

Functional Consequences of Interactions between FAK and Epithelial Membrane Protein 2 (EMP2)

Shawn A. Morales,^{1,2} Sergey Mareninov,² Paige Coulam,¹ Madhuri Wadehra,¹ Lee Goodglick,¹ Jonathan Braun,¹ and Lynn K. Gordon^{2,3}

PURPOSE. Collagen gel contraction by ARPE-19 is controlled by epithelial membrane protein 2 (EMP2) through focal adhesion kinase (FAK) activation. The purpose of this study was to test the role of EMP2 in the cellular context of FAK activation.

METHODS. The ARPE-19 cell line was recombinantly modified to increase the expression of EMP2 and was used in this study. Quantification of FAK and Src phosphorylation was determined with Western blot analysis of whole cell lysates with the use of specific antibodies for different target sites of phosphorylation. Coimmunoprecipitation of whole cell lysates with an antibody against EMP2, followed by Western blot analysis and identification of FAK, was performed. Focal adhesions and their relationship to EMP2 were identified with immunofluorescence and confocal microscopy. F-actin distribution was identified using fluorescence microscopy, and α -smooth muscle actin (α -SMA) expression was quantified with Western blot analysis and specific antibodies. Adhesion to collagen type I was determined with a binding assay.

RESULTS. EMP2 overexpression led to increased FAK phosphorylation at all measured phosphorylation sites. Coimmunoprecipitation and confocal microscopy provided evidence for a physical association between EMP2 and FAK. Increased EMP2 was also associated with altered distribution of focal adhesions, changes in actin organization, increased α -SMA expression, and increased adherence to a collagen-coated surface.

CONCLUSIONS. The EMP2-FAK association represents a novel protein-protein interaction, not previously reported, that demonstrates significant functional cellular responses in the context of *in vitro* models of proliferative vitreoretinopathy (PVR). (*Invest Ophthalmol Vis Sci.* 2009;50:4949–4956) DOI:10.1167/iov.08-3315

Focal adhesion kinase (FAK) and its activation through phosphorylation is important for cell cycle progression, proliferation, invasion, migration, survival, and contraction.¹ FAK phosphorylation can be initiated by integrin receptor ligation to extracellular matrix components. In addition, a number of nonintegrin signaling pathways promote FAK activation, in-

cluding EGF and PDGF ligation through receptor tyrosine kinase signaling and ligation of receptors for lysophosphatidic acid, bombesin, and sphingosylphosphorylcholine through G-protein-linked receptors.^{2–6} FAK activation is a complex process requiring phosphorylation at multiple sites. Phosphorylation occurs at Tyr397 and Tyr407 (N-terminal domain), Tyr576 and Tyr577 (kinase domain activation loop), and Tyr861 and Tyr925 (C-terminal domain). The initial step in FAK activation is autophosphorylation at Tyr397, creating a binding site for the SH2 domain of Src tyrosine kinase, resulting in the activation of the Src kinase domain. The remaining FAK tyrosine phosphorylation sites are preferentially phosphorylated by Src.^{7–9} Phosphorylation of FAK Tyr576 and Tyr577 in the activation loop of the kinase domain is required for maximal FAK kinase activity,^{7,10} and the phosphorylation of FAK Tyr925 creates an SH2-binding site for the Grb2 small adaptor protein.¹¹ Grb2 binding to FAK is one of several signaling pathways leading to the activation of downstream targets such as the ERK2/MAP kinase cascade. Phosphorylation at Tyr407 and Tyr861 has been implicated in epithelial-mesenchymal transdifferentiation (EMT).¹² FAK phosphorylation at Tyr861 has also been implicated in F-actin organization.¹³

Epithelial membrane protein 2 (EMP2) is a tetraspan (4-transmembrane) protein belonging to the growth arrest specific-3/peripheral myelin protein-22 (GAS3/PMP22) family. EMP2 is localized to the skin, lung, uterus, heart, thyroid, and eye.¹⁴ In the eye, EMP2 is present in multiple epithelial layers, including the cornea, ciliary body, and retinal pigment epithelium.¹⁵ EMP2 has been shown to regulate the trafficking of integrins, glycosylphosphatidyl inositol-anchored proteins, and major histocompatibility complex class I proteins.^{16–19} EMP2 is physically associated with, and modulates the function of, certain integrin isoforms in physiological settings such as blastocyst implantation and in model settings of cellular proliferation, invasion, adhesion, and metastasis.^{15–20}

The biochemical mechanisms involved in EMP2 function are uncertain. However, in other tetraspan proteins, a prominent theme is multimeric association with heterologous tetraspans and other membrane proteins involved in signaling pathways.^{21,22} This suggests that tetraspan proteins act as molecular adaptors supporting the functional assembly of signaling complexes in the membrane.²³ Perhaps best defined is a subset of tetraspan proteins shown to recruit PI-4 kinase to specific membrane locations and to induce phosphoinositide-dependent signaling.²⁴

EMP2 is known to increase collagen gel contraction of the ARPE-19 cell line through FAK activation measured by phosphorylation at Tyr576 and Tyr577.²⁵ The purpose of this study was to further explore the relationship between EMP2 and FAK activation. Here we show that EMP2 physically associates with FAK, leading to increased phosphorylation of FAK at multiple sites. This increased phosphorylation of FAK results in functional mechanical cellular alterations, leading to an increased cellular contractile capacity. These cellular alterations include increased focal adhesion density, conformational changes in the actin cytoskeleton, actin composition alterations, and increased cellular adhesive capacity. These findings suggest that

From the Departments of ¹Pathology and Laboratory Medicine and ²Ophthalmology, University of California, Los Angeles, California; and the ³Department of Surgery, Greater Los Angeles Veterans Affairs Healthcare System, Los Angeles, California.

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Corresponding author: Lynn K. Gordon, Jules Stein Eye Institute, David Geffen School of Medicine at UCLA, 100 Stein Plaza, Los Angeles, CA 90095; lgordon@ucla.edu.

EMP2 may represent a new role for tetraspans in the functional assembly of membrane signaling complexes important for the activation of FAK.

METHODS

Cell Line

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/EMP2, an EMP2-overexpressing cell line, was produced through stable infection of an EMP2 overexpressing retrovirus construct, expressing 75-fold elevated protein level of EMP2.²⁵ ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium-F12, supplemented with 10% fetal bovine serum (FBS; ATCC) at 37°C in a humidified chamber with 5% CO₂. For all experiments cells were plated and incubated overnight. All cells were approximately 75% confluent at the initiation of each experiment, unless otherwise noted.

Antibodies

All antibodies for human FAK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): goat antibody for phosphorylated FAK (p-FAK) (clone Tyr 576/577), and rabbit antibodies for FAK (clone C-20), p-FAK (Tyr 397, Tyr 407, Tyr 861, and Tyr 925). Rabbit antibodies for human p-Src (Tyr 416 and Tyr 527) were from Cell Signaling Technology (Danvers, MA). A mouse antibody recognizing human β -actin (clone 2A2.1) was from US Biological (Swampscott, MA). A mouse antibody against human α -smooth muscle actin (α -SMA; clone 1A4) was from Sigma (St. Louis, MO). Texas Red-X phalloidin (T7471), used to identify F-actin, was from Invitrogen (Carlsbad, Ca). Rabbit antisera against human EMP2 was produced after immunization of the animals with a multiple antigen peptide conjugated to the second extracellular loop of human EMP2 (EDIHDKNAKFPVTREGSYG; Research Genetics, Huntsville, AL). Horseradish peroxidase-conjugated goat anti-rabbit antibody was obtained from Southern Biotechnology Associates (Birmingham, AL). Horseradish peroxidase-conjugated goat anti-mouse was obtained from BD Biosciences (San Diego, CA). Texas Red-conjugated donkey anti-goat (705-075-147) and FITC-conjugated donkey anti-rabbit (711-095-152) antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Western Blot Analysis

Western blot analysis was performed as previously described.²⁶ Briefly, cell protein was isolated with the use of RIPA buffer containing protease and phosphatase inhibitors (Upstate, Charlottesville, VA), and the protein concentration was assayed (BCA Protein Assay; Bio-Rad, Hercules, CA). For EMP2 detection, N-linked glycans were cleaved using PNGase (New England Biolabs, Beverly, MA). Lysates were treated according to the manufacturer's instructions at 37°C for 2 hours. Ten micrograms of protein was loaded in each lane, and the proteins were fractionated by 4% to 20% SDS-PAGE gradient gel under reducing conditions. Proteins were transferred to nitrocellulose membranes (Amersham Life Sciences, Buckinghamshire, UK), and the adequacy of transfer was confirmed (Ponceau S; Sigma Chemical Co., St. Louis, MO).

The membrane was 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 α -SMA, FAK, and p-FAK (Tyr 576/577), (Tyr 397), (Tyr 407), (Tyr 861), (Tyr 925), 1:1000 for EMP2 and p-Src (Tyr 416), (Tyr 527), 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 developed with ECL (Pierce, Rockford, IL) and were quantified by scanning the blots and measuring band density with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda,

MD; available at <http://rsb.info.nih.gov/ij/index.html>). The intensity of either the β -actin or the total FAK band was used as an internal control, and all bands were analyzed in a linear range for the measurement of density by quantification. At least three independent experiments were performed and were statically evaluated using a Student's *t*-test (unpaired, one-tailed); *P* < 0.05 was considered statistically significant.

Coimmunoprecipitation

ARPE 19 and ARPE-19/EMP2 cells were plated in 10-cm dishes (Corning, Corning, NY). Cells were washed twice with PBS, lysed (1% Nonidet P-40 containing 10 μ g/mL aprotinin, 2 μ g/mL pepstatin, 0.1 mM EDTA, 10 mM HEPES, and 10 mM KCl with Complete Mini Protease Inhibitor Cocktail Tablet [Roche Applied Sciences, Mannheim, Germany]) for 30 minutes at 4°C and then sonicated for 15 seconds. Cell lysates were precleared by incubation with Protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated overnight with agarose beads and either anti-FAK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-EMP2 rabbit polyclonal antibody. The beads were washed four times in the lysis solution and twice in 62.5 mM Tris, pH 6.8. Immune complexes were eluted from the beads by boiling in Laemmli sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 2% β -mercaptoethanol) for 5 minutes. Samples were analyzed with Western blot analysis. For EMP2 detection, N-linked glycans were cleaved using PNGase (New England Biolabs). Eluates were treated according to the manufacturer's instructions at 37°C for 2 hours.

The stoichiometry of FAK and EMP2 in coimmunoprecipitation experiments was determined in the following manner. First, titrations of whole cell lysate and cognate immunoprecipitates were quantitated by densitometry. Using these data, we calculated the percentage of each protein (per input cell equivalents) in the cognate immunoprecipitate. Second, the coimmunoprecipitates were similarly analyzed to determine the percentage of each protein captured in the coimmunoprecipitate. Finally, we calculated the ratio of these two values (percentage cognate and percentage coimmunoprecipitate), representing the stoichiometry of the coimmunoprecipitated proteins.

Immunofluorescence

ARPE-19 and ARPE-19/EMP2 cells were plated overnight onto glass coverslips (Fisher Scientific, Pittsburgh, PA). Cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.075% saponin for 15 minutes. Cells were blocked with 10% normal donkey serum for 30 minutes and then were incubated overnight at 4°C in a humidified chamber with the primary antibody and washed three to four times with PBS plus 0.01% Triton X-100 (PBST). Cells were incubated for 1 hour with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG and Texas Red-conjugated donkey anti-goat at room temperature in a humidified chamber. Cells were washed with PBST, rinsed briefly with double-distilled H₂O, and mounted onto microscope slides with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

A laser scanning confocal microscope (LSM 510; Zeiss, Thornwood, NY) was used to assess the distribution and colocalization of proteins. To detect FITC-labeled and Texas Red-labeled cells, samples were excited with argon and krypton lasers at 488 and 568 nm, respectively. LSM software was used for controlling the microscope, scanning and laser modules, image recording, and analysis of image data. Colocalization analysis was performed with LSM software to generate percentage association. Highlighted pixels were determined with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). At least six fields were randomly chosen for analysis for each sample, and percentage association was an average value generated from the multiple fields. Multiple slices per field were evaluated, but only a single slice of the bottom surface of the cell, which contacts the slide and contains focal adhesions, was used for quantifying the colocalization of the focal adhesions with EMP2. In all experiments, cells were observed using a

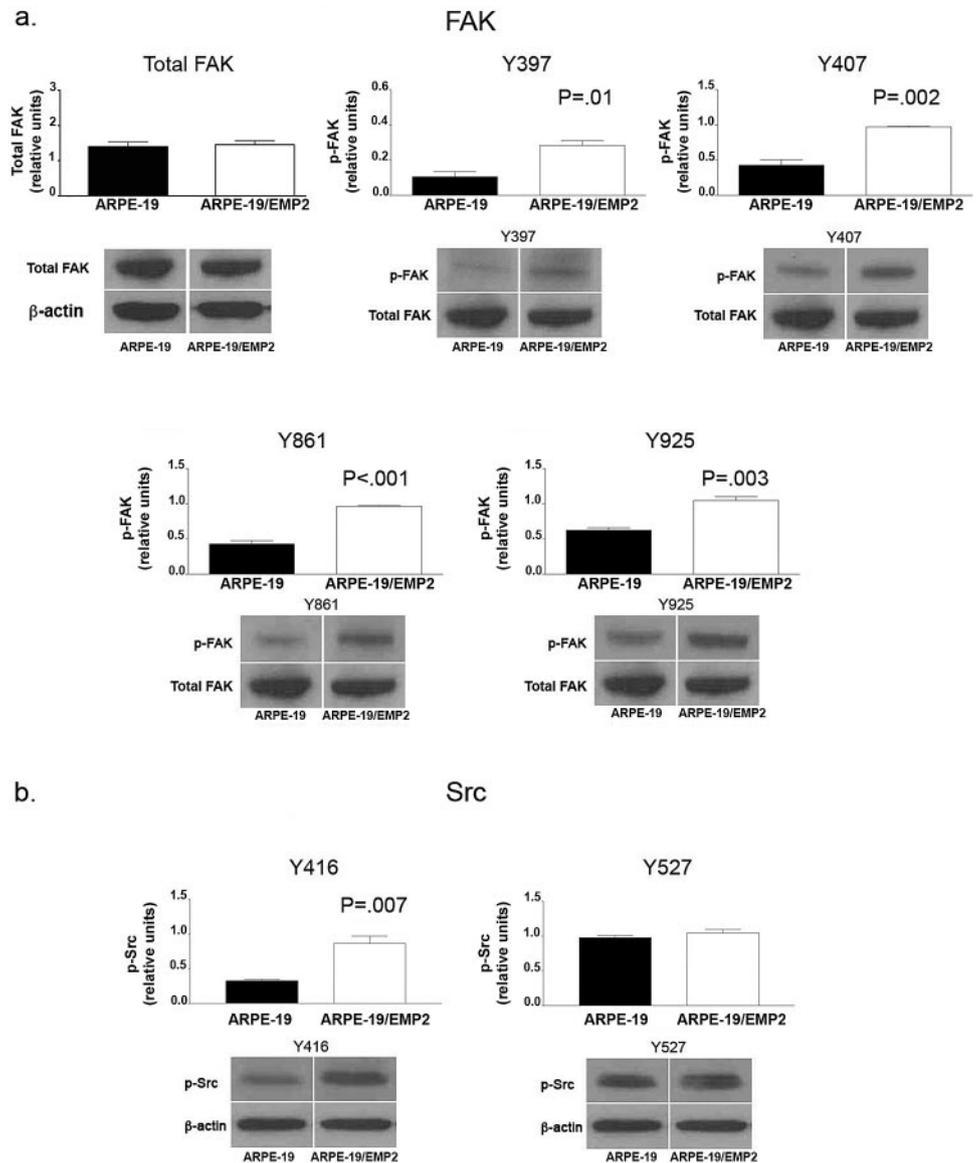


FIGURE 1. (a) Effect of EMP2 on FAK phosphorylation. Levels of total FAK, p-FAK (Y397, Y407, Y861, and Y925), and β -actin were measured by Western blot analysis in the cell lines ARPE-19 and ARPE-19/EMP2 and were quantified by densitometry. (b) p-Src (Tyr 416 and Tyr 527) and β -actin were measured by Western blot analysis in the cell lines ARPE-19 and ARPE-19/EMP2 and were quantified by densitometry. Phosphorylation level (mean + SEM) at each site was normalized to total FAK (a) or β -actin (b) and was calculated from at least three independent experiments. Representative Western blots are also shown.

60 \times oil immersion objective. Each experiment was repeated at least four times.

Adhesion Assay

ARPE-19 and ARPE-19/EMP2 cells were plated onto either a 24-well collagen-coated plate (BD Biosciences, San Diego, CA) or a screening plate precoated with fibronectin, vitronectin, collagen I, and collagen IV (CytoMatrix; Millipore, Billerica, MA) at a concentration of 2×10^5 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 and, after solubilization, absorbance was measured at 595 nm by a microplate reader (550; Bio-Rad, Hercules, CA). 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.

RESULTS

Relationship of p-FAK and EMP2 in ARPE-19 Cells

FAK phosphorylation, elaborated through Src interaction and mutual phosphorylation events, recruits signaling and adapter

proteins that drive a diverse array of cellular responses.²⁷ We previously investigated whether EMP2 overexpression in the ARPE-19 cells led to alteration in FAK/Src activity. We showed that total FAK levels were equivalent in the ARPE-19 and ARPE-19/EMP2 cells,²⁵ confirmed in Figure 1; however, overexpressing EMP2 demonstrated an almost twofold increase in the level of activated FAK (Tyr576/577) compared with the control ARPE-19 cells. 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.²⁵ Other sites of FAK phosphorylation were investigated in the ARPE-19 and ARPE-19/EMP2 cells by quantitative Western blot analysis and were normalized to total FAK (Fig. 1a). EMP2 overexpression enhanced the phosphorylation of FAK twofold to threefold at Tyr397, Tyr407, Tyr861, and Tyr925.

Src enzymatic activity is reciprocally regulated by tyrosine phosphorylation at Tyr416 (augmenting) and Tyr527 (suppressing).²⁸ Compared with control cells, overexpression of EMP2 was associated with increased Src phosphorylation at Tyr416 (Fig. 1b). This result was predicted because this activation of the Src kinase domain is associated with the phosphorylation of FAK at Tyr397. Importantly, there was no

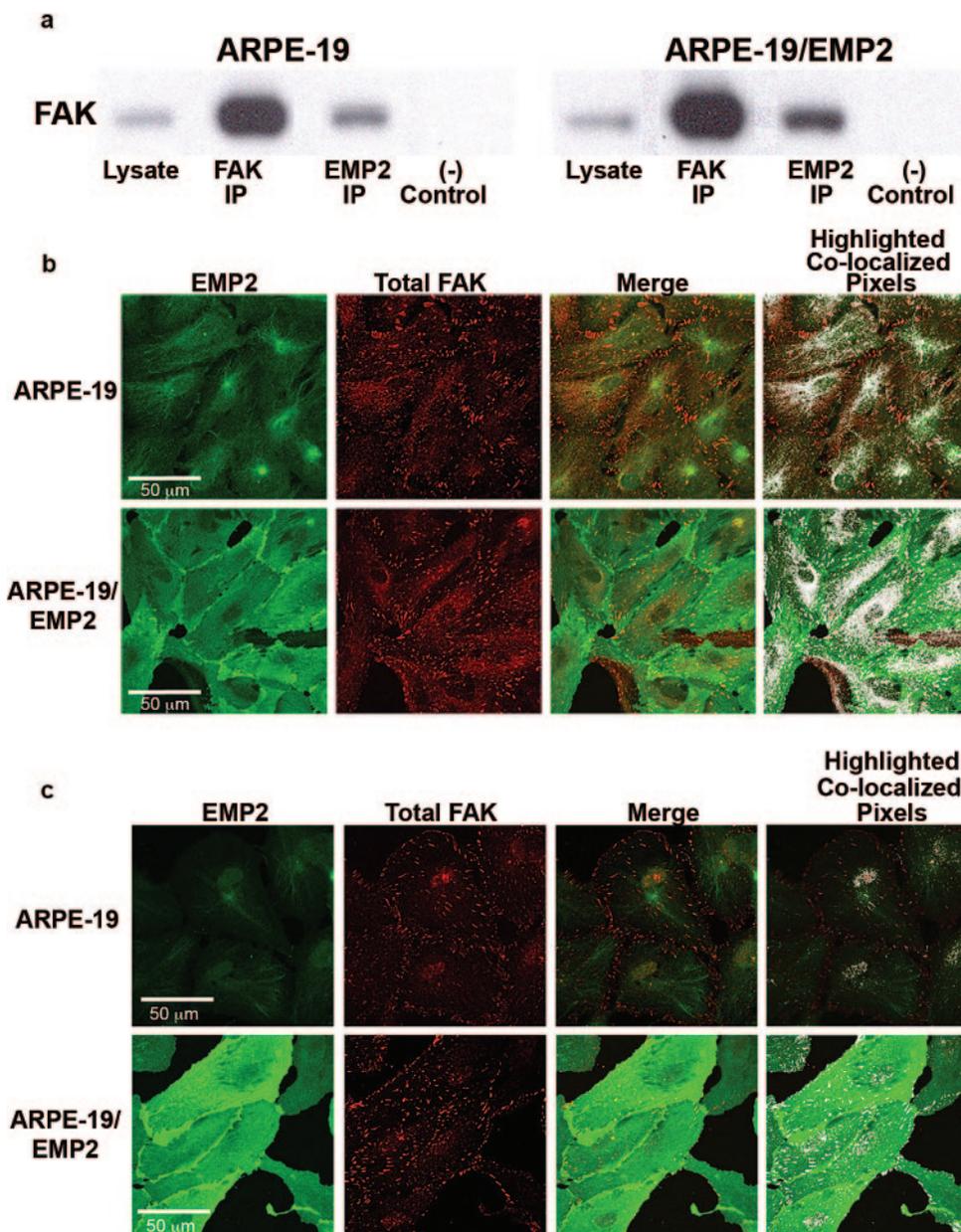


FIGURE 2. Association of FAK and EMP2. **(a)** Lysates from ARPE-19 and ARPE-19/EMP2 (10^7 cells) were immunoprecipitated using antibody to FAK, EMP2, or irrelevant rabbit antibody (– control). SDS-PAGE was performed with aliquots of the immunoprecipitates or of the original lysate (10^6 and 10^5 cell equivalents, respectively), and Western blot analysis was performed for FAK. Cognate immunoprecipitations of FAK and EMP2 (data not shown) were comparably efficient (50%–60%). EMP2 coimmunoprecipitated 25% and 30% of total FAK in ARPE-19 and ARPE-19/EMP2, respectively. Data are representative of three or more experiments. **(b)** ARPE-19 and ARPE-19/EMP2 cells were stained with antibodies to EMP2, followed by secondary FITC-conjugated donkey anti-rabbit IgG (green) and FAK, followed by secondary Texas Red-conjugated donkey anti-goat (red). The merge of these two channels is shown (center-right), as is a merge in which highlighted colocalized pixels are displayed (right). At least six fields were randomly chosen for analysis for each sample, and percentage association is an average value generated from the multiple fields. Multiple slices per field were captured. Presented is a single slice of the bottom surface of the cell, which contacts the slide and contains focal adhesions. In ARPE-19 and ARPE-19/EMP2, 68% and 96% of total FAK colocalized with EMP2, respectively. **(c)** To assess the association between EMP2 and phosphorylated FAK, ARPE-19 and ARPE-19/EMP2 cells were stained with antibodies against EMP2 and against p576/577-FAK. Bound antibody against EMP2 was identified using an FITC-conjugated secondary antibody, whereas bound antibody against p-FAK (Y576/577) staining is shown with a Texas Red-conjugated secondary antibody. A merge of these two channels is shown (white), and colocalized pixels are observed and quantified. At least six

fields were randomly chosen for analysis for each sample, and percentage association is an average value generated from the multiple fields. Multiple slices per field were captured. Presented is a single slice of the bottom surface of the cell, which contacts the slide and contains focal adhesions. In the ARPE-19 cells, 31% of phosphorylated FAK is colocalized with EMP2. In the ARPE-19/EMP2 cells, 97% of phosphorylated FAK is colocalized with EMP2. Increasing EMP2 levels results in increased phosphorylated FAK-EMP2 association.

change in phosphorylation levels at Src Tyr527, which is not in the kinase domain (Fig 1b). Taken together, these findings indicate that elevated EMP2 resulted in phosphorylation changes of FAK and Src, known to increase mechanical contractility.

EMP2 Physically Associates with FAK

EMP2 expression is required for efficient integrin-mediated cellular responses, and EMP2 physically associates with certain integrin isoforms.^{16–19} FAK is an important signaling partner of integrins, a role that includes the physical association of $\beta 1$ integrin cytoplasmic tail and the N-terminal domain of FAK in vitro.^{29,30} Therefore, we hypothesized that the positive effect of EMP2 levels on FAK phosphorylation might reflect a physical association between FAK and EMP2. To address this issue,

we examined two requirements of physical association, binding either directly or indirectly through immunoprecipitation and localization within the same cellular space through confocal microscopy.

We first evaluated this idea by testing whether EMP2 and FAK are physically associated (Fig. 2a). Immunoprecipitates using an antibody against EMP2 include readily detectable FAK in ARPE-19 and ARPE-19/EMP2 cells. To determine the stoichiometry of the association, we used three independent experiments to tabulate quantitated levels of EMP2 and FAK in the immunoprecipitates. First, titrations of whole cell lysate and cognate immunoprecipitates were quantitated by densitometry. Using this data, we calculated the percentage of each protein (per input cell equivalents) in the cognate immunoprecipitate. Second, the coimmunoprecipitates were similarly an-

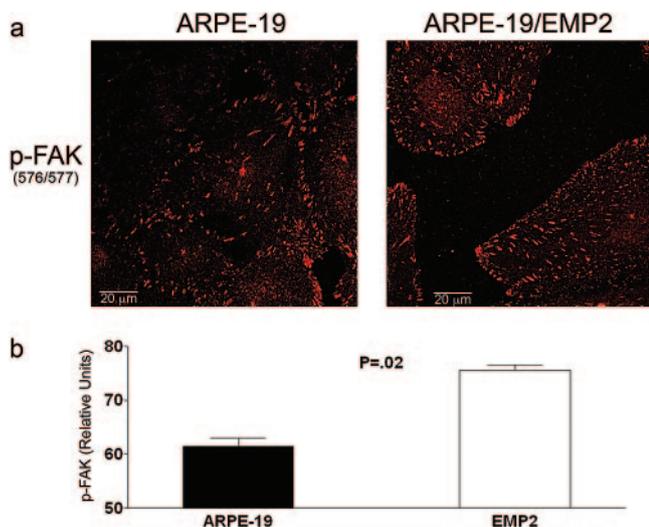


FIGURE 3. Modulating EMP2 expression affects FAK phosphorylation and localization. ARPE-19 and ARPE-19/EMP2 cells were stained with p576/577-FAK (a) primary antibody. Cells were incubated with a Texas Red-conjugated donkey anti-goat antibody and observed under a fluorescence microscope. Phosphorylated FAK (b) from at least four separate samples were quantified by calculating pixel intensity, and the data were evaluated by Student's *t*-test (unpaired, one-tailed).

alyzed to determine the percentage of each protein captured in the coimmunoprecipitate. Third, we calculated the ratio of these two values (percentage cognate and percentage coimmunoprecipitate), representing the stoichiometry of the coimmunoprecipitated proteins. Cognate immunoprecipitation of FAK (Fig. 2a) and EMP2 (data not shown) were comparably efficient (50%–60%). The levels of FAK in the EMP2 immunoprecipitates were 12% and 15% in ARPE-19 and ARPE-19/EMP2, respectively, and we calculated that 24% to 30% of total FAK was associated with EMP2 in these two cell lines.

To evaluate whether EMP2 and FAK localize within the same cellular space, their colocalization in situ was examined by confocal microscopy. Multiple levels per field were evaluated, but only a single slice of the bottom surface of the cell, which contacts the slide and contains focal adhesions, was used for quantification. EMP2 (green) and FAK (red) were detected by immunofluorescence and visualized by confocal microscopy (Fig. 2b). The merge of these two channels is shown, as well as a merge in which highlighted colocalized pixels are displayed as white signal (right). By inspection, EMP2 and FAK were prominently colocalized in both cell types. By pixel quantitation of three independent experiments, $68\% \pm 9\%$ and $96\% \pm 1\%$ of FAK present at the cell surface contacting the glass slide was colocalized with EMP2 in ARPE-19 and ARPE-19/EMP2 cells, respectively. Total FAK levels demonstrated a slight, but not significant, increase in the ARPE-19/EMP2 cell line (Fig. 1a). This confirmed our previously published data demonstrating that EMP2 levels do not affect total FAK protein expression.²⁵

To examine whether EMP2 associated with phosphorylated FAK, cells were stained with EMP2 and p576/577-FAK primary antibodies. Multiple slices per field were evaluated, but only a single slice of the bottom surface of the cell, which contacted the slide and contains focal adhesions, was used for quantification. EMP2 was detected with FITC, and phosphorylated FAK was detected with Texas Red (Fig. 2c). In ARPE-19, $31\% \pm 8\%$ of p-FAK (Y576/577) was colocalized with EMP2. A central area within the cells showed colocalization; however, identification of this subcellular region would have required additional studies. In the ARPE-19/EMP2, $97\% \pm 0.4\%$ of p-FAK (Y576/

577) was colocalized with EMP2 at this level. EMP2 and FAK (total and p-FAK (Y576/577)) were highly colocalized in situ, and elevated EMP2 increased the degree of colocalization. However, given the degree to which EMP2 was overexpressed, this might not have implied specific enrichment of FAK.

Increased EMP2 Levels Resulted in Altered Distribution of Phosphorylated FAK

ARPE-19 and ARPE-19/EMP2 (Fig. 3a) cells were stained for phosphorylated FAK (Y576/577) protein. Increased EMP2 levels resulted in altered distribution of phosphorylated FAK. In the ARPE-19 cells, phosphorylated FAK was found mainly on the cell periphery (Fig. 3a), whereas in the ARPE-19/EMP2 cells phosphorylated FAK was distributed throughout the cell (Fig. 3a). Pixel intensity from at least four separate samples was measured; by Student's *t*-test (unpaired, one-tailed), this evaluation, concordant with our previous report using Western blot analysis for protein detection,²⁵ also demonstrated a significant increase in phosphorylated FAK in the ARPE-19/EMP2 cells (Fig. 3b).

Increased EMP2 and Phosphorylated FAK Expression Leads to Altered F-Actin Distribution

Increased EMP2 expression leads to increased FAK phosphorylation at multiple sites, including Tyr861, which has been associated with F-actin organization. Accordingly, we examined whether there was a concomitant change in observable actin organization in association with increased EMP2 expression. ARPE-19 (Figs. 4a, c) and ARPE-19/EMP2 (Figs. 4b, d) cells were stained with Texas Red-X phalloidin primary antibody. Increasing EMP2 expression resulted in altered F-actin expression. F-actin was expressed along the periphery of the cell in the ARPE-19/EMP2 cells, whereas F-actin was expressed

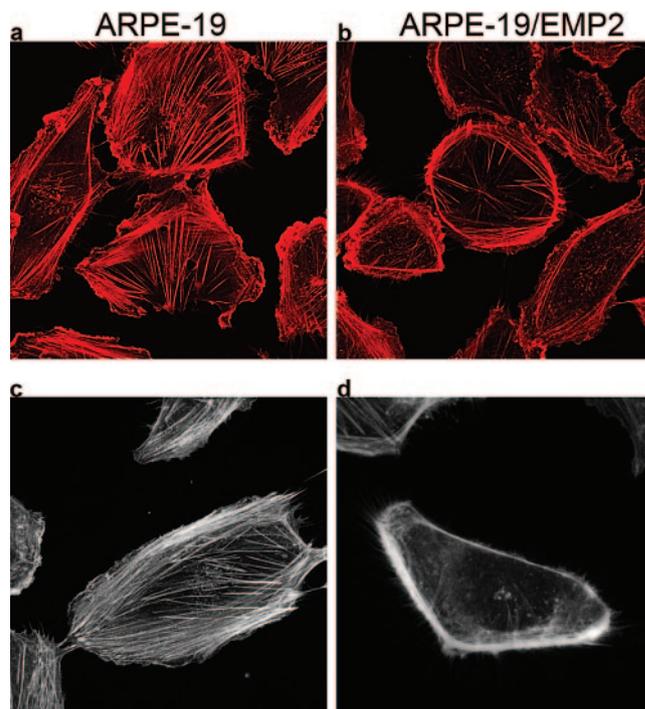


FIGURE 4. Increased EMP2 and phosphorylated FAK expression led to altered F-actin distribution. ARPE-19 (a, c) and ARPE-19/EMP2 (b, d) cells were plated overnight onto glass coverslips. Cells were fixed with paraformaldehyde, permeabilized with saponin, and stained with Texas Red-X phalloidin. Representative pictures are shown in color (a, b) and in black and white (c, d).

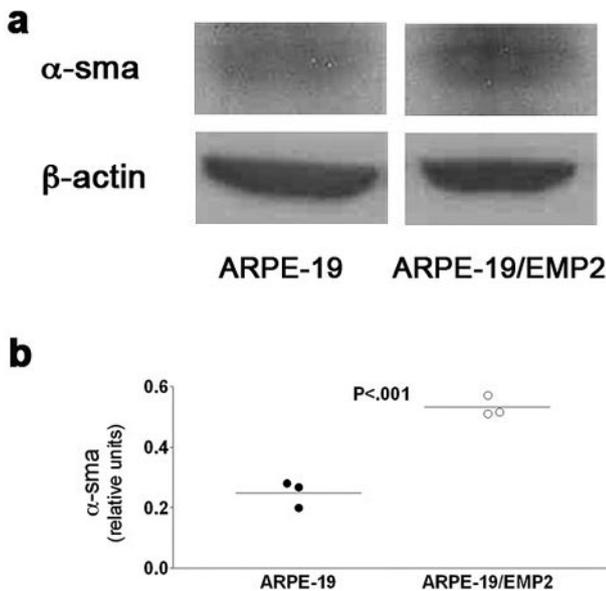


FIGURE 5. Increased EMP2 expression resulted in increased α -smooth muscle actin expression. (a) Steady state protein levels of α -smooth muscle actin (α -SMA) in the ARPE-19 and ARPE-19/EMP2 cells were measured by Western blot analysis; β -actin was used as a loading control. (b) The amount of α -SMA was calculated for each sample relative to β -actin. At least three independent experiments were performed, and the results are presented numerically. Differences in expression levels were evaluated using a Student's *t*-test (unpaired, one-tailed).

throughout the cell in ARPE-19 cells. We chose to examine actin fibers in actively growing cells, similar to those tested in the gel contraction assay. Additional studies of the arrangement in confluent cells could be conducted in the future.

Increased EMP2 Expression Resulted in Increased α -Smooth Muscle Actin Expression

Increased EMP2 expression led to increased FAK phosphorylation at Tyr861 and at Tyr407, which have both been implicated as important sites in cellular EMT. A marker for EMT is the expression of α -SMA. Levels of α -SMA were measured by Western blot analysis in ARPE-19 and ARPE-19/EMP2 cells to test whether increased EMP2 expression, with its associated FAK phosphorylation, has a functional effect on α -SMA expression (Fig. 5a). Blots were then developed with ECL to visualize bound antibody and were quantified with β -actin as an internal control (Fig. 5b). Increased EMP2 expression led to a significant increase in α -SMA expression.

Increased EMP2 Levels Led to Increased Cellular Adhesion to Collagen Types I and IV

ARPE-19/EMP2 cells showed a greater distribution of phosphorylated FAK throughout the cell than did ARPE-19 cells. We hypothesized that the increased distribution of phosphorylated FAK represented functional focal adhesions that would confer greater adhesive capacity to the ARPE-19/EMP2 cells. To test this hypothesis, we plated ARPE-19 and ARPE-19/EMP2 cells onto plates precoated with fibronectin, vitronectin, collagen I, and collagen IV. Bound cells were analyzed for crystal violet uptake with a microplate reader (550; Bio-Rad). Increasing EMP2 expression resulted in a specific increase in the attachment phase of adhesion to collagen types I and IV ($P < 0.001$), decreases in attachment to fibronectin ($P < 0.02$), and no affect in attachment to vitronectin (Fig. 6).

DISCUSSION

This study was guided by the hypothesis that EMP2, like other tetraspan family members,²³ is a molecular adaptor between certain integrin isoforms and their associated signaling modules. Our previous study identified that activation of the FAK-Src pathway is critical in producing the contraction of collagen gels in an in vitro model of proliferative vitreoretinopathy (PVR) using the RPE cell line ARPE-19.²⁶ Furthermore, our studies demonstrated that EMP2 is able to control collagen gel contraction through the activation of FAK (Y576/577).²⁵ We previously demonstrated that EMP2 associates with integrin $\beta 1$,¹⁸ and other groups have shown that FAK binds in vitro to the integrin $\beta 1$ subunits.^{8,31} In this study, we provide evidence that EMP2 enhances FAK activation and physically associates with FAK, with functional downstream consequences affecting focal adhesion density, cellular adhesive capacity, F-actin conformation, and actin composition. Accordingly, EMP2 may act as a molecular adaptor for efficient integrin-mediated FAK activation and its consequences for FAK-associated cellular functions. Although the coimmunoprecipitation and colocalization studies support either a direct or an indirect association through the formation of a multiprotein complex, additional biochemical studies are required to define the specific details of this relationship.

An important mechanism for FAK activation is integrin receptor-mediated clustering of FAK, resulting in autophosphorylation, Src recruitment, and FAK phosphorylation at multiple sites. The activated FAK/Src complex subsequently acts as a molecular scaffold for a diverse array of proteins, such as Grb2, p130^{Cas}, paxillin, Talin, CAP, Graf, Src, and the p85 subunit of PI 3-kinase, and may participate in numerous signaling pathways.^{8,31,32}

In this report we demonstrate that EMP2 associates with FAK, leading to the increased activation of FAK by enhancing focal adhesion formation, and we hypothesize that EMP2 may act as the molecular adaptor between integrin ligation and FAK

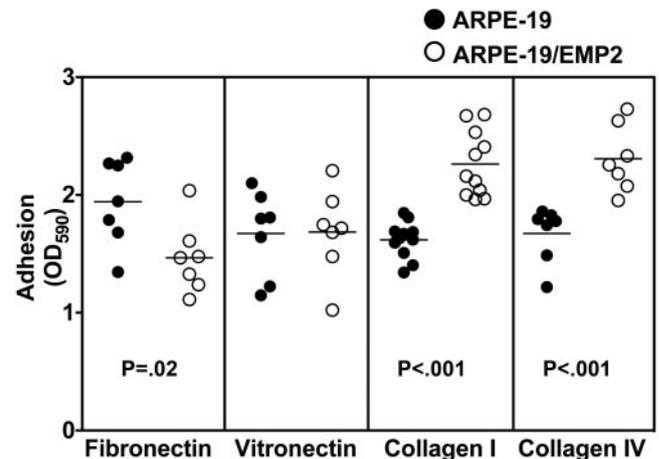


FIGURE 6. Increased EMP2 levels led to increased adhesion to collagen. ARPE-19 and ARPE-19/EMP2 cells were plated onto plates coated with fibronectin, vitronectin, collagen I, and collagen IV. Bound cells were analyzed for crystal violet uptake, and quantitative evaluation of absorbance was measured by a microplate reader (at 590 nm). Increasing EMP2 expression resulted in increased adhesion to collagen I ($P < 0.001$) and collagen IV ($P < 0.001$). Adhesion to fibronectin was increased ($P = 0.02$) in ARPE-19 cells compared with ARPE-19/EMP2 cells. Altering EMP2 levels did not affect adhesion to vitronectin. Results are presented from one experiment with at least eight replicates, and at least three independent experiments were performed with comparable results. Student's *t*-test (unpaired, one-tailed) was used for statistical analysis.

activation. We previously examined the role of EMP2 in collagen gel contraction, an in vitro correlate of PVR. PVR is a complication after surgical repair of a rhegmatogenous retinal detachment in up to 10% of patients.³³⁻³⁵ PVR represents the culmination of a complex migration of multiple cell types, including retinal pigment epithelium, into the vitreous cavity.³⁶⁻³⁹ There is evidence for EMT of RPE cells, resulting in migration, membrane formation, and an aberrant wound-healing strategy associated with contractile cellular forces leading to tractional retinal detachment. With the use of collagen gel contraction performed by ARPE-19 cells, we identified integrin receptor activation and signaling through FAK as a critically important process required for ARPE-19-mediated contraction.²⁶ We recently observed that EMP2 expression levels positively correlated with collagen gel contraction and that this phenotype was achieved through facilitating FAK activation.²⁵

EMT is a key component of PVR pathogenesis. Phosphorylation of FAK at Tyr407 and Tyr861 is identified as important in EMT progression.⁴⁰ Overexpression of EMP2 leads to increases in Tyr407 and Tyr861 phosphorylation and is thus predicted to lead to EMT progression. One marker for EMT is the upregulation of α -SMA, which was observed in the EMP2-overexpressing cell line ARPE-19/EMP2 cells. These changes, in association with increased EMP2 and increased FAK activation, are predicted to facilitate a greater contractile phenotype. The organization of F-actin may also affect the capacity of cells to contract collagen gels. F-actin expression is observed throughout the ARPE-19 cell, perhaps conferring a more rigid cellular structure, reducing the cell's ability to contract collagen gels. In ARPE-19/EMP2 cells, F-actin is found only along edges of the cell in a cortical distribution that may confer a more flexible conformation, allowing for greater contractile capacity.

Tetraspan proteins have been shown to participate in the formation of a variety of complexes to form the so-called tetraspan web,^{41,42} which is the creation of scaffolds and membrane domains that regulate signaling and sorting processes.^{41,43} These complexes can modulate the signaling, trafficking, and structural characteristics of their membrane protein constituents. When tetraspans form complexes with integrin molecules, they can modulate cell adhesion and mobility.⁴⁴ Our understanding of the tetraspan family and its biological functions is incomplete at this time. However, our report adds a piece to the tetraspan puzzle, demonstrating that EMP2 regulates FAK activation through a physical association leading to an EMT phenotype with a greater cellular contractile capacity. The EMP2-FAK association represents a novel protein-protein interaction, not previously reported, that demonstrates significant functional cellular responses in in vitro models of PVR.

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