SUPPLEMENTAL DATA

Smad4 Loss Causes Spontaneous Head and Neck Cancer with Increased Genomic Instability and Inflammation

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Supplemental Experimental Procedures

Mouse Strains. All animal experiments were performed using protocols approved by the IACUC at the Oregon Health & Science University and IACUC at the University of Colorado Denver. The inducible head-and-neck specific knockout system consists of two mouse lines, the K14.CrePR1 or K5.Cre*PR1 mice, in which Cre recombinase can be activated in head-and-neck epithelia by RU486 (1, 2), and the Smad4ff mice, in which the Smad4 gene is floxed (3). These mouse lines were crossbred to generate compound mice, allowing for homozygous Smad4 deletion (Figure S1A). Littermates were genotyped at 3 weeks of age using primer pairs P9 and P10, and grouped based on genotypes for the experiments. RU486 (100 μl of 0.2μg/μl in sesame oil) was applied in the oral cavity of 4-week-old bigenic mice daily for 5 consecutive days to induce homozygous deletion of the Smad4 gene. Monogenic (K14.CrePR1, K5.Cre*PR1, or Smad4ff) control littermates were treated with the same RU486 regimen as bigenic mice. To generate control tumors, we utilized an RU486-inducible K15.CrePR1 line (4) in combination with a knock-in mutant KrasG12D allele (5) to generate K15.CrePR1/KrasG12D mice. We also mated these mice to Smad4ff mice to generate
K15.CrePR1/KrasG12D/Smad4f/w mice. The K15 promoter also targets CrePR1 expression to head-and-neck epithelia. To generate Smad4f/f/Smad3+-/- mice for inflammation studies, we mated K14.CrePR1/Smad4f/f or K5.Cre*PR1/Smad4ff mice with our previously described germline Smad3+-/- mice (7) and RU486 was applied to 3-week-old mice using the same regimes described above. The general condition of the mice was checked at least once per week prior to the development of visible tumors. Mice with oral tumors were given soft food and monitored daily. Tumor-bearing mice were euthanized when oral tumors became ulcerated, or at the first sign of deteriorating health or pain resulting from tumors (e.g., huddled posture, vocalization, hypothermia, or ≥20% weight loss). Paired HN-Smad4+/+ littermates were euthanized at the same time and the corresponding tissue samples were dissected as controls. Necropsy was performed on each euthanized mouse to identify primary tumors and distant metastases. To dissect early preneoplastic lesions, mice with each genotype were euthanized at 4 weeks after Smad4 deletion, and head-and-neck tissue including the buccal tissue, tongue, esophagus, and forestomach were dissected.

Characterization of Head-and-Neck Epithelia-Specific Smad4 Knockout Mice. To verify Smad4 deletion in the head-and-neck epithelia, we euthanized K5.Cre*PR1/Smad4ff or K14.CrePR1/Smad4ff bigenic mice and monogenic control mice 10 days after the final RU486 treatment and extracted DNA from buccal mucosa, tongue, and esophagus. The recombinant Smad4 allele lacking exon 8 was readily detected in the above tissues by PCR in K5.Cre*PR1/Smad4ff and K14.CrePR1/Smad4ff bigenic mice, but not monogenic control littermates, using primer pairs P8 and P10 (Figure S1B).
Loss of Heterozygosity (LOH). DNA was extracted and PCR amplified using FAM-labeled forward primers for microsatellite markers adjacent to the Smad4 gene. PCR products were column purified (Promega) and analyzed using fragment length polymorphism analysis (ABI) at the OHSU MMI sequencing core to determine the size of Smad4 alleles. Two PCR products (peaks) indicated a heterozygous sample, while one product indicated a homozygous sample. LOH for a given marker was positive if the following calculation: (peak height of allele 1 of tumor/peak height of allele 2 of tumor) compared to (peak height of allele 1 of adjacent mucosa/peak height of allele 2 of adjacent mucosa) was greater than or equal to 1.5. LOH for the Smad4 locus was considered positive if either of the flanking microsatellite markers exhibited LOH. Sequences: D18S46: FAM-GAATAGCAGGACCTATCAAGAGC, CAGATTAAGTGAAAACAGCATATGTG; D18S474: FAM-TGGGGGTTTACCAGCATC, TGGCTTTCAATGTCAGAAG. 

Ras Sequencing. Genomic DNA was extracted from samples as previously described (8) and sequenced with the following primers:

Hras Exon 1 forward primer: GCAGCCGCTGTAGAAGCTATGA
Hras Exon 1 reverse primer: GTAGGCAGAGCTCACCTCTATA
Hras Exon 2 forward primer: CATGACTGTGTCCAGGACATTC
Hras Exon 2 reverse primer: TAGGCTGGTTCTGTGGATTCTC
Kras Exon 1 forward primer: TACACACAAAGGTGAGTGTTAAAAATATTGATAA
Kras Exon 1 reverse primer: AGAGCAGCGTTACCTCTATC
Kras Exon 2 forward primer: AAGATGCACTGTAATAATCCATAC
Kras Exon 2 reverse primer: ATTCAACTTAAACCCACCTATA
Sequences were uploaded and mutations were detected using Chromas Lite software.

**TUNEL Staining.** Immunofluorescence of Tdt-mediated dUTP Nick-End Labeling (TUNEL) assays was performed using the TUNEL assay kit (Promega).

**Western Blotting.** Protein was extracted from cells as previously described (9) and Western blotting was performed using primary antibodies to both Smad4 and Actin (Santa Cruz). The blot was then incubated with Alexa Fluor 700 secondary antibody to detect Smad4 and Alexa Fluor 800 secondary antibody to detect Actin (Molecular Probes), and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Supplemental Figure Legends**

*Figure S1.* Loss of heterozygosity (LOH) at the Smad4 locus occurred in 33% (6/18) of adjacent mucosa and HNSCC pairs analyzed. (A) 18 matched sample pairs of HNSCC and adjacent mucosa with case numbers at the top corresponding to samples from Figure 1A are displayed, and microsatellite markers used for PCR flanking the Smad4 locus are listed on the left side (D18S474, D18S46). Markers showing LOH, retention of heterozygosity, or that were homozygous in the adjacent mucosa sample (not informative) are displayed. *: samples exhibiting LOH in a microsatellite marker adjacent to Smad4. (B) Representative histograms of a heterozygous normal adjacent sample with paired HNSCC exhibiting loss of heterozygosity (indicated by an arrow), for both flaking microsatellite markers used (D18S474, D18S46).
**Figure S2.** Smad4 was deleted in head-and-neck epithelia. A: Schematic of mating strategy to generate bigenic K5.Cre*PR1.Smad4f/f or K14.CrePR1.Smad4f/f mice. B: An example of Smad4 genotyping PCR using primers 9 and 10 ("WT" indicates the wild-type Smad4 allele), and detection of the recombinant allele with deleted exon 8 in bigenic mice, using deletion-specific PCR with primers 8 and 10. C: Relative expression of Smad4 transcripts examined by quantitative RT-PCR (qRT-PCR). Smad4 expression was lost in HN-Smad4-/− mucosa and HNSCC. Residual expression in these tissues is due to contaminating stromal cells that retain expression of Smad4. The average expression from 5-10 samples in each group are presented. The average expression of Smad4 in the HN-Smad4+/+ samples was arbitrarily set to 100%. Error bars indicate SE. Significance was calculated using a student’s t-test: ∗ = p<0.05 in comparison with HN-Smad4+/+ mucosa. D: IHC of Smad4 in mouse buccal tissue and HNSCC. Note that the Smad4 protein was detected in the buccal epithelia of a HN-Smad4+/+ mouse orally treated with RU486. Smad4 was specifically ablated in the buccal mucosa of a HN-Smad4-/− mouse treated orally with RU486, and an HNSCC arising from a HN-Smad4-/− mouse. 5-10 samples from each group were examined and a representative picture is presented. The scale bar in the top panel represents 40 μm for all panels.

**Figure S3.** HN-Smad4-/− HNSCC exhibited increased proliferation and reduced apoptosis. A: PCNA staining of HN-Smad4+/+ mucosa, HN-Smad4-/− mucosa, and HN-Smad4-/− HNSCC. HN-Smad4-/− mucosa had increased proliferation compared to HN-Smad4+/+ mucosa, and proliferation was further increased in HN-Smad4-/− HNSCC. 5-
10 samples were analyzed and a representative picture is presented. B: TUNEL staining of \textit{HN-Smad4+/+} mucosa, \textit{HN-Smad4-/} mucosa, and \textit{HN-Smad4-/} HNSCC. Apoptosis was reduced in \textit{HN-Smad4-/} mucosa compared to \textit{HN-Smad4+/+} mucosa, and further reduced in \textit{HN-Smad4-/} HNSCC. 5-10 samples were analyzed and a representative picture is presented. The scale bar in the upper left panel represents 20 $\mu$m for all panels.

\textit{Figure S4}. Ras activation cooperates with Smad4 in HNSCC tumorigenesis. A: \textit{H-ras} sequencing traces in \textit{HN-Smad4-/} HNSCC revealed spontaneous codon 61 mutations in 4/18 cases. Examples of each type of mutation (CAA to CAC, CTA, or CGA) are presented. B: IHC for Ras revealed that Ras proteins were overexpressed in \textit{HN-Smad4-/} mucosa and HNSCC compared to \textit{HN-Smad4+/+} mucosa. C: H&E staining of \textit{HN-K-ras}^{G12D}/Smad4-/- dysplastic mucosa and HNSCC sections. The scale bar in the top panel of B represents 40 $\mu$m for all panels in B and C.

\textit{Figure S5}. Smad4 Knockdown in Normal Keratinocytes and Restoration in Smad4-deficient HNSCC cells. qRT-PCR and Western analysis of Smad4 levels in HEKn, HEKn + Smad4 siRNA, Cal27, and Cal27-Smad4 cells. The qRT-PCR values were calculated relative to HEKn cells arbitrarily set at 100% for every experiment. Samples were run in triplicate for each experiment, and the average relative expression levels from 3-4 pooled experiments are presented. *: p<0.05 for HEKn + Smad4 siRNA compared to HEKn cells, and †: p<0.05 for Cal27-Smad4 cells compared to Cal27 cells. Western blotting for Smad4 revealed that Smad4 was lost at the protein level by over 70% in HEKn + Smad4 siRNA compared to HEKn cells, and restored in Cal27-Smad4 compared to Cal27 cells.
Figure S1

### Table A

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- **●**: LOH (Loss of Heterozygosity)
- **○**: Retention of Heterozygosity
- **△**: Not informative (Homozygous)

### Table B

#### Adjacent Mucosa (Heterozygous)

- **D18S474**
- **D18S46**

#### HNSCC (LOH)

- **D18S474**
- **D18S46**
Figure S2

A

Keratin 5/14  Cre  ΔPR  LoxP  Smad4  LoxP

Wild-type Smad4  Floxed Smad4  Deleted Smad4

B

Wild-type Smad4
Floxed Smad4  Deleted Smad4

C

Smad4 Relative mRNA Expression (%)

Smad4 Relative mRNA Expression (%)

D

Smad4
Figure S3

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Figure S4

A

CAA(Gln) to CAC(His)

CAA(Gln) to CTA(Leu)

CAA(Gln) to CGA(Arg)

B

Ras

HN-Smad4+/+
Mucosa

HN-Smad4−/
Mucosa

HN-Smad4−/
HN-SCC

C

HN-Kras$^{G12D}$/Smad4+/
Mucosa

HN-Smad4−/
HN-SCC
Supplemental References


Supplemental References


