Inhibition of DNA Methylation and Methyl-CpG-Binding Protein 2 Suppresses RPE Transdifferentiation: Relevance to Proliferative Vitreoretinopathy

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PURPOSE. The purpose of this study was to evaluate expression of methyl-CpG-binding protein 2 (MeCP2) in epiretinal membranes from patients with proliferative vitreoretinopathy (PVR) and to investigate effects of inhibition of MeCP2 and DNA methylation on transforming growth factor (TGF)- β -induced retinal pigment epithelial (RPE) cell transdifferentiation.

METHODS. Expression of MeCP2 and its colocalization with cytokeratin and α -smooth muscle actin (α -SMA) in surgically excised PVR membranes was studied using immunohistochemistry. The effects of 5-AZA-2'-deoxycytidine (5-AZA-dC) on human RPE cell migration and viability were evaluated using a modified Boyden chamber assay and the colorimetric 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Expression of RASAL1 mRNA and its promoter region methylation were evaluated by real-time PCR and methylation-specific PCR. Effects of 5-AZA-dC on expression of α -SMA, fibronectin (FN), and TGF- β receptor 2 (TGF- β R2) and Smad2/3 phosphorylation were analyzed by Western blotting. Effect of short interfering RNA (siRNA) knock-down of MeCP2 on expression of α -SMA and FN induced by TGF- β was determined.

RESULTS. MeCP2 was abundantly expressed in cells within PVR membranes where it was double labeled with cells positive for cytokeratin and α -SMA. 5-AZA-dC inhibited expression of MeCP2 and suppressed RASAL1 gene methylation while increasing expression of the RASAL1 gene. Treatment with 5-AZA-dC significantly suppressed the expression of α -SMA, FN, TGF- β R2 and phosphorylation of Smad2/3 and inhibited RPE cell migration. TGF- β induced expression of α -SMA, and FN was suppressed by knock-down of MeCP2.

CONCLUSIONS. MeCP2 and DNA methylation regulate RPE transdifferentiation and may be involved in the pathogenesis of PVR.

Keywords: DNA methylation, proliferative vitreoretinopathy, retinal pigment epithelium, transforming growth factor-beta

Proliferative vitreoretinopathy (PVR) is characterized by formation of epiretinal and/or subretinal membranes, and a major cell type present in these membranes is retinal pigment epithelium (RPE).^{1–6} In PVR, the RPE contributes to the wound healing response by undergoing epithelium-mesenchyme transition (EMT) with further transdifferentiation into myofibroblasts.^{1–6} Although inflammatory factors,^{7–9} cytokines,^{10,11} growth factors,^{12–16} and extracellular matrix^{17–19} have been extensively studied for their roles in the development of PVR,^{20–28} the detailed molecular mechanisms controlling the process of RPE transdifferentiation remain unclear.

Alterations in DNA methylation status are associated with many biological processes, including wound healing and fibrosis,^{29–32} DNA repair,^{33,34} cell cycle regulation,^{35–36} inflammatory/stress response,^{37,38} apoptosis, and tumorigenesis.³⁹ DNA methylation at the 5 position of cytosine within CpG

dinucleotides epigenetically controls gene expression and maintains genome integrity. Methyl-CpG-binding protein 2 (MeCP2) is the prototypic methyl-CpG-binding protein.^{30–32} Methyl-CpG-binding proteins bind to methylated DNA through a conserved methyl-CpG-binding domain where they typically repress gene expression.^{30–32} 5-aza-2'-deoxycytidine (5-AZA-dC) is a potent inhibitor of DNA methylation that has been used to study the relevance of DNA methylation to EMT.^{29,40,41} Previous studies have demonstrated that MeCP2,⁴⁰ a reader of DNA methylation, is highly expressed in hepatic fibrosis, although treatment of hepatic stellate cells with 5-AZA-dC inhibits MeCP2, modulates MeCP2-regulated genes, and inhibits EMT in these cells.^{29,41,42}

One of the characteristics of RPE transdifferentiation is increased expression of α -smooth muscle actin (α -SMA). α -SMA-positive RPE cells have been shown to be the major cells

that mediate contraction and induce retinal detachment in PVR.^{20–22,26} Previous studies have shown that α -SMA expression in other cell types is regulated by DNA methylation; interestingly, the regulation of α -SMA by MeCP2 is mediated through the Ras GTPase activating-like protein (RASAL1).⁴²

Previously, we showed that transforming growth factor (TGF)- β is a major inducer of RPE EMT and α -SMA expression.²² Because DNA methylation is thought to be an important epigenetic regulator of the fibrotic process, we hypothesized that it might be playing an important role in the pathogenesis of PVR. Therefore, we evaluated expression of MeCP2 in human PVR membranes and determined whether inhibiting DNA methylation (by 5-AZA-dC) or MeCP2 regulated the transdifferentiation induced by TGF- β in RPE cells.

MATERIALS AND METHODS

The institutional review board of the University of Southern California approved our use of cultured human RPE cells and de-identified human PVR specimens. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from all participants.

RPE Cultures

Human fetal RPE cells were isolated from fetal human eyes of >22 weeks gestation (Advanced Bioscience Resources, Inc., Alameda, CA, USA).⁴³ The culture method used in our laboratory regularly yields >95% RPE (cytokeratin-positive) cells. Cells used were from passages 2 to 4. Transdifferentiation of RPE cell was induced using recombinant TGF- β 2 at a concentration of 10 ng/mL for 72 hours. Dose range and time of treatment of RPE with TGF- β were previously established by our laboratory.²² DNA demethylation was achieved using 5-AZA-dC (Sigma-Aldrich Corp., St. Louis, MO, USA) in concentrations ranging from 0.1 to 6 μ M for 72 hours. The 5-AZA-dC dose range associated with this time course has been previously shown to be able to induce DNA hypomethylation in human cells.⁴⁴ 5-AZA-dC-induced cell death was evaluated by trypan-blue exclusion and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays. Expression levels of ZO-1 and cytokeratin in RPE cell were determined using immunocytochemistry.

Retinal pigment epithelium-polarized monolayer culture was grown as previously described.⁴⁵ Retinal pigment epithelium cells were plated on a permeable membrane insert (12-mm diameter, 0.4-mm pore size; Transwell; Costar, Cambridge, MA, USA), and cells were cultured for at least 1 month to form differentiated monolayers with a stable transepithelial resistance (TER) of >350 ohms/cm². Transepithelial resistance was measured using an EVOM volt-ohm meter (World Precision Instruments, Sarasota, FL, USA). Retinal pigment epithelium cultures were treated with 5-AZA-dC (at 1, 2, and 6 μ M) for 4 days, and then images of the cultured cells were obtained by phase contrast microscopy (SPOT Imaging Solutions, Sterling Heights, MI, USA). Some inserts were embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (H&E). Apoptosis in the monolayer was analyzed with TUNEL assay.

Immunocytochemistry and Immunofluorescence Analysis

Ten surgically excised membranes from patients (45–70 years of age; 7 males, 3 females) with PVR were prepared for immunostaining. The snap-frozen sections were fixed in

acetone and blocked with 5% normal goat serum. Reagents were added to sections in the following sequence: anti-MeCP2 (1:100 dilution; Abcam, Inc., Cambridge, MA, USA), biotinylated secondary anti-rabbit antibody (1:400 dilution; Vector, Burlingame, CA, USA), and peroxidase-conjugated streptavidin. Red stain was developed with an amino ethyl carbazole kit (Zymed, South San Francisco, CA, USA). Intensity of the MeCP2-positive staining on each slide was scored as zero (unlabeled), 1+ (slightly labeled, <10% of cells), 2+ (moderately labeled, 10%–40% of cells), or 3+ (strongly labeled, more than 40% of cells).⁴⁶

Following treatment of RPE cells by 5-AZA-dC in confluent cultured chamber slides, the slides were stained with the following antibodies after 4% paraformaldehyde fixation: anti- α -SMA (1:100 dilution; clone 1A4; Sigma-Aldrich Corp.) or anti-FN (Chemicon International, Inc., Temecula, CA, USA) or anti-MeCP2 (Abcam), and signal was visualized as described above.

For immunofluorescent staining, PVR membranes were incubated with anti-MeCP2 (1:50 dilution; Abcam), anti- α -SMA (monoclonal mouse anti- α -SMA, 1:100 dilution, clone 1A4; Sigma-Aldrich Corp.) or anti-cytokeratin for 1 hour, and then the staining was visualized by fluorescein isothiocyanate (FITC) or rhodamine-labeled secondary antibody. RPE cells in the chamber slides were stained with anti-ZO-1 (1:50 dilution; rabbit anti-ZO-1; Life Technologies, Camarillo, CA, USA) or pan-cytokeratin antibodies (1:100 dilution; Dako, Carpinteria, CA, USA). FITC-labeled secondary anti-rabbit- or rhodamine-labeled anti-mouse antibodies were used to detect expression of ZO-1 and cytokeratin respectively. Slides were examined using microscopy (model LSM510 confocal microscope; Zeiss, Thornwood, NY, USA).

MTT Assay

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability in 96-well plates after RPE cells was transfected with MeCP2 or scrambled short interfering (si)RNA. MTT assay procedure was performed as previously detailed.⁴⁷ Absorbance at 550 nm was determined by a multiwell plate reader (Benchmark Plus; Bio-Rad, Tokyo, Japan).

Migration Assay

Migration was measured using a modified Boyden chamber assay as previously described.⁴⁸ Briefly, 5×10^4 RPE cells were seeded in the upper part of a Boyden chamber in 24-well plates after the cell treated with 5-AZA-dC (0.1, 1, 2, and 6 μ M) for 72 hours. The lower chamber was filled with a 0.4% solution of fetal bovine serum–Dulbecco's modified Eagle's medium containing 30 ng/mL recombinant hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN, USA). After a 5-hour incubation, the number of migrated cells was counted by phase-contrast microscopy. Five randomly chosen fields were counted per insert.

Real-Time PCR

Complementary (c)DNA was synthesized from 1 μ g total RNA, using avian myeloblastosis virus reverse transcriptase (QIAGEN, Germantown, MD, USA), according to the manufacturer's protocol. cDNA was subjected to quantitative PCR using a real-time PCR system (Life Technologies), using a PCR kit (QuantiTect SYBR Green; QIAGEN) for detection of RASAL1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH forward primer sequence was 5'-GAGTCAACG GATTTGGTTCGT-3' and reverse sequence was 5'-CTTGATTTTGGAGGGATCTCGC-3'; RASAL1 forward sequence

was 5'-CAGCTCCCTGAATGTTCGC-3' and reverse sequence was 5'-TCCTCATCCAGCACGTAGAAG-3'. Polymerase chain reaction running conditions were denaturing at 95°C for 10 seconds, followed by 40 cycles of denaturing at 95°C for 5 seconds, and annealing and extending at 60°C for 20 seconds. Relative change in mRNA expression was calculated by using cycle threshold ($\Delta\Delta CT$) values. Levels of mRNA were normalized relative to levels of GAPDH mRNA and reported as fold change over controls.

siRNA Transfection

MeCP2 siRNA transfection was conducted as previously described.⁴⁹ MeCP2 siRNA and scrambled siRNA were from Santa Cruz Biotechnology (Dallas, TX, USA; siRNA: MeCP2 siRNA [h2]: sc-156056 and Scrambled siRNA: sc-37007) or Sigma-Aldrich Corp. (siRNA MeCP2, SASI_Hs01_00116141 and nonsilencing control scrambled RNA Mission-SIC-001-s). Transfection was performed with 10 nM MeCP2 siRNA or scrambled siRNA, using HiPerFect Transfection Reagent (QIAGEN). Transfection efficiency was analyzed by Western blot analysis. The effects of silencing MeCP2 on expression of FN and α -SMA were determined by Western blot analysis, and viability was evaluated by MTT assay.

TUNEL Assay

Apoptotic cells were quantified by TUNEL assay (In Situ Cell Death Detection kit fluorescein; Roche Diagnostics, Indianapolis, IN, USA). The assay was performed according to the manufacturer's protocol. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and apoptotic cells were visualized in red. After cells were labeled, the staining was evaluated using fluorescence microscopy (EVOS model; Life Technologies). TUNEL-positive cells were counted under a microscope as a percentage of total cells counted.

Flow Cytometry

Retinal pigment epithelium cells in suspension were stained with FITC-conjugated mouse monoclonal antibody for α -SMA (1:200 dilution; clone 1A4 FITC-conjugated; Sigma-Aldrich Corp.) for 30 minutes in the dark and then washed twice with phosphate-buffered saline, fixed in 2% paraformaldehyde, and finally resuspended in 0.5 mL phosphate-buffered saline. Fluorescein isothiocyanate-conjugated irrelevant immunoglobulin G was applied as negative control. Samples were analyzed using a FACStar plus flow cytometer (Becton-Dickinson, Mountain View, CA, USA) with 488-nm excitation and 530-nm band pass filter for FITC. For all analyses, at least 5000 cells were assessed in each sample.

Western Blotting

Cells were lysed, and proteins were resolved on Tris-HCl 4% to 12% polyacrylamide gels (Ready Gel; Bio-Rad) at 120 V. Proteins were transferred to polyvinylidene fluoride blotting membrane (Millipore, Bedford, MA, USA), and membranes were probed with monoclonal antibody specific for α -SMA (1:1000 dilution; Sigma-Aldrich Corp.) or FN (1:500 dilution; Sigma-Aldrich Corp.) or anti-TFG- β R2 (Santa Cruz Biotechnology) or antiphosphorylated Smad2/3 (Santa Cruz Biotechnology), or MeCP2 (1:500 dilution; Abcam). Membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Vector Laboratories). Images were developed by addition of chemiluminescence detection solution (ECL; Amersham Pharmacia Biotech, Cleveland, OH, USA). After membranes were stripped, they were reprobed with anti-GAPDH antibody (Millipore) for protein loading control. For

FIGURE 1. MeCP2 expression in human PVR membranes. (A) Negative control without primary antibody. (B, C) Representative PVR membranes, showing immunohistochemical staining for MeCP2 (red chromogen) and blue nuclear counter stain. (B) Abundant MeCP2 expression was seen within cellular regions of a human PVR membrane. (C) Black arrows indicate MeCP2 staining in nuclei, and white arrows show cytoplasmic MeCP2 immunoreactivity.

quantification of Western blot bands, we scanned the bands from three independent experiments, using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA); band quantification in each experiment was obtained by determining the mean pixel density of the band (after background subtraction) and multiplying it by the band area.

MethyLight DNA Methylation Analysis

Genomic DNA was isolated from the RPE cells using a DNA isolation kit (MethyLight; QIAGEN). MethyLight PCR assays

FIGURE 2. MeCP2 double-labeling with cytokeratin and α -SMA in human PVR membrane. Localization of MeCP2 (red) and cytokeratin (green, left panel) and α -SMA (green, right panel) is shown in human PVR membranes. Yellow shows colocalization of MeCP2 with cytokeratin (lower left panel) or α -SMA (lower right panel).

were performed as previously described.^{50,51} The MethyLight RASAL1 forward primer used was 5'-TTA GAA GCG TTC GAG GAG TAT TTA TAC-3'; the reverse primer was 5'-AAA AAC AAC TCC CTA AAT ATT CGC-3'; and the probe primer was 5'-6FAM-TAA TAA AAA ACC GCG CGC TAC CTA CCA AA-BHQ-1-3', in which 6-carboxyfluorescein (6-FAM) is the fluorophore and BHQ-1 is a black hole quencher. The MethyLight RASAL1 reaction was obtained from BioSearch Technologies (Petaluma, CA, USA). MethyLight data are reported as the ratio between the relative values from real-time PCR standard curve for the methylation (KCNA2) and control (ALU) reactions. Specifically, data were reported as percentages of methylated reference (PMR), calculated as $(\{[RASAL1/ALU] \text{ sample} / [RASAL1/ALU] \text{ M.SssI}\} \times 100)$.

Statistics

All experiments were performed at least three times. Data were all normally distributed and analyzed using the Student's *t*-test, with multiple pairwise comparisons between groups adjusted by the Bonferroni correction.

RESULTS

MeCP2 Expression in PVR Membranes

All human PVR membranes displayed prominent MeCP2 immunoreactivity. MeCP2 was confined to the cellular regions of the membranes and was localized in both the nuclear and cytoplasmic compartments (Figs. 1B, 1C).⁵² MeCP2 staining was scored in PVR membranes as described in Materials and Methods, and we found the following distribution of cases: + (1 case), ++ (4 cases), and +++ (5 cases). No significant background staining was observed in the control ("no primary antibody") (Fig. 1A). Double-staining of human PVR membranes showed that MeCP2 immunoreactivity was colocalized with cytokeratin or α -SMA-positive cells (Fig. 2), indicating that many of the MeCP2⁺ cells were derived from RPE and were transdifferentiated.

Effects of 5-AZA-dC on α -SMA and FN Expression

High expression of MeCP2 in the PVR membranes suggested that DNA methylation might be playing a role in disease

FIGURE 3. Effects of 5-AZA-dC on expression levels of α -SMA- and FN-induced TGF- β 2. **(A)** α -SMA expression was detected by immunocytochemistry. Red = positive staining for α -SMA; blue = hematoxylin counter-staining of nuclei. Transforming growth factor- β treatment increased α -SMA expression compared to that of control. Pretreatment with 5-AZA-dC for 24 hours followed by Transforming growth factor- β plus 5-AZA-dC for 3 additional days caused a significant reduction of α -SMA expression, especially at a concentration of 2 μ M 5-AZA-dC or greater. **(B)** Immunocytochemical analysis of effects of 5-AZA-dC on TGF- β -induced fibronectin (FN) expression in cultured RPE cells. Red = positive staining for FN; blue = hematoxylin staining of nuclei. Fibronectin immunoreactivity was enhanced by stimulation with TGF- β . Pretreatment with 5-AZA-dC for 24 hours followed by TGF- β plus 5-AZA-dC for 3 additional days resulted in the inhibition of FN expression in a dose-dependent manner. At a 5-AZA-dC concentration of 1 μ M and greater, FN expression was much lower than that in controls. **(C)** Flow cytometry analysis of the effects of 5-AZA-dC on TGF- β -induced α -SMA expression in RPE cells. Retinal pigment epithelium cells were cultured in 6-well plates and pretreated with 5-AZA-dC for 24 hours and then with TGF- β alone or in combination with 5-AZA-dC for 3 days. α -SMA expression was analyzed using flow cytometry. The expression of α -SMA induced by TGF- β was significantly inhibited with 5-AZA-dC at a concentration of 1 μ M or greater. Mean positive cell number is shown with standard deviation. (*t*-test, **P* < 0.05; ***P* < 0.01; Bonferroni correction, *P* < 0.01). **(D)** Effects of 5-AZA-dC on TGF- β -induced α -SMA expression analyzed by Western blotting. Retinal pigment epithelium cells were pretreated with 5-AZA-dC (0.1–6 μ M) for 24 hours, followed by pretreatment with a combination of TGF- β and 5-AZA-dC for 3 days. Total protein was extracted for Western blot analysis using anti- α -SMA. GAPDH was used as protein loading control. Upregulation of α -SMA expression by TGF- β was significantly inhibited by addition of 5-AZA-dC. Densitometry results from three independent blots show inhibition of α -SMA expressions by 5-AZA-dC at a concentration of 1 μ M or greater is significant (*t*-test; **P* < 0.025; Bonferroni correction, *P* < 0.005). **(E)** Effects of 5-AZA-dC on TGF- β -induced FN expression by Western blot analysis. Retinal pigment epithelium cells were pretreated with 5-AZA-dC (0.1–6 μ M) for 24 hours, followed by a combination of TGF- β (10 ng/mL) and 5-AZA-dC for 3 days. Total protein was blotted using an anti-FN antibody. GAPDH was used for protein loading control. Transforming growth factor- β -induced FN expression was significantly inhibited by addition of 5-AZA-dC. Densitometry results from three independent blots shows inhibition of FN expression by 5-AZA-dC at a concentration of 1 μ M or greater is significant (*t*-test, **P* < 0.035; Bonferroni correction, *P* < 0.005).

FIGURE 4. Effects of 5-AZA-dC on RASAL1 gene methylation (A) and expression of RASAL1 mRNA. (B) Retinal pigment epithelium cells were treated with 5-AZA-dC (2 μ M) for 72 hours, and total RNA and genomic DNA were isolated for analysis of expression of RASAL1 mRNA and RASAL1 gene methylation by real-time PCR and MethyLight PCR, respectively. RASAL1 methylation was reduced significantly by addition of 5-AZA-dC ([A] *t*-test, **P* < 0.025). RASAL1 mRNA expression is almost 4-fold increased by treatment with 5-AZA-dC ([B] *t*-test, **P* < 0.007).

pathogenesis, thus, we were interested in seeing whether inhibition of DNA methylation was able to suppress expression of the EMT markers in RPE. Results of immunocytochemistry (Fig. 3A), flow cytometry (Fig. 3C), and Western blotting (Fig. 3D) analyses showed that TGF- β 2-induced expression of α -SMA in human RPE was inhibited significantly by 5-AZA-dC treatment (Figs. 3C, 3D; *P* < 0.025). 5-AZA-dC also caused a significant dose-dependent inhibition of FN expression (*P* < 0.035) (Figs. 3B, 3E).

Effects of 5-AZA-dC on Methylation and Expression of RASAL1

Because MeCP2 is a global reader of methylation, we evaluated effects of 5-AZA-dC on promoter methylation of key genes involved in EMT/transdifferentiation including SMA, RASAL1 (a gene known to regulate expression of SMA), peroxisome proliferator-activated receptor-gamma (PPAR- γ), and protein patched homolog 1 (PTCH1). Using methylation-specific PCR, we were unable to detect significant levels of regulated promoter methylation for SMA, PPAR- γ , or PTCH1, either in the presence or absence of 5-AZA-dC and TGF- β (results not shown). In contrast, methylation of CpG dinucleotides was present in the RASAL1 promoter and was reduced by more than half with treatment of 5-AZA-dC (2 μ M; *P* < 0.05) (Fig. 4A). Consistent with this effect, the expression of RASAL1

mRNA was 4-fold upregulated with 5-AZA-dC (2 μ M; *P* < 0.007) (Fig. 4B).

Effects of 5-AZA-dC on TGF- β R2 and TGF- β -Induced Smad2/3 Activation

Because TGF- β is a major mediator of RPE EMT, we evaluated the effect of 5-AZA-dC on TGF- β signaling pathways. Western blot analysis revealed that 5-AZA-dC significantly inhibited TGF- β R2 expression on human RPE compared to that in control cells (*P* < 0.025) (Figs. 5A, 5C). Upregulation of Smad2/3 phosphorylation induced by TGF- β was also significantly inhibited by 5-AZA-dC pretreatment at 2 μ M or above (*P* < 0.05) (Figs. 5B, 5D).

Effects of 5-AZA-dC on RPE Cell Migration

Cells that underwent EMT demonstrated a migratory phenotype. Because HGF expression was increased in PVR membranes¹⁴ and induced RPE proliferation and migration,¹⁵ we studied the effect of 5-AZA-dC on HGF-induced migration in a Boyden chamber assay. In the current study, HGF-induced migration was markedly reduced by 5-AZA-dC compared with RPE cells without 5-AZA-dC pretreatment (*P* < 0.025) (Fig. 6).

FIGURE 5. Effects of 5-AZA-dC on expression of TGF- β 2 receptor (A, C) and TGF- β -induced Smad-2/3 activation (B, D). Retinal pigment epithelium cells were pretreated with 5-AZA-dC for 3 days (A, B) and then stimulated with 10 ng/mL TGF- β for 20 minutes (B, D). Cell lysates were analyzed by Western blotting with anti-TGF- β 2 receptor (A) or antiphosphorylated Smad2 antibody (B) or anti-GAPDH antibody. Expression of TGF- β 2 receptor was inhibited by 5-AZA-dC treatment (A, C). Phosphorylation of Smad-2/3 was suppressed by 5-AZA-dC pretreatment at a concentration of 2 μ M and greater (B, D). Densitometry from three independent blots shows inhibition of the TGF- β 2 receptor ([C] *t*-test, **P* < 0.025) or Smad-2/3 activation ([D] *t*-test, **P* < 0.05).

FIGURE 6. Effects of 5-AZA-dC on HGF-induced RPE cell migration. Retinal pigment epithelium cells were treated for 3 days with 5-AZA-dC (0.1–6 μM). Migration was measured using a modified Boyden chamber assay. The migration induced by HGF was significantly inhibited at 5-AZA-dC concentrations of 1 μM or greater (*t*-test, **P* < 0.025; Bonferroni correction, *P* < 0.016).

Effects of 5-AZA-dC on Expression of MeCP2

Because 5-AZA-dC inhibits DNA methylation and MeCP2 binds methylated DNA, we evaluated the effect of 5-AZA-dC treatment on MeCP2 expression. Treatment of RPE with 5-AZA-dC (1–6 μM) resulted in significantly reduced expression of MeCP2 protein (*P* < 0.05) (Fig. 7).

Potential Toxicity of 5-AZA-dC on Cultured Human RPE

Parallel experiments were performed to determine potential toxicity of 5-AZA-dC on cultured human RPE as a control for each of the experiments described above. Toxicity was measured using TUNEL and trypan blue exclusion assays. In the above-described experiments, all significant effects on EMT-related assays were observed at either 1 and 2 or 2 μM 5-AZA-dC (Figs. 3–7). At concentrations of 1 μM and 2 μM, 5-AZA-dC caused no significant toxicity when evaluated by these assays (Supplementary Fig. S1). At 6 μM 5-AZA-dC there was a significant increase in cell death, but it was limited in extent to <5% of cells, with TUNEL assay, and 11% of cells with trypan blue exclusion (Supplementary Figs. S1A, S1C). Addition of TGF-β to 5-AZA-dC did not affect the amount of cell death (Supplementary Fig. S1D). Interestingly, addition of HGF, as used in the cell migration experiments, to the toxicity assays resulted in significantly less cell death for 6 μM 5-AZA-dC plus HGF compared to than for 6 μM 5-AZA-dC alone (Supplementary Fig. S1E).

Effects of Knock-Down of MeCP2 on Expression of α-SMA and Fibronectin

We then sought to determine whether MeCP2 independently inhibited RPE transdifferentiation. We found that knock-down of MeCP2 by its specific siRNA inhibited TGFβ-induced α-SMA (Figs. 8A, 8C) and FN (Figs. 8B, 8D) expression. A 4-fold reduction of TGFβ-induced expression of α-SMA by MeCP2 knockdown was seen when compared with cells transfected with scrambled siRNA (*P* < 0.05). Interestingly, FN expression was inhibited by MeCP2 knockdown in both non-TGFβ-treated and TGFβ-treated cells (*P* < 0.05) (Figs. 8B, 8D). There was no significant cell death observed by treatment of the cells with MeCP2-specific siRNA or scrambled siRNA under these conditions compared to control cells, as measured by MTT assay (Supplementary Fig. S2).

FIGURE 7. Effects of 5-AZA-dC on MeCP2 expression. Expression of MeCP2 was reduced by treatment with 5-AZA-dC for 72 hours (A). MeCP2 protein reduction was seen starting at 1 μM 5-AZA-dC. (B) Densitometry results from three independent blots shows maximal inhibition MeCP2 at 6 μM 5-AZA-dC exposure (*t*-test, **P* < 0.05; Bonferroni correction, *P* < 0.0125).

Effects of 5-AZA-dC on Differentiated RPE Phenotype

Because 5-AZA-dC is currently in use in clinical trials^{53,54} and our results indicate that 5-AZA-dC inhibits processes related to transdifferentiation, we wanted to know if there was any effect of 5-AZA-dC on the differentiated RPE monolayer. In highly polarized RPE monolayer cultures, the morphology of the monolayer remained the same as control after 5-AZA-dC treatment (Figs. 9A–C) and there was no drop in transepithelial resistance over 4 days of 5-AZA-dC treatment. In addition, polarized RPE cultures were highly resistant to 5-AZA-dC toxicity as measured by TUNEL stain; no TUNEL⁺ cells were seen at 1 or 2 μM 5-AZA-dC, and at 6 μM 5-AZA-dC, only rarely were TUNEL⁺ cells seen (Fig. 9D). No detectable changes in immunofluorescent expression of ZO-1 and cytokeratin were seen in polarized RPE monolayer cultures by the treatment of 5-AZA-dC for 4 days (Figs. 9E, 9F).

DISCUSSION

The overall purpose of this study was to evaluate the role of DNA methylation in processes related to RPE mesenchymal transdifferentiation and to determine its relevance to PVR. We first determined that expression of MeCP2, a DNA methylation reader, was high in the cellular regions of PVR membranes. Because these membranes contain various retinal and inflammatory cell types, we determined that many of the MeCP2⁺ cells were RPE in origin as determined by their immunoreactivity to cytokeratin; as well many of these cells were transdifferentiated as shown by their immunoreactivity to α-SMA. Thus, this set the stage to look at role of DNA methylation in the EMT process in RPE.

High expression of MeCP2 in PVR membranes suggested that DNA methylation may be playing a role in disease pathogenesis; thus, we were interested to see whether inhibition of DNA methylation was able to suppress the

FIGURE 8. Knock-down of MeCP2 inhibits expression of TGF- β -induced α -SMA (A) and FN (B) as demonstrated by Western blot analysis. Retinal pigment epithelium cells were transfected with MeCP2 siRNA or scrambled siRNA for 48 hours with or without TGF- β . GAPDH was used as protein loading control. Transforming growth factor- β -induced increase of α -SMA and FN expression levels was significantly inhibited by MeCP2 knock-down. Densitometry from three independent blots shows the inhibition of α -SMA (C, *t*-test, **P* < 0.045) and FN (D) expression are significant (*t*-test, **P* < 0.025).

expression of the EMT markers in RPE. We used the DNA methylation inhibitor 5-AZA-dC and evaluated its effects on genes and processes involved in RPE EMT, and further differentiation into motile α -SMA⁺ myofibroblastic cells. 5-AZA classically inhibits promoter methylation leading to derepression of the promoter and increased gene expression. To determine whether this was true in RPE, we evaluated promoter methylation of RASL1, a gene of the RAS-GAP family that suppresses α -SMA expression and is known to be decreased in fibrotic tissue.⁴² We found that the RASL1 promoter CpG island methylation was decreased with 5-AZA-dC treatment and associated with increased levels of the gene expression. The result suggests that RASL1 expression is under the regulation of DNA methylation in RPE.

Because DNA methylation is involved in the pathogenesis of EMT,^{29–32} we wanted to determine whether the methylation inhibitor was able alter some EMT phenotypes. The nucleotide analog 5-AZA-dC is used extensively for epigenetic therapy of neoplastic disease.^{53,54} 5-AZA-dC has a broad effect on gene expression and cell functions such as induction of apoptosis and growth arrest, as well as inhibition of angiogenesis,^{53–55} many of which are related to DNA methylation mechanisms.⁵⁶ We demonstrated here that inhibition of DNA methylation inhibits RPE migration, which is a typical cell function associated with RPE transdifferentiation.

Global DNA demethylation by 5-AZA-dC activates or represses numerous genes. Here, we were interested to see whether the methylation inhibitor could alter TGF- β signaling through its receptor and downstream Smad2/3 activation, which would suppress the cellular effects of TGF- β . Indeed, our results demonstrated that 5-AZA-dC inhibits the expression of TGF- β receptor 2 and TGF- β induced phosphorylation of Smad-2/3. We then showed that downstream TGF- β effects such as expression of α -SMA and FN were also repressed by 5-AZA-dC treatment. These results imply that RPE cell transdifferentiation, especially α -SMA and FN expression, may be subject to epigenetic regulation. Blocking α -SMA and FN expression by a DNA methylation inhibitor in RPE cells may

represent a new regulatory mechanism in the therapy of PVR.^{20–22} The ultimate mechanisms by which TGF- β induced α -SMA and FN expression are repressed are likely to be complex and indirect. For example, although 5-AZA-dC treatment led to prominent inhibition of α -SMA, evaluation of α -SMA promoter showed undetectable levels of promoter methylation, thus, indicating that 5-AZA-dC was mediating this effect indirectly, possibly through demethylation of RASL1 and/or derepression of other genes that repress TGF- β R expression.

We hypothesized that one way that 5-AZA-dC may be acting indirectly is through regulation of MeCP2 expression. More recently MeCP2 has been found to bind not only methylated CpG islands related to gene silencing but also to sites of active gene transcription.^{57,58} Because MeCP2 expression is high in PVR and is reduced in RPE after 5-AZA-dC treatment, we determined the effects of silencing MeCP2 on the expression of α -SMA and FN by using siRNA. Indeed, we found that down-regulating MeCP2 expression caused a significant suppression of the expression of α -SMA and FN induced by TGF- β . Again, it is likely that these effects are complex. Hu et al.⁵⁷ indicated that MeCP2 binds to α -SMA promoter and increased the expression of α -SMA. As such, MeCP2 may act as a transcriptional repressor or as a gene activator depending on its specific modification and the microenvironment.^{57,59,60} These observations support the suggestion that MeCP2 is an important factor that promotes the development of fibrosis in general,^{41,61–64} and that the pathogenesis of PVR may be under complex epigenetic regulation.

Additional cellular components and growth factors play important roles in the pathogenesis of PVR. Indeed, most myofibroblasts in PVR membranes are derived from RPE and retinal glial cells.⁶⁵ Results of this study suggest that the role of DNA methylation in the regulation of glial transdifferentiation would be an interesting future direction. Notably, PDGF has been shown to induce RPE transdifferentiation *in vitro*,⁶⁶ which occurs independently of TGF- β .⁶⁷ On the other hand, TGF- β activates and enhances PDGF expression in human RPE

FIGURE 9. Effects of 5-AZA-dC on the phenotype of polarized RPE monolayer cultures. Established RPE monolayers were treated with 5-AZA-dC (1, 2, and 6 μ M) for 4 days. Shape was evaluated by phase contrast microscopy (**A**) and H&E staining (**B**). Transepithelial resistance (TER) was measured using a volt-ohm meter at 4 days (**C**). Cell death was analyzed by TUNEL assay (**D**). No obvious changes in shape of the monolayer cells was revealed, either by phase contrast microscopy or H&E staining. There was no TER reduction after treatment with 5-AZA-dC for 4 days. Only extremely rare apoptotic cells were seen and only at the highest dose (6 μ M) of 5-AZA-dC. (**D**) Effects of 5-AZA-dC on expression of ZO-1 (**E**) and cytokeratin (**F**). Retinal pigment epithelium cells were treated with 5-AZA-dC (1, 2, and 6 μ M) for 4 days; immunocytochemistry showed no detectable differences between expression of ZO-1 and that of cytokeratin in the 5-AZA-dC-treated cells compared with control.

cells⁶⁸ and experimental PVR models.⁶⁸ Therefore, we focused on the role of TGF- β in pathogenesis of RPE transdifferentiation. The role of DNA methylation in PDGF-induced RPE transdifferentiation should be further studied because the inhibition of TGF- β signaling by 5-AZA-dC was unable to completely block the expression of α -SMA and FN.

Assessments of toxicity are critical when evaluating drugs with potential therapeutic use. 5-AZA-dC is currently being used in clinical trials for patients with cancer.^{53,54} In each of the experimental protocols using subconfluent human RPE cultures, there was no significant toxicity as assessed by TUNEL assay or trypan blue exclusion at levels up to and including 2 μ M 5-AZA-dC. This is important because the effects of 5-AZA-dC on inhibition of transdifferentiation were typically dose related and significantly observed at 1 and/or 2 μ M 5-AZA-dC. There was a significant increase in cell death at 6 μ M 5-AZA-dC, but overall levels of cell death remained relatively modest. It is most important to determine whether 5-AZA-dC

would have toxicity in the normal RPE monolayer. Using highly polarized human RPE monolayer cultures, we found that these cultures were extremely resistant to the toxic effects of 5-AZA-dC, suggesting that the normal RPE monolayer is unlikely to be a site of toxicity, even if 5-AZA-dC is delivered locally, as might be envisioned in therapy for patients with PVR.

Overall, the results indicate that RPE mesenchyme transdifferentiation may be mediated by the functionally interacting effects of DNA methylation and MeCP2⁶⁹ and that both may be targets for therapeutic intervention in PVR (Supplementary Fig. S3).

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