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Molecular mechanism of mammary gland involution: An update

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ABSTRACT

The mammary gland (MG) is a unique organ responsible for milk synthesis, secretion, and involution to prepare the gland for subsequent lactation. The mammary epithelial cells (MECs), which are the milk synthesizing units of the MG, proliferate, differentiate, undergo apoptosis and regenerate following a cyclic pathway of lactation – involution – lactation, fine-tuning these molecular events through hormones, growth factors and other regulatory molecules. The developmental stages of the MG are embryonic, prepubertal, pubertal, pregnancy, lactation and involution, with major developmental processes occurring after puberty. The involution stage includes interesting physiological processes such as MEC apoptosis, matrix remodeling, and the generation of cells regaining the shape of a virgin MG. Signal transducer and activator of transcription 3 (STAT3) is the established master regulator of this process and aberrant expression of STAT3 leads to subnormal involution and may induce neoplasia. Several studies have reported on the molecular mechanism of MG involution with substantial knowledge being gained about this process; however, a deep understanding of this phenomenon has yet to be attained. This review focuses deeply on the molecular details of post-lactational regression, the signaling pathways involved in the lactation-involution cycle, and the latest developments in STAT3-associated MG neoplasia. Deep insight into the involution process will pave the way towards understanding the biology, apoptosis, and oncogenesis of the MG.

1. Introduction

The mammary gland (MG) is made up of a branching network of ducts that terminate in alveoli (Jena et al., 2015). It undergoes cyclic changes of development during pregnancy, lactation and involution, and development during various functional phases is largely governed by the coordinated action of reproductive hormones and growth factors. There is more connective tissue and fat before puberty, with moderate elongation of mammary ducts having mammary epithelial cells (MECs) invaginating into the fat pad, and this invagination process does not depend on the action of hormones. The ovarian steroid hormones accelerate the extension and branching of mammary ducts at the beginning of puberty (Jena and Mohanty, 2017). Ductal branching continues during pregnancy, with intensive mitosis of MECs, which fill the fat pad in a tree-like pattern. The ducts transport milk during lactation and are lined by two layers of epithelial cells: the inner luminal layer and an outer contractile myoepithelial layer (Janjanam et al., 2013). Further branching of these ducts forms the lobules that are made up of alveoli. The innermost layer of alveoli is made up of epithelial cells that differentiate and secrete milk after parturition. When suckling ceases, milk stasis occurs and involution of the mammary epithelium is initiated. Involution is a step-wise process, characterized by intensive apoptosis of MECs, adipose tissue redevelopment (some of the fibroblasts function as preadipocytes and further differentiate into adipocytes with the accumulation of lipids (Alexander et al., 2001)) and tissue remodeling. A wide variety of genes modulate cell death, phagocytosis, matrix reorganization and the innate immune response in this process (Stein et al., 2007; Atabai et al., 2007; Watson and Kreuzaler, 2011). Tissue remodeling shapes the MG as that of a virgin female.

The MG provides an in vivo model to study the underlying molecular mechanisms of epithelial cell apoptosis during involution. Mouse genetic models have been used extensively for this purpose. In a related study on transgenic mice expressing Bax (apoptotic) protein, mice expressing ectopic Bax exhibited impaired alveologenesis, reduced epithelial cell content and STAT3 (Signal Transducer and Activator of Transcription3; apoptosis inducer) activation before normal times (Rucker et al., 2011). Similarly, transgenic mice with constitutively expressed Akt protein (a serine/threonine protein kinase and apoptosis suppressor) had a delay in the onset of apoptosis and involution (Schwertfeger and Richert, 2001). Hence, modulation of a single proapoptotic or antiapoptotic factor in vivo can affect the apoptosis of differentiated MECs.

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The mechanisms behind MG involution, as well as the associated protein molecules and pathways involved, have been discussed in a few reviews thus far (Watson and Kreuzaler, 2011; Hahm and Davidson, 1998; Watson, 2006). In the present review, we have focused on the latest findings on this process in molecular detail, with comprehensive presentation of the role of STAT3 as a master regulator of involution, its aberrant expression leading to neoplasia, and the involvement of microRNAs (miRs) during involution.

2. Stages of involution

MG involution is divided into two phases on the basis of the reversal of phase, gene expression patterns, biochemical reactions and histological studies (Lund et al., 1996). The first phase (first 48 h after weaning in mice) is characterized by milk stasis, and the predominant apoptotic pathway in this phase is the lysosomal-mediated cell death (LCD) pathway, where a stimulus causes lysosomal membrane permeabilization (LMP), resulting in lysosomal content (cathepsin proteases) release into the cytosol and extensive cell death (Hennigar et al., 2015a). The stimulus for LMP seems to be tumor necrosis factor α (TNF-α), which is produced in excess amounts in this first phase. Additionally, STAT3 signaling also up-regulates cathepsin expression and down-regulates the endogenous cathepsin inhibitor Spi2a to facilitate LCD in the first phase (Lloyd-Lewis et al.,). This phase is reversible, as resumed suckling will revive the lactation of the MG. Local factors are also responsible for the induction of programmed cell death in this phase, along with increased expression of the proapoptotic Bax gene and phosphorylation of intracellular transcription factors such as STAT5a, STAT5b and STAT3. There is enhanced production of proteins such as sulfated glycoprotein-2 (SGP-2), interleukin-1b converting enzyme (ICE) and tissue inhibitor of metalloproteinases-1 (TIMP-1), and apoptosis of differentiated MECs occurs without degradation of the extracellular matrix (ECM) (Table 1). Elevated expression of some other proteins such as transforming growth factor β3 (TGFβ3) (Nguyen and Pollard, 2000) and IL-10 (Sohn et al., 2001) also occurs in this phase. TGFβ3 induction occurs by milk stasis, which precedes the apoptosis process, suggesting its role in apoptotic events (Nguyen and Pollard, 2000). Elevated IL-10 levels trigger the expression of TRAIL (Tumor necrosis factor alpha-related apoptosis-inducing ligand) and DR4 (Death Receptor 4), which contributes to apoptosis in the initial stage of MG involution (Sohn et al., 2001). The p53 tumor suppressor gene is involved in the development of a variety of tissues. A study on its role during involution using BALB/c-p53^{null} mice revealed delayed involution of MECs in the absence of p53. However, the expression of the stromal protease stromelysin-1 was unaffected in the absence of p53, indicating that p53 participates in the 1st stage of involution without affecting the 2nd phase where stromal proteases are induced (Jerry et al., 1998). The second irreversible phase (after 48 h of weaning in mice) predominantly includes the mitochondria-mediated intrinsic apoptotic pathway (due to the withdrawal of prolactin) to facilitate the remodeling of mammary gland (Baxter et al., 2007). This phase is marked by tissue remodeling by proteinases and hormonal changes at the systemic level (Li et al., 1997). ECM remodeling occurs with extensive apoptosis on the 4th day of weaning in mice. There is increased expression of the matrix metalloproteinases gelatinase A, stromelysin-1 and serine protease urokinase-type plasminogen activator during this phase (Watson, 2006). Within the first 4 days of involution in mice, the alveolar epithelial cells engulf the apoptotic epithelial cells and repress inflammatory mediator release, thus contributing significantly to tissue homeostasis (Monks et al., 2005, 2008). A recent study showed the involvement of the BIM protein (BH3-only protein), which drives the mitochondrial apoptosis pathway in this irreversible phase (Schuler et al., 2016). The up-regulation of BIM is induced by the down-regulation of the STAT5 transcription factor rather than downstream signaling of the up-regulated STAT3 transcription factor. One of the crucial proteins in apoptosis is cathepsin D, as demonstrated by its expression patterns in mouse MG at various reproductive stages. Its level was increased in the 4th day during involution, and its overexpression in HC11 MECs induces apoptosis (Seol et al., 2006).

The metal zinc seems to activate both the LCD and mitochondria-mediated apoptosis in the involution process, where the zinc transporter ZnT2 transports zinc into lysosomal vesicles and mitochondria, thus

Table 1
Proteins up/down regulated during involution.

Name of t he protein(s)	Up/down regulation	Function	Reference
SGP-2, ICE, & TIMP-1	Up	Apoptosis of MECs in 1st phase	(Lund et al., 1996)
MMP, gelatinase-A, & stromelysin	Up	ECM remodeling in 2nd phase	(Alexander et al., 2001; Lund et al., 1996)
Bax	Up	MEC apoptosis	(Rucker et al., 2011; Li et al., 1997)
TGFβ3	Up	MEC apoptosis	(Nguyen and Pollard, 2000)
IL-10, TRAIL, & DR4	Up	MEC apoptosis	(Sohn et al., 2001)
Fas & Fas-L	Up	Tissue remodeling	(Song et al., 2000)
bcl-Xs, bak, bid and bad	Up	Proapoptotic	(Schorr et al., 1999)
bcl-2, bcl-X$_{L,}$ bfl-1 and bcl-w	Down	antiapoptotic	(Schorr et al., 1999)
calpain-1	Up	Essential for normal involution	(Dang et al., 2015)
galectin-3	Down	Antiapoptotic	(Liu et al., 2004)
STAT3	Up	Initiates involution	(Humphreys et al., 2002; Hughes et al., 2012)
BIM Protein	Up	Mitochondrial pathway of apoptosis	(Schuler et al., 2016)
STAT5	Down	Initiation of involution	(Schuler et al., 2016)
IKKβ	Up	Initiates involution	(Baxter et al., 2006)
p55α and p50α	Down	down regulate PI(3)K-Akt-mediated survival signaling	(Abell et al., 2005)
C/EBPδ and LBP	Up	apoptotic cell clearance	(Stein et al., 2004)
IL-6 & LIF	Up	Activation of STAT3	(Schere-Levy et al., 2003; Zhao et al., 2002)
cathepsin B and L	Up	lysosomal mediated cell death	(Hughes and Watson, 2012)
Cathepsin D	Up	Induces apoptosis	(Seol et al., 2006)
ZnT2 (Zinc transporter)	Up	imports Zn into vesicles and mitochondria	(Hennigar et al., 2015a)
MGP-40	Up	Induces STAT3 phosphorylation	(Anand et al., 2016)
Integrins (β1, α6, and α5)	down	Response to survival signals from BM	Prince et al., 2002; Singh et al., 2005
MFGE8	Up	Binds to apoptotic MEC	(Aoki, 2006; Prince et al., 2002; Singh et al., 2005)
Expi	Up	induces BAFF and accelerates apoptosis	(Jung et al., 2004)
Serum amyloid A (SAA)	Up	accelerates apoptosis by activating caspases	(Kho et al., 2008b)
ATF4	Up	Facilitates involution	(Bagheri-Yarmand et al., 2003)
Annexin A8	Up	Role in mouse mammary gland involution	(Stein et al., 2005)
ANXA5	Up	Epithelial apoptosis	(Rieanrakwong et al., 2016)
Claudin-1, 3, and 4	Up	Function not clear	(Baumgartner et al., 2017)

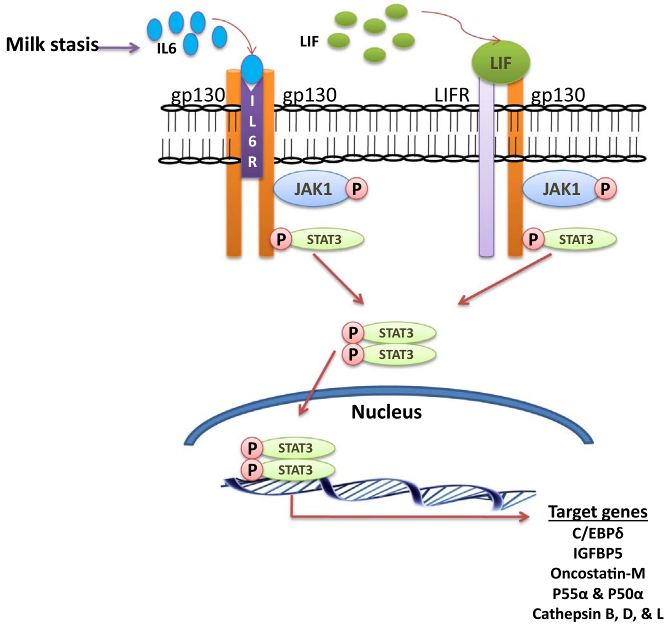

Fig. 1. Activation of STAT3 during involution. LIF and IL6 family of cytokines activate STAT3 upon milk stasis. The JAK1-STAT3 pathway induces target gene expression, which are involved in involution. Oncostatin – M also induces STAT3 activation in irreversible phase and the activation pathway is similar to that of LIF.

promoting cell death (Hennigar et al., 2015a). A recent study showed that obesity creates a pro-inflammatory microenvironment in MECs of lactating mice with increased macrophage infiltration, increasing the concentration of zinc in the endoplasmic reticulum (ER) and lysosomes (Hennigar et al., 2015b). This redistribution of sub-cellular zinc pools culminated in ER stress, autophagy in MECs and lactation failure.

The innate immune response plays a crucial role during involution (Atabai et al., 2007). Neutrophil influx occurs in the initial stage of involution, followed by macrophages and lymphocytes later on. The apoptotic cells, cell debris, and milk components are cleared off by professional phagocytes (macrophages) and nonprofessional phagocytes (MECs, endothelial cells, and fibroblasts). Protein milk fat globule EGF factor 8 (MFGE8), a major component of the milk fat globule membrane (MFGM), is highly up-regulated in involution (Aoki, 2006; Nakatani et al., 2006). MFGE8 is released in association with the exosome-like membrane vesicles (ELMV) that predominate during involution. The MFGE8 binds to apoptotic MECs during involution and is engulfed by macrophages and neighboring MECs through an RGD-integrin bridge, thus facilitating apoptotic cell clearance (Aoki, 2006).

3. STAT3: A major regulator of involution

STATs are the cytoplasmic transcription factors that mediate the effects of cytokines in the cell. They are involved in various physiological processes such as cell proliferation, differentiation, and apoptosis, etc., by integrating cytokine, hormone, and growth factor receptor signaling (Schindler et al., 1992). The STATs are actively involved in different stages of MG development, as well as neoplasia (Haricharan and Li, 2014). STAT3, a member of the STAT family, plays a major role in the involution process. This protein becomes activated in the first phase, whereas STAT5a (which is expressed at a higher level in lactation) activity decreases. Deletion experiments conducted by different researchers (Humphreys et al., 2002; Chapman et al., 1999; Hughes et al., 2012) have revealed the importance of STAT3 in involution. STAT3 inactivation through the deletion of exons 15–21 in mammary epithelium during pregnancy in mice had significant effects on the involution process (Humphreys et al., 2002). Conditional knockout of STAT3 using the Cre-lox recombination system resulted in a suppression of apoptosis in epithelial cells and delayed involution (Chapman et al., 1999). Another study in mice mammary epithelium showed impairment of the acute phase responses due to STAT3 deletion, where STAT3 modulates the immune cell profile in post-lactational regression (Hughes et al., 2012).

3.1. LIF and cytokines as activators of STAT3

Leukemia inhibitory factor (LIF) is a multifunctional glycoprotein that shows a number of biological activities in different types of cells. Its level is almost undetectable during lactation, but rises sharply during early involution, being induced by milk stasis. When supplied exogenously in a lactating gland, it induces apoptosis and phosphorylates STAT3 to its active form; thus, LIF-induced epithelial apoptosis may be partially mediated by STAT3 activation (Schere-Levy et al., 2003). LIF plays dual roles in the MG. It activates STAT3–mediated apoptosis in involution, yet it also promotes branching morphogenesis during pregnancy mediated by ERK1/2. STAT3 seems to contribute towards the down-regulation of the ERK1/2 survival pathway thus enhancing its pro-apoptotic effects (Kritikou et al., 2003). STAT3 also regulates apoptosis by inducing expression of the PI(3)K (phosphoinositide-3-OH kinase) regulatory subunits p55alpha and p50alpha to down-regulate PI(3)K-Akt-mediated survival signaling (Abell et al., 2005). STAT3 is activated by several cytokines signaling through different receptors, such as receptor-tyrosine kinases, single-chain cytokine receptors and receptors sharing the common subunit gp130 (Fig. 1). The gp130 protein is a common receptor subunit of the IL-6 family of cytokines including IL-6, IL-11, LIF, and oncostatin-M. Induction of milk stasis causes up-regulation of IL-6 (Zhao et al., 2002) and LIF (Schere-Levy et al., 2003), which signals through gp130, resulting in the activation of STAT3. Suppressor of cytokine signaling (SOCS) proteins attenuate cytokine-mediated signaling in various tissues in a negative feedback mechanism. Prolactin induces Socs3 expression in the mammary epithelium (Tam et al., 2001). Apoptosis is increased due to the deletion of SOCS3 during lactation. Therefore, SOCS3 is an important attenuator of proapoptotic pathways during lactation and involution in the MG (Sutherland et al., 2006). STAT3 is also activated by another protein, angiotensin-II, which induces post-lactational MG regression through the renin-angiotensin system (Nahmod et al., 2012).

In vitro studies in primary MECs and transfected COS-7 cells showed p44/42 MAPK and EGFR-dependent STAT3 activation. EGFR activation was gp130 dependent and independent of EGF ligands, revealing a cytoplasmic interaction and cross-talk between these two receptors, which establishes two distinct STAT3 signaling pathways arising from gp130 to be utilized in MG involution (Zhao et al., 2004).

The Janus kinases JAK1 and JAK2 have discrete roles in the activation of STAT proteins in MG during lactation to involution switching (Sakamoto et al., 2016). JAK2 mediates the activation of STAT5 in response to prolactin signaling during lactation, whereas JAK1 is a crucial link between IL-6 inflammatory cytokines and STAT3 activation for cell death of differentiated MECs and MG remodeling (Fig. 1). The reciprocal activation of STAT5 and STAT3 was found to occur at a slower rate in bovines than in rodents (Singh et al., 2016).

Apart from this, STAT3 activity is also modulated by regulatory molecules such as MGP-40, Sim-2s, and nucling. MGP-40, a chitinase-like protein, is up-regulated during involution, and induces STAT3 phosphorylation and epithelial to mesenchymal transition (EMT) (Anand et al., 2016), as studied in buffalo MECs developed in our lab. Another molecule, Singleminded-2S (Sim-2s), a basic helix-loop-helix/PAS transcription factor, has a primary role in promoting lactogenic differentiation, and it delays involution by suppressing the activity of STAT3 (Scribner et al., 2011). Another regulatory protein named nucling also regulates MG involution through the NF-κB and STAT-3 signaling pathways (Dang et al., 2015). Abnormalities in the thyroid gland seem to affect the lactation process, as premature

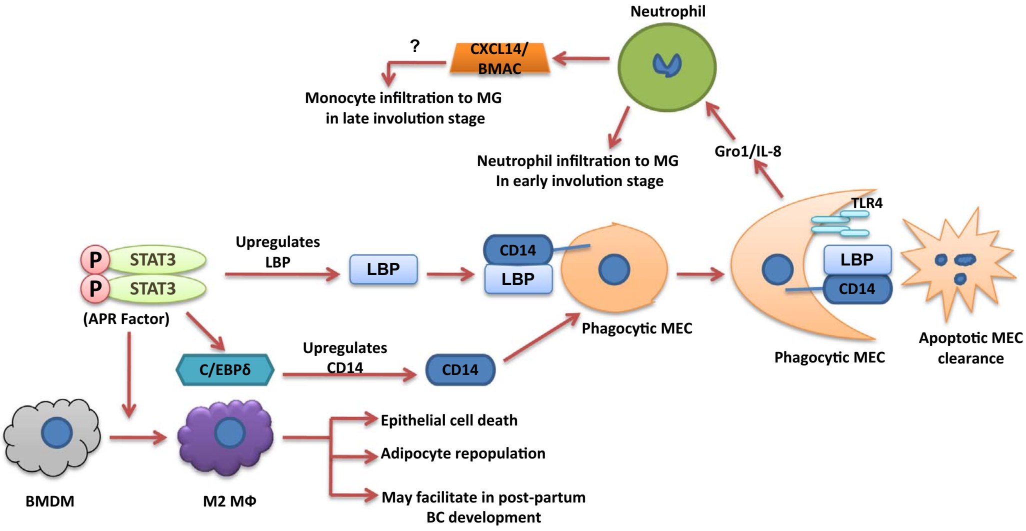

Fig. 2. Role of STAT3 in immune status of mammary gland during involution. The activated STAT3 enhances the LBP and CD14 expression in phagocytic MEC, subsequently activating the TLR4 receptor in those cells. This process induces Gro1/IL-8 (neutrophil chemoattractant) secretion from phagocytic MEC, and neutrophil recruitment in MG during early involution stage. The recruited neutrophils probably secrete the macrophage attracting chemokine CXCL14/BMAC thus recruiting the macrophages in later stage of involution. The pSTAT3 also promotes M2 macrophage production from BMDM, which phagocytose dead cells and facilitate adipocyte repopulation. LBP = LPS binding protein, APR = Acute phase response, BMDM = Bone Marrow Derived Macrophages.

mammary involution was observed in hypothyroidism cases (Campo Verde Arboccó et al., 2017, 2016). Hypothyroidism reduces the expression of prolactin receptors and reduces the expression as well as the activation of the STAT5a/b protein. The STAT5b gene has functional thyroid hormone (TH) response elements that bind to the TH receptor β differentially in a TH–dependent manner, showing its link at the hormone level. Lowered TH levels (hypothyroidism) decrease the prolactin signaling pathway and induce the LIF-STAT3 pathway, and thus, initiate the involution process (Campo Verde Arboccó et al., 2017, 2016).

3.2. STAT3 and the immune status of the mammary gland

STAT3 is a major regulator of innate immunity and the influx of inflammatory cells in the MG (Fig. 2). It polarizes the macrophages towards an alternatively activated phenotype (M2), hence, STAT3 signaling is critical for the expression of an inflammatory signature during both phases of involution and may be a key molecule for the pathogenesis of pregnancy-associated breast cancer (Hughes et al., 2012). Macrophages have no role in the initiation of apoptosis, as it has been observed that their level is very high after the initial wave of epithelial cell death. However, subsequently, the M2 population derived from bone marrow-derived macrophages (BMDM) under the influence of STAT3 plays a crucial role in epithelial cell death and redevelopment of adipocytes, as demonstrated by using the Mafia mouse model, which has a conditional deletion of the CSF-1 receptor (O'Brien et al., 2012a) (Fig. 2). The ECM of involuting MG appears to be chemotactic for M2 macrophages, where the proteolyzed fibrillar collagen mediates M2 macrophage recruitment (O'Brien et al., 2012b). The presence of M2 macrophages, fibrillar collagen accumulation, and increased M2 cytokines (IL-4 and IL-13) mimics the microenvironment of tumor progression and may facilitate postpartum breast cancer development. Immune gene expression is up-regulated during involution of the bovine MG, as revealed by cDNA microarray analysis (Singh et al., 2008). The MG provides a suitable microenvironment for an immune response increase after the completion of involution, as observed in involuting sheep MG (Tatarczuch et al., 2000). The MG after involution becomes more resistant to infections than the lactating MG or the MG during early involution. STAT3 is also called an acute phase response factor (Akira et al., 1994; Zhong et al., 1994), and it is associated with LBP (LPS-binding protein) and CD14 (LPS receptor) in an immune cascade with an acute-phase response during mouse MG involution (Stein et al., 2004). The LBP levels increase in parallel to CD14 (LPS receptor) at the beginning of involution, even without bacterial infection (Fig. 2). The LBP-CD14 interaction activates the TLR4 receptor in the phagocytic MEC, which subsequently secretes the neutrophil-attracting chemokines Gro1/IL8 through downstream signaling. In this process, the neutrophils are recruited to the MG in the early phase of involution. The recruited neutrophils probably secrete the macrophage attracting chemokine CXCL14/BMAC and recruit the macrophages at later stages of involution. LBP is a downstream target of STAT3, and CD14 is activated by C/EBPδ. Therefore, STAT3 may be involved in enhancing phagocytosis through the activation of C/EBPδ and ultimately LBP, for apoptotic cell clearance from the MG (Stein et al., 2004). C/EBPδ is a member of the CCAAT enhancer-binding protein (C/EBP) family of transcription factors and plays a role in regulating G0 growth arrest and apoptosis (Hutt et al., 2000).

3.3. STAT3 regulates milk fat globule (MFG) uptake

STAT3 seems to mediate the formation and fusion of lysosomal vacuoles, with most of the vacuoles containing triglycerides. The triglycerides (a major component of MFGs) are degraded into free fatty acids in the lysosome during involution. Various fatty acids are produced in this process, with oleic acid, palmitic acid, and stearic acid being the most abundant ones (Sargeant et al., 2014). Out of the various fatty acids, oleic acid was found to be a potent agent for lysosomal permeabilization during involution, probably due to the presence of a double bond (unsaturated fatty acid) in its structure, creating a kinked conformation. This conformation destabilizes the phospholipid membrane of lysosomes and causes cathepsin leakage (Sargeant et al., 2014).

STAT3 also promotes phenotypic switching, resulting in phagocytosis of MFGs instead of their secretion by MECs. It was found that butyrophilin 1A1 forms a coating over MFGs, a requisite for their secretion during lactation (Ogg et al., 2004). However, STAT3 mediates their uptake during involution and triglyceride degradation into fatty acids in the lysosomes. Simultaneously, STAT3 also downregulates the expression of butyrophilin1A1, ensuring the stoppage of MFG secretion.

4. Role of STAT3 in cancer progression

Breast cancer accounts for 23% of total cancer cases and 14% of total cancer-related deaths (Torre et al., 2015). It has been observed that 40% of breast cancer cases are positive for activated STAT3 (Dimri and Sukanya, 2017). The latest studies on STATs in breast carcinoma have revealed that STAT3 could be a potential target for therapy and that STAT5a/5b/6 are crucial prognostic markers for improved survival of breast carcinoma and appropriate prognosis (Wu et al., 2017). Phosphorylated STAT3 (pSTAT3), which is the active form, is associated with the development of many types of cancers in humans (Yu et al., 2009; Mankan and Greten, 2011; Debnath et al., 2012). The tumor progression via pSTAT3 occurs through the induction of tumor-promoting inflammation, pro-proliferative and anti-apoptotic gene expression, and the suppression of antitumor immunity (Kang et al., 2015).

The STAT3 protein is crucial in mediating hypoxia-induced chemoresistance in triple negative breast cancer (TNBC) cell lines (MDA-MB-231) rather than HIF-1α (Hypoxia Inducible Factor-1α). The hypoxic condition prevails in cancerous cell masses, as cell division and growth overpowers the oxygen supply to an area. The transcription factor HIF-1α is stable in hypoxic conditions and induces downstream target gene expression, and most of these genes are involved in cancer progression, aggressiveness, and chemoresistance (Semenza, 2010; Brahimi-Horn and Pouyssegur, 2006; Rohwer and Cramer, 2011). STAT3 is overexpressed in hypoxic conditions and shows chemoresistance to the drug cisplatin (which is used to treat triple negative breast cancer), independent of the effect of HIF-1α (Abyaneh et al., 2017). Additionally, STAT3 also promotes the expression of an enzyme named CSE (Cystathionine-γ-lyase), which is produced by many tissues in the body except the central nervous system and is responsible for H_2S production (Wang, 2010) and the promotion of cancerous cell proliferation (Yin et al., 2012; Cai et al., 2010). It is found that both STAT3 and CSE promote breast carcinoma development (You et al., 2017).

The STAT3 protein is immunosuppressive, whereas STAT1 induces antitumor immunity. Therefore, immunotherapy is very crucial for the treatment of cancer, and when it is combined with chemotherapy, shows promising results. It has been observed that one of the adaptor proteins, ShcA, is a vital regulator of tyrosine kinase signaling, thus promoting immunosuppression in breast cancer. This immunosuppressive activity is found to be achieved by reducing STAT1-induced antitumor immunity and enhancing STAT3-induced immunosuppressive activity (Ahn et al., 2017). The ShcA molecule is a scaffolding protein recruited to multiple tyrosine kinases, and these tyrosine kinases transduce oncogenic signals such as those found in tumor initiation, progression, and metastatic spread (Ursini-Siegel et al., 2010, 2008; Im et al., 2015).

The proteins such as annexins are Ca^{2+}-regulated phospholipid binding proteins involved in many cellular processes. The annexin A2 protein is involved in different stages of MG development and is down-regulated during lactation, whereas it is up-regulated in involution (Jena et al., 2015). This protein is also involved in breast cancer development and Tyr23 phosphorylated annexinA2 facilitates STAT3 activation in response to IL-6. Thus, STAT3 target gene (like cyclinD1 and MMP2/9) expression is increased, which has regulatory functions in cell proliferation and invasion (Yuan et al., 2017). Another regulatory molecule, ADAM12 (a disintegrin and metalloproteinase domain-containing protein 12), is found to be up-regulated in epithelial cancers, promoting cell proliferation and metastasis. It regulates endothelial cell function and promotes a pro-angiogenic microenvironment driven by activation of EGFR, STAT3, and AKT signaling in breast tumor cells (Roy et al., 2017).

Epigenetic modifications play a crucial role in regulating the activity of STAT3. It is acetylated at K685, and acetylated STAT3 (pro-tumor activity) promotes DNA methylation in the promoter region of tumor suppressor genes through an interaction with the DNMT1 enzyme (DNA (cytosine5)-methyl transferase 1) (Lee et al., 2012, 2013). Thus acetyl STAT3 promotes tumor progression by silencing tumor suppressor genes through DNA methylation.

Obesity is a predisposing factor for human cancers including breast cancer. Obesity-induced mammary hyperplasia is mediated by the leptin/leptin receptor JAK1-STAT3 pathway stimulating MEC proliferation and activated STAT3 signaling-augmented migration and invasiveness of proliferating MECs. The STAT3 activation by the long form of the leptin receptor (LEPRb) is crucial for cancer stem cell maintenance in TNBC cancers (Thiagarajan et al., 2017). It seems that the inhibition of STAT3 by the STAT3 inhibitor S31–201 reverses obesity-induced mammary hyperplasia and lessens cell invasion and migration (Park et al., 2017). The obesity-related cytokine resistin seems to promote breast cancer development by EMT and stemness in breast cancer cells through the TLR4/NF-κB/STAT3 signaling pathway (Wang et al., 2018).

5. Role of TGF-β during mammary gland involution

Transforming growth factor-β (TGF-β) is an important regulatory molecule in cell proliferation, differentiation, and apoptosis (Gordon and Blobe, 2008; Huang and Huang, 2005; Moses and Serra, 1996; Schuster and Krieglstein, 2002). Its expression level varies in different developmental stages: it is high in pregnancy, low in lactation, and again becomes higher during involution. TGF-β is the main inducer of the epithelial-mesenchymal transition (EMT) process in tissue remodeling (Serra and Crowley, 2003; Zavadil et al., 2001), and decreased levels of lactogenic hormones (prolactin and glucocorticoid) increase the level of TGF-β1 and arachidonic acid, both of which are involved in the EMT process (Fig. 4). Increased TGF-β1 also enhances arachidonic acid production to further facilitate EMT (Zhang et al., 2017). EMT is an important event in tissue remodeling where epithelial cells lose their polarity and cell attachments, thus becoming migrating mesenchymal cells that can invade surrounding cells (Kalluri and Neilson, 2003; Kalluri and Weinberg, 2009). The TGF-β cancer paradox refers to the action of TGF-β as both a tumor suppressor and tumor promoter, influenced by the cellular cross-talk of TGF-β-responsive cells (Guo et al., 2017). Involution induced by the weaning of pups can be an ideal tissue model to study the functional complexity of this molecule.

Effective apoptotic cell clearance is required in the MG, as the persistent presence of apoptotic cells has adverse effects on successive lactation, MG homeostasis, and there is also a possibility of neoplasia development. Neighboring epithelial cells actively clear the apoptotic epithelial cells by increased TGF-β signaling, which promotes phagocytic capacity. TGF-β signaling facilitates the reorganization of cell junctions and makes the neighboring cells phagocytic (Fornetti et al., 2016). The TGF-β isoform TGF-β3 is expressed in myoepithelial cells and its expression increases during involution, as it is involved in the remodeling events of the ECM during the second stage of involution in the mouse (Faure et al., 2000).

The SnoN protein, which has both pro-oncogenic and anti-oncogenic characteristics, seems to be a negative regulator of TGF-β. In a transgenic mouse model, this protein was observed to accelerate the involution process accompanied by down-regulation of phosphorylated Akt, indicating the role of SnoN in post-lactational regression (Jahchan et al., 2010).

The lactating gland is exposed to higher levels of prolactin and lower levels of TGF-β, with minimal apoptosis, but when the prolactin level drops after weaning, the TGF-β level increases, and apoptosis predominates. The interaction between prolactin, TGF-β, and Akt decides the cell fate of the MG during lactation and involution. Prolactin suppresses TGF-β-mediated apoptosis by interacting with PI3K. PI3K activates the downstream serine/threonine kinase (Akt), which supports cell survival (Bailey et al., 2004).

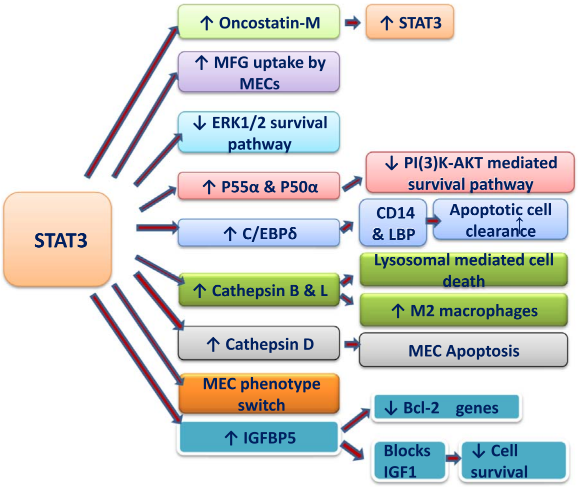

Fig. 3. STAT3 activating the target genes during MG involution.

6. IGFs and IGFBPs in involution

The action of IGFs (IGF-I and IGF-II) is regulated by 6 IGF-binding proteins (IGFBPs). IGFBP5 has been found to be essential for normal MG involution, as demonstrated by a study of IGFBP-5 knockout mice, and it can regulate MG morphogenesis in response to hormone stimulation (Ning et al., 2007). A study in our laboratory (Mohapatra et al., 2015) comparing the expression profiling of IGFBP-5 in the milk of *Bos indicus* and *Bubalus bubalus* during lactation revealed its higher expression during involution, and this expression was inversely related to the lactation length and milk production. Transcription factors such as activating transcription factors (ATFs) are basic leucine zipper containing proteins that regulate the genes involved in growth and development. ATF4 has been found to facilitate MG involution as its over-expression showed accelerated involution and impaired lactation in mice (Bagheri-Yarmand et al., 2003). There was significant nuclear translocation of activated STAT3 and up-regulation of IGFBP5 (one of the target genes of STAT3), which mediates apoptosis by sequestering the IGF (Fig. 3). IGF-II suppresses MG apoptosis during involution as seen in transgenic mice expressing IGF-II in the mammary epithelium (Moorehead et al., 2001). Phosphorylated Akt/PKB was consistent in IGF-II over-expressed cells, mediating reduced epithelial apoptosis.

7. Proapoptotic and anti-apoptotic genes in involution

The proteins encoded by the Bcl-2 family of genes are important regulators of the apoptosis process (Fig. 3) (Schorr et al., 1999). The members that have pro-apoptotic activity are bax, bcl-Xs, bak, bid and bad, whereas the anti-apoptotic proteins are bcl-2, bcl-X$_{L}$, bfl-1 and bcl-w. The coordinated action of these factors decides the apoptosis process. Bcl-x is the most abundant pro-apoptotic factor in the MG among the bcl-2 family of proteins. Conditional deletion of the bcl-x gene from mouse MECs leads to enhanced apoptosis during involution without compromising proliferation and differentiation during pregnancy and lactation (Walton et al., 2001), suggesting its requirement for controlled apoptosis in the first phase of involution and its irrelevance to mammopoiesis. Concurrent pregnancy retards the involution process after weaning, and proliferation of MECs still occurs. Expression of the anti-apoptotic factor Bfl-1 (Bcl2 family) was elevated at this time, suggesting its role in promoting cell survival during involution in concomitantly pregnant mice (Capuco et al., 2002).

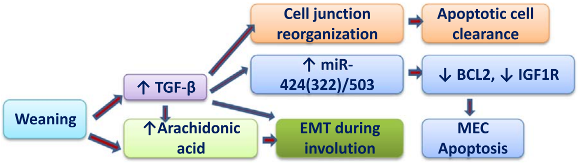

Fig. 4. Role of TGF-β during mammary gland involution.

B cell activating factor (BAFF) and a proliferation induced ligand (APRIL) are 2 members of the TNF family playing a vital role in involution. The signaling pathways activated by BAFF and APRIL lead to NF-κB activation and up-regulation of the pro-apoptotic Bax protein (Choi et al., 2007). TNF-α may induce apoptosis in lactating bovine mammary acini through Bax gene expression (Bax-dependent apoptosis) after the cessation of milking (Kim, 2000). There is a remarkable increase in the expression of the BCL2, Bax, and BCL2L1 genes, with the apoptotic index reaching its peak on day 8 of involution in the sheep MG (Colitti and Farinacci, 2009).

8. Extracellular matrix (ECM) remodeling during involution

An intact basement membrane has importance in maintaining the proper function, differentiation and morphology of epithelial cells. Cell-cell contacts and integrin-mediated signals contribute to the survival of MECs, along with support from the basement membrane (Pullan et al., 1996). Alteration of the balance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in the MG affects the morphology and function of the stroma. MMPs operate at the interface of the epithelium and stroma, and their deregulated proteolytic activity facilitates malignant transformation (Khokha and Werb, 2011). The TIMPs (TIMP-1 to TIMP-4) are important regulatory molecules, inhibiting two metzincin families of zinc-dependent proteinases, such as MMPs and the ADAMs (a disintegrin and metalloprotease) involved in pericellular proteolysis (Gomez et al., 1997; Blavier et al., 1999). TIMP-3 influences cell death, as its deficiency *(Timp-3*$^{-/-}$*)* within mammary tissue accelerates epithelial apoptosis without altering lactation (Fata et al., 2001). Accelerated differentiation of adipocytes was observed without affecting epithelial cell apoptosis, implying that MMPs alter the stromal microenvironment instead of inducing untimed epithelial apoptosis after weaning. One of the enzymes Lysil oxidase-like2 (LOXL2), which is a member of the LOX family of ECM-modifying enzymes, is involved in cancer progression. It is expressed in basal epithelial and stromal cells in the MG and seems to have a role in involution through its action as a collagen cross-linker and regulator of TIMP1 (Barker et al., 2011). Generally, the dedifferentiation of adipocytes occurs during ductal branching and alveologenesis in pregnancy, whereas redifferentiation occurs during involution. The matrix metalloproteinase MMP-3 [also known stromelysin-1 (Str1)] determines the adipocyte differentiation rate during involution, as demonstrated by a study of the MG from transgenic mice over-expressing TIMP-1 and mice having a targeted mutation in *Str1* gene (Alexander et al., 2001). The ECM signals to the cells through integrins, resulting in regulation of MMPs and TIMPs, thus orchestrating migratory, invasive and differentiating events in the MG (Werb et al., 1996). Integrins are the transmembrane proteins that serve as receptors for cell adhesion, connecting the external and internal environment of the cell (Pozzi and Zent, 2011). The cell-ECM interactions involving β1 integrin (an important member of the integrin protein family) play a crucial role in regulating the transcription of milk protein genes and maintaining differentiated MECs. Perturbation of β1 integrin function in involuting MG leads to dedifferentiation of secretory MECs, as observed in studying transgenic mice with a dominant negative β1 integrin mutant. There was a premature decrease in β-casein and whey acidic protein (WAP) mRNA levels, inactivation of STAT5, and up-regulation of the NF-κB protein (Faraldo et al., 2002). The protein laminin acts as a survival ligand for MECs and thus some specific β1 integrins suppress the cell death. The ligand-bound β1 integrin levels are decreased markedly during the transition (lactation to involution), thus becoming unable to respond to survival signals from the basement membrane, and this may contribute to the induction of apoptosis during the onset of involution (Prince et al., 2002). Expression profiling of genes involved in cell-ECM communication in the bovine MG through induced involution showed a down-regulation of integrins (β1, α6, and α5) and survival factors, whereas the pro-apoptotic factor αBax was up-regulated by 8 days of involution (Singh et al., 2005). MEC survival requires adhesion along with β1-integrin-mediated signals derived from specific ECM components. The interleukin-1 β converting enzyme (ICE) family of proteases, also termed caspases, are actively involved in MG involution. The ICEs are known as inducers of apoptosis in mammalian cells. The ECM suppresses the expression of ICEs and prevents apoptosis. When there is degradation of ECM, ICE expression increases, leading to the apoptotic cell death of MECs (Boudreau et al., 1995). The receptor-mediated interaction of cell and ECM facilitates many signaling pathways involved in cellular differentiation and function. The cell surface receptor β 1,4-galactosyl transferase-I (GalT1 long isoform) has been observed to play a critical role in involution in mice. The ECM promotes survival through integrin receptor-mediated activation of focal adhesion kinase (FAK); however, it has been suggested that surface GalT1 may negatively regulate MEC survival through linking integrin-independent FAK activation to apoptotic signaling events but not the survival pathways (de la Cruz et al., 2004). The MG microenvironment during involution has similarity to inflammatory conditions. It has been observed that inflammation can initiate tumorigenesis and the matrix of involuting MG promotes cell invasion and metastasis, explaining the increased number of incidences of pregnancy-associated breast cancer (McDaniel et al., 2006). Lactational differentiation and MG involution require plasminogen, as demonstrated in primiparous plasminogen-deficient ($plg^{-/-}$) mice, who show seriously compromised MG development and involution (Lund et al., 2000). Plasma kallikrein, a serine protease that predominantly activates plasminogen in MG, is a critical activator of MG involution and is involved in secretory epithelial cell apoptosis, followed by stromal remodeling and adipocyte replenishment of the MG through directing its differentiation. Plasma kallikrein is expressed locally and localizes to mast cells in MG (Lilla et al., 2009). The steroid hormone glucocorticoids play a major role in maintaining interepithelial attachment and act in the MG by inducing the formation of tight junctions (Zettl et al., 1992). The adhesion molecule E-cadherin belongs to a family of cadherins (single pass transmembrane glycoproteins) and mediates Ca^{2+}-dependent interepithelial membrane adhesion in secretory tissues such as the prostate and the MG. This molecule is rapidly truncated at the cytosolic domain (β-catenin binding domain) in involuting MG, resulting in the loss of the β-catenin binding domain and causing cells to dissociate and this dissociation precedes apoptosis. Thus, E-cadherin may regulate mammary homeostasis between adhesion-dependent survival and dissociation-induced apoptosis in MG (Vallorosi et al., 2000).

9. Role of hormones during involution

MG apoptosis is inhibited by endogenous progesterone and glucocorticoids during normal lactation, and either hormone alone is sufficient to prevent apoptosis (Berg et al., 2002). The transcription factor STAT5a and glucocorticoid receptors (GRs) synergize to induce the expression of milk protein genes during lactation and act as survival factors, whereas STAT3 activation mediates apoptosis during involution. Synthetic glucocorticoids, such as dexamethasone, regulate STAT5a and STAT3 signaling by inhibiting apoptosis induction when administered in the first 48 h of cessation of suckling. This delays STAT5a inactivation, weaning-triggered GR down-regulation, STAT3 activation and its entry into the MEC nucleus (Bertucci et al., 2010). Estrogen administered exogenously to the bovine MG at final milk removal showed accelerated involution of the gland (Athie et al., 1996). A reduction in the plasma prolactin level due to the withdrawal of suckling stimuli, enhanced gonadotropin-releasing hormone (GnRH) and annexin A5 (ANXA5) expression in the mammary tissue and acceleration of epithelial apoptosis has been observed. GnRH also acts as a chemoattractant for mast cells to the involuting MG, which favors tissue remodeling (Rieanrakwong et al., 2016). In an attempt to understand the mechanisms of initiation of milk secretion upon progesterone withdrawal, it was found that progesterone stimulates the synthesis of TGF-β, Wnt5b and IGFBP-5 during pregnancy (Rudolph et al., 2003). These factors interfere with prolactin and IGF-I signaling and repress milk secretion. Hence, these signaling pathways are activated upon progesterone withdrawal, which in turn activates Akt/PKB and SREBPs, resulting in a sharp increase in lipid synthesis during the onset of lactation. Study of the effects of chronic hyperthyroidism on the function of the MG during lactation (Varas et al., 2002) have revealed that thyroxin given to rats exogenously caused less prolactin, GH and oxytocin release, hence, less milk excretion. This, in turn, initiated the first stage of involution in a gland otherwise actively producing and secreting milk. The GH and prolactin deficiency resulted in a tremendous loss of MECs within 48 h of litter removal in rats. There was a minor increase in the IGFBP-4 mRNA level, along with high expression of IGFBP-5, showing that there is some cross-talk between IGFBPs during involution (Tonner et al., 1995). A similar study reported by Accorsi and coworkers (Accorsi et al., 2002) about the role of prolactin, GH, and IGF-I in MG involution in dairy cows showed that IGFBP-5 mRNA levels are highly increased in the absence of these three hormones.

One of the secreted phosphoproteins is osteopontin (which supports cell adhesion), and targeted inhibition of its expression causes abnormal morphogenesis and lactation deficiency in the MG (Nemir et al., 2000). The synthetic glucocorticoid dexamethasone has been shown to induce its expression in growing MECs. Its expression is induced during involution (Lee et al., 2000), suggesting a role in protecting a specific group of epithelial cells from apoptosis, through promoting cell adhesion, allowing them to remain to proliferate during additional pregnancies.

10. MicroRNAs in mammary gland involution

MicroRNAs (miRs) regulating MG development and neoplasia have been discussed in a recently published review (Jena, 2017), highlighting their involvement in involution and modulation of proto-oncogenes and tumor suppressor genes in cancer progression. miRs are endogenous, small (18–22 nt) RNAs that down-regulate gene expression post-transcriptionally, thus influencing the translation and stability of mRNA (Valencia-Sanchez et al., 2006; Bartel, 2009). Physiological phenomena like cell proliferation, differentiation and death are mediated through miR-mediated gene regulation (Zhao and Srivastava, 2007). The miR cluster miR-424(322)/503 is a vital class that regulates MEC involution by targeting the gene expression of BCL-2 and the IGF1 receptor (Rodriguez-Barrueco et al., 2017). The protein TGF-β regulates the function of this miR cluster, as it has been observed that TGF-β pathway activation enhances transcription of miR-424(322)/503 cluster, which subsequently down-regulates the expression of key genes, thus orchestrating epithelial remodeling. Recently, it was observed that loss of the miR-424(322)/503 cluster promotes breast cancer development and chemoresistance, due to up-regulation of the BCL2 and IGF1R target genes (Llobet-Navas et al., 2014). Mammary transplantation experiments have revealed that miR-212 and miR-132, expressed in the MG stroma exclusively, down-regulate the matrix metalloproteinase-9 (MMP-9) gene. The miR-212/132 family, an important regulator of epithelial cell-stroma interactions, plays a role in MG development during puberty (Ucar et al., 2010). miR-30 targets vimentin, which is involved in epithelial-mesenchymal transition. It down-regulates vimentin (a potential marker of breast cancer prognosis), thus inhibiting the migration and invasion of cells (Cheng et al., 2012). The miRs play roles in maintaining mammary epithelial progenitors (Ibarra et al., 2007). The miR-205 and miR-22 are overexpressed in progenitors, whereas let-7 and miR-93 are down regulated. Enforced let-7 expression induces the loss of self-renewing cells. miR-205 is expressed at a higher rate in progenitors and promotes their expansion. It regulates the expression of the tumor suppressor protein PTEN, hence, over-expression of miR-205 may be important for the etiology of cancer (Greene et al., 2010). Increased expression of miR-30b culminates in defective lactation and delayed involution in the developing MG of mice (Guillou et al., 2012).

11. Conclusion

MG involution is a very complex phenomenon, culminating in the apoptosis of MECs, mammary tissue remodeling, and regeneration of the fat pad. A number of signaling molecules, proteins, and hormones are involved in this process as demonstrated in the available literature, and much more has yet to be elucidated. This review will provide researchers ample information on what is known so far related to involution. The microenvironment of MG involution mimics the microenvironment of a developing tumor, where STAT3 has been observed to be the crucial molecule, having a role in involution and the induction of breast carcinoma development, as demonstrated by some cases of postpartum breast tumors. Hence, unraveling the molecular mechanism of involution with greater detail will guide us to understand and ultimately provide insights into cancer development and possible therapies for it.

Conflict of interest

The authors declare no conflict of interest.

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