AlkB Homolog H5 Attenuates TGF-β1-Induced Epithelial-Mesenchymal Transition of Retinal Pigment Epithelial Cells via Regulating Zinc Finger E-box Binding Homeobox 1

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ABSTRACT

There is a relationship between proliferative vitreoretinopathy (PVR) and epithelial-mesenchymal transition (EMT) of Retinal pigment epithelium (RPE). The objective of this study was to investigate the role of AlkB homolog H5 (ALKBH5) in regulating EMT of RPE cells. Reverse transcription polymerase chain reaction (RT-qPCR) was implemented to determine the expression levels of ALKBH5, Zinc finger E-box binding homeobox 1 (ZEB1), insulin-like growth factor-2 mRNA-binding protein-1 (IGF2BP1) and EMT markers. Transwell, and cholecystokinin (CCK)-8 assays were implemented to examine RPE cell migration and proliferation. RNA immunoprecipitation (RIP) assays, and Luciferase reporter and determined the interaction among ZEB1, ALKBH5 and IGF2BP1. MeRIP assay showed m6A modification of ZEB1. ALKBH5 hinders TGF-β1-induced ARPE-19 cells' EMT. Mechanistically, as an m6A demethylase, ALKBH5 suppresses the mRNA stability of EMT-linked transcription factor ZEB1 in an m6A-IGF2BP1-dependent manner. ALKBH5 modulates ZEB1 to affect ARPE-19 cells' EMT. ALKBH5 attenuates TGF-β1-induced EMT of RPE cells via regulating ZEB1. The findings in this study may inspire novel approaches for PVR treatment.

EINTRODUCTION

Being a vitreoretinal fibrosis disease, proliferative vitreoretinopathy (PVR) features the formation of proliferative membranes and can lead to repeated detachment of retina and blindness (Ma *et al*., 2021). In 1983, PVR was firstly used by Retina Society Terminology Committee for description of a disease process secondary to rhegmatogenous retinal detachment (Dai *et al*., 2020; Mudhar, 2020). Surgery is the standard treatment for PVR. Nonetheless, the success rate for PVR surgery is only 60–75%, and recurrent retinal traction causes retinal redetachment in over 25% of successful cases (Chen *et al*., 2021). Hence, it is urgently needed to pay more attention to the pathogenesis of PVR in order to improve its treatment.

During epithelial-mesenchymal transition (EMT), acquiring the phenotypes of mesenchymal cells, encompassing reinforced proliferation, migration and invasiveness (Kalluri and Weinberg, 2019; Bakir *et al*., 2020). Retinal pigment epithelium (RPE) EMT is regarded to play a key role in PVR pathogenesis (Yang *et al*., 2021). Former studies about PVR have revealed the several molecular mechanisms underlying EMT of RPE. For instance, miR-29b down-regulates p-p65 level to participates in the modulation of EMT of RPE cells (Li *et al*., 2021); miR-4516 negatively modulates OTX1 to hamper EMT of RPE cells, thus suppressing the development of PVR (Pao *et al*., 2022), interfering with KRT8 expression or suppressing its phosphorylation can impair autophagy to modulate EMT of RPE cells (Miao *et al*., 2020), artesunate reduces Smad3 and p-Smad3 levels to hinder the EMT in RPE cells, thus suppressing PVR incidence and development (Wang *et al*., 2021). Nevertheless, the mechanisms concerning RPE EMT still remain elusive, and further explorations of novel

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mechanisms are required.

AlkB homolog H5 (ALKBH5) is a N6-methyladenosine (m6A) demethylase, which has been reported to function as a regulator in multiple diseases. For example, ALKBH5 exerts regulation on tumor immune environment and targets PD-L1 mRNA intrahepatic cholangiocarcinoma (Qiu *et al.*, 2021); ALKBH5 down-regulates LYPD1 expression level through m6A modification, thus hindering malignant progression of hepatocellular carcinoma (Chen *et al.*, 2020). In addition, ALKBH5 also participates in the modulation of EMT (Sun *et al.*, 2022). However, the correlation between ALKBH5 and RPE cells EMT has never been studied before.

Herein, we selected ALKBH5 as our research object and probed into its downstream mechanisms of modulating RPE cells EMT. This study intended to identify a novel mechanism of PVR and deepen our understanding about its pathogenesis.

MATERIALS AND METHODS

Cell culture

Human RPE cell line, ARPE-19, commercially attained from ATCC, kept in DMEM/F-12 added with 10% fetal bovine serum (FBS), and maintained in 5% CO_2 at 37°C. To induce EMT, 10 ng/mL TGF-β1 was supplemented into the medium and kept for 72 h.

Cell transfection

Short hairpin RNAs (shRNAs) of ALKBH5, ZEB1 and IGF2BP1 were established to interfere with ALKBH5, ZEB1 and IGF2BP1 expression levels, with sh-NC being control. In addition, the sequences of ALKBH5, ZEB1 and IGF2BP1 used to establish their overexpression vectors, with empty vector as control. Transfection of plasmids into ARPE-19 cells was implemented by using Lipofectamine 3000.

RT-qPCR

The total RNAs were isolated from ARPE-19 cells, followed by conversion into complementary DNA (cDNA) utilizing reverse transcription. Afterwards, PCR was used to analyze the relative expression levels of E-cadherin, ZO-1, N-cadherin, α-SMA, ALKBH5, ZEB1 and IGF2BP1, standardized to GAPDH (endogenous reference). The results were calculated in accordance with $2^{-\Delta\Delta Ct}$ approach. Primer sequences were reported in Table I. We implemented bio-repeats in triplicate.

CCK-8

ARPE-19 cells were placed into 96-well culture plates, followed by 0, 24, 48 and 72 h of incubation, respectively.

After that, incubated 100 μL CCK-8 solution, and the incubation continued for another 4 h. A spectrophotometer was then employed for analysis of the absorbance at 450 nm. We implemented bio-repeats in triplicate.

Table I. The sequences of primers used in this study.

Gene	Sequence (5'-3')
E-cadherin	F: ACAAGGTGGTCCTGAGAGTTG
	R: TTTGGAGTTGCTTTTCCTCCG
ZO-1	F: CAGCCTGTGAGGCGTAGTAG
	R: TCCTTCAGCTGGTCCTCCTT
N-cadherin	F: CAGGGCCCTTTGCATTTGAC
	R: CGATCCTGTCTACGTCGGTG
α-SMA	F: TCTGCACAGGTGGCAAAGAT
	R: GGAACAGAGGTGGTTCCCTG
ALKBH5	F: TGCAAGCTCATGCAAACACC
	R: GACCCAACGTGGCAAGTCTA
ZEB1	F: GAGAGGATCATGGCGGATGG
	R: CTTTCACTGCTCCTCCCTGG
IGF2BP1	F: CAGGAGATGGTGCAGGTGTT
	R: GAGCCTTGAATTGGGCCTCT
GAPDH	F: GACAGTCAGCCGCATCTTCT
	R: GCGCCCAATACGACCAAATC

Transwell assay

In the upper chambers, serum-free medium was added to the ARPE-19 cells. Furthermore, complete medium was supplemented to the upper chambers. Subsequent to 24 h of incubation, the cells left in the upper chambers were abraded. Crystal violet solution was then utilized to stain the migrated cells left in the lower chambers. The stained cells were counted with ImageJ. We implemented bio-repeats in triplicate.

Luciferase reporter assay

The sequences of ZEB1 promoter were subcloned into pGL3 vector for the construction of pGL3-ZEB1 promoter. The reporter vectors were then subjected to co-transfection into ARPE-19 cells with vectors of ALKBH5. The relative luciferase activity was determined, with Renilla luciferase as the internal reference. We implemented bio-repeats in triplicate.

RIP assay

RIPA lysis buffer was applied to lyse ARPE-19 cells. After being conjugated with the antibodies against IGF2BP1 and immunoglobulin G (IgG), the magnetic beads were then incubated with cell lysates. Lastly, RT-qPCR was applied to measure the enrichment of RNAs precipitated by the antibodies-conjugated beads. We

implemented bio-repeats in triplicate.

Methylated RNA-binding protein immunoprecipitation (MeRIP) assay

Magna MeRIP m6A Kit (Merck Millipore, USA) was employed for the operation of MeRIP assay. After being extracted from ARPE-19 cells, the total RNAs were fragmented into 100 nucleotides. Subsequently, the samples were subjected to incubation with the m6A antibody for implementation of immunoprecipitation as per the standard instruction of manufacturer. RT-qPCR was conducted to analyze the enrichment of the RNAs containing m6A.

Statistical analysis

With SPSS software used for data analysis, all data from experiments were exhibited as the mean±SD. Student's t test was used to compare two groups, and ANOVA followed by post-hoc tests (Tukey's or Dunnett's correction) was used to compare three groups. When P-value is under 0.05, difference comparisons were considered to be significant.
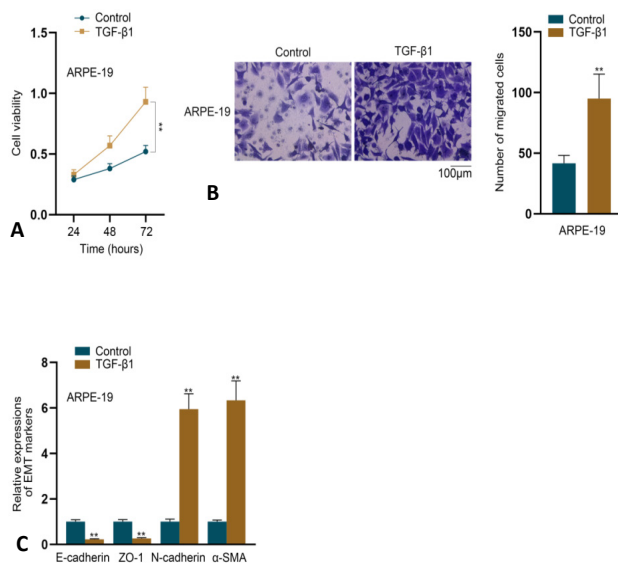

Fig. 1. TGF-β1 induces ARPE-19 EMT. (A), CCK-8 assay examined ARPE-19 cell viability in TGF-β1 and control groups; (B), Transwell assay examined ARPE-19 cell migration in TGF-β1 and control groups; (C), EMT markers expressions were unclosed by RT-qPCR in TGF-β1 and control groups.

RESULTS

Cell viability in TGF-β1 group was notably elevated relative to control group (Fig. 1A). Transwell assay uncovered that cell migration was evidently enhanced after TGF-β1 treatment as compared to control group (Fig. 1B). Additionally, the expression levels of EMT markers were determined by RT-qPCR. The outcomes unveiled that E-cadherin and ZO-1 expressions were reduced, and N-cadherin and α-SMA expressions were remarkably increased (Fig. 1C). Taken together, TGF-β1 induces ARPE-19 cells' EMT.
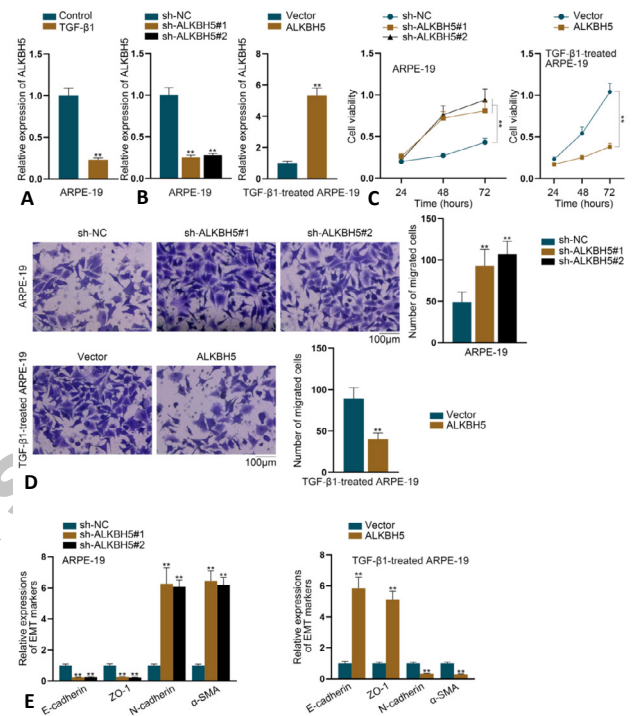

Fig. 2. ALKBH5 inhibits TGF-β1-induced ARPE-19 EMT. (A), RT-qPCR disclosed ALKBH5 expression in TGF-β1 and control groups; (B), RT-qPCR showed the interference efficiency (sh-NC, sh-ALKBH5#1 and sh-ALKBH5#2) in untreated ARPE-19 cells and the overexpression efficiency (Vector and ALKBH5) in TGF-β1-treated ARPE-19 cells; (C), CCK-8 assay examined ARPE-19 cell viability after silencing or overexpression of ALKBH5; (D), Transwell assay uncovered ARPE-19 cell migration after depletion or overexpression of ALKBH5; (E), E-cadherin, ZO-1, N-cadherin and α-SMA expressions were examined by RT-qPCR after depletion or overexpression of ALKBH5.

The results showed that ALKBH5 expression was suppressed subsequent to TGF-β1 treatment (Fig. 2A). Next, we implemented RT-qPCR to detect the interference efficiency of sh-ALKBH5#1/2 in untreated ARPE-19 cells and the overexpression efficiency of overexpression vectors of ALKBH5 in ARPE-19 cells (Fig. 2B). With these vectors, we then conducted gain/loss-of-function experiments to probe the influence of ALKBH5. As

displayed in Figure 2C, D, cell viability and migration were up-regulated after ALKBH5 depletion in ARPE-19 cells, while ALKBH5 overexpression impaired cell viability and migration in TGF-β1-treated ARPE-19 cells. Moreover, RT-qPCR was carried out to determine EMT markers after silencing or overexpression of ALKBH5. It was found that interference with ALKBH5 led to significant down-regulation in E-cadherin and ZO-1 expressions and noticeable up-regulation in N-cadherin and α-SMA expressions, while ALKBH5 enhancement exerted the opposite effects (Fig. 2E). Collectively, ALKBH5 inhibits TGF-β1-induced ARPE-19 cells' EMT.

According to the results, ZEB1 expression was up-regulated by ALKBH5 interference, while that of ZEB1 was down-regulated by ALKBH5 enhancement (Fig. 3A). The results suggested the negative regulation of ALKBH5 on ZEB1. Next, we implemented assays to explore the mechanisms of ALKBH5 underlying ZEB1 regulation. We conducted luciferase reporter assay to investigate whether ALKBH5 can combine with ZEB1 promoter. It was unearthed that the activity of pGL3-ZEB1 promoter wasn't affected by ALKBH5 (Fig. 3B). After that, RT-qPCR was implemented to examine the impacts of ALKBH5 on ZEB1 mRNA stability after α-amanitin treatment. It was unmasked that the half-life of ZEB1 was prolonged after ALKBH5 interference, while that of ZEB1 was shortened after ALKBH5 overexpression (Fig. 3C). Moreover, MeRIP results disclosed that the abundance of ZEB1 was evidently enhanced subsequent to ALKBH5 silencing in Anti-m6A group relative to Anti-IgG group, whereas that of ZEB1 was evidently attenuated subsequent to ALKBH5 overexpression (Fig. 3D). The results indicated that ALKBH5 might modulate ZEB1 mRNA stability via demethylation of ZEB1 mRNA. Previous research showed that IGF2BP1 can modulate mRNA stability in an m6A-dependent manner (Zhang *et al.*, 2021). Hence, we conjectured that ALKBH5 might modulate ZEB1 mRNA stability through IGF2BP1. After examining the efficiencies of interference and overexpression vectors of IGF2BP1 (Fig. 3E), we then carried out RT-qPCR to test the influence of IGF2BP1 on ZEB1 expression. As displayed in Figure 3F, IGF2BP1 inhibition down-regulated ZEB1 expression and its overexpression up-regulated ZEB1 expression, suggesting that IGF2BP1 might positively regulate ZEB1. RIP assay in Figure 3G proved the interaction between IGF2BP1 and ZEB1. Next, RT-qPCR was conducted to investigate the relationship among ALKBH5, IGF2BP1 and ZEB1. As displayed in Figure 3H, ZEB1 expression affected by ALKBH5 knockdown or overexpression was completely offset by IGF2BP1 silencing or overexpression. Overall, ALKBH5 suppresses ZEB1 mRNA stability in an m6A-IGF2BP1-dependent manner.
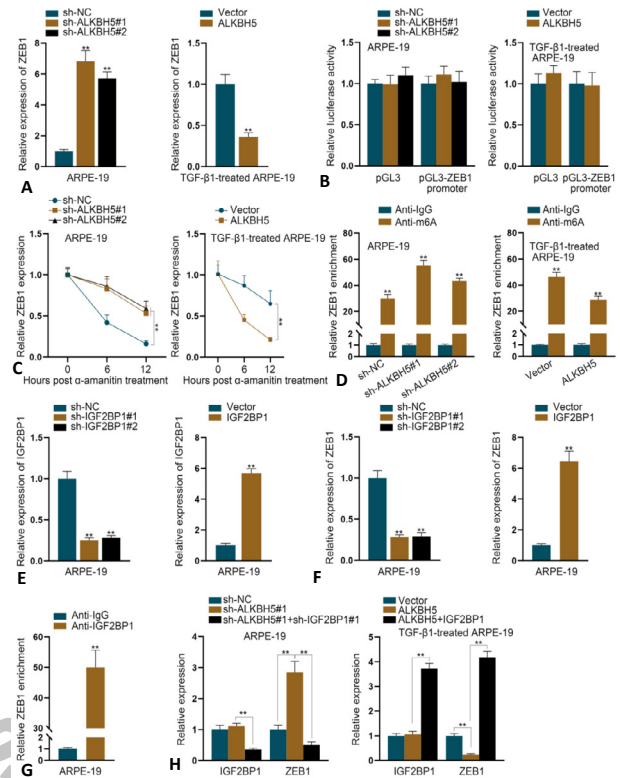

Fig. 3. ALKBH5 suppresses ZEB1 mRNA stability in an m6A-IGF2BP1-dependent manner. (A), RT-qPCR determined ZEB1 expression after depletion of ALKBH5 in untreated ARPE-19 cells and after ALKBH5 overexpression in TGF-β1-treated ARPE-19 cells; (B), Luciferase reporter assay unclosed the binding of ALKBH5 with ZEB1 promoter in ARPE-19 cells; (C), The stability of ZEB1 mRNA was determined by RT-qPCR after depletion or overexpression of ALKBH5 in ARPE-19 cells treated with α-amanitin; (D), MeRIP examined m6A modification of ZEB1 after depletion or overexpression of ALKBH5 in ARPE-19 cells; (E), RT-qPCR demonstrated the interference efficiency (sh-NC, sh-IGF2BP1#1 and sh-IGF2BP1#2) and the overexpression efficiency (Vector and IGF2BP1) in ARPE-19 cells; (F), RT-qPCR examined ZEB1 expression after depletion or overexpression of IGF2BP1 in ARPE-19 cells; (G), RIP assay validated the interaction between IGF2BP1 and ZEB1 in ARPE-19 cells; (H), RT-qPCR unclosed IGF2BP1 and ZEB1 expressions in ARPE-19 and TGF-β1-treated ARPE-19 cells under indicated transfections.

As shown in CCK-8 and transwell assays, cell viability and migration enhanced by ALKBH5 knockdown were fully reversed by ZEB1 overexpression, while ZEB1 knockdown completely rescued cell viability and migration hampered by ALKBH5 overexpression (Fig. 4A, B). Additionally, the detection of EMT markers demonstrated

that EMT enhanced by ALKBH5 knockdown was fully reversed by ZEB1 overexpression, and ZEB1 silencing could completely rescue EMT hampered by ALKBH5 overexpression **(**Fig. 4C). To sum up, ALKBH5 modulates ZEB1 to affect ARPE-19 cells' EMT.
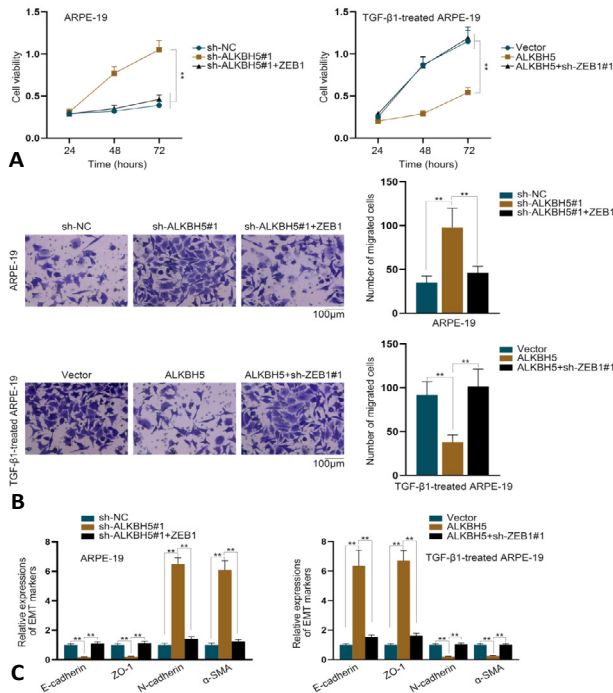

Fig. 4. ALKBH5 modulates ZEB1 to affect EMT of ARPE-19 cells. (A), CCK-8 assay examined cell viability in ARPE-19 cells transfected with sh-NC, sh-ALKBH5#1 or sh-ALKBH5#1+ZEB1 and in TGF-β1-treated ARPE-19 cells transfected with Vector, ALKBH5 or ALKBH5+sh-ZEB1#1; (B), Transwell assay examined ARPE-19 cell migration in different transfection groups; (C), EMT markers expressions were uncovered by RT-qPCR in different transfection groups.

DISCUSSION

According to the previous study, TGF-β1 can be utilized to induce EMT of RPE cells (Ma *et al*., 2021). In the current study, we also used TGF-β1 to induce ARPE-19 cells' EMT and the outcomes of CCK-8, transwell and RT-qPCR assays proved the effect of TGF-β1 on EMT. Former studies have unclosed the role of ALKBH5 in modulating EMT. ALKBH5 targets TGF-β/SMAD signaling to hinder TGF-β-induced lung cancer cell EMT (Sun *et al*., 2022). ALKBH5 facilitates EMT by demethylating FOXM1 mRNA, thus promoting the metastasis of uveal melanoma (Hao *et al*., 2021). ALKBH5 hampers EMT of trophoblast cells to modulate the occurrence of preeclampsia (He *et*

al., 2020). In line with these findings, our study proved that ALKBH5 is down-regulated in TGF-β1-treated ARPE-19 cells and impairs TGF-β1-induced ARPE-19 cells' EMT.

As a transcription factor, ZEB1 can induce EMT of cells. DCAF15 hinders EMT of hepatocellular carcinoma cells through modulating ZEB1 (Dong *et al*., 2021). CircZFPM2 interacts with miR-205-5p to regulate ZEB1, thereby promoting EMT in endometriosis (Wang *et al*., 2022). ZEB1, miR-200c and AGR2 form a negative feedback loop to regulate EMT in lung adenocarcinoma (Sommerova *et al*., 2020). Additionally, in the study of PVR, miR-194 can directly target ZEB1 to impair EMT of RPE cells (Cui *et al*., 2019). Here, we performed rescue experiments using ALKBH5 and ZEB1, proving that ALKBH5 can also target ZEB1 to modulate EMT of RPE cells.

Based on the results in the present study, we proved that ALKBH5 decreases ZEB1 mRNA stability and leads to m6A demethylation of ZEB1 mRNA. Moreover, as evidenced by luciferase reporter assay, ALKBH5 cannot interact with ZEB1 promoter, excluding the possibility of promoter methylation. Recent studies unclosed that ALKBH5 depends on m6A readers to regulate its downstream mRNA (Yang *et al*., 2022; Guo *et al*., 2020). We identified IGF2BP1 and wondered whether ALKBH5 regulates ZEB1 mRNA stability in an m6A-dependent manner through this m6A reader. IGF2BP1, as reported previously, can recognize m6A sites in PEG10 mRNA and modulate its stability in endometrial cancer (Zhang *et al*., 2021). Moreover, IGF2BP1 can enhance m6A modification of MGAT5 mRNA in order to facilitate its stability (Yang *et al*., 2021). We proved the interaction between ZEB1 and IGF2BP1 and then investigated the correlation among ALKBH, ZEB1 and IGF2BP1. It was validated that ALKBH5 suppresses ZEB1 mRNA stability in an m6A-IGF2BP1-dependent manner.

Owing to the limitation of conditions, the design of assays in our report has several limitations. The functions of ALKBH5 were only investigated at the cellular level. The lack of animal experiments may have undermined the stringency of our report. Furthermore, the upstream mechanisms of ALKBH5 need to be further explored. In the future, we will establish animal models to examine the mechanisms of ALKBH5 *in vivo*, and supplement the investigation of its upstream targets.

CONCLUSION

ALKBH5 attenuates TGF-β1-induced ARPE-19 cells' EMT via regulating ZEB1, indicating that m6A demethylase ALKBH5 is implicated in the regulation of PVR. In addition, our data uncovered that ALKBH5, in an

m6A-IGF2BP1-dependent manner, suppresses the mRNA stability of ZEB1, an EMT-related transcription factor. Our research deepens the understanding of PVR pathogenesis and provides a theoretical basis for development of novel treatment strategies.

ACKNOWLEDGMENTS

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of Yixing Eye Hospital, Jiangsu Province, China. The official letter would be available on fair request to corresponding author.

IRB approval

This study was approved by the Advanced Studies Research Board of Yixing Eye Hospital, Yixing 214200, Jiangsu Province, China.

Statement of conflict of interest

The authors have declared no conflict of interest.

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