Loss of protein kinase C delta alters mammary gland development and apoptosis

BL Allen-Petersen1,6, MR Miller2,6, MC Neville1,3, SM Anderson4, KI Nakayama5 and ME Reyland*,1,2

As apoptotic pathways are commonly deregulated in breast cancer, exploring how mammary gland cell death is regulated is critical for understanding human disease. We show that primary mammary epithelial cells from protein kinase C delta (PKCδ) –/− mice have a suppressed response to apoptotic agents in vitro. In the mammary gland in vivo, apoptosis is critical for ductal morphogenesis during puberty and involution following lactation. We have explored mammary gland development in the PKCδ –/− mouse during these two critical windows. Branching morphogenesis was altered in 4- to 6-week-old PKCδ –/− mice as indicated by reduced ductal branching; however, apoptosis and proliferation in the terminal end buds was unaltered. Conversely, activation of caspase-3 during involution was delayed in PKCδ –/− mice, but involution proceeded normally. The thymus also undergoes apoptosis in response to physiological signals. A dramatic suppression of caspase-3 activation was observed in the thymus of PKCδ –/− mice treated with irradiation, but not mice treated with dexamethasone, suggesting that there are both target- and tissue-dependent differences in the execution of apoptotic pathways in vivo. These findings highlight a role for PKCδ in both apoptotic and nonapoptotic processes in the mammary gland and underscore the redundancy of apoptotic pathways in vivo.

Cell Death and Disease (2010) 1, e17; doi:10.1038/cddis.2009.20; published online 21 January 2010

Subject Category: Cancer

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits distribution and reproduction in any medium, provided the original author and source are credited. This license does not permit commercial exploitation without specific permission.

Apoptosis is an active process of cell death that has a key role in the development and maintenance of tissue homeostasis. Mammary gland development occurs primarily in the postnatal period through the process of branching morphogenesis during puberty, and massive proliferation and secretory differentiation during pregnancy. During puberty, terminal end buds (TEBs) form at the leading edge of the growing ducts and bifurcate to produce the branched ducts characteristic of the mature virgin. TEBs are composed of a layer of highly proliferative cap cells on their distal surface, surrounding a mass of luminal body cells that undergo high levels of apoptosis resulting in luminal hollowing. Multiple B-cell leukemia/lymphoma 2 (Bcl-2) family members are expressed during branching morphogenesis, including antiapoptotic B-cell leukemia/lymphoma X (Bcl-x), Bcl-2 and B-cell leukemia/lymphoma w (Bcl-w), as well as the proapoptotic Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) proteins. Mice in which the Bcl-2 gene is overexpressed have abnormal ductal development, whereas mice in which the proapoptotic protein, Bcl-2-interacting mediator of death (Bim), is deleted show delayed apoptosis in the TEBs, supporting a role for apoptosis during ductal morphogenesis.

In addition to early luminal hollowing, apoptosis is also important for the clearance of epithelial cells during mammary gland involution. Following the cessation of lactation, large numbers of secretory mammary epithelial cells (MECs) are deleted by apoptosis, returning the gland to its prepregnancy state. Quantitative morphometric analysis of epithelial cell apoptosis and apoptotic cell clearance during mammary gland involution suggests that these processes occur rapidly after forced weaning and are largely complete by 72–96 h. Gene array studies show an early transient increase in the expression of death receptor ligands and their receptors starting 12 h after weaning, which increased expression of regulators of the intrinsic apoptotic pathway, including Apaf1, Bcl-x, Bak and Bax, and suppression of the death inhibitory proteins, Bcl-2 and Bcl-w, was observed at 24–96 h of involution. In mice, loss of Bax or overexpression of Bcl-2 results in suppression of alveolar cell apoptosis.

1Program in Cell Biology, Stem Cells and Development, School of Medicine, University of Colorado Denver, Aurora, CO 80045, USA; 2Department of Craniofacial Biology, School of Dental Medicine, University of Colorado Denver, Aurora, CO 80045, USA; 3Department of Craniofacial Biology, School of Dental Medicine, University of Colorado Denver, Aurora, CO 80045, USA; 4Department of Physiology and Biophysics, School of Medicine, University of Colorado Denver, Aurora, CO 80045, USA; 5Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan; 6These authors contributed equally to this work.

Keywords: apoptosis; mammary gland; PKCδ; thymus

Abbreviations: PKCδ, protein kinase C delta; Bcl-2, multiple B-cell leukemia/lymphoma 2; TEBs, terminal end buds; Bcl-x, B-cell leukemia/lymphoma X; Bcl-w, Bcl-2 and B-cell leukemia/lymphoma w; Bax, proapoptotic Bcl-2-associated X protein; iKO, PKCδ gene has been disrupted

Received 23.11.09; accepted 25.11.09; Edited by G Melino
whereas deletion of Bcl-x accelerates apoptosis during involution.12

Protein kinase Cδ (PKCδ) is an ubiquitously expressed isofrom of the PKC family of serine/threonine kinases.13 Studies have identified diverse roles for this signaling molecule in control of immunity,14,15 apoptosis,16 and cell migration.17 Furthermore, its reduced expression in some human tumors suggests that it may function as a tumor suppressor.18,19 Our laboratory and others have shown an essential role for PKCδ in epithelial cell apoptosis induced by genotoxins, other cell toxins, and death receptors.16,20-22 The central role PKCδ has in epithelial cell apoptosis suggests that PKCδ may contribute to the regulation of apoptosis in the mammary gland in vivo. In this study, we have explored this hypothesis using mice in which the PKCδ gene has been disrupted (δKO). We show that PKCδ regulates branching morphogenesis through nonapoptotic mechanisms in early mammary gland development. During mammary gland involution, however, the absence of PKCδ results in delayed apoptosis. Our studies also show that apoptosis in the thymus displays a similar differential sensitivity to apoptotic signals, suggesting a redundancy of apoptotic pathways in vivo.

Results

PKCδ expression in the mouse mammary gland. Development and remodeling of the mammary gland during puberty and involution require apoptosis, and thus this tissue is a useful model for exploring regulation of cell death in vivo. As shown in Figure 1, PKCδ protein is expressed at all stages of the mammary gland developmental cycle, with the highest levels seen during mid-pregnancy and during involution. As expected, no PKCδ expression was detected in tissues from δKO mice. In contrast to PKCδ, the expression of PKCα does not vary during the mammary developmental cycle (Figure 1). The mammary gland is composed of epithelial, adipose, and connective tissue. Expression of PKCδ in MECs was verified by immunoblot of primary MECs isolated from postpubertal δWT and δKO mice (data not shown). Differential regulation of PKCδ expression in the mammary gland suggests that it may contribute to the dynamic changes seen in this gland during pregnancy and involution.

Suppression of apoptosis in primary MECs from δKO mice. We have previously shown that the loss of PKCδ protects salivary gland cells in vivo from irradiation-induced cell death; however, the contribution of PKCδ to developmental or physiological programs of cell death is not known.22 To assess whether PKCδ is required for apoptosis in MECs, we investigated the effects of etoposide and integrin detachment on MECs isolated from δKO or δWT mice. Primary MECs were cultured in vitro and treated with etoposide. Activation of caspase-3 in MECs from δKO mice was suppressed by about 50% compared with MECs isolated from δWT mice, similar to what we have previously observed in primary salivary epithelial cells22 (Figure 2a). Loss of integrin engagement (anoikis) induces apoptosis in epithelial cells and may be an important apoptotic mechanism during mammary gland involution. To determine if anoikis is a PKCδ-dependent process, MECs were detached from the culture dish and plated on poly-hema-coated plates to prevent reattachment. Caspase-3 activation occurs within 4 h after plating; however, activation of caspase-3 was greatly diminished in δKO MECs as compared with δWT MECs (Figure 2b). Together, this suggests that PKCδ regulates multiple apoptotic pathways relevant to mammary gland development and maintenance.

Development of the mammary gland during puberty in δKO mice. Before the onset of puberty the mouse mammary gland consists of quiescent rudimentary ducts. Under the influence of pubertal hormones TEBs form at the leading edge of the growing ducts and undergo several bifurcation events to produce the ducts characteristic of the mature virgin. Body cells within the TEB undergo apoptosis resulting in a hollow duct for milk flow. To determine if PKCδ contributes to branching morphogenesis, we examined glands from δKO and δWT mice at 4, 5, 6 and 10 weeks of age (Figure 3). As seen in Figure 3A, the morphology of mammary glands from δKO and δWT mice at these developmental time points appears to be similar. Immunohistochemical analysis of proliferation and apoptosis in TEBs from 5-week-old δWT and δKO mice also showed comparable Ki-67 (proliferation) and active caspase-3 (apoptosis) staining (Figure 3B). However, examination of the ductal structure in mammary gland whole mounts revealed a ductal architecture that is more

---

**Figure 1**  Expression of PKCδ in the mammary gland. Mammary glands were harvested from δWT or δKO mice at the time points indicated and PKCδ or PKCα expression was determined by immunoblot analysis as described in Materials and Methods section. Blots were stripped and reprobed for total ERK expression (p44 and p42) as a loading control. Samples are as follows: 6 and 10 week virgin mice (6W, 10W); pregnancy day 5 and 15 (P5, P15); lactation day 2 (L2), and involution days 2, 4 and 6 (I2, I4 and I6). Representative data from three or more mice per time point are shown.
PKC\(\delta\) regulates apoptosis in MECs in vitro. MECs were prepared from \(\delta WT\) and \(\delta KO\) mice as described in Materials and Methods section. (a) Primary MECs were treated with etoposide for 16 h, harvested and caspase-3 activity assayed as described in Materials and Methods section; \(\delta WT\), light gray bars; \(\delta KO\), dark gray bars; Ut=untreated. Results are the average of triplicate measurements ± S.E.M. A representative experiment is shown (\(n = 3\)). \(^*\) \(P < 0.02\) by Student’s two-tailed test. (b) Primary \(\delta WT\) or \(\delta KO\) MECs were plated on polyHEMA-coated dishes to prevent attachment and harvested at the indicated times. Top, immunoblot for active caspase-3; bottom, blots were stripped and immunoblotted for ERK as control for protein loading and transfer. Representative immunoblots are shown (\(n = 3\)).

Figure 2  PKC\(\delta\) regulates apoptosis in MECs in vitro. MECs were prepared from \(\delta WT\) and \(\delta KO\) mice as described in Materials and Methods section. (a) Primary MECs were treated with etoposide for 16 h, harvested and caspase-3 activity assayed as described in Materials and Methods section; \(\delta WT\), light gray bars; \(\delta KO\), dark gray bars; Ut=untreated. Results are the average of triplicate measurements ± S.E.M. A representative experiment is shown (\(n = 3\)). \(^*\) \(P < 0.02\) by Student’s two-tailed test. (b) Primary \(\delta WT\) or \(\delta KO\) MECs were plated on polyHEMA-coated dishes to prevent attachment and harvested at the indicated times. Top, immunoblot for active caspase-3; bottom, blots were stripped and immunoblotted for ERK as control for protein loading and transfer. Representative immunoblots are shown (\(n = 3\)).

Mammary gland involution in \(\delta KO\) mice. To determine if PKC\(\delta\) contributes to apoptosis during mammary gland involution, we used a forced-weaning model in which pups were removed from dams 9 days after parturition. In this model, lactation is fully established before pup removal and involution occurs over approximately 2–3 weeks, after which the gland resembles its pregas stage. Early involution (days 1–4) is associated with suppression of milk protein genes and loss of up to 80% of the secretory epithelial cells.\(^8\) Latter stages are characterized by extensive tissue remodeling. Mammary gland tissue was harvested from \(\delta WT\) and \(\delta KO\) mice on days 1 through 8 after weaning (11 to 18) and apoptotic cells were identified by staining with an antibody that detects active caspase-3. At day 1 of involution (Figure 4a; I1), there was some shedding of apoptotic epithelial cells into the lumen, although this is more evident by involution day 2 (Figure 4a; I2). In mammary glands from \(\delta WT\) mice, the proportion of cells showing caspase-3 activation peaks at involution day 4 and declines thereafter. Meanwhile, mammary glands from \(\delta KO\) mice have peak levels of apoptosis at involution day 6, a 2-day delay compared with \(\delta WT\). Quantification of active caspase-3-positive cells (Figure 4b) shows that apoptosis is significantly reduced in mammary glands from \(\delta KO\) mice compared with \(\delta WT\) mammary glands at day 2 (36% decrease), day 4 (21% decrease) and day 8 (38% decrease) of involution. In contrast, at involution day 6, activation of caspase-3 in \(\delta KO\) mice is significantly increased relative to \(\delta WT\) mammary glands. These studies suggest that the kinetics of activation of caspase-3 during involution is delayed in the \(\delta KO\) mouse relative to \(\delta WT\) mice.

To determine if delayed activation of caspase-3 correlates with a delay or suppression of mammary gland involution, we examined mammary gland histology in \(\delta WT\) and \(\delta KO\) mice up to 21 days after weaning. Lactating glands from both genotypes show normal alveolar development and secretory activation (Figure 5, L9). Following pup withdrawal, milk accumulates within the glands (Figure 5, I1 and I2). Although the histology of mammary glands from \(\delta WT\) and \(\delta KO\) mice appears similar at these stages, lipid droplet accumulation in the \(\delta KO\) glands at I1 was consistently greater compared with glands from \(\delta WT\) mice (see inset). By involution day 3, most of the alveolar structures had collapsed (Figure 5, I3). Mid-to-late involution is characterized by remodeling of the gland, which is complete by about 21 days. A comparison of mammary gland histology at involution days 4, 6, 8, 14 and 21 in \(\delta WT\) and \(\delta KO\) mice shows a loss of secretory alveoli, a reduction in ductal structures, and an increased ratio of fat to epithelial cells. Histologically, mammary glands from \(\delta WT\) and \(\delta KO\) mice appear to be very similar, and glands from both genotypes are fully regressed by day 21 (Figure 5, I21). However, it was noted that a subset of mammary glands from \(\delta KO\) mice at involution days 4, 6 and 8 tended to have slightly more epithelial cells and to be more pleomorphic with regard to adipocyte size and shape compared with glands from \(\delta WT\) mice, suggesting that PKC\(\delta\) may have a role in the composition or organization of the mammary gland stroma.

Signal transducer and activator of transcription 3 (STAT3) is a critical regulator of mammary gland involution, and mice with a conditional deletion of STAT3 in the mammary gland show impairment of epithelial cell apoptosis and delayed involution.\(^{23–25}\) STAT3 activation, as determined by
phosphorylation on Y705, was analyzed to assess if the delay in caspase-3 activation during involution in δKO glands is due to aberrant STAT3 signaling. Similar levels of activated STAT3 are seen in mammary glands from δWT and δKO mice at L2 and I2, and slightly reduced in mammary gland tissue from δKO mice at I4 (Figure 6a). To further address whether STAT3 activation is altered in the mammary glands of δKO mice, we analyzed STAT3 Y705 phosphorylation in

Figure 3  The loss of PKCδ leads to defective mammary gland branching morphogenesis. Immunohistochemistry, whole mounts and analysis of branching frequency were performed as described in Materials and Methods section. (A) Representative pictures (×40) of mammary glands from 5, 6 and 10 weeks virgin δWT and δKO mice stained with H&E. (B) Sections (×400) through one δWT (a–c) and one δKO (d–f) TEB stained with H&E (a, d), or with antibodies to cleaved caspase-3 (b, e) or Ki67-positive cells (c, f). Inset, digital magnification of area boxed in blue to show cleaved caspase-3-positive cells (b, e) or Ki67-positive cells (c, f). (C) Representative whole mounts of 4th mammary glands from 4-, 5- and 6-week-old δWT and δKO mice stained with carmine alum. Arrows identify areas of decreased branching in δKO mammary glands as compared with δWT. (D) The distance between branch points was quantified from 4-week (n = 7), 5-week (n = 6) and 6-week (n = 5) old δWT (light gray bars) and δKO (dark gray bars) mice. The difference between branching frequency in δWT and δKO is significant over 4 weeks (*P < 0.02), 5 weeks (**P < 0.03), and 6 weeks (***P < 0.03) by Student’s two-tail t-test
primary MECs treated with leukocyte inhibitory factor (LIF) and interleukin-6 (IL-6), cytokines critical for activation of STAT3 in the mammary gland during involution. STAT3 activation was nearly identical in WT and KO MECs treated with IL-6 or LIF (Figure 6b). Taken together, our studies indicate that loss of PKCδ results in delayed activation of caspase-3 during involution, and that this is not due to a defect in STAT3 activation.

Thymic apoptosis in KO mice. Although loss of PKCδ results in a delay in caspase-3 activation in the mammary gland, involution proceeds normally in these mice. In contrast, irradiation-induced caspase-3 activation is reduced by >60% in the parotid glands of KO mice. This suggests that in vivo apoptotic pathways induced by agents that damage DNA are highly selective for PKCδ, whereas other inducers of apoptosis such as those that regulate development may be less selective. To further explore the selectivity of apoptotic pathways for PKCδ, we compared caspase-3 activation in the thymus in response to irradiation and dexamethasone treatment. Glucocorticoids have been shown to induce apoptosis and cause acute thymic involution in rodents, and this pathway of involution may be relevant physiologically in response to stress and inflammation. KO or WT mice were injected with dexamethasone, the thymus was harvested after 2 h, and active caspase-3 was assayed by immunohistochemistry. As seen in Figure 7a, dexamethasone is a potent inducer of apoptosis in the thymus of both KO or WT mice; however, no significant difference in caspase-3 activation was found. In contrast, in response to irradiation of the thymus, caspase-3 activation is potently induced in WT, but not KO mice (Figure 7b). This finding suggests that irradiation-induced apoptosis is highly dependent on PKCδ, whereas collateral apoptotic pathways may be recruited to regulate cell death in response to other signals, including developmental signals.
**Discussion**

PKCδ is required for apoptosis in response to cell injury, however, its contribution to apoptosis during development and tissue remodeling has not been addressed. In this study, we have explored a role for PKCδ in the apoptosis of primary MECs during branching morphogenesis and involution. In vitro, apoptosis in primary MECs derived from δKO mice is reduced, and in vivo, activation of caspase-3 during mammary gland involution is delayed in δKO mice. Loss of PKCδ also results in decreased branching during pubertal development of the mammary gland; however, this is likely due to a nonapoptotic function of PKCδ. These phenotypic changes are transient presumably reflecting the redundancy of apoptotic pathways in vivo and the value of proper mammary gland development to species survival.

Branching morphogenesis requires a delicate balance between apoptotic and proliferative signals to effectively establish ductal outgrowth and luminal hollowing. During puberty, mammary glands from δKO mice are similar in organization to δWT glands, with hollow lumens and correct TEB and ductal architecture. Proliferation and caspase-3 activation also appear to be similar in the TEBs of δKO and δWT mammary glands. This suggests that PKCδ either does not have a significant role in the maintenance of cell growth/death within the TEB or that loss of PKCδ is compensated for by other cell death mechanisms. Brugge and co-workers have shown that loss of the proapoptotic factor, Bim, suppresses apoptosis within the TEB resulting in transient luminal filling; however, the ducts eventually hollow out by a caspase-independent mechanism and resemble wild-type mammary glands. Similarly, overexpression of the antiapoptotic proteins Bcl2 or Bcl-XL suppresses apoptosis in three-dimensional cultures of MCF10A cells, but lumen formation still occurs. As mammary gland development and function is critical to species survival, there is likely to be evolutionary pressure to provide
alternative pathways to assure proper mammary gland function. In the context of ductal morphogenesis, these may include alternative regulators of apoptosis, as well as non-apoptotic cell death pathways such as autophagy.6 Whole mount stains of mammary glands from δKO mice during puberty show reduced branching during early ductal morphogenesis; however, by later stages these glands were indistinguishable from their WT counterparts. Altered branching morphogenesis has been observed in several other mouse models within the epithelial and stromal compartments. Although estrogen receptor,30 progesterone receptor,31 matrix metalloproteinase-232 and Src-133 have been shown to be essential for both branching and ductal outgrowth, other factors, such as eotaxin,34 primarily regulate branching. Notably, within the diverse variety of mouse models that exhibit a branching phenotype, there are very few cases in which branching morphogenesis is halted altogether, highlighting the complexity of this process. While changes in proliferation and apoptosis can affect TEB shape and its ability to drive ducts through the fat pad, ductal elongation was not affected in δKO mice. This confirms that alterations in branching were not due to deficiencies in proliferation or cell death within the TEB. More importantly, these findings implicate PKCδ in a nonapoptotic role during ductal morphogenesis.

Our previous studies show that in salivary epithelial cells PKCδ regulates apoptosis upstream of cytochrome c release and caspase activation in response to genotoxins and other cell damaging agents.21,22 In this study, we show that apoptosis induced by etoposide is similarly suppressed in primary MECs from δKO mice. Interestingly, activation of caspase-3 in anoikis, a process possibly relevant to physiological modes of apoptosis, is also suppressed in δKO MECs.
Our current studies show that PKCδ contributes to the activation of caspase-3 during mammary gland involution \textit{in vivo}; however, suppression of caspase-3 activation is much greater in cultured primary MECs from \textit{iKO} mice and in irradiated \textit{iKO} salivary glands \textit{in vivo}. This may be explained in part by the fact that tissue cell death peaks by 24 h postirradiation, while involution occurs over 1–4 days, possibly allowing activation of redundant cell death programs to insure successful completion. Alternatively, several studies indicate that cell death during involution occurs in a series of sequential steps, perhaps mediated by distinct cell death pathways. PKCδ predominantly facilitates intrinsic- or mitochondrial-mediated cell death, whereas microarray data suggest that death receptor or extrinsic cell death pathways regulate the initial wave of apoptosis during involution. If intrinsic pathways function as a back-up mechanism of cell death during involution, this may explain the relatively minor changes in involution seen in the \textit{iKO} mouse.

Nuclear accumulation of PKCδ is an early event in genotoxin-induced apoptosis that results in the activation of caspase-3 and amplification of the apoptotic signal. Our current studies suggest that apoptosis \textit{in vivo} is differentially dependent on PKCδ. This suggests that upstream regulators of apoptosis, such as PKCδ, may be differentially activated in a signal-specific manner. In this regard, we show that dexamethasone induces caspase-3 activation in the thymus to a similar extent in both \textit{iKO} and \textit{iWT} mice, while in response to irradiation, caspase-3 activation is induced in \textit{iWT} and not \textit{iKO} mice. This supports the role of PKCδ in DNA damaging pathways, whereas alternative apoptotic pathways may be recruited to facilitate cell death in response to other stimuli.

The loss of PKCδ results in small changes across both mammary gland pubertal growth and involution. This suggests that the role of PKCδ in the mammary gland is not restricted to apoptosis, as a reduction in apoptosis is not likely to explain the decreased ductal branching or decreased lactation we observe. Furthermore, subtle differences in lipid composition are evident in the mammary glands of \textit{iKO} mice, suggesting that PKCδ may have additional roles in mammary gland homeostasis. This suggests that mammary gland development and involution are complex biological processes that are essential for the maintenance and propagation of mammalian species, thus apoptosis is likely to be regulated at multiple levels to assure its proper execution.

Materials and Methods

Animals. The PKCδ \textit{iKO} mouse on the C57Bl/6 background has been previously described. Animals were maintained at the University of Colorado Denver at Anschutz Medical Campus in accordance with Laboratory Animal Care guidelines and protocols. These studies were conducted with approval of the University of Colorado Denver Institutional Animal Use and Care Committee. Wild-type littermates (\textit{iWT}) were used for all studies. For analysis of mammary gland involution, pups were removed from mothers at day 9 of lactation.

Mammary gland preparation. Mammary glands were harvested from \textit{iWT} or \textit{iKO} mice and processed for whole mount analysis or immunohistochemistry. For analysis of mammary gland whole-mounts, mammary glands (###) were spread on microscope slides and fixed overnight in 10% formalin. Tissues were hydrated, stained with carmine alum overnight, dehydrated and cleared in xylene for 2 h. Slides were analyzed on a dissecting scope and digital pictures were acquired using SPOT imaging software. For quantification of branching frequency, the total duct length of every primary branch, from nipple to distal TEB, was measured in microns and divided by the total number of branch points within the gland.

For histological and immunohistochemical analysis, contralateral 4 glands were fixed in 10% formalin, dehydrated, and embedded in paraffin. Five \textmu m sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry for active caspase-3 and Ki67 were performed as previously described using primary antibodies purchased from Cell Signaling Technology, Beverly, MA, USA. Digital images were acquired using SPOT imaging software.

Immunoblot analysis. Immunoblots were probed as previously described. Super Signal West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL, USA) was used for detection of the signal. Sources of antibodies were as follows: PKCα and PKCz (Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3, pY705STAT3, active caspase-3, Ki-67 and extracellular signal-regulated kinase (Cell Signaling Technology).

Primary cell culture. Mammary glands removed from \textit{iWT} or \textit{iKO} mice (8–14 weeks) were minced and digested in Dulbecco's modified Eagle medium/F12 (DMEM/F12) containing 2 mg/ml collagenase-B, 100 U/ml Hyaluronidase, 100 U/ml penicillin-streptomycin (pen/strep), and Gentamicin at 37°C for 3 h. Primary MECs were isolated by a series of spins at 1500 and 800 r.p.m. and then plated on collagen I (Sigma-Aldrich, St. Louis, MO, USA)-coated plates (2.5 x 10^5/cm²) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1 mg/ml fetuin (Sigma-Aldrich), 2.5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2.5 μg/ml epidermal growth factor (EGF), 25 μg/ml gentamicin and 50 U/ml Pen/Strep. After 48 h, cells were transferred to growth media (DMEM/F12 supplemented with 2.55 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2.5 μg/ml EGF, 25 μg/ml gentamicin, 50 U/ml pen/strep and 10% FBS). For induction of anokia, cells were trypsinized and plated on dishes coated with PolyHEMA (Sigma-Aldrich). Before treatment with ILF or IL-6, MECs were plated on Matrigel (BD Biosciences, Bedford, MA, USA) for 24 h in DMEM/F12 supplemented with 10% fetal calf serum, 1 mg/ml fetuin (Sigma-Aldrich), 2.5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2.5 μg/ml EGF, 25 μg/ml gentamicin and 50 U/ml pen/strep, followed by incubation in serum-free growth media for an additional 24 h. ILF was purchased from Calbiochem (San Diego, CA, USA) and IL-6 was purchased from Chemicon (Billerica, MA, USA). DMEM/F12 and FBS were purchased from HyClone (Logan, UT, USA); all other reagents for tissue culture were from Invitrogen (Carlsbad, CA, USA) unless noted otherwise.

Caspase-3 activity. Caspase-3 activity was quantified using a Biomol Quantzyme Colorimetric Assay Kit (BioMol, Plymouth Meeting, PA, USA) as previously described. Briefly, caspase-3 activity in 30 μg of cell lysate was measured by cleavage of Ac-DEVD-pNA colormetric substrate, and absorbance at 405 nm was quantified in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) at 10 min intervals for 7 h.

Apoptosis in the thymus. For dexamethasone-induced involution, 6-week-old \textit{iWT} or \textit{iKO} male mice (n = 5) were intraperitoneally injected with 0.2 mg dexamethasone (Sigma-Aldrich) dissolved in 100 μl endotoxin-free phosphate-buffered saline (PBS) or 100 μl endotoxin-free PBS alone. Two hours after injection, mice were killed and the thymus glands harvested. For irradiation-induced involution, 6-week-old \textit{iWT} or \textit{iKO} male mice (n = 4) were subjected to 2 Gy irradiation using a cobalt source. Twenty hours after irradiation, mice were killed and the thymus glands harvested and processed for histology and immunohistochemistry as described.

Miscellaneous. Etoposide was purchased from Sigma-Aldrich and dissolved in dimethylsulfoxide.

Conflict of interest

Drs. Anderson, Neville and Reyland's work has been funded by the NIH. Dr. Nakayama, Ma, Allen-Peterson and Ms. Miller declare no potential conflict of interest.

Acknowledgements. We appreciate the technical assistance of Dr. Harriet Watkin, Rachelle Kalkofen, Valerie Burns, and Andrew Lewis, and the intellectual
contributions of Drs. Pepper Schedin, Peter Henson and James McManaman. These studies were supported by grant PO1-HD38129.

1. Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia 2000; 5: 227–241.

2. Silbergstein GB. Postnatal mammary gland morphogenesis. Microsc Res Tech 2001; 52: 155–162.

3. Humphreys RC. Programmed cell death in the terminal endbud. J Mammary Gland Biol Neoplasia 1999; 4: 213–220.

4. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res 2006; 8: 201.

5. Metcalfe AO, Gilmore A, Klionska T, Oliver J, Valente JJ, Brown R et al. Developmental regulation of Bcl-2 family protein expression in the involuting mammary gland. J Cell Sci 1999; 112 (Part 11): 1771–1783.

6. Malilleau AA, Overholtzer M, Schmelzei T, Bouillet P, Strasser A, Bynge JS. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. Dev Cell 2007; 12: 221–234.

7. Stein T, Salomonis N, Gusterson BA. Mammary gland involution as a multi-step process. J Mammary Gland Biol Neoplasia 2007; 12: 25–35.

8. Monks J, Smith-Steinhart C, Zorn E, Fadok VA, Henson PM. Epithelial cells remove survival by BAFF-dependent PKCdelta-mediated nuclear signalling. J Cell Biol 2004; 166: R92–R109.

9. Schorr K, Li M, Bar-Peled U, Lewis A, Heredia A, Lewis B et al. Protein kinase C delta and mammary gland development reveals putative roles for death receptors and immune cofactors, and macromolecular interactions. J Mammary Gland Biol Neoplasia 2001; 6: 115–127.

10. Ahmed SA, Srinagananathan N. Differential effects of dexamethasone on the thymus and spleen: alterations in programmed cell death, lymphocyte subsets and activation of T cells. Immunopharmacology 1994; 28: 55–66.

11. Watson CJ. Stat transcription factors in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia 2001; 6: 115–127.

12. Watson CJ. Stat transcription factors in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia 2001; 6: 115–127.

13. Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, activation and subcellular interactions. J Biol Chem 2001; 276: 2353–2364.

14. Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, activation and subcellular interactions. J Biol Chem 2001; 276: 2353–2364.

15. Stein T, Salomonis N, Gusterson BA. Mammary gland involution as a multi-step process. J Mammary Gland Biol Neoplasia 2007; 12: 25–35.

16. Stein T, Salomonis N, Gusterson BA. Mammary gland involution as a multi-step process. J Mammary Gland Biol Neoplasia 2007; 12: 25–35.

17. Stein T, Salomonis N, Gusterson BA. Mammary gland involution as a multi-step process. J Mammary Gland Biol Neoplasia 2007; 12: 25–35.

18. D’Costa AM, Robinson JK, Maududi T, Chaturvedi V, Nickoloff BJ, Bradford AP. Analysis of protein kinase C delta (PKC delta) expression in endometrial tumors. Hum Pathol 2008; 39: 21–29.

19. D’Costa AM, Robinson JK, Maududi T, Chaturvedi V, Nickoloff BJ, Bradford AP. Analysis of protein kinase C delta (PKC delta) expression in endometrial tumors. Hum Pathol 2008; 39: 21–29.