EGF Receptor Signaling Modulates YAP Activation and Promotes Experimental Proliferative Vitreoretinopathy

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Purpose. Both epidermal growth factor receptor (EGFR) and the Yes-associated protein (YAP) signaling pathway are implicated in cell proliferation and differentiation. In this study, we explored whether the formation of proliferative vitreoretinopathy (PVR) depends on the interaction of the EGFR receptor and YAP pathway.

Methods. We studied the effects of EGFR and YAP activation on retinal fibrosis in a PVR mouse model as well as in knockout mice (conditional deletion of EGFR or YAP specifically in RPE cells). Reversal and knockdown experiments were performed to induce a model of ARPE-19 cells treated with TGF-β2 in vitro. The effect of EGFR/YAP signaling blockade on the PVR-induced cell cycle and TGF-β2–induced ARPE-19 cell activation was determined.

Results. The EGFR inhibitor erlotinib or conditional deletion of EGFR attenuated YAP activation and decreased the expression of YAP and its downstream target Cyr61 and of connective tissue growth factor in vivo and in vitro. EGFR-PI3K-PDK1 signaling induced by PVR promoted YAP activation and cell cycle progression. Furthermore, activated EGFR signaling bypassed RhoA to increase the protein levels of YAP, C-Myc, CyclinD1, and Bcl-xl.

Conclusions. Our work highlights that EGFR-PI3K-PDK1–dependent YAP activation plays a crucial role in the formation of PVR. Targeting EGFR and the YAP pathway provides promising therapeutic treatments for PVR.

Keywords: proliferative vitreoretinopathy, Yes-associated protein, epidermal growth factor receptor, Hippo signaling, retinal pigment epithelial cells

Proliferative vitreoretinopathy (PVR) is a blinding disease that can be secondary to rhegmatogenous retinal detachment and can follow surgery for rhegmatogenous retinal detachment repair or penetrating ocular trauma.[1] PVR occurs in approximately 5% to 10% of patients undergoing retinal surgeries for rhegmatogenous retinal detachment, accounting for 75% of the primary failures after surgeries.[2] Owing to recurrent detachment and PVR progression leading to retinal damage, the visual outcomes of surgery are poor. PVR is characterized by the formation of fibrotic and cellular epiretinal and subretinal membranes composed of extracellular matrix proteins and cells, including RPE cells, fibroblasts, retinal glial cells, and macrophages.[3] In fact, RPE cells and glial cells in the epiretinal and subretinal membrane seem to play an important role in the formation of PVR.[4] A complete understanding of the pathogenesis of PVR is important for the development of new therapeutic treatments for PVR patients.

Studies have shown that epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, plays a critical role in the migration and proliferation of RPE cells.[5,6] The binding of a ligand (such as EGF, heparin-binging EGF, or amphiregulin) to EGFR may activate the specific kinase domain and subsequently phosphorylate intrinsic tyrosine residues in the cytoplasmic tail.[7] A previous study showed that EGF can increase the survival rate of RPE cells and enhance the migration and proliferation of RPE cells by activating the EGFR signaling pathway.[8] Liang et al.[9] found that the activation of EGFR signaling seemed to be a crucial factor in PVR formation. However, inhibiting the activity of EGFR alone does not help to prevent the activation of RPE cells. Therefore, it is important to further explore the exact mechanism of EGFR and discover new therapeutic targets for PVR.

The Hippo signaling pathway is a highly conserved threonine/serine kinase cascade that provides insights into the control of cellular survival, death, and proliferation to regulate the size of organs.[10] Ste20-like serine/threonine protein kinase 1/2 (MST1/2), large tumor suppressor serine/threonine kinase 1/2 (LATS1/2), and its adaptor proteins Mps-one Binder 1 and Sav (also called ww45) are the core components of mammalian Hippo signaling.[11] Activated Hippo signaling leads to LATS1-stimulated phosphorylation of the downstream effector, a WW domain-containing protein (Taz), and Yes-associated protein (YAP) that causes the degradation of YAP/Taz through cytoplasmic sequestration and proteasome-stimulated inactivation.[12,13] At the molecular level, the generation of connective tissue growth

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factor (CTGF) is regulated by the YAP/Taz complex.[14] CTGF plays a crucial role in the pathogenesis and development of fibrotic retinal disease.[15,16] From a clinical and translational perspective, detecting the exact mechanism of YAP activation may help in the development of new therapies for PVR.

Previously, we demonstrated that YAP was an essential regulator of epithelial–mesenchymal transition in PVR[17] and a vital regulator of the profibrotic response in hyperglycemia-induced retinal fibrosis.[18] We also showed that targeting RhoA/YAP signaling alleviated PVR-induced retinal fibrosis and suggested an attractive novel treatment for intervening in PVR progression.[19] Based on these results, we used experimental PVR models to observe whether the formation of PVR depended on the interaction of the EGFR and YAP pathways. Our results provide direct evidence that EGFR-dependent YAP activation is closely related to the pathogenesis of PVR and will help in the identification of promising therapeutic approaches for PVR.

Methods

PVR Induction and Treatment

This study was approved by the medical ethics committee of Tianjin Eye Hospital and performed under the guidelines of the Declaration of Helsinki. The experiment was carried out according to the approved guidelines. The C57BL/6 mouse model of PVR was established by intravitreal injection of Dispase II as previously described.[19] In short, we used a 30G Hamilton syringe to intravitreally inject 3 µL of Dispase II (concentration of 0.2 U/µL; Sigma-Aldrich, St. Louis, MO, USA). Control mice received intravitreal injection of 3 µL of sterile saline solution. To inhibit YAP signaling, we administered verteporfin (100 mg/kg every other day for 4 weeks; Millipore, Billerica, MA, USA) intraperitoneally after induction of the PVR mouse model. To disturb the EGFR pathway, we administered erlotinib (80 mg/kg per day for 4 weeks; Millipore) intraperitoneally after induction of the PVR mouse model. In addition, the PVR model mice in the control groups were administered dimethyl sulfoxide (vehicle) intraperitoneally.

The gene encoding EGFR and YAP was conditionally knocked out (cKO) in RPE cells by crossing Best1-Cre mice (Jackson Laboratory, Bar Harbor, ME, USA) with mice in which the EGFR and YAP genes were flanked by the LoxP site. These strains were bred to generate RPE-specific EGFR-cKO ($EGFR^{flox/flox}$, Best1-Cre+) or RPE-specific YAP-cKO ($YAP^{flox/flox}$, Best1-Cre+) and the respective wild-type control mice as previously described.[20] To genotype the mice, a PCR analysis of the genomic DNA isolated from RPE, neural retina, and tail tips was performed using specific primers for Best1-Cre (F: 5′-ATG CCC AAG AAG AAG AAG AAG GTG TCC A-3′ and R: 5′-GGA AAA TGC CAA TGC TCT GTA-3′), mEGFR-LoxP (F: 5′-GGT CTT GAA GGC TGT CCA ACG-3′ and R: 5′-CCT CAA GAG AGC TTG GTT GGG-3′) and mYAP-LoxP (F: 5′-TGA GGA GCT TTT AGC ATT GGT GCA GT-3′ and R: 5′-AGC AGT GTG GTT ACT TTT CCA GGT T-3′).

Culture and Treatment of RPE Cells

A human RPE cell line (ARPE-19) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal bovine serum in a humidified atmosphere at 37°C with 5% CO_2. The ARPE-19 cells were used at passages two to four in all the experiments and were treated with recombinant TGF-β2 (Santa Cruz Biotechnology, Santa Cruz, CAm USA) at a concentration of 10 ng/mL to simulate the PVR environment.

To disrupt the expression of EGFR, YAP, or RhoA, the ARPE-19 cells were treated with small interfering RNA (siRNA) against EGFR, YAP, or RhoA. EGFR-siRNA, YAP-siRNA, RhoA-siRNA and nonsilencing siRNA were obtained from Invitrogen (Carlsbad, CA, USA). Lipofectamine 2000 transfection reagent (ThermoFisher Scientific, Waltham, MA, USA) was used to perform transient transfection of siRNA. To disrupt EGFR signaling, ARPE-19 cells were treated with erlotinib (100 nM; Millipore) for 24 hours. To inhibit PI3K signaling, ARPE-19 cells were treated with LY294002 (25 µM; Bio-Rad Laboratories, Hercules, CA, USA) or wortmannin (100 nM; Bio-Rad Laboratories). To disrupt PDK1 signaling, ARPE-19 cells were treated with GSK2334470 (10 µM; Bio-Rad Laboratories). To disrupt Akt signaling, ARPE-19 cells were treated with MK-2206 (10 µM; Millipore).

Proliferation and Migration Analysis of ARPE-19 Cells

The proliferation of ARPE-19 cells was measured by Cell Counting Kit-8 (CCK-8) assays (Beyotime Biotechnology, Shanghai, China). In short, ARPE-19 cells were stimulated with 10 ng/mL TGF-β2 in DMEM/F12 for 24 hours. Then, 180 µL of culture medium and 20 µL of CCK-8 solution were added to the ARPE-19 cells and cultured at 37°C for 1 hour. A spectrophotometric microplate reader was used to assess the absorbance of ARPE-19 cells in each well at 450 nm. The proliferative activity of ARPE-19 cells was represented by the optical density of each well minus the absorbance of blank wells.

The migration of ARPE-19 cells was evaluated with an Oris 96-well cell migration assay kit (Santa Cruz Biotechnology). In short, ARPE-19 cells were cultured in each well and treated with DMEM/F-12 containing 10 ng/mL TGF-β2 for 1 hour. Then, the cells were treated with 5 mM aphidicolin to inhibit cell division, cultured for 48 hours, and stained with calcein AM in PBS for another 1 hour. A Spectra microplate reader was used to evaluate the signal intensity of the stained ARPE-19 cells that migrated into the detection area.

Immunocytochemistry

Immunocytochemistry was carried out in accordance with our previous research.[21] In short, ARPE-19 cells were fixed with 4% paraformaldehyde, treated with 0.5% Triton X-100, and blocked with 10% fetal bovine serum. Next, ARPE-19 cells were cultured with monoclonal antibodies against YAP and EGFR overnight; the antibodies were obtained from Santa Cruz Biotechnology. Then, an Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (Abcam Biotechnology, Cambridge, UK) was used to probe ARPE-19 cells, and the cells were fixed with 40,6-diamidino-2-phenylindole. Images were captured with a fluorescence microscope.

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Histological and Immunohistochemical Measurement

The eyes were incubated at pH 7.4 in PBS for 2 hours, dehydrated in a graded alcohol system, and embedded in paraffin wax. The thickness of each slice was 5 µm. Masson's trichrome staining was performed according to our previous research.[18] Immunohistochemical measurements were used to prepare eye sections from the paraffin-embedded tissue, and the eye sections were incubated with YAP and EGFR antibodies, which were obtained from Abcam Biotechnology. Then, the slices were stained with biotinylated anti-rabbit or anti-mouse IgG secondary antibody (Abcam Biotechnology) for 2 hours and treated with horseradish peroxidase-conjugated streptavidin for 1 hour. The image was obtained by a Leica DMI4000B instrument (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Western Blotting Analysis

Total proteins were obtained from ARPE-19 cells and retinal samples from mice. We used the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Beyotime Biotechnology) to prepare the nuclear and cytoplasmic extract from the ARPE-19 cells or retinal samples. A bicinchoninic acid assay kit was used to measure the protein concentration. We used SDS-PAGE to separate equal amounts of protein. Specific antibodies against YAP, EGFR, p-EGFR, Mst1, Lats1, CTGF, Cry61, p-PDK1, Cyclin D1, C-Myc, Bcl-xl, and RhoA were obtained from Abcam Biotechnology. The target proteins were measured using a chemiluminescence kit (Beyotime Biotechnology). The blots were captured with a Leica DMI4000B instrument (Olympus Soft Imaging Solutions GmbH).

Statistical Analyses

The data were analyzed by SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The data are shown as the mean ± standard deviation. The Mann–Whitney *U* test was used for statistical analysis involving the comparison of two sets of data. One-way ANOVA or two-way ANOVA was used for statistical analysis to make comparisons involving three groups. A *P* value of less than 0.05 was considered to be statistically significant.

Results

EGFR and YAP Were Activated in PVR

To detect the potential role of EGFR and YAP activation in the development of PVR, we used Western blotting to examine the production of EGFR and YAP in the PVR mouse model. We found that the production of EGFR and YAP increased in the retinas of the PVR mouse model at 2 weeks and was still elevated at 4 weeks (Fig. 1A). Masson staining showed that epiretinal membrane attached to the inner retinal layer and the presence of retinal fold in the PVR-Week2 group (Fig. 1B). In the PVR-Week4 group, it showed obvious subretinal membrane attached to the RPE layer and the normal structure of retina was disrupted (Fig. 1B). Immunohistochemical measurements revealed that EGFR and YAP were detected at minimal levels in the control mice, but the production of EGFR and YAP was enhanced in the PVR mouse model at 2 and 4 weeks (Fig. 1B). In addition to the results obtained from mice with PVR, we added 10 ng/mL TGF-β2 to the medium of ARPE-19 cells to simulate the PVR environment. Immunofluorescence staining revealed that ARPE-19 cells treated with TGF-β2 showed significantly increased YAP nuclear localization and EGFR production (Fig. 1C). The Western blot results showed that treatment with TGF-β2 for 12 hours induced a great increase in YAP and EGFR at the protein level (Fig. 1D). When the Hippo pathway kinase cascade is activated, YAP is inhibited by Lats directly through phosphorylation at Ser127. We found that the production of Hippo signaling effectors, including Lats1 and Mst1, did not change significantly (Fig. 1D), which indicated that YAP was not affected by Lats1 and Mst1 in response to PVR.

YAP Activation was Dependent on EGFR Activation in PVR

We used the EGFR kinase inhibitor erlotinib to treat the PVR mouse model (Fig. 2A). Erlotinib inhibited EGFR production and decreased the levels of YAP and its target gene CTGF and of Cry61 (Fig. 2B). In addition, immunohistochemical measurements showed that erlotinib treatment attenuated the upregulated production of YAP in the PVR mouse model (Fig. 2C). We detected a marked decrease of EGFR mRNA derived from RPE, but not from neural retina or tail in the EGFR-cKO mice (Fig. 2D). Western blot analyses also showed that conditional RPE-specific EGFR-cKO markedly inhibited the increased production of YAP, CTGF, Cry61, and EGFR in the retinas of mice with PVR (Fig. 2E). Similarly, immunofluorescence examination showed that EGFR-cKO prevented the PVR-induced upregulation of YAP production (Fig. 2F).

EGFR-siRNA was used to knockdown the EGFR protein in ARPE-19 cells. Nontargeting siRNA was considered as the control group. The results showed that EGFR-siRNA greatly decreased the protein level of EGFR in ARPE-19 cells (Fig. 3A). Silencing EGFR production by siRNA or treatment with erlotinib decreased YAP nuclear translocation in ARPE-19 cells in response to TGF-β2 (Figs. 3B and 3C). YAP was distributed mainly in the cytosol and was present at minimal levels in the nucleus before the ARPE-19 cells were exposed to TGF-β2. Western blot analyses demonstrated that treatment with TGF-β2 enhanced the distribution of YAP in the nucleus and somewhat decreased the distribution of YAP in the cytosol, and this change was reversed by treatment with erlotinib (Fig. 3D).

PI3K-PDK1 Activation Mediated EGFR-dependent YAP Activation

We used two different PI3K inhibitors, namely, wortmannin and LY294002, to treat ARPE-19 cells. Our results showed that both inhibitors were able to inhibit PDK1 production and block the nuclear translocation of YAP upon treatment with TGF-β2 (Figs. 4A and 4B). An inhibitor of the PI3K downstream effector PDK1 (GSK2334470) blocked YAP enhancement and nuclear translocation, whereas an inhibitor of Akt kinase (MK-2206) had little effect on YAP production and nuclear translocation (Figs. 4C and 4D). Moreover, Western blot analyses showed that either LY294002 or GSK2334470 treatment suppressed YAP nuclear translocation upon treatment with TGF-β2 (Fig. 4E). The present data indicated that EGFR-PI3K-PDK1 signaling regulated YAP activation in ARPE-19 cells.

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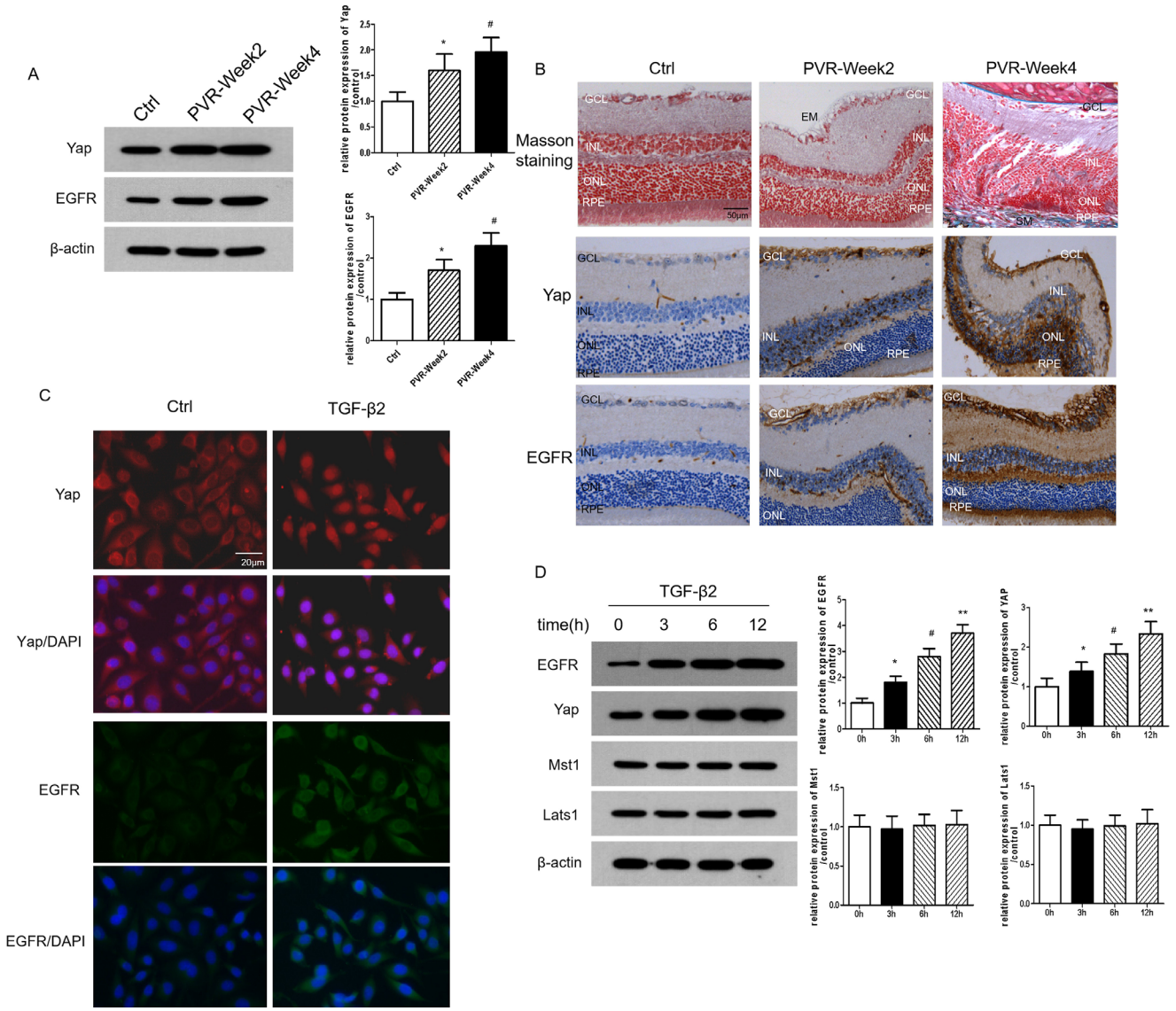

Figure 1. EGFR and YAP was activated in PVR. (**A**) Western blot assay for the expression of YAP and EGFR in the retinas of mouse. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the PVR-week2 group; $n = 3$. (**B**) Representative Masson staining and positive YAP and EGFR staining in the retinas of PVR mouse models. Scale bar, 50 μm. $n = 3$. (**C**) Expression of YAP and EGFR in ARPE-19 cells treated with TGF-β2 was determined by immunofluorescence staining. Scale bar, 20 μm. (**D**) Protein level of EGFR, YAP, Mst1, and Lats1 in ARPE-19 cells treated with TGF-β2 at different time points was detected by Western blotting. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the TGF-β2(3h) group; $^{**}P < 0.05$ versus TGF-β2(6h) group; $n = 3$.

Increased Cyclin D Expression Was Dependent on YAP Activation

The proliferation and differentiation of the surviving RPE cells are closely related to PVR progression. The transition from G1 phase to S phase depends on the activation of cyclin D–dependent kinase, which is crucial for modulating the cell cycle phase during the proliferation of cell.[22] We used the YAP inhibitor verteporfin to treat the PVR mouse model (Fig. 5A). And we detected a marked decrease in YAP mRNA derived from RPE, but not from neural retina or tail in the YAP-cKO mice (Fig. 5C). We found increased production of Cyclin D1, C-Myc, Bcl-xl, and CTGF in response to PVR, which was suppressed in verteporfin-treated mice (Fig. 5B) and RPE-specific YAP-cKO mice (Fig. 5C, D). The production of Cyclin D1, C-Myc, Bcl-xl, and CTGF was also inhibited in EGFR-cKO mice, which indicated that the expression of these genes was EGFR dependent (Fig. 5E). We used YAP-siRNA to knockdown the YAP protein in ARPE-19 cells and showed that YAP-siRNA greatly decreased the production of YAP in ARPE-19 cells (Fig. 5F). YAP knockdown greatly inhibited the proliferation of ARPE-19 cells in response to TGF-β2 compared with the nontargeting control (Fig. 5G). Further Western blot analyses showed that YAP knockdown reversed the increased production of CyclinD1, C-Myc, Bcl-xl, and CTGF in response to TGF-β2 (Fig. 5H). Scratch assays indicated that knocking down YAP also inhibited cultured ARPE-19 cell migration in response to TGF-β2 (Fig. 5I).

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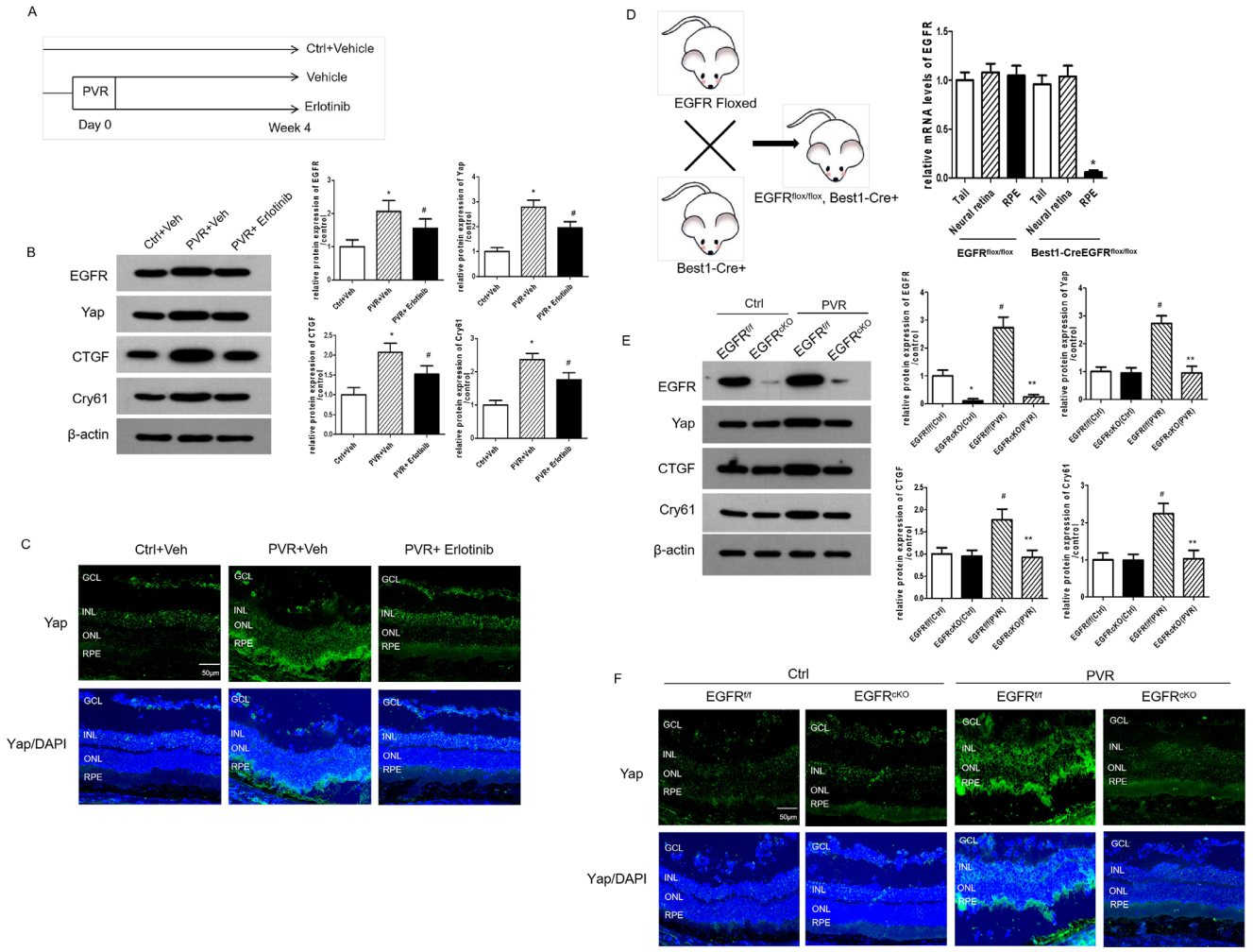

Figure 2. YAP activation was dependent on EGFR activation in PVR mice model. (**A**) The PVR mice models were randomized to treatment with erlotinib ($n = 6$) or vehicle ($n = 6$) beginning immediately after PVR induction. Control mice were also treatment with vehicle ($n = 6$). (**B**) Western blot assay for protein levels of EGFR, YAP, CTGF and Cry61 in the retinas of PVR mice with or without Erlotinib. *$P < 0.05$ versus the control group; # $P < 0.05$ versus the PVR+Veh group; $n = 3$. (**C**) Representative positive YAP staining in the retinas was examined by immunohistochemical measurement. Scale bar, 50 µm. $n = 3$. (**D**) Schematic of the generation of $EGFR^{flox/flox}$ and $EGFR^{cKO}$. And relative mRNA levels of EGFR in the RPE, neural retina and tail of $EGFR^{flox/flox}$ and $EGFR^{cKO}$ mice ($n = 3$). (**E**) Western blot assay for protein levels of EGFR, YAP, CTGF and Cry61 in the retinas of RPE-specific EGFR-cKO mice and $EGFR^{f/f}$ mice. * $P < 0.05$ versus the $EGFR^{f/f}$ group; # $P < 0.05$ versus the EGFR-cKO group; ** $P < 0.05$ versus the $EGFR^{f/f}$(PVR) group; $n = 3$. (**F**) Representative positive YAP staining in the retinas was examined by immunohistochemical measurement. Scale bar, 50 µm. $n = 3$.

Activated EGFR Signaling Could Bypass RhoA to Regulate YAP

A recent study showed that RhoA played an important role in G protein-coupled receptor–regulated YAP activation,[23] whereas another report indicated that EGFR can promote RhoA activity.[24] Thus, we determined whether activated EGFR can bypass RhoA to modulate the expression of YAP in ARPE-19 cells. Western blot analysis showed that EGF upregulated the production of YAP and RhoA in ARPE-19 cells (Fig. 6A). RhoA-siRNA was used to knockdown the RhoA protein in ARPE-19 cells, and we observed that RhoA-siRNA greatly decreased the protein level of RhoA (Fig. 6B). RhoA knockdown partially inhibited YAP production, whereas treatment with EGF still stimulated YAP production with RhoA knockdown (Fig. 6C). The CCK-8 assay showed that RhoA knockdown partially inhibited the proliferation of ARPE-19 cells; however, treatment with EGF still promoted cell proliferation with RhoA knockdown (Fig. 6D). Our mechanistic study indicated that RhoA inhibition had little effect on the EGFR pathway, but partially inhibited the protein levels of CyclinD1, C-Myc, and Bcl-xl (Fig. 6E). EGF treatment stimulated the EGFR pathway and partially increased the protein levels of CyclinD1, Bcl-xl, and C-Myc in ARPE-19 cells with RhoA knockdown (Fig. 6E). Taken together, our results indicated that activation of EGFR signaling bypassed RhoA to stimulate YAP and enhance cell proliferation in ARPE-19 cells.

Discussion

In the present study, we observed that EGFR-PI3K-PDK1–dependent YAP activation was closely related to the formation of PVR, suggesting that targeting EGFR and YAP signaling may represent a possible therapeutic approach for PVR treatment. We found the upregulated production

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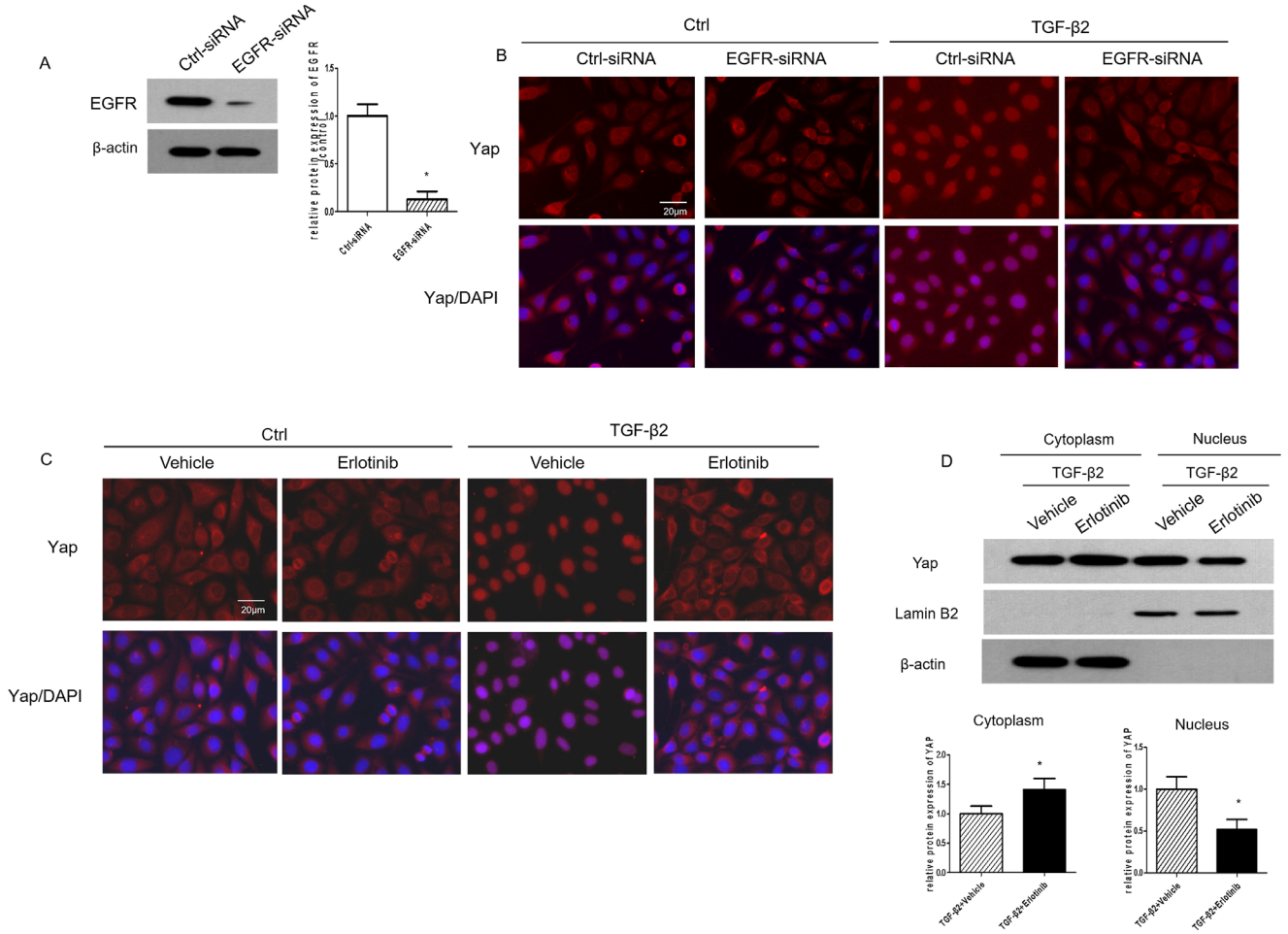

Figure 3. YAP activation was dependent on EGFR activation in ARPE-19 cells. (**A**) The expression of EGFR in ARPE-19 cells after transfection with EGFR-siRNA or Ctrl-siRNA was measured by Western blotting. $n = 3$. * $P < 0.05$ versus the Ctrl-siRNA. (**B**) Expression of YAP in ARPE-19 cells transfected with EGFR-siRNA or Ctrl-siRNA and exposure to TGF-β2 was determined by immunofluorescence staining. Scale bar, 20 μm. (**C**) Expression of YAP in ARPE-19 cells treated with or without Erlotinib and exposure to TGF-β2 was determined by immunofluorescence staining. Scale bar, 20 μm. (**D**) Expression of YAP in the cytoplasm and nucleus of ARPE-19 cells treated with or without Erlotinib and exposure to TGF-β2 was detected by Western blotting. * $P < 0.05$ versus the TGF-β2+Veh group.

of YAP in the PVR mouse model, which was an effective indicator of YAP activation. Moreover, YAP expression and nuclear translocation were enhanced in ARPE-19 cells treated with TGF-β2 in vitro. Both RPE-specific EGFR-cKO and administration of the EGFR signaling inhibitor erlotinib suppressed both nuclear translocation and YAP expression in response to PVR. Furthermore, RPE-specific YAP-cKO or treatment with the YAP signaling inhibitor verteporfin greatly decreased cell cycle progression through the downregulation of cyclin D, C-Myc, and Bcl-xl expression, as shown in Fig. 5. In response to PVR, activated EGFR signaling can bypass RhoA to regulate YAP. In summary, the present study provides novel ideas for understanding the pathogenesis of PVR.

The PVR mouse model used in the present study was not the only possible model. Several methods could be used to induce PVR in vivo, including surgical manipulation, intravitreal treatment with platelet-rich plasma, and injection of different cells.[25–27] It is worth noting that the trend of establishing a PVR model by injection of Dispase in the eyes of mice and rabbits is growing.[28] Dispase is a neutral protease obtained from *Bacillus polymyxa*.[29] Because Dispase is able to cleave the basal membrane in various tissues, it can be used to harvest and culture cells and is used as an intravitreal injection material to establish a PVR model.[30] Our previous study indicated that a PVR mouse model induced by intravitreal injection of Dispase II developed PVR-like retinal fibrosis and specific fibrogenic molecules in the retinas.[19] Savur et al.[31] also showed that this model could at least partially mimic the PVR pathology and had a higher success rate than other PVR models, as detected by histological measurement. Therefore, the PVR mouse model induced by Dispase is an ideal model for studying the pathogenesis of PVR.

Wei et al.[32] found that the correlation between EGFR signaling and the Hippo pathway was associated with the process of carcinogenesis. The correlation between these two signaling pathways and their impacts on targeted therapy in ARPE-19 cells remains unknown. In this study, we showed that the EGFR pathway was activated mainly via PI3K-PDK1 signaling and bypassed the canonical RhoA pathway instead of the Akt pathway to stimulate YAP activation. Similarly, it is worth noting that EGFR signaling could inhibit the Hippo pathway by activating PI3K-PDK1 but not AKT, thereby resulting in Lats inactivation, YAP dephos-

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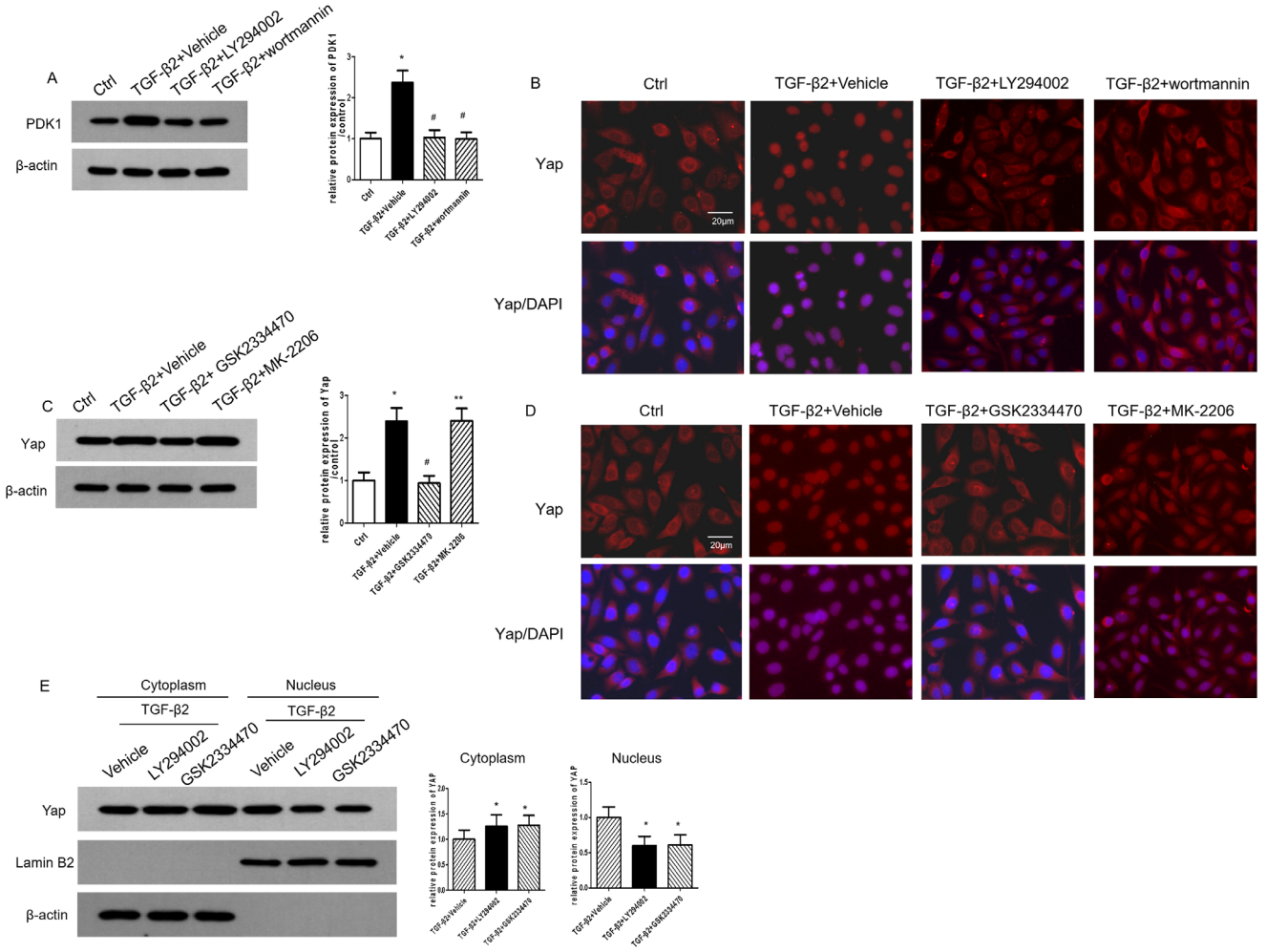

Figure 4. Pl3K-PDK1 activation mediated EGFR-dependent YAP activation. (**A**) The expression of PDK1 in ARPE-19 cells treated with LY294002 or wortmannin and exposure to TGF-β2 was measured by Western blotting. $n = 3$. * $P < 0.05$ versus the Control group; # $P < 0.05$ versus the TGF-β2+Veh group; $n = 3$. (**B**) Expression of YAP in ARPE-19 cells treated with LY294002 or wortmannin and exposure to TGF-β2 was determined by immunofluorescence staining. Scale bar, 20 μm. (**C**) The expression of YAP in ARPE-19 cells treated with GSK2334470 or MK-2206 and exposure to TGF-β2 was measured by Western blotting. $n = 3$. * $P < 0.05$ versus the Control group; # $P < 0.05$ versus the TGF-β2+Veh group; ** $P < 0.05$ versus the TGF-β2+GSK2334470 group; $n = 3$. (**D**) Expression of YAP in ARPE-19 cells treated with GSK2334470 or MK-2206 and exposure to TGF-β2 was determined by immunofluorescence staining. Scale bar, 20 μm. (**E**) Expression of YAP in the cytoplasm and nucleus of ARPE-19 cells treated with LY294002 or GSK2334470 and exposure to TGF-β2 was detected by Western blotting. * $P < 0.05$ versus the TGF-β2+Veh group.

phorylation, and nuclear translocation,[33] which is partially consistent with our reports. However, Chen et al.[34] also reported that blocking Akt1 but not PDK1 inhibited nuclear translocation and YAP expression in renal proximal tubule epithelial cells. The main cause for these differences may be the different cell types used in these two studies. We also found that the activation of EGFR signaling promoted RhoA expression and the silencing of RhoA partially reversed the EGFR-stimulated increase in YAP. Huang et al.[35] found that the activation of YAP/Taz could stimulate its upstream negative regulator RhoA, which constituted a negative feedback cycle of the Hippo pathway. Taken together, our data showed that the upregulation of PDK1 induced by EGFR may be the result of transcription-induced YAP expression, and the molecular mechanism remains to be further studied.

The activation of classic Hippo signaling results in YAP phosphorylation and subsequent sequestration of YAP in the cytoplasm, which is then degraded by proteasomes. Studies by us and others have shown that YAP activation increases in response to hyperglycemia and mediated progressive retinal fibrosis.[18,36] Given the crucial role of YAP in the cellular response to stiffness of the microenvironment, YAP was proven to be related to pathological fibrosis.[37] In fact, YAP overexpression was observed in renal, liver, and lung fibrosis.[38–40] YAP was associated with TGF-β–Smad signaling during the fibrotic process.[41] Similar to previous reports, our study showed that YAP was required for TGF-β2–induced fibrosis in ARPE-19 cells. Additionally, genetic suppression of YAP abolished TGF-β2–induced cell cycle progression. CTGF and CYR61 were proven to be downstream targets of YAP and acted as signaling molecules that were relevant for the progression of pathogenic fibrosis.[42] In addition, we observed that the activation of EGFR signaling occurred through YAP to transactivate CTGF and CYR61. This result

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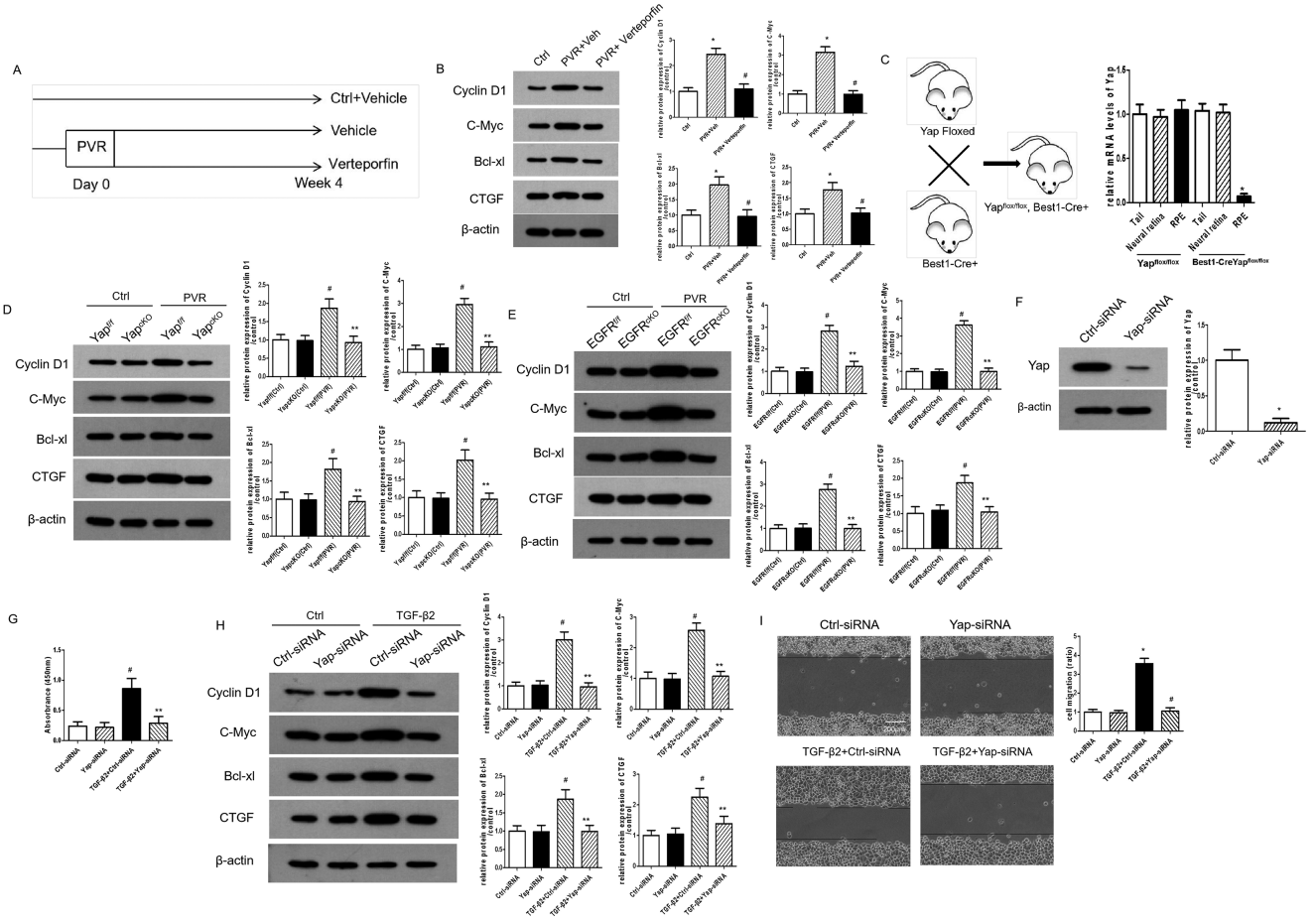

Figure 5. Increased Cyclin D Expression was dependent on YAP activation. (**A**) The PVR mice models were randomized to treatment with verteporfin ($n = 6$) or vehicle ($n = 6$) beginning immediately after PVR induction. Control mice were also treatment with vehicle ($n = 6$). (**B**) Western blot assay for protein levels of Cyclin D1, C-Myc, Bcl-xl and CTGF in the retinas of PVR mice with or without verteporfin. * $P < 0.05$ versus the control group; # $P < 0.05$ versus the PVR+Veh group; $n = 3$. (**C**) Schematic of the generation of $YAP^{flox/flox}$ and YAP^{cKO}. And relative mRNA levels of YAP in the RPE, neural retina and tail of $YAP^{flox/flox}$ and YAP^{cKO} mice ($n = 3$). (**D**) Western blot assay for protein levels of Cyclin D1, C-Myc, Bcl-xl and CTGF in the retinas of RPE-specific YAP-cKO mice and $YAP^{f/f}$ mice. # $P < 0.05$ versus the $YAP^{f/f}$ group; ** $P < 0.05$ versus the $YAP^{f/f}$(PVR) group; $n = 3$. (**E**) Western blot assay for protein levels of Cyclin D1, C-Myc, Bcl-xl and CTGF in the retinas of RPE-specific EGFR-cKO mice and $EGFR^{f/f}$ mice. # $P < 0.05$ versus the $EGFR^{f/f}$ group; ** $P < 0.05$ versus the $EGFR^{f/f}$(PVR) group; $n = 3$. (**F**) The expression of YAP in ARPE-19 cells after transfection with YAP-siRNA or Ctrl-siRNA was measured by Western blotting. $n = 3$. * $P < 0.05$ versus the Ctrl-siRNA. (**G**) Proliferation of ARPE-19 cells transfected with YAP-siRNA or Ctrl-siRNA and exposure to TGF-β2 was analyzed by CCK-8 assays. # $P < 0.05$ versus the Ctrl-siRNA group; ** $P < 0.05$ versus the TGF-β2+Ctrl-siRNA group; $n = 5$. (**H**) Western blot assay for protein levels of Cyclin D1, C-Myc, Bcl-xl and CTGF in the ARPE-19 cells transfected with YAP-siRNA or Ctrl-siRNA and exposure to TGF-β2. # $P < 0.05$ versus the Ctrl-siRNA group; ** $P < 0.05$ versus the TGF-β2+Ctrl-siRNA group; $n = 3$. (**I**) Migration of ARPE-19 cells transfected with YAP-siRNA or Ctrl-siRNA and exposure to TGF-β2 was analyzed by Scratch assays. Scale bar, 200 µm. # $P < 0.05$ versus the Ctrl-siRNA group; ** $P < 0.05$ versus the TGF-β2+Ctrl-siRNA group; $n = 3$.

was similar to the results of Rong et al.,[43] who indicated that the EGFR pathway can activate CTGF expression through YAP in lung cancer. Other reports also found that the activation of the EGFR and YAP pathways can form a positive signal cycle to promote the progression of cancer in multiple cell types, including breast epithelial cells, alveolar epithelial cells, and cerebellar granule cells.[44–46] Previously, we found that verteporfin alleviated the production of CTGF in the PVR mouse model.[17] In the present study, our in vivo results further showed that verteporfin attenuated the upregulation of the Cyclin D1, C-Myc, and Bcl-xl proteins in the PVR mouse model. Verteporfin has been clinically used as a therapeutic approach for AMD and approved by the US Food and Drug Administration.[47] Our study indicated that strategies targeting YAP represent promising therapeutic options for preventing pathogenic fibrosis in PVR.

In summary, the present study revealed that EGFR-PI3K-PDK1–dependent YAP activation plays a crucial role during PVR progression. Our findings provide direct evidence for the role of YAP and EGFR signaling in PVR inhibition and present promising therapeutic approaches for PVR treatment. The limitation of this study was the inability of the PVR mouse model by injection of Dispase to replicate PVR features such as preretinal membranes inducing retinal traction detachment. Exploring a PVR mouse model that could reproduce the clinical features of pathologic retinal fibrosis seen in patients with PVR may help to find new therapeutic approaches in the prevention of PVR. Further research is

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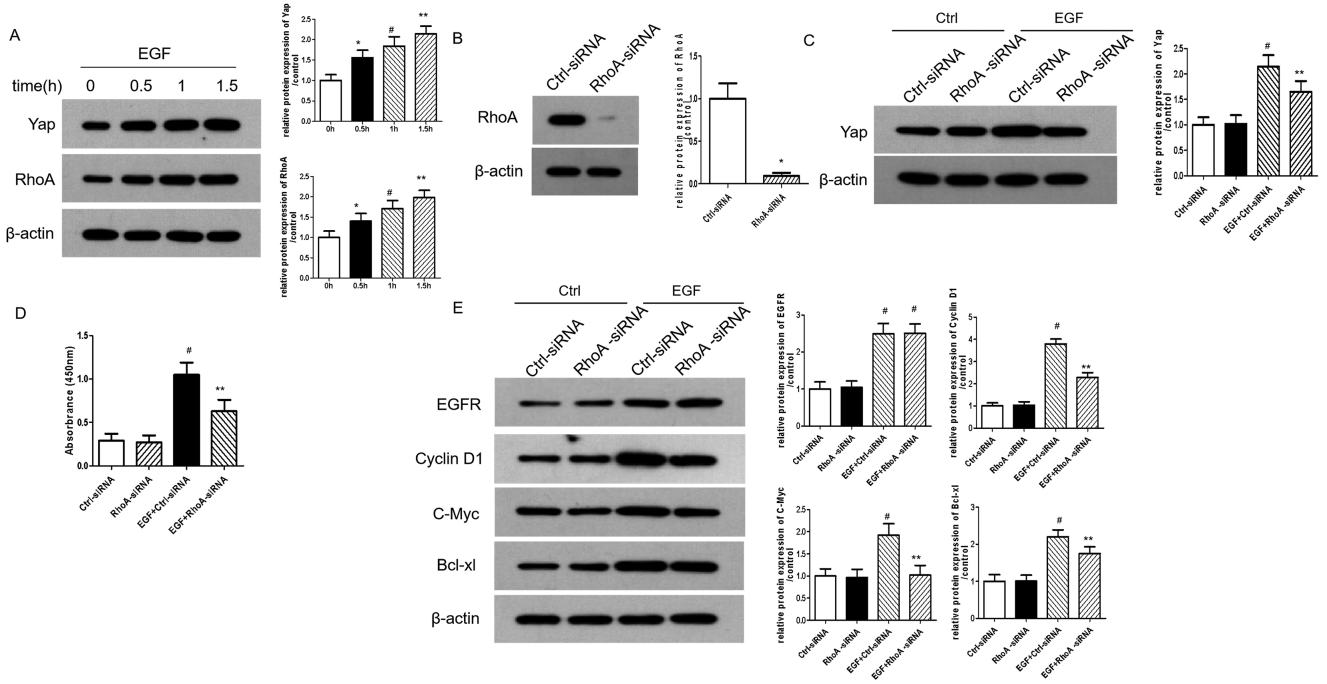

Figure 6. Activated EGFR signaling could bypass RhoA to regulate YAP. (**A**) Protein level of YAP and RhoA in ARPE-19 cells treated with EGF at different time points was detected by Western blotting. * $P < 0.05$ versus the control group; # $P < 0.05$ versus the EGF(0.5h) group; ** $P < 0.05$ versus the EGF(1h) group; $n = 3$. (**B**) The expression of RhoA in ARPE-19 cells after transfection with RhoA-siRNA or Ctrl-siRNA was measured by Western blotting. $n = 3$. * $P < 0.05$ versus the Ctrl-siRNA. (**C**) The expression of YAP in ARPE-19 cells transfected with RhoA-siRNA or Ctrl-siRNA and exposure to EGF was measured by Western blotting. # $P < 0.05$ versus the Ctrl-siRNA; ** $P < 0.05$ versus the Ctrl-siRNA+EGF; $n = 3$. (**D**) Proliferation of ARPE-19 cells transfected with RhoA-siRNA or Ctrl-siRNA and exposure to TGF-β2 was analyzed by CCK-8 assays. # $P < 0.05$ versus the Ctrl-siRNA; ** $P < 0.05$ versus the Ctrl-siRNA+EGF; $n = 5$. (**E**) Western blot assay for protein levels of EGFR, Cyclin D1, C-Myc, and Bcl-xl in the ARPE-19 cells transfected with RhoA-siRNA or Ctrl-siRNA and exposure to EGF. # $P < 0.05$ versus the Ctrl-siRNA; ** $P < 0.05$ versus the Ctrl-siRNA+EGF; $n = 3$.

needed to determine whether there is a positive feedback cycle between activated EGFR and YAP activation-mediated extracellular matrix production.

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Author Contributions: Wei Zhang conceived and designed the experiments; Wei Zhang and Jing Li performed the experiments and prepared figures; Wei Zhang and Jing Li collected the sample data; Wei Zhang contributed to the writing of the manuscript; Wei Zhang reviewed the manuscript; all authors contributed to the writing of the manuscript and discussed the manuscript at various stages.

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References

1. Mostafa I, Al-Zubaidy M, Ghareeb A, Song A. Infographic: slow-release dexamethasone in proliferative vitreoretinopathy (PVR). *Eye (Lond)*. 2022;36(2):352–353.
2. Idrees S, Sridhar J, Kuriyan AE. Proliferative vitreoretinopathy: a review. *Int Ophthalmol Clin*. 2019;59(1):221–240.
3. Sang WM, Sun Y, Warther D, Huffman K, Cheng L. New model of proliferative vitreoretinopathy in rabbit for drug delivery and pharmacodynamic studies. *Drug Deliv*. 2018;25:600–610.
4. Han H, Chen N, Huang X, Liu B, Tian J, Lei H. Phosphoinositide 3-kinase δ inactivation prevents vitreous-induced activation of AKT/MDM2/p53 and migration of retinal pigment epithelial cells. *J Biol Chem*. 2019;294(42):15408–15417.
5. Yan F, Hui Y. Epidermal growth factor receptor exists in the early stage of proliferative vitreoretinopathy. *Can J Ophthalmol*. 2012;47:e24–25.
6. Nishimura T, Végvári Á, Nakamura H, Kato H, Saji H. Mutant proteomics of lung adenocarcinomas harboring different EGFR mutations. *Front Oncol*. 2020;10:1494.
7. Penela P, Harris RC. The epidermal growth factor receptor axis and kidney fibrosis. *Cells*. 2021;30:275–279.
8. Yan F, Hui YN, Li YJ, Guo CM, Meng H. Epidermal growth factor receptor in cultured human retinal pigment epithelial cells. *Ophthalmologica*. 2007;221:244–250.
9. Liang CM, Tai MC, Chang YH, et al. Glucosamine inhibits epidermal growth factor-induced proliferation and cell-cycle progression in retinal pigment epithelial cells. *Mol Vis*. 2010;16:2559–2571.
10. Park HW, Wang Y, Li J, et al. Hippo kinases regulate cell junctions to inhibit tumor metastasis in response to oxidative stress. *Cells*. 2019;26:101233.
11. Masson C, García-García D, Bitard J, Grellier É K, Roger JE, Perron M. YAP haploinsufficiency leads to Müller cell dysfunction and late-onset cone dystrophy. *Diabetes*. 2020;11:631.
12. Yu OM, Benitez JA, Plouffe SW, et al. YAP and MRTF-A, transcriptional co-activators of RhoA-mediated gene expression, are critical for glioblastoma tumorigenicity. *Oncogene*. 2018;37:5492–5507.

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13. Hamon A, García-García D, Ail D, et al. Linking YAP to Müller glia quiescence exit in the degenerative retina. *Cell Rep*. 2019;27:1712–1725.e1716.
14. Hsu PC, Miao J, Huang Z, et al. Inhibition of yes-associated protein suppresses brain metastasis of human lung adenocarcinoma in a murine model. *J Cell Mol Med*. 2018;22:3073–3085.
15. Daftarian N, Rohani S, Kanavi MR, et al. Effects of intravitreal connective tissue growth factor neutralizing antibody on choroidal neovascular membrane-associated subretinal fibrosis. *Exp Eye Res*. 2019;184:286–295.
16. Klaassen I, van Geest RJ, Kuiper EJ, van Noorden CJ, Schlingemann RO. The role of CTGF in diabetic retinopathy. *Exp Eye Res*. 2015;133:37–48.
17. Zhang W, Li J. Yes-associated protein is essential for proliferative vitreoretinopathy development via the epithelial-mesenchymal transition in retinal pigment epithelial fibrosis. *J Cell Mol Med*. 2021;25:10213–10223.
18. Zhang W, Kong Y. YAP is essential for TGF-β-induced retinal fibrosis in diabetic rats via promoting the fibrogenic activity of Müller cells. *J Cell Mol Med*. 2020;24:12390–12400.
19. Zhang W, Han H. Targeting matrix stiffness-induced activation of retinal pigment epithelial cells through the RhoA/YAP pathway ameliorates proliferative vitreoretinopathy. *Exp Eye Res*. 2021;108677.
20. Chen J, Harris RC. Interaction of the EGF receptor and the Hippo pathway in the diabetic kidney. *J Am Soc Nephrol*. 2016;27:1689–1700.
21. Zhang W, Jiang H, Kong Y. Exosomes derived from platelet-rich plasma activate YAP and promote the fibrogenic activity of Müller cells via the PI3K/Akt pathway. *Exp Eye Res*. 2020;193:107973.
22. Lee-Rivera I, López E, Parrales A, Alvarez-Arce A, López-Colomé AM. Thrombin promotes the expression of Ccnd1 gene in RPE cells through the activation of converging signaling pathways. *Exp Eye Res*. 2015;139:81–89.
23. Park BO, Kim SH. The short-chain fatty acid receptor GPR43 modulates YAP/TAZ via RhoA. *Mol Cells*. 2021;44(7):458–467.
24. Cheng Y, Lou JX, Liu CC, et al. Microfilaments and microtubules alternately coordinate the multi-step endosomal trafficking of classical swine fever virus. *J Virol*. 2021;95(10):e02436–20.
25. Wong CW, Busoy JMF, Cheung N, Barathi VA, Storm G, Wong TT. Endogenous or exogenous retinal pigment epithelial cells: a comparison of two experimental animal models of proliferative vitreoretinopathy. *Transl Vis Sci Technol*. 2020;9:46.
26. Lyu Y, Xu W, Zhang J, et al. Protein kinase A inhibitor H89 attenuates experimental proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci*. 2020;61:1.
27. Heffer A, Wang V, Sridhar J, et al. A mouse model of proliferative vitreoretinopathy induced by intravitreal injection of Gas and RPE cells. *Transl Vis Sci Technol*. 2020;9:9.
28. Yoo K, Son BK, Kim S, Son Y, Yu SY, Hong HS. Substance P prevents development of proliferative vitreoretinopathy in mice by modulating TNF-alpha. *Mol Vis*. 2017;23:933–943.
29. Tikhonovich MV, Erdiakov AK, Gavrilova SA. Nonsteroid anti-inflammatory therapy suppresses the development of proliferative vitreoretinopathy more effectively than a steroid one. *Int Ophthalmol*. 2018;38:1365–1378.
30. Szczesniak AM, Porter RF, Toguri JT, et al. Cannabinoid 2 receptor is a novel anti-inflammatory target in experimental proliferative vitreoretinopathy. *Neuropharmacology*. 2017;113:627–638.
31. Savur F, Aydemir O, Ilhan N. The effect of infliximab and octreotide on cytokine levels experimental proliferative vitreoretinopathy. *Cutan Ocul Toxicol*. 2020;39:61–66.
32. Wei C, Li X. Determination of the migration effect and molecular docking of verteporfin in different subtypes of breast cancer cells. *Mol Med Rep*. 2020;22:3955–3961.
33. Xia H, Dai X, Yu H, et al. EGFR-PI3K-PDK1 pathway regulates YAP signaling in hepatocellular carcinoma: the mechanism and its implications in targeted therapy. *Cell Death Dis*. 2018;9:269.
34. Chen J, You H, Li Y, Xu Y, He Q, Harris RC. EGF receptor-dependent YAP activation is important for renal recovery from AKI. *J Am Soc Nephrol*. 2018;29:2372–2385.
35. Huang C, Zhou M, Zheng X. RhoA inhibitor-eluting stent attenuates restenosis by inhibiting YAP signaling. *J Vasc Surg*. 2019;69:1581–1589.e1581.
36. Lee M, Goraya N, Kim S, Cho SH. Hippo-YAP signaling in ocular development and disease. *Dev Dyn*. 2018;247:794–806.
37. Li S, Li C, Zhang Y, et al. Targeting mechanics-induced fibroblast activation through CD44-RhoA-YAP pathway ameliorates crystalline silica-induced silicosis. *Theranostics*. 2019;9:4993–5008.
38. Kim DH, Choi HI, Park JS, et al. Src-mediated crosstalk between FXR and YAP protects against renal fibrosis. *FASEB J*. 2019;33(10):11109–11122
39. Zhang C, Bian M, Chen X, et al. Oroxylin A prevents angiogenesis of LSECs in liver fibrosis via inhibition of YAP/HIF-1alpha signaling. *J Cell Biochem*. 2018;119:2258–2268.
40. Noguchi S, Saito A. YAP/TAZ signaling as a molecular link between fibrosis and cancer. *Int J Mol Sci*. 2018;19(11):3674.
41. Nagase T, Feng Y, Liang Y, et al. The signaling protein Wnt5a promotes TGFbeta1-mediated macrophage polarization and kidney fibrosis by inducing the transcriptional regulators YAP/Taz. *Int J Mol Sci*. 2018;293:19290–19302.
42. Yao M, Li J, Yuan S, et al. Role of the arecoline/YAP1/BMP4 pathway in promoting endothelial-mesenchymal transition in oral submucous fibrosis. *J Oral Pathol Med*. 2020;49(4):305–310.
43. Rong X, Liang Y, Han Q, et al. Molecular mechanisms of tyrosine kinase inhibitor resistance induced by membranous/cytoplasmic/nuclear translocation of epidermal growth factor receptor. *J Thorac Oncol*. 2019;14(10):1766–1783.
44. Hsu PC, Jablons DM, Yang CT, You L. Epidermal growth factor receptor (EGFR) pathway, Yes-associated protein (YAP) and the regulation of programmed death-ligand 1 (PD-L1) in non-small cell lung cancer (NSCLC). *Int J Mol Sci*. 2019;20(15):3821.
45. Islam SS, Uddin M, Noman ASM, et al. Antibody-drug conjugate T-DM1 treatment for HER2+ breast cancer induces ROR1 and confers resistance through activation of Hippo transcriptional coactivator YAP1. *EBioMedicine*. 2019;43:211–224.
46. Litan A, Li Z, Tokhtaeva E, Kelly P, Vagin O, Langhans SA. A functional interaction between Na,K-ATPase β(2)-subunit/AMOG and NF2/Merlin Regulates growth factor signaling in cerebellar granule cells. *Mol Neurobiol*. 2019;56:7557–7571.
47. Chen H, Deng H, Zou X, Zhao J. Hypocrellin B encapsulated in triphenyl phosphonium-modified cationic liposomes for photodynamic treatment of exudative age-related macular degeneration. *J Biomed Nanotechnol*. 2019;15:2305–2320.

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