Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 January ; 50(1): 462–469. doi:10.1167/iov.07-1598.

FAK Activation and the Role of Epithelial Membrane Protein 2 (EMP2) in Collagen Gel Contraction

Shawn A. Morales^{1,2}, Sergey Mareninov², Madhuri Wadehra¹, Lily Zhang¹, Lee Goodglick¹, Jonathan Braun¹, and Lynn K. Gordon^{3,2}

¹Departments of Pathology and Laboratory Medicine, University of California, Los Angeles, California

²Departments of Ophthalmology, University of California, Los Angeles, California

³Department of Surgery, Greater Los Angeles Veterans Affairs Healthcare System, Los Angeles, California

Abstract

PURPOSE—Proliferative vitreoretinopathy (PVR) occurs in approximately 10% of patients after retinal detachment. PVR results from a multiphase process that leads to an aberrant wound-healing strategy with contractile cellular forces and tractional retinal detachment (TRD). Epithelial membrane protein (EMP) 2 controls cell surface expression and function of integrin isoforms associated with cellular contraction in many cell types. Since EMP2 is highly expressed in retinal pigment epithelium, this study investigates the role of EMP2 in collagen gel contraction.

METHODS—EMP2 expression was recombinantly modified in the ARPE-19 cell line. Cell surface integrin expression was assessed by flow cytometry. Collagen gel contraction was assessed by using an in vitro assay and the percentage of contraction was quantified. Proliferation and migration were measured by BrdU incorporation and a wound-healing assay, respectively. Cellular invasion was investigated with polycarbonate membranes coated with collagen.

RESULTS—EMP2 expression levels correlated positively with the ability to contract collagen gels. Compared with wild-type ARPE-19 cells, the cells with increased EMP2 expression exhibited enhanced contraction ($P = 0.02$), and decreased EMP2 expression concomitantly resulted in decreased contraction ($P = 0.002$). EMP2 overexpression resulted in reduced proliferation, migration, and integrin $\alpha 1$ and $\alpha 2$ integrin expression. EMP2 overexpression was associated with a 70% increase in FAK activation ($P = 0.0003$) and relative resistance of gel contraction to inhibitors of FAK/Src activation.

CONCLUSIONS—ARPE-19-mediated collagen gel contraction is a multistep process that requires integrin ligation and activation of the FAK/Src complex. EMP2 positively modulates collagen gel contraction by ARPE-19 cells through increased FAK activation.

The 4-transmembrane (tetraspan) protein EMP2 is expressed at discrete locations in the eye, lung, heart, thyroid, and uterus.¹ In the eye, EMP2 is localized to multiple epithelial layers including the cornea, ciliary body, and retinal pigment epithelium (RPE).² In multiple nonocular cell types, EMP2 plays a critical role in selective receptor trafficking, affecting

molecules that are important in proliferation, invasion, adhesion, and metastasis.^{2–7} These studies suggest a potential central role for EMP2 in coordinately controlling diverse and important cellular processes in cells of ocular origin.

PVR is observed after rhegmatogenous retinal detachment in up to 10% of patients and is a potentially blinding complication.^{8–10} The pathophysiology underlying PVR is complex and multiple cell types, including RPE, are believed to play a critical role in this disease.^{11–14} There is evidence of an epithelial-to-mesenchymal transition resulting in cell migration, membrane formation, and an aberrant wound-healing process associated with contractile cellular forces that may lead to tractional retinal detachment. New strategies for PVR prevention require enhanced understanding of the complex pathophysiology. One in vitro correlate of PVR is collagen gel contraction.

RPE from diverse species have been used in studies of collagen gel contraction, including primary human cells,¹⁵ human ARPE-19 cells,¹⁶ bovine,¹⁷ and rabbit.¹⁸ This study was designed to test how EMP2 controls collagen gel contraction through recombinantly altering the expression of EMP2 in the ARPE-19 cell line. Our prior work¹⁹ and that of others^{16,20–26} identified specific integrin isoforms and found engagement to the collagen matrix to be critically important in collagen gel contraction. We previously identified activation of the FAK/Src pathway as essential in the ARPE-19 cell line in terms of collagen gel contraction in the presence or absence of exogenous proinflammatory stimulation. In the present study, EMP2 expression levels controlled collagen gel contraction, and increasing EMP2 was associated with enhanced FAK activation in the ARPE-19 cell line.

METHODS

EMP2 Constructs

Hammerhead ribozymes were created to cleave the human EMP2 transcripts as previously reported.³ The hRZ2 EMP2 hammerhead ribozyme, which is demonstrated to work well in transfection to reduce EMP2 expression, was used in this study. Briefly, the hRZ2 construct in pEGFP (BD-Clontech, Palo Alto, CA) was transfected into ARPE-19 and stable clones were selected.³

The full-length cDNA of human EMP2 was cloned into the retroviral vector pMSCV-IRES-GFP at the *EcoRI* site.²⁷ Expression of EMP2 was driven by the 5' long terminal repeat (LTR). This vector also enables the expression of the green fluorescence protein (GFP) through an internal ribosome entry site. High-titer helper-free retrovirus stocks were prepared by transient cotransfection of 293T cells, as previously described.²⁸

Cell Lines

ARPE-19, a spontaneously arising retinal pigment epithelial (RPE) cell line that expresses the RPE-specific markers CRALBP and RPE-65, was obtained from the American Type Culture Collection (CRL-2302; ATCC, Manassas, VA). ARPE-19 cells were cultured in DMEM-F12 medium, supplemented with 10% fetal bovine serum (FBS; ATCC) at 37°C in a humidified chamber with 5% CO₂. The culture medium was replaced twice a week. After confluence, the cultures were passaged by dissociation in 0.05% (wt/vol) trypsin. Levels of EMP2 were increased in ARPE-19 cells through stable infection of an EMP2-overexpressing retrovirus construct and selected by flow sorting of GFP-positive cells. These cells, stably infected to increase EMP2 expression are termed ARPE-19/EMP2. EMP2 levels were decreased by stable transfection of the ARPE-19 cells with 3.4 µg of pEGFP-hRZ2 ribozyme construct and transfection reagent (FuGENE 6; Roche, Basel, Switzerland). ARPE-19/Ribo cells were flow sorted for GFP-positive cells. Notably, the

transfection reagent did not produce detectable toxicity to the ARPE-19 cells, as determined by trypan blue exclusion (data not shown).

EMP2 levels were decreased by transiently transfecting ARPE-19 cells with 75 picomoles EMP2 siRNA (L-016226-00; Dharmacon, Lafayette, CO) and lipophilic transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA) and analyzed after 48 hours. As a negative control, the cells were transfected with 75 picomoles of scramble control siRNA (D-001206-13-05; Dharmacon). The EMP2 siRNA and control siRNA are a pool of four siRNAs targeting EMP2 or a pool of four nontargeting siRNAs, respectively. The level of EMP2 expression was quantified by Western blot.

Antibodies

Monoclonal antibodies (mAbs) specific for human $\alpha 1$ (clone SR84), $\alpha 2$ (clone AK-7), and $\alpha 3$ (clone C3 II.1) integrin isoforms were obtained from BD Biosciences (San Diego, CA). Rabbit antisera against human EMP2 was produced after immunization of animals with a multiple antigen peptide conjugated to the second extracellular loop of human EMP2 (EDIHDKNAKFYPVTREGSYG) (Research Genetics, Huntsville, AL).² A rabbit antibody specific for human FAK (clone C-20) and pFAK (clone Tyr 576/577) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse antibody specific for human β -actin (clone 2A2.1) was obtained from US Biological (Swampscott, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Southern Biotech (Birmingham, AL). Horseradish peroxidase-conjugated goat anti-mouse and R-PE-conjugated antibodies specific for mouse IgG were from BD Biosciences.

Flow Cytometry

The membrane expression of $\alpha 1$, $\alpha 2$, and $\alpha 3$ integrin subunits was assessed by flow cytometry. The cells were fixed, but not permeabilized, using 2% paraformaldehyde (wt/vol) in PBS for 20 minutes on ice and then incubated with primary antibody for 30 minutes on ice in PBS + 2% FCS. They were washed two times and incubated with R-PE-conjugated anti-mouse IgG antibody for 30 minutes on ice (BD Biosciences). R-PE was used at 0.25 μ g/million cells. As a negative control, cells were incubated with isotype control antibody alone. After two consecutive washes, cells were resuspended in PBS and analyzed with flow cytometry (FACScan; BD Biosciences). Integrin expression levels, calculated as mean fluorescent intensity (MFI), which is a reflection of expression in the population of cells, were determined in multiple independent experiments.

Collagen Gel Contraction

Collagen gel contraction assays were performed as previously reported.¹⁹ Briefly, collagen gels were prepared by combining collagen type I (BD Biosciences) 10 $\times$ DMEM, and DMEM/F12. The final concentration of the collagen type I mixture was 2.5 mg/mL. The collagen solution (500 μ L) was added to each well of a 24-well plate and incubated at 37°C in 5% CO₂ for 1 hour. Cultured ARPE-19 cell with modified EMP2 levels were harvested and resuspended in serum-free DMEM/F12 at a final concentration of 5 $\times$ 10⁵/mL. ARPE-19 cells with modified EMP2 levels were seeded onto the collagen gel at a concentration of 2.5 $\times$ 10⁵ cells per well and the percentage of contraction was measured at 24 hours. The area of the each gel was obtained by taking a picture of the gel using image capture (Gel Doc 2000; Bio-Rad, Hercules, CA) and quantified with NIH Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). To measure the area of the gel, we used the oval measuring tool to outline each gel. The area of the gel at time 0 was compared to the area of the gel after 24 hours, generating a percentage of contraction for each sample. Each experiment included at least six replicates, and at least three independent experiments were

performed with comparable results. A Student's *t*-test (unpaired, one-tail) was used; $P < 0.05$ was judged to be statistically significant.

Proliferation Assay

Cells were seeded on a 96-well plate and incubated overnight. The medium was removed and then replaced with either normal medium or medium that contained 25 mg/mL collagen I. The cells were then incubated for 48 hours, and proliferation was assessed by BrdU incorporation, as measured by BrdU cell proliferation assay from Calbiochem (San Diego, CA), which is a nonisotopic colorimetric immunoassay. The reaction product was quantified with a microplate reader (model 550; Bio-Rad) at a wavelength of 595 nm.

Migration Assay

ARPE-19 and ARPE-19/EMP2 cells were seeded onto a 24-well plate and incubated for 3 days until cells reached confluence. The cells were washed with PBS, serum-free medium was added, and the cells were incubated overnight. A 10 μ L pipette tip was used to make a scratch in the monolayer and the medium was removed and replaced with serum-free medium or serum-free medium that contained 50 ng/mL PDGF. Pictures of the wound were taken at various time points, and the percentage closure of the scratch was quantified with NIH Image J software. The area of the scratch was measured immediately after the wound was created. Over time, cells migrated into the cleared area; however, a gap was still visible after 24 hours. The gap size was measured and divided by the original scratch size, and this value was expressed as percentage closure.

Invasion Assay

A cell invasion assay (QCM Collagen Cell Invasion Assay; Chemicon, Temecula, CA) was used for all invasion assays performed. ARPE-19 and ARPE-19/EMP2 cells were seeded on an invasion chamber insert containing an 8- μ m pore size polycarbonate membrane coated with a thin layer of polymerized collagen. Cells that invade and migrate through the polymerized collagen layer cling to the bottom of the polycarbonate membrane. Invading cells on the bottom of the insert membrane are identified colorimetrically and quantitatively analyzed using detection at 560 nm with a microplate reader (model 550; Bio-Rad).

Collagen Production

ARPE-19 and ARPE-19/EMP2 cells were seeded onto a six-well plate at a concentration of 7×10^5 cells/mL in serum-free medium in the presence or absence of 10 ng/mL TGF- β . The medium was collected after 72 hours. Secreted collagen was measured (Sircol Collagen Assay; Accurate Chemical & Scientific, Westbury, NY). The collagen assay is a colorimetric procedure. The reaction product was quantified with the microplate reader at a wavelength of 540 nm. Each experiment included at least six replicates, and at least three independent experiments were performed. A Student's *t*-test (unpaired, one-tail) was used. $P < 0.05$ was judged to be statistically significant.

FAK/Src Inhibition

Collagen gels were prepared with collagen type I (BD Biosciences) in DMEM/F12 at a final concentration of 2.5 mg/mL. Freshly prepared collagen solution was added to each well of a 24-well plate and incubated at 37°C in 5% CO₂ for 1 hour. Cultured ARPE-19, ARPE-19/EMP2, and ARPE-19/EMP2 siRNA cells were harvested and resuspended in serum-free DMEM/F12 at a final concentration of 5×10^5 /mL. Cells were pretreated for 1 hour with various concentrations of small-molecule inhibitors. Inhibitors PP2 (FAK/Src inhibitor), and SU6656 (Src inhibitor) were used diluted in DMSO (Calbiochem). The cells were treated with DMSO alone as a vehicle control. ARPE-19, ARPE-19/EMP2, and ARPE-19/EMP2

siRNA cells were seeded onto the collagen gels at a concentration of 2.5×10^5 cells per well and the percentage of contraction was measured at specific time intervals. At least three independent experiments were performed and, where indicated, the results were evaluated for statistical significance with a Student's *t*-test (unpaired, one tail). A level of $P < 0.05$ was considered to be statistically significant.

Western Blot Analysis

Western blot analysis was performed as previously described.²⁹ Briefly, cell protein was isolated by using RIPA buffer containing protease and phosphatase inhibitors (Upstate, Charlottesville, VA) and the protein concentration determined with a protein assay (BCA; Bio-Rad). A total of 10 μ g protein was loaded in each lane and the proteins fractionated by 4% to 20% SDS-PAGE gradient gel in reducing conditions. Proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) and the adequacy of transfer confirmed by Ponceau S red staining (Sigma-Aldrich). The membrane was then blocked with nonfat milk in TBS Tween (TBST; Upstate). Blots were incubated for 1 hour with primary antibody at a dilution of 1:200 for FAK and p-FAK (Tyr 576/577), 1:1000 for EMP2, and 1:5000 for β -actin. Horseradish peroxidase-conjugated goat anti-rabbit or horseradish peroxidase-conjugated goat anti-mouse was exposed to the blots at a 1:2000 dilution. The blots were then developed with chemiluminescence to visualize bound antibody (ECL; Pierce, Rockford, IL) and quantified with β -actin as the internal control. The Western blot analyses were quantified with NIH Image J. The blots were digitized with a flatbed scanner, and the band density was measured by using Image J. To account for loading variability, β -actin was used to normalize each sample. At least three independent experiments were performed and, where indicated, the results were evaluated for statistical significance with a Student's *t*-test (unpaired, one-tail). A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Recombinant Modification of EMP2 Expression in ARPE-19 Cells

EMP2 is highly expressed in retinal pigment epithelium cells, which are believed to be important in membrane formation and contraction of collagen gels. To study the affect of EMP2 on collagen gel contraction, EMP2 levels were modified in the ARPE-19 cell line to create two additional lines of ARPE-19: an overexpressing line, designated ARPE-19/EMP2, and a stable knockdown, designated ARPE-19/Ribo. Steady state protein levels of EMP2 were measured by Western blot analysis in ARPE-19 cells, ARPE-19/EMP2 cells, and ARPE-19/Ribo cells. Experiments were performed independently at least three times with similar results, and one representative experiment is shown in Figure 1a. ARPE-19/EMP2 cells showed a 75- to 100-fold increase in EMP2 expression compared with wild-type cells when compared by using serial dilution. ARPE-19/Ribo cells showed a fivefold decrease in EMP2 expression compared with ARPE-19 cells.

An alternative method for decreasing EMP2 expression used ARPE-19 cells that were transiently transfected with siRNA specific for EMP2 (ARPE-19/EMP2 siRNA) or with a control scramble siRNA (ARPE-19/control siRNA). This technique using the specific siRNA, specifically reduced EMP2 expression to approximately 40% of the expression in control ARPE-19 (Fig. 1b). The scramble siRNA did not result in any change in EMP2 expression.

EMP2 Modification of Collagen Gel Contraction

The collagen gel contraction assay of ARPE-19/EMP2 cells, ARPE-19/Ribo cells, and ARPE-19 cells was performed at least three separate times with six replicates per sample.

Altering EMP2 levels significantly affected the cells' ability to contract the collagen gels. Increasing EMP2 expression significantly increased contraction compared with untreated wild-type cells (Fig. 2). Concordantly, decreased gel contraction was observed in the ARPE-19/Ribo cells. Additional control ARPE-19 cell lines, a retrovirally infected cell line without the EMP2 construct, and the transfected EGFP-N3 vector without ribozyme, did not alter contraction compared with the wild-type control ARPE-19 cell line (data not shown).

Effect of EMP2 on Contraction through Changes in Integrins $\alpha 1$, $\alpha 2$, or $\alpha 3$

The previous results indicated that increasing EMP2 expression results in increased collagen gel contraction. EMP2 is known to regulate intracellular trafficking and cell surface expression of specific integrin isoforms in various cell types including the mouse NIH3T3 and the human endometrial cancer line HEC1A.^{3,5} We and others demonstrated the importance of $\alpha 1$, $\alpha 2$, and $\alpha 3$ integrin engagement as an important step in collagen gel contraction in the ARPE-19 cell line^{16,19–26}) and thus hypothesized that EMP2 may increase the cell surface expression of one of these collagen-binding integrins.

The cell lines with altered EMP2 expression were evaluated for surface expression of these integrin isoforms by flow cytometry (Fig. 3). In contrast to the predicted result, cells engineered to overexpress EMP2 actually exhibited decreased cell surface expression of integrin $\alpha 1$ ($P = 0.003$) and $\alpha 2$ ($P = 0.002$) by approximately 65%. There was a slight but not statistically significant decrease in integrin $\alpha 3$ expression in the ARPE-19/EMP2 cells (Fig. 3b). This result led us to investigate other mechanisms by which EMP2 could regulate collagen gel contraction in this cell line.

Association of Changes in Proliferation, Migration, Invasion, and Collagen Production with the Effect of EMP2 on Contraction

A potential mechanism by which EMP2 may regulate collagen contraction is by increasing cell proliferation leading to increased contraction. Proliferation, assayed by BrdU incorporation, was assessed in the ARPE-19 and ARPE-19/EMP2 cells in the absence or presence of collagen stimulation (Fig. 4a). In the absence of collagen, EMP2 overexpression resulted in a mild decrease in proliferation (20%). After collagen stimulation, there was no statistically significant difference in the proliferation of either cell line, thus refuting the hypothesis that increased proliferation may lead to enhanced collagen gel contraction by ARPE-19/EMP2 cells.

To investigate the possible effect of increased EMP2 expression on migration, we performed a wound-healing assay. ARPE-19 and ARPE-19/EMP2 cells were either left untreated or treated with 50 ng/mL PDGF, and the percentage of closure, as a surrogate evaluation of wound closure, was measured at 6, 12, and 24 hours. At the 6- and 12-hour time points, in the absence or presence of PDGF, there was no difference between the two cell lines (data not shown). At the 24-hour time point, in the absence of PDGF stimulation, the ARPE-19/EMP2 cells showed a 23% decrease in gap closure compared with the ARPE-19 cells. PDGF treatment abolished this effect, resulting in equivalent wound healing in the two cell lines (Fig. 4b). Both proliferation and migration are responsible for wound closure. Although the lower proliferative rate of the ARPE-19/EMP2 cells could be responsible for the decrease in wound closure, it is also possible that the EMP2-overexpressing cells exhibit less motility. PDGF treatment, known to promote the motile phenotype, both stimulated wound closure of the ARPE-19 cells and overcame the decreased wound closure of the ARPE-19/EMP2 cells. Increased collagen gel contraction by the ARPE-19/EMP2 cells cannot be explained by increases in migration due to altered EMP2 expression.

We examined an alternative mechanism, the possibility that EMP2 expression could influence the cells' ability to invade the collagen matrix. ARPE-19 cells and ARPE-19/EMP2 cells were seeded onto polycarbonate membranes and either 10% FBS or 50 ng PDGF was used as a chemoattractant. Invasion was measured at 24 and 48 hours. There was no difference in invasion between the ARPE-19 and ARPE-19/EMP2 cells under either condition (Fig. 4c). EMP2-mediated enhancement of collagen gel contraction does not result from an altered invasive capacity.

An additional mechanism that we investigated was whether EMP2 levels affect collagen production. ARPE-19 and ARPE-19/EMP2 cells were grown in a 24-well plate for 72 hours. The medium was collected and collagen production was analyzed. There was no statistical difference in collagen production between the two cell lines (Fig. 4d). Increased cellular contractile capacity by ARPE-19/EMP2 cells cannot be explained by altered collagen production.

EMP2 Control of FAK Activation and Collagen Gel Contraction in ARPE-19 Cells

Changes in integrin expression, proliferation, migration, or invasion do not explain how increased EMP2 expression results in increased gel collagen contraction. Previously, we observed that integrin ligation and activation of FAK/Src complex are necessary for collagen gel contraction by ARPE-19 cells.¹⁹ We investigated whether EMP2 overexpression in the ARPE-19 cells led to alteration in FAK/Src activity. Although total FAK levels were equivalent in both the ARPE-19 and ARPE-19/EMP2 cells, the overexpressing ARPE-19/EMP2 cells demonstrated an almost twofold increase in the level of activated FAK compared with the control ARPE-19 cells (Fig. 5a). Multiple evaluations, in which each activated FAK was normalized to its own β -actin loading control, showed a statistically significant correlation between increased EMP2 levels and FAK activation (Fig. 5b).

Effect of EMP2 Overexpression on Resistance to Inhibition of Collagen Gel Contraction by FAK/Src Inhibitors

The observation that overexpression of EMP2 was associated with increased FAK activation prompted additional studies to test the functional significance of this observation in the context of gel contraction by using inhibitors of the FAK/Src pathway. If EMP2 effect was secondary to FAK activation, then the observed collagen gel contraction in the ARPE-19/EMP cells should show increased resistance to FAK/Src inhibition compared with the control ARPE-19 cells. To investigate this prediction, we used a range of concentrations of PP2 and SU6656, FAK/Src small-molecule inhibitors that have been demonstrated to prevent collagen gel contraction in ARPE-19 cells¹⁹ (Figs. 6, 7). Reduction of EMP2 expression with an EMP2-specific siRNA showed decreased collagen gel contraction compared with control scramble siRNA. This result was concordant with the decreased collagen gel contraction observed in the ARPE-19/Ribo cell line (Fig. 6a). The sensitivity of the collagen gel contraction assay to the FAK inhibitor PP2 correlated inversely with the EMP2 levels, there was increased sensitivity in the EMP2 siRNA-treated cells and increased resistance in the EMP2-overexpressing cells (Figs. 6a–c). The percentage inhibition of contraction, as normalized to the vehicle control, was determined for each cell line at the different concentrations of inhibitor. The concentrations of PP2 required to achieve 50% inhibition of the gel contraction were 2.5 μ M for the ARPE-19 cells, less than 1 μ M for the ARPE-19/EMP2 siRNA cells, and 5 μ M for the overexpressing ARPE-19/EMP2 cells and were consistent with the changes in FAK activation observed in these cell lines (Fig. 6d). A second inhibitor of the FAK/Src pathway, SU6656, demonstrated similar results in the ARPE-19 and the ARPE-19/EMP2 cells, thus providing independent confirmation that the

mechanism for EMP2 control of collagen gel contraction was through changes in the activation of FAK (Fig. 7).

DISCUSSION

Collagen gel contraction, an in vitro correlate for PVR, is a cellular process dependent on FAK-mediated integrin signaling.¹⁹ In this article, we demonstrate that EMP2 modulates collagen gel contraction in a process dependent on enhanced FAK activation. EMP2 regulation of FAK activation is a novel observation, but prior observations of tetraspanin-associated regulation of a variety of integrin signaling mechanisms suggests a potential shared function for this family of membrane-associated proteins.

The mechanisms by which tetraspanins regulate activation of FAK-dependent or other signaling pathways are not yet understood. Certain tetraspanins (CD9, CD53, CD81, CD82, and CD151) associate with each other or in heterocomplexes with additional membrane proteins, resulting in increased tyrosine phosphorylation.^{30–34} This implies that 4-transmembrane proteins act as molecular adaptors supporting the functional assembly of signaling complexes in the membrane.³²

Functional modulation of integrin and other cell surface receptors is a recurrently observed feature common to multiple proteins in the tetraspan families. Several tetraspanins (CD9, CD53, CD81, and CD82) participate in protein-protein interaction with integrins ($\alpha 3\beta 1$, $\alpha 4\beta 1$, and $\alpha 6\beta 1$), leading to altered adhesion and cellular activation.^{33,34} Multiple tetraspanins, including CD9, CD63, CD81, CD151, and A15/TALLA1, recruit PI-4 kinase to specific membrane locations and induce phosphoinositide-dependent signaling.³⁵ In addition, cross-linking of CD81 with anti-CD81 antibody is costimulatory for signaling through the TCR/CD3 complex.³⁴ Other relationships between changes in expression levels or cross-linking of tetraspanins have been associated with activation of signal transduction including upregulated CD53 expression or ligation, which induces JNK activation³⁶; CD9 control of adhesion, induced tyrosine phosphorylation of FAK in fibrosarcoma cells³⁷; and homophilic CD151 interactions, which induce adhesion-dependent activation of FAK, Src, and c-Jun kinases in human melanoma cells.³² In addition, homophilic protein–protein interactions of CD151 regulate integrin-dependent signaling to c-Jun through a pathway involving FAK-Src and MAP kinases.³²

RPE-mediated collagen gel contraction involves the interplay of several receptors and signaling pathways.^{16,20–26,38} The results of this study support a role for EMP2 in facilitating the activation of the FAK/Src complex leading to collagen contraction. Additional studies are needed to determine whether this effect of EMP2 may be generalized to other RPE cell lines and primary RPE cells and to test the prediction that EMP2 expression and increased activation of FAK/Src are features of in vivo PVR models or clinical PVR. Validation of this prediction may identify either EMP2 or the FAK/Src signal transduction cascade as a potential target for therapeutic intervention to prevent or treat proliferative vitreoretinopathy.

Acknowledgments

Supported by VA Merit Grant (LKG), American Health Assistance Foundation (LKG), AI52031 (SM), HD48540 (JB), and the Ruzic Foundation (JB). Lynn K. Gordon is a recipient of an RPB James S. Adams Scholars Award.

References

1. Wang CX, Wadehra M, Fisk BC, Goodglick L, Braun J. Epithelial membrane protein 2, a 4-transmembrane protein that suppresses B-cell lymphoma tumorigenicity. *Blood*. 2001; 97(12):3890–3895. [PubMed: 11389031]
2. Wadehra M, Sulur GG, Braun J, Gordon LK, Goodglick L. Epithelial membrane protein-2 is expressed in discrete anatomical regions of the eye. *Exp Mol Pathol*. 2003; 74(2):106–112. [PubMed: 12710941]
3. Wadehra M, Forbes A, Pushkarna N, et al. Epithelial membrane protein-2 regulates surface expression of alphavbeta3 integrin in the endometrium. *Dev Biol*. 2005; 287(2):336–345. [PubMed: 16216233]
4. Wadehra M, Goodglick L, Braun J. The tetraspan protein EMP2 modulates the surface expression of caveolins and glycosylphosphatidyl inositol-linked proteins. *Mol Biol Cell*. 2004; 15(5):2073–2083. [PubMed: 14978215]
5. Wadehra M, Iyer R, Goodglick L, Braun J. The tetraspan protein epithelial membrane protein-2 interacts with beta1 integrins and regulates adhesion. *J Biol Chem*. 2002; 277(43):41094–41100. [PubMed: 12189152]
6. Wadehra M, Natarajan S, Seligson DB, et al. Expression of epithelial membrane protein-2 is associated with endometrial adenocarcinoma of unfavorable outcome. *Cancer*. 2006; 107(1):90–98. [PubMed: 16736513]
7. Wadehra M, Su H, Gordon LK, Goodglick L, Braun J. The tetraspan protein EMP2 increases surface expression of class I major histocompatibility complex proteins and susceptibility to CTL-mediated cell death. *Clin Immunol*. 2003; 107(2):129–136. [PubMed: 12763482]
8. Charteris DG, Sethi CS, Lewis GP, Fisher SK. Proliferative vitreoretinopathy-developments in adjunctive treatment and retinal pathology. *Eye*. 2002; 16(4):369–374. [PubMed: 12101443]
9. Kirchhof B. Strategies to influence PVR development. *Graefes Arch Clin Exp Ophthalmol*. 2004; 242(8):699–703. [PubMed: 15309556]
10. Lewis GP, Charteris DG, Sethi CS, Fisher SK. Animal models of retinal detachment and reattachment: identifying cellular events that may affect visual recovery. *Eye*. 2002; 16(4):375–387. [PubMed: 12101444]
11. Baudouin C, Hofman P, Brignole F, Bayle J, Loubiere R, Gastaud P. Immunocytology of cellular components in vitreous and subretinal fluid from patients with proliferative vitreoretinopathy. *Ophthalmologica*. 1991; 203(1):38–46. [PubMed: 1766640]
12. Casaroli-Marano RP, Pagan R, Vilaro S. Epithelial-mesenchymal transition in proliferative vitreoretinopathy: intermediate filament protein expression in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1999; 40(9):2062–2072. [PubMed: 10440262]
13. Charteris DG. Proliferative vitreoretinopathy: pathobiology, surgical management, and adjunctive treatment. *Br J Ophthalmol*. 1995; 79(10):953–960. [PubMed: 7488586]
14. Vinore SA, Campochiaro PA, McGehee R, Orman W, Hackett SF, Hjelmeland LM. Ultrastructural and immunocytochemical changes in retinal pigment epithelium, retinal glia, and fibroblasts in vitreous culture. *Invest Ophthalmol Vis Sci*. 1990; 31(12):2529–2545. [PubMed: 1702409]
15. Hunt RC, Pakalnis VA, Choudhury P, Black EP. Cytokines and serum cause alpha 2 beta 1 integrin-mediated contraction of collagen gels by cultured retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1994; 35(3):955–963. [PubMed: 8125758]
16. Bando H, Ikuno Y, Hori Y, Sayanagi K, Tano Y. Mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways differently regulate retinal pigment epithelial cell-mediated collagen gel contraction. *Exp Eye Res*. 2006; 82(3):529–537. [PubMed: 16289050]
17. Ando A, Ueda M, Uyama M, Masu Y, Ito S. Enhancement of dedifferentiation and myoid differentiation of retinal pigment epithelial cells by platelet derived growth factor. *Br J Ophthalmol*. 2000; 84(11):1306–1311. [PubMed: 11049960]
18. Zheng Y, Bando H, Ikuno Y, et al. Involvement of rho-kinase pathway in contractile activity of rabbit RPE cells in vivo and in vitro. *Invest Ophthalmol Vis Sci*. 2004; 45(2):668–674. [PubMed: 14744913]

19. Morales SA, Mareninov S, Prasad P, Wadehra M, Braun J, Gordon LK. Collagen Gel Contraction by ARPE-19 cells is mediated by a FAK-Src dependent pathway. *Exp Eye Res.* 2007; 85(6):790–798. [PubMed: 17915217]
20. Carver W, Molano I, Reaves TA, Borg TK, Terracio L. Role of the alpha 1 beta 1 integrin complex in collagen gel contraction in vitro by fibroblasts. *J Cell Physiol.* 1995; 165(2):425–437. [PubMed: 7593221]
21. Cooke ME, Sakai T, Mosher DF. Contraction of collagen matrices mediated by alpha2beta1A and alpha(v)beta3 integrins. *J Cell Sci.* 2000; 113:2375–2383. [PubMed: 10852817]
22. Kieffer JD, Plopper G, Ingber DE, Hartwig JH, Kupper TS. Direct binding of F actin to the cytoplasmic domain of the alpha 2 integrin chain in vitro. *Biochem Biophys Res Commun.* 1995; 217(2):466–474. [PubMed: 7503723]
23. Langholz O, Rockel D, Mauch C, et al. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins. *J Cell Biol.* 1995; 131:1903–1915. [PubMed: 8557756]
24. Moulin V, Plamondon M. Differential expression of collagen integrin receptor on fetal vs. adult skin fibroblasts: implication in wound contraction during healing. *Br J Dermatol.* 2002; 147(5):886–892. [PubMed: 12410697]
25. Robbins SG, Brem RB, Wilson DJ, et al. Immunolocalization of integrins in proliferative retinal membranes. *Invest Ophthalmol Vis Sci.* 1994; 35(9):3475–3485. [PubMed: 8056523]
26. Zhang ZG, Bothe I, Hirche F, et al. Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of alpha2beta1 integrin. *J Cell Sci.* 2006; 119:1886–1895. [PubMed: 16636073]
27. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther.* 1994; 1(2):136–138. [PubMed: 7584069]
28. Pear, W. Transient transfection methods for preparation of high-titer retroviral supernatants. In: Ausubel, FM.; Brent, R.; Kingston, RE., et al., editors. *Current Protocols in Molecular Biology*. Vol. 2. New York: John Wiley & Sons; 1996. p. 9.11-9.15.
29. Wadehra M, Dayal M, Mainigi M, et al. Knockdown of the tetraspan protein epithelial membrane protein-2 inhibits implantation in the mouse. *Dev Biol.* 2006; 292(2):430–441. [PubMed: 16487956]
30. Boucheix C, Rubinstein E. Tetraspanins. *Cell Mol Life Sci.* 2001; 58(9):1189–1205. [PubMed: 11577978]
31. Hemler ME. Specific tetraspanin functions. *J Cell Biol.* 2001; 155(7):1103–1107. [PubMed: 11756464]
32. Hong IK, Jin YJ, Byun HJ, Jeoung DI, Kim YM, Lee H. Homophilic interactions of Tetraspanin CD151 up-regulate motility and matrix metalloproteinase-9 expression of human melanoma cells through adhesion-dependent c-Jun activation signaling pathways. *J Biol Chem.* 2006; 281(34):24279–24292. [PubMed: 16798740]
33. Lagaudriere-Gesbert C, Le Naour F, Lebel-Binay S, et al. Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. *Cell Immunol.* 1997; 182(2):105–112. [PubMed: 9514697]
34. Todd SC, Lipps SG, Crisa L, Salomon DR, Tsoukas CD. CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. *J Exp Med.* 1996; 184(5):2055–2060. [PubMed: 8920895]
35. Yauch RL, Hemler ME. Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem J.* 2000; 3:629–637. [PubMed: 11042117]
36. Yunta M, Oliva JL, Barcia R, Horejsi V, Angelisova P, Lazo PA. Transient activation of the c-Jun N-terminal kinase (JNK) activity by ligation of the tetraspan CD53 antigen in different cell types. *Eur J Biochem.* 2002; 269(3):1012–1021. [PubMed: 11846804]
37. Berditchevski F, Odintsova E. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J Cell Biol.* 1999; 146(2):477–492. [PubMed: 10427099]
38. Xia H, Nho RS, Kahm J, Kleidon J, Henke CA. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of

type I collagen matrices via a beta 1 integrin viability signaling pathway. J Biol Chem. 2004; 279(31):33024–33034. [PubMed: 15166238]

FIGURE 1.

Recombinant modification of EMP2 expression. **(a)** Steady state protein levels of EMP2 were measured by Western blot analysis in ARPE-19 cells (control cells), ARPE-19/EMP2 cells (increased EMP2), and ARPE-19/Ribo cells (decreased EMP2). To measure the increase in EMP2 expression, various dilutions (1:10, 1:25, 1:50, and 1:100) of ARPE-19/EMP2 cell lysates were evaluated. **(b)** ARPE-19 cells were transiently transfected with siRNA specific for EMP2 (ARPE-19/EMP2 siRNA) or with a control scramble siRNA (ARPE-19/control siRNA). Experiments were performed independently at least three times, with similar results.

FIGURE 2.

EMP2 modification affected collagen gel contraction. A collagen gel contraction assay was performed in ARPE-19/EMP2, ARPE-19/Ribo, and ARPE-19 cells. Cell lines with elevated and decreased EMP2 expression resulted in a 57% increase and 55% decrease in gel contraction, respectively ($P = 0.002$). Experiments were performed independently at least three times with similar results.

FIGURE 3.

Collagen binding integrins. Cell surface expression was measured by flow cytometry with monoclonal antibodies against $\alpha 1$, $\alpha 2$, or $\alpha 3$ integrin. **(a)** Histograms of representative experiments are presented. The isotype control staining is shown as an open tracing, and the specific staining pattern is shaded. A numerical value for the mean fluorescence intensity is presented in the *top right corner* of each panel. **(b)** The surface expression of each of these integrins was evaluated in three independent experiments and the results tabulated with the mean presented. Statistical comparison of expression of each integrin in the two cell lines was performed with Student's *t*-test (unpaired, one-tailed).

FIGURE 4.

Increased EMP levels decreased proliferation and migration but had no effect on invasion or collagen production. (a) ARPE-19 and ARPE-19/EMP2 cells were treated with normal medium or 25 mg/mL collagen I and incubated for 48 hours, and proliferation was assessed by BrdU incorporation. (b) The effect of EMP2 expression on cell migration. ARPE-19 and ARPE-19/EMP2 cells were plated in a 24-well plate, and once confluent, a scratch was made and migration was measured at various time points. ARPE-19 and ARPE-19/EMP2 cells were either left untreated or were treated with 50 ng/mL PDGF, and migration was measured after 24 hours. (c) ARPE-19 and ARPE-19/EMP2 cells were seeded on an

invasion chamber insert with 8- μ m pore size polycarbonate membrane coated with a thin layer of polymerized collagen. Either 10% FBS or 50 ng/mL PDGF was used as a chemoattractant. Invasive cells migrated through the polymerized collagen layer, clung to the bottom of the polycarbonate membrane, and were detected by staining, extraction, and measurement by a microplate reader (560 nm). (d) ARPE-19 and ARPE-19/EMP2 cells were grown in serum-free medium in the presence or absence of 10 ng/mL TGF- β for 72 hours. The media were collected and collagen production was measured by ELISA. There was no statistically significant change in collagen production in the presence or absence of TGF- β . However, TGF- β increased collagen production in each cell line compared with the untreated condition. All studies were performed at least three separate times with six wells per sample. The results were evaluated for statistical significance with a Student's *t*-test (unpaired, one-tailed). *P* < 0.05 was considered to be statistically significant.

FIGURE 5.

EMP2 overexpression increased FAK activation. Cell extracts (10 μ g protein) were fractionated by 4% to 20% SDS-PAGE gradient gel in reducing conditions, and Western immunoblots were probed with antibodies for FAK, pFAK, and β -actin. (a) Representative immunoblots; (b) experiments were performed independently at least three times with similar results. Band density, normalized to the β -actin loading control, was quantitated. Experiments were performed independently at least three times with similar results.

FIGURE 6.

EMP2 overexpression increased resistance to PP2. Cells were pretreated for 1 hour with various concentrations of the small-molecule inhibitor PP2 (FAK/Src inhibitor), and gel contraction was assessed. The experiment was performed at least three separate times with six replicates per sample, and a representative experiment is presented (a–c). Statistical analysis was performed with Student's *t*-test for each concentration of inhibitor compared with the vehicle-only control. (a) ARPE-19/EMP2 siRNA cells with decreased EMP2 expression. (b) ARPE-19 cells. (c) EMP2-overexpressing ARPE-19/EMP2 cells. (d) Each point represents the average inhibition of contraction at each concentration for each cell line

normalized to total contraction of the same cell line exposed to the vehicle control. Experiments were performed independently at least three times with similar results.

FIGURE 7.

EMP2 overexpression increased resistance to inhibition by SU6656. Cells were pretreated for 1 hour with various concentrations of the small-molecule inhibitor SU6656 (a FAK/Src inhibitor), and gel contraction was assessed. The experiment was preformed at least three separate times with six replicates per sample, and a representative experiment is presented. Statistical analysis was performed with a Student's *t*-test for each concentration of inhibitor compared with the vehicle-only control. **(a)** ARPE-19/EMP2 cells. **(b)** EMP2 overexpressing ARPE-19/EMP2 cells. **(c)** Each point represents the average inhibition of contraction at each concentration for each cell line normalized to total contraction of the same cell line exposed to the vehicle control. Experiments were performed independently at least three times with similar results.