Histochem Cell Biol (2017) 147:367–375
DOI 10.1007/s00418-016-1484-x

ORIGINAL PAPER

Notch signaling modulates proliferative vitreoretinopathy via regulating retinal pigment epithelial-to-mesenchymal transition

Jingjing Zhang[1] · Gongqiang Yuan[1] · Muchen Dong[2] · Ting Zhang[2] · Gao Hua[1] · Qingjun Zhou[2] · Weiyun Shi[1]

Accepted: 23 August 2016 / Published online: 7 September 2016

Abstract Elevated Notch signaling has been verified in a large range of fibrotic diseases developed in the kidney, liver, and lung, inducing the development of the epithelial–mesenchymal transition (EMT). The aim of this study was to observe the involvement of Notch signaling in the EMT of retinal pigment epithelial (RPE) cells and the pathogenesis of proliferative vitreoretinopathy (PVR). In vitro cultivated human RPE cells (ARPE-19) were treated with 10 ng/mL transforming growth factor (TGF)-β1 for 24, 48, and 72 h. The expression levels of ZO-1, α-SMA, vimentin, Notch1 intracellular domain (NICD1), and Hes-1 were evaluated with quantitative real-time polymerase chain reaction (qRT-PCR), immunofluorescence staining or Western blot. TGF-β1 induced EMT and the activation of Notch signaling in ARPE-19 cells. To examine the effect of Notch inhibition on TGF-β1-induced EMT and PVR formation, ARPE-19 cells were preincubated with γ-secretase inhibitor LY411575 before TGF-β1 treatment. Mouse PVR model was used for in vivo study. ARPE-19 cells were injected intravitreously with or without the LY411575 to examine the effect of Notch inhibition on PVR formation. LY411575 significantly attenuated EMT by inhibiting the Notch signaling activation in vitro. PVR was induced by intravitreal injections of ARPE-19 cells, while LY411575 inhibited mouse PVR formation in vivo. Notch signaling plays a critical role in TGF-β1-induced EMT in vitro and mice PVR model, which provides a novel insight into the pathogenesis of PVR. The specific inhibition of Notch signaling by γ-secretase inhibitor may provide a new approach for the prevention of PVR.

Keywords Notch signaling · Proliferative vitreoretinopathy · Retinal pigment epithelial cells · Epithelial–mesenchymal transition · Wound healing

✉ Qingjun Zhou
qjzhou2000@hotmail.com

✉ Weiyun Shi
weiyunshi1@163.com

[1] Shandong Eye Hospital, Shandong Eye Institute, Shandong Academy of Medical Sciences, 372 Jingsi Road, Jinan 250021, China

[2] State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong Academy of Medical Sciences, Qingdao 266071, Shandong Province, China

Introduction

Proliferative vitreoretinopathy (PVR) is a recurring and problematic blinding disorder that occurs in eyes with rhegmatogenous retinal detachment and in eyes that have recently undergone retinal detachment surgery, for which there is no pharmacologic treatment (Lei et al. 2012). The pathogenesis includes a fibrotic reaction by the retinal pigment epithelium (RPE) and other retina-derived non-neural cells (Tseng et al. 2004). RPE cells are exposed to the vitreous, activated by vitreal growth factors and cytokines, transformed into fibroblasts, and organized into membranes on the inner and/or outer surface of the retina, creating wrinkling of the retina and retinal traction, which are considered key elements in PVR (Hiscott et al. 1999; Qiu et al. 2013). This process is likely to involve epithelial-to-mesenchymal transition (EMT) and be driven by agents in the vitreous such as transforming growth factor β (TGF-β) in experimental PVR (Hoerster et al. 2014). Emerging evidence suggests that the Notch signaling pathway regulates EMT, leading to tumor invasion and metastasis, and could be down-regulated by its inhibitors or natural compounds,

resulting in the reversal of EMT to mesenchymal-to-epithelial transition (Li et al. 2013).

The Notch signaling pathway is a highly conserved cell–cell interaction mechanism that regulates cell proliferation, differentiation, apoptosis or stem cell maintenance through cell physical contact in both vertebrate and invertebrate species (Wilson and Radtke 2006). Notch signaling is composed of Notch ligands, Notch receptors, and DNA-binding proteins. There are five transmembrane Notch ligands (Jagged1, Jagged2, Dll1, Dll3, and Dll4) and four transmembrane Notch receptor isoforms (Notch1–4) in mammalian cells. CSL is a key DNA-binding protein in the Notch signaling pathway and is called CBF-1 or RBP-J which could recognize and bind specific DNA sequences (RTGGGAA) located in Notch-induced gene promoters. The pathway is activated through an interaction of a Notch receptor with a Jagged or Delta-like ligand, leading to proteolytic cleavages of Notch receptor at two distinct sites. The cleavage releases the Notch intracellular domain (NICD1) so that it can enter the nucleus and function as a transcription activator. The second cleavage is mediated by the γ-secretase complex, and an effective inhibition of Notch activation can be achieved by pharmacological inhibition of this proteolytic activity. Within the nucleus, NICD1 interacts with CSL (RBP-Jk/CBF-1) and activates transcription of downstream target genes, such as those of the Hes and Hey families (Zhu et al. 2010).

Notch signaling is of importance for eye development, and constitutive Notch activity leads to hyperproliferation and benign tumors formation, which is dependent on the presence of the transcription factor RBP-Jk (Umazume et al. 2013). Little is known about whether the Notch signaling pathway is involved in the pathogenesis of PVR. In the present study, we investigated expression change of Notch signaling in PVR formation and during the process of TGF-β1-induced EMT of RPE, and observed the influence of γ-secretase inhibitor on RPE transdifferentiation and PVR development by inhibiting Notch signaling.

Methods

Reagents and antibodies

The primary antibodies were smooth muscle α-actin (α-SMA; ab7817, Abcam), Hes-1 (ab71559, Abcam), the Notch-1 intracellular domain (NICD1; ab8925, Abcam), zona occludin-1 (ZO-1; sc-10804, Santa Cruz Biotechnology, Santa Cruz, CA), and Notch-1 (sc-6014, Santa Cruz Biotechnology). The secondary antibodies against rabbit labeled with fluorescein isothiocyanate (FITC) and mouse IgG labeled with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Primers were purchased from the Invitrogen Corporation (China). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). γ-secretase inhibitor LY411575 was purchased from Selleckehem (Houston, TX).

Cell culture

Human retinal pigment epithelial cell line ARPE-19 (Zeng et al. 2013) (ATCC, catalog No. CRL-2302, Rochkefeller, MD) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; HyClone Company, UT, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco Company, CA, USA) and penicillin/streptomycin. ARPE-19 cells were serum-starved overnight and then cultured in DMEM/F-12 with 2 % FBS and 10 ng/mL TGF-β1 treatment for 24, 48, and 72 h. Cell morphology changes were observed under an inverted microscope (Olympus CKX41, Tokyo, Japan), and the expression of ZO-1, vimentin, α-SMA, NICD1, Hes-1, Jagged-1, and RBP-Jk was examined using immunofluorescence staining, Western blot or quantitative real-time polymerase chain reaction (qRT-PCR). To examine the effect of γ-secretase inhibitor LY411575 on TGF-β1-induced EMT, ARPE-19 cells were preincubated for 1 h with LY411575 before TGF-β1 treatment.

Animal experiments

Eighty-four-week-old C57BL/6J mice were obtained from the Experiment Animal Center at the Shandong Provincial Key Laboratory of Ophthalmology of Shandong Eye Institute. All experiments adhered to the ARVO statements for the Use of Animals in Ophthalmology and Vision Research. All procedures were approved by the Ethics Committee of the Shandong Eye Institute. All surgeries were performed on only one eye of each mouse under sodium pentobarbital anesthesia, and the mice were sacrificed with an overdose of 10 % chloral hydrate.

Eighty male mice were randomly divided into three groups. Mice in control group ($n = 30$) were injected with 1 μL PBS; mice in ARPE-19 cell group ($n = 30$) received the intravitreous injection with 1 μL ARPE-19 cells (5×10^4/μL) suspended in PBS; mice in ARPE-19 cells +LY411575 group ($n = 20$) were injected with 1 μL ARPE-19 cells (5×10^4) suspended in PBS containing LY411575 (19 ng/μL). Under operating microscope, pass a 33-gauge needle attached to a 5 μL-Hamilton microsyringe (Hamilton Company, Switzerland) through the equatorial sclera, position the tip of the needle just over the optic disk and inject the cells, taking care not to disturb the lens or the retina. During injection of the RPE cells, the cells can be seen flowing from the needle tip into the vitreous and

disperse in the vitreous fluid. Meanwhile, the vitreous can become cloudy immediately.

Histology, immunohistochemistry, and whole-mount staining

ARPE-19 cells were seeded in 48-well tissue culture plates in amounts of 5×10^3 cells/well for incubation of 24 h before serum-starved overnight and then cultured in DMEM/F-12 with 2 % FBS and 10 ng/mL TGF-β1 treatment for 48 h. The expression of ZO-1, vimentin, NICD1, and Hes-1 was examined using immunofluorescence staining. Cells were incubated with primary antibodies and secondary antibodies, and nuclei were stained with DAPI (Beyotime Institute of Biotechnology, Shanghai, China). Finally, the cells were visualized and photographed using a confocal microscope (NIKON TE2000, Tokyo, Japan).

Eyes in control and ARPE-19 cell group were collected for histology at 7, 14, and 28 days after the intravitreous injection. The samples were fixed in 10 % formalin and embedded in paraffin. Continuous 4-μm sections were stained with hematoxylin and eosin (H.E. staining). The presence of RPE cells, proliferative membranes, and retinal detachment was observed by light microscopy. Meanwhile, paraffin sections and whole-mount retinas were stained by immunohistochemistry for α-SMA to visualize proliferative membranes at 14 days after the intravitreous injection (Outtz et al. 2011). Retina tissues were harvested for real-time PCR and Western blot analysis at 14 days after the intravitreous injection.

Real-time PCR analysis

The total RNA was extracted with the NucleoSpin RNA II System (Company Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol and was reverse-transcribed using PrimeScript RT reagent Kit (Takara Biotechnology Company, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green Real Master Mix and specific primers. The following PCR primers from humans were used: α-SMA: forward, 5′-GGG ACA TCA AGG AGA AAC TGT GT-3, reverse, 5′-TCT CTG GGC AGC GGA AAC-3′; Hes-1: forward, 5′-TTT GGA TGC TCT GAA GAA AGA TAG C-3′, reverse, 5′-CGG TAC TTC CCC AGC ACA CTT -3′; Jagged-1: forward, 5′-GGG AAC CCG ATC AAG GAA AT-3′, reverse, 5′-GCT CAG CAA GGG AAC AAG GA-3′; RBP-Jk: forward, 5′-GTG CTG GCG TTT GTG TAA CTT C-3′, reverse, 5′-GTG CTG GCG TTT GTG TAA CTT C-3′; and GAPDH: forward, 5′-ATG CTG GCG CTG AGT ACG T-3′, reverse, 5′-AGC CCC AGC CTT CTC CAT-3′. The following PCR primers from mice were used: α-SMA: forward, 5′-TGC CGA GCG TGA GAT TGT C-3′, reverse, 5′-CGT TCG TTT CCA ATG GTG ATC-3′; Hes-1: forward, 5′-TCC AAG CTA GAG AAG GCA GAC AT-3′, reverse, 5′-GGG TCA CCT CGT TCA TGC A-3′; and GAPDH: forward, 5′-GAC CCC TTC ATT GA CCT CAA C-3′, reverse, 5′-CTT CTC CAT GGT GGT GAA GA-3′.

Western blot

Samples were homogenized in 100 μL of ice-cold RIPA (radio-immunoprecipitation assay) lysis buffer supplemented with a proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF). The homogenates, which contained 20 μg of protein, were then separated by SDS-PAGE and transferred to nitrocellulose membrane (Thermo Fisher Scientific China, Beijing, China). The blots were probed with the following primary antibodies: α-SMA (ab7817, Abcam), vimentin (sc-5565, Santa Cruz), Hes-1 (ab71559, Abcam), NICD1 (ab8925, Abcam). Quantification of the Western blot data was performed by measuring densitometric analysis of the bands using Image J software.

Statistical analysis

All results were expressed as the means ± standard deviations (SD). Data were analyzed with the SPSS 17.0 statistical package. A one-way analysis of independent samples *t* test for two-sample comparisons was used to compare the means of two groups. A *P* value of less than 0.05 was considered statistically significant. The reported results were representative of three independent experiments.

Results

Induction of EMT and activation of Notch signaling in ARPE-19 cells by TGF-β1

Notch signaling changes were detectable during TGF-β1 induction of EMT. ARPE-19 cells exhibited a cobblestone-like morphology in the absence of TGF-β1, while after exposure to TGF-β1, ARPE-19 cells changed into spindle-shaped fibroblast-like cells, which were larger and less compact than the untreated cells (Fig. 1a). Immunofluorescence revealed that TGF-β1 treatment decreased the expression of the epithelial phenotype marker ZO-1 at endothelial cell–cell junctions and increased the expression of the mesenchymal marker α-SMA in the cytoplasm. Because the cleavage of Notch-1 is an indicator of the Notch signaling activation, and Hes-1 is a Notch downstream target, we examined NICD1 and Hes-1 as markers of the activated Notch expression by immunofluorescence, observing a significant increase in NICD1 and Hes-1 in RPE cells for TGF-β1 treatment

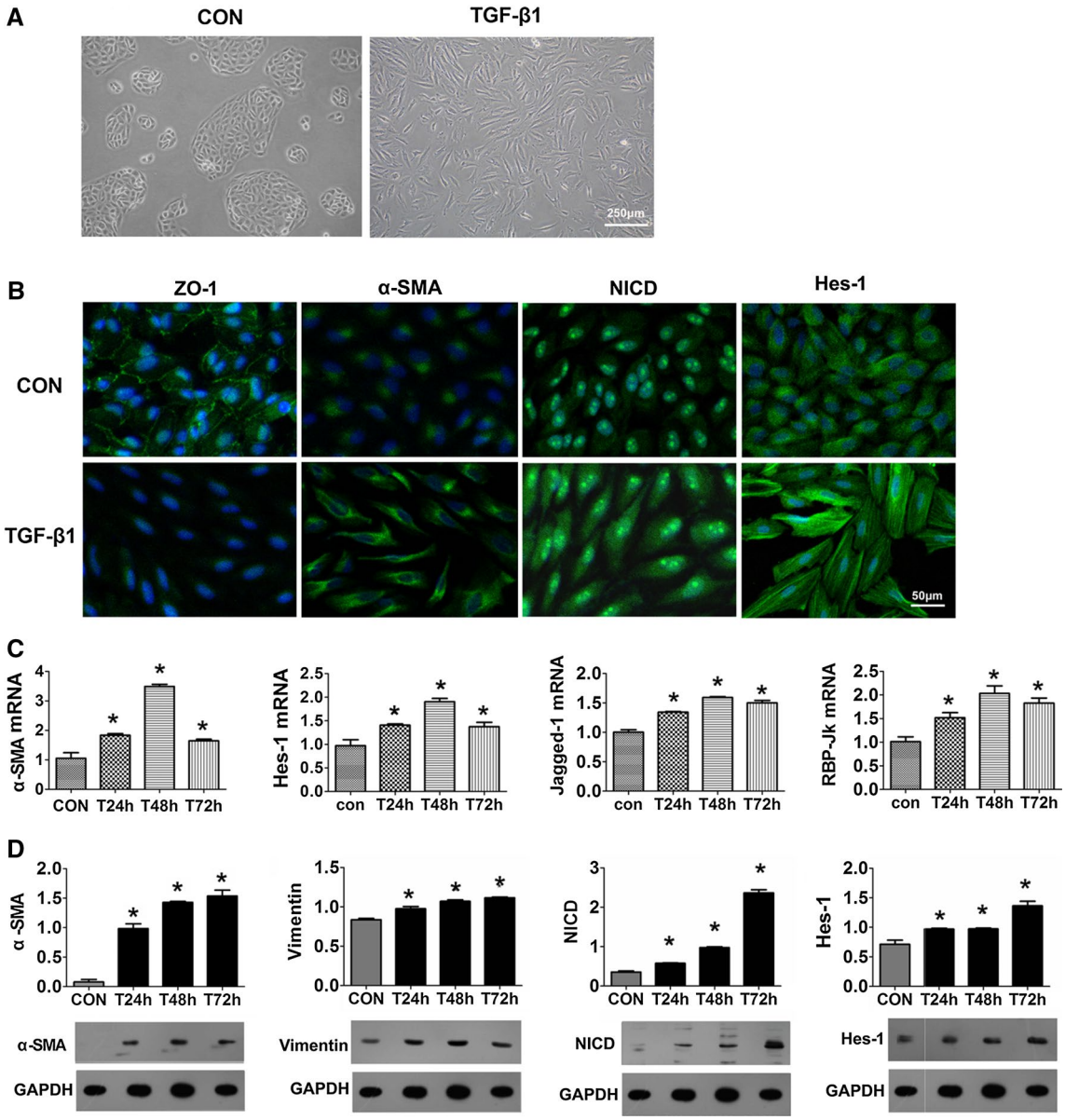

Fig. 1 TGF-β1 induces EMT and activates Notch signaling in ARPE-19 cells. **a** Cell morphological change. ARPE-19 cells exhibited a cobblestone-like morphology in the absence of TGF-β1, after exposure to TGF-β1 for 72 h. ARPE-19 cells change into spindle-shaped fibroblast-like cells, larger and less compact than untreated cells. **b** Immunoflurescence staining of ZO-1, α-SMA, NICD1, and Hes-1 in ARPE-19 cells in control group and TGF-β1 group for 72 h. Nuclei are stained with DAPI (*blue*). **c** The mRNA expression of α-SMA, Hes-1, RBP-Jk, and Jagged-1 is examined by real-time PCR after 10 ng/mL TGF-β1 exposure for 24, 48, and 72 h, and their levels peaked at 48 h. Data are given as mean ± SD ($n = 4$). *$P < 0.05$. **d** The protein levels of α-SMA, vimentin, Hes-1, and NICD1 are analyzed by Western blot at the time points as indicated after TGF-β1 (10 ng/mL) treatment. Graphic representation of relative abundance of α-SMA, vimentin, Hes-1, and NICD1 normalized to GAPDH. Data are given as mean ± SD ($n = 3$). *$P < 0.05$ versus control group

(Fig. 1b). The mRNA of α-SMA, Hes-1, RBP-Jk, and Jagged-1 was detected by qRT-PCR to significantly increase and peak at 48 h (Fig. 1c). Meanwhile, by Western blot, the protein expression of vimentin, α-SMA, NICD1, and Hes-1 was also found to significantly increase at 24, 48 and 72 h after 10 ng/mL TGF-β1 exposure (Fig. 1d). These results demonstrated that TGF-β1 induced EMT in ARPE-19 cells and activated Notch signaling in a time-dependent manner.

γ-secretase-mediated Notch signaling activation for TGF-β1-induced EMT

ARPE-19 cells were preincubated for 1 h with LY411575 (100 nM, 1 and 10 μM) or vehide (0.05 % DMSO) before TGF-β1 treatment. The mRNA levels of α-SMA were evaluated with quantitative real-time polymerase chain reaction (qRT-PCR) at 48 h. The expression of α-SMA mRNA can

be inhibited by LY411575 (100 nM, 1 and 10 μM) and significantly inhibited by concentrations of 1 μM LY411575 (Fig. 2a).

Based on the above results, we mainly investigated whether the activation of Notch signaling in TGF-β1-induced EMT was modulated by the presence of the γ-secretase inhibitor 1 μM LY411575. γ-secretase inhibitor can block Notch signaling by inhibiting the final proteolytic cleavage of the Notch receptor. First, cell morphology analysis revealed that LY411575 treatment attenuated morphological changes induced by TGF-β1 compared to vehicle-treated cells (Fig. 2b). Meanwhile, LY411575 was observed to significantly reduce the mRNA expression of Hes-1 and Jagged-1 and the protein expression of TGF-β1-induced NICD1 and Hes-1 as shown by a significant decrease in α-SMA mRNA and protein expression (Fig. 2c, d). These results indicated that the inhibition of Notch signaling mediated TGF-β1-induced EMT.

Mouse intravitreal RPE cells for the model of PVR

In this study, mice were intravitreally injected with RPE cells (5×10^4) to observe PVR evolution. Enucleated eyes were embedded in paraffin and H.E. staining showed that

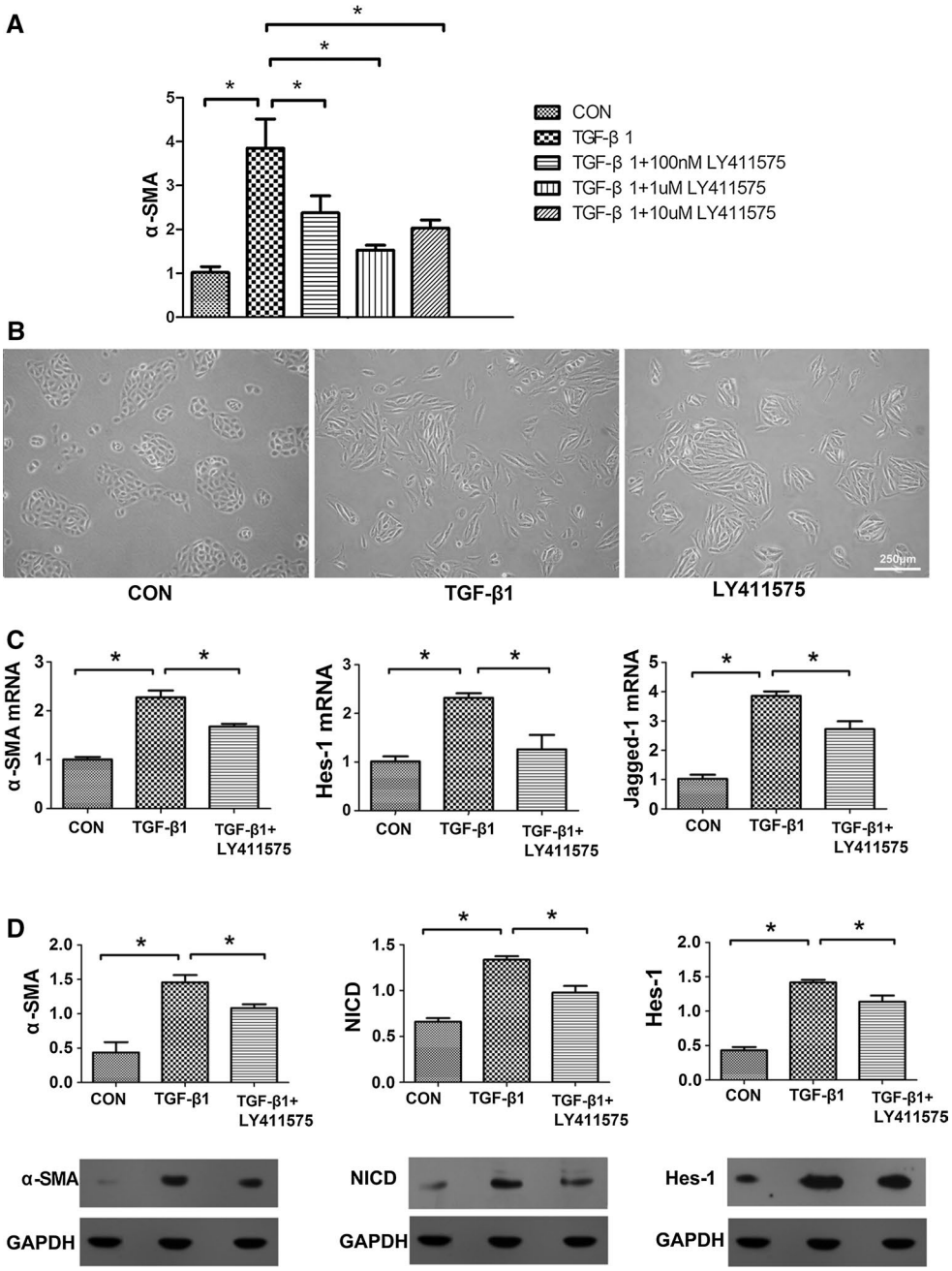

Fig. 2 γ-secretase inhibitor LY411575 inhibits TGF-β1-induced EMT in ARPE-19 cells. **a** ARPE-19 cells are treated with LY411575 (100 nM, 1 and 10 μM) and 10 ng/mL TGF-β1 for 48 h and the mRNA expression is analyzed by real-time PCR. Data are given as mean ± SD ($n = 3$). *$P < 0.05$. **b** ARPE-19 cells are treated with 1 μM LY41575 and 10 ng/mL TGF-β1 for 48 h and morphological change is examined. **c** ARPE-19 cells is treated with 1 μM LY41575 and 10 ng/mL TGF-β1 for 48 h and the mRNA expression of α-SMA, Hes-1, and Jagged-1 is analyzed by real-time PCR. Data are given as mean ± SD ($n = 3$). *$P < 0.05$. **d** ARPE-19 cells are treated with 1 μM LY41575 and 10 ng/mL TGF-β1 for 48 h and the protein levels of α-SMA, Hes-1, and NICD1 are analyzed by Western blot. GAPDH is used to verify equivalent loading

many RPE cells, which exhibited spindle-shaped fibroblast-like cells, existed in the vitreous cavity, and inflammatory cells invaded the vitreal cavity at 1 week after the intravitreal injection of ARPE-19 cells. Epiretinal membrane in all mice (100 %, 10/10) was observed at 2 weeks after an intravitreal injection of RPE cells; these proliferative membranes were associated with a tractional retinal detachment. Four weeks later, some of the mice (50 %, 5/10) developed extensive retinal detachment, and many inflammatory cells existed in the vitreous cavity. There were not proliferative membranes and retinal detachment in the control group (Fig. 3a). Immunohistochemistry analysis at 2 weeks showed that α-SMA positive labeled cells were involved in the PVR proliferative membrane (Fig. 3b), and whole-mount retinal immunofluorescence staining analyses at 14 days showed α-SMA positive labeled proliferative membrane (Fig. 3c).

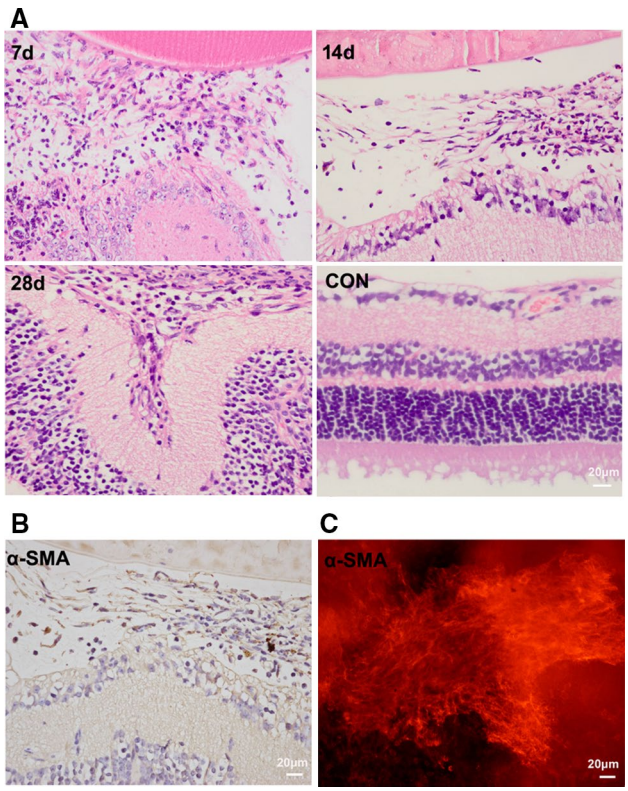

Fig. 3 Mouse PVR model induced by intravitreal injection of RPE cells. **a** HE staining: Many RPE cells, which are exhibited spindle-shaped fibroblast-like cells, existed in the vitreous cavity, and inflammatory cells invaded the vitreal cavity at 1 week. Epiretinal membranes in the vitreous cavity of all experimental mice are observed at 2 weeks. Four weeks later, some of the mice are developed extensive retinal detachment, and many inflammatory cells are existed in the vitreous cavity. There are not proliferative membranes in the control group. **b** Immunohistochemistry staining of α-SMA at 14 days after intravitreal injection of RPE cells. **c** Whole-mount retinal immunofluorescence staining of α-SMA at 14 days after intravitreal injection of RPE cells

Attenuation of mouse PVR formation by LY411575

To investigate the expression of Notch signaling components in PVR animal model, we generated a mouse model of PVR by the intravitreal injection of ARPE-19 cells. The mRNA levels of α-SMA and Hes-1 were increased in 2 weeks. Similarly, the protein levels of NICD1, Hes-1, and α-SMA were increased after the intravitreal injection of ARPE-19 cells. The effect of LY411575 on inhibiting Notch signaling was demonstrated by the decreased protein levels of NICD1 and Hes-1 and mRNA levels of Hes-1. Meanwhile, the expression of α-SMA in the protein and mRNA level remarkably attenuated in LY411575-treated mice, and PVR formation was inhibited (Fig. 4).

Discussion

Notch signaling plays an important role in choroidal neovascularization angiogenesis (Ahmad et al. 2011). Little has been known about the function of Notch signaling in PVR formation in vivo. In this study, we showed that Notch signaling was markedly activated in RPE cells after TGF-β1 treatment, as indicated by the increased expression of Hes-1, NICD1, Jagged-1, and RBP-Jk. Likely, we detected an elevated level of expression for Notch signaling components during PVR formation induced by the intravitreal injection of ARPE-19 cells. Treatment of ARPE-19 cells with the Notch inhibitor LY411575 led to a strong inhibition of TGF-β1-induced EMT. Importantly, LY411575 intravitreal injection attenuated RPE cell-induced PVR formation as demonstrated by the decreased expression of α-SMA.

The RPE, a polarized monolayer of specialized epithelium located at the outermost layer of the retina between the neural retina and choroid, is part of the outer blood-retinal barrier and plays a pivotal role in supporting photoreceptor function. Under pathological conditions, RPE cells undergo EMT, form fibroblast-like cells or myofibroblasts, and produce extracellular matrix components participating in the formation of a contractile fibrotic proliferative membrane. RPE cells are present in epiretinal and subretinal membranes and are believed to have a pro-fibrotic role in the development of PVR (Liu et al. 2013; Tamiya et al. 2010). The Notch signaling is important for the development and differentiation of RPE in mammalian eyes and might lead to tumor formation and structural damages of the eye if it is constitutively active (Bai et al. 2013; Umazume et al. 2013). In RPE cells, Jagged-1 has a higher expression level than any of the other ligands JAG2, DLL1, DLL3, and DLL4 (Liu et al. 2013). Blockage of Notch Signaling could inhibit the migration and proliferation of retinal pigment epithelial cells, which might be related to

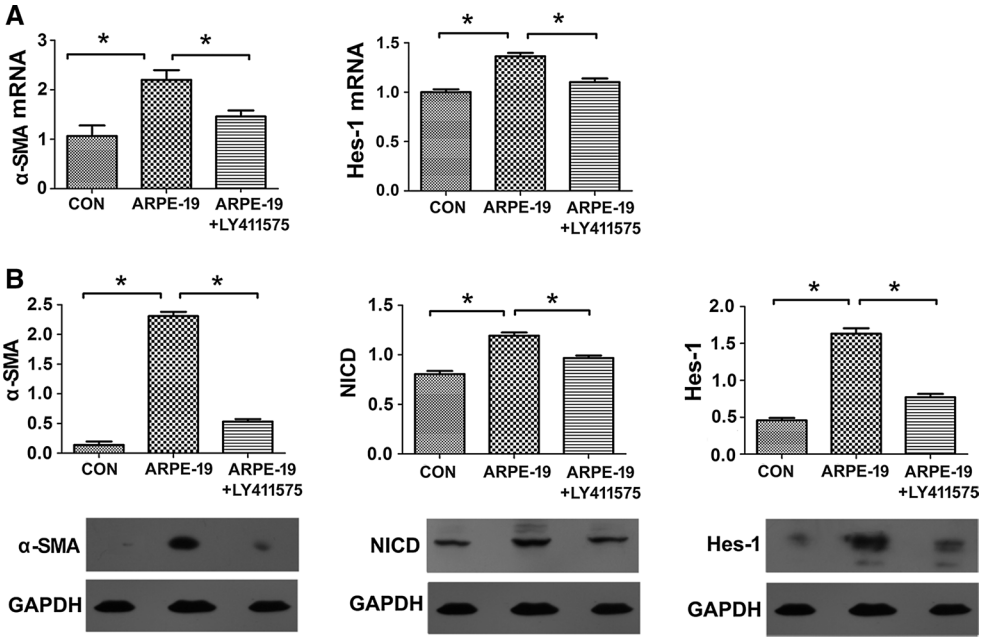

Fig. 4 γ-secretase inhibitor LY411575 attenuates mouse PVR formation. **a** Retina tissues are harvested for real-time PCR to analyze the mRNA expression of α-SMA, Hes-1, and Jagged-1. Data are given as mean ± SD ($n = 3$). *$P < 0.05$. **b** Retina tissues are harvested for Western blot analysis with antibodies against α-SMA, NICD1, and Hes-1. GAPDH is used to verify equivalent loading

a reduction in Hey2 expression levels (Liu et al. 2013). Jagged1/Notch signaling regulates RPE proliferation and specification, and plays a critical role in keeping the distal RPE from differentiating into the retina (Ou et al. 2013). The elements of the Notch signaling pathway, including Jagged-1, Notch-3, Hes-1, and Hey-1, can be up-regulated in TGF-β2-stimulated EMT in human RPE cells (Bai et al. 2013). Blockade of Notch pathway inhibited TGF-β2-induced EMT, suggesting a critical role of Notch pathway in TGF-β2-induced EMT (Chen et al. 2014). We found that Notch signaling was activated in the presence of TGF-β1, and the inhibition of Notch signaling can inhibit TGF-β1-induced EMT. Therefore, inhibition of the Notch signaling pathway may have therapeutic value in the prevention and treatment of PVR. We further observe the influence of γ-secretase inhibitor on mice PVR development by intravitreal injection of LY411575.

PVR, a scarring process that may develop with retinal detachment, is the most common cause of surgical failure in the treatment of rhegmatogenous retinal detachment. It is characterized by the formation of fibrotic proliferative membranes on the detached retina, which reduces the flexibility of retina and further results in retinal redetachment and difficulty in retinal reattachment (Yu et al. 2008). The RPE cells are a main contributor to the development of fibrotic tissue on the retina and can transform into fibroblasts in response to cues that they receive from the vitreous. Rabbit models that involve the intravitreal injection of RPE cells have been widely used (Agrawal et al. 2007). The mouse has some advantages as an animal models for PVR, although the work is not easy in mouse eyes. It would be highly desirable to have a reproducible model in mice, which would allow testing of genetic background in the pathogenesis of PVR and facilitate testing of candidate drugs to treat this disease (Agrawal et al. 2007; Soler et al. 2002). In this study, for the first time we observe in vivo mice PVR formation by intravitreal RPE cells, and analyze α-SMA expression by qRT-PCR and Western blot. We are now reporting the results of a 1-month study, demonstrating that intravitreal RPE cells induced a PVR-like condition and characterized by the appearance of epiretinal membrane and retinal folds.

This process is likely to involve EMT, a process in which epithelial cells lose their differentiated phenotypes and become mesenchymal-like cells. EMT has an important role in not only cancer progression, but also normal organ development and human pathology such as organ fibrosis and wound healing (Chen et al. 2012; Ono et al. 2012). It can be triggered by different signaling molecules, such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), bone morphogenetic proteins (BMPs), and WNTs and Snail (Barrallo-Gimeno and Nieto 2005). TGF-β1 has been elevated in the vitreous fluid during the development of PVR membranes and can trigger the influx of RPE cells into the vitreous and the EMT of RPE cells (Hoerster et al. 2014). Elevated Notch signaling has been verified in a large range of fibrotic diseases developed in the kidney, liver, and lung, inducing the development of EMT (Leask 2010; Noseda et al. 2004; Wang et al. 2010).

The human SMA promoter contains a CSL consensus binding site (TGGGAA) beginning at 64 from the cap site that is conserved in apes and rodents (Noseda

et al. 2004). Notch has recently been shown to directly regulate the expression of the mesenchymal and smooth muscle cell marker α-SMA, which is directly dependent on activation and binding of CSL to the SMA promoter (Noseda et al. 2006). TGF-β-induced EMT is a key contributor to fibrotic scar formation (Hills and Squires 2010). TGF-β1 can promote the Notch target gene Hes-1 expression during the process of EMT of primary rat mesothelial cells (RPMCs), and the γ-secretase inhibitor can dramatically inhibit TGF-β-induced EMT of RPMCs and peritoneum fibrosis (Chen et al. 2012). Elements of the Notch signaling pathway, including Jagged1 and Notch1 and 2, Hes1 and Hey1, have been identified as TGF-β1-responsive genes for example in kidney epithelia (Hills and Squires 2010; Zavadil et al. 2004). In this preliminary study, we demonstrated Notch signaling was activated during PVR formation in mice PVR model. To examine the effect of γ-secretase inhibitor LY411575 on PVR formation, C57BL/6J mice have been injected into vitreous cavity with 1 μL ARPE-19 cells (5×10^4) suspended in PBS containing LY411575 (1.9 ng/μL, 4 μM). The expression of α-SMA mRNA cannot be inhibited by LY411575 (4 μM). And then we used LY411575 (19 ng/μL, 40 μM) to inhibit PVR formation, which also referred to concentrations of γ-secretase inhibitor DAPT (16 ng/μL, 40 μM) of the intravitreous injection (Qi et al. 2012; Zhang et al. 2015). We calculate that vitreous cavity and anterior chamber volume in 4-week-old C57BL/6J mice is approximately 4 μL according to single-shot dimension measurements of the mouse eye using SD-OCT (Jiang et al. 2012). C57BL/6J mice were injected with 1 μL LY411575 (40 μM), and the concentration of LY411575 in mouse eye is approximately 10 μM, roughly equal to 10 times the concentration used in vitro. LY411575 (19 ng/μL) intravitreal injection was found to inhibit Notch signaling pathway and attenuate RPE cell-induced PVR formation.

In summary, the Notch signaling pathway plays a critical role in PVR formation by regulating EMT of RPE. The specific inhibition of Notch signaling pathway by the γ-secretase inhibitor may prevent PVR, which provides a novel insight into the pathogenesis of PVR.

Acknowledgments This study was supported by the National Natural Science Foundation of China (81470611, 81530027); Shandong Provincial Natural Science Foundation, China (JQ201518, ZR2015PH001), and Youth Foundation of Shandong Academy of Medical Sciences (2014-41). The authors thank Ms. Ping Lin for her assistance in the preparation of this manuscript.

Compliance with ethical standards

Conflict of interest None.

References

Agrawal RN, He S, Spee C, Cui JZ, Ryan SJ, Hinton DR (2007) In vivo models of proliferative vitreoretinopathy. Nat Protoc 2:67–77

Ahmad I, Balasubramanian S, Del Debbio CB, Parameswaran S, Katz AR, Toris C, Fariss RN (2011) Regulation of ocular angiogenesis by Notch signaling: implications in neovascular age-related macular degeneration. Invest Ophthalmol Vis Sci 52:2868–2878

Bai Y, Yu W, Han N, Yang F, Sun Y, Zhang L, Zhao M, Huang L, Zhou A, Wang F, Li X (2013) Effects of semaphorin 3A on retinal pigment epithelial cell activity. Invest Ophthalmol Vis Sci 54:6628–6638

Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132:3151–3161

Chen Y, Zheng S, Qi D, Zheng S, Guo J, Zhang S, Weng Z (2012) Inhibition of Notch signaling by a γ-secretase inhibitor attenuates hepatic fibrosis in rats. PLoS ONE 7:e46512

Chen X, Xiao W, Liu X, Zeng M, Luo L, Wu M, Ye S, Liu Y (2014) Blockade of Jagged/Notch pathway abrogates transforming growth factor β2-induced epithelial-mesenchymal transition in human retinal pigment epithelium cells. Curr Mol Med 14:523–534

Hills CE, Squires PE (2010) TGF-β1-induced epithelial-to-mesenchymal transition and therapeutic intervention in diabetic nephropathy. Am J Nephrol 31:68–74

Hiscott P, Sheridan C, Magee RM, Grierson I (1999) Matrix and the retinal pigment epithelium in proliferative retinal disease. Prog Retin Eye Res 18:167–190

Hoerster R, Muether PS, Vierkotten S, Hermann MM, Kirchhof B, Fauser S (2014) Upregulation of TGF-β1 in experimental proliferative vitreoretinopathy is accompanied by epithelial to mesenchymal transition. Graefes Arch Clin Exp Ophthalmol 252:11–16

Jiang M, Wu PC, Fini ME, Tsai CL, Itakura T, Zhang X, Jiao S (2012) Single-shot dimension measurements of the mouse eye using SD-OCT. Ophthalmic Surg Lasers Imaging 43:252–256

Leask A (2010) Targeting the Jagged/Notch pathway: a new treatment for fibrosis? J Cell Commun Signal 4:197–198

Lei H, Rheaume MA, Cui J, Mukai S, Maberley D, Samad A, Matsubara J, Kazlauskas A (2012) A novel function of p53: a gatekeeper of retinal detachment. Am J Pathol 181:866–874

Li Y, Ma J, Qian X, Wu Q, Xia J, Miele L, Sarkar FH, Wang Z (2013) Regulation of EMT by Notch signaling pathway in tumor progression. Curr Cancer Drug Targets 13:957–962

Liu W, Jin G, Long C, Zhou X, Tang Y, Huang S, Kuang X, Wu L, Zhang Q, Shen H (2013) Blockage of Notch signaling inhibits the migration and proliferation of retinal pigment epithelial cells. Sci World J 2013:178708

Noseda M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, Shahidi R, Dorovini-Zis K, Li L, Beckstead B, Durand RE, Hoodless PA, Karsan A (2004) Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res 94:910–917

Noseda M, Fu Y, Niessen K, Wong F, Chang L, McLean G, Karsan A (2006) Smooth muscle α-actin is a direct target of Notch/CSL. Circ Res 98:1468–1470

Ono H, Imoto I, Kozaki K, Tsuda H, Matsui T, Kurasawa Y, Muramatsu T, Sugihara K, Inazawa J (2012) SIX1 promotes epithelial-mesenchymal transition in colorectal cancer through ZEB1 activation. Oncogene 31:4923–4934

Ou J, Bharti K, Nodari A, Bertuzzi S, Arnheiter H (2013) Vax1/2 genes counteract Mitf-induced respecification of the retinal pigment epithelium. PLoS ONE 8:e59247

Outtz HH, Tattersall IW, Kofler NM, Steinbach N, Kitajewski J (2011) Notch1 controls macrophage recruitment and Notch signaling is activated at sites of endothelial cell anastomosis during retinal angiogenesis in mice. Blood 118:3436–3439

Qi X, Cai J, Ruan Q, Liu L, Boye SL, Chen Z, Hauswirth WW, Ryals RC, Shaw L, Caballero S, Grant MB, Boulton ME (2012) γ-Secretase inhibition of murine choroidal neovascularization is associated with reduction of superoxide and proinflammatory cytokines. Invest Ophthalmol Vis Sci 53:574–585

Qiu S, Jiang Z, Huang Z, Chen X, Qian X, Gao Q, Zheng H (2013) Migration of retinal pigment epithelium cells is regulated by protein kinase Cα in vitro. Invest Ophthalmol Vis Sci 54:7082–7090

Soler MVC, Gallo JE, Dodds RA, Suburo AM (2002) A mouse model of proliferative vitreoretinopathy induced by dispase. Exp Eye Res 75:491–504

Tamiya S, Liu L, Kaplan HJ (2010) Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. Invest Ophthalmol Vis Sci 51:2755–2763

Tseng W, Cortez RT, Ramirez G, Stinnett S, Jaffe GJ (2004) Prevalence and risk factors for proliferative vitreoretinopathy in eyes with rhegmatogenous retinal detachment but no previous vitreoretinal surgery. Am J Ophthalmol 137:1105–1115

Umazume K, Liu L, Scott PA, de Castro JP, McDonald K, Kaplan HJ, Tamiya S (2013) Inhibition of PVR with a tyrosine kinase inhibitor, dasatinib, in the swine. Invest Ophthalmol Vis Sci 54:1150–1159

Wang Z, Li Y, Kong D, Sarkar FH (2010) The role of Notch signaling pathway in epithelial-mesenchymal transition (EMT) during development and tumor aggressiveness. Curr Drug Targets 11:745–751

Wilson A, Radtke F (2006) Multiple functions of Notch signaling in self-renewing organs and cancer. FEBS Lett 580:2860–2868

Yu J, Liu F, Cui SJ, Liu Y, Song ZY, Cao H, Chen FE, Wang WJ, Sun T, Wang F (2008) Vitreous proteomic analysis of proliferative vitreoretinopathy. Proteomics 8:3667–3678

Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP (2004) Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J 23:1155–1165

Zeng F, Zhang M, Xu Y, Xu H (2013) ARMS2 interference leads to decrease of proinflammatory mediators. Graefes Arch Clin Exp 251:2539–2544

Zhang J, Zhou Q, Yuan G, Dong M, Shi W (2015) Notch signaling regulates M2 type macrophage polarization during the development of proliferative vitreoretinopathy. Cell Immunol 298:77–82

Zhu F, Li T, Qiu F, Fan J, Zhou Q, Ding X, Nie J, Yu X (2010) Preventive effect of Notch signaling inhibition by a γ-secretase inhibitor on peritoneal dialysis fluid-induced peritoneal fibrosis in rats. Am J Pathol 176:650–659