Rewiring Integrin-Mediated Signaling and Cellular Response with the Peripheral Myelin Protein 22 and Epithelial Membrane Protein 2 Components of the Tetraspan Web

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PURPOSE. Integrin-mediated collagen gel contraction by ARPE-19 is an in vitro model for proliferative vitreoretinopathy (PVR), an aberrant wound healing response after retinal detachment or ocular trauma. Expression of the tetraspan protein epithelial membrane protein 2 (EMP2) controls gel contraction through FAK activation. Peripheral myelin protein 22 (PMP22), another member of the tetraspan web, is closely related to EMP2. The purpose of this study was to determine whether PMP22 also controls the contractile phase associated with PVR.

METHODS. Integrin expression, adhesion, and protein expression were assessed, respectively, through flow cytometry, binding to collagen types I and IV, and Western blot analysis. Collagen gel contraction was assessed using an in vitro assay.

RESULTS. Overexpression of PMP22 in ARPE-19 cells (ARPE-19/PMP22) resulted in increased collagen adhesion. Gel contraction, however, was reduced by greater than 50% in ARPE-19/PMP22 cells ($P < 0.001$). In contrast to the FAK activation observed by increasing EMP2 expression, PMP22 overexpression led to increased AKT activation. The decrease in gel contraction by the ARPE-19/PMP22 cells was partially reversed through either PMP22 siRNA or by blockade of AKT.

CONCLUSIONS. Relative expression of EMP2 or PMP22 within the tetraspan web drives a cellular response toward a FAK- or AKT-dependent pathway, respectively. EMP2 and PMP22 differentially regulate collagen gel contraction in the ARPE-19 cell line. The implication of this finding adds a new dimension to the concept of the tetraspan web, in which the abundance of individual tetraspan family members differentially regulates signal transduction and the downstream cellular

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Peripheral myelin protein 22 (PMP22) has been shown to play an active role in epithelial biology by influencing cellular proliferation, adhesion, and migration.¹ PMP22 was initially characterized in the peripheral nervous system. PMP22 is downregulated after sciatic nerve injury in the distal nerve stump² and plays a critical role in the pathology of hereditary demyelinating neuropathies such as Charcot Marie Tooth syndrome.^{3,4} These studies have also shown that PMP22 is widely expressed and plays an important role in the basic biology of the cell, leading to diverse functional outcomes. A fresh perspective on PMP22 can be inferred from recent studies of epithelial membrane protein 2 (EMP2). PMP22 and EMP2 are both members of the tetraspan protein family and are similar with respect to amino acid identity (~40%). PMP22 and EMP2 also show a very similar expression pattern across multiple tissue types such as lung, intestine, heart, liver, brain, eye, and skeletal muscle.^{5–7} The functional significance of this shared expression is not clear, but there is evidence that these two closely related proteins interact in various cellular processes such as migration.^{1,8}

In multiple cell types, EMP2 plays a critical role in selective receptor trafficking, invasion, adhesion, and metastasis.^{7,9–13} In the uterine endometrium, EMP2 is critical for epithelial function, including blastocyst implantation¹⁴ and chlamydia invasion,¹⁵ by association with and facilitation of $\alpha v \beta 3$ integrin function. In the retinal pigment epithelium (RPE), EMP2 regulates cellular contractile capacity by facilitating FAK activation initiated by collagen-binding $\beta 1$ integrin isoforms.^{8,16,17} Contractile capacity in the RPE is assessed using the collagen gel contraction assay, an in vitro correlate for proliferative vitreoretinopathy (PVR), a potentially blinding disease. Taken together, these findings suggest that the cell biology of EMP2 involves its tuning of FAK signaling by EMP2-associated integrin isoforms.

Although PMP22 has not been previously associated with the PVR response, its role in the biology of certain epithelial cell types and its relationship to EMP2 provided the rationale for investigating the role of PMP22 in ARPE-19. Since PMP22 and EMP2 selectively associate with certain integrin isoforms, notably $\alpha 6 \beta 4$ and $\alpha v \beta 3$, respectively, we hypothesized that the specific composition of the tetraspan web tunes downstream signaling.^{9,18} To test this hypothesis in retinal epithelial cells, we conducted this study to compare PMP22 with EMP2 for their effect on integrin-associated cell responses including collagen gel contraction, which is important in the pathobiology of the RPE. We found that PMP22 expression reduces collagen gel contraction through increased activation of integrin-in-

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duced AKT signaling. This article presents evidence for the importance of members of the tetraspan web—EMP2 and PMP22—in controlling collagen gel contraction through modulating different cell signal transduction pathways.

MATERIALS AND METHODS

Cell Line

ARPE-19, a spontaneously arising retinal pigment epithelial 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). The EMP2-overexpressing cells ARPE-19/EMP2 have been previously reported and were produced through stable infection of an EMP2-overexpressing retrovirus construct.⁸ ARPE-19/PMP22, a PMP22-overexpressing cell line, was produced through stable transfection using the expression plasmid pLNCX2 for the human PMP22-MYC fusion protein⁹ using transfection reagent (FuGENE 6; Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Stable clones were selected using G418 reagent (Geneticin, 800 μ g/mL; Invitrogen, Carlsbad, CA). Additional control ARPE-19 cell lines, a retrovirally infected cell line without the EMP2 construct, and the transfected vector without the PMP22-MYC fusion protein did not alter cellular functional activity compared with the wild-type control ARPE-19 cell line (data not shown).⁸ ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12, supplemented with 10% fetal bovine serum (FBS; ATCC) at 37°C in a humidified chamber with 5% CO₂.

PMP22 levels were decreased to control levels by transient transfection of ARPE-19/PMP22 cells with 75 pmol PMP22 siRNA (L-010616-00-0005; Dharmacon, Lafayette, CO) and lipophilic transfection reagent (Lipofectamine 2000; Invitrogen) and were analyzed after 48 hours. As a negative control, cells were transfected with 75 pmol scramble control siRNA (D-001206-13-05; Dharmacon). The PMP22 siRNA and control siRNA are a pool of four siRNAs targeting PMP22 or a pool of four nontargeting siRNAs, respectively. The level of PMP22 expression was quantified using Western blot analysis.

Antibodies

Monoclonal antibodies (mAbs) specific for human α 1 (clone SR84), α 2 (clone AK-7), and α 3 (clone C3 II.1) integrin isoforms were obtained from the same manufacturer and used in flow cytometry (BD Biosciences, San Diego, CA). A rabbit antibody specific for human PMP22 (clone 193H12; Sigma Chemical Co., St. Louis, MO), p-AKT (Cell Signaling Technology, Beverly, MA), and p-FAK (clone Tyr 576/577; Santa Cruz Biotechnology, Santa Cruz, CA) were used in Western blot analysis. A mouse antibody specific for human β -actin (clone 2A2.1; US Biological) was used for Western blot analysis. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from the same manufacturer (Southern Biotech, Birmingham, AL).

Western Blot Analysis

Western blot analysis were performed as previously described.¹⁶ Protein from cultured cells was isolated, and protein concentration was determined with a BCA Protein Assay (Bio-Rad, Hercules, CA). To examine kinase activation, the cells were either treated with 50 ng/mL PDGF (PeproTech, Rocky Hill, NJ) for 30 minutes or incubated on a collagen I-coated plate for 2 hours before protein isolation. Where indicated, AKT phosphorylation was blocked by pretreating cells for 1 hour with 50 μ M of the small molecule inhibitor Ly294002 (Cell Signaling Technology, Danvers, MA). A total of 15 μ g of protein was loaded in each lane and then fractionated by 4% to 20% SDS-PAGE gradient gel under reducing conditions. Each cell lysate was examined in triplicate. The membrane was then blocked with nonfat milk in TBS Tween (Upstate, Charlottesville, VA). Blots were incubated overnight with primary antibody at a dilution of 1:500 for PMP22, 1:50 for p-FAK,

1:1000 for p-AKT, 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. Blots were then developed with enhanced chemiluminescence to visualize bound antibody (Pierce, Rockford, IL) and were quantified using β -actin as an internal control. Western blot analyses were quantified using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The blots were digitized using a flatbed scanner, and the band density was measured 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 using a Student's *t*-test (unpaired, one-tailed). A level of $P < 0.05$ was considered statistically significant.

Collagen Gel Contraction

Collagen gel contraction assays were performed as previously reported.¹⁷ Briefly, cultured ARPE-19, ARPE-19/PMP22, and ARPE-19/EMP2 cells were harvested and resuspended in serum-free DMEM/F12 at a final concentration of 5×10^5 /mL. Where indicated, AKT phosphorylation was blocked by pretreating cells with Ly294002 (Cell Signaling Technology). The cells were seeded onto the collagen gel at a concentration of 2.5×10^5 cells per well, and the percentage contraction was measured at 24 hours. In platelet-derived growth factor (PDGF)-stimulated collagen contraction assays, cells were pretreated for 30 minutes with 50 ng/mL PDGF (PeproTech). The area of each gel was obtained using image capture (Gel Doc; Bio-Rad) and was quantified using NIH Image J software. To measure the area of the gel, the oval measuring tool was used to outline the area of interest. The area of the gel at time 0 was compared to the area of the gel after 24 hours, generating a percentage 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-tailed) was used. $P < 0.05$ was judged to be statistically significant.

Flow Cytometry

The membrane expression of α 1, α 2, and α 3 integrin subunits was assessed by flow cytometry. Cells were fixed, but not permeabilized, using 2% paraformaldehyde (wt/vol) in PBS for 20 minutes on ice. Cells were incubated with primary antibody for 30 minutes on ice in PBS + 2% FCS. Then cells were washed twice and incubated with R-PE-conjugated anti-mouse IgG antibody for 30 minutes on ice (BD Biosciences, Franklin Lakes, NJ). 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 a flow cytometer (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.

Adhesion Assay

ARPE-19 and ARPE-19/PMP22 cells were plated onto a plate precoated with collagen I and collagen IV (CytoMatrix Screening; Millipore, Billerica, MA) at a concentration of 5×10^4 cells per well. The cells were incubated at 37°C in a humidified chamber with 5% CO₂ for 2 hours. The plate was then washed three times with PBS to remove any unattached cells. Bound cells were analyzed for crystal violet uptake; after solubilization, absorbance was measured at 595 nm by a microplate reader (Bio-Rad). Each experiment included at least eight replicates, and at least three independent experiments were performed with comparable results. A Student's *t*-test (unpaired, one-tailed) was used, and $P < 0.05$ was judged to be statistically significant.

FIGURE 1. Recombinant modification of PMP22 expression in ARPE-19 cells. *Filled bar:* ARPE-19; *lined bar:* ARPE-19 modified for PMP22 expression. Steady state protein levels of PMP22 were measured by Western blot analysis in the cells used in these studies. To account for loading variability, β -actin was used to normalize each sample. A PMP22-overexpressing line was created and designated as ARPE-19/PMP22 ($**P = 0.01$). PMP22 overexpression was reduced to basal levels in this overexpressing cell line using siRNA targeting PMP22 ($*P = 0.01$). ARPE-19/PMP22 cells were transiently transfected with siRNA specific for PMP22 or with a control scramble siRNA. Each experiment was performed in triplicate, and at least three independent experiments were performed. This figure shows one representative experiment. Results were evaluated for statistical significance using a Student's *t*-test (unpaired, one-tailed) in which $*P < 0.05$ was considered statistically significant.

RESULTS

Recombinant Modification of PMP22 Expression in ARPE-19 Cells

PMP22 protein expression was initially identified in ARPE-19 cells by Western blot (data not shown). To determine whether PMP22 expression contributed to a functional phenotype in terms of collagen gel contraction, PMP22 levels were modified

in this cell line (Fig. 1). A PMP22-overexpressing line was created and designated as ARPE-19/PMP22. PMP22 overexpression was reduced to basal levels in this overexpressing cell line using siRNA targeting PMP22. ARPE-19/PMP22 cells were transiently transfected with siRNA specific for PMP22 or with a control scramble siRNA. Steady state protein levels of PMP22 were measured by Western blot analysis. Experiments were performed independently at least three times with similar results, and one representative experiment is shown in Figure 1. ARPE-19/PMP22 cells showed a greater than twofold increase in PMP22 expression compared with wild-type cells. Although the control siRNA induced a small but insignificant decrease in PMP22 levels ($P = 0.3$), the PMP22-specific siRNA reduced levels of PMP22 to baseline (Fig. 1). Notably there was no change in cell viability using the PMP22 siRNA in the PMP22-overexpressing ARPE-19/PMP22 cells (data not shown); however, there was decreased viability of ARPE-19 cells treated with the PMP22 siRNA. Consequently, ARPE-19 cells lacking PMP22 were not available for additional study in these experiments (data not shown).

PMP22 Expression Differentially Alters Collagen-Binding Integrin Receptor Expression

The importance of integrin engagement, specifically $\alpha 1$, $\alpha 2$, and $\alpha 3$, has been shown to be critical for collagen gel contraction in the ARPE-19 cell line.^{17,19–26} We tested whether PMP22 expression modulates cell surface expression of these collagen-binding integrins, potentially impacting RPE biology. Overexpressing ARPE-19/PMP22 cells were evaluated by flow cytometry for surface expression of these integrin isoforms (Fig. 2). In comparison to the wild-type ARPE-19 cells, increased PMP22 expression resulted in a 60% increase ($P = 0.001$) in surface expression of integrin $\alpha 1$. However, integrin $\alpha 2$ ($P = 0.001$) and $\alpha 3$ ($P < 0.001$) showed a 44% decrease in surface expression. These results show a mixed change in collagen binding integrin receptors; therefore, a simple prediction about the functional consequence in terms of promoting collagen gel contraction is not possible given the overlapping specificity of the collagen receptors.

FIGURE 2. PMP22 expression differentially affects integrin expression. Cell surface expression was measured in ARPE-19 (*filled bar graph*) and ARPE-19/PMP22 (*lined bar graph*) cells by flow cytometry using monoclonal antibodies against $\alpha 1$ ($***P = 0.001$), $\alpha 2$ ($***P = 0.001$), or $\alpha 3$ ($***P < 0.001$) integrin. The isotype control staining (mean fluorescence intensity [MFI], 5) was performed (*open histogram*), and MFI was subtracted from the experimental samples. Surface expression of each of these integrins was evaluated in three independent experiments, and the results were tabulated. The mean is presented, and statistical comparison of the expression of each integrin in the two cell lines was performed using a Student's *t*-test (unpaired, one-tailed) in which $P < 0.05$ was considered statistically significant.

Adhesion to Collagens I and IV Is Increased in PMP22-Overexpressing Cells

To test whether the modulation of PMP22 alters collagen binding, an adhesion assay was performed. Cells were plated onto a 96-well plate coated with collagen I or collagen IV. PMP22 overexpression resulted in a 34% increase ($P = 0.02$) in adhesion to collagen I and a 27% increase ($P = 0.04$) in adhesion to collagen IV compared with ARPE-19 cells (Fig. 3).

PMP22 Modification Affects Collagen Gel Contraction

To test the hypothesis that greater adhesive capacity to collagen would result in increased collagen contraction, a collagen gel contraction assay was performed using ARPE-19 cells, ARPE-19/PMP22 cells, and ARPE-19/PMP22 cells treated with PMP22-specific siRNA or control siRNA. At least three separate experiments were conducted with six replicates per sample. ARPE-19/PMP22 cells exhibited significantly decreased contraction compared with the ARPE-19 cells (Fig. 4). Reducing PMP22 levels to baseline in ARPE-19/PMP22 using PMP22-specific siRNA restored gel contraction. Control siRNA did not alter gel contraction compared with PMP22-overexpressing cells. As a positive control, cells overexpressing EMP2 were used because these cells showed

FIGURE 3. PMP22 expression increases adhesion to collagen. Increased PMP22 levels lead to increased adhesion to collagen. ARPE-19 (filled circles) and ARPE-19/PMP22 (open triangles) cells were seeded onto plates coated with either collagen I (a) or collagen IV (b). Bound cells were analyzed for crystal violet uptake, and quantitative evaluation of absorbance was measured with a microplate reader (at 590 nm). Increased PMP22 expression resulted in increased adhesion to collagen I ($*P = 0.02$) and collagen IV ($*P = 0.04$). Results are presented from one experiment with at least six replicates, and at least three independent experiments were performed with comparable results. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis. All significant *P* values are shown.

FIGURE 4. Increased PMP22 expression leads to reduced collagen gel contraction. A collagen gel contraction assay was performed using ARPE-19 (filled bar graph), ARPE-19/PMP22 (lined bar graph), and ARPE-19/EMP2 (open bar graph) cells. ARPE-19/PMP22 cells decreased collagen gel contraction compared with wild-type cells ($***P = 0.001$). ARPE-19/PMP22 cells were treated with PMP22-specific siRNA to decrease PMP22 expression to the basal level, as shown in Figure 1. PMP22 siRNA treatment increased contractile capacity compared with untreated cells ($*P = 0.02$). Cells treated with control scramble siRNA did not show a significant difference in contraction compared with untreated cells (data not shown). Experiments were performed independently at least three times with similar results. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis. All significant *P* values are shown.

a significant increase in cellular contractile capacity, as previously reported (Fig. 4).⁸

Increased PMP22 Expression Drives Signal Transduction Away from a FAK/Src Pathway and Toward an AKT Pathway

The observations that increased PMP22 expression led to enhanced collagen adhesion but decreased collagen gel contraction prompted further exploration of the signal transduction pathways in ARPE-19/PMP22 cells. Levels of activated FAK (Fig. 5a) and AKT (Fig. 5b) were quantified in ARPE-19 cells with altered PMP22 expression. Both basal- and collagen-stimulated FAK activation were examined in ARPE-19 and ARPE-19/PMP22 cells. As a positive control for increased FAK activation, ARPE-19/EMP2 cells were included. ARPE-19/PMP22 cells showed a slight increase in basal levels of activated FAK over ARPE-19 cells. However, this increase was not statistically significant. As a control and as previously reported, EMP2 overexpression showed a substantial increase in basal FAK activation compared with ARPE-19 ($P = 0.001$) and ARPE-19/PMP22 cells ($P = 0.02$). FAK activation was also examined after collagen stimulation. Each cell line showed enhanced FAK activation after collagen engagement, but the degree of the increase was different. ARPE-19 and ARPE-19/EMP2 cells showed the greatest increase in FAK activation—nearly threefold—whereas the increase exhibited by the ARPE-19/PMP22 cells was less than onefold.

AKT activation was evaluated by Western blot in ARPE-19 and ARPE-19/PMP22 cells before and after stimulation with PDGF (Fig. 5b). In unstimulated conditions, basal AKT activation was increased twofold ($P = 0.01$) in ARPE-19/PMP22 cells compared with ARPE-19 cells. Although PDGF exposure led to increased p-AKT in the ARPE-19 cells, the PMP22-overexpressing cells had a substantially larger increase in AKT activation ($P = 0.01$) compared with ARPE-19 cells (Fig. 5b). To further confirm the relationship between increased PMP22 expression and AKT activation, ARPE-19/PMP22 cells were treated with

FIGURE 5. Increased PMP22 expression increases AKT activation. Total protein was isolated from ARPE-19 (filled bar graph), ARPE-19/PMP22 (lined bar graph), and ARPE-19/EMP2 (EMP2-overexpressing cells, open bar graph) cells. Cell extracts (15 μ g protein) were fractionated by 4% to 20% SDS-PAGE gradient gel under reducing conditions, and Western immunoblots were probed with antibodies for p-FAK (a) and p-AKT (b). To examine kinase activation, the cells were either treated with 50 ng/mL of PDGF for 30 minutes or incubated on a collagen-coated plate for 2 hours before protein isolation. Experiments were performed independently at least three times with similar results. Band density was quantified using NIH Image J software and normalized to the β -actin loading control. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis. All significant *P* values are shown (** $P \leq 0.001$; * $P \leq 0.01$; * $P < 0.05$).

PMP22-specific siRNA before exposure to PDGF, and these cells showed a reduction in PDGF-stimulated AKT activation by approximately 40% ($P = 0.03$). Control siRNA treatment did not significantly affect AKT activation (Fig. 5b). These observations demonstrated that the signaling response to various stimuli was shaped by the relative abundance of PMP22 or EMP2 protein expression. Increased PMP22 expression drove the cell to respond through AKT activation. Increased EMP2 expression drove the cell to respond through increased FAK activation.

Inhibition of AKT Activation Restores Contractile Capacity

Increased PMP22 expression clearly drove the cell to respond through an AKT pathway, leading one to ask whether AKT activation was responsible for the decreased collagen gel contraction observed in the ARPE-19/PMP22 cells in the absence of PDGF stimulation. To address this question, AKT activation

was blocked with the small molecule inhibitor Ly294002. No cell toxicity was detected with this inhibitor in these studies or as previously reported.¹⁷ After exposure to collagen, a 132% increase in AKT activation was observed in ARPE-19/PMP22 (Figs. 6a, 6b), and Ly294002 completely blocked AKT activation (Figs. 6a, 6b). ARPE-19 and ARPE-19/PMP22 cells were pretreated with AKT inhibitor and then used in a collagen gel contraction assay. AKT inhibition did not affect collagen gel contraction by the wild-type ARPE-19 cells. In contrast, AKT inhibition in the overexpressing ARPE-19/PMP22 cells increased contractile capacity by 100% compared with untreated ARPE-19/PMP22 cells (Fig. 6c). Blocking the AKT pathway restored contractile capacity to levels similar to those of wild-type ARPE-19 cells. Restoration of collagen gel contraction in

FIGURE 6. Inhibition of AKT activation restores contractile capacity. (a) Representative immunoblots. (b) AKT phosphorylation was measured by Western blot in ARPE-19/PMP22 (lined bar graph) cells. Collagen stimulation significantly increased AKT phosphorylation (** $P < 0.001$). AKT phosphorylation was abolished by pretreating the cells with a small molecule inhibitor, Ly294002, at 50 μ M for 1 hour. (c) A collagen gel contraction assay was used to examine the contractile capacity of cells after Ly294002 treatment. Blockade of AKT phosphorylation in the PMP22-overexpressing cells (lined bars), but not in the ARPE-19 cells (filled bars), led to increased gel contraction. Experiments were performed independently at least three times with similar results. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis. All significant *P* values are shown (** $P \leq 0.001$ and * $P \leq 0.01$).

the ARPE-19/PMP22 cells by AKT blockade was not accompanied by an increase in FAK activation (data not shown).

PDGF Stimulation Exacerbates the Difference in Collagen Gel Contraction between ARPE-19 and ARPE-19/PMP22 Cells

PMP22 overexpression drove the cellular response toward an AKT response and away from a FAK response. This finding raised two interesting questions: What role does PMP22 expression play in a stimulated condition of collagen gel contraction? Will the enhanced AKT activity provide a sufficient boost to overcome diminished levels of activated FAK? To address these, ARPE-19, ARPE-19/PMP22, and ARPE-19/PMP22 cells treated with PMP22-specific siRNA were tested in a collagen gel contraction assay in the presence of PDGF. PDGF modestly enhanced collagen gel contraction by the ARPE-19 cells ($P = 0.04$; Fig. 7).¹⁷ Exposure of the ARPE-19/PMP22 cells to PDGF did not result in increased collagen gel contraction. However, exposure to PMP22 siRNA, but not control siRNA, restored the PDGF response (Fig. 7). In unstimulated conditions or after exposure to PDGF, PMP22 overexpression resulted in at least a 50% reduction in contraction, (Figs. 4, 7).

DISCUSSION

Contraction of PVR membranes and the traction they cause on the retina is the cause of recurrent retinal detachment and vision loss.²⁷ Therefore, we sought to grasp a deeper understanding of the factors that modulate retinal pigment epithelial responses to conditions modeling the contractile phase of PVR. The RPE is important at maintaining homeostasis within the retina and, in turn, plays a key role in responding to pathologic alterations in the microenvironment. This study demonstrates that the signal transduction response to extracellular stimuli associated with pathologic alterations is influenced by the relative composition of the tetraspan web.

FIGURE 7. PDGF-stimulated collagen gel contraction is reduced by increased PMP22. A collagen gel contraction assay was performed using ARPE-19 (filled bar graph), ARPE-19/PMP22 (lined bar graph), and ARPE-19/PMP22 cells treated with PMP22 siRNA (hatched bar graph) or control siRNA (open bar graph). These cells were either unstimulated or stimulated with 50 ng/mL PDGF. To reduce PMP22 levels, ARPE-19/PMP22 cells were treated with PMP22 siRNA. Exposure of the ARPE-19/PMP22 cells to PDGF did not result in increased collagen gel contraction. However, exposure to PMP22 siRNA, but not to control siRNA, restored the PDGF response ($***P \leq 0.001$). In unstimulated conditions or after exposure to PDGF, PMP22 overexpression resulted in at least a 50% reduction in contraction ($***P \leq 0.001$). Experiments were performed independently at least three times with similar results. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis. All significant *P* values are shown ($*P \leq 0.05$).

The literature is replete with studies identifying multiple factors contributing to the pathogenesis of PVR, such as alteration of proinflammatory cytokine and growth factor levels, integrin receptor expression, and activation of various signal transduction pathways.^{27–35} In this report, which focuses on the contraction phase of PVR, increased PMP22 expression significantly decreased contraction compared with the ARPE-19 cells. Concordantly, reducing PMP22 levels to baseline using the PMP22-specific siRNA restored gel contraction, suggesting that the change in contraction correlated positively with PMP22 expression. Potentially limiting factors of this study were the use of the ARPE-19 cell line and uncertainty about whether the reported observations could be extended to primary retinal pigment epithelial cells and to the pathobiology of PVR and tractional retinal detachment. Certainly it is important to recognize that PVR *in vivo* is a complex process likely driven by multiple cell types, including RPE, and that additional studies would be required to confirm the observations in primary retinal pigment epithelial cells and in disease. Notably, there was a difference between the maximal gel contraction presented in our present work and that reported by previous studies of ARPE-19 and porcine RPE in primary culture (ours was lower).^{19,36} This difference, concordant with our previous publications,¹⁷ can be attributed to differences between primary cells and cell lines, measurement techniques, cell density, and methods of seeding the collagen gels. In our studies, we used a technique with a low initial seeded cell number to try to detect early pathways that are critically important in gel contraction.

Previously, we reported the activation of FAK as a critical signal transduction pathway in collagen gel contraction and demonstrated that increased EMP2 expression results in increased FAK activation.⁸ Furthermore, the increased gel contraction observed in the EMP2-overexpressing cells was directly linked to FAK activation. We also observed that integrin ligation of collagen led to activation of the FAK/Src complex, required for collagen gel contraction by ARPE-19 cells.¹⁷ In the presence of proinflammatory stimuli such as PDGF, AKT activation also plays a role in collagen gel contraction.^{17,19} In this study, increased PMP22 led to increased adhesion to collagens I and IV but functionally resulted in decreased collagen gel contraction compared with the untreated ARPE-19 cells. Despite the potential limitation that the role of PMP22 in the normal RPE or during disease states has not yet been studied, the results of this work prompt additional future investigations about the physiological role of PMP22 in RPE. The observations that increased PMP22 expression led to enhanced collagen adhesion but decreased collagen gel contraction prompted further exploration of the signal transduction pathways in ARPE-19/PMP22 cells. The change in cellular contractile capacity may be mediated through differential regulation of signal transduction pathways directly related to protein expression levels of PMP22 or EMP2. Specifically, PMP22 or EMP2 protein expression levels drive the cell toward either an AKT or a FAK pathway, respectively, leading to a divergent response to a common stimulus. The implication of this finding adds new dimension to the concept of the tetraspan web; it is not only the presence of a protein but its relative abundance that determines the cellular response.

The tetraspan web has been described as an array of diverse proteins creating a scaffold of multiple membrane domains that regulate signaling and sorting processes.^{37–39} Several sources in the literature show that tetraspans are capable of associating with each other or in heterocomplexes with additional membrane proteins, resulting in increased tyrosine phosphorylation.^{39–43} Examples of this include the recruitment and induction of PI-4 kinase, JNK-, FAK-, Src-, and MAP-kinase-dependent signaling.^{41,43,44} The implication of these findings is that

4-transmembrane proteins act as molecular adaptors, supporting the functional assembly of signaling complexes in the membrane.⁴¹ This study provides new insight regarding the complexity of tetraspan biology. Although establishing the presence or absence of individual proteins within the web may provide a general road map for potential functional outcomes, a deeper level of understanding is required to generate a high-resolution road map of potential functional outcomes.

PMP22 and EMP2 are two components of the circuitry of the tetraspan web that determine how a cell will respond to various stimuli. When PMP22 is highly expressed in ARPE-19 cells, the cells are wired to respond through an AKT-dependent pathway and away from a FAK/Src pathway. In contrast, when EMP2 is highly expressed, the cells respond primarily through a FAK/Src-dependent pathway. These findings suggest that PMP22 and EMP2 may be potentially used to regulate the activation of FAK or AKT in response to various stimuli. However, this interpretation, based on studies using cell lines engineered to express supraphysiological levels of EMP2 or PMP22, must be validated in future studies in which EMP2 and PMP22 levels are changed under physiological conditions.

PVR is a leading cause of blindness associated with failure of retinal detachment repair. In previous reports, we provide evidence for the importance of EMP2 regulation of the FAK/Src signal transduction pathway in ARPE-19 cells and potential PVR pathobiology, as demonstrated by gel contraction assays. We demonstrate that activation of the FAK/Src kinase pathway is necessary to mediate collagen gel contraction.¹⁷ In the present study, we demonstrated that PMP22, a closely related protein to EMP2, may play a pivotal role in determining how a cell responds to pathologic conditions induced by ocular trauma by steering the cells away from a FAK/Src response. The RPE is important at maintaining the homeostasis within the ocular microenvironment. Further insight into the process that determines how a cell responds to an extracellular stimulus will be important for developing future therapeutics.

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