Trichostatin A Inhibits Retinal Pigmented Epithelium Activation in an *In Vitro* Model of Proliferative Vitreoretinopathy

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Abstract

Purpose: Proliferative vitreoretinopathy (PVR) is a blinding disorder that develops after a retinal tear or detachment. Activation of the retinal pigmented epithelium (RPE) is implicated in PVR; however, the mechanisms leading to enhanced RPE proliferation, migration, and contraction remain largely unknown. This study utilized an *in vitro* model of PVR to investigate the role of acetylation in RPE activation and its contribution to the progression of this disease.

Methods: ARPE-19 cells, primary cultures of porcine RPE, and induced pluripotent stem cell-derived RPE (iPS-RPE) were utilized for cellular and molecular analyses. Cells treated with transforming growth factor beta 2 (TGFβ2; 10 ng/mL) alone or in the presence of the broad-spectrum histone deacetylase (HDAC) inhibitor, trichostatin A (TSA; 0.1 μM), were assessed for contraction and migration through collagen contraction and scratch assays, respectively. Western blotting and immunofluorescence analysis were performed to assess α-smooth muscle actin (α-SMA) and β-catenin expression after TGFβ2 treatment alone or in combination with TSA.

Results: TGFβ2 significantly increased RPE cell contraction in collagen matrix and this effect was inhibited in the presence of TSA (0.1 μM). In agreement with these data, immunofluorescence analysis of TSA-treated iPS-RPE wounded monolayers revealed decreased α-SMA as compared with control. Scratch assays to assess wound healing revealed TSA inhibited TGFβ2-mediated iPS-RPE cell migration.

Conclusions: Our findings indicate a role of acetylation in RPE activation. Specifically, the HDAC inhibitor TSA decreased RPE cell proliferation and TGFβ2-mediated cell contraction and migration. Further investigation of pharmacological compounds that modulate acetylation may hold promise as therapeutic agents for PVR.

Introduction

PROLIFERATIVE VITREORETINOPATHY (PVR) is a blinding disorder that results as a consequence of aberrant wound healing after a retinal tear or rhegmatogenous retinal detachment (RRD). PVR occurs in 5%–10% of all RRDs and is implicated in redetachment after surgery in 75% of all cases.¹ Currently, vitreoretinal surgery is the standard treatment of care; however, recurrent vitreoretinal traction can lead to secondary detachment, extensive reepithelialization of membranes, and loss of vision.² Importantly, retinal pigmented epithelium (RPE) activation after a retinal tear is implicated in PVR.^{3–5} Enhanced proliferation, migration, and contraction of RPE underlie the pathology of this disease; however, mechanisms leading to activation of these cellular processes remain largely unknown. Several studies

report that the RPE undergoes epithelial–mesenchymal transition (EMT), resulting in RPE cell activation, which migrate and proliferate to form the epiretinal membrane in PVR.^{5–8} EMT-mediated activation of signaling proteins such as β-catenin, a critical regulator of cell–cell adhesion and transcription, is also suggested to contribute to the development and progression of PVR.^{9,10} Moreover, RPE dedifferentiation into mesenchymal cells and associated collagen production contributes to fibrous tissue formation, retinal traction, and the development of PVR.^{6,7} Thus, suppression or inhibition of signaling pathways essential to the formation and development of fibrotic retinal membranes may lead to the prevention and/or treatment of PVR.

The secretion of various cytokines is known to activate RPE and promote cell proliferation and migration. Transforming growth factor beta (TGFβ) is identified as a

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cytokine implicated in both the physiological and pathological processes associated with tissue development and repair.¹¹ Of direct relevance, TGF β 2 is the predominant isoform in the posterior segment of the eye and is over-expressed in vitreous and proliferative membranes of PVR patients.^{12–14} Numerous studies have also demonstrated that TGF β stimulates α -smooth muscle actin (α -SMA) production and the transdifferentiation of RPE cells into myofibroblast-like cells.^{3,15–18} Accordingly, pharmacological compounds, which selectively inhibit TGF β 2 activity, have garnered significant interest as potential therapeutics for the treatment of PVR. After a retinal tear or detachment, RPE cells also acquire a pathological contractile function, which potentiates PVR development and recurrent retinal detachments. Collectively, increased RPE cell proliferation, migration, and contraction contribute to a poor prognosis for patients diagnosed with PVR. This study utilized an *in vitro* model of PVR to investigate pharmacological compounds that attenuate cellular processes associated with this disease. Histone deacetylase (HDAC) inhibitors are extensively evaluated in numerous experimental models of cancer, in which inhibition of deacetylation regulates cell survival, proliferation, differentiation, and apoptosis.¹⁹ In addition, recent literature demonstrates HDAC inhibitors as efficacious treatment options for choroidal angiogenesis²⁰ and retinal ischemic injury and degeneration.^{21,22} Acetylation is a critical post-translational modification that directly modulates gene transcription of histone and nonhistone proteins and is associated with the proliferation and differentiation of a variety of cell types.²³

As such, the aim of this study was to investigate whether acetylation activates cellular processes involved in the pathogenesis of PVR. We herein report that trichostatin A (TSA), a HDAC inhibitor, inhibited TGF β 2-mediated migration and contraction in RPE. In agreement with previous findings, TGF β 2 treatment increased α -SMA expression in RPE cells and this effect was blocked by TSA cotreatment. Moreover, we demonstrate that TSA treatment attenuated TGF β 2-mediated increases in interleukin (IL)-8, a key cytokine implicated in cell proliferation and migration. Collectively, these findings suggest that HDAC inhibitors may hold promise as therapeutics in the treatment of PVR through inhibition of cellular processes critical to the pathology of this disease.

Methods

ARPE19 cell culture

The immortalized ARPE19 cell line (ATCC, Manassas, VA) was cultured and maintained in complete medium [Dulbecco's modified Eagle's medium (DMEM)/F12, 10% fetal bovine serum (FBS), 5 μ g/mL gentamycin]. For *in vitro* experiments, ARPE19 cells were cultured at passages 20–24, seeded on 6-well plates at a seeding density of 2.5×10^5 cells/well, and allowed to reach 100% confluency. Next, cells were treated with the indicated compounds and assessed for protein expression through Western blotting.

Culture and differentiation of induced pluripotent stem cells

Human induced pluripotent stem (iPS) cells (IMR-90-1; WiCell Research Institute, Madison, WI) were cultured on Matrigel- (BD Biosciences, San Jose, CA) coated 6-well plates and maintained in mTeSR1 medium (Stem Cell

Technologies, Vancouver, BC, Canada). The medium was changed daily until cells were ready for passage. To initiate the differentiation protocol and generate human-induced pluripotent stem cell-derived RPE (iPS-RPE), mTeSR1 medium was replaced with differentiation medium consisting of 10% knockout serum replacement (Life Technologies, Grand Island, NY), 0.1 mM β -mercaptoethanol, 0.1 mM nonessential amino acids, 2 mM glutamine, and 10 μ g/mL gentamicin DMEM/F12. Human iPS-RPE cells have been previously characterized and shown to express RPE-specific visual cycle genes, lecithin-retinol acyl transferase, cellular retinaldehyde-binding protein, and retinal pigmented epithelium-specific protein 65 kPa (RPE65). Furthermore, 11-*cis* retinaldehyde detected in iPS-RPE culture medium demonstrates the ability of iPS-RPE to process retinoids through the visual cycle.²⁴ For iPS-RPE cell culture, half of the differentiation medium was changed every other day. Pigmented foci comprising RPE appeared and were allowed to grow large enough to be manually dissected out of culture. Pigmented iPS-RPE colonies were pooled and a single cell suspension was prepared with 0.25% trypsin. The enriched iPS-RPE was then seeded and cultured in fetal RPE (fRPE) medium composed of minimum essential medium (MEM), N1 supplement, glutamine, nonessential amino acids, taurine 0.25 mg/mL, hydrocortisone 10 ng/mL, triiodothyronine 13 ng/mL, and 15% of FBS. The seeding density at each passage after enrichment was 1×10^5 cells/cm². Cells were allowed to grow until 80% confluency and split accordingly. The culture medium was changed every 2–3 days.

Porcine RPE cell culture

Whole eye globes from 2- to 3-month-old Yorkshire pigs (Midwest Swine Research, Gibbon, MN) were placed in 10% povidone-iodine (Purdue Products LP, Stanford, CT) and rinsed 2 times with $1 \times$ phosphate-buffered saline (PBS; Life Technologies). The anterior segment of the globe and retina was removed; RPE was collected and placed in fresh fRPE complete medium. The isolated RPE was then cleaned of adherent choroidal and retinal fragments and transferred to a 50 mL conical tube. Next, RPE was rinsed one time with $1 \times$ MEM (Life Technologies), followed by mechanical disassociation with a razor blade. The disassociated RPE was then incubated with 1 mg/mL collagenase (Cat. No. C69897; Sigma, St. Louis, MO) and 1 mg/mL dispase (Cat. No. 17105-041; Life Technologies) in $1 \times$ MEM for 30 min at 37°C, with gentle agitation every 10 min. Next, the RPE was gently triturated by passage through both 18-gauge and 22-gauge needles to generate a single cell suspension. Three milliliters of fRPE complete medium was immediately added to stop enzymatic activity. Next, RPE was centrifuged at $1.5 \times 1,000$ rpm for 4 min, medium was aspirated, and cells were brought up in 3 mL fresh fRPE medium, and plated on Matrigel- (BD Biosciences) coated 6-well plates. Porcine RPE (pRPE) cells were cultured on 6-well plates for 5–6 days to allow for 80% confluency and pigment restoration. Next, RPE was subjected to the indicated drug treatments and assessed for protein expression through Western blot analysis.

Cell proliferation assay

ARPE19 and iPS-RPE cell proliferation was assessed utilizing the CyQUANT cell proliferation kit (Thermo Scientific, Grand Island, NY). Cells were seeded in 96-well

plates at a density of 1×10^3 /well. Cells were treated with the indicated compounds for 24 h. Following the manufacturer's directions, cells were then treated and lysed in Cy-QUANT GR/cell-lysis buffer. Cell proliferation was quantified in ARPE19 and iPS-RPE cells by measuring sample fluorescence (480 nm excitation).

Wound-healing assay

To model PVR and injury to the RPE monolayer, iPS-RPE cells were grown to confluency on Matrigel- (BD Biosciences) coated plates, to allow for fully pigmented monolayers, ~45 days. After maturation of the monolayer, a scratch/wound was generated with a 20 μ L pipette tip and wound healing was observed over the course of 14 days. Wounded iPS-RPE monolayers were treated with either TGF β 2 (10 ng/mL), TSA (0.1 μ M), or a combination. Over the course of 14 days, cells were replenished with fresh medium and the indicated treatments every other day. Bright field images at the leading edge of the wounded monolayer were acquired on days 1, 3, 7, and 14 postwounding with an Olympus CK2 microscope, using a 20 $\times$ objective.

Immunocytochemistry

iPS-RPE cells were cultured on fibronectin-coated inserts (Nunc, Rochester, NY). The cells were washed with PBS and fixed at room temperature in 4% paraformaldehyde for 10 min. Immunocytochemistry was performed using standard procedures with anti- β -catenin or α -SMA antibodies (ab2365, ab21027; Abcam, Cambridge, MA) and probed with the appropriate Alexa Fluor 568 and 488 secondary antibodies (Life Technologies). Bright field images were acquired with an Olympus CK2 microscope using the 20 $\times$ objective. Immunofluorescence images were acquired with an Olympus BX3 microscope equipped with a DP73 17.28 megapixel digital color camera using a 60 $\times$ oil immersion objective (Olympus Life Science, Center Valley, PA).

Collagen contraction assay

To model PVR and assess for RPE cell contractile function, collagen contraction assays were performed with pRPE cells. Collagen gels were prepared according to the manufacturer's directions (Cell Biolabs, Inc., San Diego, CA). In brief, collagen solution was mixed with 5 $\times$ concentrated DMEM and pRPE cell suspension (5×10^5 /mL). Collagen-cell mixture (0.5 mL) was added to individual wells of a 12-well plate and allowed to polymerize at 37°C for 1 h. After polymerization, 1 mL of complete medium was added to the wells and collagen gels were released from the plate by scoring the perimeter with a 20 μ L pipette tip. After the indicated drug treatments, collagen contraction was assessed at 24 and 48 h postrelease. pRPE cell contraction was measured and represented (in millimeters) as percentage of initial size of 3 independent experiments.

Western blot analysis

For Western blotting, ARPE19 cells were seeded at a density of 2.5×10^5 /well and treated with the indicated compounds. For pRPE cell analysis, cells were cultured until confluence and fully pigmented (~45 days). After indicated treatments, cells were lysed in RIPA buffer with

protease inhibitors (Santa Cruz, Santa Cruz, CA) and protein quantification was performed using the bicinchoninic acid protein assay (Thermo Scientific, Waltham, MA). Protein samples (10 μ g) were resolved through 4%–12% gradient acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (NuPage Novex; Life Technologies). Western blots were blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 and observed using anti- β -catenin, α -SMA, RPE65, microphthalmia-associated transcription factor (MITF; Abcam), acetylated α -tubulin, and α -tubulin (Cell Signaling, Bedford, MA) followed by the appropriate horseradish peroxidase-conjugated secondary antisera and enhanced chemiluminescence detection following the manufacturer's instructions (Bio-Rad, Hercules, CA).

Multiplex enzyme-linked immunosorbent assay

pRPE cells were seeded onto matrigel-coated transwell inserts with a 0.45 pore size (Corning, Lowell, MA) and grown until fully confluent and pigmented, ~14 days. On experiment day 0, cell medium was collected from the apical and basolateral chambers and the wound assay was initiated by generating a wound/scratch to the mature monolayer with a sterile 20 μ L pipette tip. Every 72 h for 18 days, conditioned apical and basolateral media were collected, replenished with fresh medium and the indicated drug treatments. At the conclusion of the experiment on day 18, apical and basolateral media were assessed for the presence of secreted cytokines and growth factors. The PCYTMG-23K-13PX porcine cytokine/chemokine magnetic bead panel (EMD Millipore, Billerica, MA) was used according to manufacturer's instructions. Experiments were conducted in triplicate and data were analyzed with the Bio-Plex Pro software (Bio-Rad).

Statistical analysis

Data were analyzed by 1-way ANOVA, with Bonferroni *post hoc* analysis. All values refer to the means $\pm$ standard error; *P* denotes the significance (**P* < 0.05, ***P* < 0.05, ****P* < 0.001) and NS indicates not significant.

Results

TSA effects in ARPE19 cells

ARPE-19 is a spontaneous immortalized human RPE cell line used extensively to conduct drug/toxicity testing and investigate signaling pathways implicated in various retina pathologies.^{25–28} A recent study utilizing the ARPE-19 cell line suggested a role of acetylation in the EMT and activation of RPE cells.⁵ Protein acetylation is a critical post-transcription modification and plays a fundamental role in regulating various physiological and pathological events.^{23,29–32} As such, we conducted initial experiments with ARPE-19 cells to assess the effect of the broad-spectrum HDAC inhibitor, TSA in this cell line. α -Tubulin, a well-known target protein modified by acetylation, is commonly utilized as a biomarker to assess HDAC activity.^{33–35} Therefore, we evaluated acetylated α -tubulin levels to determine an effective working concentration of TSA. After TSA treatment (0.1 μ M), increased levels of acetylated α -tubulin were observed as early as 3 h and this effect was sustained up to 24 h

FIG. 1. Assessment of TSA treatment in ARPE19 cells. (A) Western blot analysis of acetyl α -tubulin levels in the presence or absence of TSA (0.1 μ M) for the indicated time points. (B) Acetyl α -tubulin levels after TGF β 2 (10 ng/mL) treatment in the presence or absence of TSA (0.1 μ M). (C) Quantification of 3 independent experiments, one-way ANOVA, Bonferroni *post hoc*, * P < 0.05, ** P < 0.01, NS, not significant; TGF β 2, transforming growth factor beta 2; TSA, trichostatin A.

(Fig. 1A). Various concentrations of TSA were assessed and those exceeding 0.1 μ M were observed to be toxic to the cells (data not shown). To determine whether acetylated α -tubulin was modulated by TGF β 2, ARPE-19 cells were treated with TGF β 2 alone or in combination with TSA. Treatment with TGF β 2 (10 ng/mL) was not observed to increase acetylated α -tubulin levels and did not attenuate TSA-mediated increases in protein expression (Fig. 1B, C). Taken together, these data indicate that TSA (0.1 μ M), but not TGF β 2, was able to increase acetylated α -tubulin levels and this effect was sustained up to 24 h post-treatment.

TSA inhibits RPE cell proliferation

Increased RPE cell proliferation, after a retinal tear or detachment, is a critical event that contributes to the pathology of PVR. Therefore, to evaluate the effect of TSA on RPE activation, we conducted cell proliferation experiments with both ARPE19 and iPS-RPE cells. No significant difference in cell proliferation was observed with TGF β 2 (10 ng/mL) treatment as compared with control at this time point (24 h). Conversely, TSA (0.1 μ M) treatment significantly decreased cell proliferation in both ARPE19 and iPS-RPE cells (Fig. 2A, B). These findings demonstrate that the broad-spectrum HDAC inhibitor, TSA, inhibits cell proliferation in both the ARPE-19 cell line and iPS-RPE.

Wound healing in iPS-RPE monolayers

To further characterize the effect of TSA on cellular processes implicated in the pathology of PVR, scratch assays were conducted with iPS-RPE to determine the effect of acetylation on cell migration. These studies were conducted with iPS-RPE, rather than ARPE-19, since they exhibit the typical morphology and function of RPE²⁴ in contrast to an immortalized RPE cell line. After injury, control iPS-RPE cells demonstrated cell migration from the wound edge on day 7 and further migration into the wound by day 14 (Fig. 3). As compared with control, iPS-RPE cells treated with TGF β 2 (10 ng/mL) demonstrated substantial cell migration into the wound space on day 7. Moreover, TGF β 2-treated cells were observed to break away from the

FIG. 2. TSA decreases RPE cell proliferation. Cell proliferation was determined through CyQuant analysis in ARPE19 (A) and iPS-RPE (B) cells. Cells were seeded in 96-well plates at a cell density of 1×10^3 cells/well and treated with TGF β 2 (10 ng/mL) alone or in the presence of TSA (0.1 μ M) for 24 h. The next day, cells were lysed according to the manufacturer's instructions to determine DNA concentrations of all treatment groups. One-way ANOVA, Bonferroni *post hoc*, * P < 0.05, ** P < 0.01, iPS-RPE, induced pluripotent stem cell-derived RPE; RPE, retinal pigmented epithelium.

FIG. 3. TSA effects on wound healing and cell migration in iPS-RPE cells. Scratch assays were conducted on confluent iPS-RPE monolayers. After wounding, cells were treated with TGF β 2 (10 ng/mL), TSA (0.1 μ M), or a combination. Cell migration was observed at the indicated time points. Over the course of the experiment (14 days), cells were replenished with fresh medium and indicated treatments every other day. Figure is representative of 3 independent experiments. *Insets:* Magnification of wound edge.

wound edge and migrate independently into the wound space by day 14. Of relevance, TSA treatment (0.1 μ M) alone did not have an effect on iPS-RPE cell migration; however, TSA treatment was observed to effectively attenuate TGF β 2-mediated increases in cell migration.

EMT protein expression in wounded RPE monolayers

EMT is implicated as a major pathological process implicated in PVR, which involves activation of signaling

pathways and proteins to include β -catenin and α -SMA.^{9,10} Therefore, to determine the expression of EMT-associated proteins in wounded iPS-RPE monolayers, cells were stained with antibodies specific for β -catenin and α -SMA at the end of the wound-healing assay (14 days postscratch). At this time point, no substantial difference in β -catenin expression was observed between control and TGF β 2-treated cells (Fig. 4); however, an increase in α -SMA was observed in cells treated with TGF β 2. Treatment with TSA was observed to alter the localization of β -catenin, but did not have an effect on α -SMA expression as compared with control cells.

FIG. 4. TSA decreases EMT protein expression in iPS-RPE. iPS-RPE cells treated with TGF β 2 (10 ng/mL), TSA (0.1 μ M), or a combination were fixed in 4% paraformaldehyde and stained 14 days postwounding with antibodies specific for β -catenin (1:500) and α -SMA (1:500), followed by incubation with the appropriate Alexa Fluor secondary antibodies. *Insets:* Magnification of cell morphology. α -SMA, α -smooth muscle actin; EMT, epithelial-mesenchymal transition.

FIG. 5. Analysis of pRPE primary cultures. pRPE cells were harvested and homogenized to generate primary cell cultures of confluent, hexagonal, and pigmented monolayers. **(A)** pRPE primary cultures at days 3 and 6 (control, TSA 0.1 μ M; 24 h) postcell harvest. **(B)** Western blot analysis of cell lysates treated in the absence or presence of TSA (0.1 μ M; 24 h) and probed with antibodies targeted against α -SMA, acetyl α -tubulin, RPE65, and MITF. Figure is representative of 3 independent experiments. pRPE, porcine RPE. MITF, microphthalmia-associated transcription factor; RPE65, retinal pigmented epithelium-specific protein 65 kPa.

Interestingly, TSA was observed to block TGF β 2-mediated increases in α -SMA in wounded iPS-RPE monolayers.

Analysis of pRPE primary cultures

Although extensive research has been conducted with the ARPE19 cell line, these cells have lost both key phenotypes and functions of RPE.^{36,37} Therefore, to obtain more physiologically relevant data, primary cultures of pRPE were utilized in this study to determine the effect of acetylation on RPE activation. After cell harvest, pRPE cells were grown to confluency on matrigel-coated plates 5–6 days postharvest, which allowed for pigment restoration. pRPE cells were able to begin forming confluent, hexagonal, and pigmented monolayers by day 6 in culture (Fig. 5A). Subsequently, confluent monolayers of pRPE were treated with TSA (0.1 μ M), and cell lysates were assessed for α -SMA expression and acetyl α -tubulin. TSA treatment was observed to decrease α -SMA expression and increase acetyl α -tubulin (Fig. 5B). Interestingly, TSA treatment resulted in a shift in protein size and generation of a doublet for RPE65. Based on these findings, pRPE cells regain their unique cell morphology in culture and demonstrate significant changes in α -SMA and RPE65 protein expression, after TSA treatment.

TSA attenuates TGF β 2-mediated contraction of RPE cells

In addition to its ability to decrease epithelial cell proliferation,³⁸ TSA has been shown to decrease expression of fibrosis-related genes, to include α -SMA and collagen type I.^{39,40} Increased expression of contractile proteins in various pathologies leads to increased cell contractility and exacerbation of a given condition. After seeding in collagen matrix, baseline cell contraction was observed in control pRPE cells as compared with initial collagen size. By day 2, treatment with TGF β 2 (10 ng/mL) was observed to significantly reduce the collagen matrix size as compared with control (Fig. 6), indicating increased

contractility. Importantly, cotreatment with TSA (0.1 μ M) was observed to attenuate TGF β 2-mediated increased pRPE cell contraction. Taken together, these data demonstrate the role of TGF β 2 in potentiating RPE cell contraction and the contribution of protein acetylation in this process.

FIG. 6. TSA inhibits TGF β 2-mediated contraction of pRPE cells. Primary cultures of pRPE were trypsinized and seeded at a cell density of 2.5×10^5 cells/well in type I collagen (2.3 mg/mL). **(A)** One hour after seeding, pRPE cells embedded in collagen were treated with TGF β 2 (10 ng/mL), TSA (0.1 μ M), or in combination with TGF β 2 and assessed for cell contraction 24 and 48 h. **(B)** Quantification of 3 independent experiments, one-way ANOVA, Bonferroni *post hoc*, *** $P < 0.001$.

pRPE apical and basolateral cell secretion of cytokines

Dependent on cell type and environment, the release of various cytokines and growth factors after injury or trauma

can trigger several cellular processes. RPE cells demonstrate unique cell polarity, which function to regulate the composition of the subretinal space and support photoreceptor function in the neural retina.⁴¹ Therefore, we evaluated both pRPE apical and basolateral cell culture media collected

FIG. 7. pRPE apical and basolateral cell secretion of cytokines and growth factors. Bar graphs represent levels of GM-CSF, IL-6, IL-8, and IL-19 (ng/mL) detected in the apical (A, C, E, G) and basolateral (B, D, F, H) chambers at the indicated time points. Quantification of 3 independent experiments, one-way ANOVA, Bonferroni *post hoc*, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

from a wound assay to determine the expression and secretion of cytokines and growth factors. TGF β 2 (10 ng/mL) treatment did not have an effect on granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-6; however, expression of both cytokines was decreased by TSA treatment in basolateral culture medium (Fig. 7B, D). Of interest, TGF β 2 increased IL-8 secretion in both apical and basolateral media and these effects were attenuated by TSA treatment (Fig. 7E, F). pRPE cells also demonstrated significant increases in IL-18 secretion in apical and basolateral media after wounding and both TGF β 2 and TSA were observed to block this effect (Fig. 7G, H). Collectively, these findings demonstrate the differential effects of TGF β 2 and TSA on cytokine and growth factor secretion at the apical and basolateral pRPE cell surface.

Discussion

The limited treatment options for patients diagnosed with PVR contribute to the poor prognosis of this disorder. After retinal detachment, activation of multiple RPE cellular processes to include proliferation, migration, and contraction can lead to the development and progression of PVR. Recent studies have evaluated specific proteins and signaling pathways that directly contribute to RPE activation.^{3,6,15,42,43} As such, screening pharmacological compounds that selectively attenuate or inhibit these signaling pathways is necessary to identify therapeutics to effectively manage PVR. Utilizing *in vitro* models of PVR, the findings presented herein indicate a role of acetylation in RPE activation. Specifically, the broad-spectrum HDAC inhibitor, TSA, was observed to attenuate TGF β 2-mediated increases in iPS-RPE and pRPE cell migration and contraction, respectively. Acetylation is a critical post-translation modification that directly regulates protein stability and cellular processes such as proliferation and migration. Accordingly, protein acetylation is implicated in various human cancers,^{44,45} and HDAC inhibitors, which modulate protein acetylation, are extensively studied in numerous animal models of cancer. Promising results from these respective animal studies have led to clinical trials with isoform-specific HDAC inhibitors, which function to attenuate cancer cell proliferation and migration.

With respect to RPE activation, a recent study demonstrated TSA treatment-induced G1 phase cell arrest through inhibition of cyclin/CDK/pRb in RPE cells and decreased TGF β 2-mediated expression of contractile proteins to include α -SMA and collagen type I.⁵ In agreement, our data presented herein demonstrate that TSA decreased cell proliferation (Fig. 2). Importantly, we also demonstrate additional effects of TSA treatment on RPE cell processes.

Specifically, TSA inhibited TGF β 2-mediated RPE cell migration (Fig. 3) and contraction of collagen matrices (Fig. 6). Collectively, our findings demonstrate a role of TSA in the activation of multiple cellular processes, in addition to cell proliferation, which are suggested to contribute to the pathogenesis of PVR. After injury to the RPE cell layer, increased expression of contractile proteins leads to the development of a myofibroblast-like phenotype. These alterations in protein expression may result in increased vitreoretinal traction of RPE, subsequently leading to secondary retinal detachment. Importantly, our findings were generated from iPS-RPE and pRPE cells as opposed to ARPE-19, an immortalized cell line that has lost fundamental phenotypes and functions of RPE.^{36,37} In contrast to iPS-RPE and pRPE, ARPE-19 cells exhibit a dedifferentiated, myofibroblast-like phenotype (Fig. 8) with increased baseline cell migration and of contractile protein expression (data not shown). As such, the iPS-RPE and pRPE *in vitro* models of PVR utilized in this study provide a more physiologically relevant representation of cellular processes activated after injury and/or trauma to RPE.

Immediately after tissue injury, secretion of cytokines and/or growth factors triggers the activation of multiple signaling pathways. Although cytokines and growth factors elicit beneficial effects with regard to wound healing, aberrant expression can lead to pathophysiological consequences. Utilizing a wound-healing assay, we screened a panel of 13 cytokines and growth factors, which revealed significant differences in expression of GM-CSF, IL-6, IL-8, and IL-18 (Fig. 7). Of interest, TSA abolished TGF β 2-mediated IL-8 secretion (Fig. 7E, F) as well as inhibited IL-18 secretion compared with control cells (Fig. 7G, H). Decreased secretion of IL-8 and IL-18, cytokines involved in mediating cell proliferation and migration,^{46–48} complement our findings, demonstrating the negative effect of TSA on these processes (Figs. 2 and 3). Protein acetylation is a reversal modification that regulates gene transcription, but is also implicated in regulating protein stability and ubiquitin-mediated degradation.^{30,49,50} Based on this evidence, TSA-mediated increases in acetylation may function to stabilize and/or degrade proteins essential to RPE activation. Further studies are necessary to determine the molecular targets of TSA and characterize their role in RPE activation. Identification of the specific proteins and/or growth factors involved in the progression and development of PVR which may facilitate a greater understanding of signaling pathways involved in PVR.

In conclusion, the broad-spectrum HDAC inhibitor, TSA, blocked TGF β 2-mediated effects on RPE cell migration and contraction in an *in vitro* model of PVR. After wounding of the cell monolayer, TSA was able to inhibit the expression

FIG. 8. Confluent ARPE19, iPS-RPE, and pRPE cell culture monolayers. Phase contrast images of confluent, pigmented monolayers of ARPE19, pRPE, and iPS-RPE (40 $\times$ magnification).

of α -SMA and secretion of critical cytokines and growth factors. Specifically, TSA was observed to attenuate TGF β 2-mediated increases in IL-8 and IL-18, critical regulators of cell proliferation and migration. Collectively, these findings provide novel insight into the role of acetylation in regulating cellular processes involved in the pathogenesis of PVR as well as the potential use of HDAC inhibitors as treatment therapeutics for this disorder. Future studies to evaluate isoform-specific HDAC inhibitors are necessary to identify specific compounds, to reduce off-target effects, for the treatment/management of individuals afflicted with PVR.

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Author Disclosure Statement

No competing financial interests exist.

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