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Review

Apoptosis regulation in the mammary gland

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Abstract. Epithelial apoptosis has a key role in the development and function of the mammary gland. It is involved with the formation of ducts during puberty and is required to remove excess epithelial cells after lactation so that the gland can be prepared for future pregnancies. Deregulated apoptosis contributes to malignant progression in the genesis of breast cancer. Since epithelial cell apoptosis in the lactating mammary gland can be synchronised by forced weaning, it has been possible to undertake biochemical analysis of the pathways involved. Together with the targeted overexpression or deletion of candidate genes, these approaches have provided a unique insight into the complex mechanisms of apoptosis regulation in vivo. This review explores what is currently known about the triggers for apoptosis in the normal mammary gland, and how they link with the intrinsic apoptotic machinery.

Key words. Mammary gland; breast; apoptosis; involution; development; epithelia.

Introduction

Apoptosis is a key feature of mammary gland development and function. It has an important role in embryonic and ductal mammary development, and in epithelial homeostasis during oestrous/menstrual cycles. Moreover, apoptosis is critical for removing the milk-secreting alveolar epithelial cells at weaning so that the architecture of the tissue can be remodelled prior to future reproductive periods. The molecular mechanisms regulating some of these apoptotic events are not well understood; however, a considerable amount of information has been uncovered in the last few years about post-lactational involution. Normally involution takes place in a gradual fashion as pups slowly become weaned [1]. However, in experimental animals such as mice, forced weaning can be used as a tool to accelerate and synchronise the involution process, thereby allowing biochemical analysis. Moreover, apoptosis phenotypes resulting from specific gene deletions have been identified when mothers are unable to nurse their pups. This review will focus on how these systems have uncovered mechanisms that regulate apoptosis in normal mammary development.

Female mice have 10 mammary glands containing a mesodermally derived stromal, adipocyte-rich compartment and an ectodermally derived epithelial compartment. In the adult mouse, the epithelial component of the gland forms a network of ducts through the fat pad and provides the basic structure from which the secretory epithelium is derived in pregnancy when the mammary epithelial cells proliferate and differentiate to form polarised cells surrounding hollow lumens. During lactation these cells secrete milk into the alveolar lumen, and the expulsion of milk into the ducts is aided by contractile myoepithelia that form a basketlike mesh around each alveolus. This whole structure is surrounded by a specialized extracellular matrix (ECM), known as the basement membrane (BM).

Regulated apoptosis occurs at several stages of mammary development. In the embryo, emergence of epithelial

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buds from the ectoderm into mammary mesenchyme is the starting point for development of the ductal tree. This process occurs in both sexes, but the formation of testes and the ensuing production of testosterone in males result in its inhibition [2]. In mice this is due to apoptosis of the epithelial buds after they have emerged from the ectoderm. As the mammary ducts of pubertal female mice mature, they hollow out in a process that depends on extensive apoptosis occurring at the growing tip of a duct within the structure known as the terminal endbud [3] (fig. 1a). It is not entirely clear why apoptosis occurs at the endbud. One possibility is that cells die in order to create a lumen within the duct that is subsequently used to allow passage of milk to the nipples. However, the high level of apical mucin (e.g. muc 1) production by ductal epithelial cells, together with their basal-apical polarity and fluid transport functions, would be quite sufficient to create a luminal space. So it is possible that apoptosis occurs to remove excess cells that accumulate at the highly proliferative endbud. The mechanism of this apoptosis is not known, though it is independent of p53 and is reduced when Bcl-2 is overexpressed [3]; it may occur through insufficient survival signals because the apoptotic cells do not contact the BM. Apoptosis not only occurs in ductal morphogenesis, but also takes place in mature females, where there are cycles of proliferation and apoptosis within ductal cells during the oestrus/menstrual cycles, in a process regulated by oestrogen and progesterone [4–6].

When suckling ceases after lactation, the alveolar component of the gland involutes through a process involving both apoptosis and tissue remodelling, which rebuilds the gland to a virginlike state. Under conditions of forced weaning this process is separated into two phases, an initial apoptotic phase that begins within 12 h and lasts ~72 h (fig. 1b) and a second phase involving further apoptosis, matrix degradation and gland remodelling [7, 8]. The first phase of involution (but not apoptosis) is reversible in that lactation can be reestablished if pups are returned within the initial 2 days. During the first phase of involution, milk accumulates locally within alveolar lumens, and the levels of systemic lactogenic hormones fall. This phase is purely apoptotic, and was initially characterised by the induction of sulphated glycoprotein-2 (SGP-2) and caspase 1 [7, 9]. Degradation of nuclear DNA into fragments that form ladders on agarose gels, apoptotic morphology of epithelial cells and activation of effector caspases, which are all characteristic markers of apoptosis, are observed within 2 days of removing pups [6, 7, 9–11]. This apoptotic phase of mammary gland involution is the best characterised, and our review will focus on the mechanisms that regulate it.

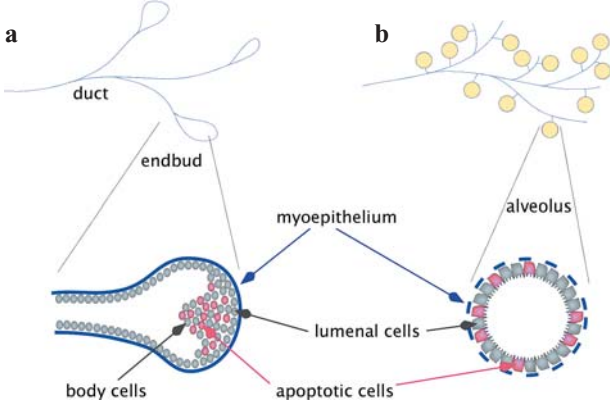

Figure 1. Major apoptotic events in mammary gland physiology. (*a*) During ductal morphogenesis, apoptosis occurs within the endbud. This proliferative structure drives forward the extension of ducts throughout puberty. Excess cells within the body cells of the endbud are removed by apoptosis. The myopepithelal cell layer is shown as a thick blue line. Luminal cells are grey, while those undergoing apoptosis are red. (*b*) Following lactation, epithelial cells within the lactating alveoli undergo apoptosis. Alveoli appear at the end of tertiary branches in lactation (yellow circles). Apoptotis occurs within the luminal cell population, beginning within 24 h of pup removal in the mouse.

As involution progresses, the second phase commences, where gland remodelling takes place through the action of matrix metalloproteinases (MMPs). The old connective tissue and BM are removed, allowing the ductal component to be reformed [12]. Apoptosis also continues through this phase, possibly through anoikis (a term for detachment-induced apoptosis) as cell-ECM interactions become perturbed. During early mammary gland involution, high levels of tissue inhibitors of metalloproteinases (TIMPs) are expressed, and these prevent excess MMP activity. The metalloproteases, gelatinase A (MMP-2) and stromelysins 1 and 3 (MMP-3 and -11) and a serine protease, urokinase-type plasminogen activator (uPA), become expressed during the second phase of involution [9, 13, 14]. As involution progresses, TIMP levels diminish, leading to increased activity of MMPs, which cause extensive remodelling of the ECM and stromal components of the tissue [13, 15].

Genetic confirmation of the involvement of TIMPs at involution comes from studies in mice lacking TIMPs. Overexpression of TIMP1 in mice expressing an autoactivating MMP-3 (stromelysin-1) transgene inhibits the unscheduled apoptosis promoted by the active MMP-3 during late pregnancy [16, 17]. Both accelerated apoptosis and involution kinetics promoted by lack of TIMP-3 (in otherwise normal mice) are restored to normal after intramammary gland implantation of pellets containing recombinant TIMP3 [18]. In contrast, mice lacking plasminogen undergo a considerably compromised involution [19].

Regulating this critical balance between MMPs and TIMPs therefore provides a mechanism to coordinate the transition between the first, largely apoptotic stage of involution and the second, which is more concerned with removal of matrix proteins and remaining cell debris,

adipocyte differentiation and resculpting the epithelial ductal tree. It is not fully known how these enzymes are involved with cellular remodelling, but one possibility is that proteolytically released fragments of ECM proteins trigger cell migration and morphogenesis. During involution, an epidermal growth factor (EGF)-like fragment is released from the BM component, laminin-5, by MMP-2 and the membrane-bound metalloproteinase, MT1-MMP, and this stimulates cell migration [20].

It has recently emerged that the expression of a wide variety of genes is altered at the onset of mammary gland involution and that different sets of genes are switched on and off during the involution programme [21–23]. Some of these include genes encoding anti-inflammatory proteins to protect the tissue from adverse effects of neutrophil invasion [24]. Macrophages also infiltrate the early involuting mammary gland, partly to remove the apoptotic debris, and some genes reflect the expression of proteins required for monocyte recruitment and for the final phagocytic stage in the apoptosis programme. The functional analysis of these genes will eventually help to dissect which proteins are required for apoptosis, immune responses and phagocytic clearing, or for rebuilding the tissue.

The majority of gland remodelling is complete within 6–10 days in the mouse, and the histology of the tissue becomes not dissimilar to that of the nonpregnant animal. One question to be resolved is where the post-lactational ductal structures come from. It is not known whether ductal cells, as opposed to alveolar cells, are refractory to apoptosis or whether stem cells are triggered to repopulate the ductal sheaths. Presumably there must be an alveolar stem cell population that also remains after involution, in order to develop new alveoli in subsequent pregnancies. This type of kinetic analysis is very difficult to follow using conventional histology, which only records 'snapshots' of development. However, emerging technologies of green fluorescent protein (GFP)-labelling cells in vivo (e. g. with mammary-specific promoters) together with multi-photon confocal microscopy on tissue in live animals, may allow such questions to be addressed in the near future. A further important question that has not been answered is how the adipose cells differentiate and repopulate the stroma. This phenomenon is partly dependent on stromelysin 1/MMP-3 and plasminogen, but the factors triggering adipose differentiation and dramatic lipid synthesis are completely unknown [25, 26]. It is also not known where the adipose cells go during late pregnancy, lactation and early involution when the epithelial component occupies the majority of the gland.

Triggers for apoptosis in vivo

Mammary-derived signals are responsible for initiating the first phase of involution since artificial addition of lactogenic hormones to mammary epithelial cells does not affect apoptosis, although it does prevent remodelling of the gland [8]. Instead, milk accumulation is likely to play a major role: sealing a single teat during lactation induces an accumulation of milk and involution in that gland, whilst the rest continue to lactate [27].

Several possible mechanisms act as the primary events in triggering the apoptosis programme and involution (fig. 2). One is that component(s) in the milk are pro-apoptotic factors but are normally removed by nursing. α-Lactalbumin, for example, stimulates apoptosis under certain conditions [28, 29]. Another is that stretch receptors become activated as the alveolar lumen expands because of continued milk production in the absence of suckling, though thus far this not been examined experimentally. For example, adhesion receptors linking the basal epithelial cell surface to the BM may become stretched [30]. Alternatively, cell-cell adhesion junctions, such as tight or adherens junctions, may become stretched or broken, leading to pro-apoptotic signals [31]. Tight junction formation is dependent on glucocorticoids, and the implantation of glucocorticoid pellets in

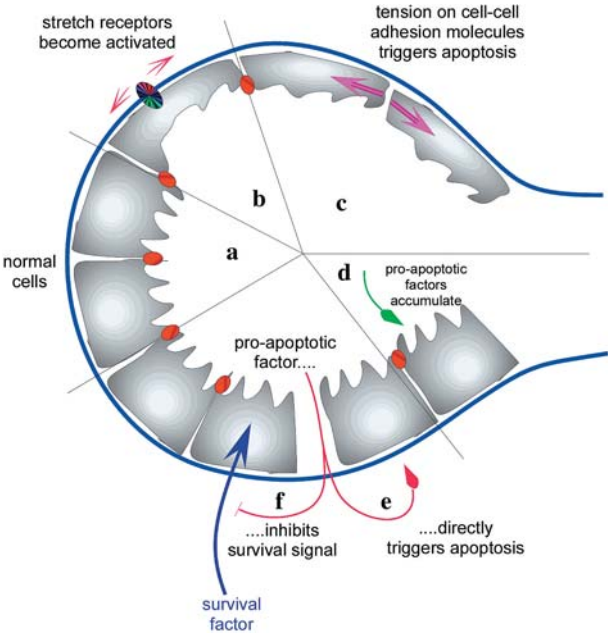

Figure 2. Possible apoptotic triggers at the onset of involution. (*a*) Normal cells are columnar, tight junctions (red ovals) seal their apical interfaces, and they interact with basally located myoepithelial cells (not shown) as well as the BM (blue line). (*b*, *c*) If milk is not removed the alveoli expand leading to luminal cell stretching. This may activate stretch receptors (*b*) or cause sufficient tension on cell-cell adhesion molecules (*c*) to induce apoptosis. (*d*) Pro-apoptotic factors that are normally excreted may accumulate and trigger apoptosis through apically located receptors (green arrow). (*e*, *f*) An unresolved mechanism may deregulate tight junctions, leading to the relocation of pro-apoptotic factors to the basal cell surface, which could either trigger apoptosis directly (*e*) or inhibit survival factors (*f*).

lactating mouse mammary glands prevents involution [32, 33]. Thus, an alternative mechanism is that falling hormone levels may lead to fragility of tight junctions and the consequent movement of factors, which are normally exposed only to the apical surface of alveolar cells, to the basal compartment where they can induce apoptosis directly or antagonise survival signals. Although the initial trigger for the first phase of involution remains undefined, both cell-autonomous and non-cell-autonomous signals are involved subsequently, and these are discussed below (table 1).

The IGF-1-PKB axis

Insulin-like growth factors (IGFs) are well established as survival ligands in several cell types and have a significant role in the mammary gland. Inhibition of the IGF signalling axis contributes to apoptosis, while extra IGFs suppress it both in vivo and in culture models. Transgenic mouse studies have been performed to determine the effect of IGFs during involution. When IGF-I with reduced affinity for IGF binding proteins (IGFBPs), des(1-3)hIGF-I, is overexpressed specifically in the mammary glands of transgenic mice under the control of the mam-

Table 1a. Stimulation of apoptosis signalling in mammary epithelium.

Extracellular triggers	
EphB4	Nikolova et al., 1998; Munarini et al., 2002
Fas L	Song et al., 2000
IGFBP5	Tonner et al., 1995, 1997 and 2002; Marshman et al., 2003
IL-6	Zhao et al., 2002
LIF	Kritikou et al., 2003; Schere-Levy et al., 2003
Milk stasis	Martin et al., 1997
PTEN	Dupont et al., 2002; Li et al., 2002
sFRP4	Wolf et al., 1997; Lacher et al., 2003
TGFβ3	Nguyen et al., 2000
TRAIL	Sohn et al., 2000
α-Lactalbumin	Hakansson et al., 1995 and 1999
Downstream factors	
AP1	Marti et al., 1994
ATF4	Bagheri-Yarmand et al., 2003
Bad	Faraldo et al., 2001; Green et al., 2004
Bax	Heermeier et al., 1996; Gilmore et al., 2000; Wang et al., 2003; Valentijn et al., 2003
Bim	Reginato et al., 2003; Wang and Streuli, unpublished
Caspase 3	Marti et al., 2000; Prince et al., 2002
Caspase 10	Green et al., 2004
Cytochrome c	Marti et al., 2001
C/ebpδ	Gigliotti et al., 1998, 2003
ESX	Neve et al., 1998
HoxA5	Raman et al., 2000
NF1C	Furlong et al., 1996; Kane et al., 2002
PKA	Marti et al., 1994
p53	Jerry et al., 1998
Smad3	Yang et al., 2002
Stat3	Chapman et al., 1999; Humphreys et al., 2002

Table 1b. Inactivation of apoptosis pathways in mammary epithelium.

Extracellular signals	
E-cadherin	Boussadia et al., 2002
EGF	Gilmore et al., 2002
Glucocorticoid	Feng et al., 1995
IGF-I	LeRoith et al., 1995; Hadsell et al., 1996; Neuenschwander et al., 1996; Farrelly et al., 1999
IGF-II	Moorehead et al., 2001
Integrins	Faraldo et al., 1998, 2001; Farrelly et al., 1999; Prince et al., 2002; Weaver et al., 2002
Laminin-rich BM	Boudreau et al., 1995; Pullan et al., 1996
Downstream factors	
Bcl-x	Walton et al., 2001
Bcl-w	Metcalfe et al., 1999
α-catenin	Nemade et al., 2004
β-catenin	Tepera et al., 2003
IRF-1	Chapman et al., 2000
NFκB	Brantley et al., 2000; Clarkson et al., 2000
PKB/Akt	Schwertfeger et al., 2001; Ackler et al., 2002
Stat5	Chapman et al., 1999; Iavnilovitch et al., 2002

mary-specific rat whey acidic protein (WAP) promoter, incomplete involution results [34]. Histological comparisons of the mammary glands from wild-type and transgenic mice at involution demonstrate marked differences, with the glands from the transgenic mice showing a lack of remodelling. In addition, mice expressing rat IGF-I under the control of the WAP promoter have reduced numbers of apoptotic cells compared with wild-type mice [35, 36]. IGF-II also has a role in the regulation of apoptosis since involution is delayed in mice expressing IGF-II under the control of the mouse mammary tumour virus (MMTV) promoter [37].

One means by which IGFs exert their survival stimulus is through phosphatidylinositol-3′-kinase (PI3K) and one of its effectors, protein kinase B (PKB, or Akt) (fig. 3a). Indeed, mammary glands from IGF-II transgenic mice have increased levels of PKB phosphorylation [37]. Involvement of the PKB pathway in vivo has been confirmed through overexpression studies. Ectopic expression of PKB under the control of the MMTV promoter delays involution [38, 39]. PTEN, a negative regulator of PI3K signalling, enhances apoptosis in the mouse mammary gland, whilst conditional deletion of PTEN delays involution [40, 41]. Although all these studies with transgenic mice are suggestive that the IGF-PKB axis may contribute to survival regulation in vivo, it will be important to confirm this genetically through the use of gene deletion. This has not been done yet, and is likely to require the generation of mice with conditional null alleles that can be targeted specifically to the mammary gland.

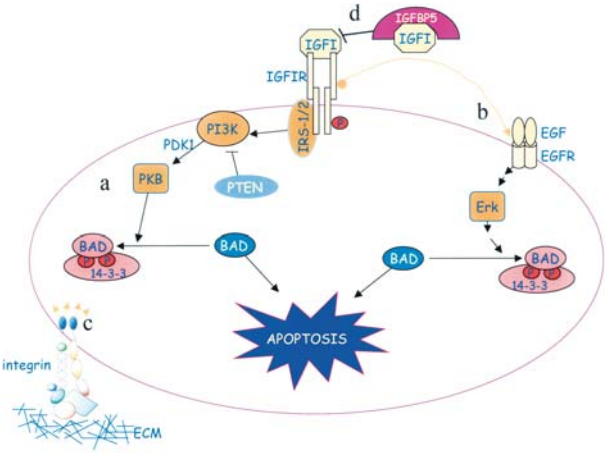

Figure 3. The IGF-PKB axis. (*a*) IGF interacts with its receptor to trigger a kinase signalling pathway, resulting in activation of PKB and Bad phosphorylation. If this pathway is blocked, Bad is dephosphorylated and apoptosis ensues. (*b*) IGF signals can also result in EGF receptor trans-activation, which also leads to Bad phosphorylation. (*c*) IGF signalling is only efficiently activated in mammary epithelial cells when they are cultured on an appropriate type of ECM, the basement membrane. Integrins organise the cytoskeleton, but also crosstalk with signalling pathways, including IRS. (*d*) IGF-mediated survival can be inhibited by IGFBP-5, which prevents receptor activation and the downstream anti-apoptotic signals.

Further evidence for a survival role of IGFs in the mammary gland comes from culture experiments. Purified primary cultures of mammary epithelial cells die in the absence of serum, but this can be prevented by IGFs [42]. The PKB pathway is activated by IGFs in mammary cells, and inhibiting the pathway at the level of PI3K prevents IGF-mediated survival. IGF also signals through Erk, although interestingly in mammary cells this occurs through transactivation of the EGF receptor [43] (fig. 3b). An EGFR antagonist, ZD1839, partially inhibits IGF-mediated survival, indicating that the Erk arm of the IGF pathway is functionally relevant. The downstream IGF effectors that feed into the apoptosis machinery have not been identified yet, but may include Bad and forkhead transcription factors [43–45].

An important finding from mammary cell culture experiments is that the IGF/insulin signalling axis is regulated by the cellular context within the tissue and depends on the ECM environment [46]. During lactation, mammary epithelial cells in vivo contact the BM, which separates cells from the stromal ECM containing collagen I. IGF-I-mediated signalling and cell survival is efficient when primary cells are plated on to BM. However, when the cells are cultured on collagen I, IGF signalling is driven only weakly and apoptosis occurs, leading to a gradual decrease in cell number over several days so that the population dies out [47]. This is due to inefficient signal transduction from the type I IGF receptor to IRS and downstream PKB signals [42]. Although the Ras-MAPK pathway is active when cells are grown on both collagen and BM, it is not sufficient to promote prolonged survival [46]. Integrins are heterodimeric α-β receptors that link ECM proteins to the cytoskeleton, providing an architectural basis for cell structure, and also link to the intracellular signalling machinery, thus directly influencing cell phenotype [48, 49]. Integrins coordinate the signals transduced by a number of growth factor receptors, and since different integrins are utilised in the adhesion of mammary cells to BM proteins and collagen I, it is likely that this provides the molecular basis for altered IGF signalling on BM and collagen I. Together there is a complex network of signals between integrins, IGF receptors and EGF receptor that modulates the IGF signalling response and determines the long-term survival outcome of mammary cells (fig. 3c). This co-ordination of signalling between IGF-I/insulin receptors and integrins is not restricted to mammary cells and occurs in other cell types [50, 51].

Since IGF proteins stimulate survival in the mammary gland, inhibition of IGF signalling is likely to represent a pro-apoptotic mechanism. Currently there are no data to indicate that the levels of IGF-I and -II are altered at the onset of involution; however, there is accumulating evidence for an important role provided by IGFBP-5 (fig. 3d). At the onset of involution, IGFBP-5 mRNA and pro-

tein levels increase dramatically within 24 h [52, 53]. This effect is stimulated by milk stasis and inhibited by prolactin. Addition of IGFBP-5 to primary mammary epithelial cell cultures directly inhibits IGF-I signalling and promotes apoptosis [44]. This suggests that IGFBP-5 produced at involution serves to sequester IGF away from the IGF-I receptor and prevent IGF-mediated survival signalling. Indeed, overexpression of IGFBP-5 in transgenic mice under the control of the β-lactoglobulin promoter, a mammary specific promoter, results in premature apoptosis characterised by increased DNA ladders and expression of caspase 3 during lactation [54].

The currently available data indicate that IGFBP-5 is a physiologically important regulator of IGF-mediated survival, and it may therefore have a major role in regulating apoptosis and involution in vivo. However, it will be important to confirm this hypothesis by examining the kinetics of apoptosis and involution in mice lacking IGFBP-5 in the mammary gland.

The LIF-Stat3 axis

The activity of the transcription factor Stat5 is vital for transcription of milk protein genes during lactation and is active during pregnancy and lactation, but downregulated during involution [55]. However, another member of the Stat family, Stat3, is activated at this time. Both these transcription factors have a critical role in controlling the progression of apoptosis since involution is delayed in the mammary glands of mice with either a conditional knockout of Stat3 or overexpressed mammary-specific Stat5 [56–58]. Thus, Stat3 and Stat5 are reciprocally activated in the mammary gland; Stat5 promotes survival and milk secretion, while Stat3 stimulates apoptosis.

Leukaemia inhibitory factor (LIF) has now been identified genetically as a physiological activator of Stat3 in the mammary gland [59, 60]. Mice lacking LIF exhibit an absence of phosphorylated Stat3 following weaning accompanied by delayed involution, and moreover, the implantation of LIF-secreting pellets into lactating mammary glands of wild-type mice increases apoptosis. Thus, a second pathway regulating apoptosis in vivo is that mediated by the LIF-Stat3 axis. The mechanism by which Stat3 drives apoptosis is currently obscure. Stat3 is a transcription factor, and it seems likely that it regulates the expression of genes directly involved in apoptosis such as components in the intrinsic or extrinsic pathways of apoptosis or in caspase activation [61]. Stat3 may also induce transcription of IGFBP-5 [56] and C/ebpδ (see below). The LIF-Stat3 axis may have a dual role during involution by activating the expression of genes that protect against inflammation [21, 22].

Interleukin (IL)-6 is also postulated to be a Stat3 activation candidate, since injection of this cytokine into mammary tissue strongly activates Stat3, and the levels of IL-6 messenger RNA (mRNA) increase dramatically after weaning induced either directly or by sealing the mammary teats. However, although genetic ablation of IL-6 results in delayed involution, this has no effect on the activation of Stat3 that occurs rapidly after weaning. Thus IL-6 appears to be another physiological activator of involution, but its mechanism for inducing apoptosis has not been established. One possibility is that it does so by targeting the mitogen-activated protein kinase (MAPK) pathway [62].

Both LIF and IL-6 therefore represent paracrine regulators of apoptosis in the mammary gland. How their expression is activated is currently unknown, but it is possible that they are induced by cell stretching while the alveolar lumens expand after nursing ceases [59].

The TGFβ3 axis

The mRNA and protein levels of transforming growth factor β3 (TGFβ3) are rapidly increased at the onset of involution, suggesting an involvement with apoptosis regulation [63]. Directed expression of TGFβ3 in lactating epithelial cells stimulates apoptosis, whereas mammary cells transplanted from TGFβ3 null mice into wild-type glands exhibit decreased cell death upon milk stasis. These studies provide genetic evidence that TGFβ3 is also involved in the promotion of apoptosis during the first phase of mammary gland involution. It is well known that TGFβ1 and other members of the superfamily (e.g. bone morphogenic proteins) regulate apoptosis, but neither these nor TGFβ2 are implicated in mammary gland apoptosis. This tissue therefore appears to have employed a specific member of the family to control involution. The signalling mediators of TGFβ are Smads, and although a variety of these transcription factors (Smad 1–5) are expressed in the mammary gland, it is not fully understood which one mediates the apoptosis response to TGFβ3, whether or not they do so by interaction with transcription factors such as Runx, or indeed how this signal might feed into the apoptosis machinery [64, 65]. Smad3 is highly expressed in mammary epithelium, and Smad3 absence results in a 30% reduction in the number of apoptotic cells during involution. So it may be that Smad3 is the key transcription factor contributing to TGFβ3-mediated apoptosis [64].

The death receptor axis

Death ligands and their receptors may also regulate involution. Fas ligand (FasL) is present in the mammary gland during pregnancy and lactation but not in virgin mammary tissue [66]. By contrast, FasL's receptor, Fas, is present in the mammary glands of virgin mice but not during pregnancy. Since the interaction of FasL with its receptor leads to a signalling cascade that feeds directly into the

apoptosis programme by activating caspases [67], the temporal disconnection between Fas and its ligand in virgin and pregnant/lactating mice suggests that they are unable to activate apoptosis at these times. However, on involution day 1 the levels of both Fas and FasL increase rapidly, thereby promoting apoptosis. Mice lacking either of these proteins exhibit an absence of mammary apoptosis at this time point [66]. Thus, by synchronising expression of ligand and receptor in vivo, apoptosis is induced. Further members of the family, including TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) and TWEAK (TNF relatedness and weak ability to induce cell death) are upregulated during mammary gland involution, suggesting the involvement of several death receptor ligands [21, 68].

These results indicate that death receptor signalling contributes to the promotion of apoptosis during mammary involution, although, as with the factors described above, they are not exclusive mediators because their absence delays involution rather than preventing it altogether.

Integrin adhesion receptors

Mammary epithelial cells are dependent upon a laminin-rich BM for survival, but die if they are cultured on fibronectin, plastic or collagen I, even in the presence of serum, EGF and IGF [47, 69]. Survival on BM matrix is mediated through β1-integrin since function-blocking anti-β1 integrin antibodies enhance mammary epithelial cell apoptosis [47, 69]. In mice expressing a dominant-negative form of β1-integrin in their mammary glands, apoptosis is increased, providing in vivo evidence that integrins have an essential survival function [70]. Integrins are heterodimers, and both α6β1 and α6β4 integrins are specific receptors for laminin. Function-blocking antibodies to the α6 integrin subunit induce apoptosis of primary mammary cultures [42], and β4-integrin has also been shown to promote mammary cell survival [71]. Both of these heterodimers therefore provide survival signals from the BM, though they may exhibit functional redundancy [72]. The mechanism of integrin requirement is not fully understood, but could involve direct links with integrin-signalling proteins such as focal adhesion kinase (see below) and indirect effects on downstream pathways. For example, MAPK and PKB are not activated properly in the mammary gland of mice expressing dominant-negative β1-integrin [73].

Thus, interactions between the laminin-rich BM and integrins constitute an essential survival axis in the mammary gland. Furthermore, there is an intriguing possibility that conformational changes within integrin subunits contribute to the repertoire of signals that activate mammary apoptosis in vivo because at the onset of involution, β1-integrin at the basal surface of alveolar epithelial cells switches to a non-ligand binding conformation [11].

Cell-cell adhesion receptors

There are several mechanisms by which cells communicate directly with one another, and in the mammary gland this includes both ligand-receptor pairs such as the Eph-Ephrin signalling system, as well as structural complexes including adherens junctions and desmosomes. Both of these have clear roles for mammary epithelial cell survival, although it is currently not known whether their activities are causally linked to the developmental transition between lactation and involution.

Ephs and ephrins are classically involved in development of the nervous system, but they are also expressed in both epithelial compartments of the mammary gland. For example, the EphB4 receptor (a receptor tyrosine kinase) is predominantly in the myoepithelial cells of the mammary gland, whereas its ligand, ephrin-B2, is limited to luminal epithelial cells [74]. The expression pattern of EphB4 suggests that it regulates alveolar development since it is downregulated in pregnancy, absent through lactation and restored during involution. At the onset of tumourigenesis, however, expression of EphB4 is increased. Interestingly, transgenic mice that express ectopic EphB4 in the luminal epithelium during lactation exhibit early apoptosis [75]. Thus, altered signalling through the Eph-ephrin system might contribute to the onset of apoptosis at involution.

Direct signalling through the cadherin system of cell-cell adhesion could also be involved in mammary apoptosis. Cadherins are transmembrane proteins that homodimerise across the intercellular space and also form lateral associations, thereby building up multimolecular aggregates called adherens junctions within the plasma membrane to link cells together. Adherens junctions associate with the actin cytoskeleton via adaptors such such as α-catenin, and contain the transcription factor β-catenin. The mammary glands of mice undergo untimely apoptosis if these structures are inappropriately perturbed. For example, dramatic apoptosis occurs after conditional inactivation of either E-cadherin or α-catenin in the mammary gland, indicating that this cell adhesion system plays a vital cell survival role [31, 76]. However, whether such alterations actually occur during normal involution has not been determined. As the alveoli expand in the absence of suckling, the luminal cells become stretched such that there is a considerably increased tension between adjacent cells. This may be detected by cadherins themselves, or alternatively through cadherin-binding proteins and their interaction with the actin cytoskeleton.

Adherens junctions also contain the transcription factor β-catenin. The levels of this protein are normally kept in check via ubiquitination, but β-catenin can be stabilised and activated by signals from the Wnt pathway. If the activity of this pathway is altered in transgenic animals, either through the expression of the β-eng dominant-nega-

tive form of β-catenin, or by interfering with signalling via a Wnt decoy receptor, premature apoptosis ensues [77, 78]. This decoy receptor, sFRP4, inhibits Wnt binding to its receptor and is normally upregulated at involution, suggesting that it may be a physiological regulator of mammary apoptosis in vivo [79]. Thus, a complex interplay between cell-cell adhesion and Wnt signalling may influence cellular homeostasis during pregnancy and lactation, but its perturbation at involution could have dramatic consequences and thereby lead to apoptosis.

Multiple signals for involution

Together these studies indicate that a number of signalling axes are altered during the induction of apoptosis at involution (fig. 4). The current genetic evidence suggests that inhibition of a single pathway does not completely prevent apoptosis from occurring. Rather, this merely delays the event for a few days. The implication is that apoptosis regulation in vivo is multifactorial and depends on the critical juxtaposition of several signalling pathways. It may be that all of these are required to carry through the full programme of epithelial apoptosis so that the gland returns to its pre-pregnant state. Whether or not this is also the case for apoptosis regulation in other tissues has not been established, though it would seem likely that it is. There are some important consequences for this in terms of the therapy of diseases where too little apoptosis occurs. This is the case for cancer, and the far-reaching implication is that therapies designed to kill cancer cells by inducing apoptosis may not work effectively unless multiple survival systems are targeted.

Downstream effectors in the apoptosis machine

In order for apoptosis to be induced, signals from the initiating events (i.e. those discussed above) need to connect to the apoptosis machinery (reviewed in [61]). Cells are normally executed following the activation of intracellular proteases known as caspases, though some caspase-independent mechanisms are now being uncovered [80, 81]. The link between initiating event and caspase activation occurs through one of two essentially independent

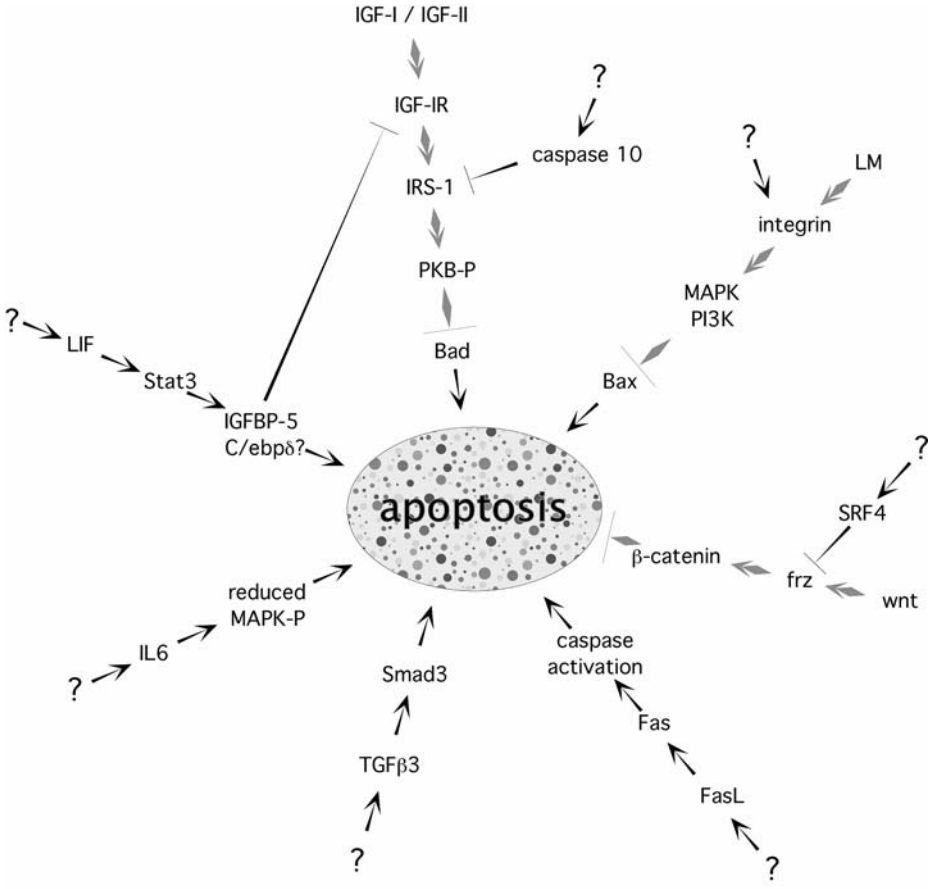

Figure 4. Apoptotic pathways during involution. Several pathways are activated and required for apoptosis during involution, but the mechanism for triggering them is not known (question marks). Some are pro-apoptotic pathways (black arrows), while others inhibit survival pathways that would otherwise be operational during lactation (grey arrows).

mechanisms, the intrinsic and extrinsic pathways, although these exhibit limited crosstalk.

The mitochondria provide control for apoptosis in the intrinsic pathway, indirectly coupling signals to caspases (fig. 5). This organelle is traditionally known to provide cells with energy to keep them alive, but it also makes execution decisions. It does so by regulating the subcellular distribution of cytochrome c and several other proteins, e.g. Smac/Diablo, that activate first the apoptosome (containing caspase 9) and subsequently the effector caspases (caspase 3, 6 and 7), which cleave intracellular proteins [81, 82]. Apoptosome activators are sequestered within the mitochondrial intramembrane space, where cytochrome c normally functions, but they can be released following the formation of transmembrane channels within the mitochondrial outer membrane by the members of the Bcl-2 class of low molecular proteins, Bax and Bak [83]. In turn, the ability of Bax and Bak to homo-oligomerize and form membrane pores depends on interactions with (i) a related set of anti-apoptotic proteins, Bcl-2, Bcl-x and Bcl-w, that bind them and prevent their function, and (ii) the pro-apoptotic Bcl-2-homology domain-3 (BH3)-containing proteins (e.g. Bad, Bim, Bid) that bind Bcl-2, -x and -w and thereby displace Bax and Bak, leading to pore formation. The anti-apoptotic proteins are normally constitutively expressed (but not always; see below), while Bax can be regulated through transcription in response to lack of survival factors or by sequestration in the cytosol and away from the mitochondria via kinase signalling pathways. Similarly, the proapoptotic function of BH3-containing proteins is regulated by both transcriptional activation and signalling events which determine their levels, localization and phosphorylation status, and thereby interaction with Bcl-2, and so on. Cells detect damage insults and homeostatic changes within their tissues, such as loss of neighbours or growth factors, via these mechanisms, and thereby commit to apoptosis.

The extrinsic pathway for apoptosis provides a more direct coupling between signal and caspase [83]. Here, death ligands activate their receptors by inducing trimerization. This leads to the formation of a plasma membrane complex, which is able to activate a specific subclass of initiator caspase (caspase 8 and 10) directly, and in turn activates downstream effector caspases thereby causing cellular execution.

Caspases

Several caspases are expressed in the mammary gland, and they become activated early in involution [84]. So far,

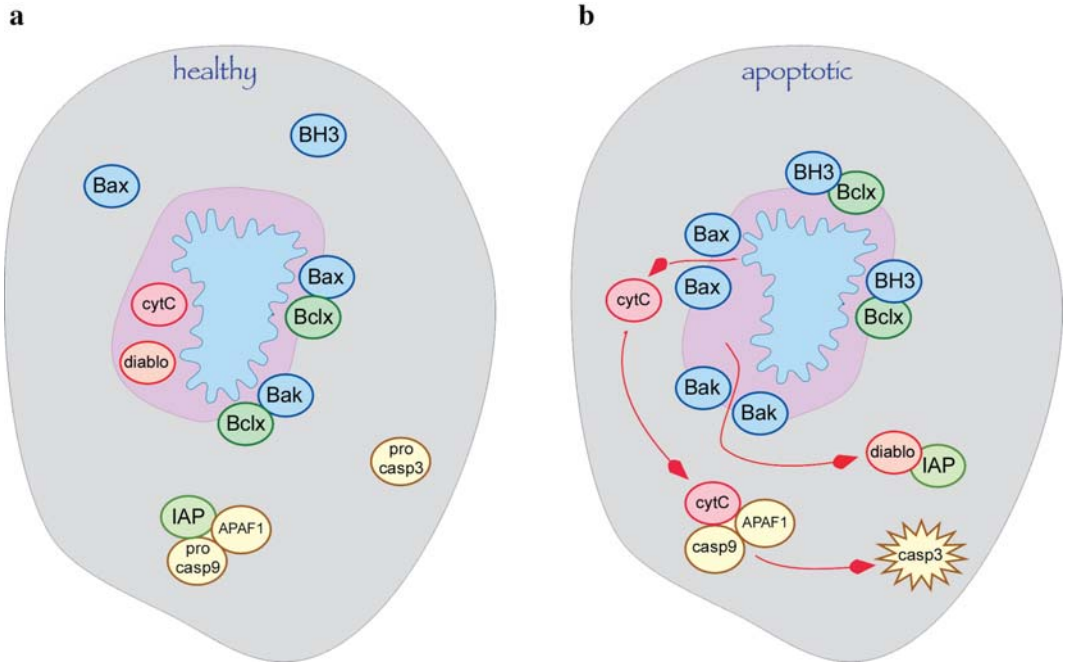

Figure 5. Intrinsic apoptosis mechanisms. (*a*) In healthy cells, caspases are inactive; IAPs help to prevent their spontaneous activation. Proapoptotic Bax and Bak are either in the wrong place to cause mitochondrial pore formation (Bax) or are inactivated through Bcl-x binding (Bax and Bak). A variety of mechanisms inhibit the action of BH3-only proteins, e.g. phosphorylation or degradation through ubiquitination. (*b*) An apoptotic signal triggers the activity or levels of BH3-only proteins (e.g. via dephosphorylation or increased transcription), which bind Bcl-2 or Bcl-x to inhibit their anti-apoptotic function. Other signals may cause Bax translocation to the mitochondrial outer membrane and conformational changes resulting in their activation. Bax or Bak then form pores that allow transfer into the cytosol of cytochrome c, which activates the apoptosome, and diablo/omi, which neutralises the caspase inhibitory effects of IAPs. Together this results in activation of apoptosis effectors such as caspase 3.

caspases 1, 3, 7, 8, 9 and 10 have been detected, and it is possible that other members of the family are present as well. The primary role for caspase 1 is the conversion of IL-1 to an active form, and any effects of this enzyme on mammary phenotype are likely to result from this rather than direct modulation of apoptosis. Caspase 8 activation fits with the observation that the Fas ligand-Fas axis is activated during involution, although this caspase can also be activated during apoptosis by caspase 3 [85]. In addition, cytochrome c is released from mitochondria on day 1 of involution [86]. Cytochrome c is required to activate the apoptosome, which contains caspase 9, indicating that the intrinsic pathway is also involved during involution. The downstream effector, caspase 3, is activated robustly in mammary involution, and cells containing the activated form of this enzyme can be detected histologically within the layer of alveolar cells prior to apoptosis and subsequent shedding into the lumen within 24 h of removing pups [11]. Caspase 3 is likely to be a major vehicle of mammary cell destruction, and interestingly, it is frequently lost or inactivated in breast cancer [87].

Caspase 10 has also been identified in mammary gland and is triggered early in involution. The mechanism of caspase 10 activation is not fully clear but appears to involve a MAPK pathway, rather than death receptors. Caspase 10 directly cleaves the adaptor proteins for IGF signal transduction, insulin receptor substrates I and II (IRS-I and II), and so a consequence of its activation is that IGF survival signals are attenuated [121]. Interestingly, caspase 10 protein levels are frequently reduced in breast cancer [88], suggesting a possible mechanism for cells to remain sensitive to IGF survival signals when they might otherwise have been triggered into apoptosis.

The involvement of caspase 8 as well as cytochrome c release indicate that mammary apoptosis requires both the extrinsic and intrinsic pathways, again supporting the hypothesis that multiple triggers lead to apoptosis induction. However, proof of this has yet to be tested with knockout models. Loss of individual caspases leads to neonatal or perinatal lethality [89], and so far mammary-specific inactivation of individual caspases using the *Cre-loxP* system has not been performed. An additional set of proteins that warrant investigation are the apoptosome regulators, including both IAPs (inhibitor of apoptosis proteins) and Smac/Diablo [90], although this has not yet been attempted.

Bcl-2 family

The Bcl-2 family of proteins control the intrinsic pathway for apoptosis, and their expression and phosphorylation are modulated during lactation and involution. There are several interesting aspects about their regulation in mammary apoptosis, though in many cases the link between the initiating event and Bcl-2 regulation has not been identified.

First, Bcl-x is an essential pro-survival member of the family that is normally expressed in mammary epithelial cells. Mice with a conditional deletion of the Bcl-x gene exhibit accelerated apoptosis during involution [91]. Bcl-2 is present in ductal epithelium but not in alveolar epithelial cells [6, 47]. Interestingly, Bcl-2 that is artificially expressed on a WAP promoter does result in involution delay, but this does not necessarily mean that it is involved in apoptosis regulation at weaning in wild-type animals [92]. Instead, Bcl-w appears to have a role here, as it is present in luminal cells during lactation but its levels are abruptly downregulated at the beginning of involution. Since Bcl-w promotes survival of mammary epithelial cells in culture, it is likely that the Bcl-w switch-off is a defining step for apoptosis induction in vivo [6]. The control is through modulation of RNA levels, but the pathway regulating Bcl-w transcription remains to be determined.

Second, both Bax and Bak are expressed in mammary epithelial cells and may be critical for involution. Little is known about Bak, other than that its levels increase during lactation and involution compared with pregnancy, possibly to prime epithelial cells for apoptosis [6]. The role of Bax, on the other hand, is more clearly understood, since it has an essential role for controlling survival mediated by cell-matrix adhesion, i.e. anoikis. The mRNA levels of Bax are increased during involution, though this does not translate into a significant increase in protein expression [6, 93]. Instead, the apoptosis function of Bax is regulated through its subcellular localization, and adhesion-driven signals keep it in the cytosol, away from a potentially dangerous location within mitochondria. In cultured mammary epithelial cells, integrin signalling, through pp125 focal adhesion kinase in adhesion complexes, is essential for cytosolic Bax distribution [94]. Loss of adhesion leads to rapid and synchronous translocation of Bax to mitochondria [95]. Mitochondrial Bax then provides the signal for apoptosis, which is suppressed in mammary cells isolated from Bax-null mice [96]. Currently it is not known to what extent either of these proteins drives involution in vivo. Bax and Bak may be redundant for each other, as studies in several cell types from mice lacking both of these regulators demonstrate that at least one of them is essential for apoptosis to proceed [97]. This has not yet been confirmed in mammary gland, but would be facilitated by the generation of mice bearing conditional-null alleles for both these genes.

The BH3-only proteins also have a role in controlling mammary gland apoptosis. Several of these proteins are present, including Bad, Bim, Bid and Bfk, and it is quite likely that others are expressed in the mammary gland [6, 95, 98, 99]. Bad is the best characterised thus far. This

protein is regulated by phosphorylation on serine residues, whence it becomes bound and sequestered by the chaperone 14-3-3. It is likely to contribute to mammary apoptosis, as its phosphorylation is inhibited at the start of involution in vivo [73, 121]. In cultured primary mammary epithelial cells the phosphorylation and apoptotic potential of Bad are controlled by both EGF and IGF signalling [43]. Moreover, mammary cells isolated from Bad-null mice are resistant to apoptosis after EGF withdrawal [100]. Bim is potently apoptotic when it is not phosphorylated. Growth factors and cytokine pathways cause its phosphorylation through the MAPK pathway, thereby suppressing its activity, while its expression can be driven after survival factors are removed in some cells types via Foxo transcription factors [101]. Bim is present in mammary epithelial cells, but its role at involution has not yet been investigated. One suggestion is that Bim mediates anoikis [99]. However, culture studies with primary mammary cultures indicate that its levels and phosphorylation are more likely to be regulated by growth factor receptors than integrins [P. Wang and C. H. Streuli, unpublished].

Since several of the BH3-only proteins are controlled directly through phosphorylation, they represent immediate apoptosis targets of kinase signalling pathways. Future studies with conditional-null alleles for BH3-only proteins will tell us which ones are really important for regulating the apoptosis machine during mammary involution. However, we argue in this article that many signals contribute to apoptosis regulation in vivo, and it is quite likely that each feeds into a separate BH3 protein.

Signalling pathways

The links between inducer and apoptosis executioner have not been fully explored yet for mammary apoptosis. We have already mentioned a role for the PI3K-PKB pathway, which may link IGF survival signals to the pro-apoptotic protein, Bad, and the extrinsic apoptosis pathway triggered by death ligands. The LIF-Stat3, TGFβ3-Smad and wnt-catenin axes are also involved, though there is no information on their downstream links to caspases (fig. 4). In addition, several other signalling pathways that feed into specific transcription factors have also been implicated in involution, although the signals that regulate them and the mechanisms by which they influence apoptosis are not well understood (table 2).

p53 was one of the first signalling proteins shown to be upregulated at the onset of involution [9]. Its functional role in mammary apotosis was initially doubted, as the glands of p53-null mice showed no change in involution kinetics [102]. However, this was subsequently suggested to be due to the allele being present in a mixed genetic background, and indeed when transferred to a uniform background, p53 deletion delays involution [103]. p53 is a transcription factor which induces expression of the cyclin-dependent kinase inhibitor, p21/WAF1, thereby inhibiting cell cycle. p21 mRNA is strongly upregulated in early mammary involution, and this is both coincident with the increase in p53 levels and dependent on the presence of the p53 gene [103]. The mechanistic link between p53 and apoptosis is through transcriptional induction of two potently pro-apoptotic BH3-only proteins, Noxa and Puma, but it is not yet known whether either of these are expressed in mammary involution in either wild-type or p53-null mice [104, 105]. p53 can also activate Bax in a transcription-independent mechanism [106]. p53 is normally associated with a DNA damage response, but can be triggered following stress. Although inactivation of p53 plays an important role in apoptosis avoidance during the progression of breast cancer, it may also be involved with apoptosis regulation in normal mammary development if cells are subject to stresses. This is an area that may warrant further investigation in relation to mammary involution.

Table 2. Transcription factors in mammary apoptosis.

	Regulatory pathway	Transcription factor
Pro-apoptotic	PKA	AP1
	?	ATF4
	Stat3	C/ebpδ
	?	ESX
	?	HoxA5
	?	NF1C
	?	p53
	TGF-β3	Smad3
	LIF	Stat3
Anti-apoptotic	Wnt	β-catenin
	glucocorticoid	glucocorticoid receptor
	?	IRF-1
	?	NFκB
	prolactin	Stat5

Several transcription factors are implicated in mammary apoptosis, however the apoptosis genes they regulate are largely unknown. Many of the signalling pathways that activate these pro- and anti-apoptotic transcription factors also remain to be fully explored.

In addition to p53, Stat3 and Smad3 as discussed above, several other transcription factors might be involved with the induction of apoptosis. The CCAAT/enhancer-binding proteins (C/ebp) comprise a family of six transcription factors, all of which have important roles in mammary gland biology [107]. The steady-state mRNA levels of two of these isoforms, C/ebpβ and C/ebpδ, increase very significantly at the beginning of involution, within 12 h of pup removal for C/ebpδ [108]. Moreover, C/ebpδ mRNA levels are not elevated after weaning in Stat3 or LIF-null mice, suggesting that this protein may act downstream in the pro-apoptotic LIF-Stat3 axis [59]. However, it is currently not clear whether either are actually involved with mammary apoptosis since, first, the C/ebpβ-null mice are sterile and it has so far not been possible to perform involution studies and, second, the kinetics of involution and the extent of apoptosis are identical in C/ebpδ-null and corresponding wild-type mice [109].

Protein kinase A (PKA) and AP-1 are also likely to play a role. PKA activity is increased rapidly following pup removal and is accompanied by increased c-fos, c-jun, junB and junD mRNA levels [110]. AP-1 is a dimer composed of Fos and Jun, and increased DNA binding activity of AP-1 is also characteristic of involution [33]. If mammary glands are resuckled after 24 h, the DNA binding activity of AP-1 returns to basal levels. Some of the genes whose expression is upregulated during involution contain a TRE element in their promoters, including stromelysin-1, c-jun and SGP-2 [33]. Thus, AP-1 activity during the first phase of involution promotes the transcription of stromelysin, which is involved subsequently in the second phase of involution. However, whether or not it contributes directly to apoptosis is not clear. Interestingly, some AP-1 components can form heterodimers with activating transcription factor-4 (ATF4), whose levels increase after weaning. Targeted expression of ATF4 to the mammary gland leads to premature apoptosis and more rapid involution, possibly through increased activation of Stat3 and subsequent transcription of IGFBP-5 [111].

An epithelial-specific Ets transcription factor, ESX (epithelial-restricted with serine box), is highly expressed during involution and may therefore be involved with the transcription of pro-apoptosis genes [112]. Similarly, one of the isoforms of nuclear factor1, NF1C, is developmentally regulated in the mammary gland, and its levels increase at involution [113]. Interestingly this isoform undergoes N-glycosylation; however, the biological role of this modification is currently unknown [114]. Overexpression of HoxA5 can induce apoptosis in breast cancer lines via p53-dependent and -independent mechanisms, but it is not clear whether this or other homeobox transcription factors are involved with regulating apoptosis during involution [115].

Some transcription factors have the opposite effects, as they suppress apoptosis. They therefore have equally important roles in the transition between lactation and involution, and some are mentioned. Stat5 is one such example, which is normally associated with milk protein gene expression, but whose levels decline after weaning. NFκB, a transcription factor that stimulates cyclin D1 activity and proliferation in pregnancy, is activated during involution [116, 117]. However, this is absent from dying cells and is only observed in cells that are not undergoing apoptosis, possibly to protect them. Moreover, in KIM-2 cells, a conditionally immortal epithelial cell line derived from mid-pregnant mammary glands [118], inhibition of the inhibitor of NFκB (IκB) results in prolonged survival due to NFκB activity. NFκB has frequently been associated as a regulator of inflammatory responses, but it also promotes survival in specific epithelial subpopulations of the mammary gland. RankL, acting through the Rank receptor, activates NFκB in the proliferative response during pregnancy, but it is not clear whether this ligand also has a pro-survival capacity [119]. Finally, the interferon-regulatory transcription factor, IRF-1 may also be involved in suppressing apoptosis because its loss through the use of genetic deletion accelerates involution kinetics in vivo [120].

Thus, a wide variety of signalling pathways leading to transcription factor regulation have a role in mammary apoptosis (table 2). Although some of these have only been identified in cell culture models, or an alteration in their levels merely correlates with involution, it remains unclear whether they have a functional role in mammary tissue. But in many cases their genetic ablation, either globally or conditionally restricted to mammary secretory epithelium, leads to altered kinetics of apoptosis in vivo, indicating that these factors really are involved at the developmental level. Currently, the signalling pathways that regulate them have not been fully explored, and their transcriptional links to apoptosis regulators have not been defined. Much work therefore needs to be done to place these pathways within a biological framework linking signals to the apoptosis machinery.

Summary

The mammary gland is uniquely privileged as an experimental system to investigate the physiological mechanisms regulating apoptosis in vivo. This is because apoptosis can be synchronised by pup removal to enable biochemical and genetic analysis, and because phenotypes are often identified in novel knockout models when litters are unable to nurse. Through the extensive experimentation analysed above, it now emerges that the regulation of apoptosis during mammary gland involution is multi-factorial. A general trend in the field has been to identify

apoptosis control mechanisms from the angle of the signalling pathway involved, and much analysis is revealing novel molecular mechanisms. Few studies, however, have approached the question of apoptosis regulation from the cell's point of view, but when one considers the mammary cell in its tissue context, the inescapable conclusion is that the control of apoptosis in normal tissue homeostasis is managed through a variety of factors that need to be carefully orchestrated: more than one 'button' needs to be pressed in order for apoptotic destruction to proceed efficiently. Thus we would argue that apoptosis in vivo is not regulated through a single signalling axis, but rather through the cooperation of multiple pathways. It will be important to determine whether this paradigm is unique to the specific developmental situation of mammary involution, or whether it represents a general concept of apoptosis regulation in vivo.

Many pathways and transcription factors have been discussed above (fig. 4, tables 1 and 2), but some recent DNA microarray studies have identified a significant number of genes whose levels are altered following pup removal, suggesting the involvement of further apoptosis-regulatory pathways [21, 22]. One concern with this type of approach in a multi-cellular tissue is that it does not allow linkage between the genes whose expression is altered and cell type, and they may therefore be involved in other processes such as adipocyte differentiation or invasion of immune cells to clear the apoptotic debris. Moreover, many apoptosis regulatory pathways are transcriptionally independent and involve kinases and GTPases together with movement of proteins from one intracellular compartment to another. Nevertheless, these studies provide a fascinating insight into clusters of genes whose expression is altered at defined stages during the involution process, and they give valuable clues about which genes to target in future genetic analyses.

There are several burning questions that arise from this new knowledge of mammary apoptosis mechanisms: First, it is still not known with certainty what the initial apoptosis trigger is at the onset of involution. A few possibilities have been discussed here, but it is not clear whether others exist or whether one or more tiggers are necessary. Second, it is important to determine how the apoptosis signalling axes in mammary gland involution are orchestrated, and to what extent they all link to each other. There are a large number of gaps in the apoptosis pathway diagram illustrated in figure 4, as well as the up- and downstream involvement of transcription factors shown in table 2. Filling these gaps is likely to be achieved only through extensive genetic analysis. Third, the connection between these pathways and the intrinsic apoptosis machinery is not known. It will, for example, be important to determine which members of the Bcl-2 family are involved. Fourth, although multiple pathways have been identified that contribute to apoptosis during involution, it has not yet been determined whether any or all of these mechanisms are involved with apoptosis during ductal development and in oestrus/menstrual cycles.

Finally, it is likely that a clear understanding of those factors influencing apoptosis in the mammary gland will provide us with valuable information for the design of future anticancer drugs. However, it has not been fully determined how the pathways identified for normal mammary epithelial apoptosis become subverted during the progression to breast cancer, and much work needs to be done to define all the potential therapeutic targets. The model we have presented indicates that the level of complexity for apoptosis triggering in normal cells is greater than one. Cancer cells probably hijack more than one apoptosis pathway in order to remain alive, and it will therefore be vital to design future therapeutic strategies that target two or more pathways simultaneously in order to destroy breast cancer cells effectively.

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