Epithelial Protein-Tyrosine Phosphatase 1B 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.

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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 (11, 18). Therefore, PTP1B is clearly an important positive component of HER2/Neu-evoked trans-
formation, 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. nonepithelial) 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 (9). 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 (19).

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 (20). 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

Mouse Mammary Tumor Virus (MMTV)-NeuNT (strain TG.NK) and severe combined immunodeficient mice (SCID)-Beige mice were purchased from Jackson Labs. PTP1Bfl/fl mice (21) were backcrossed with FVB/J (Harlan) or PTP1Bwt/wt mice containing one copy of MMTV-NeuNT and MMTV-Cre were described previously (22, 23). All mice were kept as virgins throughout the entire study.

Animal experiments

PTP1Bfl/fl or PTP1Bwt/wt mice containing one copy of MMTV-NeuNT and MMTV-Cre were monitored twice weekly for tumor onset.

PTP1Bfl/fl–MMTV-NeuNT-Actin CreERT mice and wild-type littermates PTP1Bwt/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, 10⁶ 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 homogenizer with radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl pH 7.5, 1% Triton X-100, 150 mmol/L NaCl, 0.5% Na-deoxycholate, 0.1% SDS. The following inhibitors were added to the buffer just before lysis: 10 mmol/L sodium pyrophosphate, 5 mmol/L EGTA, 2 μg/mL each of aprotinin, leupeptin, pepstatin and antipain, 2 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L β-glycerophosphate, and 2 mmol/L phenylmethylsulfonyl fluoride. 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 in 4% formaldehyde and embedded in paraffin. Sections of 4 μm were cut and dried overnight at 37°C. Staining was done 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 minutes, respectively). Primary antibodies were incubated for 1 hour at 37°C. Biotinylated secondary donkey anti-rabbit antibodies (Jackson Labs, 1:100) were then added for 30 minutes at 37°C. All sections were counterstained with Hematoxylin II (vms) and bluing reagent (vms) for 4 minutes before washing, dehydrating, and mounting. We quantified the percentage of Ki67-positive cells using the ImagePro Software. We analyzed 6 tumors/group and approximately 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 MMTV promoter, 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 epithelium 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 approximately 189 days. Interestingly, tumor onset in PTP1B^{fl/fl}-MMTV-Cre-MMTV-NeuNT mice was significantly delayed to approximately 217 days (Fig. 1B). We found that 4 of 19 tumors from PTP1B^{fl/fl}-MMTV-Cre-MMTV-NeuNT mice retained significant levels of PTP1B protein as detected by immunohistochemistry (Supplementary Fig. S1). This could reflect the known mosaic expression of Cre in this line (24) 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 and C).
Molecular Cancer Research

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<sup>fl/fl</sup>-MMTV-Cre-MMTV-NeuNT mice and PTP1B<sup>wt/wt</sup>-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 and 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<sup>fl/fl</sup> mice to MMTV-NeuNT mice and to mice expressing a tamoxifen-regulated Cre recombinase (Actin-CreERT; ref. 23). We generated 2 cohorts of mice: PTP1B<sup>fl/fl</sup>-Actin-CreERT-MMTV-NeuNT and PTP1B<sup>wt/wt</sup>-Actin-CreERT-MMTV-NeuNT mice. Upon tamoxifen injection, PTP1B was deleted in glands from PTP1B<sup>fl/fl</sup>-Actin-CreERT mice but not from PTP1B<sup>wt/wt</sup>-Actin-CreERT or PTP1B<sup>fl/fl</sup> 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. S2A and 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 (19) expressing a PTP1B shRNAmiR (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 and 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<sup>fl/fl</sup>-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. S3A and 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.

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<sup>fl/fl</sup>-Actin-CreERT mice. Immunoblots of lysates of mammary glands from PTP1B<sup>fl/fl</sup>-Actin-CreERT mice and PTP1B<sup>wt/wt</sup> mice are also shown. B, representative images of PTP1B-stained sections of mammary tumors (bottom) and adjacent nontumor (top) glands from PTP1B<sup>fl/fl</sup>-Actin-CreERT-MMTV-NeuNT and PTP1B<sup>wt/wt</sup>-Actin-CreERT-MMTV-NeuNT mice 4 weeks after tamoxifen injection. C, volume of mammary tumors from PTP1B<sup>fl/fl</sup>-Actin-CreERT-MMTV-NeuNT mice (n = 7 for weeks 1–4 and n = 4 for weeks 5 and 6) and PTP1B<sup>wt/wt</sup>-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).
Discussion

Previous in vivo studies showing that inhibition of PTP1B delays or prevents NeuNT-induced mammary tumorigenesis were done in PTP1B whole-body knockout mice. Thus, the site of action of PTP1B (epithelial vs. nonepithelial) 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 approximately 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 approximately 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 noncell autonomous role in mammary tumorigenesis. This possibility warrants further studies because (a) PTP1B is involved in immune cell signaling (25), (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, (26, 27), and (c) PTP1B regulates leptin and growth hormone signaling both of which were linked to breast cancer (28, 29).

It has been reported that whole-body knockout of PTP1B delayed HER2/Neu-induced mammary tumor onset by approximately 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 (11).
showing that PTP1B is not essential for breast tumor maintenance, might also be useful for breast cancer therapy. We now currently developed for the treatment of diabetes and obesity or inhibition delays ErbB2-induced breast cancer. Nat Rev Cancer 2011;11:354–5.

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 (11). 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 data suggest that PTP1B may not be relevant for the downstream of HER2/Neu, which is consistent with previous in vivo studies using whole-body knockouts of PTP1B (11, 18).

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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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