Original Paper

Protective Effects of Fucoïdan on Epithelial-Mesenchymal Transition of Retinal Pigment Epithelial Cells and Progression of Proliferative Vitreoretinopathy

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Key Words

Fucoïdan • Epithelial-mesenchymal transition • Retinal pigment epithelium • Proliferative vitreoretinopathy

Abstract

Background/Aims: Proliferative vitreoretinopathy (PVR) is a severe blinding complication of rhegmatogenous retinal detachment. Epithelial-mesenchymal transition (EMT) of retinal pigment epithelial (RPE) cells is thought to play a pivotal role in the pathogenesis of PVR. Fucoïdan, a marine extract, reportedly has many benefits effects in a variety of tissues and organs such as anti-inflammation, anti-oxidative stress, and anti-carcinogenesis. In this study, we investigated the potential role of fucoïdan on EMT in RPE cells and its effect on the development of PVR. **Methods:** MTS, Transwell, and collagen gel contraction assays were employed to measure the viability, migration, and contraction of RPE cells, respectively. mRNA and protein expression were evaluated via real-time quantitative PCR and western blot analysis, respectively. *In vivo*, a pigmented rabbit model of PVR was established to examine the anti-PVR effect of fucoïdan. **Results:** Fucoïdan reversed the transforming growth factor (TGF)- β 1-induced EMT of RPE cells, including the increased expression of α -smooth muscle actin (α -SMA) and fibronectin and down-regulation of E-cadherin in human primary RPE cells. Moreover, the upregulation of phosphorylated Smad2/3 induced by TGF- β 1 was suppressed by fucoïdan. Fucoïdan also inhibited the migration and contraction of RPE cells induced by TGF- β 1. *In vivo*, fucoïdan inhibited the progression of experimental PVR in rabbit eyes. Histological findings showed that fucoïdan suppressed the formation of α -SMA-positive epiretinal membranes. **Conclusion:** Our findings regarding the protective effects of fucoïdan on the EMT of RPE cells and experimental PVR suggest the potential clinical application of fucoïdan as an anti-PVR agent.

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Introduction

Proliferative vitreoretinopathy (PVR), a severe blinding complication of retinal detachment surgery, is characterized by the formation of an epi/sub-retinal membrane and traction of the reattached retina [1, 2]. The retinal pigment epithelium (RPE) is thought to play a pivotal role in the pathogenesis of PVR due to its primary appearance among several cell types established in PVR fibrotic membranes [3]. Epithelial-mesenchymal transition (EMT), which enables RPE cells to lose their epithelial properties and transform into mesenchymal cells, is considered as the fundamental mechanism underlying the formation of the PVR membrane [4, 5]. Similar to EMT in carcinogenesis, the EMT of RPE cells involves the activation of the relevant cellular pathway, rearrangement of the cytoskeleton, and disassembly of the junctions between RPE cells. Transforming growth factor (TGF)- β , a classic EMT trigger, is also found in the eye of PVR patients. Therefore, blocking the EMT of RPE cells might be an efficient way to prevent PVR. However, despite the study of the EMT of RPE cells in PVR for decades, there is no currently available drug to prevent it.

Brown algae and other marine products have long been regarded as beneficial for the human body. The exact molecules underlying these beneficial effects are being discovered gradually with the development of biopharmaceutical techniques. Fucoidan, a sulfated polysaccharide extracted from brown algae and marine invertebrates, was first isolated by Kylin in 1913. Fucoidan reportedly exerts many beneficial effects in inflammation, oxidative stress, and carcinogenesis [6-8]. In ophthalmic studies, fucoidan appears to have a therapeutic effect in several blinding diseases such as age-related macular degeneration (AMD) and diabetic retinopathy. In 2013, Yang et al. demonstrated that fucoidan alleviates diabetic retinal neovascularization and damage through the inhibition of hypoxia-inducible factor-1 α and vascular endothelial growth factor [9]. In 2015, Li et al. suggested that fucoidan normalizes reactive oxygen species and protects RPE cells against oxidative damage, which plays an essential role in the pathogenesis of AMD [10]. Fucoidan was also reported to block EMT by regulating the ERK1/2, Akt, p38, and Smad3 pathways and subsequently prevent renal interstitial fibrosis and ameliorate the progression of diabetic nephropathy [11]. However, it is still unclear whether fucoidan could inhibit the EMT of RPE cells, and its effect on the progression of PVR has not been investigated. In this study, we examined the effect of fucoidan on the EMT of RPE cells and the development of PVR.

Materials and Methods

Reagents and antibodies

Fucoidan from *Fucus vesiculosus* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant TGF- β 1 was purchased from Gibco (Carlsbad, CA, USA). The following antibodies were used for western blotting and immunofluorescence analyses: anti- α -smooth muscle actin (SMA; A2547; Sigma-Aldrich), anti-fibronectin (F7387; Sigma-Aldrich), anti-E-cadherin (610182; BD Biosciences, San Jose, CA, USA), anti- β actin (ab119716; Abcam Ltd., Cambridge, MA, USA), anti-GAPDH (ab8245; Abcam Ltd.), anti-ZO-1 (14-9776-80; Invitrogen, Carlsbad, CA, USA), anti-phospho-Smad2/3 (8828; Cell Signaling Technology, Danvers, MA, USA), and anti-Smad2/3 (3102; Cell Signaling Technology). Secondary antibodies for western blotting were IRDye 800CW and IRDye 680LT (Li Cor Biosciences, Lincoln, NE, USA). Most of the other reagents, such as salt and buffer components, were analytical grade and obtained from Sigma-Aldrich.

Ethics statements

The current research involving human participants was approved by the Ethics Committee of Shanghai Tenth People's Hospital and was in compliance with the Declaration of Helsinki. Donors' eyes were obtained from the Eye Bank of Shanghai Tenth People's Hospital. All animal experimental designs were approved by the Animal Care and Use Committee of Shanghai Tenth People's Hospital, Tongji University. This study was approved by the Science and Technology Commission of Shanghai Municipality (ID: SYXK 2011-0111). All methods were performed in accordance with the relevant guidelines and regulations.

Cell culture

Human primary RPE cells (obtained from the Eye Bank of Shanghai Tenth People's Hospital) were used in this study. Human primary RPE cells were isolated from human donors according to previously published protocols [12]. Passage 2–4 cells were used in this study. Primary RPE cells were cultured at 37°C in a 5% CO₂ humidified incubator with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). The medium was changed every 2–3 days. For further experiments, the cells were seeded and cultivated for 12 h. Then, the cells were starved with DMEM/F12 medium supplemented with 1% penicillin-streptomycin without FBS for 24 h before treatment with 10 ng/mL TGF-β1.

Cell viability assay

Primary RPE cells were seeded on 96-well plates at a density of 1.0×10^4 cells per well. RPE cells were exposed to serum-free medium containing fucoidan at different concentrations. Each experiment was performed in 6 independent wells. After 48 h, cell viability was determined using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was measured at 490 nm using a microplate spectrophotometer (Thermo, Waltham, MA, USA).

Real-time quantitative PCR

Total RNA was extracted at the indicated time points using the TRIzol reagent (Invitrogen) according to manufacturer's protocol and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc., Carlsbad, CA, USA). cDNA was synthesized using a PrimerScript™ RT Reagent Kit (Takara Clontech, Kyoto, Japan). Real-time PCR was performed in triplicate using a SuperReal PreMix Plus (SYBR Green) Kit (Takara Clontech) on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). Each reaction contained 12.5 μL of 2× SYBR® Premix Ex Taq™ (with SYBR Green I), 300 nM oligonucleotide primers synthesized by Generay Corp. (Nanjing, China), and 1 μL cDNA in a final volume of 25 μL. The thermal cycling conditions included an initial denaturation step at 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. mRNA expression was normalized to the level of β actin mRNA. The sequences for the real-time quantitative PCR primers were as follows: human E-cadherin sense: 5'-TCACGCTGTGTCATCCAACGG-3' and antisense: 5'-TAGGTGTTACATCATCGTCCGC-3'; human α-SMA sense: 5'-CAGAAGGAGATCAGGCCCTAG-3' and antisense: 5'-CGGCTTCATCGTATTCCTGTTTG-3'; human fibronectin sense: 5'-AAGACCATACCCGCCGAATG-3' and antisense: 5'-GGCATTTGGATTGAGTCCCG-3'; and human β actin sense: 5'-GGCTGTATTCCCCTCCATCG-3' and antisense: 5'-CCAGTTGGTAACAATGCCATGT-3'.

Western blot analysis

The cells were lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with phenylmethylsulfonyl fluoride and PhoSTOP EASY pack phosphatase inhibitor (Roche, Mannheim, Germany) on ice for 30 min at 48 h after treatment. The lysates were clarified by centrifugation at 12000 rpm for 5 min at 4°C. Total protein concentration was quantified by a bicinchoninic acid assay kit (Thermo Scientific). Protein (40 μg) was loaded and separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked using 5% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 45 min at room temperature to prevent non-specific binding. The membrane was then incubated with primary antibodies diluted in 2% BSA in PBS with 0.1% Tween-20 (PBS-T) at 4°C overnight. After rinsing 3 times with PBS-T, the membrane was incubated with IRDye® 680LT goat anti-rabbit or IRDye® 800CW goat anti-mouse secondary antibodies (Li Cor Biosciences) at room temperature for 1 h. After washing 3 times with PBS-T, the bound antibodies were detected using an Odyssey Infrared Imaging System (Li Cor Biosciences). Band intensities were analyzed with Odyssey software and normalized to β actin or GAPDH.

Immunofluorescence analysis

Immunofluorescence analysis of RPE cells: the cells were seeded and cultured in a 24-well plate inlaid with glass coverslips. After treatment, the cells were washed and fixed in cold acetone for 5 min. After washing 3 times with PBS, the cells were blocked with 2% BSA for 1 h at room temperature and incubated with the primary antibodies overnight at 4°C. After being rinsed 3 times with PBS, the coverslips were then

incubated with FITC-conjugated secondary antibodies for 1 h at room temperature. After counterstaining with 4, 6-diamidino-2-phenylindole (DAPI), the stained coverslips were mounted and visualized under a confocal microscope (LSM710; Carl Zeiss, Jena, Germany).

Immunofluorescence analysis of rabbit retina: prepared slides were incubated in PBS for 10 min and then permeabilized in 0.25% Triton X-100 for 10 min. After washing 3 times in PBS, the slides were blocked with 1% BSA for 30 min and then incubated with the primary antibody anti- α -SMA at 4°C overnight. After washing for 15 min in PBS, the slides were incubated with FITC-conjugated secondary antibodies for 1 h at room temperature. After counterstaining with DAPI, the stained coverslips were mounted and visualized under a confocal microscope (LSM710; Carl Zeiss).

Transwell migration assay

After treatment, the cells were trypsinized and seeded in the upper chamber of a 24-well Transwell plate (8 mm pore size; Costar, Conning, CA, USA) in 100 μ L DMEM/F12 containing 0.5% FBS at a density of 5.0×10^4 cells per chamber. The lower compartment was filled with 600 μ L DMEM/F12 containing 10% FBS. After incubation at 37°C for 18 h, the cells on the upper surface of the filter were removed and the migrated cells on the lower surface of the culture inserts were fixed with ice-cold methanol and stained with 0.1% crystal violet for 30 min. The number of migrated cells in each chamber was then determined by counting 5 random fields. All experiments were performed in triplicate.

Collagen gel contraction assay

Collagen gel contraction assays were performed as described previously [13]. In brief, 24-well culture plates were coated with 1 mL of 1% BSA for 1 h at 37°C. Primary RPE cells pre-treated by TGF- β 1 with or without fucoidan (100 μ g/mL) were harvested and suspended in serum-free DMEM/F12. Collagen I (final concentration, 2 mg/mL; Gibco), 10 $\times$ DMEM/F12, cell suspension (final density, 2.5×10^5 cells/well), sterile distilled water, and sterile 1 N NaOH (0.025-fold of the volume of collagen) were prepared and mixed on ice. The mixture (total volume, 0.5 mL) was added to the BSA-coated wells and incubated for 1 h at 37°C under 5% CO₂ to promote polymerization of the gels. The gels were freed from the sides with a pipette tip and serum-free DMEM/F12 (0.5 mL) was then added to the top of the gel. Photographs were taken after 48 h to quantify the gel contraction area using ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD, USA).

Rabbit PVR model

PVR was induced in the right eye of pigmented rabbits. Twelve rabbits were divided into 2 groups. Both groups were PVR models, one served as the control and the other was treated with fucoidan. Briefly, at 7 days before the experiment, 0.2 mL aqueous humor was removed and 0.4 mL perfluoropropane was injected into the vitreous cavity at 3 mm posterior to the corneal limbus. PBS (100 μ L) with or without fucoidan (2000 μ g/mL) containing human primary RPE cells (passage 2–3, 2.0×10^5 cells) and 50 ng PDGF-BB (R&D Systems, Minneapolis, MN, USA) were injected into the vitreous cavity on day 1. All procedures were conducted under anesthesia. Fundus examinations were conducted initially and at day 1 (before injection), day 7, and day 14 via the combination of a microscope (EZ4; Leica, Wetzlar, Germany) and a Volk lens (SuperQuad®160; Volk, Mentor, OH, USA). Representative eyes in each group were chosen to conduct optical coherence tomography (OCT) and B-ultrasonic examinations according to the condition of the fundus. PVR was classified according to Fastenberg et al. [14]. In brief, the stage of PVR was based on the findings of retinal fundus examination as follows: stage 1, intravitreal membrane; stage 2, focal traction, localized vascular changes, hyperemia, engorgement, and blood vessel elevation; stage 3, localized detachment of the medullary ray; stage 4, extensive retinal detachment, total medullary ray detachment, and peripapillary retinal detachment; and stage 5, total retinal detachment, and retinal folds and holes. The eyes were harvested on day 14 for histologic and immunofluorescence analyses.

Statistical analysis

All experiments were performed at least 3 times. The mean and standard error of the mean (SEM) were calculated for all parameters determined in this study. Data were analyzed statistically using one-way ANOVA, two-tailed Student's t test, and Mann-Whitney U test (*P < 0.05, **P < 0.01).

Results

Fucoidan inhibits EMT in human primary RPE cells by inhibiting TGF- β 1-induced Smad2/3 phosphorylation

To investigate whether fucoidan can inhibit EMT in RPE cells, we first testified the cell viability of primary RPE cells treated with fucoidan at the recommended concentrations [15]. At 48 h after exposure to fucoidan, no significant influence on cell viability was observed within the range of concentrations tested (Fig. 1A).

We next examined changes in the expression of E-cadherin, α -SMA, and fibronectin in RPE cells. Treatment with TGF- β 1 (10 ng/mL for 48 h) significantly reduced the expression of E-cadherin and increased the expression of α -SMA and fibronectin at both the mRNA and protein level. The TGF- β 1-induced decrease of E-cadherin and increase of α -SMA and fibronectin could be reversed by fucoidan at both 50 μ g/mL and 100 μ g/mL (Fig. 1B and C). The relative changes of E-cadherin, α -SMA, and fibronectin were further validated by immunofluorescence analysis. Moreover, the morphologic continuity of ZO-1 was disrupted when the cells were treated with TGF- β 1, and this phenomenon could also be prevented by fucoidan (Fig. 2). Our results suggest that fucoidan could reverse the TGF- β 1-induced EMT of RPE cells.

We then investigated the effect of fucoidan on the TGF- β signaling pathway. Smad2/3 and phosphorylated-Smad2/3 were extracted from RPE cells treated with TGF- β 1 with or

Fig. 1. Fucoidan attenuates TGF- β 1-induced EMT in human primary RPE cells. (A) Cell viability analysis of fucoidan on primary RPE cells. Primary RPE cells were treated with fucoidan at different concentrations (0, 50, 60, 70, 80, 90, and 100 μ g/mL) for 48 h and then cell viability was examined with the MTS assay. Cell viability is expressed as the percentage of the normal control, which was defined as 100%. Data are expressed as the mean $\pm$ SEM (n = 3). (B) Real-time quantitative PCR analysis. Primary RPE cells were treated with 10 ng/mL TGF- β 1 with or without fucoidan (50 μ g/mL and 100 μ g/mL) for 48 h. The mRNA expression levels of E-cadherin, α -SMA, and fibronectin were detected with real-time quantitative PCR. The data are presented as the mean $\pm$ SEM. n = 3. *P < 0.05. (C) Western blot analysis of the expression of EMT-related proteins. Primary RPE cells were treated by 10 ng/mL TGF- β 1 with or without fucoidan (50 μ g/mL and 100 μ g/mL) for 48 h. Relative protein expression (normalized to β actin) was quantified in the western blots by determining their gray scale value. The data are presented as the mean $\pm$ SEM. n = 3. *P < 0.05. Two-tailed Student's t test was used to calculate P-values. Abbreviations: EMT, epithelial-mesenchymal transition; α -SMA, α -smooth muscle actin.

Fig. 2. Immunofluorescence analysis of EMT-related proteins in human primary RPE cells. After primary RPE cells were treated with 10 ng/mL TGF- β 1 with or without fucoidan (100 μ g/mL) for 48 h, ZO-1, E cadherin, α -SMA, and fibronectin were detected using the corresponding antibody. Nuclei were stained with DAPI. The slides were examined by confocal microscopy. Original magnification: 630 $\times$, oil. Scale bar: 10 μ m. Abbreviations: N, normal control; EMT, epithelial-mesenchymal transition; ZO-1, zonula occludens-1; α -SMA, α -smooth muscle actin. (Top to bottom: ZO-1, E-cadherin, α -SMA, fibronectin.)

Fig. 3. Fucoidan attenuates TGF- β 1-induced Smad2/3 phosphorylation in primary RPE cells. Primary RPE cells were treated with 10 ng/mL TGF- β 1 with or without fucoidan (50 μ g/mL and 100 μ g/mL) for 48 h. Phosphorylated Smad2/3 was analyzed by western blotting and quantified against relative total Smad2/3 protein. (A) Western blot analysis. (B) Relative Smad2/3 protein level. (C) Relative phosphorylated Smad2/3 protein level. The data are presented as the mean $\pm$ SEM. n = 3. *P<0.05. Two-tailed Student's t test was used to calculate P-values.

without fucoidan. Significant Smad2/3 phosphorylation was detected at 1 h after TGF- β 1 treatment, while treatment with TGF- β 1 and fucoidan inhibited Smad2/3 phosphorylation (Fig. 3). Taken together, these findings suggest that fucoidan could inhibit TGF- β 1-induced EMT in human primary RPE cells by suppressing the phosphorylation of Smad2/3.

Fucoidan attenuates TGF- β 1-induced migration of RPE cells and collagen gel contraction

We examined TGF- β 1-induced functional changes using a Transwell migration assay and collagen gel contraction assay. For Transwell migration, primary RPE cells pre-treated with TGF- β 1 with or without Fucoidan (100 μ g/mL) for 48 h were seeded in the Transwell migration system. Our results suggested that TGF- β 1 significantly promoted RPE cell migration, while fucoidan treatment significantly inhibited cell migration (Fig. 4A). The gel contraction assay also indicated that TGF- β 1 facilitated the contraction of RPE cells and that fucoidan could suppress this effect (Fig. 4B).

Fucoidan inhibits the progression of PVR in vivo

We investigated the effect of fucoidan on the progression of PVR *in vivo*. Among the 12 pigmented rabbits involved in this study, 6 were assigned randomly to the PBS group

and the other 6 were assigned to the fucoidan group. Fundus examinations ensured the fitness of the retina and the visibility of the normal pigmented rabbit fundus structure before the experiment (Fig. 5E). One rabbit was excluded from the PBS group on day 7 due to endophthalmitis, and another was excluded from the fucoidan group on day 14 due to cataract. The included rabbits and detailed information of the experiment are shown in Table 1 and Fig. 5.

Representative fundus photos from the PBS group demonstrated focal traction and localized detachment of the medullary ray (Fig. 5a) on day 7 and extensive retinal detachment

Fig. 4. Fucoidan attenuates TGF- β 1-induced migration and collagen gel contraction in primary RPE cells. (A) Transwell migration analysis. After treatment with 10 ng/mL TGF- β 1 with or without fucoidan (100 μ g/mL) for 48 h, RPE cells were harvested and seeded in the Transwell system for another 18 h. The number of migrated cells was quantified by counting 3 random vision fields under a microscope (magnification $\times 200$). The data are presented as the mean $\pm$ SEM. $n = 3$. $*P < 0.05$. Scale bar: 100 μ m. (B) Collagen gel contraction analysis. After treatment with 10 ng/mL TGF- β 1 with or without fucoidan (100 μ g/mL) for 48 h, primary RPE cells were harvested and seeded in a collagen gel system for another 48 h. The extent of gel contraction was quantified. The data are presented as the mean $\pm$ SEM. $n = 3$. $*P < 0.05$. Two-tailed Student's t test was used to calculate P-values.

Fig. 5. Fucoidan inhibits the progression of experimental PVR in rabbits. Progression of PVR stages in an experimental rabbit PVR model treated with PBS or fucoidan is presented as mean $\pm$ SEM. $n = 5$ /group. $**P < 0.01$. (a-e) Ocular fundus

photographs of normal eyes and eyes with PVR after treatment with PBS or fucoidan (200 μ g). For the PBS group, the light blue arrows represent the localized detachment of the medullary ray and extensive retinal detachment on day 7 and day 14, respectively; the green arrow represents a retinal hole. For the fucoidan group, the light blue arrows represent localized vascular changes and engorgements. Details of the PVR grading procedures for Grades 1–5 for the experimental PVR model are described in the Methods section. (f–k) OCT and B-ultrasonic report of normal eyes and eyes with PVR after treatment with PBS or fucoidan (200 μ g) on day 14. The arrows represent retinal detachment. The Mann-Whitney U test was used to calculate P-values. Abbreviations: OCT, optical coherence tomography.

(Fig. 5b) with a retinal hole (green arrow) on day 14. OCT and B-ultrasonic examinations confirmed retinal detachment on day 14 (Fig. 5f–k). However, among the 5 eyes treated simultaneously with fucoidan (200 μ g), the most advanced PVR was stage 3 in 1 eye, and the other 4 eyes were all stage 2 with slight focal contraction and moderate vessel tortuosity (Fig. 5c and d). Taken together, these findings show that PVR progression was significantly inhibited in the eyes injected with fucoidan compared with the eyes injected with PBS as a control. Histological hematoxylin and eosin (H&E) staining confirmed the presence of extensive retinal detachment and an obvious epiretinal membrane in the eyes injected with PBS, while no apparent retinal detachment was seen in the eyes injected with fucoidan, despite the presence of an unsmooth retinal surface (Fig. 6A). Immunofluorescence analysis showed an α -SMA-positive epiretinal membrane in the eyes treated with PBS, while slight α -SMA staining was seen in the eyes treated with fucoidan (Fig. 6B).

Discussion

Our study showed that fucoidan inhibits the TGF- β 1-induced EMT of RPE cells through Smad2/3 signaling. We further demonstrated the suppressive effect of fucoidan on the progression of PVR in a rabbit model, suggesting that fucoidan might be a potential therapeutic agent for PVR intervention.

PVR, which is characterized by the formation of an epi/sub-retinal membrane and postoperative tractional retinal re-detachment, occurs as a complication in 5–10% of patients with rhegmatogenous retinal detachment [2]. Modern secondary surgical repair may only lead to anatomical recovery with poor functional visual acuity [16]. Despite the great efforts made by researchers, PVR remains the primary reason for the failure of retinal re-attachment surgery. The current interpretation of the pathogenesis of PVR involves the migration of cells into the vitreous cavity, where they proliferate into membranes and their subsequent contractions lead to the development of retinal wrinkles and detachment [17, 18]. The RPE, a monolayer of pigmented cells located between the nerve retina and Bruch's membrane, is an important partner for the fit of the retina, and disturbance of the RPE may ultimately lead to retinal damage and disease. Various studies have investigated the relationship and underlying mechanism between the RPE and ocular diseases [19–21]. The RPE also participates in the pathogenesis of PVR. Among several cell types established in the PVR membrane, RPE cells are regarded to play a primary role within the disease as they represent the largest cellular component [22]. Since EMT represents the dedifferentiation of the RPE and was shown to

Table 1. Summary of the stage of PVR formation

Vitreous Injection		PVR stages					
		0	1	2	3	4	5
Day1	RPE cells with PBS (n=6)	6	0	0	0	0	0
	RPE cells with Fucoidan (n=6)	6	0	0	0	0	0
Day7	RPE cells with PBS (n=5)	0	0	1	2	2	0
	RPE cells with Fucoidan (n=6)	0	4	2	0	0	0
Day14	RPE cells with PBS (n=5)	0	0	0	0	1	4
	RPE cells with Fucoidan (n=5)	0	0	4	1	0	0

Fig. 6. Fucoidan inhibits the formation of the epiretinal membrane. (A) Histological H&E staining of a normal eye (a), eye with PVR after treatment with PBS (b), or after treatment with 200 μ g fucoidan (c). **Epithelial membrane. (B) Immunofluorescence of α -SMA in a normal eye (a), eye with PVR after treatment with PBS (b, arrows represent the α -SMA-positive epiretinal membrane with massive cell proliferation), or after treatment with 200 μ g fucoidan (c, arrow represents the unsmooth surface of the retina with slight cell proliferation). Original magnification: 400 $\times$. Scale bar: 20 μ m. Abbreviations: α -SMA, α -smooth muscle actin.

underlie the development of PVR by Casaroli-Marano et al. in 1999 [4], EMT of RPE cells has been regarded as the trigger of PVR pathogenesis, and TGF- β -induced EMT in cultured RPE cells has been widely adopted to study the mechanism of PVR *in vitro* [23-26].

In our study, we tested whether inhibition of EMT could block or ameliorate the progression of PVR. We first used TGF- β to induce EMT in RPE cells *in vitro*. Both isoforms of TGF- β (1 and 2) have been used to induce EMT in a variety of cell types including RPE cells. However, which isoform exerts the greatest contribution to PVR pathogenesis is still under debate. On one hand, the concentration of TGF- β 2 is much higher than that of TGF- β 1 in PVR vitreous [27]; on the other hand, the concentration of TGF- β 1 is significantly elevated during PVR development, while that of TGF- β 2 is increased to a much lesser extent [28, 29]. Our team successfully established a model of RPE cell EMT by TGF- β 1 in 2011 [30]. In our latest published study, we compared the effect of both isoforms in inducing human RPE cell EMT. Both TGF- β isoforms exhibited a similar effect in inducing EMT of RPE cells, such as the upregulation of the epithelial marker E-cadherin and the inhibition of the mesenchymal markers α -SMA and fibronectin [31]. Therefore, we utilized TGF- β 1 to induce EMT in RPE cells in the present study.

In our experiments, TGF- β 1 was used to induce EMT in human primary RPE cells. At 48 h after TGF- β 1 treatment, the expression of the epithelial differentiation marker E-cadherin was significantly suppressed in RPE cells and accompanied with an increase in the expression of the mesenchymal markers α -SMA and fibronectin. Our data also suggested that the classical TGF- β signaling component Smad2/3 was phosphorylated under TGF- β 1 treatment in RPE cells.

Fucoidan is a heterogeneous compound of sulfated polysaccharides with a high content of L-fucose and sulfate ester groups, and it was first isolated from marine brown algae by Kylin in 1913. Fucoidan reportedly has many beneficial effects in a variety of tissues and systems such as anti-inflammation, anti-oxidative stress, and anti-carcinogenesis [32, 33]. Fucoidan also displays protective effects against several ocular diseases. For example, fucoidan reportedly attenuates diabetic retinopathy via the inhibition of vascular endothelial growth factor [9]. Fucoidan was also demonstrated to scavenge superoxide radicals and inhibit oxidative stress and protect ARPE-19 cells against oxidative damage via normalization of the generation of reactive oxygen species, indicating a beneficial role for fucoidan in AMD [10]. Recently, several studies suggested that fucoidan could inhibit EMT in various cell types. For instance, Hsu et al. found that fucoidan could regulate EMT by modulating TGFR/Smad signaling, which leads to the inhibition of breast cancer metastasis *in vitro* and *in vivo* [34]. Yan et al. found that fucoidan regulates the miR-29b/DNMT3B/MTSS1 axis and inhibits EMT in human hepatocellular carcinoma cells [35]. Chen et al. demonstrated that fucoidan inhibits EMT in HK2 cells and attenuates renal fibrosis in both type 1 and type 2 diabetic rats [11].

Similar to the results in diabetic renal fibrosis, its inhibitory effect on RPE EMT in our study suggested that fucoidan may contribute to the maintenance of the epithelial properties of RPE cells and prevent the acquisition of a mesenchymal phenotype. The retention of E-cadherin may strengthen cell-cell adhesions among RPE cells, and the elimination of α -SMA and fibronectin could prevent the release of RPE cells. The restoration of the regular morphologic features of ZO-1 suggested that fucoidan may also reinforce tight junctions to ensure that RPE cells stay *in situ*. Meanwhile, the attenuated contraction ability of RPE cells indicated that apart from protecting the RPE *in situ*, fucoidan can weaken the damage caused by released RPE cells.

Apart from the *in vitro* protective effects of fucoidan, we also performed *in vivo* experiments. Our method for the establishment of PVR involves gas vitrectomy and the injection of human primary RPE cells into the vitreous of pigmented rabbits. This method was according to Fastenberg et al. [14] and Hida et al. [36]. We chose a cell injection method to establish a PVR model due to its better reproducibility, safety, and stability compared with other methods such as lensectomy [37] or retinal cryopexy with an ocular wound [38]. For animal species, including non-human primate models, cats, mice, and rabbits, have all been adopted in previous studies [39-41]. However, considering their suitability

for the experiment, the ease of conducting the procedure and convenience for observing the development of PVR, we selected pigmented rabbits because their large eyeball is easy to manipulate, the relatively smaller size of their lens helps to avoid cataract formation, and the typical structure of the medullary wing can be used to identify definite retinal detachment. For the choice of cell type, autologous fibroblasts [42, 43], homologous fibroblasts [14, 44, 45], autologous RPE cells [46], homologous RPE cells [47], activated macrophages [48, 49], and human primary RPE cells [50] have all been used to induce PVR in pigmented rabbits. We chose human primary RPE cells to conduct the *in vivo* experiment so it was in accordance with the *in vitro* experiment. Gas vitrectomy was performed using perfluoropropane to compress the vitreous and allow the RPE cells to settle on the retinal surface rather than being trapped in the vitreous. The injection of RPE cells is more relevant to the natural pathogenesis of human PVR as it simulates the detached RPE cells in the vitreous of humans. Funduscopy, OCT, and B-ultrasonic examinations were employed to investigate the progression of PVR. On day 14, 5 rabbits in the PBS control group had developed PVR. While in the fucoidan group, 4 rabbits were stage 2 and only 1 rabbit was stage 3, due to the appearance of the localized minor detachment of the medullary ray.

Histologic H&E staining and immunofluorescence analysis were used to observe the morphologically detached retina and to evaluate cell proliferation upon the epi/sub-retinal surface. Our results showed an obvious epiretinal proliferative membrane accompanied by α -SMA-positive staining in the stage 5 eyes of the PBS control group. However, a few cells on the retinal surface with slight α -SMA staining were observed in the fucoidan group. α -SMA, one of six different actin isoforms that have been identified, is involved in cell motility, structure, and integrity. During actin stress fiber formation in EMT, the enhanced expression of α -SMA enables the target cell to acquire the abilities of migration and contraction, which may play a pivotal role in the pathogenesis of PVR. Our findings indicated that fucoidan not only inhibited the formation of the proliferative membrane but also suppressed the expression of α -SMA in the experimental PVR model.

In conclusion, these data showed that fucoidan inhibits the EMT of RPE cells and the progression of PVR. Our findings suggest the potential clinical application of fucoidan as an anti-PVR agent. However, the safety and detailed mechanism of fucoidan still need further investigation prior to its clinical administration.

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Y.Z. conceived and performed the experiments, analyzed the data, and wrote the main manuscript text; H.L. and F.W. conceived the experiments and analyzed the data; D.Z., S.Y., and H.Y. carried out the experiments. M.L., H.L., J.Z., and G.X. participated in writing and revising the manuscript. All authors reviewed the manuscript and had final approval for the submitted version.

Disclosure Statement

The authors declare to have no conflict of interests.

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