Investigating the Role of Sox2 in Stomach Tissue Homeostasis and Cancer.

Investigating the role of Sox2 in stomach tissue homeostasis and cancer

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Abstract

The transcription factor Sox2 is essential for the establishment and maintenance of multiple stem cell populations and its coding region is amplified in certain carcinomas. However, Sox2's role in stomach homeostasis and cancer is poorly understood. In this thesis, I used mouse genetics to investigate the expression pattern and function of Sox2 in the adult stomach during normal tissue homeostasis and tumorigenesis. Using a genetic lineage tracing system, I found that Sox2 expression marks a gastric stem cell population capable of self-renewal and differentiation throughout the lifetime of a mouse, raising the key question of whether Sox2 itself is required for adult stomach function. Using a combination of novel mouse models, I examined the consequences of *Sox2* loss of function on stomach regeneration as well as the susceptibility of Sox2+ cells to transformation. Surprisingly, I found that Sox2 itself is dispensable during stomach homeostasis, although *Sox2*-expressing cells readily give rise to Wnt-driven adenomas. To gain insight into the molecular function of Sox2, I performed ChIP-Seq analysis which revealed that the majority of Sox2 targets in mouse gastric stem and progenitor cells are related to tissue-specific functions such as endoderm development, Wnt signaling and gastric cancer while only a small set of genes overlaps with targets occupied by Sox2 in other stem cell populations.

SOX2 has been described as an amplified oncogene in several types of human cancers derived from the foregut endoderm including lung and esophageal squamous cell carcinomas. Unexpectedly, I found that Sox2 loss enhances stomach tumor formation and organoid growth in an Apc/Wnt-depdendent adenoma mouse model. Using a reporter assay, I further showed that altered Sox2 levels modulate Tcf/Lef-dependent transcription, providing a molecular explanation for the observed proliferation phenotypes. In summary, my genetic and molecular studies offer insight into how Sox2 regulates stomach tissue homeostasis and cancer and evidence that Sox2's mode of action is context and tissue specific.

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Chapter 1

General Introduction

1.1 Stem cells: the work horses of tissue homeostasis

Adapted from (Sarkar and Hochedlinger, 2013)

Stem cells are characterized by the capacity to continuously self-renew and the potential to differentiate into one or more mature cellular lineages. They serve to form tissues and organs during mammalian development, and they maintain ongoing cellular turnover and provide regenerative capacity in certain adult tissues. One can distinguish between pluripotent embryonic stem cells (ESCs), which give rise to all embryonic lineages, and somatic stem cells, which give rise to one or more specialized lineages within the tissues they reside in. A stem cell's decision for self-renewal or differentiation is intrinsically controlled by the interplay of cell type-specific transcription factors and chromatin regulators. Although several such molecules have been implicated in stem cell biology over the last few years, the mechanistic modes of action of these molecules remain incompletely understood.

1.2 Sox2, a regulator of cell fate decisions and proliferation in stem cell biology and disease

Adapted from (Sarkar and Hochedlinger, 2013)

The Sox gene family

Research on the Sox gene family began with the seminal discovery of the mammalian testis-determining factor, *Sry* (Gubbay et al., 1990a; Gubbay et al., 1990b; Sinclair et al., 1990). Sry carries a characteristic high-mobility-group (HMG) domain that binds DNA in a sequence-specific manner. In general, proteins containing an HMG domain with 50% or higher amino acid similarity to the HMG domain of Sry are referred to as Sox proteins (Sry-related HMG box). So far, twenty different Sox genes have been discovered in mice and humans (Schepers et al., 2002). In addition, two Sox-like genes have been identified in the unicellular choanoflagellate *Monosiga brevicollis*, suggesting that the origin of Sox proteins predates multicellularity or

possibly marks the transition of unicellular to multicellular organisms (Guth and Wegner, 2008; King et al., 2008). Sox factors that share an HMG domain with more than 80% sequence identity are divided into different groups termed A to H (Table 1.1).

Table 1.1. Mammalian Sox2 factors and their subgroups.

Group	Sox Member
SoxA	Sry
SoxB1	Sox1, Sox2, Sox3
SoxB2	Sox14 , Sox21
SoxC	Sox4, Sox11, Sox12
SoxD	Sox5, Sox6, Sox13
SoxE	Sox8, Sox9, Sox10
SoxF	Sox7, Sox17. Sox18
SoxG	Sox15
SoxH	Sox30

Individual members within a group share biochemical properties and thus have overlapping functions (Wegner, 2010). In contrast, Sox factors from different groups have acquired distinct biological functions despite recognizing the same DNA consensus motif. Target gene selectivity by different Sox factors can be achieved through differential affinity for particular flanking sequences next to consensus *Sox* sites, homo- or heterodimerization among Sox proteins, posttranslational modifications of Sox factors, or interaction with other co-factors (Wegner, 2010). This molecular versatility may explain why the same Sox factor can play very different molecular and functional roles in distinct biological contexts. Sox2 is by far the most well studied Sox factor. I review Sox2's role in stem cell biology in the context of development, tissue homeostasis, reprogramming and cancer (Figure 1.1), with the intention of demonstrating that Sox2's function is diverse and dosage and context dependent.

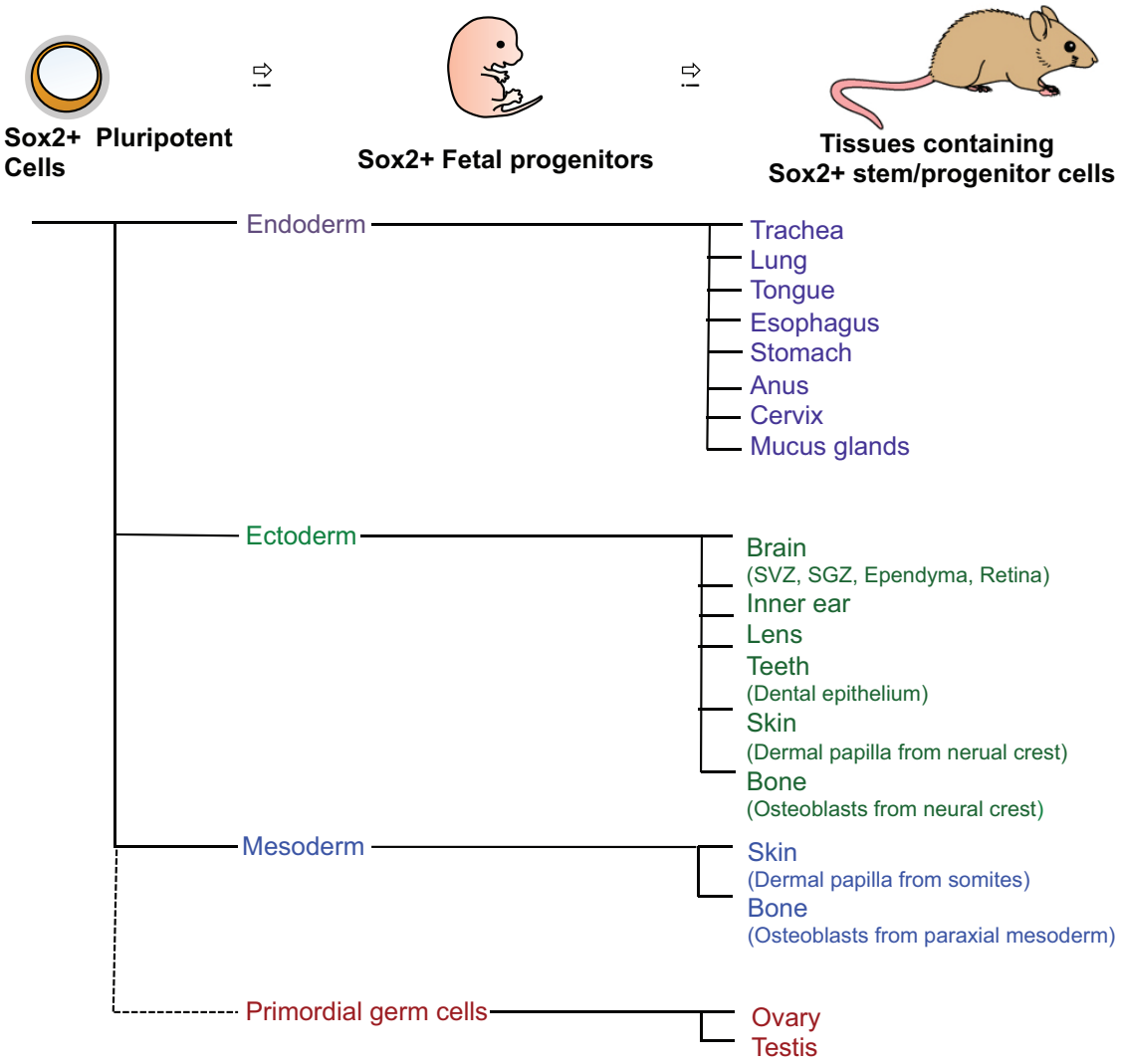


Figure 1.1. Sox2 expression in pluripotent, fetal and adult progenitor and stem cell. Sox2 is expressed throughout development, initially in pluripotent founder cells of the blastocyst and subsequently in endodermal, ectodermal and mesodermal progenitors as well as in primordial germ cells. Sox2 expression is maintained in fetal and adult tissues derived from Sox2+ fetal progenitor cells and marks stem/progenitor cells and in some cases also differentiated cells.

Sox2 in development

Sox2 plays a major role in lineage specification, morphogenesis, proliferation and differentiation in a variety of developing tissues of the fetus (Sarkar and Hochedlinger, 2013). Here, I review Sox2's role specifically in pre-implantation development and embryonic stem cells (ESCs), in nervous system development and neural progenitor cells (NPCs) and finally in endoderm development given, the relevance of these tissues and cell types to work discussed in following chapters of this dissertation.

Pre-implantation embyro

The formation of the trophectoderm (TE) and inner cell mass (ICM) within the blastocyst is the first lineage specification event in the mammalian embryo (Rossant and Tam, 2009). The ICM contains pluripotent founder cells, which give rise to all embryonic lineages, and a population of extra-embryonic endoderm (ExEn) cells that contribute to the yolk sac. Similarly, the TE contains a population of multipotent stem cells that form the extra-embryonic ectoderm and give rise to the placenta. Sox2 is initially present in both the ICM and the TE but is later confined to the ICM (Avilion et al., 2003). Zygotic deletion of *Sox2* results in early embryonic lethality due to a failure to form the pluripotent epiblast but leaves the TE unperturbed (Avilion et al., 2003). Interestingly, subsequent studies showed that maternal SOX2 protein persists in pre-implantation embryos, which might have masked a phenotype in the TE in zygotic *Sox2* mutants (Keramari et al., 2010). Indeed, depletion of both maternal and zygotic transcripts by RNAi causes an early arrest of embryos at the morula stage and a failure to form TE, suggesting that Sox2 is required for the segregation of the TE and ICM (Keramari et al., 2010). Consistent with its role in preimplantation development, *Sox2*-deficient embryos neither support the derivation of ESCs from the ICM, nor the derivation of trophoblast stem cells (TSCs) from the TE (Avilion et al., 2003). Furthermore, deletion of *Sox2* in already established ESCs results in their inappropriate differentiation into trophectoderm-like cells, indicating that Sox2 is also critical for the maintenance of ESCs (Masui et al., 2007).

Interestingly, Sox2's effect on self-renewal and differentiation of ESCs is highly dosage-dependent (Kopp et al., 2008), suggesting that its expression needs to be in equilibrium with other cofactors to maintain pluripotency. Supporting this concept is the observation that Sox2 acts cooperatively with other dosage-sensitive transcription factors, such as Oct4 and Nanog, to maintain the regulatory networks responsible for self-renewal and to repress differentiation programs in ESCs (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008; Orkin and Hochedlinger, 2011). Co-binding of these factors at targets associated with self-renewal

facilitates recruitment of the co-activator p300 and consequently transcriptional activation (Chen et al., 2008), whereas co-binding at developmental target genes causes gene silencing in concert with the repressive polycomb complex (Boyer et al., 2006). Notably, a large fraction of target genes bound by these factors contain composite *Oct4/Sox2* consensus binding sites (Masui et al., 2007; Tomioka et al., 2002), suggesting that SOX2 closely collaborates with OCT4 in order to efficiently bind to DNA and recruit other factors important for gene activation. In support of the notion that OCT4 and SOX2 jointly activate many targets is the finding that overexpression of Oct4 can partially compensate for the loss of *Sox2* (Masui et al., 2007).

Neural Development

After gastrulation of the embryo, Sox2 expression becomes largely restricted to the presumptive neuroectoderm, sensory placodes, brachial arches, gut endoderm and primordial germ cells (Avilion et al., 2003; Wood and Episkopou, 1999; Yabuta et al., 2006). Since *Sox2* deficiency causes early post-implantation lethality (Avilion et al., 2003), functional evidence for its role in the fetus has required analyses of hypomorphic and conditional mutants in *Xenopus*, chick and mouse embryos. These data have documented that the function of Sox2 is highly dosage and context dependent.

Sox2 continues to play major roles in the developing central (CNS) and peripheral nervous system (PNS) by controlling the proliferation and differentiation of fetal progenitor cells (Pevny and Nicolis; Wegner and Stolt, 2005). Sox2 expression overlaps and functions redundantly with that of other SoxB1 group factors, *Sox1* and *Sox3* in the CNS (Bylund et al., 2003; Graham et al., 2003; Wood and Episkopou, 1999). In general, overexpression of any of these SoxB1 factors promotes CNS progenitor cell proliferation, whereas depletion of these factors induces cell cycle exit and onset of differentiation (Bylund et al., 2003; Cavallaro et al., 2008; Ferri et al., 2004; Graham et al., 2003; Kishi et al., 2000; Miyagi et al., 2008). Likewise, Sox2 expression is essential for neural progenitor cell proliferation and differentiation in the retina, in part through its direct activation of the *Notch1* gene (Taranova et al., 2006).

Comparison of an allelic series of *Sox2* hypomorphic mice with conditional null mice further suggested that Sox2's effect on retinal progenitor cells (RPCs) is, like that on ESCs, highly dosage-dependent; RPCs lacking Sox2 expression lose the competence to proliferate and differentiate while reductions in Sox2 levels causes variable micropthalmia.

Surprisingly, Sox2 expression has also been reported to be important for the differentiation of subsets of neurons, indicating that its function is not always confined to the maintenance of progenitors and stem cells. For example, *Sox2* hypomorphic or knockout mice have reduced GABAergic interneurons in the newborn cortex and adult olfactory bulb (Cavallaro et al., 2008). Consistently, *Sox2* mutant NPC cultures generate beta-tubulin-positive neuronal-like cells that are poorly arborized and are negative for markers of mature neurons and GABAergic neurons (Cavallaro et al., 2008; Ferri et al., 2004). In an independent *in vitro* differentiation paradigm, Sox2 was shown to promote the maturation of migrating neural crest progenitor cells into sensory ganglia (Cimadamore et al., 2011). Collectively, these studies demonstrate that SoxB1 proteins play key roles in the development of the CNS and the PNS by controlling both the proliferation and differentiation of various progenitor cell populations. It will be important to define the mechanisms by which the same transcription factor regulates progenitor cell maintenance and differentiation within the same lineage.

Endoderm development

Sox2 plays multiple additional roles in organ specification of the foregut endoderm. *Sox2* is highly expressed in the anterior part of the foregut, giving rise to esophagus and forestomach. However, it is lowly expressed in the future trachea and posterior stomach, respectively (Que et al., 2007a). A severe decrease in Sox2 levels in hypomorphic embryos causes a transformation of esophagus into trachea, resulting in a failure to separate future trachea and esophagus (tracheoesophageal fistula) (Que et al., 2007a). Interestingly, Sox2 appears to play an independent role in defining the boundary between the keratinized forestomach/esophagus and the glandular hindstomach/intestine based on the observation that *Sox2* mutant esophagus and

forestomach exhibit histological and molecular signs of glandular stomach and intestine. Experiments regulating Sox2 dosage have further demonstrated that Sox2 is required for patterning and morphogenesis of the embryonic tongue into taste bud sensory cells (Okubo et al., 2006), branching and differentiation of primary lung bud into the lung (Gontan et al., 2008; Ishii et al., 1998) and proper differentiation of the tracheal cartilage (Que et al., 2009).

These experiments document an interesting commonality and difference in how Sox2 controls stem and progenitor cells in distinct developing tissues. A commonality among stem and progenitor cells of the retina, foregut-derived tissues and pluripotent cells is sensitivity to changes in Sox2 dose. This observation is consistent with the presence of cooperative and/or antagonistic factors whose function depends on finely tuned Sox2 levels and will be discussed below. A notable difference among these tissues is the effect *Sox2* deletion has on cell proliferation. While neural progenitors generally exit the cell cycle upon *Sox2* deletion, trachea, tongue and esophagus exhibit altered differentiation programs without changes in cell proliferation. Thus, Sox2 seems to control tissue formation in cell proliferation-dependent and independent ways that vary from tissue to tissue. Future studies of Sox2 targets in the respective cell types might give insights into the molecular mechanisms responsible for these different outcomes.

Sox2 in tissue homeostasis and regeneration

Accumulating data indicate that tissues that require Sox2 during development continue to express this factor in some adult stem and progenitor cells derived from that tissue. Below, I review the expression patterns and, where available, functional data linking Sox2 with adult stem and progenitor cells.

Using Sox2-GFP knock-in mice, Pevny and coworkers first demonstrated that *Sox2* is not only expressed in fetal neural progenitors, but also in proliferating cells in the adult CNS, specifically in neurogenic regions, such as the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus as well as the ependyma of the adult central canal

(Ellis et al., 2004). Isolated Sox2+ adult NPCs can be propagated in culture while maintaining their ability to differentiate into neurons, astrocytes and oligodendrocytes, thus documenting their self-renewal and multipotency *in vitro* (Ellis et al., 2004). The self-renewal and differentiation capacities of Sox2+ adult NPCs were verified *in vivo* by Fred Gage's group using lenti- and retroviral mediated fate mapping approaches (Suh et al., 2007). Support for a functional role of Sox2 in NPCs came from knockdown experiments *in vitro* (Cavallaro et al., 2008) and conditional deletion of *Sox2* specifically in the brain (Favaro et al., 2009; Ferri et al., 2004). These experiments revealed that Sox2 depletion in cultured NPCs attenuates their potential to form neurons whereas its absence *in vivo* causes a rapid loss of GFAP/Nestin-expressing stem/precursor cells and a decline in cell proliferation in the dentate gyrus, indicating that Sox2 marks and maintains NPCs and hence neurogenesis in the adult mouse hippocampus. Together these studies demonstrate that Sox2 regulates both developmental and adult stem cell populations in the brain.

Sox2 marks stem and progenitor cell populations in other adult tissues that depend on Sox2 expression during development. For example, Sox2+ cells have been detected in progenitors of the adult retina (Taranova et al., 2006), trachea (Que et al., 2009), tongue epithelium (Okubo et al., 2009) and dermal papilla of the hair follicle (Biernaskie et al., 2009; Driskell et al., 2009) as well as in putative progenitors of the pituitary gland (Fauquier et al., 2008). More recently, lineage tracing experiments from our lab and others have demonstrated that immature Sox2+ cells in the adult testes, forestomach, glandular stomach, trachea, anus, cervix, esophagus, lens and dental epithelium give rise to all mature cell types within these tissues (Arnold et al., 2011). Conditional *Sox2* deletion in all tracheal cells has further shown that postnatal expression of *Sox2* is required to sustain tracheal homeostasis by controlling the number of proliferating epithelial cells as well as the proportion of basal, ciliated and Clara cells. The effect of *Sox2* loss on tracheal cell proliferation thus represents an interesting difference compared with *Sox2* loss in the embryonic trachea which does not perturb proliferation (Que et

al., 2009). Deletion of *Sox2* specifically in bronchiolar Clara cells, which serve as facultative stem cells, also causes reduced cell proliferation and a gradual loss of differentiation markers for Clara, ciliated and mucous cells (Tompkins et al., 2009). This loss indicates that *Sox2* is required for the self-renewal of Clara cells and their differentiation into ciliated and mucous cells. From a molecular viewpoint, compromised bronchiolar cell proliferation might result from a de-repression of the SOX2 target gene *Smad3*, thus possibly activating the anti-proliferative Tgf-β pathway (Tompkins et al., 2009). An important question that remains to be determined is whether Sox2 expression is required for homeostasis in other Sox2+ adult tissues besides the airways and the brain.

In addition to maintaining tissue homeostasis, Sox2 is involved in tissue repair. For instance, chemically induced damage of the tracheal epithelium in mice is typically repaired within 7-10 days due to the activity of basal stem cells (Que et al., 2009). *Sox2*-deficient trachea, however, fail to undergo efficient tissue repair with severe reductions in the number of basal, ciliated and Clara cells. Peripheral nerve regeneration is another example for Sox2's role in tissue repair. Upon injury, mature adult Schwann cells re-express *Sox2*, shed their myelin sheaths and dedifferentiate to a progenitor cell-like state (Parrinello et al., 2010). Sox2 seems to play a direct role in this process by organizing Schwann cell clustering, a key event during nerve regeneration, through relocating N-Cadherin molecules (Parrinello et al., 2010). This process then enables Schwann cells to form multicellular cords to guide axon re-growth across the site of injury. It should be interesting to determine whether *Sox2* is reactivated and plays functional roles in other tissues experiencing cellular damage by promoting dedifferentiation into, or expansion of, resident progenitors.

Sox2 and disease

Sox2 deficiency in developmental disorders

SOX2 mutations have been identified in a number of developmental diseases and cancer. For example, humans carrying a heterozygous mutation for *SOX2* develop

Anophthalmia-Esophageal-Genital Syndrome (AEG). These patients have abnormalities in ectodermal and endodermal tissues including microphthalmia (small eyes), trachea-esophageal fistula, hearing loss, and brain abnormalities (Kelberman et al., 2006; Williamson et al., 2006). The heterozygous manifestation of disease in patients is consistent with the dose-dependent functions of Sox2 seen in mice. Surprisingly, however, heterozygous mutant mice are comparatively normal although they exhibit reduced pituitary size and hormone production as well as testicular atrophy and infertility with age, possibly from dose-dependent effects on pituitary and germ cell progenitors (Kelberman et al., 2006).

Sox2 dysregulation in cancer

Accumulating evidence suggests that SOX2 acts as an oncogene in some epithelial cancers. The *SOX2* locus is amplified in human squamous cell carcinomas of the lung (23%) and esophagus (15%) as well as in 27% of human small cell lung cancers analyzed (Bass et al., 2009a). Consistently, overexpression of Sox2 in the lungs and esophagus of mice induces rapid hyperproliferation (Liu et al., 2013; Rudin et al., 2012; Tompkins et al., 2011) and, in some cases, adenocarcinomas (Lu et al., 2010), although *SOX2* amplifications have not yet been described in human lung adenocarcinomas. While the function that Sox2 plays in tumorigenesis remains to be determined, recent evidence points towards pro-proliferative, pro-survial and/or anti-differentiation roles. For instance, knockdown of SOX2 in human cell lines, derived from squamous cell carcinomas and small cell lung cancer, compromises growth (Bass et al., 2009a; Rudin et al., 2012). Moreover, genetic reduction of *Sox2* levels by half in an animal model of pituitary cancer significantly reduces tumor formation (Li et al., 2012a) , and deletion of Sox2 in skin squamous carcinomas almost completely eliminated tumor initiation (Boumahdi et al., 2014). Lastly, Sox2 was shown to be critical for the proliferation and differentiation of human osteosarcoma cell lines *in vitro* and in an *in vivo* transplantation model by antagonizing WNT signaling. *SOX2* expression has also been suggested to contribute to cellular invasion in tumors of neural and neural crest origin such as glioma (Ikushima et al., 2009), melanoma (Laga et al.),

and Merkel cell carcinoma (Laga et al.), where it is overexpressed. Thus, analogous to its multiple roles in development and differentiation, Sox2 appears to function at various levels of carcinogenesis to promote tumor growth.

An important question is whether Sox2 is already expressed in the cell of origin for these tumors or whether it is activated ectopically. While it is plausible that tumors forming within Sox2+ tissues originate from a Sox2+ cell type (e.g., lungs, esophagus, neural cells, Merkel cells), unequivocal (genetic lineage tracing) evidence for this conclusion is lacking. Interestingly, two reports detected ectopic Sox2 expression in rare tumor stem cell-like populations isolated from genetically induced mouse models of squamous cell carcinoma of the skin (Beck et al., 2011; Schober and Fuchs). Another recent study demonstrated that transplantation of these ectopically Sox2 expressing cells into immunodeficient mice at limiting dilution numbers results in tumors that can be serially transplanted (Boumahdi et al., 2014), suggesting that Sox2 marks a cancer stem cell. This study also demonstrated that Sox2 and Sox2+ cells are important for the tumor propagation. Yet another study found that rare Sox2+ cells are responsible for tumor initiation and propagation in medulloblastomas (Vanner et al., 2014). Thus, Sox2 appears to have different roles in tumorigenesis and cancer maintenance in different contexts.

Mechanisms by which Sox2 controls cell fate decisions

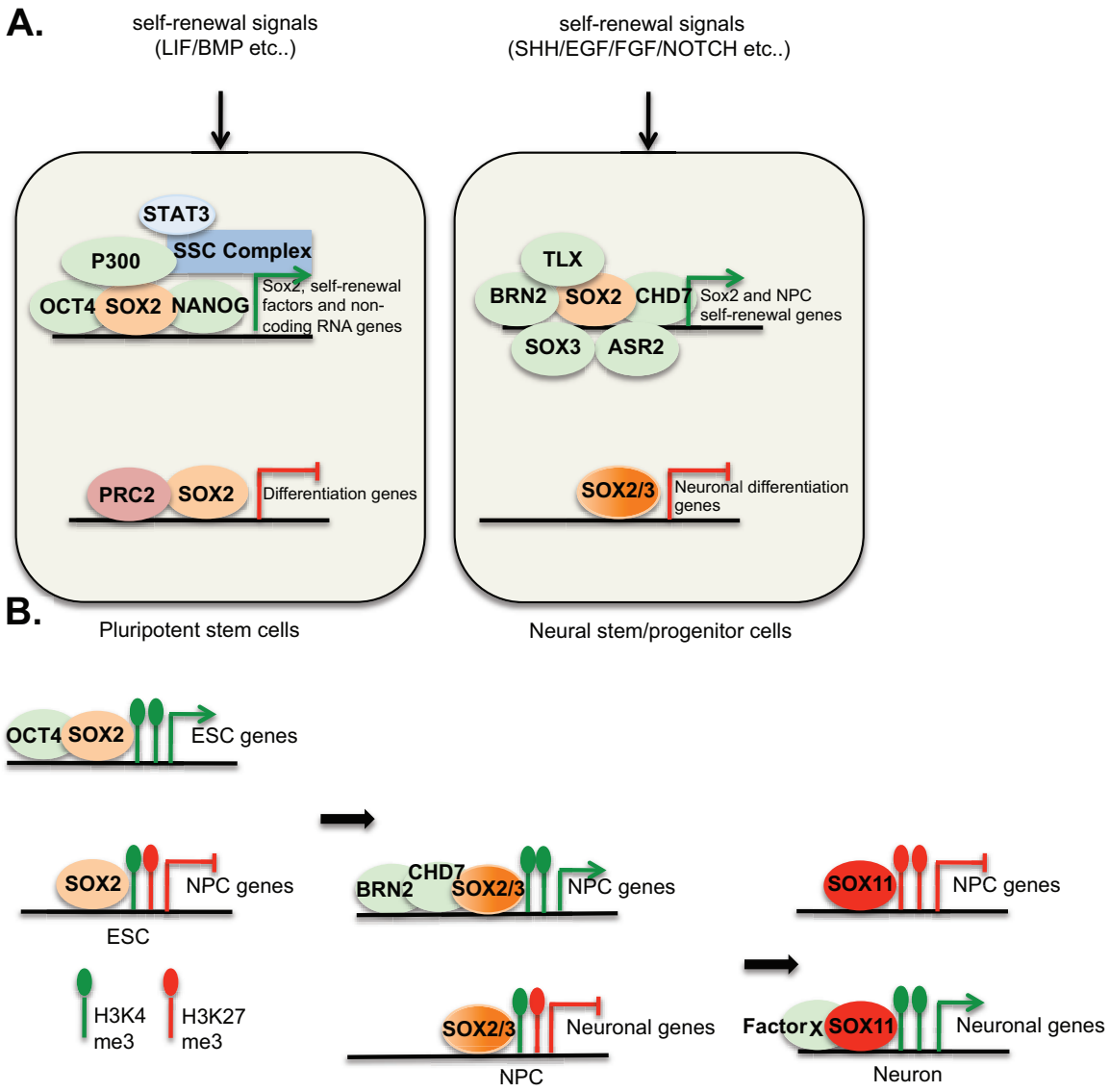


Figure 1.2. Mechanisms by which Sox2 controls self-renewal and differentiation in pluripotent and multipotent stem cells. (A) Sox2 activates self-renewal genes and represses differentiation genes in a cell type-specific manner by (i) interpreting tissue-specific signals and (ii) interacting with other cell type-specific cofactors. For example, in ESCs Sox2 occupies many targets containing Oct4-Sox2 consensus sequences and partners with downstream effectors of ESC-specific signaling pathways including Stat3 (LIF pathway). In NPCs, Sox2 occupies target genes that also contain binding sites for the brain-specific factors Brn2 and Chd7, thus activating different sets of genes. In addition, Sox2 activates its own transcription and regulates components of the signaling pathways that control self-renewal, thereby promoting maintenance of the undifferentiated state. **(B)** In addition to activating self-renewal genes and suppressing lineage-specific genes, Sox2 acts as a pioneer factor to prime stem cells for subsequent (gene activation. Sox2 occupies silent NPC genes in ESCs, which carry bivalent domains poised for gene activation. Upon differentiation into NPCs, Sox2 and Sox3 cooperate to activate self-renewal genes while keeping neuronal differentiation genes in a silent but bivalent state. When NPCs undergo terminal differentiation, Sox2 and Sox3 disengage from neuronal-specific enhancers and are replaced by Sox11.

Sox2 expression, like that of many other Sox factors, is modulated by extracellular signals and intracellular cofactors. Here, I review examples of how Sox2 expression can be positively or negatively regulated by different extracellular cues in different tissues and discuss intracellular mechanisms by which Sox2 expression is controlled in pluripotent and adult stem cells (Figure 1.2).

Extracellular regulators of Sox2 expression

Sox2 expression is positively and negatively influenced by different extracellular signals *in vivo* and *in vitro*. For instance, Fgf signaling from the surrounding ventral mesenchyme negatively regulates Sox2 expression during embryonic foregut patterning, resulting in a separation of esophagus and trachea (Que et al., 2007a). In the developing taste buds, Wnt signaling induces Sox2 expression in endodermal progenitors, causing their differentiation into taste bud cells at the expense of keratinocytes (Okubo et al., 2006). In calvarial osteoblast progenitors, however, *Sox2* is positively regulated by Fgf signaling. Upregulation of *Sox2*, in turn inhibits Wnt signaling by means of physical association of Sox2 with beta-catenin (Mansukhani et al., 2005).

In cultured pluripotent ESCs, Sox2 targets are co-occupied by Smad1 and Smad3 proteins, the downstream effectors of Tgf-β signaling that is essential for self-renewal (Chen et al., 2008; Mullen et al., 2011). Notably, one of the genes targeted by Oct4, Sox2 and Smad3 is *Lefty1*, the Tgf-β inhibitor, indicating that tight regulation of this pathway is necessary to maintain pluripotency. Similar to Tgf-β signaling in ESCs, Egf and Shh signaling stimulate Sox2 expression in NPCs (Favaro et al., 2009). Once activated, SOX2 binds to the *Egfr* and *Shh* genes amongst many other targets, thus engaging in positive feedback loops that are important for the maintenance of stem/progenitor cells (Engelen et al., 2011; Hu et al.). In agreement with this molecular link, *Sox2*-deficient NPCs fail to produce sufficient Shh, leading to loss of NPC cultures and dentate gyrus hypoplasia, respectively (Favaro et al., 2009). Remarkably, these phenotypes can be partially restored *in vitro* and *in vivo* by supplying recombinant Shh or an

Shh agonist (Favaro et al., 2009). A similar connection has been observed between Shh and Sox9 in NPCs (Scott et al., 2010). In contrast to Shh and Egf signaling, which promote Sox2 expression, thyroid hormone signaling induces differentiation of neural progenitors into neuroblasts by suppressing Sox2 expression (Lopez-Juarez et al., 2012). Specifically, thyroid receptor-alpha1 binds to a negative thyroid hormone response element within the *Sox2* enhancer, resulting in *Sox2* repression in a hormone-dependent fashion. Finally, Ephrin signaling causes Sox2 stabilization during Schwann cell regeneration, leading to N-Cadherin remodeling and subsequent Schwann cell clustering (Parrinello et al., 2010). In summary, these and several other examples (Adachi et al., 2013; Domyan et al.; Takemoto et al., 2006) demonstrate that major signaling pathways can positively or negatively control Sox2 expression levels during embryonic development, stem cell homeostasis and tissue regeneration in a context-dependent manner. Furthermore, Sox2 itself often modulates these signals by directly activating or repressing key regulators of these pathways.

Intracellular modulators of Sox2 expression in pluripotent stem cells

Once Sox2 is activated by extracellular signals, intracellular co-factors ensure that the proper set of target genes is activated in a cell-type specific fashion. One way to achieve this is to collaborate with other cell type-specific transcription factors. As discussed earlier, Sox2 physically associates with and co-occupies targets with other key pluripotency factors including Oct4 and Nanog in ESCs, thus contributing to target gene specificity. Of note, the combination and complexity of these pluripotency transcription factors at individual targets determines whether they will be activated or repressed. That is, targets bound by one or few transcription factors tend to be repressed whereas targets occupied by multiple factors tend to be expressed in ESCs (Kim et al., 2008; Sridharan et al., 2009). To ensure maintenance of the undifferentiated state of ESCs, Sox2 as well as other pluripotency factors engage in auto-regulatory loops to boost their own expression (Boyer et al., 2005).

In ESCs, Sox2 additionally requires binding of chromatin modifiers to induce expression of pluripotency-associated targets and repression of differentiation-associated targets. For example, Sox2, Oct4 and Nanog cooperate with WD repeat domain 5 (Wdr5), an effector of activating H3K4 methylation, to maintain robust expression of self-renewal genes in ESCs (Ang et al., 2011). Active Sox2 targets are also co-bound by components of the cohesion and mediator complex responsible for bridging enhancer and promoter elements to ensure efficient gene expression (Kagey et al.). Recent evidence suggests that Sox2 might even interact with ESC specific long non-coding RNAs (lncRNAs)(Ng et al.) to silence differentiation-associated genes in self-renewing ESCs.

During ESC differentiation, ESC-associated genes need to be rapidly downregulated, which is again achieved by multiple mechanisms. For example, the H3K4/K9 demethylase Lsd1 and HDACs1/2 silence active Oct4/Sox2-occupied enhancers in ESCs (Whyte et al.). Recent evidence further documents an unanticipated role for cell cycle inhibitors in transcriptional suppression of stem cell genes. The cell cycle dependent kinase inhibitor P27, which is rapidly activated as cells differentiate and thus exit the cell cycle, directly binds to and inhibits Sox2's SRR2 enhancer (Li et al., 2012a). In parallel with these transcriptional and epigenetic mechanisms, negative feedback loops kick in during differentiation that shut down the pluripotency program at the posttranscriptional level. This is exemplified by RNA miR-145, which is normally repressed by Oct4 in ESCs, and becomes activated to target Oct4, Sox2 and Klf4 RNAs for degradation when ESCs differentiate (Xu et al., 2009b). Thus, Sox2 interacts at the genic, transcript and protein levels with other core pluripotency factors, DNA repair complexes, cell cycle regulators, miRNAs, activating and repressive chromatin regulators to control specific gene expression programs that balance the decision between self-renewal and differentiation in pluripotent cells.

Intracellular modulators of Sox2 expression in adult stem cells

Similar to ESCs, Sox2 induces the expression of self-renewal pathways and inhibits the expression of differentiation genes in NPCs. Because *Oct4* and other pluripotency-associated genes are silenced in NPCs, Sox2 partners with different transcription factors to activate alternative targets. For example, Sox2 has been shown to interact with the brain-specific POU factor Brn2 to activate genes important for neural progenitors (Tanaka et al., 2004). More recently, the chromatin remodeling ATPase Chd7, which has been associated with CHARGE syndrome, was shown to physically interact and co-occupy targets with Sox2 in NPCs (Engelen et al., 2011; Lodato et al., 2013). Sox2 and Chd7 co-regulate a set of target genes of the Notch and Shh signaling pathways important for stem cell self-renewal. The nuclear receptor tailess (TLX) has been identified as another key target of Sox2 in NPCs. TLX functions as a transcriptional repressor that is important for NPC maintenance and neurogenesis in adult mice. Sox2 physically interacts with TLX and forms complexes on DNA, possibly to suppress differentiation genes (Shimozaki et al., 2012).

Sox2 expression itself is maintained in NPCs by direct transcriptional activation through Ars2, a zinc finger protein typically involved in miRNA biogenesis (Andreu-Agullo et al., 2012). Chromatin immunoprecipiation experiments have shown that Ars2, in a miRNA pathway-indepdendent manner, binds to the promoter region of *Sox2* and activates its expression. Ars2 deletion leads to a loss of NPC self-renewal and multipotency both in *in vitro* and *in vivo*. Importantly, this defect can be rescued by Sox2 overexpression (Andreu-Agullo et al., 2012). Similarly, the transcription factor myeloid ELF-1 like factor (MEF) binds to the *Sox2* locus and stimulates its expression in the context of neurospheres and glioma cells (Bazzoli et al.). Forced Sox2 expression also rescues the inability of MEF-/- cells to form neurospheres. In analogy to P27's inhibition of Sox2 expression during ESC differentiation, the cell cycle dependent kinase inhibitor P21 was shown to suppress Sox2 expression during NPC differentiation (Marques-Torrejon et al., 2013).

Posttranslational modifications, such as acetylation (Baltus et al., 2009; Sikorska et al., 2008), sumoylation (Tsuruzoe et al., 2006), phosphorylation (Jeong et al.) and arginine methylation (Zhao et al., 2011), have also been described to influence the transcriptional activity of Sox2 in ESCs or NPCs. In the case of Sox2, these modifications either cause transcriptional activation (phosphorylation, methylation) or repression (sumoylation, acetylation) by controlling Sox2's stability, nuclear-cytoplasmic localization or transactivation potential. Collectively, these results demonstrate that Sox2+ adult stem cells utilize some of the same as well as different mechanisms as ESCs to control the balance between self-renewal and differentiation. It is worth mentioning that Sox2 has been shown to collaborate with additional transcription factors in the development of other tissues. We refer to an excellent review exploring the various partners of Sox proteins for greater detail (Kondoh and Kamachi).

Sox proteins as pioneer factors

Pioneer factors are transcription factors that occupy silenced target genes in stem or progenitor cells and keep them in a poised state for activation at subsequent stages of differentiation (Zaret and Carroll). A classical example is the transcription factor Foxd3, essential for the maintenance of ESC self-renewal (Hanna et al., 2002). Foxd3 occupies the enhancer of the silent liver-specific *Alb1* gene in ESCs, thereby keeping it poised for activation upon differentiation into liver cells, when Foxa1 replaces Foxd3 to activate transcription (Xu et al., 2009a). Recent evidence exploring the genome-wide targets of different Sox factors during neural differentiation from ESCs supports the notion that Sox factors may also function as pioneer factors and thus contribute to differentiated cell fates (Bergsland et al., 2011). In ESCs, Sox2 binds to ESC specific enhancers, which are active and carry H3K4me3 marks, as well as to neural enhancers, which are silent and carry bivalent H3K4me3/H3K27me3 marks. Upon differentiation into NPCs, Sox2 collaborates with Sox3 to relocate from pluripotent to neural specific gene enhancers. These enhancers are either active in NPCs and hence carry the H3K4me3 mark or are inactive and carry bivalent marks. After neuronal differentiation, both

types of enhancers exchange their SoxB1 factors for SoxC factors, including Sox11. At the same time, previously active NPC enhancers acquire the repressive H3K27me3 mark, whereas the poised bivalent enhancers convert to a monovalent H3K4-enriched chromatin signature, resulting in gene activation. Further studies show that Sox2 can act as a pioneer factor during hematopoiesis and reprogramming(Sarkar and Hochedlinger, 2013).

Ectopic expression of Sox2 induces cellular reprogramming

Given that Sox factors play critical roles in establishing and maintaining cell types during development and in the adult, it is conceivable that their ectopic expression in heterologous cell types is sufficient to change cell fates. Indeed, Sox2 is one of the key reprogramming factors for the derivation of induced pluripotent stem cells (iPSCs) from somatic cells. Sox2 is required towards the end of reprogramming (Chen et al.; Sridharan et al., 2009), presumably by activating its own transcription as well as hundreds of pluripotency-associated targets to stabilize the pluripotent state. Sox2 expression alone or in combination with different neural transcription factors has also been reported to directly reprogram fibroblasts into neural stem cells (Han et al., 2012; Ring et al., 2012; Thier et al., 2012), suggesting that Sox2 can induce different cell fates depending on the presence of co-factors and environmental cues. Together, these findings underscore the powerful effects Sox factors have in endowing differentiated cells with immature stem cell-like properties.

Role of other Sox factors in stem cell biology

Many Sox factors act redundantly in the maintenance of stem cells (e.g., Sox1, Sox2, Sox3, Sox9 in NPCs), which may explain why certain Sox gene knockouts do not exhibit obvious phenotypes due to compensation by other Sox factors. The broad expression patterns and the partial redundancy of many Sox factors are thought to be the consequence of subfunctionalization and neofunctionalization of Sox genes resulting from an expansion of Sox

genes during vertebrate evolution (Guth and Wegner, 2008). The exact role of other Sox2 factors in stem cell biology is reviewed in (Sarkar and Hochedlinger, 2013).

Concluding Remarks

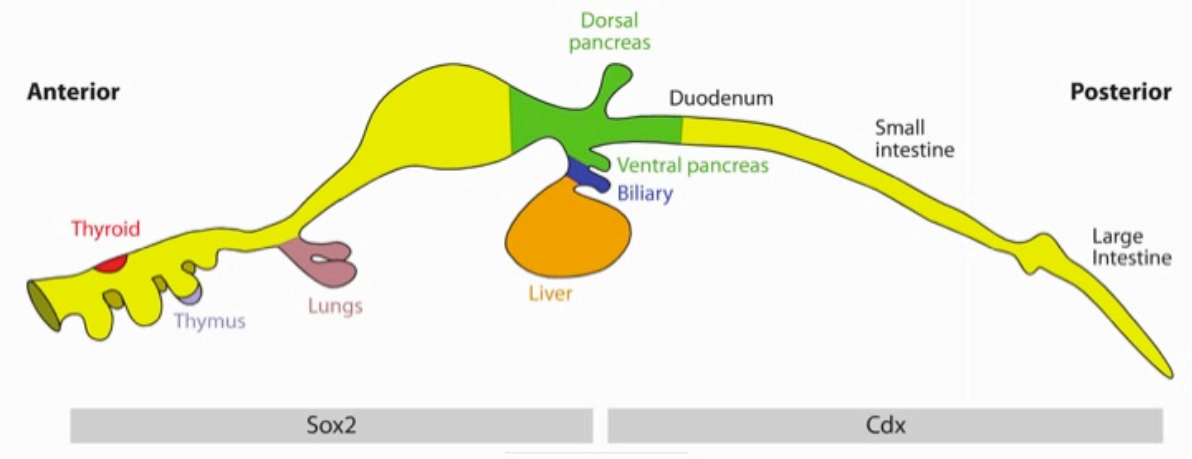
In summary, accumulating evidence implicates many Sox factors in pluripotent and multipotent stem cell biology and tissue regeneration. However, a better understanding of the mechanisms by which Sox factors induce and maintain stem cell populations should provide important insights into how tissue stem cells are generated and maintained and might lead to strategies to treat degenerative diseases or cancer affecting those tissues. Most insights into the biology of Sox factors have come from developmental studies. The finding that Sox factors are also expressed in numerous adult stem and progenitor cell populations raises interesting questions about the molecular and functional roles they play in tissue homeostasis and regeneration compared with their functions during development. In this dissertation, I describe the development and use of novel mouse models, organoid cultures and genome-wide technologies to address these fundamental questions at the mechanistic level.

1.3 The vertebrate stomach: a rapidly renewing tissue

Stomach development, in brief

In vertebrate development, the stomach forms from the foregut which develops by the end of gastrulation when endoderm is partitioned into anterior-posterior domains (Zorn and Wells, 2008). Little is known of how the foregut epithelium forms specific organ domains including the esophagus, trachea, lungs, thyroid, stomach, liver, pancreas and hepatobiliary systems (Zorn and Wells, 2008). However, evidence from global gene expression profiling from endoderm derivatives indicates that the transcription factors Sox2 and Cdx2 are important in defining the stomach and intestine domains (Sherwood et al., 2009) and suggests that Sox2 specifies the stomach and represses the intestine, while Cdx2 specifies the intestine and

represses the stomach. Support for this comes from studies of conditional *Cdx2* knockout mice during development, where loss of *Cdx2* results in a transformation of intestine towards stomach with concomitant ectopic Sox2 expression (Gao et al., 2009). Furthermore, studies of *Sox2* hypomorphic mutant mice show that dosage of Sox2 is important in establishing the boundary between the esophagus and stomach (Que et al., 2007b); *Sox2* hypomorphic mice show a transformation of keratinized forestomach into a columnar glandular-like stomach. Establishment of the Sox2/Cdx2 domains depends on production of Wnt-agonists from surrounding mesenchyme (Kim et al., 2005), which acts to locally inhibit Wnt signaling, repress Cdx2 in the epithelium and enable stomach differentiation (Figure 1.3). Thus, specification of the stomach seems to rely heavily on the localized and finely tuned expression of Sox2. Developmental events that follow stomach specification include specification of the different domains of the stomach and growth of pseudo-stratified epithelium into an elaborate glandular epithelium, and are detailed in Yasugi and Mizuno et al. (Yasugi and Mizuno, 2008). Interestingly, some of the developmental events described here can be recapituatled *in vitro* by



programming pluripotent stem cells to gastric derived organoids (McCracken et al., 2014).

Figure 1.3. Sox2 expression in foregut development. Scheme of foregut endoderm and organ primordia at ~E9.5 in mice. Sox2 and Cdx2 expression distinguishes the anterior from the posterior foregut, respectively. From Zorn and Wells (Zorn and Wells, 2008)

Stomach homeostasis

The stomach is an organ that undergoes continuous environmental assault from undigested food. The gastric epithelium, therefore, contains a stem cell population that replenishes lost or damaged cell types and maintains normal tissue homeostasis and function. The stomach can be subdivided into two domains harboring stem cell populations: the pylorus, close to the intestine, and the main body of stomach known as the corpus. Unlike humans, most vertebrate stomachs contain a third domain that resembles the squamous epithelium of the esophagus and functions mainly to store food. Stomach stem cells in the glandular stomach are thought to reside in numerous invaginations called gastric units. Individual gastric units are comprised of the pit, which is continuous with the surface epithelium, and the flask-shaped gland, which can be divided into isthmus, neck and base regions (Mills and Shivdasani, 2011). The four major functional cell types present in these glands are the gastric mucus cells secreting protective mucus, the parietal cells secreting hydrochloric acid and intrinsic factor, the chief cells with abundant zymogen granules secreting active pepsin and the enteroendocrine lineage containing several types of endocrine cells secreting hormones such as gastrin and somatostatin (Mills and Shivdasani, 2011). The precise architecture, cellular composition and turnover rate of gastric units vary markedly in the two major anatomical regions of the stomach. While, pylorus gastric glands are composed of three main cells types, mucus secreting cells, hormone-produce enteroendocrine cells and occasional parietal cells(Lee and Leblond, 1985), the corpus is comprised of longer gastric units with all four cell types described above (Karam and Leblond, 1992)(Figure 1.4). The cells in the pylorus turnover roughly every 60 days (Barker et al., 2010a); the cells in the corpus turn over every 194 days (Barker et al., 2010a; Karam, 1993; Lee and Leblond, 1985). For this reason, resident stem cells are needed to repeatedly replenish the epithelium.

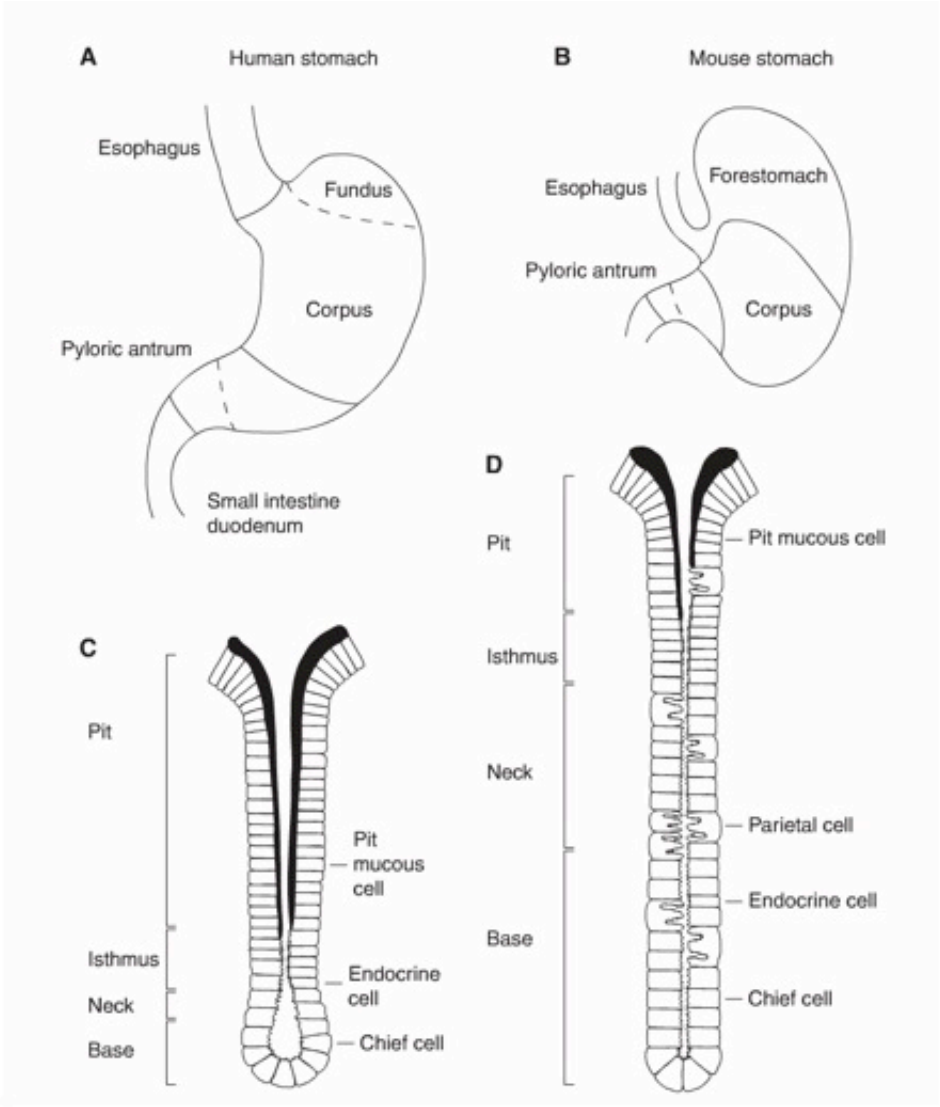


Figure 1.4. Architecture of the human and mouse stomach. (A) Human stomach. (B) Mouse stomach. (C) Gastric glands of the pyloric antrum. (D) Gastric glands of the corpus. Adapted from Arnout Schepers and Hans Clevers(Schepers and Clevers, 2012).

Previous approaches suggested the existence of adult stem cells in gastric units, which continuously replenish the various mature cell types in the epithelium. The first approach utilized was the use of ^{32}P-labeled nucleotides and bromodeoxyuridine incorporation assays in mouse stomach. The location of long-term ^{32}P-labeling in these assays suggested that the isthmus

region, found toward the upper third of the gastric units in the corpus and the lower third of units in the pylorus, was the site of cellular renewal (Karam and Leblond, 1992; Leblond et al., 1948). These studies signify that one or a few cells in the isthmus constantly regenerated cells that migrate bi-directionally up to the mucosal surface and down to the gland base as they differentiated into mature cells of the gastric unit. Furthermore, ultrastructural analyses of cells in the isthmus indicated that these cells are phenotypically undifferentiated, grandule-free proliferative cells in the isthmus region (Karam and Leblond, 1993). The second, more definitive approach utilized, was the use of in vivo lineage tracing: the permanent labeling and tracing of cells through time. Initial lineage tracing in the stomach utilized random mutagenesis of a ubiquitously expressed reporter allele (ROSA26-lacZ) in adult mice to demonstrate that rare single cells within the stomach epithelium give rise to entirely labeled glands over the long term (Bjerknes and Cheng, 2002). Subsequent experiments using gene-specific Cre drivers have demonstrated the existence of additional stem cell populations that replenish the epithelium either during normal homeostasis or injury. These include a population of cells marked by the expression of several genes encoding G protein-coupled receptors including *Lgr5* in the base of the glands in the pylorus (Barker et al., 2010b), *Cck2r* in the isthmus/+4 region of glands in the pylorus (Hayakawa et al., 2015), and *Troy* at the base of the glands in the corpus (Stange et al., 2013). These three populations can give rise to all mature cell types within their stomach domains, the corpus or the pylorus. Interestingly, both Cckr2 and Troy also mark differentiated cells, enteroendocrine and chief cells respectively. This observation raises the questions of whether expression of Cck2r and Troy mark both a stem cell population and a differentiated cell population or are Cck2r and Troy expressing cells stimulated to dedifferentiate to form an undifferentiated stem cell population. Indeed, when Troy-expressing cells are activated, they form more labeled glands when the proliferating isthmus compartment is depleted (Stange et al., 2013). Another cell population that gives rise to gastric glands, but only upon activation of an inflammatory response, is a Villin-expressing cell population, located just above the base and

below the isthmus in pylorus glands (Qiao et al., 2007). It remains to be determined whether Villin, Lgr5, and Cck2r mark the same or different stem cell populations in the pylorus and whether there is a common marker of stem cell populations in both the pylorus and corpus. In summary, these studies high-light the complexity of cell lineage relationships in the stomach and the usefulness of identification of a an endogenous marker for undifferentiated stem cell populations in both the pylorus and the corpus. I have used lineage tracing to show that Sox2 is a stem cell marker in both the pylorus and the corpus (discussed in Chapter 2). Furthermore, one would presume that dysregulation of these stem cells populations would lead to disruption of tissue homeostasis and the development of cancer. I will address this is the following section.

Stomach cancer

Stomach cancer is the third most common cause of cancer-related deaths worldwide(Stewart et al., 2014). In the United States, the five-year relative survival rate is 28% (Horner et al., 2009). Two reasons for this high mortality rate are that the disease progresses slowly and lacks symptoms, which lead to diagnosis at a stage too severe to cure the patient. Although survival rates improve to 64%, if the cancer is detected at early stages of the disease, less than 26% of gastric cancers are actually diagnosed at an early stage (Horner et al., 2009). Thus, an understanding of the etiology and pathogenesis is critical in stymieing the global fatality of gastric cancer. The majority of stomach cancers are adenocarcinomas, which can be subdivided into intestinal and diffuse types (Lauren, 1965), with the intestinal type being slightly more prevalent (Milne et al., 2009). Surprising, the sequence of events leading to stomach cancer is not fully understood. In intestinal type gastric adenocarcinomas, it is thought that Helicobacter pylori infection causes chronic inflammation of the mucosa, followed by gastric atrophy, intestinal metaplasia, dysplasia and finally adenocarcinoma (Correa, 1992; Smith et al., 2006). However, only 0.1% of individuals infected with H. Pylori will go on to develop gastric cancer (Parsonnet et al., 1997), highlighting the importance of other events in the initiation of stomach cancer. Significant progress has made in terms of cataloguing stomach cancer-specific

mutations through genome-wide sequencing efforts of human stomach adenocarcinomas (Cancer Genome Atlas Research, 2014). This has lead to the classification of four major genomic subtypes of gastric cancer including: Epstein-Barr Virus-infected tumors; tumors with microsatellite instability; genomically stable tumors; and chromosomally unstable tumors. These subtypes of tumors have distinct salient genomic features (summarized in Figure 1.5).

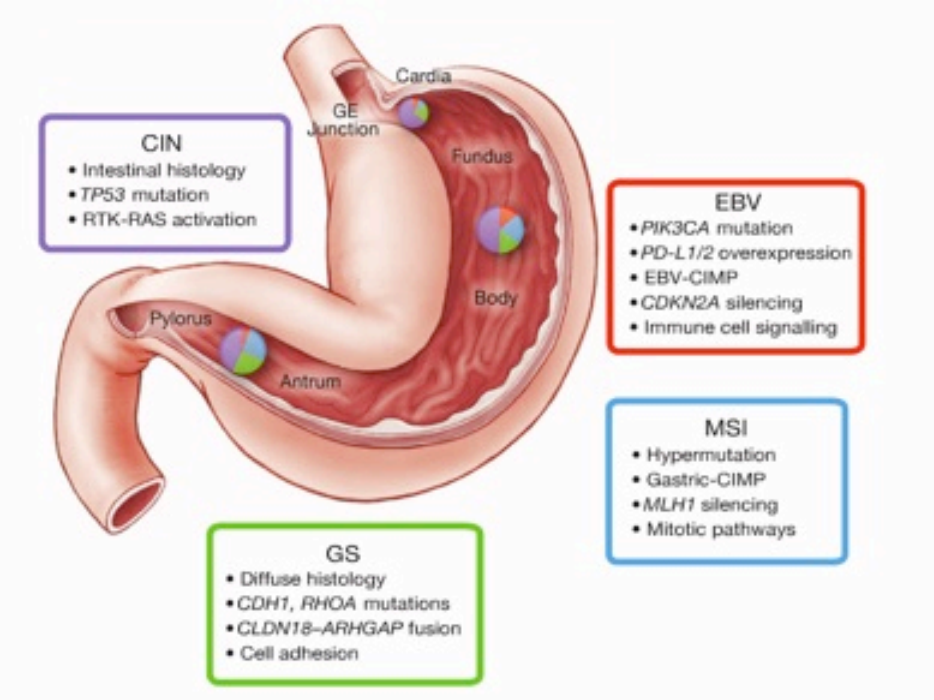


Figure 1.5. Key features of gastric cancer subtypes. This schematic lists some of the salient features associated with each of the four molecular subtypes of gastric cancer. Distribution of molecular subtypes in tumors obtained from distinct regions of the stomach is represented by inset charts (from Cancer Genome Atlas Research, 2014).

Although these advances have been made, there is still a lack of understanding of the functional roles of these stomach-cancer-specific mutations and the cell types in which they accrue during tumorigenesis remain elusive.

Given that stem cells are long-lived, it is conceivable that they may be a cell type of origin that accumulates a sufficient number of mutations to eventually lead to stomach cancer. Indeed, some preliminary studies in mouse suggest this is the case. Deletion of the tumor

suppressor *Apc* in Lgr5 expressing stomach cells gives rise to benign adenomas (Barker et al., 2010b). Furthermore, deletion of *Klf4* in Villin expressing cell gives rise to adenomas; however, only after treatment with the alkylating agent N-methyl-N-nitrosourea (Li et al., 2012b). The susceptibility of other specific cells types in the stomach to tumorigenesis has not yet been tested. Moreover, a progression of stomach adenomas to adenocarcinomas has not been observed. Accumulating evidence suggests that cancers of distinct subtypes within an organ may develop from different cells of origin which acquire the first genetic mutations that initiate cancer (Visvader, 2011). Thus, identifying the cells of origin for different cancer types would advance our understanding of the events needed to initiate different types of stomach tumorigenesis and could lead to early detection of malignancies, better prediction of tumor behavior and more targeted, cancer-type specific therapy.

Given that Sox2 is expressed in stem cell populations (see Chapter 2) in the stomach and is dysregulated in certain cancers derived from foregut tissues such as the lung and esophagus(Bass et al., 2009b), it is very plausible that *Sox2* plays a role in stomach cancer. However, evidence for this is controversial. Some studies claim that Sox2 suppresses stomach cancer. An immunohistochemistry screen for SOX2 in 203 pairs of human stomach tumors and paired tumorless tissues found that only 38% of the tumors contained SOX2 compared to 88% in paired tumorless tissues (Wang et al., 2015). Furthermore, knockdown of *SOX2* in several human stomach cell lines lead to cell growth inhibition (Otsubo et al., 2008; Wang et al., 2015). In contrast, other studies have found that human stomach tumors contain high levels of Sox2 and that Sox2 promotes cell growth in human stomach cell lines (Hütz et al., 2014; Matsuoka et al., 2012; Tian et al., 2014). Several reasons may account for these conflicting results. First, these studies did not histologically characterize the tumors they analyzed. Different types of tumors may arise from different cell types and thus may have different requirements for Sox2. Second, all functional studies of Sox2 were conducted in stomach cells lines that may have undergone genetic transformations foreign from the original tissue they were derived from. Third,

these studies did not test the genetic and molecular functions of Sox2 in an in vivo context. I address these issues and clarify the role of Sox2 in stomach cancer in Chapter 3 of this thesis.

Summary of unexplored questions in the stomach

Much progress has been made in understanding the cellular and molecular mechanisms that regulate stomach homeostasis and tumorigenesis. However there are still several unresolved questions remaining, including: 1) whether stem cells of both the corpus and pylorus can be identified and characterized by the same marker; 2) what roles do stem cells play in stomach cancer; 3) and are genes expressed in these stem cells important for the development of cancer. In this thesis, I use mouse models to study these questions.

1.4 Mouse genetics: a powerful tool to the study the functional relevance of Sox2

Genetically engineered mouse models have proven indispensable in addressing the functional importance of genes and specific cell populations in development, adult organs as well as cancers. Over the past few decades the investigation of stomach homeostasis and cancer has been, with a few exceptions, largely confined to descriptive histological rather than molecular and functional experiments. In this thesis, I use mouse models to functionally understand the roles that *Sox2* and Sox2+ stem cells play in stomach homeostasis and cancer (Figure 1.6).

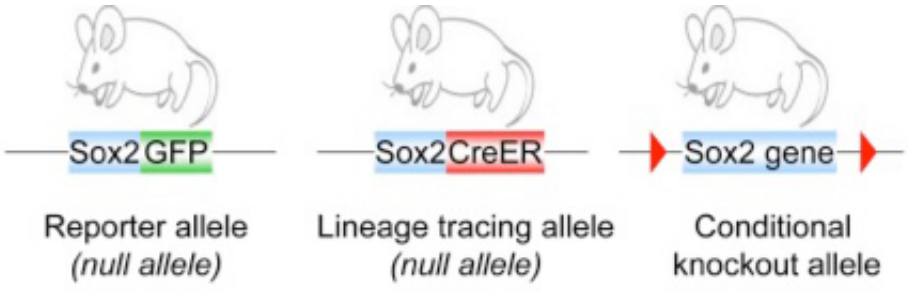


Figure 1.6. Overview of mouse models used to dissect the role of Sox2 in stomach homeostasis and cancer. (Courtesy of Konrad Hochedlinger)

Sox2 reporter mice

Reporter mice are models in which a target gene has been modified for the purpose of monitoring its promoter activity. This allows for the mapping and isolating of cells with promoter activity. In Chapter 2, I describe the use of Sox2 reporter mice, in which the green fluorescence protein (GFP) has replaced the coding sequence of *Sox2*, to identify and isolate Sox2+ stomach stem cells.

Sox2 conditional knockout mice

Conditional knockout mouse models allow for temporal and localized deletion of a gene of interest. In Chapter 3, I describe the use of a conditional knockout model for the *Sox2* gene (Sox2L/L) to delete Sox2 in stomach development, adult homeostasis and cancer.

Sox2 lineage tracing system

Lineage tracing models are powerful tools to trace and map the fate of cells and their progeny over time. To generate such a model, a hormone-inducible version of the Cre recombinase is typically expressed under the control of a promoter specific to a cell type. In Chapter 2, I describe the use of a tamoxifen-inducible Cre allele that has been knocked into the endogenous *Sox2* locus to trace Sox2+ cells and their progeny during development as well as in the adult. In Chapter 3, I demonstrate the use of this Sox2 lineage tracing system to introduce mutations specifically in Sox2+ cells and trace the effect of such mutations on the Sox2+ cell population on tissue homeostasis and on cancer.

1.6 Scope of this thesis

In this thesis, I use mouse models to define the role of Sox2 in stomach homeostasis

and tumorigenesis. In chapter 2, I use Sox2+ cell lineage tracing analyses to identify a long-term multipotent stem cell population marked by Sox2 in the two anatomically distinct regions of the glandular stomach. In chapter 3, I examine the consequences of Sox2 loss of function during stomach regeneration and tumorigenesis using novel mouse models. Surprisingly, I find that Sox2 is dispensable for normal stomach regeneration, and Sox2 acts to suppress stomach tumorigenesis when Wnt signaling is disrupted. In Chapter 3, I also examine the potential molecular function of Sox2 in the stomach through Sox2 ChIP studies, and find that Sox2 associates with gene loci distinct from other stem cells types, but specific to tissue specific loci such as endoderm development, Wnt signaling and cancer.

Altogether, my studies have elucidated the functional and molecular role that Sox2 plays in stomach stem and progenitor cells leading to important new insights into the biology of gastric regeneration and tumorigenesis and the mechanisms by which the same Sox factor may achieve differential target gene regulation in distinct stem cell populations. I expect that this data will inform future efforts to manipulate gastric stem cell populations in a regenerative setting and may lead to novel strategies to target malignant cells in gastric diseases.

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Chapter **2**

Sox2 marks stem and progenitor cells in the adult stomach.

2.1 Abstract

The transcription factor Sox2 maintains the pluripotency of early embryonic cells and regulates the formation of several epithelia during fetal development, including foregut epithelia that give rise to the adult stomach. Whether Sox2 continues to play a role in adult tissues remains largely unknown. We show here that Sox2 marks undifferentiated cells in the adult stomach. Genetic lineage tracing experiments demonstrate that Sox2-expressing cells continuously give rise to all mature cell types of the stomach, documenting their self-renewal and differentiation potentials. Developmental fate mapping reveals that Sox2+ adult stomach cells originate from fetal foregut Sox2+ tissue progenitors. Finally, we find that Sox2+ cells within gastric organoids are responsible for the propagation of these cultures. Thus, our results identify for the first time Sox2+ stomach stem cells, which regenerate the stomach epithelium and maintain stomach tissue homeostasis.

2.2 Contributing authors

Adapted from (Arnold et al., 2011)

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2.3 Author contributions

Candidate's contributions

Designed and performed experiments in figures 1.5, 1.6, 1.8 and 1.10. Prepared chapter.

Other Other's contributions

Assisted with figures 1.1, 1.2, 1.3, 1.4, 1.7 and 1.9 and histological interpretations.

2.4 Introduction

At the time of this study, it remained an unresolved question whether the self-renewal of pluripotent, fetal, and adult stem cells is controlled by the same or by different molecules. Recent findings had shown that expression of the polycomb group protein Bmi-1 (Sangiorgi and Capecchi, 2009), the HMG box transcription factor Sox9 (Furuyama et al., 2010), the telomere subunit Tert (Breault et al., 2008; Montgomery et al., 2011), and the G-protein coupled receptor Lgr5(Barker et al., 2010b; Barker et al., 2007; Jaks et al., 2008) mark various types of adult stem cells. While these observations indicated molecular commonalities among different somatic stem cells, these regulators are mostly absent in pluripotent stem cells. Another study documented that depletion of the zinc finger transcription factor Zfx1(Galan-Caridad et al., 2007) affects the self-renewal of both embryonic stem cells (ESCs) and hematopoietic stem cells, suggesting common mechanisms between pluripotent stem cells and this mesodermal stem cell type. However, *Zfx1* is also expressed in differentiated cells and its role in endodermal and ectodermal stem cell compartments has not yet been described. Lastly, several reports claimed that the pluripotency genes *Oct4* and *Nanog* are expressed in adult stem cells, but analyses of reporter animals and genetic knockout studies failed to confirm these findings(Lengner et al., 2007). In contrast, accumulating evidence suggested that Sox2, which maintains pluripotency in concert with Oct4 and Nanog, may play additional roles in fetal and adult progenitors.

Sox2 belongs to a large family of SRY related HMG box transcription factors that are important during development and cellular differentiation (Sarkar and Hochedlinger, 2013). *Sox2* is initially expressed in the inner cell mass (ICM) and extraembryonic ectoderm of blastocysts (Avilion et al., 2002). Embryos deficient for *Sox2* lack a pluripotent ICM and fail early in development, while deletion of *Sox2* in ESCs results in their inappropriate differentiation into trophectoderm-like cells (Masui et al., 2007). Forced expression of Sox2, in combination with

Oct4, Klf4, and c-Myc, endows somatic cells with pluripotency, giving rise to induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Collectively, these results underlined the importance of Sox2 in both the establishment and maintenance of pluripotent stem cells. Upon exit from pluripotency, Sox2 signaling is critical for the formation of several endodermal and ectodermal tissues during fetal development(Sarkar and Hochedlinger, 2013), including the anterior foregut endoderm which gives rise to distinct organ primordial, including those of the trachea, esophagus, stomach and lungs(Que et al., 2007).

Sox2 expression had been reported in some adult tissues(Sarkar and Hochedlinger, 2013), including the brain(Brazel et al., 2005; Ellis et al., 2003) and retina(Taranova et al., 2006), trachea(Que et al., 2009), tongue epithelium(Okubo et al., 2009), and dermal papilla of the hair follicle(Driskell et al., 2009) epithelium, as well as putative progenitors of the pituitary gland(Fauquier et al., 2008) . However, with the exception of neural stem cells(Suh et al., 2007), genetic fate mapping data to assess the lineage relationship between fetal and adult Sox2+ cells, as well as that of Sox2+ adult cells and their putative progeny, was missing.

Elegant work by Hogan and colleagues has shown that *Sox2* is highly expressed in the anterior part of the foregut, which gives rise to esophagus and forestomach, however *is* lowly expressed in the posterior stomach, which gives rise to the glandular stomach (Que et al., 2007). And although Sox2 expression is maintained in the trachea, esophagus, lungs and forestomach, it extinguishes in the distal stomach between embryonic days E16.5 and E18 (Que et al., 2007).

In this study, we have developed mouse models to (1) evaluate the Sox2 expression pattern and (2) determine the lineage relationship of Sox2+ cells. Surprisingly and in contrast to previous findings, we have identified Sox2+ cells that persist in the neonatal and adult stomach and maintain tissue homeostasis of adult stomach epithelium. In this chapter, I focus on our

studies in the glandular stomach. However, we have also identified a Sox2+ progenitors population in forestomach (Arnold et al., 2011).

2.5 Results

Sox2 is expressed in discrete cells of both the pylorus and corpus regions of the glandular stomach.

In order to systematically evaluate the expression patterns of Sox2 in adult tissues, we generated *Sox2*^{GFP} reporter mice from ESCs targeted with a previously characterized knockin construct (Ellis et al., 2004)(Figure 2.1A). Consistent with other reports (Avilion et al., 2003; Ellis et al., 2004), we detected strong GFP expression in blastocysts, neural progenitor cells (NPCs), and ESC cultures (Figure 2.1B), indicating that the knockin allele faithfully reports *Sox2* expression in previously documented Sox2+ cell types.

To detect *Sox2*^{GFP} reporter expression *in vivo*, we performed immunohistochemistry (IHC) for GFP on isolated, paraffin-embedded tissues. In contrast to previous findings, we identified Sox2GFP+ cells in gastric units of the glandular stomach, composed of the so-called anterior corpus and posterior pylorus compartments that have been suggested to contain stem cells (Bjerknes and Cheng, 2002; Karam and Leblond, 1993) (Figure 2.1D). No GFP signal was seen in liver, small intestine, colon, or pancreas, (data not shown). By FACS, we observed about 2% of all cells in the glandular stomach to be Sox2-GFP positive (Figure 2.1C). These Sox2-GFP+ cells were located both in the neck/isthmus region of glandular stomach stained with an antibody against GFP (Figure 2.1D). Single Sox2-GFP+ cells were also detected within the same neck/isthmus region of gastric units in the glandular stomach stained with an antibody against Sox2 (Figure 2.1D), demonstrating that both Sox2 mRNA and protein are produced. The frequency of Sox2-GFP+ cells is about 1-2 cells per gastric unit (data not shown). Together, our

results indicate that *Sox2* is expressed in a cell population in both the pylorus and corpus sections of the glandular stomach.

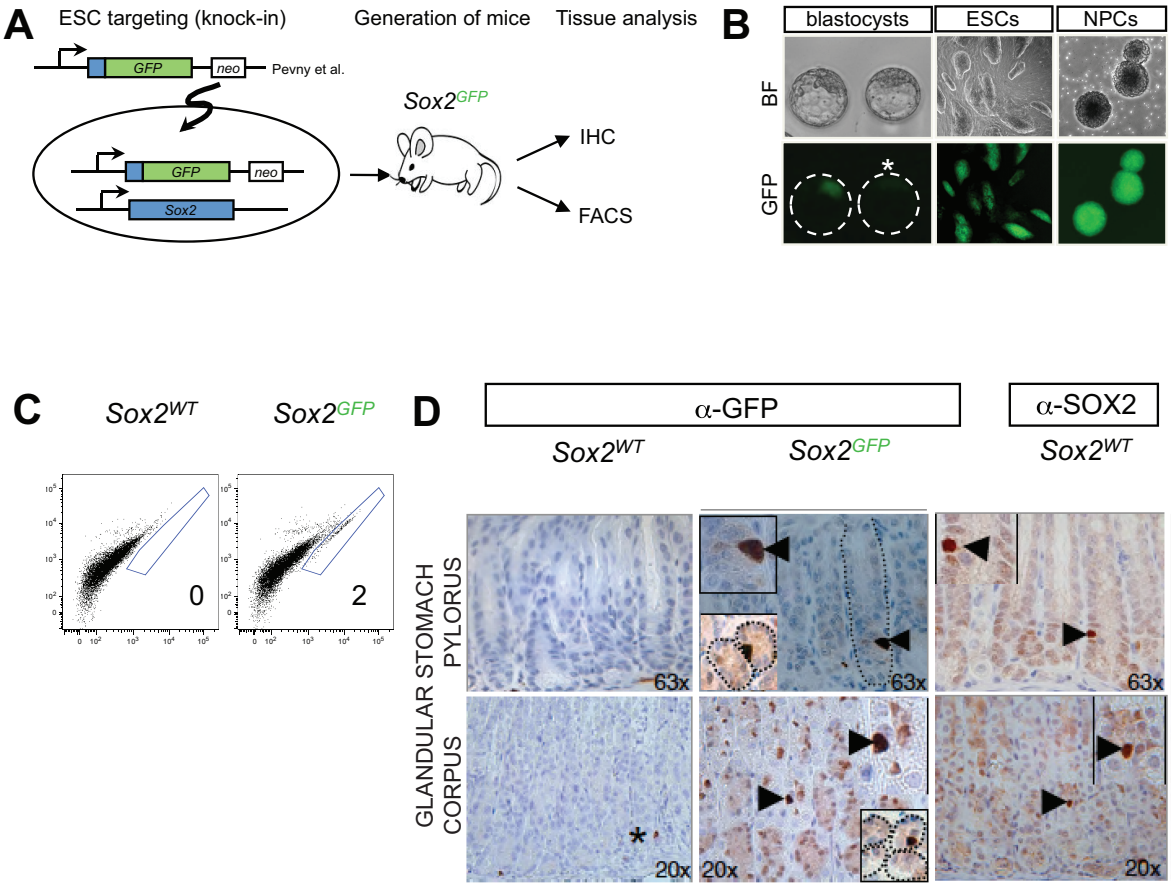

Figure 2.1. Sox2 expression in the glandular stomach. (A) Experimental outline for the generation and analysis of *Sox2*^{GFP} reporter mice. (B) *Sox2*^{GFP} expression in targeted embryonic stem cells (ESCs), the inner cell mass of blastocysts (asterisk marks GFP-negative control blastocyst), and neonatal neural progenitor cells (NPCs). BF, brightfield. (C) FACS analyses of *Sox2*^{GFP} mice and their wild type (*Sox2*^{WT}) littermate controls show GFP positive cells in the glandular stomach. (D) Immunohistochemistry (IHC) for GFP and Sox2 on glandular stomach.

Sox2 marks bona fide adult stem cells in the glandular stomach

To test whether Sox2-expressing cells do in fact contain adult stem cells, we devised a genetic lineage tracing approach. In brief, we engineered ESCs to harbor a tamoxifen (TAM)-inducible Cre allele (CreER-T2) in the endogenous *Sox2* locus (Figures 2.2A,B). Additional targeting of a ROSA26-lox- STOP-lox (lsl)-EYFP reporter allele(Srinivas et al., 2000) into these cells and subsequent exposure to the TAM analog 4-OHT gave rise to around 20% EYFP+ cells

in treated, but not in untreated, ESC populations, thus validating the inducibility and specificity of the system (Figure 2.2C). Mice were generated from these ESCs to establish a stable colony of *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ animals for further analysis. Untreated *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ mice and mice treated with solvent (corn oil) alone showed extremely rare and thus negligible spontaneous labeling events in the stomach (data not shown).

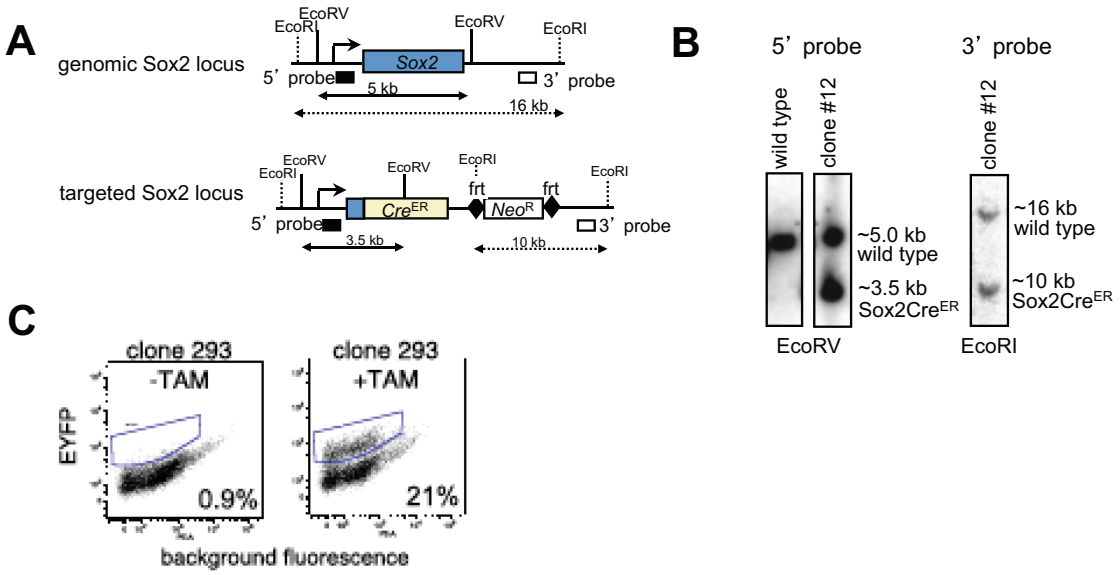

Figure 2.2. Sox2 genetic lineage tracing system. (A) Targeting strategy for generating *Sox2*^{CreER} ESCs and mice. Restriction sites, southern blot analysis probes and expected restriction fragment lengths are indicated. (B) Southern blot analyses with 5' and 3' probes, as indicated in (A), to verify correct targeting. (C) FACS analysis for EYFP expression on ESCs, carrying the *Sox2*^{CreER} and *ROSA26*$^{lsl\text{-}EYFP}$ alleles, 2 weeks after exposure to 4-OHT.

In order to permanently label Sox2-expressing cells and their progeny in vivo, we initially injected TAM intraperitoneally on 4 consecutive days into a cohort of 3- to 6-week-old *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ mice (Figure 2.3A). Tissues were isolated immediately after the treatment (''pulse'') or at different time points thereafter (''chase''). If Sox2 expression marks stem cells, we would expect to find permanent labeling of both the putative Sox2+ stem cells and differentiated progeny (Figure 2.3B). However, if Sox2+ cells are short-lived progenitors, we would expect to see transient labeling of cells, which should disappear over time due to their replenishment by unlabeled stem cells.

In the stomach, analysis of Sox2-CreER; ROSA26-lsl-EYFP lineage tracing mice immediately after the 4 day administration of TAM showed the appearance of individual dispersed EYFP+ cells as well as small patches of EYFP+ cells, consistent with the notion that some rare Sox2+ cells had expanded over the 4 day pulse period (Figure 2.3C, middle rows). Importantly, 15–22 months after the pulse, entire glands were fully labeled in both the corpus and pylorus, suggesting that Sox2-expressing cells can self-renew and give rise to the mature cell types of the glandular stomach (Figure 2.3C, right rows). It is worth mentioning that fully labeled corpus glands were observed less frequently than fully labeled pyloric glands at all time points and especially soon after the pulse (1–3 months), in agreement with a reported slower turnover rate of corpus cells (3–194 days) compared with pyloric cells (1–60 days) (Barker et al., 2010a).

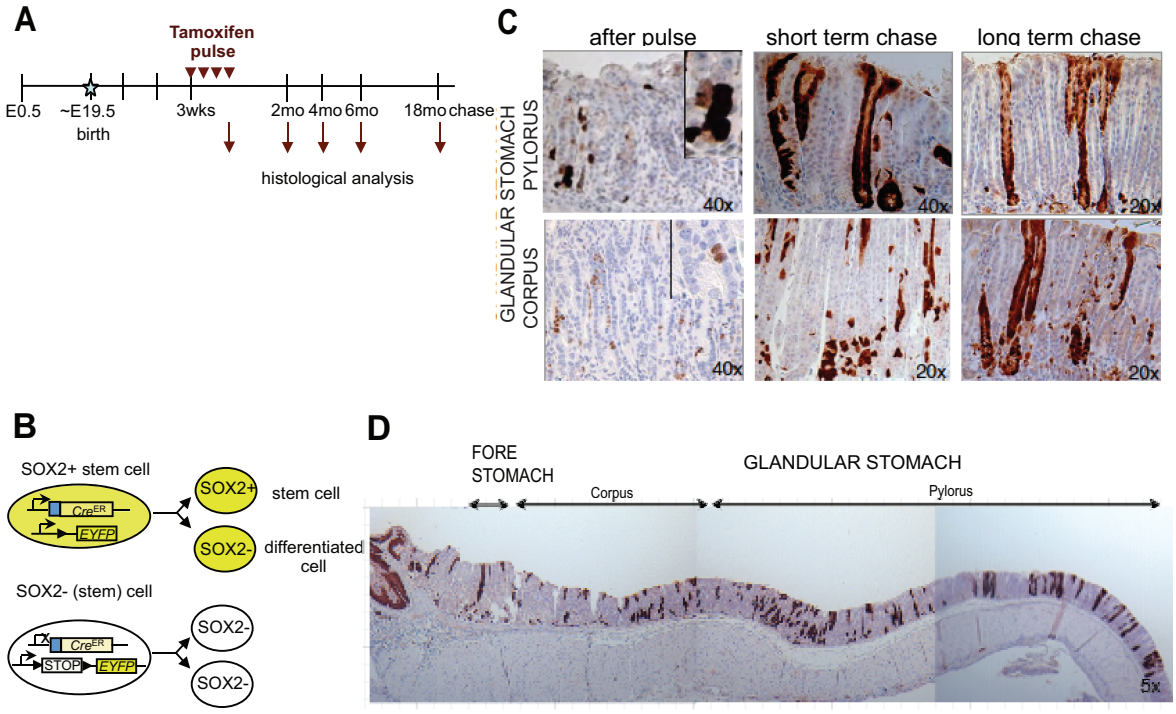

Figure 2.3. **Lineage tracing analysis of Sox2 expressing cells in the glandular stomach in young adult mice.** (A) Experimental outline for pulse-chase experiment. Tamoxifen (TAM) was given intraperitoneally on four consecutive days to a cohort of *Sox2*^{CreER}, *ROSA26*$^{lsl\text{-}EYFP}$ mice ("pulse"). Animals were sacrificed at the indicated time points and tissues analyzed by FACS and IHC for EYFP. E, embryonic day; mo, months. (B) Possible outcomes of lineage tracing assay. If cells that express Sox2

Figure 2.3. (Continued) are stem cells, they should activate the EYFP reporter upon TAM administration and give rise to permanently labeled Sox2+ stem cells as well as differentiated progeny. Sox2-negative cells will remain unlabeled. (C) IHC for EYFP, detected with an α-YFP antibody. Note individual cells or small patches of EYFP+ cells immediately after the pulse, which gradually expand over 1 and 6 months. (D) EYFP staining of stomach epithelium from adult *Sox2*^{CreER}*;Rosa26*$^{lsl\text{-}EYFP}$ mice induced with TAM and analyzed 5 months later. Note permanent labeling of gastric units in both the pyloric and corpus regions of the stomach.

To exclude the possibility that the observed labeling patterns were due to marking of transient postnatal progenitor cells, we repeated pulse-chase experiments on 6-month-old mice and analyzed tissue sections 12 months later. Consistent with the prior results, we detected fully labeled EYFP+ tissue clones glandular stomach (Figure 2.4). However, we noticed a lower frequency of labeling in older mice compared with that in young mice, due to a decrease in Sox2+ stem cells, reduced efficiency of the lineage tracing system with age, or both.

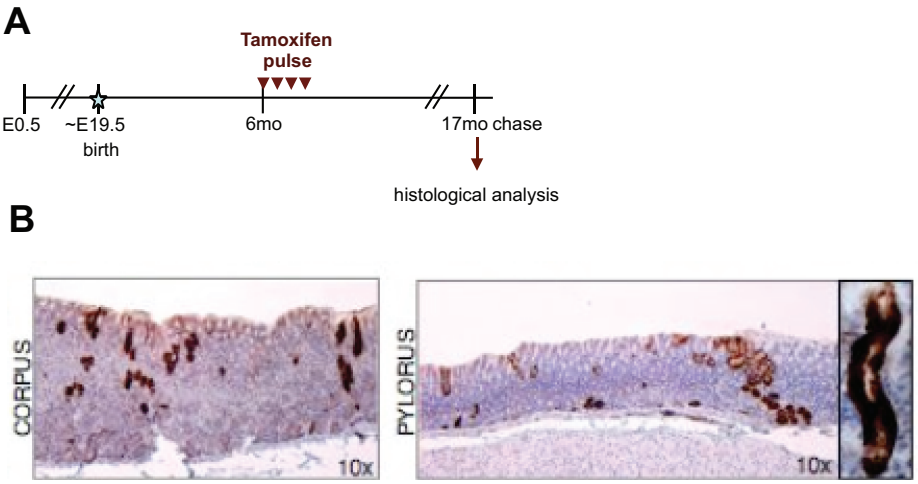

Figure 2.4. Lineage tracing analysis of Sox2 expressing cells in the glandular stomach of old adult mice. . (A) Experimental outline for pulse-chase experiment. (B) IHC for EYFP on glandular stomach sections of 17 month-old *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ mice 12-month after a 4-day TAM pulse given at 6 months of age.

Sox2+ stomach stem cells are multipotent and self-renew

Gastric glands of the stomach are thought to contain multipotent stem cells that can, depending on the region, produce three or four common cell types throughout life (Mills and Shivdasani, 2011).These are the so-called mucus cells, parietal cells, enteroendocrine cells,

and chief cells, with the latter cell type only found in the corpus. To further characterize Sox2+ stomach cells and their differentiated progeny, we first performed double labeling experiments with antibodies recognizing the major differentiated cell types of the stomach.

This analysis revealed that Sox2-GFP+ cells were negative for all tested differentiation markers in pylorus and corpus (Figure 2.5), supporting the notion that they are uncommitted stem cells.

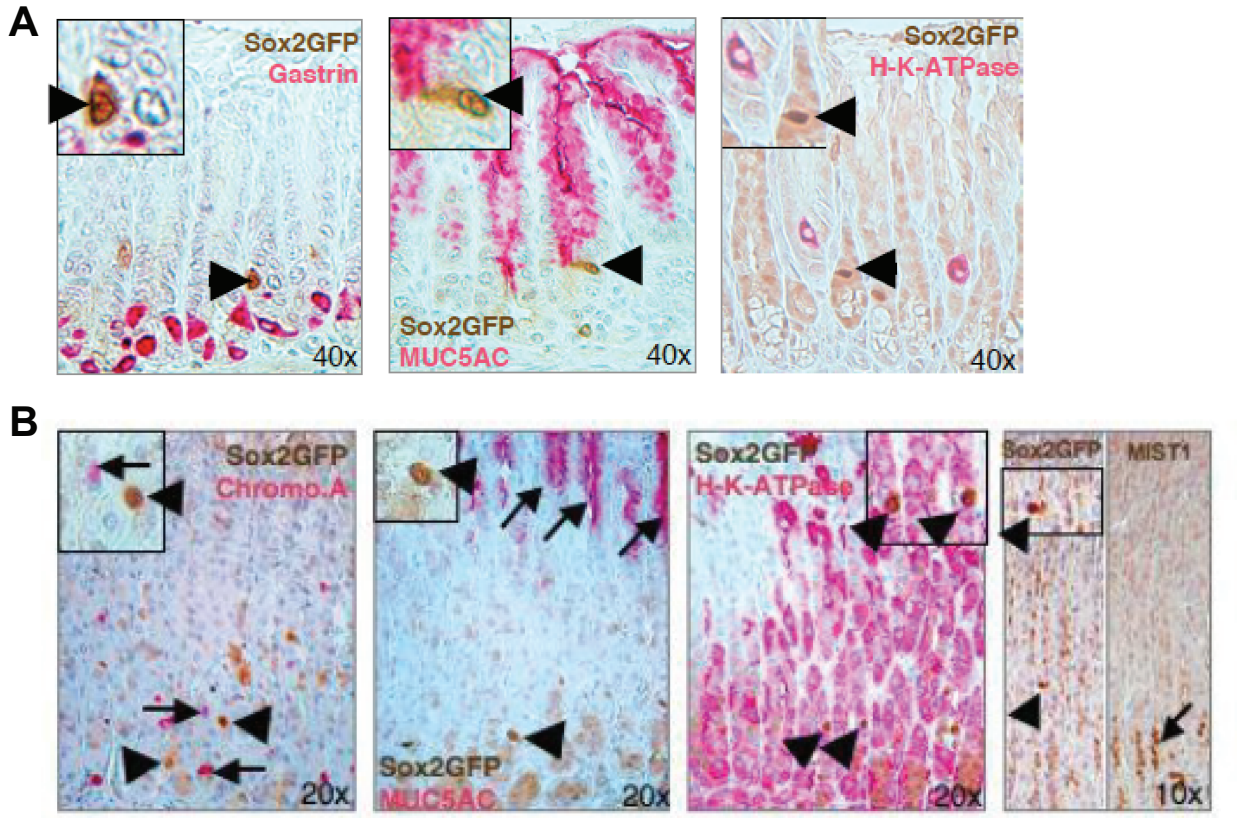

Figure 2.5. **Sox2GFP+ cells mark un-differentiated cells in gastric glands of the glandular stomach.** Co-staining for GFP and gastric markers on pylorus sections(A) and corpus sections from *Sox2*^{GFP} mice (B). Sox2-GFP+ cells (arrowheads) do not express differentiated cell markers gastrin (enteroendocrine cells, left); MUC5AC (mucus cells, middle); H-K-ATPase (parietal cells, right); MIST1(Chief cells, right).

In contrast, analysis of EYFP+ glands descending from lineage-traced Sox2+ cells 14 months after the pulse showed costaining of EYFP+ cells with markers of enteroendocrine cells, mucus cells, parietal cells, and chief cells (Figure 2.6 and Figure 2.7).

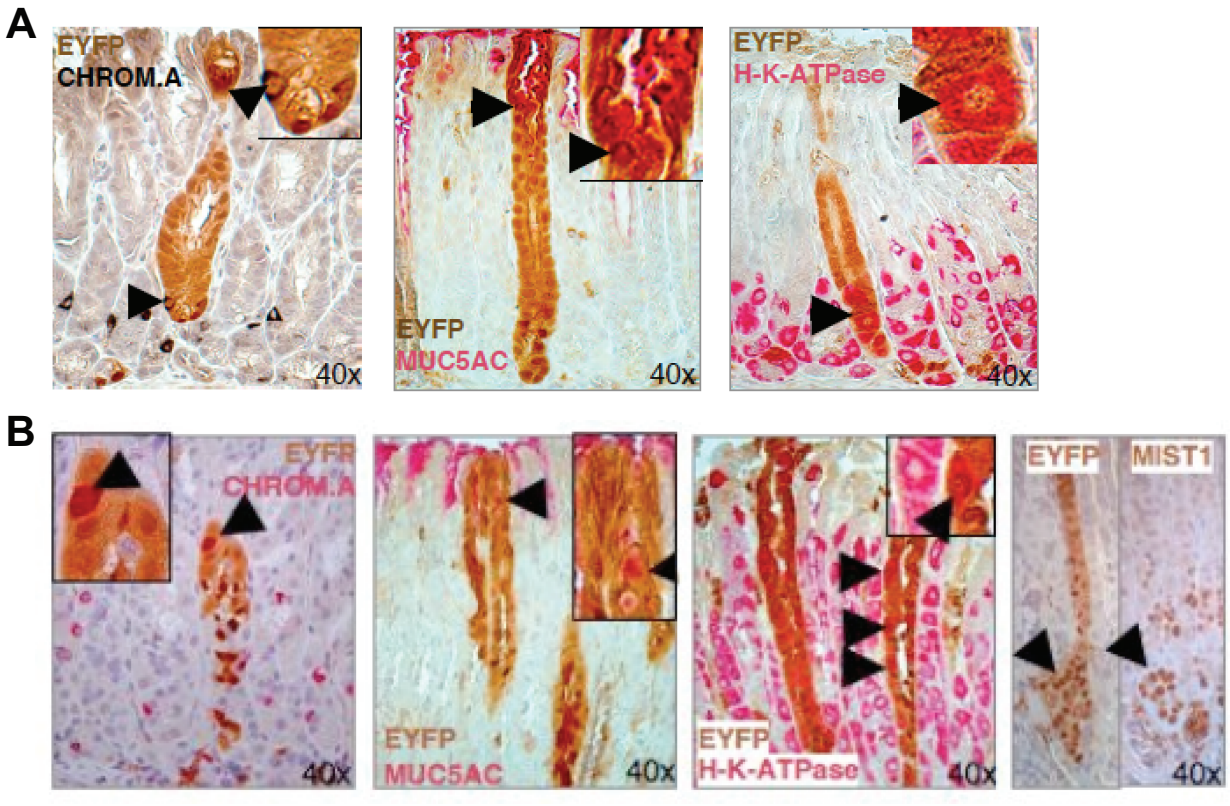

Figure 2.6. Self-renewal and multipotency of Sox2+ stomach stem cells. Co-staining of lineage tracing sections of *Sox2*^{CreER}*; Rosa26*$^{lsl\text{-}EYFP}$ mice 14 month after the TAM pulse with differentiation markers on the pylorus(A) and corpus (B). Co-localization (arrowheads) was detected between EYFP+ cells and enteroendocrine cells (Chromogranin A), mucus cells (MUC5AC), parietal cells (H-K-ATPase) and corpus specific chief cells (Mist1).

These data corroborate the conclusion that Sox2+ cells are multipotent stem cells in both the pylorus and corpus of the glandular stomach. Our observation that entirely labeled glands were still detectable up to 460 days after the pulse indicates that Sox2+ stem cells have self-renewal potential (Figures 2.7).

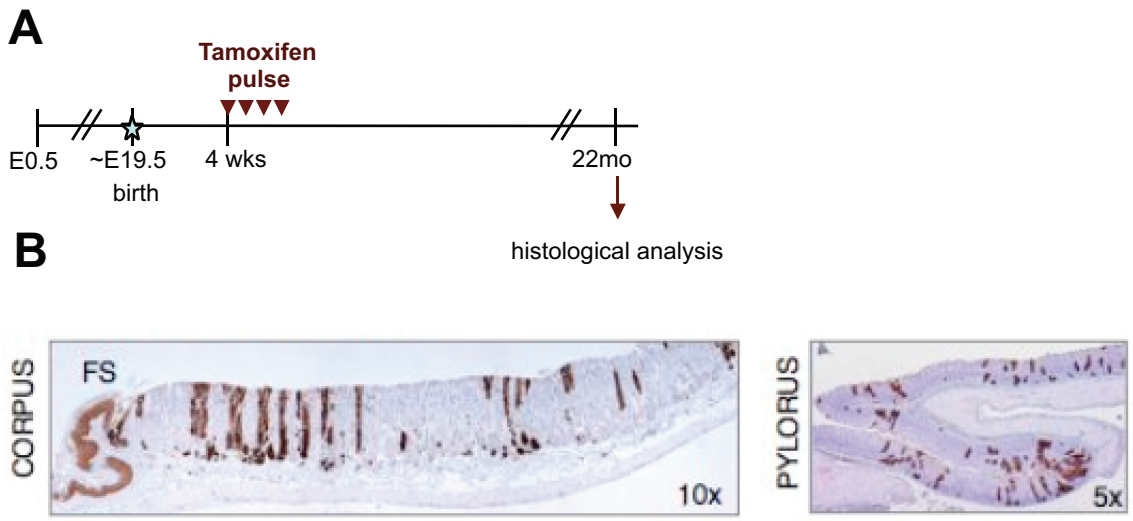

Figure 2.7. Long term lineage tracing analysis of Sox2 expressing cells in the glandular stomach. (A) Experimental outline for pulse-chase experiment. (B) IHC for EYFP on tissue sections of *Sox2*^{CreER}; *Rosa26*$^{lsl\text{-}EYFP}$ mice 22 months after the TAM pulse. Overview of long-term labeling in corpus and pylorus of glandular stomach. FS, forestomach

A previous study has shown that Lgr5+ cells function as multipotent stem cells of the pylorus (Barker et al., 2010b), raising the question of whether there is overlap in the expression patterns of Sox2 and Lgr5. Lgr5-GFP+ cells were confirmed to be present at a frequency of three or four cells at the base of each gland, while we observed either one or two Sox2-GFP+ cells above the base of each gland (2.1D). Interestingly, IHC for both Sox2 and GFP on pyloric sections of Lgr5$^{GFP\text{-}ires\text{-}CreER}$ mice showed no overlap between the expression of these two markers, suggesting that Lgr5 and Sox2 mark different types of stem cells in the pylorus (Figure 2.8A). However, we cannot exclude the existence of double-positive cells expressing low levels of the respective other marker that was beyond the threshold for IHC detection.

Colabeling experiments of Sox2-GFP pyloric sections for GFP and the proliferation marker Ki67 further showed that roughly 50% of the Sox2+ cells are actively cycling (n = 45 Sox2-GFP nuclei), indicating that half of the Sox2+ cells may be quiescent under homeostatic conditions (Figure 2.8B) . Analysis of *Sox2*^{CreER}; *ROSA26*$^{lsl\text{-}EYFP}$ mice receiving only a single dose of TAM after 1 day and 1 week confirmed our observations that some Sox2+ cells are

cycling and give rise to EYFP+ patches of cells after 1 week while some Sox2+ cells appear to be slow cycling and remain as singly labeled EYFP+ cells, respectively (Figure 2.8C).

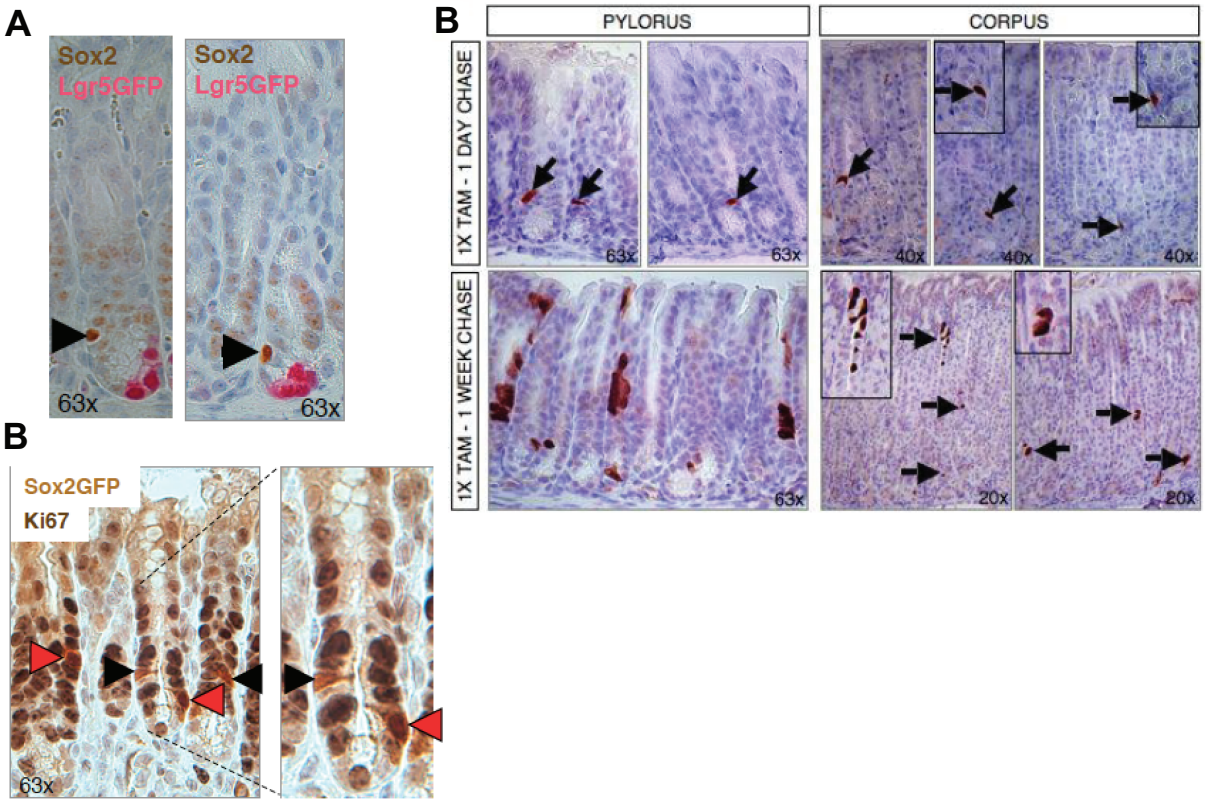

Figure 2.8. Characterization of Sox2+ stomach stem cells. (A) Localization analysis for Sox2 and *Lgr5*$^{EGF\text{-}ires\text{-}CreERT2}$ in pylorus. Sox2-high cells (arrowheads) and Lgr5-EGFP+ cells (pink) are different cell populations. (B) Co-localization analysis between proliferation marker Ki67 and Sox2-GFP. Original magnifications are as indicated. (C) Single, low-dose TAM treatment of Sox2^{CreER}; Rosa26$^{lsl\text{-}EYFP}$ mice and short term analysis (1 day and 1 week post-induction) of marked cells in corpus and pylorus. Note individually labeled EYFP+ cells in both corpus and pylorus at positions reflecting Sox2 expression patterns after 1 day of chase (marked by arrows). While some labeling events persist as single EYFP+ cells after 1 week of chase, other EYFP+ cells have already expanded into patches with some cellular offspring having migrated.

Fetal Sox2+ cells give rise to adult Sox2+ stem cell compartments

Given that Sox2 is already expressed and plays important roles in fetal development, we wondered whether Sox2+ cells emerging in the embryo are the precursors for the observed Sox2-expressing tissues in the adult. We first reevaluated Sox2 expression in E15.5 fetuses by IHC. Consistent with previous observations, we saw Sox2 signal in the developing stomach

(Figure 2.9A)

To determine whether these fetal Sox2+ cells give rise to the corresponding Sox2+ adult tissue, we injected pregnant females carrying *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ embryos with TAM at E13.5 or E14.5 and analyzed their tissues as adults (Figure 2.9B). Similar to results obtained with adult labeling, we detected EYFP expression in the glandular stomach, (Figure 2.9C and data not shown). Moreover, we saw EYFP expression in rare crypts of the duodenum, which was never seen in adults and is in agreement with reported Sox2 expression at the boundary between fetal glandular stomach and duodenum (Que et al., 2007, data not shown). We also noticed that the labeled stomach samples usually contained clusters of three to ten adjacent gastric units, suggesting that fetal or early postnatal progenitors had undergone clonal expansion following their genetic marking. Together, these results show that early Sox2+ fetal progenitors are the precursors for Sox2+ adult stem cells and that some tissues (duodenum and submandibular gland) appear to express Sox2 only transiently during fetal development.

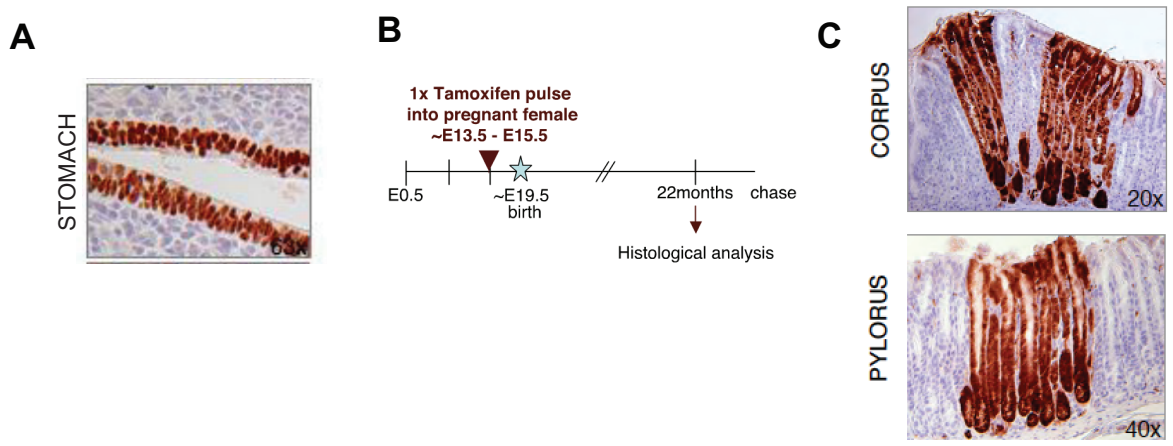

Figure 2.9. Fetal Sox2+ cells give rise to adult Sox2+ stem cells. (A) IHC for Sox2 on E15.5 stomach section. Sox2+ cells were detected in the developing stomach. (B) Experimental strategy for embryonic lineage tracing. (C) IHC for EYFP on stomachs from *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ mice that received one pulse of tamoxifen at E14.5 and were chased for 22 months.

Sox2 expressing cells maintain gastric organoids *in vitro*

Over the last few years, a three-dimensional organ bud culture system, termed organoid culture, for the GI tract has been developed(Barker et al., 2010b; Barker et al., 2007). By culturing whole gastric glands in matrigel and growth factors (including Noggin, EGF, Respondin1, Wnt3A and Fgf10), gastric glands form organoids that are expandable over long periods of time(Barker et al., 2010b). We derived organoids from $Sox2^{GFP}$ mice to determine the number of Sox2 expressing cells within these cultures. Surprisingly, 80% of cells within the organoid cutlures were Sox2-GFP+. To determine if the Sox2-GFP+ organoid cell population contained cells required for organoid propagation, we used FACS to sort for Sox2GFP+ and Sox2GFP- cells. We plated these two cell populations at equal numbers to compare their organoid repopulation potential. Sox2GFP+ cells had a 10 fold increase in repopulation potential compared to Sox2GFP- cells. Therefore, we conclude the Sox2 expressing cell population in gastric organoids contains the stem cells required for organoid propagation.

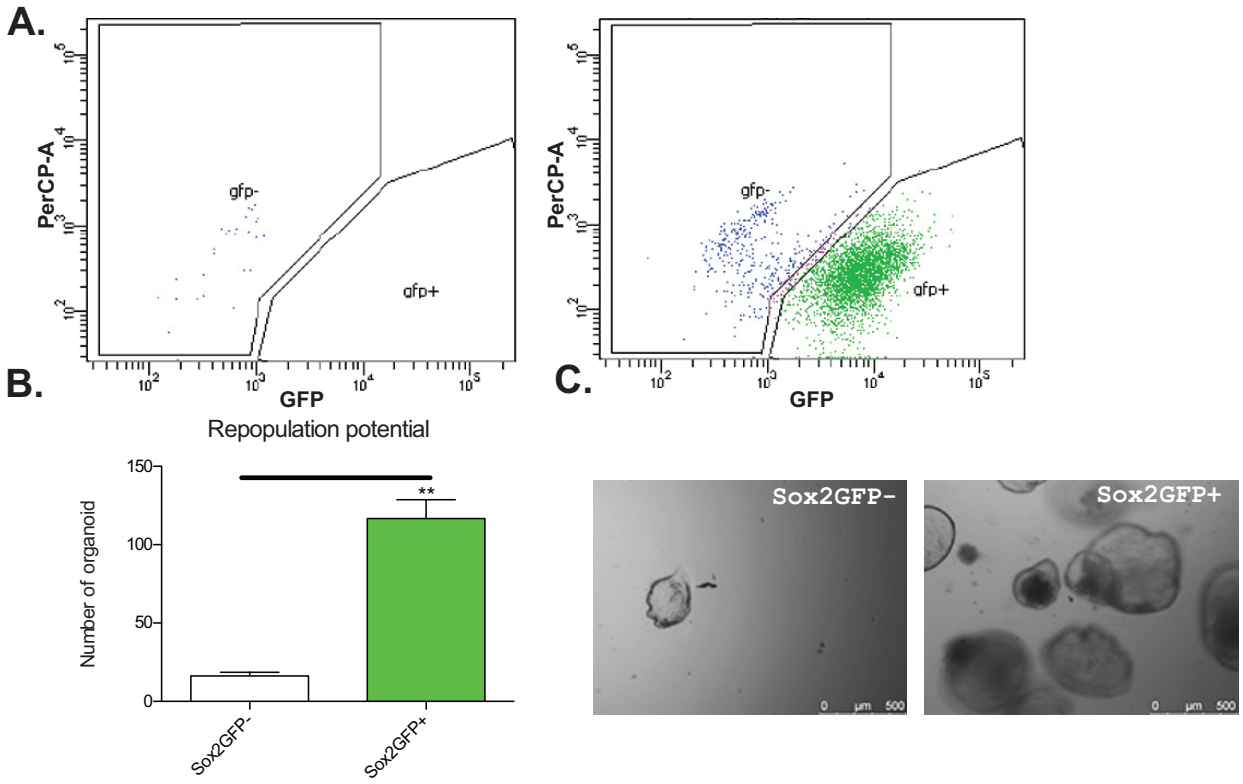

Figure 2.10. Sox2 expression in organoid cultures. (A) FACS analyses of organoids derived from pylorus of *Sox2*^{GFP} mice and their wild type (Sox2^{WT}) littermate controls. (B) Left, repopulation assay for sorted Sox2GFP+ and Sox2GFP- cells from organoids derived from *Sox2*^{GFP} mice. Right, bright field images of sorted Sox2GFP- and SoxGFP+ cells 7 days after re-plating.

2.6 Discussion

In this study, we have identified expression of the stem cell factor *Sox2* in the glandular stomach, where it has not previously been characterized. Importantly, we provide unequivocal fate mapping evidence that Sox2+ cells contain long-term stem cells in this tissue.

To our knowledge, Sox2 is the only transcriptional regulator that is commonly expressed between ESCs, fetal progenitors, and a number of adult stem cells and may therefore point toward molecular similarities in the regulation of pluripotent and different adult stem cells. Our developmental lineage tracing data show that Sox2+ adult stem cells originate from early Sox2+

epithelial progenitors in fetal development. These results suggest that Sox2 expression plays key roles at multiple stages of prenatal and postnatal development. While it is initially required for the establishment of pluripotent founder cells within the embryo, it subsequently controls the formation of fetal endodermal primordia and eventually becomes confined to a discrete cell population in the adult glandular stomach where it marks multipotent stem cells.

Recent elegant fate mapping studies have found that Lgr5 expression marks stem cells in several epithelial tissues (Barker et al., 2010a; Jaks et al., 2008)including the pyloric stomach (Barker et al., 2010b) raising the question of whether the same or different stem cells are labeled when compared with Sox2+ cells. Based on our coexpression results, we conclude that Sox2-GFP expression labels a different population of cells in the glandular stomach than Lgr5-GFP expression does. Another pronounced difference between Lgr5+ and Sox2+ stem cells is that Lgr5+ cells are seen in the intestine and pyloric stomach, whereas Sox2+ cells are found in both the pylorus and corpus compartment of the adult glandular stomach, but not in the intestine. A previous study has identified a rare population of Villin-expressing cells in antral glands that may coincide with the Sox2-expressing cells described here. In contrast to our study and the report by (Barker et al., 2010b) however, lineage tracing experiments showed that these Villin+ cells only give rise to entirely labeled gastric units in response to interferon treatment(Qiao et al., 2007). Future studies will be needed to conclusively establish whether Villin+, Lgr5+, and Sox2+ cells are different types of stem cells that independently replenish the stomach or whether these cells are hierarchically related to each other. The notion of distinct stomach stem cells is reminiscent of recently identified stem cell markers in the small intestine that seem to label different cell populations (Barker et al., 2007; Montgomery et al., 2011; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Tian et al., 2011). This may reflect either functional differences of these epithelia or their distinct developmental origin despite a remarkably similar structure.

In summary, our results establish Sox2 as a marker of adult stomach stem cells, and suggest that common target genes and pathways may be activated that are crucial for maintaining the self-renewal and differentiation potential of these cells and other Sox2+ stem cells populations. Given the recent recognition that Sox2 can act as an oncogene in squamous cell carcinoma of the lung and esophagus(Bass et al., 2009), it should be interesting to investigate further whether the Sox2-expressing cells, identified here, can act as tumor-initiating cells, and whether Sox2 expression itself may be oncogenic in the stomach.

2.7 Materials and methods

Mice

V6.5 ESCs were targeted with knockin constructs containing CreERT2 (Feil et al., 1997), puro-delta-TK (Chen and Bradley, 2000), or EGFP (Ellis et al., 2004) alleles under the control of endogenous Sox2 regulatory elements using standard protocols. Correct insertion of constructs was verified by Southern blot, and correctly targeted clones were injected into BDF1 blastocysts and transferred into pseudopregnant females. Resultant chimeric mice were bred with 129SvJae mice and germline offspring were bred to establish stable lines. All animal experiments were approved by the IACUC committee and conform to the regulatory standards.

TAM Induction

Sox2^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ ESC clones were treated with one dose of 100 nM 4-OH TAM (Calbiochem), and cells were analyzed by FACS after 2 weeks. Two milligrams TAM (Sigma), dissolved in corn oil, was given daily to 3- to 6-week-old *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ animals on 4 consecutive days if not otherwise stated. Mice were analyzed by IHC on day 5 (pulse time point), and at different time points thereafter. *Sox2*^{CreER}*; ROSA26*$^{lsl\text{-}EYFP}$ littermates injected with corn oil alone were used as controls. *Sox2*^{WT}*; ROSA26*$^{lsl\text{-}EYFP}$ animals were used as staining

control. For lineage tracing **i**n the embryo, a single pulse of 2 mg TAM plus 1 mg Progesteron was given to pregnant females at E14.5 and resultant offspring were analyzed at 22 months of age.

Tissue Preparation and IHC

Tissues were harvested, fixed in 10% formalin or bouins solution overnight, washed in PBS, embedded in paraffin and sectioned onto slides. Sections were then rehydrated, and antigen retrieval was performed by incubating sections in either citrate buffer (pH 6) or EDTA (pH 9) in a pressure cooker following the manufacturer's instructions. For staining with diaminobenzidine tetrahydrochloride, sections were processed using Vectastain Elite Kits (Vector Labs). For staining with alkaline phosphatase, sections were processed using Vectastain ABC-AP Kits (Vector labs). For double staining, the nuclear antibody staining was performed first followed by cytoplasmic antibody staining. Antibodies used include GFP/YFP (1/1500, JL-8, Clontech); Sox2 (1/2500, AB5603, Millipore), and p63 (1/300, sc-8431, Santa Cruz), Ki67 (1/200, ab15580, Abcam), MUC5AC (1/100, ab3649, Abcam); Gastrin (1/500, sc-7783, Santa Cruz); H-K-ATPASE (D032-3H, MBL), and Chromogranin A (1/400, ab15160, Abcam).

Neural progenitor cell isolation

NPCs were isolated from P1 pups and cultured as monolayers with poly-ornithin (PO), or as neurospheres without PO as previously described (Eminli et al., 2008).

FACS analysis

For FACS analysis on tissues, small tissue pieces were minced with scalpels and treated with Collagenase Type VI (Invitrogen) at 4C for 15-30 min, followed by a 20-40 min incubation at 37C (incubation times were adjusted depending on the age of the mouse and the

type of tissue). To obtain single cell suspensions, tissues were resuspended in cell dissociation buffer (Invitrogen) by thorough pipetting and filtered through a 70um filter. DMEM/5% FBS was added to the filtrate to stop Collagenase activity, samples were spun down for 5 min, 1500 rpm at room temperature and pellets were washed twice with dissociation buffer, resupended in 1xPBS and filtered through a 45um filter before they analysis by FACS. If antibody staining was required, cell suspensions were incubated with the respective antibody diluted in 1xPBS/ 2% FBS at 4C in the dark for 1 hour (ckit, CD117, clone 2B8, eBioscience), washed twice with 1xPBS and filtered through a 45 μM filter prior to analysis. Propidium iodide (PI, Invitrogen) was added to detect dead cells.

Organoid derivation and culture

Gastric gland units were isolated from 6-week-old mouse pylorus stomach essentially as described (Barker et al., 2010b). Whole gastric glands were embedded in Matrigel (BD Bioscience) and plated in 24-well plates. After polymerization of Matrigel, gastric culture medium (Advanced DMEM/F12 Supplemented with B27, N2, nAcetylcysteine Invitrogen), and 50 ng/ml EGF [Peprotech], Gastrin (10 nM [Sigma-Aldrich]), 100 ng/ml FGF10 [Preprotech], 100 ng/ml Noggin [Peprotech], 25% Wnt3A Conditioned media, and 25% Rspondin Conditioned media was overlaid. For the first 2 days after seeding, the media was supplemented with 10 μM ROCK inhibitor Y-27632 (Stemgent) to prevent anoikis.

Organoid repopulation assay

Organoids were derived from $Sox2^{GFP}$ and $Sox2^{WT}$ littermate mice and passaged once. Organoids were then dissociated in Accutase (Life Technologies) for 10 min at room temperature, filtered through a 45 μM mesh and analyzed and sorted using flow cytometry. Propidium iodide (PI, Invitrogen) was added to detect dead cells. Sox2-GFP+ and Sox2-GFP- cells were sorted from $Sox2^{GFP}$ mice, and plated at a density of 1,000 cells per well according

the protocol detailed above.

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Chapter 3

Sox2 suppresses gastric tumorigenesis by modulating Wnt/β-catenin activity

3.1 Abstract

The transcription factor Sox2 plays crucial roles in mammalian development, stem cell maintenance, and tumorigenesis. We previously showed that Sox2 expression marks adult stem and progenitor cells in the stomach, raising the question of whether Sox2 itself is important during gastric epithelial homeostasis and disease. Unexpectedly, we find that Sox2 is dispensable for gastric stem cell and epithelial self-renewal. ChIP-Seq analysis reveals that the majority of Sox2 targets in gastric stem and progenitor cells are related to tissue-specific functions such as endoderm development, Wnt signaling and gastric cancer. Consistent with this finding, Sox2+ cells are highly susceptible to tumorigenesis in an Apc/Wnt-driven mouse model. However, Sox2 loss enhances rather than impairs tumor formation in *Apc* deficient gastric cells *in vivo* and *ex vivo*. Moreover, Sox2 loss increases Tcf/Lef-dependent transcription in stomach organoids, providing a mechanistic basis for the observed effect on tumorigenesis. 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 developmental and cancer-associated pathways.

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3.3 Author contributions

Candidate's contributions

Designed all experiments. Performed all experiments. Performed all data analyses. Prepared figures and chapter.

Other Other's contributions

Assisted with generation of mice, Wnt reporter assay, Sox2 ChIP-seq, bioinformatics and histological interpretations.

3.4 Introduction

The transcription factor Sox2 has been widely studied in the context of development, pluripotency and cellular reprogramming (Avilion et al., 2002; Sarkar and Hochedlinger, 2013; Takahashi and Yamanaka, 2006). Sox2 is essential in early embryogenesis, controlling self-renewal and differentiation of a number of embryo-derived stem cell populations, including embryonic stem (ESC)(Masui et al., 2007) and neural progenitor (NPC) cells (Graham et al., 2003). Consistent with this finding, ChIP-Seq analyses in ESCs and NPCs indicate that SOX2 activates self-renewal genes while suppressing genes associated with differentiation (Adachi et al., 2013; Lodato et al., 2013). We recently showed that Sox2 continues to be 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).

The gastric epithelium in mice and humans mainly consists of flask-like glandular units that contain mucus-, acid-, zymogen- and hormone-producing cells required to digest food (Mills and Shivdasani, 2011). The mouse, but not the human, stomach also contains a keratinized rostral region called the forestomach. Both glandular and keratinized portions of the stomach undergo continuous replenishment throughout life. Sox2 is expressed in the basal layer of the forestomach and at the base of gastric glands, which are thought to represent stem and progenitor cell compartments. Lineage-tracing experiments further demonstrated that the Sox2-expressing pool contains multipotent stem cells in the glandular stomach and unipotent stem cells in the forestomach (Arnold et al., 2011). Whether Sox2 expression serves simply as a marker of gastric stem cells or is also functionally important remains unresolved. It is also unclear whether Sox2 targets similar or different sets of genes in gastric stem cells compared to ESCs or NPCs to control self-renewal and differentiation.

Gastric cancer is the third most frequent causes of cancer-related deaths worldwide and

is incurable when metastases are present (Stewart et al., 2014). Although genome-wide sequencing efforts have catalogued numerous gastric-cancer-specific mutations, the functional roles 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 (Bass et al., 2009; Rudin et al., 2012), esophagus (Bass et al., 2009), pituitary gland (Andoniadou et al., 2013), skin (Boumahdi et al., 2014), retina (Li et al., 2012), and stomach. While Sox2 is overexpressed or amplified in most of these tumors, consistent with an oncogenic function, Sox2's role in gastric cancer remains controversial (Hütz et al., 2014; Matsuoka et al., 2012; Otsubo et al., 2011; Wang et al., 2015; Zhang et al., 2010).

To better understand the mechanisms by which Sox2 may contribute to stomach homeostasis and cancer, we sought to determine its functions in the normal and malignant gastric epithelium using novel loss-of-function mouse models. We also assessed the susceptibility of Sox2+ stem/progenitor cells to initiate tumors and defined the repertoire of Sox2 target genes in gastric epithelial cells relative to other Sox2-expressing stem cell populations.

3.5 Results

Sox2 is dispensable for normal stomach homeostasis

To determine whether *Sox2* is required during adult stomach homeostasis, we generated a conditional knockout allele, $Sox2^{fl}$, using conventional gene targeting (Figure 3.1A). To delete *Sox2* in adult tissues, we crossed homozygous $Sox2^{fl/fl}$ mice with animals expressing tamoxifen-inducible *Cre* recombinase from the *Rosa26* locus ($Rosa26^{CreER}$). We then gave 6 week-old $Rosa26^{CreEr}$; $Sox2^{fl/fl}$ mice tamoxifen to induce Cre-mediated excision of *Sox2* and sacrificed animals at different times (Figure 3.1B); we refer to these animals as Sox2 KO mice and wild type controls as Sox2 WT mice. Both the keratinized forestomach and the glandular stomach in

Sox2 KO animals displayed nearly complete loss of Sox2 by immunoblot analysis and immunohistochemistry (Figure 3.1C, D, E), indicating high loop-out efficiency. Surprisingly, we observed no morphological changes in either region of the stomach, even weeks or several months after *Sox2* deletion (Figure 3.1D, E). Other tissues that contain Sox2+ cells (e.g., lung, salivary gland, uterus, anus, testes) also lacked histologic abnormalities and we observed no excess mortality in Sox2 KO mice (data not shown). Thus, *Sox2* is dispensable for viability or adult stomach homeostasis, in striking contrast to its essential roles in early embryos and embryonic or neural stem cell populations.

To probe *Sox2*'s function independently in gastric epithelial regeneration and differentiation, we generated organoids, which self-renew and differentiate in culture (Barker et al., 2010), recapitulating gastric cell behaviors *ex vivo*. Organoids cultured from *Rosa26*^{CreEr}*;Sox2*$^{fl/fl}$ gastric glands and treated with 4-hydroxytamoxifen showed morphology and growth similar to solvent-treated control organoids and TAM-treated control organoids (Figure 3.1F, data not shown). These data confirm that *Sox2* is dispensable for gastric epithelial self-renewal.

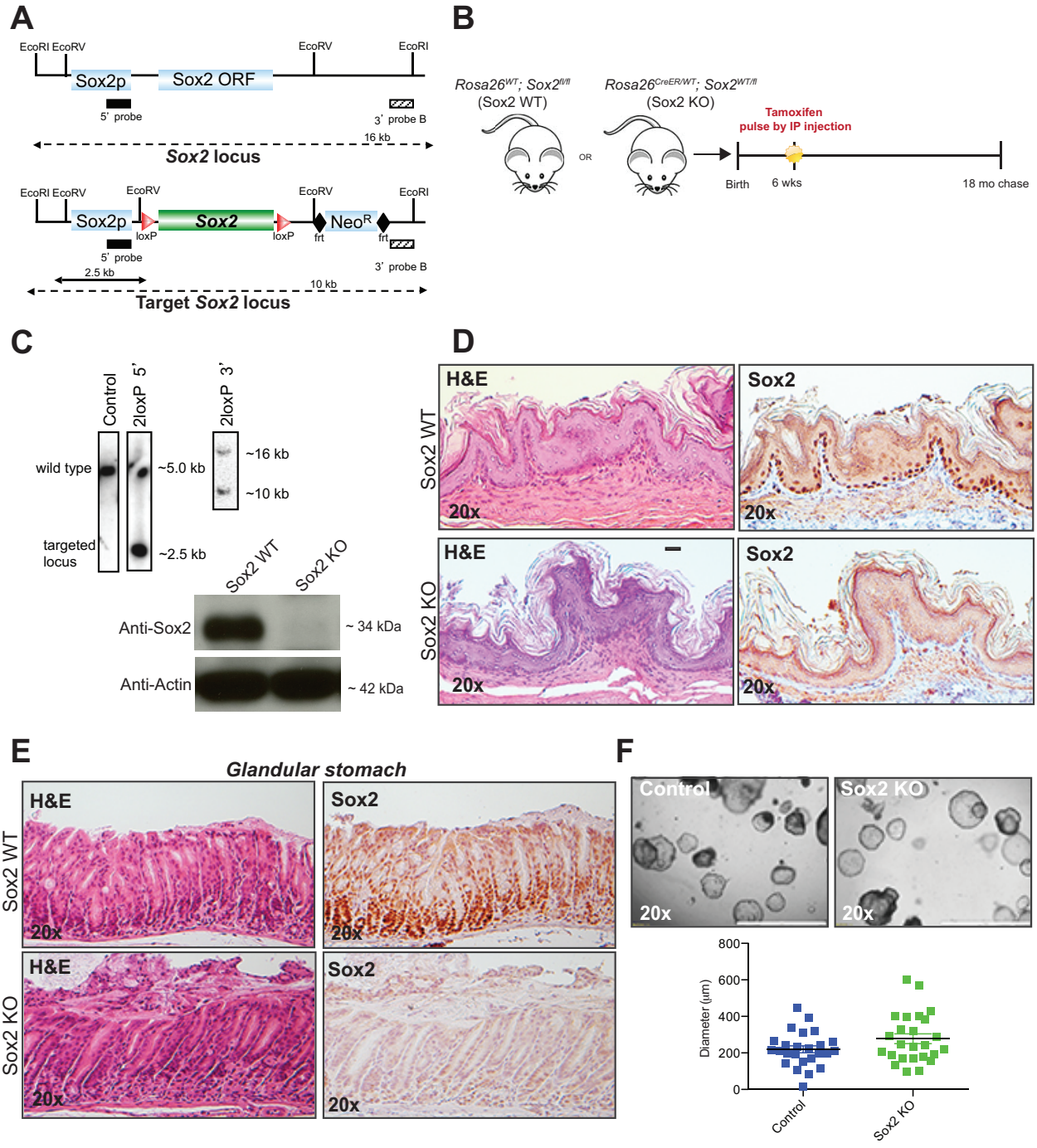

Figure 3.1. *Sox2* is dispensable during stomach homeostasis. (A) Targeting strategy for Sox2$^{fl/fl}$ ESCs and mice. Restriction sites, southern blot analysis probes and expected restriction fragment lengths are indicated. (B) Genetic strategy used to induce conditional deletion of *Sox2* in mice. (C) Top, Southern blot analyses with 5' and 3 probes to verify correct targeting. Bottom, western blot analyses on stomach isolated from tamoxifen induced Sox2 WT and Sox2 KO tissue. (D-E) IHC for Sox2 in Sox2 WT and Sox2 KO mice 18 months after tamoxifen induction. (F) Left, sample bright field images of organoids isolated from Sox2 KO glandular stomach and treated with ETOH (Control) or 4OHT (Sox2 KO). Right, organoid

Figure 3.1. (Continued) diameter measurements in Control and Sox2 KO organoid lines (n=25). Abbreviations: wks, weeks; mo, months.

In the above studies, we used a new antibody for Sox2 from Neuromics. By conducting immunohistochemistry with this antibody on stomach isolated from both Sox2 WT and tamoxifen induced Sox2 KO mice, we surprising confirmed the presence of Sox2 protein throughout the entire epithelium of the pylorus (Figures 3.1E). This contrasted with our previously published IHC for Sox2 using an antibody from Chemicon, which marked 1-2 cells per gastric gland, and lead us to investigate the reason for this discrepancy (Figure 3.2). IHC for Sox2 marked 1-2 cells in +3/+4 position of gastric glands in Sox2GFP reporter mice (Figure 3.21A), in Sox2 lineage-tracing mice pulsed with low dose tamoxifen for 1 day (Figure 3.2B) and in wild type mice stained with the Chemicon antibody (Figure 3.2C). Interestingly, these cells overlapped with cells that bound more strongly to the Neuromics antibody (Figure 3.2D inset). Thus we conclude that our Sox2GFP reporter model marks a $Sox2^{high}$ population of cells. Such discrepancy between reporter expression and gene expression has also been observed in stem cell populations in the intestine (Li et al., 2014).

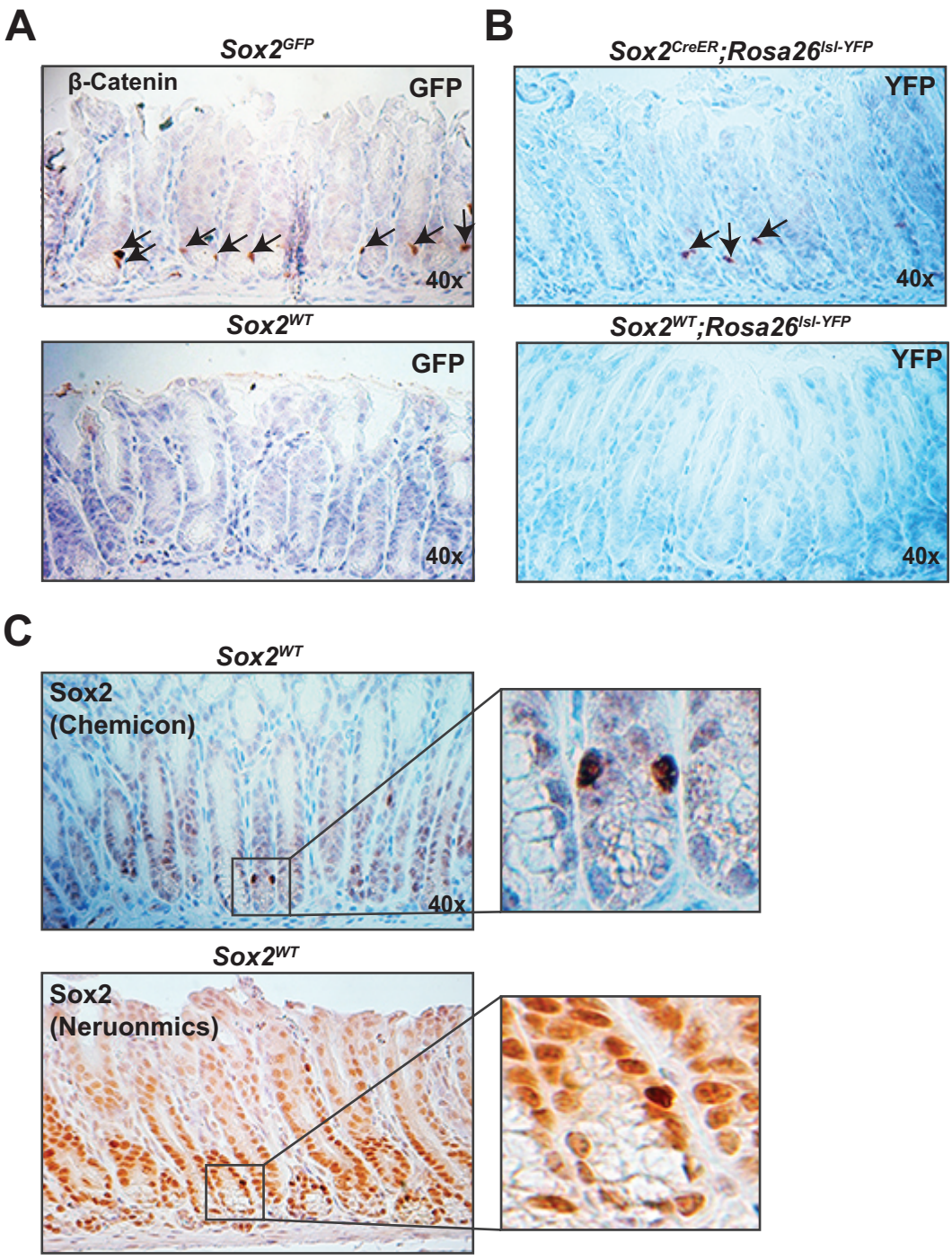

Figure 3.2. Comparison of Sox2 reporter, lineage tracing and antibody staining. (A) IHC for GFP on pylorus of stomach from wild type ($Sox2^{WT}$) and $Sox2^{GFP}$ littermate mice. (B) IHC for YFP on pylorus from wild type ($Sox2^{WT}$; $Rosa26^{lsl\text{-}YFP}$) and Sox2 lineage tracing ($Sox2^{CreER}$; $Rosa26^{lsl\text{-}YFP}$) littermate mice 1 day after a single, low-dose of tamoxifen treatment. (C) IHC for Sox2 on pylorus from wild type mice using two different antibodies. Arrows point to labeled cells. Insets highlight cells highly expressing Sox2.

Sox2 occupies loci related to endoderm development and gastric cancer

To investigate Sox2's possible functions in the glandular stomach, we identified target genes by chromatin immunoprecipitation and sequencing (ChIP-seq) on gastric glands isolated from tamoxifen-treated Sox2 WT and Sox2 KO mice (Defined in Figure 3.1C). We mapped more than 7,000 high-confidence binding sites in Sox2 WT gastric glands, compared to 887 unique targets in Sox2 KO glands (Figure 3.3A), indicating highly specific ChIP. Bound sites were highly enriched for the classical Sox consensus motif *ACAAAG* (93% of all sites), implying direct occupancy of most sites (Figure 3.3A). Consistent with Sox2's role as a transcriptional regulator, ~15% of sites mapped to promoters (0 kb to 3 kb upstream of transcription start sites, TSSs) and roughly 40% of sites mapped to introns (Figure 3.3B).

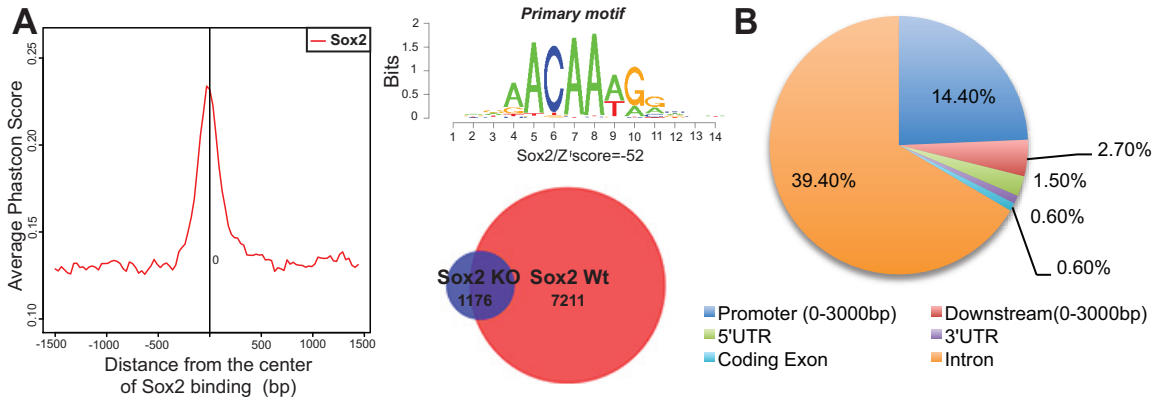

Figure 3.3 Sox2 ChIP-Seq on gastric glands. (A) High sequence conservation (left) and significant enrichment of the canconical Sox2 sequence motif (right, top) among Sox2 ChIP sites in pyloric gastric glands. (right, bottom) Venn diagram demonstrating enrichment of Sox2 targets in gastric glands from Sox2 WT compared to tamoxifen treated Sox2 KO mice (described in Figure 3.1) (B) Pie-chart distribution across the genome revealing Sox2 binding mainly in promoters and intergenic regions.

Gene Ontology (GO) analysis using the GREAT algorithm revealed enrichment of genes related to digestive tract and foregut morphogenesis and glandular epithelial differentiation among TSS-proximal Sox2 targets (0-5 kb upstream of TSSs) and of genes associated with the alimentary system when including TSS-distal target sites (5-30 kb from TSSs) (Figure 3.4A). In

agreement with these enriched categories, we detected Sox2 binding near key regulators previously associated with endoderm development and gut differentiation, including *Gata6* and *Cdx2* (Figure 3.4B). Of interest, Sox2 binds its own promoter in the stomach, suggesting an autoregulatory feedback mechanism akin to ESCs and NPCs (Figure 3.4B). 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 3.4A). We further observed an enrichment for signaling pathways mutated in gastric cancer, such as components of the Akt, ErbB2, Vegf and Wnt cascades (Figure 3.4A) and the ChIP-Seq data confirmed prominent Sox2 peaks at a number of candidate loci associated with these pathways (e.g., *Apc, ErbB2, Vegfa*)(data not shown).

Sox2 binding sites in the stomach mucosa showed hardly any overlap with binding sites previously identified in murine NPCs and ESCs (Figure 3.4C), which suggests that Sox2 is directed to tissue-specific genes by associating with cell type-specific cofactors, as was observed in NPCs and ESCs (Sarkar and Hochedlinger, 2013). Sequences near gastric Sox2 binding sites were enriched for motifs recognized by the glandular stomach-expressed transcription factors Jun, Gata6, Klf4, and Foxa2, which and may confer target gene selectivity (Figure 3.4D). Altogether, these data reveal that Sox2 occupies target genes related to endoderm development, stomach function and gastric cancer, showing little overlap with Sox2 binding sites in ESCs and NPCs.

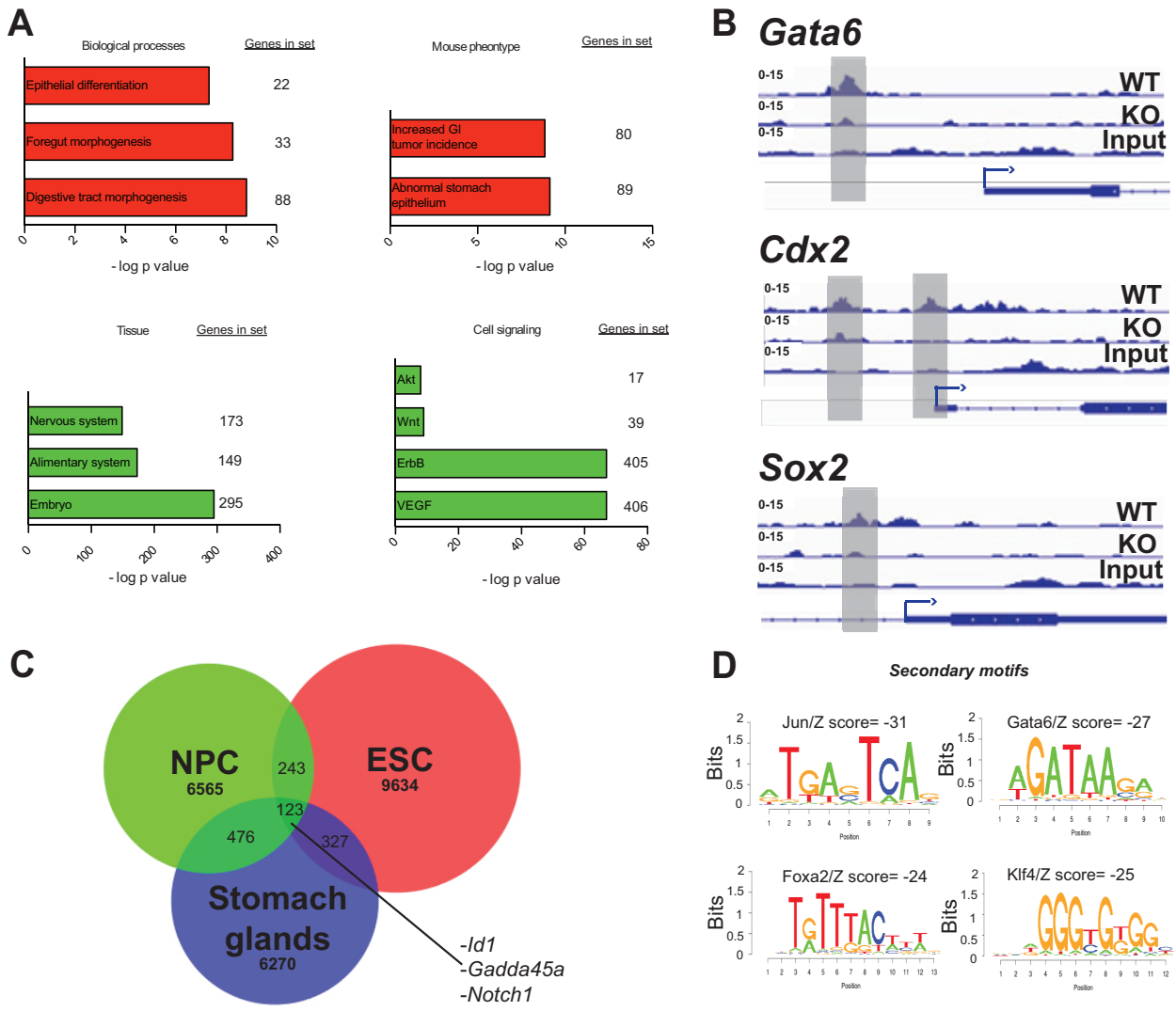

Figure 3.4 Sox2 occupies loci related to endoderm development and gastric cancer. (A) GO analysis for categories enriched in Sox2 ChIP data set from stomach glands. X-axis reflects negative log base 10 of binomial raw p-vale for enrichment versus a whole genome background. Red represents GO on proximal promoter elements, Green on distal regulatory elements. Numbers to right indicate genes in category. (B) Wiggle tracks from Sox2 ChIP–seq. (C) Venn diagram representing the overlap of Sox2 occupancy in neural progenitor cells (NPCs), embryonic stem cells (ESCs) and pylorus gastric glands. Genes listed are common Sox2 targets among all 3 cells types. (D) Enriched sequence motifs found next to Sox2 peaks.

Rapid and widespread adenoma formation from SOX2+ gastric cells.

As this ChIP-Seq analysis revealed Sox2 occupancy at a number of genes implicated in cell proliferation and stomach cancer, we considered that Sox2+ stem and progenitor cells might serve as a source for gastric tumors. To test this hypothesis, we acutely inactivated the *Apc* tumor suppressor gene 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 (Apc^{Min}) develop adenomas in the distal stomach through loss of heterozygosity (Tomita et al., 2007). We crossed $Apc^{fl/fl}$ mice (Colnot et al., 2004) with mice carrying both a tamoxifen-inducible *Cre* allele (*CreER*) in the endogenous *Sox2* locus and, to monitor excision efficiency, a loxP-flanked EYFP reporter in the *Rosa26* locus to ($Sox2^{CreEr}$;$Rosa26^{lslYFP}$)(Srinivas et al., 2000)(Figures 3.5A). 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 3.5A) and verified deletion of *Apc* in Sox2+ cells through the appearance of small groups of cells with activated β-CATENIN 3 days later (Figure 3.5B). These cells replicated rapidly forming multiple micro-adenomas within 1 month and large pyloric adenomas within 1 year (Figure 3.5B). 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 3.5C), confirming that they originated from Sox2+ cells. Moreover, tumors continued to express Sox2 in a heterogeneous pattern, indicating the presence of cells with stem/progenitor cell characteristics (Figure 3.5D). Collectively, these data demonstrate that Sox2+ cells are the cells of tumor origin in a Wnt-driven mouse model.

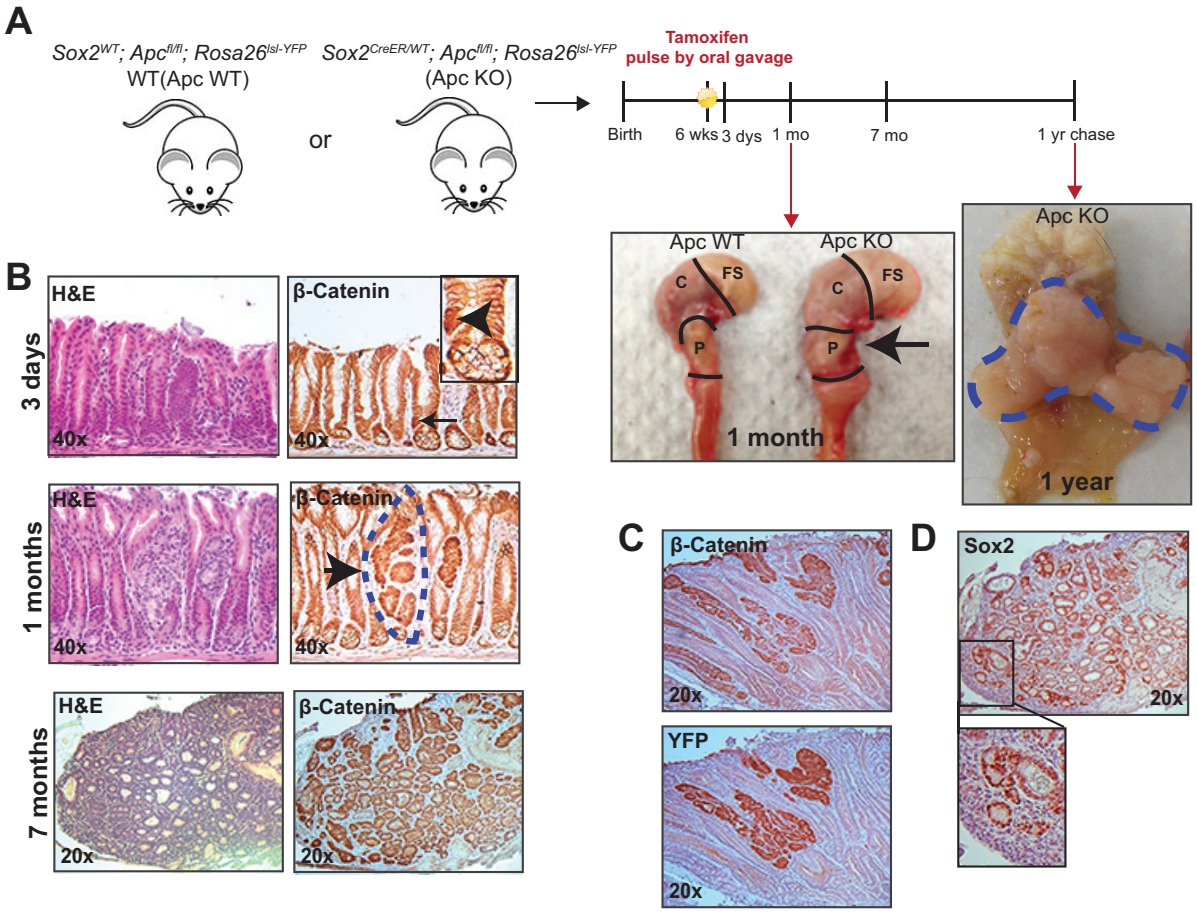

Figure 3.5. Wnt-driven transformation of adult Sox2+ cells initiates tumor formation in the distal stomach (A) Genetic strategy used to determine tumor-initiating potential of Sox2+ cells, and stomachs from Apc WT and Apc KO mice 1 month and 1 year after tamoxifen induction. Arrow indicates enlarge pylorus in Apc KO stomach. Blue dotted line highlights large tumors in pylorus of Apc KO stomach. (B) IHC for Wnt effector protein, β-Catenin, in Apc KO pylorus 3 days, 1 month and 7 months after tamoxifen induction. Arrows point to cells/tumors with accumulation of β-Catenin. Blue dotted line highlights microadenoma. IHC for YFP(C) and Sox2 (D) on adenoma in Apc KO pylorus 7 months after tamoxifen induction. Abbreviations: wks, weeks; mo, months; FS, forestomach; C, corpus; P, pylorus.

Sox2 suppresses tumorigenesis via Wnt-dependent transcription

Given that SOX2 binds genes associated with gastric cancer in the normal stomach mucosa (Figure 3.5) and that SOX2 acts as an oncogene in other tissues (Bass et al., 2009), we next we sought out to determine whether SOX2 was highly expressed in gastric cancer. To this end, we acquired and performed immunohistochemistry on normal human gastric mucosa and 18 gastric adenocarcinoma specimens. SOX2 expression at the base of normal human gastric

glands recapitulated the pattern observed in mouse stomach (Figure 3.6A). However, SOX2 expression was absent or extremely low in 13 of the 18 gastric tumors (Figure 3.6A, Table 3.1). These findings were surprising and suggest that *SOX2* may be silenced during the progression of normal human mucosa to gastric cancer.

Table 3.1. Aggregate analysis of IHC for SOX2 in 18 human gastric cancers. IHC 0: negative, +: weak, ++: moderate, +++: strong.

IHC score	Histologic Type	Histologic Differentiation
+++	Intramucosal	Moderate
+	Signet-ring cell	Poor
+	Signet-ring cell	Poor
0	Signet-ring cell	Poor
++	Diffuse	Poor
+	Diffuse	Poor
++	Mix	Poor
0	Mix	Poor
+++	Intestinal	Moderate
++	Intestinal	Moderate to poor
0	Intestinal	Moderate to poor
0	Intestinal	Moderate
0	Intestinal	Moderate to poor
0	Adenocarcinoma	Poor
0	Adenocarcinoma	Poor
0	Adenocarcinoma	Poor
0	Adenocarcinoma	Moderate to poor
0	Adenocarcinoma	Moderate to poor

Given our findings that SOX2 expression is lost in human gastric cancer, we turned to mice to conduct further functional studies to understand why silencing of SOX2 may be beneficial for tumorigenesis. To this end, we mated $Sox2^{fl/fl}$ mice (Figure 3.6B) to $Sox2^{CreEr};Apc^{fl/fl}$ animals to generate *Apc* knockout (Apc KO) and *Apc/Sox2* double knockout (DKO) mice (Figure 3.6B). In contrast to the expected decrease, we observed a 3-fold increase in the number of adenomas in DKO mice compared to Apc KO mice (Figure 3.6C,D). To confirm this result by independent means, we generated gastric organoids from these animals and observed a 3-fold increase in the number of colonies growing after the first cell passage from DKO cultures compared to Apc KO cultures (Figure 3.6E,F). These results indicate that Sox2 functions as a tumor suppressor rather than an oncogene during Wnt-driven adenomagenesis in

the glandular stomach.

These findings raise the possibility that Sox2 may interfere directly with expression of Wnt/β-catenin target genes. We tested this hypothesis using a lentivirus-based Tcf/Lef reporter assay using organoids derived from Sox2 WT, Sox2 KO, Apc KO and DKO mice (Fuerer and Nusse, 2010). Organoids infected with a lentivirus carrying a Tcf/Lef-dependent luciferase reporter 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, consistent with the absence of homeostatic defects in Sox2 KO mice, we observed 10-fold higher Tcf/Lef activity in Apc KO organoids and 25-fold increase in DKO organoids (Figure 3.6G,H). We therefore conclude that Sox2 suppresses tumorigenesis, at least in part, by restraining hyperactivated Wnt signaling in the context of an *Apc* mutation.

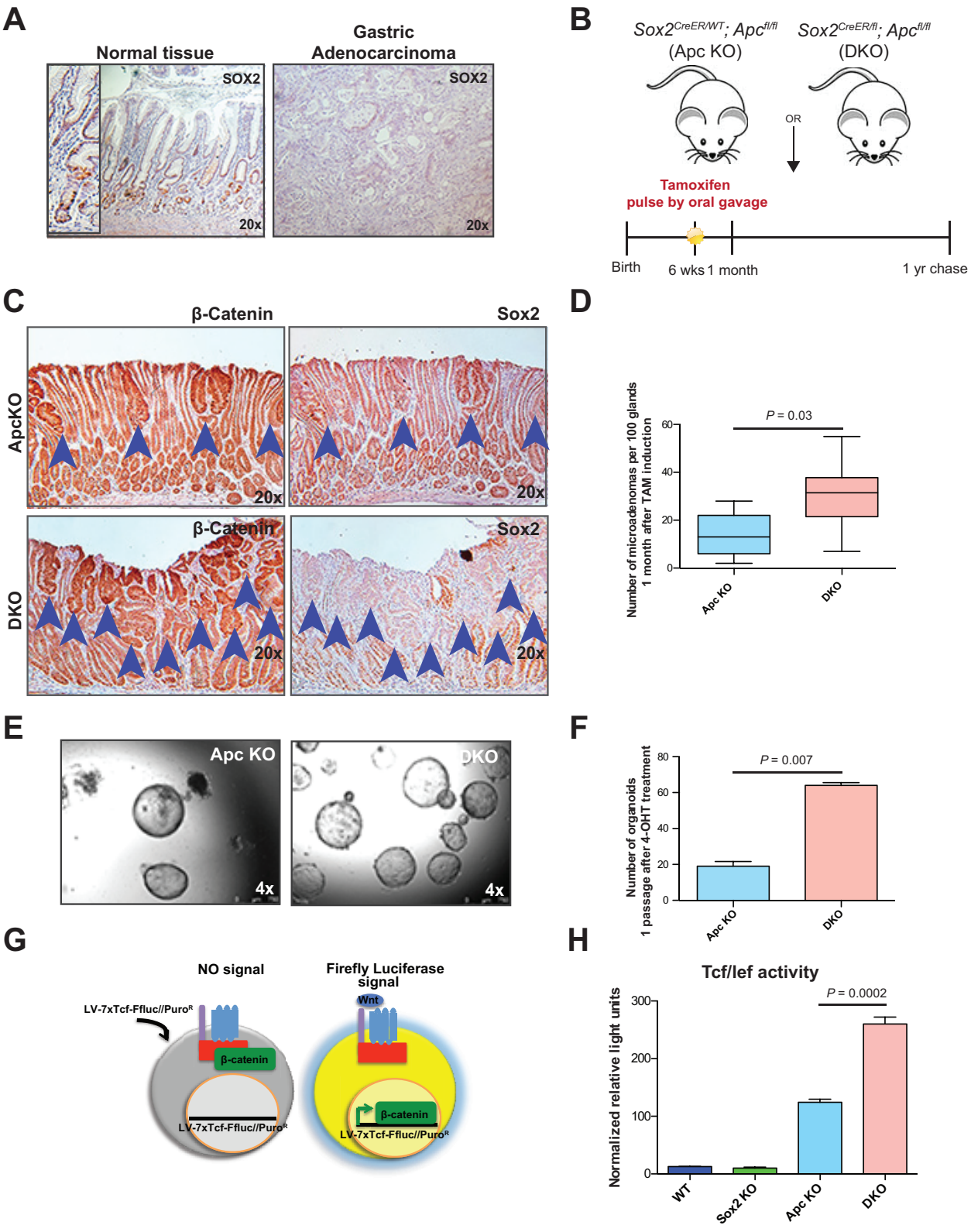

Figure 3.6. Sox2 suppresses Wnt-driven adenomagenesis.

Figure 3.6. (Continued) (A) IHC for Sox2 on normal human stomach and adenocarcinoma. (B) Genetic strategy used to study role of *Sox2* in Wnt-driven adenomagenesis. (C) IHC for β-Catenin and Sox2 in Apc KO and DKO mice 1 month after tamoxifen induction. (D) Quantification of number of adenomas in Apc KO and DKO mice 1 month after tamoxifen induction (n=11 Apc KO mice, n=14 DKO mice). (E) Bright field images of organoids isolated from adult Apc KO and DKO mice and treated with 4-OHT. (F) Organoid repopulation potential in Apc KO and DKO organoid lines treated with 4-OHT. Abbreviations wks, weeks; mo, months, yr, year. (G) Model for Wnt reporter assay. Organoids were infected with a lentivirus containing the 7x-Tcf-Ffluc/Puro^R reporter. Addition of puromycin allowed for selection and expansion of infected cells only. Wnt signaling was measured by expression of Firefly luciferase. (H) Luciferase activity in LV-7x-Tcf-Ffluc/Puro^R infected organoids.

3.6 Discussion

We have dissected the molecular and functional roles of *Sox2* and Sox2-expressing cells in gastric epithelial homeostasis and tumorigenesis. Our finding that *Sox2* is dispensable for normal tissue renewal in both the squamous and glandular parts of the stomach mucosa is 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 (Adachi et al., 2013; Sarkar and Hochedlinger, 2013). We surmise that other Sox proteins can compensate in the absence of Sox2, and at least one other Sox family member, Sox9, is expressed in the glandular stomach (Barker et al., 2010). As *Sox9* deletion perturbs intestinal proliferation and differentiation, it will be interesting to assess the consequence of combined loss of *Sox2* and *Sox9* in the normal gastric epithelium.

The cells of origin for gastric cancer remain unknown. Although loss of the *Apc* tumor suppressor in Lgr5+ gastric stem cells can give rise to isolated adenomas within a few weeks (Barker et al., 2010), the frequency of adenomas and 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 represents a useful system to study early events in tumorigenesis and to identify facilitators of gastric cancer progression.

We further conclude that Sox2 functions 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 associated with tumor progression in the esophagus, lungs, retina, skin, and pituitary. However, our results are consistent with the previous observation that Sox2 expression is reduced during gastric cancer development in patients, which we confirmed in our study. Sox2's effect on cell proliferation and tumorigenesis is thus highly context-dependent.

How might Sox2 suppress tumors in the context of oncogenic stimuli? In the glandular stomach, Sox2 appears to compete directly with Wnt/b-catenin signaling, based on our finding that *Sox2* loss promotes Tcf/Lef-dependent transcription in *Apc*-deficient gastric organoids. While this mechanism seems insufficient to perturb the self-renewal of wild type gastric stem and progenitor cells, likely due to compensation by other Sox factors or alternative mechanisms, Sox2 may limit cell proliferation and tumor growth when Wnt/β-Catenin signaling is hyperactivated, as in *Apc*-mutant cells. Our finding that high Sox2 levels repress Wnt/β-Catenin activity and proliferation in the stomach is consistent with previous reports, which established links between Sox factors and Wnt signaling in other tissues (Hagey and Muhr, 2014; Kormish et al., 2010). In addition to modulation of Tcf/Lef transcriptional activity, *Sox2* loss may contribute to tumorigenesis by reducing expression of tumor suppressors or by de-repressing oncogenic targets of Sox2 regulation. In gastric epithelial cells Sox2 occupies many loci that are mutated in gastric cancers (e.g., *VegfA, ErbB2, Fgfr2, Gata4/6, Kras, Catnnb, Smad2*) (Cancer Genome Atlas Research, 2014).

Comparison of Sox2 target genes in different stem cell populations is important for understanding how the same transcription factor achieves such functional versatility. While Sox2 shows evidence for autoregulatory feedback in all examined stem cell populations (gastric, neural and embryonic stem cells), the vast majority of Sox2 targets differ between these stem

cell populations. These specific binding patterns are likely the consequence of cell-type specific cofactors that associate with Sox2 and target distinct gene sets. Whereas recent evidence shows Sox2 associations with Oct4 in ESCs, Brn2 in NPCs, p63 in esophageal cancer cells and Tcfap2l in TSCs, our ChIP-Seq data point to a small number of candidate cofactors in the stomach, including the gastric transcription factors Jun, Gata6, Foxa2 and Klf4. It will now be interesting to test if Sox2 physically associates with these proteins and co-occupies key targets in gastric progenitors.

Altogether, our study elucidates the functional and molecular role of Sox2 in stomach stem and progenitor cells, yielding new insights into (i) the basis of gastric regeneration and tumorigenesis, (ii) molecular links between Sox2 and Wnt/β-Catenin signaling, and (iii) mechanisms by which the same Sox factor may control different target genes in distinct stem cell populations. These data will inform efforts to manipulate gastric stem cell populations for regenerative therapy and may lead to novel strategies to target gastric cancer.

3.7 Materials and methods

Mice

Sox2$^{fl/fl}$ mice were generated by homologus recombination in embryonic stem cells targeting a knock-in construct containing the *Sox2*$^{fl/fl}$ allele under the control of endogenous *Sox2* regulatory elements. Correct insertion was verified by Southern blot analysis, and correctly targeted clones were injected into BDF1 blastocysts and transferred into pseudo-pregnant females. Resultant chimeric mice were bred with 129SvJae mice and germline offspring were bred to establish stable lines. *Rosa*^{CreER} (Ventura et al., 2007) *Sox2*^{CreER}; *Sox2*^{GFP} (Arnold et al., 2011) and *Lgr5*$^{GFP;Ires\text{-}CreERT2}$*; Apc*$^{fl/fl}$ *(Barker et al., 2010)* mice have been previously described and were obtained from Jackson Laboratories or provided by K. Haigis. All animal studies were

carried out following approved guidelines of the animal protocol of the Massachusetts General Hospital Cancer Center.

Treatments of mice

All treatments were initiated on adult mice 6-8 weeks old. *Collagen*$^{Sox2\text{-}ires\text{-}tdTomato}$*;Rosa*^{M2rtTA} chimeric mice were fed water containing doxycycline (Sigma, 2 mg/ml, supplemented with sucrose at 10 mg/ml) for 2 months. *Rosa*^{CreER}*; Sox2* $^{fl/fl}$ mice were injected intraperitoneally with 1mg of tamoxifen (Sigma) for five consecutive days. For short term lineage tracing, *Sox2*^{CreER}*; Rosa26*$^{lsl\text{-}YFP}$ mice were injected intraperitoneally with 2 mg of tamoxifen. All other mice were fed 1mg tamoxifen by oral gavage.

Tissue preparation and immunohistochemistry

Mouse issues 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). HE and IHC were preformed using the same protocol described in Chapter 2. Primary antibodies and dilutions used include goat anti-Sox2 (1/200, Neuromics), rabbit anti-Sox2 (1/2500, Chemicon), rabbit anti-Ki67 (1/200, Abcam), mouse anti-p63(1/300, Santa Cruz); mouse anti-β-Catenin (1/100, BD Transductions Labs).

Western blot analysis

Cell extracts were run in 15% SDS–PAGE gels. The gels were run at 90V until proteins were separated (2 h) and transferred to PVDF membranes (Bio-Rad) by running overnight at 20 volts, 4 °C in transfer apparatus(Bio-Rad). The membranes were washed in PBS-T (PBS 1 0.1% Tween) and blocked in 5% milk in PBS-T for 1 hour. The membranes were then incubated with goat anti-SOX2 (R&D) and mouse anti-β-Actin antibody(Abcam) overnight at 4 °C, washed and incubated in horseradish-peroxidase-conjugated anti secondary antibodies for 1 hour at room

temperature. Immunoblots were visualized using ECL reagent (Santa Cruz).

Gastric pylorus organoid culture

Gastric gland units were isolated from 6-week-old mouse pylorus stomach essentially as described (Barker et al., 2010). Whole gastric glands were embedded in Matrigel (BD Bioscience) and plated in 24-well plates. After polymerization of Matrigel, gastric culture medium (Advanced DMEM/F12 Supplemented with B27, N2, nAcetylcysteine Invitrogen), and 50 ng/ml Egf [Peprotech], Gastrin (10 nM [Sigma-Aldrich]), 100 ng/ml FGF10 [Preprotech], 100 ng/ml Noggin [Peprotech], 3μM CHIR99021 (Stemgent), 25% WNT3A Conditioned media, and 25% RSPONDIN Conditioned media was overlaid. For the first 2 days after seeding, the media was supplemented with 10 μM ROCK inhibitor Y-27632 (Stemgent) to prevent anoikis. To induce *Sox2* and *Apc* knockout, organoids were grown in media containing 100nM 4-OH tamoxifen (Calbiochem) for 1 week. To induce chimeric $Collagen^{Sox2\text{-}ires\text{-}tdTomato}$; $Rosa^{M2rtTA}$ organoids, 1mg/ml puromycin was added for 3 days to cultures to select for targeted cells then 2μg/ml doxycycline was added to induce ectopic expression of Sox2.

Chromatin immunoprecipitation sequencing

Gastric glands were isolated from the pylorus and crosslinked with 1% formaldehyde for 10 min at room temperature. ChIP-seq was performed as previously described (Sulahian et al., 2014), using four micrograms of goat anti-Sox2 (AF2018, R&D). We used Cistrome tools (www.cistrome.org/) to call and annotate peaks, generate wiggle files and conservation plots, identify enriched sequence motifs and linked genes, and compare data across ChIP-seq libraries. Wiggle traces were generated using the Integrative Genome Viewer. Gene ontology analysis was performed using the GREAT alogorithm (http://great.stanford.edu/). Sox2 ChIP-seq libraries were obtained from (Engelen et al., 2011) for NPCs and (Marson et al., 2008) for ESCs.

Wnt reporter assay

A puromycin-selectable lentivirus that facilitates the expression of firefly luciferase via 7 tcf binding sites was generated as well as a lentivirus that facilitates the constitutive expression of renilla luciferase. Both lentiviruses were used to infect wild type, Sox2 KO, Apc KO, and DKO gastric organoids, as previously described (Koo et al., 2013). Once infected, organoids were selected for 7 days with 1ug/mL puromycin. Following selection, organoids corresponding to each genotype were expanded and grown in gastric media with Wnt3A, Rspondin, and CHIR99021 removed. Firefly and luciferase activity was measured at 0 and 72 hours using a dual-Luciferase assay kit and luminometer (Promega, Madison, WI).

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Chapter 4

Discussion and future directions

4.1 Review of projects in this thesis

Sox2 is a well-established regulator of development. However its role in adult tissue homeostasis and cancer is still relatively unknown (discussed in Chapter 1). Applying mouse genetics and ChIP-seq methodologies, I studied the role of Sox2 in tissue homeostasis and cancer of the stomach. I focused on studying the stomach for several reasons. First, the mechanisms by which stomach homeostasis is maintained remains elusive. Furthermore, the cell of origin and genetic and molecular basis of stomach cancer are poorly understood. Thus, my work addresses several areas of need. Below, I review my results and the implications for these findings (Figure 4.1). I also propose future research directions and experiments that could address new questions that have emerged from these findings.

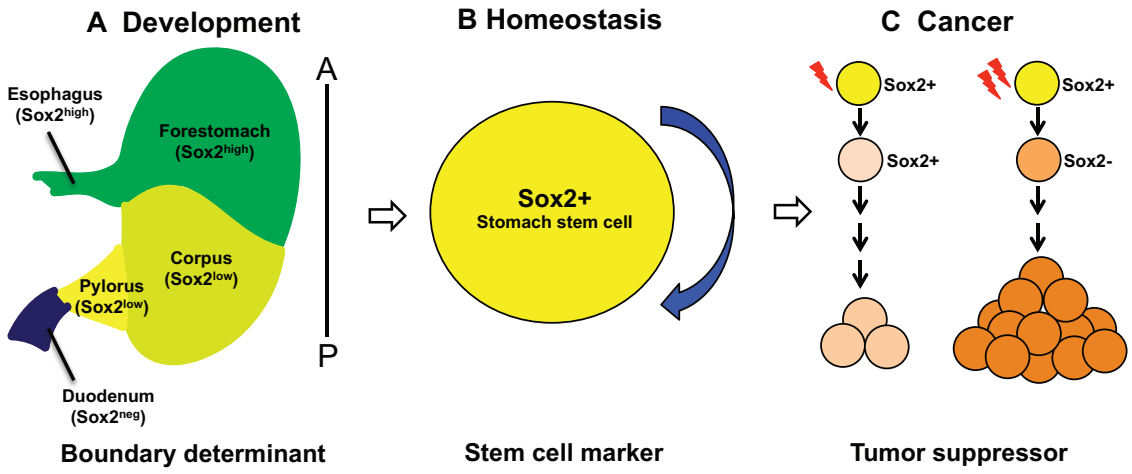

Figure 4.1 Thesis overview: Role of Sox2 in stomach development, tissue homeostasis and cancer. (A) In the development, previous studies have shown that the expression of Sox2 is important for establishing the boundary between the anterior stomach and the posterior stomach. (B) In normal stomach homeostasis, my studies have identified Sox2 as a marker of a multipotent stem cell population. (C) In stomach cancer, my studies have demonstrated that Sox2 is silenced most human adenocarcinomas and acts to suppress tumorigenesis when Wnt signaling is disrupted. Abbreviations: A, anterior, P, posterior.

Sox2 marks multipotent stomach stem cells

The stomach is an organ that requires continuous regeneration due to environmental assault from undigested material. Resident stem cell populations drive this renewal, however

the characteristics of these stem cells are poorly understood. In Chapter 2, I describe the use of mouse genetic reporter and lineage tracing studies to define and characterized a novel Sox2 expressing stem cell population in both domains of mouse glandular stomach (Arnold et al., 2011), the corpus and the pylorus. In the glandular stomach, the Sox2+ stomach stem cell population is located in the isthmus region of gastric gland, and is capable of replenishing all differentiated cell types in both the pylorus and corpus over the entire lifetime of a mouse (15 months). Interestingly, I detected Sox2 expression at the base of normal human gastric glands in a pattern that recapitulates Sox2 expression patterns in the mouse stomach, suggesting that Sox2 may mark a gastric stem cell population in humans as well. Finally, I found that Sox2 expressing cells within organoid cultures derived from gastric glands are responsible for the propagation of these cultures. All together, these studies have identified novel stem cell populations marked by Sox2 in the different domains of the stomach, and compels further study of stem cell behavior in the context of stomach homeostasis and cancer. Furthermore the development of a Sox2GFP reporter mice and the establishment of a cell culture system enriched for Sox2+ gastric stem cells provide a method to isolate and exploit the regenerative potential of these cells *in vitro*.

Recently, studies using mouse reporter alleles and lineage tracing systems identified different markers of stem cells in the intestinal tract, including Lgr5 (Barker et al., 2007), Bmi1 (Sangiorgi and Capecchi, 2008), Hopx (Takeda et al., 2011), Lrig1 (Anne et al., 2012) , mTert (Montgomery et al., 2011) and Id1(Zhang et al., 2014). Comparative lineage tracing and cell ablations studies (Takeda et al., 2011; Tian et al., 2011; Yan et al., 2012) as well as single-cell transcriptional profiling studies(Li et al., 2014) suggest that these markers can be divided into two stem cell populations; while Lgr5 marks actively cycling crypt base columnar cells (CBCs), Bmi1, Hopx, mTert, Lrig1 and Id1 appear to mark reserve stem cells around the +4 position from the crypt base (Li and Clevers, 2010; Potten et al., 2002; Yan et al., 2012). These findings suggest a two-stem cell system (Carlone and Breault, 2011; Li and Clevers, 2010), where in

normal homeostasis, the reserve stem cell gives rise to an active CBC, which bears the proliferative burden necessary to maintain homeostasis.

Like the intestine, studies using mouse reporter alleles and lineage tracing systems have identified several different stem cell populations in the stomach. These include Lgr5 (Barker et al., 2010), which marks a multipotent stem cell at the base of gastric crypts in the pylorus and Cck2r receptor (Hayakawa et al., 2015), which marks a multipotent stem cell population at the +4 position of gastric glands in the pylorus. Our studies show that cells expressing high levels of Sox2 do not co-overlap with the Lgr5-GFP+ population, suggesting that Sox2+ stem cells and Lgr5+ stem cells may be separate stem cell populations (Chapter 2). However, further experiments such as single cell transcriptional profiling of Sox2+, Lgr5+ and Cck2r+ cells will be needed to clarify if these are indeed separate populations. Furthermore, lineage tracing and cell ablation experiments could reveal the relationship between Sox2+, Lgr5+ and Cck2r+ stem cells and determine if a two-stem cell system exists in the stomach as well, where Lgr5+ cells mark a more proliferative stem cell compartment and Sox2+ cells or Cck2r+ cells, or both Sox2+ and Cck2r+ cells mark a more reserve populations.

Another question of interest is how Sox2+ stomach stem cells respond following injury. Several injury-responsive stem cell populations have been identified in the stomach. These include Villin-expressing cells in the pylorus, which reportedly give rise to gastric units in response to interferon treatment (Qiao et al., 2007), and Troy-expressing chief cells in the corpus, which are activated to replenish the epithelium in response to 5-fluoruracil treatment (Stange et al., 2013). Whether Sox2+ stem cells are also activated during these types of injuries to expand and replenish the epithelium remains to be determined.

Sox2 occupies distinct genomic regions in ESCs, NPCs and stomach epithelium

Given that Sox2 is expressed in ESCs, NPCs, TSCs and a population of cells in the stomach epithelium that contain multipotent stem cells, I investigated whether Sox2 plays a similar molecular role in all three stem cell populations. I expected Sox2 to occupy common

genomic regions in all three cell types important for the regulation of genes involved in stem cell self-renewal and proliferation. Based on ChIP-seq analysis, I observed Sox2 binding within it's own regulatory regions in all three Sox2-expression stem cell populations, suggesting a common autoregulatory feedback loop. However, the majority of all other Sox2 bound genomic regions were distinct in the three cell types (Chapter 3). This corroborates the finding that Sox2 binds distinct regions in ESCs and NPCs (Lodato et al., 2013), and suggests that Sox2 functions in a tissue specific manner to specify distinct cell states (Lodato et al., 2013).

One method by which Sox2 could selectively bind to specific targets in the stomach is through the cooperation with cofactors that bind to Sox2 and bind to DNA in the vicinity of the Sox2 binding site (Kondoh and Kamachi, 2010). These cofactors tend to be cell type specific and can direct Sox2 to specific Sox2 binding sites that may be important for a given cell state (Kondoh and Kamachi, 2010; Sarkar and Hochedlinger, 2013). For example, Sox2 associates with the Pou family member, Oct4, in ESCs to regulate genes important for pluripotency, but associates with the Pou factor, Brn2, in NPCs to regulate genes important for neural differentiation (Lodato et al., 2013). Although, Sox2 cofactors have been well established in ESCs and NSCs, little is known about Sox2 protein interactions in endoderm-derived tissue. My ChIP-seq results identified enrichment of several transcription factor motifs, and thus potential cofactors for Sox2, surrounding Sox2 peaks in the Sox2 ChIP-seq analysis (Chapter 3). These include motifs recognized by the transcription factors Gata6, Foxa2, and Klf4. Interestingly, all of these proteins are important regulators of endoderm development (Bossard and Zaret, 1998; Jacobsen et al., 2002; Katz et al., 2005; Rojas et al., 2010), and would be good candidates to test as potential cofactors for Sox2 in the stomach. Coimmunoprecipitation of Sox2 and these transcription factors from stomach epithelium would clarify if Sox2 protein binds directly or indirectly to Gata6, Foxa2 and Klf4. Comparative analyses between Sox2 ChIP-seq data and Gata6, Foxa2 and Klf4 ChIP-seq data from stomach epithelium would unveil whether these factors bind to similar regions of DNA in stomach epithelium as would be expected for cofactors.

Furthermore, comparative loss of function and over expression experiments coupled with RNA-sequencing, would provide further functional evidence that Sox2 together with these factors co-regulate genes to specify cell fate in the stomach epithelium.

Sox2 is dispensable for stomach epithelial homeostasis

Sox2 is a master regulator of stem cell renewal and cell proliferation in several different stem cell types (Sarkar and Hochedlinger, 2013), however it's function in adult tissue homeostasis is unknown. Although Sox2 marks a mulitpotent stem cell population in the adult stomach, my data show that loss of Sox2 does not change stomach tissue composition nor does it affect organoid growth (Chapter 3). These results are surprising and indicate that there maybe other factors that are functionally redundant or compensate for loss of Sox2. Sox2 belongs to a subfamily of Sox factors, SoxB1 factors, which share biochemical and functional properties. Although other SoxB1 members, Sox1 and Sox3, are not expressed in the stomach (data not shown), another more distantly related Sox factor, Sox9, is highly expressed in the Lgr5+ stem cell compartment (Barker et al., 2010). Conducting RNA-sequencing that compares the transcriptional differences between wild type and Sox2 null gastric tissue could unveil which factors or pathways may compensate for the loss of Sox2.

Sox2+ cells are potent cells of origin in Wnt driven tumorigenesis

Accumulating evidence suggests that cancer of distinct subtypes within an organ may develop from different cells of origin, which acquire the first genetic hits that initiates cancer (Visvader, 2011). Identifying the cells of origin for different cancer types holds tremendous therapeutic potential by facilitating earlier detection of malignancies, better prediction of tumor behavior and more targeted, cancer-type specific therapies. To identify cells of origin in solid tumors, lineage tracing systems have been utilized to follow certain cell populations as they undergo transformation. However, a major caveat with several of the existing lineage tracing systems for the stomach is that the Cre recombinase driver used is also expressed in the more

rapidly dividing intestine. This often leads to a fatal tumor burden in the intestine before tumors in the stomach can fully develop. I showed that our Sox2+ cell lineage tracing system marks cells in the stomach but not in the intestine (Chapter 2). This allowed me to use the Sox2+ cell lineage tracing system to conduct long term tracing, up to 1 year after tumor initiation in Sox2+ cells. Indeed, my Sox2+ cell lineage tracing studies demonstrated that Sox2+ cells are important cells of origin in stomach cancer when Wnt signaling is disrupted (Chapter 3). Given that animals survived for one year, our mouse model may represent a useful system in which to study the earliest events of tumor formation and to identify additional facilitators of gastric cancer progression. Finally, I established an organoid cell culture system for transformed Sox2+ stomach stem cells. Establishment of this culture system could prove to be a useful *in vitro* system to conduct drug screens aimed at identifying more targeted therapies got cancers with a similar histology to adenomas originating from Sox2+ cells. With the establishment of these models, now several different questions can be tested to clarify Sox2's role in stomach cancer including: 1) do other tumor-initiating events, such as activation of Kras or deletion of *p53* in Sox2+ cells gives rise to more aggressive gastric cancers; 2) how Sox2+ cells relate to other cells of origin in the stomach and 3) what pathways make Sox2+ cells susceptible to tumorigenesis.

Sox2 is a tumor suppressor in gastric cancer

As summarized in Chapter 1, several studies demonstrate that Sox2 promotes proliferation of different types of cancer and acts as an oncogene in human esophageal and lung squamous cell carcinoma. However there is much controversy with regard to the role of Sox2, itself, in stomach cancer. Surprisingly, my data showed that overexpression of Sox2 does not activate cell proliferation in the glandular stomach (Chapter 3). Furthermore, I showed that loss of Sox2 enhances adenoma formation and organoid growth when Wnt signaling is activated (Chapter 3). I also found a correlation between loss of Sox2 and human gastric cancer (Chapter 3). Together, these results demonstrate for the first time that Sox2 can suppress

tumorigenesis. I hypothesize that Sox2 acts mechanistically in its tumor suppressor role by attenuating Wnt signaling. SOX2 ChIP-seq studies in stomach epithelial cells supported this hypothesis by demonstrating that SOX2 binds to enhancers of several important Wnt regulatory genes, including *Tcf4*, *Tel1* and *Ctnnb1* (Chapter 3 and Appendix). Furthermore, Wnt reporter assays in organoids demonstrated that Wnt signaling increased dramatically in cells lacking Sox2 and wild type Apc compared to cells with only one type of mutation. These results supports the notion that the presence of Sox2 may be limiting for efficient proliferation and tumor growth when Wnt/b-catenin signaling is hyperactivated, as is the case in Apc-mutant cells. Of note, this type of Wnt signaling repression by Sox2 has been suggested by another study in NPCs (Hagey and Muhr, 2014). In summary, my studies have demonstrated, for the first time, that Sox2 acts as a tumor suppressor by regulating Wnt signaling in an Apc model of tumorigenesis.

My studies suggest that Sox2 acts to inhibit Wnt signaling, however there may be additional mechanism by which Sox2 suppresses tumorigenesis. For example, Sox2 deletion may change the identity of the cell of origin, in stomach cancer, to one that is more susceptible to transformation. Such a potential change in cell fate can be tested by conducting short-term lineage tracing experiments of cells that have lost Sox2 in adenomas. These cells can then be isolated and characterized by RNA sequencing to determine if their transcriptome resembles the transcriptome of the cell of origin or another cell type. Another possibility is that Sox2 may regulate the transcription of genes important for tumorigenesis. Indeed, I noticed that Sox2 binds nearby genes that are reportedly mutated in gastric cancer (e.g., *VegfA, ErbB2, Fgfr2, Gata4/6, Kras, Apc, Smad2,* data not shown). Thus, it would be of interests to test if the expression of these genes changes when Sox2 is deleted during tumorigenesis.

It is striking that Sox2 can act both as an oncogene and a tumor suppressor in different types of cancer, suggesting that the role of transcription factors in cancer initiation events may depend on several factors including the other oncogenic mutations involved in cell

transformation and the tissue and cell type of origin. Therefore, clarifying the mechanism by which Sox2 either promotes or suppresses tumorigenesis may allow for better predictions of how disruption of Sox2 will affect the outcome of specific types of cancer. I provide evidence that Sox2 suppresses tumorigenesis by regulating Wnt signaling. In contrast, Sox2 promotes tumorigenesis in the lung, esophagus and skin by binding to, and perhaps transcriptionally activating, genes involved in tumour stemness, survival, proliferation, adhesion, and invasion (Bass et al., 2009; Boumahdi et al., 2014). In this way, Sox2 can have contrasting effects on different types of tumorigenesis (Figure 4.2).

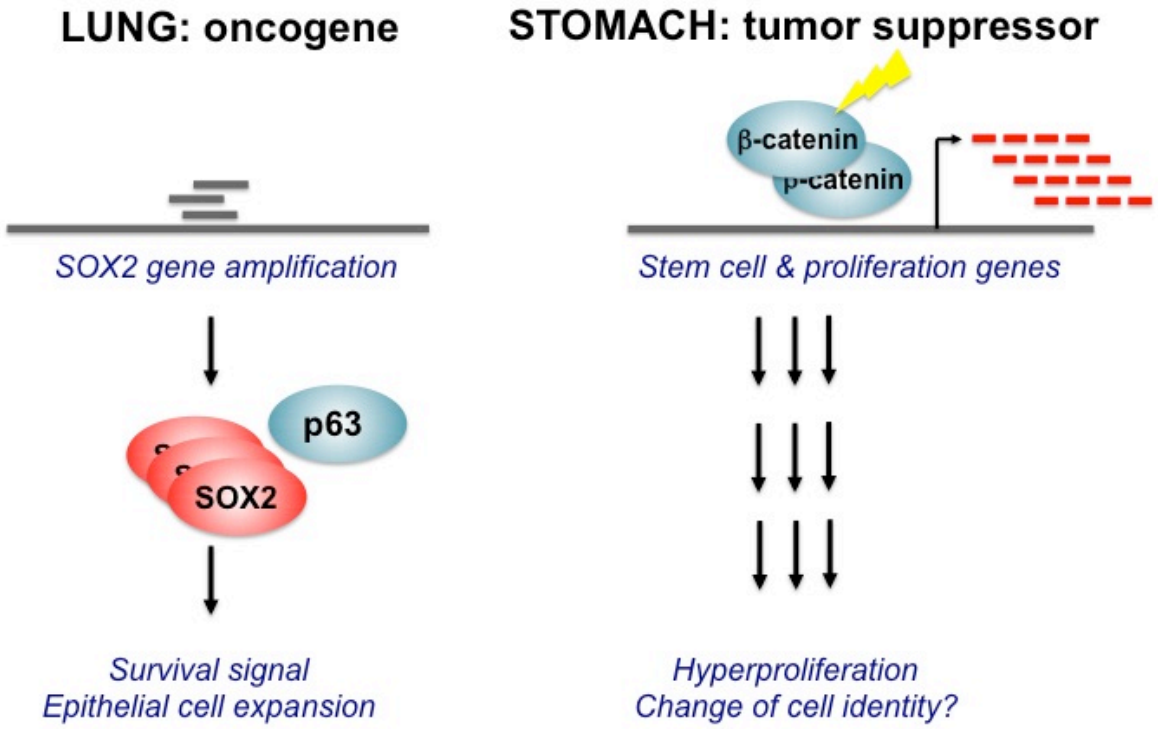

Figure 4.2. Model for context-dependent role of Sox2 in cancer. (Left) In about ~30% of human squamous cell lung carcinomas, *SOX2* is an amplified. SOX2 acts with P63 to promote cell survival signals and epithelial cell expansion in these cancers (Bass et al., 2009; Watanabe et al., 2014). (Right) In human stomach adenocarcinomas, Sox2 is often lost and may act as a tumor suppressor by regulating stem cell and proliferation genes (discussed further in Chapter 3). (Courtesy of Konrad Hochedlinger)

4.2 Conclusions

In this thesis, I discuss the role of Sox2 in stomach tissue homeostasis and tumorigenesis. My findings have elucidated the cellular, functional and molecular role that Sox2 plays in stomach stem and progenitor cells, leading to important new insights into (i) the biology of gastric regeneration and tumorigenesis, (ii) the molecular links between Sox proteins and Wnt-beta-catenin signaling, and (iii) the mechanisms by which the same Sox factor may achieve differential target gene regulation in distinct stem cell populations. My findings reveal that the function of Sox2 is highly tissue and context dependent, and that caution should be taken if Sox2 is used as a target for therapeutic application such as the treatment of cancer.

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Appendix

Supplementary information for Chapter 3

A.1 Supplementary data

Sox2 suppresses forestomach papilloma formation

In addition to looking for adenoma formation in the pylorus of tamoxifen treated Sox2$^{CreERT2/wt}$;*Apc*$^{fl/fl}$ (Apc KO) mice and Sox2$^{CreERT/fl}$;*Apc*$^{fl/fl}$ (DKO) mice (described in Chapter 3), we also looked for signs of tumorigenesis in the forestomach. Forestomachs from Apc KO mice, 7 months after tamoxifen treatment, contained hyperproliferative zones (Figure A.1), however they did not contain any noticeable tumors. In contrast, forestomachs from (DKO) mice contained papilomas (Figure A.2). Thus, Sox2 suppresses tumorigenesis when Wnt signaling is disrupted in the forestomach in addition to the glandular stomach.

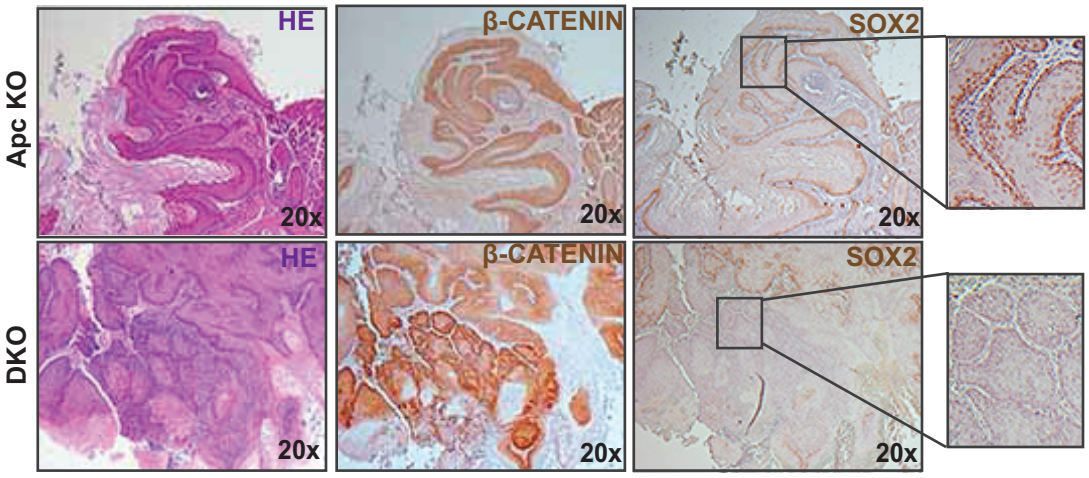

Figure A.1. Long-term loss of Sox2 inhibits forestomach papilloma formation. H&E staining and IHC for β-Catenin and Sox2 on consecutive sections from forestomach tissue isolated from *Sox2*$^{CreER/WT}$; *APC*$^{flox/flox}$ (Apc KO) and *Sox2*$^{CreER/flox}$; *APC*$^{flox/flox}$ (DKO) mice 7 months after TAM induction. Images show forestomach papilloma in DKO forestomach and hyperproliferation in Apc KO forestomach. Insets highlight Sox2+ basal cells in the Apc KO forestomach and Sox2- basal cells in DKO forestomach

The majority of Sox2 targets in ESCs, NPCs, and gastric epithelium have unknown functions in stem cells

Given that Sox2 is expressed in many different stem cell populations, we were curious to see which common set of genes Sox2 targets, and perhaps regulates, in these different stem cell populations. We were able to gain access to Sox2 ChIP-seq data from ESCs(Marson et al., 2008) and NPCs(Engelen et al., 2011) and compared these data sets to Sox2 ChIP-seq data from gastric epithelium using Cistrome tools (www.cistrome.org/). I found Sox2 targeted regulatory regions of 36 common genes in ESCs, NPCs, and gastric epithelium (Table A.1). With the exception of Notch1, the function of most of these genes in stem cell populations is unknown. It would be interesting to test the function of these genes in ESCs, NPCs and Sox2+ stomach stem cells, given that they are commonly regulated in all three stem cell types.

Table A.1 Common targets of Sox2 in ESCs, NPCs, and gastric epithelium.

Gene	Description	Function in stem cells	Citation
Acap1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	NA	
Arhgap12	Rho GTPase activating protein 12	NA	
Arrdc3	Arrestin domain containing 3	NA	
C2cd3	C2 calcium-dependent domain containing 3	NA	
Cabyr	Calcium binding tyrosine-(Y)-phosphorylation regulated	NA	
Ccar1	Cell division cycle and apoptosis regulator 1	Regulates adipocyte differentiation	(Ou et al., 2014)
Cenpl	Centromere protein L		
Crip2	Cysteine rich protein 2		
Etv1	Ets variant 1	Knockdown suppresses mesenchymal differentiation	(Chen et al., 2014)
Fgd4	FYVE, RhoGEF and PH domain containing 4		
Gadd45a	Growth arrest and DNA-damage-inducible, alpha	Loss leads to increased hematopoietic stem cell proliferation after injury	(Chen et al., 2014)
Gfod1	Glucose-fructose oxidoreductase domain containing 1		

Table A.1. (Continued)

Gene	Description	Function in stem cells	Citation
Hbp1	**HMG-box transcription factor 1**		
Id1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Loss leads to embryonic stem cell differentiation; marks intestinal stem cells and regulates stem cell response to injury	(Romero-Lanman et al., 2012);(Zhang et al., 2014)
Klf7	Kruppel-like factor 7 (ubiquitous) [*Mus musculus*	NA	
Lyrm5	LYR motif containing 5	NA	
Marcks	Myristoylated alanine rich protein kinase C substrate	Required for chemotaxis of mesenchymal stemc ells	(Miller et al., 2010)
Muc1	Mucin 1, cell surface associated	NA	
N4bp2	NEDD4 binding protein 2	NA	
Notch1	Notch 1	Crucial regulator of multiple stem cell populations	Reviewed in (Liu et al., 2010)
Oaz2-ps	Ornithine decarboxylase antizyme 2, pseudogene	NA	
Pask	PAS domain containing serine/threonine kinase	NA	
Plekha3	Pleckstrin homology domain containing, family A	NA	
Psmd8	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	NA	
Rps7	Ribosomal protein S7	NA	
Rsbn1	Round spermatid basic protein 1	NA	
Smg7	SMG7 nonsense mediated mRNA decay factor		
Stmn1	Stathmin 1	NA	
Tbce	Tubulin-specific chaperone E	NA	
Trim8	Tripartite motif containing 8	NA	
Txndc12	Thioredoxin domain containing 12 (endoplasmic reticulum)	NA	
Ylpm1	YLP motif containing 1	NA	
Ywhag	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	NA	
	Zinc finger protein 113	NA	
Zfp146	Zinc finger protein 146	NA	
Zfp428	Zinc finger protein 428	NA	

Sox2 targets Wnt pathways genes in gastric epithelium

GO analysis of Sox2 ChIP-seq data from gastric epithelium, using the GREAT algorithm, revealed that Spx2 bound to regulatory regions of genes in the Wnt pathway (Table A.2). Although our Wnt reporter assays (Chapter 3) indicate that Sox2 may repress Wnt signaling by binding to Tcf binding sites and repressing transcription, it remains to be determined which Wnt genes Sox2 regulates.

Table A.2. Wnt pathways targets of Sox2 in gastric epithelium

Gene	Description	Peak location (distance to TSS)
Aes	Amino-terminal enhancer of split	-13100, -10448
Apc	Adenomatosis polyposis coli	+14611
Bcl9	B-cell CLL/lymphoma 9	-10785
Btrc	Beta-transducin repeat containing E3 ubiquitin protein ligase	-156
Ccnd1	Cyclin D1	+10237
Ccnd2	Cyclin D2	-1642
Ctbp2	C-terminal binding protein 2	-14580
Ctnnb1	Catenin (cadherin associated protein), beta 1	-14003, +18735
Ctnnbip1	Catenin beta interacting protein 1	-27987
Cxxc4	CXXC finger protein 4	-19019, -3915, +3297, +3576
Fbxw11	F-box and WD repeat domain containing 11	-6451
Frat1	Frequently rearranged in advanced T-cell lymphomas 1	-758, +8136, +9010
Fzd3	Frizzled homolog 3	-4053
Fzd4	Frizzled homolog 4	-23108, -12452
Fzd5	Frizzled homolog 5	-579
Gsk3a	Glycogen synthase kinase 3 alpha	-596
Gsk3b	Glycogen synthase kinase 3 beta	+11619

Table A.2. (Continued)

Gene	Description	Peak location (distance to TSS)
Nlk	Nemo like kinase	-547
Ppp2ca	Protein phosphatase, catalytic subunit, alpha isoform	-20735, +25843
Rpl13a	Ribosomal protein L13A	-258
Senp2	SUMO/sentrin specific peptidase 2	-13857, -11298
Sfrp4	Secreted frizzled-related protein 4	+8019
Slc9a3r1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	-127
Tcf7l1	Transcription factor 7 like 1 (T cell specific, HMG box)	-6090
Tle1	Transducin-like enhancer of split 1, homolog of Drosophila E(spl)	+1840
Tle4	Transducin-like enhancer of split 4, homolog of Drosophila E(spl)	+22420
Wnt11	Wingless-type MMTV integration site family, member 11	+11821

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