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Knockdown of survivin results in inhibition of epithelial to mesenchymal transition in retinal pigment epithelial cells by attenuating the TGFβ pathway

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ABSTRACT

Proliferative vitreoretinopathy (PVR) is a common complication of open globe injury and the most common cause of failed retinal detachment surgery. The response by retinal pigment epithelial (RPE) cells liberated into the vitreous includes proliferation and migration; most importantly, epithelial to mesenchymal transition (EMT) of RPE plays a central role in the development and progress of PVR. For the first time, we show that knockdown of BIRC5, a member of the inhibitor of apoptosis protein family, using either lentiviral vector based CRISPR/Cas9 nickase gene editing or inhibition of survivin using the small-molecule inhibitor YM155, results in the suppression of EMT in RPE cells. Knockdown of survivin or inhibition of survivin significantly reduced TGFβ-induced cell proliferation and migration. We further demonstrated that knockdown or inhibition of survivin attenuated the TGFβ signaling by showing reduced phospho-SMAD2 in BIRC5 knockdown or YM155-treated cells compared to controls. Inhibition of the TGFβ pathway using TGFβ receptor inhibitor also suppressed survivin expression in RPE cells. Our studies demonstrate that survivin contributes to EMT by cross-talking with the TGFβ pathway in RPE cells. Targeting survivin using small-molecule inhibitors may provide a novel approach to treat PVR disease.

1. Introduction

Proliferative vitreoretinopathy (PVR) is a common complication of open globe injury and the most common cause of failed retinal detachment surgery. It is characterized by the formation and contraction of epiretinal membranes (ERM) and causes rhegmatogenous retinal detachment (RRD) [1]. PVR occurs in 5%–10% of all RRD, and it is a major risk factor for redetachment after surgery [2]. PVR also occurs in other ocular disorders including large retinal tears or injuries, and endoresection of tumors. Currently, the standard treatment for PVR is surgery by restoring normal anatomy and the ciliary body function, including scleral buckling, vitrectomy, membrane peeling, and retinotomies. However, the success rate is only 40–80% [2]. Therefore, understanding the molecular mechanisms of PVR and developing new therapies including pharmacological intervention are essential to better treat this disease.

There are several cell types identified in ERMs including retinal pigment epithelial (RPE) cells, astrocytes, microglia, macrophages, and Muller cells [3,4]. RPE cells are primary contributors in forming ERM by transforming into fibroblastic and myofibroblastic phenotypes via epithelial to mesenchymal transition (EMT) [5]. However, the molecular mechanisms underlying EMT in RPE cells remain elusive. Previous studies indicated that multiple signaling pathways were involved in EMT of RPE cells including Wnt/β-catenin [6], TGFβ [7], Notch [8], HIF1α [9], ERK1/2 [10], and p38MAPK [11]. Activation of those pathways promoted EMT in RPE cells.

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family encoded by the BIRC5 gene, functions by inhibiting the caspase activation and promotes cell growth, which is required for embryonic development [12]. Survivin was extensively studied in various human cancers and highly expressed in cancer cells but rarely expressed in normal corresponding cells, thus making it a drug target for cancer therapy. Several studies demonstrated that survivin promoted EMT in cancer cells by participating in multiple signaling pathways: TGFβ, ERK1/2, and PI3/AKT [13–15]. Survivin was expressed in RPE cells as a survival factor [16,17] and induced by Activin A, a member of the TGFβ superfamily [18] and TGFβ in RPE cells [19]. However, the function of survivin and how survivin contributes to EMT in RPE cells is little understood.

In this study, we investigated the role of survivin in ARPE19 cells by using lentiviral CRISPR/Cas9 nickase vector-mediated gene editing and inhibition of survivin expression using a small-molecule survivin inhibitor, YM155. For the first time, we show that survivin regulates EMT by activating the TGFβ pathway in RPE cells, and thus, survivin inhibitors may provide a novel approach to treat PVR.

2. Materials and methods

2.1. Cell culture

ARPE-19 cells were obtained from the American Type Culture Collection and maintained at 37 °C in a 5% CO_2 in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and antibiotics (Penicillin– Streptomycin solution, Invitrogen; Carlsbad, CA). Cells were cultured to 90% confluence and serum-starved for 12 h before survivin inhibitor treatment. HEK293 FT cells were purchased from Invitrogen and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% glutamine and 1% nonessential amino acids. TGFβ1 was purchased from Sigma (St. Louis, MO).

2.2. Lentiviral vector production

The lentiviral CRISPR/Cas9 nickase-mediated BIRC5 gene editing vectors were described previously [13]. Survivin knockdown (KD) stable cell lines were generated by transducing the ARPE-19 cells with the lentiviral CRISPR/Cas9 nickase BIRC5 vector and lentiCas9-blast Cas9 nickase vectors and selected with 5 μg/ml puromycin or 10 μg/ml blasticidin. LentiCas9-blast was used as the control vector without gRNAs.

2.3. Immunofluorescent staining

Cells were fixed for 5 min in 4% paraformaldehyde and washed three times with 0.1% Tween 20 in PBS (PBST), and then incubated with blocking buffer (5% normal goat serum, 3% bovine serum albumin, and 0.1% Triton-X 100 in PBS) for 1 h. Cells were incubated with the primary antibodies to cytokeratin-7 (Abcam, Cambridge, MA) and β-catenin (1:200 dilution, Cell Signaling, Danvers, MA) overnight at 4 °C and then washed three times with PBST. Secondary antibodies, Alexa 488 or 594 conjugated goat anti-rabbit or mouse (1:200 dilution, Life Technologies), were added and incubated for 1 h at room temperature. Cell nuclei were counterstained with DAPI (Vector Laboratories, Inc.; Burlingame, CA). Images were taken using a Nikon inverted fluorescence microscope.

2.4. Cell migration assay

The cell migration assay was performed using a modified transwell chamber (BD Falcon™, San Jose, CA). These chambers were inserted into 24-well cell culture plates. ARPE-19 cells transduced with lentiviral BIRC5 Cas9 nickase gRNAs and control vectors (3×10^4) in 300 μl serum-free DMEM were added to the upper chamber. DMEM containing 6 ng/ml TGFβ in DMEM was added into the lower chamber of each well and incubated for 24 h. The medium and nonmigrated cells in the upper chamber were removed, while the migrated cells on the lower side of the membranes were fixed with methanol and stained with crystal violet. Pictures were taken at 20× magnification, and cells from at least three different fields were counted.

2.5. MTT assay

ARPE-19 cells (5000/well), transduced with lentiviral CRISPR/Cas9 nickase for BIRC5 editing and control vectors, were plated into 96-well plates and cells and were then cultured at 2% DMEM with or without 6 ng/ml TGFβ for different time points (24, 48 and 72 h). OD570 was measured at indicated time points by adding 10 μl of MTT reagent into each well and incubated for ~4 h and then terminated by adding 100 μl detergent reagent to incubate at 22 °C in the dark for 2 h. Cell proliferation was compared between control and BIRC5 KD cells with or without TGFβ treatment.

2.6. Western blot

ARPE-19 cells were collected and lysed in RIPA buffer (Thermo Scientific; Rockford, IL) containing 1% Halt Proteinase Inhibitor Cocktail (Thermo Scientific; Rockford, IL). An equal amount of protein was loaded onto 10% SDS-PAGE gels and transferred onto nitrocellulose membranes, which were then blocked with 5% nonfat milk for 1 h and incubated with primary antibodies against survivin, N-cadherin, vimentin (Cell Signaling), cytokeratin-7(Abcam) and GAPDH (Sigma; St. Louis, MO) for 24 h at 4 °C. The membranes were washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Protein bands were visualized using chemiluminescence by exposing on X-ray film.

2.7. Statistical analysis

Significant differences were determined from independent experiments performed in triplicate and presented as means ± S.D. using Student's t-test. $p < 0.05$ was considered to be a significant difference.

3. Results

3.1. Knockdown of BIRC5 using lentiviral CRISPR/Cas9 nickase-mediated editing or inhibition of survivin using the small-molecule survivin inhibitor YM155 leads to suppression of EMT in RPE cells

To investigate the role of BIRC5 in RPE cells, we generated BIRC5 KD and control stable cells by transducing ARPE-19 cell line with lentiviral BIRC5 CRISPR/Cas9 nickase and control vectors, respectively. In BIRC5 KD cell line, survivin expression was significantly reduced compared to control cells. EMT markers were also significantly altered in survivin KD compared to control cells, including epithelial cell marker cytokeratin-7 upregulation and mesenchymal markers N-cadherin, β-catenin, and vimentin downregulation, indicating that knockdown of survivin inhibited EMT in RPE cells (Fig. 1A). We also examined EMT markers by performing immunofluorescent staining of mesenchymal marker β-catenin and epithelial marker cytokeratin-7. β-catenin was stained in cell membranes with weak fluorescence, whereas cytokeratin-7 was strongly stained in cytoplasm and cell membrane in BIRC5 KD compared to control cells (Fig. 1B and C). Immunofluorescent