TIMP3 Regulates Mammary Epithelial Apoptosis with Immune Cell Recruitment Through Differential TNF Dependence

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Abstract

Post-lactation mammary involution is a homeostatic process requiring epithelial apoptosis and clearance. Given that the deficiency of the extracellular metalloproteinase inhibitor TIMP3 impacts epithelial apoptosis and heightens inflammatory response, we investigated whether TIMP3 regulates these distinct processes during the phases of mammary gland involution in the mouse. Here we show that TIMP3 deficiency leads to TNF dysregulation, earlier caspase activation and onset of mitochondrial apoptosis. This accelerated first phase of involution includes faster loss of initiating signals (STAT3 activation; TGFβ3) concurrent with immediate luminal deconstruction through E-cadherin fragmentation. Epithelial apoptosis is followed by accelerated adipogenesis and a greater macrophage and T-cell infiltration in Timp3−/− involuting glands. Crossing in Tnf deficiency abrogates caspase 3 activation, but heights macrophage and T-cell influx into Timp3−/− glands. The data indicate that TIMP3 differentially impacts apoptosis and inflammatory cell influx, based on involvement of TNF, during the process of mammary involution. An understanding of the molecular factors and wound healing microenvironment of the postpartum mammary gland may have implications for understanding pregnancy-associated breast cancer risk.

Introduction

Tissue involution is a postnatal process that offers a unique window to study physiological cell death. Involution occurs in the thymus [1], prostate [2], uterus and mammary gland [3]. These tissues are subject to physiological cues that instruct them to undergo apoptosis to maintain cell number and homeostasis. In humans, the mammary gland undergoes extensive involution after the cessation of lactation [4], and this process has been widely studied in murine models. Post-lactational mammary gland involution serves to remodel the highly structured secretory gland into one that resembles the virgin state so that the differentiation and involves widespread apoptosis and tissue remodeling, with extracellular matrix turnover and structural reorganization of the gland [19]. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) regulate matrix turnover during pubertal morphogenesis [20,21], gestation, lactation [22], and involution [23,24,25]. While the first phase of involution is considered to be protease-independent [11], the second protease-dependent stage has decreased levels of TIMP and activated MMPs which initiate the deconstruction of the surrounding stroma therefore facilitating gland remodeling. At the same time, MMP activity has also been proposed to influence adipogenesis [8]. Synchronization of
multiple compartments is essential for maintaining the functional integrity of the mammary gland during the phases of involution.

Interestingly, microarray and histological studies have revealed that inflammatory mediators are present during mammary gland involution [9,10]. These arrays suggest the death receptor family involvement in the first phase of involution and the involvement of monocyte and lymphoid cytokines during the second phase [26]. Macrophages in particular have been found in the involuting glands of several species, and respond to the chemotactic signals provided by the remodeling matrix [27]. The involuting mammary gland microenvironment has been likened to wound healing and cancer environments [28]. Macrophages are known to play a role in involution, the wound healing response, and are also implicated in promoting breast cancer metastasis [27,29]. Given that pregnancy associated breast cancers (PABC) have a poor patient outcome [30,31,32], a better definition of molecular factors that differentially regulate apoptosis from inflammatory cell recruitment will enhance our understanding of the processes underscoring physiological mammary involution and mammary cancer progression.

Both gain- and loss-of-function studies underscore the complex apoptotic role of TIMP3 [33]. TIMP3 is a negative regulator of inflammation in specific tissues [34,35,36] and in systemic responses to endotoxin [37]. TIMP3 deficient mice have accelerated post-lactation involution and fail to reinitiate lactation after 48hrs [23]. In this study, we ask whether TIMP3 functions to integrate the distinct programs of apoptosis and inflammation during homeostatic remodeling of post-lactational mammary involution. We identify the molecular and cellular events underlying the accelerated mammary gland involution in Timp3−/− mice. TIMP3 deficiency leads to a loss of the survival signal provided by E-cadherin-mediated cell to cell contact as well as an accelerated first phase of involution. A far greater and sustained influx of macrophages also occurs, which is coupled with an infiltration of T-cells in Timp3−/− involuting glands. Combining the TIMP3 and TNF deficiencies, we further dissect the TNF dependency of these molecular and cellular events. Our results show that TIMP3 is a critical physiological regulator for both apoptosis and inflammatory cell recruitment during murine mammary gland involution.

Results

Higher TNF levels and accelerated apoptosis during mammary involution in Timp3−/− mice

TIMP3 is the only TIMP capable of inhibiting the TNF alpha converting enzyme (TACE/ADAM17) [38,39]. Timp3−/− mice have increased TNF levels and signaling owing to increased TACE activity in several systems [34,35,37]. We measured mammary TNF levels, which were detectable at the end of lactation, and again on day 4 of involution (4i) in wild-type mice. Timp3−/− mammary gland also displayed two distinct periods of TNF increase. It was 4-fold higher than wild-type at 10L, reappeared earlier and stayed higher than in wild-type tissue (Fig. 1A). Fas and FasL levels did not differ between wild-type and Timp3−/− glands throughout involution (data not shown). Death receptor-mediated apoptosis induced by TNF occurs through activation of the initiator caspase, caspase 8 [40]. Consistent with the elevated TNF levels in Timp3−/− glands, the cleaved form of caspase 8 was detectable at 10L and elevated earlier than wild-type (Fig. 1B).

We next examined the executioner arm of mitochondrial apoptosis, a multiprotein complex known as the apoptosome, which is comprised of cytochrome c, Apaf-1, and procaspase 9 [41,42,43]. These components showed an earlier temporal shift upon the loss of TIMP3 (Fig. 1B). Notably, Apaf-1 was present on 1i and 2i coinciding with the timing of maximal epithelial apoptosis in Timp3−/− mammary glands (Fata et al. 2001), but absent in wild-type tissue. Caspases 3 and 9 were also clearly activated earlier in Timp3 null tissue as indicated by the presence of their cleaved forms (Fig. 1B). The loss of mitochondrial membrane integrity results in the release of cytochrome c into the cytoplasm [44]. Immunofluorescence showed mitochondrial cytochrome c release within luminal epithelial cells at 1i in Timp3−/− glands, but only at 3i in wild-type (Fig. 1C, arrows). The number of epithelial cells shed into lumens (Fig. 1C, arrowheads) with cytochrome c release was present in greater numbers in Timp3−/− glands compared to wild-type at 1i. We also observed apoptosis-inducing factor (AIF) expression to be earlier than wild-type in Timp3−/− involuting glands (Fig. 1B). The levels of second mitochondria-derived activator of caspase (SMAC), a mitochondrial-derived inhibitor of IAPs (inhibitors of apoptosis) [45], decreased sooner in Timp3−/− involuting glands (Fig. 1B). Thus, an earlier progression to apoptosis was indicated by the rapid appearance of several caspases and the loss of mitochondrial integrity in Timp3−/− involuting mammary glands.

Mapping of first phase of involution and adipogenic signals

Murine mammary gland involution occurs in two phases where involution is reversible in the first 48 hours. Timp3 null mice fail to re-establish lactation after 48 hours of involution [23]. STAT3 is known to be involved in this early phase and STAT3 loss, or loss of its upstream activators, abrogates the first phase and delays the onset of involution [17,46,47]. We observed that total STAT3 was upregulated earlier in Timp3−/− mammary gland. STAT3 was phosphorylated immediately upon induction of involution in both wild-type and Timp3−/− mammary glands, but activation of STAT3 was turned off earlier in Timp3−/− and not seen past 3i (Fig. 2). Milk stasis during involution also rapidly induces TGFβ-3 levels in the mammary gland to induce apoptosis [15]. We observed that Tgfβ-3 expression lasted until 2i in wild-type glands, but was undetectable after 1i in Timp3−/− glands (Fig. 2) consistent with the rapid completion of the first phase of involution.

In mammary involution, adipogenesis is important for the re-establishment of the pre-pregnant state filling out the space left behind by epithelial apoptosis [8]. We therefore examined markers of adipocyte differentiation: CEBPb for early [40] and PPARγ for late adipocyte differentiation, and ALBP as a marker for adipocytes [49]. Generally, an earlier and increased levels of PPARγ and ALBP coincident with decreased CEBPb levels indicated faster onset of the adipogenic program (Fig. 2).

E-cadherin breakdown compromises epithelial architecture in Timp3−/− mammary glands

We determined whether TIMP3 loss affected E-cadherin-mediated cell-cell contact since this adhesion molecule is processed by MMPs [50,51], and we earlier reported an aberrant MMP2 activation during mammary involution in Timp3−/− mice [23]. E-cadherin breakdown was evident by 2i in wild-type mammary gland. On the other hand, an immediate and far greater fragmentation of E-cadherin occurred at 1i in Timp3−/− mammary tissue (Fig. 3), which was even present at day 10 of lactation (10L), suggesting that this tissue may have compromised epithelial cell-cell contact during lactation. Also notable was the overall elevated level of E-cadherin in Timp3−/− glands. There
Figure 1. Increased TNF levels and apoptotic signaling in Timp3−/− involuting glands. (A) TNF levels as assessed by ELISA at different involution timepoints starting from day 10L until day 7i revealed higher levels of TNF levels in Timp3−/− glands (n = 3) compared to wild-type (WT, n = 3) both prior to the onset of involution and as an earlier rebound. (B) Representative western blot analyses of lysates from different involution timepoints probed for caspase 8 (Casp 8), Apaf-1, cytochrome c (Cyt c), caspase 9 (Casp 9; top band represents pro-caspase 9, bottom band represents cleaved caspase 9; ND = not determined for 7i), cleaved caspase 3 (Casp 3), Apoptosis-inducing factor (AIF), and Second mitochondria-
derived activator of caspase (SMAC). (C) Immunofluorescence and co-localization of mitochondria (MitoTracker) and cytochrome c revealed earlier release of cytochrome c from the mitochondria (red signal, arrows) in Temp3\(^{-/-}\) luminal cells, as well as cells that have detached and were shed into the lumen by 1i, than in wild-type at 3i. Bar graphs are expressed as mean ± SEM (* = P<0.02). Scale bar = 30 μm.

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was a corresponding disappearance of β-catenin by 4i in Temp3\(^{-/-}\) involuting glands (Fig. 3) in contrast to wild-type tissue, suggesting the earlier destabilization of E-cadherin-mediated adhesions consistent with compromised cell-cell contact in this tissue. Altogether the data illustrated in Figures 1, 2, and 3 provide a map of key molecular signals involved in physiological apoptosis and adipogenesis in the mammary gland, where TIMP3 deficiency leads to an earlier deconstruction and remodeling of the epithelial compartment.

Re-establishment of survival signaling by recombinant TIMP3

Next we asked whether TIMP3 plays a direct role in inhibiting E-cadherin breakdown and TNF bioactivity during mammary involution. TIMP3-containing slow-release Elvax pellets were implanted distal to the lymph node in the fourth inguinal mammary fat pad at the onset of involution and tissue immediate to the pellet was collected at 3i. Reconstitution of TIMP3 rescued E-cadherin fragmentation in Temp3\(^{-/-}\) mice (Fig. 4A). The increased TNF level in Temp3\(^{-/-}\) glands was also significantly reduced (P<0.01) by this manipulation (Fig. 4B). Furthermore, activation of caspase 8 was reduced in recombinant cell-containing null tissue (Fig. 4C). These data show that TIMP3 protein could rescue the alterations associated with accelerated mammary involution in Temp3\(^{-/-}\) mice.

Temp3 deficiency initiates inflammatory and immune cell recruitment in involuting glands

Historically, studies in dairy animals (cow, sheep and goat) have described neutrophil and macrophage infiltration during the process of mammary gland involution [52,53,54]. Microarray studies in the mouse also support an inflammatory component to involution [9,10]. Since we previously identified TIMP3 as a negative regulator of inflammation in several systems [34,55], we assessed the infiltration of inflammatory cells into involuting mammary glands. Neutrophil influx occurred during involution and was comparable between wild-type and Temp3\(^{-/-}\) involuting glands (data not shown). In contrast, macrophage numbers were comparable during lactation, but increased up to 4-fold (P<0.01) in Temp3\(^{-/-}\) involuting glands at early stage involution (1i and 2i) when compared to wild-type glands, and rapidly declined thereafter at 3i (Fig. 5A). The maximal macrophage influx in wild-type glands was at 3i, however these did not reach levels seen in Temp3\(^{-/-}\) glands. This represented an abrupt and immediate initiation of macrophage infiltration in mammary glands lacking TIMP3.

The presence of lymphocytes has been indicated in the involuting mammary glands of ruminants [56,57], but has yet to be studied in the murine model. Given that lymphocytic infiltrates were found in aged Temp3\(^{-/-}\) livers [34], we measured the infiltration of B and T-cells in involuting mammary glands. CD3-positive T-cells were present in alveolar and ductal epithelium, including interalveolar and periductal areas, at low levels in wild-type but were elevated in Temp3\(^{-/-}\) glands throughout involution. The number of T-cells in involuting Temp3-deficient glands peaked at 3i and was sustained until 5i. The wild-type tissue displayed similar kinetics but a smaller increase of these cells. The T-cell infiltration exhibited different kinetics compared to the macrophage influx, temporally occurring subsequent to macrophage infiltration (Fig. 5B). B cells were very sparse in the mammary gland and remained comparable between wild-type and Temp3\(^{-/-}\) glands, as assessed by anti-B220 immunohistochemistry (data not shown).

The role of TNF in mammary involution of the Temp3 deficient gland

TIMP3 regulates the bioactivity of the inflammatory cytokine TNF since it is the physiological inhibitor of the TNF sheddase TACE. We have shown TIMP3 to be a critical regulator of physiological systems that depend on TNF such as liver regeneration and apoptosis [34,58] and the innate immune response to endotoxins [36,37]. We therefore asked whether increased apoptosis and inflammatory reaction during mammary involution in Temp3\(^{-/-}\) mice was dependent on increased TNF
bioactivity, by combining the Timp3 and Tnf deficiencies. For these experiments we used Timp3 mice that we backcrossed seven times into the C57BL/6 background to match the Tnf deficient mice, whereas the data in Figures 1, 2, 3, 4, and 5 were generated using pure FVB/N Timp3 mice. Activation of caspase 3 is a measure of apoptosis downstream of death receptor signaling by TNF, and its positivity has been previously reported in regressing mammary glands [59]. The percentage of mammary epithelial cells that stained positive for active caspase 3 as quantified by histomorphometry of single (Timp3+/− or Tnf−/−) and compound (Timp3−/−/Tnf−/−) knockouts in the C57BL/6 background at 1i and 3i (Fig. 6A). The higher number of activated caspase 3-positive cells in Timp3−/− glands compared to wild-type confirmed greater apoptosis in C57BL/6 Timp3−/− mice at 1i similar to FVB/N Timp3−/− strain [23]. Also as expected, the apoptosis index was higher in wild-type at 3i compared to Timp3−/− mice, which coincide with the expected peak of epithelial apoptosis in involuting wild-type glands. Deletion of Tnf in Timp3 null mice resulted in significantly reduced cleaved caspase 3 positive cells compared to Timp3−/− at 1i, showing that the lack of TNF rescued the accelerated apoptosis in Timp3−/− glands (P<0.05; Fig. 6A). A control group with TNF deletion alone also showed reduced numbers of epithelial cells with activated caspase 3 at 3i, also confirming the role of the TNF pathway during normal mammary involution.

We next examined whether the greater inflammatory cell influx seen during mammary involution of Timp3−/− mice depended on TNF (Fig. 5, 6B, C). Intriguingly at 1i, the number of F4/80-positive macrophages was increased by at least 4-fold in Timp3−/−/Tnf−/− when compared to Tnf−/− glands, and up to 8-fold (P<0.01) when compared to Timp3−/− glands (Fig. 6B, 5C). At 3i, the overall numbers of macrophages subsided and became comparable between single and compound knockout groups, but still remained higher than that in wild-type glands (Fig. 6B). Examination of CD3-positive T-cells revealed a significant 3-fold increase (P<0.05) at 1i following compound removal of TIMP3 and TNF (Fig. 6C). This trend of increased T-cell recruitment was maintained at 3i in Timp3−/−/Tnf−/− involuting glands (P<0.05; Fig. 5C). Contrary to our expectation the inflammatory and immune cell influx was not ameliorated by adding Tnf deficiency to Timp3-deficient mice. This series of experiments indicated that TNF is required for accelerated mammary epithelial apoptosis but not immune cell influx of Timp3 null involuting glands.

**Discussion**

The rapid mammary involution established in the Timp3 null mouse provides a model to study the different phases and cellular compartments of this remodeling tissue. We analyzed several key molecules essential for epithelial cell survival and explored inflammatory cells during mammary gland regression, as temporally mapped in Figure 7. The loss of the extracellular metalloproteinase inhibitor, TIMP3, accelerated the first phase of involution leading to an early onset of the irreversible second phase [23]. We observed extensive E-cadherin fragmentation and an immediate onset of TNF-induced apoptosis coinciding with...
pSTAT3 activation and TGFβ3 expression, which are recognized initiators of involution. The second phase was marked by early adipogenesis and prominent immune cell infiltration. The ability of TIMP3 to differentially impact specific aspects of inflammation that have been proposed during involution[26], such as death receptors and ligands during the first phase and cytokine/chemokines dependent immune cell influx during the second phase, is revealed upon its combination with TNF deficiency.

An early event in Timp3−/− mammary gland involution is E-cadherin fragmentation and the loss of β-catenin resulting in the loss of a critical survival signal that provides structural integrity. Targeted deletion of E-cadherin in the mammary gland leads to unscheduled apoptosis during pregnancy such that the gland resembles involuting tissue [60]. Vallorasi et al. have shown that
the truncation of E-cadherin is present at the initiation of mammary involution [61]. Synthetic MMP inhibitors prevent E-cadherin processing [50,62] and we have found increased MMP2 activation in a variety of Timp3 deficient systems [35,63,64]. In particular, the immediate activation of MMP2 at the onset of mammary involution in Timp3 deficient glands [23] coincides with E-cadherin fragmentation, which is mitigated by reconstitution with recombinant Timp3. The presence of fragmentation products at 10L suggests that epithelial structures may even be compromised during lactation in Timp3 null mice. We propose that the loss of E-cadherin triggers an impending change in mammary architecture that sensitizes to apoptosis. Beyond mammary gland involution, another in vivo model has demonstrated that the loss of TIMP3 sensitizes tissues to cell death pathways. During liver regeneration the loss of TIMP3 initiates an apoptosis program due to excessive TNF [34]. In this study, TNF dysregulation was concurrent with increased caspase 8 activation and accompanied by an accelerated timeline of mitochondrial apoptosis in Timp3−/− involuting mammary glands. The immediate peak of apoptosis in Timp3−/− glands was attenuated by the genetic ablation of TNF, thereby confirming the role of TNF in the first phase of involution.

The loss of TIMP3 created an environment with greater macrophage infiltration in comparison to wild-type mammary glands. It is likely that this inflammatory influx does not initiate apoptosis, because the peak of macrophage infiltration follows the peak of apoptosis in both wild-type and Timp3−/− glands. Apoptotic cell bodies must be removed by phagocytosis either by neighboring epithelial cells or by professional phagocytes such as macrophages in order to maintain tissue homeostasis during mammary involution [65,66,67,68]. The number of CD3+ T-cells were also elevated and sustained in Timp3−/− glands following the influx of macrophages. To date, lymphocyte infiltration has only been described in bovine and ovine mammary gland involution [56] and their function in the mammary gland remains to be defined. We observed a pronounced macrophage and T-cell influx upon the compound loss of Timp3 and TNF, indicating a TNF-independent mechanism for their recruitment into a Timp3-deficient microenvironment. Compound Timp3 and TNF deficiency in the heart also leads to a 5-fold greater neutrophil infiltration in an otherwise non-inflammatory system [35]. Since involution related inflammation has been theorized to play a role in PABC, this separation of epithelial apoptosis from inflammation provides insight into the mechanisms of inflammation during this critical period.

Our results highlight the pleiotropic ability of the extracellular metalloproteinase inhibitor TIMP3 during mammary gland involution. TIMP3 influences distinct processes through TNF-dependent and TNF-independent pathways with its loss accelerating and amplifying specific involution events. TIMP3 acts as a safeguard against untimely physiological cell death, unscheduled inflammatory response, and premature loss of mammary gland function. A greater understanding of apoptotic and inflammatory regulators provides insight into PABC-related risk factors.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with all efforts made to minimize suffering. The protocol was approved by the University Health Network Animal Care Committee (Animal Use Protocol #812).
Mice and Experimental Involution

For the majority of the analyses, wild-type and Timp3−/− mice were in FVB/N background. For the compound knockout studies with TNF, wild-type, Timp3−/−, Tnf−/−, and Timp3−/−/Tnf−/− mice were all in C57/BL6 background. Forced experimental involution was performed as previously described [23] according to guidelines established by the Canadian Council for Animal Care under protocols approved by the Ontario Cancer Institute Animal Care Committee.

Antibodies and Reagents

The antibodies used were: Apaf-1 (Upstate); caspase 9 and activated caspase 3 (Cell Signaling); E-cadherin, β-catenin, cytokeratone c, SMAC, and Mitotracker CD3, F4/80, B220 (BD Biosciences); anti-neutrophil antibody (Serotec); AIF (gift from Dr. J. Penninger); caspase β (gift from Dr. R. Hakem). Recombinant human TIMP3 was prepared according to the manufacturer’s specifications (R&D Systems).

Protein collection, western blotting, and TNF ELISA

Mammary gland collection and lystate preparation were performed as previously described [23]. A total of 3 independent, non-sibling females were used for each timepoint per genotype. Protein quantification was performed using DC Biorad Assay as per manufacturer’s instructions. Equal loading was rigorously assessed and adjusted for, based on amido black staining of newly transferred gels and silver staining of SDS-PAGE gels that were electrophoresed in parallel. The amido black-stained gels and silver-stained gels were included and labeled as ‘Protein’ in the Figures. For western blotting experiments, 50 μg of total protein were loaded onto SDS-PAGE gels. Densitometry analyses were performed using ImageQuant and Northern Eclipse software. TNF ELISA was performed as previously described [34].

Pellet implantation

50 μg of recombinant human TIMP3 (R&D Systems) were loaded onto pellets as per manufacturer’s instructions. Implantation of pellets was done as previously described [23].

Confocal microscopy and Immunocytochemistry

Two photon confocal microscopy was used for all immunofluorescence and immunocytochemistry analyses. Exposure time, gain, and offset parameters were first empirically determined and then kept constant throughout. Preparation of mammary glands for immunofluorescence and immunocytochemistry were performed as previously described [23]. Depending on the primary antibody used, control IgG antibodies and secondary antibodies alone were used for controls to assess specificity of primary antibodies.

Immunohistochemistry and Histomorphometry

Preparation of mammary glands for immunohistochemistry were performed as previously described [23], using 1:50 dilutions of anti-activated caspase 3 (Cell Signaling); anti-CD3, anti-F4/80, and anti-B220 (BD Pharmingen); and anti-neutrophil (Serotec). Histomorphometric counts of were performed on the fourth inguinal gland, using 10 random fields distal to the lymph node.

Statistical Analyses

Statistical analyses were performed for observations that have at least three mice per group. Student’s t test was performed for statistical analyses between two groups (WT and Timp3−/−). One-way ANOVA was performed for statistical comparison between four groups (WT, Timp3−/−, Tnf−/−, and Timp3−/−/Tnf−/−), followed by Tukey test for pair-wise statistical analysis.

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Author Contributions

Conceived and designed the experiments: CVH RK. Performed the experiments: CVH. Analyzed the data: CVH HWJ RK. Wrote the paper: HWJ RK.

References

1. Garcia-Suarez O, Perez-Perez M, Germana A, Esteban I, Germana G (2003) Involvement of growth factors in thymic involution. Microse Res Tech 62: 514–523.
2. Kwong J, Choi HL, Huang Y, Chan FL (1999) Ultrastructural and biochemical observations on the early changes in apoptotic epithelial cells of the rat prostate induced by castration. Cell Tissue Res 298: 125–136.
3. Niles-Hamilton M, Liu Q, Ryan J, Bendickson L, Leapot P, et al. (2003) Tissue involution and the acute phase response. Ann N Y Acad Sci 95: 94–108.
4. Howard BA, Gusterson BA (2000) Human breast development. J Mammary Gland Biol Neoplasia 5: 119–137.
5. Green KA, Streuli CH (2004) Apoptosis regulation in the mammary gland. Cell Mol Life Sci 61: 1867–1883.
6. Hemighausen L, Robinson GW (2005) Information networks in the mammary gland. Nat Rev Mol Cell Biol 6: 715–725.
7. Wiersman BS, Werb Z (2002) Stromal effects on mammary gland development and breast cancer. Science 296: 1046–1049.
8. Alexander CM, Selvarajan S, Madgett J, Werb Z (2001) Stromelysin-1 regulates adipogenesis during mammary gland involution. J Cell Biol 152: 693–703.
9. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ (2004) Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Res 6: R92–R109.
10. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, et al. (2004) Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving ILBP, CD14 and STAT3. Breast Cancer Res 6: R75–R91.
11. Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, et al. (1996) Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and dependent pathways. Development 122: 181–193.
12. Stein T, Salomonis N, Guerterson BA (2007) Mammary gland involution as a multi-step process. J Mammary Gland Biol Neoplasia 12: 25–35.
13. Vaeola AM, Ip MM (1996) Tumor necrosis factor-alpha: a multifunctional regulator of mammary gland development. Endocrinology 137: 4915–4924.
14. Song J, Sapi E, Brown W, Nilsen J, Tartaro K, et al. (2000) Roles of Fas and Fas ligand during mammary gland remodeling. J Clin Invest 106: 1209–1220.
15. Nguyen AV, Pollard JW (2000) Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. Development 127: 3107–3118.
16. Humphreys RC, Bier B, Zhao L, Ruz R, Levy D, et al. (2002) Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. Endocrinology 143: 3641–3650.
17. Chapman RS, Lourenc RC, Tummer E, Flint DJ, Selbert S, et al. (1999) Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev 13: 2604–2616.
18. Marti A, Ritter PM, Jager R, Lazar H, Baltzer A, et al. (2001) Mouse mammary gland involution is associated with cytochrome c release and caspase activation. Mech Dev 104: 89–98.
19. Fata JE, Werb Z, Bisell MJ (2004) Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. Breast Cancer Res 6: 1–11.
20. Fata JE, Leco KJ, Moorehead RA, Martin DC, Khokha R (1999) Timp-1 is a key regulator of mammary gland development. Endocrinology 137: 4915–4924.
21. Wiseman BS, Stermitz MD, Lund LR, Alexander CM, Mott J, et al. (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. J Cell Biol 162: 1123–1133.
22. Sympson CJ, Talhouk RS, Alexander CM, Chin JR, Clift SM, et al. (1994) Targeted expression of stemline-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. J Cell Biol 125: 601-693.

23. Fata JF, Leco KJ, Voura EB, Yu HY, Waterhouse P, et al. (2001) Accelerated apoptosis in the Timp-3-deficient mammary gland. J Clin Invest 108: 831-841.

24. Khokha K, Werb Z (2011) Mammary gland reprogramming: metalloproteinases couple form with function. Cold Spring Harb Perspect Biol 3.

25. Talhouk RS, Bisell MJ, Werb Z (1992) Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J Cell Biol 118: 1271–1292.

26. Watson CJ (2009) Immune cell regulators in mouse mammary development and involution. J Anim Sci 87: 35–42.

27. O'Brien J, Lyons T, Monk J, Lacia MS, Wilson RS, et al. (2010) Alternatively activated macrophages and collagen remodeling characterize the postpartum invading mammary gland across species. Am J Pathol 176: 1241–1255.

28. Schedin P (2006) Pregnancy-associated breast cancer and metastasis. Nat Rev Cancer 6: 291–291.

29. Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 induces apoptosis in mammary tumors to malignancy. J Exp Med 193: 727–740.

30. Lyons TR, Schedin PJ, Borges VF (2009) Pregnancy and breast cancer: when they collide. J Mammary Gland Biol Neoplasia 14: 87–90.

31. Stensheim H, Moller B, van Dijk T, Fossa SD (2009) Cause-specific survival for women diagnosed with cancer during pregnancy or lactation: a registry-based cohort study. J Clin Oncol 27: 45–51.

32. Mathelin C, Annane K, Treviser A, Chenard MP, Tomasetto C, et al. (2008) Pregnancy and post-partum breast cancer: a prospective study. Anticancer Res 28: 2447–2452.

33. Woessner JF, Jr. (2001) That impish TIMP: the tissue inhibitor of metalloproteinases-3. J Clin Invest 108: 799–800.

34. Mohammed FF, Smookler DS, Taylor SE, Di Grappa M, Kassiri Z, et al. (2004) Abnormal TNF activity in Timp3−/− mice leads to chronic hepatic inflammation and failure of liver regeneration. Nat Genet 36: 969–977.

35. Kassiri Z, Oudit GY, Sanchez O, Dawood F, Mohammed FF, et al. (2005) Combination of tumor necrosis factor-alpha ablation and matrix metalloproteinase inhibition prevents heart failure after pressure overload in tissue inhibitor of metalloproteinase-3 knock-out mice. Circ Res 97: 509–510.

36. Malmoodi M, Sahebjam S, Smookler D, Khokha R, Mort JS (2005) Lack of tissue inhibitor of metalloproteinases-3 results in an enhanced inflammatory response in antigen-induced arthritis. Am J Pathol 166: 1733–1740.

37. Smookler DS, Mohammed FF, Kassiri Z, Duncan GS, Mak TW, et al. (2006) Tissue inhibitor of metalloproteinase-3 regulates TNF-dependent systemic inflammation. J Immunol 178: 721–725.

38. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, et al. (1998) TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435: 39–44.

39. Lee MH, Knauper V, Becherer JD, Murphy G (2001) Full-length and N-TIMP-3 regulation of mammary involution. J Cell Physiol 187: 276: 4972–4980.

40. Hurley WL, Finkelstein E (1986) Bovine leukocytes in mammary secretions during involution: surface protein changes. Am J Vet Res 47: 2410–2420.

41. Steinhusen U, Weiske J, Badeck V, Tauber R, Bommert K, et al. (2001) Enhanced metastatic dissemination to multiple organs by melanoma and breast cancer cells. J Pathol 203: 5528–5537.

42. Vallorosi CJ, Day KC, Zhao X, Rashid MG, Rubin MA, et al. (2000) Truncation of the beta-catenin binding domain of E-cadherin precedes epithelial apoptosis during prostate and mammary involution. J Biol Chem 275: 3328–3334.

43. Steinhusen U, Weiske J, Badeck V, Tauber R, Bommert K, et al. (2001) Cleavage and shedding of E-cadherin after induction of apoptosis. J Biol Chem 276: 4972–4980.

44. Cruz-Munoz W, Sanchez OH, Di Grappa M, English JL, Hill RP, et al. (2006) Enhanced metastatic dissemination to multiple organs by melanoma and lymphoma cells in timp-3+/− mice. Oncogene.

45. English JL, Kassiri Z, Koskiirta I, Akinson SJ, Di Grappa M, et al. (2006) Individual timp deficiencies differentially impact pro-MMP-2 activation. J Biol Chem.

46. Atabai K, Fernandez R, Huang X, Ueki I, Kyle A, et al. (2005) Mife8 is critical for mammary gland remodeling during involution. Mol Biol Cell 16: 5528–5537.

47. Santhial M, Hunter DM, Strunk KE, Earp HS, Cook RS (2010) Epithelial cell-directed effrocytosis in the post-partum mammary gland is necessary for tissue homeostasis and future lactation. BMC Dev Biol 10: 122.

48. Altenberg JL, Korsu D, Leishman I, Hansel L, et al. (2005) Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammalian alveolar epithelial cells and repress inflammatory mediator release. Cell Death Differ 12: 107–114.

49. Hanayama R, Nagata S (2005) Impaired invasion of mammary glands in the absence of milk fat globule EGF factor 8. Proc Natl Acad Sci U S A 102: 16086–16091.