Endophilin A2 Promotes TNBC Cell Invasion and Tumor Metastasis

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Abstract

Triple-negative breast cancers (TNBCs) are highly aggressive cancers that lack targeted therapies. However, EGFR is frequently activated in a subset of TNBCs and represents a viable clinical target. Because the endocytic adaptor protein Endophilin A2 (SH3GL1/Endo II) has been implicated in EGFR internalization, we investigated Endo II expression and function in human TNBCs. Endo II expression was high in several TNBC cells compared with normal breast epithelial cells. Stable knockdown (KD) of Endo II was achieved in two TNBC cell lines, and although cell viability was unaffected, defects in receptor-mediated endocytosis were observed. EGFR signaling to Erk and Akt kinases was impaired in Endo II KD cells, and this correlated with reduced rates of EGFR internalization and cell motility. Endo II KD cells also displayed defects in three dimensional (3D) cell invasion, and this correlated with impaired extracellular matrix degradation and internalization of MT1-MMP. Endo II silencing also caused a significant reduction in TNBC tumor growth and lung metastasis in mammary orthotopic tumor xenograft assays. In human breast tumor specimens, Endo II expression was highest in TNBC tumors compared with other subtypes, and at the level of gene expression, high Endo II was associated with reduced relapse-free survival in patients with basal-like breast cancers. Together, these results identify a positive role for Endo II in TNBC tumor metastasis and a potential link with poor prognosis.

Implications: Endophilin A2 and related adaptor proteins represent important signaling hubs to target in metastatic cancers. Mol Cancer Res; 13(6): 1–12. ©2015 AACR.

Introduction

Breast cancer continues to be the most prevalent and second most fatal cancer for women within the developed world (1). Triple-negative breast cancers (TNBC) lack expression of estrogen receptor (ER), progesterone receptor (PgR), and HER2, and currently lack targeted therapies. TNBCs are often aggressive with frequent invasion of lymph nodes and development of metastases (2). These properties of TNBCs are often due to activation of EGFR, which leads to downstream activation of signaling pathways promoting cell growth, survival, motility, and invasion through basement membranes (3, 4). This invasive phenotype is dependent on downstream activation of Erk and Akt kinases (5, 6), which signal to key regulators of cell motility, proliferation, and survival (7). Sustained signaling to Erk and Akt pathways has been linked to internalization of EGFR via clathrin-mediated endocytosis (CME) and signaling from endosomes (8, 9). Thus, a more complete understanding of EGFR internalization mechanisms in EGFR-driven cancers may yield important new targets relevant to TNBC and other cancers. One such study used an RNAi-based screen to identify Annexin A2 as a novel regulator of EGFR trafficking, signaling, and metastasis in TNBC cells (10).

Another protein family implicated in EGFR internalization is the Bin1/Amphiphysin/Rvs (BAR) domain-containing protein family (11). BAR proteins both sense and promote membrane curvature via their BAR domains, and recruit key regulators of endocytosis and actin polymerization to these membranes via their SH3 domains (12, 13). Indeed, several BAR proteins have been implicated in regulating cell motility, invasion, and metastasis in EGFR-driven cancer models (14–19). Another BAR protein of potential relevance to metastatic TNBC is Endophilin A2 (hereafter called Endo II). Endo II has been implicated in promoting internalization of EGFR (20) and membrane type-1 matrix metalloproteinase-14 (MT1-MMP; ref. 21). Tyrosine phosphorylation of Endo II within its SH3 domain (Y315) is mediated by Src kinase in complex with focal adhesion kinase (FAK). Endo II phosphorylation by Src prevents MT1-MMP internalization in fibroblasts by disrupting binding to Dynamin (21). Recently, disruption of this FAK/Src/Endo II regulatory loop was shown to affect tumor growth and metastasis in mouse mammary tumors derived from the Polyoma virus middle T antigen model (22). Silencing of Endo II caused a partial reversal of the epithelial–mesenchymal transition (EMT) phenotype and reduced numbers of tumor-initiating cells (TIC; ref. 22). Although these studies in mice models identify Endo II as a candidate regulator of breast cancer metastasis, further studies in human breast cancers are required. This is especially true since receptor cargos of Endo II, such as MT1-MMP and EGFR, are key drivers of TNBC metastasis (23).

In this study, we report on the role of Endo II in regulating EGFR internalization, signaling, and motility of human TNBC cell models. Endo II expression was high in TNBC compared with cell lines representative of luminal subtypes or normal mammary...
epithelial cells. Lentiviral shRNA–mediated silencing of Endo II in two TNBC cell lines (HCC 1806, MDA-MB-231), resulted in reduced internalization of EGF-R, and diminished downstream signaling to Erk and Akt kinases. These signaling defects correlated with impaired cell motility, invasion, MT1-MMP internalization, and extracellular matrix (ECM) degradation in Endo II knockdown (KD) cells. In mammary orthotopic xenograft assays, we observed reduced tumor growth and lung metastases for Endo II KD TNBC cells compared with control cells. Lastly, profiling of human TNBCs revealed high Endo II expression in this subtype, and a correlation with worse relapse-free survival in patients with basal-like breast cancer, which is a major subset of TNBCs.

Materials and Methods

Cell lines and antibodies

Normal-like MCF-10A and all breast cancer cell lines (MCF-7, T-47D, ZR-75-1, BT-474, MDA-MB-453, SK-BR-3, MDA-MB-231, HCC 1806, Hs578T, MDA-MB-468, Du4475, BT-20, HCC 38) were obtained and cultured according to the ATCC guidelines. Media and supplements were from Sigma-Aldrich. The following antibodies were used in this study: anti-Endophilin II [Santa Cruz Biotech (SCBT); H-60; rabbit], anti-RasGAP (rabbit) (24), anti-pEGFR [Cell Signaling Technologies (CST); Y1068; 1H12; mouse], anti-ERK1 (SCBT; K-23; rabbit), anti-pERK (SCBT; E-4; mouse), anti-Actin (SCBT; C4; mouse), anti-MMP14 (Abcam; ab15580; rabbit), anti-Ki67 (Abcam; ab15580; rabbit).

Generation of stable Endo II KD cell lines

Lentiviral shRNA constructs in pGIPZ vector were obtained (Open Biosystems) for a nontargeting (NT) control shRNA and two Endo II shRNAs. Lentiviral transduction of HCC 1806 and MDA-MB-231 cells with NT or Endo II shRNAs and selection with puromycin was performed as previously described (15). Endo II KD1 cells correspond with clone V3LHS_345454, containing target sequence 5′AGAACTGCTTCTTCAGCCC3′. Endo II KD2 cells correspond with clone V3LHS_345456, containing target sequence 5′AGAACGTCGTCTCTAGCCGG3′.

Cell lysis and immunoblotting

Breast cancer cell lines were lysed with NP-40 lysis buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% Nonidet P-40; 10 μg/mL aprotinin; 10 μg/mL leupeptin; 1 mmol/L Na3VO4; 100 μmol/L phenylmethylsulfonyl fluoride). The following antibodies and concentrations were used for immunoblot (IB) analysis: Endo II (1:2,000); RasGAP (1:2,000); β-actin (1:2,000); pEGFR (1:200); pEGFR (1:1,000); Erk (1:200); pErk (1:200); Akt (1:1,000); pAkt (1:1,000); MMP14 (1:500).

EFG degradation assays

Coverslips are coated with a mixture of 0.2% gelatin and TRITC ester (8:1 ratio) for HCC 1806 cells as previously described (26). For MDA-MB-231, coverslips with TRITC–fibronectin were prepared, as previously described (15). For both cell lines, cells were seeded onto coverslips with complete media and incubated overnight, then fixed and counterstained with AlexaFluor530 Phalloidin (Life Technologies). Coverslips were mounted onto slides with Mowiol and imaged at 100× on

Cell growth and viability assays

Cell viability assays were done as previously described (25). Briefly, the HCC 1806 parental cells, NT cells, and Endo II KD cells were seeded onto 96-well plates (2,500 cells in 100 μL/well), and after 18 hours AlamarBlue (Life Technologies) was added to the growth medium (10 μL/well). After 6 hours, the spectral absorbance at 595 nm was measured using a Multiskan Spectrum plate reader (Thermo). Cell growth curves were generated by seeding HCC 1806 and MDA-MB-231 cells onto 12-well plates (5,000 cells/well) in triplicate. Once a day for 4 days following seeding, cells were trypsinized, diluted to 1 mL in PBS, and counted using a Coulter Counter (Beckman-Coulter).

Cell migration assays

HCC 1806 and MDA-MB-231 cells (50,000) were seeded in transwell inserts (8 μm pore; BD Falcon), and allowed to migrate toward either media supplemented with either serum (MDA-MB-231 cells) or 50 ng/mL EGF in the lower chamber. Cells on the filter were fixed, and nuclei were stained with DAPI. Cells were removed from the upper surface of the filters before mounting in Mowiol (Sigma), and imaging by epifluorescence microscopy. Migrating cells were scored using Image-Pro Plus 6 software (Media Cybernetics).

Spheroid invasion assays

HCC 1806 cell invasion was investigated using the Cultrex 96 Well 3D Spheroid BME Cell Invasion Assay, as per the manufacturer’s instructions (cat #3500-096-K; Trevigen). Briefly, 3,000 cells were resuspended in spheroid formation matrix solution and pelleted in a 96-well round bottom plate and images acquired on day 3. Serum-supplemented invasion matrix was added to each well, and images were acquired after 7 days. The area of each spheroid was measured on day 3 (preinvasion) and day 10 (postinvasion) using Image-Pro Plus 6 software (Media Cybernetics), and the difference was used to calculate total area of cell invasion.

ECM degradation assays

Coverslips were coated with a mixture of 0.2% gelatin and TRITC ester (8:1 ratio) for HCC 1806 cells as previously described (26). For MDA-MB-231, coverslips with TRITC–fibronectin were prepared, as previously described (15). For both cell lines, cells were seeded onto coverslips with complete media and incubated overnight, then fixed and counterstained with AlexaFluor530 Phalloidin (Life Technologies). Coverslips were mounted onto slides with Mowiol and imaged at 100× on

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a BX51 epifluorescence microscope (Olympus). Degradation area and total cell areas were scored using Image-Pro Plus 6 software (Media Cybernetics).

Surface biotinylation assays
Surface biotinylation assays were performed as previously described (18). Briefly, MDA-MB-231 cells were seeded on six-well plates, and the next day, cells were chilled on ice before addition of biotin. Samples were either kept on ice (total surface) or incubated at 37°C for 15 minutes, followed by once again chilling on ice. All cells except those in the surface level group were incubated with a biotin stripping buffer. All cells were then lysed and their lysates incubated with streptavidin-coated beads (Roche). Both lysates and streptavidin pull down fractions were analyzed by IB as indicated in figure legend.

Tumor xenograft assays
Mammary orthotopic xenograft assays were performed using MDA-MB-231 and HCC 1806 cell models and Rag2-/-:IL2R-gc-/- (BALB/c) mice, as described previously (15). Briefly, mammary fat pads were injected with 1.5 x 10^6 NT or Endo II KD cells in a 50% Matrigel (BD Biosciences) mixture. Mammary tumors were allowed to grow for 5 weeks before animal sacrifice and tissue harvesting. Primary tumor homogenates were prepared by suspension of primary tumor in NP-40 lysis buffer followed by homogenization. Whole lungs were imaged by epifluorescence microscopy to detect GFP-positive metastatic nodules before formalin fixation and paraffin embedding. Sections (5 μm) were prepared and stained with hematoxylin/eosin (H&E). For tail vein injections, 2 x 10^5 MDA-MB-231 NT or Endo II KD cells in 200 μL serum-free medium were injected into the tail vein of Rag2-/-:IL2R-gc-/- mice. At 2 weeks after injection, animals were sacrificed and tissues harvested and analyzed as above. All animals were housed in a specific pathogen-free facility (Queen's University Animal Care Services), and procedures were approved by the Queen's University Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines.

Immunohistochemistry staining and scoring
Human breast cancer tissue microarrays (BR10010b, T087; US Biomax) were stained using the Discovery XT Staining System (Ventana Medical Systems, Inc.). Antigens were retrieved with an EDTA pH 8.0 solution and incubated with rabbit anti-Endo II (1:100) or rabbit anti-Ki67 (1:1000) antibody. Endo II immunohistochemistry (IHC) staining was visualized using DAB and a hematoxylin counterstain. Microarrays were scanned using the Aperio CS digital slide scanner (Queen's Laboratory for Molecular Pathology) and analyzed with ImageScope software (Aperio). As a
control, Endo II antibody was precleared with beads coated with GST-Endo II linker-SH3 construct. The depleted antibody and mock-depleted antibody were tested by IP/IB using MDA-MB-231 cell lysates. HSC staining of human tumors using precleared Endo II antibody was used to calculate the minimum threshold for subsequent staining with Endo II antibody. Core sections selected for scoring were of similar areas, and included only epithelial-like cells. H-scores were calculated based on positive pixel intensity according to the formula: (% weak positive) + (% positive × 2) + (% strong positive × 3).

Bioinformatics
For analysis of Endo II transcripts in breast cancer cell lines, we used openly available Affymetrix microarray datasets (www.biorxiv.org). For analysis of Endo II transcripts in breast cancer microarray studies, a Kaplan–Meier curve for relapse-free survival was created using Kaplan–Meier Plotter (www.kmplot.com) for 199 basal-like breast cancer patients (ER+/PgR+/HER2−) grouped as above or below median expression levels of the sh3gl1 gene. Hazard ratio (with 95% confidence interval) and log-rank P-values were calculated.

Statistical analysis
Unless otherwise specified, all experiments were performed in triplicate and presented as the means ± standard error (SEM). The Student two-tailed t-test was used to compare NT and KD cell lines, with significant differences defined by \( P < 0.05 \), unless otherwise stated in figure legends.

Results
Endo II is highly expressed in TNBC cell lines
Endo II has been implicated in a FAK/Src kinase signaling axis in fibroblasts and mouse mammary tumor cell lines that regulates MT1-MMP internalization (21, 22). To begin testing the role of Endo II in human breast cancer, we profiled Endo II expression in a panel of human breast-derived cell lines. This panel included an immortalized normal-like breast epithelial cell line (MCF-10A), luminal A (MCF-7, T-47D), luminal B (ZR-75-1, BT-474), HER2 (MDA-MB-453, SK-BR-3), and TNBC cell lines (MDA-MB-231, HCC 1806, Hs578T, MDA-MB-468, Du4475, BT-20, HCC 38). IB analyses revealed that Endo II was expressed at high levels in HER2 and TNBC cell lines, compared with MCF-10A or luminal cell lines (Fig. 1A; p120RasGAP served as a loading control). Similar results were observed at the level of Endo II gene expression, based on microarray results for 51 normal-like and breast cancer cell lines (27, 28), with a trend toward higher transcript levels in TNBC and HER2 cell lines (MDA-MB-231, HCC 1806, Hs578T, MDA-MB-468, Du4475, BT-20, HCC 38). In all assays, Endo II expression was greater than levels of Endo II remaining in these cell lines (NT, KD1, KD2, respectively). Results are representative of 3 independent experiments (\( ^* ^* ^* ^* ^* ^* \), \( P < 0.001 \)).

Endo II promotes EGFR internalization in TNBC cell lines
Previous studies have implicated Endo II in promoting early and late events in CME, such as vesicle scission and clathrin-coated vesicle uncoating (20, 30–32). To test the potential effects of Endo II KD on CME in HCC 1806 cell lines (NT, KD1, KD2), we incubated them with Texas Red–labeled transferrin (TR-Tfn). After 3 minutes, extracellular TR-Tfn was removed, and cell nuclei were stained with DAPI before imaging by confocal microscopy. Although NT and Endo II KD cell lines both showed internalization of TR-Tfn (Fig. 2A), we observed a significant reduction in Tfn-positive vesicles in Endo KD1 and KD2 cell lines compared with NT cells (Fig. 2B). The magnitude of these defects in CME was proportional to levels of Endo II remaining in these cell lines (Fig. 1B and C), and therefore supports that Endo II silencing causes these defects rather than potential off-target effects of individual shRNAs.

EGFR is frequently activated in TNBC, and internalization of activated EGFR regulates signaling and degradation (10, 33). To test whether Endo II regulates EGFR internalization, we
analyzed the effects of Endo II KD on uptake of Texas Red-labeled EGF (TR-EGF). Although EGF uptake was observed in both NT and Endo II KD TNBC cell lines, fewer EGF-positive vesicles were observed in Endo II KD cells (Supplementary Fig. S2A and S2C). Quantification of these results revealed a significant reduction in EGF uptake in Endo II KD cells for both HCC 1806 and MDA-MB-231 cell lines, compared with NT controls (Supplementary Fig. S2B and S2D). Together, these results support a role for Endo II in promoting early events in receptor endocytosis in TNBC cells.

Endo II regulates EGFR signaling in TNBC

Because internalization of EGFR has been linked to sustained activation of Erk and Akt signaling pathways (10, 33), we tested the potential effects of Endo II on EGFR signaling in TNBC cells. A time course of EGF treatment was performed on serum-starved HCC 1806 NT or KD1 cells, and EGFR signaling was analyzed by IB. To test the extent of EGFR activation, autophosphorylation of EGFR at Y1068 (pEGFR) was analyzed and showed similar kinetics of activation in NT and KD cells (Fig. 3A). Densitometric analyses revealed a small increase in relative pEGFR levels in

![Figure 3](https://example.com/figure3.jpg)

Figure 3.
Endo II promotes EGFR signaling to Erk and Akt kinases in TNBC cells.

A, HCC 1806 NT and KD cells were treated with EGF (50 ng/mL) for the indicated times (minutes). Lysates were subjected to IB analysis for the indicated proteins and their phosphorylated forms. B, graph depicts relative levels of pEGFR, pErk, and pAkt compared with total levels observed in 3 independent experiments. (*, P < 0.05; **, P < 0.01; ***, P < 0.001)
Endo II KD cells, compared with control, but not at all timepoints (Fig. 3B). Next, we investigated EGFR signaling to Erk and Akt kinases, which regulate myriad responses to EGF, including cell proliferation, survival, motility, and invasion (34, 35). Interestingly, Endo II KD cells displayed a dramatic reduction in EGF-induced phosphorylation of Erk (pErk) and Akt (pAkt) kinases compared with NT control cells (Fig. 3A). Densitometric analyses from multiple experiments revealed significant reductions in pErk and pAkt levels at each time point of EGF treatment in Endo II KD cells (Fig. 3B).

Endo II promotes TNBC cell motility and invasion of ECM

To test whether defects in EGFR internalization and signaling in Endo II KD cells alter the highly invasive phenotypes of these TNBC cell lines, we tested them in chemotaxis and invasion assays. Using transwell migration chambers, and EGF as chemoattractant, we observed a significant reduction in chemotaxis of HCC 1806 cells upon silencing of Endo II (Fig. 4A). Similar assays were performed in MDA-MB-231 NT, KD1, and KD2 cells using serum as the chemoattractant, which also revealed a role for Endo II in promoting TNBC cell motility (data not shown). To test the potential effects of Endo II KD on TNBC cell invasion, we compared HCC 1806 NT and KD cells for their ability to form spheroids in 3D culture and invade the surrounding ECM. We observed similar growth of NT and Endo II KD cells as spheroids at day 3, but following overlay with ECM permissive of cell invasion for 7 days, we observed a dramatic reduction in 3D cell invasion (Fig. 4B and C). Together, these results demonstrate a key role for Endo II in promoting TNBC cell motility and invasion of ECM.

Endo II promotes invadopodia formation and ECM degradation in TNBC cells

Previous studies in Src-transformed fibroblasts and mouse mammary tumor cells have implicated Endo II in promoting internalization of MT1-MMP and ECM degradation by invadopodia (21, 22). Endo II inactivation following phosphorylation by FAK/Src complexes was shown to promote the invasive phenotypes in these models. To test effects of Endo II KD on invadopodia formation in human TNBC cells, we plated NT and KD cells on coverslips coated with a thin layer of TRITC-labeled ECM proteins, such as gelatin (for HCC 1806 cells) or fibronectin (for MDA-MB-231 cells). After 24 hours, cells were fixed and filamentous actin (F-actin) was stained before imaging by epifluorescence microscopy. In these assays, invadopodia are visualized as F-actin dots over areas of ECM degradation, and these F-actin dots were observed in the perinuclear region of NT and KD MDA-MB-231 cells (Fig. 5A). Although no significant differences were observed in F-actin dots (Fig. 5B), we observed a significant defect in ECM degradation in Endo II KD cells (Fig. 5C). We observed very similar results in parallel studies with HCC 1806 NT and KD cells (Supplementary Fig. S3). Together, these results implicate Endo II in promoting invadopodia maturation and ECM degradation in TNBC cells.

In fibroblasts, overexpression of a gain-of-function mutant in Endo II (Y315F), lacking the Src phosphorylation site, was shown to increase MT1-MMP internalization and reduce ECM degradation (21). To address whether Endo II KD alters MT1-MMP internalization in TNBC cells, surface biotin assays were performed to compare levels of cell surface and internalized pools of MT1-MMP. In the highly invasive MDA-MB-231 cells, Endo II KD cells exhibited increased levels of MT1-MMP on the surface of Endo II KD cells in these assays (Fig. 5D; 4°C condition). Upon shifting the cells to 37°C to allow endocytosis to proceed, we observed defects in internalization of MT1-MMP in Endo II KD cells (Fig. 5D). As a positive control, we analyzed EGFR internalization and observed similar defects in Endo II KD cells (Fig. 5D). These results are consistent with Endo II promoting MT1-MMP internalization in human TNBC cells, and the loss of endosomal pools of MT1-MMP and EGFR may prevent mobilization to invadopodia precursors in these cancer cells.

Figure 4.
Endo II promotes TNBC cell motility and invasion. A, Transwell migration assay using EGF-induced migration of HCC 1806 NT and KD cells. Results are representative of 3 independent experiments (*, P < 0.05). B, NT and Endo II KD cells are imaged upon formation of spheroids (preinvasion) and a week after addition of a Matrigel layer (postinvasion). C, quantification of invasion area was determined by subtracting preinvasion from postinvasion area using imaging software. Results are representative of 3 independent experiments (*, P < 0.05).
Endo II promotes tumor growth and metastases in mice

To better characterize the contributions of Endo II to disease progression in TNBC, we performed mammary orthotopic xenograft assays using Endo II KD TNBC cells and their controls injected into Rag2<sup>−/−</sup>:IL-2Rγ<sup>−/−</sup> mice. At 5 weeks after injection, mice were sacrificed and several tissues along with primary tumors were harvested. For both MDA-MB-231 and HCC 1806 cell models, Endo II silencing caused a significant reduction in tumor growth (Fig. 6A; Supplementary Fig. S4A). This defect in tumor growth was not due to differences in cell growth rates in vitro (Fig. 1D), or with expression of the Ki67 marker of proliferating tumor cells in vivo (Fig. 6B; Supplementary Fig. S4B). To establish whether the silencing of Endo II remained intact in vivo, we prepared homogenates from primary tumors for IB analysis. This showed a substantial reduction in Endo II within the primary tumors for KD cells compared with NT controls (Fig. 6C; Supplementary Fig. S4C). Lungs from the mice were harvested and imaged by fluorescence microscopy to detect GFP-positive TNBC metastases. In both models, Endo II KD resulted in less GFP<sup>+</sup> lung metastases (Fig. 6D; Supplementary Fig. S4D). To quantify these results, lung tissue sections were stained with H&E, and metastatic nodules were quantified (Fig. 6D and E; Supplementary Fig. S4D and S4E). We observed a significant reduction in lung metastases in both TNBC cell models upon Endo II silencing (Fig. 6E; Supplementary Fig. S4E). These results were likely not explained by differences in tumor size for Endo II KD group, because we observed no strong correlation between number of metastases and mass of the primary tumors in these TNBC models (Supplementary Fig. S5). To test whether Endo II functions in early or late events in metastasis, we compared the lung seeding efficiency of MDA-MB-231 NT and KD cells. We observed a significant defect in Endo II KD cells in lung seeding efficiency compared with NT control cells (Supplementary Fig. S6). These results implicate Endo II in promoting TNBC metastasis during early events within.
the mammary gland (orthotopic model) and following dissemination in the peripheral blood (tail vein model).

High Endo II expression in TNBC correlates with poor clinical outcome

Since our functional studies of Endo II in TNBC cell and tumor models support a key role for Endo II in TNBC, we extended our study of Endo II to examine its expression in human breast tumor samples. Endo II protein levels were analyzed by IHC staining of tissue microarrays containing 50 invasive ductal carcinomas. To test the specificity of the Endo II IHC signal, we depleted the antisera with a GST–Endo II fusion protein, or with GST alone as a control. The GST-Endo II depletion resulted in a nearly complete loss of recovery of Endo II by immunoprecipitation from MDA-MB-231 cells (Supplementary Fig. S7A). This depleted antibody also showed very little signal in IHC staining of human breast tumor samples compared with control Endo II antibody (Supplementary Fig. S7B). We went on to profile Endo II expression in human breast tumors and grouped them according to molecular subtypes of luminal (ER⁺/PgR⁺), HER2 (HER2⁺), and triple-negative (ER⁻/PgR⁻/HER2⁻). Although all tumors showed detectable Endo II expression, the highest levels were observed in triple-negative tumors (Fig. 7A). To quantify and analyze these results, we converted our IHC signals to H-Scores using imaging software. When organized by subtype, we observed a trend toward higher Endo II expression in TNBC tumors compared with luminal or HER2 cases (Fig. 7B). It is worth noting that this result is consistent with our profiling of Endo II levels in breast cancer cell lines isolated from TNBCs (Fig. 1A; Supplementary Fig. S1). To extend this study of Endo II expression to samples with known outcomes, we queried results of gene expression data for basal-like breast cancer patients using KM Plotter (36), and this revealed significantly higher risk of relapse for patients with tumors characterized by high levels of Endo II mRNA expression (Fig. 7C). Similar results were not observed in patients with other breast cancer subtypes (data not shown). Together, these results suggest that high levels of Endo II expression may contribute to TNBC progression and metastasis, which is supported by our functional studies of Endo II in TNBC xenograft models.

Discussion

Dynamic changes in membrane shape and composition are central to many biologic processes (37). Invasive cancer cells take advantage of these processes during cancer cell invasion and tumor metastasis (38). This study adds to a growing list of membrane shaping BAR proteins that have been implicated in cancer metastasis (14, 15, 19, 22, 39, 40). Through stable silencing of Endo II in TNBC cell models, we provide evidence that Endo II promotes internalization of EGFR and downstream signaling to Erk and Akt pathways. Endo II KD cells display defects in cell motility and invasion that correlated with a striking defect in ECM...
degradation activity in two TNBC cell models. The lack of mature invadopodia in Endo II KD cells may be due to defects in MT1-MMP internalization and subsequent trafficking in the absence of Endo II. Consistent with role of invadopodia in promoting tumor progression and metastasis in TNBC (23, 41), we also show that Endo II promotes metastasis in TNBC tumor xenograft models. Lastly, we identify a link between elevated Endo II expression and an increased risk of relapse in basal-like breast cancer patients.

Endo II has recently been studied in mammary carcinoma cells isolated from the MMTV-PyMT model crossed with FAK knock-in mice with mutations to the SH3 domain–binding motif for Endo II (+/PA-MT and PA/PA-MT; ref. 22). Endo II association with FAK/Src kinases is retained in +/PA-MT cells, which allows phosphorylation (pY315)-mediated inactivation of Endo II and reduced MT1-MMP internalization (21, 22). The +/PA-MT cells have high surface levels of MT1-MMP and low E-cadherin levels compared with PA/PA-MT cells. Endo II silencing had no effect on surface MT1-MMP in +/PA-MT cells, but did promote MT1-MMP surface expression in PA/PA-MT cells (22). Although it is difficult to make direct comparisons between our human TNBC models and those in the Fan and colleagues study, we have also observed increased surface levels of MT1-MMP with Endo II silencing. However, this did not correlate with increased ECM degradation activity in Endo II KD TNBC cells, as reported in Src-transformed fibroblasts (21). In fact, we observe impaired ECM degradation in Endo II KD TNBC cells. This likely relates to the complex trafficking of MT1-MMP from the cell surface to late endosomes and regulated delivery to invadopodia precursors (42, 43). Because our TNBC cell models are not engineered to disrupt the FAK interaction with Endo II, we hypothesize that Endo II can largely escape inhibition by Src, and thereby promote endocytosis of MT1-MMP at membrane sites lacking activation of the FAK/Src complex. Here, we show that internalization of MT1-MMP is
impaired in Endo II KD MDA-MB-231 cells subjected to surface biotin assays. We hypothesize that lack of an endosomal pool of MT1-MMP may limit subsequent delivery to invadopodia precursors in Endo II KD TNBC cells. It is worth noting that the Endo II binding partner Dynamin has recently been implicated in the formation and fission of tubuleovascular carriers from this intracellular pool of MT1-MMP (44). Like our findings here for Endo II, Dynamin was also identified as a positive regulator of invadopodia formation and cell invasion in MDA-MB-231 cells (45). In future, the Endo II KD TNBC cell models that we have developed can be used to define the cellular compartments of MT1-MMP that are most affected by Endo II silencing.

In this study, we identified a role for Endo II in promoting internalization of EGFR, which is a key pathway linked to TNBC tumor metastasis (46, 47). Our results are consistent with a recent study showing that Endo II associates with Lamellipodin, an actin filament elongation protein, to promote internalization of EGFR in HeLa cells (20). Recent studies also implicate Endo II and Lamellipodin in mediating a distinct, non–clathrin-dependent, fast endocytosis pathway for ligand-activated receptors (48, 49). Although we have not been able to confirm Lamellipodin interaction with Endo II in TNBC cells (data not shown), it will be interesting to test for potential defects in membrane recruitment of Lamellipodin in Endo II KD cells. It is worth noting that Lamellipodin was shown to localize to the leading edge of MDA-MB-231 cells in a phosphoinositide (PI(3,4)P2)-dependent manner (50). Since PI(3,4)P2 is enriched in invadopodia precursors (51), it will be interesting to test whether Lamellipodin also localizes to invadopodia to regulate actin polymerization, and whether this depends on Endo II. Endo II was also recently identified as an interacting partner for the proline-rich region of Tks4 adaptor protein (52). It is worth noting that lung metastasis of tumor cells requires Tks4-induced invadopodia formation and maturation (53). Future studies of this Tks4–Endo II axis will be required to understand their roles in formation or function of invadopodia in TNBC cells (52). Another recently described binding partner of Endo II is the exchange factor for Arf6 (EFA6) whose activity is enhanced by Endo II leading to increased Arf6GTP signaling at cell protrusions (54). Interestingly, Arf6 was previously shown to promote Erk activation, invadopodia formation, and cell invasion in melanoma and breast cancer models (55, 56). Given the defects in EGFR signaling to Erk that we have observed in Endo II KD cells, it will be interesting to test whether this is due to altered Arf6 activation, or altered transist time of EGFR within signaling endosomes. A recent study of proteins involved in EGFR internalization provides further support for compartmentalized signaling of EGFR from endosomes (10). In their study, Annexin A2 silencing enhanced EGF uptake, Akt activation, and lung metastasis in xenograft models. In our study, Endo II silencing led to defects in EGFR internalization, Akt activation, and lung metastasis in xenograft assays. Because both Erk and Akt pathways play key roles in tumor progression, it will be interesting to define the gene expression signature of TNBCs in Endo II KD tumors.

Our analysis of Endo II expression in human breast tumors revealed a trend toward high Endo II expression in TNBC cases, compared with luminal and HER2 tumors. These results are consistent with our results in surveying breast cancer cell lines. Considering the importance of receptor cargos (MT1-MMP, EGFR) and signaling pathways (Akt, Erk) that are regulated by Endo II in TNBC cell models, it will be interesting to perform parallel studies of these signaling proteins in tumors with varying levels of Endo II. Since our tumor xenograft studies identify Endo II as a metastasis-promoting factor, it will be important to profile Endo II expression and the above pathways in paired samples of primary tumors and lymph node or distant metastases. Given the evidence that FAK/Src kinases phosphorylate Endo II at Y315 leading to loss of binding to SH3 domain partners (21), it would be very interesting to profile Endo II pY315 expression and localization within TNBC cells and tumors. However, this will require preparation of new phospho-specific antibodies for Endo II. For a widely expressed gene, it is interesting that expression of the 3k3g1 gene encoding Endo II at levels above or below the median (within the primary tumor) was associated with risk of relapse in basal-like breast cancer patients. These results will spur further testing of this association at the Endo II protein level in patient cohorts with known clinical outcomes. Given that some of the pathways regulated by Endo II are being considered in targeted therapies, the relative levels of Endo II expression in these patient samples may be relevant to the response to therapy. It is also worth noting that in the rapidly emerging field of antibody–drug conjugates for cancer therapy, biomarkers related to the endocytic pathways may be useful to predict response, since receptor internalization is a necessary step for release of these anticancer drugs (57).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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