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Epidermal Growth Factor Receptor (EGFR)-mediated Positive Feedback of Protein-tyrosine Phosphatase ϵ (PTPϵ) on ERK1/2 and AKT Protein Pathways Is Required for Survival of Human Breast Cancer Cells*

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Background: To investigate the functional role of PTPϵ in human breast cancer cell lines.
Results: PTPϵ was up-regulated in human breast cancer cells in an EGFR- and ERK1/2-dependent manner. PTPϵ displayed a positive role in survival of human breast cancer cells.
Conclusion: PTPϵ generates a positive feedback regulatory loop required for survival of human breast cancer cells.
Significance: PTPϵ could be a putative target in breast cancer treatment.

Increased tyrosine phosphorylation has been correlated with human cancer, including breast cancer. In general, the activation of tyrosine kinases (TKs) can be antagonized by the action of protein-tyrosine phosphatases (PTPs). However, in some cases PTPs can potentiate the activation of TKs. In this study, we have investigated the functional role of PTPϵ in human breast cancer cell lines. We found the up-regulation and activation of receptor PTPϵ (RPTPϵ) in MCF-7 cells and MDA-MB-231 upon PMA, FGF, and serum stimulation, which depended on EGFR and ERK1/2 activity. Diminishing the expression of PTPϵ in human breast cancer cells abolished ERK1/2 and AKT activation, and decreased the viability and anchorage-independent growth of the cells. Conversely, stable MCF-7 cell lines expressing inducible high levels of ectopic PTPϵ displayed higher activation of ERK1/2 and anchorage-independent growth. Our results demonstrate that expression of PTPϵ is up-regulated and activated in breast cancer cell lines, through EGFR, by sustained activation of the ERK1/2 pathway, generating a positive feedback regulatory loop required for survival of human breast cancer cells.

In human breast cancer cells, the tyrosine kinases (TKs)[3] from the human epidermal growth factor receptor (HER) family are major drivers of oncogenesis, as a consequence of their increased Tyr phosphorylation and activity (1). Conversely, a group of protein-tyrosine phosphatases (PTPs) counteract the action of HER kinases and are candidate breast cancer suppressor proteins (2). Src-family kinases (SFKs) are non-receptor TKs whose activity is also relevant for breast cancer tumorigenesis (3). However, most of PTPs that dephosphorylate SFKs, including the related receptor PTPs PTPRA/PTPα and PTPRE/PTPϵ, have a positive role on SFK activity, suggesting an oncogenic role for these PTPs (4–7). PTPϵ is present in cells as a receptor type (RPTPϵ) or as a cytosolic (cytPTPϵ) protein, encoded by different mRNAs (8–10). Alternative translation initiation from both RPTPϵ and cytPTPϵ mRNAs renders a third PTPϵ cytosolic protein product, p67-PTPϵ; and calpain cleavage generates in cells another cytosolic PTPϵ protein species, named p65-PTPϵ (11). In leukocytes, an additional cytosolic isoform lacking the C-terminal portion has been reported (12). RPTPϵ is located at the plasma membrane and is highly glycosylated in the extracellular region, while the cytPTPϵ, p67 and p65 are mainly localized in the cytosol (8, 11, 13, 14). Both RPTPϵ and cytPTPϵ can form dimers, making the phosphatase inactive, and removing of the 22 N-terminal residues causes enzyme inactivation by constitutive dimerization of the protein (15). In mammary tumor cells, as well as in HEK293 cells, RPTPϵ can be phosphorylated at its C-terminal Tyr-695 residue by HER2, which reduces dimerization and augments RPTPϵ activity to dephosphorylate and activate Src (16). On the other hand, phosphorylation by epidermal growth factor receptor (EGFR) of cytPTPϵ on the equivalent Tyr (Tyr-638) residue, increases cytPTPϵ association to tubulin from microtubules, reducing its phosphatase activity and localization near the plasma membrane (17). Finally, in osteoclasts, integrin activation also triggers Src-dependent cytPTPϵ phosphorylation on Tyr-638, which results in increased Src dephosphorylation and activation (18).

PTPϵ-deficient mice develop normally, breed well and are fertile, although they show functional abnormalities in bone marrow-derived macrophages (19) and defects in osteoclast subcellular organization and function (20). Abnormalities in

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[3] The abbreviations used are: TK, tyrosine kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide; Dox, doxycycline; PTP, protein-tyrosine phosphatase; SFK, Src-family kinase; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide; siNS, nonspecific silenced cell; siPTPϵ, PTPϵ-specific siRNA; HER, human epidermal growth factor receptor; FGF, fibroblast growth factor; FBS, fetal bovine serum; EGFR, epidermal growth factor receptor; BCCL, breast cancer cell line.

cellular morphology, together with reduced intracellular K^+ content and increased Ca^{2+}-activated K^+ channel activity were also documented in red cells from PTPε-deficient mice (21). In addition, at an early post-natal age these mice displayed hypomyelination in the sciatic nerve axons, most likely as a result of hyperphosphorylation of voltage-gated potassium channel Kv2.1 in Schwann cells (22, 23).

RPTPε is overexpressed in mouse mammary tumors initiated by activated Ras or activated HER2 (9). Other tumor types from the Ras-transgenic mice did not overexpress RPTPε, suggesting a specific linkage with mammary epithelial tumors. In mammary tumor cells where HER2 is expressed, the up-regulation of PTPε, together with its activation upon phosphorylation by HER2, facilitates the dephosphorylation of Src (at Tyr-530), Yes and Fyn, and activation of these SFKs (24, 25). Transgenic mice overexpressing RPTPε under the mouse mammary tumor virus (MMTV) promoter developed mammary gland hyperplasia, in which foci of transformed cells were observed, accompanied by residual milk production following several cycles of pregnancy. Additionally, these mice developed sporadic mammary tumors more frequently than wild type control mice (26). Cells from mammary epithelial tumors initiated by HER2 in PTPε knock-out mice confirmed these findings by appearing less transformed morphologically and with reduced cell proliferation (25). Together, these findings suggest that PTPε is necessary for the fully transformed phenotype of HER2-induced mouse mammary tumor cells (4).

Expression of PTPε is induced in several types of cancer cells in response to various stimuli, including IL-1, TNFα, NGF, FGF, IL-6, PDGF, and PMA (8, 27–31). In cells where HER2 is not present or expressed at low levels (such as NIH3T3 or HEK293 cells), PTPε dephosphorylates the adaptor protein Shc, which decreases ERK1/2 activation (32, 33). In these cells, PTPε formed a molecular complex with ERK1/2, possibly impeding their nuclear translocation (32). A negative regulatory role on ERK1/2 activation has also been reported for cytPTPε in lymphocytes (12, 31), and for RPTPε in primary hepatocytes, through dephosphorylation of the insulin receptor (34). In vascular smooth muscle cell lines, RPTPε dephosphorylates PDGF receptor-β, inhibiting PDGF-mediated cell signaling (27), and in M1-leukemia cells, cytPTPε dephosphorylates components of the JAK-STAT signaling pathway, which inhibits terminal differentiation and apoptosis by IL-6 (35).

The alteration of PTPε expression in human cancer has been poorly documented. Global genomic analysis identified PTPRE as a gene deleted in glioblastoma and whose expression is down-regulated in malignant pheochromocytoma (36, 37). In breast cancer, DNA microarray analysis revealed up-regulation of PTPε in invasive breast carcinomas *versus* normal breast tissue (38). On the other hand, human HER2-positive *versus* HER2-negative breast carcinomas, as well as normal fibroadenomas *versus* invasive ductal carcinomas, displayed down-regulation of PTPε expression (39). These findings support a role for PTPε both as a tumor suppressor and as an oncogene in human cancer. However, the putative role of PTPε in human cancer, including breast cancer, has not been experimentally addressed. In this study we have analyzed the expression and function of the different PTPε isoforms in human breast cancer cell lines. Our results reveal the existence of an EGFR-induced pro-survival positive feedback loop on the ERK1/2 and AKT pathways exerted by PTPε, and suggest an anti-apoptotic and oncogenic role for PTPε in human breast cancer.

EXPERIMENTAL PROCEDURES

cDNAs, Plasmids, Reagents, and Antibodies—The mammalian expression plasmids to generate double stable Tet-On cell lines, pTRE2hyg-cytPTPε and pTRE2hyg-RPTPε, wild type (wt) and mutants, were obtained by PCR (to remove the flag epitope) and subcloning from pCDNA3-flag-cytPTPε and pCDNA3-flag-RPTPε cDNAs (23). Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and epidermal growth factor (EGF, Invitrogen) were used at 50 ng/ml, during the indicated times, except for LNCaP cells, in which PMA was used at 1 ng/ml. Basic fibroblast growth factor (bFGF, Invitrogen) was used during the indicated times, at 10 or 20 ng/ml in MCF-7 and MDA-MB-231 cells, respectively. 10% of fetal bovine serum (FBS, Invitrogen) stimulation was performed on overnight serum-starved cells. The signaling pathways inhibitors used were: PD98059 (MEK-1 inhibitor, 20 μM; Calbiochem), SB203580 (p38 inhibitor, 10 μM; Calbiochem), SP600125 (JNK inhibitor, 20 μM; Biomol), GF109203X (PKC inhibitor, 1 μM; Biomol), wortmannin (PI3K inhibitor, 100 nM; Sigma), AG1478 (EGFR inhibitor, 10 μM; Calbiochem). Cells were pretreated with all inhibitors for 1 h before PMA stimulation, except for wortmannin, where pretreatment lasted for 4 h. The monoclonal antibodies used were anti-GAPDH, (Santa Cruz Biotechnology), anti-pERK1/2 [$pThr^{202}$ + $pTyr^{204}$] (New England Biolabs) and anti-caspase-8 (Cell Signaling). The polyclonal antibodies used were: anti-PTPε (raised against residues 154–167 from murine RPTPε cDNA (9)), anti-pY695 PTPε (16), anti-cleaved-PARP, anti-pAKT [$pThr^{308}$ + $pSer^{473}$] and anti-AKT (Cell Signaling), anti-EGFR (Santa Cruz Biotechnology), and anti-ERK1/2 (Santa Cruz Biotechnology). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit (Oncogene), anti-mouse (Promega), and anti-sheep (Sigma) IgG antibodies. Dilution of antibodies was done in NET-gelatin buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin). Quantification of protein band intensities from immunoblots was made using ImageJ 1.40g, from at least two different experiments.

Cell Culture, RNA Interference, Cell Lysis, and Immunoblot—All parental human breast cancer cell line were obtained from ATTC, and were grown as indicated (40). LNCaP prostate adenocarcinoma cell lines was grown in Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. HT-29 colon carcinoma cells were grown in McCoy 5A Medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. SH-SY5Y neuroblastoma cell line was grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate. To generate double-stable cell lines, MCF-7/Tet-On cell line was transfected with the pTRE2hyg plasmids (Clontech) using Fugene HD (Roche Diagnostics). To induce PTPε expression in MCF-7/Tet-On cell lines, cells were pre-

treated with 100–500 ng/ml doxycycline (Dox; Sigma) 24 h before processing for further treatment or analysis. Silencing of gene expression by RNA interference in MDA-MB-231 cells and in parental MCF-7 cell lines was performed by transfection of validated siRNAs (Ambion and Qiagen) with Lipofectamine 2000 (Invitrogen). For flow cytometry analysis, soft agar colony-growth and cell proliferation/viability MTT analysis, cells were processed 24–72 h post-transfection. Whole cell protein extracts were prepared by cell lysis and immunoblot as indicated (40).

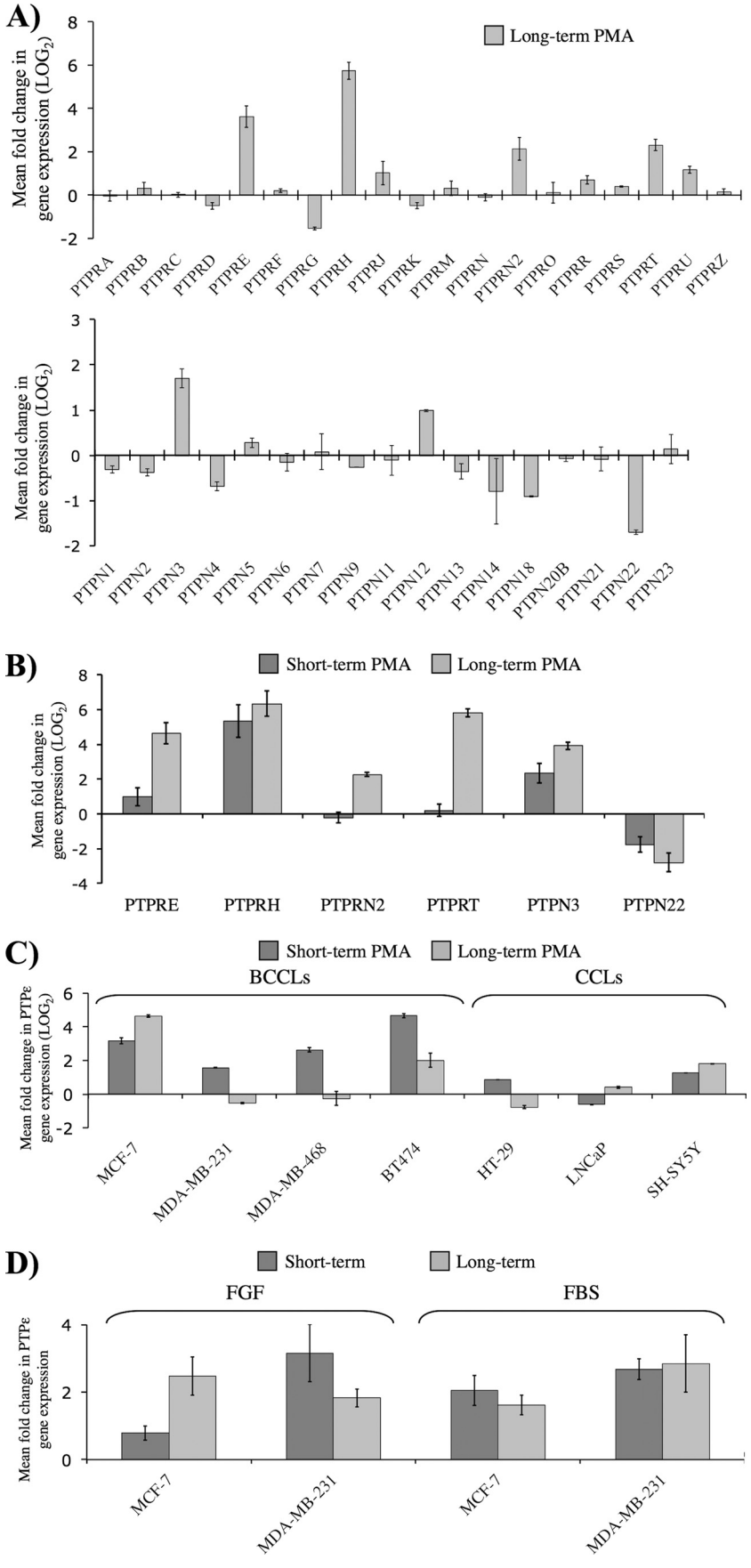
Gene Expression Profiling by DNA Microarray and Semi-quantitative and Quantitative Real-time PCR—DNA microarray analysis of gene expression was performed using total RNA from MCF-7 cells treated or not with PMA for 4 days, as previously described (40). Semi-quantitative RT-PCR or quantitative real-time PCR (qPCR) were performed using total RNA from cell lines treated or not with PMA, FGF, or serum (FBS) for different times. RNA was purified using illustra RNAspin mini purification kit (GE Healthcare). The breast tissue RNA was purchased from Clontech (Human Total RNA Master Panel II). 1 μg total RNA was subjected to reverse transcription (RT) using RevertAidTM reverse transcriptase, oligo(dT)18 primers, and RiboLock and RNase inhibitor (all from Fermentas). PCR reactions were performed using TaqDNA polymerase (Roche), as described (40). To assess the expression of the different PTPε isoforms by semi-quantitative RT-PCR, PCRs were performed on the synthesized cDNA samples (100 ng/reaction) using sets of isoform-specific primers (cytoplasmic forward: 5′-AGCAACAGGAGTAGCTTTTCC-3′; receptor forward: 5′-CGGGCGCCTCCCAGCCGC-3′; cytoplasmic and receptor reverse: 5′-CCAGTTGGCTCAGAATCACCC-3′) and β-actin primers (as a control). qPCR reactions were performed using Lightcycler 480 (Roche), with the corresponding SYBRGreenI Master (Roche), and validated primer sets (Qiagen) specific for the PTPs, EGFR, or the reference genes HPRT, ACTB, and HMBS. All quantifications were normalized to the reference gene data. Relative quantification was performed using the comparative $2(-\Delta\Delta C_t)$ method, or as the LOG_2 $(2(-\Delta\Delta C_t))$, according to the manufacturer's instructions.

Cell Functional Assays—Cell cycle phase distribution and cell death were determined by flow cytometry analysis after propidium iodide (PI) labeling. Cells were plated at a density of 4×10^5 cells per well (6-well plates), and grown in complete medium for 24 h. Cells were trypsinized and permeabilized, and labeled with PI (red fluorescence; Sigma-Aldrich). For soft agar colony formation assays, cells were plated at a density of 2500 cells per well (12-well plates) in 0.5 ml of complete media containing 0.35% cell culture tested agar (Sigma-Aldrich), onto a solidified bottom layer of 0.5 ml of complete media with 0.4% agar. Colonies were stained after 2–3 weeks with 0.05% crystal violet, and were photographed at X4 and ×40 magnification. Quantification of the number of colonies was made using ImageJ 1.40g, from triplicate plates. Cell proliferation/viability was determined using the MTT assay, according to the manufacturer's protocol (Roche). Cells were plated at a density of 3000 cells per well (96-well plates) with complete medium for 24 h. Then cells were incubated for 1–4 days and collected for processing. The absorbance was measured at 570 nm. Data are presented as the average absorbance ± S.D. corrected for background, from at least three different experiments.

RESULTS

Differential Expression of Receptor and Cytosolic PTPε in Human Breast Cancer Cells—To search for PTPs involved in the control of cell growth in breast cancer, a comprehensive analysis of the expression of the complete panel of human classical PTPs (36 genes) was performed. mRNA was obtained from MCF-7 human breast carcinoma cells, grown under control conditions or in the presence of the phorbol ester PMA, a PKC activator that causes prolonged activation of the MAP kinases ERK1/2 (41, 42), and PTP expression was measured by DNA microarray gene-expression analysis (Fig. 1*A*). As shown, significant mRNA up-regulation was observed for PTPRE/PTPε, PTPRH/SAP1, PTPRN2/PTP-IA-2β, PTPRT/RPTPρ, and PTPN3/PTPH1. Conversely, PTPN22/LYP mRNA was significantly down-regulated. These results were validated by quantitative real-time PCR (qPCR), using oligonucleotides specific for the above mentioned PTPs and mRNA from MCF-7 cells treated with PMA for short-term (6 h) or long-term (72 h) (Fig. 1*B*). PTPRE/PTPε has been related with mammary carcinogenesis in mice, although scarce information is available on its role in human cancer. Thus, we focused our study on PTPRE/PTPε. Quantitative expression of PTPε mRNA was tested in several breast cancer cell lines (BCCLs) upon short- and long-term PMA treatment (Fig. 1*C*, *left panel*). Up-regulation of PTPε was observed in both MCF-7 and BT474 cells after short- and long-term PMA treatment. A more moderate up-regulation of PTPε was observed in MDA-MB-231 and MDA-MB-468 cells upon short-term PMA stimulation. In other human adenocarcinoma cells, such as colon HT-29 and prostate LNCaP cells, no significant up-regulation was observed upon PMA treatment, whereas in the SH-SY5Y human neuroblastoma cells, PTPε was moderately up-regulated (Fig. 1*C*, *right panel*). This indicates that PTPε up-regulation upon PMA treatment is not exclusive to MCF-7 cells, and it is selectively achieved in different BCCLs and CCLs. We also analyzed PTPε expression in MCF-7 and MDA-MB-231 cells upon other ERK1/2 activating stimuli, such as fibroblast growth factor (FGF) or serum (FBS). Up-regulation of PTPε was observed in both MCF-7 and MDA-MB-231 cells after short- and long-term treatment with FGF or serum, demonstrating that PTPε up-regulation in these cells is achieved by various stimuli (Fig. 1*D*).

Next, we investigated the identity of the PTPε mRNAs and proteins in the distinct human breast cancer cell lines grown in the absence and in the presence of PMA. Semiquantitative RT-PCR was performed, using oligonucleotides specific for cytPTPε and RPTPε mRNAs (Fig. 2*A*). MCF-7 cells treated with PMA expressed the RPTPε mRNA, whereas MDA-MB-231 expressed RPTPε constitutively and displayed cytPTPε up-regulation upon PMA treatment. In other breast cancer cell lines, the expression and induction of PTPε isoforms was variable. We also monitored the relative abundance of cytPTPε and RPTPε mRNAs on human breast tissue (Fig. 2*A*). As shown, human breast tissue expressed both cytPTPε and RPTPε


A)
Long-term PMA
Mean fold change in gene expression (LOG2)
PTPRA PTPRB PTPRC PTPRD PTPRE PTPRF PTPRG PTPRH PTPRJ PTPRK PTPRM PTPRN PTPRN2 PTPRO PTPRR PTPRS PTPRT PTPRU PTPRZ
PTPN1 PTPN2 PTPN3 PTPN4 PTPN5 PTPN6 PTPN7 PTPN9 PTPN11 PTPN12 PTPN13 PTPN14 PTPN18 PTPN20B PTPN21 PTPN22 PTPN23
B)
Short-term PMA
Long-term PMA
PTPRE PTPRH PTPRN2 PTPRT PTPN3 PTPN22
C)
BCCLs
CCLs
Mean fold change in PTPε gene expression (LOG2)
MCF-7 MDA-MB-231 MDA-MB-468 BT474 HT-29 LNCaP SH-SY5Y
D)
Short-term
Long-term
FGF
FBS
Mean fold change in PTPε gene expression
MCF-7 MDA-MB-231 MCF-7 MDA-MB-231

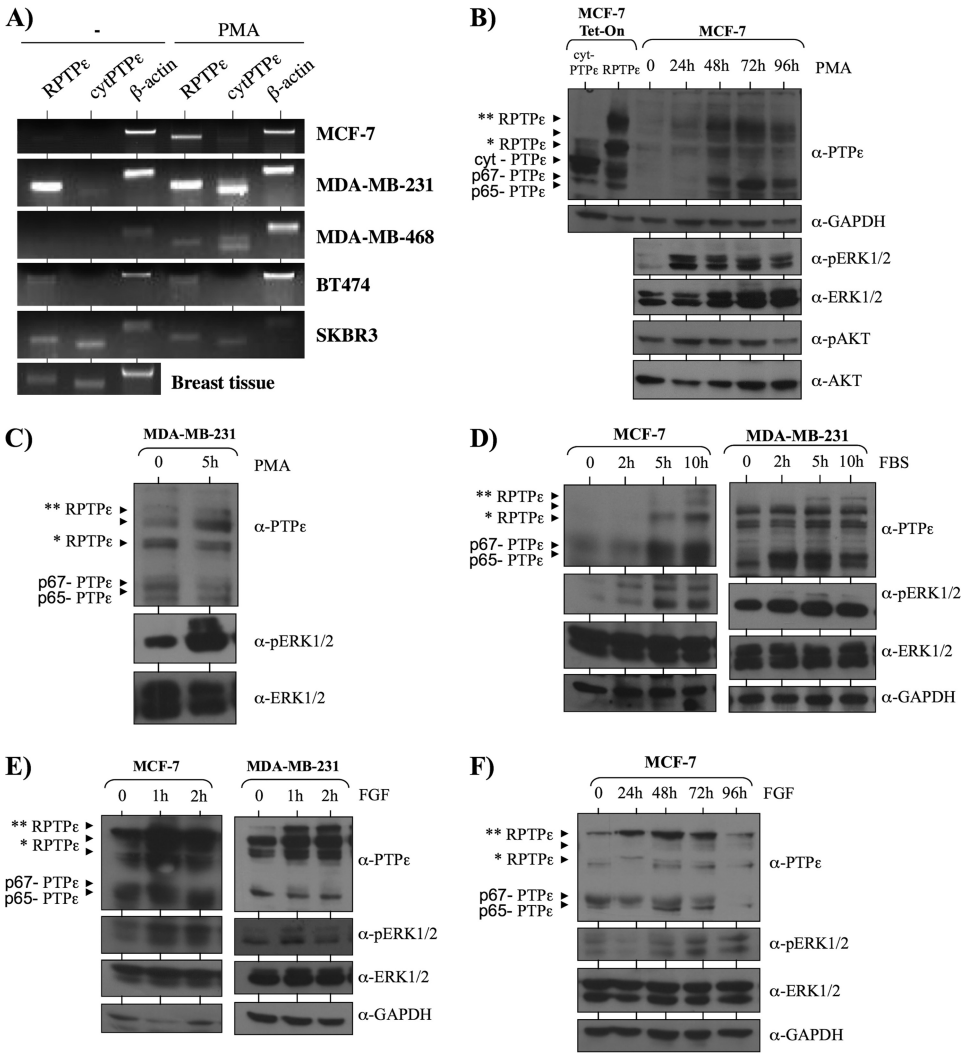


FIGURE 2. **PTPε isoform expression in human breast cancer cell lines treated with PMA, serum, or FGF.** *A*, relative mRNA expression of PTPε isoforms in human BCCLs. Semi-quantitative RT-PCR was performed from mRNA from the different BCCLs grown in the absence or in the presence of PMA (6 h), as well as from human breast tissue, using primers specific for cytPTPε, RPTPε, or β-actin. *B*, up-regulation of RPTPε protein in MCF-7 cells upon PMA treatment. MCF-7 cells were left untreated or were treated with PMA for different times, followed by immunoblot analysis using anti-PTPε antibody (α-PTPε). In the *left*, the migration of recombinant PTPε isoforms (overexpressed in MCF-7 cells using pTREhyg-cytPTPε and pTREhyg-RPTPε) is illustrated. The *arrows* indicate the migration of the different PTPε species. *, non-glycosylated RPTPε; **, fully glycosylated RPTPε. Note the increase in RPTPε protein content from MCF-7 cells treated with PMA. ERK1/2 and pERK1/2 levels, and AKT and pAKT levels, were monitored in parallel using specific antibodies, as indicated. *C*, PTPε protein expression in MDA-MB-231 cells upon PMA treatment. Cells were left untreated or were treated with PMA for 5 h, followed by immunoblot analysis as in *B*. Note the high constitutive expression of RPTPε in MDA-MB-231 cells. Levels of ERK1/2 and pERK1/2 were also determined. *D*, up-regulation of RPTPε protein in MCF-7 and MDA-MB-231 cells upon serum treatment. Cells were left untreated or were treated with serum (FBS) for different times. *E*, up-regulation of RPTPε protein in MCF-7 and MDA-MB-231 cells upon FGF treatment. Cells were left untreated or were treated with FGF for different times. *F*, up-regulation of RPTPε protein in MCF-7 cells after long-term FGF treatment. Cells were left untreated or were treated with FGF for different times. Actin or GAPDH content were included as loading controls. In all the panels, a representative immunoblot is shown from at least three different experiments.

mRNAs, in line with what observed in human breast cancer cell lines.

Next, the expression of PTPε protein in MCF-7 and MDA-MB-231 cells was investigated, using a specific anti-PTPε antibody that recognizes all four isoforms of PTPε (11) (Fig. 2, *B* and *C*). MCF-7 cells displayed increased expression of the RPTPε, p67, and p65 isoforms, upon PMA treatment, which was sustained up to 96 h. This kinetics was delayed with respect to the

FIGURE 1. **PTPε mRNA is up-regulated in human breast cancer cell lines treated with PMA, serum, or FGF.** *A*, DNA microarray analysis of the expression of classical PTPs on PMA-treated MCF-7 cells. PTP gene expression was obtained from normalized data, and calculation of Log_2 fold-change ± S.D. was performed dividing values from PMA-treated (4 days) to control untreated cells. *B*, qPCR analysis of expression of classical PTPs in MCF-7 cells treated with PMA. Validated gene specific primer sets for the 6 different PTPs were used for the qPCR, and a primer set for HPRT was used as a normalization reference gene. mRNA levels of PTPRE/PTPε, PTPRH, PTPRN2, PTPRT, PTPN3, and PTPN22 were calculated from MCF-7 cells untreated or treated with PMA for short-term (6 h) or long-term (72 h). Relative expression values are shown in Log_2 as fold change ± S.D. of treated cells *versus* untreated cells. *C*, qPCR analysis of PTPε expression in human cancer cell lines treated with PMA. PTPε mRNA levels were calculated as in *B*, from breast cancer cell lines (BCCLs) MCF-7, MDA-MB-231, MDA-MB-468, BT474, and SKBR3, and from cancer cell lines (CCLs), HT-29 (colon adenocarcinoma), LNCaP (prostate adenocarcinoma), and SH-SY5Y (neuroblastoma). *D*, qPCR analysis of PTPε expression in MCF-7 and MDA-MB-231 cells treated with FGF or serum. PTPε mRNA levels were calculated as in *B*, from MCF-7 and MDA-MB-231 cells untreated or treated with serum (FBS) or FGF for short-term (2 h) or long-term (7 h). Relative expression values were calculated as in *B*, and shown as fold change ± S.D.

kinetics of activation of ERK1/2, as monitored by pERK1/2 content (Fig. 2*B*). High levels of RPTPε, p67, and p65 proteins were observed in untreated MDA-MB-231 cells, which were modestly increased upon PMA treatment. Despite our mRNA results, we did not detect expression of cytPTPε protein isoform, in consistence with previous reports showing non-overlapping protein expression of cytPTPε and RPTPε isoforms (8, 10). Remarkably, the high basal content of RPTPε in MDA-MB-231 cells correlated with high basal ERK1/2 activation (Fig. 2*C*). We also tested PTPε protein expression upon cell stimulation with FGF or serum. RPTPε was up-regulated in MCF-7 and MDA-MB-231 cells upon these stimuli (Fig. 2, *D*, *E*, and *F*). In general, we observed a correlation between RPTPε up-regulation and ERK1/2 activation, suggesting a functional relation between PTPε and ERK1/2 activity.

PTPε Up-regulation in Breast Cancer Cells Depends on the EGFR and ERK1/2 Pathways—Using specific inhibitors we tested the involvement of distinct signaling pathways, including ERK1/2, p38, JNK, PI3K/AKT, and PKC pathways, in the up-regulation of RPTPε in MCF-7 and MDA-MB-231 cells treated with PMA (Fig. 3*A*, and data not shown). As shown, the PKC inhibitor GF109203X strongly diminished the PMA-triggered up-regulation of RPTPε mRNA and protein (Fig. 3*A*, and data not shown), in agreement with previous reports that identified PKC as the major upstream target of PMA in MCF-7 and MDA-MB-231 cells (43–47). The ERK1/2 pathway-specific inhibitor PD98059 partially inhibited the up-regulation of RPTPε, both at the mRNA and protein level (Fig. 3*A*). In contrast, the p38-specific SB203580, the JNK-specific SP600125, and the PI3K-specific wortmannin inhibitors slightly increased RPTPε up-regulation (data not shown).

Up-regulation of EGFR, but not of other HER family members in MCF-7 cells treated with PMA was observed by DNA microarray analysis, which was confirmed by qPCR (Fig. 3*B*, *left panels*). Also, FGF and serum stimulation triggered the up-regulation of EGFR in MCF-7 and MDA-MB-231 cells (data not shown). Increase in EGFR protein expression in PMA-treated MCF-7 cells was also observed by immunoblot (Fig. 3*B*, *right panel*). This prompted us to investigate the putative role of EGFR in PTPε up-regulation. Treatment of cells with the AG1478 EGFR inhibitor prevented the up-regulation RPTPε by PMA, as well as the activation of ERK1/2 and AKT (Fig. 3*C*). Experiments with stable MCF-7 cell lines overexpressing RPTPε (see below) demonstrated that RPTPε is phosphorylated in tyrosine 695. Remarkably, this phosphorylation was prevented in the presence of the AG1478 EGFR inhibitor (Fig. 3*D*). Together, these results indicate that EGFR activity is required for RPTPε up-regulation and activation.

Ectopic Expression of PTPε in MCF-7 Cells Enhances Induced ERK1/2 Phosphorylation, and Affects Colony Formation in Soft Agar—Stable MCF-7 clones were generated overexpressing, in a doxycycline (Dox)-dependent, inducible-manner, cytPTPε or RPTPε wild type or catalytically inactive/substrate-trapping mutants (cytPTPε wt, cytPTPε D245A, RPTPε wt, and RPTPε D302A). Overexpression of ectopic PTPε was efficiently induced upon Dox treatment, although some leakage expression was observed in the absence of Dox, especially in the case of cytPTPε (Fig. 4*A*). Ectopic overexpression of wild type RPTPε or cytPTPε enhanced the pERK1/2 levels upon EGF stimulation, when compared with EGF-treated, control empty vector cell line. On the other hand, in the catalytically inactive substrate-trapping mutant cell lines, a decrease in pERK1/2 levels was observed, suggesting a dominant negative effect on ERK1/2 activation for these mutations (Fig. 4*A*). Similar results were obtained upon stimulation with PMA or serum (data not shown).

The functional consequences of PTPε overexpression in MCF-7 cells were also investigated at the cellular level. Because of PTPε leakage expression in our MCF-7 cells, in the functional studies with the PTPε clones we have compared empty vector (EV) clones with PTPε clones, always in the presence of Dox. The capacity of colony formation in soft agar of the cell lines was tested. MCF-7 cells overexpressing wild type RPTPε or cytPTPε displayed enhanced capacity of colony formation in soft agar, while those overexpressing cytPTPε D245A or RPTPε D302A displayed a decrease in colony formation in soft agar (Fig. 4*B*). Because PTP substrate-trapping mutants bind their substrates also in the presence of wild-type PTPs (48), our results suggest that endogenous PTPε may play as positive role in cell growth.

Silencing of PTPε Decreased Breast Cancer Cell Viability and Colony Formation in Soft Agar, and Abolished ERK1/2 and AKT Activation upon Stimulation—To investigate further the functional role of PTPε in breast cancer cells, PTPε expression was down-regulated in MCF-7 cells using specific siRNA. Down-regulation of PTPε (siPTPε; ~80% efficiency as measured by qPCR and immunoblot) induced robust cell death when compared with nonspecific silenced cells (siNS), as indicated by the morphology of the cells (Fig. 5*A*). This was confirmed by flow cytometry, measuring cell death, and sub-G0 cell cycle distribution by PI staining, and by immunoblot analysis of apoptotic cell markers. Increased cell death and retention in G0-G1 phase was observed on the siPTPε-silenced cells after 72 h of silencing, when compared with control cells (Fig. 5*B*). We also detected, upon silencing of PTPε, the presence of markers for cells undergoing apoptosis, such as cleaved caspase-8 and cleaved PARP (Fig. 5*B*), indicating that silencing of PTPε in MCF-7 cells triggers caspase-mediated apoptotic pathways. Formation of colonies in soft agar was also decreased upon silencing of PTPε in MCF-7 cells, when compared with nonspecific silenced cells (siNS) (Fig. 5*C*). Cell proliferation and viability of the cells was measured by MTT assay, using two different siRNAs (siPTPε #1 and siPTPε #2). After silencing for 48 and 72 h, diminished cell viability was observed in siPTPε cells, when compared with control-silenced cells (Fig. 5*D*). Finally, silencing of PTPε in MDA-MB-231 also caused less proliferation and viability, when compared with siNS cells (Fig. 5*E*). Together, these data suggest that PTPε is required for the survival of BCCLs.

The effect of PTPε silencing on MCF-7 cell signaling was tested by immunoblotting. To this end, 48 h after transfection of siRNAs, cells were treated for 24 h with PMA (Fig. 6*A*). Silenced PTPε cells showed diminished pERK1/2 and pAKT levels when compared with PMA-treated, control-silenced cells. Interestingly, upon silencing of PTPε, lower basal levels of total AKT were observed. However, the basal ERK1/2 levels

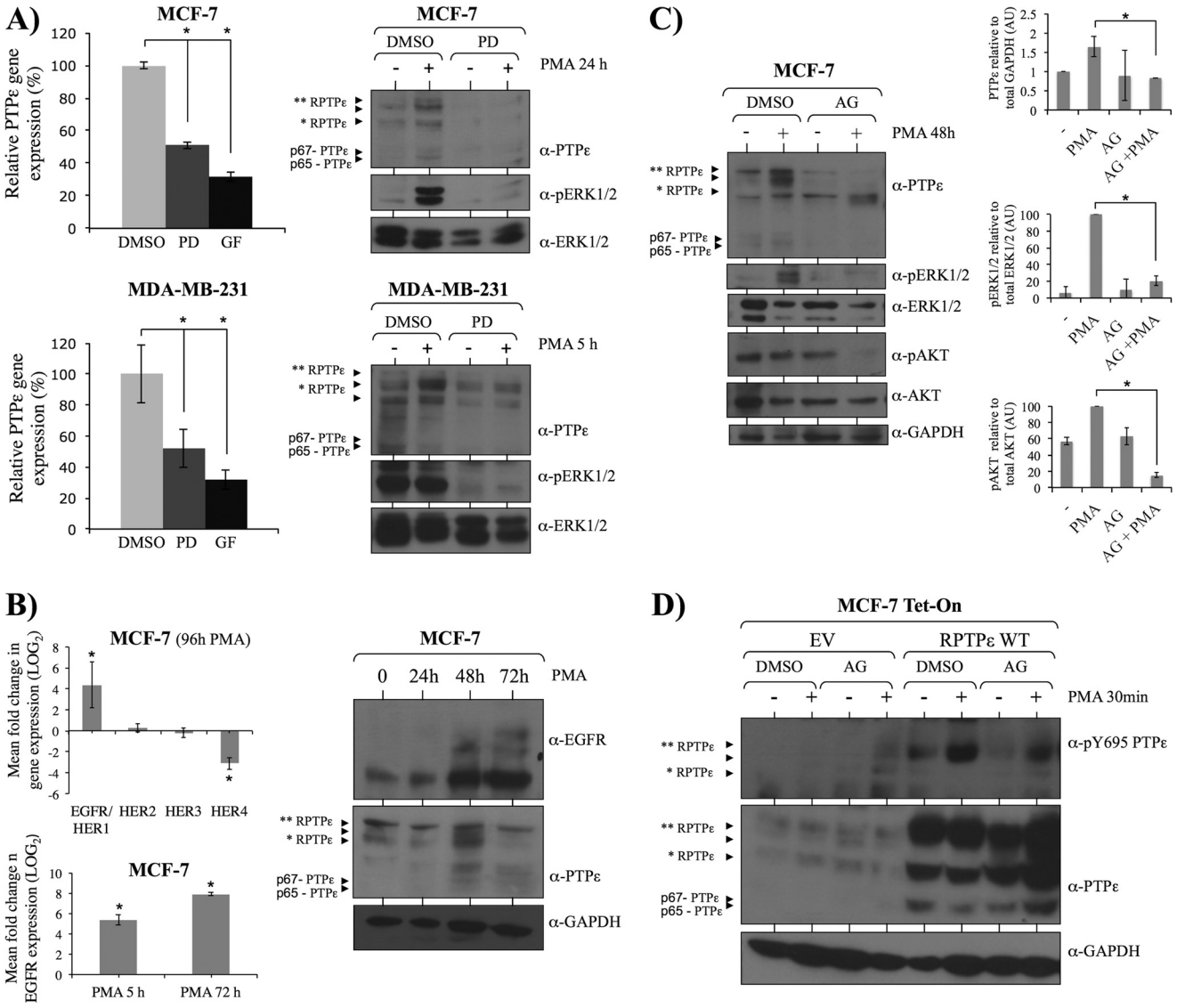


FIGURE 3. **Induced PTPε up-regulation in human breast cancer cells requires the activation of EGFR and ERK1/2 pathways.** *A*, inhibition of ERK1/2 pathway decreases the up-regulation of RPTPε mRNA and protein in MCF-7 and MDA-MB-231 cells upon PMA treatment. Cells were pre-treated with DMSO, or with the indicated inhibitors prior to PMA treatment, and harvested after PMA treatment. In the *left panel*, the fold change in PTPε mRNA levels upon PMA treatment in the presence of DMSO or the inhibitors was measured by qPCR, and relative expression values (%) are shown. In the *right panel*, RPTPε protein levels in untreated and PMA-treated cells, after treatment with the inhibitors, were monitored by immunoblot. *, non-glycosylated RPTPε; **, fully glycosylated RPTPε. *PD*, MEK1 inhibitor (PD98059); *GF*, PKC inhibitor (GF109203X). *B*, EGFR is up-regulated in MCF-7 cells upon PMA treatment. In the *upper left panel*, DNA microarray analysis of the expression of HER family members in MCF-7 cells treated with PMA. In the *lower left panel*, qPCR analysis of EGFR expression in MCF-7 cells treated with PMA. Results are shown as in *A*. In the *right panel*, immunoblot analysis of EGFR protein expression in MCF-7 cells treated with PMA. *C*, inhibition of EGFR activity decreases the up-regulation of RPTPε in MCF-7 cells upon PMA treatment. Cells were pre-treated with DMSO or with AG1478 EGFR inhibitor (*AG*), prior to PMA treatment, and harvested after 48 h of PMA treatment. PTPε, pERK1/2, ERK1/2, pAKT, and AKT levels were analyzed by immunoblot. Quantification of PTPε expression relative to GAPDH, pERK1/2 relative to total ERK1/2, and pAKT relative to total AKT, are shown as arbitrary units (*AU*) in the *right panels*. In *A*, *B*, and *C*, data represent the mean values ± S.D., statistically significant results are marked with: *, $p < 0.005$. *D*, RPTPε tyrosine phosphorylation in MCF-7 cells depends on EGFR activity. Empty vector (EV) MCF-7-Tet-On cells, or MCF-7-Tet-On cells expressing RPTPε wt were generated, and ectopic expression of PTPε was induced with Dox. Cells were pre-treated with DMSO or with AG, prior to PMA treatment, and harvested after 30 min of PMA treatment. RPTPε phosphorylated on Tyr-695 residue and total RPTPε levels were analyzed by immunoblot using specific antibodies. In *B*, *C*, and *D*, GAPDH content is included as a protein loading control.

were not affected by the silencing. Together, these results demonstrate that endogenous PTPε exerts in MCF-7 cells a positive regulation of ERK1/2- and AKT-mediated responses to PMA.

DISCUSSION

The pTyr content in cells is tightly regulated by the actions of TKs and PTPs, which play important roles in breast cancer (1, 2). In this work, we have analyzed the expression of classical PTPs in the MCF-7 human breast cancer cell line grown in the presence of PMA, a pleiotropic agent that activates PKC and ERK1/2. Using DNA microarray technology and semi-quantitative and quantitative PCR methods, we show that five classical PTP mRNAs were up-regulated in MCF-7 cells treated with PMA, *i.e.* PTPRE/PTPε, PTPRH/SAP1, PTPRN2/PTP-IA-2β, PTPRT/PTPρ, and PTPN3/PTPH1. PTPRT and PTPN3 transcripts have also been found to be up-regulated in breast cancer samples (49, 50). Only one PTP mRNA, PTPN22/LYP, was down-regulated in this system. Interestingly, alterations on

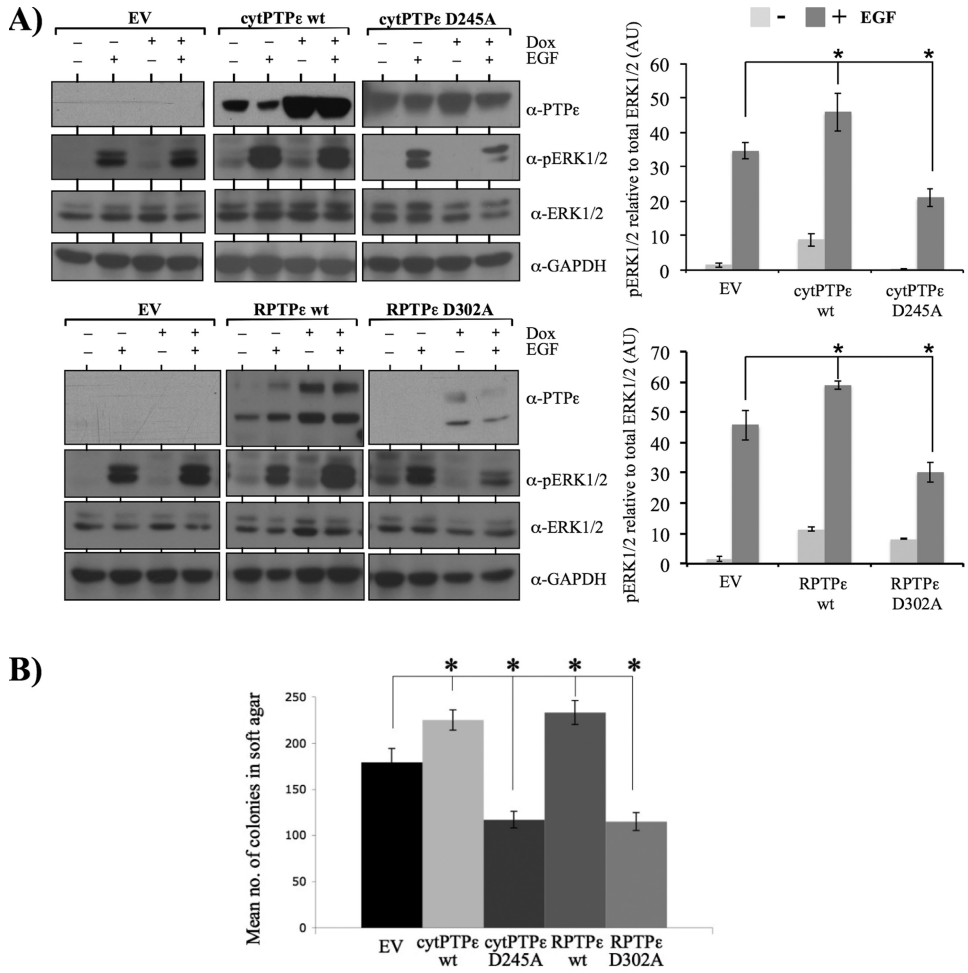


FIGURE 4. **Functional analysis of stable MCF-7 cell lines expressing PTPε.** *A*, MCF-7-PTPε cells display higher activation of ERK1/2 upon EGF stimulation. Empty vector (*EV*) MCF-7-Tet-On cells, or MCF-7-Tet-On cells expressing cytPTPε wild type (*wt*), cytPTPε D245A, RPTPε wt, or RPTPε D302A, were generated, and ectopic expression of PTPε was induced with Dox. Cells were incubated in the absence or in the presence of EGF for 5 min, and levels of PTPε, pERK1/2, and ERK1/2 were determined by immunoblot. GAPDH expression is included as a loading control. A representative immunoblot is shown out of at least three different experiments from at least two different clones. Quantification of pERK1/2 relative to total ERK1/2 levels is shown in arbitrary units (*AU*) in the right panels from doxycyline-induced cells. *B*, MCF-7-PTPε cells form more colonies in soft agar. Stable cell lines expressing cytPTPε or RPTPε, wt or catalytically inactive substrate-trapping mutants (D245A and D302A) were pre-treated for 24 h with Dox before plating in soft agar for the formation of anchorage-independent colonies. Cells were grown for 2 weeks in soft agar, and photographs of representative plates were taken. Quantification of the number of colonies, using ImageJ 1.40g, from triplicate plates, is shown. In *A* and *B*, data represent the mean values ± S.D., statistically significant results are marked with: *, $p < 0.005$.

PTPN22 function are associated with the unbalanced regulation of SFKs during autoimmune diseases (51), but the links between PTPN22 and breast cancer remain to be explored.

From our screening, we found PTPε of special interest since this PTP had previously been related with the transformation of mouse mammary tumors (4). However, the association of PTPε with human breast cancer has not been studied in detail. We found up-regulation of PTPε mRNA by PMA in several breast cancer cell lines, but PTPε expression was not induced in the LNCaP prostate or HT-29 colon cancer cell lines. Immunoblot analysis showed that the PTPε isoform mainly induced in MCF-7 and MDA-MB-231 cells was the receptor form, RPTPε, together with the two smaller isoforms: p65 and p67. We also found up-regulation of RPTPε by serum and FGF in MCF-7 and MDA-MB-231 cells. However, the patterns of basal and induced expression of PTPε isoforms in other breast cancer cells were variable, suggesting that expression of PTPε is tightly regulated in mammary cells. In mouse mammary tumors initiated by MAPK upstream activators, such as constitutively active forms of HER2 and Ras, high levels of RPTPε were observed (9). In line with these findings, we have found that up-regulation of both RPTPε mRNA and protein, in MCF-7 and MDA-MB-231 cells, required the activation of EGFR and ERK1/2 pathways. Interestingly, EGFR is up-regulated and activated upon PMA-treatment (52, 53); and our results), suggesting a link between EGFR activity and PTPε up-regulation. At this regard, our results using the AG1468 EGFR inhibitor indicate that EGFR activity is required for up-regulation and phosphorylation of RPTPε. Because EGFR is a current target for triple negative breast cancer therapy, it would be of interest to analyze the status of RPTPε in EGFR-altered breast cancer samples.

In mouse mammary tumor cells from transgenic mice overexpressing RPTPε in the mammary tissue, RPTPε dephosphorylates and activates Src, which favors a transformed phenotype. No consistent changes in Src phosphorylation and activation

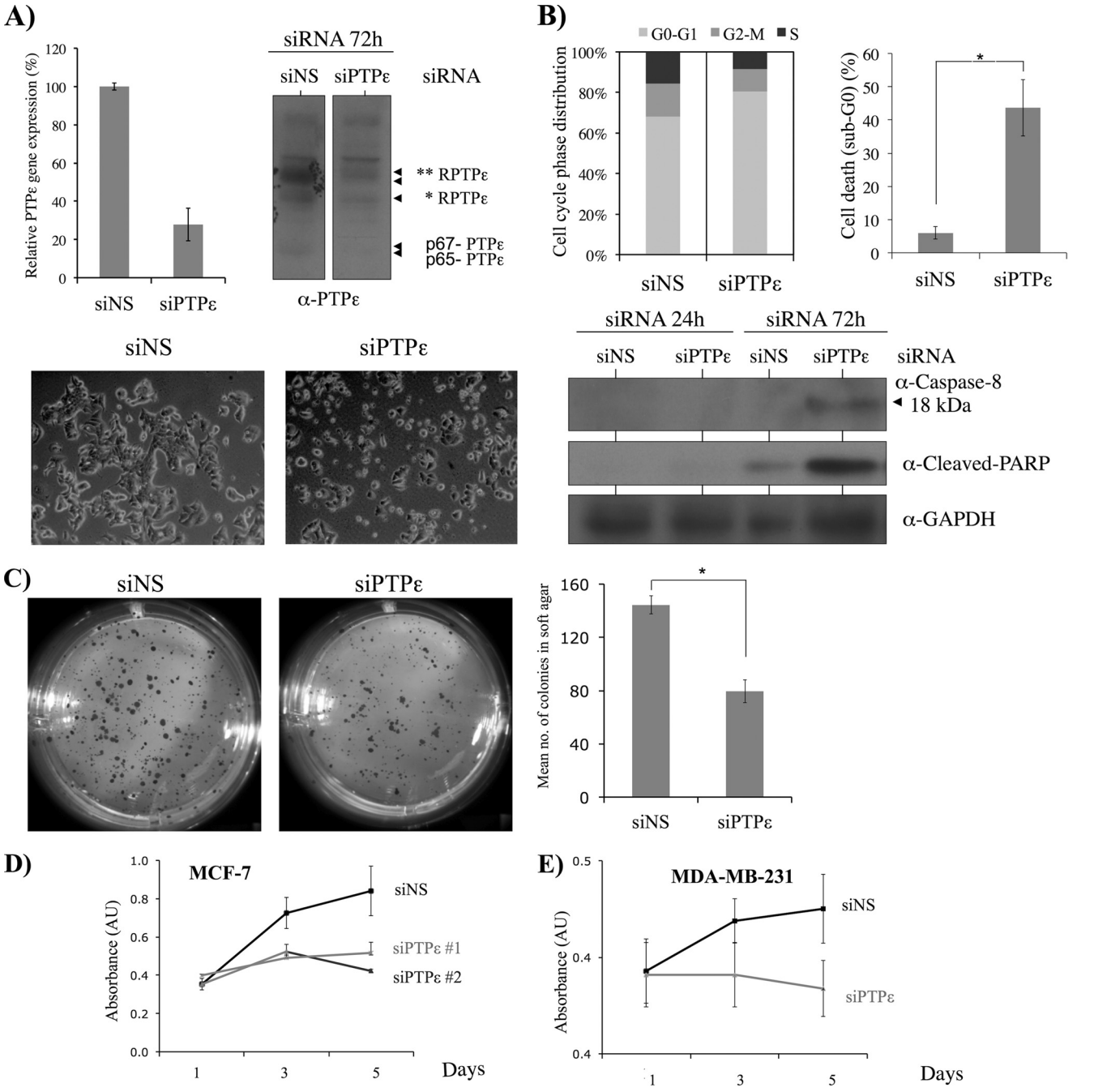


FIGURE 5. **Functional analysis of MCF-7 cells after silencing of PTPε by RNA interference.** *A*, morphological changes in PTPε-silenced MCF-7 cells. Cells were transfected with siNS or with siPTPε. 72 h after transfection cells were photographed (*bottom panel*), and then were harvested for PTPε mRNA and protein expression analysis (*top panels*). *B*, silencing of PTPε in MCF-7 cells increases retention of the cells in G0-G1 cell cycle phase and induces apoptotic markers and cell death. MCF-7 cells were transfected with siNS or with siPTPε for 72 h, and then processed for cell cycle analysis by PI staining and flow cytometry analysis. Distribution of % of cells in cell cycle phase G0-G1, G2-M, and S is shown on the *upper left panel*, and sub-G0 representing cell death is shown in the *upper right panel*. Data are shown from the mean of two independent experiments. In the *bottom panel*, cells were silenced as above, and the levels of cleaved caspase-8 and cleaved PARP were determined by immunoblot. *C*, silencing of PTPε in MCF-7 cells decreases colony formation in soft agar and viability. In the *left panel*, MCF-7 cells were transfected with siNS or with siPTPε, and cells were plated in soft agar for the formation of anchorage independent colonies. Cells were grown for 2 weeks in soft agar, and photographs of representative plates were taken. In the *right panel*, quantification of the number of colonies, using ImageJ 1.40g, from triplicate plates, is shown. Results represent the mean values ± S.D. *, $p < 0.005$. *D*, silencing of PTPε decreases viability in MCF-7 cells. MCF-7 cells were transfected with siNS or with two different PTPε specific siRNAs (siPTPε#1 and siPTPε#2), and cell viability was measured during 4 days by the MTT assay. *E*, silencing of PTPε decreases viability in MDA-MB-231 cells. MDA-MB-231 cells were transfected with siNS or siPTPε-specific siRNAs, and cell viability was measured by MTT as above. In *B* and *C*, data represent the mean values ± S.D., statistically significant results are marked with: *, $p < 0.005$.

were detected in our studies when RPTPε overexpressing or silenced MCF-7 cells where compared with empty vector-expressing or control-silenced MCF-7 cells (data not shown). The possibility that other SFKs could be targets of PTPε in this cell system needs to be explored. In this regard, we have identified Lyn and Fyn as SFKs whose mRNAs are up-regulated in MCF-7 cells upon PMA stimulation (data not shown). Alternatively, it is possible that the molecular mechanism of PTPε action in the

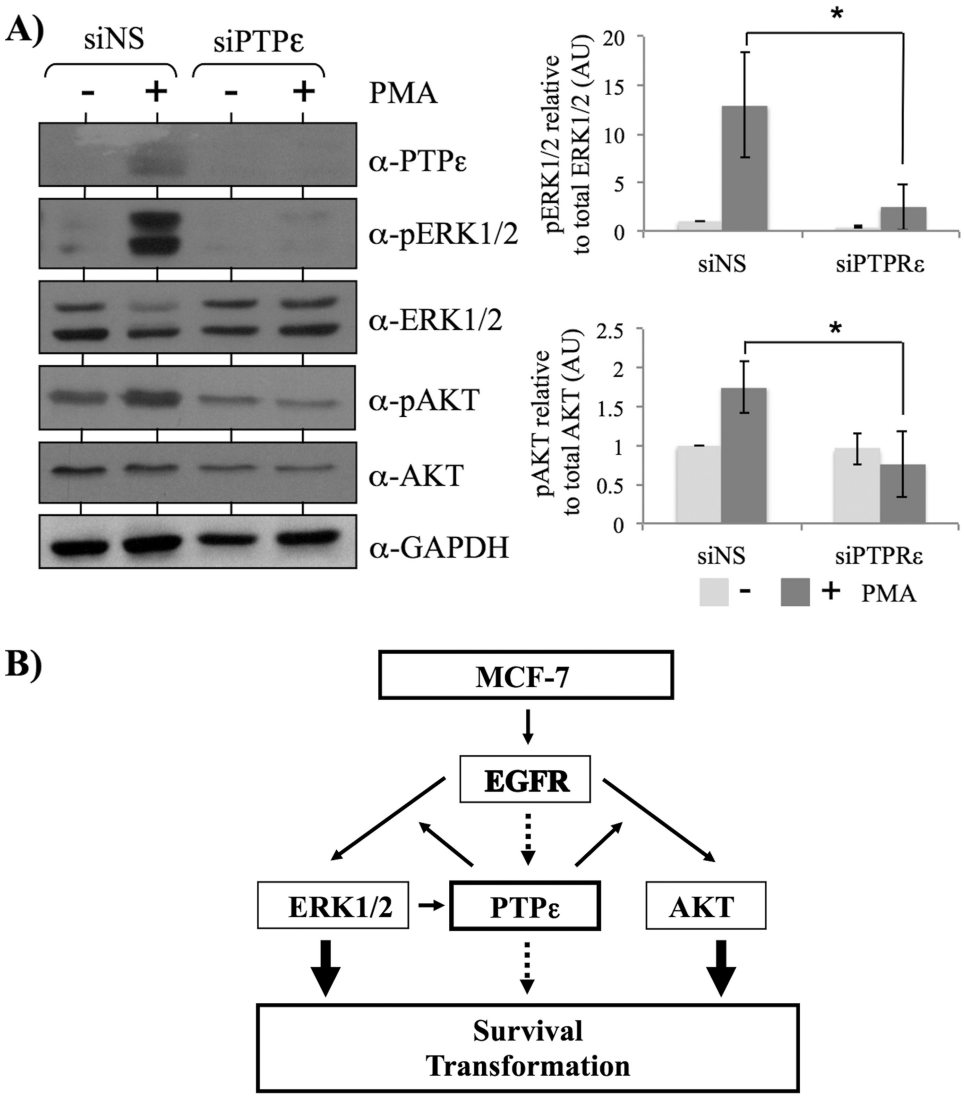


FIGURE 6. *A*, silencing of PTPϵ decreased the activation of ERK1/2 and AKT in PMA-treated MCF-7 cells. Cells were transfected with siNS or with siPTPϵ. 48 h after transfection, cells were kept untreated or treated with PMA for 24 h, before harvesting. PTPϵ, pERK1/2, ERK1/2, pAKT, and AKT levels were analyzed by immunoblot. A representative immunoblot is shown out of at least three different experiments. GAPDH content is included as a protein loading control. Quantification of relative pERK1/2 relative to total ERK1/2 levels, and pAKT relative to total AKT levels, are shown as arbitrary units (*AU*) in the *right panels*. Data represent the mean values ± S.D., statistically significant results are marked with: *, $p < 0.005$. *B*, scheme of the feedback regulatory mechanism of PTPϵ, and its role in the control of survival and transformation of MCF-7 cells. EGFR up-regulation and activation is required for PTPϵ induction and activation. Low levels of PTPϵ diminish ERK1/2 and AKT activation, which reduces cell survival. EGFR and ERK1/2 activation induces PTPϵ expression, which results in further activation of ERK1/2 and enhancement of survival and transformation. The possibility that PTPϵ affects cell survival and/or transformation through other pathways is indicated with the *lower dashed arrow*.

MCF-7 human cell line is distinct from its mechanism in mouse mammary tumor cells.

We observed an increase in ERK1/2 activation when catalytically active PTPϵ was overexpressed, and a decrease in ERK1/2 and AKT activation upon PTPϵ silencing. This suggests that the expression and phosphatase activity of PTPϵ is required to maintain the activation of ERK1/2 and AKT pathways in MCF-7 cells. This also suggests that PTPϵ could dephosphorylate and activate upstream components in the ERK1/2 and AKT pathways, and that PTPϵ exerts its function in a positive feedback loop for the activation of both pathways in MCF-7 cells (Fig. 6*B*). In contrast, in other cell types, such as NIH3T3, HEK293, lymphocytes, or primary hepatocytes, PTPϵ inhibited the activation of ERK1/2 (31, 32, 34, 53). Interestingly, analogous findings have been reported for PTP1B. PTP1B is a positive regulator of HER2 signaling, favoring the activation of ERK1/2 and PI3K/AKT pathways in breast cancer cells, and PTP1B knock-out mice display attenuated mammary tumorigenesis and malignancy (54–56). However, in other cell types, PTP1B inhibits ERK1/2, and PI3K/AKT pathways (57).

In support of a positive role for PTPϵ in survival and transformation of breast cancer cells, we have found that MCF-7 cells overexpressing PTPϵ displayed enhanced formation of colonies in soft agar, whereas the PTPϵ substrate-trapping mutant expressing cells showed decreased colony formation. Silencing of PTPϵ in MCF-7 cells also decreased the formation of colonies, likely as a result of the decreased survival and viability detected in these cells. In this regard, silencing of the closely related PTPα induced apoptosis in estrogen receptor

(ER)-negative MDA-MB-231 breast cancer cells, but not in MCF-7 cells (58). This suggests a differential involvement of PTPα and PTPϵ in the growth control of distinct human breast cancer cell types.

We have found up-regulation of RPTPϵ expression in different human breast cancer cell lines upon different cell-growth conditions. Interestingly, we did not detect expression of cytPTPϵ in the absence of RPTPϵ, suggesting the existence of internal regulatory loops of PTPϵ expression. In MCF-7 and MDA-MB-231 cells, we have identified a positive feedback regulatory loop of RPTPϵ expression upon the control of the EGFR and ERK1/2 signaling pathways. Our functional results in MCF-7 cells and other breast cancer cell lines suggest a positive role for PTPϵ in cell growth and survival, which makes PTPϵ a suitable candidate target for breast cancer therapy. The development of specific PTPϵ inhibitors and/or specific antibodies against the RPTPϵ extracellular region will be necessary to test the consequences of inhibiting PTPϵ in breast cancer.

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