Akt1 Ablation Inhibits, whereas Akt2 Ablation Accelerates, the Development of Mammary Adenocarcinomas in Mouse Mammary Tumor Virus (MMTV)-ErbB2/Neu and MMTV-Polyoma Middle T Transgenic Mice

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Abstract

Ample evidence to date links the phosphatidylinositol 3-kinase-regulated protein kinase Akt with the induction and progression of human cancer, including breast cancer. However, there are three Akt isoforms with limited information about their specificity during oncogenesis. This study addresses the role of the three isoforms in polyoma middle T (PyMT) and ErbB2/Neu-driven mammary adenocarcinomas in mice. The effects of ablation of Akt1, Akt2, and Akt3 on the induction and the biology of these tumors were dramatically different, with ablation of Akt1 inhibiting, ablation of Akt2 accelerating, and ablation of Akt3 having a small, not statistically significant, inhibitory effect on tumor induction by both transgenes. Whereas PyMT-induced tumors are all invasive, Akt1−/−Neu–induced tumors are more invasive than Akt2−/−Neu–induced tumors. Invasiveness, however, does not always correlate with metastasis. Ablation of individual Akt isoforms does not affect the development of the mammary gland during puberty or the expression of the transgenes. Akt ablation, therefore, influences tumor induction by modulating transgene-induced oncogenic signaling. Immunostaining for Ki-67 and cyclin D1 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays on tissue sections revealed that the delay of tumor induction in Akt1 knockout mice is due to the inhibitory effects of Akt1 ablation on cell proliferation and survival. Given that these animal models exhibit significant similarities to human breast cancer, the results of the present study may have significant translational implications because they may influence how Akt inhibitors will be used in the treatment of human cancer. [Cancer Res 2007;67(1):167–77]

Introduction

ErbB2/Neu is a member of the epidermal growth factor (EGF) receptor family that is overexpressed in approximately one-third of all the cases of human breast cancer. Overexpression of Neu activates multiple signaling pathways and is associated with poor prognosis (1). One of the pathways activated by Neu is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (2). The same pathway is also activated by polyoma middle T (PyMT), a viral oncogene (3). Expression of either Neu or PyMT in the mammary gland of transgenic mice from mouse mammary tumor virus long terminal repeat (MMTV LTR)–driven transgenes causes mammary adenocarcinomas (4, 5).

Akt is a serine-threonine protein kinase that is activated by a variety of stimuli via PI3K-dependent mechanisms (6–11). Being an effector of PI3K, Akt regulates a variety of cellular functions, including survival, proliferation, migration, and intermediary metabolism (7, 11–14). There are three Akt isoforms, which are very similar in sequence and which seem to be regulated similarly and to have overlapping functions. However, in animal studies, the ablation of individual Akt isoforms causes distinct phenotypes. Akt1−/− mice are small and exhibit perinatal lethality (15, 16), Akt2−/− mice develop insulin-resistant diabetes (17, 18), and Akt3−/− mice have small brains and exhibit mild neurologic defects (19, 20). Moreover, whereas Akt2−/−/Akt3−/− mice are viable and fertile, Akt1−/−/Akt2−/− mice die perinatally with severe musculoskeletal and adipose tissue defects (21) and Akt1−/−/Akt3−/− mice die at embryonic day 10.5 to embryonic day 11.5 with severe defects in the cardiovascular and nervous systems (22).

A recent study addressing the specificity of Akt isoforms showed that knocking down Akt1 activates extracellular signal-regulated kinase and promotes the induction of epithelial-mesenchymal transition (EMT) by insulin-like growth factor-I (IGF-I) or EGF in immortalized human mammary epithelial cells in culture. Knocking down Akt2 on the other hand reversed the prosurvival and proliferative effects of IGF-I, suppressed EMT induced by knocking down Akt1 in IGF-1 receptor–overexpressing cells, and inhibited the migration of EGF-stimulated cells (23). These data suggested that Akt1 may selectively inhibit, whereas Akt2 may selectively enhance, the migration of immortalized mammary epithelial cells in culture. This conclusion is supported by the results of two additional recent studies. One of these studies showed that ectopic expression of constitutively active Akt1 reduces the expression of nuclear factor of activated T cells and cyclooxygenase-2 and inhibits the motility of breast cancer cells, whereas down-regulation of Akt1 by transfection of small interfering RNA has the opposite effect (24, 25). The other study showed that overexpression of the activated form of Akt1, which phosphorylates TSC2 (26), promotes TSC2 degradation, leading to the reduction of RhoGTPase activity and cell motility and invasiveness (27). The preceding studies were carried out in cultured cells. However, the effects of Akt1 in cell migration and invasiveness agreed with the results of earlier studies addressing the effects of a constitutively active Akt1 T308D/S473D (Akt1D/D) transgene on mammary oncogenesis by a mutant PyMT decoupled from the PI3K (28) or by activated Neu (29). The results of the
former study showed that Akt1<sup>D/D</sup> compliments the mutant PyMT in tumor induction and tumor growth but not in cell invasiveness and metastasis (28). The results of the latter study showed that Akt1<sup>D/D</sup> synergizes with Neu also in tumor induction and tumor growth (29). However, the tumors developing in double transgenic mice were less invasive and less metastatic, suggesting that activated Akt1 inhibits tumor invasiveness and metastasis.

The present report addresses the specificity of Akt isoforms by focusing on the effects of their ablation on PyMT- and Neu-driven mammary oncogenesis in mice. The MMTV-Neu (5) and the MMTV-PyMT (4) transgenes were crossed into the Akt<sup>1<sup>-/-</sup></sup>, Akt<sup>2<sup>-/-</sup></sup>, and Akt<sup>3<sup>-/-</sup></sup> genetic backgrounds, and the resulting transgenic/wild-type (wt) mice and transgenic/Akt<sup>1<sup>-/-</sup></sup>, Akt<sup>2<sup>-/-</sup></sup>, or Akt<sup>3<sup>-/-</sup></sup> littersmates were monitored for the development of mammary adenocarcinomas. The results showed that ablation of Akt1 inhibited cell proliferation and survival and delayed tumor induction by both transgenes. This effect was unique for the ablation of Akt1 because ablation of Akt2 accelerated whereas ablation of Akt3 had a small, not statistically significant inhibitory effect on Neu- and PyMT-induced oncogenesis.

Overall, the data presented in this report agree with the results of earlier studies suggesting that Akt1 may inhibit, whereas Akt2 may enhance, the local invasiveness of breast cancer cells. However, in the context of the complex environment of the whole animal, Akt1 promotes, whereas Akt2 inhibits, tumor induction and tumor growth, an outcome that could not have been predicted from the results of the earlier in vitro studies. The present study provides clues on the role of individual Akt isoforms in mammary oncogenesis and may therefore influence how Akt inhibitors will be used for the treatment of breast cancer.

**Materials and Methods**

Mice. Akt1<sup>-/-</sup> mice have been described previously, Akt2<sup>-/-</sup> mice were provided by Dr. M. Birnbaum (University of Pennsylvania, Philadelphia, PA), and Akt3<sup>-/-</sup> mice were provided by Dr. Thomas Ludwig (Columbia University, New York, NY; refs. 17, 19, 20). MMTV-c-Neu mice (5) were purchased from Charles River Laboratories (Wilmington, MA), and MMTV-PyMT mice (4) were purchased from The Jackson Laboratory (Bar Harbor, ME). PyMT and MMTV-Neu transgenic mice were crossed with Akt1<sup>1<sup>-/-</sup></sup>, Akt2<sup>2<sup>-/-</sup></sup>, or Akt3<sup>3<sup>-/-</sup></sup> animals. Following the breeding strategy described in Supplementary Data, we established heterozygous MMTV-PyMT and homozygous MMTV-Neu transgenic mice in the wt, Akt1<sup>1<sup>-/-</sup></sup>, Akt2<sup>2<sup>-/-</sup></sup>, and Akt3<sup>3<sup>-/-</sup></sup> genetic backgrounds. Experimental and control virgin mice were monitored twice weekly by palpation for tumor induction.

Whole mounts of the mammary gland and histology. The left inguinal and thoracic mammary glands were dissected, and whole mounts were prepared as described (30). Tissues were paraffin embedded, sectioned, and stained with H&E. A pathologist (S.P.N.) analyzed the histologic sections blindly. The code was broken only after all the data were compiled.

Immunohistochemistry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays. To identify cells expressing Neu, or Ki-67, formalin-fixed mammary gland sections were immunostained with the anti-Neu rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or with the anti-Ki-67 rabbit monoclonal antibody SP1 (NeoMarkers, Fremont, CA). To address the expression of cyclin D1, formalin-fixed tissue sections were stained with an anti-cyclin D1 rabbit monoclonal antibody (NeoMarkers), and terminal deoxynucleotidyl trans-ferase–mediated dUTP nick end labeling (TUNEL) staining was carried out on formalin-fixed, paraffin-embedded sections with the ApopTag kit (InterGen, Purchase, NY).

**Results**

All three Akt isoforms are expressed in the mammary gland. Before addressing the role of individual Akt isoforms in mammary oncogenesis, we wanted to determine their relative expression in the mammary gland. To this end, Western blots of total cell lysates of mammary tissue from adult 10-week-old wt, Akt1<sup>1<sup>-/-</sup></sup>, Akt2<sup>2<sup>-/-</sup></sup>, and Akt3<sup>3<sup>-/-</sup></sup> mice were probed with antibodies specific for Akt1, Akt2, and Akt3. The results showed that all Akt isoforms are expressed at easily detectable levels in the mammary gland. Mammary glands of Akt<sup>1<sup>-/-</sup></sup>, Akt<sup>2<sup>-/-</sup></sup>, and Akt<sup>3<sup>-/-</sup></sup> females do not express Akt1, Akt2, or Akt3, respectively, but express normal levels of the remaining Akt isoforms (Fig. 1A).

Akt1 ablation significantly delays, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in MMTV-ErbB2/Neu and MMTV-PyMT transgenic mice. To address the role of the three Akt isoforms in mammary oncogenesis, we crossed the Neu and the PyMT transgenes into the Akt<sup>1<sup>-/-</sup></sup>, Akt<sup>2<sup>-/-</sup></sup>, and Akt<sup>3<sup>-/-</sup></sup> genetic backgrounds. The Kaplan-Meier curves in Fig. 1B show the timing of tumor detection for each mouse strain. The results of this experiment were remarkable in that they showed that Akt1 ablation inhibits, whereas Akt2 ablation accelerates, tumor induction by both transgenes. Akt3 ablation seems to also slightly inhibit tumor induction. However, as of to date, the difference between wt and Akt3<sup>-/-</sup> mice has not reached statistical significance (P = 0.086 for the PyMT transgenic mice and P = 0.1089 for the Neu transgenic mice; Fig. 1B).

Akt1 is up-regulated, whereas Akt2 is down-regulated, in tumors arising in MMTV-ErbB2/Neu and MMTV-PyMT transgenic mice. If ablation of Akt1 inhibits tumor development in Neu and PyMT transgenic mice, one would predict that mammary adenocarcinomas developing in Akt wt transgenic mice would express Akt1. To address this question, we probed Western blots of tumors arising in Neu and PyMT transgenic, Akt<sup>1<sup>-/-</sup></sup>, and Akt1<sup>1<sup>-/-</sup></sup>, Akt2<sup>2<sup>-/-</sup></sup>, and Akt3<sup>3<sup>-/-</sup></sup> mice with antibodies specific for the three Akt isoforms. The normal mammary gland of a 10-week-old virgin wt mouse was used as a control. The results showed that in PyMT- and Neu-induced tumors Akt1 and Akt3 were slightly up-regulated, whereas Akt2 was down-regulated. However, the down-regulation of Akt2 was more pronounced in tumors arising in Akt wt than in Akt1<sup>1<sup>-/-</sup></sup> or Akt3<sup>3<sup>-/-</sup></sup> mice, suggesting that Akt2 expression may be modulated to compensate for the loss of Akt1 or Akt3 (Fig. 1C; Supplementary Fig. S1). The results of this experiment are consistent with the hypothesis that Akt1, which promotes tumor induction (Fig. 1B), may also be required for tumor growth.

Because mammary adenocarcinomas are epithelial neoplasms, the high levels of Akt1 and the low levels of Akt2 in tumors arising

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3 C. Mao et al. Unequal contribution of Akt isoforms in the DN to DP thymocyte transition, submitted for publication.
Akt Isoforms in Mammary Oncogenesis

Figure 1. Akt1 ablation significantly delays, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in PyMT (MMTV-PyMT) and Neu (MMTV-Neu) transgenic mice. Expression of Akt isoforms in the normal and neoplastic mammary gland. A, all three Akt isoforms are expressed in the mammary gland. Western blots of lysates derived from mammary glands of virgin wt, Akt1+/−, Akt2−/−, and Akt3−/− female mice were probed with isoform-specific antibodies for Akt1, Akt2, and Akt3 or with an anti-α-tubulin antibody. B, Kaplan-Meier curves showing the latency of tumor induction in wt, Akt1+/−, Akt2−/−, and Akt3−/− mice carrying the PyMT or the Neu transgene. Virgin transgenic mice carrying two copies of the Neu transgene, or a single copy of the PyMT transgene, were observed for up to 65 wks. The age of tumor induction for each animal was that at which the tumor was first palpable. n, number of mice analyzed for each strain. Because this experiment was carried out in a mixed genetic background, the control mice for each transgenic/Akt−/− strain were the Akt+/+ littermates of the experimental mice. Representative Western blots of lysates derived from mammary glands of virgin wt mice and from mammary tumors developing in PyMT or Neu transgenic mice in the Akt+−, Akt2−/−, and Akt3−/− genetic backgrounds. The blots were probed with isoform-specific antibodies for Akt1, Akt2, Akt3 and with an anti-α-actin antibody. Quantitative data on the expression of Akt1 and Akt2, combining the results of three experiments, are shown in Supplementary Fig. S1. D, Akt2 is expressed primarily in the stroma of the normal mammary gland. Total wt mammary gland lysates were electrophoresed in parallel with lysates of primary cultures of wt mammary epithelia. Western blots of the lysates were probed with anti-Akt1, anti-Akt2, anti-Akt3, and anti-α-actin antibodies. The total mammary gland and the epithelial cell lysates probed with each antibody had been electrophoresed in the same gel.
suggesting that Akt1 and Akt3 are expressed in both the epithelia and the stroma. However, the expression of Akt2 in the epithelium was very low, suggesting that Akt2 is expressed primarily in the stroma (Fig. 1D). These findings suggest that Akt1 may have a selective advantage during oncogenesis. The low levels of Akt2 on the other hand may reflect the cellular pattern of expression of this isoform.

**Histopathology and invasive and metastatic potential of mammary adenocarcinomas developing in wt, Akt1<sup>-/-</sup>, Akt2<sup>-/-</sup>, and Akt3<sup>-/-</sup> mice.** The data in Fig. 2 and Table 1 suggest that ablation of different Akt isoforms causes different selective pressures that may ultimately affect the histopathology of the developing tumors. However, overall, the tumors developing in mice with different Akt backgrounds exhibit significant overlaps in histopathology (Fig. 2A and B; Table 1; see figure legend for details). All tumor-bearing animals were analyzed for metastatic foci in the lung. Pulmonary metastases of PyMT-driven adenocarcinomas were almost universally parenchymal, whereas metastatic foci of Neu-driven adenocarcinomas were primarily intravascular (Fig. 2A, A2 and Fig. 2B, B2; Table 1). A histopathologic feature that should be highlighted here is the invasiveness of Neu-induced tumors, which is apparently slightly enhanced in tumors developing in Akt1<sup>-/-</sup> mice.
Akt1 does not inhibit the expression of MMTV promoter-driven transgenes. Transgenes driven by the MMTV promoter are induced in the mammary gland stochastically during puberty. Mutations that interfere with the induction of transgenes that encode oncogenic proteins would be expected to inhibit the oncogenic potential of these transgenes. To determine whether the ablation of Akt1 interferes with the induction of MMTV LTR-driven transgenes, we used immunohistochemistry and semiquantitative RT-PCR to examine the expression of Neu in three 10- to 12-week-old Neu/wt mice and in three Neu/Akt1−/− mice. Immunohistochemical slides were analyzed for the number of positive cells in four randomly selected fields from each mouse. A representative sample of the immunohistochemical data (Fig. 3C) shows that the ablation of Akt1 does not affect the induction of the transgene. The cumulative number of Neu-positive cells in the four randomly selected fields (Fig. 3D, left) and RT-PCR-based analyses (Fig. 3D, right) comparing Neu/Akt1wt and Neu/Akt1−/− mammary gland tissues for the expression of the MMTV-Neu transgene confirmed this conclusion.

The data in Fig. 3 show that mutations in any of the three Akt isoforms do not affect mammary gland development or the expression of MMTV LTR-driven transgenes. We therefore conclude that the profound effects of mutation of individual Akt isoforms on tumor induction by Neu or PyMT are due to changes in the transduction of Neu- or PyMT-induced oncogenic signals.

The ablation of Akt1 is associated with a decrease in the number and size of hyperplastic and transformed foci in the mammary gland of MMTV-PyMT transgenic mice. The tumor incidence in Neu/Akt1−/− mice is very low with only 3 mice out of a group of 18 developing detectable tumors after 65 weeks of observation. Because of this, and because the effects of Akt1 ablation on tumor incidence and latency in Neu- and PyMT-driven tumors are similar, almost all subsequent experiments addressing the mechanism by which Akt1 ablation interferes with tumor

### Table 1. Histopathology and metastatic potential of MMTV-PyMT and MMTV-ErbB2/Neu mammary adenocarcinomas developing in wt, Akt1−/−, Akt2−/−, and Akt3−/− mice

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<td>Akt1−/−</td>
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<td>Fibrosis</td>
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<td>Invasion</td>
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<td>Metastasis</td>
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NOTE: For PyMT: All the tumors arising in PyMT/wt and PyMT/Akt3−/− mice were characterized by a predominantly solid growth pattern, interspersed with areas of papillary, glandular, and cribriform growth in some of the mice. Only some of the tumors arising in PyMT/Akt1−/− and PyMT/Akt2−/− mice on the other hand exhibited a solid growth pattern. All the tumors arising in PyMT/Akt2−/− mice exhibited papillary growth. PyMT/Akt1−/− induced tumors tended to exhibit a more malignant histologic grade than PyMT-induced tumors arising in other genetic backgrounds. The tumors arising in both the wt and the Akt knockout mice were all invasive. However, invasiveness did not correlate with metastasis in that PyMT/Akt1−/− and PyMT/Akt2−/− tumors were significantly less metastatic (P < 0.0021 and P < 0.00228, respectively). Serial sections of the lungs of tumor-bearing mice sacrificed when their primary tumors approached 2 cm in diameter. For Neu: Mammary tumors arising in Neu/wt mice were shown to exhibit a predominantly solid growth pattern, and their histologic grade was evenly divided between grades 2 and 3. Papillary differentiation was noted only in the tumors arising in Neu/Akt2−/− mice, which exhibited a distinct solid papillary growth pattern. The differences in the frequency of invasiveness between Neu-induced tumors in wt and Akt2−/− or Akt3−/− mice were not statistically significant. Interestingly, at the time of this report, only three Neu/Akt1−/− mice had developed tumors and all three of them were invasive, suggesting that tumors arising in mice lacking Akt1 may be more invasive than tumors arising in mice of all other genetic backgrounds (P < 0.0350). Because we had only three Akt1−/− tumors to date, we cannot exclude the possibility that ablation of Akt1 in the Neu model may also increase the rate of metastasis.

The ablation of Akt1, Akt2, or Akt3 does not interfere with mammary ductal outgrowth. Resistance of mutant mice to the oncogenic potential of transforming genes could be the result of developmental defects that eliminate or limit the number of available target cells. To determine whether Akt1 ablation may affect the target cell number, we examined whether it is required for mammary gland development. To this end, we examined the mammary gland architecture of 10-week-old, wt, Akt1−/−, Akt2−/−, and Akt3−/− mice (four mice per group) using whole-mount analysis. The results revealed no differences in ductal architecture between the groups. A representative sample of the results is shown in Fig. 3A.

Similar analyses were carried out on 6- to 8-week-old PyMT and on 10- to 12-week-old Neu transgenic, Akt wt, Akt1−/−, and Akt2−/− mice. Analysis of four mice from each group revealed again no differences in the branching morphology and the ductal outgrowth between wt and Akt knockout mice carrying either of the two transgenes (Fig. 3B).
induction were carried out on the PyMT transgenic model. The ablation of Akt1 may interfere with tumor induction by suppressing the rate of appearance of transformed foci, by inhibiting their rate of growth, or both. To distinguish between these possibilities, we compared whole mounts of the mammary gland of 8- and 12-week-old PyMT/wt and PyMT/Akt1−/− mice and 30-week-old PyMT/Akt1−/− mice (three mice per group). This analysis revealed that, at early time points, PyMT/Akt1−/− mice harbor fewer and smaller

Figure 3. The ablation of Akt1, Akt2, or Akt3 does not interfere with mammary ductal outgrowth or with the expression of MMTV-driven transgenes. A, mammary gland whole mounts were prepared from virgin wt, Akt1−/−, Akt2−/−, and Akt3−/− mice at 10 wks of age. The mammary epithelial tree was visualized by carmine red staining. B, the branching morphology and the ductal outgrowth in young PyMT or Neu, Akt wt, Akt1−/−, and Akt2−/− mice are indistinguishable. The mammary epithelial tree was visualized with carmine red staining as above. C, mammary gland tissue sections of 12-week-old wt and Akt1−/− mice carrying the MMTV-Neu transgene were immunostained with an anti-Neu antibody. Negative control mammary gland tissue sections derived from the same Neu/wt mice were stained only with the secondary antibody. D, left, ablation of Akt1 does not inhibit the expression of MMTV-driven transgenes. Graphical representation of the cumulative data obtained from the experiment shown in (C). The bars show the mean percentage of positive cells ± the standard error of the mean in tissue sections of mammary glands derived from three mice in each group. The number of positive cells in each mouse was obtained by scoring for such cells in four randomly selected fields per mammary gland. Right, ablation of Akt1 does not inhibit the expression of the MMTV-driven Neu transgene. Neu expression was analyzed by RT-PCR using pooled mammary gland RNAs derived from four animals of each of the indicated genotypes. β-Actin was used as an internal loading control.
hyperplastic foci than PyMT/wt mice. At subsequent time points, the number of foci in the Akt1−/− mice was increased, but their size continued to be smaller (Fig. 4A). The small size of hyperplastic foci in PyMT/Akt1−/− mice suggested that the ablation of Akt1 might interfere with the transduction of transgene-generated signals that control growth. Their small number in the early stages of oncogenesis may also be a reflection of their slow growth. Foci that grow slowly may indeed be difficult to detect early because it may take them longer to grow to a detectable size. However, the small number of hyperplastic foci in PyMT/Akt1−/− mice may also be interpreted to suggest that ablation of Akt1 inhibits genetic and/or epigenetic events involved in tumor initiation.

The ablation of Akt1 interferes with mammary tumor growth in MMTV-PyMT and MMTV-ErbB2/Neu transgenic mice. To directly address the effects of ablation of individual Akt isoforms on the growth of mammary tumors developing in PyMT and Neu transgenic mice, wt and Akt1, Akt2, and Akt3 knockout animals carrying these transgenes were monitored for tumor growth starting immediately after the tumors were first detected. To monitor the tumor growth, we measured the diameter of the tumor weekly using a caliper. The results agreed with the results of the preceding experiment in that they showed that the number of mammary epithelial cells expressing cyclin D1 in Akt1−/− mice in the early stages of neoplastic development was significantly lower than in WT mice. The ablation of Akt1 is associated with a decrease in the number and size of hyperplastic and transformed foci in the mammary gland of PyMT transgenic mice. Representative mammary gland whole mounts from 8-week-old PyMT/WT and PyMT/Akt1−/− mice were stained for the Ki-67 proliferation marker and they were compared with similarly stained lesions of mammary gland tissue sections of three 8-week-old and three 12-week-old PyMT/wt mice, showing that the number of mammary epithelial cells expressing cyclin D1 in Akt1−/− mice is lower than in WT mice (see figure legend for details).

Figure 4. The ablation of Akt1 inhibits tumor growth in MMTV-PyMT and MMTV-Neu transgenic mice. A, ablation of Akt1 is associated with a decrease in the number and size of hyperplastic and transformed foci in the mammary gland of PyMT transgenic mice. Representative mammary gland whole mounts from 8- and 12-week-old PyMT transgenic, Akt1−/−, and Akt wt mice. Representative mammary gland whole mount from a 30-week-old PyMT/Akt1−/− mouse. At 30 weeks of age, all the PyMT/wt mice had died with rapidly growing and highly metastatic mammary adenocarcinomas. Note that the hyperplastic lesions were larger in 12-week-old wt mice than in 12-week-old Akt1−/− mice. Mammary gland whole mounts were prepared from three 8-week-old littermate pairs, three 12-week-old littermate pairs, and three 30-week-old old mice. B, left, ablation of Akt1 inhibits and ablation of Akt2 accelerates the growth of mammary adenocarcinomas in PyMT transgenic mice; right, ablation of Akt1 inhibits the growth of mammary adenocarcinomas in Neu transgenic mice. All tumors exhibit approximately the same minimal size at the time of diagnosis (time 0). The slopes of the curves indicate the average rate of growth of tumors arising in mice of the indicated Akt genotypes. The size differences between wt and Akt1−/− or wt and Akt2−/−PyMT-induced tumors at 4 weeks were statistically significant (P < 0.0069 and P < 0.0049, respectively). The size differences between Neu-induced tumors arising in Akt2−/− mice and Akt1−/− mice at 4 and 6 weeks were also statistically significant (P < 0.0046 and P < 0.0396, respectively). For Neu-induced tumors arising in Akt2−/− mice, we do not currently have sufficient data to reliably plot their growth over time. Please note that all comparisons were made between tumors arising in wt and Akt1−/− or wt and Akt2−/− mice. Comparisons between tumors arising in Akt1−/− and Akt2−/− mice would be difficult to interpret because of the different genetic backgrounds of these mice.


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stages of oncogenesis is lower than in the wt mice (Fig. 5B). Interestingly, comparison of the preneoplastic and early neoplastic lesions of 6-week-old PyMT/wt and PyMT/Akt2−/− mice revealed a higher number of cyclin D1–expressing cells in lesions from mice lacking Akt2 (Fig. 5B, right).

The preceding data addressed the role of Akt1 and Akt2 in the expression of the proliferation marker Ki-67 and/or the expression of cyclin D1 in the early stages of oncogenesis in PyMT transgenic mice. To determine the role of Akt1 in the expression of these markers in established tumors, we stained advanced mammary adenocarcinomas derived from 3-week-old PyMT/wt and three 27-week-old PyMT/Akt1−/− mice for either Ki-67 or cyclin D1. The results showed that the expression of both markers may be slightly higher in advanced tumors developing in wt as opposed to Akt1 knockout mice. However, the observed differences were not statistically significant at this stage (Fig. 5C). Therefore, tumor cells of advanced PyMT/Akt1−/− tumors adapt to the loss of Akt1 and proliferate at rates that are comparable with the proliferation rates of tumor cells of advanced PyMT/wt tumors.

Akt1 ablation promotes apoptosis of mammary epithelia in MMTV-PyMT transgenic mice. To address the role of Akt1 in cell survival in hyperplastic and early neoplastic foci developing in PyMT transgenic mice, mammary glands of 3-week-old and 32-week-old PyMT/wt and PyMT/Akt2−/− mice were analyzed for apoptosis using the TUNEL assay. This analysis detected a larger than 2-fold increase in the number of apoptotic cells in hyperplastic and early neoplastic lesions in Akt1−/− mice (Fig. 6A, left and B). Similar observations were made when we analyzed advanced tumors from the same category of mice (Fig. 6A, right and B). The analysis of the advanced tumors also revealed foci of apoptotic cell clusters, which were limited to the tumors developing in Akt1 knockout mice (Fig. 6C).
These observations suggest that ablation of Akt1 may limit the rate of growth of preneoplastic lesions and established tumors by inhibiting both cell proliferation and cell survival.

**Discussion**

The biological specificity of individual Akt isoforms is a question of major importance in cancer research. Experiments presented in this report addressed this question in the context of Neu- and PyMT-induced mammary oncogenesis in mice. The results revealed dramatic differences between isoforms. Thus, whereas ablation of Akt1 inhibited, ablation of Akt2 accelerated tumor development by both oncogenes. Ablation of Akt3 had a small, not statistically significant, protective effect on tumor induction. In agreement with these data, tumors arising in wt mice express high levels of Akt1 and low levels of Akt2.

There was overlap in histopathology between Neu-induced and PyMT-induced tumors arising in wt and Akt1/C0/C0, Akt2/C0/C0, and Akt3/C0/C0 mice. However, tumors arising in mice of different Akt-deficient genetic backgrounds tended to exhibit trends toward different histopathologic patterns. The histopathologic trends observed in transgenic mice lacking different Akt isoforms suggest that the ablation of different isoforms may generate isoform-specific selective pressures that promote the development of biologically distinct neoplasms. However, the biological boundaries between the neoplasms arising in Akt1−/−, Akt2−/−, and Akt3−/− mice were not clearly defined.

The development of the mammary gland during puberty in all Akt knockout mice was indistinguishable from the development of the mammary gland in wt mice. Moreover, the expression of the transgene was not affected by the ablation of Akt1, Akt2, or Akt3. These data combined, suggest that the observed differences in Neu- or PyMT-induced tumorigenesis in Akt1−/−, Akt2−/−, and Akt3−/− mice are due to differences in the transduction of Neu and PyMT oncogenic signals among the three Akt isoforms.

Akt1 ablation delays the induction of detectable mammary tumors in both the Neu and the PyMT transgenic mice. This may be interpreted to suggest that the ablation of Akt1 inhibits tumor initiation. Another interpretation, which does not exclude the preceding one, is that it inhibits tumor growth and that the delayed detection may be due to the slow growth of preneoplastic and early neoplastic foci. The observation that the ablation of Akt1 correlates with a decrease in the size of hyperplastic and transformed foci in the mammary gland of PyMT transgenic mice provided support for the latter hypothesis. Experiments addressing the differences in the rate of growth of tumors developing in wt and Akt1 knockout mice confirmed this hypothesis and suggested that the ablation of Akt1 may also inhibit the growth of established tumors. The growth inhibition of established tumors caused by the ablation of Akt1

![Figure 6](image_url)

**Figure 6.** Akt1 ablation promotes apoptosis of mammary epithelia in MMTV-PyMT transgenic mice. A, apoptotic cells in hyperplastic and early neoplastic lesions and advanced neoplasms developing in PyMT/wt and PyMT/Akt1−/− mice were detected with the TUNEL assay in a panel of slide-mounted, methyl green-stained mammary tissue sections. The TUNEL assay uses horseradish peroxidase-conjugated anti-digoxigenin antibodies to detect digoxigenin-labeled DNA ends. B, cumulative data derived from the experiments shown in A. The bars show the mean percentage ± SE of the mean of TUNEL-positive cells in early lesions and in advanced neoplasms of PyMT/wt and PyMT/Akt1−/− mice. The number of TUNEL-positive apoptotic cells was measured in sections of mammary glands derived from three mice of each genotype (four randomly selected fields per mouse). C, advanced neoplasms developing in Akt1−/− mice exhibit foci of apoptotic cell clusters. Apoptosis was again detected by the TUNEL assay.
suggests that inhibition of Akt1 may not only have a protective effect in tumor initiation but that it may also have a therapeutic effect in growing tumors.

Evidence presented in this report showed that the dramatic delay in tumorigenesis induced by the ablation of Akt1 is due to the inhibition of cell proliferation and to the promotion of apoptosis caused by the loss of Akt1-transduced signals. In addition to the scattered apoptotic cells, tumors arising in Akt1 knockout mice were shown to also contain focal areas of enhanced apoptosis, which may develop because of hypoxia in sections of the developing tumor. The more frequent appearance of these apoptotic areas in tumors arising in Akt1 knockout mice could be the result of either impaired angiogenesis or enhanced apoptosis in hypoxic areas of the Akt1−/− tumors.

The effects of ablation of individual Akt isoforms in oncogenesis by Neu and PyMT could be either cell autonomous or stroma dependent. Although we have not yet addressed this question directly, our data show that Akt2 is primarily expressed in the stroma of the mammary gland. This suggests that the ablation of Akt2 may accelerate tumor induction and may enhance tumor growth by changing the expression of stroma-derived molecules that regulate the growth of the mammary epithelium. This hypothesis will be addressed directly in future studies.

The ablation of individual Akt isoforms modulates differentially the local invasiveness and metastatic potential of Neu- and PyMT-induced mammary tumors. Thus, Neu-induced tumors seem to exhibit different degrees of invasiveness depending on the Akt genotype of the mouse. Interestingly, the tumors arising in Akt1 knockout mice exhibit the highest degree of invasiveness (P < 0.0350). This agrees with the results of earlier studies on the role of individual Akt isoforms in cell migration in culture (23–25, 27) as well as with the results of earlier studies addressing the ability of constitutively active Akt1 (Akt1D/D) to compliment a mutant PyMT transgene that is uncoupled from PI3K (28) and to synergize with an activated Neu transgene (29). PyMT-induced tumors differ from Neu-induced tumors in that they are all locally invasive independent of the Akt genotype of the mouse. The frequency of distant metastases, however, is significantly lower in tumors arising in Akt1 and Akt2 knockout mice. The uncoupling of the two processes in mice of different genetic backgrounds suggests that the two may be functionally distinct.

The earlier studies on the role of different Akt isoforms on cell migration in culture had been interpreted to suggest that inhibition of Akt1 may be undesirable because it may increase the invasiveness and metastatic potential of tumor cells. Our data agree with the observation that ablation of Akt1 may increase the invasiveness of tumor cells. However, they show that the increased invasiveness does not always correlate with an increase in metastatic potential. Given that the Akt1−/− tumor cells survive poorly and proliferate slowly in the primary site, the decreased metastatic potential of these tumors may be due to the fact that Akt1−/− tumor cells may not survive or they may grow slowly following their transport into a metastatic site. Consequently, inhibition of Akt1 may protect animals harboring Neu- and PyMT-induced mammary adenocarcinomas despite the fact that Akt1 activity inhibits tumor invasiveness, whereas Akt2 activity may have the opposite effect.

Despite the similarities of some of the data generated by the earlier in vitro studies and the studies presented here, the full range of our data led us to opposite conclusions about the likely therapeutic potential of inhibition of different Akt isoforms. The difference in data interpretation between the present in vivo study and the earlier in vitro studies is due, at least in part, to the nature of the present study, which extends the biological variables that could be affected by the ablation of Akt1, Akt2, and Akt3. Another possibility is that the regulation of different Akt isoforms and the ultimate consequences of Akt signals in tumors growing in animals depend on the integration of complex autocrine and paracrine stimuli that may be missing in the in vitro systems. These systems indeed lack the full range of interactions between the stroma and the epithelial cells, they are devoid of the immune system, and they lack hormonal and other factors that function at a distance from the site they are produced.

The hypothesis that Akt2 activity may promote whereas Akt1 activity may inhibit Neu- and PyMT-driven tumorigenesis, which was suggested by the earlier studies, seemed to be supported by clinicopathologic data showing that Akt2 is frequently overexpressed in human breast cancer (32). However, although Akt2 may be overexpressed more frequently than Akt1, mutations enhancing the cellular levels of D3 phosphorylated phosphoinositides, which are common in human breast cancer, activate not only Akt2 but also Akt1 and Akt3. More important, a recent large study on 402 estrogen receptor-α–positive breast carcinomas revealed that overexpression of Akt2 is associated with a lower rather than a higher rate of relapse in the course of tamoxifen treatment (33). The latter observation agrees with the data presented in this report because it suggests that Akt2 activity may have a protective effect in breast cancer.

Further confirmation of the in vivo animal data presented in this report, in human breast cancer, will have significant translational implications because it will dramatically affect how Akt inhibitors may be used for the treatment of breast cancer.

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Akt Isoforms in Mammary Oncogenesis

Akt1 Ablation Inhibits, whereas Akt2 Ablation Accelerates, the Development of Mammary Adenocarcinomas in Mouse Mammary Tumor Virus (MMTV)-ErbB2/Neu and MMTV-Polyoma Middle T Transgenic Mice

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