

Review

Transforming growth factor- β and breast cancer Transforming growth factor- β /SMAD signaling defects and cancer

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Abstract

Transforming growth factor- β (TGF- β) is a tumor suppressor, the function of which is compromised in many types of human cancer, including breast cancer. The tumor suppressive effects of TGF- β are caused by potent inhibition of cell proliferation due to cell cycle arrest in the G1 phase. Such antiproliferative responses are mediated by a signaling system that includes two types of cell surface receptors and intracellular signal transducers, the SMAD proteins. Different molecular mechanisms can lead to loss of antiproliferative TGF- β responses in tumor cells, including mutations in components of the signaling system and inhibition of the SMAD signaling pathway by aberrant activities of various regulatory molecules. Some of these mechanisms will be discussed, with emphasis on their potential involvement in breast tumorigenesis.

Keywords: breast cancer, growth inhibition, SMAD proteins, transforming growth factor (TGF)- β , tumor suppressor

Introduction

The transforming growth factor- β (TGF- β) family of polypeptide growth factors regulates cellular processes, including cell division, differentiation, motility, adhesion, and death, in virtually all tissues ([1] and references therein). TGF- β is an important regulator of normal mammary gland development and function, as well as of the development and progression of breast tumors. TGF- β potently inhibits cell cycle progression of epithelial cells, including those of the lobules and ducts of the mammary gland, and it thereby controls epithelial cell proliferation and regression during mammary gland development, and during and after lactation in the adult gland [2].

In breast cancer, TGF- β has been suggested to play a dual role [3]. It acts as a tumor suppressor in early stages

of the disease when it inhibits the outgrowth of carcinomas *in situ* via its antiproliferative functions. This has been demonstrated in transgenic mouse models, in which overexpression of TGF- β 1 (one isoform of TGF- β) is targeted to the mammary gland, and tumor formation is induced by concomitant overexpression of TGF- β and administration of a chemical carcinogen [4]. In later stages of the disease, TGF- β is believed to promote tumor progression, in part by enhancing tumor cell motility and invasiveness [5,6] and the capacity to form metastases [6–8]. Tumor promoting functions of TGF- β correlate with increased secretion of TGF- β by the cancer cells during tumor progression [3].

This apparent switch of the role of TGF- β in the regulation of tumorigenesis is reflected in changes of tumor cell

responsiveness. Similar to other types of carcinomas, many malignant breast carcinoma cells have lost most or all sensitivity to TGF- β -induced growth inhibition, while tumor cells derived from early stages of the disease are usually inhibited [9]. This loss of antiproliferative responsiveness thereby predisposes to or causes cancer progression.

TGF- β induces growth inhibition by arresting cells in the G1 phase of the cell cycle, leading in some circumstances to terminal differentiation or induction of apoptosis [10]. G1 arrest is achieved by several mechanisms that may act in a complimentary fashion in the same cell, and which include the transcriptional upregulation of the cyclin-dependent kinase inhibitors p15^{Ink4B} and p21^{cip1/waf1}, the downregulation of the cyclin-dependent kinase activating phosphatase Cdc25A, and the downregulation of the proto-oncogene c-Myc. Together, these events lead to a hypophosphorylated, activated state of the pRB tumor suppressor protein, and thereby to the arrest of the cell cycle in G1.

The antiproliferative response to TGF- β depends on a signaling pathway that is initiated by the ligand-activated TGF- β receptor complex on the cell surface and is transduced into the nucleus by signaling mediators, the SMAD proteins [1,11] (Fig. 1). In this pathway, TGF- β binds to a specific pair of type I and type II receptor serine/threonine kinases, leading to the transphosphorylation and activation of the type I receptor (T β R-I) by the type II receptor (T β R-II). Activated T β R-I then phosphorylates a specific subset of SMAD proteins, Smad2 and Smad3, which subsequently translocate into the nucleus. On their way to the nucleus, these receptor-activated SMAD proteins associate with the related Smad4 protein. Once in the nucleus, SMAD proteins form functional transcription complexes in association with DNA binding factors, coactivators, or corepressors [12].

Resistance to TGF- β -induced growth inhibition in breast carcinoma cells may be caused by a number of mechanisms, some of which are just starting to become clear. In this review, I will focus on molecular events that directly interfere with the TGF- β /SMAD signaling pathway.

Inactivating mutations in components of the TGF- β /SMAD signaling pathway

Disruption of the TGF- β /SMAD signaling pathway by mutation has been observed in several types of human cancer. T β R-II is inactivated by mutation in a majority of colon and gastric cancers with microsatellite instability [13^{**},14,15] and, to a smaller percentage, also in microsatellite stable colon cancers [16]. In comparison, mutations in T β R-II are relatively rare in cancers of the pancreas, liver, and pituitary gland, and in myelodysplastic syndrome or endometrial cancers with microsatellite instability [14,17–20]. Mutations or structural alterations in T β R-II have not been found in primary human breast carci-

nomas [20,21^{*},22,23] or breast carcinoma cell lines [24]. Inactivation of T β R-II by mutation therefore appears to be specifically selected for in gastrointestinal cancers [25].

Homozygous deletions of T β R-I were found in a small percentage of pancreatic and biliary adenocarcinomas [26], and a large deletion mutation was detected in T β R-I in one case of anaplastic large cell lymphoma [27]. Furthermore, a polymorphism resulting in a deletion of three residues from a nine alanine stretch has been observed in some colorectal and cervical carcinomas, and homozygous carriers of this polymorphism may be at enhanced risk for cancer development [28,29]. In breast cancer, a point mutation (S387Y) in T β R-I, which diminishes its signaling capacity, has been reported in 2 of 31 primary carcinomas (6%) and 5 of 12 lymph node metastases (42%), indicating that this mutation may represent an important event in the progression of breast cancer to malignancy [21^{*}]. This view has been challenged, however, by another study in which no mutation at this site was detected in 20 cases of breast cancer metastases [30^{*}].

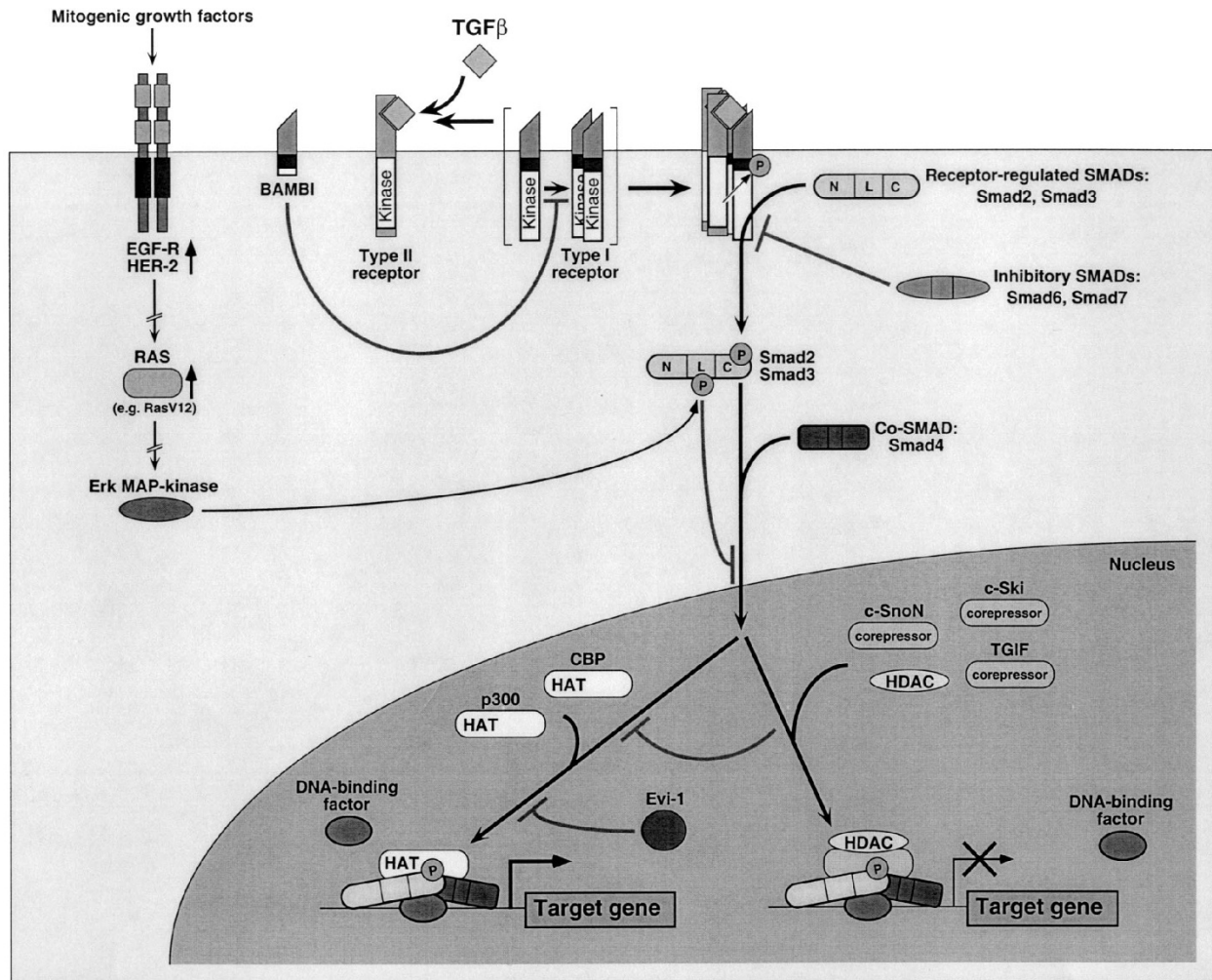
Inactivating mutations in SMAD genes are found in a number of human cancers, with the highest frequency in pancreatic and colon carcinomas. Smad4/DPC4 was originally isolated as a tumor suppressor gene on chromosome 18q21 that is deleted or mutated in nearly half of all human pancreatic carcinomas [31^{**}], and much less frequently in other cancers [32–37]. Smad2, which is also located on 18q21, is mutated in a small number of colon, head and neck, and lung carcinomas [38^{**},39–42], but appears to be unaffected in other types of carcinomas as well as leukemias and lymphomas [34,35,43–50]. In breast cancer, inactivating mutations in Smad2 have not been reported and they are rare in Smad4 [37,51]. Inactivating mutations in Smad3 have not been observed in any of a large number of tumors, including those from gastrointestinal, breast, lung, ovarian, and pancreatic cancers [39, 43,44,48,52–54].

Why inactivating mutations in T β R-II and Smad4 are involved in significant proportions of gastrointestinal and pancreatic cancers, respectively, but are rare in breast (and other) cancers is currently unclear. It suggests, however, that activities of these molecules might be required for the development and/or progression of breast cancer, and therefore mutational inactivation would not confer a selective advantage.

Reduced expression of TGF- β /SMAD signaling components

In some breast cancer cell lines, limited expression of T β R-II has been correlated with the lack of TGF- β responsiveness [55^{*},56,57^{*}]. Stable expression of T β R-II in such cell lines can restore TGF- β -induced growth inhibition, indicating that all other signaling components are functional [55^{*},57^{*}]. Vari-

Figure 1



This model schematically illustrates the various molecular mechanisms that can contribute to negative regulation of SMAD signaling and thereby to a loss of transforming growth factor (TGF)β-induced growth inhibition. Inactivating mutations or loss of expression of components of the TGFβ/SMAD signaling pathway are not depicted here but may also be important. For details, see text.

able expression levels of TβR-II were observed in primary epithelial cell cultures derived from malignant breast tissue [58]. In contrast, another study detected no loss of TβR-II expression in primary breast carcinomas [21*].

In a limited number of cases, reduced expression of TβR-I has been suggested as the cause for loss of TGF-β-mediated growth inhibition in pancreatic or colon carcinoma cell lines [59–61] or cells from patients with chronic lymphocytic leukemia [62]. However, normal TβR-I expression was observed in the vast majority of primary epithelial cell cultures derived from malignant breast tissue [58].

In a panel of both estrogen-responsive and estrogen-insensitive breast carcinoma cell lines, *Smad2*, *Smad3*, *Smad4*, and *TβR-I* were all expressed at the mRNA level, with the exception of one cell line (MDA-MB-468) with a homozy-

gous deletion of *Smad4* [63]. *TβR-II* mRNA could not be detected in two of the cell lines tested (ZR-75-1 and T47D).

Taken together, these results suggest that limited or no presence of functional Smad4, TβR-I or TβR-II, due to mutation or aberrant expression, may contribute to loss of TGF-β growth inhibition in a small percentage of breast carcinomas. Aberrations in functional expression of Smad2 and Smad3 are unlikely to play a significant role. Alternative mechanisms to abolish growth inhibitory responses to TGF-β must therefore evolve in most breast carcinomas.

Inhibition of the TGF-β/SMAD pathway by Ras/MAP-kinase signaling

TGF-β can override the proliferative effects of EGF and other Ras-activating mitogens in normal epithelial cells. However, cells harboring oncogenic Ras mutations often

show a loss of TGF- β antiproliferative responses. Oncogenic Ras can achieve inhibition of TGF- β signaling in mammary epithelial cells by negatively regulating Smad2 and Smad3; that is, by inhibiting their TGF- β -induced nuclear accumulation and transcriptional activity [64**]. Acting via Erk MAP kinases, Ras causes phosphorylation of Smad2 and Smad3 at specific sites in the central portions of the proteins. These sites are separate from the carboxy terminal sites targeted by T β R-I. Mutation of these MAP kinase sites in Smad3 yields a Ras-resistant form that can partially rescue the growth inhibitory response to TGF- β in Ras-transformed cells [64**,65]. EGF, which activates MAP-kinase transiently, induces a less extensive phosphorylation and cytoplasmic retention of Smad2 and Smad3 [64**]. These results have suggested a mechanism for counterbalanced regulation of Smad2 and Smad3 by TGF- β and Ras signals in normal cells, and for the inhibition of antiproliferative TGF- β functions by hyperactive Ras in cancer cells. In addition to such direct effects on SMAD signaling, Ras can also interfere with TGF- β functions at other levels; for example, by upregulating the activities of G1 phase cyclin-dependent kinases [66].

Even though oncogenic Ras mutations are relatively rare in breast cancers (found in about 5% of all cases [67]), about one-third of all breast cancers display overexpression or amplification of the HER-2/Neu receptor tyrosine kinase [68–70]. In addition, the related EGF receptor is also overexpressed in a significant number of cases [71]. Stimulation of the Ras/MAP-kinase pathway is a major component of the proliferative signals by these receptors. Amplification and overexpression of HER-2/Neu and EGF-R have been correlated with aggressive tumor phenotype and poor clinical prognosis [69,72]. It is plausible, therefore, that one function of oncogenic Ras mutations and elevated HER-2/Neu or EGF-R activity in breast carcinomas is to impede the growth inhibitory function of TGF- β via phosphorylation of SMAD proteins by Erk MAP-kinase. Consistent with such a model, an inverse correlation between the presence of oncogenic Ras mutations and the ability of TGF- β to inhibit cell proliferation has been observed in a panel of carcinoma cell lines, derived in this case from colon cancers [64**].

Apparently conflicting results with this model are presented in a study which proposes that EGF and HGF signal positively through the Smad2 protein in certain cell lines, and that Smad2 thereby functions as a common effector of receptor tyrosine kinase and receptor serine/threonine kinase signaling [73]. The observed effects are suggested to be mediated by phosphorylation of Smad2 at sites that are also phosphorylated in response to TGF- β but are different from the carboxy terminal sites targeted by T β R-I. A clear evaluation of these results will have to await the mapping and mutational analysis of the involved phosphorylation sites. Interactions

between TGF- β signaling and various MAP-kinase pathways are currently the subject of intense investigation, fueled by observations that factors of the TGF- β family and receptor tyrosine kinase activating factors synergize with or antagonize each other's actions during developmental processes, and that under certain conditions, TGF- β factors elevate MAP-kinase activities in cultured cells [74].

An interesting example of a tumor cell line that features more than one mechanism of inhibition of TGF- β /SMAD signaling has recently been described [65]. The colon carcinoma cell line SW480.7 lacks expression of a functional Smad4 protein and also harbors an activating mutation in the *Ki-Ras* oncogene. As expected, this cell line does not show antiproliferative responses to TGF- β . Furthermore, exogenous expression of Smad4 does not rescue these responses [65]. Only the concomitant expression of Smad4 and a Ras-resistant Smad3 mutant protein restored antiproliferative TGF- β responses, indicating that both lack of Smad4 and inhibition of Smad3 through oncogenic Ras signaling contribute to the repression of TGF- β /SMAD signaling in this cell line [65]. It is likely that these two mechanisms have evolved at different stages of tumor development, with the *Ki-Ras* mutation being an earlier event than the *Smad4* mutation [75].

Altered expression of TGF- β /SMAD inhibitory molecules

Several molecules have been identified that can interfere with SMAD signaling by competitive protein–protein interactions either in the cytoplasm or the nucleus. Two members of the SMAD family, Smad6 and Smad7, inhibit the formation of transcriptionally active SMAD complexes by ligand-induced association with the receptor complex [76**–78**] or Smad4 [79**]. Association with the receptor complex prevents the interaction between activated T β R-I and its SMAD substrates, thereby blocking the TGF- β -induced phosphorylation and activation of Smad2 and Smad3. One physiological function of these inhibitory SMAD proteins might be to provide a negative feedback regulation of TGF- β family signaling [78**,80,81]. Northern blot and *in situ* hybridization analyses indicated that Smad6 and Smad7 are overexpressed in pancreatic cancer tissues and cell lines, suggesting that elevated expression of inhibitory SMAD proteins may contribute to the loss of growth inhibitory TGF- β responses in pancreatic carcinomas [82,83*]. In other studies, however, no increased expression of Smad7 in a panel of pancreatic carcinoma cell lines [44] and no clear differences in Smad6/7 immunostaining of epithelial cells from normal and tumor tissues of the colon were observed [84]. There have been no reports as yet concerning expression levels of Smad6 and Smad7 in breast tissues.

Very recently, a novel mechanism of interference with certain TGF- β responses emerged. Several SMAD-inter-

acting proteins were identified that serve as transcriptional corepressors in the TGF- β response [85**–87**,88,89**,90**]. Two of these, the related proto-oncoproteins c-Ski and c-SnoN, associate with Smad2, Smad3, or Smad4 in the nucleus to repress the ability of TGF- β -activated SMAD complexes to activate transcription [85**–87**,88,89**]. Both c-Ski and c-SnoN achieve this repression by recruitment of the transcriptional corepressors N-CoR and mSin3 [91], which in turn associate with histone deacetylases, thereby leading to the formation of a repressive SMAD complex on the target promoter. Similar to a model proposed for another SMAD corepressor, TGIF [90**], the recruitment of c-Ski and c-SnoN is likely to compete with binding of the transcriptional coactivators p300 or CBP that possess histone acetyl transferase activity. The acetylation state of core histones plays a critical role in transcriptional regulation [92]. Thus, the balance between SMAD corepressors and coactivators might be important in determining the transcriptional activities of nuclear SMAD complexes. Overexpression of c-Ski is thought to be sufficient for oncogenic activation [93], and elevated expression of c-Ski was detected in several tumor cell lines derived from neuroblastoma, melanoma and prostate cancer [94,95]. A thorough analysis of expression levels of c-Ski or c-SnoN in normal and cancerous breast tissue has not been reported, but the recent identification of their involvement in TGF- β signaling warrants closer investigation of this issue. Furthermore, a recent study suggested that c-Ski expression is upregulated in response to estrogen signaling in epithelial cells of the uterus [96], raising the possibility that c-Ski might also be involved in the interactions between estrogen and TGF- β in breast cancer.

A nuclear zinc-finger protein, Evi-1 is involved in leukemic transformation of hematopoietic cells subsequent to chromosomal translocations that lead to expression of an AML1/Evi-1 fusion product under the control of the AML1 promoter [97]. The biological functions of Evi-1 are not well defined, but one way of contributing to a transformed phenotype may be through its ability to inhibit TGF- β /SMAD signaling [98**]. Evi-1 is proposed to associate specifically with the Smad3 protein, thereby preventing DNA binding and transcriptional activity of Smad3 containing complexes. Evi-1 or the AML1/Evi-1 fusion protein can suppress TGF- β -induced growth inhibition when expressed in lung epithelial or myeloid cells [98**,99]. It has been suggested that Evi-1 might also be involved in solid tumors since overexpression has been observed in ovarian cancer samples [100] and Evi-1 causes transformation when exogenously expressed in fibroblasts [101,102]. Aberrant expression of Evi-1 in breast cancer, however, has not been reported to date.

Another recent addition to the growing list of molecules that can regulate TGF- β /SMAD signaling by direct association with one of the signaling components is the

pseudoreceptor BAMBI [103**]. BAMBI was isolated by an expression screen for molecules involved in BMP4 (a member of the TGF- β family) signaling. BAMBI is related to the TGF- β family type I receptors but lacks an intracellular kinase domain. Its intracellular domain, however, contains the homodimerization interface that allows BAMBI to form complexes with type I receptors, thereby preventing the formation of functional type I receptor homodimers [103**]. Compared with nonmetastatic melanoma cell lines and normal tissues, the expression of BAMBI in certain metastatic melanoma cell lines is strongly reduced [104]. It will be important to confirm this observation in primary melanoma tissue and in other types of tumors, such as breast cancer, in which a loss of BAMBI may enhance the tumorigenic activity of TGF- β .

Interference with TGF- β /SMAD responses downstream of SMAD proteins

The antiproliferative response of tumor cells to TGF- β can also be affected by aberrant expression or inactivation of cell cycle regulators that function downstream of or independently of the TGF- β /SMAD pathway. Such alterations are frequently found in human breast tumors and they include increased expression of CyclinD1, CyclinE, MDM2, or c-Myc, decreased expression of p16^{INK4A} or p27^{Kip1}, and mutations in pRB or p53 [68]. For more detailed discussion of these alterations, see [68,105].

Conclusions

A number of different molecular mechanisms have been described that might contribute to the development of resistance to the growth inhibitory effects of TGF- β in breast carcinoma cells. More work needs to be carried out to determine the relative significance of these various mechanisms for the etiology of breast cancer. Each individual case of breast cancer might feature a specific combination of these mechanisms. The nature of this combination is likely to influence the course of the disease by determining the extent of resistance to TGF- β -induced growth inhibition at different stages and by the manifestation of other TGF- β responses that may contribute to the invasive and metastatic potential of the tumor cells. For example, while inactivating mutations in *T β R-II* should abolish all TGF- β responses, inhibition of SMAD signaling by various mechanisms will block SMAD-dependent responses, such as growth inhibition, to varying extents and will allow potential SMAD-independent TGF- β effects to remain intact. The continued elucidation of TGF- β signaling and its molecular and functional interactions with oncogenic events involved in breast cancer has the potential to lead to novel ways of treating the disease; for example, by selectively restoring tumor suppressive TGF- β functions and/or inhibiting tumor promoting TGF- β functions.

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- of special interest
- of outstanding interest

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