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Transforming growth factor- β s and mammary gland involution; functional roles and implications for cancer progression

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Abstract

During rodent mammary gland involution there is a dramatic increase in the expression of the transforming growth factor- β isoform, TGF- β 3. The TGF- β s are multifunctional cytokines which play important roles in wound healing and in carcinogenesis. The responses that are activated in the remodeling of the gland during involution have many similarities with the wound healing process and have been postulated to generate a mammary stroma that provides a microenvironment favoring tumor progression. In this review we will discuss the putative role of TGF- β during involution, as well as its effects on the mammary microenvironment and possible implications for pregnancy-associated tumorigenesis.

Keywords

TGF- β ; involution; microenvironment; wound-healing response; pregnancy-associated breast cancer

Introduction

Upon weaning, the mammary gland undergoes a highly-coordinated tissue remodeling process (involution) which returns it to a state similar to that of the virgin gland. Studies of involution in the mouse mammary gland after forced weaning have divided the process into 3 stages (1). In the initial phase which lasts for approximately 48 h, the cessation of milk secretion coincides with the loss of the differentiated phenotype of alveolar cells which begin to undergo apoptosis (2). During the second stage of involution, when the process becomes irreversible, proteolytic breakdown of the basement membrane causes a collapse of the alveoli (3) and clearance of residual milk proteins and apoptotic cells by phagocytes (1). The final stage involves the regrowth of the stromal adipose tissue with remodeling of the extracellular matrix (ECM) and lipid accumulation.

It has been proposed that mammary gland involution, which activates cellular apoptosis, matrix remodeling, inflammation and angiogenesis, has many similarities with the wound healing response, and may contribute to the aggressive nature of pregnancy-associated breast cancers (4;5). Preclinical studies have clearly demonstrated a tumor-promoting effect of wounding (6), including in breast cancer models (7). Furthermore, clinical gene expression studies have shown that patients whose breast cancer lesions express a 'wound-response signature' have a decreased overall survival and distant metastasis-free survival compared to patients whose tumors do not express this signature (8). The ability of the multifunctional cytokine, transforming growth factor- β (TGF- β), to regulate many of the biological processes associated both with the wound healing response and with cancer progression is provocative in this context

(reviewed in ⁹;10). In this review we will discuss the putative role of TGF- β during involution, as well as its effects on the mammary microenvironment, and the possible implications for pregnancy-associated tumorigenesis and/or parity protection.

TGF- β Background

TGF- β s as context-dependent regulators of a wide range of biological functions

TGF- β s are multifunctional cytokines regulating a number of diverse biological responses important in development, the maintenance of cellular homeostasis and response to injury (11–13). A central role for TGF- β in adult homeostasis is supported by the observation that dysregulation of the TGF- β pathway is associated with a wide range of pathological conditions, including autoimmunity, vascular diseases, fibrotic disorders and cancer (14). TGF- β s play particularly complex roles in carcinogenesis, with much pre-clinical and clinical data supporting the hypothesis that they suppress tumorigenesis in the early stages of the process, and then promote progression later on (15;16).

TGF- β s are the founding members of a large superfamily consisting of over 40 ligands, including activins, inhibins, nodals, bone morphogenetic proteins (BMPs), and growth and differentiation factors (17). There is evidence for expression of several of these superfamily members in various stages of mammary gland development (18–20), however, the TGF- β s are the only members to have been studied extensively in the involution process and are the focus of this review. Mammals express three highly homologous isoforms of TGF- β (TGF- β s 1, 2, and 3) which share 70 % sequence identity in the biologically active C-terminal region, and which all bind to the same receptor complex and activate the same downstream signaling pathways. Most cell types express one or more of the three isoforms of TGF- β , with TGF- β 1 being the most abundant and intensively studied isoform, and essentially all cells express TGF- β receptors. Thus TGF- β s are uniquely well-positioned to coordinate the functioning of many different cell types in a local tissue microenvironment.

The three TGF- β isoforms show distinct temporo-spatial patterns of expression *in vivo* and germline knock-out of the individual isoforms results in very different developmental phenotypes (21). Most of the observed differences are likely to be the result of different temporo-spatial patterns of isoform expression, but some may also reflect intrinsically different biological activities. The role of TGF- β isoforms during palate development provides an excellent example of these concepts. While TGF- β s 1 and 2 are expressed mainly in the mesenchyme of the developing palate, TGF- β 3 expression is confined to the epithelial cells at the medial edge of the palate, the cell type that is critical for palatal fusion (22). Consistent with the spatial restriction of TGF- β 3 to these cells, knock-out of TGF- β 3, but not TGF- β 1 and - β 2, inhibits palatal fusion (23). However, knock-in of the TGF- β 1 gene into the TGF- β 3 locus only partially rescues the cleft palate phenotype, suggesting that the two isoforms may elicit intrinsically different biological responses in addition to differing in their temporo-spatial expression patterns (24). Indeed, while the isoforms nearly always show essentially identical biological activities *in vitro*, a few differences have been noted. For example, TGF- β s1 and 3 inhibit migration of bovine aortic endothelial cells and TGF- β 2 does not (25), while TGF- β 3 induces migration of confluent fibroblasts and TGF- β s 1 and 2 do not (26). Similarly, TGF- β 3, but not TGF- β 1, protects keratinocytes against TPA-induced cell death, while TGF- β 1, but not TGF- β 3, promotes basal apoptosis in keratinocytes (27).

TGF- β s regulate a broad spectrum of biological activities, including cell growth and differentiation, apoptosis, cell migration, immune cell function, angiogenesis and ECM production (11;12). The ability of TGF- β to coordinate a variety of responses in a number of cell types is central to its role as an important mediator of wound healing, and nicely demonstrates the multifunctionality of this growth factor (9). Degranulation of platelets at the

site of a wound releases a large bolus of TGF- β 1 which contributes to the recruitment of inflammatory cells and fibroblasts into the wound bed. TGF- β acts on the inflammatory cells to induce production of a variety of cytokines, including additional TGF- β . TGF- β promotes the differentiation of fibroblasts into myofibroblasts which are contractile, motile and produce the majority of ECM proteins. In addition to stimulating ECM production, TGF- β 1 also inhibits ECM degradation by down-regulating the expression of matrix-degrading enzymes and increasing expression of matrix metalloproteinase (MMP) inhibitors in fibroblasts (13). The net effect is the accumulation of ECM needed for wound closure.

TGF- β signaling mechanisms provide insight into multifunctionality and contextuality

Studies on the TGF- β signaling pathway are shedding light on the molecular mechanisms underlying the remarkable multifunctionality and contextuality of TGF- β action. The active forms of all three TGF- β ligands bind to a constitutively active serine-threonine kinase transmembrane receptor (T β RII) which complexes with and phosphorylates the type I receptor (T β RI) on residues in the juxtamembrane domain (reviewed in ^{28,29}). In the canonical TGF- β signaling pathway (Figure 1), the activated T β RI phosphorylates receptor-activated Smad proteins (R-Smads) on a C-terminal SSXS motif, allowing them to form an oligomeric complex with Smad4, a common mediator Smad. This complex translocates to the nucleus where it interacts with Smad binding elements in the DNA and with other transcription and chromatin remodeling factors to regulate target gene expression. Generally, TGF- β induces phosphorylation of the R-Smads, Smads 2 and 3, while BMPs phosphorylate Smads 1, 5, and 8, but recent evidence suggests that under certain conditions TGF- β s may also activate BMP Smads (30;31). Furthermore, TGF- β s can also activate a number of non-canonical signaling cascades, including the MAPK, PI-3 kinase/Akt and S6 kinase pathways, and Smads are also substrates for MAPKs (32–34). Like the BMP Smads, Smad2 and 3 may serve as a platform for integrating signal inputs from multiple kinase pathways (35), and the complex phosphorylation patterns of R-Smads mediated by a number of intracellular kinases may contribute to the remarkable context-dependence of the biological actions of TGF- β .

Multilayered regulation of TGF- β biological activity creates challenges for interpretation of expression data

Since TGF- β s regulate such diverse biological responses, the activity of these ligands is subject to a complex set of regulatory mechanisms in order to ensure the appropriate temporal and spatial activation of signals necessary to maintain cellular homeostasis. The multilayered nature of these regulatory mechanisms has important practical implications for the interpretation of TGF- β expression data, as discussed below. Both TGF- β s 1 and 3 are subject to extensive translational regulation (36;37), so RNA levels of TGF- β s 1 and 3 may not correspond directly to protein levels. Furthermore, TGF- β is secreted in a biologically inactive, latent form which must undergo an activation process before it can bind to its receptor and initiate signaling (38). The activation of TGF- β is mediated primarily by interaction with thrombospondin, certain proteases, or integrins and appears to be highly regulated *in vivo* (38). Detection of TGF- β protein by tissue extraction and Western blotting or by immunohistochemistry generally shows levels of total, not active, TGF- β , so determination of TGF- β expression by these methods may not accurately reflect its local biological activity, and these caveats should be considered when interpreting most published data. Importantly, while active TGF- β is a master regulator of ECM synthesis in wound healing and fibrosis, the composition of the ECM can also regulate the bioavailability of TGF- β (39). The large latent complex of TGF- β binds to fibrillin (40), a component of elastic fibers. Degradation of microfibrils by MMPs or genetic alterations in fibrillin can activate latent TGF- β , and a specific fibrillin-1 fragment can bind to deposited fibrillin and release bioactive TGF- β (41;42). Myofibroblast contraction also can release active TGF- β from the ECM especially in the microenvironment of a stiff matrix which is present in wound granulation tissue (43;44). Thus

changes in matrix composition such as those that may occur during wound healing or involution are likely also to affect local bioavailability of TGF- β .

Expression of TGF- β during involution

Expression and roles for TGF- β in mammary gland development and functional differentiation

Several reports have used *in situ* hybridization and immunohistochemistry to describe the expression of TGF- β isoforms in the rodent mammary gland during development, pregnancy, lactation, and involution (45–47). As discussed in the previous paragraph, the demonstrated discordance between TGF- β RNA and protein levels can make interpretation of these studies difficult, however agreement between the results obtained by *in situ* hybridization and immunohistochemistry give a reasonable indication of local production of TGF- β . These studies show that during mammary gland development TGF- β s 1 and 3 are expressed in the stromal cells of the cleared mammary fat pad and in the epithelium of the mammary end buds during branching morphogenesis, with TGF- β 3 also being expressed in myoepithelial cells. At these stages, the TGF- β s are thought to play a role in dictating spacing of the ductal tree, in preventing precocious lobular-alveolar development in virgin animals and possibly in regulating the mammary stem cell (45;⁴⁸;49). TGF- β 2 is expressed at low levels in the virgin gland and is only significantly expressed during pregnancy. TGF- β 1 expression is similar in the virgin and pregnant mammary gland, whereas TGF- β 3 expression increases in ducts and alveoli during pregnancy. However, the mRNA expression of all three isoforms, as well as the protein expression of TGF- β 3 as determined by immunostaining, is greatly reduced during lactation. Since TGF- β s inhibit the synthesis of milk casein proteins, as determined by Western blotting, in explant cultures of the pregnant mammary gland in response to lactogenic hormones (50), the relatively high levels of TGF- β present during pregnancy may inhibit milk production, with the sudden decrease in TGF- β mRNA and protein expression at parturition then permitting milk secretion.

Expression of TGF- β s in involution

During involution in the mouse, the expression of TGF- β s 1 and 2 mRNA returns from the low level seen in lactation back to that of the virgin gland (46). In contrast, TGF- β 3 mRNA shows a dramatic and progressive increase during involution, with a maximum 5–6-fold increase above that in the virgin gland and a 3-fold increase compared to pregnancy (46;51) (Figure 2). Increased expression of TGF- β 3 mRNA is seen as early as 3 h after pup removal (52), peaks at 2–4 days and continues at high levels for up to 9 days post-weaning (46;⁵¹;53). At one day post-weaning, the majority of TGF- β 3 expression is located in the lobular-alveolar cells as shown by immunohistochemistry (52). However at 4 days post-weaning, *in situ* hybridization and immunohistochemistry show that TGF- β 3 expression is most abundant in the myoepithelial cells (46). This change in the localization of TGF- β 3 may relate to differences in its roles in early and late involution as discussed in the next section. Interestingly, this is one of the few examples in the literature where the TGF- β 3 isoform specifically has been implicated in a post-natal biological process.

Milk stasis seems to play a critical role in inducing the dramatic increase in TGF- β 3 expression at weaning. In teat sealing experiments TGF- β 3 mRNA is expressed in the sealed gland, but not in the contralateral feeding gland of the same mouse (52). Weaning results in a rapid decrease in the pituitary hormones prolactin and oxytocin, but exogenous administration of these hormones does not affect TGF- β 3 mRNA levels in the absence of suckling, suggesting that milk stasis *per se* and not changes in lactogenic hormones, induces TGF- β 3 expression upon weaning. The underlying mechanism of this effect is currently not known. The rapidity of the increase in TGF- β 3 mRNA in involution (significantly increased within 3h) is intriguing,

and suggests that this isoform might contribute to early “priming” of the gland for involution should milk stasis continue.

It is not known whether the high levels of TGF- β 3 seen during involution in the mouse mammary gland also occur in other species. There are reports of increased expression of TGF- β 1 mRNA and protein in the bovine, porcine, and caprine involuting glands as compared to glands in the early lactation phase (54–56), but expression of other TGF- β isoforms was not examined. Importantly, there are no reports regarding the expression of TGF- β isoforms in involuting gland in humans, and even in animal models most of the studies have been correlative expression studies, with all the caveats for interpreting mRNA and protein levels in terms of bioactive TGF- β that were discussed earlier. Additional studies are needed to document the expression of TGF- β isoforms and their activation states in involution, especially in the regressing gland in humans.

Functions of TGF- β in involution

Apoptosis

Most of what is known about the biological roles of TGF- β s in involution comes from the elegant functional studies of Nguyen and Pollard (52). In wild-type C57Bl/6 mice expression of TGF- β 3 mRNA began to rise as early as 3 h after pup removal, significantly preceding the peak of alveolar apoptosis in the mammary gland which occurred at approximately 48 h after pup removal (46;⁵⁷;58). Given the known ability of TGF- β s to induce apoptosis in many target cells *in vitro* (59), it was reasonable to propose that the elevated TGF- β 3 might be critical for inducing epithelial cell apoptosis during involution. Transgenic over-expression of TGF- β 3 driven by the β -lactoglobulin promoter to force expression to the lobular-alveolar cells at the end of pregnancy and through lactation, resulted in elevated levels of apoptotic cells at d 1–3 post-parturition (52), suggesting that TGF- β 3 is capable of inducing apoptosis of mammary epithelial cells (MEC) *in situ*. TGF- β 3 null mice die at birth (23), so to address whether endogenous TGF- β 3 was responsible for inducing apoptosis in the involuting gland, mammary glands from TGF- β 3 null and wild-type mice were transplanted into wild-type hosts, and these mice were mated and sacrificed one day after the birth of their pups (52). Since the transplanted mammary glands are not connected to the nipple, milk rapidly accumulates and they begin to undergo the first stage of involution despite normal circulating hormone levels and lactation from intact host glands. There was a 70% reduction in apoptosis in the TGF- β 3 null mammary glands as compared to corresponding wild-type glands, which strongly suggests that endogenous TGF- β 3 plays a major causal role in the initial stage of alveolar apoptosis following pup removal.

The importance of TGF- β signaling in the early stages of involution was addressed in a number of other studies in genetically engineered mouse models. When mammary epithelium from Smad3 null or wild-type mice was transplanted into the cleared fat pads in nude mice, there was an ~40% reduction in apoptotic cells at days 1 and 2 after parturition in involuting Smad3 null glands compared to wild-type glands (60). Similarly, transgenic mice over-expressing a dominant negative type II TGF- β receptor from the MMTV-LTR (MMTV-DNIIR mice) showed delayed involution after normal weaning (61), and in teat-sealing experiments to induce milk stasis and involution, alveolar structures failed to collapse in the transgenic mice by day 3 after teat sealing (62). A similar phenotype of delayed involution with persistence of distended uncollapsed alveolar structures at day 3 was seen in a model that used Cre recombinase driven by the whey acidic protein promoter to delete T β RII specifically in the lobulo-alveolar epithelium (63). There was a corresponding delay in the wave of apoptosis, with higher apoptosis seen in knock-out compared to wild-type glands at days 7 and 10 post-involution.

The above results are consistent with a critical role for TGF- β 3, signaling through the TGF- β receptor complex and the canonical Smad3 pathway, in inducing apoptosis and alveolar collapse in the early stages of involution. In contrast, transgenic mice expressing a constitutively active T β RI driven by the MMTV promoter showed a 3-fold *decrease* in apoptotic cells in the mammary gland at 3 days post-weaning as compared to control mice (64). The reason why TGF- β signaling appears to promote survival of MECs during involution in this study, while promoting apoptosis in other studies, is unclear. Perhaps the high signaling flux generated by the constitutively active receptor recruits pathways that are not normally active under physiological conditions.

Several studies have proposed molecular mechanisms that may mediate the pro-apoptotic effects of TGF- β during involution. Sealed glands in MMTV-DNIIR mice showed a decrease in caspase-3 activation accompanied by an increase in phosphorylation of Akt when compared to wild-type sealed glands (62), suggesting that endogenous TGF- β may promote apoptosis in part by decreasing activation of the Akt pro-survival pathway. In support of this mechanism, the expression of dominant-negative Akt in HC11 mouse mammary epithelial cells *in vitro* blocked the protective effects of prolactin on TGF- β -induced apoptosis (62). Additional mechanisms have also been proposed. Microarray analysis show that expression of the TGF- β target, TGF- β -stimulated clone 22 (Tsc-22) is increased 12–24 h after pup removal (65). TGF- β induces expression of Tsc-22 mRNA and transient over-expression of Tsc-22 induces apoptosis in MCF-7 cells (65). Like TGF- β 3, Tsc-22 is expressed in the myoepithelial cells of the virgin and pregnant gland, but in the luminal epithelium during involution, consistent with an important functional relationship between these two molecules (65). Interestingly, down-regulation of Tsc-22 is observed in salivary gland and brain cancers (66;67), suggesting that decreases in Tsc-22-mediated apoptosis may contribute to the carcinogenic process. In mammary glands transgenically over-expressing TGF- β 3 (52) there is increased expression of the pro-apoptotic factor, Bax, which has been shown to contribute to apoptosis in the early phase of involution (68). TGF- β 1 also induces apoptosis and Bax expression in rat liver epithelial cells (69) and primitive hematopoietic cells (70). Furthermore, TGF- β 3 overexpression was associated with increased nuclear localization of phospho-Stat3, and it has been proposed that the JAK/Stat pathway may mediate apoptosis during involution (71). The mechanisms whereby TGF- β 3 might activate the Stat3 pathway are currently not clear.

Tissue remodeling and immune cell infiltration

The later phases of involution involve influx of immune cells, loss of basement membrane, removal of dead cells and then tissue remodeling to restore the pre-pregnant glandular architecture (3;⁵;65). While the experimental evidence is compelling for a key functional role for TGF- β 3 in driving apoptosis of lobular-alveolar cells in the initial phase of involution, to date no studies have convincingly addressed the functional roles of TGF- β s in the later stages. However, the fact that TGF- β 3 mRNA levels do not peak until day 4 of involution, and then persist at above virgin levels until at least day 8, strongly suggests that TGF- β 3 is likely to play an important role in these later phases. Northern blot and microarray studies have identified genes whose expression is altered during the later phase of involution (3;⁷²;73), and many of these are involved in the wound healing cascade and are known TGF- β targets and/or regulators of TGF- β activity (5). Thus it is tempting to speculate that TGF- β 3 may contribute to many aspects of the wound-healing type of response that occurs in involution, as discussed in more detail below.

Influx and activity of inflammatory cells—Microarray studies on the involuting gland have shown that genes of the acute phase response are upregulated early in involution (73). Thus, although involution involves programmed as opposed to unscheduled tissue destruction, it may nevertheless evoke a transient local inflammatory response. At the histological level,

increased numbers of neutrophils and macrophages, which are likely recruited to remove cellular debris, are apparent on days 3 and 4 post-weaning (73), and in addition there is an influx of plasma cells and eosinophils, whose role in the involution process is not currently known. In wound healing, TGF- β 1 has been shown to be a potent chemoattractant for both neutrophils (74) and monocytes (75), and TGF- β 3 has similar chemotactic effects on monocytes *in vitro* (76). Even though other pro-inflammatory mediators, such as cytokines and ECM fragments are upregulated once the tissue destruction phase is underway, increased TGF- β 3 levels at the earlier time points could contribute to the initial recruitment of these phagocytic cells into the regressing gland. The recruiting effects of TGF- β on inflammatory cells are dose-dependent, and at higher concentrations recruitment and migration are inhibited (75). This dose-dependency may restrict the pro-inflammatory activity of TGF- β to the early time points when TGF- β levels are still relatively low. Furthermore, TGF- β s can also have anti-inflammatory effects, as suggested by the autoimmune-like multifocal inflammatory phenotype of the TGF- β 1 null mouse (77). Mechanisms for suppression of inflammation include down-regulation of MHC expression, inhibition of proliferation and effector function of a number of different immune cell types, and induction of inhibitory regulatory T-cells (78). Thus the high levels of TGF- β seen at the later stages of involution could actually be important in limiting the inflammatory response to the programmed tissue degradation, thereby preventing uncontrolled tissue destruction and the development of undesirable autoimmune responses.

Tissue degradation and remodeling—The remodeling phase of involution involves the destruction and removal of the secretory alveolar cells and underlying basement membrane, followed by extensive remodeling of the ECM and restoration of the adipose stroma. TGF- β inhibits adipocyte differentiation *in vitro* (79), so it is unlikely that TGF- β 3 contributes to adipocyte repopulation, but there are many reasons to think it may have a central role in the fibroblast recruitment and remodeling of the ECM. ECM remodeling is a hallmark activity of the TGF- β s that is seen in wound healing and many fibrotic disorders (9). Early experiments showed that subcutaneous injection of TGF- β 1 into neonatal mice induces fibroblast accumulation and ECM production (80). TGF- β 1 is chemotactic for fibroblasts *in vitro* (81) and TGF- β 3 can have similar effects (82). However, in more biologically relevant 3-dimensional matrices, TGF- β 3 is the only isoform which induces motogenesis of dermal fibroblasts (83), so induction of TGF- β 3 during involution could play a critical role in fibroblast recruitment. Both TGF- β s 1 and 3 can promote differentiation of quiescent fibroblasts into activated fibroblasts which secrete ECM proteins (84).

Data-mining by Schedin and colleagues of published gene expression datasets in involuting mouse glands (5) has shown major changes in expression of wound healing and ECM genes at day 2 of involution. Most of these genes are direct targets of TGF- β and/or regulate the bioavailability of TGF- β (see Table 1). These include CTGF, itself a potent regulator of ECM; structural components of the ECM including many collagens, collagen accessory proteins such as decorin, lumican and biglycan, and other fibrillar components such as fibrillin and elastin; secreted glycoproteins such as thrombospondin and SPARC; and matrix degrading enzymes including MMPs 3 and 15 and ADAM family proteases. Thus a compelling argument could be made for a major role of TGF- β in recruiting and activating fibroblasts to remodel the ECM in the involuting gland. However, clearly this question needs to be addressed experimentally.

Figure 3 provides a summary of the defined and putative roles of TGF- β during different phases of involution in the rodent. There is a dramatic induction of TGF- β expression at the beginning of involution and relatively high levels are maintained into the later stages. As shown, TGF- β has the potential to modulate almost all of the biological processes that are a part of involution, many of which are related to its role as a regulator of wound healing.

Effects of parity and involution on tumorigenesis

Parity has a dual effect on breast cancer risk. While in general parity appears to have a protective effect, in the period immediately following parturition, there is an elevated risk of breast cancer in all age groups. This phenomenon is known as “pregnancy-associated breast cancer” and is associated with more aggressive, poor-prognosis forms of the disease. Schedin and colleagues have proposed a mechanistic explanation for this effect (4), suggesting that the tissue remodeling response that occurs during involution may alter the state of the mammary gland stroma to one which promotes tumor cell growth and dissemination. In support of this hypothesis, they have shown that ECM isolated from nulliparous glands promoted ductal organization of normal MEC and suppressed invasion of mammary tumor cells, while ECM isolated from involuting glands did not support ductal development in normal cells, but did promote invasiveness of tumor cells (85). Furthermore, when breast cancer cells (MDA-MB-231) were mixed with involution ECM and injected into the mammary fat pad of nude mice, metastases to the lung, liver, and kidney were increased as compared to cells injected with nulliparous ECM, even though the sizes of the primary tumors were similar (85).

Even though the increased risk of breast cancer remains for 10 years post-partum in humans (86), the aggressive nature of pregnancy-associated breast cancers appears to decrease with increasing time post-parity, as there is an inverse relationship between survival and the time between birth and breast cancer diagnosis (87). The mechanisms that contribute to the poor prognosis of pregnancy-associated breast cancers are not well understood. It is possible that exposure of a pre-existing initiated, pre-malignant or early malignant MEC present during pregnancy to the combination of pregnancy hormones and involuting stroma may have pro-progression effects that take years to manifest themselves as a metastatic lesion. Alternatively, involution may durably change the post-partum stroma in such a way that it promotes progression of initiated cells years after parturition. There are very few studies which have characterized the stroma of the completely regressed gland. In the rat at 2 months post-weaning, protein levels of fibronectin, laminin, tenascin, and clusterin, along with expression and activity of MMPs 2, 3, and 9 appear to be similar to those in the virgin gland (88;89), suggesting that the regressed stroma has returned to a quiescent state, though there may be persistent changes in collagen (Schedin, unpublished). It is obvious that additional studies are needed, especially in humans, to determine the persistence of altered mammary stroma post-involution and its possible contribution to the aggressive nature of pregnancy-associated breast cancers.

At later times after parturition, with elapsed time to the cross-over depending on the age at first pregnancy, the net effect of parity is to decrease breast cancer risk (86). This parity protection effect has been ascribed to the differentiating effect of pregnancy hormones on the mammary epithelium, as the effect of pregnancy can be mimicked in rodents simply by treatment with high levels of 17 β -estradiol and progesterone (90). Thus the epidemiologic and experimental data are consistent with a comparatively short-lived promoting effect of pregnancy on breast cancer progression, possibly driven by involution-related alterations in mammary stroma, followed by a long-lasting protective effect, probably driven by changes in the mammary epithelium elicited by exposure to pregnancy hormones.

Roles for involution-induced TGF- β in pregnancy-associated breast cancer?

As is the case for parity, the effects of TGF- β s on breast cancer progression are complex. A wealth of pre-clinical and clinical data support the hypothesis that TGF- β s function initially as tumor suppressors but then promote progression in the later stages of carcinogenesis (15; 91). Tumor suppressor effects in the early stages include inhibition of cell proliferation, induction of apoptosis or replicative senescence, and maintenance of genomic stability in the target epithelium (reviewed in 92). However, as the carcinogenic process progresses, many

cancer cells selectively lose responsiveness to the tumor suppressor effects of TGF- β , accompanied by an increased expression of the TGF- β ligand in the tumor bed (examples in 92). At this later stage, the elevated TGF- β levels now promote progression and metastasis by a number of mechanisms, involving both the tumor parenchyma and cells of the tumor stroma. TGF- β can induce an epithelial-to mesenchymal transition, which enhances migration and invasion and may induce a more “stem-cell” like phenotype in the transformed cell (93). TGF- β promotes differentiation of fibroblasts to a myofibroblast-like “cancer-associated fibroblast” phenotype, with resulting changes in ECM composition, the production of MMPs, cytokines, and chemokines that promote tumor cell proliferation and invasion, as well as neoangiogenesis to support metastatic growth (15). Additionally, TGF- β has profound immunosuppressive effects, suppressing the generation and/or activity of cytotoxic T-cells and natural killer cells, thereby allowing the tumor to escape the host immune response (78;94).

Clearly TGF- β has the potential to play multiple roles in carcinogenesis. In the current context however, the most relevant possibility to consider is that increased expression of TGF- β during involution may contribute to the wound-like and tumor-promoting environment of the regressing gland, and hence to the development and poor prognosis of pregnancy-associated breast cancers. Interestingly wound healing is one of the few biological processes for which dramatically different responses to different TGF- β isoforms have been experimentally demonstrated, and these differences are crucial to the discussion since during involution in rodents, TGF- β 3 expression increases significantly more than does that of TGF- β s 1 or 2.

It has been suggested that TGF- β 1, which is present at high levels in platelets, has been utilized in evolution to promote the rapid, but scarring, closure of wounds so as to minimize infection (95). The trade-off of this protective effect is that the organization of the local ECM is permanently altered to a non-native form, that among other altered properties, may promote tumorigenesis. Indeed, the incidence of skin cancers is known to be higher at scar sites, as in burn victims (96;97). *In vitro*, TGF- β s 1 and 3 show similar activities associated with desmoplasia, including recruitment and activation of inflammatory cells and fibroblasts, increase in ECM production and enhancement of blood vessel formation (78;⁹⁸;99). However, the effects of TGF- β 1 and TGF- β 3 on wound healing *in vivo* appear to be dramatically different from each other. In contrast to wounds made in adult tissues, wounds made in mammalian embryos heal without scarring, and this effect is associated with high levels of TGF- β 3 and low levels of TGF- β s 1 and 2 (100;101). Furthermore, when cutaneous wounds are made in rodents and treated with exogenous TGF- β 3, the wounds heal with reduced scarring and show decreased numbers of monocytes and macrophages, as well as decreased ECM deposition, suggesting that TGF- β 3 can actively oppose the pro-desmoplastic effects of TGF- β 1 *in vivo* (95). TGF- β 3 is being used in a clinical setting for scar prevention (102), suggesting that the opposing effects of TGF- β 1 and TGF- β 3 in wound healing are also seen in humans.

Since TGF- β 3 prevents scarring at cutaneous sites, it is tempting to speculate that one important role of TGF- β 3 in involution may in fact be to limit stromal activation and thus prevent scarring or pathological desmoplasia from occurring in the mammary gland as a consequence of this dramatic, but physiological, tissue remodeling event, allowing repeated cycles of lactation and involution. By extension, TGF- β 3 may be critical for limiting the tumor promoting effect of involution in driving pregnancy-associated breast cancer. Both TGF-1 and TGF- β 3 are polymorphic genes (103;104), so one can expect variation in the relative levels of these two proteins across the human population. Since both isoforms appear to be present during the involution phase, it is conceivable that the relative levels of these two TGF- β s during this phase can qualitatively affect the composition of the involution stroma and hence breast cancer progression. This logic generates the testable hypothesis that genetic combinations that favor a high TGF- β 3/TGF- β 1 ratio might protect against pregnancy-associated breast cancer by limiting and/or resolving the wound-like changes in the mammary stroma.

There are currently no published studies that look at gene expression signatures specifically in pregnancy-associated breast cancer, so we can only look to clues as to the possible role of TGF- β 3 using more general breast cancer datasets. When publically available clinical cancer microarray datasets (www.oncomine.org) are analyzed with respect to TGF- β 3 mRNA expression, there are 6 independent breast cancer studies that show a highly statistically significant negative correlation between TGF- β 3 mRNA levels and increasing tumor grade (Figure 4A). Furthermore, TGF- β 3 is present in a 70 gene signature which predicts clinical outcome in breast cancer, with high expression of TGF- β 3 mRNA being associated with good outcome (105) (Figure 4B). Thus these correlative data are consistent with a protective role for TGF- β 3 in breast cancer progression, but obviously there is no demonstration of causality in these studies and the relevance to involution and pregnancy-associated breast cancer is not clear.

It is possible that the elevated TGF- β 3 that is associated with decreased breast cancer stage and good prognosis is independent of the TGF- β 3 that is involved in the involution process. In investigating the phenomenon of parity protection, D'Cruz et al (53) identified a 38 gene signature which reproducibly distinguished between nulliparous and parous glands one month post-involution in several strains of mice and rats. The parous glands showed a general decrease in expression of growth-promoting genes and increased expression of genes associated with differentiation and immune cell function, as well as an increase in TGF- β 3 and three of its transcriptional targets, clusterin, Eta-1, and Id-2. Moreover, TGF- β 3 was part of a 5 gene signature that remained up-regulated 30 wks post-involution, suggesting there may be persistent changes in the regressed gland compared to the nulliparous gland. However, the authors also showed that parity protection and the persistent induction of TGF- β 3 could be generated by simply treating the rodents with pregnancy hormones, without the need for full term pregnancy and lactation. These observations suggested that the parity protection phenomenon and associated upregulation of TGF- β 3 is independent of the upregulation of TGF- β 3 that occurs during involution. The authors proposed that TGF- β 3 might contribute to parity-induced protection against breast cancer by virtue of the known ability of TGF- β s to inhibit proliferation and induce apoptosis of MECs (53). Clearly, there is a critical need for functional studies *in vivo* to clarify the potentially opposing roles of the various TGF- β isoforms in involution, pregnancy-associated breast cancer and parity protection.

Conclusions

Evidence suggests that TGF- β 3 plays a causal role in promoting apoptosis in early stages of involution in mouse models, though it is unknown if TGF- β 3 is an important functional player in the human breast. The elevated expression of TGF- β 3 in the later stages of involution in rodents is consistent with an ongoing role for TGF- β 3 in the remodeling phase which has many parallels to the wound healing process. Since TGF- β 3 opposes TGF- β 1 in cutaneous wound healing, it is tempting to speculate that one critical role of TGF- β 3 during late involution may be to limit the development of a pathological desmoplastic response, and thus to limit the tumor-promoting effects of the involution stroma. The association of TGF- β 3 with lower tumor grade and better outcome in clinical microarray studies of breast cancer is consistent with this hypothesis, but may reflect protective roles for TGF- β 3 at other stages of the parity process. There is an urgent need for pre-clinical studies to address the functional roles of the TGF- β isoforms in involution and their possible involvement in the tumor-promoting effects of the involution stroma, as well as for careful examination of TGF- β isoform expression in the involuting human breast and specifically in the subset of breast cancers that are pregnancy-associated.

Abbreviations

ADAM	A disintegrin and metalloproteinase domain
BMP	bone morphogenetic protein
CTGF	connective tissue growth factor
DNIIR	dominant-negative TGF- β type II receptor
ECM	extracellular matrix
MAPK	mitogen-activated protein kinase
MEC	mammary epithelial cells
MMP	matrix metalloproteinases
MTV	mouse mammary tumor virus
PI-3	phosphatidylinositol-3
SPARC	secreted acidic cysteine rich glycoprotein
T β RI	TGF- β type I receptor
T β RII	TGF- β type II receptor
TGF- β	transforming growth factor- β
Tsc-22	TGF- β -simulated clone 22

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Figure 1.

Overview of the TGF- β /Smad signaling pathway. Latent TGF- β , bound to the ECM, undergoes an activation step (see text), releasing the TGF- β ligand which binds to the constitutively active Type II receptor at the cell surface. The Type I receptor is recruited into the complex where it is phosphorylated. The activated Type I receptor phosphorylates R-Smads (Smads 2 and 3) at the C-terminal serine residues and the R-Smads complex with the common mediator Smad4. This complex translocates to the nucleus where it regulates transcription of target genes and binds to a variety of transcription factors (TF).

Figure 2.

Quantitation of TGF- β mRNA levels in the mammary gland from a virgin mouse (V), pregnant mouse at 15 d gestation (P), lactating mouse (L), and days 2, 4, 6, and 8 of involution. (♦ TGF- β 1, ■ TGF- β 2, ▲, TGF- β 3). Taken from Ref.(46) with kind permission of Springer Science +Business Media.

Figure 3.

Involvement of TGF- β in different phases of rodent mammary gland involution. Milk stasis initiates involution as well as induces expression of TGF- β which in turn has effects on the processes indicated by arrows. A solid arrow indicates that a direct effect by TGF- β has been demonstrated in involution. A dashed arrow indicates a possible effect by TGF- β inferred from its biological activities in other systems. Double arrowheads indicate that these processes are also likely to result in production of additional TGF- β .

Figure 4.

TGF- β 3 mRNA expression in clinical breast cancer datasets. A. Inverse relationship between TGF- β 3 mRNA expression and the histopathologic tumor grade. Data is shown for the study of Miller et al.(135), but a similar relationship was seen in 6 independent studies, using two different array platforms. B. Inverse relationship between TGF- β 3 mRNA and metastasis at 5 years. Data is taken from the Van't Veer study (105). Datamining of clinical breast cancer array datasets was done using Oncomine analysis tools (www.oncomine.org). Boxes represent median and interquartile values, while whiskers represent the range. N represents the number of patients in each group.

Table 1ECM and wound healing genes changed at involution day 2 and their relationship to TGF- β^a

Gene name	TGF- β Target	TGF- β Modulator	Reference
TGF- β 1	X ^b	X ^c	(104;106)
TGF- β 2	X	X	(104;107)
TGF- β 3	X	X	(108)
CTGF	X		(109)
Procollagen, type I, α 1	X		(110)
Procollagen, type I, α 2	X		(111)
Procollagen, type III, α 1	X		(111)
Procollagen, type IV, α 5	X	X	(112;113)
Procollagen, type V, α 1	X		(114)
Procollagen, type V, α 2	X		(114)
Procollagen, type VI, α 1	X		(111)
Procollagen, type VI, α 3	X		(111)
Procollagen, type XV	X		(115)
Procollagen, type IX, α 1			
Procollagen, type IX, α 2			
Procollagen, type XIII, α 1	X		(116)
Elastin	X	X	(117;118)
Fibrillin 1	X	X	(119;120)
Microfibrillar assoc. protein 5			
Decorin	X	X	(121;122)
Lumican	X		(123)
Biglycan	X	X	(121;124)
MMP3	X		(111)
MMP15	X		(125)
ADAM 10	X		(126)
ADAM 5	X		(127)
ADAM 9	X		(127)
SPARC-like 1			
TIMP2			
Thrombospondin	X	X	(128;129)
SPARC	X	X	(130;131)
Cartilage assoc. protein			
Fibulin 2			
Periostin	X	X	(132;133)
Amyloid β precursor prot.	X		(134)
Amyloid β precursor-like protein 2			

^aGene list taken from Scheiden et al. (5)^bGene expression altered by TGF- β ^cModulator alters TGF- β expression or activation