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Chapter 1

Apoptosis and Clearance of the Secretory Mammary Epithelium

Jamie C. Stanford and Rebecca S. Cook

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1. Introduction

The development of the mammary gland occurs in four distinct phases: embryogenesis, puberty, pregnancy, and a post-lactational phase involving profound levels of cell death and tissue remodeling. This post-lactational phase is termed post-lactational involution. During embryogenesis, a solid epithelial bud is generated in the embryonic ectoderm. As this bud continues to grow in cell number, the epithelial bud invaginates into the underlying mesenchyme forming the nascent mammary epithelium. The mammary epithelium grows as solid epithelial cords, lengthening distally and branching to form the rudimentary epithelial network. At puberty, ductal elongation continues in a proximal-to-distal direction, and side branches appear along the ducts. The side branches also lengthen distally, and continue to branch. This pattern of distal growth and branching fills the mouse mammary fat pad with an extensively branched epithelium by the end of puberty [6]. Similar to what is seen during embryonic mammary development and patterning, the mammary ducts developing during puberty originally appear in solid epithelial cords. Apoptosis canalizes the luminal space within the ducts, allowing a patent conduit for milk to traverse through the breast epithelium [1, 7]. Ultimately, the rodent mammary epithelium is comprised of a continuous, branching network leading from the nipple to primary ducts and smaller ductules that terminate in terminal end buds (TEBs), blunt ends or alveoli. The inner luminal cells are separated from the basement membrane by an outer myoepithelial layer. Myoepithelial cells secrete basement membrane components to which the epithelium attaches, and that physically separates the epithelium from the stromal compartment.

Many morphological similarities exist between the mouse mammary gland and the human breast, although some distinctions exist. In the human breast, the cluster of epithelial acini arising from a single terminal duct, referred to as the terminal duct lobular unit (TDLU), is thought to be the milk-producing unit of the mammary gland. Therefore, the post-pubertal

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human breast harbors cells capable of milk production even in the absence of pregnancy whereas the rodent mammary gland does not. However, profound expansion and differentiation of the TDLU population in the human breast is still required in order to render lactation successful.

This expansion of the alveolar epithelium during pregnancy occurs in response to both local and systemic factors that drive mammary alveolar proliferation. In rodents, the entire secretory epithelium of the mammary gland develops during the gestation period of approximately three weeks, signifying a rapid 10-fold increase in epithelial content of the mammary gland. The mammary gland produces colostrum and then milk upon partuition. However, once offspring are weaned, the milk-producing lobuloalveolar cells are no longer necessary. Rather than being maintained, these secretory cells undergo programmed cell death in an exquisitely controlled and rapid process, while leaving the ductal epithelium intact. Dying cells are cleared from the post-lactational mammary gland rapidly and without causing acute inflammation, removing up to 90% of the total mammary epithelial content within the time span of just one week in rodent models. This returns the mammary gland to an almost pre-pregnant state, so that the changes in the mammary gland that accompany pregnancy, lactation and involution may again occur with each successive pregnancy.

The process of involution is complex requiring two distinct phases, the initiation of extensive cell death to remove the milk-producing epithelial cells, followed by the controlled influx of macrophages and other immune cell types to breakdown extracellular matrix, remodel blood vessels, replenish the adipocyte population in the mammary fatpad, and to phagocytically remove dead cells, residual milk, and debris. This review will focus primarily on the events controlling cell death that occur within the first days of post-lactational involution.

2. Body

2.1. Signaling mechanisms that control post-lactational apoptosis in the breast

In recent years, molecular regulation of post-lactational involution has been studied primarily in the mouse mammary gland, due in large part to the relatively rapid gestation and nursing period in mice, and to the extensive use of genetically engineered mouse models. These models, coupled with advances in transcriptional profiling have provided a detailed analysis of the dynamic cellular and molecular events occurring during the earliest days of involution, when the majority of programmed cell death occurs.

2.2. Milk stasis

Using teat-sealing to block milk delivery in a single mouse mammary gland, investigators demonstrated that a complex multi-step process initiating massive epithelial apoptosis is triggered by local stimuli produced in the sealed mammary gland, but not by changing levels of circulating hormones that are available to the remaining nine mouse mammary glands [8, 9]. These studies revealed that milk stasis is a primary trigger of post-lactational

cell death in the mammary gland [8]. Accumulation of milk within the secretory luminal space might initiate cell death by causing a mechanical stretch of these cells, or of cell-cell junctions [10]. It is clear that mechanical stress, including cell stretching, can initiate biological responses in several epithelial and endothelial cell types, and may activate signaling pathways known to trigger cell death in the post-lactational mammary gland. For example, cell stretching induces STAT3 phosphorylation, inhibition of the survival factor AKT, and expression of Leukemia Inhibitory Factor (LIF), each of which are critical during early post-lactational involution for the induction of cell death, as discussed below. Another potential explanation of milk stasis-induced cell death is that accumulation of milk components, such as calcium, may trigger cell death [11]. In support of this hypothesis, transcription of the plasma membrane protein calcium-ATPase 2 (PMCA2), which transports 60–70% of milk calcium [12], is dramatically and rapidly reduced during involution, perhaps due to self-limiting negative feedback in an effort to control potentially toxic divalent cation levels [13]. Loss of the gene encoding PMCA2 (*Atp2b2)* in mice caused precocious alveolar cell death at lactation. Interestingly, PMCA2 expression is also regulated by enforced shape changes in mammary epithelial cells [13]. Stanniocalcin-1 (STC-1), a newly discovered mammalian hormone that accumulates nearly 3-fold upon milk stasis [14], has recently been implicated as an inducer of post-lactational involution [15].

2.3. STAT3

Transcriptional profiling studies of the mouse mammary gland at specific time points during lactation and post-lactational involution demonstrated that a specific subset of genes is dramatically induced within 12 hours of pup withdrawal, presumably in response to milk accumulation [4, 16]. It was hypothesized that this gene subset may represent potential 'master regulators' of programmed cell death in the post-lactational mammary gland. This idea has been largely confirmed using genetically engineered mouse models that disrupt key expression events, resulting in a delay in post-lactational programmed cell death.

The transcription factor Signal Transducer and Activator of Transcription (STAT) 3 was conditionally deleted in the mammary epithelium of genetically engineered mice, revealing its critical role in initiating the earliest events in post-lactational apoptosis [17-19]. While it has been known for some time that STAT3 regulates the expression of pro-inflammatory genes involved in the acute phase response (the early inflammatory response to tissue injury) [20, 21], and that many inflammation-related genes are expressed during post-lactational involution [4, 22, 23], these studies were the first to demonstrate the molecular similarities that exist between the involuting mammary gland and the traditional wound healing scenario [23], despite the fact that involution-induced transcriptional responses are directed primarily by epithelial cells, while wound healing-induced transcriptional responses are directed by cells of the immune system.

STAT3 is widely expressed, and is activated by tyrosine phosphorylation in response to numerous cytokines and growth factors [e.g. interleukin-6 (IL-6), IL-10, IL-17, IL-23, EGF] and tyrosine kinases (c-Src, Met, ErbB-2) [24]. Tyrosine phosphorylation of STAT3 allows

STAT3 dimers to translocate from the cytoplasm to the nucleus, where STAT3 binds to sequence-specific DNA elements in the promoters of STAT3 target genes. STAT3 activates gene transcription of many inflammation related genes, and can also repress the transcription of others. Although STAT3 transcriptional activity is associated with cell survival in several cell types including lymphomas and solid tumor epithelial cells [25-28], STAT3 takes on a different role in the post-lactational mammary gland, where STAT3 is required to initiate cell death. In the absence of STAT3, cell death was abrogated for at least 6 days after pup withdrawal, despite milk stasis [18, 19]. Conversely, loss of suppressor of cytokine signaling (SOCS)-3, a negative regulator of STAT3, accelerated involution by increasing the rate of cell death following pup withdrawal [29, 30].

A number of genes regulated by STAT3, such as CAAT/enhancer binding protein (C/ebp)δ, oncostatin M (OSM), OSM receptor (OSMR), and insulin-like growth factor (IGF) binding protein (IGFBP)-5, are also required in the post-lactational mammary epithelium to initiate cell death [31, 32]. OSM, a cytokine normally produced by macrophages but in this case produced by the mammary epithelium, is required during post-lactational involution, since OSMR knockout mice exhibited delayed involution [33]. Loss of C/ebpδ, a transcription factor involved in the acute phase response, also delayed mammary gland involution [31]. Because STAT3, and many target genes activated by STAT3, are critical triggers of cell death during post-lactational involution, it is likely that STAT3 lies at the apex of a transcriptionally-activated signaling cascade that is required to initiate cell death in the post-lactational mammary epithelium. This role of STAT3 as an apoptosis inducer lies in contrast with observations that STAT3 is frequently activated in several cancer entities [28], correlating with heightened malignancy [34, 35]. Further, constitutive STAT3 activity promotes tumor formation in skin [36] and lung [37, 38]. The apparent discrepancy may be related to tissue specificity of STAT3 activity, or the activity of STAT3 in the tumor microenvironment (for example, in inflammatory cells) versus its role in the epithelial compartment of the tumor.

2.4. NF-κB

Although the role of STAT3 in the induction of post-lactational apoptosis is clear, STAT3 alone is insufficient to induce involution in the absence of nuclear factor-κB (NF-κB) signaling [39]. NF-κB comprises a family of five structurally and functionally related transcription factors [40]. Based on their transactivation properties, NF-κB proteins are divided into two classes: Class I consists of RelA/p65, RelB, and c-Rel, while Class II includes NF-κB1/p50 and NF-κB2/p52. Each can dimerize in almost any combination but only class I proteins possess the C-terminal transactivation domains required for NF-κB-mediated transcription of target genes. Under basal conditions, NF-κB dimers are sequestered in the cytoplasm bound to the protein Inhibitor of κB (IκB). Several signaling pathways can activate the IκB kinases (IKKs) that phosphorylate IκB, thus liberating NF-κB dimers and allowing their nuclear translocation, where they bind to specific DNA sequences in target genes.

Among this family of transcription factors, two NF-κB subunits, RelA (p65) and p50 are expressed at different levels in the mammary epithelium throughout mammary gland

development. Furthermore, NF-κB activity as measured *in vivo* using a transgenic NF-kB reporter model demonstrated two major peaks of NF-κB-mediated trans-activation: one that occurs during pregnancy, and another that occurs during involution [41]. These data are consistent with reports showing that NF-κB induced transcription of pro-survival genes [42-45], and other reports showing that NFκB activated transcription of pro-apoptotic genes [46-48]. Therefore, it is possible that NF-κB might drive cell growth and survival in the breast epithelium in some cases, but may regulate breast epithelial cell death in others. In support of the idea that NF-κB might promote cell death, increased NF-κB activity is rapidly induced after weaning, with strong increases seen within one hour of milk stasis in mouse mammary glands. NF-κB activity remains elevated through the first four days of murine post-lactational involution. Furthermore, loss of NF-κB signaling in a genetically engineered mouse model of conditional IKK-β disruption decreased post-lactational NF-κB signaling, resulting in decreased caspase-3 cleavage and delayed post-lactational apoptosis [39], confirming the importance of NFκB signaling in post-lactational cell death of the secretory epithelium. Conversely, constitutively active IKK-β increased NF-κB signaling, thus causing accelerated induction and higher rates of apoptosis during post-lactational involution [49]. Even in the absence of milk stasis, constitutively active IKK-β was capable of inducing apoptosis in the mouse secretory mammary epithelium, and therefore interfered with successful lactation by nursing dams.

2.5. Akt/PI3K

Intense interest in survival signaling pathways has revealed that phosphatidyl inositol 3-kinase (PI3K) is a potent regulator of cell survival [50, 51]. Cancer cells frequently utilize PI3K signaling to promote cell survival under conditions of hypoxia, nutrient stress, or even to escape the cytotoxic effects of therapeutic anti-cancer treatments. It is clear, however, that non-transformed cells also use the PI3K signaling pathway to promote cell survival, and that increased PI3K signaling can interfere with physiological cell death [52]. PI3K is a heterodimer comprised of p110 (the catalytic domain) and p85 (the regulatory domain) [50, 53]. Under basal conditions, p85 represses the catalytic activity of p110. However, SH2 domains in p85 interact with phosphorylated tyrosines within YxxM motifs of receptor tyrosine kinases (RTKs) such as the insulin-like growth factor (IGF)-1 receptor (IGFR) or adaptor proteins, such as the insulin receptor substrate proteins. This relieves p85-mediated inhibition of p110, allowing p110 to phosphorylate phosphatidyl inositol 2-phosphate (PIP2), thus generating PIP3, a powerful membrane-associated second messenger that recruits pleckstrin homology (PH)-domain containing proteins to the cell membrane. PDK1 and Akt are two PH-domain containing proteins recruited to the membrane in response to RTK activation [52, 54, 55]. PDK1 is a serine-threonine kinase that phosphorylates Akt, another serine-threonine kinase that stimulates cell survival by interacting with members of the Bcl2 family of apoptosis regulators [56-58], which are also involved in the induction of cell death during involution [59-61]. For example, mammary-specific loss of the Bcl2 family member Bax, a known cell death inducer, decreased apoptosis during early involution [60, 62-64], while overexpression of Bax within the secretory mammary epithelium increased

post-lactational apoptosis and promoted precocious STAT3 activity [7, 65]. Loss of the anti-apoptotic protein Bcl-xL in the mammary gland during post-lactational involution accelerated cell death [66, 67]. By inactivating Bax and activating Bcl-xL, Akt activity increases cell survival in the secretory mammary epithelium.

The role of PI3K/Akt signaling in suppressing post-lactational apoptosis is supported by genetically engineered mouse models that result in increased PI3K/Akt signaling. For example, a mouse model in which mammary-specific expression of myristoylated p110α [68], a modified p110α that is restricted to the cell membrane, resulted in aberrantly elevated PI3K activity in the mammary epithelium and delayed post-lactational involution. Similarly, mammary-specific transgenic overexpression of Akt1 or Akt2 promoted cell survival and delayed post-lactational involution in mice [69, 70]. Conversely, ablation of Akt1, but not the ablation of Akt2 or Akt3, promoted apoptosis and accelerates involution [71], demonstrating isoform-specificity in the gene-dosage effects of Akt (overexpression versus ablation), and highlights the importance of Akt1 in the post-lactational mammary gland. Other studies demonstrated that Akt signaling is sustained during lactation by prolactin signaling [72-74]. This observation was confirmed in an independent transgenic mouse model of mammary-specific STAT5 activation, in which STAT5 activity, when aberrantly sustained through post-lactational involution, upregulated Akt1 transcription and impaired apoptosis. These studies suggest that high levels of prolactin-induced STAT5 activity, as seen during lactation, maintains Akt1 expression and activity to promote cell survival, but when lactation ceases STAT5-induced Akt expression must be depleted in order for cells to undergo apoptosis [75].

Like prolactin signaling, other ligand-activated signaling cascades are capable of driving PI3K/Akt signaling during lactation, and if not turned off, can delay post-lactational apoptosis. For example, cell signaling initiated by IGF-1, which activates IGFR thus causing tyrosine phosphorylation of insulin receptor substrate proteins [76], potently activates the PI3K/Akt signal transduction cascade in the mammary epithelium during lactation. Overexpression of IGF-1 in the mouse mammary gland delayed post-lactational involution, suggesting that suppression of IGF-mediated cell survival is required for apoptosis to occur, and supporting the idea that PI3K signaling must be interrupted to initiate post-lactational apoptosis [77]. IGF-1 bio-availability is tightly controlled by IGF binding proteins (IGFBPs), which can sequester IGFs in the extracellular microenvironment of the mammary epithelium [78]. Consistent with the ability of IGF-1 to interfere with post-lactational cell death, one of the earliest transcriptional events during post-lactational involution is the upregulation of IGFBP-2 mRNA (4-fold), IGFBP-4 (6-fold) and IGFBP-5 mRNA (50-fold) [79]. This profound increase in IGFBP-5 is also seen at the protein level, and is conserved across several species. Increased expression of IGFBPs may limit IGF1-induced signaling, thus limiting IGF1-induced PI3K/Akt signaling [77, 80, 81]. Transgenic overexpression of IGFBP-5 in the mouse mammary gland increased caspase-3 cleavage (an indicator of apoptosis) and decreased the expression of the pro-survival factors Bcl-2 and Bcl-xL [80-84], suggesting that IGFBP-5 is pro-apoptotic. An IGF-1 analogue which binds weakly to IGFBP-5 partially overcame IGFBP-5-induced cell death during post-lactational involution,

suggesting that IGFBP-5 was acting, at least in part, by inhibiting IGF action. Conversely, *Igfbp5* null mammary glands exhibit delayed post-lactational apoptosis [78].

2.6. TGFβ3

While prolactin, IGF-1, and several RTK-activating ligands can activate PI3K/Akt signaling to promote cell survival, other ligands are capable of inducing cell death during post-lactational involution, such as leukemia inhibitor factor (LIF) [85-87], serotonin [88], Fas ligand (FasL) [89] TRAIL [90], and transforming growth factor (TGF)-β3. Transcripts encoding TGF-β3, but not TGF-β1 or TGF-β2, substantially increase in the milk-producing cells during post-lactational involution [91, 92]. This rapid induction of TGF-β3 transcription in the secretory mammary epithelium occurs as early as 3 hours after pup withdrawal in response to milk stasis [93], and is among the most rapid gene expression changes occurring in response to post-lactational involution, suggesting that TGFβ3 might be an initiating signal for cell death during involution. It would be interesting to determine the impact of mechanical stress on expression from the TGFβ3 promoter. Consistent with the proposed role for elevated TGF-β3 in inducing apoptosis during involution, transgenic over-expression of TGF-β3 in the secretory cells of the mouse mammary gland accelerated apoptosis during early post-lactational involution. Conversely, loss of TGFβ3 reduced post-lactational apoptosis by nearly 70% [93], suggesting that autocrine TGF-β3 signaling initiates cell death following pup removal.

The importance of the TGFβ3-induced signaling pathway for post-lactational apoptosis has been further investigated in genetically engineered mouse models. For example, loss of the TGFβ-regulated transcription factor Smad3 decreased post-lactational apoptosis by nearly 40% [94]. Similarly, loss of TGF-β receptor type II (TβRII) in the mammary epithelium, or transgenic expression of dominant negative (DN) TβRII decreased apoptosis during early post-lactational involution [95-97], consistent with a critical role for TGF-β3 signaling through TβRII and Smad3 to induce apoptosis during early involution. However, there is some discrepancy regarding the role of TGFβ signaling during involution, as transgenic expression of constitutively active TβRI decreased apoptotic cells in the mammary gland [98]. Perhaps elevated TβRI signaling activates signaling pathways not normally active under physiological conditions.

2.7. Stromal-epithelial interactions

The signaling pathways described above focus on those events occurring within the secretory epithelium that are responsible for initiating cell death during post-lactational involution. However, it is becoming more apparent that stromal cells contribute substantially to post-lactational apoptosis [99-103]. This was recently demonstrated in a transgenic mouse model referred to as MAFIA (macrophage Fas-induced apoptosis) [104]. Macrophages from MAFIA mice express a modified Fas receptor that, in response to a dimerization-inducing small molecule (AP20187), triggers Fas-mediated apoptosis. Depletion of macrophages immediately prior to weaning impaired apoptosis within the secretory mammary epithelium, despite milk stasis and STAT3 activation [105]. These results demonstrate that macrophages

are necessary to initiate apoptosis in the mammary epithelium. The underlying mechanism remains unclear at this point. However, it is possible that macrophages respond to signals emanating from mechanically stressed epithelia by producing factors that may activate the signaling pathways necessary for induction of apoptosis, or repress signaling pathways that may otherwise limit apoptosis. The transcriptional signatures generated from mouse mammary glands during involution were derived from whole tissue RNA, which would include not only epithelia but also the dynamic stromal components of the mammary gland. Therefore, it is possible that many expression events detected during early post-lactational involution are occurring within macrophage populations.

Mast cells are also heavily recruited to the mammary gland during post-lactational involution [106], and like macrophages, are critical for epithelial apoptosis during mammary gland involution [107]. Specifically, mast cells produce plasma kallikrien (PKaI), the primary activator of plasminogen in the mammary gland. Expression of PKaI rapidly increases during involution, and while PKaI, plasminogen, and other serine proteases undoubtedly have a major role in tissue remodeling during later stages of involution, evidence suggests that PKaI also drives epithelial apoptosis. Inhibition of mast cell-derived PKaI during post-lactational involution impaired epithelial cell death, suggesting that mast cells are vital for triggering apoptosis in the post-lactational mammary gland. Interestingly, in the absence of STAT3 within the mammary epithelium, mast cells and macrophages are not recruited to the mammary gland during post-lactational involution [108], suggesting that recruitment of stromal cells to the involuting mammary gland is initiated by early apoptotic signaling events occurring within the epithelial compartment (**Fig. 1**).

2.8. Lysosomal membrane polarization

Although most studies suggest that mammary gland involution occurs by apoptosis, it has been proposed recently that several morphological features of the involuting mammary gland may resemble necrosis rather than apoptosis [109]. These include cytoplasmic swelling, lack of membrane blebbing, and lack of nuclear fragmentation. Using mice deficient for both caspase 3 and 6, it was shown that mammary gland involution could proceed in the absence of these two classical activators of apoptosis, suggesting that perhaps alternative mechanisms of programmed cell death may exist in the post-lactational mammary gland. The authors proposed that STAT3 activity could upregulate expression of lysosomal cathepsins, which may leak from lysosomes to activate cell death pathways [110, 111]. In support of this idea, cathepsin L is upregulated strongly with the onset of mammary involution [112]. Mice treated with a specific cathepsin L inhibitor during the first three days of involution demonstrated reduced cell death as compared to untreated mice [112]. Cathepsin-induced cell death can be simulated by ectopic addition of reactive oxygen species (ROS) to cultures of mammary epithelial cells. Interestingly, the ROS nitric oxide (NO) can trigger mammary gland involution after weaning [113] in mice. While the role of apoptotic cell death in the mammary gland is widely accepted, investigators should be aware of alternative cell death pathways that contribute to programmed cell death during involution.

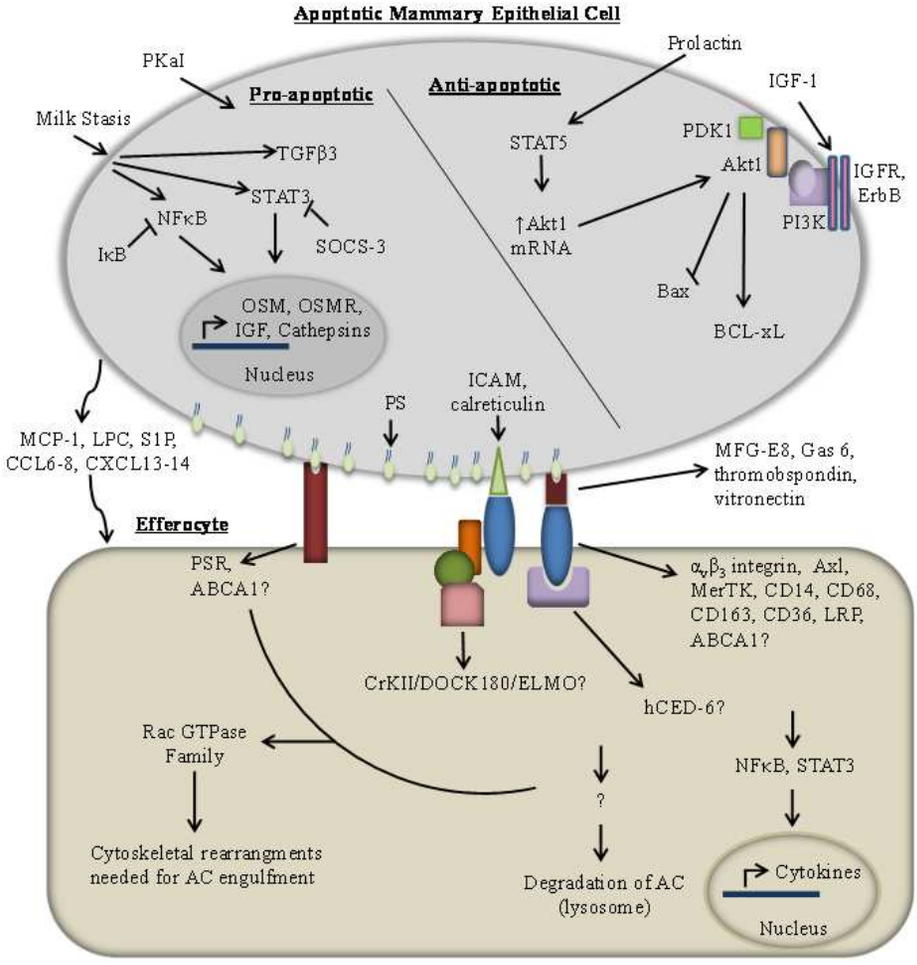

Figure 1. Apoptosis in the post-lactational mammary epithelial cell (MEC) is initiated by three molecular signals that are each required: TGFβ3, NFκB, and STAT3. Their loss impairs post-lactational apoptosis, despite continued milk stasis. Apoptotic MECs are cleared from the mammary gland by efferocytosis, or the phagocytic engulfment of dying cells. In the post-lactational mammary gland, MECs and macrophages engulf neighboring apoptotic cells. The phagocyte uses cell surface receptors to recognize, bind, and engulf apoptotic cells. These receptors include MerTK, Axl, αvβ3 integrin and others. Intracellular signaling pathways that regulate cytoskeletal rearrangements (such as Rac signaling) are necessary for efferocytosis. Once engulfed into vesicles, apoptotic cells are degraded by the lysosomal pathway. Efferocytosis activates NF-κB and STAT3, upregulating cytokines that are critical for post-lactational mammary remodeling.

2.9. Pathologies of the breast due to aberrant regulation of apoptosis

In the clinical setting, post-lactational involution of the secretory epithelium begins with milk stasis, at which point the secretory cells undergo apoptosis. Clearance of dying cells and residual milk is accomplished by phagocytes within the breast [114]. Regrowth of stromal adipose tissue and continued tissue remodeling returns the breast to a relatively quiescent state comprised of morphological structures similar to those found in nulliparous women. Rarely, the process of involution may be delayed. Failure to remove unnecessary lactational cells may result in symptomatic inflammatory tissue damage. Delayed involution in the human breast is characterized by the maintenance of secretory structures, loss of post-

lactational apoptosis, and infiltration of the breast by inflammatory cells. Focal calcification may also be present [5]. Ductal distention of accumulated milk can be painful. Stagnant milk can be a source for infection and mastitis, to which the gland would respond with secretion of acute inflammatory cytokines and recruitment of leukocytes [115]. Similar to this clinical scenario, mouse models of delayed post-lactational cell death commonly develop mastitis [49, 108, 116].

EMS receptor	Bridging Molecule (BM)	EMS	Mammary Tissue and Cell Line Expression
PSR	-	PS [117]	Primary mouse mammary epithelia [117]
TIMs	-	PS [118-124]	unknown
BAI1	-	PS [125]	unknown
Stabilin-2	-	PS [126, 127].	unknown
ABCA1	unknown	PS [128, 129]	Bovine [130], mouse, human mammary [129]
$\alpha_v\beta_3$ integrin	Vitronectin [131] Thrombospondin [132] MFG-E8 [133]	PS [131, 132]	Human MCF10A, MCF-7, and MDA-MB-231 cells [134] bovine [135], mouse mammary gland [136, 137]
Tyro3	Gas6 [138] Protein S [139, 140]	PS [138]	unknown
Axl	Gas6 [138, 141]	PS [138, 141]	Human breast [142]
MerTK	Gas6 [138] Protein S[140]	PS [138, 140]	Mouse mammary [116]
CR3/CR4	unknown	C3bi [143]	unknown
CD14	unknown	ICAM [144]	Bovine [145], canine [146], mouse [4], and human mammary [144, 147]
CD68	unknown	unknown	Human, mouse mammary macrophages [148]
CD163	unknown	unknown	Human breast [148]
CD36	Thrombospondin [132]	unknown	MDA-MB-435, MDA-MB-231 human cells in mouse mammary [149]
LRP	β_2GP1 [150] C1q [151]	PS [150] Calreticulin [151]	Rat mammary gland [152] Normal, transformed mammary epithelia [153]
Marco [154]	unknown	unknown	unknown

Table 1. Key Factors Involved in Efferocytosis

Recent data garnered from mouse models of delayed post-lactational involution suggest that deregulation of post-lactational apoptosis may facilitate mammary tumor formation [34, 70, 73, 155-157]. Observations made in human populations also suggest that altered post-lactational involution may associate with tumor formation in the breast [100-102, 158]. This may reflect micro-environmental influences, or may be a function cell death-dependent removal of unnecessary breast epithelial cells in a regulated fashion. In many cancers, intrinsic cell deaths mechanisms become suppressed, contributing to the net growth of the transformed cell population. For example, activation of STAT5 in post-lactational mouse mammary glands delays apoptosis, and results in formation of mammary tumors that express estrogen and progesterone receptors (ER+PR+), as well as activated STAT3 and STAT5 [159]. Moreover, post-lactational transcriptional programs initiated by NF-κB and STAT3 not only support cell death, but also enhance tumor formation and progression by inducing expression of pro-tumorigenic cytokines [23, 24, 28, 34, 160-162]. Indeed, the transiently increased risk of developing breast cancer in the five years following a pregnancy may be greatly influenced by a deregulated tumor microenvironment developed in the post-lactational breast [101].

While post-lactational involution and age-related lobular involution are distinct processes, recent studies indicate that both are related to breast cancer development. Clinical studies show that completion of lobular involution may reduce future breast cancer incidence [163-166]. With aging, there is a gradual loss of breast epithelial tissue that typically begins in peri-menopause, which then accelerates during menopause. Lobular involution is characterized by the apoptosis-mediated decrease in the size and complexity of the ductal tree and of the TDLU. This is distinct from post-lactational involution, which occurs very rapidly by comparison. However, similar mechanisms controlling apoptosis of the breast epithelium may occur in these two distinct models of involution.

Lobular involution, like post-lactational involution, may inversely correlate with breast cancer risk, since premenopausal women who underwent partial or complete lobular involution had a substantially decreased incidence of breast cancer, while postmenopausal women who showed delayed lobular involution were found to have a correspondingly elevated breast cancer incidence [164]. While much remains to be learned about how lobular involution is regulated, some clinical studies and animal models suggest that IGF-1 may inhibit involution of lobules in the breast [163]. Clinically, a cross-sectional study among 472 women demonstrated that higher IGF-1 levels associated with incomplete lobular involution, supporting the idea that IGF-1/PI3K/Akt-induced survival pathways prevent physiologic cell death, leading to pathological consequences.

2.10. Introduction to efferocytosis in the breast

Following apoptosis, one final event is needed to truly complete the life of the cell. This final step is phagocytic engulfment of apoptotic cells or 'efferocytosis'. The term 'efferocytosis' was recently coined by Hensen et al. to distinguish phagocytic apoptotic cell removal from phagocytic pathogen removal [167]. While both processes are executed by phagocytes, they

result in distinctly different biological responses, one characterized by a dampened acute inflammatory response and upregulation of tolerogenic and wound healing effectors (efferocytosis), while the other is characterized by a pro-inflammatory response (pathogen removal).

Efferocytosis is a carefully regulated process involving recruitment of phagocytes to the apoptotic cell, recognition of the apoptotic cell by the phagocytes, engulfment of the apoptotic cell by the phagocyte, and final breakdown of apoptotic cell components. If disrupted, apoptotic cells will undergo necrotic lysis, leading to acute inflammation, tissue damage and autoimmunity. Therefore, efferocytosis is critical for tissue homeostasis. However, recent discoveries indicate that the normal process of efferocytosis may be undesirable under certain pathological conditions, such as in the tumor microenvironment. We will discuss apoptotic cell clearance in the normal post-lactational breast and in the breast tumor microenvironment.

2.11. The process of efferocytosis

In general, clearance of apoptotic cells is often executed by macrophages and dendritic cells (DCs), but can also be performed by fibroblasts, endothelial and epithelial cells. A cell that engulfs an apoptotic cell through phagocytic mechanisms is called an efferocyte, regardless of its origin. Studies performed in cell culture and *in vivo* demonstrate that MECs and macrophages are both capable of engulfing apoptotic MECs during post-lactational involution of the secretory mammary epithelium [114, 116, 168].

Macrophages, the 'professional phagocytes' of the immune system, must infiltrate the mammary gland in response to the physiological presence of apoptotic cells during involution. Large quantities of bone marrow-derived and spleen-derived macrophages infiltrate the post-lactational mouse mammary gland in response to STAT3 activation and apoptosis of the mammary secretory epithelium. It is thought that apoptotic cells may release soluble chemo-attractants, or 'find me' signals, which recruit macrophages to the post-lactational mammary gland. For example, Monocyte Chemo-attractant Protein-1 (MCP-1/CCL2) is released from apoptotic cells in an NF-κB-dependent manner [169]. Interestingly, MCP-1 expression is strongly induced in the mammary gland at day 2 of involution, a time point that follows NF-κB-induced cell death, and that precedes macrophage influx in the post-lactational mammary gland. These observations are consistent with NF-κB-induced apoptosis followed by NF-κB-induced expression of an efferocyte chemo-attractant, although this has not yet been demonstrated. Additional chemokines including CX3CL1, CCL6, CCL7, CCL8, and CXCL14, are induced during post-lactational involution and may be signals that recruit macrophages to the involuting mammary gland to clear the accumulating apoptotic cell burden [170, 171] [4, 16].

Histological evidence of apoptotic cells within cytoplasmic vacuoles of mammary macrophages confirms that mammary macrophages engulf apoptotic cells during involution. Once present within the mammary gland, the macrophage identifies apoptotic

cells, scanning for signals that are present on the apoptotic cell but not a healthy cell, often referred to as an 'eat me' signal (EMS, **Table 1**). The earliest and most recognized EMS is surface exposure of phosphatidylserine (PS). Healthy cells actively maintain PS on the inner plasma membrane leaflet. At the onset of apoptosis, PS is presented to the outer leaflet thus acting as a marker for a dying cell that requires engulfment [172-174]. EMSs are recognized by macrophages that express EMS receptors on their cell surface. EMS receptors may bind directly to the EMS on the dying cell. For example, the PS receptor (PSR), a transmembrane protein expressed by macrophages [128] and MECs [129], directly binds PS. Brain angiogenesis inhibitor 1 (BAI1) also binds PS directly, and is important for macrophage-mediated efferocytosis [125], but has not yet been studied in the mammary gland. Stabilin-2 and members of the T cell immunoglobin and mucin (TIM) family of receptors also directly bind PS [118-124, 126, 127], demonstrating mechanistic redundancy in the efferocytic pathways. It should be noted, however, that PS is an insufficient EMS, as macrophages fail to recognize live cells in which PS is forced to the outer leaflet [173, 175].

While some EMS receptors bind apoptotic cell EMSs directly, other EMS receptors bind an extracellular bridging molecule that simultaneously binds the EMS and the EMS receptor (**Table 1**). For example, the bridging ligand milkfat globule epidermal growth factor-like 8 (MFG-E8) binds to PS on apoptotic cells [133, 176, 177], while binding $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expressed by macrophages. Growth arrest specific gene 6 (Gas6) and Protein S [178, 179] are bridging molecules that bind to the EMS receptors MerTK, Axl, and Tyro3 [180] expressed by macrophages, while simultaneously binding PS on the apoptotic cell.

Once the apoptotic cell is bound to the macrophage, intracellular signaling pathways must remodel the actin cytoskeleton to drive phagocytic ingestion of the apoptotic cell. Most of these events have been mapped out in *Caenorhabditis elegans* (*C. elegans*), which are discussed in detail within comprehensive reviews by Reddien et al. and Ravichandran et al. [181, 182]. In mammalian macrophages, intracellular signaling networks that regulate actin cytoskeletal dynamics are required for apoptotic cell engulfment. For example, a protein complex comprised of CrkII, DOCK180, and ELMO causes activation of the Rac GTPase, a master regulator of actin cytoskeletal dynamics. Actin-dependent membrane extensions physically engulf the apoptotic cell [183-187]. Rac-mediated actin rearrangements are countered by RhoA [188], which prevents the formation of cell extensions needed for efferocytosis [189]. The engulfed apoptotic cell is then consumed by the efferocyte, primarily through lysosomal degradation. Many signaling factors that drive apoptotic cell engulfment also enhance lysosomal degradation of apoptotic cells. For example, Rac is essential for maturation of phago-lysosomes in macrophages [190-193].

In addition to efferocytosis in the mammary gland, macrophages are also critical for cytokine modulation and extracellular matrix remodeling during the second phase of involution, underscoring the important role of macrophages in the post-lactational breast. Given their known role as professional phagocytes and their massive influx to the post-lactational mammary gland, it is perhaps not surprising that efferocytosis by macrophages occurs in the post-lactational breast. What is more surprising is that apoptotic cell clearance occurs on a profound scale prior to the influx of macrophages to the involuting mammary

gland. Recent evidence demonstrated that MECs are the primary efferocytes of the breast during the earliest stages of post-lactational involution, the first three days prior to the influx of macrophages. The ability of MECs to act as efferocytes ensures a rapid response to the massive level of apoptosis that occurs during post-lactational involution. Loss of MEC-mediated efferocytosis impairs post-lactational homeostasis, resulting in chronic mammary inflammation, scarring and inhibition of future lactation.

Interestingly, MECs utilize many of the same EMS receptors used by macrophages to recognize apoptotic MECs. For example, MerTK is critical for MEC-mediated efferocytosis during post-lactational involution. MerTK loss from the mouse mammary epithelium causes apoptotic cell accumulation and milk stasis [116], despite the presence of wild-type macrophages. Interestingly, mRNA and protein expression of MerTK is dramatically upregulated by post-lactational day 1 within the luminal mammary epithelium. Similarly, the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed in the early post-lactational mammary epithelium. The $\alpha_v\beta_5$ bridging ligand, MFG-E8, is simultaneously induced [194], increasing the physical interaction between MFG-E8 and PS [136] and driving the clearance of membrane-coated milk components from the involuting mammary gland [195].

2.12. Physiological and pathological consequences of efferocytosis

After the efferocyte removes the apoptotic cell, transcriptional events in the efferocyte result in cytokine, chemokine and growth factor production. The combined profile of the factors produced by the efferocyte promotes wound healing through enhanced tissue remodeling, angiogenesis, proliferation and resolution of acute inflammation. The efferocytosis-induced wound healing cytokine profile contrasts sharply to the cytokine profile produced in response to phagocytosis of pathogens, which is characterized by acute inflammatory cytokines [196]. In fact, efferocytosis is thought to be a key step in resolving or dampening acute inflammatory cytokine expression following tissue injury or pathogen exposure, resulting in repair and homeostasis [197]. Microarray analyses of mammary glands harvested at early post-lactational involution time points displayed a pronounced wound healing expression signature [3, 4, 158, 168, 198, 199], consistent with transcriptional changes that result from efferocytosis. The prominent role of efferocytosis in re-establishing mammary homeostasis following widespread apoptosis of the secretory epithelium was shown by experiments in which loss of efferocytosis resulted in apoptotic cell accumulation, sustained milk stasis within ductal lumens, inflammation and scarring [116]. These pathological changes impaired lactation in future pregnancies.

Although key to re-establishing homeostasis in the post-lactational mammary gland [116], recent evidence indicates that efferocytosis may support a more malignant tumor microenvironment [101, 102, 105, 106, 158, 198, 200-202]. Researchers are beginning to address the overlapping roles of efferocytic and metastasis-promoting cytokines. In agreement with this idea, mouse studies show that breast tumors grew more rapidly, invaded more readily, and formed distant metastases more efficiently when implanted in the post-lactational mammary gland as compared to implantation into a nulliparous

mammary gland [101]. One explanation for this observation is that post-lactational efferocytosis promotes breast tumor malignancy through production of wound healing cytokines, which are known to drive breast cancer growth and invasion. In support of this idea, MFG-E8 [203] and its receptor $\alpha_v\beta_{3/5}$, as well as Gas6 and its ligand MerTK [116, 204] are frequently overexpressed in breast cancers. One recent study demonstrated that the pro-tumorigenic cytokine IL-6 induces expression of MerTK, enhancing the ability of macrophages to engulf apoptotic cells and increasing production of wound healing cytokines such as IL-4 and IL-10 [205]. Recently published data implicates MerTK in breast cancer metastasis [206].

This observation has clinical relevance to pregnancy associated breast cancers (PABCs), defined as breast cancers that arise during the 5 years following a pregnancy. PABCs are among the most malignantly aggressive breast cancers, and are thus associated with poor prognosis. A better understanding of the processes outline above will undoubtedly expand the therapeutic options for these patients.

3. Conclusion

Altogether, these data support the hypothesis that targeting mediators of efferocytosis may limit pro-tumorigenic cytokine production. Moreover, it is becoming increasingly apparent that many factors within the mammary gland cooperate to ensure apoptosis and apoptotic cell clearance, highlighting the complexity of these processes and the need for more detailed investigations. Due to the dominant role of apoptosis and efferocytosis in maintaining tissue homeostasis, especially during post-lactational involution, the mammary gland provides an ideal platform for future study.

Author details

Jamie C. Stanford and Rebecca S. Cook
Department of Cancer Biology, Vanderbilt University, Nashville, TN, USA

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