Abstract

Deregulated transforming growth factor-β (TGFβ) signaling is a common feature of many epithelial cancers. Deletion of TGFβ receptor type 2 (TGFBR2) in fibroblast specific protein-1 (FSP1)-positive stromal cells induces squamous cell carcinoma in the murine forestomach, implicating fibroblast-derived hepatocyte growth factor (HGF) as the major driver of the epithelium carcinogenesis. Prior to cancer development, hyperproliferative FSP1+ fibroblasts lacking TGFBR2 accumulate in the forestomach, disrupting the regulatory signaling cross-talk with the forestomach epithelium. Here, concurrent loss in TGFBR2 and SMAD4 completely abrogates the development of forestomach cancer. Bone morphogenic protein-7 (BMP7) was highly upregulated in forestomach cancer tissue, activating Smad1/5/8 signaling, cell proliferation, and HGF production in TGFBR2-deficient FSP1+ fibroblasts. This stimulation by BMP7 was lost in the combined TGFBR2 and SMAD4 double knockout fibroblasts, which included a profound decrease in HGF expression. Thus, Smad4-mediated signaling is required to initiate epithelial carcinogenesis subsequent to TGFBR2 deletion in FSP1+ fibroblasts.

Implications: These findings reveal a complex cross-talk between epithelial cells and the stroma, wherein Smad4 is required to elicit squamous cell carcinomas in the forestomach of mice with TGFBR2-deficient stromal cells. Mol Cancer Res; 1–11. ©2018 AACR.

Introduction

TGFβ signaling acts as a potential tumor suppressor in early-stage carcinogenesis, but is commonly upregulated and could stimulate tumor progression in advanced cancers (1). Deregulated TGFβ/BMP signaling in solid tumors has emerged as a dynamic component of tumorigenesis, cancer progression, and metastasis (1). Disrupted TGFβ/BMP signaling in cancer may be attributed to the frequently reported TGFBR2 and SMAD4 mutations in various gastrointestinal (GI) carcinomas (1–4). These mutations accumulate in the transforming epithelial cells during adenoma to carcinoma transition (1, 3, 5), but genetically engineered mouse modeling studies show that such loss-of-function mutations, in the epithelium, cannot initiate cancer development alone and require additional carcinogenic stimuli to elicit cancer (1). Accordingly, conditional deletion of TGFBR2 or SMAD4 in epithelial cells was not sufficient to generate spontaneously arising tumors in the GI tract of genetically engineered mouse models (GEMM; ref. 1). Deregulated TGFβ signaling in the stroma may, however, partake in early events of epithelial carcinogenesis, possibly via mutagenesis or epigenetic modifications (6, 7). Specifically, targeted deletion of TGFBR2 in FSP1+ stromal cells led to the development of squamous cell carcinoma in the forestomachs of mice (6).

The importance of potential genetic and epigenetic defects accumulating in mesenchymal stromal cells in neoplastic conversion of epithelium and carcinogenesis remains an area of active investigation (7–13). The delicate paracrine signaling control between the epithelium and its supporting stroma is nevertheless critical for tissue homeostasis, and impaired TGFβ signaling in FSP1+ fibroblasts resulted in highly penetrant forestomach carcinomas in mice, implicating engagement of Met signaling in cancer cells via enhanced HGF production by FSP1+ fibroblasts (6). Our study aimed to delineate the impaired TGFβ signaling circuitry in FSP1+ fibroblasts in the emergence of forestomach cancer. We report here the obligatory Smad4-mediated signaling in FSP1+ fibroblasts lacking TGFBR2 to give rise to epithelial carcinogenesis in the forestomach.

Materials and Methods

Mice

Tg(S100a4-cre)1Egn (FSP1-Cre) and Tg(S100a4-EGFP)M1Egn (FSP1-GFP) mice were a kind gift from E.G. Neilson (Northwestern; ref. 6). Tgbr2sim1.2Him (Tgbr2loxPlox) mice were a kind gift from F. Neilson.
gift from H. L. Moses (Vanderbilt; ref. 14). Smad4<sup>tm1.1Rdp</sup> (Smad4<sub>fl<sub>ox/red<sup>/></sub></sub>) mice (15) were kindly provided by R.A. DePinho (MDACC). R26R-LSL-EYFP (R26R-EYFP) reporter mice (16) were kindly provided by B.G. Neel (Harvard). The αSMA-Cre and αSMA-RFP mice were previously described (17, 18). Mice strains used in this study include control (wt) mice which include both Cre-negative littermates of TGFBR2<sup>fl<sub>ox</sub></sub> and TGFBR2/Smad4<sup>fl<sub>ox</sub></sub> strains or Cre-positive littermates with heterozygous loss of Smad4 (gift from H. L. Moses (Vanderbilt; ref. 14). Smad4<sup>tm1.1Rdp</sup> Eikesdal et al.

Forestomach fibroblast primary cell culture

The forestomachs of wild-type (wt), TGFBR2<sup>fl<sub>ox</sub></sub>, and TGFBR2/Smad4<sup>fl<sub>ox</sub></sub> mice were collected, minced into small pieces, and digested with 400 units/ml of collagenase IV (Worthington) in Dulbecco Minimal Essential Medium (DMEM, Cellgro) at 37°C for 24 hours in a cell incubator. Next day, the medium was replaced with DMEM, supplemented with 20% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Cellgro). The cells were grown at 37°C in a humidified chamber and 5% CO<sub>2</sub> under sterile tissue culture conditions and passaged when they reached 80% confluence. The resulting fibroblast cell cultures at passages 4 to 6 were used for all the experiments. The fibroblast cultures were confirmed for the presence of FSP1 protein by immunostaining.

Antibodies

For immunohistochemistry. Goat anti-BMP7 (Santa Cruz Biotechnology, sc-6899, dilution: 1:25), goat anti-ALK2 (R&D, AF637, 1:50), rabbit anti-ALK3 (Santa Cruz Biotechnology, sc-20736, 1:150), goat anti-ALK6 (Santa Cruz Biotechnology, sc-5679, 1:150), rat anti-CD45 (R&D, MAB114, 1:50), rabbit anti-C5 (Abcam, ab24647, 1:1200), rabbit anti-CXCR2 (Abcam, ab53120, 1:200), rabbit anti-Met (phosphorylated Tyr1001, Abcam, ab1024, 1:50), rabbit anti-S100A4/FSP1 (gift from E.G. Neilson, 1:450), rabbit anti-HGF (Santa Cruz Biotechnology, sc-7949, 1:50), rabbit anti-Ki67 (Abcam, ab15580, 1:500), and rabbit anti-p63 (phosphorylated Ser160/162, Cell Signaling Technology, sc-3101S, 1:100). BMP7, HGF, phosphorylated Akt, phosphorylated-Smad1/5, and PTEN are monoclonal antibodies; all other antibodies are polyclonal.

For immunofluorescence. Anti-ALK6 (Santa Cruz Biotechnology, sc-25455, 1:50), anti-C5 (Abcam, ab24647, 1:300), anti-Met (phosphorylated Tyr1001, Abcam, ab61024, 1:50), anti-FSP1 (gift from E.G. Neilson, 1:150), anti-HGF (Santa Cruz Biotechnology, sc-7949, 1:50), anti-Tgfbr2 (Santa Cruz, sc-220, 1:100), anti-S100A4/FSP1 (DAKO, A5114, 1:4,000) or anti-Ki67 (Thermo Scientific, RM-9106-S, 1:500). Anti-Ki67 is rabbit monoclonal; all other antibodies are polyclonal.

For Western blot. Rabbit anti-Akt (Sigma, A2066, 1:2500), rabbit anti-Akt (phosphorylated Ser473, Cell Signaling Technology, #4060, 1:1000), mouse anti-BMP7 (Sigma, B2555, 1:2000), rabbit anti-Erk1/2 (phosphorylated Thr202/Tyr204, Cell Signaling Technology, #9101, 1:500), mouse anti-HGF (Assay Designs, #905-165, 1:5000), rabbit anti-Pten (Cell Signaling Technology, #9559, 1:1000), rabbit anti-Smad1/5 (phosphorylated Ser463/465, Cell Signaling Technology, #9516, 1:1000), rabbit anti-Smad2 (phosphorylated Ser465/467, Cell Signaling Technology, #3101S, 1:1000). BMP7, HGF, phosphorylated Akt, phosphorylated-Smad1/5, and PTEN are monoclonal antibodies; all other antibodies are polyclonal.

Immunohistochemistry of tissue sections

Tissues were fixed in formalin, paraffin embedded, and 4 μm sections were prepared. Briefly, sections were deparaffinized and rehydrated, before antigen retrieval at 98°C for 1 hour in 0.01 mol/L citrate buffer (pH 6.0). After blocking with diluted serum from the secondary antibody host for 30 minutes, the slides were incubated overnight (+4°C) with the primary antibody. After blocking endogenous peroxidase activity for 20 minutes with 3% hydrogen peroxide (Sigma), a biotinylated anti-goat or anti-rabbit secondary antibody was applied for 30 minutes (Vector Laboratories). The antigen–antibody complex was revealed by incubating with avidin–biotin–peroxidase (ABC) for 30 minutes according to the manufacturer’s instructions (Vetcstain ABC Kit, Vector Laboratories). Staining was visualized by incubation with diaminobenzidine tetrahydrochloride (Vector Laboratories) for 2 to 10 minutes, as appropriate. The sections were then counterstained with hematoxylin (Fisher) where appropriate, dehydrated and mounted with Entellan (Electron Microscopy Services). Parallel sections were run for all the experiments without primary antibody, to ensure the specificity of the immunoreactions. The above protocol was also used for ALK6 and FSP1 and CD45 and FSP1 double staining, with the following modifications: Blocking was performed with diluted donkey serum. The goat anti-ALK6 or rat anti-CD45 antibody was added as the first antibody and incubated overnight at 4°C, followed by a biotinylated donkey anti-goat or anti-rabbit secondary antibody (Jackson ImmunoResearch) and ABC reagent. Staining was visualized by incubating for 20 minutes with 3-amin-9-ethylcarbazole (AEC, Vector Laboratories), before washing thoroughly and adding an avidin–biotin blocking solution (Vector). Thereafter, the tissue sections were blocked again with diluted donkey serum, before adding a rabbit anti-FSP1, polyclonal antibody for 60 minutes, followed by a biotinylated goat anti-rabbit antibody. The antigen–antibody complex was revealed by incubation with avidin–biotin–alkaline phosphatase for 30 minutes according to the manufacturer’s instructions (Vetcstain ABC-AP Kit, Vector Laboratories). Staining was visualized by incubating for 30 minutes with Vector Blue (Vector Laboratories). The sections were then dehydrated and mounted with Vectamount AQ (Vector Laboratories).

Immunofluorescence analyses of cultured cells

Primary forestomach fibroblasts were grown to subconfluence on 8-well BD Falcon culture slides and fixed in ice-cold methanol (–20°C). After blocking with 1% donkey serum, the cells were incubated with the primary antibody overnight at 4°C. Subsequently, the immunoreactions were detected by rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch), mounted with Vectashield with DAPI (Vector Laboratories), and analyzed by fluorescence microscopy. Parallel wells were run for all the experiments without primary antibody, to ensure the specificity of the immunoreactions.

Imaging of EYFP signal and CK5 immunofluorescence

Mouse tissues were fixed overnight at 4°C in 4% paraformaldehyde (PFA); then transferred into 30% sucrose/phosphate
buffered saline (PBS, Cellgro) for at least 24 hours. After rinsing with PBS, the tissues were embedded in OCT compound (Sakura) and frozen in liquid nitrogen. Cryosections (10 μm) were prepared, blocked with 1% bovine serum albumin (BSA, Sigma), before incubating with a rabbit anti-Ck5 antibody for 60 minutes. Thereafter a TRITC-conjugated donkey anti-rabbit secondary antibody (The Jackson Laboratory) was applied for 60 minutes to detect the Ck5-positive cells. The sections were then mounted with Vectashield with DAPI and analyzed by fluorescence microscopy. EYFP-positive cells were visualized directly, without the need for antibody staining.

**Imaging of FSP1-GFP and αSMA-RFP signal**

Tissues from FSP1-GFP;αSMA-RFP double transgenic and wt mice were fixed in 4% PFA overnight at 4°C and then transferred into 30% sucrose/PBS for at least 24 hours. After rinsing with PBS, the tissues were embedded in OCT compound and frozen in liquid nitrogen. Cryosections (7.5 μm) were prepared, hydrated with a brief wash in PBS, mounted with Vectashield with DAPI and analyzed by fluorescence microscopy. Both the GFP and RFP transgene could be visualized directly by fluorescence microscopy.

**Coimmunofluorescence staining for FSP1 and Ki67**

Coimmunofluorescence staining was performed using tyramide signal amplification (TSA) technology (PerkinElmer), as described previously (19). Tissues were fixed overnight in formalin, paraffin embedded, and 5 μm sections were prepared. After deparaffinization and rehydration, tissues were fixed to the slides with formaldehyde:methanol (1:1) prior to antigen retrieval for 30 minutes in citrate buffer (pH 6.0) heated to 98°C in the EZ Retriever microwave (BioGenex). After cooling, tissues were blocked with 1% BSA for 30 minutes at room temperature (RT) followed by primary antibody (FSP1) incubation (1 hour at RT). A secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody was applied subsequently (10 minutes at RT), followed by incubation with the TSA reagent (Opal 690, 1:50, 10 minutes at RT). A second antigen retrieval of 30 minutes (citrate buffer, pH 6.0, 98°C) followed and the same protocol was executed for the Ki67 antibody (TSA reagent: Opal 520, 1:200).

Immunofluorescence pictures (400×) were taken of representative forestomach epithelium and stroma by one observer (H. Sugimoto), and the pictures were given random numbers. The number of Ki67-positive cells in the forestomach were counted by another observer (H. Eikesdal), blinded to the mouse genotype. Ki67-positive cells in the epithelium (FSP1 negative), and in the stroma (FSP1 positive) were summarized for the whole tissue section framed within the picture, and from at least three separate mice per genotype. After unblinding by the first observer (H. Sugimoto), the number of Ki67-positive cells in the epithelium (FSP1 negative) and the stroma (FSP1 positive) were compared between the three genotypes.

**RT-PCR analyses**

Primary forestomach fibroblasts were plated on 6-well plates (Falcon), at 150,000 cells per well, and starved 24 hours in DMEM with 0.1% FBS. Thereafter, the cells were either harvested directly, or the media were changed to DMEM with 0.1% FBS with100 ng/mL of BMP7, and the cells were treated for 24 or 48 hours before harvesting protein. The protein lysates were fractionated using SDS–PAGE gel electrophoresis, transblotted to PVDF membranes by semidry technique, before performing coomassie staining to ensure equal protein loading. Thereafter, the membranes were blocked with 5% fat-free dry milk for 60 minutes, before immunoblotting overnight with the primary antibody. The immobilized antibody was detected using the appropriate horse-radish peroxidase–conjugated secondary antibody (Sigma) and ECL (Pierce). The immunoreaction was visualized using Hyblot autoradiography films (Denville). Immunoblot for actin were performed for all samples to ensure equal protein loading. Uncropped Western blots are displayed in Supplementary Fig. S5. The intensity of the bands from uncropped blots was quantified using an Eppendorf BioPhotometer. Reverse transcription of 0.5 μg of RNA from each sample was performed with Superscript II reverse transcriptase (Invitrogen) or Multiscribe reverse transcriptase (Applied Biosystems), followed by RNASate treatment, before running PCR (35 cycles with the below primers.

**Primer sequence.**

<table>
<thead>
<tr>
<th>Primer forward</th>
<th>Primer reverse</th>
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<tbody>
<tr>
<td>Activin A forward</td>
<td>TGGAGCAGACCTCGAGATCTAC</td>
</tr>
<tr>
<td>Activin A reverse</td>
<td>AAAGCATGACTGCGGACCTC</td>
</tr>
<tr>
<td>Activin B forward</td>
<td>TCCTGAGATCATCAGTTGACG</td>
</tr>
<tr>
<td>BMPF2 reverse</td>
<td>ATCTGTACGCAGCTATGAGC</td>
</tr>
<tr>
<td>BMPF4 reverse</td>
<td>AAGGAGAACTCTACTGCGTC</td>
</tr>
<tr>
<td>BMPF6 reverse</td>
<td>ACAGCTCTTGAGACGGCGAAT</td>
</tr>
<tr>
<td>BMPF7 reverse</td>
<td>TGACAGAAGAGTGTGCCAGAG</td>
</tr>
<tr>
<td>HGF forward</td>
<td>ACCAAATCTGGCGCTTCTG</td>
</tr>
<tr>
<td>[ß-actin forward</td>
<td>TGGATTTGCTGTTGCTGATATC</td>
</tr>
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</table>
| [ß-actin reverse      | AGTTCTGATGAGCAGCCAG  | Role of Mesenchymal Stroma in Gastric cancer

**Western blot**

Cells and tissues were homogenized and lysed with protein lysis buffer (50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Protein concentrations were measured by a bicinchnonic acid assay (Pierce), and 30 μg of protein was loaded per lane for the whole tissue immunoblots and 15 μg of protein was loaded per lane for the cell culture immunoblots. For the BMP7 stimulation experiment, primary forestomach fibroblasts were plated on 6-well plates (Falcon), at 150,000 cells per well, and starved 24 hours in DMEM with 0.1% FBS. Thereafter, the cells were either harvested directly, or the media were changed to DMEM with 0.1% FBS with100 ng/mL of BMP7, and the cells were treated for 24 or 48 hours before harvesting protein. The protein lysates were fractionated using SDS–PAGE gel electrophoresis, transblotted to PVDF membranes by semidry technique, before performing coomassie staining to ensure equal protein loading. Thereafter, the membranes were blocked with 5% fat-free dry milk for 60 minutes, before immunoblotting overnight with the primary antibody. The immobilized antibody was detected using the appropriate horse-radish peroxidase–conjugated secondary antibody (Sigma) and ECL (Pierce). The immunoreaction was visualized using Hyblot autoradiography films (Denville). Immunoblots for actin were performed for all samples to ensure equal protein loading. Uncropped Western blots are displayed in Supplementary Fig. S5. The intensity of the bands from uncropped blots was quantified using ImageJ software. The areas under each peak for the proteins of interest were normalized to those of actin in each blot.

**Chromogenic in situ hybridization (CISH)**

Sense and antisense oligonucleotide probes (Operon) for HGF and BMP7 RNA were designed and labeled with digoxigenin using the DIG oligonucleotide 3’-End Labeling Kit (Roche). Glass slides were coated with a 2% solution of 3-aminopropyl-triethoxy-silane (Sigma) and washed with acetone and DEPC-water before use. Cryosections (14 μm) were applied to the coated slides, fixed immediately with 4% PFA, permeabilized with 0.2 mol/L HCl and protease K, fixed again in 4% PFA, and then incubated 3 hours

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in hybridization buffer (50% formamide, 2× SSC, 50 mmol/L phosphate buffer, 1× Denhardt’s solution, 5% sodium dextran). Thereafter, the sections were incubated in hybridization buffer with either the sense or antisense DIG-labeled probes, overnight at 37°C. The next day the sections were washed in washing buffer (100 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl), and incubated 30 minutes with blocking buffer (Roche DIG High Prime DNA Labeling and Detection Starter kit I), before adding an alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 60 minutes at 37°C. After repeated washes, the sections were equilibrated in detection buffer (100 mmol/L Tris HCl, pH 9.5, 100 mmol/L NaCl), and the immunoreaction was visualized by incubating with NBT/BCIP color substrate (Roche) for 3 hours. The color reaction was stopped using TE buffer, and the sections were mounted using Vectashield. Parallel sections were run with sense and antisense oligonucleotide probes, with or without DIG antibody, and with or without NBT/BCIP color substrate, to ensure the specificity of the in situ RNA hybridization and of the immunoreaction.

**Sequence of the CISH oligonucleotide primers.**

| HGF sense | CAGTGTCCAGATGAGTGTGACCTGTC
| HGF antisense | CGACGATCTGATCACTTGGAAACACTG
| BMP7 sense | AGCGATTTGACAACGAGACCTTCCAGATCACAGTCTATCAGTGTCCTCAG
| BMP7 antisense | CTGGAGACCGCTGATAGGCTGTGACCTGTC

**E10 epithelial cell proliferation assays.**

Primary forestomach fibroblasts were grown to subconfluence in DMEM with 20% FBS. Thereafter, the media were changed to DMEM with 0.1% FBS, and the cells were incubated for 48 hours. Then, the conditioned media were sterile filtered (0.22 μm, Fisherbrand) and stored at −80°C until further use. E10 epithelial cell lines were used to determine the influence of conditioned fibroblast media on epithelial cell function. The E10 lung epithelial cell line was a kind gift from A. Malkinson (UCHSC). The cells were subsequently profiled by STR analysis, tested negative for mycoplasma, and were grown in CMRL-1066 Medium (Gibco), supplemented with L-glutamine (0.15 g/L, Cellgro), 10% FBS, and 100 μg/mL of penicillin, and 100 μg/mL of streptomycin. The cells were seeded at 1,800 cells per well in 96-well plates (Falcon) and allowed to attach overnight in CMRL-1066 with 10% FBS. Next, the E10 cells were serum starved in DMEM with 0.1% FBS for 24 hours, before culturing for 48 hours with the conditioned fibroblast media, with or without the Met inhibitor SU11274 (10 μmol/L, kind gift from Pfizer). The surviving cell number was assessed by methylene blue assay, as described previously (20). Multiple rows of 8 wells were subsequently analyzed. Cells were regularly tested and negative for mycoplasma.

**BMP7 stimulation of forestomach fibroblast proliferation.**

Primary forestomach fibroblasts were seeded at 2,000 cells per well in 96-well plates and allowed to attach overnight in DMEM with 20% FBS. Thereafter, the cells were serum starved in DMEM with 0.1% FBS for 24 hours, before treating for 48 hours with 0, 1, 10 or 100 ng/mL recombinant human BMP7 (BMP7, R&D) in DMEM with 0.1% FBS. The surviving cell number was assessed by methylene blue assay, as previously described (20).

**Statistical analyses.**

The data are presented as the mean ± SEM. GraphPad prism was used to generate graphs and perform statistical analyses, and the statistical tests used are listed for each respective panel in the figure legends. All source data are provided for every panel.

**Results.**

**Loss of Smad4-mediated signaling in FSP1+ stromal cells lacking TGFBR2 abrogates forestomach cancer development.**

We faithfully replicated the findings by Bhowmick and colleagues, wherein conditional loss of TGFBR2 in FSP1+ stromal cells (Tgfrb2loxP/loxP, FSP1-Cre mice, entitled TGFBR2 conditional knockout, TGFBR2KO mice) resulted in squamous cell carcinomas of the forestomach, with 100% penetrance at 1.5 months of age, and moribundancy between 1 and 2 months of age (Fig. 1A–C, ref. 6). Strikingly however, when these mice were further crossed onto the Smad4loxP/loxP background to generate mice with FSP1+ stromal cells lacking both TGFBR2 and Smad4 (Tgfrb2loxP/loxP, Smad4loxP/loxP-FSP1-Cre, hereafter referred to as TGFBR2/Smad4KO), they no longer presented with cancer and had a normal life span, similar to control (wt) mice (Fig. 1A–C).

In contrast to the TGFBR2/Smad4KO mice, TGFBR2/Smad4KO mice showed no evidence of forestomach carcinogenesis and presented with normal forestomach histology (Fig. 1C). Smad4 conditional loss in FSP1+ stromal cells (Smad4loxP/loxP, FSP1-Cre; Smad4KO) did not result in forestomach cancer (Fig. 1A and C), and these mice presented with mild cartilage developmental defects as previously described (21). Cre-negative controls (Tgfrb2loxP/loxP; Smad4loxP/loxP) were phenotypically normal (Fig. 1A). Notably, FSP1+ stromal cells appeared equally abundant in the forestomach of wt and TGFBR2/Smad4KO mice (Fig. 1C). These results indicated that the loss of Smad4 in FSP1+ stromal cells lacking TGFBR2 did not result in loss of FSP1+ cells in the forestomach, but rather changed their downstream signaling, leading to a phenotypic, fully penetrant, reversal of the oncogenic potential of FSP1+ stromal cells acting on the epithelium.

**Mesenchymal FSP1+ stromal cells specifically induce forestomach cancer.**

In the forestomach tumors of TGFBR2KO mice, the squamous cell carcinoma cells were characterized by cytokeratin 5 and phosphorylated p63-positive staining (Supplementary Fig. S1A), whereas the FSP1 immunolabeling was localized to the stroma (Fig. 1C). Systematic analysis of the healthy gastrointestinal tract of double transgenic αSMA-RFP; FSP1-GFP mice demonstrated that FSP1+ stroma cells were abundantly found immediately below the epithelium, whereas αSMA+ stromal cells were mostly located in the deeper smooth muscle layers (Fig. 1D; Supplementary Fig. S1B). Lineage tracing of FSP1+ cells in normal forestomach of adult mice, using FSP1-Cre; R26R-LSL-EYFP mice, indicated that FSP1 was largely expressed in the stroma and not the epithelium of the forestomach (Fig. 1E). Combined immunolabeling for FSP1 and the pan-leucocyte marker CD45 indicated that a subset of the FSP1+ stromal cells were leukocytes (Supplementary Fig. S1C), in agreement with previous reports (22, 23). While inflammation could affect forestomach cancer progression (24), leucocyte infiltration in the forestomach of TGFBR2KO mice was not a dominant feature of the histopathologic findings.

We additionally tested whether other mesenchymal stromal cells in the forestomach could also impart tumorigenesis via specific deletion of TGFBR2. We used the α-smooth muscle actin...
Figure 1. SMAD4 loss in TGFBR2ΔKO FSPI⁺ stroma abrogates forestomach cancer. A, Forestomach squamous cell carcinoma incidence rates and (B) survival of mice with the indicated genotypes. Log-rank (Mantel-Cox) test. ***, P < 0.001. wt, n = 7; TGFBR2ΔKO, n = 38; TGFBR2/SMAD4ΔKO, n = 14 mice. C, Hematoxylin and eosin (H&E) staining and FSP1 immunolabeling of forestomach tissue of mice with the indicated genotypes. Scale bar, 50 μm. D, Visualization of FSP1-GFP and αSMA-RFP fluorescent gene product in sections of the forestomach of FSP1-GFP;αSMA-RFP double transgenic mice. L: lumen, E: epithelium, S: stroma, SM: smooth muscle; *, green autofluorescence in the epithelial keratin layer. DAPI (blue): nuclei. Scale bar, 50 μm. E, EYFP visualization (green) and immunolabeling for cytokeratin 5 (CK5) detected with TRITC-conjugated secondary immunolabeling in sections of the forestomach tissue from R26-EYFP; FSP1-Cre⁺ mice. DAPI (blue); nuclei; E, epithelium; S, stroma. See accompanying source data.
(αSMA)-Cre mice (17) to conditionally delete TGFBR2 in the smooth muscle layer of the forestomach. As previously reported, the Tgfbr2<sup>ΔSMA</sup> mice were generated with no obvious phenotypic defects (17). Histologic analyses of the forestomach revealed no evidence of neoplasia (Supplementary Fig. S1D). These results support that deregulated TGFβ signaling specifically in the mesenchymal FSP1<sup>+</sup> stroma most proximal to the forestomach epithelium leads to forestomach carcinoma.

**Loss of TGFβR2 in FSP1<sup>+</sup> stromal cells induces epithelial proliferation via HGF/Met signaling**

Analysis of forestomachs from TGFBR2<sup>−/−</sup> mice prior to the emergence of carcinoma (mice of 2 and 4 weeks of age) indicated a progressive, robust induction of FSP1<sup>+</sup> stromal cell proliferation beneath the forestomach epithelium (Fig. 2A–E; Supplementary Fig. S2). Elevated HGF was noted in TGFBR2<sup>−/−</sup> forestomach tumor lysates when carcinomas had developed, and this was not seen in TGFBR2/SMAD4<sup>−/−</sup> forestomachs (Fig. 2F and G; Supplementary Fig. S1E–S1G). These data suggested that HGF production by TGFBR2-deficient fibroblasts participated in neoplasia of the forestomach. RT-PCR was used to assess gene expression of known growth factors secreted by fibroblasts to directly affect epithelial cells, such as EGF, IL6, CTGF, and others (25–35). Out of all tested growth factors, HGF was found specifically upregulated in TGFBR2<sup>−/−</sup> forestomach fibroblasts (Supplementary Fig. S3A). Increased secreted HGF levels by TGFBR2<sup>−/−</sup> forestomach-derived fibroblasts expanded in vitro, compared with wt control forestomach fibroblasts, were validated using a mouse angiogenesis cytokine array (Supplementary Fig. S3B–S3C). Immunolabeling analysis showed that TGFBR2<sup>−/−</sup> forestomach fibroblasts, in contrast to wt and TGFBR2/SMAD4<sup>−/−</sup> forestomach fibroblasts, had upregulated HGF expression (Fig. 2H). Notably, all fibroblasts expressed FSP1, and a specific loss of TGFβR2 expression in TGFBR2/SMAD4<sup>−/−</sup> and TGFBR2<sup>−/−</sup> forestomach fibroblasts was confirmed (Fig. 2I).

The impact of elevated HGF production by TGFBR2<sup>−/−</sup> forestomach fibroblasts was evaluated in the E10 normal mouse lung epithelial cell line (Fig. 2J). Conditioned media from TGFBR2<sup>−/−</sup> forestomach fibroblasts induced E10 cell Met phosphorylation, whereas conditioned media of TGFBR2/SMAD4<sup>−/−</sup> forestomach fibroblasts failed to induce Met phosphorylation (Fig. 2J). Critically, TGFBR2<sup>−/−</sup> forestomach fibroblasts conditioned media induced E10 cell proliferation in a Met-signaling dependent manner, with SU11274 Met inhibitor abrogating these effects (Fig. 2I). While our transcriptomic analysis and cytokine array analysis of conditioned media from purified forestomach fibroblasts indicate that additional cytokines and growth factors could play a role in the induction of forestomach epithelial cell proliferation and emergence of forestomach carcinoma (Supplementary Fig. S3A–S3C), HGF emerged as a critical player in forestomach carcinogenesis because rescuing the malignant phenotype by additional SMAD4 deletion normalized HGF production and Met phosphorylation (Fig. 2G and K).

**BMP7/HGF imbalance in forestomach and epithelium paracrine signaling aggravates forestomach carcinogenic response**

Given that stromal SMAD4 deletion led to a complete rescue of the TGFBR2<sup>−/−</sup> forestomach carcinoma (Fig. 1A–C), Smad4-dependent signaling, implicating bone morphogenetic protein (BMP) and/or activin receptor-mediated signaling, thus emerged as critical for carcinogenesis of the forestomach epithelium. Within the TGFβ superfamily, BMPs and activins signal via BMP and activin receptors, respectively, to activate regulatory Smads, which use Smad4 to translocate to the nucleus (37). Forestomach lysates from TGFBR2<sup>−/−</sup> mice showed increased levels of phosphorylated Smad1/5 and Smad2 (normalized to actin), while this was not observed in forestomach lysates from wt and TGFBR2/SMAD4<sup>−/−</sup> mice (Fig. 3A; Supplementary Fig. S3D). We also assessed the expression levels of alternative ligands of the TGFβ superfamily (BMP2, BMP4, BMP6, BMP7, Activin A and Activin B) in the forestomach that could activate Smad4 signaling (Fig. 3B). Activin A and BMP7 were both upregulated in TGFBR2<sup>−/−</sup> forestomachs in contrast to TGFBR2/SMAD4<sup>−/−</sup> forestomachs (Fig. 3B). Activin A, however, failed to induce HGF expression in TGFBR2<sup>−/−</sup> forestomach fibroblasts (Supplementary Fig. S4A), whereas BMP7 exposure robustly induced HGF expression in TGFBR2<sup>−/−</sup> fibroblasts in vitro (Fig. 3C). Transcriptomic analysis as well as BMP7-dependent HGF induction thus implicated BMP7 as a key signaling molecule able to induce HGF in fibroblasts lacking TGFBR2 but with intact SMAD4. BMP7 protein level was markedly elevated in forestomachs of TGFBR2<sup>−/−</sup> mice, concomitant with increased HGF expression, possibly contributing to the BMP7/HGF imbalance in forestomach and epithelium paracrine signaling.
during forestomach cancer progression (Fig. 3D; Supplementary Fig. S4B). In situ hybridization demonstrated that HGF expression localized to forestomach stromal cells, while BMP7 was produced in the epithelium (Fig. 3E).

BMP ligands are known to signal via ALK2, ALK3, or ALK6 to activate Smad1/5/8, bind Smad4, and translocate to the nucleus (3, 36). BMP7 preferentially signals via ALK2 and ALK6 and is known to stimulate mesenchymal cell growth (32, 37). BMP receptor IB (ALK6) was expressed on stroma cells, as well as on the tumor epithelium, and double staining demonstrated that the ALK6 receptor was present on FSP1⁺ cells (Fig. 3F). Moreover, the ALK6 receptor was strongly expressed on the in vitro fibroblasts (Fig. 3G). The two alternative BMP-specific coreceptors, ALK2 and ALK3, exhibited weaker immunolabeling (Supplementary Fig. S4C).

BMP7 can also signal via non-Smad dependent pathways, including FAK, ERK1/2, JNK, and p38 MAPK-mediated signaling, depending on BMP7 concentration (38, 39). Phosphorylated Akt and Erk1/2 levels were elevated in TGFB2/SMAD4KO fibroblasts, possibly as a compensatory mechanism for lack of Smad4 (Supplementary Fig. S4D). Critically however, exposure of forestomach fibroblasts to high levels of BMP7 indicated enhanced proliferation in wt and TGFB2cKO fibroblasts, in contrast to TGFB2/SMAD4cKO fibroblasts (Fig. 4A), supporting Smad4 signaling is required for BMP7 to stimulate fibroblast proliferation (Fig. 4A) and HGF production (Fig. 3C). Altogether our studies support a model wherein carcinogenesis of the forestomach epithelium may ensue from perpetual HGF production by FSP1⁺ fibroblasts lacking TGFB2. HGF stimulates epithelial Met signaling and induces epithelial cell proliferation (Fig. 4B). HGF production is in part promoted by epithelial-derived BMP7/ALK6 signaling via Smad4 in fibroblasts (Fig. 4B). The deregulated BMP7/HGF signaling between fibroblasts and epithelial cells enables carcinogenesis of the forestomach epithelium, wherein
loss of TGFRB2 in FSP1+ stromal cells promotes HGF production due to increased dependence on epithelial cell–derived BMP7 signaling via ALK6 and Smad4 (Fig. 4B). Interestingly, the observed BMP7/HGF cross-talk is only active when TGFBR2 is deleted in FSP1+ and not αSMA+ stromal cells (Fig. 4C; Supplementary Fig. S4E).

Discussion

The cross-talk between epithelial cells and the stroma is extensive, and while loss of TGFRB2 in epithelial cells is not sufficient to elicit carcinogenesis (1, 40), deletion of TGFRB2 in FSP1+ stromal cells mediates epithelial carcinogenesis (6), pointing at the regulatory role of stromal Tgfbr2 signaling toward the epithelium. The current results demonstrate that in TGFRB2-deficient stromal cells, Smad4 is required to elicit squamous cell carcinomas in the forestomach. Furthermore, the present data demonstrate that BMP7 signaling via Smad1/5/8 upregulates HGF production from forestomach stromal cells to promote epithelial cell proliferation via Met activation. The occurrence of carcinomas exclusively in the forestomach is not explained by the current results, but the hyperproliferative squamous epithelium in the upper part of the stomach, with high acidity and continuous mechanical erosion from undigested food may impart a particular dependence on the stroma in this organ to prevent epithelial carcinogenesis. As such, the human correlate to squamous cell carcinomas (SCC) of the forestomach in mice is SCCs developing in the lower part of the esophagus (41). Although human esophagus SCCs have a poor prognosis with today’s standard of care, HGF activation is involved in the invasive process of this cancer entity (42), and the therapeutic potential of BMP7 or HGF inhibition should be explored further clinically.

It was previously reported that conditional deletion of BMP receptor 2 (BMPR2) in FSP1+ fibroblasts or disruption of BMPR2 in breast epithelial cells promotes breast cancer metastasis in mice expressing the MMTV-PyVT oncogene (43, 44). In contrast, when BMPR2 was deleted in nestin-positive stromal cells intestinal polyps developed (45), and inhibition of BMP signaling in all cell compartments with the BMP antagonist DMH1 suppressed breast cancer metastasis (46). BMP signaling in cancer is thus highly context dependent. Here, we propose that a cross-talk circuit exists in TGFRB2cKO forestomachs consisting of BMP7 secreted by the cancer cells and HGF secreted by the stroma (Fig. 4B), which is a known signaling circuit in prostate cancer (47). Cross-talk between the cancer epithelial cells and the stroma is important for tumor progression, and apart from HGF and BMP7, numerous other growth factors are known to be secreted by these two cell compartments (30, 48–50). Importantly, the
predominant source of HGF production in epithelial cancers is the tumor stroma (48), which was confirmed by in situ hybridization in the current work. Our collective analyses indicated a dominant role for HGF deregulated signaling in TGFBRR2-deficient foretostomach fibroblasts, leading to the activation of epithelial Met receptors, thereby stimulating epithelial proliferation. Furthermore, we report that BMP7 is produced by the epithelial cells to activate BMP receptor signaling in the fibroblasts, and requiring Smad4 to promote HGF production. In contrast, Tgfbr2 binds the TGFβ receptor type II in microsatellite stable colon cancers. Cancer Res 1999;59:320–4.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Role of Mesenchymal Stroma in Gastric cancer


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