Epithelial Protein-Tyrosine Phosphatase 1B (PTP1B) Contributes to the Induction of Mammary Tumors by HER2/Neu but is not Essential for Tumor Maintenance

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Abstract

Protein-tyrosine phosphatase 1B (PTP1B), a well-established metabolic regulator, plays an important role in breast cancer. Using whole-body PTP1B knockout mice, recent studies have shown that PTP1B ablation delays HER2/Neu-induced mammary cancer. Whether PTP1B plays a cell-autonomous or a non-cell-autonomous role in HER2/Neu-evoked tumorigenesis and whether it is involved in tumor maintenance was unknown. We generated mice expressing HER2/Neu and lacking PTP1B specifically in the mammary epithelium. We found that mammary-specific deletion of PTP1B delays the onset of HER2/Neu-evoked mammary tumors, establishing a cell autonomous role for PTP1B in such neoplasms. We also deleted PTP1B in established mouse mammary tumors or depleted PTP1B in human breast cancer cell lines grown as xenografts. PTP1B inhibition did not affect tumor growth in either model showing that neither epithelial nor stromal PTP1B is necessary for tumor maintenance. Taken together, our data show that despite the PTP1B contribution to tumor onset, it is not essential for tumor maintenance. This suggests that PTP1B inhibition could be effective in breast tumor prevention.

Keywords

PTPN1; PTP1B; Tyrosine Phosphatases; HER2; Breast Cancer

Introduction

Breast cancer is one of the most common malignancies in women, with ~400,000 deaths annually worldwide (1). The receptor tyrosine kinase c-ErbB2 (HER2/Neu), a member of the epidermal growth factor receptor family, is overexpressed in ~20% of breast cancers (2). Transgenic expression of activated forms of HER2/Neu (NeuNT) in the mammary gland causes mammary adenocarcinoma (3, 4). Furthermore, the success of the anti-HER2 monoclonal antibody trastuzumab (Herceptin) in clinics highlights the importance of HER2/ Neu in human breast cancer. In addition to tyrosine kinases such as HER2/Neu, tyrosyl phosphorylation is also regulated by protein-tyrosine phosphatases (PTPs) (5, 6). Because

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they antagonize the action of tyrosine kinases, PTPs were initially thought to play a negative (signal-attenuating) role in signaling and consequently a tumor-suppressing role in cancer. Recent studies, however, have shown that certain PTPs can also enhance signaling and, thus promote oncogenesis (7).

Protein-tyrosine phosphatase 1B (PTP1B) has signal-attenuating properties downstream of insulin and leptin signaling (8). Knockout mice for PTP1B are insulin- and leptin hypersensitive due to inhibitory effects of PTP1B downstream of the insulin and leptin pathways (9, 10). While PTP1B decreases insulin signaling by dephosphorylating the insulin receptor and IRS proteins, it decreases leptin action by dephosphorylating Jak2. Moreover, PTP1B also attenuates growth hormone-mediated Jak2-Stat signaling, providing another possible mechanism for PTP1B roles in obesity (11). These observations have identified PTP1B as an important target in diabetes and obesity and stimulated the development of PTP1B inhibitors (6).

PTP1B is also involved in oncogenesis, as first suggested by the amplification and overexpression of *PTPN1*, the gene encoding PTP1B, in breast cancer (12, 13). In contrast to its activity in insulin and leptin receptor signaling, PTP1B was shown to positively regulate IGF-1- and PDGF-induced RAS/ERK signaling in immortalized fibroblasts (14, 15). However, the role of PTP1B downstream of HER2/Neu and other oncogenes was initially controversial, as it was found to enhance or to attenuate the transforming effects depending on the test system (16, 17). Thus, the precise role of PTP1B in breast cancer has remained ill-defined. We and others have shown that global deletion of PTP1B in mice delays or protects against HER2/Neu-induced mammary cancer, depending on the particular HER2/Neu allele and mouse strain studied (18, 19). Therefore, PTP1B is clearly an important positive component of HER2/Neu-evoked transformation, raising the possibility that PTP1B inhibition could be useful for treating breast cancer. However, it is not clear whether this is a direct effect of PTP1B deletion on HER2/Neu signaling in the mammary epithelium or an indirect consequence of the salutary metabolic effects of PTP1B deficiency.

Here, we addressed the site of action of PTP1B (i.e., epithelial vs. non-epithelial) and assessed whether PTP1B is involved in tumor maintenance. We have used a combination of mouse genetics and reverse genetics to address these questions and found that inhibition of PTP1B in the mammary epithelium delays mammary tumor onset, whereas inhibition of PTP1B in established mammary tumors does not affect their growth.

Materials and Methods

Reagents

The pLXSN-NeuNT construct was from L. Petti (Albany Medical College). Rabbit polyclonal anti-mouse PTP1B antibodies were described elsewhere (10). Commercial antibodies included anti-Her2 (Calbiochem), Erk2 (Santa Cruz), phospho-Erk1/2, phospho-Src Y416, Src (Cell Signaling) and Ki-67 (Neomarkers). The dox-inducible lentiviral vector was described elsewhere (20).

Three-Dimensional Cultures

MCF10A cells (from J. Brugge, Harvard Medical School) were infected with pLXSN-NeuNT and pools of cells grown and stained as previously described (21). For experiments with inducible miRs, 500 ng/ml of dox was added to the medium 1 day after seeding the cells and refreshed every 2 days.

Transgenic mice

MMTV-NeuNT (strain TG.NK) and SCID-Beige mice were purchased from Jackson Labs. PTP1B$^{fl/fl}$ mice (22) were backcrossed with FVB/J (Harlan) mice for six generations. MMTV-Cre and Actin-CreERT mice were described previously (23, 24). All mice were kept as virgins throughout the entire study.

Animal Experiments

PTP1B$^{fl/fl}$ or PTP1B$^{wt/wt}$ mice containing one copy of MMTV-NeuNT and MMTV-Cre were monitored twice weekly for tumor onset.

PTP1B$^{fl/fl}$ - MMTV-NeuNT - Actin CreERT mice and wild-type littermates PTP1B$^{wt/wt}$ - MMTV-NeuNT - Actin CreERT were monitored twice weekly for tumor onset. Once palpable tumors were formed, the mice were injected every day intraperitoneally with 0.5 mg of Tamoxifen (Sigma) for a total of 10 days.

For xenograft studies, 10^6 MCF10A-NeuNT cells or 500,000 MDA-MB-231 cells (from ATCC) were suspended in a 100-μl mixture of Basement Membrane Matrix Phenol Red-free (BD Biosciences) and PBS 1:1 and injected orthotopically into SCID-Beige mice. Expression of CTRL or PTP1B miR was induced by doxycycline (Sigma) in the drinking water (2 g/l in a 5% sucrose solution, refreshed every 2 days). Tumor volume was measured every 5 days using calipers.

Protein Analysis

Snap-frozen mammary glands or mammary tumors were lysed in a tissue homogeniser with RIPA buffer containing 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS. The following inhibitors were added to the buffer just before lysis: 10 mM sodium pyrophosphate, 5 mM EGTA, 2 μg/ml each of aprotinin, leupeptin, pepstatin and antipain, 2mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycero-phosphate and 2 mM PMSF. Tumor lysates were resolved by SDS-PAGE, transferred to Immobilon-FL membranes (Millipore) and immunoblotted with the indicated antibodies.

Immunohistochemistry

Excised mammary glands or tumors were fixed with Formal-Fix (Thermoscientific) for 24 h at 4°C. Fixed samples were processed and embedded in paraffin. Sections of 4 μm were cut and dried overnight at 37°C. Staining was performed automatically using a Discovery XT automated stainer (Ventana Medical Systems (vms). In brief, for Ki-67 (1:100) and PTP1B (1:100) antibodies, the Research IHC DABMAP XT procedure was used with mild CC1 and Protease 1 (vms) pretreatment (for 4 min), respectively. Primary antibodies were incubated for 1 h at 37°C. Biotinylated secondary donkey anti-rabbit antibodies (Jackson Labs, 1:100) were then added for 32 min at 37°C. All sections were counterstained with Hematoxylin II (vms) and bluing reagent (vms) for 4 min before washing, dehydrating and mounting. We quantified the percentage of Ki67-positive cells using the ImagePro Software. We analyzed 6 tumors/group and ~10,000 cells/tumor.

Statistical Analysis

Survival curves were generated using the Kaplan-Meier method and significance evaluated with the log-rank test. Paired data were evaluated by Student's t-test and tumor growth was analyzed by Wilcoxon rank sum test using JMP software.

Results

Epithelial Specific Deletion of PTP1B Delays Mammary Tumor Onset

To determine whether epithelial expression of PTP1B is important for mammary tumor onset, we crossed PTP1B^{fl/fl} mice to MMTV-Cre mice, in which expression of the Cre recombinase is under the control of the Mouse Mammary Tumor Virus Promoter (MMTV), and to MMTV-NeuNT mice, which express the activated HER2/Neu oncogene NeuNT. We generated PTP1B^{fl/fl} - MMTV-Cre - MMTV-NeuNT mice and PTP1B[wt/wt] - MMTV-Cre - MMTV-NeuNT mice. As expected, immunohistochemistry analysis showed specific deletion of PTP1B in the mammary glands of PTP1B^{fl/fl} - MMTV-Cre, but not PTP1B[wt/wt] - MMTV-Cre, mice (Fig. 1A). Tumor latency in PTP1B[wt/wt] - MMTV-Cre- MMTV-NeuNT mice was ~189 days. Interestingly, tumor onset in PTP1B^{fl/fl} - MMTV-Cre - MMTV-NeuNT mice was significantly delayed to ~217 days (Fig. 1B). We found that 4 out of 19 tumors from PTP1B^{fl/fl} - MMTV-Cre - MMTV-NeuNT mice retained significant levels of PTP1B protein as detected by immunohistochemistry (Supplementary Fig. 1). This could reflect the known mosaic expression of Cre in this line (25) and/or a selective growth advantage of tumor cells that retain PTP1B. In any event, these mice were excluded from the analysis. Our data show that the absence of PTP1B in mammary epithelium delays tumor onset, arguing for a cell-autonomous role of PTP1B in the initiation of mammary tumors of this subtype.

Epithelial Specific PTP1B Deletion Does not Affect the Growth of Mammary Tumors

To gain insight into the involvement of PTP1B in tumor growth, we stained mammary tumors from PTP1B^{fl/fl} - MMTV-Cre - MMTV-NeuNT mice and PTP1B[wt/wt] - MMTV-Cre - MMTV-NeuNT mice for the proliferation marker Ki67. No differences between tumors with and without PTP1B were found (Fig. 2A). Consistently, the absence of PTP1B did not affect tumor volume or the number of tumors per mouse (Fig. 2B, C).

We then assessed changes in the phosphorylation of key signaling molecules downstream of HER2/Neu. Immunoblotting of lysates from mammary tumors showed differences in the phosphorylation status of Erk between some PTP1B^{fl/fl} - MMTV-Cre - MMTV-NeuNT mice and PTP1B[wt/wt] - MMTV-Cre - MMTV-NeuNT littermates, but these changes were not consistent across all animals. Furthermore, there were no consistent differences in the phosphorylation of Akt, pY-416Src or p70S6k (Fig. 2D, E and data not shown). These data indicate that tumors developing in the absence of PTP1B activated other pathways circumventing the effect of PTP1B on tumor growth.

PTP1B is not Essential for Tumor Maintenance

Previous studies and our new data show that PTP1B is important for tumor onset but do not reveal whether PTP1B is involved in tumor maintenance. To address this question, we crossed PTP1B^{fl/fl} mice to MMTV-NeuNT mice and to mice expressing a tamoxifen-regulated Cre recombinase (Actin-CreERT) (23). We generated two cohorts of mice: PTP1B^{fl/fl} - Actin-CreERT - MMTV-NeuNT and PTP1B[wt/wt] - Actin-CreERT - MMTV-NeuNT mice. Upon tamoxifen injection, PTP1B was deleted in glands from PTP1B^{fl/fl} - Actin-CreERT mice but not from PTP1B[wt/wt] - Actin-CreERT or PTP1B^{fl/fl} mice (Fig. 3A). To determine whether PTP1B affects tumor maintenance, we administered tamoxifen to mice once tumors became palpable and found that tamoxifen treatment led to PTP1B deletion (Fig. 3B). Surprisingly, deletion of PTP1B had no effect on tumor growth, proliferation or apoptosis (Fig. 3C and Supplementary Fig. 2A, B) and is, thus, not essential for the maintenance of HER2/Neu-induced mammary tumors.

To further assess a potential role of PTP1B in tumor maintenance, we generated a doxycycline (dox)-inducible lentiviral vector (20) expressing a PTP1B shRNA[miR] (PTP1B miR) to knockdown PTP1B in the transformed breast epithelial cell line MCF10A-NeuNT. As control, we used cells expressing a lentiviral vector targeting firefly luciferase (CTRL miR). Dox treatment suppressed PTP1B expression (79.8%) in cells infected with PTP1B miR but not in cells infected with CTRL miR (Fig. 4A). In the absence of dox, MCF10A-NeuNT cells expressing CTRL miR or PTP1B miR formed invasive structures when grown in 3D culture (Fig. 4B). Knockdown of PTP1B by dox treatment 1 day after seeding cells in 3D cultures did not affect the invasiveness of MCF10A-NeuNT cells expressing PTP1B miR (Fig. 4B, C). We then injected MCF10A-NeuNT CTRL miR or PTP1B miR cells into the fat pad of immunodeficient mice and, once tumors became palpable, administered dox to achieve PTP1B knockdown. Consistent with our results using the inducible Cre in PTP1B^{fl/fl} - Actin-CreERT - MMTV-NeuNT mice (Fig. 3C), we found no difference in the maintenance of MCF10A-NeuNT tumors at this level of PTP1B knockdown (Fig. 4D). Immunoblotting of protein lysates obtained from these tumors at the end of the experiment confirmed knockdown of PTP1B upon dox administration (Fig. 4D). Similar results were obtained upon dox-inducible knockdown of PTP1B in xenografts of the MDA-MB-231 breast cancer cell line and in shRNA-mediated constitutive knockdown of PTP1B in MCF10A-NeuNT cells (Supplementary Fig. 3A, B and data not shown). Taken together, these data show that, once tumors are formed, knockdown of PTP1B does not affect tumor growth, which suggests that PTP1B is not essential for breast tumor maintenance.

Discussion

Previous *in vivo* studies showing that inhibition of PTP1B delays or prevents NeuNT-induced mammary tumorigenesis were performed in PTP1B whole-body knockout mice. Thus, the site of action of PTP1B (epithelial vs. non-epithelial) remained unclear. We have now deleted PTP1B specifically in the mammary epithelium and discovered that epithelial PTP1B is important for NeuNT-evoked mammary cancer.

There was a delay of ~28 days in the onset of mammary tumors when PTP1B was deleted in the mammary epithelium of nulliparous MMTV-NeuNT mice in the FVB/J background. The magnitude of this delay differs from earlier studies. Using nulliparous mice in a mixed genetic background (FVB/J, 129Sv, C57B6/J), we showed previously that PTP1B deletion delayed tumor onset by ~86 days in about one-third of cases and completely protected the remaining mice against NeuNT-evoked mammary tumors (18). This difference in tumor onset may be attributable to the different genetic backgrounds of the mice, but a further, more interesting possibility is that PTP1B also plays a non-cell autonomous role in mammary tumorigenesis. This possibility warrants further studies because a) PTP1B is involved in immune cell signaling (26), b) the level of circulating insulin is lower in PTP1B knockout mice than in wild-type littermates and increased insulin levels have been associated with a high risk of developing breast cancer (27, 28), and c) PTP1B regulates leptin and growth hormone signaling both of which were linked to breast cancer (29, 30). It has been reported that whole-body knockout of PTP1B delayed HER2/Neu-induced mammary tumor onset by ~57 days in multiparous mice in an FVB/J background. In this case, the mice expressed an in-frame deletion in the extracellular domain of HER2/Neu (NDL2, Neu deletion in extracellular domain 2 mice) and tumor onset was assessed in multiparous mice. These factors may explain the observed difference in tumor onset (19).

In the present study, we found no differences in the growth rate of tumors, or in the number of tumors per animal expressing or lacking PTP1B. There also were no consistent changes in the phosphorylation status of Erk, Akt, c-Src or p70S6k. These data suggest that PTP1B may not be relevant for the progression of the disease once tumors are formed, most likely

because the tumors activate other oncogenic pathways not requiring PTP1B. Studies in cancer cell lines grown as monolayers or 3D cultures have suggested that PTP1B knockdown suppresses activation of c-SRC (31, 32). In the present study, epithelial deletion of PTP1B did not affect c-Src activation downstream of HER2/Neu, which is consistent with previous *in vivo* studies using whole-body knockouts of PTP1B (18, 19).

Clearly, PTP1B plays an important role in tumor onset downstream of HER2/Neu but the question of whether PTP1B is involved in the maintenance of established mammary tumors had until now not been answered. Studies using an inhibitor targeting PTP1B did not report its effect on established mammary tumors (19). Here we report studies using mouse genetics and xenograft models which have shown that neither epithelial nor stromal PTP1B is required for tumor maintenance. A similar discrepancy between the effects on tumor onset and maintenance was observed upon deletion of Jak2 in mammary tumors (33).

Earlier studies showing that PTP1B is required for tumor onset raised the exciting possibility that PTP1B inhibitors, as currently developed for the treatment of diabetes and obesity, might also be useful for breast cancer therapy. We now show that PTP1B is not essential for breast tumor maintenance in HER2/Neu-evoked mammary tumors but that inhibitors of PTP1B may be relevant as chemopreventive agents in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

PTP   protein-tyrosine phosphatase

MMTV   mouse mammary tumor virus

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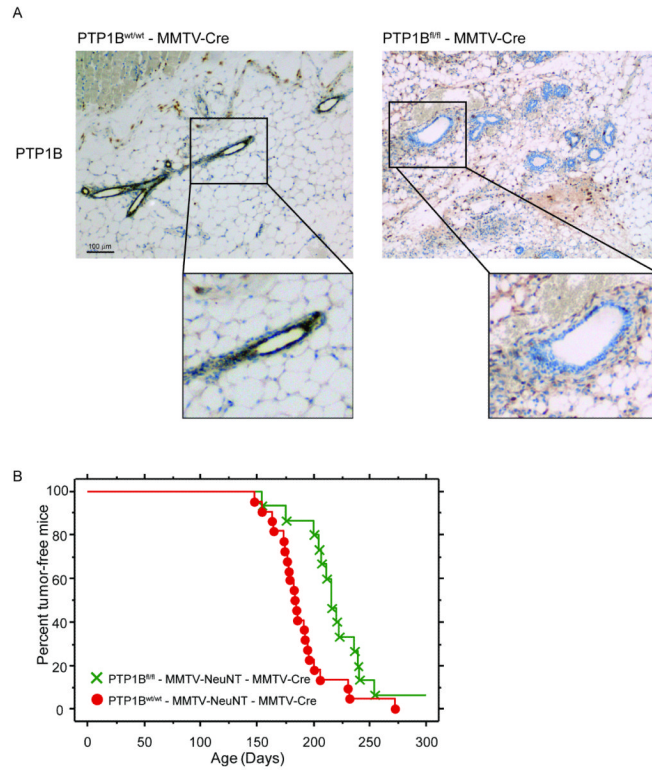
Figure 1. MMTV-Cre-mediated deletion of PTP1B delays NeuNT-induced mammary tumor onset

A. PTP1B is deleted specifically in the mammary epithelial cells of PTP1B$^{fl/fl}$ - MMTV-Cre mice. Immunohistochemical staining of PTP1B in the mammary glands of PTP1B$^{fl/fl}$ - MMTV-Cre and PTP1B$^{wt/wt}$ - MMTV-Cre mice. PTP1B staining is shown in brown. Inserts show a higher magnification of the boxed areas.

B. Epithelial-specific deletion of PTP1B delays NeuNT-induced mammary tumor onset. Kaplan-Meier curves showing mammary tumor onset in PTP1B$^{wt/wt}$ - MMTV-Cre - MMTV-NeuNT mice (n=22) and PTP1B$^{fl/fl}$ - MMTV-Cre - MMTV-NeuNT mice (n=15). PTP1B$^{wt/wt}$ - MMTV-Cre - MMTV-NeuNT mice developed palpable tumors within a mean probability of 189 days, whereas PTP1B$^{fl/fl}$ - MMTV-Cre - MMTV-NeuNT mice developed tumors by 217 days. P=0.006, log-rank test.
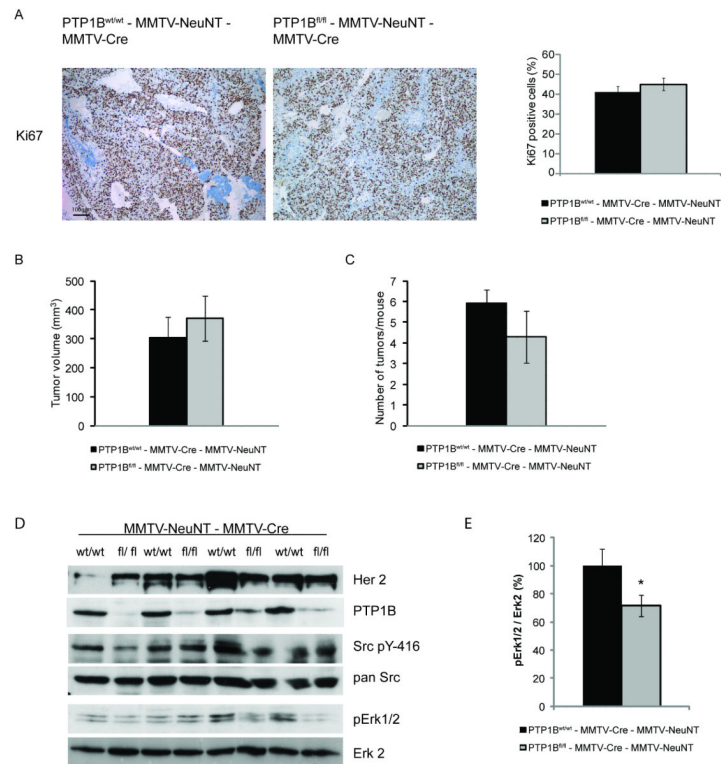
Figure 2. Epithelial deletion of PTP1B does not affect proliferation and signaling of NeuNT-induced mammary tumors

A. Immunohistochemical analysis of mammary tumors from PTP1B$^{wt/wt}$ - MMTV-Cre - MMTV-NeuNT and PTP1B$^{fl/fl}$ - MMTV-Cre - MMTV-NeuNT mice 5 weeks after their onset. Representative images of Ki67-stained sections of mammary tumors as indicated. Bar graph showing the quantification of Ki67 staining in mammary tumors (n=6). P=0.41 student's t-test.

B, C. Tumor volume and the number of tumors per mouse of PTP1B$^{wt/wt}$ - MMTV-Cre - MMTV-NeuNT (n=12) and PTP1B$^{fl/fl}$ - MMTV-Cre - MMTV-NeuNT mice (n=6) 30-35 days after tumor onset. *P=0.56* for tumor volume and *P=0.24* for the number of tumors per mouse, student's t-test.

D. Immunoblots of mammary tumor lysates from PTP1B$^{wt/wt}$ - MMTV-Cre - MMTV-NeuNT and PTP1B$^{fl/fl}$ - MMTV-Cre - MMTV-NeuNT mice 5 weeks after tumor onset.

E. Densitometric quantification of pErk1/2 normalized to Erk2 levels (n=4). *P=0.03*, student's t-test.
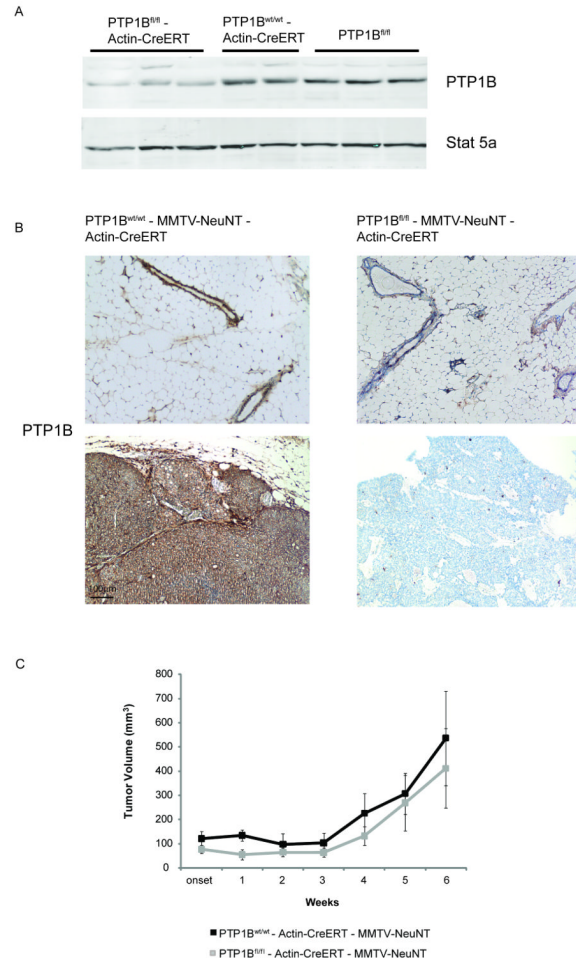
Figure 3. Deletion of PTP1B after overt tumor development does not affect tumor growth
A. Deletion of PTP1B in the mammary gland following tamoxifen injection into PTP1B$^{fl/fl}$ - Actin-CreERT mice. Immunoblots of lysates of mammary glands from PTP1B$^{wt/wt}$ - Actin-CreERT mice and PTP1B$^{fl/fl}$ mice are also shown.
B. Representative images of PTP1B-stained sections of mammary tumors (*upper panel*) and adjacent non-tumor (*lower panel*) glands from PTP1B$^{fl/fl}$ - Actin-CreERT - MMTV-NeuNT and PTP1B$^{wt/wt}$ - Actin-CreERT - MMTV-NeuNT mice 4 weeks after tamoxifen injection.
C. Volume of mammary tumors from PTP1B$^{wt/wt}$ - Actin-CreERT - MMTV-NeuNT mice (*n*=7 for weeks 1-4 and n=4 for weeks 5 and 6) and PTP1B$^{fl/fl}$ - Actin-CreERT - MMTV-NeuNT mice (*n*=8 for weeks 1-4 and n=3 for weeks 5 and 6) after tamoxifen injection (*P*=0.91 at week 4, *P*=0.85 at week 6, Wilcoxon rank sum test).
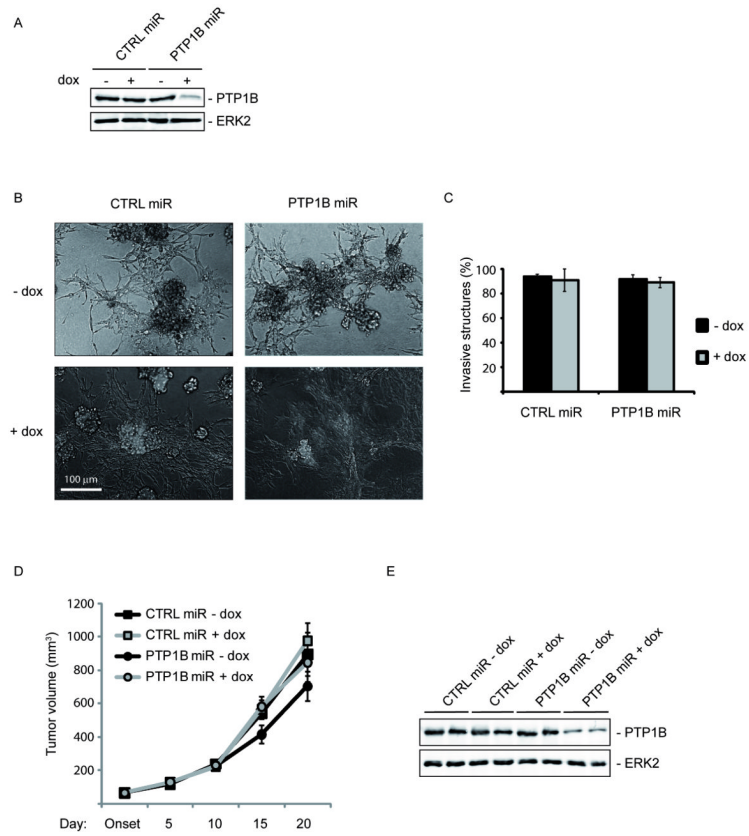
Figure 4. PTP1B is not essential for tumor maintenance
A. Immunoblotting of lysates from pools of MCF10A-NeuNT cells expressing a firefly control (CTRL) or PTP1B miR after 5 days of culture in the presence or absence of dox. Dox treatment of MCF10A-NeuNT cells deleted PTP1B (79.8%) in cells expressing PTP1B miR, but not in control cells.
B. PTP1B knockdown does not affect invasiveness of MCF10A-NeuNT cells. MCF10A-NeuNT cells expressing control or PTP1B miR were grown in 3D cultures in the presence or absence of dox. Phase contrast images showing the invasive structures.
C. Bar graph showing the mean percentage of MCF10A-NeuNT invasive structures ±SEM (n=3; P=0.85 student's t-test).
D. Growth curves of MCF10A-NeuNT tumors in the presence or absence of PTP1B, showing the mean tumor volume (mm^3) ±SEM (n=8; P=0.42 student's t-test).
E. Immunoblot of lysates from MCF10A-NeuNT tumors in the presence or absence of PTP1B.