The deficiency of *Akt1* is sufficient to suppress tumor development in *Pten*$^{+/-}$ mice

Mei-Ling Chen,[1,6] Pei-Zhang Xu,[1,6] Xiao-ding Peng,[1] William S. Chen,[1] Grace Guzman,[2] Ximing Yang,[3] Antonio Di Cristofano,[4] Pier Paolo Pandolfi,[5] and Nissim Hay[1,7]

[1]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607, USA; [2]Department of Pathology, University of Illinois at Chicago, Chicago, Illinois 60607, USA; [3]Department of Pathology, Northwestern Medical School, Chicago, Illinois 60611, USA; [4]Human Genetics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111, USA; [5]Cancer Biology and Genetics Program, Department of Pathology, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

The tumor suppressor PTEN is frequently inactivated in human cancers. A major downstream effector of PTEN is Akt, which is hyperactivated via PTEN inactivation. It is not known, however, whether diminished Akt activity is sufficient to inhibit tumorigenesis initiated by Pten deficiency. Here we showed that the deficiency of *Akt1* is sufficient to dramatically inhibit tumor development in *Pten*$^{+/-}$ mice. Akt1 deficiency had a profound effect on endometrium and prostate neoplasia, two types of human cancer, in which PTEN is frequently mutated, and also affected thyroid and adrenal medulla tumors and intestinal polyps. Even haplodeficiency of Akt1 was sufficient to markedly attenuate the development of high-grade prostate intraepithelial neoplasia (PIN) and endometrial carcinoma. These results have significant implications for cancer therapy.

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The tumor suppressor PTEN is a phospholipids phosphatase, which dephosphorylates the D3 position of the inositol ring of phosphoinositides, the products of phosphatidylinositol 3-kinase (PI3K). PTEN therefore negates the activity of PI3K and inhibits the activities of its downstream effectors. One major downstream effector of PI3K and PTEN is the serine/threonine kinase Akt, also known as protein kinase B (PKB). Mammalian cells contain three genes that encode three Akt isoforms (Akt1–3). The cDNAs of the three genes have >85% sequence identity, and their protein products share the same structural organization. All three Akt isoforms possess conserved threonine and serine residues (T308 and S473 in Akt1) that are critical for the full activation of Akt. All Akt isoforms seem to have identical or similar substrate specificity, and it is not clear whether they possess different functional specificities in vivo. (Kandel and Hay 1999; Brazil and Hemmings 2001).

PTEN is frequently inactivated in human cancers (Sansal and Sellers 2004). In particular, a high incidence of PTEN inactivation has been observed in prostate and endometrial cancers, glioblastoma, and melanoma. Thus, it is important to identify therapeutic strategies that counteract PTEN inactivation to inhibit cancer. One attractive therapeutic target is Akt. It is not known, however, whether inactivation of Akt is sufficient to alleviate the impact of PI3K activation mediated by PTEN deficiency, as other targets of PI3K, including Rho GTPases, Tec family kinases, and PDK1 as well as other AGC kinase family members (Cantley 2002; Wymann and Marone 2005), could also contribute to the genesis of cancer. Furthermore, because of the pleiotropic activities of Akt, it is not known whether ablating Akt activity to the extent needed to inhibit cancer will have severe physiological consequences. Here we used mouse genetics to address these questions.

Haplodeficiency of *Pten*, in mice, elicits a wide range of tumors with a high tumor incidence in prostate, endometrium, thyroid, adrenal medulla, and the intestine (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). We therefore crossed *Pten*$^{+/-}$ mice with *Akt1*$^{-/-}$ mice, which are not impaired in their lifespan, to examine the ability of *Akt1* deficiency to inhibit tumor development in *Pten*$^{+/-}$ mice. We found that *Akt1* deficiency is sufficient to inhibit prostate neoplasia, endometrial carcinoma, thyroid and adrenal medulla tumors, and intestinal polyps markedly in *Pten*$^{+/-}$ mice, with the most profound effects in the prostate, endometrium, and small intestine. In these cases, even haplodeficiency of *Akt1* was sufficient to inhibit tumor development significantly. Finally, we showed that *Akt1* deficiency is sufficient to markedly reduce lymphoid hyperplasia in *Pten*$^{+/-}$ mice. Thus, our results imply that it is possible to inhibit Akt activity partially to the extent that it could be used as an approach for cancer therapy without severe physiological consequences.

Results and Discussion

To determine whether partial ablation of Akt activity could inhibit tumor development induced by *Pten* deficiency without severe physiological consequences, we crossed *Pten*$^{+/-}$ mice, which develop diverse types of tumors, with *Akt1*$^{-/-}$ mice, which do not show a decreased lifespan (and possibly increased lifespan) (Supplementary Fig. S1; data not shown) despite spontaneous apoptosis in certain organs (Chen et al. 2001). The resulting *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice were generated in the C57BL/6/129sv (1:1) background. *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice were inter crossed to generate *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{-/-}$, and wild-type mice.

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[6]These authors contributed equally to this work.
[7]Corresponding author.
E-MAIL nhay@uic.edu; FAX (312) 355-2032.
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Akt1 deficiency is sufficient to inhibit the development of PIN in Pten heterozygous mice

PTEN is frequently mutated in human prostate cancer (Sansal and Sellers 2004), and $Pten^{+/-}$ mice frequently develop prostate intraepithelial neoplasia (PIN) (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). We therefore determined PIN development in the three prostate lobes of 11-mo-old $Pten^{+/-}$, $Pten^{+/-}$/$Akt1^{+/-}$, and $Pten^{+/-}Akt1^{-/-}$ mice using histopathological criteria as well as BrdU incorporation and K14 staining.

For the histopathological analysis, the grades of PIN were defined according to previously described criteria (Shappell et al. 2004) (Supplementary Fig. S2a). In the high-grade lesions (PIN3 and PIN4), we often found diminished PTEN staining and increased plasma membrane staining of Akt phosphorylated at Ser473 (pAkt) (Fig. 1A; Supplementary Fig. S2b), demonstrating reduced expression or loss of the wild-type *Pten* allele with concomitant activation of Akt in the lesions. The strong plasma membrane staining of pAkt was often correlated with strong PCNA staining (Supplementary Fig. S2c), indicating that cells in which Akt was activated were in a proliferative state. We monitored PIN3 and PIN4 and observed that all $Pten^{+/-}$ mice had PIN3 and PIN4 lesions in the anterior lobe (~16% with PIN3 and ~84% with PIN4 lesions) (Fig. 1B). Even haplodeficiency of *Akt1* markedly reduced the frequency of PIN4 lesions in the anterior lobe to ~29%, although the number of mice with PIN3 lesions was increased to 50%. We observed a dramatic reduction in PIN4 lesions in $Pten^{+/-}Akt1^{-/-}$ mice, of which only ~4% and 11% had PIN4 and PIN3 lesions, respectively. Similar results were found in the dorsolateral lobe (Fig. 1B). In the ventral lobe, ~82% and 4% of $Pten^{+/-}$ mice developed PIN4 and PIN3 lesions, respectively, whereas only ~24% of $Pten^{+/-}Akt^{+/-}$ mice developed PIN4 lesions and 18% developed PIN3 lesions. $Pten^{+/-}Akt1^{-/-}$ mice did not develop PIN3 and PIN4 lesions at all in the ventral lobe (Fig. 1B), and ~46% of these mice were completely free of lesions in the ventral lobe.

PIN3 and PIN4 lesions are also characterized by the presence of mitotic figures. We therefore analyzed proliferation in the prostate by measuring the level of BrdU incorporation. There was a significant increase ($P < 0.01$) in BrdU incorporation in all-prostate lobes of the $Pten^{+/-}$ mice when compared with wild type (Fig. 1C). In contrast, BrdU incorporation in $Pten^{+/-}Akt1^{-/-}$ mice was indistinguishable ($P > 0.05$) from that seen in wild-type mice.

It was previously reported that high grades PIN in $Pten^{+/-}$ mice are characterized by increased expression of K14 in the lumen (Park et al. 2002). In the normal prostate, K14-positive cells were confined to the basal layer, whereas in high-grade PIN, there were many large K14-positive cells in the lumen of the duct (Supplementary Fig. S2d). We therefore used the intense K14 staining in the lumen of the ducts as an independent criterion for neoplastic lesions in the mouse prostate. We found that 85% of $Pten^{+/-}$ mice tested had intense K14 staining in the lumen of the anterior lobe and ~43% of $Pten^{+/-}$ mice tested had intense K14 staining in the lumen of the dorsolateral lobe. In contrast, no intense K14 staining was observed in $Pten^{+/-}Akt1^{-/-}$ mice (Supplementary Fig. S2d). Thus the K14 staining recapitulates the histopathological grading. The strong K14 staining also indicates

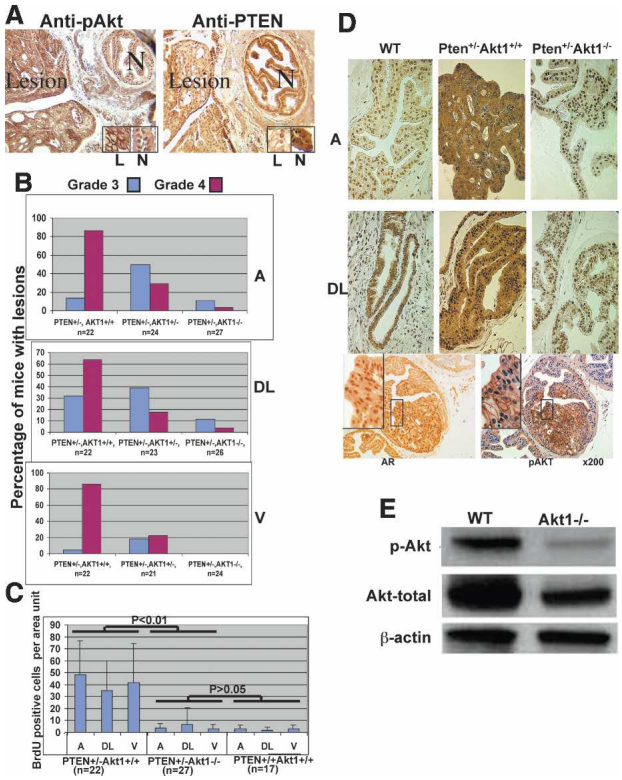

Figure 1. Akt1 deficiency impairs the development of high-grade PIN in $Pten^{+/-}$ mice. (*A*) Paraffin-embedded anterior lobe sections derived from $Pten^{+/-}$ mice were stained with either anti-pAkt or anti-PTEN. A PIN4 lesion (Lesion) and normal gland (N) are shown. (*Insets*) High magnification of an area for the lesion (L) or normal gland (N). (*B*) Incidence of PIN3 and PIN4 lesions in the three prostate lobes of $Pten^{+/-}$, $Pten^{+/-}Akt1^{+/-}$, and $Pten^{+/-}Akt1^{-/-}$ mice. The number of mice in each group is indicated. (*C*) BrdU incorporation in the prostate lobes of $Pten^{+/-}$, $Pten^{+/-}Akt1^{+/-}$, and $Pten^{+/-}Akt1^{-/-}$ mice. The number of mice in each group is indicated in parentheses. BrdU-positive cells were counted as described in Materials and Methods. *P* values were calculated for each prostate lobe in each genotype. (*D*) Increased expression of androgen receptor in PIN4 lesions in the anterior and dorsolateral lobes correlates with increased pAkt in the lesions. (*Top* panels) Immunostaining with anti-AR of paraffin-embedded sections derived form the anterior (A) and dorsolateral (DL) lobes of wild-type, $Pten^{+/-}$, and $Pten^{+/-}Akt1^{-/-}$ mice. (*Bottom* panel) Immunostaining with anti-AR or anti-pAkt of serial sections derived from the anterior lobe of $Pten^{+/-}$ mice. (*Insets*) High magnifications. (*E*) Immunoblot of protein extracts derived from wild-type and $Akt1^{-/-}$ mouse prostates using anti-pAkt, anti-pan-Akt, and anti-β-actin.

that the neoplastic cells may have originated from stem cells in the basal epithelium layer.

We also found that androgen receptor (AR) expression was dramatically elevated in the PIN4 lesions of $Pten^{+/-}$ prostate as manifested by the strong staining with anti-AR antibodies (Fig. 1D). Unlike AR localization in wild-type and $Pten^{+/-}Akt1^{-/-}$ mice, AR was not confined only to the nucleus in $Pten^{+/-}$ mice. Elevated AR expression was directly correlated with activation of Akt in the neoplastic lesions and was not present when *Akt1* was deleted (Fig. 1D). Increased expression of AR in neoplastic cells supports the notion that these cells are of epithelial origin. These results also suggest that activation of Akt can eventually lead to elevation of AR expression and may explain why AR is overexpressed in human prostate tumors that do not contain AR gene amplification. The

expression of AR in the neoplastic lesions suggests that they might be sensitive to hormone deprivation treatment. However, in light of the recent observation that elevated AR expression in human prostate tumors correlates with increased resistance to AR antagonists (Chen et al. 2004), these observations imply that prostate tumors in which Akt is activated may be more resistant to hormone therapy and that treatment of these tumors could benefit from the combination of Akt ablation and hormone therapy.

We then analyzed the activities of the two downstream effectors of Akt, FOXO and mTOR. The high-grade PIN lesions showed strong cytoplasmic staining for FOXO1 and strong staining for the phosphorylated S6 (pS6) ribosomal protein, which was used to indicate mTOR activity. FOXO1 staining was confined to nuclei in *Pten*$^{+/-}$*Akt1*$^{-/-}$ prostate sections but was mostly cytoplasmic in *Pten*$^{+/-}$ lesions (Supplementary Fig. S2e), indicating that it is phosphorylated and inactivated. S6 phosphorylation was dramatically reduced in *Pten*$^{+/-}$*Akt1*$^{-/-}$ prostate sections (Supplementary Fig. S2f), suggesting that mTOR activation as well as cytoplasmic localization of FOXO1 are associated with neoplasia and proliferation induced by the activation of Akt.

To determine the relative expression of the Akt isoforms in the different prostate lobes, we conducted RT–PCR analysis of the three *Akt* genes: *Akt1* was the major expressed isoform in all three prostate lobes (Supplementary Fig. S2g). Based on this analysis of mRNA expression, we estimate that *Akt1* deficiency reduced total Akt activity by ~50%. This was further corroborated by the amounts of total and phosphorylated Akt protein in the prostates of *Akt1*$^{-/-}$ mice (Fig. 1E).

Taken together, these results demonstrate that the deficiency of *Akt1* alone is sufficient to inhibit prostate tumor development in *Pten*$^{+/-}$ mice and that even haplodeficiency of *Akt1* can significantly attenuate the development of prostate neoplasia induced by *Pten* deficiency. As *Pten* deficiency has been observed in many primary human prostate cancer tumors as well as prostate cancer cell lines, these results should have an important impact on prostate cancer therapy.

Akt1 deficiency inhibits development of endometrial carcinoma in Pten$^{+/-}$ *mice*

Mutations in and deletions of *PTEN* are the most common genetic changes occurring in endometrial carcinomas (Ellenson and Wu 2004). Female *Pten*$^{+/-}$ mice have a high incidence of endometrial neoplasia by 9 mo of age (Di Cristofano et al. 1998; Podsypanina et al. 1999). Female mice were sacrificed for analysis when they reached 40 wk of age, and uterus sections were subjected to histopathological analyses. (The development of severe lymphoid hyperplasia in female *Pten*$^{+/-}$ mice ultimately leads to their death before 1 yr of age [Di Cristofano et al. 1998; Podsypanina et al. 1999], precluding analysis of older female mice.)

Endometrial epithelial lesions were classified according to the following grades (Supplementary Fig. S3a): normal, simple hyperplasia (SH), mild atypical hyperplasia (AH1), moderate atypical hyperplasia (AH2), complex atypical hyperplasia (AH3), focal carcinoma in situ (CIS), and invasive carcinoma (CA).

In thirty-one 40-wk-old female *Pten*$^{+/-}$ mice examined, we found no mouse with a normal endometrium

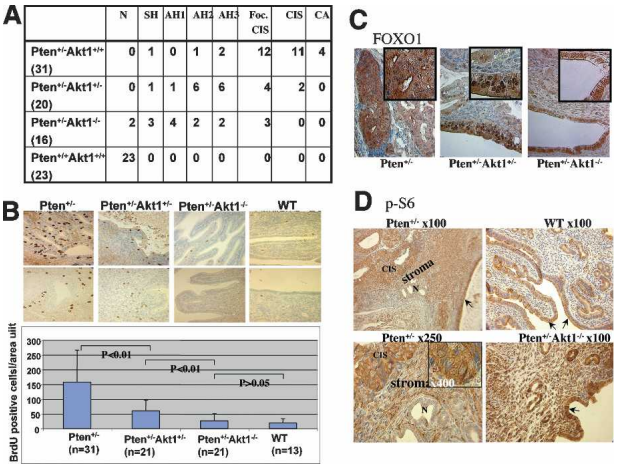

Figure 2. Akt1 deficiency is sufficient to inhibit the development of endometrial carcinoma in *Pten*$^{+/-}$ mice. (*A*) Incidence of endometrial neoplasia in *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{-/-}$, and wild-type mice. Total number of mice examined in each group is indicated in parentheses. (*B*) BrdU incorporation in the uteri of *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{-/-}$, and wild-type mice. (*Top* panels) Representative areas of BrdU-positive cells. (*C*) Localization of FOXO1 in the uteri of *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{+/-}$, and *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice. Representative sections immunostained with anti-FOXO1, showing strong cytoplasmic staining in the lesion of *Pten*$^{+/-}$ uteri, both cytoplasmic and nuclear staining in *Pten*$^{+/-}$*Akt1*$^{+/-}$ uteri, and mostly nuclear staining in *Pten*$^{+/-}$*Akt1*$^{-/-}$ uteri. (*Insets*) High magnifications. (*D*) Immunostaining with anti-pS6 of uterus sections derived from *Pten*$^{+/-}$, wild-type, and *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice. Representative sections showing strong staining of pS6 in the luminal epithelium and the lesions in *Pten*$^{+/-}$ uteri (100×, 250×, and 400×) that are confined only to the luminal epithelium (indicated by arrows) of wild-type and *Pten*$^{+/-}$*Akt1*$^{-/-}$ uteri. CIS, stroma, and a normal gland (N) are indicated.

(Fig. 2A). In most cases (74%), the highest-grade lesions were in the category of focal CIS or CIS, and in four mice (~13%) the highest grade observed was invasive carcinoma. In contrast, in 20- to 40-wk-old female *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice examined, there were no invasive carcinomas, and focal CIS or CIS occurred in only six mice (33%; Fig. 2A). Twelve *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice (60%) had only low-grade lesions in the category of AH1 and AH2. The lesion grades were further reduced in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (Fig. 2A). Two of 16 *Pten*$^{+/-}$*Akt1*$^{-/-}$ females (12.5%) had a normal endometrium, and there were no mice with CIS or invasive carcinomas. In most of the *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (~69%), the endometrium displayed only low-grade lesions (simple hyperplasia to AH3). The highest-grade lesion was focal CIS, which was observed in only three mice (~19%). Thus, the histopathological analyses clearly demonstrate that the reduction in Akt1 is directly correlated with a marked attenuation in the genesis of endometrial neoplasia and progression to endometrial carcinoma.

To establish these results further, we analyzed BrdU incorporation in the uteri of female mice with the different genotypes (Fig. 2B). BrdU incorporation was markedly elevated in *Pten*$^{+/-}$ mice when compared with wild-type mice. This elevated BrdU incorporation was significantly reduced in *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice and was further diminished in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice, as BrdU incorporation was observed mostly in CIS and invasive carcinoma.

We have quantified Akt activity in the endometrium using anti-phospho-Ser473 of Akt (Supplementary Fig.

S3b) and found that 80% of the *Pten*$^{+/-}$ mice examined displayed activation Akt in the endometrium, whereas this was markedly reduced in *Pten*$^{-/+}$*Akt1*$^{+/-}$ mice and further reduced in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice. As described above for prostate lesions, we again analyzed the activities of the two downstream effectors of Akt, FOXO1 and mTOR, in the uteri of mice with different genotypes. We found strong cytoplasmic staining of FOXO1 in high-grade lesions (AH3 and higher) (Fig. 2C; data not shown). Thus, in most lesions in *Pten*$^{+/-}$ uteri, we observed strong cytoplasmic FOXO1 staining. In most lesions in the uteri of *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice, we observed both cytoplasmic and nuclear staining of FOXO1 and, in most lesions in *Pten*$^{+/-}$*Akt1*$^{-/-}$ uteri, there was only nuclear staining of FOXO1 (Fig. 2C). Cytoplasmic staining for pS6 was confined to the luminal epithelium in wild-type mice (Fig. 2D). Strong cytoplasmic staining of pS6 was observed in high-grade lesions in *Pten*$^{+/-}$ mice, indicating hyperactivation of mTOR in the lesions. This strong cytoplasmic staining was diminished in *Pten*$^{+/-}$*Akt1*$^{-/-}$ uteri and was confined to the luminal epithelium as in wild-type mice, although we frequently observed nuclear pS6 staining in *Pten*$^{+/-}$*Akt1*$^{-/-}$ uteri (Fig. 2D).

In summary, the absence of Akt1 is sufficient to inhibit the development to endometrial carcinoma in *Pten*$^{+/-}$ mice dramatically, and even haplodeficiency of *Akt1* is sufficient to inhibit the development to endometrial carcinoma markedly. *Akt1* is the most highly expressed *Akt* isoform in the uterus (Fig. 3E; Supplementary Fig. S5), and we estimate that the complete deletion of *Akt1* could reduce total Akt activity in the endometrium by ~50%. Therefore, the *Akt1* haplodeficiency would reduce Akt activity in the endometrium by only ~25%. Thus, even a relatively small reduction in total Akt activity substantially reduces the progression to endometrial carcinoma in *Pten*$^{+/-}$ mice. As *PTEN* mutations are the most common genetic changes occurring in human endometrial carcinoma (Ellenson and Wu 2004), even partial inhibition of Akt activity by small-molecule inhibitors could be used as an efficient therapy for endometrial carcinoma.

Interestingly, we observed a significant decrease in estrogen receptor α (ERα) nuclear staining in high-grade lesions and carcinoma (Supplementary Fig. S3c). In wild-type mice, strong nuclear staining of ERα was observed only in the glands that were in the proliferative phase (Supplementary Fig. S3c, panel A), whereas this was not observed in the secretory glands (Supplementary Fig. S3c, panel B). During tumor progression, however, we observed that ERα expression is gradually diminished in the neoplastic cells (Supplementary Fig. S3c, panels C,D). This diminished nuclear staining of ERα was reciprocally correlated with the activation of Akt as measured by pAkt and pS6 staining (Supplementary Fig. S3d). Because proliferation of epithelial cells in the endometrium is dependent on estrogen receptor activity, these results suggest that *Pten* deficiency allows neoplastic cells to proliferate independently of estrogen receptor. This has implications for hormonal therapy of endometrial cancer exhibiting *Pten* deficiency.

The effect of Akt1 deficiency on the development of adrenal medullary and thyroid neoplasia, intestinal polyps, and lymphoid hyperplasia in Pten$^{+/-}$ *mice*

Consistent with previous results (Di Cristofano et al. 1998; Podsypanina et al. 1999), we found that most male *Pten*$^{+/-}$ mice sacrificed at 11 mo of age and all female *Pten*$^{+/-}$ mice sacrificed at 40 wk of age developed bilateral adrenal medullary tumors. The development of adrenal neoplasia was accelerated in female mice when compared with male mice. The size of the adrenal medulla, which was directly correlated with the size of the tumors in *Pten*$^{+/-}$ mice, was significantly decreased in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (Supplementary Fig. S4a,b). In addition, the adrenal medulla tumors in *Pten*$^{+/-}$ mice had high numbers mitotic figures; these were significantly reduced in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (data not shown). This was further corroborated by the analysis of BrdU incorporation (Fig. 3A).

In an attempt to categorize the neoplastic lesions observed in the histological analysis of the adrenal medulla, we utilized the grading criteria shown in Supplementary Figure S4b, whereas normal gland is a grade 0 and neoplastic glands were in the categories of grades 1–3. Mitotic figures and BrdU-positive cells were observed almost exclusively in grade 3 lesions. The percentage of male mice displaying grade 1 lesions was similar in both *Pten*$^{+/-}$ and *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (Fig. 3B). While the percentage of *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice displaying grade 2 lesions was slightly higher than in *Pten*$^{+/-}$ mice, the percentage of male *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice that had grade 3 lesions was >50% less than what was observed for male *Pten*$^{+/-}$ mice. The number of female *Pten*$^{+/-}$ mice that had grade 2 and 3 lesions was markedly higher than that seen in male mice. The percentage of female *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice that had grade 2 lesions was

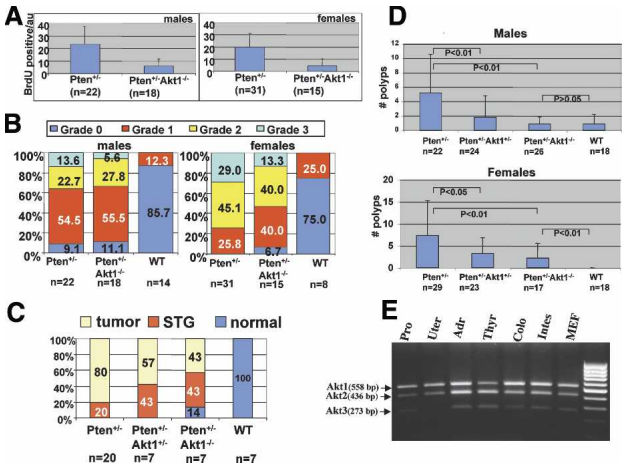

Figure 3. Effect of Akt1 deficiency on tumor development in the adrenal and thyroid glands and on the number of polyps in the small intestine of *Pten*$^{+/-}$ mice. (*A*) Quantification of BrdU incorporation in the adrenal medulla of *Pten*$^{+/-}$ and *Pten*$^{+/-}$*Akt1*$^{-/-}$ 11-mo-old male or 40-wk-old female mice. (*B*) Histopathological grades of the neoplastic lesions in adrenal medullas derived from *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{-/-}$, and wild-type 11-mo-old male or 40-wk-old female mice. (*C*) Incidence of neoplastic lesions in thyroid glands derived from *Pten*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{+/-}$, *Pten*$^{+/-}$*Akt1*$^{-/-}$, and wild-type 11-mo-old male mice. (*D*) The deficiency of *Akt1* markedly reduced the number of polyps in the small intestine of *Pten*$^{+/-}$ mice. Quantification of the number of intestinal polyps in 11-mo-old male mice (*top* panel) or 40-wk-old female mice (*bottom* panel). The number of polyps ± SD per mouse is shown. (*E*) Relative expression of *Akt1*, *Akt2*, and *Akt3* mRNAs in prostate (lane *1*), uterus (lane *2*), adrenal gland (lane *3*), thyroid (lane *4*), colon (lane *5*), intestine (lane *6*), and in mouse embryo fibroblasts (lane *7*).

similar to that observed in female *Pten*$^{+/-}$ mice, whereas the percentage of female *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice with grade 1 lesions was markedly higher, and, as was observed for male mice, female *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice had a markedly reduced incidence of grade 3 lesions (Fig. 3B). Thus, although a complete ablation of *Akt1* did not significantly reduce the total incidence of neoplastic lesions in the adrenal medulla of *Pten*$^{+/-}$ mice, it did markedly reduce the incidence of high-grade lesions. Haplodeficiency of *Akt1* did not reduce the development of neoplasia in the adrenal medulla in *Pten*$^{+/-}$ mice, because we did not observe a reduction in the incidence and grades of neoplasia in *Pten*$^{+/-}$*Akt1*$^{+/-}$ (data not shown). We also did not find a significant decrease in the size of the adrenal medulla (Supplementary Fig. S4a,b), mitotic figures, or BrdU incorporation in *Pten*$^{+/-}$*Akt1*$^{+/-}$ mice (data not shown). Overall, the deficiency of *Akt1* did not reduce tumorigenesis in the adrenal medulla to the same extent as was observed in the prostate and the endometrium. This could be attributed to the relatively lower level of *Akt1* expression (relative to *Akt2*) in the adrenal medulla, when compared with their relative expression in the prostate and uterus (Fig. 3E; Supplementary Fig. S5). Alternatively, other downstream effectors of PI3K could be more critical than Akt for the development of adrenal medulla tumors.

Pten$^{+/-}$ mice have a high frequency of neoplastic lesions in the thyroid glands (Di Cristofano et al. 1998; Podsypanina et al. 1999). We therefore analyzed the effect of *Akt1* deficiency on the development of thyroid tumors in *Pten*$^{+/-}$ mice. All *Pten*$^{+/-}$ mice developed neoplastic lesions in the thyroid (Fig. 3C). There are two different degrees of lesions. Transformed glands that tend to blend into the surrounding normal-looking thyroid follicles were defined as separated transformed glands (STG), to distinguish them from aggregated transformed glands with nodule formation, which, based on their morphology, are consistent with thyroid adenomatoid nodules or multiple adenomas. These latter lesions were defined here simply as tumors. We examined thyroid lesions in 11-mo-old male mice. In *Pten*$^{+/-}$ mice, 80% developed thyroid tumors and only 20% had STG (Fig. 3C). There was a gradual decrease in the percentage of mice with thyroid tumors, which was correlated with reduced *Akt1* expression. In contrast, the percentage of mice with lower-grade lesions (STG) was increased when *Akt1* level was reduced. When *Akt1* was completely deleted, the incidence of thyroid tumors was decreased by about twofold, and ~14% of mice were free of any neoplastic lesions in the thyroid (Fig. 3C). Thus, *Akt1* deficiency significantly reduced the incidence of thyroid tumors in *Pten*$^{+/-}$ mice, despite the localized lower level of *Akt1* expression relative to *Akt2* (Fig. 3E; Supplementary Fig. S5).

As was previously reported (Di Cristofano et al. 1998; Podsypanina et al. 1999), we found that *Pten*$^{+/-}$ mice had a relatively high number of polyps in the small intestine. These polyps seemed to be adenomatous polyps (tubular adenoma) or hamartomatous polyps, and no invasive lesions could be detected. We monitored the mice for incidence and number of polyps in the small intestine. Almost 80% of 11-mo-old male mice had polyps, and slightly more than 90% of 40-wk-old females had polyps in the small intestine. The incidence of polyps was reduced in both males and females *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice to ~60%. The number of polyps per mouse was substantially decreased in both males and females *Pten*$^{+/-}$*Akt1*$^{-/-}$ (Fig. 3D). Interestingly, even haplodeficiency of *Akt1* was sufficient to reduce the percentage of polyp-positive mice almost to the same extent as was observed with complete *Akt1* deficiency as well as the average number of polyps (Fig. 3D). Because 11-mo-old male mice spontaneously develop polyps in the small intestine (Fig. 3D), the decrease in the incidence and number of polyps observed when *Akt1* was deleted was more substantial than that observed in female mice (Fig. 3D). As estimated from the RT–PCR analysis (Fig. 3E; Supplementary Fig. S5), *Akt1* is the major *Akt* isoform expressed in the small intestine and may explain the marked reduction in the number of polyps observed when *Akt1* was deleted.

Because *Pten*$^{+/-}$ mice develop lymphoid hyperplasia and expansions of both B and T cell populations, we examined the effect of Akt1 deficiency on the proliferation in lymph nodes (Supplementary Fig. S6a) and spleen (Supplementary Fig. S6b). In agreement with previous observations (Podsypanina et al. 1999), we found an increase in BrdU incorporation in the lymph nodes and spleen of *Pten*$^{+/-}$ and this was diminished in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice to a level, which was not significantly different than in wild-type mice.

Finally, we found that ~32% of female *Pten*$^{+/-}$ mice examined (Supplementary Fig. S6c) had nodules composed of tropoblasts, which resemble placental-site tropoblastic tumors (PSTT). This was almost completely diminished in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (Supplementary Fig. S6c). In addition, we found that ~19% of female *Pten*$^{+/-}$ mice developed benign and malignant adenomyoepithelioma in the mammary glands (Supplementary Fig. S6d,e). These tumors, which were found in multiple mammary glands (up to seven large tumors in one mouse) and in multiple foci, are composed of luminal epithelium and large number of spindle and polygonal myoepithelial cells as they were stained with both anti-K14 and anti-smooth muscles antigen (SMA) (Supplementary Fig. S6d). These tumors were completely eliminated in *Pten*$^{+/-}$*Akt1*$^{-/-}$ mice (Supplementary Fig. S6e).

Concluding remarks

Here we have used a mouse model system to demonstrate that *Akt1* deficiency, which does not shorten the lifespan of mice, can markedly decrease the incidence and development of tumors in *Pten*$^{+/-}$ mice in all tissues tested, with the most dramatic inhibition occurring in the prostate, endometrium, and small intestine. In these cases, even haplodeficiency of *Akt1* was able to reduce tumorigenesis. In the prostate, endometrium, and the intestine, *Akt1* was the predominantly expressed *Akt* isoform, and its deficiency reduced total Akt activity by ~50%. Thus, haplodeficiency of *Akt1* could reduce total Akt activity by only ~25%. Even a small reduction in total Akt activity was therefore sufficient to inhibit the development of tumors induced by PTEN inactivation. Furthermore, even in the adrenal medulla and (particularly) thyroid, where *Akt1* was not the predominantly expressed isoform and Akt2 is expressed to the same or higher level of Akt1 (Fig. 3E; Supplementary Fig. S5), the reduction in tumorigenesis mediated by *Akt1* deficiency was still effective. Thus, our results show that a deficiency of *Akt1* was most effective as an inhibitor of neo-

plasia induced by PTEN inactivation. We cannot exclude the possibility, however, that Akt1 has a higher affinity than Akt2 for certain protein targets that affect tumorigenesis. It therefore remains to be determined whether *Akt2* deficiency is more effective than *Akt1* deficiency in the inhibition of adrenal medulla and thyroid tumors in $Pten^{+/-}$ mice.

Since Akt1 was ablated prior to the development of tumors in $PTEN^{+/-}$ mice, it does not completely recapitulate potential therapy to ablate Akt activity after the development of tumors. Further studies using conditional Akt1 knockout mice are required to address this issue. Nevertheless our results have important implications for cancer therapy, as they indicate that partial inhibition of Akt activity or inhibition of individual Akt isoforms could be used for cancer therapy without severe side effects. Although the three Akt isoforms are highly homologous, which makes it difficult to design specific inhibitors for the individual isoforms, it was recently reported that such specificity can be obtained using small molecule inhibitors (DeFeo-Jones et al. 2005; Lindsley et al. 2005).

Akt is very frequently activated in human cancer not only via inactivation of PTEN, which occurs at a high frequency but also through activation of Ras, amplification and activation of the catalytic subunit of PI3K, and activation of growth factor receptors (Hay 2005). Thus, Akt is activated in a large number of human cancers, and therefore using partial ablation of Akt activity as a therapeutic approach may be not limited to tumors in which PTEN is inactivated.

Materials and methods

Source of mice
The $Pten^{+/-}$ mice and $Akt1^{-/-}$ mice used in these studies were previously described (Di Cristofano et al. 1998; Chen et al. 2001).

Histology and immunocytochemistry
Histology and immunocytochemster were done as previously described (Peng et al. 2003). For details, see Supplemental Material.

Immunofluorescence staining, laser scanning confocal microscopy, immunoblotting, and RT–PCR analysis
See Supplemental Material.

BrdU incorporation assay
Mice were given i.p. injections of 0.5 mg of BrdU per 10 g of body weight for 1 h before sacrificing. Tissues were collected and processed as described above. After dewaxing and rehydration, paraffin sections were digested by pepsin followed by digestion with EcoRI and Exonuclease III. Slides were then incubated with anti-BrdU and processed for immunohistochemistry. BrdU-labeled cells were counted from five fields at 250× magnifications for prostate and uterus and 400× magnifications for adrenal medulla. All counts were taken from the highest-labeled area of all sections.

Data analysis Data were entered using Microsoft Excel spreadsheets. *P* values were calculated with a Student's *t*-test. Error bars represent the standard deviation.

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