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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The objective of this research is to elucidate the roles of the phosphatidylinositol 3'-OH kinase (PI3K) and its downstream target Akt kinase in the induction of mammary tumors. To assess the role of Akt in mammary development and tumorigenesis, we 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 activated ErbB-2 or 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 also contributes to ErbB-2 mediated mammary tumorigenesis as bi-transgenics co-expressing Akt-DD and activated ErbB-2 transgenes show higher rates of tumorigenesis correlated with increased proliferation and overexpression of cyclin D1. These observations indicate that Akt activation can impede mammary gland regression and contribute to tumor progression by providing survival and proliferation signals.

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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 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 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. Furthermore, PI3K may contribute to ErbB-2 mediated mammary tumorigenesis as mammary tumors from mice expressing activated ErbB-2 overexpress ErbB-3, which possesses 7 consensus PI3K binding sites(*3*). Based on these observations, we are directly assessing the importance of activation of the PI3K and its downstream target the Akt serine/threonine kinase(*4*) in mammary tumorigenesis and metastasis through the use of transgenic mice expressing constitutively active forms of PI3K or Akt. Although we have not 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, activated Akt contributes to ErbB-2 tumorigenesis, as co-expression of activated Akt and an activated ErbB-2 in the mammary gland increases mammary tumor formation correlated with increased cellular proliferation. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing important cell survival and proliferation signals 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. Our results show that Akt-DD has no detectable effect on virgin mammary gland development and female virgin Akt transgenic do not develop mammary tumors after a year of observation. Furthermore, multiparous Akt females, which have undergone multiple periods of high transgene expression, also fail to exhibit tumors. We investigated whether Akt-DD could affect the apoptotic process of mammary gland involution and found that 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 revealed that mammary glands derived from the involuting FVB glands exhibited elevated levels of apoptotic cell death

relative to that of the Akt strains (compare Appendix 1, Fig. 4A to 4B). 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 tumorigenesis in these models, 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.

We measured the degree of apoptotic cell death in mammary glands derived from the bi-transgenic mice and found 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) Thus, the dramatic acceleration of mammary tumorigenesis exhibited by these strains is likely due to suppression by Akt of elevated apoptosis in the MTY315/322F mammary epithelium.

We further 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 target its expression(*8, 9*). Western blot analysis revealed no decreases in p27/Kip1 levels in the bi-transgenics (Appendix 1, Fig. 7B, third panel).

Another potential target for PI3K/Akt kinase axis is the cell cycle machinery(*10*). Immunoblot analysis revealed that the bi-transgenic tissues 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 transcript as these samples expressed comparable levels (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-transgenics (Appendix 1, Fig. 7B, first and second panels).

The proposal outlined experiments to examine the contribution of Akt activation to ErbB-2 mediated mammary tumorigenesis. Transgenic mice co-expressing Akt-DD and the NDL2-5 (activated ErbB-2) (*14*) transgenes were generated (Appendix 2, Fig. 1). The results indicate that

bi-transgenics show reduced latency of mammary tumor formation (Appendix 2, Fig. 2) with a 50% of the animals showing tumor formation at 104 days (n=32) as opposed to 185 days (n=20) in the NDL2-5 animals alone. Indeed, wholemount analysis reveals that these bi-transgenic animals form lesions as early as 10 weeks of age (Appendix 2, Fig. 2). Histological analysis of these tumors reveals them to be atypical cystic hyperplasias with numerous foci of large cysts lined by an irregular multi-layered atypical epithelium (Appendix 2, Fig. 2). I have used these lesions to derive clonal cell lines and confirmed expression of both HA-AktDD and ErbB-2 (Appendix 2, Fig. 1).

Examination of levels in the bi-transgenic Akt7XNDL2-5 animals by TUNEL analysis revealed no significant differences between strains (data not shown), but examination of the Ki67 proliferation index of the mammary gland epithelium by immunohistochemistry revealed that at early time points mammary epithelial cells from bi-transgenic Akt7XNDL2-5 animals exhibit a qualitatively higher proliferation index than the NDL2-5 strain at the same age (Appendix 2, Fig. 3). Therefore it appears that Akt may be promoting early tumor formation through effects on cell cycle in this cross.

To determine the molecular basis of these phenotypes I examined the status of various downstream targets of Akt. Consistent with the TUNEL results, analysis of FKHR phosphorylation levels failed to reveal any increase of FKHR phosphorylation levels on Serine 256 in these animals as compared to controls (data not shown). Similar to the previous cross, examination of p27/Kip1 levels by Western blot revealed no apparent decreases in p27/Kip1 levels in the bi-transgenic animals as compared to NDL2-5 and FVB/n controls (Appendix 2, Fig. 4). However, analysis of Cyclin D1 levels revealed it to be overexpressed in both late stage NDL2-5 and bi-transgenic mammary glands. More significantly however, Cyclin D1 is overexpressed in early stage bi-transgenics but is not in NDL2-5 mammary glands of the same age (Appendix 2, Fig. 4). Thus it is possible that the effects on proliferation in the bi-transgenic strain are mediated by Cyclin D1. Preliminary analyses indicate that the differences in Cyclin D1 protein are not due to increased levels of cyclin D1 transcript since it appears that samples from the various strains expressed comparable levels of transcript (data not shown). To examine the mechanism of this Cyclin D1 overexpression I am examining GSK3 phosphorylation levels; preliminary data indicate that there is no decrease in GSK3 phosphorylation levels in the bi-transgenics (data not shown).

Another route by which Akt may post-transcriptionally affect Cyclin D1 levels is through the translation initiation complex. In order to examine this I have exposed the Akt7XNDL2-5 derived cell lines to the translational inhibitor rapamycin (for a review of translation initiation, see (*15*)). Immunoblot analysis of Cyclin D1 levels in these rapamycin treated cell lines failed to reveal any decreases in Cyclin D1 levels in the Akt&XNDL2-5 derived cell lines suggesting that the upregulation of Cyclin D1 levels in the bi-transgenics may not be rapamycin sensitive (Appendix 2, Fig. 5). I have examined 4ebp-1 phosphorylation levels as readout of mTOR activity to determine if the rapamycin dose is effective but these studies remain inconclusive at this time. However, as significant decreases in Cyclin D1 levels were observed in the NAFA activated NeuNT tumor derived cell line it remains possible that the dose employed in this experiment was insufficient to fully inhibit mTOR activity. To address this possibility I am repeating this experiment with a higher dose. I will also continue to attempt to examine 4ebp-1 phosphorylation levels to determine the efficacy of the rapamycin dose.

Cyclin D1 is known to promote cell cycle progression via its ability to promote phosphorylation of Rb by CDK4 to allow exit from the G1 phase of the cell cycle. To determine

if the overexpressed Cyclin D1 was functioning in this manner I examined the phosphorylation status of Rb in the mammary glands of the transgenic strains. Rb phosphorylation levels were higher in early stage Akt7XNDL2-5 mammary glands than in age-matched NDL2-5 samples (Appendix 2, Fig. 5) revealing that Cyclin D1 is acting to phosphorylate Rb in this context. Thus the overexpressed Cyclin D1 is functional.

(Tasks 6 & 7)

As noted in the original proposal, I proposed to subcutaneously transplant primary cells from the various transgenic lines into syngeneic FVB/n animals, track tumor growth, metastasis to the lung and quantitate levels by Southern analysis against the transgene. Although these studies have been initiated they have not been completed at this time.

(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.

TRAINING ACCOMPLISHMENTS

During the course of this traineeship, I have acquired skills pertaining to the construction and analysis of transgenic mouse models of breast cancer including molecular biological techniques, biochemical analyses and immunohistochemistry. I have also gained a great deal of knowledge regarding the histopathology of normal and tumorigenic mammary gland tissue, the progression of metastatic disease, and knowledge of the variety of tumor types arising in the mammary glands of both humans and transgenic mouse models. In addition, 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

- Characterization of mice expressing activated Akt in the mammary epithelium demonstrating lack of tumorigenesis and defects in apoptosis during involution.
- 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 and co-expressing Akt and NDL2 (activated ErbB-2)
- Molecular and biological characterization of mice co-expressing activated Akt mice and activated ErbB-2, demonstrating proliferative effect of the transgene and revealing molecular targets involved in tumor formation.

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. 34, 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. 35, Appendix 4)
- Oral presentation—Oncogene Meeting, Salk Institute, San Diego, California, USA, June 22-25, 2000 (see abstract, p. 36, 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 both PyV mT and ErbB-2 mediated mammary tumorigenesis via its effects on these cellular apoptosis and proliferation. Importantly, Akt effects tumorigenesis without affecting metastasis in at least one of these crosses. 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*).

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*). Furthermore, our finding that the combination of Akt-DD and MTY315/322F or NDL2 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.

Although 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 we feel that the results of the studies outlined above have greatly increased our knowledge of the importance of 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

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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.

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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-

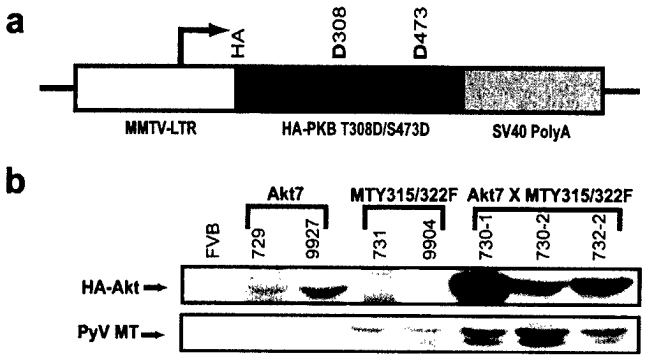

FIG. 1. Activated Akt transgene expression. (a) Structure of the MMTV/Akt transgene. The Bluescript vector backbone is represented by a thin line on either side of the expression cassette, with the white region corresponding to the MMTV-LTR derived from plasmid pAp, the black portion corresponding to the hemagglutinin tag, the dark grey region corresponding to the Akt (HAPKBT308D/S473D) cDNA with aspartate substitutions at amino acid positions 308 and 473, and the mid-grey region corresponding to the transcriptional processing sequences derived from the SV40 early transcription unit. The transcription start site is indicated by an arrow. (b) Immunoblot analysis of expression of HAPKB and PyV mT in bitransgenic Akt7 × MTY315/322F strains. Note that Akt7 × MTY315/322F tumor samples coexpress both Akt and PyV mT proteins. The numbers above each lane indicate individual mouse identification numbers.

lated with the phosphorylation of FKHR, a member of the forkhead class of transcription factors, and induction of cyclin D1. Together these observations suggest that activation of Akt provides complementary cell survival signals that are required for mammary tumorigenesis.

MATERIALS AND METHODS

Generation and identification of transgenics. The cDNA encoding HAPKB308D473D was subcloned into pMMTV-SV40Pa (p206) (18). This construct was prepared and injected as previously described (35). Transgenic progeny were identified by Southern analysis using the *Eco*RI-*Bam*HI (3900 to 4775) fragment of p206 (18) as a probe. Akt-MTY315/322F bitransgenics were generated by crossing MMTV/MTY315/322F males to MMTV/Akt7 females and were subsequently identified by identical Southern analysis.

Histology and apoptosis assays. Lower left mammary fat pad tissues were fixed in 4% paraformaldehyde, blocked in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined. Whole-mount preparations were prepared from the lower right mammary fat pad as previously described (35). In situ apoptosis assays were performed with the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Intergen) as described previously (35).

TABLE 1. MMTV/Akt transgene expression in mammary gland[a]

Line	Expression[b]
Akt1	−
Akt2	−
Akt3	−
Akt4	−
Akt5[c]	ND
Akt6	−
Akt7	++
Akt8	−
Akt9	+
Akt10	+
Akt11[c]	ND

[a] Expression of the Akt transgene in the mammary gland was determined via RNase protection analysis on 20 μg of total RNA with a probe directed against the SV40 poly(A) region of the transgene.
[b] Relative levels of transgene expression: ND, no data; −, not detected; +, low; ++, high.
[c] Strain did not pass transgene.

TABLE 2. MMTV/Akt transgene expression in MMTV/Akt7 tissues[a]

Sex	Tissue	Expression[b] in strain: Akt7	Akt10
Female	Mammary gland	++	+
	Brain	−	−
	Heart	−	−
	Kidney	−	−
	Liver	−	−
	Lung	−	−
	Ovary	−	−
	Salivary	−	−
	Spleen	−	−
	Thymus	−	−
Male	Epididymis	++++	+++
	Seminal vesicles	++	++++
	Testes	−	−

[a] Expression of the Akt transgene was determined by Western blot analysis using the HA-11 monoclonal antibody (Babco) on 250 μg of total protein lysate precleared in protein G-Sepharose.
[b] Relative levels of transgene expression: −, not detected; +, low; ++, intermediate; +++, high; ++++, very high.

RNA isolation and analysis. RNA was isolated from mammary glands and analyzed by RNase protection using simian virus 40 (SV40) polyadenylation-specific (SPA) and PGK-1 ribonucleotide protection probes as previously described (35).

Protein extraction and analysis. Tissue from various organs was flash frozen in liquid nitrogen and stored at −80°C or immediately lysed. Protein lysates were prepared as previously described (35). All immunoblots and immunoprecipitations were carried out as previously described (35) with the following exceptions. Antihemagglutinin (anti-HA) immunoblot analyses of mammary tissue from the FVB/n, Akt7, MTY315/322F, and Akt7 × MTY315/322F strains were performed on 250 μg of total protein lysate. Lysates were precleared in protein G-Sepharose and subjected to anti-HA immunoblot analysis with HA-11 monoclonal antibody (Babco) (1:1,000). PyV mT was immunoprecipitated from 2 mg of total protein lysate with 2 μg of mouse monoclonal Pab762 (courtesy of S. Dilworth) and subjected to anti-mT immunoblot analysis with rat monoclonal Pab701 (1:1,000). Anticytokeratin immunoblot analysis was carried out on 250 μg of total protein lysate using Troma-1 rat monoclonal antibody from ascites (1:50). Cyclin D1 analysis was carried out on 50 μg of total protein using the anti-cyclin D1 72-13G monoclonal antibody from Santa Cruz. FKHR analysis was carried out on 50 μg of total protein using the anti-FKHR N-18 polyclonal antibody from Santa Cruz and the anti-phospho-FKHR (Ser256) antibody from New England Biolabs.

Akt immunoblotting was carried out on total lysate using the anti-Akt antibody from New England Biolabs. Akt kinase activity assays were carried out on immunoprecipitates from total lysate using the anti-PH domain PKB antibody from UBI and the cross-tide peptide as substrate. Glycogen synthase kinase 3 (GSK-3) analysis was carried out on total lysate using anti-GSK-3 antibodies from New England Biolabs.

RESULTS AND DISCUSSION

To further assess the importance of the PI3K/Akt signaling pathway in PyV mT-induced tumorigenesis and metastasis, we derived transgenic mice that express a constitutively active version of Akt (HAPKB308D473D or Akt-DD), which mimics the active phosphorylated state of the protein (1), in the mammary gland (Fig. 1a). To distinguish between the transgene-derived and endogenous Akt protein, an HA epitope tag was placed in frame at the amino terminus of the activated Akt protein (Fig. 1a). Initially, 11 activated MMTV/Akt founder lines were derived. Nine of these lines passed the transgene to their offspring, and a screen for expression of the activated Akt

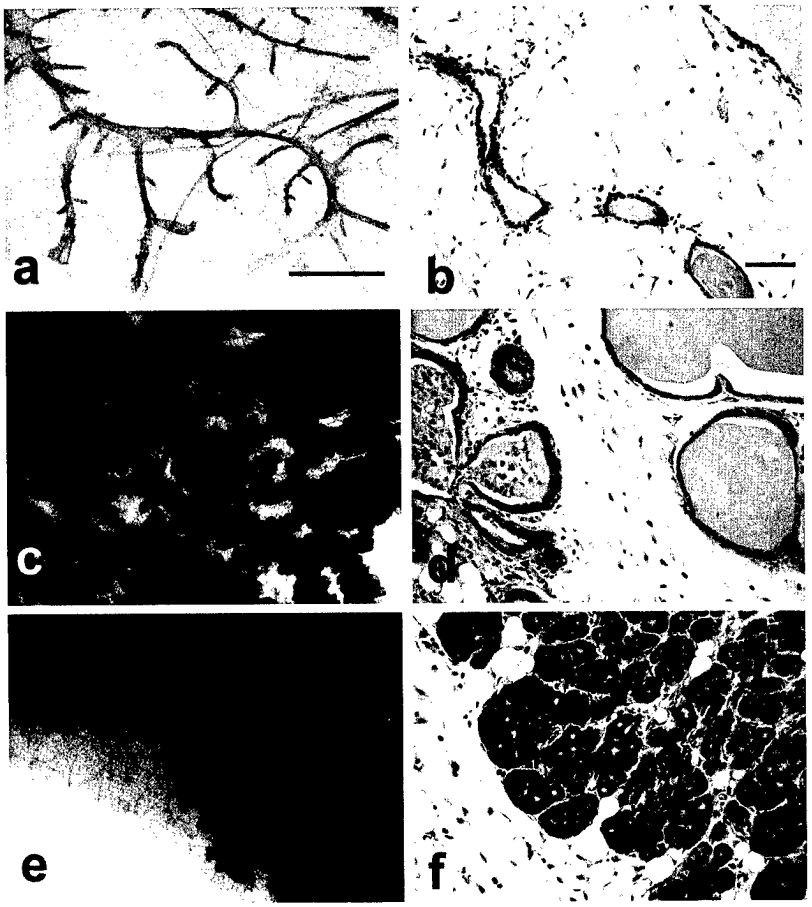

FIG. 2. Coexpression of Akt and mutant PyV mT oncogene results in the induction of multifocal mammary tumors. These digital images illustrate the histological patterns observed in the Akt7 (a and b), MTY315/322F (c and d), and Akt7 × MTY315/322F (e and f) bigenic mice. Note that the whole-mount preparations (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

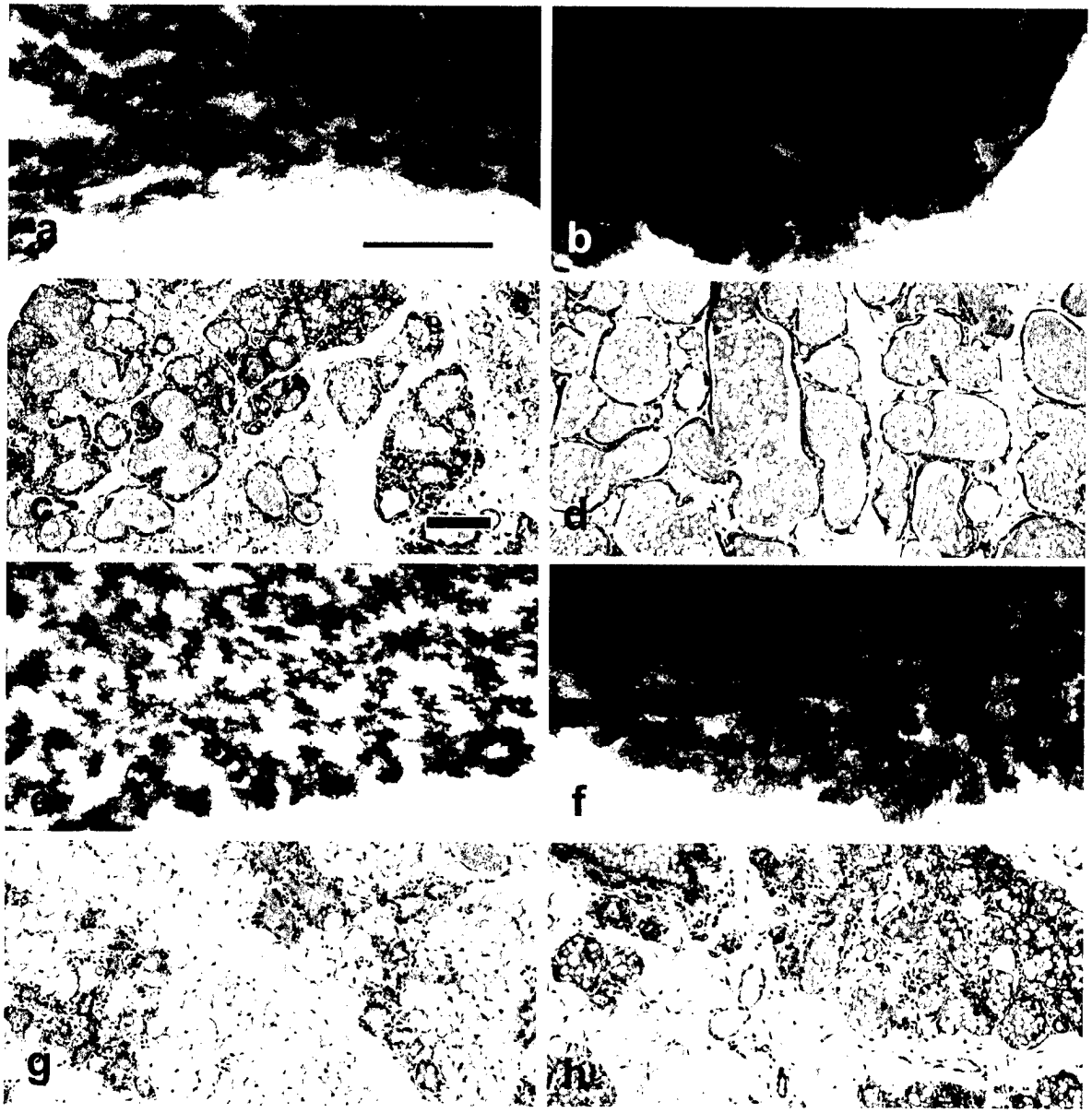

FIG. 3. Mammary epithelial expression of Akt results in defect in mammary gland involution. Digital images of the involution patterns in wild-type (a, c, e, and g) and Akt7 (b, d, f, and h) mammary glands. The images compare whole-mount preparations (a, e, b, and f) of the mammary gland (scale bar = 1 mm) with the histological pattern (c, d, g, and h) (scale bar = 0.1 mm) on days 1 (a to d) and 3 (e to h) of involution. Note the delayed involution in the Akt7 mouse mammary gland (b to h).

derived from the involuting FVB/n glands exhibited elevated levels of apoptotic cell death relative to mammary epithelium of the Akt7 strain (compare Fig. 4a and b). 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.

Although these data suggest that the Akt-DD mutant can interfere with apoptotic cell death during mammary gland involution, its role in mammary tumorigenesis is unclear. To explore whether active Akt expression could complement the defect in tumorigenesis exhibited by transgenic mice expressing the mutant PyV mT decoupled from the PI3K/Akt signaling pathway, bitransgenics expressing both the Akt transgene and the mutant mT transgene (MTY315/322F) were derived (Fig. 1b) and monitored for tumor formation by physical palpation. The results of these analyses revealed that bitransgenic mice developed multifocal mammary tumors with 100% penetrance with an average latency of 46 days (Fig. 5a). In contrast, physical palpation of two independent cohorts of female mice carrying the mutant PyV mT transgene alone revealed a significant delay in the onset of tumor formation with average latencies of 123 and 119 days, respectively (Fig. 5a). In addition, these tumors were focal in nature, arising next to hyperplastic mammary epithelium. Consistent with these kinetic analyses, whole-mount analyses of virgin mammary glands of bitransgenic mice revealed a dramatic difference in the extent of tumor growth (compare Fig. 2e and f to c and d). In contrast to the diffuse cystic hyperplasias exhibited by the mutant PyV

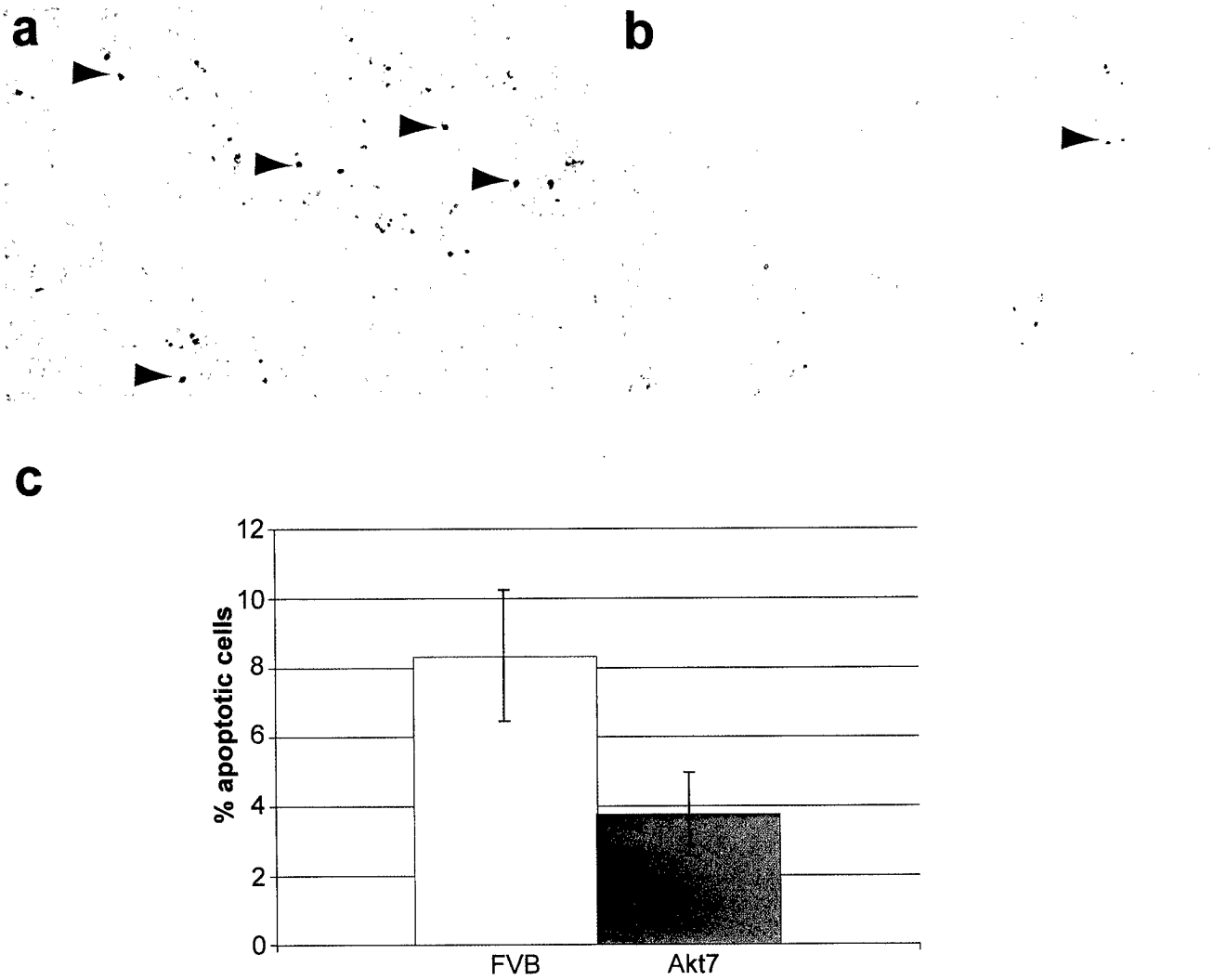

FIG. 4. Mammary epithelial expression of Akt results in decreased mammary gland apoptosis during involution. (a and b) TUNEL analysis of involuting mammary glands from FVB/n (a) and Akt7 (b) at 3 days postparturition. Arrows indicate representative apoptotic cells. (c) Mammary apoptotic indices of FVB/n 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

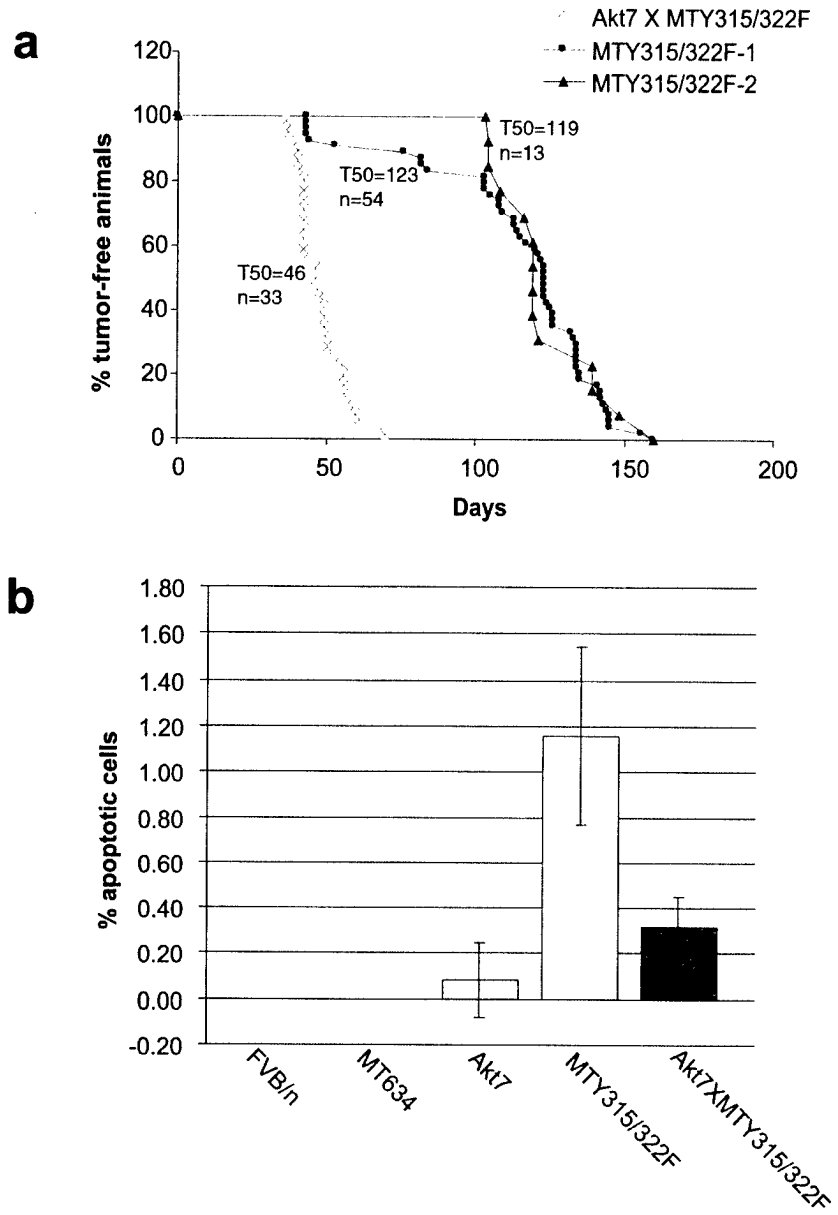

FIG. 5. Mammary tumor kinetics and apoptotic indices in transgenic strains. (a) Mammary tumor kinetics of MTY315/322F and Akt7 × MTY315/322F strains. Two different kinetics curves are shown for the MTY315/322F strain, from the original published data (MTY315/322F-1) and confirmatory data us (MTY315/322F-2), to account for possible differences in palpation technique between researchers. The age indicated is that at which a mammary tumor is first palpable in each transgenic strain. The number of animals analyzed for each strain (*n*) and the median age at which tumors were palpable are also shown. (b) Mammary apoptotic indices of FVB/n, MT634 (wild-type mT), Akt7, MTY315/322F, and Akt7 × MTY315/322F strains. Values shown represent the percentage of total cells stained positive for apoptosis by TUNEL assay in virgin female mice at 10 to 12 weeks of age.

points. The penetrance of the metastatic phenotype is comparable to the 30% metastasis levels exhibited by the parental mutant PyV strains. In contrast, 100% of mice expressing wild-type mT show multiple lung metastases at comparable time points and tumor loads (18). These observations argue that while expression of active Akt can complement the defect in mammary tumor progression, it is unable to rescue the defect in metastatic progression.

To further explore the molecular basis for the observed cooperative interaction between activated Akt and the mutant PyV mT oncogene, we assessed the status of some of the known targets of Akt, including BAD (9), I-κ-B (20), and the FKHR forkhead transcription factor (34).

No significant differences in either BAD-Ser136 phosphorylation or I-κ-B levels were observed between the various transgenic strains (data not shown). Caspase 9, another Akt substrate (4), was not examined, as the Akt phosphorylation site found in human caspase 9 is absent in mouse caspase 9 (17). However, analysis of protein lysates from mammary tissues of 8-week-old virgin FVB/n, Akt7, mutant PyV mT, and bigenic mice subjected to immunoblot analyses with phospho-specific antisera to serine 256 of FKHR (Fig. 7a) revealed that

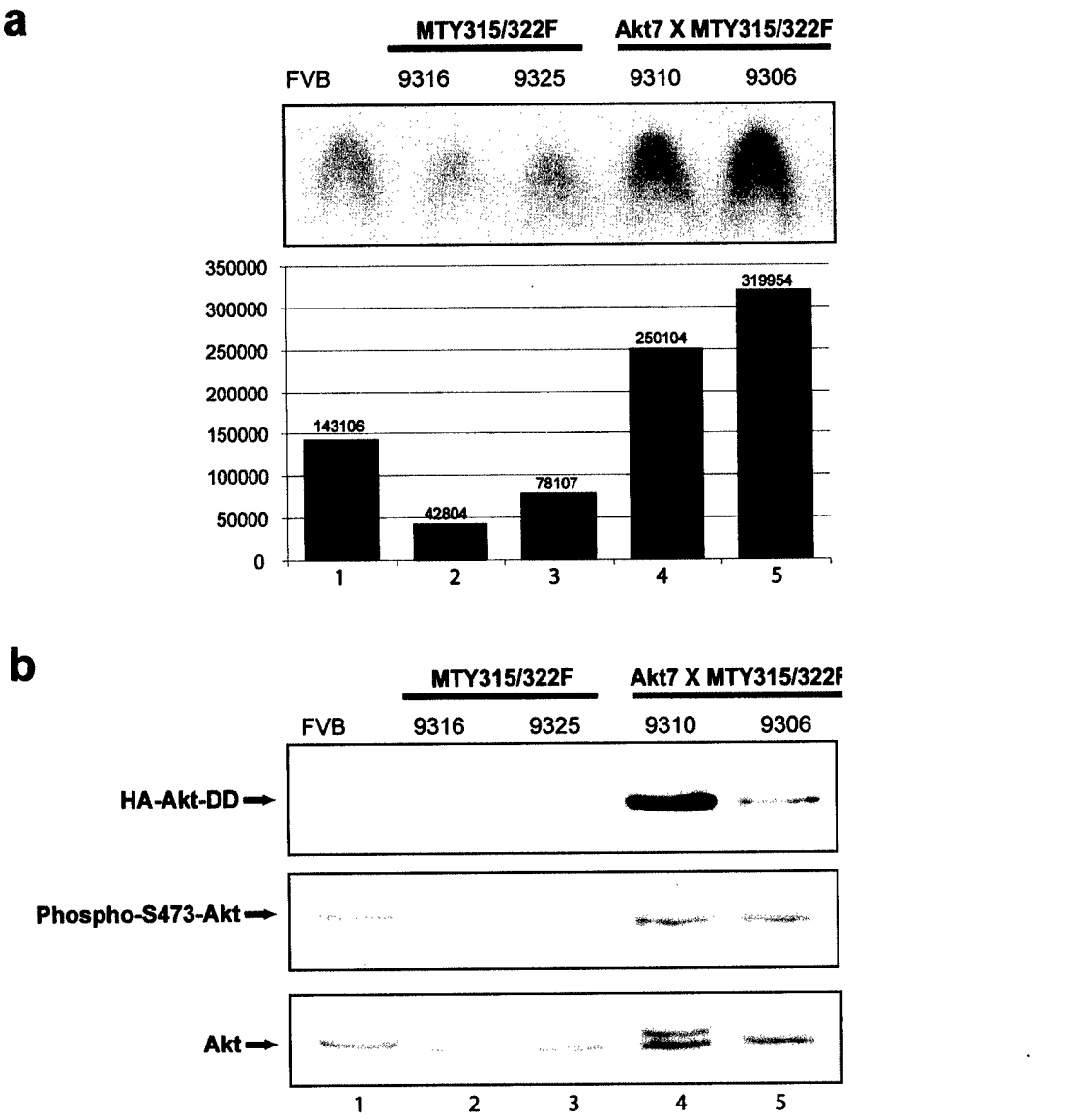

FIG. 6. Akt kinase activity in transgenic strains. (a) Total Akt kinase activity analysis in 8- to 10-week-old virgin females from FVB/n (lane 1), MTY135/322F (lanes 2 and 3), and bitransgenic Akt7 × MTY315/322F (lanes 4 and 5) strains. Assays were conducted using the cross-tide peptide as an Akt kinase 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

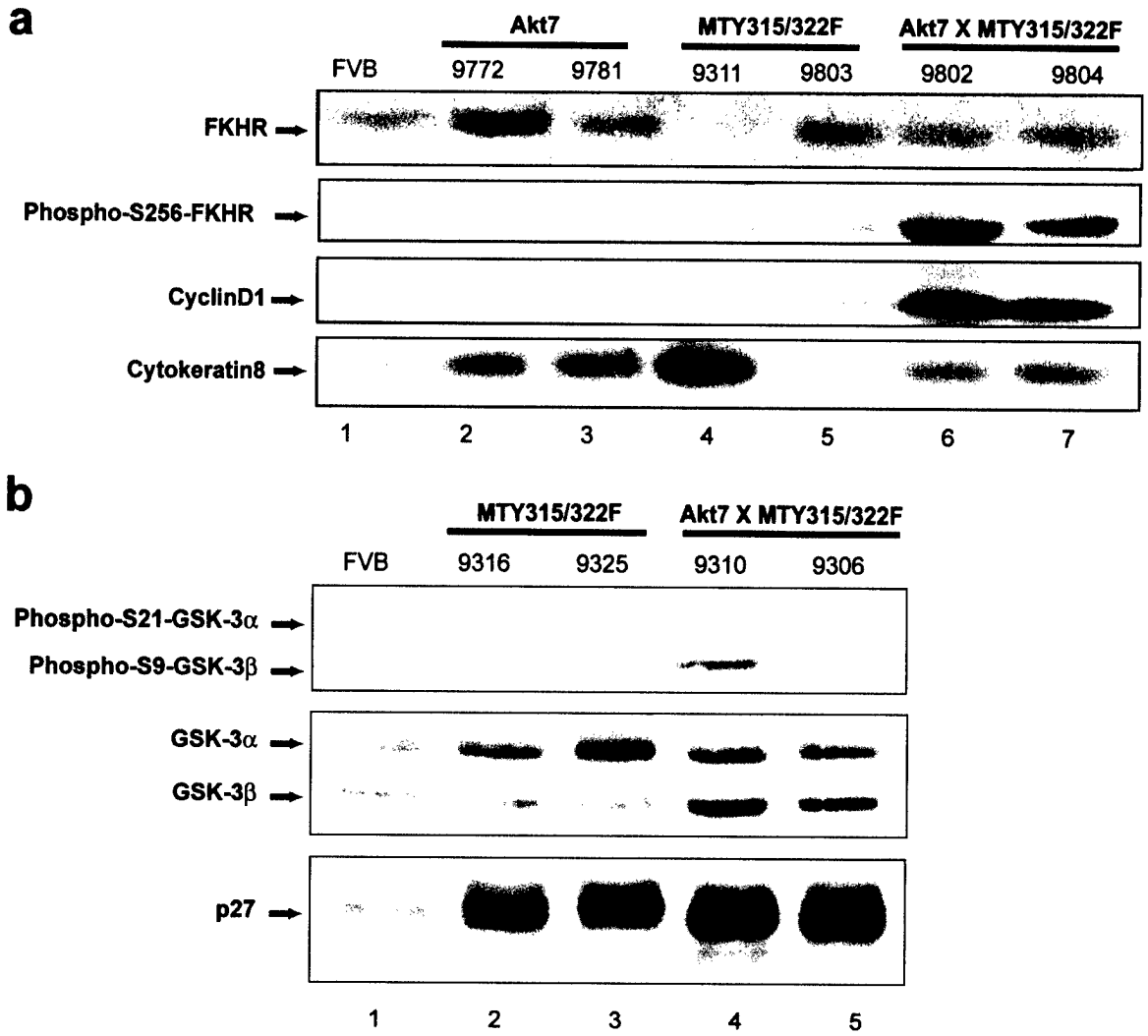

FIG. 7. Coexpression of activated Akt and MTY315/322F results in FKHR phosphorylation at serine 256 and increased cyclin D1 levels but does not affect GSK-3 phosphorylation or p27 levels. (a) Immunoblot analysis of expression of FKHR, phospho-FKHR (Ser256), cyclin D1, and cytokeratin in 8- to 10-week-old virgin females from FVB/n (lane 1), Akt7 (lanes 2 and 3), MTY135/322F (lanes 4 and 5), and bitransgenic Akt7 × MTY315/322F (lanes 6 and 7) strains. All tissues were derived from 8- to 10-week virgin mammary glands. The arrows indicate the migration of FKHR (upper panel), phospho-FKHR (Ser256) (second panel), cyclin D1 (third panel), and cytokeratin proteins (lower panel). The numbers above each lane indicate individual mouse identification numbers. (b) Immunoblot analysis of expression of phospho-S21-GSK-3α, phospho-S9-GSK-3β, GSK-3α/β, and p27 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 virgin mammary glands. The arrows indicate the migration of phospho-S21-GSK-3α and phospho-S9-GSK-3β (upper panel), GSK-3α/β (middle panel), and p27 (lower panel). The numbers above each lane indicate individual mouse identification numbers.

pathway by expression of the PTEN tumor suppressor results in downregulation of cyclin D1 expression and cell cycle arrest (32, 36). To determine whether the levels of cyclin D1 could be influenced by Akt activation, the identical set of mammary tissues were subjected to immunoblot analyses with cyclin D1-specific antibodies. The results of these analyses revealed that the bitransgenic tissues coexpressing both Akt-DD and the mutant PyV mT oncogene exhibited dramatically elevated levels of cyclin D1 (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). Taken together these observations suggest that activation of FKHR and cyclin D1 proteins are involved in promoting tumor progression in these strains.

The studies outlined above provide compelling evidence that expression of activated Akt is involved in promoting tumor progression by providing a critical cell survival pathway. Consistent with this contention, mammary epithelial expression of Akt can result in profound delays in mammary gland involution, a process involving extensive apoptotic cell death. Moreover, coexpression of activated Akt can suppress the elevated rates of apoptotic cell death that are observed in mammary epithelial hyperplasias induced by the mutant PyV mT decoupled from the PI3K signaling pathway. However, because mammary epithelial expression of activated Akt does not result

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

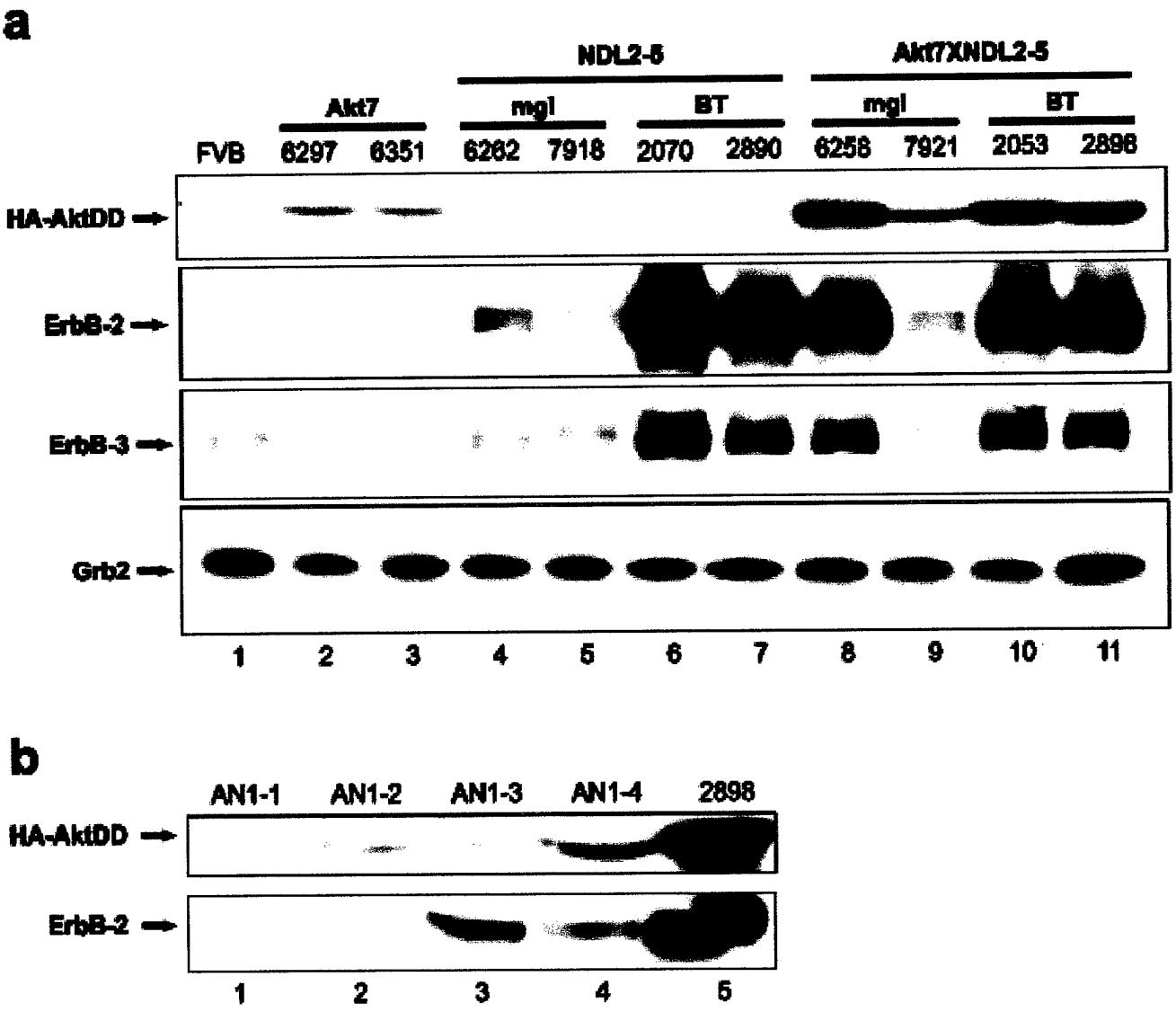

Figure 1 - Activated Akt transgene is expressed in Akt7XNDL2-5 bitransgenics and tumor derived cell lines.

(a) Immunoblot analyses of expression of HA-AktDD, ErbB-2 and ErbB-3 in mammary glands from 8-10 week old virgin females from FVB (lane1), MMTV/Akt7 (lanes 2 and 3), MMTV/NDL2-5 (lanes 4 and 5), MMTV/NDL2-5XAkt7 (lanes 8 and 9), and breast tumors from 40-42 week old MMTV/NDL2-5 virgin females (lanes 6 and 7) and 23-24 week old virgin MMTV/NDL2-5XAkt7 females (lanes 10 and 11). The arrows indicate the migration of HA-AktDD (upper panel), ErbB-2 (upper middle panel), ErbB-3 (lower middle panel) and a Grb2 loading control (lower panel). The numbers above each lane indicate mouse identification numbers.

(b) Immunoblot analyses of expression of HA-AktDD and ErbB-2 in MMTV/NDL2-5XAkt7 tumor derived cell lines (lanes 1-4) and breast tumor from a 24 week old virgin MMTV/NDL2-5XAkt7 female (lane 5). The arrows indicate the migration of HA-AktDD (upper panel) and ErbB-2 (lower panel). The identity of each cell line and mouse is indicated above each lane.

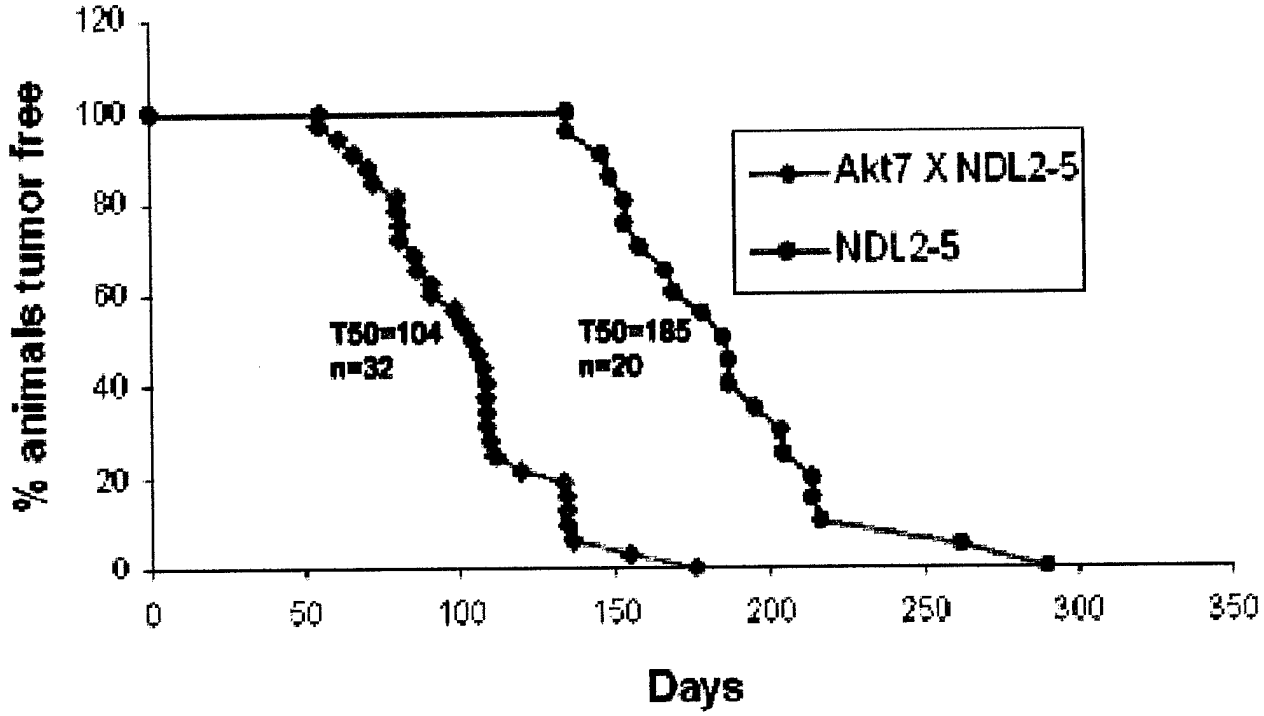

Figure 2 - Tumor Kinetics in Transgenic Strains

Mammary tumor kinetics of MMTV/NDL2-5 and MMTV/NDL2-5XAkt7strains. Age indicated is that at which a mammary tumor is first palpable in each transgenic strain. The number of animals analyzed for each strain (n) and the median age at which tumors are palpable is also shown.

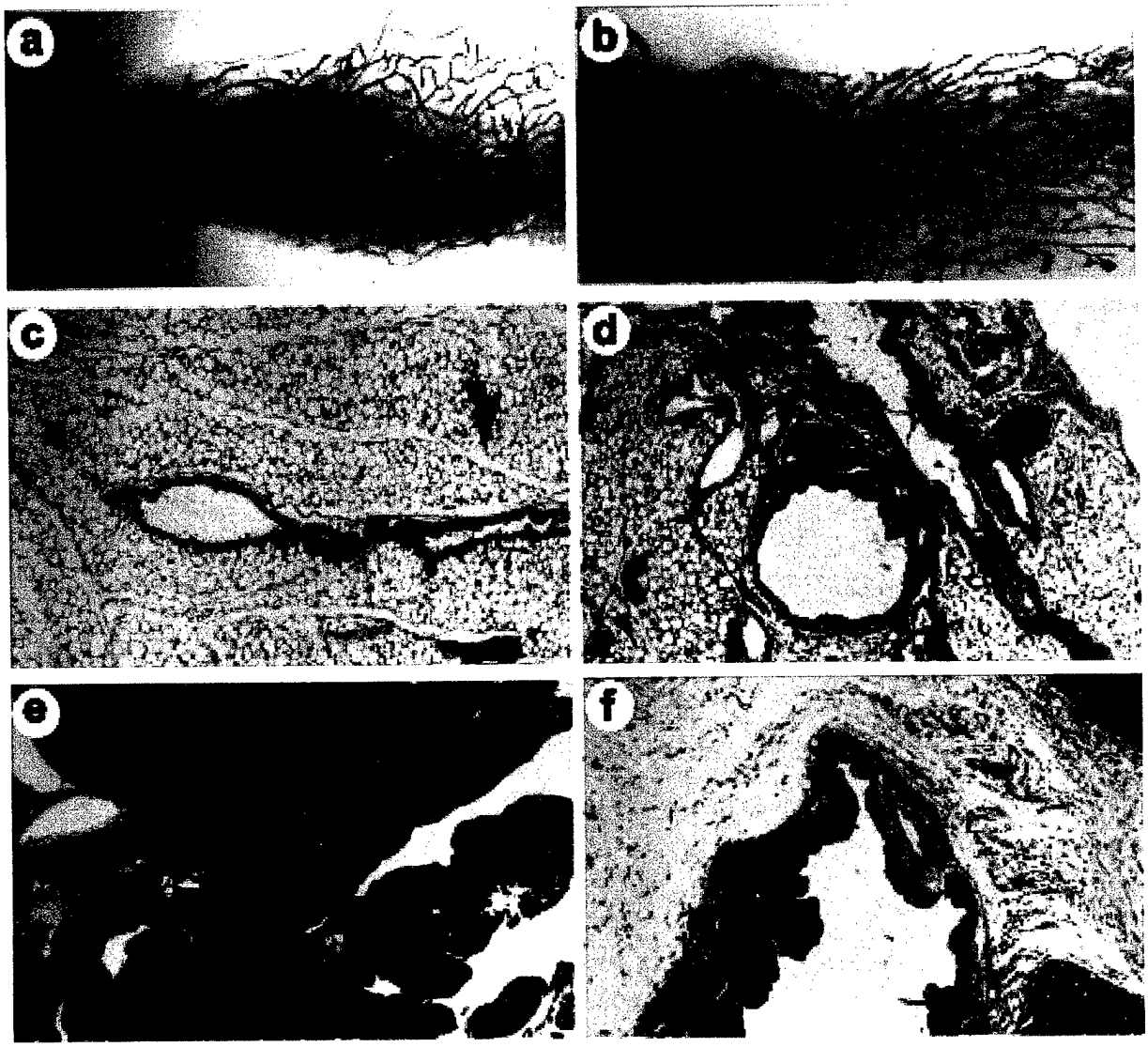

Figure 3 - Coexpression of HA-AktDD and NDL2 results in the induction of multifocal mammary tumors as early as 10 weeks of age.

(a-f) These digital images illustrate the histological patterns observed in the MMTV/NDL2-5 (b,d and f) and MMTV/NDL2-5XAkt7 bigenic mice (c, e and g)). Note that the wholemount preparations **(a,b)** demonstrate that the MMTV/NDL2-5 strain has a relatively normal mammary tree with some small lateral side buds **(a)** compared to the cystic numerous lateral side buds and atypical focal lesions observed in the MMTV/NDL2-5XAkt7 strain at the same age **(b)** (10 weeks). The histological patterns seen at high magnification demonstrate that the NDL2-5 strain has a normal epithelium **(c)**, while the bigenic strain at the same age (13 weeks) shows numerous areas of lobular development and cysts with atypical hyperplasia **(d)**. At more advanced ages **(e and f)** the NDL2-5 strain (41 weeks) shows the development of nodular tumors with the characteristic erbB2 cytology **(e)**. In contrast, the bigenic strain (20 weeks) shows the formation of large cysts lined by irregular multi-layered atypical epithelium that is papillary in some areas **(f)**.

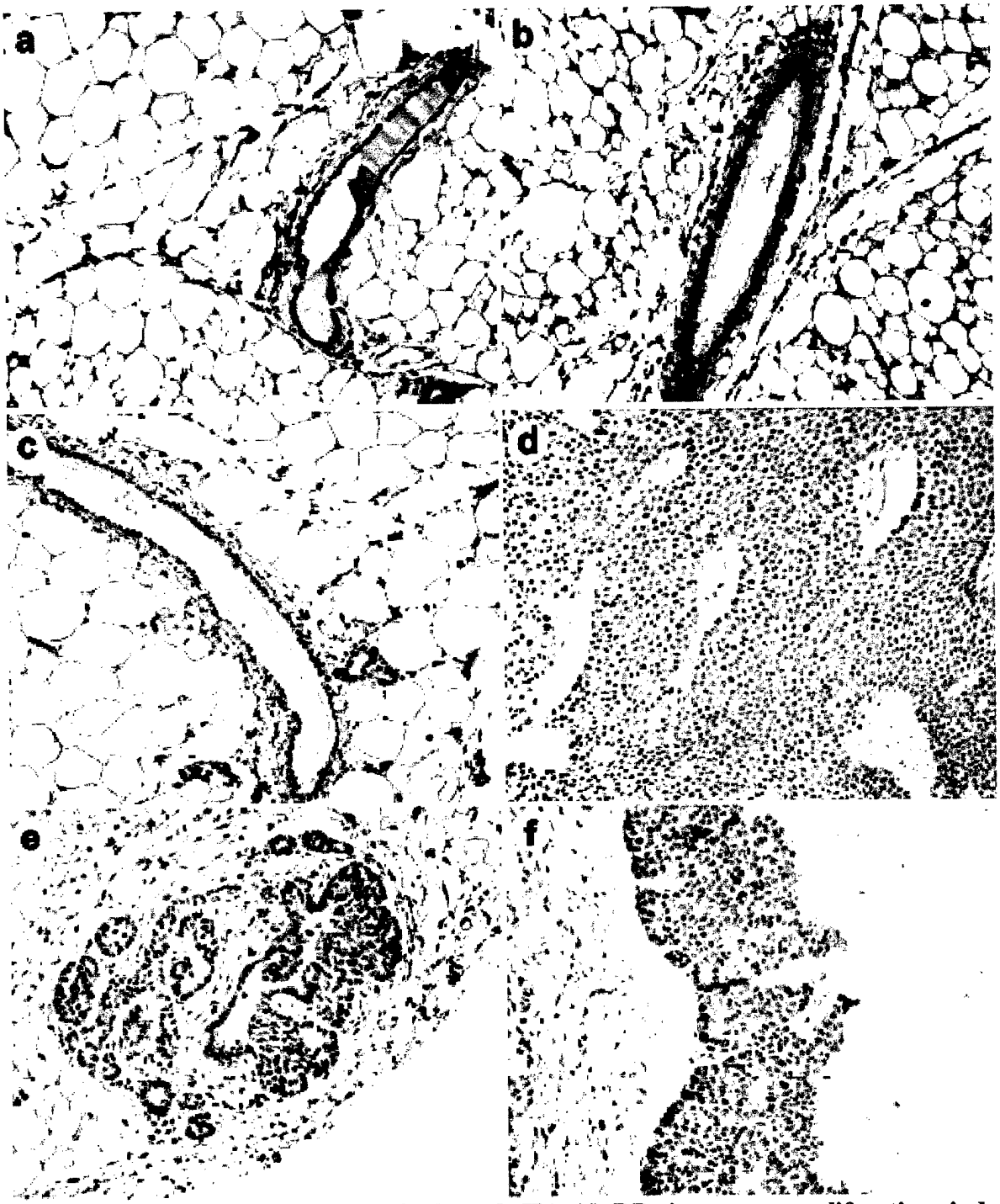

Figure 4 - Co-expression of NDL2 and HA-AktDD increases proliferation index of mammary epithelial cells.

Ki67 immunohistochemical analysis of virgin mammary glands from FVB/n **(a)**, MMTV/Akt7 **(b)**, MMT/NDL2-5 **(c and d)** and MMTV/NDL2-5XAkt7 **(e and f)** strains. at 10 weeks **(a, b, c, e)**, 20 weeks **(f)** and 33 weeks **(d)** of age. Cells were stained brown for Ki67 with DAB and counterstained blue-green with methyl green. Note the increased staining in the 10 weeks MMTV/NDL2-5XAkt7 sample **(e)** as compared to the age-matched MMTV/NDL2-5 sample **(c)**.

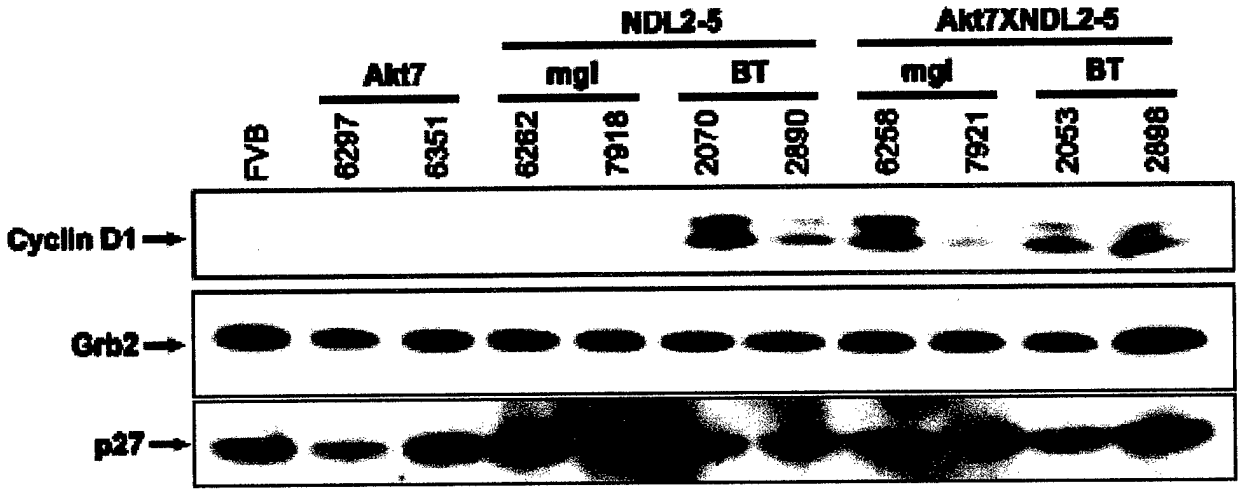

Figure 5 - Cyclin D1 is overexpressed in MMTV/NDL2-5XAkt7 mammary glands.
Immunoblot analyses of expression of Cyclin D1, Grb2 and p27 in mammary glands from 8-10 week old virgin females from FVB (lane1), MMTV/Akt7 (lanes 2 and 3), MMTV/NDL2-5 (lanes 4 and 5), MMTV/NDL2-5XAkt7 (lanes 8 and 9), and breast tumors from 33-40 week old MMTV/NDL2-5 virgin females (lanes 6 and 7) and 23-32 week old virgin MMTV/NDL2-5XAkt7 females (lanes 10 and 11). The arrows indicate the migration of Cyclin D1(upper panel), Grb2 loading control (middle panel) and p27 (lower panel). The numbers above each lane indicate mouse identification numbers.

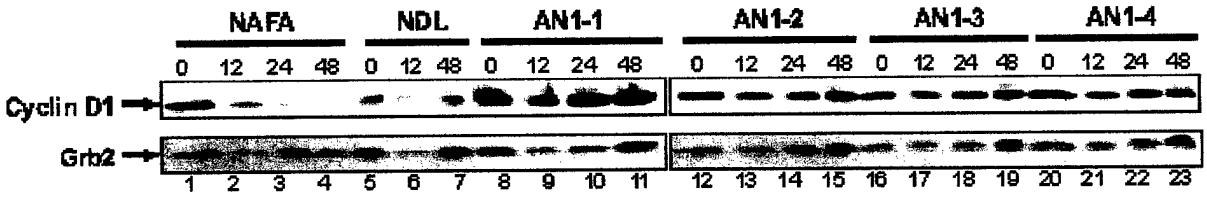

Figure 6 – Effect of Translation Inhibition on Cyclin D1 levels in MMTV/NDL2-5XAkt7 mammary gland derived cell lines.

Immunoblot analyses of expression of Cyclin D1 and Grb2 (loading control) in MMTV/N202 (lanes 1 and 7), MMTV/NDL1-2 (lanes 2 and8) and MMTV/NDL2-5XAkt7 tumor derived cell lines (lanes 3-6 and 9-12) treated with 40ng/ml of rapamycin for 0 to 48 hours. The arrows indicate the migration of Cyclin D1 (upper panel) and Grb2 (lower panel). The numbers above each lane indicate the number of hours of rapamycin treatment and the identity of each cell line is indicated above these numbers.

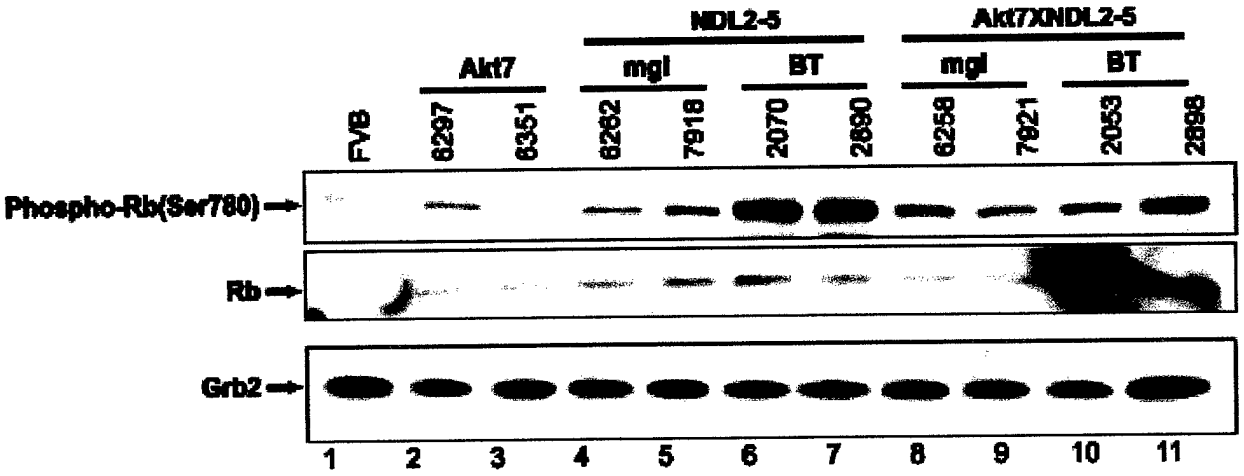

Figure 7 - Cyclin D1 is functional in MMTV/NDL2-5 and bitransgenic MMTV/NDL2-5XAkt7 mammary glands.

(a) Immunoblot analyses of expression of Cyclin D1, Phospho-Rb(Ser780) and Rb in mammary glands from 8-10 week old virgin females from FVB (lane1), MMTV/Akt7 (lanes 2 and 3), MMTV/NDL2-5 (lanes 4 and 5), MMTV/NDL2-5XAkt7 (lanes 8 and 9), and breast tumors from 33-40 week old MMTV/NDL2-5 virgin females (lanes 6 and 7) and 23-32 week old virgin MMTV/NDL2-5XAkt7 females (lanes 10 and 11). The arrows indicate the migration of Cyclin D1(upper panel), Phospho-Rb(Ser780) (middle panel) and Rb(lower panel). The numbers above each lane indicate mouse identification numbers.

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

adenocarcinomas that histologically resemble human comedocarcinomas (Muller *et al.*, 1988; Bouchard *et al.*, 1989; Cardiff and Muller, 1993; Guy *et al.*, 1996; Stocklin *et al.*, 1993).

Although these studies suggest a significant role for ErbB-2 in human breast cancer progression, the lack of a comparable mutation in human breast cancers suggests that the primary mechanism operating in human breast cancer is the overexpression of wild-type ErbB-2 and not its mutational activation (Lemoine *et al.*, 1990; Slamon, 1989; Zoll *et al.*, 1992). Consequently, a more relevant model in which a wild-type *neu* cDNA was expressed under MMTV control was generated to test the oncogenic potential of the wild-type receptor. These mice develop focal mammary tumors of similar comedocarcinoma-type morphology after an average of 7 months which frequently metastasize to the lung (Guy *et al.*, 1992). Further examination of the ErbB-2 status in these tumors revealed that tumors but not the adjacent normal mammary epithelium carried sporadic mutations in *neu* which resulted in its constitutive activation (Siegel *et al.*, 1994). These mutations were comprised of multiple in frame deletions, insertions or point mutations in the extracellular domain of *neu* and promoted the transforming ability of *neu* through the formation of intermolecular di-sulfide bonds (Siegel and Muller, 1996). To directly explore the importance of these activated forms of Neu, transgenic mice carrying altered Neu receptors were derived. Females from these lines develop multiple mammary tumors that frequently metastasize to the lung with a mean onset between that of the normal and point-activated alleles (Siegel *et al.*, 1999). Interestingly, tumor progression in these strains was associated with elevated levels of tyrosine-phosphorylated Neu and ErbB-3 (Siegel *et al.*, 1999). Consistent with these observations, a survey of primary human breast tumors revealed frequent co-expression of both ErbB-2 and ErbB-3 transcripts (Siegel *et al.*, 1999). These results suggest that ErbB-3 may be the critical heterodimerization partner for Neu in breast cancer progression.

Although the transmembrane point mutation has not been detected in primary human breast cancers overexpressing ErbB-2, studies have detected an alternative splice form in human breast cancers and breast cancer-derived cell lines (Kwong and Hung, 1998; Siegel *et al.*, 1999). Significantly, this splice isoform closely resembles the *neu* deletion mutants observed in the transgenic line overexpressing wildtype *neu* with a 16 amino acid deletion in the juxta-transmembrane region of ErbB-2. Like the sporadic *neu* mutants, this splice isoform is oncogenic due to its capacity to form constitutively active dimers. Conceivably the observed high rate of mutations observed in the transgene in the Neu transgenic strain reflects the fact that the transgene was originally derived from a *neu* cDNA that is incapable of undergoing alternative splicing. Further studies will be required to assess the significance of this splice isoform in the pathogenesis of human breast cancer. Taken together these transgenic studies strongly implicate the activation of ErbB-2 through receptor dimerization as a critical step in mammary tumorigenesis.

Role of growth factors in breast cancer progression

EGFR ligands

As alluded to above, the expression of EGFR family members plays a critical role in the induction of mammary cancers. Another way to activate members of the EGFR receptor family is through elevated expression of their ligands. Indeed, expression of EGFR ligands TGF-α and amphiregulin can be detected in erbB-2 induced mammary tumors (Kenney *et al.*, 1996). Transforming factor alpha is a peptide hormone first isolated from retrovirus-transformed cells and subsequently identified in the conditioned media of breast cancer-derived cell lines and and in invasive ductal carcinomas. TGF-α possesses strong homology to EGF and like EGF acts as a ligand for the EGFR. TGF and EGFR expression has been found to coincide with normal mammary epithelial proliferation *in vivo* (reviewed in Humphreys and Hennighausen, 2000). Early studies with transgenic mice expressing TGF-α weakly in the mammary gland showed increased cellular proliferation and fat pad developmental defects (Sandgren *et al.*, 1990). These mice displayed mammary epithelial hyperplasias and dysplasias after multiple pregnancies (Sandgren *et al.*, 1990). Transgenic mice expressing TGF-α under MMTV control showed developmental defects and hyperplasias in virgin mice (Matsui *et al.*, 1990). These hyperplasias were observed to progress towards dysplasias with multiple pregnancies and increasing age, with 40% of multiparous animals showing tumors at 16 months of age (Halter *et al.*, 1992). More dramatic effects were achieved by targeting TGF-α to the mammary gland with the WAP promoter. Mice expressing TGF-α under WAP control showed increased incidence and shorter latency of tumor formation as compared to the MMTV models (Sandgren *et al.*, 1995). The relationship between mammary development and tumorigenesis is highlighted by the observation that these mice also displayed significant delays in mammary gland involution (Sandgren *et al.*, 1995). As these mice still required multiple pregnancies for tumors to form, it is possible that this delay in involution may act to provide an expanded population of proliferating epithelial cells predisposed to transformation (Humphreys and Hennighausen, 2000). However, the long latency observed once again indicates that additional genetic events are necessary for tumor progression in this model. In this regard, it is interesting to note that levels of Cyclin D1 were increased in tumors from the WAP-TGF-α mice (Sandgren *et al.*, 1995).

Another class of EGFR ligands that has been implicated in the induction of mammary cancers are members of the heregulin family (Chang *et al.*, 1997). Mammary specific expression of one of the neuregulin isoforms in transgenic mice initially resulted in generation of terminal end bud hyperplasias (Krane and Leder, 1996). However these transgenic mice eventually developed focal mammary tumors and co-expressed constitutively tyrosine phosphorylated ErbB-2 and ErbB-3 receptors. These observations reinforce the importance of ErbB-2 and ErbB-3 heterodimers in the induction of mammary cancers.

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.

Transgenic mouse models of multistep carcinogenesis

One of the major lessons the study of these transgenic models has illustrated is that expression of a single activated oncogene or loss of tumor suppressor gene is not sufficient to convert the mammary epithelial cell to the malignant phenotype. To assess the relative contribution of these genetic events to mammary tumorigenesis, investigators have performed genetic crosses between separate transgenic strains harboring these different genetic lesions. One of the first examples of this approach involved the interbreeding of MMTV/v-Ha-*ras* strains with the MMTV/c-*myc* mice (Sinn *et al.*, 1987). In contrast to either parental strain, bi-transgenic mice expressing both c-*myc* and activated *ras* developed focal mammary tumors with a dramatically shortened latency period. Although these experiments demonstrated that C-myc and activated Ras could cooperate to accelerate mammary tumor formation, both the focal nature of the tumors and the latency period suggested that additional genetic events were required to transform the mammary epithelium to full malignancy.

Another example of this approach is illustrated by crossing the MMTV/c-*myc* strain to mice expressing TGF under MMTV control (Amundadottir *et al.*, 1995). These bi-transgenics exhibited mammary tumors by a mean time of 66 days as compared to 298 days in MMTV/c-*myc* mice. TGF-α mice developed no observable tumors in this study. Significantly, the entire mammary gland was malignant in the bi-transgenics, demonstrating that these two signals are likely sufficient for mammary tumorigenesis to proceed. Interestingly, it appears that both transgenes contribute to mammary epithelial proliferation but TGF-α also acts to prevent c-*myc* induced apoptosis in these tumors (Amundadottir *et al.*, 1995).

Given the ability of EGFR family members to heterodimerize to transduce signals within the cell and the proven importance of Neu and the EGFR ligand TGF-α in mammary tumorigenesis, crosses were generated between transgenics carrying these transgenes under the transcriptional control of MMTV (Muller *et al.*, 1996). Bi-transgenic mice co-expressing TGFα and Neu exhibited accelerated tumor kinetics resulting in multifocal tumors involving the entire mammary gland. In contrast to the parental Neu transgenic strain, mutations could not be detected within the Neu transgene. Conceivably, activation of intrinsic Neu tyrosine kinase activity is achieved in the bi-transgenic tumors by trans-phorylation of Neu by EGFR rather through selection of somatic mutations in the transgene. Interestingly, similar results were obtained in crosses between MMTV/Neu transgenic mice and mice carrying a mutant p53 (*p53 172 R-H*) under WAP control (Li *et al.*, 1997). Again, bi-transgenic mice developed multifocal mammary tumors with a dramatically shorter latency period without evidence of somatic mutations in the transgene. It is possible that inactivation of the p53 tumor suppressor pathway also obviates the selection of activating mutations in the transgene by activating Neu through an independent pathway. In this regard, it is interesting to note that both p53 ablation and TGF-α overexpression are potent anti-apopotic signals.

Consistent with these studies, inactivation of p53 appears to be a critical event in other transgenic model systems. For example, mice deficient in p53 have been crossed to a variety of transgenics including MMTV/Wnt1 (Donehower *et al.*, 1995), MMTV/c-*myc* (Elson and Leder, 1995), MMTV/v-Ha-*ras* (Hundley *et al.*, 1997), WAP/IGF-1 (Hadsell *et al.*, 2000) transgenic lines as well as BRCA1 heterozygotes (Cressman *et al.*, 1999) and the conditional BRCA1 knockouts (Xu *et al.*, 1999). With the exception of the MMTV/c-*myc* and MMTV/v-Ha-*ras* strains which died due to extensive lymphomas, inactivation of p53 resulted in a dramatic acceleration of mammary tumor progression. Mammary tumors found in these bi-transgenics also displayed increased aneuploidy as compared to those found in the mono-transgenic alone (Donehower *et al.*, 1995; Hadsell *et al.*, 2000). These results suggest that an absence of p53 predisposes mammary epithelial cells to genetic instability and tumor formation in the presence of some other initiating event such as a growth signal. This necessity for a proliferative signal balanced with an anti-apoptotic is a common theme in many transgenic models. For instance, overexpression of the cell survival factor, Bcl-2, in the mammary glands of WAP/Tag mice also accelerates tumor formation (Furth *et al.*, 1999). Taken together these studies suggest that suppression of apoptotic cell death is a critical step in mammary tumorigenesis in these transgenic models.

The role of hormones in mammary tumor progression

Many hormones affect the development of the mammary gland and have been tied to breast development and cancer progression. Currently, mouse models exist to address the roles of three of these hormones; prolactin, estrogen, and progesterone. The effects of these hormones on breast cancer have been studied through the use of knockout technologies directed against either their receptors or the hormone itself. Through crosses to established transgenic strains, the contribution of these hormones to the tumorigenesis phenotype is being examined. Together with other lactogenic hormones, prolactin provides signals that drive the development of the mammary gland. Mice lacking the prolactin receptor show defects in mammary gland development with terminal end buds failing to differentiate into proper lobuloalveoli (Brisken *et al.*, 1999). Mice lacking the prolactin hormone itself show arrested mammary gland development. Crosses between the prolactin knockouts and mice expressing the viral oncogene polyomavirus middle T antigen (PyV mT) under MMTV control show slower induced tumor growth than in mice expressing PyV mT alone (Wennbo and Tornell, 2000).

The roles of the steroid hormones estrogen and progesterone in mouse models of breast cancer have both been recently studied. Mice lacking the estrogen receptor alpha, when crossed to MMTV/Wnt1 mice, show greatly decreased tumor kinetics (50%) while having no effect on the formation of early hyperplasias characteristic of the MMTV/Wnt1 strain. Similarly, progesterone receptor function is necessary for mammary gland maturation in normal mammary development (Brisken *et al.*, 1999) as well as for tumorigenesis in a carcinogen-induced mammary tumor model (Lydon *et al.*, 1999). Interestingly, both progesterone

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.

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

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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.

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.