

Protein tyrosine phosphatase 1B restrains mammary alveologenesis and secretory differentiation

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SUMMARY

Tyrosine phosphorylation plays a fundamental role in mammary gland development. However, the role of specific tyrosine phosphatases in controlling mammary cell fate remains ill defined. We have identified protein tyrosine phosphatase 1B (PTP1B) as an essential regulator of alveologenesis and lactogenesis. PTP1B depletion increased the number of luminal mammary progenitors in nulliparous mice, leading to enhanced alveoli formation upon pregnancy. Mechanistically, *Ptp1b* deletion enhanced the expression of progesterone receptor and phosphorylation of Stat5, two key regulators of alveologenesis. Furthermore, glands from *Ptp1b* knockout mice exhibited increased expression of milk proteins during pregnancy due to enhanced Stat5 activation. These findings reveal that PTP1B constrains the number of mammary progenitors and thus prevents inappropriate onset of alveologenesis in early pregnancy. Moreover, PTP1B restrains the expression of milk proteins during pregnancy and thus prevents premature lactogenesis. Our work has implications for breast tumorigenesis because *Ptp1b* deletion has been shown to prevent or delay the onset of mammary tumors.

KEY WORDS: PTP1B (Ptpn1), Stat5, Mammary gland, Stem cell, Progenitor cell, Mouse

INTRODUCTION

The epithelium of rodent and human mammary glands is hierarchically organized, encompassing cells at various differentiation stages (Stingl et al., 2006b; LaBarge et al., 2007; Visvader, 2009; Visvader and Smith, 2011). The results of serial transplantation of mammary gland fragments into cleared mouse mammary fat pad suggested the existence of mammary stem cells (Deome et al., 1959; Faulkin and Deome, 1960). Direct evidence was provided by the finding that serial transplantation of fragments from mouse mammary tumor virus-infected mammary glands yields clonal outgrowths with the same viral insertion site through five transplant generations (Kordon and Smith, 1998; Bruno and Smith, 2011). Other studies have used cell surface markers to enrich for, and isolate, mammary stem cells, progenitor cells, and more differentiated luminal and myoepithelial cells (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006a; Asselin-Labat et al., 2007; Regan et al., 2011). Notably, these cell subpopulations display different functional attributes: mammary stem cells [MaSCs, also called mammary repopulating units (MRUs)] are able to repopulate a cleared mammary fat pad. Progenitor cells display a high capacity for colony formation and proliferation *in vitro*. By contrast, terminally differentiated cells are not able to repopulate the mammary gland or to form colonies *in vitro* (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006a; Asselin-Labat et al., 2007). Recent lineage-tracing studies have questioned the existence of adult multipotent MaSCs and have instead suggested the existence of unipotent luminal and myoepithelial progenitor cells in the adult gland (Van Keymeulen et al., 2011).

The mammary gland undergoes functional differentiation during pregnancy. In the early stages, epithelial cells undergo extensive proliferation and form alveoli (alveologenesis) (Briskin, 2002), while in later stages of pregnancy alveolar cells secrete milk proteins (lactogenesis) (Neville et al., 2002; Hennighausen and Robinson, 2005; Briskin and Rajaram, 2006). Progesterone induces the expansion of MaSCs and the formation of mammary alveoli via activation of the Rankl (Tnfsf11) pathway, which in turn elicits the proliferation of epithelial cells (Asselin-Labat et al., 2010; Joshi et al., 2010). Prolactin (Prl) controls alveologenesis and lactogenesis via binding to its receptor [prolactin receptor (Prl-R; Prlr)] and activation of the Jak2/Stat5 pathway. Activated Stat5 translocates to the nucleus and induces expression of its target genes (e.g. milk proteins) (Gouilleux et al., 1994; Wartmann et al., 1996; Groner, 2002). Although Prl-R, Jak2 and Stat5 are regulated by tyrosine phosphorylation, little is known about how protein tyrosine phosphatases regulate this pathway and affect breast cell fate and differentiation *in vivo*.

Protein tyrosine phosphatase 1B (PTP1B; also known as Ptpn1), a ubiquitously expressed phosphatase, is an established negative regulator of insulin and leptin signaling and a leading target for the treatment of diabetes and obesity (Elchebly et al., 1999; Klamann et al., 2000). PTP1B is also involved in breast cancer. Whole-body or mammary-specific deletion of *Ptp1b* delays or prevents mammary tumor onset evoked by Her2 (also known as Neu and ErbB2) (Bentires-Alj and Neel, 2007; Julien et al., 2007; Balavenkatraman et al., 2011).

In contrast to its well-studied involvement in metabolism and cancer, the role of PTP1B in breast development remains unclear. Early *in vitro* studies suggested that both Jak2 and Stat5 are PTP1B substrates (Myers et al., 2001; Aoki and Matsuda, 2002). In the present study, we asked whether PTP1B controls mammary cell fate commitment and/or alveologenesis and lactogenesis *in vivo*. Using *Ptp1b* knockout mice, we have found that PTP1B depletion increases the number of mammary

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progenitor cells in nulliparous mice, induces precocious formation of alveoli, and enhances the expression of milk proteins during pregnancy.

MATERIALS AND METHODS

Mice

All animal experiments were performed according to Swiss guidelines governing animal experimentation and were approved by the Swiss veterinary authorities. *Ptp1b*^{-/-} mice (Klaman et al., 2000) were backcrossed to an FVB background for at least seven generations. Twelve-week-old female mice were mated and pregnancy scored by the observation of a vaginal plug and confirmed by the presence of fertilized eggs or embryos when mammary glands were collected at pregnancy days 3, 7 or 10. Mammary glands from nulliparous mice were collected when mice were in estrus, as determined by a vaginal plug after an overnight mating with a male.

Whole-mounts and histological analysis

For whole-mounts and histology, inguinal and thoracic mammary glands were dissected at the indicated time points. Following fixation with methacarn solution for 4 hours, tissues were hydrated, stained with Carmine Alum, and cleared with xylene. After analysis, the tissues were processed for paraffin sectioning and stained with Hematoxylin and Eosin (H&E).

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on methacarn-fixed or 4% paraformaldehyde (PFA)-fixed, paraffin-embedded tissue sections using the following antibodies: Ki67 (Lab Vision), rabbit anti-milk serum (Marte et al., 1995), pStat5 (Cell Signaling Technology), Stat5 (Santa Cruz Biotechnology), estrogen receptor (ER; Esr1) (Santa Cruz Biotechnology) and progesterone receptor (PR; Pgr) (Thermo Scientific). Immunohistochemistry was carried out with the Discovery XT Staining Module (Ventana Medical Systems), except for ER and PR immunohistochemistry, which were performed manually. All sections were counterstained with Hematoxylin (J.T.Baker). Quantification of pStat5, PR and ER was performed by counting cells from at least 20 fields at a magnification of 20× and at least 2000 nuclei per sample. The number of positive cells was expressed as a percentage of the total number of Hematoxylin-stained cells. Quantification of epithelial density and proliferation index were performed on mammary gland sections stained with periodic acid Schiff (Ventana Medical Systems) and Hematoxylin, and scanned with Miramax Scan (Carl Zeiss). For epithelial density, the area covered by epithelial cells (excluding lumen and blood vessels) was measured and the ratio of epithelial area over total organ area was calculated using Definiens software as described (Stoelzle et al., 2009). The same protocol was followed for the proliferation index using the area covered by Ki67-positive epithelial cells over total area of epithelial cells. At least three mice per genotype were scanned for each developmental stage.

Immunofluorescence was performed on 4% PFA-fixed, paraffin-embedded tissue sections stained with Rankl; tissues were then incubated with Alexa Fluor 546 anti-goat IgG (Molecular Probes, Invitrogen), stained with DAPI (Boehringer Mannheim), mounted in ProLong Gold antifade reagent (Invitrogen), and analyzed with an LSM 700 scanning head and Zen 2010 software (Carl Zeiss).

Crystal Violet staining was performed on cells grown in 24-well BD Primaria plates (BD Biosciences). The numbers and sizes of Crystal Violet-stained colonies per well were quantified using ImagePro software (Media Cybernetics). Three wells per genotype were examined in four independent experiments.

Immunoblotting

Protein lysates were extracted from inguinal mammary glands using RIPA buffer [50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EGTA, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM PMSF and protease inhibitor cocktail (Pierce)]. Proteins (50 µg) were resolved on SDS-PAGE (Bio-Rad) and transferred to a PVDF membrane (Immobilon-FL, Millipore).

Membranes were blocked in PBS with 5% skimmed milk powder and incubated with PTP1B (Klaman et al., 2000), pStat5 (Cell Signaling) and Stat5a (Transduction Laboratories) antibodies. Antibody binding was visualized by incubation of secondary antibodies comprising Alexa Fluor 680 anti-mouse IgG, Alexa Fluor 680 anti-rabbit IgG (Molecular Probes, Invitrogen), IRDye 800 anti-mouse IgG and IRDye 800 anti-rabbit IgG (Rockland), and examined with an Odyssey infrared imaging system (Li-Cor Bioscience).

Real-time PCR

Total RNA was isolated from frozen mammary glands using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and then treated with the TURBO-DNase Kit (Applied Biosystems). cDNA synthesis was performed using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was performed on 30-60 ng cDNA using the TaqMan Gene Expression Assay (Applied Biosystems) for *Wap* (Mm00839913_m1), β -casein (Mm00839664_m1), cytokeratin 18 (Mm01601702_g1), *Rankl* (Mm00441906_m1), *Pr* (Mm00435628_m1), *Er* (Mm00433149_m1) and *Gapdh* (Rodent Gapdh Control Reagents VIC Probe, Applied Biosystems) on an ABI Prism 7000 (Applied Biosystems) according to the manufacturer's instructions.

Mammary cell preparation, cell sorting and cell culture

Inguinal mammary glands were dissected from 10-week-old virgin females or pregnant mice at gestation day 10, mechanically disaggregated, and digested with collagenase (Sigma) and trypsin (Sigma) for 1 hour at 37°C (Sleeman et al., 2006). The resulting organoids were processed to single-cell suspensions by digestion with HyQTase (HyClone) for 10-15 minutes at 37°C and filtered through a 40-µm cell strainer (Falcon). Cells were stained as previously described (Sleeman et al., 2006) with the following antibodies: FITC-CD24, PE-CD49f (Itga6), PE-Cy7-CD45 (Ptpc) (Pharmingen), APC-Sca1 (Ly6a), biotinylated-CD61 (Itgb3) (Biolegend) and streptavidin-PE-Cy5.5 (eBioscience). FACS analysis and cell sorting were carried out on a MoFlo cell sorter (Beckman Coulter).

Colony-forming assays were performed by plating freshly sorted cells (500 cells) on irradiated 3T3-L1 feeder cells in Multiwell BD Primaria plates for 7 days in DMEM/Ham's F12 mix (Invitrogen) with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 5 µg/ml bovine pancreatic insulin (Sigma, cell culture tested solution) and 10 ng/ml cholera toxin (Sigma). Aldefluor assay was performed according to the manufacturer's instructions (Stemcell Technologies).

Hormone treatment

Six-week-old female mice were ovariectomized and treated 10 days later every 24 hours by subcutaneous injection of 17 β -estradiol (Sigma; 4 ng/g body weight) in corn oil (Sigma) and sacrificed 48 hours later. For treatment with estrogen and progesterone, ovariectomized mice were injected with 17 β -estradiol and 48 hours later injected with 17 β -estradiol plus progesterone (Sigma; 100 µg/g body weight) daily for 72 hours.

Chemicals

NVP-BSK805 (Novartis, Switzerland) was freshly prepared in NMP/PEG 300/Solutol HS15 (5%/80%/15%). Twelve-week-old mice were treated every 24 hours by oral gavage (120 mg/kg body weight) for 5 consecutive days. Glands were collected and fixed 4 hours after the final treatment.

Microarray analysis

RNA was isolated from three biological replicates per condition using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop 1000 and the quality of the RNA assessed using the Agilent 2100 bioanalyzer and RNA Nano Chip. Aliquots (100 ng) of extracted total RNA were amplified using the Ambion WT Expression Kit and the resulting sense-strand cDNA was fragmented and labeled using the Affymetrix GeneChip WT Terminal Labeling Kit. Affymetrix GeneChip arrays were hybridized following the GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix) with a hybridization time of 16 hours. The Affymetrix Fluidics protocol FS450_0007 was used for washing. Scanning was performed with Affymetrix GCC Scan Control

version 3.0.0.1214 on a GeneChip Scanner 3000 with autoloader. Probe sets were summarized and probeset level values normalized with the justRMA() function from the R (version 2.12.0)/Bioconductor (version 2.6) package affy using the CDF environment MoGene-1_0-st-v1.r3.cdf (as provided by Bioconductor). Differentially expressed genes were identified using the R package limma (Gentleman et al., 2004) and by selecting genes with a minimum absolute \log_2 fold change of 1.5 and $P < 0.05$, using the method of Benjamini and Hochberg for multiple testing correction. Microarray data are available at GEO under accession number GSE41768.

Statistical analysis

Statistical significance was determined by two-tailed Student's *t*-test. For FACS analysis, a paired two-tailed Student's *t*-test was performed.

RESULTS

Ptp1b deletion accelerates alveologensis

Tyrosine phosphorylation plays an important role in mammary gland alveologensis. To determine whether PTP1B regulates this process, we analyzed mammary glands of PTP1B-deficient and wild-type (WT) female mice at different developmental stages. Whole-mounts and histological analysis showed significant changes in the structure of PTP1B-depleted compared with control glands (Fig. 1A). In nulliparous mice at estrus, H&E and Ki67 staining revealed a twofold increase in epithelial cell density and threefold more Ki67-positive cells in glands lacking PTP1B than in WT glands (Fig. 1B-D). During early stages of pregnancy, *Ptp1b*^{-/-} glands showed an overall increase in the number of epithelial cells and alveolar structures (Fig. 1B,C). These results demonstrate that PTP1B constrains cell proliferation and alveologensis during estrus and early pregnancy.

Increased progenitor cell number in mammary glands from *Ptp1b*^{-/-} mice

To test whether the enhanced epithelial density found in PTP1B-deficient mice is a consequence of an increase in the stem/progenitor cell subpopulations, we characterized mammary epithelial cells (MECs) from *Ptp1b*^{+/+} and *Ptp1b*^{-/-} mice phenotypically using Sca1, CD24 and CD49f markers (Sleeman et al., 2006; Stingl et al., 2006a), which have been shown to enrich for MaSCs (CD24^{lo} Sca1⁻ CD49f⁺), luminal progenitor cells (CD24^{hi} CD61⁺), more differentiated luminal cells (CD24^{hi} CD61⁻) and myoepithelial cells (CD24^{lo} CD49f^{lo}) (Asselin-Labat et al., 2007; Sleeman et al., 2007). We found no significant differences in the proportion of MECs bearing the stem cell phenotype (CD24^{lo} Sca1⁻ CD49f⁺) or in the proportions of total luminal epithelial cells (CD24^{hi} Sca1⁻ and CD24^{hi} Sca1⁺) in *Ptp1b*^{-/-} versus *Ptp1b*^{+/+} mice (Fig. 2A; supplementary material Fig. S1A). Limiting dilution transplantation experiments showed no difference in the capacities of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} MECs to repopulate the mammary gland and, thus, that *Ptp1b* ablation does not alter the properties of mammary repopulating cells (supplementary material Fig. S1B).

We then investigated whether *Ptp1b* deletion alters mammary colony-forming capacity (Stingl et al., 2006a). Freshly isolated MECs from *Ptp1b*^{+/+} and *Ptp1b*^{-/-} glands were cultured on feeder cells and the number of colonies quantified. *Ptp1b*^{-/-} MECs formed approximately twice as many colonies as *Ptp1b*^{+/+} MECs, which suggested an increase in progenitor cells in glands lacking PTP1B (Fig. 2B). Furthermore, the colonies formed by *Ptp1b*^{-/-} MECs were larger (Fig. 2B), consistent with our results showing increased proliferation in glands from *Ptp1b*^{-/-} compared with *Ptp1b*^{+/+} mice (Fig. 1D). The increase in progenitor cell number was further tested by FACS analysis for CD61, an epithelial progenitor marker

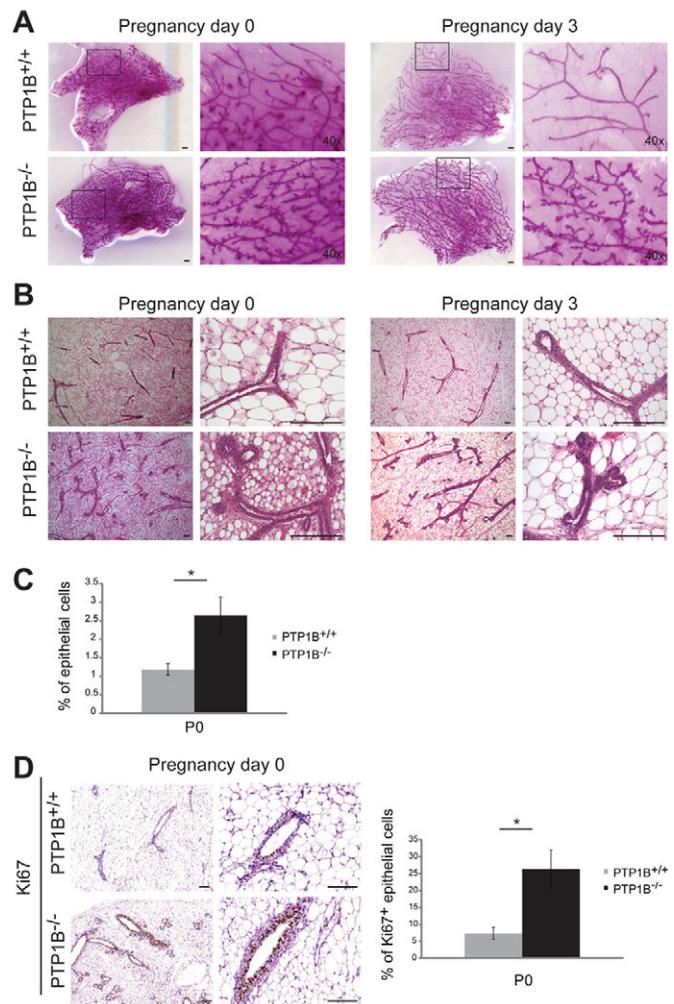


Fig. 1. Alveolar development is accelerated in PTP1B-deficient mammary glands. (A) Whole-mounts of *Ptp1b*^{-/-} mammary tissues showing precocious alveolar formation compared with *Ptp1b*^{+/+} mice. Boxed regions are magnified in images on the right. (B) H&E-stained histological sections of *Ptp1b*^{+/+} and *Ptp1b*^{-/-} mammary tissues. (C) The percentages of epithelial cells in *Ptp1b*^{+/+} and *Ptp1b*^{-/-} glands from nulliparous mice at estrus. (D) (Left) Ki67-stained histological sections. (Right) Proliferating (Ki67⁺) cells as a percentage of total epithelial cells per gland. Pregnancy day 0 (P0) refers to nulliparous mice at estrus. Error bars indicate mean \pm s.e.m.; * $P < 0.05$ by Student's *t*-test; C, D, $n = 4$. Scale bars: 1 mm in A; 100 μ m in B, D.

(Asselin-Labat et al., 2007). We found a significant increase in the CD24^{hi} CD61⁺ population in *Ptp1b*^{-/-} MECs compared with *Ptp1b*^{+/+} MECs (29.63% CD24^{hi} CD61⁺ cells in *Ptp1b*^{-/-} MECs versus 19.86% in *Ptp1b*^{+/+} MECs) (Fig. 2C).

Several studies have suggested that high aldehyde dehydrogenase (ALDH) activity is a property of stem and/or progenitor cells in human and mouse mammary tissues (Ginestier et al., 2007; Cohn et al., 2010; Eirew et al., 2012). Using the Aldefluor assay, we found a higher ALDH activity in MECs from *Ptp1b*^{-/-} than *Ptp1b*^{+/+} mice (supplementary material Fig. S1C), further supporting an increase in the proportion of mammary progenitor cells in PTP1B-deficient glands. Together, these results show that PTP1B restrains the number of mammary progenitor cells in nulliparous mice.

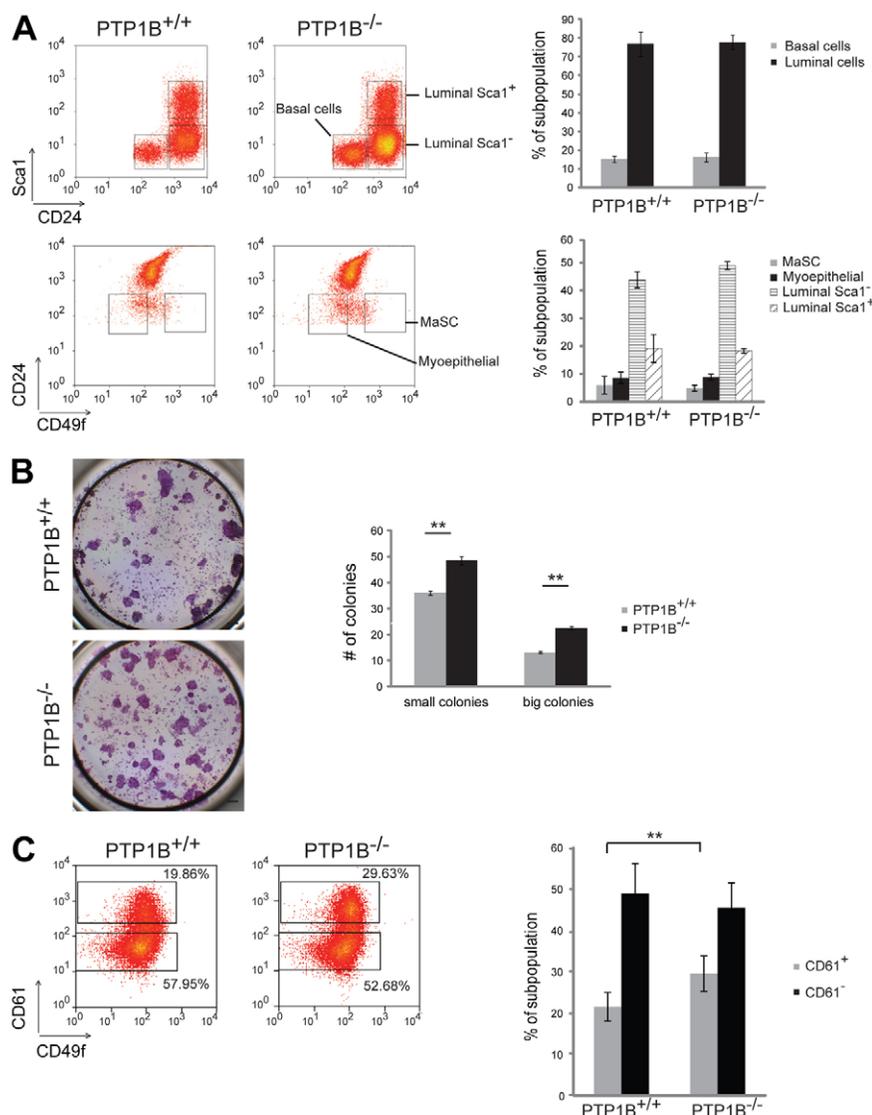


Fig. 2. PTP1B depletion increases the proportion of mammary progenitors.

(A) (Top row, left) Flow cytometry dot plots of mammary epithelial cells (MECs) from nulliparous mice at estrus. Luminal cells: CD24^{hi} Sca1⁺ and CD24^{hi} Sca1⁻. Basal cells: CD24^{lo} Sca1⁻. (Top row, right) The percentages of luminal and basal cell subpopulations of MECs from *Ptp1b*^{+/+} and *Ptp1b*^{-/-} nulliparous mice at estrus. (Bottom row, left) Dot plots of myoepithelial (CD24^{lo} Sca1⁻ CD49f⁺) and mammary stem cell (MaSC) (CD24^{lo} Sca1⁻ CD49f⁻) populations. (Bottom row, right) The percentages of myoepithelial and MaSC populations of *Ptp1b*^{+/+} and *Ptp1b*^{-/-} glands from nulliparous mice at estrus. Error bars indicate mean \pm s.e.m. ($n=6$). (B) (Left) Colony formation assay of MECs from nulliparous mice at estrus. Scale bar: 1 mm. (Right) The number of *Ptp1b*^{+/+} and *Ptp1b*^{-/-} colonies. Small refers to colonies <8000 μm^2 and big to colonies >8000 μm^2 ($n=4$, ** $P<0.01$ by Student's *t*-test). (C) (Left) Flow cytometry dot plots of luminal cells (CD24^{hi} Sca1⁺ and CD24^{hi} Sca1⁻) of MECs from nulliparous mice at estrus stained for the progenitor marker CD61. (Right) The percentages of CD61⁺ and CD61⁻ populations of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} MECs. Error bars indicate mean \pm s.e.m. ($n=4$, ** $P<0.01$ by paired two-tailed Student's *t*-test).

PTP1B negatively regulates ER activity

To investigate the molecular mediators of the observed increase in epithelial density and mammary progenitors in mammary glands from *Ptp1b*^{-/-} mice, we performed gene expression profiling of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands from mice at estrus. The absence of PTP1B increased the expression of several components of the cell cycle machinery: including cyclin B2, cyclin A2, cyclin-dependent kinase 1 and topoisomerase 2A (Fig. 3A; supplementary material Fig. S2A, Table S1). These results, combined with the increased proliferation observed by immunohistochemistry (Fig. 1D), support a role for PTP1B in the regulation of epithelial cell proliferation.

Further, analysis of the expression profiles of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands revealed increased expression of several estrogen-responsive genes (supplementary material Fig. S2A): *Pr*, amphiregulin, *Expi* (*Wfdc18*), *Egr2* and *c-Myb*. Furthermore, quantitative RT-PCR and immunohistochemistry analysis revealed an increase in PR expression in glands lacking PTP1B (Fig. 3B,C). Similarly, we found increased expression of *Rankl*, an established downstream target of PR (Fata et al., 2000; Beleut et al., 2010), in glands deficient in PTP1B (Fig. 3B; supplementary material Fig. S2B). These data suggest that PR-induced expression of *Rankl*

might account for the increased number of MECs observed in glands from *Ptp1b*^{-/-} mice.

We then tested whether overexpression of ER and/or estrogen accounts for the increased transcription of ER targets in glands from *Ptp1b*^{-/-} mice, but found no difference in ER expression between *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands (Fig. 3B,C) and no difference in plasma levels of estrogen between *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice at estrus (supplementary material Fig. S3A). Further analysis showed no differences in the plasma levels of progesterone in *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice (supplementary material Fig. S3A). Thus, *Ptp1b* deletion appears to increase mammary cell proliferation by enhancing the responsiveness of the mammary gland to normal levels of circulating estrogen and progesterone. To test this possibility directly, we assessed the effects of 17 β -estradiol treatment alone or in combination with progesterone on *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice that were previously depleted of endogenous steroid hormones by ovariectomy. Treatment with 17 β -estradiol for 48 hours increased the expression of PR in glands from *Ptp1b*^{-/-} ovariectomized mice compared with ovariectomized WT littermates (Fig. 3D). These data show that *Ptp1b* deletion increases ER activity in nulliparous glands. Furthermore, treatment of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice with 17 β -estradiol and progesterone for 72 hours resulted in enhanced

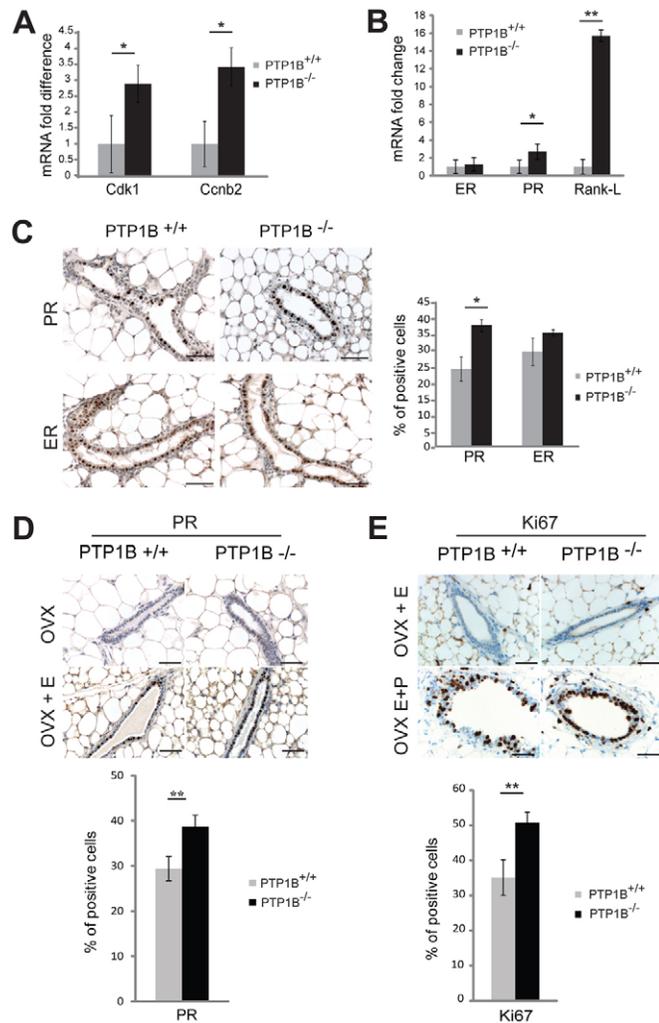


Fig. 3. Absence of PTP1B increases the expression of cell cycle and estrogen-responsive genes. (A) Fold changes in *Cdk1* and *Ccnb2* mRNA as assessed by quantitative real-time PCR. (B) *Er*, *Pr* and *Rankl* mRNA fold changes as assessed by quantitative real-time PCR. (C) (Left) ER- and PR-stained sections of mammary glands from nulliparous mice at estrus. (Right) The percentages of PR- and ER-positive epithelial cells. (D) (Top) Mammary glands from ovariectomized (OVX) mice treated (or otherwise) with 17 β -estradiol (+E) for 48 hours and stained for PR. (Bottom) The percentages of PR-positive epithelial cells in 17 β -estradiol-treated glands (22 images from two mice per genotype were quantified). (E) (Top) Mammary glands from ovariectomized mice treated with 17 β -estradiol alone (+E) or together with progesterone (E+P) for 72 hours and stained for Ki67. (Bottom) The percentages of Ki67-positive epithelial cells in the 17 β -estradiol plus progesterone-treated glands (30 images from four mice per genotype were quantified). Error bars indicate mean \pm s.e.m.; * P <0.05, ** P <0.01 by Student's *t*-test; A-C, n =3. Scale bars: 50 μ m.

expression of *Rankl* and proliferation of *Ptp1b*^{-/-} epithelial cells compared with WT (Fig. 3E; supplementary material Fig. S2B). Thus, PTP1B restrains epithelial cell proliferation by negatively regulating ER activity and PR expression.

PTP1B depletion increases Stat5 phosphorylation

Genetic depletion of Stat5 revealed that this transcription factor enhances the proliferation of epithelial cells in response to estrogen and progesterone stimuli, increases the number of

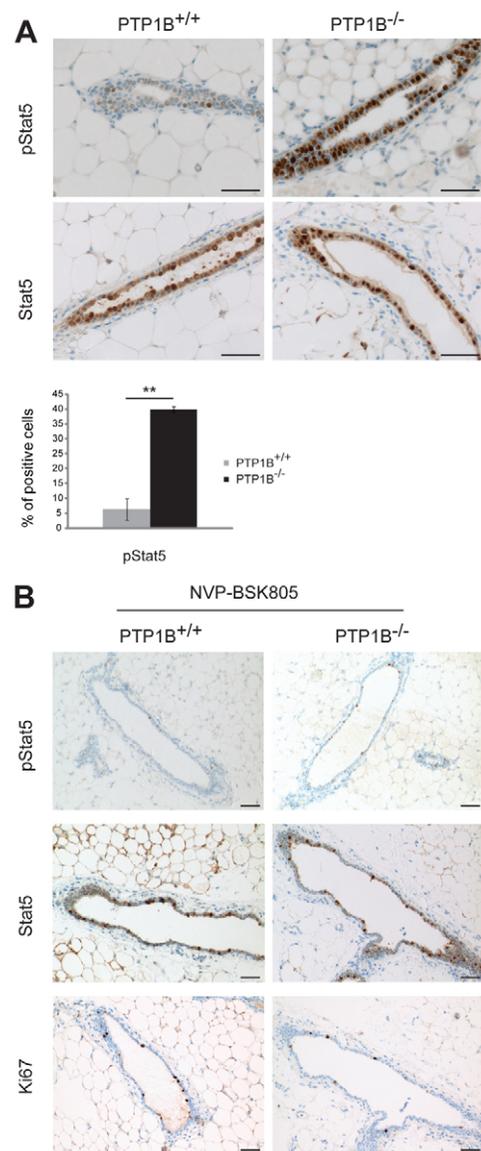


Fig. 4. Absence of PTP1B increases Stat5 phosphorylation. (A) (Top) Mammary gland sections from nulliparous mice at estrus stained for pStat5 and Stat5. (Bottom) The percentage of cells positive for nuclear pStat5. Error bars indicate mean \pm s.e.m.; n =4; ** P <0.01 by Student's *t*-test. (B) Mammary gland sections from NVP-BSK805-treated mice stained for pStat5, Stat5 or Ki67. Scale bars: 50 μ m.

mammary luminal progenitor cells, and promotes alveologenesis (Miyoshi et al., 2001; Cui et al., 2004; Yamaji et al., 2009). The *in vitro* data suggesting Stat5 as a potential PTP1B substrate (Aoki and Matsuda, 2002) raise the possibility that Stat5 is hyperactivated in glands lacking PTP1B. To test this, we stained control and *Ptp1b* knockout glands for Stat5 and phosphorylated Stat5 (pStat5) and found a dramatic increase in pStat5 in the absence of PTP1B (Fig. 4A). We then tested whether Jak2/Stat5 inhibition blocks the increased epithelial cell proliferation observed in *Ptp1b*^{-/-} glands. Treatment of *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice with NVP-BSK805 [a selective Jak2 inhibitor that results in Stat5 dephosphorylation (Baffert et al., 2010)] inhibited Stat5 phosphorylation and, notably, significantly reduced proliferation in *Ptp1b*^{-/-} glands (Fig. 4B).

We next investigated whether overexpression of Prl and/or Prl-R or hyperphosphorylation of Jak2 accounts for the increased pStat5 in glands from *Ptp1b*^{-/-} mice. We found no difference in the plasma levels of Prl, in Prl-R expression or in Jak2 expression and phosphorylation between *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands (supplementary material Fig. S3A-D). This suggests that PTP1B acts via the Stat5 pathway in constraining epithelial cell proliferation.

PTP1B depletion accelerates mammary gland differentiation during pregnancy

We next assessed the consequences of *Ptp1b* deletion on mammary gland development at later stages of pregnancy. Similar to the phenotype at pregnancy day 3 (Fig. 1A,B), whole-mounts and H&E staining of glands showed that the absence of PTP1B also results in the increased formation of alveolar structures at pregnancy days 7 and 10 (Fig. 5A-C).

FACS analysis of MECs isolated from *Ptp1b*^{+/+} and *Ptp1b*^{-/-} mice at pregnancy day 10 showed an increase in the luminal CD24^{hi} Sca1⁻ population, which is enriched in milk-expressing cells (Sleeman et al., 2006). No changes were observed in the other

subpopulations (Fig. 5D). Furthermore, histological analysis revealed that the alveolar structures in *Ptp1b*^{-/-} but not *Ptp1b*^{+/+} glands were precociously distended, displayed lipid droplets and expressed milk proteins, which are all characteristics of differentiated alveoli (Fig. 5A,B, Fig. 6A). Given the higher number of alveoli in glands lacking PTP1B, the observed increase in milk protein expression might be caused by enhanced expression and/or by an increase in the number of milk-producing cells. To distinguish these possibilities, we assessed expression of the genes encoding the early pregnancy milk protein β -casein and the late pregnancy milk protein whey acidic protein (*Wap*), normalized to the expression of epithelial markers cytokeratin 8 and 18 in glands from control and *Ptp1b*^{-/-} mice. PTP1B-depleted alveoli not only expressed milk proteins precociously but also expressed a higher level of milk proteins per cell. PTP1B depletion resulted in 5.5-fold and 4.9-fold increases in the levels of β -casein and *Wap*, respectively (Fig. 6B; data not shown).

Next, we assessed the molecular mechanism underlying the precocious lactogenesis seen in *Ptp1b*^{-/-} glands. Immunoblotting revealed increased phosphorylation of Stat5, a well-established inducer of milk protein expression during pregnancy (Wakao et al.,

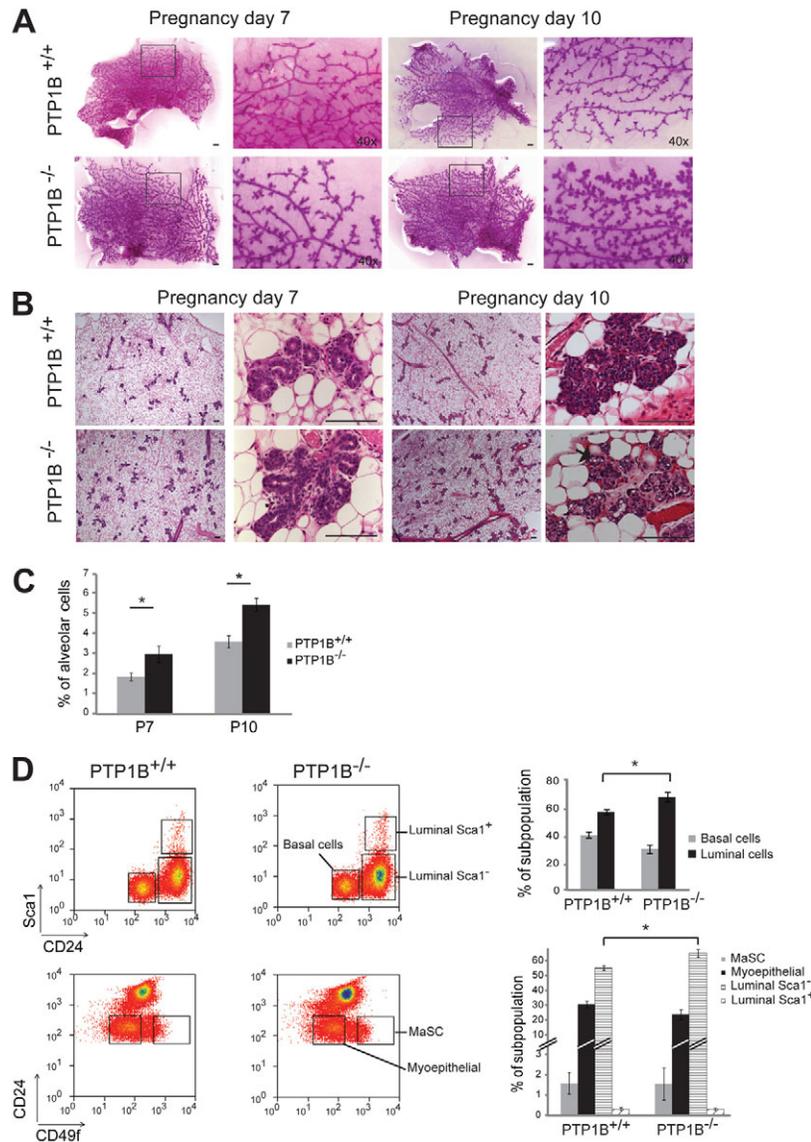


Fig. 5. Precocious secretory differentiation in

PTP1B-deficient mammary glands. (A)

Whole-mounts of *Ptp1b*^{+/+} and *Ptp1b*^{-/-} mammary tissues at pregnancy days 7 and 10. Boxed regions are magnified in images on the right. (B) H&E-stained histological sections of *Ptp1b*^{+/+} and *Ptp1b*^{-/-} mammary tissues at pregnancy days 7 and 10. The arrow points to lipid droplets. (C) The percentages of alveolar cells in *Ptp1b*^{+/+} and *Ptp1b*^{-/-} glands at pregnancy days 7 and 10. (D) (Top row) Dot plots (left) and percentages (right) of luminal (CD24^{hi} Sca1⁺ and CD24^{hi} Sca1⁻) and basal (CD24^{lo} Sca1⁻) cell populations from *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands at pregnancy day 10. (Bottom row) Dot plots (left) and percentages (right) of myoepithelial (CD24^{lo} Sca1⁻ CD49f⁺) and stem cell (CD24^{lo} Sca1⁻ CD49f⁺) populations. Error bars indicate mean \pm s.e.m.; **P*<0.05 by Student's *t*-test; C, *n*=4; D, *n*=3. Scale bars: 1 mm in A; 100 μ m in B.

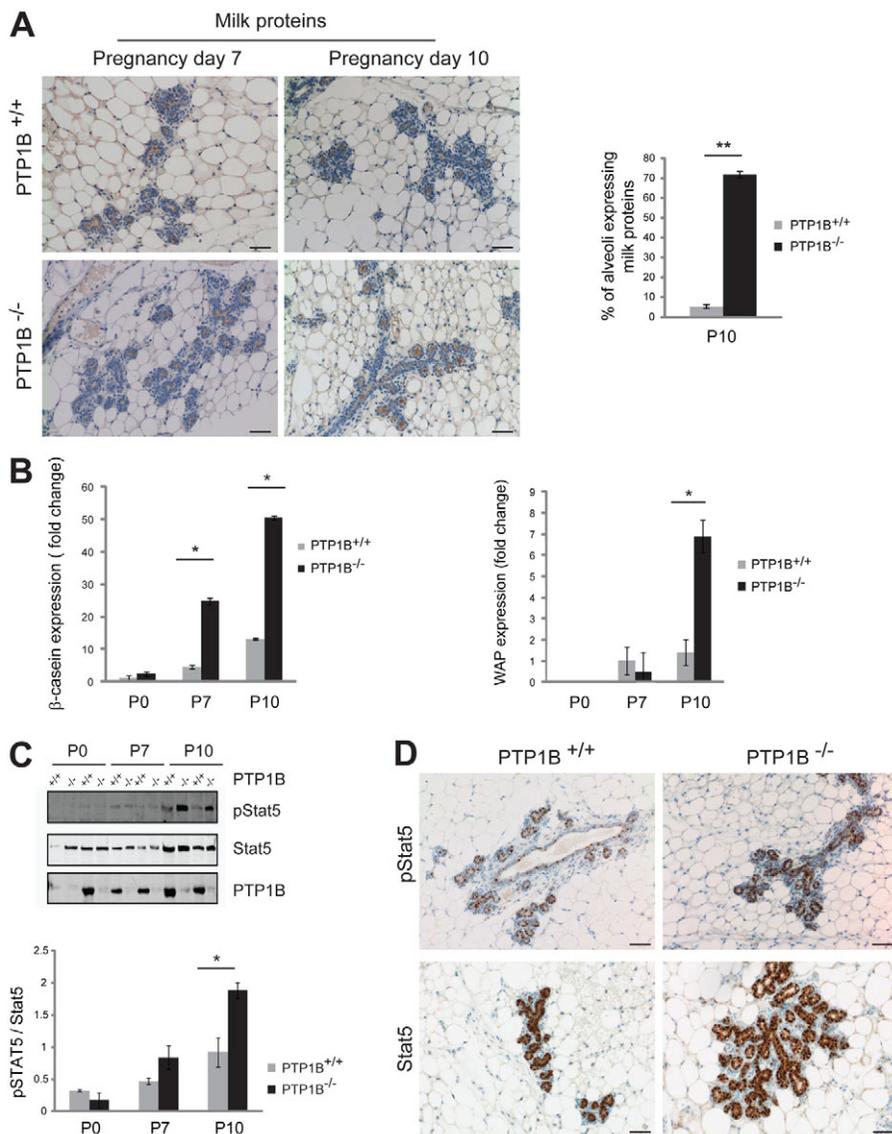


Fig. 6. Activation of Stat5 induces precocious lactogenesis in *Ptp1b*^{-/-} glands.

(A) (Left) Mammary gland sections stained for expression of total milk proteins. (Right) Milk protein-expressing alveoli as a percentage of total alveoli per gland. (B) Fold changes in β -casein and *Wap* mRNA from mice as assessed by quantitative real-time PCR. P0, nulliparous mice at estrus; P7 and P10, pregnancy day 7 and pregnancy day 10. (C) Immunoblots of lysates from glands as indicated (top) and the ratio of pStat5/Stat5 (bottom). (D) Mammary gland sections stained for Stat5 and pStat5 at pregnancy day 10. Error bars indicate mean \pm s.e.m.; * P <0.05, ** P <0.01 by Student's *t*-test; A, n =4; B, C, n =3. Scale bars: 50 μ m.

1994; Liu et al., 1997), in PTP1B-deficient glands compared with controls (Fig. 6C). To exclude the possibility that the observed changes in pStat5 were due to changes in the stroma and not in epithelial cells, we performed immunostaining against pStat5. We found that pStat5 in epithelial cells of glands lacking PTP1B was markedly increased (Fig. 6D). We then tested whether Jak2 expression and/or phosphorylation is altered in glands lacking PTP1B and found no difference in pJak2 between *Ptp1b*^{-/-} and *Ptp1b*^{+/+} glands at pregnancy day 10 (supplementary material Fig. S3C,D).

To investigate whether *Ptp1b* deletion affects involution, we analyzed glands from *Ptp1b*^{-/-} and *Ptp1b*^{+/+} mice 5 days after cessation of suckling and observed no differences (supplementary material Fig. S3E).

These data show that PTP1B depletion precociously increases Stat5 phosphorylation, thus triggering the expression of milk proteins. This suggests that PTP1B expression constrains lactogenesis during pregnancy.

DISCUSSION

Tight regulation of mammary alveologenesis and lactogenesis is fundamental for lactating species. In this study, we have shown that

the tyrosine phosphatase PTP1B constrains these important processes. Alveologenesis is a developmental program characterized by the expansion and differentiation of mammary progenitor cells into alveolar cells. Loss of PTP1B increases the number of progenitor cells in nulliparous mice. This enhances the pool of cells able to generate alveolar structures and, thus, results in the increased alveolar density observed in *Ptp1b*^{-/-} glands during early pregnancy.

Several factors influence mammary gland alveologenesis. Mechanistically, we found that lack of PTP1B induces the expression of several estrogen-responsive genes in nulliparous glands, including *Pr* and its downstream target *Rankl*. PR plays a key role in epithelial cell proliferation and alveolar formation during early pregnancy (Lydon et al., 1995; Brisken et al., 1998; Mulac-Jericevic et al., 2003; Obr and Edwards, 2012). Therefore, the precocious alveolar development observed in *Ptp1b*^{-/-} glands might be mediated by the overexpression and activation of PR, which then precociously initiates alveologenesis.

Estrogen and progesterone have been shown to regulate the number and/or activity of MaSCs via a paracrine mechanism involving the Rank and Wnt pathways (Asselin-Labat et al., 2010;

Joshi et al., 2010; Axlund and Sartorius, 2012). In glands lacking PTP1B, we observed an increase in ER and PR activity associated with an increase in the number and activity of progenitor cells but not of MaSCs. The discrepancy between our results and those reported previously might be due to differences in the degrees of ER and PR activation in the two models.

In addition to increasing PR expression, PTP1B depletion increased the phosphorylation of Stat5, a key regulator of mammary luminal progenitor cells and alveologenesis (Yamaji et al., 2009), suggesting that PTP1B restrains the number of mammary progenitor cells and regulates alveologenesis via Stat5 dephosphorylation. Stat5 has been shown to promote the proliferation of epithelial cells in response to estrogen and progesterone stimuli, which indicated that they act in a common pathway (Miyoshi et al., 2001; Cui et al., 2004). Conceivably, PTP1B depletion increases ER activity and PR expression, which in turn activates Stat5 and leads to increased alveologenesis. *In vitro* studies have demonstrated that PTP1B can directly dephosphorylate Stat5 (Myers et al., 2001), raising an alternative possibility that lack of PTP1B independently increases PR expression via ER and Stat5 phosphorylation. These two possibilities are not mutually exclusive.

But how does lack of PTP1B increase ER activity? *In vitro* studies have shown that PTP1B dephosphorylates ER at tyrosine 537, a residue known to inhibit estrogen binding and to reduce transcriptional activity of ER when phosphorylated (Arnold et al., 1997). These data raise the possibility that PTP1B activates the estrogen pathway by regulating the phosphorylation of ER.

Our results also support a role for PTP1B in lactogenesis. PTP1B depletion induces the precocious expression of milk proteins due to an increase in Stat5 phosphorylation. Indeed, Stat5 is a well-established regulator of lactogenesis, mediating Prl-induced milk expression (Wakao et al., 1994; Liu et al., 1997). Taken together, our results suggest a role for PTP1B as a temporal regulator of mammary gland development that downregulates the progesterone and Stat5 pathway(s) and thus prevents the inappropriate onset of alveologenesis and lactogenesis during pregnancy.

Mammary gland development and differentiation are regulated by a complex mechanism involving several different pathways (Hennighausen and Robinson, 2005; Briskin and O'Malley, 2010). We cannot exclude the possibility that PTP1B acts via other pathways in constraining mammary gland alveologenesis and lactogenesis. For example, PTP1B is a well-known regulator of the insulin and leptin pathways in other organs, and mice lacking PTP1B are insulin and leptin hypersensitive (Elchebly et al., 1999; Klaman et al., 2000). In the light of reports of a role for insulin, Igf1 and Igf2 in mammary gland morphogenesis (Kleinberg et al., 2000; Briskin et al., 2002; Hovey et al., 2003; Berlato and Doppler, 2009), PTP1B might also regulate mammary gland morphogenesis via its inhibitory effects on these pathways.

Epidemiological studies have shown that early menarche, late menopause and late age of first pregnancy are all risk factors for developing sporadic breast cancer (Medina, 2005). Conversely, early full-term pregnancy (<24 years) decreases lifetime breast cancer risk. Clearly, the hormonal milieu and breast development cycles, possibly through changes in the differentiation state of breast stem/progenitor cells, affect the susceptibility of the breast to oncogenic transformation. Our finding that *Ptp1b* deletion induces precocious differentiation of the mammary gland raises the possibility that the cells of origin of Her2-evoked mammary tumors are decreased in *Ptp1b*^{-/-} mice. This would explain why deletion

of *Ptp1b* delays or prevents mammary tumor formation in MMTV-NeuNT and MMTV-NDL1 mice (Bentires-Alj and Neel, 2007; Julien et al., 2007; Balavenkatraman et al., 2011). An exploration of this possibility is now warranted.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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