



JAM-C prevents ocular fibrosis by suppressing the TAZ/KLF6 pathway

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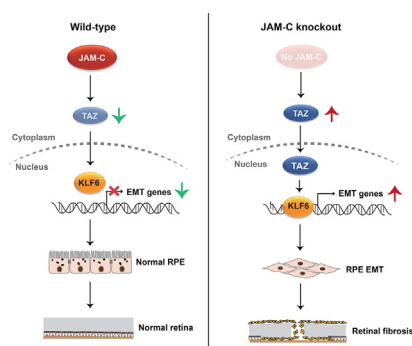
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HIGHLIGHTS

- JAM-C is a critical molecule to prevent ocular fibrosis.
- JAM-C level decreased markedly in the vitreous humor of patients with ocular fibrosis.
- Genetic deletion of JAM-C exacerbated ocular fibrosis in different mouse models.
- JAM-C prevents RPE EMT and ocular fibrosis by suppressing the TAZ/KLF6 axis.
- JAM-C delivery by AAV significantly alleviated ocular fibrosis in different mouse models.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Ocular fibrosis is one of the leading causes of irreversible visual impairment or blindness. Currently, there is no effective drug available for such diseases. Therefore, understanding the underlying mechanisms is a prerequisite for finding better therapeutic strategies.

Objectives: This study aims to investigate the role of the junctional adhesion molecule C (JAM-C) in ocular fibrosis.

Methods: The protein levels of JAM-C were determined in the vitreous humor samples of patients with ocular fibrosis using ELISA. *Jam-c* genetic deletion mice and ocular fibrosis mouse models were generated to study the role of JAM-C *in vivo*. EMT, proliferation, migration, and gel contraction capacities in RPE cells were examined after JAM-C knockdown by siRNAs. RNA sequencing, co-IP, ChIP-qPCR, and luciferase reporter assay were performed to investigate the underlying mechanisms. Subretinal injection of adeno-associated virus, immunofluorescence, western blot were performed to evaluate the potential of JAM-C in preventing ocular fibrosis in different mouse models.

Results: Markedly reduced JAM-C expression was found in patients with ocular fibrosis. Genetic deletion of *Jam-c* in mice exacerbated ocular fibrosis, and JAM-C knockdown triggered the EMT process in RPE cells. Mechanistically, we reveal that JAM-C inhibits ocular fibrosis by suppressing the nuclear

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localization and function of TAZ, which otherwise binds to KLF6 to promote its expression and activity to initiate the EMT cascade. Importantly, AAV-mediated JAM-C augmentation alleviated ocular fibrosis in different mouse models.

Conclusion: Our findings unveil a novel function of JAM-C in preventing ocular fibrosis by inhibiting the TAZ/KLF6 pathway, and suggest new therapeutic possibilities for the treatment of fibrotic diseases.

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Introduction

Ocular homeostasis is essential for maintaining normal visual function and can be disrupted by various pathologies, such as fibrosis. Ocular fibrosis is a leading cause of irreversible vision loss, characterized by the excessive scar formation that damages the normal ocular structure and function, resulting in numerous ocular diseases, such as proliferative vitreoretinopathy (PVR), age-related macular degeneration (AMD), diabetic retinopathy, retinopathy of prematurity, and neovascular glaucoma [1,2]. Moreover, ocular fibrosis represents a substantial impediment to various regenerative therapies, including cell therapies [3]. Currently, there is no pharmaceutical agent can be utilized for the treatment or prevention of ocular fibrosis [4]. It is therefore urgent to elucidate the underlying molecular mechanisms involved for the better treatment of such diseases.

PVR is a major complication of rhegmatogenous retinal detachment and is characterized by fibrosis formation, with the growth of fibrotic membranes on both sides of the retina and in the vitreous [5]. The contractile membranes often cause tractional retinal detachment, compromising surgical outcomes and resulting in vision loss [6]. Neovascular AMD (nAMD) accounts for about 90 % of AMD-associated blindness and is characterized by the presence of choroidal neovascularization (CNV) [7]. Subretinal fibrosis is the end-stage complication of nAMD leading to irreversible vision loss with a 62.7 % cumulative incidence at 10 years [8]. Retinal pigment epithelial (RPE) is a monolayer of polarized and differentiated epithelial cells essential to maintain the normal morphology and function of the retina in multiple processes, such as the formation of blood-retinal barrier, phagocytosis, transportation of nutrients and oxygen, maintenance of visual cycle, and production of various factors required for the normal function of the eye [9]. Impaired RPE structure leads to RPE dysfunction, resulting in various ocular pathologies. Epithelial-mesenchymal transition (EMT) in RPE is a pivotal step driving ocular fibrosis [7,10]. However, the mechanisms underlying RPE EMT are not well understood.

Junctional adhesion molecules (JAMs) are essential cell-to-cell adhesion factors to maintain epithelial tight junctions and establishing epithelial cell polarity [11,12]. The JAM family comprises seven proteins, including three classical JAMs (JAM-A, JAM-B, and JAM-C) and four related proteins (JAM-4, CAR, JAM-L, and ESAM) [11]. JAM-C is abundantly expressed in the eye and mainly exists

as a transmembrane protein [13]. Yet, JAM-C also exists in a soluble form in endothelial or neural stem cells after being cleaved by metalloproteinases, such as ADAM10 [14,15]. Previous studies have shown that JAM-C inhibits human trophoblast differentiation by suppressing the EMT process and contribute to preeclampsia development [16]. On the other hand, JAM-C was shown to promote liver fibrosis induced by autoimmune pathologies in mice [17]. Although JAM-C is particularly enriched in tight junctions of the RPE layer and promotes RPE junction formation and polarization [13,18], the role of JAM-C in ocular fibrosis remains unclear.

In this study, we show that JAM-C level decreased in the vitreous humor of patients with ocular fibrosis. Using different ocular fibrosis mouse models and RPE-specific JAM-C deficient mice, we reveal that JAM-C inhibits ocular fibrosis by suppressing the EMT-promoting TAZ/KLF6 pathway. Importantly, JAM-C overexpression reduced ocular fibrosis in different mouse models. Collectively, our data highlight the significance of JAM-C in preventing ocular fibrosis pathogenesis, and suggest a potential new therapeutic avenue for the treatment of fibrosis-related diseases through modulating JAM-C activity.

Results

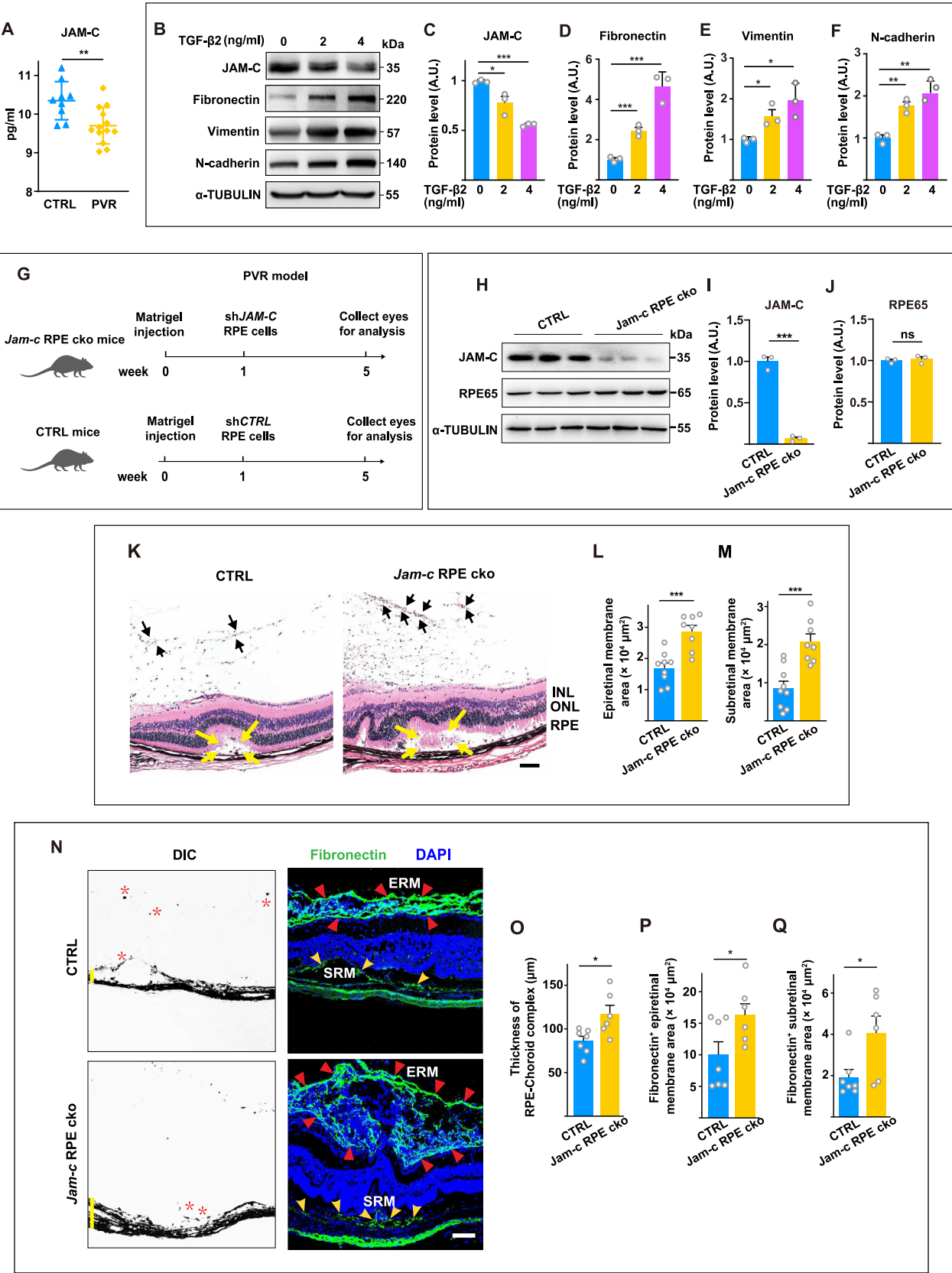
JAM-C level decreases in patients with ocular fibrosis

JAM-C is highly expressed in the eye [13,18]. However, it is still unclear whether it plays a role in ocular fibrosis. Given that the accentuated fibrosis is a main character of PVR, hereafter we used a PVR model to study the function of JAM-C in ocular fibrosis. Using the vitreous humor of PVR patients, we found that JAM-C level significantly decreased in PVR patients as revealed by ELISA (Fig. 1A, Supplementary Table 1 and 2). RPE EMT is a critical pathology in PVR [10]. We therefore investigated JAM-C levels in RPE undergoing EMT, and found that JAM-C levels gradually decreased during TGF- β 2-induced EMT in RPE cells [17] in a dose-dependent manner, whereas the levels of the EMT markers fibronectin, vimentin and N-cadherin increased (Fig. 1B–F). These data thus suggested a potential role of JAM-C in ocular fibrosis.

Genetic deletion of JAM-C exacerbates ocular fibrosis

We next investigated the role of JAM-C in ocular fibrosis utilizing a modified PVR mouse model [19] (Fig. 1G). The results demon-

Fig. 1. Genetic deletion of *Jam-c* exacerbates ocular fibrosis. (A) ELISA results showing lower levels of JAM-C in the vitreous humor of PVR patients. $n = 9$ for CTRL and $n = 13$ for PVR. (B–F) RPE cells were treated with TGF- β 2 (2 and 4 ng/ml) for 48 h and subjected to Western blot for indicated proteins. $n = 3$. (G) Diagram showing the generation of the PVR mouse model in *Jam-c* RPE cko and CTRL mice. (H–J) Western blot showing the protein levels of JAM-C and RPE marker (RPE65) in the RPE-choroid complex of CTRL and *Jam-c* RPE cko mice. $n = 3$. (K–M) H&E staining showing epiretinal (black arrows) and subretinal (yellow arrows) membrane formation in *Jam-c* RPE cko and CTRL mice in a PVR model (K). RPE: retinal pigment epithelium, INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar: 50 μ m. The area of the membranes (K) are shown in L and M. $n = 9$ for CTRL and $n = 8$ for *Jam-c* RPE cko. (N–Q) DIC (differential interference contrast) and immunostaining of fibronectin in *Jam-c* RPE cko and CTRL mice in a PVR model (N). Green: fibronectin; Blue: DAPI. Red asterisks: misplaced melanosome; Orange arrows: subretinal membrane (SRM); Red arrows: epiretinal membrane (ERM); Yellow lines: RPE-choroid complex. Scale bar: 50 μ m. The thickness of RPE-choroid complex and the fibronectin-positive membrane area (N) are shown in O–Q. $n = 7$ for CTRL and $n = 6$ for *Jam-c* RPE cko. Data are shown as mean $\pm$ SD. Statistical significance was assessed by one-way ANOVA in C–F, and Student's *t*-test in A, I–J, L–M, and O–Q. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



strated that RPE-specific *Jam-c* deletion (*Jam-c* RPE cko, Fig. 1H–J) led to more severe fibrosis as shown by the presence of more epiretinal (black arrows, Fig. 1K, L) and subretinal fibrotic membranes (yellow arrows, Fig. 1K, M), mispositioned melanosomes in the subretinal space and vitreous (asterisks, Fig. 1N), and increased thickness of the RPE-choroid complex in *Jam-c* deficient mice (yellow lines, Fig. 1N, O). The more severe fibrosis was also supported by the markedly enhanced fibronectin levels in both the vitreous and subretinal space of *Jam-c* deficient mice (green, Fig. 1N, P, Q). Consistently, the elevated fibronectin levels were also observed in another PVR model induced by dispase [20] (Supplementary Fig. 1A–C). Furthermore, in a laser-induced subretinal fibrosis model in nAMD [21] (Supplementary Fig. 1D), *Jam-c* deletion increased fibronectin levels in the CNV lesions (Supplementary Fig. 1E–F). Overall, these results shown that loss of JAM-C promotes ocular fibrosis.

JAM-C knockdown leads to RPE EMT

We used yet another loss-of-function approach to investigate the effect of JAM-C on RPE cells by knocking down JAM-C using two different siRNAs (Fig. 2A). JAM-C knockdown increased the levels of EMT markers fibronectin and N-cadherin, while decreased the level of epithelial marker E-cadherin in both an ARPE-19 cell line (Fig. 2B–F) and primary human RPE cells (Fig. 2G–L). Immunofluorescence staining further confirmed a higher fibronectin level in JAM-C deficient RPE cells (Fig. 2M–N). Moreover, JAM-C knockdown increased RPE proliferation and migration (Fig. 2O–Q, Supplementary Fig. 2), two hallmarks in the process of RPE EMT [22]. Functionally, JAM-C knockdown markedly increased collagen gel contraction of the RPE cells (similar to PVR membrane contraction, Fig. 2R–S). Thus, loss of JAM-C led to EMT of RPE cells.

JAM-C reduces TAZ protein stability and nuclear translocation

To explore how JAM-C exerts the protective role in RPE cells, we performed RNA sequencing (RNA-Seq) using JAM-C-overexpressing RPE cells (Supplementary Fig. 3A–B, Supplementary Table 6). We found the Hippo pathway was mostly enriched for the differentially expressed genes (DEGs) by KEGG analysis (Supplementary Fig. 3C–D). Protein-protein interactions (PPI) analysis showed that TAZ (transcriptional co-activator with the PDZ-binding motif) is a key hub gene with the highest correlation with the other Hippo pathway members (Supplementary Fig. 3E). TAZ is an important factor regulating EMT [23], and its phosphorylation promotes its protein degradation, thereby inhibiting its nuclear entry and activation [24,25]. We found that JAM-C knockdown upregulated TAZ and decreased its phosphorylation (Fig. 3A–D). By contrast, the expression of YAP, another Hippo pathway effector [23], was not affected by JAM-C knockdown (Fig. 3A, E). A cycloheximide (CHX) chase assay further confirmed that JAM-C knockdown increased TAZ protein stability (Fig. 3F–H). It is known that protein interaction between junction proteins and TAZ can regulate TAZ's subcellular localization and activity [26]. A co-immunoprecipitation (co-IP) assay demonstrated that JAM-C co-precipitated with TAZ but not YAP (Fig. 3I). To further explore the binding sites between JAM-C and TAZ, we generated Flag-tagged JAM-C full-length (FL) and a truncate without the cytoplasmic domain (JAM-C Δ Cyto) (Fig. 3J). Co-IP results showed that JAM-C FL co-precipitated with endogenous TAZ, while the interaction between JAM-C Δ Cyto and TAZ was very weak (Fig. 3K), suggesting that the cytoplasmic domain of JAM-C is essential for the interaction with TAZ [27]. A subcellular fractionation assay shown that JAM-C knockdown promotes the nuclear translocation of TAZ (Fig. 3L–N), which was confirmed by immunofluorescence staining (Fig. 3O–P). Noteworthy, overexpression of TAZ in RPE cells (Sup-

plementary Fig. 3F–G) led to EMT of RPE cells as shown by the elongated fibroblast-like morphology of the cells (Supplementary Fig. 3H) and the increased levels of many EMT markers (Supplementary Fig. 3I). These data show that JAM-C keeps TAZ in check by inhibiting its expression and nuclear translocation.

TAZ promotes KLF6-mediated transcription activation to induce RPE EMT

To investigate the mechanism underlying TAZ's function in the process of RPE EMT, we performed RNA-seq using TAZ-overexpressing RPE cells and identified 1780 upregulated and 1828 downregulated DEGs respectively (Fig. 4A, Supplementary Table 7). Since TAZ is a transcription co-activator to facilitate the activities of other transcription factors (TFs) [28], all the TFs were screened in the list and we mainly focused on KLF6, since it is a potential TF important for EMT by bioinformatics analysis [29]. Indeed, TAZ overexpression upregulated KLF6 (Fig. 4B), whereas TAZ depletion markedly downregulated KLF6 (Fig. 4C–E, Supplementary Fig. 3J–L). We then investigated whether TAZ affected KLF6 function and found that overexpression of TAZ enhanced KLF6-induced promoter activity of fibronectin gene (*FN1*) in a luciferase assay (Fig. 4F). Moreover, overexpression of TAZ significantly enhanced the binding of KLF6 to *FN1* promoter in a chromatin immunoprecipitation (ChIP)-qPCR assay (Fig. 4G), leading to the increased *FN1* expression (Fig. 4H). Notably, TAZ depletion markedly attenuated the upregulated KLF6 and fibronectin levels induced by JAM-C knockdown in RPE cells (Fig. 4I–N). These data thus show that TAZ could reverse JAM-C's anti-EMT function via enhancing KLF6's transcription activation.

KLF6 mediates JAM-C depletion-induced RPE EMT

The role of KLF6 in RPE EMT is currently undetermined. Thereby, we knocked down KLF6 by siRNA, which markedly reduced EMT and cell proliferation of the RPE cells (Fig. 5A–F, Supplementary Fig. 4A–B). Importantly, KLF6 knockdown reversed the loss-of-JAM-C-induced proliferation of RPE cells in a CCK-8 assay (Fig. 5G–J), and abolished loss-of-JAM-C-induced upregulation of fibronectin (Fig. 5K–L). Together, these data demonstrate that KLF6 is the downstream negative regulator of JAM-C to promote RPE EMT.

JAM-C overexpression ameliorates ocular fibrosis

We explored the potential of JAM-C in inhibiting ocular fibrosis using adeno-associated viruses (AAV)-mediated RPE specific (under hVMD2 promoter) [30] JAM-C overexpression (JAM-C OE) in PVR mouse model (Fig. 6A–D). We found that JAM-C overexpression inhibited ocular fibrosis as shown by the decreased number of misdistributed melanosomes (asterisks, Fig. 6E) and reduced thickness of the RPE-choroid complex (yellow lines, Fig. 6E, F). Consistently, fibronectin levels also markedly decreased in the vitreous and subretinal regions in mice with JAM-C overexpression (green, Fig. 6E, G, H), indicating the reduction of fibrotic membrane formation. Similarly, JAM-C overexpression reduced ocular fibrosis in both the dispase-induced PVR model (Supplementary Fig. 5A–C) and a subretinal fibrosis model (Supplementary Fig. 5D–F) as shown by fibronectin staining. Moreover, we verified that KLF6 levels were decreased along with fibronectin in JAM-C overexpressing mice with PVR (Fig. 6I–N), whereas it increased in *Jam-c* knockout PVR model (Supplementary Fig. 5G–I). Overall, these data suggested that JAM-C overexpression ameliorates ocular fibrosis via inhibiting KLF6 and indicated that JAM-C is a potential therapeutic target for fibrosis.

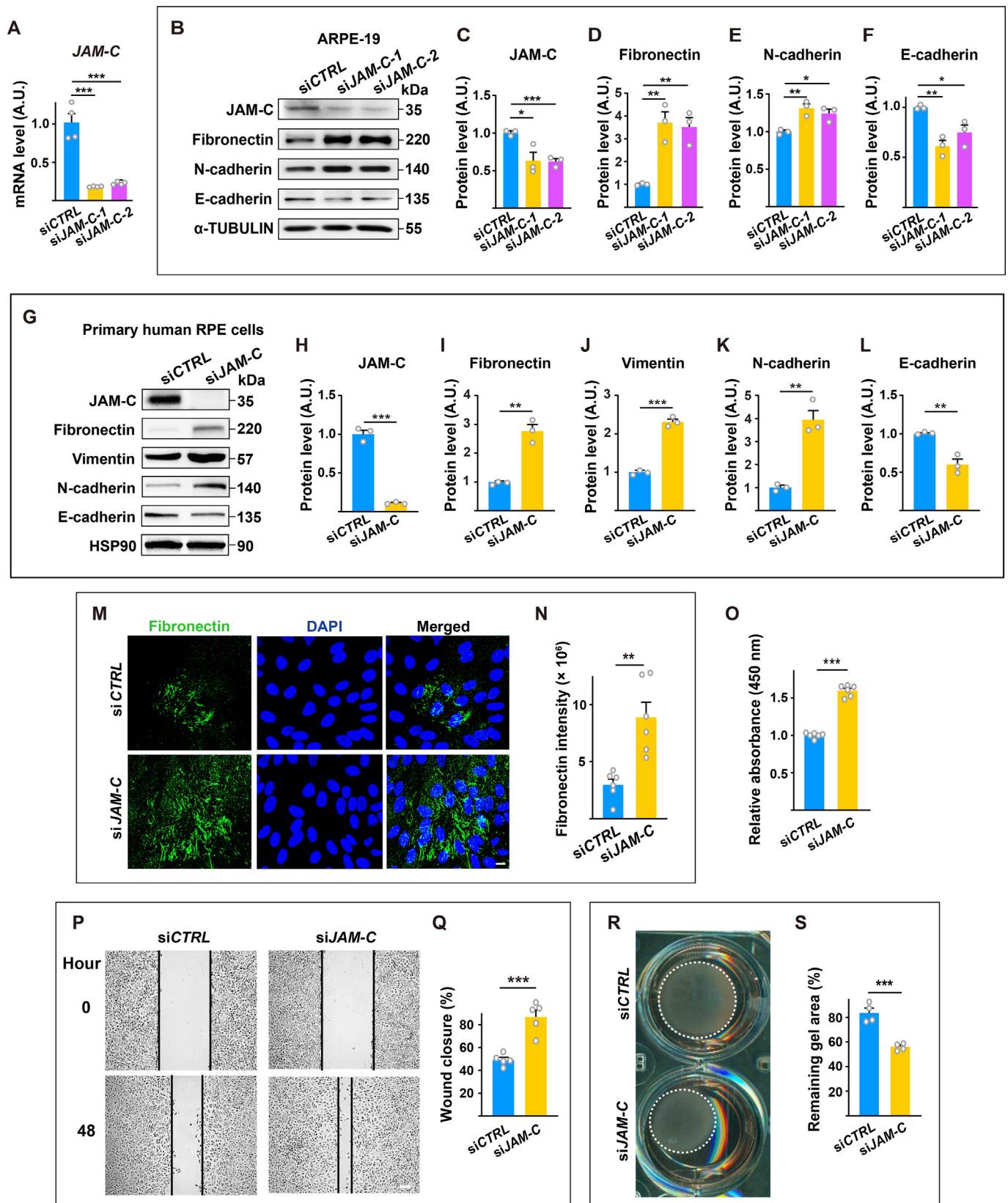
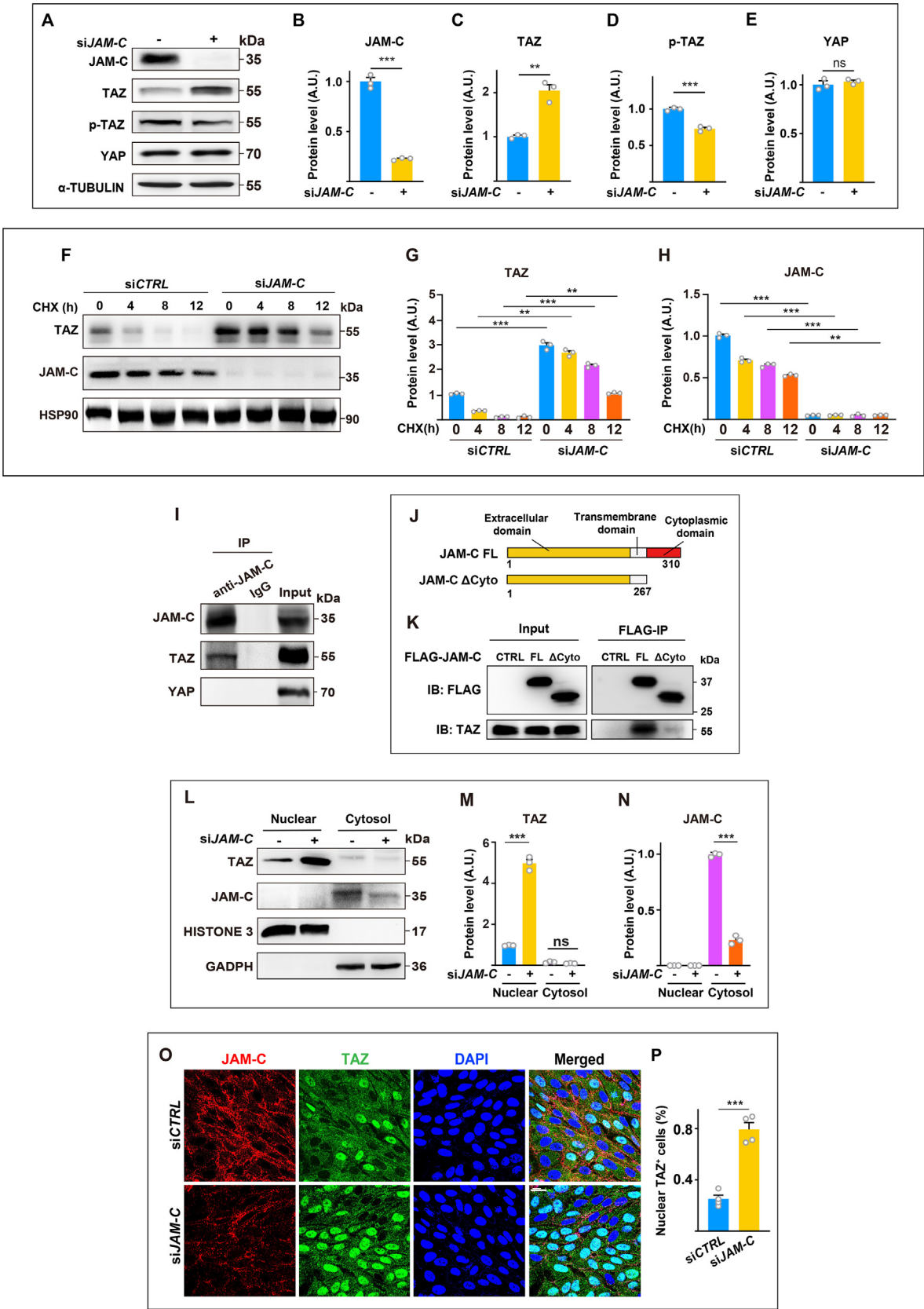


Fig. 2. JAM-C knockdown results in RPE EMT. (A) qRT-PCR results showing the knockdown efficiency of JAM-C by two different siRNA oligos in RPE cells. $n = 4$. (B-F) Western blot showing the protein levels of JAM-C, mesenchymal markers (fibronectin, N-cadherin) and the epithelial marker E-cadherin in ARPE-19 cells with JAM-C knockdown. $n = 3$. (G-L) Western blot showing the protein levels of JAM-C, mesenchymal markers (fibronectin, vimentin, N-cadherin) and the epithelial marker E-cadherin in primary human RPE cells with JAM-C knockdown. $n = 3$. (M-N) Immunostaining of fibronectin in RPE cells with JAM-C knockdown (M). Nuclei are stained by DAPI. Scale bar: 10 μ m. Quantification of fibronectin staining intensity is shown in N. $n = 6$. (O) CCK8 assay results showing the cell proliferation status after JAM-C knockdown in RPE cells at 72 h. $n = 6$. (P-Q) Wound healing assay results showing the migration potential of RPE cells after JAM-C knockdown (P). Scale bar: 200 μ m. Quantification of wound closure areas are shown in Q. $n = 5$. (R-S) Gel contraction assay results showing the cell contraction ability after JAM-C knockdown (R). Quantification of the relative remaining areas of the gels are shown in S. $n = 4$. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA in A, C-F, and Student's t -test in H-L, N, O, Q and S. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



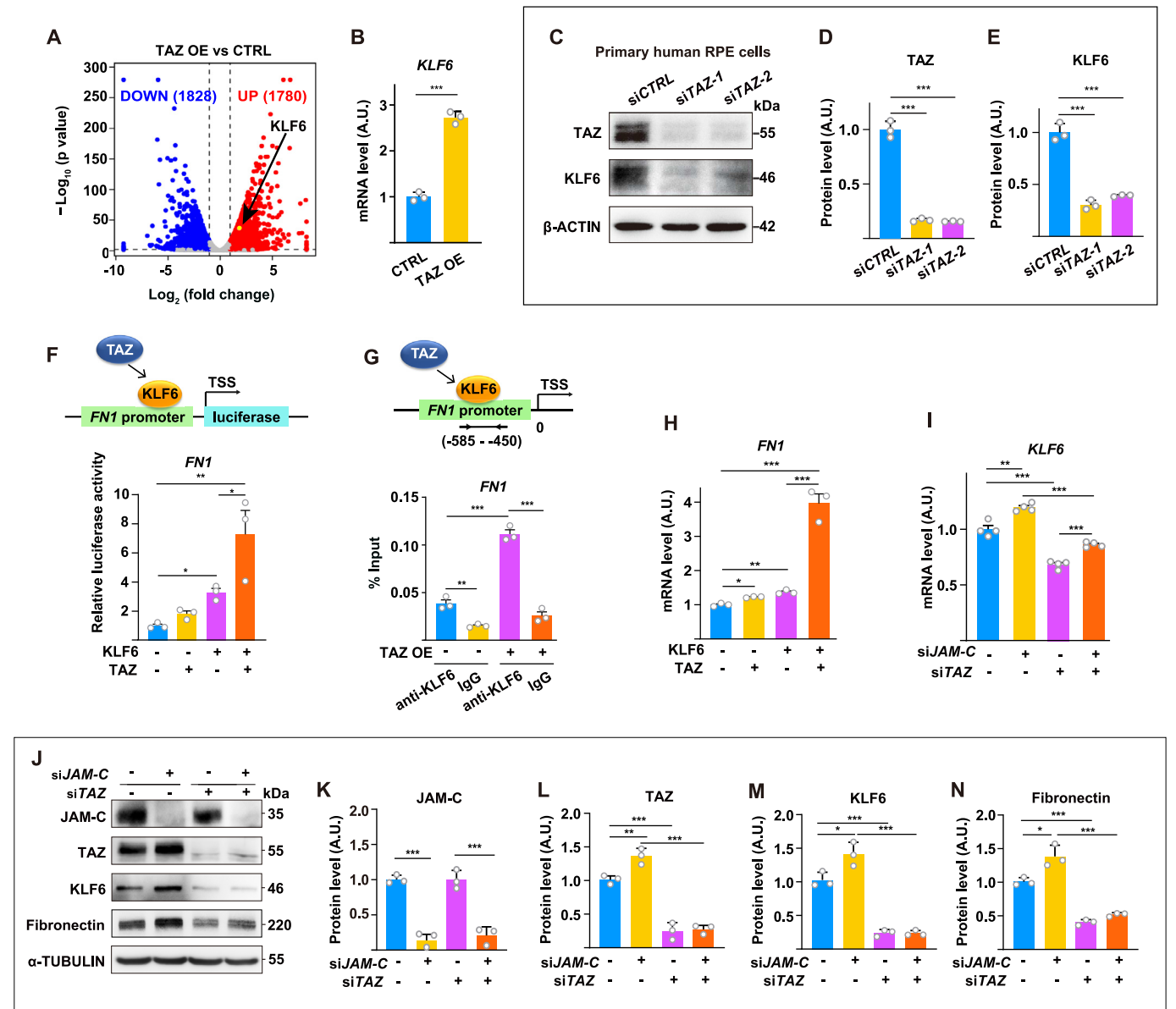
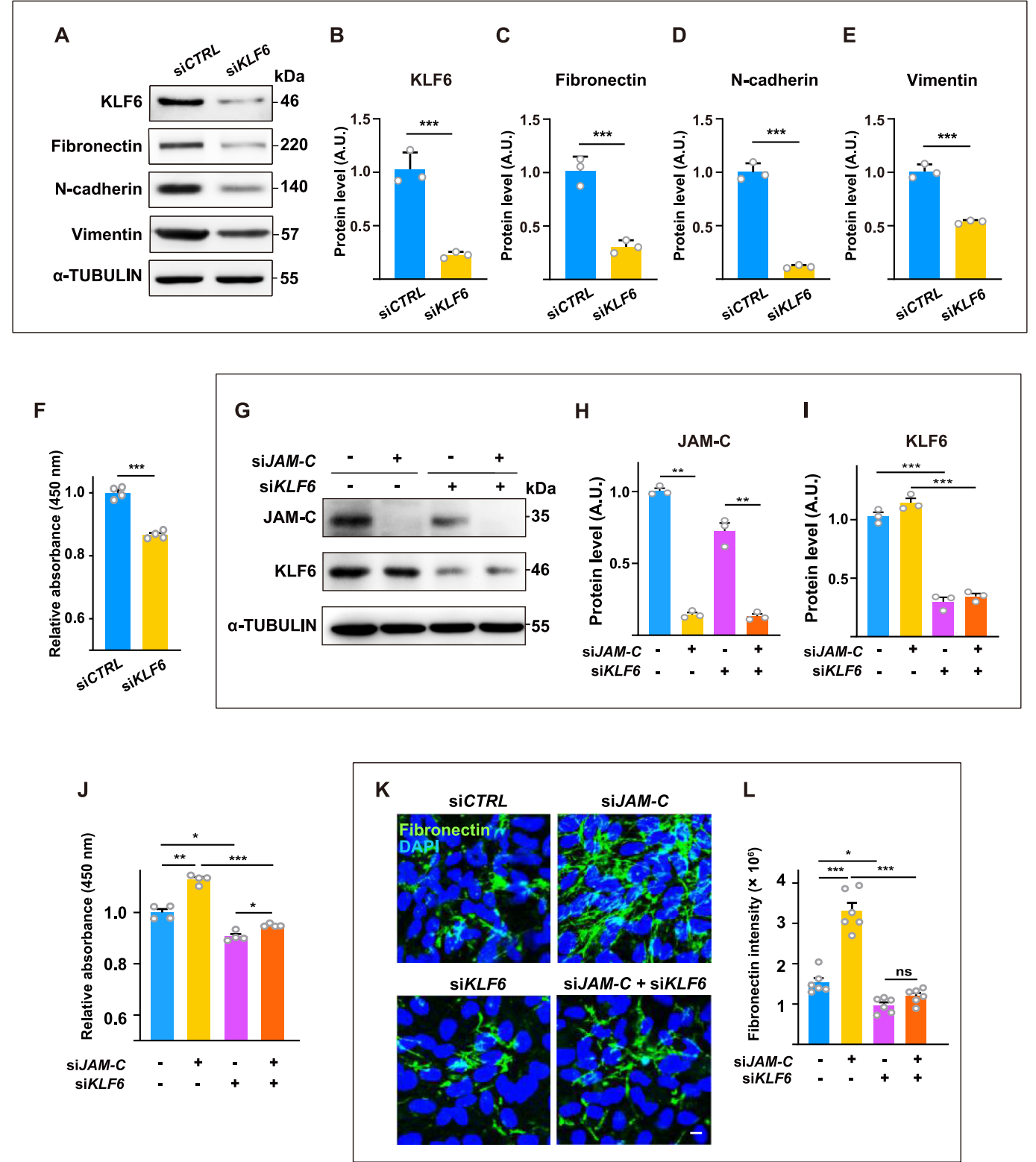


Fig. 4. TAZ increases KLF6's expression and pro-EMT function. (A) Volcano plot showing the up- and down-regulated genes in TAZ overexpressing RPE cells. (B) qRT-PCR showing KLF6 level in TAZ overexpressing RPE cells. $n = 3$. (C-E) Western blot showing the protein levels of TAZ and KLF6 after TAZ knockdown in primary human RPE cells. $n = 3$. (F) Dual luciferase reporter gene assay showing the luciferase activity of FN1 promoter after the overexpression of TAZ and/or KLF6 in RPE cells. $n = 3$. (G) ChIP-qPCR assay detected the enrichment of KLF6 at FN1 promoter region in RPE cells. $n = 3$. (H) qRT-PCR results showing the expression level of FN1 in RPE cells overexpressing KLF6 and/or TAZ. $n = 3$. (I) qRT-PCR result showing KLF6 mRNA level after knockdown of JAM-C and/or TAZ in RPE cells. $n = 4$. (J-N) Western blot showing the protein levels of JAM-C, TAZ, KLF6 and fibronectin after the knockdown of JAM-C and/or TAZ in RPE cells. $n = 3$. Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t -test in B and one-way ANOVA in D-I, K-N. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. JAM-C inhibits TAZ activation in RPE. (A-E) Western blot showing the expression of JAM-C, TAZ, p-TAZ and YAP after JAM-C knockdown in RPE cells. $n = 3$. (F-H) Western blot analysis for TAZ after JAM-C knockdown and treatment with 25 $\mu\text{g}/\text{ml}$ Cycloheximide (CHX) in RPE cells. $n = 3$. (I) Co-immunoprecipitation (co-IP) using anti-JAM-C antibody or IgG followed by Western blot showing the interaction of JAM-C, TAZ and YAP in RPE cells. $n = 3$. (J) Schematic representation of full-length JAM-C and its ΔCyto truncate. (K) Results of co-immunoprecipitation (co-IP) followed by Western blot showing the interaction between Flag-tagged JAM-C FL but not ΔCyto and TAZ. $n = 3$. (L-N) Western blot showing the subcellular portion of TAZ in RPE cells after JAM-C knockdown. GAPDH and HISTONE 3 were used as cytoplasmic or nuclear controls respectively. $n = 3$. (O-P) Immunostaining of JAM-C (red) and TAZ (green) in RPE cells after JAM-C knockdown. Nuclei are stained by DAPI. Scale bar: 20 μm . Quantification of the percentage of TAZ⁺ cells in the nucleus are shown in P, $n = 4$. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA in G, H, M-N and Student's t -test in B-E, and P. ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



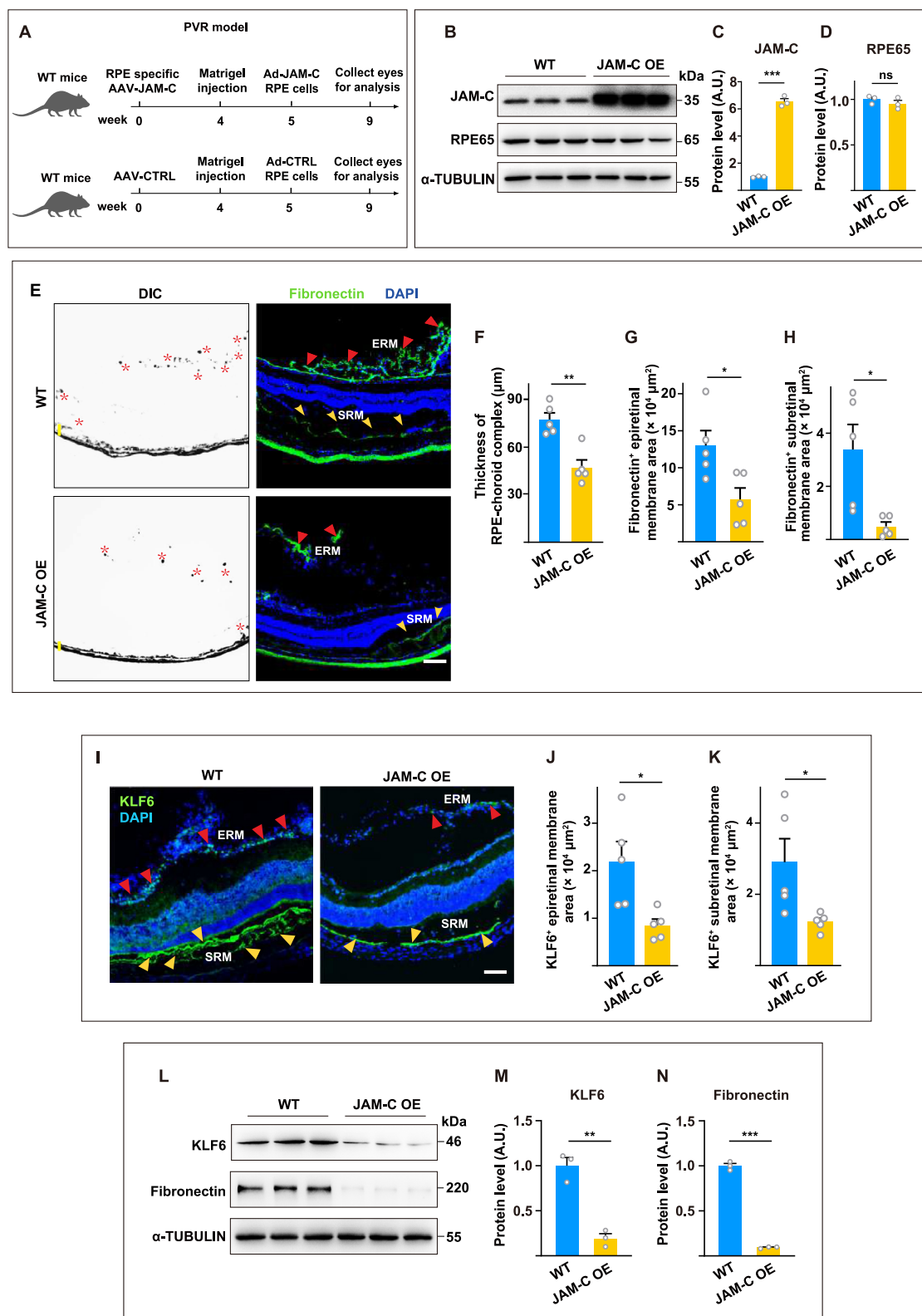


Fig. 6. JAM-C overexpression alleviates ocular fibrosis. (A) Diagram showing the generation of the PVR model in WT mice with RPE-specific overexpression of JAM-C or CTRL. AAV, adeno-associated virus. Ad, adenovirus. (B–D) Western blot showing the protein levels of JAM-C and the RPE marker RPE65 in the RPE-choroid complex from mice with JAM-C OE in PVR models. $n = 3$. (E–H) DIC and immunostaining of fibronectin in the RPE-choroid complex from mice with JAM-C OE in PVR model (E). Green: fibronectin, Blue: DAPI. Red asterisks: displaced melanosome, orange arrows: subretinal membrane (SRM), red arrows: epiretinal membrane (ERM), yellow lines: thickness of RPE-choroid complex. Scale bar: 50 μ m. Quantification of the thickness of RPE-choroid complex and the fibronectin-positive membrane areas in E are shown in F–H. $n = 5$ for each group. (I–K) Immunostaining of KLF6 in the retina of mice with JAM-C OE in a PVR model (I). Green: KLF6, Blue: DAPI, Red arrows: epiretinal membrane (ERM) Orange arrows: subretinal membrane (SRM). Scale bar: 50 μ m. Quantification of the KLF6 positive areas in I are shown in J and K. $n = 5$ for each group. (L–N) Western blot showing the protein levels of KLF6 and the EMT marker fibronectin in the RPE-choroid complex of mice with JAM-C OE in PVR models. $n = 3$. Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t -test in C–D, F–H, J–K, M–N. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Discussion

Fibrosis can cause severe dysfunction of many organs, including heart, kidney, lung, and liver, and contributes to about 45 % of all causes of death world-wide [31]. Ocular fibrosis is detrimental to visual function and accounts for the vision loss of millions of individuals globally [2]. Currently, there is no effective pharmaceutical agent available to slow or stop ocular fibrosis progression. Here, we provide the first evidence that JAM-C is a pivotal molecule to prevent ocular fibrosis by suppressing the EMT-promoting activities of the TAZ/KLF6 axis (Fig. 7).

JAM-C is widely distributed at the tight junctions [11] and is important for cell polarity, self-renewal, barrier function, angiogenesis, leukocyte transmigration, and tissue development [11,12,32,33]. JAM-C is detected in RPE cells [13] and plays important parts in RPE regulation, including junction formation, polarization, migration, and survival [18,34,35]. Yet, little is known regarding whether it is involved in ocular fibrosis. Here we not only show that JAM-C within RPE cells is an inhibitor of ocular fibrosis, but also reveal that soluble JAM-C is decreased in the vitreous humor of PVR patients. To date, little is known regarding the signaling transduction initiated by soluble JAM-C. Given that the membrane bound JAM-C interacts with other adhesion receptors, such as JAM molecules and integrins [12,36], it may be possible that soluble JAM-C could bind to other receptors on the surface of RPE cells to suppress the TAZ/KLF6 axis.

In this study, we found that JAM-C interacts with TAZ in RPE cells, and the cytoplasmic domain of JAM-C is crucial for this interaction. We further unveiled that JAM-C ablation induced TAZ activation by reducing its phosphorylation, which promotes TAZ

stability and nuclear translocation, resulting in RPE EMT. Consistently, the phosphorylation state and protein stability of TAZ have been reported to be essential for its activity in many other cell types [24,25,37]. Noteworthy, we were not able to detect the interaction between YAP and JAM-C in RPE cells, and YAP expression was not affected by JAM-C knockdown. Our study suggests that although both YAP and TAZ are crucial regulators for EMT in various cell types [23], they could be modulated by different factors in RPE cells. For instance, YAP was induced by EGFR [38] and RhoA [39] to promote RPE EMT. By contrast, a previous study showed that TAZ but not YAP is abundantly expressed in primary mouse RPE cells. In addition, TAZ displays a stronger binding capacity than YAP to the EMT transcription factor Zeb1, leading to RPE cell proliferation and EMT [40]. Thus, future studies are needed to better understand how TAZ and YAP respond to different signaling pathways.

In addition, we reveal here that TAZ promotes KLF6 expression and facilitates KLF6-mediated transcription activation of fibronectin in RPE cells. Given that TAZ can also bind to transcription factors to initiate the transcription of downstream target genes, or compartmentalize transcription factors via phase separation to activate transcription [41–46], the precise transcriptional program coordinated by TAZ with KLF6 in RPE requires further investigation. In addition, the time-resolved fluorescence resonance energy transfer (TR-FRET) assay was used to screen small-molecule inhibitors with potent anti-tumor activity by blocking the interaction between TAZ/YAP and all TEAD proteins [47]. This approach could be useful to discover anti-ocular fibrosis drugs that could disrupt the interaction between TAZ and KLF6. KLF6 belongs to the KLF family [48], which is implicated in modulating EMT in many epithelial cell

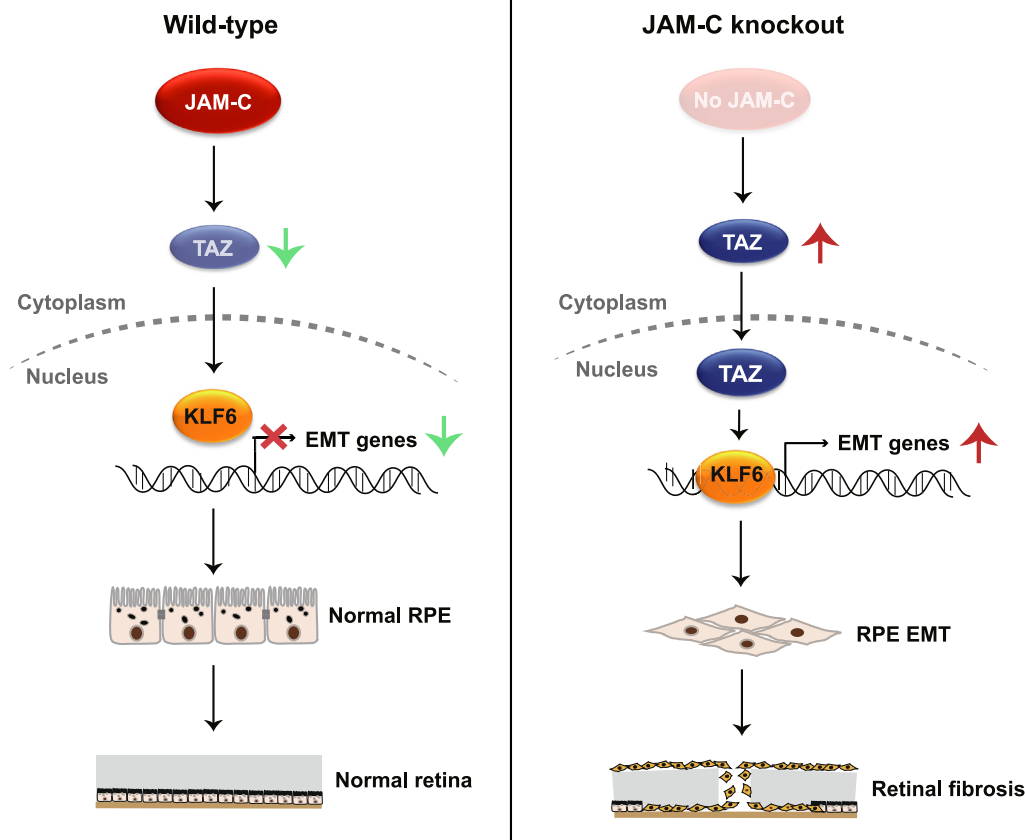


Fig. 7. JAM-C prevents RPE EMT and ocular fibrosis by suppressing the TAZ/KLF6 axis. In wild-type mice, JAM-C reduces TAZ expression, stability and nuclear transportation to inhibit its EMT-promoting function to prevent EMT and fibrosis, thus maintaining normal RPE and retina. In *Jam-c* knockout mice, in the absence of JAM-C, TAZ expression, stability and nuclear translocation increases, leading to the upregulation of KLF6 and KLF6-induced expression of many EMT causing genes, resulting in RPE EMT and fibrosis.

types [49,50]. However, little is known regarding whether it is involved in RPE EMT. We found that KLF6 knockdown suppressed RPE cell proliferation and the expression of EMT genes in RPE cells, and KLF6 depletion abolished JAM-C knockdown-induced RPE EMT, demonstrating KLF6 as a pro-EMT regulator in RPE cells. Indeed, in the epiretinal and subretinal fibrotic membranes of PVR patients, KLF6 was the most abundant of the KLF family (<https://www.eyescriptome.com/index.php>), supporting an important role of KLF6 in RPE EMT and ocular fibrosis. Future studies using samples from patients with nAMD, diabetic retinopathy, or retinopathy of prematurity, may help verify whether JAM-C/TAZ/KLF6 could serve as potential biomarkers for ocular fibrosis.

In summary, in this work, we show that JAM-C is downregulated in the vitreous humor of patients with ocular fibrosis and reveal the suppressive effect of JAM-C on TAZ/KLF6 axis to prevent RPE EMT. Importantly, we find that AAV-mediated delivery of JAM-C ameliorated fibrosis in different murine ocular fibrosis models. Given that AAV has the advantages to provide a long-lasting gene expression and lacks pathogenicity [51], JAM-C-expressing AAV might be a promising approach for the treatment of ocular fibrotic diseases.

Methods

Human sample collection and enzyme-linked immunosorbent assay (ELISA)

Demographic information and PVR classification [52] of the patients are summarized in Supplementary Table 1. Patients with idiopathic epithelial membranes, idiopathic macular holes and ectopia lentis were used as controls (Supplementary Table 2). The exclusion criteria included complicated ocular diseases, such as malignant tumors, severe uveitis, diabetic retinopathy, or laser coagulation within three months [53]. The vitreous humor was collected during vitreoretinal surgery using standard surgical procedures, which was then centrifuged to collect supernatant for ELISA analysis to measure JAM-C level in accordance with the manufacturer's protocol (AE98563Hu, AMEKO, Shanghai, China).

Cell culture, transfection, and adenovirus infection

Primary human RPE cells (ScienCell) were cultured in epithelial cell medium (ScienCell, 4101) consisting of 500 mL of basal medium, 5 mL epithelial cell growth supplement (ScienCell, 4152), 5 mL penicillin/streptomycin solution (ScienCell, 0503) and 10 mL fetal bovine serum (ScienCell, 0010). The ARPE-19 cells (human RPE cell line, ATCC, Manassas, VA, USA) were cultured in DMEM/F12 medium (10-092-CV, Corning, NY, USA) containing 2.48 mM L-glutamine, 15 mM HEPES, 1000 mg/L of glucose and other supplements essential for cell growth, the medium was complemented with 10 % (vol/vol) fetal bovine serum (Corning) and 1 % (vol/vol) penicillin/streptomycin (ScienCell, Carlsbad, CA, USA) for cell growth.

For siRNA-mediated knockdown, RPE cells were seeded in 6-well plates and grown to approximately 70–80 % confluence. For each well, JAM-C siRNA, TAZ siRNA or control siRNA respectively (30 pmol, RiboBio, Huangpu, Guangzhou, China) were mixed with 100 μ L Opti-MEM (100 μ L, Gibco, Semitic, New York, USA), and Lipofectamine RNA-iMAX (6 μ L, Invitrogen, Carlsbad, CA, USA) was mixed with another 100 μ L of Opti-MEM, the two mixtures were incubated at room temperature for 5 min each. The two mixtures were then combined and incubated for 15 min at room temperature before adding to the wells. The cells were used for further analyses 48 h after transfection. The sequences of the siRNAs are as the following: *siJAM-C-1*: GAACTGTCTGCATCATTA, *siJAM-C-2*:

GCTACTTCATCAACAATAA, *siTAZ-1*: GACATGAGATCCATCACTA, *siTAZ-2*: GGAUACAGGAGAAAACGCA, and *siKLF6*: GCCTAGAGCTGGAACGTTA.

For shRNA-mediated knockdown in mice, 1.5 μ g of human JAM-C shRNA plasmid or control shRNA plasmid (Sigma-Aldrich, St. Louis, MO, USA) was transfected into RPE cells using the JetPEI method (Polyplus-transfection, Illkirch, France) following the manufacturer's instructions. After 3 days, puromycin (1 μ g/mL, Beyotime Biotechnology, Songjiang, Shanghai, China) was added to select the shRNA-expressing cells. Transfected cells were collected for intravitreal injection. The sequence of the shJAM-C is GAGCAGGAGATGGAAGTCTAT.

For adenovirus (Ad)-mediated gene overexpression [54], 1×10^{10} viral particles of Ad-JAM-C, Ad-TAZ or Ad-CONTROL respectively (Vigene Biosciences, Shandong, China) were used to transduce RPE cells into a 10-cm dish. After transfection for 48 h, cells were collected for RNA sequencing analysis or PVR model set up (details in Matrigel and RPE-induced PVR model).

Mice

Mice were kept in the animal facility of the Zhongshan Ophthalmic Centre, Sun Yat-sen University and maintained on a 12/12 h light/dark cycle. *Jam-c^{flox/flox}* mice and *BEST1-Cre* mice were kindly provided by Dr. Triantafyllos Chavakis (National Cancer Institute, NIH, USA) and Dr. Joshua L Dunaief (University of Pennsylvania, USA) respectively. To generate RPE-specific *Jam-c*-deficient (*Jam-c^{fl/fl}*; *BEST1-Cre⁺*) mice, *Jam-c^{flox/flox}* mice were bred with *BEST1-Cre* mice. PCR and sequencing were performed to genotype the mice.

Matrigel and RPE-induced PVR model

The Matrigel and RPE-induced PVR model was described previously with some modifications [19]. For the PVR model using *Jam-c* RPE cko mice, see illustration in Fig. 1G. Specifically, the *Jam-c* RPE cko mice were injected subretinally with Matrigel (1.5 μ L/eye, 356231, BD biocoat). After one week, the mice were injected intravitreally with shJAM-C RPE cells (1×10^4 cells/1 μ L/eye). Control mice were injected with Matrigel for one week followed by shCTRL RPE cells injection. After four weeks, the eyes were collected for analysis.

For the PVR model using WT mice, see illustration in Fig. 6A. Specifically, the WT mice were injected subretinally with AAV2/8 expressing JAM-C under a RPE-specific hVMD2 promoter [55] (1 μ L/eye, 2×10^{13} vg/mL, OBIO, China). After four weeks, the mice were injected with Matrigel for one week followed by adenovirus (Ad)-mediated JAM-C overexpressing RPE cells for four weeks. For the control group, WT mice were injected subretinally with AAV-hVMD2-mCherry. After four weeks, the mice were injected with Matrigel for one week followed by Ad-CTRL RPE cells for four weeks. The eyes of the mice were then collected for analysis.

Disperse-induced PVR model

The disperse-induced PVR model was generated as previously described [56]. Briefly, eight-week-old mice were injected intravitreally with 1 μ L of 0.005 U disperse (17105041, GIBCO) using a 5 μ L microsyringe (7632-01, Hamilton). After 3 weeks, the eyes were harvested for further analysis.

Laser-induced subretinal fibrosis model

The laser-induced subretinal fibrosis model was generated as previously described [21]. Briefly, eight-week-old mice were subjected to laser photocoagulation (532 nm, 180mW, 90 ms, Novus

Spectra, Japan). Four laser spots in each eye were created around the optic disc using a contact lens, and subretinal bubble formation at the time of laser application indicated successful rupture of the Bruch's membrane. Laser with bleeding was excluded. After 35 days, the eyes were collected for further analysis.

H&E staining

Mice eyeballs were fixed overnight in 4 % paraformaldehyde overnight. Dehydrated specimens were fixed in paraffin and sectioned at 5 μ m thickness, and then deparaffinised and rehydrated for H&E staining to observe histological changes.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. 1 μ g RNA was reverse transcribed into cDNA using a cDNA synthesis kit (KR116, TIANGEN, Beijing, China). qRT-PCR was performed using the qPCR SYBR[®] green master mix (Q711, Vazyme, China) and an ABI QuantStudio 6 Flex device (Life Technologies, Gaithersburg, MD, USA). Results were normalised to GAPDH transcripts. A delta-delta Ct method was used to calculate the relative fold change in gene expression. The sequences of the qRT-PCR primers are listed in Supplementary Table 3.

Co-IP and Western blotting

For the co-IP assay, cells were harvested and lysed for 30 min on ice with lysis buffer (NP40, P0013F, Beyotime Biotechnology) containing protease inhibitor and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were incubated with JAM-C antibody (AF1189, R&D Systems, MN, USA), or IgG (AB-108-C, R&D Systems) at 4 °C overnight, followed by Protein A/G-Sepharose (sc-2003, Santa Cruz, USA) incubation for 1.5 h at 4 °C. The complexes were washed three times in lysing buffer and collected. For the co-IP assay of protein with FLAG tag, anti-DYKDDDDK G1 Affinity Resin (L00432, GenScript) was used to capture the complex, followed by incubation with 3 $\times$ flag peptide (P9801, Beyotime) at 4 °C for 2 h to washout the light and heavy chains. The immunoprecipitates were centrifuged and collected. After processing with 5 $\times$ loading buffer, the samples were separated by 10 % SDS-PAGE.

For Western blotting, cells were washed twice with ice-cold PBS and then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5 % sodium deoxycholate, 1 % NP-40, and 0.1 % SDS) with protease and phosphatase inhibiting cocktails (Thermo Fisher Scientific). The cell lysates were then centrifuged and the supernatants were collected. Protein concentrations were determined by means of the BCA protein assay kit (Beyotime Biotechnology). Protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane (Merck Millipore, MA, USA). The membrane was incubated overnight at 4 °C with the following primary antibodies: anti-fibronectin (F3648, Sigma-Aldrich), anti-JAM-C (AF1189, AF1213, R&D systems), anti-vimentin (AB92547, Abcam, MA, USA), anti-N-cadherin (AB12221, Abcam), anti-E-Cadherin (AB11512, Abcam), anti-TAZ (4883S, Cell Signaling Technology), anti-p-TAZ (sc-17610, Santa Cruz), anti-YAP (4883S, Cell Signaling Technology), anti-KLF6 (14716-1-AP, Proteintech, China), anti-FLAG (14793S, Cell Signaling Technology), anti-HSP90 (4874S, Cell Signaling Technology), anti- α -TUBULIN (102-25843, Ray Antibody Biotech), anti-GAPDH (102-10016, Ray Antibody Biotech, Beijing, China), anti- β -ACTIN (130-10255, Ray Antibody Biotech) and anti-Histone H3 (GB13102-1, Servicebio, China). Membranes were then incubated with anti-rabbit, anti-goat, or anti-mouse horseradish peroxidase-conjugated antibodies (MultiSciences Biotech Co. Ltd,

China) for 1 h at room temperature, and developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) or SuperSignal Pico (Thermo Fisher Scientific). A GBOX-CHEMI-XX8 instrument (Syngene, Ballenger Creek, MD, USA) was used to visualize the bands.

Immunofluorescence staining

The cells were fixed in 4 % paraformaldehyde for 20 min, rinsed in PBS, permeabilized with 0.3 % Triton X-100 (9002-93-1, Sigma-Aldrich) in PBS for 30 min, and rinsed three times with PBS. The cells were then blocked using 5 % bovine serum albumin (BSA, B2064, Sigma-Aldrich) at room temperature for 1 h, and incubated overnight at 4 °C with the indicated primary antibodies, including anti-JAM-C (AF1189, R&D Systems) and anti-fibronectin (F3648, Sigma-Aldrich) and anti-TAZ (HPA007415 Sigma-Aldrich). The next day, sections were incubated with secondary antibodies (Invitrogen) for 1 h at room temperature and mounted with DAPI (D9542, Sigma-Aldrich). Immunofluorescence imaging was performed using a fluorescence microscope (Carl Zeiss, Jena, Germany).

For immunostaining, eyeballs were fixed in 4 % paraformaldehyde (158127, Sigma-Aldrich) 2 h at 4 °C. After washing with PBS and removing the cornea and lens, the eyeballs were dehydrated with sucrose (10, 20, and 30 %; V900116, Sigma-Aldrich) for 1 h each respectively. Eyeballs were then frozen in tissue-tek O.C.T. compound (4583, SAKURA, CA, USA) and sectioned (10 μ m) using a freezing microtome (LEICA, Wetzlar, Germany). Sections were blocked with 0.5 % Triton X-100 (T8787, Sigma-Aldrich) in PBS and 5 % donkey serum (D9663, Sigma-Aldrich) for 1 h at room temperature, and incubated overnight at 4 °C with primary antibodies. The primary antibodies used were anti-fibronectin (F3648, Sigma-Aldrich), and anti-KLF6 (14716-1-AP, Proteintech). The next day, the sections were washed with PBS, incubated with relative secondary antibody and mounted with DAPI. The images were taken on a LSM 710 confocal fluorescence microscope (Carl Zeiss).

Cell migration assay

RPE cells were plated in a 6-well plate at a density of 1.5×10^5 cells/well, maintained until approximately 85 % confluence, and then transfected with JAM-C or control siRNAs for 24 h. The medium was changed with serum-free DMEM/F12 (Corning) after treatment with Mitomycin C (1 μ M, Sigma-Aldrich) for 1 h. The cell monolayer was scraped with a 200 μ L tip and washed twice with serum-free DMEM/F12 (Corning). The cells were cultured in serum-free DMEM/F12 (2 mL) for 48 h. The percentage of wound closure was analysed using ImageJ software (v1.49).

CCK-8 assay

RPE cells were plated in a 48-well plate at a density of 5×10^3 cells/well and incubated overnight, followed by transfection with JAM-C, KLF6, or control siRNA for 72 h. Cells were changed with 100 μ L fresh DMEM/F12 medium. 10 μ L/well of Cell Counting Kit-8 (CCK-8) solution was added to the medium and incubated for 1 h at 37 °C, followed by measurement at 450 nm using an ELx800 Absorbance microplate reader (BioTek, Vermont, USA).

Gel contraction assay

The 24-well plates were coated with 1 % BSA (1 mL/well) for 1 h at 37 °C. RPE cells were transfected with siJAM-C or siCTRL for 24 h and resuspended in serum-free DMEM/F12. The cell suspension (2.5×10^5 cells/well), Collagen I (2 mg/mL, 200110-10, Xiangbo Biological, China), ddH₂O, and 0.025 M NaOH were combined on

ice and then added to the BSA-coated well and incubated for 20 min at 37 °C until the gel is solidified. The gel was separated from the walls of the wells using a micropipette. 500 µL serum-free DMEM/F12 was added to the top of the gel. Photographs were taken after 48 h. The ratio of gel contraction area was quantified using ImageJ software.

Subcellular fractionation

RPE cells were seeded in 10-cm dishes and transfected with JAM-C or control siRNAs for 48 h. Cells were lysed in cytoskeletal CSK buffer (50 mM NaCl, 10 mM Pipes pH 6.8, 3 mM MgCl₂, 300 mM sucrose, 0.5 % (vol/vol) Triton X-100) with protease inhibitor cocktail (B14002, Biotool, China), 1 mM DTT, and 1 mM PMSF) for 30 min, followed by centrifugation at 12000g for 10 min at 4°C. The supernatant was saved as the cytoplasmic fraction. The remaining fraction was washed three times with CSK buffer and solubilized in RIPA buffer as the nuclear fraction for Western blot analysis.

Cycloheximide (CHX) assay

RPE cells were seeded in a 6-well plate and transfected with JAM-C or control siRNAs for 24 h. Then cells were treated with CHX (25 µg/mL, Cell Signaling Technology) for indicated time points before they were harvested for Western blotting analysis.

Dual luciferase reporter assay

To construct the expression plasmids, the cDNA of TAZ and KLF6 were extracted from RPE cells and cloned into the pCDH-CMV-MCS-EF1-Puro expression vector using a cloning kit (SC612, Gene-Sand, China). To construct the firefly luciferase vector, the promoter region (–2000 to 100 bp from the transcriptional start site) of the human *FN1* gene was synthesized and cloned into the PGL3.0 luciferase reporter vector. RPE cells were seeded at a density of 5×10^4 cells/well in a 48-well plate. TAZ or KLF6 expressing plasmid (alone or together) were transfected into the cells with the *FN1* luciferase vector using Lipofectamine 2000. The pCMV vector expressing the Renilla luciferase reporter gene was used as a control. After 48 h, Renilla and firefly luciferase activities were measured using a dual luciferase reporter gene assay kit (11402ES60, YEASEN, China). The relative firefly luciferase activity was normalized to that of Renilla. The primers used are listed in Supplementary Table 4.

ChIP-qPCR

RPE cells were seeded in 15-cm dishes followed by transfection with ad-TAZ or Ad-GFP for 48 h. After fixation with 1.42 % formaldehyde and quenching with glycine, the cells were washed with PBS and suspended in IP buffer (0.5 % (vol/vol) NP40, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 % (vol/vol) Triton X-100, 50 mM EDTA) with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific), followed by sonication and pre-clearance with IgG and Protein A/G agarose beads at 4 °C overnight. After washing with IP buffer, the complex of nuclear proteins/DNA was incubated with anti-KLF6 antibody (14716–1-AP, Proteintech, 1:100 dilution) or IgG control (2729 s, Cell Signaling Technology, 1:300 dilution) and retrieved by Protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz). After washing with ChIP buffer and TE buffer, the samples were eluted and reverse cross-linked at 65 °C overnight, followed by DNA purification with a DNA purification & recovery kit (DP214, TIANGEN). The DNA was then amplified using qPCR and analysed. The results were normalized to those from the input DNA. The sequences of the ChIP-qPCR primer were designed

according to the predicted sequence in the promoter region of *FN1* as predicted by JASPAR (<https://jaspar.elixir.no>) and are listed in Supplementary Table 5.

RNA sequencing (RNA-seq)

RPE cells were treated with adenoviruses expressing JAM-C, TAZ, or GFP for 48 h and collected for RNA sequencing by PersonalBio (Shanghai, China). Illumina NovaSeq 6000 (Shanghai Personal Biotechnology Cp. Ltd, China) was used to construct the cDNA libraries. The RNA-seq reads were trimmed to remove poly-A. Unqualified reads were eliminated using FASTP (v0.22.0). Using HISAT2 (v2.1.0), the remaining reads were matched to the *Homo sapiens* reference genome GRCh38. Reads were pooled at the gene level using HTSeq (v0.9.1). The values for gene expression were calculated as fragments per kilobase per million fragments (FPKM), which were obtained by adding a pseudo-count of 1 and log2 to transform the results. These FPKM values were utilised to create volcano plots using the PersonalBio online tool (<https://www.genesccloud.cn/chart>). Paired differential gene expression analyses with fold change > 2 and p-value < 0.05 were performed using the DESeq R package. Gene ontology and KEGG enrichment analyses of differential genes were assessed with ClusterProfiler (v4.6.0) and topGO (v2.50.0), respectively. To screen for hub genes, we used STRING (<https://cn.string-db.org/>) to establish a protein–protein interaction (PPI) network and Cytoscape software (v3.10.1) [57] for visualization. Node scores were ranked using the CytoHubba plug-in and calculated using the DMNC algorithm. The RNA sequencing data is accessible in GEO database (GSE251997). DEG lists are in Supplementary Table 6 and 7.

Statistics

GraphPad Prism (GraphPad Software, Inc.) was used for statistical analyses except for RNA-seq data. PersonalBio online tool (<https://www.genesccloud.cn/chart>) was used for the generation of volcano plots for DEGs in RNA-seq. Data are represented as mean ± SD. For comparisons between two groups, Student's t tests were used. For comparisons among more than two groups, one-way ANOVA analysis was used.

Ethics statement.

All experiments involving animals were conducted according to ethical policies and procedures approved by the Animal Care and Use Committee of Sun Yat-Sen University in Guangzhou, China (Approval no. Z2021002). All the clinical samples were approved by the Ethics Committee of the Wuxi People's Hospital in Jiangsu, China (Approval no. KY22095), and in accordance with the Declaration of Helsinki. All patients were informed and gave written consent.

Compliance with Ethics Requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the Wuxi People's Hospital in Jiangsu, China, approval number: KY22095) and in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients for being included in the study.

All animal experiments performed in this work were approved by the Animal Use and Care Committee of Zhongshan Ophthalmic Center at the Sun Yat-Sen University, Guangzhou, China. All animal care and use followed the guidelines of the Animal Care and Use Committee of Sun Yat-Sen University.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2025.05.037>.

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