Forced Involution of the Functionally Differentiated Mammary Gland by Overexpression of the Pro-Apoptotic Protein Bax

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Summary: The mammary gland is a developmentally dynamic, hormone-responsive organ that undergoes proliferation and differentiation within the secretory epithelial compartment during pregnancy. The epithelia are maintained by pro-survival signals (e.g., Stat5, Akt1) during lactation, but undergo apoptosis during involution through inactivation of cell survival pathways and upregulation of pro-apoptotic proteins. To assess if the survival signals in the functionally differentiated mammary epithelial cells can override a pro-apoptotic signal, we generated transgenic mice that express Bax under the whey acidic protein (WAP) promoter. WAP-Bax females exhibited a lactation defect and were unable to nourish their offspring. Mammary glands demonstrated: (1) a reduction in epithelial content, (2) hallmark signs of mitochondria-mediated cell death, (3) an increase in apoptotic cells by TUNEL assay, and (4) precocious Stat3 activation. This suggests that upregulation of a single pro-apoptotic factor of the Bcl-2 family is sufficient to initiate apoptosis of functionally differentiated mammary epithelial cells in vivo. genesis 49:24–35, 2011. **© 2010 Wiley-Liss, Inc.**

Key words: mammary gland; epithelium; Bax; apoptosis; involution; lactation

INTRODUCTION

Mammary gland development is classically compartmentalized into four distinct phases: virgin, pregnancy, lactation, and involution. The contributions of hormones, signaling pathways, and genes that give rise to the mammary gland, from the progression of the rudimentary anlagen during embryonic development through the postnatal stages described above, has been intensely studied (Hennighausen and Robinson, 2005). Although estrogen and progesterone are the predominant hormones responsible for development of the gland in the virgin and early pregnancy phases, prolactin directs the proliferation and differentiation of the epithelia during mid-pregnancy through the prolactin receptor PRL-R/Jak2/Stat5 signaling pathway. Jak2-Stat5 signaling is persistent through the lactation phase for transcriptional activation of milk protein genes, and drops at the lactation-involution transition phase. Involution proceeds through: (1) an initial reversible phase marked by programmed cell death of secretory epithelium without remodeling of the lobuloalveolar structure, followed by (2) an irreversible phase characterized by remodeling of the gland by proteases (e.g., matrix metalloproteinases, MMPs) and macrophages (Lund *et al.*, 1996).

Forced involution, initiated at lactation day 10, triggers apoptosis in response to milk stasis within the alveolar lumen and the precipitous decline in lactogenic hormones (e.g., prolactin), (Feng *et al.*, 1995). The lactation–involution transition is regulated, in part, by the contrasting activation of Stat5 and Stat3. Stat5 phosphorylation is maintained through lactation but drops precipitously at the onset of involution, while Stat3 phosphorylation is initiated as the gland enters involution (Liu *et al.*, 1996; Philp *et al.*, 1996). Conditional deletion of *Stat5* results in the loss of mammary epithelium

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during mid-pregnancy and lactation, suggesting that this transcription factor is required for cell survival. In contrast, transgenic overexpression of Stat5 or gene ablation of *Stat3* delays the involution process (Chapman *et al*., 1999; Iavnilovitch *et al*., 2002). Similarly, loss of Socs3, a negative regulator of the Jak-Stat pathway, leads to an increased rate of involution via increased Stat3 activity (Sutherland *et al*., 2006). In addition to the Jak-Stat pathway, genetic modifications within the IGF and Akt1 signaling pathways reveal their importance. Transgenic IGF-1 or IGF-2 mice have delayed involution, while IGFBP5 overexpressors have increased apoptosis (Moorehead *et al*., 2001; Neuenschwander *et al*., 1996; Tonner *et al*., 2002). Downstream in the IGF signaling pathway, overexpression of PTEN or Akt1 in transgenic models displayed accelerated or delayed involution phases, respectively (Ackler *et al*., 2002; Dupont *et al*., 2002; Schwertfeger *et al*., 2001). Recently, Stat5 has been demonstrated to transcriptionally regulate Akt1, and conditional expression of Stat5 leads to persistent Akt1 activation and delayed involution (Creamer *et al*., 2010). In addition, PTEN ablation increased the survival of alveolar cells similar to the Akt1 overexpression (Li *et al*., 2002).

Programmed cell death (PCD), an important physiological process that is necessary for development, is classified either as either: Type I (apoptosis), Type II (autophagy), or Type III (non-lysosomal vesiculate degradation). Apoptosis can be generally stratified into a three-tiered molecular cascade system consisting of "elicitor" death receptors (e.g., TNFR1, TNFR2, Fas) (Ashkenazi and Vishva, 1998; Singh *et al*., 1998), Bcl-2 family member "mediators" (e.g., Bcl-2, Bcl-X_L, Bcl-X_S, Bax, Bcl-W), (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Yang *et al*., 1995) and the caspase "executioners" (Alnemri *et al*., 1996; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). The mammary gland is an advantageous organ to profile the stages of involution, as a forced involution allows for the synchronization of the biochemical processes. In the context of cell death programs, the involution phase exhibits one of the most dramatic physiological responses, with around 80% of the mammary epithelial cells undergoing programmed cell death (Marti *et al*., 1994). The cohort of signaling pathways and cell death genes necessary for this transitional period has been reviewed extensively (Baxter *et al*., 2007; Green *et al*., 2004; Stein *et al*., 2007). Although microarray and proteome analyses have revealed a predicted cast of proteins and highlighted biochemical pathways involved in involution, the most revealing studies have stemmed from mouse models (Clarkson *et al*., 2004; Stein *et al*., 2004).

Bcl-2 family members are considered the gatekeepers of the mitochondrial-mediated apoptosis, with the fate of the cell dependent upon the relative levels of the individual proteins. Bcl-2-like proteins can be subdivided into two distinct groups, those that are pro-apoptotic (e.g., Bax, Bcl-X_S, Bid, and Bad) and anti-apoptotic (e.g., Bcl-2, Bcl-X_L, and Bcl-W). *Bcl-X_L* and *Bax* are the two most prominent Bcl-2 family members expressed in mammary tissue. *Bcl-X_L* mRNA levels are low in virgin mammary tissue, but increase during pregnancy in parallel with Stat5 activation and cell differentiation. *Bcl-X_L* mRNA levels decrease during lactation, but increase again sharply within 48 h of involution. Similarly, *Bax* mRNA levels increase with the onset of involution (Heermeier *et al*., 1996). Within the Bcl-2 family, several models highlight their influence on the maintenance of the secretory epithelium. We have shown previously that the mammary gland-specific deletion of *Bcl-X_L* resulted in an increase in apoptosis and hastened involution (Walton *et al*., 2001). In the Bax-deficient mammary gland, involution is initially delayed but resembles the wild-type gland at involution day 10 after remodeling is completed (Schorr *et al*., 1999). Although Bax-deficient mammary glands have a delayed early involution, the loss of Bax does not rescue the accelerated cell death in *Bcl-X_L*-null mammary epithelium in our conditional knockout of *Bcl-X_L* (Walton *et al*., 2001). These studies show a cell- and developmental-specific dependence on Bax-mediated apoptosis. Ablation of *Bax* or enforced expression of Bcl-2 (WAP-Bcl-2 transgenic) delays but does not prevent involution, and promotes mammary tumor development (Furth *et al*., 1999; Jager *et al*., 1997; Schorr *et al*., 1999; Shibata *et al*., 1999).

To date, it has not been addressed whether upregulation of a single pro-apoptotic factor is sufficient to initiate involution and remodeling of the functionally differentiated mammary epithelium. To address this issue, we generated transgenic mice that express Bax under a mammary gland-specific promoter that is upregulated during secretory differentiation. Wap-Bax transgenic females display a lactation defect due to an increase in cell death and premature involution. This suggests that upregulation of Bax can override the pro-survival signaling functions of Stat5 and Akt1 in the lactating mammary gland.

RESULTS

WAP-Bax Transgenic Mice Exhibit Impaired Alveologenesis

Coinjection of the WAP-Bax transgene (Fig. 1a) and a K14-agouti construct gave five independent founder lines out of 72 progeny as determined by PCR analysis (Fig. 1b). Previous characterization of coinjected transgenes with the K-14 agouti demonstrated 95% cointegration, which allowed for visual determination of transgene inheritance (Kucera *et al*., 1996). All WAP-Bax lines also carried the K-14 agouti transgene, making it

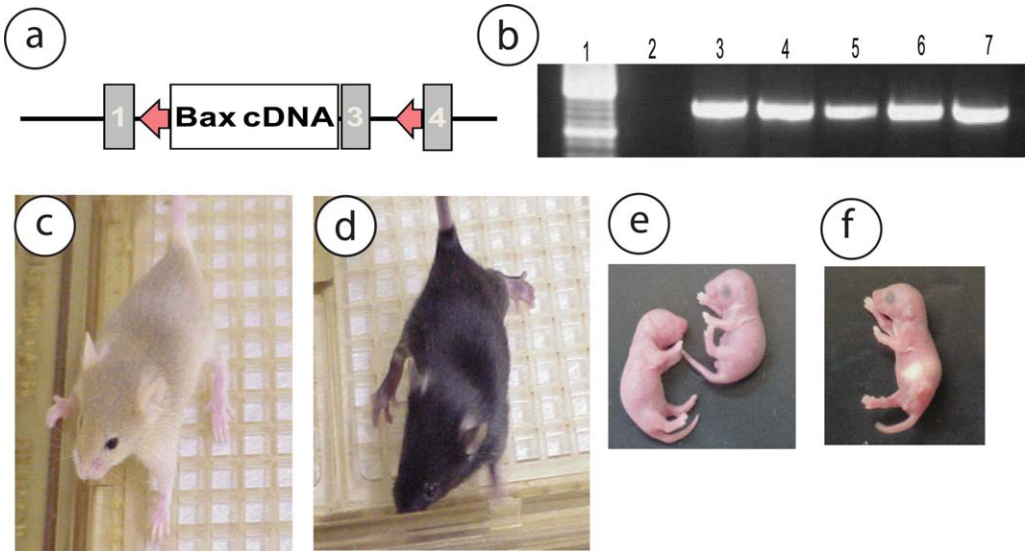

FIG. 1. Generation of WAP-Bax transgenic lines. WAP-Bax transgenic mice were produced by pronuclear injection of the WAP-Bax and K14-agouti constructs. (**a**) Schematic of WAP-Bax transgene, showing the *Bax* cDNA cloned into a WAP promoter-based plasmid. The WAP promoter, portions of Exons 1 and 3, Intron 3, 4, and downstream sequence are present. (**b**) Gel electrophoresis of Bax PCR products from negative control or transgenic WAP-Bax lines IR5, IR15, IR20, IR32, and IR62. Lanes: 1, 100 bp ladder; 2, wild-type control; 3, line IR5; 4, line IR15; 5, line IR20; 6, line IR32; 7, line IR62. (**c**) Coat color of bi-transgenic WAP-Bax; K14-agouti mice. (**d**) Coat color of non-transgenic littermate. (**e**) Neonates (Day 1 of lactation) from WAP-Bax IR15 dam, which have failed to recover milk after suckling. (**f**) Neonates from WAP-Bax IR15 dam fostered onto control lactating dam.

possible to distinguish bi-transgenic mice by a phaeomelanin (yellow) coat color (Fig. 1c,d). Q-PCR was performed to establish copy number of the WAP-Bax transgenic lines, with a range of 1–3 copies of the transgene per line. Upon establishing germline transmission of the WAP-Bax transgene, F1 females were mated with control C57 males to determine effects on mammary gland development. Out of the five lines, two lines that demonstrated protein expression (IR5, IR15) could not support litters at lactation day 1 (L1). Neonates from WAP-Bax dams did not have milk "spots," but could be fostered onto control lactating females (Fig. 1e,f). Therefore, the defect was not a suckling defect with the neonates but the transgenic dams.

Whole mount analysis showed that control mammary glands at lactation day 1 predominantly consisted of secretory epithelia (Fig. 2a). In addition, there was a distinct "rounded" morphology to the alveoli. In contrast, WAP-Bax mammary glands demonstrated a reduced epithelial compartment and altered "star-shaped" alveoli (Fig. 2b,c). Hematoxylin-eosin staining of paraffin-embedded mammary glands showed normal, uniform secretory epithelia in the control glands, but reduced epithelia with altered morphology in the postpartum WAP-Bax transgenic glands (see Fig. 3). Epithelia in the mutant glands displayed increased signs of apoptosis; condensed cells had pyknotic nuclei with many being released into the lumen (Fig. 3d).

Activation of an Apoptotic Program in Response to Expression of Bax

To determine the activation of the apoptotic program, a series of immunohistochemistry (IHC) experiments were performed. For these experiments, the IR15 line, which showed the highest expression and greatest loss of epithelia, was used. At lactation day 1, expression of Bax was confined to the epithelial compartment of WAP-Bax mammary glands, but not detected in the controls (see Fig. 4). Upon Bax-mediated permeabilization of the mitochondria outer membrane, it was expected that cytochrome C is released, culminating in the activation of caspase 3 downstream of the apoptosome. Using IHC, we detected cytochrome C, apaF1 localization, and active caspase 3 within the cytosol of mammary epithelial cells of WAP-Bax transgenic females (see Fig. 5). This clearly suggests that overexpression of Bax is sufficient to elicit a classical mitochondria-mediated apoptotic program of functionally differentiated mammary epithelial cells.

Secretory Mammary Epithelial Cells in WAP-Bax Females are Lost due to Apoptosis

Upregulation of Bax normally coincides with the first phase of involution, which precedes the actual remodeling process 2–3 days later (Heermeier *et al*., 1996; Schorr *et al*., 1999). To confirm that continuous

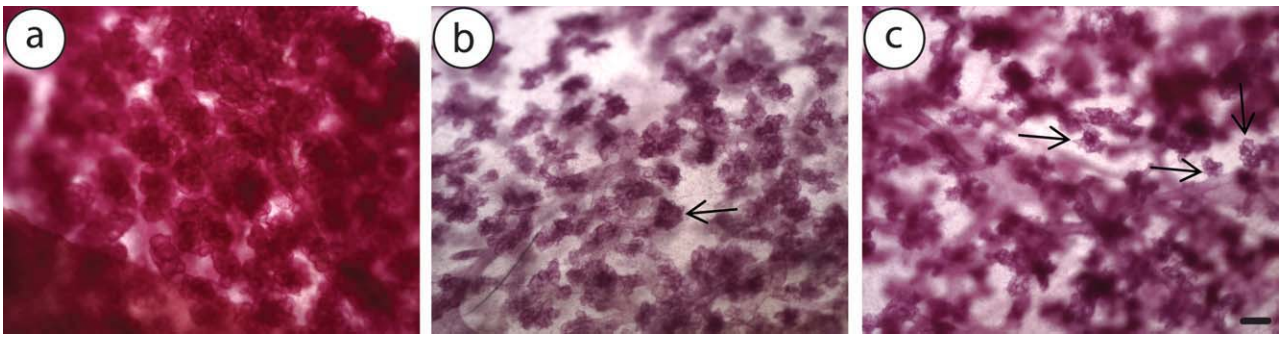

FIG. 2. Expression of Bax under regulation of the WAP promoter results in reduced epithelial content in the postpartum mammary gland. Whole mounts from lactation day 1 control (**a**) and WAP-Bax transgenic mice (**b, c**) show reduced epithelial compartments in the transgenic mice. In addition, the wild-type alveoli have a rounded morphology compared to patches of collapsed, "star-shaped" alveoli in the transgenic mice (denoted by arrows). Fp = fat pad. Bar = 0.5 mm.

expression of exogenous Bax is sufficient to induce the second and terminal stage of apoptosis, we performed TUNEL staining (see Fig. 6). As expected, mammary glands of postpartum control females did not reveal apoptotic cells. In contrast, mammary glands of WAP-Bax transgenic mice had high levels of TUNEL-positive cells in the epithelial compartment. Transgenic lines IR62 (Fig. 6b) and IR32 (Fig. 6f), which expressed less Bax, had also fewer TUNEL-positive cells compared to lines IR5 and IR15 (Fig. 6c–e).

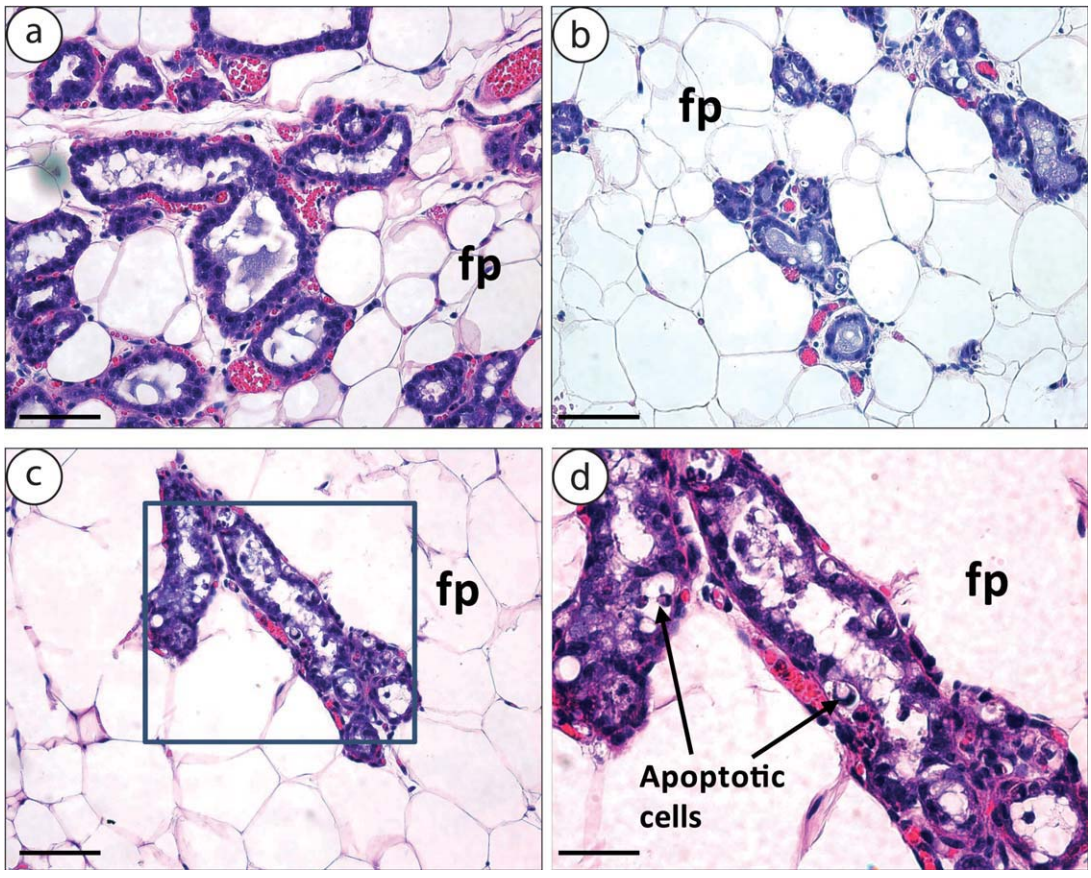

FIG. 3. Presence of apoptotic cells in mammary glands of WAP-Bax transgenic females at lactation day 1. Hematoxylin-eosin staining of lactation day 1 mammary glands was performed on control (**a**) and WAP-Bax mice (**b–d**). (a) Control glands show uniform epithelial morphology and large lipid droplets in the lumina. (b) WAP-Bax glands show reduced epithelia and increased fat pad, and lumina have smaller lipid droplets. (c) Altered morphology is apparent in the epithelium and at higher magnification (d). Apoptotic cells are present as characterized by pyknotic nuclei (arrows). Fp = fat pad. Bar (a–c) = 50 μM. Bar (d) = 25 μM.

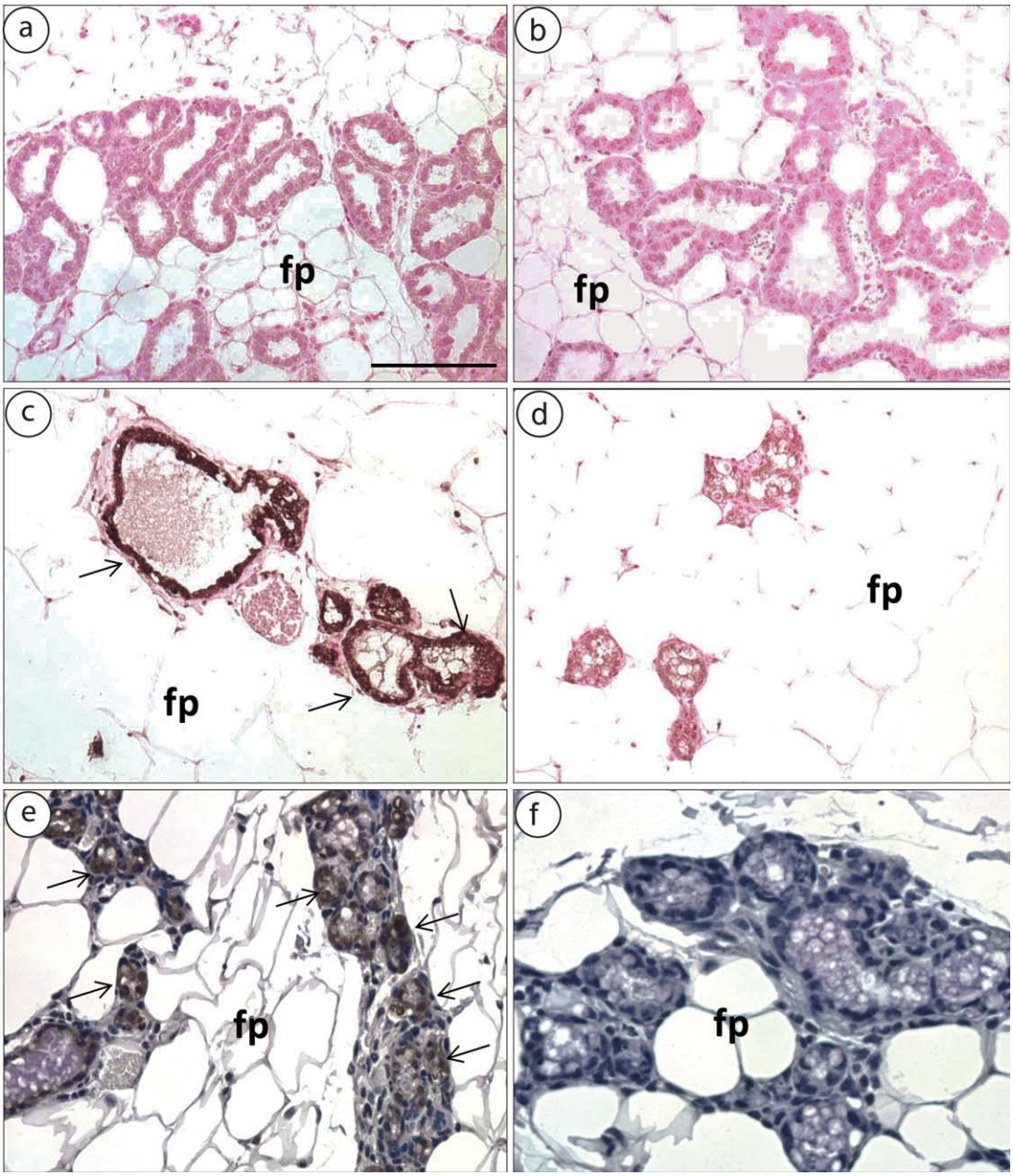

FIG. 4. Expression of Bax under regulation of the Wap promoter is confined to the epithelial compartment of the developing mammary gland in postpartum females. Bax IHC shows localized expression in WAP-Bax but not control lactation day 1 mammary glands. (**a**) Bax protein is not found to be expressed in the control gland using IHC; (**b**) negative control for wild-type gland. (**c**) and (**e**) reveal Bax confined to the epithelial compartment in WAP-Bax glands; (**d**) and (**f**) represent negative IHC controls. Counterstain was performed with nuclear fast red (a–d) or hematoxylin (e, f). Arrows point to positive cells/alveoli. Fp = fat pad. Bar = 100 μM.

Clusterin is a glycoprotein that is found to be expressed at high levels during pregnancy and involution, but is suppressed during lactation (French *et al*., 1996). Because of its expression profile, it is a marker for the lactation-involution transition. Clusterin was found by IHC in the WAP-Bax mammary glands and not in the control glands (see Fig. 7). More importantly, nuclear pStat3 was not present in control glands but was found in transgenic glands (see Fig. 8). pStat5 was expressed in control and transgenic glands as well. In line IR15, where most of the epithelia was already lost, pStat3 and pStat5 were not detected. Overall, these data suggest that the lactation defect in WAP-Bax females is due to a "forced involution" process and loss of the secretory epithelia due to classical pStat3-mediated apoptosis.

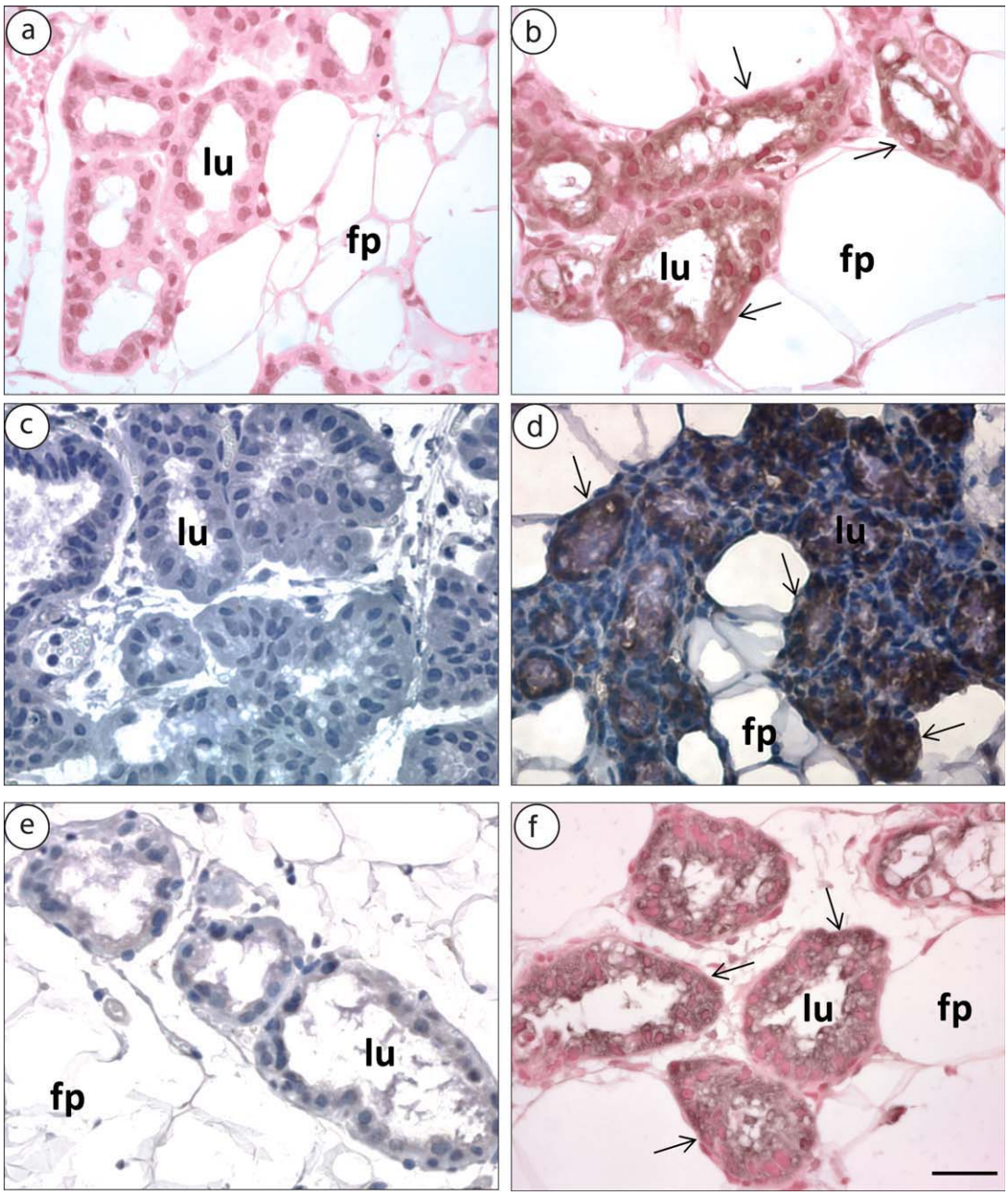

FIG. 5. Misexpression of Bax in the secretory mammary epithelium causes mitochondria-triggered caspase activation. WAP-Bax glands showed IHC staining for cytochrome C (**b**), apaF1 (**d**), and active caspase 3 (**e, f**), whereas control glands did not show cytochrome C (**a**) or apaF1 staining (**c**). (e) WAP-Bax gland revealed active caspase-3 IHC staining in the alveoli (denoted with arrow) but not in the duct (du). Counterstain was performed with nuclear fast red (a, b, f) or hematoxylin (c, d, e). Arrows point to positive cells/alveoli. Fp = fat pad; du = duct; lu = lumen. Bar (a, b, e, f) = 25 μM. Bar (c, d) = 50 μM.

DISCUSSION

Programmed cell death (PCD) is a requisite cellular mechanism for development and homeostasis. Apoptosis, Type I PCD, can proceed via extrinsic or intrinsic pathways. Extrinsic pathways rely on apoptosis initiation by receptors at the cell surface, while intrinsic pathways from internal signals such as cellular damage (Chipuk and Green, 2005). Both pathways can converge on the mitochondria to mediate the cell death program. Bax, a pro-apoptotic protein from the Bcl-2 protein family, can induce pore formation in the mitochondria that ultimately leads to mitochondria outer membrane permeabilization (MOMP). Once MOMP

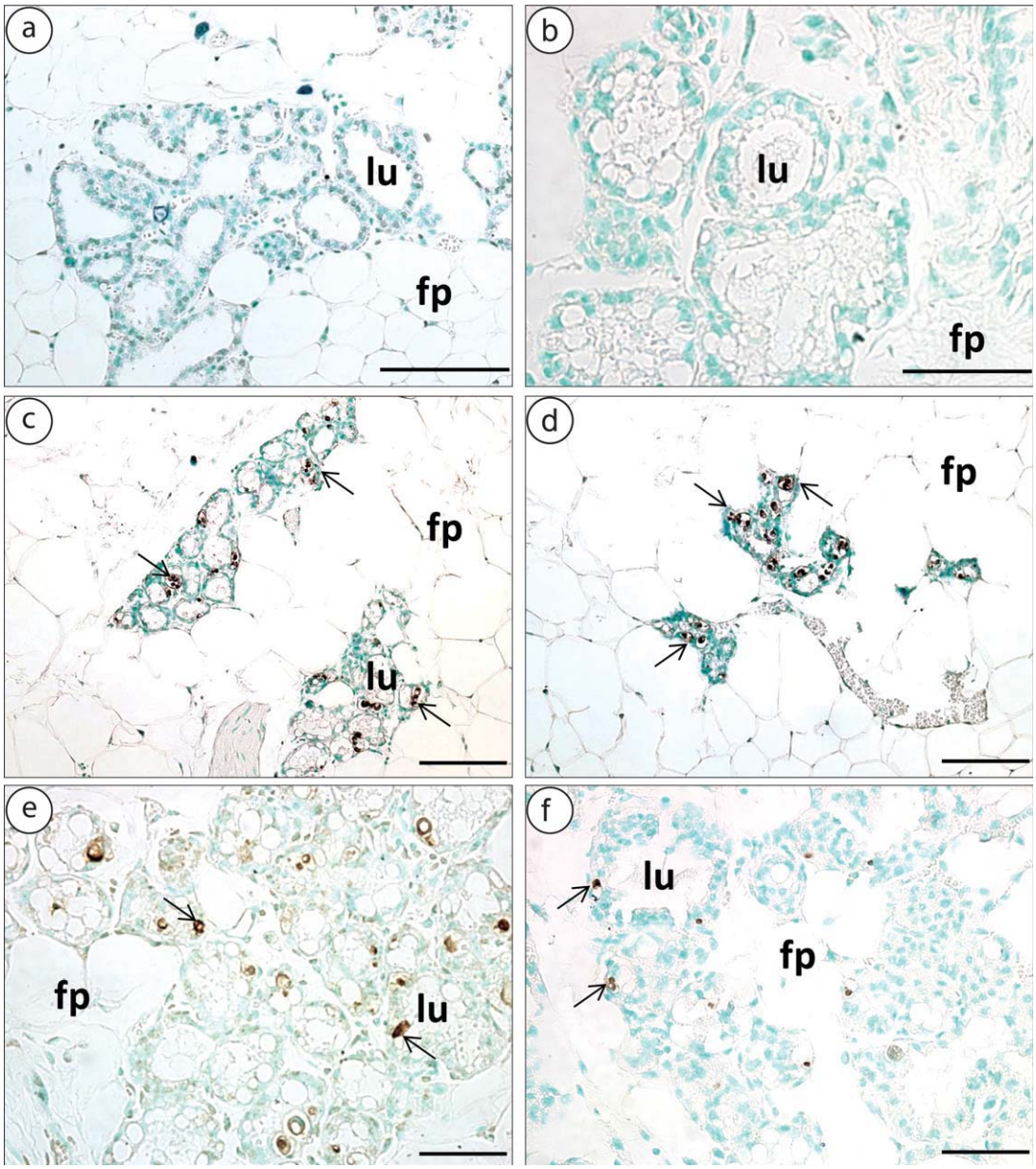

FIG. 6. Premature involution of secretory mammary epithelial cells in postpartum WAP-Bax transgenic females. WAP-Bax glands showed increased levels of apoptosis compared to control gland. (**a**) Wild-type lactation day 1 mammary gland with no TUNEL-positive cells. (**b**) WAP-Bax line IR62. (**c, d**) WAP-Bax line IR15. (**e**) WAP-Bax line IR5. (**f**) WAP-Bax line IR32. Lines IR5 and IR15 (c–e), which have lactation defects, have increased levels of TUNEL-positive cells compared to lines IR62 and IR32 (b and f). Counterstain was performed with methyl green. Arrows point to positive cells. Fp = fat pad; lu = lumen. Bar = 100 μM.

occurs, cytochrome C is released from the damaged mitochondria, thus triggering apoptosome formation, caspase activation, and DNA degradation. Alternatively, release of additional mitochondrial proteins (e.g., AIF, OMI, and EndoG) can lead to necrosis through caspase-independent events (Chipuk and Green, 2005; Portier and Taglialatela, 2006).

Other genetic studies from mouse models have revealed roles for Bax in regulating apoptosis in reproductive tissues. *Bax*-null mice have phenotypes within the testis and ovary. Males are sterile from the developmental failure of primary spermatocytes to mature into secondary spermatocytes, thus prompting a wave of apoptosis in the adolescent testis (Knudson *et al*., 1995). Females are endowed with three times the population of primordial follicles compared to wild-type females. In addition, the reproductive lifespan of $Bax^{-/-}$ females is longer due to reduced atresia (Perez *et al*., 1999). Bax is

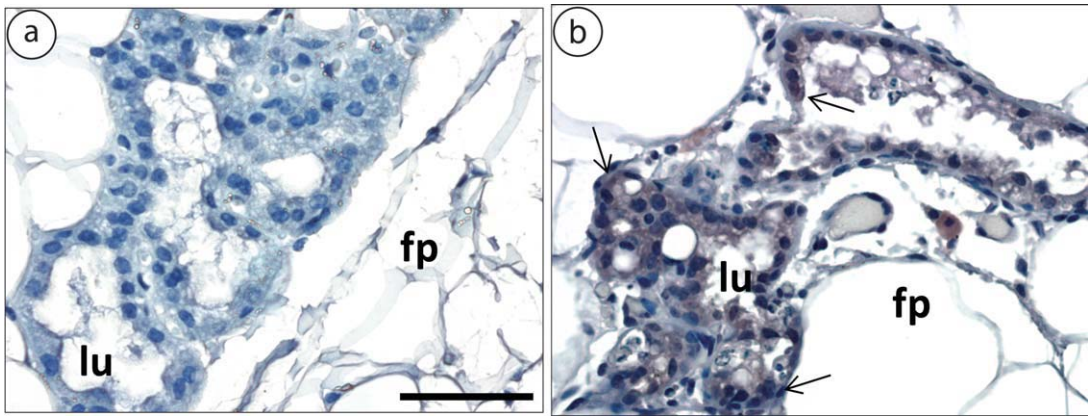

FIG. 7. Clusterin expression in the lactating mammary gland. Clusterin expression, a marker for involution, was not found in the control lactation day 1 gland (**a**) but was present in the WAP-Bax gland (**b**). Counterstain was performed with hematoxylin. Arrows point to positive cells/alveoli. Fp = fat pad; lu = lumen. Bar = 50 μM.

also involved with primordial germ cell (PGC) development in the fetus. Bax insures the apoptotic death of ectopic PGCs that fail to arrive at the indifferent gonad (Stallock *et al*., 2003). Moreover, once PGCs colonize the fetal gonads, we have shown that Bax and Bcl-X_L regulate the survival of the germ cells (Rucker *et al*., 2000). Reduction of Bcl-X_L in PGCs leads to a sterile "Sertoli cell only" phenotype in the male, and a 30-fold reduction in primordial follicle in the male. The loss of germ cell populations is restored by the concomitant loss of Bax in PGCs. Previously we have shown that the secretory epithelia of the mammary gland are also impacted by the loss of *Bax* or *Bcl-X_L*. These studies show a cell- and developmental-specific dependence on Bax-mediated apoptosis.

Our WAP-Bax transgenic model demonstrated a "forced involution" and premature cessation of lactation at parturition. The cell death we find is due to a caspase-dependent induction of apoptosis, as determined by active caspase-3 IHC and TUNEL assay. Although Stat5 and Akt1 promote cell survival in the mammary gland during pregnancy and lactation, the ectopic overexpression of Bax overcomes these signals. Bax is regulated by Akt1 phosphorylation at Ser^{184}, which serves to maintain the cytosolic localization of Bax and prevent its mitochondrial targeting (Gardai *et al*., 2004). Since Akt1, Bcl-2, and Bcl-x_L are all cleaved by caspases, initial caspase-3 activation would provide a positive feedback loop to inhibit these pro-survival proteins (Bachelder *et al*., 1999; Cheng *et al*., 1997; Clem *et al*., 1998; Widmann *et al*., 1998). It is unclear as to the mechanism of pStat3 activation, although either induction of c-Src or caspase-mediated degradation of Socs3 could lead to premature Stat3 phosphorylation. An additional cell survival mechanism that the mammary gland would utilize is autophagy. Autophagy is active in the L1 murine mammary glands (unpublished observations; EBR, ANH), which normally would serve to sequester damaged mitochondria into autophagosomes for turnover. Apparently, the two transgenic WAP-Bax lines with lactation defects have overcome the normal capacity of autophagy as a survival pathway within the epithelia. Interestingly, PUMA-dependent activation of Bax has been shown to concurrently induce autophagy of mitochondria (mitophagy) and a pro-apoptotic response (Yee *et al*., 2009). Thus, our WAP-Bax model may not only have an increase in Bax-mediated apoptosis but also Bax-dependent autophagy. Quantitation of autophagosomes in the WAP-Bax model with the transgenic GFP-LC3 autophagy reporter line would establish whether both of these processes are coordinately activated (Mizushima *et al*., 2004). Some cells could be lost from autophagic cell death, although the abundance of pyknotic nuclei and TUNEL-positive cells argue for apoptosis as the central programmed cell death mechanism.

There are two classical stages of mammary gland involution: the programmed cell death-driven reversible phase and the remodeling-driven irreversible phase. However, gene expression studies of the early phase has demarcated seven signature profiles: (1) a 12-h peak, (2) a 24-h peak, (3) a 24-h increase with persistent expression, (4) a 24-h peak with a slow decrease, (5) a 72- to 96-h peak, (6) a delayed expression for 48 h, and (7) a delayed expression with peak after 96 h (Baxter *et al*., 2007). *Bax* mRNA is found in the fourth group with a 24-h peak and slow decrease. As previously stated, this is the developmental window when Bax has an effect on involution. From our study, Bax also has a dramatic effect on the survival of the secretory epithelia around parturition. This highlights the dynamic plasticity of the mammary gland; epithelia poised with the requisite proteome to carry out programmed cell death given the proper internal signal or environmental cue. In summary, overexpression of the pro-apoptotic pro-

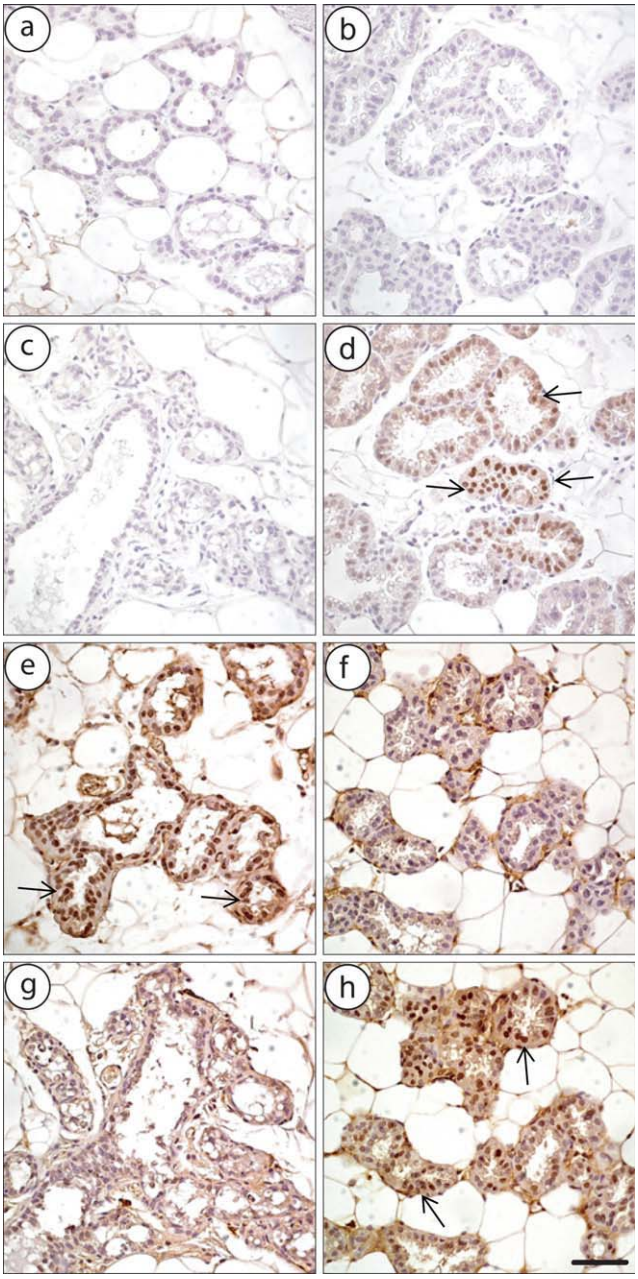

FIG. 8. pStat3 and pStat5 expression in the WAP-Bax mammary gland. pStat3 (**a**–**d**) and pStat5 (**e**–**h**) immunolocalization in wildtype (a, e), IR15 (c, g), and IR20 (d, h) glands at L1. Negative control (no primary Ab) is shown for pStat3 (b) and pStat5 (f). Arrows show nuclear localization of pStat5 and pStat3. Counterstain was performed with hematoxylin. Bar = 50 μM.

tein Bax in the mammary gland resulted in a lactation defect because of increased levels of apoptosis within the functionally differentiated mammary epithelial cell compartment. This WAP-Bax transgenic model of "forced involution" demonstrated that upregulation of a single pro-apoptotic factor of the Bcl-2 family is sufficient to initiate programmed cell death of functionally differentiated mammary epithelial cells in vivo.

METHODS

Generation of Transgenic Mice

The WAP-Bax construct was digested with *Not*I and gel purified with the Qiagen Gel Extraction Kit. The keratin 14 (K14)-agouti transgene (Kucera *et al.*, 1996) was digested with *Cla*I and also gel purified. Transgenes were co-injected at a final concentration of 2 μg ml^{-1} into pronuclear-staged FVB embryos. Pups (3 weeks old) were genotyped for the Wap-Bax transgene with the following PCR primers and reaction conditions: forward (5′-TAG AGC TGT GCC AGC CTC TTC-3′); reverse (5′-GAC ACA GTC GAC TCA GAA CAT CTT CTT CCA G-3′); cycling conditions of 94°C for 5 min (1 cycle), 94°C for 30 s, 58°C for 30 s, 72°C for 1 min (32 cycles), and a final step of 72°C for 5 min. The product of 650 base pairs was resolved on a 1.5% agarose gel. Founders were backcrossed with C57/BL6 mice for five generations. WAP-Bax transgene copy number was determined using Exon 3 and 4 specific primers paired with an Exon 3-specific TaqMan probe (5′-6FAM ATG CGT CCA AGA AGC TGA GCG TAMRA-3′ at 200 nM final) with cycling conditions of 50°C for 2 min (one cycle), 95°C for 10 min, 95°C 15 s, 60°C for 1 min (40 cycles) on an ABI Prism 7700 thermocycler. Copy number was normalized to the endogenous Bcl-X_L gene (two copies).

Isolation and Preparation of Mammary Glands

At lactation day 1 of the second pregnancy, transgenic and nontransgenic females were anesthetized with 2.5% (v/v) avertin and then euthanized. Mammary glands were surgically removed and fixed in 10% buffered formalin overnight at 4°C. Tissues were placed in 70% ethanol, paraffin embedded, sectioned onto Superfrost slides (Fisherbrand).

Staining of Mammary Gland Whole Mounts

Mammary glands were harvested and fixed overnight in 10% (v/v) neutral buffered formalin. Samples were rehydrated in 70% (v/v) ethanol for 30 min and then distilled water for 15 min. Staining was performed overnight with carmine alum followed by dehydrating the following day (35% ethanol for 30 min; 50% ethanol for 30 min; 70% ethanol for 30 min; 95% ethanol for 30 min; 100% ethanol overnight). Clearing was done with xylene overnight, and then mammary glands were compressed between slides overnight, released and expanded 6 h before cover slipping with Permount.

Immunohistochemistry

Mammary glands (lactation day 1) were fixed in 10% (v/v) neutral buffered formalin solution (Sigma) overnight, transferred to 70% ethanol, paraffin-embedded and then sectioned at 5 μm. Sections were deparaffinized through ethanol, rehydrated in water, and placed in 0.03% (v/v) hydrogen peroxide in methanol for 30 min at room temperature. Sections were rinsed in 1× PBS, treated with Target Unmasking Solution (Vector) at 100°C for 10 min, slow cooled an additional 10 min, and finally rinsed in 1× PBS. Blocking was performed with 10% serum at room temperature for 30 min followed by the addition of Bax antibody, (1:200; B.D. PharMingen 13686E), Cytochrome C (1:50, B.D. PharMingen SC7159), Caspase-3 (1:50, Santa Cruz 556425), phospho-Stat5a/6 (Tyr 694/699; 1:100, Millipore 05-495), phospho-Stat3 (Tyr 705; Cell Signaling 9145) and Active Caspase-3 (1:50, B.D. PharMingen 559565) for overnight incubation at 4°C. Upon washing in 1× TBS, peroxidase-conjugated secondary antibody (1:400) was added for 1 h at room temperature. After rinsing in 1× TBS, samples were incubated in ABC solution and treated with DAB (Vector Laboratory) according to manufacturer's protocol). Slides were counterstained with Methyl Green 0.1%, Nuclear Fast Red, or Hematoxylin QS (Vector laboratories) and mounted with Permount (Sigma).

Measurement of Apoptosis Mammary Gland Epithelial Cells

For quantitation of apoptotic cells, the TUNEL-based Apoptag assay was performed according to the manufacturer on paraffin-embedded sections (Intergen). Briefly described, mammary gland tissue sections were deparaffinized, quenched in 3% hydrogen peroxidase and incubated with terminal transferase. After applying the anti-digoxigenin conjugate, the color was developed in DAB peroxidase substrate for 6 min. Samples were counterstained with methyl green (Vector Labs).

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