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PRECLINICAL STUDY

Inhibition of breast cancer cell migration by activation of cAMP signaling

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Abstract Almost all deaths from breast cancer arise from metastasis of the transformed cells to other sites in the body. Hence, uncovering a means of inhibiting breast cancer cell migration would provide a significant advance in the treatment of this disease. Stimulation of the cAMP signaling pathway has been shown to inhibit migration and motility of a number of cell types. A very effective way of selectively stimulating cAMP signaling is through inhibition of cyclic nucleotide phosphodiesterases (PDEs). Therefore, we examined full expression profiles of all known PDE genes at the mRNA and protein levels in four human breast cancer cell lines and eight patients' breast cancer tissues. By these analyses, expression of almost all PDE genes was seen in both cell lines and tissues. In the cell lines, appreciable expression was seen for PDEs 1C, 2A, 3B, 4A, 4B, 4D, 5A, 6B, 6C, 7A, 7B, 8A, 9A, 10A, and 11A. In patients' tissues, appreciable expression was seen for PDEs 1A, 3B, 4A, 4B, 4C, 4D, 5A, 6B, 6C, 7A, 7B, 8A, 8B, and 9A. PDE8A mRNA in particular is prominently expressed in all cell lines and patients' tissue samples examined. We show here that stimulation of cAMP signaling with cAMP analogs, forskolin, and PDE inhibitors, including selective inhibitors of PDE3, PDE4, PDE7, and PDE8, inhibit aggressive triple negative MDA-MB-231 breast cancer cell migration. Under the same conditions, these agents had little effect on breast cancer cell proliferation. This study demonstrates that PDE inhibitors inhibit breast cancer cell migration, and thus may be valuable therapeutic targets for inhibition of breast cancer metastasis. Since PDE8A is expressed in all breast cancer samples, and since dipyridamole, which inhibits PDE8, and PF-04957325, a selective PDE8 inhibitor, both inhibit migration, it suggests that PDE8A may be a valuable novel target for treatment of this disease.

Keywords Breast cancer · Cyclic nucleotide phosphodiesterase · Cell migration · cAMP signaling

Introduction

Deaths from breast cancer almost always arise from metastasis of the transformed cells to other sites in the body [1]. Hence, uncovering a means of inhibiting breast cancer metastasis would provide a significant advance in the treatment of this disease. Stimulation of cAMP signaling has been shown to inhibit migration and motility of a number of cell types, including fibroblasts [2], epithelial cells [3], endothelial cells [4], melanoma cells [5], colon cancer cells [6], pancreatic cancer cells [7, 8], bladder cancer cells [9], and cervical cancer cells [10]. Selective elevation of cAMP in breast cancer cells could, therefore, inhibit migration and metastasis of breast cancer cells, and thereby provide an effective means to treat this disease, either alone, or in combination with other established treatments. It is now appreciated that cAMP signaling is compartmentalized in cells, primarily through selective expression of PDEs [11–14]. PDEs can be anchored to

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signaling complexes at specific intracellular locations so as to achieve targeted cAMP degradation and the creation of localized intracellular cAMP gradients. This allows specific PDE isoforms to control specific cellular functions. Thus alterations in the expression of particular PDE forms will confer specific functional changes and may contribute to underlying molecular pathologies of cellular responses to protect against detrimental inputs. There is tremendous multiplicity of PDEs. They comprise a superfamily of related enzymes encoded by 21 different genes, grouped into 11 different gene families (PDEs 1–11), based on similarity of sequence, mode of regulation, and preference for cAMP and/or cGMP as substrate [15–18]. With the existence of multiple transcription initiation sites, as well as alternatively spliced forms of many of these PDE genes, more than 100 different forms of PDE have been identified. A detailed analysis of PDE expression in human breast cancer tissues has not been reported but analysis of PDE activity in the human breast cancer cell line MCF-7 showed activity corresponding to the PDE4 gene family to be predominantly expressed [19]. It was also shown that under certain conditions treatment of MCF-7 cells with the PDE4 selective inhibitors, DC-TA-46 and rolipram inhibit their growth [19, 20] and that isobutylmethylxanthine (IBMX) and rolipram inhibit lysophosphatidic acid-induced chemotactic migration of MDA-MB-435 cells [21]. Additionally, the combination of forskolin and the PDE4 inhibitor, CI-1044, was shown to reduce SK3-dependent Ca^{2+} entry and inhibit migration of MDA-MB-435 cells [22].

Cell motility and migration involves the extension of a leading edge protrusion or lamellipodium, the establishment of new adhesion sites at the front, cell body contraction, and detachment of adhesions at the rear [23, 24]. All these steps involve the assembly, the disassembly, or the reorganization of the actin cytoskeleton. Rho GTPases are one group of proteins that has clearly been shown to play a pivotal role in regulating this process [23, 24]. Rho GTPases are activated by many different stimuli, with integrin clustering or engagement being a main stimulus. One of the principal functions of Rho is to promote the formation of stress fibers in the cell. Stress fiber formation is regulated by the state of myosin light chain (MLC) phosphorylation. Phosphorylation of MLC allows myosin to interact with actin to produce contractility. Phosphorylation of MLC is regulated both by the Ca^{++}-calmodulin-dependent myosin light chain kinase, and also by Rho kinase (ROCK), a downstream effector of Rho. Additionally, ROCK also phosphorylates MLC phosphatase, preventing MLC dephosphorylation. Rho can be directly phosphorylated and inhibited by cAMP-dependent protein kinase (PKA) [25], and it was shown that PDE4 inhibitors profoundly inhibit the migration of fibroblasts through PKA-mediated inhibition of RhoA, a major isoform of Rho, preventing the activation of ROCK and stress fiber formation [2]. In breast cancer cells, RhoA is activated by engagement of the α6β4 integrin receptor and this activation of RhoA in breast cancer cells appears to be critical for its motility and function [21, 26]. Specifically it was shown that lamellae formation and chemotactic migration of MDA-MB-435 cells are inhibited or gated by cAMP, and a critical function of the α6β4 integrin is to lower the cAMP concentrations by increasing the activity of PDE4 [21]. Additionally it was shown that the integrin effector, focal adhesion kinase (FAK), forms a complex with RACK1 and PDE4D5, and that this complex acts to recruit specific components of the cAMP signaling system to nascent integrin adhesions and to the leading edge of polarizing cells [27]. Indeed, earlier studies had shown elevated expression of FAK in human breast cancers and the requirement of FAK for breast cancer progression and metastasis [28], which may result from its complex with PDE4D5 and the subsequent modulation of cAMP signaling.

In our previous studies with endothelial cells and T lymphocytes, we showed that PDE8A regulates integrin surface expression, cell adhesion, and cell migration [29, 30]. In our studies presented here, we show the complete expression profile of all known PDE genes at both the mRNA and protein levels in estrogen receptor-positive and estrogen receptor-negative cell lines as well as in patients' breast cancer tissues. We find expression of a considerable number of PDE genes, with expression of PDE8A mRNA, in particular, prominently expressed in all breast cancer cells and tissues examined. We also show that selective inhibition of PDE3, PDE4, PDE7, or PDE8 inhibits breast cancer cell migration. Inasmuch as PDE8A was recently shown to bind in a complex with and regulate raf-1 [31], which is at the apex of the MEK-ERK pathway, controlling many fundamental biological processes including cell proliferation, survival, and transformation, as well as impacting on cell migration, PDE8A in particular may be an important metastatic target.

Materials and methods

Breast cancer cells and tissues

Breast cancer/uninvolved paired tissue array (catalog no. BRC481) was obtained from Pantomics (San Francisco, CA, USA). Tissues were classified as tumor or uninvolved based upon H&E staining and immunohistochemistry using anti-cytokeratin antibody and staged according to the standard TNM classification by a certified pathologist. Human breast adenocarcinoma estrogen receptor-positive MCF-7 and T-47D, and estrogen receptor-negative MDA-

MB-231 and MDA-MB-435 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). There is controversy as to the origin of the MDA-MB-435 cell line in that it is identical to the M14 melanoma cell line at the karyotypic level; however, evidence of secretion of milk proteins and the presence of two X chromosomes from this cell line would indicate that it is derived from the mammary epithelium of a human female thus making it uncertain and controversial as to whether these cells originated from human breast adenocarcinoma or melanoma [32, 33]. Breast cancer tumor tissues for microarray analysis were obtained from the Neag Cancer Center of the University of Connecticut Health Center (UCHC) in a deidentified manner. All procedures involving human tissue were approved by the UCHC Institutional Review Board.

Materials and methods for cell culture, microarray, cell proliferation assay, and immunofluorescence assay, performed as described previously [34–36], as well as the transwell migration assay and wound healing assay are presented in a Supplementary Materials and Methods document.

Results

PDE mRNA expression in breast cancer cell lines

Expression of the mRNA for all PDE genes was examined in the human breast adenocarcinoma estrogen receptor-negative cell lines, MDA-MB-231 and MDA-MB-435, and in the estrogen receptor-positive cell lines, T47D and MCF-7, by microarray analysis and qRT-PCR. Figure 1a shows the expression of mRNA for PDE genes by microarray analysis. Significant expression was seen in at least one or more of these cell lines for PDEs 1C, 2A, 3A, 3B, 4A, 4B, 4D, 5A, 7A, 7B, 8A, 8B, 9A, and 10A (Fig. 1a; Table S2). Significant expression was also seen for the light-activated PDE genes, PDEs 6A, 6B, and 6C, which encode the rod α, rod β, and cone α' PDE6 catalytic subunits, respectively. Further documentation of this PDE6 expression, which was previously thought to be photoreceptor specific, and commentary on its possible function in breast cancer was the focus of a separate report [35]. Figure 1b and Table S3 show the expression of mRNA for PDE genes by qRT-PCR. Except for PDE3A, which showed no expression in any of the cell lines by qRT-PCR, expression of mRNA in at least one or more of these cell lines was seen by qRT-PCR for the same PDE genes as that seen by microarray analysis, although by qRT-PCR the relative abundance of expression appeared greater for some PDE genes than that seen by microarray. PDE8A mRNA in particular appeared to be prominently expressed by both qRT-PCR, as well as by microarray analysis, in all cell lines examined.

Expression of PDE protein in breast cancer cell lines

Using antibodies specific for members of all 11 PDE gene families, the expression and localization of PDE protein was examined in the same four breast cancer cell lines, MDA-MB-231, MDA-MB-435, MCF-7, and T47D. As shown in Fig. 2a, by immunofluorescence, protein expression was seen for PDEs 1C, 3B, 4A, 4B, 4C, 4D, 5A, 7A, 8A, 9A, 10A, and 11A. Expression of PDE6B protein by immunofluorescence and Western immunoblot analysis was reported earlier [35]. Figure 2a also shows that the localization of the expression of these different PDEs within the cells differs greatly from one PDE to another, indicating that different PDEs can localize differently, even within the same cell. PDEs in these breast cancer cells exhibit localizations, as determined by immunofluorescence, as follows: PDE1C shows mostly nuclear staining; PDE3B is mostly perinuclear, with possible localization to golgi; PDE4A appears to be localized to vesicles, such as endosomes or lysosomes; PDE4B shows a diffuse staining pattern; PDE4C appears to be mainly nuclear; PDE4D shows surface blebbing expression and dense nuclear staining; PDE5A appears to be mainly nuclear in MDA-MB-231 and MDA-MB-435 cells, and expressed throughout the cell in the estrogen receptor-positive cells; PDE7A shows punctate staining in vesicles; PDE8A, like PDE5A, appears to be mainly nuclear in MDA-MB-231 and MDA-MB-435 cells, and exhibits a diffuse whole cell distribution in the estrogen receptor-positive cells, although some vesicular expression of PDE8A is seen as well; PDE9A shows diffuse staining throughout most cells; PDE10A also shows diffuse staining throughout most cells; and PDE11A shows dense nuclear staining.

We also examined protein expression of PDEs in breast cancer cells by Western immunoblot analysis. Expression of PDE4 at the protein level had already been shown by activity analysis in MCF-7 and MDA-MB-435 cells [19, 21], and we therefore examined protein expression of several other PDEs that had not been reported before in breast cancer cells. As shown in Fig. 2b, Western blot analysis further shows the expression of PDEs 3B, 7A, and 8A at the protein level in MCF-7 and MDA-MB-231 cells.

Treatment of the MDA-MB-231 cells with PDE inhibitors resulted in a dramatic translocation and sequestration of some PDEs into foci or aggregates, similar to that reported for PDE4A4 in CHO cells [37]. As shown in Fig. 2c, this was seen for PDE4A when treated with the PDE4-selective inhibitor, rolipram, or the nonselective inhibitor, IBMX; for PDE7A when treated with the PDE7-selective inhibitor, spiroquinazolinone, or the nonselective

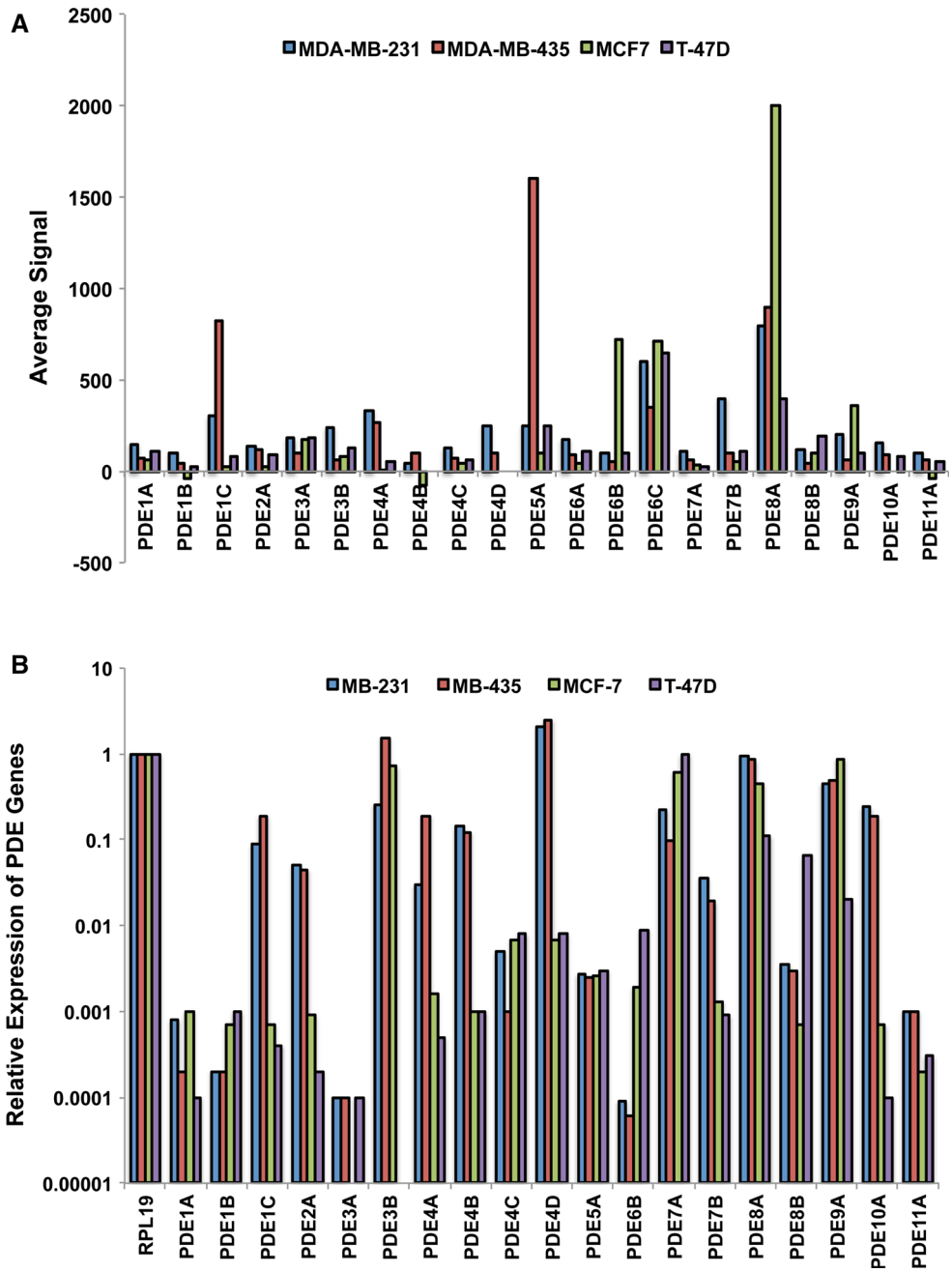

Fig. 1 Expression of PDE mRNA in breast cancer cell lines. **a** Microarray analysis of PDE mRNA expression. **b** qRT-PCR analysis of PDE mRNA expression

inhibitor, IBMX; and for PDE8A when treated with the PDE8-selective inhibitor, PF-04957325, or the nonselective inhibitor, dipyridamole. Some differences exist, however, between these observations in MDA-MB-231 cells and those in CHO cells, in that the redistribution of PDE4A4 into foci in CHO cells occurred upon treatment with rolipram, but not with IBMX [37]. Further studies with the CHO system have shown that the redistribution of PDE4A4 into reversible protein aggregates or foci occurs through its binding to p62, a multi-domain scaffold protein linked to autophagy and proteasome degradation pathways [38–40]. Whether this is also true for the redistribution of these PDEs in the MDA-MB-231 cells will need to be determined.

Microarray analysis of PDE mRNA in patients' breast cancer tissue

PDE gene expression was analyzed by microarray analysis in breast tumor tissue from eight breast cancer patients. As shown in Fig. 3 and Table S4, significant expression was seen in at least one or more of these samples for PDEs 1A,

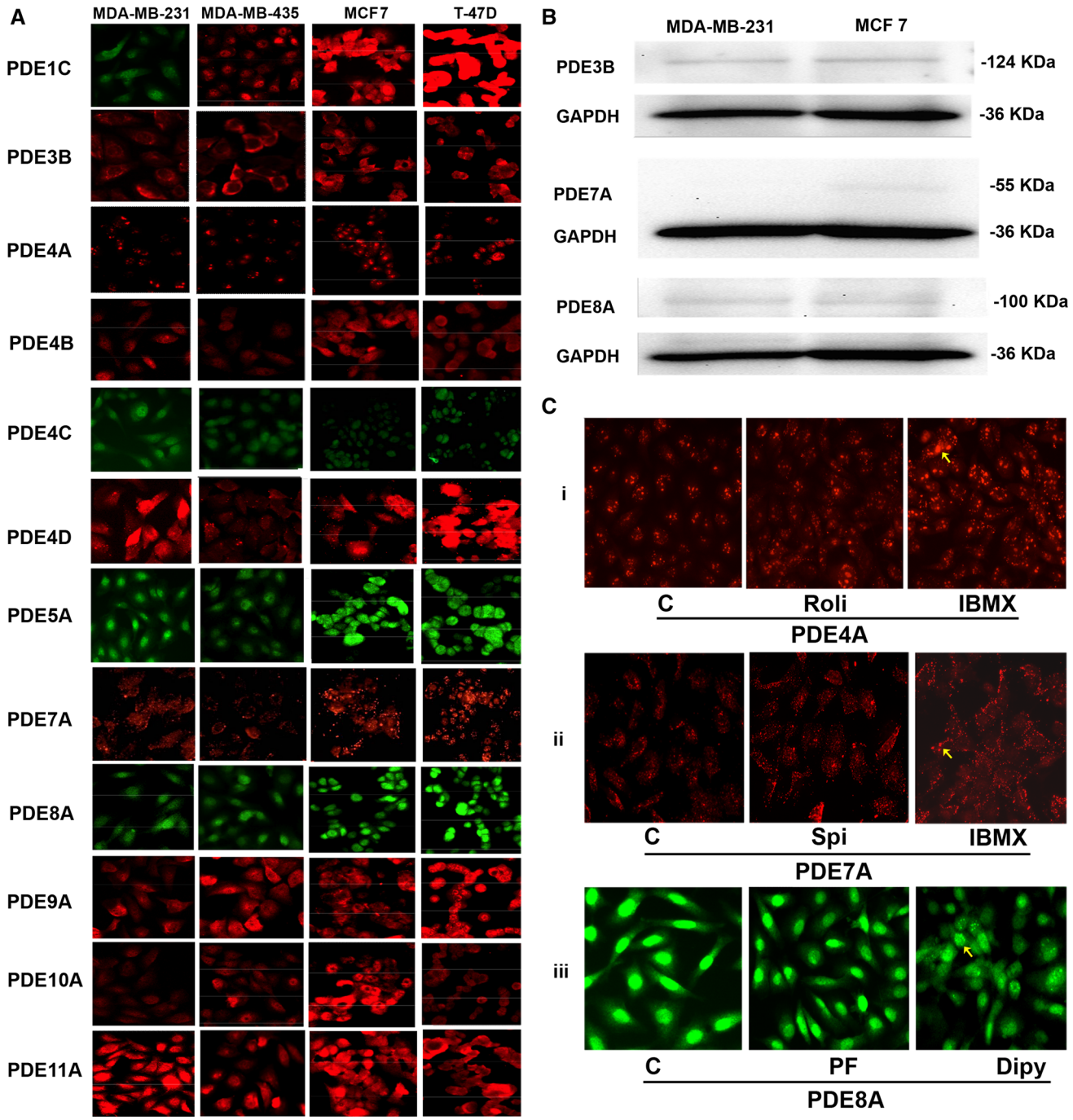

Fig. 2 Expression of PDE protein in breast cancer cell lines by immunofluorescence and Western immunoblot analysis. **a** MDA-MB-231, MDA-MB-435, MCF-7, and T-47D cells were each stained with primary antibodies specific for 12 different PDE genes and secondary Alexa Fluor conjugated antibodies as described in Methods. Results were visualized and photographed by fluorescent microscopy at ×400 magnification. **b** MDA-MB-231 and MCF-7 cells were analyzed for expression of PDEs 3B, 7A, and 8A by Western immunoblot analysis using antibodies specific for each of these PDE genes as described in Methods. **c** MDA-MB-231 cells were treated with vehicle (C) or with PDE inhibitors as indicated for 24 h and stained with anti-PDE antibodies selective for (*i*) PDE4A, (*ii*) PDE7A, and (*iii*) PDE8A; the *arrows* show representative examples of the foci that formed after PDE inhibitor treatment. The PDE inhibitors used were rolipram (Roli) 10 μM, isobutylmethylxanthine (IBMX) 500 μM, spiroquinazolinone (Spi) 1 μM, dipypridamole (Dipy) 100 μM, and PF-04957325 (PF) 10 μM

1B, 1C, 2A, 3A, 3B, 4A, 4B, 4D, 5A, 6A, 6B, 6C, 7A, 7B, 8A, 8B, 9A, and 10A. As observed with the breast cancer cell lines, PDE8A mRNA was also highly expressed in breast cancer tissues as well, from all eight patients, although very high expression of PDE5A mRNA was also seen in two of the eight patients (Fig. 3 insert).

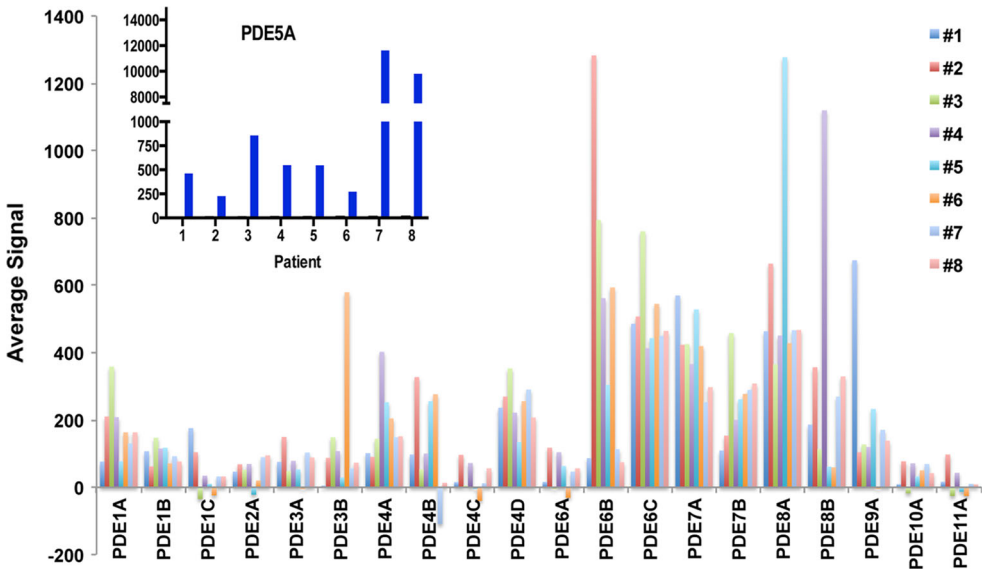

Fig. 3 Expression of PDE mRNA in patients' breast cancer tissues. mRNA expressions for all 21 known PDE genes were analyzed in breast cancer tumor tissues from eight patients by Illumina microarray analysis

Expression of PDE protein in breast cancer tumors

Using PDE gene specific antibodies capable of detecting PDE protein expression by immunohistochemistry (Table S1), the expression of PDE protein was examined in sixteen cases of invasive breast cancer, including ductal, poorly differentiated and lobular carcinoma, and surrounding tissue, by immunohistochemistry. As seen in Fig. 4, considerable expression of PDE protein representing PDEs 3B, 4A, 4B, 4D, 5A, 7A, and 8A was seen in these breast cancer tissue samples, regardless of what type of breast carcinoma they represented. The sixteen samples contained eleven cases of ductal carcinoma, three cases of lobular carcinoma, and two cases of poorly differentiated carcinoma. Figure 4 shows PDE protein expression in a representative example of each of these types of cancer. Considerable PDE expression is seen in all of these, and expression is seen in the uninvolved tissue surrounding these tumors as well. In controls, where primary PDE antibody was omitted, no labeling was seen at all.

Effect of stimulation of cAMP signaling on breast cancer cell migration

In order to test the effects of stimulation of the cAMP signaling pathway on breast cancer cell migration, a transwell assay was set up using MDA-MB-231 cells. Using this assay, serum stimulated the migration of the cells through a collagen-coated 8 μm membrane by 5.5-fold (Fig S1). Agents capable of stimulating cAMP signaling were then tested for their effects on migration using this system. As shown in Fig. 5a1, the cell permeable cAMP analogs, 8-bromo-cAMP (8-Br-cAMP), 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP), and dibutyryl cAMP (db-cAMP), all significantly inhibited MDA-MB-231 breast cancer cell migration by 20–30 %. As shown in Fig. 5a2, the nonselective PDE inhibitors, IBMX and dipyridamole, and selective inhibitors of PDE3 (milrinone), PDE4 (piclamilast and rolipram), PDE7 (spiroquinazolinone), and PDE8 (PF-04957325), all significantly inhibited migration of the cells by 20–50 %, and this inhibition was potentiated by addition of the adenylyl cyclase activator, forskolin, yielding inhibitions in the range of 40–75 %. Also as shown in Fig. 5a2, when dipyridamole and rolipram were added together, the degree of inhibition was additive, and moreover, the inhibition by dipyridamole and rolipram was reversed by the PKA antagonist, Rp-cAMPS, indicating that this inhibition is most likely mediated by PKA.

A second migration assay, a wound healing assay, was also employed. As shown in Fig. 5b1, b2, the nonselective PDE inhibitors, dipyridamole and IBMX, the adenylyl cyclase activator, forskolin, the PDE3-selective inhibitor milrinone, the PDE7-selective inhibitor spiroquinazolinone, and the PDE8-selective inhibitor PF-04957325, all significantly inhibited migration of MDA-MB-231 cells in the range of 15–55 %.

Effect of stimulation of cAMP signaling on breast cancer cell proliferation

Agents capable of stimulating cAMP signaling were examined for their effects on proliferation of breast cancer cells. The nonselective PDE inhibitors, IBMX and dipyridamole, and the PDE4-selective inhibitors, rolipram and piclamilast, had no significant effect on proliferation of either MDA-MB-231 cells (Fig. 6a) or MDA-MB-435 cells (Fig. 6b) in the presence or absence of forskolin. The cell permeable cAMP analogs, 8-Br-cAMP, 8-CPT-cAMP, and

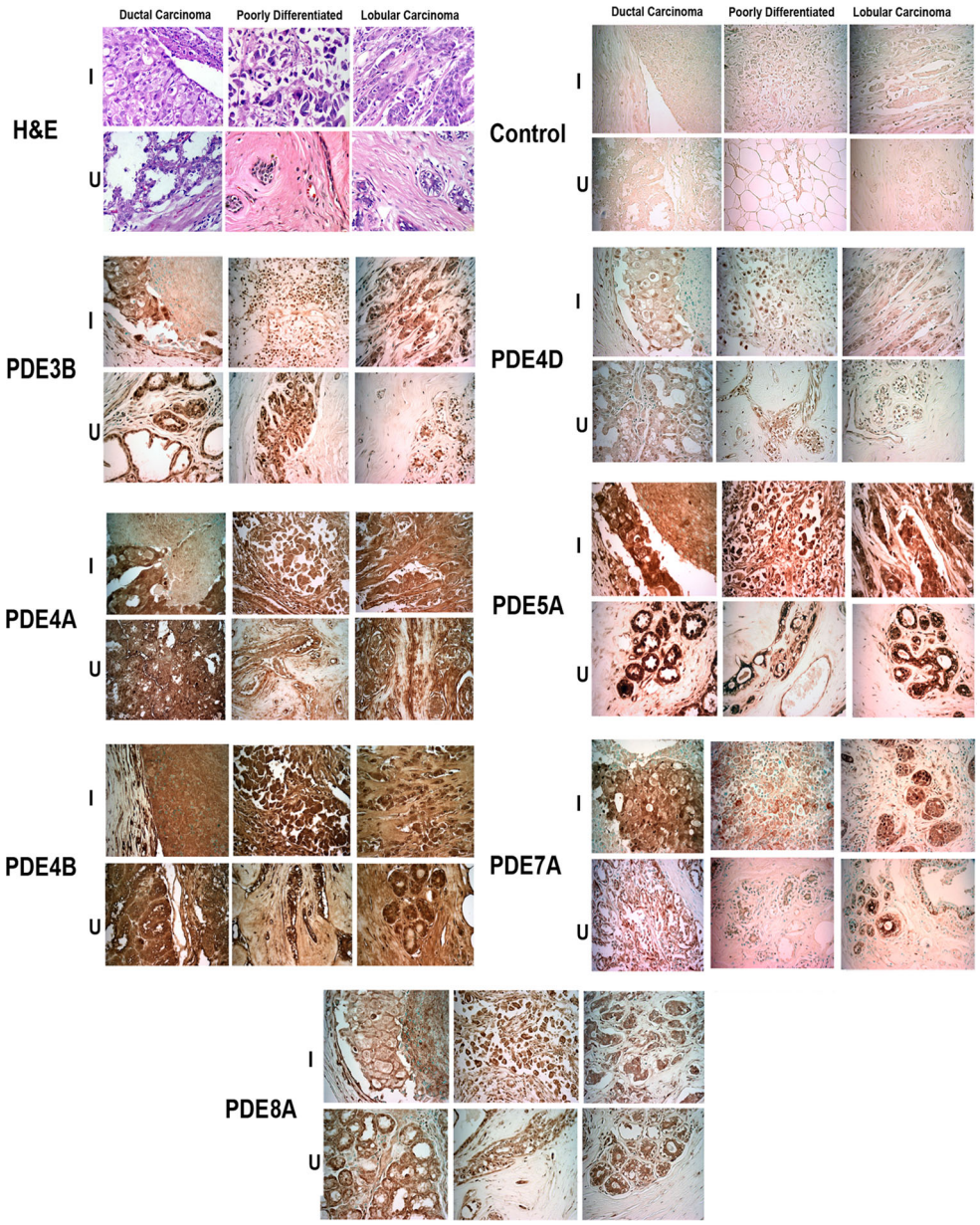

Fig. 4 Expression of PDE proteins in patients' breast cancer tissue. Immunohistochemistry with seven different PDE antibodies was performed on paraffin sections from sixteen cases of invasive (I) breast cancer, which included eleven cases of ductal, three cases of lobular, and two cases of poorly differentiated carcinoma, with corresponding uninvolved (U) tissue. All sixteen samples were positive for PDE expression and a representative sample of each of the three types of carcinoma with its corresponding uninvolved tissue is shown. H&E staining was provided by Pantomics and control represents staining without primary antibody to assure signal specificity

db-cAMP also had no significant effect on proliferation of MDA-MB-231 cells (Fig. 6c) or MDA-MB-435 cells (Fig. 6d). The PDE8-selective inhibitor, PF-04957325, also had no significant effect on proliferation of MDA-MB-231 or MDA-MB-435 cells at concentrations up to at least 10 μM (Fig. 6e).

Discussion

Breast cancer accounts for the highest number of cancer deaths among women. It is estimated that in 2015 in the United States, 231,840 new cases of invasive breast cancer will arise resulting in 40,290 deaths [41]. The cAMP signaling pathway is intimately involved in regulation of cell movement and migration, and although the effects of activation of cAMP signaling on cell migration can be complex, evidence in a number of systems has shown that activation of cAMP signaling leads to inhibition of cell motility, movement, and migration [29, 30, 42, 43]. Indeed, activation of cAMP signaling has already been shown to inhibit migration of a wide range of cells including fibroblasts [2], epithelial cells [3], endothelial cells [4], melanoma cells [5], colon cancer cells [6], pancreatic cancer cells [7, 8], bladder cancer cells [9], and cervical cancer cells [10]. The aim of this study was to investigate the activation of cAMP signaling on breast cancer cell motility. One means of activating cAMP signaling is

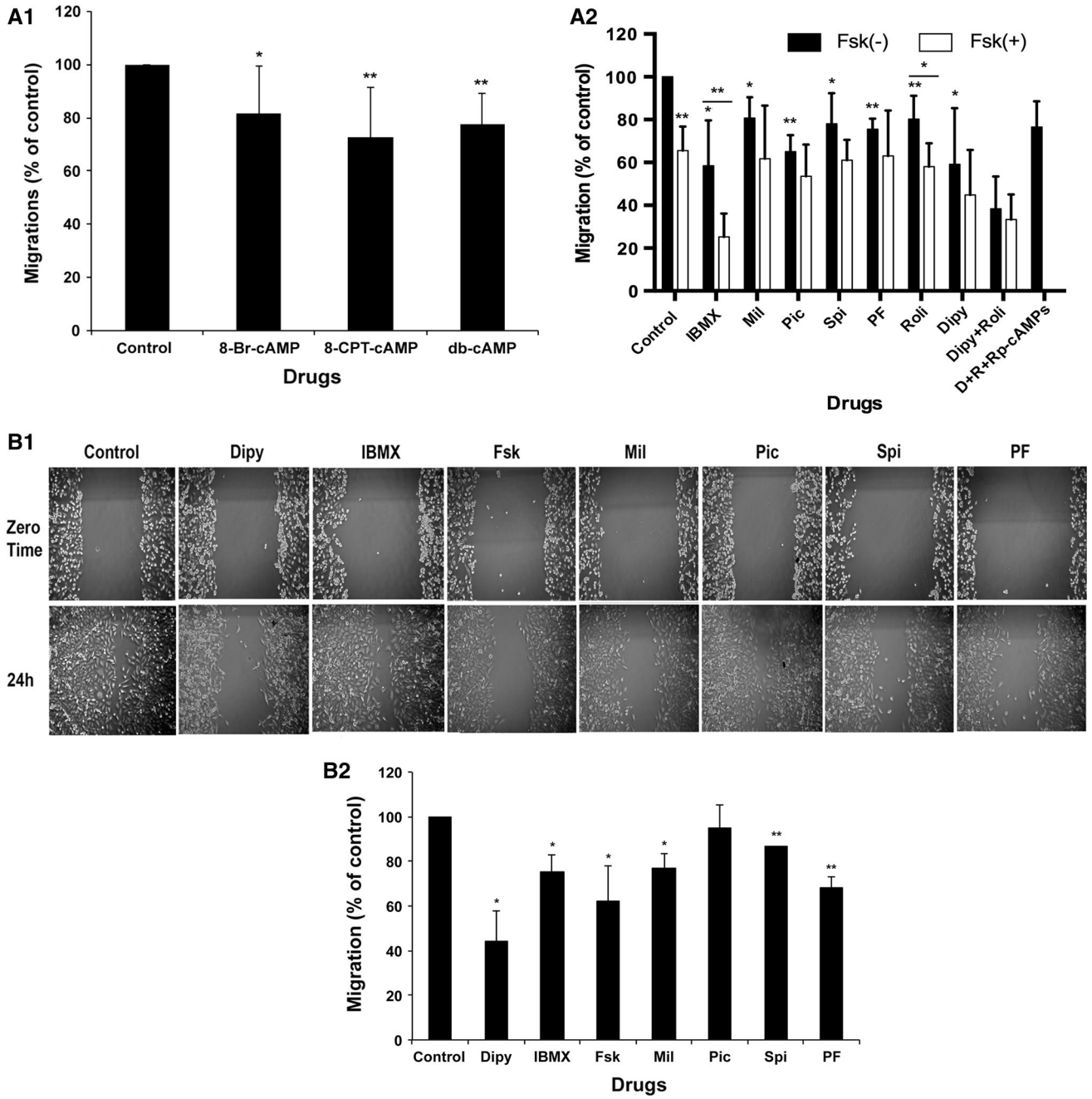

Fig. 5 Effect of activation of cAMP signaling on MDA-MB-231 breast cancer cell migration. **a** Effect of cAMP analogs and PDE inhibitors with and without forskolin determined by transwell assay. MDA-MB-231 cells were grown in the absence (Control) or presence of 8-Br-cAMP 500 μM, 8-CPT-cAMP 500 μM, and db-cAMP 500 μM (**a1**); or isobutylmethlxanthine (IBMX) 500 μM, milrinone (Mil) 10 μM, piclamilast (Pic) 1 μM, spiroquinazolinone (Spi) 1 μM, PF-04957325 (PF) 1 μM, rolipram (Roli) 10 μM, dipyridamole (Dipy) 100 μM, and dipyridamole 100 μM + rolipram 10 μM (Dipy + Roli) with or without 10 μM forskolin (Fsk) and dipyridamole 100 μM + rolipram 10 μM + Rp-cAMPS 1 mM (D+R+Rp-cAMPS) (**a2**) for 24 h. Cells that migrated from the upper chamber to the lower chamber were labeled with calcein-AM dye and counted using a fluorescence plate reader. **b** Effect of PDE inhibitors and forskolin determined by wound healing assay. MDA-MB-231 cells migration was performed by wound healing assay. MDA-MB-231 cells were grown in vehicle (Control) or in dipyridamole (Dipy) 100 μM, isobutylmethlxanthine (IBMX) 500 μM, forskolin (Fsk) 10 μM, milrinone (Mil) 10 μM, piclamilast (Pic) 1 μM, spiroquinazolinone (Spi) 1 μM, or PF-04957325 (PF) 10 μM. The scratched zone in the wound healing assay was pictured by an inverted microscope (**b1**), quantified by NIH ImageJ software, and presented as percent of control (**b2**). All the migration data are presented as the mean ± SD of triplicate wells from three independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$

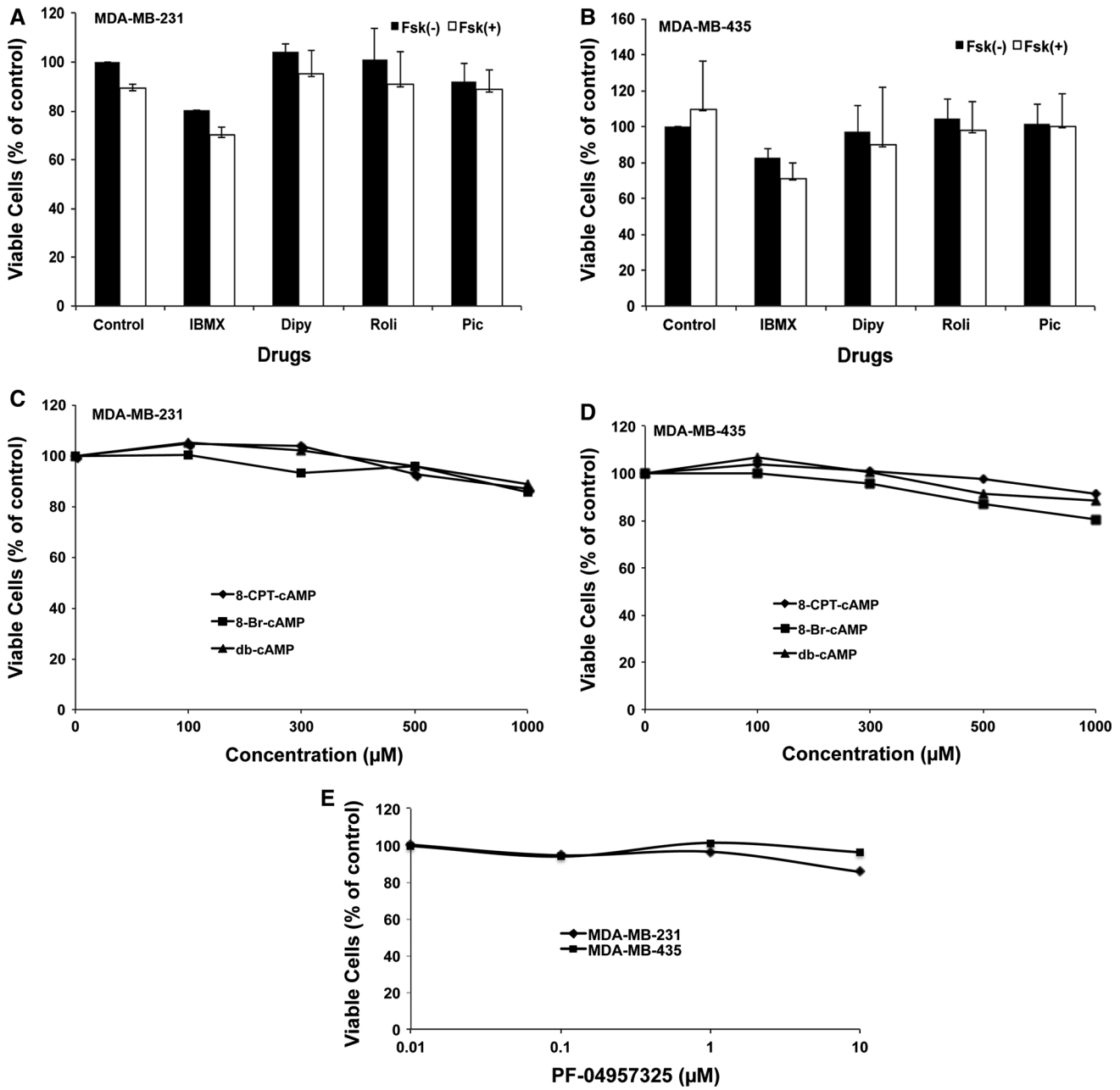

Fig. 6 Effect of activation of cAMP signaling on breast cancer cell proliferation. Effect of PDE inhibitors with and without forskolin on **a** MDA-MB-231 cells and **b** MDA-MB-435 cells. Effect of cAMP analogs on **c** MDA-MB-231 cells and **d** MDA-MB-435 cells. Effect of PF-04957325 on **e** MDA-MB-231 and MDA-MB-435 cells. Breast cancer cells were treated for 72 h and viable cells determined by MTS assay. The concentration of all drugs used was the same as those for migration assays given in Fig. 5. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ and $^{*}P < 0.05$

through inhibition of the PDE enzymes, which degrade cAMP, thus elevating cAMP levels and activating the cAMP signaling pathway. It is understood now that PDEs control not only the steady state levels of cAMP, but also the temporal and spatial dynamics of cAMP signaling, which is highly compartmentalized within distinct cellular regions [11–14]. As a result, PDEs are becoming increasingly important as selective therapeutic targets for treating an increasing number of important illnesses [44, 45]. In this study, we provide the first complete analysis of PDE expression in human breast cancer cells and tissues at both the mRNA and protein levels. We find a large number of PDEs to be expressed in breast cancer cells and tissues, including PDEs 1A, 1C, 2A, 3B, 4A, 4B, 4C, 4D, 5A, 6B, 6C, 7A, 7B, 8A, 8B, 9A, 10A, and 11A. PDE8A in particular was prominently expressed, at the mRNA level, in

all of the established breast cancer cell lines and patients' tissues examined, although as a result of compartmentalization, even PDEs with lower mRNA expressions can have profound effects on cell function and pathophysiology. It is of note that expression of PDE8A in the form of an AKAP13-PDE8A fusion transcript is also highly recurrent in colorectal cancer [46]. We demonstrate in this study that breast cancer cell migration is significantly inhibited by activation of cAMP signaling through either application of cell permeable cAMP analogs or by treatment with PDE inhibitors. Use of the nonselective PDE inhibitors, dipyridamole or IBMX, as well as selective inhibitors for PDE3, PDE4, PDE7, and PDE8 inhibited breast cancer cell migration, and this inhibition was potentiated by stimulation of adenylyl cyclase with forskolin.

The cyclic nucleotides, cAMP and cGMP, have both been reported to have effects on breast cancer cell proliferation and apoptosis. Elevation of cGMP levels through inhibition of the cGMP specific PDEs, PDE5 or PDE9, both inhibits proliferation and induces apoptosis of breast cancer cells [47, 48]. Effects of cAMP have been more controversial, with cAMP reported to either stimulate or inhibit proliferation, depending on the state of the cells studied, the concentrations of the agents and the particular conditions used, such as the presence or absence of estradiol in the serum [49, 50]. Evidence also suggests that the state of expression of the membrane form of estrogen receptor alpha may also determine whether cAMP stimulates or inhibits proliferation of these cells [51]. Under the conditions used in our study, we find no significant effect of cAMP analogs or cAMP PDE inhibitors on proliferation of the breast cancer cells, indicating that the reduction of cell migration observed is functionally selective and not due to a lowering of cell numbers. Similar to these findings, another study also showed that forskolin significantly inhibited leptin-induced migration of MDA-MB-231 cells at levels in which it had no effect on cell proliferation [52].

Several studies have shown that the chemokine, CXCL12, acting through its cognate receptor, CXCR4, regulates the directional trafficking and invasion of breast cancer cells to sites of metastasis [53–56]. CXCL12 and its receptor CXCR4 were first identified in the context of trafficking and homeostasis of T lymphocytes. Studies from our laboratory have shown that PDE8A regulates integrin expression and adhesion of T cells to vascular endothelial cells and the CXCL12-induced chemotaxis of activated lymphocytes, and that it is necessary to inhibit PDE8A in order to inhibit CXCL12-directed chemotaxis of activated lymphocytes [29, 30]. Similar effects were seen for PDE4 inhibitors in models of CXCL12-dependent growth and metastasis of brain tumors [57]. Additionally, caffeine, a nonselective PDE inhibitor, was found to inhibit metastasis in a highly metastatic autochthonous transgene-induced mammary tumor model [58]. These observations, as well as our finding in this study that PDE8A is prominently expressed at the mRNA level in all breast cancer cells and tissues examined, and the recent finding that PDE8A binds to and regulates raf-1 [31], which controls many fundamental biological processes, suggests that PDE8A may provide an excellent novel target for inhibiting breast cancer metastasis. Of note, the nonselective PDE inhibitor, dipyridamole, which in contrast to the nonselective methylxanthine PDE inhibitors, is capable of inhibiting PDE8A, and which we show to significantly inhibit breast cancer cell migration, was recently shown to inhibit breast cancer tumorigenesis and metastasis in animal models [59–61], further suggesting that PDE8A may represent an excellent new target for breast cancer treatment.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that all experiments reported in this publication were performed in compliance with all current laws and regulations of the United States of America.

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