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Choreographing Metastasis to the Tune of LTBP

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Abstract

Latent Transforming Growth Factor beta (TGF β) Binding Proteins (LTBPs) are chaperones and determinants of TGF β isoform-specific secretion. They belong to the LTBP/Fibrillin family and form integral components of the fibronectin and microfibrillar extracellular matrix (ECM). LTBPs serve as master regulators of TGF β bioavailability, functioning to incorporate and spatially pattern latent TGF β at regular intervals within the ECM, and actively participate in integrin-mediated stretch activation of TGF β in vivo. In so doing they create a highly patterned sensory system where local changes in ECM tension can be detected and transduced into focal signals. The physiological role of LTBPs in the mammary gland remains largely unstudied, however both loss and gain of LTBP expression is found in breast cancers and breast cancer cell lines. Importantly, elevated LTBP1 levels appear in two gene signatures predictive of enhanced metastatic behavior. LTBP may promote metastasis by providing the bridge between structural and signaling components of the epithelial to mesenchymal transition (EMT).

Keywords

LTBP; TGF β ; Fibronectin; Metastasis; Mammary; Breast cancer

Introduction

TGF β s are small peptides (25 kDa dimer) belonging to a superfamily of over thirty related disulphide-linked dimeric growth factors that includes the bone morphogenetic proteins (BMPs), inhibins and activins, and growth and differentiation factors (GDFs). TGF β s are multifunctional: they inhibit proliferation, enhance ECM synthesis, promote apoptosis, downregulate cell-cell and upregulate cell-matrix adhesion receptor expression to favor cell movement, and regulate diverse immune responses. All three mammalian TGF β isoforms (TGF β 1-3) are produced in dynamic temporal-spatial expression patterns during mammary

development and the mammary cycle [1–4]. TGF β signaling within the mammary stroma inhibits HGF and induces Wnt5a expression, which acts in a paracrine fashion to attenuate ductal morphogenesis during puberty [5, 6]. Luminal TGF β signaling prevents precocious alveolar production and differentiation in virgin mice and TGF β 1 suppresses proliferation of ductal ER-positive cells in an autocrine manner [1, 3, 7, 8]. Physiological upregulation of TGF β 3 expression promotes alveolar apoptosis during involution [9]. In keeping with the above roles, gain of TGF β signaling results in ductal hypoplasia and compromised alveologenesis, whereas loss or suppression of signaling leads to precocious ductal extension and alveologenesis, hyperbranching and delayed involution depending on the specific cell-type targeted [10–13]. Importantly, TGF β exerts biphasic effects in breast cancer. By maintaining cells in a quiescent state during normal homeostasis TGF β functions as a breast tumor suppressor. However, during malignant progression, as cells lose or block the TGF β growth inhibition pathway, alternative TGF β signaling routes can induce epithelial to mesenchymal transition (EMT) and, as a consequence, promote metastatic dissemination and acquisition of stem cell properties [14, 15]. In addition to its role in breast cancer, TGF β signaling also contributes significantly to the development of radiation-induced breast fibrosis [16].

A key feature that distinguishes TGF β 1-3 from most other members of the TGF β superfamily, and which is central to understanding their biology, is their secretion in a latent form [17, 18]. All three TGF β s are widely expressed and act through a common receptor system that is found on most cells to trigger intracellular signal transduction [14, 17]. Thus specificity of TGF β action lies not in transcriptional regulation of ligand or receptor expression but is instead determined by three key posttranscriptional events: 1) regulation of latent TGF β secretion, 2) spatial targeting, localization and deposition of latent TGF β within the ECM and 3) subsequent temporal activation, involving release or steric exposure of the mature TGF β ligand from its latent complex [18]. Latent TGF β binding proteins (LTBPs) play critical roles in each of these three events and thus are master regulators of the specific focal presentation of TGF β to its receptors and render the matrix poised for conversation with overlying cells.

LTBPs: Essential TGF β Chaperones and Determinants of TGF β Isoform-Specific Secretion

LTBP1, as its name suggests, was discovered as a large molecular weight protein (125–160 kDa) covalently complexed to latent TGF β [19]. This association forms within the biosynthetic compartment creating what is referred to as the large latent complex (LLC) (see Fig. 1) [20]. LTBP1 is synthesized in molar excess of pro-TGF β and forms disulphide bonds with cysteine 33 in the TGF β 1 propeptide domain while in the endoplasmic reticulum [21, 22]. Here, TGF β also dimerizes via three disulphide bonds: two between the propeptides and one between the TGF β domains [19, 20, 23]. Once in the Golgi apparatus, TGF β propeptides become glycosylated and are cleaved by furin but remain tightly associated with the mature TGF β dimer (25 KDa) to sterically hinder and regulate subsequent interaction with its receptor. Hence, the cleaved TGF β propeptide dimer is referred to by its functional property as the latency associated peptide (LAP). Importantly, in the absence of LTBP, LAP forms aberrant disulphide bonds within TGF β , resulting in delayed secretion of a misfolded, denatured and inactivatable form of the latent complex [24–26]. Thus, the initial function of LTBP1 is that of a chaperone, which ensures correct folding and efficient secretion of latent TGF β [20, 24].

LTBPs coevolved with the TGF β family and appear to have emerged in sea urchins [27]. Cloning and analysis of the human databases has revealed the presence of four genes, *LTBP1-4*, encoding a family of closely related proteins [28–32]. Genbank analyses also

reveal the presence of pseudogenes for human *LTBP1* and *3* and mouse *Ltbp1*, *2* and *4*. *LTBP2* and *LTBP3* are transcribed from single promoters, whereas long and short isoforms of *LTBP1* and *LTBP4* are generated by transcription from two independent promoters. Extensive alternative splicing has been reported for all LTBP, resulting in the expression of multiple tissue-specific variants with altered binding properties for TGF β and/or ECM proteins (see Fig. 2) [2, 27]. All four LTBP mRNAs are produced in mouse mammary gland (Pinderhughes and Cowin unpublished results) and database analyses reveal that they are also expressed to varying degrees in human breast tissues and normal as well as breast cancer cell lines (see Table 1). LTBP1-4 differ in their binding affinities for specific TGF β -LAP domains (see Table 2) [21, 22, 33, 34]. LTBP1 and 3 covalently bind to all three TGF β -LAPs, whereas LTBP4 associates specifically with TGF β 1-LAP [33]. In contrast, LTBP2 is incapable of binding TGF β -LAP and thus has no effect on TGF β secretion. Differences in LTBP1, 3 and 4 expression patterns and TGF β preferences, can therefore regulate not only the folding and rate of secretion but also influence which particular TGF β species emerges from the cell.

The LTBP/Fibrillin Family: Integral Components of the ECM that Spatially Pattern Latent TGF β

The role of LTBP in TGF β biology does not end at the cell surface. LTBP remains bound to secreted latent TGF β and play an essential role in determining the spatial pattern of latent TGF β deposition within the ECM (see Fig. 1). LTBP associates with at least two elements—fibronectin and microfibrils—and themselves form integral components of the ECM [35–37]. LTBP shows extensive sequence similarity with fibrillins, which form the major component of microfibrils that ensheath elastic fibers [27, 38–40]. LTBP and fibrillins are organized into an N-terminal region, a hinge and a C-terminal rod that is stiffened by Ca²⁺ association with multiple EGF-like repeats (see Fig. 2). Fibrillins are larger and in general contain twice as many EGF-like and 8-cysteine containing repeats (8-cys) as found in LTBP. 8-cys repeats are found exclusively in LTBP and fibrillins forming a hallmark of this family [27]. These structural domains come in two forms. One 8-cys repeat in LTBP1, 3 and 4 is structurally modified by a two-amino acid insertion that facilitates disulphide bond formation with LAP C33. All other 8-cys domains lack this modification [22, 33]. Thus LTBP1, 3 and 4 bind to LAP-TGF β whereas LTBP2 and fibrillins fail to do so [21, 22, 33, 34].

Upon exiting the cell, fibrillins initially associate with fibronectin and require this protein for further assembly. They copolymerize in a head to tail fashion, associate laterally in a staggered manner and become crosslinked to form microfibrils. Microfibrils have a ~10 nm diameter ‘beads-on-a-string’ appearance and provide a scaffold for incorporation of elastin and numerous other components which assemble to form elastic fibers [38, 41–43]. In addition to providing docking sites for LTBP, fibrillins also capture other TGF β superfamily members, such as BMPs, and thus are viewed as multifaceted signaling platforms within the ECM [43].

LTBP initially associate with fibronectin through specific sequences in their N-terminal region [36, 37]. Fibronectin is required for LTBP incorporation into ECM and this association is promoted by transglutaminase [37, 44–46]. At later stages LTBP colocalize with microfibrils through specific interactions between the LTBP C-terminal domain and the fibrillin-1 N-terminus [35, 47]. Thus, LTBP is a structural element of both the fibronectin ECM and microfibril-associated complexes and theoretically can bridge these two ECM components via independent N- and C-terminal interactions (see Fig. 1). However, it has been proposed that fibronectin provides a template or guide for LTBP1 and 4 deposition and subsequent transfer to microfibrils [43]. Regardless, through these two types of interaction

LTBPs incorporate and sequester silos of latent TGF β poised for activation at regular coordinates within the ECM. In so doing they create a highly patterned sensory system where local changes in ECM tension can be detected and transduced into focal signals. Of note, among other responses, TGF β signals elicit further matrix and integrin production. Thus, LTBP creates a mechano-sensory net that spatially senses and generates a highly localized feedback response to the physical tractile requirements of the cellular microenvironment.

An important facet of LTBP biology in this regard is that LTBP binding to the matrix occurs independently of its ability to interact with TGF β . A corollary of this is that factors that increase LTBP expression in excess of TGF β , or factors that regulate expression of isoforms that are unable to interact with TGF β , will theoretically induce a dominant negative effect on TGF β bioavailability. LTBP1 and 4 are both synthesized in excess of proTGF β , whereas LTBP3 and TGF β expression and secretion are tightly coregulated [48]. LTBP2 and some variants of other LTBPs cannot associate with TGF β but compete with LTBP1 for the same sites on fibrillins and thus might competitively reduce latent TGF β incorporation into the ECM. Additional proteins, for example fibulins 2, 4 and 5, also compete with LTBP1 for fibrillin association [38].

LTBPs Play a Significant Role in the Physiological and Pathological Activation of TGF β In Vivo

In vitro studies have identified a number of factors that can liberate TGF β from latent complexes. Denaturing conditions, for example, heat, acid, chaotropic agents and detergents all activate TGF β and TGF β is also activated by irradiation and reactive oxygen species [49–52]. Activation by plasmin has been described in co-cultures of smooth muscle and endothelial cells, with steps involving localization of the LLC to the ECM, release by proteolytic cleavage, binding of sugar moieties in LAP to mannose-6-phosphate receptors on the cell surface and liberation of TGF β by plasmin cleavage of LAP [53]. In this type of activation, LTBPs are perceived as functioning to concentrate latent TGF β on the ECM and/or the cell surface [44, 54]. Although platelet derived TGF β becomes activated when blood clots are dissolved with plasmin, the general physiological relevance of this mechanism has been called into question by the finding that plasminogen null mice do not phenocopy TGF β null mice [55]. However, plasmin may act redundantly with other proteolytic mechanisms [43, 56]. Finally, thrombospondin 1 has been shown to bind simultaneously to LAP and TGF β , altering their affinity for one another [57]. Thrombospondin 1 null mice phenocopy some aspects of TGF β null mice supporting the relevance of this mechanism in vivo [58, 59].

A series of studies have provided strong evidence for the physiological importance of integrin-mediated activation of TGF β [60, 61]. LAP1 and 3 contain an integrin-binding RGD sequence that in LAP1 bind integrins α v β 1, α v β 3, α v β 5 α v β 6 and α v β 8, as well as α 8 β 1 and in LAP3 bind α v β 6 and α v β 8 [60–66]. Integrins α v β 3, α v β 5 and α v β 6, α v β 8, activate TGF β signaling in vitro [61, 64, 67–70]. The physiological relevance of α v β 6 and α v β 8 integrin-mediated TGF β activation in vivo is strongly supported by the fact that α v, β 6 and β 8 integrin null mice phenocopy aspects of TGF β 1 and TGF β 3 null mice [60, 65, 71–77]. For example, both TGF β null and integrin β 6 null mice show lung and skin inflammation and are resistant to bleomycin-induced fibrosis, a pathology that is associated with excess TGF β signaling [65]. Finally, knock-in mice homozygous for the LAP1^{RGE/RGE} allele completely recapitulate the TGF β 1 null phenotype [78]. Thus integrin binding is essential for TGF β signaling during development and in fibrosis.

Two distinct integrin-mediated roles in TGF β activation have been proposed. In one model, integrin α v β 8 is proposed to recruit the cell surface protease, MT1-MMP, into close proximity with LAP, facilitating LAP cleavage and liberating TGF β [63, 79]. Of note, in the integrinprotease recruitment model LTBP1s are relegated to a passive role in presenting latent TGF β as a substrate. A second model proposes a more active participatory role for LTBP1s (see Fig. 1c). Here, the binding of integrins to the RGD sequence in LAP, which, in turn, is bound to LTBP1, is proposed to create a bridge between the actin cytoskeleton and the ECM (see Fig. 1c) [65, 67]. As a consequence, traction generated by actin stress fibers within or by tensile force outside of the cell contorts LAP and exposes TGF β to its receptor. To date three integrins, α v β 3, α v β 5, and α v β 6 have been shown to activate TGF β independently of proteases [65, 67, 68]. Myoepithelial cells in the mammary gland express α 1, α 5 and α v and both myoepithelial and luminal cells express α 2, α 3, α 6, β 1 and β 4. Significantly, cultured mammary cells express α v β 3 and α v β 5 [80]. In vitro experiments have shown that integrin-dependent but protease-independent TGF β signaling requires 1) an intact integrin C-terminal binding domain for cytoskeletal binding partners and is abrogated by agents that disrupt actin in polymerization or block myofibroblast contraction, 2) a mechanically stiff matrix that allows force generation [61, 65, 67, 68]. Several lines of evidence support the critical involvement of LTBP1s in stretch-induced signaling: a requirement for the LTBP1 bridge has been suggested by experiments indicating that TGF β signaling is promoted by stretching myofibroblasts and demonstrated directly by showing that deletion of the LTBP1 ECM-binding hinge domain abrogates signaling, and expression of a recombinantly engineered minimal LTBP1 ECM-binding hinge domain coupled to the LAP binding domain is sufficient to support TGF β activation and signaling [67, 68]. Recent studies have shown that mice homozygous for a knock-in mutant allele of TGF β 1 harboring a mutation in C33S of LAP1 (TGF β 1^{C33S/C33S}), which abrogates its association with LTBP1, recapitulate key aspects of the TGF β 1 null phenotype. These mice display multi-organ inflammation, lack of skin Langerhans cells, and tumors of the stomach, rectum and anus [81]. Collectively, these data establish that LTBP1 is required for, and positively regulates, TGF β 1 and 3 signaling in many tissues in vivo. As LAP2 lacks the RGD sequence motif necessary for integrin association, TGF β 2 activation must involve other mechanisms [60]. Intriguingly, LTBP1 and 2 and all fibrillins also contain RGD sequences (see Fig. 2). Integrins α 5 β 1, α v β 3 and α v β 6 bind to fibrillin 1, however to date, association between integrins and LTBP1s has not been reported and the RGD sequences in LTBP1s are not conserved across species [60, 82–87].

LTBP1s are Required for and Regulate TGF β Signaling

The importance of the precise ECM incorporation and patterning of TGF β can best be appreciated in the phenotypes of knock-out mice and in human pathologies arising from mutations that disrupt LTBP1/fibrillin multi-protein assemblies (see Table 3) [88–90]. Individual LTBP1 isoform-specific knock-out mice show distinct phenotypes (see Table 4). Loss of LTBP1L results in embryonic and early postnatal lethality due to malformation of the major heart vessels and valves [91, 92]. LTBP13 knock-outs show craniofacial abnormalities, osteosclerosis and osteoarthritis and lung alveolar septation defects [93, 94]. LTBP14 hypomorphs present with severe pulmonary emphysema, secondary cardiomyopathy and are predisposed to colonic prolapse and colorectal cancer [95]. These mice show disruption of the elastic fibers [95]. Each of these mutants show evidence of loss of specific TGF β deposition and signaling activity. However, further studies on LTBP14 null mice have suggested that the lung developmental defects arise from excessive TGF β signaling and can be corrected by administering TGF β antagonists or genetically reducing expression of TGF β 2 [96]. In contrast, their elastic fiber defects cannot be corrected and appear to result from TGF β -independent functions of LTBP14 as a microfibril-associated component and facilitator of elastic fiber formation [96]. Collectively, these studies indicate that LTBP1, 3

and 4 are required for normal TGF β function and regulate TGF β signaling in tissue-specific and isoform-specific ways. A degree of functional redundancy between the LTBP isoforms is probable, as each LTBP null phenotype displays only a subset of the effects observed in individual TGF β null mice (see Table 4) [60]. All LBTPs likely have additional TGF β -independent functions. This is certainly true for LTBP2, which is unable to interact with TGF β . LTBP2 nulls show early embryonic lethality due to implantation failure. LTBP2 has been proposed to regulate elastogenesis by targeting of fibulin-5 to microfibrils [97, 98]. In humans, LTBP2 mutations are associated with glaucoma and defects in lens development (see Table 3) [90].

Mutations in fibrillin have been linked to several genetic syndromes and studied most intensively in Marfan syndrome, a disease associated with tall stature, muscle weakness, lens dislocation, heart valve deformities and aortic aneurism. Although originally thought to arise from fragmentation of elastin fibers resulting from lack of fibrillin expression or incorporation into microfibrils, analysis of mouse models hypomorphic for fibrillin have shown that Marfan pathology is associated with elevated TGF β signaling and can be ameliorated by administration of TGF β antagonists [43, 99]. In Marfan syndrome, excessive TGF β activation is proposed to result from insufficient sites within the ECM to sequester and quench TGF β [43]. In contrast mutations in the integrin-binding RGD domain of fibrillin-1 that impair association with integrins α v β 3 and α v β 6 produce Stiff Skin Syndrome characterized by enhanced fibrillin 1, elastin and LTBP4 deposition. In this syndrome, excess TGF β signaling is thought to arise due to latent TGF β production exceeding the available sites on the ECM [60].

LBTPs in Mammary Development

Surprisingly, given their central function in controlling the bioavailability of a regulator of breast development and homeostasis, the role of LBTPs in the mammary gland is largely unstudied. Ltpb1L has been studied in the mammary gland by using a knock in mouse (Ltpb1^{+LZ}) where *lacZ* has been inserted downstream of the *Ltpb1L* promoter. Analysis of this reporter mouse reveals that Ltpb1L-LacZ is expressed in a dynamic spatio-temporal pattern throughout mammary development as summarized in Fig. 3 (Pinderhughes, Hiremath and Cowin unpublished results). Ltpb1L-LacZ becomes strongly expressed in the embryonic mammary region around E12.5. At this stage it is found exclusively within the condensing mammary mesenchyme surrounding the epithelial buds. Robust mesenchymal expression continues under the developing nipple throughout embryonic development. Postnatally, the reporter expression continues in stromal cells underneath the nipple shield and becomes most intensely expressed in smooth muscle at the nipple base. In addition to this mesenchymal expression, Ltpb1L-LacZ appears within luminal epithelia, coinciding spatially and temporally with the onset of lumen formation at E17.5. During puberty, Ltpb1L-LacZ expression is found exclusively throughout the luminal epithelial layer of the developing ductal system. However, it is notably absent from the cap and body cells of the terminal end buds, which are multilayered foci of active proliferation that provide the powerhouse for ductal extension. Within these structures, Ltpb1L-LacZ is restricted to a few body cells immediately facing the luminal cavity. Luminal epithelial expression of Ltpb1L-LacZ continues within the permanent ductal system and appears within all of the developing side-branches but is notably absent from both the developing and differentiated alveoli during pregnancy. During lactation Ltpb1L-LacZ disappears altogether but becomes robustly expressed within 24 h of pup weaning. It is strongly expressed coincident with the wave of alveolar apoptosis that occurs during the earliest stage of involution and becomes restricted again to the luminal cells of the permanent ductal tree at later stages.

These data show that a single *Ltbp* gene is active in a very specific and dynamic manner during mammary development. The pattern of *Ltbp1L-lacZ* expression suggests that *Ltbp1L* and any associated $\text{TGF}\beta$ is positioned to contribute towards embryonic mammary mesenchyme signaling. There are no published reports of $\text{TGF}\beta$ expression during embryonic mammary development. However, $\text{TGF}\beta 1$ and 3 mRNAs have been detected in the mammary mesenchyme in microarrays of microdissected mammary buds and thus represent potential partners (Beatrice Howard personal communication). These same analyses have revealed $\text{TGF}\beta$ receptors in both epithelial and mesenchymal compartments. Thus, *Ltbp1* may present $\text{TGF}\beta$ for autocrine signaling during the process of mammary mesenchyme condensation. This event phenotypically resembles some aspects of fibrosis, a matrix-building process that is promoted by $\text{TGF}\beta$ activity [16]. Alternatively, *Ltbp1* may be presenting $\text{TGF}\beta$ for paracrine signaling to the mammary bud and later to the overlying developing nipple. Adult nipples are sites of considerable elastin synthesis and undergo extensive matrix remodeling during the course of each pregnancy. Elastin and many other genes, which encode ECM proteases are known targets of $\text{TGF}\beta$ signaling. The coincidence of *Ltbp1L-LacZ* expression in ducts with lumen formation suggests possible roles in polarity and luminal cavitation, as well as in maintaining the general growth restraint and quiescence of luminal cells lining the mammary ducts. The dramatic surge in expression during early stages of involution exactly mirrors that of $\text{TGF}\beta 3$ elevation and indicates potential roles in promoting apoptosis [9]. Preliminary data show *Ltbp1* is deposited around luminal cells as well as in the underlying periductal stroma. Thus, *Ltbp1* is positioned to affect luminal, myoepithelial and stromal populations. Given that *Ltbp* and $\text{TGF}\beta$ must be synthesized within the same cell in order to form a large latent complex, the pattern of *Ltbp1L-LacZ* expression points to $\text{TGF}\beta 3$ as the most likely partner. All four *Ltbp* mRNAs can be detected in the mammary gland. Important questions remain concerning their specific sites of expression and deposition and how these contribute to regulating synthesis of the periductal ECM and $\text{TGF}\beta$ bioavailability within what has been termed this instructive platform [43].

LTBPs are Increased and Decreased in Breast Cancers and Cell Lines

Currently, a systematic analysis on breast tissue and breast cancer cell lines is needed. Although sparse, the literature nevertheless shows that both loss and gain of LTBP expression is found in breast cancers (see Table 1). This biphasic effect mirrors that of $\text{TGF}\beta$ in breast cancer and underscores the importance of maintaining physiological levels of LTBP expression for homeostasis of the normal mammary gland. Three publications have reported significant changes in LTBP expression in breast cancers. In one, LTBP4 mRNA expression was downregulated in five human mammary adenocarcinomas when compared with matched healthy mammary tissues from the same patient. Intriguingly, a drop in $\text{TGF}\beta 1$ expression and deposition and a compensatory increase in $\text{TGF}\beta 2$ accompanied reduction in LTBP4 [100]. Two further studies of canine breast tumors also showed a significant drop in LTBP4 protein expression when compared to normal breast tissue. In one, LTBP4 mRNA expression decreased in a graded manner during tumor progression with a concomitant increase in LTBP1 mRNA [101, 102]. LTBP4 was one of only three genes within a forty-nine gene signature that accurately distinguished benign from malignant tumors [101]. In silico analysis of expression profiling data available on the Oncomine database reveals three further reports where LTBP cDNAs were included in gene expression profiles from breast cancer tissues and compared to normal breast tissues (see Table 1). Collectively, these data show that LTBP isoform expression relative to one another is dysregulated in breast cancer and suggest that further study is warranted on larger cohorts.

A similar situation was found in human breast cancer cell lines (see Table 1). MCF7 and HS578T breast carcinoma cell lines show lower LTBP4 mRNA and protein levels compared

to a non-transformed variant cell line, Hs578Bst. Oncomine analyses show that all four LTBP1s are expressed to variable degrees in a wide range of breast cell lines (see Table 1). Consistent with the observations in human breast tumors, LTBP4 was absent from most breast cancer cell lines and in general LTBP1 expression appears inversely related to that of LTBP2 and 3 [103–105].

Two studies provide evidence that increasing LTBP1 may promote metastasis. Massague and colleagues set out to identify tissue-specific metastatic signatures within primary breast cancers that could predict the propensity to metastasize to brain or to lungs. To do this they took sublines of metastatic MDA-MB-231 cells, injected them into immunocompromised mice. After several rounds of in vivo selection for tissue-specific metastasis they compared the gene profiles of the aggressively metastatic subclones displaying specific tropism to that of parental cells. Microarray profiling revealed 18 gene and 17 gene signatures that could stratify patients who were at high risk for developing lung or brain metastasis respectively. These signatures were clinically relevant and identified patients with significantly worse outcome. Importantly, LTBP1 was one of only six genes that were common to both of these metastasis signatures. Thus LTBP1 elevation appears to play a fundamental role in enhancing metastatic behavior [106–108].

How might both loss and gain of LTBP1 be linked to breast cancer? First, this feature is highly reminiscent of the biphasic effects of TGF β on breast cancer and is likely linked to LTBP1's role in TGF β biology. As LTBP1 is required for efficient secretion of TGF β , then decreased LTBP1 expression is predicted to significantly reduce the amount of latent TGF β emerging from cells. This in turn would critically reduce the pool of extracellular latent TGF β available for activation, thereby limiting growth restraining signals and unleashing cell proliferation.

In contrast, elevating LTBP1 could produce one of three effects. First, it is predicted to lead to full LLC occupancy of the available ECM binding sites. Second, increasing LTBP1 could result in secretion of free LTBP (without its TGF β partner) a scenario predicted to lead to competitive occupancy of ECM binding sites and displacement of LLC complexes. Both of these events would cause the gland to become pathologically awash in untethered LLC that is highly susceptible to protease activation. This scenario, described as “an overflowing sink”, is akin to mechanisms proposed to account for excessive TGF β activation in stiff skin syndrome associated with fibrillin mutations [60]. A third potential scenario (see Figs. 1c and 4a) is that increased LTBP1 will lead to LLC occupying not only all of the usual mature microfibrillar binding sites but also becoming backlogged and entrenched on what would, under normal conditions, be transient sites on fibronectin. A necessary corollary of this model is that aberrant long-term occupancy of fibronectin may provide a mitogenic signal to the cell.

It has been hypothesized that cells respond not to individual signals from growth factors acting in isolation but rather to combinations of signals that are dictated by the context of particular matrices [109]. This theory posits that different elements of the ECM, by virtue of their unique protein domain arrangements, bring specific complements of receptors into cis alignment such that “cells hear chords and not single notes” [109]. Fibronectin has long been associated with events involving cell migration and metastasis and it is both the initial binding partner for LTBP1 and a TGF β target gene [110]. Thus, the integrin-LLC bridge provides the cell with extra footholds on fibronectin and simultaneously generates the stretch-mediated TGF β signals that will reinforce migratory behavior by inducing transcription of fibronectin as part of the EMT program. In breast cancer cell lines expressing elevated LTBP1, we speculate that fibronectin and LTBP1 engage in a fatal pas de deux that, to take the musical analogy further, strikes the chord for metastatic migration.

Conclusions

In conclusion, LTBP_s regulate the secretion, localization, presentation and activation of their specific latent TGF β partners. As binding sites for LTBP_s within the ECM are finite homeostasis depends upon the delivery of critical concentrations of LLC. Diminished occupancy likely results in insufficient TGF β leading to unbridled cell proliferation, whereas excess occupancy is likely to enhance metastatic properties by promoting TGF β -induced EMT. Little is currently understood about the role of individual LTBP_s in the mammary gland. The importance of further studies on this topic is warranted by their important functions in regulating TGF β —a major breast tumor suppressor and oncogene—and by their appearance in multiple gene signatures predictive of enhanced metastatic behavior.

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Abbreviations

BMP	Bone morphogenetic protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
GDF	Growth and differentiation factor
LAP	Latency associated protein
LLC	Large latent complex
LTBP	Latent TGF β binding protein
MAGP	Microfibril-associated glycoprotein
MT1-MMP	Membrane type 1 metalloproteinase
RER	Rough endoplasmic reticulum
SLC	Small latent complex
TGF	Transforming growth factor

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Figure 1.

Assembly and function of latent-TGFβ. **a** The tripartite large latent complex (LLC) is formed early in the rough endoplasmic reticulum (RER), via disulphide bonding first between two TGFβ pro-protein monomers to form pro-TGFβ, and then between pro-TGFβ and the 8-cys domain of LTBP. In the Golgi, furin mediates the cleavage of pro-TGFβ into dimeric TGFβ and its propeptide, latency associated protein (LAP). These two homodimers associate non-covalently to form the small latent complex (SLC). LTBP acts as a chaperone, ensuring proper folding and disulphide bonding between LAP and TGFβ. LAP is also glycosylated in the Golgi at mannose-6-phosphate groups, which may be important in extracellular signaling. The mature LLC is then secreted. **b** The LLC positions itself within the extracellular matrix (ECM) first by LTBP binding through its N-terminus (N) to fibronectin and later through its C-terminus (C) binding to N-terminus of fibrillin in microfibrils. This maintains extracellular TGFβ in its latent state. **c** Latent TGFβ is activated via integrins in the stretch mechanism. Upon mechanical signal from the actin cytoskeleton, integrins bind to RGD sites on LAP. As LTBP maintains its hold on the ECM during this process, the two opposing forces allow a conformational change in LAP that releases TGFβ from the complex, activating it and allowing it to bind its receptor, TGF-βR

Figure 2.

Members of the LTBP/Fibrillin family. Four LTBP and three fibrillins are known. LTBP have internal promoter initiation sites (black arrows) and undergo alternative splicing giving rise to splice variants (pink dotted lines). LTBP and fibrillin contain 8-Cys repeats (red globules) and the EGF-like repeats. EGF-like repeats can either bind to calcium (grey rods), giving the protein its rigidity, or can remain unbound (green rods). Hybrid domains (brown globules) have combined features of 8-Cys and EGF-like repeats. The small latent TGFβ-LAP complex (SLC) binds to a specific 8-Cys domain found only in LTBP 1, 3 and 4, adjacent to the fibrillin binding C-terminal region. LTBP also have a central hinge region for additional flexibility. The hinge and the hybrid domains form a major site for ECM attachment and play a role in the stretch response when interacting with integrins. Fibrillins contain a unique C-terminal conserved region (blue rods and purple octagons). RGD domains (yellow arrowheads) present in LAP and in fibrillins are functional and bind to integrins. Although present in LTBP their integrin binding function has not been established

Figure 3.

Expression pattern of LTBP-1 during mammary development, using LTBP1L-LacZ mouse model. **a** LTBP1L-LacZ (*blue*) is expressed in the mammary mesenchyme surrounding the embryonic (E) E12.5 (*left*) mammary bud, and persists at this site under the developing nipple E17.5. LTBP1L-LacZ becomes additionally expressed within the luminal epithelium of the rudimentary sprout as the lumen first forms. **b** During puberty, LTBP1L-LacZ is expressed in quiescent ductal luminal cells but not in the terminal end buds. **c** During pregnancy, LTBP1L-LacZ expression is high in the luminal cells of ducts and side branches, but is absent from secretory structures such as alveoli. **d** LTBP1L-LacZ is not expressed during lactation, but is seen in a subset of cells during involution

Figure 4.

Model for potential LTBP interactions with the ECM in mammary gland. Integrins are multifunctional proteins, which can specify cellular responses in multiple ways. **a** Cells use integrin $\alpha 5 \beta 1$ (brown) to bind directly to fibronectin (FN) in the ECM. Mammary cells express $\alpha V \beta 5$ (yellow) and $\alpha V \beta 3$ (orange), which in other cells have been shown to bind to the RGD domains (yellow arrowheads) of LAP. **b** $\alpha V \beta 5$ (yellow) and $\alpha V \beta 3$ (orange) interactions can also form between the cell surface and fibrillins. The integrin-LLC association with fibronectin and fibrillins provide extra footholds between the cell and the matrix. Different matrix molecules assemble distinct complements and arrangements of integrins. In the case of fibronectin it is hypothesized that stretch mediated integrin activation of TGF β will stimulate the EMT transcriptional program to reinforce migratory behavior

Table 1

Cell lines and breast cancer tissues expressing various LTBP isoforms

Description	LTBP 1	LTBP 2	LTBP 3	LTBP 4
Breast Adenocarcinoma Cell Lines				
AU565	+++	-	+	-
SKBR-3	++	-	-	-
MCF-7 *	++	++	+++	++
MDA-MS-231	++	+	-	-
MDA-MB-468	-	+	+	-
MDA-MB-436	-	-	+++	-
MDA-MB-361	-	-	++	-
HCC-1428	-	-	+++	-
CAL-85-1	-	++++	++++	-
Breast Carcinoma Cell Lines				
Hs578T *	+++	+++	-	-
BT20	+	++	+	-
MDA-MB-453	++	-	+++	-
DU4475	++	++	++++	+
Ductal Breast Carcinoma Cell Lines				
HCC1954	++++	+	-	-
HCC38	-	+++	-	-
HCC1143	-	++++	+++	+
HCC1937	-	++++	-	-
T47D	++	-	-	-
JIMT1	-	++++	-	-
ZR-75-1	+++	-	-	-
Invasive Ductal Carcinoma Cell Lines				
HCC70	+	-	+	-
BT549	+++	++++	++	-
BT474	++	-	-	-
HDQP1	-	++++	-	-
HCC1187	-	++++	-	-
HCC1599	++	-	-	+
Metaplastic Breast Carcinoma Cell Lines				
HCC1569	++	-	++++	-

Breast Cancer Studies (reported in literature)				
Mauel et al. (2007)	N.D.	N.D.	N.D.	↓
Klopfleisch et al (2010)	↑	N.D.	N.D.	↓
Klopfleisch et al (2010)	↑	N.D.	N.D.	↓

Breast Cancer Studies (Oncomine Database Search)				
Richardson et al (2006)	↑ (4-fold)	↓ (3-fold)	↓ (2-fold)	↓ (2-fold)
Karnoub et al (2007)	↓ (1-fold)	No change	↓ (1-fold)	↓ (2-fold)
Ramaswamy et al (2001)	↓ (3-fold)	↑ (2-fold)	N.D.	↑ (1-fold)

Breast cancer cell lines were analyzed using the Oncomine database for the expression of LTBP isoforms (1–4) and classified as breast adenocarcinomas, breast carcinomas, ductal breast carcinomas, invasive ductal carcinomas and metaplastic breast carcinomas. Cell lines have been designated as of luminal (yellow) or basal (blue) origin based on the classification of Minn et al. Cell lines of unknown origins are shown in white. LTBP expression in breast cancer tissues reported in literature as well as those analyzed using the Oncomine database are also shown (orange).

* Represents cell lines included in Muel et al. 2007

Table 2

LTBPs show different preferences for binding to TGF β -LAP isofoms

	TGF β 1-LAP	TGF β 2-LAP	TGF β 3-LAP	BMPs
LTBP-1	+	+	+	–
LTBP-2	–	–	–	–
LTBP-3	+	+	+	–
LTBP-4	+	–	–	–
Fibrillin-1,2,3	–	–	–	+

Table 3**Mutant mouse models involved in TGF β latency and activation**

Mutant	Knockout mouse phenotype	Effect on TGFβ	References
LTBP 1 (L)	Perinatal mortality Developmental defects in heart valves and vessels	Decrease in TGF β signaling	Todorovic et al. [91]
LTBP 2	Embryonic lethal, E3.5-E6.5	Non-TGF β related essential role	Shipley et al. [97]
LTBP 3	Skeletal abnormality, osteoarthritis Decreased body size Pulmonary Emphysema	Temporary decrease in TGF β signaling	Colarossi et al. [94], Dabovic et al. [93]
LTBP 4 (S)	Rectal prolapse with colorectal tumors Pulmonary emphysema Deformed elastin fibers	Decreased TGF β signaling Increased TGF β signaling	Sterner-Kock et al. [95] Dabovic et al. [96]
TGF β -1	Multiorgan inflammation Premature death	–	Shull et al. [71]
TGF β -2	Perinatal mortality Cardiac, lung, limb, spinal cord defects	–	Sanford et al. [73]
TGF β -3	Perinatal mortality Cleft palate Delayed lung development	–	Kaartinen et al. [72]
TGF β ^(C33S/C33S)	Multiorgan inflammation Stomach and colorectal tumors Premature death	Disrupts LAP binding site to LTBP Decreased TGF β signaling	Yoshinaga et al. [81]
TGF β -1 ^(RGE/RGE)	Multiorgan inflammation Phenotypically similar to TGF β -1 mutant	Disrupts integrin binding to LAP Prohibits integrin-mediated TGF β activation	Yang et al. [78]
α V β 6 (Itg β 6 ^{-/-})	Lung inflammation Delayed-onset emphysema	Inhibits α V β 6 integrin-mediated TGF β activation Decreases TGF β signaling	Puthawala et al. [111]
α V β 8 (Itg β 8 ^{-/-})	Embryonic or perinatal mortality Impaired vasculogenesis Defect in brain vascular morphogenesis Cleft palate	Inhibits α V β 8 integrin-mediated TGF β activation Decreases TGF β signaling	Aluwihare et al. [76]
α V subunit (Itg α V ^{-/-})	Embryonic or perinatal mortality Cleft Palate Cerebral hemorrhage	Inhibits integrin-mediated TGF β activation	Yang et al. [78]

Table 4

Human pathology related to LTBP mutations

Mutant	Human phenotype	Reference
LTBP-1	No related Pathology	Robertson et al. [27]
LTBP-2	Primary congenital glaucoma Impaired lens development	Ali et al. [90]
LTBP-3	Tooth abnormalities Short stature Scoliosis	Noor et al. [88]
LTBP-4	Severe systemic developmental defects	Robertson et al. [27], Urban et al. [89]