Alterations in the Smad pathway in human cancers

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TABLE OF CONTENTS

1. ABSTRACT

Members of the TGF-beta superfamily exhibit various biological activities, and perturbations of their signaling are linked to certain clinical disorders including cancer. The role of TGF-beta signaling as a tumor suppressor pathway is best illustrated by the presence of inactivating mutations in genes encoding TGF-beta receptors and Smads in human carcinomas. This perspective is further supported by studies of tumor development in mouse models after modulation of receptors and Smads. TGF-beta also controls processes such as cell invasion, immune regulation, and microenvironment alterations that cancer cells may exploit to their advantage for their progression. Consequently, the output of a TGF-beta response is highly situation dependent, across different tissues, and also in cancer in general. Understanding the mechanisms of TGF-beta superfamily signaling is thus important for the development of new ways to treat various types of cancer. This review focuses on recent advances in understanding the Smad dependent TGF-beta pathway as it relates to human carcinogenesis.

2. INTRODUCTION

The TGF-beta super-family of growth factors comprises seven genes in *Drosophila melanogaster* and at least 30 genes in mammals, including 3 TGF-beta isoforms, 4 activin beta-chains, the protein nodal, 10 bone morphogenetic proteins (BMPs) and 11 growth and differentiation factors (GDFs). Most mammalian cells express different members of this receptor family, some of which may be shared by different TGF-beta ligands. All these ligands are synthesized as dimeric pre-proproteins (1). Dimerization requires the pro-domains (1, 2) and thus occurs intracellularly, before cleavage by proteases of the subtilisin like proprotein convertase (SPC) family (3, 4). The mature, fully processed dimeric growth factors are subsequently secreted. TGFβs are secreted as latent forms while still non-covalently attached to their propeptide. They require a further activation step to release the active ligand (5), which involves the metalloprotease BMP1 (also known as Tolloid in *D. melanogaster*) (6). In contrast to this theme, the nodal precursor was recently shown to bind to
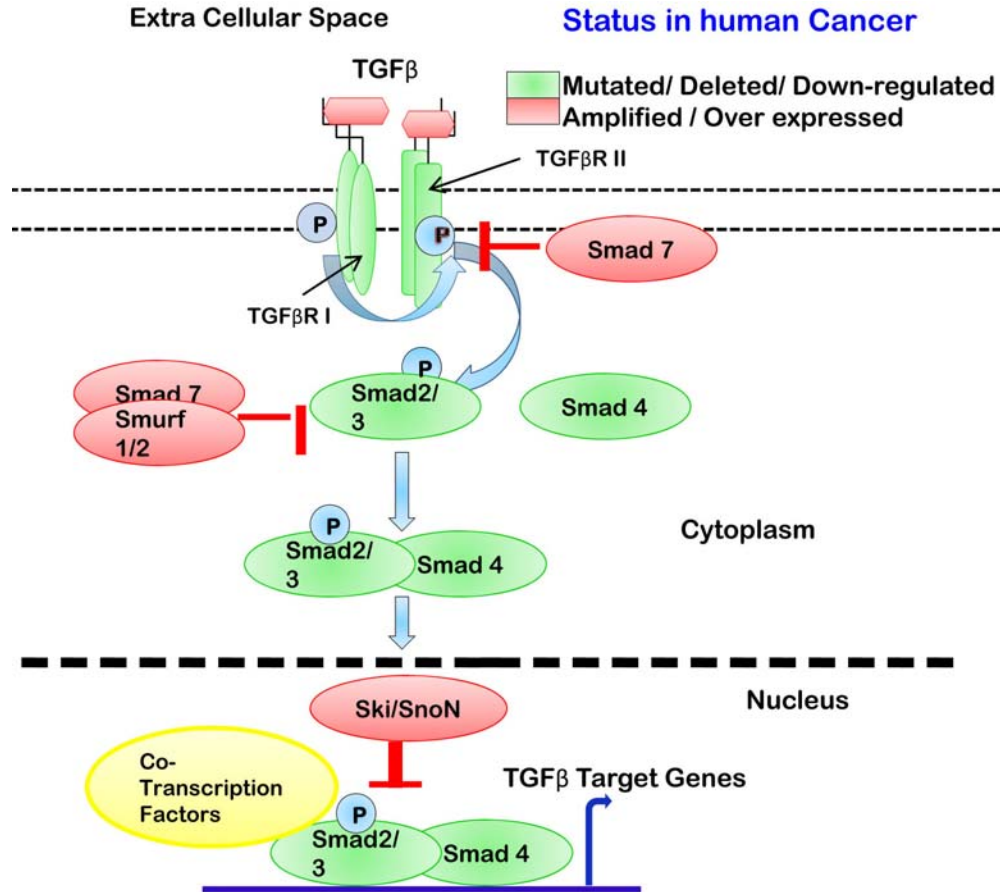
Figure 1. Alterations of the Smad dependent TGF-beta pathways in Cancer. The components that are mutated, deleted or down-regulated, are shown in green, while the components that are amplified or over-expressed, are shown in red**.**

receptors and to activate signaling without being processed (7).

The common feature of TGF-beta superfamily ligands is the so called 'cysteine knot' (8), a structural motif in the mature protein that is formed by three intramolecular disulphide bonds between six strictly conserved Cys residues. Except for GDF3, GDF9 and BMP15, all ligands use an additional conserved Cys residue to form an intermolecular disulphide bond for stabilization of the dimer. Although homo dimers seem to be the prevalent form, additional combinatorial variety occurs through hetero dimerization as in activin beta A–beta B heterodimers, nodal–BMP4 and nodal–BMP7(9)**.**

Ligands of the TGF-beta superfamily of growth factors regulate many cellular functions including cell growth, adhesion, migration, cell-fate determination and differentiation, and apoptosis. Malfunctions in signaling downstream of TGF-beta are implicated in serious human diseases such as cancer, fibrosis, wound-healing disorders, and several hereditary conditions such as familial primary pulmonary hypertension and hereditary Haemorrhagic Telangiectasia (HHT). The growth inhibitory effect of TGF-beta signaling in epithelial cells explains its role as a

tumor suppressor in carcinomas, although TGF-beta expression by tumor cells contributes to cancer progression as well. The current model of induction of signaling responses by TGF-β-related factors (Figure 1) is a linear signaling pathway from the type II to the type I receptor kinase to Smad activation, resulting in ligand-induced transcription (10-12).

3. RECEPTORS

TGF-beta binds to two distinct receptor types, known as type II and type I receptors (13,14). Both type II and type I receptors are required for signal transduction. In addition, some cell surface proteins, including betaglycan (also known as TGF-beta type III receptor), endoglin, and the EGF-CFC family proteins, containing a divergent EGF-like motif and a novel cysteine-rich CFC motif, act as co-receptors for certain TGF-beta super family ligands.

Both type II and type I receptors contain serine/ threonine kinase domains in their intracellular portions. The type II receptor kinases are constitutively active. When they bind to the ligand they form hetero-tetrameric complexes composed of two molecules each of type II and type I receptors (15, 16). In the hetero-tetrameric receptor I
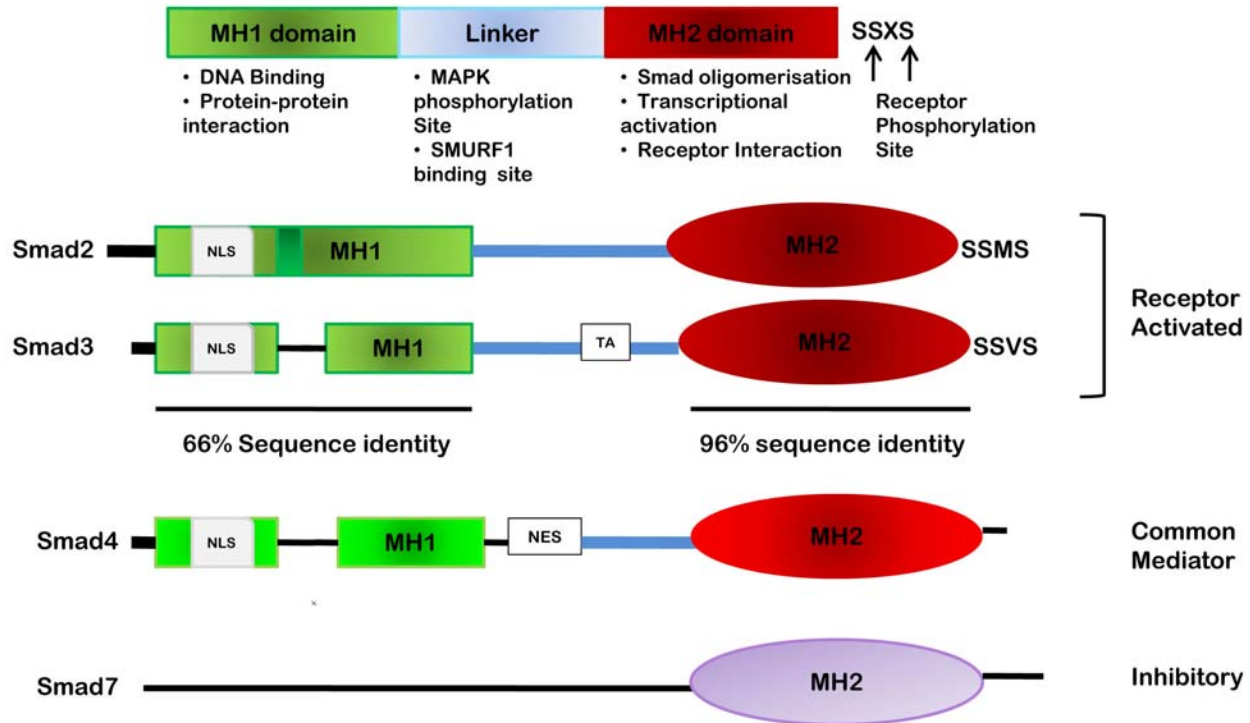
Figure 2. Domain structure of Smads. MH1 domain of Smad2 contains an additional 30 amino acids denoted by dark green box. Smad3 contains a trans-activation (TA) in its linker region. Smad4 contains Nucleus Export Signal (NES) in its linker region. Smad2,3 and Smad4 contains a Nucleus Localization Signal (NLS) in their MH1 domain. Smad 7 lacks MH1 domain. Parts of the figure are reproduced with permission from Ref.122.

and II complexes, type II receptor kinases transphosphorylate the GS-domain of type I receptor, which is located between the transmembrane domain and the kinase domain of type I receptor (Figure 1). The GS domain type I receptor kinases are activated after getting phosphorylated and phosphorylate intracellular substrates. Thus, the type I receptor acts as a downstream component of type II receptors in the signaling pathway, and determines the specificity of the intracellular signals induced by the TGF-beta superfamily cytokines.

Five type II receptors and seven type I receptors are present in mammals. The TGF-beta type II receptor (TβRII) is the specific receptor for TGF-betas. Activin type II and type IIB receptors (ActR-II and ActR-IIB) serve as type II receptors for activins, but are shared with other TGF-beta superfamily members, including nodal and BMPs. BMP type II receptor (BMPR-II) and AMH type II receptor (AMHR-II) specifically bind to BMPs and AMH, respectively.

4. SMADS

The only well-characterized signaling effector pathway that is initiated by activated TGF-beta receptors is provided by the Smads, a small family of structurally related proteins (Figure 2) (10, 11, 17–21). Smads function as signal transducers of TGF-beta family members in organisms ranging from worms to humans. Smad proteins are major signaling molecules acting downstream of the serine/threonine kinase receptors (11, 14). The term Smad is derived from the founding members of this family, the *Drosophila* protein MAD (Mothers Against Decapentaplegic) and the *Caenorhabditis elegans* protein SMA (Small body size). Smads are classified into three subclasses, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads are further divided into two subclasses; Smad2 and Smad3 are referred to as activin/TGF-beta activated R-Smads and AR-Smads, and are activated by activin, nodal and TGF-beta type I receptors, ALK-4, -5 and -7. There are eight vertebrate Smads: Smad1 to Smad8. Smad2 and Smad3 are activated through carboxy-terminal phosphorylation by the TGF-beta and activin receptors TbRI and ActRIB, whereas Smad1, Smad5 and Smad8 are activated by ALK-1, ALK-2, BMP-RIA/ALK-3 and BMP-RIB/ALK-6 in response to BMP1–4 or other ligands.

Smad proteins are 500 amino acids in length and consist of two globular domains coupled by a linker region (8) (Figure 2). The N-terminal domain, or "Mad-homology 1" (MH1) domain and the C-terminal MH2 domain, are highly conserved in all R-Smads and Smad4 but not in Smads 6 and 7. The linker region is quite divergent between the various subgroups. The mitogen-activated protein kinase phosphorylation sites (22, 23) and sites for recognition by the ubiquitin ligase SMURF1 (24) are located in the linker region. Both the MH1 and the MH2
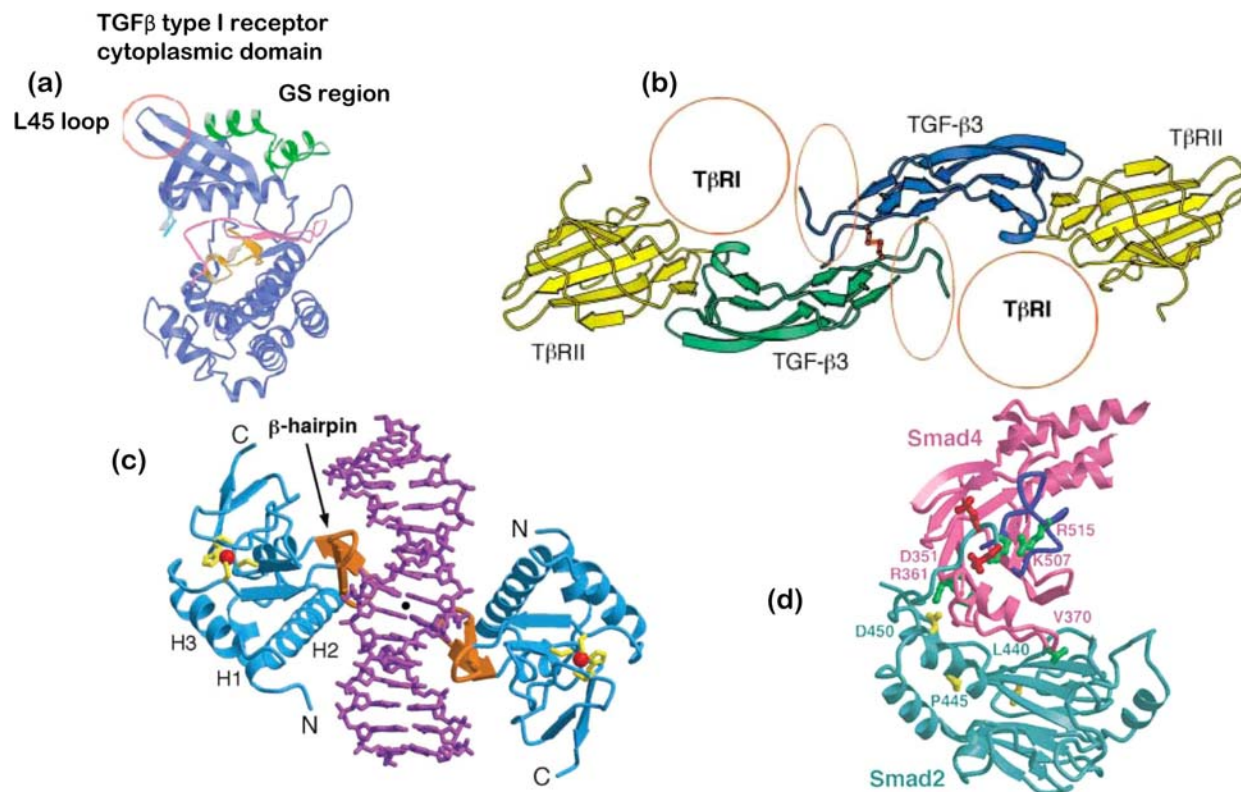
Figure 3. (a) Crystal Structure of the cytoplasmic domain of TGFbeta receptor 1 (b) Structure of the cytoplasmic domain of TGFß receptor I Ref.125. The predicted TβR-I binding sites are shown by red circles (c) Overall structure of the Smad3 MH1 domain bound to SBE. The palindromic DNA and the MH1 domain are colored purple and cyan, respectively. The DNA binding motif is highlighted in *orange.* The bound zinc atom is shown in *red, and* its coordinating residues are colored yellow. Parts of the figure are adapted from Ref.124. (d) A proposed interface between Smad2 (cyan) and Smad4 (pink). In this model, the phosphorylated C terminus of Smad2 interacts with the highly conserved loop-strand pocket of Smad4. Parts of the figure are reproduced with permission from Ref.123.

domains can interact with select sequence-specific transcription factors, whereas the C terminus of the R-Smads interacts with and recruits the related co-activators CREB-binding protein (CBP) or p300 (14, 20, 25). The MH1 domain plays a role in R- and Co-Smad nuclear import, cytoplasmic anchoring, DNA binding, and regulation of transcription. However, Smad2 cannot bind DNA directly owing to a small insert encoded by an extra exon (26). The MH2 domain regulates Smad oligomerization, cytoplasmic anchoring, and transcription of target genes. The MH1 and MH2domains bind to a number of proteins including ubiquitination adaptors and substrates, transcriptional co-activators and co-repressors, and a number of transcription factors.

In the basal state, Smads stay in the cytoplasm. The Smad2 protein is retained in the cytoplasm by an interaction with the protein SARA (Smad anchor for receptor activation) (27). Activated type I receptors associate with specific R-Smads and phosphorylate them on a conserved SSXS motif (where S is serine and X can be any amino acid) at the COOH-terminus of the proteins (Figure 2). When the activated TGF-beta receptor recognizes R-Smads, the specificity of this recognition is

determined by the sequence of the L45 loop on the receptor kinase domain (in red circle) and the sequence of the L3 loop (purple) in the Smad MH2 domain (28) (Figure 3). The L3 loop is a short, conserved sequence that differs in only two amino acids between the Smad1, 5, 8 subgroup and the Smad2, 3 subgroup. The differences in surface structures between these two versions of the L3 loop are sufficient for Smad discrimination by the receptor (29). Smad1 recognition by receptors of the ALK1 subgroup also requires the α-helix 1. The phosphorylated R-Smad dissociates from the receptor and forms a heteromeric complex with the co-Smad Smad4, and together the heteromeric complex moves to the nucleus. Once in the nucleus, the activated Smads contact DNA through the MH1 domain (30) and activate transcription through the MH2 domain (31).

5. TGF-BETA IN CANCER

Cellular homeostasis is tightly controlled by the various pathways that regulate cell proliferation and cell death. Breaking this balance is often associated with cancer development. The transforming growth factor-beta (TGF-β) pathway plays an important role in cellular homeostasis by

regulating cell growth inhibition, cellular senescence, differentiation, and apoptosis. Alteration of some of the components of the TGF-beta receptors and the intercellular signal transducers, the Smads, has been observed in human tumors. These alterations can be deletions or mutations, or downregulation of components that act positively in the pathway. Alternatively, it can be due to the amplification or overexpression of inhibitors of the pathways. Deregulated TGF-beta signaling is known to be involved in a variety of human cancers, including those of the colon, pancreas, breast, lung, and prostate. Epithelial-mesenchymal transition (EMT) is the differentiation switch by which polarized epithelial cells differentiate into contractile and motile mesenchymal cells. Cell motility and invasive capacity are activated upon EMT. TGF-beta induces tissue fibrosis and EMT through activation of Smad and non-Smad signaling pathways.

6. SMAD ALTERATIONS IN CANCERS

Similar to type I and type II receptors, the importance of the Smad proteins to function as tumor suppressors has been demonstrated by the discovery of somatic mutations of these genes within certain cancers. Mutations of the Smad2- and Smad4-encoding gene sequences, but not those in Smad3 or the inhibitory Smad6 or Smad7, have been detected in several carcinomas. This is not a common feature though. As in the case of the TGF-beta receptors, however, decreased expression of the Smad family members is observed in human cancers and may account for TGF-beta resistance. We will discuss the role of each Smads reported to be involved in cancer individually.

6.1. Smad2

The gene that encodes Smad2 is located at 18q21. It has also been proposed to be a putative tumor suppressor target for 18q LOH. Mutation of Smad2 occurs at very low frequency, in 8% cervical cancers, about 8% colorectal cancers, 3% Hepatocellular Carcinoma (HCC) and 2% Non Small Cell Lung Cancer (NSCLC) (32-37). To date only two cases of homozygous deletion have been observed in colon cancer. Most of the mutations that are observed in Smad2 are missense mutations either in the MH1 or MH2 domains. These mutations have been proposed to affect Smad2 phosphorylation (D450E, P445H) (36, 38) nuclear translocation (P445H) (39), to increase Smad2 auto inhibition (R133C) (40), or to decrease protein stability (L440R, Q407R) (36, 41). Occurrence of Smad2, but not Smad3, mutations can be rationalized by the crucial role of Smad2 in the TGF-β–induced expression of p21CIP1 or p15INK4B CDK inhibitor (42).

Smad2 is proposed to be a tumor suppressor. Apart from undergoing LOH in various types of cancer, reduced expression of Smad2 from human breast cancer cell lines resulted in enhanced tumorigenicity with a reduction in metastasis (43). Knockout of the Smad2 gene in mice results in early embryonic lethality at embryonic day 7.5 -12.5, suggesting that it is very important for development and hence possibly has some role in the

development of cancer (44-47). This lethality is due to the restriction of the site of primitive streak formation and the failure to establish an anterior–posterior axis within the epiblast or formation of the ectoderm, mesoderm, and endoderm (45-47). As expected Mouse embryonic fibroblasts (MEFs) derived from Smad2 deficient mice show differential activation of multiple Smad reporters and genes involved in the Smad positive and negative feedback loops. For example activation of a luciferase reporter that contains an ARE from the Xenopus Mix.2 gene promoter (ARE-luciferase) is strongly suppressed in Smad2 null MEFs. However, activation of a luciferase reporter with four repeats of the Smad binding element (SBE4-luc) is not dependent on the expression of Smad2. Microarray analysis of Smad2 null MEFs suggests that Smad2 is the essential mediator of TGF-beta signaling. This is supported by the data which shows that TGF-beta does not induce expression of Pai-1 or p15 in the Smad2 deficient MEFs (48, 49). The reduction of TGF-beta sensitivity in the Smad2 deficient MEFs can be attributed the delay in p15 up-regulation and the failure to down-regulate c-Myc by TGF-beta (49, 50). In HaCaT cells the same result is observed. Specifically, silencing of Smad2 only partially inhibits the reduction of phosphorylated Rb and repression of c-myc and induction of cyclin –dependent kinase inhibitors p15 and p21, by TGF-beta treatment (42). Consequently, silencing of Smad2 in HaCaT cell reduces the inhibitory response of TGF-β.

6.2. Smad3

The Smad3 gene is located on 15q21-q22. Inactivating mutations of Smad3 have not been identified in human tumors. There is a greater frequency of loss of expression of Smad3 in human tumors. The expression of Smad3 is lost in tumor samples, compared with surrounding mucosa, in three out of eight gastric cancers and in two out of nine gastric cell lines that have lost some TGF-beta responsiveness (52). Tumor suppressive activity of TGF-beta in those two cell lines was restored by reintroducing Smad3, suggesting the specificity of action of Smad3. This observation also suggests that Smad3 might be a target for epigenetic inactivation during gastric tumorigenesis. Loss of Smad3 expression in choriocarcinoma cells has been linked to down regulation of TIMP-1, and this may allow the enhanced activity of MMPs classically identified to have a role in tumor invasion (53). The absence of Smad3 expression might impair TGF-β–mediated immunosuppression and contribute to immune or inflammatory responses that predispose to cancer formation.

Knockout of the Smad3 gene in mice is not embryonic lethal (54, 48, 55). Knockout mice generated by different groups have yielded varied outcomes. In one knockout model, the mice die between 1 and 8 months due to compromised immune function and the formation of colorectal adenocarcinomas (54). These colorectal tumors are invasive, metastasize to the lymph nodes, and are the cause of death in 100% of the animals by 30 weeks of age (54). These would suggest that Smad3 is very important in the suppression of cancer. In contrast, these adenocarcinomas were not detected in Smad3 null mice

generated by two other groups (48, 55). This discrepancy could be due to differences in genetic background or in environmental factors associated with animal husbandry. However, as mice with different genetic backgrounds did not show any malignancy, it is likely that the putative tumor suppressor function of Smad3 is dependent on other factors, (48, 55). Recently, it was shown that infection of Smad3 null mice with Helicobacter pylori resulted in chronic inflammation and colon cancer in these animals (56). Smad3 null mice are commonly characterized by thymic involution, enlarged lymph nodes, and formation of bacterial abscesses adjacent to mucosal surfaces (55). The null mice also exhibit forelimb malformations and exhibit accelerated wound healing characterized by an increased rate of re-epithelialization and reduced inflammation (48, 57). Interestingly, Smad3 deficient mice are protected against cutaneous injury induced by ionizing radiation (58).

Knockout of Smad3 in MEFs results in only weak growth inhibition by TGF-beta in culture, compared to wild-type (WT) fibroblasts, which are growth inhibited by TGF-beta (48, 49). The plausible reason for the reduction of TGF-beta sensitivity in the Smad3 deficient MEFs could be the delay in p15 up regulation and the failure to down regulate c-Myc by TGF-(49, 50). Furthermore, in human lung epithelial cells, the expression of Smad3 is down-regulated by TGF-β, and over expression of Smad3 induced apoptosis (59). In addition to the studies performed in MEFs, the function of Smad3 in TGF-beta signaling is illustrated by RNAi-mediated silencing of Smad3. In the HaCaT cell line, RNAi-mediated silencing of Smad3 blocks the growth inhibitory response to TGF-β. Mechanistically, this could be due to either a smaller increase in type 2 transglutaminase (TGase2) or p21 protein levels, or by inhibiting the decrease in ID1, phosphorylated Rb, and MYC protein levels after TGF-β1 treatment (42).

Expression of hTERT stimulates cell proliferation toward immortality (60-64) and contributes to tumor formation (65, 66). hTERT is a key element in telomerase activation, telomere maintenance and tumor development. In A549 and MCF-7 cells, TGF-beta represses the expression of hTERT gene expression. Recent studies illustrate the prominent role of TGF-beta in regulating telomerase expression and identify Smad3 and E2F-1 as critical mediators of TGF-beta effects in both normal and cancer cells (67).

6.3. Smad4

The Smad4 gene is located on chromosome18q. LOH on chromosome 18q is found in about 30% of breast, prostate, neuroblastoma and cervical cancers. The LOH of 18q is more frequent in some cancers namely, HNSCC (40%), NSCLC (56%), colon cancer (60%), gastric cancer (61%) and up to 90% of pancreatic tumors (51, 68, 69). This means that genetic alteration of Smad4 in pancreatic tumors is as common as mutations in K-Ras (80%), p53 (70%) and CDKN2A (p16INK4a) (80%) (70). Inactivation of the genes encoding Smad4 occurs by loss of entire chromosome segments, small deletions, and frameshift, nonsense or missense mutations (71). Smad4 mutations

occur primarily in pancreatic carcinomas, in which the Smad4 gene was first identified as *DPC4* (deleted in pancreatic carcinomas) (72), and in colon carcinomas, and less frequently in other types of carcinomas. Whereas biallelic inactivation of Smad4 often occurs in pancreatic and colon carcinomas, haplo-insufficiency of the Smad4 locus may also contribute to progression of cancer (73, 74).

The notion that Smad4 acts as a tumor suppressor is supported by the occurrence of *MADH4* mutations in the germ line of a subset of juvenile polyposis families (75). Studies in which mice carried an inactivated allele of Smad4 support a role for this gene in tumor suppression. Whereas homozygous inactivation of Smad4 leads to early embryonic lethality, heterozygous mice are viable but develop intestinal polyps that can progress to carcinomas (73, 76, 77). When combined with an inactivated allele of the adenomatous polyposis coli (*Apc*) gene, simultaneous loss of the wild type alleles at both loci results in the development of multiple polyps and in progression to heterogeneous invasive adenocarcinomas (76). Although Smad4 is generally perceived as essential for TGF-beta responses, the loss of Smad4 function may not abolish TGF-beta responsiveness. This is supported by the observation that mouse fibroblasts derived from Smad4 $^{-/-}$ embryos (78), as well as some Smad4-deficient tumor cell lines (79, 80) retain at least some TGF-beta responses. Consistent with the above observation that TGF-β–induced synthesis of fibronectin can still occur in the absence of Smad4 (81). Thus, the high frequency of Smad4 deletions in tumors might represent a selective disruption rather than a complete abrogation of TGF-beta signaling. Inactivating Smad4 mutations have been found in conjunction with mutations in TGFβR2 or TGFβR1 (82). This observation strongly suggests that Smad4 tumor suppressive action is unrelated to TGF-beta signaling. In addition, loss of Smad4 expression may enhance Ras signaling and progression to undifferentiated carcinoma (83), further emphasizing the crosstalk between Smad4 and Ras signaling.

Recently, Smad4 has been shown to have a role in metastasis also. Smad4 knockdown inhibited TGF-β-induced epithelial to mesenchymal transition (EMT) of NMuMG cells as measured by morphologic transformation from epithelial to fibroblast-like cells, formation of stress fibers, inhibition of E-cadherin expression, and gain of expression of various mesenchymal markers (84). Knockdown of Smad4 in MDA-MB-231 breast cancer cells strongly inhibited the frequency of bone metastasis in nude mice by 75% and significantly increased metastasis-free survival (84). Matrix metalloproteinases (MMPs) appear to play a critical role in TGF-β-induced migration and invasion (85-87). Recently, a study from the same group showed that TGF-beta caused invasion in premalignant and highly malignant breast cancer cells by inducing expression of MMP2 and MMP9 in a Smad3/Smad4 dependent manner (88). Although previous studies have suggested a pro-metastatic role of Smad signaling in breast cancer and melanoma metastasis to lung and bone, in the case of colon cancer the scenario appears to be opposite. In patients with colorectal cancer (CRC), mutations or reduced levels of Smad4 have been correlated with reduced survival (89, 90). Results from our group indicate that loss of Smad4

expression in CRC enhances tumorigenicity and metastasis to the liver. Thus, Smad4 signaling plays a critical anti-metastatic role in CRC (91). Taken together, it is clear that Smad4 plays an important role in both tumor suppression and progression of cancer depending on the stage and tumor type.

7. UPREGULATION OF ANTAGONISTIC SMADS

Theoretically, inhibitors of the inhibitor of tumor formation would actually support tumor formation and as a result assist in cancer development. There are several known inhibitors of the TGF-beta signaling pathway, for example, the inhibitory Smads, Smad6 and Smad7, the E3 ubiquitin ligases, Smurf1 and 2, and the transcriptional repressors, Ski and SnoN. As expected hypothetically, over expression of these inhibitors is known to inhibit the tumor suppressive functions of TGF-beta by decreasing TGF-beta signaling and inhibiting TGF-β-induced growth arrest in tissue culture cell lines. Over expression and/or amplification of some of these inhibitory components have been detected in a subset of human tumors.

7.1 Smad7

The gene encoding Smad7 is located in the 18q21 region. This gene has been mapped between the genes encoding Smad2 and Smad4 within a 4 Mb cluster (92). Interestingly, when the frequency of deletion of the genes was evaluated in 233 DNA samples of colorectal cancers, Smad7 showed deletion in 48% of the cases. The frequency of deletion of Smad7 is much less than the frequency observed in the case of Smad2 and Smad4 (64 and 66% respectively). The Smad7 locus also appears to be more frequently amplified (10%) than the Smad2 (6%) and Smad4 (7%) loci (93). Therefore, retention and even amplification of Smad7 seems to be a selected event during the progression of colorectal tumors. This finding is in agreement with the frequent over expression of Smad7 observed in endometrial and thyroid follicular carcinomas (94, 95). Smad7 also assists TGF-beta in promoting tumorigenesis by modulating processes such as immune regulation, and microenvironment modification that cancer cells may exploit for their own benefit (96). There have been studies that suggest that Smad7 can promote tumorigenesis. Smad7 blocks TGF-beta mediated growth inhibition. A previous study from our group has shown that Smad7 also inhibits apoptosis in pancreatic as well as in colon cancer (97, 98). In a xenograft model, when primary keratinocytes were co-transfected with Smad7 and H-ras, mixed with dermal fibroblasts, and grafted into nude mice, they progressed into skin squamous cell carcinomas, while control cells did not (99). On the contrary, Smad7 has been reported to inhibit endometrial carcinomas, thyroid follicular tumors, and hepatocellular carcinomas (100-102).

In addition, Smad7 also plays a role in cancer progression by modulating cell invasion and metastasis. Over-expression of Smad7 in mouse mammary carcinoma JygMC(A) cells inhibits their metastasis (103). The inhibition of metastasis occurs via up-regulation of E-cadherin and down-regulation of N-cadherin, leading to a reduction in cell migration (104). A similar result was observed in a study which showed that Smad7 over expression inhibits the formation of osteolytic metastases by human breast cancer and melanoma cells (105-107). In the case of colon cancer, data from our group had shown that abrogation of Smad signaling by Smad7 induced liver metastasis in a splenic injection model (108). Therefore, the role of Smad7 in the process of tumor formation is very complicated and varies depending on tumor types and their microenvironments.

8. NON-SMAD TGF-BETA ANTAGONISTS

The non-Smad antagonists include the E3 ubiquitin ligases, Smurf1 and 2, and the transcriptional repressors, Ski and SnoN. There is evidence for over expression of the TGF-beta antagonist, Smurf2 in human tumors. In one study comprising 80 patients with esophageal squamous cell carcinoma (SCC), high levels of Smurf2 expression were detected by immunohistochemical staining in 56.3% of the surgical specimens (109). SKI also interacts with Smads to repress TGF-beta signaling proving its role as an antagonist of TGF-beta (110). SKI and the closely related gene, SnoN, can be both deleted or amplified in human tumors. In 179 colorectal cancer samples, partial or complete allelic loss was found in 41.5 and 55.2% for SKI and SnoN, respectively, whereas amplification was found in 10.1 and 15.1%, respectively (109). $SKI^{-/+}$ and $SnoN^{-/+}$ heterozygous mice show an increased number of lymphomas compared to wild-type mice when challenged with carcinogens (111,112). Increased expression of SKI/SnoN has been detected in many different tumor types including breast cancer, melanoma, and esophageal SCC (113–117), suggesting that it is in fact an oncogene. However, there are some deletions observed for the chromosome loci 1p36 and 3q36 where the SKI and SnoN genes are located, which would suggest that they act as tumor suppressors.

9. TARGETING THE SMAD PATHWAY

TGF-beta is a powerful multifunctional regulator of cell proliferation and differentiation. Hence, perturbation of its signaling plays a crucial role in various clinical disorders, including cancer. Since perturbation in the TGF-beta pathway plays an important role in promoting tumorigenesis (depending on the stage of the cancer) and cancer progression, there has been considerable effort in therapeutically targeting this pathway. As most of the activity of the TGF-beta signaling is mediated through the Smads, selective disruption of Smad protein–protein interactions is a potential target for therapeutics. To date this problem has been approached in two different ways. One is the development of 'aptamers'. These aptamers are oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Aptamers can be used to disrupt protein-protein interactions (111). Peptide aptamers that interact specifically with Smad proteins have been developed that inhibit TGF-beta responses. There are two classes of Smad interacting aptamers that have been developed thus far. The first class of Smad-interacting peptide aptamers is specific

and it interferes with the ability of Smad2 and Smad3 to interact with their transcriptional co-activators p300/CBP and their co-repressors TGIF, Ski/SnoN, FoxH1, and LEF1. In the first class of aptamers the Smad interaction motifs are introduced into the scaffold proteins and are expressed in HepG2 cells. Expression of aptamer/scaffold protein complexes specifically inhibited Smad mediated gene expression that was dependent on the Smad-protein interaction, whereas they do not inhibit other TGF-beta responses (119). The second class of Smad-interacting peptide aptamers is more general and blocks TGF-beta-induced signaling and EMT by inhibiting the interaction of Smad2 and Smad3 with its cytoplasmic anchoring partner SARA (Smad Anchor for Receptor Activation). This suppression of TGF-beta signaling is achieved by inhibition of Smad nucleo-cytoplasmic shuttling and complex formation with Smad4 (120). One of the pitfalls of the two classes of aptamers that were developed is that they are not selective between Smad2 and Smad3, because the proteins that were targeted bind both Smad2 and Smad3. In the future, peptide aptamers could be developed that are specific for Smad2 versus Smad3-interacting proteins. These Smad-specific aptamers may be more effective because a specific Smad response could be blocked. However, the specific TGF-beta responses that are mediated by Smad2 or Smad3 are not completely understood. As a result, determining how Smad2 versus Smad3 regulates the TGF-beta signaling pathway may enable us to develop better strategies for cancer therapies. In the future, the development of other aptamers that block Smad2 and Smad3 nuclear export or protein degradation might also prove to be effective means to target general Smad-mediated responses.

10. CONCLUSIONS

The role of TGF-beta in cancer biology is complex and involves aspects of tumor suppression as well as tumor promotion. The ability of TGF-beta to potently inhibit the proliferation of epithelial, endothelial, and haematopoietic cell lineages is central to the tumor-suppressive mechanism. Despite our remarkable progress in unraveling the TGF-β–Smad signaling pathway, many important issues remain unresolved. Our knowledge about the mechanisms of intracellular Smad signal transduction stems predominantly from experiments performed in cell-culture systems. One of the current challenges is to understand the functional importance of modulatory, context-dependent inputs into the core pathway. In order to understand how modulatory inputs shape a functional response a strong focus on endogenous signaling in intact tissues to complement functional studies in tissue culture will be required. One other critical issue is how activated R-Smads are de-phosphorylated, leading to recycling of Smads. What are the phosphatases that are involved in the de-phosphorylation of R-Smads? Since Smads activate or repress transcription of genes in the context of chromatin, it is important to understand the effects of Smad signaling on chromatin remodeling. Just as continuous nucleo-cytoplasmic shuttling of Smads facilitates sensing of TGF-beta receptor activity; endocytosis of TGF-beta receptor can also alter TGF-beta signaling. Experimental approaches need to be improved to better understand the roles of

endocytosis and intracellular routing of the TGF-beta receptor. It is possible that the Smad signaling pathway is activated through some signaling cross-talk (121). Are Smads the only signal transducers to receive signals directly from TGF-beta receptors that lead to changes in transcription? The mechanisms through which non-Smad signaling pathways are activated by the receptors and what these pathways contribute to the cellular response need to be better defined. However, there are still many questions that remain to be answered. What is the basis for the tissue specificity seen for the alterations in different components of the TGF-beta super family pathways? For instance, why is Smad4 predominantly mutated or deleted in colorectal and pancreatic cancers, but not in cancers derived from other organs? What triggers the switch between TGF-beta acting as a tumor suppressor, to it acting as a tumor promoter? Understanding the molecular events that are involved in TGF-beta function in normal cells and its lack of function in tumor cells should identify novel therapeutic targets in human cancers. Further studies will undoubtedly elucidate whether and precisely how certain Smads in the context of a given cell type can dictate the ultimate response to TGF-beta in human cancer.

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