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Smad4: gatekeeper gene in head and neck squamous cell carcinoma

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Unchecked cell growth is a hallmark of cancer. During oncogenesis, cancerous cells become resistant to the TGF-β signaling pathway that usually keeps cell growth in check. The role of a critical mediator of this pathway, Smad4, in head and neck squamous cell carcinoma (HNSCC) remains unclear. In this issue of the *JCI*, Bornstein and colleagues report that Smad4 expression is decreased in malignant HNSCC and, surprisingly, also in normal-appearing buccal mucosa adjacent to HNSCC (see the related article beginning on page 3408). They also show that targeted conditional deletion of *Smad4* in the head and neck epithelium of mice is alone sufficient to initiate spontaneous HNSCC, in conjunction with DNA repair gene dysregulation, genetic instability, and inflammation. These findings point to a novel function for *Smad4* as a guardian gene that maintains genomic stability.

The vast majority of head and neck cancers are squamous cell carcinomas (HNSCCs), all of which arise from a mucosal surface. HNSCCs can include cancers of the mouth, larynx, pharynx, tongue, lip, or nasal cavity but traditionally do not include cancers of the thyroid, esophagus, or skin. The malignancy is more prevalent in males and in individuals who smoke or chew tobacco and/or consume alcohol (1, 2). Certain viral agents, such as human papilloma virus types 16 and 18, increase the risk of developing HNSCC in the oral cavity (1, 2). In spite of considerable advances in our understanding of the molecular alterations that occur in this malignancy, the 5-year survival rate has stubbornly remained at approximately 50%, due to resistance to therapy; cancer recurrence following surgical resection even when followed by chemo- or radiotherapy; and the development of second, unrelated malignancies (1–3).

Key genetic alterations known to exist in HNSCC include (a) overexpression of the growth factor receptor EGFR; (b) mutations in the tumor suppressor gene *p53*; (c) mutation or overexpression of the oncogenes *K-ras* or *H-ras*; (d) increased levels of the cell-cycle regulator and proto-oncogene cyclin D1, the cytokine IL-6, the transcription factor runt-related transcription factor 2 (RUNX3), and the inflammatory mediator COX2; (e) excessive activation of PI3K/Akt, STAT3, and NF-κB pathways fundamental to cell proliferation and survival; (f) germline mutations in the Fanconi anemia/breast cancer susceptibility gene (Fanc/Brca) pathway, which coordi-

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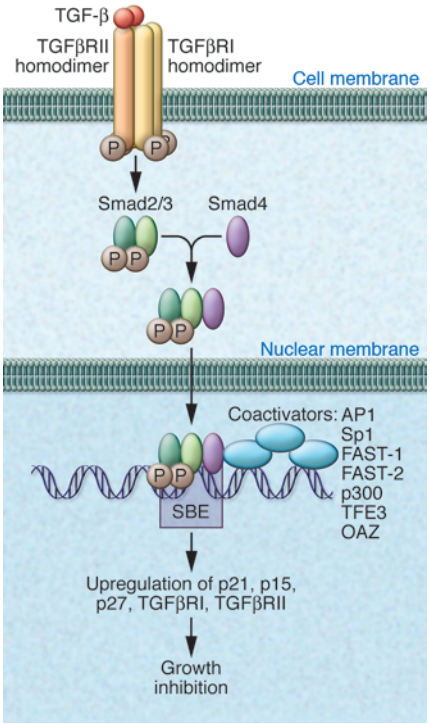

Figure 1
TGF-β signaling. A TGF-β dimer is shown binding to a homodimer of TGFβRII, which then recruits a TGFβRI homodimer. The heterotetrameric complex allows TGFβRI to undergo serine-threonine phosphorylation, which activates its kinase activity and phosphorylates Smad2 and Smad3. These phosphorylated Smad proteins associate with Smad4, and the resultant complex translocates to the nucleus, where it can interact with coactivators such as AP1, Sp1, FAST-1 and the related transcription factor FAST-2, p300, TFE3, and OAZ (8–10) and corepressors (not shown) to modulate gene transcription. For example, genes encoding p21, p15, and p27 are upregulated through interactions between Smad2/3/4-coactivator complexes and Smad-binding elements (SBE). The upregulated proteins then act to inhibit cell-cycle progression and proliferation.

nates DNA cross-link repair; (g) susceptibility to loss of heterozygosity (LOH; the loss of normal function of one allele of a gene when the other allele has already been inactivated); and (h) multiple alterations in TGF-β signaling pathways (2–6).

Overview of TGF-β signaling

To better understand the study reported by Bornstein and colleagues in this issue of the *JCI* (7), it is important to review the signaling pathways that are activated by TGF-βs; these are encoded by genes that belong to the TGF-β superfamily, which includes three mammalian TGF-β isoforms, activins, inhibins, myostatin, growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs). TGF-βs regulate many cellular processes; enhance mesenchymal cell proliferation; inhibit epithelial cell proliferation; promote leukocyte infiltration; are chemotactic toward fibroblasts, monocytes, and blood polymorphonuclear cells; regulate the ECM; and support communications between the ECM and cellular cytoskeleton (8).

TGF-β signaling is mediated by the type I and type II TGF-β receptors (TGFβRI and TGFβRII, respectively) (Figure 1), which are transmembrane proteins with principally serine-threonine kinase activity (8–10). There are five different type II receptors that bind different members of the TGF-β superfamily, occasionally binding to more than one member of the family. For example, there are two type II activin receptors, termed A and B, that bind activin, nodal, and myostatin, and there is one type II BMP receptor that binds BMPs and GDFs (10). By contrast, there is only one TGFβRII, and it only binds dimeric TGF-β ligand (10). The ligand-bound TGFβRII homodimer associates with a TGFβRI homodimer, and the resultant heterotetrameric complex then initiates a signaling cascade (8–10) (Figure 1). TGFβRI is phosphorylated within its GS region (i.e., SGSGSG sequence) by TGFβRII, allowing it to phosphorylate downstream receptor-regulated Smads (R-Smads) — Smad2 and Smad3 — at their C-terminal SSXS motif (10, 11).

Once activated, R-Smads oligomerize with the common mediator Smad4, and the complex translocates to the nucleus, where, along with coactivators and corepressors, it regulates gene transcription (8–11) (Figure 1). Smad4 can then interact with protein complexes to modulate gene expression. Smad4, which lacks a C-terminal SSXS domain, associates (through a Smad-binding motif [CAGAC]) with many Smad-interacting DNA-binding proteins, including coactivators such as activator protein 1 (AP1), specificity protein 1 (Sp1), forkhead activin signal transducer 1 (FAST-1) and the related transcription factor FAST-2, p300, transcription factor for immunoglobulin heavy chain enhancer 3 (TFE3), and Olf-1/EBF–associated zinc finger (OAZ) (8–11). In contrast to the role of Smad4, Smad1, -3, -5, and 8 generally mediate the actions of BMPs, whereas inhibitory Smads (Smad6 and Smad7) act to suppress Smad2/3 activation (8–11).

Deleterious role of TGF-β pathways in cancer

It is well established that components of the TGF-β pathway act as tumor suppressors (11). Nonetheless, TGF-β actions are context dependent, and increased TGF-β expression has been observed frequently in the setting of perturbations in TGF-β signaling pathways and in turn contributes to tumor growth in many epithelial cell–derived cancers (10, 11). TGF-β's tumor-promoting activity has been principally attributed to its paracrine effects: (a) enhancing tumor angiogenesis; (b) altering components of the ECM; (c) causing aberrant epithelial-mesenchymal interactions; (d) enhancing substratum adhesiveness, which facilitates tumor metastasis; and (e) suppressing cancer-directed immune responses (10, 11).

There is also a strong correlation between malignant progression and loss of sensitivity of the cancer cells to the growth-inhibitory effects of TGF-βs. This resistance to TGF-β–mediated growth inhibition may be due to a variety of alterations in TGF-β signaling pathways: (a) decreased expression of TGFβRII, as in gastric cancer, or TGFβRI, as in prostate and pancreatic cancers (12–14); (b) mutations in the genes encoding both receptors, as in colon and gastric cancers (15, 16); (c) mutations in the *Smad4* gene, as in pancreatic cancer (17); (d) Smad4 underexpression, as in HNSCC (7); or (e) overexpression of inhibitory Smad7, as in pancreatic cancer (18). Taken together, these observations support the concept that potent paracrine actions on tumor growth, which are exerted by high levels of TGF-β, are often associated with perturbations in TGF-β tumor suppressor pathways.

Smad4 haploinsufficiency and HNSCC

In this issue of the *JCI*, Bornstein and colleagues (7) point out that loss of the Smad4 protein in patients with HNSCC is known to correlate with depth of invasion of the cancer cells, a more advanced pathologic stage, enhanced regional metastases, and decreased patient survival. Other alterations known to occur in the TGF-β pathway in HNSCC include decreased expression or mutation of TGFβRII, increased TGF-β1 expression, and decreased levels of activated Smad2 (6, 19, 20). However, (a) the relevance of these molecular alterations to Smad4-associated oncogenesis in HNSCC is not well understood; (b) it is not known whether Smad4 loss contributes to HNSCC initia-

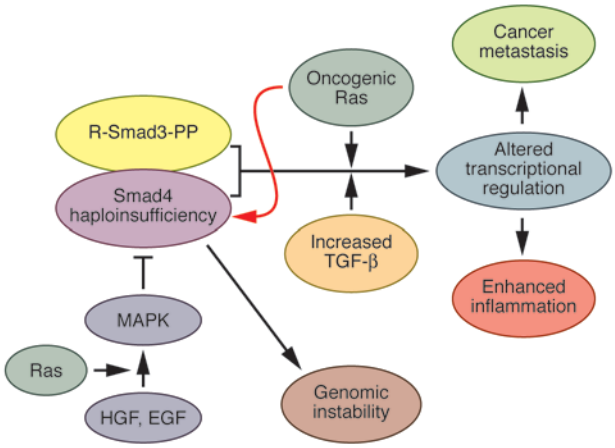

Figure 2

Consequences of Smad4 haploinsufficiency. As shown by Bornstein et al. in their study in this issue of the *JCI* (7), attenuated Smad4 levels in HNSCC lead to genomic instability, upregulation of phosphorylated R-Smad3 (R-Smad3-PP), excessive Ras activation (through overexpression or mutation), increased production of TGF-βs, and enhanced inflammation. In theory, oncogenic Ras can enhance Smad4 degradation (red line), and ligands such as EGF or HGF can activate the Ras/MAKP pathway to further modulate Smad4 function. The net result is altered transcriptional regulation of numerous genes leading to enhanced cancer spread and metastasis.

tion and/or progression; and (c) previously studied animal models have not mimicked spontaneous human HNSCC. Accordingly, Bornstein et al. evaluated Smad4 expression in patients with HNSCC and studied a new mouse model of HNSCC.

Bornstein and colleagues (7) initially determined that the cancers in patients with HNSCC, as well as in the adjacent grossly normal buccal mucosa, expressed decreased levels of Smad4, which implied that Smad4 loss occurs early during the development of HNSCC in humans. The observation that Smad4 expression is also decreased in the normal buccal mucosa adjacent to head and neck cancer is an unexpected and exciting finding. In view of the fact that low levels of Smad4 have been associated with attenuated responsiveness to TGF-β–mediated growth inhibition, this observation suggests that Smad4 haploinsufficiency can lead to an enhanced propensity for head and neck carcinogenesis.

The mechanisms underlying decreased Smad4 expression in both HNSCC and the adjacent grossly normal mucosa are not readily evident (7) and cannot be explained by LOH alone. Additionally, it is not clear whether Smad4 levels within the tumor mass were decreased both in the cancer cells and in the stromal compartment, or only in the cancer cells. Nonetheless, in future studies it will be worth determining whether there is attenuation of Smad4 transcription rates or decreased *Smad4* mRNA stability in HNSCC cancer cells and evaluating Smad4 expression in the different cell types within the tumor mass by in situ hybridization. Another exciting area of research would be to determine whether *Smad4* mRNA levels are suppressed by noncoding microRNAs that are known to have the ability to target coding mRNAs. For example, the microRNA-23b cluster was recently reported to suppress Smad4 expression (21).

Importantly, Bornstein and colleagues next used an inducible head and neck–specific knockout system, which, after RU486 application to the oral cavity in 4-week-old mice, caused *Smad4* deletion in head and neck epithelial cells (*HN-Smad4*$^{-/-}$ mice) (7). They observed the spontaneous development of oral tumors in 74% of *HN-Smad4*$^{-/-}$ mice, some of which developed lymph node metastases. By contrast, tumors did not form in either the *HNSmad4*$^{+/-}$ or the *HN-Smad4*$^{+/+}$ mice. These observations provide more direct evidence in support of the concept that *Smad4* deficiency leads to an enhanced propensity for head and neck carcinogenesis.

The ability of *Smad4* deficiency to cause HNSCC (7) was somewhat surprising, given the previous observation that in a mouse model of HNSCC in which TGFβRII was deleted in head and neck mucosa, attenuation of growth inhibition did not lead to malignant transformation unless there was concomitant expression of mutated *K-ras* or *H-ras* (6). The authors therefore postulated that the loss of Smad4 facilitated *K-ras* or *H-ras* activation (7). They sequenced both genes in 18 HNSCC tumors that have arisen in the *HN-Smad4*$^{-/-}$ mice and determined that four tumors exhibited oncogenic *H-ras* mutations at codon 61. Inasmuch as *H-ras* is more frequently mutated at codons 12 and 13, this observation suggests that Smad4 loss in head and neck epithelial cells in mice may specifically lead to codon 61 *H-ras* mutations, and this possibility is worthy of further exploration.

Bornstein and colleagues (7) also determined that tumors with wild-type *Ras* exhibited increased Ras protein levels, in agreement with the overall hypothesis that spontaneous Ras activation is a potential initiation event for HNSCC formation in *HN-Smad4*$^{-/-}$ tumors. To test this hypothesis, the authors generated mice with heterozygous deletion of *Smad4* together with heterozygous *HN-K-ras*^{G12D} mutation in head and neck epithelia (*HN-K-ras*^{G12D}*Smad4*$^{+/-}$ mice) and observed that these mice developed HNSCC. By contrast, *HN-Smad4*$^{+/-}$ mice did not develop tumors. Therefore, in this mouse model, Smad4 haploinsufficiency did not lead to cancer formation, except in the presence of oncogenic *K-ras*, underscoring differences between the mouse model and HNSCC in humans.

Smad4 and genomic stability

To explain their findings with respect to the high incidence of *Ras* mutation or overexpression in the inducible head and neck specific–knockout system (*HN-Smad4*$^{-/-}$ mice), Bornstein and colleagues (7) postulated that *Smad4* deletion in head and neck epithelium resulted in genomic instability. Indeed, they observed decreased expression of mRNA moieties encoding members of the Fanconi anemia (FA) family of proteins and Rad51, which are known to participate in the DNA repair pathway by regulating homologous recombination (22). Thus, there were variable but significant decreases in the levels of mRNA encoding Brca1, FancA, FancD2, and Rad51 in both the HNSCC lesions and adjoining mucosa of *HN-Smad4*$^{-/-}$ mice, indicating that these changes most likely occurred as a result of Smad4 deletion and prior to tumor formation.

Two additional lines of evidence supported the concept that Smad4 deficiency leads to genomic instability in HNSCC. First, there was an increase in centrosome number in the HNSCC lesions of the *HN-Smad4*$^{-/-}$ mice (7). Second, as determined by array

comparative genomic hybridization, the tumors in the *HN-Smad4*$^{-/-}$ mice exhibited chromosomal losses that are syntenic to human chromosomal regions 19p13 and 17q21 and are common in human HNSCC (4). Taken together, these findings indicate that Smad4 has a gatekeeper function, maintaining genomic stability in oral mucosa.

TGF-β1 can cooperate with Ras proteins to promote epithelial-mesenchymal transformation (EMT), a process whereby epithelial cells acquire mesenchymal markers; cell-cell junctional integrity is lost; the epithelial adhesion molecules β-catenin and E-cadherin become mislocalized principally to the nucleus and cytoplasm, respectively; and cellular invasiveness is enhanced (11). Inasmuch as one of the consequences of *Smad4* deletion in *HN-Smad4*$^{-/-}$ mice was increased expression of TGF-β1, it is likely that the initiating event occurring as a consequence of excessive Ras activation was further fueled by elevated TGF-β1 levels.

HNSCC and inflammation

Excessive Ras activation may also redirect TGF-β actions toward tumor promotion and a proinflammatory profile (11). In this regard, Bornstein and colleagues (7) show that the stroma adjacent to both the normal buccal mucosa and to the HNSCC in the *HN-Smad4*$^{-/-}$ mice exhibited leukocyte infiltration comprising in part macrophages, granulocytes, and T lymphocytes (including Th17 cells, which represent a subset of proinflammatory T lymphocytes that are activated by TGF-β1 in mice). Moreover, there was increased cytokine expression, including elevated levels of monocyte chemoattractant protein–1 (MCP-1), MCP-2, and macrophage inflammatory protein–2 (MIP-2), which are known to be upregulated by TGF-β1 overexpression in keratinocytes (23). These alterations occurred in spite of the loss of nuclear phospho-Smad2 (pSmad2), which points to deficient Smad2-dependent signaling. However, this defect was counterbalanced by increased nuclear staining for Smad3, implying selective aberrant activation of Smad3-dependent pathways. To test for this possibility, *HN-Smad4*$^{-/-}$ mice were crossed into a *Smad3*$^{+/-}$ background, yielding *HN-Smad4*$^{-/-}$*Smad3*$^{+/-}$ mice (7). The lesions in these mice exhibited reduced leukocyte infiltration in comparison with the *HN-Smad4*$^{-/-}$ animals, implicating TGF-β–activated Smad3 pathways in the cancer-associated inflammatory process.

Surprisingly, there was also increased staining for pSmad1/5/8 (7). It is not clear, however, whether activation of these Smads was due to excessive activation of BMP-dependent pathways, since the expression of BMP and/or BMP receptors was not examined, or to TGF-β acting through an alternate pSmad1/5 pathway, as recently reported in certain cell types, including epithelial cells (24).

Possible implications

The present findings in HNSCC (7), which were largely focused in a mouse model of human HNSCC, underscore the potential importance of *Smad4* as a "guardian gene" and raise the possibility that cancers in which there is concomitant loss of *Smad4* and excessive *ras* oncogene activation may become especially susceptible to marked genomic instability. For example, *Smad4* was originally identified as a mutated/deleted gene in pancreatic ductal adenocarcinoma (PDAC), and its original name was *deleted in pancreatic cancer, locus 4* (19). PDAC is also characterized by a greater than 90% mutation rate in the *K-ras* oncogene (25). The concomitant loss of Smad4, observed in 50% of PDAC cases, and high frequency of oncogenic *K-ras* in this cancer could explain the marked genomic instability observed in the malignancy. In view of the known ability of oncogenic Ras to induce Smad4 degradation (26), and of tyrosine kinase pathways that activate Ras to modulate Smad4 function (27), it is also possible that once a mucosal cell exhibits low levels of Smad4 and its Ras pathways become excessively active, a vicious cycle is established that leads to a further lowering of Smad4 levels as a consequence of Ras-induced Smad4 degradation (Figure 2). In conjunction with the ensuing genomic instability, which elicits multiple molecular alterations including a pro-oncogenic inflammatory profile, these alterations can then promote disease aggressiveness, invasion, and metastasis.

Future directions

It is not known why Smad4 levels are low in both HNSCC and the adjacent grossly normal mucosa (7). Clearly, future studies are necessary to examine Smad4 transcriptional regulation in head and neck mucosa, the potential role of microRNAs in this regulation, the role of endogenous inhibitors of transcription such as noncoding antisense RNA, and the role of translational modifications such as ubiquitination and sumoylation (28). Similarly, it is not clear exactly how a full dose of Smad4 acts to maintain genomic stability. Differential gene expression studies in relation to different levels of Smad4 may shed light on this issue.

K-ras and *H-ras* mutations are more common in Asian populations with HNSCC, whereas in the United States, HNSCC is more frequently associated with elevated *K-ras* and *H-ras* levels (6). In view of the important *ras-Smad4* interactions, additional studies should be conducted to determine whether these regional differences are due to specific dietary, environmental, or genetic factors. Similarly, in view of the inflammatory response observed as a result of *ras-Smad4* interactions, and the activation of Smad1/5 pathways, it will be important to determine whether other noncanonical TGF-β signaling molecules, such as JNK and p38 MAPK kinase, are activated in HNSCC and whether reactive oxygen species or specific cytokines contribute to the inflammatory response.

Future studies may take advantage of the inducible head and neck–specific Smad4-knockout mouse model (7) to evaluate chemoprevention strategies and to test a variety of therapeutic mechanisms that concomitantly target Ras, Smad4, and TGF-β signaling pathways, in an attempt to suppress genomic instability, aberrant proliferation, and lymph node metastasis.

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