

ORIGINAL RESEARCH—BASIC

Regulation and Function of Sox2 and CD44 Expression in the Stomach by Bone Morphogenetic Protein Signaling



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BACKGROUND AND AIMS: The transcription factor Sox2 and the adhesion molecule CD44 are implicated in gastric metaplasia and neoplasia. *Lgr5* is a cellular receptor that marks gastric progenitors involved in epithelial homeostasis. We reported that inhibition of bone morphogenetic protein (BMP) signaling in the stomach activates aberrant cells that lead to spasmolytic polypeptide-expressing metaplasia (SPEM), a preneoplastic metaplasia. We investigated the role of BMP-regulated transcriptional programs in gastric metaplasia/SPEM and the localization and function of CD44 and Sox2 in the context of inhibition of BMP signaling. **METHODS:** Deletion of *Cd44*, *Sox2*, and of *BMP Receptor 1a* (*Bmpr1a*) in *Lgr5* cells was achieved by crossing *Lgr5-Cre* mice to mice expressing floxed alleles of *Cd44*, *Sox2*, and *Bmpr1a*. The H^+/K^+ -ATPase β -subunit promoter was used to express the BMP inhibitor noggin in the corpus. Lineage tracing studies were conducted by crossing *Lgr5-Cre* mice to mice expressing *Nog* and *tdTomato*. Morphology of the mucosa was analyzed by hematoxylin and eosin staining and immunohistochemistry. Single cell RNA sequencing was performed in both control and H^+/K^+ -*Nog* mice. **RESULTS:** Sox2⁺ cells were detected in the antrum but not in the corpus/lesser curvature of wild type mice. Conversely, expression of Sox2 was seen in *Lgr5*⁺ cells along the lesser curvature of *Lgr5-Cre*; *Bmpr1a*^{flx/flx} mice and in CD44⁺ cells in the corpus of H^+/K^+ -*Nog* mice. Noggin induced SPEM-signature genes in clusters expressing pit, chief, parietal, and mucus cell markers. Deletion of *Cd44* and *Sox2* in *Lgr5* cells attenuated the SPEM phenotype. **CONCLUSION:** Inhibition of BMP signaling affects gastric epithelial transcriptomes and cell plasticity, underscoring the relevance of this mechanism in the development of metaplasia and in the regulation of CD44 and Sox2.

postulated that chronic inflammation and mucosal injury can alter the normal homeostatic mechanisms of the gastric epithelium leading to the development of metaplastic and dysplastic changes and, ultimately, to neoplasia.^{1,2} Both Intestinal Metaplasia and spasmolytic polypeptide-expressing metaplasia (SPEM), which is characterized by the aberrant expression of Trefoil Factor 2 (TFF2) at the base of glands of the oxyntic mucosa, have been associated with inflammation-induced gastric neoplasms.²⁻⁹

Several studies have identified, based on expression of molecular markers, populations of gastric epithelial cells that can self-renew and that can exhibit multi-lineage differentiation capacity, such as those expressing *Lgr5*, *Sox2*, *Aqp5*, *villin*, *Prom1/Cd133*, *Cd44*, *Dck1/Dcamk1*, *Troy*, *Mist-1*, *Tff2*, and *Runx1*.¹⁰⁻¹⁷ It has been suggested that alterations in the biological behavior of these cells might lead to their transformation and to the initiation of tumor growth.¹⁰ *Lgr5*-, *Cd44*-, and *Sox2*-expressing cells, in particular, have been the focus of numerous investigations that have underscored their importance in the pathogenesis of gastric metaplasia, dysplasia and neoplasia.

In the stomach, cells with *Lgr5* transcriptional activity have been identified at the base of antral glands, along the lesser curvature of the oxyntic mucosa and in the corpus.^{13,15,18} Recent studies have suggested that they represent a subpopulation of chief cells¹⁹ that following injury acquire stem cell functions to promote repair of the gastric epithelium.¹⁹

Keywords: Gene Expression; RNA-Seq; Cell Signaling; Cell Differentiation

Introduction

The mechanisms responsible for the development of metaplasia, dysplasia, and neoplasia in the stomach have been only partially characterized. It has been

Abbreviations used in this paper: BMP, bone morphogenetic protein; GFP, Green Fluorescent Protein; HBSS, Hank Balanced Salt Solution; H&E, hematoxylin and eosin; PC, parietal cell; QRT-PCR, quantitative real-time polymerase chain reaction; SPEM, spasmolytic polypeptide-expressing metaplasia; TFF2, Trefoil Factor 2.

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An increased number of *Lgr5*-expressing cells has been detected in patients with gastritis and intestinal metaplasia and in gastric carcinomas.^{19,20} In support of these findings, studies have also shown that *Lgr5*-expressing chief cells can be a major cell-of origin of gastric tumors.²⁰

In agreement with these observations, *Lgr5* cells have been shown to express growth promoting and pro-inflammatory peptides,¹⁵ molecules known to participate in the regulation of cell proliferation and in the response of the gastric mucosa to inflammatory stimuli. The adhesion molecule CD44, in particular, has been shown to be expressed in *Lgr5* cells and to play an important role in the pathogenesis of gastric metaplasia and neoplasia.^{15,21–25}

The transcription factor Sox2 can be normally detected in nuclei of cells located at the base of antral glands and, to a lesser extent, in the isthmus of glands of the body. Sox2-expressing cells have been shown to function as multipotent stem cells with self-renewal potential. Sox2⁺ve cells appear to play an important role during development since ablation of *Sox2* leads to embryonic lethality.^{11,26,27} In addition, it has been suggested that Sox2 is involved in the pathophysiology of metaplasia and neoplasia in the stomach.^{28–30}

The factors that control the number and function of *Lgr5*-, *Cd44*-, and of *Sox2*-expressing cells during the development of gastric metaplasia, dysplasia, and neoplasia have been poorly characterized.

The bone morphogenetic proteins (BMPs) are regulatory peptides that exert important effects on the growth and differentiation of gastrointestinal tissues.^{31–33} Our laboratory previously demonstrated that transgenic expression in the oxyntic mucosa of the mouse stomach of the BMP inhibitor noggin leads to the development of SPEM, to parietal cell (PC) loss and to increased epithelial cell proliferation.^{31,32,34}

The BMPs have also been shown to represent physiologically relevant inhibitors of gastrointestinal inflammation.^{31,34–37} Indeed, studies from our laboratory have demonstrated that expression of noggin in the stomach increases *Helicobacter*-induced inflammation and epithelial cell proliferation leading to the accelerated development of dysplasia.^{34,37}

In recently published reports, we also observed that that inhibition of BMP signaling achieved by either deletion of *Bmpr1a* in *Lgr5* cells or by expression of *noggin* in the oxyntic mucosa leads to induction of CD44 in epithelial cells derived from *Lgr5*⁺ve progenitors. Moreover, we demonstrated that SPEM can arise in proliferating CD44⁺ve cells.³⁷

Since the function of BMP signaling in the control of gastric epithelial homeostasis has been only partially elucidated, we undertook studies to investigate the role of BMP-regulated transcriptional programs in gastric metaplasia and the localization and function of CD44 and Sox2 in the context of inhibition of BMP signaling in the stomach.

Material and Methods

Mice

The *H*⁺/*K*⁺-*noggin* (*H/K-nog*) transgenic mice were generated in our laboratory and they were previously described.³² Pathogen-free C57BL/6 mice, *Lgr5-EGFP-ires-CreERT2* (*Lgr5-Cre*) mice, in which the expression of both *Cre* and *Green Fluorescent Protein* (*GFP*) is driven by endogenous *Lgr5* regulatory sequences,¹⁵ and *Sox2*^{fllox/fllox} mice, aged 4- to 10-week, were purchased from Jackson Laboratory (Bar Harbor, ME). *Bmpr1a*^{fllox/fllox} mice were received from the laboratory of Dr Yuji Mishina, (University of Michigan).³⁸ Mouse genotyping was described elsewhere.^{32,37} *Cd44*^{fllox/fllox} mice were obtained from the laboratory of Dr Orian-Rousseau (Karlsruhe Institute of Technology, Germany).³⁹ Specific inhibition of BMP signaling in *Lgr5* cells was achieved by crossing *Lgr5-Cre* mice to *Bmpr1a*^{fllox-fllox} mice to generate *Lgr5-Cre; Bmpr1a*^{fllox-fllox} mice.³⁷ For lineage tracing studies in the presence of Noggin, *H*⁺/*K*⁺-*Nog* mice were crossed to *Rosa26-Tom* reporter mice to generate *H*⁺/*K*⁺-*Nog; Rosa26-Tom* mice. *Lgr5-Cre* mice were then crossed to *H*⁺/*K*⁺-*Nog; Rosa26-Tom* mice to generate *Lgr5-Cre; H*⁺/*K*⁺-*Nog; Rosa26-Tom* mice.³⁷

To delete either *Sox2* or *Cd44* in *Lgr5* cells, we crossed *Lgr5-Cre* mice to either *Sox2*^{fllox-fllox} mice or to *Cd44*^{fllox-fllox} mice to generate *Lgr5-Cre; Sox2*^{fllox-fllox} mice and *Lgr5-Cre; Cd44*^{fllox-fllox} mice, respectively. In order to assess the function of *Cd44* and *Sox2* in *Lgr5* cells in the presence of inhibition of BMP signaling, we crossed mice with floxed alleles of *Cd44* and *Sox2* to *H*⁺/*K*⁺-*Nog* mice to generate *Cd44*^{fllox-fllox; H⁺/*K*⁺-*Nog* and *Sox2*^{fllox-fllox; H⁺/*K*⁺-*Nog* mice. These mice were then crossed to *Lgr5-Cre* mice to generate *Lgr5-Cre; Cd44*^{fllox-fllox; H⁺/*K*⁺-*Nog* mice and *Lgr5-Cre; Sox2*^{fllox-fllox; H⁺/*K*⁺-*Nog* mice, which express *noggin* in the presence of deletion of either *Cd44* or *Sox2* in *Lgr5* cells. *Cre* was activated by 1 intraperitoneal injection of tamoxifen (0.1 mg/g body weight). All mice received tamoxifen injections. The mice were maintained on a C57BL/6 background. In all experiments, animals were fasted overnight with free access to water before tissue collection. Mice were housed under specific pathogen-free conditions in automated watered and ventilated cages on a 12-h light/dark cycle, in the animal maintenance facility at the University of Michigan. All animal experiments were approved by the University of Michigan Animal Care and Use Committee.}}}}

Single Cell Isolation and RNA Sequencing

Isolation of gastric epithelial cells from control mice and from *Nog*-expressing mice was performed as previously described⁴⁰ with modifications. Samples of gastric mucosa were placed in Mg²⁺-, Ca²⁺-free Hank Balanced Salt Solution (HBSS), 5% fetal bovine serum, 2 mM ethylenediaminetetraacetic acid, shaken for 20 min at 37 °C, and filtered through a 100 mm cell strainer. After filtration, the samples were placed in HBSS containing 0.1U/mL Liberase (Roche, Basel, Switzerland) and 40 mg/mL DNase I (Sigma, St. Louis, MO). The solutions were shaken for 10 min at 37 °C, filtered through a 100 mm cell strainer, and spun at 1500 rpm for 5 min. After 1 wash with HBSS, the pellets were resuspended in 500 mL of dead cell removal reagent (phosphate-buffered saline/2% fetal bovine serum/1 mM calcium chloride) and transferred to 12 mm × 75mm polystyrene round-bottom tubes. Dead cells

were removed using the EasySep Dead Cell Removal kit (catalog number 17899, STEMCELL Technologies, Cambridge, MA), according to the manufacturer's instructions. Cells were collected in 5 mL tubes, spun down, and resuspended in 200 μ L HBSS/2% fetal bovine serum.

Single cell RNA sequencing was performed by the Advanced Genomics Core at the University of Michigan. Single cell suspensions were subjected to counting and viability checks on the LUNA F $\times$ 7 Automated Cell Counter (Logos Biosystems) and diluted to a concentration of 700–1000 cells/mL. Single cell libraries were generated using the 10x Genomics Chromium Controller with 3' Gene Expression reagents following the manufacturer's protocol (10x Genomics). Final library quality was assessed using the LabChip GX (PerkinElmer). Libraries were subjected to paired-end sequencing according to the manufacturer's protocol (Illumina NovaSeq 6000). Bcl2fastq2 Conversion Software (Illumina) was used to generate demultiplexed Fastq files and the CellRanger Pipeline (10x Genomics) was used to align reads and generate count matrices. Count matrices were processed via Seurat (version 5.1.0).⁴¹ For quality control, cells expressing between 200 and 7500 genes and <20% mitochondrial counts and <15% ribosomal counts were selected for analysis. Counts were globally scaled and then log transformed. The top 2000 differentially expressed genes were selected to be used for downstream analysis. After scaling and running principle component analysis on the data, cells were clustered with uniform manifold approximation and projection with 20 principle components and a resolution of 0.6. Clusters were annotated using ScType⁴² with published gastric cell markers.⁴³ Genes which were at least 0.1 log-fold different between groups and present in at least 0.01% cells of either group were tested for differential expression. Genes and isoforms were annotated with NCBI Entrez GeneIDs and text descriptions. The Molecular Signatures Database Hallmark 2020,⁴⁴ was queried using the program Enrichr^{45–47} for analysis of sets of significantly differentially expressed genes in the selected cell clusters to identify enriched functional categories. Definition of the transcriptional diversity of the epithelial cells was assessed using cytoTRACE.⁴⁸

Gastric Epithelial Cell Isolation and Flow Cytometry

These studies were carried out according to previously reported methods³⁷ (see [Supplementary Materials and Methods](#)).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis

RNA was isolated from isolated cells and from full thickness samples of the corpus of mice using TRIzol reagent (Invitrogen), followed by DNase treatment and purification with the RNeasy Mini kit (Qiagen).³² Quantitative real-time polymerase chain reaction (QRT-PCR) was performed according to previously published methods³² using primer sequences and protocols that were obtained from commercially available sources. *Gapdh*, *Cd44*, and *Bmpr1a* primers were obtained from Integrated DNA Technologies (Coralville, IA). *Bmpr1a* QRT-PCR primers span exons 2–3 of the gene. The sequences are as follows: primer 5'-CTTGGCAATGACTTTCACCTG-3 binds in exon 3 (284–304 in

Genebank NM-009758.4), primer 5'-TCGCTTTGAA-TACTGTCTTGGAA-3' binds in exon 2 (170–191 in Genebank NM-009758.4). The deletion in the *Bmpr1a* floxed mice from the laboratory of Y. Mishina deletes a portion of exon 2. Accordingly, these primers will not amplify a product in the recombinant mice. QRT-PCR primers for *Cd44*^{flox-flox} mice were as follows: forward primer: 5GGG AGC ATT GGC TGA CAA CA 3, reverse primer: 5 TTA CTC TGA TCA TGG CTC TC 3.³⁹ QRT-PCR primer sequences for *Sox2*^{flox-flox} mice were obtained from Jackson laboratories (Bar Harbor, ME): forward primer: TGG AAT CAG GCT GCC GAG AAT CC, reverse primer: TCG TTC TGG CAA CAA GTG CTA AAG C.

Histochemical Analysis and Image Acquisition

Tissue fixation, generation of paraffin sections, hematoxylin and eosin (H&E) staining, and immunostaining were performed according to previously published methods.^{32,34,35,37} Sections were stained with the following primary antibodies: anti-H⁺/K⁺-ATP-ase α -subunit (1:200) (Medical and Biological Laboratories, Nagoya, Japan, catalog number: DO31–3), anti-Ki67 (1:500) (Thermo Scientific, Waltham, MA, catalog number: MA5-14520), antiretro fluorescent protein (1:200) (Rockland/Biomol, Hamburg, Germany, catalog number: 600–401–379), anti-Sox2 (1:20) (R&D, catalog number AF2018), anti-intrinsic factor (1:1000) (gift from Dr Jason Mills, Washington University, St. Louis, MO), antimouse CD44 (1:200) (BD Biosciences, San Jose, CA, catalog number: 550538), and with Alexa 488-conjugated anti-GFP (1:200) (Invitrogen, Carlsbad, CA, catalog number A21311). In some experiments, the slides were stained for 1 h at room temperature with Alexa 488-conjugated Griffonia (Bandeiraea) simplicifolia lectin II (GS II) (1:1000) (Invitrogen, Carlsbad, CA, catalog number: L-21415). For immunofluorescence analysis, Alexa 555-donkey antirabbit (1:500), Alexa 594-donkey anti rat (1:500) and Alexa 488-donkey antirat and antimouse (1:500), secondary antibodies were used (Molecular Probes, Eugene, OR). ProLong Gold Antifade reagent with DAPI (Invitrogen, Carlsbad, CA) was used for nuclear counterstain and mounting medium. Control experiments were performed by incubating the slides in the presence of the secondary antibodies without the primary antibodies (data not shown). Visualization of slides was performed with a Nikon Eclipse E 800 fluorescence microscope for all other studies.

For histologic scoring of metaplasia/SPEM, sections from the stomachs of at least 3 separate animals from each of the 4 treatment groups were examined and scored as previously described.^{34,37,49} Slides containing sections of well-oriented gastric glandular mucosa were examined in a blinded fashion. Only fields that contained full-thickness gastric mucosa that was oriented perpendicularly were analyzed. All well-oriented fields were scored. Each field was scored separately for the presence or absence and severity of epithelial metaplasia.⁴⁹ Metaplasia was defined as loss of normal differentiated morphology which in mice, as previously described, appears morphologically as SPEM. Severity of the changes was graded using a scale from 1 (mild) to 3 (severe) as follows: score = 1 was defined as loss of PCs with replacement by mucus-type cells; score = 2 was defined as anisocytosis/karyosis, loss of polarity, abnormal shape, and/or multiple epithelial layers; score = 3 was defined as dilation, irregularity, disorganization of glands, with cellular dysplasia.

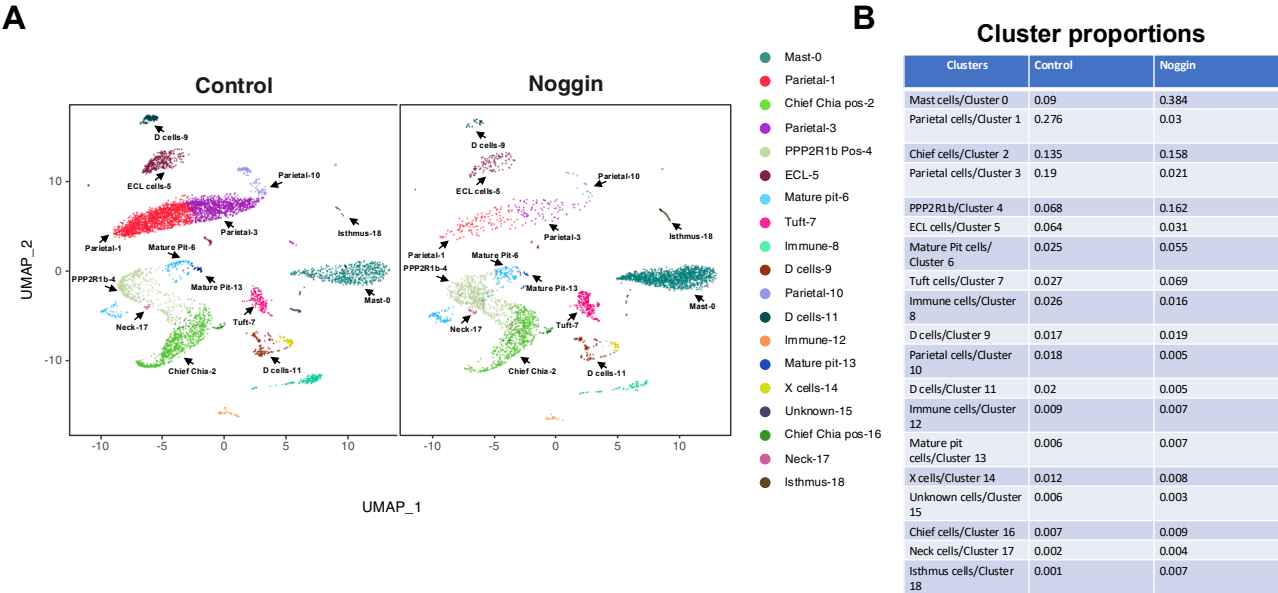


Figure 1. Clustering of isolated gastric epithelial cells in the presence and absence of noggin. (A) Transcripts of individual cells from control and noggin-expressing mice were sequenced and the data from each group were combined and subjected to unbiased uniform manifold approximation and projection (UMAP) dimensionality reduction, in 19 color-coded clusters. (B) Table depicting cluster proportions of the isolated cells in the presence and absence of noggin. Similar results were obtained in one other separate experiment.

Each field was scored, and the final score was calculated and expressed as a weighted average of all fields with that lesion as follows: sum of the number of fields for each score x the score, divided by the total number of fields.

Data Analysis

Data are expressed as means ± standard error. Statistical analysis was performed using Student *t*-test. In the experiments analyzing differential expression of SPEM markers in gastric epithelial cell clusters, we used the Wilcoxon test with Benjamini and Hochberg correction for multiple comparisons. *P* values <.05, were considered to be significant.

Results

To investigate the role of BMP-regulated transcriptional programs in the development of gastric metaplasia, we performed single cell RNA sequencing on samples of epithelial cells isolated from the stomachs of both control and *H⁺/K⁺-Nog* mice. As shown on Figure 1A and B, we defined 19 clusters of cells isolated from the mucosa of the corpus. The major clusters identified cells expressing markers of pit-, parietal-, mucus (PPP2R1b)-, chief-, tuft-, ECL-, D-, and mast-cell differentiation. We also noted a few small clusters of immune cells and of cells expressing markers of neck- and isthmic-cell differentiation. Expression of noggin caused changes in the clustering profile of the cells. In particular, noggin predominantly induced an increase in the size of the pit-, mucus (PPP2R1b)-, tuft-, and mast-cell clusters and a decrease in the size of the parietal-

and entero-endocrine-cell clusters, suggesting that BMP signaling has broad effects on the differentiation and fate of multiple gastric epithelial cell lineages.

To better define the characteristics of the transcriptomes of the single cells we analyzed the data using cytoTRACE, a program which determines the complexity and the transcriptional diversity of cells.⁴⁸ As shown on in Figure 2A, expression of noggin induced an increase in the cytoTRACE score. Correlation analysis of the transcripts indicated, as shown in Figure 2B, that high cytoTRACE scores were associated with the expression of genes involved in protein synthesis and cell proliferation, while low scores were linked to decreased representation of genes such as *chia 1* and the *H⁺/K⁺-ATPase*, which are markers of chief and PC differentiation.

We examined if these changes involved differential expression of genes known to be associated with the development of SPEM in clusters of epithelial cells, which have been shown to participate in the development of this pathological process.^{1-4,6-10} As shown in Figure 3, noggin induced an increase in the expression of *Tff2*, *Cd44*, *Muc6*, *Gkn3*, and *Aqp5* transcripts in cells belonging to the mucus (PPP2R1b)-, pit-, parietal-, and chief-cell clusters. Interestingly, the plots also indicated that noggin could influence the expression of *Sox2* and *Sox9* in the Pit-cell clusters and of *Cd44* in the isthmal-cell cluster. Thus, inhibition of BMP signaling in the gastric epithelium causes broad alterations in the normal differentiation programs of cells of the oxyntic mucosa which appear to cluster with cells with more transcriptionally diverse and less differentiated features.

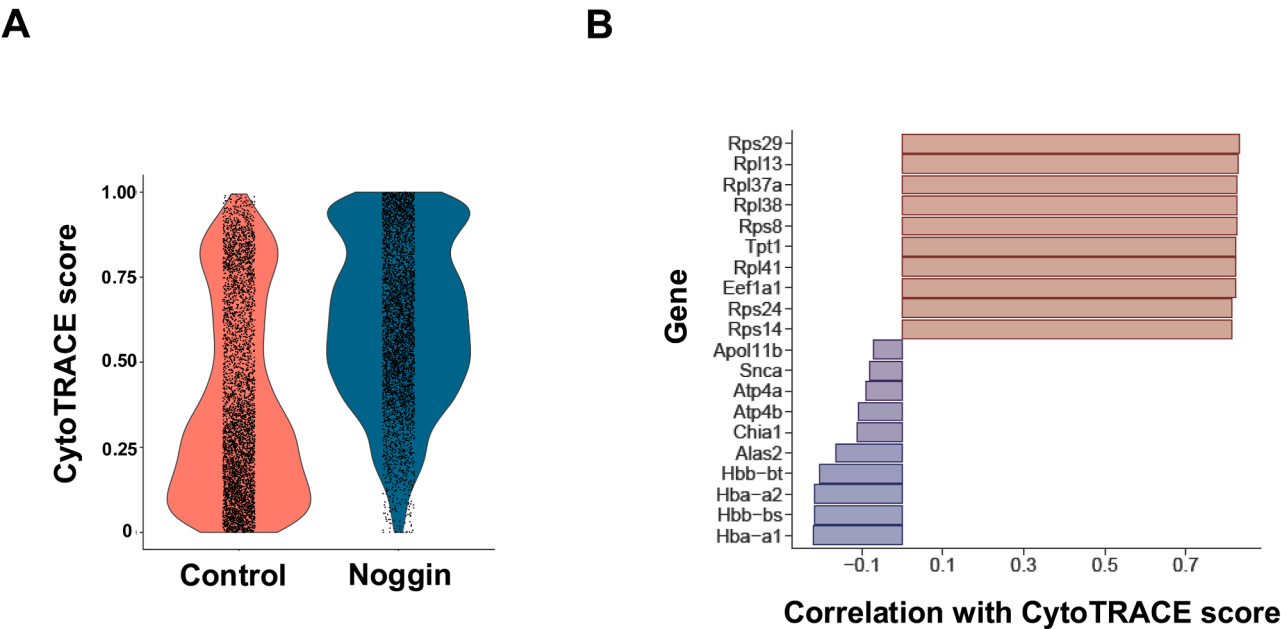


Figure 2. CytoTRACE analysis of gastric epithelial cells. (A) CytoTRACE score of transcriptomes of isolated gastric epithelial cells in the presence and absence of noggin. (B) Correlation analysis between cytoTRACE score and gene expression in cells isolated from control and noggin mice (Positive score = More stem like. Negative score = More differentiated).

We next examined the signaling pathways activated by noggin in gastric epithelial cells. Analysis of the number of significantly differentially expressed genes in the clusters indicated that, only PCs belonging to clusters 1 and 3; chief cells belonging to cluster 2; mucus cells, ECL cells, tuft cells, and pit cells belonging to cluster 13, expressed enough differentially expressed genes to conduct pathway analysis. As shown in Figure 4, noggin induced increased expression of molecules involved in the regulation of cellular apoptosis and in the response

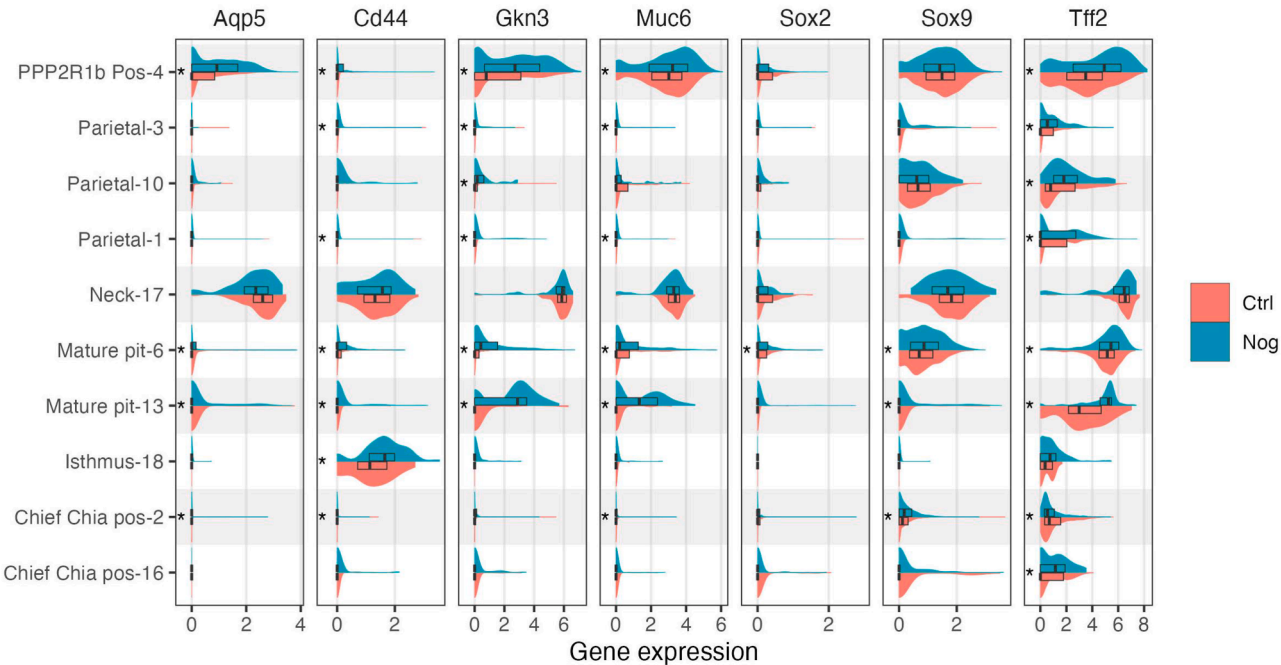


Figure 3. Noggin-induced progenitor cell cluster-specific differential gene expression. Violin plots of Aqp5, Cd44, Gkn3, Muc6, Sox2, Sox9, Tff2 in the PPP2R1b Pos-4, Parietal-3, Parietal-10, Parietal-1, Neck-17, Mature pit-6, Mature pit-13, Isthmus-17, Chief Chia pos-2, and Chief Chia pos-16 clusters in the presence and absence of noggin. Boxes are interquartile ranges (IQRs); bold lines are medians; * $P < .05$ (Benjamini-Hochberg-adjusted P values).

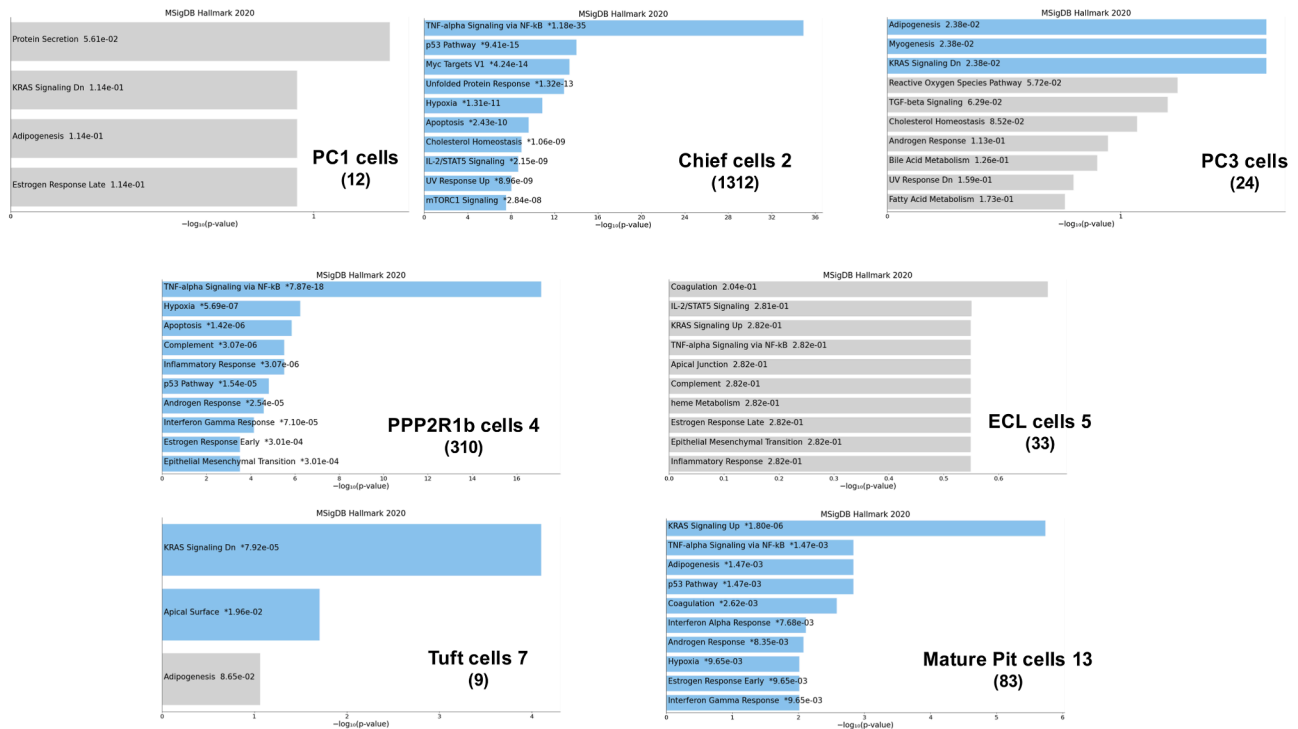


Figure 4. Pathway analysis of gene transcripts in clusters of epithelial cells from control and noggin mice. Pathway analysis of significantly up-regulated transcripts ($P < .05$) in control and noggin mice was performed using the Molecular Signatures Database 2020 (MSigDB) Hallmark list. Numbers in parenthesis indicate the number of significantly upregulated genes per cluster.

to inflammatory mediators, cellular stress, KRAS, and mTORC1 signaling.

The transcription factor Sox2 and the cell adhesion molecule CD44 play an important role in the development of gastric metaplasia and neoplasia.^{11,21-25,28-30} In addition, Sox2 has been shown to be involved in gastric development.^{26,27} While we and others have shown that CD44 is expressed in Lgr5 cells,^{15,37} reports have suggested that Sox2 and Lgr5 might label different populations of gastric progenitors.¹¹ Accordingly, we sought to investigate the localization and regulation of Sox2 and CD44 in the context of inhibition of BMP signaling in the stomach. To define the localization of Sox2 in the gastric mucosa, we stained sections of both the antrum and of the lesser curvature of *Lgr5-Cre* mice with anti-Sox2 and anti-GFP antibodies, which mark cells with Lgr5 transcriptional activity.³⁷ Analysis of sections of the antrum revealed robust expression of Sox2 in the nuclei of Lgr5/GFP + ve cells located at the base of the glands (Figure 5A). In contrast, we could only observe a few rare GFP + ve cells in sections of the lesser curvature without any Sox2 staining (Figure 5B). We next investigated if inhibition of BMP signaling in Lgr5 cells affects the expression of Sox2. For these studies, sections of the lesser curvature of *Lgr5-Cre;Bmpr1a^{fllox-fllox}* mice were stained with antibodies directed against both Sox2 and GFP. As shown in Figure 5B, analysis of the sections demonstrated the appearance of double stained cells in mice that exhibited deletion of BMP

receptor 1a in Lgr5 cells. Thus, inhibition of BMP signaling induces Sox2 in Lgr5 cells located along the lesser curvature of the oxyntic mucosa. Lineage tracing studies conducted in *Lgr5-Cre;H⁺/K⁺-Nog;Rosa26-Tom* mice that express both noggin and tomato in the oxyntic mucosa of the lesser curvature, demonstrated, in agreement with previously published reports,³⁷ the appearance of tomato⁺ cells in the mucosa of the lesser curvature.³⁷ As shown in Figure 5C, staining of the sections with anti-Sox2 antibodies revealed expression of Sox2 in nuclei of tomato⁺ cells, confirming the notion that Sox2⁺ cells can arise from cells with *Lgr5* transcriptional activity.

To assess the function of CD44 and Sox2 in Lgr5 cells in the presence of inhibition of BMP signaling, we examined the consequences of deletion of *Cd44* and *Sox2* in these cells, in mice expressing noggin in the mucosa of the lesser curvature. In order to confirm successful *Cre*-induced deletion of *Cd44* and *Sox2* in Lgr5 cells, we isolated GFP⁺ cells from the gastric mucosa of *Lgr5-Cre*, *Lgr5-Cre;Sox2^{fllox-fllox}* and *Lgr5-Cre;Cd44^{fllox-fllox}* mice using flow cytometry. As shown in Supplemental Figure 1, GFP + ve cells from *Lgr5-Cre;Cd44^{fllox-fllox}* and *Lgr5-Cre;Sox2^{fllox-fllox}* mice exhibited diminished expression of *Cd44* and *Sox2* mRNA when compared to cells isolated from control, *Lgr5-Cre* mice.

We examined the morphological changes induced by deletion of *Cd44* in Lgr5 cells in mice that express the BMP inhibitor noggin in the oxyntic mucosa. Microscopic and morphometric analysis of H&E-stained sections of the

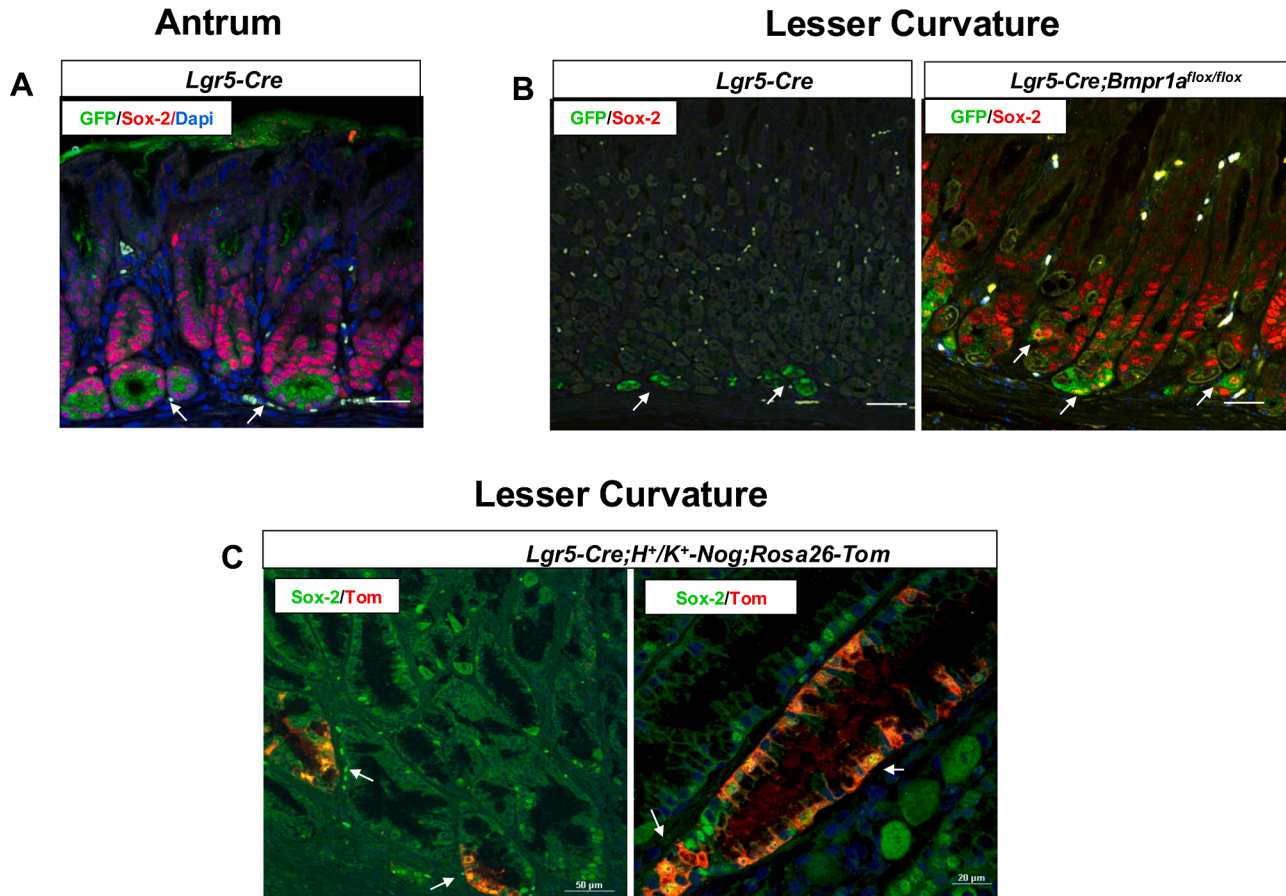


Figure 5. Regulation of Sox2-positive cells and of cells with *Lgr5* transcriptional activity by BMP signaling. 1-2-month old *Lgr5-Cre* mice and *Lgr5-Cre;Bmpr1a^{lox/lox}* mice were treated with 1 i.p. injection of tamoxifen (0.1 mg/g body weight). Animals were analyzed 5 months after tamoxifen injection. Paraffin sections of the antrum (A) and of the lesser curvature of *Lgr5-Cre* mice and of *Lgr5-Cre;Bmpr1a^{lox/lox}* mice (B) were stained with Alexa 488-conjugated anti-GFP antibodies (green) together with an anti-Sox2 antibody and an Alexa 555-conjugated secondary antibody (red). Size bar 50 μm. Similar results were obtained in one other separate experiment. Arrows point to GFP/Sox2 double-labeled cells in A and in B (right panel) and to GFP + ve cells in B (left panel). *Lgr5-Cre;H⁺/K⁺-Nog;Rosa26-tdTom* mice were treated with 1 i.p. injection of tamoxifen (0.1 mg/g body weight) and analyzed 5 months after tamoxifen injection. Gastric paraffin sections of the lesser curvature of *Lgr5-Cre;H⁺/K⁺-Nog;Rosa26-tdTom* were stained with antitomato (RFP) primary antibodies and Alexa 555-conjugated secondary antibodies (red) together with anti-Sox2 antibodies and Alexa 488-conjugated secondary antibodies (green) (C). Size bar 50 μm in the left panel, 20 μm in the right panel. Similar results were observed in one other mouse. RFP, red fluorescent protein.

mucosa of the lesser curvature of *Cd44^{lox/lox};H⁺/K⁺-Nog* mice demonstrated metaplastic (SPEM) changes characterized by a significant reduction in the number of cells that had the morphological appearance of PCs, and by an increase in the number of light-staining cells. In contrast, deletion of *Cd44* in *Lgr5* cells led to attenuation of the severity of the morphological changes induced by noggin (Figure 6A and B).

To better define the effect of deletion of *Cd44* on noggin-induced SPEM, we stained sections of the oxyntic mucosa of the lesser curvature with antibodies directed against the H^{+},K^{+} -ATPase α -subunit, the mitotic marker Ki-67, Intrinsic Factor and with the lectin griffonia simplicifolia lectin II that normally binds mucus neck cells. As shown in Figure 6C, expression of noggin led to a reduction in the number of H^{+},K^{+} -ATPase-positive cells and to an increase in the

number of proliferating cells which were located in all sections of the glands. In addition, noggin induced the development of SPEM, which was characterized by the expansion toward the base of the glands of aberrant cells that could be costained with anti-intrinsic factor antibodies and with the lectin griffonia simplicifolia lectin II. Deletion of *Cd44* restrained the proliferative response of the epithelium with restriction of the proliferative compartment to the isthmus of the glands. Moreover, this intervention attenuated the metaplastic changes induced by noggin leading to repopulation of the glands with H^{+},K^{+} -ATPase-positive cells and to restriction of griffonia simplicifolia lectin II binding to mucus cells located in the neck region of the glands.

We then examined if, deletion of *Cd44* in *Lgr5* cells, affects the expression of Sox2. First, we investigated the pattern of expression of Sox2 and CD44 in the mucosa of

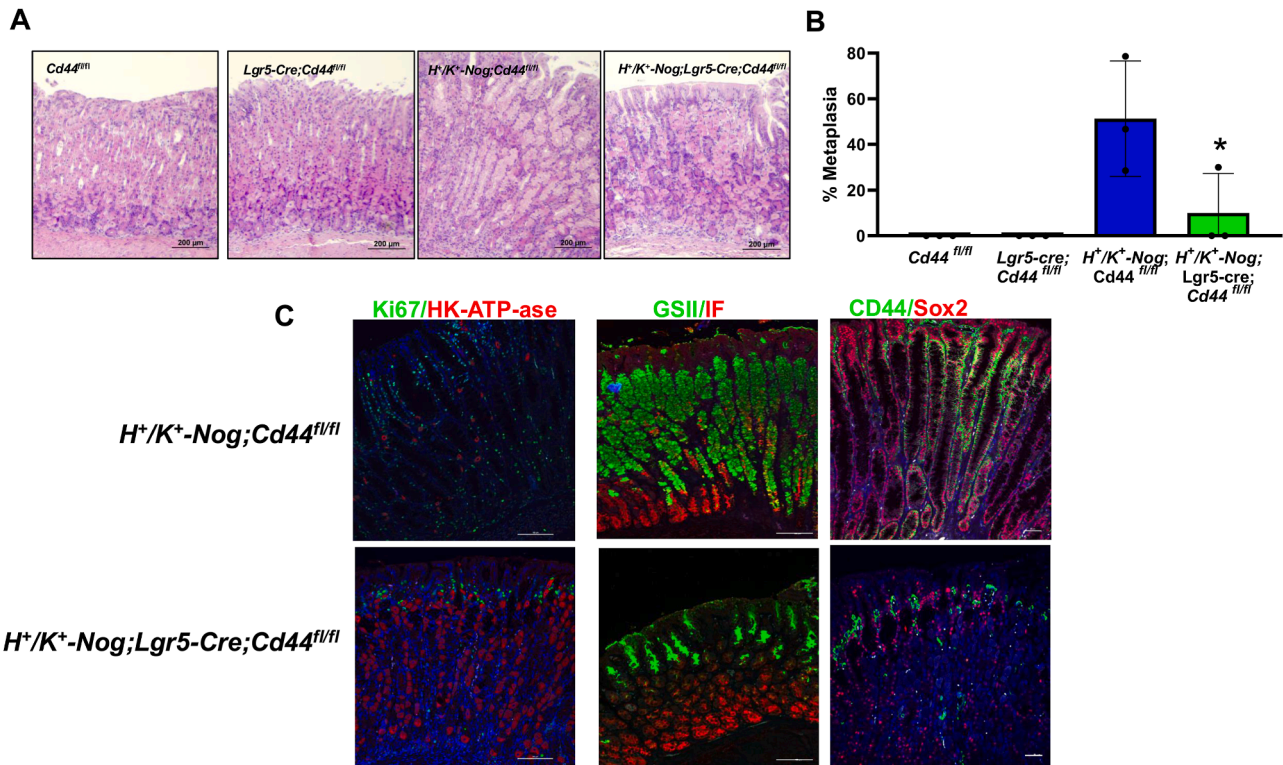


Figure 6. Metaplasia in the gastric epithelium of *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice. One- to 2-month old *Cd44^{flox-flox}*, *Lgr5-Cre;Cd44^{flox-flox}*, *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice were treated with 1 i.p. injection of tamoxifen (0.1 mg/g body weight). Animals were analyzed 3 months after tamoxifen injection. (A) representative H&E-stained paraffin sections of the lesser curvature of *Cd44^{flox-flox}*, *Lgr5-Cre;Cd44^{flox-flox}*, *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice. Size bars 200 μ m. (B) Graph bars represent the percent of fields affected by metaplasia. Values are shown as means $\pm$ standard error. * $P < .05$. (C) Paraffin sections of the lesser curvature of *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice were stained with the H⁺,K⁺-ATPase α -subunit primary antibody and an Alexa 488-conjugated secondary antibody (green) and with an anti-Ki67 primary antibody and an Alexa 555-conjugated secondary antibody (red). Paraffin sections of the lesser curvature of *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice were stained with the Lectin GSII (green) an anti-IF primary antibody and an Alexa 555-conjugated secondary antibody (red). Paraffin sections of the lesser curvature of *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice were stained with the anti-CD44 primary antibody and an Alexa 488-conjugated secondary antibody (green) and with an anti-Sox2 primary antibody and an Alexa 555-conjugated secondary antibody (red). Size bar 100 μ m in the left and central panels, 50 μ m in the right panels. Similar results were observed in at least 3 other groups of *H⁺/K⁺-Nog;Cd44^{flox-flox}* and *Lgr5-Cre;Cd44^{flox-flox};H⁺/K⁺-Nog* mice.

H⁺/K⁺-Nog mice. As shown in Figure 6C (right upper panel), noggin-induced inhibition of BMP signaling led to robust expression of CD44 in Sox2-expressing cells, indicating colocalization of these proteins in the metaplastic gastric mucosa. As depicted in the bottom right panel, deletion of CD44 in *Lgr5* cells led to a significant attenuation of the metaplastic phenotype and to decreased expression of Sox2, suggesting the CD44 regulates Sox2 in the presence of inhibition of BMP signaling.

To define more precisely the role of Sox2 in metaplasia, we examined the morphological changes induced by deletion of *Sox2* in cells with *Lgr5* transcriptional activity in mice that express noggin in the oxyntic mucosa. As shown in Figure 7A, depicting representative H&E-stained sections, the mucosa of the lesser curvature of *Sox2^{flox-flox};H⁺/K⁺-Nog* mice demonstrated the development of metaplastic

(SPEM) changes characterized by a significant reduction in the number of cells that had the morphological appearance of PCs, and by an increase in the number of light-staining cells. Deletion of *Sox2* in *Lgr5* cells led to a significant attenuation of the metaplastic phenotype (Figure 7B) confirming the notion that Sox2 participates in the development of SPEM.

To better assess the role of Sox2 in the pathophysiology of SPEM and in the regulation of CD44, we stained the sections with both anti-Sox2 and anti-CD44 antibodies. As shown in Figure 7C, as previously reported, noggin led to robust expression of CD44 in Sox2-expressing cells. Deletion of *Sox2* in *Lgr5* cells led to decreased expression of both Sox2 and CD44, and to attenuation of the metaplastic phenotype, confirming the notion that both CD44 and Sox2 contribute to the pathophysiology of noggin induced SPEM.

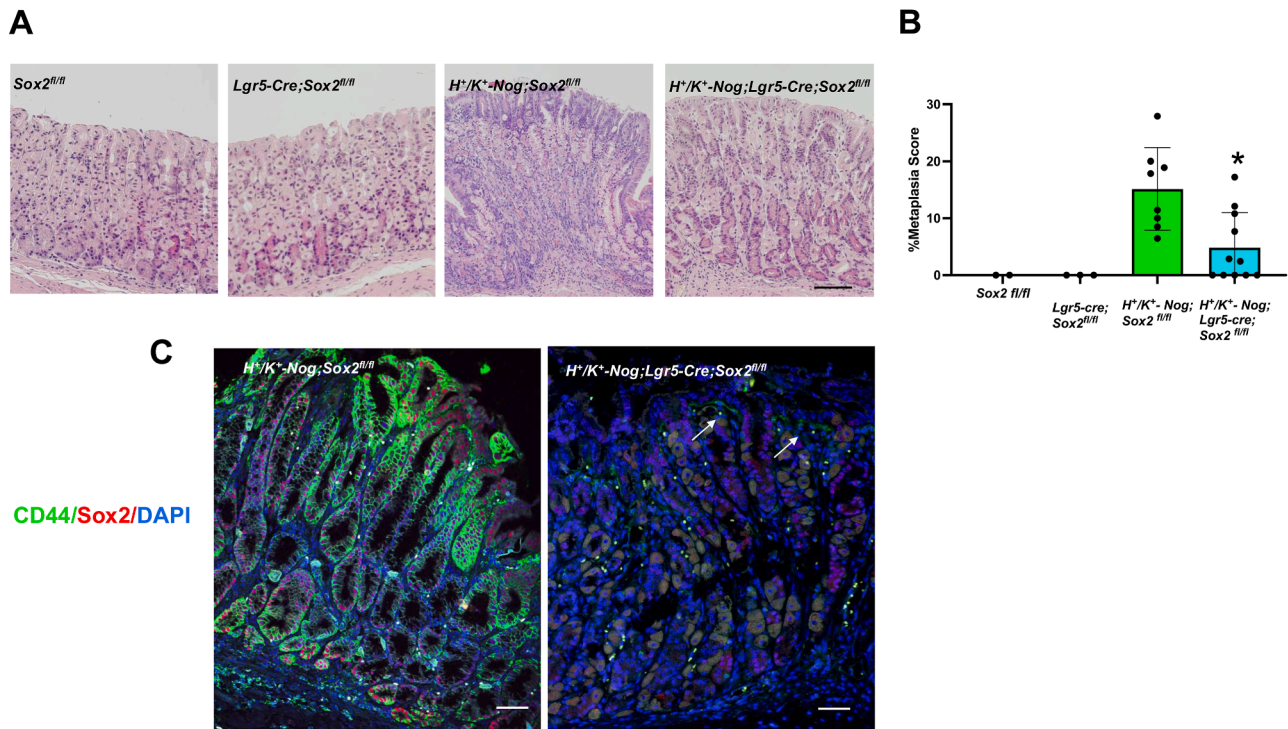


Figure 7. Metaplasia in the gastric epithelium of *Lgr5-Cre;Sox2^{lox-fox};H^{+/K⁺}-Nog* mice. One- to 2-month old *Sox2^{lox-fox}*, *Lgr5-Cre;Sox2^{lox-fox}*, *H^{+/K⁺}-Nog;Sox2^{lox-fox}* and *Lgr5-Cre;Sox2^{lox-fox};H^{+/K⁺}-Nog* mice were treated with 1 i.p. injection of tamoxifen (0.1 mg/g body weight). Animals were analyzed 3 months after tamoxifen injection. (A) representative H&E-stained paraffin sections of the lesser curvature of *Sox2^{lox-fox}*, *Lgr5-Cre;Sox2^{lox-fox}*, *H^{+/K⁺}-Nog;Sox2^{lox-fox}* and *Lgr5-Cre;Sox2^{lox-fox};H^{+/K⁺}-Nog* mice. Size bars 200 μ m. (B) Graph bars representing the percent of fields affected by metaplasia. Values are shown as means $\pm$ standard error. * $P < .05$. (C) Paraffin sections of the lesser curvature of *H^{+/K⁺}-Nog;Sox2^{lox-fox}* and *Lgr5-Cre;Sox2^{lox-fox};H^{+/K⁺}-Nog* mice were stained with the CD44 primary antibody and an Alexa 488-conjugated secondary antibody (green) and with an anti-Sox2 primary antibody and an Alexa 555-conjugated secondary antibody (red). Size bar 50 μ m. Similar results were observed in at least 2 other groups of *H^{+/K⁺}-Nog;Sox2^{lox-fox}* and *Lgr5-Cre;Sox2^{lox-fox};H^{+/K⁺}-Nog* mice. Arrows point to rare, isthmal CD44+ve cells.

Discussion

In this article, we report a series of novel observations that underscore the importance of BMP signaling in the regulation of gastric epithelial homeostasis. We present evidence that inhibition of BMP signaling induces profound changes in the transcriptional programs of the gastric epithelium and that CD44 and Sox2 are BMP-regulated molecules that play an important role in the development of metaplasia (SPEM).

Our study shows that inhibition of BMP signaling leads to an increase in the transcriptional diversity of gastric epithelial cells and to inhibition of programs of cell differentiation. Indeed, the gastric mucosa of the noggin transgenic mice exhibits, compared to control mice, a higher CytoTRACE score, a value that predicts the differentiation state, or stemness of cells based on its gene expression signatures. In agreement with this finding, we observed an increase in the percentage of cells expressing markers of mucus- and pit-cell differentiation and a decrease in that of cells expressing markers of PC differentiation. Interestingly, noggin also induced the expression of SPEM marker genes in several gastric epithelial cells clusters and it activated signaling

pathways that regulate cell proliferation, apoptosis, and inflammation. The observation that the expression of some of these molecules was also increased in the PCs clusters confirms previously published reports from our laboratory that have suggested that inhibition of BMP signaling has profound effects on the normal mechanisms that regulate PC differentiation.⁵⁰ Taken together, these findings support the notion that inhibition of BMP signaling in the mucosa of the corpus promotes a switch to less differentiated and more “stem-like” transcriptional programs that play an important role in the development of metaplastic changes of the stomach.

Another interesting observation of our study is that inhibition of BMP signaling leads to an increase in the number of cells expressing markers of mast-cell differentiation in the gastric mucosa. One attractive hypothesis is that mast cells are BMP signaling-regulated inflammatory cells. Since mast cells have been implicated in the pathophysiology of gastric cancer,^{51,52} it is conceivable that inhibition of BMP signaling could lead to the development of a pro-oncogenic environment characterized by an increased number of mast cells in the gastric mucosa. Additional studies will be needed to explore these interesting possibilities.

The specific cellular localization of Sox2 in the stomach has been the focus of some controversy. According to 1 published immunohistochemical analysis of the gastric mucosa, *Lgr5* and *Sox2* label different populations of gastric progenitor cells.¹¹ In contrast to those observations, we provide evidence that Sox2 is expressed in *Lgr5* cells. Moreover, we show that loss of BMP signaling upregulates the expression of this protein in cells with *Lgr5* transcriptional activity, which has been shown to express CD44¹⁵ and to play an important role in the pathophysiology of metaplasia and neoplasia in the stomach.^{19,20} The observation that Sox2 colocalizes with CD44 further suggests that these proteins are likely to exert cooperative functions in the pathophysiology of metaplasia during situations of mucosal injury and inflammation.

Recent reports have indicated that another member of the Sox family of transcription factors, Sox9, plays an important role in the pathophysiology of gastric metaplasia and neoplasia.⁵³ That study also showed that Sox9 is coexpressed with Sox2 in a subpopulation of gastric epithelial cells and that oncogenic transformation of these cells leads to the development of gastric neoplasms. Thus, it is possible that Sox2 cooperates with Sox9 in the regulation of gastric epithelial cell growth, suggesting that multiple Sox proteins could be involved in the response of the gastric epithelium to oncogenic transformation. Additional studies are needed to better define the role of BMP signaling on the expression and regulation of Sox9.

Another significant result of our study is the observation that deletion of *Cd44* in cells with *Lgr5* transcriptional activity leads to attenuation of the metaplastic changes induced by noggin and to decreased expression of Sox2 in the gastric epithelium in the context of inhibition of BMP signaling. These findings underscore the importance of CD44 in the pathophysiology of metaplasia, and they propose a functional link between CD44 and Sox2. Previous reports have suggested that during the inflammatory response, CD44 induces the activation of a signal transduction pathway that involves the sequential activation of STAT3 and ERK.²³ Thus, it is possible that Sox2 represents an important target of this signaling cascade and that induction of Sox2 expression could be responsible for the activation of genes involved in the regulation of cell proliferation and in the response to injury and inflammation.

Several studies have investigated the role of Sox2 in the pathophysiology of gastric metaplasia and neoplasia. Aberrant expression of Sox2 has been reported in gastrointestinal tumors,^{28–30} where Sox2 has been shown to exert pro-oncogenic functions. Some reports, however, have suggested that Sox2 can inhibit the growth of gastric cancers, presumably through its ability to restrain Wnt-driven tumorigenesis.¹¹ Our observations appear to favor the hypothesis that expression Sox2 is necessary for the development of metaplasia and that activation of BMP signaling inhibits the expression of this transcription factor. Interestingly, our mice that exhibit decreased expression of Sox2 in *Lgr5* cells did not develop neoplasia. Accordingly, it

is conceivable that loss of Sox2 is not sufficient to determine the development of gastric cancer. This observation would be in agreement with the notion that gastric cancer is a complex multistep process that requires the interplay of multiple signaling mechanism and transcription factors.

Conclusion

Our results indicate that inhibition of BMP signaling induces the expression of CD44 and Sox2 in *Lgr5* cells. Moreover, we demonstrated that BMP signaling regulates the function of *Lgr5* cells through CD44- and Sox2-mediated mechanisms. These findings underscore the relevance of BMP signaling, CD44 and Sox2 in the regulation of epithelial homeostasis, and they shed new insights in the mechanisms that lead to the development of metaplasia in the stomach.

Supplementary Materials

Material associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.gastha.2026.101075>.

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Andrea Todisco: Study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content; statistical analysis, obtained funding, study supervision, and writing of the manuscript. Justin Colacino: Acquisition of data, analysis and interpretation of data, statistical analysis, and critical revision of the manuscript for important intellectual content. Nicholas Lesniak: Acquisition of data, analysis and interpretation of data, statistical analysis, and critical revision of the manuscript for important intellectual content. Lei Peng: Acquisition of data, analysis and interpretation of data, technical support, and statistical analysis. Xuan Li: Acquisition of data, analysis and interpretation of data, technical support, and statistical analysis. Tsutomu Takeda: Acquisition of data, analysis and interpretation of data, technical support, and statistical analysis. Xia Wei: Acquisition of data, analysis and interpretation of data, technical support, and statistical analysis. Maria Mao: Acquisition of data, analysis and interpretation of data, technical support, and statistical analysis. Veronique Orian-Rousseau: Acquisition of data and critical revision of the manuscript for important intellectual content. Kathryn A. Eaton: Acquisition of data, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content.

Conflicts of Interest:

The authors disclose no conflicts.

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Ethical Statement:

The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:

Data generated from this study will be submitted to PubMed Central for access to the public.

Reporting Guidelines:

We will follow the guidelines and policies that have been put forth by the NIH regarding the distribution of model organisms and experimental results.