Lipid rafts remodeling in estrogen receptor–negative breast cancer is reversed by histone deacetylase inhibitor

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Abstract
Recently, we have found dramatic overexpression of ecto-5′-nucleotidase (or CD73), a glycosylphosphatidylinositol-anchored component of lipid rafts, in estrogen receptor–negative [ER(−)] breast cancer cell lines and in clinical samples. To find out whether there is a more general shift in expression profile of membrane proteins, we undertook an investigation on the expression of selected membrane and cytoskeletal proteins in aggressive and metastatic breast cancer cells. Our analysis revealed a remarkably uniform shift in expression of a broad range of membrane, cytoskeletal, and signaling proteins in ER(−) cells. A similar change was found in two in vitro models of transition to ER(−) breast cancer: drug-resistant Adr2 and c-Jun-transformed clones of MCF-7 cells. Interestingly, similar expression pattern was observed in normal fibroblasts, suggesting the commonality of membrane determinants of invasive cancer cells with normal mesenchymal phenotype. Because a number of investigated proteins are components of lipid rafts, our results suggest that there is a major remodeling of lipid rafts and underlying cytoskeleton in ER(−) breast cancer.

To test whether this broadly defined ER(−) phenotype could be reversed by treatment with differentiating agent, we treated ER(−) cells with trichostatin A, an inhibitor of histone deacetylase, and observed reversal of mesenchymal and reappearance of epithelial markers. Changes in gene and protein expression also included increased capacity to generate adenosine and altered expression profile of adenosine receptors. Thus, our results suggest that during transition to invasive breast cancer there is a significant structural reorganization of lipid rafts and underlying cytoskeleton that is reversed upon histone deacetylase inhibition. [Mol Cancer Ther 2006;5(2):238–45]

Introduction
Establishment and clinical use of tumor markers that define invasive and metastatic breast carcinoma is critical for more individualized therapeutic strategies in the future. Because breast cancer is becoming an increasingly heterogeneous disease, the task of defining specific cancer cell phenotypes is especially challenging. Several individual breast cancer markers have been established for target-specific pharmacologic intervention. The clinically proven therapies include targeting estrogen receptor (ER) and progesterone receptor and more recently Erb2 and epidermal growth factor receptor (EGFR). Major differences in expression profiles of wide number of genes has been documented in ER(−) and ER(+) carcinomas (1). Both in in vitro studies and in the clinic, these differences were associated with either more motile and invasive phenotype or more aggressive course of the disease in the case of ER(−) breast carcinoma (2). Membrane proteins and associated cytoskeletal mediators the communication with the extracellular milieu and the composition of membrane proteins is critical for cell behavior in general and invasive and metastatic properties in particular.

Among different membrane microdomains, lipid rafts are one of the least understood subcellular elements. Although their lipid composition has been investigated in several studies (3–7), protein components were not systematically compared between invasive and noninvasive cells. Because several in vitro models of breast cancer exemplify transition to more invasive and metastatic state, we have chosen this model to study changes in lipid rafts composition after loss of ER expression. Our recent finding that ER(−) breast cancer cells express high level of ecto-5′-nucleotidase, a glycosylphosphatidylinositol-anchored protein and a marker of lipid rafts, provides an early argument for the lipid raft remodeling during transition to more aggressive breast carcinoma (8). In this study, we aimed to analyze whether there is a consistent alteration in expression of cytoskeletal membrane and lipid raft protein components across a wider population of ER(+) and ER(−) cells that would suggest a coordinate expression consistent with the motile and invasive phenotype. Within this phenotype, we also investigated the expression of genes and proteins involved in adenosine generation and signaling. Although limited by the availability of suitable antibodies, our unique focus on expressed proteins, rather than mRNA profile, allowed us to directly relate protein expression with the specific cell phenotype.

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Materials and Methods

Cell Lines

Breast cancer ER(+) cell lines MDA-MB-474, ZR-75-1, MCF-7 and negative SK-BR-3, MDA-MB-468, MDA-MB-435s, MDA-MB-231, BT-549, Hs578t, nontransformed MCF-10A, c-Jun-transformed MCF-7/c-Jun clone 2-33 and control MCF-7/neo clone 7-1, and human fibroblasts WI-38 were obtained from either Tissue Culture Facility at Lineberger Comprehensive Cancer Center/University of North Carolina, American Type Culture Collection (Manassas, VA), or developed as described before (9). The Adr2 and A DR MCF-7 Adriamycin-resistant cell sublines were from Dr. Y.M. Rustum (Roswell Park Cancer Institute, Buffalo, NY). Cells were maintained in MEM supplemented with Eagle salts, NaPyr, nonessential amino acids, and 10% fetal bovine serum (most cell lines); in McCoy’s supplemented with 15% fetal bovine serum (SK-BR-3 cells); in Leibowit L-15 supplemented with 10% fetal bovine serum (MDA-MB-468 cells); in mammary epithelial growth medium supplemented with bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, and 10% fetal bovine serum (BioWhittaker medium for MCF-10A cells) in CO2/O2 atmosphere at 37°C, except for MDA-MB-468 cells that were grown at ambient atmosphere at 37°C. All media contained penicillin and streptomycin. Original cell stocks were grown at ambient atmosphere at 37°C (BioWhittaker medium for MCF-10A cells) in CO2/O2 atmosphere at 37°C, except for MDA-MB-468 cells that were grown at ambient atmosphere at 37°C. All media contained penicillin and streptomycin. Original cell stocks were stored in liquid nitrogen and each sample was kept in culture for no more than 15 passages.

Reagents

All general reagents were ACS or the highest purity commercially available. The following antibodies were used. Rabbit polyclonal anti-ecto-5’-nucleotidase antibodies (for Western blot) were generated as described before (10) or purchased from BD Biosciences (PharMingen, San Jose, CA; for immunofluorescence). Anti-Ga12 sc-409, Ga13 sc-410, Gαs I41720, integrin α1 I41720, integrin α5 I55220, PKCβ1 P17720, PKCδ P65620, PKCδ P15120, moesin M36820, EBP50 E83020, ezrin M36820, gelsolin G37820, and flotillin-1 G15120, moesin M36820, and c-Src GD11 were from Upstate (Lake Placid, NY); rabbit anti-uPAR (399R) were from American Diagnostica, Inc. (Greenwich, CT); anti-β-actin was from Oncogene (Boston, MA); antifilamin antibody MAB1678, integrin αV AB1930, talin MAB1676, and vimentin AB1620 were from Chemicon (Temecula, CA); and anti-intestinal alkaline phosphatase antibodies were from Biogenesis (Kingston, NH).

Lipid Raft Isolation

Cell lysates were prepared by mixing equal volumes of cell pellets with 2% Triton X-100 on ice for 1 minute and subsequent dilution twice with PBS and twice further with 35% Nycodenz [5-(N-2,3-dihydroxpropylacetamido)-2,4,6-triiodo-N,N-bis(2,3-dihydroxypropyl)-isophtalamide] in PBS. At each step, mixing was achieved by pipetting the lysate up and down several times with Eppendorf pipettor. A modified procedure for density gradient centrifugation using Nycodenz from Sigma-Aldrich (St. Louis, MO) was used to fractionate Triton X-100–soluble and Triton X-100–insoluble membrane and cytoskeletal subdomains and complexes (11). For the purpose of centrifugation, cell lysates containing 3 to 4 mg total protein were diluted 2-fold with 35% Nycodenz. Density step gradient was generated by applying 0.5 mL aliquots of diluted concentration of Nycodenz (35%, 25%, 22.5%, 20%, 17.5%, 15%, 12%, 8%, and 4%) sequentially into Beckman (Palo Alto, CA) 13 × 51 mm polyyallomer tubes. Lysates were placed in the middle of Nycodenz gradient premixed in 17.5% Nycodenz. Tubes were centrifuged at 46,000 × g for 4 hours in a Beckman 55 Ti rotor at 4°C. Following centrifugation, 0.5 mL fractions were carefully withdrawn and small pellet was resuspended in PBS containing 0.5% SDS and 1% Triton X-100 (fraction 10). Total of 10 fractions and control input lysate were analyzed for the distribution of proteins by Western blot. Typically, components of light lipid rafts and caveolae distributed into first four fractions: Soluble cell components, including cytosolic proteins, remained in fractions 5 and 6 and cytoskeleton-associated high-density fractions were distributed in fractions 7 to 9.

Western Blotting

Cell extracts, obtained by scraping cells in PBS in the presence of protein phosphatase and protease inhibitors and lysing with ice-cold 1% Triton X-100/PBS, were loaded on the SDS-PAGE at 30 μg per lane. Separated proteins were transferred onto Immobilon-P 0.45 μm/L (Millipore, Bedford MA) polyvinylidene difluoride membrane and used for probing with specific antibodies. Two buffer systems were used during incubations with antibodies: PBS supplemented with 5% Carnation fat-free dry milk and 0.2% Tween 20 or 25 mmol/L Tris (pH 8.4) supplemented with 130 mmol/L NaCl, 5 mmol/L potassium phosphate, 5% fat-free dry milk, and 0.2% Tween 20. Blots were reused several times after mild stripping by air drying overnight at room temperature when necessary. Secondary antibodies conjugated to horseradish peroxidase and BM Chemiluminescence Western Blotting kit (Roche, Indianapolis IN) were used to develop images on Kodak (Rochester, NY) X-Mat Blue XB-1 film.

Reverse Transcription-PCR

Extraction of total RNA was done using TRI reagent from Molecular Research Center (Cincinnati, OH). Reverse
transcription-PCR assays were done using Advantage RT and Titanium Taq PCR kits from Clontech BD Biosciences (Mountain View, CA). The following PCR oligonucleotides for adenosine receptor subtypes were used: A1 forward: AATTGCTGTGGACCGCTACCTC, reverse: CGACACCTTCTTGTGAGCTG; A2a forward: TTGACCGCTACATTGCATCCG, reverse: GAAGATCCGCAAATAGACACC; A2b forward: ACCAACTACTTCCTGGTGTCC, reverse: GCAGCTTTCATTCGTGGTTCC; A3 forward: ATCGCTGTGGACCGATACTTG, reverse: AATGCACCTGTCTCTTTGAAG. Amplification reactions were run at the following conditions: 1 minute each at 94°C, 62°C, and 72°C, total 40 cycles. The size of PCR products were as follows: AR1 344 bp, AR2a 305 bp, AR2b 374 bp, AR3 352 bp, glyceraldehyde-3-phosphate dehydrogenase 983 bp, and trypsin inhibitor 407 bp.

Results
We have done a broad survey of the expression of membrane, cytoskeletal, and signaling proteins in breast cancer cell lines that represent ER(+) and ER(-) phenotypes. The cell panel consists of well-characterized cell lines, which invasive and metastatic potential have been defined in previous studies (12, 13), thus enabling us to correlate protein expression with specific cell phenotypes. In this cell panel, MCF-10A cells is an example of nontumorigenic mammary epithelial control cells and SK-Br-3 and MDA-MB-468 represent ER(-) cells that are less tumorigenic than MDA-MB-435s, MDA-MB-231, BT-549, and HS578t cells (12). Recently, we showed dramatic up-regulation of ecto-5'-nucleotidase, a glycosylphosphatidylinositol-anchored membrane ecto-protein, in ER(-) breast cancer cells (8). Here, we compared the expression of ecto-5'-nucleotidase with other lipid raft components, such as CD24 and alkaline phosphatase. The expression of proposed breast cancer marker CD24 concurred with the ER receptor status and was also found in SK-Br-3 and MDA-MB-468 cells. A reciprocal pattern was found for intestinal alkaline phosphatase (Fig. 1A). The broader membrane and cytoskeletal protein survey revealed an alteration in expression of specific proteins in ER(+) and ER(-) cell lines with somewhat variable expression in SK-Br-3 and MDA-MB-468 cells (Fig. 1A). Although E-cadherin was expressed mostly in ER(+) and control cells, other adhesion receptor integrins β1, α5, and αV; CD44; and OB-cadherin and N-cadherin tended to coexpress with ecto-5'-nucleotidase in ER(-) cells (Fig. 1B). Similarly, cytoskeletal

Figure 1. Differential expression of membrane and cytoskeletal proteins in hyperplastic MCF-10A cells and in a panel of breast cancer cell lines. A, representative cytoskeletal and lipid raft–associated proteins. eN, ecto-5'-nucleotidase; ALP, alkaline phosphatase. B, representative membrane and signaling proteins with roles in adhesion. C, representative cytoskeleton-associated proteins. Note that moesin (bottom) and ezrin (top band on the bottom panel) are detected simultaneously using M36820 antibodies from Transduction Laboratories. Cell lysates were prepared in enough quantity so different proteins could be compared using the same lysate. They were loaded onto each lane (30 µg) and processed for SDS-PAGE and Western blotting procedure as described in Materials and Methods.
protein vimentin and partially smooth muscle actin, in contrast to several cytotkeratins, coexpressed with ecto-5'-nucleotidase in ER(−) cells. Lamin B1 expression was independent of cell type; however, α-tubulin tended to express at higher levels in more aggressive ER(−) cells (Fig. 1A). Analysis of proteins associated with cytoskeleton show that although EBP50 and gelsolin were associated with less invasive cells, fimbrin, talin, filamin, and especially fascin and moesin tended to express at higher level in more invasive breast cancer cells (not shown). Interestingly, caveolin-1 expression strongly coincided with ecto-5'-nucleotidase, further suggesting that, in addition to ecto-5'-nucleotidase lipid rafts, caveolae may have specific function in more invasive cells. Other membrane or membrane-associated signaling molecules, such as EGFR (not shown), lyn, and PKCα, also showed similar expression pattern. On the other hand, FAK, PKCζ, PKCδ, and integrin α3 did not display cell-specific expression (not shown).

This comprehensive analysis helped us subcategorize our breast cancer cell panel into three distinct profiles. BT474, ZR-75-1, and MCF-7 cells having epithelial and epithelial-435s, MDA-MB-231, BT-549, and Hs578t having more mesenchymal features. Significantly, cell lines SK-BR-3 and MDA-MB-468 fall in between: Although losing many epithelial markers, such as E-cadherin and certain cytotkeratins, they did not yet acquire full set of mesenchymal features, and, as have been shown previously, are less tumorigenic (12). Interestingly, during long cell culture (>20 cell passages), we occasionally observed temporal expression of ecto-5'-nucleotidase in SK-BR or lower expression in MDA-MB-468 cells, but no change in expression in other cell lines, suggesting some intrinsic phenotypic plasticity in these particular cells. Despite their less aggressive phenotype, this could explain metastatic behavior of MDA-MB-468 cells in mouse xenograft model (14). The MCF-10A cell line, on the other hand, expresses more complex mixture of epithelial and mesenchymal markers, and thus may likely have myoepithelial origin.

To further test whether transition to ER(−) status and more invasive phenotype will show similar shift in expression profile, we used two independent in vitro models of breast cancer progression. Development of drug resistance and overexpression of c-Jun in MCF-7 cells were both shown to correlate with the loss of ER expression and transition to more invasive and tumorigenic phenotype (9, 15). Results presented in Fig. 2 show that in these two models, there is a very similar shift in expression profile to that in ER(−) cells shown in Fig. 1A-C. To further analyze whether the expression of membrane or cytoskeletal proteins may differentiate between “cancer metastatic” and normal motile phenotypes, we included in this survey lysates from normal human fibroblasts WI-38. Normal fibroblasts are commonly used to represent mesenchymal phenotype. This comparison revealed that drug-resistant, c-Jun-transformed, and all other invasive cells shown in Fig. 1A-C exhibit the expression pattern of membrane and cytoskeletal proteins that is remarkably similar to fibroblasts. In addition to data presented in Fig. 2, we observed up-regulation of Src, lyn, and EGFR and down-regulation of Lck in ER(−) cells and in fibroblasts. The expression of fimbrin and flotillin-1 did not show, however, significant variation in these cells (not shown).

To determine which membrane, cytoskeletal, and signaling proteins were associated with lipid rafts, we used the modified density gradient centrifugation with Nycodenz (11) and used MDA-MB-231 and MCF-7 cell lysates as representative for ER(−) and ER(+) phenotypes. These two cell lines are very well characterized in previous studies and therefore are better suited for comparative analysis. Furthermore, MDA-MB-231 cells were shown to reexpress ERα and E-cadherin upon trichostatin A treatment (16), suggesting an intrinsic plasticity important for the purpose of this work. Other cell lines shown in Fig. 1, both ER(−) and ER(+), were also tested for lipid raft distribution of selected membrane proteins and the results were similar to MCF-7 and MDA-MB-231 cells (not shown). Preliminary experiments also determined that cholesterol, an important component of lipid rafts, distributed mostly with low-density fractions 2 to 4 (here defined as lipid rafts). As shown in Fig. 3A, ecto-5'-nucleotidase and flotillin-1, two independent markers of lipid rafts, were found predominantly in low-density fractions. On the other hand, α-tubulin (disassembled to monomers under low-temperature conditions) and adenosine kinase were distributed predominantly to fractions 5 to 7, which represent Triton X-100 soluble cell extracts (not shown). Vimentin,
beta-actin, and lamin B1 were distributed mostly to fractions 5 to 10, which represent heavier Triton X-100–soluble and Triton X-100–insoluble (cytoskeletal) fractions of cell lysate (not shown).

Among signaling membrane-associated proteins, src-family members, with the exception of c-yes, distributed almost exclusively to lipid rafts (Fig. 3A). In contrast, protein kinase Ca was found exclusively in soluble cell compartment and neither associated with lipid rafts nor with insoluble cytoskeleton (Fig. 3A). CD44 and Gai2, known lipid raft components, also distributed almost exclusively to lipid rafts. On the other hand, Gas and Gp2 distributed in significant part to soluble and cytoskeletal compartments (Fig. 3B). Integrin beta1, EGFR, and OB-cadherin proteins highly expressed in MDA-MB-231 cells were distributed in all three cell compartments and FAK was found only in soluble and cytoskeletal fractions. Analysis of cell lysates from ER(+) breast cancer MCF-7 cells that express very low levels of ecto-5'-nucleotidase also showed lipid raft distribution of this protein, along with glycosylphosphatidylinositol-linked CD24, src, lyn, and Gai, suggesting that this distribution is cell type independent (not shown).

Previous studies showed that histone deacetylase inhibitors caused shift in expression pattern of selective protein groups. In breast cancer cells, trichostatin A has been shown to induce the expression of ERa groups. In breast cancer cells, trichostatin A has been shown to cause shift in expression pattern of selective protein distribution. In breast cancer cells, trichostatin A has been shown to cause shift in expression pattern of selective protein distribution (not shown). However, epithelial markers, such as cytokeratins (detectable with K8-13 antibody), E-cadherin, ERalpha, EBPs0, and gelsolin, were up-regulated. The expression of CD24, another marker of lipid rafts in ER(+) cells, was also significantly increased. The expression of several other proteins, including lyn (not shown) and vimentin, was only slightly down-regulated; however, at 48 hours of drug treatment, there was increased incidence of apoptosis that may have masked further changes in gene expression. Along with the complete disappearance of CD44 standard form, we have noticed an appearance of a higher molecular form, probably a splice variant (18), that was also seen in untreated MDA-MB-468 cells (Fig. 1A).

Because ecto-5'-nucleotidase, the major adenosine-producing enzyme in epithelial cells (19), was dramatically down-regulated in ER(−) cells exposed to trichostatin A (Figs. 1A and 4A), we tested the expression of adenosine receptors in MDA-MB-231 cells treated with trichostatin A. As shown in Fig. 4B, expression of adenosine receptors was unevenly affected by trichostatin A: Although A2a and A2b were unchanged, the expression of A1 was decreased and A3 was increased during this trichostatin A–induced transition to epithelial phenotype (Fig. 4B). Thus, increased capacity to produce adenosine seem to correlate with increased signaling through A1 receptors in aggressive breast cancer cells and this feature is reversed by histone deacetylase inhibitors.

**Discussion**

Analysis of lipid components of membranes in breast cancer cells was done previously and revealed significantly altered ratio of phospholipids (3), increased level of gangliosides, and components of lipid rafts (4–6) in ER(−) cells. Furthermore, increased circulating levels of gangliosides were found in breast cancer patients when compared with healthy individuals (7). Although association of few

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**Figure 3.** Distribution of proteins from MDA-MB-231 cells after density gradient centrifugation. A and B, analysis of proteins associated with lipid rafts and cytoskeleton in MDA-MB-231 cells. Other conditions as in Fig. 1.
Our analysis of the expression of membrane, cytoskeletal, and associated proteins in MDA-MB-231 cells were treated with 0.5 μmol/L trichostatin A for 48 h, harvested, and processed as described in Materials and Methods. Note that some Western blots were exposed much longer than shown in Fig. 1, which caused some proteins, such as cytokeratins, EBP50, or gelsolin, to be detected in control MDA-MB-231 cells. Each experiment was repeated at least twice with similar result. A, effect of trichostatin A on the expression of selected membrane, cytoskeletal, and associated proteins in MDA-MB-231 cells. Cells were treated with 0.5 μmol/L trichostatin A for 48 h, harvested, and processed as described in Materials and Methods. Note that some Western blots were exposed much longer than shown in Fig. 1, which caused some proteins, such as cytokeratins, EBP50, or gelsolin, to be detected in control MDA-MB-231 cells. Each experiment was repeated at least twice with similar result. B, effect of trichostatin A on the expression of adenosine receptor mRNAs determined by reverse transcription-PCR (40 cycles). The sizes of PCR products are as follows: AR1 344 bp, AR2a 305 bp, AR2b 374 bp, AR3 352 bp, glyceraldehyde-3-phosphate dehydrogenase 983 bp, and trypsin inhibitor 407 bp.

Figure 4.

We found that several membrane proteins residing in lipid rafts are differentially expressed in invasive breast cancer cells, suggesting that there is a major remodeling of this membrane microdomain during breast cancer progression. Glycosylphosphatidylinositol-linked proteins are typically considered markers of lipid rafts. In our study, they show most dramatic shift in expression profile. The complete down-regulation of CD24 in ER(−) cells and the emergence of ecto-5′-nucleotidase and alkaline phosphatase most likely have physiologic consequences. CD24 is a new marker for breast and ovarian carcinoma (28, 29) that coincides with ER(+) status (30). This mucin-like heavily glycosylated protein was shown to be a ligand for P-selectin and proposed to mediate rolling in endothelium (31). On the other hand, ecto-5′-nucleotidase and alkaline phosphatase, which seem to replace CD24 during breast cancer progression, both have phosphohydrolase activity and participate in dephosphorylation of extracellular nucleotides (mostly AMPs) and generation of signaling adenosine (32). Adenosine, the product of both ecto-5′-nucleotidase and alkaline phosphatase enzymatic activities (19), acting through a family of receptors, has well-established roles in adhesion, growth regulation, vasodilation, and angiogenesis. These activities are likely to be relevant for breast cancer progression (reviewed in refs. 32, 33). Although ecto-5′-nucleotidase was also reported to participate in cell adhesion (34, 35), it is at present unclear how ecto-5′-nucleotidase would specifically contribute to invasive cell behavior. Nonetheless, increased capacity to generate adenosine on one hand, and increased expression of A1 and decreased expression of A3 on the other hand, support the view that adenosine signaling may have distinct roles in epithelial and mesenchymal cells. Recent demonstration of increased ecto-5′-nucleotidase expression and adenosine formation upon wnt-1 pathway activation further collaborate this notion (36).
Inhibitors of histone deacetylases, including trichostatin A, have potent effects on global gene expression, inhibit cell proliferation, and induce differentiation and apoptosis (37–39). Although histone deacetylase inhibitors caused changes in many different cell types, mesenchymal cells were proposed to be particularly sensitive (40). In particular, previous studies have shown that MDA-MB-231 cells induced the expression of E-cadherin and ERα after treatment with histone deacetylase inhibitor trichostatin A (16). Given the known genome-wide effects of histone deacetylase inhibitors, such response suggested that trichostatin A may have induced differentiation, which in the context of mesenchymal cells may be regarded as mesenchymal to epithelial transdifferentiation. Indeed, the expression of most markers that differentiate ER(−) and ER(+) cells were affected in a reciprocal manner. Thus, our expression profiling of a broader set of membrane and cytoskeletal proteins provide a strong evidence for the mesenchymal to epithelial transdifferentiation after treatment of breast cancer cells of mesenchymal phenotype with trichostatin A. Previous studies have shown that histone deacetylase inhibitors increased the expression of metastasis suppressors, such as breast metastasis suppressor I (41), tissue inhibitor of metalloproteinase 3 (42), and nm23 (43) and thereby may have therapeutic activity in metastatic phase of breast and other carcinomas. In this context, our results suggest that such antimetastatic activity, at least in the subset of ER(−) breast cancer cells, may be due to the reversion of intrinsically invasive and metastatic mesenchymal phenotype.

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References


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