Akt is required for Stat5 activation and mammary differentiation

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Abstract

Introduction: The Akt pathway plays a central role in regulating cell survival, proliferation and metabolism, and is one of the most commonly activated pathways in human cancer. A role for Akt in epithelial differentiation, however, has not been established. We previously reported that mice lacking Akt1, but not Akt2, exhibit a pronounced metabolic defect during late pregnancy and lactation that results from a failure to upregulate Glut1 as well as several lipid synthetic enzymes. Despite this metabolic defect, however, both Akt1-deficient and Akt2-deficient mice exhibit normal mammary epithelial differentiation and Stat5 activation.

Methods: In light of the overlapping functions of Akt family members, we considered the possibility that Akt may play an essential role in regulating mammary epithelial development that is not evident in Akt1-deficient mice due to compensation by other Akt isoforms. To address this possibility, we interbred mice bearing targeted deletions in Akt1 and Akt2 and determined the effect on mammary differentiation during pregnancy and lactation.

Results: Deletion of one allele of Akt2 in Akt1-deficient mice resulted in a severe defect in Stat5 activation during late pregnancy that was accompanied by a global failure of terminal mammary epithelial cell differentiation, as manifested by the near-complete loss in production of the three principal components of milk: lactose, lipid, and milk proteins. This defect was due, in part, to a failure of pregnant Akt1-/Akt2+/ mice to upregulate the positive regulator of Prlr-Jak-Stat5 signaling, Id2, or to downregulate the negative regulators of Prlr-Jak-Stat5 signaling, caveolin-1 and Socs2.

Conclusions: Our findings demonstrate an unexpected requirement for Akt in Prlr-Jak-Stat5 signaling and establish Akt as an essential central regulator of mammary epithelial differentiation and lactation.

Introduction

The serine/threonine kinase Akt is a critical downstream effector in multiple signal transduction pathways and regulates cellular proliferation, survival, and metabolism. Consistent with this, Akt is inappropriately activated in a wide range of human cancers [1,2]. Three Akt isoforms (Akt1, Akt2, and Akt3) are present in mammals, and targeted deletion of each gene has revealed distinct as well as overlapping functions in cellular physiology. Akt1-/- mice exhibit increased perinatal mortality and a modest growth defect. Akt2-/- mice are viable but develop insulin resistance and a diabetes-like phenotype, whereas Akt3-/- mice have normal glucose homeostasis but decreased brain size [3-7].

Given the high degree of homology among Akt isoforms, the possibility that these three proteins play redundant roles has been addressed by generating mice deficient for multiple isoforms. This has revealed that Akt1-/-;Akt2-/- mice display perinatal lethality, reduced growth, and defects in skin and bone development, as well as adipogenesis [8]. Akt1-/-;Akt3-/- mice die during embryonic development at embryonic day 12, and Akt1-/-;Akt3+/ mice exhibit developmental abnormalities in multiple organs that result in the death of 90% of mice shortly after birth [9]. In contrast, Akt2-/-;Akt3-/- mice are born in Mendelian ratios, but are significantly smaller than wild-type littermates [10]. These results indicate that individual Akt isoforms play both unique and overlapping roles in development and physiology.
and further suggest that a critical threshold of Akt activity may be required to produce a given cellular output.

Similar to Akt, the Stat5 pathway plays a central role in regulating cellular function and is activated in response to a wide range of stimuli, including growth factors, cytokines, and hormones. Stat5 regulates cell proliferation, survival, and differentiation through direct activation of target genes. Stat5 is encoded by two closely related genes, Stat5a and Stat5b. While constitutive deletion of both genes leads to embryonic lethality [11], conditional knockouts have revealed tissue-specific functions of Stat5 [11-14].

The best-characterized role of Stat5 in normal physiology is the regulation of pregnancy-induced mammary epithelial development, where it is essential for the proliferation, differentiation, and survival of mammary epithelial cells [11]. Analysis of a variety of Stat5 mutant alleles in mice has revealed that Stat5 deficiency in the mammary gland leads to a near-complete loss of lobuloalveolar development, a reduction in expression of milk protein genes during pregnancy, and lactation failure [11,15-18]. Stat5 deletion during pregnancy results in the death of differentiated mammary epithelial cells, with further experiments suggesting that the requirement for Stat5 is required for the survival of alveolar luminal progenitor cells during pregnancy-induced lobuloalveolar development [11,19].

Constitutive activation of Akt in the mammary epithelium promotes the precocious accumulation of intracellular lipid droplets during pregnancy and delays postweaning involution by inhibiting apoptosis [20-22]. Consistent with its upstream role as a negative regulator of Akt activity, Pten-deficient mice exhibit delayed mammary involution and reduced apoptosis [23], whereas forced expression of Pten in the mammary gland results in impaired lactation due to decreased mammary epithelial proliferation and increased apoptosis during pregnancy [24].

We recently investigated the role of Akt in mammary development by examining mice bearing targeted deletions in either Akt1 or Akt2 [25]. We found that loss of both alleles of Akt1 results in failure of the coordinated metabolic response required for the establishment of lactation at parturition, including increased glucose uptake and lipid synthesis, which in turn results in decreased milk production. In contrast, deletion of both alleles of Akt2 had no discernible effect on lactation. Notably, despite the requirement for Akt1 in the metabolic control of the lactating mammary gland, mammary epithelial differentiation, proliferation, and survival were unaffected during pregnancy in Akt1-/- mice.

In light of the overlapping functions of Akt isoforms, we considered the possibility that Akt may play an essential role in regulating mammary epithelial development that is not evident in Akt1-/- mice due to compensation by other Akt isoforms. To address this issue, we interbred mice bearing targeted deletions in Akt1 and Akt2 in order to determine the effect on mammary differentiation. We find that deletion of one allele of Akt2 in Akt1-/- mice results in a severe defect in terminal mammary epithelial differentiation and lactation failure due to a loss of prolactin-mediated Stat5 activation. Notably, this defect occurred in the absence of changes in pregnancy-induced lobuloalveolar development, including proliferation, apoptosis or acinar formation. As such, the defects observed in Akt1-/-;Akt2-/- mice reflect the abrogation of Stat5 signaling and the molecular program of terminal differentiation in the mammary gland, a program that is intact in Akt1-deficient mice. Our observations demonstrate an unexpected requirement for Akt in Prl-Jak-Stat5 signaling in the mammary gland, and establish Akt as an essential regulator of differentiation, metabolism and lactation in the mammary gland.

Materials and methods

Animals

Akt1-/- and Akt2-/- mice of C57BL/6 genetic background were generated and provided by Dr Morris Birnbaum (Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA, USA) [5,6]. Mice were housed and maintained according to Institutional Animal Use and Care Committee guidelines. For timed pregnancies, the morning of the observed virginal plug was counted as day 0.5. At given time points, animals were killed by carbon dioxide asphyxiation and the mammary gland tissues were harvested and snap-frozen on dry ice, fixed in 4% paraformaldehyde in 1 × PBS (4% paraformaldehyde (PFA)) or frozen in Optimal Cutting Temperature (OCT) compound for further analysis.

The determination of pup weight, milk volume and pup mortality among various knockout mice has been described previously [25,26]. All experiments and experimental methods related to the use of animals were approved by the University of Pennsylvania Institutional Animal Use and Care Committee.

Antibodies

The following rabbit polyclonal antibodies were used in the study: phospho-S6 (Ser235/236), S6, and Akt (Cell Signaling Technology, Danvers, MA, USA), suppressor of cytokine signaling 2(Socs2) (Zymed Laboratories, South San Francisco, CA, USA), inhibitor of DNA binding 2 (Id2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse milk-specific proteins (Nordic Immunological Laboratories, Tilburg, Netherlands). The following mouse monoclonal antibodies were used: phospho-Stat5a/b (Tyr694/Tyr699) and Stat5a/b.
Mammary gland whole-mount and histological analysis
The abdominal mammary glands fixed in 4% PFA were either stained in carmine/aluminum potassium sulfate for whole mounts as previously described [27] or embedded in paraffin wax. For histological analysis, 5 μm tissue sections were cut, dewaxed in xylene, rehydrated, and stained with H & E.

Immunofluorescence analysis
After paraffin-embedded 5 μm tissue sections were cleared and rehydrated, sections were subjected to antigen retrieval performed by heating treatment in an antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA). Subsequently, sections were incubated in blocking solution consisting of 5% BSA and 10% (v/v) normal goat serum in PBS at room temperature for 1 hour. The primary antibodies phospho-Stat5 (1:100), Npt2b (1:300) and anti-CK8 (1:100) were then applied and incubated at 4°C overnight. Appropriate fluorescein-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used: phospho-S6 (Ser235/236) (1:1,000), S6 (1:1,000), Akt (1:1,000), phospho-Stat5α/β (Tyr694/Tyr699) (1:500), Stat5α/b (1:500), mouse milk-specific proteins (1:20,000), β-tubulin (1:1,000), caveolin-1 (1:1,000), Socs2 (1:150), Id2 (1:100), and Elf5 (1:100). Chemiluminescence was detected with horseradish peroxidase-conjugated goat anti-rabbit or mouse secondary antibodies at a dilution of 1:5,000, and was developed in the ECL plus system based on the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA). All experiments were independently repeated three times. Only the representative images are shown. Densitometry for western blots was carried out with the Photoshop program.

Northern blot, in situ hybridization and quantitative RT-PCR analysis
Total RNA isolation from snap-frozen abdominal and inguinal mammary tissues without lymph nodes, preparation of radioactively labeled cDNA probes, northern blots and in situ hybridization were performed as previously described [28]. The cDNA probes for northern hybridization correspond to β-casein (nucleotides 181 to 719), whey acidic protein (WAP) (nucleotides 131 to 483), v-casein (nucleotides 83 to 637), α-lactalbumin (nucleotides 174 to 700) and CK18 (nucleotides 589 to 1287). The sequence of an oligomer used to detect 18S rRNA is CGGAATCTAAGGCGGTATCTG.

Single-stranded cDNA for quantitative RT-PCR analysis was generated by the high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the TaqMan-based ABI 7900HT fast real-time PCR system according to the manufacturer’s instructions (Applied Biosystems). The probes used were Aldoc Mm01298111_g1, Fads1 Mm00507605_m1, Elovl5 Mm00506717_m1, Prlr Mm00599957_m1, and cytokeratin 18 Mm01601706_g1.

Western blot analysis
Frozen abdominal and inguinal mammary tissues (lymph nodes removed) were homogenized in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM sodium fluoride (NaF), 3 mM sodium pyrophosphate, and 5 mM β-glycerol phosphate) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and were subjected to western blot analysis, performed as described previously [25]. The following primary antibodies were used: phospho-S6 (Ser235/236) (1:1,000), S6 (1:1,000), Akt (1:1,000), phospho-Stat5α/b (Tyr694/Tyr699) (1:500), Stat5α/b (1:500), mouse milk-specific proteins (1:20,000), β-tubulin (1:1,000), caveolin-1 (1:1,000), Socs2 (1:150), Id2 (1:100), and Elf5 (1:100). Chemiluminescence was detected with horseradish peroxidase-conjugated goat anti-rabbit or mouse secondary antibodies at a dilution of 1:5,000, and was developed in the ECL plus system based on the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ, USA) followed by exposure to X-ray film (Kodak, Rochester, NY, USA). All experiments were independently repeated three times. Only the representative images are shown. Densitometry for western blots was carried out with the Photoshop program.

Lactose analysis
Lactose in the mammary gland was measured by subjecting 20 μg tissue lysates to the lactose assay kit as per the manufacturer’s instructions (MBL, Woburn, MA, USA). The lactose level was calculated by subtracting the free galactose level from the total galactose level.

Mammary gland culture
The whole-organ culture of the mammary gland was as described previously but with slight modifications [26]. Briefly, the lymph node-free abdominal and inguinal glands of Akt1+/−;Akt2+/− mice and Akt1−/−;Akt2−/− mice
at day 18.5 of pregnancy were aseptically collected and minced into fine pieces (~2 mm). Tissues were incubated in Waymouth’s serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mM HEPES, 4 mM glutamine, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. The tissues were replaced with fresh culture medium daily to remove hormones carried from mice, and the mammary tissues were cultured for 5 days followed by growth-factor starvation overnight. The tissues treated with 0.2 μg/ml prolactin for the indicated periods were collected.

**Statistical analysis**

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was calculated by Student’s t test unless otherwise indicated.

**Results**

**Akt is required for lactation**

Homozygous deletion of either Akt1 or Akt2 has no effect on mammary epithelial cell differentiation [25]. Akt1 is required in an isoform-specific manner, however, for coordinating multiple metabolic pathways in the mammary gland during the transition from pregnancy to lactation [25]. The inability of lactating Akt1−/− mice to secrete normal amounts of milk arises from a failure to upregulate Glut1, glucose uptake, and lipid synthesis, as well as a failure to downregulate lipid catabolism [25]. In contrast, Akt2 is entirely dispensable for the metabolic response of the mammary gland to lactation.

Since Akt1 and Akt2 are expressed in the same cell types in the mammary gland [25], we considered the possibility that they might have compensatory roles in development. As deletion of one Akt3 allele in Akt1-deficient mice results in perinatal lethality, we generated mice with combined loss of Akt1 and Akt2 alleles. Because Akt1−/−;Akt2−/− mice die shortly after birth, we compared the ability of Akt1−/−;Akt2−/− and Akt1+/−; Akt2−/− mice to support the growth and survival of nursing pups.

In agreement with our previous report [25], pups nursed by Akt1−/−;Akt2−/− mothers, but not those nursed by Akt1+/−;Akt2−/− mothers, exhibited growth retardation compared with pups nursed by wild-type mice (Figure 1a). Deletion of one allele of Akt2 on an Akt1-deficient background exaggerated the growth defect observed in pups nursed by Akt1-deficient mice (P < 0.0001). In contrast, deletion of one Akt1 allele on an Akt2-deficient background had no effect on the ability of lactating mothers to support pups (Figure 1a). By postpartum day 4, pups nursed by Akt1+/−;Akt2−/− mice weighed significantly less than those nursed by Akt1−/−; Akt2−/− mice. By postpartum day 9, the average weight of pups nursed by Akt1+/−;Akt2−/− mothers was two-thirds that of pups nursed by Akt1+/−;Akt2+/− mothers and one-half that of pups nursed by Akt1+/−;Akt2−/− mothers (Figure 1a).

In addition to the dramatic growth defect observed in pups nursed by Akt1−/−;Akt2−/− mothers, these pups also displayed markedly increased perinatal mortality. By postpartum day 9, 70% of pups nursed by Akt1−/−;Akt2−/− mice had died, compared with 30% of pups nursed by Akt1+/−;Akt2−/− mice and 2% of pups nursed by wild-type mice (Figure 1b).

Consistent with the severe growth retardation and increased mortality of pups nursed by Akt1−/−;Akt2−/− mice, milk production was significantly decreased in these mice (Figure 1c). Compared with wild-type mice, Akt1+/−;Akt2−/− mice displayed a nine-fold reduction in oxytocin-stimulated milk secretion (P = 0.0001) (Figure 1c), a reduction even more profound than the four-fold reduction observed in Akt1−/−;Akt2+/− mice (Figure 1c). These observations suggest that milk production in the lactating mammary gland is influenced by allele dosages of Akt.

**Akt is required for mammary differentiation during pregnancy and lactation**

Milk production is the culmination of an orchestrated series of developmental events. Exposed to the hormonal milieu of pregnancy and lactation, mammary epithelial cells proliferate to form alveoli and differentiate into milk-secreting cells. Akt1+/−;Akt2+/− mice exhibit a lactation defect, yet mammary glands from these mice undergo normal alveologenesis and secretory differentiation during pregnancy [25]. Since Akt1−/−;Akt2−/− mice displayed a more severe lactation defect than Akt1+/−; Akt2−/− mice, we examined alveolar development and differentiation in these mice.

Examination of carmine-stained whole mounts of mammary glands harvested from late-term pregnant and lactating mice revealed a defect in the expansion of lobuloalveolar structures in Akt1−/−;Akt2−/− mice compared with Akt1+/−;Akt2−/− or wild-type mice (Figure 2a). By day 9 of lactation, Akt1+/−;Akt2+/− mammary epithelia failed to form fully-expanded alveolar secretory units (Figure 2a). Analysis of histological sections revealed that alveoli of Akt1−/−;Akt2−/− mice and Akt1+/−;Akt2−/− mice were markedly less distended with milk compared with those of wild-type mice from day 18.5 of pregnancy through day 9 of lactation (Figure 2b). By comparison, both Akt1+/−;Akt2−/− and Akt1+/−; Akt2−/− mammary epithelia displayed normal alveologenesis (Figure 2a,b). Evaluation of mammary sections for bromodeoxyuridine (BrDU) incorporation and TUNEL staining revealed normal rates of proliferation and apoptosis in Akt1−/−;Akt2−/− glands during mid-to-late pregnancy (data not shown). This observation suggests that
the defect in lobuloalveolar expansion in Akt1−/−;Akt2+/− mice is due to defective functional differentiation of mammary epithelial cells, rather than reduced proliferation or survival.

The morphological and histological signs of reduced milk secretion in Akt1−/−;Akt2+/− mammary glands suggested that the alveolar epithelia failed to undergo functional differentiation. To determine the differentiation status of Akt1−/−;Akt2+/− mammary epithelia we examined the expression of milk protein genes. Sequential upregulation of early (β-casein), mid (WAP and α-lactalbumin), and late (ε-casein) milk protein genes is a hallmark of secretory differentiation of the mammary epithelium [27]. Northern analysis of mammary gland mRNA at day 18.5 of pregnancy demonstrated that expression of β-casein, WAP, and ε-casein was not altered in Akt1−/−;Akt2+/− mice or in Akt1+/+;Akt2+/− mice. Expression of these genes was significantly reduced, however, in Akt1−/−;Akt2+/− mice (Figure 3a). Consistent with these data, immunoblotting analysis revealed markedly reduced levels of milk proteins in the mammary gland of Akt1−/−;Akt2+/− mice at day 18.5 of pregnancy (Figure 3b).

The failure of mammary glands from Akt1−/−;Akt2+/− mice to express milk proteins suggested a defect in secretory differentiation. Npt2b, a Na-Pi co-transporter, is a marker of secretory differentiation and is highly expressed in the lactating, but not nulliparous, mammary gland [17,25,28,29]. We therefore examined the expression of Npt2b in mammary tissue from lactating mice of differing Akt genotypes. Whereas Npt2b expression was appropriately upregulated in lactating mammary epithelia of wild-type mice, Akt1−/−;Akt2+/− mice,
Akt1+/−;Akt2+/− mice, and Akt1+/−;Akt2−/− mice, Npt2b expression failed to be upregulated in lactating Akt1−/−;Akt2+/- mice (Figure 3d). Notably, total Akt protein expression was significantly lower in the mammary glands of Akt1−/−;Akt2+/- mice compared with Akt1+/−;Akt2−/− mice (0.26 ± 0.05 vs. 1.04 ± 0.13; Figure 3b,c), indicating that Akt1 is the predominant form of Akt in the mammary gland at day 18.5 of pregnancy. Consistent with this notion, total Akt expression was significantly reduced by deletion of one allele of Akt1 in Akt2+/- mice (0.71 ± 0.12 for Akt1+/−;Akt2−/− mice vs. 1.04 ± 0.13 for Akt1+/−;Akt2+/− mice), but not by deletion of one allele of Akt2 in Akt1−/− mice (0.29 ± 0.04 for Akt1−/−;Akt2+/− mice vs. 0.26 ± 0.05 for Akt1+/−;Akt2+/− mice) (Figure 3b,c). Taken together, these results indicate that the severe lactation defect observed in Akt1−/−;Akt2−/− mammary glands could be due to the observed decreases in steady-state levels of transcription for milk protein genes (Figure 3a), or due to decreased mRNA expression coupled with decreased rates of translation for milk proteins. To address this issue, we examined levels of phospho-S6, a direct downstream target of the mTOR pathway, in the late

Figure 2 Akt1+/−;Akt2+/− mice exhibit defective expansion of mammary gland during late pregnancy and lactation (a) Whole-mount carmine-stained mammary glands from Akt1+/−;Akt2+/− mice, Akt1+/−;Akt2−/− mice, Akt1+/−;Akt2−/− mice, and Akt1+/−;Akt2−/− mice at day 18.5 of pregnancy and days 2 and 9 of lactation. (b) H & E-stained sections of mammary glands from mice with indicated Akt genotypes at day 18.5 of pregnancy (top) and day 9 of lactation (bottom). Scale bars: (a) 2 mm, (b) 100 μm.

Akt regulates the production of essential milk components in the lactating mammary gland
Milk is a complex mixture of proteins, lipids and carbohydrates [30]. Our finding that milk protein expression is reduced in Akt1+/−;Akt2−/− mice (Figure 3a,b), together with our previous finding that Akt1 is required for lipid synthesis during lactation [25], led us to examine potential roles for Akt in the biosynthesis of each of the three components of milk.

The cellular capacity for protein synthesis is regulated by the mammalian target of rapamycin (mTOR) pathway. The decrease in milk proteins observed in Akt1+/−;Akt2−/− mammary glands could be due to the observed decreases in steady-state levels of transcription for milk protein genes (Figure 3a), or due to decreased mRNA expression coupled with decreased rates of translation for milk proteins. To address this issue, we examined levels of phospho-S6, a direct downstream target of the mTOR pathway, in the late
pregnant mammary gland. Levels of phospho-S6 were significantly reduced in the mammary glands of Akt1\(^{--}\);Akt2\(^{+/-}\) mice compared with either wild-type mice (\(P < 0.001\)) or Akt1\(^{+/-}\);Akt2\(^{++}\) mice (\(P = 0.03\)), indicating that mTOR activity is decreased in the Akt1\(^{--}\);Akt2\(^{+/-}\) mammary gland (Figure 3b,c).

Lactose in mammary alveolar cells is synthesized from glucose and galactose by the lactose synthase complex, which consists of \(\alpha\)-lactalbumin and galactosyltransferase [31]. In Akt1\(^{--}\);Akt2\(^{+/-}\) mammary tissue, a modest decrease in intraepithelial lactose levels was evident (Figure 3e), presumably due to reduced glucose uptake coupled with normal levels of \(\alpha\)-lactalbumin (Figure 3a) [25]. In contrast, lactose levels were dramatically reduced in Akt1\(^{+/-}\);Akt2\(^{+/-}\) mice during lactation, reflecting decreased glucose uptake coupled with a marked
decrease in α-lactalbumin expression ($P = 0.0003$ and $P = 0.03$ compared with wild-type mice and Akt1/−/−; Akt2+/+ mice, respectively) (Figure 3a,e).

We previously showed that Akt1 contributes to lipid biosynthesis in the lactating mammary gland by regulating expression of genes involved in lipid metabolism, such as Scd2, Scd3 and Dgat2 [25]. To extend this analysis, we examined expression of Aldoc, Fads1 and Elovl5, which are preferentially expressed in the mammary epithelium, are upregulated during lactation and have been implicated in fatty acid synthesis [32]. Quantitative RT-PCR analysis demonstrated that the expression levels of Aldoc, Fads1 and Elovl5 were lower in Akt1/−/−; Akt2+/+ mice compared with wild-type mice, and were further reduced in Akt1/−/−; Akt2−/− mice (Figure 4). In aggregate, these findings demonstrate that Akt is required for production of the three main components of milk - milk proteins, lipid, and lactose - in the lactating mammary gland.

**Akt is required for Stat5 activation**

The Prlr-Jak2-Stat5 signaling pathway plays a critical role in alveolar morphogenesis and differentiation [11,16,18,33,34], as illustrated by the fact that Stat5-deficient mice fail to form alveoli during pregnancy and do not express milk protein genes [17]. Although alveolar formation in Akt1/−/−; Akt2−/− mice occurred normally, the defect in mammary epithelial differentiation observed in Akt1/−/−; Akt2−/− mice led us to hypothesize that a functional relationship might exist between the Akt and Stat5 pathways.

To address this hypothesis, we evaluated Stat5 activity in the mammary glands of Akt1/−/−; Akt2−/− pregnant mice by determining the fraction of epithelial cells with nuclear phospho-Stat5a/b. Mammary tissue was harvested from mice with various Akt genotypes at day 18.5 of pregnancy and immunofluorescence was performed for phospho-Stat5a/b and cytokeratin 8. This revealed that the fraction of mammary epithelial cells with nuclear phospho-Stat5a/b was markedly diminished during pregnancy in Akt1/−/−; Akt2−/− mice compared with Akt1/−/−; Akt2+/+ mice, Akt1+/+; Akt2−/− mice, Akt1+/+; Akt2+/+ mice, or wild-type mice (Figure 5a,b). The percentage of mammary epithelial cells with nuclear phospho-Stat5a/b was 10-fold lower in Akt1/−/−; Akt2−/− mice compared with wild-type mice or Akt1+/+; Akt2−/− mice ($P < 0.0001$ and $P = 0.0007$, respectively). Consistent with this, immunoblotting revealed markedly decreased levels of phospho-Stat5a/b in the mammary glands of pregnant Akt1/−/−; Akt2−/− mice (Figure 5c,d). In contrast, phospho-Stat5a/b levels were unaffected by deletion of Akt1 or Akt2 alone (Figure 5c,d).

Of note, phospho-Stat5 levels were decreased in Akt1+/+; Akt2−/− mice, although not to the extent observed in Akt1/−/−; Akt2−/− mice as normalized phospho-Stat5 levels in Akt1+/+; Akt2−/− mice remained ~60% higher than in their Akt1/−/−; Akt2−/− counterparts (Figure 5d).

Interestingly, despite the modest decreases in phospho-Stat5 levels observed in Akt1/−/−; Akt2−/− mice by western blotting, the fraction of alveolar epithelial cells exhibiting nuclear phospho-Stat5 observed by immunofluorescence was unaffected in these mice (Figure 5b,d). Consistent with the nuclear localization of phospho-Stat5 representing the most reliable indicator of its functional status as a transcription factor, milk production, milk protein gene expression, mTOR activity, Npt2b upregulation, and expression of lipid synthetic enzymes were all normal in Akt1+/+; Akt2−/− mice. As such, the normal nuclear localization and function of

**Figure 4 Expression of lipid synthetic enzymes is markedly decreased in Akt1−/−; Akt2−/− mice.** Relative expression of Aldoc, Fads1 and Elovl5 mRNA in mammary glands from Akt knockout mice at day 9 of lactation (n = 4 for each genotype). Average expression values normalized to cytokeratin 18 ± standard error of the mean are shown. Statistical differences in expression were calculated by comparing each group with Akt1+/+; Akt2−/− mice except the indicated P values shown between Akt1−/−; Akt2−/− mice and Akt1+/+; Akt2−/− mice. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5 Stat5a/b activation is reduced in the mammary glands of Akt1<sup>−/−</sup>;Akt2<sup>+/−</sup> mice. (a) Immunofluorescence analysis of phospho-Stat5a/b (p-Stat5) expression in mammary tissues from day 18.5 pregnant mice with the indicated Akt genotypes. Luminal epithelial cells and nuclei were counterstained with anti-CK8 (green) and Hoechst 33258 (blue), respectively. Scale bars represent 50 μm. (b) Quantitative analysis of nuclear p-Stat5 in mammary epithelial cells of late pregnant mice (n = 3 mice per genotype). Statistical analysis in differential activity was calculated by comparing each group with Akt1<sup>+/−</sup>;Akt2<sup>+/−</sup> mice, except for the indicated P value shown between Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice and Akt1<sup>−/−</sup>;Akt2<sup>+/−</sup> mice. *P < 0.0001. (c) Representative western analysis of p-Stat5a/b and total Stat5a/b expression in mammary tissues from day 18.5 pregnant mice with indicated Akt genotypes. β-Tubulin levels served as a loading control. (d) Quantitative analysis of p-Stat5/Stat5 in late-pregnant mice (n = 6 mice per genotype). The ratio was normalized to Akt1<sup>+/−</sup>;Akt2<sup>+/−</sup> mice. Statistical analysis in differential activity was calculated by comparing each group with Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice, except for the indicated P values shown between Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> and Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice. *P < 0.001. (e) Relative expression of Prl mRNA in mammary glands from Akt knockout mice at day 18.5 of pregnancy (n = 4 for each genotype). Average expression values normalized to cytokeratin 18 ± standard error of the mean are shown.
phospho-Stat5 in Akt1+/−;Akt2+/− mice despite modest reductions in total phospho-Stat5 levels could reflect a threshold requirement for Akt for phospho-Stat5 nuclear localization, effects of Akt deletion on phospho-Stat5 levels in the adipose stroma, or the intriguing possibility that Akt may affect the nuclear localization of Stat5 by mechanisms other than its activation by phosphorylation.

Quantitative RT-PCR analysis revealed that that Prlr expression in Akt1−/−;Akt2−/− mice was comparable with that observed in wild-type mice (Figure 5e). This indicates that reduction in Stat5 activity in Akt1−/−;Akt2−/− mice is not due to a defect in prolactin receptor expression. Together, these findings demonstrate that Akt is required for Stat5a/b activation in the mammary gland during pregnancy.

Akt regulates the expression of regulators of prolactin-Jak-Stat5 signaling

A number of molecules have been identified that regulate activity of the Prlr-Jak-Stat5 pathway. For example, caveolin-1 and Socs2 function as suppressors of prolactin-induced phosphorylation of Stat5a/b in mammary epithelial cells, whereas Id2 positively regulates Stat5a/b signaling [35-39]. Id2 is also essential for mammary gland development during pregnancy and lactation [37,38]. In addition, recent experiments have demonstrated that Elf5 expression in the mammary epithelium can rescue the alveolar defect observed in Prlr−/− mice and can drive pregnancy-associated mammary differentiation [35,40].

To elucidate the basis of the requirement for Akt in Prlr-Jak-Stat5 signaling, we examined levels of caveolin-1, Socs2, and Id2 in day 18.5 pregnant glands from mice bearing targeted deletions in Akt1 and Akt2. This analysis revealed that expression of the negative regulators of Stat5 signaling, caveolin-1 and Socs2, were markedly increased in Akt1−/−;Akt2−/− mice, whereas expression of the positive regulator of Stat5 signaling, Id2, was markedly decreased compared with genetic controls (Figure 6a,b). In contrast, Elf5 expression was unaffected in Akt1−/−;Akt2−/− mice, despite their severe lactation defect (Figure 6a,b). In addition, although animal-to-animal variation was evident (Figure 6a), caveolin-1, Socs2 and Id2 levels did not differ between wild-type mice and Akt2-deficient mice (Figure 6b).

Consistent with a causal relationship between changes in the expression of Stat5 regulatory molecules and changes in phospho-Stat5 levels, expression of caveolin-1 and Socs2 were highest - and expression of Id2 was lowest - in Akt1−/−;Akt2−/− mice compared with other genotypes (Figure 6a). Changes in caveolin-1, Socs2 and Id2 expression were not observed in Akt1−/−;Akt2+/− mice, however, despite modest reductions in phospho-Stat5 levels. This suggests the possibility that additional regulatory molecules downstream of Akt may play a role in modulating Prlr-Jak2-Stat5 signaling.

To address the hypothesis that Akt-mediated decreases in caveolin-1 and Socs2 expression, along with increases in Id2 expression, are required for prolactin-induced Stat5a/b activation and mammary differentiation during pregnancy, we evaluated the extent of prolactin-induced Stat5a/b activation in vitro in mammary glands harvested from Akt1−/−;Akt2+/− mice and Akt1+/−;Akt2−/− mice at day 18.5 of pregnancy. Intact mammary tissues were used instead of isolated mammary epithelial cells since dissociation of the mammary gland results in loss of caveolin-1 expression (unpublished observations), despite the fact that caveolin-1 is highly expressed in both the mammary epithelium and adipocytes of the virgin mammary gland [39]. This observation suggests that the architecture of the mammary gland is important in maintaining caveolin-1 expression, which is in turn consistent with our observation that mammary epithelial cell lines express only low levels of caveolin-1 compared with the mammary gland (unpublished observations).

Addition of prolactin to organ cultures containing mammary tissue from Akt1−/−;Akt2−/− mice or Akt1−/−; Akt2−/− mice revealed that peak levels of Stat5a/b activation in Akt1−/−;Akt2+/− glands were less than one-half of those observed in wild-type mice (Figure 6c). These results indicate that prolactin-induced Stat5a/b activation in the pregnant mammary gland is markedly blunted in Akt1−/−;Akt2−/− mice. In aggregate, these findings suggest that Akt potentiates prolactin-induced Stat5a/b activation and mammary epithelial differentiation, at least in part by downregulating the known suppressors of Stat5a/b phosphorylation, caveolin-1 and Socs2, and by upregulating the positive regulator of Stat5a/b signaling, Id2.

Discussion

The prolactin-Jak-Stat5 pathway has long been recognized as a central mediator of pregnancy-induced lobuloalveolar development and lactation, which together constitute a developmental transition that is essential for the survival of mammals. Accordingly, the role of this pathway in mammary development has been intensively studied. In the present manuscript we describe a previously unrecognized requirement for Akt in Prlr-Jak-Stat5 signaling. Mice lacking one allele of Akt2 and both alleles of Akt1 displayed a severe lactation defect due to the global impairment of alveolar epithelial cell differentiation. Consistent with their failure to terminally differentiate, pregnant Akt1−/−;Akt2−/− mice fail to upregulate Npt2b or phospho-Stat5a/b and display markedly reduced synthesis of each of the three major components of milk during lactation. Notably, epithelial cell
proliferation, cell survival and the formation of architecturally normal acini during pregnancy were unaffected by Akt deletion, reinforcing that the lactation defect observed in Akt1-/-;Akt2+/- mice results from a defect in differentiation, rather than from a failure to form acinar structures. In aggregate, these findings establish an essential but heretofore unrecognized role for Akt in epithelial differentiation.

Despite the fact that both Akt1-/-;Akt2+/+ mice and Akt1-/-;Akt2+/- mice exhibit defects in lactation, the molecular basis of their lactation phenotypes is strikingly different. The isoform-specific defect in lactation observed in Akt1-/-;Akt2+/+ mice results from a defect in differentiation, rather than from a failure to form acinar structures. In aggregate, these findings establish an essential but heretofore unrecognized role for Akt in epithelial differentiation.

Figure 6 Akt regulates the expression of key regulators of Stat5 activity. (a) Representative western analysis of expression of proteins that regulate mammary differentiation. Mammary protein lysates from mice with indicated Akt genotypes at day 18.5 of pregnancy. β-Tubulin levels served as a loading control. (b) Quantitative analysis of individual molecule expression in late-pregnant mice (n = 6 mice per genotype). The ratios in each group were normalized to Akt1+/+;Akt2+/+ mice. Statistical analysis was calculated by comparing each group with Akt1+/+;Akt2+/+ mice, except for the indicated P values shown between Akt1+/+;Akt2+/+ mice and Akt1-/-;Akt2+/+ mice. *P < 0.05. **P < 0.01. ***P < 0.001. n.s., not significant. (c) Representative western analysis of Stat5a/b activation in Akt1+/+;Akt2+/+ and Akt1-/-;Akt2+/+ mammary tissues at day 18.5 of pregnancy treated with prolactin (0.2 μg/ml) for the indicated periods (top panel). Graph shows the ratios of phospho-Stat5 (p-Stat5)/Stat5 at the indicated timepoints calculated from three independent experiments (bottom panel).

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Acly, Scd2, and Scd3. These molecular defects result in a profound inability of terminally differentiated mammary epithelial cells to take up glucose or to synthesize normal amounts of lipid. Nevertheless, mammary epithelial differentiation is normal in lactating Akt1-/- mice, as demonstrated by physiologically normal levels of Stat5 activation, normal upregulation of the terminal differentiation marker Npt2b, normal downregulation of the virgin-specific transporter NKCC1, normal expression of all major milk proteins, and normal intraepithelial lactose levels.

In contrast, our current study demonstrates that deletion of one allele of Akt2 in Akt1-deficient mice results in a severe defect in mammary epithelial differentiation that is due to a failure to activate Stat5. In contrast to Akt1-deficient mice, late-pregnant Akt1+/+;Akt2+/+ mice exhibit dramatically reduced Prrr-Jak-Stat5 signaling, as well as markedly reduced milk protein expression,
lactose levels, lipid synthesis, expression of the terminal differentiation marker Npt2b, and mTOR activity.

The lactation defect observed in Akt1−/− mice is thus due to a metabolic defect that results from a failure to upregulate Glut1 and other Akt1-specific target genes. This defect occurs in the context of normal Prlr-Jak-Stat5 signaling and normal mammary epithelial differentiation. In contrast, the lactation defect in Akt1−/−;Akt2−/− mice is due to a profound defect in mammary epithelial differentiation that results from a failure to activate Stat5. While the outward phenotypes (that is, lactation defect) of Akt1−/− mice and Akt1−/−;Akt2−/− mice are similar at a superficial level, the molecular phenotypes as well as the molecular basis for these phenotypes are profoundly different.

The defect in Stat5 activation observed in Akt1−/−; Akt2−/− mice is due, at least in part, to a failure to up regulate the positive regulator of Prlr-Jak-Stat5 signaling, Id2, or to downregulate the negative regulators of prolactin-Jak-Stat5 signaling, caveolin-1 and Socs2. In addition, our findings suggest that Akt most likely regulates the expression or activity of other molecules that modulate Prlr-Jak-Stat5 pathway activity. Together, these findings provide a molecular basis for this previously unrecognized connection between the Akt and Stat5 pathways.

Notably, mammary epithelial proliferation and apoptosis rates were unaffected in Akt2−/−;Akt2−/− mice during pregnancy, suggesting that Akt is essential for Stat5-dependent secretory differentiation of mammary epithelium, but possibly not for Stat5-dependent alveolar development or acinar formation; that is, whereas Stat5 deficiency in the mammary gland results in a failure of lobuloalveolar development as well as secretory differentiation, Akt1−/−;Akt2−/− mice exhibit only a defect in secretory differentiation. Nevertheless, it is possible that deletion of all four Akt1 alleles would result in a defect in prolactin-Stat5-mediated epithelial proliferation and, thereby, lobuloalveolar development similar to that observed in Stat5-deficient mice. Given the perinatal lethality of combined germline deletion of Akt1 and Akt2, however, mammary-specific deletion of these genes may be required to determine the role of the remaining Akt2 allele in mammary epithelial proliferation and differentiation.

Elf5 has been shown recently to regulate alveolar cell differentiation by acting downstream of the prolactin receptor [41]. Since Elf5 expression was unchanged in the mammary glands of Akt1−/−;Akt2−/− mice despite their dramatic reduction in Stat5 activity, our data suggest that the impact of Akt deletion on Prlr-Jak-Stat5 signaling is not mediated by Elf5. Rather, our findings suggest that Akt and Elf5 may act via parallel pathways, that Elf5 alone is not sufficient to compensate for loss of Prlr signaling, and that factors other than Prlr signaling may regulate Elf5 [19,41].

Maroulakou and colleagues have reported that Akt1 deficiency delayed, whereas Akt2 deficiency facilitated, mammary epithelial differentiation during pregnancy and lactation [42]. In contrast, consistent with our prior study, the findings described here confirm that deletion of either Akt1 or Akt2 alone has no appreciable effect on secretory differentiation during pregnancy or lactation. Moreover, our finding that deletion of one allele of Akt2 in Akt1-deficient mice results in a pronounced defect in mammary epithelial differentiation that is not observed in mice deficient for Akt1 alone strongly suggests that Akt2 synergizes with - rather than antagonizes - the pro-differentiation effects of Akt1 on mammary epithelial cells. Whether this discrepancy is explained by the mixed genetic background of mice used in the former study or other factors remains to be determined.

**Conclusions**

As we have previously described, Akt1 is required for the orchestrated metabolic response of the mammary gland to lactation. We now report that the combined action of Akt1 and Akt2 is required for mammary epithelial differentiation during pregnancy and lactation due to their requirement for Stat5 activation. Together, our findings establish Akt as an essential and central regulator of epithelial differentiation and metabolism during lactation. As Akt is a common downstream effector of many receptor tyrosine kinases, the finding that the Akt and Stat5 signaling pathways are functionally interconnected has important implications for the numerous biological processes regulated by these pathways. In particular, both pathways regulate cell survival and cell proliferation, and have been implicated in mammary tumorigenesis in rodents and in humans [43-45]. Consequently, our findings predict that therapeutic blockade of the Akt pathway may be effective in treating Prlr-Jak-Stat5-driven tumors, whereas blockade of Prlr-Jak-Stat5 signaling may be effective in treating Akt-driven tumors. In light of the fact that normal pathways of differentiation and development are frequently usurped during the process of carcinogenesis, we predict that combined therapeutic approaches targeting crosstalk between the Akt and Prlr-Jak-Stat5 pathways may be particularly effective in the case of breast cancer.

**Abbreviations**

Ady: ATP citrate lyase; Aldoc: aldolase C; BrdU: bromodeoxyuridine; BSA: bovine serum albumin; Dgat2: diacylglycerol O-acyltransferase 2; Elf5: E74-like factor 5; Elov5: elongation of very-long-chain fatty acids protein 5; Fads1: fatty acid desaturase 1; Gata3: GATA binding protein 3; H & E: hematoxylin and eosin; Id2: inhibitor of DNA binding 2; Jak2: Janus kinase 2; mTOR: mammalian target of rapamycin; NKCC1: sodium/potassium/chloride cotransporter isoform 1; Npt2b: Na-Pi cotransporter 2b; PBS: phosphate-
buffered saline, Prlr: prolactin receptor; qRT-PCR: quantitative real-time polymerase chain reaction; Scd: stearyl-coenzyme A desaturase; SEM: standard error of the mean; Socs2: suppressor of cytokine signaling 2; Stat5: signal transducer and activator of transcription 5; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; WAP: whey acidic protein.

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Authors’ contributions

CCC participated in the design, execution and analysis of experiments and participated in drafting the manuscript. RBB participated in the execution and analysis of mouse experiments. DBS participated in the execution and analysis of immunofluorescence experiments. CPP participated in the execution of mouse experiments. RHH participated in the execution of mouse and molecular experiments. JAW participated in the analysis of data and drafting of the manuscript. MIB participated in the mouse experiments and drafting of the manuscript. LAC participated in the design and analysis of experiments and drafting of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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