Podocalyxin Increases the Aggressive Phenotype of Breast and Prostate Cancer Cells In vitro through Its Interaction with Ezrin

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Abstract

Podocalyxin is an anti-adhesive transmembrane sialomucin that has been implicated in the development of more aggressive forms of breast and prostate cancer. The mechanism through which podocalyxin increases cancer aggressiveness remains poorly understood but may involve the interaction of podocalyxin with ezrin, an established mediator of metastasis. Here, we show that overexpression of podocalyxin in MCF7 breast cancer and PC3 prostate cancer cell lines increased their in vitro invasive and migratory potential and led to increased expression of matrix metalloproteases 1 and 9 (MMP1 and MMP9). Podocalyxin expression also led to an increase in mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) activity. To determine the role of ezrin in these podocalyxin-dependent phenotypic events, we first confirmed that podocalyxin formed a complex with ezrin in MCF7 and PC3 cells. Furthermore, expression of podocalyxin was associated with a changed ezrin subcellular localization and increased ezrin phosphorylation. Transient knockdown of ezrin protein abrogated MAPK and PI3K signaling as well as MMP expression and invasiveness in cancer cells overexpressing podocalyxin. These findings suggest that podocalyxin leads to increased in vitro migration and invasion, increased MMP expression, and increased activation of MAPK and PI3K activity in MCF7 and PC3 cells through its ability to form a complex with ezrin. [Cancer Res 2007;67(13):6183–91]

Introduction

Podocalyxin is a transmembrane sialomucin structurally related to the vascular endothelium marker CD34 that is expressed on the surface of a wide range of cells including kidney podocytes, vascular endothelia cells, hematopoietic stem cells, and platelets (1–4). Podocalyxin is best characterized in the kidney where it functions as an anti-adhesion molecule that maintains open filtration pathways between neighboring podocyte foot processes through the charge-repulsive effects of its large highly anionic extracellular domain (5, 6). In canine kidney (MDCK) cells, podocalyxin overexpression leads to the inhibition of cell-cell interaction as shown by decreased cell-cell adhesion, decreased tight junction-dependent transepithelial resistance, and redistribution of cell junction proteins (6). Decreased cell-cell interaction is a prominent feature of cancer cells that display a more metastatic phenotype, suggesting a possible role for anti-adhesive molecules, such as podocalyxin, in cancer progression (7).

The role for podocalyxin in cancer remains unclear. However, the gene encoding podocalyxin (PODXL or PCLP1) has been reported to be a downstream target of both WT1 and TP53 (8). Recently, podocalyxin has been implicated in the development of more aggressive forms of cancer. High levels of podocalyxin protein expression correlated with poor outcome in a subset of breast carcinomas, and podocalyxin is commonly expressed by blasts in acute myeloid and acute lymphoblastic leukemia (9, 10). Furthermore, gene expression studies revealed a positive correlation between expression of PODXL and expression of ETS1, a regulator of angiogenesis, in human vascular endothelial cells (11). PODXL has also been implicated in more aggressive forms of prostate cancer (12). The PODXL gene maps to chromosome 7q32–q33 (13), a region that has been linked to aggressive forms of prostate cancer and that exhibits a high frequency of allelic imbalance in prostate tumors (14–16). Recently, PODXL variants were found to be associated with both the risk of prostate cancer and prostate tumor aggressiveness (12). Thus, podocalyxin is a candidate for playing a critical role in cancer development and aggressiveness.

The mechanism underlying the role of podocalyxin in cancer may involve its interaction with the actin-binding protein ezrin. In the podocyte, podocalyxin is linked to the cytoskeleton via a complex comprised of ezrin and the bridging protein NHERF (Na+/H+ exchanger regulatory factor (17, 18). Additional data support the direct interaction of podocalyxin and ezrin (19). Ezrin is a member of the ezrin/radixin/moesin (ERM) family of proteins that regulate cytoskeletal-related functions such as cell adhesion, cell survival, and cell motility, all of which are important in tumor progression (20–22). The importance of ezrin as a positive regulator of metastasis has been established in murine osteosarcoma, rhabdomyosarcoma, and mammary carcinoma models (23–25). Furthermore, high expression of ezrin is associated with a more aggressive phenotype in human osteosarcomas, rhabdomyosarcomas, and melanomas as well as in endometrial cancers (24, 26–28). Recently, abnormal ezrin localization has been associated with adverse tumor characteristics in breast cancer (29). Ezrin has been shown to bind directly to phosphatidylinositol 3-kinase (PI3K) and influence a number of signaling pathways that affect cellular functions related to tumorigenesis and metastasis, including the mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK-ERK1/2), PI3K-AKT, and Rho pathways (21, 23, 30). Ezrin-mediated effects on AKT and ERK1/2 activity have been linked to the ability of ezrin to promote tumor progression and metastasis (23, 25, 31). NHERF gene mutations have also been implicated in breast cancer (32).

In the present study, we show that in MCF7 breast cancer and PC3 prostate cancer cells, podocalyxin expression correlates with increased migration and invasion, increased matrix metalloprotease (MMP) expression, and increased MAPK and PI3K activity.
We also show that podocalyxin and ezrin form a complex in these cells and that overexpression of podocalyxin correlates with changes in the phosphorylation state and subcellular localization of ezrin. Finally, we show that these podocalyxin-related changes in signaling events and invasiveness are ezrin-dependent events.

**Materials and Methods**

**Cell lines.** MCF7 and MDA-MB-231 breast cancer cells were grown in DMEM supplemented with 5% FCS, 1% t-glutamine, 1% penicillin, and 1% streptomycin in 5% CO2 at 37 °C. PC3 prostate cancer cells were grown in DMEM supplemented with 10% FCS, 1% t-glutamine, 1% penicillin, and 1% streptomycin in 5% CO2 at 37 °C. Cells were routinely passaged at sub-90% confluence using a solution of 0.05% trypsin, 0.33 mM EDTA-4Na in HBSS without calcium or magnesium. Full-length PODXL cDNA was cloned into pcDNA3.1 (Invitrogen) and stably transfected into MCF7 and PC3 cells by electroporation using a Bio-Rad GenePulser followed by clonal selection in media containing 500 μg/mL G418 (Invitrogen). Control cells were transfected with empty pcDNA3.1 vector and selected in the same manner. Transient transfections with a full-length antisense ezrin construct, a dominant-negative ezrin (T567A mutant) construct (gifts from Dr. Chand Khanna, CCR National Cancer Institute, NIH; ref. 23), and/or empty pcDNA3.1 (Invitrogen) were done using LipofectAMINE 2000 Reagent (Invitrogen) in accordance with the manufacturer’s recommendations. The total concentration of DNA used in all transient transfections was maintained at 10 μg by titration of full-length antisense ezrin construct or dominant-negative ezrin (T567A) construct with empty vector DNA. Transfection efficiencies were 40% for MCF7 and 45% for PC3 cells as determined by transfection with green fluorescent protein vector pcDNA3.1/NT-GFP-Topo (Invitrogen).

**Western blotting and immunodetection.** Cells at 70% confluence were rinsed in 1× PBS and lysed by repeated freeze-thaw in buffer containing 50 mMol/L Tris (pH 8.0), 150 mMol/L NaCl, 1% Triton X-100, 1× proteinase inhibitor cocktail (Roche Diagnostics), 1 mMol/L sodium fluoride, and 1 μMol/L sodium orthovanadate. To detect protein phosphorylation state, cells were deprived of serum for 20 h before lysis. For detection of Tyr353 phosphorylated ezrin (pEzrin), cells at 70% confluence were deprived of serum for 20 h and then treated with 50 μMol/L sodium orthovanadate for 3 h before cell lysis. To determine the phosphorylation state of ezrin at Thr558, cell lysates were immunoprecipitated with 2 μg ezrin antibody (BD Biosciences) and 20 μL pre-blocked Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 16 h at 4 °C. The immunoprecipitate was then separated by SDS-PAGE, electroblotted, and probed with pEzrin (Thr558)/radixin (Thr564)/moesin (Thr567) antibody (1:1,000 dilution), Cell Signaling). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). In experiments using protein lysates from cells in which ezrin was transiently knocked down by the introduction of antisense construct or in which dominant-negative ezrin (T567A) had been introduced, lysates were collected 56 h after transfection.

Proteins were separated by using 10% SDS-PAGE gels followed by electroblotting to Immobilon-P membranes (Millipore) using a transfer buffer consisting of 25 mmol/L Tris, 192 mmol/L glycine, 0.01% SDS, and 20% methanol at pH 8.3. Membranes were blocked by incubation in 1× PBS containing 5% fat-free dry milk for 1 h at room temperature. Blots were incubated with the following primary antibodies: podocalyxin (1:10,000 dilution; clone 3D3; Santa Cruz Biotechnology), ezrin (1:5,000 dilution; BD Biosciences), actin (1:1,000 dilution; clone AC-15; Sigma), pEzrin (Thr558)/radixin (Thr564)/moesin (Thr567) antibody (1:1,000 dilution), Cell Signaling), pEzrin (Thr558)/radixin (Thr564)/moesin (Thr567) antibody (1:1,000 dilution; Cell Signaling), ezrin/radixin/moesin (1:1,000 dilution; Cell Signaling), phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204; 1:1,000 dilution; clone E10; Cell Signaling), total ERK1/2 (1:1,000 dilution; Cell Signaling), phosphorylated AKT (pAKT; Ser473; 1,100 dilution; Cell Signaling), or total AKT (1:1,000 dilution; Cell Signaling). Incubation with primary antibodies specific to podocalyxin, ezrin, and actin were carried out for 1 h at room temperature in a dilution buffer containing 1× PBS, 0.1% Tween 20, and 5% fat-free dry milk. Incubations with antibodies against pEzrin, pERK1/2, total ERK1/2, pAKT, and total AKT were carried out for 16 h at 4 °C in a dilution buffer containing 5% bovine serum albumin in 1× PBS and 0.1% Tween 20. Blots were subsequently probed with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences) diluted 1:5,000 in 1× PBS, 0.1% Tween 20, and 5% fat-free dry milk. Signals were visualized using the ECL Plus detection system (Amersham Biosciences) according to the manufacturer’s instructions. Relative quantitations of Western blots were done using Scion Image Software for Windows (Scion Corp.).

**Invasion assay.** Matrigel-precocated Transwell chambers with PET membranes containing 8-μm pores (BD Biosciences) were soaked in DMEM and incubated for 60 min at 37 °C. After the chambers were rehydrated, 1 × 105 cells in 0.5 mL serum-free culture medium were added to the upper compartment of the Transwell chamber. DMEM (0.5 mL) supplemented with 10% FCS as a chemoattractant was added to the lower chamber. As a control, an equal number of uncoated BD control chambers were seeded with cells in parallel. After 48 h (MCF7 cells) or 24 h (PC3 cells) of incubation, non-invaded cells in the upper compartment were removed using a cotton-tipped swab. Invaded cells were stained with the Diff-Quik stain kit (BD Biosciences) and photographed (×10 magnification). Cells were counted in three unique fields of each triplate membrane. Data were expressed as the percentage of cells that invaded through the Matrigel matrix-coated membrane relative to the cells that migrated through the control membrane. For inhibitor studies, cells were treated with 10 μMol/L LY294002 (Calbiochem), or DMSO (Sigma) as a vehicle control, and invasion was assayed after 48 h (MCF7 cells) or 24 h (PC3 cells) of incubation. For experiments using transiently transfected clones, invasion assays were initiated 16 h following transfection. Data were averaged from two independent experiments.

**Wound migration assay.** Confluent MCF7 or PC3 cells grown in 10-cm² dishes were wounded using sterile pipette tips (T = 0 h), washed twice with 1× PBS, and grown in DMEM with 5% FCS. At T = 0, 24, 48, and 72 h MCF7 cells were photographed under a phase-contrast microscope (×10 magnification). PC3 cells were monitored for migration at T = 0, 24, and 48 h.

**Migration assay.** Migration was analyzed in BD Falcon 24-well plates receiving 8-μm pore size PET membrane BD Biocoat cell culture inserts (BD Biosciences). The upper compartment was seeded with 1 × 105 cells in DMEM without serum. The lower compartment was filled with 0.5 mL DMEM supplemented with 10% FCS as a chemoattractant. After 48 h (MCF7 cells) or 24 h (PC3 cells) at 37 °C, the remaining cells were removed from the upper side of the membrane with a cotton swab, and cells that had migrated and attached to the lower side were stained with Diff-Quik kit (BD Biosciences) and photographed (×10 magnification). Cells were counted in three fields of triplicate membranes. Data were averaged from two independent experiments.

**Soft agar growth assay.** Cells (1 × 106) were grown in triplicate on 10-cm² dishes in a suspension of 0.6% low melting point agarose (Life Technologies) and DMEM supplemented with 10% FCS. After 2 weeks, the plates were photographed under a phase-contrast microscope and assayed for colony number and size.

**Proliferation assay.** Cells (5 × 104) were dispensed in triplicate to 96-well plates containing serum supplemented with 5% FCS. Cell proliferation was measured over a 7-day period using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit (Promega).

**Coimmunoprecipitation.** Cells at 70% confluence were rinsed in 1× PBS and lysed in buffer containing 50 mMol/L Tris (pH 8.0), 150 mMol/L NaCl, 1% Triton X-100, 1× proteinase inhibitor cocktail (Boehringer), 1 mMol/L sodium fluoride, and 1 μMol/L sodium orthovanadate. Protein concentrations were determined using the BCA protein assay kit (Pierce). 500 μg total protein was immunoprecipitated with 2 μg anti-ezrin rabbit polyclonal antibody (Upstate) or 2 μg rabbit IgG (Rockland Immunochemicals) and 20 μL pre-blocked Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 16 h at 4 °C. The precipitates were separated by SDS-PAGE followed by immunoblotting with anti-ezrin mouse monoclonal antibody (1:1,000 dilution; BD Biosciences) and anti-podocalyxin mouse monoclonal antibody Clone 3D3 (1:2,000 dilution; Santa Cruz Biotechnology).
Subcellular colocalization. Cells were grown on glass coverslips until 30% confluent and fixed with acetone for 5 min. After blocking, cells were incubated with anti-ezrin rabbit polyclonal antibody (1:100 dilution; Upstate) alone or in combination with anti-podocalyxin mouse monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology). Secondary antibodies were Texas red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG used at 1:200 dilutions. Cells were counterstained with 4',6-diamidino-2-phenylindole and visualized under fluorescent microscopy (∼100 magnification).

Gelatin zymography. Cells were grown to 70% confluence, washed twice with 1× PBS and once with serum-free DMEM, and incubated in serum-free medium. After 24 h, conditioned medium was collected and concentrated 20-fold using Amicon Ultra 10,000 MWCO centrifugal filter devices (Millipore). Media protein concentrations were determined using the BCA protein assay kit (Pierce). Protein samples were prepared in non-reducing sample buffer containing 0.625 mmol/L Tris-HCl, 10% glycerol, 2% SDS, and 2% bromphenol blue. Proteins, 40 μg per lane, were separated by SDS-PAGE on gels containing 2 mg/mL gelatin type A (Sigma). Gels were renatured for 1 h at room temperature in a solution containing 50 mmol/L Tris/HCl (pH 8.0), 5 mmol/L CaCl2, and 2.5% Triton X-100. Gels were incubated overnight at 37°C in a buffer containing 50 μmol/L Tris-HCl (pH 8.0), 5 μmol/L CaCl2, 0.5% Triton X-100, and 0.02% NaN3. The gels were fixed in a solution of 20% methanol and 5% acetic acid for 30 min at room temperature. Gels were stained with Coomassie blue R-250 (Sigma) and destained in a solution of 40% methanol and 15% acetic acid to reveal gelatinolytic activity. For inhibitor studies, cells were treated with 10 μmol/L LY294002, 30 μmol/L PD98059, or DMSO (as vehicle control), and conditioned media were collected after 24 h of incubation. For studies in which ezrin protein levels were transiently knocked down, culture medium was changed 3 h after transfection and collected after conditioning for 24 h.

Real-time PCR quantification. Total RNA was extracted from cell lines with TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Following DNase I treatment using the DNA-free kit (Ambion), cDNA was generated from total RNA using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Real-time quantitative PCR experiments were done using the SYBR Green PCR Core kit (Applied Biosystems) according to the vendor’s instructions using an ABI 7900HT (Applied Biosystems) real-time PCR instrument. The following primer pairs were used to amplify MMP1, MMP2, MMP9, MT1-MMP (MMP14), and actin: MMP1 forward, 5'-TGGACCTGGAAGAAATCTGTGC-3' and reverse, 5'-GCATGGTCTC-3'; MMP2 forward, 5'-AGCTGACAAGTTGTTGCTG-3' and reverse, 5'-GGATTGACTGCT-3'; MMP9 forward, 5'-TCTCATGCCTCAGCTAACATGCT-3' and reverse, 5'-AATCCGCAGATCTCCATACATCT-3'; MT1-MMP forward, 5'-CCATCTGGCACCCTTTATC-3'; and reverse, 5'-TATCAGGAACGAAAGCCGGG-3'; actin forward, 5'-TGCGCAAGAATTGCGAGGCAG-3' and reverse, 5'-TTATCCCTAATCTGCTGGG-3'. All primers were designed using Primer Express v2.0 software (Applied Biosystems). Transcript expression levels were normalized using actin levels as an endogenous control. Cycle conditions were 95°C for 10 min followed by 45 cycles of 95°C for 10 s and 60°C for 60 s. Relative quantitation of gene transcripts was accomplished using the comparative CΔt method.

Results

Podocalyxin expression increases the in vitro invasive and migratory potential of cancer cells. To investigate the potential role of podocalyxin in invasion and migration, we compared the phenotype of MCF7 and PC3 cells stably expressing podocalyxin or empty vector. Full-length podocalyxin or the empty pcDNA3.1 vector was stably introduced into MCF7 and PC3 cells (Fig. 1A). Podocalyxin expression had no effect on ezrin expression (Fig. 1A, middle). Podocalyxin expression also had no effect on the expression of two other ERM protein family members: radixin and moesin (data not shown). The effect of podocalyxin expression on the invasive potential of MCF7 and PC3 cells was examined using Matrigel-coated Transwell chamber assays. As shown in Fig. 1B, podocalyxin expression increased the in vitro invasive potential of MCF7 cells by 2.5- to 4-fold in the three MCF7/PODXL transfectants compared with the empty vector control. Podocalyxin expression also increased the in vitro invasive potential of PC3 cells 1.8- to 2.7-fold compared with the vector control (data not shown). In a wound-healing assay, cells were grown to confluence before wounding with a sterile pipette tip and monitored every 24 h for 3 days for migration into the wound area (Fig. 1C). All MCF7/PODXL transfectants showed increased wound healing at each time point compared with the pcDNA vector control cells. PC3/PODXL transfectants also showed increased wound healing compared with empty vector controls over a 48-h period (data not shown). Migration was also assayed quantitatively using culture inserts with 8-μm pores (Fig. 1D). Migration of MCF7/PODXL cells was increased by 40% to 60% compared with vector control cells. The effect of podocalyxin on anchorage-independent growth of MCF7 cells was measured by growth in soft agar. Podocalyxin expression had no effect on the number or size of colonies developing over a 2-week period (data not shown). Podocalyxin expression was also found to have no effect on the proliferation of MCF7 cells grown in DMEM supplemented with 5% FCS or PC3 cells grown in DMEM supplemented with 10% FCS. Cell doubling times for the MCF7 clones were 40 h, whereas the PC3 clones doubled in 24 h (data not shown).

Podocalyxin coimmunoprecipitates and colocalizes with ezrin. Podocalyxin is known to interact with the cytoskeletal linker protein ezrin in kidney podocytes, but this complex has not been shown in cancer cells (17). We first tested for the presence of podocalyxin/ezrin complexes in MCF7 transfectants by coimmunoprecipitation. Lysates from MCF7/PODXL transfectants and empty vector controls were immunoprecipitated with ezrin antibody. The precipitates were separated by SDS-PAGE and probed for either ezrin or podocalyxin (Fig. 2A, top). Podocalyxin was coimmunoprecipitated with ezrin in all MCF7/PODXL transfectants but was not detectable in immunoprecipitates from MCF7/pcDNA vector control cells (Fig. 2A, lane 2). Neither ezrin nor podocalyxin were precipitated with a nonspecific (IgG) antibody (Fig. 2A, lane 1), showing the interaction to be specific. To ensure that podocalyxin and ezrin could form a complex in cancer cells that endogenously express both of these proteins, we confirmed the coimmunoprecipitation of podocalyxin and ezrin in MDA-MB-231 cells (Supplementary Fig. S1). A reciprocal set of experiments using podocalyxin antibody to immunoprecipitate ezrin confirmed the presence of complexes containing podocalyxin and ezrin in MCF7/PODXL cells (data not shown).

To determine whether ezrin and podocalyxin colocalized, MCF7 and PC3 clones were examined by fluorescent microscopy. Ezrin and podocalyxin were found to colocalize in both MCF7/PODXL (Fig. 2B, top) and PC3/PODXL (Fig. 2B, bottom) cells. Cytoplasmic ezrin staining was seen in both pcDNA control and PODXL-expressing cells (Fig. 2B and C), with additional strong, focal ezrin staining primarily at cell boundaries evident in PODXL-expressing clones but not in pcDNA control cells (Fig. 2B and C). Focal staining of podocalyxin was seen primarily along cellular boundaries in MCF7/PODXL and PC3/PODXL cells, and these areas of staining overlapped with areas of intense ezrin staining (Fig. 2B).

Phosphorylation of Tyr353 is associated with changes in the subcellular localization of ezrin, regulates the interaction between...
ezrin and the p85 subunit of PI3K, and is required for activation of the PI3K/akt survival pathway by ezrin (24, 33). We therefore tested whether phosphorylation of ezrin at Tyr353 was changed in response to podocalyxin expression. Western blots with an antibody specific for ezrin phosphorylated on Tyr353 showed that phosphorylation of this residue was greatly increased in MCF7/PODXL and PC3/PODXL cells (Fig. 2D). Ezrin may also be phosphorylated at Thr567. Phosphorylation of this residue is reported to disrupt intramolecular association and is associated with changes in ezrin localization and the formation of actin rich structures near the cell membrane (34, 35). Expression of podocalyxin was also found to lead to increased phosphorylation of ezrin on Thr567 in both MCF7 and PC3 cells (Supplementary Fig. S2).

**Podocalyxin expression increases the activity of the MAPK and PI3K pathways.** Interaction between podocalyxin and ezrin in cancer cells suggests that podocalyxin may increase cancer cell aggressiveness by modulating signaling pathways influenced by ezrin. To test this theory, we analyzed the activity of two pathways previously shown to be influenced by ezrin: the PI3K and MAPK pathways (21, 23). Activity of the MAPK pathway was assayed by measuring the amount of phosphorylated, active ERK1/2 kinase. PI3K pathway activity was determined by measuring the amount of phosphorylated, active AKT. Western blot analysis showed that ERK1/2 phosphorylation was increased by 1.6- to 2.3-fold in MCF7/PODXL cells compared with MCF7/pcDNA control cells (Fig. 3A). Expression of podocalyxin in PC3 cells resulted in a 2.6- to 2.7-fold increase in ERK1/2 phosphorylation (data not shown). AKT phosphorylation was also enhanced 2.4- to 2.5-fold in MCF7/PODXL cells compared with MCF7/pcDNA cells (Fig. 3A) and 7.5- to 10-fold in PC3/PODXL cells compared with PC3/pcDNA cells (data not shown).
We next tested whether ezrin was required for the increase in ERK1/2 and AKT phosphorylation seen in MCF7/PODXL and PC3/PODXL cells. Ezrin expression was transiently knocked down using an antisense ezrin construct kindly provided by Dr. Chand Khanna. This construct has previously been used successfully to reduce ezrin protein levels in osteosarcoma cells (23). When podocalyxin-expressing MCF7 or PC3 transfectants were transiently transfected with 5 or 10 g of full-length antisense ezrin construct, ezrin protein levels were reduced by 40% or 80%, respectively, in MCF7 cells and 40% or 60%, respectively, in PC3 cells (Fig. 3B, top). Knockdown of ezrin protein using 5 or 10 g of the antisense ezrin construct resulted in 40% and 70% reductions, respectively, in ERK1/2 phosphorylation in MCF7/PODXL cells along with 40% reduction in PC3/PODXL cells (Fig. 3B, top middle). Transient knockdown of ezrin resulted in 50% and 90% reductions in AKT phosphorylation in MCF7/PODXL cells and 60% and 70% reductions in AKT phosphorylation in PC3/PODXL cells (Fig. 3B, bottom middle). A dominant-negative ezrin construct (T567A), kindly provided by Dr. Chand Khanna, was used to confirm that ezrin activity is required for altered signaling activity in PODXL-expressing cells. Expression of dominant-negative ezrin in MCF/PODXL clones resulted in up to 80% reduction in AKT phosphorylation in MCF7/PODXL clones (data not shown) in agreement with data from experiments with the antisense ezrin construct. However, phosphorylation of ERK1/2 in PODXL-expressing clones was increased up to 40% by introduction of dominant-negative ezrin (data not shown). This unexpected effect of dominant-negative ezrin (T567A) on ERK1/2 phosphorylation has been reported elsewhere (36) and suggests an alternate mechanism through which ezrin influences MAPK signaling. These data indicate that the increased activity of the MAPK and PI3K pathways induced by podocalyxin expression is mediated through ezrin.

**Podocalyxin expression results in increased expression and activity of MMPs.** Increased cellular invasion is often accompanied by increased activity of proteins that degrade the extracellular matrix such as members of the MMP family. In addition, the MAPK and PI3K pathways have been shown to regulate expression of MMP family members (31, 37, 38). Therefore, we assessed the activity and expression of several members of the MMP family in MCF7/PODXL and PC3/PODXL cells. Supernatants from cells grown in the absence of serum were concentrated 20-fold and subjected to gelatin zymography to measure the activity of MMP2 and MMP9. MCF7/PODXL and PC3/PODXL cells showed an increase in MMP9 activity compared with pcDNA control cells (Fig. 4A). MMP2 activity was unaffected (data not shown).

We next measured mRNA expression levels of MMP9, MMP2, MMP1, and MT1-MMP (MMP14) by real-time quantitative PCR. MMP9 gene expression was increased 2.5- to 5.5-fold in the MCF7/PODXL cells compared with empty vector control cells (Fig. 4B).
and transcript levels correlated well with observed MMP9 activity levels. In addition, MMP1 mRNA expression was up-regulated 7.5- to 14-fold in PODXL transfectants (Fig. 4C). There were no changes in expression of MMP2 and MT1-MMP (MMP14) mRNAs between MCF7/PODXL cells compared with empty vector control cells (data not shown). To determine whether the ability of podocalyxin to induce MMP expression in PODXL-expressing cells was dependent on ezrin, we measured MMP activity and expression in MCF7/PODXL and PC3/PODXL cells in which ezrin protein levels were transiently knocked down via transfection with a full-length antisense ezrin construct (see Fig. 3B). Transient knockdown of ezrin protein levels resulted in a dose-dependent reduction in both the amount of active MMP9 secreted (Fig. 5A) and the levels of MMP9 and MMP1 transcripts (Supplementary Fig. S3A and B). These data indicate that ezrin is required for the increased expression and activity observed in PODXL-expressing cells.

Inhibition of MAPK or PI3K activity attenuates MMP expression and activity in MCF7 and PC3 cells. We next sought to determine if either of the ezrin-influenced signaling pathways (MAPK or PI3K) was responsible for the induction of MMP expression in PODXL-expressing cells. To accomplish this, we used inhibitors targeted to each of these pathways: 10 µmol/L LY294002 and 30 µmol/L PD98059 specifically and effectively inhibited the PI3K or MAPK kinase 1 (MEK1) pathways in MCF7 and PC3 cells (Supplementary Fig. S4A). Treatment with PD98059 resulted in a decrease of between 33% and 50% in MMP9 expression in all MCF7/PODXL cells (Supplementary Fig. S4B). MMP1 expression was also noticeably decreased in MCF7/PODXL cells treated with either LY294002 (Supplementary Fig. S4C) or PD98059 (Supplementary Fig. S4D). Together, these results suggest that signaling through both the MEK1 and PI3K pathways are required for the increased expression and activity of MMP1 and MMP9 seen in PODXL-expressing cells.

Activity of both MEK1 and PI3K is required for the podocalyxin-dependent increased invasiveness of MCF7/PODXL and PC3/PODXL cells. We next asked if increased activity of the ezrin-related signaling pathways (MAPK and PI3K) was responsible for the enhanced in vitro invasive potential of PODXL-expressing cells. PODXL-expressing cells and empty vector control cells were seeded onto Matrigel-coated Transwell chambers and treated with PD98059, LY294002, or DMSO and allowed to invade. The presence of either PD98059 or LY294002 significantly inhibited invasion of podocalyxin transfectants (Fig. 6A and B). Invasion of MCF7/pDNA and PC3/pDNA vector control cells was resulted in a sharp decrease in MMP9 activity. Likewise, when MCF7 (Fig. 5C) and PC3 (data not shown) cells were treated with the MEK1 inhibitor PD98059, a strong decrease in MMP9 activity was observed by zymography in all transfectants. LY294002 treatment also resulted in a decrease of between 45% and 53% in MMP9 mRNA expression in all three MCF7/PODXL cells (Supplementary Fig. S4A). Treatment with PD98059 resulted in a decrease of between 33% and 50% in MMP9 expression in all MCF7/PODXL cells (Supplementary Fig. S4B). MMP1 expression was also noticeably decreased in MCF7/PODXL cells treated with either LY294002 (Supplementary Fig. S4C) or PD98059 (Supplementary Fig. S4D). Together, these results suggest that signaling through both the MEK1 and PI3K pathways are required for the increased expression and activity of MMP1 and MMP9 seen in PODXL-expressing cells.

Activity of both MEK1 and PI3K is required for the podocalyxin-dependent increased invasiveness of MCF7/PODXL and PC3/PODXL cells. We next asked if increased activity of the ezrin-related signaling pathways (MAPK and PI3K) was responsible for the enhanced in vitro invasive potential of PODXL-expressing cells. PODXL-expressing cells and empty vector control cells were seeded onto Matrigel-coated Transwell chambers and treated with PD98059, LY294002, or DMSO and allowed to invade. The presence of either PD98059 or LY294002 significantly inhibited invasion of podocalyxin transfectants (Fig. 6A and B). Invasion of MCF7/pDNA and PC3/pDNA vector control cells was
only marginally affected by treatment with either inhibitor as may be expected because these cells exhibit reduced basal activity of ERK1/2 or AKT. Finally, to confirm that ezrin is required for the increased invasiveness exhibited by podocalyxin-overexpressing cells, we measured the ability of MCF7/PODXL and PC3/PODXL cells to invade when ezrin was transiently knocked down. Transient knockdown of ezrin with 10 μg antisense ezrin construct severely reduced the invasive ability of MCF7/PODXL and PC3/PODXL clones, resulting in invasive capacities equivalent to those measured in pcDNA control cells treated with no antisense construct (Fig. 6C and D).

**Discussion**

Recent studies have implicated podocalyxin in the development of more aggressive forms of breast and prostate cancers (9, 12). Here, we show for the first time that podocalyxin expression increases the in vitro migratory and invasive properties of MCF7 breast cancer and PC3 prostate cancer cells, leads to increased MMP1 and MMP9 expression, and leads to increased MAPK and PI3K activity. We further show that these podocalyxin-dependent effects are mediated at least in part through its interaction with ezrin.

The known anti-adhesive properties of podocalyxin suggest at least one mechanism through which podocalyxin may increase the aggressiveness of cancer cells. Recent work has shown that overexpression of podocalyxin in breast cancer cells disrupts cell-cell contact (9). A second possible mechanism relies on the property of podocalyxin to form a complex with the metastasis promoter ezrin, an interaction that has been shown in kidney podocytes (17). Here, we establish for the first time that podocalyxin is able to form a complex with ezrin in breast and prostate cancer cells. In MCF7 and PC3 cells overexpressing podocalyxin, both podocalyxin and ezrin localize to cell boundaries. The anti-adhesive properties of podocalyxin suggest that high concentrations of podocalyxin along cellular boundaries might inhibit the formation of proper cell-cell interactions. Ezrin is thought to play an active role in targeting molecules to specific cell

![Figure 4. Podocalyxin expression leads to increased MMP9 and MMP1 expression. A, zymogram showing increased secretion of MMP9 by MCF7/PODXL and PC3/PODXL transfectants. Cells were grown for 24 h in the absence of serum. Supernatants were collected, concentrated, and used for gelatin zymography. B and C, histograms showing the correlation between podocalyxin expression and increased production of MMP9 (B) and MMP1 (C) mRNA using real-time PCR analysis. mRNA was extracted from the same MCF7/PODXL cells as in (A), and real-time quantitative PCR analyses were done.](image)

![Figure 5. Increased MMP activity in MCF7/PODXL and PC3/PODXL cells is dependent upon ezrin expression and activity of PI3K and MEK1. A, zymogram showing transient knockdown of ezrin protein levels resulted in decreased secretion of MMP9 in MCF7/PODXL and PC3/PODXL transfectants. Ezrin protein levels were knocked down in MCF7/PODXL and PC3/PODXL cells by transient transfection of an antisense ezrin construct. Supernatants were collected, concentrated, and used for gelatin zymography. Representative results for MCF7/PODXL1 and PC3/PODXL1 cells. B and C, zymograms showing that secretion of MMP9 by MCF7/PODXL cells is dependent on PI3K and MEK1 activity. Cells were grown for 24 h in serum-free medium in the presence or absence of the PI3K inhibitor LY294002 (B; LY) or the MEK1 inhibitor PD98059 (C; PD). Supernatants were collected, concentrated, and used for gelatin zymography.](image)
Our data suggest that the redistribution of podocalyxin to cell boundaries relies on interaction with ezrin and the underlying cytoskeleton.

Podocalyxin expression was also found to result in changes in the phosphorylation state of ezrin at tyrosine residue 353 and threonine residue 567. Phosphorylation of ezrin Tyr353 has been seen in response to treatment with epidermal growth factor followed by a change in the subcellular localization of ezrin (33). Phosphorylation at Tyr353 is also required for interaction of ezrin and the p85 subunit of PI3K and ezrin-regulated modulation of the PI3K pathway (21). Phosphorylation at Thr567 is thought to allow for activation of ezrin by reduction of intramolecular association and is associated with changes in localization of ezrin and the formation of actin-rich structures (34, 35).

Our observation that podocalyxin expression correlated with a change in the subcellular localization of ezrin and changes to ezrin phosphorylation suggests that podocalyxin might influence ezrin-regulated signaling pathways (i.e., the PI3K and MAPK pathways). In support of this, we found that podocalyxin expression correlated with increased PI3K and MAPK activity. Transient knockdown of ezrin protein by expression of antisense ezrin RNA abrogated the increase in podocalyxin-dependent changes in MAPK and PI3K activities, providing further support for the hypothesis that the ability of podocalyxin to increase migration and invasiveness of MCF7 and PC3 cells is dependent upon its interaction with ezrin.

The PI3K and MAPK pathways are believed to affect metastasis through a number of mechanisms including induction of members of the MMP family (37, 38, 40). We observed an increase in MMP9 secretion as well as an increase in MMP9 and MMP1 mRNA levels in PODXL-overexpressing cells. This podocalyxin-dependent induction of MMP expression required ezrin and ezrin-related signaling as shown following knockdown of ezrin protein and chemical inhibition of the PI3K and MAPK pathways. Inhibition of either the MAPK or PI3K pathways or knockdown of ezrin levels not only led to a decrease in MMP expression but also to a decrease in the in vitro invasive potential of MCF7/PODXL cells.

Taken together, our results support the hypothesis that podocalyxin increases the in vitro migration and invasive properties of breast and prostate cancer cells through its interaction with ezrin. This complex may be important for targeting podocalyxin to specific membrane sites where the anti-adhesive properties of podocalyxin prevent cell-cell interaction. Moreover, by binding to ezrin and tethering this protein near the plasma membrane, podocalyxin may also affect ezrin-dependent migration and invasion.

**Figure 6.** Inhibition of MEK1 or PI3K activity decreases invasion of MCF7 and PC3 cells expressing podocalyxin. A and B, histogram showing that the increased in vitro invasion exhibited by MCF7/PODXL clones (A) and PC3/PODXL clones (B) requires MEK1 and PI3K activity. Cells (1 × 10⁵) were seeded into the upper well of an invasion chamber in serum-free DMEM. DMEM supplemented with 10% serum was used as a chemoattractant in the lower chamber. Cells were grown for 48 h (MCF7) or 24 h (PC3) in the presence or absence of the inhibitors PD98059 and LY294002. The invasion index was calculated as the number of cells invading through the invasion chamber relative to the number of cells moving through a control chamber normalized to the DMSO-treated MCF7/pcDNA or PC3/pcDNA cells, respectively. Data are summarized as histograms from two independent experiments. C and D, histogram showing that the increased in vitro invasion exhibited by MCF7/PODXL clones (C) and PC3/PODXL clones (D) requires ezrin. Cells were transiently transfected with antisense ezrin construct (0, 5, or 10 μg) and/or empty vector (10, 5, or 0 μg to maintain total DNA concentration at 10 μg for all transfections). Sixteen hours after transfection, cells (1 × 10⁵) were seeded into the upper well of an invasion chamber in serum-free DMEM. DMEM supplemented with 10% serum was used as a chemoattractant in the lower chamber. Cells were grown for 48 h (MCF7) or 24 h (PC3). The invasion index was calculated as the number of cells invading through the invasion chamber relative to the number of cells moving through a control chamber normalized to MCF7/pcDNA or PC3/pcDNA cells treated with 0 μg antisense construct, respectively.
signaling events, leading to increased activities of the MAPK and PI3K pathways, induction of MMPs, and ultimately leading to a more invasive phenotype. Our findings suggest that podocalyxin and the podocalyxin/ezrin complex may represent potential targets for the treatment of some metastatic breast and prostate cancers.

References


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