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Postlactational Involution: Molecular Mechanisms and Relevance for Breast Cancer Development

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Abstract

Mammary gland tissue changes appearance and functionality in different sequential steps. The tissue of virgin, pregnant, or lactating mammary glands changes controlled by finely regulated physiological processes. A fourth stage (involution), triggered upon weaning, involves remodeling, and the gland regresses to resemble a prepregnant stage. This highly complex process characterized by a high degree of epithelial cell death and tissue remodeling can be divided into phases, which can be independent of each other. The present article describes a variety of signaling pathway components, transcription factors, and mRNA stabilization proteins that play a role in the regulation of cell fate during the involution process. These molecular actors are finely related in health to trigger the delicate mechanism that govern involution after weaning, leaving the gland in a latent stage until needed again. Importantly, it has been shown that this process may contribute to cancer development in the years following childbirth, mainly because of the involvement of inflammatory and remodeling factors.

Keywords: mammary gland involution, transcription factors, inflammatory cytokines, STATs, cell death, TNF-α, mRNA stability, breast cancer

1. Introduction

Upon weaning, the mammary gland recovers a morphology similar to its prepregnant state through an intricate process known as postlactational involution. During this phase, a high proportion of epithelial cells die, the basal membrane is partially digested, and the adipose tissue reoccupies the space left by the regressed alveoli. In the mouse, mammary gland involution has been described as a two-step process according to its reversibility [1]. The first reversible phase is induced by *local factors* and lasts approximately 48 h during which it can be reversed through resuckling. At this stage, proapoptotic factors are upregulated, while survival factors

are reduced. This reversible phase is followed after 72 h by a nonreversible phase where widespread apoptosis and tissue remodeling takes place. This later phase requires *systemic factors and local* proteases such as MMP-3/Stromelysin-1 and MMP11/Stromelysin-3, MMP2/Gelatinase A, ICE (interleukin-1 beta converting enzyme), and Urokinase-type plasminogen activator [2].

In 1997, Li et al. [3] defined the role of local factors as compared with systemic hormones during the first and second stages of involution. When milk release was disrupted in the presence of systemic lactogenic hormones, they demonstrated that local signals were sufficient to induce alveolar cell death. These authors demonstrated that a variety of procedures successfully triggered mammary involution, although none of them prevented the presence of circulating lactogenic hormones. For example, sealing of the teats, mammary gland transplants unable to release milk due to the absence of a teat connection or inactivation of the oxytocin gene efficiently induced mammary cell death. On the other hand, in these scenarios, systemic hormones were able to preserve lobular-alveolar structure, although they did not prevent apoptosis. This chapter reviews the discovery of the local factors involved in the process of involution as well as the finding of the mechanisms involved in their ability to induce cell death shortly after weaning. In addition, the generation of a pro-oncogenic microenvironment during mammary involution, which is able to facilitate breast cancer progression, is also discussed.

2. Involvement of STAT3 signaling

The studies referred above found that cell death correlated with the induction of proapoptotic genes as *bax*, decreased expression of milk proteins, dephosphorylation of STAT5a and 5b (main transcription factors that mediate prolactin triggered signaling), and activation of STAT3. Signal transducer and activator of transcription (STATs) are a family of latent transcription factors, which are activated in response to a variety of cytokines and growth factors. This family of signaling molecules has been implicated in cell growth, differentiation, survival, and apoptosis. STAT3 and STAT5 have reciprocal patterns of activation throughout a mammary developmental cycle, suggesting that STAT5 may be a survival factor and STAT3 a death factor for differentiated mammary epithelium. Chapman et al. [4], using the lox/Cre recombination system, showed a decrease in epithelial apoptosis and a dramatic delay of the involution process upon forced weaning in conditional KO mice, in which STAT3 was specifically deleted in the lactating mammary gland. In addition, early activation of STAT1 and induction of p53 and p21 expression was observed, which suggested a potential compensatory mechanism for induction of eventual involution in the STAT3 null mammary glands. These results demonstrated the importance of STAT factors in signaling the initiation of physiological apoptosis *in vivo* and highlighted the utility of the lox/Cre system for addressing the function of genes with an embryonic lethal phenotype, specifically in the mammary gland.

STAT3 is the most ubiquitous of the members of this family of proteins, is activated by many different cytokines and growth factors, and plays many roles in different physiological processes. In addition, this protein is, the first STAT family member found to be constitutively activated in a variety of neoplastic tissues. It was determined that STAT3 modulates the

expression of various target genes involved in cell-cycle regulation, angiogenesis, and apoptosis inhibition. Because of this, silencing or inhibiting STAT3 reduces tumor cell proliferation and survival in both animal and human studies. However, in the involuting mammary gland, STAT3 signaling induces cell death [5].

By 2003, it had been shown that STAT3 is the main factor involved in the initiation of apoptosis of mammary cells after weaning, but the mechanism of its activation remained unclear. In 2002, based on the hypothesis that IL-6 is the activating cytokine for STAT3, Hennighausen's group showed that expression of IL-6 increases during early involution together with STAT3 and p44/42 MAPK activation. Besides, it was shown that IL-6 treatment activated STAT3 in the mammary gland of virgin and lactating mice. In addition, IL-6-, STAT3-, and Bax-null mice showed similar mammary phenotypes, that is a significant delay in postlactational involution. Nevertheless, it was demonstrated that STAT3 activation during involution was independent of the IL-6 levels in the mammary after weaning. In contrast, the increase of p44/42 MAPK (ERK1/2) phosphorylation at the onset of involution was dependent on the presence of this cytokine. This suggested that either IL-6 does not induce STAT3 *in vivo* or its absence is compensated for by other cytokines, such as leukemia-inhibitory factor (LIF) [6].

By that time, there was no evidence in the literature reporting LIF expression and/or activities in the normal mammary gland tissue. Therefore, LIF expression profile was analyzed during the successive stages of mammary gland development, function, and involution. The results demonstrated that LIF is expressed in the mammary gland at low levels in postpubertal, adult virgin, and pregnant mice. But, expression of this protein almost disappear during lactation to then show a significant increase a few hours after weaning, maintaining these high levels during the following days. We demonstrated that LIF expression in the gland is induced by milk stasis and not by the decrease of circulating lactogenic hormones after weaning. In addition, implantation of LIF containing pellets in lactating glands resulted in a significant increase of STAT3 phosphorylation and epithelium apoptosis. We then concluded that LIF-regulated expression in the mouse mammary gland may play a relevant role during the first stage of mammary gland involution and that LIF-induced mammary epithelium apoptosis could be mediated, at least partially, by STAT3 activation [7].

Shortly after our paper was published, Christine Watson's lab also demonstrated that LIF is the physiological activator of STAT3, as they report that pSTAT3 is absent and C/EBPdelta (a well-known STAT3 target) is not upregulated in involuting transplanted mammary glands of LIF double knock-out (LIF(−/−) mice). Similarly to what was observed in the STAT3-null glands, LIF(−/−) mammary glands exhibit delayed involution, reduced apoptosis, and elevated levels of p53 [8].

STAT3 activation and LIF expression have not been observed only in the involuting mammary gland. It was determined that autocrine/paracrine LIF present in conditioned medium from primary cultures of mouse mammary tumors was also able to induce activation of that transcription factor and to increase cell survival in mammary tumor cell lines. However, although LIF blocking antibody prevented STAT3 phosphorylation, inhibition of STAT3 increased cell survival. These results indicated that LIF is overexpressed in mouse mammary tumors, where it acts as the main STAT3 activator. Nevertheless, the data also suggested that the positive

LIF effect on tumor cell survival was not dependent on STAT3 activation, which seemed to inhibit tumor cell viability as it does in involuting mammary epithelium [9]. Kritikou et al. showed that pERK1/2 is significantly reduced in LIF(−/−) glands during pregnancy [8], suggesting that at this stage, LIF mediates its effects through pERK1/2. Therefore, it is possible that LIF proliferative effects on mammary tumors depend on ERK ½ activation. In addition, although it has been reported that STAT3 acts a potent oncogene in different tumor types, it was also demonstrated that the biological role of this factor is modulated by the stage of tumor progression [10]. Similarly, it can be proposed that in well-to-moderately differentiated mammary tumors, STAT3 activation induces cell death as observed in nontumorigenic mammary cells after lactation. This activity might be altered in more aggressive or less differentiated tumors, as it has been shown that STAT3 constitutive activation is very common in basal breast cancer [11], which have worse prognosis than luminal tumors. However, our results imply that in the development of therapeutic strategies for blocking STAT3 in breast cancer cells, the strong dependence on the cellular context that this factor activity displays should be taken into account.

Mechanical stress is a relevant factor to induce adaptive responses in multiple cell types [12–16]. Importantly, the signaling pathways triggered by this stimulus in those different examples also play a relevant role during mammary gland involution. Therefore, it was proposed that upon weaning, milk accumulation may cause cell stretching that, in turn, would induce the initiation of the molecular cascades that lead to the remodeling process of the lactating gland. To address this issue, we designed a new practical device that allowed us to evaluate the effects of radial stretching on the HC11 nontumorigenic mammary epithelial cell line cultured on flexible silicone membranes. The results showed that, as previously observed in other cell types, mechanical stress induced ERK1/2 phosphorylation and c-Fos expression induction, as well as LIF secretion, STAT3 activation, and AKT phosphorylation inhibition. Therefore, mechanical strain is able to induce weaning-associated events in cultured mammary epithelial cells [17].

STAT3 is essential, but not sufficient for the onset of apoptosis during mammary involution, as expression of a constitutively active *Akt*, a downstream effector of the phosphoinositide-3-OH kinase (PI3K) pathway, provides an overriding survival signal after lactation [18]. However, AKT downregulation depends on STAT3 activation, since PI(3)K regulatory subunits p55α and p50α (each of them, when overexpressed, reduces levels of activated AKT) are induced by that transcription factor during mammary involution. In fact, it has been shown that STAT3 binds directly to the promoters of p55α and p50α subunits *in vivo* and in STAT3 KO mice, upregulation of p55α and p50α is abrogated, levels of activated AKT are sustained, and apoptosis is prevented [19]. In addition, it was shown that deletion of both p55α and p50α subunits reduced cell death as well as expression and activity of cathepsin L during mammary involution. This protease participates in lysosomal-mediated programmed cell death (LM-PCD), which is upregulated during normal involution by activated STAT3. Furthermore, involution is delayed in cathepsin L-deficient mice, suggesting that the p55α/p50α subunits mediate cell death in part by elevating the level of cathepsin L. Surprisingly, it was found that during involution, p55α/p50α localize to the nucleus where they bind to chromatin and regulate transcription of a subset of inflammatory/acute phase genes that are also STAT3 targets. Therefore, these findings revealed that postlactational regression of the

mammary gland is accomplished through a nonclassical, lysosomal-mediated pathway of cell death, in which PI3K regulatory subunits participate as main regulators [20]. In fact, it has been demonstrated that cell death of mammary epithelium after weaning does not depend on the activation of executioner caspases 3, 6, and 7, although it requires STAT3 for cathepsin B and L induction as well as for the downregulation of their endogenous inhibitor Spi2A [21].

Global gene expression changes during involution have been profiled by microarray analysis, which allowed characterization of clusters of genes with distinct expression profiles during the first 4 days of involution. Such expression profiling led to the observation that one of the most strikingly upregulated genes in the absence of STAT3 is the serpin Spi2a. Interestingly, during mammary involution, STAT3 not only regulates LM-PCD by inhibiting serpin Spi2a, inducing the expression of cathepsins B and L, and the regulatory subunits p55α/p50α, but also by the uptake of secreted MFGs that lead to the formation and fusion of large lysosomal-like vacuoles, which are toxic to epithelial cells. Upon re-entry, the MFG (mammary fat globules) triglycerides are metabolized to free fatty acids, including oleic acid, that can distort membranes and result in leakage of cathepsins from lysosomes. Therefore, STAT3 promotes a phenotypic switch from secretion to phagocytosis of MFGs, the latter function delivering triglyceride to vacuoles with the ensuing consequences of LMP and cell death [22].

3. NF-κB signaling

It is clear that there are multiple mechanisms of regulation in early involution that synergise to ensure efficient induction of cell death, phagocytosis, suppression of inflammation, and remodeling of the architecture of the gland. Clarkson and Watson identified clusters of genes that are transcriptional targets of either NF-κB or STAT3, or indeed both, during early involution [23]. For example, among the NFκB targets, the TNF superfamily of death receptor (DR) ligands have been detected. These proteins induce apoptosis through binding to their receptor, which recruits caspase 8 (via FADD) and activates executioner caspases, finally leading to cell death [24]. Specifically, *Tnf*, *Tnfsf4*, *Tnfsf6*, *Tnfsf7*, *Tnfsf10*, and *Tnfsf12* are induced transiently at 12 h after weaning, and the proteins Fas ligand, TNF-α, TWEAK, and TRAIL are able to activate extrinsic apoptosis through their cognate receptors Fas, TNFR-1, TNFR-2, DR3, and DR4. The genes for the first two of these receptors (*Fas* and *Tnfrsf1a*) were also induced, and maximally coexpressed, within 24 h of weaning. NF-κB activity also correlated with the rapid activation of these TNF superfamily ligands [25].

Particularly, about TNF-α, our results have shown that this factor, through TNF-α receptor-2 (TNFR2) binding induces LIF expression mediated by ERK1/2 activation in nontumorigenic mouse mammary epithelial cells. In addition, the AP-1 has been implicated in this signaling cascade, since blocking the activity of this transcription factor resulted in a significant reduction of TNF-α induced LIF expression. Therefore, TNF-α may contribute to mammary gland involution by, among other activities, eliciting LIF expression through ERK1/2 and AP1 activation [26].

The NF-κB family of transcription factors primarily plays anti-apoptotic roles. DNA binding activity of this transcription factor is markedly upregulated within 3 h of forced involution and is suggested to promote survival of a subpopulation of mammary epithelial cells [25]. This hypothesis is consistent with the paradigm of NF-κB–mediated suppression of TNF-α cytotoxicity in TNF-α–responsive cells. NF-κB activity is mediated by a multiprotein signaling complex called the IκB kinase (IKK), which consists of two catalytic subunits: IKK1/α, IKK2/β and a regulatory subunit, NEMO (NF-kappa-B essential modulator). Activation of this complex leads to phosphorylation of the IκB proteins; phospho-IκB is rapidly ubiquitinated and degraded via the 26S-proteasome releasing NF-κB and unmasking its nuclear localization signal, allowing its activity as transcription regulator of many target genes [27]. NF-κB then inhibits the death signal by trans-activating genes that promote resistance to apoptosis. The effect of this negative feedback mediated by NF-κB is the modulation of apoptosis in response to the TNF-α death signal. However, deletion of the gene encoding IKK2 resulted in delayed apoptosis and remodeling, as well as blockade of caspase 3 activation in the postlactational mammary gland. This failure to induce cell death was associated with reduced expression of TNF and its receptor TNFR1, which are known NF-κB targets. In addition, the observed high levels of active AKT together with downregulation of TWEAK, another DR ligand, also contributed to retard the involution process in these genetically engineered mice [28]. These results suggest that NF-κB may provide either proapoptotic or antiapoptotic signals during involution, depending on the timing and cellular context in which this transcription factor is activated.

4. Gene expression regulation at the level of mRNA stability

It has been demonstrated that the stability of many messenger RNAs (mRNAs) encoding onco-proteins, chemokines, cytokines, and other inflammatory mediators is controlled by AU-rich elements (AREs), sequences located within the 3′-UTR of many transcripts [29, 30]. ARE-directed control of mRNA decay is mediated, in part, through interactions with specific ARE-binding proteins (AUBPs). One such protein is tristetraprolin (TTP), which accelerates the decay of targeted transcripts [31]. During inflammation, TTP plays a relevant role destabilizing different mRNAs, participating in glucocorticoid-mediated anti-inflammatory activity [32–34] and inhibiting NF-κB signaling [35]. The relevance of TTP as a negative regulator of these processes has been demonstrated by the severe chronic inflammation displayed by multiple tissues in TTP-KO mice, which was mostly due to the dramatic increase of TNF-α levels [32].

Several reports indicate that TTP participates in the inhibition of tumor progression. It has been shown that TTP mRNA levels are significantly decreased in many tumor types, including breast cancer [36]. We have also reported that TTP expression is lower in all breast cancer types compared with normal mammary tissue, and high levels of this protein negatively correlate with cancer cell aggressiveness. Interestingly, we have also determined that in the mouse mammary gland, expression of this protein reaches the highest level during lactation, and can be induced in culture by treatment with lactogenic hormones [37].

These studies reveal a new potential biological role for this tumor suppressor protein in mammary epithelium, since TTP might protect the tissue from inflammatory and/or remodeling activities that would trigger involution of the gland. Interestingly, our unpublished results show that by reducing TTP expression in the differentiated mammary epithelium, cell death is induced in the midst of lactation without requirement of additional stimuli. Then, TTP is not (or at least not only) a mechanism of surveillance, which prevents an eventual increase of inflammatory factors that might lead lactation to a halt, but it actually functions as a survival factor in the mammary epithelium, since reducing its levels is enough to induce cell death and involution of this tissue.

5. Mammary involution and cancer

Breast cancer is the most frequent malignancy diagnosed in association with pregnancy [38–40]. In addition, different studies have demonstrated an increase in breast cancer risk in the years immediately following giving birth [41–43]. Importantly, not only a rise in breast cancer incidence during the postpartum years has been observed, but also a higher risk for poor outcomes in women diagnosed during that timeframe [44]. However, it has been well established that pregnancy provides lifetime protection for women who are under 35 years at first birth [45–47]. Therefore, pregnancy would exert two opposite effects on breast cancer development: induces protection, which is associated with the differentiation of mammary epithelium, and increases risk, through alteration of tissue microenvironment.

Postpartum breast cancers have been referred to as type II Pregnancy-Associated Breast Cancer (PABC) to distinguish these cancers from those diagnosed during pregnancy [45]. A physiological window unique to type II PABC is mammary gland involution and, in an effort to distinguish why type II PABC patients have worse prognoses, the normal postpartum breast microenvironment has been investigated for potential tumor-enhancing attributes. These studies reveal that postpartum involution utilizes wound-healing programs for gland remodeling, including increases in matrix metalloproteinase activity, release of bioactive fragments of extracellular matrix (ECM) termed matricryptins, accumulation of fibrillar collagen, and influx of immune cells, which also generates tumor-promotional microenvironments [48–51]. *In vivo* experiments showed that tumors from xenografts of breast cancer cells exposed to the postpartum involution microenvironment have increased growth, invasion, and metastasis compared to those growing in nulliparous hosts. In addition, these implants showed augmented fibrillar collagen accumulation and high cyclooxygenase-2 (COX-2) expression [52]. Coincidently, in the postlactating mammary gland, inhibition of COX-2 reduced the collagen fibrillogenesis, as well as tumor development and cancer cell invasiveness [53]. Therefore, it has been proposed that women at high risk for postpartum breast cancer might benefit from treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) during postpartum, which would reduce COX-2 expression and its consequences on the behavior of breast initiated cells.

Without doubt, immune response plays a primordial role in mouse mammary involution, since molecular profiling of that phase is consistent with acute phase, innate and adaptive

immune responses [51–54]. Interestingly, after weaning, mammary epithelial cells themselves express transcripts traditionally associated with immune cells [55, 56] and acquire phagocytic capability [57]. Therefore, it has not been possible to completely determine which cell types and in what part are responsible for the observed immune-like gene signatures. Particularly, there is not much data about the participation of adaptive immune cells, but innate immune cell populations have been partially characterized. Specifically, it has been observed that granulocyte infiltration in the mouse gland on the first day of involution suggest the involvement of this cell type in early involution [58]. In addition, resident macrophages seem to be required for this phase, while infiltrating macrophages are important during the remodeling stage [52]. It was observed that on this last phase, macrophages express low iNOS, high arginase-1, and the mannose receptor, which is consistent with alternative activation or M2 polarization of these cells [59]. Importantly, this phenotype correlated with breast tumor promotion in patients [60] and murine mammary tumor progression [61].

The earlier mentioned STAT3 and NF-κB signaling pathways, as well as others involving transforming growth factor beta (TGF-β) and the retinoid acid receptors (RARs)/retinoid X receptors (RXRs), participate in mammary gland involution as well as in breast cancer development. After weaning, target genes of RARα/p300 and RelA/p65, which belong to the NF-κB protein family, are induced, and high activity of the proteins coded by these genes, e.g., *MMP9*, *Capn1*, and *Capn2*, has been detected in breast cancer cells. Calpains belong to a family of calcium-dependent intracellular cysteine proteases involved in a wide variety of physiological and pathological processes. These proteases are heterodimers, consisting of a small regulatory subunit, encoded by CAPN4 gene, common for both members, and a large catalytic subunit encoded by either CAPN1 or CAPN2. During mammary gland involution and cancer progression, these proteins are relevant for modifying the extracellular matrix, allowing tissue remodeling and/or cell invasion. In addition, calpains also cleave intracellular proteins located in the cell membrane, lysosomes, mitochondria, and nuclei, favoring cell death during involution and cell anchoring loss during tumor progression [62].

6. Conclusion

As in other physiological processes, the use of conditional knockout mice and the application of high-throughput techniques have been very useful to understand that normal postlactation mammary gland involution relays on the fine-tune coordination of multiple signaling pathways. Although involution is a physiologically normal, developmentally orchestrated tissue-remodeling process, it shares striking similarities with pathologically induced wound-healing and tumor-promotional microenvironments. This highlights the relevance of further investigating this process, since it may yield novel therapeutic targets or prognostic markers for breast cancer. Importantly, studying this particular phase of mammary gland biology may help us to pinpoint subtle changes in a pro-oncogenic

environment that determines the derailment from normal physiological to pathological tissue behavior.

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