Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation

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Abstract **Expression of constitutively activated Akt in the mammary glands of transgenic mice results in a delay in post-lactational involution. We now report precocious lipid accumulation in the alveolar epithelium of mouse mammary tumor virus-myr-Akt transgenic mice accompanied by a lactation defect that results in a 50% decrease in litter weight over the first 9 days of lactation. Although ductal structures and alveolar units develop normally during pregnancy, cytoplasmic lipid droplets appeared precociously in mammary epithelial cells in early pregnancy and were accompanied by increased expression of adipophilin, which is associated with lipid droplets. By late pregnancy the lipid droplets had become significantly larger than in nontransgenic mice, and they persisted into lactation. The fat content of milk from lactating myr-Akt transgenic mice was 65–70% by volume compared to 25–30% in wild-type mice. The diminished growth of pups nursed by transgenic mothers could result from the high viscosity of the milk and the inability of the pups to remove sufficient quantities of milk by suckling. Transduction of the CIT3 mammary epithelial cell line with a recombinant human adenovirus encoding myr-Akt resulted in an increase in glucose transport and lipid biosynthesis, suggesting that Akt plays an important role in regulation of lipid metabolism.**—Schwertfeger, K. L., J. L. McManaman, C. A. Palmer, M. C. Neville, and S. M. Anderson. **Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation. *J. Lipid Res.* 2003.** 44: **1100–1112.**

Supplementary key words transgenic mice • glucose transport • milk fat globule • lipid biosynthesis

Milk lipid is an important source of both calories and essential fatty acids for the newborn. The unique demands of lactation make the lactating mammary gland among the most active triglyceride-synthesizing organs in the body. For example, a fully lactating woman secretes 800 ml of milk containing 4% fat per day; thus, the mammary gland synthesizes about 32 g of triglyceride daily or nearly 6 kg fat during a typical 6 month lactation (1). The mammary gland of the lactating mouse performs an even more prodigious feat: in the FVB strain used in the experiments described in these studies, the mammary glands are estimated to secrete about 5 ml of milk per day containing about 30% lipid, 1 or 1.5 g of lipid per day. Over the course of 20 days of lactation, the dam thus secretes about 30 g of milk lipid, equivalent to her entire body weight!

When one considers that the capacity for synthesizing this amount of lipid must be developed during a 20 day pregnancy and activated precisely at the time of birth, the regulatory mechanisms in both the developmental (pregnancy) and secretory (lactation) phases become extremely interesting. Mammary gland development during pregnancy is characterized by extensive proliferation of alveolar structures accompanied by differentiation of mammary epithelial cells that occurs progressively throughout pregnancy and can be monitored temporally by the regulated expression patterns of specific genes (2–4). For example, increased expression of β-casein is observed early in pregnancy, whereas expression of whey acidic protein (WAP) occurs during mid-pregnancy (2). In addition, cytoplasmic lipid droplets can be observed in the epithelial cells of the developing alveoli during mid and late pregnancy (5, 6) surrounded by the protein adipophilin (ADPH) (7).

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Abbreviations: ADPH, adipophilin; GLUT1, glucose transporter-1; MMTV, mouse mammary tumor virus; WAP, whey acidic protein; WGA, wheat germ agglutinin.

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At parturition, the mammary gland begins copious milk production in response to both a decrease in the level of progesterone caused by the loss of the placenta and the high levels of prolactin produced by the pituitary (5). During lactation, mammary epithelial cells synthesize triglycerides using fatty acids obtained both from the blood stream and by de novo synthesis from glucose (8). The cytoplasmic lipid droplets formed move toward the apical surface of the epithelial cells and are budded into the lumen. This process results in secretion of the milk lipid globule surrounded by a membrane composed of both apical plasma membrane and intracellular components (9). The major proteins identified in the milk lipid globule membrane include butyrophilin (10, 11), xanthine oxidase (12), and ADPH (13). There is good evidence that glucose uptake by mammary epithelial cells for use during de novo fatty acid synthesis, as well as synthesis of lactose, is mediated by the glucose transporter, glucose transporter-1 (GLUT1). Expression of GLUT1 increases during lactation, and a change in its subcellular localization has been observed in mammary epithelial cells during lactation (14–16).

Akt, also known as protein kinase B, is a serine/threonine protein kinase that is activated in response to a wide variety of stimuli. Numerous studies have shown that Akt suppresses apoptosis in response to a number of stimuli, including growth factor withdrawal, detachment from extracellular matrix, UV irradiation, cell cycle discordance, and Fas ligand (17–23). In addition to its role in the suppression of apoptosis, Akt activation is now thought to be involved in regulating insulin-mediated lipid synthesis and glucose uptake in adipocytes. For example, expression of constitutively activated Akt in 3T3-L1 fibroblasts resulted in spontaneous differentiation into adipocytes associated with increased glucose uptake and lipid accumulation in the absence of insulin (24, 25). Further studies in 3T3-L1 adipocytes reveal that Akt may regulate expression levels and/or localization of the glucose transport proteins (24, 26–28).

Akt is activated in response to a number of hormones and growth factors that are involved in mammary gland development, including epidermal growth factor (EGF) (22), insulin-like growth factor I (29, 30), growth hormone (31), RANK ligand (also known as osteoprotegerin-ligand) (32, 33), prolactin (34), and integrins (35), suggesting that Akt may represent a central signaling molecule in mammary gland development and function. To test this hypothesis, we generated transgenic mice that express a constitutively active form of Akt (myr-Akt) under the control of the long terminal repeat of the mouse mammary tumor virus (MMTV) promoter (36). We have previously demonstrated that myr-Akt expression results in delayed involution corresponding with a delay in the onset of apoptosis, consistent with the antiapoptotic function of Akt. Similar results have been obtained by other investigators who used different transgenic models overexpressing Akt (37, 38). Further analyses of mammary glands from the myr-Akt transgenic mice presented here suggest that Akt may also contribute to regulation of milk secretion by modifying the formation and/or accumulation of cytoplasmic lipid droplets in mammary epithelial cells.

MATERIALS AND METHODS

Animals

The generation of the MMTV-myr-Akt transgenic mice has been previously described (36). Additional FVB female mice were obtained from Taconic Laboratory (Germantown, MD). All animals were maintained by the laboratory Animal Care Facility of the University of Colorado Health Sciences Center under protocols approved by the Institutional Animal Care and Use Committee.

Tissue collection

Mammary gland tissues were collected from both wild-type and transgenic female mice during pregnancy and lactation. The left fourth (inguinal) mammary glands were removed, snap frozen in liquid nitrogen for RNA and protein extraction, and stored at −70°C until extraction. The right inguinal mammary glands were removed and fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin and sectioned at 5 microns either to be stained with hematoxylin and eosin or to be used for immunofluorescence. For the pregnancy timepoints, female mice were mated and checked daily for the presence of a vaginal plug. The day of the observed plug was recorded as P1, and tissues were collected at P8, P12, or P18. At least six FVB and six transgenic mice were analyzed per timepoint. For the lactation timepoints, female mice were mated and the resulting litters were normalized to eight pups each. The day of parturition was recorded as L1, and tissues were collected at L2, L9, and L15. At least three FVB and three transgenic mice were analyzed per timepoint. If any of the pups present in these normalized litters died, they were replaced with another pup of the same age obtained from another litter. In studies where the pups were weighed daily to assess weight gain, only the original pups in the normalized litters were weighed; replacement pups were not included in the weight gain analysis since they were not initially nursed by the mother being analyzed.

Northern blot analysis

RNA was extracted from homogenized tissue using Trizol (Life Technologies, Gaithersburg, MD). Ten microgram amounts of total RNA were denatured and analyzed on 1% agarose gels containing 6% formaldehyde and transferred onto GeneScreen (NEN Life Science, Boston, MA) in 10× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) according to manufacturer's protocols. The blots were incubated in prehybridization solution [5× SSPE (1.7 M NaCl, 0.05 M Na_2HPO_4, and 0.005 M EDTA), 50% deionized formamide, 5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 1% SDS, and 10% Dextran sulfate)] containing 100 μg/ml heat-denatured sheared salmon sperm DNA at 42°C for 2 h. Following prehybridization, the blots were incubated in hybridization solution, which consists of prehybridization solution containing 3×10^6 cpm/ml of labeled probe overnight at 42°C. The blots were then washed twice in 2× SSPE, 1% SDS, for 15 min each at 65°C, followed by two washes in 0.2× SSPE, 1% SDS, for 15 min each at 50°C. Probes for the Northern blots were radiolabeled using random primers (Prime-It II, Stratagene, San Diego, CA) and [α-^{32}P]dCTP (NEN Life Science) according to manufacturer's recommendations. Following hybridization, the blots were analyzed by phosphorimager analysis and quantified using Imagequant software (Molecular Dynamics, Piscataway, NJ). The Akt probe was obtained from Dr. Phil Tsichlis

(Kimmel Cancer Center, Temple University, Philadelphia, PA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was from the collection of this laboratory. Northern blots were performed in duplicate with RNA extracted from at least three different mice per timepoint.

Immunoblot analysis

Protein was extracted from frozen tissue by homogenizing in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% DOC, 0.1% SDS, 1 mM DTT, 5 mM sodium orthovanadate, 100 μg/ml PMSF, and a protease inhibitor cocktail (Sigma # P 8340, St. Louis, MO)]. The samples were then boiled for 10 min, chilled on ice, and sonicated until homogeneous. Protein assays were performed using the Pierce Coomassie Plus protein assay reagent (Pierce Chemical Co., Rockford, IL). Fifty microgram amounts of total protein were separated on 8% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA), and immunoblotted with a 1:400 dilution of the horseradish-peroxidase-conjugated anti-hemagglutinin (HA) antibody, clone 3F10 (Roche, Indianapolis, IN). Bound antibodies were detected using enhanced chemiluminescence according to manufacturer's recommendations (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence and lipid droplet analysis

Mammary glands were removed from mice at the indicated times, fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections were cut at 5 microns. Following dehydration with graded alcohols, microwave antigen retrieval was performed for 20 min in 10 mM sodium citrate, pH 6.0. The sections were permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% normal goat serum in PBS, and incubated with the indicated antibodies. Mammary gland sections from Days 8, 12, and 18 of pregnancy were stained with an anti-adipose differentiation-related protein rabbit polyclonal antibody (Dr. Thomas Keenan, Virginia Polytechnic Institute and State University, Blacksburg, VA) at a dilution of 1:200. Mammary gland sections from Days 2 and 9 of lactation were stained with an anti-WAP antibody obtained from Dr. Floyd Schanbacher (Department of Animal Industries, Ohio Agricultural Technical Institute, Ohio State University, Wooster, OH), at a dilution of 1:200. The sections were then incubated with Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:150 dilution, Oregon-Green 488-conjugated wheat germ agglutinin (WGA) (Molecular Probes, Eugene, OR) at a 1:250 dilution, and 0.6 μg/ml 4′,6′-diamidino-2′-phenylindole (DAPI) (Sigma). Images were collected using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO) on a Nikon Diaphot TMD microscope equipped for fluorescence with a Xenon lamp and filter wheels (Sutter Instruments, Novato, CA), fluorescent filters (Chroma, Brattleboro, VT), cooled CCD camera (Cooke, Tonawanda, NY), and stepper motor (Intelligent Imaging Innovations, Inc.).

The number and volume of individual lipid droplets stained by ADPH were determined by deconvolution of the fluorescent images followed by quantitation of the lipid droplets using masking algorithms on SlideBook (Intelligent Imaging Innovations, Inc.). Calculated values were based on the analysis of 10 to 30 randomly selected alveoli.

Milk collection and analysis

Female mice were mated to male mice at 10 weeks of age, and the resulting litters were normalized to eight pups each. At Day 10 of lactation, the mice were anaesthetized using 0.4 mg/g avertin. Immediately prior to milking, the mice were injected with 2.5 units of oxytocin to induce milk let down. The milk was removed from the mammary glands using suction. For analysis of fat content, whole milk was drawn into capillary tubes and centrifuged at 3,000 rpm for 20 min. The amount of fat that collected into the top layer was calculated as a percentage of the total material present in the tube. For milk protein analysis, protein concentration was determined using the Pierce Coomassie Plus protein assay reagent (Pierce Chemical Co.). Fifty microgram amounts of total protein were separated on a 5–15% SDS-polyacrylamide gradient gel. The gel was stained with Coomassie blue (Sigma) for 2 h, destained overnight in destain solution (50% methanol, 10% acetic acid), and dried under vacuum for 2 h at 80°C. Milk samples were collected and analyzed from a total of three wild-type and three transgenic mice.

Cell culture and adenoviral transductions

CIT-3 cells, obtained from Dr. Margaret Neville (University of Colorado Health Sciences Center, Denver, CO) were grown in a 50:50 mixture of DMEM and F12 medium containing 2% heat-inactivated fetal bovine serum, 5 ng/ml EGF, 10 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. All media components were obtained from GIBCO Life Sciences (Gaithersburg, MD) except the fetal calf serum, which was obtained from HyClone (Orem, UT). Adenoviral constructs were produced as described previously (39). For adenoviral transductions, confluent CIT-3 cells were transduced with the indicated multiplicity of infection (MOI) of adenovirus for 36 h. For the [^{3}H]2-deoxyglucose uptake studies, the cells were then incubated in serum-free, glucose-free DMEM for 1 h. The cells were then incubated with 10 μCi of [^{3}H]2-deoxyglucose (NEN Life Science) for 15 min. The cells were washed three times with ice-cold PBS, harvested with 5% trichloroacetic acid, and counted. DNA was isolated from duplicate wells by lysing in buffer containing proteinase K followed by incubation at 37°C for 6 h, phenol-chloroform extraction, and quantitation. All experiments were performed in duplicate three times. To examine Oil Red O staining, the cells were transduced as described above and incubated in normal growth medium for 4 days. The cells were washed in PBS and fixed for 1 h in 10% formalin. The cells were then washed with water and stained with Oil Red O for 2 h as described previously (40). The cells were then washed extensively with water and allowed to dry overnight. The Oil Red O was extracted using isopropanol, transferred to microtiter plates, and analyzed at 490 nm.

RESULTS

Developmental pattern of *Akt* expression in the mammary gland

To determine the expression pattern of endogenous Akt throughout mammary gland development in the wild-type mouse, we performed Northern blot analysis of *Akt* mRNA. The Northern blot was first probed with a cDNA probe for *Akt1* (**Fig. 1A**), then reprobed with a GAPDH-specific probe (Fig. 1B), and levels of *Akt* expression were normalized to GAPDH levels (Fig. 1C). The decrease in GAPDH expression observed during lactation has been observed in other experiments and may reflect dilution of GAPDH RNA by the presence of the copious milk protein RNAs, or may result from a readjustment of glycolytic pathways during lactation (M. C. Neville, unpublished observations). The cDNA probe used in these hybridizations cross-reacts with *Akt1*, *Akt2*, and *Akt3*; therefore, the results

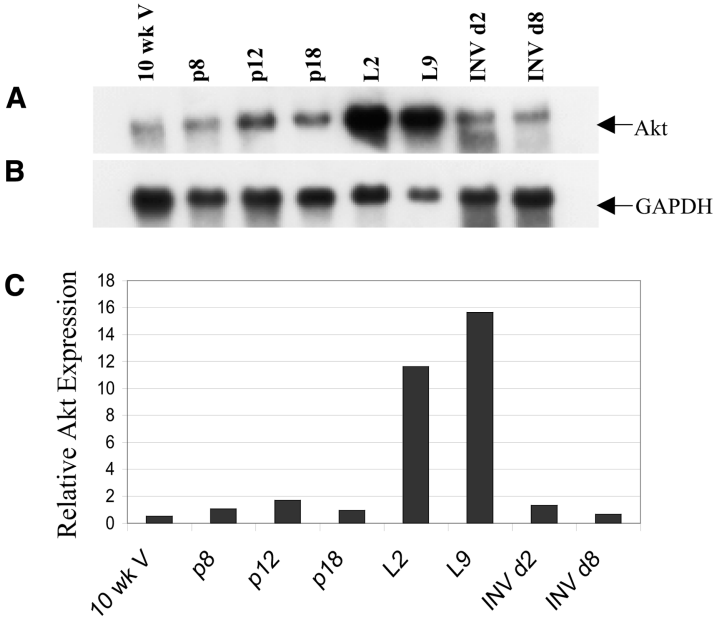

Fig. 1. Expression of *Akt* through mammary gland development. Northern blot analysis of total RNA extracted from mammary glands of three wild-type (FVB) mice per time point at 10 weeks of age (10 wk V); Days 8, 12, and 18 of pregnancy (P8, P12, and P18); Days 2 and 9 of lactation (L2, L9); and Days 2 and 8 of involution forced by pup removal at Day 2 (INV d2, INV d8). A: The blot was probed with an *Akt*-specific probe. B: The blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe as a loading control. C: *Akt* expression was quantified using phosphorimager analysis, normalized to GAPDH levels, and shown relative to the level observed in the 10 week virgin.

shown in Fig. 1A reflect the contribution of all three RNAs. Expression of *Akt* was relatively low during puberty, pregnancy, and involution, but increased 12-fold between Day 18 of pregnancy and Day 2 of lactation (Fig. 1C). This high level was maintained on Day 9 of lactation (Fig. 1A). By in situ hybridization (unpublished observations) and immunofluorescence (36), *Akt* was expressed in mammary epithelial cells during lactation. These data suggest an important role for Akt during lactation.

Expression of myr-Akt in mammary glands from MMTV-myr-Akt transgenic mice

To examine the potential effects of Akt in mammary gland development, we generated transgenic mice that preferentially express an activated form of Akt, myr-Akt, in mammary epithelial cells under the control of the MMTV promoter (36). The expression of the HA-tagged myr-Akt transgene was characterized in mammary glands from transgenic mice throughout mammary gland development by immunoblot analysis using an anti-HA antibody detected using enhanced chemiluminescence. As previously reported, two bands representing the myr-Akt protein are consistently observed (36). Expression of the myr-Akt transgene increased during pregnancy (**Fig. 2A**), likely reflecting both the hormonal responsiveness of the MMTV promoter and an increase in the number of epithelial cells during pregnancy. There was a marked jump in the amount of myr-Akt protein at the onset of lactation, as indicated by the need for a shorter exposure time to detect the transgene at this time point (Fig. 2B). The change in expression levels of myr-Akt between late pregnancy and lactation is more dramatic than expression profiles typically exhibited by MMTV-driven transgenes and may reflect the genomic site of integration in this founder line (line 1173). Probing these immunoblots with antiphospho-Akt (either serine473 or threonine473) yields an identical pattern of expression, because the myr-Akt transgene is constitutively active (unpublished observations). We have previously shown that the level of transgene expression, as determined by immunoblotting with the anti-HA antibody, also correlates with the extent of phosphorylation of endogenous GSK3, a substrate for Akt (36).

Histological analysis of cytoplasmic lipid droplets in mammary epithelial cells from transgenic mice during mid and late pregnancy

To determine whether the expression of myr-Akt results in a phenotype during pregnancy, we examined mammary glands from both wild-type and transgenic mice throughout pregnancy. **Figure 3** shows hematoxylin- and eosin-stained sections of mammary glands from wild-type

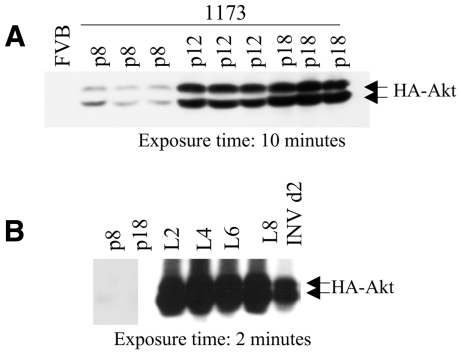

Fig. 2. Expression of myr-Akt protein in mammary glands from transgenic mice is high during lactation, resulting in a lactation defect observed by a decrease in average pup weights. A: Immunoblot analysis of myr-Akt expression in mammary glands from wild-type (FVB) and transgenic (1173) mice at Days 8, 12, and 18 of pregnancy. B: Immunoblot analysis of myr-Akt expression in mammary glands from transgenic mice at Days 8 and 18 of pregnancy (p); Days 2, 4, 6, and 8 of lactation (L); and at Day 2 of involution (INV d2). The exposure time is indicated below each immunoblot.

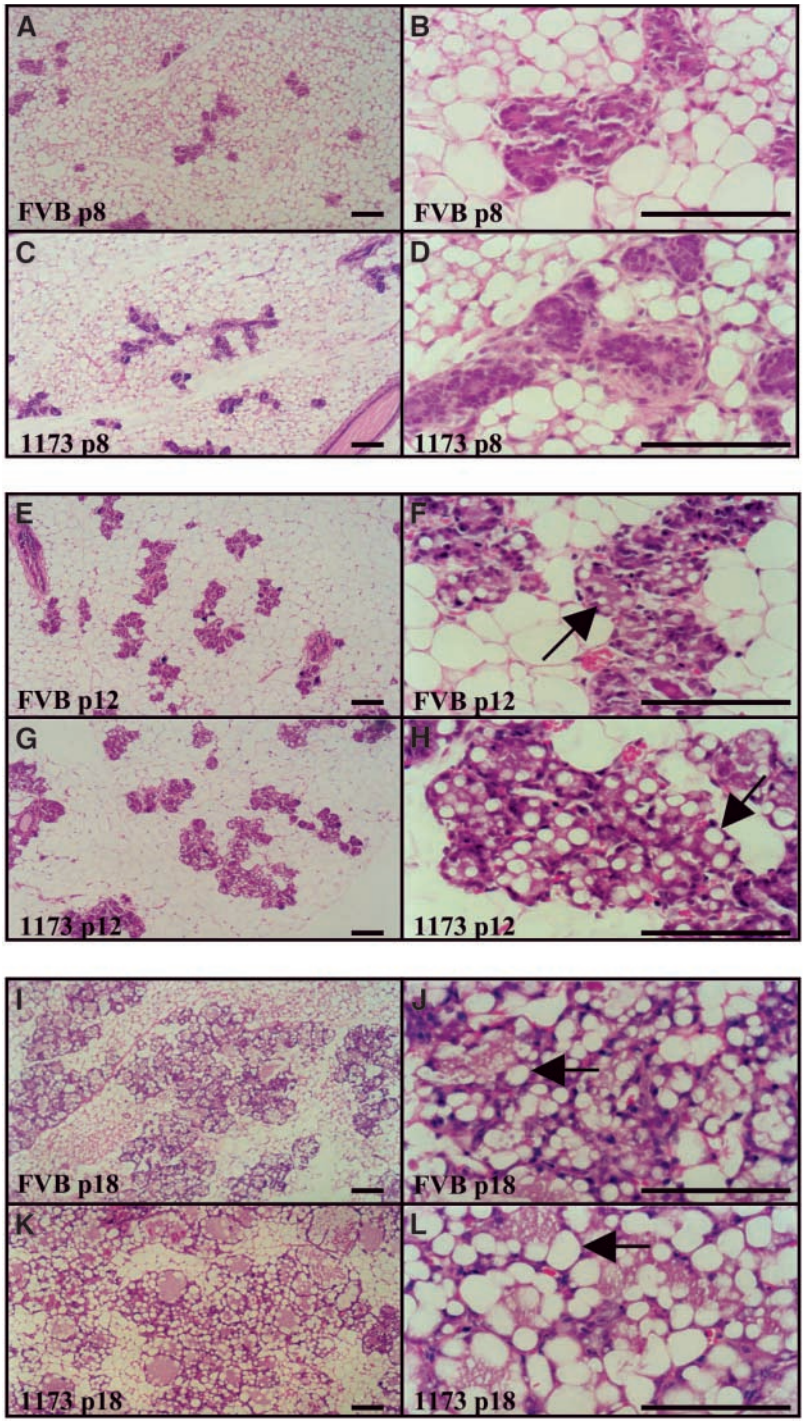

Fig. 3. Mammary gland development during pregnancy in wild-type FVB and mouse mammary tumor virus (MMTV)-myr-Akt transgenic mice. Mammary glands were removed from both wild-type (FVB) and transgenic (1173) mice at Days 8, 12, and 18 of pregnancy (p8, p12, and p18), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A, B: FVB mice at Day 8 of pregnancy. C, D: Transgenic mice at Day 8 of pregnancy. E, F: FVB mice at Day 12 of pregnancy. G, H: Transgenic mice at Day 12 of pregnancy. I, J: FVB mice at Day 18 of pregnancy. K, L: Transgenic mice at Day 18 of pregnancy. Scale bars represent 100 microns. Cytoplasmic lipid droplets are indicated by the arrows in F, H, J, and L.

(Fig. 3A, B, E, F, I, and J) and transgenic (Fig. 3C, D, G, H, K, and L) mice during early, mid, and late pregnancy. Lower magnification views of the mammary glands were used to examine the overall pattern of alveolar development (Fig. 3A, C, E, G, and K), and higher magnification views were used to examine individual alveoli (Fig. 3B, D, F, H, J, and L). At Day 8 of pregnancy, alveolar development is comparable in wild-type (Fig. 3A) and transgenic mice (Fig. 3C). No dramatic morphological differences were seen at higher magnification at this stage (compare

Fig. 3B and D). Alveolar development also appeared comparable at Day 12 of pregnancy between mammary glands from wild-type (Fig. 3E) and transgenic mice (Fig. 3G), and examination at a higher magnification revealed that cytoplasmic lipid droplets were apparent in the epithelial cells at mid pregnancy in both strains (arrows, Fig. 3F, H). However, the cytoplasmic lipid droplets were significantly larger in the transgenic than in the wild-type mice. At Day 18 of pregnancy, the proportion of alveoli and adipose tissue was similar in the transgenic mice (Fig. 3K) compared with the wild-type mice (Fig. 3I); however, even at low magnification, the cytoplasmic lipid droplets were larger in the transgenic mice, and the lumina expanded, possibly due to the presence of these large lipid droplets (arrows, Fig. 3J, L).

To further examine cytoplasmic lipid droplet accumulation in mammary epithelial cells of myr-Akt transgenic mice during pregnancy, we stained sections of mammary glands at Days 8 and 18 of pregnancy using an anti-ADPH-specific antibody (**Fig. 4A**, **B**, **D**, **F**). ADPH is found in the protein coat of cytoplasmic and secreted-milk lipid droplets (7, 41). As shown in Fig. 4A–F, little ADPH staining was observed in sections from wild-type mice at Day 8 of pregnancy (Fig. 4A). In comparison, some ADPH staining was consistently observed in the alveoli from transgenic mice at this timepoint (Fig. 4D). Quantitation of cytoplasmic lipid droplet volume confirmed the increase in lipid accumulation in transgenic mice (Fig. 4G). At Day 18 of pregnancy, ADPH was abundant in alveoli from both wild-type (Fig. 4B) and transgenic mice (Fig. 4E). Quantitation revealed an increase in both the number and volume of cytoplasmic lipid droplets in mammary epithelial cells from transgenic mice compared with those from wild-type mice during pregnancy (Fig. 4G). These data confirm the observations made on the hematoxylin- and eosin-stained sections and indicate that expression of myr-Akt in mammary epithelial cells results in precocious lipid accumulation early in pregnancy, as well as an increase in the number and size of cytoplasmic lipid droplets produced during late pregnancy.

Histological analysis of mammary glands from wild-type and transgenic mice during lactation

To determine whether myr-Akt expression results in a lactation phenotype, mammary gland morphology was ex-

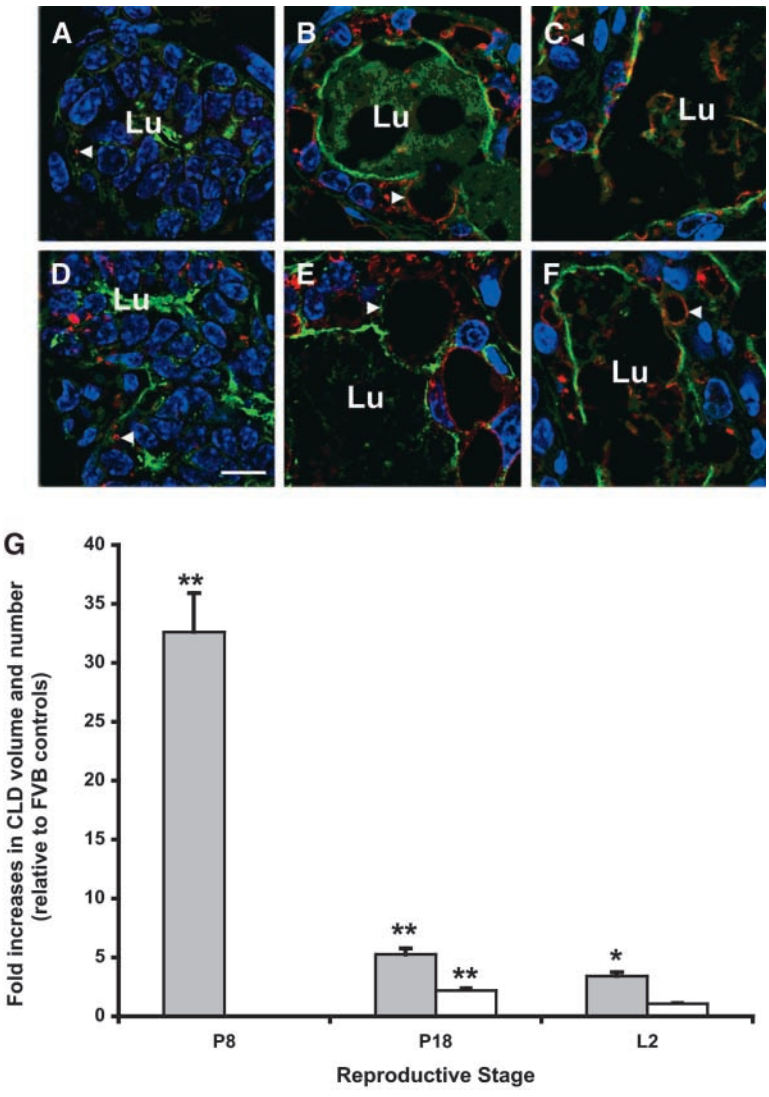

Fig. 4. Myr-Akt expression increases lipid droplet size and accumulation in mammary alveoli. A–F: Lipid droplets (arrowheads) in alveoli of mammary glands from FVB (A–C), and myr-Akt (D, F) mice at P8 (A, D), P18 (B, E), and L2 (C, F) were identified by staining for adipophilin (ADPH) (red). The sections were also stained with wheat germ agglutinin (WGA) (green) to outline the luminal space, and 4′,6′-diamidino-2′-phenylindole (DAPI) (blue) to stain the nuclei. Lu, lumen. Scale bar represents 10 microns. G: The volume (gray bars) and number (white bars) of cytoplasmic lipid droplets in mammary glands from transgenic mice normalized to levels in wild-type (FVB) mice as determined by quantitation of ADPH staining. Statistical significance is indicated by the asterisk; double asterisk values have are significant at the $P < 0.0001$, while the single asterisk values are significant at $P < 0.001$. Values without asterisks are not significantly different from controls. Mammary tissue from at least two animals was used in the analysis. Between 10 and 30 randomly chosen alveoli were analyzed in each animal. Error bars represent SEM.

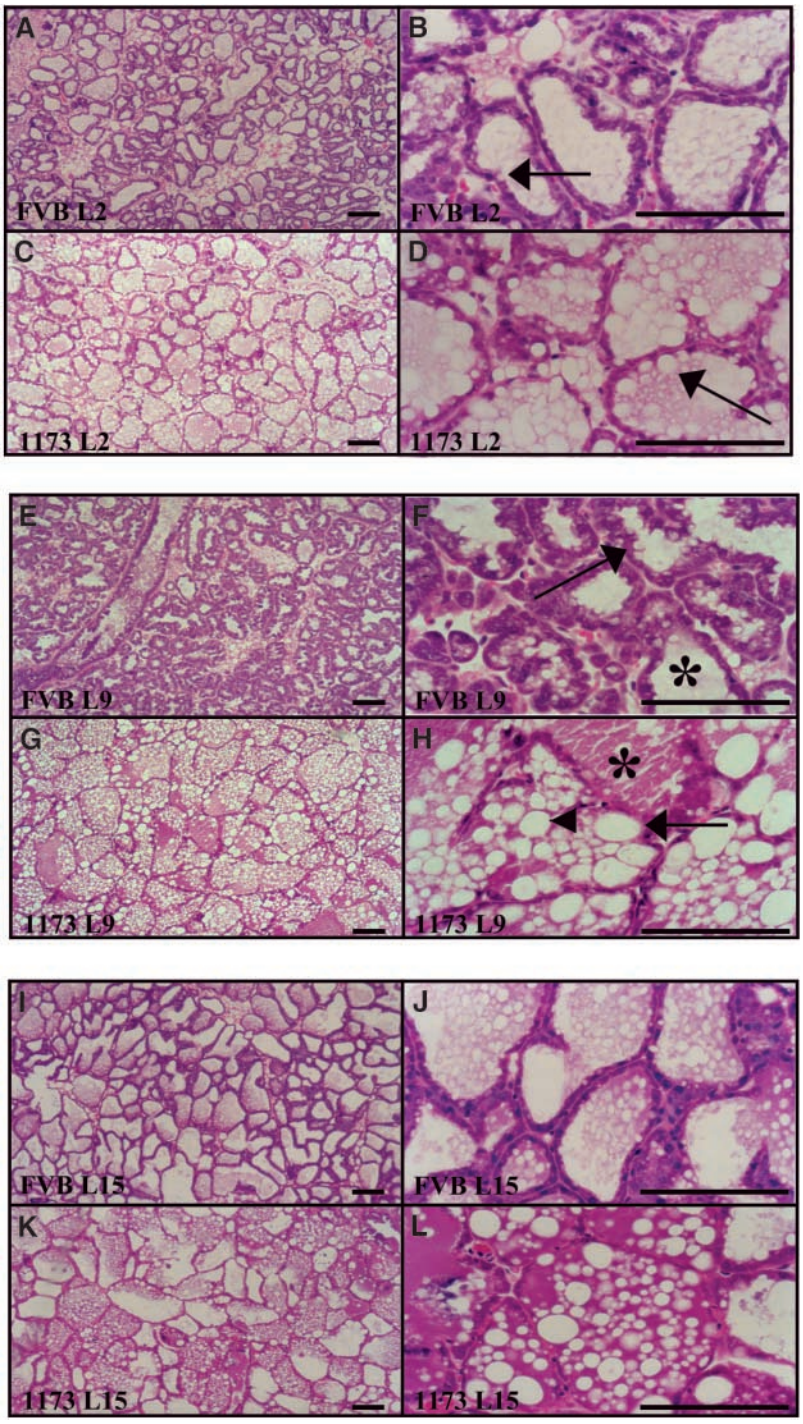

Fig. 5. Increased size of lipid droplets in mammary glands from myr-Akt transgenic mice during lactation. A, B, C, D: Sections from FVB (A, B) and transgenic (C, D) mice at Day 2 of lactation. E, F, G, H: Sections from FVB (E, F) and transgenic (G, H) mice at Day 9 of lactation. An asterisk denotes a lumen in F. The lumina contain excess proteinaceous material (asterisk) in H, I, J, K, and L. Mammary gland sections from FVB (I, J) and transgenic (K, L) mice at Day 15 of lactation. K, L: The arrows point to milk lipid droplets in the process of being secreted in B, D, F, and H. In H, the arrowhead indicates a large luminal milk droplet. Magnification bars represent 100 microns.

amined during lactation using hematoxylin- and eosin-stained sections from both wild-type and transgenic mice (**Fig. 5**). At Day 2 of lactation, the alveoli have expanded to fill the fat pad in mammary glands from both wild-type and transgenic mice, and the lumina are highly distended (Fig. 5A, C). In addition, cytoplasmic lipid droplets can be observed in the epithelial cells (arrow, Fig. 5B), although they are smaller than those observed during pregnancy (see arrow, Fig. 3J). At Day 2 of lactation in mammary glands from transgenic mice, the alveoli appear to be slightly distended

(Fig. 5C), and the cytoplasmic lipid droplets appear to be slightly larger than those observed in the wild-type mice (arrow, Fig. 5D). At Day 9 of lactation in mammary glands from wild-type mice, the alveoli have filled the majority of the fat pad (Fig. 5E), and small lipid droplets are observed in the lumen close to the epithelial cells (arrow, Fig. 5F). In contrast, the alveoli in mammary glands from transgenic mice appear large and distended compared with alveoli from wild-type mice (Fig. 5G). 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 (arrow, Fig. 5H), as well as in the lumen (arrowhead, Fig. 5H). In addition, there appears to be more proteinaceous material that stains a deep red in the alveolar lumina (asterisk, Fig. 5H) compared with wild-type alveoli (asterisk, Fig. 5F), possibly a result of milk stasis since we have seen a similar staining pattern in other transgenic models with lactation defects (M. C. Neville, unpublished observations) (42). Similarly, at Day 15 of lactation, the larger lipid droplets and proteinaceous material persist in mammary glands from transgenic mice compared with those from wild-type mice (compare Fig. 5I, J, and Fig. 5K, L).

To further examine cytoplasmic lipid droplet production in mammary glands from myr-Akt transgenic mice, sections were stained using the ADPH antibody. Analysis of cytoplasmic lipid droplets during lactation reveals that the increased size persists in mammary glands from transgenic mice (Fig. 4C) compared with those from wild-type mice (Fig. 4F). To confirm these observations, cytoplasmic lipid droplet volume and number were quantitated and found to be increased in the transgenic mice compared with wild-type mice (Fig. 4G). It appears that during lactation, the increase in the volume of the lipid droplets is more pronounced than the increase in the number of lipid droplets. These results, along with the histological analyses, suggest that expression of myr-Akt in mammary epithelial cells is increasing lipid synthesis and/or accumulation, consistent with the observations made during pregnancy.

Expression of myr-Akt in the mammary gland results in a defect in lactation

At birth, the pups from the transgenic mothers appeared normal compared with those from wild-type mothers, and when fostered onto wild-type dams, growth was normal. However, only a small amount of milk, if any, was observed in the stomachs of 1-day-old pups nursed by the transgenic mothers (unpublished observations), and within one day of parturition, they appeared smaller than pups from wild-type mothers. When the rate of growth was quantitated by weighing every 24 h for 9 days after parturition, a significant decrease in the average pup weight was observed compared with the weights of pups nursed by wild-type mice, as shown

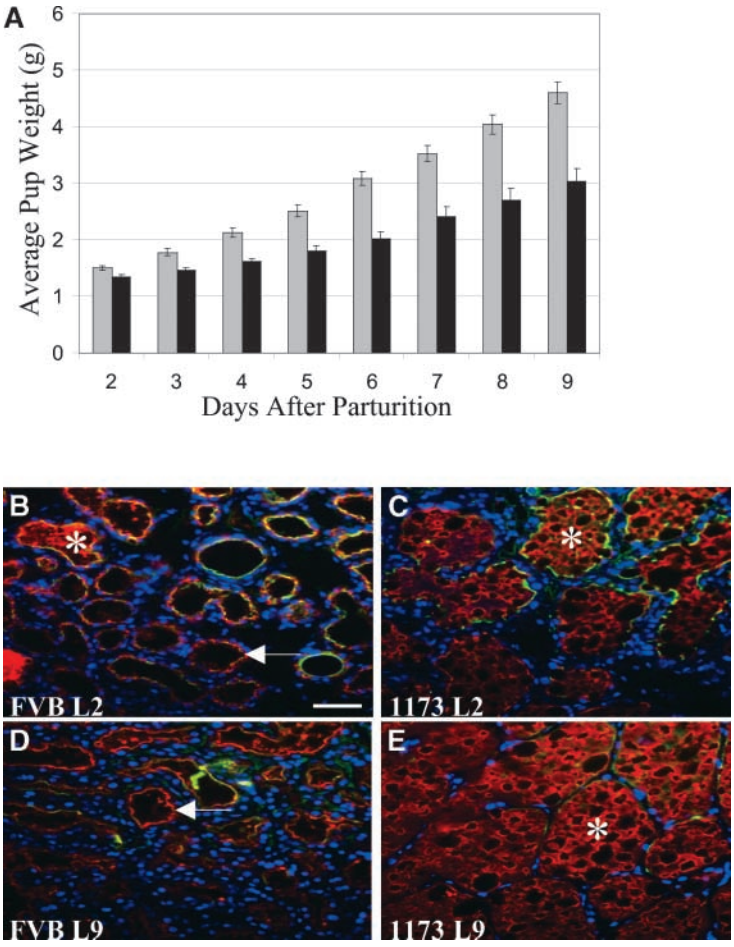

Fig. 6. Effect of MMTV-Myr-Akt on pup growth. A: Pups nursed by both wild-type (gray bars) and transgenic (black bars) mice were weighed every 24 h starting 48 h after parturition. Litters were normalized to eight pups each 24 h after parturition. If any of the pups present in these normalized litters died, they were replaced with another pup of the same age obtained from another litter in our colony. Only the original pups in the normalized litters were weighed each day; replacement pups were not included in the weight gain analysis since they were not initially nursed by the mother being analyzed. A total of 200 pups nursed by FVB mice and 192 mice nursed by transgenic mice were weighed. Error bars represent SEM. B–E: Staining for whey acidic protein (WAP) (red) in sections of mammary glands from wild-type (FVB) and transgenic (1173) mice. WGA (green) staining outlines the luminal space, and DAPI (blue) identifies the nuclei. B: Mammary gland section from a wild-type mouse at Day 2 of lactation. WAP is primarily observed around the luminal borders (arrow), although it is occasionally observed throughout the lumen (asterisk). C: Mammary gland from a transgenic mouse at Day 2 of lactation. WAP is observed throughout the majority of the lumina (asterisk). D: Mammary gland section from a wild-type mouse at Day 9 of lactation. WAP is observed at luminal borders (arrow). E: Mammary gland section from a transgenic mouse at Day 9 of lactation. WAP staining is observed throughout the lumen (asterisk). Magnification bar represents 100 microns.

in **Fig. 6A**. As additional evidence that the difference in weight was not a direct effect of the transgene upon the pup, all litters contained both wild-type and transgenic pups, and all the pups within each litter were approximately the same weight at birth and during lactation, indicating that the genotype of the pup was not a determining factor. Finally, both wild-type and transgenic mothers were observed to exhibit normal maternal behaviors such as nest building, crouching, and pup retrieval, suggesting that decreased pup weights are due neither to defects in pup development nor in maternal behavior. These observations are consistent with a lactation defect in myr-Akt transgenic mice.

Milk stasis during lactation in mammary glands from transgenic mice

To further examine the cause of the lactation defect, immunofluorescence was performed using an anti-WAP-specific antibody on mammary gland sections from both wild-type and transgenic mice at Days 2 and 9 of lactation (Fig. 6B). WAP expression is visualized by red staining; WGA, which stains the apical surfaces of the epithelial cells, is visualized by green staining; and DAPI-stained nuclei are represented by blue staining. WAP protein expression in mammary gland sections from wild-type mice was observed at the apical surfaces of the epithelial cells during lactation (arrows, Fig. 6B, D). In some cases, WAP expression can be detected throughout the lumen, which is possibly due to the presence of milk that has not been recently removed (asterisk, Fig. 6B). However, in mammary gland sections from transgenic mice, WAP expression is consistently detected throughout the lumen during lactation (asterisks, Fig. 6C, E), indicating that the milk is not being removed from the mammary gland. These results are consistent with the indication in the hematoxylin- and eosin-stained sections that the mammary glands from the myr-Akt transgenic mice are undergoing milk stasis during lactation. We cannot rule out the possibility that milk proteins are normally found in the luminal space of the mammary glands from normal mice and that this protein is lost during the processing of the tissue for histological analysis. Even if this were the case, the presence of WAP in the luminal space of mammary glands from transgenic mice (Fig. 6C, E) supports the conclusion that the composition of the milk produced by the MMTV-myr-Akt transgenic mice is altered, and that its presence in these sections is consistent with a greater viscosity.

Increased percentage of milk fat in milk from myr-Akt transgenic mice

To further examine the lactation defect observed in lactating myr-Akt females, milk was collected from wild-type and transgenic mice and analyzed for both the percentage of milk fat as well as milk protein composition. Following collection, the milk was centrifuged in capillary tubes, and the amount of fat was measured and expressed as the percentage of total volume of material present in the tube. Milk collected from wild-type Akt mice contained 20–30% milk fat (**Fig. 7A**); however, milk collected from transgenic mice contained 65–70% milk fat (Fig. 7A).

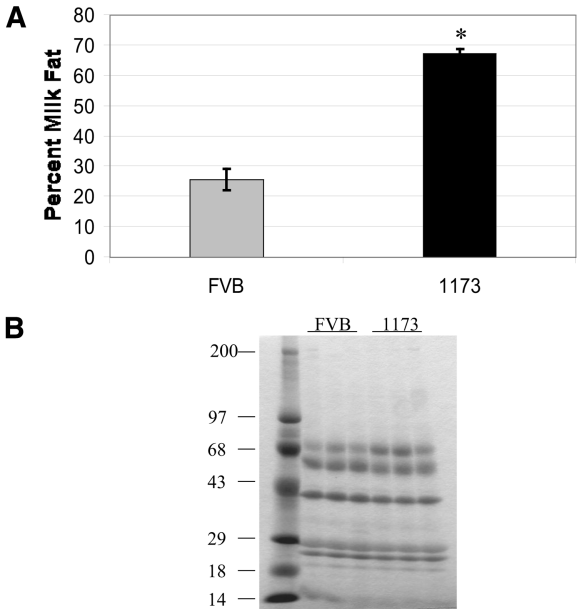

Fig. 7. Increased fat content and normal protein composition in milk from myr-Akt transgenic mice. A: The percentage of milk fat present in milk collected from FVB and transgenic (1173) mice at Day 10 of lactation. Milk samples from three FVB and three transgenic mice were analyzed in triplicate. Error bars represent SEM. Statistical significance is indicated by the asterisk, $P < 0.001$. B: Equal amounts of protein from whole milk were analyzed on a 5–15% gradient SDS-polyacrylamide gel and stained with Coomassie blue. Each lane represents analysis of milk from a different mouse; milk was from three different transgenic mice, and three different control mice were analyzed. Sizes of molecular weight markers are indicated on the side of the gel.

The protein composition of milk from wild-type and transgenic mice was also examined. Fifty microgram amounts of whole milk protein were analyzed on a gradient polyacrylamide gel and stained with Coomassie blue to visualize the proteins. As shown in Fig. 7B, the overall composition of the milk proteins appeared to be similar in milk from wild-type and transgenic mice, although it should be noted that the total protein concentration of milk from transgenic mice was significantly higher than in milk from wild-type mice (data not shown). These data indicate that expression of myr-Akt in the mammary gland results in increased milk fat, with no obvious defect in milk protein composition.

Myr-Akt induces lipid formation in mammary epithelial cells

If myr-Akt is inducing increased milk lipid synthesis, we hypothesize that it should affect lipid synthesis in a tissue culture model of the mammary epithelium. To test this hypothesis, we examined the effects of myr-Akt expression on glucose uptake and lipid accumulation in CIT-3 cells (43, 44), which were derived from the normal mouse mammary epithelial cell line, Comma 1D (45). Following adenovirus-mediated expression of myr-Akt in CIT-3 cells, the ability of the cells to take up [^{3}H]2-deoxyglucose was analyzed. As shown in **Fig. 8A**, the expression of myr-Akt at an MOI of 10 resulted in increased uptake of 2-deoxyglucose. Expression

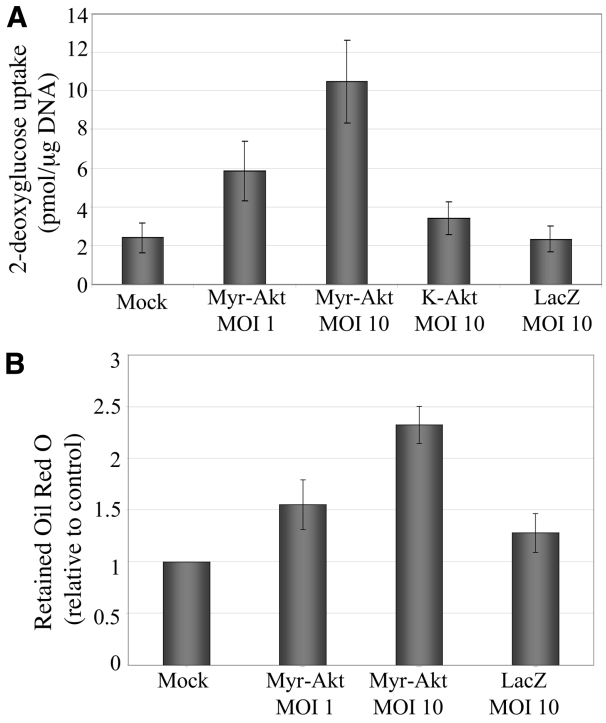

Fig. 8. Expression of myr-Akt induces 2-deoxyglucose uptake and lipid production in CIT-3 cells. A: CIT-3 cells were transduced with the following adenoviruses: activated Akt (myr-Akt), kinase-inactive Akt (K-Akt), and LacZ. The ability of the cells to take up [^{3}H]2-deoxyglucose was assessed following a 1 h incubation in glucose-free medium using a 15 min 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.

of LacZ at an MOI of 10 does not affect 2-deoxyglucose uptake (Fig. 8A), indicating that this observation is specific for myr-Akt rather than an effect of adenoviral transduction. We also examined the ability of myr-Akt to induce the formation of cytoplasmic lipid droplets in the CIT-3 cells. Following a 48 h transduction, cells transduced with either myr-Akt or LacZ were stained with Oil Red O, which stains accumulated triglycerides (40). 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 (40). As shown in Fig. 8B, expression of myr-Akt resulted in increased Oil Red O staining at an MOI of 10 compared with 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, results that are consistent with the phenotype observed in the myr-Akt transgenic mice.

DISCUSSION

Our results show that overexpression of an activated form of Akt in the mammary glands of transgenic mice induces increased lipid synthesis in both pregnancy and lactation and is associated with a lactation defect that retards pup growth. The timing of the observed phenotype is clearly related to the temporal expression of the transgene protein, as determined by immunoblotting. Overexpression of this transgene in a tissue culture model of the mammary epithelium led to an increase in both lipid synthesis and glucose transport, suggesting that the increase in lipid synthesis might be a function of increased cellular glucose in the presence of the transgene.

Alveolar development during pregnancy is regulated primarily by the hormones progesterone and prolactin (46–49), and possibly by other growth factors such as EGF (50, 51) and fibroblast growth factor (FGF) (52). Prolactin, EGF, insulin-like growth factor-I (IGF-I), and FGF have been shown to induce activation of Akt (22, 29, 34, 53, 54). Therefore, we predicted at the outset of these studies that expression of activated Akt in mammary epithelial cells might enhance alveolar development. However, examination of the mammary glands from the myr-Akt transgenic mice revealed no obvious differences in the growth of the alveolar structures. It is possible that expression of the transgene in the mammary gland during pregnancy is not high enough to increase alveolar proliferation, or that it plays no role in this process.

One indication of mammary differentiation during pregnancy is the appearance of cytoplasmic lipid droplets at about mid pregnancy. These droplets grow in size with further progression of the pregnancy and are expelled from the cytoplasm into the lumen around parturition. Histological examination of the mammary alveoli during pregnancy and lactation suggested an increase in both the size and number of cytoplasmic lipid droplets during pregnancy. We also observed an increase in the size of these droplets, as well as luminal milk fat globules, during lactation in the MMTV-myr-Akt transgenic mice. To quantify these effects, we studied sections stained by immunofluorescence with an antibody specific to ADPH, which is a protein that is expressed in the protein coat of both cytoplasmic lipid droplets and the milk fat globule membrane (13). Use of this antibody allowed us to quantify lipid droplet size and number in wild-type and transgenic mice and showed clearly that lipid synthesis and cytoplasmic lipid droplet formation is enhanced in the transgenic mice. Although these data suggest that Akt may play a role in inducing or maintaining differentiation, it is important to note that expression of endogenous Akt is low until late pregnancy and lactation (4) (Fig. 1), when the mammary epithelial cells are already differentiated. This finding suggests either that only low levels of activated Akt are required to bring about differentiation and/or lipid biosynthesis or that Akt may not be a critical regulatory component during pregnancy. In fact, it is possible that Akt is necessarily repressed in the epithelium of the pregnant mammary gland in order to maintain a low level of lipid synthesis. However, it is important to note that expression of Akt during pregnancy was examined only at the mRNA level; it remains possible that the phosphorylation and subsequent activation of Akt is proportionally higher during pregnancy.

In addition to the delayed involution observed in the myr-Akt transgenic mice that we have previously described (36), these mice also exhibit a defect in lactation. This lactation defect is characterized by an ~50% decrease in the weight of pups over the first 9 days after birth, and the death of a significant number of pups over the first 5 days (unpublished observations). There was no difference in the handling of the pups from litters of normal versus transgenic mothers that could account for the differences in neonate mortality. A number of transgenic and knockout mouse models exist that exhibit a lactation defect that is the result of insufficient development of the alveolar structures during pregnancy. These include the prolactin receptor heterozygous null mice (48), STAT5a knockout mice (55), OPGL knockout mice (33), and HIF1α knockout mice (56). In contrast, development of the alveolar structures appears to occur relatively normally in the myr-Akt transgenic mice, suggesting that there is another explanation for the lactation defect. For this reason, milk was collected and analyzed from both wild-type and transgenic mice during lactation. Milk from wild-type mice consists of 20–30% milk fat, whereas milk collected from the MMTV-myr-Akt transgenic mice contained 65–70% milk fat. It is important to note that the milk collected from the transgenic mice was viscous, and only a small amount of milk (50 μl) could be obtained compared with wild-type mice (1 ml). Therefore, the analysis of milk fat could only be performed on milk that could be extracted, indicating that the true percentage of milk fat in the remaining milk that was not collected could be even higher. This finding suggests a possible explanation for the lactation defect; the milk may be so viscous that the newborn pups may be physically unable to remove the milk from the mammary gland, leading to milk stasis. It is possible that lower levels of prolactin and oxytocin could contribute to the lactation defect observed. In this context, it should be noted, however, that in our previous work we did not detect a change in the amount of β-casein or WAP RNA in lactating mice, and in fact these RNAs were present longer in the mammary glands from transgenic mice undergoing involution than in corresponding mammary glands from control mice (36). If the presence of β-casein or WAP RNA is a reflection of the level of prolactin, then it would be expected that the level of prolactin is relatively normal. It remains possible that the levels of oxytocin are decreased; however, it is hard to imagine how a transgene expressed in the mammary gland would alter expression of oxytocin in the pituitary gland. The described results were observed in founder line 1173. A second founder line, line 1176, has 3- to 4-fold lower amounts of the myr-Akt transgene protein, as determined by immunoblotting, and does not display the lactation defect; however, the suppression of apoptosis observed during involution is also diminished (36). We have observed changes in the size of lipid droplets in histological sections that suggest some of the features of the phenotype we have described in line 1173 may also be present in line 1176 (unpublished observations); however, we have not extensively characterized these mice.

The accumulation of abnormally staining proteins, as well as the distended alveoli, provide further indication that milk stasis is occurring during lactation in the transgenic mice almost immediately after parturition. Others have shown that milk stasis can induce the initial phase of involution characterized by apoptosis of the secretory epithelial cells (57). For example, milk stasis induced by teat sealing or forced involution results in distended alveoli, accumulation of milk and lipid in the alveolar lumen and subsequent epithelial cell apoptosis within 48 h, and alveolar collapse within 72 h (58). Although the alveoli from transgenic mice exhibit milk stasis, interestingly, they do not appear to be undergoing involution. Possibly, the myr-Akt transgene is also suppressing apoptosis. We have previously shown that expression of the myr-Akt transgene delays involution following pup withdrawal. Our current data suggest that the myr-Akt transgene suppresses apoptosis even when milk stasis first occurs shortly after parturition, and essentially rescues the epithelium from involution until the pups are strong enough to extract at least some of the very viscous milk. A similar phenomenon was shown to occur in Stat 3 knockout mice, which also show delayed involution (59). These mice could reinitiate lactation up to 6 days after pup removal, compared with only 2 days in the wild-type strain.

Akt has been shown to regulate insulin-mediated glucose uptake and lipid synthesis in adipocytes (25, 26, 60). Based on the results obtained from the myr-Akt transgenic mice, we examined the ability of myr-Akt to induce glucose uptake and lipid synthesis in mammary epithelial cells. Adenoviral expression of myr-Akt in the mouse mammary epithelial cell line, CIT-3, resulted in both increased uptake of 2-deoxyglucose and synthesis of lipid droplets. Although the mechanism of Akt enhancement of glucose uptake is not fully understood, many studies have suggested that Akt affects glucose transporter function. Specifically, Akt increases both GLUT4 translocation to the membrane and transcription of GLUT1 (24, 26, 27, 61). Several glucose transporters are expressed in the mammary epithelium, including GLUT1 (14–16), GLUT12 (62), and GLUT8 (M. C. Neville, unpublished observations). GLUT1 has been most extensively studied; its expression increases throughout late pregnancy and peaks during lactation, when the requirement for glucose uptake is high (14). Therefore, it is possible that myr-Akt affects the expression or localization of glucose transport proteins such as GLUT1 in mammary epithelial cells. In this context, it is interesting to note that blocking the production of prolactin by the pituitary with bromocriptine, either alone or in combination with anti-growth hormone antibody, decreased glucose transport in the rat mammary gland, and that treatment with IGF-I did not reverse this effect (63). Furthermore, prolactin is reported to stimulate a 5-fold increase in GLUT1 levels in mammary gland explants (64), and a 3-fold increase in GLUT1 levels in mammospheres (65). Prolactin has also been shown to stimulate lipid biosynthesis in mammary explants (66). However, the relation between glucose transport and triglyceride synthesis has not been studied in the mammary

gland, and the possibility that Akt is acting more directly on regulators of fatty acid synthesis such as SREBP-1c (67) has not been investigated.

The results presented here demonstrate that expression of myr-Akt in mammary glands of transgenic mice affects mammary epithelial cell differentiation by inducing precocious development of cytoplasmic lipid droplets during pregnancy. In addition, the results described suggest that myr-Akt may mediate both the suppression of apoptosis induced by milk stasis as well as lipid synthesis during lactation. These observations and studies in a tissue culture model of the mammary epithelium raise the interesting possibility that Akt may have multiple functions in the mammary gland, including suppression of apoptosis, induction of differentiation, and regulation of glucose uptake resulting in lipid synthesis.

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