Podocalyxin-like 1 promotes invadopodia formation and metastasis through activation of Rac1/Cdc42/cortactin signaling in breast cancer cells

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Metastatic disease is the leading cause of cancer mortality. Identifying biomarkers and regulatory mechanisms is important toward developing diagnostic and therapeutic tools against metastatic cancer. In this study, we demonstrated that podocalyxin-like 1 (PODXL) is overexpressed in breast tumor cells and increased in lymph node metastatic cancer. Mechanistically, we found that the expression of PODXL was associated with cell motility and invasiveness. Suppression of PODXL in MDA-MB-231 cells reduced lamellipodia formation and focal adhesion kinase (FAK) and paxillin phosphorylation. PODXL knockdown reduced the formation of invadopodia, such as inhibiting the colocalization of F-actin with cortactin and suppressing phosphorylation of cortactin and neural Wiskott–Aldrich syndrome protein. Conversely, overexpression of PODXL in MCF7 cells induced F-actin/cortactin colocalization and enhanced invadopodia formation and activation. Invadopodia activity and tumor invasion in PODXL-knockdown cells are similar to that in cortactin-knockdown cells. We further found that the DTHL motif in PODXL is crucial for regulation of cortactin phosphorylation and Rac1/Cdc42 activation. Inhibition of Rac1/Cdc42 impeded PODXL-mediated cortactin activation and FAK and paxillin phosphorylation. Moreover, inhibition of PODXL in MDA-MB-231 cells significantly suppressed tumor colonization in the lungs and distant metastases, similar to those in cortactin-knockdown cells. These findings show that overexpression of PODXL enhanced invadopodia formation and tumor metastasis by inducing Rac1/Cdc42/cortactin signaling network.

Introduction

Tumor metastasis is the leading cause of cancer mortality; identifying genes involved in tumor migration/invasion is paramount to the development of effective treatments. Degradation of the extracellular matrix (ECM) by tumor cells is an important step in tumor invasion and metastasis. Tumor cells secrete proteases that degrade ECM components. In addition, formation of invadopodia allows cells to coordinate ECM degradation, thereby facilitating cell migration and invasion (1–3). Invadopodia are actin-rich membrane protrusions driven by clusters of intracellular components, which include focal adhesion kinase (FAK), paxillin, neural Wiskott–Aldrich syndrome protein (N-WASp) and members of the Rho GTPase family, such as Cdc42 and Rac1. Activation and/or phosphorylation of these molecules promote invadopodia formation (4,5). Cortactin plays a crucial role in orchestrating activation of actin-nucleating factors and actin assembly during invadopodia formation. Cortactin binds to filamentous (F)-actin and facilitates the binding of other actin regulatory proteins, including N-WASp and Arp2/3, which further supports nucleation of a branched F-actin network (6–8). Expression of cortactin promotes cell motility by enhancing ECM secretion and the persistence of lamellipodia, a second type of actin-based protrusion, which are localized in the leading edge of motile cell and act in cell movement (9,10). The association between cortactin and invadopodia was demonstrated to play a critical role in tumor invasiveness and metastasis (11–13).

Podocalyxin-like 1 (PODXL), also known as Gp135, was initially identified in podocytes of renal glomeruli that are instrumental in kidney development (14,15). The structure of PODXL is closely related to CD34 and endoglycan, a sialomucin in the plasma membrane (16,17). Expression of PODXL was identified in podocytes, hematopoietic progenitors, vascular endothelia and embryonic stem cells (18–20). It has been reported that PODXL disrupts cell–junction complex localization and decreases tight junction-dependent transepithelial resistance (21,22). In addition, the association of PODXL with actin-binding proteins such as Na+/H+ exchanger regulatory factors and Ezrin was reported to be involved in the antiadhesive and migratory capabilities of tumor cells (23,24). Recent studies showed that increased expression of PODXL is correlated with poor prognoses in certain types of cancer (25–28), in particular, high expression of PODXL is associated with lymphatic invasion in breast cancers (28). Although the expression of PODXL has been reported to promote antiadhesive and migratory abilities, whether PODXL participates in tumor metastases remains unclear. Hence, the role of PODXL and its detail regulatory mechanism in tumor metastasis still need to be elucidated.

In the current study, we provide evidence demonstrating the critical functional role of PODXL in promoting invadopodia formation and tumor metastases. PODXL was found to induce Rac1/Cdc42/cortactin signaling cascade and to regulate invadopodia formation, leading to promotion of tumor invasiveness and metastasis.

Materials and methods

Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). MCF-7 cells were grown in modified Eagle medium supplemented with non-essential amino acid and sodium pyruvate. MDA-MB-231 cells were grown in Dulbecco’s modified Eagle medium-F12. All culture media were supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin (Gibco) and were grown at 37°C in a 5% CO2 atmosphere. Cells were cultured and stored according to the suppliers’ instructions and used at passages 10 to 20. Once resuscitated, cell lines were routinely authenticated through cell morphology monitoring, growth curve analysis and identity verification using short tandem repeat profiling analysis to ensure no contamination.

Plasmid construction

Full-length human PODXL complementary DNA (PODXL-wt) was obtained from Origene (Rockville, MD) and was subcloned into the pCMV6-DDK vector. PODXL-related mutants (PODXL-ACT and PODXL-ADTHL) were generated from pCMV6-PODXL-wt-DDK. Full-length human cortactin (cortactin-wt) was obtained from Origene and subcloned into the pCMV6-green fluorescent protein (GFP) vector. Cortactin-related mutants (cortactin-CT, cortactin-N, cortactin-SH3 and cortactin-ASH3) were generated from the pCMV6-cortactin-GFP vector. All primers are listed in the Supplementary Table S1, available at Carcinogenesis Online, and the reading frame sequence was verified.

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Lentivirus production

Small-hairpin RNA vectors were obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). To generate stable PODXL- and cortactin-knockdown cell lines, HEK293T packaging cells were cotransfected with the packaging plasmid (pCMV-AR8.91), envelope (pMDG) and hairpin pLKO-RNAi vectors using a PolyJet Transfection Kit (SignaGen Laboratories, Ijamsville, MD). At 48 h post-transfection, virus-containing supernatants were collected, mixed with fresh media containing polybrene (8 μg/ml) and incubated with target cells for another 48 h. Transduced cells were selected with puromycin (4 μg/ml) for 7 days.

Animal models

For the orthotopic spontaneous metastasis assay, MDA-MB-231 (1 × 10^6) cells were mixed with Matrigel (1:1) in a volume of 100 μl and were injected into the fourth mammary fat pad of 6- to 8-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which were then monitored for 10–12 weeks. Primary tumor growth after an orthotopic injection was measured using calipers every 3 days during the experiments and was calculated using the following equation: length × width^2 / 2. For the experimental lung metastasis assay, tumor cells were injected into a tail vein of NOD/SCID mice for 8 weeks. All dissected lungs were embedded with paraffin, and tissue sections were stained with hematoxylin and eosin. Metastatic tumor colonies were counted under a microscope. To examine macrometastases, MDA-MB-231-eGFP cells were orthotopically inoculated into NOD/SCID mice. Mice were sacrificed at 12 weeks after inoculation, and tissues were excised and observed under a fluorescence stereomicroscope to detect macrometastases. All animals were cared for in a specific pathogen-free room and treated in accordance with the animal care protocol approved by the Academia Sinica Animal Committee.

Rac1/Cdc42 activity

Rac1/Cdc42 activity was detected using a Rac1/Cdc42 activation assay kit (Millipore, Bedford, MA), according to the manufacturer’s instructions. In brief, cells were lysed with lysis buffer and directly incubated with PAK-1 PBD agarose at 4°C for 1 h on a rotator. Beads were washed three times with wash buffer, resuspended in 5x Laemmli reducing sample buffer and subjected to Western blotting. Wild-type (Addgene12975) and dominant negative (T17N) Cdc42 (Addgene12601) plasmids were kindly provided by Dr Klaus M.Hahn (MD). The infection titers of Rac1 and Cdc42 were obtained from Merck (Merck KGaA, Darmstadt, Germany).

Real-time PCR

Total RNA was extracted using an RNaseasy Plus Mini kit (Qiagen) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using the resulting cDNA, LightCycler 480 System (Roche, Basel, Switzerland) and a LightCycler 480 System (Roche). The results were expressed as fold change relative to the control sample, using the equation ΔΔCt. GAPDH was used as internal controls for normalization. Primer sequences are listed in the Supplementary Table S2, available at Cancergenesis Online.

Western blotting and immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer (150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate and 50mM Tris-HCl at pH 7.3) containing protease and a phosphatase inhibitor cocktail (Roche). For immunoprecipitation, cell lysates were preclarified with agarose-protein G for 1 h and incubated overnight at 4°C with the appropriate protein G-conjugated primary antibodies. Beads were washed three times with radioimmunoprecipitation assay buffer and were boiled in sample buffer (50mM Tris-HCl at pH 6.8, 2% sodium dodecylsulfate, 0.1% bromphenol blue and 10% glycerol). Equal amounts of proteins were separated on sodium dodecylsulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 1% bovine serum albumin/Tris Buffered Saline 20 and overnight incubation with specific primary antibodies against PODXL (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), FAK (1:2000, Millipore), phospho-FAK (1:2000, Millipore), paxillin (1:2000, Millipore), phospho-paxillin (1:500, Millipore), DDK (1:5000, Origene), GFP (1:10000, Origene), N-WASP (1:1000, In Vivo Biosciences, Sunnyvale, MD), phospho-cortactin (1:2000, Cell Signal Technology) or α-tubulin (1:5000, Sigma, St. Louis, MO). Membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (1:5000, Jackson ImmunoResearch) for 1 h at room temperature, and proteins were detected using an enhanced chemiluminescence kit (Millipore, Temecula, CA).

Transwell migration and invasion assays

Cells (10^5) were seeded in a transwell insert (8 μm filters, Corning) coated with Matrigel (BD Biosciences, La Jolla, CA) or left uncoated for different periods of time. After incubation, cells were fixed with 4% paraformaldehyde for 10 min. The un-invaded cells were removed by a cotton swab; invaded cells were stained with 4',6-diamino-2-phenylindole, imaged under an inverted fluorescent microscope (Zeiss). Data were derived from six random pictures.

Time-lapse migration assay

Cells at 2 × 10^6 were seeded on a Chamber slide magnetic chamber with complete growth media and were monitored using an inverted light microscope (Zeiss HAL100 reflected-light microscope) under a temperature and CO2 control system. Images were captured every 10 min for 6 h. The distance of migration was defined as the movement of the cell center per unit of time and was measured using MetaMorph software (Universal Imaging, West Chester, PA).

Immunohistochemistry

Human colon and breast cancer tissue microarrays were purchased from Biomax. The use of tissue microarrays was approved by the Human Subject Research Ethics Committee, Institutional Review Board, Academia Sinica (AS-IRB02-100067). Slides were deparaffinized, rehydrated and heated in sodium citrate buffer (0.01M, pH 6.0) for antigen retrieval. Slides were immersed in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and incubated with an anti-PODXL antibody (1:250, Atlas) for 1 h. After washing three times with Phosphate Buffered Saline Tween 20, a detection reagent (Super Sensitive Polymer-HRP HIC Detection System, BioGenex) and DAB chromogen were applied, followed by counterstaining with hematoxylin. The tissue microarrays were scanned by TissueFAX (TissueGnostics, Vienna, Austria), which were performed by two blind independent observers.

Invadopodia assay and gelatin degradation

To detect invadopodia, cells were stained with rhodamine-conjugated phaloidin (1:350, Molecular Probe, Invitrogen) and a fluorescein isothiocyanate-conjugated anticoartcin antibody (Millipore), before being observed under a confocal microscopy (TCS SP5; Leica, Wetzlar, Germany). To measure invadopodia activity, cells were seeded onto Millicell EZ slides (Millipore) coated with fluorescein isothiocyanate-conjugated gelatin, according to the instructions of the Gelatin Invadopodia Assay Kit (Millipore). After 16–48 h of incubation, slides were fixed, permeabilized with Triton-X 100 (0.1% in phosphate-buffere saline) and stained with rhodamine-conjugated phaloidin or cortactin. Nuclei were counterstained with 4',6-diamino-2-phenylindole (1:500). Slides were examined and photographed under a confocal microscope. The activity of invadopodia was defined by the average number of degradation puncta per cell in at least eight random fields (30).

Bioinformatic analysis

Gene expression data obtained from NCI-60 cancer cell lines using Affymetrix HU-133A and U133B chips were downloaded from the CellMiner database (http://discover.nci.nih.gov/cellminer/), and heat maps were drawn using Microsoft Excel. P values are given for the medium-rank analysis.

Statistical analysis

All data were derived from at least three independent experiments. Values were expressed as the mean ± standard error of the mean. Significant differences were determined using two-tailed, unpaired Student’s t-test, unless otherwise specified. P values of (*) < 0.05 and (**) < 0.01 were considered significant. The correlation coefficient was assayed by a Pearson correlation.

Results

Expression of PODXL is associated with breast cancer motility and the metastatic capability

To evaluate the clinical relevance of PODXL to cancer malignancy, we used immunohistochemistry analysis to examine PODXL protein expression in 80 breast tumor tissues and their matched adjacent normal counterparts. Results showed that the expression of PODXL was significantly increased in tumor tissues (P < 0.001) (Figure 1A). We further analyzed matched samples from primary and lymph node metastatic breast tumors and detected an increased potential of PODXL upregulation in lymph node metastatic tumor sites (n = 50, P < 0.001) (Figure 1B), suggesting that increased expression of PODXL might be associated with tumor metastasis.

Tumor metastasis encompasses several processes, such as migration/invasion, extravasation and metastatic colonization. To elucidate the effect of PODXL on tumor metastasis, we investigated whether PODXL expression was correlated with the migratory ability. We analyzed gene profiles from an NCI60 cancer cell panel database to study the correlation between PODXL and
Fig. 1. Overexpression of PODXL is associated with tumor invasiveness and metastasis. (A) Immunohistochemical analysis of PODXL expression in breast tumor (T) and matched adjacent normal tissues (N). Representative images were obtained from three individual patients. Scale bar = 50 μm. *P* values were computed by a chi-square test, *n* = 80. (B) Immunohistochemical analysis of PODXL expression in primary tumors (T) and matched lymph-node metastatic tumor tissues (M) of breast cancer. Representative images were obtained from three individual patients. Scale bar = 50 μm. *P* values were computed by a chi-square test, *n* = 50. (C) Expression of PODXL was correlated with mesenchymal traits in cancer cells. The heat map depicts relative expressions of PODXL, mesenchymal trait genes (vimentin and CD44) and an epithelial trait gene (CDH1) from a database (Affy HG-133) of NCI60 cancer cell line panels. Orange indicates high expression, and blue indicates low expression. Correlation coefficients were computed by Pearson correlation tests. (D) Real-time PCR and Western blot analyses of PODXL knockdown efficiency in MDA-MB-231 cells. *Mw* denotes molecular weight. (E) Transwell analysis of tumor migration and invasion in MDA-MB-231/mock (LKO) and PODXL-knockdown (shPODXL-3) cells. (F) Knockdown of PODXL suppresses migration of MDA-MB-231 cells. Cell migration was tracked and recorded by time-lapse observations. Representative images show the migratory tracks of five cells over 8h. The migratory velocity was analyzed by calculating the average distance per hour of 20 cells.
motility-related genes, such as E-cadherin (CDH1), CD44 and vimentin. We found that PODXL expression was positively correlated with CD44 (correlation coefficient = 0.425, P = 0.006) and vimentin (correlation coefficient = 0.335, P = 0.03), both of which are characteristic of basal-like and mobile cells (Figure 1C). Conversely, CDH1, an adhesion and luminal-like cell marker, was negatively correlated with PODXL (correlation coefficient = -0.530, P = 0.001) (Figure 1C). A recent study also reported that increased expression of PODXL is correlated with basal-like phenotype of breast cancer (28). In agreement with previous study, we found PODXL expression was positively correlated with CD44 and vimentin. In addition, a higher level of PODXL in basal-like MDA-MB-231 cells exhibited a greater migratory capability than did luminal-like MCF7 cells (Supplementary Figure S1A, available at Carcinogenesis Online). Conversely, knockdown of PODXL in MDA-MB-231 cells resulted in diminished cell migration and invasion capabilities (Figure 1D and 1E). MDA/PODXL shRNA-1 and shRNA-2 showed similar effects on cell migration as that of MDA/PODXL shRNA-3 cells (data not shown). Time-lapse observations also revealed that suppression of PODXL significantly decreased cell migratory rate (Figure 1F). Conversely, overexpression of PODXL in MCF7 cells increased cell migration and invasion (Supplementary Figure S1B, available at Carcinogenesis Online), suggesting that expression of PODXL is crucial for tumor mobility.

PODXL regulates invadopodia formation

We observed that suppression of PODXL impacted cell morphology in MDA-MB-231 cells. The morphology of mock cells displayed a spindle-like appearance, whereas the morphology of PODXL-knockdown cells showed irregular characteristics and a rounded and swelling appearance (Figure 2A, left panel). Quantitative data showed that suppression of PODXL drastically increased cell area, consistent with our findings. These results suggest that PODXL might play a role in actin architecture (Figure 2A, upper right). We further stained F-actin, and the results showed that a dynamic lamellar structure was observed in mock cells but was lost in PODXL-knockdown cells (Supplementary Figure S2A, available at Carcinogenesis Online). Actin-enriched lamellipodia, which refer to migratory cells, were observed at the leading edge of lamella in mock cells (Figure 2A, middle and right panels). The percentage of cells with lamellipodia among PODXL-knockdown cells decreased from 12.6 to 3.1%, compared with that of mock cells. In addition, knockdown of PODXL resulted in loss of actin polarity and directionality (Figure 2A, lower panel). Given that FAK and paxillin are crucial for lamellipodia formation, we examined the effects of PODXL on FAK and paxillin. The results showed that knockdown of PODXL decreased phosphorylation of FAK and paxillin (Figure 2B, left panel), whereas the phosphorylation of FAK and paxillin was increased in PODXL-overexpressing cells (Supplementary Figure S2B), available at Carcinogenesis Online, suggesting that the induction of FAK/paxillin activation may be involved in PODXL-mediated lamellipodia formation. Because the formation of invadopodia plays an important role in the degradation of local ECM regions, which facilitates tumor invasion, we wondered whether PODXL affected invadopodia formation. MDA-MB-231 cells were co-stained with F-actin and cortactin, which are often used as indicators of invadopodia precursors. In mock cells, we found that most of the F-actin and cortactin were colocalized at the cell periphery, implying nascent focal adhesion in migratory cells (Figure 2C, arrow indicated). We also observed that some of the F-actin/cortactin colocalizations formed a punctate-like structure in the cytoplasm, implying an invadopodia precursor (Figure 2C, triangle indicated). However, in PODXL-knockdown cells, colocalization of F-actin/cortactin was lost at both the membrane edge and in the cytoplasm (Figure 2C). Moreover, the phosphorylation of cortactin and N-WASp, two important mediators in invadopodia formation, was decreased in PODXL-knockdown cells (Figure 2B, right panel). To further verify that PODXL can regulate invadopodia function, we used fluorescein isothiocyanate–conjugated gelatin degradation assay to monitor invadopodial activity. As shown in Figure 2D, accumulation of cortactin in the perinuclear membrane and membrane edge were found to be colocalized with the gelatin degradation puncta in the mock cells. The z-stack projection images showed the invasion of cortactin into the gelatin layer, implying invadopodial protrusions. However, the colocalization of cortactin with gelatin degradation puncta was blocked in MDA/shPODXL cells (Figure 2D). Conversely, overexpression of PODXL in MCF7 cells induced colocalization of F-actin and cortactin in both the cell periphery and cytoplasm, compared with mock cells (Figure 2E). PODXL overexpression promoted the invasion of F-actin and cortactin into the gelatin layer (Supplementary Figure S3, available at Carcinogenesis Online) and increased gelatin degradation as well (Figure 2F). These data indicate that PODXL expression plays a crucial role in invadopodia formation and activation.

Suppression of PODXL downregulates cortactin-mediated invadopodia activation

Expression of cortactin is crucial for invadopodia formation and activation through assembling actin-nucleating molecules. To further confirm the importance of PODXL in invadopodia-mediated tumor invasiveness, we compared PODXL-knockdown alone with cortactin-knockdown cells. Results showed that suppression of cortactin significantly inhibited tumor invasion (Supplementary Figure S4, available at Carcinogenesis Online). In addition, suppression of PODXL alone significantly diminished invadopodial activity, similar to that in cortactin-knockdown cells (Figure 3A). Moreover, suppression of both PODXL and cortactin markedly abrogated invadopodal activity (Figure 3A) and the invasive ability (Figure 3B). However, inhibition of gelatin degradation in PODXL-knockdown cells was rescued when cortactin was reintroduced (Figure 3C). We also observed that the reintroduction of cortactin (AS2_Cortactin) into PODXL-knockdown cells restored cytoskeleton polarity (data not shown). These data suggest that cortactin participates in PODXL-mediated invadopodia formation and activation.

Rac1/Cdc42 participates in PODXL-mediated cortactin activation

We further investigated the molecular mechanism by which PODXL induces invadopodia formation. Immunoprecipitation-Western blot analysis revealed that PODXL was associated with cortactin (Figure 4A). Immunofluorescence analysis showed that some of the cortactin colocalized with PODXL in the plasma membrane (Figure 4B). These findings were confirmed by co-immunoprecipitation of DDK-tagged PODXL with cortactin-GFP (Figure 4C). Previous studies showed that the DTHL residue in the C-terminal tail of PODXL is required for binding of the Ezrin/Na+/H⁺ exchanger regulatory factor complex, which supports actin reorganization (23,31). As such, we tested whether the DTHL motif of PODXL is required for the association with cortactin. Co-immunoprecipitation experiments showed that deletion of the DTHL motif of PODXL (PODXL-ΔDTHL) resulted in the loss of binding between the protein and cortactin (Figure 4D). Similar results were found after deletion of the intracellular domain (PODXL-ΔCT) of PODXL (Figure 4D), suggesting that the DTHL residue participates in the association with cortactin. To further map the region of cortactin required for association with PODXL, a series of truncation mutants was generated (Figure 4E). Co-immunoprecipitation experiments revealed that PODXL associated with the N-terminal domain, but not the SH3 or the C-terminal domain of cortactin (Figure 4E). In addition, overexpression of wild-type PODXL (PODXL-wt) increased cortactin phosphorylation (Figure 4F) and invadopodia activation as well (Figure 4G); however, these activations were reduced in both PODXL-ΔDTHL and PODXL-ΔCT transfectants (Figure 4F and G).

Because a previous study reported that the DTHL motif in PODXL is required for Rac1 activation (23), and Rac1 and Cdc42 can regulate cortactin activation, we next examined whether Rac1 and/or Cdc42
Fig. 2. Expression of PODXL is associated with lamellipodia and invadopodia formation. (A) Knockdown of PODXL induced morphological change in MDA-MB-231 cells. Bright-field images of cell morphology in MDA/LKO and MDA/shPODXL-3 cells (left panel), scale bar = 10 μm. Immunofluorescent images of the F-actin structure by rhodamine-conjugated phalloidin staining under a confocal microscope at a 200-fold magnification (middle panel) and a 400-fold magnification (right panel). Scale bar = 10 μm. The F-actin-enriched area in the leading edge of cell was defined as lamellipodia (arrows indicated), and percentage of cells with lamellipodia was quantified (lower right). Data were derived from six random fields and are expressed as the mean ± SE. To define cell morphological change, quantification of cell area from 50 cells was analyzed by Metamorph software (upper right). (B) Western blot analysis of the phosphorylation of FAK (pY397) and paxillin (pY118) (left panel) and phosphorylation of cortactin (pY421) and N-WASp (pY256) (right panel) expressions in PODXL-knockdown MDA-MB-231 cells. Mw denotes molecular weight. (C) Immunofluorescent analysis of F-actin (red) and cortactin (green) colocalization in MDA/LKO and MDA/shPODXL-3 cells. Enlarged pictures are shown in the lower panel. Arrows indicate colocalization of F-actin/cortactin in the cell periphery, and triangles indicate F-actin/cortactin colocalization in the cytoplasm. The percentage of cells with F-actin/cortactin colocalization was quantified (right panel). Data were derived from six random fields and are expressed as the mean ± SE. (D) Suppression of PODXL inhibited invadopodia function. MDA-MB-231 cells were seeded onto FITC–gelatin (green) coated cover slide for 24 h, and cells were stained with cortactin antibody (red). Arrows indicate colocalization of cortactin and gelatin degradation puncta. Scale bar = 20 μm. Z-stack projection shows the invasion of cortactin into the gelatin layer. (E) Immunofluorescent analysis of F-actin and cortactin colocalization in MCF7/mock and MCF7/PODXL-4 cells. The high-power magnification images are shown in the left panel. Arrows indicate colocalization of F-actin and cortactin in the cell periphery, and triangles indicate F-actin/cortactin colocalization in the cytoplasm. Representative images show high-power magnification (left panel) and lower magnification fields (upper right). Scale bar = 10 μm. The percentage of cells with F-actin/cortactin colocalization was quantified (lower right). (F) MCF7 cells were stably expressed with DDK-tagged PODXL-1 and PODXL-4, and invadopodia activity was measured by the gelatin degradation assay. Arrows indicate gelatin degradation puncta. The percentage of gelatin degradation was quantified as described in Materials and methods. Data were derived from eight random fields and are expressed as the mean ± SE (upper right). Protein expression was verified by Western blot analyses using anti-DDK or anti-PODXL antibodies (lower right).
Fig. 3. Expression of PODXL is required for invadopodia formation and gelatin degradation. (A) Evaluation of invadopodia activity by a fluorescent gelatin degradation assay in MDA/shPODXL-3, MDA/shcortactin-3 and MDA/shPODXL+shcortactin cells. The high-power magnification of gelatin degradation images (left panel) were performed by plating cells in 12h incubation. Scale bar = 10 μm. To quantify gelatin degradation, cells were plated for 24h incubation and were subsequently observed at low-power magnification to count degraded puncta (right panel). Arrows indicate gelatin degradation puncta. Scale bar = 100 μm. (B) The invasive abilities in MDA/shPODXL-3, MDA/shcortactin-3 and MDA/shPODXL+shcortactin cells were assessed by transwell assay. (C) Ectopic expression of cortactin (AS2_Cortactin) restored invadopodia activity in PODXL-knockdown cells. MDA/shPODXL-3 cells were stably expressed with cortactin (AS2_Cortactin), and invadopodia activity was assessed by a gelatin degradation assay. Representative images were shown in left panel. Scale bar = 100 μm.
Podocalyxin-like 1 regulates invadopodia formation

Results showed that knockdown of PODXL in MDA-MB-231 cells abrogated Rac1 and Cdc42 activity (Figure 5A). Similar results were found in cortactin-knockdown and PODXL/cortactin-knockdown cells (Figure 5A). In addition, we found that PODXL induced Cdc42 activity, whereas it was diminished in cells transfected with PODXL-ΔDTHL or PODXL-ΔCT (Figure 5B). Moreover, overexpression of the dominant-negative Cdc42 (T17N) impeded PODXL-elicited cortactin phosphorylation and reduced the association of cortactin with PODXL (Figure 5D). These data suggest that the DTHL motif in PODXL might be important in triggering Rac1/Cdc42/cortactin-mediated invadopodia activation.

PODXL induces activation of FAK and paxillin through Rac1/Cdc42 signaling

The aforementioned data identified that suppression of PODXL downregulated FAK and paxillin phosphorylation (Figure 2B), and both are critical for lamellipodial induction. It is still unclear how PODXL regulates FAK and paxillin. 293T cells were transfected with different mutants of PODXL, and results showed that PODXL-wt induced phosphorylation of FAK and paxillin (Figure 5E). However, phosphorylation of FAK and paxillin decreased in cells transfected with PODXL-ΔDTHL or PODXL-ΔCT (Figure 5F). Treatment of cells with Src inhibitor (PP2) blocked phosphorylation of FAK and paxillin. Similar results were
observed in cells treated with the Src-specific inhibitor (SU6656), which has high affinity and selectivity for Src kinase, relative to PP2 which are also inhibitors of PDGFR and FAK (Supplementary Figure S5, available at Carcinogenesis Online). These data indicate that Src may participate in PODXL-induced FAK and paxillin activation, and that activation of Rac1/Cdc42 by PODXL is crucial for FAK and paxillin activation.

Inhibition of PODXL suppresses breast tumor metastasis

To demonstrate that PODXL indeed contributes to tumor metastasis, MDA-MB-231/shPODXL cells were intravenously injected into tail vein of NOD/SCID mice. Results showed that knockdown of PODXL effectively suppressed tumor colonization and nodules formation in mice lungs (Figure 6A). Moreover, MDA-MB-231-eGFP cells were orthotopically inoculated into NOD/SCID mice for 12 weeks, and systemic metastases were observed. In the control group, six of seven mice contained metastatic tumors in the lungs, and four of seven mice had macrometastases in the brain, liver, or spleen. However, suppression of PODXL significantly inhibited tumor dissemination to both the lungs and other tissues (Figure 6B). No preferential organ for metastasis was observed in PODXL-knockdown cells (Figure 6B). Knockdown of PODXL significantly impeded tumor dissemination but had modest inhibition on primary tumor volumes (Supplementary Figure S6A, available at Carcinogenesis Online). We did not observe significant growth inhibition in PODXL knockdown cells in the in vitro culture condition according to the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay (data not shown), suggesting that other factors might regulate tumor growth in the tumor microenvironment. Analysis of mRNA level in the primary tumor tissues showed that the pro-metastatic cytokines and chemokines, including IL1β, IL8, IL24, LOX and LOXL4, were downregulated in PODXL knockdown tumor tissues (Supplementary Figure S6B, available at Carcinogenesis Online). Several studies have reported that these molecules act as chemoattractants to support tumor growth in the tumor microenvironment, and that promotes tumor progression. These data indicate that PODXL participates in not only promotion of invadopodia activity but also regulation of metastasis gene expression. Moreover, suppression of PODXL showed a similar inhibitory effect as cortactin-knockdown on tumor metastasis (Figure 6C). Taken together, these results emphasize that PODXL expression is critical for tumor metastasis.
Discussion

Previous studies have reported that overexpression of PODXL is associated with poor prognoses features. In breast cancer, high expression of PODXL is associated with lymphatic invasion. However, it is still unclear whether PODXL participates in tumor metastasis, and the role of PODXL and its underlying mechanism are still unknown. In the current study, we found that increased expression of PODXL was detected in lymph node metastatic breast cancer cells. We further provided evidence showing that PODXL promotes invadopodia activity through Rac1/Cdc42-mediated FAK and cortactin signaling. Knockdown of PODXL significantly suppressed tumor dissemination. These data provide direct evidence demonstrating that PODXL plays an important role in tumor metastases.

PODXL is located on chromosome 7q32-q33, a region that is frequently associated with allelic imbalances and aggressive prostate cancer (32). In addition to prostate cancer, upregulation of PODXL is associated with poor prognoses of breast, colorectal and ovarian cancers (25–27). The molecular mechanism underlying the role of PODXL to the contribution of tumor progression is still unclear. We found that PODXL expression was closely correlated with the mesenchymal cell markers, CD44 and vimentin (Figure 1C), which are frequently associated with cell motility. Suppression of PODXL inhibited tumor migratory and invasive capabilities (Figure 1E and F), and restricted tumor dissemination (Figure 6A–C). Clinical analyses also revealed that expression of PODXL was increased in lymph node metastatic cancers (Figure 1B). These data strongly indicate that PODXL expression contributes to tumor metastasis.
The formation of invadopodia endows cells with the ability to erode local ECM regions via trafficking matrix metalloproteinases to the tips of invadopodia (1,13). Cortactin plays a central role in the formation of lamellipodia and invadopodia through gathering of the actin nucleation factors, Arp2/3 and N-WASP, which further enhances elongation of branch actin (6,9,33,34). Cortactin acts as a bridge to cluster actin filament and N-WASP. N-WASP is responsible for carrying out actin nucleation through interaction with Arp2/3. The activation of cortactin and N-WASP enhances actin polymerization, and the colocalization of cortactin and F-actin promotes filipodia and invadopodia formation. Colocalization of cortactin with F-actin at the cell periphery is referred to as nascent focal adhesion (9), whereas their colocalization in the cytoplasm indicates the formation of invadopodia precursors (5). Cdc42 and Rac1 play important roles in cortactin activation, cortactin-mediated actin polymerization and the formation of lamellipodia (35,36). They also enhance actin polymerization by promoting the binding of N-WASP to F-actin (37–39). Activation of Rac1 has been reported to be involved in the PODXL-mediated anti-adhesive characteristic (23). The roles of Cdc42/Rac1 and cortactin in PODXL-induced cell invasiveness, however, are still unclear.

We found that PODXL expression was crucial for actin reorganization. Suppression of PODXL significantly induced cell morphological and behavior changes (Figure 2A). PODXL knockdown MDA-MB-231 cells showed irregular and rounded appearance, compared with mock cells, which showed spindle-like cell shape. Rac1/ Cdc42, cortactin and N-WASP are important molecules involved in the nucleation of actin polymerization. In addition, FAK and ERM proteins, including Ezrin, participate in the linkage of actin filaments to the plasma membrane. The formation of actin filaments maintains cell shape, structure and locomotion; however, these molecules are suppressed by PODXL silencing, suggesting that PODXL is crucial for cytoskeleton maintenance.

Reciprocal activation of Rac1/Cdc42 and FAK was reported to be involved in cell spread and extracellular signal-regulated kinase phosphorylation (40,41). In addition, FAK phosphorylates cortactin at tyrosines Y421, Y466 and Y482 (42), and the association of cortactin with the FAK/Src complex regulates cell motility (43,44). Activation of Src also plays an important role in modulating cortactin phosphorylation and invadopodia formation. We found that Cdc42/Rac1 is important to PODXL-induced cortactin phosphorylation. Inhibition of Cdc42/Rac1 and Src downregulates PODXL-induced FAK and paxillin phosphorylation. These data support the notion that PODXL activates Rac1/Cdc42/cortactin and the FAK signaling network, leading to promotion of lamellipodia and invadopodia formation.

Previous studies demonstrated that the association of Ezrin with PODXL regulates tumor mobility. In addition, activation of Rac1 by PODXL via binding of the Rac1 guanine exchange factor, ARHGEF7, contributes to cell aggressiveness (23). Rac1 can trigger cortactin translocation to the cell periphery (45), probably through PAK1, downstream of Rac1/Cdc42. Activation of Rac1/Cdc42 leads to triggering of the reorganization of cortactin in the lamella spreading site and its association with N-WASP. The mechanism underlying PODXL-mediated invadopodia formation, however, is still unclear. We found that the DTHL motif in PODXL is crucial for Rac1/Cdc42 activation and subsequent phosphorylation of cortactin. The N-terminal domain of cortactin is required for binding of Arp2/3 and subsequent F-actin nucleation (7), whereas phosphorylation at tyrosine 421 in the C-terminal domain is involved in the Rac1-mediated cortical actin network (46). Rac1/Cdc42 was reported to participate in cortactin-mediated lamella spreading in platelet cells (35). We propose that activation of Rac1/Cdc42 is required for PODXL to induce the phosphorylation and translocation of cortactin to cell membranes. Because activation of cortactin regulates Rac1/Cdc42 activity, the reciprocal activation of cortactin and Rac1/Cdc42 by PODXL enhances lamellipodia and invadopodia formation. However, it is worthwhile deciphering the direct binding region between PODXL and cortactin with in vitro binding assay.

Our data are the first to report that PODXL regulates invadopodia through Rac1/Cdc42-mediated cortactin activation. PODXL is important to cell adhesion, migration and invasion by cooperating with actin reorganization factors. Our unpublished data also found that PODXL regulates genes involved in ECM degradation, wound healing and the epithelial–mesenchymal transition, which are characteristics of tumor metastases. However, the detailed regulatory mechanism still needs to be investigated. Taken together, we identified that overexpression of PODXL is strongly associated with tumor malignancy progression, it could be a potential biomarker for cancer diagnosis, and as such, PODXL might be an attractive therapeutic target for cancer treatment.

**Supplementary material**

Supplementary tables S1 and S2 and figures S1–S6 can be found at http://carcin.oxfordjournals.org/.

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**References**

Podocalyxin-like 1 regulates invadopodia formation


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