Significance of PELP1 in ER-Negative Breast Cancer Metastasis

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Abstract

Breast cancer metastasis is a major clinical problem. The molecular basis of breast cancer progression to metastasis remains poorly understood. PELP1 is an estrogen receptor (ER) co-regulator that has been implicated as a proto-oncogene whose expression is deregulated in metastatic breast tumors and whose expression is retained in ER-negative tumors. We examined the mechanism and significance of PELP1-mediated signaling in ER-negative breast cancer progression using two ER-negative model cells (MDA-MB-231 and 4T1 cells) that stably express PELP1-shRNA. These model cells had reduced PELP1 expression (75% of endogenous levels) and exhibited less propensity to proliferate in growth assays in vitro. PELP1 downregulation substantially affected migration of ER-negative cells in Boyden chamber and invasion assays. Using mechanistic studies, we found that PELP1 modulated expression of several genes involved in the epithelial mesenchymal transition (EMT), including MMPs, SNAIL, TWIST, and ZEB. In addition, PELP1 knockdown reduced the in vivo metastatic potential of ER-negative breast cancer cells and significantly reduced lung metastatic nodules in a xenograft assay. These results implicate PELP1 as having a role in ER-negative breast cancer metastasis, reveal novel mechanism of coregulator regulation of metastasis via promoting cell motility/EMT by modulating expression of genes, and suggest PELP1 may be a potential therapeutic target for metastatic ER-negative breast cancer.

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Introduction

Endocrine therapy has been shown to have a positive effect on the treatment of advanced metastatic disease (1, 2). Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs with tumors recurring as metastasis, which is the leading cause of death from breast cancer. Tumor metastasis comprises a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location (3) and involves a multistep cascade of coordinated cell adhesion and contractility, as well as proteolytic remodeling of the extracellular matrix (4, 5). The process of migration is orchestrated through the activation of biochemical pathways that involve multiple cytoskeleton proteins (5). Even though substantial information is available on the process of metastasis, a critical need to identify novel targets that can be used to curb the progression of breast cancer metastasis still exists.

During the past 20 years, studies have extensively focused on the role of 2 nuclear receptors, the estrogen receptor (ER) and the progesterone receptor (PR). The presence of ER and PR in ER-positive tumors can explain the biology of the tumor; but, what drives ER-negative metastatic tumors is not known (6). With the recent advances in detection technologies, the potential importance of several additional nuclear receptors (NR), including estrogen-related receptor alpha (ERRα), glucocorticoid receptor (GR), and androgen receptor (AR), are being appreciated in breast cancer (7–9). Emerging evidence also suggests that NR action is complex and requires functional interactions with coregulators (10, 11). As a modulator of NR functions, coregulators are likely to play a role in breast cancer progression. Coregulators function as “master genes” sensing the physiologic signals and activating the appropriate set of genes and thus have the potential to control the expression of subsets of genes to produce a desired function such as cell growth (12). With the enormous potential of coregulators as master regulators, their deregulation is likely to provide cancer cells an advantage in growth and metastasis (13). Understanding how NR coregulators play a role in metastasis will be useful in maximizing treatment opportunities for metastatic breast cancer.
Proline glutamic acid–rich protein (PELP1) was initially identified as an ER coregulator (14). Recent studies showed that PELP1 functions as a general coregulator for a number of nuclear receptors, including ERβ, ERR, GR, and AR (15). In the nuclear compartment, PELP1 interacts with histones and histone-modifying enzymes and, thus, plays a role in chromatin remodeling (16). PELP1 also couples NRs to several cytosolic signaling axes, such as Src-MAPK, PI3K-Akt, and EGFR/Her2 (15), thus functioning as a mediator of NR extranuclear actions. PELP1 expression is deregulated in breast cancer and exhibits oncogenic potential (17). Its expression is maintained in ER-negative breast tumors (17, 18). Although these studies suggested that PELP1 has potential to participate in hormonal driven pathologies, whether PELP1 plays a role in initiation and progression of ER-negative breast cancer remains unknown.

In this study, we examined whether the proto-oncogene PELP1 contributes to the metastatic potential of ER-negative breast cancer cells. Using in vitro and in vivo xenograft models, we provide evidence that shows PELP1 plays a role in ER-negative breast cancer invasion and metastasis by modulating expression of the genes involved in epithelial mesenchymal transition (EMT). Our results suggest that PELP1 plays a critical role in promoting cell motility/EMT and suggest PELP1 may be a potential therapeutic target for metastatic ER-negative breast cancer.

Materials and Methods

Cell cultures and reagents

MDA-MB-231 and 4T1 cells were purchased from the American Type Culture Collection and maintained in RPMI-1640 supplemented with 10% FBS (Hyclone Laboratories Ltd). The PELP1 antibody was purchased from Bethyl laboratories.

Generation of PELP1-shRNA model cells

Breast cancer cells stably expressing PELP1-shRNA were generated using validated human and mouse specific Lentiviral PELP1-shRNA particles (Sigma) for use on MDA-MB-231 and 4T1 cells, respectively. Stable clones were selected in puromycin selection (1 μg/mL) and pooled clones were used for all the studies. Lentiviral particles expressing non-targeted short hairpin RNA (shRNA) were used to generate control cells. Transient knockdown of PELP1 was achieved using On-TargetPlusSMARTpool siRNA (L-004463-00-0050) purchased from ThermoScientific and by using oligofectamine transfection (Invitrogen).

Microarray studies

The Human EMT RT² Profiler PCR Array that profiles the expression of 84 key genes was purchased from SABiosciences. Total RNA isolated from the MDA-MB-231 cells was used for screening by real-time PCR as per the manufacturer’s instructions. Target genes whose expression was differentially regulated (at least 2-fold difference) by PELP1 under expression were selected and validated using real-time PCR with primers used for validation of PELP1-regulated genes were purchased from RealTimePrimers.

Cell migration, invasion, and MMP reporter gene assays

The cell migration and invasion assays were carried out by using the caloriemetric cell migration assay kit (Chemicon) and the BD Biocat growth factor reduced Matrigel invasion chamber kit (BD Biosciences), respectively, as described (19). The PGL3-MMP9-Luc and MMP2-Luc plasmids were described earlier (19). Reporter gene assays were done by transient transfection using the FuGENE6 method (Roche) as described (20). Each transfection was carried out in 6-well plates in triplicate and normalized with the β-gal activity and total protein concentration.

Gelatin zymography

The model cells expressing control or PELP1-shRNA were cultured in a low percentage of serum containing RPMI medium and then the culture supernatant was used to determine the enzymatic activity of matrix metalloproteinase 2 (MMP2) and MMP9 by using SDS-PAGE gelatin zymography as described (21) using Novex Zymogram Gels (Invitrogen). Recombinant MMP2 and MMP9 were purchased from R&D systems and used as positive controls.

Nude mice studies

Model cells (1 × 10⁶) in serum-free medium were injected into left cardiac ventricle of 6-week-old female athymic nude mice (n = 10 per group) as described (21). MDA-MB-231 cells and MDA-MB-231–PELP1-shRNA cells were transfected with green fluorescent protein (GFP)-Luc plasmid to monitor metastasis with whole animal imaging (22). The mice were monitored daily for adverse effects and the body weight was recorded every 3 days. The Xenogen Small-Animal Imaging System was used for subcellular imaging in live mice once a week. On day 21, mice were euthanized, and the total weight and the number of micrometastatic tumor nodules in the lungs were counted with an inverted microscope. Bone tissues (tibia and femora) were fixed in 10% neutral buffered formalin (Fisher Scientific) for 48 hours at room temperature, decalcified in 10% EDTA, and embedded in paraffin. Sections were stained with hematoxylin, eosin, orange G, and phloxine. Histomorphometrical analysis was carried out to obtain the trabecular bone volume and tumor burden in the tibia using BioQuant Osteo, 2010, version V10.3.6 software.

Statistical analysis

Statistical differences among groups were analyzed with either t test or ANOVA as appropriate using SPSS software. P values less than 0.05 were considered significant.

Results

PELP1 knockdown reduces cell proliferation of ER-negative breast cancer cells

We used 2 ER-negative model cells: MDA-MB-231 (human) and 4T1 (mouse). Earlier studies showed that

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these models cells metastasize efficiently to sites affected in human breast cancer (23, 24) and both cells express high levels of PELP1. To establish the significance of the PELP1 axis, we knocked down PELP1 expression using lentiviral mediated transduction of PELP1-shRNA. Pooled clones stably expressing PELP1-shRNA were selected by puromycin. Real-time quantitative reverse transcription PCR (qRT-PCR) and Western analysis showed that PELP1 expression in MDA-MB-231–PELP1-shRNA and 4T1–PELP1-shRNA model cells was reduced by 70% to 80% (Fig. 1A).

We next examined whether PELP1 downregulation affects proliferation of breast cancer cells in vitro using the Cell Titer-Glo assay. Both PELP1-shRNA model cells showed substantially less cellular proliferation than the control shRNA-transfected cells (Fig. 1B). We also validated these results using another PELP1 siRNA that targeted a different site on PELP1 than the PELP1-shRNA targeted site. Transient knockdown using PELP1 siRNA resulted in an 80% reduction of endogenous PELP1 (Supplementary Fig. S1). In proliferation assays, transient knockdown of PELP1 expression also substantially decreased the proliferation of MDA-MB-231 cells (Supplementary Fig. S1). Collectively, these results indicate that the proto-oncogene PELP1 has potential to regulate the cell proliferation of ER-negative breast epithelial cells.

**PELP1 signaling axis is needed for optimal cell migration and invasion of ER-negative breast cancer cells**

PELP1 expression is deregulated in metastatic tumors (17). However, whether PELP1 plays a role in the metastasis of ER-negative cells remains unknown. To examine the significance of PELP1 in ER-negative cell metastasis, we carried out in vitro migration assays and an invasion assays using Boyden chamber assay. In the migration assays, PELP1 knockdown resulted in significantly less migration in both the MDA-MB-231 and 4T1 cells than in the control vector-transfected cells (Fig. 2A). PELP1 knockdown also significantly reduced the invasion potential of both the MDA-MB-231 and 4T1 cells (Fig. 2B). The results observed in PELP1-shRNA stable clones were also validated by using
transient knockdown of PELP1 by siRNA that targeted a different site on PELP1 than the PELP1-shRNA target site. PELP1 siRNA, but not control siRNA, treatment significantly reduced the ability of MDA-MB-231 cells to migrate in the Boyden chamber assays (Supplementary Fig. S2). Collectively, these results suggested that PELP1 has the potential to modulate migration and invasion of ER-negative breast cancer cells.

**PELP1 knockdown promotes alterations in the expression of the EMT markers**

EMT is a key process that is implicated in tumor metastases. PELP1 is a coregulator that plays a critical role in NR genomic functions via chromatin remodeling and by modulating the histone code at the NR target promoters (16). Because PELP1 knockdown ER-negative breast cancer cells exhibited less migration and invasion, we examined whether the lack of expression of PELP1 affects the expression of genes involved in EMT by using a focused microarray approach. The EMT microarray contains 84 genes including cell surface receptors; cytoskeletal genes mediating cell adhesion, migration, motility, and morphogenesis; genes controlling cell differentiation, development, growth, and proliferation; and signal transduction and transcription factor genes that cause EMT and all of its associated processes. Total RNA isolated from the MDA-MB-231 and MDA-MB-231–PELP1-siRNA cells were used for the array analysis. The results from the screen suggested that PELP1 downregulation substantially reduced the expression of a number of genes, including MMP2, MMP3, MMP9, SMAD2, SNAIL, TWIST1, VIM, ZEB1, and ZEB2 compared with their expression in control siRNA-transfected cells (Fig. 3A). We validated the changes seen in the array study by measuring gene expression of the top 8 genes that had significant reductions by carrying real-time qPCR in the MDA-MB-231–PELP1-shRNA stable clones (Fig. 3B). PELP1 regulation of EMT-related genes was also independently confirmed in the MDA-MB-231 cells by the transient expression of PELP1 siRNA (Fig. 3C). Furthermore, qRT-PCR analysis using the 4T1 cells expressing PELP1 siRNA also showed that PELP1 downregulation significantly affected the expression of EMT genes compared with the genes in the control siRNA cells (Supplementary Fig. S3A). Furthermore, qRT-PCR and Western analysis of 4T1 model cells showed upregulation of E-cadherin (CDH1) under conditions of PELP1 knockdown (Supplementary Fig. S3B). These results suggested that PELP1 has potential to modulate expression of genes involved in the EMT/metastasis.

**PELP1 knockdown modulates expression and activities of MMPs**

Because PELP1 downregulation altered expression of MMPs in ER-negative breast cancer and because MMPs are involved in EMT and all of its associated processes (17), we examined whether the reduced expression of MMPs correlates with functional activity. We determined MMP activity in both the MDA-MB-231 and 4T1 cells expressing control or PELP1-siRNA by using gelatin zymography. MMP2 and MMP9 activities were lower in PELP1 knockdown cells than in control cells (Fig. 4A). To elucidate the mechanism by which PELP1 regulates MMP2 and MMP9 expression, we carried out promoter-Luc assays using the
previously published MMP2 and MMP9 reporter genes (25, 26). In both the MDA-MB-231 and 4T1 model cells, knockdown of PELP1 significantly reduced the MMP2 and MMP9 reporter gene activation (Fig. 4B). We examined further whether the PELP1-mediated regulation of MMPs is due to PELP1 recruitment to the promoter regions of MMPs by using a chromatin immunoprecipitation (ChIP)-based assay. Our ChIP results showed that PELP1 is uniquely recruited to the proximal promoter region of MMP9 and MMP2 (Fig. 4C) and no recruitment in the distal regions was observed (data not shown). PELP1 associates with acetylases (p300, CBP) and demethylases (KDM1) and was observed (data not shown). PELP1 associates with acetylases (p300, CBP) and demethylases (KDM1) and participates in epigenetic modifications that are required for optimal transcriptional activation (15). To examine whether lack of PELP1 promotes inhibitory marks, we examined for metastasis. Subcellular imaging of whole lung viewed (ChIP) on days 0, 7, 14, and 21. The presence of the luciferase signal within the whole animal indicated that there was a greater propensity of metastasis signal both in the dorsal and ventral views (Fig. 5A) of mice injected with control MDA-MB-231 cells than with the corresponding MDA-MB-231–PELP1-shRNA 2. Compared with control MDA-MB-231–shRNA cells injected mice, nude mice injected with PELP1-shRNA cells had a significant reduction in tumor metastatic signal (91%, P < 0.0001) in the dorsal view and a reduction in the ventral view (84%, P < 0.0001; Fig. 5B). The body weights were not significantly different between control shRNA and PELP1-shRNA treatment groups (data not shown), however, mice injected with the control MDA-MB-231 shRNA cells had slower mobility (probably because of metastasis) than the mice injected with PELP1-shRNA cells on day 21. To further examine the role of PELP1 in dissemination of tumor cells in vivo, the lungs and bones were collected after day 21 and histologically examined for metastasis. Subcellular imaging of whole lung GFP fluorescence revealed that a significant reduction (88%, P < 0.0001) in tumor nodule formation in the

Significance of the PELP1 axis in the metastatic potential of ER-negative cells in vivo

To examine whether blockage of PELP1 axis in vivo reduces metastasis potential of ER-negative breast cancer cells, we carried out studies in nude mice using the MDA-MB-231 and MDA-MB-231–PELP1-shRNA cells that were transfected with GFP-Luciferase vectors (Supplementary Fig. S4). For this assay, model cells (1 × 10^7 in 0.1 mL PBS/mouse) were injected into the left cardiac ventricle of nude mice (10 animals each group, total 20 mice) to monitor metastasis with whole animal imaging. The Xenogen non-invasive optical imaging system was used for whole animal imaging on days 0, 7, 14, and 21. The presence of the luciferase signal within the whole animal indicated that there was a greater propensity of metastasis signal both in the dorsal and ventral views (Fig. 5A) of mice injected with control MDA-MB-231 cells than with the corresponding MDA-MB-231–PELP1-shRNA 2. Compared with control MDA-MB-231–shRNA cells injected mice, nude mice injected with PELP1-shRNA cells had a significant reduction in tumor metastatic signal (91%, P < 0.0001) in the dorsal view and a reduction in the ventral view (84%, P < 0.0001; Fig. 5B). The body weights were not significantly different between control shRNA and PELP1-shRNA treatment groups (data not shown), however, mice injected with the control MDA-MB-231 shRNA cells had slower mobility (probably because of metastasis) than the mice injected with PELP1-shRNA cells on day 21. To further examine the role of PELP1 in dissemination of tumor cells in vivo, the lungs and bones were collected after day 21 and histologically examined for metastasis. Subcellular imaging of whole lung GFP fluorescence revealed that a significant reduction (88%, P < 0.0001) in tumor nodule formation in the

Figure 3. PELP1 knockdown affects expression of selective genes involved in EMT. A, RNA isolated from MDA-MB-231–siRNA negative control and MDA-MB-231–PELP1-siRNA-expressing cells were hybridized to the human EMT Array. Representative genes downregulated upon PELP1 depletion are shown. B, real-time qPCR validation of the changes in gene expression in MDA-MB-231 cells stably expressing negative control shRNA or PELP1-shRNA. C, MDA-MB-231 cells were transiently transfected with control or PELP1-specific siRNA and the expression of indicated genes was analyzed by real-time qPCR. *P < 0.05, t test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
PELP1-shRNA treatment groups compared with the control group (Fig. 5C). X-ray imaging and corresponding quantitation of tibia and femur bones analysis elucidated that a portion of bone loss was observed in the MDA-MB-231 control mice and not in the PELP1-shRNA groups (Fig. 5D). Collectively, these results suggested that PELP1 knockdown cells exhibited less propensity to metastasize to distant organs.

Discussion

Breast cancer is the most frequently diagnosed cancer in women. Tumor metastasis remains a significant problem and is the main cause of patient fatality. In this study, we examined whether the proto-oncogene PELP1 contributes to metastatic potential of ER-negative breast cancer cells and determined whether blocking the PELP1 signaling axis has a therapeutic effect. We found that (a) PELP1 knockdown affected the cell proliferation of ER-negative breast cancer cells; (b) PELP1 knockdown substantially affected the ability of both MDA-MB-231 and 4T1 cells to migrate in Boyden chamber and invasion assays; (c) downregulation of PELP1 reduced the activity of MMPs in ER-negative metastatic cells; (d) PELP1 modulated the expression of genes involved in EMT; and (e) downregulation of PELP1 in vivo by PELP1-shRNA treatment groups compared with the control (Fig. 5C). X-ray imaging and corresponding quantitation of tibia and femur bones analysis elucidated that a portion of bone loss was observed in the MDA-MB-231 control mice and not in the PELP1-shRNA groups (Fig. 5D). Collectively, these results suggested that PELP1 knockdown cells exhibited less propensity to metastasize to distant organs.

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Figure 4. PELP1 regulates the expression and activities of MMPs in ER-negative cells. A, gelatin zymography analysis of activity of MMP2 and MMP9 in the MDA-MB-231 and 4T1 cells that were transfecte...
significantly reduced ER-negative breast metastasis in a nude mice model. Collectively, these results suggest that PELP1 signaling confers growth and metastatic advantages to ER-negative breast epithelial cells.

To appreciate the mechanisms by which breast cancers develop into metastasis, it is necessary to understand the molecular mechanism(s) involved in metastasis. Recent advances suggest that nuclear receptor interacting coregulators may play roles in growth and metastasis of ER-negative tumors by modulating transcription of target genes (27, 28). For example, the ER coregulator AIB1 can promote breast cancer metastasis by the activation of PEA3-mediated MMP2 and MMP9 expression (29). PELP1 was initially identified as a novel ER coregulator (14). Recent studies showed that PELP1 functions as a general coregulator for a number of nuclear receptors, including ER, ERR, PR, GR, AR, and transcription factors, such as E2F, FHL2, and STAT3 (15). The ability of PELP1 to interact with a wide variety of nuclear receptors and transcription factors suggests that PELP1 deregulation in metastatic cells may provide advantage by modulating a set of genes required for metastasis. Accordingly, the results from our gene array experiment

Figure 5. PELP1 downregulation reduces the metastatic potential of ER-negative cells in vivo. A, whole animal imaging analysis of mice (n = 10 per group) treated with MDA-MB-231 and MDA-MB-231-PELP1-shRNA. Both dorsal and ventral views are shown. B, luciferase signals were quantitated by using the software Living Image 3.2. C, number of tumor nodules in the lung was measured in the control and PELP1-shRNA–treated mice groups (n = 10 per group). D, bone loss was detected by X-ray imaging analysis and bone and tumor volumes in sections of tibiae were quantified using histomorphometry analysis as described in Materials and Methods. *, P < 0.05, t test.
indicated that PELP1 plays a critical role in the expression of a number of genes involved in EMT and metastasis.

PELP1 expression is deregulated in breast cancer. Our earlier studies using breast tumor prognostic arrays showed that node-positive and metastatic tumors have greater PELP1 expression than in node-negative specimens (17). Curiously, in this study, PELP1 overexpression is equally observed in both ERα-positive and negative grade2/3, node-positive and metastatic tumors, suggesting that PELP1 may have functions independent of the ER in metastatic cells (17). A recent study of a larger number of patients (n = 1,162) with invasive breast cancers found that high PELP1 expression is associated with tumor clinical parameters, and implicated PELP1 protein expression as an independent prognostic predictor of shorter breast cancer–specific survival (18). In this study, using in vitro and in vivo models, we have provided the first evidence that PELP1 signaling plays a critical role in ER-negative breast cancer proliferation and metastasis. Earlier studies using ovarian cancer xenograft model indicated that PELP1 knockdown reduces primary tumorigenesis (19). Our findings that PELP1 reduces proliferation and metastasis of ER-ve model cells suggests that PELP1 signaling can also be potentially used to therapeutically target ER-negative breast cancers.

In the nuclear compartment, PELP1 interacts with histones and histone-modifying enzymes, and thus plays a role in chromatin remodeling activities at target genes (30). Previous studies from our laboratory showed that PELP1 facilitates target gene activation by histone modification via lysine demethylase 1 (LSD1/KDM1; ref. 16). Our results using the MMP9 and MMP2 promoter reporter gene assays suggest that PELP1 has potential to modulate transcription of these genes. ChIP analysis showed that PELP1 is recruited to the MMP9 and MMP2 promoters and that PELP1 status has potential to influence the inhibitory histone marks at MMP promoter. Furthermore, EMT gene array analysis indicated that PELP1 has potential to modulate several regulatory genes involved in the EMT process including TWIST, SNAI1, and ZEB. These findings suggest that PELP1 modulation of genes involved in EMT and invasion as one possible mechanism by which it promotes tumor progression in ER-negative breast metastasis.

Earlier studies showed that PELP1 interacts with several proteins involved in cytoskeleton remodeling, including Src kinase (31), PI3K (32), four-and-a-half LIM protein 2 (33), and ILK1 (34). PELP1 modulates functions of metastasis-associated antigen 1 (MTA1), a protein implicated in metastasis. PELP1 also interacts with the MTA1-associated coactivator and promotes ER-transactivation functions in a synergistic manner (35). PELP1 is shown to modulate expression of MTA3, a gene implicated in the invasive growth of human breast cancers. These earlier studies suggest that PELP1 interactions with the ER, cytoskeletal kinases, and MTA family members contribute to PELP1-driven ER-positive metastasis. Our current results add new information that PELP1 also has potential to modulate migratory properties of ER-negative breast cancer cells. In vitro migration and invasion assays using 2 different model cells showed that PELP1 signaling is essential for optimal cell migration. Furthermore, in vivo xenograft-based assays showed that PELP1 plays a critical role in in vivo metastasis of ER-negative breast cancer cells.

In summary, our results show that the NR coregulator PELP1 plays a critical role in ER-negative breast cancer cell migration and modulates expression of several genes involved in EMT/metastasis. Even though earlier studies found that PELP1 plays a role in the proliferation of hormone-driven tumors, this study shows that it also has the potential to promote metastasis of ER-negative breast epithelial cells. Furthermore, our study provides the first in vivo evidence that the PELP1 axis is a potential therapeutic for blocking ER-negative breast cancer metastasis. Exploring the role of the PELP1 axis in metastasis is a novel area and understanding how PELP1 plays a role in metastasis will be useful in maximizing treatment opportunities for metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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