Retinal Cell Biology

Regulation of ADAM10 and ADAM17 by Sorafenib Inhibits Epithelial-to-Mesenchymal Transition in Epstein-Barr Virus–Infected Retinal Pigment Epithelial Cells

Ga Bin Park,[1,2] Daejin Kim,[1,2] Yeong Seok Kim,[1] Jin Woo Kim,[3] Hook Sun,[3] Kug-Hwan Roh,[4] Jae Wook Yang,[2,5] and Dae Young Hur[1,2]

[1]Department of Anatomy, Inje University College of Medicine, Busan, Republic of Korea
[2]Ocular Neovascular Disease Research Center, Inje University Busan Paik Hospital, Busan, Republic of Korea
[3]Department of Plastic Surgery, Inje University Busan Paik Hospital, Busan, Republic of Korea
[4]Department of Microbiology and Immunology, Inje University College of Medicine, Busan, Republic of Korea
[5]Department of Ophthalmology, Inje University Busan Paik Hospital, Busan, Republic of Korea

Correspondence: Jae Wook Yang, Department of Ophthalmology, Inje University Busan Paik Hospital, Bokjiro-75, Jin-gu, Busan 614-735, Republic of Korea; eyeyang@inje.ac.kr.
Dae Young Hur, Department of Anatomy, Inje University College of Medicine, Bokjiro-75, Jin-gu, Busan 614-735, Republic of Korea; dyhur@inje.ac.kr.

GBP and DK contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: November 11, 2014
Accepted: July 6, 2015

Citation: Park GB, Kim D, Kim YS, et al. Regulation of ADAM10 and ADAM17 by sorafenib inhibits epithelial-to-mesenchymal transition in Epstein-Barr virus–infected retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2015;56:5162–5173. DOI:10.1167/iovs.14-16058

Purpose. The a-disintegrin-and-metalloprotease (ADAM) family proteins are widely expressed in the different layers of the retina throughout development. The effect of ADAM proteins on the epithelial-to-mesenchymal transition (EMT) in proliferative vitreoretinopathy (PVR) or AMD is yet to be elucidated. In this study we used Epstein-Barr virus (EBV)-transformed adult retinal pigment epithelial (ARPE) cells to investigate how sorafenib, a multikinase inhibitor, modulates ADAM proteins to control EMT.

Methods. Epithelial to mesenchymal transition and related mechanisms in EBV-infected ARPE cells were determined by RT-PCR, Western blot, invasion assay, ELISA assay, and gene silencing with siRNA.

Results. Mesenchymal-like ARPE/EBV cells exhibited considerably increased cellular migration and invasion compared with ARPE cells and produced EMT-related cytokines. Sorafenib significantly inhibited production of TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α and blocked the activation of migration-related signaling molecules, such as HIF-1α, p-STAT3, MMP2, and Ang-1. The expression of mature ADAM10, ADAM17, and cleaved Notch 1 proteins in ARPE/EBV cells was downregulated after treatment with sorafenib through the regulatory activity of nardilysin (NRD-1). Gene silencing of NRD-1 in ARPE/EBV cells attenuated secretion of EMT-related cytokines and expression of ADAM10 and 17 and upregulated epithelial markers.

Conclusions. Sorafenib controls the mesenchymal characteristics of EBV-infected ARPE cells. Nardilysin and ADAM family proteins might be new targets for the prevention or control of EMT in retinal diseases.

Keywords: RPE cells, Epstein-Barr virus, sorafenib, epithelial–mesenchymal transition, ADAM, nardilysin

Epithelial to mesenchymal transition (EMT) is an essential morphologic conversion that occurs in adults during wound healing, tumor progression, and organ fibrosis.[1] Epithelial to mesenchymal transition is characterized by the disassembly of cell–cell contacts, remodeling of the actin cytoskeleton, and separation of cells, and generates fibroblast-like cells that express mesenchymal markers and have migratory properties.[2–4] This transition is characterized by loss of epithelial proteins such as E-cadherin and the acquisition of new mesenchymal markers, including vimentin and α-smooth muscle actin (α-SMA).[5]

Epithelial to mesenchymal transition has been linked to the optical conditions of proliferative vitreoretinopathy (PVR) and wet AMD. Proliferative vitreoretinopathy is a dynamic scarring process that develops with some cases of retinal detachment (RD) and is characterized by the formation of fibrotic tissue.[6,7] After detachment from their basement membrane, RPE cells can attach to the vitreous or the retinal surface and undergo EMT. During this process, the RPE cells lose their epithelial morphology and transform into fibroblast-like cells.[8,9] Wet AMD is characterized by the formation of choroidal neovascularization (CNV).[10] Upon CNV, RPE cells have been shown to lose their junctional integrity.[11] Although inflammatory or angiogenesis-related cytokines, such as VEGF and connective tissue growth factor (CTGF), are known to trigger EMT changes, little is known about the molecular mechanisms of EMT in PVR and CNV.

Sorafenib (SRF) (Nexavar; Bayer HealthCare Pharmaceuticals, Inc., Whippany, NJ, USA), the first oral multikinase inhibitor that blocks multiple signaling pathways, was initially approved as an oral agent for the treatment of advanced renal cell carcinoma.[12] Recently, SRF has also been approved for the treatment of hepatocellular carcinoma (HCC).[13] Recent studies show that SRF has antifibrotic activity in vitro. Sorafenib inhibits the activation, growth, and collagen accumulation of hepatic stellate cells (HSCs),[14,15] and also suppresses TGF-β1-induced EMT of HCC through blocking the upregulation of Snail and recovering the expression of E-cadherin.[16,17] Signal transducer and activator of transcription 3 (STAT3) cooperates

Copyright 2015 The Association for Research in Vision and Ophthalmology, Inc.

Downloaded from iovs.arvojournals.org on 07/01/2019

Table. Specific Primer Sequences Used for RT-PCR

Target	Primers, 5′ → 3′	
	Sense	Antisense
EBNA1	GAGCGGGGAGATAATGTACA	TAAAAGATGGCCGGACAAGG
EBNA2	AACCCTCTAAGACTCAAGGC	ACTTTCGTCTAAGTCTGCGG
LMP1	CACGACCTTGAGAGGGGCCCA	GCCAGATGGTGGCACCAAGTC
LMP2A	ATGACTCATCTCAACACATA	CATGTTAGGCAAATTGCAAA
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC
TGF-β	GGACACCAACTATTGCTTCAG	TCCAGGCTCCAAATGTAGG
HIF-1α	TGATTGCATCTCCATCTCCTACC	GACTCAAAGCGACAGATAACACG
VEGF	AGGAGGGCAGAATCATCACG	CAAGGCCCACAGGGATTTTCT
STAT3	ACCTGCAGCAATACCATTGAC	AAGGTGAGGGACTCAAACTGC
MCP-1	AATGCCCCAGTCACCTGCTGTTAT	GCAATTTCCCCAAGTCTCTGTATC
PEDF	CAGAAGAACCTCAAGAGTGCC	CTTCATCCAAGTAGAAATCC
N-cadherin	CACCCAACATGTTTACAATCAACAATGAGAC	CTGCAGCAACAGTAAGGACAAACATCCTATT
E-cadherin	GACGCGGACGATGATGTGAAC	TTGTACTGTTGTGGATTGAAG
Vimentin	GGAAGAGAACTTTGCCGTTGAA	GTGACGAGCCATTTCCTCCTT
ZO-1	CCAGAATCTCGGAAAAGTGC	ACCGTGTAATGGCAGACTCC
α-SMA	ATCACCATCGGAAATGAACG	CTGGAAGGTGGACAGAGAGG
Snail	CAGATGAGGACAGTGGGAAAGG	ACTCTTGGTGCTTGTGGAGCAG
MMP2	TGGCAAGTACGGCTTCTGTC	TGGCAAGTACGGCTTCTGTC
β-actin	ATCCACGAAACTACCTTCAA	ATCCACACGGAGTACTTGC

with hypoxia-inducible factor 1-alpha (HIF-1α), and the subsequent accumulation of HIF-1α in the nucleus upregulates Twist-related protein 1 (TWIST1) and TGF-β1 expression.[18,19] Interestingly, HSCs show increased expression of the sheddases a-disintegrin-and-metalloprotease 10 (ADAM10) and ADAM17 that are associated with the severity of liver fibrosis in patients with chronic liver diseases.[20,21] The ADAM family is the major protein family that mediates ectodomain shedding through activity similar to that of α-secretase.[22] Both ADAM10 and ADAM17 are widely expressed in the different layers of the retina throughout the whole embryonic period, whereas ADAM12 is mainly expressed in the ganglion cell layer at a later stage.[23] The ADAM10 activated by TGF-β1 specifically cleaves E-cadherin in its ectodomain.[24] Furthermore, the shedding of E-cadherin by ADAM10 modulates the subcellular localization of β-catenin and its downstream signaling.[25] Both ADAM10 and ADAM17 have an important role in maintaining the epidermal integrity through regulation of Notch-mediated signaling.[26,27] Nardilysin (N-arginine dibasic convertase [NRD-1]), a metalloendopeptidase of the M16 family, promotes releasing of the precursor forms of various growth factors and cytokines by enhancing the protease activities of ADAM proteins.[28] However, the role of ADAM family proteins or NRD-1 in PVR or CNV and the relationship between EMT and changes in EMT-related proteins in RPE cells are not yet fully understood.

Ocular manifestations of Epstein-Barr virus (EBV) infection as a cause of intraocular inflammation are rare and typically mild[29]; however, they can involve various parts of the eye, including the conjunctiva, cornea, retina, uvea, and optic nerve.[30,31] We previously developed EBV-infected human corneal epithelial cells (EBV-HCECs) as a model of viral keratitis.[32] The EBV-HCECs exhibit morphologic change into a spindle-like shape and express several mesenchymal markers.[32] In this study, we established adult retinal pigment epithelial (ARPE) cells that secrete TGF-β and VEGF after transformation by EBV infection. These cells lose expression of E-cadherin and N-cadherin, which is the most common adherens junction (AJ) proteins in RPE cells.[33,34] They also gain expression of mesenchymal markers, including vimentin and α-SMA. We investigated the molecular mechanisms of EMT in PVR or CNV conditions using these EBV-transformed ARPE cells as model of RD or AMD. We also focused on the development of new possible treatments or therapeutic targets by investigating the effects of the multikinase inhibitor SRF on the regulation of ADAM proteins and EMT-triggering cytokines.

Materials and Methods

Cell Culture and Reagents

Primary human retina pigment epithelial (HRPEpi) cells were purchased from ScienCell (Carlsbad, CA, USA). A human retinal pigment epithelial cell line, ARPE-19, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DME/F12 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone) and antibiotics under a humidified atmosphere with 5% CO_2. Sorafenib (BAY43-9006, Nexavar) was purchased from LC Laboratories (Woburn, MA, USA). GI254023X, Marimastat, and ONO4817 were purchased from TOCRIS Biosciences (Bristol, UK).

Preparation of EBV Virions and Generation of EBV-Infected HRPEpi and ARPE-19 Cells

Cell-free EBV virions were prepared from the B95-8 cell line (EBV type I; ATCC), which has been described previously.[32] After HRPEpi and ARPE-19 cells (2×10^5 cells/T25 flask/4 mL media) were completely attached, an equal volume of EBV supernatant (4 mL, 73 ± 11 colony-forming units per milliliter) was added. The cultures were incubated for durations of 1 day to 4 weeks.

Reverse-Transcription PCR

After infection for 2 weeks, total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA was transcribed into cDNA using oligo(dT) primers (Bioneer, Daejeon, Korea) and reverse transcriptase. To investigate the expression of EBV genes in EBV-infected ARPE-19 and parental ARPE-19 cells, PCR amplification was performed using specific primer sets (Table; Bioneer) and Prime Taq Premix (GeNet Bio, Chungnam, Korea). The PCR products were analyzed by

Downloaded from iovs.arvojournals.org on 07/01/2019

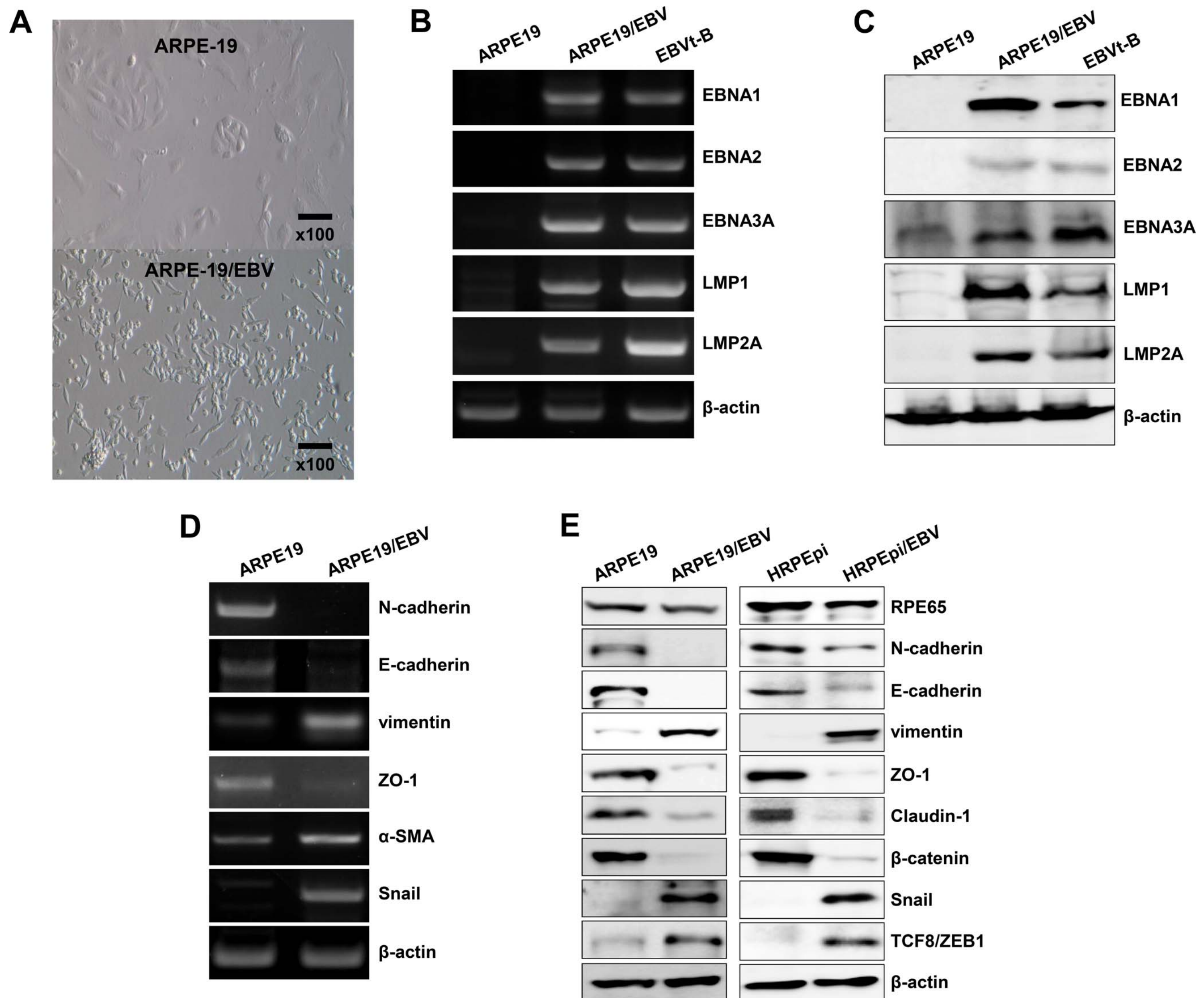

FIGURE 1. Epstein-Barr virus induces EMT-like transformation in the human RPE cell line ARPE-19. (**A**) Mesenchymal morphology elicited by EBV infection. Morphology was observed under an inverted phase-contrast microscope. Magnification bar is 100 μm. Photographs were taken at ×100 magnification using a digital camera (Olympus, Tokyo, Japan). (**B**, **C**) Messenger RNA (**B**) and protein (**C**) levels of EBV-related gene expression in EBV-infected and uninfected ARPE-19 cells measured using RT-PCR and Western blotting, respectively. Epstein-Barr virus–transformed B cells were used as a positive control. (**D**, **E**) Effect of EBV infection on expression of epithelial and mesenchymal markers, as analyzed by RT-PCR (**D**) and Western blotting (**E**). The HRPEpi is a human primary retina epithelial cell; RPE65 is an RPE-specific marker; β-actin served as the loading control. Data are representative of three independent experiments.

agarose gel electrophoresis and visualized with ethidium bromide under UV light using the multiple Gel DOC system (Fujifilm, Tokyo, Japan). Data were analyzed using ImageJ 1.38 software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Experiments were performed in triplicate.

Immunoblotting

After infection for 4 weeks, cells were harvested, lysed, and subjected to SDS-PAGE and immunoblotting using standard techniques. Primary antibodies against the following proteins were used: phospho-Stat3 (Tyr^{705}), Stat3, HIF-1α, metalloproteinase (MMP)-2, MMP-9, E-cadherin, N-cadherin, *β*-catenin, Vimentin, Snail, TCF8/Zeb1, PARP, ZO-1, Claudin-1, cleaved Notch, and *β*-actin (Cell Signaling Technology, Beverly, MA, USA); phospho-Fyn (Thr^{12}), Epstein-Barr nuclear antigen (EBNA)-2, EBNA-3A, latent membrane protein (LMP)-1, LMP-2A, Ang-1, VEGF, nardilysin, ADAM10, ADAM12, and ADAM17 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); EBNA-1 (Thermo Scientific, Rockford, IL, USA); RPE65 and PEDF (Abcam, Cambridge, UK); and *β*-tubulin (BD Biosciences, San Diego, CA, USA).

Enzyme-Linked Immunosorbent Assay

At 24 hours, the conditioned media were collected and the concentration of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 secreted by ARPE-19 or EBV-infected ARPE-19 cells was measured using the Single Analyte ELISArray Kit (Qiagen) according to the manufacturer's instructions. The VEGF, TNF-α, and active TGF-β1 were quantified using the Single Cytokine ELISA Assay Kit (R&D Systems, Minneapolis, MN, USA). Data are expressed as the mean value for biological replicates ± SD.

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

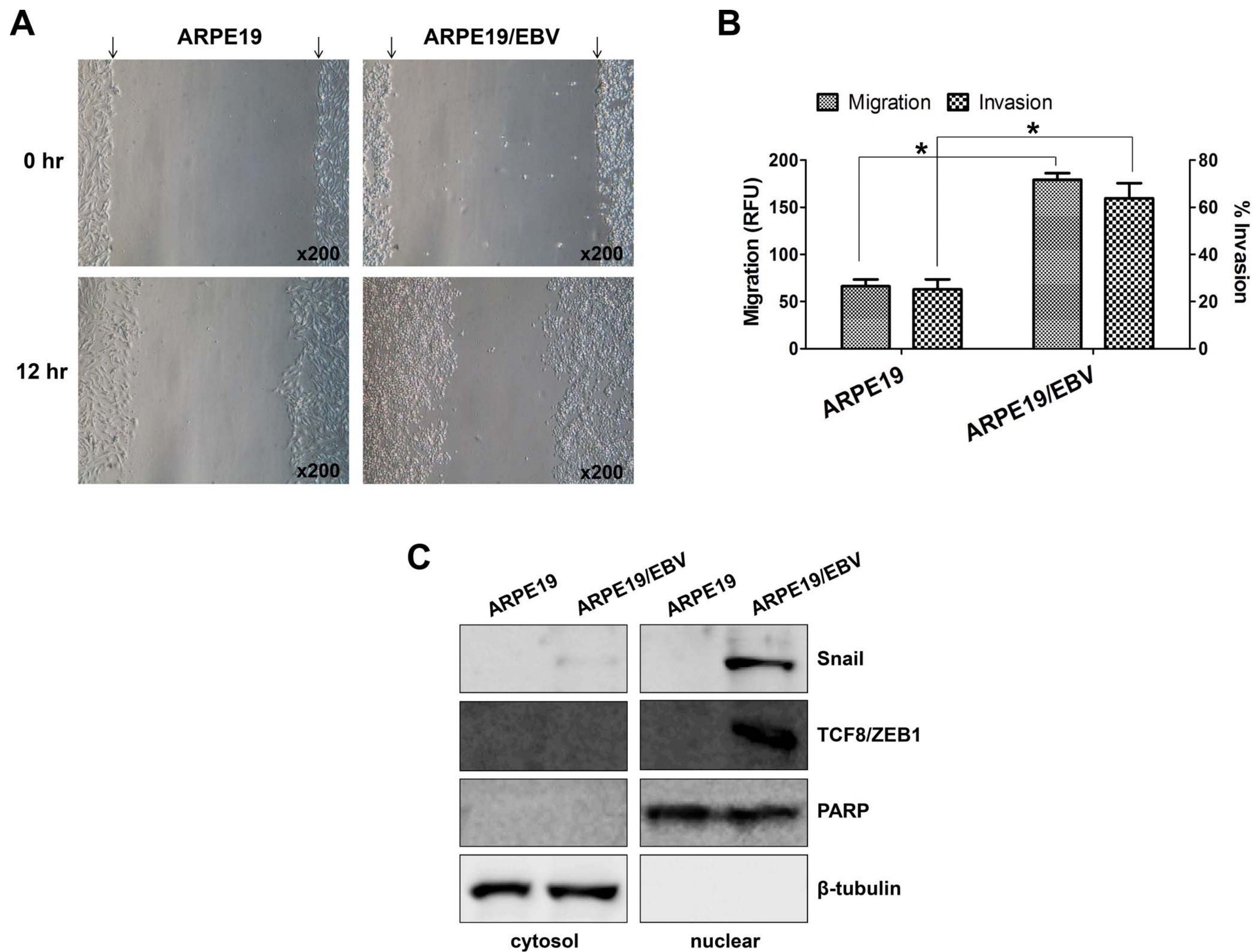

FIGURE 2. Epstein-Barr virus increases cell migration and invasion and induces nuclear translocation of Snail and TCF8/Zeb1. (**A**) Cell motility of ARPE-19 cells was increased by EBV infection as measured with a wound-healing assay. Epstein-Barr virus–infected and uninfected ARPE-19 cells were wounded (0 h) and maintained for 12 hours in complete medium. *Arrows* point to the edges of the wounds. Wound closure (measured after 12 hours) was faster in EBV-infected ARPE-19 cells than in uninfected ARPE-19 cells. (**B**) The migratory capacity of ARPE-19 cells was increased by EBV infection, as determined by a transwell migration assay kit. Epstein-Barr virus also enhanced invasiveness, as detected by a BME cell invasion assay kit. Each value is the mean ± SD of three determinations. *$P < 0.05$. (**C**) Cytosolic extracts (*left*) or nuclear extracts (*right*) were analyzed by Western blotting using antibodies against Snail and TCF8/Zeb1. The nuclear marker PARP and the cytosol marker β-tubulin were used to check the purity of each fraction. Data are representative of three independent experiments.

Wound Healing and E-Cadherin Repressors Assay

Wound-healing assays were performed to measure the migration ability of ARPE-19 cells. Epstein-Barr virus–infected or uninfected ARPE-19 cells were plated in six-well plates. After the cell layers had reached confluence, we inflicted a uniform wound, such as a straight line in each well using a 200-μL micropipette tip and washed the wounded layers with PBS to remove all cell debris. The cells were cultured in 5% CO_2 at 37°C, and images were taken at 0 and 24 hours after scratching using an inverted phase-contrast microscope (Olympus, Tokyo, Japan) at ×100 magnification. After infection for 4 weeks, cytosol and nuclear cellular fractions were prepared to detect E-cadherin repressor proteins using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. All fractions were stored at –80°C until use. Cytosolic extracts or nuclear extracts were analyzed by Western blotting using antibodies against Snail and TCF8/Zeb1.

Detection of Migration and Invasion

After infection for 4 weeks, transendothelial migration of EBV-infected ARPE-19 cells was detected using a CytoSelect Tumor Transendothelial Migration Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions. The fluorescence (in relative fluorescence units [RFU]) of migrated cells was measured using a microplate reader. The invasion assay was performed using the CultureCoat 96-well Medium BME Cell Invasion Assay Kit (R&D Systems) according to the manufacturer's instructions. Invaded cells were stained with calcein AM and quantified using a microplate reader.

Statistical Analysis

Data were expressed as the mean ± SD. Statistical analysis was conducted using one-way ANOVA. A P value less than 0.05 was considered to be statistically significant.

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

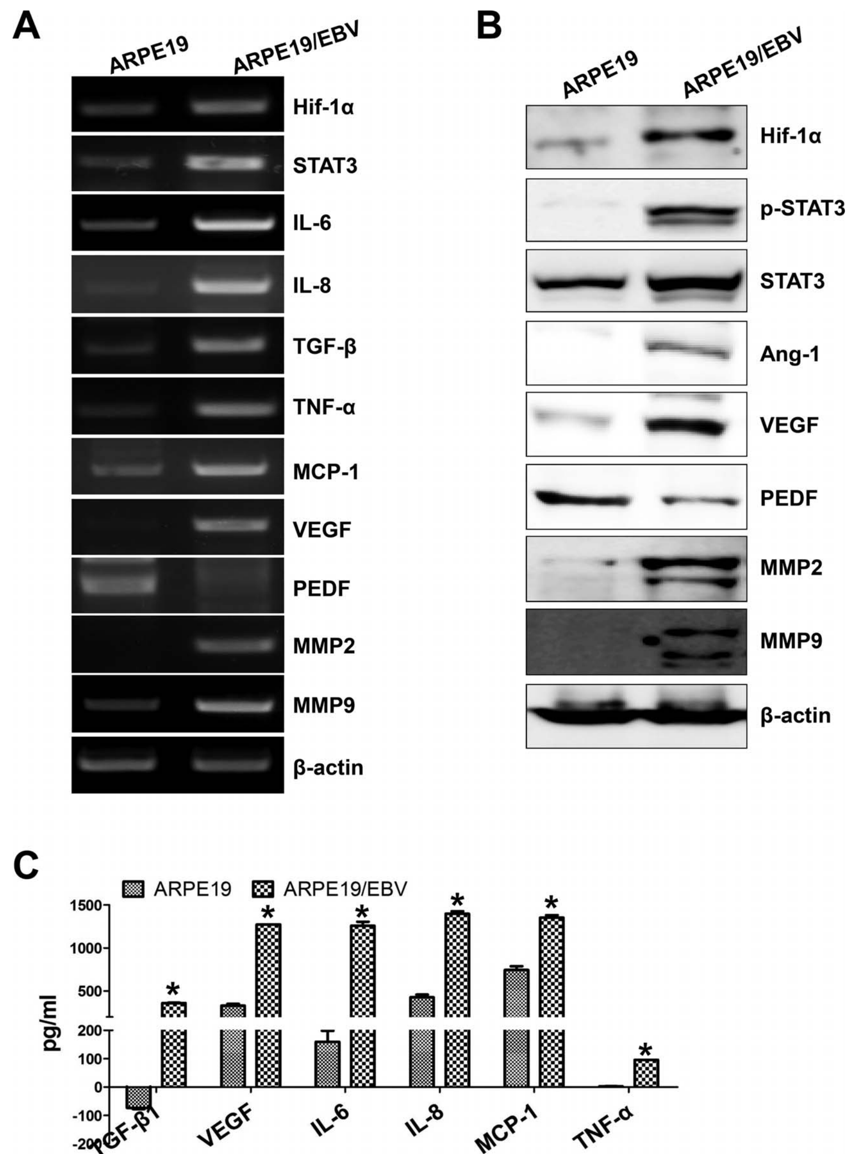

FIGURE 3. Epstein-Barr virus promotes the production of proinflammatory cytokines through upregulation of various signaling molecules. (**A**, **B**) Total RNA and protein were extracted from EBV-infected and uninfected ARPE-19 cells. Reverse-transcriptase PCR (**A**) and Western blot (**B**) analysis of HIF-1α, STAT3, VEGF, PEDF, IL-6, IL-8, TNF-α, TGF-β, MCP-1, Ang-1, MMP2, and MMP9 were performed; β-actin served as the loading control. (**C**) Concentrations of TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α in the culture supernatants of ARPE-19 and ARPE/EBV cells were quantified by ELISA assay. Cells were seeded into six-well plates (1×10^5/well) and incubated for 24 hours. *$P < 0.05$. Data are presented as the mean of three independent experiments and error bars represent SDs of the means.

RESULTS

ARPE Cells Show Mesenchymal Characteristics and Enhanced Migration and Invasion After EBV Infection

We developed EBV-infected HRPEpi (HRPEpi/EBV) and ARPE-19 (ARPE/EBV) cells to determine whether ARPE cells similarly show EMT after transformation with EBV. As expected, EBV-infected RPE cells (HRPEpi/EBV and ARPE/EBV) acquired a fibroblast-like, mesenchymal appearance (Fig. 1A; Supplementary Fig. S1A) and stably expressed EBV-related viral mRNA or proteins, including EBNA1, EBNA2, EBNA3A, LMP1, and LMP2A (Figs. 1B, 1C; Supplementary Fig. S1B). The expression of mRNA and proteins encoding epithelial markers (such as E-cadherin, ZO-1, claudin-1, and β-catenin) was downregulated in HRPEpi/EBV and ARPE/EBV cells, whereas mesenchymal markers (such as Vimentin and Snail) were upregulated (Figs. 1D, 1E). The expression of N-cadherin, the most common AJ proteins in ARPE cells, was reduced at mRNA and protein levels after infection with EBV (Figs. 1D, 1E). Next, we examined whether the mesenchymal characteristics of ARPE/EBV cells were associated with enhanced cell migration and invasion. A scratch assay and migration chamber assay revealed that cellular migration and invasion activities were enhanced approximately 2-fold in ARPE/EBV cells compared with those of ARPE cells (Figs. 2A, 2B). We also observed that the E-cadherin repressors Snail and TCF8/ZEB1 were present in the nuclear fraction of ARPE/EBV, but not ARPE cells (Fig. 2C). Our data suggest that the mesenchymal-like ARPE cells generated by EBV infection acquire EMT phenotypes and could be an in vitro model of pathologic PVR and CNV conditions.

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

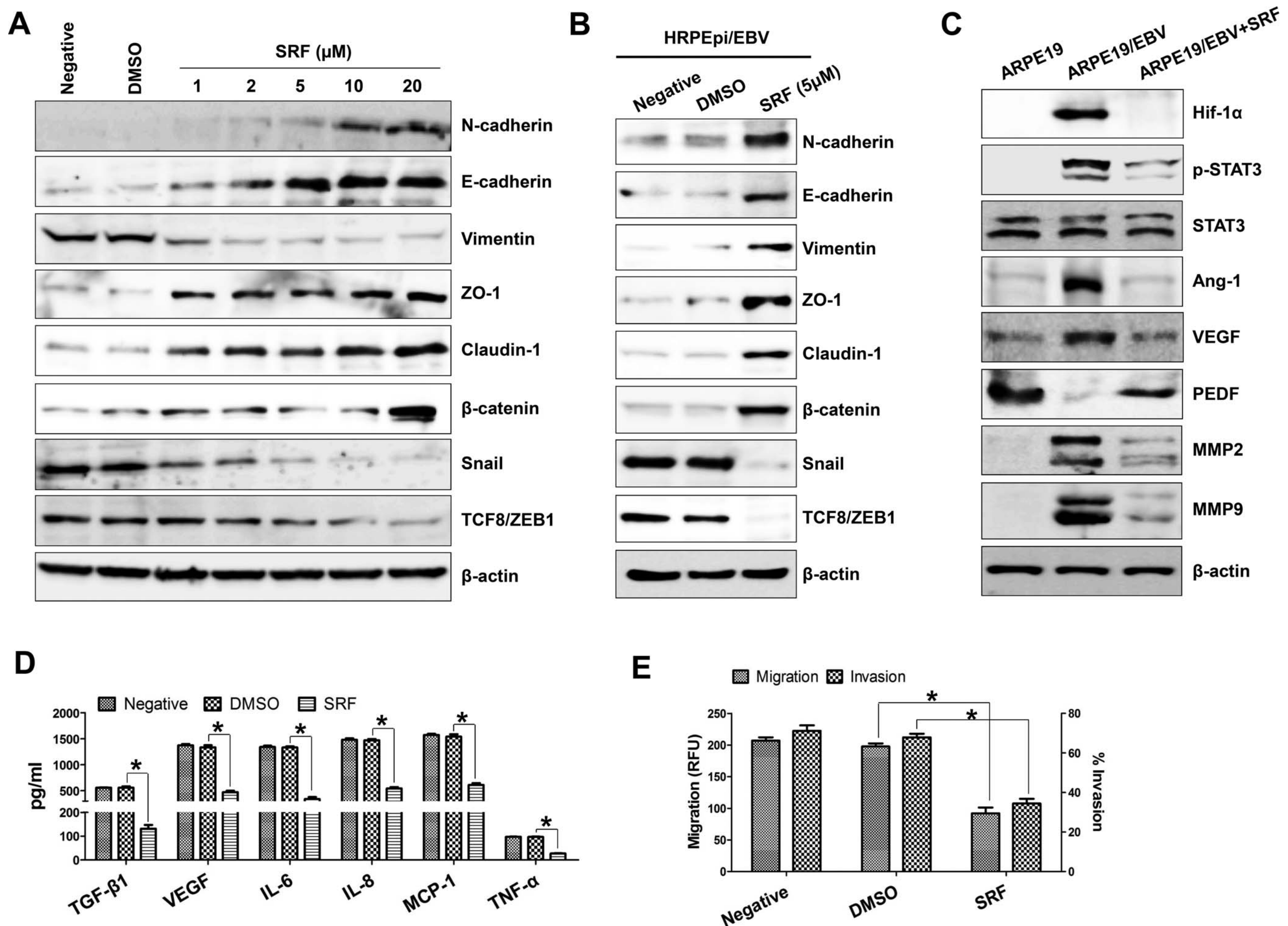

FIGURE 4. Sorafenib inhibits the EMT through regulation of cytokine secretion and related signaling pathways. (**A**) The ARPE/EBV cells were treated with 1, 2, 5, 10, and 20 μM SRF for 24 hours. Cells were harvested and Western blotting was performed using an antibody to EMT markers; β-actin served as the loading control. (**B**) The HRPEpi/EBV cells were treated with 5 μM SRF for 24 hours. The cells were collected and immunoblotting was performed using an antibody to EMT markers; β-actin served as the loading control. (**C**) Expression of EBV-induced proinflammatory and proangiogenic signaling molecules was inhibited by SRF treatment. Cells of ARPE-19, DMSO-treated ARPE/EBV, and SRF-treated (5 μM, 24 hours) ARPE/EBV were harvested and subjected to Western blot analysis with the indicated antibodies. (**D**) After treatment with 5 μM SRF, concentrations of TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α in the culture supernatants of ARPE19/EBV cells were quantified by ELISA assay. Cells were seeded into six-well plates (1×10^5/well) and incubated for 24 hours. $^*P < 0.05$. Data are presented as the mean of three independent experiments and error bars represent SDs of the means. (**E**) The migratory capacity and invasiveness of ARPE/EBV cells was inhibited by SRF as detected by transwell migration assay and BME cell invasion assay. $^*P < 0.05$. Each value is the mean ± SD of three determinations.

ARPE/EBV Cells Produce EMT-Related Cytokines Through Upregulation of Various Signaling Molecules

To determine the effect of inflammatory or angiogenesis-related cytokines on EMT in ARPE/EBV cells, we compared the expression level of these cytokines in ARPE/EBV and ARPE cells. First, to investigate whether EBV infection affects the cellular expression of signaling proteins involved in the induction of EMT-related cytokines, we examined the mRNA and protein levels of HIF-1α, phosphorylated STAT3 (p-STAT3), MMP-2, and MMP-9. The mRNA and protein levels of HIF-1α, p-STAT3, Angiopoietin-1 (Ang-1), MMP2, and MMP9 were upregulated concomitant with VEGF production in ARPE/EBV cells compared with the expression level of these molecules in ARPE cells (Figs. 3A, 3B). Next, culture media of each cell line were collected and the concentration of secreted TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α was measured using a sandwich ELISA method. The TGF-β1 and TNF-α, critical cytokines for EMT, were barely detectable in the culture media of ARPE cells. However, the production of these cytokines by ARPE/EBV cells was approximately 2- to 5-fold higher than that in ARPE cells (Figs. 3A, 3C). Our data suggest that ARPE/EBV cells unconventionally produce EMT-related cytokines and signaling molecules to regulate cell migration and invasion.

Sorafenib Inhibits the EMT Through Regulation of Cytokine Secretion and Related Signaling Pathways

To investigate whether SRF affects the EMT characteristics of ARPE/EBV cells and related signaling pathways, we first conducted dose-response experiments to investigate the effect of SRF on retinal vascularization using a mouse model of laser-induced neovascularization. Treatment with intravitreal SRF at daily interval for 5 days considerably inhibited the neovascularization (Supplementary Fig. S2). Next, we examined the changes in epithelial or mesenchymal markers in the presence of SRF. In ARPE/EBV cells, SRF markedly recovered expression

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

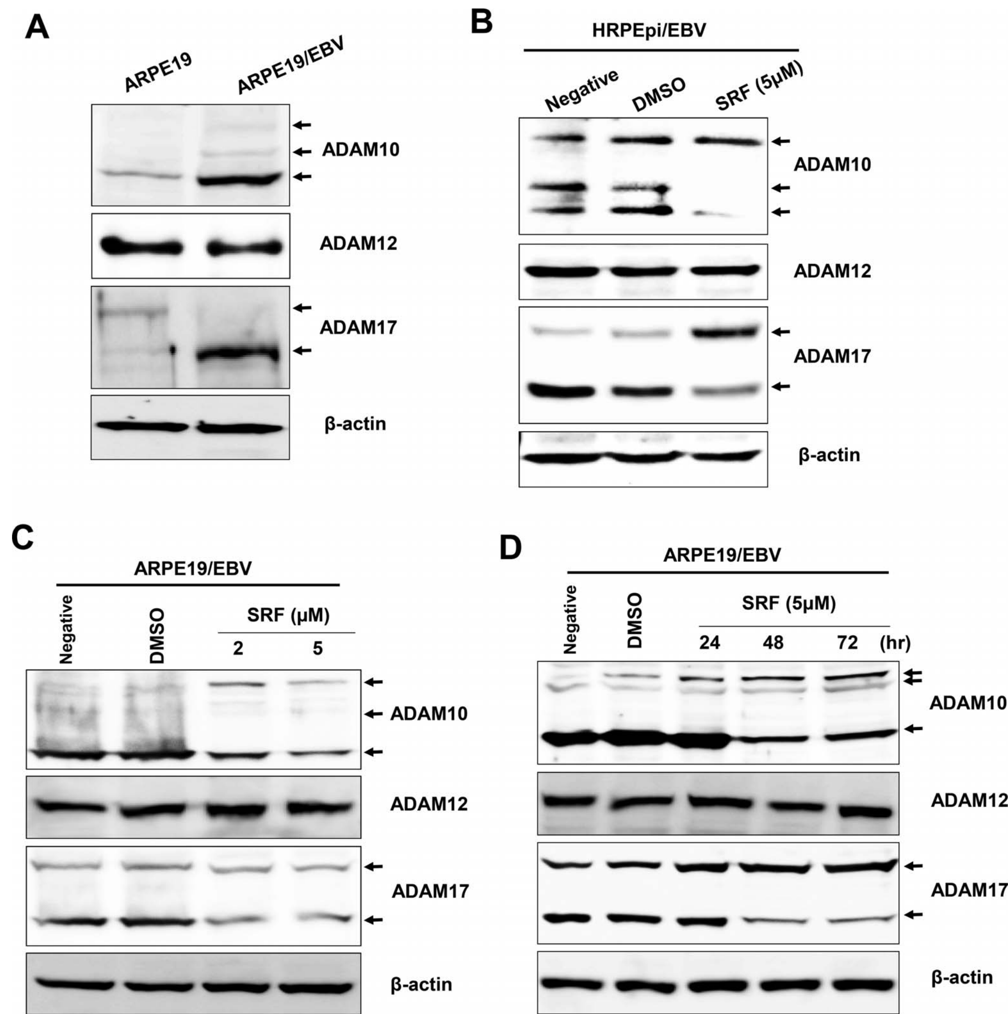

FIGURE 5. Epstein-Barr virus activates ADAM10 and ADAM17 in ARPE19 cells. (**A**) Epstein-Barr virus–infected and uninfected ARPE19 cells were harvested and subjected to Western blot analysis with the indicated antibodies. Epstein-Barr virus infection increased the activity of ADAM10 and ADAM17, but not ADAM12. (**B**) Effect of SRF on the activity of ADAM family proteins in HRPEpi/EBV cells. Sorafinib efficiently blocked the expression of mature ADAM10 and ADAM17. The ARPE/EBV cells were treated with 2 μM and 5 μM SRF for 24 hours (**C**), and with 5 μM SRF for 24, 48, and 72 hours (**D**). After SRF treatment, cells were harvested and subjected to Western blot analysis with the indicated antibodies; β-actin served as the loading control. Data are representative of three independent experiments.

of epithelial markers such as E-cadherin, N-cadherin, ZO-1, claudin-1, and β-catenin, whereas the expression of mesenchymal markers, including vimentin, Snail, and TCF/ZEB1, decreased in a dose-dependent manner (Fig. 4A). Sorafenib also reversed the epithelial characteristics of EBV-infected primary human retina pigment cells (HRPEpi/EBV) (Fig. 4B). Migration-related signaling pathways involving HIF-1α, p-STAT3, MMP-2, MMP-9, and Ang-1 were also blocked after treatment of ARPE/EBV cells with SRF (Fig. 4C). Productions of EMT-related cytokines, including TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α were significantly inhibited after treatment with SRF (Fig. 4D). Migration activity was also blocked after treatment of ARPE/EBV cells with SRF (Fig. 4E). Our data suggest that SRF might control the effect of inflammatory or angiogenesis-related cytokines on EMT in ARPE/EBV cells.

ADAM10 and 17 Control the Mesenchymal Phenotype and Cell Migration Activity of ARPE/EBV Cells

Although the expression of ADAM in the retina during development has been extensively investigated, the role of ADAM family proteins on AMD or RD is still poorly understood. To examine whether SRF affects the activity of ADAM family proteins, we analyzed the expression of ADAM proteins in ARPE/EBV cells by immunoblot analysis. Expression of the mature forms of ADAM10 and ADAM17, a close homologue of ADAM10, was significantly increased in ARPE/EBV cells compared with ARPE cells (Fig. 5A). The expression of mature ADAM10 and ADAM17 was blocked in SRF-treated HRPEpi cells (Fig. 5B). Treatment with SRF also downregulated the generation of cleaved form ADAM10 and ADAM17 in a dose-dependent manner at 48 hours in ARPE/EBV cells (Figs. 5C, 5D). Next, to investigate whether inhibition of ADAM protein activity influences secretion of EMT-related cytokines and EMT characteristics of ARPE/EBV cells, we cultured ARPE/EBV cells in the presence of various ADAM inhibitors, MMP inhibitors, and SRF. GI254023X (ADAM10 inhibitor, 10 μM) and Marimastat (ADAM17 inhibitor, 50 nM) recovered the expression of epithelial markers and downregulated expression of mesenchymal markers compared with nontreated ARPE/EBV cells. Treatment with SRF or ONO4817 (MMP inhibitor) also blocked the EMT characteristics of ARPE/EBV cells (Fig. 6A). Inhibitors of ADAM10 and 17 modulated cytokine secretion and

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

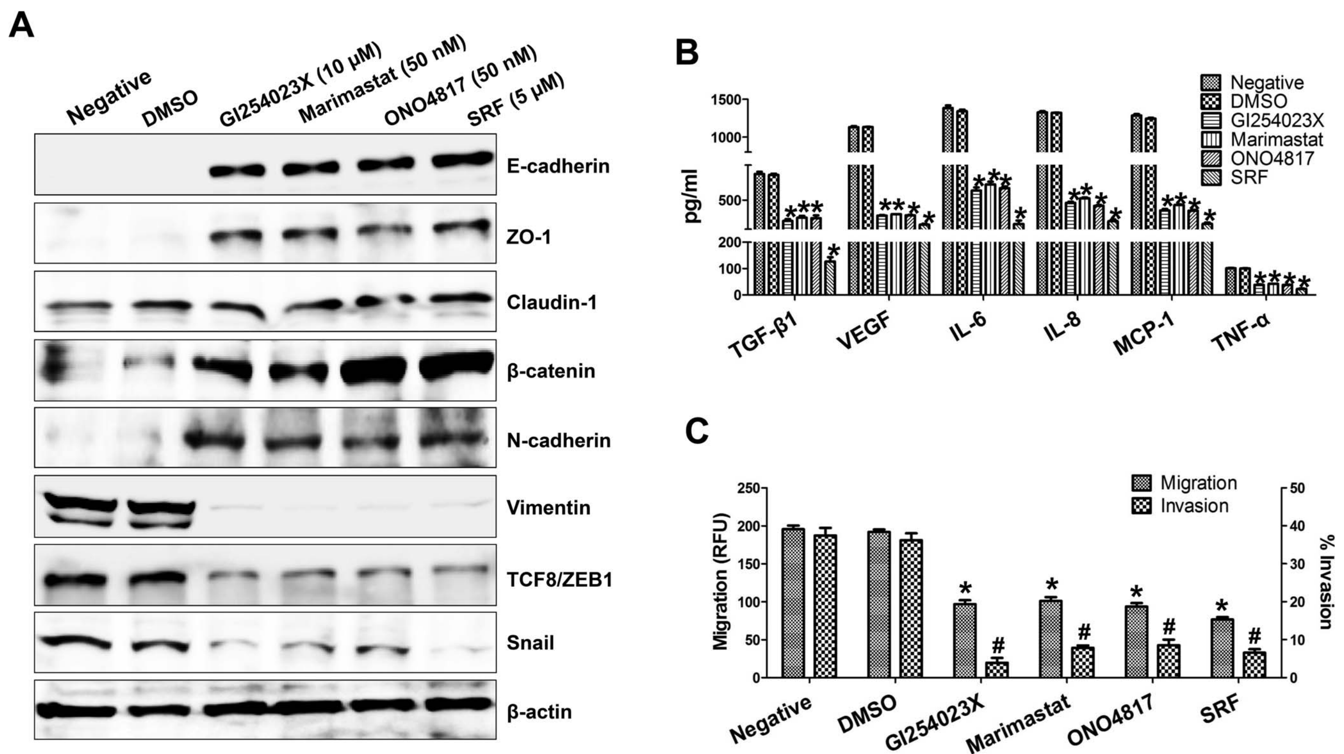

FIGURE 6. Both ADAM10 and ADAM17 regulate mesenchymal phenotype and cell migration activity of ARPE/EBV cells. The ARPE/EBV cells were preincubated with the ADAM10 inhibitor GI254023X (10 μM), the ADAM17 inhibitor Marimastat (50 nM), the pan-MMP inhibitor ONO4817 (50 nM or 5 μM) for 24 hours. (**A**) Western blots were performed using the indicated antibodies; β-actin served as the loading control. (**B**) Concentrations of TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α in the culture supernatants of ARPE/EBV cells were quantified by ELISA assay. Cells were seeded into six-well plates (1×10^5/well) and incubated for 24 hours. $^*P < 0.05$. Data are presented as the mean of three independent experiments and error bars represent SDs of the means. (**C**) The migratory capacity and invasiveness of ARPE/EBV cells were inhibited by GI254023X, Marimastat, ONO4817, or SRF as detected by transwell migration assay and BME cell invasion assay. $^*P < 0.05$. Each value is the mean ± SD of three determinations.

migration activity in ARPE/EBV cells (Figs. 6B, 6C). Together, our data suggest that SRF controls the EMT activity of ARPE/EBV cells through regulation of ADAM family proteins.

Knockdown of NRD-1 Expression Attenuates Expression of ADAM10 and ADAM17 and the Mesenchymal Characteristics of ARPE/EBV Cells

To investigate whether SRF affects the activity of ADAM family proteins through regulation of NRD-1 in ARPE/EBV cells, we first showed that the expression of NRD-1 was increased by infection with EBV, but was downregulated after treatment with SRF (Figs. 7A, 7B). Next, we analyzed the expression of ADAM proteins after silencing NRD-1 with small-interfering RNA (siRNA) in ARPE/EBV cells and showed that the expression of mature ADAM10 and ADAM17 was downregulated (Fig. 7C), whereas expression of epithelial markers was recovered (Fig. 7D). Gene silencing of ADAM10 and ADAM17 also upregulated the epithelial characteristics of ARPE/EBV cells (Fig. 7E). Production of EMT-related cytokines (TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α) was significantly attenuated (Fig. 7F) and migration and invasion activities were decreased (Fig. 7G) after NRD-1 knockdown in ARPE/EBV cells. We also observed that overexpressing ADAM10 and ADAM17 in ARPE/EBV cells induced the expression of cleaved Notch (Fig. 7A); however, SRF or transfection with siRNA-ADAM10 and siRNA-ADMA17 efficiently blocked the expression of cleaved Notch (Figs. 7B, 7E). Our data suggest that SRF modulates ADAM family activity through regulation of NRD-1 expression in ARPE/EBV cells.

DISCUSSION

Proliferative vitreoretinopathy is a common complication of posterior segmental ocular trauma and surgical procedures. Epithelial to mesenchymal transition of RPE cells is the key event leading to visual impairment after retinal damage. Additionally, CNV associated with wet AMD is a leading cause of irreversible blindness.[10] In this study, we developed EBV-infected ARPE19 cells as a model of retinal disease to investigate the effect of several drugs on PVR or wet AMD in vitro. During EMT, epithelial cells lose intracellular junctions, dissociate from surrounding cells, acquire mesenchymal-like characteristics, and become able to migrate away from their original location.[35] Migration of RPE cells is the major cause of severe eye diseases such as PVR, an ocular fibrotic disease. Recently, several studies have revealed the exact molecular mechanisms of EMT in RPE cells after exposure to various cytokines.[36–39] There is also clinical and experimental evidence that RPE and glial cells contribute to the final outcome of PVR.[40,41] Proliferative vitreoretinopathy involves a process of fibrocellular proliferation in the vitreous cavity and on both surfaces of the retina that may lead to the formation of contractile epiretinal membranes.[7,42,43] Fibrotic changes in the foveal CNV lesion result in permanent visual impairment in patients with wet AMD.[44] Inflammatory or angiogenesis-related cytokines, such as VEGF, CTGF, TNF-α, and TGF-β, which are known to trigger EMT changes, are expressed in CNV tissues.[45–47] Generally, binding of such ligands to the appropriate receptor upregulates the expression of EMT-regulating transcription factors, including SNAI1, SNAI2, ZEB1, ZEB2, and TWIST.[48] These findings led us to propose that tyrosine kinase

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

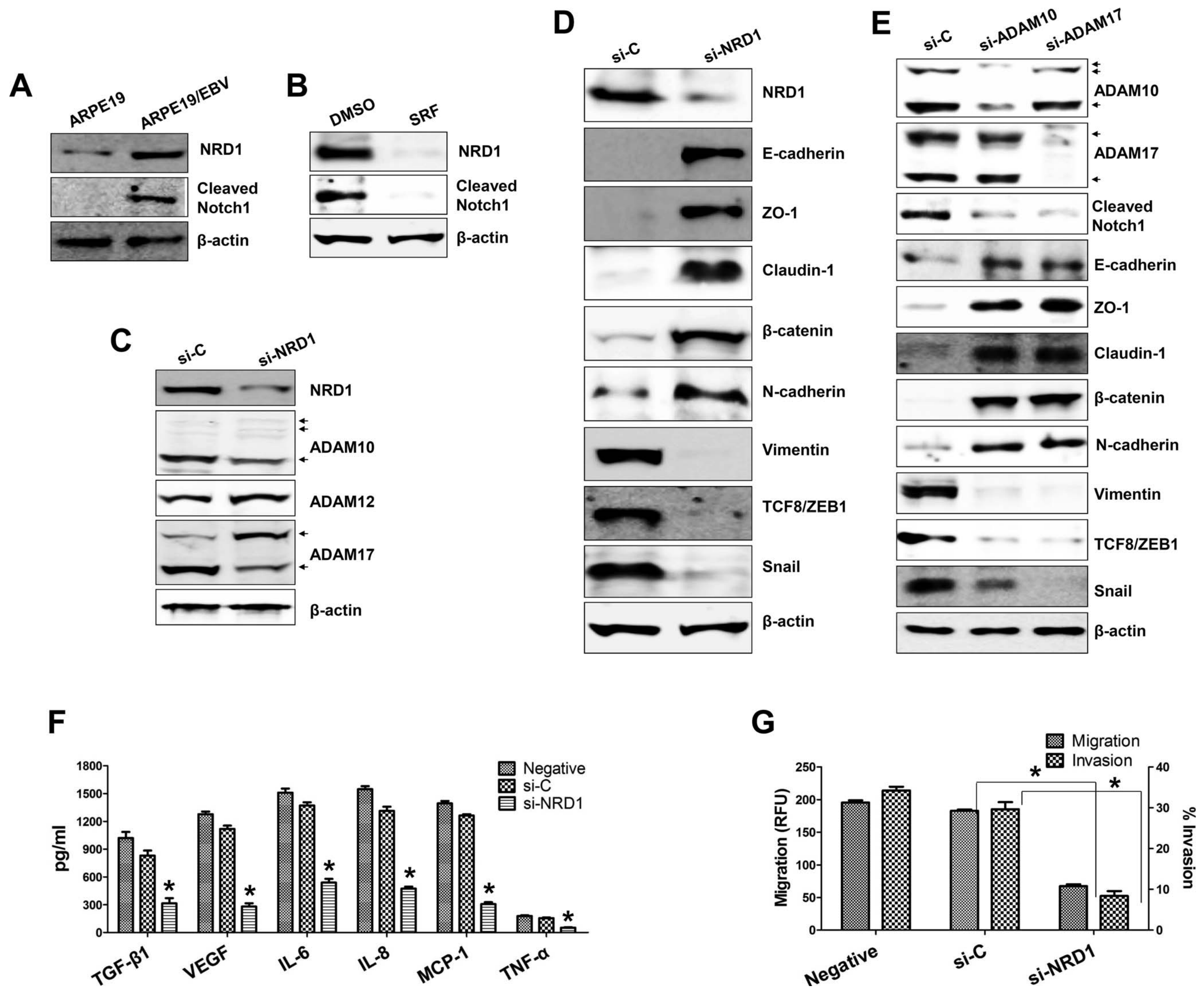

FIGURE 7. Silencing of NRD-1 attenuates ADAM10 and ADAM17 expression and the mesenchymal characteristics of ARPE/EBV cells. (**A**) Epstein-Barr virus infection significantly increased NRD-1 and cleaved Notch1 expression in ARPE19 cells. (**B**) Nardilysin and cleaved Notch1 expression in ARPE/EBV cells was inhibited after treatment with SRF. (**C**, **D**) After transfection with either NRD-1-siRNA or control-siRNA for 48 hours, protein levels of ARPE/EBV cells were detected by Western blotting with antibody to NRD-1, ADAM proteins (**C**), and EMT markers (**D**); β-actin was used as a loading control. (**E**) Gene silencing with ADAM10-siRNA or ADAM17-siRNA not only decreased the expression of mesenchymal markers and cleaved Notch1 but also recovered the expression of epithelial markers in ARPE/EBV cells. (**F**) After transfection with either NRD-1-siRNA or control-siRNA, ARPE/EBV cells were seeded into six-well plates (1×10^5/well) and incubated for 48 hours. Concentrations of TGF-β1, VEGF, IL-6, IL-8, MCP-1, and TNF-α in the culture supernatants of ARPE/EBV cells were quantified by ELISA assay. *$P < 0.05$. Data are presented as the mean of three independent experiments and error bars represent SDs of the means. (**G**) After transfection with either NRD-1-siRNA or control-siRNA for 48 hours, the migratory capacity and invasiveness of ARPE/EBV cells were inhibited by the knockdown of NRD-1 expression as detected by transwell migration assay and BME cell invasion assay. *$P < 0.05$. Each value is the mean ± SD of three determinations.

inhibitors could be used to control the EMT in RPE cells and might inhibit fibrotic scar formation in AMD. Tyrosine kinase inhibitors are particularly attractive agents because they inhibit fibrotic cellular changes and might therefore also prevent PVR.[49,50] Sorafenib is a small molecule that inhibits the kinase activities of Raf-1, B-Raf, VEGFRs, PDGFR-b (platelet-derived growth factor receptor b), Flt-3, and c-KIT.[51] There are also data suggesting that SRF has antifibrotic activities. Sorafenib can inhibit the activation, growth, and collagen accumulation of hepatic HSCs in vitro.[14,15] Sorafenib inhibits the secretion of VEGF, which is associated with progression of AMD in ARPE cells.[52] Astrocytes in optic nerve that are exposed to light also reduce the secretion of VEGF as well as platelet-derived growth factor (PDGF) after treatment with SRF.[53] These results suggest that SRF has effect on the retinal neovascularization in both RPE cell and glial cells of retina. Recently, dasatinib, a tyrosine kinase inhibitor approved by the Food and Drug Administration, was shown to significantly inhibit PVR-related changes in RPE in vitro and prevent traction RD in an experimental PVR model in swine. Dasatinib was also shown to prevent RPE cell migration and EMT.[49] However, these reports did not reveal the mechanisms by which these drugs inhibit PVR-related changes in RPE cells. In the current study, we showed that the multikinase inhibitor SRF controlled migration and invasion of ARPE19/EBV cells by regulating the secretion of EMT-related cytokines and transcription factors. Thus, our data on the inhibition of EMT and cell migration by SRF may provide a possible explanation for its activity in tumor control and

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

reduced cancer metastasis. Transforming growth factor-β can promote EMT during carcinogenesis and enhance the migratory and invasive properties of tumor cells.[54] Sorafenib inhibits STAT3 phosphorylation in a variety of tumors, including medulloblastoma, cholangiocarcinoma, and HCC.[55–57] Moreover, SRF also inhibits TGF-β–induced STAT3 phosphorylation during TGF-β–mediated EMT in mouse hepatocytes.[16] However, although our data showed that migration-related signaling molecules, such as HIF-1α, p-STAT3, and MMP2, were downregulated after treatment with SRF in ARPE/EBV cells, further studies are needed to investigate the precise signaling pathway that initiates the process of EMT in retinal diseases.

Both ADAM10 and ADAM17 (also known as TNF-α–converting enzyme or TACE) show very prominent expression in all epithelial tissues and are required for proper epithelial tissue development in mice.[58,59] Both ADAM10 and ADAM17 are also essential for adult epidermis maintenance through activation of Notch pathway.[26,27] The ADAM10/E-cadherin interaction represents a common regulatory mechanism in inflammatory epidermal diseases, which are characterized by loss of E-cadherin expression and loss of epithelial integrity.[24] In contrast, downregulation of ADAM10 leads to a more scattered cell phenotype, which is accompanied by the induction of Slug and the loss of E-cadherin, as observed during EMT.[60] ADAM10 is coexpressed with classic cadherins in the developing retina.[23] Although ADAM10-mediated E-cadherin shedding generally affects epithelial cell–cell adhesion and cell migration, there are no specific data on the modulation of ADAM10-mediated EMT processes in ocular disease. Our results suggest that ADAM family proteins might be involved in the migration and invasion of RPE cells through the control of E-cadherin expression and secretion of EMT-related cytokines.

Nardilysin is a zinc peptidase of the M16 family that is localized diffusely in the cytoplasm and is secreted to the cell surface by a currently undetermined mechanism.[61,62] Nardilysin binds to the extracellular domain of ADAM17 and directly enhances its catalytic activity.[63,64] In this study, knockdown of NRD-1 downregulated the EMT process in ARPE19/EBV cells through regulation of ADAM proteases and the secretion of EMT-related cytokines. Our results suggest that regulation of NRD-1 and ADAM family proteins by the multikinase inhibitor SRF might be a new therapeutic approach to control PVR or CNV and provide experimental evidence supporting the application of ADAM inhibitors to prevent blindness from other retina-related ocular diseases.

Acknowledgments

Supported by a grant from the Korea Healthcare Technology R&D Project of the Ministry of Health and Welfare Affairs, Republic of Korea (Grant HI12C0005).

Disclosure: **G.B. Park**, None; **D. Kim**, None; **Y.S. Kim**, None; **J.W. Kim**, None; **H. Sun**, None; **K.-H. Roh**, None; **J.W. Yang**, None; **D.Y. Hur**, None

References

1. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest*. 2009;119:1417–1419.
2. Baum B, Settleman J, Quinlan MP. Transitions between epithelial and mesenchymal states in development and disease. *Semin Cell Dev Biol*. 2008;19:294–308.
3. Guan F, Handa K, Hakomori SI. Specific glycosphingolipids mediate epithelial-to-mesenchymal transition of human and mouse epithelial cell lines. *Proc Natl Acad Sci U S A*. 2009; 106:7461–7466.
4. Lee J, Choi JH, Joo CK. TGF-β1 regulates cell fate during epithelial-mesenchymal transition by upregulating survivin. *Cell Death Dis*. 2013;4:e714.
5. Willis BC, Liebler JM, Luby-Phelps K, et al. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol*. 2005;166:1321–1332.
6. Pastor JC, de la Rúa ER, Martín F. Proliferative vitreoretinopathy: risk factors and pathobiology. *Prog Retin Eye Res*. 2002; 21:127–144.
7. Yu J, Liu F, Cui SJ, et al. Vitreous proteomic analysis of proliferative vitreoretinopathy. *Proteomics*. 2008;8:3667–3678.
8. Casaroli-Marano RP, Pagan R, Vilaró S. Epithelial-mesenchymal transition in proliferative vitreoretinopathy: intermediate filament protein expression in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1999;40:2062–2072.
9. Tamiya S, Liu L, Kaplan HJ. Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. *Invest Ophthalmol Vis Sci*. 2010; 51:2755–2763.
10. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. *N Engl J Med*. 2008;358:2606–2617.
11. Bailey TA, Kanuga N, Romero IA, et al. Oxidative stress affects the junctional integrity of retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2004;45:675–684.
12. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med*. 2007;356:125–134.
13. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. 2008;359:378–390.
14. Wang Y, Gao J, Zhang D, et al. New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. *J Hepatol*. 2010;53:132–144.
15. Hong F, Chou H, Fiel MI, Friedman SL. Antifibrotic activity of sorafenib in experimental hepatic fibrosis: refinement of inhibitory targets, dosing, and window of efficacy in vivo. *Dig Dis Sci*. 2013;58:257–264.
16. Chen YL, Lv J, Ye XL, et al. Sorafenib inhibits transforming growth factor β1-mediated epithelial-mesenchymal transition and apoptosis in mouse hepatocytes. *Hepatology*. 2011;53: 1708–1718.
17. Nagai T, Arao T, Furuta K, et al. Sorafenib inhibits the hepatocyte growth factor-mediated epithelial mesenchymal transition in hepatocellular carcinoma. *Mol Cancer Ther*. 2011;10:169–177.
18. Tsuzuki Y, Fukumura D, Oosthuyse B, et al. Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha-> hypoxia response element-> VEGF cascade differentially regulates vascular response and growth rate in tumors. *Cancer Res*. 2000;60:6248–6252.
19. Cho KH, Jeong KJ, Shin SC, et al. STAT3 mediates TGF-β1-induced TWIST1 expression and prostate cancer invasion. *Cancer Lett*. 2013;336:167–173.
20. Bourd-Boittin K, Basset L, Bonnier D, et al. CX3CL1/fractalkine shedding by human hepatic stellate cells: contribution to chronic inflammation in the liver. *J Cell Mol Med*. 2009;13: 1526–1535.
21. Fujita T, Maesawa C, Oikawa K, et al. Interferon-gamma down-regulates expression of tumor necrosis factor-alpha converting enzyme/a disintegrin and metalloproteinase 17 in activated hepatic stellate cells of rats. *Int J Mol Med*. 2006;17:605–616.
22. Allinson TM, Parkin ET, Turner AJ, Hooper NM. ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res*. 2003;74:342–352.

Downloaded from iovs.arvojournals.org on 07/01/2019

23. Yan X, Lin J, Rolfs A, Luo J. Differential expression of the ADAMs in developing chicken retina. *Dev Growth Differ*. 2011;3:726–739.
24. Maretzky T, Scholz F, Köten B, et al. ADAM10-mediated E-cadherin release is regulated by proinflammatory cytokines and modulates keratinocyte cohesion in eczematous dermatitis. *J Invest Dermatol*. 2008;128:1737–1746.
25. Maretzky T, Reiss K, Ludwig A, et al. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc Natl Acad Sci U S A*. 2005;102:9182–9187.
26. Weber S, Niessen MT, Prox J, et al. The disintegrin/metalloproteinase Adam10 is essential for epidermal integrity and Notch-mediated signaling. *Development*. 2011;138:495–505.
27. Murthy A, Shao YW, Narala SR, et al. Notch activation by the metalloproteinase ADAM17 regulates myeloproliferation and atopic barrier immunity by suppressing epithelial cytokine synthesis. *Immunity*. 2012;36:105–119.
28. Kanda K, Komekado H, Sawabu T, et al. Nardilysin and ADAM proteases promote gastric cancer cell growth by activating intrinsic cytokine signaling via enhanced ectodomain shedding of TNF-α. *EMBO Mol Med*. 2012;4:396–411.
29. Matoba AY. Ocular disease associated with Epstein-Barr virus infection. *Surv Ophthalmol*. 1990;35:145–150.
30. Peponis VG, Chatziralli IP, Parikakis EA, et al. Bilateral multifocal chorioretinitis and optic neuritis due to Epstein-Barr virus: a case report. *Case Rep Ophthalmol*. 2012;3:327–332.
31. Kim S, Barañano D, Grossniklaus H, Martin D. Epstein-Barr infection of the retina: case report and review of the literature. *Retin Cases Brief Rep*. 2011;5:1–5.
32. Park GB, Kim D, Kim YS, et al. The Epstein-Barr virus causes epithelial-mesenchymal transition in human corneal epithelial cells via Syk/src and Akt/Erk signaling pathways. *Invest Ophthalmol Vis Sci*. 2014;55:1770–1779.
33. Rak DJ, Hardy KM, Jaffe GJ, McKay BS. Ca++-switch induction of RPE differentiation. *Exp Eye Res*. 2006;82:648–656.
34. Van Aken EH, De Wever O, Van Hoorde L, et al. Invasion of retinal pigment epithelial cells: N-cadherin, hepatocyte growth factor, and focal adhesion kinase. *Invest Ophthalmol Vis Sci*. 2003;44:463–472.
35. Guarino M. Epithelial-mesenchymal transition and tumour invasion. *Int J Biochem Cell Biol*. 2007;39:2153–2160.
36. Bastiaans J, van Meurs JC, van Holten-Neelen C, et al. Thrombin induces epithelial-mesenchymal transition and collagen production by retinal pigment epithelial cells via autocrine PDGF-receptor signaling. *Invest Ophthalmol Vis Sci*. 2013;54:8306–8314.
37. Chen X, Ye S, Xiao W, Luo L, Liu Y. Differentially expressed microRNAs in TGFβ2-induced epithelial-mesenchymal transition in retinal pigment epithelium cells. *Int J Mol Med*. 2014;33:1195–1200.
38. Chen X, Xiao W, Wang W, et al. The complex interplay between ERK1/2, TGFβ/Smad, and Jagged/Notch signaling pathways in the regulation of epithelial-mesenchymal transition in retinal pigment epithelium cells. *PLoS One*. 2014;9:e96365.
39. Xiao W, Chen X, Liu X, et al. Trichostatin A, a histone deacetylase inhibitor, suppresses proliferation and epithelial-mesenchymal transition in retinal pigment epithelium cells. *J Cell Mol Med*. 2014;18:646–655.
40. Charteris DG, Sethi CS, Lewis GP, Fisher SK. Proliferative vitreoretinopathy-developments in adjunctive treatment and retinal pathology. *Eye (Lond)*. 2002;16:369–374.
41. Bringmann A, Wiedemann P. Involvement of Müller glial cells in epiretinal membrane formation. *Graefes Arch Clin Exp Ophthalmol*. 2009;247:865–883.
42. Li H, Kernt H, Wang F, Gu Q, Xu X. Snail involves in the transforming growth factor β1-mediated epithelial-mesenchymal transition of retinal pigment epithelial cells. *PLoS One*. 2011;6:e23322.
43. García S, López E, López-Colomé AM. Glutamate accelerates RPE cell proliferation through ERK1/2 activation via distinct receptor-specific mechanisms. *J Cell Biochem*. 2008;104:377–390.
44. Constable I, Shen WY, Rakoczy E. Emerging biological therapies for age-related macular degeneration. *Expert Opin Biol Ther*. 2005;5:1373–1385.
45. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2004;242:91–101.
46. He S, Jin ML, Worpel V, Hinton DR. A role for connective tissue growth factor in the pathogenesis of choroidal neovascularization. *Arch Ophthalmol*. 2003;121:1283–1288.
47. Oh H, Takagi H, Takagi C, et al. The potential angiogenic role of macrophages in the formation of choroidal neovascular membranes. *Invest Ophthalmol Vis Sci*. 1999;40:1891–1898.
48. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*. 2007;7:415–428.
49. Umazume K, Liu L, Scott PA, et al. Inhibition of PVR with a tyrosine kinase inhibitor, dasatinib, in the swine. *Invest Ophthalmol Vis Sci*. 2013;54:1150–1159.
50. Imai K, Loewenstein A, Koroma B, Grebe R, de Juan E Jr. Herbimycin A in the treatment of experimental proliferative vitreoretinopathy: toxicity and efficacy study. *Graefes Arch Clin Exp Ophthalmol*. 2000;238:440–447.
51. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 2004;64:7099–7109.
52. Kernt M, Neubauer AS, Liegl RG, et al. Sorafenib prevents human retinal pigment epithelium cells from light-induced overexpression of VEGF, PDGF and PlGF. *Br J Ophthalmol*. 2010;94:1533–1539.
53. Kernt M, Liegl RG, Rueping J, et al. Sorafenib protects human optic nerve head astrocytes from light-induced overexpression of vascular endothelial growth factor, platelet-derived growth factor, and placenta growth factor. *Growth Factors*. 2010;28:211–220.
54. Padua D, Massagué J. Roles of TGFbeta in metastasis. *Cell Res*. 2009;19:89–102.
55. Yang F, Van Meter TE, Buettner R, et al. Sorafenib inhibits signal transducer and activator of transcription 3 signaling associated with growth arrest and apoptosis of medulloblastomas. *Mol Cancer Ther*. 2008;7:3519–3526.
56. Blechacz BR, Smoot RL, Bronk SF, et al. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatter proof 2. *Hepatology*. 2009;50:1861–1870.
57. Chen KF, Tai WT, Liu TH, et al. Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. *Clin Cancer Res*. 2010;16:5189–5199.
58. Blobel CP. ADAMs: key components in EGFR signaling and development. *Nat Rev Mol Cell Biol*. 2005;6:32–43.
59. Peschon JJ, Slack JL, Reddy P, et al. An essential role for ectodomain shedding in mammalian development. *Science*. 1998;282:1281–1284.
60. Doberstein K, Pfeilschifter J, Gutwein P. The transcription factor PAX2 regulates ADAM10 expression in renal cell carcinoma. *Carcinogenesis*. 2011;32:1713–1723.
61. Pierotti AR, Prat A, Chesneau V, et al. N-arginine dibasic convertase, a metalloendopeptidase as a prototype of a class of

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 07/01/2019

processing enzymes. *Proc Natl Acad Sci U S A*. 1994;91:6078–6082.

62. Hospital V, Chesneau V, Balogh A, et al. N-arginine dibasic convertase (nardilysin) isoforms are soluble dibasic-specific metalloendopeptidases that localize in the cytoplasm and at the cell surface. *Biochem J*. 2000;349:587–597.
63. Hiraoka Y, Ohno M, Yoshida K, et al. Enhancement of alpha-secretase cleavage of amyloid precursor protein by a metalloendopeptidase nardilysin. *J Neurochem*. 2007;102:1595–1605.
64. Nishi E, Hiraoka Y, Yoshida K, Okawa K, Kita T. Nardilysin enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor through activation of tumor necrosis factor-alpha-converting enzyme. *J Biol Chem*. 2006;281:31164–31172.

Downloaded from iovs.arvojournals.org on 07/01/2019