Multiple roles for Sox2 in the developing and adult mouse trachea

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The esophagus, trachea and lung develop from the embryonic foregut, yet acquire and maintain distinct tissue phenotypes. Previously, we demonstrated that the transcription factor Sox2 is necessary for foregut morphogenesis and esophagus development. We show that Sox2 is also required for the normal development of the trachea and lung. In both the embryo and adult, Sox2 is exclusively expressed in the epithelium of the trachea and airways. We use an Nkx2.5-Cre transgene and a Sox2 floxed allele to conditionally delete Sox2 in the ventral epithelial domain of the early anterior foregut, which gives rise to the future trachea and lung buds. All conditional mutants die of respiratory distress at birth, probably due to abnormal differentiation of the laryngeal and tracheal cartilage as a result of defective epithelial-mesenchymal interaction. About 60% of the mutants have a short trachea, suggesting that the primary budding site of the lung shifts anteriorly. In the tracheal epithelium of all conditional mutants there are significantly more mucus-producing cells compared with wild type, and fewer basal stem cells, ciliated and Clara cells. Differentiation of the epithelium lining the conducting airways in the lung is abnormal, suggesting that Sox2 also plays a role in the differentiation of embryonic airway progenitors into specific lineages. Conditional deletion of Sox2 was then used to test its role in adult epithelium maintenance. We found that epithelial cells, including basal stem cells, lacking Sox2 show a reduced capacity to proliferate in culture and to repair after injury in vivo. Taken together, these results define multiple roles for Sox2 in the developing and adult trachea.

KEY WORDS: Sox2, Foregut development, Trachea, Epithelial-mesenchymal interaction, Epithelial differentiation, Injury-repair, Mouse

INTRODUCTION

The anterior foregut of the mammalian embryo initially consists of a simple epithelial tube surrounded by mesoderm. Through a wellorchestrated series of developmental processes this simple tube gives rise to the esophagus, trachea and lungs, each containing epithelial and mesenchymal cells with a characteristic organization and phenotype. A major challenge is to define the genetic and cellular mechanisms underlying the patterning, morphogenesis and differentiation of these different foregut-derived organ systems. Work over the past few years has uncovered important roles for signaling pathways and downstream transcription factors that mediate the reciprocal interactions between the epithelium and mesenchyme (Cardoso and Lu, 2006; Miller et al., 2004; Shannon and Hyatt, 2004). Previous studies from our lab provided evidence that the transcription factor Sox2 plays a key role in the early dorsoventral patterning of the foregut and in the subsequent development of the esophagus (Que et al., 2007). We now extend these studies to identify crucial roles for Sox2 in the development of the tracheal epithelium and, indirectly, in the differentiation of the surrounding mesenchyme.

Sox2 belongs to a family of evolutionarily conserved transcription factors containing a Sry-related high mobility group (HMG) box. In the early mouse embryo and in human and mouse embryonic stem (ES) cells, Sox2 synergizes with its partners to regulate the transcription of many other transcription factors, including Nanog and Oct4 (Pou5f1 – Mouse Genome Informatics) that function within a network to maintain self-renewal and pluripotency (Chen et al., 2008b). Later in development, Sox2 is expressed in the endodermal epithelium of the tongue, esophagus, trachea and lung, and plays crucial roles in the differentiation and morphogenesis of these organ systems (Gontan et al., 2008; Ishii et al., 1998; Okubo et al., 2006; Que et al., 2007). In the case of foregut development, Sox2 is initially expressed at highest levels in the dorsal epithelium of the undivided tube that will give rise to the esophagus. It is expressed at relatively lower levels in the ventral region that will give rise to the trachea and lung buds (Que et al., 2007). We have shown a dose-dependent role for Sox2 in the separation of the foregut into the esophagus and trachea. Sixty percent of Sox2<sup>−/−</sup> hypomorphic mutants with barely detectable levels of Sox2 protein have a defect known as esophageal atresia and tracheal-esophageal fistula (EA/TEF) (Que et al., 2006; Que et al., 2007). In the remaining 40% of hypomorphic mutants with ~17% of wild-type Sox2 levels, the foregut does separate into esophagus and trachea. However, the esophagus has an abnormal phenotype resembling a condition in humans known as mucus metaplasia (Que et al., 2006). Consistent with an important role of Sox2 in the differentiation of the mucus-producing cells, reduced levels of SOX2 have been found in regions of intestinal metaplasia in both the esophagus and antral mucosa of human patients with Barrett’s esophagus (Chen et al., 2008a; Tsukamoto et al., 2004).

At birth, the trachea is surrounded by a segmental array of C-ring cartilages along the ventral side and by smooth muscle dorsally. The tracheal lumen is lined by a pseudostratified epithelium composed of three major cell populations [basal, ciliated, and secretory (Clara-like) cells] and two minor ones (neuroendocrine and mucus-producing cells). Several signaling pathways and downstream transcription factors play crucial roles in specifying these different cell lineages during development. Perturbation of these signaling molecules and transcription factors inevitably affects the development of the trachea (Metzger et al., 2008; Mucenski et al., 2005). For example, loss of the transcription factor p63 (Tcf1 –
Mouse Genome Informatics) results in the depletion of basal cells in the trachea and the development of an epithelium composed mainly of ciliated cells (Daniely et al., 2004). By contrast, when Sox2 is overexpressed in the lung epithelium, p63-positive cells are ectopically present in the peripheral airways, accompanied by an increased number of neuroendocrine cells and reduced branching (Gontan et al., 2008). Although the emerging evidence suggests that Sox2 plays important roles in the development of the respiratory system, the exact role of this transcription factor in the trachea remains unclear.

To address this problem we have employed an Nkx2.5-Cre transgene that is expressed in the early ventral foregut to delete Sox2. In the majority of conditional mutants, separation of the foregut into esophagus and trachea and branching morphogenesis of the lung proceeds normally. However, the trachea is shorter than normal, with abnormal differentiation of mesenchymal and epithelial cells. With a temporally controllable CreER system, we further show that Sox2 is required for maintenance of the tracheal epithelium in the adult mouse. Loss of Sox2 impairs the ability of epithelial cells to repopulate the denuded trachea after injury.

MATERIALS AND METHODS

Mouse

The mouse line with a Sox2<sup>cond</sup> allele in which the only exon of Sox2 is flanked by loxp sites was derived by Taranova et al. (Taranova et al., 2006) and maintained on a mixed (129/SvEv × C57Bl/6) genetic background. No abnormal phenotype was observed in unrecombined homozygotes at any stage. The Nkx2.5-Cre mouse line was generated by Dr Robert Schwartz (Moses et al., 2001). Git(Rosa26)<sup>cre</sup>/<sup>fl</sup> (referred to as R26R) and Tg(CMV-cre/ESR1)<sup>119</sup> (referred to as CMV-CreER) lines were obtained from the Jackson Laboratory. To activate Cre recombinase, Tamoxifen (Tmx, 0.2 mg/g body weight, dissolved in corn oil) was administered intraperitoneally (Rawlins et al., 2007). Airway injury was initiated by exposing adult male mice to 500 ppm SO<sub>2</sub> in air for 3 hours (Rawlins et al., 2007).

Tissue preparation and immunostaining

The trachea and lungs were dissected in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 3-4 hours at 4°C. After dehydration they were embedded in paraffin for sectioning and sectioning see Que et al. (Que et al., 2006).

For Periodic acid-Schiff (PAS) and alcian blue staining

For Periodic acid-Schiff staining, sections were treated with or without amylase from Aspergillus oryzae, Fluka 10065: 0.5% in distilled water at 37°C for 15 minutes, then stained in Schiff’s reagent (Sigma, S-5133). For alcian blue staining, sections were treated with 3% acetic acid solution for 3 minutes, stained in alcian blue for 30 minutes and counterstained with eosin. For whole-mount staining of cartilage, dissected tracheas were fixed in 95% ethanol for 18 hours followed by overnight staining with 0.03% alcian blue dissolved in 80% ethanol and 20% acetic acid.

Cultures of tracheal epithelial cells

Epithelial cells were isolated from tracheas of adult CMV-CreER mice after three Tmx injections over a period of 5 days (one injection every other day). For isolation, the trachea was washed and then treated with 20 U/ml Dispase (BD Biosciences, Bedford, MA, USA) in PBS for 22 minutes at room temperature. Then the epithelial layer was peeled off with fine forceps and incubated in 0.1% trypsin with 0.04% EDTA (diluted with PBS from 0.5% trypsin with 0.2% EDTA stock solution, GibcoB) for 18 minutes at 37°C to dissociate it into single cells. The cells were filtered through a cell strainer (pore size 40 μm, BD Biosciences) and cultured in 35 mm culture dishes coated with 120 μl 1.75 mg/ml rat tail type I collagen (R&D Systems) at 37°C in 5% CO<sub>2</sub>. The cell density was 8×10<sup>5</sup> cells/dish. The medium contained DMEM/F-12 (1:1, GibcoB), Insulin-Transferrin-Selenium-A Supplement (1:100 diluted from stock solution, Invitrogen 51300-044), 100 U/ml penicillin G, 100 μg/ml streptomycin, 4 mM L-glutamine (GibcoB), 10 ng/ml heparin (Sigma) and 30 μg/ml bovine pituitary extract (Invitrogen) with 20 ng/ml Fg2 and 25 ng/ml Egf (R&D Systems). Medium was refreshed every three days. For each experiment, cultures were repeated at least three times. After culture, cells were stained with Wright blue or fixed with 4% PFA for 10 minutes at 4°C and then stained with antibodies.

BrdU labeling

For embryonic (E) day 18.5 embryos, BrdU (Amersham Bioscience, UK) was injected intraperitoneally into pregnant females at a dose of 10 μl/g body weight. For postnatal (P) day 0 newborns and adults the same dose of BrdU was injected intraperitoneally. After 2 hours, tissues were collected and fixed in 70% ethanol for 1 hour and then stained with 4% PFA in PBS (pH 7.4). For immunohistochemistry, anti-BrdU antibody (1:500, mouse monoclonal, Sigma clone Bu33) was used, in combination with a MOM Kit (Vector Labs).

Quantitative analysis

For calculating the proportion of proliferating (BrdU positive or Ki67 positive), basal (p63 positive), Clara (Scgb1a1 positive), ciliated (acetylated α-tubulin positive) and mucus-producing (alcian blue positive) cells in the total epithelium, longitudinal sections (ten sections per trachea) through the same level of each trachea were selected for measurement. Tissue images were obtained using an Axiophot light microscope (Carl Zeiss, Germany) or a Leica ASMDW laser scanning confocal microscope. Cells were manually counted on a z-series of optical sections, and multiple optical sections were examined to distinguish cell boundaries.

RT-PCR

Total RNA was extracted from each tissue by RNeasy (Qiagen) and cDNA was synthesized from 500 ng total RNA by SuperScript (Invitrogen). Semi-quantitative PCR for specific genes was performed using the following primer sets (5’-3’): Hoxa5, AGCAGCAGAGGGGTTGGG, GCCGTTACAGTGGGTTGGA; Raldh2, ATCCACCGACCTCA-ATCC, GCAGTTGGTTGCTGTGTT; Shh, GTCCCCCTGCTC-CCGTCTCA, GTTGAGAGAATGAAACAT; Pocl, CCTGGCTCT- GTAGTACCGTT, CATTTGAGAACCACCAAGCACG; Sox2, AACG-GTCGCCCCACCTACAGC, CAGGGGCGATTGCTGTATTGG; Sox9, GAGGCCACAGAAGACAGCTCA, CGCGCGTGGAGAAGAAG- CAT; and β-actin, GTGTCACCCAGGGCATTGATGG, GCAATGCT- CTGGTGATCTGTTG.

RESULTS

Expression of Sox2 during trachea and lung development

We have previously reported a dynamic expression of Sox2 in the epithelial cells of the early foregut. At E9.5, before foregut separation, both Sox2<sup>Egfp</sup> and Sox2 protein are expressed at...
Deletion of Sox2 using Nkx2.5-Cre in the early ventral foregut endoderm results in a perinatally lethal phenotype

In the trachea of Sox2 hypomorphic (Sox2<sup>EGFP</sup>/COND) mutants (Que et al., 2007), Sox2 protein can still be detected in the epithelium by immunohistochemistry, even though the level of protein is dramatically decreased compared with wild type (data not shown). To study the effect of complete Sox2 deletion in the ventral epithelium that gives rise to the trachea and lung, we used the Nkx2.5-Cre transgene in combination with the floxed Sox2<sup>COND</sup> allele. Nkx2.5-Cre drives recombination in the ventral epithelial cells of the undivided foregut earlier than E9.5. This is shown by lacZ expression in Nkx2.5-Cre;R26R embryos (Fig. 1G). In addition, recombination of the reporter is also seen in the ventral mesenchyme where Nkx2.5-Cre is active (Fig. 1G). At E10.5, E12.5 and E16.5 lacZ expression is maintained in the trachea and lung, which are derived from the ventral foregut (Fig. 1H,I; see Fig. S1D in the supplementary material). In both tissues, lacZ is expressed in the epithelial and mesenchymal compartments. However, it is important to note that Sox2 expression is confined to the epithelium (see Fig. 1F), so that the Nkx2.5-Cre expression in the mesenchyme of Sox2<sup>COND/COND</sup> embryos is functionally irrelevant.

To delete Sox2 in the ventral epithelium of the foregut, Sox2<sup>COND/COND</sup> mice were mated with Nkx2.5-Cre;Sox2<sup>EGFP</sup> mice. None of the Nkx2.5-Cre;Sox2<sup>EGFP</sup> control embryos examined at any stage has visible defects in foregut and cartilage development, showing that Cre by itself is not toxic. By contrast, at P0 all Nkx2.5-Cre;Sox2<sup>COND/COND</sup> compound mutants gasp for air and die within 3 hours, apparently owing to the abnormal development of laryngeal and tracheal cartilages (see below). As expected, at E9.5 Sox2 protein is undetectable in the ventral foregut epithelium of compound mutants (Fig. 1J), and only very few Sox2-positive cells remain in the trachea and distal airways at E15.5 (Fig. 1K).

Analysis of Nkx2.5-Cre;Sox2<sup>COND/COND</sup> embryos showed that the overall size and shape of conditional mutant lungs, as well as the size and wet weight of individual lobes are similar to Nkx2.5-Cre;Sox2<sup>COND/+</sup> controls (Fig. 2A and data not shown). However, 60% of conditional mutants have a shorter trachea and longer main bronchi than controls (Fig. 2B). Measurement of the individual lengths of the trachea and left main bronchus indicates a significant difference in control versus mutant (1.9 mm versus 1.3 mm for trachea, P<0.01; 1.0 mm versus 1.5 mm for left main bronchus, P<0.01; n=5 for each group; Fig. 2C). Nevertheless, the combined length of the trachea and the left main bronchus is not significantly different (P>0.05).

We previously reported the formation of TEF in 60% of Sox2<sup>EGFP/COND</sup> hypomorphic mutants on the mixed (129/SvEv X C57BL/6) background (Que et al., 2007). In the present study 10% (3/30) of mutants at P0 also have TEF. Significantly, all those mutants were generated by mating the same Nkx2.5-Cre;Sox2<sup>COND/+</sup> males (129/SvEv X C57BL/6; see Fig. S2B in the supplementary material). This suggests that modifier loci in different genetic backgrounds can alter the severity of the phenotype caused by conditional deletion of Sox2 in the ventral epithelium before foregut separation.

Abnormal cartilage development in tracheal mesenchyme of Nkx2.5-Cre;Sox2<sup>COND/COND</sup> embryos

Skeletal preparations and histological analysis revealed disorganized laryngeal and tracheal cartilages in compound mutants (Fig. 2D and see Fig. S2A,B in the supplementary material). The cricoid and thyroid cartilages are the two major
components of the larynx, with the thyroid cartilage normally being the larger of the two. In all mutants analyzed by whole-mount alcin blue staining at P0 (n=12), the thyroid cartilage was variably abnormal in shape (Fig. 2D and see Fig. S2A,B in the supplementary material). In some mutants, the cricoid cartilage was larger than the thyroid cartilage due to fusion with the first tracheal ring. This defect resembles the cartilage defects in Hoxa5 null mutants (Aubin et al., 1997). In addition, the tracheal cartilage rings lose their classic C-shape, and in mutants with a short trachea the number of cartilage rings is reduced (10-11 in wild type and 3-8 in mutant; Fig. 2D). In comparison with the reduced size of the cartilage, the dorsal domain of smooth muscle cells extends more ventrally in the mutant trachea at E15.5 and P0 (Fig. 2E,F; see Fig. S2C,D in the supplementary material).

Although Sox2 is also deleted in the major airways of the lung (Fig. 1K), patterning of the smooth muscle in both the airways and blood vessels is not altered (data not shown).

Previous studies have shown that several genes regulate the differentiation of laryngeal and tracheal mesenchymal cells (Aubin et al., 1997; Chisaka and Capecchi, 1991; Mendelsohn et al., 1994; Vermot et al., 2003). Using PCR primers for transcripts of such genes, we were unable to detect any significant change of expression of Hoxa5 and Aldh2 (Aldh1a2 – Mouse Genome Informatics) in E11.5 mutant tracheae by RT-PCR (Fig. 2I). However, the level of Shh expression is greatly reduced after Sox2 ablation. Consistent with reduced Shh signaling, the transcript levels of its downstream targets Sox9 and Ptc1 (Pch1 – Mouse Genome Informatics) are also downregulated (Fig. 2I). At the protein level, a reduction of Shh is detected as early as E9.5 in the unseparated foregut, whereas the expression of Shh in the floor plate, notochord and primary lung buds is unaffected (Fig. 2G,H and data not shown).

**Deletion of Sox2 in the epithelium leads to abnormal differentiation, including excessive generation of mucus-producing cells**

The epithelium of the newborn mouse trachea contains ciliated, secretory (Clara-like) and mucus-producing columnar cells. It also contains a few neuroendocrine cells and a population of p63-positive basal cells making up about 30% of the total epithelium. There is strong evidence that these basal cells are the stem cells of the trachea (Hong et al., 2004; Schacht et al., 2003; Schoch et al., 2004). Previously, we have shown a reduction of p63-positive basal cells in the separated esophagus and forestomach of Sox2 hypomorphic mutants (Que et al., 2007). We also noted that the proportion of p63-positive basal cells within the total epithelium is reduced in the tracheae of these mutants at E18.5 and P0 (data not shown). In agreement with this, Nkx2.5-Cre;Sox2COND/COND mutants have a lower proportion of p63-positive cells than wild type. This difference can be detected as early as E13.5 (Fig. 3A-G). A similar reduction is seen using two other basal cell markers, keratin 5 and T1-α (Schacht et al., 2003; Schoch et al., 2004) (Fig. 3E,F and data not shown).

In addition, the proportions of ciliated (acetylated α-tubulin-positive) and Clara (Scgb1a1-positive) cells are both greatly reduced in P0 mutants (Fig. 4A,B; P<0.01). By contrast, the proportion of alcin blue- and PAS-positive mucus-producing cells is significantly increased (Fig. 4C,F; P<0.01), and these cells are also reactive for Muc5ac staining (Fig. 4G,H). Other studies have implied that the transcription factor Foxa2 represses the expression of the Muc5ac gene (Wan et al., 2004). Consistent with this, the proportion of Foxa2-positive cells is reduced in the mutant compared with the wild-type control (Fig. 4I,J and see Fig. S2E,F in the supplementary material). Further colocalization analysis revealed that the alcin blue-positive cells and Foxa2-positive cells are two distinct populations (see Fig. S2G in the supplementary material). In addition, transmission EM analysis of mutant tracheae reveals many epithelial cells containing apically located membrane-bound vesicles, typical of mucus-containing vesicles (see Fig. S2H in the supplementary material) (Evans et al., 2004). When quantitatively different cell populations we noted that ~15% cells are not positive for any of the tracheal epithelial markers p63, Scgb1a1, acetylated α-tubulin or calcitonin gene-related peptide (CGRP) (Fig. 4K). Of note, in larger bronchi and mutant lungs in which Sox2 has been deleted (Fig. 1K) we also observed a significant reduction of p63-positive, ciliated and Clara cells, but no increase in alcin blue-positive cells (see Fig. S2L in the supplementary material and data not shown).
In spite of these dramatic changes in the cellular composition of the trachea, we detected no significant change in the rate of proliferation of the total epithelium after deleting Sox2, as judged by BrdU labeling after a 2-hour pulse at E18.5 and P0 (n=3 for each group; see Fig. S3A-E in the supplementary material). Sox2 is required for the in vitro proliferation of adult tracheal epithelial cells

Studies have shown the importance of Sox2 in the self-renewal and proliferation of stem/progenitor cells in the adult brain and pituitary gland (Fauquier et al., 2008; Ferri et al., 2004; Suh et al., 2007). We therefore asked whether Sox2 is also crucial for the homeostasis of the adult tracheal epithelium. To address this question, we first established a culture system that allowed us to grow adult tracheal epithelial cells in vitro. Single cells derived from enzymatically isolated epithelium (8×10^4/35 mm plate) were cultured in serum-free medium with Fgf2 and Egf (see Materials and methods). Under these conditions the epithelial cells attached, proliferated and formed small patches. Subsequently, cells in many of the patches flattened and ceased proliferation. By day 10 there were approximately 40 colonies/35 mm dish, predominantly of two types; those containing small basal cells (p63-positive) and those containing flattened Clara cells (Scgb1a1-positive; Fig. 5A). By day 15, only basal cell colonies (4-7 colonies/35 mm plate) survived and propagated. These basal cells expressed high levels of p63 and Sox2 (Fig. 5B).

We then used the ubiquitously expressed CMV-driven CreER to conditionally delete Sox2 in the adult following Tamoxifen (Tmx) injection. In this system, after Tmx induction Cre recombinase is translocated into the nucleus to excise the targeted gene. Control experiments show that CreER drives recombination in 80% of tracheal epithelial cells in CMV-CreER:R26R mice after three injections over a period of 5 days (see Fig. S4D,E in the supplementary material).

With the same Tmx injection scheme we deleted Sox2 in the tracheal epithelium of the CMV-CreER;Sox2^COND/COND compound mutants (Fig. 5C-E). At the time of harvesting (2 days after final injection) the number of p63-positive basal cells, Clara and ciliated cells is unchanged compared with wild-type controls (CMV-CreER and CMV-CreER;Sox2^COND) or CMV-CreER;Sox2^COND mutants without Tmx injection (see Fig. S4F-I in the supplementary material). The tracheal epithelial cells were then isolated from both conditional mutant and wild type, and the same number cultured (8×10^4/35 mm plate). By day 5, in the control group 140±13, 146±21 and 34±10 patches were p63-positive, Scgb1a1-positive and p63-Scgb1a1 double positive, respectively (n=3, see Fig. S4A-C in the supplementary material). Up to 70% of these colonies had a cell number larger than 15 and ~1.6% of the total epithelial cells were positive for the proliferation marker phosphorylated Histone H3 (see Fig. S4H in the supplementary material). By day 15, each 35 mm culture dish contained 4-7 basal cell colonies that were positive for p63 (n=5, Fig. 5K,M). By contrast, loss of Sox2 led to about a fourfold reduction in the number of cell patches compared with
controls at day 5 ($n=3$, $P<0.01$; Fig. 5J). The average size of the colonies was also smaller, and only 42% of patches had a cell number larger than 15. The proliferation index was 0.7%, calculated by staining for phosphorylated Histone H3 (see Fig. S4K in the supplementary material). The number of colonies positive for p63, Scgb1a1, and p63 and Scgb1a1, was 37±13, 50±11 and 9±3, respectively ($n=3$). When examined at day 10, only very few cells were positive for phosphorylated Histone H3 and, by day 15, all of these colonies disappeared ($n=5$, Fig. 5L,O).

**Sox2 is required for the homeostasis and repair of adult tracheal epithelial cells in vivo**

To test the role of Sox2 in long-term maintenance of the adult epithelium, we analyzed the tracheas of CMV-CreER;Sox2COND/COND mutants 3 months after Tmx exposure ($n=4$; Fig. 6A). CMV-CreER;Sox2COND/COND mice treated with Tmx served as controls. At the time of harvest (90 days after the final injection), Sox2 protein is undetectable in the tracheal epithelium of mutants (Fig. 6B,C). Moreover, the epithelium is composed of flattened cells that are more loosely packed than controls, as judged by morphology and internuclear distance (Fig. 6D-E). The number of epithelial cells per unit length of trachea (500 μm) is reduced from 160±11 in wild type to 114±5 in mutants ($n=4$). The proportion of cells in the total epithelium positive for the proliferation marker Ki67 (Mki67 – Mouse Genome Informatics) is decreased by 72% in mutants compared with controls ($n=4$) (Fig. 6J-L). Consistently, we also observed a decreased rate of BrdU incorporation in mutants after a 2-hour labeling period (see Fig. S4L-O in the supplementary material).
material). However, there is no significant apoptosis in mutants as detected by cleaved caspase 3 antibody staining (data not shown). Sox2 depletion in the adult also altered the composition of the tracheal epithelium, and a reduction of the proportion of basal, ciliated and Clara cells was observed in mutants (Fig. 6F-I). The proportion of basal cells in the total epithelium decreases by 24.6% compared with controls. However, unlike the embryonic trachea, no significant increase of alcian blue-positive or mucus-producing cells is noticed after Sox2 deletion, and approximately 20% of the epithelial cells are not positive for any markers examined, including CGRP.

The ability to repair after injury is an important feature of the tracheal epithelium. To investigate whether loss of Sox2 affects this property, we used a model in which SO2 induces damage to the tracheal epithelium. Normally, the epithelium starts to regenerate 24 hours after injury, and is fully recovered by 7-10 days (Rawlins et al., 2007). Two days after the final of three Tmx injections, CMV-CreER;Sox2-COND/COND (n=3) and CMV-CreER;Sox2-COND-/+ (n=3) control male mice were exposed to SO2, and the tracheae were harvested 15 days later (17 days after final injection; Fig. 7A). As before, Sox2 protein is undetectable in the CMV-CreER;Sox2-COND/COND compound mutants (data not shown). Strikingly, the repair of the trachea is impaired after Sox2 deletion (Fig. 7B-C). Examination of sections shows that in some parts of the trachea the epithelial cells are disorganized and the underlying mesenchyme is actively proliferating (data not shown), and inflammatory cells are still present (Fig. 7C). In wild-type trachea, regenerated epithelial cells lining the airways are tightly packed (Fig. 7B′-B″) and have differentiated normally into basal, ciliated and Clara cells (Fig. 7E,F). By contrast, the tracheal epithelium in mutants is flattened and loosely packed, in particular on the dorsal surface (Fig. 7C′-C″). Proliferation, as indicated by Ki67 expression, is also decreased (Fig. 7D,E). Moreover, Sox2 deletion affects the differentiation of the regenerated epithelium. The numbers of basal, ciliated and Clara cells are greatly reduced (Fig. 7G,I), and the proportion of p63-positive cells is decreased by 17.2%. However, we were unable to detect significant increase of alcian blue-positive cells after regeneration in mutants compared with wild type (data not shown).

DISCUSSION

In this article we extend our previous studies on the role of Sox2 in foregut development to show that it also has crucial functions in the developing and adult trachea. Conditionally deleting Sox2 during embryonic development affects the differentiation of both epithelium and mesenchyme. Loss of Sox2 in the adult perturbs the homeostasis of the tracheal epithelium and impairs the repair process after injury.

Role of Sox2 in laryngeal and tracheal development involves epithelial-mesenchymal interactions

All of the Nkx2.5-Cre;Sox2-COND/COND mutants die at birth with abnormal laryngeal cartilage. Up to 60% of the mutants also have a shortened trachea and deformed tracheal cartilage. Previously, several genes, including Shh, Hoxa5 and Raldh2, have been implicated in regulating the early development of the tracheal and laryngeal cartilages (Chisaka and Capeschi, 1991; Litingtung et al., 1999; Mendelsohn et al., 1999; Pecipelli et al., 1998; Vermot et al., 2003). Neither Hoxa5 nor Raldh2 appears to be affected by the loss of Sox2. However, we did observe decreased levels of Shh protein and RNA in the mutant epithelium of the E9.5 foregut and E11.5 trachea. We also observed decreased transcript levels of Shh downstream targets Ptc1 and Sox9, which are expressed in the mesenchyme to regulate its differentiation (Miller et al., 2004; Miller et al., 2001). Given the similar defects in foregut separation and tracheal cartilage in Shh and Sox2 mutants (Litingtung et al., 1999), we suggest that these two molecules fall into the same regulatory pathway. In addition, we noted that the domain of smooth muscle expands into the ventral side of the mutant trachea. It is possible that a threshold level of Shh is required for the differentiation of the mesenchyme towards cartilage versus smooth muscle in the trachea.

Up to 60% of conditional mutants have shortened a trachea and longer main bronchi than controls, although the total length is the same. One explanation for this phenotype is a defect in the positioning of the primary lung buds during foregut development. The retinoic acid signaling pathway has been shown to induce lung budding through inhibiting TGFβ in the ventral foregut (Chen et al., 2007). This inhibition allows for the localized high expression of Fgf10, which in turn induces the formation of primary lung buds (Desai et al., 2004). Deletion of Fgf10 abrogates the lung budding process (Sekine et al., 1999). We and others have shown that Fgf10...
negatively regulates Sox2 expression in the ventral foregut and lung (Nyeng et al., 2008; Que et al., 2007), and we noticed a gradual decrease of Sox2 expression from anterior to posterior in the ventral foregut (unpublished observations). In addition, at sites where the primary lung buds are formed, Sox2 expression is lost (Sherwood et al., 2008). We therefore propose that downregulation of Sox2 by Fgf10 at a specific site within the ventral foregut is necessary to initiate lung budding. Consequently, as noticed in the present study, Sox2 deletion throughout the ventral foregut leads to an anterior shift in the budding site in some mutants. However, a more complex network must exist that includes other signaling molecules, as truncated tracheas also occur in mutants with a disrupted Wnt signaling pathway (Li et al., 2002; Li et al., 2008).

Recent studies by us and others have shown that Wnt signaling, as evidenced by TOP-Gal expression, is active in the ventral foregut endoderm at E9.5 (Li et al., 2008; Okubo and Hogan, 2004; Shu et al., 2005). In light of a recent report that Sox2 forms a complex with β-catenin to regulate transcription of genes such as cyclin D1 (Chen et al., 2008c), it is tempting to speculate that Sox2 could similarly work with Wnt signaling to determine the budding site of the lung. That said, we cannot exclude the possibility that the truncated trachea results from the disorganized patterning of mesenchyme. This might in turn affect the morphogenesis of the epithelium, although we were not able to detect significant differences in proliferation between mutants and wild-type tracheas.

Role of Sox2 in the development of the tracheal epithelium

Sox2 is crucial for the development of several tissues, including brain, retina and tongue (Miyagi et al., 2008; Okubo et al., 2006; Suh et al., 2007; Taranova et al., 2006). Loss of Sox2 function results in cell cycle exit and differentiation of retinal progenitor cells (Taranova et al., 2006). Here, we show that Sox2 deletion in the early ventral foregut epithelium results in abnormal differentiation of tracheal epithelium, but it seems to occur without an effect on cell proliferation. This observation is consistent with our previous findings in the embryonic tongue and esophagus (Okubo et al., 2006; Que et al., 2007), Ferri et al. (Ferri et al., 2004) also notice that Sox2 is not required for the proliferation of the embryonic neural epithelium, and they suggest that Sox1 and Sox3, two other members of the SoxB1 family, play compensatory roles (Ferri et al., 2004).

We observed a significant decrease in the proportion of basal, ciliated and Clara cells after deleting Sox2. By contrast, more mucus-producing cells (Muc5ac-positive) are present in the mutant trachea. We also observed that up to 15% of the epithelial cells lack expression of any of the lineage specific markers examined. These results emphasize that Sox2 is required for the normal differentiation of the epithelium in the developing trachea. Recently Gontan et al. (Gontan et al., 2008) reported that Sox2 overexpression in embryonic lung epithelium also affects the differentiation, and significantly more neuroendocrine cells and p63-positive cells are present compared with wild type (Gontan et al., 2008). In future studies it will be important to determine the response of tracheal epithelium to increased Sox2 levels.

Role of Sox2 in maintenance of the adult trachea

Although Sox2 is not needed for the proliferation of the embryonic epithelium, it plays crucial roles in the maintenance of adult tracheal epithelium. Our first evidence comes from in vitro culture. We noted that after Sox2 deletion both the number and size of cell colonies are reduced, and no p63-positive basal cells continue to proliferate or form large colonies of undifferentiated cells. This suggests that Sox2 is needed for the self-renewal of these cells, which have been shown by in vivo lineage tracing to be stem cells (Hong et al., 2004). However, Sox2 does not appear to only affect basal cells, as the survival and proliferation of differentiated lineages, which transiently form colonies of large flattened cells, are also reduced. Since the starting population for the culture is a mixture of different cell types, we are unable to dissect out the contribution of Sox2 to each lineage. In the future studies, one could use CreER expressed in specific cell types such as basal or Clara cells to determine the effect of deleting Sox2 in the adult.

Our injury-repair mouse model has lent further support to the importance of Sox2 for maintaining adult tracheal epithelium. In mice without Sox2, the repair after SO2 injury is severely hampered. Loss of Sox2 not only decreases proliferation of the epithelium but also affects differentiation. Similar to what we have observed at steady state in mutants without Sox2, the numbers of basal, ciliated and Clara cells in mutants also decrease after regeneration. Several lines of evidence have shown that a network of transcription factors important for embryonic development can be reused during injury-repair processes in the respiratory system (Cardoso and Whitsett, 2008). Our data suggest that Sox2 is a potential key player in this network.

In summary, here we present new evidence that Sox2 plays multiple roles in both the developing and adult trachea. In the embryo, Sox2 is required for specifying the lung budding site and the subsequent differentiation of both tracheal mesenchyme and epithelium. Conditionally deleting Sox2 leads to a reduction in the number of basal, ciliated and Clara cells, whereas the number of mucus-producing cells is increased (Fig. 8). In the adult, Sox2 continues to play important roles and it regulates both the proliferation and differentiation of the epithelium at steady state and following injury (Fig. 8). In future studies it will be important to determine the downstream effectors and target genes of Sox2 in the respiratory system.

Fig. 8. Model for the roles of Sox2 in developing and adult trachea. At embryonic stage, Sox2 is required for the differentiation of tracheal mesenchyme and epithelium. Conditional deletion of Sox2 results in a decreased number of basal, Clara and ciliated cells, and more mucus-producing cells in the developing trachea. In the adult, Sox2 regulates both proliferation and differentiation at steady state and following injury. Sox2 deletion leads to reduced proliferation of tracheal epithelium both in vivo and in vitro. Loss of Sox2 also impairs the regeneration process of the trachea after inhaled SO2-induced injury.
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Supplementary material
Supplementary material available online at http://dev.biologists.org/cgi/content/full/136/11/1899/DC1

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