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**Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation**

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Smad4 is a central mediator of TGF-β signaling, and its expression is downregulated or lost at the malignant stage in several cancer types. In this study, we found that Smad4 was frequently downregulated not only in human head and neck squamous cell carcinoma (HNSCC) malignant lesions, but also in grossly normal adjacent buccal mucosa. To gain insight into the importance of this observation, we generated mice in which Smad4 was deleted in head and neck epithelia (referred to herein as HN-Smad4–/– mice) and found that they developed spontaneous HNSCC. Interestingly, both normal head and neck tissue and HNSCC from HN-Smad4–/– mice exhibited increased genomic instability, which correlated with downregulated expression and function of genes encoding proteins in the Fanconi anemia/Brca (Fanc/Brca) DNA repair pathway linked to HNSCC susceptibility in humans. Consistent with this, further analysis revealed a correlation between downregulation of Smad4 protein and downregulation of the Brca1 and Rad51 proteins in human HNSCC. In addition to the above changes in tumor epithelia, both normal head and neck tissue and HNSCC from HN-Smad4–/– mice exhibited severe inflammation, which was associated with increased expression of TGF-β1 and activated Smad3. We present what we believe to be the first single gene–knockout model for HNSCC, in which both HNSCC formation and invasion occurred as a result of Smad4 deletion. Our results reveal an intriguing connection between Smad4 and the Fanc/Brca pathway and highlight the impact of epithelial Smad4 loss on inflammation.

**Introduction**

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide (1). Despite recent advances in cancer biology and therapy, the 5-year survival for patients with HNSCC has remained 50% for the past 20 years (2). Several genetic alterations in HNSCC have been reported, including loss of genetic material on 9p (encoding p16), 17p (encoding p53) (1), 18q (encoding DCC, Smad4, and Smad2) (3–5), and the type II TGF-β receptor (TGFBR2) (6). Several oncogenic signaling pathways are also upregulated in HNSCC, including Ras, EGFR/Stat3, and PI3K/PTEN/Akt pathways (1,7). Among them, Ras activation plays a role in HNSCC initiation but is insufficient for malignant conversion (8,9). In contrast, loss of TGFBR2 is insufficient to initiate HNSCC formation but is a potent promoter in HNSCC tumorigenesis (8). In general, little is known about which somatic changes in HNSCC lesions play a causal role. To date, the best-studied germline mutations that lead to increased susceptibility to HNSCC are in genes belonging to the Fanconi anemia/Brca (Fanc/Brca) pathway (10).

Smad4 was originally identified as a tumor suppressor in pancreatic cancer (11) and subsequently characterized as a key mediator of TGF-β signaling (12). Termed the “common Smad,” Smad4 plays an instrumental role in TGF-β/BMP signaling by forming complexes with receptor-activated Smads, i.e., Smad-2 and -3, or Smad-1 and -5. The Smad complexes then translocate to the nucleus to regulate gene expression of Smad targets involved in a wide variety of cancer-related processes including proliferation, apoptosis, and inflammation (13). Somatic inactivation of Smad4 is a frequent event in multiple tumor types (14). Smad4 deletion in murine tissues, in combination with other genetic alterations that cause tumor initiation, resulted in cancer lesions of the colon (15, 16), pancreas (17, 18), forestomach (19), and liver (20). Thus, Smad4 loss appears to play an important role in malignant progression. However, Smad4 deletion in mice also resulted in spontaneous cancer formation of the stomach (21), skin (22–24), and mammary gland (23), suggesting that Smad4 loss has both initiation and promotion effects on tumorigenesis in these tissues. Other than that they cause loss of expression of TGF-β target genes associated with growth inhibition, little is known about the molecular mechanisms underlying Smad4 loss–associated tumorigenesis.

With respect to the role of Smad4 in HNSCC, the region on chromosome 18q where Smad4 is located is frequently lost at the genetic level in HNSCC (25), and loss of heterozygosity (LOH) at the Smad4 locus has been reported at approximately 50% (3, 4). Additionally, loss of the Smad4 protein is common in HNSCC, and this was correlated with depth of invasion and pathologic stage (26) as well as regional metastases and decreased survival (27). However, it is unknown at which stage Smad4 is downregulated in human HNSCC and whether Smad4 loss plays a causal role in HNSCC development and progression. To address these questions, we examined Smad4 levels in both grossly normal mucosa adjacent to HNSCC and neoplastic lesions of HNSCC patients. Unlike TGFBR2,
which was downregulated only in malignant HNSCC, Smad4 was downregulated in both grossly normal mucosa and malignant HNSCC lesions compared with normal head and neck tissues. We deleted Smad4 specifically in murine head and neck epithelia and found that Smad4-knockout mice developed spontaneous HNSCC with evidence of increased genomic instability and inflammation.

Results

Smad4 downregulation occurred at an early stage in human HNSCC. To determine which stage Smad4 was downregulated in human HNSCC, we analyzed SMAD4 mRNA levels in 36 pairs of human HNSCC and mucosa samples adjacent to each HNSCC by quantitative RT-PCR (qRT-PCR), using normal mucosa samples from sleep apnea patients as controls. Among these samples, 86% (31 of 36) of HNSCC samples exhibited downregulation of SMAD4 mRNA to less than 50% of Smad4 levels in control mucosa samples. In addition, 67% (24 of 36) of adjacent mucosa samples exhibited more than 50% downregulation of Smad4, indicating that Smad4 loss occurred early in cancer development (Figure 1A). We then performed Smad4 immunohistochemistry (IHC) on these samples and found that overall, Smad4 protein staining patterns correlated with their mRNA levels, i.e., while Smad4 stained strongly in the normal control group, Smad4 staining was significantly reduced or lost in adjacent mucosa samples and HNSCCs (Figure 1B). To determine whether Smad4 reduction was due in part to genetic loss, we performed an LOH analysis using HNSCC samples that lost SMAD4 mRNA, with paired adjacent mucosa samples as controls, and found that 33% (6 of 18) of sample pairs exhibited LOH at the Smad4 locus (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI38854DS1).

Deletion of Smad4 in murine head and neck epithelia resulted in spontaneous HNSCC that mimicked human HNSCC. The high frequency of Smad4 downregulation in human HNSCC, particularly at the stage prior to tumor formation, prompted us to investigate whether Smad4 loss played a causal role in HNSCC tumorigenesis. We used our inducible head and neck–specific knockout system (8, 9), which allows Smad4 deletion (HN-Smad4–/–) in head and neck epithelial cells upon RU486 application to the oral cavity (Supplemental Figure 2). We induced Smad4 deletion in murine head and neck epithelia at 4 weeks of age. In total, 35 HN-Smad4–/–, 9 HN-Smad4+/–, and 23 HN-Smad4+/+ mice (all on the C57BL/6 background) were monitored for tumor formation for up to 80 weeks. Beginning at 29 weeks of age, HN-Smad4–/– mice began to develop oral tumors. By 80 weeks, 74% (26 of 35) developed spontaneous oral tumors, and 12% (3 of 26) of tumor-bearing mice harbored regional lymph node metastases prior to being euthanized. Most tumor-bearing mice required euthanasia prior to the development of metastatic disease, due to difficulties with food intake or excessive bleeding, which are problems often encountered in human HNSCC patients. No tumors were observed in either HN-Smad4+/– or HN-Smad4+/+ mice (Figure 2A).

Tumors generated in HN-Smad4–/– mice were derived from the buccal mucosa (Figure 2B) and palate (data not shown). Histologically, HN-Smad4–/– HNSCCs exhibited regions ranging from moderately to poorly differentiated squamous cell carcinoma (SCC).
The precursor lesions displayed histology ranging from hyperplasia to dysplasia (Figure 2C), which is reflective of the histological stages seen during human head and neck cancer development. HNSCC cells displayed enlarged nuclei and increased mitoses (Figure 2D). Keratin staining revealed that the adjacent buccal mucosa of HN-Smad4+/− HNSCCs expressed keratin 13 (K13), which is a marker of oral epithelia (28), not hyperplastic epidermis, verifying that the tumors were derived from the oral cavity rather than the skin (Figure 2E). However, HN-Smad4−/− HNSCCs exhibited patchy loss of this differentiation marker, which is associated with malignant conversion (Figure 2E) (8). Histological sections of enlarged regional lymph nodes demonstrated HNSCC metastases. As shown in Figure 2F, keratin pearls, which are pathognomonic for SCCs, are adjacent to lymphatic tissue. Keratin staining of these lymph nodes (Figure 2G) further verified that metastases arose from HNSCC, as K13, as well as the general keratinocyte marker K14, both stained the lymph node section.

As a central mediator of TGF-β signaling, Smad4 mediates the well-documented tumor suppressor roles of TGF-β, such as growth inhibition and apoptosis, which are reflected in HN-Smad4+/− mucosa and HNSCC. Proliferating cell nuclear antigen (PCNA) staining for proliferative cells and TUNEL staining for apoptotic cells revealed increased cell proliferation and reduced apoptosis in HN-Smad4−/− mucosa and HNSCC compared with HN-Smad4+/− mucosa (Supplemental Figure 3). However, these changes did not explain why Smad4 gene deletion alone was sufficient for HNSCC formation, as similar alterations were also observed in Tgfb2−/− head and neck keratinocytes, which required additional Ras activation for HNSCC tumorigenesis (8). To determine whether loss of Smad4 facilitated Ras activation, we sequenced KRAS and HRAS genes in HN-Smad4+/− HNSCCs. From a total of 18 tumors examined, 4 exhibited HRAS mutations at codon 61. Among the tumors with HRAS mutations, 2 changed CAA to CTA, 1 changed CAA to CGA (Supplemental Figure 4A). All of these mutations are associated with G12D Ras activation (29). No KRAS mutation was detected in HN-Smad4+/− HNSCCs. While the minority of HNSCCs exhibited Ras mutation, similar to our previous observation in human HNSCC, tumors without a Ras mutation exhibited increased Ras protein levels (Supplemental Figure 4B). These data suggest that spontaneous Ras activation via mutation or overexpression could provide an initiation event for HNSCC formation in at least a subset of HN-Smad4+/− tumors. To test this, we generated mice with heterozygous deletion of Smad4 together with heterozygous HN-K-rasG12D mutation in the head and neck epithelia (HN-K-rasG12D/Smad4−/−) using a breeding strategy similar to that in our previous report (8). HN-K-rasG12D/Smad4−/− mice devel-
we used papillomas induced by head and neck–specific genomic instability. Since instability (32), we examined whether Brca1 and Rad51 in HN-Smad4+/- mucosa and HNSCC, compared with HN-Smad4+/- mucosa by qRT-PCR. Means of 5–10 samples from each group are presented, and error bars indicate SEM. The average expression of Smad4 in the HN-Smad4+/- samples was arbitrarily set at 100%. *P < 0.05 versus HN-Smad4+/- mucosa. (B) Decreased Brca1 and Rad51 protein by IHC in HN-Smad4+/- mucosa and HNSCC compared with HN-Smad4+/- mucosa. Five to 10 samples per group were analyzed, and a representative image is presented. Scale bar: 40 μm (all panels).

![Figure 3](http://www.jci.org)

**Figure 3** Downregulation of Fanc/Brca transcripts in HN-Smad4+/- mucosa and HNSCC. (A) Decreased relative mRNA expression of Brca1, FancA, FancD2, and Rad51 in HN-Smad4+/- mucosa and HNSCC, compared with HN-Smad4+/- mucosa by qRT-PCR. Means of 5–10 samples from each group are presented, and error bars indicate SEM. The average expression of Smad4 in the HN-Smad4+/- samples was arbitrarily set at 100%. *P < 0.05 versus HN-Smad4+/- mucosa. (B) Decreased Brca1 and Rad51 protein by IHC in HN-Smad4+/- mucosa and HNSCC compared with HN-Smad4+/- mucosa. Five to 10 samples per group were analyzed, and a representative image is presented. Scale bar: 40 μm (all panels).
The effect of Smad4 loss or that other mechanisms regulate these molecules in HNSCC cancer cells. However, the latter is likely the case, as shown by an additional experiment in which we used siRNA to knock down Smad4 in normal primary human epidermal keratinocytes (HEKn). In response to approximately 75% Smad4 knockdown 48 hours after Smad4 siRNA treatment (Supplemental Figure 5), expression levels of Brca1, FancA, FancD2, and Rad51 were all reduced by approximately 50% in HEKn cells (Figure 5B).

Figure 4
Abnormal centrosomes, increased genomic aberrations, and increased MMC sensitivity in HN-Smad4–/– mucosa and HNSCC. (A) Immunofluorescence for pericentrin (green or yellow). HN-Smad4–/– mucosa and HNSCCs have increased abnormal centosome numbers compared with HN-Smad4+/+ mucosa and control HN-Smad4–/– tumors (HN-KrasG12D papillomas), respectively. K14 (red) was used to counterstain epithelial cells. 3–5 samples per group were analyzed, and a representative image is presented. Arrows highlight cells with at least 3 centrosomes. The histogram summarizes quantification of centrosome numbers. 100–200 cells per group were analyzed. Error bars indicate SEM. †P < 0.05 versus HN-Smad4+/+ tumors; *P < 0.05 versus HN-Smad4+/+ mucosa. Scale bar: 10 μm (all panels). (B) Chromosome 4 aCGH of 3 HN-Smad4–/– HNSCCs indicates that HN-Smad4–/– HNSCCs have several consistent genomic aberrations. The boxed region represents 2 copies of loss at chromosome 4qA5. (C) MMC sensitivity assay. Percent cell viability at increasing MMC concentrations indicates that Smad4–/– cells were significantly more sensitive to MMC than Smad4+/+ cells. The experiment was run in triplicate, and error bars indicate SEM. P < 0.05 for all data points, other than 0 ng/ml, versus Smad4+/+ cells. (D) Chromosome breakage assay. Plot of average chromosome breaks per cell for Smad4+/+ and Smad4–/– cells at increasing MMC concentrations indicates that Smad4–/– cells have increased chromosome breaks compared with Smad4+/+ cells. The experiments were run in triplicate, and error bars indicate SEM. P < 0.001 for all data points, other than 0 ng/ml, versus Smad4+/+ cells.
To address whether rescued expression of Brca1 and Rad51 in Cal27 cells had functional consequences, we examined Brca1 and Rad51 DNA repair nuclear foci formation by immunofluorescence staining using the Cal27 and Cal27-Smad4 cells. Under normal conditions, Brca1 and Rad51 localize to sites of MMC-induced DNA damage with other members of the Fanc/Brca pathway to form DNA repair nuclear foci (38). After MMC treatment, only 10%–20% of Cal27 cells were able to form Brca1 and Rad51 foci, whereas Cal27-Smad4 cells exhibited a 3- to 4-fold increase in the number of cells able to form Brca1 and Rad51 foci (Figure 5C). These data suggest that Smad4 level affects not only expression levels of Fanc/Brca molecules, but also their function.

To determine whether Smad4 protein loss led to downregulation of Brca1 and Rad51 in human head and neck tissues, we assessed whether there was a correlation between Smad4 loss and Brca1 or Rad51 loss using IHC. While inflammatory cells were rarely detected in the stroma of HN-Smad4+/+ buccal tissues, both the stroma adjacent to HN-Smad4−/− buccal mucosa and the tumor stroma of HN-Smad4−/− HNSCC exhibited leukocyte infiltration comprising, in part, macrophages, granulocytes, and T lymphocytes (Figure 7A). Interestingly, Th17 cells, which represent a subset of proinflammatory T lymphocytes that are activated by TGF-β1 in mice (40, 41), were also detected in HN-Smad4−/− mucosa and HNSCC (Figure 7A).

We then assessed which inflammatory cytokines were activated in HN-Smad4−/− buccal mucosa and HNSCC, using a “Mouse Chemokines & Receptors” Superarray. Inflammatory cytokines were undetectable or present at very low levels in HN-Smad4−/− mucosa, but several cytokines were readily detected in HN-Smad4−/− mucosa and HNSCC (Figure 7B). Among them, MCP-1, MCP-2, and MIP-2 have been shown to be elevated by TGF-β1 overexpression in keratinocytes (42). These data are consistent with our previous observation that TGF-β1 overexpression alone in head and
The Journal of Clinical Investigation

Research Article

The Journal of Clinical Investigation

November 2009

Volume 119
Number 11

HNSCCs expressed significantly more TGF-β1 (514 pg/mg total protein) than HN-Smad4+/+ buccal mucosa (84 pg/mg total protein) (Figure 8A). Interestingly, HN-Smad4–/– buccal mucosa also exhibited significantly more TGF-β1 (156 pg/mg total protein) than HN-Smad4+/+ mucosa (Figure 8A), indicating that the Smad4 loss–associated increase in TGF-β1 occurred early on in tumorigenesis. To determine whether increased TGF-β1 conferred downstream signaling activation, we performed immunostaining of signaling Smads in HN-Smad4–/– mucosa and HNSCC. Interestingly, while loss of nuclear phospho-Smad2 (pSmad2) was evident in HN-Smad4–/– mucosa and HNSCC, increased nuclear staining for Smad3 and pSmad1/5/8 was observed in HN-Smad4–/– buccal mucosa and HNSCC compared with HN-Smad4+/+ mucosa (Figure 8B).

Based on our previous study suggesting that Smad3 is a key mediator in TGF-β1–associated inflammation (44), we assessed whether increased TGF-β1 and Smad3 nuclear staining represented functional activation that contributed to inflammation in HN-Smad4–/– tissues. We crossed HN-Smad4–/– mice into a Smad3–/– background (44) to generate compound HN-Smad4–/–/Smad3–/– mice. Smad4 deletion was induced at 3 weeks, and mice were euthanized at 4 weeks of age for excision of head and neck tissues. HN-Smad4–/– tissues exhibited inflammation 1 week after Smad4 was deleted, with infiltrated leukocytes consisting mainly of macrophages and granulocytes, as well as sporadic T lymphocytes (CD3+) (Figure 8C). Interestingly, leukocyte infiltration in HN-Smad4–/– tissues was markedly reduced in HN-Smad4–/–/Smad3–/– tissues (Figure 8C), suggesting that Smad3-dependent TGF-β1 signaling contributed at least in part to inflammation in HN-Smad4–/– tissues.

Discussion

Early-stage Smad4 loss plays a causal role in HNSCC tumorigenesis. In the current study, we found that SMAD4 mRNA levels were significantly reduced in human HNSCCs and in grossly normal buccal mucosa adjacent to HNSCC. Among them, we detected 33% LOH at the Smad4 locus, which is similar to previous reports (3, 4). In these cases, genetic loss of the first allele may contribute to at least 50% of Smad4 downregulation, as mice with heterozygous deletion of Smad4 exhibited approximately 50% loss of transcript and protein (22–24). Smad4 levels can be further downregulated if the remaining allele is mutated. Additionally, epigenetic, posttranscriptional, and posttranslational modifications could contribute to further loss of the remaining Smad4 expression. In contrast to other cancer types in which Smad4 is downregulated or lost at malignant stages, frequent Smad4 downregulation in grossly normal buccal mucosa adjacent to HNSCC suggests an early role of Smad4 reduction in HNSCC tumorigenesis. This notion is further supported by spontaneous HNSCC formation in mice with Smad4 deletion specifically in head and neck epithelia. This is somewhat surprising, since patients...
with juvenile polyposis coli, a syndrome characterized by multiple benign colon polyps as a result of germline heterozygous Smad4 loss, do not exhibit an increased risk for HNSCC. This could reflect the tissue specificity of the germline mutation and indicates that additional molecular alterations in keratinocytes must be required for HNSCC formation in HN-Smad4⁻/⁻ mice. Interestingly, grossly normal HN-Smad4⁻/⁻ mucosa exhibited increased abnormal centrosome numbers and reduced expression of Fanc/Brca genes, suggesting that homozygous Smad4 loss, through initiation of genomic instability, may generate secondary oncogenic changes necessary for tumorigenesis. For example, we found that a subset of HN-Smad4⁻/⁻ HNSCCs exhibited Ras mutations, which have been associated with HNSCC initiation (8, 9). Since mice with head and neck–specific TGFβR2 deletion do not develop spontaneous HNSCC unless a mutant Ras is introduced (8), spontaneous Ras mutation appears to be infrequent in mouse head and neck epithelia but could be a consequence of Smad4 loss–mediated genomic instability. While neither heterozygous deletion of Smad4 in the current study nor K-ras mutation alone (8) are able to lead to HNSCC formation, K-rasHG12D/Smad4⁻/⁻ mice rapidly developed HNSCC, indicating that early genetic Ras activation is able to initiate HNSCC formation with heterozygous Smad4 loss. These data also suggest that single-copy loss of Smad4 in the setting of other oncogenic changes, i.e., the “field cancerization” observed in HNSCC patients (1), is sufficient for HNSCC tumorigenesis. Thus, approximately 50% loss of Smad4 mRNA at either the genomic or transcriptional level could be sufficient for tumorigenesis in these cases. Further, although we have not detected EGFR amplification in HN-Smad4⁻/⁻ HNSCCs as seen in human patients (1), Ras activation could exert similar effects. It remains to be determined whether other genetic alterations found in HN-Smad4⁻/⁻ HNSCCs also cause tumor initiation.

Smad4 loss contributes to defects in the Fanc/Brca pathway and genomic instability. It has been shown that Fanconi anemia patients, who carry germline mutations in Fanc/Brca pathway genes, have a high incidence of HNSCC at a young age (10). Moreover, Fanc/Brca pathway–associated genes are downregulated in sporadic HNSCC (6, 35, 45, 46). Additionally, mice with epithelia-specific heterozygous knockout of Brca1 developed oral SCCs, indicating that Brca1 loss plays an important role in HNSCC tumorigenesis (47). In the current study, downregulation of Fanc/Brca pathway genes was detected in both grossly normal buccal mucosa and HNSCC lesions of HN-Smad4⁻/⁻ mice. Smad4 knockdown led to reduction in Fanc/Brca gene expression, and restoration of normal Smad4 in a Smad4-deficient HNSCC cell line increased expression of Fanc/Brca pathway genes. Additionally, the majority of Smad4-deficient mucosa and HNSCC clinical samples demonstrated loss of both Brca1 and Rad51. Thus, our data suggest a causal role for Smad4 loss in down-regulation of the Fanc/Brca pathway. It remains to be determined whether Smad4 regulates any of the Fanc/Brca family members via direct transcriptional activation, or via other regulators downstream of Smad4. Nevertheless, Smad4 loss appeared to be sufficient to cause functional defects in the Fanc/Brca pathway. This is evidenced by increased sensitivity to MMC killing and chromosome breakage in Smad4⁻/⁻ primary cells and defects in Rad51/Brca1 nuclear foci formation in Smad4-deficient HNSCC cells. Defects in the Fanconi pathway lead to increased genomic instability in Fanconi anemia patients (38) and could be responsible for the increased abnormal centromere numbers and genomic aberrations observed in HN-Smad4⁻/⁻ HNSCC. Consistent with our observations, both Brca1-deficient cells (31) and cells expressing a dominant-negative form of Rad51 (30) exhibited increased centrosome numbers and chromosomal aberrations. In contrast to our current study, TGF-β1-treated Mv1Lu cells show decreased Rad51 protein and Rad51 nuclear foci in a Smad4-dependent manner (48). It is possible that Smad4 functions differently in different cell types. Additionally, increased TGF-β1/Smad3 in HN-Smad4⁻/⁻ tissues could contribute to downregulation of the Fanc/Brca pathway. In support of the latter hypothesis, Smad3 has been shown to bind to the Brca1 protein and inhibit Brca1 nuclear foci formation and DNA repair efficiency (49). Thus, genomic instability coupled with hyperproliferation and reduced apoptosis may allow HN-Smad4⁻/⁻ cells to escape from DNA damage–induced cell death during tumorigenesis.

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Smad4⁺</th>
<th>Smad4⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brcα1 loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa adjacent to HNSCC</td>
<td>0/8 (0%)</td>
<td>12/13 (92%)⁺</td>
</tr>
<tr>
<td>HNSCC</td>
<td>0/8 (0%)</td>
<td>21/24 (88%)⁺</td>
</tr>
<tr>
<td>Rad51 loss</td>
<td></td>
<td></td>
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<tr>
<td>Mucosa adjacent to HNSCC</td>
<td>0/8 (0%)</td>
<td>12/13 (92%)⁺</td>
</tr>
<tr>
<td>HNSCC</td>
<td>0/8 (0%)</td>
<td>20/24 (83%)⁺</td>
</tr>
</tbody>
</table>

⁺P < 0.05 versus Smad4⁺.
Figure 7
Increased inflammation and inflammatory chemokines in HN-Smad4−/− lesions. (A) Immunostaining of HN-Smad4+/+ mucosa, HN-Smad4−/− mucosa, and HN-Smad4−/− HNSCCs with leukocyte markers. Macrophages (Mφ) were stained with antibodies against F4/80; granulocytes with antibodies against Ly6G; T cells with antibodies against CD11b; and Th17 cells with antibodies against IL-17. The black dotted lines indicate the boundary between mucosa and stroma. HN-Smad4−/− mucosa has increased inflammatory markers in the underlying stroma compared with HN-Smad4+/+ mucosa, and HN-Smad4−/− HNSCCs have marked inflammation in the tumor stroma. 5–7 samples from each group were analyzed, and a representative image is presented. Scale bar: 40 μm (all panels). (B) Pathway-specific “Mouse Chemokines & Receptors” Superarray revealed increased inflammatory molecules in HN-Smad4−/− mucosa and HNSCC compared with HN-Smad4+/+ mucosa. Three samples per group of HN-Smad4+/+ mucosa, HN-Smad4+/+ mucosa, and HN-Smad4−/− HNSCC were examined, and a representative blot from each group is presented. Genes with visible differences compared with HN-Smad4+/+ mucosa are labeled to the left with numbers that correspond to gene IDs quantified in C. (C) Relative mRNA expression (fold change) for each gene was calculated as optical density above adjacent background for HN-Smad4−/− mucosa and HN-Smad4−/− HNSCC samples compared with HN-Smad4+/+ samples. Gene names and IDs corresponding to numbers on the blot in B are displayed below. Error bars indicate SEM. *P < 0.05 versus HN-Smad4+/+ mucosa samples.
Smad2 and Smad4, Smad3 has a tumor-promoting role under certain circumstances (55). We have shown that Smad3 is important in mediating TGF-β–induced inflammation and expression of activating protein 1 (AP-1) family members (44). In the current study, inflammation in HN-Smad4+/− tissues was markedly attenuated in a Smad3-heterozygous background, suggesting that Smad3 mediates, at least in part, increased TGF-β1–associated inflammation. In addition to inducing changes in cytokines critical for HNSCC tumorigenesis (56, 57), inflammation and the resultant production of reactive oxygen species have been linked to the generation of genomic instability (58). Thus, Smad4 loss–associated inflammation may also contribute to the genomic instability observed in our model.

In summary, we found that Smad4 was downregulated at the pretranslational level early in human HNSCC tumorigenesis, which correlated with downregulation of Brca1 and Rad51. We present a model wherein Smad4 loss results in spontaneous HNSCC formation. Our results indicate that Smad4 loss contributes to increased genomic instability in tumor epithelia. This effect, coupled with abrogation of TGF-β–induced growth inhibition and apoptosis but enhancement of TGF-β–mediated inflammation, could allow expansion of genetically damaged cells during HNSCC tumorigenesis. Our study also suggests potential therapeutic strategies for HNSCC with Smad4 loss. For instance, since Fanc/Brca-defective cells exhibit increased sensitivity to radiation (59) and poly(ADP-ribose) polymerase inhibitor–induced cell death (38, 60), our study will encourage future investigation into whether these approaches, coupled with control of excessive inflammation, will be effective treatments for patients with Smad4-deficient HNSCC. We believe the mouse model presented here will be an ideal tool for testing novel therapeutic interventions.

Methods

For experimental procedures not described herein, see Supplemental Methods.

Patients. HNSCC and case-matched adjacent tissue samples were surgically resected between the years 2000 and 2005 from consenting patients at the OHSU Department of Otolaryngology under an Institutional Review
Board-approved protocol. Tissues examined in this study included 21 oral cavity SCCs, 9 larynx SCCs, 5 oropharynx SCCs, 1 nasal cavity SCC, and case-matched tissues adjacent to tumors. Seven normal oropharynx samples from sleep apnea patients were used as normal controls.

**Cell culture and transfections.** Primary Smad4+/− and Smad4−/− cell lines were generated from neonatal compound K5.CrePR1/Smad4+/− or mono- genic Smad4−/− or Smad4+/− control mice after being exposed in utero to RU486 to accomplish Smad4 deletion in bionic mice. Cell lines were generated by CellINtex (Millipore) and were cultured in keratinocyte-specific medium Cnt07 (Chemicon). Human tongue SCC line Cal27 was purchased from ATCC and cultured in DMEM (ATCC) with 10% FBS. To create Cal27 lines stably expressing Smad4 (Cal27-Smad4), Cal27 cells were transfected with pcDNA Flag-Smad4M purchased from Addgene, using Lipofectamine 2000 Transfection Reagent (Invitrogen), and selected in G418 (Sigma-Aldrich) for 4 weeks. To knock down Smad4 in HEK cells (HEK cells plus Smad4 siRNA), HEK cells were treated with Stealth RNAi against Smad4 (5′-GGUGAUGUUUGGACUGGCUCUA-3′) from Invitrogen using XtremeGENE siRNA Transfection Reagent (Roche) for 48 hours, then harvested.

**Histology and immunostaining.** Samples were fixed in 10% neutral buffered formalin, embedded, sectioned, and stained with H&E as we have previously described (8). Primary antibodies included Smad4, Smad3 (Santa Cruz Biotechnology Inc.); pSmad2 and total Akt1 (Cell Signaling Technology); Ly-6G (eBioscience); F4/80 (Caltag Laboratories); CD11b, IL-17 (BD Biosciences—Pharmingen); and Ras (Abcam) as we have previously described (8). Sections were counterstained with hematoxylin. Evaluation of Smad4, Brc1, and Rad51 staining of human HNSCC samples was performed by 2 independent investigators using the methods described previously (8). Double immunofluorescence staining was performed as we have described previously (8). Primary antibodies included K13 (RDI), Keratin 14 (Fitzgerald), Pericentrin (Covance), pSmad1/5/8 (Cell Signaling Technology), and PCNA (Santa Cruz Biotechnology Inc.). The sections were incubated with Alexa Fluor 488-conjugated (green) or Alexa Fluor 594-conjugated (red) secondary antibodies (Invitrogen). Centrosome immunofluorescence staining was captured using confocal microscopy with a Leica SP2 confocal microscope (Leica Microsystems Inc.).

**qRT-PCR.** Total RNA was isolated using TRIzol (Invitrogen) and further purified using a QIAGEN RNeasy Mini kit as previously described (8). One hundred nanograms of RNA from each sample was subjected to qRT-PCR using Brilliant II QRT-PCR 1-Step Master Mix (Stratagene) and TaqMan Assays-on-Demand probes (Applied Biosystems). An 18S, 1 protein. Statistical differences between 2 groups of data were analyzed using a 2-tailed Student’s t test with the exceptions of centrosome quantification in Figure 4A, foci quantification in Figure 5C, and the data in Table 1, which were calculated using a 2-tailed Fisher’s exact test; and the chromosome breakage data in Figure 4D, which was calculated using a χ² test. The data are presented as mean ± SEM. P values less than 0.05 were considered significant.

**Acknowledgments**

The authors thank Yuexin Li and Donna Wang for their technical assistance. This work was supported by NIH grants to X.-J. Wang and by an Oregon Medical Research Foundation grant to S.-L. Lu. S.-L. Lu is an investigator of the THANC (Thyroid, Head and Neck Cancer) Foundation. S. Bornstein and S. Malkoski are recipients of NIH training grants.

Received for publication February 9, 2009, and accepted in revised form August 5, 2009.

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