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13. ABSTRACT *(Maximum 200 Words)*

Akt is a serine/threonine protein kinase that suppresses apoptosis in response to a wide variety of stimuli (4-6;8;10-12). Overexpression of Akt has been identified in certain types of cancers (1-3:13:17). Prolactin induces activation of Akt; results described in this summary demonstrate that prolactin may induce activation of Akt through a src-like kinase→Cbl→ PI3K→ Akt pathway. Akt is activated in response to a number of growth factors involved in mammary gland development{198,15,139,473,263,196,197}, we hypothesized that overexpression of Akt in the mammary gland may result in the suppression of apoptosis, possibly leading to tumorigenesis. We generated mice that express a constitutively active from of Akt in the mammary gland. Upon examination of the mammary gland during development in these transgenic mice, we have shown that Akt can suppress apoptosis during mammary gland involution. Our data also suggest that Akt may be involved in lipid synthesis during pregnancy and lactation. Finally, although the presence of myr-Akt in the mammary gland does not result in high levels of spontaneous tumors, it is possible that Akt overexpression may result in the induction of a preneoplastic state and in the presence of other mutations, may result in tumorigenesis.

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Table of Contents

Introduction

Although cancer is typically thought of as the result of uncontrolled cellular proliferation, it can also involve the inability of cells to undergo apoptosis at the correct time. Therefore, numerous studies have focused on the roles of various anti-apoptotic molecules in the induction of cancer. Akt is a serine/threonine protein kinase that has been shown to suppress apoptosis in response to a wide variety of stimuli (4-6;8;10-12). In addition, overexpression of Akt has been identified in certain types of cancers, such as gastric adenocarcinomas (17), and ovarian and pancreatic carcinomas (1-3;13). Our initial studies focused on the ability of prolactin to induce activation of Akt and on the identification of signaling pathways that mediate this activation. Results described in this summary demonstrate that prolactin may induce activation of Akt through a src-like kinase→Cbl→ PI3K→ Akt pathway. Because Akt is activated in response to a number of growth factors that are involved in mammary gland development {198,15,139,473,263,196,197}, we hypothesized that overexpression of Akt in the mammary gland may result in the suppression of apoptosis, possibly leading to tumorigenesis. We have generated mice that express a constitutively active from of Akt in the mammary gland under the control of the mouse mammary tumor virus (MMTV) promoter. Upon examination of the effects of this transgene on the mammary gland during development, we have shown that Akt can suppress apoptosis during mammary gland involution. Surprisingly, our data also suggest that Akt may also be involved in lipid synthesis during pregnancy and lactation. Finally, although the presence of myr-Akt in the mammary gland does not result in high levels of spontaneous tumors, it is possible that Akt overexpression may result in the induction of a preneoplastic state and in the presence of other mutations, may result in tumorigenesis.

Body

The goal of Aim 1 is to identify the upstream signaling molecules that mediate prolactin-induced activation of Akt. Because tyrosine phosphorylation of the prolactin receptor is thought to be critical for the activation of signaling pathways by prolactin (9;15), we obtained a panel of receptor mutants in which the tyrosine residues were sequentially mutated to phenylalanine residues (14). The mutant receptors were stably transfected into 32Dcl3 cells, selected with 1 mg/ml neomycin, and single cell derived clones were isolated by growth in soft agar. Each cell line was examined for the ability to activate Akt following prolactin stimulation. The only cell lines that were capable of activating Akt were the lines containing the wild-type receptor (32D/long) and the mutant receptor containing the tyrosine 580 residue (32D/8FY580) (Figure 1), suggesting that this tyrosine residue may be responsible for mediating Akt activation.

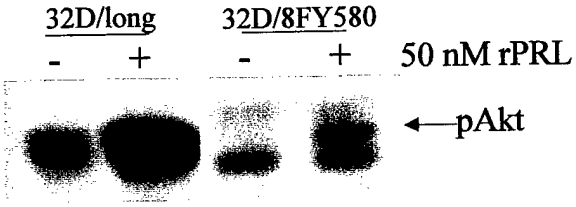


Figure 1: Activation of Akt by 32D cells expressing the wild-type and 8FY580 prolactin receptors. 32D/long and 32D/8FY580 cells were cultured in media containing 2% charcoal stripped serum for 18 hours and were then stimulated with 50 nM rPRL for 30 minutes. The cells were lysed, and equal amounts of proteins were analyzed on an 8% polyacrylamide gel. Immunoblot analysis was performed with an anti-phospho-Akt antibody.

We have also shown that prolactin may mediate activation of Akt through src-like kinases and phosphatidylinositol 3-kinase (PI3K) in Nb2 cells using specific inhibitors. In a previous update, we demonstrated that treatment of the Nb2 cell line with a specific inhibitor of src-like kinases, PP1, results in decreased Akt phosphorylation. We have also examined the phosphorylation state of Cbl, an adaptor protein involved in the PI3K pathway that must be phosphorylated, following PP1 treatment of Nb2 cells. As shown in Figure 2, treatment of cells with PP1 results in decreased tyrosine phosphorylation of Cbl following prolactin stimulation. These studies suggest that prolactin may mediate activation of Akt through a src-like kinase→Cbl→PI3K→Akt pathway in Nb2 cells. However, further experiments are required to confirm these results. Experiments utilizing mutant constructs of the various components of this pathway were described in the original proposal. However, we have not yet developed the optimal method for expressing these constructs in Nb2 cells.

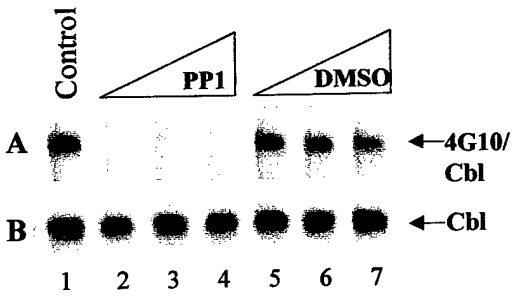


Figure 2: Inhibition of Cbl phosphorylation following PP1 treatment of Nb2 cells. Nb2 cells were incubated overnight in the absence of PRL. Nb2 cells were then stimulated with PRL alone (lane 1), and incubated in the presence of either PP1 (25, 50, 100 μM) (lanes 2-4) or equivalent amounts of DMSO (lanes 5-7) for 4 hours. Cbl was immunoprecipitated using an anti-Cbl antibody and analyzed on a 7.5% SDS-PAGE gel. A) The proteins were transferred to a membrane and immunoblotted with phosphotyrosine antisera (4G10). B) The immunoblot in A was re-probed with an antibody to Cbl to indicate equal loading.

The goal of Aim 2 is to examine the effects of a constitutively active form of Akt on cell survival in various cell lines. To examine this, we had proposed to stably express constitutively active Akt (myr-Akt) in Nb2 cells and 32Dcl3 cells expressing the human form of the prolactin receptor and examine the effects of this construct on cell survival following prolactin withdrawal. Despite extensive efforts to produce a cell line that stably expresses constitutively

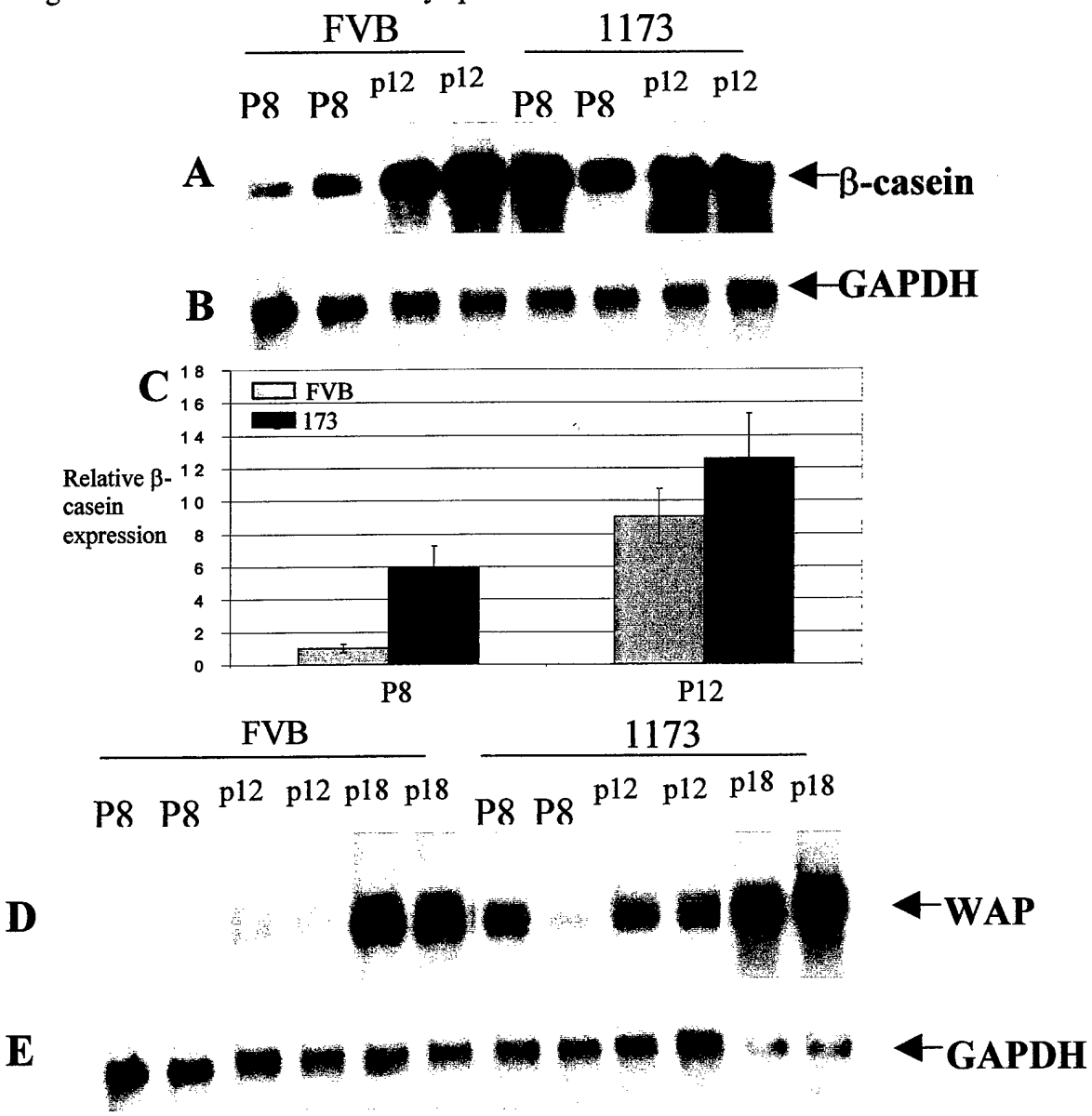
active Akt, we have been unable to generate this line. However, we were able to generate stable cell lines that express wild-type Akt (data not shown). We have also attempted to express myr-Akt in Nb2 cells using adenoviral transduction. However, attempts to express high levels of myr-Akt in these cells have resulted in toxicity. In contrast, studies shown in the previous update demonstrated that adenoviral transduction of myr-Akt into the mammary epithelial cell line, HC-11, results in multiplicity of infection (MOI)-dependent expression of the myr-Akt construct. In addition, we have been able to transfect these cells with myr-Akt cDNA, which also results in detectable levels of expression (data not shown).

The goal of Aim 3 is to determine the effects of myr-Akt expression in mammary glands of transgenic mice. In a previous update, generation of the MMTV-myr-Akt transgenic mice, expression of myr-Akt in the mammary gland, and the effects of myr-Akt on the involution process were discussed. These studies have been continued by examining mammary gland morphology in virgin mice, and during pregnancy and lactation using either whole mount or histological analyses. Early data suggested that expression of myr-Akt is not detectable until lactation in mammary glands from transgenic mice. However, further analysis demonstrates that following longer exposure times of the immunoblots, we can detect transgene expression in mammary glands from virgin and pregnant animals.

Analysis of mammary gland morphology of virgin mice using both whole mounts and histology demonstrates that there are no apparent differences between mammary glands of wild-type mice and mammary glands of transgenic mice at this stage (data not shown). Mammary glands from wild-type and transgenic mice were also analyzed at days 8, 12, and 18 of pregnancy. At day 8 and 12 of pregnancy, alveolar development appears comparable between mammary glands from wild-type mice and mammary glands from transgenic mice (data not shown). However, examination of sections from day 12 at higher magnification reveals some differences in the individual alveoli in mammary glands from transgenic mice compared to those from wild-type mice. In the alveoli from wild-type mice, small lipid droplets are apparent in the alveoli during mid-pregnancy. The lipid droplets are also apparent in the alveoli from transgenic mice; however, they appear to be larger in size. The increased size of lipid droplets is also observed at day 18 of pregnancy in alveoli in transgenic mice. Overall development of the alveoli does not seem to be affected in the transgenic mice. These data suggest that in addition to suppressing apoptosis, myr-Akt may be involved in regulating the synthesis and/or secretion of lipid droplets in developing alveoli during pregnancy.

To further examine the effects of myr-Akt on the mammary gland during pregnancy, expression levels of the milk protein genes β-casein and WAP were examined during pregnancy. β-casein gene expression is an early marker of differentiation and can be detected during early pregnancy. Northern blot analysis using a β-casein specific probe was performed on RNA extracted from mammary glands of both wild-type and transgenic mice at days 8, 12, and 18 of pregnancy. Expression of β-casein in mammary glands from wild-type mice is observed at day 8 of pregnancy and increases by day 12 of pregnancy (Figure 3A). However, it appears that there is more β-casein expression in mammary glands from transgenic mice than wild-type mice at day 8 of pregnancy (Figure 3A). Expression levels of β-casein were analyzed using a phosphorimager, normalized to GAPDH levels (Figure 3B), and β-casein levels were graphed relative to GAPDH (Figure 3C). Consistent with the observations from the autoradiograph, β-casein expression is increased approximately six-fold in mammary glands from transgenic mice at day 8 of pregnancy. WAP gene expression was also examined in mammary glands from transgenic mice at days 8, 12, and 18 of pregnancy. WAP expression is an intermediate marker

of differentiation and is not detected in mammary glands from wild-type mice at day 8 of pregnancy in any sample (Figure 3D and data not shown), even upon overexposure (data not shown). However, in mammary glands from transgenic mice, WAP expression is detectable at day 8 of pregnancy (Figure 3D). WAP expression is consistently detected in every sample examined at this stage, although the level of expression is variable (Figure 3D) and re-probing with a GAPDH specific probe indicates that this variability is not due to a loading artifact (Figure 3E). Expression of WAP increases in mammary glands from transgenic mice by day 12 of pregnancy and remains high through day 18 of pregnancy (Figure 3D). Phosphorimager analysis and normalization of WAP levels to GAPDH levels confirms the observations of the autoradiograph (Figure 3F). These data suggest that precocious expression of milk protein genes is observed in mammary glands of the myr-Akt transgenic mice, suggesting that myr-Akt may be inducing differentiation of the mammary epithelial cells.



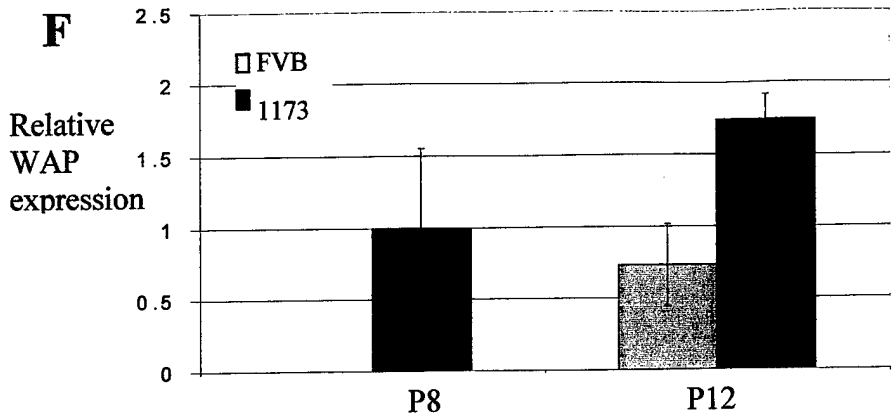


Figure 3: Precocious expression of milk protein genes during pregnancy in mammary glands from MMTV-myr-Akt transgenic mice. Northern blot analysis of total RNA from mammary glands of wild-type (FVB) and transgenic (1173) mice at days 8, 12, and 18 of pregnancy. A) The blot was probed with a β-casein specific probe and B) re-probed with a GAPDH specific probe. C) Phosphorimager analysis of β-casein expression relative to GAPDH expression. D) The blot was probed with a WAP specific probe and E) re-probed with a GAPDH specific probe. F) Phosphorimager analysis of WAP expression relative to GAPDH expression. Error bars represent SEM

To further characterize the MMTV-myr-Akt transgenic mice, mammary gland morphology during lactation was examined using hematoxylin and eosin stained sections from both wild-type and transgenic mice. At day 9 of lactation in mammary glands from transgenic mice, the alveoli have filled the mammary fat pad, but they appear larger and distended compared to the alveoli from wild-type mice. In addition, the lipid droplets observed in the alveoli are larger in size and can be found around the edges of the lumen, possibly in the process of being secreted from the epithelial cells, as well as in the lumen. There also appears to be more proteinaceous material in the alveolar lumina compared to wild-type alveoli, suggesting that milk stasis may be occurring. Similarly, at day 15 of lactation, the alveoli from transgenic mice remain distended. The lipid droplets are also larger and the proteinaceous material persists in alveoli from transgenic mice.

To further examine the role of Akt in cytoplasmic lipid droplet synthesis and accumulation in mammary epithelial cells, we examined the effects of myr-Akt expression on glucose uptake and lipid accumulation in CIT-3 cells, which were derived from the mouse mammary epithelial cell line, Comma 1D. Following adenovirus-mediated expression of myr-Akt in CIT-3 cells, the ability of the cells to uptake [^{3}H]-2-deoxyglucose was analyzed. As shown in Figure 4A, the expression of myr-Akt at a multiplicity of infection (MOI) of 10 results in increased uptake of 2-deoxyglucose. Expression of LacZ at an MOI of 10 does not affect 2-deoxyglucose uptake (Figure 4A), indicating that this observation is specific for myr-Akt rather than an effect of adenoviral transduction. Because increased glucose transport could lead directly to lipid droplet synthesis, we also examined the ability of myr-Akt to induce the formation of cytoplasmic lipid droplets in the CIT-3 cells. Following a 48 hour transduction, cells transduced with either myr-Akt or LacZ were stained with oil red O, which stains accumulated triglycerides. Following extensive washing, the oil red O was extracted from the cells using isopropanol and the amount of staining was quantitated by measuring the absorbance at 490 nm. As shown in Figure 4B, expression of myr-Akt results in increased oil red O staining at an MOI of 10 compared to both mock-transduced and LacZ expressing cells. These results suggest that myr-Akt expression can induce both the uptake of 2-deoxyglucose and the formation of cytoplasmic lipid droplets in mammary epithelial cells, which is consistent with the phenotype observed during lactation in the myr-Akt transgenic mice.

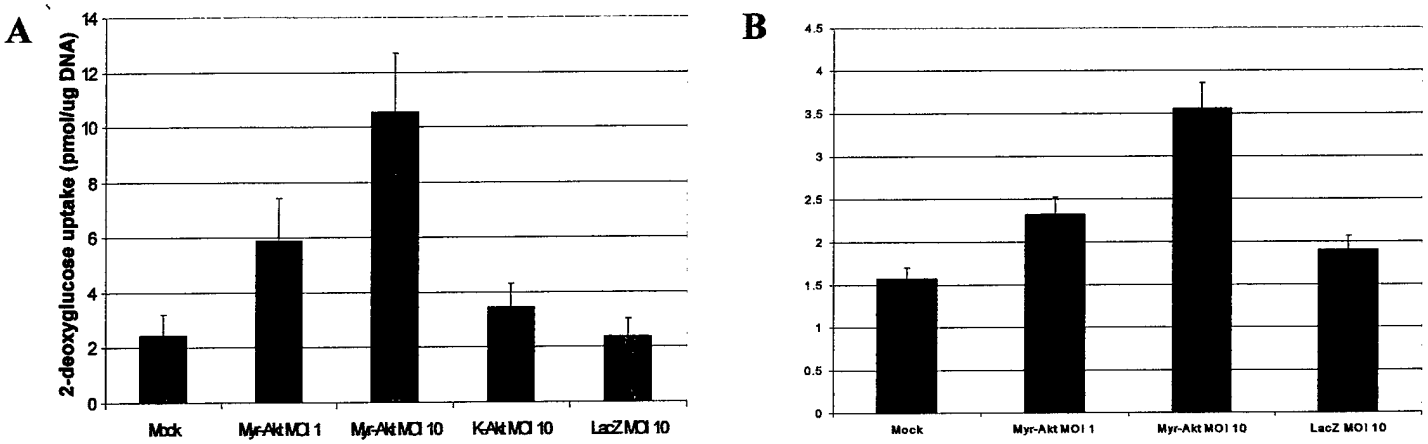


Figure 4: Expression of myr-Akt induces 2-deoxyglucose uptake and lipid production in CIT-3 cells. A) CIT-3 cells were transduced with the following adenoviruses: activated Akt (myr-Akt), kinase inactive Akt (K-Akt), and LacZ. The ability of the cells to uptake ^{3}H-2-deoxyglucose was assessed following a 1 hour incubation in glucose-free medium and a 15 minute incubation with labeled 2-deoxyglucose. B) CIT-3 cells were transduced with either activated Akt (myr-Akt) or LacZ adenovirus. The cells were stained with Oil Red O, washed extensively, and the amount of staining was quantitated by collecting the retained Oil Red O with isopropanol and reading the samples at 490 nm.

We further examined the lactation phenotype by analyzing the composition of milk from the transgenic mice. Although protein composition of milk from transgenic mice is comparable to that from wild-type mice (data not shown), there is a significant increase in the percentage of fat in milk from transgenic mice (67%) compared to milk from wild-type mice (25%) (data not shown). These results suggest that expression of myr-Akt in mammary epithelial cells is affecting lipid synthesis and/or secretion, consistent with the observations made during pregnancy.

Data described in a previous update demonstrates that expression of myr-Akt results in delayed involution and a delay in the onset of apoptosis following forced weaning. We have observed that although the mammary glands eventually regress, there appears to be more epithelium present in mammary glands from transgenic mice compared to those from wild-type mice (see Schwertfeger et al., 2001, Figure 4). To further examine this observation, the amount of epithelium was quantitated at day 21 of involution. There is approximately a 2-fold increase in the amount of epithelium present in mammary glands from transgenic mice compared to those from wild-type mice. It is possible that increased epithelium may result in the subsequent development of hyperplasias in mammary glands from transgenic mice following the first or subsequent pregnancies, however, this possibility remains to be examined.

The formation of spontaneous tumors in MMTV-myr-Akt transgenic mice appears to be infrequent. Two poorly differentiated mammary adenocarcinomas have been identified during pregnancy (data not shown) and during involution that express high levels of myr-Akt transgene. In addition, two hyperplasias have been identified in mammary glands during pregnancy and involution (data not shown), although the presence of myr-Akt transgene in these lesions has not yet been examined. Although it is apparent that expression of myr-Akt is not strong enough to induce tumorigenesis on its own, it is possible that expression of myr-Akt may induce a preneoplastic state in the mammary gland and that other events are required to induce tumorigenesis.

Key Research Accomplishments

- Prolactin-induced Akt activation may be mediated by the tyrosine 580 residue on the prolactin receptor.
- Experiments using the chemical inhibitor PP1 indicate that prolactin-induced activation of Akt may be mediated through a src-like kinase→Cbl→ PI3K→ Akt pathway.
- Expression of myr-Akt in the transgenic mice is observed in mammary glands from virgin and pregnant mice.
- Analysis of mammary glands from transgenic mice during pregnancy and lactation suggests that Akt may be involved in the lipid synthesis pathway in mammary epithelial cells. In addition, mammary gland involution and the onset of apoptosis is delayed in transgenic mice, suggesting that Akt may suppress apoptosis in the mammary gland.
- More epithelium is present in mammary glands from transgenic mice following weaning, suggesting that not all of the epithelial cells are undergoing apoptosis during involution.
- A small number of hyperplasias and tumors have been identified in mammary glands from transgenic mice, suggesting that Akt may induce a preneoplastic state in the mammary gland, although its expression alone is probably not sufficient for the induction of tumorigenesis.

Reportable Outcomes

Abstracts:
Schwertfeger, K.L., Richert, M.M., McManaman, J.L., Lewis, M.T., and Anderson, S.M. "Delayed Mammary Gland Involution and a Defect in Lactation in Transgenic Mice Expressing Activated Akt.".
Presented as a poster at the Mammary Gland Gordon Conference, June, 2001.

Schwertfeger, K.L., McManaman, J.L., Richert, M.M. and Anderson, S.M. "Expression of Constitutively Active Akt in the Mouse Mammary Gland: Effects on Apoptosis and Lipid Synthesis".
Presented as an oral presentation (#53-3) by KLS at the 83rd Meeting of the Endocrine Society, June 2001.

Publications:
Richert, M.M.*, **Schwertfeger, K.L.***, Ryder, J.W., and Anderson, S.M. An Atlas of Mouse Mammary Gland Development. *Journal of Mammary Gland Biology and Neoplasia.* 5(2), 227-241, 2000.
*These authors contributed equally

Schwertfeger, K.L., Richert, M.M., and Anderson, S.M. Mammary Gland Involution is Delayed by Activated Akt in Transgenic Mice. *Molecular Endocrinology.* 15(6), 867-881, 2001.

Conclusions

The initial aims outlined in this grant proposal focus on determining the signaling pathways involved in mediating prolactin-induced activation of Akt and examining the effects of activated Akt on various cell lines. We have preliminary evidence that prolactin can potentially activate Akt through src-like kinases, Cbl, and PI3K in Nb2 cells. However, these results are based on experiments using chemical inhibitors, which raises questions concerning specificity of the inhibitors. Further experiments will be performed using dominant negative and constitutively active mutants of the various signaling molecules in the proposed pathway to determine whether these molecules are responsible for mediating prolactin-induced activation of Akt in Nb2 cells. In addition, these experiments should also be performed in other cell types to be certain that the molecules involved in this signaling pathway are not cell type specific. These results of these experiments contribute to the scientific understanding of how prolactin and potentially other growth factors induce activation of specific signaling pathways and mediate the suppression of apoptosis.

The final aim of the grant focuses on the generation and characterization of the MMTV-myr-Akt transgenic mice. We have identified mice that express the myr-Akt transgene in the mammary gland throughout development. Our initial experiments, which were described in the previous report, demonstrate that expression of myr-Akt in the mammary gland delays involution and suppresses apoptosis of the mammary epithelial cells, indicating that Akt may suppress apoptosis in the mammary gland. In addition, we have also identified another mammary gland phenoype in the MMTV-myr-Akt mice, which is characterized by an increase in the size of lipid droplets during pregnancy and lactation and increased fat in milk from transgenic mice. Interestingly, Akt has been previously shown to mediate lipid synthesis in 3T3-L1 adipocytes {260,247,257}. Therefore, it appears in addition to the suppression of apoptosis, Akt may also be involved in milk fat synthesis during lactation. Further experiments will be performed to examine the ability of myr-Akt to suppress apoptosis and induce lipid synthesis in mammary epithelial cells in culture. Although the MMTV-myr-Akt transgenic mice do not exhibit high rates of mammary tumors, we have identified a small number of tumors and hyperplasias at different times during development. Therefore, it is unlikely that Akt can induce tumorigenesis on its own, but may induce a preneoplastic state in the mammary gland and may induce tumorigenesis in the presence of other mutations. To further examine the potential roles of Akt in inducing tumorigenesis, we will examine mammary glands following one or multiple pregnancies.

The results from the MMTV-myr-Akt transgenic mice contribute to the scientific understanding of the roles of Akt in the mammary gland. Although we had predicted that expression of the transgene in the mammary gland would likely lead to the suppression of apoptosis, we did not predict the potential role for Akt in lactation. Further analyses of tumor development in these mice will lead to better understanding of the roles of cell survival and possibly oncogene cooperativity in mammary tumor development.

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Delayed Mammary Gland Involution and a Defect in Lactation in Transgenic Mice Expressing Activated Akt. Schwertfeger, K.L., Richert, M.M., McManaman, J.L., Lewis, M.T., and Anderson, S.M.

Apoptosis is known to occur in the mammary gland at several developmental time points including in the terminal end buds during puberty and, most dramatically, during involution following weaning. It is not clear what signaling molecules regulate apoptosis during involution. We have focused upon the anti-apoptotic serine/threonine protein kinase Akt (also known as protein kinase B) which is activated by insulin-like growth factor I, epidermal growth factor, prolactin, and estrogen, all of which are important in regulating mammary gland development. Using immunoblot analysis, we show that Akt is expressed in the virgin, pregnant, lactating, and involuting mammary gland. Immunofluorescence analysis using Akt1 and Akt2 specific antibodies indicate differential expression patterns of these two proteins. To examine the role of Akt in regulating mammary gland involution we have generated transgenic mice which express a constitutively activated mutant of Akt referred to as Myr-Akt under the control of the mouse mammary tumor virus promoter. Founder mice were established and the expression of the transgene has been determined using both RT-PCR and immunoblot analysis. Morphological analysis indicates that involution is delayed in transgenic mice following forced weaning. Secretory alveolar structures remain intact for at least 6 days after withdrawal of the pups, and there is a delay in the onset of epithelial cell apoptosis, demonstrating an effect of activated Akt on tissue remodeling. A less dramatic phenotype was also observed in a second transgenic line corresponding to a reduced level of transgene expression. In addition to suppressed involution, there is an effect on lactation since the growth of pups nursed by transgenic females (as determined by weight) is decreased by nearly 50% compared to litters nursed by normal females over the first eight days of life. Histological analysis and immunofluorescence indicate that lipid droplets in the mammary glands from lactating transgenic mice are much larger than in mammary glands from normal mice, and the composition of milk fat is higher in milk from the transgenic mice. These data suggest that Akt is a critical regulator of mammary involution and may also influence lactation by affecting lipid synthesis and/or secretion.

Expression of Constitutively Active Akt in the Mouse Mammary Gland: Effects on Apoptosis and Lipid Synthesis. Schwertfeger, K.L., McManaman, J.L., Richert, M.M. and Anderson, S.M.

The serine/threonine protein kinase Akt has been shown to suppress apoptosis in a number of systems as well as mediate insulin-stimulated glucose uptake and lipid synthesis. In the mouse mammary gland, Akt is expressed at high levels during lactation, when milk lipid synthesis is high, and decreases at the onset of involution, when apoptosis occurs. This expression pattern suggests potential roles for Akt in both lipid synthesis and suppression of apoptosis in the mammary gland. To examine the role of Akt in mammary gland development, transgenic mice were generated that express a constitutively active form of Akt (myr-Akt) under the control of the MMTV promoter. Immunoblot analysis shows transgene expression throughout mammary gland development, with highest levels of expression during lactation and early in involution. We have shown that expression of the myr-Akt transgene results in delayed involution, consistent with the anti-apoptotic function of Akt. In addition, the transgenic mice exhibit a defect in lactation, indicated by decreased pup weight in litters nursed by transgenic mothers. Histological analysis of mammary glands from pregnant and lactating mice indicates an increased size of milk fat droplets in mammary glands from transgenic mice compared to those from wild-type mice. Although milk protein composition appears normal, the percentage of milk fat is dramatically increased in milk from transgenic mice (67%) compared to milk from wild-type mice (25%). Experiments are currently in progress to examine the downstream effects of Akt that could result in the lactation phenotype. In addition, gene array analysis is being utilized to identify differentially regulated genes during lactation in mammary glands from transgenic and wild-type mice, which should elucidate the downstream effects of Akt in this phenotype. In conclusion, analysis of the expression of myr-Akt in the mammary glands of transgenic mice suggests a dual function for Akt in the mammary gland as both a regulator of apoptosis and a mediator of lipid synthesis.