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Protein Kinase C Induces Motility of Breast Cancers by Upregulating Secretion of Urokinase-Type Plasminogen Activator through Activation of AP-1 and NF-κB

Daniel Sliva,*[,1] Denis English,†[,]‡ Denise Lyons,* and Frank P. Lloyd, Jr.*
**Cancer Research Laboratory and †Experimental Cell Research Program, Methodist Research Institute, Clarian Health Partners Incorporated, Indianapolis, Indiana 46202; and ‡School of Allied Health Sciences, Indiana University School of Medicine, Indianapolis, Indiana 46202*

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Cell migration is a crucial process in cancer metastasis that does not require extracellular matrix degradation—a characteristic of cell invasion. The urokinase-type plasminogen activator (uPA) system is responsible for invasion through uPA enzymatic activity and for migration through the binding of uPA to the uPA receptor (uPAR). Constitutively high levels of uPA are characteristic of the highly metastatic breast cancer cells MDA-MB-231, but the mechanisms underlying constitutive uPA expression are not fully characterized. In this report we show that inhibition of protein kinase C (PKC) represses constitutive (nonstimulated) migration of MDA-MB-231 cells. Bisindolylmaleimide I (Bis I) inhibits cell migration and constitutive activation of transcription factors AP-1 and NF-κB, suggesting that PKC is responsible for increased migration of MDA-MB-231 cells. It is clear that the inhibition of PKC occurs at the transactivation levels of AP-1 and NF-κB because Bis I did not affect constitutive DNA binding of AP-1 and NF-κB. Furthermore, we show that Bis I did not affect the levels of IκBα, suggesting that PKC-mediated cell migration is IκBα independent. Finally, we demonstrate that constitutive secretion of uPA is repressed by Bis I, implying an important role for AP-1 and NF-κB in cell migration. Our data demonstrate a connection among PKC, constitutively active AP-1 and NF-κB, constitutive secretion of uPA, and cell migration of highly invasive breast cancer cells. Thus, PKC controls cell motility by regulating expression of uPA through the activation of AP-1 and NF-κB. The disruption of PKC, AP- 1, and NF-κB signaling in breast cancer may be used to develop therapies for breast cancer prevention and intervention by reducing the secretion of uPA.

[1] To whom correspondence and reprint requests should be addressed at Cancer Research Laboratory, Methodist Research Institute, 1633 North Capitol Avenue, MT 350, Indianapolis, IN 46202. Fax: (317) 962-7468. E-mail: dsliva@clarian.org.

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Tumor invasion and metastasis are multifaceted processes involving cell adhesion, proteolytic degradation of tissue barriers, and cell migration (1). Several types of proteolytic enzymes are responsible for the degradation of the extracellular matrix, namely, matrix metalloproteinases (MMPs), cysteine proteases, and serine proteases (2–4). The urokinase-type plasminogen activator (uPA) is a serine protease that cleaves the extracellular matrix and stimulates the conversion of plasminogen to plasmin (5). Plasmin can mediate invasion directly by degrading matrix proteins such as collagen IV, fibronectin, and laminin or indirectly by activating matrix metalloproteinases MMP-2, -3, and -9 and uPA (6–9). Furthermore, uPA is also involved in cell adhesion and migration (5). Increased expression and secretion of uPA characterizes the malignant phenotype of cells, and overexpression of uPA in breast cancers is a strong indicator of poor prognosis (10, 11). Expression of uPA is controlled by a variety of extracellular signals, such as phorbol ester, the oncogenes v-Src and v-Ras, growth factors, cytokines, protein kinase C, and others (for review, see Ref. 12).

The importance of PKC expression in breast cancer was recently demonstrated, and PKC activity has been shown to be higher in breast cancers than in normal breast tissue (13, 14). Furthermore, high levels of PKCα and -γ were correlated with enhanced uPA secretion in estrogen receptor-negative, highly invasive, and chemotherapy-resistant MDA-MB-231 breast cancer cells (15). Transfection of poorly invasive, nonmetastatic MCF-7 breast cancer cells with the PKCα gene resulted in more aggressive neoplastic phenotype, and the induction of PKC with PMA in MCF-7 cells re-

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sulted in increases in both invasiveness and uPA expression (16, 17).

Expression of the uPA gene is regulated through the uPA promoter, which contains functional binding sites for the transcription factors AP-1, NF-κB, and PEA3 (18, 19). Constitutive activation of NF-κB and AP-1 has been detected in some breast cancer cell lines (20–22), and NF-κB can further induce transcription of AP-1-regulated genes through the interaction of NF-κB with AP-1 (23). Furthermore, we have recently demonstrated that phosphatidylinositol 3′-kinase (PI3K) and NF-κB, both of which are constitutively active, regulate migration of breast cancer cells by the secretion of uPA (24).

In this report we show that the migration of the highly invasive breast cancer cells MDA-MB-231 is repressed by the inhibition of PKC. The inhibition of PKC resulted in a decrease in constitutive activation of AP-1 and NF-κB in the reporter gene assay, whereas DNA binding of NF-κB and AP-1 was not affected. In addition, inhibition of PKC suppressed the secretion of uPA from MDA-MB-231 cells. Our data suggest that secretion of uPA is regulated by PKC through the constitutively active transcription factors AP-1 and NF-κB and that this signaling pathway is responsible for the high migratory potential of breast cancer cells.

MATERIALS AND METHODS

Cell culture and materials. The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from ATCC (Manassas, VA), and were maintained as previously described (24). Media and supplements came from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Transwell polycarbonate chambers (8-μm pore size) came from Costar (Cambridge, MA). Bis I, Gö6976, and calphostin C were obtained from Calbiochem (San Diego, CA).

Cell migration assay. MDA-MB-231 cells were harvested and preincubated with PKC inhibitors, as indicated below. Chemokinesis was assessed and analyzed as previously described (25). Data points represent averages ± SD of 3 individual filters within one representative experiment repeated at least twice.

DNA transfection and chloramphenicol acetyltransferase (CAT) assay. MDA-MB-231 cells were transfected with the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions with the NF-κB-CAT and AP-1-CAT reporter constructs, and with β-galactosidase expression vector pCH110 (gifts from Dr. H. Nakshatri, Indiana University School of Medicine, Indianapolis, IN) (21). Forty-eight hours after transfection cells were treated with PKC inhibitors for 6 h at 37°C. Cells were harvested and cell extracts were prepared, and used in a liquid CAT assay with [^{14}C]chloramphenicol as described (24). Data points represent averages ± SD of three to six independent transfection experiments.

Gel electrophoretic mobility shift assay (GEMSA). Nuclear extracts were prepared as previously described (26). Oligonucleotide probes containing consensus sequences for NF-κB and AP-1 binding sites were purchased from Promega Corp. (Madison, WI). A gel electrophoretic mobility shift assay (GEMSA) was performed with ^{32}P-labeled NF-κB or AP-1 probes according to the manufacturer's instructions (Promega Corp.).

Expression of IκBα and uPA secretion. MDA-MB-231 cells were treated with PKC inhibitors for 6 h, and whole cell extracts were prepared as described previously (27). IκBα and actin expression was determined in the extracts (25 μg) by Western blot analysis (24). The secretion of uPA was determined in concentrated DMEM medium from MDA-MB-231 cells untreated or treated with PKC inhibitors I for 48 h as described (24).

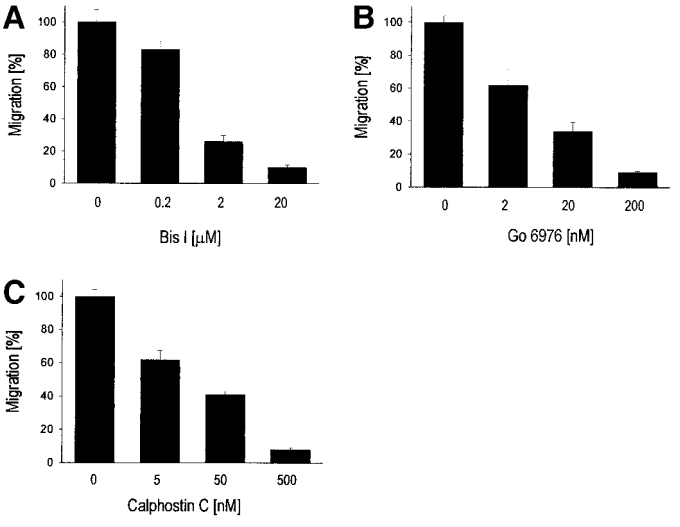

FIG. 1. Migration of MDA-MB-231 cells is repressed by the inhibition of PKC. MDA-MB-231 cells were harvested and preincubated with (A) 0–20 μM Bis I, (B) 0–200 nM Gö6976, or (C) 0–500 nM calphostin C for 1 h at 37°C. Migration was assessed as described under Materials and Methods. Data points represent averages ± SD of three individual filters within one representative experiment repeat at least twice.

RESULTS

Inhibition of PKC Represses Migration of MDA-MB-231 Cells

We have previously shown that constitutively active PI3K is responsible for the increased migratory potential of the highly invasive and chemotherapy-resistant breast cancer cells MDA-MB-231 (24). Because MDA-MB-231 cells demonstrated a significantly higher level of PKC activity than poorly invasive MCF-7 cells (16), we examined whether spontaneous cell migration is directly linked to the increased activity of PKC. MDA-MB-231 cells were pretreated for 1 h with the PKC inhibitors Bis I (0–20 μM), Gö6976 I (0–20 μM), and calphostin C (0–500 nM), and cell motility was determined after an additional 3 h of incubation. As shown in Fig. 1, Bis I, Gö6976, and calphostin C inhibited migration of MDA-MB-231 cells in a dose-dependent manner. Thus, inhibition of PKC suppresses spontaneous migration of MDA-MB-231 cells.

PKC Controls Transactivation of NF-κB and AP-1 in MDA-MB-231 Cells

PKC has been shown to be involved in the activation of NF-κB in different systems by a variety of stimuli (28–31). Because NF-κB stimulated migration of MDA-MB-231 cells, and NF-κB induced transcription

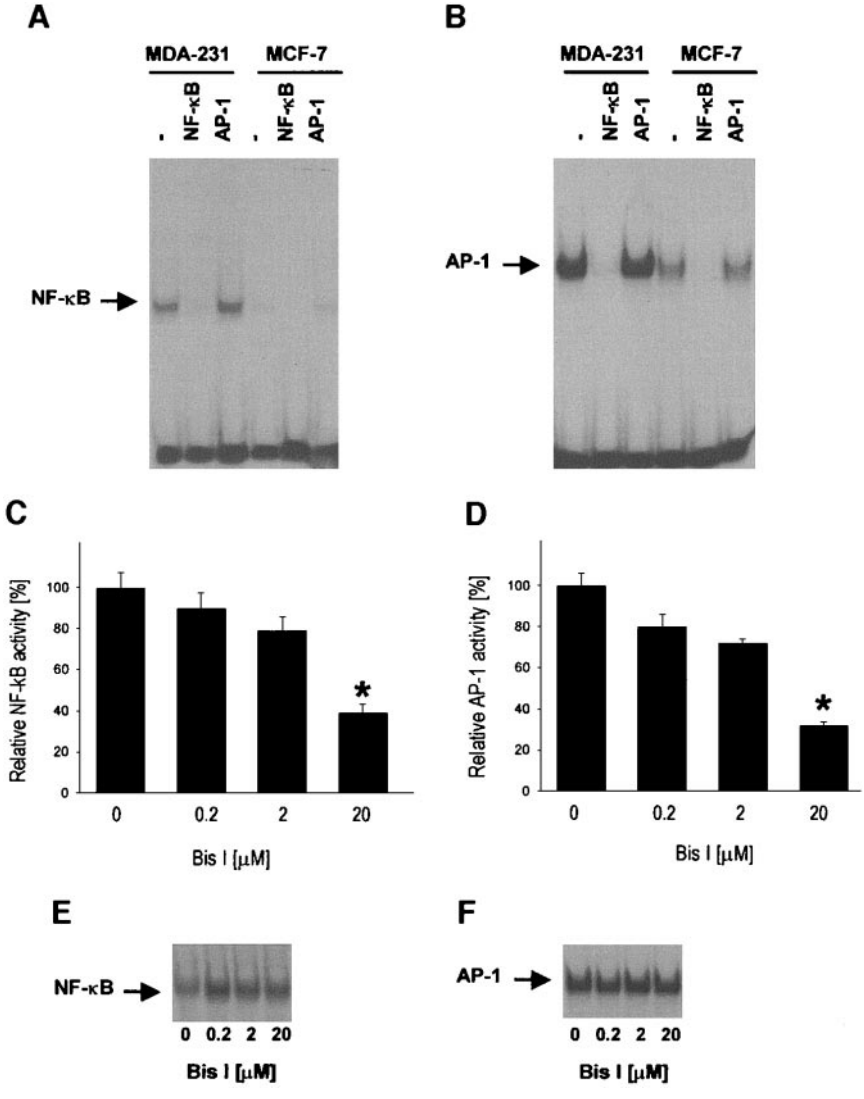

FIG. 2. DNA-binding and transactivation of NF-κB and AP-1 in MDA-MB-231 cells treated with Bis I. DNA-binding in MDA-MB-231 and MCF- cells. Nuclear extracts were incubated with a (A) ^{32}P-labeled NF-κB probe or (B) a ^{32}P-labeled AP-1 probe in the presence or absence of cold NF-κB and AP-1 oligonucleotides as indicated. Gel electrophoretic mobility shift assays were performed as described under Materials and Methods. The specific NF-κB and AP-1 complexes are indicated by an arrow. Transactivation of NF-κB and AP-1 CAT. MDA-MB-231 cells were transfected with (C) 1 μg NF-κB-CAT or (D) 2.5 μg AP-1-CAT reporter constructs and 3 μg β-galactosidase plasmid. Forty-eight hours after transfection, the cells were treated with 0–20 μM Bis I for 6 h at 37°C, and CAT activity in equal number of β-galactosidase units was determined. Data are means ± SD of triplicate determinations, $*P < 0.001$. Similar results were obtained in at least two to five additional experiments. DNA-binding after Bis I treatment. MDA-MB-231 cells were treated with 0–20 μM Bis I for 6 h at 37°C. Nuclear extracts were prepared and incubated with (E) a ^{32}P-labeled NF-κB probe or (F) a ^{32}P-labeled AP-1 probe. Gel electrophoretic mobility shift assays were performed as described above. The specific NF-κB and AP-1 complexes are indicated by an arrow.

of AP-1-regulated genes after the binding of NF-κB to AP-1 (23, 24), we were interested in whether PKC is involved in the constitutive activation of NF-κB and AP-1. To confirm that NF-κB and AP-1 are constitutively active in breast cancer cell lines, we prepared and incubated nuclear extracts from MDA-MB-231 and MCF-7 cells with specific probes for NF-κB and AP-1. Gel shift analysis showed that nuclear extracts from MDA-MB-231 have higher constitutive DNA-binding activity of NF-κB and AP-1 compared to nuclear extracts from MCF-7 cells (Figs. 2A and 2B). Thus, we hypothesized that inhibition of PKC would abolish constitutive activation of NF-κB and AP-1 in MDA-MB-231 cells.

Cells were transiently transfected with a reporter NF-κB-CAT or AP-1-CAT plasmids and treated with Bis I 48 h after transfection. Exposure of MDA-MB-231 cells to increased concentrations of Bis I (0–20 μM) significantly inhibited constitutive transactivation of NF-κB and AP-1, as assessed by the CAT-reporter gene assay (Figs. 2C and 2D). To compare the inhibition of constitutive transactivation of NF-κB and AP-1 with

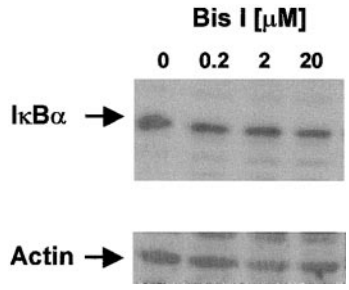

FIG. 3. Bis I does not alter the expression of IκBα in MDA-MB-231 cells. MDA-MB-231 cells were treated with 0–20 μM Bis I for 6 h at 37°C. Cell extracts were subjected to Western blot analysis with anti-IκBα antibody. The identical blot was reprobed with anti-actin antibody. Results are representative of three separate experiments.

their respective DNA-binding activity, we analyzed nuclear extracts from MDA-MB-231 cells treated with Bis I by gel shift analysis. Surprisingly, Bis I which inhibited constitutive activation of NF-κB and AP-1 in a reporter gene assay, did not affect DNA-binding of NF-κB (Fig. 2E) or AP-1 (Fig. 2F). Therefore, it is clear that the inhibition of NF-κB and AP-1 occurs mostly at the transactivation level because the constitutive DNA-binding activity of NF-κB and AP-1 was not affected.

PKC Inhibition of Constitutive NF-κB Activation Is IκBα Independent

We have recently shown that overexpression of IκBα abolishes cell motility and constitutive NF-κB activation in MDA-MB-231 cells (24). Therefore, we investigated whether the inhibition of PKC would directly affect the levels of IκBα and would repress cell migration by sequestering NF-κB in cytoplasm. MDA-MB-231 cells were treated with Bis I (0–20 μM), and the cell lysates were subjected to SDS–PAGE and Western blot analysis with IκBα antibody. Despite the dramatic decrease in cell motility and constitutive NF-κB activation, which implies an increase in the expression of IκBα, inhibition of PKC did not change the levels of IκBα (Fig. 3). These results suggest that PKC controls cell motility and activation of NF-κB in MDA-MB-231 cells by a distinct signaling pathway, which is independent of IκBα.

Inhibition of PKC Represses Constitutive Expression of uPA in MDA-MB-231 Cells

Given that uPA is directly involved in migration of breast cancer cells and inhibition of PI3K suppresses uPA secretion (24), we were interested whether the inhibition of PKC would have the same effect on the secretion of uPA as the inhibition of PI3K. As shown above, our data demonstrate that by inhibiting PKC, we can inhibit constitutive activation of AP-1 and NF-κB and cell motility of MDA-MB-231 cells. Therefore, we hypothesize that by inhibiting AP-1 and NF-κB in the promoter region of uPA we will repress the expression of uPA, which is responsible for the migration of MDA-MB-231 cells. Media from MDA-MB-231 cells treated with increased concentrations of Bis I (0–20 μM) were collected, concentrated, and subjected to Western blot analysis with anti-uPA antibody. As seen in Fig. 4, constitutive secretion of uPA from MDA-MB-231 cells was significantly repressed with PKC inhibitor Bis I in a dose-dependent manner.

Taken together, our data indicate that constitutive expression and secretion of uPA, which is responsible for the high migratory potential of breast cancer cell line MDA-MB-231 can be suppressed by the inhibition of PKC.

DISCUSSION

We have recently demonstrated that constitutively active PI3K is responsible for the enhanced motility of the highly invasive human breast cancer cells MDA-MB-231 through the transactivation of NF-κB and uPA secretion (24). Because PKC is downstream of PI3K in a proposed signaling pathway of EGF-induced cell proliferation in MDA-MB-231 cells (32), we were interested in whether the inhibition of PKC would suppress cell migration by the same mechanism as PI3K. In the present study, we show that PKC is responsible for the enhanced motility of human breast cancer cells MDA-MB-231. We demonstrate that inhibition of PKC reduced constitutive transactivation of AP-1 and NF-κB and repressed the secretion of uPA and cell migration. The results suggest that, by activating AP-1 and NF-κB, PKC induces the expression and secretion of uPA, which is responsible for the augmented motility of MDA-MB-231 cells.

The role of constitutively active PKC in the invasiveness of cancer cells is well established. A variety of PKC isoforms, Ca^{2+}-dependent (PKCα and -γ) and Ca^{2+}-independent (PKCμ), have been linked to the invasive potential of breast cancer cells (33, 34). In addition, total cytosolic PKC activity and uPA secretion are significantly increased in breast cancer cells with a metastatic phenotype (16). In our study, we show that inhibition of PKC inhibited the migration of MDA-MB-

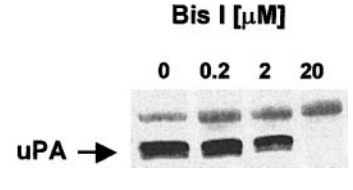

FIG. 4. Bis I inhibits constitutive secretion of uPA from MDA-MB-231 cells. Medium from MDA-MB-231 cells treated with 0–20 μM Bis I for 48 h was concentrated as described under Materials and Methods, and secretion of uPA was detected by Western blot analysis with anti-uPA antibody. Results are representative of three separate experiments.

231 cells. It is clear that the inhibition of migration is mediated through the transcription factors AP-1 and NF-κB because PKC inhibitor Bis I suppressed constitutive activation of AP-1 and NF-κB in a reporter gene assay. Although both nonmetastatic MCF-7 cells and highly invasive MDA-MB-231 cells showed constitutive DNA-binding activity of AP-1 with significantly higher activity in MDA-MB-231 cells, the DNA binding of AP-1 was not affected by the inhibition of PKC. In addition, constitutive DNA-binding activity of NF-κB in highly invasive MDA-MB-231 cells was not affected by the inhibition of PKC. Therefore, the effect of PKC inhibition on transcription factors AP-1 and NF-κB occurs mostly at the transactivation level because the constitutive DNA-binding activity of AP-1 and NF-κB was not affected by Bis I treatment. A decrease in NF-κB transcriptional activity independent of DNA-binding activity in MDA-MB-231 cells was recently reported by us and others (24, 35).

While Bis I, Gö6976 and calphostin C inhibit cell migration, only Bis I inhibits transactivation of AP-1 and NF-κB and uPA secretion (Figs. 2C and 2D, Fig. 4, and data not shown), suggesting that specific isoforms of PKC are responsible for distinct pathways. While calphostin C, a specific inhibitor of PKC, is nonselective, Bis I and Gö6976 are more selective (36). Therefore, the inhibition of PKCα by both Bis I and Gö6976 repressed cell migration, while constitutive transactivation of AP-1 and NF-κB and uPA secretion was reduced only Bis I treatment. Because Bis I inhibits the atypical PKCζ (36), and both PKCα and PKCζ are expressed in MDA-MB-231 cells (15), we hypothesize that PKCζ is responsible for the activation of AP-1 and NF-κB. Furthermore, the involvement of PKCζ in the transcriptional activity of NF-κB has been recently demonstrated (37, 38). In our study, significant inhibitory effects of Bis I were found at concentrations of 2 to 20 μM, which is consistent with the reported inhibitory constant (IC_{50} = 5.8 μM) for PKCζ (36). Therefore, our data suggest that although both PKCα and PKCζ are involved in cell migration of breast cancer cells, transactivation of AP-1 and NF-κB is regulated by PKCζ alone. Alternatively, other signaling molecules can be involved in cell migration and transactivation of AP-1 and NF-κB, since Davies *et al.* (39) recently showed that Bis I can inhibit other protein kinases such is MAPKAP-K1b, MSK1, and S6K1.

Transcription factors AP-1 and NF-κB control the expression of uPA, and highly invasive breast cancer cells show increased constitutive expression and secretion of uPA (16, 40). Cell migration does not require extracellular matrix degradation, which is a characteristic of cell invasion, although cell migration is a basic step in metastatic spread. Because uPA is responsible for extracellular matrix degradation and also for cell migration through the interaction with uPA receptor (uPAR) (5), the signaling pathways involved in cell migration are of particular interest. We have recently shown that nonmetastatic MCF-7 cells do not secrete uPA and that constitutive uPA secretion from MDA-MB-231 was suppressed by the PI3K and NF-κB inhibitors (24). In the present study we demonstrate the inhibition of constitutive uPA secretion by PKC inhibitor Bis I. Therefore, PKC is responsible for the constitutive activation of AP-1 and NF-κB, which regulate expression and secretion of uPA, resulting in the high migratory potential of breast cancer cells. Our data are in accord with other studies, suggesting AP-1 or NF-κB as potential targets for the cancer treatment (41, 42).

In summary, constitutively active PI3K activates PKC, which is responsible for the constitutive activation of AP-1 and NF-κB, which in turn regulate expression and secretion of uPA, resulting in the high migratory potential of breast cancer cells. Therapeutic interventions able to disrupt PI3K, PKC, and AP-1 and NF-κB signaling may decrease uPA secretion with subsequent reduction of the invasive and metastatic potential of breast cancers.

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