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Transforming growth factor- β signaling: emerging stem cell target in metastatic breast cancer?

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Abstract

In most human breast cancers, lowering of TGF β receptor- or Smad gene expression combined with increased levels of TGF β s in the tumor microenvironment is sufficient to abrogate TGF β s tumor suppressive effects and to induce a mesenchymal, motile and invasive phenotype. In genetic mouse models, TGF β signaling suppresses de novo mammary cancer formation but promotes metastasis of tumors that have broken through TGF β tumor suppression. In mouse models of “triple-negative” or basal-like breast cancer, treatment with TGF β neutralizing anti-bodies or receptor kinase inhibitors strongly inhibits development of lung- and bone metastases. These TGF β antagonists do not significantly affect tumor cell proliferation or apoptosis. Rather, they de-repress anti-tumor immunity, inhibit angiogenesis and reverse the mesenchymal, motile, invasive phenotype characteristic of basal-like and HER2-positive breast cancer cells. Patterns of TGF β target genes upregulation in human breast cancers suggest that TGF β may drive tumor progression in estrogen-independent cancer, while it mediates a suppressive host cell response in estrogen-dependent luminal cancers. In addition, TGF β appears to play a key role in maintaining the mammary epithelial (cancer) stem cell pool, in part by inducing a mesenchymal phenotype, while differentiated, estrogen receptor-positive, luminal cells are unresponsive to TGF β because the *TGFBR2* receptor gene is transcriptionally silent. These same cells respond to estrogen by downregulating TGF β , while antiestrogens act by upregulating TGF β . This model predicts that inhibiting TGF β signaling should drive the differentiation of mammary stem cells into ductal cells. Consequently, TGF β antagonists may convert basal-like or HER2-positive cancers to a more epithelioid, non-proliferating (and, perhaps, non-metastatic) phenotype. Conversely, these agents might antagonize the therapeutic effects of anti-estrogens in estrogen-dependent luminal cancers. These predictions need to be addressed prospectively in clinical trials and should inform the selection of patient populations most likely to benefit from this novel anti-metastatic therapeutic approach.

Keywords

Breast cancer; Transforming growth factor- β ; Metastasis; Antibody; Receptor kinase inhibitor; Cancer stem cells

TGF β in health and disease

Physiological functions of TGF β

The TGF β family of polypeptides comprises a group of highly conserved dimeric proteins with a molecular weight of approximately 25 kDa [1]. They are ubiquitously expressed in eukaryotes and typically secreted into the extracellular milieu in an inactive form, where they become locally activated in response to the appropriate stimuli [2-4]. As shown in Fig. 1A, the TGF β signaling pathway is highly conserved from lower organisms, such as *D. melanogaster*, to man. TGF β binds to the type II TGF β receptor (T β R-II) kinase. The type I receptor (T β R-I) is then recruited into the ligand/T β R-II complex and phosphorylated and activated by the T β R-II kinase. The activated T β R-I receptor then phosphorylates receptor-associated Smad2 and Smad3, which, in turn, form complexes with the common Smad, Smad4, and accumulate in the nucleus. In the nucleus, activated Smad complexes, along with co-activators and cell-specific DNA-binding factors, regulate gene expression and ultimately cell cycle and tissue repair [5]. It is important to realize that, besides this classical pathway, the T β R-II receptor is capable of partnering with other members of the type I receptor family (Fig. 1B), including Alk-1, Alk-2 and Alk-3 [6-9]. In these cases, TGF β signals can also activate the BMP Smads 1, 5 and 8. These alternate pathways normally appear to be restricted to certain cell- or tissue types, such as, e.g., endothelial, neuronal and epidermal cells, in which they activate distinct genetic programs [6-9]. However, in the context of cancer, this second pathway can become constitutively activated and drive epithelial-to-mesenchymal transitions (EMT), cell motility and invasiveness [6].

In self-renewing epithelia, which are the most common sites of origin of cancer, TGF β appears to exert two major functions, tissue homeostasis and the response to tissue injury:

Growth control and tissue homeostasis—In self-renewing tissues in general, and epithelia in particular, TGF β plays a key role in maintaining the balance between self renewal and cell differentiation and loss [10]. This process probably involves a basal level of “endogenous” TGF β signaling, which protects against the development of early neoplastic lesions. For example, using a transgenic mouse model, Cui et al. [11] showed that constitutive expression of TGF β 1 in suprabasal keratinocytes protects against 12-tetradecanoylphorbol-13-acetate (TPA)-induced hyperplasia and subsequent cancer development. Moreover, TGF β protects keratinocytes against DNA damage [12,13]. Thus, TGF β 1 signaling plays an important role in the endogenous homeostatic regulatory machinery in the mouse epidermis. Consistent with this, non-neoplastic epithelial cells in culture often express a low level of endogenous phosphorylated Smad2 (pSmad2). Furthermore, when these cells are treated with chemical T β R-I kinase inhibitors, pSmad2 becomes dephosphorylated and cell growth is stimulated [14]. Similarly, pSmad2 is detectable in normal lining and ductal epithelia, including the mammary gland, in human as well as mouse tissues [15-17]. Even though most of the TGF β secreted into the extracellular matrix remains latent, these observations suggest that a small amount becomes activated at the cell surface of lining and ductal epithelial cells, presumably to control normal cell proliferation and differentiation in an autocrine manner. Finally, it is likely through this homeostatic function that TGF β suppresses tumor development, and that loss of homeostatic cell growth control is an early event in epithelial carcinogenesis. This is clearly illustrated by mice that are homozygous for a hypomorphic allele of the latent TGF β binding protein, LTBP-4. These animals fail to express pSmad2 precisely in those

epithelia that normally express this particular LTBP isoform, such as colon and lung [18]. Moreover, these mice are prone to develop colon cancer, supporting the notion of a failure of TGF β 's homeostatic function. Finally, in vivo, most human breast-, colon- and head-and-neck cancers continue to express pSmad2 [15-17]. As these tumors are actively growing, they have presumably escaped from TGF β -mediated homeostatic growth control.

Besides its role in cell cycle control, TGF β also maintains tissue homeostasis by regulating apoptosis and perhaps others forms of cell death. For example, TGF β 3 plays a key role in mediating the massive apoptosis of mammary glandular epithelium during postlactational involution, an effect that appears to be mediated by Smad3 [19-21]. In addition, genotoxic stress and DNA damage induced by ionizing radiation or cytotoxic chemotherapy are associated with dramatic activation of TGF β [22,23]. Ionizing radiation-induced DNA damage elicits a cellular program of damage control coordinated by the kinase activity of the ATM protein for which TGF β is required [24]. Recently, Kirshner et al. [22] reported that inhibiting TGF β signaling in mammary epithelial cells using a chemical T β R-I receptor kinase inhibitor attenuated ATM autophosphorylation and significantly reduced its kinase activity, while adding back TGF β 1 restored functional ATM and downstream DNA damage responses. These studies have uncovered a critical link between activation of TGF β 1 in the microenvironment and ATM, which directs epithelial cell genotoxic stress responses and, indirectly, tissue integrity. Thus, in addition to its role in homeostatic control of cell cycle and -survival, TGF β 1 plays a complex role in regulating responses to genotoxic stress, the failure of which could contribute to the development of cancer. Conversely, inhibiting TGF β may be used to our advantage in cancer therapy, as these agents may sensitize tumors to DNA damage and promote cell death [25].

Besides its homeostatic role in self-renewing epithelial tissues, TGF β also plays a key role in immune homeostasis [26-28]. By regulating lymphocyte proliferation, differentiation, and survival, TGF β maintains the delicate balance between the immune defense against foreign pathogens and the suppression of the immune system to maintain self-tolerance and prevent runaway autoimmune disease. In addition, TGF β controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis and activation of leukocytes in the periphery, including lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes. Through these dual effects on immune cells, TGF β prevents the development of autoimmune diseases on the one hand, without compromising the immune response to pathogens on the other. Conversely, the immunopathology associated with hyperactivation of the TGF β pathway in the context of cancer promotes tumor progression, which is one of the main reasons this pathway has attracted attention as a novel therapeutic target [26,28-31].

Finally, TGF β signaling plays a key role in maintaining vascular integrity [32-36]. This is perhaps best illustrated by genetic syndromes in which germline mutations in TGF β superfamily receptors give rise to a variety of vascular defects, including hereditary hemorrhagic telangiectasias and aortic aneurysm syndromes. Interestingly, the developmental defects in large vessels seen in Marfan's and other aortic aneurysm syndromes are associated with seemingly paradoxically increased levels of TGF β /Smad signaling [35].

In summary, TGF β plays a key role in maintaining homeostasis in many different tissues, and loss of this essential cellular control function is the biological basis for several fundamental features of the neoplastic phenotype [37].

Response to tissue injury, tissue repair—The second major function of TGF β is to orchestrate and mediate the local response to tissue injury. Wounding results in brisk local activation of TGF β , which induces epithelial cells to detach from each other, assume a

fibroblastoid and motile phenotype (epithelial-to-mesenchymal transition, EMT), and to produce extracellular matrix (ECM) components of what later becomes a scar [38]. Normally, this process is self-limited in space and time, allowing epithelial cells to revert back to their cohesive epithelioid phenotype [39]. However, in chronic inflammatory conditions, persistent activation of TGF β causes epithelial cells to be permanently converted to myofibroblasts, resulting in the loss of epithelial structures and tissue fibrosis [40,41]. Similarly, as we will review below, persistent activation of TGF β signaling in the context of cancer appears to drive invasion and metastasis [42].

TGF β in genetic mouse models of mammary development and cancer

Genetically engineered mouse models have been particularly informative to understand the different roles of TGF β in mammary gland development and mammary cancer. These studies have examined the effects of either attenuating basal (endogenous) TGF β signaling or of increasing/activating TGF β signaling.

To evaluate the effects of *attenuating endogenous TGF β signaling* on the growth and differentiation of the mammary gland *in vivo*, Tang et al. [43] generated mice with a heterozygous deletion of the TGF β 1 gene. These TGF β 1^{+/-} mice expressed only 10-30% of wild-type TGF β 1 protein levels, and displayed an accelerated development of the mammary ductal tree during puberty and an increased proliferation in the mammary epithelium in response to hormonal stimulation. These findings illustrated the important role endogenous TGF β 1 plays in limiting proliferation of the ductal epithelium in response to ovarian hormones [44]. However, in spite of a proliferative mammary gland phenotype, these mice were not predisposed to spontaneous tumor formation. In subsequent studies, Yang et al. [45] developed transgenic mice that expressed a soluble type II TGF β receptor:Fc fusion protein (Fc:T β RII) under control of the mammary gland-selective mouse mammary tumor virus (MMTV) promoter/enhancer. Biologically significant levels of antagonist were detectable in the serum and most tissues of this mouse line. Nonetheless, similar to the TGF β 1^{+/-} heterozygote mice, these mice did not develop spontaneous mammary tumors during their lifetime.

In order to selectively attenuate TGF β signaling in the mammary gland epithelium, Gorska et al. [46] targeted expression of a truncated, kinase-defective dominant negative type II TGF β receptor (DNT β RII) to mammary epithelial cells using the MMTV promoter/enhancer. Virgin female transgenic mice displayed mammary epithelial hyperplasia. In addition, these mammary glands exhibited unscheduled alveolar development and expression of the milk protein, β -casein, in the absence of pregnancy. An essentially identical phenotype was seen in transgenic mice that expressed a full-length T β R-II antisense RNA under control of the MMTV promoter [47]. Thus, impaired responsiveness of the mammary gland epithelium to endogenous TGF β s results in inappropriate alveolar development and differentiation, consistent with the idea that endogenous TGF β normally serves to maintain homeostasis in the mammary glands of virgin animals. In a subsequent study, Gorska et al. [48] showed that *MMTV-DNT β RII* mice can develop spontaneous mammary tumors, but these were mostly carcinomas *in situ* and arose after a prolonged latency.

On the other hand, when *MMTV-DNT β RII* were cross-bred to MMTV-transforming growth factor- α (TGF α) transgenic mice, mammary tumors developed with a much shorter latency, similar to that seen in *MMTV-TGF α* single transgenic mice. The major difference in mammary tumors arising in *MMTV-TGF α* compared to bi-genic *MMTV-DNT β RII/MMTV-TGF α* was the marked suppression of tumor invasion by DNT β RII transgenic expression. This suggested that TGF β signaling in the tumor cells is required for EMT-mediated mammary cancer cell invasion.

Even though complete inactivation of TGF β signaling by deletion or mutation of one of the canonical TGF β signaling pathway components (receptors or Smads) is rarely seen in human breast cancer (Fig. 3A), several genetic mouse models have been constructed to determine what the consequences for mammary gland development and carcinogenesis would be. Forrester et al. [49] conditionally deleted the type II TGF β receptor gene, *TGFBR2*, in the mammary epithelium. Absence of *TGFBR2* in the mammary epithelium resulted in lobular-alveolar hyperplasia in the developing mammary gland and increased apoptosis, similar to that seen in the *MMTV-DNT β RII* mice, but no spontaneous tumor formation. However, when *TGFBR2*^{null} mice were mated to the MMTV-polyomavirus middle T antigen (*PyVmT*) transgenic mouse model of metastatic mammary cancer, tumor development was greatly accelerated, associated with disproportional increase in the number of pulmonary metastases.

In addition to autocrine cell autonomous effects on the tumor cells, TGF β is believed to promote tumor growth and progression by exerting paracrine effects on the host tumor microenvironment, particularly by activating stromal cells, suppressing anti-tumor immunity and stimulating angiogenesis [31,50-52]. Bieri et al. [53] recently ablated *TGFBR2* expression specifically within mouse mammary alveolar progenitor cells by using a whey acidic protein (WAP) promoter driven Cre recombinase. Crossbreeding with *MMTV/PyVmT* transgenic mice was used to produce mammary tumors in the absence or presence of *TGFBR2* in the mammary epithelium. As expected, absence of TGF β signaling significantly decreased tumor latency. In addition, deleting the *TGFBR2* gene in the epithelial cells resulted in a greater than 5-fold increase in the number of pulmonary metastasis. Furthermore, *TGFBR2*^{null} tumors were larger and associated with an abundant fibrovascular stroma accompanied by increased recruitment of a F4/80⁺ macrophage population. The recruitment of F4/80⁺ cells correlated with increased expression of known inflammatory genes including *GRO1/CXCL1*, *CXCL5*, and *PTGS2/COX-2*, which have also been associated with lung metastases of human breast cancer [54]. In addition, *TGFBR2*^{null} mammary carcinomas were infiltrated by Gr-1⁺CD11b⁺ myeloid cells [55]. This recruitment of Gr-1⁺CD11b⁺ cells into *TGFBR2*^{null} tumors was mediated by *SDF-1/CXCR4* and *CXCL5/CXCR2* chemokine/chemokine receptor axes. In addition, this infiltration of Gr-1⁺CD11b⁺ cells into the invasive front of the tumors was associated with an increased abundance of TGF β 1 in *TGFBR2*^{null} tumors and facilitated tumor cell invasion and metastasis through a process involving matrix metalloproteinase activation. Finally, primary *TGFBR2*^{null} tumors and corresponding pulmonary metastases were enriched for cells expressing the *KRT5*⁺/dNp63⁺ mammary stem cell markers, suggesting that the absence of TGF β signaling in this subset of carcinoma cells might contribute to metastasis. Together, these results indicate that complete absence of TGF β signaling in mammary epithelial progenitors accelerates tumor initiation, progression, and metastasis through regulation of both intrinsic cell signaling and adjacent stromal-epithelial interactions in vivo, perhaps, in part, because of the associated increased local production of TGF β 1. Several important points can be made here. First, complete abrogation of the TGF β signaling pathway results in derepression of genes normally partly suppressed by endogenous TGF β signaling. Many of these genes turn out to encode cytokines and chemokines that then attract various host immune cell populations, including F4/80⁺ macrophages and Gr-1⁺CD11b⁺ myeloid cells. Secondly, these *TGFBR2*⁺ epithelial cells retain their epithelioid morphology and are unable to undergo EMT in response to TGF β . Moreover, carcinomas that arise on the *PyVmT* background have been shown to share many features of human luminal type breast cancer [56]. A distinguishing feature of these tumors is the high expression of *XBPI*, which is a human luminal tumor-defining gene. These tumors also expressed tight junction structural component genes, including *Occludin*, *Tight Junction Protein 2 and 3*, E-cadherin (*CDH1*) and the luminal keratins, *KRT8* and *-18*. Moreover, based on gene set enrichment analysis (GSEA), the murine MMTV-*PyVmT* carcinomas appeared to resemble most closely the human luminal cancer profile and were anti-correlated with the human basal-like subtype. Interestingly, histological analysis demonstrated that these carcinomas invade

not via transition to a mesenchymal phenotype (EMT), but display so-called cohesive invasion (Harold Moses, Personal Commun.). An unresolved question is whether the high levels of TGF β 1 associated with the *WAP/PyVmT/TGFBR2^{null}* tumors contributes to their highly metastatic phenotype, or represents a compensatory tumor suppressive host cell response. From the point of view of clinical development of TGF β pathway antagonists, this is an important question to address, in order to determine whether or not breast cancers that are *TGFBR2* low (i.e., the luminal subtypes) would benefit from this type of treatment strategy.

In fact, reality may even be more complex, in that TGF β signaling may be switched on and off in different cell populations at different times, even within the same tumors. For example, direct visualization of cell migration in tumors by in vivo videomicroscopy has allowed Sahai et al. [57] to detect both cohesive and single cell, amoeboid, migration within the same tumors. Interestingly, the amoeboid, single cell migration was the one predominantly associated with a low proliferation rate as well as the ability to disseminate to secondary sites, where these cells seemingly reverted back to a cohesive, epithelioid phenotype [57]. Most interesting from our perspective is that, in tumors derived from metastatic rat mammary adenocarcinoma 13762NF MTLn3 cells, active TGF β signaling, as evidenced by Smad2 phosphorylation and nuclear localization, appeared to be confined to the population of cells displaying the amoeboid migratory and metastatic behavior, while TGF β signaling was turned off in the majority population of cohesive, epithelioid cells. Moreover, inhibition of TGF β signaling in the tumor cells by expressing a dominant-negative *TGFBR2* abrogated the single-cell amoeboid migratory behavior (Eric Sahai, Personal Commun.). Thus, these studies suggest that the ability of a subpopulation of MTLn3 cancer cells to reversibly turn on TGF β signaling, is associated with EMT, detachment from a cohesive cell mass, and migration to secondary sites. Moreover, this transient state was associated with a slower than average proliferation rate. Consistent with these findings, Welch et al. [58] had previously shown that these same MTLn3rat mammary adenocarcinoma cells acquired a hypermetastatic phenotype in vivo if they were exposed to TGF β in vitro prior to tailvein injection. Of note, MTLn3 cells and tumors express both basal and luminal cytokeratins, suggesting the presence of both precursor and more differentiated luminal cell populations [59]. It is tempting to speculate that the same tumor cell subpopulation that responds to TGF β by acquiring an EMT-mediated invasive phenotype also possesses stem cell properties, as recently suggested by Mani et al. [60] (see section “TGF β plays a key role in regulating self-renewal and differentiation within normal and malignant breast tissue”).

To determine the effects of constitutive *activation* of TGF β signaling, several groups have generated transgenic mice that produce a constitutively active form of TGF β 1. Constitutive expression of bioactive TGF β 1 driven by an MMTV promoter was associated with marked reduction in total ductal tree volume, first observed at 7 weeks, soon after estrous begins, and most apparent at 13 weeks, as ductal growth in the normal mammary gland declines [61]. However, during pregnancy, alveolar outgrowths did develop from the hypoplastic ductal trees and lactation occurred [61]. In addition, these mice were resistant to 7,12-dimethyl-benz[α]anthracene (DMBA)-induced mammary tumor formation [62]. Furthermore, in cross-breeding of these mice with mice that overexpress the epithelial mitogen, TGF α , and that develop mammary tumors at a high rate, there was a marked suppression of mammary tumor formation [62]. Similar results were obtained when Booth et al. [63] crossed TGF α transgenic mice with WAP promoter-driven TGF β 1 transgenic mice. Compared to single transgenic TGF α expressing mice, these bi-transgenic mice were resistant to mammary tumorigenesis. Moreover, transplantation studies showed that bi-transgenic mammary tissue was highly resistant to tumor formation, even after multiple pregnancies. In addition, rates of apoptosis during post-lactational involution were decreased in both TGF α and bi-transgenic TGF α /TGF β 1 mice compared to wild type and WAP-TGF β 1 transgenics. In aggregate, these studies indicate that, when TGF β 1 and TGF α are co-expressed, TGF β 1 suppresses the mitogenic and survival functions of TGF α in the context of mammary development and tumorigenesis. Most

importantly, overexpression of TGF β 1 in vivo can markedly suppress de novo TGF α - or carcinogen-driven mammary tumor development.

The dual tumor suppressive and prometastatic roles of TGF β are further illustrated by two independent studies in which transgenic mice that expressed an activated *neu* gene in the mammary gland were crossed with strains that expressed either constitutively active TGF β 1 or an activated TGF β type I receptor (*TGFBRI*, Alk5) gene [64,65]. In both cases, primary tumor development was markedly delayed, and tumor growth was slower than in single *neu* transgenics [64,65]. However, the carcinomas that did arise in bi-transgenic MMTV-Alk^(T204D) $\times$ *neu* mice were more metastatic than those occurring in MMTV-*neu* single transgenics [66].

In aggregate, genetic mouse models have provided strong support for a tumor-suppressive role for epithelial TGF β signaling in mammary gland tumorigenesis. In addition, some of these studies have suggested that TGF β signaling might play an important role in EMT-mediated spontaneous metastasis, primarily to the lungs. Moreover, the phenotypes observed in these transgenic mouse strains are generally consistent with the notion that attenuating the TGF β signaling pathway in the epithelial cell compartment is not associated with an increased predisposition to form spontaneous mammary tumors, but does increase tumor incidence and shortens latency in the context of independent strong oncogenic events. Conversely, while constitutive activation of the TGF β pathway can still suppress early stages of mammary cancer formation even in the context of a strong oncogene, it appears to enhance the metastatic potential of the carcinomas that do develop once they have broken through the growth suppressive barrier provided by TGF β signaling. A key point is to distinguish between alterations in the level of TGF β ligand and of the cellular components of the signaling separately. We have recently shown that these two variables have very different effects on the transcriptional profile of epithelial cells [67]. Our findings indicated that a quantitative reduction in the level of receptor expression or activity eliminates a set of genes from the TGF β -regulated transcriptional program that are primarily involved in maintaining homeostasis and development. When, as is often the case in advanced cancers, the level of active TGF β within the tumor microenvironment rises, a third genetic program becomes activated that is primarily involved in the cellular response to noxious external stimuli, including stress, infection and injury. This gene set includes a large number of chemokines, cytokines, cytokine regulators, matrix metalloproteases and mediators of inflammation that play key roles in invasion, tumor angiogenesis and metastasis. In summary, our working model, which is entirely consistent with the transgenic mouse studies summarized above, is that two major changes in TGF β signaling occur during tumor development: the first is associated with a global reduction in receptor signaling and results in loss of homeostatic control and of TGF β 's tumor suppressive activity, while the second is associated with overproduction of bioactive TGF β , resulting in activation of a pro-invasive, -angiogenic, and -metastatic TGF β -regulated gene expression program that induces a mesenchymal and highly metastatic tumor cell phenotype. As we will discuss below (section "Alterations of TGF β signaling in breast cancer-loss of homeostasis and activation of EMT"), this scenario probably applies to the vast majority of human breast cancers, in which TGF β s are often over-produced and all of the canonical TGF β signaling pathway components are still expressed (albeit often at lower than normal levels) (Fig. 3A). On the other hand, complete inactivation of the signaling pathway by deletion or mutation of TGF β receptor- or Smad genes is rarely seen in human breast cancer [42] and the clinical implications of genetic mouse models in which the *TGFB2* gene has been deleted in the mammary epithelium remain to be clarified.

Alterations of TGF β signaling in breast cancer-loss of homeostasis and activation of EMT

Escape from TGF β -mediated homeostatic control

As summarized above, TGF β suppresses tumor development by maintaining the balance between cell renewal and cell differentiation and -loss [10], and loss of this homeostatic function is commonly an early event in carcinogenesis (see [50] for review). In addition, not only do many cancers retain the ability to engage the TGF β -dependent tissue injury response, but, in some, it becomes constitutively activated [68]. In addition, there is mounting evidence that activation of the TGF β signaling pathway in an autocrine fashion results in a state of constitutive EMT and a highly invasive and metastatic phenotype [66,69-71]. Thus, in this context, TGF β signaling acquires the properties of an oncogene. This shift appears to be brought about by attenuation of receptor signaling [67] resulting in the clonal expansion of tumor cells that have escaped from TGF β -mediated growth inhibition on one hand, combined with an increasing constitutive production and release of TGF β by the tumor cells and activation of latent TGF β within the microenvironment, which result in a constitutive EMT/mesenchymal phenotype (reviewed in [50,51]).

Escape from TGF β -mediated growth inhibition—Mammary epithelial cell lines range from being exquisitely sensitive to being completely refractory to TGF β -mediated growth inhibition (reviewed in [72]). For example, spontaneously immortalized human mammary epithelial cells (HMEC) were found to be nearly as sensitive to TGF β as primary HMEC, even when stably transfected with individual viral oncogenes, such as v-*mos*, v-*Ha-ras*, or SV40 virus large T antigen [73]. However, fully transformed and tumorigenic HMEC obtained by transfection with the combination of v-*Ha-ras* and SV40 large T antigen were no longer growth inhibited by TGF β [73]. An important point is that, even though modest differences in TGF β sensitivity have been reported among breast carcinoma cell lines (see below), on average, the IC₅₀ of TGF β for neoplastic cells is considerably higher and the maximally achievable inhibition of growth is consistently lower than for primary HMEC (reviewed in [72]). Consistent with these in vitro studies, Zugmaier et al. [74] reported that treating MDA-MB-231 human breast cancer-bearing mice with TGF β did not result in any suppression of tumor growth in vivo. Interestingly, the degree to which human breast cancer cells may still be partly growth inhibited by TGF β appears to depend, to some extent, on whether cells are grown in monolayer (2D) or in soft-agar (3D) culture. By and large, TGF β appears to be a more potent inhibitor of anchorage-independent growth than of growth in monolayer culture [75-77]. This suggests that, in this case, TGF β may affect cell growth indirectly by the changes it induces in the microenvironment, rather than by direct inhibition of cell cycle progression.

Extensive cross-talk between estrogen- and TGF β signaling pathways—A large body of experimental data underscores the extensive cross-talk between the estrogen- and TGF β signaling pathways. A number of studies have suggested that estrogen receptor (ER)-negative human breast carcinoma lines were relatively more sensitive to growth inhibition by TGF β than ER-positive lines [75-77]. Moreover, Arteaga et al. [75] reported that ER-negative cell lines expressed TGF β receptors, while these were undetectable in ER-positive lines, a finding that was recently confirmed in primary human breast cancer cells [78]. However, other investigators were not able to confirm these observations [15,79]. One possible confounding factor is that TGF β -sensitivity may primarily be a function of the estrogen-dependence of the breast cancer cells, rather than the expression of hormone receptors. For example, estrogen-dependent MCF-7 breast cancer cells were found to be quite sensitive to TGF β -mediated growth inhibition, while estrogen-independent MCF-7 sublines were refractory to TGF β [79].

Growth of ER-positive MCF-7 cells is stimulated by estradiol as well as by progestins, such as norethindrone, in a dose-dependent manner, and this effect can be blocked by treatment with 4-hydroxy-tamoxifen (4-OH-T) [80,81]. Moreover, estradiol- or norethindrone-induced growth stimulation was accompanied by a dramatic decrease in TGF β 2 and -3 mRNA levels, whereas the level of TGF β 1 mRNA was not affected [79-81]. The inhibitory effect of estradiol or norethindrone on TGF β 2 and -3 mRNA could be blocked by treatment with 4-OH-T [80, 81]. These studies suggested that the differential regulation of TGF β expression by estradiol or norethindrone on one hand and 4-OH-T on the other might be, at least in part, responsible for the growth stimulation induced by norethindrone and growth suppression by 4-OH-T [80, 81]. In fact, over two decades ago, Knabbe et al. [76] proposed the hypothesis that the antitumor effects of tamoxifen and other anti-estrogens on invasive and metastatic breast cancer might be mediated by TGF β . This hypothesis was based on the observation that treatment of human ER-positive MCF-7 breast cancer cell lines with tamoxifen in vitro induced the production and secretion of TGF β s by these tumor cells, which, in turn, was able to slow down the proliferation of co-cultivated ER-negative MDA-MB-231 breast cancer cells [76]. Consistent with this idea, Manni et al. [82] demonstrated that estradiol was required to support MCF-7 cell soft agar colony formation in the absence of serum, and that this effect was mediated by TGF α and insulin-like growth factor-1 (IGF-1). Moreover, even though treatment with TGF β had no effect on MCF-7 cell growth in 2D culture, it inhibited colony formation in soft agar in a dose-dependent manner to a degree comparable to that observed with 4-OH-T. Furthermore, the growth inhibitory effect of 4-OH-T was completely reversed by an anti-TGF β antibody. These observations suggested that, at least in 3D cultures of MCF7 cells, TGF β might act on a small TGF β -responsive progenitor cell population. This view is remarkably consistent with recent findings reported by Shipitsin et al. [78]. These investigators examined gene expression- and genetic profiles of cells isolated from cancerous and normal breast tissue using the cell surface markers, *CD44* and *CD24*. Most tumors contained cell populations that were either predominantly ER-positive, *CD44*⁺, *CD24*⁺ or ER-negative, *CD44*⁺, *CD24*^{lo}. Moreover, the *TGFBR2* gene was selectively expressed in ER-negative *CD44*⁺, *CD24*^{lo} mammary epithelial precursors but was epigenetically silenced in differentiated, ER-positive *CD44*⁺, *CD24*⁺ luminal cells. Thus, differentiation into luminal cells appeared to be associated with inactivation of TGF β signaling [78]. Furthermore, while TGF β induces benign mammary epithelial cells to undergo epithelial-to-mesenchymal transition (EMT) [83-85], treatment of ER⁻, *CD44*⁺, *CD24*^{lo}, *TGFBR2*⁺ mammary cancer cells with a TGF β receptor kinase inhibitor caused their mesenchymal phenotype to revert to a much more epithelioid one. Thus, it is quite possible that (anti)estrogens directly regulate proliferation of differentiated ER-positive cell populations, which, in turn, produce growth factors and TGF β that ultimately regulate the proliferation of a coexisting putative small ER-negative progenitor or stem cell population (Fig. 2).

Tamoxifen treatment not only induces TGF β 2 mRNA expression [86], but also causes activation of latent TGF β 1 in culture medium [87]. These effects of tamoxifen were dependent on the expression of functional ER in the breast cancer cells, as induction of TGF β 2 expression was not seen in anti-estrogen resistant MCF7 or T47D cell variants, nor in ER-negative cell lines, such as MDA-MB-231 [81,88]. Moreover, besides tamoxifen, other ER modulators induced TGF β 2 expression in MCF7 cells as well. In general, steroidal ER antagonists, such as fulvestrant, were more potent inducers of TGF β 2 than triphenylethylene type inhibitors, such as tamoxifen, 4-OH-T, droloxifen and toremifene [89,90]. In addition, ER modulators induced transcription of *TGFBR2* mRNA as well as phosphorylation of Smad2 to a degree proportional to their growth inhibitory effects [86,91]. Furthermore, several clinical studies showed that tamoxifen treatment of patients with breast cancer was associated with an increase in TGF β plasma levels, and that this correlated with a clinical response to tamoxifen treatment [92]. In addition, MacCallum et al. [93] demonstrated that a clinical response to neo-adjuvant tamoxifen was associated with an increased TGF β 2 mRNA level in tumor tissue. Similarly,

others have reported induction of TGF β 1 protein in breast cancer epithelial or stromal cells following tamoxifen therapy [94,95].

Thus, in aggregate, there is a substantial body of experimental and clinical evidence to support the notion that the cytostatic effect of ER modulators may be explained by the autocrine or paracrine activation of TGF β s, which, in turn, inhibit progenitor cell growth. In addition, there is some evidence to suggest that tamoxifen may act in an ER-independent manner on host stromal cells. Thus, Colletta et al. [96,97] reported that tamoxifen induced the production of TGF β by human fetal fibroblasts. Similarly, van Roozendaal et al. [98] showed that treatment with 4-OH-T increased latent TGF β 1 secretion by cancer-associated fibroblasts in vitro, but not by fibroblasts from normal adjacent breast tissue. Consistent with this, Butta et al. [94] demonstrated that treatment of women with tamoxifen induced TGF β production by stromal tissue surrounding the tumor mass, but not in the breast carcinoma itself.

A key point here is that tamoxifen-responsiveness in vivo may depend not only on whether or not the breast cancer cells express ER, but on whether or not the stem cell population is still sensitive to TGF β -mediated growth arrest. Thus, in some cases, ER-positive tumors might become resistant to anti-estrogens because the tumor stem cells no longer respond to TGF β -mediated cell cycle arrest. In this situation, constitutive activation of TGF β signaling may even contribute to anti-estrogen resistance. For example, the combination of high levels of TGF β 1 mRNA with low ER expression in breast tumors has been associated with clinical resistance to pre-operative tamoxifen therapy [99]. Moreover, Arteaga et al. [100] found that TGF β 2 could mediate anti-estrogen resistance in MCF7-derived LCC2 human breast cancer cells. Growth of these MCF7/LCC2 cells was paradoxically stimulated by tamoxifen in vitro, and this was not affected by the pan-TGF β neutralizing antibody, 2G7. However, in vivo, tamoxifen stimulated the growth of MCF7/LCC2 tumors, and simultaneous treatment with 2G7 abrogated tamoxifen-dependent growth. This reversal of tamoxifen resistance by 2G7 in vivo was only observed in nude mice but not in mice that lacked natural killer (NK)-cell function, suggesting that immune mechanisms were involved in the antitumor effects of 2G7. Moreover, antisense TGF β 2 oligodeoxynucleotides enhanced the NK sensitivity of MCF7/LCC2 cells in the presence of tamoxifen. These data suggested that host NK function mediated, in part, the antitumor effect of tamoxifen and that TGF β 2 can abrogate this mechanism, thereby contributing to tamoxifen resistance [100].

In summary, there clearly is a differential sensitivity to the anti-proliferative effect of TGF β between non-neo-plastic HMEC and breast cancer cells in vitro as well as in vivo. Immortalization of primary HMEC per se is not associated with the acquisition of TGF β -resistance, but the development of a fully transformed phenotype is, suggesting that there is a linkage between these two events. Furthermore, TGF β may mediate the anti-tumor effects of tamoxifen, while the malignant progression of breast cancer appears to be associated with the increased autocrine production and secretion of TGF β , which can eventually contribute to tamoxifen resistance. The simplest way to reconcile these seemingly contradictory observations is to postulate that, in the early stages of breast cancer development, the mammary epithelial stem cell population is still sensitive to growth inhibition by TGF β (and, thus, sensitive to tamoxifen as long as there is an ER-positive subpopulation), but that escape from TGF β -mediated growth arrest and the associated higher levels of TGF β production by the tumor cells are later events in breast cancer progression, perhaps associated with greater invasive and/or metastatic potential and tamoxifen-resistance. However, one has to be careful not to equate escape from TGF β -mediated cell cycle control (and tumor suppression) with metastasis, as there are clearly separate processes. Thus, estrogen-dependent cancers can still metastasize, and metastases that become clinically manifest after prolonged periods of dormancy are typically responsive to estrogen deprivation. If the therapeutic effects of estrogen deprivation are, in fact, mediated by TGF β , as the evidence summarized above appears to

indicate, treatment of these patients with a TGF β antagonist would likely be detrimental. On the other hand, one would also predict that when these cancers become refractory to anti-estrogens (estrogen-independent), this may be due to escape from TGF β mediated growth inhibition. In this context, if we can demonstrate that the metastatic process is dependent on TGF β signaling, it may be rational to intervene with TGF β antagonists.

Constitutive activation of the tissue injury response and the induction of a mesenchymal phenotype drive metastasis

Our working model [67], which is entirely consistent with the animal and in vitro models summarized above, is that two major changes in TGF β signaling occur during breast cancer development: the first is associated with a global reduction in receptor (or, perhaps, Smad) signaling, which results in loss of homeostatic control and of TGF β 's tumor suppressive activity, while the second is associated with overproduction of bioactive TGF β , resulting in activation of a pro-invasive, -angiogenic, and -metastatic TGF β -regulated gene expression program, which induces a highly mesenchymal, motile, invasive and metastatic phenotype.

TGF β signaling can drive breast cancer metastasis—Welch et al. [58] were the first to draw attention to TGF β 's potential role in mammary tumor metastasis: pre-treatment of MTLn3 rat mammary adenocarcinoma cells with TGF β 1 in vitro resulted in a dose-dependent increase in lung metastases following tail vein injection into syngeneic F344 rats. This effect correlated with an increased propensity of the cells to invade a reconstituted basement-membrane in vitro without affecting cell growth, and could be blocked using a neutralizing anti-TGF β antibody [58]. Conversely, McEarchern et al. [101] reported that expressing a dominant negative truncated TGF β type II receptor in highly metastatic 4T1 murine mammary carcinoma cells resulted in diminished TGF β signaling and significantly restricted the ability of 4T1 cells to establish distant metastases. Along the same line, Yin et al. [70] showed that TGF β also promotes human breast cancer metastasis in a cell autonomous manner. Expression of a dominant-negative mutant of the T β R-II receptor in the human breast cancer cell line, MDA-MB-231, inhibited the extent of experimental bone metastases. Reversal of the dominant-negative signaling blockade by overexpressing a constitutively active T β R-I receptor in these breast cancer cells increased production of parathyroid hormone-related protein (PTHrP) by the tumor cells and enhanced their osteolytic bone metastases. In similar studies, Tang et al. [102] showed that introducing a dominant-negative T β R-II gene into highly metastatic MCF10Ca1 mammary carcinoma cells resulted in a reduction in experimental pulmonary metastases. More recently, using genetic depletion experiments, several groups have demonstrated that Smad4 [103,104] as well as Smad2 and 3 [105] contribute to the formation of osteolytic bone metastases by MDA-MB-231 cells. Similarly, Tian et al. [106] used the MCF10Ca model to show that interference with endogenous Smad2/3 signaling enhanced the malignant potential of xenografted premalignant and well-differentiated tumor cells but strongly suppressed experimental lung metastases of more aggressive carcinoma cell lines. Overexpression of Smad3 in the same cells had the opposite effects. Thus, Smad2/3 as well as Smad4 appear to mediate TGF β 's tumor prometastatic signals. In aggregate, these studies indicated that, even though human breast carcinoma cell lines cells are refractory to TGF β -mediated growth suppression, the remaining TGF β signaling activity is able to drive the formation of macrometastases in several different secondary sites, including bone and lungs [70,102,103]. Thus, cell autonomous effects of TGF β appear to be required for metastasis of these human breast cancer cells, independently of its effects on host tissues.

Different sets of TGF β target genes involved in organ-specific metastasis—By comparing individual metastatic MDA-MB-231 sub-clones, Kang et al. [103,107,108] identified a set of ~100 genes whose expression levels varied as a function of bone-tropism. Most of these genes encoded secreted and cell surface proteins. Functional validation studies

showed that four of these genes (*IL-11*, *CTGF*, *CXCR4*, *MMP-1*) acted cooperatively to cause osteolytic metastases. Importantly, each of these four genes are TGF β targets [67]. In addition, some animals developed skeletal metastases following a prolonged period of dormancy. Cell lines derived from these “post-dormancy” metastases retained clear bone-tropism when re-injected into secondary animals, but displayed a gene expression profile that was quite distinct from that found in the “primary” bone metastases [109]. Most striking was the fact that, in contrast to the “primary” bone metastases, the post-dormancy clones did not express the TGF β response gene signature (Y. Kang, Personal Communication). These findings raised the question whether primary bone metastases might be more dependent on activation of their TGF β signaling by TGF β released from bone [110] than post-dormancy metastases. However, our recent in vivo treatment experiments indicate that this is not the case, suggesting that TGF β plays its prometastatic role mainly within the host microenvironment rather than through cell autonomous effects (Ge et al. manuscript in preparation).

In similar studies, van der Pluijm et al. [111] examined the gene expression in MDA-MB-231 metastases at different sites, as well as in surrounding mouse host tissues. PTHrP is believed to enhance osteoclastic bone resorption. Consistent with this, PTHrP mRNA expression was strongly up-regulated in bone metastases, compared to brain- or lung metastases. In the same set of MDA-MB-231-derived bone and soft tissue metastases, the expression of VEGF-A, -B, and -C was strongly up-regulated in the bone/bone marrow metastases compared to those in brain or lung. Importantly, VEGF is also a direct TGF β target gene in epithelial cells [67]. Consistent with these observations, treatment of tumor bearing mice with the 2G7 anti-TGF β neutralizing antibody significantly reduced circulating VEGF levels [112] (Genentech, US Patent Application 2005/0276802 A1).

Finally, Hiraga et al. [113] recently examined the role of cyclooxygenase-2 (*PTGS2/COX2*), in breast cancer bone metastasis. COX-2 protein was clearly over-expressed in bone metastases from patients with various types of cancers. Moreover, MDA-MB-231 cells expressed COX-2 when they metastasized to bone but not when growing in the mammary fatpad, suggesting that the bone microenvironment specifically induced COX-2 expression. Importantly, *PTGS2/COX2* is also a major transcriptional target of TGF β in MDA-MB-231 and other epithelial cells [67]. Moreover, COX-2 expression was impaired in MDA-MB-231 cells that overexpressed dominant-negative T β R-II receptors, associated with decreased bone metastases and reduced osteoclastic bone resorption. Similarly, treatment with COX-2 inhibitors significantly suppressed bone metastases with decreased osteoclast number and increased apoptosis in MDA-MB-231 cells. These results indicate that bone-derived TGF β may up-regulate *PTGS2/COX2* expression in breast cancer cells, thereby increasing prostaglandin E(2) production, which in turn, stimulates osteoclastic bone destruction, leading to the progression of lytic bone metastases [113]. In summary, a set of at least seven genes, *IL-11*, *CTGF*, *CXCR4*, *MMP-1*, *PTHrP*, *VEGF* and *PTGS2/COX2*, have been identified as drivers of human breast cancer bone metastases in the MDA-MB-231 model, and each of these genes are transcriptionally regulated by and dependent on TGF β signaling in vivo. Thus, these studies provide powerful support for targeting the TGF β signaling pathway to prevent or inhibit bone metastases by basal-like breast cancer cells.

It is important to note that, while the role of TGF β has been well-established in the development of osteolytic metastases by the basal-like MDA-MB-231 breast cancer cells, little is known about the role of this pathway in the development of osteoblastic metastases that are more commonly seen in breast cancer. In this regard, it is intriguing that T47D human breast carcinoma cells, which give rise to osteoblastic metastases in 3-6 months following intracardiac injection [114,115], express estrogen receptors but are entirely unresponsive to TGF β by virtue of the fact that the *TGFBR2* gene is transcriptionally silent. Thus, these cells faithfully reproduce the phenotype of the differentiated, *ER*⁺, *CD44*⁺, *CD24*⁺, *TGFBR2*⁻ luminal breast

cancer cell population recently described by Shipitsin et al. [78]. Consequently, if TGF β contributes to the formation of osteoblastic metastases in this case, it must do so entirely via paracrine mechanisms. In contrast to MDA-MB-231 cells that rely on PTHrP, the bone tropism of ER-positive MCF7, ZR75-1 and T47D cells appeared to be dependent on endothelin-1 (*EDNI*) [115,116]. Interestingly, *EDNI* gene expression is also strongly induced by TGF β [67,117,118]. Thus, one might speculate that T47D bone metastases may be driven by release of TGF β from bone matrix followed by induction of endothelin-1 in a stromal cell population, which in turn, stimulates osteoblast activity.

Besides bone-tropic sublines, Minn et al. [119] have also used in vivo selection to isolate MDA-MB-231 clones that are highly lung tropic. The gene expression signature of lung-tropic clones was distinct from that of bone-tropic clones. Nine genes were identified not only as markers but also as functional mediators of lung-specific metastasis. Perhaps most importantly from our perspective, several of these genes are also TGF β targets, including *GRO1/CXCL1*, *MMP2*, *ID1* and *PTGS2/COX2*. This suggests that the lung microenvironment also contains active TGF β , which provides a selective advantage for highly TGF β -responsive tumor cells, but that establishing a metastasis in the lung requires a different set of metastasis effectors than in the bone microenvironment. Interestingly, Gupta et al. [120] showed that combined treatment with the anti-EGFR antibody, cetuximab, the broad-spectrum MMP inhibitor GM6001 and the COX-2 inhibitor celecoxib reduced the rate of primary tumor growth accompanied by vascular defects that precipitated tissue hypoxia and tumor cell apoptosis, diminished the presence of circulating tumor cells, and prevented the progression of micrometastases to macrometastases in the lungs of MDA-MB-231 bearing mice. The TGF β -dependence of the effector proteins raises the question whether the therapeutic efficacy could be further potentiated by the addition of TGF β pathway antagonists.

Cooperation between TGF β and ras gene activation in driving mammary cancer progression

Using polarized EpRas Ha-*ras*-transformed murine mammary epithelial cells as their model system, Janda et al. [121] demonstrated that the Ha-*ras* oncogene cooperates with TGF β to cause EMT, which is characterized by a spindle-like cell morphology, loss of epithelial markers and cell cohesion, and induction of mesenchymal markers. Interestingly, treatment with TGF β alone induced loss of cell-cell contacts and a spindle-like cell phenotype that was fully reversible after factor withdrawal and was not associated with a sustained mesenchymal phenotype. Using specific inhibitors and effector-specific Ha-*ras* mutants, these investigators showed that a hyperactive Raf/mitogen-activated protein kinase (MAPK) was required for EMT, whereas activation of phosphatidylinositol 3-kinase (PI3K) causes scattering and protects from TGF β -induced apoptosis. Either hyperactivation of the PI3K pathway or the Raf/MAPK pathway was sufficient for xenograft tumor formation, while full-blown EMT and metastasis required simultaneous activation of both pathways. Consistent with these findings, inhibiting TGF β signaling by expressing a dominant-negative T β R-II receptor caused a reversal of EMT (mesenchymal to epithelial transition), inhibition of in vitro invasiveness and resulted in a significant reduction in primary tumor growth and metastatic capability in vivo [69]. Thus, EMT appears to be a close in vitro correlate of metastasis, as both require synergism between TGF β - and Raf/MAPK signaling [69,121]. These studies clearly illustrate that the TGF β and Ha-*ras* pathways must cooperate to induce EMT, mammary cancer cell motility and invasiveness in vitro, and metastasis in vivo, and provide a rationale for combining TGF β targeted agents with agents that inhibit Ras-dependent mitogenic pathways.

Cooperation between TGF β and HER2/neu in driving mammary cancer progression

Several in vitro studies have demonstrated that overexpression of an activated HER2/*neu* (erbB2) oncogene in untransformed MCF10A human mammary epithelial cells converts TGF β from a neutral or even anti-migratory factor to a strongly pro-migratory and -invasive

factor [122-124]. These apparently synergistic actions of the TGF β and HER2/*neu* signaling pathways in vitro have been further substantiated by crossbreeding of transgenic mice that expressed an activated *neu* gene in the mammary gland with strains that expressed either a constitutively active TGF β 1 or an activated T β R-I gene [64-66]. In both cases, tumors that developed in bigenic animals gave rise to a significantly higher number of pulmonary metastases than those from *neu* single transgenic mice. These findings suggest that, once cells break through TGF β 's anti-proliferative barrier, TGF β becomes a strong pro-metastatic factor. Consistent with this idea, bigenic tumors expressed higher levels of phosphorylated Smad2, Akt, mitogen-activated protein kinase (MAPK), and p38, as well as higher Rac1 activity than tumors expressing *neu* alone [64]. In terms of the underlying mechanisms mediating the synergy between the HER2/*neu* and TGF β signaling pathways, Northey et al. [125] have mapped the synergistic effect of TGF β -induced motility and invasion to signals emanating from tyrosine residues 1226/1227 and 1253 of HER2/*neu*, specifically involving the ShcA adaptor protein, which interacts with HER2/*neu* through these residues. In aggregate, these studies provide further rationale for clinical development of combination treatment regimens using TGF β pathway antagonists and inhibitors of specific growth factor pathways, such as HER2/*neu*.

In summary, tumor-associated TGF β appears to endow neoplastic mammary epithelial cells with a selective advantage because of the altered responsiveness of the tumor cells themselves to TGF β (tumor cell-autonomous effects) as well as TGF β 's actions on the supporting host cell infrastructure. In aggregate, the mounting experimental evidence favoring an oncogenic, prometastatic role of TGF β signaling has generated considerable enthusiasm for the development of TGF β pathway antagonists as a novel anti-metastatic treatment for breast [126-128].

TGF β and human breast cancer

TGF β production associated with breast cancer progression

Several studies have shown that TGF β s can be detected in human breast cancer specimens. Moreover, tumor tissue appears to express higher levels of TGF β than the corresponding normal tissue, and the association of TGF β s with cancer appears to be strongest in the most advanced stages of tumor progression. For example, TGF β 1, -2 and -3-specific mRNAs could be detected in the majority of primary breast cancers [129] and appeared to be more abundant than in normal breast tissue [130]. In addition, several studies have reported immunostainable TGF β 1, -2, and -3 to be associated with the majority of primary human breast carcinomas [131-135]. Furthermore, a significantly greater fraction of invasive carcinomas expressed immunodetectable TGF β than in situ carcinomas [131], and the strongest staining was observed in invasive carcinomas with associated lymph node metastases [133,136], suggesting that there may be a semi-quantitative relationship between TGF β production and tumor progression.

Besides these reports of increased TGF β expression in breast carcinomas, plasma levels of TGF β have also been reported to be elevated in patients with breast cancer [137], to correlate with disease stage [138], and to decrease following resection of the primary tumor [139]. Most recently, Grau et al. [140] examined the association between circulating TGF β levels and overall and disease-free survival. TGF β levels were measured in plasma samples of recently diagnosed breast cancer patients in the population-based case-control Shanghai Breast Cancer Study. The median follow up time was 7.2 years. Compared with the patients with the lowest quartile of plasma TGF β , patients with the highest quartile of plasma TGF β had a significantly worse overall- and disease-free survival, while this was not the case for patients with the second and third quartiles of plasma TGF β levels, independently of stage of disease. Thus, the highest circulating levels of TGF β were associated with worse survival independently of stage of disease. In addition, Baselga et al. [141] recently reported plasma TGF β 1 levels in 23 patients

with breast cancer and bone metastases. Plasma TGF β 1 levels were elevated in more than half of the cancer patients and positively correlated with increased platelet factor 4 (PF4) levels, PTHrP, von Willebrand Factor (vWF) and interleukin (IL)-10. Whether or not elevated TGF β plasma levels are indicative of TGF β -dependent metastatic disease and, therefore, predictive of response to TGF β pathway antagonists are questions that are actively being pursued in ongoing clinical trials.

Alterations of TGF β signaling pathway components in breast cancer

In an early study, TGF β type I receptor (*TGFR1*) mRNA was detected in normal breast and in 90% of breast cancers. *TGFR2* and -3 receptor mRNAs were expressed less frequently [142]. Gobbi et al. reported that loss of T β R-II protein expression correlated both with progression to and with high histological grade of invasive breast carcinomas [143,144]. Subsequently, Buck et al. [145] examined expression of T β R-I and -II by immunohistochemistry in 246 invasive breast cancer specimens. Expression of T β R-I was strongly correlated with tumor size and nodal status but only weakly with overall survival. In contrast, T β R-II expression was inversely correlated with overall survival, but only in patients with ER-negative cancers. Thus, survival of patients with ER-negative, T β R-II⁺ tumors was significantly shortened, while loss of T β R-II expression in the ER-negative subgroup was associated with a better overall survival, comparable to that of patients with ER-positive cancers. Moreover, within the ER-positive subset, T β R-II expression had no impact on patient outcome [145]. These findings are remarkably consistent with those reported by Shipitsin et al. [78]. In a study of 286 patients, these investigators reported that the *TGFR2* and 15 TGF β -regulated genes (including *CTGF*, *COL1A1*, *FNI*, *SERPINE1*, *MMP9*) were most strongly expressed in ER-negative, *CD44*⁺, *CD24*^{lo} tumors, and were associated with a significant shortening of distant metastasis-free survival [146]. Consistent with this observation, Padua et al. [147] recently reported that expression of a 153 TGF β response gene signature predicted for the development of lung metastases and poor outcome of ER-negative but not ER-positive breast cancers.

Even though two studies failed to detect structural alterations of the *TGFR2* gene in primary invasive breast cancers [148,149], Lucke et al. [150] found several *TGFR2* mutations in breast cancer specimens. Similarly, even though Baxter et al. [151] found no evidence for *TGFR1* gene mutations, we reported that one particular C to A transversion mutation resulting in a serine to tyrosine substitution at codon 387 (S387Y) was present in two of 31 primary breast carcinomas and 5 of 12 lymph node metastases. This *TGFR1* mutant has an attenuated ability to mediate TGF β -dependent effects on gene expression as compared with wild-type T β R-I by preventing receptor sumoylation [149,152]. Thus, *TGFR1* and -2 gene mutations are occasionally found in breast cancer but these are clearly low frequency events. However, these mutations can have quite dramatic phenotypic properties. For example, we have recently described a cancer-associated mutation in the *TGFR2* gene that confers a dual phenotype in which Smad2/3 signaling is lost, while Smad1/5 signaling is constitutively activated and drives cell motility and invasion [6].

Besides *TGFR2*, Smad4 expression is reduced in a small subset of breast cancer compared to normal glandular epithelium [15,153]. In our own hands the absence of either Smad4 or phosphorylated Smad2 expression in lymph node positive breast cancer was strongly associated with a shorter overall survival [15]. On the other hand, nuclear Smad3 accumulation in primary invasive breast cancers correlated with high histological grade, although the effect on patient survival was unclear [154]. Thus, loss of Smad expression and/or activation does occur in breast cancer but is a low frequency event and its clinical significance remains uncertain.

In aggregate, these studies demonstrate that the vast majority of human breast cancers express all of the canonical TGF β signaling pathway components albeit often at lower than normal

levels (Fig. 3A), while complete inactivation of the signaling pathway by deletion or mutation of TGF β receptor-or Smad genes are rarely seen in breast cancer. Moreover, these findings are consistent with the model that partial retention of TGF β signaling is associated with EMT-mediated invasion, and an aggressive metastatic phenotype predominantly in the ER-negative subtypes of breast cancer, while complete inactivation of the TGF β signaling pathway is associated with lack of EMT, cohesive invasion, and lower propensity for metastasis [42,67].

TGF β plays a key role in regulating self-renewal and differentiation within normal and malignant breast tissue

Breast cancer initiating cells have a mesenchymal phenotype and active TGF β signaling—Al-Hajj et al. [155] recently used cell surface markers to isolate a subpopulation of breast cancer cells that was able to initiate tumor growth in immune deficient mice. These so-called cancer-initiating cells (CIC) or breast cancer stem cells had a $CD44^+$, $CD24^{-/lo}$, lin $^-$ phenotype. More recently, *ALDH1* was identified as marker that further narrows down the breast cancer stem cell phenotype [156,157]. The cell population with this phenotype is highly enriched in tumor initiating cells. Moreover, the tumors that arose from these CICs contained additional $CD44^+$, $CD24^{lo}$ tumorigenic cells as well as phenotypically diverse mixed populations of non-tumorigenic cells similar to those present in the initial tumor. Thus, CICs were operationally defined of cells that have self-renewal capability as well as the ability to give rise to more differentiated (non-tumorigenic) cell progeny. Following up on these initial observations, Shipitsin et al. [78] examined gene expression- and genetic profiles of cells isolated from cancerous and normal breast tissue using the cell surface markers, *CD44* and *CD24*. Most tumors contained cell populations that were either predominantly ER-positive, $CD44^+$, $CD24^+$ or ER-negative, $CD44^+$, $CD24^{lo}$. Genes that were co-expressed with $CD44^+$ included *VIM*, *CTGF*, *SERPINE1*, *SPARC*, as well as *TGFBR2*. In fact, many of the genes actively transcribed by $CD44^+$ cells were associated with a mesenchymal phenotype and/or were TGF β targets [67]. Moreover, the presence of this gene signature correlated with decreased patient survival. In contrast to the ER-negative, $CD44^+$, $CD24^{lo}$, TGFBR2 $^+$ cell population, in differentiated ER-positive, $CD44^+$, $CD24^+$ luminal cells, the TGF β signaling pathway was inactive because of epigenetic transcriptional silencing of the *TGFBR2* gene. Thus, differentiation of mammary epithelial progenitors into luminal cells appears to be associated with shutting down TGF β signaling [78]. Perhaps most interestingly, treatment of ER-negative, $CD44^+$, $CD24^{lo}$, TGFBR2 $^+$ metastatic mammary cancer cells with the dual TGF β receptor kinase inhibitor, LY2109761, resulted in a reversal of their mesenchymal to the more epithelioid $CD44^+$, $CD24^+$ phenotype. Conversely, it has been known for some time that TGF β can induce benign mammary epithelial cells to undergo epithelial-to-mesenchymal transition (EMT), an effect that can be blocked by treatment with TGF β receptor kinase inhibitors [83-85]. Thus, in aggregate, these studies have provided strong evidence for a critical role of TGF β and TGF β signaling in regulating the dynamics of cell populations within normal and malignant breast tissues by favoring stem-cell self renewal and inhibiting the commitment to differentiation.

Fillmore and Kuperwasser [158] recently tested the hypothesis that established breast cancer cell lines might have retained the hierarchical differentiation programs found in primary breast tumors. Using the same *CD44* and *CD24* cell surface markers, these investigators demonstrated that, similar to primary human breast cancers, human breast carcinoma cell lines were either predominantly $CD44^+$, $CD24^+$ or $CD44^+$, $CD24^{lo}$ or contained mixtures of these two cell types. However, contrary to their expectation, the frequency of tumor initiating cells was quite similar across these cell lines, indicating that the $CD44^+$, $CD24^{lo}$ versus $CD44^+$, $CD24^+$ phenotypes were more representative of the basal versus luminal cancer subtypes than that they reflected the presence or absence of stem cell properties. Further analysis demonstrated that each of the human breast carcinoma cell lines, independently of their predominant phenotype, also

contained much smaller subpopulations of $CD44^+/CD24^{-/lo}/ESA^+$ cells, which were highly enriched for progenitor cells capable of initiating tumor growth in the mammary fatpad. Furthermore, this $CD44^+/CD24^{-/lo}/ESA^+$ subpopulation was able to self-renew, to reconstitute the parental cell culture with $CD44^+/CD24^+/ESA^+$ and $CD44^+/CD24^{-/lo}/ESA^-$ cells, to retain BrdU label, and to preferentially survive treatment with cytotoxic chemotherapy. Most importantly, this stem cell-like subpopulation could be identified across a number of different cell lines, including those with predominantly luminal or basal-like phenotypes. Along similar lines, Horwitz et al. [159] recently examined xenografts derived from luminal (ER-positive, $CD44^+$, $CD24^+$, $TGFBR2^-$) T47D breast cancer cells. Even though these xenografts were predominantly composed of luminal-like ER-positive, PR-positive, $KRT5^-$ cells, they also contained a small subpopulation of cells that were $CD44^+$ and ER-negative, PR-negative, $KRT5^+$. Most importantly, this $CD44^+$, $KRT5^+$ cell fraction was highly enriched for cells that were clonogenic in vitro and able to re-initiate tumor growth in vivo compared with the $CD44^-$ cell fraction. During expansion of $CD44^+$ -derived colonies in vitro, the ER-negative, PR-negative, $KRT5^+$ population was present from the outset but did not expand with increasing colony size, while the number of differentiated ER-positive, PR-positive, $KRT5^-$ cells expanded linearly with colony growth. Similarly, tumors originating in vivo from $CD44^+$ cells contained a small static ER-negative, PR-negative, $CD44^+$, $CD24^{lo}$, $KRT5^+$ population, an intermediate ER-negative, PR-negative, $KRT5^-$ population, and an expanding ER-positive, PR-positive, $KRT5^-$ cell population. These findings strongly suggest that luminal breast tumors might contain a minority $CD44^+$, ER-negative, PR-negative, $KRT5^+$ (presumably $TGFBR2^+$) population that has the capacity to generate the majority of fully differentiated ER-positive, PR-positive, $KRT5^-$, $KRT18^+$ cells. Interestingly, these observations bring to mind T47D cells that were isolated by prolonged estrogen deprivation in vitro [88]. These cells no longer expressed ER, but expressed elevated levels of TGF β mRNAs. One might speculate that estrogen deprivation resulted in selective expansion of the $CD44^+$, ER-negative, PR-negative, $KRT5^+$ progenitor like population, a model that is readily testable.

Neve et al. [160] recently characterized a larger panel of human breast cancer cell lines both by gene expression profiling and comparative genomic hybridization. Results obtained were highly consistent with those reported by Fillmore et al. [161]. Unsupervised clustering of transcriptional profiles revealed several major groups, which appear to coincide with those described for clinical tumor samples. In this study, basal-like cancer lines were characterized by low ER and high *CAVI* expression. This group contained at least two major subdivisions, termed basal-like (BL) A ($KRT5^-$, $KRT14^+$) and B (VIM^+). The BL_A closely matched the basal-like subset initially defined by Perou et al. [162], whereas the BL_B cluster exhibited a stem cell-like expression profile and preferentially expressed *CD44*, *MSN*, *TGFBR2*, *CAVI*, *CAV2*, *VIM*, *SPARC* and *AXL*, while *CD24* expression was low. These observations have been independently confirmed by Charafe-Jauffret et al. [163]. These investigators interrogated 31 human breast cancer cell lines and found that the cells clustered into Luminal, Basal and Mesenchymal subgroups. In this study, basal-like lines were also characterized by overexpression of *CD44*, *MSN*, *TGFBR2*, *CAVI*, *CAV2*, *EGFR*, *MET*, *LYN*, *ETS1*, and various annexins, as well as a number of collagen genes, *MMP1*, *PLAU*, *TIMP1*, *FNI*, and *SPARC*. The latter are TGF β -regulated genes involved in EMT, ECM remodeling and invasion. Moreover, a subset of basal-like cancers termed the “mesenchymal” subtype characterized by high vimentin (*VIM*) and low *CD24* expression, correspond well to the group designated as the BL_B cluster described by Neve et al. [160]. BL_B/mesenchymal cells were shown in both studies to resemble “basal-myoepithelial” cells in the expression of mesenchymal gene products, but lacked a number of cytokeratins seen in myoepithelial cells. Blick et al. [164] examined expression of markers for EMT (*VIM*, *CDH1*, *CDH2*, *FNI*) across these cell lines. *VIM* was absent in the majority of luminal lines and clearly over-expressed in the BL_B, but also quite widely expressed also in BL_A cell lines. E-Cadherin (*CDH1*), on the other hand, was an important discriminator between BL_A (which all expressed levels close to the median) and

BL_B, which had reduced expression. Surprisingly, eight of the 25 Luminal cell lines also showed reduced expression of *CDH1*. N-Cadherin (*CDH2*) and fibronectin (*FNI*), classically used to monitor EMT, each showed relative enrichment in the BL_B subgroup, compared with the other subgroups, although *FNI* expression was relatively high in several luminal lines. Several *CDH2*-negative lines clustered as mesenchymal, most notably MDA-MB-231. On the whole, there was a reasonable concordance between expression of EMT drivers and the BL_B/Mesenchymal phenotype. *SNAI2* was overexpressed in quite a few BL_A lines as well as most lines in the BL_B group. *TWIST* was expressed in most BL_B lines, but surprisingly, also in several luminal lines. High relative expression of both *ZEB1* and *TCF4* was limited to BL_B lines. All members of BL_B had a high relative expression of *ELK3*, as did a few members of BL_A, but no luminal cell lines. *ELF3*, also an ETS-domain protein, was markedly underexpressed by most BL_B cell lines. Recently the high mobility group A2 protein (HMGA2), which is induced by the Smad pathway during TGFβ-mediated EMT, was shown to integrate transcriptional input for the expression of *SNAIL*, *SNAI2*, *TWIST* and inhibitor of differentiation 2 (*ID2*), each of which has been implicated in EMT. Thus, the complex interplay seen amongst EMT-driving transcription factors may be coordinated by HMGA2. In summary, a subset of human breast cancer cell lines (termed BL_B or Mesenchymal) display a constitutive mesenchymal phenotype as defined by their morphology in 2D and 3D culture, gene expression patterns (including retention of *TGFBR2* expression), loss of epithelial cell markers and expression of mesenchymal markers, ability to invade as single cells and with great metastatic potential. These cell lines seem to be derived from and recapitulate the phenotype of a subset of basal-like breast cancers that are associated with an activated TGFβ response gene signature, high propensity for metastasis and poor outcome.

A series of elegant studies recently reported by Mani et al. [60] have provided further support for a key association between mesenchymal and stem cell phenotypes and the role of TGFβ therein. Treatment with TGFβ or ectopic expression of either the *TWIST* or *SNAIL* transcription factors induced non-tumorigenic, immortalized HMEC to acquire a fibroblast-like, mesenchymal appearance, associated with downregulation of epithelial markers (such as E-cadherin (*CDH1*), and upregulation of mesenchymal markers (such as N-cadherin (*CDH2*), vimentin (*VIM*), and fibronectin (*FNI*)). Most importantly, all three treatments were associated with a shift in the cell population from predominantly *CD44*⁻, *CD24*⁺ cells to predominantly *CD44*⁺, *CD24*^{lo} cells. Moreover, this phenotypic shift was associated with a dramatic (30-40-fold) enrichment in the number of cells capable of giving rise to mammospheres, a functional assay for the stem cell phenotype. Further experiments demonstrated that the putative stem cells gave rise to diverse cell populations that expressed either basal cytokeratins (*KRT14*⁺) or luminal cytokeratins (*KRT8* or *18*⁺), both in mammospheres and in 2D culture. To address the possibility that induction of an EMT could force the conversion of *CD44*⁻, *CD24*⁺ cells to stem cell-like *CD44*⁺, *CD24*^{lo}, *SNAIL* or *TWIST* were selectively expressed in *CD44*⁻, *CD24*⁺ cells. The majority of these *CD44*⁻, *CD24*⁻ cells underwent EMT, as judged by their morphology, and gave rise to *CD44*⁺, *CD24*^{lo} cells. In addition, the mesenchymal-appearing cells derived de novo by inducing EMT in *CD44*⁻, *CD24*⁺ cells formed >10-fold more mammospheres than *CD44*⁻, *CD24*⁺ cells infected with a control vector, suggesting the possibility that luminal cells can revert to a more primitive stem cell state.

To address the possibility that forcing EMT could also promote the generation of cancer stem cells from more differentiated neoplastic cells, *SNAIL* or *TWIST* were overexpressed in immortalized HMEC that had been fully transformed by introduction of an activated form of the *HER2/neu* oncogene or with a *Ha-Ras* oncogene. In both models, induction of EMT dramatically increased the frequency of mammosphere forming cells (i.e., stem cells), as well as the number of colonies in soft agar, suggesting that EMT is associated with both anchorage-independent growth and a stem cell-like phenotype. However, following EMT, xenografted *HER2/neu*-driven transformants failed to form tumors more efficiently than untreated cells in

vivo, which suggested that long-term maintenance of the EMT/stem cell state depends on continuous EMT-inducing signals, at least in this experimental model. In contrast, the majority of the mice that were injected with *TWIST*- or *SNAIL*-expressing V12H-Ras-transfected cells did form tumors in mice. Thus, in this model, expression of either the *TWIST* or *SNAIL* EMT-inducing transcription factor was sufficient to increase the number of tumor-initiating cells by approximately two orders of magnitude [165]. Although TGF β was only used to induce EMT in the first set of experiments, and we do not know whether the *TGFBR2* gene was re-activated with induction of EMT and the acquisition stem cell phenotype, these questions could readily be addressed.

The CD44 and TGF β signaling pathways are directly linked—Activation of TGF β and TGF β receptor signaling appear to be tightly linked to the CD44 signaling pathway at several different levels. First, CD44-deficient mice develop increased lung inflammation following bleomycin-induced lung disease, similar to the phenotype observed in both the TGF β 1 and α_5 integrin knock out mice [166-168]. Even more intriguing is the notion that CD44 may regulate activation of TGF β , thereby localizing its effects to areas of active injury [168, 169]. Thus, Yu and Stamenkovic [169] reported several years ago that CD44 provides a cell surface docking receptor for proteolytically active matrix metalloproteinase-9 (MMP-9), and that CD44 and MMP-9 form a complex on the surface of TA/St mouse mammary carcinoma cells that activates latent TGF β and is required for tumor invasion. Disruption of the CD44/MMP-9 complex by expression of soluble CD44 resulted in the loss of tumor invasiveness and abrogated tumor cell survival in host lung parenchyma following intravenous injection into syngeneic mice [170]. To determine whether activation of latent TGF β by the CD44/MMP-9 complex was responsible for tumor cell survival in the lung microenvironment, TA3 cells overexpressing dominant-negative soluble CD44 (TA3sCD44), which compromises native CD44 function and the ability of TA3 cells to develop metastases, were transfected with constitutively active or latent TGF β 2 and tested for their ability to form tumors in syngeneic mice. Expression of the constitutively active, but not the latent, form of TGF β 2 rescued TA3sCD44 cells from apoptosis during lung colonization. These observations provided direct evidence that the functional axis composed of CD44, MMP-9 and TGF β is essential for activation of latent TGF β , which, in turn, is required for tumor cell survival and metastatic colony formation in the lung microenvironment [170]. Moreover, the importance of CD44 for activation of latent TGF β has been substantiated by several recent studies using CD44 deficient mice [171,172]. CD44 deficient mouse embryo fibroblasts exhibited migration that was characterized by increased velocity but loss of directionality, compared with CD44 wild type fibroblasts. In terms of mechanism of action, CD44 wild type cells generated more active TGF β than CD44 deficient cells and CD44 promoted the activation of TGF β via an MMP-dependent mechanism, as it could be blocked in the presence of the general MMP inhibitor, GM6001. Reconstitution of CD44 expression completely rescued the phenotype of CD44 deficient cells, whereas exposure of CD44 deficient cells to exogenous active TGF β rescued the defect in stress fibers and migrational velocity, but was not sufficient to restore directionality of migration. These results suggest that CD44 may be critical for the recruitment of fibroblasts to sites of injury and the function of fibroblast-derived TGF β in tissue remodeling and fibrosis. Consistent with this idea, CD44 deficient mice developed more tubular damage, associated with decreased proliferation and increased apoptosis of tubular epithelial cells but less renal fibrosis following ureteral obstruction. In addition, impaired influx of macrophages and decreased accumulation of myofibroblasts was observed in the obstructed kidney of CD44 deficient mice compared with wild type mice, consistent with reduced TGF β activity and induction of EMT. Indeed, Smad2/3 activation was reduced in the obstructed kidney of CD44 deficient mice. These results tie together the role of chronic TGF β activation in renal fibrosis secondary to obstruction with the necessary presence of CD44 [171].

Besides its critical role in TGF β activation, CD44 also seems to enhance TGF β receptor signaling in MDA-MB-231 human breast cancer cells [173]. Co-immunoprecipitation experiments demonstrated that MDA-MB-231 cells selectively expressed the CD44 variant 3 (CD44v3) and that CD44v3 and the T β R-I (and to a lesser extent, T β R-II) receptors were present in a single complex in vivo. Moreover, the cytoplasmic domain of CD44 bound to T β RI with high affinity. Furthermore, binding of hyaluronan to CD44 in MDA-MB-231 cells stimulated T β R-I kinase activity, resulting in Smad2/3 phosphorylation and PTH-rP production. Most importantly, activated T β R-I kinase phosphorylated CD44, which enhanced its binding interaction with the cytoskeletal protein, ankyrin, leading, in turn, to increased tumor cell migration. In aggregate, these studies have demonstrated that CD44 is instrumental not only in activating latent TGF β , but also directly physically interacts with the TGF β receptor complex, resulting in further activation of multiple signaling pathways required for tumor cell migration and, possibly, metastasis.

Unifying model of the role of TGF β signaling in normal and malignant breast tissue—In aggregate, the studies summarized in sections “Breast cancer initiating cells have a mesenchymal phenotype and active TGF β signaling” and “The *CD44* and TGF β signaling pathways are directly linked” are remarkably consistent with a model of TGF β action in normal mammary gland epithelium in which TGF β plays a major role in maintaining the stem cell population by counterbalancing the differentiation into luminal, basal or myoepithelial cells (Fig. 2). The model assumes the existence of a primitive multipotent stem cell population that is ER-negative, PR-negative, *CD44*⁺, *CD24*^{lo}, *ESA*⁺, *KRT5*⁺, *ALDH1*⁺, *TGFBR2*⁺ and possibly expresses mesenchymal markers. When these cells divide, they give rise either to phenotypically identical daughter stem cells or to precursors of differentiated progeny (luminal, basal, or myoepithelial). Luminal cells are epithelioid (*ESA*⁺, *KRT8/18*⁺, *CDH1*⁺), express ER and PR, are *CD44*⁺, *CD24*⁺, and the *TGFBR2* gene is transcriptionally silent (*TGFBR2*[−]). The basal and myoepithelial progeny express basal cytokeratins (*KRT5/6/14*⁺) and/or myoepithelial markers (α SMA, p63), lack *ESA* (*ESA*[−]), and appear to be enriched for *CD44*⁺, *CD24*^{lo} cells. It is likely that these cells continue to express *TGFBR2*, but this remains to be demonstrated. One of the fundamental determinants of the proportions of these different subpopulations in normal or cancer tissue is the ratio of stem cell divisions that give rise to daughter stem cells versus differentiated progeny [174]. We propose that exposure to TGF β leads to enrichment of the cell population with multipotent *CD44*⁺, *CD24*^{lo} clonogenic/tumor initiating cells that express mesenchymal markers (EMT) [60], while treatment with a TGF β receptor kinase inhibitor leads to enrichment with *CD44*⁺, *CD24*⁺, *TGFBR2*[−] non-clonogenic differentiated luminal cells [78]. Interestingly, several groups have demonstrated that TGF β /T β R-I kinase/Smad2/3 signaling is required to keep human embryonic stem cells in their pluripotent, undifferentiated state [175,176], and functions downstream of the Wnt signaling pathway, which is involved in stem cell maintenance in several different tissues, including the mammary gland [177]. Thus, we propose the model that TGF β signaling is a key determinant of the fate of stem cell progeny, and favors self-renewal of the stem cell pool in the normal mammary gland. This model predicts that constitutive activation of TGF β signaling associated with invasive and metastatic breast cancer might contribute to steadily enriching the cancer stem cell pool, thereby indirectly enhancing the potential for metastasis, drug resistance and poor patient survival. In this context, it is noteworthy that EMT of normal as well as malignant human epithelial cells appears to be tightly associated with the stem cell state [60].

Superimposed onto these population dynamics within the mammary gland epithelium is the role of systemic estrogens and progestins in regulating TGF β signaling (see section “Extensive cross-talk between estrogen- and TGF β signaling pathways”). The ER/PR-positive luminal cells respond to estrogen by producing mitogenic growth factors and by lowering TGF β production. Our model predicts that these conditions would favor commitment of stem cell daughter cells to become more differentiated progenitor cells. Moreover, because the

TGFBR2 gene is transcriptionally silenced in these luminal daughter cells, this would further favor their clonal expansion. Conversely, anti-estrogens induce TGF β production, which, even if it inhibits the rate of stem cell proliferation, would favor their self-renewal over commitment to differentiation.

At the biochemical level, one might envision a circuitry in which estrogen signaling regulates the production and secretion of (latent) TGF β by ER-positive cells into the glandular microenvironment, which then becomes activated at the cell surface of neighboring *CD44*⁺ cells, thereby inducing or maintaining the stem cell phenotype, while, at the same time, restricting the proliferation of this same population. Once these *CD44*⁺ cancer stem cells break through the barrier of TGF β -mediated growth arrest, their ability to activate latent TGF β via *CD44* and MMPs would select for continued expansion of the cancer stem cell pool by restricting the differentiation to luminal cells, and, consequently, promote invasion, dissemination and metastasis.

Thus, our model predicts that treatment of basal-like cancers with TGF β antagonists may have anti-tumor effects by converting the putative stem cell population into a luminal, non-proliferating (and, perhaps, non-metastatic), phenotype (mesenchymal-to-epithelial transition, MET). Conversely, these agents might antagonize the therapeutic effects of anti-estrogens in estrogen-dependent luminal cancers. However, once these cells become estrogen-independent because they escape from TGF β -mediated growth arrest, our model provides a rationale for including TGF β pathway antagonists in the treatment program. Each of these predictions is testable in model systems as well as in clinical trials.

Norton and Massagué [178] recently proposed the hypothesis that transitions of epithelial cancer cells to a motile mesenchymal phenotype (EMT) are not only required for invasion and dissemination to distant sites, but may also occur locally within the primary tumor and its capillary bed. The attraction of this model is that it would explain a number of different characteristics of cancer, including histologic heterogeneity within a primary tumor mass, the development of satellite nodules around the primary tumor and, in some cases, multifocality. In a broader sense, this model implies that metastasis is a dynamic ongoing process and predicts that primary metastases can seed secondary metastases. These questions are currently being addressed experimentally and preliminary results appear to validate this hypothesis (Joan Massagué, Personal communication). Perhaps most important are the therapeutic implications of this model. It is generally assumed that, once metastases have been established, agents that target the process of dissemination (EMT, cell migration, invasion, colonization at secondary sites) would be of little clinical value. However, if the Norton-Massagué hypothesis turns out to be correct, such agents might be of much greater therapeutic value than is generally believed. In this regard, TGF β pathway antagonists might represent the first of this new class of agents to undergo clinical evaluation.

TGF β signaling in breast cancer subtypes—As noted earlier, Shipitsin et al. [78] reported that TGF β signaling pathway genes, particularly *TGFBR2*, were selectively expressed in tumor-isolated *CD44*⁺ *CD24*^{lo} cells, and transcriptionally silenced in the *CD44*⁺ *CD24*⁺ cell population. Moreover, these investigators reported that the 15 TGF β -regulated genes (including *CTGF*, *COL1A1*, *FN1*, *SERPINE1*, *MMP9*) most strongly expressed in *CD44*⁺ tumors were also associated with a shortened distant metastasis-free survival [146]. Recently, investigators from MSKCC demonstrated that a 153 gene TGF β gene response signature (TBRS) is highly expressed in approximately one-third of primary human breast cancers, equally distributed across the ER-positive and ER-negative subsets [147]. Moreover, expression of this TBRS in ER-negative cancers strongly predicted for the development of lung metastases and poor outcome. These findings are remarkably consistent with the demonstration by Buck et al. [145] that survival of patients with ER-negative, *TGFR2*⁺ tumors

was significantly shorter than those with ER-negative, *TGFBR2*⁻ cancers. In contrast, expression of the TBRS in ER-positive cancers did not affect outcome [147], and neither did expression of *TGFBR2* [145].

At our own institution, Alexe et al. [179] recently developed a highly robust classifier of gene expression profiles of human breast cancer using principal component analysis and consensus ensemble clustering. Clustering applied to breast cancer expression profiles consistently divide samples along an ER⁺/ER⁻ split, which might result in a misclassification of some samples. This is particularly pertinent to the HER2-overexpressing subset, which includes both ER-positive and -negative cases. In order to circumvent this problem, Alexe et al. [179] first identified *HER2*⁺ tumors based on co-amplification of three or more of the genes located on the *HER2/neu* amplicon: *HER2/neu*, *GRB7*, *STARD3*, and *PPARB*. After separating *HER2*-positive from *HER2*-negative samples, each set was analyzed separately by principal component analysis and consensus ensemble clustering. This analysis robustly identified a total of six major breast cancer subtypes, which include two subsets of *HER2*-positive tumors, two subsets of *HER2* negative, ER-negative “basal-like” tumors, and two subsets of ER-positive, *HER2*-negative “luminal” tumors [179]. Interestingly, the *HER2*-positive group could be easily divided both by gene expression profile and by histopathology into two very distinct subsets: *HER2*(I) tumors contain an abundant lymphocytic infiltrate, strongly express a lymphocyte-associated gene signature, and are associated with an excellent clinical outcome. In contrast, the *HER2*(NI) subset lacks a lymphocytic infiltrate and lymphocyte-associated gene signature, and is associated with a strong desmoplastic reaction and poor clinical outcome. Similarly, basal-like 1 (BA1) subset tumors are associated with an interferon/interleukin response gene signature not found in the BA2 subset, and are associated with a better prognosis than BA2 tumors. We then posed the question whether the 153-gene TBRS described by Padua et al. [147] (TBRS^{MSKCC}) as well as a similar 92-gene signature developed in our own laboratory [67] (TBRS^{CINJ}) is associated with any particular breast cancer subsets. As shown in Fig. 3B, the TBRS^{MSKCC} was strongly positively associated with tumors in the *HER2*(NI), BA2 and LA1 subsets. These results were validated across three independent publicly available breast cancer expression data sets from different centers [147,180,181]. Moreover, the TBRS^{CINJ} gave identical results to that developed by Padua et al. [147]. The principal difference between the two instruments is that the TBRS^{MSKCC} includes a large number of ESTs, whereas the TBRS^{CINJ} signature was developed using only annotated genes, which will facilitate clinical validation.

The relationships between TBRS^{MSKCC} expression and overall survival were explored using data from patients with lymph node negative disease, who did not receive adjuvant systemic therapy (Fig. 3C). The expression of a TBRS was associated with poor overall survival. However, in subset analysis, TBRS was not associated with a difference in overall survival within the luminal A subset, although there was trend towards a negative effect in the luminal B subset. Similar trends were found between TBRS expression and survival in both two basal-like cancer subsets. As previously reported by Alexe et al. [179], patients with a *HER2*(I) cancer had a significantly better outcome than those with a *HER2*(NI) cancer. However, among the *HER2*(NI) cases, the expression of a TBRS predicted for extremely poor survival, while survival of patients whose tumors did not express the TBRS was essentially indistinguishable from those with *HER*(I) disease. These results suggest that activation of the TGFβ pathway largely accounts for the poor prognosis of patient with *HER2*(NI) breast cancer, and that this particular subset may derive particular benefit from treatment with TGFβ pathway antagonists. This idea is readily testable in a clinical trial.

In spite of the striking associations we uncovered between the TBRS and breast cancer subtypes, one has to be cautious how one interprets these observations, as the biological significance of a positive TBRS remains to be determined. A positive association of the TBRS

with a breast cancer may reflect dependence of the tumor on this signaling pathway (by, e.g., driving invasion and/or metastasis), particularly in the HER2(NI) and, perhaps, BA2 subclusters. On the other hand, expression of a TBRS by good prognosis luminal A tumors may indicate that, in the context of ER-positive, presumably *TGFBR2*⁻ cancer cells, the expression of a TBRS reflects a strong response of stromal, immune and other host cells to TGF β . In this context, it is intriguing that luminal-type *PyVmt*-driven mammary carcinomas that develop on a *TGFBR2*^{null} genetic background are specifically associated with a strong chemokine-driven immune cell infiltrate [53,55]. Thus, in the context of ER-negative cancers, the expression of a TBRS might represent a bio-marker that predicts for response to TGF β pathway antagonists, while, for ER-positive cancers, treatment with a TGF β targeted agent may accelerate tumor growth and be detrimental to the patient. These critical questions will need to be carefully addressed in upcoming clinical trials of these agents in breast cancer and other diseases.

Preclinical therapeutic studies of TGF β antagonists against mammary cancer in vivo

TGF β pathway antagonists in clinical development

Two approaches for targeting TGF β /Smad signaling are in early stages of clinical development (Table 1). These include (1) neutralization of TGF β ligands and (2) selective inhibition of TGF β receptor kinases. Extensive preclinical studies in a number of different syngeneic as well as xenograft models of metastatic breast cancer suggest that both types of agents have similar anti-metastatic properties against skeletal and lung metastases [126].

Experimental models

Given TGF β 's pleiotropic effects on both tumor cells and host cells, and its presumed role in tumor metastasis, detailed assessment of anti-tumor effects of TGF β antagonists can only be accomplished by using models of metastatic mammary cancer (Table 2). This has limited these studies to a handful of model cells lines, including the murine metastatic mammary cancer cell lines 4T1 (Balb/C), EMT6 (Balb/C) and R3T (129S1), and the human metastatic MDA-MB-231, MDA-MB-435, MCF10ACA1A and MX-1 cell lines that are inoculated into immunodeficient mice. It should be noted that each of these cell lines are representative of so-called basal-like breast cancer, as they are ER-negative, PR-negative, HER2/neu-negative, *CD24*^{lo}, and express *CD44* as well as mesenchymal markers [160,163,182,183]. The only model of spontaneous mammary cancer that efficiently gives rise to metastases is the *MMTV/PyVmt* transgenic mouse model [184,185]. In contrast to the basal-like carcinoma cell lines, carcinomas that arise on the *PyVmt* background have been shown to share many features of human luminal type breast cancer, even though they do not express ER [56]. A distinguishing feature of these tumors is the high expression of *XBPI*, which is a human luminal tumor-defining gene. These tumors also expressed tight junction structural component genes, including Occludin, Tight Junction Protein 2 and 3, E-cadherin (CDH1) and the luminal keratins *KRT8* and *-18*.

In terms of metastasis assays, some of the studies have employed spontaneous metastasis assays in which cells are injected orthotopically into the mammary fatpad and metastases originate at the primary injection site, while others have used experimental metastasis assays, in which the tumor cells are injected directly into either the venous or arterial circulation to give rise to pulmonary or extrapulmonary metastases, respectively. Finally, in most of the preclinical studies conducted and reported to date, treatment with TGF β antagonists was initiated shortly after tumor cell inoculation. It is important to realize that, under these conditions, it is difficult to ascribe any observed anti-metastatic effect to either inhibition of micrometastatic tumor colony formation or suppression of growth of metastatic lesions.

Single agent activity of TGF β antagonists

TGF β neutralizing antibodies—GC1008 (human IgG4 kappa, Genzyme, Inc.), 1D11 (mouse IgG1), and 2G7 (mouse IgG2b, Genentech®) are all high-affinity monoclonal antibodies capable of neutralizing all three major mammalian isoforms of TGF β (i.e., 1, 2, and 3). TGF β is highly conserved between species, and both the human clinical compound, GC1008, and its murine counterparts, 1D11 and 2G7, have similar TGF β -neutralizing properties. However, 1D11 and 2G7 are not immunogenic in the mouse, and this property permits prolonged administration to mice.

The anti-tumor effects of the murine 1D11 and 2G7 antibodies have been tested in several different preclinical syngeneic and xenograft mammary cancer models (Table 2). Arteaga et al. [186] were the first to show that intraperitoneal injections of 2G7 (Genentech®), suppressed intra-abdominal tumor growth as well as lung metastases of MDA-MB-231 human breast cancer cells that had been inoculated intraperitoneally. Carano et al. [112] (US Patent 2005/0276802 A1) demonstrated that treatment of mice with 2G7 following mammary fatpad inoculation of syngeneic 4T1 carcinoma cells reduced the volume of lung metastases by ~50%. Interestingly, 2G7 treatment was associated with a normalization of plasma VEGF levels, suggesting the possibility that 2G7's effect was mediated at least in part via inhibition of angiogenesis. In addition, treatment of spontaneously arising mammary carcinomas in *PyVmt* transgenic mice with 2G7 also caused a significant tumor growth delay [112] (US Patent 2005/0276802 A1). In experiments reported by Pinkas et al. [187], 4T1 mammary carcinoma cells were inoculated by intracardiac left ventricular injection to give rise to bone metastases. Treatment with the 1D11 anti-TGF β antibody demonstrated a significant reduction in the number of lytic bone lesions as well as a survival benefit [187]. Using 4T1 cells in an experimental lung metastasis assay, Nam et al. showed that treatment with 1D11 significantly suppressed both the number and size of tumor metastases to the lungs [188-190]. Overall, in this syngeneic model, the therapeutic effects seen with the TGF β neutralizing antibodies against skeletal or pulmonary metastases appear to be of a similar order of magnitude.

In our own laboratory, we have carried out a number of in vivo treatment experiments to assess the efficacy of the murine anti-TGF β monoclonal antibody, 1D11 in experimental metastasis models using bone-tropic as well as lung-tropic MDA-MB-231 human breast cancer cells (Ge et al. manuscript in preparation). For this purpose, we used sublines of basal cell-like MDA-MB-231 human breast carcinoma cells that preferentially metastasize to lungs (MDA-231-4175TR) or bone (MDA-231-SCP2TR and 2860TR). Treatment with 1D11 was able to block TGF β -induced phosphorylation of the receptor-associated Smads, Smad 2 and -3, in each of these cell lines. While 1D11 had no effect on cell growth in vitro, it did inhibit TGF β -stimulated tumor cell migration and -invasiveness into Matrigel®. To determine the effects of 1D11 on bone- or lung metastasis, cells were inoculated into athymic nude mice, and treatment with 1D-11 was initiated the following day. Treatment with 1D-11 antibody significantly reduced the burden of MDA-231-SCP2TR or 2860TR-derived metastases to bones as well as MDA-231-4175TR-derived metastases to lungs by ~40%. In aggregate, these results support the notion that TGF β plays a role in both bone- and lung metastases of basal-like breast cancer, and that inhibiting TGF β signaling results in a therapeutic effect independently of the tissue-tropism of the metastatic cells.

Soluble TGF β receptor fusion constructs—Studies using transgenic mice expressing a soluble T β R-II receptor:Fc fusion protein (Fc:T β RII) driven by a MMTV-LTR promoter/enhancer have demonstrated anti-metastatic activity without increasing primary tumor incidence [45]. This suggested that Fc:T β R-II might selectively neutralize the undesirable TGF β associated with metastasis, while sparing the homeostatic function of TGF β in normal tissues. Subsequently, Muraoka et al. [191] reported that systemic administration of Fc:T β RII

to *MMTV/PyVmT* transgenic mice inhibited the development of lung metastases. Fc: T β RII also inhibited the development of lung metastases from 4T1 and EMT-6 mouse mammary tumors inoculated into the mammary fatpad of syngeneic BALB/c mice.

Along similar lines, Bandyopadhyay et al. [192] examined whether expression of a truncated soluble extracellular domain of the TGF β type III receptor (sT β R-III) might antagonize the tumor-promoting activity of TGF β by sequestering active TGF β produced by the cancer cells. Conditioned medium of MDA-MB-231 human breast cancer cells engineered to produce sT β R-III effectively neutralized active TGF β 1 and -2. Moreover, tumor incidence and growth rates of sT β R-III-expressing MDA-MB-231 clones in athymic nude mice were significantly lower than those of control cells. In addition, while orthotopically inoculated control cells gave rise to spontaneous lung metastases, none of the sT β R-III-expressing cell-inoculated mice developed lung metastases [192]. Similar results were obtained using a second human breast carcinoma cell line, MDA-MB-435 [193]. Furthermore, Matrigel® plug assays showed that the blood volume in Matrigel® plugs containing sT β R-III-expressing cells was significantly lower than that those containing the respective control cells, and this correlated with a decrease in microvessel counts. Moreover, treatment of human endothelial cells with a recombinant sT β R-III significantly inhibited their ability to form a capillary web structure in Matrigel. These results suggested that sT β R-III-mediated tumor suppression may be due, at least in part, due inhibition of angiogenesis [194]. In aggregate, these studies have demonstrated that soluble receptors may selectively neutralize the undesirable TGF β associated with metastasis, while sparing the regulatory roles of TGF β in normal tissues.

TGF β anti-sense oligonucleotides—Anti-sense oligonucleotides against the TGF β ligands have been shown to induce tumor regression in a number of different syngeneic in vivo tumor models, apparently by enhancing anti-tumor cytotoxic T-cell activity. For example, Park et al. [195] stably expressed antisense TGF β 1 in EMT6 murine mammary tumor cells using a retroviral vector. These EMT6as-TGF β 1 cells secreted less than half the amounts of TGF β 1 produced by tumor cells transduced with a control vector, and culture medium from these cells also exhibited a decreased capacity to inhibit alloantigen-specific cytotoxic T-cell responses in vitro. Furthermore, tumor growth in mice injected with EMT6as-TGF β 1 tumor cells was inhibited compared to mice injected with control tumor cells. These results demonstrated that expression of antisense TGF β 1 by transduced EMT6 cells decreased their tumorigenicity, presumably by eliminating immune suppression. An anti-sense oligonucleotide directed against TGF β 2, AP-12009, is currently undergoing clinical trials for patients with malignant gliomas and pancreatic cancer [196-199].

Chemical T β R kinase inhibitors—Several different chemical classes of T β R kinase inhibitors have been developed and have been shown to block TGF β -induced Smad phosphorylation, reporter gene activation, and cellular responses of mammary epithelial cells in vitro, including cell cycle arrest and EMT, at submicromolar concentrations [14,200]. These serine-threonine kinase inhibitors bind to the ATP binding site of the T β R-I kinase and may maintain the enzyme in its inactive configuration [201]. We have examined the effects of the orally bio-available compound, SD-208 (Scios, Inc.), on metastatic mouse mammary carcinoma cell lines (R3T and 4T1) [83]. When orthotopically injected into the mouse mammary fatpad, both R3T and 4T1 cells form primary tumors that efficiently metastasize to lungs and other organs. Up to 60 mg/kg SD-208 could be administered safely by daily gavage for up to 84 days. Moreover, treatment of syngeneic R3T or 4T1 tumor-bearing mice with orally administered SD-208 inhibited primary tumor growth as well as the number and size of lung metastases. In contrast, SD-208 failed to inhibit R3T tumor growth or metastases in athymic nude mice. Moreover, in vitro anti-4T1 cell cytotoxic T-cell responses of splenocytes from drug-treated animals were enhanced compared with cells from control animals. In addition, SD-208 treatment resulted in a decrease in tumor angiogenesis. Moreover, SD-208

as well as other T β R-I kinase inhibitors are also capable of inhibiting human breast cancer-derived bone- and lung metastases in nude mice in vivo [202,203], indicating that its therapeutic effect can be attributed, at least in part, to actions on tumor cells themselves or on non-immune host cells. Thus, SD-208s therapeutic effects can be attributed, in part, to enhancing antitumor cytotoxic T-cell activity, inhibiting tumor angiogenesis and cell-autonomous effects on the tumor cells themselves.

Investigators at Eli Lilly & Co., have recently developed a series of chemical inhibitors of the T β R-I and T β R-II kinase domains on a pyrazole backbone [128,204-209]. Biochemical and cellular analysis confirmed the highly selective nature of these compounds, including the clinical candidate, LY2157299 (currently in phase I clinical trial). Moreover, these compounds showed in vivo anti-tumor activity against 4T1 mouse and MX-1 human mammary carcinomas [210]. Plasma levels of LY2157299, the fraction of phosphorylated Smad2 and -3 (pSmad) in tumor tissue, and tumor size were used to derive a semi-mechanistic pharmacokinetic-pharmacodynamic model of drug action. Plasma drug levels were linked to intratumoral pSmad dephosphorylation, and this, in turn, to tumor growth inhibition [210]. In efforts to expand the structure-activity relationships of the quinoline domain of dihydropyrrolopyrazole series, these same investigators have recently developed a second orally bioavailable compound with demonstrated antitumor efficacy against MX-1 sub-cutaneous xenografts, LY2109761, which is representative of T β R kinase inhibitors that have activity against both the T β R-I and -II receptors [209]. In order to investigate the possible clinical utility of LY2109761 in metastatic breast cancer, we examined its effects on experimental metastases of bone- and lung-tropic sublines of basal cell-like MDA-MB-231 human breast carcinoma cells. LY2109761 was able to block TGF β -induced phosphorylation of the receptor-associated Smads, and to induce dephosphorylation of activated Smads. While anchorage-dependent cell growth was not affected, TGF β -mediated in vitro stimulation of cell migration and invasion was inhibited by LY2109761. Beginning one day following tumor cell injection, mice inoculated with lung-tropic MDA-231-4175TR or with bone-tropic SCP2TR cells were treated with LY2109761 twice daily by gavage for 5 weeks. LY2109761 treatment reduced the burden of lung- as well as bone metastases by ~40%.

Bandyopadhyay et al. [202] recently reported the efficacy of another pyrazole-based TGF β type I receptor kinase inhibitor (Biogen Idec HTS466284, Eli Lilly LY364947), on early systemic bone- and lung metastases of highly metastatic human MDA-MB-435-F-L breast carcinoma cells. Systemic administration of the kinase inhibitor via intraperitoneal injection effectively reduced the number and size of lung metastases in both orthotopic and experimental metastasis assays without affecting primary tumor growth. In addition, kinase inhibitor treatment reduced the incidence of widespread early skeletal metastases [202]. Along similar lines, Ehata et al. [211] recently investigated the effects of a novel TGF β type I receptor kinase inhibitor, Ki26894, on bone metastasis of a highly bone-tropic human MDA-MB-231-D breast cancer cells. Treatment with Ki26894 blocked TGF β signaling in MDA-MB-231-D cells, as detected by suppression of phosphorylation of Smad2 and inhibition of TGF β -responsive target gene activity. Moreover, Ki26894 decreased the motility and the invasion of MDA-MB-231-D cells induced by TGF β in vitro. X-ray radiography revealed that systemic Ki26894 treatment initiated 1 day before the inoculation of MDA-MB-231-D cells into the left ventricle of BALB/c nu/nu female mice resulted in decreased bone metastases and prolonged survival compared to vehicle-treated mice. In aggregate, these studies suggest that TGF β plays a role in both bone- and lung metastases of basal-like breast cancer, and that inhibiting TGF β signaling results in a therapeutic effect independently of the tissue-tropism of the metastatic cells. Moreover, the neutralizing antibody, 1D11 (section "TGF β neutralizing antibodies"), and the chemical T β R kinase inhibitor, LY2109761, appear to inhibit metastases to a similar degree, in that both cause an approximately one-third to one half reduction in metastatic load.

In summary, these preclinical studies have provided convincing evidence that targeting the TGF β pathway using either ligand traps or receptor kinase inhibitors can inhibit both early lung and bone metastases in several different syngeneic as well as allogeneic mammary cancer models and suggest that the primary target is metastatic clonogenic potential. In addition, in vivo activity against particular cell lines seems to correlate with an in vitro mesenchymal phenotype in which TGF β drives EMT, cell motility and cell invasion. Furthermore, both autocrine effects of TGF β on the tumor cells themselves as well as its effects on host cells are likely to be contributing to the therapeutic activity of TGF β antagonists in vivo. These findings are consistent with the concept that TGF β signaling plays several different roles in the complex interplay between tumor and host cells that constitute the pre-metastatic niche. The signaling pathway appears to be fundamentally altered in tumor cells in such a way that the tumor cells interpret incoming signals as pro-invasive, while they are no longer growth inhibited. This results in the secretion of TGF β -induced metastasis-effector proteins, which exert pro-metastatic actions on the host microenvironment. In addition, the stromal compartment becomes modified (“activated”) in response to TGF β , and this stromal response also contributes to preparing the metastatic niche to further support tumor cell survival. Thus, a microenvironment rich in bioactive TGF β would provide a selective pressure that favors growth and invasion of tumor cells with this particular phenotype.

Combination therapy approaches

Combinations with cytotoxic agents or ionizing radiation—Several studies point to a role of TGF β in mediating resistance to DNA damaging agents. Thus, Ohmori et al. [212] first reported that treatment of MDA-MB-231 human breast tumor cell spheroids grown in three dimensional cultures using the 2G7 murine neutralizing anti-TGF β antibody potentiated cisplatin (CDDP)-mediated cytotoxicity, associated with a greater than ten-fold reduction in CDDP IC₅₀. These results suggested that tumor cell-associated TGF β might protect cells from DNA damage and that postchemotherapy administration of TGF β inhibitors may facilitate progression beyond G₁/S, potentially increasing the efficacy of cytotoxic chemotherapy. Along the same lines, Teicher et al. [213] examined the putative role of TGF β in in vivo resistance by administration of TGF β -neutralizing antibody to animals bearing EMT-6 mammary carcinoma or its alkylating agent resistant sublines, EMT-6/CTX or EMT-6/CDDP. Treatment of tumor-bearing animals with the 2G7 pan-TGF β neutralizing antibody by intraperitoneal injection daily on days 0-8 post-tumor cell implantation increased the sensitivity of the parental EMT-6 tumors to cyclophosphamide (CTX) and CDDP and markedly increased the sensitivity of the EMT-6/CTX tumor to CTX and the EMT6/CDDP tumor to CDDP, as determined by ex vivo cell survival assays. Animals bearing the EMT-6/CTX and EMT-6/CDDP tumors had higher serum lactate levels than control or parental EMT-6 tumor-bearing animals, which were decreased by the anti-TGF β regimen, suggesting that TGF β may play a role in regulating autophagy. Thus, treatment with TGF β -neutralizing antibodies restored drug sensitivity in the alkylating agent-resistant tumors, altering both the tumor and host metabolic states.

Investigators at Genzyme, Inc. have also examined the anti-tumor effects of the pan-TGF β neutralizing antibody, 1D11, in combination with various common chemotherapeutics against mammary cancer models. The combination of 1D11 with CDDP resulted in long-term survivors in the 4T1 murine breast cancer experimental bone metastasis assay. More recently, these same investigators demonstrated at least additive dose-dependent effects of 1D11 against several human tumor xenografts (including breast and renal cell) when combined with a variety of cytotoxic agents, including paclitaxel, CDDP, doxorubicin, or CTX. Similarly, scientists at Genentech have shown that the anti-TGF β mouse monoclonal antibody, 2G7, potentiates the efficacy of docetaxel in 4T1 spontaneous lung metastasis assays [112] (US Patent 2005/0276802 A1).

Bandyopadhyay et al. [214] determined whether the efficacy of doxorubicin in the inhibition of tumor growth and lung metastasis could be improved by simultaneous treatment with a pyrazole-based TGF β type I receptor kinase inhibitor (Biogen Idec HTS466284, Eli Lilly LY364947). In these studies, murine breast cancer 4T1 cells were inoculated into both inguinal mammary fat pads of Balb/c mice. Treatment with doxorubicin with or without kinase inhibitor was initiated either within 2 days of tumor cell inoculation or once the average tumor volume reached 60-70 mm³. Results from both sets of experiments indicated that, while the T β RI inhibitor alone failed to inhibit tumor growth, it significantly enhanced doxorubicin's antitumor activity. The analysis of lung metastases with the early treatment protocol revealed remarkable reduction in the number of lung metastases following combination treatment in comparison to either vehicle or doxorubicin alone. In contrast, when treatment was initiated after the appearance of palpable tumors, there was no significant difference in the number of lung metastases between the treatment groups and placebo control, suggesting that TGF β plays its main role initial metastatic colonization rather than controlling the growth of macrometastases.

From a mechanistic standpoint, it has been known for some time that ionizing radiation is a potent inducer of TGF β activation in normal and neoplastic tissue [215]. Ionizing radiation-induced DNA damage elicits a cellular program of damage control coordinated by the kinase activity of the ATM protein for which TGF β is required [24]. Recently, Kirshner et al. [22] reported that inhibiting TGF β signaling in mammary epithelial cells using a chemical T β R-I receptor kinase inhibitor attenuated ATM autophosphorylation and significantly reduced its kinase activity, while adding back TGF β 1 restored functional ATM and downstream DNA damage responses. These studies have uncovered a critical link between activation of TGF β 1 in the microenvironment and ATM, which directs epithelial cell genotoxic stress responses and, indirectly, tissue integrity. Thus, in addition to its role in homeostatic control of cell cycle and -survival, TGF β 1 plays a complex role in regulating responses to genotoxic stress, the failure of which could contribute to the development of cancer. Conversely, inhibiting TGF β may be used to our advantage in cancer therapy, as these agents may sensitize tumors to DNA damage and promote cell death [25].

Support for this concept was recently provided by Biswas et al. [23]. Using the *MMTV/PyVmT* transgenic model of metastatic breast cancer, these investigators showed that administration of ionizing radiation (or doxorubicin) increased circulating levels of TGF β 1 as well as the number of circulating tumor cells and lung metastases. These effects were abrogated by administration of the neutralizing pan-TGF β antibody, 2G7. Circulating PyVmT-expressing tumor cells did not grow *ex vivo* in the presence of the TGF β antibody, suggesting that autocrine TGF β provides a survival signal for these cells. Ionizing radiation failed to enhance lung metastases in mice bearing tumors that lacked the *TGFBR2* receptor, suggesting that the increase in metastasis was due, at least in part, to a cell-autonomous effect of TGF β on the cancer cells themselves. These data implicate TGF β induced by ionizing radiation or cytotoxic chemotherapy as a pro-metastatic signal in tumor cells and provide further rationale for the simultaneous use of DNA damaging agents in combination with TGF β inhibitors to suppress tumor growth and lung metastases.

Combination with immunotherapy—Even though preclinical studies of TGF β antagonists in syngeneic mammary cancer models have demonstrated their ability to stimulate anti-tumor immunity, it's surprising that little has been reported in terms of combination immunotherapy [216]. Kobie et al. [217] showed that exposure to TGF β inhibited the ability of dendritic cells to present antigen, stimulate tumor-sensitized T lymphocytes, and migrate to draining lymph nodes. Conversely, neutralization of TGF β using the pan-TGF β -neutralizing monoclonal antibody 2G7 enhanced the ability of dendritic cell-based vaccines to inhibit the growth of established 4T1 murine mammary tumors. Treatment of 4T1 tumors transduced with an antisense TGF β 1 transgene (4T1-asT) with the combination of dendritic cells and 2G7

monoclonal antibody inhibited tumor growth and resulted in complete regression of tumors in 40% of the mice. These results demonstrated that neutralization of TGF β in tumor-bearing mice dramatically enhanced the efficacy of dendritic cell-based vaccines. If these results can be validated in other model systems, this approach should be considered for clinical development.

Combinations with oncolytic viruses—Seth et al. [218] have recently proposed a novel approach to cancer gene therapy in which the oncolytic effects of an infectious adenoviral vector are combined with selective expression of a soluble fusion protein composed of the type II TGF β receptor II extracellular domain and the Fc portion of IgG (Fc:T β RII) to antagonize active TGF β in the tumor microenvironment. An oncolytic adenovirus expressing Fc:T β RII, was constructed by homologous recombination. Infection of MDA-MB-231 and MCF-7 human breast cancer cells with this virus produced Fc:T β RII, which was released into the medium. The conditioned medium containing Fc:T β RII bound TGF β 1 and inhibited TGF β -dependent transcription in target cells. Infection of MDA-MB-231, MCF-7, and 76NE human breast cancer cells with the adenovirus resulted in high levels of viral replication, comparable to that of a wild-type virus, while there was no replication in non-proliferating normal cells. Direct injection of virus into MDA-MB-231 human breast carcinoma xenografts caused tumor regression in more than 85% of the animals [218]. In further studies in which Fc:T β RII cDNA was cloned into a conditionally replicating adenoviral vector showed that it is possible to construct an oncolytic virus expressing Fc:T β RII, in which both viral replication and transgene expression remain intact, and which retained its oncolytic activity in a human tumor xenograft model, indicating that it could be developed as a potential anticancer agent [219].

Combinations with antagonists of other oncogenic pathways—Several different genetic mouse models of mammary cancer have demonstrated an apparent synergy between the TGF β signaling pathway and growth factor pathways in driving metastasis (summarized in section “Cooperation between TGF β and ras gene activation in driving mammary cancer progression”). These studies provide a strong rationale for examining the therapeutic effects of combinations of TGF β targeted agents with drugs that target the HER2/neu or EGF signaling pathways. However, no preclinical studies along these lines have yet been reported.

A large body of experimental and clinical evidence supports the notion that the therapeutic effects of antiestrogen therapy are mediated, at least in part, by TGF β (see section “Extensive cross-talk between estrogen- and TGF β signaling pathways”). Thus, in this context, one would predict that combination therapy of a TGF β pathway antagonist with an anti-estrogen would be antagonistic. On the other hand, once breast cancers have become resistant to anti-estrogens and/or estrogen-independent, it may be rational to combine the two modalities. This idea is supported by early studies by Arteaga et al. in which tamoxifen resistance could be overcome by treatment with a TGF β neutralizing antibody [100]. Tamoxifen paradoxically stimulated proliferation of the LCC2 tamoxifen-resistant variant of MCF7 human breast cancer cells (MCF7/LCC2) in vitro, and this was not affected by the pan-TGF β neutralizing antibody, 2G7. However, treatment with tamoxifen caused accelerated growth of MCF7/LCC2 xenograft tumors in nude mice, and, in this case, the addition of 2G7 caused complete growth arrest. This reversal of tamoxifen resistance in vivo by 2G7 did not occur in beige/nude mice, which lack natural killer-cell function, suggesting that immune mechanisms may be involved in these antitumor effects. Consistent with this, antisense TGF β 2 oligodeoxynucleotides enhanced the sensitivity of MCF7/LCC2 cells to natural killer cells in the presence of tamoxifen. In addition, tamoxifen sensitive MCF7/LCC1 tumors were markedly more sensitive to tamoxifen in natural killer-active than in deficient mice. These data support the notion that host natural killer cell function mediates, in part, the antitumor effect of tamoxifen and that TGF β 2 may abrogate this mechanism, thus contributing to tamoxifen resistance [100]. If these findings can be confirmed

and validated in other model systems, combination trials of anti-estrogens and TGF β antagonists may be considered in the clinical setting of anti-estrogen resistance.

Mechanisms of action of TGF β antagonists in animal models—a triple-edged sword

In aggregate, the preclinical studies demonstrating anti-tumor activity of TGF β pathway-targeted agents in a variety of syngeneic and allogeneic mammary cancer models have suggested that these agents have several different mechanisms of action. These include cell-autonomous as well as host cell effects. The latter appear to include immune-mediated as well as anti-angiogenic mechanisms of action.

Tumor cell-autonomous mechanisms of action of TGF β pathway antagonists—

As summarized in section “Single agent activity of TGF β antagonists,” both major classes of TGF β pathway antagonists are able to significantly reduce the metastatic burden of human breast carcinoma cells inoculated into athymic nude mice. These studies suggest that these agents are acting, at least to some extent, by inhibiting the cell autonomous effects of TGF β that drive EMT-mediated invasiveness and metastasis. Because most of these studies were conducted in nude mice, they certainly demonstrate that these therapeutic effects were achieved independently of T-cell immunity, although they do not rule out an effect of the antagonists on NK cell activity. Perhaps the most compelling evidence in favor of a cell autonomous effect of a TGF β antagonist was recently provided by Nam et al. [190]. Using the 4T1 syngeneic model of experimental lung metastasis, these investigators compared the anti-metastatic activity of the pan-TGF β neutralizing antibody, 1D11, against parental 4T1 cells with that on 4T1 cells in which the intrinsic TGF β signaling pathway had been attenuated by the introduction of a dominant-negative T β R-II receptor. Interestingly, the introduction of a dominant-negative T β R-II receptor was sufficient to strongly suppress their metastatic ability and ablated most of the metastasis-suppressing effect of 1D11. The investigators estimated that ~60% of 1D11's anti-metastatic activity requires an intact TGF β response in the tumor cells themselves.

Immune-mediated mechanisms of action of TGF β pathway antagonists—

Inoculation of MDA-MB-231 human breast cancer cells into athymic nude mice decreased mouse spleen natural killer (NK) cell activity [186]. Intraperitoneal injections of the pan-TGF β neutralizing antibody, 2G7, following intraperitoneal inoculation of tumor cells suppressed intra-abdominal tumor and lung metastases, whereas a non-neutralizing anti-TGF β control antibody had no effect. Histologically, both 2G7-treated and control tumors were identical. Intraperitoneal administration of 2G7 resulted in a marked increase in mouse spleen NK cell activity. In contrast, 2G7 did not inhibit MDA-MB-231 primary tumor or metastases formation, nor did it stimulate NK cell-mediated cytotoxicity in beige NK-deficient nude mice. Finally, serum-free conditioned medium from MDA-MB-231 cells inhibited the NK cell activity of human blood lymphocytes, and this inhibition could be blocked by 2G7 but not by a nonspecific control antibody. These data supported a possible role for tumor cell TGF β in the progression of mammary carcinomas by suppressing NK cell-mediated host immune surveillance [186].

More recently, using 4T1 cells in an experimental lung metastasis assay, Nam et al. showed that treatment with 1D11 strongly suppressed the number of metastases to the lungs [188]. 1D11's anti-tumor activity in vivo appeared to be dependent on a synergistic combination of cell autonomous effects on the tumor cells and effects on the host microenvironment. The latter include suppression of angiogenesis as well as bone sialoprotein (Bsp), which reduces the ability of TGF β to induce local collagen degradation and invasion and allows for complement-mediated tumor cell lysis. In addition, treatment with 1D11 increased infiltration of NK cells and T cells at metastatic sites, and enhanced expression of coactivators (NKG2D) and cytotoxic

effectors (perforin and granzyme B) on CD8⁺T-cells. On the tumor cells, increased expression of an NKG2D ligand (Rae1 γ) and of a death receptor (TNFRSF1A) contributed to enhanced immune cell-mediated recognition and lysis.

In the same series of studies, depletion of CD8⁺ T cells alone was found to cause a paradoxical reduction in 4T1 tumor size, both at the primary site and in 4T1 metastases that arose in the lung following tail vein [189]. Further analysis demonstrated that CD8⁺ splenocytes from tumor-bearing mice actively promoted 4T1 tumor growth by producing interleukin (IL)-17 in response to TGF β , and that treatment of mice with 1D11 in vivo reduced IL-17 expression both in the tumor and the locoregional lymph nodes. IL-17 suppressed apoptosis of tumor cells in vitro, and knockdown of the IL-17 receptor in 4T1 mouse mammary cancer cells enhanced apoptosis and decreased tumor growth in vivo. Thus, in addition to suppressing immune surveillance, tumor-induced TGF β may actively subvert the CD8⁺ arm of the immune system into directly promoting tumor growth by an IL-17-dependent mechanism. In summary, elevated TGF β expression in the tumor microenvironment modulates a complex web of intercellular interactions that increase the number of metastases. In addition, an IL-17-dependent mechanism can increase tumor size. TGF β antibodies reversed these effects, and the absence of major toxicity of TGF β antagonism in any one cell compartment and the avoidance of autoimmune complications is reassuring for the further development of these agents for clinical use [188-190].

Effects of TGF β pathway antagonists on tumor angiogenesis—Several studies have reported that treatment with TGF β pathway antagonists inhibit tumor angiogenesis. Thus, Ge et al. [83] found that tumor angiogenesis, as reflected by CD34-positive microvessel density, was significantly reduced in the R3T murine mammary tumors in animals treated with the T β R-I kinase inhibitor, SD-208 compared to control animals, while tumor cell proliferation (Ki-67 staining), or apoptosis (TUNEL) were unaffected. Similarly, Nam et al. [190] found that treatment of 4T1 murine mammary tumors with 1D11 was associated with a statistically significant decrease in microvessel density in the primary tumor, although not in lung metastases. Consistent with these findings, treatment of 4T1 tumor bearing mice with the 2G7 anti-TGF β neutralizing antibody significantly reduced circulating VEGF levels [112] (Genentech, US Patent Application 2005/0276802 A1). These findings indicate that, besides enhancing anti-tumor immunity, TGF β pathway antagonists also have modest anti-angiogenic properties in vivo.

In summary, the evidence to date indicates that TGF β antagonists exert their therapeutic effects through at least three different mechanisms, including enhancement of anti-tumor immunity, inhibition of angiogenesis, and reversal of the invasive/metastatic phenotype of the tumor cells themselves. Nam et al. [190] were able to estimate the relative contributions of cell autonomous versus host cell effects of the pan-TGF β neutralizing antibody, 1D11, by comparing its efficacy against wild type 4T1 murine mammary cancers with that against 4T1 tumors expressing a dominant-negative T β R-II receptor. Combining data from multiple experiments conducted in syngeneic mice, they estimated that 75% of the antibody efficacy was dependent on the activity of CD8⁺ cytotoxic T-cells, whereas 35% was dependent on the presence of NK cells, and ~60% required an intact TGF- β response in the tumor cell itself. Because the sum of these effects was >100%, the data suggest that treatment with 1D11 enhances antitumor immune responses by a cooperative mechanism that involves several different cellular compartments, including the CD8⁺ T cells, NK cells, and the tumor cells themselves.

Potential risks of inhibiting the TGF β signaling pathway

Newborn mice genetically deficient in TGF β 1 or T cell TGF β receptors display profound immune dysregulation, as evidenced by increased lymph node size, expression of markers of

memory/activation on T cells, inflammation in a variety of tissues and the development of autoantibodies [220,221]. Thus, one major potential concern of long-term TGF β pathway neutralization is the development of severe immunopathology. To address this concern, Ruzek et al. [222] examined the effects of daily administration of up to 2.5 mg/kg of anti-TGF β (1D11) to adult mice for 3 weeks. Comprehensive hematological and histopathological evaluation showed no evidence of pathology. In a second study, the antibody treatment period was extended to 12 weeks, and mice were treated with 50 mg/kg of anti-TGF β (1D11) three times per week. Many parameters of immune status were assessed, including natural killer (NK) cell activity, lymphocyte proliferative responses, phagocytic activity, phenotypic assessment of leukocyte subsets, and serum measurements of pro-inflammatory cytokines, auto-antibodies and immunoglobulin isotypes. In addition, histopathological assessment of heart, lungs, liver, kidney, salivary glands, skin, spleen and lymph nodes was also performed. Very few of the multiple immune parameters examined showed detectable changes in anti-TGF β -treated mice. Changes that were observed were primarily restricted to the spleen and included increased spleen cell recoveries, increased percentages of macrophages, decreased percentages of NK cells, decreased phagocytic activity, decreased proliferative responses to mitogens and slight increases in activated T and B cells. Many of these same parameters examined in the lymph nodes were not altered by the anti-TGF β treatment. The thymus was decreased in size, but altered only slightly in one population of developing T cells. Most of the changes observed were modest and returned to control levels following discontinuation of treatment. The only serological finding was a selective increase in IgA levels in anti-TGF β -treated mice. Finally, there was no evidence of increased inflammation in any of the peripheral tissues examined. In conclusion, even though modest changes in some of the immunological parameters were observed, these were few and typically reversible following discontinuation of treatment. Moreover, these changes were minor compared to those found in genetically engineered mouse strains deficient in TGF β 1 or with TGF β unresponsive T or B cells [220, 221]. Thus, long-term antibody-mediated neutralization of TGF β in normal mice was not associated with any significant immune dysregulation.

A second concern of chronic administration of a TGF β pathway antagonist is the inhibition of TGF β 's tumor suppressive function and the increased risk of secondary malignancies. In this regard, it should be noted that prolonged administration of 1D11 to *Apc*^{min} mice, a model of familial adenomatous polyposis, was associated with an increased frequency of transition from adenoma to invasive adenocarcinoma, quite reminiscent of the phenotype of *Apc*^{min}/*Smad4* compound heterozygote mice [223,224]. Other than this, there have been no reports suggesting that prolonged administration of or exposure to a TGF β pathway antagonist is associated with an increased frequency of spontaneous invasive tumor development. In fact, it is reassuring that, in transgenic mice that constitutively express a soluble T β R-II receptor:Fc fusion protein (Fc:T β RII) in the mammary gland, primary tumorigenesis was not enhanced in spite of lifetime exposure of mammary glands to the soluble TGF β antagonist [45]. Nevertheless, this possibility will have to be carefully monitored in ongoing and future clinical trials of these agents.

Alterations in TGF β signaling have been implicated in a number of inherited or acquired cardiovascular diseases [225,226]. Hence, a third concern is that chronic treatment with TGF β antagonists might be associated with cardiovascular toxicity. To address this question, Marathe et al. [227] treated Sprague-Dawley rats for 14 days with the chemical TGF β receptor kinase inhibitor, LY2109761. Valvular lesions characterized by leaflet thickening, increased numbers of interstitial cells, and accumulation of myxoid stroma were observed in animals treated with LY2109761, but were not seen in rats receiving vehicle only. Interstitial cells in LY2109761-treated valves had increased α -SMA immunoreactivity and prominent nuclear expression of phosphorylated Smad2. These preliminary findings suggest that administration of LY2109761 may cause paradoxical myofibroblast activation of TGF β signaling in heart

valves of Sprague-Dawley rats. The observed phenotype, both in terms of the valvulopathy and the activation of pSmads was strikingly similar to that seen in animal models of Marfan syndrome as well as humans with Marfan-like syndromes due to either fibrillin or *TGFBR* gene mutations [35,228]. In addition, the valvulopathy seen in LY2109761 treated rats was also reminiscent of that seen in *Tgfb2* and -3 double knockout mice, which is associated with hyperphosphorylation of BMP Smads 1 and -5 [229]. Thus, patients who will be treated with TGF β pathway antagonists, particularly receptor kinase inhibitors, will have to be carefully monitored for this unusual type of cardiovascular toxicity.

In summary, chronic neutralization of TGF β does not appear to significantly affect its homeostatic functions in epithelial, immune or vascular compartments. Toxicity appears to become manifest only in the context of any type of tissue remodeling, tissue injury, pathogen exposure, or on a background of genetic cancer predisposition. In contrast, continuous administration of chemical TGF β receptor kinase inhibitors does appear to affect vascular integrity, perhaps by compensatory upregulation of TGF β ligands. For this reason, the ongoing phase I clinical trial of LY2157299 has been amended to employ an intermittent rather than a continuous dosing schedule.

Clinical trials of TGF β pathway targeting agents

Summary of phase I clinical trials

The following three clinical TGF β pathway antagonists are currently in early phases of clinical development (Table 1).

AP 12009 (Antisense Pharma GmbH)—AP 12009 is an antisense oligodeoxynucleotide specifically directed against TGF β 2 mRNA, resulting in reduced production of the TGF β 2 isoform. AP 12009 was initially administered by intra-tumor infusion in patients with high-grade malignant gliomas with encouraging results [197]. Preliminary results of an ongoing phase I/II study using systemic i.v. administration of AP 12009 for patients with stage IV pancreatic carcinoma, malignant melanoma, or colorectal carcinoma were recently reported [230]. In this ongoing open-label, multicenter phase I/II study (AP 12009-P001), patients are receiving AP 12009 intravenously according to a cohort dose escalation design. To date, 17 patients in four cohorts have been treated with AP 12009 administered as a continuous infusion for 7 days every other week. Dose limiting toxicities (thrombocytopenia, rash) were observed at a dose of 240 mg/m²/day. One patient with stage IV pancreatic carcinoma who received 80 mg/m²/day experienced a complete regression of liver metastases and is alive at 128 weeks. Because progression-free survival of patients who received 80 mg/m²/day was better than in any other cohort, this dose will be used in phase II trials of the 7-day every other week treatment schedule. Meanwhile, a further dose escalation using a modified treatment schedule of 4 days of AP 12009 administration, followed by a treatment-free interval of 10 days for each treatment cycle, is ongoing.

GC1008 (Genzyme, Inc.)—GC1008 is a human IgG4 kappa monoclonal antibody capable of neutralizing all mammalian isoforms of TGF β (i.e., 1, 2, and 3). GC1008 is a high-affinity antibody with Kds of 1.8, 2.8, and 1.4 nM for TGF β 1, 2, and 3, respectively. A single-dose phase I study of GC1008 for patients with idiopathic pulmonary fibrosis is currently open to enrollment. In addition, Morris et al. [231] recently reported results of an ongoing, first in cancer, multi-center trial examining the safety and effectiveness of GC1008. Patients with advanced melanoma or renal cell carcinoma, who had failed ≥ 1 prior therapy, were treated at one of six dose-cohort levels (0.1, 0.3, 1, 3, 10, or 15 mg/kg) using a 3 + 3 design. Patients who did not experience any dose-limiting toxicity within 28 days of the first treatment received three additional doses given 2 weeks apart. The patients who achieved stable disease, partial response, or a complete response were eligible for extended treatment with GC1008. Twenty-

two patients (21 with melanoma, one with renal cell carcinoma) have been treated, including 6 patients at 15 mg/kg. No dose-limiting toxicities were observed and 15 mg/kg was determined to be the maximal safe dose. Adverse events possibly related to GC1008 included skin rash (Grade 1 in 2 patients, Grade 2 in 1 patient, grade 3 in 2 patients), fatigue (Grade 1), headache (Grade 1, 2), epistaxis, gingival bleeding (Grade 1), and gastrointestinal symptoms (Grade 1, 2). The only serious adverse event considered possibly related to treatment is a well-differentiated squamous cell cancer of the skin in a patient with a prior history of this cancer. One patients with melanoma skin metastases achieved a partial response lasting >52 weeks on extended treatment. Four patients with malignant melanoma achieved stable disease (ranging from 22 to 31 weeks) and also received extended treatment. Three of these patients had mixed responses with improvement of some of their liver or lung metastases. A phase II expansion study for patients with metastatic melanoma has been initiated, and phase II studies for patients with stage IV breast cancer are planned.

LY2157299 (Eli Lilly & Co.)—A multi-center phase I trial of the first clinical selective chemical TGF β type I receptor kinase inhibitor, LY2157299 (Eli Lilly & Co.) is currently ongoing. Calvo-Aller et al. [232] recently reported the clinical experience on the first seven patients with advanced/metastatic malignancies who were treated on this study. Cohorts of three patients each were used to determine the safety and pharmacokinetics at daily doses of 40 and 80 mg. The first seven patients (three and four patients at the 40 and 80 mg dose, respectively) enrolled on this study included three with colon cancer, and one patient each with prostate cancer, adrenocortical carcinoma, breast cancer and malignant melanoma. LY2157299 was well tolerated at both dose levels and no drug-related grade three or four toxicities were observed. The pharmacokinetic and pharmacodynamic profiles of LY2157299 were determined using the pharmacodynamic assay validated in a phase 0 study by Baselga et al. [141] and found to be consistent with the preclinical-based PK/PD modeling described by Farrington et al. [233]. Analysis of the two cohorts showed that absorption of LY2157299 was rapid, and the AUC proportional to the drug dose. The mean $t_{1/2}$ was estimated to be 6.5 h, and no definitive accumulation of LY2157299 was observed over the 56-day twice-daily dosing period. In summary, daily oral administration of LY2157299 was safe and well tolerated at the two dose levels and the pharmacokinetic profile was consistent with the prediction derived from the preclinical PK/PD model [141,233]. Because of concerns raised in animal studies regarding the possibility of serious valvulopathy with continuous dosing (see section “Potential risks of inhibiting the TGF β signaling pathway”), the remainder of this phase I trial is being conducted using an intermittent LY2157299 dosing schedule. Moreover, eligibility has been restricted to patients with high-grade malignant gliomas. This study is expected to be completed by the end of 2008.

Key questions that need to be addressed in future clinical trials of TGF β pathway antagonists

Pharmacodynamic measures of TGF β pathway inhibition in vivo—Assessing the pharmacodynamic properties of a potential targeted therapeutic agent through the use of a downstream biomarker is essential. Ideally, one would like to assay tumor tissue directly but limited accessibility and invasiveness of the sampling procedure poses major challenges to achieve this in clinical practice. For trials of chemical TGF β receptor kinase inhibitors, Farrington et al. [233] developed an assay that makes use of ex vivo TGF β 1 stimulation of PBMCs coupled with the measurement of phosphorylated Smad2 using a sandwich ELISA. This assay has been validated in a phase 0 clinical study and is being used to gain insight into TGF β signal modulation by both large and small molecules in ongoing clinical trials [141].

For clinical trials of ligand traps, such as the GC1008 pan-TGF β neutralizing antibody, the use of radiolabeled antibody itself as detector of tumor associated target protein and measure of target inactivation are being considered. Recent (pre)clinical studies with ^{124}I - and ^{89}Zr -labeled

anti-VEGF monoclonal antibodies, such as bevacizumab, have provided proof-of-concept that this type of reagent can be used to detect a secreted protein target, such as VEGF, in the tumor microenvironment with a high degree of accuracy and specificity using immuno-positron emission tomography (PET), especially when combined with CT or MRI anatomic imaging [234–236]. Clinical trials are ongoing to determine whether uptake of tracer doses of ^{89}Zr -bevacizumab in human tumors can serve as a pharmacodynamic marker of drug action, can predict for response to bevacizumab therapy, and can be used as an early biomarker of response to therapy. Given the remarkable parallels between VEGF and TGF β as therapeutic targets, one might envision the development of ^{89}Zr -GC1008 for immuno-PET/CT imaging to address these same key questions.

Selection of patient population-predictors of clinical response—Given tumor heterogeneity (TGF β -dependent and -independent), an unselected breast cancer patient population is likely to have a low rate of response to TGF β antagonists. Therefore, it is imperative that tools be developed to identify tumors that are driven by active TGF β signaling (pathway addiction) as well as accurate predictive biomarkers of clinical response to TGF β pathway antagonists. In principle, one could take one of two approaches to address these critical issues: One option is to build a predictor based on cell line- or animal data, and then validate this “biomarker” prospectively in a clinical trial. However, because of the inherent difficulties in simulating the behavior of human tumors using experimental animal model systems, especially for a multi-functional target such as TGF β , and the limited number of available animal models, this may not be the most attractive option. Moreover, in a prospective validation trial in a non-selected patient population, the overall clinical response rate will still be very low, even if the predictor turns out to be valid. An alternative, perhaps more attractive approach, is to use one's “best guess” based on *all* available experimental and clinical data to define a patient population likely to be *enriched* for responders, and use the trial itself to develop a more accurate predictor. Given the remarkable consistency across clinical, biological and experimental data reviewed above, this may be the most efficient approach for further development of these agents for the treatment of breast cancer. As phase II trials of GC1008 and LY2157299 are being considered for patients with metastatic breast cancer, it is important to stress the fact that the clinical and experimental data summarized in sections “Alterations of TGF β signaling in breast cancer-loss of homeostasis and activation of EMT” and “TGF β and human breast cancer” suggest that ER-negative (either basal-like or HER2-positive) breast cancers appear to be driven by TGF β and, therefore, more likely to benefit than estrogen-dependent luminal cancers. In addition, it is possible that ER-positive tumors with intrinsic or acquired anti-estrogen resistance (estrogen-independent) acquire a phenotype that is similar to that of the basal or HER2-positive subtypes. The clinical studies reviewed in section “TGF β and human breast cancer” suggest that subsets of basal-like, HER2-positive and luminal breast cancers are characterized by a high TGF β response gene expression signature. Preclinical genetic and pharmacological studies suggest that, at least in mouse models of metastatic basal-like or HER2-positive mammary cancer, the TGF β pathway plays a key role in maintaining or enriching the stem cell pool and in establishing metastases at secondary sites (section “Alterations of TGF β signaling in breast cancer-loss of homeostasis and activation of EMT”). Finally, while the therapeutic effects of anti-estrogens in ER-positive breast cancer appear to be mediated, at least in part, by TGF β , clinical resistance to anti-estrogens as well as cytotoxic chemotherapy may be overcome by TGF β neutralization. Thus, based on this collective evidence, it would appear most prudent to primarily focus phase II trials of TGF β pathway antagonists on patients with stage IV ER-negative basal-like and HER2-positive breast cancer. In addition, one might consider including patients with ER-positive but estrogen-independent (i.e., anti-estrogen resistant) disease, while patients with estrogen-dependent breast cancer should be specifically excluded because of the potential antagonism between anti-estrogens and TGF β antagonists. Most importantly, all clinical trials need to incorporate validation

studies of putative predictive biomarkers, such as, e.g., *TGFBR2* status and the TBR5 tumor gene expression signature in BA1 versus BA2 and in HER2(NI) versus—I subsets, pSmad2 expression, or ⁸⁹Zr-GC1008 immuno-PET/CT imaging.

Phase II trial design-single agents versus combination studies—Even though some preclinical studies support the idea that TGFβ pathway antagonists might potentiate the efficacy of HER2 or other growth factor antagonists, anti-angiogenic therapy, vaccine- and other immunotherapies, and, perhaps, overcome the resistance to cytotoxic chemotherapy and/or anti-estrogens, these combination therapy approaches need to undergo more extensive validation before subjected to clinical testing in humans. Having said this, the collective preclinical experience using TGFβ antagonists in combination with cytotoxic chemotherapy in mammary cancer models is probably sufficiently mature to warrant including a cytotoxic agent in phase II trials of TGFβ inhibitors.

Mechanisms of action of TGFβ pathway antagonists in vivo—Just as important in utilizing phase II trials to validate putative predictive biomarkers is to include studies that address the potential mechanisms of action of these new agents in man. As described in section “Preclinical therapeutic studies of TGFβ antagonists against mammary cancer in vivo,” animal studies suggest that TGFβ pathway inhibitors exert their anti-tumor effects by multiple mechanisms, including cell autonomous effects, as well as potent immune stimulatory and anti-angiogenic actions. Determining which of these is operative in humans and to which extent, is essential to inform the design of future combination therapy trials. Phase II studies should include, at the very least, serial measures of tumor-specific immunity, angiogenesis, and tumor cell phenotype.

Summary and conclusions

The vast majority of human breast cancers express all of the canonical TGFβ signaling pathway components albeit often at lower than normal levels, while complete inactivation of the signaling pathway by deletion or mutation of TGFβ receptor- or Smad genes are rarely seen in breast cancer. On the other hand, overexpression of TGFβs is associated with late stage disease and/or poor outcome. This constellation of events, i.e., lowering of TGFβ receptor- or Smad gene expression combined with increased levels of TGFβs in the tumor microenvironment, is sufficient to abrogate TGFβ's tumor suppressive effects and activating it's ability to induce a mesenchymal, motile and invasive phenotype. Genetic mouse models in which TGFβ signaling is either attenuated or constitutively activated support the notion that TGFβ signaling suppresses de novo mammary cancer formation but promotes metastasis of tumors that do form once they have broken through TGFβ's tumor suppressive barrier. In addition, this pro-metastatic activity of TGFβ is synergistic with activation of the *Ha-ras* as well as HER2/neu pathways. Treatment of mice with TGFβ neutralizing antibodies shortly following inoculation of mammary tumor cells strongly inhibits development of either lung- or bone metastases in syngeneic models, and modestly in human MDA-MB-231 nude mouse xenograft models, but either no or modest primary tumor growth retardation in syngeneic models, and no effect or even modest growth stimulation in xenograft models. Chemical type I TGFβ receptor (TβR-I) kinase inhibitors exert quantitatively similar effects on mammary cancer metastases and also appear to be able to slow growth of human breast cancer xenografts. In terms of mechanisms of action, these TGFβ targeted agents do not significantly affect tumor cell proliferation or apoptosis. Rather, they appear to act by at least three complementary mechanisms, including derepression of anti-tumor immunity, inhibition of angiogenesis and cell autonomous reversal of the mesenchymal, motile, invasive phenotype characteristic of basal-like and HER2-positive breast cancer cells.

Because tumor-associated activation of TGF β signaling comes into play relatively late in tumor development, and affects primarily the tumor cell's ability to invade and establish metastases, preclinical studies necessarily have had to rely on mouse models of breast cancer that recapitulate part or all of the metastatic cascade. Only a limited number of breast cancer models fulfill these criteria, and few of these are estrogen-dependent. In fact, almost all evidence of preclinical efficacy of TGF β antagonists was obtained using "triple-negative" or basal-like breast cancer models. A robust and independently validated signature of TGF β upregulated genes is clearly associated with poor prognosis subsets of basal-like- and HER2-positive breast cancers, as well as with a good prognosis subset of luminal breast cancers. These findings suggest that the presence of a TGF β response signature might drive tumor progression in estrogen-independent cancer, but may reflect a suppressive host cell response in estrogen-dependent luminal cancers. Consistent with this idea is the mounting experimental evidence that TGF β plays a key role in maintaining the mammary epithelial stem cell pool, both in normal and malignant breast tissue, in part by inducing epithelial-to-mesenchymal transitions, while differentiated, estrogen receptor-positive, luminal cells are unresponsive to TGF β by virtue of epigenetic silencing of the *TGFBR2* receptor gene. This same cell population responds to estrogen by downregulating TGF β , while the anti-proliferative and proapoptotic actions of anti-estrogens appear to be mediated by TGF β . Based on this proposed model, we predict that inhibiting TGF β signaling in mammary stem cells should drive the differentiation into luminal or basal ductal cells. Consequently, treatment of basal-like or HER2-positive cancers with TGF β antagonists may have anti-tumor effects by converting the cells into a more epithelioid, non-proliferating (and, perhaps, non-metastatic) phenotype (mesenchymal-to-epithelial transition, MET). Conversely, these agents might antagonize the therapeutic effects of anti-estrogens in estrogen-dependent luminal cancers. These predictions need to be addressed prospectively in clinical trials, but should inform the selection of patient populations most likely to benefit from this novel anti-metastatic therapeutic approach.

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Abbreviations

4-OH-T, 4-Hydroxy-tamoxifen
 ATM, Ataxia teleangiectasia mutated protein
 CIC, Cancer initiating cells
 DC, Dendritic cells
 DMBA, 7,12-Dimethylbenz[α]anthracene
 DNT β RII, Dominant negative TGF β type II receptor
 EMT, Epithelial to mesenchymal transition
 ER, Estrogen receptor
 EST, Expressed sequence tag
 Fc:T β RII Fc-soluble TGF β type II receptor fusion protein
 HMEC, Human mammary epithelial cells
 IHC, Immunohistochemistry
 MMTV, Mouse mammary tumor virus
 NK, Natural killer cells
 PBMC, Peripheral blood mononuclear cells
 PR, Progesterone receptor
 PyVmT, Polyoma virus middle T antigen
 TBRS, TGF β response gene signature
 TGFBR1, TGF β type I receptor gene

TGFBR2, TGF β type II receptor gene
 TGF α , Transforming growth factor- α
 TGF β , Transforming growth factor- β
 TPA, 12-Tetradecanoyl-phorbol-13-acetate
 T β R, TGF β receptor
 WAP, Whey acidic protein

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Fig. 1.

A The TGFβ/Smad signaling pathway is highly conserved from fruitfly (left) to man (right). Currently available TGFβ pathway antagonists are shown in red next to their molecular targets. AP-12009, GC-1008 and LY2157299 are currently undergoing early phase clinical trials. Preclinical studies of TGFβ antagonists in cancer have recently been reviewed in [127,187, 237-239]. These studies have provided convincing evidence that targeting the TGFβ pathway can inhibit tumor growth and metastasis in at least some cancer models. These findings demonstrate that the neoplastic phenotype can be critically dependent on TGFβ signaling, so-called pathway addiction. Besides cancer, preclinical studies of chemical TGFβ receptor antagonists have demonstrated therapeutic activity in animal models of idiopathic pulmonary

fibrosis [240], liver disease [241,242], renal fibrosis [243] and Marfan syndrome [244]. **B** TGF β superfamily of ligands and associated signaling receptors. The two major subgroups of ligands activate either the Smad2/3 or the Smad1/5 pathways that control two largely non-overlapping genetic programs

Fig. 2.

Unifying model of TGFβ signaling in breast epithelium. Implications for cancer treatment using TGFβ pathway antagonists. The model assumes the existence of a primitive multipotent stem cell population that is ER-negative, PR-negative, *CD44⁺*, *CD24^{lo}*, *ESA⁺*, *KRT5⁺*, *TGFBR2⁺* and possibly express mesenchymal markers. When these cells divide, they give rise either to phenotypically identical daughter stem cells or to precursors of differentiated progeny (luminal, basal, or myoepithelial). Luminal cells are epithelioid (*ESA⁺*, *KRT8/18⁺*, *CDH1⁺*), express ER and PR, are *CD44⁺*, *CD24⁺*, and the *TGFBR2* gene is transcriptionally silent (*TGFBR2⁻*). The basal and myoepithelial progeny express basal cytokeratins (*KRT5/6/14⁺*) or myoepithelial markers (*αSMA*, *p63*), lack *ESA* (*ESA⁻*), and appear to be enriched for *CD44⁺*, *CD24^{lo}* cells. Whether these cells do or do not continue to express *TGFBR2* is unclear. One of the fundamental determinants of the proportions of these different subpopulations in normal or cancer tissue is the ratio of stem cell divisions that give rise to daughter stem cells versus differentiated progeny [174]. We propose that exposure to TGFβ leads to enrichment of the cell population with multipotent *CD44⁺*, *CD24^{lo}* clonogenic/tumor initiating cells that express mesenchymal markers (EMT) [60], while treatment with a TGFβ receptor kinase inhibitor leads to enrichment with *CD44⁺*, *CD24⁺*, *TGFBR2⁻* nonclonogenic differentiated luminal cells [78]. Thus, we would like to propose the idea that TGFβ signaling is a key determinant of the fate of stem cell progeny, and responsible for maintenance of the stem cell pool in the normal mammary gland. Moreover, this model suggests the hypothesis that the constitutive activation of TGFβ signaling associated with invasive and metastatic breast cancer might contribute to steadily enriching the cancer stem cell pool, thereby indirectly enhancing the potential for

metastasis and poor patient survival. In this context, it is noteworthy that EMT of normal as well as malignant human mammary epithelial cells appears to be tightly associated with the stem cell state [60]. Superimposed on to these population dynamics at the tissue level is the role of systemic estrogens and progestins in regulating TGF β signaling (see section “Extensive cross-talk between estrogen- and TGF β signaling pathways”). The ER/PR-positive luminal cells respond to estrogen by producing growth factors and lowering TGF β production. These conditions would favor the commitment of stem cell daughter cells to become more differentiated progenitor cells. Moreover, because the *TGFBR2* gene is transcriptionally silenced in these luminal daughter cells, this would further favor their clonal expansion. Conversely, anti-estrogens induce TGF β production, which, even if it inhibits the rate of stem cell proliferation, would favor their self-renewal over commitment to differentiation. Once cancer stem cells break through the barrier of TGF β -mediated growth arrest, the presence of active TGF β would select for continued expansion of the cancer stem cell pool, and, consequently, metastasis. This model predicts that treatment of basal-like cancers with TGF β antagonists may have anti-tumor effects by converting the putative stem cell population into a luminal, non-proliferating (and, perhaps, non-metastatic), phenotype (mesenchymal-to-epithelial transition, MET). Conversely, these agents might antagonize the therapeutic effects of anti-estrogens in estrogen-dependent luminal cancers. However, once these cells become estrogen-independent because they escape from TGF β -mediated growth arrest, our model provides a rationale for including TGF β pathway antagonists in the treatment program. Each of these predictions is testable in model systems as well as in clinical trials

Fig. 3.

Association between TGF β pathway gene expression and TGF β target gene response signature (TBRS) and human breast cancer subsets. **A** We first posed the question whether or not expression of genes involved in the canonical TGF β signaling pathway were associated with particular breast cancer subsets as defined by Alexe et al. [179]. Gene expression profiles from 200 breast cancer specimens described by Wang et al. [146] and from 47 breast cancer specimens reported by Richardson et al. [245] were examined. The samples in these datasets were first classified into the six subtypes of breast cancer identified based on a nearest centroid approach as described by Alexe et al. [179]. Expression data were preprocessed and normalized as previously described (MAS 5.0 normalization followed by max median compression of

probeset IDs to unigene symbols, followed by standard normalization of genes across samples; integration of the two standard normalized datasets was performed by applying the Distance Weighted Discrimination (DWD) approach [179]. The identification of differential expression of TGF β -pathway genes in breast cancer subtypes versus normal samples was performed based on a non-parametric (Wilcoxon) *t*-test with multiple re-sampling to correct for biased distribution sizes. Boxplots of gene expression intensities across breast cancer subtypes and normal samples are shown. *TGFB1* was significantly up-regulated in the HER2 + (I) and LA subsets ($P < 0.01$), the BA1 subset ($P = 0.03$) and the HER2 + (NI) ($P = 0.04$). *TGFB2* was not significantly up- or down regulated in any of the subtypes. *TGFB3* was significantly down-regulated in the basal subtypes, but not significantly up-regulated in any other subtype. *TGFBR1* (ALK5) was up-regulated in BA1 ($P = 0.04$), both HER2 + subclusters (HER2 + (I) $P = 0.03$; HER2 + (NI) $P = 0.013$). *TGFBR2* was significantly down-regulated in each breast cancer subtype, most notably in the LA, BA2, and HER2 + (NI) subclusters, which are most strongly associated with the TBRS (see **B**). **B** We then posed the question whether the 153-gene TBRS described by Padua et al. [147] (TBRS^{MSKCC}) as well as a similar 92-gene signature developed in our own laboratory [67] (TBRS^{CINJ}) were associated with any particular breast cancer subsets, as defined by Alexe et al. [179], using Gene Set Enrichment Analysis (GSEA) [246,247]. Given a list of genes, ranked by the correlation of their genome-wide expression profiles with one of several phenotypes, GSEA seeks to estimate the significance of the over-representation of an independently defined set of genes, *S*, in the highly correlated or anti-correlated genes in the list. To evaluate this degree of 'enrichment' the GSEA method calculates an Enrichment Score (ES) by walking down the list, increasing a cumulative sum when a gene is in *S* and decreasing it if a gene is not in *S*. The size of the increment depends on the gene/phenotype correlation. The ES is the maximum deviation from zero of the cumulative sum and can be interpreted as a weighted Kolmogorov-Smirnov statistic. The genes in the gene set *S* that appear in the ranked list before the point where the running sum achieves the ES are called the leading-edge subset and are particularly important in evaluating the results of GSEA analysis. The significance of a gene set's ES is estimated by an empirical phenotype-based permutation test procedure. When an entire database of gene sets is scored, an adjustment must be made to the resulting *P*-values to account for multiple hypotheses testing. GSEA normalizes the ES for each gene set to account for the variation in set sizes, yielding a normalized enrichment score (NES), and calculates a false discovery rate (FDR) corresponding to each NES. The FDR gives an estimate of the probability that a set with a given NES represents a false positive finding; it is computed by comparing the tails of the observed and permutation-computed null distributions for the NES. For each tumor, a number between -1 and +1 was derived, indicating the likelihood that TGF β signaling is active in that tumor. Each bar represents an individual tumor specimen. Three different breast cancer expression profile data sets were analyzed. These included the GSE_2034 data of 286 specimens described by Wang et al. [146], the GSE_4992 data of 249 specimens described by Ivshina et al. [181] and the GSE_7390 data on 165 specimens reported by Desmedt et al. [180]. Based on the GSEA scores, the TBRS was significantly associated with the poorprognosis BA2 and HER2(NI) subclusters, as well as with the good prognosis LA subset. Moreover, results obtained using the TBRS^{CINJ} signature [67] closely matched those obtained with the TBRS^{MSKCC} [147] (Correlation coefficient ~0.80). **C** The relationships between TBRS^{MSKCC} and overall survival were explored on patients with lymph node negative disease, who did not receive adjuvant systemic therapy. Cases from Wang et al. [147] and Desmedt et al. [180] were pooled for this analysis. *Red lines*: TBRS^{MSKCC}-negative; *Blue lines*: TBRS^{MSKCC}-positive; *Grey lines*: Combined groups. The expression of a TBRS was associated with poor survival. However, in subset analysis, TBRS was not associated with a difference in overall survival in the luminal A subset, although there was trend towards a negative effect in the luminal B subset. Similar trends were found between TBRS expression and survival in both two basal-like cancer subsets. Patients with a HER2(I) cancer had a significantly better outcome than those with a HER2(NI)

cancer. This result validates the results previously reported by Alexe et al. [179]. However, among the HER2(NI) cases, the expression of a TBRS predicted for extremely poor survival, while survival of patients whose tumors did not express the TBRS was essentially indistinguishable from those with HER(I) disease. These results suggest that activation of the TGF β pathway largely accounts for the poor prognosis of patient with HER2(NI) breast cancer, and that this particular subset may benefit the most from treatment with TGF β pathway antagonists

Table 1

TGFβ pathway antagonists in clinical development

Agent	Class	Type	Molecular target(s)	Clinical trials	Clinical indication	Ref.
AP 12009	Ligand trap	Antisense oligonucleotide	TGFβ2	Phase II	Pancreas Colorectal Melanoma	[196,198,199]
Belagenpumatucel-L	Ligand trap	TGF-β2 antisense-modified tumor cell vaccine	TGFβ2	Phase II	Non-small cell lung cancer	[248,249]
CAT-152 (lerdelimumab)	Ligand trap	Humanized monoclonal antibody (IgG4)	TGFβ2	Phase III	Glaucoma	[250,251]
CAT-192 (metelimumab)	Ligand trap	Humanized monoclonal antibody (IgG4)	TGFβ1	Phase I-II	Systemic sclerosis	[252]
GC1008	Ligand trap	Human monoclonal antibody (IgG4)	TGFβ1, -2, -3	Phase I-II	Melanoma	[231]
LY2157299	Receptor serine-threonine kinase inhibitor	Small molecule	TβR-I/Alk5 (TGFβ) Alk4, Alk7 (Activin)	Phase I	Grade III-IV malignant glioma	[232]

Table 2

In vivo models of metastatic mammary cancer

Cell line	Genetic background	Spontaneous metastasis	Experimental metastasis	Oncogene(s)	TGFβ signaling
R3T	129S1 mice	LNN, Lungs	Bone	PyVmT, H-ras ^{mt}	Normal
4T1	Balb/C mice	Lungs	Bone (lytic), Lungs	Unknown	Smad4 null
EMT6	Balb/C mice	NT	Lungs	Unknown	NT
MMTV/PyVmT	FVB	Lungs	NT	PyVmT	NT
MDA-MB-231	Human	No	Bone (lytic)	Unknown	TβR-II mutant
MDA-MB-435	Human	LNN, Lung	NT	Unknown	Normal
MCF10A/CAla	Human	Lungs	Lungs	H-ras ^{mt}	NT
T47D	Human	No	LNN, Bone (blastic)	Unknown	TβR-II null
ZR75-1	Human	No	Bone (blastic)	Unknown	NT
MCF7	Human	No	Bone (blastic)	Unknown	NT
MX-1	Human	NT	NT	Unknown	NT

LNN, Lymph nodes; NT, Not tested; PyVmT, Polyoma middle T antigen