Development 129, 4159-4170 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV20009

DEVELOPMENT AND DISEASE

Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland

Gang Li[1,4], Gertraud W. Robinson[2], Ralf Lesche[1,*], Hilda Martinez-Diaz[1], Zhaorong Jiang[3], Nora Rozengurt[3], Kay-Uwe Wagner[2], De-Chang Wu[4], Timothy F. Lane[5], Xin Liu[3], Lothar Hennighausen[2] and Hong Wu[1,†]

[1]Department of Molecular and Medical Pharmacology, and Howard Hughes Medical Institute, [3]Department of Pathology and Laboratory Medicine, [5]Jonsson Comprehensive Cancer Center, UCLA School of Medicine, 650 Circle Drive South, Los Angeles, CA 90095-1735, USA
[2]Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0822, USA
[4]Institute of Radiation Medicine, Beijing, PR China
*Present address: Epigenomics AG, Kastanienallee 24, 10435 Berlin, Germany
†Author for correspondence (e-mail: hwu@mednet.ucla.edu)

Accepted 4 June 2002

SUMMARY

PTEN tumor suppressor is frequently mutated in human cancers, including breast cancers. Female patients with inherited PTEN mutations suffer from virginal hypertrophy of the breast with high risk of malignant transformation. However, the exact mechanisms of PTEN in controlling mammary gland development and tumorigenesis are unclear. In this study, we generated mice with a mammary-specific deletion of the *Pten* gene. Mutant mammary tissue displayed precocious lobulo-alveolar development, excessive ductal branching, delayed involution and severely reduced apoptosis. *Pten* null mammary epithelial cells were disregulated and hyperproliferative. Mutant females developed mammary tumors early in life. Similar phenotypes were observed in *Pten*-null mammary epithelia that had been transplanted into wild-type stroma, suggesting that PTEN plays an essential and cell-autonomous role in controlling the proliferation, differentiation and apoptosis of mammary epithelial cells.

Key words: PTEN, Mammary development, Cowden's disease, Ductal growth, Apoptosis

INTRODUCTION

It has long been speculated that phosphatases, because of their ability to counteract the action of kinases, may function as tumor suppressors. PTEN (phosphatase and tensin homolog deleted from chromosome 10, or *MMAC1/TEP1*) is the first phosphatase identified as a tumor suppressor (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). Deletions and mutations in the *PTEN* gene are found at high frequency in many sporadic human cancers, including glioblastomas, as well as endometrial, prostate, and breast cancers (Dahia, 2000). It is estimated that the overall frequency of *PTEN* mutation in sporadic human cancer is similar to that of the tumor suppressor gene *TP53* (Stokoe, 2001). Studies also indicate that germline *PTEN* mutations are the cause of three inherited autosomal dominant disorders: Cowden syndrome (CS), Lhermitte-Duclos disease (LD) and Bannayan-Zonana syndrome (BRR) (Liaw et al., 1997; Marsh et al., 1999; Nelen et al., 1997). Patients with Cowden syndrome develop benign hamartomatous lesions throughout their bodies and have a higher risk of developing breast and thyroid cancers later in their life.

PTEN contains a sequence motif similar to the catalytic domain found in dual specificity phosphatases, whose in vitro substrate specificities include highly acidic phospho-serine, threonine, and tyrosine residues (Li and Sun, 1997; Myers et al., 1997). Both in vivo and in vitro studies indicated that the major target of PTEN is the lipid phosphotidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), a direct product of PI 3-kinase (Maehama and Dixon, 1998; Stambolic et al., 1998; Sun et al., 1999). Crystal structural analysis of PTEN further confirmed its lipid as well as protein phosphatase activities (Lee et al., 1999). Loss of PTEN function either in embryonic stem cells (ES cells) or in human cancer cell lines resulted in accumulation of PtdIns(3,4,5)P_3 and activation of its downstream signaling molecule, Akt/PKB. The activated PI 3-kinase/Akt pathway in turn stimulates cell cycle progression, cell survival and cell migration (Liliental et al., 2000; Stambolic et al., 1998; Sun et al., 1999). Even though PTEN has been shown in vitro to possess protein phosphatase activity, whether PTEN also functions as a protein phosphatase and regulates signaling pathways other than the PI 3-kinase/Akt pathway in vivo is largely unknown.

Breast cancer is one of the most common malignancies

associated with CS. Accumulated evidence indicated that PTEN is a crucial negative regulator of breast tumorigenesis and loss of PTEN is associated with a poor outcome of breast cancer (Depowski et al., 2001). Family-based analysis suggests a lifetime risk of developing breast cancer in 25-50% of affected CS females (Schrager et al., 1998). Although somatic *PTEN* mutations are detected only in a smaller fraction of breast cancer cases (12%) (Dahia, 2000), loss of heterozygosity (LOH) at the *PTEN* locus (10q23) is frequently found (30%-40%) (Feilotter et al., 1999; Garcia et al., 1999). Immunohistochemical studies suggest that loss of PTEN protein expression is a common event in breast cancer (33%-48%), with strong correlation with lymph node metastasis, loss of estrogen receptor staining, and disease related death (Depowski et al., 2001; Perren et al., 1999). Thus, epigenetic mechanisms might be responsible for a number of cases in which PTEN levels are downregulated or even totally ablated in the absence of a detectable mutation.

Several *Pten*-null mice were generated. In all cases, homozygous mutant embryos died during early embryogenesis at E6.5-9.5, and heterozygous animals develop a broad range of tumors, including breast cancer, partially resembling the spectrum of neoplasia observed in CS patients (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). After crossing *Pten*$^{+/-}$ mice to *MMTV-Wnt1* transgenic mice, Li et al. found that compound heterozygous females developed mammary tumors earlier than their parental strains, suggesting that the PTEN-controlled signaling pathway may cooperate with that of WNT1 in mammary tumor development (Li et al., 2001). However, the early embryonic lethality of conventional *Pten*$^{-/-}$ mice precluded further studies of PTEN function in normal mammary gland development.

To directly address the roles of PTEN in mammary gland development and tumorigenesis, we generated conditional *Pten* gene knock-out mice by flanking exon 5, which encodes the phosphatase domain of PTEN, with LoxP sequences (Lesche et al., 2002). We showed that deletion of the *Pten* gene in mammary epithelium caused increased cell proliferation, hyper-branched ductal structure, precocious development, delayed involution and severely impaired apoptosis. PTEN-deficient mammary epithelium also displayed remarkable neoplastic changes. These observations underscored the essential functions of PTEN during normal mammary gland development and in suppressing breast cancer formation.

MATERIALS AND METHODS

Generation of mice and genotyping.

Mammary-specific *Pten* deletion was achieved by crossing mice homozygous for the floxed-*Pten* alleles (*Pten*$^{loxp/loxp}$) (Lesche et al., 2002) with two independent MMTV-Cre transgenic mice (T. F. L., unpublished result) (Wagner et al., 2001). Mice were genotyped by PCR analysis (Lesche et al., 2002) using genomic DNA prepared from tail biopsies, as illustrated in Fig. 1A. Littermates with genotypes *Pten*$^{loxp/loxp}$*;MMTV-Cre*$^{-/-}$ (controls) and *Pten*$^{loxp/loxp}$*;MMTV-Cre*$^{+/-}$ (mutants) were used for further analysis.

Extraction of RNA and northern blot hybridization.

RNA was prepared using Trizol (Gibco BRL). Total RNA was separated on 1.0% agarose gels in the presence of formaldehyde, transferred to Hybond-N$^+$ Nylon membranes (Amersham pharmacia biotech) and hybridized in QuikHyb® hybridization solution (Stratagene). Probes (β-casein, and α-casein) were labeled with [^{32}P]dCTP using random priming kit (Prime-It® II, Stratagene).

Whole mounts, histology and immunohistochemistry analysis

The first inguinal glands were dissected at the indicated ages and were spread on a glass slide. After fixation with Carnoy's fixative for 2-4 hours, the tissues were hydrated and stained in Carmine alum overnight as previously described (http://mammary.nih.gov/tools/histological/Histology/index.html#a1). Samples were then dehydrated, cleared with xylene and mounted.

Hematoxylin and Eosin (HE) staining was performed according to standard procedures. Immunohistochemical analysis was performed on formalin-fixed paraffin sections following antigen retrieval (BioGenex). PTEN/MMAC1 antibody (AB-2, 1:50, Neomarkers), VECTASTAIN ABC Kit (Rabbit IgG, 1:200, Vector Laboratories), and AEC (3-amino-9-ethylcarbazole) substrate (BioGenex) were used for detection. Slides were counterstained with Hematoxylin. Antibodies for cytokeratins 5 and 6 (Babco; 1:200) and β-catenin (Transduction Laboratories; 1:100) were detected by immunofluorescence with appropriate secondary antibodies conjugated with FITC and Texas Red, respectively.

BrdU labeling and TUNEL assays

Four hours before sacrifice, mice were injected with 100 μg/g of body weight of BrdU (Sigma). Mammary glands were prepared, fixed overnight in 10% neutralized buffered formalin and embedded in paraffin. BrdU uptake was detected using BrdU In-Situ Detection Kit (BD PharMingen). Slides were counterstained with Hematoxylin.

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Boehringer-Mannheim) was performed according to the manufacturer's instructions. Briefly, mammary glands were fixed overnight in 10% neutralized buffered formalin, paraffin-embedded and sectioned at 10 μm; sections were incubated for 20 minutes in 20 μg/ml proteinase K, rinsed in PBS and incubated in terminal deoxynucleotidyl transferase [TdT] and labeled nucleotide mixture for 1 hour at 37°C in a humidified chamber. Sections were rinsed and mounted using Vectashield mounting medium with DAPI (Vector Labs). Ten randomly selected 200× magnification fields were measured. Quantification of the number of TUNEL-positive cells was performed by the Image-Pro (Media Cybernetics) counting function combining with manual splitting. Statistical analysis was performed using the Student's *t*-test.

Antibodies and western blot analysis

Protein was extracted from frozen mammary glands using RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium dexoycholate, 0.1% SDS and protease inhibitor cocktail tablet [Roche]) and fractionated on SDS-polyacrylamide gels. Western blots were then probed with the primary antibody. The sources of antibodies used in this study are as follows: α-PTEN, α-AKT, α-phospho-AKT (Ser473), α-phospho-BAD (Ser136), α-phospho-FKHR (Thr24)/FKHRL1 (Thr32), α-phospho-STAT3 (Tyr705), and α-STAT3 (Cell Signaling Technology); α-STAT5 (Santa Cruz Biotechnology); α-keratin 18 (Chemicon); α-β-actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies and LumiGLO™ reagent (Cell Signaling Technology) were used to detect specific binding, and signals were captured by X-ray film.

Mammary epithelium transplantation.

Small pieces of mammary tissue from *Pten*$^{loxp/loxp}$*;MMTV-Cre*$^{+/-}$ and control mice were transplanted into the cleared inguinal fat pads of 3-week old athymic *nu/nu* NCr recipients. Each host carried tissue from a mutant and control mouse in the contra-lateral fat pads such that both transplants were exposed to the identical conditions during the experiment. After 8 weeks the hosts were sacrificed and the

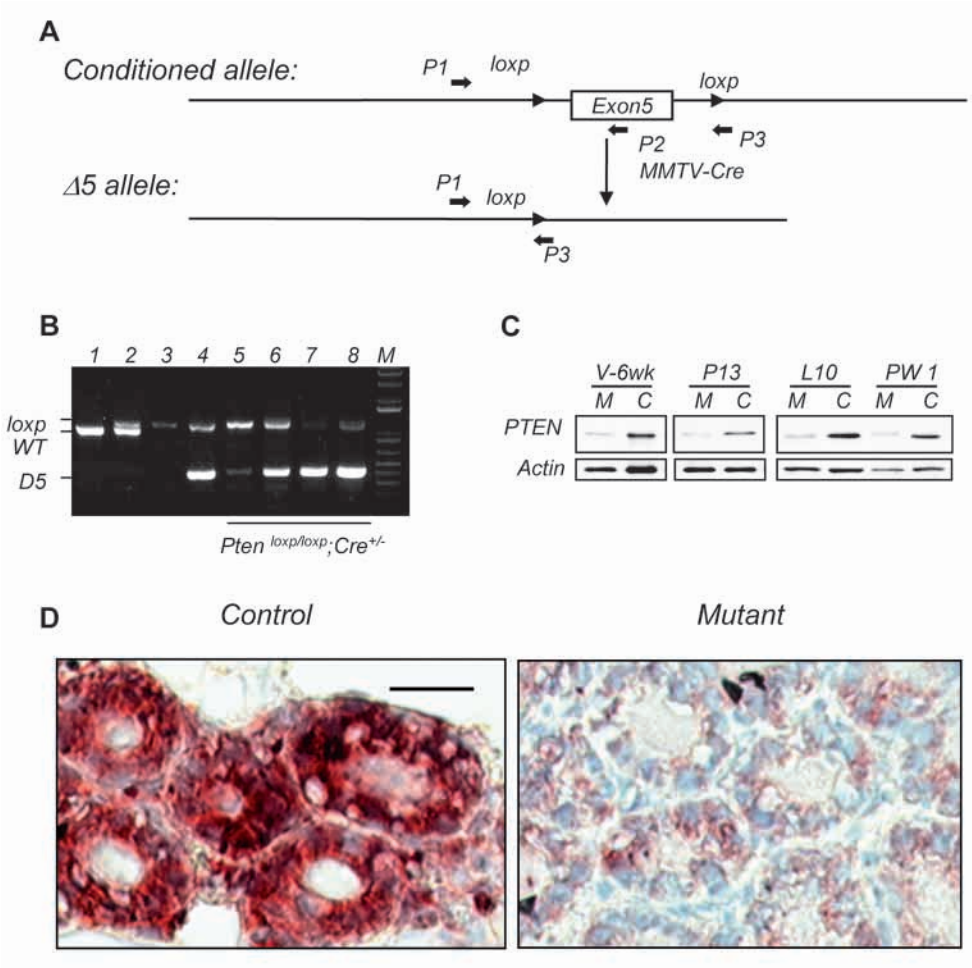

Fig. 1. Conditional deletion of *Pten* in mammary gland. (A) LoxP sequences are inserted on both sides of exon 5. After crossing with *MMTV-Cre*$^{+/-}$ transgenic mice, exon 5 is deleted in the mammary gland. P1, P2 and P3 localize the PCR primers used for genotyping. The predicted PCR products are: loxp=1100 bp; wild type=1000 bp; Δ5=300 bp. (B) PCR screen for Cre-mediated *Pten* exon5 deletion in *Pten*$^{loxp/loxp}$*/MMTV-Cre*$^{+/-}$ mammary glands at different developmental stages. Lanes 1-4, control DNAs isolated from wild type (WT), *Pten*$^{loxp/+}$, *Pten*$^{loxp/loxp}$ and *Pten*$^{loxp/\Delta5}$ mice; lane 5-8, DNAs prepared from 5 week virgin, 8 week virgin, 3 days after weaning, and resting multi-parous mammary glands. (C) Decreased PTEN protein expression in the *Pten*$^{loxp/loxp}$*/MMTV-Cre*$^{+/-}$ mammary gland. Total cell lysates from mutant (*Pten*$^{loxp/loxp}$*/MMTV-Cre*$^{+/-}$, M) and control (*Pten*$^{loxp/loxp}$*/MMTV-Cre*$^{-/-}$, C) mice of 6 week-old virgin (V-6wk), pregnancy day 13 (P13), lactation day 10 (L10), and post-weaning day 1 (PW1) were subjected to western blot analysis, using α-PTEN antibody and α-actin antibody for loading controls. (D) Deletion of PTEN in mammary epithelial cells. Immunohistochemical staining for PTEN is conducted on mutant and control mammary glands at day 13 of pregnancy. Scale bar: 20 μm.

transplants were harvested (virgin) or the hosts were mated and tissues were harvested within 12 hours of delivery of pups.

RESULTS

Conditional deletion of *Pten* in the mammary gland

To study the roles of PTEN in mammary gland development and mammary tumor formation, and to overcome the early embryonic lethality caused by conventional knock-out strategies, we generated a conditional *Pten* knock-out mouse with LoxP sequences flanking exon 5 of the *Pten* gene (Lesche et al., 2002). In contrast to multiple tumor formation observed in *Pten*$^{+/-}$ and early embryonic lethality in *Pten*$^{-/-}$ animals, mice homozygous for the floxed-*Pten* allele (*Pten*$^{loxp/loxp}$) are viable with no signs of tumor development up to 2 years, suggesting that introduction of the LoxP sites has no effects on the normal function of PTEN. *Pten*$^{loxp/loxp}$ mice were crossed into two independent lines of transgenic mice expressing MMTV-Cre transgenes. One line expresses Cre more specifically in mammary epithelium (T. F. L., unpublished results). Mice that carry two floxed *Pten* alleles and the mammary-specific *MMTV-Cre* transgene were fertile and the females had normal sized litter and were able to nurse their babies. Thus all analyses, except the ones shown in Fig. 4 and Fig. 7B, were conducted by using this line. The second line expresses Cre earlier in ductal and alveolar mammary epithelium as well as in other cell types (Wagner et al., 2001) and this line was used for the mammary gland transplantation experiment.

To confirm *Pten* deletion in mammary tissue, we used PCR, western blot, and immunochemical strategies. As shown in Fig. 1B, exon 5 deletions could be detected in virgin mammary tissue as early as 5 weeks (lane 5), and the percentage of deletion accumulated with age and was further exacerbated by pregnancy (lanes 6-8). This observation is consistent with the known regulation of the MMTV-LTR since it is upregulated by steroid and peptide hormones during estrus and pregnancy. As a direct result of *Pten* deletion, the overall PTEN protein levels were significantly reduced in mammary tissue of virgin, pregnant, and lactating females (Fig. 1C). Immunohistochemical analyses indicated that PTEN is normally localized in both the nucleus and cytoplasm of epithelial cells lining the alveoli (Fig. 1D, control), as well as ductal and stromal cells (not shown) in the *Pten*$^{loxp/loxp}$ mammary tissue. In contrast, PTEN immunostaining was significantly reduced or completely lost in the majority of cells in *Pten*$^{loxp/loxp}$*;MMTVCre*$^{+/-}$ mammary tissue (Fig. 1D, mutant).

Pten deletion leads to accelerated ductal extension, excessive side-branching and precocious lobulo-alveolar development during puberty and pregnancy

Mouse mammary development begins at embryonic day (E) 10.5 with a thickening in the epidermis that gradually forms an

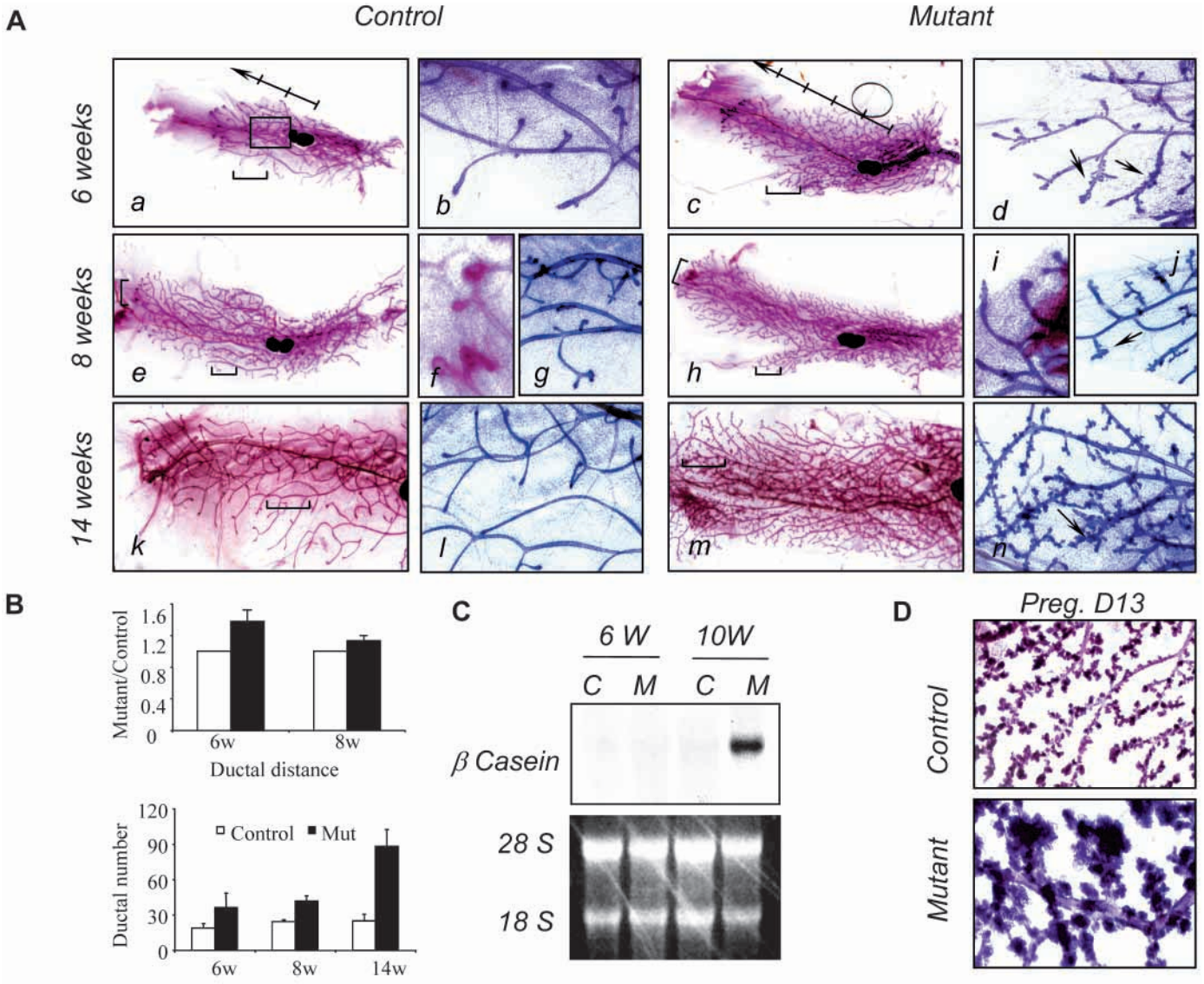

Fig. 2. Accelerated ductal extension, excessive side-branching and precocious lobulo-alveolar development in mutant mammary glands. (A) Whole-mount preparations of control and mutant mammary glands at different developmental stages. Carmine Red-stained whole mounts of inguinal mammary glands from control (a, b, e-g, k and l) and mutant (c, d, h-j, m and n) mice at 6 (a-d), 8 (e-j), 14 (k-n) weeks of age. Images in b, d, f, g, i, j, l, and n show higher magnifications of the areas bracketed in a, c, e, h, k and m. Note the lobulo-alveolar development on mutant glands (arrows in d, j, and n). Scaled arrows in a and c indicate the distances between the centers of the lymph node and the end of the TEBs. Box in a indicates the area where the number of branches was counted. (B) Quantitative representation of distances from the center of the lymph node to the far end of TEBs (top), and the number of branching points within the comparable boxed area (see Aa. 8 mm^2) at different stages analyzed (n=4~5). (C) Northern blot analysis of β-casein mRNA levels in control (C) and mutant (M) mammary glands. RNA was extracted from virgin mammary glands at 6 weeks (6W) and 10 weeks (10W). Northern blots were probed with cDNAs encoding α– and β-casein. One representative of two independent experiments is shown. The amount of ribosomal RNAs was used as loading controls. (D) Enlarged lobulo-alveolar in mutant mammary gland compared with control at 13 days after pregnancy.

epithelial bud. In newborn mice, a rudimentary system of small ducts is present, which occupies only a small portion of the mammary fat pad and grows slowly. Accelerated ductal extension and branching commences with the onset of estrogen secretion at about 3-4 weeks of age when terminal end buds (TEBs) appear that serve as sites of active cell proliferation during ductal growth. Once epithelial growth has extended throughout the fat pad, the TEBs disappear and ductal growth enters a quiescent stage in the virgin female until pregnancy. With the onset of pregnancy, increases in progesterone, prolactin and/or placental lactogen stimulate more side-branching and an extensive lobulo-alveolar epithelial cell proliferation and terminal differentiation which leads to the onset of milk secretion at parturition (Hennighausen and Robinson, 2001; Muller and Neville, 2001).

To investigate the effect of PTEN loss on mammary development, we examined whole-mount preparations from littermates with matching estrous cycles at different developmental stages. During puberty, the mutant ducts grew much faster than controls: the distance between the end of

TEBs and the center of the lymph node of mutant ducts was approximately 1.4 (n=5) times longer than control's at 6 weeks (scaled arrows in 2Aa and 2Ac; quantified in Fig. 2B upper panel), leading to earlier occupancy of the fat pad and disappearance of the TEBs in the mutant glands at 8 weeks (Fig. 2Ah and 2Ai). In contrast, TEBs in the control glands were still evident at the same developmental stage (Fig. 2Af).

Some of the morphological changes observed in the absence of PTEN were reminiscent of normal mammary development seen during early pregnancy. For instance, mutant ducts developed excessive side branches or small protrusions at 6 weeks as compared to the smooth surface seen in the control gland (Fig. 2Ab,Ad). To provide a more quantitative comparison, the number of branching points within an 8 mm^2 area near the lymph node (box in Fig. 2Aa) was counted. In the control mice, the number of side branches reaches a plateau after 8 weeks when the mammary epithelium occupies the fat pad. The mutant mammary glands contained more side branches at 6-weeks old, and the number of side branches continuously increased even after the disappearance of the TEBs, leading to more than a 2-fold increase in the number of side branches at 14 weeks (Fig. 2Ak,Am; quantified in 2B lower panel). The virgin mutant gland also contained many lobulo-alveolar buds, which are normally associated with hormonal stimulation during pregnancy (comparing 2Al and 2An). To investigate if this precocious alveolar development was accompanied by functional differentiation, we examined the expression levels of two milk-specific proteins, α- and β-casein, in the virgin mammary gland. As expected, no casein expression could be detected in the control virgin gland. In contrast, both α- (data not shown) and β-casein were expressed as early as 10 weeks in the mutant virgin gland (Fig. 2C). However, no changes in the phosphorylation status of STAT5 could be detected in the mutant virgin mammary glands (data not shown). This precocious phenotype continues to pregnant stage as mutant mammary gland displayed much bigger lobules at pregnancy day 13 (Fig. 2D); HE staining also revealed hyperplastic growth in the mutant glands (data not shown). Taken together, these observations suggest that PTEN serves as an important negative regulator for mammary gland development and deletion of *Pten* in the mammary gland leads to an accelerated mammary epithelium proliferation and differentiation.

PTEN negatively controls mammary epithelial cell proliferation

During mammary gland development, the cells in TEBs are responsible for the outgrowth or extension of the mammary ducts while cells in the ductal epithelium structures are crucial for side-branch formation and subsequent mammary cycles during each pregnancy. The fact that the loss of PTEN led to faster ductal growth and more side-branching prompted us to investigate whether PTEN negatively controls mammary epithelial cell growth using the nucleotide substitution method. A 2.8-fold increase in the number of bromodeoxyuridine (BrdU) pulse-labeled cells was observed in the mutant TEBs region at 5 weeks (Fig. 3A, 38.3% versus 13.7%, P<0.001, n=3), providing an explanation for the faster ductal extension observed in the mutant mice. An even more remarkable difference was observed in 12-week-old virgin mammary glands: the control mammary gland had entered a relative quiescent stage and only 0.027 $BrdU^+$ cells per duct section could be identified (Fig. 3B, upper panel; n=146); In contrast, the mutant ductal epithelium never stopped proliferation as there were 0.87 cells per duct section that could be pulse-labeled by BrdU (Fig. 3B, lower panel; n=202), representing a more than 32-fold increase in the number of proliferating cells over corresponding wild-type sections. This increased cell proliferation is similar to our previous study on $Pten^{-/-}$ neural cells (Groszer et al., 2001), suggesting that PTEN's control of cell proliferation is general rather than a cell type-specific mechanism.

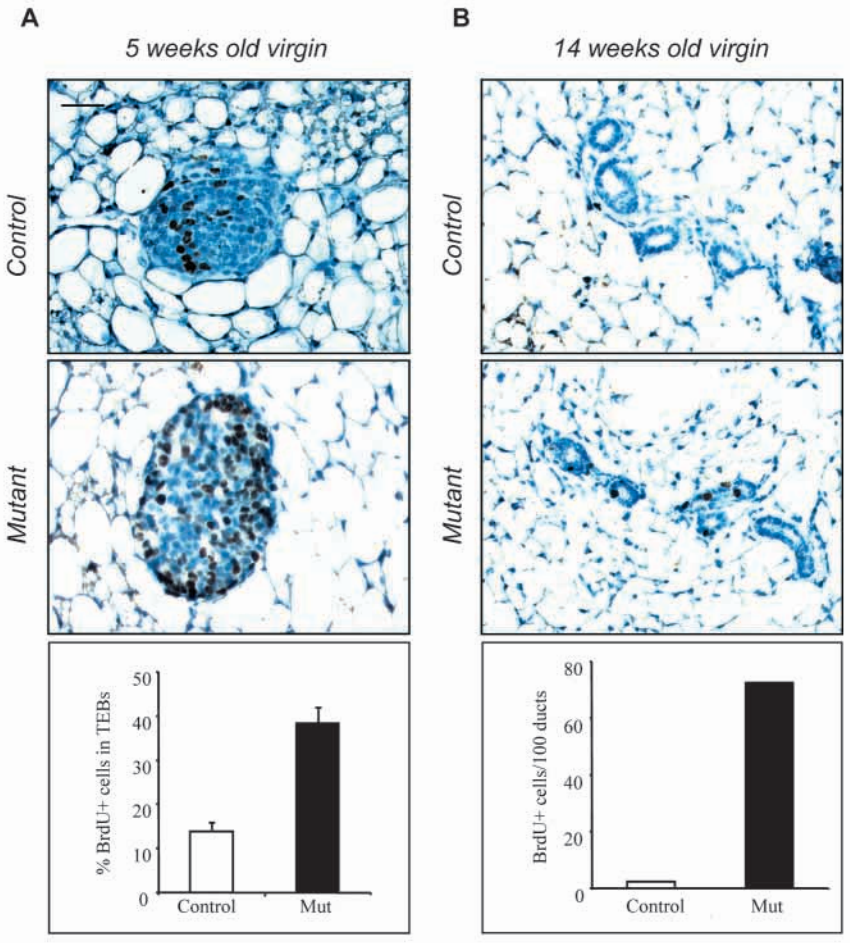

Fig. 3. Increased BrdU incorporation in the mutant mammary glands. Five-week-old (A) and 14-week-old (B) virgin control and mutant mice were pulse-labeled with BrdU. The numbers of $BrdU^+$ cells were counted in the TEB region (for 5-week-old samples) or in the ductal areas (for 14-week-old samples). The proliferation index for 5-week-old mice was calculated as follows: [number of $BrdU^+$-labeled cells]/[total number of cells]×100; Error bars represent standard errors of the mean, P<0.01, χ^2-test, n=3. For 14-week-old mice, all of the BrdU-labeled cells from the whole sections were counted; values were expressed as [number of $BrdU^+$-labeled cells]/[100 ductal structures]. Bar: 40 μm.

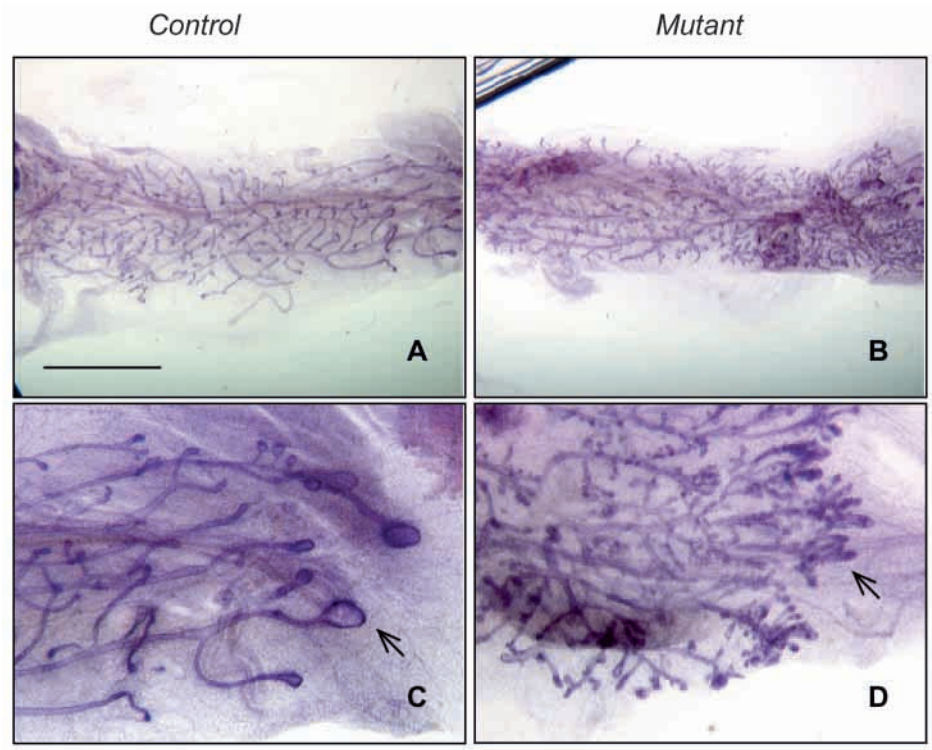

Fig. 4. Abnormal ductal development of transplanted mutant mammary epithelium. Whole-mount analysis of mammary epithelial transplants (A and C, control epithelium; B and D, mutant epithelium) from virgin females. At higher magnification, more and feather-like irregular side-branches are seen in mutant ductal and terminal structures (arrow in D) while control transplants display smooth ducts and normal terminal end buds (arrow in C). Scale bar: for A and B, 300 μm; for C and D, 200 μm.

PTEN controls mammary epithelium growth in a cell-autonomous manner

Development of the mammary gland occurs via epithelial-stromal interaction, and PTEN is localized in both epithelial and stromal cells. To determine whether the aforementioned phenotype was due to a cell-autonomous function of PTEN in mammary epithelium, or indirectly regulated by surrounding stromal cells or systemic cues, we transplanted control and PTEN-null mammary epithelium into wild-type fat pads free of endogenous mammary epithelia. To ensure complete deletion of *Pten* in the donor mammary epithelium, we took advantage of the second MMTV-Cre transgenic line, which expresses Cre earlier and at a higher level (Wagner et al., 2001). The resulting mammary epithelium was used for the transplantation experiment. As shown in Fig. 4, the transplanted mutant mammary epithelium displayed much more side-branching formation in the wild-type environment, suggesting that PTEN controls mammary epithelium proliferation and differentiation in a cell autonomous manner. Of note, the feathery hyperbranched phenotype is similar to reported MMTV-Wnt1 transgenic mice (see Fig. 6A and Discussion).

Pten deletion leads to severe defects in mammary gland involution

The entire alveolar epithelial compartment of the mammary gland undergoes remodeling after weaning and will finally assumes a virgin-like state. We investigated whether PTEN influences mammary gland involution after cessation of lactation. Involution is a two-step process: while the first stage is characterized by a loss of differentiation and programmed cell death of the secretory epithelium, the second stage involves protease-mediated remodeling. By day 1 of involution, the alveoli in the control glands had started to collapse while the alveoli of the mutant mice retained the general appearance of a lactating gland (Fig. 5A, upper panels). Two days later, the collapse of alveoli was more apparent (Fig. 5A, second left panel) with apoptotic cells shed into the lumina of the control gland, as demonstrated by TUNEL assay (in green, Fig. 5B, upper left). In addition, adipocytes began to reappear (data not shown). In contrast, the collapse of alveoli was not apparent in the mutant mice as the gland still contained intact alveolar structures (Fig. 5A, second right panel), resulting in 1.5 times fewer cells per field of view (Fig. 5C, right panel; $P<0.01$). Quantitative analysis indicated that there were 2.6-fold less apoptotic cells in the mutant gland (Fig. 5B, upper right; $P<0.01$). By day 21 after weaning, the control glands had completely returned to a state similar to virgin mammary gland (Fig. 5A, third left panel), and the majority of tissue was made up of adipocytes (Fig. 5A, lower left panel). However, some lobular-like structures still remained in the mutant glands as demonstrated by whole mounts (Fig. 5A, arrow in third right panel), and milk-like liquid could be found after probing such structures. HE staining revealed that those structures were composed of alveoli with big lumina (Fig. 5A, lower right panel). Taken together, these observations indicate that the normal involution and apoptosis process are severely impaired in *Pten*-deleted mammary glands.

Molecular analysis of involuting mammary glands in the absence of PTEN

The delayed involution process in mutant mammary glands was accompanied by sustained expression of a milk-specific gene. As shown in Fig. 6A, β-casein expression decreases in the control gland to almost undetectable levels by day 6 of involution. In contrast, β-casein expression in the mutant mammary glands remains high even at day 6 post weaning. Northern blot analysis also demonstrated that the mutant mammary tissues produce higher levels of cyclin D1 and c-myc (Fig. 6A), two known down stream targets of WNT signaling pathway, consistent with the feathery hyperbranched phenotype shown in Fig. 4.

To further explore the molecular mechanisms for impaired apoptosis and involution in the mutant mammary glands, we assessed the status of some known targets of PTEN controlled

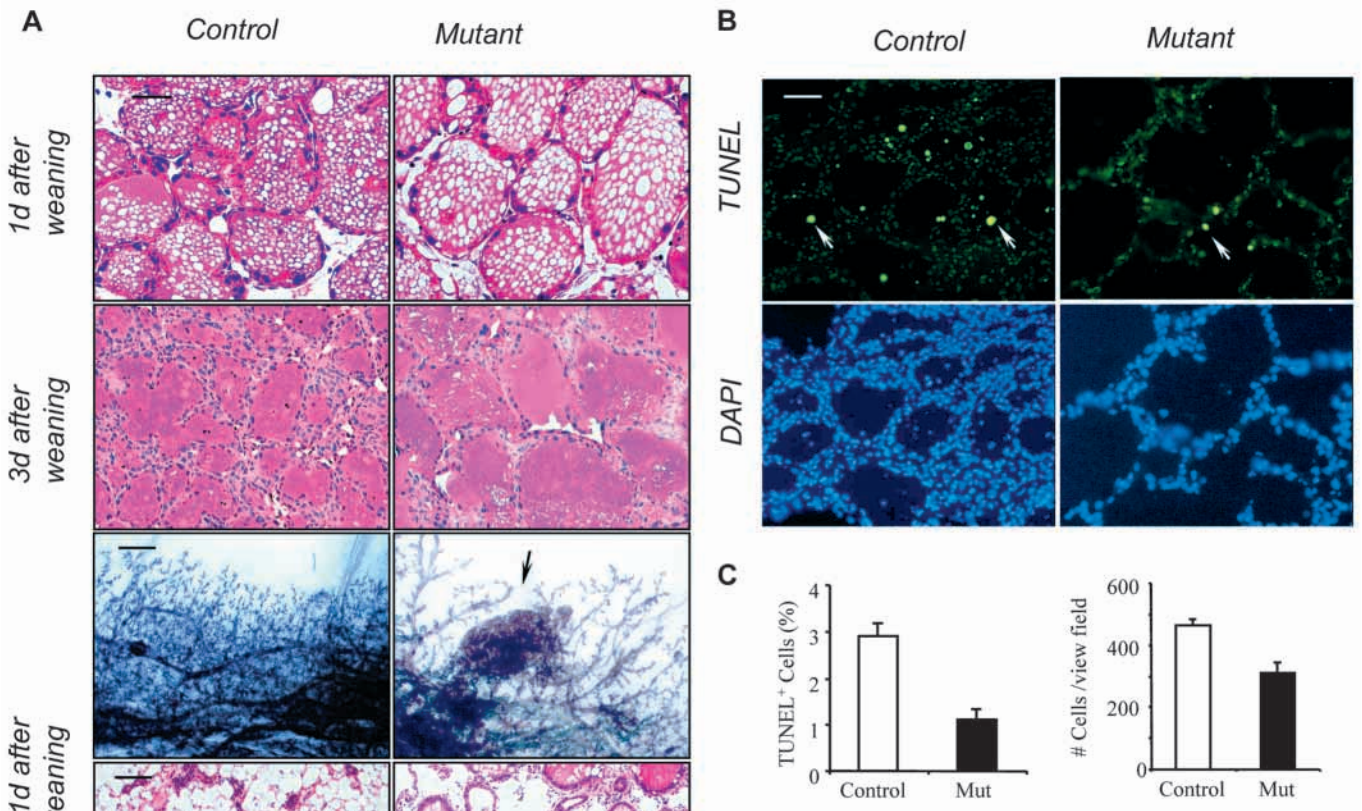

Fig. 5. Involution is severely affected in the mutant mammary glands. (A) HE staining of mammary glands sections. (Left panels) Mammary glands from control mice. (Right panels) Mammary glands from mutant mice. (Upper panels) Day 1 of involution; (second panels) day 3 of involution; scale bar: 40 μm; (third panels) Carmine Red-stained whole mounts of mammary glands at day 21 of involution; scale bar: 800 μm; (bottom panels) HE staining of sections from the corresponding whole-mounts samples; scale bar: 80 μm. (B) TUNEL analysis of control (upper left panels) and mutant (upper right panel) involuting mammary glands 3 days post parturition. TUNEL-positive cells are in green. Lower panels show DAPI staining of the corresponding sections. Scale bar: 40 μm. (C) Quantification of TUNEL-positive cells (left). Ten 200× magnification fields of view were randomly counted. The apoptosis index was calculated as following: ([number of TUNEL positive cells]/[total number of cells])×100. The total cell numbers per 400× magnification fields of view was also quantified and graphed (right panel). Statistical analysis was performed using Student's *t*-test. Error bars represent standard errors of the mean.

signaling pathways. One of the primary targets of PTEN is PtdIns(3,4,5)P_3, a direct product of PI 3-kinase. Loss of PTEN function results in accumulation of PtdIns(3,4,5)P_3 and activation of its downstream effectors, such as AKT/PKB (Stambolic et al., 1998; Sun et al., 1999). Activated AKT/PKB is a well characterized survival factor in vitro and prevents cells from undergoing apoptosis by inhibiting the pro-apoptotic factors BAD (del Peso et al., 1997), caspase 9 (Cardone et al., 1998), as well as the nuclear translocation of Forkhead transcription factors. The levels of endogenous AKT change from lactating to involution: it is high during lactation and decreases following weaning in both control and mutant mammary glands. However, AKT phosphorylation or activation was significantly higher in the mutant gland, especially during lactation (Fig. 6A, L10). As consequences of AKT hyperphosphorylation, Bad and FKHR phosphorylation were increased in the mutant mammary gland (Fig. 6B). The biggest difference in BAD phosphorylation was seen 3 days after weaning (I-3), and its phosphorylation became almost undetectable in both control and mutant glands 6 days after weaning (Fig. 6B). Interestingly, phosphorylated FKHR disappeared at involution day 3 but reappeared at involution day 6 (Fig. 6B).

The transcription factors STAT5 and STAT3 are required for mammary gland development and involution, respectively; and their phosphorylation status during lactation and involution are inversely correlated (Chapman et al., 1999; Liu et al., 1997; Miyoshi et al., 2001). To test whether loss of PTEN would influence the signaling pathways controlling the level as well as activation of these two STAT molecules, we analyzed the endogenous protein levels and phosphorylation status of STAT3 and STAT5 by western blots. As shown in Fig. 6C, both STAT5 and STAT3 protein levels were more or less stable from lactation day 10 to involution day 6, and no significant differences could be detected when comparing the mutant and control glands. Both mutant and control glands also displayed similar patterns of STAT5 and STAT3 phosphorylation during lactation and involution: STAT5 phosphorylation was high

during lactation but rapidly decreased after the commencement of involution. In contrast, activated STAT3 was not detectable during lactation but rapidly increased to a higher level in the control tissue compared to the mutant tissue at involution day 1. This most likely reflects the delayed involution in PTEN-deficient epithelium. These results suggest that PTEN or PTEN-controlled signaling pathways have no significant role in regulating either the expression or the phosphorylation status of the STAT5 and STAT3 molecules.

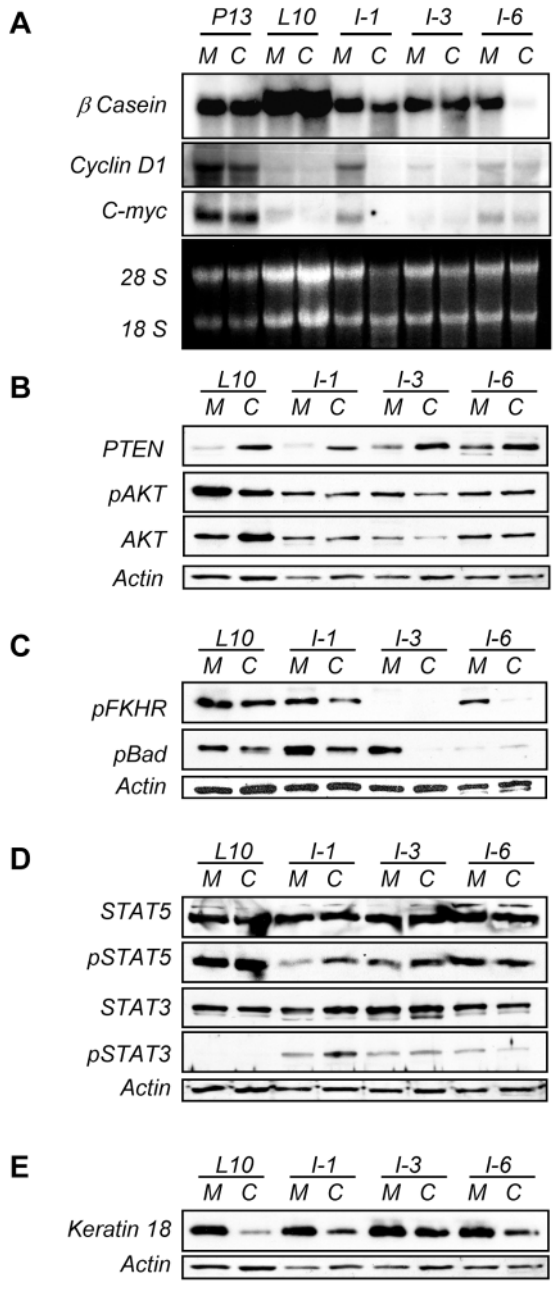

Fig. 6. Analysis of down-stream signaling targets of PTEN controlled pathway. (A) Northern blot analysis of β-casein, cyclin D1, and c-myc mRNA levels in control (C) and mutant (M) mammary glands. RNA was extracted from pregnancy day 13 (P13), lactation day 10 (L10), and involution day 1, 3 and 6 glands, respectively (I-1 to I-6). One representative of two independent experiments is shown. The amount of ribosomal RNAs was used as loading control. (B-E) Western blot analysis. Total cell lysates from lactation day 10 (L10), involution day 1 (I-1), day 3 (I-3), and day 6 (I-6) glands were examined. Mutant and control are labeled as M, mutant; C, control. α-actin antibody was used as loading control.

Keratin 18 is a marker of mammary luminal epithelial cells and its levels were significantly increased in the mutant glands. This difference persisted from lactation day 10 to involution day 6 (Fig. 6D), although actin levels were quite consistent among all the samples. Recent studies suggest that keratin 18 can attenuate TNF-induced cell death through association with TRADD (Caulin et al., 2000; Inada et al., 2001). Even though there is no evidence so far that TNF is involved in mammary gland involution, a possible contribution of up-regulated keratin 18 to impaired apoptosis in PTEN-deficient mammary glands warrants further investigation.

PTEN deficiency leads to mammary tumor formation

The abnormal mammary gland development and involution caused by *Pten* deletion leads to a high frequency of breast tumor formation in mutant animals. The latency of mammary tumor development in females carrying heterozygous *Pten* deletion is 10 months (R. L., N. Kertesz, D. Freeman, H. M.-D., M. Groszer, J. Gao, N. R., R. Cardiff, X. L. and H. W., unpublished data). However, females with mammary-specific *Pten* deletion develop tumors as early as 2 months (Fig. 7C). Multiple neoplastic changes could be detected in the whole mounts (Fig. 7Aa). In the virgin mutant transplants, focal ductal hyperplasia containing papillary structures could be observed (Fig. 7Ab, arrow). Mutant glands after one pregnancy showed intra-luminal focal cellular hyperplasia (Fig. 7Ac, arrows), and dysplasia (Fig. 7Ad) with areas of atypical proliferation and mitotic figures (Fig. 7Ae, arrow). Some glands contained what appears to be squamous epithelium and keratinous debris (Fig. 7Af, arrows). Tumor incidence was significantly increased with shortened latencies in multiparous females. Histological features of the tumors varied from benign fibroadenomas (Fig. 7Ag) to pleiomorphic adenocarcinomas (Fig. 7Ah). Often, this variety of lesions could be found within the same mass. Immunohistochemistry analysis revealed up-regulated expression of two cytokeratins. Cytokeratin 5 is normally expressed in myoepithelial cells of the mammary duct but its expression was significantly increased in the hyperplastic areas of virgin and terms transplants (Fig. 7Bc and Be). Cytokeratin 6 expression is associated with hyperproliferation or renewing epithelia (Smith and Chepko, 2001). Interestingly, up-regulated cytokeratin 6 expression was detected only in epithelial cells that protruded into the lumen of the virgin mutant transplants (Fig. 7Bd, arrows), which may represent the initiation of focal ductal epithelial hyperplasia seen in Fig. 7Ab. Thus, the BrdU incorporation studies (Fig. 3) and tumor analyses demonstrated that the primary effect of *Pten* deletion in mammary epithelium is disregulated cell proliferation.

DISCUSSION

In 1962, Lloyd and Dennis published the first case report on a “rare, familial, developmental disease” referred to as ‘Cowden’s disease’, after the family name of the proposita. The clinical findings included “bilateral virginal hypertrophy of the

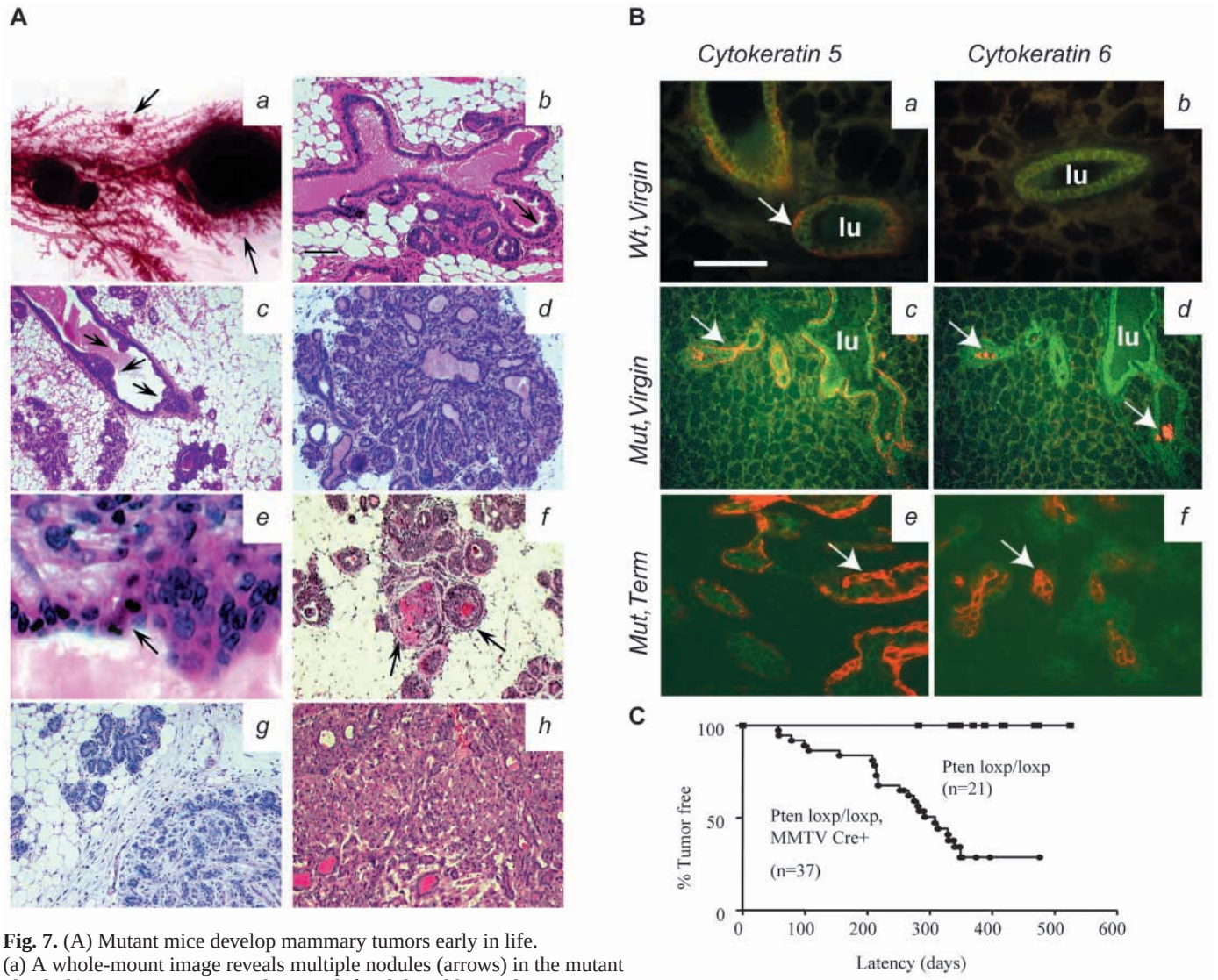

Fig. 7. (A) Mutant mice develop mammary tumors early in life. (a) A whole-mount image reveals multiple nodules (arrows) in the mutant gland. (b) Virgin mutant transplants with focal ductal hyperplasia containing papillary structures (arrow). (c) Mutant glands after one pregnancy showed intra-luminal focal hyperplasia. Note the ductal epithelial cells contain multi-layers and protrude into the lumen (arrows). (d,e) Mutant gland after one pregnancy with dysplastic changes (d) and pleomorphic nuclei and mitotic figures (arrow in e). (f) Mutant gland with squamous epithelium and keratinous debris within (arrows). (g,h) Tumors from multiparous mutant mammary glands show various histological features, ranging from well differentiated fibroadenoma (g) to pleomorphic adenocarcinoma (h). Scale bar: b, 80 μm; c, 160 μm; d,f,g,h, 80 μm; e, 16 μm. (B) Up-regulated cytokeratin 5 and 6 expressions in the mutant mammary tissue transplants. Cytokeratin 5 (left panels) and cytokeratin 6 (right panels) immuno-staining (in red) of normal virgin (a,b), hyperplastic areas of mutant virgin (c,d) and term glands (e,f). All sections were counter-stained with β-catenin antibody (in green). Cytokeratin 5 expression is low in myoepithelial cells of normal glands, and is upregulated in hyperplastic areas (arrow in c). Cytokeratin 6 is absent in normal alveoli and is expressed only in epithelial cells that protrude into the lumen of hyperplasic areas (arrows in d). Broader ranges of cytokeratin 5 and 6 expressions can be detected in the term mutant glands (e,f). Bar for a,b,e,f, 80 μm; c,d, 250 μm. (C) Tumor incidence of $Pten^{loxp/loxp};MMTVCre^{+/-}$ (n=37) and $Pten^{loxp/loxp}$ (n=21) females.

breasts ….and early malignant degeneration" (Lloyd and Dennis, 1962). In 1997, the link between Cowden's disease and *PTEN* mutation was formally established (Liaw et al., 1997; Nelen et al., 1997). However, the exact mechanisms of PTEN control of mammary gland development are unclear. In this study, we showed that conditional deletion of *Pten* in the mammary gland resulted in dramatically increased ductal branching and BrdU incorporation, accelerated lobulo-alveolar-like precocious differentiation, decreased cell apoptosis and impaired involution process, suggesting that PTEN is one of the important factors controlling mammary epithelial cells during normal mammary gland development and mammary gland cycling.

PTEN and mammary epithelial cell proliferation

Enhanced ductal extension, increased ductal side-branching, together with increased BrdU incorporation, suggest that mammary epithelial cell proliferation was disregulated in the absence of PTEN. Loss of PTEN leads to an increase in the intracellular concentration of PtdIns(3,4,5)P_3, mimicking the

effect of constitutive PI3K activation. PtdIns(3,4,5)P_3 accumulation can lead to the activation of various protein kinases, including AKT/PKB serine/threonine kinase. As one of the central players in the PTEN regulated pathway, hyperactivated AKT has been shown to promote cell proliferation, possibly through down regulation of the cyclin-dependent kinase inhibitor p27 as well as upregulation and stabilization of cyclin D1 (Blume-Jensen and Hunter, 2001). AKT activation is also important for cell survival. However, can the observed phenotypes in *Pten* conditional deleted mammary gland be explained solely by activation of AKT pathway?

Three independent groups have generated transgenic mice expressing constitutively active AKT in the mammary gland under the control of MMTV promoter (Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2001). Activation of the AKT pathway led to involution defects, consistent with the phenotype in our *Pten* conditional knockout mice. However, no significant differences could be observed in ductal growth and epithelial differentiation when comparing transgenic and control virgin females, despite the high expression levels of constitutively active form of AKT (Hutchinson et al., 2001). This discrepancy could be due to insufficient levels of activated AKT. Alternatively, PTEN may control multiple pathways, including the AKT pathway. Interestingly, comparing other mouse models (Deng and Brodie, 2001; Dunbar and Wysolmerski, 2001), the phenotypes described in this study are very similar to MMTV-Wnt1 transgenic mice in which increased side-branching, feathery-like morphology, and precocious lobulo-alveolar development were reported (Tsukamoto et al., 1988).

PTEN and mammary epithelium differentiation

Two possible mechanisms could contribute to the observed precocious lobulo-alveolar development. One possibility is that loss of PTEN could render epithelial cells more sensitive to extracellular signals, no matter if it is a proliferation or a differentiation signal. In fact, within the estrous cycles which begin at about 4 weeks of age, progesterone secretion can stimulate some mammary epithelial cells in virgin mice to undergo a transient differentiation process: including milk-specific gene expression and alveolar budding during proestrus and estrus stages (Robinson et al., 1995). Thus, more epithelial cells would undergo proliferation and differentiation in the PTEN-deficient mammary gland during each estrous cycle, leading to the precocious phenotype. Our observation that mature virgin mammary glands of mutant mice had many more BrdU-labeled cells seems to support this possibility. The second mechanism could be impaired cell death, since transient epithelium differentiation is balanced by cell death during metestrus and diestrus phases of the hormonal cycle under the normal situation. Because PTEN negatively regulates cell death pathways (see below), less apoptosis during metestrus and diestrus could lead to accumulation of the differentiated cells. To test this hypothesis, we performed TUNEL analysis on mature virgin mammary glands. However, the number of TUNEL-positive cells was too low to provide any statistically significant comparison (data not shown).

PTEN and mammary gland involution

Involution is severely affected in the mutant mammary glands as illustrated by histological analysis and TUNEL assay. Strikingly, some big lobulo-alveolar structures still exist 21 days after weaning in the mutant glands, demonstrating that PTEN plays an important role in mammary gland involution. PTEN deficiency leads to decreased apoptosis through hyperactivating the AKT pathway (Fig. 6). Activated AKT can inhibit the function of the death-promoting molecules such as BAD, Caspase 9, FKHR, and FKHRL1, leading to cell survival (Datta et al., 1999).

STAT protein activation, in response to cytokine stimulation (such as prolactin), are also crucial in the cyclical cellular proliferation, differentiation, and regression of mammary epithelial cells (Chapman et al., 1999; Liu et al., 1997; Miyoshi et al., 2001). Specifically, STAT5 is highly phosphorylated during late pregnancy and throughout lactation but its phosphorylation levels sharply decline at the onset of involution (Liu et al., 1996). *Stat5a* knockout mice have impaired lobulo-alveolar differentiation during pregnancy and fail to lactate (Liu et al., 1997). Mice lacking both *Stat5a* and *Stat5b* fail to develop secretory mammary epithelium during pregnancy (Miyoshi et al., 2001). In contrast, STAT3 activation is hardly detectable during lactation, but is strongly induced at the onset of involution. *Stat3* mammary gland specific conditional knockout mice have suppressed epithelial apoptosis and delayed mammary gland involution (Chapman et al., 1999). It has been suggested that the coordinated regulation of STAT3 and STAT5 function may be a driving force in the induction of apoptosis in the involution process. The overall protein levels of STAT3 and STAT5 are comparable between normal and *Pten*-deficient mammary gland, while a higher phosphylation of STAT3 in the control gland at day 1 of involution is consistent with the delay in involution seen in the mutant tissue. These results suggest that the pathways regulating STAT5 and STAT3 remain more or less intact in the PTEN-deficient mammary gland.

Interestingly, keratin 18 was significantly increased in mutant glands at various stages, ranging from lactation day 10 to involution day 6. This cannot simply be explained by reduced epithelial cell death in the mutant glands during involution since keratin 18 expression was also high during lactation stage. Keratin 8 and 18 (K8/18) are the major components of intermediate filament (IF) proteins of simple or single-layered epithelia. Recent data showed that normal and malignant epithelial cells deficient in K8/18 were nearly 100 times more sensitive to tumor necrosis factor (TNF)-induced cell death. K18 could bind TRADD (TNF receptor type 1 (TNFR1)-associated death domain protein) and the cytoplasmic domain of TNFR2. It has been suggested that K18 might inhibit TNF-induced apoptosis by sequestering TRADD to attenuate interactions between TRADD and activated TNFR1, or by moderating TNF-induced, Jun N-terminal kinase (JNK) intracellular signaling and NFB activation (Caulin et al., 2000; Inada et al., 2001). Our data suggest that PTEN may be involved in the regulation of K18 expression. However, further experiments are needed to elucidate the significance of this observation.

PTEN, Cowden's disease and mammary tumor

The proposita of the first reported Cowden's diseases "had difficulty with her breast since her menarche at age of 12 years, when the breast had rapidly and progressively enlarged to an

abnormal degree with the development of multiple, occasionally tender modules of varying size" (Lloyd and Dennis, 1962). Mice with mammary-specific deletion of *Pten* suffered from increased cell proliferation at early puberty stage, progressively increased ductal number and mass, precocious lobuloalveolar development, and intra-lumina focal hyperplasia and dysplasia. Thus, the developmental abnormalities in PTEN-deficient virgin mammary glands are similar to the breast phenotypes of Cowden's disease. These results suggest that disregulated mammary epithelial cell proliferation may be the initial cause of the mammary gland abnormalities in the Cowden patients. PTEN is also crucial in controlling apoptosis during involution, leading to progressively malignant transformation in multiparous females.

It was initially suggested that *PTEN* was frequently mutated in late stages or more advanced tumor types, such as glioblastoma, which associated PTEN function with tumor progression. However, this hypothesis did not withstand extensive analysis when other tumor types were included. It is now known that *PTEN* abnormalities are present in early cancerous conditions (Ali, 2000), and PTEN may function as a gatekeeper or landscaper for the normal cellular function. The fact that patients with Cowden's disease develop multiple harmartomas in tissues derived from all three major embryonic germ layers further suggests that Cowden's disease may represent a stem cell and developmental disease.

Recent studies suggest that cancers may be viewed as an aberrant organ initiated by 'cancer stem cells' (Reya et al., 2001). Like normal stem cells, cancer stem cells have the capacity for indefinite proliferation and the ability to give rise to new abnormal tissues through self-renewal and differentiation, analogous to normal stem cells. The key difference between normal and cancer stem cells may be disregulated stem cell self-renewal and proliferation/survival in the cancer stem cells, through accumulated mutations. We have shown previously that PTEN negatively regulates neural stem cell renewal and proliferation, both in vivo and in vitro (Groszer et al., 2001). Interestingly, the differentiation program of the mutant stem cells is not changed, which fits well the concept of 'cancer stem cells'. Mammary gland morphogenesis and cycling are also highly regulated processes, which are controlled by the interaction of epithelial/stromal lineages as well as by the stimulation of multiple steroid and peptide hormones and growth factors. Mammary epithelial stem/progenitor cells, even though still poorly defined, are distributed throughout the mammary structures since transplanting any fragment of mammary duct to a parenchyma-free fat pad leads to the formation of a new mammary gland. Thus, mammary stem/progenitor cell proliferation is subject to a high degree of control. In this study, we demonstrated that *Pten*-deficient mammary epithelium cells acquired disregulated cell proliferation and death without blocking the differentiation process. Whether PTEN regulates mammary epithelium stem cell proliferation and self-renewal warrants further investigation.

We thank Bei Zhang for valuable technical assistance and members of our laboratories for helpful comments on the manuscript. H. W. is an Assistant Investigator of the Howard Hughes Medical Institute (HHMI). G. L. is supported by HHMI, U01CA84128-03 (H. W.), and Chinese State Key Program of Basic Research G1998051208 (D. W. and G. L.). R. L. was supported by HHMI and the Deutsche Forschungsgemeinschaft. This work was supported by the following grants from the NIH: NS38489 (X. L.), P50CA86306 (H. W.), and DOD: PC991538 (H. W.).

REFERENCES

Ackler, S., Ahmad, S., Tobias, C., Johnson, M. D. and Glazer, R. I. (2002). Delayed mammary gland involution in MMTV-AKT1 transgenic mice. *Oncogene* **21**, 198-206.

Ali, I. U. (2000). Gatekeeper for endometrium: the PTEN tumor suppressor gene. *J. Natl. Cancer Inst.* **92**, 861-863.

Blume-Jensen, P. and Hunter, T. (2001). Oncogenic kinase signalling. *Nature* **411**, 355-365.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S. and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**, 1318-1321.

Caulin, C., Ware, C. F., Magin, T. M. and Oshima, R. G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J. Cell Biol.* **149**, 17-22.

Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* **13**, 2604-2616.

Dahia, P. L. (2000). PTEN, a unique tumor suppressor gene. *Endocr. Relat. Cancer* **7**, 115-129.

Datta, S. R., Brunet, A. and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905-2927.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**, 687-689.

Deng, C. X. and Brodie, S. G. (2001). Knockout mouse models and mammary tumorigenesis. *Semin. Cancer Biol.* **11**, 387-394.

Depowski, P. L., Rosenthal, S. I. and Ross, J. S. (2001). Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. *Mod. Pathol.* **14**, 672-676.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C. and Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression. *Nat. Genet.* **19**, 348-355.

Dunbar, M. E. and Wysolmerski, J. J. (2001). Mammary ductal and alveolar development: lesson learned from genetically manipulated mice. *Microsc. Res. Tech.* **52**, 163-170.

Feilotter, H. E., Coulon, V., McVeigh, J. L., Boag, A. H., Dorion-Bonnet, F., Duboue, B., Latham, W. C., Eng, C., Mulligan, L. M. and Longy, M. (1999). Analysis of the 10q23 chromosomal region and the PTEN gene in human sporadic breast carcinoma. *Br. J. Cancer* **79**, 718-723.

Garcia, J. M., Silva, J. M., Dominguez, G., Gonzalez, R., Navarro, A., Carretero, L., Provencio, M., Espana, P. and Bonilla, F. (1999). Allelic loss of the PTEN region (10q23) in breast carcinomas of poor pathophenotype. *Breast Cancer Res. Treat.* **57**, 237-243.

Groszer, M., Erickson, R., Scripture-Adams, D. D., Lesche, R., Trumpp, A., Zack, J. A., Kornblum, H. I., Liu, X. and Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science* **294**, 2186-2189.

Hennighausen, L. and Robinson, G. W. (2001). Signaling pathways in mammary gland development. *Dev. Cell* **1**, 467-475.

Hutchinson, J., Jin, J., Cardiff, R. D., Woodgett, J. R. and Muller, W. J. (2001). Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. *Mol. Cell. Biol.* **21**, 2203-2212.

Inada, H., Izawa, I., Nishizawa, M., Fujita, E., Kiyono, T., Takahashi, T., Momoi, T. and Inagaki, M. (2001). Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J. Cell Biol.* **155**, 415-426.

Lee, J. O., Yang, H., Georgescu, M. M., di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P. and Pavletich, N. P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323-334.

Lesche, R., Groszer, M., Gao, J., Wang, Y., Messing, A., Sun, H., Liu, X.

and Wu, H. (2002). Cre/*loxP*-mediated inactivation of the murine Pten tumor suppressor gene. *Genesis* **32**, 148-149.

Li, D. M. and Sun, H. (1997). TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.* **57**, 2124-2129.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947.

Li, Y., Podsypanina, K., Liu, X., Crane, A., Tan, L. K., Parsons, R. and Varmus, H. E. (2001). Deficiency of Pten accelerates mammary oncogenesis in MMTV-Wnt-1 transgenic mice. *BMC Mol. Biol.* **2**, 2.

Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M. et al. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.* **16**, 64-67.

Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H. and Wu, H. (2000). Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Curr. Biol.* **10**, 401-404.

Liu, X., Robinson, G. W. and Hennighausen, L. (1996). Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. *Mol. Endocrinol.* **10**, 1496-1506.

Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* **11**, 179-186.

Lloyd, K. M. and Dennis, M. (1962). Cowden's disease: A possible new symptom complex with multiple system involvement. *Ann. Intern. Med.* **58**, 136-142.

Maehama, T. and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13375-13378.

Marsh, D. J., Kum, J. B., Lunetta, K. L., Bennett, M. J., Gorlin, R. J., Ahmed, S. F., Bodurtha, J., Crowe, C., Curtis, M. A., Dasouki, M. et al. (1999). PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Hum. Mol. Genet.* **8**, 1461-1472.

Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K. U., Oka, T., Rosen, J. M., Robinson, G. W. and Hennighausen, L. (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J. Cell Biol.* **155**, 531-542.

Muller, W. J. and Neville, M. C. (2001). Introduction: Signaling in mammary development and tumorigenesis. *J. Mammary Gland Biol. Neoplasia* **6**, 1-5.

Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. and Tonks, N. K. (1997). P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. USA* **94**, 9052-9057.

Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C. F., Fryns, J. P., Sijmons, R. H., Woods, D. G. et al. (1997). Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum. Mol. Genet.* **6**, 1383-1387.

Perren, A., Weng, L. P., Boag, A. H., Ziebold, U., Thakore, K., Dahia, P. L., Komminoth, P., Lees, J. A., Mulligan, L. M., Mutter, G. L. et al. (1999). Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *Am. J. Pathol.* **155**, 1253-1260.

Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E. and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc. Natl. Acad. Sci. USA* **96**, 1563-1568.

Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111.

Robinson, G. W., McKnight, R. A., Smith, G. H. and Hennighausen, L. (1995). Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development* **121**, 2079-2090.

Schrager, C. A., Schneider, D., Gruener, A. C., Tsou, H. C. and Peacocke, M. (1998). Clinical and pathological features of breast disease in Cowden's syndrome: an underrecognized syndrome with an increased risk of breast cancer. *Hum. Pathol.* **29**, 47-53.

Schwertfeger, K. L., Richert, M. M. and Anderson, S. M. (2001). Mammary gland involution is delayed by activated Akt in transgenic mice. *Mol. Endocrinol.* **15**, 867-881.

Smith, G. H. and Chepko, G. (2001). Mammary epithelial stem cells. *Microsc. Res. Tech.* **52**, 190-203.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P. and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39.

Stambolic, V., Tsao, M. S., Macpherson, D., Suzuki, A., Chapman, W. B. and Mak, T. W. (2000). High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/– mice. *Cancer Res.* **60**, 3605-3611.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T. et al. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**, 356-362.

Stokoe, D. (2001). Pten. *Curr. Biol.* **11**, R502.

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 6199-6204.

Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W. et al. (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr. Biol.* **8**, 1169-1178.

Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. and Varmus, H. E. (1988). Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619-625.

Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R. and Hennighausen, L. (2001). Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res.* **10**, 545-553.