Overexpression of the Tumor Suppressor Gene Phosphatase and Tensin Homologue Partially Inhibits Wnt-1–Induced Mammary Tumorigenesis

Hong Zhao,[1] Yongzhi Cui,[2] Joelle Dupont,[1] Hui Sun,[1] Lothar Hennighausen,[2] and Shoshana Yakar[1]

[1]Diabetes Branch and [2]Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

Abstract

The tumor suppressor phosphatase and tensin homologue (PTEN) is involved in cell proliferation, adhesion, and apoptosis. PTEN overexpression in mammary epithelium leads to reduced cell number and impaired differentiation and secretion. In contrast, overexpression of the proto-oncogene *Wnt-1* in mammary epithelium leads to mammary hyperplasia and subsequently focal mammary tumors. To explore the possibility that PTEN intersects with Wnt-induced tumorigenesis, mice that ectopically express PTEN and Wnt-1 in mammary epithelium were generated. PTEN overexpression resulted in an 11% reduction of Wnt-1–induced tumors within a 12-month period and the onset of tumors was delayed from an average of 5.9 to 7.7 months. The rate of tumor growth, measured from 0.5 cm diameter until the tumors reached 1.0 cm diameter, was increased from 8.4 days in Wnt-1 mice to 17.7 days in Wnt-1 mice overexpressing PTEN. Here we show for the first time *in vivo* that overexpression of PTEN in the Wnt-1 transgenic mice resulted in a marked decrease in the insulin-like growth factor (IGF)-I receptor levels leading to a reduced IGF-I–mediated mitogenesis. Moreover, the percentage of BrdUrd-positive epithelial nuclei was decreased by 48%. β-Catenin immunoreactivity was significantly decreased and the percentage of signal transducer and activator of transcription 5a (stat5a)–positive mammary epithelial cells was increased by 2-fold in Wnt-1 mice overexpressing PTEN. The present study shows that PTEN can partially inhibit the Wnt-1–induced mammary tumorigenesis in early neoplastic stages by blocking the AKT pathway and by reducing the IGF-I receptor levels in mammary gland. This study identifies the PTEN as a therapeutic target for the treatment of mammary cancer and presumably other types of cancer. (Cancer Res 2005; 65(15): 6864-73)

Introduction

Wnt-1 protein is a member of a large family of secreted proteins (1). In mice the Wnt-1 protein is exclusively expressed in the developing central nervous system where it is required for the development of midbrain and cerebellum and in the adult mouse it is also expressed in the testes (1). The Wnt proteins bind to their seven transmembrane frizzled receptors and transmit a signal to cytoplasmic phosphoproteins of the disheveled family, which then inhibit the activity of the constitutively active glycogen synthase kinase 3β (2). This sequence of events leads to stabilization and increased levels of β-catenin, which is known to associate with membrane-bound E-cadherin and several DNA binding proteins such as T-cell factors/lymphoid enhancer transcription factors (3, 4). Translocation of β-catenin into the nucleus results in transactivation of number of genes, such as c-*myc* and *cyclin D1*, and therefore drives the cell cycle and promotes proliferation (5, 6).

Continuous activation of the Wnt-1 signaling pathway in mammary epithelium induces the development of mammary hyperplasia and adenocarcinoma (7). However, a reduction of Wnt signaling results in a rapid disappearance of Wnt-initiated invasive primary tumors as well as pulmonary metastases (8). Tumor regression does not require p53 and occurs even in highly aneuploid tumors. However, the absence of p53 is associated with incomplete tumor regression and acceleration of recurrence of fully regressed mammary tumors because loss of p53 results in Wnt-independent tumor growth. Therefore, p53 plays an important role in suppressing tumor recurrence (8).

The tumor suppressor gene product of the phosphatase and tensin homologue (*pten*) gene is a lipid phosphatase, which reduces the cellular levels of phosphatidylinositol-3-phosphate (PI3P) by antagonizing the activity of phosphoinositol-3 kinase (PI3K; refs. 9–11). At the cellular level, loss of PTEN activity leads to accumulation of cytosolic PI3P and activation of the AKT pathway, which results in increased cell growth, survival, invasiveness, and metabolism (12–15). On the other hand, it has been shown that overexpression of PTEN in cell culture down-regulates cyclin D1 expression and results in cell cycle arrest (14, 15). In a previous study, we showed that overexpression of PTEN in a PC3 prostate cancer cell line resulted in a marked reduction in the levels of insulin-like growth factor (IGF)-I receptor and a significant reduction in its expression on the cell surface (16). In that study, we showed that PTEN overexpression affected IGF-I receptor synthesis at the posttrancriptional level, which might explain, at least in part, the reduction in cell proliferation in those cells.

PTEN was the first phosphatase identified to be frequently mutated or to show somatic deletions in various human cancers. The majority of cancer-related mutations have been mapped within the conserved catalytic domain of PTEN, suggesting that the phosphatase activity of PTEN is required for its tumor suppressor function (17–19). Deletion of both PTEN alleles in the mouse resulted in early embryonic lethality (20) and heterozygous PTEN mice developed hyperplastic or neoplastic changes in many organs at an early age (20, 21). When heterozygous PTEN mice were crossed with Wnt-1 transgenic mice, ductal carcinomas appeared earlier than either Wnt-1 or heterozygous PTEN alone (22). Moreover, loss

Requests for reprints: Shoshana Yakar, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bldg. 10, Room 8D12, 9000 Rockville Pike, Bethesda, MD 20892-1758. Phone: 301-496-7112; Fax: 301-402-4900; E-mail: ShoshanaY@intra.niddk.nih.gov.

of heterozygosity of PTEN occurred in the majority of those tumors, suggesting a selective growth advantage in cells that lack PTEN.

In our previous study, we showed that overexpression of PTEN specifically in the mammary gland resulted in a marked decrease in mammary epithelial cell proliferation, a profound increase in epithelial cell apoptosis, and, as a consequence, mammary tissue hypoplasia which led to incomplete functional differentiation and failure in lactation (23). To test the functional significance of a possible crosstalk between the inhibitory PTEN signaling pathway and the stimulatory Wnt-1 signaling pathway, we employed a transgenic approach. Mice were generated that express both the *PTEN* and the *Wnt-1* transgenes in mammary epithelium under the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) promoter. This study describes a new link between the Wnt-1 and PTEN signaling pathways and shows that overexpression of PTEN inhibits the growth and progression of Wnt-1–induced mammary tumors. This study also suggests that PTEN can inhibit the Wnt-1 signaling pathway through inhibition of the cellular accumulation of β-catenin, down-regulation of cyclin D1, and reduction in the IGF-I receptor levels which can also be part of the mitogenic processes driven by the *Wnt-1* transgene.

Materials and Methods

Animal husbandry and genotyping. Generation of MMTV-Wnt-1 transgenic mice (24) and MMTV-PTEN mice (23) has been described previously. Male Wnt-1 transgenic mice (a mixture of FVB, SJL, and C_{57}BL/6) were crossed with PTEN transgenic females (C_{57}BL/6) to generate all four genotypes used in this study: wild-type (WT), PTEN, Wnt-1, and PTEN-Wnt-1. Genotyping of PTEN and Wnt-1 transgenic mice was determined using PCR as previously described (23, 24). Mice were monitored weekly for tumor formation. Most tumors were found when they were ~0.5 cm and harvested for further analysis when they were 2.0 cm in diameter. Tissues were fixed in 4% phosphate-buffered paraformaldehyde overnight and paraffin embedded for further investigation. All procedures were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH (Bethesda, MD).

RNA isolation and Northern blots. Total RNA from the left inguinal mammary gland (gland number 4) was extracted at 8 to 12 weeks of age using TRIzol reagent according to the instructions of the manufacturer (Invitrogen Corp., Carlsbad, CA). Twenty micrograms of total RNA were separated in 0.8% formaldehyde gels and blotted on nylon membranes. Expression of PTEN and Wnt-1 mRNAs was analyzed using the entire 1.2-kb human PTEN cDNA and the Wnt-1 cDNA (Upstate, Inc., Lake Placid, NY) as probes, respectively. RNA levels were corrected to keratin 18 mRNA levels and were quantified using a PhosphoImager apparatus (Fujifilm Medical Systems USA, Inc., Stamford, CT).

Whole mounts, histology, and immunohistochemistry. The morphology of the mammary glands was examined using the whole-mount technique (25). To prepare mammary gland sections, inguinal glands were fixed in 4% phosphate-buffered paraformaldehyde overnight, and then transferred to 70% ethanol. Tissues were embedded in paraffin and sectioned at 5 μm. After deparaffinization, rehydration, and antigen retrieval by heating in antigen unmasking solution (Vector Laboratories Burlingame, CA), primary antibodies [β-catenin (1:100), Transduction Laboratories, Lexington, KY; signal transducer and activator of transcription (Stat) 5 (1:100), Santa Cruz Biotechnology, Santa Cruz, CA] were applied to the sections. After incubation at 37°C for 1 hour or 4°C overnight, the sections were washed with PBS and incubated with Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-mouse secondary antibodies (1:400; Molecular Probes, Eugene, OR) at 37°C for 30 minutes. Slides were studied using a Zeiss Axioscop.

Cell proliferation assay. Mice were injected with 20 μL/g body weight of BrdUrd reagent (3 mg/mL; Amersham Biosciences, Piscataway, NJ) for 2 hours. Mammary glands were dissected and fixed in 4% paraformaldehyde overnight. The tissues were then transferred to 70% ethanol and embedded in paraffin. For detection of incorporated BrdUrd, 5-μm sections were processed according to the instructions of the manufacturer (Amersham Life Science, Arlington Heights, IL).

Protein extraction and Western blotting. Mammary gland proteins were extracted in a buffer containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% Nonidet P40 containing protease inhibitors (2 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL aprotinin), and phosphatase inhibitors (100 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and 2 mmol/L sodium orthovanadate). Lysates were centrifuged at 48,000 × *g* for 60 minutes at 4°C. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the instruction of the manufacturer. Samples containing 50 μg of protein were boiled for 4 minutes in reducing Laemmli sample buffer containing 80 mmol/L DTT and subjected to electrophoresis on 10% SDS-polyacrylamide gels. Proteins were then transferred from gels to nitrocellulose membranes. The membranes were blocked with 5% insulin-free bovine serum albumin in TBS-Tween 20 buffer, and proteins were detected using various antibodies, as indicated in the figure legends [E-cadherin antibody (Transduction Laboratories); β-catenin, phospho-extracellular signal-regulated kinase (ERK)-1/2, phospho-Stat5, and matrix metalloproteinase-9 antibodies (Cell Signaling Technology, Inc., Beverly, MA); IGF-I receptor, phospho-Akt, Akt, and ERK2 antibodies (Santa Cruz Biotechnology); cyclin D1 antibody (NeoMarkers, Fremont, CA); keratin 8 antibody (Covance, Berkely, CA); and actin antibody (Sigma Chemicals, St. Louis, MO)]. After extensive washings, immune complexes were detected with horseradish peroxidase conjugated with specific secondary antiserum (Amersham Corp., Arlington Heights, IL) followed by enhanced chemiluminescence reaction. Blots were analysed by densitometry and quantified with MacBas V2.52 software (Fuji PhotoFilm).

Quantitative real-time PCR. Total RNA was extracted from mouse mammary gland and cDNA was synthesized using oligo(dT) primers with reverse transcription-PCR kit according to the instructions of the manufacturer (Invitrogen). Real-time PCR was done with the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer in ABI PRISM 7900HT sequence detection systems (Applied Biosystems, Foster City, CA). The primers that were used for real-time PCR are as follows: Sca-1 forward: 5′-TTGCCTTTATAGCCCCTGCT-3′,

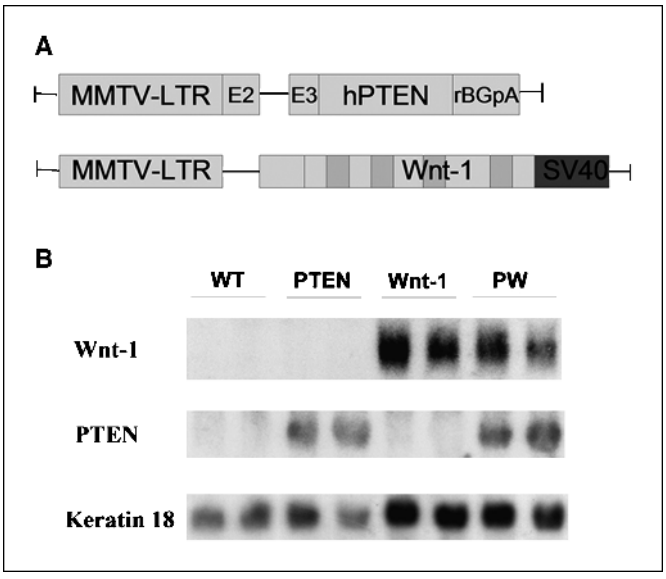

Figure 1. *PTEN* and *Wnt-1* transgenes are expressed under the MMTV-LTR promoter specifically in the mammary epithelia. *A,* schematic representation of the DNA constructs that were used to generate the PTEN and Wnt-1 transgenic mice. *B,* mRNA expression of *PTEN* and *Wnt-1* transgenes in mouse mammary tissue. Keratin 18 was used as a loading control. *PW,* PTEN-Wnt-1.

Sca-1 reverse: 5′-GTCATGAGCAGCAATCCACA-3′; keratin 6 forward: 5′-CATTGGAGGTGGAGCTGGTA-3′, keratin 6 reverse: 5′-GATGGTGACCTCTTGGATGC-3′; keratin 8 forward: 5′-TCCAGACTTCACCATGTCCA-3′, keratin 8 reverse: 5′-AAAGCTGGAAGAGCTGATGC-3′. Real-time PCR cycler conditions were 95°C, 15 min/45 × (94°C 15 seconds, 60°C 30 seconds, 72°C 15 seconds). The results were normalized against keratin 8 gene expression.

Statistical analysis. Results are expressed as mean ± SE. Statistically significant differences at $P < 0.05$ were determined using a one-factor ANOVA followed by a *t* test.

Results

Overexpression of the *Wnt-1* and *PTEN* transgenes in mouse mammary epithelium. PTEN and Wnt-1 transgenic mice express the *PTEN* and *Wnt-1* genes under the control of the MMTV-LTR promoter specifically in mammary epithelium (Fig. 1*A*). Crossing of the two transgenic lines resulted in four groups that were analyzed in this study: WT, PTEN, Wnt-1, and PTEN-Wnt-1 transgenic mice. Northern blot analysis, shown in Fig. 1*B*, shows overexpression of the *Wnt-1* transgene in Wnt-1 and PTEN-Wnt-1 transgenic mice and overexpression of the *PTEN* transgene in PTEN and PTEN-Wnt-1 transgenic mice.

Overexpression of phosphatase and tensin homologue in Wnt-1 transgenic mice decreased mammary epithelial cell proliferation and mammary hyperplasia. To determine the effect of inactivation of the AKT pathway (by PTEN overexpression) on Wnt-1–induced mammary hyperplasia, the PTEN transgenic mice were crossed with the Wnt-1 transgenic mice. Inguinal mammary glands from mice bearing the *Wnt-1* transgene in the presence or absence of the *PTEN* transgene were analyzed at 8 and 16 weeks of age. Figure 2 shows whole-mount staining of mammary glands at 16 weeks of age. Virgin WT female mice exhibited normal mammary ductal morphogenesis as reflected by the presence of an ordered ductal network with extensive side branching. Similarly, ductal development in the PTEN mice at the virgin stage was normal, although less secondary and ternary side branches were observed. In contrast, Wnt-1 transgenic mice displayed extensive mammary gland hyperplasia reflected by excessive ductal side branching and by the development of aberrant lobular structures (Fig. 2). However, overexpression of the *PTEN* transgene in the Wnt-1 transgenic mice resulted in reduced mammary gland hyperplasia reflected by reduced ductal side branching when compared with mammary tissue from Wnt-1 transgenic mice.

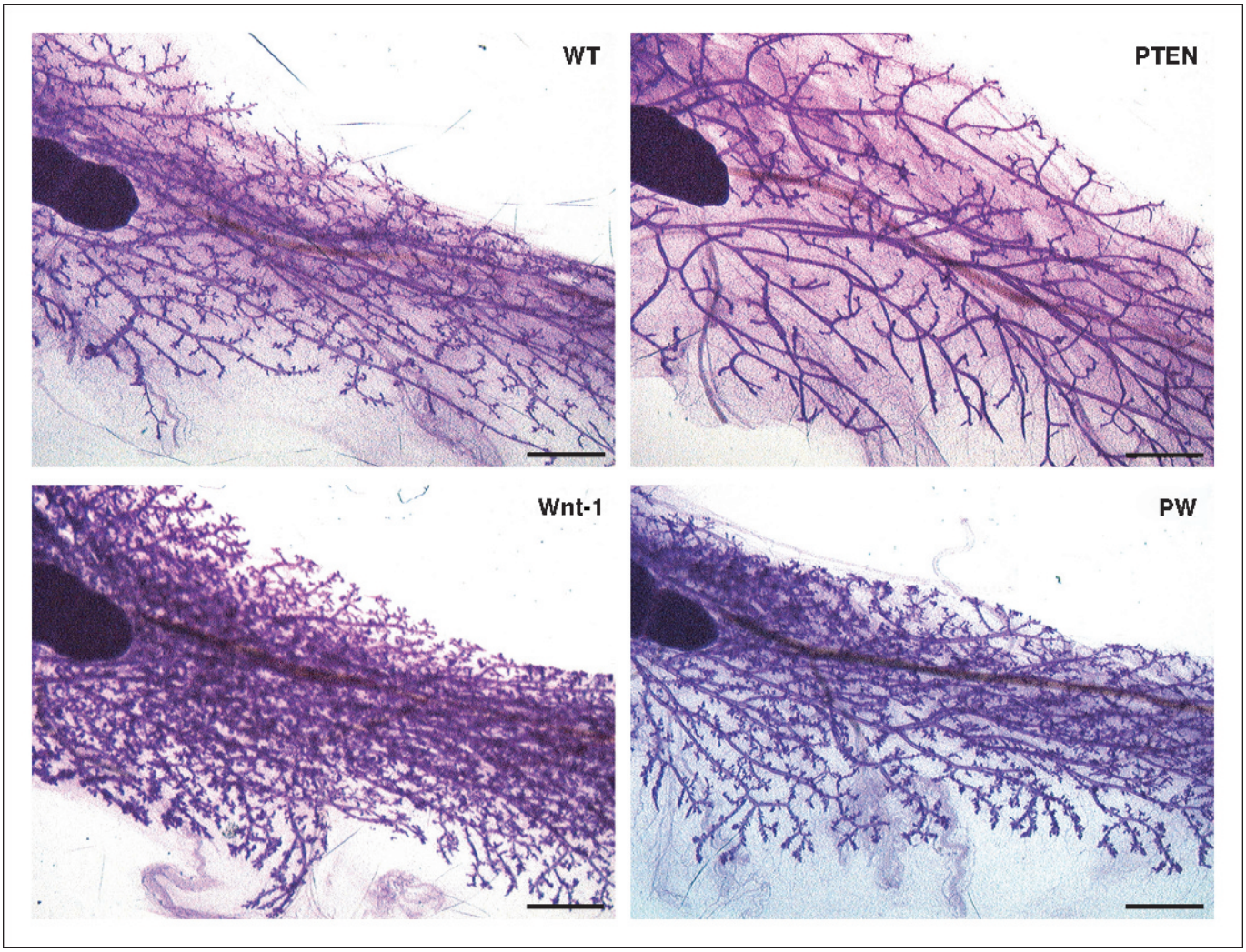

Figure 2. PTEN overexpression decreased mammary hyperplasia in Wnt-1 transgenic mice. Whole-mount staining was done on mammary glands harvested from 4-month-old WT, PTEN, Wnt-1, and PTEN-Wnt-1 mice. Glands were spread on glass slides, fixed, and subjected to carmine staining; $n = 4$ to 5 mice per group. *Bar*, 500 μm.

To verify whether reduced mammary gland hyperplasia in PTEN and PTEN-Wnt-1 mice was due to a decreased rate of epithelial proliferation, BrdUrd incorporation assay was done. Based on the immunohistochemical staining (Fig. 3*A*), the number of BrdUrd-positive nuclei in mammary epithelium of Wnt-1 transgenic mice was 2-fold higher than in WT mice. Interestingly, Wnt-1 transgenic mice overexpressing the *PTEN* transgene showed a 48% decrease in BrdUrd incorporation compared with Wnt-1 mice. In addition, a 5-fold decrease in the number of BrdUrd-positive nuclei was observed in mammary epithelium of PTEN mice compared with control mice (Fig. 3*B*). Additionally, at the basal state, both Akt and ERK1/2 phosphorylation was markedly reduced in PTEN-Wnt-1 as compared with Wnt-1 mice (Fig. 3*C*), suggesting a reduction in overall proliferative activity. However, when stimulated with IGF-I, there is a marked increase in Akt phosphorylation in both PTEN-Wnt-1 and Wnt-1 mice. In contrast, ERK1/2 phosphorylation remains low even in IGF-I stimulated state in PTEN-Wnt-1 mice. Taken together, these data suggest that overexpression of the *PTEN* transgene in mice harboring the *Wnt-1* transgene leads to decreased Wnt-1–induced mammary hyperplasia by decreasing cell proliferation.

Overexpression of phosphatase and tensin homologue in Wnt-1 transgenic mice decreased tumor incidence, delayed tumor onset, and decreased tumor growth rate. To determine the effect of PTEN overexpression on Wnt-1–induced mammary tumor incidence and latency, the ages at which mammary tumors (≥0.5 cm diameter) appeared and the time required for them to reach a size of 2 cm diameter were recorded. As shown in Fig. 4*A*, WT and PTEN transgenic mice were free of tumors during 12 months of follow-up. In contrast, the majority of mice (89% for Wnt-1 and 78% for PTEN-Wnt-1) harboring the *Wnt-1* transgene developed mammary tumors. Thus, those that expressed the *PTEN* transgene together with the *Wnt-1* transgene exhibited a significantly lower tumor incidence. As shown in Fig. 4*B*, 50% of the Wnt-1 transgenic mice developed mammary tumors at 5.9 months of age. In contrast, the onset of tumors in PTEN-Wnt-1 mice was delayed, with 50% of these mice developing tumors by 7.7 months of age. The rate of tumor growth was expressed as the time required for tumors to grow from 0.5 to 1 cm in diameter (doubling time). As shown in Fig. 4*C*, Wnt-1 tumors required 8.9 days to reach 1 cm diameter in size. However, PTEN overexpression in Wnt-1 mice required 17.7 days to reach a 1 cm diameter, suggesting that *PTEN*

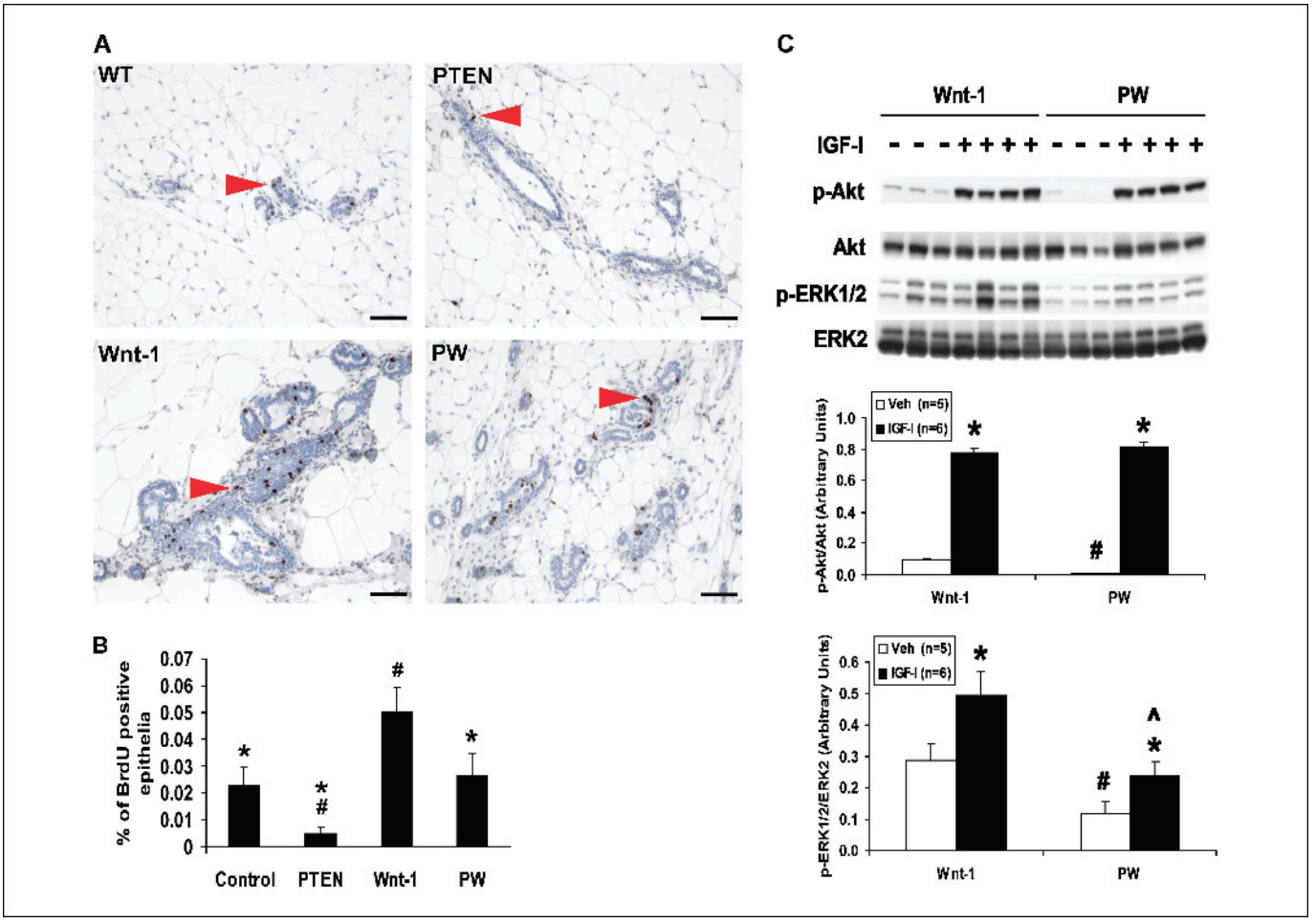

Figure 3. Cell proliferation evaluated by BrdUrd staining is decreased in mammary epithelium of Wnt-1 transgenic mice overexpressing PTEN. WT, PTEN, Wnt-1, and PTEN-Wnt-1 mice were injected with BrdUrd and sacrificed 2 hours later. *A,* BrdUrd-labeled nuclei were detected by immunostaining (*red arrowheads*). *B,* 2,000 to 3,000 nuclei were counted in sections obtained from mice at 2 months of age. *Columns,* mean (n = 3-4 in each group); *bars,* SE. #, $P < 0.05$, versus WT group. *, $P < 0.05$, versus Wnt-1. *Bar*, 50 μm. *C,* basal Akt and ERK1/2 phosphorylation is decreased in mammary epithelia of PTEN-Wnt-1 mice. Representative Western blot analysis and quantification of phospho-Akt and phospho-ERK1/2 in response to IGF-I stimulation. *Columns,* mean; *bars,* SE. *, $P < 0.05$, for IGF-I versus Vehicle; #, $P < 0.05$, for PTEN-Wnt-1 + Vehicle versus Wnt-1 + Vehicle group. ^, $P < 0.05$, for PTEN-Wnt-1 + IGF-I versus Wnt-1 + IGF-I group.

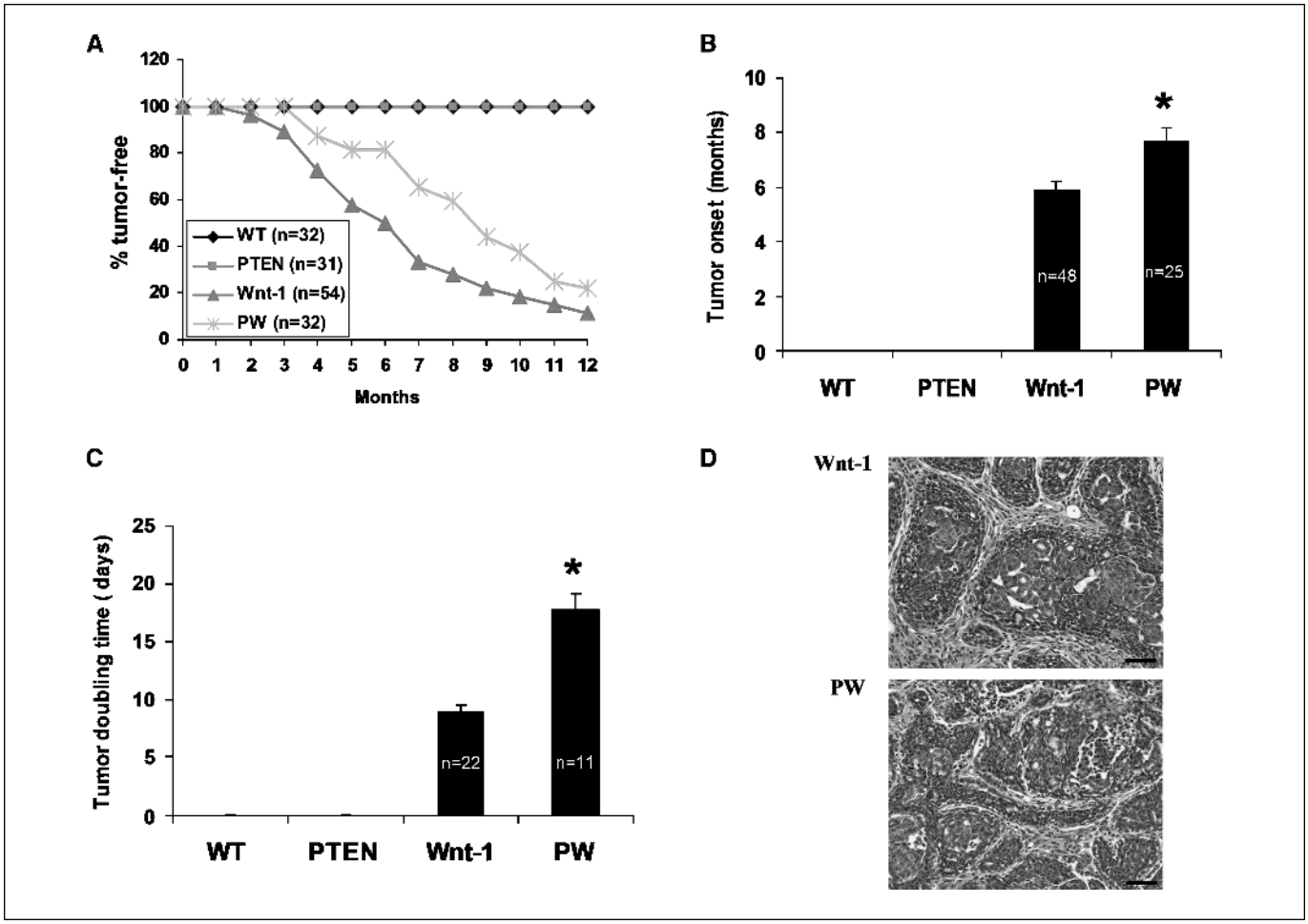

Figure 4. PTEN overexpression decreased tumor incidence (*A*), delayed tumor onset (*B*), and decreased the rate of tumor growth (*C*) in Wnt-1 transgenic mice. Tumor formation was monitored by weekly visual inspection and palpation from 2 to 12 months of age. No tumors arose in WT or PTEN group. Average tumor onset was calculated against the age when tumors were found. Rate of tumor growth was measured from tumors 0.5 cm till they reached 1.0 cm in diameter and expressed as tumor doubling time calculated from tumor size 0.5 till 1.0 cm in diameter. *Columns,* mean; *bars,* SE. *, $P < 0.05$, for Wnt-1 versus PTEN-Wnt-1 group. *D,* mammary tumors obtained from Wnt-1 or PTEN-Wnt-1 mice showed similar morphologic features of mammary adenocarcinoma. Sections from mammary glands removed from Wnt-1 and PTEN-WNT-1 mice were analyzed by H&E staining; $n = 10$ to 15 mice per group. *Bars*, 50 μm.

transgene reduced the rate of Wnt-1–induced mammary tumor growth.

The tumors that arose in both Wnt-1 and PTEN-Wnt-1 transgenic mice were lobuloalveolar adenocarcinoma as was previously reported for the Wnt-1 transgenic mice (Fig. 4*D*).

Overexpression of phosphatase and tensin homologue in Wnt-1 transgenic mice led to an increase in signal transducer and activator of transcription 5a–positive epithelial cells and a decrease in β-catenin staining in hyperplastic mammary tissue. Histologic analysis of mammary tissue from Wnt-1 and PTEN-Wnt-1 transgenic mice revealed hyperplastic structures in both genotypes (Fig. 5*A*). In Wnt-1 transgenic mice, there was little evidence of normal alveoli and many of the epithelial structures had expanded and showed strong β-catenin staining (Fig. 5*A*). Western blot analysis of β-catenin revealed a marked decrease in β-catenin in PTEN-Wnt-1 as compared with Wnt-1 mice (Fig. 5*B*). In contrast, overexpression of PTEN in Wnt-1 transgenic mice resulted in reduced accumulation of β-catenin in the hyperplastic nodules, and many alveoli had undergone differentiation as evidenced by increased Stat5a levels (Fig. 6*A* and *B*). Additionally, Western blot analysis of Stat5a phosphorylation showed a significant increase in Stat5a phosphorylation in PTEN-Wnt-1 as compared with Wnt-1 mice (Fig. 6*C*). These results suggest that destabilization of β-catenin in the Wnt-1 transgenic mice due to overexpression of PTEN decreases the number of normal alveolar epithelia that undergo transdifferentiation, and may eventually result in squamous metaplasia in the developing tumors. Although increased Stat5a does suggest more differentiation of epithelial cells in PTEN-Wnt-1 mice, it is also important to assess the levels of stem cell markers in mammary epithelia as it is believed that cancer cells might arise from stem or progenitor cells. The putative stem cell markers Sca-1 and keratin 6, which seem to be preferentially expressed in mammary stem or progenitor cells, were analyzed by real-time PCR. As shown in Fig. 6*D*, Sca-1 levels were significantly reduced in PTEN-Wnt-1 as compared with Wnt-1 mice, and keratin 6 had a tendency of reduction in PTEN-Wnt-1 mice.

Overexpression of phosphatase and tensin homologue in Wnt-1 transgenic mice led to a decrease in insulin-like growth factor-I receptor, cyclin D1, matrix metalloproteinase-9, and E-cadherin levels in the hyperplastic mammary tissue. In view of our previous study where we detected a marked reduction in

IGF-I receptor protein levels when PTEN was overexpressed in a PC3 prostate cancer cell line (16), we analyzed the IGF-I receptor protein levels in the PTEN-Wnt-1 double transgenic mice. Interestingly, in the PTEN-Wnt-1 mammary model we also detected a reduction in IGF-I receptor levels in mammary tissue compared with the Wnt-1 mice (Fig. 7*A*). This reduction can partially account for the significant decrease in BrdUrd incorporation in the mammary gland of PTEN-Wnt-1 transgenic mice (Fig. 3*A*) that was described above. Cyclin D1, MMP-9, and E-cadherin, which are implicated in the Wnt-1 signaling pathway, are also inhibited when PTEN is overexpressed in mammary gland of Wnt-1 transgenic mice (Fig. 7*B-D*). This inhibition is most likely through inhibition of glycogen synthase kinase 3β phosphorylation and β-catenin translocation to the nucleus. Taken together, reduction in the levels of IGF-I receptor, mitogen-activated protein kinase (MAPK) and Akt phosphorylation, cyclin D1, β-catenin, MMP-9, and E-cadherin could account for reduced cell proliferation and reduced number of cells that are more susceptible to transformation in the PTEN-Wnt-1 double transgenic mice.

Discussion

In this study, we present several lines of evidence supporting the notion that the tumor suppressor PTEN inhibits Wnt-1–induced cellular growth through inhibition of cyclin D1 and β-catenin and down-regulation of the IGF-I receptor. However, we also show that PTEN cannot completely suppress the proliferative processes driven by *Wnt-1* transgene, despite the fact that both PTEN and Wnt-1 are expressed under the same promoter in the same cell type. This observation suggests that the proliferative drive of the mammary epithelial cells is composed of many molecular mechanisms leading to malignancy and cannot be completely blocked by one tumor suppressor gene but, rather, only inhibited to some degree. It is necessary to recognize that the incomplete blockage of tumorigenesis could also be due to incomplete overlap of the cells that express these two genes. Because Wnt-1 is a secreted protein, its paracrine signaling might also contribute to the observed tumorigenesis.

Wnt-1 signaling is known to play a critical role in the proliferation of a variety of cell types such as hematopoetic stem cells, keratinocyte stem cells, and colon cells (26–30). Deregulation of the Wnt-1 pathway has been shown to be a cancer-predisposing factor in many tissues (2). In the mammary gland, it is believed that progenitor cells are most likely the precursors to cancer (31) and, therefore, activation of the Wnt signaling pathway increases the number of precursor cells that can undergo transformation and initiate genetic lesions, which lead to mammary tumors. PTEN, on the other hand, decreases the potential number of cells that can undergo transformation by inhibiting cell proliferation and increasing apoptosis (32). Indeed, in this study, we show, as have others (33), that in Wnt-1 transgenic mice there is a marked increase in epithelial cell proliferation and a significant increase in tumor incidence and tumor growth. Overexpression of the tumor suppressor PTEN in the Wnt-1 transgenic mice led to decreased epithelial cell proliferation and decreased hyperplasia, resulting in a significant decrease in mammary tumor incidence and growth. However, it is important to note that PTEN was not able to completely block epithelial cell proliferation. This could be due to

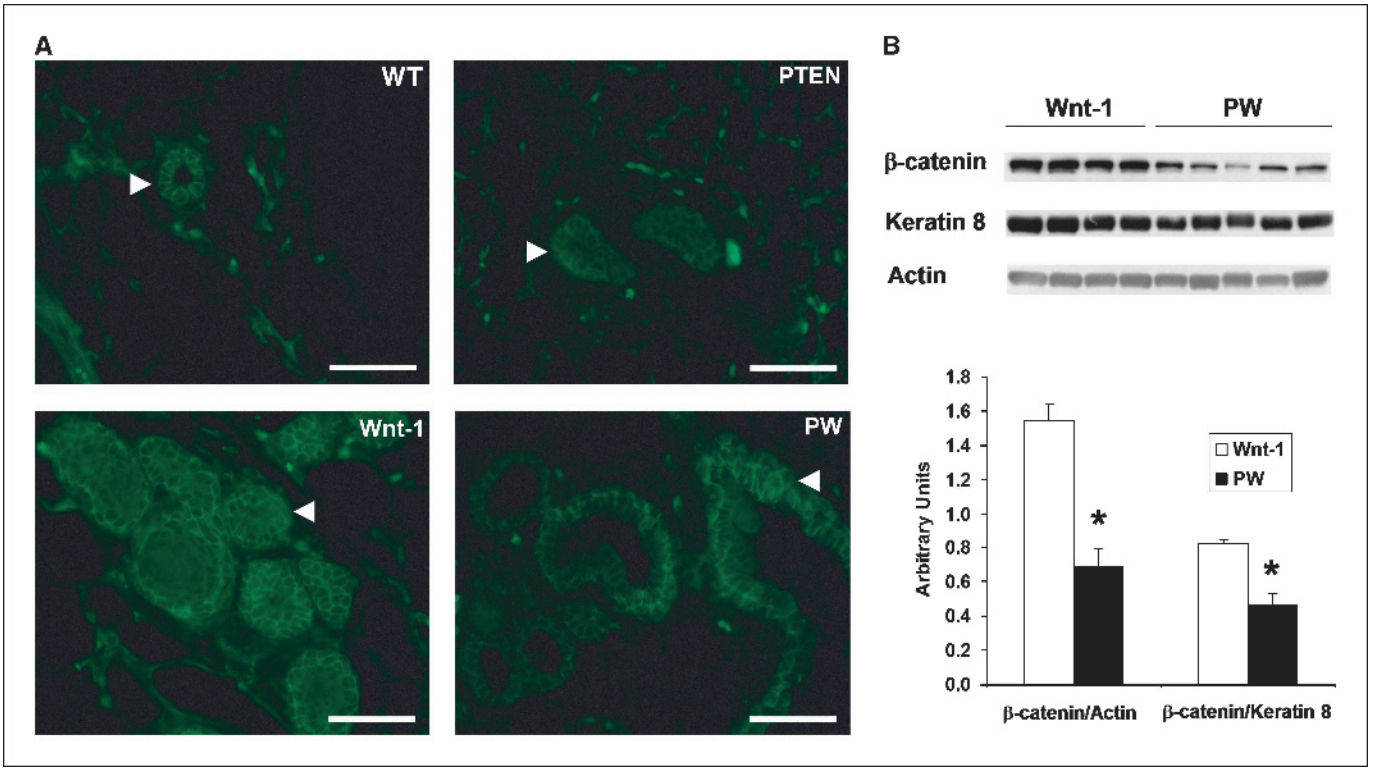

Figure 5. PTEN overexpression in Wnt-1 transgenic mice decreased β-catenin expression in the mammary tissue. *A,* sections of mammary glands removed from WT, PTEN, Wnt-1, and PTEN-WNT-1 mice were analyzed by β-catenin immunofluorescence staining (*green*). β-Catenin antibody was used as described in Materials and Methods; $n = 4$ to 5 in each group. *Bar*, 50 μm. *B,* representative Western blot analysis and quantification of β-catenin in mammary epithelia of Wnt-1 and PTEN-Wnt-1 mice. *Columns,* mean ($n = 4$-5 in each group); *bars,* SE. *, $P < 0.05$, versus Wnt-1 group.

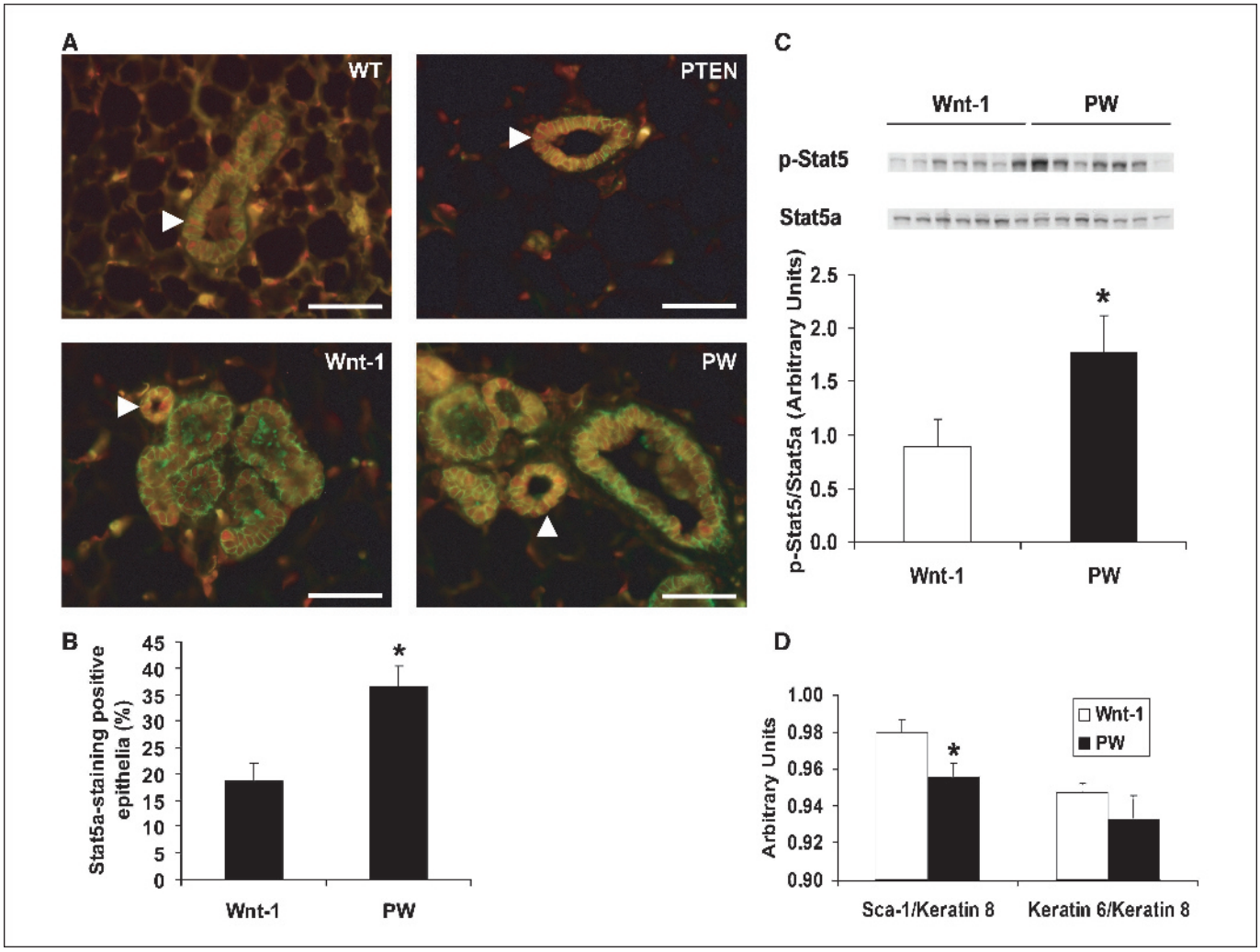

Figure 6. PTEN overexpression in Wnt-1 transgenic mice increased Stat5a expression and phophorylation in mammary tissue. *A,* sections of mammary glands removed from WT, PTEN, Wnt-1, and PTEN-WNT-1 mice were analyzed by Stat5a immunofluorescence staining (*red*; *white arrowheads*). Stat5a antibody was used as described in Materials and Methods. E-cadherin (*green*) was used to stain the cell membrane. *B,* quantification of Stat5a-positive cells; 1,800 to 2,000 nuclei were counted in sections obtained from mice at 2 to 3 months of age. *Columns,* mean (n = 4-5 in each group); *bars,* SE. *, $P < 0.05$, for Wnt-1 versus PTEN-WNT-1 group. *Bar*, 50 μm. *C,* representative Western blot analysis and quantification of phospho-Stat5a in mammary epithelia of Wnt-1 and PTEN-Wnt-1 transgenic mice. *Columns,* mean (n = 7 in each group); *bars,* SE. *, $P < 0.05$, versus Wnt-1 group. *D,* RNA levels of the putative stem cell markers Sca-1 and keratin 6 in Wnt-1 and PTEN-Wnt-1 mice. Mouse mammary glands were harvested at 16 weeks of age, and RNA was isolated and subjected to real-time PCR analysis. *Columns,* mean (n = 4 in each group); *bars,* SE. *, $P < 0.05$, versus Wnt-1 mice.

simultaneous activation of different molecular mechanisms that drive cell proliferation or due to generation of a subset of tumor cells that have lost PTEN expression.

β-Catenin is an integral part of the Wnt pathway (2). Upon binding of the Wnt proteins to the transmembrane Frizzled receptor, there is an activation of a signaling cascade leading to the stabilization of β-catenin, its translocation to the nucleus, and activation of the lymphoid enhancer transcription factors and T-cell factors (2). The role of β-catenin in mammary gland development has been studied *in vivo* in mice expressing a truncated form of β-catenin (34, 35). These experiments show that β-catenin signaling determines alveolar cell fate, survival, and proliferation. Overexpression of β-catenin activators, such as Wnt-1, Wnt-3, and Wnt-10b, leads to stimulation of alveolar development (34, 36). In contrast, overexpression of β-catenin supressors, such as axin and dominant-negative β-catenin (37, 38), results in impaired alveolar development. In the present study, we show that PTEN decreased mammary tumor incidence and growth stimulated by the *Wnt-1* transgene. These data are supported by immunohistochemistry exhibiting a marked reduction in β-catenin staining in the double transgenic PTEN-Wnt-1 mice, and by Western blotting showing a significant reduction in β-catenin and cyclin D1 levels, which are implicated in the Wnt-1 signaling pathway. These results indicate that PTEN reduced the cytosolic levels of PI3P by antagonizing the activity of PI3K, which could then lead to an increase in the activity of glycogen synthase kinase 3β and eventually to destabilization of β-catenin and reduction in its transcriptional activation of the *cyclin D1* gene.

The histopathology and the molecular features of mammary tumors can vary depending on the different oncogenes that initiated them (39–44). There are oncogenes that can only transform progenitor cells but cannot arrest them at this stage or induce their differentiation (31). In contrast, there are

oncogenes that transform differentiated cells exclusively (31). In the present study, we show that PTEN overexpression in the Wnt-1 transgenic mice led to a significant increase in Stat5a-positive epithelial cells as compared with Wnt-1 transgenic mice; Stat5a is a well-known marker of differentiation. However, it remains to be determined whether the Wnt-1 signaling pathway induces mammary tumors at a specific stage of differentiation and, therefore, it is hard to determine at what stage PTEN inhibits the Wnt-1 signaling. It is also unclear whether the MMTV promoter is differentially activated in progenitor versus differentiated cells, and so it is also unclear whether these Stat5a-positive cells represent an arrested subpopulation of transformed mammary epithelial cells. In this study, we also show that activation of the Wnt-1 signaling pathway leads to an increase in stem or progenitor cell markers (Sca1 and keratin6), which reflects an increased population of undifferentiated cells that might be more susceptible for tumorigenesis. PTEN overexpression in the Wnt-1 transgenic mice resulted in a decrease in the levels of these markers.

The IGF-I receptor is a tyrosine kinase receptor, which on ligand binding undergoes phosphorylation and transmits its signal to multiple intracellular substrates such as insulin receptor substrates, PI3K, and MAPK (45). Activation of the PI3K/AKT and the Ras/Raf/MAPK pathways is considered to mediate the mitogenic effect of the IGF-I receptor (46). IGF-I receptor is universally expressed in various hematologic cancers, such as multiple myoloma, lymphoma, and leukemia, and in solid tumors, such as breast, prostate, and lung (47). Expression of functional IGF-I receptor is required for neoplastic transformation in diverse tumorigenesis models. Overexpression of the tumor suppressor PTEN, which inhibits the IGF-I receptor signaling by dephosphorylation of PI3P and inactivation of the AKT pathway, leads to a decrease in cell proliferation and an increase in cell apoptosis (15). Moreover, it has been shown that expression of PTEN in PTEN-deficient glioma cells inhibited cell growth, which was similar to the effect of inactivation of the IGF-I receptor in these cells (48). Interestingly, in the present study, overexpression of PTEN in the Wnt-1 transgenic mice led

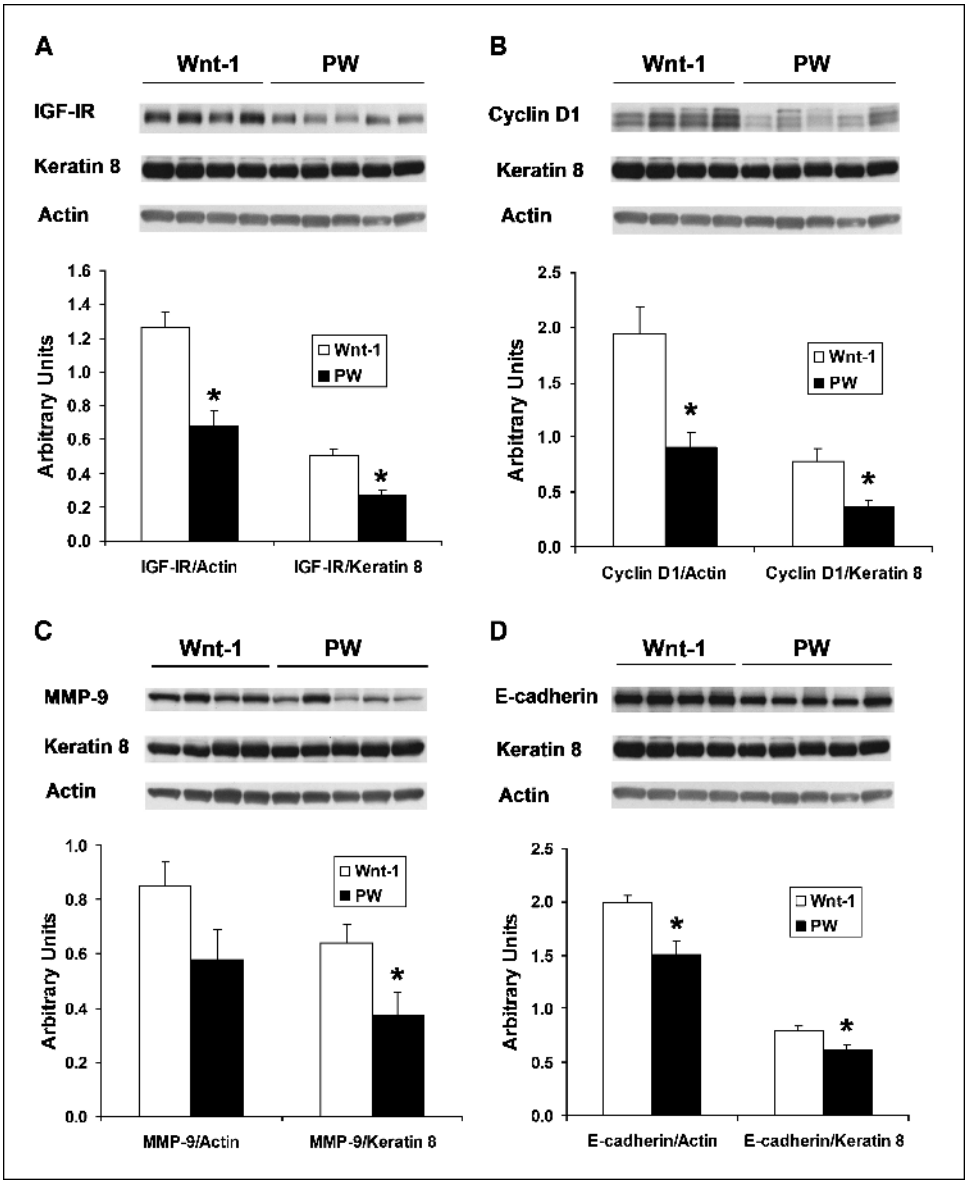

Figure 7. PTEN expression inhibited IGF-I receptor, cyclin D1, MMP-9, and E-cadherin expression in the mammary tissue of PTEN-Wnt-1 double transgenic mice. *A,* IGF-I receptor; *B,* cyclin D1; *C,* MMP-9; *D,* E-cadherin protein levels in Wnt-1 and PTEN-Wnt-1 double transgenic mice. Immunoreactive bands were normalized to keratin 8 and actin. *Columns,* mean (n = 4-5 in each group); *bars,* SE. *, $P < 0.05$, versus Wnt-1 group.

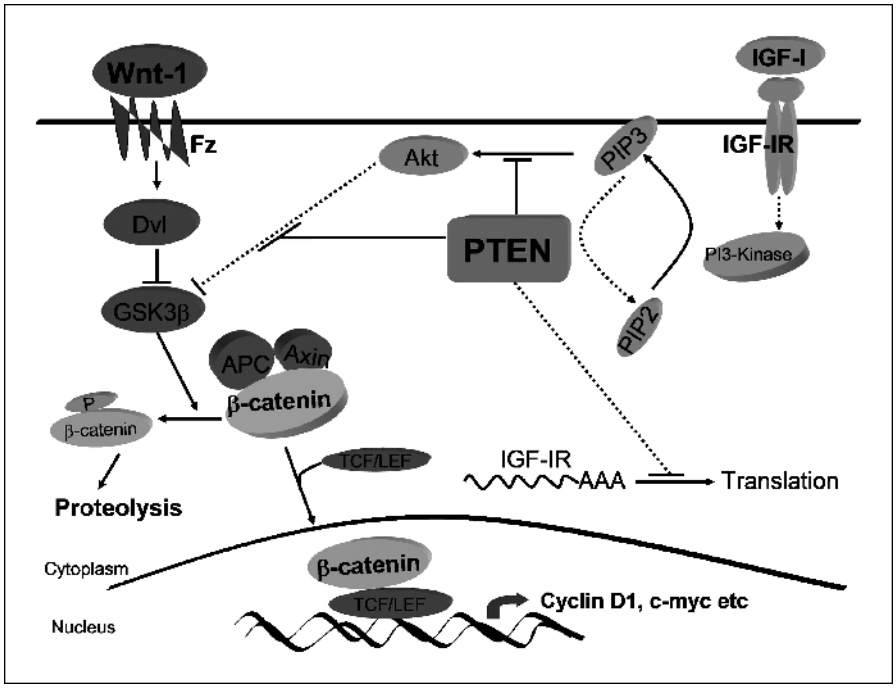

Figure 8. Schematic demonstrating the signaling cascade induced by the oncogene Wnt-1 and the inhibitory effect of the tumor suppressor PTEN on that pathway.

to a marked decrease in the IGF-I receptor levels as compared with Wnt-1 transgenic mice. These data are in accord with our previous (*in vitro*) study, which showed a significant reduction in IGF-I receptor protein levels when PTEN was overexpressed in PC3 prostate cancer cells (16).

A schematic summary of the data is presented in Fig. 8. Wnt-1 overexpression leads to activation of the Wnt-1 signaling pathway, which drives proliferation. PTEN overexpression, on the other hand, blocks the AKT pathway which can lead to partial inhibition of Wnt-1–induced tumorigenesis through destabilization of β-catenin and a reduction in cyclin D1 protein levels (49, 50). PTEN also inhibits the IGF-I receptor translation leading to a reduced IGF-I–mediated mitogenesis as reflected by reduction in the basal phosphorylation of both Akt and MAPK.

Acknowledgments

Received 1/24/2005; revised 5/03/2005; accepted 5/16/2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004; 20:781–810.
2. Polakis P. Wnt signaling and cancer. Genes Dev 2000; 14:1837–51.
3. Behrens J, von Kries JP, Kuhl M, et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996;382:638–42.
4. Roose J, Huls G, van Beest M, et al. Synergy between tumor suppressor APC and the β-catenin-Tcf4 target Tcf1. Science 1999;285:1923–26.
5. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998; 281:1509–12.
6. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 1999;96:5522–27.
7. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 1982;31:99–109.
8. Gunther EJ, Moody SE, Belka GK, et al. Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev 2003;17:488–501.
9. Lee JO, Yang H, Georgescu MM, et al. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell 1999;99:323–34.
10. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273:13375–78.
11. Myers MP, Pass I, Batty IH, et al. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci U S A 1998;95: 13513–18.
12. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–41.
13. Stiles B, Gilman V, Khanzenzon N, et al. Essential role of AKT-1/protein kinase B α in PTEN-controlled tumorigenesis. Mol Cell Biol 2002;22:3842–51.
14. Weng L, Brown J, Eng C. PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways. Hum Mol Genet 2001;10:237–42.
15. Weng LP, Brown JL, Eng C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Hum Mol Genet 2001;10:599–604.
16. Zhao H, Dupont J, Yakar S, Karas M, LeRoith D. PTEN inhibits cell proliferation and induces apoptosis by down-regulating cell surface IGF-I receptor expression in prostate cancer cells. Oncogene 2004;23:786–94.
17. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–47.
18. Li J, Simpson L, Takahashi M, et al. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res 1998;58:5667–72.
19. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15: 356–62.
20. Suzuki A, de la Pompa JL, Stambolic V, et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol 1998;8:1169–78.
21. Podsypanina K, Ellenson LH, Nemes A, et al. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A 1999; 96:1563–68.
22. Li Y, Podsypanina K, Liu X, et al. Deficiency of Pten accelerates mammary oncogenesis in MMTV-Wnt-1 transgenic mice. BMC Mol Biol 2001;2:2.
23. Dupont J, Renou JP, Shani M, Hennighausen L, LeRoith D. PTEN overexpression suppresses proliferation and differentiation and enhances apoptosis of the mouse mammary epithelium. J Clin Invest 2002;110: 815–25.
24. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 1988;55:619–25.

25. Cui Y, Miyoshi K, Claudio E, et al. Loss of the peroxisome proliferation-activated receptor γ (PPARγ) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. J Biol Chem 2002;277:17830–35.

26. Alonso L, Fuchs E. Stem cells in the skin: waste not, Wnt not. Genes Dev 2003;17:1189–200.

27. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409–14.

28. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–50.

29. Polesskaya A, Seale P, Rudnicki MA. Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. Cell 2003; 113:841–52.

30. Andl T, Reddy ST, Gaddapara T, Millar SE. WNT signals are required for the initiation of hair follicle development. Dev Cell 2002;2:643–53.

31. Li Y, Welm B, Podsypanina K, et al. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A 2003;100: 15853–8.

32. Stiles B, Groszer M, Wang S, Jiao J, Wu H. PTENless means more. Dev Biol 2004;273:175–84.

33. Vincan E. Frizzled/WNT signalling: the insidious promoter of tumour growth and progression. Front Biosci 2004;9:1023–34.

34. Miyoshi K, Rosner A, Nozawa M, et al. Activation of different Wnt/β-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. Oncogene 2002;21: 5548–56.

35. Imbert A, Eelkema R, Jordan S, Feiner H, Cowin P. Δ N89 β-catenin induces precocious development, differentiation, and neoplasia in mammary gland. J Cell Biol 2001;153:555–68.

36. Robinson GW, Hennighausen L, Johnson PF. Side-branching in the mammary gland: the progesterone-Wnt connection. Genes Dev 2000;14:889–94.

37. Hatsell S, Rowlands T, Hiremath M, Cowin P. β-Catenin and Tcfs in mammary development and cancer. J Mammary Gland Biol Neoplasia 2003;8145–58.

38. Luo W, Lin SC. Axin: a master scaffold for multiple signaling pathways. Neurosignals 2004;13:99–113.

39. Morrison BW, Leder P. neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. Oncogene 1994;9:3417–26.

40. Rosner A, Miyoshi K, Landesman-Bollag E, et al. Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. Am J Pathol 2002;161:1087–97.

41. Cardiff RD, Anver MR, Gusterson BA, et al. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene 2000;19:968–88.

42. Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001;344:539–48.

43. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406: 747–52.

44. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869–74.

45. LeRoith D. Insulin-like growth factor receptors and binding proteins. Baillieres Clin Endocrinol Metab 1996; 10:49–73.

46. LeRoith D, Roberts CT Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003; 195:127–37.

47. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 2004;5: 221–30.

48. Seely BL, Samimi G, Webster NJ. Retroviral expression of a kinase-defective IGF-I receptor suppresses growth and causes apoptosis of CHO and U87 cells in-vivo. BMC Cancer 2002;2:15.

49. Fukumoto S, Hsieh CM, Maemura K, et al. Akt participation in the Wnt signaling pathway through Dishevelled. J Biol Chem 2001;276:17479–83.

50. Detera-Wadleigh SD. Lithium-related genetics of bipolar disorder. Ann Med 2001;33:272–85.