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The objective of this research is to elucidate the roles of the phosphatidylinositol 3'-OH kinase (PI3K) and its downstream targets such as the Akt kinase in the induction of mammary tumors. To assess the role of Akt in mammary development and tumorigenesis, we have generated transgenic mice that express an activated Akt (Akt-DD) in the mammary epithelium. Although expression of Akt-DD interferes with apoptosis during normal mammary gland involution, mammary tumors are not observed in these strains. To explore the role of Akt in mammary tumorigenesis, mice co-expressing Akt–DD and a mutant form of Polyomavirus middle T (PyV mT) antigen de-coupled from PI3K/Akt signaling were generated. Co-expression of Akt-DD with mutant PyV mT resulted in dramatic acceleration of mammary tumorigenesis. This acceleration was further correlated with reduced apoptotic cell death, phosphorylation of FKHR and overexpression of cyclin D1. However, activation of Akt does not to affect metastatic progression. Akt activation may also be able to contribute to ErbB-2 mediated mammary tumorigenesis as bi-transgenics co-expressing Akt-DD and activated ErbB-2 transgenes show higher rates of tumorigenesis. Taken together these observations indicate that Akt activation can impede mammary gland regression and contribute to tumor progression by providing an important cell survival signal.

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## INTRODUCTION

The research objective outlined in the original proposal was to elucidate the roles of the phosphatidylinositol 3'-OH kinase (PI3K) and its downstream signaling partners in the induction of mammary tumors. The work is based on observations in transgenic mice that overexpress a mutant Polyomavirus middle T (PyV mT) oncogene that is decoupled from the the PI3K signaling pathway (MTY315/322F) under control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) in the mammary gland. In contrast to transgenic mice expressing wildtype PyV mT which rapidly develop multifocal metastatic mammary tumors, transgenic strains expressing MTY315/322F develop extensive mammary epithelial hyperplasias that are highly apoptotic [1,2]. Although mammary tumors do eventually develop in this strain, they are focal and form with considerably longer latency than in the wildtype strain. Furthermore, in contrast to the 100% metastasis observed in the wildtype strain, a greatly reduced number of the MMTV-MTY315/322F tumors metastasize to the lung. Based on these observations, we are directly assessing the importance of activation of the PI3K and its downstream targets such as the Akt serine/threonine kinase and the small GTPase Rac [3,4] in mammary tumorigenesis and metastasis through the use of transgenic mice expressing constitutively active forms of PI3K or Akt. Although we have not at this time succeeded in deriving mice expressing constitutively active PI3K we have generated mice that express constitutively active Akt (Akt-DD) in the mammary gland. Although expression of Akt-DD interferes with the apoptotic process of normal mammary gland involution, tumors were not observed in these strains. However, co-expression of Akt-DD with MTY315/322F resulted in a dramatic acceleration of mammary tumorigenesis correlated with reduced apoptotic cell death. Importantly, we did not observe an associated restoration of wildtype metastasis levels in the bi-transgenic strain. Furthermore, preliminary results indicate that activated Akt may contribute to ErbB-2 tumorigenesis, as co-expression of activated Akt and an activated ErbB-2 in the mammary gland greatly increases mammary tumor formation. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal but does not promote metastatic progression. It is hoped that the knowledge generated by these studies will enhance our understanding of the genetic process of breast cancer yielding new targets for therapy and enable better assessments of risk factors in human breast cancer progression.

## RESEARCH ACCOMPLISHMENTS

### 1) Final characterization of the MMTV/activated Akt transgenics. (Task 2)

One goal of the proposal was to further characterize the developmental effects of the activated Akt transgene (Akt-DD) on the mammary glands of the MMTV-Akt7 strain. Akt-DD had no detectable effect on virgin mammary gland with female virgin Akt transgenic mice having yet to develop mammary tumors after a year of observation. This observation is now further supported by the observation that multiparous Akt females, which would have undergone multiple periods of high transgene expression, have also failed to exhibit tumors. Given Akt's role in promoting cellular survival, we investigated whether Akt-DD could affect the apoptotic process of mammary gland involution. Mammary glands from wild type and activated Akt strains were examined at 1, 3 and 7-days post-parturition. In contrast to wild type control animals which exhibited extensive involution at 1 and 3-days post-parturition (Appendix 1, Fig. 3A,C,E,G), the Akt animals displayed a dramatic defect in mammary gland involution (Appendix 1, Fig. 3B,D,F,H). However, the Akt mammary glands eventually underwent full involution at 7-days post-parturition (data not shown). TUNEL analyses were conducted on

involuting mammary epithelium derived from FVB and Akt strains (Appendix 1, Fig. 4) and the results revealed that mammary glands derived from the involuting FVB glands exhibited elevated levels of apoptotic cell death relative to mammary epithelium of the Akt strains (compare Appendix 1, Fig. 4A to 4B). Taken together, these observations argue that activation of Akt can interfere with normal mammary gland involution by attenuating apoptotic death in the involuting mammary gland.

**2) Characterization of the bi-transgenics generated by interbreeding the MMTV/activated Akt, MMTV/-MTY315/322F and MMTV/activated ErbB-2 strains. (Tasks 4 & 8)**

Another goal of the original research was to explore the contribution of Akt activation in PyV mT and ErbB-2 mediated mammary tumorigenesis. As stated in the original proposal, to explore whether Akt-DD expression could complement the defect in tumorigenesis exhibited by transgenic mice expressing the MTY315/322F bi-transgenics expressing both Akt-DD and MTY315/322F were derived and monitored for tumor formation. The results of these analyses revealed that bi-transgenic mice developed multifocal tumors with shorter latency than observed in the MTY315/322F strain. In agreement with these analyses these lesions could be subcutaneously transplanted into syngeneic recipients. To confirm that bi-transgenics expressing MTY315/322F and Akt-DD exhibited elevated Akt kinase activity, we examined the total Akt kinase activity against a peptide substrate in virgin FVB, MTY315/322F and bi-transgenic mammary glands. These studies revealed an approximately five-fold increase in the total Akt kinase activity in the bi-transgenic mammary glands as compared to those of MTY315/322F transgenics (Appendix 1, Fig. 6A). The minimal increases in endogenous Akt phosphorylation (Appendix 1, Fig. 6B) would suggest that the majority of the Akt kinase activity is derived from the activated mutant.

As the mammary epithelial hyperplasias associated with the mutant PyV mT strains possess elevated levels of apoptotic cell death, we measured the degree of apoptotic cell death in mammary glands derived from the bi-transgenic mice. The results revealed that mammary epithelial expression of Akt-DD resulted in a dramatic repression of the high rates of apoptotic cell death in MTY315/322F tissue (Appendix 1, Fig. 6B). Taken together, these observations argue that the dramatic acceleration of mammary tumorigenesis exhibited by these strains is due to the ability of activated Akt to suppress the elevated apoptotic cell death displayed by mutant PyV mT mammary epithelium.

To further explore the molecular basis for the observed cooperative interaction between Akt-DD and MTY315/322F, we assessed the status of some of the known targets of Akt, including BAD [5], I-kappa-B [6] and the FKHR forkhead transcription factor [7]. No significant differences in either BAD-Ser136 phosphorylation or I-kappa_B levels were observed between the various transgenic strains (data not shown). However immunoblot analysis with phospho-specific antisera to serine 256 of FKHR-1 revealed that the mammary tissue samples derived from bi-transgenic animals expressed elevated levels of phosphorylated FKHR protein relative to other control transgenic tissue samples (Appendix 1, Fig. 7A). To further explore this observation we examined the status of p27/Kip1 as forkhead transcription factors have been shown to target expression of the cell cycle regulator p27/Kip1 [8,9]. However examination of p27/Kip1 levels by Western blot revealed no apparent decreases in p27/Kip1 levels in the bi-transgenic animals as compared to MTY315/322F and FVB/n controls (Appendix 1, Fig. 7B, third panel).

Another potential target for PI3K/Akt kinase axis is the cell cycle machinery as it has been demonstrated that suppression of the PI3K signaling pathway by the PTEN tumor

suppressor results in down regulation of cyclin D1 expression and cell cycle arrest [10]. Immunoblot analysis revealed that the bi-transgenic tissues co-expressing both Akt-DD and the mutant PyV mT oncogene exhibited dramatically elevated levels of cyclin D1 (Appendix 1, Fig. 7A, third panel). The differences in cyclin D1 protein were not due to increased levels of cyclin D1 transcripts since these samples expressed comparable levels of cyclin D1 transcript (data not shown). A potential mechanism for the increased levels of cyclin D1 was suggested by the ability of Akt and MAP kinases to phosphorylate and inhibit glycogen synthase kinase-3 (GSK3) [11,12], which has been shown to target cyclin D1 for proteasomal degradation [13]. However, analysis of GSK3 phosphorylation showed no significant increases in the bi-transgenic strain as compared to FVB/n and MTY315/322F controls, once differences in GSK3 levels were accounted for (Fig. 7B, first and second panels).

The original proposal also outlined experiments to examine the contribution of Akt activation to ErbB-2 mediated mammary tumorigenesis. To pursue these studies, transgenic mice co-expressing Akt-DD and the NDL2-5 activated ErbB-2 [14] transgenes have been generated. Preliminary results indicate that activated Akt may contribute to ErbB-2 tumorigenesis, as bi-transgenics show reduced latency of mammary tumor formation (Appendix 2, Fig. 1).

**1) Metastasis studies. (Tasks 6 & 7)**

As noted in the original proposal, simple histological assessment of metastasis is not beyond doubt, as it does not account for differences in tumor kinetics and load amongst the transgenic strains. Therefore I proposed to subcutaneously transplant primary cells from the various transgenic lines into syngeneic FVB/n animals and track tumor growth and metastasis to the lung. Metastasis levels would be quantitated using either Southern or PCR analysis against the transgene. These studies have been initiated and only the final quantitation remains to be completed. Matrigel invasion experiments will also be used to corroborate these *in vivo* studies.

To support these primary tumor cell studies, I have also derived cell lines from mammary tumors derived from bi-transgenics co-expressing Akt-DD and MTY315/322F (Appendix 2, Fig. 2). These cell lines will be used for similar in vivo and cell culture experiments as outlined above.

As the small GTPase Rac is a target of PI3K that has been implicated in cell motility signaling [15], I proposed to extend the above studies to study the role of Rac in PyV mT mediated metastasis. To do so, I have derived retroviral vectors expressing activated, dominant negative and wildtype versions of Rac and RhoA as well as activated PI3K and Akt controls. These retroviral vectors also contain an internal ribosome entry site (IRES) following the transgene, which allows the expression the potent selectable marker blastocydin in tandem with the transgene, allowing easy selection of cells expressing the construct. These studies will be initiated in the fall of 2001.

**(Tasks 1, 3 and 5)**

The original proposal also outlined experiments to examine the capacity of PI3K activation to affect mammary tumorigenesis alone and to determine its contribution to PyV mT and ErbB-2 mediated mammary tumorigenesis using transgenic mice expressing activated PI3K in the mammary gland. However, as we have been unable to derive the MMTV/activated PI3K transgenics at this time, these studies have not yet been initiated. Although the initial report indicated a potential hyperplastic phenotype in one activated PI3K strain, subsequent expression analysis by RNase protection against both the SV40 poly A (SPA) region of the transgene and an internal region of the PI3K transgene failed to show any expression of the transgene. We are currently deriving more lines to screen for transgene expression using a newly prepared injection

fragment. It is anticipated that the mice will be derived before the end of 2001 and characterized in the next year.

## TRAINING ACCOMPLISHMENTS

Over the past 2 years, I have acquired skills pertaining to the construction and analysis of transgenic mouse models of breast cancer. These skills include molecular biological techniques as well as biochemical analyses and immunohistochemistry. In addition to these basic laboratory skills, I have gained a great deal of knowledge regarding the histopathology of normal and tumorigenic mammary gland tissue. I have a better understanding of the progression of metastatic disease, and have acquired a greater knowledge of the variety of tumor types arising in the mammary glands of both humans and transgenic mouse models. In addition, since Akt overexpression impacts the involution state of the mammary gland, I now have a much greater understanding of mammary gland development and the molecular mechanisms behind the specialized functions of this tissue. Furthermore, the opportunity to submit a manuscript of my work and write a review has greatly increased my knowledge of the state of the field of breast cancer research, the scientific process and allowed me to improve my writing skills.

## KEY RESEARCH ACCOMPLISHMENTS

- Further characterization of mice expressing activated Akt in the mammary epithelium demonstrating defects in apoptosis during involution.
- Further molecular and biological characterization of mice co-expressing activated Akt and MTY315/322F in the mammary epithelium demonstrating anti-apoptotic effect of the transgene and revealing molecular targets involved in tumor formation.
- Generation of cell lines from mammary tumors derived from bi-transgenics co-expressing activated Akt and MTY315/322F.
- Interbreeding of the MMTV/activated Akt mice with MMTV/activated erbB-2 mice, to assess role of Akt in erbB-2-induced mammary tumorigenesis.

## REPORTABLE OUTCOMES

- Manuscript – "Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression." Hutchinson J, Jin J, Cardiff R, Woodgett J, Muller W., Mol Cell Biol, 2001 Mar;21(6):2203-12. (see reprint, Appendix 1).
- Manuscript – "Transgenic mouse models of human breast cancer." Hutchinson, J & Muller W., Oncogene, 2000 Dec 11;19(53):6130. (see reprint, Appendix 3)
- Oral presentation—Canadian Breast Cancer Research Initiative, Reasons for Hope Meeting, LeConcorde Hotel, Quebec City, Quebec, May 3-5, 2001 (see abstract, p. 33, Appendix 4)
- Abstract – The Fifth Conference on Signaling in Normal and Cancer Cells, Banff Centre for Conferences, Banff, Alberta, March 2-6, 2001 (see abstract, p. 34, Appendix 4)
- Oral presentation—Oncogene Meeting, Salk Institute, San Diego, California, USA, June 22-25, 2000 (see abstract, p. 35, Appendix 4)

## CONCLUSIONS

Funding for this project was provided in order to assess the roles of the phosphatidylinositol 3'-OH kinase (PI3K) and its downstream signaling partners in the induction of mammary tumors. The most important result after one year of funding is the demonstration that the activation of Akt in the mammary gland can adversely affect mammary gland involution

and partially contribute to PyV mT mediated mammary tumorigenesis via its effects on these processes through its effects on cellular apoptosis. Importantly, Akt has these effects on tumorigenesis but does not affect metastasis. These results are directly relevant to the understanding of the molecular mechanisms behind invasive breast cancer, as the overexpression of the PTEN tumor suppressor has been shown to induce apoptosis and cell cycle arrest through Akt-dependent pathways in a breast cancer cell line [16]. Preliminary results with bi-transgenics co-expressing Akt-DD and activated ErbB-2 support the notion that

Consistent with its role in promoting tumorigenesis, Akt activation resulted in the phosphorylation and inactivation of FKHR, a transcription factor involved in promoting apoptosis [17]. Interestingly, we observed little to no effect on such Akt targets as BAD, I-kappa-B or GSK 3. These results may reflect the different nature of the tissues and signals involved in these experiments. Furthermore, our finding that the combination of Akt-DD and MTY315/322F resulted in a non-transcriptional upregulation of cyclin D1 suggests that the concerted activation of both cell survival and proliferative signaling pathways may be a common requirement for oncogenic transformation of primary cells.

The inability to generate mice expressing activated PI3K in the mammary gland has delayed our ability to fully pursue a portion of the proposed research. However, we are currently actively pursuing the derivation of these mice, and expect to derive expressers shortly. To circumvent these delays I am currently pursuing *in vivo* transplant studies employing cells transfected with a retrovirus expressing the activated PI3K construct. These studies, should provide answers to the questions posed in the original proposal.

In summary, the results of these combined studies has increased our knowledge of the importance of PI3K and Akt in mammary tumorigenesis and metastasis. It is hoped that these results will aid in the assessment of patient's risk level at the time of diagnosis and may be used to assess therapeutic targets for the treatment of breast cancer.

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## Appendix 1

Manuscript published in Molecular and Cellular Biology.

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# Activation of Akt (Protein Kinase B) in Mammary Epithelium Provides a Critical Cell Survival Signal Required for Tumor Progression

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**Activation of Akt by the phosphatidylinositol 3′-OH kinase (PI3K) results in the inhibition of proapoptotic signals and the promotion of survival signals (L. P. Kane et al., Curr. Biol. 9:601–604, 1999; G. J. Kops et al., Nature 398:630–634, 1999). Evidence supporting the importance of the PI3K/Akt signaling pathway in tumorigenesis stems from experiments with transgenic mice bearing polyomavirus middle T antigen under the control of the mouse mammary tumor virus long terminal repeat promoter. Mammary epithelium-specific expression of polyomavirus middle T antigen results in the rapid development of multifocal metastatic mammary tumors, whereas transgenic mice expressing a mutant middle T antigen decoupled from the phosphatidylinositol 3′-OH kinase (MTY315/322F) develop extensive mammary gland hyperplasias that are highly apoptotic. To directly assess the role of Akt in mammary epithelial development and tumorigenesis, we generated transgenic mice expressing constitutively active Akt (HAPKB308D473D or Akt-DD). Although expression of Akt-DD interferes with normal mammary gland involution, tumors were not observed in these strains. However, coexpression of Akt-DD with MTY315/322F resulted in a dramatic acceleration of mammary tumorigenesis correlated with reduced apoptotic cell death. Furthermore, coexpression of Akt-DD with MTY315/322F resulted in phosphorylation of the FKHR forkhead transcription factor and translational upregulation of cyclin D1 levels. Importantly, we did not observe an associated restoration of wild-type metastasis levels in the bitransgenic strain. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal but does not promote metastatic progression.**

The growth and development of the mammary gland is regulated by a complex set of factors including hormones, cell-substratum interactions, and growth factors and their associated receptors. Activation of growth factor receptors leads to the recruitment of a number of cytoplasmic signaling molecules, including the phosphatidylinositol 3′-OH kinase (PI3K). Recruitment of the PI3K to the cell membrane by these activated growth factors or docking molecules then results in the activation of a number of molecules. PI3K-dependent generation of phosphatidylinositol 3′ phosphate provides docking sites for several Pleckstrin homology (PH) domain-harboring molecules including Akt (also known as protein kinase B [PKB]) as well as its upstream kinases, PDK1 and the proposed PDK2 (2, 16). These latter enzymes phosphorylate Akt at threonine 308 and serine 473, respectively, causing full Akt activation (1, 2). Activation of Akt subsequently results in the inhibition of proapoptotic signals from such proteins as BAD (9), caspase 9 (4), and the forkhead transcription factor family (3, 22, 34) and the promotion of survival signals from such proteins as NF-κB (20). Although evidence suggests roles for PI3K and Akt in normal mammary development (15) and tumorigenesis (5, 30, 31, 35), the role of these signaling molecules in these processes remains to be elucidated.

Evidence supporting the importance of the PI3K/Akt signaling pathway in tumorigenesis stems from experiments with transgenic mice bearing polyomavirus (PyV) middle T antigen (mT) under the control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR). The MMTV-LTR is transcriptionally active throughout mammary development, and its transcriptional activity increases during pregnancy (26). Mammary epithelium-specific expression of PyV mT results in the rapid development of multifocal metastatic mammary tumors (18) due to its ability to associate with and activate the Src family kinases, PI3K, and the Shc adapter protein (6, 7, 14). In contrast to the rapid tumor progression observed in transgenic mice carrying the PyV mT oncogene (MT634), transgenic mice expressing a mutant mT decoupled from the PI3K pathway (MMTV/MTY315/322F) develop extensive mammary gland hyperplasias that are highly apoptotic (35). Focal mammary tumors do eventually arise in these strains and are further correlated with upregulation of the ErbB-2 and ErbB-3 growth factor receptors (35). In addition, these tumors show defects in metastatic progression (35).

The defects in tumor progression in the mutant mT strain suggested that Akt may play important roles in tumorigenesis by inhibiting apoptosis and/or promoting metastasis. In this report we show that activation of Akt alone can interfere with the apoptotic process of mammary gland involution and promote tumor progression by providing an important cell survival signal but does not promote metastasis. The dramatic acceleration of tumor progression in these strains was further corre-

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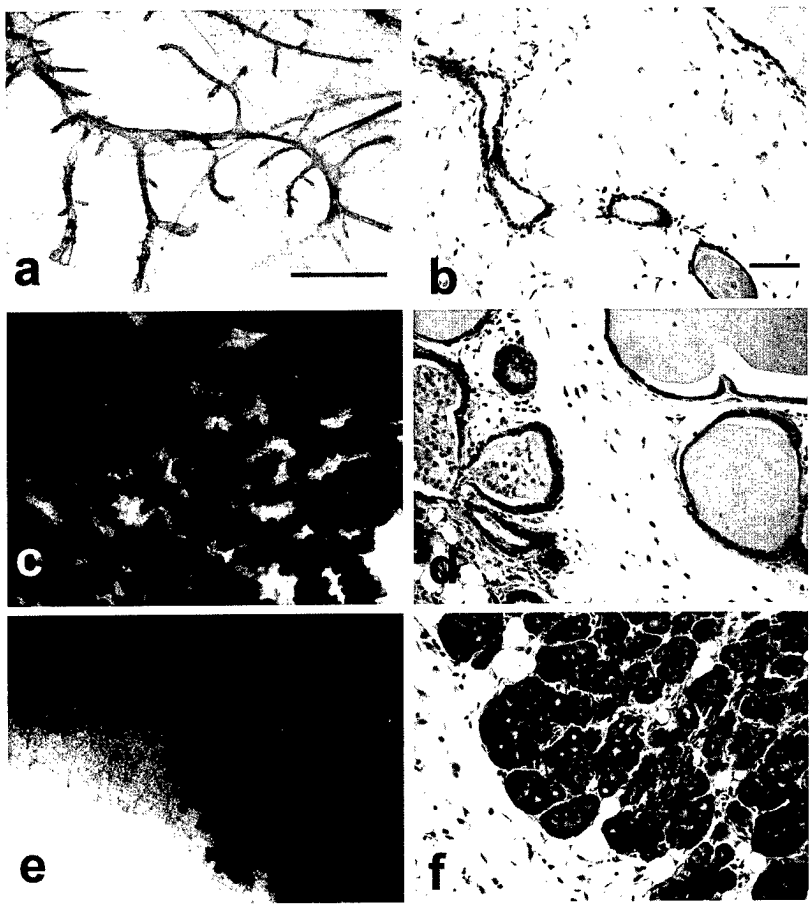


FIG. 2. Coexpression of Akt and mutant PyV mT oncogene results in the induction of multifocal mammary tumors. These digital images illustrate the histological patterns observed in the Akt7 (a and b), MTY315/322F (c and d), and Akt7 × MTY315/322F (e and f) bigenic mice. Note that the whole-mount preparations (a, c, and e) demonstrate that the Akt strains have a relatively normal mammary tree (a) compared to the cystic hyperplasias seen in the MTY315/322F strains at the same age (c) (8 weeks) (scale bar = 1 mm). In contrast, the bigenic mammary gland does not fill the fat pad (e) and is a solid mass at this age (f). The histological patterns seen at high magnification (scale bar = 0.01 mm) demonstrate that the Akt7 strain has a normal epithelium (b), while the MTY315/322F strain has a cystic hyperplasia of the ducts and glands without significant atypia (d). In contrast, the Akt7 × MTY315/322F cross has acinar or lobular hyperplasia with low-grade atypia at 8 weeks (f). Normal mammary gland morphologies for the FVB strain can be viewed at the following website: http://ccm.ucdavis.edu/tgmouse/wmtable.htm.

transgene revealed expression in the mammary gland in three of these lines (Table 1). The tissue specificity of transgene protein product expression of two of these lines (MMTV/Akt7 and MMTV/Akt10) was determined, and the higher expresser (MMTV/Akt7) was chosen for further study (Table 2). To confirm that activated Akt protein product was expressed in the mammary epithelium of transgenic mice, multiple mammary tissue extracts from the Akt7 line were subjected to anti-HA immunoblot analysis. The results revealed that virgin mammary glands from these strains were expressing significant levels of the transgene-derived Akt protein (Fig. 1b).

To ascertain whether elevated expression of activated Akt could interfere with normal mammary gland development, whole-mount analyses of both virgin and involuting mammary glands were conducted. Virgin female glands from MMTV/Akt strains were histologically and morphologically identical to FVB/n female controls (Fig. 2a and b). Consistent with these observations, female virgin Akt transgenic mice have yet to develop mammary tumors after a year of observation. This observation is further supported by the observation that multiparous Akt transgenic females, which would have undergone multiple periods of high transgene expression, have also failed to exhibit tumors. Given the importance of apoptotic cell death in mammary gland involution, we next examined whether mammary gland involution was adversely affected in the activated Akt strain. To explore this possibility, mammary glands from the wild-type and activated Akt strains were examined at 1, 3, and 7 days postparturition. In contrast to wild-type control animals, which exhibited extensive involution at 1 and 3 days postparturition (Fig. 3a, c, e, and g), the Akt7 animals displayed a dramatic defect in mammary gland involution (Fig. 3b, d, f, and h). However, the Akt7 mammary glands eventually underwent full involution at 7 days postparturition (data not shown), likely due to a drop in the hormonally responsive MMTV-driven transgene expression in the activated Akt strain.

To assess whether the observed delay in mammary gland involution was due to a defect in the induction of apoptotic cell death, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analyses were conducted on involuting mammary epithelium derived from FVB/n and Akt7 strains (Fig. 4). The results revealed that mammary glands

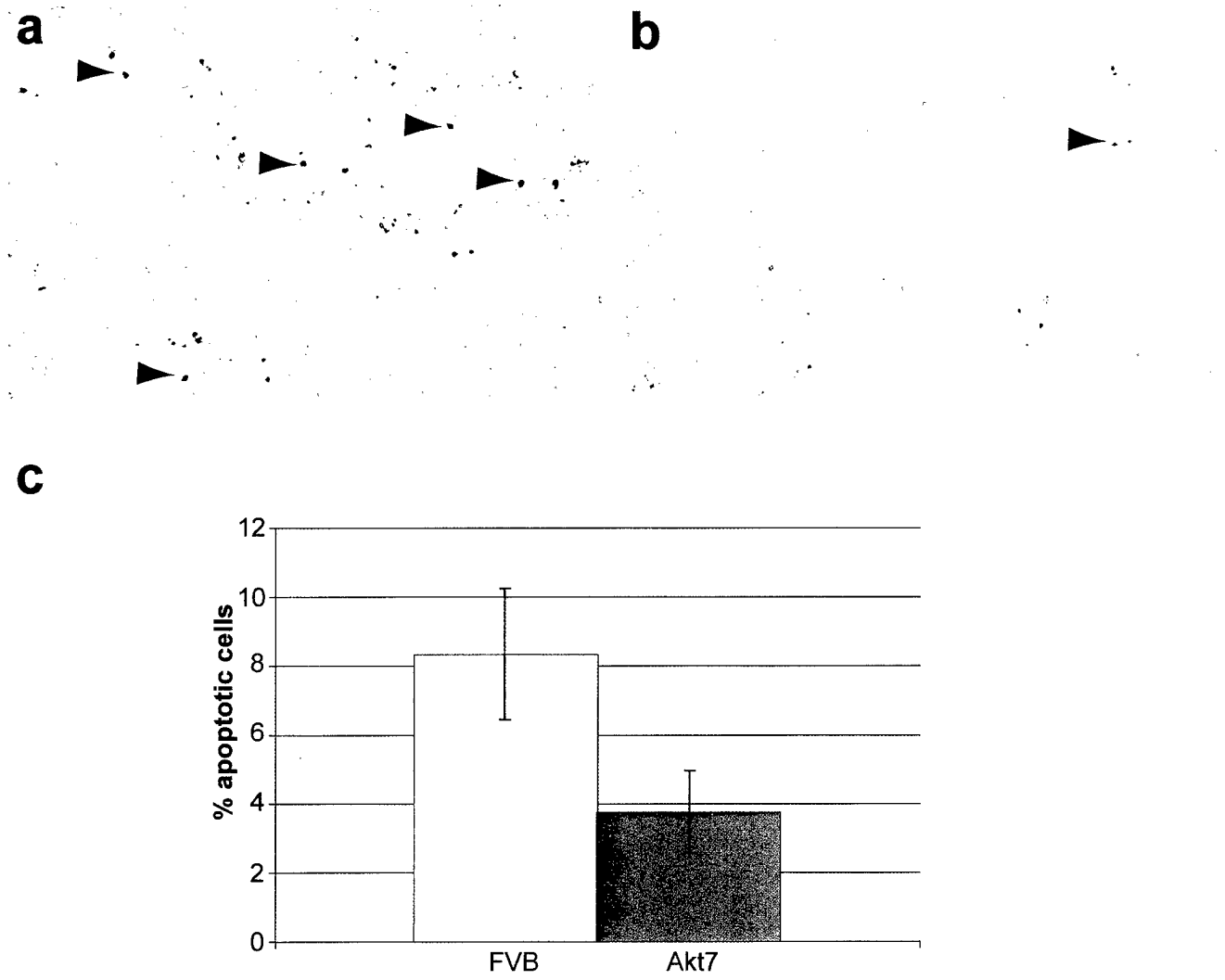


FIG. 4. Mammary epithelial expression of Akt results in decreased mammary gland apoptosis during involution. (a and b) TUNEL analysis of involuting mammary glands from FVB/n (a) and Akt7 (b) at 3 days postparturition. Arrows indicate representative apoptotic cells. (c) Mammary apoptotic indices of FVB/n and Akt7 at 3 days postparturition. Values shown represent the percentage of total cells stained positive for apoptosis by TUNEL assay in age-matched singly parous female mice at 15 weeks of age.

mT strains, female transgenic mice coexpressing the mutant PyV mT and activated Akt transgenes exhibited polyclonal differentiated carcinomas. In agreement with these analyses, these lesions could be subcutaneously transplanted into syngeneic recipients. To confirm that bitransgenics expressing MTY315/322F and activated Akt exhibited elevated Akt kinase activity, we examined the total Akt kinase activity against a peptide substrate in virgin FVB/n, MTY315/322F, and bitransgenic mammary glands. These studies revealed an approximately fivefold increase in the total Akt kinase activity in the bitransgenic mammary glands as compared to those of MTY315/322F transgenics (Fig. 6a). The minimal increases in endogenous Akt phosphorylation (Fig. 6b) would suggest that the majority of the Akt kinase activity is derived from the activated mutant. However, these results do not completely preclude a mechanism whereby endogenous Akt is in some way activated via the combination of Akt-DD and MTY315/322F and contributes to tumor formation.

As the mammary epithelial hyperplasias associated with the mutant PyV mT strains exhibit elevated levels of apoptotic cell death, we measured the degree of apoptotic cell death in mammary glands derived from the mutant PyV mT or bitransgenic mice. The results revealed that mammary epithelial expression of activated Akt resulted in a dramatic repression of the high rates of apoptotic cell death in PyV mT mutant tissue decoupled from the PI3K (Fig. 5b). Taken together, these observations argue that the dramatic acceleration of mammary tumorigenesis exhibited by these strains is due to the ability of activated Akt to suppress the elevated apoptotic cell death displayed by mutant PyV mT mammary epithelium.

Although the active, transgenic Akt is able to complement the mutant PyV mT strains for the induction of mammary tumors, only 20% of the tumor-bearing mice have developed lung metastases more than 8 weeks after the initial palpation of the mammary tumor ($n$ = 10) at tumor loads comparable to those observed in mice expressing wild-type mT at similar time

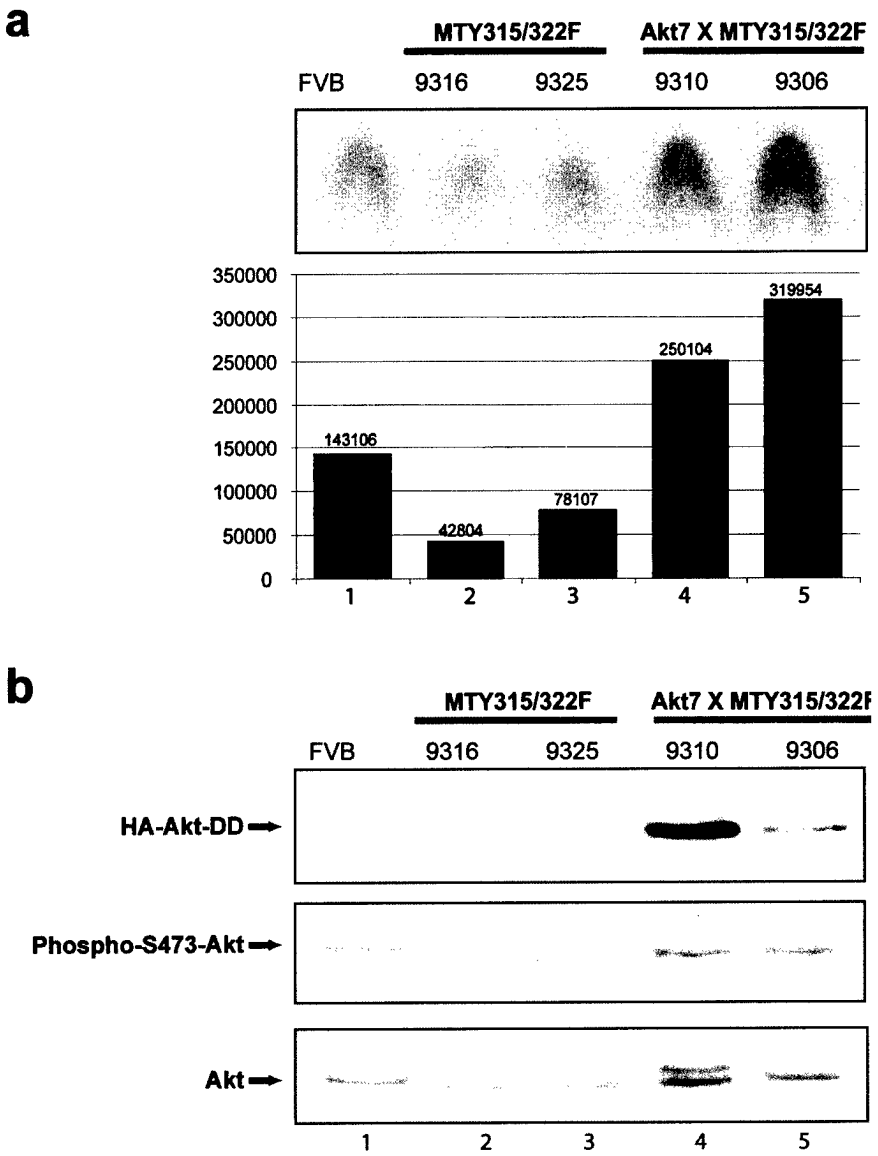


FIG. 6. Akt kinase activity in transgenic strains. (a) Total Akt kinase activity analysis in 8- to 10-week-old virgin females from FVB/n (lane 1), MTY135/322F (lanes 2 and 3), and bitransgenic Akt7 × MTY315/322F (lanes 4 and 5) strains. Assays were conducted using the cross-tide peptide as an Akt kinase substrate. Kinase activities were quantified by phosphorimager analysis and are represented here both graphically and numerically. (b) Immunoblot analysis of expression of HA-Akt-DD, phospho-S473-Akt, and Akt in 8- to 10-week-old virgin females from FVB/n (lane 1), MTY135/322F (lanes 2 and 3), and bitransgenic Akt7 × MTY315/322F (lanes 4 and 5) strains. All tissues were derived from 8- to 10-week-old virgin mammary glands. The arrows indicate the migration of transgenic HA-Akt-DD (upper panel), phospho-S473-Akt (middle panel), and total Akt (bottom panel). The numbers above each lane indicate individual mouse identification numbers.

the mammary tissue samples derived from the bitransgenic animals expressed elevated levels of phosphorylated FKHR protein relative to the other tissue samples (second panel). The differences in the phosphorylation status of FKHR proteins were not due to levels of FKHR protein, since most of the tissues expressed comparable levels of FKHR protein (upper panel). In addition, the differences in the phosphorylation status could not be due to variation in epithelial content, since these samples expressed comparable levels of cytokeratin 8 (lower panel). Consistent with these observations, we have demonstrated an identical pattern of FKHR phosphorylation in a second independent cohort of samples (data not shown). To further explore this observation we examined the status of p27 (Kip1), as forkhead transcription factors have been shown to target expression of the cell cycle regulator p27 (13, 23, 24). In particular, adenoviral expression of a constitutively active version of FKHR in the human renal cancer cell line 786-O cells induces expression of p27 (24). However examination of p27 levels by Western blotting revealed no apparent decreases in p27 levels in the bitransgenic animals as compared to MTY315/322F and FVB/n controls (Fig. 7b, lower panel). This apparent discrepancy may be due to the different nature of the tissues and signals involved in these experiments.

Nevertheless, another potential target for the PI3K/Akt kinase axis is the cell cycle machinery. Indeed, it has previously been demonstrated that suppression of the PI3K signaling

in the induction of mammary tumors itself, tumorigenesis requires the constitutive activation of other signaling pathways that are recruited by the mutant PyV mT oncogene, including the Src family kinases and Shc/Grb2/Ras pathway. Consistent with this view, we have observed that efficient phosphorylation of the FKHR protein requires the concerted activation of both Akt and the mutant PyV mT oncogene (Fig. 7). A similar requirement for coactivation of Akt and mutant PyV mT was also noted for the induction of cyclin D1. In this regard, it has recently been reported that the cooperation of Ras and Akt are required for the efficient transformation of primary glial cells (19). A potential mechanism for the increased levels of cyclin D1 was suggested by the ability of Akt and mitogen-activated protein kinases to phosphorylate and inhibit GSK-3 (8, 33), which has been shown to target cyclin D1 for proteasomal degradation (12). However, analysis of GSK-3 phosphorylation showed no significant increases in the bitransgenic strain as compared to FVB/n and MTY315/322F controls, once differences in GSK-3 levels were accounted for (Fig. 7b, upper and middle panels). Even so, these results suggest that the concerted activation of both cell survival and proliferative signaling pathways may be a common requirement for oncogenic transformation of primary cells.

Although our studies suggest that activated Akt can cooperate with these signaling pathways to efficiently induce mammary tumorigenesis, the observed low rates of metastasis suggest the involvement of other Akt-independent signals downstream of middle T in the potent metastatic phenotype exhibited by wild-type PyV mT. While these signals are in all likelihood PI3K dependent, we cannot exclude the possibility that signaling molecules other than PI3K may bind to and be activated via the 315 and 322 phosphorylated tyrosine residues. However, PI3K activation does modulate the activity of members of the Rho family of GTP-binding proteins (21, 25, 27, 29) and the integrin-linked kinase (11). This modulation is highly relevant, as the roles of these sets of signaling molecules in cell migration and adhesion implicates them in metastatic progression (10, 28). Further exploration of these PI3K-dependent pathways will provide important insight into the molecular basis of the metastatic phenotype.

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**Appendix 2**

Figures

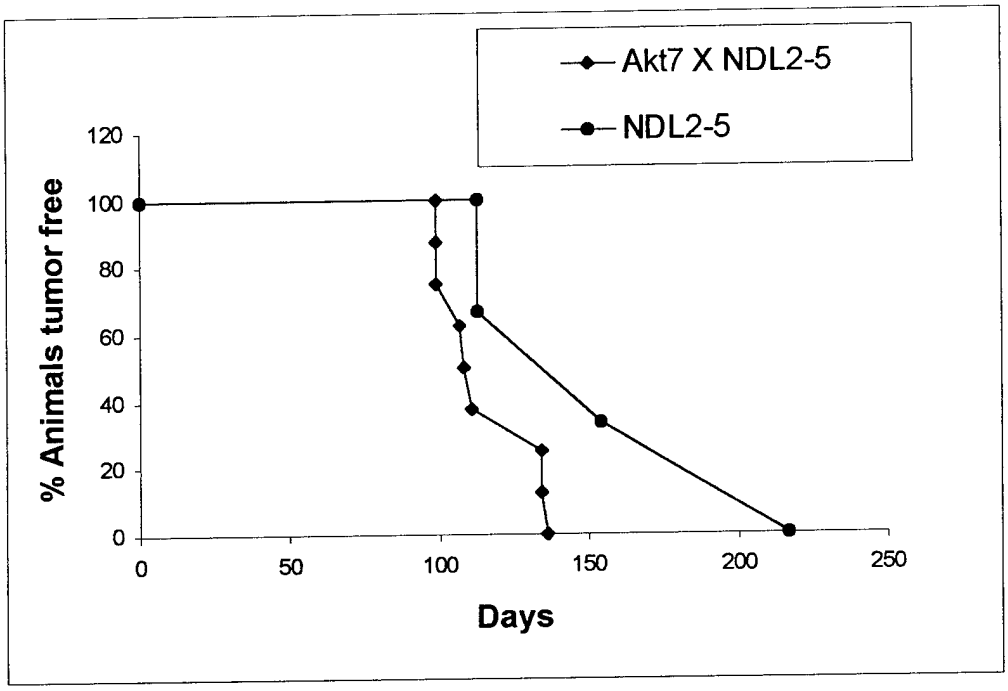


**Figure 1 – Mammary tumor kinetics in transgenic strains.**
Mammary tumor kinetics of MMTV/NDL2-5 (n=3) and MMTV/Akt7 X MMTV/NDL2-5 (n=8) strains. The age indicated is that at which a mammary tumor is first palpable in each transgenic strain.

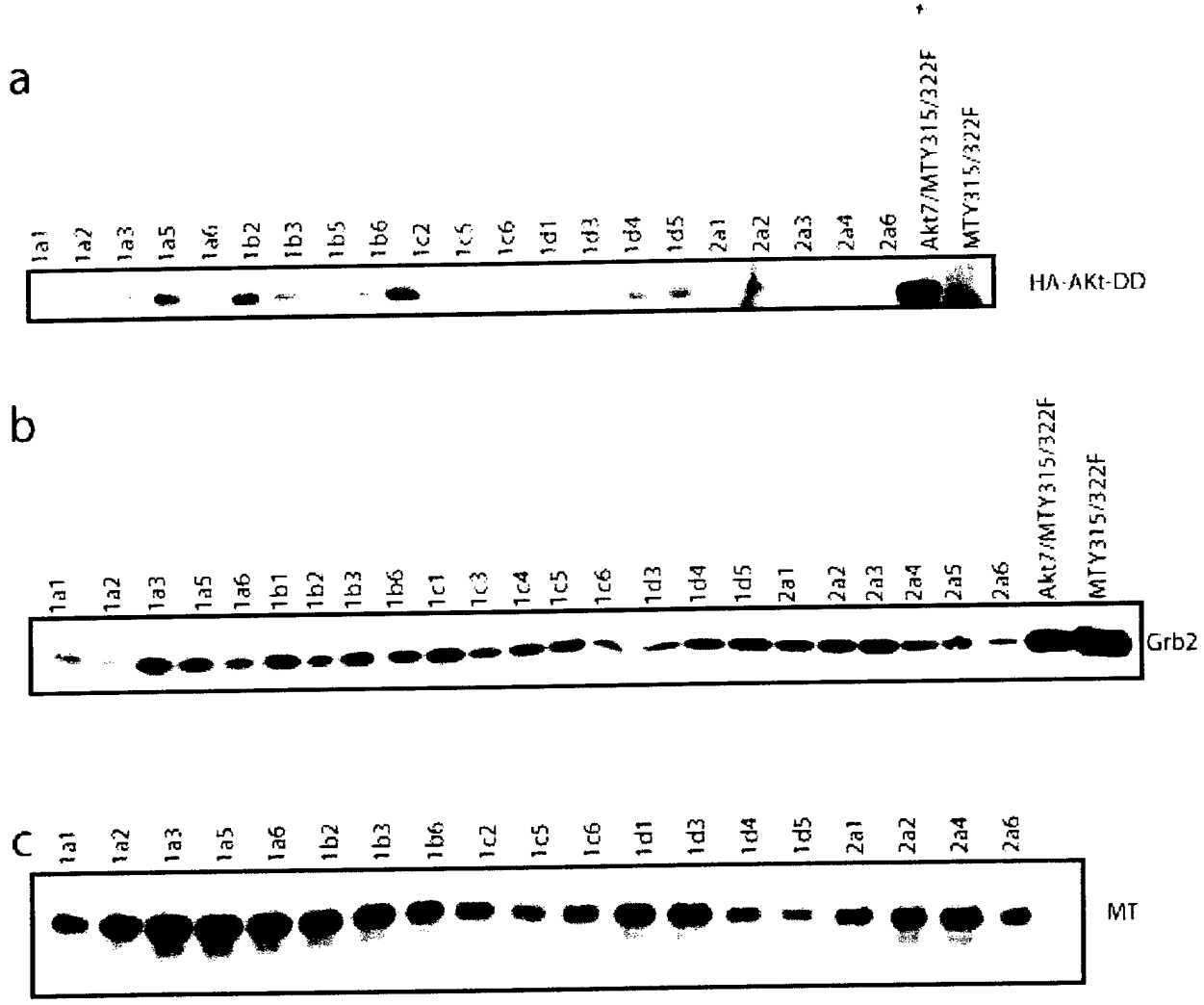


**Figure 2 – Transgene expression in AktXMTY315/322F cell lines**

a) Immunoblot analysis of expression of HA-Akt-DD in AktXMTY315/322F cell lines.

b) Immunoblot analysis of expression of Grb-2 (loading control) in AktXMTY315/322F cell lines.

c) RNase protection analysis of expression of MTY315/322F in AktXMTY315/322F using a probe directed against the MT transgene.

## Appendix 3

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# Transgenic mouse models of human breast cancer

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**The pathogenesis of human breast cancer is thought to involve multiple genetic events, the majority of which fall into two categories, gain of function mutations in proto-oncogenes such as c-*myc*, cyclin D1, ErbB-2 and various growth factors which are involved in supporting cell growth, division and survival, and loss of function mutations in so called 'tumor suppressor' genes, such as *p53*, which are involved in preventing unrestrained cellular growth. A number of mouse systems exist to address the significance of these mutations in the pathogenesis of breast cancer including transgenic mice expressing high levels of a specific gene in target tissues and knockout mice in which specific genes have been ablated via homologous recombination. More recently, the combination of these techniques to create bigenics as well as the use of 'knockin' and conditional tissue specific gene targeting strategies have allowed the models more reflective of the human disease to be devised. Studies with these models have not only implicated particular genetic events in the progression of the disease but have emphasized the complex, multi-step nature of breast cancer progression. These models also provide the opportunity to study various aspects of the pathogenesis of this disease, from hormonal effects to responses to chemotherapeutic drugs. It is hoped that through the combined use of these models, and the further development of more relevant models, that a deeper understanding of this disease and the generation of new therapeutic agents will result.** *Oncogene* (2000) **19,** 6130–6137.

**Keywords:** Transgenic mice; knockout mice; mammary gland; cancer; oncogenes; tumor suppressors

## Introduction

The pathogenesis of breast cancer is thought to involve multiple genetic events. Karyotypic and epidemiological analyses of mammary tumors at various stages suggest that breast carcinomas become increasingly aggressive through the stepwise accumulation of genetic changes (Dupont and Page, 1985). The majority of genetic changes found in human breast cancer fall into two categories, gain of function mutations in proto-oncogenes, which are involved in supporting cell growth, division and survival, and loss of function mutations in so called 'tumor suppressor' genes, which are involved in preventing unrestrained cellular growth. The majority of gain of function mutations in human primary breast cancers involve amplifications in one of three chromosomal regions, the c-*myc* and *erbB-2* proto-oncogenes or the chromosomal band 11q13 (Lidereau *et al.*, 1988). Loss of function mutations in primary human breast cancers include changes in the known tumor suppressor *p53* as well as in the familial cancer markers of the BRCA gene family. Additionally, multiple regions of loss of heterozygosity (LOH) are observed in primary human breast cancers (Bieche and Lidereau, 1995; Callahan *et al.*, 1992; Garcia *et al.*, 1999). It is thought that these regions of LOH affect as yet unidentified putative tumor suppressors. Indeed allelic loss of the PTEN region has been noted in a subset of aggressive breast cancers (Garcia *et al.*, 1999).

A number of mouse systems exist to address the significance of these mutations in the pathogenesis of breast cancer. On the most basic level, the use of transgenic mice expressing high levels of a specific gene in a target tissue allows the involvement of a given gene in the pathogenesis of breast cancer to be addressed. Alternatively, the ablation of specific genes via homologous recombination also allows researchers to determine the role of a gene in breast cancer progression. More recently, the combination of these techniques to create bigenics as well as the use of 'knock-in' and conditional tissue specific gene targeting strategies have allowed the creation of models more reflective of the human disease to be devised.

## Transgenic mouse models of gain of function mutations

A number of transgenic promoters have been employed to target transgene expression to the mammary gland. A majority of the transgenics generated have employed either the mouse mammary tumor virus long terminal repeat (MMTV) or the whey acidic protein promoter (WAP). The MMTV-LTR is active throughout mammary development and its transcriptional activity increases during pregnancy (Pattengale *et al.*, 1989). In contrast, the WAP promoter is only active in the mid-pregnant mammary gland. Thus, it is apparent that the phenotypes exhibited by WAP and MMTV transgenics may depend upon the developmental stage of the individual mouse examined. Other less common promoters employed include the 5′ flanking region of the C3(1) component of the rat prostate steroid binding protein, beta-lactalbumin, metallothionein and tetracycline responsive promoters.

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## Models for genetic regions amplified in human breast cancer

### c-myc

The c-*myc* gene encodes for a transcription factor that is frequently amplified in human tumors (Berns *et al.*, 1992; Bieche and Lidereau, 1995; Escot *et al.*, 1986). Multiple transgenic studies in which the *myc* gene was overexpressed under the control of mammary specific promoters have indicated an important role for *myc* in the progression of breast cancer (Leder *et al.*, 1986; Schoenenberger *et al.*, 1988; Stewart *et al.*, 1984). The first of these studies used the MMTV promoter to over-express *myc* in the mammary glands of mice and resulted in spontaneous mammary adenocarcinomas in two distinct lines by 4 to 8 months of age (Stewart *et al.*, 1984). A second transgenic study also using the MMTV promoter to overexpress *myc* resulted in the formation of locally invasive mammary tumors in four multiparous females by 10 to 19 months of age (Leder *et al.*, 1986). Interestingly, in one of MMTV/*myc* transgenic strains, c-*myc* expression was detected in a wide range of tissues. Despite the broad pattern of tissue specific expression, these mice developed a limited subset of tumor types including mammary tumors. Thus elevated expression of c-*myc* appears capable of inducing tumors in selected tissue sites.

Elevated expression of *myc* in the mammary gland has also been achieved by placing the c-*myc* oncogene under the transcriptional control of the WAP promoter (Schoenenberger *et al.*, 1988). In these transgenic strains, 80% of female transgenics develop multiple tumors affecting single or multiple glands after two pregnancies at ages as early as 2 months (Schoenenberger *et al.*, 1988). Together they demonstrate that c-*myc* can induce mammary tumor formation when overexpressed in the mammary gland. However, the fact that overexpression of c-*myc* does not result in transformation of the entire mammary gland, as normal mammary epithelium is also present in these strains, reveals that additional genetic events are required for the development of overt mammary carcinomas. In this sense, these models accurately reflect the nature of the progression of human breast cancer.

### *Cyclin D1*

Cyclins regulate the activation of cyclin-dependent kinases (CDK's) allowing cell cycle progression, S phase entry and DNA replication. A variety of lines of evidence have linked cyclins to the progression of breast cancer. Foremost, the *cyclin D1* gene is found within the 11q13 region, which is amplified in 15–20% of primary human breast cancers (Bieche and Lidereau, 1995; Brison, 1993). Overexpression of *cyclin D1* under control of the MMTV promoter results in proliferative abnormalities in the mammary gland, with significant lobulo-alveolar development shortly after sexual maturity is reached. Significantly, eight of 12 mice from three distinct transgenic lines developed focal mammary tumors with a mean onset of 18 months (Wang *et al.*, 1994). As with the c-*myc* transgenic models, the long latency and focal nature of these tumors suggests that although *cyclin D1* can promote mammary tumorigenesis, additional genetic changes are needed for the development of overt mammary carcinomas. Consistent with this view, mammary epithelial expression of *cyclin D1* has been implicated as an important event in mammary tumor induced by activated Src kinases, integrin linked kinase (ILK) and ErbB-2 (Lee *et al.*, 1999, 2000; Radeva *et al.*, 1997). Conversely germline inactivation of *cyclin D1* results in impaired mammary epithelial gland development (Fantl *et al.*, 1999). Collectively these observations suggest that *cyclin D1* plays a critical role in both normal mammary gland development and mammary tumorigenesis.

### *ErbB-2*

ErbB-2 is a member of the EGFR family of receptor tyrosine kinases (RTKs). This family is comprised of four closely related type 1 RTKs that include the EGFR, ErbB-2 (Neu, HER2), ErbB-3 (HER3), and ErbB-4 (HER4) (Hynes and Stern, 1994; Olayioye *et al.*, 2000). Signaling in these receptors involves the formation of homo and hetero-dimers in response to ligand stimulation. This dimerization results in the phosphorylation of specific tyrosine residues on the receptor. These phosphorylated tyrosines then offer docking sites for the SH2 and SH3 (PTB) domains of various endogenous signaling molecules that are able to interact with the receptor and transduce the signal (Dankort and Muller, 2000; Hynes and Stern, 1994). Originally, *erbB-2* was described as the oncogene *neu* found in chemically induced neuroblastomas in rats (Schechter *et al.*, 1984). Neu possesses a valine-glutamic acid substitution in its transmembrane domain that results in the constitutive aggregation and activation of the receptor in the absence of ligand (Bargmann *et al.*, 1986a,b; Dankort and Muller, 2000; Stern *et al.*, 1986; Xie *et al.*, 1995).

The importance of ErbB-2 in primary human breast cancer is highlighted by the fact that 20–30% of human breast cancers express elevated levels of ErbB-2 due to the genomic amplification of the *erbB-2* proto-oncogene (Slamon, 1987, 1989). Furthermore, its amplification and subsequent overexpression strongly correlates with a negative clinical prognosis in both lymph node positive (Hynes and Stern, 1994; Mansour *et al.*, 1994; Ravdin and Chamness, 1995) and node-negative (Andrulis *et al.*, 1998) breast cancer patients. Further evidence that overexpression of ErbB-2 results in an aggressive tumor type stems from studies showing that elevated ErbB-2 expression is observed in many *in situ* and invasive human ductal carcinomas but is rarely observed in benign breast disorders such as hyperplasias and dysplasias (Allred *et al.*, 1992, Mansour *et al.*, 1994). Significantly, ErbB-2 overexpression may be useful not only as a prognostic marker but as a predictive marker as well as HER-2 overexpression predicts tamoxifen resistance of the primary tumor (reviewed in Pegram *et al.*, 1998).

Multiple transgenic mouse studies have confirmed a direct role for ErbB-2 in mammary tumorigenesis each with their own level of relevance to the human disease. MMTV-driven overexpression of the oncogene *neu* or an analogous ERbB-2 transgene engineered to possess a similar activating mutation within the transmembrane domain results in the formation of mammary

*Hepatocyte growth factor (HGF)*

Another growth factor thought to play an important role in modulating the biological behavior of mammary epithelial cells is the hepatocyte growth factor. HGF and its receptor tyrosine kinase Met are involved in the development of the normal mammary gland (Niemann *et al.*, 1998; Yang *et al.*, 1995). Several studies have also shown overexpression of both Met and HGF in human breast cancers (Lamszus *et al.*, 1997; Tuck *et al.*, 1996; Yamashita *et al.*, 1994). Two studies have generated mice which express an activated form of the Met receptor under control of the metallothionein promoter with varying results (Jeffers *et al.*, 1998; Liang *et al.*, 1996). Mice displayed either hyperplastic nodules progressing to tumors between 11 and 14 months of age (Liang *et al.*, 1996) or induction of metastatic mammary tumors (Jeffers *et al.*, 1998). Consistent with these results, generation of mice expressing HGF under metallothionein control developed tumors of various types, the majority mammary tumors. Together, these studies support a role for HGF in mammary tumor progression.

*Fibroblast growth factors*

Early studies with MMTV insertion sites revealed frequent proviral activation of members of the fibroblast growth factor (FGF) family including Fgf3 (Dickson *et al.*, 1984; Peters *et al.*, 1983), Fgf4 (Peters *et al.*, 1989) and Fgf8 (Kapoun and Shackleford, 1997; MacArthur *et al.*, 1995). Direct evidence supporting a role for these growth factors derives from studies of a number of transgenic models. Mammary epithelial expression of Fgf3 (*int2*) results in induction of wide spread mammary epithelial hyperplasias that eventually progress towards full malignancy (Muller *et al.*, 1990). In addition to Fgf3, transgenic mice expressing either Fgf8 (Daphna-Iken *et al.*, 1998) or Fgf7 (Kitsberg and Leder, 1996) under MMTV develop pregnancy-dependent mammary hyperplasias that progress to tumors.

## The role of tumor suppressors in mammary tumor progression

Recent transgenic studies have also highlighted the role of LOH in breast cancer progression. Studies in multiple transgenic mice lines including MMTV/v-Ha-*ras* (Radany *et al.*, 1997), MMTV/wild-type *neu* (Ritland *et al.*, 1997), MMTV/c-*myc* (Weaver *et al.*, 1999) and MMTV/activated *neu* (Cool and Jolicoeur, 1999) have demonstrated that tumors from these animals also show LOH. Significantly, amongst the many areas affected by LOH in these tumors, all showed LOH affecting markers in chromosome 4, an area that contains regions syntenic to human chromosomal regions frequently lost in human breast cancers (1p32-36 and 9p21-22). This further validates these transgenics as models of events involved in human breast cancer. Although it is thought that these LOH mutations affect tumor suppressor genes, many of the loci affected have yet to be identified. However, two types of loss of function mutations that frequently occur in primary human breast cancers are those that affect the known tumor suppressor p53 and the BRCA gene family.

BRCA1 and BRCA2 have been strongly implicated in the pathogenesis of familial or heritable breast cancer. In fact, germline mutations in BRCA1 have been detected in 90% of familial breast/ovarian cancers and almost 50% of familial cases involving breast cancer alone (reviwed in Alberg and Helzlsouer, 1997; Paterson, 1998). The p53 tumor suppressor has also been frequently investigated, both in the context of breast cancer and cancer in general. In fact, *p53* is the most commonly altered gene by deletion or mutation in human breast cancer (Elledge and Allred, 1994). The advent of gene targeting in embryonic stem cells has enabled researchers to directly assess the importance of both p53 and the BRCA family in mammary tumorigenesis. One problem with this approach is that these mutations either effect viability or life span of the mouse. For instance, mice homozygous for BRCA1 mutations die early during embryongenesis (Liu *et al.*, 1996). Heterozygotes for BRCA1 are no more informative as they are not pre-disposed to develop mammary tumors (Liu *et al.*, 1996). Similarly, although mice homozygous for null *p53* do develop a diverse array of tumors, mammary tumors are rarely observed (Donehower *et al.*, 1992). Studies with the p53 knockouts are further complicated by the formation of extensive lymphomas and thymic tumors that result in the death of the animal at an early age (Donehower *et al.*, 1992). To circumvent these limitations, mice carrying a mutant p53 $172^{Arg\text{-}His}$ under WAP control were generated (Li *et al.*, 1998). These mice display low tumor incidence but exhibit increased tumor incidence as compared to controls in response to chemical carcinogens.

Recent technological advances have also allowed the drawbacks of knockouts, such as embryonic lethality, to be circumvented. Using a powerful modification of the original knockout technique, conditional mutants may be generated which excise the gene of interest in a tissue-specific manner via combination of the Cre-Lox recombination system with the knockin approach. The basis for this system is the ability of the Cre recombinase to excise genetic material flanked by *loxP* sequences from the genome. This can be achieved at the transgenic level through the generation of mice carrying mammary-targeted Cre recombinase under either the MMTV or WAP promoters (for a review of the Cre-Lox system in mice, see Sauer, 1998). These mice are then crossed with mice which have been engineered through homologous recombination techniques to possess *loxP* sequences flanking a critical region of the gene of interest.

This advanced technique has allowed the question of the role of BRCA in mammary tumorigenesis to be properly addressed. In the case of the BRCA1 conditional knockout, Cre-mediated excision of exon 11 of *brca1* in mouse mammary epithelium initially caused increased apoptosis and abnormal ductal development (Xu *et al.*, 1999). Eventually after a long latency, mammary tumors formed which were further associated with genetic instability characterized by aneuploidy, chromosomal rearrangements or alteration of the *p53* locus (Xu *et al.*, 1999), supporting the view that BRCA1 is involved in DNA repair and maintenance of genomic integrity.

and estrogen have been shown to induce the production of cyclin D1 in murine mammary epithelial cells (Said *et al.*, 1997). These results indicate that hormonal effects may play an important role in mammary cancer progression. Further studies using a wider number of established models should greatly increase our knowledge of their precise roles and effects.

One of the primary limitations to many of these transgenic models discussed is their dependency on strong viral, hormonally sensitive promoters such as WAP and MMTV. Consequently, it is difficult to properly address the interactions between the oncogene-coupled signaling pathways and endocrine hormones that affect mammary gland development. This problem is being addressed by the use of knockins in which transgenics are generated which express oncogenes of interest from their endogenous promoters. This is achieved through the use of a modified homologous recombination approach by which oncogenes of interest are introduced into their endogenous loci.

A combination of both tissue specific recombination and knock-in technologies has enabled researchers in this lab to place the activated *neu* under the endogenous *erbB-2* promoter (Andrechek *et al.*, 2000). To prevent the early embryonic lethality that may have resulted from expression of this cDNA, a silencer cassette containing a neo cassette flanked with *loxP* sites was placed between the *erbB-2* promoter and the activated *neu* allele. This resulted in expression of the endogenous ErbB-2 until the silencer cassette was excised by mammary epithelial specific expression of the Cre recombinase resulting in mammary epithelial specific expression of the activated ErbB-2 allele (Andrechek *et al.*, 2000). Expression of this allele in the mammary gland resulted in accelerated lobuloalveolar development and tumor formation after a long latency period (Andrechek *et al.*, 2000). Significantly, normal levels of expression of the activated allele from the endogenous *erbB-2* promoter were not sufficient for tumorigenesis as all tumors showed amplification (2–22 copies) of the activated *neu* allele relative to normal mammary tissue (Andrechek *et al.*, 2000). Thus like ErbB-2 positive human tumors, mammary tumorigenesis in this mouse model required amplification of the *erbB-2* locus. This model thus holds great promise for relevant studies of the pathogenesis of ErbB-2 positive human breast cancer.

## Conclusions

It is evident from the models outlined above that it is important to consider many factors when assessing the applicability of a mouse model for breast cancer research to human breast cancer. The nature of the genetic change, the characteristics of the promoter used to target transgene expression, the status of endogenous signaling pathways, the spectrum of additional mutations that may arise during tumor progression in the transgenic, the number of transgenic lines examined and the reliability of the phenotype amongst them, the transgenic's genetic background and the molecular pathology and histology are all important indicators of the relevancy of the model to the human disease.

While no single genetically engineered mouse can offer a complete model of the wide assortment of human neoplasms found in human breast cancer, it is hoped that these multiple approaches will enable us to develop insights into the complex molecular events involved in tumorigenic progression of the breast. One common theme evident from these studies is the involvement of genes necessary for normal mammary gland development in the progression of this disease. Another emergent theme is the complex, multi-step nature of all stages of breast cancer progression from initial tumor formation to final metastasis. Fortunately, researchers now have many models available to them to study these steps in a controlled and rational manner. Furthermore these models provide the opportunity to study many various aspects of the pathogenesis of this disease, from hormonal effects to responses to chemotherapeutic drugs. It is hoped that through the combined use of these models, and the further development of more relevant models that a deeper understanding of this disease and the generation of new therapeutic agents will result.

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## Appendix 4

Abstracts from conferences.

Activation of Akt/PKB in mammary epithelium interferes with mammary gland involution and provides a critical cell survival signal required for tumor progression.

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The mammary gland undergoes a regulated cycle of proliferation, differentiation and apoptosis and disruption of this cycle can lead to the occurrence of many abnormalities including tumorigenesis. In conjunction with hormones and cell-substratum interactions, the growth, differentiation and apoptosis of mammary epithelial cells is regulated by growth factors and their receptors. Activation of these receptors leads to the recruitment of a number of cytoplasmic signaling molecules including the phosphatidylinositol 3'-OH kinase (PI3K) which plays an important role in coupling these growth factor receptors to cell survival pathways via the Akt/PKB (Protein Kinase B) serine/threonine kinase. Evidence supporting the importance of the PI3K/Akt signaling pathway in mammary tumorigenesis stems from experiments with transgenic mice bearing polyomavirus middle T antigen (PyV mT) under the control of the mouse mammary tumor virus-long terminal repeat promoter (MMTV-LTR). Mammary epithelial specific-expression of PyV mT results in the rapid development of multifocal metastatic mammary tumors whereas transgenic mice expressing a mutant mT de-coupled from the phosphatidylinositol 3'-OH kinase (MTY315/322F) develop extensive mammary gland hyperplasias that are highly apoptotic. To directly assess the role of Akt in mammary epithelial development and tumorigenesis, we generated transgenic mice expressing constitutively active Akt (Akt-DD). Although expression of Akt-DD interferes with normal mammary gland involution, tumors were not observed in these strains. However, co-expression of Akt-DD with MTY315/322F resulted in a dramatic acceleration of mammary tumorigenesis correlated with reduced apoptotic cell death. Importantly, we did not observe an associated restoration of wildtype metastasis levels in the bi-transgenic strain. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal but does not promote metastatic progression.

Activation of Akt/PKB in mammary epithelium interferes with mammary gland involution and provides a critical cell survival signal required for tumor progression.

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The mammary gland undergoes a regulated cycle of proliferation, differentiation and apoptosis and disruption of this cycle can lead to the occurrence of many abnormalities including tumorigenesis. In conjunction with hormones and cell-substratum interactions, the growth, differentiation and apoptosis of mammary epithelial cells is regulated by growth factors and their receptors. Activation of these receptors leads to the recruitment of a number of cytoplasmic signaling molecules including the phosphatidylinositol 3'-OH kinase (PI3K) which plays an important role in coupling these growth factor receptors to cell survival pathways via the Akt/PKB (Protein Kinase B) serine/threonine kinase. Evidence supporting the importance of the PI3K/Akt signaling pathway in mammary tumorigenesis stems from experiments with transgenic mice bearing polyomavirus middle T antigen under the control of the mouse mammary tumor virus-long terminal repeat promoter (MMTV-LTR). Mammary epithelial specific-expression of polyomavirus middle T antigen results in the rapid development of multifocal metastatic mammary tumors whereas transgenic mice expressing a mutant middle T antigen de-coupled from the phosphatidylinositol 3'-OH kinase (MTY315/322F) develop extensive mammary gland hyperplasias that are highly apoptotic. To directly assess the role of Akt in mammary epithelial development and tumorigenesis, we generated transgenic mice expressing constitutively active Akt (Akt-DD). Although expression of Akt-DD interferes with normal mammary gland involution, tumors were not observed in these strains. However, co-expression of Akt-DD with MTY315/322F resulted in a dramatic acceleration of mammary tumorigenesis correlated with reduced apoptotic cell death. Furthermore, co-expression of Akt-DD with MTY315/322F resulted in phosphorylation of the FKHR forkhead transcription factor and translational upregulation of cyclin D1 levels. Importantly, we did not observe an associated restoration of wildtype metastasis levels in the bi-transgenic strain. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal but does not promote metastatic progression.

Mammary Epithelial Expression Of Akt/Pkb Affects Mammary Gland Involution And Tumor Progression.

**John Hutchinson[1], Jin Jing[2], Robert Cardiff[3], Jim Woodgett[2] & William Muller[1].**

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**In conjunction with hormones and cell-substratum interactions, the growth and differentiation of mammary epithelial cells is regulated by growth factors and their receptors. Activation of these receptors leads to the recruitment of a number of cytoplasmic signaling molecules to the cell membrane including the phosphatidylinositol 3'-OH kinase (PI3K). Recruitment and activation of PI3K by these docking molecules results in the activation of a number of Peckstrin homology domain harbouring molecules including the Akt serine/threonine kinase. Akt plays roles in coupling these receptors to critical cell survival pathways through inhibition of pro-apoptotic signals from BAD, caspase-9 and the forkhead transcription factor family as well as the promotion of survival signals from NF□-B. Although evidence suggests roles for the PI3K and Akt/PKB in normal mammary development and tumorigenesis there is no direct evidence tying them to these processes in the mammary gland. To assess the role of Akt in mammary epithelial development and tumorigenesis, we generated transgenic mice expressing activated Akt in the mammary epithelium. Although Akt interferes with the normal apoptotic process of mammary gland involution, mammary tumors were not observed in this strain after more than a year of observation. However, co-expression of activated Akt with a mutant form of Polyomavirus middle T (PyV mT) antigen de-coupled from the PI3K signaling pathway results in a dramatic acceleration of mammary tumorigenesis in this strain. This acceleration was further correlated with reduced apoptotic cell death in mammary epithelium expressing the mutant form of PyV mT. Importantly, associated wildtype PyV mT levels were not observed. These observations suggest that activation of Akt can contribute to tumor progression by providing a cell survival signal but that Akt/PKB does not contribute to metastasis.**