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BMP7 Signaling in *TGFBR2*-deficient Stromal Cells Provokes Epithelial Carcinogenesis

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Abstract

Deregulated transforming growth factor- β (TGF β) signaling is a common feature of many epithelial cancers. Deletion of *TGF β 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 crosstalk 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

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Conflict of interest

The authors have no conflict of interest.

Author contribution

R.K. conceptually designed the experimental strategy, provided intellectual input and helped to write the manuscript. V.S.L. and H.P.E. provided intellectual input, analyzed the data from the experiments, performed statistical analyses, prepared figures and wrote the manuscript. Y.T. and J.L.C. performed the mouse breeding, crossings and performed necropsy analyses. L.M.B. cross-validated reagents and PCR primer sequences, performed densitometry analyses, immunostainings of tissues, and provided intellectual input. H.S. performed necropsy analyses and immunohistochemistry of tissue sections. Y.T. and H.P.E. made the epithelial and fibroblast primary cell lines. H.P.E. and A.K. performed immunostaining analyses, RNA extraction, RT-PCR, protein extraction and western blots of cell lines and tissues from the mice. Y.T., H.P.E. and A.K. performed *in vitro* experiments to assess how growth factors influenced fibroblasts and epithelial cell lines. H.P.E. and K.K. designed and performed the *in situ* hybridization experiment.

decrease in HGF expression. Thus, Smad4-mediated signaling is required to initiate epithelial carcinogenesis subsequent to *TGFBR2* deletion in FSP1⁺ fibroblasts.

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 were not sufficient to generate spontaneously arising tumors in the GI tract of genetically engineered mouse models (GEMMs) (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) (6). Tgfb^{tm1.2Hlm} (Tgfb^{loxE2}) mice were a kind gift from H. L. Moses (Vanderbilt) (14). Smad4^{tm1.1Rdp} (Smad4^{loxE8/9}) 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 employed in this study include control (wt) mice which include both Cre-negative littermates of *TGFBR2*^{ΔKO} and *TGFBR2*/*SMAD4*^{KO} strains or Cre-positive littermates with heterozygous loss of *TGFBR2*. The FSP1-GFP and αSMA-RFP strains were bred to generate the double transgenic strain. Both

male and female young and adult mice were studied. The genetic backgrounds were C57Bl/6, Balb/c, sv129 or a mixture of these backgrounds. Animal studies were carried out at the Beth Israel Deaconess Medical Center and at the MD Anderson Cancer Center and approved by the Institutional Animal Care and Use Committee of each institution.

Forestomach fibroblast primary cell culture

The forestomachs of wild-type (wt), *TGFBR2*^{CKO} and *TGFBR2/SMAD4*^{CKO} mice were collected, minced into small pieces, and digested with 400 units.ml⁻¹ 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⁻¹ of penicillin and 100 µg.ml⁻¹ of streptomycin (Cellgro). The cells were grown at 37°C in a humidified chamber and 5% CO₂ under sterile tissue culture conditions and passaged when they reached 80% confluency. The resulting fibroblast cell cultures at passages 4–6 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, sc-20736, 1:150), goat anti-ALK6 (Santa Cruz, sc-5679, 1:150), rat anti-CD45 (R&D, MAB114, 1:50), rabbit anti-CK5 (Abcam, ab24647, 1:1200), rabbit anti-CK20 (Abcam, ab53120, 1:200), rabbit anti-Met (phosphorylated Tyr1001, Abcam, ab61024, 1:50), rabbit anti-S100A4/FSP1 (gift from E.G. Neilson, 1:450), rabbit anti-HGF (Santa Cruz, sc-7949, 1:50), rabbit anti-Ki67 (Abcam, ab15580, 1:500) and rabbit anti-p63 (phosphorylated Ser160/162, Cell Signaling, #4981, 1:100). Anti-CD45 is a monoclonal antibody; all other antibodies are polyclonal.

For immunofluorescence—Anti-ALK6 (Santa Cruz, sc-25455, 1:50), anti-CK5 (Abcam, ab24647, 1:300), anti-Met (phosphorylated Tyr1001, Abcam, ab61024, 1:50), anti-FSP1 (gift from E.G. Neilson, 1:150), anti-HGFα (Santa Cruz, sc-7949, 1:50), anti-Tgfr2 (Santa Cruz, sc-220, 1:100), anti-S100A4/FSP1 (DAKO, A5114, 1:4000) or anti-Ki67 (Thermo Scientific, RM-9106-S, 1:500). Anti-Ki67 is rabbit monoclonal; all other antibodies are rabbit polyclonal.

For western blots—Rabbit anti-Actin (Sigma, A2066, 1:2500), rabbit anti-Akt (phosphorylated Ser473, Cell Signaling, #4060, 1:1000), mouse anti-BMP7 (Sigma, B2555, 1:2000), rabbit anti-ERK1/2 (phosphorylated Thr202/Tyr204, Cell Signaling, #9101, 1:500), mouse anti-HGF (Assay Designs, #905-165, 1:5000), rabbit anti-PTEN (Cell Signaling, #9559, 1:1000), rabbit anti-Smad1/5 (phosphorylated Ser463/465, Cell Signaling, #9516, 1:1000), rabbit anti-Smad2 (phosphorylated Ser465/467, Cell Signaling, #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 μ m sections prepared. Briefly, sections were deparaffinized and rehydrated, before antigen retrieval at 98°C for 1 hour in 0.01 M 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 (Vectastain® ABC Kit, Vector). Staining was visualized by incubation with diaminobenzidine tetrahydrochloride (Vector) for 2–10 minutes, as appropriate. The sections were then counterstained with haematoxylin (Fisher) where appropriate, dehydrated and mounted with Entellan (Electron Microscopy Services). Parallel sections were run for all the experiments without primary antibody, to assure the specificity of the immunoreactions. The above protocol was also used for ALK6 & FSP1 and CD45 & 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-rat secondary antibody (Jackson ImmunoResearch) and ABC reagent. Staining was visualized by incubating for 20 minutes with 3-amino-9-ethylcarbazole (AEC, Vector), 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 (Vectastain® ABC-AP Kit, Vector). Staining was visualized by incubating for 30 minutes with Vector Blue (Vector). The sections were then dehydrated and mounted with Vectamount AQ (Vector).

Immunofluorescence analyses of cultured cells

Primary forestomach fibroblasts were grown to sub-confluency on eight-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 (Vectorlabs) and analyzed by fluorescence microscopy. Parallel wells were run for all the experiments without primary antibody, to assure 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 μ m) 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 (Jackson) 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 α SMA-RFP signal

Tissues from FSP1-GFP; α SMA-RFP double transgenic and wt mice were fixed in 4% PFA overnight at 4°C; 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 μ m) 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.

Co-immunofluorescence staining for FSP1 and Ki67

Co-immunofluorescence staining was performed using Tyramide Signal Amplification (TSA) technology (Perkin Elmer), as described previously (19). Tissues were fixed overnight in formalin, paraffin-embedded and 5 μ m sections prepared. After deparaffinization and rehydration, tissues were fixed to the slides with formaldehyde:methanol (1:10) 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 $\times$) were taken of representative forestomach epithelium and stroma by one observer (H.S.), and the pictures were given random numbers. The number of Ki67 positive cells in the forestomach were counted by another observer (H.P.E.), 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.S.), 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 media was changed to DMEM with 0.1% FBS with or without Activin A (50 ng.ml⁻¹) or 100 ng.ml⁻¹ BMP7 (R&D), and the cells were treated for 24 hours. Then the cells were washed with warm (37°C) PBS, and RNA was isolated with Trizol, as described in the Invitrogen product manual, followed by DNase (Invitrogen) treatment. RNA concentration was measured using an Eppendorf BioPhotometer. Reverse transcription of 0.5 μ g of RNA from each sample was performed with Superscript IITM reverse transcriptase (Invitrogen) or MultiscribeTM reverse

transcriptase (Applied Biosystems), followed by RNase treatment, before running PCR (35 cycles) with the below primers.

Primer sequence

Activin A forward:	TGGAGCAGACCTCGGAGATCATC
Activin A reverse:	AAGCACTAGACTGGCACCCTC
Activin B forward:	TCCGAGATCATCAGCTTTCAG
Activin B reverse:	TGCGGATGCGATGTCTGCTATCG
BMP2 forward:	ATCTGTACCGCAGGCACTCAGG
BMP2 reverse:	TGTGTGGTCCACCGCATCACAG
BMP4 forward:	AGCCATGCTAGTTTGATACCTG
BMP4 reverse:	AAGCAGAGCTCTCACTGGTCC
BMP6 forward:	TGCTGGATCTCTACAACGCCCTG
BMP6 reverse:	ACAGTCCTGTAGACGCGGAATC
BMP7 forward:	TGGACAACGAGGTGCACTCCAG
BMP7 reverse:	TGGTTGCTGGTGGCTGTGATATC
HGF forward:	ACCAAATTCTGCCGGTCTGTG
HGF reverse:	ATCATGGAATTCCAAGGCTGGC
β -actin forward:	TGGCAATTGTACCAACTGGG
β -actin reverse:	AGTTTCATGGATGCCACAGG

Western blot

Cells and tissues were homogenized and lysed with protein lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Protein concentrations were measured by a bicinchoninic acid (BCA) assay (Pierce), and 30 μ g of protein was loaded per lane for the whole tissue immunoblots and 15 μ g 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 was changed to DMEM with 0.1% FBS with 100 ng/ml⁻¹ 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 semi-dry technique, before performing coomassie staining to assure 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 horseradish 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 assure equal protein loading. Uncropped western blots are displayed in Suppl. Fig. 5. The intensity of the bands from uncropped blots were 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 anti-sense 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 μ m) were applied to the coated slides, fixed immediately with 4% PFA, permeabilized with 0.2M HCl and proteinase K, fixed again in 4% PFA, and then incubated 3 hours in hybridization buffer (50% formamide, 2 $\times$ SSC, 50mM phosphate buffer, 1 $\times$ Denhard's solution, 5% sodium dextran). Thereafter, the sections were incubated in hybridization buffer with either the sense or anti-sense DIG-labeled probes, overnight at 37°C. The next day the sections were washed in washing buffer (100 mM Tris HCl, pH 7.5, 150 mM 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 mM Tris HCl, pH 9.5, 100 mM NaCl) and the immunoreaction was visualized by incubating with NBT/BCTP color substrate (Roche) for three hours. The color reaction was stopped using TE-buffer and the sections were mounted using Vectashield. Parallel sections were run with sense and anti-sense oligonucleotide probes, with or without DIG antibody, and with or without NBT/BCTP color substrate, to assure the specificity of the *in situ* RNA hybridization and of the immunoreaction.

Sequences of the CISH oligonucleotide primers

HGF sense:	CAGTGTTCAGAAAGTTGAATGCATGACCTGC
HGF anti-sense:	GCAGGTCATGCATTCAACTTCTGAACACTG
BMP7 sense:	AGCGATTTGACAACGAGACCTTCCAGATCACAGTCTATCAGGTGC TCCAG
BMP7 anti-sense:	CTGGAGCACCTGATAGACTGTGATCTGGAAGGTCTCGTTGTCAAA TCGCT

E10 epithelial cell proliferation assays

Primary forestomach fibroblasts were grown to sub-confluency 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 μ m, 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⁻¹, Cellgro), 10% FBS, 100 units.ml⁻¹ of penicillin and 100 μ g.ml⁻¹ 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 μ M, kind gift from Pfizer). The surviving cell number was assessed by methylene blue assay, as described previously (20). Multiple rows of eight 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⁻¹ 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 is presented as the mean $\pm$ SEM. GraphPad prism was used to generate graphs and perform statistical analyses, and the statistical tests employed 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 TGFR2 abrogates forestomach cancer development

We faithfully replicated the findings by Bhowmick *et al.*, wherein conditional loss of *TGFR2* in FSP1⁺ stromal cells (*Tgfr2*^{flxE2}; FSP1-Cre mice, entitled *TGFR2* conditional knockout, *TGFR2*^{CKO} mice) resulted in squamous cell carcinomas of the forestomach, with 100% penetrance at 1.5 months of age, and moribundancy between 1 to 2 months of age (Fig. 1A–C) (6). Strikingly however, when these mice were further crossed onto the *Smad4*^{flxE8-9} background to generate mice with FSP1⁺ stromal cells lacking both *TGFR2* and *SMAD4* (*Tgfr2*^{flxE2}; *Smad4*^{flxE8-9}; FSP1-Cre, hereafter referred to as *TGFR2/SMAD4*^{CKO}), they no longer presented with cancer, and had a normal life span, similar to control (wt) mice (Fig. 1A–C).

In contrast to the *TGFR2*^{CKO} mice, *TGFR2/SMAD4*^{CKO} mice showed no evidence of forestomach carcinogenesis and presented with normal forestomach histology (Fig. 1C). *SMAD4* conditional loss in FSP1⁺ stromal cells (*Smad4*^{flxE8&9}; FSP1-Cre: *SMAD4*^{CKO}) did not result in forestomach cancer (Fig. 1A, C), and these mice presented with mild cartilage developmental defects as previously described (21). Cre-negative controls (*Tgfr2*^{flxE2} and *Smad4*^{flxE8-9}) were phenotypically normal (Fig. 1A). Notably, FSP1⁺ stromal cells appeared equally abundant in the forestomach of wild-type (wt) and *TGFR2/SMAD4*^{CKO} mice (Fig. 1C). These results indicated that the loss of *SMAD4* in FSP1⁺ stromal cells lacking *TGFR2* 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 *TGFR2*^{CKO} mice, the squamous cell carcinoma cells were characterized by cytokeratin 5 and phosphorylated p63 positive staining (Suppl. Fig. 1A), whereas the FSP1 immunolabeling was localized to the stroma (Fig. 1C). Systematic analysis of the healthy gastrointestinal tract of double transgenic α SMA-RFP; FSP1-GFP mice demonstrated that FSP1⁺ stroma cells were abundantly found immediately below the epithelium, whereas α SMA⁺ stromal cells were mostly located in the deeper smooth muscle

layers (Fig. 1D, Suppl. Fig. 1B). Lineage tracing of FSP1⁺ cells in normal forestomach of adult mice, employing 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 (Suppl. Fig. 1C), in agreement with previous reports (22,23). While inflammation could affect forestomach cancer progression (24), leucocyte infiltration in the forestomach of *TGFBR2*^{crKO} mice was not a dominant feature of the histopathological findings.

We additionally tested whether other mesenchymal stromal cells in the forestomach could also impart tumorigenesis via specific deletion of *TGFBR2*. We employed the α -smooth muscle actin (α SMA)-Cre mice (17) to conditionally delete *TGFBR2* in the smooth muscle layer of the forestomach. As previously reported, the *Tgfb2*^{flloxE2}; α SMA-Cre progeny was born with no obvious phenotypic defects (17). Histological analyses of the forestomach revealed no evidence of neoplasia (Suppl. Fig. 1D). These results support that deregulated TGF β signaling specifically in the mesenchymal FSP1⁺ stroma most proximal to the forestomach epithelium leads to forestomach carcinoma.

Loss of TGFBR2 in FSP1⁺ stromal cells induces epithelial proliferation via HGF/Met signaling

Analysis of forestomachs from *TGFBR2*^{crKO} mice prior to the emergence of carcinoma (mice of 2 and 4 weeks of age) indicated a progressive, robust induction of FSP1⁺ stromal cell proliferation beneath the forestomach epithelium (Fig. 2A–E, Suppl. Fig. 2). Elevated HGF was noted in *TGFBR2*^{crKO} forestomach tumor lysates when carcinomas had developed and this was not seen in *TGFBR2/SMAD4*^{crKO} forestomachs (Fig. 2F and G, Suppl. Fig. 1E–G). 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 (see references (25–35)). Out of all tested growth factors, HGF was found specifically upregulated in *TGFBR2*^{crKO} forestomach fibroblasts (Suppl. Fig. 3A). Increased secreted HGF levels by *TGFBR2*^{crKO} forestomach-derived fibroblasts expanded *in vitro*, compared to wt control forestomach fibroblasts, was validated using a mouse angiogenesis cytokine array (Suppl. Fig. 3B–C). Immunolabeling analysis showed that *TGFBR2*^{crKO} forestomach fibroblasts, in contrast to wt and *TGFBR2/SMAD4*^{crKO} forestomach fibroblasts, had upregulated HGF expression (Fig. 2H). Notably, all fibroblasts expressed FSP1, and a specific loss of *TGFRB2* expression in *TGFBR2/SMAD4*^{crKO} and *TGFBR2*^{crKO} forestomach fibroblasts was confirmed (Fig. 2H).

The impact of elevated HGF production by *TGFBR2*^{crKO} forestomach fibroblasts was evaluated in the E10 normal mouse lung epithelial cell line (Fig. 2I–J). Conditioned media from *TGFBR2*^{crKO} forestomach fibroblasts induced E10 cell Met phosphorylation, whereas conditioned media of *TGFBR2/SMAD4*^{crKO} forestomach fibroblasts failed to induce Met phosphorylation (Fig. 2I). Critically, *TGFBR2*^{crKO} forestomach fibroblasts conditioned media induced E10 cell proliferation in a Met signaling dependent manner, with SU11274 Met inhibitor abrogating these effects (Fig. 2J). 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 (Suppl. Fig. 3A–C), HGF emerged as a critical player in forestomach carcinogenesis since rescuing the malignant phenotype by additional *SMAD4* deletion normalized HGF production and Met phosphorylation (Fig. 2G, 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^{CKO}* forestomach cancer (Fig. 1A–C), Smad4-dependent signaling, implicating bone morphogenic protein (BMP) and/or activin receptor-mediated signaling, thus emerged as critical for carcinogenesis of the forestomach epithelium. Within the TGFβ superfamily, bone morphogenic proteins (BMPs) and activins signal via BMP and activin receptors, respectively, to activate regulatory Smads, which use Smad4 to translocate to the nucleus (3). Forestomach lysates from *TGFBR2^{CKO}* 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^{CKO}* mice (Fig. 3A, Suppl. Fig. 3D). 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^{CKO}* forestomachs in contrast to *TGFBR2/SMAD4^{CKO}* forestomachs (Fig. 3B). Activin A however failed to induce HGF expression in *TGFBR2^{CKO}* forestomach fibroblasts (Suppl. Fig. 4A), whereas BMP7 exposure robustly induced HGF expression in *TGFBR2^{CKO}* 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 *TGFRB2* but with intact *SMAD4*. BMP7 protein level was markedly elevated in forestomachs of *TGFBR2^{CKO}* mice, concomitant with increased HGF expression, during forestomach cancer progression (Fig. 3D, Suppl. Fig. 4B). *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 co-receptors, ALK2 and ALK3, exhibited weaker immunolabeling (Suppl. Fig. 4C).

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 *TGFBR2/SMAD4^{CKO}* fibroblasts, possibly as a compensatory mechanism for lack of Smad4 (Suppl. Fig. 4D). Critically

however, exposure of forestomach fibroblasts to high levels of BMP7 indicated enhanced proliferation in wt and *TGFBR2^{ΔKO}* fibroblasts, in contrast to *TGFBR2/SMAD4^{ΔKO}* 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 *TGFRB2*. 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 crosstalk is only active when *TGFBR2* is deleted in FSP1⁺ and not αSMA⁺ stromal cells (Fig. 4C and Suppl. Fig. 4E).

Discussion

The crosstalk between epithelial cells and the stroma is extensive, and while loss of *TGFBR2* in epithelial cells is not sufficient to elicit carcinogenesis (1,40), deletion of *TGFBR2* in FSP1⁺ stromal cells mediates epithelial carcinogenesis (6), pointing at the regulatory role of stromal Tgfr2 signaling towards the epithelium. The current results demonstrate that in *TGFBR2*-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 up-regulates 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 (SCCs) of the forestomach in mice is SCCs developing in the lower part of the esophagus (41). While 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 FSP⁺ 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 crosstalk circuit exists in *TGFBR2^{ΔKO}* 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).

Crosstalk 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 *TGFBR2*-deficient forestomach 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, Tgfr2 binds the TGF β ligand and mediates signaling via Smad2/3 & Smad4 to inhibit cell proliferation in normal tissues, by up-regulating *p21* and down-regulating *c-Myc* transcription (4,51). Accordingly, our data demonstrate that stromal *SMAD4* is indispensable for epithelial carcinogenesis when *TGFBR2* is deleted in FSP1⁺ fibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMP	bone morphogenic protein
CCL5	chemokine (C-C motif) ligand 5
CTGF	connective tissue growth factor
EGF	epidermal growth factor
FGF	fibroblast growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HB-EGF	Heparin-binding EGF-like growth factor
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IL6	interleukin-6
KGF	keratinocyte growth factor
NGF	nerve growth factor

SDF1α	stromal cell-derived factor 1 α
SFRP1	Secreted frizzled-related protein 1
TNC	tenascin C
TGF	transforming growth factor
TSA	Tyramide Signal Amplification

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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 *TGFR2*-deficient stromal cells.

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Figure 1. *Smad4* Loss in *TGFBR2*^{cKO} FSP1⁺ stroma abrogates forestomach cancer

(A) Forestomach squamous cell carcinoma incidence rate and (B) survival of mice with the indicated genotypes. Log-rank (Mantel-Cox) test. **** $p < 0.001$. wt, $n = 7$; *TGFBR2*^{cKO}, $n = 38$; *TGFBR2/SMAD4*^{cKO}, $n = 14$ mice. (C) Hematoxylin-eosin (H&E) staining and FSP1 immunolabeling of forestomach tissue of mice with the indicated genotypes. Scale bar: 50 μ m. (D) Visualization of FSP1-GFP and α SMA-RFP fluorescent gene product in sections of the forestomach of FSP1-GFP; α SMA-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 μ m. (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.

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Figure 2. Smad4 signaling is required for TGFB2^{cko} stromal proliferation and HGF production

(A–B) Immunolabeling (A) and quantification (B) for FSP1, HGF and Ki67 expression in the forestomachs of 2-week-old wt and TGFB2^{cko} mice. n=2–3 distinct mice per group, unpaired two-tailed t test. (C–D) Immunolabeling (C) and quantification (D) for FSP1, HGF and Ki67 expression in the forestomachs of 4-week-old wt and TGFB2^{cko} mice. n=2–3 distinct mice per group, unpaired two-tailed t test. Red arrow points to FSP1⁺ fibroblasts in the pre-cancerous forestomach stroma of TGFB2^{cko} mice. In A and C, DAB (brown) was used as the color substrate for the immunohistochemistry, and nuclear hematoxylin

counterstaining was omitted for the Ki67 immunolabeling. S: stroma. Scale bar: 100 μ m. **(E)** Immunolabeling for FSP1 and Ki67 and quantification of Ki67 positive epithelial and stromal cells in the forestomachs of 6-week-old wt, *TGFBR2^{CKO}* and *TGFBR2/SMAD4^{CKO}* mice. L: lumen. S: stroma. E: epithelium (for *TGFBR2^{CKO}*: squamous cell carcinoma). Scale bar: 60 μ m. The quantification of Ki67 positive cells was based on three distinct mice per group, and the groups were compared by one-way ANOVA. See accompanying source data. **(F)** Western blots analyses for HGF and BMP7 protein levels in 4-week-old wt and *TGFBR2^{CKO}* forestomach whole tissue lysates, 30 μ g of protein loaded per lane, each lane represents a distinct mouse. **(G)** Western blot analysis for HGF in the forestomach lysate of 6-week-old mice with the listed genotypes, 30 μ g of protein loaded per lane, each lane represents a distinct mouse. **(H)** Immunolabeling for FSP1, HGF and Tgfbr2 in cultured forestomach fibroblasts isolated from mice with the listed genotypes. DAPI (blue): nuclei; omitted in HGF panel. Red insets: negative control (Secondary antibody alone). Blue insets: display the merged image while the larger image omits the DAPI signal to clearly show the nuclear localization. Scale bars: 50 μ m. **(I)** Immunolabeling for phosphorylated Met (p-Met) in E10 lung epithelial cell incubated with conditioned media from cultured forestomach fibroblasts isolated from mice with the listed genotypes. DAPI (blue): nuclei. Red inset: negative control (Secondary antibody alone). Scale bars: 50 μ m. **(J)** E10 lung epithelial cell proliferation (methylene blue absorbance) after exposure to conditioned media of the forestomach fibroblasts isolated from mice with the listed genotypes, with and without the Met inhibitor, SU11274 (10 μ M). Unpaired two-tailed t test. The data is presented as the mean $\pm$ SEM. * p <0.05, ** p <0.01, ns: not significant. See accompanying source data. **(K)** Immunolabeling for HGF and p-Met. Color substrate: 3, 3'-diaminobenzidine (DAB, brown). Scale bar: 20 μ m. S: stroma.

Figure 3. BMP7 signaling is elevated in tumors precipitated by TGFB2^{ckO} stroma

(A) Western blots analyses for Smad2 and Smad1/5 phosphorylation levels in forestomach tissue lysate from approximately 6-week-old mice with the listed genotypes, 30 µg of protein loaded per lane, each lane represents a distinct mouse. (B) RT-PCR electrophoretic product for the listed genes in the forestomach of approximately 6-week-old mice with the listed genotypes. Buffer: no template control. (C) RT-PCR electrophoretic product for HGF in the forestomach fibroblasts from approximately 6-week-old mice with the listed genotypes with or without (0.1% FBS) stimulation with BMP7 (100 ng.ml⁻¹). (D) Western blot analysis for BMP7 in forestomach tissue lysate from approximately 6-week-old mice

with the listed genotypes, 30 µg of protein loaded per lane, each lane represents a distinct mouse. The actin blot is the same as depicted in Fig. 2G. **(E)** *In situ* hybridization for *HGF* and *BMP7* mRNA (NBT/BCTP substrate, purple) in sections of the forestomach of the listed mice. L: lumen, S: stroma, E: epithelium/cancer cells, M: smooth muscle. Arrows point to stromal HGF and cancer cell BMP7 expression, respectively. Scale bars: upper panel (HGF): 50 µm, lower panel (BMP7): 20 µm. **(F)** Immunolabeling for BMP7, ALK6, ALK6 & FSP1. Color substrate: 3, 3'-diaminobenzidine (DAB, brown); for ALK6 & FSP1: 3-amino-9-ethylcarbazole (AEC, red) and Vector Blue. Scale bar: 20 µm. S: stroma. Arrowhead points to FSP1⁺ fibroblast with ALK6 immunoreactivity. **(G)** Immunofluorescence for ALK6 in cultured forestomach fibroblasts from approximately 6-week-old mice with the listed genotypes. Scale bars: 50 µm. Red inset: negative control, scale bar: 20 µm.

Figure 4. BMP7/HGF imbalance drive *TGFBR2^{cko}* stromal proliferation and forestomach carcinogenesis

(A) Cell proliferation (methylene blue absorbance) of wt (gray), *TGFBR2^{cko}* (red) or *TGFBR2/SMAD4^{cko}* (blue) forestomach fibroblasts cultured *in vitro* with the indicated treatments. The data is normalized by setting the 0.1% FBS control at 1. Unpaired one-tailed t test compared to 0.1% FBS control, statistical analyses carried out on untransformed data. The data is presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, ns: not significant. See accompanying source data. (B) Diagram of the proposed crosstalk between the *TGFBR2*-deficient FSP1⁺ fibroblasts (blue) and the overlying epithelium (black). FSP1⁺ fibroblasts produce HGF to activate Met receptors on overlying epithelial cells, causing epithelial cells to produce BMP7, which stimulates ALK6 receptors on FSP1⁺ fibroblasts to produce HGF. (C) Comparison of BMP7 and HGF protein levels in forestomach whole tissue lysates of wt, *TGFBR2^{flx}E2*; α SMA-Cre and *TGFBR2^{flx}E2*; FSP1-Cre (*TGFBR2^{cko}*) mice. Western blots, 30 μ g of protein loaded per lane. BMP7 and HGF are highly upregulated in the *TGFBR2^{cko}* forestomach cancer tissue.