Sox2 Suppresses Gastric Tumorigenesis in Mice

Graphical Abstract

Highlights
- Sox2 targets epithelial, developmental, and cancer genes in gastric progenitors
- Sox2 is dispensable for gastric stem cell self-renewal and epithelial homeostasis
- Sox2+ cells are potent cells of origin in Wnt-driven adenoma model
- Sox2 acts as a tumor suppressor by modulating Wnt-related and intestinal genes

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In Brief
Sarkar et al. explore the role of the stem cell factor Sox2 in gastric homeostasis and tumorigenesis. Surprisingly, they find that Sox2 is dispensable for epithelial regeneration, while it inhibits tumorigenesis in an adenoma mouse model. Mechanistically, Sox2 appears to suppress tumorigenesis by restraining Wnt/β-catenin signaling and repressing an intestinal program.

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Sox2 Suppresses Gastric Tumorigenesis in Mice

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SUMMARY

Sox2 expression marks gastric stem and progenitor cells, raising important questions regarding the genes regulated by Sox2 and the role of Sox2 itself during stomach homeostasis and disease. By using ChIP-seq analysis, we have found that the majority of Sox2 targets in gastric epithelial cells are tissue specific and related to functions such as endoderm development, Wnt signaling, and gastric cancer. Unexpectedly, we found that Sox2 itself is dispensable for gastric stem cell and epithelial self-renewal, yet Sox2+ cells are highly susceptible to tumorigenesis in an Apc/Wnt-driven mouse model. Moreover, Sox2 loss enhances, rather than impairs, tumor formation in Apc-deficient gastric cells in vivo and in vitro by inducing Tcf/Lef-dependent transcription and upregulating intestinal metaplasia-associated genes, providing a mechanistic basis for the observed phenotype. Together, these data identify Sox2 as a context-dependent tumor suppressor protein that is dispensable for normal tissue regeneration but restrains stomach adenoma formation through modulation of Wnt-responsive and intestinal genes.

INTRODUCTION

Sox2 is a transcription factor that has been widely studied in the context of development, pluripotency, and cellular reprogramming (Sarkar and Hochedlinger, 2013). During development, Sox2 controls the self-renewal and differentiation of a number of embryonic stem cells (ESCs) (Masui et al., 2007) and neural progenitor cells (NPCs) (Graham et al., 2003). Consistent with this finding, chromatin immunoprecipitation sequencing (ChIP-seq) analyses in ESCs and NPCs indicate that Sox2 activates self-renewal genes, while suppressing genes associated with differentiation (Lodato et al., 2013). In addition to its role in development, Sox2 is expressed in a number of adult tissues, including the salivary gland, uterus, anus, testes, and stomach, where it marks stem and progenitor cell populations (Arnold et al., 2011). Whether Sox2 expression simply serves as a marker of adult stem and progenitor cells or is also functionally important remains largely unexplored. It is also unclear whether Sox2 targets similar or different sets of genes in adult stem and progenitor cells compared to ESCs to control self-renewal and differentiation. We chose the glandular stomach as a model system to address some of these questions as it constantly regenerates and contains a population of Sox2+ stem and progenitor cells.

The gastric epithelium in mice and humans consists of flask-like glandular units that contain mucus-, acid-, hormone-, and enzyme-producing cells required to digest food (Mills and Shivdasani, 2011). The glandular stomach is further subdivided into the antrum and corpus, which exhibit different ratios of the four principal cell types and distinct rates of epithelial turnover. Sox2 is expressed at the base of antral glands, areas thought to represent stem and progenitor cell compartments (Arnold et al., 2011). Indeed, lineage-tracing experiments demonstrated that the Sox2-expressing cells contain multipotent stem cells in the glandular stomach (Arnold et al., 2011). However, the biological role of Sox2 itself in this cell population remains to be determined.

Gastric cancer is the third most frequent cause of cancer-related deaths worldwide and is incurable when metastases are present (Stewart et al., 2014). Although genome-wide sequencing efforts have cataloged numerous gastric-cancer-specific mutations, the functional significance of these mutations and the cell types in which they act remain unknown. Dysregulation of Sox2 is associated with tumors in various tissues, including the lung, esophagus, pituitary gland, skin, and retina (Boumahdi et al., 2014; Kareta et al., 2015; Sarkar and Hochedlinger, 2013; Bass et al., 2009). While SOX2 is overexpressed or amplified in most of these tumors, consistent with an oncogenic function, Sox2’s role in gastric cancer remains controversial. For
example, some reports have found a positive correlation between SOX2 expression and gastric cancer progression in patients, consistent with a tumor-promoting role of SOX2 (Hült et al., 2014; Matsuoka et al., 2012). However, independent studies noticed a downregulation of SOX2 expression with gastric cancer progression indicative of a possible tumor suppressor function (Otsubo et al., 2008, 2011; Wang et al., 2015; Zhang et al., 2010).

To better understand the mechanisms by which Sox2 expression may contribute to stomach homeostasis and cancer, we sought to determine its functions in the normal and malignant gastric epithelium using a loss-of-function mouse model, as well as an in vitro organoid system. We also have used mouse models to assess the susceptibility of Sox22+ stem/progenitor cells to initiate tumors and employed genome-wide approaches to define downstream targets of Sox2 in gastric epithelial cells relative to other Sox2-expressing stem and progenitor cell populations.

RESULTS

Sox2 Occupies Loci Related to Endoderm Development and Gastric Cancer

Using a fluorescent knockin reporter and genetic lineage tracing, we previously showed that Sox2 expression marks rare epithelial cells in the glandular stomach that function as somatic stem cells (Arnold et al., 2011). The same stem cells could be visualized by immunohistochemistry (IHC) with a Sox2-specific antibody, which we confirmed in this study (Figures S1A and S1B). We previously deemed the rarity of Sox22+ cells to be incompatible with assays to interrogate genome-wide targets of Sox2 using ChIP-seq analysis. However, when we re-examined Sox2 expression patterns in the antrum with two novel Sox2 antibodies, we detected not only this rare population of Sox2high cells but also a more abundant population of Sox2low cells surrounding the stem cells and coinciding with the proliferative progenitor cell compartment (Figure 1A, S1A, and S1B). In situ hybridization confirmed that Sox2 is expressed as a gradient in gastric epithelial cells with a discernible population of Sox2high cells (Figure S1C). This finding is reminiscent of a continuous, but weak, expression of stem cell markers across progenitor cell populations in the brain and intestine (Hagey and Muhr, 2014; Muñoz et al., 2012). We therefore reasoned that the observed Sox2 expression gradient in stomach glands might allow us to procure sufficient material to examine Sox2 targets in the gastric stem and progenitor cell compartment by using ChIP-seq analysis.

We performed ChIP-seq on isolated mouse gastric glands from wild-type mice and mapped more than 7,000 high-confidence binding sites (Figures S2A and S2B). Consistent with Sox2’s role as a transcriptional regulator, ~15% of sites mapped to promoters (0–3 kb upstream of transcription start sites [TSSs]), and roughly 80% of sites mapped to intergenic regions and introns, which typically contain enhancers (Figure 1B). Bound sites were highly enriched for the classical Sox consensus motif ACAAGG (93% of all sites), implying direct occupancy of most sites (Figure 1C). Gene ontology (GO) analysis using the GREAT algorithm revealed enrichment of genes related to digestive tract, foregut morphogenesis, and epithelial differentiation among regulatory regions (Figure 1D, left). In agreement with these enriched categories, we detected Sox2 binding near key regulatory genes previously associated with endoderm development and gut differentiation, including Gata6 and Cdx2 (Figure 1E; Table S1) (Zorn and Wells, 2009). Of interest, Sox2 binds to its own promoter in the stomach, suggesting an autoregulatory feedback mechanism akin to ESCs and NPCs (Boyer et al., 2005; Lodato et al., 2013) (Figure 1F). In addition to targets associated with differentiation and development, GREAT analysis showed overrepresentation of processes related to gastric disease, such as increased GI tumor incidence and abnormal stomach epithelium (Figure 1D, left). We further observed enrichment for signaling pathways mutated in gastric cancer, such as components of the Akt, ErbB2, Vegf, and Wnt cascades (Figure 1D, right).

Notably, Sox2 binding sites in the stomach mucosa showed hardly any overlap with binding sites previously identified in murine NPCs and ESCs (Figure 1F) even though the same antibody was used for ChIP-seq analysis (Engelen et al., 2011; Marson et al., 2008). This observation suggests that Sox2 is directed to tissue-specific genes by associating with cell-type-specific cofactors, similar to observations in NPCs and ESCs (Sarkar and Hochedlinger, 2013). Consistently, genomic regions adjacent to Sox2 binding sites were enriched for motifs recognized by the glandular stomach-expressed transcription factors Foxa2 (69% of all Sox2 targets), Klf4 (60%), Jun (35%), and Gata6 (32%), which may confer target gene selectivity (Figure 1G, top). Expression patterns of Klf4 and Gata6 in the gastric epithelium partially overlapped with those of Sox2 expression, further suggesting co-regulation of targets by these factors (Figure 1G, bottom). Altogether, these data reveal that Sox2 occupies target genes related to endoderm development, stomach function, and gastric cancer and that there is little overlap with Sox2 binding sites in ESCs and NPCs.

Sox2 Is Dispensable for Normal Stomach Homeostasis

Given that Sox2 occupies loci associated with endoderm development and epithelial differentiation, we next determined whether Sox2 itself is required during adult stomach homeostasis. Toward this end, we generated a conditional knockout (KO) allele, Sox2flfl, using conventional gene targeting (Figures 2A and 2B) and crossed homozygous Sox2flfl mice with mice expressing tamoxifen-inducible Cre recombinase from the ubiquitously expressed Rosa26 locus (Rosa26CreERT2). We then gave 6-week-old Rosa26CreERT2; Sox2flfl mice tamoxifen to induce Cre-mediated excision of Sox2 and sacrificed animals at different times (Figure 2C); we refer to these animals as Sox2 KO mice and wild-type controls as Sox2 WT mice. The glandular stomach in Sox2 KO animals displayed near-complete loss of Sox2 by RNA and protein analysis (Figure 2D, left), indicating high loop-out efficiency. Furthermore, ChIP-seq analysis for Sox2 on gastric glands isolated from tamoxifen-induced Sox2 KO animals mapped only a few targets compared to binding sites detected in Sox2 WT gastric glands (Figures S2A and S2B), demonstrating the absence of chromatin-associated Sox2 protein in Sox2 KO mice and confirming the specificity of our Sox2 ChIP assay (Figure 1). We presume that the residual ChIP-seq signal in Sox2 KO samples is due to inefficient loopout.

Surprisingly, we observed no obvious morphological changes in the glandular stomach, even weeks or several months after
Sox2 deletion (Figure 2E). Consistently, the gastric epithelium of both Sox2 WT and KO mice contained the expected pattern of proliferative (Ki67+) progenitors and differentiated cells, including enteroendocrine (Gastrin+) and secretory (Muc5AC+) cells (Figure 2F). To probe Sox2’s function in gastric epithelial regeneration and differentiation independently, we generated organoids, which self-renew and differentiate in culture (Barker et al., 2010), recapitulating gastric cell behaviors in vitro.
Figure 2. Sox2 Is Dispensable for Stomach Homeostasis

(A) Targeting strategy to generate Sox2^{fl/fl} ESCs and mice. Restriction sites, Southern blot probes and expected restriction fragment lengths are indicated.

(B) Southern blot analyses with 5' and 3' probes to verify correct targeting in ESCs.

(C) Breeding strategy to generate Sox2^{ko} and control animals.

(D) Top: western blot analyses on tamoxifen induced Sox2 WT and KO antrum. Bottom: Expression of Sox2 in tamoxifen induced Sox2 WT and KO antrum and organoid samples using RNA-seq analysis. FDRs (false discovery rates) are listed. Abbreviations: st, stomach; org, organoid.

(E and F) Immunohistochemistry for Sox2, Gastrin, Muc5AC and Ki67 on antrum isolated from Sox2 WT and KO mice 18 months after tamoxifen induction.

(G) Sample bright-field images of Sox2 KO organoids four passages after treatment with EtOH (control) or 4-OHT (Sox2 KO).

(H) Organoid diameter measurements in control and Sox2 KO organoid lines (n = 25; p = 0.0920; t test) four passages after EtOH and 4-OHT treatment.

(I) Venn diagram representing overlap between differentially expressed genes (RNA-seq; fold change > 2; FDR < 0.05) and genes with Sox2 occupancy within 10 kb of the TSS (ChIP-seq) when comparing Sox2 WT and KO antrum.

(J) MA plot highlighting differentially expressed genes (red; fold change > 2; FDR < 0.05) and gastric differentiation markers (purple) between Sox2 WT and KO antrum.

(K) Expression of Sox factors in Sox2 WT and KO antrum 2 weeks after tamoxifen induction.

See also Figure S2 and Tables S2 and S3.
Organoids cultured from Rosa26CreER, Sox2fl/fl gastric glands and treated with 4-hydroxytamoxifen lost Sox2 RNA and protein expression (Figure 2D, right panels), yet showed morphology and growth similar to solvent-treated or 4-OHT-treated control organoids (Figures 2G and 2H). Other tissues that reportedly contain Sox2+ cells (e.g., lung, uterus, and anus) also lacked overt abnormalities and we observed no excess mortality in Sox2 KO mice (data not shown). Thus, Sox2 is dispensable for viability, gastric epithelial regeneration and differentiation, in striking contrast to its essential roles during development (Avilion et al., 2003; Que et al., 2007; Sarkar and Hochdelinger, 2013).

To determine whether loss of Sox2 in the stomach epithelium results in any measurable transcriptional dysregulation, we performed RNA sequencing (RNA-seq) of gastric glands isolated from the antrum of Sox2 WT (n = 3) and Sox2 KO (n = 3) animals. We detected 59 genes that were dysregulated more than 2-fold in Sox2 KO stomachs compared to WT stomachs (42 upregulated and 17 downregulated genes; p < 0.05) (Figures 2I and 2J; Table S2). GO analysis showed that differentially expressed genes are involved in extracellular functions, signaling, and secretion (data not shown). Of note, 17% (10/59) of dysregulated genes had nearby Sox2 binding sites (~10 kb from the TSS), suggesting direct regulation by Sox2 (e.g., Trefl1, Itin1, and Atp6v0d2) (Figure 2I). Moreover, six of the Sox2 targets were upregulated, while four of the Sox2 targets were downregulated in Sox2 KO antrum, implying that Sox2 contributes to both gene activation and repression (Table S3). However, only 0.5% (10/2,121) of all TSS-associated Sox2 peaks were transcriptionally dysregulated, which raises the possibility that other Sox family transcription factors may compensate for the absence of Sox2 (Figure 2I). Indeed, we detected RNA expression of Sox4, Sox9, Sox13, Sox18, and Sox21 in the antrum of WT mice, although none of these factors were upregulated in Sox2-deficient mice (Figures 2K and S2G). These data show that loss of Sox2 in the gastric epithelium results in only modest transcriptional changes, and this is not accompanied by compensatory upregulation of other Sox factors.

**Rapid and Widespread Adenoma Formation from Sox2+ Gastric Cells**

Because our ChIP-seq analysis also revealed Sox2 occupancy at a number of genes implicated in cell proliferation and stomach cancer, we considered that Sox2+ cells might serve as a source for gastric tumors. To test this hypothesis, we targeted the Wnt pathway by acutely inactivating Apc, the tumor suppressor and inhibitor of β-catenin, in Sox2+ cells. APC mutations are present in up to one-third of human gastric cancers (Cervantes et al., 2007) and mice heterozygous for an Apc mutation (ApcMinn) develop adenomas in the antrum of the stomach through loss of heterozygosity (Tomita et al., 2007). We crossed Apcfl/fl mice with mice carrying both a tamoxifen-inducible Cre allele in the endogenous Sox2 locus (Sox2CreER) and a loxP-flanked EYFP reporter in the Rosa26 locus (Sox2CreER; Rosa26CreER; Rosa26EYFP) (Figure 3A). To avoid compromised viability as a result of Apc deletion in other Sox2-expressing tissues, we delivered tamoxifen (1 dose of 1 mg) locally by oral gavage (Figure 3A) and verified deletion of Apc in Sox2+ cells through the appearance of small groups of cells with elevated levels of nuclear β-catenin 3 days later (Figures 3B and S3A). These β-catenin+ cells replicated rapidly, forming multiple micro-adenomas within 1 month and large adenomas within 1 year (Figures 3B and 3C). However, these lesions did not progress to invasive adenocarcinomas over the course of 1 year (n = 2), suggesting that additional events are necessary for tumor progression. Adenomas expressed the EYFP reporter (Figure 3D), confirming that they originated from Sox2+ cells. Moreover, tumors continued to express Sox2, implying the presence of cells with stem/progenitor cell characteristics (Figure 3E). Collectively, these data demonstrate that Sox2+ cells are potent cells of origin in a Wnt-driven gastric tumor model.

Lgr5 expression identifies a cell population at the very base of gastric glands, which also contains stem cells and gives rise to adenomas in an Apc-dependent tumor model (Barker et al., 2010; Radulescu et al., 2013). To compare the efficiency of tumor formation from Lgr5+ and Sox2+ cells, we crossed Sox2CreER mice or Lgr5GFP-ires-CreER mice to Apcfl/fl mice, treated double-transgenic animals with tamoxifen and scored tumor formation 4 weeks later (Figure 3F). Remarkably, we detected ~10 times more adenomas in Sox2CreER, Apcfl/fl mice compared to Lgr5GFP-ires-CreER, Apcfl/fl mice (Figures 3G and 3H). However, since Sox2CreER and Lgr5GFP-ires-CreER mice appear to exhibit different loopout efficiencies (Figure S3C), we cannot exclude that increased tumor numbers in Sox2CreER, Apcfl/fl mice is partly due to Apc deletion in more stem or progenitor cells. In contrast to induced Sox2CreER, Apcfl/fl animals, Lgr5GFP-ires-CreER, Apcfl/fl mice that developed gastric adenomas could not be analyzed beyond 3–4 weeks post-tamoxifen treatment because of a significant intestinal tumor burden that required us to sacrifice animals. Altogether, our data identify Sox2CreER, Apcfl/fl mice as a useful gastric tumorigenesis model since they produce more adenomas that can be examined for a longer period of time compared to Lgr5GFP-ires-CreER, Apcfl/fl mice.

**Sox2 Loss Enhances Gastric Tumorigenesis**

Next, we revisited the functional role of Sox2 in gastric tumorigenesis since previous reports are conflicting. We performed immunohistochemistry for SOX2 on 18 human gastric adenocarcinoma specimens and matching normal human gastric mucosa samples. We detected SOX2+ cells at the base of human gastric glands in the antrum, recapitulating expression patterns observed in mouse stomach (Figure 4A). However, Sox2 expression was either absent or extremely low in 13 out of 18 gastric tumors (Figures 4A; Table S4), suggesting that SOX2 overexpression may be selected for during gastric cancer progression. To corroborate this notion, we reanalyzed publicly available expression and epigenetic data collected from over 300 gastric cancer specimens (Cancer Genome Atlas Research Network, 2014). While there was no evidence for SOX2 mutations in this dataset, we found that gastric tumors of the microsatellite instability (MSI) subtype (Cancer Genome Atlas Research Network, 2014), which are most prevalent in the antrum, showed reduced SOX2 expression and increased DNA methylation at the SOX2 locus relative to normal tissue or other gastric cancer subtypes (p < 0.05) (Figures 4B and 4C). These results are consistent with our histological observations of human specimens and thus strengthen the interpretation.
Figure 3. Efficient Tumor Formation following Wnt-Driven Transformation of Adult Sox2+ Gastric Cells

(A) Breeding strategy to determine tumor-initiating potential of Sox2+ cells.

(B) IHC for β-catenin in Apc KO antrum 3 days, 1 month, and 7 months after tamoxifen induction. Arrows point to cells or tumors with nuclear accumulation of β-catenin. Blue dotted line highlights microadenoma.

(C) Stomachs from Apc WT and Apc KO mice 1 month and 1 year after tamoxifen induction. Arrow indicates enlarged antrum in Apc KO stomach. Blue dotted line highlights large tumors in antrum of Apc KO stomach.

(D and E) IHC for YFP (D) and Sox2 (E) on adenoma from Apc KO antrum 7 months after tamoxifen induction.

(F) Breeding strategy to compare tumor-initiating potential of Sox2+ and Lgr5+ cells.

(G) Quantification of adenomas developing in Lgr5CreER and Sox2CreER mice 1 week (p = 0.0006; t test) and 1 month (p < 0.0001; t test) after tamoxifen induction.

(H) IHC for β-catenin in Lgr5CreER and Sox2CreER antrum 1 week after induction. Abbreviations: FS, forestomach; C, corpus; A, antrum; I, intestine.

See also Figure S3.
that epigenetic silencing may be a common mechanism to attenuate SOX2 expression during gastric tumorigenesis within the antrum.

To directly test the functional consequence of Sox2 expression on gastric tumorigenesis, we mated Sox2fl/fl mice to Sox2CreER; Apcfl/fl animals to generate Apc KO and Apc/Sox2 double-knockout (DKO) mice (Figure 4D). We observed adenoma formation with nuclear accumulation of β-catenin in both Apc KO and DKO mice (Figure 4E) and loss of Sox2 specifically in DKO adenomas (Figures 4E and S3B). Notably, we detected a 3-fold increase in the number of adenomas in tamoxifen treated DKO mice compared to Apc KO mice, which is in agreement with the observation that SOX2 expression inversely correlates with human gastric cancer growth (Figures 4E and 4G). To confirm this result by independent means, we generated gastric organoids from these animals and observed again a 3-fold increase in the number of organoids that grew after 4-hydroxytamoxifen treatment of DKO cultures compared to Apc KO cultures (Figures 4F and 4H). These results suggest that SOX2 functions as a context dependent tumor suppressor rather than an oncogene during Wnt-driven adenomagenesis in the antrum of the glandular stomach.

Figure 4. Sox2 Loss Enhances Wnt-Driven Adenomagenesis
(A) IHC for SOX2 on normal human stomach and stomach adenocarcinoma.
(B) Expression of SOX2 in human gastric cancer subtypes (p < 0.05, Kruskal-Wallis test).
(C) Methylation of SOX2 locus in human gastric cancer subtypes CIN, EBV, GS, and MSI (p < 0.05, Kruskal-Wallis test).
(D) Breeding strategy to study role of Sox2 in Wnt-driven adenomagenesis.
(E) IHC for β-catenin and Sox2 in Apc KO and DKO mice 1 month after tamoxifen induction.
(F) Bright-field images of organoids isolated from adult Apc KO and DKO mice and treated with 4-OHT.
(G) Quantification of adenomas in Apc KO and DKO mice 1 month after tamoxifen induction (n = 11 for Apc KO mice; n = 14 for DKO mice; p = 0.03; t test).
(H) Organoid repopulation potential of Apc KO and DKO organoid lines treated with 4-OHT (n = 3 organoid lines; p = 0.007; t test).
See also Figure S4 and Table S4.
Sox2 Restrains Wnt Signaling in Apc-Deficient Gastric Cells

The above-mentioned findings raise the possibility that Sox2 may interfere directly with expression of Wnt/β-Catenin target genes, providing a plausible explanation for increased tumorigenesis in the absence of Sox2. In agreement with this idea, we found local co-enrichment of motifs recognized by Tcf3/Tcf4 and Sox2 within our Sox2 ChIP-seq peaks (Figures 5A and S4A); Tcf proteins are effectors of canonical Wnt/β-catenin signaling. The observed enrichment for Tcf motifs appears to be specific since Smad2 sites, which respond to the unrelated Bmp signaling pathway, are rarely found next to Sox2 targets even though both Smad2 and Tcf3/Tcf4 are abundantly expressed in the gastric epithelium (data not shown). In addition to the overall co-enrichment of Tcf and Sox2 sites, we found that Sox2 occupies several key genes involved in the Wnt/β-catenin cascade, including Tcf4, Apc, and Ctnnb1 (p values from Wilcoxon test).

(Figure 5B; Table S1). These data suggest that gastric Wnt targets and Sox2 targets may be co-regulated globally.

To functionally support the hypothesis that Sox2 suppresses Wnt/β-catenin signaling, we determined expression of the Wnt target Lgr5 in the antrum of Sox2 WT, Sox2 KO, Apc KO, and DKO mice using in situ hybridization (Figure S4B). Although we were unable to detect differences in Lgr5 expression between Sox2 KO and WT stomachs, DKO mice showed a trend for increased Lgr5 signal compared to Apc deficient mice. This observation suggests that Sox2 suppresses Wnt-responsive genes specifically in the context of Apc-dependent Wnt activation. Attempts to confirm this result by global gene expression analysis of Sox2 KO and DKO tissue was unsuccessful because of the heterogeneity of DKO tumors relative to Sox2 KO epithelium. We therefore performed an alternative lentivirus-based Tcf/Lef reporter assay (Fuerer and Nusse, 2010) by using organoids derived from Sox2 WT, Sox2 KO, Apc KO, and DKO mice. Organoids infected with this lentivirus were first selected in puromycin to ensure homogeneous expression of the reporter construct, followed by analysis of luciferase activity. While Tcf/Lef transcriptional activity was comparable between Sox2 WT and Sox2 KO organoids, we observed 10-fold higher Tcf/Lef activity in Apc KO organoids and 25-fold increase in DKO
organoids (Figure 5D). These findings further suggest that Sox2 suppresses tumorigenesis, at least in part, by restraining hyperactivated Wnt signaling in the context of an Apc mutation.

Considering that genetic loss of Sox2 leads to hyperactive Wnt signaling in Apc-deficient gastric cells in mouse, we reasoned that epigenetic silencing of Sox2 might be common in human gastric cancers with hyperactive Wnt signaling. We therefore reanalyzed the Sox2 expression and DNA methylation status across gastric tumors harboring mutations in genes that activate WNT/β-catenin signaling including RNF43, CTNNB1, and APC (Cancer Genome Atlas Research Network, 2014). This analysis indeed revealed reduced Sox2 expression and increased Sox2 methylation across all examined gastric cancer samples harboring mutations in RNF43 (p < 0.05 for expression; p < 0.05 for methylation) and a similar trend in samples harboring mutations in CTNNB1 (p = 0.12; p = 0.31) or APC (p = 0.09; p = 0.15) (Figure 5E), although this was not significant. These results show that Sox2 tends to be epigenetically and transcriptionally silenced in human gastric tumors with WNT-activating mutations, suggesting that Sox2 may suppress Wnt signaling not only in mouse but also in human gastric tumor cells.

To assess whether Sox2 expression is sufficient to attenuate WNT signaling in human gastric cancer cells carrying mutations in components of WNT pathway, we compared Tcf/Lef reporter activity between a gastric cancer cell line harboring a homozygous APC mutation (HUG1N) and a gastric cancer cell line with no known mutations in the WNT pathway (SNU-1) (Barretina et al., 2012). Briefly, we transduced these cell lines with separate lentiviral vectors carrying the Tcf/Lef reporter, a doxycycline-inducible Sox2 transgene and the rtTA transactivator. Transgenic Sox2 induction in these lines was confirmed by western blot analysis before measuring reporter activity (Figure 5F). As expected, HUG1N cells produced a robust reporter signal whereas SNU-1 cells produced only basal reporter signal in the absence of doxycycline (Figure 5G). However, doxycycline exposure of HUG1N cells reduced Tcf/Lef activity by 2- to 3-fold, whereas it had no effect on reporter activity in SNU-1 cells (Figure 5G). These data complement our results on Sox2 KO organoids and provide supportive evidence that Sox2 expression restrains Tcf/Lef activity in a well-characterized human gastric cancer cell line with activated WNT signaling.

**Sox2 Modulates Intestinal and Metaplasia-Associated Genes**

We next wondered whether Sox2 interferes with other aspects of gastric tumorigenesis beyond its role in Wnt/β-catenin signaling. Intestinal metaplasia of the stomach epithelium is considered to be a precursor lesion to gastric cancer and involves the upregulation of intestinal genes and the silencing of gastric genes. As epigenetic silencing of Sox2 coincides with intestinal metaplasia (Camilo et al., 2015; Tsukamoto et al., 2004) and precedes gastric cancer in patients, we wondered whether Sox2 might be directly involved in maintaining the gastric expression program and suppressing the intestinal expression program. While we did not detect major transcriptional changes in the Sox2 KO stomach (Figure 2J), closer inspection of the dysregulated genes revealed an association with intestinal biology and metaplasia. For example, the gene *Intelectin1* (Itlin1), which is expressed in intestinal goblet cells and intestinal metaplasia (Zheng et al., 2012) but absent in the normal gastric mucosa, was upregulated 45-fold in Sox2-deficient gastric tissue (Figures 6A and S5A). We also observed ectopic expression of the intestinal mucin Zg16 and the colon-expressed lysosomal H⁺ transporting ATPase subunit Atp6v0d2, which has been associated with gastric cancer (Fukamachi et al., 2014), in Sox2 KO stomach samples (Figure 6A). Conversely, we detected reduced expression in Sox2 KO stomach of Gsdmc2, Gsdmc3, and Gsdmc4, members of the gadermin family of genes (Figure 6B and S5A), which are frequently silenced in intestinal type gastric cancers (Saeki et al., 2007).

To probe whether Sox2-deficient gastric cells activate other, more established markers of intestinal identity, we examined the expression status of the intestinal epithelial marker Cdx2 as well as the intestinal stem cell marker Olfm4. Indeed, we noticed a modest upregulation of both genes in 2 of 3 examined Sox2 KO antrum samples (Figure S5B), although the differences were not statistically significant. However, when we analyzed these intestinal markers in our gastric organoid system, we observed a pronounced increase in Olfm4 RNA levels in the Sox2 KO sample compared to a wild-type control (Figure 6C). Of note, Olfm4 expression has also been associated with intestinal metaplasia and its suppression in established gastric cancer cell lines reportedly attenuates cell growth (Jang et al., 2015; Liu et al., 2012). To confirm upregulation of Olfm4 upon Sox2 loss with an independent assay, we generated a viral construct containing an EGFP reporter driven by a 1,168 bp fragment containing the Olfm4 promoter. We detected increased Olfm4-EGFP expression in Sox2 KO organoids compared to control organoids by both live fluorescence (Figure 6D) and flow cytometry (Figure 6E), thus supporting the conclusion that this marker of intestinal stem cells and early stages of gastric cancer is activated upon Sox2 loss.

Finally, we determined whether genes upregulated in the Sox2 KO stomach are also elevated in human gastric cancer. Indeed, we found a significant increase of *Olfm4* and *ATP6V0D2* levels in stomach cancer samples compared to normal tissue (Figure 6F). Collectively, these results suggest that Sox2 depletion may contribute to tumorigenesis not only by activating Wnt signaling but also by derepressing a subset of genes associated with intestinal homeostasis and metaplasia (Figure 7).

**DISCUSSION**

We have dissected the molecular and functional roles of Sox2 and Sox2-expressing cells in gastric epithelial homeostasis and tumorigenesis. A main conclusion of our study is that Sox2 is dispensable for normal tissue renewal in the adult glandular mouse stomach. This result was unexpected in the light of Sox2’s requirements in embryonic development and maintenance of other types of stem cells such as ESCs, NSCs and trophoblast stem cells (Sarkar and Hochedlinger, 2013). We surmise that other Sox proteins can compensate in the absence of Sox2. Considering that several other Sox family members are expressed in the glandular stomach, it will be interesting to assess the consequence of their combined loss in the normal gastric epithelium. For example, we expect that co-deletion of Sox2 and Sox9 might exert a homeostatic phenotype given that Sox9 is also expressed in the progenitor cell compartment of...
the stomach and its loss in the intestinal epithelium reportedly causes crypt hyperplasia and secretory cell defects by deregulating Wnt signaling (Blache et al., 2004; Mori-Akiyama et al., 2007; Sinner et al., 2007).

Our mouse models also allowed us to revisit the cellular origin of gastric tumors, which remains elusive. Although loss of the Apc tumor suppressor in Lgr5+ gastric stem cells gives rise to isolated adenomas within a few weeks (Barker et al., 2010), their potential to progress could not be evaluated owing to the overgrowth of intestinal tumors. In contrast, our data identify Sox2-expressing gastric epithelial cells as an efficient source of Wnt-driven tumors, giving rise to multiple micro-adenomas that grow substantially over many months. As these animals survive for over a year, our mouse model may represent a useful system to study early events in tumorigenesis and to identify facilitators of gastric cancer progression. It will be important to test in the future whether qualitative and/or quantitative differences between Lgr5+ and Sox2+ gastric cells underlie increased adenoma formation in Sox2CreER, Apcfl/fl mice relative to Lgr5GFP-IRES-CreER, Apcfl/fl mice.

The presented data further help to clarify the functional role of Sox2 in gastric tumorigenesis, which remains controversial. We conclude that Sox2 acts as a tumor suppressor by restraining the growth of Apc mutant cells in vivo and in vitro. This finding was also unexpected because SOX2 amplifications and overexpression are generally associated with tumor progression in the esophagus, lungs, retina, skin, and pituitary. However, our results are consistent with previous reports, which showed that SOX2 expression is reduced during gastric cancer development in patients (Otsubo et al., 2008; Wang et al., 2015; Zhang et al., 2010), and we confirmed this observation here with an independent set of primary human gastric tumors, as well as a recently published collection of over 300 cancer samples (Cancer Genome Atlas Research Network, 2014). The recent finding that Sox2 heterozygosity leads to a 15-fold increase in papillary tumors originating from bronchiole cells suggests that Sox2 may function as a tumor suppressor in other tissues as well (Xu et al., 2014). These results indicate that Sox2’s effect on cell proliferation and tumorigenesis is highly context dependent.

Our data suggest that Sox2 loss influences gastric tumorigenesis by at least two mechanisms. First, Sox2 seems to limit cell proliferation and tumor growth by dampening hyperactivate Wnt/b-catenin signaling, as in Apc mutant cells. This link between Sox factors and Wnt/b-catenin activity is supported by previous reports in alternative systems (Blache et al., 2004;
Hagey and Muhr, 2014; Kormish et al., 2010). Second, Sox2 loss may contribute to tumorigenesis by derepressing a subset of genes associated with intestinal identity including the stem cell marker Olfm4. Critically, several of these genes are dysregulated in gastric intestinal metaplasia. We surmise that these transcriptional changes prime gastric cells for increased tumor growth in vitro and in vivo once potent tumor suppressors such as Apc are lost. Additional work is certainly required to fully understand the mechanisms by which Sox2 influences Wnt/β-catenin signaling and intestinal gene expression in gastric tumorigenesis.

Comparison of Sox2 target genes in different stem cell populations is important for understanding how the same transcription factor achieves such functional versatility. Surprisingly, the vast majority of Sox2 targets differ between gastric, neural, and embryonic stem and progenitor cell populations. These unique binding patterns are likely the consequence of cell-type-specific cofactors that associate with Sox2 and target distinct gene sets (Sarkar and Hochedlinger, 2013). Indeed, our ChiP-seq data point to a small number of candidate cofactors in the stomach, including the transcription factors Jun, Gata6, Foxa2, and Klf4. Of note, the expression pattern of Gata6 and Klf4 partially overlaps with that of Sox2 in the gastric epithelium, suggesting that they may collaborate within the same cells. It will now be interesting to test if Sox2 physically associates with any of these proteins and co-occupies key targets in gastric progenitors.

Altogether, our study elucidates functional and molecular roles of Sox2 in stomach stem and progenitor cells, yielding insights into (1) the basis of gastric tumorigenesis, (2) molecular links between Sox2, Wnt/β-catenin signaling and intestinal cell fate, and (3) mechanisms by which the same Sox factor may control different target genes in distinct stem and progenitor cell populations. These data will inform efforts to manipulate gastric stem cell populations for regenerative therapy and may lead to strategies to target gastric cancer.

**EXPERIMENTAL PROCEDURES**

**Mice**

Sox2<sup>fl/fl</sup> mice were generated by homologous recombination in embryonic stem cells using standard technology. Correctly targeted clones were injected into BDF1 blastocysts and transferred into pseudo-pregnant females. The resultant chimeric mice were bred with 129Sv/Jae mice, and germline offspring were bred to establish stable lines. Sox2<sub>CreER</sub> (Ventura et al., 2007), Sox2<sub>CreER</sub> (Arnold et al., 2011), Lgr5<sup>GFP-Ires-CreERT2</sup> (Barker et al., 2010), and Apc<sup>fl/fl</sup> (Colnot et al., 2004) mice have been previously described and were obtained from Jackson Laboratories or provided by K. Haigis. All animal studies were carried out following the approved guidelines of the animal protocol of the Massachusetts General Hospital Cancer Center.

**Treatments of Mice**

All treatments were initiated on adult 6- to 8-week-old mice. Rosa<sub>CreER</sub>; Sox2<sup>fl/fl</sup> mice were injected intraperitoneally with 1 mg of tamoxifen (Sigma) for five consecutive days. All other mice were administered a single dose of 1 mg tamoxifen by oral gavage.

**Tissue Preparation and Immunohistochemistry**

Mouse tissues were harvested, fixed in 10% formalin overnight, and then processed for IHC. Human tissues were obtained and prepared as previously described (Sulahian et al., 2014). H&E and IHC stains were performed using standard procedures. For antibody descriptions, see the Supplemental Experimental Procedures.

**ChiP Sequencing and RNA Sequencing**

For details, see the Supplemental Experimental Procedures.

**Organoid Culture and Reporter Assays**

For details, see the Supplemental Experimental Procedures.

**Statistical Analysis**

Data analysis and graph generation were performed using GraphPad Prism (v.6) (GraphPad Prism Software). Statistics for all mouse and organoid experiments were analyzed using Student’s t test, as indicated in figures. Data are displayed as mean ± SD. n is the number of biological replicates unless otherwise specified. Human gastric tumor data were analyzed using the Wilcoxon signed-rank test.

**ACCESSION NUMBERS**

The accession number for the RNA-seq and the ChiP-seq data reported in this paper is GEO: GSE83966.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.034.
AUTHOR CONTRIBUTIONS

A.S. performed the majority of experiments. A.J.H. designed and conducted Wnt and OLFM4 reporter assays and prepared RNA for RNA-seq. R.S. and R.A.S. performed ChIP-seq and human tissue histology. A.A. and R.S. conducted bioinformatics analyses on RNA-seq and ChIP-seq data. X.X. and A.J.B. analyzed The Cancer Genome Atlas database. N.D. and M.R. performed in situ hybridization. C.S. and R.M. created the OLFM4 expression construct. K.F. and M.A.Y. assisted with organoid and mouse experiments. K.A. generated Sox2(+) mice. R.B. analyzed mouse tissue histology. A.S., A.J.H., and K.H. analyzed results. A.S. and K.H. conceived the experiments and wrote the paper.

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