Extranuclear Functions of ER Impact Invasive Migration and Metastasis by Breast Cancer Cells

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Abstract

The molecular basis of breast cancer progression to metastasis and the role of estrogen receptor (ER) signaling in this process remain poorly understood. Emerging evidence suggests that ER participates in extranuclear signaling in addition to genomic functions. Recent studies identified proline-, glutamic acid–, and leucine-rich protein-1 (PELP1) as one of the components of ER signalosome in the cytoplasm. PELP1 expression is deregulated in metastatic breast tumors. We examined the mechanism and significance of ER-PELP1-mediated extranuclear signals in the cytoskeletal remodeling and metastasis. Using estrogen dendrimer conjugate (EDC) that uniquely activate ER extranuclear signaling and by using model cells that stably express PELP1 short hairpin RNA (shRNA), we show that PELP1 is required for optimal activation of ER extranuclear actions. Using a yeast two-hybrid screen, we identified integrin-linked kinase 1 (ILK1) as a novel PELP1-binding protein. Activation of extranuclear signaling by EDC uniquely enhanced E2-mediated ruffles and filopodia-like structures. Using dominant-negative and dominant-active reagents, we found that estrogen-mediated extranuclear signaling promotes cytoskeleton reorganization through the ER-Src-PELP1-phosphoinositide 3-kinase-ILK1 pathway. Using in vitro Boyden chamber assays and in vivo xenograft assays, we found that ER extranuclear actions contribute to cell migration. Collectively, our results suggest that ER extranuclear actions play a role in cell motility/metastasis, establishing for the first time that endogenous PELP1 serves as a critical component of ER extranuclear actions leading to cell motility/invasion and that the ER-Src-PELP1-ILK1 pathway represents a novel therapeutic target for preventing the emergence of ER-positive metastasis.

Introduction

The estrogen receptor (ER) is implicated in breast cancer progression. The majority of human breast cancers start out as ER positive (1) and a large portion of metastases retain their ER (2). Although initial endocrine therapy has a positive effect on the treatment of advanced metastatic disease (3), acquired resistance to endocrine therapies frequently occurs, with tumors recurring as metastases, which is the leading cause of death from breast cancer. Tumor metastasis consists of a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location and involves a multistep cascade (4). The process of migration is orchestrated through the activation of biochemical pathways that involve multiple cytoskeleton proteins (5). Although substantial information is available on the process of metastasis, the role of E2-ER signaling in breast metastasis remains controversial.

ER extranuclear signaling has been linked to rapid responses to E2 through stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (PI3K) pathways in the cytosol. Emerging evidence suggests that ER participates in extranuclear signaling through the formation of a multiprotein complex collectively called a "signalosome" (6). The use of novel ligands that uniquely activate extranuclear signals showed that extranuclear pathways have distinct biological outcomes (7). The molecular mechanism(s) of ER extranuclear signaling and the pathobiology of ER extranuclear actions remain unknown.

Proline-, glutamic acid–, leucine-rich protein-1 (PELP1; ref. 8) is also known as the modulator of the nongenomic actions of the ER (9). PELP1 plays important roles both in the genomic (10) and the nongenomic actions of the ER (11, 12). Recent evidence also suggests that PELP1 couples ER to several signaling pathways such as Src-MAPK, PI3K-AKT, and epidermal growth factor receptor (EGFR)-signal transducers and activators of transcription 3 (12, 13), and that PELP1

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expression is deregulated in metastatic breast tumors (14). Although these studies suggested that PELP1 has tumorigenic potential, whether PELP1-mediated extranuclear signaling plays a role in cell invasion and/or metastasis has not yet been defined.

In this study, we examined whether PELP1-mediated ER extranuclear signaling play a role in cytoskeletal remodeling, invasion, and metastasis. Our results suggest that ER extranuclear signaling has the potential to contribute to the tumor cell motility and that targeting the ER-PELP1 axis represents a novel therapeutic target to combat breast cancer progression to metastasis in ER-positive breast tumors.

**Materials and Methods**

**Cell cultures and reagents.** MCF7 cells were purchased from the American Type Culture Collection. ZR75 cells were maintained as previously described (15). Antibodies against vinculin, actin, and the steroid hormone 17β estradiol were purchased from Sigma. Green fluorescent protein (GFP)-epitope antibody was purchased from Clontech and anti-β-tubulin antibody was purchased from EMD BioSciences. PELP1 antibody was purchased from Bethyl Laboratories. Antibodies against phospho-AKT, phospho-MAPK, phospho-GSK3, phospho-Src, and PI3K inhibitor LY294002 were purchased from Cell Signaling. Dusatminib was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. MCF7 cells stably expressing PELP1 short hairpin RNA (shRNA) were generated using FuGENE-6 transfection (Roche) and by G418 selection (300 μg/mL). PELP1-specific shRNA and control shRNA vectors were purchased from SuperArray. MCF7-PELP1cyto cells were earlier described (12).

**Plasmids, generation of mutants, and transfection.** PELP1-Src mutant (PELP1SrcMT) that contains a mutation in the SH2 binding site and the Src phosphorylation site Y410F was generated by site-directed mutagenesis using the Quick Change Lightning Mutagenesis kit (Stratagene). Plasmids for the myr-p110 subunit of PI3K (active PI3K) and SrcY527F (active Src kinase) were described earlier (16, 17). RFP-myr-p110 (Active PI3K) was constructed by PCR-based cloning of open reading frame of myr-p110 into pDsRed-monomer-N1 vector. Expression vector for integrin αvβ3 with E2 for short time periods. Compared with shRNA control. Western blot analysis of total lysates revealed that the PELP1-shRNA clones downregulated PELP1 expression to ∼80% of the level seen in the parental and the vector-transfected cells (Fig. 1A). To examine the significance of endogenous PELP1 in the activation of ER extranuclear signaling pathways, we measured the activation of signaling pathways including Src, AKT, and MAPK after treating cells with E2 for short time periods. Compared with shRNA vector–transfected cells, PELP1-shRNA–expressing cells had significantly less Src, AKT, and MAPK activation (Fig. 1B). To further establish the role of PELP1 in E2-mediated

A poly amido amine dendrimer (G-6) was used for the preparation of EDCs. The EDCs were characterized by nuclear magnetic resonance analysis and the data were found to be consistent with that of the published report (7). A small aliquot was FITC labeled by using the Sure Link Fluorescein (FITC) labeling kit (KPL).

**Cell migration and metastasis assays.** Wound healing and Boyden chamber assays were performed as described (21). For determining in vivo metastatic potential, xenograft studies were performed as described (22). Briefly, 1 × 10^6 model cells in 100 μL PBS were injected into the tail vein or left cardiac ventricle of 5- to 6-week-old ovariectomized nude mice (n = 5) that were each implanted with one E2 pellet (60-d release, 0.72 mg, Innovative Research of America). After 8 weeks, the mice were euthanized and metastatic nodules on the surface of lung and liver were identified by color and counted under a fluorescent microscope.

**Western blotting and immunoprecipitation.** Cell lysis, immunoprecipitation, and Western blot analysis with phospho antibodies were performed as described (23).

**ILK kinase assays.** Exogenously expressed GFP-ILK1 or endogenous ILK1 was immunoprecipitated and was used as a source of ILK enzyme. In vitro kinase assays using MBP protein were performed in HEPES buffer (50 mmol/L HEPES, 10 mmol/L MgCl_2, 10 mmol/L MnCl_2, 1 mmol/L NaF, and 0.2 mmol/L Na_3VO_4) containing immunoprecipitated ILK1 enzyme, 10 μCi of [γ-32P] ATP, and 25 μmol/L ATP in 30 μL reaction.

**Immunofluorescence studies.** The immunofluorescence studies were performed as previously described (13). Secondary antibodies conjugated with Alexa 488 (green) or Alexa 546 (red), or Alexa 633 (Blue) dye was used to recognize different primary antibodies (Molecular Probes). The filamentous actin (F-actin) status was analyzed by phalloidin staining.

**Results**

**PELP1 knockdown affects E2-ER-mediated extranuclear signaling and cytoskeletal reorganization.** To study the in vivo significance of PELP1 in the extranuclear actions of ER, we established MCF7 breast cancer model cells that stably expressed PELP1shRNA that specifically downregulate endogenous PELP1. MCF7 cells were transfected with shRNA vector and negative control shRNA vector (that express shRNA targeting scrambled artificial sequence with no sequence identity to the human genome) were used as a control. Western blot analysis of total lysates revealed that the PELP1-shRNA clones downregulated PELP1 expression to ∼80% of the level seen in the parental and the vector–transfected clones (Fig. 1A). To examine the significance of endogenous PELP1 in the activation of ER extranuclear signaling pathways, we measured the activation of signaling pathways including Src, AKT, and MAPK after treating cells with E2 for short time periods. Compared with shRNA vector–transfected cells, PELP1-shRNA–expressing cells had significantly less Src, AKT, and MAPK activation (Fig. 1B). To further establish the role of PELP1 in E2-mediated...
Figure 1. Activation of ER extranuclear signaling promotes actin reorganization. A, MCF7 control or MCF7-PELP1-shRNA cells were lysed and the expression of PELP1 was analyzed by Western blotting. B, MCF7 vector control and MCF7-PELP1-shRNA cells were cultured in 5% DCC serum containing medium treated with or without E2. The activation of signaling pathways was analyzed by Western blotting of total protein lysates with phospho-specific antibodies. Densitometric analysis of the Western blots of phospho bands from triplicate samples were performed and corrected with the values of respective total bands. Columns, mean of triple determinations; bars, SEM. *, \( P < 0.05 \); **, \( P < 0.001 \). C, MCF7 cells were treated with FITC-labeled EDC for 45 min and localization of EDC was analyzed by confocal microscopy (left). MCF7 and MCF7-PELP1-shRNA cells were treated with EDC and activation of signaling pathways was analyzed by Western blotting (middle). Quantitation of the bands was as described in B (right). D, MCF7 or MCF7-PELP1-shRNA cells were treated either with E2 or EDC and the F-actin status was analyzed by phalloidin staining and visualized by confocal microscopy.
nongenomic actions, we used EDC (nanoparticles coated with estrogen) that uniquely localize in the membrane/cytoplasm (Fig. 1C, left) and preferably activate ER extranuclear signaling (7). MCF7 cells that express vector or PELP1-shRNA were treated with EDC for 2 and 5 minutes, and signaling was analyzed by phospho-specific antibodies. EDC addition uniquely promoted the activation of Src and MAPK pathways. However, knockdown of PELP1 by shRNA significantly affected the EDC-mediated increase in Src and MAPK activation (Fig. 1C, middle and right). These results suggest that E2-mediated extranuclear actions play a key role in the activation of Src and MAPK and that the functional PELP1 signaling axis is needed for E2-mediated extranuclear signaling. Because Src and PI3K play important role in cytoskeletal functions, cell attachment, and migration, we asked whether E2-ER extranuclear actions contribute to cytoskeletal reorganization leading to cell migration. MCF7 cells that expressed vector or PELP1-shRNA were treated with either E2 or EDC for 10 minutes and cytoskeletal changes were analyzed by confocal microscopy. E2 or EDC addition uniquely promoted actin reorganization with filopodia and ruffle formations. However, knockdown of PELP1 by shRNA substantially affected actin reorganization by E2 or EDC with little ruffles/filopodia formations and predominantly showed cortical actin and stress fibers (Fig. 1D). These studies show that ER extranuclear actions have the potential to promote cytoskeletal changes leading to ruffle and filopodia formation.

**Src kinase plays a critical role in PELP1-mediated E2 extranuclear signaling leading to cytoskeletal reorganization.** PELP1 acts as a scaffolding protein coupling ER with Src kinase, leading to the activation of ER-Src-MAPK and ER-Src-AKT pathways (11). Earlier studies also revealed that PELP1 interacts with c-Src SH3 domain through its NH₂-terminal PXXP motif and Src phosphorylates PELP1 at its COOH terminal (tyrosine 920) domain (9). To establish the significance of Src kinase in PELP1-mediated E2-ER extranuclear signaling, we generated a PELP1 mutant construct (PELP1SrcMT) that contains a mutation in the Src-SH3 binding site on PELP1 (ProXXPro is mutated to AlaXXAla) and a mutation in Src phosphorylation site (Tyr 920 is mutated to Phe; Fig. 2A). The PELP1SrcMT is unable to interact with Src kinase and thus functions as a DN mutant of PELP1. As expected, PELP1 wild-type (WT) but not the PELP1SrcMT interacted with Src kinase (Fig. 2B). Transient expression of PELP1SrcMT substantially effected the E2-mediated cytoskeletal reorganization in a DN fashion (Fig. 2B, right) and also interfered with the E2-mediated activation of Src and MAPK (Fig. 2C). Because Src kinase seems to play a key role in E2 extranuclear signaling, we examined the effect of inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine kinases (24). For these studies, we used MCF7 control cells or MCF7-PELP1WT model cells that overexpress PELP1 and exhibit increased E2-ER extranuclear signaling. Pharmacologic inhibition of Src kinase using dasatinib abolished the E2-mediated activation of AKT and MAPK pathways both in MCF7 as well as in PELP1-overexpressing MCF7 cells (Fig. 2D). Collectively, these results suggest that Src kinase play an important role in PELP1-mediated E2 extranuclear actions.

**Integrin-linked kinase 1 is a novel PELP1-interacting protein.** To identify the novel components of the PELP1 signalsome that contribute to ER extranuclear signaling leading to cytoskeletal reorganization, we performed a yeast two-hybrid screen using a mammary gland cDNA expression library. One of the positive clone sequences matched with that of ILK1. The specificity of ILK1 and PELP1 interaction was confirmed further using cotransformation followed by a survival assay in selection medium using yeast cells that stably expressed histidine, tryptophan, leucine nutrient reporter genes under the control of GAL response elements. The GBD-PELP1- and GAD-ILK1-transformed colonies grew in the medium lacking adenine, histidine, tryptophan, and leucine, whereas the cells cotransformed with the control GBD vector and GAD-ILK1 did not grow (Fig. 3A, left). Deletion experiments revealed that the possible interaction of ILK1 and PELP1 involved amino acids 601 to 886 (Fig. 3A, right). To further verify the interaction between ILK1 and PELP1, we transfected T7-tagged PELP1 and GFP-tagged ILK1 into MCF7 cells. Total lysates were subjected to immunoprecipitation with PELP1-tagged antibody followed by Western blotting with ILK1. Results showed that PELP1 can interact with ILK1 in vivo. (Fig. 3B, left). Similarly, the PELP1 and ILK1 interaction was also observed in PELP1cyto model cells that express PELP1 exclusively in the cytoplasm, suggesting the physiologic significance of such an interaction in the cytoplasm (Fig. 3B, right). Confocal analysis of EDC-treated MCF7 cells showed colocalization of PELP1 with ILK1 upon EDC treatment (Fig. 3C). Coimmunoprecipitation assay results showed that PELP1 interaction with ILK1 is dependent on ligand (Fig. 3D, left). Immunoprecipitation of PELP1 also showed the presence of ILK1 in the precipitates along with Src, ER, and the P85 subunit of PI3K, which are the known PELP1 signalsome components (Fig. 3D, right). These data suggest that ILK1 is a novel component of the PELP1 signalsome.

**ILK1 couples E2-mediated PELP1 signaling to cytoskeleton.** Because ILK1 is a novel component of PELP1 signalsome, we examined the significance of ILK1 in PELP1 signaling using DN and active ILK1 constructs (18), and monitored the formation of motility-related structures including the formation of stress fibers, lamellipodia (membrane ruffles), and filopodia (microspikes). Expression of DN ILK1 into MCF7 cells significantly reduced the formation of actin structures by E2 treatment (Fig. 4A, top). Accordingly, overexpression of DA ILK1 rescued the formation of F-actin structures including ruffles and filopodia in MCF7-PELP1shRNA cells (Fig. 4A, bottom). Overexpression of Src kinase failed to rescue the cytoskeleton defects in PELP1-shRNA clones (data not shown), suggesting that PELP1 is downstream of Src kinase. Because the Src kinase phosphorylation of PELP1 promotes downstream signaling by coupling with the PI3K axis, we introduced a membrane-targeted, GFP-tagged PI3K, a myristoylated subunit of p110 that functions as an active PI3K, into PELP1-shRNA cells. The expression of membrane-targeted PI3K rescued the actin structures...
in PELP1-shRNA cells (Fig. 4B, top). However, cotransfection of membrane-targeted PI3K along with DN ILK1 inhibited the active PI3K-mediated rescue of actin structures (Fig. 4B, bottom), indicating that ILK1 functions downstream of PI3K in the PELP1 signaling axis. We also measured whether the expression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42, a known downstream target of ILK1, res...
whereas Src inhibitor dasatinib has no effect on the PELP1-mediated activation of ILK1 (Fig. 4D, right). Earlier studies have shown that ligand-induced phosphorylation of PELP1 by Src is critical for PELP1 coupling to the PI3K pathway by p85-SH2 domain (9). Similarly, overexpression of PELP1 (a situation that occurs in tumors) is also shown to constitutively activate PI3K pathway by p85-SH3 domain–mediated interactions (12), suggesting that PELP1 can potentially interact with and activate PI3K through two distinct mechanisms. Because in this experiment (Fig. 4D) we have used PELP1 overexpression, inhibition of ILK1 activity by PELP1 in the presence of PI3K inhibitor but not in the presence of Src kinase inhibitor suggests that the direct interactions of PELP1 with PI3K may lead to ILK1 activation. However, in the physiologic context, Src kinase does play a role in the ligand-mediated activation of ILK1 by promoting PELP1–PI3K–ILK1 complex formation.

**PELP1 is needed for optimal cell migration promoted by E2 extranuclear actions.** We examined whether E2-mediated extranuclear actions contribute to cell migration. In Boyden chamber assays, parental MCF7 cells showed low motility and EDC further increased the migratory potential of those cells. The knockdown of PELP1 expression by small interfering RNA substantially reduced EDC-mediated cell motility (Fig. 5A). Interestingly, model cells expressing DN ILK1 also failed to migrate upon EDC stimulation (Fig. 5A). We also examined whether EDC-mediated cell migratory potential can be blocked by pharmacologic inhibition of Src kinase. Dasatinib effectively blocked the EDC-mediated cell migration in Boyden chamber assays (Fig. 5B). Similarly, dasatinib also inhibited E2-mediated cell migration in wound-healing assays (Fig. 5C).

**PELP1 overexpression enhances the in vivo metastatic potential of ER-positive ZR75 cells.** Because PELP1 expression is deregulated in metastatic tumors (14), we hypothesized that PELP1 overexpression may play a role in metastasis by promoting E2 extranuclear actions. We performed a proof-of-principle experiment using ER-positive ZR75 cells that exhibit poor metastasis in nude mice models. ZR75 cells were stably transfected with a GFP control or PELP1WT-GFP vector. PELP1WT-GFP cells had 3-fold higher expression of PELP1 than the control cells (data not shown). Mice injected with GFP control cells showed none to one metastatic nodule. However, PELP1-overexpressing cells had an increased propensity for metastases with 8 to 12 nodules identified in lungs (4 of 5 mice) and 6 to 8 nodules in liver (4 of 5 mice; Fig. 5D). To validate these findings further, we also injected GFP-vector and GFP-PELP1WT cells through a cardiac route into nude mice. Earlier studies found that this route facilitates bone metastasis (25). GFP-PELP1WT–overexpressing

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**Figure 3.** ILK1 is a novel PELP1-binding protein. A, confirmation of PELP1 interaction with ILK1 is shown in a yeast-based growth assay (left). Identification of the domain of interaction between PELP1 and ILK1 using a yeast-based growth assay (right). B, MCF7 cells that express T7-tagged PELP1WT (left) or T7-PELP1cyto mutant (right) were treated with EDC and the PELP1 and ILK1 interaction was confirmed by immunoprecipitation assay. C, MCF7 cells were treated with or without EDC for 5 min and the colocalization of PELP1 and ILK1 was analyzed by confocal microscopy. D, MCF7–T7-PELP1cyto cells were treated with or without EDC and total protein lysates were immunoprecipitated with T7-tagged antibody. The presence of PELP1, Src, ILK1, ER, and p85 in the immunoprecipitates was analyzed by Western blotting.
Figure 4. PELP1-ILK1 axis plays a productive role in E2-mediated cytoskeleton reorganization. Only one representative image for each experimental condition is shown and the results are representative of three independent replicates. A, MCF7 breast cancer cells were transfected with DN-ILK1 (red). After 72 h, the cells were treated with EDC for 10 min and F-actin (green) status was analyzed by confocal microscopy (top). MCF7-PELP1-shRNA cells were transiently transfected with DA-ILK1. F-actin status was verified by confocal microscopy (bottom). B, MCF7-PELP1-shRNA cells were transfected with constitutively active RFP-P110α subunit of PI3K (red) without (top) or with DN-ILK1 (bottom, blue) and F-actin (green) changes were analyzed by confocal microscopy. C, MCF7-PELP1-shRNA cells were transiently transfected with DA-CDC42 (green) without (top) or with DN-ILK1 (bottom, blue) and F-actin status was verified by confocal microscopy. D, the ability of PELP1 to enhance ILK1 activity was measured by an in vitro kinase assay by incubating immunoprecipitated GFP-ILK1 with increasing of amounts of GST-PELP1 (left). MCF7 cells were transfected with ILK1 expression vector with or without PELP1 expression vector and the ability of PELP1 to enhance ILK1 downstream signaling was analyzed by Western blotting (left). MCF7 and MCF7-PELP1-shRNA cells were treated with or without EDC. ILK1 was immunoprecipitated and kinase activity was measured using an in vitro kinase assay (middle). Cells were transfected with ILK1 expression vector with or without PELP1 expression vector. After 72 h, cells were treated with the PI3K inhibitor (LY294002, 50 μmol/L) or Src kinase inhibitor (dasatinib, 100 nmol/L). ILK1 was immunoprecipitated and the ILK1 activity was measured by an in vitro kinase assay (right).
Figure 5. E2-mediated extranuclear actions promote cell migration and metastasis. A, MCF7 cells, MCF7 cells transfected with DN-ILK1, and MCF7 cells stably transfected with PELP1-shRNA were treated with or without EDC and the migratory potential was analyzed by using Boyden chamber assay. Photomicrographs of migrated cells in various treatments (right). Columns, mean from three independent experiments performed in triplicate wells; bars, SEM. **, P < 0.001. B, MCF7 cells were treated with EDC in the presence or absence of Src inhibitor dasatinib (100 nmol/L). The cell migratory potential was analyzed by using the Boyden chamber assay. Columns, mean from three independent experiments performed in triplicate wells; bars, SEM. *, P < 0.05. C, wound-healing assay was performed in the presence or absence of E2 and in presence or absence of dasatinib. D, ZR75 cells expressing GFP-vector or GFP-PELP1WT were injected into nude mice either through the tail vein (left) or cardiac route (middle) and metastases were recorded after 8 wk. MCF7 cells expressing control GFP-vector or GFP+PELP1cyto were injected into nude mice (n = 5) through the tail vein (right). Representative images of metastatic nodules as observed by fluorescence microscope are shown. Columns, mean number of tumor nodules; bars, SEM; **, P < 0.0001.
cells, but not GFP vector–expressing cells, had metastases in the bone (Fig. 5D, middle). To examine the significance of PELP1 extranuclear signaling in metastasis, we have repeated xenograft assay using PELP1cyto cells (12) that uniquely express PELP1 in the cytoplasm and are shown to excessively promote ER extranuclear signaling. Similar to PELP1WT cells, PELP1cyto cells also showed increased propensity to metastasize compared with MCF7 control cells (Fig. 5D, right). These results further suggest that ER extranuclear actions have potential to promote metastasis.

**Discussion**

The pathologic significance of ER extranuclear signaling and its role in the progression to metastasis of breast cancer remain unknown. In this study, using estrogen dendrimers, DN reagents, and pharmacologic inhibitors of ER extranuclear signaling, we found that ER extranuclear actions play an important role in cell motility and metastases. In addition, we established for the first time that endogenous PELP1 play a critical role in coupling ER extranuclear signaling to cell motility through the ER-Src-PELP1-ILK-Rac/CDC42 pathway. The proto-oncogene c-Src is a multifunctional intracellular tyrosine kinase implicated in the regulation of a variety of processes including proliferation, differentiation, survival, and motility (26). Src interacts with multiple cellular factors including human EGFR 2, EGFR, and ER, and breast tumors overexpress Src kinase (27). PELP1 acts as a scaffolding protein coupling the ER with Src kinase leading to the activation of the ER-Src-MAPK pathway (11). Our data suggest that PELP1 and Src kinase play an essential role in the activation of ER extranuclear signaling leading to cytoskeleton reorganization and migration. Because breast tumors overexpress Src kinase, deregulation of PELP1 seen in breast tumors can contribute to the activation of Src, leading to the progression to metastasis. Pharmacologic inhibition of Src using dasatinib significantly inhibited E2-mediated extranuclear actions and reduced E2-mediated migratory potential. These results suggest that the ER-Src-PELP1 axis is a novel target for preventing the emergence of metastatic cells and that dasatinib may have therapeutic utility in blocking ER-positive metastases.

ErbB has been implicated in breast cancer progression and a majority of the human breast cancers starts out as hormone-dependent. Some evidence suggests that the extranuclear effects of estrogen can regulate different cellular processes, such as proliferation, survival, and apoptosis (28). Our results using EDC shows that ER extranuclear signaling has potential to promote cytoskeletal changes, leading to increased cell migration. Findings from these studies also showed that E2 extranuclear signaling promotes the formation of signaling complexes that contain PELP1, ER Src, and ILK1 and that extranuclear signaling from this axis play important roles in cytoskeletal rearrangements, motility, and metastasis.

We identified ILK1 as a novel interacting protein of PELP1 and showed that ILK1 functions as a downstream effector of ER extranuclear signaling, leading to cytoskeletal reorganization. ILK1 is known to play an important role in cytoskeleton reorganization and in the activation of Rho GTPases (Rac and CDC42). These effects are reversible upon inhibition of ILK protein expression (29). The ability of PELP1 to modulate the ILK1 pathway and its potential deregulation in metastatic breast cancer suggest that the modulation of ILK1 pathway may represent one potential mechanism by which PELP1 promotes metastasis in breast cancer cells.

PELP1 is a key component of the ER signalsome in the cytoplasm and is shown to play a role in ER extranuclear actions (8, 9). PELP1 expression seems to be predominantly in the cytoplasm in a subset of breast tumors. Previous studies showed that PELP1 cytoplasmic localization excessively promotes ER extranuclear signaling and that such deregulation contributed to tamoxifen therapy resistance (12). A recent study showed that patients whose tumors had high levels of cytoplasmic PELP1 had a tendency to respond poorly to tamoxifen compared with patients whose tumors had low levels of cytoplasmic PELP1 (30). In this study, using ligands that uniquely activate ER extranuclear signaling (EDC), and PELP1shRNA or dominant mutants that block PELP1 signaling, we found that E2-driven PELP1-mediated ER extranuclear actions can promote the cell migratory potential.

Endocrine therapy has also been shown to have a positive effect on the treatment of advanced metastatic disease (3). A few earlier studies suggested a negative effect of ER signaling on motility and invasion of cells (31, 32), whereas several recent studies showed a positive effect of ER signaling on motility (32, 33). Many metastatic tumors retain ER (34), and if primary tumors are ER positive, >80% of lymph node metastases and 65% to 70% of distant metastases retain ER (2, 35). A clinical correlation has also been reported between ER-positive tumors and the development of bone metastasis (34, 36). Similarly, ER signaling has been shown to enhance lung metastasis by promoting host-compartment response (37). PELP1 expression is deregulated in metastatic tumors (14) and PELP1 protein expression is an independent prognostic predictor of shorter breast cancer–specific survival and its elevated expression is positively associated with markers of poor outcome (38). Our data suggest that ER extranuclear signaling plays a role in metastasis and PELP1 deregulation commonly seen in metastatic tumors may play a role in metastasis by enhancing ER extranuclear signaling.

In summary, our data provide the first evidence showing the significance of ER extranuclear signaling to the metastatic potential of breast cancer cells. Our findings also identified ILK1 as a novel component of ER-PELP1 signalsome that connects ER signaling to cytoskeleton. We hypothesize that the ER-Src-PELP1-PI3K-ILK1 pathway represents a novel target to prevent the emergence of ER-positive metastatic cells through blockage of ER extranuclear signals in combination with endocrine therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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References

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