Autocrine prolactin induced by the Pten–Akt pathway is required for lactation initiation and provides a direct link between the Akt and Stat5 pathways

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Extrapituitary prolactin (Prl) is produced in humans and rodents; however, little is known about its in vivo regulation or physiological function. We now report that autocrine prolactin is required for terminal mammary epithelial differentiation during pregnancy and that its production is regulated by the Pten–PI3K–Akt pathway. Conditional activation of the PI3K–Akt pathway in the mammary glands of virgin mice by either Akt1 expression or Pten deletion rapidly induced terminal mammary epithelial differentiation accompanied by the synthesis of milk despite the absence of lobuloalveolar development. Surprisingly, we found that mammary differentiation was due to the PI3K–Akt-dependent synthesis and secretion of autocrine prolactin and downstream activation of the prolactin receptor (Prlr)–Jak–Stat5 pathway. Consistent with this, Akt-induced mammary differentiation was abrogated in Prl–/–, Prlr–/–, and Stat5–/– mice. Furthermore, cells treated with conditioned medium from mammary glands in which Akt had been activated underwent rapid Stat5 phosphorylation in a manner that was blocked by inhibition of Jak2, treatment with an anti-Prl antibody, or deletion of the prolactin gene. Demonstrating a physiological requirement for autocrine prolactin, mammary glands from lactation-defective Akt1–/–;Akt2+/– mice failed to express autocrine prolactin or activate Stat5 during late pregnancy despite normal levels of circulating serum prolactin and pituitary prolactin production. Our findings reveal that PI3K–Akt pathway activation is necessary and sufficient to induce autocrine prolactin production in the mammary gland, Stat5 activation, and terminal mammary epithelial differentiation, even in the absence of the normal developmental program that prepares the mammary gland for lactation. Together, these findings identify a function for autocrine prolactin during normal development and demonstrate its endogenous regulation by the PI3K–Akt pathway.

[Keywords: Pten; Akt; Stat5; mammary gland; lactation; differentiation]

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molecules that regulate Stat5 activity, including ErbB2, ErbB4, Pak1, caveolin-1, Socs2, Id2, and Elf5, result in abnormal mammary epithelial differentiation during pregnancy and lactation (Jones and Stern 1999; Jones et al. 1999; Mori et al. 2000; Long et al. 2003; Wang et al. 2003; Zhou et al. 2005; Harris et al. 2006).

In addition to stimulating the Jak2–Stat5 pathway, Prl also activates PI3K–Akt signaling (Acosta et al. 2003; Chakravarti et al. 2005). These two signaling cascades mediate distinct aspects of the production of the three major components of milk: lactose, lipids, and milk proteins (Anderson et al. 2007). For example, while Stat5 regulates milk protein gene transcription in the mammary gland, Akt controls glucose transport, the translation of milk protein mRNAs, and the biosynthesis of lactose and lipids. Consequently, despite the fact that Stat5 activation and mammary epithelial differentiation occur normally in Akt1-deficient mice, loss of Akt1 results in impaired lactation at parturition due to an inability of mammary epithelial cells to up-regulate glucose uptake and lipid synthesis (Boxer et al. 2006). In contrast, deletion of one allele of Akt2 in Akt1-deficient mice results in a more severe lactation defect that is attributable, at least in part, to a failure of pregnant Akt1−/−Akt2−/− mice to up-regulate the positive regulator of Prlr–Jak–Stat5 signaling, Id2, or down-regulate the negative regulators of Prlr–Jak–Stat5 signaling, caveolin-1 and Socs2 (Chen et al. 2010). These and other findings suggest that Akt regulates Stat5 signaling in the mammary gland during pregnancy.

To further characterize the role of the PI3K–Akt pathway in regulating mammary differentiation, we generated mice in which Akt1 can be inducibly activated or Pten can be conditionally deleted in the mammary epithelium. We now report that acute activation of the PI3K–Akt pathway in virgin mice induces rapid Stat5 activation, terminal mammary epithelial differentiation, and milk production that are mediated by the secretion of autocrine prolactin. Remarkably, these changes occur in the absence of lobuloalveolar development or the complex hormonal changes that accompany pregnancy. Consistent with this, we found that the inability of Akt1−/−; Akt2−/− mice to activate Stat5 and initiate lactation at parturition is due to their inability to synthesize and secrete autocrine prolactin. Our findings provide the first demonstration that PI3K–Akt signaling regulates the synthesis and secretion of autocrine prolactin in the mammary gland, identify an in vivo physiological function for autocrine prolactin, and reveal a direct connection between the Akt and Stat5 signaling cascades.

Results

**Akt1 rapidly induces secretory differentiation in the virgin mammary gland in the absence of lobuloalveolar development**

To explore the role of the PI3K–Akt pathway in regulating mammary differentiation, we generated doxycycline-inducible Akt1 transgenic mice, MMTV-rtTA;TetO-Akt1 [MTB/tAkt1], which conditionally express an activated allele of Akt1 in the mammary epithelium (Gunther et al. 2002, Boxer et al. 2006). Administration of doxycycline [2 mg/mL] to 6-wk-old virgin MTB/tAkt1 mice for 96 h induced expression of myristoylated Akt1 in the mammary ductal epithelium (Fig. 1A). Morphological analysis of carmine-stained mammary gland whole mounts revealed that epithelial ducts in the mammary glands of virgin doxycycline-induced MTB/tAkt1 mice were increased in caliber compared with MTB controls but did not appear to contain alveoli (Fig. 1B). Surprisingly, hematoxylin and eosin (H&E)-stained tissue sections from MTB/tAkt1 virgin mice induced with doxycycline for 96 h were morphologically similar to mammary tissues harvested from lactating mice in that mammary ducts were distended with lipid-laden, proteinaceous material similar to that found within alveoli during lactation (Fig. 1C).

Northern analysis of mammary tissues from virgin MTB/tAkt1 mice in which Akt1 had been activated for 96 h revealed that milk protein genes normally up-regulated during early pregnancy (β-casein) or mid-pregnancy (Wap) were expressed at levels comparable with those observed in mid- to late-pregnant wild-type mice (Fig. 1D). MTB/tAkt1 mice acutely induced with doxycycline also expressed the late milk protein gene α-casein, which is normally not expressed until the onset of lactation (Fig. 1D). Quantitative RT–PCR indicated that expression levels of β-casein, Wap, α-lactalbumin, and α-casein mRNAs were increased by 235-fold, 142-fold, 45-fold, and 19-fold, respectively, compared with virgin MTB controls, although levels were lower than those observed in wild-type mice at day 2 of lactation (14-fold, 132-fold, 12-fold, and 391-fold less, respectively) [Fig. 1E; data not shown]. Consistent with these findings, immunoblotting revealed that α-casein and β-casein proteins were expressed in the mammary glands of doxycycline-induced MTB/tAkt1 virgin mice at levels comparable with those found in lactating wild-type mice (Fig. 1F). Immunofluorescence staining confirmed the colocalization of β-casein and phospho-Akt expression in the ductal epithelium of doxycycline-induced MTB/tAkt1 mice (Fig. 1G).

The Na-K-Cl cotransporter Nkcc1 is expressed at high levels in ductal epithelial cells of virgin mice and is down-regulated during pregnancy (Shillingford et al. 2002a,b, 2003). Consistent with the ability of Akt to rapidly induce a lactation-like state in virgin animals, only rare NKCC1-staining cells were detected in the mammary glands of doxycycline-induced MTB/tAkt1 virgin mice at levels comparable with those found in lactating wild-type mice (Fig. 1F). Conversely, the transporter Npt2b is not expressed in the mammary epithelium of virgin mice but is expressed throughout the alveolar epithelium of lactating mice [Miyoshi et al. 2001]. Concordant with the differentiated appearance of mammary glands in which Akt1 had been induced, mammary epithelial cells in doxycycline-induced MTB/tAkt1 mice exhibited strong Npt2b staining [Fig. 1I]. In aggregate, these findings demonstrate that Akt1 activation rapidly induces secretory differentiation in mammary epithelial cells in vivo.

In wild-type mice, β-casein is expressed predominantly in alveolar secretory subunits during early and mid-pregnancy. Only in late pregnancy—after >2 wk of lobuloalveolar...
Akt activation induces secretory differentiation and milk production in the virgin mammary gland. (A) Immunofluorescence analysis for expression of p-Akt and the luminal epithelial marker cytokeratin 8 (CK8) in the mammary glands of MTB and MTB/tAkt1 mice induced with doxycycline for 96 h. Nuclei were counterstained with Hoechst 33258 (blue). Bars, 50 μm. (B) Whole-mount staining of the mammary glands of MTB and MTB/tAkt1 mice induced with doxycycline for 96 h. Bars, 5 mm. (C) H&E-stained sections of mammary tissue from induced (+Dox) and uninduced (−Dox) MTB/tAkt1 mice and lactating MTB control mice. Bars, 100 μm. (D) Northern analysis of milk protein gene expression in mammary glands from doxycycline-induced MTB and MTB/tAkt1 mice. Wild-type FVB mice at the indicated developmental stages are shown as controls for the temporal expression patterns of individual milk protein genes. 28S rRNA served as a loading control. (E) Quantitative RT–PCR analysis of milk protein gene expression in mammary glands from induced MTB and MTB/tAkt1 mice. Gene expression levels were normalized to cytokeratin 18. Error bars represent mean ± standard error of the mean (SEM). (F) Immunoblotting analysis of total milk proteins in mammary glands from MTB and MTB/tAkt1 mice induced with doxycycline for 96 h and wild-type FVB mice at the indicated developmental stages. β-Tubulin served as a loading control. (G) Immunofluorescence analysis of β-casein and CK8 expression in the mammary glands of MTB and MTB/tAkt1 mice induced with doxycycline for 96 h. Nuclei were counterstained with Hoechst 33258 (blue). Bars, 50 μm. (H, I) Immunofluorescence analysis of NKCC1 [H] and Npt2b [I] expression in the mammary glands of MTB and MTB/tAkt1 mice induced with doxycycline for 96 h as well as lactating wild-type controls. Arrows indicate epithelial cells that have down-regulated NKCC1 expression. Nuclei were counterstained with Hoechst 33258 (blue). Bars, 100 μm.

Figure 1.
development induced by exposure to hormones of pregnancy—does β-casein become expressed in ductal cells? Notably, we found that acute Akt1 activation rapidly induced ductal epithelial cells to express β-casein without the antecedent formation of alveolar structures. This suggested that Akt’s ability to induce milk protein expression is not an indirect consequence of Akt-induced lobuloalveolar development, but rather may be due to Akt’s ability to directly control epithelial secretory differentiation and the production of each of the major components of milk.

Pten deletion induces secretory differentiation in the virgin mammary gland

We next examined whether activation of endogenous Akt at physiological levels would also induce secretory differentiation in the mammary glands of virgin mice. To address this, we generated mice in which the tumor suppressor Pten, an inhibitor of the PI3K–Akt pathway, could be conditionally deleted in the mammary epithelium in response to doxycycline treatment. Pten\(^{fl/fl}\) mice (Groszer et al. 2001) were bred to MTB/TetO-Cre (TTC1) mice, in which Cre expression is under the control of the tet operator, to generate MTB/TTC1;Pten\(^{fl/fl}\) mice or MTB/Pten\(^{fl/fl}\) controls. As predicted, Pten expression was markedly decreased in the mammary epithelium of virgin MTB/TTC1;Pten\(^{fl/fl}\) mice in which Cre expression was induced with 2 mg/mL doxycycline for 2 wk, whereas Pten expression persisted in the mammary epithelium and induced with 2 mg/mL doxycycline for 2 wk, whereas Pten expression persisted in the mammary epithelium and induced with 2 mg/mL doxycycline for 2 wk, whereas Pten expression persisted in the mammary epithelium and induced with 2 mg/mL doxycycline for 2 wk. Consistent with this, we found that acute Akt1 activation rapidly induced ductal cells to express β-casein without the antecedent formation of alveolar structures. This suggested that Akt’s ability to induce milk protein expression is not an indirect consequence of Akt-induced lobuloalveolar development, but rather may be due to Akt’s ability to directly control epithelial secretory differentiation and the production of each of the major components of milk.

Akt1 induces the synthesis of all major milk components in virgin mice

Our previous observation that the biosynthesis of all three major components of milk—lactose, lipids, and milk proteins—is greatly reduced in Akt1\(^{-/-}\);Akt2\(^{-/-}\) mice (Chen et al. 2010) led us to examine whether Akt-induced mammary epithelial differentiation is sufficient to induce the production of lactose and lipids as well as milk proteins in the virgin mammary gland.

Lactose synthesis from glucose and galactose in mammary epithelial cells during lactation is catalyzed by lactose synthase. The activity and specificity of this enzyme is regulated by its cofactor, α-lactalbumin [Permyakov and Berliner 2000]. Our observation that Akt1 activation in virgin MTB/tAkt1 mice induced the expression of α-lactalbumin mRNA 45-fold compared with control MTB mice, coupled with our prior observation that Akt1 activation in virgin MTB/tAkt1 mice markedly up-regulates expression of the glucose transporter Glut1 [Boxer et al. 2006], suggested that Akt1 activation might be sufficient to induce lactose synthesis in virgin mice. As predicted, MTB/tAkt1 mice induced with doxycycline for 96 h exhibited a 50-fold increase in lactose levels (Fig. 2D).
During lactation, Akt1 is required for maximal lipid synthesis by means of its regulation of the expression of key metabolic enzymes (Boxer et al. 2006). Consistent with this, staining for lipids revealed that Akt1-expressing cells in the mammary glands of doxycycline-induced MTB/tAkt1 mice contained abundant cytoplasmic lipid droplets, whereas MTB control cells did not [Fig. 2E]. Akt1 activation also induced mammary epithelial-specific expression of the fatty acid synthesis genes Aldoc, Fads1, and Elovl5 (Supplemental Fig. S2). Together, our results indicate that Akt1 activation is sufficient to rapidly induce production of the three main components of milk—milk proteins, lipid, and lactose—in the mammary glands of virgin mice.

Akt induces Stat5 activation

The Prlr–Jak2–Stat5 signaling pathway is required for terminal differentiation of the alveolar epithelium during pregnancy, as deletion of Prlr, Jak2, or Stat5 leads to decreased Stat5 activation, lobuloalveolar development, and milk protein gene expression [Liu et al. 1997; Ormandy et al. 1997; Teglund et al. 1998; Cui et al. 2004; Wagner et al. 2004]. To test the possibility that Akt-induced mammary epithelial differentiation is mediated by this pathway, we examined Stat5 activity in doxycycline-induced virgin MTB/tAkt1 as well as MTB/TTC1;Pten<sup>0/0</sup> mice.

Immunoblotting revealed that Akt activation rapidly induced high levels of phospho-Stat5α/β in mammary tissues from doxycycline-induced virgin MTB/tAkt1 as well as MTB/TTC1;Pten<sup>0/0</sup> mice that was absent in MTB control mice [Fig. 3A,B]. Consistent with these findings, nuclear phospho-Stat5α/β was detected by immunofluorescence in most, although not all, ductal epithelial cells expressing p-Akt in MTB/tAkt1 and MTB/TTC1;Pten<sup>0/0</sup> mice but not in ductal epithelial cells in control MTB mice [Fig. 3C,D].

To determine whether the ability of Akt1 to induce Stat5 activation in MTB/tAkt1 mice is due to its action in the mammary gland or requires systemic hormonal alterations, we transplanted MTB/tAkt1 mammary glands into wild-type recipient mice. Four weeks following transplantation, recipient mice were induced with doxycycline for 96 h. Activation of Akt in transplanted MTB/tAkt1 glands resulted in rapid Stat5 phosphorylation, milk protein production, and distension of mammary ducts with milk in a manner similar to that observed in intact MTB/tAkt1 mice (Supplemental Fig. S3A,B).

To confirm that the ability of Akt1 to induce Stat5 activation is due to Akt transgene expression in mammary epithelial cells, we injected isolated mammary epithelial cells from MTB/tAkt1 mice into the cleared fat pads of wild-type recipients, allowed ductal outgrowth for 6 wk, and then induced recipient mice with doxycycline for 96-h. Activation of Akt in the transplanted epithelial cells of recipient mice resulted in rapid Stat5 phosphorylation, milk protein production, and distension of mammary ducts with milk (Supplemental Fig. S3C,D).

Akt1 activation also induced rapid Stat5 phosphorylation in explanted MTB/tAkt1 glands induced with doxycycline in vitro (Supplemental Fig. S4A). Similarly, phospho-Stat5α/β levels were elevated in vitro in mammary glands harvested from MTB/TTC1;Pten<sup>0/0</sup> mice that had been treated with doxycycline to induce deletion of Pten [Supplemental Fig. S4B]. Together, our observations indicate that activation of the Pten–Akt pathway results in rapid Stat5 activation in mammary epithelial cells in vivo and in vitro in a manner that is cell-intrinsic.

Akt-induced mammary differentiation requires Prlr and Stat5α/β

To determine whether Akt-induced mammary differentiation in virgin mice is mediated by Stat5, we asked whether genetic disruption of the Stat5α/β genes would block mammary differentiation induced by sustained Akt activity. Akt1 expression was induced for 96 h in 6-wk-old
virgin MTB/tAkt1 mice that were either wild type or heterozygous or homozygous for hypomorphic alleles of both the Stat5a and Stat5b genes (Stat5a/b+/+, Stat5a/b−/−, Stat5a/b−/−). Consistent with our earlier findings, H&E staining revealed that mammary ducts in MTB/tAkt1;Stat5a/b+/+ and MTB/tAkt1;Stat5a/b−/− mice were distended with proteinaceous material containing lipid droplets similar to that observed in the mammary glands of lactating mice (Fig. 4A). In contrast, mammary ducts in MTB/tAkt1;Stat5a/b−/− mice were compact and lacked a differentiation phenotype (Fig. 4A). Western analysis revealed that milk protein expression induced by Akt1 was dramatically attenuated in MTB/tAkt1;Stat5a/b−/− mice and was accompanied by reduced levels of phosphorylation of the truncated Stat5a/b proteins (Fig. 4B).

Further evidence that Akt activation is unable to induce mammary epithelial differentiation in MTB/tAkt1;Stat5a/b−/− mice came from immunofluorescence analyses for NKCC1 and Npt2b expression. In contrast to the differentiated patterns of NKCC1 and Npt2b expression observed in the mammary epithelium of doxycycline-induced MTB/tAkt1 mice, attenuation of Stat5 activity in MTB/tAkt1;Stat5a/b−/− mice blocked Akt activation-induced differentiation of mammary epithelium as indicated by the failure to down-regulate NKCC1 or up-regulate Npt2b (Fig. 4C). In aggregate, these results demonstrate that mammary glands from induced MTB/tAkt1;Stat5a/b−/− virgin mice fail to undergo secretory differentiation following Akt activation.

Increasing prolactin levels during pregnancy result in Stat5 activation through binding to Prlr, activation of Jak2, and Jak2-directed Stat5 phosphorylation (Farth et al. 2011). However, Stat5 phosphorylation can also occur downstream from pathways other than Prlr. To further explore the requirement for the Prlr–Jak2–Stat5 signaling pathway in Akt activation-induced mammary differentiation, we generated bistransgenic MTB/tAkt1 mice that were wild type or heterozygous or homozygous for null alleles of Prlr (MTB/tAkt1;Prlr+/+, MTB/tAkt1;Prlr−/−, and MTB/tAkt1;Prlr−−). As with Stat5 hypomorphism, genetic ablation of Prlr resulted in the abrogation of Akt-induced mammary epithelial differentiation, as evidenced by dramatic attenuation of Akt-induced milk protein expression, Stat5a/b phosphorylation, and mammary ductal distension in MTB/tAkt1;Prlr−− mice (Fig. 4D,E). Immunofluorescence analysis confirmed that Prlr deletion also abrogated Akt-induced differentiated patterns of expression for NKCC1 and Npt2b (Fig. 4F). Taken together, these findings establish that Prlr and Stat5a/b are required for Akt-induced mammary epithelial differentiation in virgin mice.

We next examined whether Akt-induced lipid and lactose biosynthesis were dependent on Prlr–Stat5 signaling. Following Akt activation, the mammary epithelium of MTB/tAkt1 mice lacking either Stat5a/b or Prlr failed to accumulate cytoplasmic lipid droplets or induce lactose synthesis (Fig. 4G,H). This latter defect was likely due to the 80% reduction in Akt-induced α-lactalbumin mRNA expression observed in mutant mice (Supplemental Fig. S5A) rather than to a defect in glucose transport, since Glut1 expression and localization were unaffected by Stat5 or Prlr deletion (Supplemental Fig. S5B). Together, these results demonstrate that Akt-induced production of each of the three major components of milk requires a functional Prlr–Jak–Stat5 pathway despite the fact that the Akt-mediated regulation of Glut1 occurs independently of Prlr and Stat5.

**The requirement for Stat5a/b and Prlr for Akt-induced differentiation is intrinsic to the mammary gland**

Mice bearing germline mutations in Stat5a/b or Prlr exhibit developmental abnormalities in the mammary gland as well as other organs (Bole-Feyso et al. 1998; Cui et al. 2004). To rule out the possibility that the block in Akt-induced mammary differentiation observed in MTB/tAkt1;Stat5a/b−/− and MTB/tAkt1;Prlr−− mice might be secondary to systemic effects of Stat5a/b or Prlr deletion, we induced Akt1 in mammary glands cultured in vitro and examined milk protein expression as a surrogate marker for mammary differentiation.

Following treatment with doxycycline for 4 d, explanted glands from MTB/tAkt1 mice expressed milk proteins as well as elevated levels of phospho-Stat5a/b (Supplemental Fig. S6A,B). In contrast, Akt activation in mammary tissue from Stat5a/b−/− and Prlr−− mice failed to induce milk protein synthesis (Supplemental Fig. S6A,B). These data demonstrate that the defect in Akt-induced mammary differentiation in MTB/tAkt1;Stat5a/b−/− and MTB/tAkt1;Prlr−− mice is due to a requirement for Stat5 activation in the mammary gland rather than to systemic alterations in these mice.

**Akt induces expression of a secreted factor responsible for Stat5 activation and milk protein expression**

The finding that Akt activation in the mammary gland results in rapid Stat5 phosphorylation and expression of milk proteins suggested that Akt might directly mediate activation of the Stat5 signaling pathway. Indeed, Akt activation induced in vitro by doxycycline treatment of explanted MTB/tAkt1 mammary tissue was capable of inducing Stat5 phosphorylation and milk protein expression when cultured in the absence of prolactin (Fig. 5A). In contrast, at least 50 ng/mL exogenous prolactin was required to induce Stat5 phosphorylation in uninduced mammary tissues from MTB/tAkt1 mice.

Since Akt is a serine/threonine kinase, we reasoned that it was unlikely to directly phosphorylate Stat5 at Tyr694 or Tyr699. Rather, we hypothesized that Akt might induce the secretion of a factor capable of activating Stat5 via an autocrine or paracrine loop. To test this hypothesis, we incubated the nontransformed mammary epithelial cell line HC11 with conditioned medium (CM) harvested from explanted MTB/tAkt1 mammary glands induced with doxycycline in vitro. When applied to HC11 cells, CM from doxycycline-induced MTB/tAkt1 glands induced Stat5 phosphorylation, whereas CM
Figure 4. Akt-mediated differentiation of the virgin mammary gland requires Stat5a/b and Prlr. (A) H&E-stained mammary sections from doxycycline-induced virgin MTB and MTB/tAkt1 wild-type mice or mice heterozygous or homozygous for a hypomorphic allele of Stat5a/b. Bars, 200 μm. (B) Immunoblotting analysis of mammary protein lysates corresponding to mice in A. The faster-migrating p-Stat5a/b and Stat5a/b bands represent the N-terminal-truncated Stat5a/b protein encoded by the hypomorphic allele of Stat5a/b. β-Tubulin served as a loading control. (C) Immunofluorescence analysis of NKCC1 (top) and Npt2b (bottom) expression in mammary tissues from doxycycline-induced 6-wk-old virgin MTB/tAkt1 mice of the indicated Stat5a/b genotypes. Luminal epithelial cells were coimmunostained with CK8. Arrows denote mammary epithelial cells in which NKCC1 expression was not detected. Mammary tissues from MTB mice homozygous for mutant alleles of Stat5a/b served as negative controls. Nuclei were counterstained with Hoechst 33258 (blue). Bars, 100 μm. (D) H&E-stained mammary sections from doxycycline-induced virgin MTB/tAkt1 wild-type mice and mice heterozygous or homozygous for a null allele of Prlr. Bars, 200 μm. (E) Immunoblotting analysis of mammary protein lysates corresponding to mice in D. (F) Immunofluorescence analysis of NKCC1 (top) and Npt2b (bottom) expression in mammary tissues from doxycycline-induced 6-wk-old virgin MTB/tAkt1 mice of the indicated Prlr genotypes. Luminal epithelial cells were coimmunostained with CK8. Arrows denote mammary epithelial cells in which NKCC1 expression was not detected. Mammary tissues from MTB mice homozygous for mutant alleles of Prlr served as negative controls. Nuclei were counterstained with Hoechst 33258 (blue). Bars, 100 μm. (G) Nile red staining of cytoplasmic lipid droplets in the mammary glands of doxycycline-induced virgin MTB/tAkt1, MTB/tAkt1,Stat5a/b−/−, and MTB/tAkt1,Prlr−/− mice. Bars, 50 μm. (H) Lactose levels in mammary glands from doxycycline-induced virgin MTB/tAkt1, MTB/tAkt1,Stat5a/b−/−, and MTB/tAkt1,Prlr−/− mice. MTB,Stat5a/b−/− and MTB,Prlr−/− mice are included as controls. Error bars indicate mean ± SEM.
Levels of prolactin mRNA in the mammmary glands of induced MTB/tAkt1 mice were detectable but unchanged compared with MTB mice [Fig. 6D]. In addition, circulating levels of prolactin were unchanged in doxycycline-induced MTB/tAkt1 mice [Fig. 6E], suggesting that the observed increase in prolactin in the mammmary gland was not the result of an increase in systemic prolactin levels. When considered together, our findings demonstrate that the increase in prolactin protein levels observed in the mammmary gland following Akt activation is not due to systemic changes in circulating prolactin or increased autonomous prolactin mRNA expression in the mammmary gland, but is instead the result of Akt-induced post-transcriptional regulation of prolactin expression in mammmary epithelial cells.

We next wished to determine whether the mammmary-derived factor present in CM from doxycycline-induced MTB/tAkt1 glands that was capable of activating Stat5 when applied to HC11 cells was indeed prolactin. To address this, CM harvested from MTB/tAkt1 mammary tissues was incubated with an anti-prolactin antibody for 30 min. When compared with CM incubated with an IgG control, CM that had been neutralized with a prolactin antibody exhibited a reduced ability to stimulate Stat5 phosphorylation [Fig. 6F]. CM also failed to stimulate Stat5 phosphorylation when HC11 cells were pretreated for 1 h with the Jak2-specific inhibitor NVP-BSK805 in accordance with the requirement for Jak2 in prolactin-induced Stat5 activation [Fig. 6G; Baffert et al. 2010].

The above findings strongly suggested that prolactin is the Akt-inducible factor in CM that is responsible for Stat5 activation. However, given the incomplete neutralization of the phospho-Stat5-inducing activity in CM by the anti-prolactin antibody tested, coupled with the fact that Jak2 can be activated by pathways other than ligand-bound Prlr, we sought further proof supporting this possibility. Therefore, we used mice constitutively deficient for Prlr to definitively address the role of prolactin or increased autonomous prolactin mRNA expression in mammary epithelial cells.

Instead of detecting prolactin, we intended to detect the presence of Jak2. To this end, the conditioned medium was incubated with an anti-prolactin antibody coupled to beads to which Stat5 was immunoprecipitated from HC11 cells incubated for 20 min with CM harvested from wild-type MTB/tAkt1 mammary tissues induced with doxycycline ex vivo. When compared with CM incubated with an IgG control, the ability of CM harvested from doxycycline-induced explanted mammary glands from MTB/tAkt1;Prlr−/− mice to stimulate Stat5 phosphorylation was reduced to levels below those observed in the presence of CM harvested from wild-type MTB/tAkt1 explanted mammary glands. Taken together, these results indicate that Akt activation in mammary epithelial cells induces the secretion of a factor that activates Stat5 in an autocrine or paracrine manner and that production of this factor does not require Stat5a/b or Prlr.

**Akt induces the expression and secretion of autocrine prolactin in the mammmary gland**

Since we had found that the ability of Akt1 to induce Stat5 phosphorylation and mammary epithelial differentiation in mice requires the presence of Prlr, we considered the possibility that these effects of Akt1 are mediated by the autocrine production of prolactin. To test this hypothesis, we first examined prolactin expression in the mammary gland following Akt1 activation in vivo. As expected, prolactin protein was highly expressed in the pituitary glands of wild-type FVB mice but was not detected in the mammary glands of virgin MTB or MTB;Ptenfl/fl mice [Fig. 6A,B]. In contrast, prolactin protein was clearly detectable in the mammary glands of doxycycline-induced MTB/tAkt1 mice as well as doxycycline-induced MTB/TTC1;Ptenfl/fl mice [Fig. 6A,B]. Prolactin expression in the mammary glands of induced MTB/tAkt1 mice occurred in the epithelial compartment [Fig. 6C], which is also the site of Akt1 induction.

Interestingly, levels of prolactin mRNA in the mammmary glands of induced MTB/tAkt1 mice were detectable but unchanged compared with MTB mice [Fig. 6D]. In addition, circulating levels of prolactin were unchanged in doxycycline-induced MTB/tAkt1 mice [Fig. 6E], suggesting that the observed increase in prolactin in the mammmary gland was not the result of an increase in systemic prolactin levels. When considered together, our findings demonstrate that the increase in prolactin protein levels observed in the mammmary gland following Akt activation is not due to systemic changes in circulating prolactin or increased autonomous prolactin mRNA expression in the mammmary gland, but is instead the result of Akt-induced post-transcriptional regulation of prolactin expression in mammmary epithelial cells.

**Figure 5. Akt induces expression of a secreted factor responsible for Stat5 activation and milk protein expression.** (A) Immunoblotting analysis of protein lysates from MTB/tAkt1 mammary glands treated in vivo with the indicated doses of prolactin in the absence (−Dox) or presence (+Dox) of 2 μg/mL doxycycline. (B) Immunoblotting analysis of Stat5a/b immunoprecipitated from HC11 cells incubated for 20 min with CM harvested from wild-type MTB/tAkt1 mammary tissues induced with doxycycline for 4 d ex vivo (−Dox) or untreated (−Dox). Lysate from HC11 cells treated with 1 μg/mL prolactin is shown as a positive control. (C) Immunoblotting analysis of Stat5a/b immunoprecipitated from HC11 cells incubated for 20 min with CM harvested from wild-type or Stat5a/b−/− MTB/tAkt1 mammary tissues induced with doxycycline ex vivo.
mammary tissues to activate Stat5 when applied to HC11 cells was also completely abrogated by Prl deletion (Fig. 7B). Together, these findings demonstrate that Akt-induced Stat5 activation and mammary differentiation are mediated by the Akt-induced autocrine production of prolactin by the mammary gland.

To extend these findings, we transplanted mammary epithelial fragments from MTB/tAkt1 or MTB mice into the cleared mammary fat pads of Prl−/− mice. After allowing 4 wk for ductal outgrowth, recipient mice were induced with doxycycline for 4 d. Examination of carmine-stained whole mounts confirmed that epithelial fragments from donor mice grew to a comparable extent in the fat pads of recipient Prl−/− mice (Supplemental Fig. S7A). Notably, induction of Akt in chimeric mammary glands composed of donor MTB/tAkt1-derived epithelium and either recipient Prl−/− or Prl+/+ stroma resulted in comparable levels of Stat5 activation and milk protein expression (Supplemental Fig. S7A,B). This demonstrates that Akt-induced Stat5 phosphorylation and mammary differentiation are due to autocrine prolactin production in the mammary epithelium and do not require prolactin produced by mammary stromal cells or other tissues.

Down-regulation of endogenous Akt in the mammary gland impairs the physiological production of autocrine prolactin

In light of our finding that Akt pathway activation is sufficient to induce the production of autocrine prolactin, we also wished to determine whether endogenous Akt is required for the physiological production of autocrine prolactin in the mammary gland. In particular, we sought to establish whether the Akt-induced production of autocrine prolactin plays a physiological role in mammary gland development. In support of this possibility, late-pregnant Akt1−/−;Akt2−/− mice exhibit a defect in mammary epithelial differentiation (Chen et al. 2010) that could ostensibly result from a defect in their ability to express autocrine prolactin. Indeed, immunoblotting revealed that autocrine prolactin expression was completely abrogated in the mammary glands of late-pregnant Akt1−/−;Akt2−/− mice (Fig. 7C,D). In contrast, pituitary prolactin mRNA expression and serum prolactin levels...
Autocrine prolactin controls mammary differentiation

In order to confirm that the defect in autocrine prolactin production and Stat5 activation observed in Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice at day 18.5 of pregnancy is due to deletion of Akt in the mammary gland rather than endocrine alterations caused by Akt deletion in other tissues, we transplanted Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> or wild-type mammary epithelial fragments into the cleared fat pads of 3-wk-old wild-type or Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice. After permitting 4 wk for ductal outgrowth, transplant recipients were set up for mating to collect mammary tissues at day 18.5 of pregnancy.

Analogous to our observations in intact Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice, whole-mount staining and histological analysis revealed that alveolar epithelium derived from Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mammary glands failed to differentiate during late pregnancy when transplanted into wild-type mice, whereas alveolar epithelium derived from the mammary glands of wild-type mice differentiated normally when transplanted into Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice (Supplemental Fig. S9A,B). In accordance with this, mammary prolactin expression, Stat5 activation, and expression of milk proteins were dramatically reduced in alveolar epithelium derived from Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> glands regardless of the genotype of the transplant recipient, whereas autocrine prolactin production, Stat5 activation, and expression of milk proteins were not changed when transplanted into wild-type mammary glands transplanted into Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice (Supplemental Fig. S9C,D). Taken together, these findings demonstrate that Akt activation in the mammary epithelium is required for the production of autocrine prolactin, Stat5 activation, and mammary differentiation in a manner that is intrinsic to the mammary epithelium.

**Discussion**

Prolactin is classically viewed as an endocrine hormone whose multiple reproductive and metabolic functions depend on its delivery from the anterior pituitary, where it is produced, to peripheral target tissues via the circulation. In recent years, it has become evident that prolactin is also expressed in several extrapituitary tissues and cell types in humans, including the mammary gland as well as some breast and prostate cancer cell lines (Ben-Jonathan et al. 2008; Bernichtein et al. 2010). However, in contrast to our extensive understanding of the pleiotropic actions of endocrine prolactin, remarkably little is known about the physiological functions of autocrine prolactin in vivo or the pathways that regulate its production.

The pathways that regulate the synthesis and secretion of autocrine prolactin in vivo are almost entirely unknown, with the sole exception of studies demonstrating the estradiol-induced transcriptional up-regulation of autocrine prolactin in rat trigeminal neurons (Diogenes et al. 2006)—a mechanism analogous to that by which estradiol up-regulates prolactin transcription in pituitary lactotrophs—and androgen-dependent regulation of autocrine prolactin expression in the rat prostate (Nevalainen et al. 1997). We now report that the in vivo production of autocrine prolactin in the mammary gland is regulated by the Pten–Akt pathway. Conditional activation of the Akt

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**Figure 7.** Prl is required for Akt1-mediated Stat5 activation and mammary differentiation. [A] Immunoblotting analysis of the indicated proteins in protein lysates from virgin MTB/tAkt1 and MTB/tAkt1,Prl<sup>−/−</sup> mammary tissues induced with doxycycline for 4 d ex vivo (+Dox) or untreated (−Dox). β-Tubulin levels served as a loading control. [B] Immunoblotting analysis of Stat5a/b immunoprecipitated from HC11 cells treated for 20 min with CM from MTB/tAkt1 and MTB/tAkt1,Prl<sup>−/−</sup> mammary tissues induced with doxycycline ex vivo. [C] Immunoblotting analysis of prolactin expression in the mammary glands of mice bearing the indicated Akt genotypes at day 18.5 of pregnancy. β-Tubulin served as a loading control. [D] Quantification of prolactin expression in the mammary glands of mice bearing the indicated Akt1 and Akt2 genotypes at day 18.5 of pregnancy (n = 6 per genotype) normalized to β-tubulin expression. Prolactin/β-tubulin ratios were normalized to those in wild-type mice. Error bars indicate mean ± SEM. Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> vs. wild type, *P < 0.001; Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> vs. Akt1<sup>−/−</sup>;Akt2<sup>−/+</sup>, *P = 0.04, as indicated.

did not differ between Akt1<sup>−/−</sup>;Akt2<sup>−/+</sup> and control mice (Supplemental Fig. S8A; data not shown).

In agreement with our observation that Akt induced up-regulation of autocrine prolactin in MTB/tAkt1 mice occurs at the post-transcriptional level, prolactin mRNA expression levels were unchanged in the mammary glands of late-pregnant Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice compared with controls (Supplemental Fig. S8B). Moreover, consistent with the presence of normal phospho-Stat5 levels in the mammary glands of lactating—as opposed to late-pregnant—Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> mice, prolactin protein levels were comparable in the mammary glands of lactating Akt1<sup>−/−</sup>;Akt2<sup>−/−</sup> and wild-type mice (Supplemental Fig. S8C). In aggregate, our findings indicate that endogenous Akt activity is required for autocrine prolactin production in the mammary gland during late pregnancy.

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pathway in the mammary epithelium of virgin mice by either Akt1 expression or Pten deletion rapidly induced the production of autocrine prolactin. In contrast to findings in rat trigeminal neurons, Akt-induced up-regulation of autocrine prolactin occurred at the post-transcriptional level, revealing a previously unsuspected mechanism for the induction of prolactin protein expression.

Analogous to the paucity of information regarding the regulation of autocrine prolactin expression, little information currently exists regarding its physiological functions. While autocrine prolactin mRNA expression has been demonstrated in a variety of normal tissues, including the uterus, skin, white fat, and placenta (Ben-Jonathan et al. 2008; Bernichtein et al. 2010), no function for autocrine prolactin produced by these tissues has been demonstrated either in vivo or in vitro.

Autocrine prolactin mRNA expression in the mammary gland has been detected in late-pregnant and lactating rats [Kurtz et al. 1993; Steinmetz et al. 1993; Iwasaka et al. 2000; Ben-Jonathan et al. 2008]. In this regard, the sole report of a function for autocrine prolactin in a normal tissue arises from studies comparing proliferation rates in recombined mouse mammary glands composed of either Prl1<−/−> or Prl1<+/−> epithelium transplanted into wild-type mammary stroma. These studies identified a 2.8-fold lower level of alveolar epithelial proliferation at parturition in the absence of any detectable morphological alterations in glands derived from Prl1<−/−> mice [Naylor et al. 2003]. The impact of prolactin deletion on epithelial differentiation was not investigated. At present, this role in proliferation at the time of parturition constitutes the only defined physiological function for autocrine prolactin in normal tissues [Bernichtein et al. 2010].

We now report that autocrine prolactin produced by mammary epithelial cells is required for their terminal differentiation during late pregnancy as well as the initiation of lactation at parturition, a critical developmental transition essential for energy output to support survival of mammalian offspring. Conditional activation of the Akt pathway in the mammary glands of virgin mice by either Akt1 expression or Pten deletion rapidly induced terminal mammary epithelial differentiation accompanied by the synthesis of milk despite the absence of lobuloalveolar development. Mammary differentiation was due to the Akt-dependent synthesis and secretion of autocrine prolactin and downstream activation of the Prlr–Jak–Stat5 pathway. Consistent with the Akt-dependent secretion of autocrine prolactin, cells treated with CM from mammary glands in which Akt had been activated underwent rapid Stat5 phosphorylation in a manner that was blocked by treatment with an anti-Prl antibody, inhibition of Jak2, or deletion of the prolactin gene. Furthermore, Akt-induced mammary differentiation was abrogated in Prl1<−/−>, Prlr<−/−>, and Stat5<−/−> mice.

Supporting a physiological requirement for autocrine prolactin during development, the mammary glands of lactation-defective Akt1<−/−>:Akt2<+/−> mice failed to express autocrine prolactin or activate Stat5 during late pregnancy despite normal levels of serum and pituitary prolactin, resulting in a failure to initiate secretory activation or appropriately up-regulate the production of milk proteins, lactose, and lipids. In further support of a mammary epithelial-intrinsic mechanism of action, Akt1<−/−>;Akt2<−/−> mammary epithelial fragments transplanted into wild-type mice failed to activate Stat5 and exhibited defective mammary differentiation during late pregnancy, whereas wild-type mammary epithelium transplanted into Akt1<−/−>;Akt2<−/−> recipients activated Stat5 and differentiated normally. In aggregate, our findings demonstrate that Akt pathway activation is necessary and sufficient to induce autocrine prolactin production, Stat5 activation, and terminal mammary epithelial differentiation, even in the absence of the developmental program that normally prepares the mammary gland for lactation. Together, these findings reveal a function for autocrine prolactin in mammary differentiation, demonstrate its endogenous regulation by the PI3K–Akt pathway, and provide a direct link between Akt activation and the Jak–Stat5 pathway.

Notably, the requirement for PI3K–Akt pathway activity for autocrine prolactin production as well as the requirement for autocrine prolactin itself in mammary epithelial differentiation appears to be specific to late pregnancy and parturition, since lactating Akt1<−/−>;Akt2<−/−> mice exhibit normal mammary differentiation characterized by normal levels of autocrine mammary prolactin, milk production, and Stat5 activity [Supplemental Fig. S8C, data not shown]. As such, our data indicate that autocrine prolactin is required for activation of the Jak2–Stat5 pathway during a specific development stage of pregnancy at which pituitary prolactin is unable to provide this function.

It is intriguing that pituitary prolactin fails to compensate for the loss of local prolactin production in Akt1<−/−>; Akt2<−/−> mice during late pregnancy. Serum prolactin levels in rats fluctuate during the first half of pregnancy with daily nocturnal and diurnal surges, whereas pituitary prolactin is suppressed thereafter by rising levels of placental lactogen I [Ben-Jonathan et al. 2008; Bernichtein et al. 2010]. If the release of pituitary prolactin is similarly suppressed in mice during the second half of pregnancy, low circulating levels of prolactin may be insufficient to induce sustained Stat5 activation in Akt1<−/−>;Akt2<−/−> mice, which are relatively resistant to prolactin-induced Stat5 activation due to decreased expression of the positive regulator of Prlr–Jak–Stat5 signaling [Id2] and increased expression of the negative regulators of Prlr–Jak–Stat5 signaling [caveolin-1 and Socs2] [Chen et al. 2010]. As such, local prolactin production may be required to overcome this resistance.

Given the dramatic morphological changes that occur in the rodent mammary gland during the 3-wk period of pregnancy to prepare for lactation, it is striking that activated Akt1 can effect similar changes in the mammary glands of virgin mice within 4 d. Importantly, mammary epithelial differentiation induced by Akt activation is not merely an artifact of gross overexpression of an Akt transgene, as virgin mice deleted for mammary epithelial Pten also underwent mammary epithelial differentiation as a consequence of autocrine prolactin production. Pre-
sumably, the greater extent of mammary epithelial differentiation induced by expression of an activated Akt transgene than by *Pten* deletion reflects the more pronounced activation of Akt by expression of exogenous myr-Akt than by activation of endogenous Akt via *Pten* deletion.

Notably, our results indicate that, even in the absence of hormones of pregnancy or lobuloalveolar development, Akt activation is sufficient to drive the terminal differentiation of mammary ductal epithelium, resulting in large-scale synthesis of milk proteins, lipid, and lactose within 96 h. This is particularly striking given that *β-casein* is normally not expressed in ducts until day 18.5 of pregnancy, although it is expressed in alveoli beginning at day 6.5 of pregnancy. Indeed, Akt1 activation was was robustly induced in the mammary glands of virgin *Stat5a/b−/−* and *Prlr−/−* mice despite the inability of Akt to activate Stat5 or induce differentiation in these mice [Supplemental Fig. S10]. This indicates that Akt is an upstream transcriptional regulator of Elf5 in a manner that does not require Prlr–Jak–Stat5 signaling and that Elf5 expression alone is not sufficient to substitute for the differentiative effects of Prlr signaling during secretory activation. In aggregate, our findings indicate that the ability of Akt to induce terminal mammary epithelial differentiation and secretory activation is due to its ability to stimulate autocrine prolactin production and activate Stat5, rather than its ability to up-regulate Elf5.

Notably, beyond normal mammary epithelial cells, autocrine prolactin is also synthesized and secreted by some human breast cancer cell lines (Ginsburg and Vonderhaar 1995) and has been identified in human breast cancers [Clevenger et al. 2003]. As such, it has been proposed to play a direct role in mammary tumorigenesis that may be distinct from any roles played by endocrine prolactin secreted from the pituitary gland [Bernichtein et al. 2010]. For example, during cancer progression, autocrine prolactin may provide locally derived mitogenic and pro-survival stimuli as well as induce cell migration and facilitate angiogenesis [Clevenger et al. 2003; Wagner and Rui 2008]. However, to date, no functional in vivo evidence exists demonstrating a role for autocrine prolactin in the growth of mammary tumors in either xenograft or autochthonous mouse models. In this regard, our observations identifying the PI3K–Akt pathway as an upstream regulator of autocrine prolactin production has intriguing potential implications for the pathogenesis and treatment of human cancer, since the PI3K–Akt pathway is one of the most common activated oncogenic pathways in human cancer [Altomare and Testa 2005]. As sustained Akt activation may regulate multiple cellular processes through Stat5 signaling, this study raises the possibility that autocrine prolactin may play a role in cancer progression. Further characterization of the relationship between the Akt and Prlr–Jak–Stat5 pathway as an upstream regulator of autocrine prolactin production has intriguing potential implications for the pathogenesis and treatment of human cancer, since the PI3K–Akt pathway is one of the most common activated oncogenic pathways in human cancer [Altomare and Testa 2005]. As sustained Akt activation may regulate multiple cellular processes through Stat5 signaling, this study raises the possibility that autocrine prolactin may play a role in cancer progression. Further characterization of the relationship between the Akt and Prlr–Jak–Stat5 pathway as an upstream regulator of autocrine prolactin production has intriguing potential implications for the pathogenesis and treatment of human cancer, since the PI3K–Akt pathway is one of the most common activated oncogenic pathways in human cancer [Altomare and Testa 2005]. As sustained Akt activation may regulate multiple cellular processes through Stat5 signaling, this study raises the possibility that autocrine prolactin may play a role in cancer progression.
pathways will be required in order to determine whether therapeutic approaches aimed at blocking the interaction between these pathways will be beneficial in the treatment of human cancers.

Materials and methods

Animals

Ptent/0 (Groszer et al. 2001) and Prl−/− (Ormandy et al. 1997) mice were obtained from The Jackson Laboratory. Stat5a/b−/− and Prl−/− mice were obtained from Dr. James Ihle [St. Jude Children’s Research Hospital] and Dr. Nelson Horserman [University of Cincinnati], respectively [Horserman et al. 1997; Teglund et al. 1998]. Mice were backcrossed onto the FVB background prior to experimental use [Ptent/0; N6; Prl−/−; N17, Stat5a/b−/−; N10, and Prl−/−; N5]. Akt1−/− and Akt2−/− mice were provided by Dr. Morris Birnbaum [University of Pennsylvania] (Cho et al. 2001a,b). MMTV/rtTA/TetoA-Akt1 (MTB/Akt1) mice expressing myrAkt1 in a mammary epithelial-specific manner were generated by crossing tAkt1 mice provided by Dr. Craig Thompson [Memorial Sloan-Kettering Cancer Center] and Dr. Jeffrey Rathmell [Duke University] with MMTV/rtTA (MTB) mice as previously described (Gunther et al. 2002; Boxer et al. 2006). Teto-O-TurboCre (TTC1) mice were generated by transgenic injection of a plasmid in which the Turbo-Cre recombinase coding region [gift of Dr. Timothy Ley, Washington University School of Medicine] was subcloned downstream from the tet operator in TMILA (Gunther et al. 2003).

For timed pregnancies, the morning of the observed vaginal plug was counted as day 0.5. To induce expression of Akt1 protein, mice induced with doxycycline were incubated in a digestion medium containing 2 mg/mL collagenase A, 100 U/mL hyaluronidase, and DMEM 2 mg/mL doxycycline for 4 d in vitro were harvested and passed through a 0.45-µm filter, followed by concentration using an Amicon Ultra-4 centrifugal device with 3-kDa cut point. Lactose in the mammary gland was measured by using the lactose assay kit according to the manufacturer’s instructions (MBL International).

Mammary gland culture and isolation of mammary epithelia and adipose tissue

The number 5 inguinal glands and the proximal portion of the number 4 inguinal mammary glands of 3-wk-old donor mice were implanted into the cleared fat pads of recipient mice. To reduce variation between recipient mice, control mammary tissues were transplanted into the contralateral cleared fat pad. Prior to sacrificing recipient mice at 4 wk post-transplantation, mice were induced with 2 mg/mL doxycycline for 4 d or set up for mating. Engrafted mammary glands were harvested for whole-mount and histological analyses or frozen for immunoblotting analysis.

Primary mammary epithelial cells (4.5 × 10^5) freshly isolated as described above from MTB/tAkt1 mice were resuspended in 50 µL of 30% Matrigel in PBS and injected into the cleared fat pads of 3-wk-old wild-type recipient mice. Contralateral fat pads were injected with control MTB mammary epithelial cells. Six weeks post-transplantation, mice were induced with 2 mg/mL doxycycline for 4 d, and reconstituted mammary glands were harvested for analysis.

Morphological analysis

Whole-mount, histological, and immunofluorescence analyses of inguinal mammary glands were performed as described (Gunther et al. 2002; Boxer et al. 2006). The following primary antibodies were used: Glut1 [1:5000, provided by Dr. Morris Birnbaum, University of Pennsylvania]; NKCC1 [1:1000, provided by Dr. Jim Turner, National Institute of Dental and Craniofacial Research]; Npt2b [1:300, provided by Dr. Jurg Biber, University of Zurich]; cytokeratin 8 [1:100, Developmental Study Hybridoma Bank, University of Iowa, Iowa City, IA], β-casein [1:200, provided by Dr. Mina Bissell, Lawrence Berkeley National Laboratory, Berkeley, CA]; phospho-Akt (Ser473, 1:50) and Pten [1:100, Cell Signaling Technology], and phospho-Stat5a/b [Tyr694/Tyr699, 1:50, Upstate Biotechnology]. Stained sections were examined using a Leica microscope [model DM5000B, Leica Microsystems] equipped with a digital camera [Leica DFC350FX].

Immunoblotting and Prl neutralization

Immunoblotting analysis was performed as described (Boxer et al. 2006). The following primary antibodies were used: phospho-Akt (Ser473, 1:1000), Akt [1:1000], and phospho-Stat5a/b [Tyr694/Tyr699, 1:1000] from Cell Signaling Technology; phospho-Stat5a/b [Tyr694/Tyr699, 1:500] and adiponectin [1:1000] from Millipore; mouse milk-specific proteins [1:20,000] from Nordic Immunological Laboratories; β-tubulin [1:1000] from BioGenex; Stat5a/b [1:500] from BD Biosciences; E-cadherin [1:500] from Zymed Laboratories; and Stat5a/b [2 µg for immunoprecipitation], prolactin [1:200], and β-actin [1:1000] from Santa Cruz Biotechnology.

The anti-mouse prolactin antibody used to neutralize CM was obtained from R&D Systems. The Jak2 inhibitor NVP-BSK805 [Baffert et al. 2010] was provided by Novartis Institutes for Biomedical Research.

Analysis of cytokines and lactose

Serum prolactin was measured by a mouse prolactin ELISA kit according to the manufacturer’s instructions [RayBiotech]. CM from MTB/tAkt1 mammary tissues treated or not treated with 2 µg/mL doxycycline for 4 d in vitro were harvested and passed through a 0.45-µm filter, followed by concentration using an Amicon Ultra-4 centrifugal device with 3-kDa cut point. Lactose in the mammary gland was measured by using the lactose assay kit according to the manufacturer’s instructions (MBL International).

Transplantation of mammary tissue and primary mammary epithelial cells

To generate cleared fat pads, the proximal portion of the number four inguinal gland containing the rudimentary mammary epithelial tree of 3-wk-old recipient mice was removed as de-
RNA analysis

Total RNA isolation from snap-frozen tissues, preparation of radioactively labeled cDNA probes, Northern blotting, in situ hybridization, and quantitative RT–PCR analysis were performed as described (Marquis et al. 1995; Chen et al. 2010). The cDNA probe for Northern hybridization detection of Prl corresponded to nucleotides 31–836. The exposure time for in situ hybridization experiments was 2 d. TaqMan-based probes for quantitative RT–PCR were purchased from Applied Biosystems. Probes used were B-casein Mm00839664_m1, Wap Mm008389913_m1, a-lactalbumin Mm00495258_m1, e-casein Mm00 839674_m1, Aldoc Mm01298111_g1, Fads1 Mm00507605_m1, Elolv5 Mm00506717_m1, Tph Mm00446973_m1, and cytokeratin 18 Mm01601706_g1. Statistical analysis was calculated by a two-tailed Student’s t-test.

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Autocrine prolactin induced by the Pten–Akt pathway is required for lactation initiation and provides a direct link between the Akt and Stat5 pathways

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