

MicroRNA-124 Controls Transforming Growth Factor β 1-Induced Epithelial-Mesenchymal Transition in the Retinal Pigment Epithelium by Targeting RHOG

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PURPOSE. MicroRNA-124 (miR-124) is thought to be involved in the epithelial-mesenchymal transition (EMT) of RPE. We investigated the regulation of TGF- β 1-induced EMT by miR-124 in human RPE cells (ARPE-19).

METHODS. Expression of miR-124 was evaluated after TGF- β 1 treatment by quantitative RT-PCR. Phenotypic alterations were analyzed by Western blot analysis and immunocytochemical staining. Target validation was performed by a luciferase reporter assay to identify the putative target of miR-124.

RESULTS. The expression level of miR-124 was downregulated during the progression of EMT. Overexpression of miR-124 upregulated the levels of zonular occludens 1 and occludin, and downregulated those of fibronectin, α -smooth muscle actin, and vimentin. Furthermore, inhibition of endogenous miR-124 increased and decreased the levels of mesenchymal and epithelial factors, respectively. TargetScan predicted two well-conserved and two vertebrate-only conserved miR-124 target sequences in the 3' untranslated region (UTR) of the *Ras homology Growth-related (RHOG)* mRNA. Direct targeting of this 3' UTR by miR-124 was demonstrated using a luciferase assay. Silencing of *RHOG* using a specific siRNA had identical effects on EMT regulation. Overexpression of miR-124 repressed TGF- β 1-induced RPE cell-collagen gel lattice contraction by altering cell spreading/cell-to-cell adhesion.

CONCLUSIONS. This study describes the regulation of EMT in RPE cells by TGF- β 1/miR-124/RHOG signaling and suggests that the supplement of miR-124 exogenously would be a valuable therapeutic approach for the prevention or treatment of proliferative vitreoretinopathy.

Keywords: epithelial-mesenchymal transition (EMT), microRNA-124 (miR-124), retinal pigment epithelium (RPE), Ras homology Growth-related (RHOG), transforming growth factor beta (TGF- β)

Proliferative vitreoretinopathy (PVR) is a vision-threatening scarring process that occurs as the terminal situation of a pathologic process initiated by dispersions of RPE cells into the vitreous.¹⁻³ In addition to RPE cells, TGF- β 1/ β 2, the extracellular matrix (ECM), and epithelial-mesenchymal transition (EMT) are major components of the pathobiology of PVR.²⁻⁴ Garweg et al.³ distinguished the three different pathobiological processes of PVR as the migration of RPE cells, their proliferation, and matrix contraction. The vitreal cavity is a large ECM reservoir, so exposure of the RPE to vitreous components moves to transdifferentiation as a necessity.

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate the expression of various target genes.^{5,6} Some miRNAs are regulators of tumor physiologies and also may have key roles in neuronal differentiation.⁷⁻¹² MicroRNA-124 (miR-124) is nervous system-specific and is the most well-characterized miRNA in the brain. This miRNA is derived from three independent genes (*miR-124-1*, *miR-124-2*, and *miR-124-3*) and is expressed during neuronal differentiation.^{7,13} Recent

reports demonstrated that downregulation of *miR-124-1*, resulting in depletion of mature miR-124, affects the structure of neurons by regulating Ras homology Growth-related (RHOG).^{8,10,14} As a member of the RHO family of small GTPases, RHOG is a key regulator of the actin cytoskeleton, and in our previous studies,¹⁵⁻¹⁷ we unraveled the importance of cytoskeletal rearrangement by RHO during RPE transdifferentiation.

Transforming growth factor- β is a potent inducer of EMT.²⁻⁴ Although there is no evidence of a direct relationship between RHOG and TGF- β -induced EMT, Ras-related C3 botulinum toxin substrate 1 (RAC1), a downstream target of RHOG, is a known regulator of the EMT process.^{18,19} Therefore, we hypothesized that miR-124 may affect EMT through the regulation of RHOG and subsequent effects on the actin cytoskeleton. Analyses of the miRNA expression profile of the murine eye revealed that miR-124 is expressed at much lower levels in the RPE than in other retinal layers.^{20,21} Whole retinal layers originate from the neuroepithelium,²² and a lack of miR-



124 in the RPE could contribute to its vulnerability to dedifferentiation, especially when exposed to vitreal fluid during retinal detachment. Moreover, the majority of central nervous system (CNS) and retinal layers commonly originate from the neuroectoderm, this small RNA expression discrepancy of RPE from these tissue implicated miR-124 has unknown role to be elucidated in RPE physiology.

We demonstrated that miR-124 regulates RPE fibroblastoid transdifferentiation by targeting the *RHOG* 3' UTR during EMT. The results presented suggest that *RHOG*, under the control of miR-124, has a novel role in EMT, and that this nervous system-specific miRNA regulates the differentiation of RPE cells, which have the same developmental origin as nerve cells.

MATERIALS AND METHODS

Cell Line and Cell Culture

An immortalized human RPE cell line, ARPE-19, was purchased from the American Type Culture Collection (Manassas, VA, USA) and primary human RPE (H-RPE) cell line was purchased from Lonza (Basel, Switzerland). The ARPE-19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 Nutrient Mixture Ham (Welgene, Daegu, Korea) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Welgene) and 1% (vol/vol) penicillin/streptomycin (Welgene). Primary human RPE cells were cultured in RPE cell basal medium (RtEBM; Lonza). These cells were maintained at 37°C in a humidified 5% CO₂ incubator.

TGF- β 1-Mediated Induction of EMT

Cellular transdifferentiation was promoted by adding 10 ng/mL human recombinant TGF- β 1 (Peprotech, Offenbach, Germany) to ARPE-19 cells at 80% to 90% confluency. Cellular phenotypic changes were observed by phase contrast microscopy (CK30; Olympus, Shinjuku, Japan) after incubation with TGF- β 1 for 24 to 48 hours. All TGF- β 1 stimulations were performed under serum-free conditions.

RNAi and Reagents

The mirVana miRNA-124 mimic, miRNA negative control (NC) mimic, mirVana miR-124 inhibitor, and miRNA NC inhibitor were purchased from Life Technologies (Carlsbad, CA, USA). The AccuTarget Fluorescein-labeled miRNA NC mimic was purchased from Bioneer (Daejeon, Korea). The NC small interfering RNA (siRNA) and predesigned *RHOG*-specific siRNA were purchased from Qiagen (Venlo, Limburg, The Netherlands). The Hs_miR-124a_1 miScript Primer Assay, Hs_RNU6-2_11 miScript Primer Assay, Hs_miR-16_2 miScript Primer Assay, miScript II Reverse Transcriptase, and QuantiTect SYBR Green PCR Master Mix also were obtained from Qiagen. Alexa Fluor 488-conjugated phalloidin and the Lipofectamine RNAi-MAX and Lipofectamine 3000 transfection reagents were purchased from Life Technologies. Cellmatrix Collagen gel solution was obtained from Nitta Gelatin (Osaka, Japan). The following primary antibodies were used: anti-*RHOG* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-fibronectin (Santa Cruz Biotechnology), anti-vimentin (Santa Cruz Biotechnology), anti- α -smooth muscle actin (SMA; Santa Cruz Biotechnology), anti-RAC1 (EMD Millipore, Billerica, MA, USA), anti-zonula occludens 1 (ZO-1; Abcam, Cambridge, UK), anti-occludin (Santa Cruz Biotechnology), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA). All other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Transfection of Cells With MiRNA or SiRNA

At 24 hours before transfection, ARPE-19 cells were trypsinized, seeded into 6-well dishes, and incubated until they reached 70% to 80% confluence. The cells were transfected with the NC siRNA, the *RHOG*-specific siRNA, miRNA mimics, or miRNA inhibitors (50 nM each) for 6 hours. The medium containing the transfection reagents then was replaced with fresh medium supplemented with 10% FBS. After an overnight incubation, the culture medium was changed to serum-free medium with or without TGF- β 1 (10 ng/mL).

Quantitative Analysis of Relative MiRNA Expression

After removal of culture medium and three rinses with phosphate-buffered saline (PBS), total RNA was extracted from cells using TRIzol reagent (Life Technologies). A 1 μ g aliquot total RNA was reverse transcribed using miScript II Reverse Transcriptase. Real-time quantitative PCR (qPCR) was performed using QuantiTect SYBR Green PCR Master Mix and the StepOnePlus PCR system (Applied Biosystems, Foster City, CA, USA). The expression level of miRNA-124 was normalized to that of the control RNU6 RNA or miR-16 using the 2^{- $\Delta\Delta$ C_T} method.²³ The expression level of miRNA-124 in the absence or presence of TGF- β 1 (10 ng/mL) in primary human RPE cells also was evaluated.

Western Blotting

Cells were lysed using radioimmunoprecipitation buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.5, and 2 mM EDTA pH 8.0). Equal quantities of total protein samples were separated via 8% to 15% SDS-PAGE. After electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane, which was blocked with 5% skimmed milk solution for 1 hour. After an overnight incubation with the primary antibody, the membrane was incubated with the secondary antibody for 1 hour. The expression level of GAPDH was used as a loading control for band densitometry analyses.

Phalloidin Staining and Immunocytochemistry

Cells were seeded onto 4-well cell culture slides (SPL Life Sciences, Pocheon, Korea) and transfected with the miRNA or siRNA 24 hours later. The cells then were incubated in the presence or absence of TGF- β 1 for up to 24 hours. After three rinses with PBS, the cells were fixed with 4% paraformaldehyde for 30 minutes. The fixed cells were incubated with Alexa Fluor 488-conjugated phalloidin and Hoechst 33342 solution, respectively. For immunocytochemical evaluations, the cells were permeabilized with 0.4% (vol/vol) Triton X-100 for 15 minutes, and then blocked with 1% (wt/vol) bovine serum albumin in 0.1% (vol/vol) Tween-20/PBS for 30 minutes. The cells were incubated with the primary antibodies for 12 hours at 4°C, followed by Alexa Fluor 488/594-conjugated secondary antibodies for 1 hour at room temperature (Molecular Probes; Life Technologies). Each slide was observed using an upright fluorescent microscope (Axio Imager 2; Zeiss, Cologne, Germany) and images were taken using AxioVision SE64 version 4.9.1.0 software (Zeiss).

Collagen Gel Contraction Assay

Cells in 60-mm dishes were transfected with the miR-124 or NC mimic for 6 hours and then harvested using a 0.25% Trypsin-EDTA solution. Two parts of the cell suspension were mixed

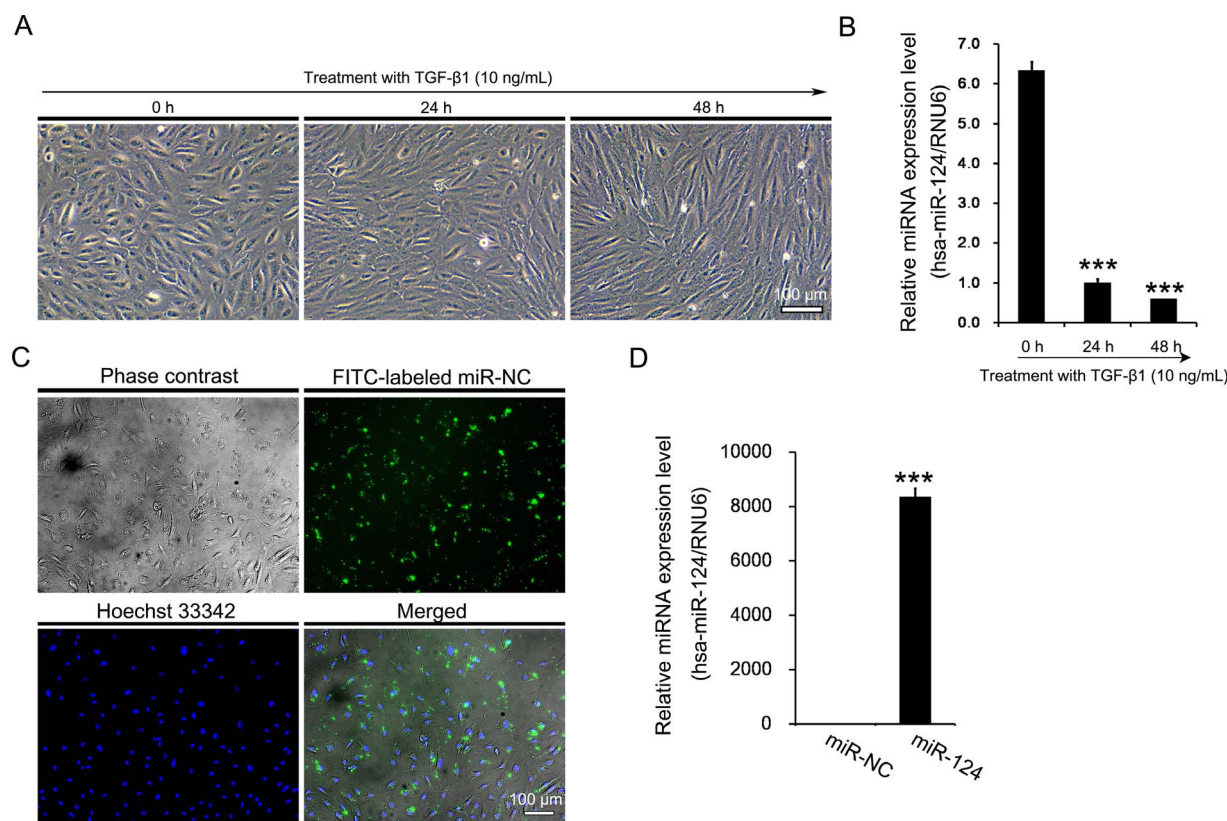


FIGURE 1. (A) Fibroblastoid differentiation of RPE cells after treatment with TGF- β 1 (10 ng/mL) for the indicated times. (B) The expression levels of miR-124 in RPE cells treated with TGF- β 1 for the indicated times, determined using qPCR analyses. The expression levels of miR-124 were normalized to those of RNU6. Data are represented as the mean $\pm$ SD of three replicates. *** P < 0.001 by a 1-way ANOVA, followed by Tukey's HSD post hoc test. (C) Verification of the efficient transfection of RPE cells with a fluorescein-labeled miRNA NC mimic. (D) The expression levels of miR-124 in RPE cells transfected with a NC or miR-124 mimic (50 nM), as determined by qPCR analyses. The expression levels of miR-124 were normalized to those of RNU6. Data are represented as the mean $\pm$ SD of three replicates. *** P < 0.001 by an independent t -test.

with eight parts of cold collagen gel solution, and then 100 μ L cell-collagen gel mixture was dispensed into 48-well plates. After polymerization for 1 hour at 37°C, 100 μ L medium containing 20 ng/mL TGF- β 1 was added to the top of the collagen lattice, and the samples were incubated for 24 hours. Each gel was observed under phase contrast microscopy; the collagen gel area in each well was measured using images taken after the 24 hours incubation. To evaluate cell spreading or cell-to-cell adhesion, each cell-collagen gel mixture was stained with hematoxylin and eosin (H&E) and observed by upright microscopy (DM5500B; Leica, Biberach, Germany). Images were taken using LAS imaging software (Leica).

Quantification of the Fluorescence Profile of RAC1

Cells were transfected with the miR-124 or NC mimic and conventional immunostaining procedures were performed. After acquiring images of RAC1 localization, the image files were analyzed with ImageJ (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Lines were drawn along the area of interest across individual RPE cells transfected with the miR-124 or NC mimic. Analysis was performed using the "Plot Profile" function of the "Analyze" category in ImageJ.

Plasmid Transfection and Luciferase Assays

The pLightSwitch *RHOG* 3' UTR plasmid and random control luciferase plasmid were purchased from Switchgear Genomics (Carlsbad, CA, USA). Cells were seeded into 96-well plates 24

hours before transfection and were cultured to 70% confluence. The cells then were cotransfected for 6 hours with the random control or *RHOG* 3' UTR luciferase plasmid and the miR-124 mimic, NC mimic, miR-124 inhibitor, or NC inhibitor using Lipofectamine 3000 reagent. After a 24-hour incubation, the cells were lysed with buffer-substrate solution (LightSwitch assay reagent LS010; Switchgear Genomics) for 30 minutes, and then the luciferase activity in each well was detected using a luminometer (Centro LB 960; Berthold, Technologies GmbH, Calmbacher, Germany). All experiments were performed at least three times.

Statistics

Data were collected from biological triplicates ($n = 3$). To identify statistically significant differences, the data were evaluated using an independent t -test or 1-way ANOVA followed by Tukey's HSD post hoc test. P < 0.05 was considered significant.

RESULTS

MiR-124 Expression is Reduced During the Transdifferentiation of RPE Cells

After exposure to 10 ng/mL TGF- β 1, the phenotype of cells progressed to a spindle-shaped appearance (Fig. 1A). Alongside the progression of transdifferentiation, real-time qPCR analyses revealed that exposure to TGF- β 1 reduced the level of

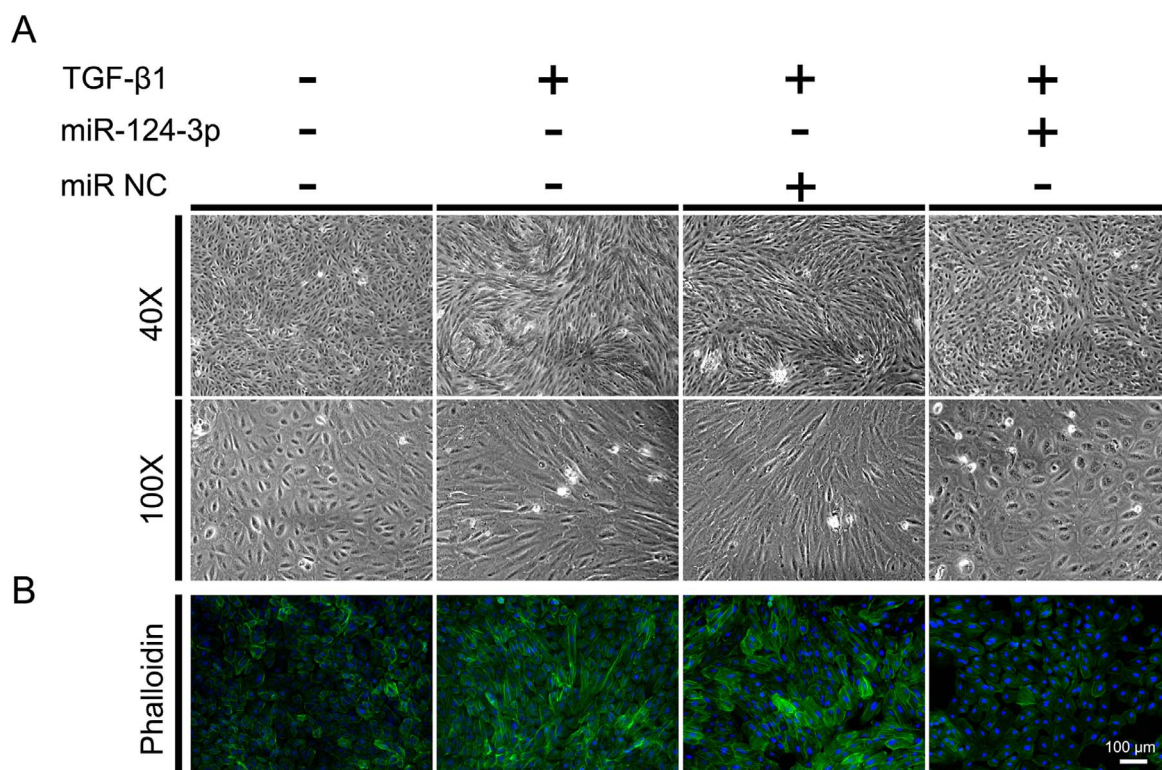


FIGURE 2. (A) Morphologic alterations in RPE cells transfected with a NC or miR-124 mimic (50 nM) and incubated in the presence or absence of TGF-β1 (10 ng/mL). The cells were examined by phase contrast microscopy. (B) The effect of overexpression of miR-124 on stress fiber formation in RPE cells treated with or without TGF-β1, as determined via immunofluorescent staining of filamentous actin using Alexa Fluor 488-conjugated phalloidin.

endogenous miR-124 significantly; at the 24- and 48-hour time-points, the expression level of this miRNA was less than 20% and less than 10% of the control, respectively (Fig. 1B). Endogenous miR-124 expression normalized by miR-16 expression showed a similar pattern; it was reduced gradually after TGF-β1 treatment of ARPE-19 cells (Supplementary Fig. S1). Treatment with TGF-β1 (10 ng/mL) significantly reduced endogenous miR-124 expression in primary human RPE cells (Supplementary Fig. S2).

Effective Delivery of Synthetic MiR-124 Into RPE Cells

To evaluate the efficiency of the transfection procedures, ARPE-19 cells were transfected with a fluorescein-labeled NC miRNA and examined by inverted fluorescence microscopy (Axio Observer D1; Zeiss). Fluorescein-labeled miRNAs were observed in 80% to 90% of the cell population (Fig. 1C). In addition, a qPCR analysis revealed that the level of miR-124 in cells transfected with a miR-124 mimic was 8000-fold higher than that in cells transfected with a NC mimic (Fig. 1D).

MiR-124 Inhibits Fibroblastic Alterations in RPE Cells

After incubation with 10 ng/mL TGF-β1 for 24 hours, ARPE-19 cells were transformed and showed a fibroblastoid appearance; however, overexpression of miR-124 inhibited the progression of these fibroblastic alterations. Like cells incubated in the absence of TGF-β1, those overexpressing miR-124 showed an epithelial morphology (Fig. 2A). Phalloidin staining revealed stress fiber formation due to cytoskeletal rearrangement during

TGF-β1-induced EMT, but this effect was reversed effectively by overexpression of miR-124 (Fig. 2B).

MiR-124 Negatively Regulates the Expression and Localization of EMT-Related Factors

The effects of transfection of ARPE-19 cells with the NC or miR-124 mimic on TGF-β1-induced alterations of various EMT-related factors were evaluated. Quantitative immunoblot analyses revealed that, compared to those transfected with the NC mimic, TGF-β1-treated ARPE-19 cells expressing the miR-124 mimic had significantly lower levels of fibronectin, vimentin, and α-SMA, all of which are representative of fibroblastic proteins, and significantly higher levels of ZO-1 and occludin, both of which represent epithelial proteins (Figs. 3A, 3C). Furthermore, opposing results were obtained when cells were transfected with a miR-124-specific or NC inhibitor (Fig. 3B). These results also were confirmed by immunocytochemistry, which showed that overexpression of miR-124 downregulated the levels of the fibroblastic phenotype proteins and upregulated the levels of the epithelial phenotype proteins (Fig. 3D).

Identification of Putative MiR-124 Target Sites in the *RHOG* 3' UTR

To identify putative regulatory targets of miR-124, we performed an in silico analysis using a web-based program (TargetScan, version 6.2; available in the public domain at <http://www.targetscan.org>). *RHOG* (NM_001665) is a well-known regulatory gene that is related to cytoskeletal rearrangement, which is a crucial component of EMT. A search of the 3' UTR of the human *RHOG* gene revealed two

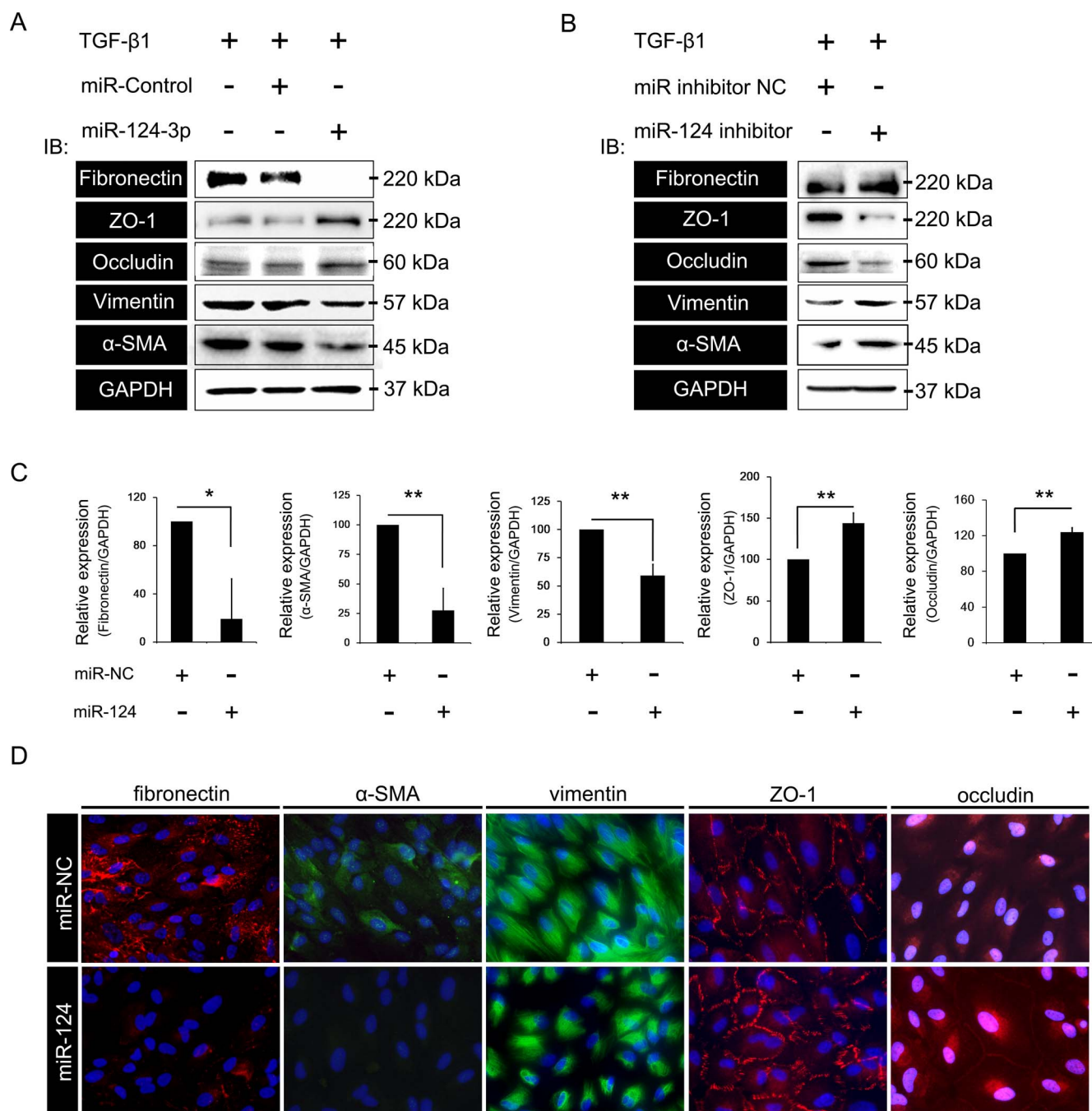


FIGURE 3. (A) Immunoblot analyses of the effects of overexpressing miR-124 on the expression levels of the factors altered during TGF- β 1-induced EMT of RPE cells. (B) Immunoblot analyses of the effects of inhibiting miR-124 on the expression levels of the factors altered during TGF- β 1-induced EMT of RPE cells. (C) Quantification of the immunoblot data shown in (A). Three independent experiments were performed. Data are represented as the mean $\pm$ SD. * P < 0.05 and ** P < 0.01 by independent t -tests. (D) Representative images of immunostained fibroblastic (fibronectin, vimentin, and α -SMA) and epithelial (ZO-1 and occludin) factors in RPE cells transfected with the NC or miR-124 mimic and treated with TGF- β 1 for 24 hours.

evolutionarily highly conserved and two poorly conserved (vertebrates only) miR-124 target sequences (Fig. 4A).

MiR-124 Targets the *RHOG* 3' UTR Directly in ARPE-19 Cells

Next, we used a luciferase assay to determine whether the *RHOG* 3' UTR is an actual target of miR-124 in ARPE-19 cells. The luciferase activity in cells cotransfected with a random

control reporter plasmid and a NC mimic was comparable to that in cells cotransfected with the random control plasmid and a miR-124 mimic (Fig. 4B). However, in cells cotransfected with the *RHOG* 3' UTR reporter plasmid, overexpression of miR-124 reduced the luciferase activity by approximately 85% (Fig. 4B). By contrast, the luciferase activity of the *RHOG* 3' UTR reporter in cells transfected with a miR-124 inhibitor was 4-fold higher than that in cells cotransfected with a NC inhibitor (Fig. 4B).

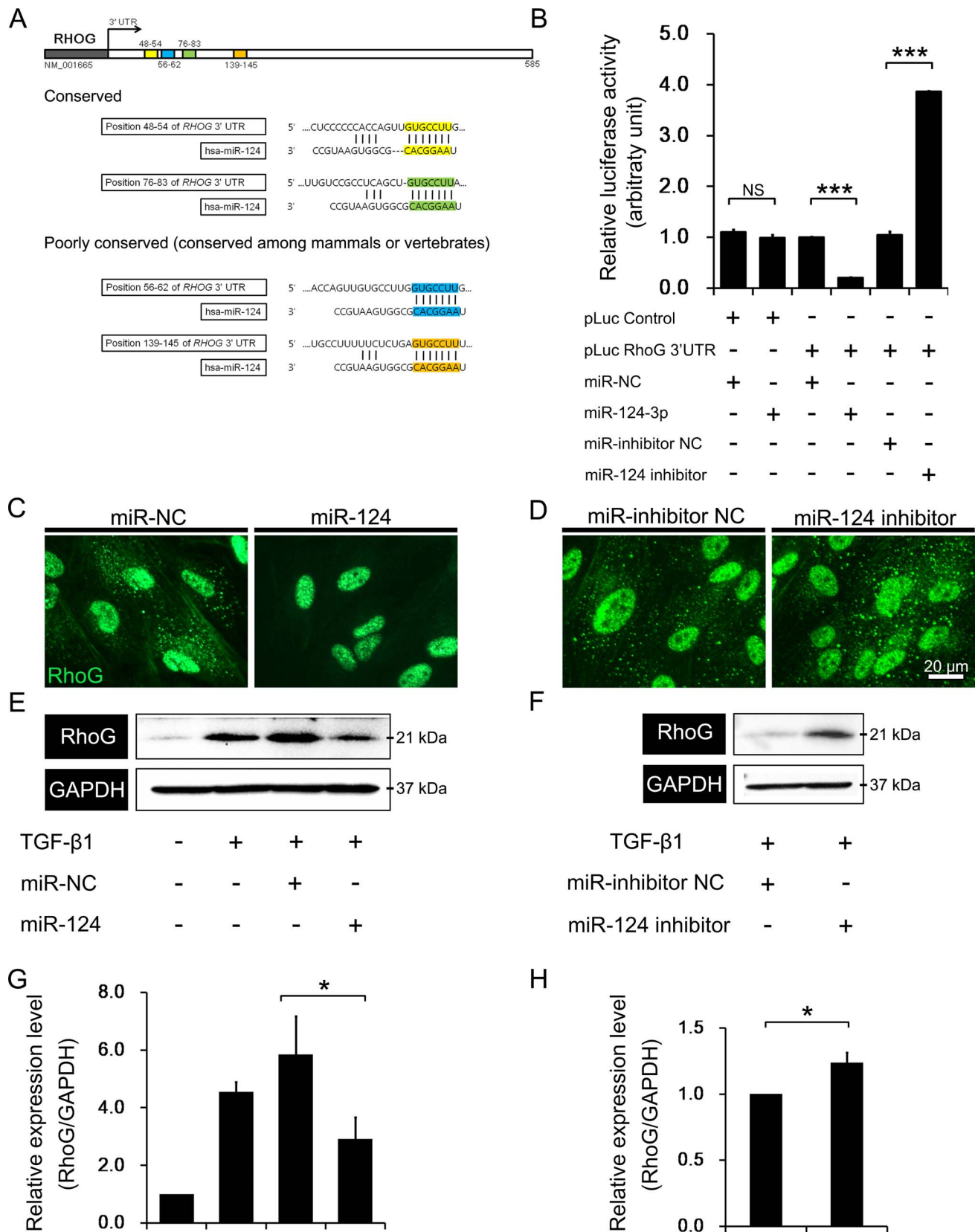


FIGURE 4. Validation of the *RHO G* 3' UTR as a target of miR-124 and regulation of *RHO G* expression and localization patterns by overexpression of this miRNA. (A) Putative miR-124 seed matches within the 3' UTR of the *RHO G* mRNA. The web-based program TargetScan predicted two seed matches that were perfectly conserved across various species and another two seed matches that were conserved in vertebrates only. (B) Relative activities of a control luciferase reporter and a luciferase reporter fused to the 3' UTR of *RHO G* in RPE cells cotransfected with a NC or miR-124 mimic or inhibitor. Data are represented as the mean $\pm$ SD of three replicates. (C, D) Localization of *RHO G* in RPE cells transfected with a NC or miR-124 mimic or inhibitor. (E, F) Immunoblot analyses of RPE cells transfected with a NC or miR-124 mimic or inhibitor and treated with or without TGF- β 1. (G, H) Quantification of the data shown in (E) and (F), respectively. (B, G, H) NS, nonspecific; * P < 0.05 and *** P < 0.001 via independent t -tests (B, H) or 1-way ANOVA followed by Tukey's HSD post hoc test (G).

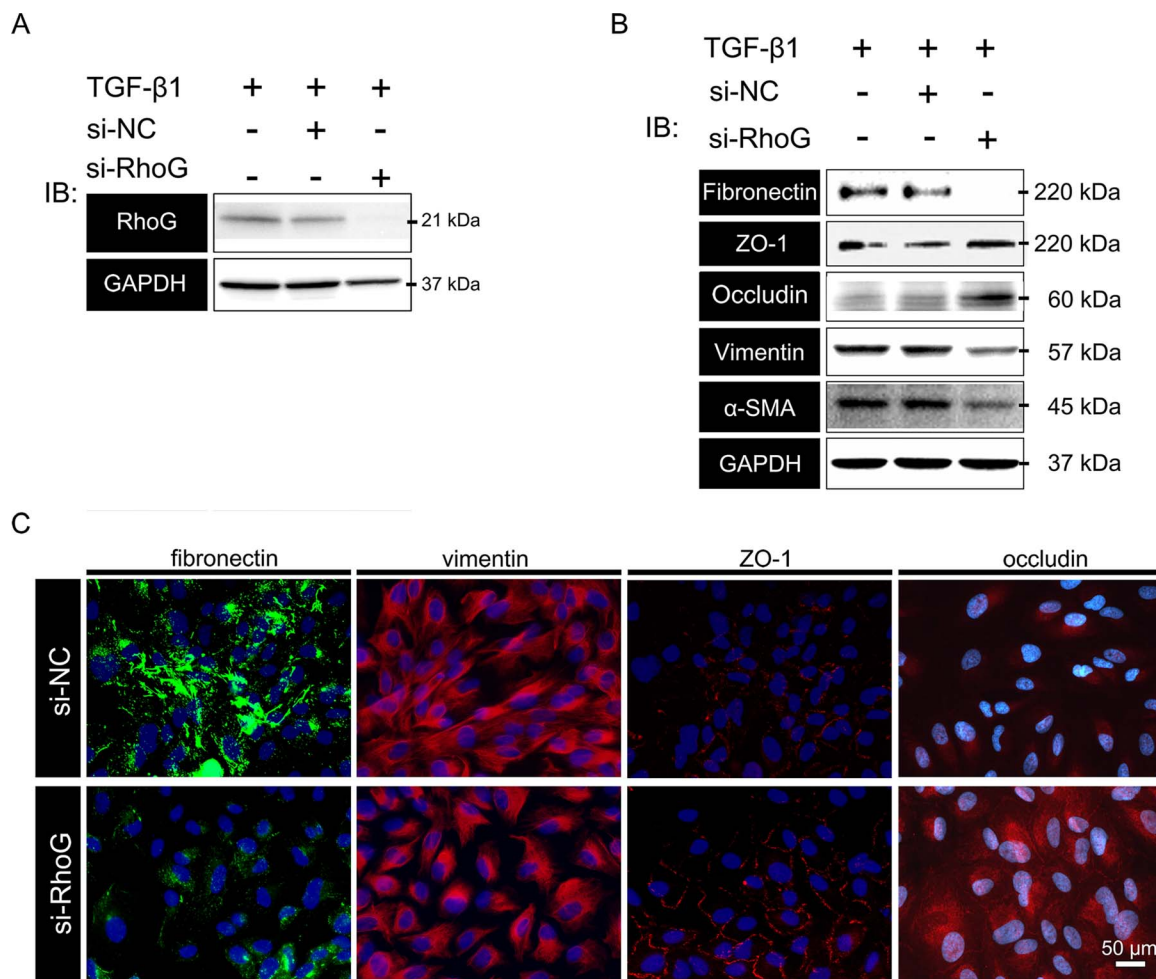


FIGURE 5. The effects of knockdown of RHO on EMT-related factors in RPE cells. (A) Representative images of immunostained fibroblastic (fibronectin and vimentin) and epithelial (ZO-1 and occludin) factors in TGF-β1-treated RPE cells transfected with a NC or *RHO*-specific siRNA. (B) Knockdown of RhoG expression in RPE cells transfected with a NC or *RHO*-specific siRNA. (C) Immunoblot analyses of the effects of RHO knockdown on the expression levels of fibroblastic (fibronectin, vimentin, and α-SMA) and epithelial (ZO-1 and occludin) factors during TGF-β1-induced EMT of RPE cells.

Exogenous Overexpression of MiR-124 in RPE Cells Downregulates the Expression and Localization of RHO

In the presence of 10 ng/mL TGF-β1, cells transfected with a miR-124 mimic had lower levels of RHO that those transfected with a NC mimic, whereas those transfected with a miR-124 inhibitor to antagonize endogenous miR-124 had higher levels of RHO than those transfected with a NC inhibitor (Figs. 4C–F). These results were confirmed by quantitative analyses of three independent immunoblotting experiments, which indicated statistically significant effects of miRNA overexpression or inhibition on RHO levels (Figs. 4G, 4H).

Knockdown of RHO Negatively Regulates the Expression and Localization of EMT-Related Factors

We examined whether silencing of *RHO* could downregulate EMT. Transfection of ARPE-19 cells with a *RHO*-specific siRNA effectively knocked down RHO expression (Fig. 5A). In the presence of TGF-β1, knockdown of RHO downregulated the levels of fibronectin and vimentin, and upregulated

those of ZO-1 and occludin (Fig. 5B). Immunostaining of these factors showed similar effects of RHO knockdown on their localization patterns in cells (Fig. 5C).

MiR-124 Downregulates RAC1, a Downstream Target of RHO

RAC1 is an important modulator of cytoskeletal alterations induced by the vitreous in RPE cells,^{24,25} and is positioned downstream of RHO in its signaling pathway.^{18,19} Immunostaining and immunoblot analyses revealed that overexpression of miR-124 in RPE cells reduced the distribution of RAC1 throughout the cytoplasm (Fig. 6A) and the expression level of the RAC1 protein (Fig. 6B). RAC1 expression was reduced not only in general, but also at the edges of the cytoplasm (Fig. 6C).

Overexpression of MiR-124 Reduces RPE–Collagen Gel Mixture Lattice Contraction

In PVR, matrix contraction is a key pathology that contributes to retinal shrinkage; therefore, we examined whether overexpression of miR-124 reduces the contraction of RPE–collagen gel lattices. In the presence of TGF-β1, the collagen gel matrix shrunk to approximately half of the area of untreated gels;

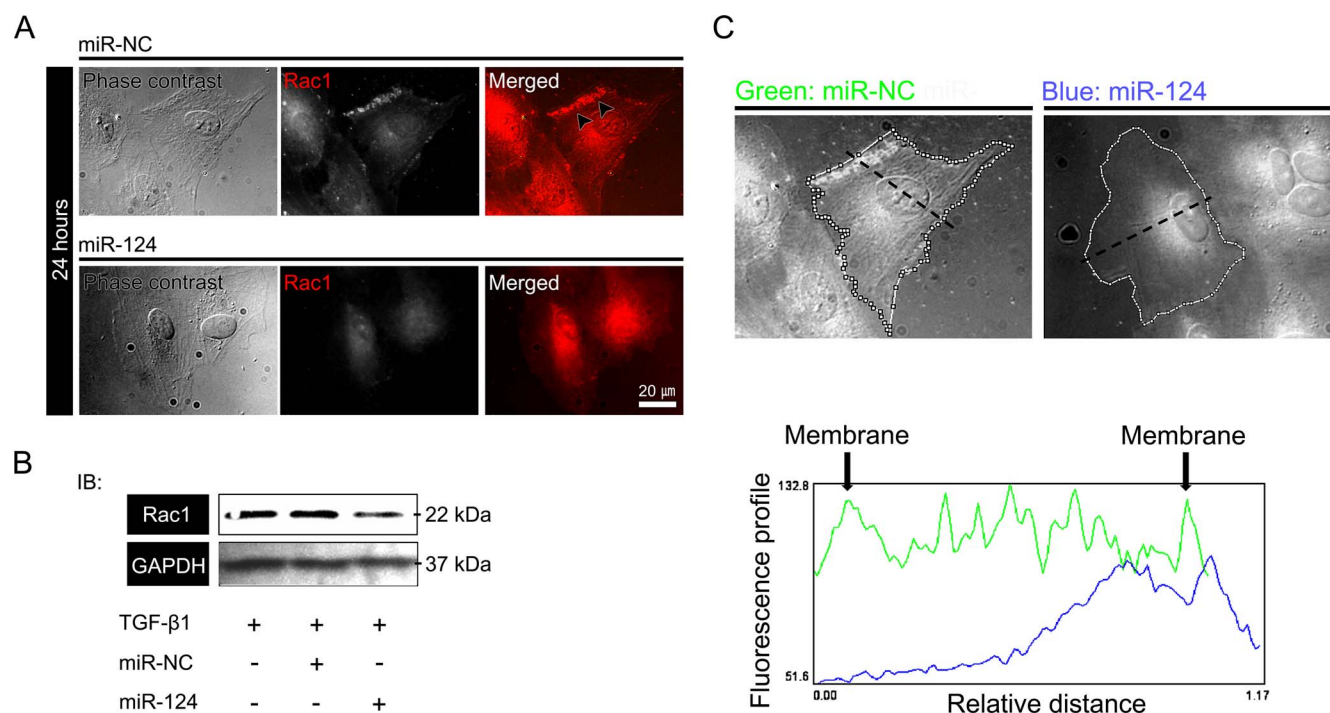


FIGURE 6. The localization pattern and expression of RAC1 protein after transfection of RPE cells with a NC or miR-124 mimic. **(A)** Immunostaining of RPE cells showing the effect of overexpression of miR-124 on the localization of RAC1 in the presence of TGF- β 1. **(B)** Immunoblot analysis of RAC1 protein levels in TGF- β 1-treated RPE cells overexpressing miR-124 or a NC mimic. **(C)** Alterations in the localization pattern of RAC1 after overexpression of miR-124 in RPE cells.

however, this TGF- β 1-induced shrinkage was reduced by overexpression of miR-124 (Fig. 7A). This result was statistically significant in three independent experiments (Fig. 7B). H&E staining of the cell-collagen gel matrices revealed that overexpression of miR-124 inhibited cell spreading within the gel and reduced fibroblastic band formation by impeding cell-to-cell adhesions (Fig. 7C).

DISCUSSION

Epithelial mesenchymal transition, a major pathologic mechanism in ocular tissues, involves cellular transdifferentiation of the retina, cornea, and lens, resulting in a reduction of the integrity or clarity of the visual axis.²⁶ Myofibroblastic alterations of fibroblasts and epithelial cells occur in tissues such as the kidney and liver; by contrast, in the lens and retina in particular, fibrotic lesions are derived from the transdifferentiation of epithelial cells.^{27,28} During embryonic development, the RPE and neural retina both originate from the neuroectoderm.²² The loose attachment of these two layers results in vulnerability to separation, which can lead to retinal detachment.²⁶ Proliferative vitreoretinopathy is a catastrophic sequela of rhegmatogenous retinal detachment, and TGF- β -related fibroblastoid differentiation is implicated in this process.^{1,3,29} Despite numerous efforts concentrated on its prevention or treatment, PVR still is an ongoing issue. In recent years, various studies have described regulatory roles of miRNAs in developmental processes and cellular physiology^{30,31}; hence, regulatory effects of miRNAs on EMT may have a pivotal role in TGF- β signaling, although the mechanisms of regulation of this pathway during EMT are largely unknown.^{32,33}

The human pre-miR-124a-1 locus is located in the chromosome 8p23.1 region, and its partial deletion compromises

cerebral development and may be related to neuropsychiatric disorders.^{34–36} In addition, recent studies demonstrated that miR-124 is expressed mainly in CNS tissues and is a crucial regulator of CNS development.^{9,10,14,37} Like CNS tissues, retinal cells originate from the neural ectoderm, and miR-124 is expressed at high levels in the majority of retinal layers.^{20,21} In fact, mouse miR-124 is expressed strongly in the neural retina from the prenatal stage to adulthood.²¹ Notably, miR-124 expression is undetectable in RPE cells, which are implicated in the pathology of PVR.²¹ Hence, we hypothesized that the lack of or low expression level of miR-124 in RPE cells renders them vulnerable to transdifferentiation induced by various insults. To test this hypothesis, we quantitatively measured the expression level of miR-124 in the ARPE-19 cell line and primary human RPE cells, and found that it was reduced dramatically during TGF- β 1-induced EMT progression, respectively. Based on this result, we concluded that miR-124 is related to the homeostasis or differentiation of RPE cells and has a crucial role in signaling cascades involving TGF- β 1.

To screen for potential regulatory targets of miR-124, we searched the available literature. Recently, miR-124 was implicated in neuronal differentiation and cancer pathophysiology^{8,9,38,39} via its effects on various target genes, such as *EZH2*, *FOXQ1*, *SERP1*, and *RHOG*. *RHOG* was selected as a potential target of interest because it is a pivotal signaling molecule that controls actin remodeling, as well as an upstream regulator of RAC1, which modulates TGF- β 1-induced epithelial cell plasticity.^{15,18,40} We reported previously that actin remodeling by RHO signaling has a key role in transdifferentiation of the RPE.^{16,17} In addition, targeting of *RHOG* by miR-124 in neuronal cells was reported recently.⁸ Here, the *RHOG* 3' UTR was identified as the third most probable target of miR-124 by the bioinformatics program TargetScan. Based on these findings, we examined the

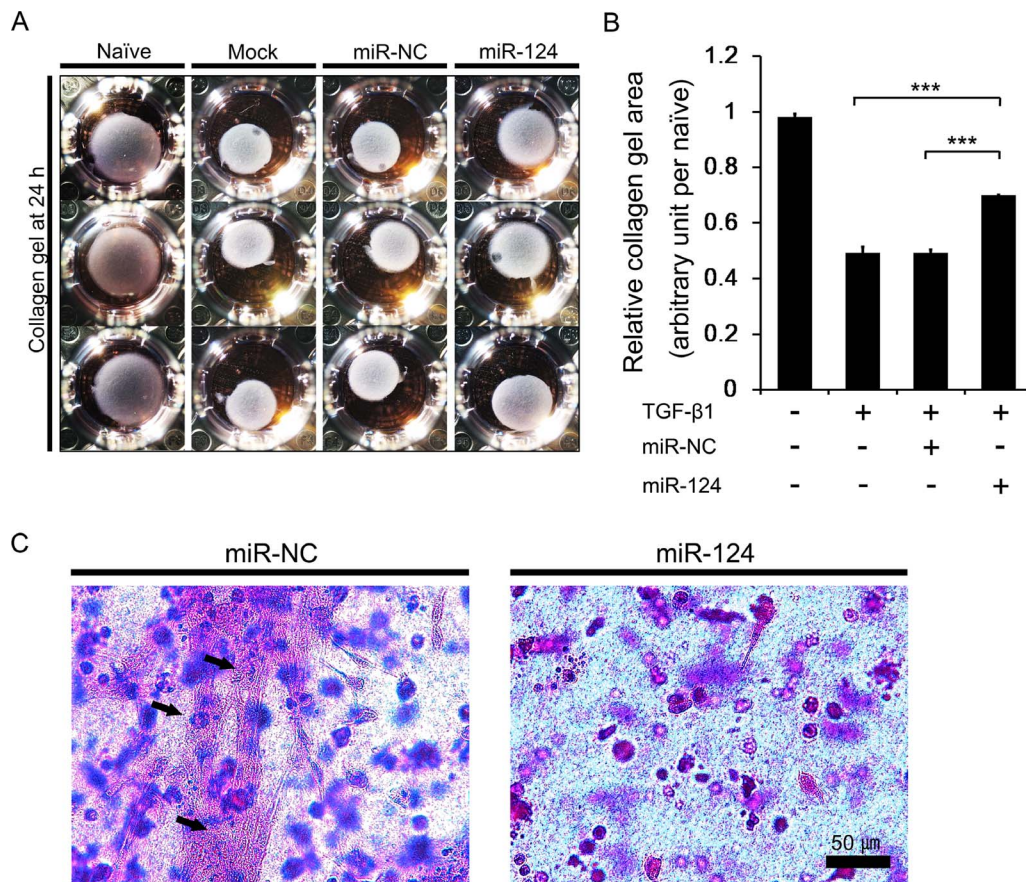


FIGURE 7. Collagen gel contraction assay in the presence or absence of TGF-β1 and overexpression of miR-124. (A) Transforming growth factor-β1-induced contraction of the collagen gel-RPE cell mixture, and the effects of overexpression of miR-124 or a NC miRNA mimic on this response. Naïve, not transfected with a miRNA and not treated with TGF-β1. Mock, not transfected with a miRNA mimic, but treated with TGF-β1 (10 ng/mL). (B) Quantification of the mean collagen gel areas in the matrices shown in (A). Data are represented as the mean $\pm$ SD of three replicates. *** P < 0.001 by a 1-way ANOVA followed by Tukey's HSD post hoc test. (C) Microscopic observations of H&E-stained collagen gel-RPE cell mixtures. Fibroblastic band formation is evident across the collagen gel (arrows).

regulation of TGF-β1-induced EMT by RHOG/miR-124 in RPE cells.

Transforming growth factor-β1 treatment induced spindle-shaped morphologic alterations in RPE cells, and concomitant overexpression of exogenous miR-124 inhibited these structural changes. Stress fiber formation caused by actin remodeling is a crucial change that occurs during EMT and is related to RHOG; therefore, we also evaluated the effect of miR-124 on stress fiber formation. Retinal pigment epithelial cells overexpressing miR-124 had reduced levels of TGF-β1-induced stress fibers and showed an epithelial phenotype similar to that of non-TGF-β1 treated cells. In support of these findings, immunoblot assays revealed that overexpression of miR-124 reduced the levels of fibroblastoid markers and increased those of epithelial factors. By contrast, transfection of RPE cells with an miR-124 inhibitor had the opposite effects. These results suggested that the endogenous homeostasis of miR-124 in RPE cells is a key factor during EMT; it may be possible to control EMT during PVR progression by artificial overexpression of miR-124.

Direct targeting of the *RHOG* 3' UTR by miR-124 was confirmed using a luciferase reporter assay. Cotransfection of RPE cells with synthetic miR-124 reduced the luciferase activity. By contrast, cotransfection with an miR-124 inhibitor increased the luciferase activity by 4-fold, presumably by blocking endogenous targeting of the *RHOG* 3' UTR by miR-

124. Moreover, in accordance with the results described above, TGF-β1 induced the upregulation of RHOG expression and increased its localization in the cytoplasm, and inhibition of miR-124 enhanced RHOG expression further. Based on these results, we proposed that miR-124 regulates RHOG during EMT induced by TGF-β1. The observed inhibitory effects of miR-124 on RAC1 expression/localization suggest that miR-124 contributes to the EMT process via the TGF-β1/RHOG/RAC1 pathway.

Retinal pigment epithelium or fibroblastoid cells are the major producers of several types of collagen and fibronectin; these cells contribute to the formation of contractile membranes by introducing various cytokines^{41,42} and are involved in the control of cellular adhesion and phenotypes.⁴³ Transforming growth factor-β-mediated contraction of RPE-collagen lattices is implicated in the pathophysiology of PVR.⁴⁴ Treatment with TGF-β1 induced a marked contraction of the RPE-collagen matrix, and this effect was reduced by overexpression of miR-124 in the present study. Microscopic observations of gel lattices revealed that cell spreading and cell-to-cell adhesion were markedly impeded by miR-124, and this inhibited the formation of contractile bands, suggesting that miR-124 may be a useful therapeutic agent for PVR.

Single miRNAs can target hundreds, if not thousands, of genes; therefore, we confirmed that RHOG is likely responsible for the effects of miR-124 on EMT of the RPE by silencing *RHOG* using a specific siRNA. As expected, knockdown of

RHOG mimicked the effects of overexpression of miR-124 in RPE cells. Our results provide insights into the pivotal role of RHOG in EMT and its regulation by miR-124. Using bioinformatics programs that predict protein-protein interactions, we identified a high possibility of interaction of RHOG with two Sma and Mad-related proteins, namely SMAD4 and SMAD5, and identified a relationship between SMAD4 and RHOG during EMT by *RHOG* silencing experiments (data not shown). Hence, it would be interesting to investigate the detailed crosstalk between the SMAD and RHOG signaling cascades.

In conclusion, we described a novel regulatory effect of miR-124 on TGF- β 1-induced EMT in RPE cells, and demonstrated that this regulatory effect is mediated via downregulation of RHOG. These findings suggest that miR-124 may be a novel therapeutic target for PVR.

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