Two sides of the story? Smad4 loss in pancreatic cancer versus head-and-neck cancer

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Abstract

TGFβ signaling Smads (Smad2, 3, and 4) were suspected tumor suppressors soon after their discovery. Nearly two decades of research confirmed this role and revealed other divergent and cancer-specific functions including paradoxical tumor promotion effects. Although Smad4 is the most potent tumor suppressor, its functions are highly context-specific as exemplified by pancreatic cancer and head-and-neck cancer: in pancreatic cancer, Smad4 loss cannot initiate tumor formation but promotes metastases while in head-and-neck cancer Smad4 loss promotes cancer progression but also initiates tumor formation, likely through effects on genomic instability. The differing consequences of impaired Smad signaling in human cancers and the molecular mechanisms that underpin these differences will have important implications for the design and application of novel targeted therapies.

Keywords

Fanconi Anemia; juvenile polyposis Smads; pancreatic cancer; squamous cell carcinomas; TGFβ signaling

Introduction

Mammalian Smads were discovered in 1996 as the intracellular signaling mediators of transforming growth factor β (TGFβ) and bone morphogenic protein (BMP) superfamily molecules and were named after their non-mammalian homologs (the Sma genes of C. elegans and the Mad (Mothers against decapentaplegic) gene from Drosophila) [1–3]. Smads regulate a variety of critical processes including embryonic development, fibrosis, tumor development, immune function, and wound healing [4]. TGFβ/BMP ligands initiate intracellular actions through transmembrane receptors that phosphorylate intracellular signaling molecules of the Smad family [5]. TGFβ signaling is mediated by receptor-specific Smads2/3 while BMP signaling is mediated by receptor-specific Smads1/5/8 (Fig. 1). Phosphorylated receptor Smads heterotrimerize with the common Smad (Smad4) and translocate into the nucleus where they bind Smad binding elements (SBEs) within the

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regulatory elements of TGFβ/BMP responsive genes [5]. Inhibitory Smads (Smad6 and Smad7) are also upregulated after TGFβ/BMP ligand binding, antagonize receptor-specific Smad actions through a variety of mechanisms, and serve as an endogenous negative feedback system to receptor Smad signaling [6]. Smad binding at SBEs modulates gene expression though recruitment of cell- or tissue-specific co-activators or co-repressors to target gene promoters [5,7].

Because TGFβ inhibits epithelial cell growth and promotes differentiation, defects in TGFβ/BMP signaling frequently promote tumor growth, and numerous molecules in these pathways including TGFβRI, TGFβRII, and Smad4 are established tumor suppressors [8]. However, epithelial cells with impaired TGFβ signaling frequently release additional TGFβ into the tumor microenvironment which can paradoxically promote tumor growth and progression by stimulating angiogenesis and inflammation within the stromal compartment where TGFβ signaling remains intact [9]. TGFβ/BMP ligands can also signal through non-canonical pathways including ERK, p38/JNK (c-jun activated kinase), Rho/Rac, and phosphatidylinositol-3-kinase (PI3K)/AKT; these pathways can be independent of, or cooperate with, Smad signaling [10].

Since Smads 2, 3, and 4 are all required for TGFβ-induced growth arrest [11], these molecules were suspected tumor suppressors soon after their discovery. For Smad4, this role was clearly substantiated by the identification of Smad4 as "Deleted in Pancreatic Cancer 4 (DPC4)" which lies within a region of chromosome 18q frequently lost in pancreatic cancer [12] and the observation that germ-line Smad4 mutations cause juvenile polyposis (JP), a condition characterized by intestinal polyp formation early in life and a lifetime gastrointestinal cancer risk of ~50% [13]. Studies in the past two decades have shown that Smad signaling is highly tissue and tumor specific. This review compares and contrasts the role of Smad4 loss in cancers arising from simple epithelia (as exemplified by pancreatic cancer) with squamous carcinomas arising from stratified epithelia (as exemplified by head-and-neck and esophageal cancer) to highlight the different consequences of impaired Smad signaling in human cancer.

The role of Smad4 loss in pancreatic cancer

Pancreatic cancer, the 4th leading cause of cancer death in the US with a 5-year survival of ~6% [14], progresses though a series of pre-neoplastic lesions with characteristic genetic alterations [15]. Activating Kras mutations are the most common initiating event and are found in >95% of pancreatic cancers [16]. Kras activation is typically followed by loss of tumor suppressors, including p16[INK4a] [17], p53 [18] and Smad4 [12] that then allow progression of pre-neoplastic lesions to full malignancy. Smad4 loss occurs late in the development of pancreatic cancer [19] and has been associated with metastatic disease [20]. Although both Smad4 mutation [21] and reduced expression [22] have been independently associated with reduced survival, the relationship between reduced Smad4 expression and worse clinical outcome has not been consistently observed [23].

Although Smad4 was initially identified as DPC4 [12] and is lost in at least 55% of pancreatic cancers through a combination of loss of heterozygosity (LOH) [12] and intragenetic mutation [21], in the presence of multiple other mutations (Kras activation, p16[INK4] loss, p53 loss), the role of Smad4 loss in human pancreatic cancer can only be evaluated at a correlative level. Since the rate of reduced Smad4 immunostaining (57%) is similar to that of genetic loss (deletion (45%) plus mutation (38%), see Table 1), genetic loss appears to explain the bulk of reduced Smad4 expression in pancreatic cancer. Germline Smad4 mutations are not associated with pancreatic tumors in humans [13], suggesting that Smad4 loss does not initiate human pancreatic cancer formation.

Several groups have used pancreatic-specific Cre recombinase strategies to study the role of Smad4 loss in both initiating and promoting pancreatic cancer development. Smad4 deletion mediated by the pancreas-specific Pdx1-Cre driver [24] or Ptf1a(p48)-Cre driver [25] had no discernible effect on pancreatic development and did not initiate pancreatic tumors formation in mice out to 70 weeks of age. However, Smad4 loss markedly promoted tumor development initiated by Kras[G12D] activation and Kras[G12D].Smad4−/− tumors exhibited both increased proliferation and tumor stromal formation [26]. Interestingly, Smad4 deletion increased the proportion of well-differentiated tumors initiated by a combination of Kras activation and p16[Ink4a] deletion [26], suggesting that TGFβ/Smad4 signaling promotes epithelial-mesenchymal transition (EMT) and that Smad4 loss prevents de-differentiation and EMT. Nevertheless, Smad4−/− tumors metastasized more frequently than Smad4+/+ tumors, suggesting that EMT does not always correlate with metastasis and is not the primary form of tumor invasion driven by Smad4 loss [27]. Two other studies confirmed that Smad4 deletion accelerated Kras[G12D]-initiated pancreatic tumor formation and also reported that Pdx-Cre.Kras[G12D].Smad4−/− mice develop squamous cell carcinomas in the forestomach or distal esophagus as well as mucinous pancreatic lesions [27,28]. Mechanistically, Kras[G12D].Smad4−/− pancreatic tumor cells exhibited no genomic instability [27]. In another study, Smad4 deletion accelerated growth and progression of hyperplastic pancreatic lesions initiated by PTEN deletion, perhaps through mechanisms involving activated Notch signaling and trans-differentiation [29]. In sum, these studies demonstrate that Smad4 loss cannot initiate pancreatic tumor formation, but promotes pancreatic tumor progression and increases metastases independent of TGFβ-mediated EMT.

The role of Smad4 loss in upper aerodigestive tract malignancies: head-and-neck squamous cell carcinoma (HNSCC) and esophageal squamous cell carcinoma (ESCC)

Squamous cell carcinomas (SCCs) arising from stratified squamous epithelial tissues of the upper aerodigestive track cause at least 20,000 deaths per year in US [14]. It has been consistently reported that Smad4 LOH occurs in ~30–50% of HNSCC and ESCC [30–35]. However, Smad4 point mutations are uncommon, occurring in <5% of these malignancies [30,36] (compared to ~35% of pancreatic cancer and ~12% of colon cancer; see Table 1). The significantly higher rates of LOH compared to mutation are consistent with genetic abnormalities in human cancer *i.e.*, for an average size gene, LOH occurs at a rate of 10^{-4} per cell division while point mutations occur at a rate of 10^{-7} per cell division [37]. In both HNSCC and ESCC, reduced Smad4 immunostaining is associated with more aggressive tumor behavior [38–41]. However, reports of reduced Smad4 expression in human HNSCC and ESCC vary significantly from as low as 12% (Xie, et al., in press; M. Reiss personal communication) to as high as 86% [34]. These differences are likely explained by a combination of the criteria used to define reduced Smad4 expression and the control group against which Smad4 expression was compared (*i.e.*, adjacent non-malignant tissue versus normal tissue from unrelated individuals). For instance *complete* Smad4 protein loss was reported in 12% of HNSCC (Xie, et al., in press; M. Reiss personal communication) and 63% of ESCC [40,41] while *reduced* Smad4 protein has been reported in 35% of HNSCC [38,39] (see Table 1). When we compared HNSCC and non-malignant adjacent mucosa *with oral mucosa from patients without cancer*, we found reduced Smad4 mRNA expression in 31/36 (86%) of HNSCC samples and in 24/36 (67%) of non-malignant adjacent mucosa [34]. Importantly, Smad4 immunostaining correlated with reduced mRNA expression [34]. We used a 50% reduction of mRNA expression to classify individual samples as "reduced" based on the finding that Smad4 haploid insufficiency promoted tumor development in our HNSCC animal model [34] (see below). Using these same criteria, had we compared Smad4

mRNA expression in tumors to adjacent non-malignant mucosa where Smad4 reduction has already occurred in some cases, we would have reported significantly less Smad4 reduction in HNSCC (17/36 or 47%) [34]. Since loss of at least one Smad4 copy has been consistently reported in 30–50% of HNSCC [31–34], reduced Smad4 immunostaining in less than 30% of HNSCC cases should raise questions about antibody specificity. Further, it remains to be determined if patient population, etiological factors (*e.g.*, tobacco vs. HPV), or sample storage method or length of time potentially contribute to the variation reported with reduced Smad4 in HNSCC.

In contrast to pancreatic cancer, our finding of Smad4 downregulation in preneoplastic oral mucosa suggests that Smad4 downregulation is an <u>early</u> event in human HNSCC development. To understand the role of Smad4 loss in HNSCC, we deleted Smad4 specifically in mouse oral epithelia and found that Smad4 loss caused spontaneous HNSCC formation [34]. Although deletion of a single Smad4 allele did not initiate HNSCC formation, it markedly accelerated HNSCC development initiated by oncogenic Kras^{G12D} activation [34], suggesting that Smad4 haploid insufficiency with a commensurate 50% Smad4 protein reduction could promote oncogene-initiated tumor development. Similarly, Smad4 haploid insufficiency also promoted the development of ESCC from PTEN deletion initiated hyperplasia [42]. With the observation that Smad4 downregulation occurs early in HNSCC development, these studies show that, in contrast to pancreatic cancer, Smad4 loss can initiate HNSCC formation.

One of our most intriguing findings may explain why Smad4 deletion initiated HNSCC development: in oral mucosa and HNSCC, Smad4 deletion down-regulates the Fanc/Brca (Fanconi Anemia Complementation/Breast cancer susceptibility) genes critical for maintaining genomic stability [34]. Germline mutations within this pathway cause Fanconi Anemia (FA) and while many FA patients die of chromosome instability-associated bone marrow failure as children [43], bone marrow transplants have allowed more FA patients to survive to adulthood where they subsequently they have a risk of HNSCC 500–700 fold higher than the general population and develop tumors at a median age of 27 years [44]. Smad4-loss dependent downregulation of Fanc/Brca expression and subsequent genomic instability may allow the accumulation of the genetic defects required for HNSCC initiation. Our study showed that downregulation of Fanc/Brca genes occurred within pre-malignant Smad4−/− oral epithelium, well before the development of overt malignancy, and Smad4−/− HNSCC had increased genomic instability as evidenced by increased centrosome number, genomic aberrations by aCGH, and sensitivity to the DNA damaging agent mitomycin C [34], all of which are characteristic of FA cells [44]. In addition, forced Smad4 expression in a Smad4-deficient cell line increased Brca1 and Rad51 expression with a commensurate increase in the appearance of these molecules at DNA repair nuclear foci [34]. Importantly, in human HNSCC and non-malignant adjacent oral epithelium, reduced Smad4 immunostaining correlated with reduced immunostaining for both Brca1 and Rad51 [34], suggesting that Fanc/Brca downregulation after reduced Smad4 expression also occurs in human HNSCC. Since mice with single Fanc gene deletions do not develop HNSCC [45], it is possible that downregulation of multiple Fanc/Brca genes after Smad4 loss eliminates functional compensation among Fanc/Brca complementation groups, hence facilitating HNSCC initiation. Our study also suggests intrinsic differences in the role of Smad4 regulation of Fanc/Brca, and consequently genomic stability, in the upper and lower digestive tract (or stratified versus simple epithelia layers). Although Smad4 loss is common late in pancreatic and colon cancer, targeted Smad4 deletion to the pancreas did not initiate pancreatic cancer [24,25], perhaps because Smad4 does not regulate Fanc/Brca genes in simple epithelium; a hypothesis is supported by the observation that Smad4−/− pancreatic tumors did not exhibit genomic instability [27]. An alternative hypothesis, which is supported by the observation that FA patients do not have an increased incidence of colon or

pancreatic cancer, despite their susceptibility to HNSCC [43], is that, like hematopoietic cells, simple epithelial cells with Fanc/Brca down-regulation (regardless of whether mediated by Smad4 loss or not) may undergo apoptosis that prevents them from developing into cancer.

Another potential mechanism related to the initiation capacity of Smad4 loss in HNSCC is Smad4 loss-associated inflammation. Mice with Smad4 deletion in T cells developed inflammation in the gastrointestinal tract and lower intestinal malignancies reminiscent of juvenile polyposis (JP) as well as oral SCCs [46]. Similarly, our murine Smad4−/− HNSCC have increased expression of inflammatory cytokines with an associated infiltration of neutrophils, T cells, and Th17 cells [34]. Interestingly, Smad4 loss-associated submucosal inflammation was abrogated in a Smad3+/− background, suggesting that inflammation in Smad4−/− HNSCC requires Smad3-dependent TGFβ signaling. Supporting this notion, Smad4−/− HNSCC expressed more TGFβ1 ligand and had evidence of increased signaling through both Smad3 and phospho-Smad1/5/8 in tumor epithelial cells [34], consistent with increased BMP signaling after TGFβ signaling disruption [47]. Hence, it is likely that Smad3-dependent inflammation after Smad4 deletion reflects Smad3-mediated chemokine/cytokine production by epithelial cells. Consistent with our study, loss of pSmad2/3 staining in human HNSCC was associated with improved survival (Xie, et al., in press; M. Reiss personal communication), further supporting the tumor promoting effect of epithelial Smad3 activation in human HNSCC. These studies also highlight the context-specific nature of TGFβ signaling in immune response, as Smad3 loss in T cells abrogates TGFβ-induced immune suppression [48] while Smad3 loss in epithelial cells abrogates pro-inflammatory TGFβ actions [34].

Consistent with the finding in pancreatic cancer that Smad4 is required for TGFβ-mediated EMT but that EMT is not always required for metastasis, Smad4−/− SCCs do not undergo early-stage EMT [49] but retain the ability to metastasize [34]. Similarly, although ESCC induced by simultaneous PTEN and Smad4 deletion retain terminal differentiation markers early in carcinogenesis, they also metastasize [42]. That Smad4−/− tumors retain the ability to metastasize in the absence of EMT, suggests that Smad4 loss promotes metastases through other mechanisms such as increased inflammation and angiogenesis within the tumor associated stroma. Overall the phenotype of murine Smad4−/− HNSCC (reduced FA/Brca expression, lack of EMT) suggests that human HNSCC with reduced Smad4 expression may be more sensitive to specific therapies. For example, epidermal growth factor receptor (EGFR) inhibitors are more effective in HNSCCs that retain E-cadherin expression [50] and poly(ADP-ribose) polymerase (PARP) inhibitors are more effective in tumors with Fanc/Brca defects [51,52].

Smad4 loss in cancers similar to pancreatic cancer: colon cancer, cholangiocarcinoma, and prostate cancer

Colorectal cancer, is the 2nd leading cause of cancer death with ~140,000 new cases and ~50,000 deaths per year in the US [14], and also progresses through a series of pre-neoplastic lesions with well-known genetic alterations [53,54]. Polyp formation is typically initiated by APC (adenomatous polyposis coli) gene inactivation [55,56]; Kras mutation and p53 inactivation then allow adenoma growth and progression to carcinoma [57,58]. Similar to pancreatic cancer, Smad4 loss occurs through a combination of genetic deletion or mutation, is present in ~40–50% of colon cancers, occurs around the transition from benign to invasive carcinoma [59], and is associated with metastasis, advanced disease, and reduced survival [60–63]. Although germline Smad4 mutations are found in ~20% of juvenile polyposis (JP), a rare autosomal disease characterized by numerous intestinal polyps and a lifetime colon cancer risk of ~50% [13], not all germline Smad4 mutations cause JP [64].

In mice, homozygous germline Smad4 deletion is lethal between embryonic days 6.5 and 8.5 due to failure of mesoderm induction [65,66]. Consistent with JP caused by heterozygous Smad4 loss in humans, mice with heterozygous germline Smad4 deletion also develop inflammatory polyps in the glandular stomach and duodenum beginning at about 1y of age [67,68]. These polyps occur after loss of the second Smad4 allele, have an inflammatory stromal component that resembles the polyps seen in human JP [69], and can eventually degenerate into carcinoma [67,68]. The long latency of inflammatory polyp formation suggests that epithelial Smad4 loss alone may be insufficient for tumor initiation. Supporting this notion, Smad4 deletion in T cells causes intestinal inflammation and intestinal tumors that are also reminiscent of the lesions seen in human JP [46], suggesting that Smad4 signaling in T cells plays a critical role in tumor suppression, particularly in the GI tract, and that tumorigenesis in JP or heterozygous germline Smad4 deletion may be related to Smad4 loss in the immune system as opposed to Smad4 loss in epithelial cells [70]. Similar to pancreatic cancer, Smad4 loss plays an important role in tumor promotion in colon cancer. Heterozygous Smad4 loss promotes the development of colon cancers initiated by germline APC gene deletion [66] and APC+/−Smad4−/− tumors recruit immature CD34+/CCR1+ myeloid cells to the tumor front that then promote tumor invasion [71], again suggesting inflammation as an important component of colon cancer initiation. In aggregate, both clinical and animal studies suggest that Smad4 loss in simple epithelia may not initiate cancer formation, but clearly promotes cancer growth and progression. Conversely, while JP patients with heterozygous Smad4 mutations are markedly susceptible to colon cancer, they do not have an increased incidence of HNSCC or other cancers arising from squamous epithelia [13], implying that loss of one Smad4 allele does not select mutant Fanc/Brca cells for outgrowth or is insufficient to downregulate the Fanc/Brca pathway and cause tumor formation in head-and-neck tissue.

Other mouse models of Smad4 deletion have deepened and supported the findings outlined above. For example, Smad4 deletion targeted to the liver with an albumin-Cre driver did not initiate cholangiocarcinoma formation but homozygous Smad4 deletion promoted development of cholangiocarcinoma initiated by PTEN deletion [72]. This suggests the role of Smad4 in cholangiocarcinoma and pancreatic cancer may be similar; that Smad4 immunostaining is lost in 45% of cholangiocarcinomas and is associated with more aggressive tumor behavior supports this idea [73]. A similar pattern occurs in prostate cancer. Prostate specific Smad4 deletion did not initiate cancer formation but increased both prostate cancer progression initiated by PTEN deletion and the frequency of metastases to local lymph nodes and lung [74]. Similar to SCCs [75,76], cyclin D1 upregulation may mediate tumor promotion after Smad4 loss in pancreatic cancer [74]. In human prostate cancer, Smad4 LOH occurs in ~25% of cases [77] and Smad4 mutations are uncommon [77], but promoter methylation may contribute to reduced Smad4 expression [78].

Smad4 loss in cancers similar to HNSCC: SCCs arising from skin and mammary glands

Similar to HNSCC, we found Smad4 LOH in 57% of human skin SCCs with reduced Smad4 immunostaining in 70% [49]. Although it remains to be determined whether Smad4 downregulation occurs early in skin SCC, various mouse models show that Smad4 loss can initiate skin SCC. Keratinocyte-specific Smad4 deletion mediated by K5-Cre interrupted hair follicle cycling causing hyperproliferative hair follicles, progressive hair loss, and predominantly well-differentiated skin SCCs consistent with both the role of Smad4 as an in initiator in stratified epithelia and the critical role of Smad4 in mediating TGFβ-dependent EMT [75]. Smad4 deletion driven by mouse mammary tumor virus (MMTV)-Cre caused a similar skin phenotype with hair loss, disruption of hair follicle differentiation, and the formation of well-differentiated SCC [76]. Our unpublished data reveal that Smad4 loss

initiates tumor formation by disrupting DNA repair mechanisms similar to that observed in HNSCC. Similar to HNSCC, Smad4 loss activates survival factors including increased AKT signaling, activation of cyclin D1, and increased c-myc expression [75,76] which promotes the growth of Smad4−/− skin stem cells resulting the development of multiple cancer types including sebaceous adenomas and basal cell carcinomas [75,76]. Smad4 loss also promotes PTEN deletion-initiated skin tumor formation [75]. Similar to HNSCC, Smad4−/− skin SCC has increased angiogenesis and inflammation [79]. Interestingly, Smad4 deletion targeted to the mammary gland (by either MMTV-Cre or WAP-Cre) causes mammary abscesses and mammary tumors with transdifferentiation to squamous histology and blockade of EMT [80]. These data clearly demonstrate that Smad4 loss initiates SCC formation and promotes tumor progression through increased inflammation and angiogenesis while preventing TGFβ-dependent EMT.

Smad2 loss and cancer

Smad2 maps to 18q21, near the Smad4 locus [81]; however, Smad2 genetic losses have not been described in pancreatic cancer. Although Smad2 mutations occur infrequently in colon cancer (~5%) [81,82], reduced Smad2 immunostaining has been associated with shortened survival [83]. Germline Smad2 deletion in mice is embryonic lethal due to gastrulation failure [84–87], but Smad2 heterozygotes are viable, fertile, and do not develop spontaneous tumors [88]. Smad2 deletion has not been specifically targeted to either the pancreas or colon but Smad2+/−APC+/− compound heterozygotes had larger and more invasive colonic tumors than APC+/− controls without an increase in tumor number [88]. These studies suggest that while Smad2 functions as a tumor suppressor in cancers arising from simple epithelia, Smad2 loss alone is insufficient to initiate tumor formation.

Smad2 downregulation is more common in SCCs than in simple epithelial tumors. Although Smad2 mutations and deletions are infrequent in both primary HNSCC and HNSCC cell lines [32,89,90], Smad2 LOH was detected in 63% of HNSCC cell lines [33]. Similarly, we found that 70% of skin SCCs (and 100% of poorly differentiated skin SCCs) had reduced Smad2 immunostaining [49]. Among poorly differentiated skin SCCs, 94% had a >50% reduction in Smad2 mRNA expression compared to normal skin; of these samples, 67% exhibited Smad2 LOH [49]. These observations show that while Smad2 mutations and deletions are uncommon in SCCs, Smad2 LOH is frequent and associated with reduced Smad2 expression and de-differentiation. That reduced Smad2 expression is far more common in poorly differentiated skin SCC [49] suggests a role in SCC progression rather than initiation. Like Smad4, the incidence of reduced Smad2 immunostaining in human SCC varies widely from 14% of HNSCC with absent Smad2 immunostaining [38] to 70% of skin SCCs with reduced Smad2 immunostaining [49].

To understand the role of Smad2 in skin carcinogenesis we targeted Smad2 deletion to epithelial cells using a keratin 5 (K5) promoter [49,91]. While neither K5.Smad2−/− nor K5.Smad2+/− mice developed spontaneous skin tumors, when subjected to a two-stage chemical skin carcinogenesis protocol both K5.Smad2−/− and K5.Smad2+/− animals exhibited accelerated tumor formation with earlier malignant conversion, increased EMT, and reduced differentiation [49]. Similarly, germline Smad2+/− mice did not develop spontaneous tumors, but had increased susceptibility to chemical skin carcinogenesis and also develop more poorly differentiated tumors [92]. These data suggest that Smad2 exhibits haploid insufficiency with respect to tumor suppression, a criteria that should be taken into consideration when analyzing Smad2 downregulation in human cancer samples. Mechanistically, we found that Smad2 loss increased Smad3/4 binding at the Snail SBE, leading to increased Snail expression and reduced E-cadherin [49]. In addition, both Smad2−/− skin SCCs and Smad2−/− non-malignant murine skin had increased angiogenesis

through a similar mechanism involving increased hepatocyte growth factor (HGF) expression [91] and human skin and head and neck SCC with reduced Smad2 expression also have higher HGF expression [91]. In contrast to Smad4−/− SCCs, Smad2−/− SCCs did not have increased expression of either TGFβ or VEGF [91]. This suggests that tumors with reduced Smad2 expression may be susceptible to HGF-targeted treatment, a hypothesis supported by our study showing that short term treatment of Smad2−/− skin with a c-Met (HGF receptor) inhibitor reduces Smad2 loss-associated angiogenesis [91].

Smad2 mutations also occur infrequently (<5%) in lung cancer, cervical cancer, and hepatocellular carcinoma [93–95]. Targeted Smad2 disruption in T cells reduced Th17 cell differentiation [96,97] and Smad2 deletion in B cells prevented immunoglobulin class switching [98] but tumors were not described in any of these models. This is in contrast to the tumor formation seen after Smad4 deletion in T cells [46] and suggests different roles for Smad2 and Smad4 in immune cell homeostasis and tumor suppression. Other studies have also consistently shown that Smad2 deletion in various tissues (central nervous system, kidney, ovary, liver) is insufficient to initiate tumor formation [99–102]. Similar to our observation in skin carcinogenesis, Smad2 deletion in hepatocytes did not affect liver development or cause tumors but Smad2−/− hepatocytes undergo spontaneous EMT and have increased migration and proliferation *in vitro* [102]. In sum, Smad2 loss alone is insufficient for tumor initiation but promotes tumor progression through increased EMT and angiogenesis.

The role of Smad3 in cancer

Smad3 is located at 15q22 and mutations are associated with familial thoracic aortic aneurysms [103]. Smad3 mutations were not initially found in either human colon cancer or pancreatic cancer [104], though subsequent analyses identified a low frequency of Smad3 mutations in colon cancer [82]. The clinical significance of Smad3 mutations is unclear as other reports describe increased Smad3 immunostaining in colon cancer [105] and increased phospho-Smad3 immunostaining in ulcerative colitis lesions [106], suggesting that *activated* TGFβ/Smad3 signaling may be present in, and contributing to, these inflammatory lesions. While one of three independently described germline Smad3 knockout mouse models developed metastatic colon carcinomas [48,107,108], a later study suggested that colon tumor formation in these animals required chronic helicobacter infection that drove local inflammation [109]. Although Smad3 germline deletion increased multiplicity of APC initiated tumors in the distal colon [110], it is unclear whether this related to Smad3 loss in colonic mucosa or Smad3 loss in immune cells, particularly since Smad3−/− animals exhibited chronic intestinal inflammation [48] that could have promoted APC-initiated tumors.

Similarly, Smad3 mutations are rare in human HNSCC [30] and reduced Smad3 immunostaining is uncommon (0–7%) in HNSCC, ESCC, and skin SCC (Xie, et al., in press; M. Reiss personal communication) [49,111]. Smad3 may be tumor suppressive in keratinocytes as suggested by the observation that Smad3 deletion increased SCC formation of grafted, v-H-ras-transduced keratinocytes [112]. However, Smad3+/− and Smad3−/− mice are resistant to chemical skin carcinogenesis [92,113] and we found that Smad3−/− skin tumors have reduced inflammation [113], again suggesting that Smad3 mediates TGFβ-induced inflammation. Keratinocyte molecular profiling demonstrates that Smad3 mediates expression of both tumor suppressive and tumor promoting TGFβ-responsive genes [114]; however, on balance, it appears that the inflammatory actions of Smad3 are more potent than its tumor suppressive actions. Smad3 deletion accelerates both skin and oral wound healing by reducing local inflammation [115,116] but conversely, impairs colonic wound healing [117]. Because these models all employed germline Smad3 deletion, it cannot be

discerned if the observed effects are related to Smad3 loss in the epithelium or in the immune cells recruited to these different anatomic locations. Nevertheless, these studies highlight the context-specific nature of Smad3 function in inflammation.

The role of Smad3 in other malignancies is less clear. Smad3 mutations are infrequent in human lung and breast cancer [82,104,118]; however, reduced Smad3 mRNA or protein expression has been seen in endometrial cancer, gastric cancer, and parathyroid adenomas [119–121]. The best evidence for Smad3 as a tumor suppressor in human cancer comes from pediatric T cell acute lymphoblastic leukemia where there is no Smad3 protein expression despite the absence of Smad3 mutations [122]. Animal data is similarly sparse. Forced Smad3 expression in hepatocytes protects against chemical liver carcinogenesis by promoting apoptosis through Bcl2 [123], suggesting a tumor suppressive role for Smad3 in mouse liver carcinogenesis. In contrast, Smad3 deletion slows gonadal tumor development in inhibin null mice [124], suggesting a tumor promoting role in this setting. In sum, to better define the roles of Smad3 in various malignancies will require additional studies more specifically targeting Smad3 loss or overexpression to specific cell types or tissues.

Implications of signaling Smad status in cancer therapy

Research on TGFβ signaling Smads in cancer has revealed their diverse roles at many levels: individual signaling Smads have different roles in a given cancer, while the same Smad can have different roles depending on the cancer type. Because the levels and functions of Smads are tightly regulated, reduced expression of one Smad can critically affect this balance such that while individual Smads (particularly Smads 2 and 4) are generally tumor suppressive, hyperactivation of Smad2, 3, and 4 can promote EMT [49,125] and angiogenesis [79, 91] (see Fig. 2). Additionally, alterations in each Smad could affect the balance between Smad-dependent and Smad-independent TGFβ signaling, and many of the Smad-independent TGFβ pathways are potentially oncogenic [10]. These dynamic and complex relationships make directly targeting Smads for cancer therapy challenging. However, the downstream or alternative pathways activated after Smad loss are potential therapeutic targets, meaning that Smad status is a biomarker for drug susceptibly. For example, Brca mutant breast cancers are more sensitive to PARP inhibitors that prevent DNA repair and induce DNA damage-associated cell death [51,52]. Since Smad4−/− keratinocytes have reduced Fanc/Brca expression and are similarly sensitive to DNA damage, it will be interesting to see whether HNSCC with reduced Smad4 expression are similarly sensitive to PARP inhibitors. Of note, this type of targeted therapy may not be applicable to all Smad4 deficient cancers, specifically pancreatic and colon cancers, where Smad4 loss is not associated with genomic instability [27]. Smad4−/− SCCs also have increased TGFβ-mediated inflammation and VEGF-driven angiogenesis [34,79], suggesting that targeting the tumor stroma with a TGFβ receptor inhibitor or a VEGF inhibitor might be effective. Finally, we have also shown that short-term treatment with a c-Met inhibitor reduces Smad2 loss-associated angiogenesis [91]; it remains to be seen if c-Met inhibitors have clinical efficacy in human tumors with reduced Smad2 expression.

Future perspectives

Although the frequency of genetic alteration or reduced Smad expression are comparable between cancers arising from simple (pancreatic/colon) and stratified epithelial layers (SCCs), numerous studies show that the consequences of reduced Smad expression (particularly Smad4) differ significantly among these malignancies and suggest that this could substantially alter the therapeutic strategies employed to target these cancers. Well controlled clinical trials that include measurement of relevant biomarkers will be required to determine which of these basic scientific observations are ultimately relevant to cancer

treatment. Additionally, studies in other cancer types will be required to determine which model of Smad4 loss they follow. For example, will breast cancers harboring Smad4 mutations [126] be as sensitive to PARP inhibitors as Brcamutant breast or ovarian cancers? Similar questions exist for lung cancer where Smad4 mutations also occur [127], but the Smad4 loss phenotype has not been determined. In addition, Smad4 being more frequently inactivated in HPV-positive tumors [128], suggests that different mutagens could affect TGFβ signaling and hence impact targeted treatment paradigms. In sum, the TGFβ/Smad field is moving closer to translating outstanding basic science discoveries into therapeutic cancer strategies.

Acknowledgments

Abbreviations

APC	adenomatous polyposis coli
BMP	bone morphogenic protein
Brca	breast cancer susceptibility gene
DPC4	Deleted in Pancreatic Cancer 4
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ESCC	esophageal squamous cell carcinoma
FA	Fanconi Anemia
Fanc	Fanconi anemia complementation
HGF	hepatocyte growth factor
HNSCC	head-and-neck squamous cell carcinoma
HPV	human papilloma virus
LOH	loss of heterozygosity
JP	juvenile polyposis
MMTV	mouse mammary tumor virus
PARP	poly(ADP-ribose) polymerase
PTEN	phosphatase and tensin homolog deleted on chromosome 10
SBE	Smad binding element
SCCs	squamous cell carcinomas
TGFβ	transformation growth factor β
VEGF	vascular endothelial growth factor

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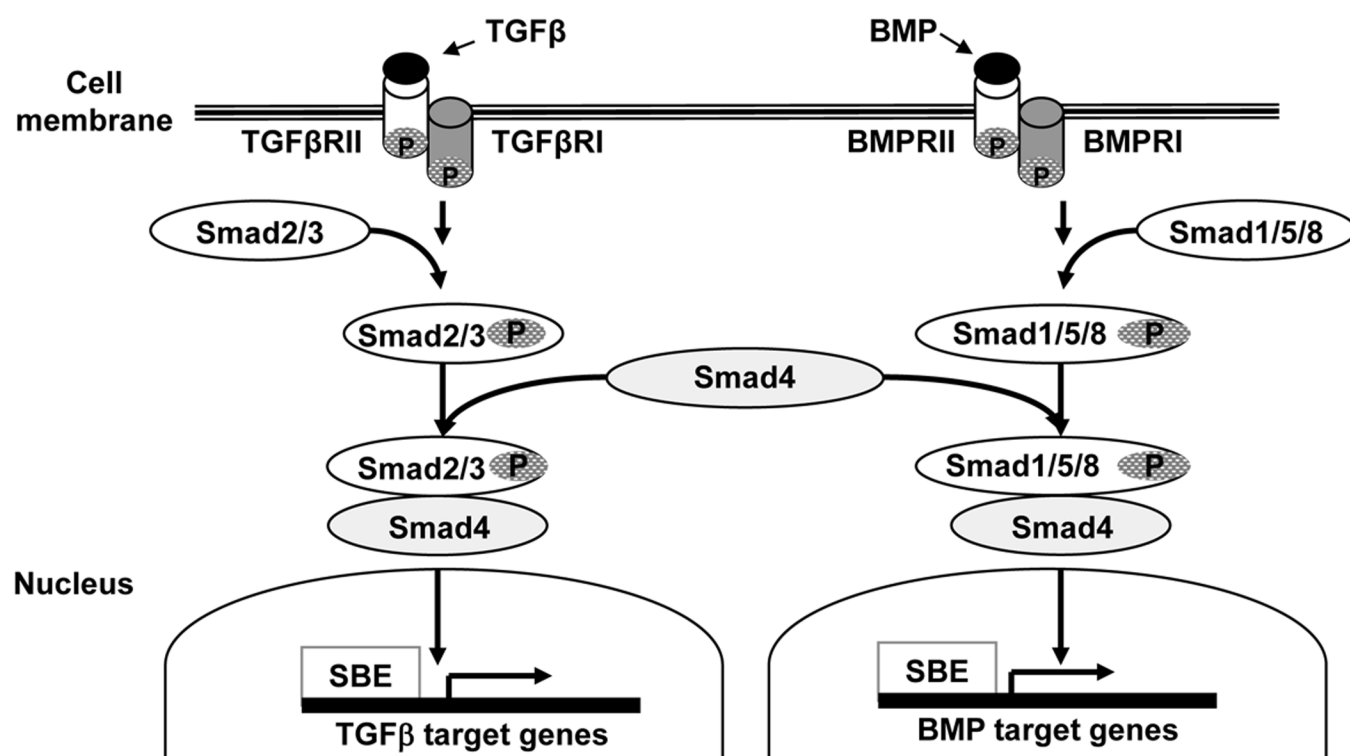
Figure 1. TGFβ/BMP signaling
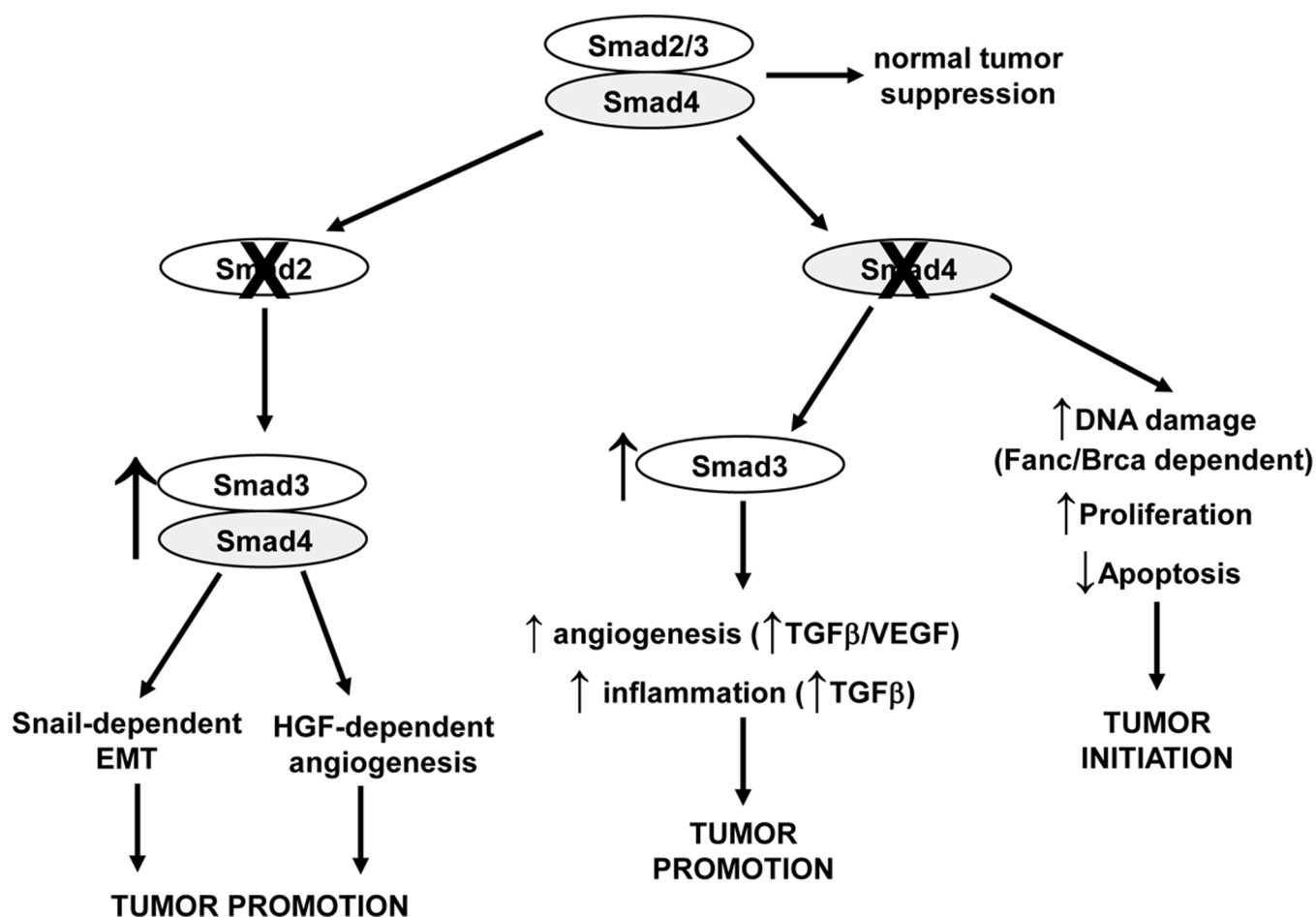TGFβ/BMP ligands mediate their signals through Smad family members.

Figure 2. Roles of Smads identified in SCCs
In non-malignant epithelial tissues, Smads function as tumor suppression. Smad2 loss
promotes tumor progression through increased EMT and increased HGF while Smad4 loss
promotes tumor progression through increased angiogenesis and inflammation mediated by
increased VEGF and TGFβ expression, respectively. In addition, Smad4 loss initiates SCC
formation in stratified epithelial layers via downregulation of Fanc/Brca family members.

Table 1

Mechanisms of reduced Smad4 expression in pancreatic cancer compared to HNSCC and ESCC.

Pancreatic cancer	N/T (Reduced)	% (Reduced)	Associations/Notes	Ref
Loss 18q	25/84	30%		[12]
	15/25	60%		[128]
	15/35	42%	Poor prognosis (w/ loss of 12q and 17p)	[129]
	40/89	44%	Reduced survival	[21]
	105/233	**45%**	**Total (from the above reports)**	
Mutation	6/27	22%		[12]
	20/38	53%		[15]
	4/12	33%		[130]
	30/77	**38%**	**Total (from the above reports)**	
mRNA	6/21	29%		[131]
IHC	9/18	50%		[132]
	39/59	66%		[133]
	138/249	55%	Reduced survival	[22]
	63/119	52%	Improved survival after resection	[134]
	8/34	24%	Reduced differentiation, stage IV	[135]
	75/88	85%	Nodal involvement, reduced survival	[131]
	40/65	61%	Metastases	[20]
	65/124	52%	Increased vessel density, improved survival	[136]
	437/756	**57%**	**Total (from the above reports)**	
HNSCC				
LOH 18q	>5/32	>15%		[30]
	7/15	47%		[31]
	8/23	34%		[32]
	20/47	54%		[33]
	6/18	33%		[34]
	47/135	**35%**	**Total (from the above reports)**	
Mutation	1/32	3%		[30]
mRNA	31/36	86%	vs normal controls	[34]
IHC	38/170	22%	LN metastases	[38]
	61/108	61%	Grade, LN metastases	[39]
	41/117	**35%**	**Total (from the above reports)**	
ESCC				
LOH 18q	5/14	35%		[35]
mRNA	97	NA	Non-response to chemo	[137]
	70	NA	Higher stage, worse prognosis	[41]
IHC	174/258	67%	LN mets, higher stage	[40]

Pancreatic cancer	N/T (Reduced)	% (Reduced)	Associations/Notes	Ref
	41/80	51%	Invasion, higher stage	[41]
	215/338	**63%**	**Total (from the above reports)**	

N/T: Number of reduced cases vs. total cases examined. Pancreatic data are for pancreatic ductal adenocarcinoma only (not mucinous cystic neoplasms and intraductal papillary mucinous neoplasms). In some instances insufficient data were provided to calculate a frequency of reduction.