Akt1 and Akt2 Play Distinct Roles in the Initiation and Metastatic Phases of Mammary Tumor Progression

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Abstract

The phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway is often dysregulated in cancer. Our previous studies have shown that coexpression of activated Akt1 with activated ErbB2 or poloma virus middle T antigen uncoupled from the PI3K pathway (PyVmT Y315/322F) accelerates mammary tumor development but cannot rescue the metastatic phenotype associated with these models. Here, we report the generation of transgenic mice expressing activated Akt2 in the mammary epithelium. Like the mouse mammary tumor virus–Akt1 strain, mammary-specific expression of Akt2 delayed mammary gland involution. However, in contrast to Akt1, coexpression of Akt2 with activated ErbB2 or PyVmT Y315/322F in the mammary glands of transgenic mice did not affect the latency of tumor development. Strikingly, Akt2 coexpression markedly increased the incidence of pulmonary metastases in both tumor models, demonstrating a unique role in tumor progression. Together, these observations argue that these highly conserved kinases have distinct biological and biochemical outputs that play opposing roles in mammary tumor induction and metastasis. [Cancer Res 2009;69(12):5057–64]

Introduction

The Akt family of serine/threonine kinases consists of three members: Akt1, Akt2, and Akt3. Akt has been implicated in a number of cellular processes, including proliferation, cell survival, and metabolism (reviewed in ref. 1). Several studies have shown that expression of Akt1 in the mammary epithelium, while incapable of inducing mammary tumors, results in a profound involution defect (2–5). Coexpression of an activated Akt1 (Akt1-DD) with either polyoma virus middle T antigen (PyVmT) or PyVmT Y315/322F (5) resulted in increased involution latency in both tumor models (3, 6). Akt1 coexpression did not affect the incidence of lung metastases in PyVmT Y315/322F transgenic mice and decreased lung metastases in the NDL model (3, 6). Akt1 coexpression did not alter the latency of mammary tumor development but cannot rescue the metastatic phenotype associated with these models. Here, we report the generation of transgenic mice expressing activated Akt2 in the mammary epithelium. Like mouse mammary tumor virus–Akt1, mammary-specific expression of Akt2 delayed mammary gland involution. However, in contrast to Akt1, coexpression of Akt2 with activated ErbB2 or Poloma virus middle T antigen uncoupled from the PI3K pathway (PyVmT Y315/322F) or activated ErbB2 (NDL) results in decreased tumor latency in both tumor models (3, 6). Akt1 coexpression did not affect the incidence of lung metastases in PyVmT Y315/322F transgenic mice and decreased lung metastases in the NDL model (3, 6). Conversely, germline deletion of Akt1 in NDL and PyVmT mouse models resulted in a profound mammary tumor induction defect (7, 8). Interestingly, germline deletion of Akt2 resulted in slightly accelerated mammary tumor induction in both models (8). Together, these observations suggest that Akt1 and Akt2 may have distinct roles in mammary tumor induction in both ErbB2 and PyVmT mammary tumor models.

Whereas these observations suggest that each Akt isoform may have distinct effects, the in vivo consequence of ectopic expression of activated Akt2 has not yet been examined. To directly explore this, we generated transgenic mice expressing a constitutively active Akt2 (Akt2-DD) from the mouse mammary tumor virus (MMTV) promoter. Like MMTV-Akt1 mice, we show that mammary-specific expression of activated Akt2 impairs mammary gland involution as a result of attenuated apoptotic cell death. To address the role of Akt2 in mammary tumorigenesis, activated Akt2 transgenic mice were interbred with separate strains of MMTV-PyVmT Y315/322F and MMTV-NDL transgenic mice. Unlike Akt1, the coexpression of Akt2 did not alter the latency of mammary tumor formation; however, activated Akt2 coexpression resulted in a marked increase in the incidence of lung metastases in both models. Furthermore, we have isolated clones of an ErbB2-driven mammary tumor cell line differing in their metastatic capacity and the highly metastatic clones expressed elevated Akt2 protein. In addition, ectopic expression of activated Akt2 increased invasion in all clones, whereas short interfering RNA (siRNA) knockdown of Akt2 in the highly metastatic clones impaired invasion. Collectively, the results of this study show that Akt1 and Akt2 perform distinct and nonredundant functions in mammary tumorigenesis and metastasis.

Materials and Methods

Transgenic mice. MMTV-Akt1-DD (3), MMTV-PyVmT Y315/322F (9), and MMTV-NDL (10) transgenic mice have been described previously. HA-tagged human Akt2-DD (T389D/S474D) cDNA was cloned downstream of the MMTV promoter/enhancer and followed by the SV40 PolyA sequence in the p206 vector. The fragment was linearized by SalI/Sphi digestion and purified using the QiAspin Gel extraction kit (Qiagen) as per the manufacturer’s protocol. DNA fragments were injected into one-cell zygotes of FVB/n mice at the McGill Transgenic Core Facility and implanted into pseudopregnant females. Potential founder animals were screened by PCR and validated by Southern blot. MMTV-Akt1-DD and MMTV-Akt2-DD mice were interbred with MMTV-PyVmT Y315/322F and MMTV-NDL mice and routine genotyping was performed by PCR. Experimental and control mice were monitored for tumor formation by physical palpation. All animals were maintained in accordance with the guidelines of the Royal Victoria Hospital Animal Care Committee.

Plasmid construction. HA-Akt1-DD and HA-Akt2-DD were subcloned from p206 into pmSCVpuro (Clontech) as an XhoI/EcoRI fragment. All constructs were verified by sequencing.

Tissue harvesting, immunoblotting, and immunoprecipitations. Mammary gland and mammary tumor tissues were flash frozen in nitrogen and lysates prepared as described previously (9). For cell lines, extracts were prepared in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L, 1% Trition X-100, protease inhibitor cocktail (Roche)]. Immunoblotting was performed as described previously.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L EGTA (pH 8.0), 1.5 mmol/L MgCl₂, 10 mmol/L sodium fluoride, 10 mmol/L sodium PIP supplemented with 1 mg/mL aprotinin and leupentin and 1 mmol/L sodium orthovanadate. Antibodies for immunoblots included HA (Covance), Neu (Oncoimmune Research Products), estrogen receptor α (EBio; clone AER311, Upstate), PyVimT (11), Akt1 (2H10, Cell Signaling), Akt2 (catalog no. 2962, Cell Signaling), growth factor receptor binding protein 2 (C-23, Santa Cruz Biotechnology), and β-actin (clone AC-15, Sigma). For immunoprecipitations, cell lysate was incubated overnight with anti-pAkt (catalog no. 9271 from Cell Signaling for immunoprecipitations immunoblotted for Akt1 and catalog no. 4051 from Cell Signaling for immunoprecipitations immunoblotted for Akt2). Protein G beads (GE Healthcare) were added and rotated for an additional 3 h. The immunoprecipitates were washed five times and analyzed by SDS-PAGE. All membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (The Jackson Laboratory) and visualized using enhanced chemiluminescence (Amersham).

Histology. Tissues were fixed in 10% buffered formalin and blocked in paraffin. Embedded tissues were sectioned at 4 μm and were H&E stained. For lung examinations, five-step sections were performed at 50-μm intervals and the slides were scanned using a ScanScope XT Digital Slide Scanner (Aperio). Mammary gland whole mounts were prepared as previously described (9).

Immunohistochemistry and in situ apoptosis assays. For immunohistochemistry, tissue sections were deparaffinized in xylene and antigen retrieval was performed in 10 mmol/L sodium citrate (pH 6) using a pressure cooker. Blocking was performed using Power Block Universal Blocking Agent (Biogenex); primary antibody incubations were performed in 2% bovine serum albumin and processed using the Elite IgG VectaStain ABC kit (Vector Laboratories) according to the manufacturer’s instructions. Eρα antibody was purchased from Novoceastra (6F11). Stained sections were scanned using a ScanScope XT Digital Slide Scanner and a nuclear algorithm was performed on 10 independent fields. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were conducted using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) as per the manufacturer’s protocol.

Real-time reverse transcription-CR. Total RNA was isolated from 8-wk virgin mammary glands and mammary tumor tissue using the Lipid Tissue RNeasy Midi and RNeasy Midi kits, respectively, from Qiagen as per the manufacturer’s instructions. For quantitative real-time reverse transcription-PCR (RT-PCR), 50 ng of total RNA were used with the Quantitect SYBR Green RT-PCR kit (Qiagen) and the LightCycler (Roche). Each amplification reaction was performed in triplicate and transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. Primer sequences used for antibody blotting. Briefly, the nonspecific binding sites on nitrocellulose were blocked by Re-Blot (mild, Chemicon International) and I-Block (Applied Biosystems). The endogenous peroxidase, avidin, and biotin were blocked by hydrogen peroxidase and a Biotin Blocking system (Dako cytometry). Each slide was then incubated with a primary antibody (Supplementary Table S1) in the appropriate dilution. The signal was captured by biotin-conjugated secondary antibody and amplified by tyramide deposition. The analyte was detected by avidin-conjugated peroxidase reactive to its substrate chromogranin aminobenzidine. Subsequently, the slides were individually scanned, analyzed, and quantitated using MicroVigene software (VigeneTech, Inc.). This software provides automated spot identification, background correction, individual spot intensity determination (expressed in logarithmic units), and curve fitting using a logistic fit model such as ln(y) = a + (ba) / (1 + exp(c – d – ln(x))).

The raw spot signal intensity data in .txt files from MicroVigene were processed by the R package SuperCurve (version 0.997; ref. 12) developed by the Department of Bioinformatics and Computational Biology at the M. D. Anderson Cancer Center, currently available as version 1.01 at the repository. This program fits a single curve using all samples (i.e., all dilution series) on each slide by using signal intensity as the response variable and the dilution series as an independent variable. The assumption is that that the dilution series of all samples on any given slide stained with a specific antibody fall on the same curve. A fitted curve (called “supercurve”) is thus plotted with the signal intensities—both observed and fitted—on the Y axis and the log 2 concentration of each protein on the X axis using the nonparametric, monotone-increasing B-spline model. The protein concentrations are derived from a supercurve for each sample lysate on the slide and then normalized by median polish. Each total and phosphoprotein measurement is subsequently corrected for loading using the average expression of all measured proteins in each sample.

Results

Generation of MMTV-activated Akt2 transgenic mice. To further assess the importance of the PI3K/Akt pathway in mammary gland development and tumorigenesis, we derived transgenic mice expressing an activated Akt2 (Akt2-DD) in the mammary epithelium. Akt2-DD cDNA was placed downstream of the MMTV promoter to direct transgene expression primarily to the epithelial cells of the mammary gland (Supplementary Fig. S1A). Akt2 transgene expression in the seven founder lines was examined at the RNA level by quantitative RT-PCR and protein expression was validated by immunoblotting for HA-tagged Akt2-DD (Supplementary Fig. S1B and C). Two founder lines expressed significant amounts of the transgene (lines 2-1 and 2-3) and the Akt2-1 line was used in subsequent studies.

Activated Akt2 expression delays mammary gland involution through an attenuation of apoptotic cell death. Given...
the importance of Akt signaling as a negative regulator of apoptosis, we first examined the effect of activated Akt2 expression on mammary gland involution, a process characterized by extensive apoptotic cell death. Mammary glands of age-matched wild-type and Akt2 transgenic mice were analyzed at days 1, 3, 7, and 10 postparturition. In contrast to the rapid involution observed in the wild-type controls, Akt2 transgenic mice showed delayed mammary gland involution, which could be observed by both histologic and whole-mount analyses (Supplementary Fig. S2A). However, by day 10 postparturition, Akt2 mammary glands had essentially completed the involution process and were indistinguishable from controls (data not shown). To explore whether this involution defect was due to differences in the induction of apoptosis, the mammary glands were analyzed by TUNEL staining. In accordance with the involution delay, Akt2 transgenics showed decreased apoptosis at day 3 compared with wild-type controls. However, at day 7 of involution, where the control glands showed very little apoptosis, Akt2 mammary glands still displayed significant levels (Supplementary Fig. S2B). These results argue that expression of an activated Akt2 interferes with the involution process by inhibiting apoptotic cell death.

Akt1 promotes mammary tumor induction, whereas Akt2 promotes metastasis in transgenic mice expressing a PyVmT uncoupled from the PI3K pathway. Although Akt2 attenuated apoptotic cell death in the mammary epithelium, the in vivo effect of expressing activated Akt2 in mammary tumorigenesis is unknown. Like MMTV-Akt1 mice (3), mammary epithelial expression of activated Akt2 was not sufficient for mammary tumor formation. To address the role of Akt2 in mammary tumorigenesis, we assessed whether activated Akt2 expression could accelerate mammary tumorigenesis in a strain of mice expressing PyVmT uncoupled from the PI3K pathway (PyVmT Y315/322F) in the mammary epithelium. Cohorts of bigenic mice expressing the PyVmT Y315/322F and Akt2 transgenes

Figure 1. Activated Akt2 does not affect mammary tumor onset in PyVmT Y315/322F mice but partially restores the metastatic defect associated with uncoupling PyVmT from PI3K. A, cohorts of virgin female mice were monitored for tumor formation by physical palpation. T50 represents the time at which 50% of the mice had palpable mammary tumors and n is the number of animals analyzed for each strain. B, H&E-stained lung sections were scored for metastatic lesions. *, P = 0.038 (Fisher’s exact test). C, representative H&E-stained mammary tumors. Bar, 0.5 mm. D, lysates of mammary tumors from PyVmT Y315/322F, PyVmT Y315/322F/Akt1, and PyVmT Y315/322F/Akt2 mice were immunoblotted for PyVmT and HA-tagged activated Akt1 or Akt2. β-Actin was detected as a control for loading. The PyVmT Y315/322F lysates were identical for the left and right panels, allowing for direct comparison.
and single transgene controls were monitored for tumor formation. PyVmT Y315/322F/Akt2 bigenic mice developed mammary tumors at 100% penetrance at a latency of 123 days (Fig. 1A). This tumor onset is similar to the latency observed for the PyVmT Y315/322F strain, indicating that unlike Akt1, Akt2 does not affect mammary tumor induction in the PyVmT Y315/322F mouse model. To directly compare the effect of Akt1 and Akt2, a separate cohort of PyVmT Y315/322F/Akt1 transgenic mice was generated. In accordance with our previous observations (3), Akt1 coexpression dramatically decreased tumor latency in the PyVmT Y315/322F model (Fig. 1A). We then examined whether Akt2 coexpression had any effect on the ability of the PyVmT Y315/322F tumor cells to metastasize. To ensure equal tumor-bearing time, all mice were sacrificed 8 weeks following initial tumor detection. At this end point, no significant difference in tumor burden in mice of different genetic combinations was noted (data not shown). Akt1 coexpression did not affect the incidence of lung metastases; however, Akt2 coexpression resulted in an approximately 2-fold increase in the proportion of mice with lung metastases (Fig. 1B). Unlike PyVmT Y315/322F/Akt1 mammary tumors, which display a more differentiated histology, PyVmT Y315/322F and PyVmT Y315/322F/Akt2 tumors displayed a similar pathology (Fig. 1C). Immunoblots showed no significant difference in expression of the PyVmT Y315/322F oncogene and all bigenic tumors expressed the activated Akt isoform (Fig. 1D). These results argue that in this mouse model where oncogene-dependent activation of PI3K is impaired, ectopic expression of activated Akt1 and Akt2 have opposing effects on either tumor onset or metastatic spread.

Akt1 promotes mammary tumor induction but impairs metastasis, whereas Akt2 promotes metastasis in activated ErbB2 transgenic mice. To further substantiate the distinct roles of Akt1 and Akt2 in mammary tumorigenesis, MMTV-Akt2 mice were interbred with MMTV-activated ErbB2 (NDL) mice. NDL/Akt2 mammary tumors were immunoblotted for ErbB2 and HA-tagged activated Akt1 or Akt2. β-Actin was detected as a control for loading. The NDL lysates were identical for the left and right panels, allowing for direct comparison.

Figure 2. Activated Akt2 does not affect mammary tumor onset in NDL mice but increases lung metastases. A, cohorts of virgin female mice were monitored for tumor formation by physical palpation. T50 represents the time at which 50% of the mice had palpable mammary tumors and n is the number of animals analyzed for each strain. B, H&E-stained lung sections were scored for metastatic lesions. *, P < 0.001; **, P = 0.039 (Fisher’s exact test). C, representative H&E-stained mammary tumors. Bar, 0.5 mm. D, lysates from NDL, NDL/Akt1, and NDL/Akt2 mammary tumors were immunoblotted for ErbB2 and HA-tagged activated Akt1 or Akt2. β-Actin was detected as a control for loading. The NDL lysates were identical for the left and right panels, allowing for direct comparison.
The slight difference in ErbB2 protein was not due to erbb2 transcript differences (Supplementary Fig. S3), likely reflecting alternative posttranscriptional regulation. Together, these observations suggest that similar to the PyVmT model, Akt1 plays a critical role in the induction of NDL mammary tumors, whereas Akt2 selectively modulates the metastatic phase of mammary tumorigenesis.

**Endogenous Akt2 promotes invasion in mammary tumor-derived cell lines.** One important issue raised by the above studies is whether the effects of the Akt1 and Akt2 isoforms and the ErbB2 and PyVmT oncogenes are due to their constitutive activation state or high expression levels driven by a strong viral promoter. To further establish the physiologic importance of the Akt isoforms, we took advantage of a mammary tumor cell line derived from mice expressing activated ErbB2 under its endogenous promoter (13). Like human ErbB2-induced mammary tumor progression, tumorigenesis in this strain of mice is associated with selective amplification of a core erbB2 amplicon comprising 10 genes (reviewed in ref. 14). One characteristic feature of mammary tumors and their derived cell lines in this strain is a very low rates of spontaneous metastasis (13–15). Given the heterogeneity of the derived cell line (TM15), clonally expanded cells were injected into the mammary fat pad of athymic mice and lungs scored for metastatic lesions at tumor end point. Although the primary tumors grew at comparable rates, striking differences in the number of lung metastases were observed for the different clonal cell lines (Fig. 3A and B). Clones 6 and 13 consistently formed fewer metastatic lesions, compared with clones 7 and 10. We then examined Akt1 and Akt2 expression levels and found the highly metastatic clones displayed up-regulated Akt2 expression compared with the low metastatic clones (Fig. 3C). Interestingly, the low metastatic clones 6 and 13 had the highest pAkt levels (Fig. 3C). However, from pAkt immunoprecipitates, the elevated pAkt in the low metastatic clones was shown to be due to elevated Akt1 phosphorylation, whereas highly metastatic clones showed markedly higher Akt2 phosphorylation (Fig. 3C). To establish the effect of ectopic activated Akt expression, stable cell lines expressing Akt1-DD or Akt2-DD were generated for *in vitro* invasion assays. All clones expressing Akt2-DD displayed increased invasion through Matrigel in transwell assays compared with empty vector controls, whereas Akt1-DD expression had little effect on invasion (Supplementary Fig. S4). Furthermore, siRNA knockdown of endogenous Akt2 in the highly metastatic clones impaired invasion (Fig. 3D; Supplementary Fig. S5). In contrast, siRNA down-regulation of endogenous Akt1, the predominantly phosphorylated isoform in clones 6 and 13, did not affect their invasive ability (Supplementary Fig. S5A–B). Together with the transgenic mouse data above, these results strongly suggest that elevated Akt2 positively mediates breast cancer cell invasion and metastasis.

**Akt1 and Akt2 expression in mammary tumors result in distinct signaling perturbations.** Given the distinct roles of Akt1 and Akt2 in mammary tumor induction and metastasis, we explored differences in signaling pathway activation in tumors of...
A protein array approach (16–18) was used using lysates of mammary tumors derived from five independent animals of each genotype and a number of proteins were found to be present and/or phosphorylated at different levels (Fig. 4). Not surprisingly, all NDL-based tumors displayed elevated Her2 and pHer2 levels compared with those of the PyVmT Y315/322F model. Unexpectedly, the protein arrays suggested that ERα was differentially expressed in the tumor models. In contrast to low ERα protein in NDL and NDL/Akt2 mammary tumors, NDL/Akt1 tumors displayed elevated ERα levels (Fig. 5A). We also examined ERα subcellular localization by immunohistochemistry and found a 3-fold increase in the proportion of the different genotypes. A protein array approach (16–18) was used using lysates of mammary tumors derived from five independent animals of each genotype and a number of proteins were found to be present and/or phosphorylated at different levels (Fig. 4). Not surprisingly, all NDL-based tumors displayed elevated Her2 and pHer2 levels compared with those of the PyVmT Y315/322F model. Unexpectedly, the protein arrays suggested that ERα was differentially expressed in the tumor models. In contrast to low ERα protein in NDL and NDL/Akt2 mammary tumors, NDL/Akt1 tumors displayed elevated ERα levels (Fig. 5A). We also examined ERα subcellular localization by immunohistochemistry and found a 3-fold increase in the proportion of

Figure 5. NDL/Akt1 mammary tumors express elevated ERα. A, lysates of NDL, NDL/Akt1, and NDL/Akt2 mammary tumors were immunoblotted for ERα. β-Actin was detected as a control for loading. The NDL lysates were identical for the left and right panels, allowing direct comparison. B, representative images of tumor sections stained with ERα-specific antibody. Bar, 50 μm. C, the percentage of ERα-positive nuclei was determined for 10 fields for each tumor section. Columns, mean of four independent tumors for each genotype; bars, SD. *P = 0.045 (Student’s t test).
Akt2 Promotes Metastasis

Akt1 and Akt2, two isoforms of the Akt serine/threonine kinase, have distinct roles in tumor promotion and metastasis. Akt1 promotes mammary tumor induction, whereas Akt2 promotes metastasis. This was demonstrated using transgenic mice expressing activated Akt1 or Akt2 in the mammary epithelium. Activated Akt2 expression did not affect pubertal mammary gland development nor did it affect the ability of transgene carrying females to lactate and nurse their pups. Activated Akt2 expression, however, affected mammary gland involution due to delayed apoptotic cell death. This was shown using in vitro and in vivo studies. Akt2-null mammary glands also displayed accelerated differentiation during pregnancy. However, transplantation experiments showed this effect to be nonepithelial cell autonomous. Alternatively, a precise level of Akt2 activation may be required for proper mammary gland involution and increasing or decreasing Akt2 levels may affect this delicate balance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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