

Research article

Deficiency of *Pten* accelerates mammary oncogenesis in MMTV-*Wnt-1* transgenic mice

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Abstract

Background: Germline mutations in the tumor suppressor *PTEN* predispose human beings to breast cancer, and genetic and epigenetic alterations of *PTEN* are also detected in sporadic human breast cancer. Germline *Pten* mutations in mice lead to the development of a variety of tumors, but mammary carcinomas are infrequently found, especially in mice under the age of six months.

Results: To better understand the role of *PTEN* in breast tumor development, we have crossed *Pten* heterozygous mice to MMTV-*Wnt-1* transgenic mice that routinely develop ductal carcinomas in the mammary gland. Female *Wnt-1* transgenics heterozygous for *Pten* developed mammary tumors earlier than *Wnt-1* transgenics that were wild type for *Pten*. In most tumors arising in *Pten* heterozygotes, the *Pten* wild-type allele was lost, suggesting that cells lacking *Pten* function have a growth advantage over cells retaining a wild type allele. Tumors with LOH contained high levels of activated AKT/PKB, a downstream target of the PTEN/PI3K pathway.

Conclusions: An animal model has been developed in which the absence of *Pten* collaborates with *Wnt-1* to induce ductal carcinoma in the mammary gland. This animal model may be useful for testing therapies specific for tumors deregulated in the PTEN/PI3K/AKT pathway.

Introduction

Cancer evolves from the accumulation of mutations and the deregulation of two classes of genes, oncogenes and tumor suppressor genes. The large majority of breast cancers arise in epithelia of the breast, and they are thought to evolve from hyperplasia with atypia to carcinoma in situ, invasive carcinoma, and, finally, metastatic disease. The molecular mechanisms leading to breast malignancies are unclear, but many genetic abnormalities and epigenetic factors have been implicated, includ-

ing changes affecting known tumor suppressor genes (*p53*, *BRCA1*, *BRCA2*, *PTEN/MMAC1/TEP1*) and proto-oncogenes (*neu/ErbB2/HER2*, *ErbB1/EGFR*, *PRAD-1/cyclin D1*, *Mdm2*, and *c-myc*) [reviewed in ref. 1].

The gene product of *PTEN* (*phosphatase and tensin* homolog deleted from chromosome 10) is a lipid phosphatase that reverses the activities of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating the D3 position of its lipid products, phosphati-

diinositol-3, 4 bis-phosphate (PtdIns(3,4)P₂), phosphatidylinositol-3,5 bis-phosphate (PtdIns(3,5)P₂), and 3,4,5-tri-phosphate (PtdIns(3,4,5)P₃) [2, 3], the elevated levels of which are linked to the transforming ability of PI3K [reviewed in ref. 4].

PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ recruit AKT (also known as protein kinase B [PKB]) to the membrane through the N-terminal pleckstrin homology (PH) domain of AKT [reviewed in ref. 5]. Membrane localization renders AKT permissive to phosphorylation by phosphatidylinositol-dependent kinases, PDK1 and PDK2, at Thr308 within the activation loop and at Ser473 near the carboxyl terminus. Phosphorylation activates the kinase domain of AKT, which, in turn, phosphorylates target proteins on serine and threonine residues. Substrates of AKT include glycogen synthase kinase 3 (GSK3), p70/S6 kinase, BAD, Caspase 9, 4E-BP1, IκB kinase α and members of the Forkhead transcription family [reviewed in ref. 6, 7]. Constitutive membrane localization of AKT leads to its deregulated activation and can induce oncogenic transformation [8, 9].

All three human members of the AKT family are over-produced in various human cancers. *AKT1* is over-expressed in gastric adenocarcinomas and breast cancer cell lines, *AKT2* is amplified and over-expressed in pancreatic, ovarian, and breast cancers, and *AKT3* is upregulated in estrogen receptor-deficient breast cancer and in androgen-independent prostate cell lines [reviewed in ref. 6].

Consistent with the role of PTEN as a lipid phosphatase, *PTEN*-deficient human and mouse cells have elevated levels of intracellular PtdIns(3,4,5)P₃ [10, 11] and demonstrate constitutive activation of AKT in the absence of growth factors [12,13,14]. The relationship between components of this pathway is also conserved in flies and worms.

A germline mutation in *PTEN* is associated with multi-neoplastic, autosomally dominant syndromes in humans, such as Cowden disease and Bannayan-Zonana syndrome (also known as Bannayan-Riley-Ruvalcaba syndrome), and 20-50% of affected females in these families develop breast cancer [15,16,17]. The *PTEN* gene is also frequently deleted or mutated in a variety of sporadic human malignancies, such as glioblastoma and carcinomas of the prostate, endometrium and breast. Although somatic mutations in the gene have been detected in only about 1% of breast cancers, loss of heterozygosity (LOH) at the *PTEN* locus (10q23) is frequently found (approximately 40%), and 33% of breast tumors have no detectable, or reduced, levels of the protein product [18,19,20,21,22].

Three independent mouse lines carrying inactivating *Pten* mutations have been made [23,24,25]. All mice homozygous for these *Pten* mutations die during early embryogenesis. By 6 months of age, heterozygous mice develop tumors of the lymphoid system, colon, uterus, and adrenal medulla with very high penetrance, and tumors of the prostate and thyroid with lower penetrance. While benign mammary lesions, usually fibroadenomas, are found in female mice after 7 months of age, few animals develop ductal carcinomas [KP and RP, unpublished; 26], which account for over 85% of human breast cancers.

In an effort to develop a model for studying the effects of *Pten* inactivation in mammary oncogenesis, we have introduced an additional genetic alteration, an MMTV-*Wnt-1* transgene, by breeding *Pten* heterozygotes [25] with MMTV-*Wnt-1* transgenic (TG) mice [27]. Female mice harboring the *Wnt-1* transgene develop extensive mammary hyperplasia early in life, and 50% of them develop mammary ductal carcinomas by 6 months of age [27]. This transgene has been shown to collaborate with *Fgf-3* and inactivation of *p53* to accelerate the appearance of mammary tumors [28, 30]. After a mutant allele of *Pten* was crossed into *Wnt-1* TG mice, we found that ductal carcinomas appeared earlier than either alteration alone and that LOH at the *Pten* locus occurred in the majority of these tumors, suggesting a selective growth advantage in cells that lack *Pten* functions.

Results

Wnt-1 TG mice have been used to assess the functions of a number of genetic lesions in breast carcinogenesis because tumors appear with predictable kinetics. The median age at which tumors are detected is 5-6 months in MMTV-*Wnt-1* TG females [27, 28, 30, 31]. After one mutated allele of *Pten* was bred into this transgenic line, tumors appeared significantly earlier than in sibling females harboring only a MMTV-*Wnt-1* transgene ($P < 0.001$, Fig. 1A). Fifty percent of *Pten*^{+/-}, *Wnt-1* TG female mice formed tumors by 3.5 months, 2.5 months earlier than *Wnt-1* TG mice (Fig. 1A). While it took one year for the entire cohort (15) of *Wnt-1* TG females with a wild type *Pten* background to develop tumors, all *Wnt-1* TG, *Pten*^{+/-} females showed tumors by 5 months of age. Five each of *Pten*^{+/-} mice without a *Wnt-1* transgene were monitored for 7 months and 10.5 months, but none developed any palpable mammary lesions. These observations differ from a recent report that one of the other two *Pten*^{+/-} lines develops fibroadenomas and adenocarcinomas in the mammary tissue after 7 months of age [26]. The lack of a mammary phenotype in our studies could be due to differences in the targeting constructs, the genetic backgrounds, or even environmental agents.

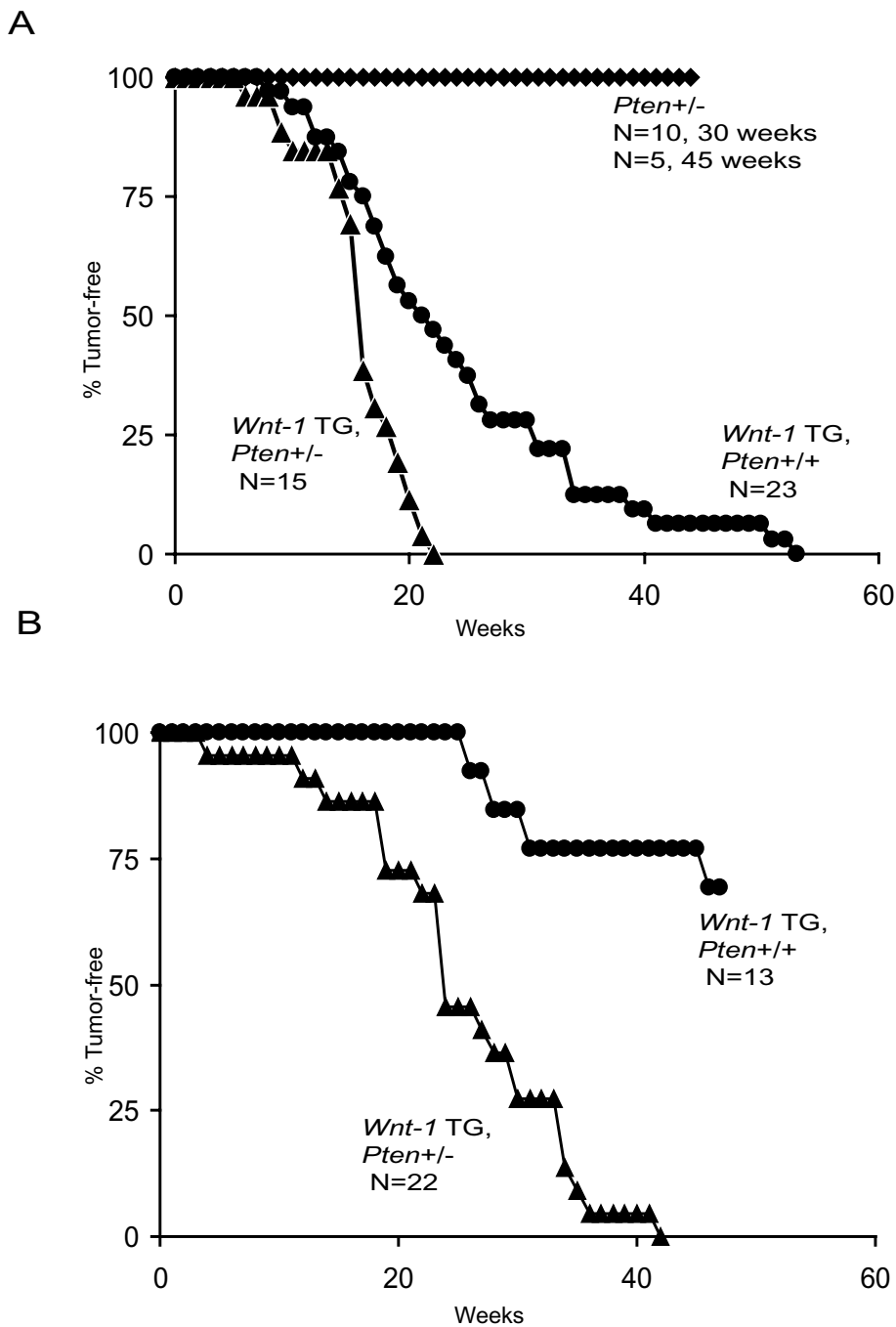


Figure 1

Pten heterozygosity accelerates tumor appearance in *Wnt-1* TG females (A) and males (B). Percentages of mice that were tumor-free, as determined by weekly visual inspection and/or palpation, were plotted against the age when tumors were found. Genotypes and sizes are as indicated. For the *Pten*^{+/-} female group without the *Wnt-1* transgene, 10 mice were monitored to 30 weeks of age; among the 10 mice, 5 were kept for another 15 weeks of age. (A) All mice showed at least one adenocarcinoma. (B) The *Wnt-1* TG, *Pten*^{+/+} cohort (13) had 1 mammary and 3 salivary tumors, and the *Wnt-1* TG, *Pten*^{+/-} cohort (22) had 2 mammary, 6 muzzle, and 14 salivary tumors.

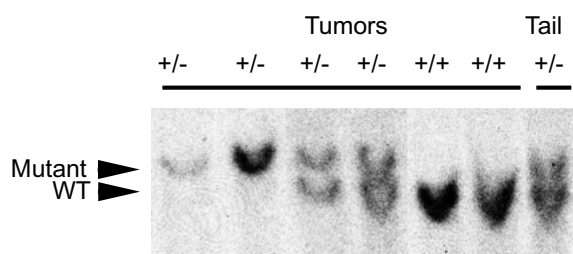


Figure 2

Example of LOH analysis. Blots of tumor and tail DNAs, digested with *Pst*I, were probed with a 32 P labeled *Pten* probe. Two bands could be observed, representing wild type and mutant alleles, as indicated at the left. Genotypes of mice are shown at the top.

By one year of age, approximately 15% of *Wnt-1* TG males have been reported to develop mammary tumors [27, 28, 31]. In our cross, one of the four tumors from *Wnt-1* TG males, in an otherwise wild type background, was a mammary carcinoma; the other three tumors were salivary in origin, with a similar histopathology to the mammary lesion. The appearance of tumors was greatly accelerated in the *Pten* heterozygous background (Fig. 1B). Fifty percent of males developed tumors by 5.5 months of age, and all developed tumors within 10 months. As in the case of *Wnt-1* TG male mice without *Pten* mutations, the majority of tumors (14/22) arose in the salivary tissue; only a small number of tumors (2/22) were mammary in origin, and, interestingly, some (6/22) were bilateral or unilateral muzzle tumors of epithelial origin, similar histopathologically to lesions found in mammary and salivary tissues.

To determine if the wild type allele of *Pten* was inactivated in tumors arising in a *Pten*^{+/-} background, 11 tumors from *Wnt-1* TG, *Pten*^{+/-} mice were examined for LOH by Southern hybridization. Seven tumors had lost the wild type *Pten* allele (see Fig. 2 for an example of the analysis). This result suggests that, in tumorigenesis, cells that lack a functional allele of *Pten* have a significant proliferative advantage over the cells that retain the normal copy of *Pten*.

Histological examination showed that tumors from *Wnt-1* TG mice, regardless of *Pten* status, were adenocarcinomas with cribriform, cystic and focal papillary growth patterns. However, 3 of the 6 tumors that were *Wnt-1* TG, *Pten*^{+/-} with LOH appeared to have a higher nuclear grade, more cellular stroma and a more infiltrative pattern compared to the tumors without a *Pten* defect (Fig. 3), suggesting a more aggressive phenotype.

In human cancer, a tumor suppressor gene can be inactivated by mutations, chromosomal loss, promoter silencing, and other means. To determine if the *Pten* protein was produced in *Wnt-1* TG, *Pten*^{+/-} tumors that did not undergo LOH, sections were analyzed using an immunohistochemical assay with a polyclonal rabbit antibody. Abundant amounts of *Pten* were detected in mammary ducts from wild type mice. *Pten* was detected, at reduced levels, in both hyperplastic ducts and mammary tumors that did not have LOH at the *Pten* allele (Fig. 4), suggesting that *Pten* was, indeed, made from the second allele. Similar low levels of *Pten* were also detected in mammary ductal epithelial cells in *Pten* heterozygous mice that did not have a *Wnt-1* transgene, suggesting that the *Wnt-1* transgene does not influence *Pten* expression. As expected, *Pten* was not detected in tumors that had undergone LOH at the *Pten* locus (Fig. 4E).

Inactivation of *Pten* promotes membrane recruitment of AKT and its subsequent activation by phosphorylation at Ser473 and Thr308 [10, 11, 13]. All three human isoforms of AKT have been found to be deregulated in human breast cancer cells [32,33,34]. To determine if activated AKT was increased in tumors that had a *Pten* defect, these sections were stained using an antibody that specifically detects AKT1 phosphorylated at Ser 473 (and AKT2 and AKT3 phosphorylated at equivalent sites). Tumors that were *Wnt-1* TG, *Pten*^{+/-} with LOH displayed staining of phosphorylated AKT (Fig. 5A). Surprisingly, the staining was patchy, suggesting additional factors may be required for AKT activation. As expected, phosphorylated AKT was undetectable in hyperplastic ducts, in tumors from *Wnt-1* TG, *Pten*^{+/+} mice, and tumors from *Wnt-1* TG, *Pten*^{+/-} mice that retained the wild type allele (Fig. 5B & 5C).

Several apoptotic pathways may be inhibited upon activation of AKT, promoting maintenance and survival of tumor cells. Cellular proliferation may also be enhanced by activated AKT, which inhibits p27 and GSK-3 (leading to stabilization of cyclin D1) [35]. However, tumors from *Wnt-1* TG, *Pten*^{+/+} mice have low apoptotic rates (40 ± 16 per digital camera field using a 20x objective) and high proliferation rates ($53\% \pm 11$). Neither apoptotic nor proliferation rates were affected by the status of *Pten* when measured by the TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling) assay, which labels the ends of fragmented DNA present in apoptotic cells, and immunohistochemical staining for Ki67, a cell proliferation marker (data not shown).

Most *Wnt-1* TG mice (38/49) developed only one palpable tumor regardless of *Pten* status. Fifty-three of 55 single sections from *Pten*^{+/+} glands that were free of a

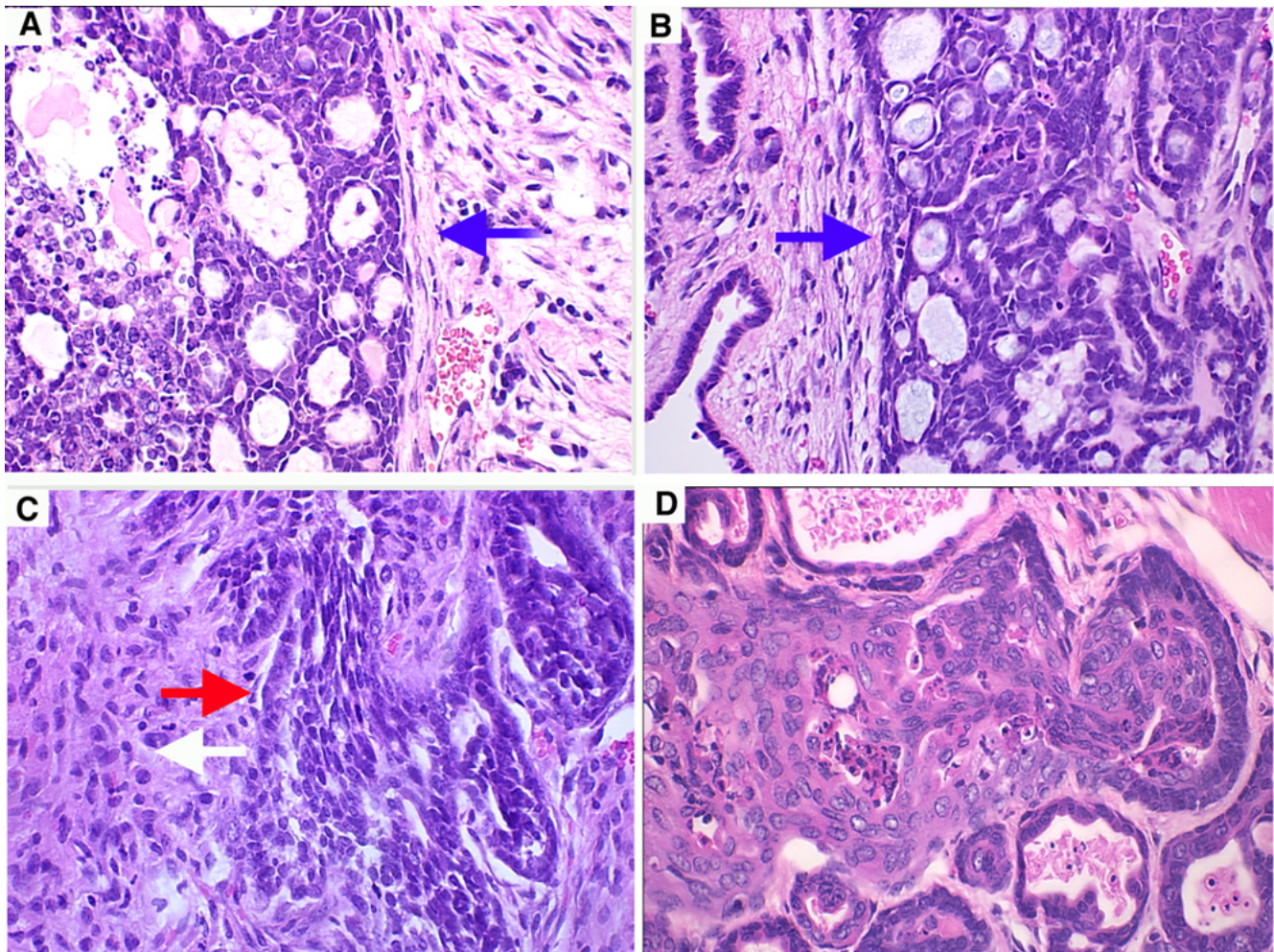


Figure 3

Representative H+E sections of mammary tumors from progeny females of the MMTV-*Wnt-1* and *Pten*^{+/-} cross. Sections were from tumors that were *Wnt-1* TG⁺, *Pten*^{+/+} (A); *Wnt-1* TG⁺, *Pten*^{+/-} (B); and *Wnt-1* TG⁺, *Pten*^{+/-} with LOH (C and D). A 40x objective was used. Blue arrows indicate the smooth boundaries of tumors, red arrow marks the infiltrating front of the tumor, and white arrows label the highly cellular stroma. (D) shows a part of an intraductal squamous lesion.

palpable mass did not show any lesions more advanced than ductal hyperplasia. However, at the time of sacrifice, the majority (50/56) of glands from *Wnt-1* TG, *Pten*^{+/-} mice had intra-ductal squamous lesions of various sizes (Fig. 3D). These squamous lesions were usually multi-focal and located both at the periphery of the adenocarcinomas and in other mammary glands free of adenocarcinomas.

Since *Pten* mutations are usually found in advanced tumors, and the protein has been suggested to inhibit cell spreading, migration, and invasion [36, 37], it is possible that the tumors from *Wnt-1* TG, *Pten*^{+/-} mice may have a higher potential to metastasize than those from mice carrying a *Wnt-1* transgene in a wild type *Pten* background. *Wnt-1* TG mice occasionally show lymphoid or pulmonary metastasis [27], and serial sectioning of lung

tissues from the animals reported here revealed microscopic metastatic foci in two of five lungs from *Wnt-1* TG mice bearing primary tumors 1.5-2.0 cm in size. However, *Wnt-1* TG mice and the *Wnt-1* TG, *Pten*^{+/-} mice harbored metastatic foci at similar frequencies (5/10). (Statistical evaluation was not performed because the sample sizes were too small.)

Discussion

Mouse models are valuable research tools for the study of the molecular mechanisms of oncogenesis and for the testing and screening of cancer therapeutic agents targeted to specific oncogenic lesions. In order to produce such a model for studying the effects of *Pten* mutations in breast cancer, we have crossed *Wnt-1* TG mice to mice heterozygous for *Pten*. Mice transgenic for MMTV-*Wnt-1* and heterozygous for *Pten* developed mammary aden-

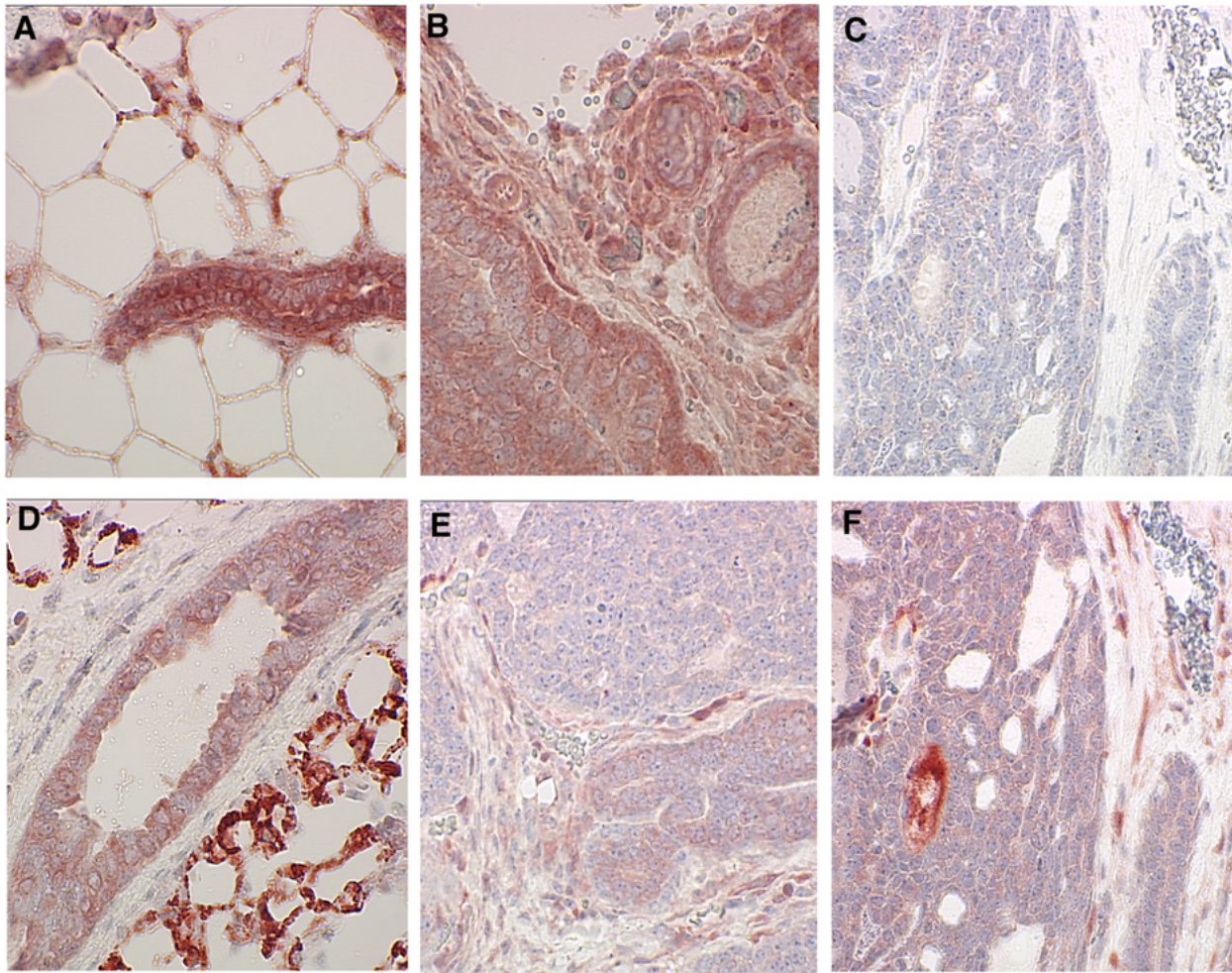


Figure 4

Immunohistochemical staining for Pten. Sections of mammary glands (A, D) and tumors (B, C, E, F) were stained for Pten (A, B, D, E, F) or control rabbit Ig (C) and counterstained with hematoxylin. Genotypes of mice were *Pten*^{+/+} (A); *Wnt-1* TG, *Pten*^{+/+} (B); and *Wnt-1* TG, *Pten*^{+/-} (C-F). (E) and (C, F) were from tumors with or without LOH, respectively. A 40x objective was used.

ocarcinomas much earlier than mice carrying either lesion alone. The majority of tumors in *Wnt-1* TG, *Pten*^{+/-} mice had lost the wild type allele of *Pten*, suggesting that the complete inactivation of *Pten* function provides an additional growth advantage to these tumors. LOH at the *Pten* locus has recently been reported in mammary tumors arising in *Pten* heterozygous mice [26], but only 4% of the mice developed tumors before the age of 30 weeks, and, in our cross, none of the *Pten*^{+/-} mice without the *Wnt-1* transgene had a palpable mammary tumor before the age of 30 weeks. LOH at the *Pten* locus has also been reported in adrenal and lymphatic tumors, but not in uterine tumors and prostate lesions in *Pten*^{+/-} mice [KP & RP, submitted; 38].

Activated AKT was more abundant in tumors that were *Wnt-1* TG, *Pten*^{+/-} with LOH, as has been shown for uterine tumors in *Pten* heterozygous mice (KP, RP, submitted). The presence of areas unstained with antibody specific for phosphorylated AKT suggests that additional factors other than Pten may influence the activation of AKT.

Neither apoptosis nor proliferation, as determined by the TUNEL assay and KI67 staining, was significantly different between tumors heterozygous for *Pten* (with or without LOH) and those without a *Pten* defect. This result may seem paradoxical since increased phosphorylated AKT, due to inactivation of *Pten*, suppresses apoptosis and promotes proliferation. However, tumors in *Wnt-1* TG mice in a wild type *Pten* background already have

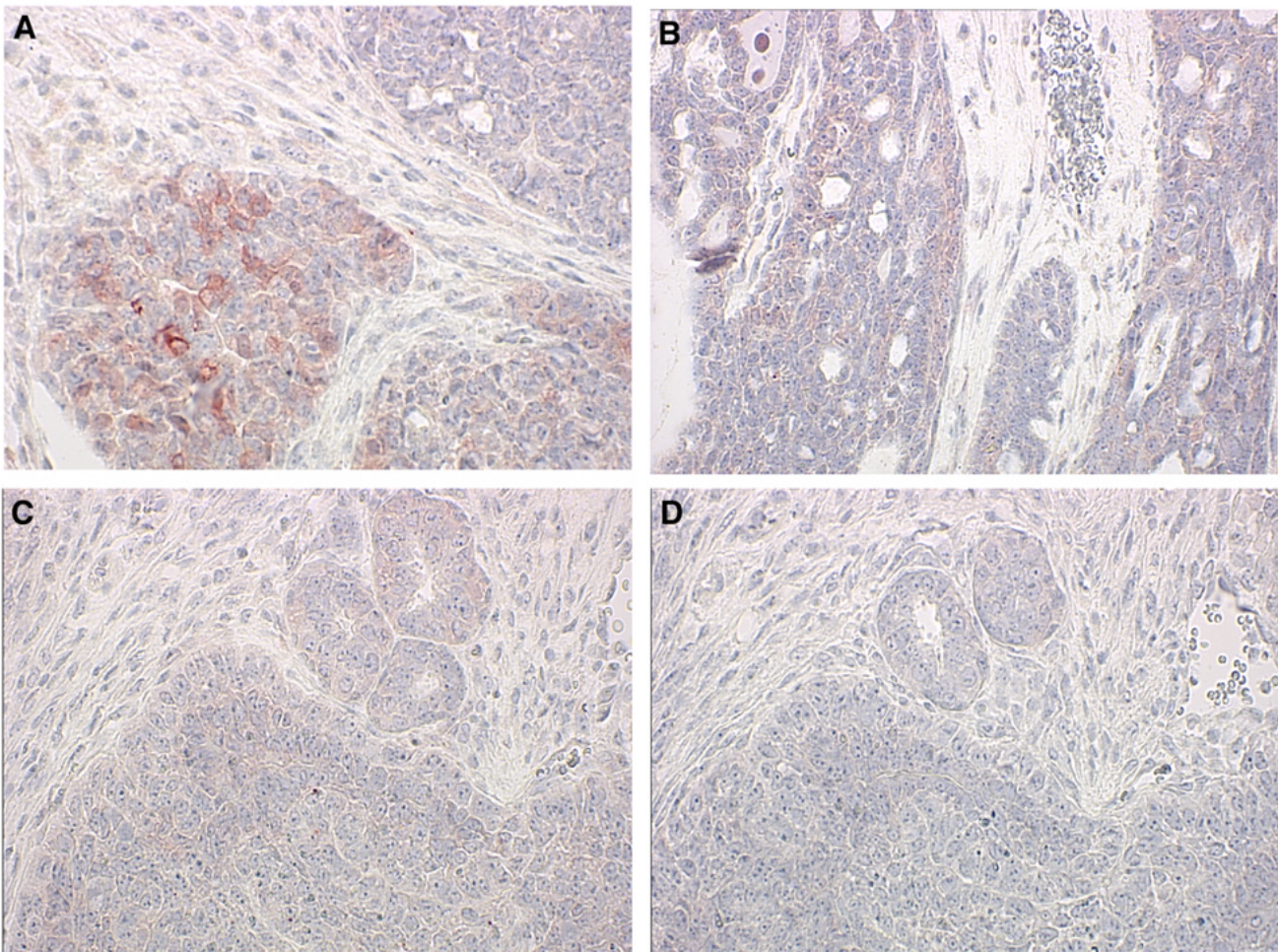


Figure 5

Immunohistochemical staining for phosphorylated Akt. Sections of tumors were stained for phospho-Akt (A, B, C) or control rabbit IgG (D) and counterstained with hematoxylin. Genotypes of mice were *Wnt-1* TG⁺, *Pten*^{+/-} (A, B); *Wnt-1* TG⁺, *Pten*^{+/+} (C, D). (A) and (B) were from tumors with or without LOH, respectively. A 40x objective was used.

highly suppressed apoptosis and accelerated proliferation rates, and any incremental changes, if present, may be difficult to detect with our current assays. Nonetheless, a seemingly minor difference in either apoptosis or proliferation could have a dramatic impact on tumor growth over time.

Accelerated appearance of mammary adenocarcinomas in the presence of a *Pten* mutation suggests a synergy between *Pten* inactivation and the *Wnt-1* transgene. The apparent cooperation of these two genetic factors is further strengthened by our finding that the majority of tumors arising in a *Pten*^{+/-} background have lost the wild type allele. To date, heterozygous inactivation of any of the other tumor suppressor genes implicated in breast cancer, including *P53*, *Bracl*, *Bracl2*, and *Rb*, has failed to cooperate with *Wnt-1* in promoting the appearance of mammary tumors [reviewed in ref. 39]. Therefore, it is

possible that the signal pathway controlled by *Pten* is one of the preferred partners of the *Wnt-1* pathway in cancer progression, although it remains possible that LOH at the *Pten* locus is more likely to occur than for the other alleles crossed into *Wnt-1* TG mice. Even though *Wnt-1* has not been directly implicated in human cancers, many components of the Wnt signaling pathway (such as β -catenin, APC, *c-myc*, and *cyclin D1*) are involved in human tumors including breast cancer [reviewed in ref. 40].

One of the goals of mouse modeling efforts is to provide an opportunity for pre-clinical testing of generalized or mechanism-specific therapeutics in a defined genetic setting. Many human breast tumors have reduced levels of PTEN, hence inhibitors of the cell signaling pathways affected by PTEN could be tested in the model we have developed. One such candidate is the rapamycin analog

CCI779, which has been suggested to inhibit the AKT signaling pathway, probably through inhibition of mTOR (target of rapamycin), a potential target of AKT [reviewed in ref. 41]. CCI779 has been shown to have inhibitory effects on the growth of *Pten*-deficient tumors in the uterus and prostate in mice (KP & RP, submitted; M. Neshat & C. Sawyers, submitted). Since tumors arise rapidly after a copy of *Pten* is inactivated, and with predictable kinetics, this model may be useful for testing the efficacy of these therapies.

Results presented here and published earlier [15,16,17,18, 26] have clearly demonstrated the significant impact of *Pten* on mammary oncogenesis. Future testing will examine the tumorigenic effects caused by other kinds of genetic damage in the signaling pathways affected by *Pten*. The TVA-directed gene delivery system [reviewed in ref. 42] can be used to introduce viral vectors carrying genes encoding an activated form of PI3K, AKT, or candidate substrates for AKT into the mammary tissue of *Wnt-1* TG mice. This would allow the testing of therapeutic interventions directed to specific steps of the signaling pathway regulated by *Pten*.

Materials and methods

Mice

MMTV-*Wnt-1* TG mice [27] and those that carry a mutant allele of *Pten* [25] have been described. Male *Wnt-1* TG mice (a mixture of FVB, SJL, and C57BL/6) were crossed with *Pten* heterozygous females (a mixture of C57BL/6 and 129SvJ) to generate all three genotypes used in this paper: *Wnt-1* TG, *Pten*^{+/-}; *Wnt-1* TG, *Pten*^{+/+}; and *Pten*^{+/-}. Genotyping for the *Wnt-1* transgene was done using PCR as described [27]; PCR using primers from the neo cassette was performed to detect the *Pten* knockout allele. Mice were monitored weekly for tumor formation by visual inspection and/or palpation; most tumors were found when they were approximately 0.5 cm. Most of tumors were harvested for further analysis when they were 1.5 cm in diameter. Tissues were fixed in 10% buffered formalin and paraffin-embedded. For histological survey, one H-E section per block was analyzed.

Immunohistochemical staining and TUNEL assay

Formalin-fixed and paraffin-embedded sections were deparaffined and rehydrated in several changes of xylene, ethanol, and water. Antigen-unmasking was achieved by boiling the slides for 15 minutes in citrate buffer, pH 6.0, or 1 mM EDTA. Endogenous peroxidase activity was inactivated by a 5 minute incubation in 3% hydrogen peroxide, when needed. Immunohistochemical stainings were performed using Vector ABC kits (Vector Laboratories) following the manufacturer's recommendations and as described [25]. Rabbit anti-

bodies against *Pten*, Ki67, and AKT phosphorylated at Ser 473 were from Neomarkers, Dako, and New England BioLabs, respectively. The TUNEL assay was performed as described [25].

Southern hybridization

DNAs extracted from tissues and tumors were digested with *Pst*I, followed by electrophoresis, and blotted to Nylon+ membrane. Hybridization was done in ExpressHyb solution (Clontech) at 60°C using a ³²P dCTP-labeled probe that was synthesized from a 0.9 Kb template located in intron 5 of *Pten* [25] using the RTS RadPrime DNA labeling system (Life Technologies). The probe was incubated with Cot-1 DNA (Life Technologies) at 70°C for 10 minutes before addition to the hybridization solution.

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