results provide important information suggesting that fisetin could be used as a treatment agent for PVR by inhibiting AKT expression that depends on MMP-9. Previous studies have indicated that MMP-9 inhibition is followed by the regulation of MMP-9 promoter activity by transcription factors such as NF-κB, AP-1, and SP1 [41-43]. In this study, we found that EGF specifically stimulated the nucleus and promoted Sp1 expression, and fisetin inhibited EGF-stimulated expression of nuclear Sp1 in ARPE-19 cells. On one hand, previous reports have suggested that Sp1 is a key regulator of the MMP-9 promoter [44] and AKT is associated with regulating the expression of transcriptional factor Sp1 [45,46]. The present results suggested that EGF strongly induced the transcriptional activity of Sp1 and direct binding to the MMP-9 promoter, which was prevented by fisetin in EGF/AKT-mediated Sp1 transcription. To our knowledge, this is the first evidence regarding the importance of Sp1 for

MMP-9 regulation in RPE cells. Therefore, we hypothesize that the EGF/AKT/SP1/MMP-9 pathway plays an important role in regulating the cell migratory activities in RPE cells.

In conclusion, we found that fisetin prevents human PRE cells against EGF-induced migration via AKT regulated on MMP-9 transcription activity (Figure 6) and thus, may be helpful for the treatment of abnormal migration of RPE cells in PVR. Further studies to investigate the effects of fisetin on RPE in vivo are warranted. The findings of this study suggest that fisetin has the potential to be helpful for the treatment of the abnormal migratory activities of RPE cells in PVR.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words "Appendix 1."

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