Isoform-specific requirement for Akt1 in the developmental regulation of cellular metabolism during lactation

Robert B. Boxer,[1,2,3,7] Douglas B. Stairs,[1,2,3,7] Katherine D. Dugan,[1,2,3] Kathleen L. Notarfrancesco,[1,2,3] Carla P. Portocarrero,[1,2,3] Blaine A. Keister,[1,2,3] George K. Belka,[1,2,3] Han Cho,[6] Jeffrey C. Rathmell,[1,3] Craig B. Thompson,[1,3,4] Morris J. Birnbaum,[4,5] and Lewis A. Chodosh[1,2,3,4,*]

[1] Department of Cancer Biology
[2] Department of Cell and Developmental Biology
[3] Abramson Family Cancer Research Institute
[4] Department of Medicine
[5] Howard Hughes Medical Institute
University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104
[6] Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[7] These authors contributed equally to this work.
*Correspondence: chodosh@mail.med.upenn.edu

Summary

The metabolic demands and synthetic capacity of the lactating mammary gland exceed that of any other tissue, thereby providing a useful paradigm for understanding the developmental regulation of cellular metabolism. By evaluating mice bearing targeted deletions in *Akt1* or *Akt2*, we demonstrate that Akt1 is specifically required for lactating mice to synthesize sufficient quantities of milk to support their offspring. Whereas cellular proliferation, differentiation, and apoptosis are unaffected, loss of Akt1 disrupts the coordinate regulation of metabolic pathways that normally occurs at the onset of lactation. This results in a failure to upregulate glucose uptake, Glut1 surface localization, lipid synthesis, and multiple lipogenic enzymes, as well as a failure to downregulate lipid catabolic enzymes. These findings demonstrate that Akt1 is required in an isoform-specific manner for orchestrating many of the developmental changes in cellular metabolism that occur at the onset of lactation and establish a role for Akt1 in glucose metabolism.

Introduction

The developmental program that prepares the mammary gland for lactation is among the most critical and highly conserved in mammals, as the provision of nutritional support is essential for the survival of offspring from this class of vertebrates. Lactation represents the terminally differentiated state of the mammary gland and is the culmination of an orchestrated series of developmental events occurring during pregnancy and early lactation that involve mammary epithelial proliferation, differentiation, and secretory activation.

Although mammary epithelial cells do not proliferate during lactation, other than in the initial 24 hr, the metabolic demands and synthetic capacity of the lactating mammary gland exceed that of any other tissue. During a typical 21-day course of lactation, a lactating mouse will produce its entire body weight in lipid as well as three times its total body weight in milk, whose principal constituents are lipid (30%), protein (15%), and lactose (3%) (Schwertfeger et al., 2003). To accomplish this, two distinct developmental programs must be executed: first, alveolar epithelial cells must proliferate and differentiate to form lobuloalveolar structures that will ultimately serve as the site of milk synthesis during lactation, and second, during the transition from pregnancy to lactation, mammary epithelial cells must coordinately upregulate multiple biosynthetic pathways—as well as downregulate catabolic pathways—in order to have the metabolic capacity to synthesize large quantities of milk. As such, the lactating mammary gland provides an ideal setting in which to study the developmental regulation of cellular metabolism under highly anabolic, yet nonproliferative, conditions.

Prior analyses of protein kinase expression patterns during mammary gland development revealed that expression of the serine-threonine kinase *Akt1* is markedly upregulated during pregnancy and lactation, suggesting a potential developmental role for this kinase (Chodosh et al., 2000). In addition, constitutive activation of Akt1 in the mammary glands of transgenic mice induces precocious lipid accumulation during pregnancy, suggesting that Akt upregulation during pregnancy and lactation may help meet the increased synthetic demand for lipid (Schwertfeger et al., 2003). Nevertheless, while these experiments suggest a role for Akt in the developmental regulation of milk synthesis and metabolism in the lactating mammary gland, they do not address whether Akt is required for these processes, nor do they determine which Akt isoforms may be important.

Mice bearing targeted deletions in *Akt1*, *Akt2*, or *Akt3* have been used to evaluate the physiologic roles of Akt family members (Chen et al., 2001, 2004; Cho et al., 2001a, 2001b; Easton et al., 2005; Garofalo et al., 2003; Tschopp et al., 2005) and have identified redundant as well as specific functions for Akt isoforms. For example, among the three Akt family members, only deletion of *Akt2* has thus far been shown to result in impaired metabolism in mice. $Akt2^{-/-}$ mice display a diabetes mellitus-like syndrome characterized by hyperglycemia and hyperinsulinemia (Cho et al., 2001a; Garofalo et al., 2003). In contrast, glucose homeostasis is normal in mice deficient for Akt1 or

Akt3 (Chen et al., 2001; Cho et al., 2001b; Easton et al., 2005; Tschopp et al., 2005). *Akt1* null mice instead display mild growth retardation and increased thymic and testicular apoptosis, whereas *Akt3* null mice display reduced postnatal brain growth (Chen et al., 2001; Cho et al., 2001a; Garofalo et al., 2003).

Akt1 has, however, been implicated in the control of cellular metabolism in other contexts (Plas and Thompson, 2005). Whereas Akt2 regulates insulin-mediated Glut4 translocation in liver, skeletal muscle, and fat, Glut1 expression is ubiquitous and is upregulated in response to growth factor and oncogene-mediated activation of Akt (Barnes et al., 2005; Rathmell et al., 2003). Overexpression of Akt1 in hematopoietic cells increases Glut1 translocation and enhances phosphorylation of glucose by hexokinase, the rate-limiting step of glycolysis, by promoting translocation of this enzyme to the mitochondrial outer membrane (Gottlob et al., 2001; Plas et al., 2001). In addition to glucose metabolism, Akt1 has also been implicated in regulating protein synthesis (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002) as well as lipid synthesis (Berwick et al., 2002; Kohn et al., 1996; Magun et al., 1996; Schwertfeger et al., 2003).

To investigate the developmental roles played by specific Akt isoforms in the response to metabolic stress, we evaluated the ability of mice bearing targeted deletions in *Akt1* or *Akt2* to lactate. Our studies demonstrate that Akt1 is required—in an isoform-specific manner—for orchestrating many of the dramatic developmental changes in metabolism that occur in the mammary gland at the transition from pregnancy to lactation. Specifically, the mammary glands of *Akt1* null mice fail to upregulate glucose uptake and trapping, membrane localization of Glut1, lipid synthesis, activation of ATP-citrate lyase, and expression of stearoyl-CoA desaturases. In addition, Akt1 loss results in the inappropriate derepression of enzymes involved in the β-oxidation of fatty acids. Thus, in the absence of Akt1, the coordinate developmental regulation of multiple synthetic pathways that is essential for the successful transition to the lactating state fails to occur. As a result, nursing mothers are unable to support the growth or survival of their offspring. Together, these studies identify an isoform-specific requirement for Akt1 in the developmental regulation of cellular metabolism and establish a role for Akt1 both in glucose metabolism and as a central regulator of the response to metabolic stress.

Results

Isoform-specific regulation of Akt expression during mammary gland development

To begin evaluating the contributions of specific Akt isoforms to the metabolic response of the mammary gland to the synthetic demands of lactation, mRNA expression patterns of *Akt1*, *Akt2*, and *Akt3* and were analyzed by northern hybridization at 12 time points encompassing mammary gland development. Dilutional effects of milk protein gene expression, as exemplified by the apparent decrease in β-*actin* mRNA levels during late pregnancy and lactation (Figure 1A), were accounted for by normalizing *Akt* gene expression to that of β-*actin* (Figure 1B). This analysis revealed that *Akt1* mRNA levels in the mammary gland increased during late pregnancy and lactation and then returned to baseline during early postlactational involution (Figures 1A and 1B; Chodosh et al., 2000). In contrast, expression levels of *Akt2* and *Akt3* decreased during early pregnancy and remained low throughout pregnancy, lactation, and early involution before returning to baseline late in postlactational involution.

Immunoblotting with antibodies specific for Akt1 or Akt2 confirmed the distinct developmental expression patterns of these isoforms. Akt1 protein expression increased during pregnancy and lactation, whereas Akt2 expression decreased during these same stages of development (Figure 1C). In agreement with previous reports, total Akt and the level of activated phospho-Akt(Ser473) decreased at the onset of postlactational involution concomitant with the onset of widespread epithelial apoptosis (Figure 1C; Schwertfeger et al., 2001). Notably, homozygous deletion of *Akt1* resulted in a marked decrease in total Akt protein content in lactating mammary tissue, whereas homozygous deletion of *Akt2* did not (Figure 1C).

Western analysis of epithelial and adipose tissue isolated from 5-week-old nulliparous mice was performed to explore whether the differences in the developmental expression profiles of Akt1 and Akt2 in the mammary gland may reflect differences in their localization within the mammary gland. These experiments revealed that Akt1 and Akt2 are each expressed within both the epithelial and stromal compartments of the mammary gland, and with a similar ratio of expression between the two compartments (Figure 1D). These findings demonstrate that the apparent differential developmental regulation of Akt1 and Akt2 expression in the mammary gland is not simply a consequence of their expression in different cellular compartments or of their exclusive expression within a cellular compartment that expands or contracts during mammary development. Rather, our results suggest that Akt1 and Akt2 are expressed within the same cell types in the virgin mammary gland and at similar relative ratios between the epithelial and stromal compartments. Together, the distinct developmental expression profiles of Akt family members in the mammary gland raise the possibility that these kinases play isoform-specific functions in this tissue during pregnancy and lactation.

Akt1, but not Akt2, is required for pup growth and survival during lactation

To evaluate the roles played by specific Akt isoforms during mammary gland development, lactating mice bearing targeted deletions in either *Akt1* or *Akt2* were analyzed for their ability to support nursing litters of pups. As early as postpartum day 2, pups nursed by $Akt1^{-/-}$ mothers weighed significantly less than those nursed by $Akt1^{+/+}$ or $Akt1^{+/-}$ mothers (Figure 2A). This difference became more exaggerated during the postpartum period. By 9 days postpartum, the average weight of pups nursed by $Akt1^{-/-}$ mice was two-thirds that of pups nursed by $Akt1^{+/+}$ mice (Figure 2A). In contrast, pups born to $Akt2^{-/-}$ mice did not exhibit any impairment in weight gain.

In addition to the growth retardation observed in pups born to $Akt1^{-/-}$ females, these pups also exhibited increased mortality. By 9 days postpartum, 25% of pups born to $Akt1^{-/-}$ females had died, compared to the less than 2% mortality of pups born to $Akt1^{+/+}$, $Akt1^{+/-}$, or $Akt2^{-/-}$ females (Figure 2B). The reduced growth and viability of litters nursed by $Akt1^{-/-}$ females was not due to abnormal maternal behavior since $Akt1^{-/-}$ females built nests and suckled their pups appropriately. Thus, maternal Akt1 is specifically required for the survival of offspring during nursing.

Notably, the observed defect in pup growth and viability in $Akt1^{-/-}$ mice was dependent upon the genotype of the mother

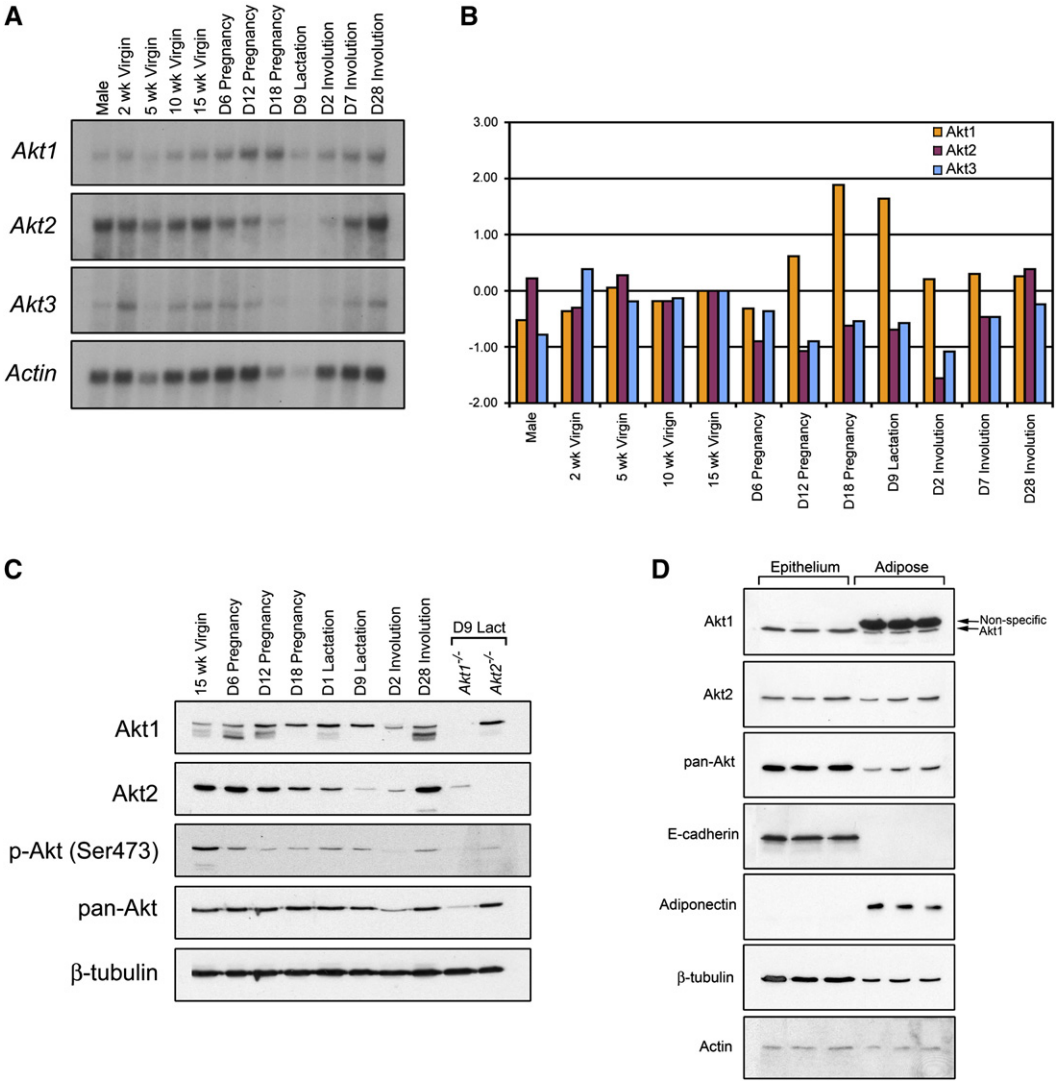

Figure 1. Akt isoforms are differentially regulated during mammary gland development

A) Northern analysis of *Akt* isoform expression during mammary gland development. Independent RNA samples isolated from mammary glands of female FVB mice at 12 developmental time points were electrophoresed on replicate blots and analyzed by northern hybridization using cDNA probes from the 3′ untranslated regions of *Akt1*, *Akt2*, *Akt3*, and β-*actin*. The decreased β-*actin* signals at D18.5 of pregnancy and D9 of lactation reflect dilution by the abundant expression of milk protein genes at these developmental stages.

B) Phosphorimager quantification of *Akt1*, *Akt2*, and *Akt3* expression from northern blots in (**A**). Bars depict the signal intensity for each *Akt* isoform normalized to β-*actin*.

C) Immunoblot analysis of Akt isoform protein expression during mammary gland development. Replicate blots were probed with antibodies specific for Akt1, Akt2, phospho-Akt(Ser473), pan-Akt, and β-tubulin. Lysates from lactating $Akt1^{-/-}$ and $Akt2^{-/-}$ mice are included as controls for antibody specificity.

D) Immunoblot analyses of epithelial and stromal tissue isolated from the mammary glands of 5-week-old mice demonstrating expression of Akt1, Akt2, and cellular markers specific for epithelial and stromal compartments of the mammary gland. The location of a nonspecific band detected by the anti-Akt1 antibody in cultured adipose tissue is indicated by an arrow.

rather than the genotype of the pups. Wild-type pups born to wild-type mice but fostered to $Akt1^{-/-}$ mice at the time of parturition exhibited reduced growth, whereas pups born to $Akt1^{-/-}$ mice grew normally when nursed by wild-type mice (Figure 2C). These observations indicate that Akt1, but not Akt2, is required for lactating mice to support the growth and survival of their litters.

Milk secretion is impaired in $Akt1^{-/-}$ mice

Pups nursed by $Akt1^{-/-}$ mice were found to have less milk in their stomachs, suggesting that their increased mortality and growth retardation was due to an inability of $Akt1^{-/-}$ mothers to secrete normal amounts of milk. To address this directly, milk was collected from lactating mice following administration of oxytocin to stimulate milk release. Lactating $Akt1^{-/-}$ mice displayed a 4-fold reduction in oxytocin-stimulated milk secretion compared to $Akt1^{+/+}$ mice ($p = 0.0002$) (Figure 2D). In contrast, oxytocin-stimulated milk secretion was normal in $Akt2^{-/-}$ mice (Figure 2D). These results demonstrate an isoform-specific requirement for Akt1 in the mammary gland for the secretion of normal amounts of milk during lactation.

Western analysis using a polyclonal antibody raised against milk proteins demonstrated that the milk protein compositions of milk collected from lactating $Akt1^{+/+}$, $Akt1^{+/-}$, and

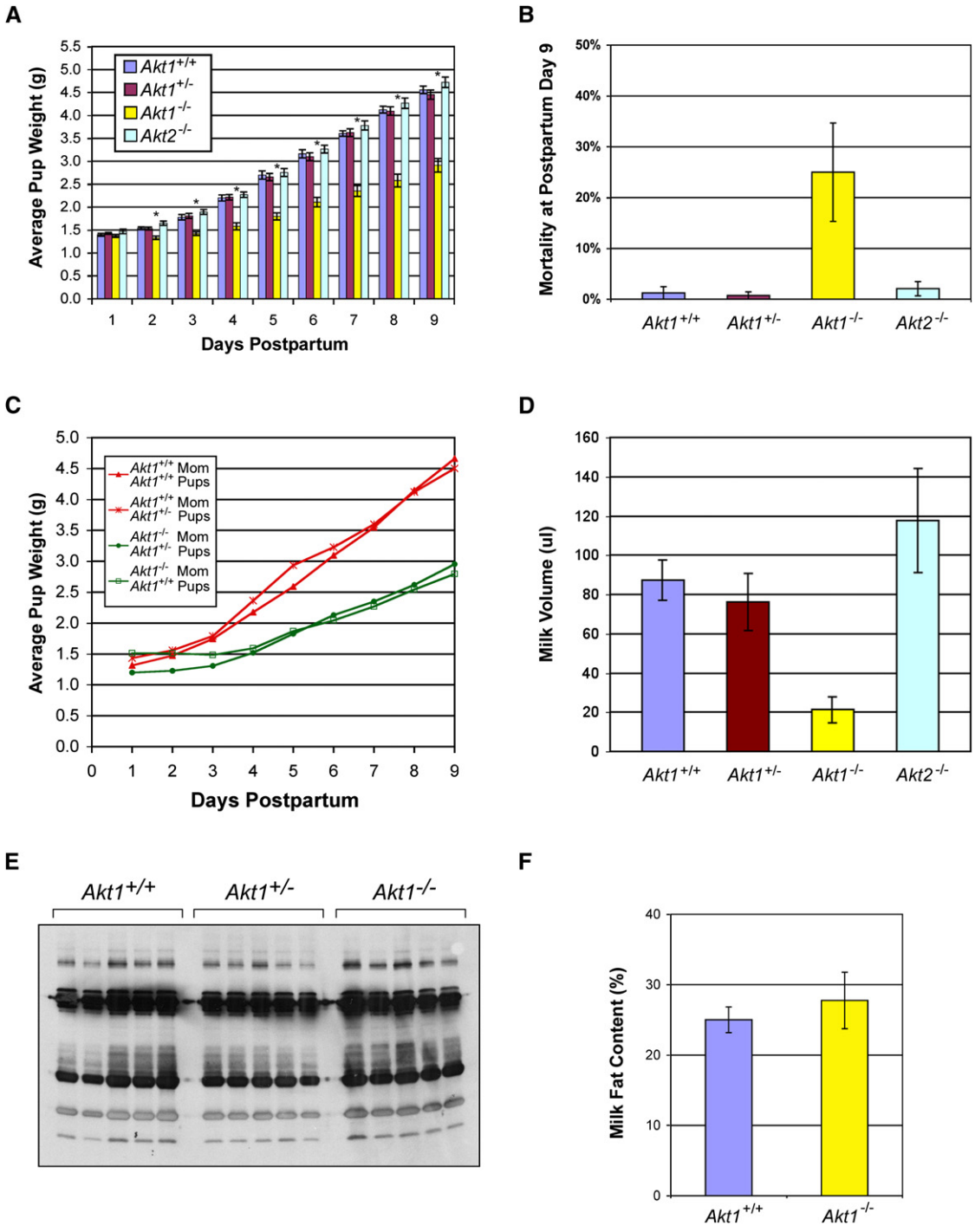

Figure 2. Increased mortality and reduced growth of pups nursed by *Akt1*$^{-/-}$ females is associated with decreased amounts of milk of normal composition

A) Analysis of postpartum weight gain in pups nursed by *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ females. Graph depicts average daily pup weights for litters born to mice of the following genotypes: *Akt1*$^{+/+}$ (n = 9), *Akt1*$^{+/-}$ (n = 17), *Akt1*$^{-/-}$ (n = 10), and *Akt2*$^{-/-}$ (n = 12). *Akt1*$^{-/-}$ versus *Akt1*$^{+/+}$: $p < 0.0007$ at time points indicated by asterisk. Error bars in (**A**), (**B**), (**D**), and (**F**) represent SEM.

B) Pup mortality in litters nursed by mice lacking Akt1 or Akt2. Pup mortality was determined for litters from female *Akt1*$^{+/+}$ (n = 10), *Akt1*$^{+/-}$ (n = 17), *Akt1*$^{-/-}$ (n = 10), and *Akt2*$^{-/-}$ (n = 12) mice as the percentage of litters that died within the first 9 days postpartum. *Akt1*$^{-/-}$ versus *Akt1*$^{+/+}$: p = 0.026; *Akt1*$^{-/-}$ versus *Akt1*$^{+/-}$: p = 0.003.

C) Reduced weight gain in pups nursed by *Akt1*$^{-/-}$ mice is independent of pup genotype or the genotype of their birth mother. On the day of parturition, litters from *Akt1*$^{-/-}$ females were exchanged with those of *Akt1*$^{+/+}$ females. Pup weights were measured postpartum and compared to those for control litters of mice that were not exchanged. Pup growth is shown for representative litters.

D) Milk secretion following oxytocin stimulation. The volume of milk collected from number three mammary glands following oxytocin stimulation was compared for *Akt1*$^{+/+}$ (n = 17), *Akt1*$^{+/-}$ (n = 11), *Akt1*$^{-/-}$ (n = 9), and *Akt2*$^{-/-}$ (n = 5) mice at day 9 of lactation. *Akt1*$^{-/-}$ versus *Akt1*$^{+/+}$, *Akt1*$^{-/-}$ versus *Akt1*$^{+/-}$, *Akt1*$^{-/-}$ versus *Akt2*$^{-/-}$: $p < 0.001$.

E) Milk protein analysis. Equal volumes of milk collected at day 9 of lactation from *Akt1*$^{+/+}$ (n = 5), *Akt1*$^{+/-}$ (n = 5), and *Akt1*$^{-/-}$ (n = 5) mice were analyzed by immunoblotting using a polyclonal antibody raised against milk proteins.

F) Milk fat analysis. The percentage of lipid by volume was measured for milk samples collected at day 9 of lactation from *Akt1*$^{+/+}$ (n = 5) and *Akt1*$^{-/-}$ (n = 5) mice.

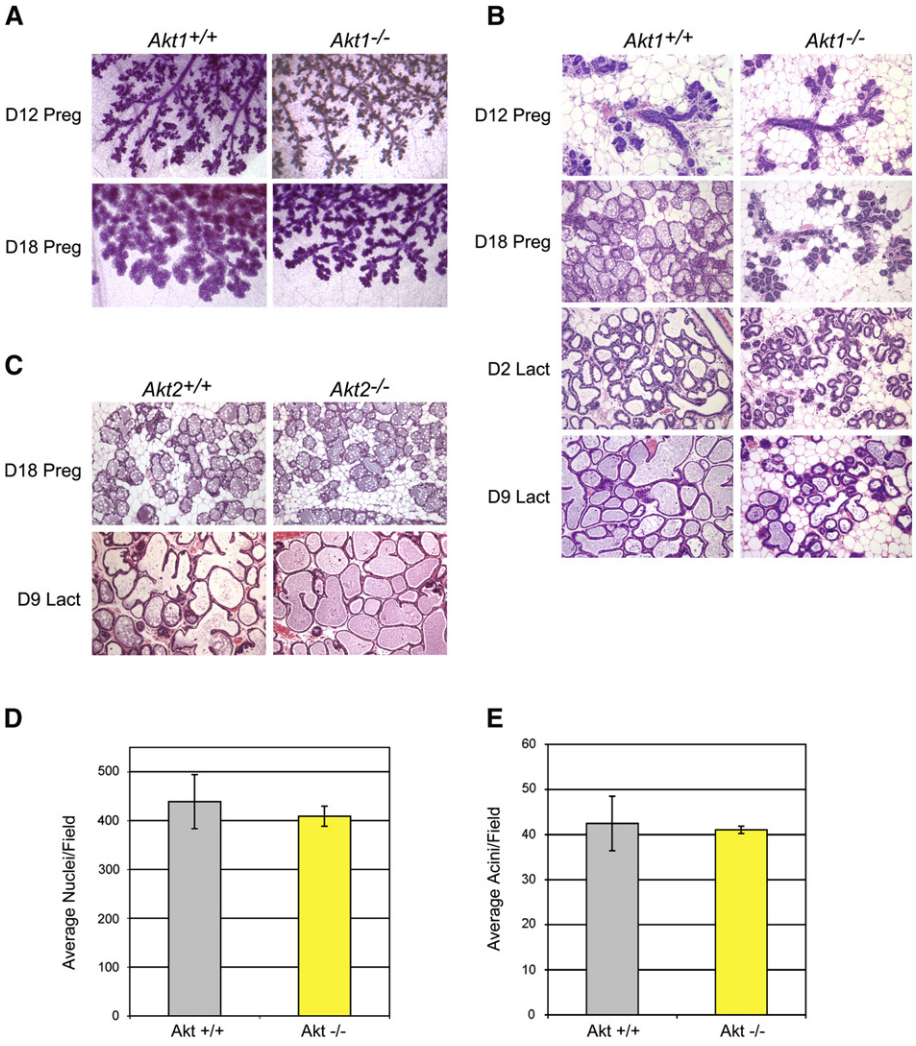

Figure 3. Normal lobuloalveolar development but reduced milk secretion in mammary glands of *Akt1*$^{-/-}$ mice

A) Carmine-stained whole-mounted mammary glands from *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at days 12.5 and 18.5 of pregnancy. 16× magnification.

B) Hematoxylin-and-eosin-stained sections of mammary glands from *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at days 12.5 and 18.5 of pregnancy and days 2 and 9 of lactation showing decreased distention of mammary ducts and alveoli in *Akt1*$^{-/-}$ mice. 200× magnification.

C) Hematoxylin-and-eosin-stained sections of mammary glands from *Akt2*$^{+/+}$ and *Akt2*$^{-/-}$ mice at day 18.5 of pregnancy and day 9 of lactation. 200× magnification. Results are representative of sections from at least three animals per group.

D) Quantification of epithelial nuclei at day 9 of lactation in the mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice. For (**D**) and (**E**), the mean of five fields of view from each of three mice per genotype is shown. Error bars represent SEM.

E) Quantification of the number of alveoli at day 9 of lactation in the mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice.

Akt1$^{-/-}$ mice were indistinguishable (Figure 2E). Similarly, the lipid content of milk samples did not differ between *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice (Figure 2F). Together, these data suggest that, while total milk production is decreased in *Akt1*$^{-/-}$ mice, the milk that is produced has a normal composition.

Deletion of *Akt1* results in a functional rather than structural defect in the mammary gland during pregnancy and lactation

Milk production during lactation is preceded by lobuloalveolar development during pregnancy. A central feature of lobuloalveolar development is the massive expansion of the mammary epithelial compartment via cellular proliferation that gives rise to increased branching of mammary ducts and formation of numerous alveolar secretory units (Borst and Mahoney, 1982). Since Akt is known to promote cellular proliferation and survival, we wished to determine whether the defect observed in milk production in lactating *Akt1*$^{-/-}$ mice resulted from a defect in lobuloalveolar development.

Analysis of carmine-stained whole mounts of mammary glands harvested from mid- to late-term pregnant mice (postcoital days 12.5 and 18.5, respectively) revealed that the number and organization of lobuloalveolar structures in *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice were comparable (Figure 3A). Similarly, histological sections of mammary glands from *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice were indistinguishable at day 12.5 of pregnancy, as were the numbers of epithelial cells present in mammary sections from *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at days 12.5 and 18.5 of pregnancy (Figure 3B and data not shown). Consistent with this, functional evaluation of the mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice during pregnancy and lactation by BrdU incorporation and TUNEL incorporation revealed indistinguishable rates of proliferation and apoptosis, respectively (data not shown).

As predicted based upon the unperturbed cellular dynamics of lobuloalveolar development in *Akt1*$^{-/-}$ mice during pregnancy, no differences were observed in the number of epithelial cells per alveolus or the number of alveoli present in mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at day 9 of lactation (Figures 3B, 3D, and 3E). However, analysis of histological sections revealed that alveoli in *Akt1*$^{-/-}$ mice were markedly less distended with milk than in *Akt1*$^{+/+}$ littermates at day 18.5 of pregnancy (Figure 3B). This reduction in alveolar distention in *Akt1*$^{-/-}$ mammary glands persisted throughout lactation (Figure 3B). In contrast, both the formation of lobuloalveolar structures and secretion of milk into the ductal lumen were unaffected in *Akt2*$^{-/-}$ mice at day 18.5 of pregnancy and day 9 of lactation (Figure 3C).

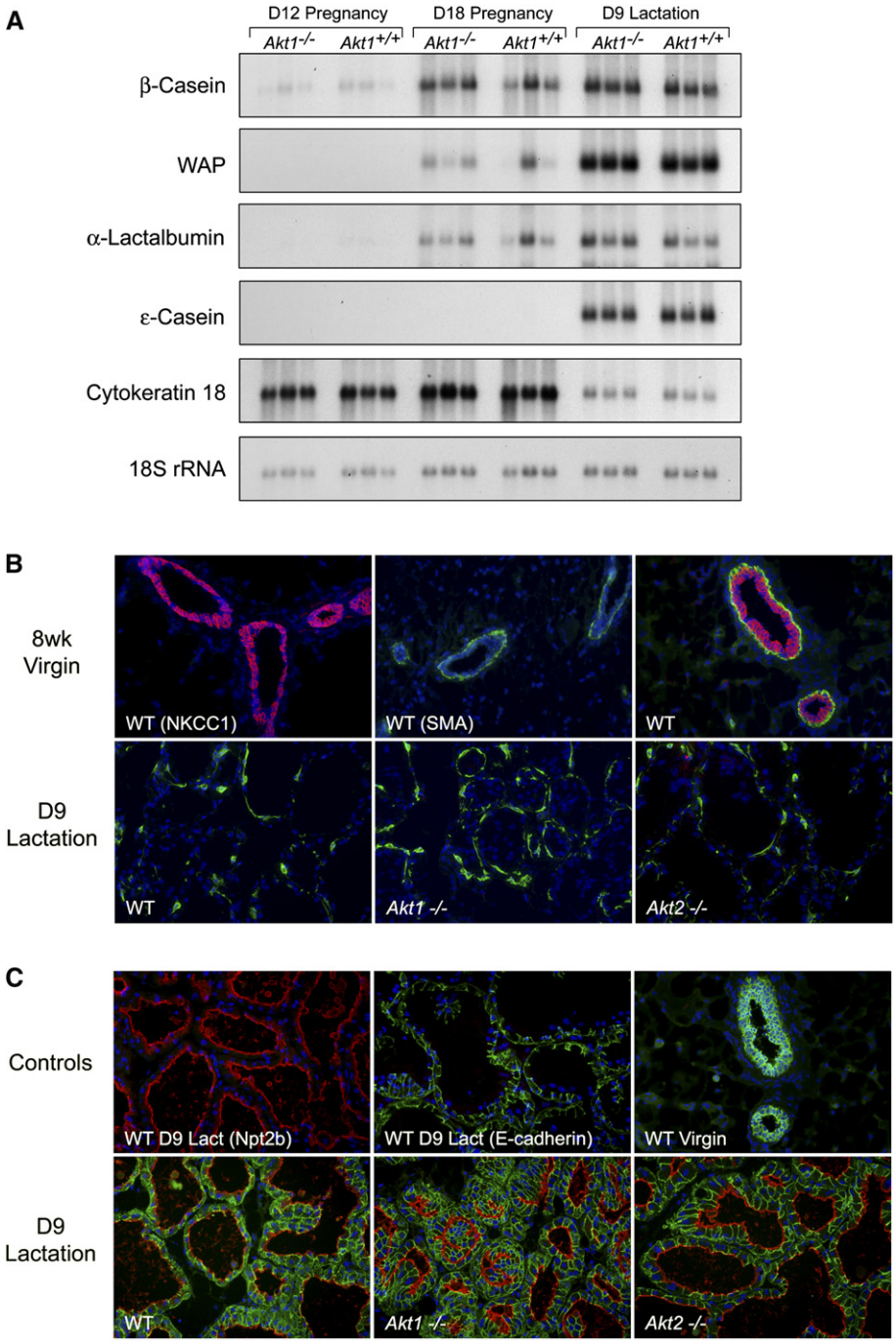

Figure 4. Secretory differentiation occurs normally in *Akt1*$^{-/-}$mice

A) Northern analysis of milk protein gene expression in mammary glands from *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at days 12.5 and 18.5 of pregnancy and day 9 of lactation showing the normal temporal expression of β-*casein*, *WAP*, α-*lactalbumin*, and ε-*casein*. *Cytokeratin 18* and 18S rRNA are included as controls for epithelial content and RNA loading, respectively.

B) NKCC1 (red) and smooth muscle actin (SMA) (green) immunofluorescence staining in wild-type (WT) virgin and day 9 lactation mammary sections from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ female mice. As expected, NKCC1 immunostaining, which is characteristic of nonsecretory luminal mammary epithelial cells, is observed in virgin but not lactating mammary glands. SMA staining identifies myoepithelial cells, which are positioned outside of luminal cells. Controls show staining reactions on WT virgin mammary sections in which the primary antibodies against SMA (top left) or NKCC1 (top center) were omitted. Nuclei were counterstained with Hoechst 33258 (blue). 400× magnification.

C) Npt2b (red) and E-cadherin (green) immunofluorescence staining in WT virgin and lactating WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice. Nuclei were counterstained with Hoechst 33258 (blue). As expected, the apical membranes of luminal mammary epithelial cells from lactating mice stain positively for Npt2b, a marker for secretory alveolar cells, whereas Npt2b staining is absent in virgin tissue (top right). Controls show staining reactions on lactating mammary sections in which the primary antibodies against E-cadherin (top left) or Npt2b (top center) were omitted. 400× magnification.

Taken together, these observations strongly suggest that the isoform-specific requirement for Akt1 during lactation is due to a functional defect in milk production rather than a structural defect in mammary gland development.

Secretory differentiation occurs normally in *Akt1*$^{-/-}$ mice

We next considered whether the failure of *Akt1*$^{-/-}$ mice to secrete normal amounts of milk was due to a defect in mammary epithelial differentiation. Secretory differentiation in the mammary gland is marked by the sequential upregulation of early, mid, and late milk protein genes (Rosen et al., 1999). Northern analysis of mammary RNA from four time points during pregnancy and lactation demonstrated that expression of early (β-casein), mid (whey acidic protein and α-lactalbumin), and late (ε-casein) epithelial differentiation markers was indistinguishable between *Akt1*$^{-/-}$ and *Akt1*$^{+/+}$ mice (Figure 4A).

Similar to milk protein gene expression, developmental regulation of the expression and localization of the solute transporters NKCC1 and Npt2b has been used to identify defects in mammary epithelial cell differentiation (Shillingford et al., 2003). Consistent with previous reports, NKCC1 staining on the basolateral surface of ductal epithelial cells was easily detected in mammary sections from wild-type virgin mice (Figure 4B). In contrast, NKCC1 staining was absent—as

expected—in sections from lactating *Akt1*$^{+/+}$, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice (Figure 4B).

Conversely, while Npt2b staining was absent in epithelial cells from nulliparous mammary glands, the apical membranes of secretory alveoli in *Akt1*$^{+/+}$ and *Akt2*$^{-/-}$ mice stained intensely for Npt2b at day 9 of lactation as expected (Figure 4C; Shillingford et al., 2003). Notably, despite the fact that secretory alveoli in *Akt1*$^{-/-}$ mice were not distended with milk, expression of Npt2b was appropriately upregulated during lactation and properly localized to the apical membrane in a manner similar to that observed in wild-type mice (Figure 4C). As such, the proper developmental regulation and localization of Npt2b and NKCC1 observed in *Akt1*$^{-/-}$ mice, when considered together with the appropriate temporal induction of milk protein gene expression, strongly suggests that the lactation defect observed in *Akt1*$^{-/-}$ mice is not due to a global defect in mammary epithelial differentiation.

Loss of Akt1 results in decreased glucose uptake during lactation

In addition to the regulation of milk protein gene transcription and solute transporters, the differentiated function of the mammary gland also requires the coordinate developmental upregulation of multiple biosynthetic pathways during late pregnancy and early lactation. In particular, the enormous energetic and synthetic demands of lactation require high levels of glucose transport to provide adequate substrate and energy to support the synthesis of milk proteins, lactose, and lipids. Accordingly, the developmental transition from pregnancy to lactation is accompanied by marked increases in Glut1 expression and its localization to the basal membrane of mammary epithelial cells (Camps et al., 1994).

To determine whether the lactation defect in *Akt1*$^{-/-}$ mice was associated with decreased glucose uptake, we evaluated the expression and localization of Glut1 by immunofluorescence. Whereas Glut1 expression was abundant on the basolateral surface of mammary epithelial cells from lactating *Akt1*$^{+/+}$ and *Akt2*$^{-/-}$ mice, Glut1 was virtually absent from the cell surface of lactating *Akt1*$^{-/-}$ mammary epithelial cells (Figure 5A). Conversely, immunofluorescence analysis for Glut1 in mammary sections from bitransgenic *MMTV-rtTA/TetO-myrAkt1* (MTB/tAkt1) mice, in which expression of a constitutively activated allele of *Akt1* was induced by doxycycline administration for 96 hr (R.B.B. and L.A.C., unpublished data), demonstrated a marked, acute increase in the expression and cell-surface localization of Glut1 in response to Akt activation (Figure 5B). Together, these data indicate that Akt1 is a critical regulator of Glut1 expression and localization in the mammary gland in vivo, which in turn suggests that Akt1 may regulate glucose uptake in this developmental context.

To directly examine the rate of glucose uptake and capture in lactating mammary glands in vivo, positron emission tomography was used to evaluate the accumulation of [^{18}F]fluorodeoxyglucose ([^{18}F]FDG). In accordance with the dramatic increase in metabolism that occurs during lactation, [^{18}F]FDG uptake was markedly elevated in the mammary glands of lactating compared to nulliparous wild-type mice (Figure 5C). In contrast, no increase in [^{18}F]FDG uptake was evident in the mammary glands of lactating *Akt1*$^{-/-}$ mice (Figure 5C). Lactating *Akt2*$^{-/-}$ mice, however, exhibited high levels of [^{18}F]FDG uptake comparable to those observed in lactating wild-type mice (Figure 5C). These observations demonstrate that Akt1 is required in an isoform-specific manner for the developmental-stage-specific increase in glucose uptake and trapping that occurs in the mammary gland at the onset of lactation.

Intraepithelial levels of lactose and milk proteins are normal in lactating *Akt1*$^{-/-}$ mice

The above findings demonstrated that the inability of Akt1-deficient mice to secrete normal amounts of milk during lactation is accompanied by a failure to upregulate glucose uptake in the mammary gland in a developmentally appropriate manner. The marked increase in glucose delivery to mammary epithelial cells that occurs at the onset of lactation is required to provide adequate substrate and energy to support the synthesis of milk proteins, lipids, and lactose. As the predominant carbohydrate in milk, lactose is one of the key osmotic regulators of milk secretion. Indeed, homozygous deletion of the regulatory subunit of lactose synthetase *α-lactalbumin*, whose expression is rate limiting for lactose synthesis, results in the formation of milk that is markedly reduced in volume and highly viscous such that it cannot be removed from the mammary gland by suckling pups (Stinnakre et al., 1994).

Arguing against such a mechanism for the impairment of lactation in *Akt1*$^{-/-}$ mice, however, the upregulation of *α-lactalbumin* expression at day 18.5 of pregnancy was unaffected by *Akt1* deletion (Figure 4A). Additionally, staining of mammary sections with the lactose-specific lectin ECA revealed similar levels of lactose in lactating *Akt1*$^{-/-}$ and *Akt1*$^{+/+}$ glands (Figure 6A). Thus, despite decreased glucose uptake and trapping in lactating *Akt1*$^{-/-}$ mice, steady-state levels of the principal sugar in milk—which is itself derived from glucose—were largely unaffected by loss of Akt1.

As noted above, the temporal regulation of milk protein gene expression during pregnancy was unaltered in *Akt1*$^{-/-}$ mice. However, despite transcription of milk protein genes during pregnancy, large-scale production of milk proteins does not occur until after parturition and requires the developmental upregulation of protein synthetic pathways, such as those regulated by mTOR. Since glucose represents a major source of energy in the lactating cell, and since Akt functions upstream of mTOR, we considered the possibility that lactation failure in *Akt1*$^{-/-}$ mice was due to defects in the developmental activation of the mTOR pathway and milk protein synthesis.

In contrast to this expectation, levels of the Ser235/236-phosphorylated form of the mTOR downstream effector S6 were appropriately upregulated in the mammary glands of *Akt1*$^{-/-}$ mice during lactation to levels indistinguishable from those in wild-type mice (Figure 6B, compare nulliparous MTB and lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ samples). These findings suggest that mTOR activity is largely unaffected by the absence of Akt1. Moreover, immunoblotting and immunofluorescence analyses demonstrated normal levels of milk proteins in the mammary gland and normal packaging of milk proteins into casein micelles (Figures 6C and 6D). Consistent with this, electron microscopy of epithelial cells from lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice revealed comparable numbers of ribosomes that were engaged appropriately on the endoplasmic reticulum (Figure 6E, lower panels). In aggregate, these findings indicate that milk protein synthesis—including the activation of the mTOR pathway—as well as lactose synthesis are largely unaffected by the deletion of *Akt1*.

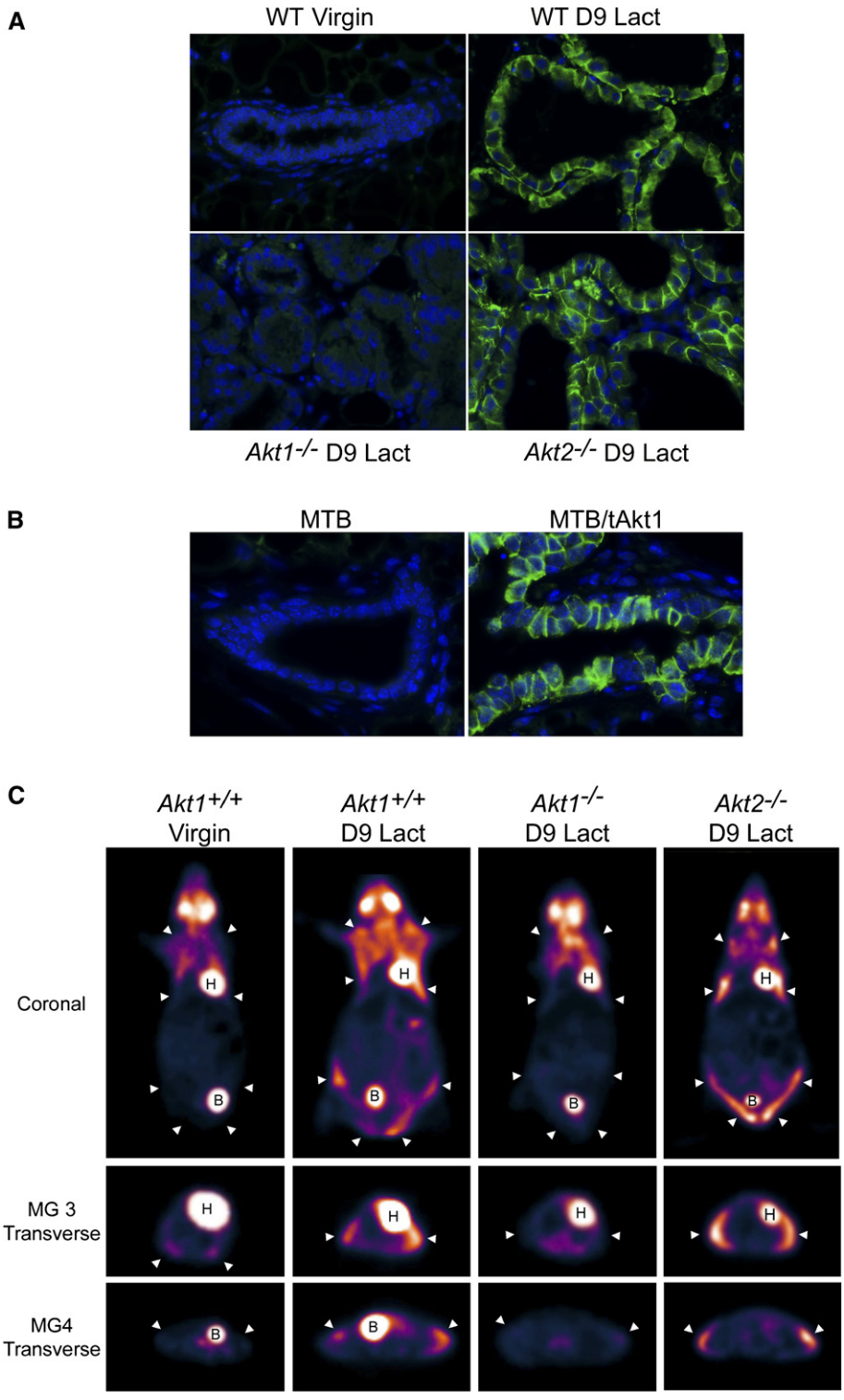

Figure 5. Decreased glucose uptake in the mammary glands of lactating *Akt1*$^{-/-}$ mice

A) Immunofluorescence analysis of Glut1 expression during lactation. Mammary sections from virgin WT (top left), lactating WT (top right), *Akt1*$^{-/-}$ (bottom left), and *Akt2*$^{-/-}$ (bottom right) mice were immunostained for Glut1 (green). Nuclei were counterstained with Hoechst 33258 (blue). 400× magnification.

B) Immunofluorescence analysis of Glut1 expression in inducible Akt1 transgenic mice. Mammary sections from control MTB (left) and MTB/tAkt1 (right) mice were immunostained for Glut1 (green). Nuclei were counterstained with Hoechst 33258 (blue). 400× magnification.

C) [^{18}F]FDG uptake during mammary gland development. Virgin and day 9 lactating *Akt1*$^{+/+}$, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were administered [^{18}F]FDG by tail-vein injection 2 hr prior to positron emission tomography (PET) scanning. Coronal and transverse images are shown for representative mice. Positions of mammary glands are indicated by arrows. Normal uptake in heart (H) and bladder (B) are indicated.

Akt1 is required for the upregulation of lipid synthesis in the lactating mammary gland

In addition to its requirement for protein and lactose synthesis, glucose also provides both energy and substrate for lipid production in the mammary gland. Indeed, lipid represents the most abundant constituent in milk, as a lactating mouse produces its entire body weight in lipid while nursing a single litter. Accordingly, the production of adequate amounts of lipid during lactation constitutes the principal metabolic stress on mammary epithelial cells. In turn, lipid is itself a critical dietary source of energy and substrate for rapidly growing offspring in the neonatal period.

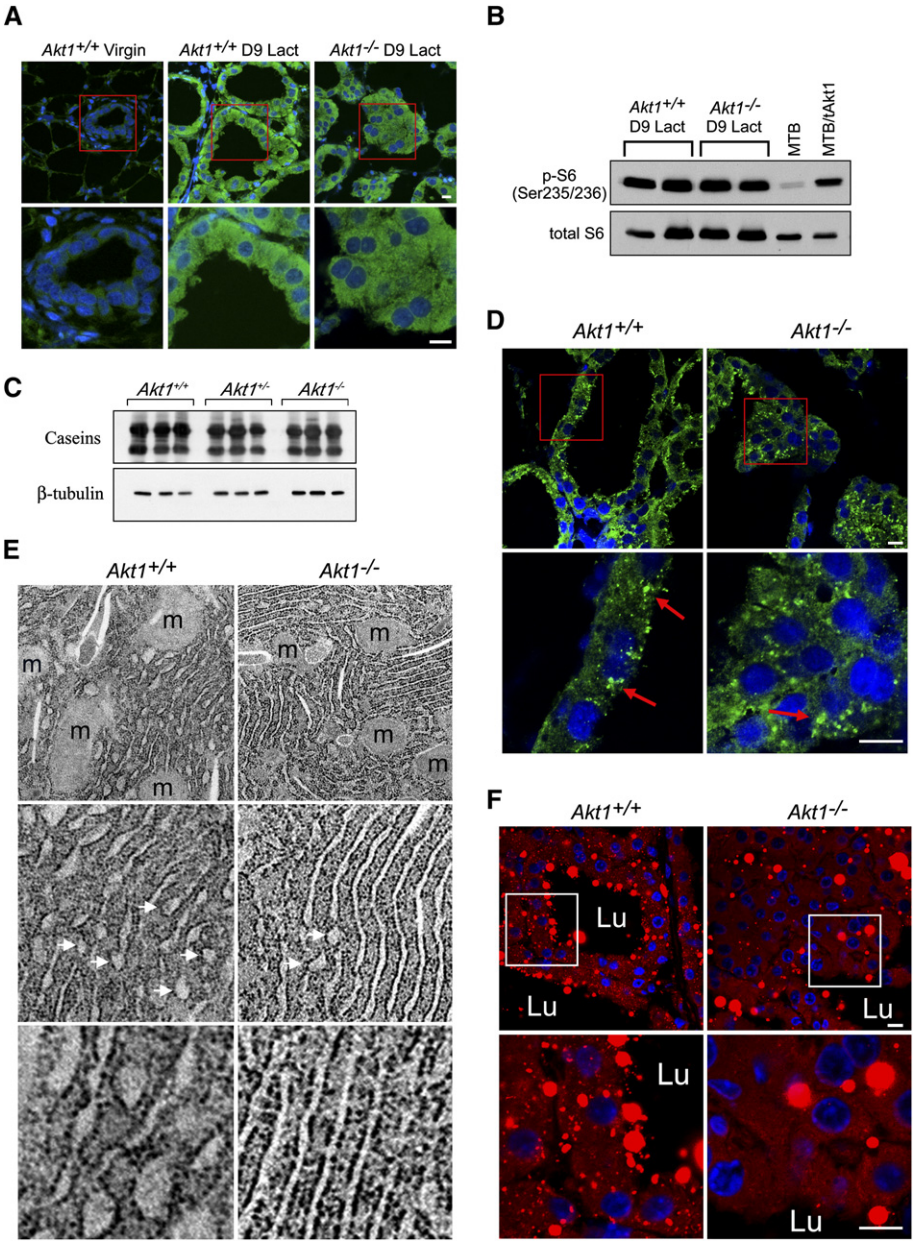

Figure 6. Normal lactose and protein but decreased lipid content in mammary epithelial cells from lactating *Akt1*$^{-/-}$ mice

A) Lactose-specific lectin staining of mammary epithelial cells. Mammary sections from virgin *Akt1*$^{+/+}$ mice (left) and day 9 lactating *Akt1*$^{+/+}$ (center) and *Akt1*$^{-/-}$ (right) mice stained for lactose using a Texas red-labeled ECA lectin. Magnified areas are shown in red boxes. Scale bars = 10 μm.

B) Western analysis of phospho-S6(Ser235/236) and total S6 expression in mammary protein lysates from lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice. Lysates from MTB and doxycycline-induced MTB/tAkt1 mice are shown as controls.

C) Western analysis of casein expression in mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at day 9 of lactation. β-tubulin levels are shown as a loading control.

D) Immunofluorescence analysis of casein micelles in mammary glands of *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice at day 9 of lactation. Scale bars = 10 μm.

E) Electron microscopy of mammary gland sections from lactating *Akt1*$^{+/+}$ (left) and *Akt1*$^{-/-}$ (right) mice. Lipid vesicles budding from the endoplasmic reticulum are indicated by white arrows. m, mitochondria. Top, middle, and bottom panels are at 50,000×, 100,000×, and 200,000× magnification, respectively.

F) Nile red staining of cytoplasmic lipid droplets in mammary sections from lactating *Akt1*$^{+/+}$ (left) and *Akt1*$^{-/-}$ (right) mice. Magnified areas are shown in white boxes. Lu, alveolar lumen. Scale bars = 10 μm.

Since Akt has previously been implicated in the regulation of fatty-acid synthesis (Gagnon et al., 1999; Porstmann et al., 2005; Van de Sande et al., 2002), we sought to determine whether the impaired production of milk observed in lactating *Akt1*$^{-/-}$ mice was associated with a defect in lipid production. Examination by electron microscopy of mammary sections from lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice revealed numerous small lipid vesicles budding from the endoplasmic reticulum of mammary epithelial cells in lactating *Akt1*$^{+/+}$ mice, whereas few lipid-containing vesicles were observed in lactating *Akt1*$^{-/-}$ mice (Figure 6E). In contrast, equivalent numbers of ribosomes and mitochondria were observed in cells from lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice. Consistent with the reduction in budding lipid vesicles observed by electron microscopy, visualization of lipid by Nile red staining revealed markedly fewer intracellular lipid droplets within epithelial cells from lactating *Akt1*$^{-/-}$ compared to *Akt1*$^{+/+}$ mice (Figure 6F). These data suggest that Akt1 is required for the developmental upregulation of lipid synthesis in the lactating mammary gland.

Akt1 is required for the developmental regulation of multiple lipid synthetic enzymes

To meet the demand for increased de novo lipid synthesis in the lactating mammary gland, the activities of multiple lipogenic enzymes are coordinately upregulated at the onset of lactation both transcriptionally as well as through phosphorylation and allosteric control (Barber et al., 1997). For example, microarray analysis revealed marked increases in stearoyl-CoA desaturase 2 (*Scd-2*) mRNA expression as well as modest but statistically significant increases in fatty-acid synthase (*Fasn*) and stearoyl-CoA desaturase 1 (*Scd-1*) expression in wild-type mice at the transition from pregnancy to lactation (Figure 7A).

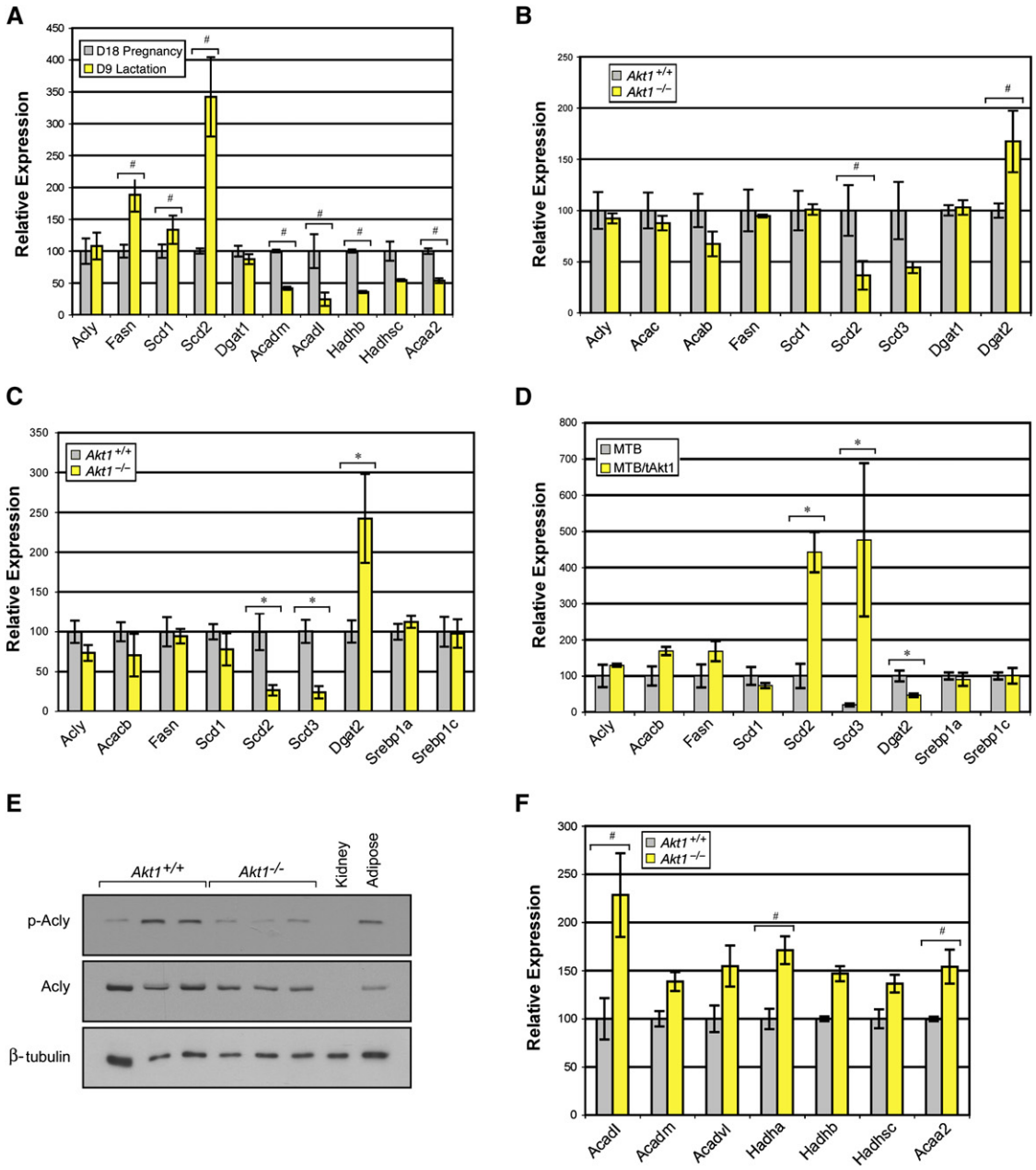

Figure 7. Akt1 mediates developmental changes in the expression and activity of enzymes involved in lipid metabolism

Microarray (**A**, **B**, and **F**), quantitative RT-PCR (**C** and **D**), and western analysis (**E**) of regulatory changes in lipid synthetic and catabolic enzymes in the mammary gland are shown.

A) Microarray analysis of mammary gene expression changes for enzymes involved in lipid metabolism that occur at the transition from pregnancy to lactation. Three pooled RNA samples isolated from mammary glands of wild-type female FVB mice at day 18.5 of pregnancy and day 9 of lactation were hybridized to MGU74Av2 Affymetrix arrays. For (**A**), (**B**), and (**F**), graphs depict average signal intensities, and error bars represent SEM. Gene expression changes determined to be statistically significant by ChipStat (Master et al., 2005) are indicated by a #.

B) Microarray analysis of gene expression for lipogenic enzymes in mammary glands of $Akt1^{+/+}$ and $Akt1^{-/-}$ mice at day 9 of lactation. Independent RNA samples from three mice of each genotype were hybridized to MOE430A Affymetrix arrays.

C) Quantitative RT-PCR analysis of genes involved in lipid synthesis. cDNA generated from total RNA samples of $Akt1^{+/+}$ (n = 7) and $Akt1^{-/-}$ (n = 5) mammary glands at day 9 of lactation were analyzed by quantitative PCR. For (**C**) and (**D**), average expression values normalized to *Tbp* ± SEM are shown. Significant differences in expression as measured by Student's t test ($p < 0.05$) are indicated by an asterisk (*).

D) Quantitative RT-PCR analyses of genes involved in lipid synthesis are shown for cDNA generated from MTB (n = 4) and MTB/tAkt1 (n = 4) mice induced with doxycycline for 4 days.

E) Western analysis of phospho-Acly and total Acly expression in mammary glands from $Akt1^{+/+}$ and $Akt1^{-/-}$ mice at day 9 of lactation. β-tubulin is shown as a loading control. Protein lysates from kidney and adipose tissue are also shown as negative and positive controls, respectively.

F) Microarray expression analysis for enzymes involved in β-oxidation of fatty acids in mammary glands of $Akt1^{+/+}$ and $Akt1^{-/-}$ mice at day 9 of lactation. Total RNA from mammary glands of $Akt1^{+/+}$ (n = 3) and $Akt1^{-/-}$ (n = 3) mice during lactation were analyzed by microarray as in (**B**).

In addition to the developmental upregulation of enzymes required for fatty-acid synthesis, the transition from pregnancy to lactation is also accompanied by the coordinate downregulation of multiple catabolic enzymes involved in the mitochondrial β-oxidation of fatty acids, including the acyl-coenzyme A dehydrogenases *Acadm* and *Acadl*, the *Hadhb* and *Hadhsc* subunits of the mitochondrial trifunctional protein, and acetyl-coenzyme A acyltransferase 2 (*Acaa2*) (Figure 7A). As such, the coordinate developmental regulation of gene expression that occurs at the onset of lactation promotes the net synthesis of lipid by upregulating lipid synthetic enzymes while downregulating lipid catabolic enzymes.

To explore the mechanism by which Akt1 deficiency results in impaired lipid accumulation in the lactating mammary gland, we performed a microarray expression screen in *Akt1*$^{-/-}$ and *Akt1*$^{+/+}$ mice to identify alterations in the expression of key enzymes regulating fatty acid and triglyceride metabolism. Consistent with the observed decrease in intracellular lipid in *Akt1*$^{-/-}$ mammary epithelial cells, expression of stearoyl-CoA desaturase 2 (*Scd2*) and stearoyl-CoA desaturase 3 (*Scd3*) were markedly decreased, and the β isoform of acetyl-CoA carboxylase (*Acacb*) was modestly decreased in the mammary glands of lactating *Akt1*$^{-/-}$ mice (Figure 7B). *Scd1* expression was unaffected by *Akt1* deletion (Figure 7B). In contrast, expression of diacylglycerol acyltransferase 2 (*Dgat2*), which catalyzes the final step in the production of triacylglycerol, was increased.

To confirm the above changes in gene expression, quantitative RT-PCR was performed on RNA from the mammary glands of lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice (Figure 7C). Consistent with microarray results, lactating Akt1-deficient mice exhibited marked decreases in *Scd2* and *Scd3* expression, a significant increase in *Dgat2* expression, and a trend toward decreased expression of *Acacb*. Again, no change was observed for *Scd1* expression (Figure 7C). Moreover, significant alterations in the expression of *Fasn* and ATP-citrate lyase (*Acly*) were not detected (Figures 7B and 7C), despite previous reports of their transcriptional regulation by Akt in other cell types (Porstmann et al., 2005; Van de Sande et al., 2002). These findings suggest that Akt1 may play a role in the coordinate upregulation of lipid synthetic enzymes at the onset of lactation and identify *Scd2*, *Scd3*, and *Dgat2* as transcriptional targets of Akt1.

To further evaluate a causal role for Akt1 in the transcriptional regulation of enzymes involved in lipid biosynthesis, the effect of acute Akt1 activation on the expression of these enzymes was determined in vivo in the mammary glands of MTB/tAkt1 bitransgenic mice induced to express activated Akt1 by the administration of doxycycline for 4 days. In vivo activation of Akt1 in the mammary epithelium resulted in a 4-fold induction of *Scd2*, a 40-fold induction of *Scd3*, and no induction of *Scd1* (Figure 7D). Additionally, consistent with our observations in *Akt1*$^{-/-}$ mice, expression of *Dgat2* was reduced by more than 50% in the mammary glands of induced bitransgenic mice (Figure 7D). Modest Akt1-induced increases in *Acacb* and *Fasn* expression were also observed.

The robust, inverse changes in *Scd2* and *Scd3* expression that occur in response to Akt1 loss versus Akt1 activation in the mammary gland suggest that Akt1 may play a relatively direct role in regulating stearoyl-CoA desaturase expression, as is further implied by the rapidity with which *Scd* expression is induced following Akt1 induction in MTB/tAkt1 mice. Given that *Scd* mRNA levels are the main determinant of Scd activity in the mammary gland (Singh et al., 2004) and that Scd activity correlates with a cell's capacity for triglyceride synthesis (Listenberger et al., 2003), we conclude that Akt1-mediated upregulation of *Scd* expression may constitute an important mechanism for upregulating triglyceride synthesis during the transition from pregnancy to lactation.

Akt overexpression has previously been implicated in the induction of SREBP1c (Fleischmann and Iynedjian, 2000; Porstmann et al., 2005), a transcription factor that regulates the expression of several of the lipogenic enzymes that we found were altered in *Akt1*$^{-/-}$ mice. Accordingly, quantitative RT-PCR analysis of *SREBP1a* and *SREBP1c* expression levels were performed using probes specific for each isoform (Porstmann et al., 2005). No difference in expression was detected for either *SREBP1* isoform between lactating *Akt1*$^{-/-}$ and *Akt1*$^{+/+}$ mice or when Akt1 was transiently activated in the mammary glands of MTB/tAkt1 mice (Figures 7C and 7D). These findings strongly suggest that Akt1-mediated regulation of lipogenic enzymes in the lactating mammary gland is independent of SREBP1.

Acly, the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA, is activated through phosphorylation by Akt (Berwick et al., 2002). Acly hydrolyzes citrate produced in mitochondria and transported to the cytoplasm, thereby generating oxaloacetate and acetyl-CoA, which serve as essential substrates for NADPH generation and fatty-acid synthesis, respectively. As predicted, immunoblotting performed for phospho-Acly(Ser454) revealed that phospho-Acly levels were reduced in the mammary glands of lactating *Akt1*$^{-/-}$ mice compared to wild-type mice (Figure 7E). This indicates that loss of Akt1 results in decreased Acly activation in the mammary gland, suggesting that impaired Acly activation may contribute to the decreased synthesis of lipid observed in *Akt1*$^{-/-}$ mice.

In addition to the coordinate upregulation of lipid synthetic enzymes that normally occurs at the onset of lactation, coordinate repression of enzymes involved in the β-oxidation of fatty acids is also observed (Figure 7A). Presumably, such a shift would prevent fatty acids from being catabolized by β-oxidation at the same time that epithelial cells are synthesizing them for secretion into milk. Notably, comparison of mammary expression profiles of lactating *Akt1*$^{+/+}$ and *Akt1*$^{-/-}$ mice revealed the inappropriate derepression of multiple enzymes involved in the β-oxidation of fatty acids (Figure 7F) that are otherwise downregulated at the onset of lactation (Figure 7A). These included several acyl-coenzyme A dehydrogenases (*Acadm*, *Acadl*, and *Acadvl*), both subunits of the mitochondrial trifunctional protein (*Hadha* and *Hadhb*), and *Acaa2* (Figure 7F). These observations indicate that Akt1 is required not only for the upregulation of lipid synthetic enzymes that occurs at the transition from pregnancy to lactation but also for the repression of enzymes involved in the β-oxidation of fatty acids. This, in turn, suggests that Akt1 not only acts to promote the synthesis of fatty acids during lactation but also concurrently suppresses their catabolism.

Discussion

Nutritional support of offspring represents one of the most critical and highly conserved functions found in mammals, so much so that mammals are defined by their ability to produce

milk. To meet the synthetic demands of lactation, mammary epithelial cells must coordinately upregulate pathways controlling carbohydrate, protein, and lipid synthesis as well as the generation of an adequate supply of energy and reducing equivalents to support these anabolic processes. In this manuscript, we demonstrate that Akt1 is essential for this metabolic response.

The isoform-specific requirement that we have observed for Akt1 in the developmental regulation of cellular metabolism in the mammary gland is consistent with the upregulation of Akt1 expression that occurs during pregnancy and lactation. In contrast, Akt2 is dispensable for this developmental response to metabolic stress. Notably, despite Akt1's established role in the regulation of cellular proliferation and apoptosis, the failure of *Akt1*$^{-/-}$ mice to lactate does not arise from a failure of mammary epithelial expansion during pregnancy or from structural defects in the mammary gland. Rather, the developmental defects observed in Akt1-deficient mice arise from a failure to coordinately upregulate multiple metabolic pathways at the transition from pregnancy to lactation. This suggests that the developmental regulation of cellular metabolism represents a distinct facet of the developmental program that results in secretory activation and lactation in the mammary gland. Moreover, the failure of Akt1-deficient mice to lactate demonstrates that these coordinated metabolic changes are no less essential for the development of the mammary gland than the structural changes that occur in the gland during lobuloalveolar development.

The transition from pregnancy to lactation in the mammary gland is normally accompanied by a coordinated gene expression program that promotes net lipogenesis by upregulating lipid synthesis while downregulating lipid catabolism. This developmental program is disrupted by the loss of Akt1, thereby leading to a defect in lactation. Specifically, loss of Akt1 at this developmental transition results in a failure to upregulate glucose uptake and trapping, a failure to upregulate Glut1, a failure to localize Glut1 to the cell surface, a failure to upregulate lipid synthesis, and a failure to upregulate the expression and activation of multiple lipogenic enzymes, including Acly, stearoyl-CoA desaturase 2, and stearoyl-CoA desaturase 3. In addition, the repression of enzymes involved in the β-oxidation of fatty acids that normally occurs at the onset of lactation fails to occur in the absence of Akt1. In aggregate, our findings indicate that Akt1 is required for orchestrating the metabolic response of mammary epithelial cells to the nutritional stress of lactation.

In vitro studies have implicated the PI3K/Akt pathway in regulating glucose transporter expression and localization in a variety of cell types and in response to multiple signals (reviewed in Plas and Thompson, 2005; Welsh et al., 2005). Previously, the requirement for Akt family members in glucose metabolism had been ascribed almost exclusively to Akt2 (Cho et al., 2001a; Garofalo et al., 2003). In contrast, our studies demonstrate that Akt1 is absolutely required—and that Akt2 is entirely dispensable—for translocation of Glut1 to the cell surface and upregulation of glucose uptake and trapping at the onset of lactation in the mammary gland. As such, these current findings identify a developmental-stage-specific function for Akt1 and reveal a requirement for Akt1 in the regulation of glucose metabolism in vivo. Moreover, these effects are isoform specific, as the upregulation of Glut1 and glucose uptake that accompanies lactation occurs normally in mice lacking Akt2.

Once taken up by the lactating mammary epithelial cell, glucose is either used in the synthesis of lactose or processed by glycolysis to provide energy, reducing equivalents, and carbon subunits for synthetic pathways. The data presented in this manuscript indicate that upregulation of lipid synthesis—the most abundant component of milk—is severely impaired in the absence of Akt1. Our findings are consistent with overexpression studies implicating Akt1 in the control of lipogenesis (Gagnon et al., 1999; Magun et al., 1996; Porstmann et al., 2005; Schwertfeger et al., 2003) and extend these findings by demonstrating that Akt1 is required for lipid synthesis in a physiologically relevant context.

In lipogenic cells with high rates of glucose uptake, mitochondria produce citrate from glycolytically derived pyruvate by means of a truncated TCA cycle (Baggetto, 1992). Citrate is then preferentially exported to the cytosol via the tricarboxylate transporter (Kaplan et al., 1993) and cleaved by Acly to form oxaloacetate and acetyl-CoA (Srere, 1959), the requisite building block for de novo fatty-acid synthesis. Loss of Akt1 in lactating mammary epithelial cells not only reduces the supply of carbon units through decreased glucose uptake but also results in the reduced phosphorylation and activation of Acly, thereby further impairing fatty-acid synthesis. These data, along with recent evidence demonstrating that knockdown of Acly activity impairs Akt1-mediated leukemogenesis, suggest that Acly is an important mediator of Akt activity in vivo (Bauer et al., 2005).

Loss of Akt1 also resulted in decreased expression of *Scd2* and *Scd3* in the lactating mammary gland. These enzymes catalyze the conversion of saturated fatty acids to monounsaturated fatty acids during the final phases of fatty-acid synthesis and are normally upregulated at the onset of lactation (Miyazaki and Ntambi, 2003). Consistent with a role for the diminished upregulation of *Scd2* and *Scd3* in impaired lipid synthesis in lactating *Akt1*$^{-/-}$ mice, targeted deletion of *Scd2* in mice reduces triglyceride synthesis, and targeted deletion of *Scd1* results in decreased triglyceride synthesis and body adiposity as well as increased hepatic expression of enzymes involved in the β-oxidation of fatty acids (Miyazaki et al., 2005; Ntambi et al., 2002). When taken together with the rapid induction of *Scd2* and *Scd3* expression that we observe in vivo following activation of Akt1, our findings identify these steroyl-CoA desaturases as likely transcriptional targets of Akt1.

In addition to the failed upregulation of lipid synthetic enzymes, multiple enzymes regulating mitochondrial β-oxidation—which are normally repressed during lactation—were inappropriately derepressed in the mammary glands of *Akt1*$^{-/-}$ mice. It can be speculated that, were such alterations to result in increased fatty-acid oxidation, they would further contribute to the decreased net synthesis of lipids observed in mammary epithelial cells lacking Akt1.

Beyond the reduced lipid content of the lactating mammary gland in Akt1-deficient mice, the absolute quantities of milk proteins and lactose produced during lactation were also reduced by loss of Akt1 as a consequence of the marked decrease in milk volume. Potentially, this could reflect specific effects of Akt1 on glucose uptake, which is required for the synthesis of lactose and nonessential amino acids. Nevertheless, steady-state levels of lactose and milk proteins were grossly normal within lactating mammary epithelial cells of *Akt1*$^{-/-}$ mice, whereas steady-state lipid levels were markedly reduced. The predominance of the lipid synthetic defect observed in lactating *Akt1*$^{-/-}$ mice may reflect the fact that lipid represents the chief component of milk by weight, more than twice that of protein

and ten times that of lactose, and likely constitutes the principal metabolic stress for lactating mammary epithelial cells. Since *Akt1*$^{-/-}$ mice produce only a fraction of the milk produced by their wild-type counterparts, potential defects in metabolic pathways required for the synthesis of milk components other than lipid may be masked. As such, given the marked reduction in glucose uptake and trapping engendered by Akt1 loss, it is possible that protein and lactose synthesis would be further impaired were *Akt1*$^{-/-}$ mice able to generate normal quantities of milk.

Interestingly, unlike mammary epithelial cells in lactating *Akt1*$^{-/-}$ mice, which have a reduced lipid content, the milk generated by *Akt1*$^{-/-}$ mice is normal in composition, though reduced in volume. This observation raises the intriguing possibility that a metabolic feedback mechanism may exist coupling lipid synthesis to protein and lactose synthesis in the lactating mammary gland in order to maintain the lipid content of milk above a threshold level. If this is the case, however, such a mechanism would likely be specific for lipid since mice homozygous for targeted deletions of α-lactalbumin produce milk that is devoid of lactose but rich in fat and protein (Stinnakre et al., 1994) and mice homozygous for targeted deletions of β-casein produce milk with reduced protein concentration (Kumar et al., 1994). Nevertheless, while a specific mechanism by which a defect in lipid synthesis might regulate the synthesis or secretion of other macromolecules has yet to be elucidated, given the physiological complexity of the lactating mammary gland, systems biology considerations alone suggest that feedback loops interconnecting diverse metabolic pathways are likely to exist.

To date, few isoform-specific functions for Akt1 and Akt2 have been identified, and differing phenotypes in mice deficient for individual Akt family members have typically been attributed to tissue-specific differences in the expression of different isoforms. One notable exception has been the ability of Akt family members to regulate glucose uptake and metabolism, which has previously been ascribed almost exclusively to Akt2. Indeed, among the three Akt family members, only deletion of *Akt2* has thus far been shown to result in impaired metabolism in mice. Consistent with this, siRNA-mediated knockdown of Akt2—but not Akt1—in 3T3-L1 cells results in impaired glucose uptake (Jiang et al., 2003). Similarly, reintroduction of Akt2—but not Akt1—into Akt2-deficient adipocytes restores insulin-induced glucose uptake (Bae et al., 2003).

In contrast, our studies demonstrate that Akt1 is absolutely required—and that Akt2 is entirely dispensable—for the developmental-stage-specific upregulation and translocation of Glut1 to the cell surface and for the upregulation of glucose uptake and trapping. In addition, deletion of *Akt1* causes a lipid synthetic defect in the lactating mammary gland that is severe enough to result in growth defects and pup mortality, whereas mice lacking Akt2 lactate normally and support their young. As such, the identification of this unexpected metabolic phenotype in Akt1-deficient mice reveals a developmental-stage-specific function for Akt1 and—contrary to prior expectations—clearly establishes a role for Akt1 in glucose metabolism in vivo. Additionally, the expression of both Akt1 and Akt2 within the epithelium and stroma of the mammary gland suggests that the presence of a lactation defect in *Akt1*$^{-/-}$, but not *Akt2*$^{-/-}$, mice results from the loss of isoform-specific activities of Akt1 rather than differences in the localization of Akt isoforms in the mammary gland. As such, our data demonstrate that Akt1 acts as a central metabolic regulator in the mammary gland during lactation and suggest that its ability to do so may be due to isoform-specifc functions of this Akt family member.

Finally, it is worth noting that the metabolic demands of the lactating mammary epithelial cell bear many similarities to those of tumor cells in that pathways controlling energy generation as well as carbohydrate, protein, and lipid synthesis must be coordinated to support the anabolic processes central to cell growth. In this regard, Akt1's ability to act as a central regulator of nutrient uptake and utilization is consistent with the frequent observation of constitutive activation of the PI3K/AKT pathway in a wide range of human cancers, including those of the breast (Altomare and Testa, 2005). Transformed cells commonly exhibit increased rates of glucose uptake as part of a shift to increased glycolysis and reduced oxidative phosphorylation (Warburg et al., 1924). Increased glycolytic metabolism in tumors as determined by FDG-PET is associated with increased tumor aggressiveness and poor prognosis (Buck et al., 2004; Detterbeck et al., 2004). Beyond promoting cell survival through maintenance of mitochondrial potential (Rathmell et al., 2003), this adaptation facilitates the utilization of glucose in multiple biosynthetic pathways required for cell growth, including the synthesis of lipid for use in membranes (Bauer et al., 2004, 2005). Our findings demonstrating a physiological requirement for Akt1 in the control of glucose and lipid metabolism further implicate Akt1 activation as a potential mediator of the Warburg effect and reinforce the importance of efforts to design pharmacologic agents that will inhibit this pathway in tumors.

Experimental procedures

Animals and milk collection

Akt1$^{-/-}$ (Cho et al., 2001b) and *Akt2*$^{-/-}$ (Cho et al., 2001a) mice were backcrossed a minimum of ten generations onto a C57BL/6 background. Bitransgenic mice that inducibly express Akt1 were generated by crossing *MMTV-rtTA* (MTB) mice (Gunther et al., 2002) to *TetO-Akt1* mice bearing a myristoylated *Akt1* allele fused to a tetracycline-inducible minimal promoter. Transgenic animals were administered doxycycline (2 mg/ml; Sigma) in their drinking water.

For milk collection, pups were removed for 3 hr at day 9 of lactation and dams were injected with 10 units of oxytocin to induce milk letdown. Milk was manually removed from the number three mammary glands. For analysis of fat content, whole milk was centrifuged in capillary tubes as described (Schwertfeger et al., 2003).

RNA analysis

RNA isolation and northern hybridization were performed as described, as was preparation of biotinylated cRNA, hybridization to MGU74Av2 and MOE430A Affymetrix arrays, and analysis using GeneChip MAS 5.0 (Master et al., 2002). Microarray data have been deposited in MIAMEXPRESS (accession numbers E-MEXP-891 and E-MEXP-892). Northern blots were quantified using a Molecular Dynamics phosphorimager.

Quantitative RT-PCR using TaqMan assays (Applied Biosystems) were performed using an ABI Prism sequence detection system (Applied Biosystems). Probes used were as follows: *Acly*, assay ID Mm00652520_m1; *Acacb*, assay ID Mm01204657_m1; *Fasn*, assay ID Mm00662319_m1; *Scd1*, assay ID Mm00772290_m1; *Scd2*, Assay ID Mm00485951_g1; *Scd3*, assay ID Mm00470480_m1; *Dgat2*, assay ID Mm00499530_m1; *Tbp*, assay ID Mm00446973_m1; *Srebp1a* and *Srebp1c* (Shimomura et al., 1997). Gene expression levels were normalized to *Tbp*.

Epithelial and adipose tissue isolation

Mammary tissues were minced and digested in a collagenase solution (1 mg/ml collagenase, 1 mg/ml fatty-acid-free BSA in EBSS) for 3 hr at 37°C using

10 ml of solution per gram of tissue. Tissue fragments were allowed to settle to the bottom of the tube for 15 min, the collagenase solution was replaced, and the incubation was continued for an additional hour. After pelleting at 1000 rpm for 4 min, epithelial cells were washed three times and lysed in sample loading buffer. Adipose tissue was treated identically except for the omission of collagenase from the digestion medium.

Quantification of epithelial nuclei and alveoli

Epithelial nuclei and alveoli were quantified on mammary sections from day 9 lactating mice stained for CK8 primary antibody with Hoechst 33258 nuclear counterstaining. Five images per animal and three animals per genotype were captured for analysis. Cell counts were obtained using ImagePro Plus 5.0 (Media Cybernetics).

Western analysis

Western blotting was performed as described (Gardner et al., 2000) with the following antibodies: phospho-Akt(Ser473), Akt, Akt1 (2H10), Akt2 (5B5), phospho-Acly(Ser454), Acly, phospho-S6(Ser235/236), and S6 (Cell Signaling); β-tubulin (clone DM-1, InnoGenex); and actin (Santa Cruz). Membranes for milk protein western blots were blocked in 3% BSA, 1× PBS, and 0.05% Tween 20 (PBST-BSA) and probed with polyclonal rabbit antisera to mouse milk-specific proteins (Nordic Immunological Laboratories).

Fluorescence and immunofluorescence

Ten micrometer mammary sections embedded in OCT medium were fixed in acetone (NKCC1, Npt2b) or 4% PFA for 10 min (casein, Glut1, CK8) at room temperature. Primary antibodies were incubated on sections overnight at 4°C using the rabbit polyclonal antibodies NKCC1 (1:1,000, gift from Dr. Jim Turner, NIDCR), Npt2b (1:300, gift from Dr. Jurg Biber, University of Zurich), and Glut1 (1:5,000, Dr. Morris Birnbaum, University of Pennsylvania) or the monoclonal antibodies rat anti-E-cadherin (1:100; Zymed), mouse anti-smooth muscle actin (1:400, Sigma), and cytokeratin 8 (1:100, Developmental Studies Hybridoma Bank, University of Iowa) or anti-casein (clone RB4, gift from Dr. Mina Bissell, Lawrence Berkeley National Laboratory). Following incubation with appropriate Alexa 488 and Alexa 568 (Molecular Probes) fluorescent-conjugated secondary antibodies (1:1,000), sections were counterstained with Hoechst 33258 (1:10,000) and mounted with Fluoromount-G (Southern Biotechnology Associates).

Lipid and lactose staining were performed on 10 μm OCT-embedded sections fixed in 4% PFA. For lipids, sections were stained with Hoechst 33258 and coverslipped with a drop of Nile red solution (1:200 dilution in 75% glycerol). For lactose, following CK8 antibody and Alexa 488 incubation, sections were stained with Texas red-labeled ECA lectin (EY laboratories) and Hoechst 33258 and coverslipped with Fluoromount-G.

Electron microscopy

Minced tissues were fixed in 2.5% glutaraldehyde and 4% PFA and post-fixed with 2% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate (pH 7.2). After embedding in Polybed resin (Polysciences, Inc.), 60–70 nm sections were placed on 200-mesh copper grids and stained with 1% uranyl acetate in 50% ethanol and bismuth subnitrate (1:50). Images were viewed and captured using a JEOL 1010 transmission electron microscope equipped with a Hamamatsu CCD camera and AMT imaging software.

FDG-PET imaging

Mice were administered [^{18}F]FDG (0.4–0.6 mCi) via tail-vein injection 2 hr prior to PET scanning. Mice were anesthetized via inhalation with isofluorane immediately prior to and during scanning. PET scanning and image processing were performed as described (Surti et al., 2005).

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Accession numbers

Microarray data have been deposited in MIAMEXPRESS with the accession numbers E-MEXP-891 and E-MEXP-892.