

Expression and Function of Phosphodiesterase Type 5 in Human Breast Cancer

Cell Lines and Tissues: Implications for Targeted Therapy.

Stefania Catalano^{1*}, Antonella Campana^{1*}, Cinzia Giordano², Balázs Győrffy³, Roberta Tarallo⁴, Antonio Rinaldi⁴, Giuseppina Bruno⁴, Aurora Ferraro⁵, Francesco Romeo⁵, Marilena Lanzino¹, Fabio Naro⁶, Daniela Bonofiglio¹, Sebastiano Andò^{1,2#}, Ines Barone^{1#}.

Author Affiliations:

¹Department of Pharmacy, Health and Nutritional Sciences, ²Centro Sanitario, University of Calabria, Rende (CS), Italy; ³MTA TTK Lendület Cancer Biomarker Research Group, Semmelweis University 2nd Dept. of Pediatrics, Budapest, Hungary; ⁴Laboratory of Molecular Medicine and Genomics, Department of Medicine and Surgery, University of Salerno, Baronissi (SA), Italy; ⁵Division of Anatomic Pathology, Annunziata Hospital, Cosenza (CS), Italy; ⁶Department of Anatomical, Histological, Forensic, and Orthopedic Sciences, Sapienza University, Rome, Italy.

* Joint first authors

Joint senior authors

Running Title: PDE5 enhances breast cancer cell invasive potential.

Key Words: breast cancer, PDE5, Rho GTPases, targeted therapy, biomarkers

Correspondence and requests should be addressed to:

Prof. Sebastiano Andò: Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende (CS), 87036, ITALY, Tel: +39 0984 496201, Fax: +39 0984 496203, e-mail: sebastiano.ando@unical.it

Dr Ines Barone: Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende (CS), 87036, ITALY Tel: +39 0984 496216, Fax: +39 0984 496203, e-mail: inesbarone@virgilio.it, ines.barone@unical.it

Disclosure of Potential Conflict of Interest: No potential conflicts of interest were disclosed.

Word Count: 4996

Number of Figures and Tables: 6 Figures, 2 Suppl. Figures, 2 Suppl. Tables.

First Submission to: Clinical Cancer Research, August 6, 2015

Second Submission to: Clinical Cancer Research, November 12, 2015

Translational Relevance

Although early detection and conventional therapies have changed the natural history of breast cancer, many patients die from progressive disease. Dissection of the mechanisms underlying breast cancer progression may identify early markers for invasive tumors as well as specific targeted therapeutics, providing further improvement in the clinical outcomes of patients. This study recognizes a novel function for phosphodiesterase (PDE) type 5 in controlling malignant breast epithelial cell behavior, and provides important clinical implications that can impact the prediction of the risk, and the treatment of breast cancer patients. First, since enhanced PDE5 expression promotes the invasive potential of breast cancer cells and predicts a worse survival in patients, this enzyme may represent a valuable molecular candidate with prognostic significance. Moreover, being PDE5 a known druggable target, our findings may be promising for antitumor therapy with reduced adverse effects. Certainly, future studies could clarify the role of PDE5 in breast cancer.

Abstract

Purpose: By catalyzing cGMP hydrolysis, phosphodiesterase (PDE) 5 is a critical regulator of its concentration and effects in different (patho)physiological processes, including cancers. Being PDE5 a known druggable target, we investigated the clinical significance of its expression in breast cancers and the underlying mechanisms by which it may contribute to tumor progression.

Experimental Design: PDE5 expression was evaluated in seven breast cancer cells by RT-PCR and immunoblotting. To examine PDE5's impact on cancer phenotype, MCF-7 cells, expressing lower levels of the enzyme, were engineered to stably overexpress PDE5. Proliferation was evaluated by MTT assays, motility and invasion by wound-healing/transmigration/invasion assays; transcriptome-profiling by RNA-sequencing; Rho GTPase signaling activation by GST-pulldown assays and immunoblotting. Clinical relevance was investigated by immunohistochemistry on tissues and retrospective studies from Metabric-cohort.

Results: PDE5 is differentially expressed in each molecular subtypes of both breast cancer cell lines and tissues, with higher levels representing a startling feature of HER2-positive and triple-negative breast cancers. A positive correlation was established between elevated PDE5 levels and cancers of high-histological grade. Higher PDE5 expression correlated with shorter patient survival in retrospective analyses. On molecular level, stable PDE5 overexpression in Luminal-A-like MCF-7 cells resulted in enhanced motility and invasion through Rho GTPase signaling activation. Treatment of PDE5-stable clones with selective ROCK or PDE5 inhibitors completely restored the less motile and weak invasive behavior of control-vector cells.

Conclusion: PDE5 expression enhances breast cancer cell invasive potential, highlighting this enzyme as a novel prognostic candidate and an attractive target for future therapy in breast cancers.

Introduction

In 2012, an estimated 1,67 million new cases of invasive breast cancer were diagnosed among women and approximately 522,000 patients were expected to die from breast cancer world-wide (1). Despite advances in surgery, radiation and therapy, metastatic disease represents the most important contributor to breast cancer-related mortality (2). Thus, novel marker and therapeutic target exploration is critical to the early detection, metastasis prevention, and effective treatment of human breast cancers.

Phosphodiesterases (PDEs) are metallo-hydrolases which catalyze the breakdown of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) into their biologically inactive 5'-derivatives, thus modulating the amplitude and duration of their intracellular signalings. In most tissues, PDE5 is the predominant isoform responsible for cGMP hydrolysis and its activity is tightly controlled by cGMP itself (3). cGMP regulates a myriad of biological processes, including cell growth and adhesion, energy homeostasis, neuronal signaling, and muscle relaxation (4, 5). In addition, dysregulation of cGMP homeostasis was observed in various (patho)physiological conditions, including cancers. Indeed, cGMP signaling, through activation of downstream effectors (i.e., cGMP-dependent protein kinase G-PKG, cyclic-nucleotide-gated ion channels) and/or crosstalk with cAMP pathways, appears to play an important role in promoting apoptosis and inhibiting proliferation of certain epithelial cells (6, 7). Interestingly, PKG expression or cGMP levels are reduced in cancer cells and tissues compared to their normal counterparts (8, 9). In the last ten years, overexpression of PDE5 has been described in multiple human carcinomas, including bladder, lung and breast cancers (10-13). The increased expression of PDE5 in human malignancies coupled with the efficacy and high tolerability profiles of PDE5 inhibitors (i.e. sildenafil and vardenafil) in the treatment of erectile dysfunction and pulmonary hypertension have led to an increased interest in investigating PDE5-targeted drugs in cancer management (14, 15). Indeed, several *in vitro* observations have shown anti-proliferative and pro-apoptotic effects of

PDE5 inhibitors in cancer cell lines, as those of the breast (13, 14, 16, 17). Another PDE5 inhibitor, the nonsteroidal anti-inflammatory drug exisulimid inhibited growth and induced apoptosis in human tumor cells through cGMP elevation and PKG activation (12, 18, 19). PDE5 inhibitors also increased the efficacy of chemotherapy agents in cancer models (20). Recently, it has been reported that modulation of host immune response represents an additional mechanism by which PDE5 inhibitors may block tumor progression (21). However, despite these studies, neither the expression of PDE5 in breast cancer cell lines and tissues nor the underlying regulatory molecular mechanisms by which PDE5 expression may contribute to breast cancer progression have been deeply studied. Being PDE5 a well-characterized druggable target, in this study we propose to examine PDE5's impact on breast cancer phenotype *in vitro*, as well as to assess its clinical relevance in breast cancer patients.

Materials and Methods

Reagents, antibodies and plasmids

Sildenafil/Y-27632 were from Sigma; PDE5A/p-c-Myc^{Thr58/Ser62}/c-Myc/p-IκB-α^{Ser32/36}/IκB-α/NF-κBp65/Laminb/GAPDH antibodies from Santa Cruz Biotechnology; RhoA-C/Cdc42/Rac1-3 from Life Technologies; Cofilin activation/Myosin Light Chain 2 antibody sampler kits from Cell Signaling. pEGFP-C1 vector and the fusion protein expression vector pEGFP-PDE5A were provided by Dr F. Barbagallo (Sapienza-University, Rome-Italy). Scrambled and 4-unique 29mer PDE5A shRNA constructs in pGFP-C-shLenti vector were from Origene.

Cell culture

MCF-7/T47D/ZR-75/SKBR3/BT-20 and MDA-MB-468/MDA-MB-435 breast cancer cell lines were from American-Type-Culture-Collection and Interlab-Cell-Line-Collection, respectively, where they were authenticated, stored following suppliers and used within six-months after frozen-

aliquot resuscitations. To generate PDE5A-overexpressing MCF-7 cells, cells were transfected with pEGFP-PDE5A vector using Fugene6-reagent (Promega). Stable clones were selected with G418 antibiotic (1mg/ml, Life Technologies), and positive clones were identified using fluorescence microscopy and immunoblot analysis. Cells were authenticated, every six-months (last examined in February 2015), by short-tandem-repeat profiling, morphology, doubling-times, and tested for mycoplasma-negativity (MycoAlert, Lonza).

Transient transfection

For overexpression studies, T47D cells were transfected with pEGFP-C1 or pEGFP-PDE5A vectors and for gene silencing, MDA-MB-468 cells with scrambled or PDE5A shRNA constructs using Fugene6. After 48h, cells were harvested and used in different experimental procedures.

RT-PCR assays

PDE5 and 36B4 gene expression were evaluated by reverse transcription (RT)-PCR method as described (22). Primers: forward 5'-ACTTGCATTGCTGATTGCTG-3' and reverse 5'-TTGAATAGGCCAGGGTTTTG-3' (*PDE5A*); forward 5'-CAAATCCCATATCCTCGTCC-3' and reverse 5'-CTCAACATCTCCCCCTTCTC-3' (*36B4*).

Immunoblot analysis

Cell extracts were resolved by SDS-PAGE, as described (22). Immunoblots show a single representative of three-separate experiments.

Fluorescence microscopy

Cells were fixed with 4% paraformaldehyde and permeabilized with PBS+0.2% TritonX-100. 4',6-Diamidino-2-phenylindole (DAPI, Sigma) was used for nuclei detection. Fluorescence was photographed with OLYMPUS-BX51 microscope, 100X-magnification.

MTT cell proliferation assays

Three days after seeding, cell proliferation was assessed using 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reagent/MTT (Sigma) and expressed as fold change relative to empty vector-transfected cells. Data represent three-independent experiments, performed in triplicate.

Wound-healing assays

Cell monolayers were scraped and subjected to various experimental conditions. Wound closure was monitored over 24h, cells were fixed and stained with Comassie-Brillant-Blue. Pictures represent one of three-independent experiments (10X-magnification, phase-contrast microscopy).

Transmigration assays

Cells under the various experimental conditions were placed in upper compartments of Boyden-chambers (8 μ m-membranes, Corning). Bottom well contained regular-growth media. After 24h, migrated cells were fixed and stained with DAPI. Migration was quantified by viewing five-separate fields/membrane (10X-magnification) and expressed as mean numbers of migrated cells. Data represent three-independent experiments, assayed in triplicate.

Invasion assays

Matrigel-based invasion assay was performed in Boyden-chambers (8 μ m-membranes) coated with Matrigel (BD Biosciences, 0.4 μ g/ml), as described (23). After 24h, invaded cells were quantified as reported for transmigration assays.

RNA library preparation and sequencing

Total RNA was extracted from vector- and PDE5-expressing cells and libraries were prepared and sequenced as described in Supplementary Materials and Methods.

Rho GTPase activation assays

Rho GTPases activation was determined by active Rho and Cdc42 pull-down and detection kits, following manufacturers (Life Technologies).

Phalloidin staining

Polymerized actin stress fibers were stained with Alexa Fluor 568-conjugated phalloidin, following manufacturers (Life Technologies). Cell nuclei were counterstained with DAPI. OLYMPUS-BX51 microscope (100X-magnification) was used for imaging.

Patients and tissue specimens

A total of 35 primary breast carcinomas and three non-neoplastic breast tissues were analyzed in this study. These carcinomas were obtained from patients who had undergone initial surgery and signed informed consent between 2012-2014 at Annunziata Hospital (Cosenza, Italy). The patients' age at diagnosis varied from 33 to 85 years (mean, 60.6 years; median, 57 years). Characteristics of the patient cohort are reported in Supplementary Table 1. Fresh tissues were formalin-fixed/paraffin-embedded after surgical removal. Sections were stained with H&E to select samples consisting of at least 50% tumor cells and to establish the histologic type and grade (Supplementary Table 1). The clinical investigation conformed the Declaration of Helsinki of 1975 and was approved by ethics and institutional human subjects committees at Annunziata Hospital.

Classification of molecular subtypes

In breast cancer, immunohistochemistry has been used as a surrogate for molecular classification by gene expression profiling in large population-based studies, showing an acceptable level of accuracy for determining molecular phenotypes (24-26). Breast subtype classification is described in Supplementary Materials and Methods.

Immunohistochemical analysis

PDE5A expression in non-neoplastic and neoplastic breast tissues was detected as described in Supplementary Materials and Methods.

Database setup for retrospective study

The entire database contains 1988 patients (average overall survival: 8.07 years, ER-positive patients: 76%, lymphnode-positive patients: 47.3%). Illumina gene-chips published by the Metabric-consortia were downloaded from the EGA repository (27). The raw gene-chip data were imported into R and summarized using beadarray package (28). Quantile normalization was performed using preprocessCore package (29).

Statistical analysis

Data were analyzed for statistical significance using two-tailed student's Test, GraphPad-Prism4. Standard deviations/S.D. are shown. For RNA sequencing bioinformatics analysis, sequencing reads (50-60 million reads/sample on average) were trimmed, quality filtered and aligned, including junction-spanning reads back, to the human genome hg19 (Homo sapiens Ensembl GRCh37) using Tophat v.2.0.10 (30). HTSeq (31) was used to compute read counts across each gene, which were then used as input to R package DESeq2 (32). DESeq2 was used to normalize read counts for library size and dispersion followed by tests for differential gene expression. Significant differentially expressed genes were determined using false discovery rate (FDR) cutoff ≤ 0.05 and at least 1.5-fold change between conditions. Functional analyses were performed with Ingenuity Pathway Analysis suit (Ingenuity Systems). For immunohistochemistry, the correlations between PDE5 and grading/ER/PR/HER2 status were examined with Mann-Whitney-U test, between PDE5 and breast cancer subtypes by Kruskal-Wallis test (GraphPad-Prism4). Kaplan-Meier survival plot, hazard ratio with 95% confidence intervals and logrank P value were calculated and plotted in R as

described (33). Cox proportional hazard regression was computed to compare the association between gene expression, clinical variables including ER/HER2/lymphnode status and survival in multivariate analysis using WinSTAT 2014 for Microsoft Excel (Robert Fitch Software). Statistical significance was set at $p < 0.05$.

Results

PDE5 expression varies among different breast cancer cell subtypes.

On the basis of gene expression signatures in breast cancer patients, researchers have currently identified at least four major molecular and clinically distinct subtypes of neoplasm: Luminal A (ER-positive) and B (ER-positive/HER2-enriched), HER2-positive and basal-like (34, 35). Thus, we first aimed to evaluate mRNA and protein expression levels of PDE5 in breast cancer cell lines of different molecular subtypes ($n=7$) (36, 37) by RT-PCR and immunoblot analyses. As shown in Fig. 1A and B, PDE5 expression was detected at very low levels in luminal A-type (MCF-7/T47D) breast cancer cells. Luminal B-like (ZR-75) cells exhibited a modest induction in PDE5 expression in respect with Luminal A-like cells. Notably, higher PDE5 levels were observed in HER2-overexpressing (SKBR3) and basal-like (BT-20/MDA-MB-468/MDA-MB-435) breast cancer cells. These results may suggest that high PDE5 expression may be associated with more aggressive breast cancer phenotypes.

PDE5 overexpression affects motility and invasion of MCF-7 breast cancer cells.

In order to explore PDE5's role in breast cancer growth and progression, a breast tumor cell line that expresses the lowest levels of this enzyme, MCF-7, was chosen to generate breast cancer *in vitro* models exhibiting forced PDE5 overexpression. For direct visualization of PDE5 cellular location, the corresponding cDNA was cloned in frame with enhanced-green-fluorescent-protein (EGFP) in the mammalian expression vector pEGFP-C1 and stable clones were screened using

fluorescence microscopy (Fig. 1C). Parental MCF-7 breast cancer cells are shown along with one clone stably expressing EGFP (MCF-7 e.v.) and three clones expressing cytoplasmic EGFP-PDE5 (PDE5 1/2/3). This was further evaluated by immunoblotting detection, showing the presence of an exogenous PDE5 band (EGFP-tagged, ~125 kDa) in protein extracts from PDE5-overexpressing cells (Fig. 1D). We used these experimental models to first investigate whether PDE5 overexpression may cause any changes in cellular phenotypes, including proliferation, migration, and invasion. Anchorage-dependent growth assays revealed a slight, but significant increase in cell proliferation in all three PDE5 clones compared to vector-expressing cells (Fig. 1E). We then evaluated the ability of PDE5 overexpression to influence cell migration in wound-healing scratch assays and found that PDE5-expressing cells moved the farthest in either direction to close the gap compared with vector-expressing cells (Fig. 1F). Given the evident enhancement of motility in PDE5-overexpressing cells, the capacity of cells to migrate across uncoated membrane in transmigration assays or invade an artificial basement membrane Matrigel in invasion assays was tested. Although vector-expressing cells exhibited little motile and no invasive behavior *in vitro*, our data clearly showed that PDE5 overexpression significantly increased both motility and invasion of MCF-7 cells (Fig. 1G and H).

To evaluate the effects of PDE5 inhibition on breast tumor cell migratory and invasive properties, cells were treated with the specific PDE5 inhibitor sildenafil (Fig. 2A-C). We found that sildenafil treatment was able to completely restore in PDE5 1/2/3 clones the less motile and weak invasive behavior similar to that of control MCF-7 e.v. cells. Interestingly, although at less extent, sildenafil caused a significant decrease in motility and invasion of vector-expressing cells (by 73% and 65%, respectively). This may suggest that PDE5 activity is required for controlling migration and invasion processes also of malignant breast epithelial cells expressing low levels of the enzyme.

Role of PDE5 in motility and invasion of T47D and MDA-MD-468 breast cancer cells.

To extend the results obtained, we transfected vector and PDE5-expression plasmids in T47D luminal A-like breast cancer cells (Fig. 3A). As previously shown for MCF-7 cells, we found a significant increase in both migratory and invasive cell potential when PDE5 was overexpressed and treatment with the selective PDE5 inhibitor sildenafil completely abrogated these effects (Fig. 3B and C). Again, PDE5 inhibition was associated with a significant reduction of motility and invasion also in vector-expressing cells (Fig. 3B and C). In addition, as a third confirmatory model we silenced PDE5 expression in MDA-MB-468 breast cancer cells, that express high levels of PDE5 compared to MCF-7 and T47D cells (Fig. 3D). Motility and invasion were significantly reduced in PDE5sh-transfected cells compared to control shRNA-transfected cells (Fig. 3E and F). Thus, PDE5 may be an important determinant of breast tumor cell motility and invasion.

PDE5-overexpressing cells exhibit increased Rho GTPase activation.

To gain insights into the biological properties of the highly migratory and invasive PDE5-expressing breast cancer cells, quantitative transcriptome profiling of vector- and PDE5-overexpressing MCF-7 cells was carried out by RNA-sequencing analysis. Comparison of the whole transcriptome of the two cell models revealed 4425 differentially expressed genes (FDR<0.05), of which 611 were up-regulated and 468 were down-regulated in response to PDE5 overexpression (fold change \geq 1.5, Supplementary Table 2). These genes were next subjected to Ingenuity Pathway Analysis (IPA) to rank enriched biological processes (Fig. 4A), and to calculate the activation z-score of molecular and cellular functions and pathways (Fig. 4B and C). In line with our previous experiments, cellular movement was the most significantly overrepresented biological process in PDE5-expressing cells. In addition, many differentially-expressed genes were involved in cell death and survival, cell-to-cell signaling and interaction, cell growth and proliferation, cellular development, thus concurring to identify the five top enriched functional categories in PDE5 clones (Fig. 4A). Interestingly, analyzing the functions involving differentially-expressed genes, migration of cells and cell movement resulted to be highly activated by PDE5

overexpression (Fig. 4B). Then, considering the most affected pathways by PDE5 overexpression, we found marked changes in the activity of Rho GTPase family, with RhoA, Cdc42 and Rac signalings showing activation z-score of 1.9, 1.342, and 0.302, respectively (Fig. 4C). The predicted activation of these canonical pathways was confirmed using molecule activity predictor analysis of IPA (Fig. 4D and Supplementary Fig. 1). An enriched Rho GTPases signaling profile is consistent with the enhanced migration and invasion capabilities of PDE5-expressing cells, as these proteins are known to govern cell cytoskeleton organization, migration, and metastasis dissemination (38). To validate the gene expression profile identified by RNA sequencing, we compared expression and activation of Rho family of GTPases in vector and PDE5 clones. Increased protein levels of Rho A-C, Cdc42 and Rac 1-3 (Fig. 5A, *input panel*) along with increased levels of activated forms (Fig. 5A, *GTP-bound panel*) were detected in PDE5-overexpressing cells. In the GTP-bound form, these proteins are able to interact with effector molecules (i.e. mainly the Rho kinase ROCK and the p21-activated kinase PAK1) to induce phosphorylation of LIM Kinase (LIMK), which is able to inhibit (by phosphorylation) Cofilin, leading to stabilization of filamentous actin structures (39). ROCK has also been shown to phosphorylate the regulatory Myosin Light Chain (MLC), which enhances its binding to F-actin (40). Consistent with increased Rho GTPase activation, the levels of phosphorylated LIMK, Cofilin and MLC are greatly increased in PDE5 clones compared to e.v. cells (Fig. 5B and C). Among other downstream targets of Rho signaling, c-Myc and NF- κ B are important for cell motility and invasion (39, 41, 42). In PDE5-overexpressing cells, we detected elevated levels of the activated form of c-Myc (phospho c-Myc), whereas we did not observe NF- κ B activation, as evidenced by no changes in phosphorylation of the inhibitor of NF- κ B I κ B α as well as in NF- κ B nuclear translocation (Supplementary Fig. 2). To further investigate the role of Rho GTPases activation on cytoskeletal organization in our model systems, we evaluated the formation of stress fibers, since they provide the contractile force required for motility (Fig. 5D). In vector-expressing cells, actin filament remained diffusely distributed in the cytoplasm; while overexpression of PDE5 resulted in visible stress fibers. According to these results, treatment with

the selective ROCK inhibitor Y-27632 was able to significantly reduce both migration and invasion of PDE5 clones (Fig. 5E and F). Collectively, our data strongly suggest that the molecular and cellular functions identified with IPA are biologically relevant and PDE5 may regulate motility and invasion through activation of the Rho family of GTPases.

PDE5 expression in human breast cancers.

To evaluate the clinical significance of PDE5 in human breast tumors, we analyzed its expression levels in patient-derived tissues (n=35) by immunohistochemistry analysis. The characteristics of all patients are listed in Supplementary Table 1. We found that ~85% of cases showed cytoplasmic staining for PDE5 in cancer cells. Interestingly, ER/PR-positive tumors exhibited weak (n=5) or intermediate (n=15) PDE5 staining, while the strongest staining intensity was observed among HER2-positive or triple negative (TN) samples (n=15). In contrast, in non-neoplastic (NN) breast tissues (n=3), a weak to missing PDE5 expression was detected in the cytoplasm. Representative images of PDE5 staining patterns are shown in Figure 6A. Accordingly, PDE5 expression had a significant inverse correlation with ER and PR status (Fig. 6B and C) and a significant positive correlation with HER2 status (Fig. 6D). Of note, as the staining intensity of PDE5 increases, grading of cancer cells also tended to increase ($p = 0.001$), indicating that the higher expression of PDE5 might be related with a higher malignancy phenotype.

We then correlated PDE5 expression with the different subtypes of breast carcinoma and found that its cytoplasmic expression was significantly different among the four molecular subtypes (Fig. 6E). In particular, in HER2 and triple-negative subtypes, PDE5 expression was the highest, followed by the luminal B-type and the luminal A-type, that showed the lowest percentage of cells with PDE5 expression. Since HER2 and TN types are correlated with a generally more aggressive tumor phenotype and poorer prognosis (26), these results suggest that PDE5 overexpression may be correlated with poor prognosis.

High levels of PDE5 are associated with shorter survival in breast cancer patients.

To strengthen the results obtained in human breast tumor tissues, we conducted retrospective analyses of the correlation between PDE5 expression and survival in patients with breast cancer. In the univariate analysis, the Kaplan-Meier overall survival (OS) curve obtained from a cohort of 1988 patients indicated that increased expression levels of PDE5 are associated with a statistically significant shorter OS when compared to those tumors expressing low levels of PDE5 ($p=0.014$, $HR=1.2$, Fig. 6F). Interestingly, significant difference could also be observed between OS for ER-positive patients having high or low PDE5 levels ($p=7.4E-04$, $HR=1.4$, Fig. 6G), suggesting a role for PDE5 in predicting disease progression in ER-positive tumors that according to immunohistochemistry may have lower levels of the enzyme compared to ER-negative ones. In addition, PDE5 retained its significance when performing a multivariate analysis including PDE5 expression, ER, HER2 and lymphnode status in the entire database (PDE5: $p=6.6E-03$, $HR=1.24$, ER status: $p=6.6E-05$, $HR=0.69$, HER2 status: $p=1E-05$, $HR=1.6$, lymph node status: $p=1.1E-06$, $HR=2.24$). Therefore, high levels of PDE5 expression may independently predict poor outcome among breast cancer patients.

Discussion

Despite vast improvement in the overall survival rate of breast cancer patients, advanced metastatic disease remains a life-threatening stage of cancer. One of the major challenges in mammary cancer research is to identify key proteins that are directly involved in pathways promoting tumorigenesis and tissue invasion. These proteins can then be explored as early markers for invasive tumors as well as potential targets for the development of therapeutic strategies aimed at controlling and curing malignant disease. In this report, we show that phosphodiesterase type 5 (PDE5) is differentially expressed in human breast cancer subtypes, with a significant positive correlation with tumor grading. Importantly, high PDE5 levels predict a worse prognosis for

patients at 8-year median follow-up. In experimental breast cancer models, PDE5 overexpression increased motile and invasive properties of cells through activation of the Rho family of GTPases, highlighting the potential benefit of therapeutic targeting PDE5.

Breast cancer is a complex and highly heterogeneous disease due to its diverse morphological features, variable clinical outcomes and disparate therapeutic responses. By using hierarchical clustering analysis of gene expression profiling, Perou et al. were able to identify molecularly-defined and clinically-distinct classes of breast cancer (Luminal, HER2-enriched, basal-like and normal-like) (34, 35). Due to the prognostic and predictive values of this molecular classification in clinical setting, attempts have been made to identify surrogate markers that would allow subtype identification using the more familiar immunohistochemical approach. Accordingly, the combined evaluation of histopathological grade and immunohistochemical parameters (ER/PR/HER2) would approximate the molecular classification of Luminal A, Luminal B, HER2-enriched and triple-negative breast cancers (24-26). Despite the lack of complete overlapping, the panelists of the last St. Gallen Consensus have endorsed the use of the immunohistochemical assays to identify breast cancer subtypes and allow the physicians to tailor properly the systemic interventions (43). Here, we found a significant increase in PDE5 expression in breast tumors when compared to non-neoplastic breast tissues. Although ~85% of the tumor entities examined showed cytoplasmic staining for PDE5, our results clearly indicate that PDE5 is differentially expressed between each molecular breast cancer subset. In particular, the lowest expression of this enzyme is detected in the more favorable Luminal A subtypes of breast tumors, whereas its overexpression is closely related to breast cancers of high histological grade including triple-negative and HER2-positive molecular subtypes. Accordingly, PDE5 levels have a negative correlation with hormone receptor status, and a positive one with HER2 status and tumor grading. The clinical significance of PDE5 overexpression is strongly supported by its significant association with a shorter overall survival time in retrospective studies. This association is also significant in patients with ER-positive diseases, advocating the utility of PDE5 as a predictor of breast cancer prognosis in ER-positive tumors that

immunohistochemically may have lower PDE5 levels compared to ER-negative ones. In line with findings on patients, PDE5 expression also varies among different breast cancer cell line models. Taken together, our results suggest that the differential expression of PDE5 may contribute to breast cancer heterogeneity and, being PDE5 a known druggable target, it could help to integrate subsets of aggressive breast cancer into clinically meaningful subtypes.

At the present time, the selective PDE5 inhibitors sildenafil (Viagra and Revatio-Pfizer) and tadalafil (Cialis-Eli Lilly; Adcirca-United Therapeutics) have Food and Drug Administration approval for the treatment of erectile dysfunction (ED) as well as pulmonary artery hypertension; whereas vardenafil (Levitra and Vivanza-Bayer) and avanafil (Stendra-Vivus) are approved only for ED. However, due to their favorable toxicity profile (44), these agents are being investigated in a wide range of other potential medical and surgical applications, including neurological and cardiovascular disorders, transplant and reconstructive surgery, and several clinical trials are currently in progress (15). In the last ten years, various PDE5 inhibitors have been also reported to inhibit growth and induce apoptosis in many cancer cell lines, without affecting normal epithelial cells (12-14, 16, 18, 19). Recently, two reports have suggested a role for PDE5 inhibition in influencing cancer cell motility (17, 45). Indeed, tadalafil and sildenafil reduced the capacity of thyroid cancer cells to migrate at lower doses than those used to block proliferation (17). Similarly, down-regulation of PDE5 in the aggressive human breast cancer cell line MDA-MB-231T resulted in no difference in cell proliferation and reduced motility *in vitro* and *in vivo* (45). Here, we show that genetic and pharmacological inhibition of PDE5 significantly decreases migration and invasion of different human breast cancer cell lines. In contrast, overexpression of PDE5 strongly increases motility and invasion of MCF-7 cells and sildenafil treatment completely restores in PDE5 stable clones the less motile and weak invasive behavior similar to that of control cells. Interestingly, although at less extent, a reduction of migration and invasion was observed after treatment with sildenafil also in vector cells, expressing lower levels of the enzyme, further highlighting a role for PDE5 in controlling migration and invasion processes of malignant breast epithelial cells. Increased

formation of actin stress fibers and enhanced contractility are common features of motile cells in 2D culture conditions (46). Indeed, immunofluorescent staining of polymerized actin (F-actin) reveals an induced formation of stress fibers in cells bearing PDE5 overexpression. Accordingly, ingenuity pathway analysis on PDE5 modulated genes by RNA-sequencing highlights cellular movement as the most significantly represented biological process. Moreover, most of the genes differentially regulated by PDE5 overexpression are significantly involved in migration of cells. Taken together, these results indicate that PDE5 may be important for sustaining malignant motile and invasive behavior of breast cancers.

The acquisition of a remodeled cytoskeleton and a motile phenotype are important steps in tissue invasion and metastasis establishment, and the Rho family of GTP-binding proteins have been reported to mediate these processes. Although at least 20 Rho family proteins have been identified in humans, the most widely characterized molecules for their effects on cell migration are Rho A-C, which regulate stress fibers and focal adhesion formation, and Rac 1-3 and Cdc42, which control membrane ruffling, and filopodium formation (38). In the GTP-bound form, these proteins are able to interact with effector molecules to regulate actin cytoskeleton assembly and organization through phosphorylation of specific substrates, such as Cofilin or Myosin Light Chain, as well as to modulate a variety of other biochemical signalings involved in cell transformation and metastasis, including the c-Myc pathway (39-41). In breast tumors, Rho expression and/or activity are frequently increased (47). On the other hand, a complex signaling interplay between cGMP and Rho GTPase pathways has been described. For instance, cGMP-PKG cascade inhibits RhoA in various cell types (48, 49). Similarly, vardenafil prevents RhoA membrane translocation/activation, and decreases activity of its downstream effector ROCK in human bladder smooth muscle cells, inhibiting endothelin-1-induced migration (50). On the molecular level, increased levels of total and activated Rho A-C, Cdc42 and Rac 1-3 are detected in PDE5-overexpressing cells compared with vector cells. Phosphorylation levels of Rho GTPase downstream targets, including LIM Kinase, Cofilin, Myosin Light Chain and c-Myc, are also greatly increased in PDE5 clones compared to

vector cells. Moreover, transcriptome analysis identifies an enriched Rho GTPase signaling profile in PDE5-overexpressing cells. Consistent with these findings, a selective ROCK inhibitor significantly reduces both migration and invasion of PDE5 clones.

In conclusion, results of this study highlight a novel role for PDE5 in controlling malignant breast epithelial cell behavior and provide the first indications for the clinical relevance of this enzyme in human breast cancers. Since PDE5 overexpression greatly enhances the invasive potential of breast cancer cells and reduces survival in patients, it is tempting to speculate that this enzyme may represent a novel prognostic biomarker candidate. In addition, having already addressed the delivery, stability and toxicity issues of PDE5 inhibitors in other diseases, our findings may offer promising insights into future cancer treatments by providing the rationale to implement safer and more efficacious drugs in the adjuvant therapy for improving clinical care and reducing mortality from breast cancer. This may assume particular significance in triple-negative breast cancers in which PDE5 may be an attractive target. Based on these observations, it is evident that the impact of PDE5 in the prediction of the risk, and the treatment of breast cancer patients deserves further attention.

Grant Support:

This work was supported by PROGRAMMA "FUTURO IN RICERCA" Anno 2012 # RBFR12FI27 to IB, and #RBFR12W5V5_003 to RT.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359-86.
2. Kraljevic Pavelic S, Sedic M, Bosnjak H, Spaventi S, Pavelic K. Metastasis: new perspectives on an old problem. *Mol Cancer* 2011;10:22.
3. Zoraghi R, Bessay EP, Corbin JD, Francis SH. Structural and functional features in human PDE5A1 regulatory domain that provide for allosteric cGMP binding, dimerization, and regulation. *J Biol Chem* 2005;280:12051-63.
4. Francis SH, Busch JL, Corbin JD, Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 2010;62:525-63.
5. Rehmann H, Wittinghofer A, Bos JL. Capturing cyclic nucleotides in action: snapshots from crystallographic studies. *Nat Rev Mol Cell Biol* 2007;8:63-73.
6. Fajardo AM, Piazza GA, Tinsley HN. The role of cyclic nucleotide signaling pathways in cancer: targets for prevention and treatment. *Cancers (Basel)* 2014;6:436-58.
7. Fallahian F, Karami-Tehrani F, Salami S, Aghaei M. Cyclic GMP induced apoptosis via protein kinase G in oestrogen receptor-positive and -negative breast cancer cell lines. *FEBS J* 2011;278:3360-9.
8. Karami-Tehrani F, Fallahian F, Atri M. Expression of cGMP-dependent protein kinase, PKGIalpha, PKGIbeta, and PKGII in malignant and benign breast tumors. *Tumour Biol* 2012;33:1927-32.
9. Hou Y, Gupta N, Schoenlein P, Wong E, Martindale R, Ganapathy V, et al. An anti-tumor role for cGMP-dependent protein kinase. *Cancer Lett* 2006;240:60-8.
10. Karami-Tehrani F, Moeinifard M, Aghaei M, Atri M. Evaluation of PDE5 and PDE9 expression in benign and malignant breast tumors. *Arch Med Res* 2012;43:470-5.
11. Puzstai L, Zhen JH, Arun B, Rivera E, Whitehead C, Thompson WJ, et al. Phase I and II study of exisulind in combination with capecitabine in patients with metastatic breast cancer. *J Clin Oncol* 2003;21:3454-61.
12. Whitehead CM, Earle KA, Fetter J, Xu S, Hartman T, Chan DC, et al. Exisulind-induced apoptosis in a non-small cell lung cancer orthotopic lung tumor model augments docetaxel treatment and contributes to increased survival. *Mol Cancer Ther* 2003;2:479-88.
13. Piazza GA, Thompson WJ, Pamukcu R, Alila HW, Whitehead CM, Liu L, et al. Exisulind, a novel proapoptotic drug, inhibits rat urinary bladder tumorigenesis. *Cancer Res* 2001;61:3961-8.
14. Savai R, Pullamsetti SS, Banat GA, Weissmann N, Ghofrani HA, Grimminger F, et al. Targeting cancer with phosphodiesterase inhibitors. *Expert Opin Investig Drugs* 2010;19:117-31.
15. Kloner RA, Comstock G, Levine LA, Tiger S, Stecher VJ. Investigational noncardiovascular uses of phosphodiesterase-5 inhibitors. *Expert Opin Pharmacother* 2011;12:2297-313.
16. Sarfati M, Mateo V, Baudet S, Rubio M, Fernandez C, Davi F, et al. Sildenafil and vardenafil, types 5 and 6 phosphodiesterase inhibitors, induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 2003;101:265-9.
17. Sponziello M, Verrienti A, Rosignolo F, De Rose RF, Pecce V, Maggisano V, et al. PDE5 expression in human thyroid tumors and effects of PDE5 inhibitors on growth and migration of cancer cells. *Endocrine* 2015;50:434-41.
18. Tinsley HN, Gary BD, Keeton AB, Zhang W, Abadi AH, Reynolds RC, et al. Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G. *Mol Cancer Ther* 2009;8:3331-40.
19. Li N, Xi Y, Tinsley HN, Gurpinar E, Gary BD, Zhu B, et al. Sulindac selectively inhibits colon tumor cell growth by activating the cGMP/PKG pathway to suppress Wnt/beta-catenin signaling. *Mol Cancer Ther* 2013;12:1848-59.

20. Shi Z, Tiwari AK, Patel AS, Fu LW, Chen ZS. Roles of sildenafil in enhancing drug sensitivity in cancer. *Cancer Res* 2011;71:3735-8.
21. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 2006;203:2691-702.
22. Gu G, Barone I, Gelsomino L, Giordano C, Bonofiglio D, Statti G, et al. Oldenlandia diffusa extracts exert antiproliferative and apoptotic effects on human breast cancer cells through ERalpha/Sp1-mediated p53 activation. *J Cell Physiol* 2012;227:3363-72.
23. Barone I, Catalano S, Gelsomino L, Marsico S, Giordano C, Panza S, et al. Leptin mediates tumor-stromal interactions that promote the invasive growth of breast cancer cells. *Cancer Res* 2012;72:1416-27.
24. Collins LC, Marotti JD, Gelber S, Cole K, Ruddy K, Kereakoglow S, et al. Pathologic features and molecular phenotype by patient age in a large cohort of young women with breast cancer. *Breast Cancer Res Treat* 2012;131:1061-6.
25. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* 2006;295:2492-502.
26. Yang XR, Sherman ME, Rimm DL, Lissowska J, Brinton LA, Peplonska B, et al. Differences in risk factors for breast cancer molecular subtypes in a population-based study. *Cancer Epidemiol Biomarkers Prev* 2007;16:439-43.
27. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012;486:346-52.
28. Dunning MJ, Smith ML, Ritchie ME, Tavare S. beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics* 2007;23:2183-4.
29. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185-93.
30. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 2013;14:R36.
31. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 2015;31:166-9.
32. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.
33. Mihaly Z, Kormos M, Lanczky A, Dank M, Budczies J, Szasz MA, et al. A meta-analysis of gene expression-based biomarkers predicting outcome after tamoxifen treatment in breast cancer. *Breast Cancer Res Treat* 2013;140:219-32.
34. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature* 2000;406:747-52.
35. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001;98:10869-74.
36. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res* 2011;13:215.
37. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006;10:515-27.
38. Schmitz AA, Govek EE, Bottner B, Van Aelst L. Rho GTPases: signaling, migration, and invasion. *Exp Cell Res* 2000;261:1-12.
39. Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem J* 2000;348 Pt 2:241-55.
40. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003;4:446-56.

41. Liu S, Goldstein RH, Scepanky EM, Rosenblatt M. Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. *Cancer Res* 2009;69:8742-51.
42. Helbig G, Christopherson KW, 2nd, Bhat-Nakshatri P, Kumar S, Kishimoto H, Miller KD, et al. NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem* 2003;278:21631-8.
43. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, et al. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* 2011;22:1736-47.
44. Nehra A. Erectile dysfunction and cardiovascular disease: efficacy and safety of phosphodiesterase type 5 inhibitors in men with both conditions. *Mayo Clin Proc* 2009;84:139-48.
45. Marino N, Collins JW, Shen C, Caplen NJ, Merchant AS, Gokmen-Polar Y, et al. Identification and validation of genes with expression patterns inverse to multiple metastasis suppressor genes in breast cancer cell lines. *Clin Exp Metastasis* 2014;31:771-86.
46. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. *Science* 2003;302:1704-9.
47. Vega FM, Ridley AJ. Rho GTPases in cancer cell biology. *FEBS Lett* 2008;582:2093-101.
48. Sauzeau V, Rolli-Derkinderen M, Marionneau C, Loirand G, Pacaud P. RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. *J Biol Chem* 2003;278:9472-80.
49. Sawada N, Itoh H, Yamashita J, Doi K, Inoue M, Masatsugu K, et al. cGMP-dependent protein kinase phosphorylates and inactivates RhoA. *Biochem Biophys Res Commun* 2001;280:798-805.
50. Morelli A, Filippi S, Sandner P, Fibbi B, Chavalmane AK, Silvestrini E, et al. Vardenafil modulates bladder contractility through cGMP-mediated inhibition of RhoA/Rho kinase signaling pathway in spontaneously hypertensive rats. *J Sex Med* 2009;6:1594-608.

Figures Legends

Figure 1. Impact of PDE5 overexpression on breast cancer cell proliferation, motility and invasion. **A**, RT-PCR analysis for PDE5 and 36B4 (internal standard) mRNA levels in Luminal A-type (MCF-7/T47D), Luminal B-like (ZR-75), HER2-overexpressing (SKBR3) and basal-like (BT-20/MDA-MB-468/MDA-MB-435) breast cancer cells. **B**, Immunoblotting for PDE5 expression in indicated cells. GAPDH, control for loading. **C**, Fluorescence-microscopic analysis to visualize EGFP-fluorescence in MCF-7 cells stably transfected with pEGFP-C1 (e.v.) or fusion protein expression pEGFP-PDE5A vectors (PDE5 1/2/3). MCF-7 cells, negative control. *Insets*: DAPI, nuclear staining. **D**, Immunoblotting showing EGFP-PDE5 expression in e.v. and PDE5 1/2/3 MCF-7 cells. GAPDH, control for loading. MTT growth (**E**), wound-healing (**F**, *insets*: time 0), transmigration (**G**) and invasion (**H**) assays in cells under basal nonstimulated conditions. * $P < 0.05$, ** $P < 0.005$.

Figure 2. Effects of sildenafil on motility and invasion of PDE5-overexpressing MCF-7 breast cancer cells. Wound-healing (**A**, *insets*: time 0), transmigration (**B**) and invasion (**C**) assays in cells treated with vehicle (-) or sildenafil (Sild, 10 μ M). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

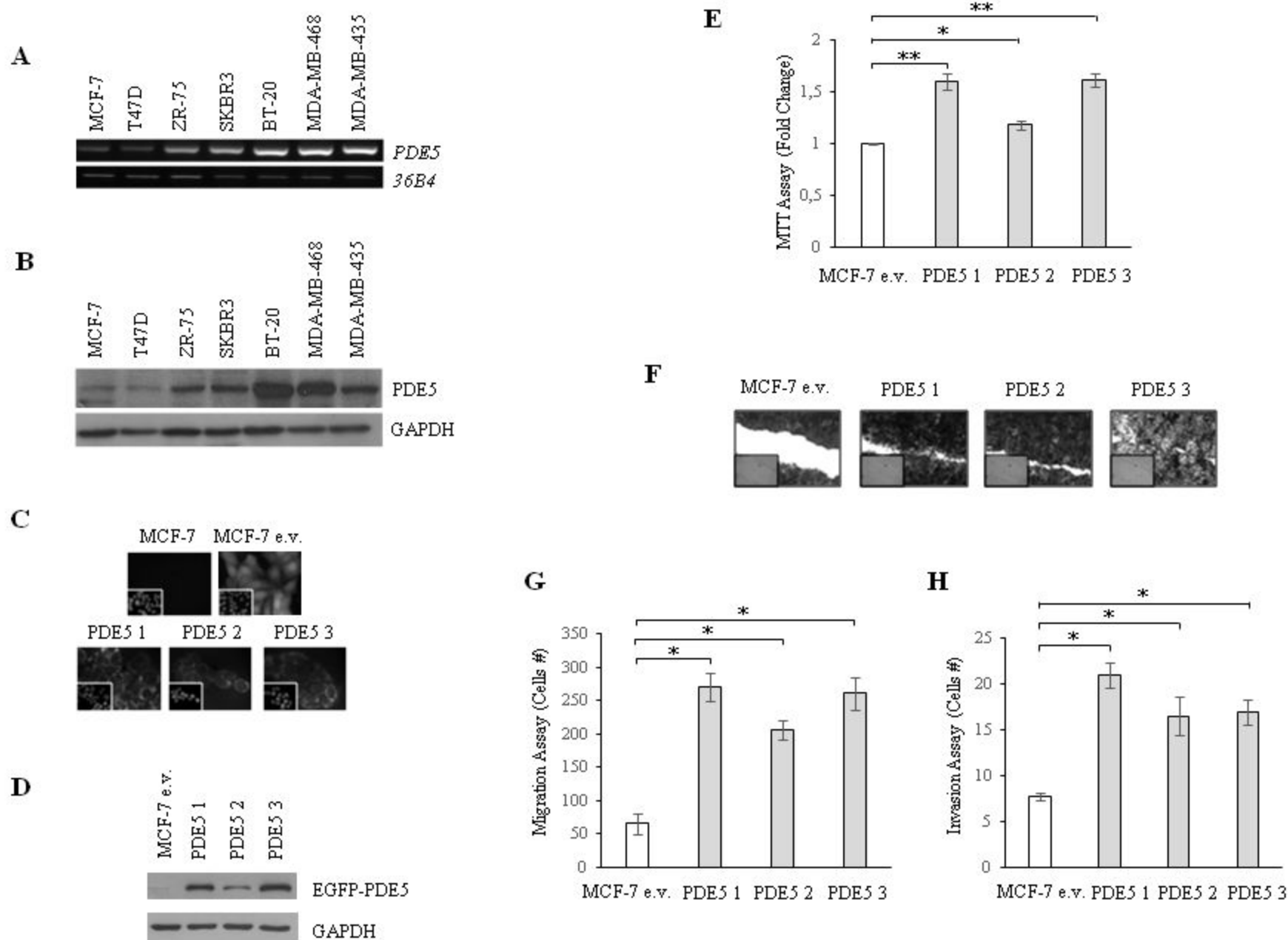
Figure 3. Influence of PDE5 on motility and invasion of T47D and MDA-MB-468 breast cancer cells. **A**, RT-PCR (*upper panel*) and immunoblot (*lower panel*) analyses for PDE5 expression in vector (e.v.) and PDE5-expressing T47D cells. 36B4, internal standard. GAPDH, control for loading. Transmigration (**B**) and invasion (**C**) assays in cells treated with vehicle (-) or sildenafil (Sild, 10 μ M). **D**, RT-PCR (*upper panel*) and immunoblot (*lower panel*) analyses for PDE5 expression in MDA-MB-468 cells transfected with control scrambled-shRNA (CTRLsh) and PDE5 shRNA (PDE5sh) constructs. 36B4, internal standard. GAPDH, control for loading.

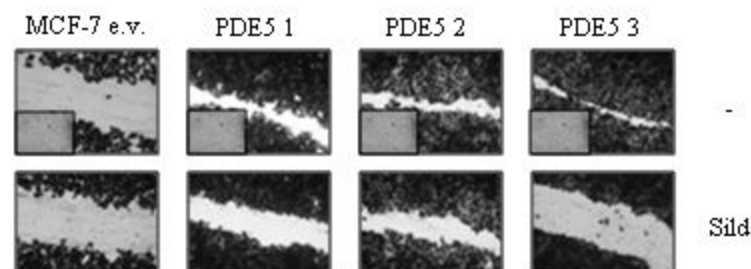
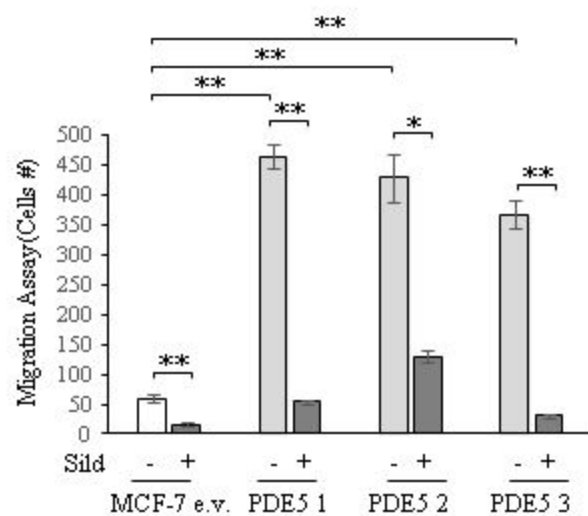
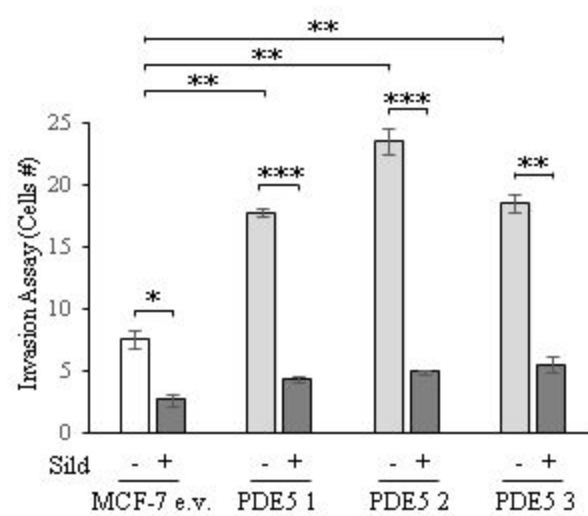
Transmigration (**E**) and invasion (**F**) assays in CTRLsh and PDE5sh transfected MDA-MB-468 cells. ** $P < 0.005$.

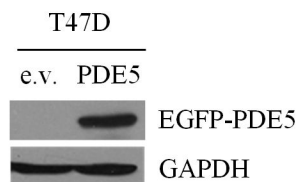
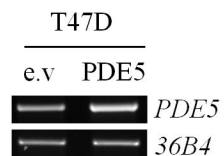
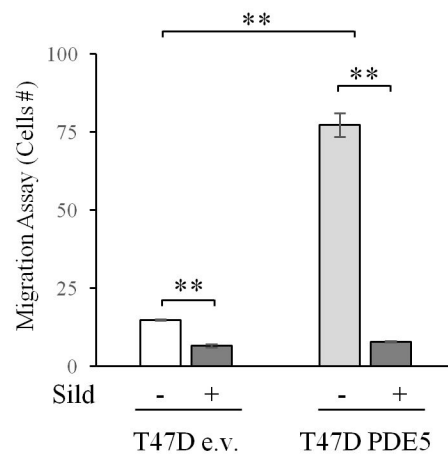
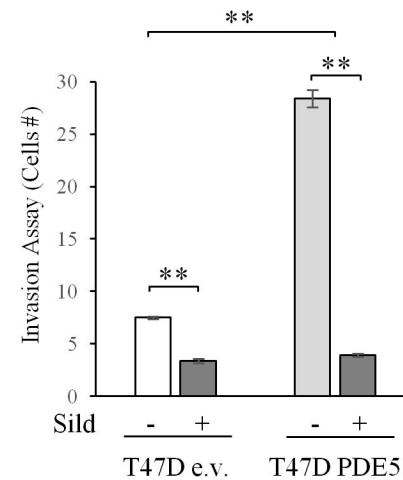
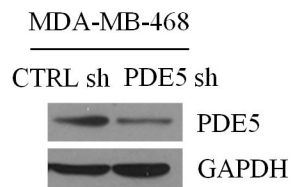
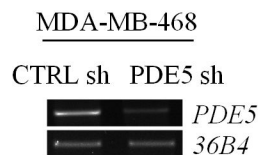
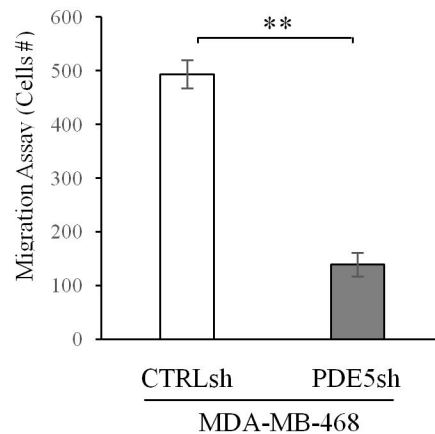
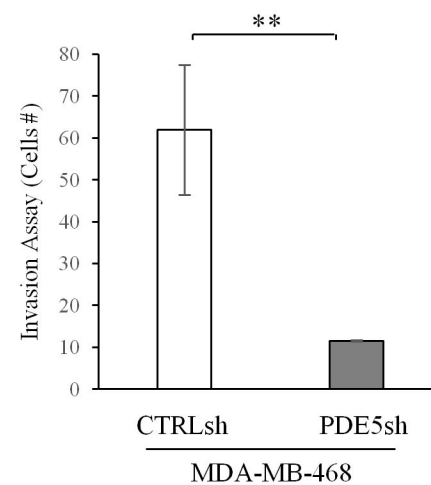
Figure 4. Biological processes, functions and pathways identified from RNA sequencing data in PDE5-overexpressing MCF-7 cells. Ingenuity Pathway Analysis (IPA) used to identify biological processes significantly associated with differentially-expressed genes (**A**) and to calculate activation z-score of biological functions (**B**) and pathways (**C**). **D**, Predicted activation of RhoA signaling pathway by molecule activity predictor analysis of IPA in PDE5-overexpressing versus vector-transfected MCF-7 cells.

Figure 5. Rho GTPase activation in PDE5-overexpressing cells. **A**, *Input panel*, immunoblotting for Rho A-C, Cdc42 and Rac 1-3 expression. *GTP-bound panel*, activation assays of Rho A-C, Cdc42 and Rac 1-3. Immunoblotting showing phosphorylated LIM Kinase (pLIMK1^{Thr508}/pLIMK2^{Thr505}), Cofilin (p-Cofilin^{Ser3}) (**B**), Myosin Light Chain (pMLC^{Thr18/Ser19}) (**C**) and total proteins from whole-cell lysates. GAPDH, control for loading. **D**, Phalloidin staining of F-actin (stress fibers, red). DAPI, nuclear staining. *Insets*: Stress fibers with higher resolution. Transmigration (**E**) and invasion (**F**) assays in cells treated with vehicle (-) or ROCK inhibitor (Y-27632, 10 μ M). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

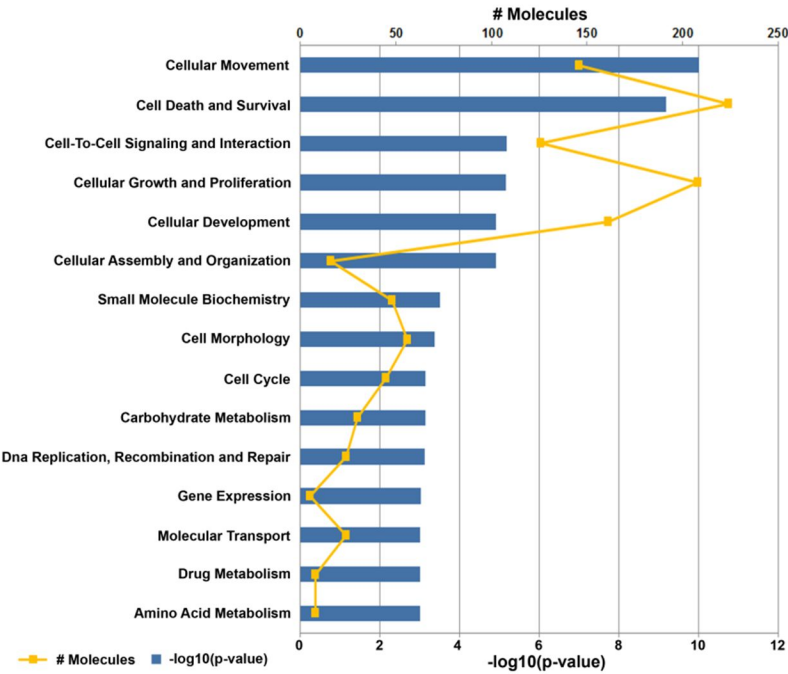
Figure 6. Role of PDE5 in breast cancer patients. **A**, Immunohistochemical detection of PDE5 expression in nonneoplastic breast tissues (NN), ER/PR-positive (+), HER2-overexpressing (HER2) and triple-negative (TN) breast cancer tissues. Representative fields were photographed at 20X-magnification (*insets*: details of PDE5 subcellular localization). PDE5 immunohistochemical expression was correlated to ER (**B**), PR (**C**), HER2 (**D**) status or molecular subtypes (**E**) of breast cancer patients. Kaplan-Meier survival analysis relating PDE5 levels and overall survival in all patients (**F**) or in patients with ER-positive breast cancers (**G**).



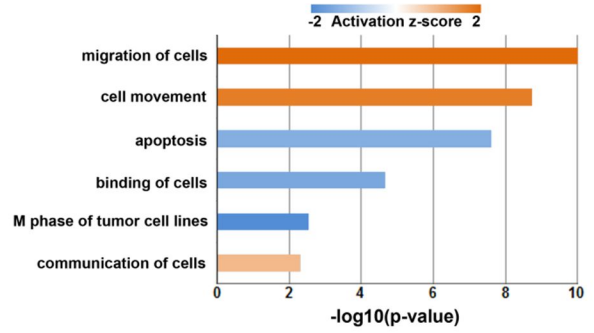
A**B****C****Figure 2**

A**B****C****D****E****F****Figure 3**

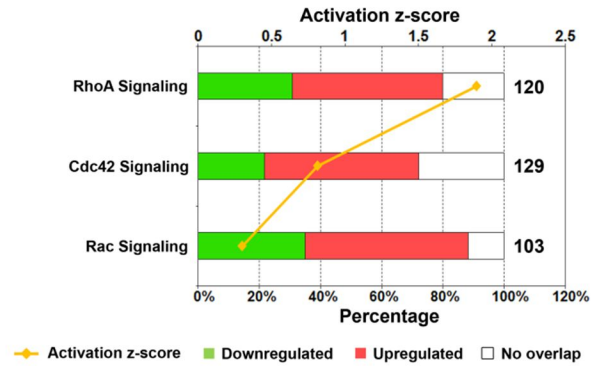
A



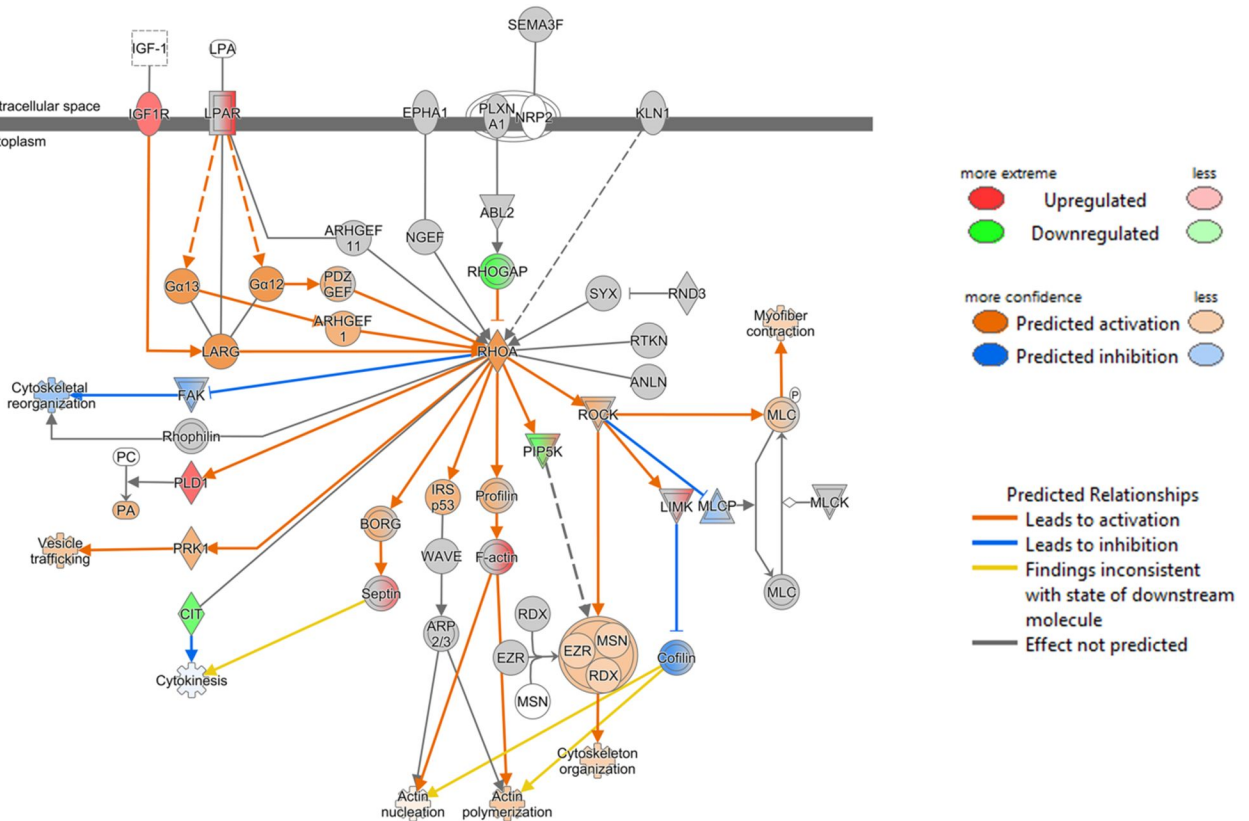
B

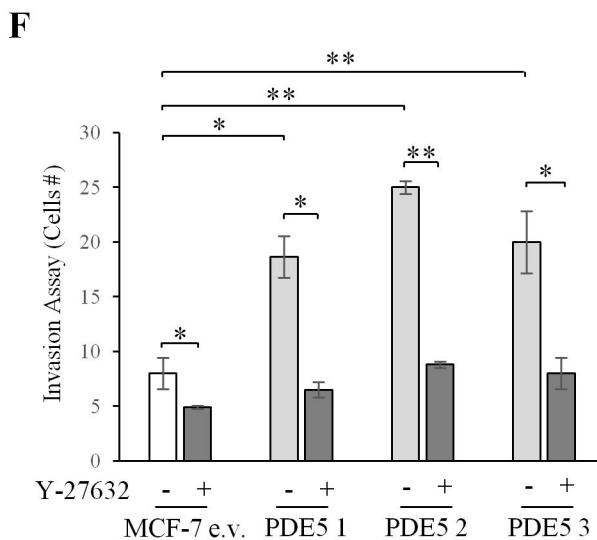
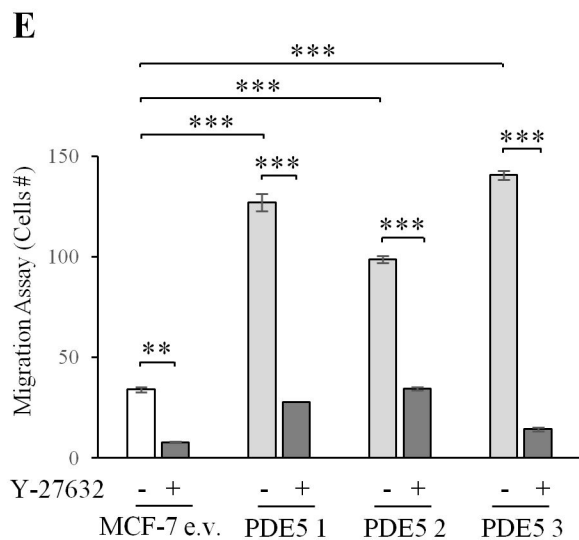
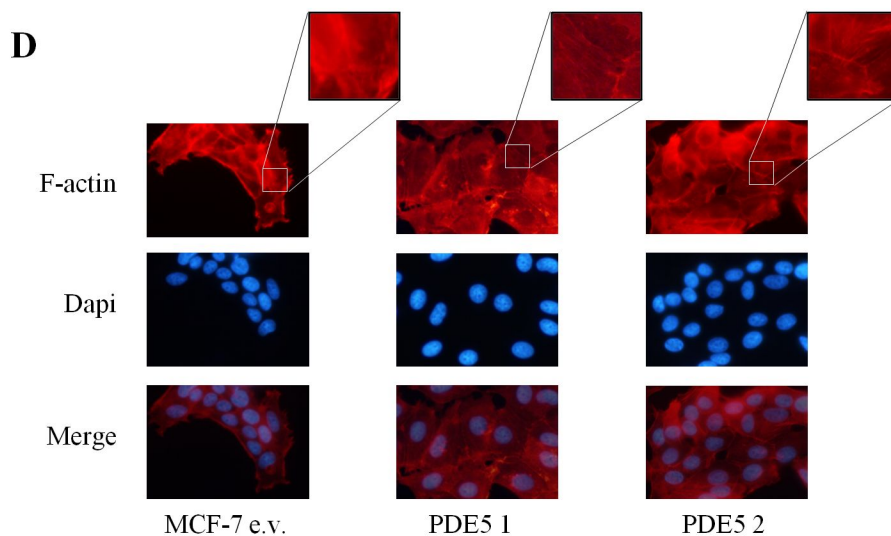
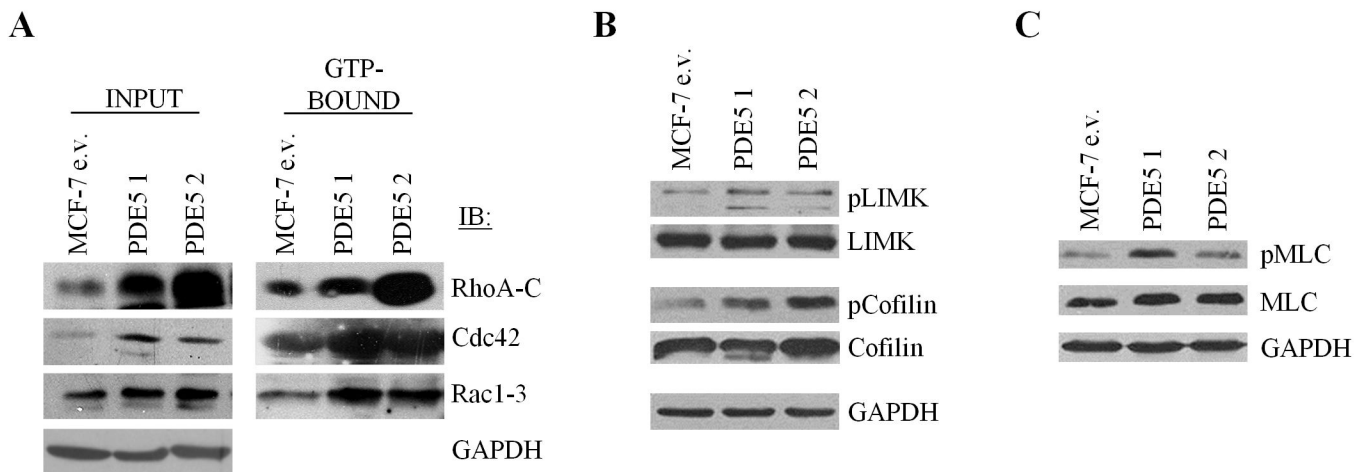


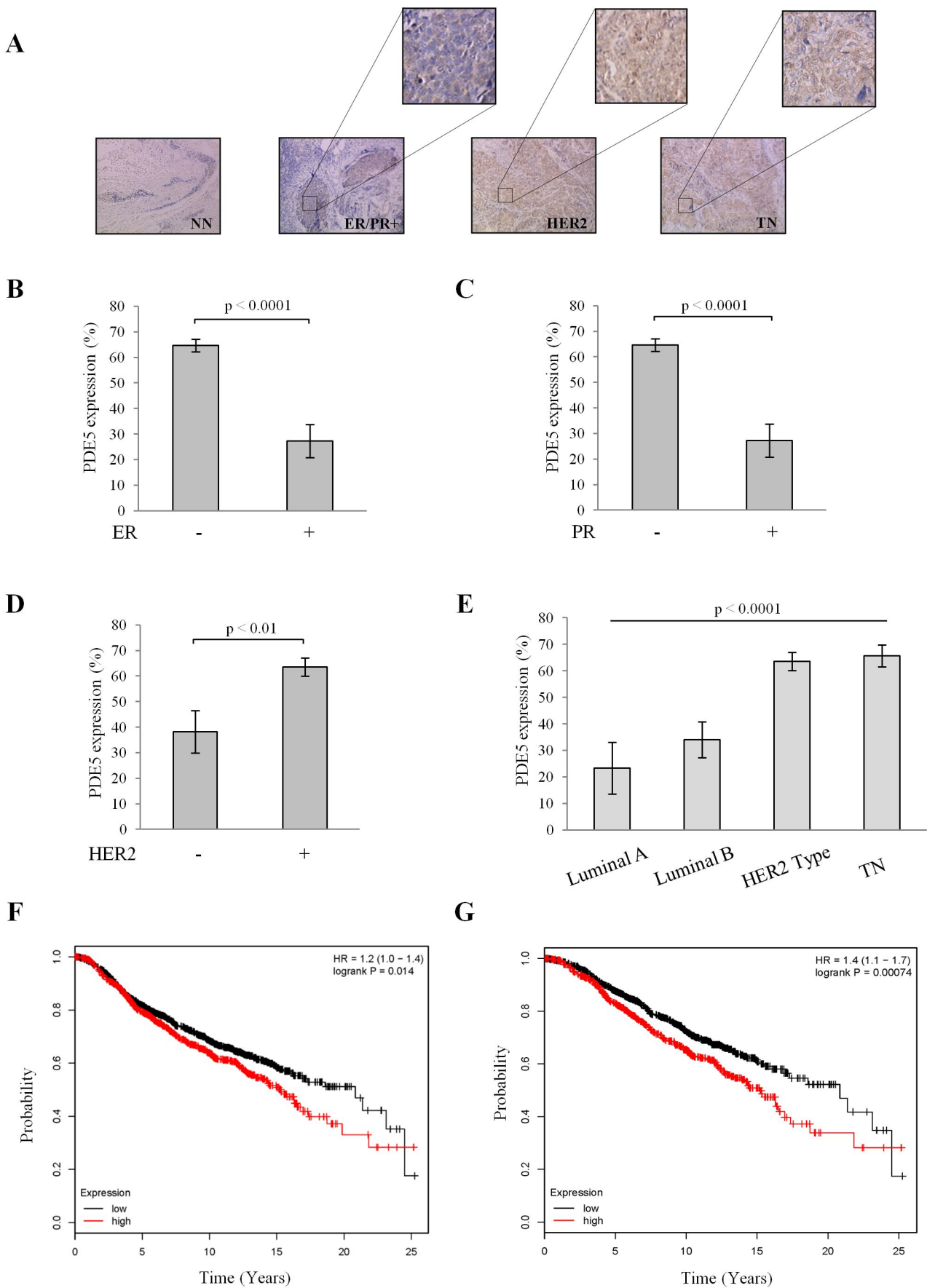
C



D







Clinical Cancer Research

Expression and Function of Phosphodiesterase Type 5 in Human Breast Cancer Cell Lines and Tissues: Implications for Targeted Therapy

Ines Barone, Stefania Catalano, Antonella Campana, et al.

Clin Cancer Res Published OnlineFirst December 14, 2015.

Updated version	Access the most recent version of this article at: doi: 10.1158/1078-0432.CCR-15-1900
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/12/12/1078-0432.CCR-15-1900.DC1.html
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .