Cell Stem Cell
Article

Sox2 Cooperates with Inflammation-Mediated Stat3 Activation in the Malignant Transformation of Foregut Basal Progenitor Cells

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http://dx.doi.org/10.1016/j.stem.2013.01.007

SUMMARY

Sox2 regulates the self-renewal of multiple types of stem cells. Recent studies suggest it also plays oncogenic roles in the formation of squamous carcinoma in several organs, including the esophagus where Sox2 is predominantly expressed in the basal progenitor cells of the stratified epithelium. Here, we use mouse genetic models to reveal a mechanism by which Sox2 cooperates with microenvironmental signals to malignantly transform epithelial progenitor cells. Conditional overexpression of *Sox2* in basal cells expands the progenitor population in both the esophagus and forestomach. Significantly, carcinoma only develops in the forestomach, where pathological progression correlates with inflammation and nuclear localization of Stat3 in progenitor cells. Importantly, co-overexpression of Sox2 and activated Stat3 (Stat3C) also transforms esophageal basal cells but not the differentiated suprabasal cells. These findings indicate that basal stem/progenitor cells are the cells of origin of squamous carcinoma and that cooperation between Sox2 and microenvironment-activated Stat3 is required for Sox2-driven tumorigenesis.

INTRODUCTION

Stem/progenitor cells are important for maintaining tissue homeostasis, and their aberrant regulation contributes to tumor initiation and cancer progression (Barker et al., 2009; Schepers et al., 2012). This suggests that signaling molecules and transcription factors such as Sox2, which are important for stem cell maintenance, need to be strictly regulated. Recently, genomic studies have shown that abnormal levels of Sox2 correlate with squamous cell carcinoma (SCC) in the lung and esophagus (Bass et al., 2009; Gen et al., 2010). However, the mechanisms underlying this association remain largely unexplored.

Sox2 plays a critical role in maintaining embryonic stem cells as well as adult stem cells in multiple tissues (Arnold et al., 2011; Avilion et al., 2003; Masui et al., 2007; Que et al., 2009; Sarkar and Hochedlinger, 2013). Sox2 is also required for the self-renewal of cancer stem cells (also known as tumor initiating cells) in several malignancies, including glioblastoma and breast cancer (Gangemi et al., 2009; Leis et al., 2012). Moreover, recently Sox2 has been identified as a direct target of Myeloid Elf-1-like factor (MEF, also known as ELF4) in glioblastoma cancer stem cells, and Sox2 overexpression could rescue the decrease in neurosphere formation seen in cells lacking *MEF* (Bazzoli et al., 2012). We previously demonstrated that Sox2 regulates the proliferation and differentiation of epithelial progenitor cells in the developing mouse esophagus and forestomach, which are both lined by a similar stratified keratinized epithelium (Que et al., 2007). In the adult, Sox2 is predominantly expressed in all of the basal progenitor cells in these tissues (this study and Arnold et al., 2011). Intriguingly, recent clinical studies have revealed that *SOX2* gene amplification and protein overexpression frequently occur in SCC of human foregut-derived tissues including the lung and esophagus (Bass et al., 2009; Gen et al., 2010). Conditional Sox2 overexpression in adult mouse lung epithelium leads to tumor formation in one study (Lu et al., 2010). In another study, Sox2 overexpression in the same cell population results in hyperplasia but not tumor formation, and the reason for this discrepancy remains undetermined

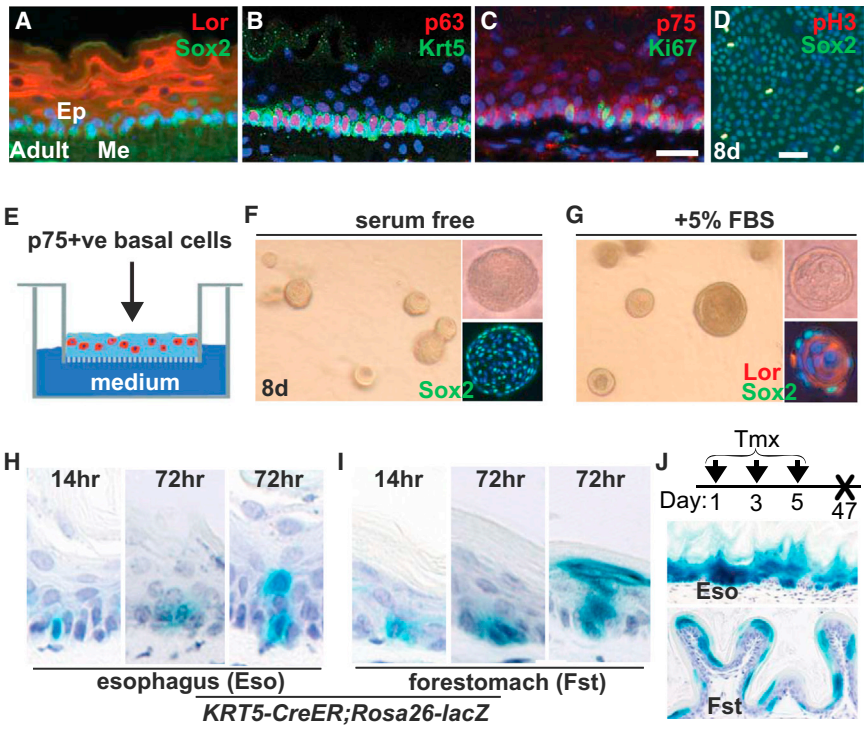

Figure 1. Sox2-Positive Basal Progenitor Cells Self-Renew and Differentiate In Vitro and In Vivo

(A) Sox2 and Loricrin are expressed in the basal and suprabasal layers of the stratified epithelium, respectively. Nuclei are counterstained with DAPI.

(B) Krt5 and p63 are also expressed in the basal cells.

(C) Proliferating (Ki67+) cells are limited to the basal layer, labeled by p75 (nerve growth factor receptor).

(D) Isolated basal progenitor cells proliferate to form colonies and maintain the expression of Sox2.

(E) Schematic of the 3D culture system in which single p75-positive basal cells are embedded and grown in matrix.

(F) Single basal cells proliferate to form esophageospheres and maintain high levels of Sox2 expression in serum-free medium after being cultured for 8 days.

(G) Cells inside the sphere differentiate into squamous epithelium (Loricrin+) in the presence of 5% fetal bovine serum.

(H) A single low dose of Tamoxifen (0.1 mg/g body weight) labels individual cells in the basal layer of the *KRT5-CreER;Rosa26-lacZ* esophagus 14 hr after injection (left). Labeled basal cells divide and remain in basal layer (middle) and daughter cells differentiate and migrate upward (right) 72 hr after injection.

(I) Similarly, *KRT5-CreER* labels individual basal progenitor cells in the forestomach with a low dose of Tamoxifen.

(J) Three doses of Tamoxifen (0.25 mg/g body weight) induce genetic recombination in ~75% and ~56% epithelial cells of the *KRT5-CreER;Rosa26-lacZ* esophagus and forestomach, respectively (n = 3). Abbreviations: Lor, Loricrin; Krt, Keratin; Tmx, Tamoxifen. Scale bar represents 50 μm. See also Figure S1.

(Tompkins et al., 2011). In other important studies using human immortalized airway epithelial cells, SOX2 overexpression alone is insufficient to drive transformation, and this outcome requires co-overexpression of additional genes such as *FoxE1* or *FgfR2* IIIb (Bass et al., 2009). Therefore, synergistic cooperation between multiple genes/pathways appears to be required for SOX2 overexpression to drive tumor initiation. However, how the cooperation is executed in an in vivo setting and whether the oncogenic role of Sox2 is specific for stem/progenitor cells has yet to be determined.

Inflammation is frequently observed in human esophageal SCC biopsies and facilitates tumor formation in the esophagus and forestomach of animal models (Stairs et al., 2011; Taccioli et al., 2012). However, the mechanism by which inflammation promotes tumor initiation in these tissues remains elusive. Tissue-specific overexpression of the inflammatory factor IL-1β in the glandular mouse hindstomach induces severe inflammation, with increased levels of IL-6, and promotes adenocarcinoma in this region through the activation of both the Stat3 and NF-κB pathways (Tu et al., 2008). In addition, deletion of the intercellular adhesion molecule *p120-catenin* disrupts epithelial integrity and leads to SCC in the forestomach. The pathological progression of the SCC is also accompanied by the accumulation of inflammatory cells and increased nuclear localization of phosphorylated Stat3 (p-Stat3) in tumor cells (Stairs et al., 2011), but how this increased Stat3 activation is involved in SCC formation has not been determined.

Here, we use mouse models in combination with in vitro assays to investigate the mechanism by which Sox2 overexpression drives SCC formation. We show that conditional Sox2 overexpression increases proliferation and inhibits differentiation of basal progenitor cells in the stratified epithelium. Nevertheless, Sox2 overexpression alone is insufficient for driving SCC formation. Rather, this outcome is associated with microenvironment-activated Stat3, which cooperates with Sox2 to drive malignant transformation of progenitor cells.

RESULTS

Sox2- and Krt5-Positive Basal Progenitor Cells Both Self-Renew and Differentiate In Vitro and In Vivo

We demonstrated previously that Sox2 is required for the development of the stratified epithelium of the mouse esophagus and forestomach (Jacobs et al., 2012; Que et al., 2007). In the adult, Sox2 is expressed prominently in the basal layer of these two tissues (Figures 1A and 2E) (Arnold et al., 2011). All of these cells also express the intermediate filaments Keratin 5 (Krt5) and Keratin 14 (Krt14) (data not shown), as well as p63 (Figure 1B), a transcription factor important for epithelial stratification (Wang et al., 2011). Ki67 immunostaining indicates that these basal cells are highly proliferative compared to nondividing suprabasal cells (Figure 1C). When purified by fluorescence-activated cell sorting (FACS) with the basal cell surface marker p75 (nerve growth factor receptor [NgfR], Figure 1C) and cultured in the presence of 20 ng/ml Fgf2 and Egf, Sox2+ve basal cells maintain self-renewal capability and form large colonies with a colony-forming efficiency of 4% (Figure 1D). In addition, isolated single basal cells proliferate to form organoids

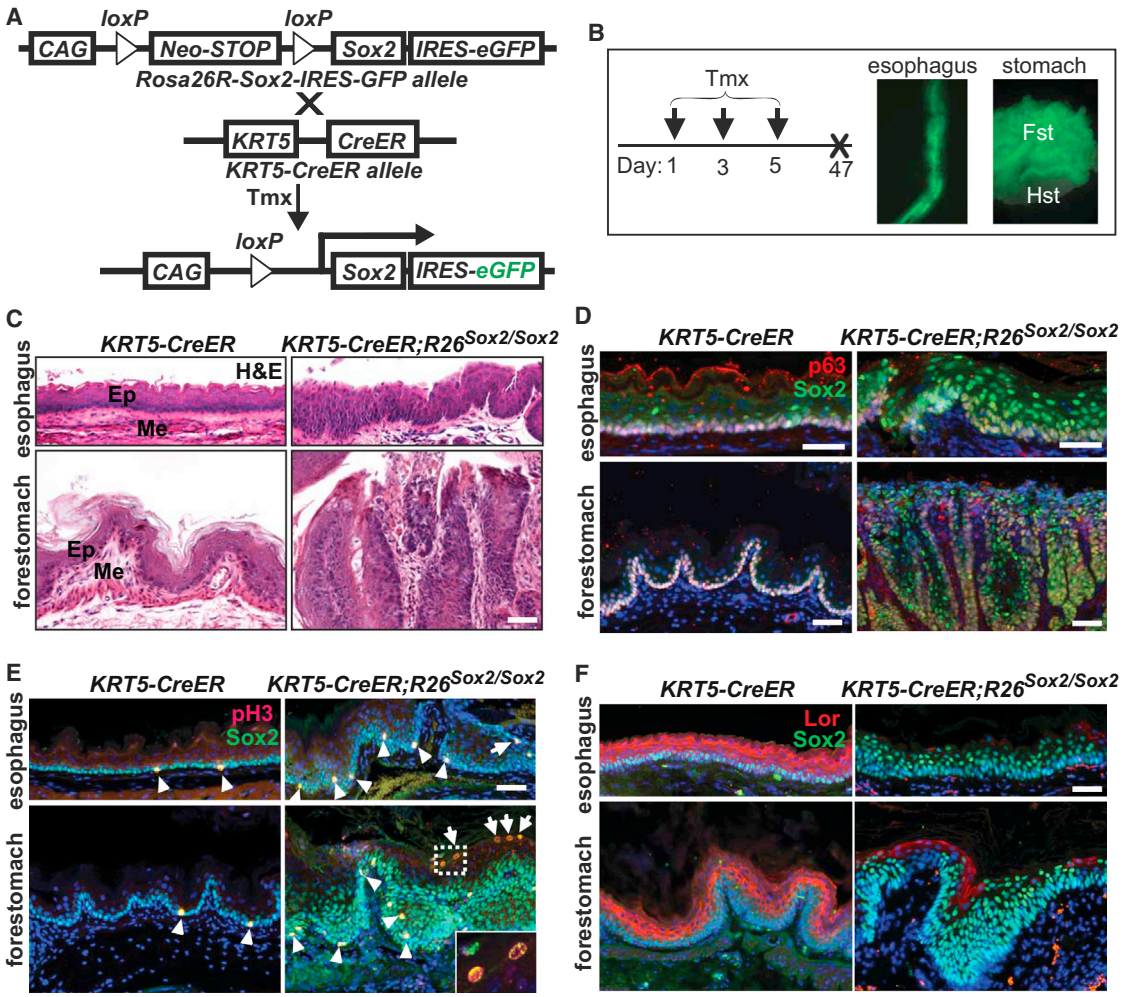

Figure 2. Sox2 Overexpression Promotes Self-Renewal and Inhibits the Differentiation of Basal Progenitor Cells

(A) Schematic for the generation of *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ (*KRT5-CreER;R26*$^{Sox2/Sox2}$) compound mutants. The *CAG* promoter is a combination of the cytomegalovirus (CMV) early enhancer element and chicken *beta-actin* promoter.

(B) Three doses of Tamoxifen induce Sox2 overexpression in the esophagus and forestomach as indicated by GFP expression.

(C) Sox2 overexpression leads to epithelial hyperplasia in the esophagus and forestomach as shown by hematoxylin and eosin (H&E) immunohistochemistry.

(D) Sox2 overexpression expands p63+ve progenitor cell populations in the esophagus and forestomach.

(E) Sox2 overexpression leads to increased cell proliferation as indicated by phosphorylated Histone H3 (arrowheads). Note that proliferating cells are present in the surface layer (arrows). The corner insert is a higher magnification of the boxed region.

(F) Sox2 overexpression inhibits the differentiation of basal progenitor cells in the esophagus and forestomach. Note that in the mutants the presence of patches of epithelial cells negative for the differentiation marker Loricrin. Refer to Figure S1F for individual fluorescent channels. Abbreviations: Fst, forestomach; Hst, hindstomach; Ep, epithelium; Me, mesenchyme. Scale bar represents 50 μm. See also Figure S1.

(esophageospheres) when embedded in extracellular matrix in a three-dimensional (3D) culture system (Figure 1E). Consistent with these findings, only FACS-sorted GFP-positive epithelial cells from the esophagus of a *Sox2GFP* "knockin" mouse line form esophageospheres (see Figure S1A available online). In the absence of serum, basal cells self-renew to form solid spheres in which ~95% of the cells maintain high levels of Sox2 protein (Figure 1F). When dissociated and reseeded under the same culture conditions, single progenitor cells can reform spheres for four passages (data not shown). By contrast, the addition of 5% serum to the sphere culture induces the differentiation of cells in the center toward a Loricrin+ve squamous cell fate, while Sox2+ve cells are confined to the periphery (Figure 1G). This demonstrates that adult Sox2+ve basal cells are progenitors that both self-renew and differentiate in vitro.

To test whether Sox2+ve basal cells can self-renew and differentiate in vivo, we exploited a *KRT5-CreER* transgenic mouse line to perform lineage tracing in combination with the *Rosa26-lacZ* reporter allele (Jovov et al., 2011; Rock et al., 2009). By using a low dose of Tamoxifen to label individual basal cells in the esophagus and following their behavior for 3 days, we found that single cells can divide to duplicate themselves and also differentiate to form columns of squamous epithelium (Figure 1H). Similar results were observed in the forestomach (Figure 1I). These findings are consistent with previous lineage-tracing results using the *Sox2-CreER* mouse line (Arnold et al.,

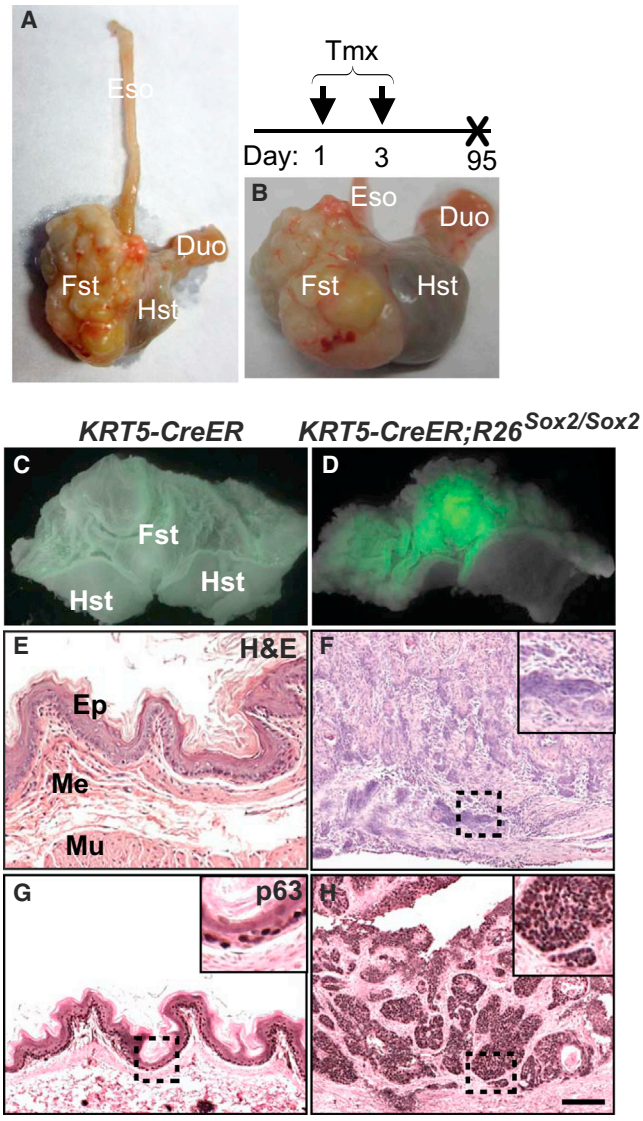

Figure 3. Sox2 Overexpression Leads to the Development of Invasive Squamous Cancer in the Forestomach

(A and B) Tumors are formed in the forestomach but not the esophagus at 3 months after Sox2 induction with two doses of Tamoxifen. (B) is a magnified view of the stomach in (A).

(C and D) Tumor mass is positive for GFP (included in the *Sox2* overexpression construct) (D). No fluorescent signal is observed in the non-Sox2 overexpression control (C).

(E and F) Well-organized stratified epithelium, mesenchyme, and muscle in the forestomach of the wild-type control (E), and cancer cells invade into the muscle layer as shown by H&E staining (F).

(G and H) In the normal forestomach, the basal cell marker p63 is expressed in the basal layer (G), and cancer cells in the mutant forestomach are positive for p63 (H). Inserts are high magnification of the boxed region in the sections. Abbreviations: Eso, esophagus; Fst, forestomach; Hst, hindstomach; Duo, duodenum; Ep, epithelium; Me, mesenchyme; Mu, muscle. Scale bar represents 50 μm. See also Table S1.

2011) and recent in vivo lineage-tracing data (Doupé et al., 2012). In addition, three injections with a higher dose of Tamoxifen gave genetic recombination in ~75% and ~56% of basal cells in the esophagus and forestomach, respectively (n = 3, Figure 1J), showing that the *KRT5-CreER* allele is an efficient tool for genetic manipulation of the basal progenitor cell population in these tissues.

Conditional Overexpression of Sox2 Results in Basal Progenitor Cell Hyperplasia in Both the Esophagus and SCC in the Forestomach

SOX2 overexpression has been implicated in the formation of esophageal SCC (Bass et al., 2009; Gen et al., 2010). To directly test the oncogenic potential of Sox2 in vivo, we generated *KRT5-CreER;Rosa26*$^{CAG\text{-}loxp\text{-}stop\text{-}loxp\text{-}Sox2\text{-}IRES\text{-}Egfp}$ compound mutants (hereafter referred to as *KRT5-CreER;Rosa26*^{Sox2}) in which Sox2 is overexpressed in basal progenitor cells and their derivatives after Tamoxifen injection (Figure 2A). The conditional *Rosa26*^{Sox2} allele contains *IRES-eGFP*, which serves as an indicator for Sox2 overexpression at the cellular level (Figures 2A and 2B). Three injections with Tamoxifen (0.25 mg/g body weight) result in Sox2 overexpression in 71% and 63% of basal cells in the esophagus and forestomach, respectively, with an insignificant difference between the esophagus and forestomach ($p > 0.05$). The Sox2 protein levels in the Sox2-overexpressing progenitor cells (GFP+ve) isolated from both the esophagus and forestomach are comparable but apparently higher than wild-type basal progenitor cells (Figure S1B and data not shown). While no apparent phenotypic changes were observed in *Rosa26*$^{Sox2/Sox2}$ or *KRT5-CreER;Rosa26*$^{Sox2/+}$ (heterozygous) compound mutants 6 months after three Tamoxifen injections (data not shown), Sox2 overexpression in *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ (homozygous) mutants disrupts epithelial structure within 6 weeks (Figure 2C). Basal cell populations (Sox2 and p63 double positive) in both the esophagus and forestomach are expanded (Figure 2D) and proliferating cells (phosphorylated Histone H3 positive) are present in the upper layers of the epithelium (Figure 2E). The proliferation index for the esophageal epithelium increases 2.3-fold after Sox2 overexpression (Figure S1C), and this correlates with increased expression of the cell-cycle protein Cyclin D1 (Figure S1D). When cultured in the 3D system, these Sox2 overexpressing progenitor cells form larger spheres than wild-type controls (Figure S1E). Moreover, Sox2 overexpression inhibits the differentiation of basal progenitor cells into squamous epithelium in the esophagus and forestomach, leading to the presence of patches of undifferentiated cells (Figures 2F and S1F).

Although epithelial hyperplasia is initially seen in both the esophagus and forestomach after Sox2 overexpression, further pathological progression to SCC was observed only in the forestomach (n = 34; Figures 3A and 3B) (Table S1). Severe skin lesions were also noticed in the *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ mutants and necessitated euthanasia of the animals. When Sox2 overexpression was induced by only two doses of Tamoxifen, the skin lesions were less severe. While no tumors were found in the esophagus over 30 weeks, multiple SCC nodules ($n \geq 4$) containing GFP+ve and p63+ve cells developed in the forestomach within 13 weeks (Figures 3A–3H) (Table S1). Significantly, the cells invade into the muscularis externa in 14 out of 23 mice that have tumors at week 13 (Figures 3E and 3F) and 3 out of 3 at week 30 (Table S1), confirming their tumorigenic phenotype. Multiple invasive SCC nodules also develop in the skin at week 30 (Figure S2A).

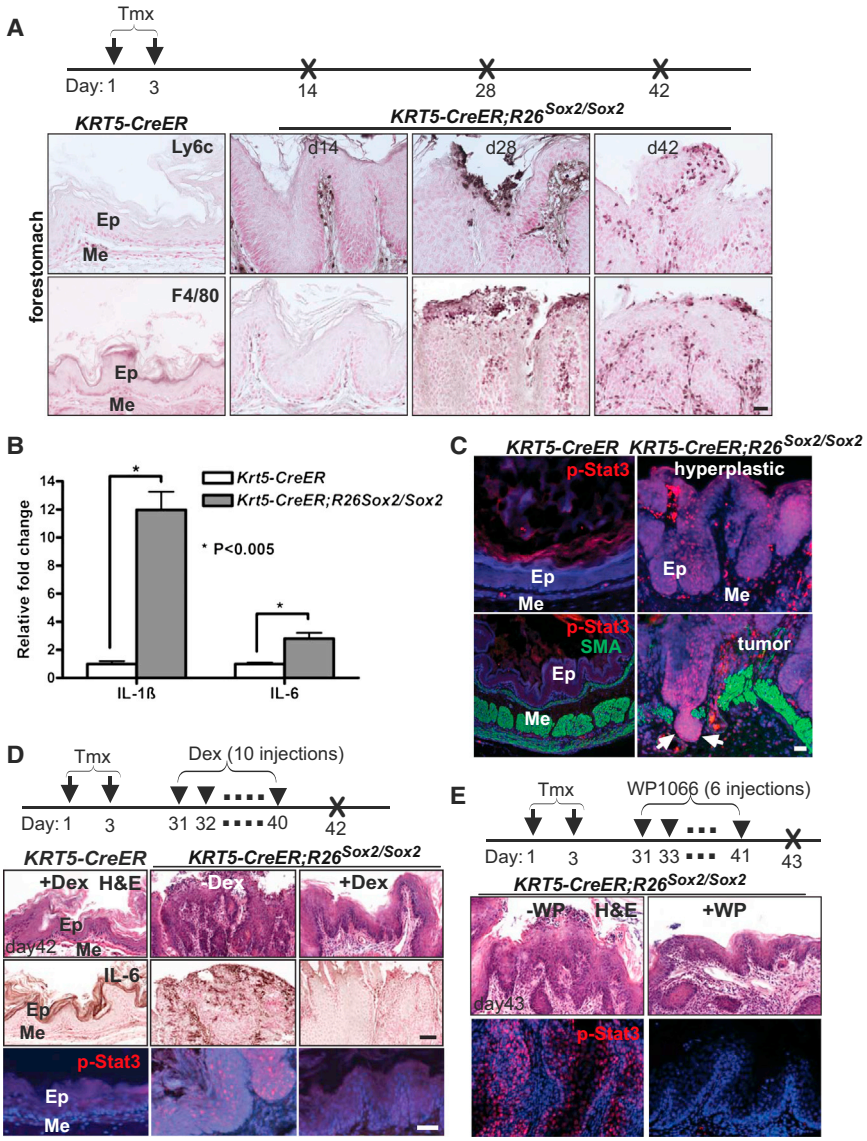

Figure 4. Tumor Initiation in the Forestomach Involves Inflammatory Signaling after Sox2 Overexpression

(A) Progressive accumulation of neutrophils (Ly6c+) and macrophages (F4/80+) in the epithelium and mesenchyme of the hyperplastic forestomach. Two injections of Tamoxifen were used to induce Sox2 overexpression. While a small number of neutrophils and macrophages are present in the mesenchyme at day 14, extensive infiltration of inflammatory cells appears in the damaged epithelium at day 28 and day 42.

(B) Quantitative real-time RT-PCR (n = 3) analysis shows increased transcript levels of IL-1β and IL-6 in the hyperplastic tissues after Sox2 overexpression. Data are represented as mean ± SEM.

(C) p-Stat3 levels are increased in the nuclei of the hyperplasic and invading SCC epithelium in the mutants, as indicated by immunostaining. Note that tumor cells invade into the muscle layer, which is positive for smooth muscle actin staining (arrows).

(D) Inhibition of inflammation with serial injections of Dexamethasone in the hyperplastic forestomach after Sox2 overexpression. Note that the levels of IL-6 and p-Stat3 are decreased upon Dexamethasone injections.

(E) Inhibition of Stat3 signaling with serial injections of the Stat3 inhibitor WP1066 in the hyperplastic forestomach. Note that Stat3 phosphorylation has largely been suppressed by the inhibitor in the epithelium and mesenchyme of the forestomach harvested 2 days after the final injection. Abbreviations: SMA, smooth muscle actin; Dex, Dexamethasone. Scale bar represents 50 μm. See also Figure S2 and Tables S2, S3, and S4.

Taken together, these results reveal that Sox2 overexpression in basal progenitor cells promotes proliferation and inhibits differentiation in both the esophagus and forestomach. Significantly, Sox2 overexpression induces invasive SCC formation only in the forestomach, suggesting that either region-specific intrinsic differences in tumor susceptibility or microenvironmental factors are important for the malignant transformation of Sox2 overexpressing progenitor cells.

Inflammation with IL-6/Stat3 Pathway Activation Facilitates Tumor Formation in the Forestomach of the *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ Mice

We next investigated why SCC only develops in the forestomach after Sox2 overexpression and not in the esophagus. When retrospectively examining pathological progression, we observed small numbers of neutrophils and macrophages in the mesenchyme of the forestomach 2 weeks after Sox2 overexpression. As hyperplasia develops 4–6 weeks after Sox2 induction, the inflammatory cells progressively accumulate in both the mesenchyme and epithelium (Figure 4A). This appears to correlate with the loss of differentiated cells and exposure of progenitor cells to the bile acid-rich environment in the stomach. Significantly, such inflammation never occurs in the esophagus at any time examined (Figure S2B). We then compared gene expression between the hyperplastic forestomach and the normal tissue of *KRT5-CreER* controls (n = 3 for each) 6 weeks after Sox2 induction by microarray analysis. Transcript levels of the endogenous *Rosa26* gene in the mutants decreased 14.2-fold, as expected from recombination (Table S2). This is consistent with the overexpression of Sox2 protein, as validated by immunostaining (Figures 2F and S1F). Of the 84 genes significantly upregulated ($p < 0.05$, fold change ≥ 1.5) upon Sox2 overexpression (Table S3), many are associated with tissue damage and inflammation, including *S100A8* (227-fold), *Ccl20* (21-fold), *Cxcr3* (17-fold), and *IL-1β* (9-fold) (Table S4). The increase of *IL-1β* transcript levels was validated with independently prepared RNAs using quantitative real-time RT-PCR (Figure 4B). Although the microarray data showed no significant change, qPCR detected a 2.8-fold increase in IL-6 transcript levels in the hyperplastic epithelia (Figure 4B) and this upregulation was confirmed by

immunohistochemistry (Figure 4D, middle). These findings corroborate previous observations that overexpression of IL-1β in glandular stomach tissue is accompanied by increased levels of IL-6 (Quante et al., 2012; Tu et al., 2008). Interestingly, when FACS-sorted Sox2 overexpressing forestomach and esophageal cells (GFP+ve) were cultured in the presence of 10 ng/ml IL-6, the cells around the periphery of the esophageospheres became protrusive and irregular and correlated with decreased levels of E-Cadherin (Figure S2C and data not shown), suggesting that IL-6-mediated signaling promotes invasiveness of Sox2 overexpressing progenitor cells.

IL-6 is a potent activator of the Janus kinase (Jak)/Stat3 signaling pathway, which is involved in tumor formation in several tissues, including the forestomach (Stairs et al., 2011). Consistently, we found that p-Stat3 levels are increased in the hyperplastic forestomach epithelium 6 weeks after Tamoxifen injection and the total protein level of Stat3 is also moderately increased upon Sox2 overexpression (Figure S2D). Immunostaining further confirmed that p-Stat3 is enriched in the nuclei of hyperplastic forestomach epithelium (Figure 4C) and that this expression pattern is maintained in invasive SCC tumor cells (Figure 4C). Of note is that we did not observe a similar increase in nuclear p-Stat3 in the esophagi of the same mice in which either forestomach hyperplasia or tumor was found (data not shown).

To test whether inflammation after Sox2 overexpression is important for tumor initiation in the forestomach, we injected the immunosuppressant Dexamethasone after Sox2 induction (Figure 4D). Serial Dexamethasone treatment reduces the inflammatory reaction in the hyperplastic forestomach and also dampens the accumulation of IL-6 and nuclear localization of p-Stat3 in the epithelial layers (Figures 4D and S2E). More importantly, Dexamethasone treatment dramatically reduces tumor incidence (1/8 versus 6/8, $p < 0.01$) when examined at 13 weeks after Sox2 overexpression (Figure S2F). Similarly, when phosphorylation of Stat3 is inhibited with a specific inhibitor WP1066 (Iwamaru et al., 2007), tumor incidence is also decreased (1/9 versus 8/11, $p < 0.01$) (Figures 4E and S2G). These findings support a model in which some unique feature of the forestomach microenvironment, for example, exposure to acid secreted by the hindstomach as previously shown (Quante et al., 2012), leads to inflammation when combined with the disruption of epithelial integrity caused by Sox2 overexpression. Moreover, this inflammation, mediated through the IL-1β/IL-6/Stat3 pathway, is required for the malignant transformation of basal progenitor cells.

Activated Stat3 and Sox2 Synergistically Transforms Mouse and Human Esophageal Basal Progenitor, but Not Differentiated Suprabasal Cells

Unlike in the mouse, the human esophagus is immediately adjacent to the acid-producing stomach and the formation of human esophageal SCC has recently been linked to acid reflux from this organ (Pandeya et al., 2010; Uno et al., 2011). Although our findings demonstrate that microenvironment-activated inflammation and Stat3 are important for the malignant transformation of basal progenitor cells, it is also possible that intrinsic differences between esophagus and forestomach contribute to the contrasting tumor susceptibility after Sox2 overexpression. We therefore asked whether a combination of Sox2 and activated Stat3 is capable of transforming basal progenitor cells of the mouse esophagus. Sox2-overexpressing basal progenitor cells (GFP+ve) from the esophagi of *KRT5-CreER; Rosa26*$^{Sox2/Sox2}$ mice were isolated and infected with a constitutively activated Stat3 (*Stat3C*) lentivirus (Figures 5A and 5B). These cells were then injected into the flanks of the immunodeficient NOD/SCID gamma (NSG) mice (1×10^6 cells/injection). No tumor was observed in mice injected with esophageal basal cells overexpressing either Sox2 or Stat3C alone (three mice for each group). In contrast, progenitors co-overexpressing Sox2 and Stat3C give rise to SCCs (GFP+ve) within a 5 week observation period in all of the three mice (Figures 5C and 5D). The experiment was repeated once and the same results were obtained. The tumor cells express p63, indicating their basal progenitor cell identity, and proliferating cells (pH3+ve) are present in the tumor mass (Figure 5E). We have also isolated and co-overexpressed Sox2 and Stat3 in the differentiated suprabasal cells (p75 negative), but no tumor formation was observed from injecting these cells into three NSG mice (1×10^6 cells/injection) after a 5 week observation period.

We next asked whether the combined overexpression of Sox2 and activated Stat3 can similarly transform human esophageal epithelial cells. We used EPC2, an immortalized human esophageal epithelial cell line. EPC2 cells express moderate levels of Sox2 and p63 (Figure S3A) and can proliferate and differentiate to form stratified epithelium in 3D organotypic culture (Andl et al., 2003; Okawa et al., 2007). When embedded in extracellular matrix, single EPC2 cells are also able to self-renew to form spheres (Figure S3B), suggesting EPC2 cells maintain some progenitor cell functions. EPC2 cells infected with lentivirus harboring *Sox2-IRES-GFP* and *Stat3C* have a higher sphere-forming efficiency than control cells that overexpress either *Sox2-IRES-GFP* or *Stat3C* alone (9.2% for co-overexpression, 6.4% and 7.8% for Sox2 and Stat3C overexpression alone, respectively; Figures 5F and 5G). When 1×10^6 cells were injected into NSG mice, none of the control groups (three mice per group) induced tumor formation. In contrast, prominent tumor masses (GFP+ve) were observed in three out of three mice injected with EPC2 cells co-overexpressing Sox2 and Stat3C (Figures 5G–5I). Histological analysis indicates that they are invasive squamous cell cancer (Figure 5J). As expected, the nuclei of the tumor cells express high levels of Sox2 and moderate levels of p63 (Figures 5K and 5L).

Together, these findings demonstrate that high levels of Sox2 and activated Stat3 are able to transform basal progenitor cells but not the differentiated suprabasal cells isolated from the mouse esophagus. Moreover, the combination of these two transcription factors can also malignantly transform human esophageal progenitor-like cells, leading to squamous cell carcinoma when implanted into immunodeficient mice.

shRNA-Mediated Knockdown of *STAT3* and *SOX2* Reduces the Growth of Mouse and Human Squamous Cancer Cells

High levels of Sox2 are needed for maintaining breast and brain cancer stem cells (tumor initiating cells) and tumor growth (Gangemi et al., 2009; Leis et al., 2012). To test whether Sox2 is similarly required for the maintenance of SCC, we isolated tumor

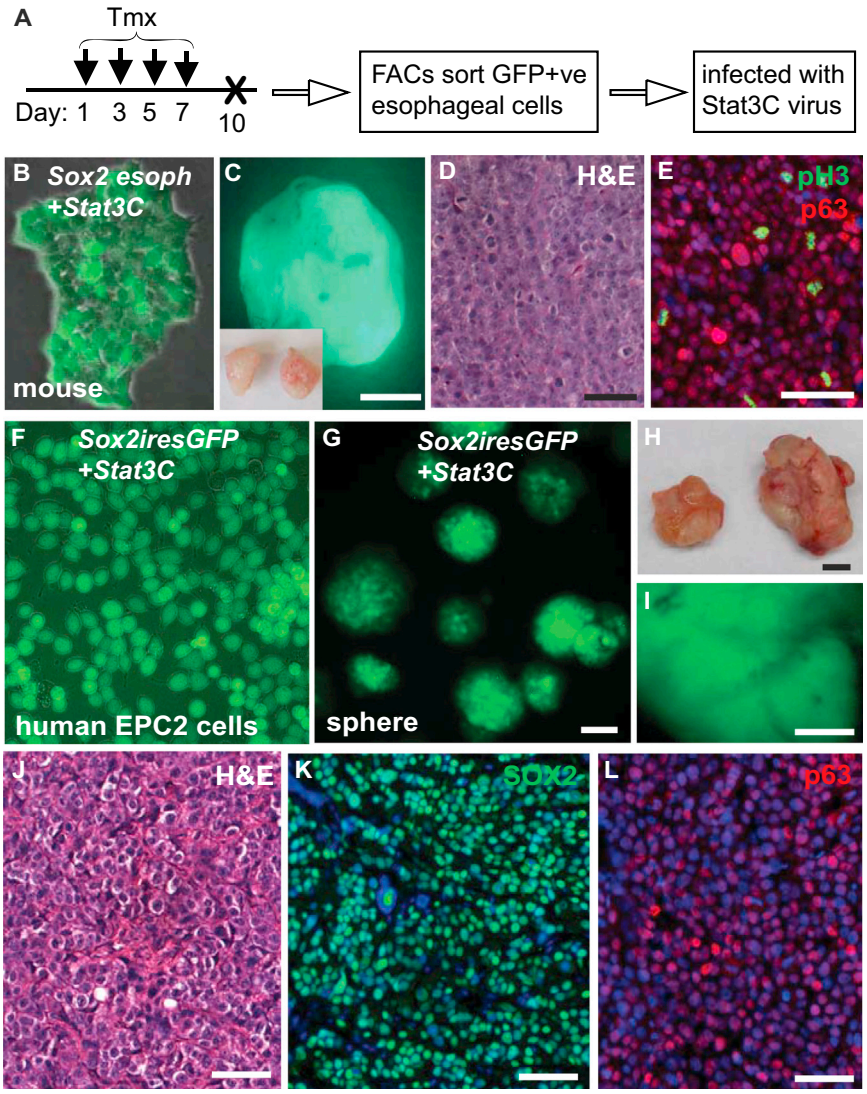

Figure 5. Co-overexpression of Sox2 and Constitutively Activated Stat3 Leads to Malignant Transformation of Mouse Esophageal Basal Progenitors and Human Esophageal Progenitor-like EPC2 Cells

(A) Schematic of the experimental procedure for the transformation of Sox2-overexpressing esophageal basal progenitor cells with Stat3C lentivirus.

(B) Representative colony of Sox2 overexpressing (GFP+ve) esophageal basal progenitor cells infected with Stat3C lentivirus. Basal progenitor cells were isolated from the esophagus of the *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ mice after four doses of Tamoxifen.

(C) Representative GFP+ve tumors developed in the immunodeficient NSG mice after injecting Sox2 and Stat3C co-overexpressing mouse esophageal basal progenitor cells.

(D) Tumors initiated by Sox2 and Stat3C co-overexpressing basal progenitor cells are squamous cell carcinomas, as indicated by H&E staining.

(E) Tumor cells express basal progenitor marker p63 and some of tumor cells are at proliferative state (phosphorylated Histone H3+ve).

(F) EPC2 cells transformed with *Sox2-IRES-eGFP* and *Stat3C* express GFP as visualized with immunofluorescence microscopy.

(G) Sox2 and Stat3C co-overexpressing EPC2 cells form spheres with high levels of GFP as visualized with immunofluorescence microscopy.

(H and I) Representative GFP-positive tumors form in the NSG mice after inoculation of Sox2 and Stat3C co-overexpressing EPC2 cells. Two representative tumors formed by the transformed EPC2 cells are shown (H). The tumor is positive for GFP under the fluorescent microscope (I).

(J) H&E staining to show that tumors initiated by Sox2 and Stat3C co-overexpressing EPC2 cells are squamous cell carcinomas.

(K) Tumor cells express high levels of Sox2 in the nuclei.

(L) Tumor cells express the basal progenitor cell marker p63. Scale bar represents 3 mm in (C), (H), and (I) and 50 μm in (D), (E), (G), (J), and (L). See also Figure S3.

cells from the forestomach of the *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ mice and performed Sox2 knockdown with a lentivirus-based system. *Sox2* knockdown reduces the size of the tumor spheres formed in the 3D culture system and is accompanied by an increased number of apoptotic cells (Figures 6A and S4A). Similarly, *Stat3* knockdown also reduces the size of the tumor spheres (Figures 6A and S4A). When injected into NSG mice (1×10^6 cells/injection), the tumors formed by the *Sox2* or *Stat3* knockdown cells are apparently smaller than those formed by the nonknockdown tumor cells (0.31 ± 0.078 g [*Sox2* knockdown], 0.24 ± 0.046 g [*Stat3* knockdown], and 0.66 ± 0.070 g [nonknockdown control], n = 6 for each group, Figure 6B). The weight difference of *Sox2* knockdown versus control or *Stat3* knockdown versus control is significant ($p < 0.05$).

A previous study has shown that *SOX2* knockdown reduces the proliferation of the nontumorigenic esophageal SCC line TE10 (Bass et al., 2009). *SOX2* knockdown using the same system inhibits proliferation in the tumorigenic esophageal SCC line KYSE450 and leads to small esophageospheres (Figures 6C, 6D, and S4B). Sox2 knockdown also increases the number of apoptotic cells within the esophageospheres (Figure S4B). Similarly, shRNA-mediated *STAT3* knockdown also reduces the proliferation and increases the apoptosis of KYSE450 cells (Figures 6C, 6D, and S4B) and combined knockdown of *SOX2* and *STAT3* leads to a further decrease in cell proliferation (Figures 6C and 6D). Moreover, individual knockdown of *SOX2* and *STAT3* in KYSE450 cells leads to smaller tumors than in mock controls (Figure 6E) and combined knockdown of *SOX2* and *STAT3* further reduces xenograft weight in NSG mice (Figure 6E). In aggregate, individual or combined knockdown of *SOX2* and *STAT3* in a tumorigenic esophageal SCC cell line reduces cell proliferation and tumor growth when injected into NSG mice.

Increased Protein Levels of p-STAT3 and SOX2 Correlate with a Poor Prognosis for Human ESCC

Upregulated SOX2 expression is associated with poor differentiation of esophageal SCC in human patients (Gen et al., 2010). In

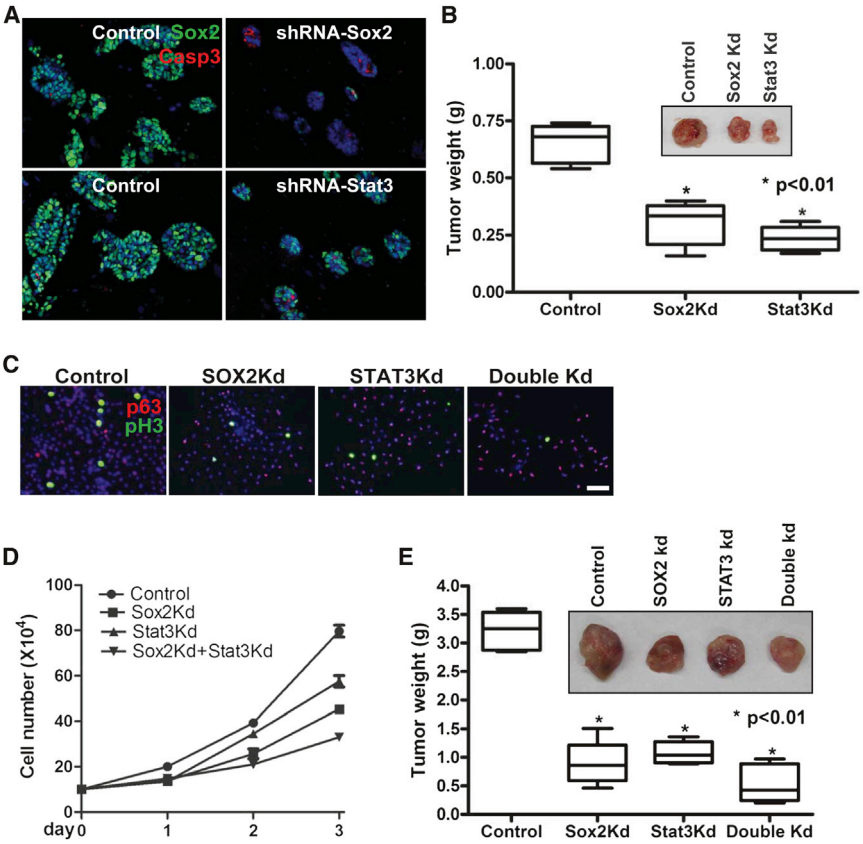

Figure 6. shRNA-Mediated Knockdown of *Sox2* and *Stat3* Reduces the Proliferation of Mouse Forestomach and Human Esophageal Squamous Cancer Cells

(A) Lentiviral shRNA knockdown of *Sox2* or *Stat3* increases the number of apoptotic cells and reduces the size of spheres formed by mouse forestomach squamous cancer cells.

(B) Knockdown of Sox2 or Stat3 in forestomach cancer cells significantly reduces tumor weight after injection into immunodeficient NSG mice. Control is a representative tumor generated by forestomach cancer cells infected with virus containing scramble controls ($n = 6$ for each group, $p < 0.01$), and data are represented as mean ± SEM.

(C and D) Lentiviral shRNA knockdown of *SOX2*, *STAT3*, or both reduces the proliferation of KYSE450 cells. The proliferating cells are indicated by the phosphorylated Histone H3 (pH3) immunostaining (C). Cell numbers in (D) were calculated by the average of cells in three wells for each group, and data are represented as mean ± SEM.

(E) Knockdown of *SOX2*, *STAT3*, or both in KYSE450 cells significantly reduces tumor weight after injection into immunodeficient NSG mice. Control is a representative tumor generated by KYSE450 cells infected with virus containing scramble controls ($n = 6$ for each group, $p < 0.01$), and data are represented as mean ± SEM. Scale bar represents 50 μm. See also Figure S4.

the human esophagus, SOX2 protein is highly enriched in basal progenitor cells that express p75 (Figures 7A and 7B) and ~8% of basal cells are positive for p-STAT3 (Figure 7C, also see Figure S5). Through immunohistochemical examination of 83 primary human esophageal SCC samples (from 74 male and 9 females, refer to Tables S5 and S6 for other clinical parameters), we found that 45 (54.2%) and 38 (45.8%) samples are positive for SOX2 and p-STAT3 staining, respectively. Interestingly, 31 (37.3%) samples are positive for both SOX2 and p-STAT3 (Figures 7D–7F). High levels of p-STAT3 and SOX2 in esophageal SCC correlate with a poor 5 year survival as compared to SOX2 positive only group (14.3% versus 42.9%, $p < 0.01$) (Figure 7G). Of note is that patients with high levels of STAT3, including the STAT3 and SOX2 double-positive group, have a worse prognosis than STAT3-negative patients (11.6% versus 37.1%, $p < 0.05$). Taken together, these results suggest that a simultaneous increase in SOX2 and p-STAT3 protein levels serves as a marker of poor prognosis for patients with esophageal SCC.

DISCUSSION

It has been proposed that mutations accumulated in stem/progenitor cells can have catastrophic consequences for tissue integrity and homeostasis over the long term compared with mutations in cell populations committed to terminal differentiation (Barker et al., 2009; Visvader and Lindeman, 2012). In this study, we provide genetic evidence that links basal progenitor cells with the etiology of squamous cell carcinoma in the foregut. We identify a mechanism by which the transcription factor Sox2 cooperates with inflammation to transform basal progenitor cells and initiate invasive squamous cell carcinoma (Figure 7I).

Stem/progenitor cells are located in the basal layer of the stratified epithelium in the esophagus (Kalabis et al., 2008; Seery and Watt, 2000). A recent study combining lineage tracing and mathematic modeling suggests that the stratified esophageal epithelium is maintained by a single type of basal stem/progenitor cell (Doupé et al., 2012). The progenitor marker we use here, p75, has previously been used to isolate mouse tracheal basal progenitor cells and human esophageal progenitor cells (Okumura et al., 2003; Rock et al., 2009). Our immunostaining results confirm that like Sox2 and Krt5, p75 is expressed in all basal progenitors in the mouse and human esophagus. Purified p75+ve mouse basal progenitor cells form large colonies of cells in monolayer culture that continue to express the basal cell markers Sox2 and p63. When grown in matrix, single progenitor cells can proliferate to form spheres and the undifferentiated cells can regenerate spheres when dissociated and re-embedded, a property that has also been observed in progenitor cells isolated from the trachea and neural tissue (Reynolds and Weiss, 1992; Rock et al., 2009). Upon addition of serum, progenitor cells in the center of the sphere differentiate into squamous cells. Our lineage-tracing results using *KRT5-CreER* are consistent with previous findings using the *Sox2-CreER* mouse line (Arnold et al., 2011), confirming that basal cells are progenitor cells for the maintenance of the esophageal and forestomach epithelium. We recently established a new *Sox2-CreER* "knockin" mouse line to confirm that Sox2+ve basal progenitor cells self-renew and differentiate to maintain the epithelium.

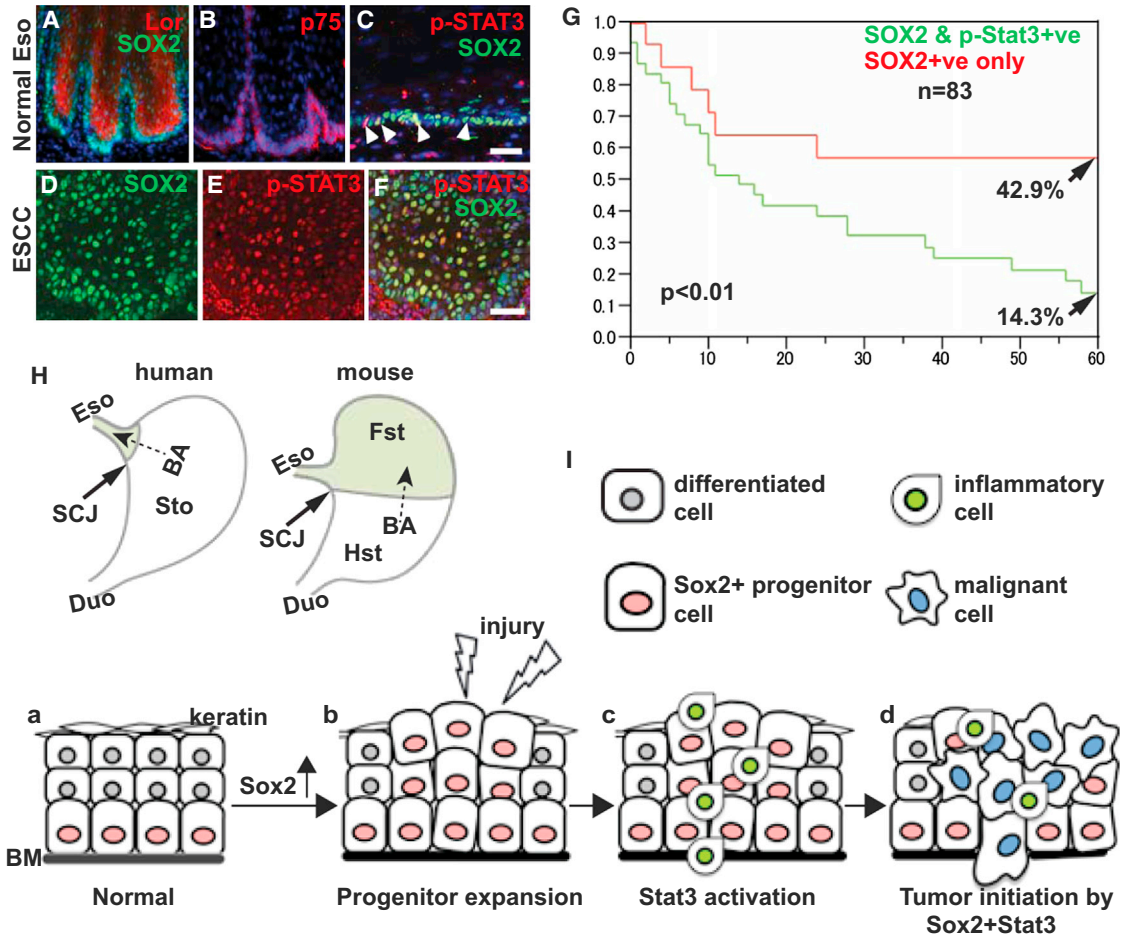

Figure 7. High Levels of SOX2 and p-STAT3 Are Associated with a Poor Prognosis of Human ESCC and Model Figure

(A) SOX2 is enriched in basal cells, and LORICRIN is expressed in differentiated suprabasal cells of human esophageal biopsies.

(B) Basal cells are labeled by p75.

(C) p-STAT3 is colocalized with SOX2 in ~8% of basal cells (arrowheads). Refer to Figure S5 for individual fluorescent channels.

(D–F) Representative ESCC sample that has high levels of SOX2 (D) and p-STAT3 (E). (F) shows overlapping view of the two individual fluorescent channels.

(G) High levels of p-STAT3 in SOX2+ve esophageal SCCs predict a poor 5 year survival rate. Overall survival curves were plotted according to the Kaplan-Meier method, and p value was calculated using log rank test.

(H) Schematic of the squamous-columnar junction that separates the squamous (green) and columnar (white) tissues in the human and mouse upper gastrointestinal tract. Bile acid can cross the junction and induce injuries under pathological conditions.

(I) Schematic in which Sox2 overexpression cooperates with inflammation-activated Stat3 to malignantly transform basal progenitor cells. (Ia) Sox2 is expressed in the basal progenitor cells of the normal esophagus and forestomach. (Ib) Sox2 overexpression expands basal progenitor cells by promoting proliferation and inhibiting differentiation. (Ic) Bile acid reflux induces injury and inflammation in the expanded Sox2+ve progenitor cells. (Id) Sox2 and inflammation-activated Stat3 cooperate to transform basal progenitor cells and initiate invasive esophageal SCC. Inhibition of inflammation or Stat3 signaling reduces tumor incidence. Note that the top keratin layer is absent in the human esophagus. Abbreviations: Lor, Loricrin; SCJ, squamous-columnar junction; BA, bile acid; Eso, esophagus; Fst, forestomach; Hst, hindstomach; Duo, duodenum; BM, basement membrane. Scale bar represents 50 μm. See also Figure S5 and Tables S5 and S6.

(See Figure S1G for lineage-tracing results. The details of this mouse line will be described elsewhere.) Similar to the findings by Arnold et al. (2011), ablation of basal cells disrupts tissue integrity of the esophagus and forestomach in the *Sox2-CreER; Rosa26*^{DTA} mice (Figure S1H), confirming that basal cells are stem/progenitor cells required for tissue maintenance.

Genomic amplification and protein overexpression of SOX2 has been identified in SCCs of several human tissues including the lung, oral cavity, and esophagus (Bass et al., 2009; Freier et al., 2010; Gen et al., 2010). We have provided direct evidence that Sox2 overexpression promotes the proliferation of basal progenitor cells in both the esophagus and forestomach. Notably, when Sox2 is overexpressed in the differentiated suprabasal cells of the esophagus and forestomach using the *Krt10-Cre* mouse line, the proliferation status in these cells remains unchanged and no tumor formation is observed (J.Q., unpublished data), suggesting that the ability to promote cell proliferation by Sox2 overexpression is specific to progenitor cells. This concept is further supported by our evidence that a combination of Sox2 and Stat3C overexpression can only transform esophageal basal progenitor cells but not the differentiated suprabasal cells (discussed below).

We found that increased levels of Sox2 are unable to transform progenitor cells in the esophagus, while tumor formation in the forestomach requires cooperation with inflammatory signaling that can be suppressed by Dexamethasone. In the mouse, the forestomach is in direct continuity with the hindstomach, which secretes acid, while the esophagus is protected by a sphincter. The acidic forestomach environment presumably injures the hyperplastic epithelium, which contains regions of undifferentiated progenitor cells and reduced levels of Loricrin after Sox2 overexpression. This is followed by inflammation and increased levels of cytokines IL-1β and IL-6. In the human, there is no keratinized forestomach expressing Sox2 and the esophagus is also protected by a sphincter (Figure 7H). However, bile acid may reach this tissue from the bile duct or stomach as a result of gastroesophageal reflux (Figure 7H), a disorder affecting 30% of normal populations. In either case, exposure to bile acid is associated with pathological changes and even malignancy. Although it is bile acid-mediated injury that is more commonly associated with the pathobiology of adenocarcinoma (Souza, 2010), recent studies suggest that acid reflux also increases the risk of esophageal SCC (Pandeya et al., 2010; Uno et al., 2011). Our previous study showed that acid refluxate disrupts epithelial integrity accompanied by Adam10-mediated E-Cadherin cleavage in human biopsies. Conditional deletion of *E-cadherin* in the mouse forestomach leads to a leaky epithelium and basal cell hyperplasia (Jovov et al., 2011). In addition, bile acid reflux precipitates inflammation and promotes the incidence of SCC after carcinogen treatment in the esophagus and forestomach in animal models that have undergone esophageoduodenal anastomosis surgery (Chen et al., 2007; Hao et al., 2009). Our current study provides further evidence that inflammatory signaling is required for malignant transformation of basal progenitor cells and that inhibition of inflammation with Dexamethasone reduces tumor incidence.

Stat3 is a key player in mediating inflammation-driven tumorigenesis in multiple gastrointestinal organs (Corcoran et al., 2011; Grivennikov et al., 2009; Quante et al., 2012; Stairs et al., 2011). Activation of Stat3 through inflammatory cytokines in mouse models has been implicated in the development of esophageal cancers, including SCC and adenocarcinoma (Quante et al., 2012; Stairs et al., 2011). However, there is no direct evidence that activated Stat3 drives esophageal tumor initiation. Overexpression of Stat3C with the *KRT5* promoter induces hyperproliferation of epidermal epithelium but not tumor formation in *KRT5-Stat3C* transgenic mice (Chan et al., 2008). Although Stat3C overexpression transforms immortalized mouse fibroblasts (Bromberg et al., 1999), we found that Stat3C overexpression alone is unable to transform mouse esophageal progenitors or immortalized human EPC2 esophageal progenitor-like cell line and requires the cooperation of Sox2 overexpression to generate tumorigenic cells. Sox2 and Stat3 are essential components of the core circuitry that regulates self-renewal of mouse embryonic stem cells. Studies combining chromatin immunoprecipitation (ChIP) with genomic sequencing demonstrate that Stat3 and Sox2 along with other pluripotency transcription factors are probably clustered in multiple transcription factor-binding loci (MTL), where they coregulate common targets (Chen et al., 2008). Our data also suggest that Sox2 and Stat3 cooperate in the transformation of basal progenitor cells and that disruption of this cooperation with a specific Stat3 inhibitor reduces tumor incidence.

In summary, we provide multiple lines of evidence supporting the idea that Sox2/Krt5/p75-positive basal progenitor cells in the esophagus and forestomach are able to self-renew and differentiate and that ectopic Sox2 overexpression promotes the expansion of the progenitor pool. Furthermore, Sox2 cooperates with inflammation signaling to transform progenitor cells and leads to the formation of SCC. In clinical samples, increased levels of SOX2 and p-STAT3 correlate with a poor 5 year survival rate. Perturbing *SOX2* and *STAT3* expression reduces the proliferation and survival of tumorigenic esophageal SCC cells both in vitro and in a xenograft model. Cooperative oncogenic lesions malignantly transform cells by modulating downstream gene expression and signaling circuitry (Hanahan and Weinberg, 2000; McMurray et al., 2008). Identifying these target genes in Sox2 and Stat3 co-overexpressing esophageal progenitor cells will provide further insights into the pathobiology and new therapeutic avenues for more effective treatment of esophageal cancer.

EXPERIMENTAL PROCEDURES

Cell Isolation for the Mouse Esophagus and Forestomach Tumor and FACS Analysis

Muscle layers were stripped off the mouse esophagus and the remaining tissue was incubated in Dispase (BD Biosciences, 16 U/ml) in PBS (30 min) at room temperature. Digestion was stopped by addition of Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS). Epithelium was peeled off with forceps, cut into small pieces, and incubated in 0.1% trypsin, 1.6 mM EDTA for 20 min at 37°C. Digestion was stopped with 5% FBS-containing medium followed by gentle pipetting and passage through a 40 μm cell strainer. Similarly, forestomach tumor tissues were cut into small pieces and digested with Trypsin but followed by passing through a syringe with 21G needle for three times and then by passing through a strainer. For FACS with p75 antibody, cells were diluted to 1×10^6 cells/ml in 2% FBS, 2% BSA in PBS, and incubated in 18 μg/ml rabbit anti-mouse p75 antibody (1:100) or IgG isotype control followed by washing and incubation in allophycocyanin-conjugated or Alexa Fluor 488 donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch Laboratories). Cells were then washed with DMEM + 2% BSA and propidium iodide added to a final concentration of 200 ng/ml before sorting. GFP-positive cells were also sorted from the esophagus and forestomach of the *Sox2-GFP* or *KRT5-CreER;Rosa26*$^{Sox2/Sox2}$ mice following the previously described protocol (Rock et al., 2009). Cell sorting was performed on FACS Vantage SE or FACS Aria II and data analyzed with FACS Diva (BD Biosciences). Cells were collected in DMEM with 2% BSA and cultured immediately.

Cell Culture and Sphere Culture

FACS-sorted mouse basal progenitor or forestomach tumor cells were plated onto rat type IV collagen (BD Biosciences)-coated tissue culture dishes in MTEC/Plus containing 20 ng/ml Egf and Fgf2 (Que et al., 2009). Human esophageal SCC cell line KYSE450 was kindly provided by Dr. Yutaka Shimada at Kyoto University and maintained in 10% FBS RPMI medium. The EPC2 cell line was hTERT immortalized with functionally intact p53 and p16 and maintained as previously described (Okawa et al., 2007).

FACS-sorted mouse esophageal, human EPC2, or KYSE450 cells were resuspended in MTEC/Plus containing 20 ng/ml Egf and Fgf2, mixed 1:1 with growth factor-reduced Matrigel (BD Biosciences), and pipetted into a 12-well 0.4 μm Transwell insert (Falcon). MTEC/Plus medium (1 ml) was added to the lower chamber and refreshed every other day. For esophageosphere-reforming assay, cell balls were collected and incubated in 0.25% Trypsine with EDTA (GIBCO) for 12 min at 37°C to dissociate into single cells and then replated in the matrix. For the formation of spheres using sorted

KRT5-CreER;Rosa26$^{Sox2/Sox2}$ forestomach tumor or KYSE450 cells, drug-selected scramble control, Sox2, or Stat3 knockdown cells were dispersed into single cells and cultured with same matrix and medium. All cultures were maintained at 37°C, 5% CO_2. For inducing differentiation, 5% FBS was added to MTEC medium from day 1 in culture. To use IL-6 to treat the esophageospheres formed by FACS-sorted Sox2-overexpressing esophageal or forestomach epithelium, we initially cultured cells for 8 days to form spheres, and then we maintained the culture for another 5 days in the presence of 10 ng/ml IL-6. Whole-mount fluorescent pictures were taken with Leica DMI3000 inverted microscope. Samples were fixed in 4% paraformaldehyde in PBS, embedded in 3% UltraPure low-melting point agarose and then paraffin, and were sectioned.

Statistical Analysis

Data are expressed as mean ± SD or mean ± SEM (for tumor weight). Differences between two samples were analyzed by Student's t test. p values of 0.05 or less were considered statistically significant. Differences among three or more groups were analyzed using ANOVA (SAS 9.2 version, SAS Institute).

SUPPLEMENTAL INFORMATION

Supplemental Information includes fives figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.01.007.

ACKNOWLEDGMENTS

This work was initiated in Dr. Brigid Hogan's laboratory at the Department of Cell Biology, Duke University. We are grateful to Dr. Brigid Hogan and Dr. Mark Noble, Dr. Hartmut Land, Dr. Craig Jordan, and Dr. Dirk Bohmann at the Department of Biomedical Genetics in the University of Rochester for helpful discussion and critical reading of the manuscript. We also wish to thank Ian Jacobs and other members of the Que laboratory for discussion and technical assistance. This work was supported by the following grants: NIDDK K99/R00 DK082650 (J.Q.), NCI U54 CA156735 (X.C.), NCI P01-CA098101 (and its Molecular Pathology/Imaging and Molecular Biology Core Facilities, A.R.), NCI U01-CA143056 (A.R.), and American Cancer Society (A.R.).

Received: May 11, 2012
Revised: December 5, 2012
Accepted: January 10, 2013
Published: March 7, 2013

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