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REVIEW

Roles of TGFβ in metastasis

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The TGFβ signaling pathway is conserved from flies to humans and has been shown to regulate such diverse processes as cell proliferation, differentiation, motility, adhesion, organization, and programmed cell death. Both *in vitro* and *in vivo* experiments suggest that TGFβ can utilize these varied programs to promote cancer metastasis through its effects on the tumor microenvironment, enhanced invasive properties, and inhibition of immune cell function. Recent clinical evidence demonstrating a link between TGFβ signaling and cancer progression is fostering interest in this signaling pathway as a therapeutic target. Anti-TGFβ therapies are currently being developed and tested in preclinical studies. However, targeting TGFβ carries a substantial risk as this pathway is implicated in multiple homeostatic processes and is also known to have tumor-suppressor functions. Additionally, clinical and experimental results show that TGFβ has diverse and often conflicting roles in tumor progression even within the same tumor types. The development of TGFβ inhibitors for clinical use will require a deeper understanding of TGFβ signaling, its consequences, and the contexts in which it acts.

***Keywords*:** cytostasis, tumor-suppression, EMT, breast cancer, metastasis, prognostics

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Introduction

Metastasis is the final stage in tumor progression and is thought to be responsible for up to 90% of deaths associated with solid tumors [1]. This multifaceted process consists of a series of steps whereby cancer cells enter the circulation, disseminate to distal capillary beds, enter a parenchyma by extravasation, adapt to the new host microenvironment, and eventually grow into lethal tumor colonies in distal organs. Adding to the complexity of this process, metastasis often follows characteristic organ distribution patterns that reflect inherent differences within the disseminating cells of distinct tumors [2, 3]. Breast cancer, for example, preferentially spreads to the bones, lungs, liver, and brain, whereas prostate cancer almost exclusively colonizes the bones [4]. Much has been learned about the processes that initiate and sustain general tumor growth; however, the mechanisms that enable metastasis, particularly tissue-tropic metastasis, remain largely unknown. Despite the complexity of the problem, researchers have begun to dissect many aspects of this metastatic cascade. Indeed, recent work has identified the key mediators of extravasation, microenvironment remodeling, homing, invasion, migration, and survival [3]. Interestingly, the TGFβ pathway has been implicated in many of these metastatic processes and has been shown to dramatically impact the ability of tumor cells to spread throughout the body [5-8].

Although TGFβ has been implicated in tumor progression, studies have uncovered a complicated and context-dependent picture regarding the function and utility of this cytokine. Analysis from clinical tumor samples reveals that TGFβ-mediated signaling is indeed strongly implicated in the regulation of cancer. Retrospective studies have shown that in various human tumor types, components of the TGFβ signaling pathway, namely *TGFBR2*, *TGFBR1*, and *SMAD4*, are commonly inactivated either through mutation or through allelic loss of heterozygosity (LOH) [9]. Indeed, *TGFBR2*-inactivating mutations are frequently found in cancers associated with microsatellite instability (MSI) [10]. MSI arises from defects in the mismatch repair system. The *TGFBR2* gene contains a 10-base pair poly-adenine repeat, which is prone to replication errors specifically in MSI+ cancers. Poly(A) tract *TGFBR2* mutations accumulate in a vari-

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ety of tumors, including MSI+ gastrointestinal cancers, biliary cancers, lung adenocarcinomas, gliomas, and universally in colon cancer patients with inherited mutations in mismatch repair genes. In addition to the mutations of the TGFβ receptors, the downstream transducers of the TGFβ pathway, the Smads, have also been altered in cancer tissues. Smad2 and Smad4 are located on chromosome 18q, which is often mutated or completely lost in pancreatic and colon cancers [11, 12]. For pancreatic tumors, the minimal lost region has been mapped to 18q21, which contains three candidate tumor suppressors, *DCC* (deleted in colorectal carcinomas), *SMAD4/DPC4* (deleted in pancreatic carcinomas, locus 4, which encodes Smad4), and *MADR2/JV18-1* (MAD-related gene 2, which encodes Smad2). Of these three genes, the *SMAD4* gene has been identified as a genetic target of this 18q LOH in about 50% of pancreatic tumors. These results provide evidence that many core components of the TGFβ pathway function as tumor suppressors that cancers must bypass by selective mutations.

However, the TGFβ pathway is also thought to act as a tumor promoter. Consistent with this notion, increased TGFβ1 expression by tumor cells correlated with tumor progression in non-small cell lung carcinoma (NSCLC), colorectal cancer, prostate cancer, and gastric carcinoma [13-16]. Additionally, intense TGFβ staining has also been positively correlated with metastasis in breast carcinoma, prostate cancer, and colorectal cancer. TGFβ staining is shown to be stronger in the invading local lymph node metastases compared with the paired primary tumor site in both colorectal and breast cancer [Ref. [9] and references therein].

These retrospective clinical studies highlight the dichotomous role of the TGFβ pathway in human cancers. The clinical evidence suggests that pancreatic, colon, and gastric tumors selectively eliminate the core components of the TGFβ signaling pathway, effectively shutting down all signaling. However, tumors such as breast cancer, skin cancer, and gliomas exhibit much lower levels of mutations in these core factors and may in fact derive a selective advantage from the TGFβ pathway. As seen in the clinical data, expression of TGFβ ligands has been associated with tumor progression markers. From these observations, TGFβ's role in human cancers appears as both complex and context-dependent. Depending on the tumor type and the stage of tumor progression, TGFβ may provide either potent tumor-suppressive or tumor-promoting functions. Studying the effects and consequences of TGFβ signaling will enable a deeper understanding of the significant morbidity and mortality associated with cancer, particularly in regard to metastasis.

TGFβ signaling

In humans, the TGFβ superfamily represents a diverse set of growth factors, including bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), activins, TGFβ's, nodal, and anti-mullerian hormone (AMH). Most members of this family exist in variant forms, with the TGFβ cytokine consisting of three isoforms: TGFβ1, TGFβ2, and TGFβ3. The TGFβ ligands are synthesized within the cell as dimeric pro-hormones [17]. Latent dimeric forms are secreted into the extracellular matrix, where they are cleaved by furins and other convertases to form active signaling molecules [18, 19]. Activated TGFβ cytokines can then signal by bringing together two pairs of receptor serine/threonine kinases, the type I and type II receptors, forming a heteromeric complex. The human genome encodes seven type I receptors (ALKs 1-7) and five type II receptors (ActR-IIa, ActR-IIB, BMPRII, AMHRII, and TβRII) that are paired in different combinations as receptor complexes for various members of the TGFβ family. The TGFβ1 ligand preferentially signals through the TβR-II type II receptor and the ALK5 type I receptor. In addition to these two classes of receptors, type III receptors such as betaglycan aid the TGFβ ligands to more efficiently bind to their cognate TGFβ receptors [20].

On binding the ligands, the constitutively active type II receptor comes in close contact with the type I receptor and phosphorylates a 30-amino-acid regulatory segment called the GS region, located immediately upstream of the kinase domain [21]. Phosphorylation of the type I receptor disrupts the interaction between the kinase domain and a TGFβ signaling inhibitor, FKBP12. With the release of FKBP12 from the active site, the Smads transcription factors are then able to form a complex with the receptor [22, 23]. Activated receptor complexes propagate canonical TGFβ signaling through phosphorylation of the receptor-associated Smads at their carboxy-terminal. In the absence of phosphorylation, the Smads are transcriptionally inactive.

Humans express eight Smad proteins. Five Smads act as substrates for the TGFβ-family receptors (Smad1, 2, 3, 5, and 8) and are known as the receptor-associated Smads (R-Smads). Of these, Smad2 and 3 mediate the TGFβ branch of signaling, whereas the BMP branch exclusively utilizes Smad1, 5, and 8. Smad4 is referred to as the Co-Smad and serves as a common partner for the R-Smads. Finally, Smad6 and 7 are inhibitory Smads that serve as decoys interfering with the Smad-receptor and Smad-Smad interactions. Structurally, the R-Smads and Smad4 share two homologous protein domains, MH1 and MH2. The MH1 domain is located at the amino-ter-

minal and is responsible for DNA binding. The carboxy-terminal MH2 domain mediates Smad-receptor, Smad-Smad, and Smad-transcription factor interactions [24]. These domains are separated by a less conserved linker region, which can be phosphorylated by multiple inputs and is thought to serve as an integrating center for inputs from other signaling pathways [25-27]. For example, phosphorylation of the Smad1 linker region by MAPK leads to the recruitment of the HECT-domain ubiquitin ligase Smurf1. Recruitment of this ligase results in degradation of the Smad, thereby terminating TGFβ-mediated signaling events [27].

Once phosphorylated at the carboxy-terminal, the R-Smads lose their affinity to cytoplasmic retention proteins such as SARA (Smad anchor for receptor activation), thereby exposing their nuclear import signal [28]. Through interactions with nucleoporins, the Smads are shuttled into the nucleus and associate with Smad4 to regulate expression of potentially hundreds of genes [29, 30]. As the Smad MH1 domains bind weakly to DNA, R-Smads and Smad4 must collaborate with additional DNA-binding co-factors in order to achieve high DNA affinity and selectivity for specific target genes. The Smad binding partners include the forkhead, homeobox, zinc-finger, bHLH, and AP1 family of transcription factors [24, 31]. Each of these Smad4-R-Smad-transcription factor complexes recruits co-activators, repressors, and chromatin remodeling factors to specific sequence elements in the regulatory regions of the target genes. Cells from different lineages or under distinct influences express a varying set of Smad-interacting transcription factors. The cellular context in which the TGFβ signal is occurring will therefore determine the specific genes that are induced within particular cells. This context-dependent TGFβ signaling explains how a relatively simple signal transduction pathway can elicit such a diverse set of biological responses (Figure 1) [32-34].

TGFβ tumor-suppressive functions

One of the primary functions of TGFβ is to limit epithelial proliferation and halt pre-malignant growth. However, experiments in mice reveal that TGFβ is not a universal proliferation regulator; rather TGFβ elicits its anti-proliferative effects in specific contexts. For example, tissue-specific inactivation of *TGFBR2* in mouse models rarely leads to spontaneous tumor formation with little to no pathology in mouse mammary epithelium, oral cavity esophagus, forestomach, pancreas, intestine, and skin [35-39]. Instead, TGFβ's potent anti-proliferative effects only become apparent under conditions of tissue injury or oncogenic stress. Injured skin that lacks either *SMAD3* or *TGFBR2* expression is shown to heal faster with a rapid rate of keratinocyte proliferation and migration [36, 40]. In cases of oncogenic stress, multiple examples demonstrate that deletions of *TGFBR2* and *SMAD4* strongly accelerate the malignant progression of cancerous lesions. Indeed, *TGFBR2* deletion favors carcinoma-formation in intestinal polyps initiated by *APC* inactivation or chemical mutagenesis [39, 41], mammary tumors initiated by polyoma middle T (PyMT) expression [35], and pancreatic lesions initiated by *KRAS* [37]. Conversely, the expression of a constitutively activated *TGFBR1* in the context of ErB2/HER2-driven mammary tumors results in delayed tumor formation and smaller tumor size [42, 43]. These results demonstrate that active TGFβ signal is able to constrain cancerous growths driven by distinct sets of stimuli. These findings are in line with the previously described clinical observations that *TGFBR2* and *SMAD4* mutations emerge during the adenoma to carcinoma transition [44, 45].

The cytostatic response

TGFβ's effects on tumor proliferation are largely driven by the induction of a cytostatic program. Through genome-wide analysis, a coherent picture has emerged detailing the gene responses used to organize the TGFβ cytostatic program in multiple epithelial cell lines. At the core of this mechanism are the TGFβ-mediated inductions of the CDK inhibitors p21Cip1 and p15Ink4b [46, 47]. Equally important are the TGFβ-mediated repressions of c-Myc, a transcription factor that promotes cell growth and proliferation, and ID1, 2 and 3, which are nuclear factors that prevent cell differentiation [29]. Thus, TGFβ mediates a dual effect on the cell cycle by simultaneously inhibiting the CDK functions and eliminating proliferative drivers. This mechanism has been observed not only in epithelial cells but also in hematopoietic progenitor cells, indicating that this mechanism is widely used by cells in our bodies [48].

TGFβ regulates the expression of these cell cycle regulators, *p15INK4b*, *p21CIP1*, *c-MYC*, and *ID*, through the canonical Smad-signaling pathway [29, 32, 49]. Recent research has identified many of the transcriptional complexes that mediate these specific responses. In the case of the CDK inhibitors, an activated Smad-FoxO transcriptional complex mediates *p21CIP1* induction, whereas induction of *p15INK4b* requires a Smad-FoxO-C/EBPβ transcriptional complex [32, 50]. TGFβ also mediates repression of c-MYC and ID family members through the Smad-E2F4/5-C/EBPβ and Smad-ATF3 complexes, respectively [51]. Besides stimulating proliferation, c-Myc is involved in the regulation of the CDK inhibitors. In collaboration with the zinc-finger protein

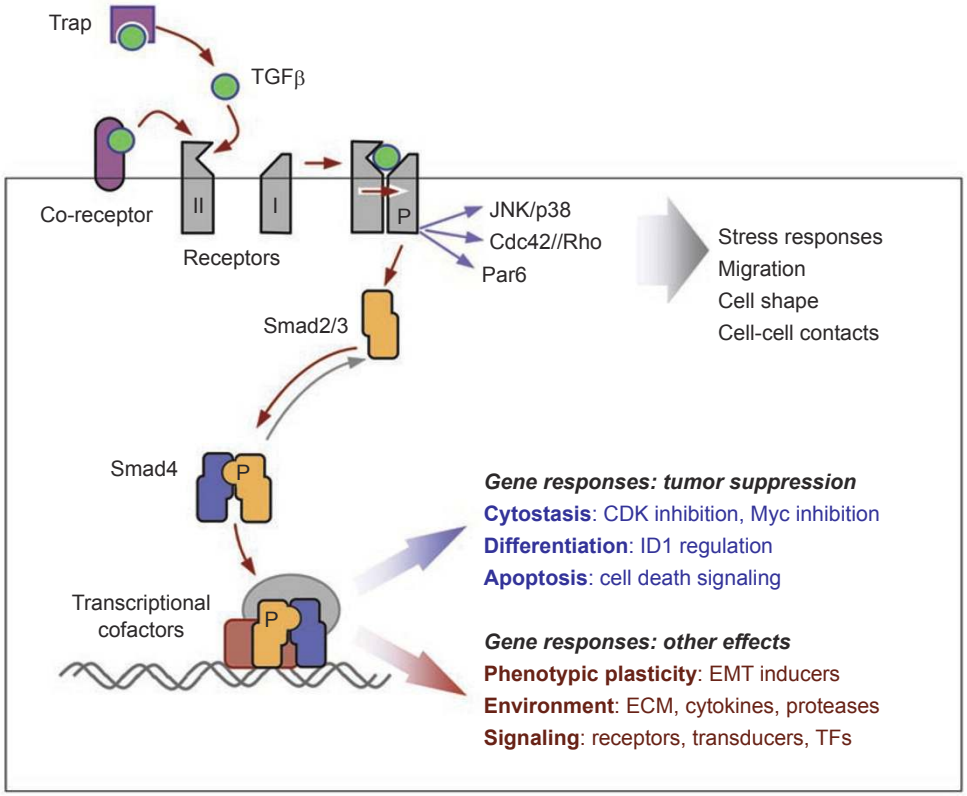

Figure 1 Signaling arms of the TGFβ pathway. The TGFβ signaling begins with the binding of the TGFβ ligand to the type I and type II TGFβ receptors at the surface of the cell. The formation of this heteromeric complex initiates a phosphorylation event wherein the type II receptor phosphorylates the type I receptor, thereby activating the complex. TGFβ can then signal through the Smad pathway or other pathways. The canonical Smad pathway is activated by receptor-mediated phosphorylation of Smad transcriptional factors. This arm of the pathway can elicit a broad range of traditional TGFβ responses such as cytostasis, apoptosis, EMT induction, and protease activation. Additionally, TGFβ can signal through JNK/p38 MAP kinases, Cdc42/Rho small G-proteins, and the cell-junctional complex regulator Par6. These mediators have been shown to elicit stress responses, enhance migration, and alter cell-cell contacts.

Miz1, c-Myc binds to the promoters of both *p21CIP1* and *p15INK4b* and interferes with their TGFβ-induced transcriptional activation. The TGFβ-mediated c-Myc downregulation relieves this interference, thereby rendering *p21CIP1* and *p15INK4b* available for activation [50, 52].

Pro-apoptotic functions

In addition to its role in regulating the cell cycle, TGFβ can limit cancer formation and maintain tissue homeostasis through its influence on apoptotic pathways. Depending on unknown cell-autonomous and environmental factors, TGFβ has been shown to paradoxically induce or suppress apoptosis [53]. Unlike the coherent mechanisms that regulate TGFβ-induced cell cycle arrest, no uniform program has been elucidated for the activation of the apoptotic program in epithelial tissues. However, there have been several Smad-dependent and -independent mechanisms described for a variety of cell lines. Examples include the expression of the death-associated protein kinase (DAPK), which is increased during TGFβ-induced apoptosis in hepatoma cells [54]. In hematopoietic cells, TGFβ-induced apoptosis relies on Smad-dependent upregulation of SHIP (SH2-domain-containing inositol-5-phosphatase) expression, which inhibits signaling by the survival protein kinase AKT [55]. Recent work has also uncovered additional components of the TGFβ cell-death network. The adaptor protein DAXX has been shown to be required for TGFβ-induced apoptosis and is thought to physically associate with TGFBR2, providing an example of a Smad-independent mechanism [56]. The various components of the TGFβ apoptotic program link the TGFβ signal to the core components of the cell-death machinery. This signal ultimately results in activation of pro-apopotic caspases as well as changes in the expression, localization and

activation of both pro- and anti-apoptotic members of the BCL2 family [57].

Evading the tumor-suppressive mechanisms

Cancer progression requires tumors to evade the body's natural defenses against unchecked cell growth. Given TGFβ's cytostatic and apoptotic functions, it is not surprising to find multiple tumors with inactivating mutations in the TGFβ pathway. As previously mentioned, large subsets of pancreatic, colorectal, gastric, and head and neck cancers decapitate the pathway by mutational inactivation of its core constituents: the receptors and the Smad transcription factors (Figure 2A). However, breast cancers, melanomas, and prostate cancers prefer to retain the core signaling aspects and instead amputate the tumor-suppressive arm of TGFβ signaling (Figure 2B). Indeed, mutations in the core components are rarely seen in these cancers. Although these mutations may occur in breast and prostate cancers, the cancer cells that retain the aspects of TGFβ signaling gain advantages that enable them to dominate the tumor.

Cancer cells can lose this tumor-suppressive arm through alterations in the Smad transcriptional complexes that mediate the cytostatic gene responses. These responses, however, must be altered in combination. Due to functional redundancies, TGFβ remains a powerful growth inhibitor in cells that lack either p15Ink4b or the c-Myc response alone [49, 58]. However, the combined loss of these two gene responses results in an effective evasion of cytostasis [59]. Using breast cancer as a model system, the mechanisms that result in deficient TGFβ cytostatic gene responses have been recently worked out. Work done in both breast cancer cell lines and clinical samples derived from breast cancer patients shows a functional core signaling pathway and proper regulation of certain canonical gene targets. However, these cells have a partial to complete loss of TGFβ-induced cytostasis. These samples fail to initiate inductions of p15INK4b or repression of c-MYC expression in response to TGFβ.

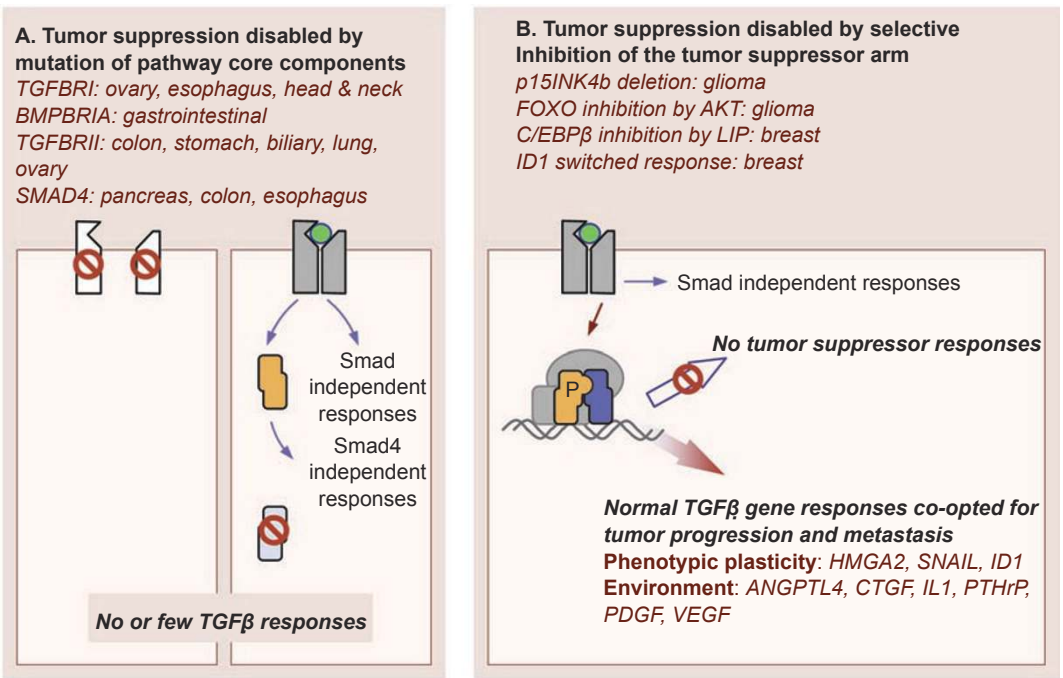

Figure 2 Evading TGFβ tumor-suppressive action. TGFβ signaling can elicit potent tumor-suppressive responses in normal and pre-malignant cells. Tumor progression requires a loss of such suppressive responses, and this is accomplished by either of two general mechanisms: **(A)** Tumors of the colon, ovaries, esophagus, and pancreas "decapitate" the pathway by mutational inactivation of its core constituents: the receptors and the Smad transcription factors. This strategy effectively eliminates most or all of TGFβ signaling and, consequently, eliminates its tumor-suppressive activity. **(B)** Alternatively, breast cancers, melanomas, and prostate cancers frequently retain functional TGFβ signal transduction components but selectively "amputate" the tumor suppressor arm downstream of these components. In so doing, these tumors can use to their advantage some of the remaining TGFβ responses, including activation of *ID1* and *ANGPTL4* in breast cancers, and of *PDGF* in gliomas.

As mentioned earlier, TGFβ regulates these genes through a Smad transcriptional complex that contains the Smad-transcription partner C/EBPβ. C/EBPβ exists as two isoforms, LIP and LAP, which function as transcriptional inhibitors and activators, respectively. In the breast cancer samples that selectively lost the cytostatic response, the predominant form of C/EBPβ was the inhibitory isoform, LIP [32]. Without the LAP co-activator, TGFβ fails to induce the key cytostatic genes and therefore cannot limit cell growth. Independent studies have established an association between the expression of the inhibitory isoform of C/EBPβ and tumor aggressiveness in breast cancer [60]. An alternative mechanism has recently been identified for gastric cancer cells. Recent work has shown that E2F1 can enhance the expression of the microRNA cluster *miR-106b-25*. Expression of this microRNA cluster inhibits *p21CIP1* and the pro-apoptotic mediator *BIM*, thus blocking both the cell cycle arrest and apoptotic responses initiated by TGFβ [61]. These results illustrate the multiple mechanisms that tumors utilize to selectively turn off the distinct arms of the TGFβ pathway. This capacity to evade the cytostatic program enables the corrupt use of the TGFβ pathway in tumor progression.

Tumor-promoting functions

Cancers that can selectively shut down the tumor-suppressive arm of the TGFβ signaling pathway are free to take advantage of its many pro-tumorigenic properties. Recent research using mouse models and cell culture systems has begun to investigate the dual nature of TGFβ's influence on tumor progression. Indeed, overexpression of TGFβ1 in the skin of mice initially yielded fewer benign skin papillomas in response to carcinogen treatment, consistent with the tumor-suppressive functions of TGFβ. However, the skin tumors that eventually emerged were found to be more locally invasive and aggressive when compared with the control mice [62]. In addition to the skin carcinogenesis models, the dual role of TGFβ signaling has been observed in various other tumor models, including metastatic colon cancer, prostate cancer, and breast cancer [53]. Recent research has made significant progress in defining the cellular and molecular events that mediate the pro-tumorigenic effects on both the microenvironment and the tumors cells. Among the various functions that TGFβ can provide, it prominently enhances cell invasion, migration, and evasion of immunity. Each of these functions plays a prominent role in the ability of TGFβ to enhance tumor progression and eventually aid in the metastatic process.

Immune suppression/evasion

One of the key components of the anti-tumor defense is the immune system. As tumors emerge, the body uses T lymphocytes and natural killer cells to recognize the rogue cancer cells and specifically eliminate them. Cancer cells that have bypassed the tumor-suppressive functions of TGFβ can take advantage of its potent immunosuppressive functions to dampen this surveillance system. In studies using transgenic mice, expression of the dominant-negative TGFBR2 in either $CD4^+$ or $CD8^+$ T cells eliminated both thymoma- and melanoma-cell line-derived tumors more effectively than non-transgenic controls. These results implicate these T lymphocyte subsets as critical targets for negative regulation by TGFβ [63]. Recent work has identified the molecular mechanisms that mediate TGFβ's anti-immune effects in $CD8^+$ T cells. Acting through the Smad pathway, TGFβ represses production of cytolytic factors, including the pore-forming protein perforin, the caspase-activating secreted factors granzyme A and B, and the pro-apoptotic cytokines Fas-ligand and IFNγ [64]. TGFβ can also impair T-cell activation by inhibiting the function of antigen-presenting cells (APCs) such as dendritic cells. During an immune response, dendritic cells mature and acquire the ability to effectively stimulate T cells. This activation process, however, is blocked by TGFβ [65]. Additional targets of TGFβ-mediated immune evasion include natural killer cells and neutrophils [66-68]. Collectively, evidence from both xenograft and transgenic models demonstrates a critical role for TGFβ in enabling cancer progression through the suppression of the host immune system.

Angiogenesis

Tumor angiogenesis is critical for the growth and dissemination of tumor cells. The recruitment of endothelial cells and vessels enables a fast-growing tumor to receive the nutrients and oxygen needed for growth. Moreover, these vessels can also serve as access points for the hematogenous spread of tumor cells throughout the body. Through its effects on local angiogenic cytokine networks, TGFβ can induce a pro-angiogenic environment and stimulate angiogenesis. Indeed, *in vitro* studies reveal that several key angiogenic mediators such as vascular endothelial growth factor (VEGF) and connective-tissue growth factor (CTGF) are direct targets of the TGFβ signaling pathway [69, 70]. Hypoxic conditions present at the core of a tumor in conjunction with TGFβ signaling can induce robust levels of VEGF mRNA through the activation of hypoxia-inducible factor 1 (HIF1) and Smad proteins. These transcription factors have been shown to interact and induce expression of the

VEGF [70]. Additionally, TGFβ can regulate the expression, secretion, and activity of matrix metalloproteases MMP-2 and MMP-9, and downregulate the expression of the protease inhibitor TIMP in the tumor and endothelial cells [71, 72]. Through these metalloprotease activities, TGFβ can enhance the migratory and invasive properties of endothelial cells required for angiogenesis.

Mouse models defective in TGFβ signaling components further demonstrate the importance of TGFβ signaling in normal vasculature development. Targeted inactivation of TGFβ pathway components such as TGFβ1, TβRII, and TβRI/ALK5 showed clear defects in angiogenesis leading to the death of these animals [73-75]. Additionally, mouse models have also revealed a role for tumor cell-secreted TGFβ in tumor angiogenesis. Increased expression of TGFβ in either prostate carcinoma cells or Chinese hamster ovary cells resulted in robust angiogenic responses, which could be blocked by TGFβ neutralizing antibodies [76]. These results indicate that TGFβ's effect on both the tumor cells and the surrounding environment can stimulate tumor angiogenesis in a variety of settings [71].

Epithelial-mesenchymal transdifferentiation

TGFβ can also enhance the migratory and invasive properties of cancer. Epithelial cell migration requires the loss of cell-cell contacts and acquisition of fibroblastic characteristics. This process is known as the epithelial-mesenchymal transition (EMT) and is important for embryonic development [77]. TGFβ has long been known to be a major inducer of EMT particularly in heart formation and palate fusion in mice as well as in some mammary cell lines, and in mouse models of skin carcinogenesis [77, 78]. In human cancers, pathology sections contain areas with characteristics of EMT particularly at the invasion front, a location that is rich in stromal TGFβ and other cytokines that may cooperate in the induction of EMT.

TGFβ promotes EMT by a combination of Smad-dependent transcriptional events and Smad-independent effects on cell-junction complexes. One of the key targets for repression during EMT is the cell-cell adhesion receptor, E-cadherin. E-cadherin is commonly downregulated in many cancers, and its overexpression can suppress invasion by tumor cells. TGFβ-induced EMT often coincides with loss of E-cadherin expression [79, 80]. Recent work has identified that TGFβ signaling through Smad-mediated expression of HMGA2 (high mobility group A2) is important for the induction of Snail and Slug, which are zinc-finger transcription factors known to repress the E-cadherin gene [53]. Independently, TGF-BR2-mediated phosphorylation of Par6 promotes the dissolution of cell-junction complexes [81]. Therefore, TGFβ-dependent EMT in cancer cells is mediated, in part, by the ability of TGFβ to induce the expression of E-cadherin gene repressors as well as its ability to alter the cell junctions.

However, in addition to its role in enhancing invasion and migration, recent evidence suggests a surprising new function for EMT in breast cancer progression. EMT-inducing factors such as Twist, Snail, and even TGFβ may also promote the expression of cell surface markers of presumptive tumor-propagating cells, also referred to as "cancer stem cells" [82]. Indeed, earlier work has shown that putative breast cancer stem cells identified by the CD44hi/CD24lo marker overexpress components of the TGFβ pathway. Furthermore, treatment with a TβRI-kinase inhibitor induced these putative stem cells to take on a more epithelial phenotype and shed their mesenchymal traits [83]. These data suggest that CD44hi/CD24lo cells may utilize the TGFβ pathway to maintain its tumor-propagating phenotype and by extension may represent a tumor cell population that has undergone EMT. In the most recent studies, immortalized human mammary epithelial cells were forced to undergo an EMT induction resulting in the expression of both mesenchymal and stem cell markers. The treatment resulted in an increased ability to form mammospheres, a function that is thought to be associated with stem cell phenotype, as well as increases in the tumorigenicity of the cells in experimental mouse models. The authors argue that, during the process of tumor metastasis, disseminated cancer cells would need to acquire self-renewal capacities similar to those exhibited by normal stem cells in order to initiate and propagate macroscopic metastases. This notion raises the possibility that the EMT process, which is thought to enable cancer cell dissemination, may also impart a self-renewal capability to disseminating cancer cells [82]. Evidence presented in these studies points at this possible connection; however, future studies will be required to further investigate this connection both in experimental systems and, importantly, in human diseases.

A role for TGFβ in metastasis

In addition to the tumor-promoting functions described above, there is growing experimental evidence that TGFβ can influence the metastatic process (Figure 3). However, the extent of TGFβ's influence on metastasis and its mechanisms of action remain largely unclear. Evidence from clinical studies and experimental systems paints a complicated context-dependent role for TGFβ in metastasis.

TGFβ and bone colonization

In the most vivid example of TGFβ's role in metastasis, recent research has uncovered a prominent role for TGFβ in bone metastases, a common site of dissemination for both breast and prostate cancers. The bone microenvironment consists of a rich store of multiple growth factors including TGFβ. Metastatic cells that reach this tissue release pro-metastatic cytokines that in turn activate osteoclast differentiation. Once activated, osteoclasts function to degrade the bone matrix and release the stored TGFβ. Histological analysis demonstrates that 75% of human bone metastasis biopsies show nuclear phosphorylated-Smad2 in the metastatic cells. These observations indicate functional and active TGFβ signaling in human breast cancer samples [84]. In experimental metastasis assays, bone-tropic MDA-231 cells were transduced with a retroviral vector expressing a reporter gene under the control of a TGFβ-sensitive promoter. Using this reporter, the researchers were able to see active TGFβ-Smad signaling specifically in the bone [84]. To test the requirement of the TGFβ pathway, tumor cells were generated with a knockdown of Smad4, expression of inhibitory Smad7, or expression of dominant-negative *TGFBR2*. These perturbations block TGFβ signaling and dramatically decrease bone metastases in both breast cancer and melanoma models, further implicating TGFβ in the bone metastatic process [84-86].

TGFβ is able to promote these aggressive bone metastases through specific gene inductions. The TGFβ-Smad-signaling pathway induces the production of pro-osteolytic factors, such as parathyroid hormone-related protein (PTHrP) [86, 87]. TGFβ-induced PTHrP stimulates production of RANK ligand, enabling osteoclast differentiation and promoting bone metastases [88]. Additional factors that may enable TGFβ-mediated bone metastasis include members of a previously described Bone Metastasis Signature [69]. Among these genes, *IL11* and *CTGF* are osteolytic genes that are induced by TGFβ-Smad signaling. CTGF is an extracellular mediator of invasion and angiogenesis, whereas IL11 stimulates the expression of osteoclastogenic factors RANK ligand (RANKL) and GM-CSF in osteoblasts. By promoting osteoclast

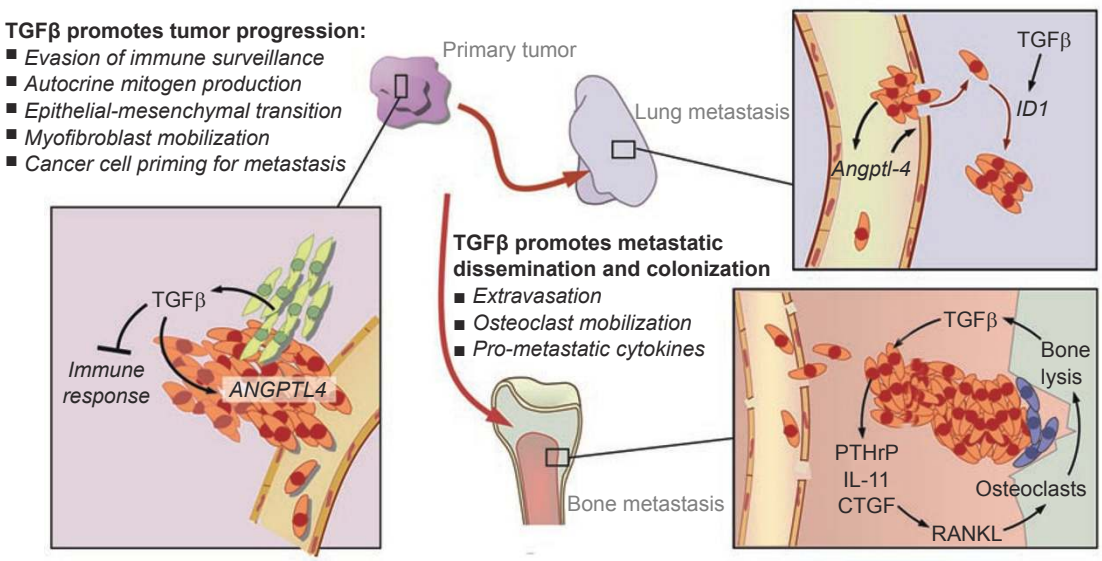

Figure 3 Multiple roles for TGFβ in breast cancer metastasis. TGFβ in tumors may be produced by cancer cells or by stromal components such as myofibroblasts and infiltrating myeloid progenitor cells. TGFβ may support tumor progression through the evasion of immune surveillance, enhanced production of mitogens, or the mobilization of myofibroblasts. Additionally, TGFβ can prime departing tumor cells for metastasis. In ER− breast tumors, TGFβ can induce the expression of genes including *Angiopoietin-like 4* (*ANGPTL4*; primary breast tumor inset). The tumor cells subsequently enter the circulation, with enhanced Angptl4 production. This event primes cancer cells for seeding of lung metastasis, as Angptl4 disrupts vascular endothelial junctions when the cancer cells lodge in lung capillaries (lung metastasis inset). Once inside the pulmonary parenchyma, ER− breast cancer cells may utilize local TGFβ to induce other genes such as *Inhibitor of Differentiation/DNA binding 1* (*ID1*), which enhances tumor reinitiation. Entry of circulating tumor cells into the bone marrow does not benefit from Angptl4 because the marrow capillaries are naturally fenestrated (bone metastasis inset). Osteoclast activity releases TGFβ stored in the bone matrix, which can then act on the growing cancer cells to stimulate the production of parathyroid hormone-related protein (PTHrP), interleukin-11, and connective tissue growth factor (CTGF). These factors act on osteoblasts to stimulate the release of RANK ligand (RANKL), which mediates osteoclast mobilization, perpetuating an osteolytic metastasis cycle.

functions and further bone degradations, bone metastases set up a vicious cycle wherein TGFβ from the stroma stimulates metastatic cells to activate osteoclasts, which go on to further release TGFβ, thereby perpetuating the bone metastatic lesions. TGFβ can therefore exert a pro-metastatic function and facilitate the establishment of metastatic lesions once tumors have reached a secondary site like the bone.

Mouse models of TGFβ and metastasis

In further investigating the role of TGFβ in metastasis, mouse models of metastasis have revealed that systemic inhibition of the TGFβ signaling pathway negatively affects metastasis formation. Pathway inhibition was achieved using a variety of modalities, including small-molecule inhibitors, soluble-TGFβ receptors and antibodies against the TGFβ ligands. In the first example, a small-molecule inhibitor of the type I TGFβ receptor was administered to immunocompromised mice implanted with a human breast cancer cell line. Intraperitoneal injections of the inhibitor effectively reduced the number and size of the lung and bone metastases in both orthotopic and experimental metastasis models [89]. Using an alternative method of pathway inhibition, soluble TGFβ receptors were either administered to tumor-bearing animals or co-expressed as transgenes in a transgenic model of metastatic breast cancer. In each of these cases, the soluble TGFβ type II receptor served to trap the TGFβ ligand and decrease the cancer's metastatic capacity [42, 90]. Lastly, pan-TGFβ neutralizing antibodies have been used in a variety of tumor models and show concordant results of diminishing breast cancer spread in mouse models. In one example, anti-TGFβ antibodies were administered to tumor-bearing mice undergoing radiation treatment. Irradiation of the tumors resulted in increased incidence of lung metastases, a process that is blocked by the neutralizing TGFβ-antibody [91]. Additionally, other groups have shown that the spread of a transplantable model of metastatic breast cancer, 4T1 breast cancer cells, can be efficiently suppressed by administering an antibody that targets all three isoforms of the TGFβ ligand. This work went on to show that TGFβ neutralizing antibodies can have multiple cooperative effects on angiogenesis, immune cell function, and tumor cell viability, eventually leading to effective tumor control and reductions in metastasis [92]. These results illustrate the capacity to target the TGFβ pathway in order to effectively inhibit metastatic events. Additionally, this research highlights the possible use of anti-TGFβ therapies in metastatic breast cancer patients. One concern with these types of therapies is the potential for detrimental side effects. Given the importance of TGFβ in normal tissue homeostasis, broad inhibition is predicted to affect a wide array of normal cell functions. However, long-term treatments with TGFβ inhibitors like the soluble TGFβ receptor traps do not seem to significantly alter animal morbidity [90].

Systemic inhibition of TGFβ, however, affects the entire tumor microenvironment from the cancerous epithelium to the stromal cells. To directly test the role of TGFβ signaling in each of these compartments, several groups have sought to target the TGFβ pathway within the tumor cells as well as stromal fibroblasts. However, depending on the tumor models used, conflicting results have emerged regarding the role of TGFβ signaling in cancer and metastasis. Consistent with the previous anti-TGFβ therapy experiments, several independent groups have found that expression of activated type I receptors or dominant-negative Smad transcription factors in the carcinoma cells affects a primary tumor's capacity to initiate and establish metastasis [42, 43, 93, 94]. In one of the first examples, transgenic mice were generated expressing an activated TGFβ type I receptor or a dominant-negative TGFβ type II receptor under the control of the mouse mammary tumor virus promoter (MMTV), a promoter that directs expression specifically in the mammary glands. When crossed with mice expressing an activated form of the Neu receptor, an epidermal growth factor receptor family tyrosine kinase found to be amplified in >30% of human breast cancers [95], activated TGFβ type I receptor increased the latency of mammary tumor formation as well as enhanced the frequency of extravascular lung metastasis. Intriguingly, this work suggests that TGFβ can enhance the extravasation of breast cancer cells from pulmonary vessels in order to facilitate the metastatic process [43]. Conversely, expression of the dominant-negative type II receptor decreased the latency of Neu-induced mammary tumor formation, while significantly reducing the incidence of extravascular lung metastases. These observations along with the drug studies suggest that TGFβ can promote the formation of lung metastases.

However, given the clinical and experimental evidence that TGFβ acts as a tumor suppressor, other groups have argued that TGFβ functions as an inhibitor of epithelial tumor growth and metastasis. Researchers generated a conditional knockout of *TGFBR2* in both the mammary epithelium and the tumor-associated fibroblasts. In their studies, loss of *TGFBR2* in either mammary epithelial cells or fibroblasts increased tumor formation and enhanced many markers of tumor progression. Indeed, knockout of *TGFBR2* in the fibroblasts of the tumor microenvironment resulted in upregulation of HGF, MSP, TGF-α, and other secreted factors that significantly en-

hanced the adjacent epithelial cells to proliferate [5, 96]. Surprisingly, these studies show that *TGFBR2* knockout animals developed significantly more pulmonary metastases compared with control mice [35, 97-99]. In a recent report, researchers have shown that targeted deletion of *TGFBR2* in mouse mammary epithelium initiates the recruitment of myeloid immune suppressor cells through the CXCL5 axis. Results from *in vitro* coculture and *in vivo* coinjection of tumor cells with these myeloid cells suggest a role of these myeloid cells in tumor invasion and in enhancing lung metastases through the expression of metalloproteases that facilitate tumor cell invasion. Interestingly, they also show that these *TGFBR2* knockout tumors have high levels of TGFβ1 most likely secreted by myeloid suppressor cells located at the invasive edge of the tumors. These authors argue that the TGFβ may provide an additional boost to tumor progression by dampening the immune response to the tumors [99]. Corroborating these mouse model results, other researchers demonstrated that expression of a dominant-negative *TGFBR2* in tumor cells enhanced metastasis in a mouse prostate tumor model [100]. These results conflict with the previously described research and suggest that the role of TGFβ in metastasis depends on multiple factors, including the tumor-initiating mutation, method of TGFβ inactivation, and the timing of the TGFβ signal. Whereas certain tumor mouse models show an active role of TGFβ in metastasis promotion, others have shown that TGFβ may in fact inhibit tumor metastasis through its effects on both the epithelial and stromal compartments.

Clinical correlates of TGFβ and distal relapse

The controversy regarding the role of TGFβ in breast cancer metastasis has led to a variety of interpretations, each yielding different answers. It should be noted, however, that the eventual goal of this type of research is to try to understand how TGFβ affects human disease. To this end, researchers have turned to clinical samples to see whether there are correlations between TGFβ signaling and metastasis. As mentioned earlier, the TGFβ receptors and the Smad transcription factors are tumor suppressors that frequently suffer inactivation in gastrointestinal, pancreatic, ovarian, and hepatocellular carcinomas and subsets of gliomas and lung adenocarcinomas [9, 101]. However, in breast carcinoma, glioblastoma, melanoma, and other types of cancer, selective losses of TGFβ-mediated growth inhibitory responses often accrue through alterations downstream of Smad, leaving the rest of the TGFβ pathway operational and open to cooption for the advantage of tumor progression [51]. Indeed, clinical correlations between pre- or post-operative plasma levels of TGFβ and metastatic disease have been reported in many studies on colorectal, prostate, bladder, breast, pancreatic, or renal cancers, and on myeloma and lymphoma [102]. Additionally, low-level expression of TGFβ receptors in the estrogen receptor-negative (ER−) breast tumors is associated with better overall outcome [103], whereas overexpression of TGFβ1 is associated with a high incidence of distant metastasis [104].

However, many of these studies rely on examining the expression of TGFβ signaling components in clinical samples. One caveat with immunohistochemical analysis of components of the TGFβ signaling pathway is that it does not take into account the eventual outcome of TGFβ signaling, the gene expression changes. Indeed, mutations or alterations downstream of the signaling components may very well prevent an appropriate response to TGFβ signals. To circumvent this caveat, researchers looked at the TGFβ response status of various clinical samples by using a bioinformatics tool termed the TGFβ response signature (TBRS) [105]. This signature was identified in epithelial cell lines and defined as the set of genes whose expression collectively changes upon TGFβ treatment. By interrogating large clinical cohorts, it was found that approximately 40% of human breast tumors could be designated as TBRS positive or seen as actively responding to TGFβ signals. Indeed, this status correlated with high expression of many activators and mediators of the TGFβ signaling pathway, namely TGFβ1, TGFβ2, LTBP1, SMAD3, and SMAD4. Surprisingly, the TBRS status in human breast cancer samples was found to be associated with lung relapse but not bone relapse, specifically in the ER− but not in ER+ primary tumors.

TGFβ primes for metastasis to the lung

The above results imply that active TGFβ signaling in the primary tumor selectively enhances lung metastases, but only in the specific context of ER− tumors. To test the requirement of TGFβ signaling in the metastasis of ER− breast cancer, a derivative of the MDA-MB-231 ER− breast cancer cell line was used in a xenograft mouse model of metastasis. Abrogating the TGFβ signaling pathway either through expression of a dominant-negative TGFβ receptor or through reductions in expression of the SMAD4 transcription factor blunted the cancer cell's ability to metastasize to the lung from an established primary tumor. In understanding how this signaling event at the primary tumor enhanced distant metastases, a novel metastatic mechanism was proposed, wherein departing cells are primed by the TGFβ signal to efficiently and specifically colonize the lung. Central to this process is the vascular remodeling gene, *angiopoietin-like 4* (*ANGPTL4*), which was identified as a canonical target of TGFβ signaling in multiple breast

cancer samples. Interestingly, this gene can enact a disruption of vascular cell-cell junctions and induce lung vasculature permeability. ANGPTL4's vascular remodeling function was shown to aid cancer cells as they travel through a well-organized vascular barrier like that in the lung [105]. The bone microenvironment, on the other hand, is designed to enable hematopoietic cells to easily shuttle back and forth. Unlike the lung environment, the bone vasculature is organized in sinusoids that contain fenestrated capillary beds [106]. Therefore, tumor cells that exhibit enhanced skills at breaching tight vascular barriers would gain a significant advantage in colonizing lung while gaining little advantage in colonizing bone. With this mechanism, a new paradigm for TGFβ action in metastasis is set up. Whereas initial reports suggested that TGFβ actions are restricted to the bone microenvironment, for example, through initiating angiogenesis, this new model suggests that TGFβ can act at a distance. The cytokine relay between TGFβ and ANGPTL4 enables the actions of TGFβ to project throughout the body, enhancing the reach and impact of TGFβ signaling and metastasis.

Conclusions

During the course of tumor progression, the acquisition of metastatic characteristics often bodes the onset of significant cancer-associated morbidity and mortality. Although powerful treatments are being developed to fight the growth of primary tumors, new therapies are required to tackle the ever-emerging problem of metastasis. Given the clinical and experimental evidence showing TGFβ's role in the metastatic process, TGFβ has become an attractive candidate for anti-metastasis therapies. In fact, the pharmaceutical industry is investing in therapies that can effectively target the TGFβ pathway. Small-molecule inhibitors targeting the receptor kinases, large-molecule neutralizing antibodies, as well as nucleic acid-based therapies are being developed to inhibit the TGFβ pathway with the eventual goal of using these therapies on cancer patients [107]. However, as noted above, TGFβ has a complex role in tumor progression. Depending on the tumor type and the stage in tumor progression, TGFβ can act as a tumor suppressor or tumor promoter. This extensive body of work highlights the need to faithfully identify patient subpopulations that may benefit from the otherwise potentially harmful anti-TGFβ therapies. As with any medication, there is a small inherent risk of developing deadly complications from the novel TGFβ-based therapies. However, the potential of developing these complications may be outweighed by the enormous benefits gained by using these powerful new therapies. Through better stratification tools, future physicians will be able to discern which patients would actually benefit from the potentially life-saving therapies, while at the same time sparing those whose tumors would never respond to these targeted therapies because of the risks and morbidity associated with the therapies. To properly stratify these patient subpopulations, new and robust diagnostic tools must be developed. The TBRS described above is one example of such a tool that can be expanded and further developed to meet this need. The TBRS in combination with the Lung Metastasis Signature is able to identify patients who are at high risk of developing lung metastasis [105, 108]. However, further work is required to better classify the TGFβ response using similar microarray tools and to test the effectiveness and the predictive power of such bioinformatics tools.

Much of the work presented above has focused on the role of TGFβ in breast cancer. However, TGFβ's role in metastasis need not be limited to this disease; indeed a variety of other cancers may take advantage of very similar mechanisms and as a result these diseases may also benefit from modulating the TGFβ pathway. Cancers such as bladder cancer, endometrial cancer, sarcomas, and melanomas should also be tested to determine the extent and the role of TGFβ signaling. The new metastasis mechanisms identified in breast cancer and the tools developed to investigate their role could be easily employed to study the participation of TGFβ in disease progression of a variety of other cancers.

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