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# SOX2-DEPENDENT TRANSCRIPTIONAL CONTROL OF AIRWAY DIFFERENTIATION IN THE MOUSE LUNG

Belinda J. Hernandez

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# SOX2-DEPENDENT TRANSCRIPTIONAL CONTROL OF AIRWAY DIFFERENTIATION IN THE MOUSE LUNG

By:

Belinda Judith Hernandez, B.S.

APPROVED:

---

Jichao Chen Ph.D, Advisory Professor

---

Richard R. Behringer Ph.D

---

Elsa R. Flores, Ph.D

---

Nami McCarty, Ph.D

---

Eric J. Wagner, Ph.D

APPROVED:

---

Dean, The University of Texas Graduate School of Biomedical Sciences at Houston

# SOX2-DEPENDENT TRANSCRIPTIONAL CONTROL OF AIRWAY DIFFERENTIATION IN THE MOUSE LUNG

A

Thesis

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

In Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

By

Belinda Judith Hernandez, B.S.

Houston, Texas

December 2014

## Dedication

I dedicate this work to my mother, father, brother, and sister.

## **Acknowledgments**

First, I would like to thank my mentor Dr. Jichao Chen for his guidance, patience, teachings, and support. I learned a lot from Dr. Chen during my graduate studies. He has helped me become a better science researcher.

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## Abstract

### SOX2-Dependent Transcriptional Control of Airway Differentiation in the Mouse Lung.

Belinda Judith Hernandez, B.S.

Advisory Professor: Jichao Chen, Ph.D.

The lung is a highly branched tree-like tubular system that results from more than 20 generations of the conducting airways and consists of 300 million alveoli for gas exchange. Airway branches form via branching morphogenesis and then mature into conducting airways, in which the number and distribution of different cell types need to be precisely controlled. The conducting airways contain four lung cell types: club cells, ciliated cells, basal cells, and neuroendocrine cells. SOX2 is a well-known conducting airway marker. SOX2 is a transcription factor that is known to be important in embryonic development and induction of pluripotent stem cells. We hypothesized that SOX2 controls cell differentiation in the conducting airways. To determine the role of SOX2 in the conducting airways, we used various mouse models to determine if SOX2 controls cell differentiation. SOX2 was deleted in the lung epithelium by using *Sox2*<sup>EGFP/CKO</sup> and *Shh*<sup>Cre/+</sup>. We observed a decrease in club cell, basal cell, and neuroendocrine cell differentiation when Sox2 is absent in the conducting airways. SOX2 overexpression was done by using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* in the lung epithelium, Sox9 distal progenitor cells, alveolar type 1 cells, and alveolar type 2 cells. We found that SOX2 expression in the alveoli region reprograms alveoli cells into conducting airway cells. Mutant alveolar type 1 and type 2 cells expressing SOX2 are able to generate basal-like cells. Further, through

microarray analysis we found SOX21, a transcription factor, significantly down regulated in the absence of Sox2. Sox21 has a unique expression pattern in the conducting airways. The expression of SOX21 is uniform in the proximal airways and sporadic in the distal airways. Together, these data suggest SOX2 controls cell differentiation in the conducting airways. Our data regarding how Sox2 controls airway cell differentiation in the mouse lung gives insight regarding direct differentiation from an immature cell to a specialize cell.

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## **1. Introduction**

### **1.1 The Lung Structure**

The lung is an essential organ in humans that is required for breathing and provides a defense mechanism against infection. According to Morrisey and Hogan, the function of the lung is to transport oxygen from the environment into the bloodstream, and to release carbon dioxide from the bloodstream to the environment. The lung provides a line of defense by the secretion and clearance of mucus (1). The lung is a three-dimensional tree-like tubular system that consists of more than 20 generations of conducting airways and 300 million alveoli. Henry Grey describes the lung starts with formation of the trachea and separates into the two mainstem bronchi. The two mainstem bronchi branch out to form branch generations consisting of intralobar bronchioles (2). Upon the completion of lung branching, grape-like structures known as the alveoli start to form. The lung has five lobes: the right cranial, right medial, right caudal, accessory, and left lobe. The lung has two compartments: the conducting airways and gas exchange region (1). The conducting airways contain basal cells, ciliated cells, club cells, goblet cells and neuroendocrine cells. The gas exchange region known as the alveoli, which contains alveolar type 1 and type 2 cells (3).

### **1.2 Lung Development**

The respiratory system begins with the anterior foregut separating longitudinally to form tubes: the esophagus dorsally and the trachea ventrally (3). According to literature studies from Rock and Hogan, the formation of the primary lung buds arise from the ventral foregut endoderm from different morphogenetic processes (4,5). Branching morphogenesis occurs when the primary left and right buds develop into a tree-like structure containing epithelial tubules (4). One of the earliest markers for lung specification is NKX2.1, in which NKX2.1 null mice do not undergo complete lung development (6). According to Rackley and Stripp, there are 5 stages in lung development: embryonic, pseudoglandular, canalicular, saccular, and alveolar (7). In the embryonic stage, the lung starts to form in the mouse at

E9.5 or in humans at week 4 during pregnancy (4,6). The pseudoglanular stage occurs at E11-16 in the mouse or human week 5-17 during pregnancy, in which the lung epithelial tubes undergo branching morphogenesis (8). There is proximal to distal patterning to form the lung bronchial tree (5). The process of branching morphogenesis requires signaling between the epithelium and mesenchyme. Epithelial and mesenchyme signals include genes: TGF- $\beta$ , BMP, SHH, WNT, FGF, and EGF (9). The canalicular stage occurs in mouse at E16-E17 or human week 16-25 during pregnancy, airway epithelium cells undergo cell differentiation into the four lung cell types; club cells, ciliated cells, basal cells, and neuroendocrine cells (8). The sacular stage occurs in the mouse E17 to birth or human week 26- 36 during pregnancy, the distal epithelium cells differentiate into alveolar type 1 and type 2 cells. In the sacular stage, there is formation of saccules and the gas exchange region (4, 9). The alveolar stage occurs in the mouse at birth – P20 or human pregnancy week 36 to 3 years old, the majority of the gas exchange surface is formed (7,9). Lung development is under temporal control in order to form the conducting airway cells and gas exchange region to provide proper lung function.

### **1.3 Conducting Airway Cells**

The conducting airways are exposed to infection when inhaling airborne particles from the environment (10). The conducting airways provide a first line of host defense, which functions from multiciliated and secretory cells that drive mucociliary clearance from inhaled microorganisms, based on a review from Rock and Hogan (3). Mucus clearance is an innate mechanism in the conducting airways that requires a mucous layer and pericellular liquid layer, in order for trapped particles to move through the airways with the help of ciliated cells (11). The conducting airways include the trachea, mainstem bronchi, and intralobar bronchioles. The trachea and bronchi region are lined by pseudostratified cells. The intralobar bronchioles are lined by simple columnar epithelial cells. The conducting airways are composed of four lung cell types: club cells, ciliated cells, basal cells, and neuroendocrine cells (3, Figure 1). The cell proliferation in the conducting airways is low based on a study done from S.L. Kauffman (12).

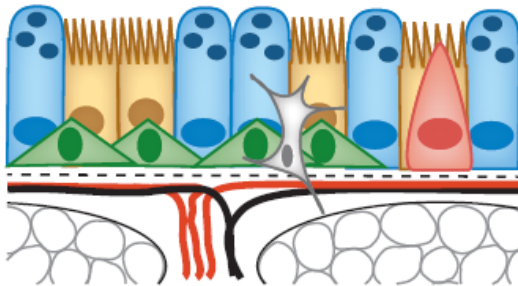
Neuroendocrine cells are found at branching points and near the bronchiolar alveolar duct junction (BADJ). Neuroendocrine cells appear in the airways as solitary cells and in clusters, known as neuroepithelial bodies (13). Neuroendocrine cells secrete calcitonin and sense stimuli in the airways such as hypoxia and nicotine (13).

Ciliated cells in the airways are motile and require FOXJ1 for ciliated cell differentiation (14). Ciliated cells help clear the airways of bacteria (15). Ciliogenesis in the lung is characterized by FOXJ1 and Acetyl tubulin expression (15).

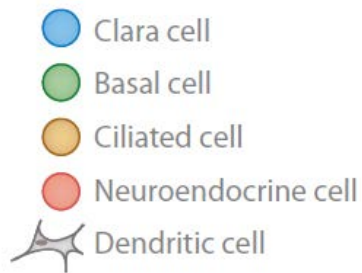
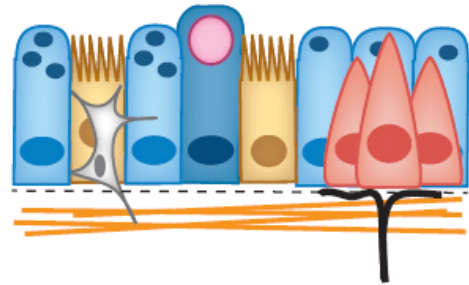
Club cells are cuboidal cells that protect the airways by secreting secretoglobin (SCGB1A1) and Clara cell secreting protein (CCSP) in the mucociliary process (16). In other lab studies, club cells resistant to naphthalene that reside close to neuroepithelial bodies are able to regenerate club cells. Club cell markers that co-localize with neuroendocrine cell markers support the idea that naphthalene resistant club cells can serve as progenitor cells to regenerate club cells in the airways. For club cell regeneration to occur, club cells must exist in the neuroepithelial body environment (17). Club cells expressing Scgb1a1 are able to self-renew and generate ciliated cells in the airways (18).

Basal cells are found in the trachea and at the mainstem bronchi. Basal cells are stratified epithelial cells and are identified in the lung by transcription factor, P63 (19). Basal cells are attached to the columnar epithelium of the basal lamina, and interact with underlying mesenchymal cells. Basal cells have the capacity to act as progenitors of the airways, but have a low rate of proliferation. Basal cells are identified by P63, cytokeratin 5 (KRT5), and cytokeratin 14 (KRT14) markers (20). Basal cells are involved in inflammatory response by upregulating expression of receptors for migratory inflammatory cells and lymphocytes (20). Based on a lung injury model with naphthalene, basal cells have the capacity to generate club cells, ciliated cells, and basal cells (21). In the trachea, basal cells are able to self-renew when there is injury (22).

**a** Trachea and bronchi



**b** Intralobar bronchioles

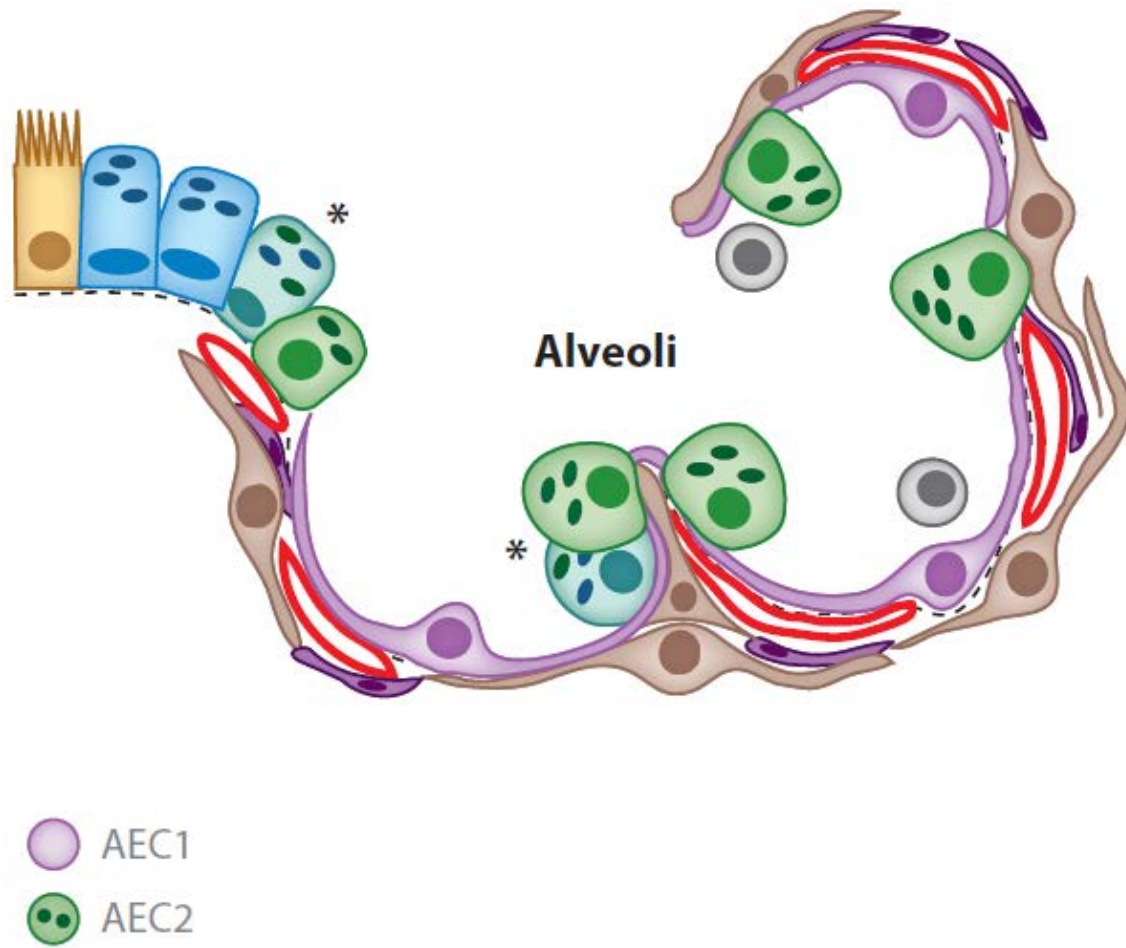


**Figure 1.1: The distribution of specialized cells in the conducting airways.**

(A) The tracheobronchial regions of the mouse respiratory system are lined by pseudostratified epithelium containing basal cells, ciliated cells, and secretory (club) cells. (B) The intralobar bronchioles are lined by simple columnar epithelium containing ciliated cells, club cells, and mucus-producing goblet cells, which are sparse in the airways (3).

## 1.4 Alveoli Cells

The gas exchange region also known as the alveoli contain alveolar type 1 and type 2 epithelial cells (Figure 2). Alveolar type 1 cells are squamous and cover 95 to 97% of total surface area. Aquaporin 5 and T1 $\alpha$  are known markers for alveolar type 1 cells (23). Alveolar type 1 cells are known not proliferate, but there is evidence that alveolar type 2 cells can proliferate (24). Alveolar type 2 cells are cuboidal in shape and secrete surfactant proteins: SP-A, SP-B, SP-C, and SP-D (25). Surfactant proteins are composed of phospholipids that decrease surface tension and prevent the lung from collapsing (26). There is evidence that alveolar type 2 cells can give rise to alveolar type 1 and type 2 cells upon lung injury (27, 28).



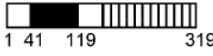

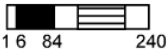
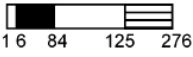
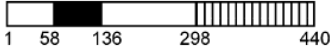
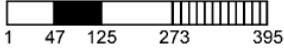
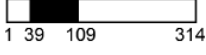

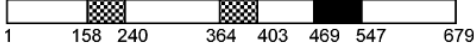
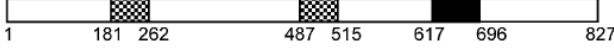
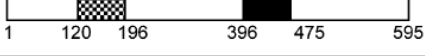
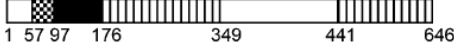
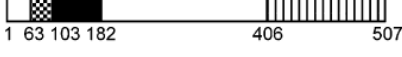
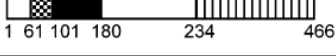
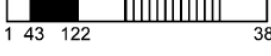
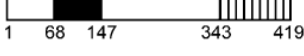
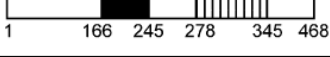
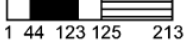
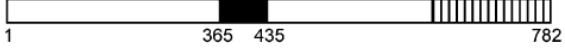


**Figure 1.2: The distribution of type 1 and type 2 cells in the gas exchange region.**

The alveoli region is lined by squamous alveolar epithelial type 1 cells (AEC1) and cuboidal alveolar epithelial type 2 cells (AEC2).

## 1.5 SOX Genes

SOX genes are transcription factors that are classified based on their high mobility group domain box (HMG). The HMG domain box of Sox genes is 50 % similar to the HMG domain box of the sex-determining gene of the Y chromosome (SRY) (29). The high mobility group domain box (HMG) is composed of non-histone nuclear proteins that contain functional sequence motifs, which are the main site of interaction between HMG protein and DNA (30). Once the HMG domain binds to DNA, the DNA bends to allow other transcription factors to bind and form an active transcriptional complex to start transcription (31). The Sox name comes from SRY-related HMG box and a number is given to the SOX gene based on the order of discovery (31). There are twenty SOX genes that have been identified and are placed into a group or subgroup (32, Table 1). SOX genes are placed in a group based on their similarity of > 80% of their HMG domain box (33). SOX genes are known to be important in embryonic development process such as neural development, lung development, skeletal muscle, and hair follicle development (34, Table 2).

Group	Gene	Locus	Schematic
A	<i>Sry</i>	YC3	
B1	<i>Sox1</i>	8 A1-A2	
	<i>Sox2</i>	3 A2-B	
	<i>Sox3</i>	X A7.3-B	
B2	<i>Sox14</i>	9 E3.3	
	<i>Sox21</i>	14 E4	
C	<i>Sox4</i>	13 A3-A5	
	<i>Sox11</i>	12 A3	
	<i>Sox12</i>	2 G3	
D	<i>Sox5</i>	6 G3	
	<i>L-Sox5</i>	6 G3	
	<i>Sox6</i>	7 F1	
	<i>Sox13</i>	1 E4	
E	<i>Sox8</i>	17 A3	
	<i>Sox9</i>	11 E2	
	<i>Sox10</i>	15 E1	
F	<i>Sox7</i>	14 C3	
	<i>Sox17</i>	1 A1	
	<i>Sox18</i>	2 H4	
G	<i>Sox15</i>	11 B3	
H	<i>Sox30</i>	11 B1.1	



**Table 1.1 The Classification of SOX Genes.**

The HMG domain of SOX genes is 50 % similar to the HMG domain of SRY. Each SOX gene is placed on a group based on > 80 % of HMG box domain similarity. The boxes are HMG box domains (dark), transactivation domain (vertical strips), transrepression domain (horizontal stripes), and dimerization domain (checkers) (31).

Gene	Sox Group <sup>a</sup>	Major Known (or Deduced) Functions <sup>b</sup>
<i>Sry</i>	A	Testis determination
<i>Sox1</i>	B1	Lens development, (neural determination)
<i>Sox2</i>	B1	Neural induction, (lens induction, pluripotency)
<i>Sox3</i>	B1	(Neural determination, lens induction)
<i>Sox4</i>	C	Heart, lymphocyte, thymocyte development
<i>Sox5</i>	D	Chondrogenesis
<i>Sox6</i>	D	Chondrogenesis, (cardiac myogenesis)
<i>Sox7</i>	F	(Development of vascular and many other tissues)
<i>Sox8</i>	E	(Development of many tissues)
<i>Sox9</i>	E	Chondrogenesis, sex determination
<i>Sox10</i>	E	Neural crest specification
<i>Sox11</i>	C	(Neuronal, glial maturation)
<i>Sox12<sup>e</sup></i>	C	(Development of many tissues)
<i>Sox13</i>	D	(Development of arterial walls, pancreatic islets)
<i>Sox14</i>	B2	(Interneuron specification, limb development)
<i>Sox15<sup>g</sup></i>	G	(Myogenesis)
<i>Sox17</i>	F	Endoderm specification
<i>Sox18</i>	F	Vascular and hair follicle development
<i>Sox21</i>	B2	(CNS patterning)
<i>Sox30</i>	H	(Male germ cell maturation)

**Table 1.2** The role of development for each SOX gene (32).

## 1.6 Sox2 gene

SOX2 is transcription factor and has a HMG box domain that is 50% similar to the HMG domain of SRY (35). SOX2 belongs to the SoxB1 group along with SOX1 and SOX3 (31). SOX2 is known to be important in embryonic development, such as maintaining neural progenitor cells in the central nervous system, sensory progenitors in the inner ear, and development of taste bud sensory cells (36, 37, 38). SOX2 is one of the four factors in combination with Oct3/4, c-Myc, and Klf14 that are able to reprogram fibroblasts into pluripotent cells (39). In the chicken embryo, SOX2 expression is seen at day 3 of embryonic development from the rostral gut epithelium to the stomach and the lung (35). In the mouse lung epithelium, SOX2 is specifically expressed in the proximal airways and absent in the gas exchange region (40). In the mouse lung at E9.5, SOX2 is localized in endoderm cells of the undivided foregut (41). An abnormal lung phenotype in the absence of SOX2 is tracheoesophageal fistula (TEF), in which the trachea and the esophagus fail to separate (41). In a lung trachea study, SOX2 was deleted using *NKX2.5-Cre* and the result was a decrease in club cells, ciliated cells, and basal cells (42). Based on lung injury study with SO<sub>2</sub> and SOX2 deletion, there is a significant decrease in the number of basal cells, ciliated cells, and club cells (42). The lung injury study suggests that SOX2 is important for trachea cell regeneration and cell differentiation (42). SOX2 deletion in postnatal lungs using *Scgb1a1-Cre* suggests that SOX2 expression is necessary for differentiation and maintenance of club cells and ciliated cells in the conducting airways (43). SOX2 overexpression is observed in human squamous cell lung tumors and Sox2 mRNA expression levels are elevated in non-small cell lung cancer (NSCLC) (44, 45).

## **1.7 Therapeutic Implications**

Based on the literature, SOX2 is not only a conducting airway marker due to the affect it has on cell differentiation. In the absence of SOX2, there is a decrease in cell differentiation in the conducting airways. SOX2 overexpression leads to tumor formation (44.45). SOX2 is a transcription factor that can be studied to give insight how to help patients with genetic lung defects. Cells isolated from patients with genetic lung defects can be reprogrammed by transcription factors into specialized cells (46). Once the cells have been reprogrammed and are able to differentiate into specialized cells, the new specialized cells can be transferred back to the patient (47). Induced pluripotent stem cells can be used as a treatment method to convert immature cells into specialized lung epithelial cells (47). By understanding the role of SOX2 in cell differentiation in the mouse lung, SOX2 can provide insight by direct differentiation from an immature cell to a specialize cell.

## **1.8 Hypothesis**

Airway branches form via branching morphogenesis and then mature into conducting airways, in which the number and distribution of different cell types need to be precisely controlled. Based on the literature, SOX2 is an early marker well-known for the conducting airways and before cell differentiation. The focus of my study is to use the mouse as a model to specifically study the lung epithelium during embryonic lung development to determine which one of the four lung cell types requires SOX2, does SOX2 control cell differentiation, and is SOX2 cell autonomous in the conducting airways? My hypothesis is SOX2-dependent transcriptional network controls cell differentiation in the conducting airways.

## 2. Materials and Methods

### 2.1 Mice

*Sox2*<sup>CKO/CKO</sup> mice have two loxP sites flanking the SOX2 promoter and *Sox2*<sup>EGFP/+</sup> have the SOX2 coding sequence was replaced with EGFP (48). *Sox2*<sup>EGFP/+</sup>; *Rosa*<sup>tdt/tdt</sup>; *Shh*<sup>Cre/+</sup> male mice were purchased from the Jackson Laboratory. *Sox2*<sup>EGFP/+</sup> male mice were purchased from the Jackson Laboratory. *Sox9*<sup>CreER/+</sup> male mice were purchased from the Jackson Laboratory. The *Sox9*<sup>CreER/+</sup> contains a 5' internal ribosome entry sequence (IRES) followed by a *CreER* integrated into the 3' UTR of the endogenous mouse *Sox9* gene (49). *Rosa*<sup>Sox2/Sox2</sup> mice were obtained from Dr. Jianwen Que at the University of Rochester. The overexpression of SOX2 was generated by using the Rosa26 locus. The SOX2 overexpression allele construct is *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)*. The removal of the flanked loxP sites leads to SOX2 expression and express green fluorescence (45). *Sox2*<sup>CreER/+</sup> male mice were obtained from the Jackson Laboratory. The *Sox*<sup>CreER/+</sup> mouse construct has the SOX2 open reading frame replaced with a *CreER* fusion gene (50). For SOX2 expression in alveolar type 1 and type 2 cells, we used *Hopx*<sup>CreER/+</sup> for alveolar type 1 cells and *Sftpc*<sup>CreER/+</sup> for alveolar type 2 cells. The *Hopx*<sup>CreER/+</sup> construct has a *CreER* knock in targeted to the 3' untranslated region of the *Hopx* locus following an internal ribosomal entry sequence (IRES) (51) and the *Sftpc*<sup>CreER/+</sup> construct has a *IRES-CreER* cassette and a PGK neo cassette flanked with FRT sites recombined in the 3' UTR of *Sftpc* (52).

### 2.2 Embryo Fixation

E18.5 embryos and postnatal lungs were fixed for 3 hours with 0.5% paraformaldehyde (PFA), washed with 1X PBS twice for 5 minutes, and placed in 1X PBS overnight at 4 °C in a rocker.

### 2.3 Tissue Immunofluorescence

Lungs were treated in PBS conditions or in bleach conditions depending on the primary antibody being used for the experiment. For PBS treated lungs, the lungs were separated into lobes

and placed in 30% sucrose/PBS overnight. The next day, the lungs were embedded in OCT and frozen in a block with dry ice and 100% ethanol.

Lung samples treated in bleach condition were washed with a methanol gradient wash first. The methanol gradient treatment consisted of 25% methanol in PBS for 5 minutes, 50% methanol in PBS for 5 minutes, 75% methanol in PBS for 5 minutes, 100% methanol in PBS for 5 minutes, and placed in bleach overnight at 4 ° C in a rocker. The next day, the lung samples were washed for 1 hour in 100% methanol and treated with methanol gradient wash. The methanol gradient wash treatment consisted of 75% methanol in PBS for 5 minutes, 50% methanol in PBS for 5 minutes, 25% methanol in PBS for 5 minutes, 1X PBS for 5 minutes , and 30% sucrose in PBS overnight in 4 ° C in a rocker. The next day, the lungs were embedded in OCT and frozen in a block with dry ice and 100% ethanol.

Lung tissue sections were cut at 10 microns. The tissue sections were washed three times with 1X PBS over a period of 10 minutes to wash away OCT. Tissue sections were blocked for 1 hour with 5% donkey serum in PBS + 0.3% TritonX-100 at room temperature. Tissue sections were incubated overnight at 4 ° C with primary antibodies (Table 3) in PBS + 0.3% TritonX-100. On the second day, the tissue sections were washed with 1X PBS for one hour and half. After 1X PBS wash, tissue sections were incubated at room temperature for 2 hours with secondary antibodies and DAPI in 1XPBS + PBSTT=PBS + 1% TritonX-100 + 1% Tween-20, 1X PBS wash for 1 hour, and coverslips were mounted.

## **2.4 Whole Mount Immunofluorescence**

Lung samples were treated in PBS or bleach condition prior to staining. For postnatal lungs, strips were cut and placed in 1XPBS. Lungs samples were blocked for 2 hours in 2.5% donkey serum in PBS + 0.3% TritonX-100 at room temperature in a rocker. Lung samples were incubated overnight at 4 ° C degrees with primary antibodies (Table 3) in PBS + 0.3% TritonX-100. On the second day, the lung samples were washed six times with PBSTT over a period of six hours, incubated overnight at 4 ° C with secondary antibodies. On the third day, the lung samples were washed six times with PBSTT over a

period of six hours, washed with 1X PBS twice for 5 minutes, incubate for 2 hours with 4% PFA for fixation, wash with 1X PBS for 1 hour, lungs were placed on slides, and coverslips were mounted.

For Benzyl Alcohol Benzyl Benzoate (BABB) treated lungs, after whole mount staining and 4% PFA fixation, the lung samples were incubated for 2 overnights with 100% methanol at 4° C in a rocker. On the third day, the lung samples were incubated overnight with BABB at 4° C.

## 2.5 Confocal Microscopy

Lung tissue sections and whole mount lung samples were imaged using an Olympus Fluoview FV1000 confocal microscope. The filters used for imaging the samples were Alexa 488, CY3, CY5, and DAPI. For lung tissue sections, images were taken with 40X oil magnification, 1.5 zoom magnification, and image size of 1024 x 1024. For whole mount lung samples, images were taken with 40X oil magnification, and 2.0 zoom magnification, and image size of 512 x 512. For BABB treated whole mount lung samples, images were taken with 10X magnification, 2.0 zoom magnification, and image size 512 x 512.

## 2.6 Fluorescence-activated cell sorting (FACS)

The embryonic stages collected for FACS were E14.5, E16.5, and E18.5. The SOX2 embryonic control's genotype is *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>+/+</sup> and shows green fluorescence. The mutant SOX2 embryonic mutant's genotype is *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>Cre/+</sup> and shows green and red fluorescence. Lungs were taken from embryos of interest and dissected in PBS at room temperature. Lungs were minced < 1mm with forceps. The tissue was transferred into a 5mL round tube containing 1.35 mL pre-warmed PBS + 150 µL 20 mg/mL Collagenase Type 1 (Worthington CLS-1 dissolved in PBS) + 15 µL 20mg/mL DNaseI (Worthington D dissolved in PBS). Lungs were digested at 37 ° C for 40 minutes and inverted to mix every 10 minutes. 1.5 mL Trypsin/EDTA (Invitrogen, 25200-056) was pre-warmed while lungs are digested at 37 ° C. After 40 minutes, pre-warmed Trypsin/EDTA was added and mixed 30

times with pipettor. Lung samples were digested at 37 ° C for 10 minutes and inverted to mix after 5 minutes. Lung digest was stopped with 300 µL of FBS (Invitrogen, 10082-139). Samples were centrifuged at 5000 rpm for 1 minute. Samples were washed with 2mL ice cold PBS + 1% FBS. 2 mL of red blood lysis buffer was added to samples and placed at room temperature for 3 minutes. Samples were centrifuged at 5000 rpm for 1 minute. Samples were washed with 2 mL ice cold PBS + 1% FBS. Samples were centrifuged at 5000 rpm for 1 minute. Final volume of sample was 500 µL ice-cold PBS + 1% FBS. FACS cells were collected in ice-cold PBS + 1% FBS. Cells were centrifuged at 2000 rpm for 5 minutes at 4 ° C. Cells were placed 1mL of trizol and stored at -80 ° C.

## 2.7 Microarray Analysis

Total RNA was extracted from E14.5, E16.5, and E18.5 FACS cells. RNA was purified using RNeasy Micro kit (Qiagen, 74004). 500 ng/µL of total RNA was used to for library preparation. The Illumina TotalPrep RNA Amplification Kit was used to prepare libraries for microarray analysis. Microarray services were obtained from the M.D. Anderson Core Facility.

## 2.8 Statistical Analysis

Statistical analysis was done by using the student t-test. For SOX2 deletion using *Shh<sup>Cre/+</sup>*, we used confocal images of immunostained lung sections to count the number of basal cells in the trachea epithelium. We counted P63<sup>+</sup> cells, KRT5<sup>+</sup> cells, and the total number of DAPI cells in the trachea epithelium. The percentage of P63 or KRT5 cells was obtained by: P63<sup>+</sup> cells or KRT5<sup>+</sup> cells/total number of DAPI cells For SOX2 overexpression using the *Shh<sup>Cre/+</sup>*, we used confocal images of immunostained lung sections to count the number of basal cells in the lung epithelium. We counted P63<sup>+</sup> cells or KRT5<sup>+</sup> cells that co-localized with SOX2 and the total number of SOX2 cells. The percentage of P63 or KRT5 cells was obtained by: P63<sup>+</sup> cells or KRT5<sup>+</sup> cells/total number of SOX2<sup>+</sup> cells. P-values less than 0.05 were considered significant.



**Table 2.1 Primers Set for Genotyping.**

Gene	Forward Primer	Reverse Primer
<i>Sox2</i> <sup>CKO/CKO</sup>	TGGAATCAGGCTGCCGAGAATCC	TCGTTCTGGCAACAAGTGCTAAAGC
<i>CreER</i>	ATACCGGAGATCATGCAAGC	AGGTGGACCTGATCATGGAG
<i>Cre</i>	GTTTCGCAAGAACCTGATGGACA	CTAGAGCCTGTTTTGCACGTTT
<i>Rosa</i> <sup>Sox2/Sox2</sup>	CTACCTGAGCACCCAGTCCG	ATGACACCTACTCAGACAATGC
<i>Hopx</i> <sup>CreER/+</sup>	CGAGGGGATCAGATGAAGAA	CCAAAAGACGGCAATATGGT
<i>SPC</i> <sup>CreER/+</sup>	GCTTCACAGGGTCGGTAG	CAACTCACAACTGGGCACTG
<i>SPC</i> <sup>CreER/+</sup> WT Reverse		GAGGCACCGCTCCGCGAG
<i>Sox2</i> <sup>CreER/+</sup>	TGGGCGGCATGGTGCAAGTT	CGGTGCTAACCAAGCGTTTTTC

**Table 2.2 Primary Antibodies for Immunofluorescence Staining.**

Name	Anti-	Concentration	Company
SOX2	goat	1:250	Santa Cruz Biotechnology
SOX21	goat	1:100	R&D systems
CCSP	rabbit	1:2500	Seven hills Bioreagents
CGRP	rabbit	1:500	Enzo Lab Sciences
AC.TUB	mouse	1:2500	Sigma Aldrich
FOXJ1	mouse	1:250	eBioscience
P63	rabbit	1:100	Santa Cruz Biotechnology
KRT5	rabbit	1:500	Fisher Scientific/Thermo
GFP	chicken	1:2500	Abcam
ECAD	rat	1:1000	Invitrogen
ABCA3	rabbit	1:1000	Seven hills Bioreagents
LAMP3	rat	1:1000	Imgenex
SPC	rabbit	1:1000	Millipore
RAGE	rat	1:1000	R&D systems

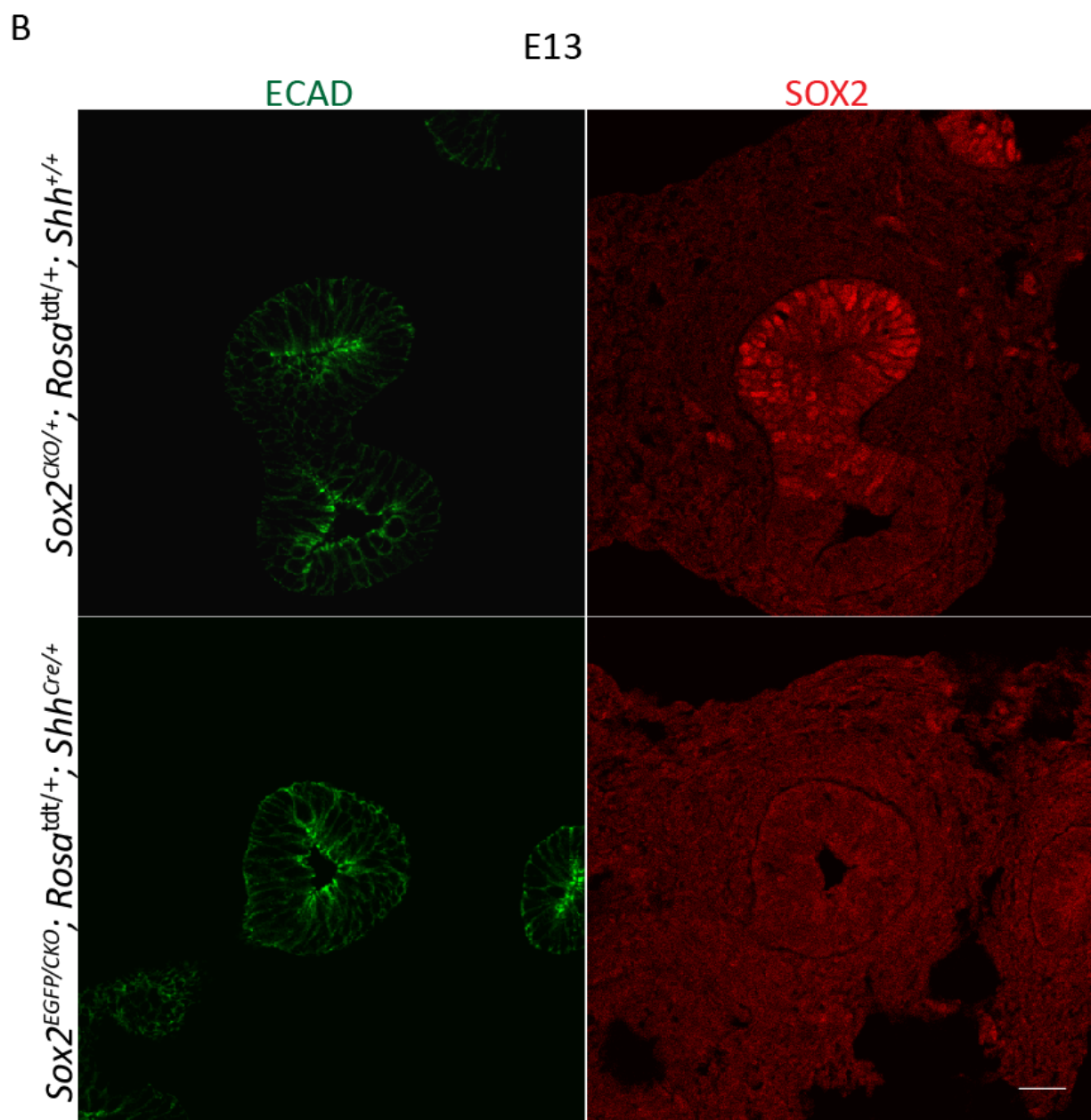
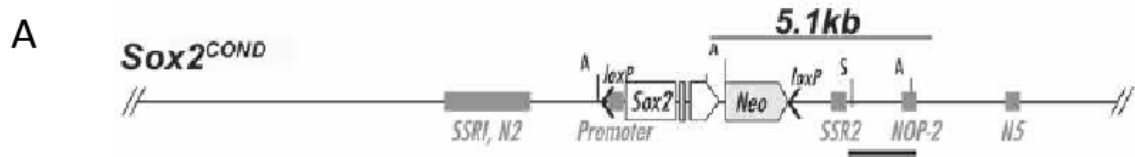
### 3. Results I: Cell differentiation in the conducting airways is decreased in the absence of SOX2.

#### 3.1 SOX2 deletion decreases cell differentiation in the trachea.

SOX2 is well-known conducting airway marker in the lung epithelium. To investigate if SOX2 controls airway differentiation during embryonic lung development, we deleted SOX2 expression in the lung epithelium. A SOX2 knock out mouse model cannot be used in this study, because embryos die before birth and do not undergo further development (53). We used SOX2 conditional knock out female mice (48, Figure 3.1A). The mouse breeding setup was: ♀  $Sox2^{CKO/CKO}; Rosa^{+/+}; Shh^{+/+}$  x ♂  $Sox2^{EGFP/+}; Rosa^{tdt/tdt}; Shh^{Cre/+}$ .  $Sox2^{EGFP/+}$  labels conducting airways. To delete SOX2 in the lung epithelium, we used  $Shh^{Cre/+}$ .  $Rosa^{tdt/tdt}$  is a reporter used to confirm Cre activity. There were four possible genotype outcomes from the breeding scheme and each with a 25% frequency. The SOX2 controls are  $Sox2^{CKO/+}; Rosa^{tdt/+}; Shh^{Cre/+}$ ,  $Sox2^{CKO/+}; Rosa^{tdt/+}; Shh^{+/+}$ , and  $Sox2^{EGFP/CKO}; Rosa^{tdt/+}; Shh^{+/+}$ . The SOX2 mutant embryo is  $Sox2^{EGFP/CKO}; Rosa^{tdt/+}; Shh^{Cre/+}$ . A fluorescence microscope was used to screen mutant and control embryos. The controls are:  $Sox2^{EGFP/CKO}; Rosa^{tdt/+}; Shh^{+/+}$  has green fluorescence,  $Sox2^{CKO/+}; Rosa^{tdt/+}; Shh^{Cre/+}$  has red fluorescence, and  $Sox2^{CKO/+}; Rosa^{tdt/+}; Shh^{+/+}$  has no fluorescence. The mutant  $Sox2^{EGFP/CKO}; Rosa^{tdt/+}; Shh^{Cre/+}$  has green and red fluorescence. To confirm our screening method for control and mutant embryos, we performed immunofluorescence staining on E13 lungs. The mouse model for SOX2 deletion does delete Sox2 in the conducting airways (Figure 3.1B). Cell differentiation of the four lung cell types; club cells, ciliated cells, basal cells, and neuroendocrine cells occur in a salt and pepper pattern. We harvested embryonic lungs at E19, which is the stage that airway differentiation begins. To determine which lung cell type requires SOX2, we used E19 trachea sections to study the proximal region of the lung. Immunofluorescence staining of E19 trachea sections allowed us to identify which lung cell type markers co-localize with SOX2 and expresses in the absence of SOX2. The first lung cell type we studied in the trachea epithelium was basal cell differentiation using P63 and Keratin 5 (KRT5) as basal cell markers. In the trachea region, basal cell differentiation is decreased

when SOX2 is absent (Figure 3.2A and 3.2B). We performed quantification analysis on basal cell expression in the trachea epithelium to calculate the portion of basal cells expressed in the presence and absence of SOX2. The percentage of P63<sup>+</sup> cells was calculated by counting the number of P63 cells over the total number of DAPI cells in the trachea epithelium. We counted three E19 trachea sections for both control and mutant lungs (n=3). The percentage of P63 positive cells for the control was 24% ± 5% from a total number of 28 P63<sup>+</sup> cells and 114 DAPI cells. The percentage of P63 positive cells for the mutant was 1% ± 1% from a total number of 1 P63<sup>+</sup> cell and 49 DAPI cells. The percentage of P63 positive cells in the trachea for the control and mutant is significant, p-value < 0.5. We used another basal cell marker, KRT5, to identify mature basal cells. We performed quantification analysis on KRT5 expression in the trachea epithelium to calculate portion of basal cells in the presence and absence of SOX2. The percentage of KRT5<sup>+</sup> cells was calculated by counting the number of KRT5 cells over the total number of DAPI cells in the trachea epithelium. We counted three E19 trachea sections for both control and mutant lungs (n=3). The percentage of KRT5 positive cells for the control was 40% ± 4% from a total number of 64 KRT5<sup>+</sup> cells and 159 DAPI cells. The percentage of the mutant was 3% ± 2% from a total of 5 KRT 5<sup>+</sup> cells and 121 DAPI cells. The percentage of KRT5 positive cells in the trachea for the control and mutant is significant, p-value < 0.5. Based on the quantification results from both basal markers P63 and KRT5, SOX2 is necessary for basal cell differentiation. The second and third lung cell types we studied in the trachea epithelium were club cells and ciliated cells. To determine if SOX2 is required for club cell and ciliated cell differentiation in the trachea, we used Clara cell secreting protein (CCSP) and acetylated tubulin (AC.TUB) as markers. We observed an absence of club cell differentiation in the mutant compared to the control. Ciliated cell differentiation was not affected by the absence of SOX2 (Figure 3.3). The data suggests that SOX2 is required for club cell differentiation in the trachea. In the trachea, ciliated cell differentiation is able to occur in the absence of SOX2 (Figure 3.3). We observed a decrease in basal cell and club cell differentiation in the trachea. We need to investigate beyond the

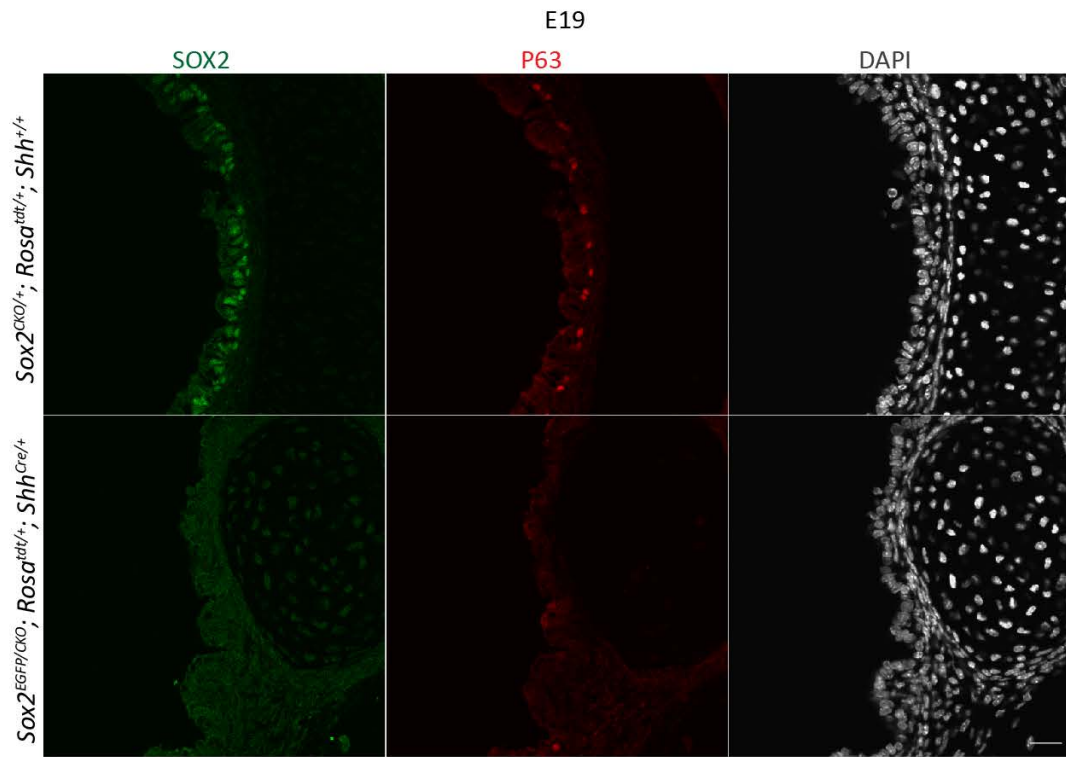
trachea such as the lobular bronchioles to determine if there is a decrease in cell differentiation similar to the trachea.



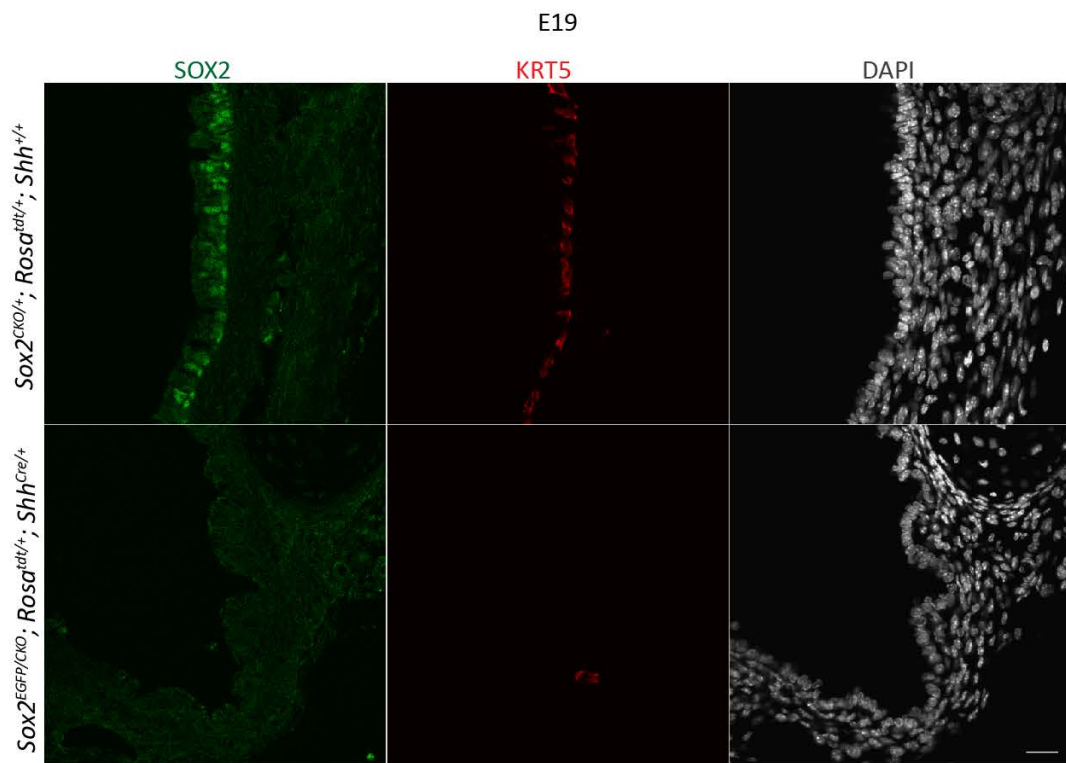
**Figure 3.1 SOX2 deletion mouse model.**

A) A schematic diagram construct of *Sox2*<sup>CKO/CKO</sup> (48). B) Confocal images of immunostained E13 lung sections. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. ECAD (green) labels the epithelium and SOX2 (red) labels the conducting airways. SOX2 is absent in mutant. The mouse model for SOX2 deletion does show the absence of SOX2 in the mutant embryo. Scale bar: 20  $\mu$ m.

A



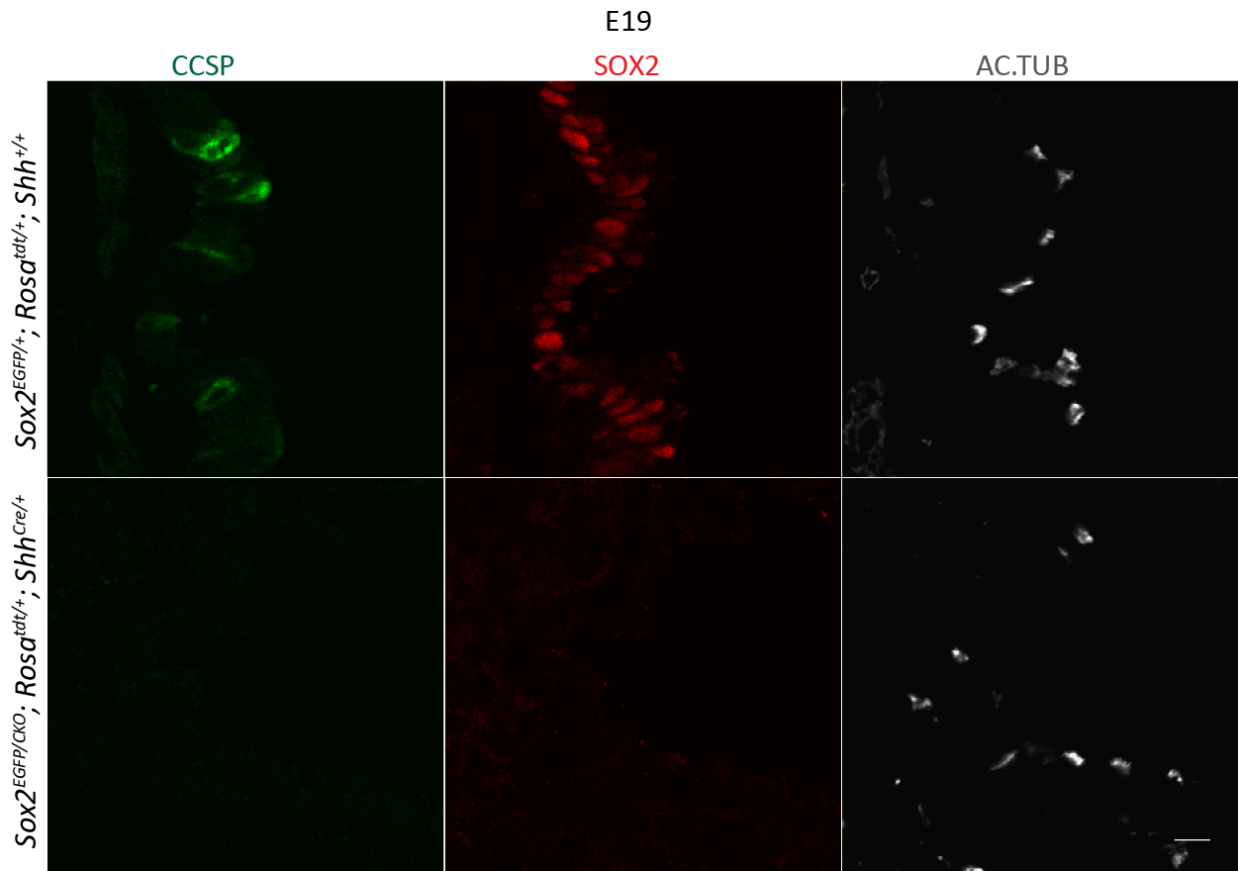
B



**Figure 3.2 Basal cell differentiation is decreased in the SOX2 mutant trachea.**

Confocal images of immunostained E19 trachea sections. A-B) The top panels are the SOX2 control lungs and the bottom panels are the SOX2 mutant lungs. SOX2 (green) labels conducting airway cells and DAPI (gray). A) We used P63 (red) to label basal cells. In the absence of SOX2, basal cell expression is decreased in the mutant lung compared to the control. B) A second basal cell marker, KRT5 (red) was used to visualize basal cell differentiation. Basal cell differentiation is decreased when SOX2 is absent in the lung. Scale bar: 20  $\mu\text{m}$ .



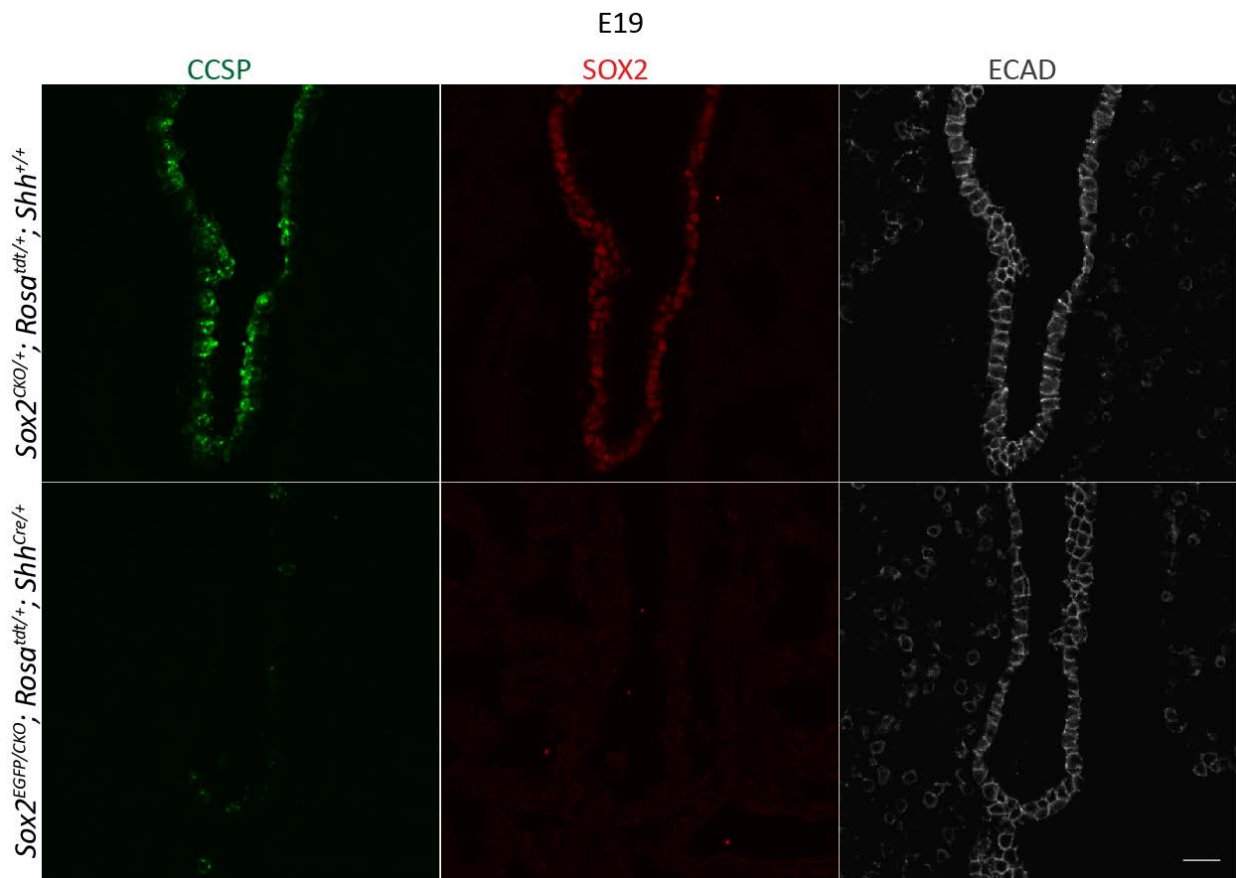


**Figure 3.3 In the SOX2 mutant trachea, club cell differentiation does not occur but ciliated cell differentiation does occur.**

Confocal images of immunostained E19 trachea sections. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. CCSP (green) labels club cells, SOX2 (red) labels conducting airway cells, and AC.TUB (gray) labels ciliated cells. CCSP expression is absent in the mutant trachea. Club cell differentiation requires SOX2. Ciliated cell differentiation occurred in the presence and absence of SOX2. Scale bar: 40  $\mu$ m.

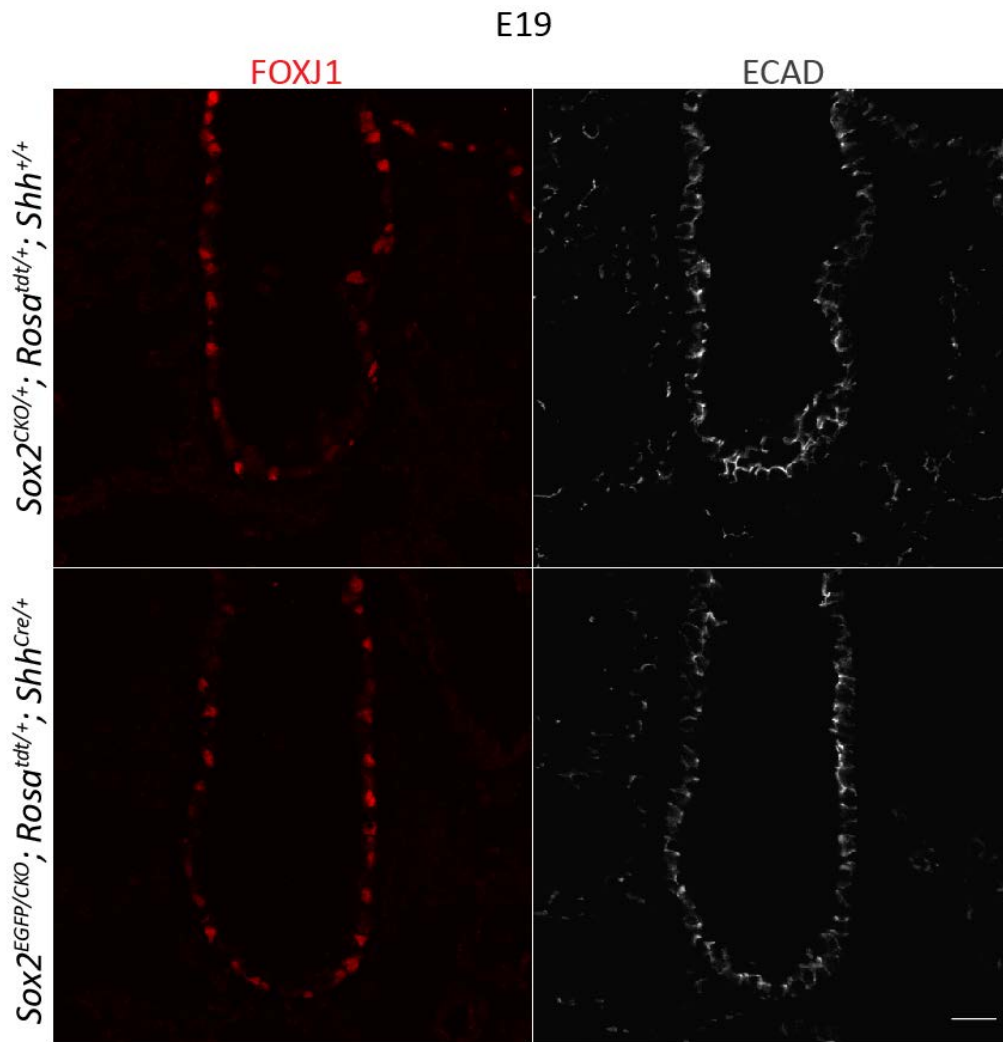
### **3.2 Club cell differentiation is blocked and ciliated cell differentiation occurs but at low frequency in the absence of SOX2.**

In the trachea epithelium, club cell differentiation is decreased in the mutant trachea compared to the control trachea. To determine if there is a decrease in conducting airway cell differentiation in the whole lung, we examined the epithelium lobular bronchiole regions. The first lung cell type we studied was club cells. Club cell differentiation in the SOX2 mutant is decreased compared to the SOX2 control based on CCSP expression (Figure 3.4). In the absence of SOX2, club cell differentiation is blocked with occasional escapers. Escapers are cells that express CCSP, but do not express SOX2. The occasional escapers may be immature cells that have not fully differentiated into club cells and remain as immature cells. The second lung cell type we studied was ciliated cells. We used FOXJ1, a nuclear marker for ciliated cells, to study the first step of ciliated cell differentiation. Based on our results, we did not detect a difference in FOXJ1 expression between control and mutant lungs (Figure 3.5). Next, we used AC.TUB as a marker to study the second step ciliated cell differentiation. We observed low expression levels of AC.TUB in the SOX2 mutant, which suggests ciliated cell differentiation occurs at low frequency (Figure 3.6). The data suggests the first step of ciliated cell differentiation is normal based on FOXJ1 expression, but the second step of ciliated cell differentiation is moderately affected based on AC.TUB expression. The data suggests SOX2 controls cell fate of conducting airway cells, whether cells can become club cells or ciliated cells. In the absence of SOX2, club cell differentiation is severely comprised and ciliated cell differentiation is moderately affected. The last lung cell type we studied in the lobular bronchiole region was neuroendocrine cells. We used calcitonin gene related protein (CGRP) as a neuroendocrine cell marker. Based on the literature, neuroendocrine cells appear in the conducting airways in solitary form or in the form of clusters. In the SOX2 mutant lung, CGRP expression is completely absent compared to the SOX2 control lung (Figure 3.7). Based on the lung sections, SOX2 is required for neuroendocrine cell differentiation. In the lobular bronchioles, club cell differentiation is reduced and ciliated cell differentiation at low efficiency in the absence of SOX2. Neuroendocrine cells are not formed in the lobular bronchioles when SOX2 is absent.



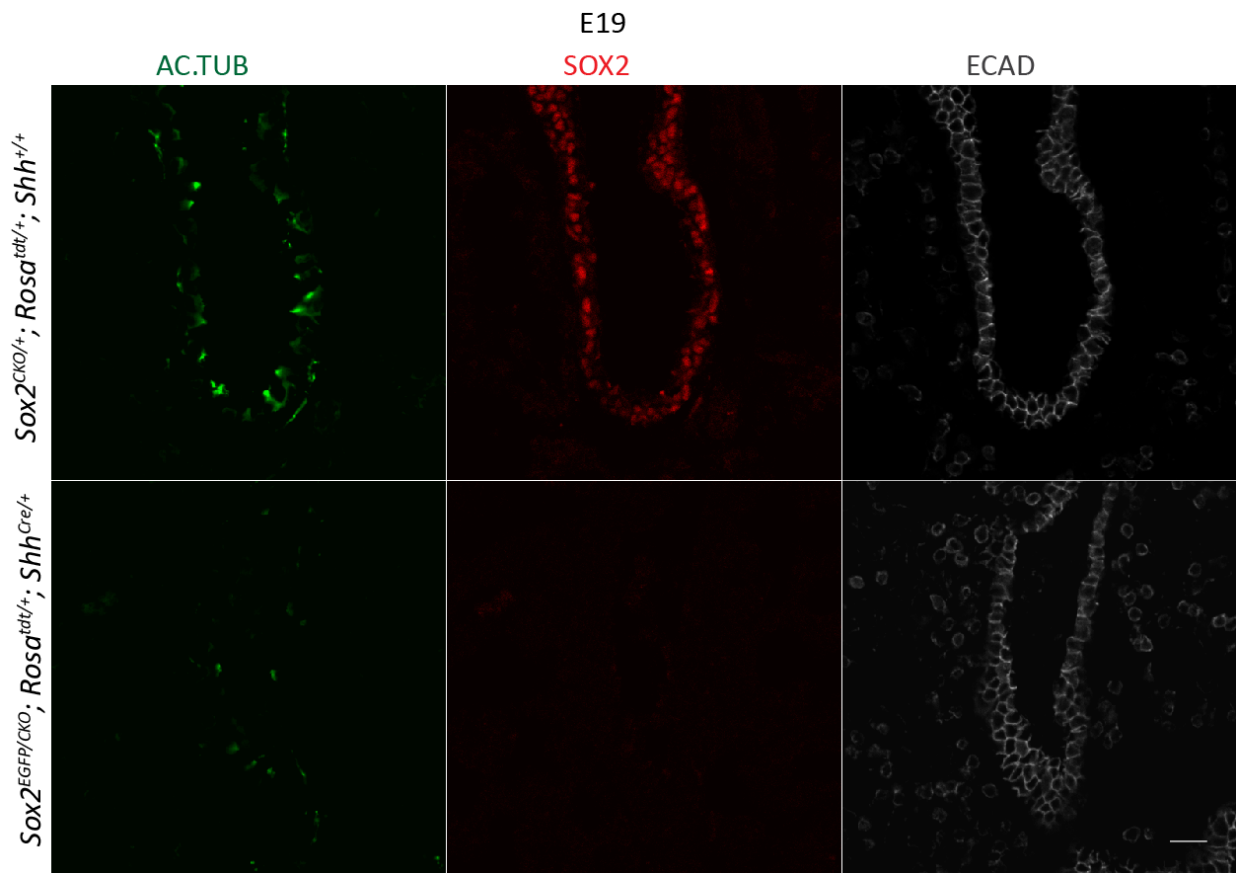
**Figure 3.4 Club cell differentiation is severely comprised in the SOX2 mutant.**

Confocal images of immunostained E19 lung tissue sections. CCSP (green) labels club cells, SOX2 (red) labels the conducting airways, and ECAD (gray) labels the epithelium. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. The level of CCSP expression in the SOX2 mutant is decreased compared to the SOX2 control. Scale bar: 20  $\mu$ m.



**Figure 3.5 The first step of ciliated cell differentiation is normal in the SOX2 mutant.**

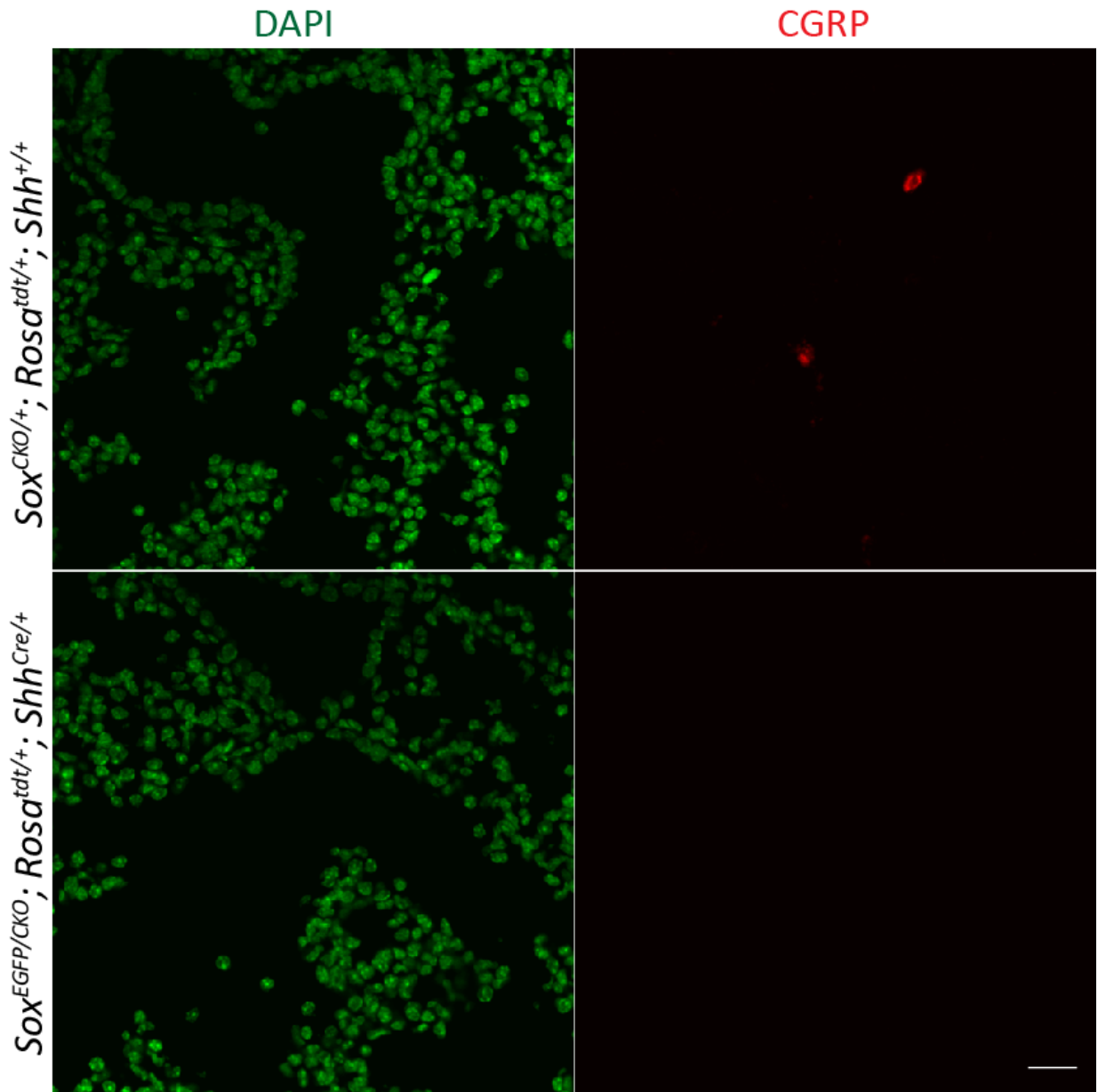
Confocal images of immunostained E19 lung tissue sections. FOXJ1 (red) labels ciliated cells and ECAD (gray) labels the epithelium. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. We did not observe a difference in FOXJ expression level between control and mutant lungs. Scale bar: 20 $\mu$ m.



**Figure 3.6 Ciliated cell differentiation is moderately affected in the SOX2 mutant.**

Confocal images of immunostained E19 lung tissue sections. AC.TUB (green) is acetyl tubulin and labels ciliated cells. SOX2 (red) labels the conducting airways, and ECAD (gray) labels the epithelium. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. Immunostained E19 lung sections allow us to visualize AC.TUB expression in the conducting airways. In the SOX2 mutant, AC.TUB expression signal is weak but there is ciliated cell differentiation. When SOX2 is absent in the conducting airways, ciliated cell differentiation occurs at low efficiency. Scale bar: 20  $\mu$ m.

E19

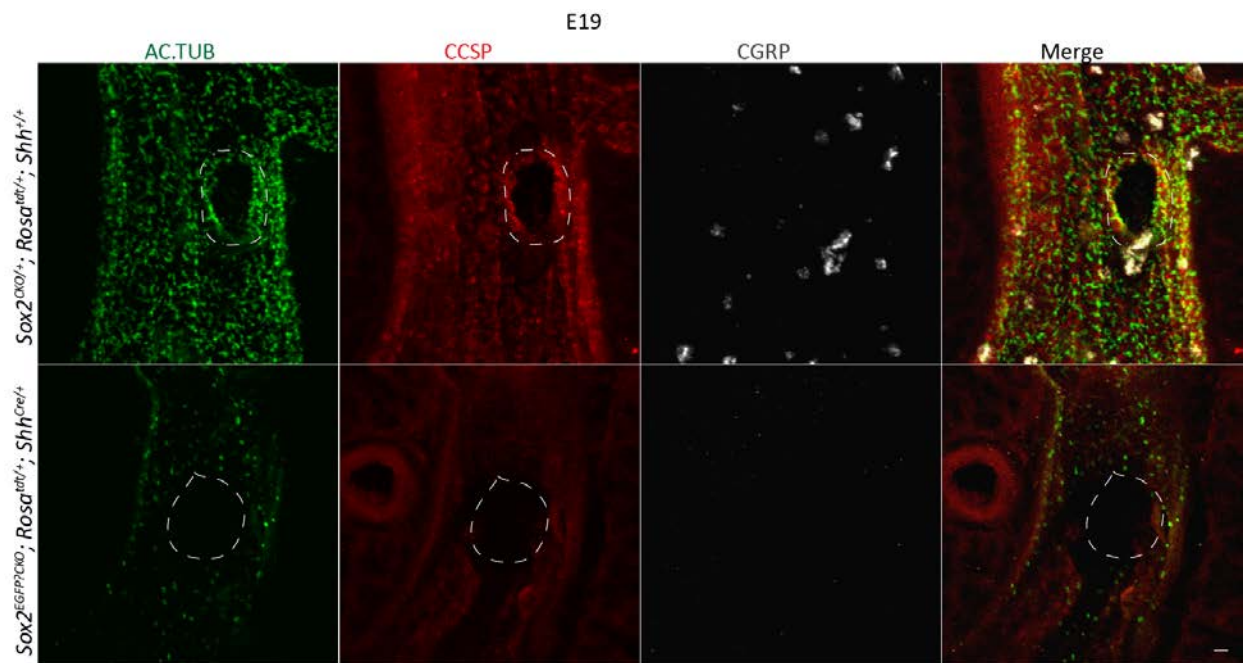


**Figure 3.7 Neuroendocrine cell differentiation does not occur in the SOX2 mutant.**

Confocal images of immunostained E19 lung tissue sections. DAPI (green) and CGRP (red) labels neuroendocrine cells. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. In the SOX2 mutant, neuroendocrine cells are absent when SOX2 is absent. Scale bar: 20  $\mu$ m.

### **3.3 SOX2 is required for neuroendocrine cell differentiation.**

Based on immunofluorescence results from E19 lung sections, neuroendocrine cell differentiation requires SOX2. The E19 lung sections in this study were random lobular bronchiole regions. We decided to analyze the same region for SOX2 control and SOX2 mutant lungs. We decided to specifically analyze the proximal region close to the mainstem bronchi and capture all the cells in the bronchus. We used E19 left lobes from Sox2 control and Sox2 mutant lungs for whole mount staining. Whole mount staining allowed us to identify the bronchus between lateral branch 2 (L2) and lateral branch 1 (L1). We were able to image the same region for SOX2 control and SOX2 mutant left lobes by imaging the bronchus region between L2 and L1. Z-stack confocal images were taken from the bronchus between L2 and L1. The lung tube was imaged from top to bottom in order to capture all the cells. We studied three lung cell types; club cells, ciliated cells, and neuroendocrine cells. Ciliated cell differentiation was moderately affected in the SOX2 mutant (Figure 3.8), which is similar to the results from E19 lung sections in figure 3.6. Club cell differentiation is decreased in the SOX2 mutant lung compared to the SOX2 control. CGRP expression is absent in the Sox2 mutant lung. We show SOX2 is required for neuroendocrine cell differentiation.



**Figure 3.8 Club cell and neuroendocrine cell differentiation requires SOX2 but ciliated cell differentiation does not.**

Confocal z-stack projection of immunostained E19 left lobes. E19 left lobes were bleached before staining. After whole mount staining, the left lobes were cleared with Benzyl Alcohol Benzyl Benzoate (BABB) in order visualize the conducting airways. The bronchus between L2 and L1 was imaged from top to bottom in order to capture all the cells. The SOX2 control lung had a z-stack of 71 images and SOX2 mutant lung had a z-stack of 35 images. Images were taken at 10X, magnification of 3.0, and step size of 2  $\mu\text{m}$ . AC.TUB (green) labels ciliated cells, CCSP (red) labels club cells, and CGRP (gray) labels neuroendocrine cells. Immunostained E19 left lobes allow us to visualize and capture images of SOX2<sup>+</sup>, AC.TUB<sup>+</sup>, CCSP<sup>+</sup>, and CGRP<sup>+</sup> cells in the conducting airways. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. Ciliated cell differentiation occurred at low frequency and club cell differentiation is reduced in the absence of SOX2. Neuroendocrine cell differentiation does not occur when SOX2 is absent. Scale bar: 50 $\mu\text{m}$ .

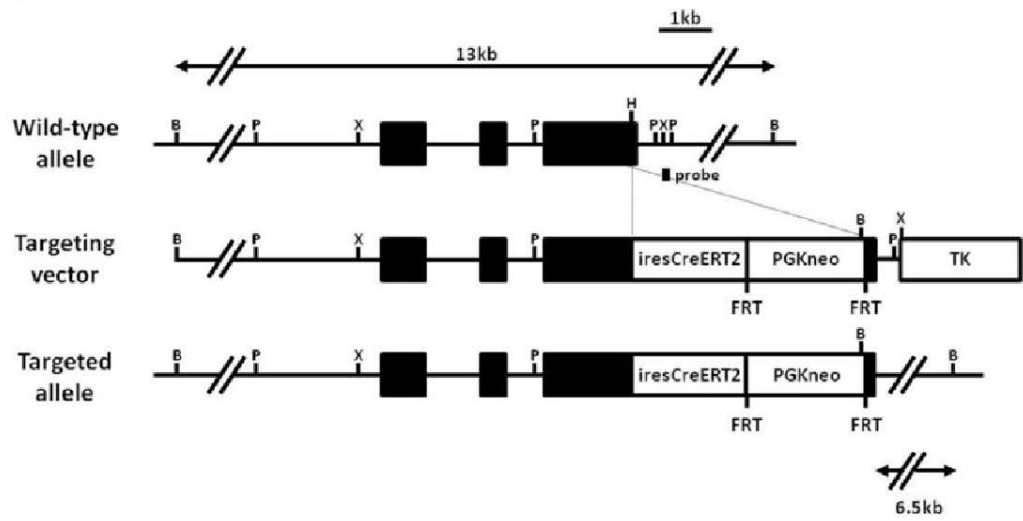


### 3.4 SOX2 is required cell- autonomous for club cell differentiation.

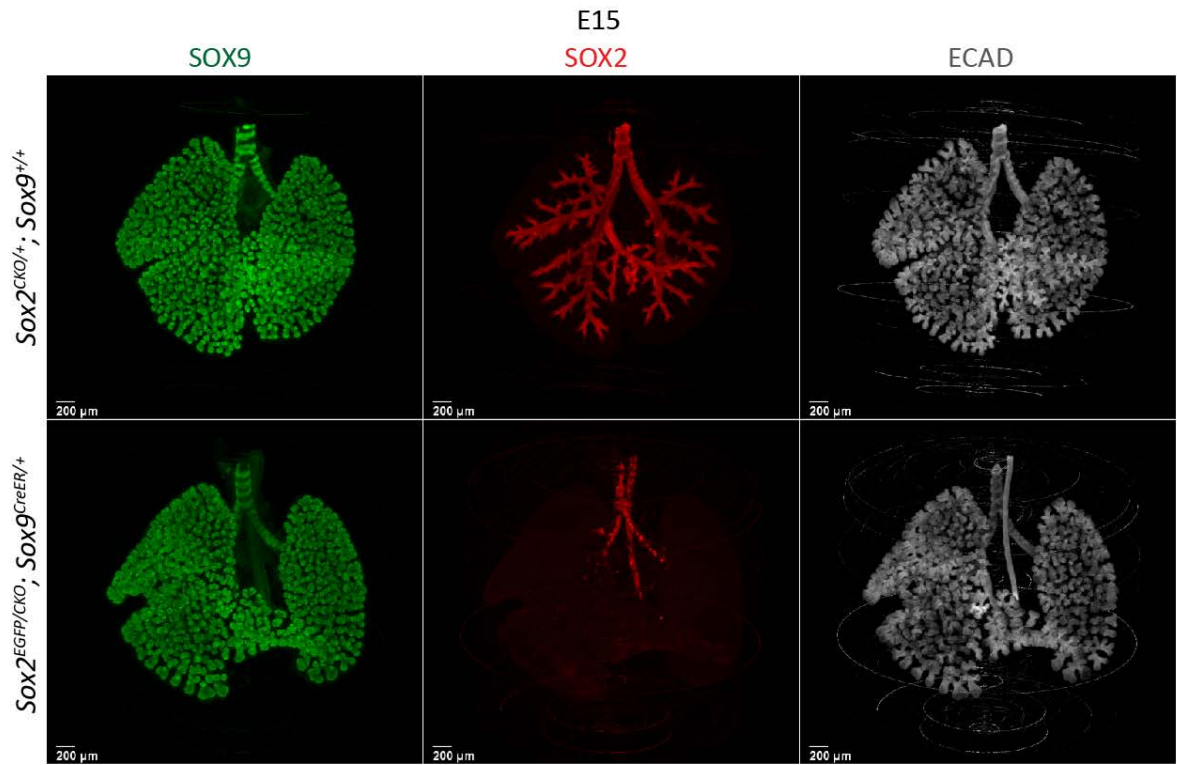
To determine if SOX2 signaling is cell-autonomous or non-autonomous in the conducting airways, we used a mosaic model to study SOX2 positive cells and SOX2 negative cells. The mosaic model will help determine how SOX2 control and mutant cells behave next to each other. We used *SOX9<sup>CreER/+</sup>* to induce SOX2 recombination in SOX9 progenitor cells. SOX9 progenitor cells are considered to give rise to future conducting airway cells. SOX9 is known to be important in branching morphogenesis. After lung branching is established, the conducting airways are developed. The *CreER* is integrated into the 3' UTR of the endogenous mouse *Sox9*. The allele construct consists of an internal ribosome entry sequence (IRES) followed by *CreER* (49, Figure 3.9A). The mouse breeding setup was ♀ *Sox2<sup>CKO/CKO</sup>* x ♂ *Sox2<sup>EGFP/+</sup>; Sox9<sup>CreER/+</sup>*. Pregnant female mice were injected via intraperitoneal with 2.0 mg of tamoxifen at E11, and harvest at E19. We injected female mice with tamoxifen at E11, because E11 is the stage when the lung starts to form during development. Pregnant female were harvested at E19, because E19 is the stage when airway differentiation begins. The SOX2 mutant of interest is *Sox2<sup>EGFP/CKO</sup>; Sox9<sup>CreER/+</sup>*. The SOX2 controls are *Sox2<sup>EGFP/CKO</sup>; Sox9<sup>+/+</sup>*, *Sox2<sup>CKO/+</sup>; Sox9<sup>+/+</sup>*, and *Sox2<sup>CKO/+</sup>; Sox9<sup>CreER/+</sup>*. We genotyped E19 embryos for *Sox9<sup>CreER/+</sup>* by using *CreER* primers (Table 4). We screened E19 embryos for GFP fluorescence to identify *Sox2<sup>EGFP/CKO</sup>* using a fluorescence microscope and genotyped for *Sox2<sup>CKO/CKO</sup>*. We show our mosaic mouse model for SOX2 recombination does work (Figure 3.9B). In the E15 SOX2 mutant lung, we detected patches of SOX2 positive cells in the conducting airways. We analyzed the trachea region to identify which lung cell types are SOX2 positive and SOX2 negative. The two lung cell types we studied in the trachea epithelium were club cells and ciliated cells. In the SOX2 mutant trachea, CCSP expression is not present in SOX2 positive cells and SOX2 negative cells (Figure 3.10). Club cell differentiation is inhibited by SOX2 negative and SOX2 positive cells. SOX2 negative cells may inhibit SOX2 positive cells, which leads to block club cell differentiation. In the second SOX2 mutant trachea, CCSP expression is present when there is continuous expression of SOX2. Ciliated cell expression is detected in both SOX2 positive cells and SOX2

negative cells. Ciliated differentiation occurred at low frequency. Based on our SOX2 trachea results, club cell differentiation is blocked in the absence of SOX2. To further address if SOX2 is cell-autonomous in the conducting airways, we use an E19 Left lobe from a SOX2 mutant lung and imaged the bronchus between L2 and L1. The bronchus tube was imaged from top to bottom in order to capture all cells. The SOX2 mutant lung is our internal control. We two lung cell types we studied in the left lobes were club cells and ciliated cells. We observed SOX2 positive cells co-localize with CCSP positive cells (Figure 3.11). We did not observe SOX2 negative cells co-localize with CCSP positive cells. Our data suggests that SOX2 is required cell-autonomously for club cell differentiation in the conducting airways. Based on our loss of function experiments, SOX2 controls cell differentiation in the conducting airways. Basal cell, ciliated cell, club cell, and neuroendocrine cell differentiation are affected at different levels when SOX2 is absent in the conducting airways. The next question is if SOX2 is sufficient to induce airway differentiation? To determine the cell fate of conducting airway cells we need to manipulate SOX2 expression in the lung epithelium by inducing SOX2 where it is not normally present.

A

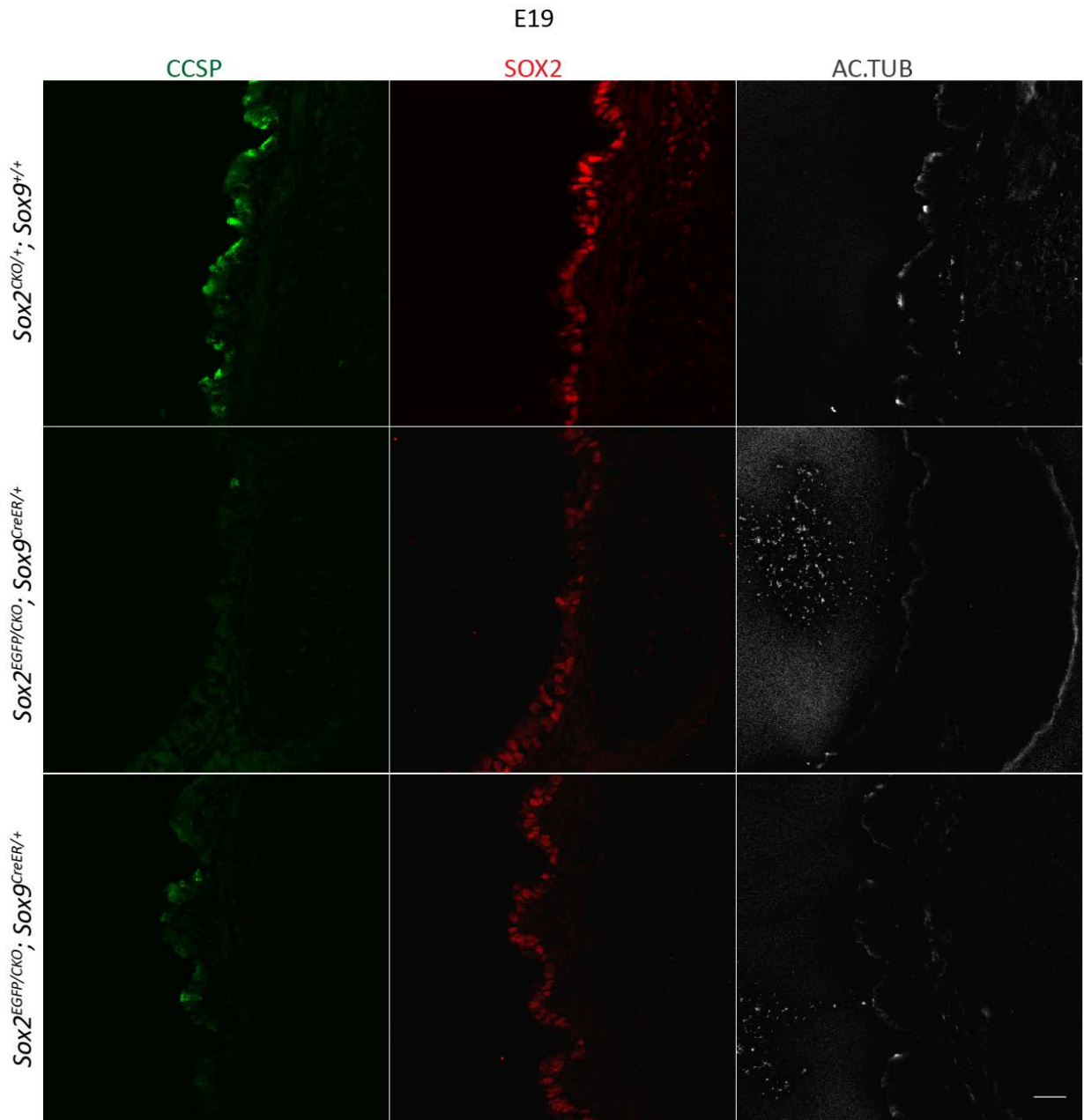


B



**Figure 3.9 SOX2 mosaic model.**

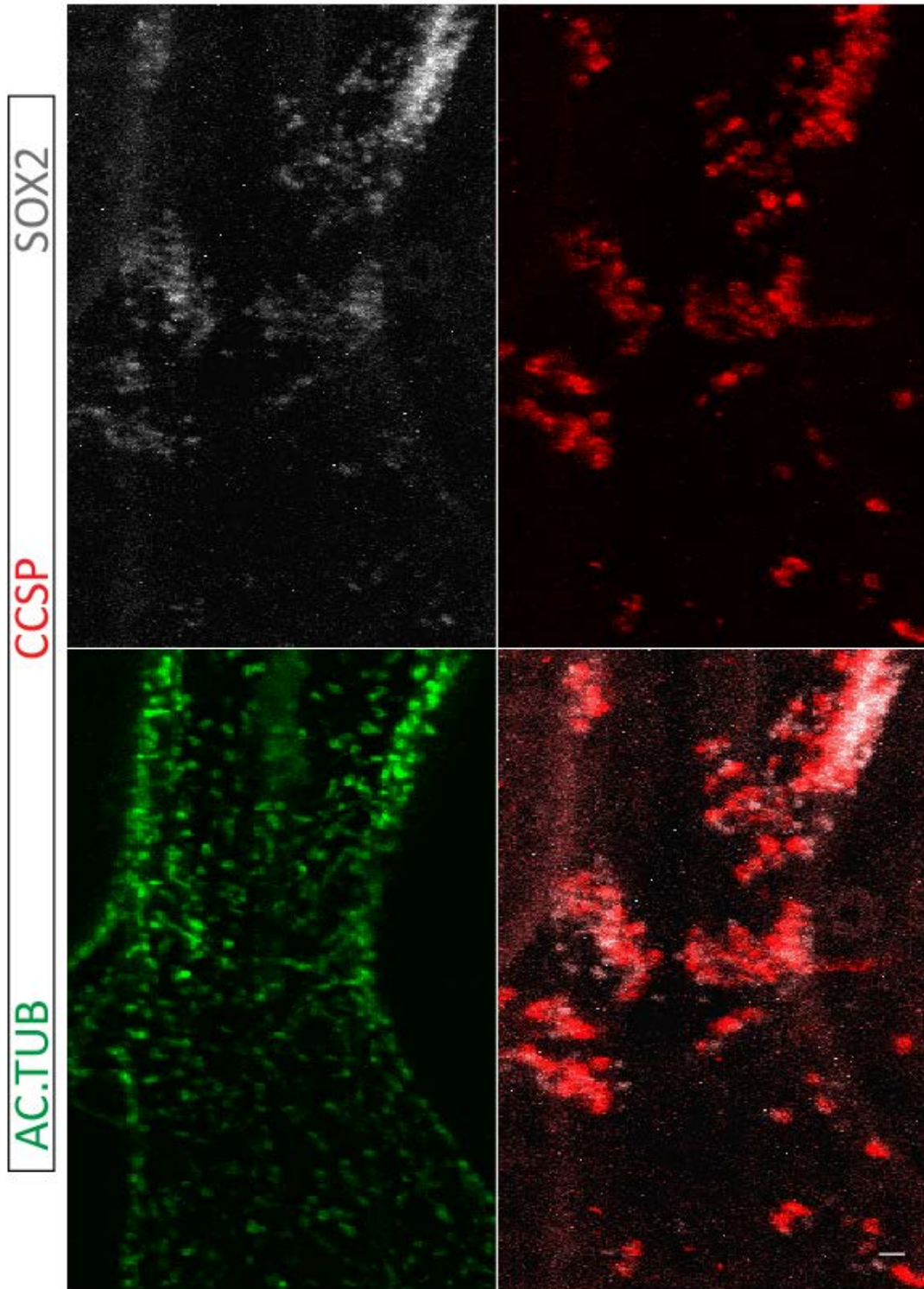
A) A schematic diagram of the *Sox9<sup>CreER/+</sup>* mouse construct (49). B) Optical projection tomography (OPT) scans of immunostained E15 whole lungs. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. SOX9 (green) labels distal progenitor cells, SOX2 (red) labels conducting airway cells, and ECAD (gray) labels the lung epithelium. The mosaic mouse model works by showing recombination of SOX2 cells. We observed small patches of SOX2<sup>+</sup> cells and SOX2 negative cells in the conducting airway. Scale bar: 200  $\mu\text{m}$ .



**Figure 3.10 In the trachea, SOX2 is required cell-autonomous for club cell differentiation.**

Confocal images of immunostained E19 lung tissue sections. CCSP (green) labels club cells, SOX2 (red) labels the conducting airways, and ECAD (gray) labels the epithelium. Immunostained E19 lung sections allow us to visualize co-localization of CCSP<sup>+</sup> cells and AC.TUB<sup>+</sup> cells with SOX2 positive and SOX2 negative cells. The top panel is the SOX2 control trachea. The middle and bottom panels are the SOX2 mutant tracheas. In the middle panel, SOX2 negative cells do not express CCSP nor do SOX2 positive neighbor cells. In the bottom panel, continuous expression of SOX2 leads to club cell differentiation. Scale bar: 20µm.

E19 Sox2<sup>EGFP/CKO</sup>; Sox9<sup>CreER/+</sup>



**Figure 3.11 SOX2 is required cell-autonomously for club cell differentiation in the conducting airways.**

Confocal z-stack projection of an immunostained SOX2 mutant E19 left lobe. The E19 left lobe was bleached before staining. After whole mount staining, the left lobe was treated with BABB in order to visualize the conducting airways. The bronchus between L2 and L1 for the SOX2 mutant (z-stack of 52 images) was imaged from top to bottom in order to capture all cells. Images were taken at 10X, magnification of 3.0, and step size of 2  $\mu\text{m}$ . AC.TUB (green) labels ciliated cells, CCSP (red) labels club cells, and SOX2 (gray) labels conducting airways. The SOX2 mutant is our internal control. SOX2 positive cells co-localize with CCSP and SOX2 negative cells did not co-localize with CCSP. Scale bar: 20  $\mu\text{m}$ .

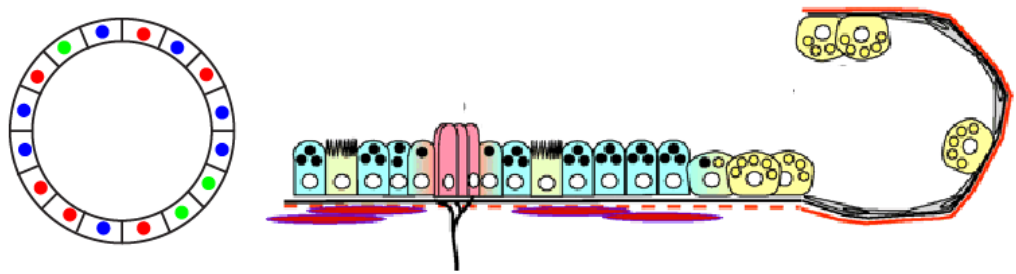


#### 4. Results II: SOX2 controls cell differentiation of conducting airway cells.

##### 4.1 Sox2 overexpression in the lung epithelium.

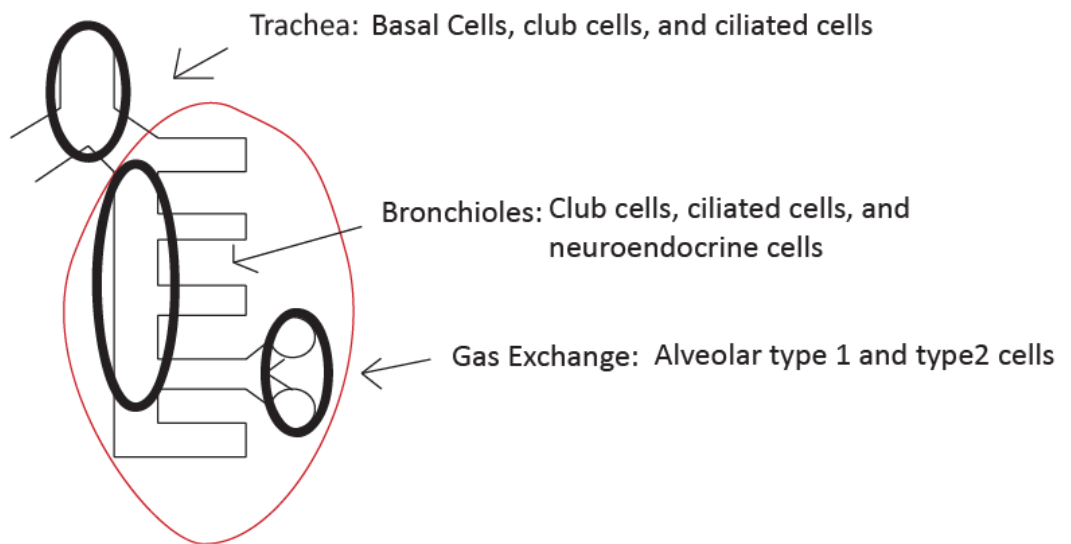
The conducting airways are tubes that are alive. The airway tubes are composed of living cells, and within a tube there are different types of cells (Figure 4.1A). The lung contains airway tubes with different types of cells. The mouse lung has three different regions (Figure 4.1B). The trachea region consists of basal cells, club cells, and ciliated cells. The bronchioles consist of club cells, ciliated cells, and neuroendocrine cells. The gas exchange region also known as the alveoli consists of alveolar type 1 and type 2 cells. We want to identify the gene that controls the type and the distribution of cells in the conducting airways. Based on the SOX2 loss of function experiments in this study, SOX2 has an effect on cell differentiation. In the absence of SOX2, neuroendocrine cell differentiation does not occur. Club cell differentiation and basal cell differentiation is decreased in the absence of SOX2. To determine to what extent SOX2 controls the distribution of different cell types, we overexpressed SOX2 in the conducting airways and gas exchange region. SOX2 overexpression will help us determine if there is a change in the distribution pattern of conducting airways. Is SOX2 sufficient to induce airway differentiation? We used a mouse model to express SOX2 in the gas exchange region, where SOX2 is not normally present. SOX2 overexpression was achieved by using a *Rosa26* locus. The SOX2 overexpression allele construct is *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* referred as *Rosa<sup>Sox2/Sox2</sup>*. The removal of the flanked loxP sites leads to SOX2 expression and expresses green fluorescence (45, Figure 4.2A). SOX2 was overexpressed in the lung epithelium by using *Shh<sup>Cre/+</sup>* and *Rosa<sup>Sox2/Sox2</sup>*. The mouse breeding was: ♀ *Rosa<sup>Sox2/Sox2</sup>* x ♂ *Shh<sup>Cre/+</sup>*. The mutant embryo of interest is *Rosa<sup>Sox2/+</sup>; Shh<sup>Cre/+</sup>*. The controls are *Rosa<sup>Sox2/+</sup>*. We genotyped E19 embryos for *Rosa<sup>Sox2/+</sup>* and *Shh<sup>Cre/+</sup>* (Table 4). We harvested E19 lungs because it is the stage when cell differentiation occurs. We used E19 control and mutant embryos to confirm SOX2 overexpression. We used confocal images of immunostained E19 lung sections to observe SOX2 expression in the gas exchange region (Figure 4.2B). We were able to overexpress SOX2 in the conducting airways and in the gas exchange region.

A



B

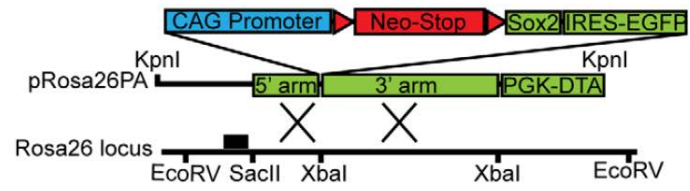
Normal Distribution Pattern of Lung Cell Types in the Mouse Lung



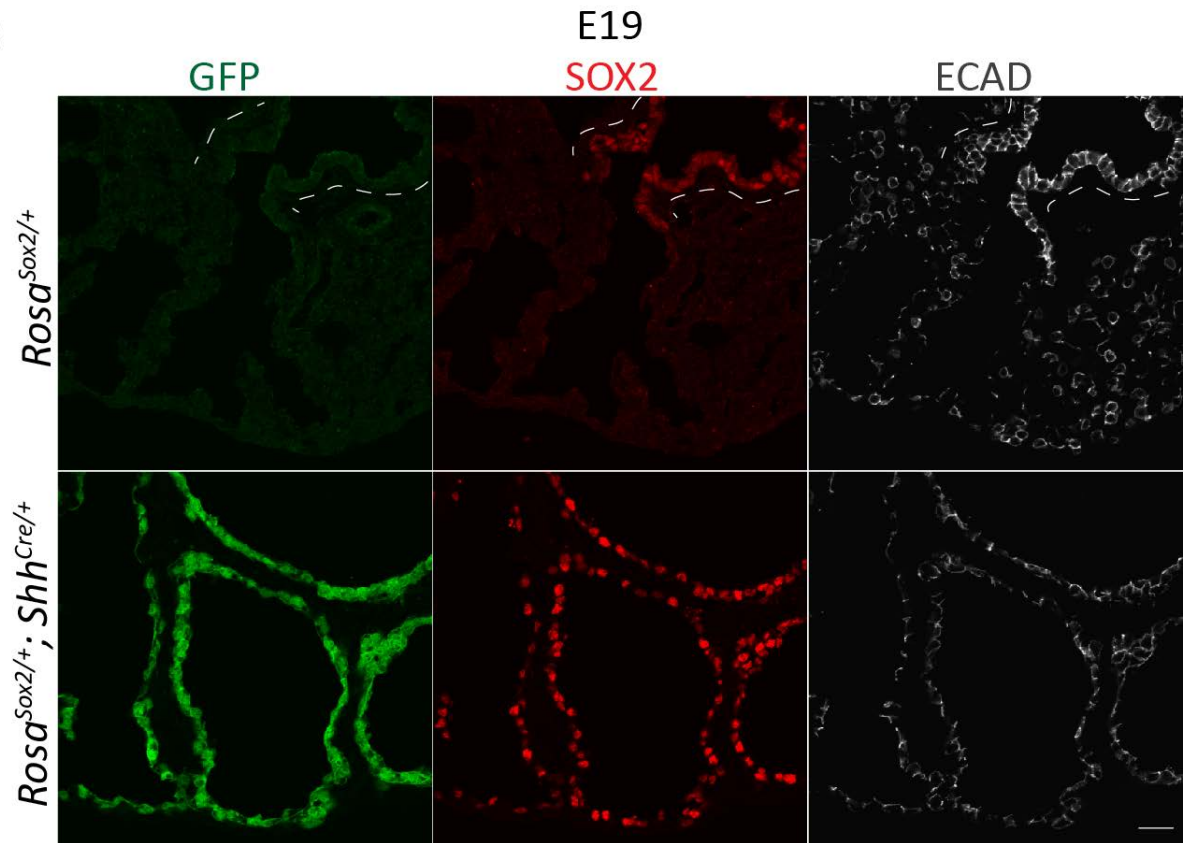
**Figure 4.1 Cell differentiation in the mouse lung.**

A) A representation of a slice section of an airway tube with different types of color. The different colors represent the different cell types within a tube. The lung is an organ that has a tubular structure (53). Within the conducting airways, there are different lung cell types. The distal part of the lung is the gas exchange region, which contains alveolar type 1 and type 2 cells. B) A picture diagram of the three regions of the mouse lung with a distribution pattern of different lung cell types.

A



B



**Figure 4.2 SOX2 overexpression in the entire lung epithelium.**

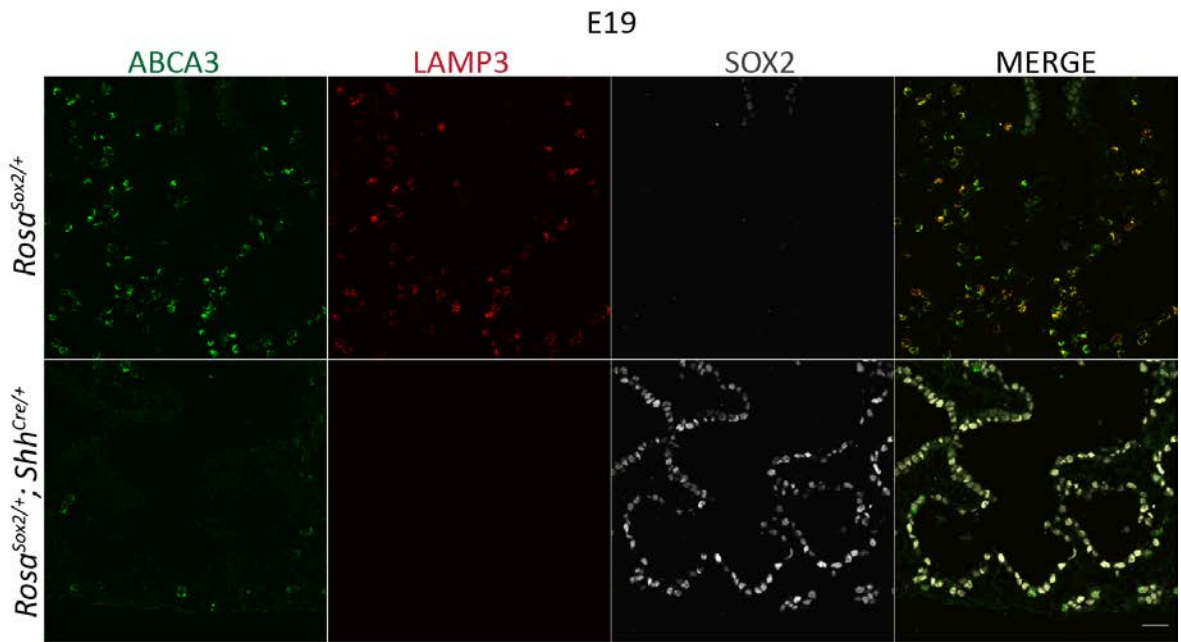
A) A schematic diagram of *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* allele (45). B) Confocal images of immunostained E19 lung sections. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. The dashed lines outline the conducting airways. GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, SOX2 (red) labels the conducting airways, and ECAD (gray) labels the epithelium. GFP positive cells express SOX2 cells in the SOX2 mutant lung. Scale bar: 20  $\mu\text{m}$ .

## 4.2 Sox2 ectopic expression in the gas exchange region suppresses type 1 and type 2 alveolar cells.

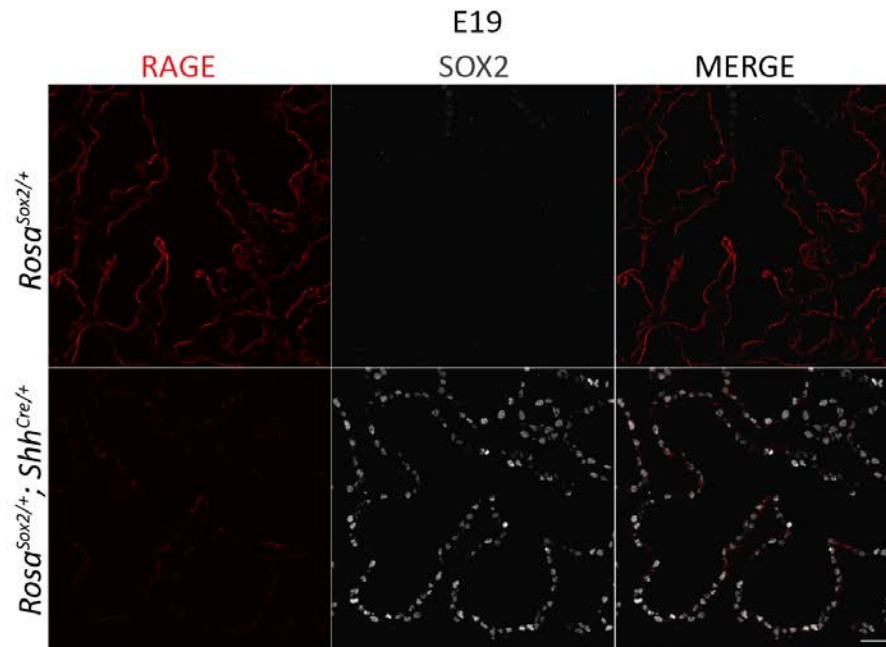
We were able to express SOX2 in the gas exchange region in the mouse. The question is when SOX2 is expressed in the gas exchange region is there alveolar type 1 and type 2 cell differentiation? At E19, alveolar type 1 and type 2 cells start to differentiate. We used E19 lungs to determine if there is alveolar type 1 cell differentiation and alveolar type 2 cell differentiation. We used confocal images of immunostained E19 lung sections of alveolar type 2 cell markers; ABCA3 and LAMP3 (Figure 4.3A). We also stained for RAGE, an alveolar type 1 cell marker. When SOX2 is expressed in the gas exchange region, alveolar type 1 cell differentiation and alveolar type 2 cell differentiation is suppressed (Figure 4.3A and 4.3B). These results suggest that SOX2 triggers a signal in the alveoli by establishing conducting airway compartment and not the gas exchange compartment. SOX2 expression in the gas exchange region overrides alveolar cell differentiation. SOX2 dominates the gas exchange region by overriding alveolar cell differentiation and sends a signal that it is the conducting airway compartment. To further address if SOX2 overrides alveolar cell differentiation in the gas exchange region, we used another mouse model to specifically express SOX2 in the gas exchange region. When we overexpressed SOX2 using *Shh<sup>Cre/+</sup>*, we observed large airway branches and a lower number of branches. We used *Sox9<sup>CreER/+</sup>* and *Rosa<sup>Sox2/Sox2</sup>* to express SOX2 in SOX9 progenitor cells to avoid branching defects and have temporal control. SOX9 positive cells are found in the distal area of the lung (1). The *Sox9<sup>CreER/+</sup>* consists of a 5' internal ribosome entry sequence (IRES) followed by a *CreER* integrated into the 3' UTR of the endogenous mouse *Sox9* gene (49). The mouse breeding setup was: ♀ *Rosa<sup>Sox2/Sox2</sup>* × ♂ *Sox9<sup>CreER/+</sup>*. The mutant embryo of interest is *Sox9<sup>CreER/+</sup>; Rosa<sup>Sox2/+</sup>*. The control is *Rosa<sup>Sox2/+</sup>*. Pregnant female mice were injected intraperitoneally with 2.0mg of tamoxifen at E15 and harvested at E19. We genotyped E19 embryos for *Rosa<sup>Sox2/+</sup>* and *Sox9<sup>CreER/+</sup>* (Table 4). We injected pregnant female mice at E14 because E14 is the stage after lung formation. We were able to express SOX2 in the gas exchange region with no branching defects (Figure 4.4). We used E19 lung sections for immunofluorescence staining to determine if there is alveolar type 1 cell and alveolar type 2 cell differentiations. In the

mutant lung, SOX2 inhibits alveolar type 1 cell and alveolar type 2 cell differentiation (Figure 4.5A and 4.5B). A few SOX2 positive cells do express alveolar type 2 cell markers (Figure 4.5A). RAGE, an alveolar type 1 marker, wraps around SOX2 positive cells but does not co-localize with Sox2 (Figure 4.5B). We show SOX2 expression in the gas exchange region inhibits alveolar cell differentiation. SOX2 signaling dominates the gas exchange region by overriding the alveolar cell differentiation and setting it up as the conducting airways.

A



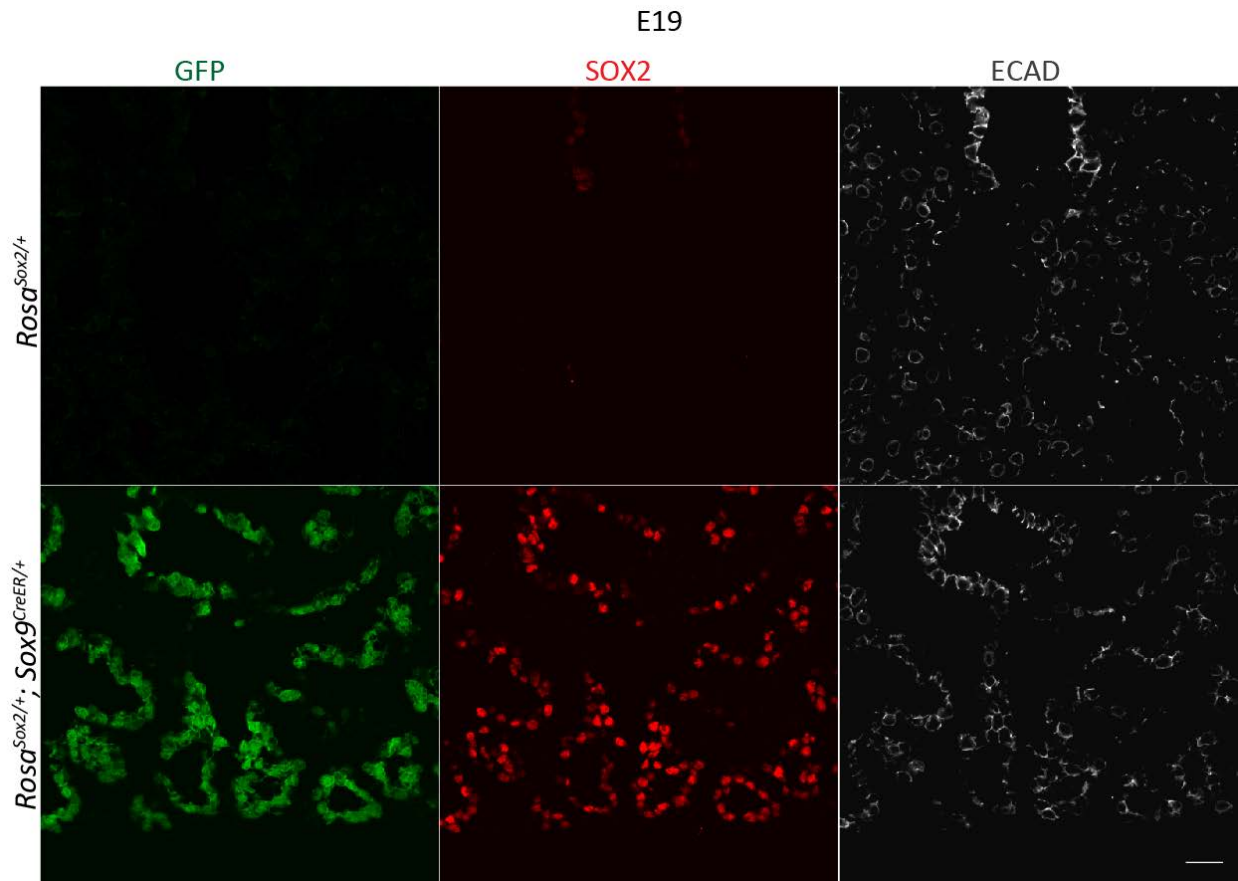
B





**Figure 4.3 SOX2 ectopic expression in the gas exchange region suppresses alveolar type 1 cell differentiation and alveolar type 2 cell differentiation.**

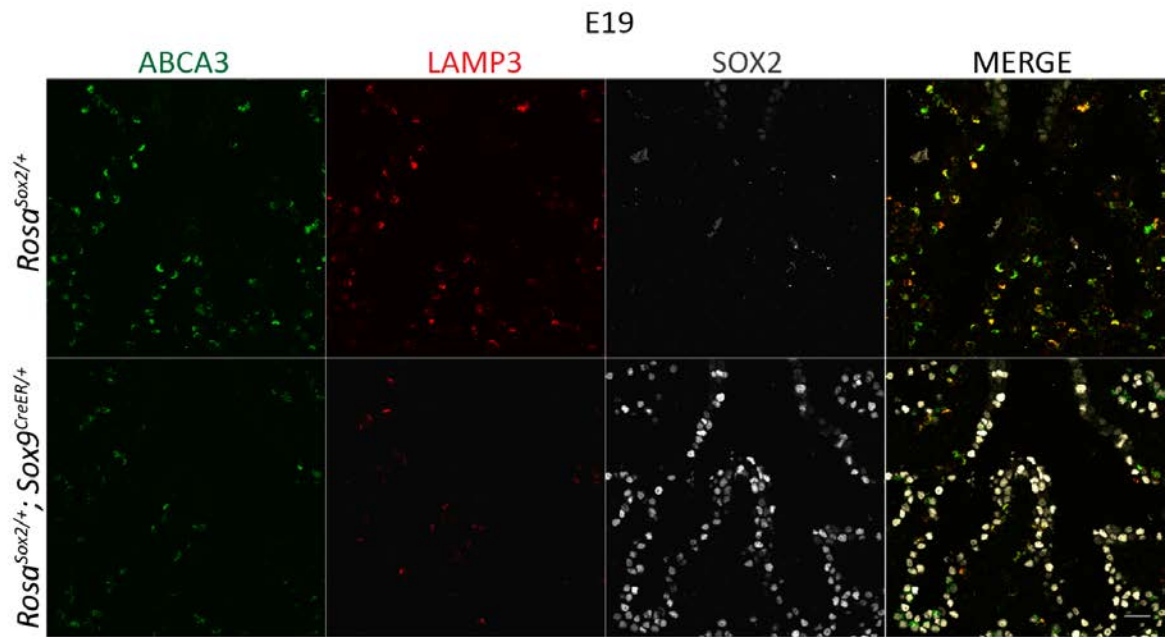
Confocal images of immunostained E19 lung sections. (A and B) The top panels are the SOX2 control lungs and bottom panels are the SOX2 mutant lungs. A) To visualize co-localization of alveolar type 2 cell expression with SOX2 (gray), we used ABCA3 (green) and LAMP3 (red). B) To visualize co-localization of SOX2 (gray) with RAGE (red), an alveolar type 1 cell marker. (A and B) SOX2 inhibits alveolar type 1 cell differentiation and alveolar type 2 cell differentiation in the gas exchange region. Scale bar: 20 $\mu$ m.



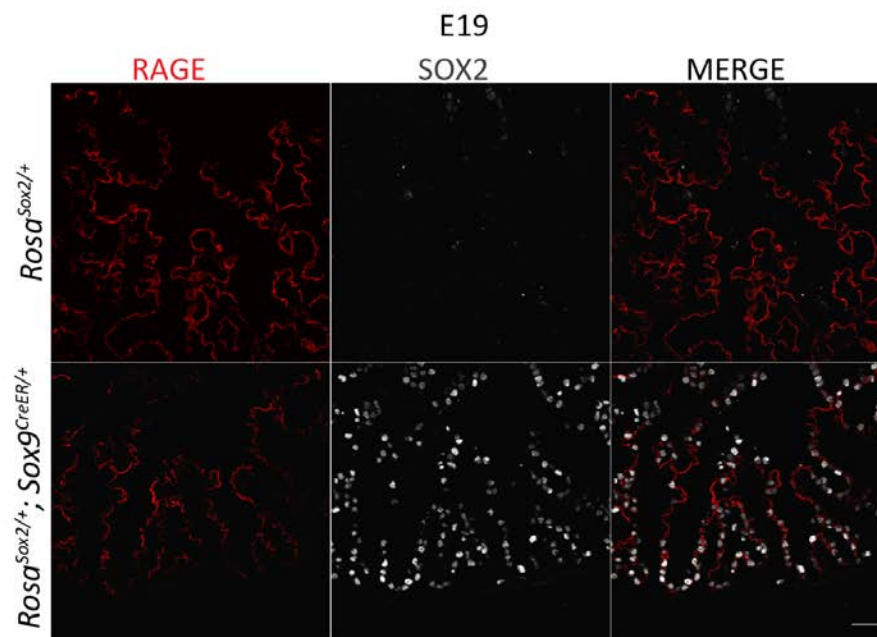
**Figure 4.4 SOX2 ectopic expression specifically in the gas exchange region.**

Confocal images of immunostained E19 lung sections. Pregnant female mice were injected intraperitoneally with 2.0 mg Tamoxifen at E15 and harvest at E19. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, SOX2 (red) labels the conducting airways, and ECAD (gray) labels the epithelium. SOX2 was specifically express in the gas exchange region with no abnormal branching defects. Scale bar: 20  $\mu\text{m}$ .

A



B



**Figure 4.5 SOX2 ectopic expression in the distal tips reduces alveolar type1 cell differentiation and alveolar type 2 cell differentiation.**

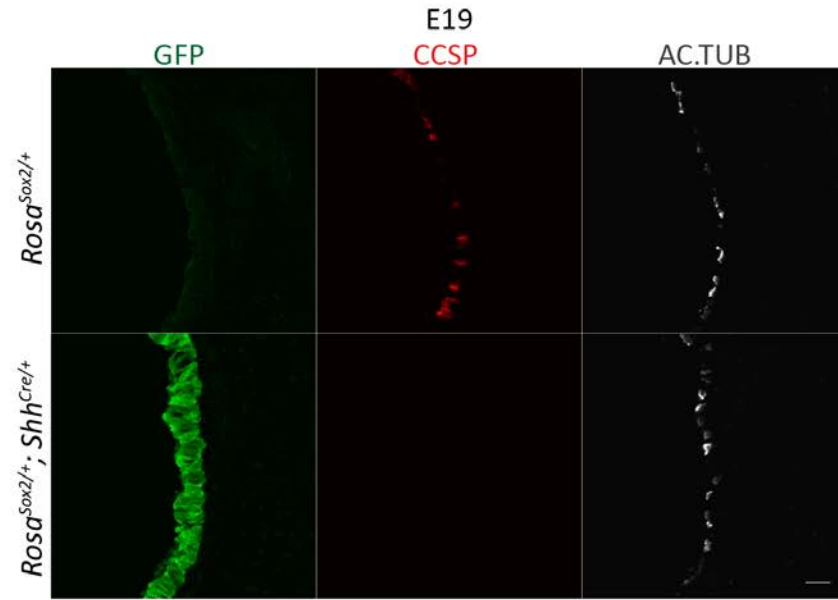
Confocal images of immunostained E19 lung sections. (A and B) The top panels are the SOX2 control lungs and the bottom panels are the SOX2 mutant lungs. A) To visualize co-localization of SOX2 (gray) with alveolar type 2 cells, we used ABCA3 (green) and LAMP3 (red). B) To visualize co-localization of SOX2 (gray) with alveolar type 1 cells, we used RAGE (red). (A and B) SOX2 expression in distal progenitor cells suppresses alveolar cell differentiation. Scale bar: 20  $\mu$ m.

### 4.3 SOX2 overexpression does not extend the pattern of club cells and ciliated cells in the conducting airways

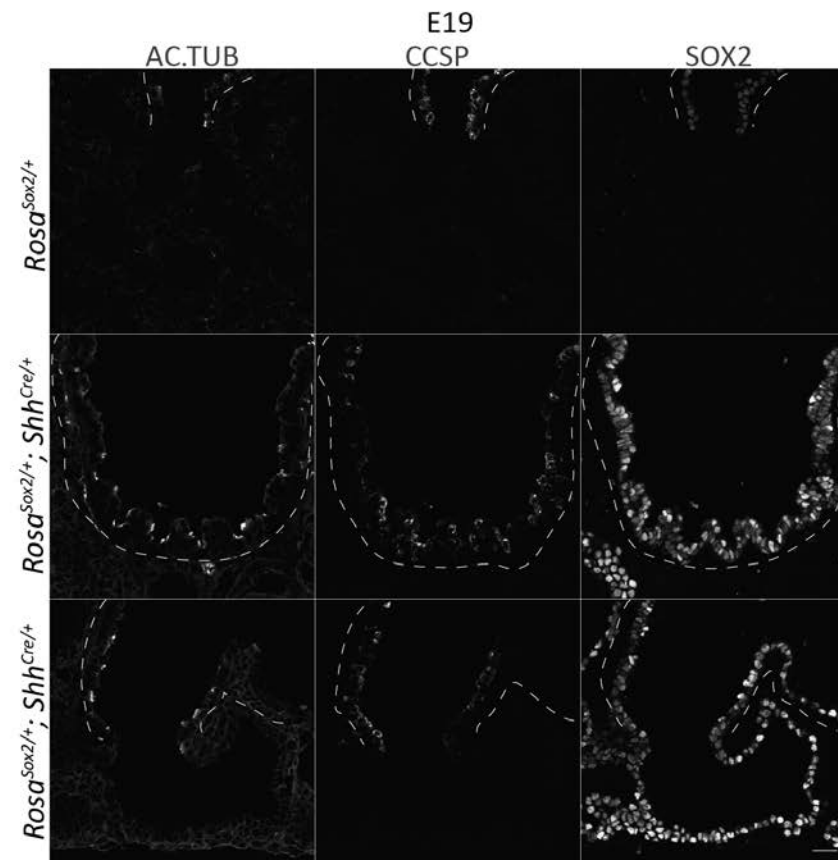
In our previous experiments, SOX2 overexpression throughout the entire lung epithelium including the gas exchange region inhibits alveolar cell differentiation. The next question to address is if SOX2 overexpression will extend the distribution pattern of conducting airway cells into the gas exchange region. We used the *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* allele and *Shh<sup>Cre/+</sup>* to overexpress SOX2 in the lung epithelium. We used the SOX2 overexpression mouse model (Figure 4.2) to study the distribution pattern of club cells and ciliated cells. We harvested E19 mouse embryos because that is the embryonic stage when conducting airway cells have differentiated. We analyzed trachea region first to determine if there is a change in the distribution pattern of conducting airway lung cell types in any of the three different regions of the lung. Interestingly, no club cells were detected in the trachea when SOX2 is overexpressed (Figure 4.6A). We did not observe a difference in the distribution and expression of ciliated cells between the control and mutant lungs. Ciliated cell differentiation is not affected by the increase of SOX2, but there is an effect in club cell differentiation in the trachea. Next, we analyzed the lobular bronchioles and the gas exchange region. SOX2 overexpression in the entire lung epithelium did not extend club cell expression or ciliated cell expression in the gas exchange region (Figure 4.6B). The distribution pattern of club cells and ciliated cells was restricted only in the conducting airways. The amount increase of SOX2 cells in the lung did not change the distribution pattern in the lobular bronchioles or extend the distribution pattern of club cells and ciliated cells into the alveoli. The data suggests that as long as SOX2 is present in the lung there is club cell differentiation and ciliated differentiation in the lobular bronchioles. Based on the SOX2 loss of function experiments, club cell differentiation is blocked when SOX2 is absent. When SOX2 is overexpressed in the lung, there is a change in club cell differentiation in the trachea but not in the lobular bronchioles. SOX2 is required for club cell differentiation. Ciliated cell differentiation was not blocked in the absence of SOX2. The distribution pattern of ciliated cells did not change when Sox2 was overexpressed in the lung. In the absence of SOX2, more ciliated cells are present than club cells. The

data suggests that SOX2 has control on ciliated cell differentiation. In a normal lung, SOX2 controls the salt and pepper pattern of club cells and ciliated cells in the trachea and the bronchioles. SOX2 is required to coordinate the distribution pattern of club cells and ciliated cells in the conducting airways.

A



B



**Figure 4.6 SOX2 overexpression in the lung epithelium does not extend the pattern of club cells and ciliated into the gas exchange region.**

Confocal images of immunostained E19 lung sections. A) E19 trachea sections of the SOX2 control (top panel) and SOX2 mutant (bottom panel). GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, CCSP (red) labels club cells, and AC.TUB (gray) labels ciliated cells. SOX2 overexpression induces GFP expression. CCSP is absent in the mutant trachea. B) E19 lung sections are in gray scale for the SOX2 control (top panel) and SOX2 mutants (middle and bottom panels). The dashed lines outline the bronchioles in the conducting airways. The middle panel is a lobular bronchiole section of the SOX2 mutant lung. The bottom panel is a section of the gas exchange region of the SOX2 mutant lung. Club cell and ciliated cell differentiation was not detected in the mutant gas exchange region. Scale bar: 20  $\mu\text{m}$ .

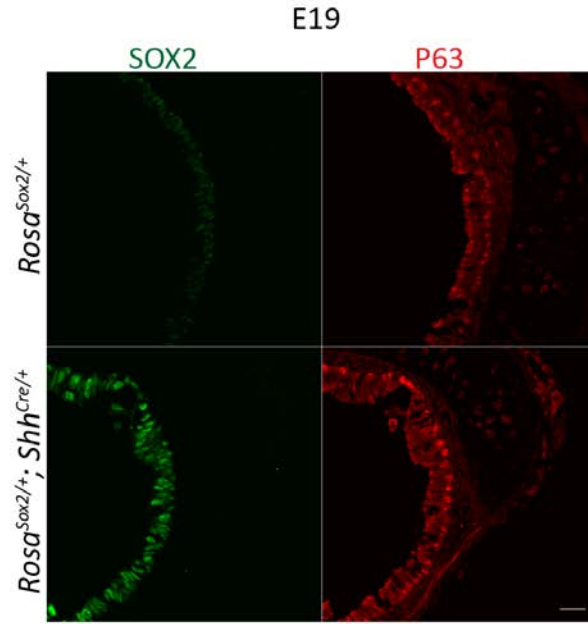


#### 4.4 SOX2 overexpression in the lung epithelium induces basal cell differentiation in the gas exchange region.

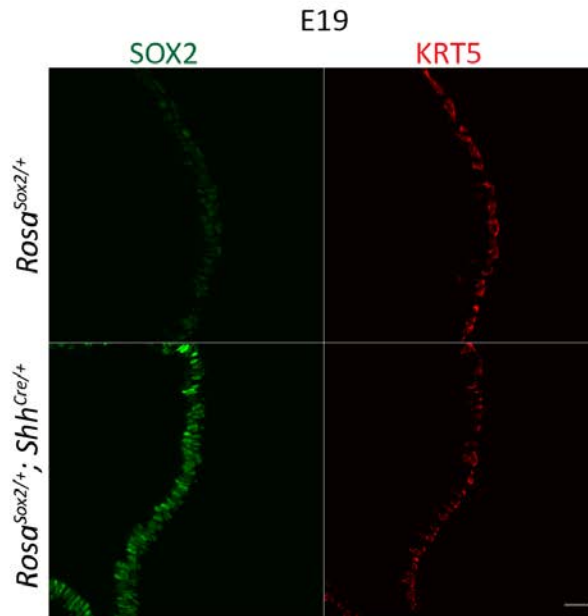
We have studied two conducting airway cell types; club cells and ciliated cells. We did not detect an extension of the club cells and ciliated cells into the gas exchange region. We did not detect club cell differentiation in the trachea when SOX2 is overexpressed. We decided to study another lung cell type, basal cells. We used the *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* allele and *Shh<sup>Cre/+</sup>* to overexpress SOX2 in the lung epithelium to determine the expression pattern of basal cells. We analyzed the trachea first, the proximal region of the lung, to determine the expression pattern of basal cells. We did not detect a difference in basal cell expression between the control and mutant trachea (Figure 4.7A). We perform quantification analysis to calculate the portion of SOX2 cells in the trachea epithelium that express P63. We counted the number of P63 cells that co-localize with SOX2. The percentage of basal cells was calculated by P63<sup>+</sup> cells over the total number of SOX2 cells. We counted three E19 trachea sections from both control and mutant lungs (n=3). The percentage of basal cells for the control is 17% ± 2% from a total number of 28 P63<sup>+</sup> cells and 164 SOX2<sup>+</sup> cells. The percentage of basal cells for the mutant is 18% ± 3% from a total number of 44 P63<sup>+</sup> cells and 251 SOX2<sup>+</sup> cells. The percentage of basal cells in the trachea was not significant, p-value > 0.5. We used another basal cell marker, Keratin 5 (KRT5), to study basal cell differentiation. Keratin 5 is considered to be a marker for mature basal cells. We did not detect a difference in Keratin 5 expression between the control and mutant trachea (Figure 4.7B). The data suggests that basal cells in both control and mutant tracheas contain mature basal cells by expressing both P63 and KRT5. Next, we analyzed the lobular bronchioles and the gas exchange region. In the lobular bronchioles, we detected rare P63<sup>+</sup> cells in the mutant. Interestingly, P63<sup>+</sup> cells were present in the mutant gas exchange region (Figure 4.8A). Basal cells are known to be expressed in the trachea and the mainstem bronchi. Quantification analysis was done to calculate the portion of SOX2 cells that express P63 in the gas exchange region for both the mutant and control lungs. We counted three E19 lung sections from both mutant and control lungs (n=3). The

percentage of P63<sup>+</sup> cells for the control was 0%. The percentage of P63<sup>+</sup> cells for the mutant was 29% ± 0.21% from a total of 120 P63<sup>+</sup> cells and 575 SOX2<sup>+</sup> cells. The percentage of P63 cells in the gas exchange region was significant, p-value < 0.5. To further study basal cells in the gas exchange region, we used another basal cell marker, KRT5. In the lobular bronchioles, we detected rare KRT5<sup>+</sup> cells in the mutant lung and none in the control lung. In the gas exchange region, we detected a few KRT5<sup>+</sup> cells in the mutant (Figure 4.8B). We performed quantification analysis of KRT5<sup>+</sup> in the gas exchange region for both control and mutant lungs to calculate the portion of SOX2 cells that are able to express KRT5. The percentage of KRT5<sup>+</sup> cells in the control lung was 0%. The percentage of KRT5<sup>+</sup> cells for the mutant lung is 4% ± 1% from a total number of 16 KRT5<sup>+</sup> cells and 492 SOX2<sup>+</sup> cells. The percentage of KRT5 cells in the gas exchange region was not significant, p-value > 0.5. A small percentage of basal cells are fully matured basal cells by expressing both basal cell markers, KRT5 and P63. In the mutant lung, there are few basal cells that are able to have complete basal cell properties. The remaining basal cells that only express P63 are not mature cells and the cell fate of these cells is unknown. The data suggests that SOX2 can extend the distribution pattern of basal into the gas exchange region. The basal cell distribution pattern in the mutant lung is in the trachea and the gas exchange region. In the literature, there are two types of P63 isoforms. The two types of P63 isoforms are P63 transactivation (TA) and lacking the transactivation domain, delta N (ΔN). We analyzed E19 lung sections from mutant and control lungs to identify which type of P63 isoform is expressed in the mutant gas exchange region. In the lobular bronchioles, we detected P63 TA expression in the control lung and not in the mutant lung (Figure 4.9). In the gas exchange region, we detected P63 ΔN expression in the mutant lung and not in the control lung (Figure 4.10). P63 TA expression is present only in the bronchioles and not in the gas exchange region. P63 ΔN expression is present in the bronchioles of the control lung and in the gas exchange region of the mutant lung. The basal cells that are found in the mutant gas exchange region are P63 ΔN. Basal cell expression in the mutant gas exchange region suggests that SOX2 is establishing the conducting airway compartment by beginning with basal cell differentiation.

A



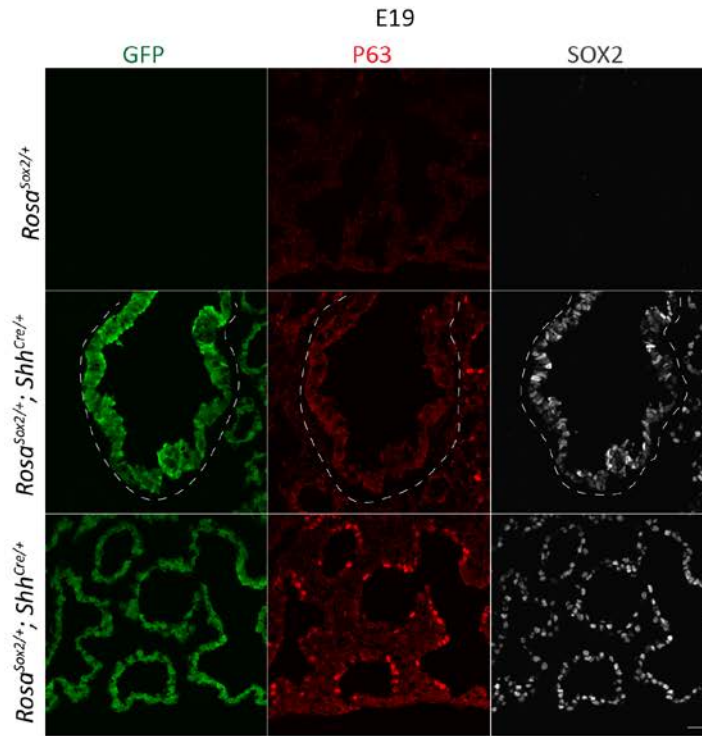
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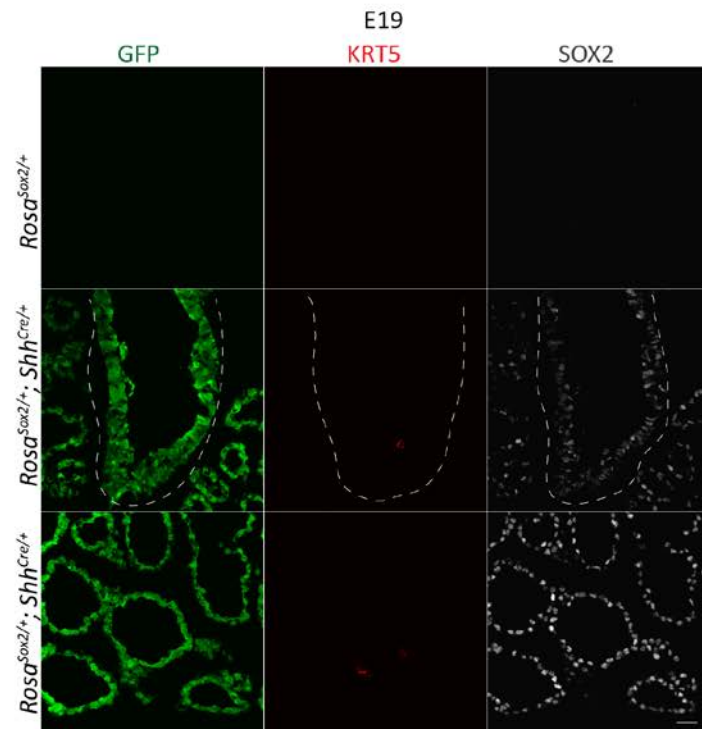
**Figure 4.7 The pattern of basal cells in the trachea is unchanged when SOX2 is overexpressed.**

Confocal images of immunostained E19 trachea sections. (A and B) The top panels are the SOX2 control lungs and the bottom panels are the SOX2 mutant lungs. P63 (red in A) and KRT5 (red in B) are basal cell markers and SOX2 (green) marks the conducting airways. (A and B) we can visualize the pattern of basal cells in the trachea. We did not detect a difference in P63 and KRT5 expression between control and mutant lungs. Scale bar: 20  $\mu\text{m}$ .

A



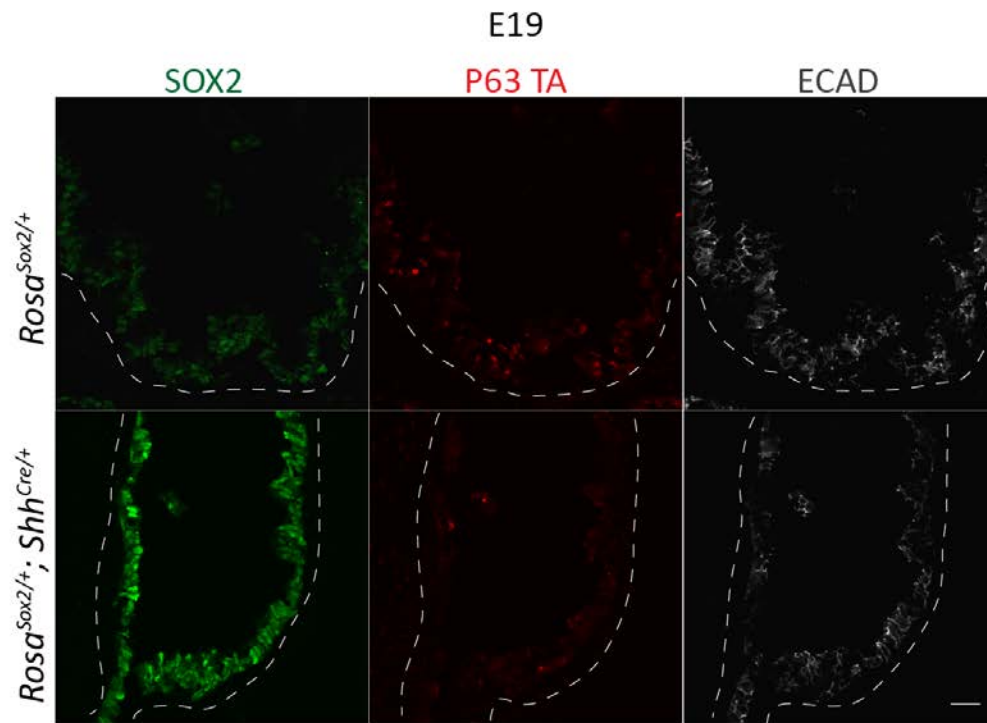
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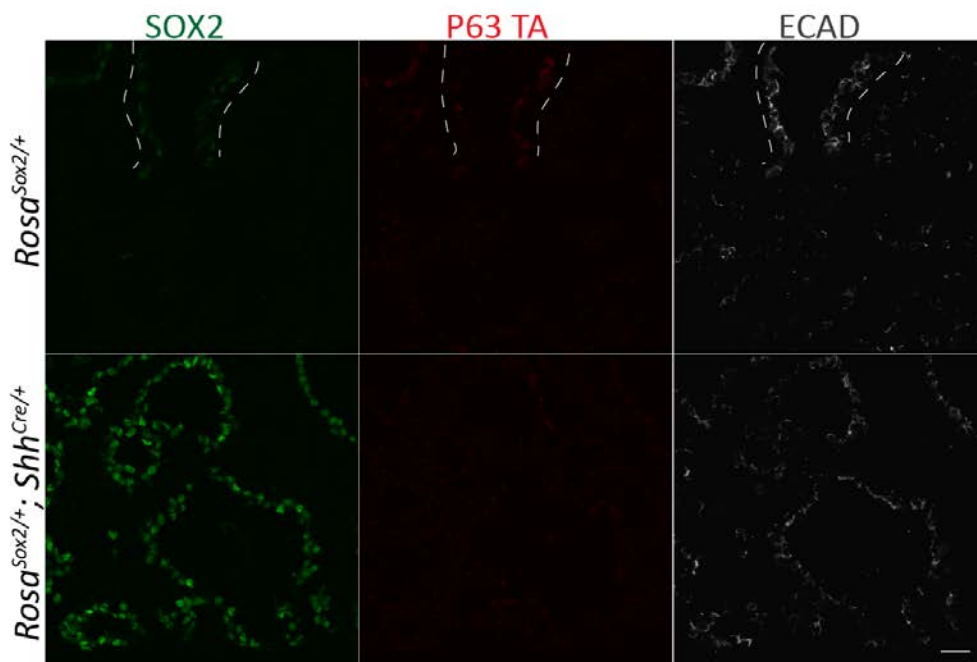
**Figure 4.8 SOX2 ectopic expression in the gas exchange region generates basal-like cells.**

Confocal images of immunostained E19 lung sections. (A and B) The top panels are the SOX2 control lungs. The middle panels and the bottom panels are the SOX2 mutant lungs. The middle panel shows the lobular bronchioles. The dashed lines outline the conducting airways. The bottom panel shows the gas exchange region. GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele and SOX2 (gray) labels the conducting airway. (A) We identify basal cells by P63 (red) expression. (B) We identify basal cells by KRT5 (red) expression. SOX2 expression in the gas exchange region induces basal cell differentiation. Scale bar: 20  $\mu$ m.

A



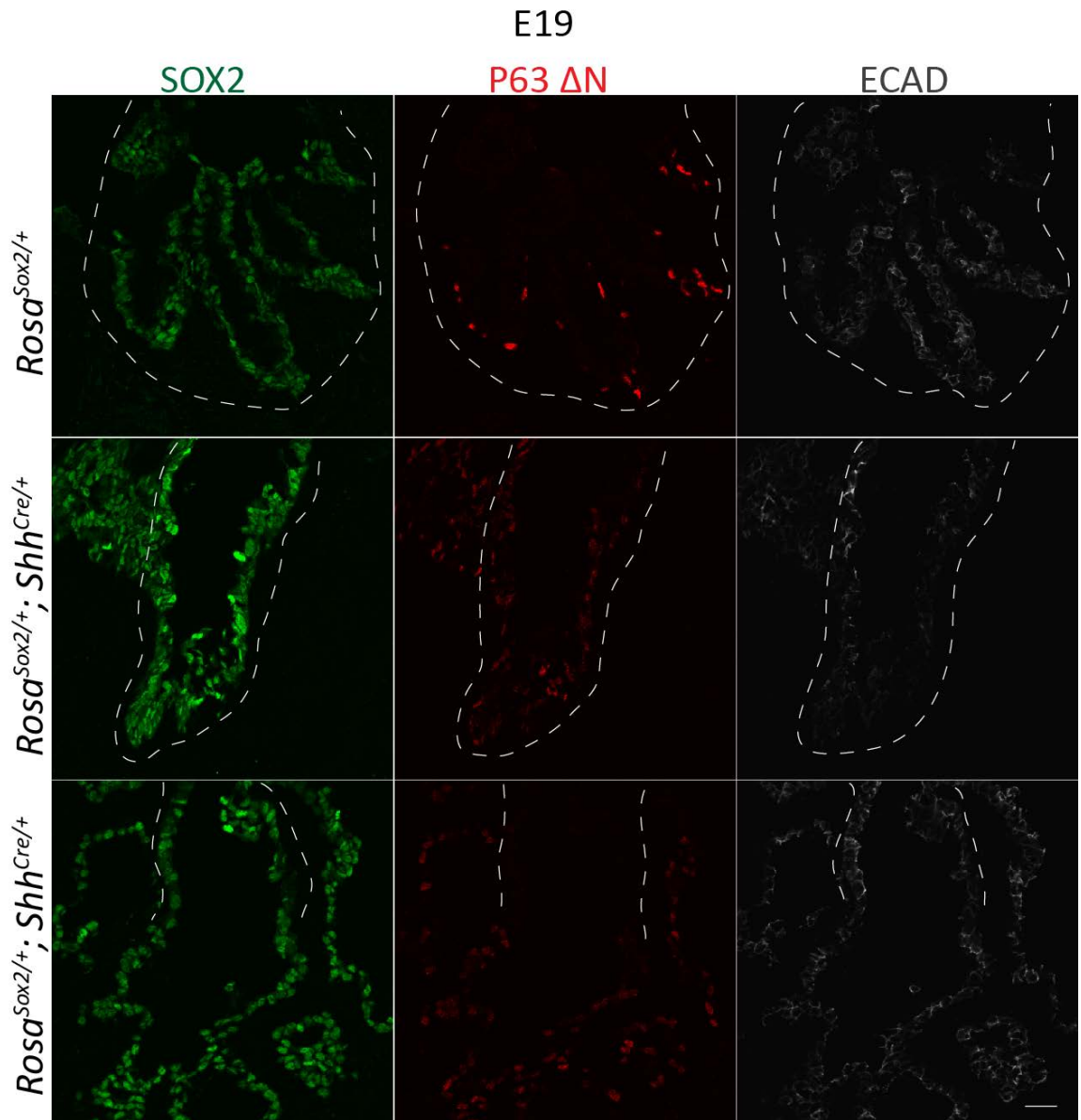
B



**Figure 4.9 P63 TA is expressed in the lobular bronchioles.**

Confocal images of immunostained E19 lung sections. (A-B) The top panels are the SOX2 control lungs and bottom panels are the SOX2 mutant lungs. The dashed lines outline the conducting airways. SOX2 (green) labels the conducting airways, P63 TA (red), and ECAD (gray) labels the epithelium. (A) We show P63 TA expression in the lobular bronchioles. (B) P63 TA expression is absent in the gas exchange region in both the control and mutant lungs. Scale bar: 20  $\mu\text{m}$ .





**Figure 4.10 SOX2 expression in the alveoli expresses P63 ΔN.**

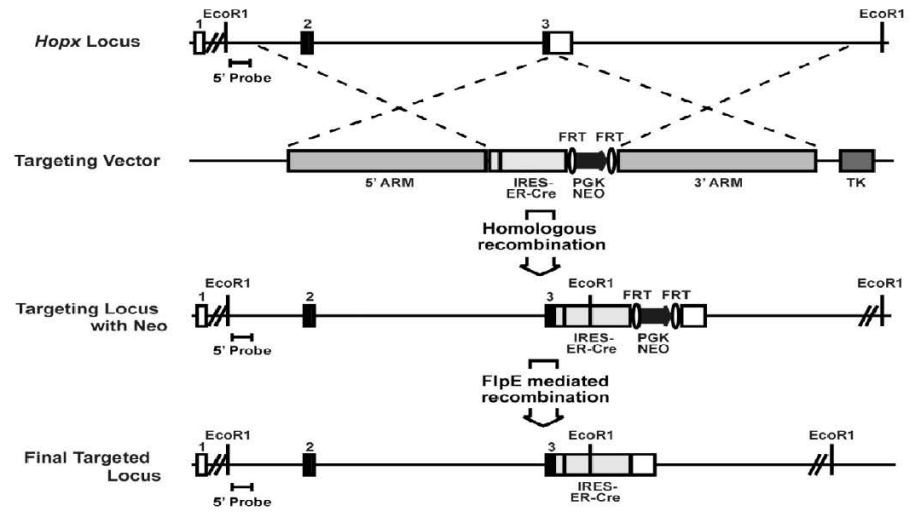
Confocal images of immunostained E19 lung sections. The top panel is the SOX2 control lung. The middle and the bottom panel are the SOX2 mutant lungs. The middle panel is a lobular bronchiole section and the bottom panel is a section from the gas exchange region. The dashed lines outline the conducting airways. SOX2 (green) labels the conducting airways, P63 ΔN (red), and ECAD (gray) labels the epithelium. P63 ΔN is expressed when SOX2 is present in the lobular bronchioles and gas exchange region. Scale bar: 20 μm.

#### 4.5 Cell division of alveolar type 1 cells can occur when SOX2 expression is induced in committed alveolar type 1 cells.

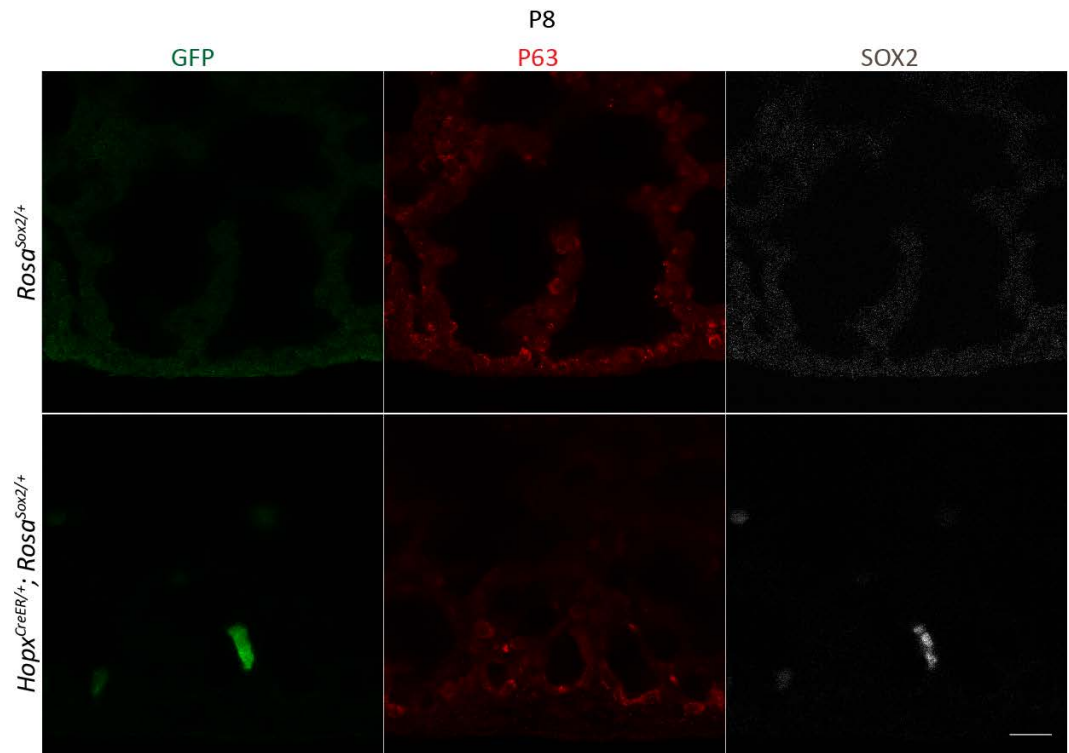
In our study, SOX2 expression in the gas exchange region suppresses alveolar type 1 cell differentiation and alveolar type 2 cell differentiation during embryonic lung development. Also, SOX2 expression in the gas exchange region can induce basal cell differentiation. To further address what is the cell fate of mutant alveoli cells expressing SOX2, we analyzed Sox2 expression in alveolar type 1 cells. Alveolar type 1 cells are squamous, cover 97% of the gas exchange region, and are known to be non-proliferative (23, 24). What will happen to the cell morphology and cell fate of alveolar type 1 cells when we express SOX2 specifically in alveolar type 1 cells? What do alveolar type 1 cells become when they express SOX2? We decided to study the cell morphology and cell differentiation of alveolar type 1 cells when we expressed SOX2 specifically in committed alveolar type 1 cells. We describe committed cells as cells that have made a decision to become alveolar type 1 cells. We used the *Hopx*<sup>CreER/+</sup> allele, which it has a construct of a *CreER* knock in targeted to the 3' untranslated region of the *Hopx* locus following an internal ribosomal entry sequence (IRES) (51, Figure 4.11A). *Hopx* is a marker for type 1 alveolar cells (28). To express SOX2 in alveolar type 1 cells, we used *Rosa*<sup>Sox2/Sox2</sup> and *Rosa*<sup>mT/mG</sup> as a reporter. To test if there is a change in committed type 1 alveolar cells, we used *Hopx*<sup>CreER/+</sup> to have temporal control. The breeding setup was: ♀ *Hopx*<sup>CreER/+</sup>; *Rosa*<sup>mT/mG</sup> x ♂ *Rosa*<sup>Sox2/Sox2</sup>. We genotyped pups at birth for *Rosa*<sup>Sox2/+</sup> and *Hopx*<sup>CreER/+</sup> (Table 4). We screened for red fluorescence under a fluorescence microscope for *Rosa*<sup>mT/mG</sup>. We injected 0.25 mg tamoxifen via intragastric at postnatal day 4. We harvested at postnatal day 8 (P8) and postnatal day 21 (P21). When we introduced SOX2 expression in committed type 1 alveolar cells during early postnatal development, we observed cell division in *Hopx* lineage traced cells from P8 to P21 and a change in cell shape (Figure 4.11B and 4.12). *Hopx*<sup>CreER/+</sup> labeled cells that expressed SOX2 were cuboidal in shape. From P8 to P21, there was an increase of SOX2 positive cells. At P8 after 4 days of SOX2 induction, we detected four SOX2<sup>+</sup> cells (Figure 24B). At P21, there were more SOX2 positive cells than P8 and SOX2<sup>+</sup> cells were in form of

clusters (Figure 4.12). Basal cell differentiation occurred among SOX2<sup>+</sup> clusters. We interpret these results that there is requirement of time for basal cell differentiation to occur. At P21, after 17 days of inducing SOX2 there was basal cell differentiation. In early postnatal lungs, mutant alveolar type 1 cells expressing SOX2 were able to change cell fate into conducting airway cells such as basal cells. From the time of SOX2 induction at P4 to P21, alveolar type 1 cells were able throughout time become conducting airway cells. Basal cell differentiation occurred in an environment where there were clusters of SOX2 positive cells (Figure 4.12). Next, we decided to study alveolar type 1 cells in late postnatal lung development. In late postnatal lung development, alveolar type 1 cells are mature cells. We describe mature cells as cells that have become alveolar type 1 cells. We injected 4 week old mice with 2.0 mg tamoxifen intraperitoneally. Mice were harvested 1 week and 3 weeks after tamoxifen injection. In late postnatal lung development, there is cell division based on the increase of SOX2 positive cells from 5 weeks to 7 weeks (Figure 4.13A and 4.13B). We detected fewer SOX2<sup>+</sup> cells in late postnatal lungs compared to early postnatal lungs. However, we did not detect basal cell differentiation in late postnatal lungs when SOX2 is present. One possible explanation is that it is too late to change the cell fate of mature alveolar type 1 cells due to a timing factor. The change of cell fate requires more time, for example, inducing SOX2 at P4 gave alveolar type 1 cells time to change cell fate compared to the time of SOX2 induction in late postnatal development. In late postnatal lungs, SOX2 is not able to override the cell program in alveolar type 1 cells. We show that alveolar type 1 cells are able to proliferate when SOX2 is induced in alveolar type 1 cells and have the capacity to induce basal cell differentiation in early postnatal lung development.

A

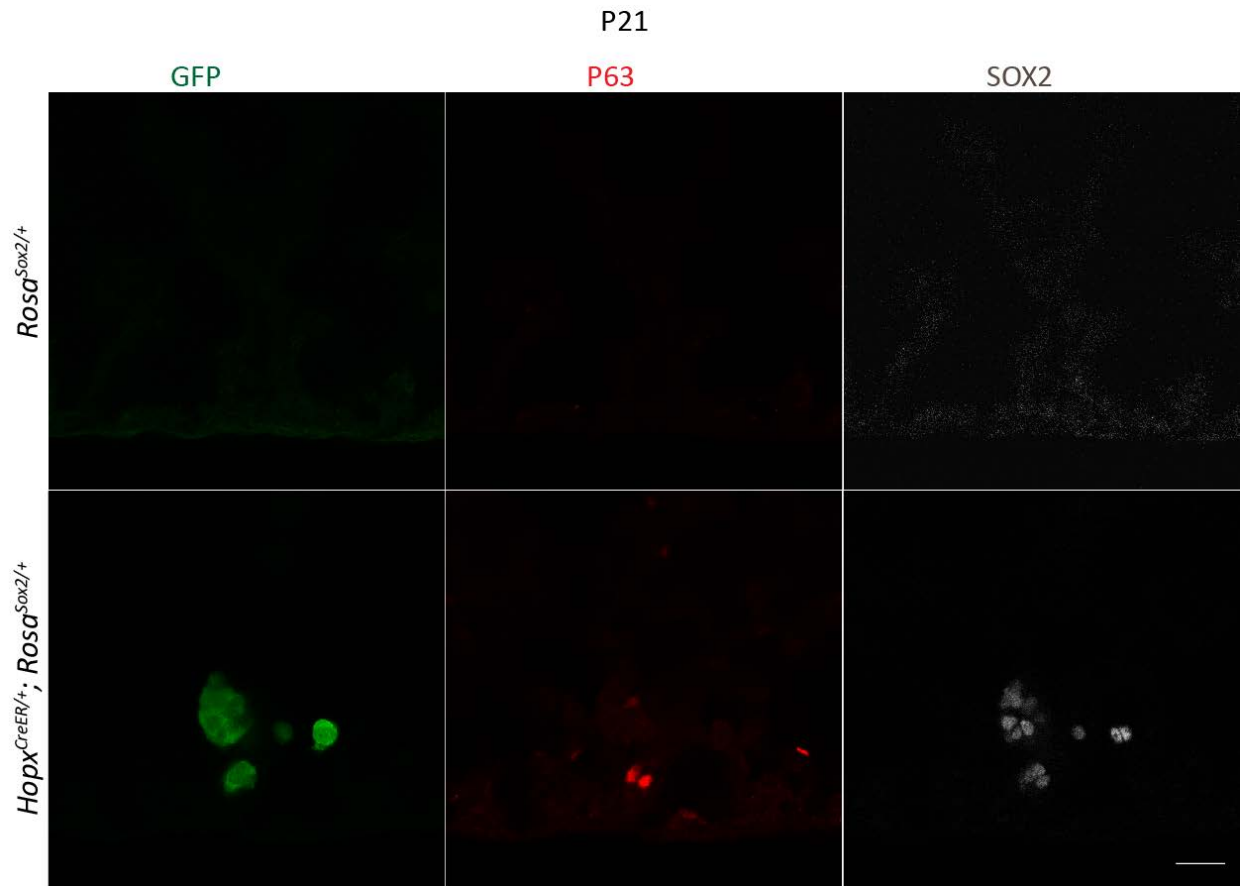


B



**Figure 4.11 Alveolar type 1 cells containing SOX2 are able to undergo cell division.**

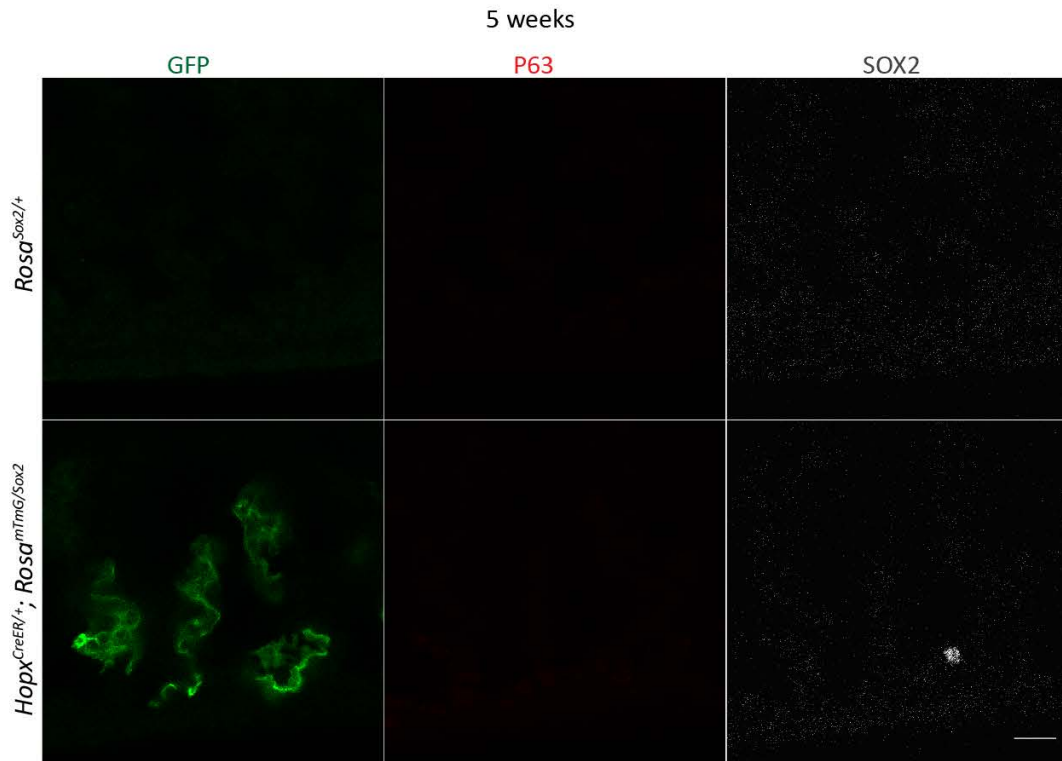
A) A schematic diagram of the  $Hopx^{CreER/+}$  mouse construct (51). B) Confocal images from z-stack sections of immunostained lung strips of postnatal day 8 mice. Mice were injected with 0.25mg tamoxifen via intragastric at 4 days after birth. The top panel is the control lung (z-stack of 33 images) and the bottom panel is the mutant lung (z-stack of 32 images). GFP (green) labels SOX2 cells from the  $Rosa^{Sox2/Sox2} IRES-GFP$  allele, P63 (red) labels basal cells, and SOX2 (gray) labels the conducting airway cells. There is a change in cell morphology of mutant cells from squamous into a cuboidal shape. Alveolar type 1 cells are able to undergo cell division when they express SOX2 within the cell, but there is no basal cell differentiation. Scale bar: 20  $\mu\text{m}$ .



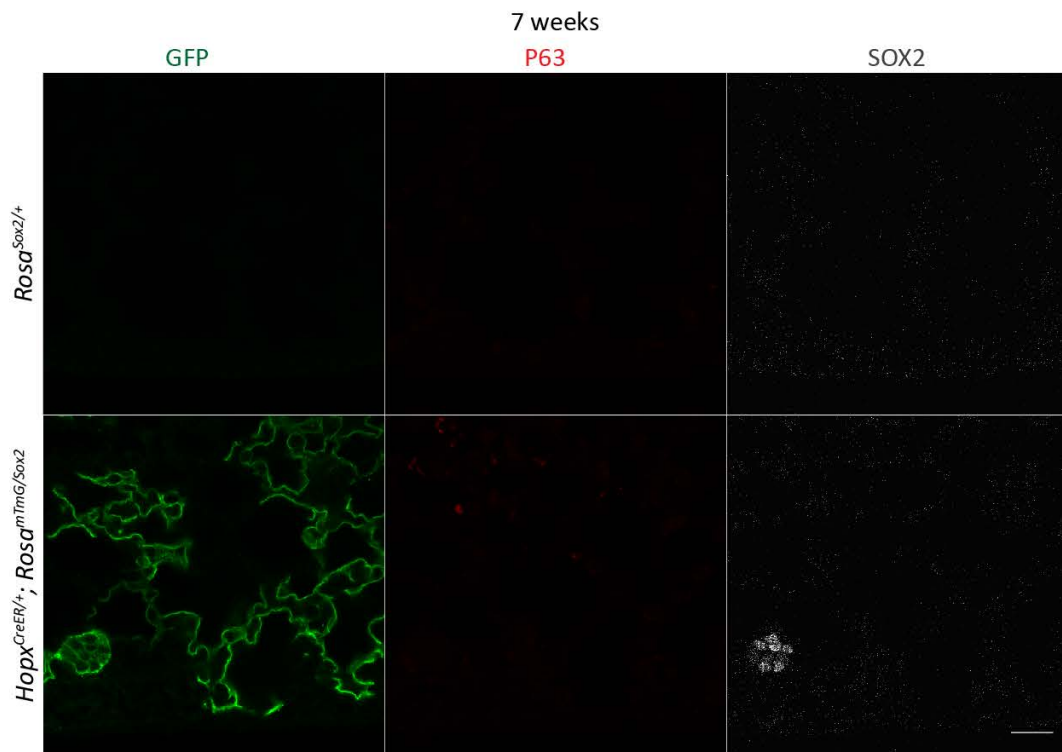
**Figure 4.12 SOX2 ectopic expression in alveolar type 1 cells can induce basal cell differentiation.**

Confocal images from z-stack sections of immunostained lung strips of postnatal day 21 mice. Mice were injected with 0.25mg tamoxifen via intragastric at 4 days after birth. The top panel is the control lung (z-stack of 30 images) and the bottom panel is the mutant lung (z-stack of 25 images). GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, P63 (red) labels basal cells, and SOX2 (gray) labels the conducting airway cells. Alveolar type 1 cells are able to induce basal cell differentiation when they express SOX2 and generate more SOX2 cells in form of a cluster. Scale bar: 20  $\mu$ m.

A



B



**Figure 4.13 SOX2 expression in alveolar type 1 cells during late lung postnatal development is sufficient for alveolar type 1 cells to undergo cell division.**

Confocal images from z-stack sections of immunostained lung strips of 5 weeks and 7 weeks old mice. Mice were injected with 2.0 mg tamoxifen intraperitoneally at 4 weeks of age. A-B) The top panels are the control lungs and the bottom panels are the mutant lungs. GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele and GFP cells from *Rosa<sup>mTmG/+</sup>*, P63 (red) labels basal cells, and SOX2 (gray) labels the conducting airway cells. A) The 5 week control has a z-stack of 41 images and the mutant has a z-stack of 37 images. SOX2 expression is induced in alveolar type 1 cells but at low efficiency. B) The 7 week control has a z-stack of 44 images and the mutant has a z-stack of 41 images. We observed a small cluster of SOX2 positive cells arise from mutant alveolar type 1 cells. Scale bar: 20  $\mu$ m.

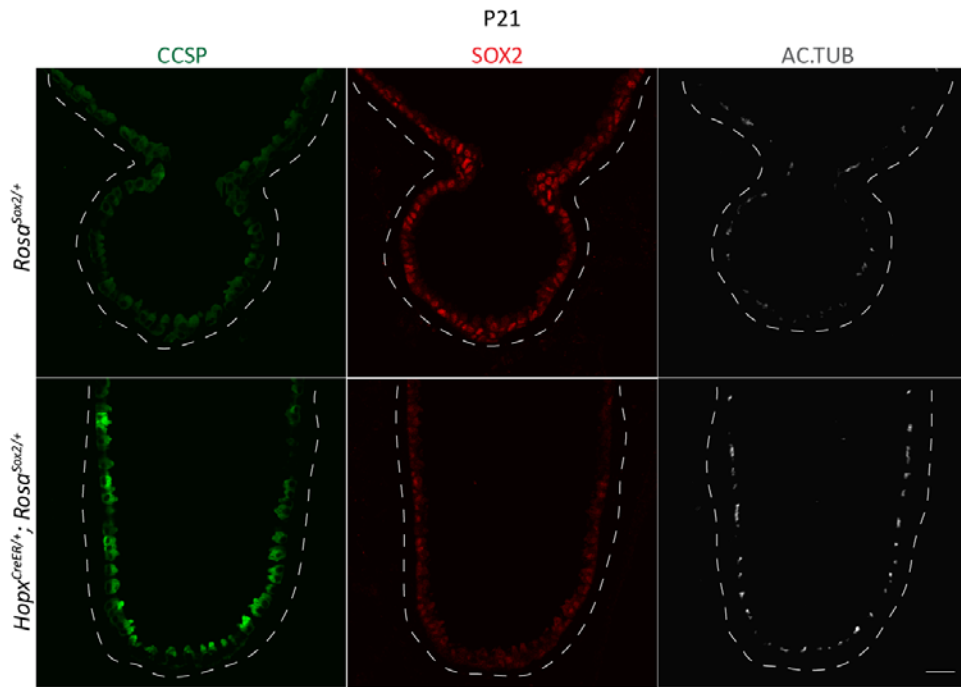


#### 4.6 SOX2 expression in alveolar type 1 cells can induce club cell differentiation.

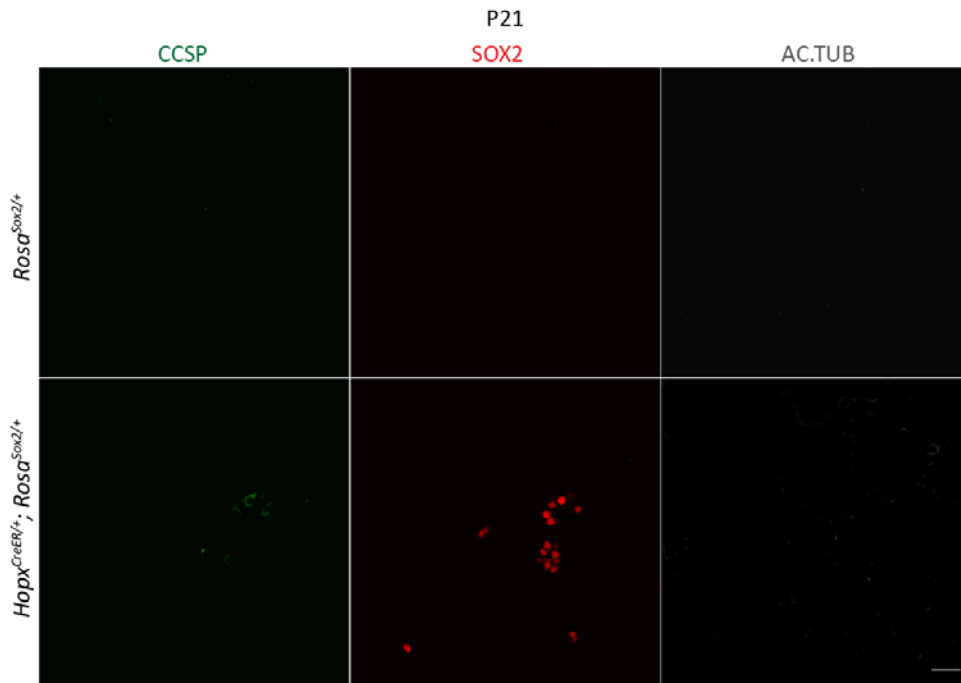
In our previous results, we show mutant alveolar type 1 cells expressing SOX2 can undergo cell division and induce basal cell differentiation. We decided to study cell differentiation of the other conducting airway cell types; club cells, ciliated cells, and neuroendocrine cells when SOX2 is expressed specifically in committed alveolar type 1 cells. *Hoxp*<sup>CreER/+</sup>; *Rosa*<sup>Sox2/+</sup> postnatal day 4 mice were injected via intragastric with 0.25mg Tamixofen and harvest at postnatal day 21. We analyzed confocal images of immunostained postnatal day 21 lung sections to study club cell, ciliated cell, and neuroendocrine cell differentiation. We analyzed the lobular bronchiole region from both mutant and control lung sections. We did not observe a significant difference in club cell and ciliated cell expression between control and mutant lung sections (Figure 4.14A). We did not expect to observe a difference in club cell and ciliated cell expression in the conducting airways because alveolar type 1 cells are not found in the conducting airways. We analyzed the gas exchange region from both mutant and control lung sections. Club cell expression is present in mutant alveolar type 1 cells (Figure 4.14B). Club cell expression in the mutant lung is weak compared to the club cell expression in the control conducting airways. The club cells' low level expression in the mutant lung suggests mutant cells are not fully mature club cells. Ciliated cell expression was not detected in the mutant gas exchange region. Next, we studied neuroendocrine cell differentiation in mutant alveolar type 1 cells. We used CGRP, a neuroendocrine cell marker, to determine if SOX2 positive cells co-localize with CGRP. Neuroendocrine cell differentiation occurred only in the conducting airways and not in the gas exchange region (Figure 4.15). SOX2 expression in alveolar type 1 cells did not induce neuroendocrine cell differentiation in the alveoli region. SOX2 is required in the conducting airways to induce neuroendocrine cell differentiation, ciliated cell differentiation, and club cell differentiation. SOX2 expression in alveolar type 1 cells were able to give rise to a few club cells, but not all SOX2 positive cells gave rise to club cells. Thus far, inducing SOX2 alone in alveolar type 1 cells can lead to basal cell differentiation, club cell

differentiation, and alveolar type 1 cell proliferation. Our studies in alveolar type 1 cells show that SOX2 is not only a conducting airway marker, but can lead to cell differentiation of conducting airway cells.

A



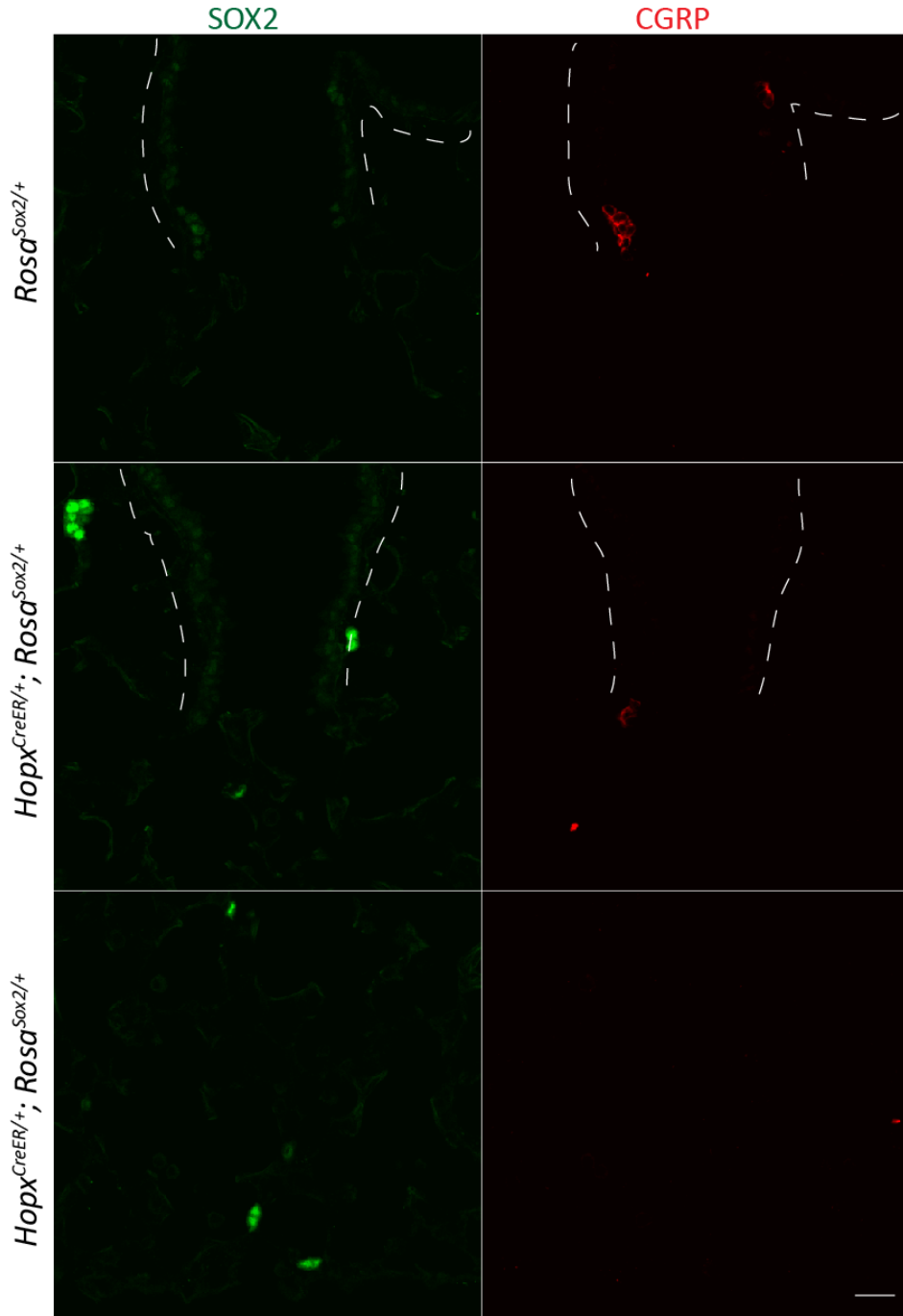
B



**Figure 4.14 Club cell differentiation occurs at low frequency in mutant alveolar type 1 cells.**

Confocal images of immunostained postnatal 21 day lung sections. A-B) Top panels are the control lungs and the bottom panels are the mutant lungs. CCSP (green) labels club cells, SOX2 (red) labels conducting airway cells, and AC.TUB (gray) labels ciliated cells. A) We did not detect a difference in club cell and ciliated cell expression between the control and mutant conducting airways. B) Clusters of alveolar type 1 cells expressing SOX2 can induce club cell expression but at low frequency. Ciliated cell expression is absent in the control and mutant gas exchange region. Scale bar: 20  $\mu\text{m}$ .

P21



**Figure 4.15 Neuroendocrine cell differentiation occurs only in the conducting airways.**

Confocal images of immunostained postnatal day 21 lung sections. The top panel is a control lung section. The middle and bottom panels are the mutant lung sections. The dashed lines outline the conducting airways. SOX2 (green) labels conducting airway cells and CGRP (red) labels neuroendocrine cells. Neuroendocrine cell expression was detected only in the conducting airways in both the control and mutant lungs. Scale bar: 20  $\mu\text{m}$ .

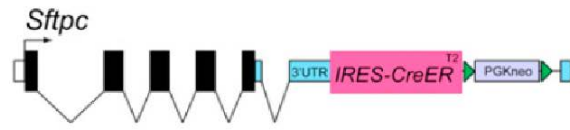
#### 4.7 Alveolar Type 2 cells expressing SOX2 lose alveolar type 2 cell properties.

We have seen in our study that SOX2 expression in alveolar type 1 cells leads to a change in cell shape and express conducting airway cells such as; basal cells and club cells. Mutant alveolar type 1 cells expressing SOX2 became cuboidal in shape and gained conducting airway cell properties. So far, we have studied one part of the gas exchange region. The next question is if SOX2 expression in alveolar type 2 cells will change their cell fate into conducting airway cells and lose their alveolar type 2 properties? To address this, we used *Sftpc*<sup>CreER/+</sup> that contains an *IRES-CreER* cassette and a PGK neo cassette flanked with FRT sites recombined in the 3' UTR of *Sftpc* (52, Figure 4.16A). The surfactant protein C (*Sftpc*<sup>CreER/+</sup>) allele in this study is referred as *SPC*<sup>CreER/+</sup>, which labels alveolar type 2 cells. We used *Rosa*<sup>Sox2/Sox2</sup> to express SOX2 in alveolar type 2 cells and *Rosa*<sup>mTmG/+</sup> as a reporter. The *SPC*<sup>CreER/+</sup> allele allowed us to have temporal control and test changes in committed alveolar type 2 cells when we express SOX2. We studied early and late postnatal lung development. The breeding setup was: ♀ *SPC*<sup>CreER/+</sup>; *Rosa*<sup>mTmG/+</sup> × ♂ *Rosa*<sup>Sox2/Sox2</sup>. We genotyped pups at birth for *Rosa*<sup>Sox2/+</sup> and *SPC*<sup>CreER/+</sup> (Table 4). We screened for red fluorescence under a fluorescence microscope for *Rosa*<sup>mTmG/+</sup>. For early postnatal lung development, we studied cells that are committed to become alveolar type 2 cells. For late postnatal lung development, we studied mature alveolar type 2 cells which have alveolar type 2 characteristics. Early postnatal mice were injected via intraperitoneal with 0.25mg Tamoxifen at postnatal day 3 and harvest at postnatal day 10. Late postnatal mice were injected intraperitoneally with 2.0 mg Tamoxifen at 4weeks of age and harvest 10 days after Tamoxifen injection. We cut strips from right cranial and left lobes for whole mount staining. We used z-stack confocal images of immunostained lung strips for early and late postnatal lungs to capture cells in the gas exchange region. SOX2 expression in alveolar type 2 cells decreases SPC expression, an alveolar type 2 cell marker. We did not detect a difference in alveolar type 1 cell expression between control and mutant lungs (Figure 4.16B and 4.17). We detected a decrease in alveolar type 2 cell expression for one marker, SPC. We decided to analyze another set of alveolar type 2 cell markers, LAMP3 and ABCA3. Mutant alveolar type

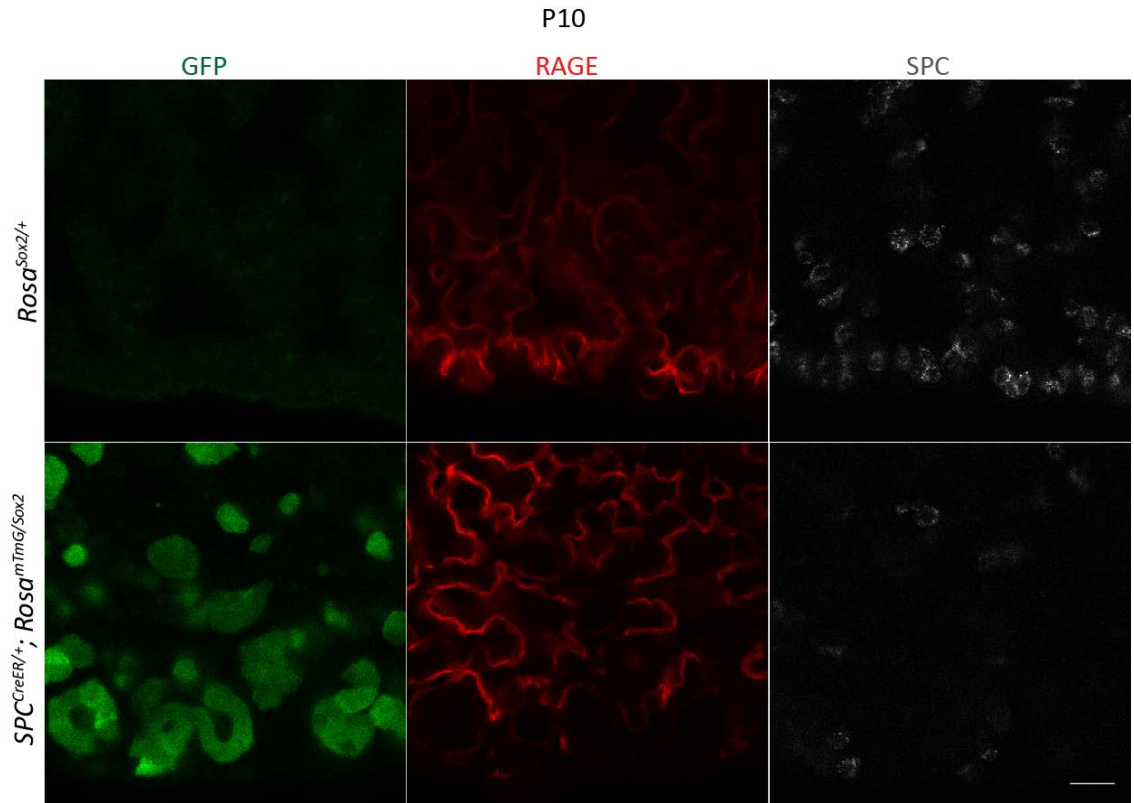
2 cells expressing SOX2 did not express LAMP3 and ABCA3 (Figure 4.18A and 4.18B). In early and late postnatal lung development, SOX2 expression in alveolar type 2 cells can change the cell fate of alveolar type 2 cells. In early and late postnatal lungs, SOX2<sup>+</sup> cells did not co-localize with LAMP3 and ABCA3. SOX2 expression in alveolar type 2 cells change in cell fate of these alveolar type 2 cells into conducting airways regardless if SOX2 induction is at an early or late stage. Alveolar type 2 cells lose their properties when they express SOX2, but do these cells differentiate into specialized conducting airway cells?



A

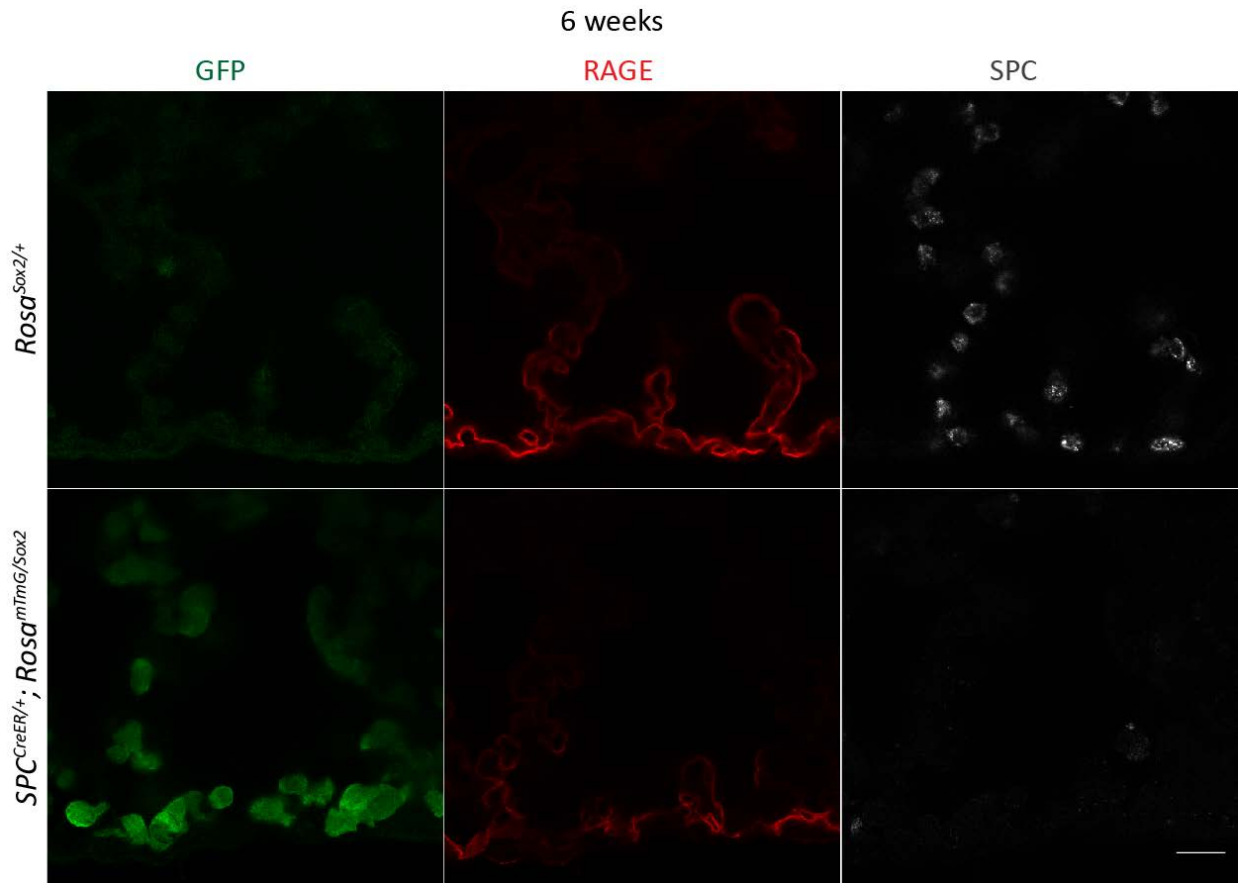


B



**Figure 4.16 In early lung development, SOX2 expression in alveolar type 2 cells inhibits alveolar type 2 cell expression.**

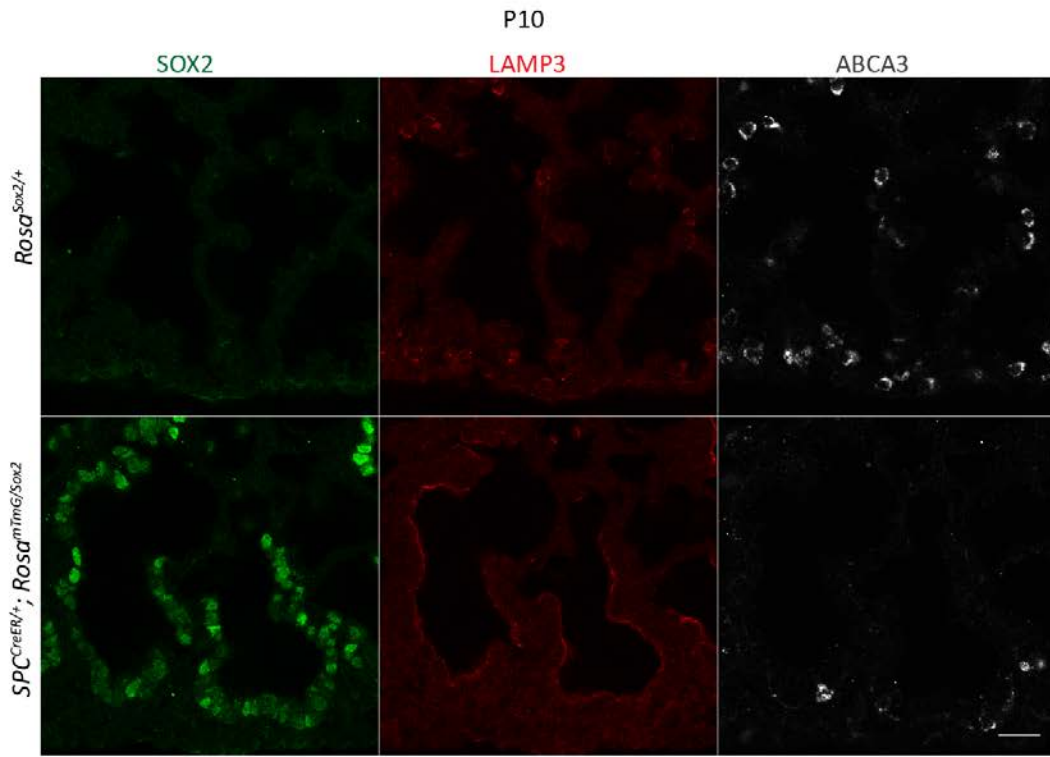
A) A schematic diagram of the SPC<sup>CreER/+</sup> allele construct (52). B) Confocal images from z-stack sections of immunostained lung strips of postnatal day 10 mice. The top panel is the control lung (z-stack of 28 images) and the bottom panel is the mutant lung (z-stack of 38 images). GFP (green) labels mG positive cells from *Rosa<sup>mTmG/+</sup>* and SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, RAGE (red) labels alveolar type 1 cells, and SPC (gray) labels alveolar type 2 cells. We observed a decrease in SPC expression in the mutant. Alveolar type 2 cells expressing SOX2 lose alveolar type 2 cell properties based on the decrease of SPC expression in the mutant. Scale bar: 20  $\mu$ m.



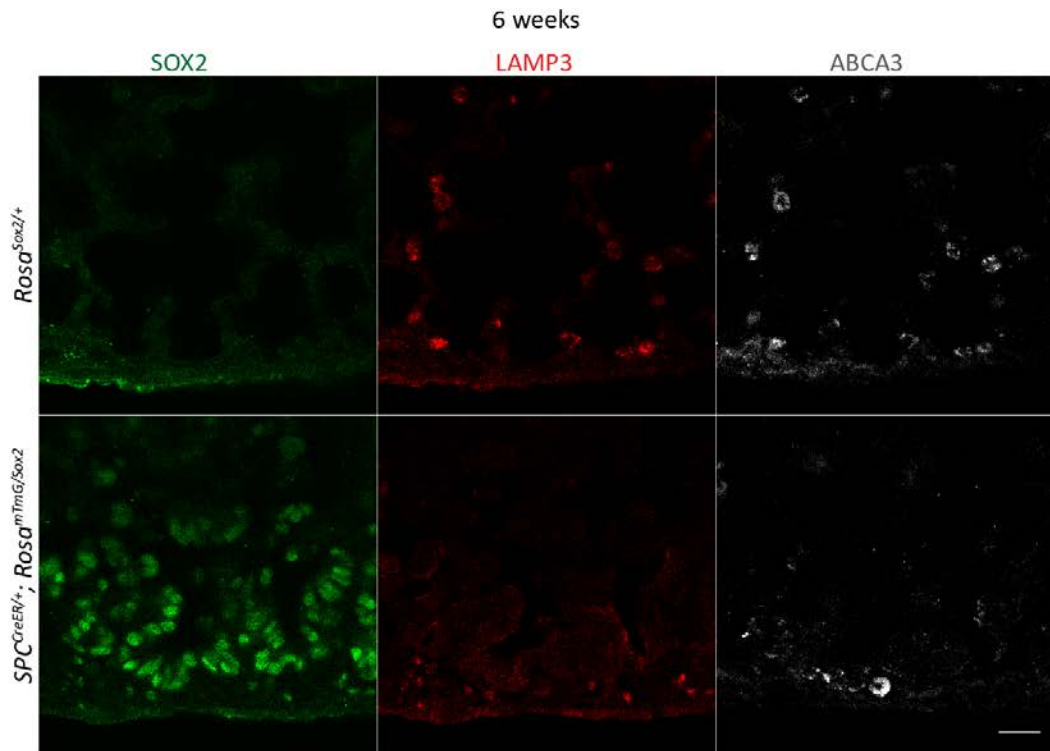
**Figure 4.17 Inducing SOX2 expression in alveolar type 2 cells during late postnatal lung development inhibits alveolar type 2 cell expression.**

Confocal images from z-stack sections of immunostained lung strips from 6 week old mice. The top panel is the control lung (z-stack of 26 images) and the bottom panel is the mutant lung (z-stack of 31 images). GFP (green) labels mG positive cells from *Rosa<sup>mTmG/+</sup>* and SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele. RAGE (red) labels alveolar type 1 cells, and SPC (gray) labels alveolar type 2 cells. Mutant alveolar type 2 cells expressing SOX2 lost alveolar type 2 cell properties based on the decrease of SPC expression. Scale bar: 20  $\mu$ m.

A



B



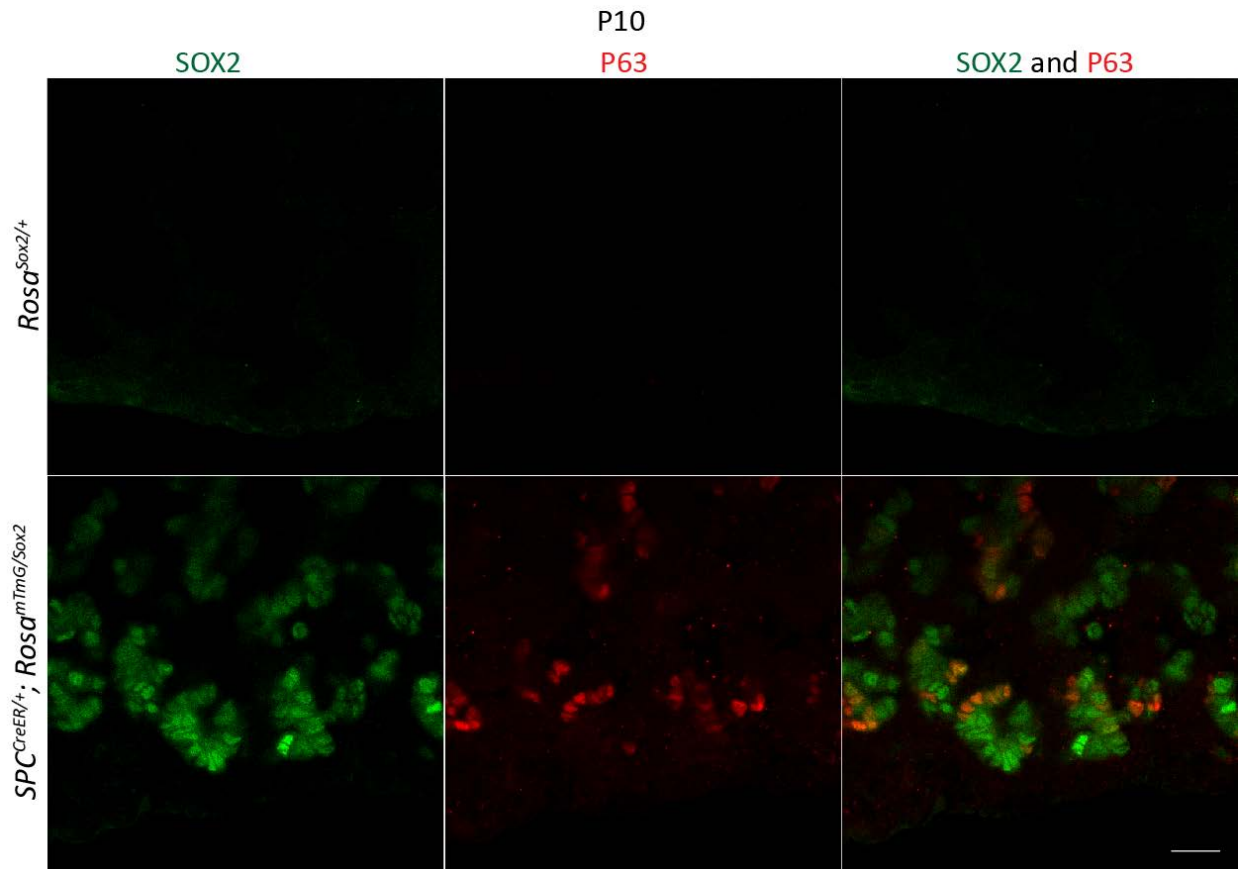
**Figure 4.18 SOX2 ectopic expression in alveolar type 2 cells leads to a loss of alveolar type 2 cell fate.**

Confocal images from z-stack sections of immunostained lung strips of postnatal day 10 mice and 6 week old mice. A-B) The top panels are the control lungs and the bottom panels are the mutant lungs. SOX2 (green) labels conducting airway cells, LAMP3 (red) and ABCA3 (gray) labels alveolar type 2 cells. A) Confocal images of early postnatal lung development at postnatal day 10. The control lung has a z-stack of 27 images and the mutant lung has a z-stack of 37 images. SOX2 expression in alveolar type 2 cells inhibits alveolar type 2 cell differentiation. B) Confocal images of late postnatal lung development in mice at 6 weeks of age. The control lung has a z-stack of 23 images and the mutant lung has a z-stack of 30 images. SOX2 expression in alveolar type 2 cells inhibits alveolar type 2 cell differentiation. Scale bar: 20  $\mu\text{m}$ .

#### 4.8 Alveolar type 2 cells expressing SOX2 are able to induce club cell and basal cell differentiation

From our previous experiments, we have demonstrated that when we induced SOX2 during early and late postnatal lung development there is inhibition of alveolar type 2 cell differentiation. We analyzed early postnatal lungs to identify which type of specialized cells do mutant alveolar type 2 cells become. We used *SPC<sup>CreER/+</sup>* and *Rosa<sup>Sox2/Sox2</sup>* to express SOX2 in alveolar type 2 cells. We used *Rosa<sup>mTmG/+</sup>* as a reporter. Early postnatal mice were injected intraperitoneally with 0.25mg Tamoxifen at postnatal day 3 and harvest at postnatal day 10. We cut strips from right cranial and left lobes for whole mount staining. We used z-stack confocal images of immunostained lung strips to visualize cells in the gas exchange region. Mutant alveolar type 2 cells expressing SOX2 are able to induce basal cell differentiation based on the co-localization of SOX2 and P63 (Figure 4.19). Also, mutant alveolar type 2 cells that express SOX2 lost their alveolar type 2 cell characteristics (Figure 4.18A and 4.18B), and a subset of mutant alveolar type 2 cells that are SOX2<sup>+</sup> express P63<sup>+</sup> cells (Figure 4.19). We analyzed other conducting airway cell markers to identify what do the other non-basal SOX2<sup>+</sup> alveolar type 2 cells become. We used confocal images of immunostained postnatal day 10 lung sections to visualize co-localization of SOX2<sup>+</sup> cells with conducting airway cell markers. Club cell and ciliated cell expression was present in the conducting airways of control and mutant lungs. Interestingly, club cells were present in the mutant gas exchange region (Figure 4.20). Next, we studied neuroendocrine cell differentiation in control and mutant lungs. Neuroendocrine cell differentiation was restricted only in the conducting airways in both control and mutant lungs. Neuroendocrine cell differentiation did not occur in the mutant gas exchange region (Figure 4.21). SOX2 expression in alveolar type 2 cells changes the cell fate of alveolar type 2 cells into conducting airway cells. Mutant alveolar type 2 cells expressing SOX2 lose their properties due to their loss of alveolar type 2 cell marker expression (Figure 4.18 and 4.19B). At P10, which is 7 days of inducing SOX2 expression in alveolar type 2 cells was enough time to induce club cell and basal cell differentiation. SOX2<sup>+</sup> alveolar type 2 cells did not generate ciliated cells and

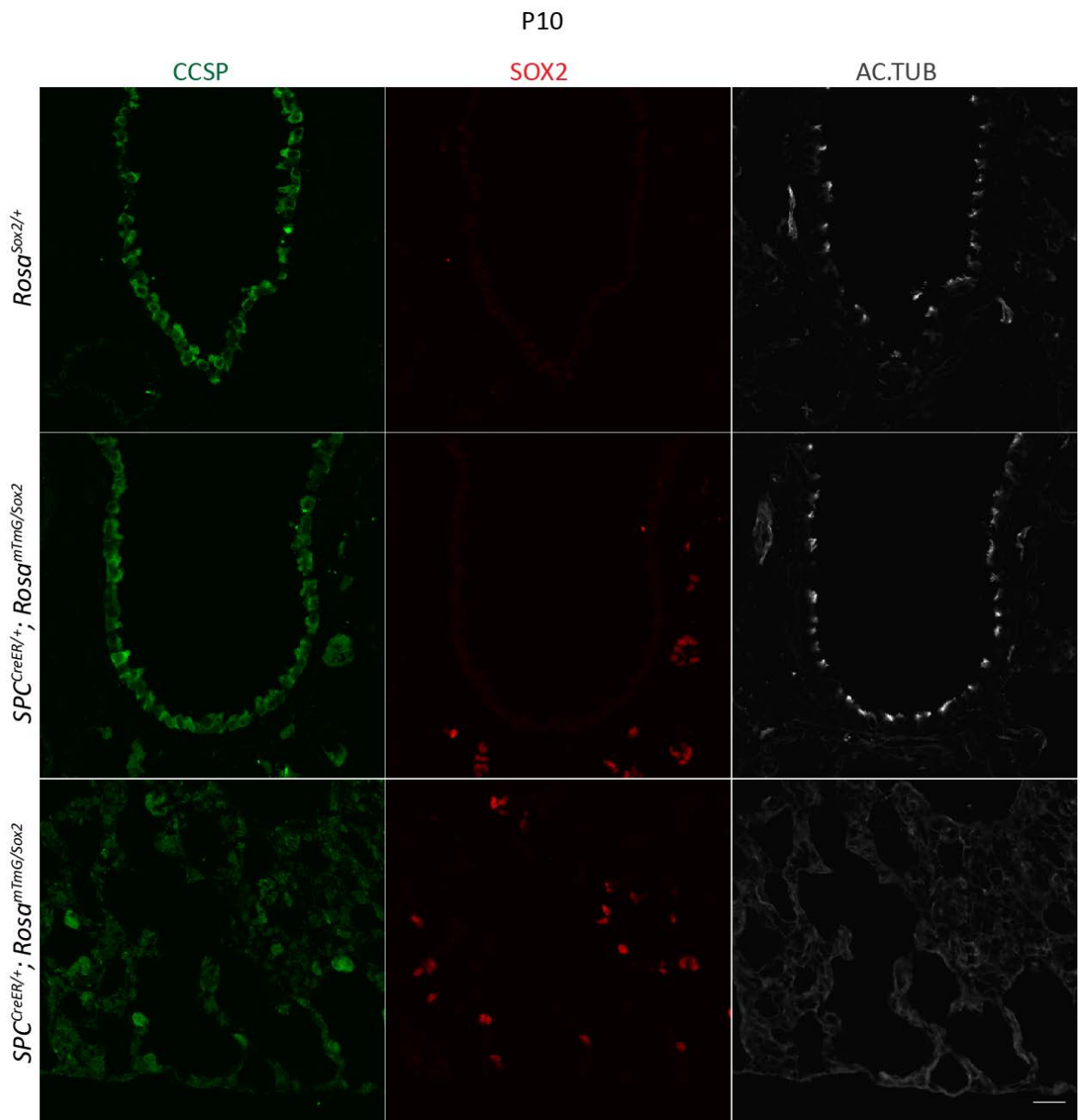
neuroendocrine cells. One possible explanation is that 7 days of inducing SOX2 is not enough time to induce conducting airway cells in a salt and pepper pattern in the gas exchange region.



**Figure 4.19 Mutant alveolar type 2 cells expressing SOX2 are able to induce basal cell differentiation.**

Confocal images from z-stack sections of immunostained lung strips of postnatal day 10 mice. The top panel is the control lung (z-stack of 25 images) and the bottom panel is the mutant lung (z-stack of 40 images). SOX2 (green) labels the conducting airway cells and P63 (red) label basal cells. A subset of SOX2 positive cells expressed P63. Mutant alveolar type 2 cells expressing SOX2 have the capacity to generate basal cells. Scale bar: 20  $\mu$ m.





**Figure 4.20 Ciliated cell differentiation is absent in the gas exchange region, but club cell differentiation does occur when SOX2 is induced in alveolar type 2 cells.**

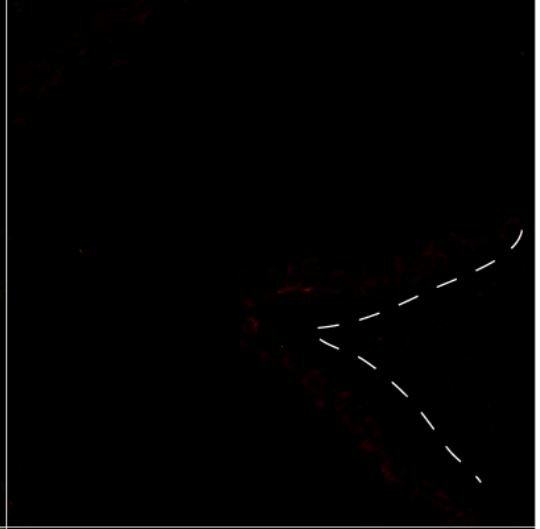
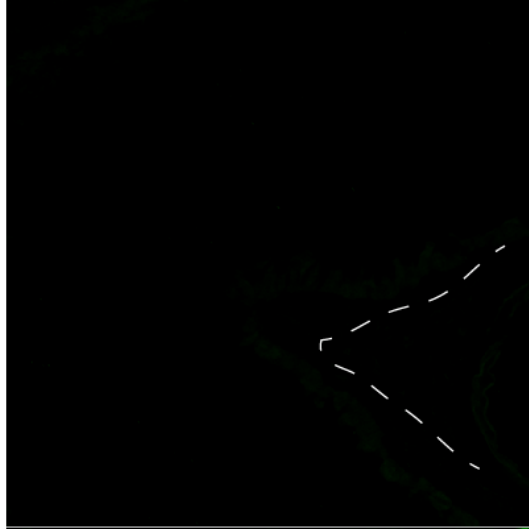
Confocal images of immunostained of postnatal day 10 lung sections. The top panel is a control lung section. The middle and the top panels are mutant lung sections. CCSP (green) labels club cells, SOX2 (red) labels conducting airway cells, AC.TUB (gray) labels ciliated cells. CCSP and AC.TUB expression is present in the conducting airways of both control and mutant lungs. Club cell differentiation occurs in the mutant gas exchange region, but ciliated cell differentiation. SOX2<sup>+</sup> cells in the gas exchange region were able to generate club cells but not ciliated cells. Scale bar: 20 μm.

P10

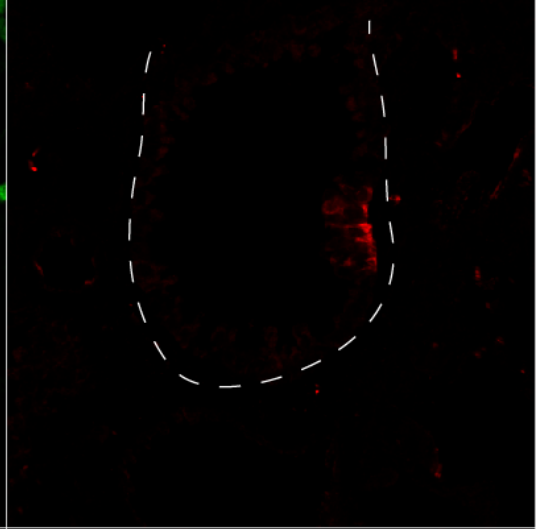
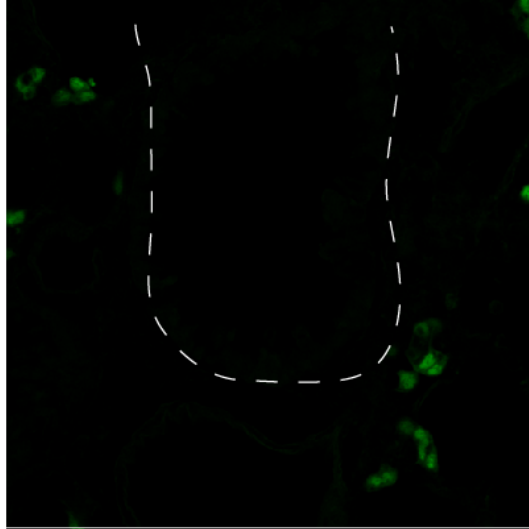
SOX2

CGRP

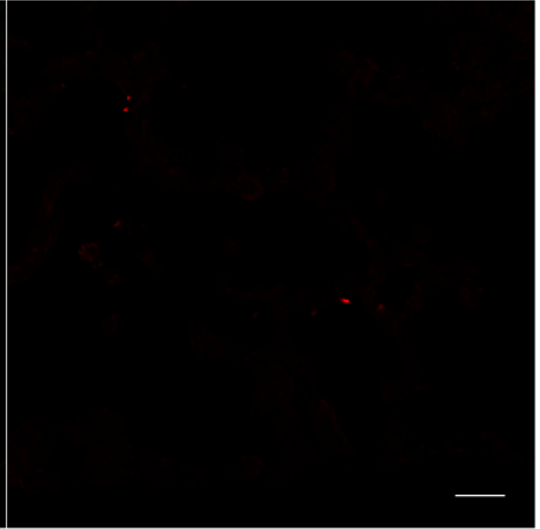
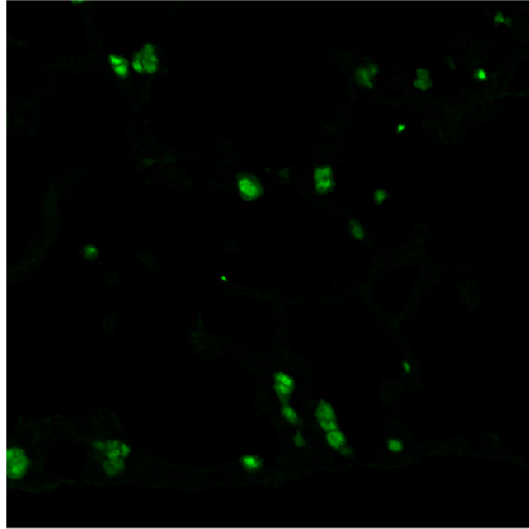
*Rosa<sup>Sox2/+</sup>*



*SPC<sup>CreER/+</sup>; Rosa<sup>mTmG/Sox2</sup>*



*SPC<sup>CreER/+</sup>; Rosa<sup>mTmG/Sox2</sup>*



**Figure 4.21 SOX2 expression in alveolar type 2 cells does not induce neuroendocrine cell differentiation.**

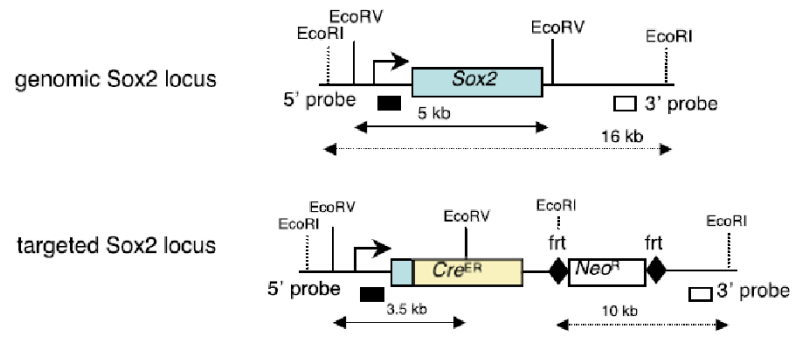
Confocal images of immunostained postnatal day 10 lung sections. The top panel is the control lung section. The middle and bottom panels are the mutant lung sections. SOX2 (green) labels the conducting airway cells and CGRP (red) labels neuroendocrine cells. In early postnatal lungs, CGRP expression is restricted only in the conducting airways of control and mutant lungs. Neuroendocrine cell differentiation did not occur in the mutant gas exchange region. Scale bar: 20  $\mu\text{m}$ .

#### 4.9 An increase in SOX2 expression in the conducting airways does not change the distribution pattern of conducting airway cells.

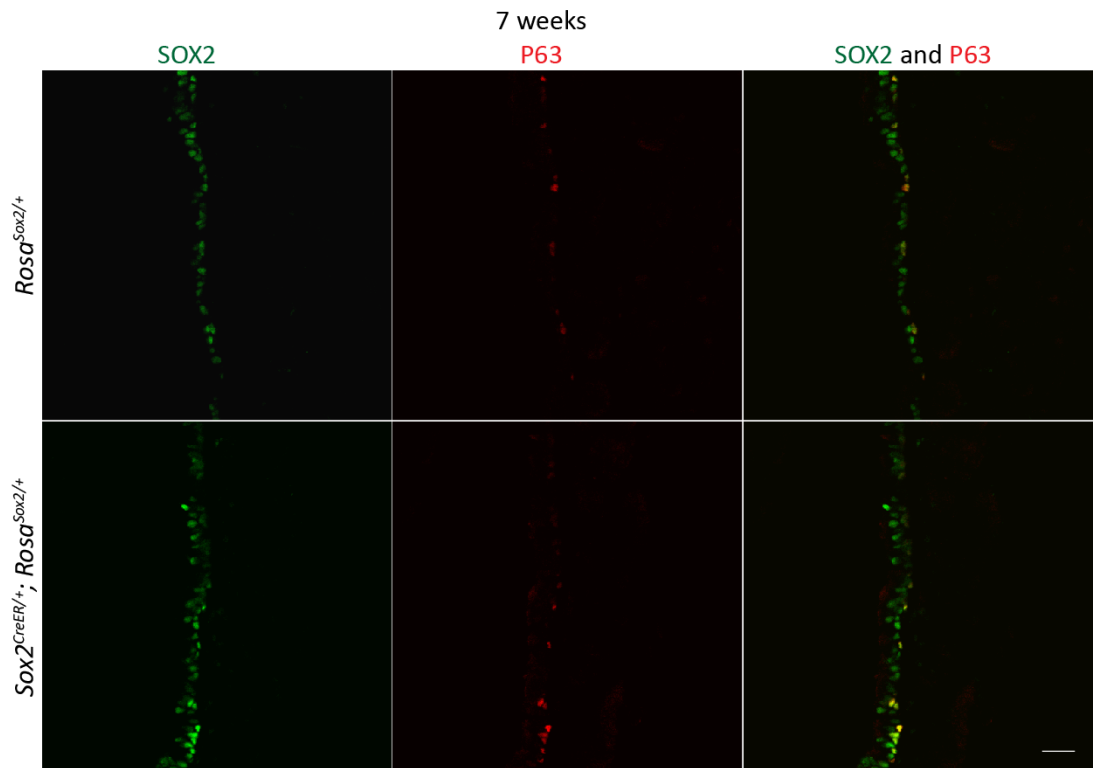
In our study, we were able to overexpress SOX2 the entire lung epithelium by using *Shh*<sup>Cre/+</sup>, induce SOX2 in SOX9 progenitor cells, alveolar type 1 cells, and alveolar type 2 cells. So far, we expressed SOX2 in the gas exchange region where it normally is not present, but we have not overexpressed SOX2 specifically in the conducting airways. To specifically overexpress SOX2 in the conducting airways, we used *Rosa*<sup>Sox2/+</sup> and *Sox2*<sup>CreER/+</sup> to generate more SOX2 cells in the conducting airways. The *Sox2*<sup>CreER/+</sup> construct contains the SOX2 open reading frame replaced with a *CreER* fusion gene (50, Figure 4.22A). The breeding setup was: ♀ *Rosa*<sup>Sox2/+</sup>; *Sox2*<sup>CreER/+</sup> x ♂ *Rosa*<sup>Sox2/Sox2</sup>. We studied SOX2 overexpression in late postnatal lung development to determine if generating more SOX2 cells will change the expression level of specialized conducting airway cells such as: club cells, ciliated cells, basal cells, and neuroendocrine cells. Mice were injected intraperitoneally with 2.0 mg Tamoxifen at 4 weeks of age and harvest 3 weeks after Tamoxifen injection. We analyzed confocal images of immunostained 7 week old lung sections to visualize a change in expression level of specialized conducting airway cells when SOX2 is overexpressed. To determine a change in basal cell expression, we used P63 as marker to label basal cells in both mutant and control lungs. After generating more SOX2 cells for 3 weeks, we did not detect a difference in basal cell expression between control and mutant lungs (Figure 4.22B). The increase of SOX2 in the mutant lung was detected based on the increase signal intensity of SOX2 compared to the control lung (Figure 4.22B). We used acetyl tubulin (AC.TUB) to label ciliated cells and CCSP to label club cells in control and mutant lungs. In control and mutant conducting airways, we did not see a difference in club cell and ciliated cell expression (Figure 4.23). The number of club cells, ciliated cells, and basal cells did not increase by generating more SOX2 in the conducting airways. To determine if there is an increase in neuroendocrine cells, we used CGRP to label neuroendocrine cells in both the control and mutant lungs. Interestingly, the mutant lung contained larger clusters of neuroendocrine cells compared to the control lung (Figure 4.24). In the

mutant lung, the large clusters of neuroendocrine cells are associated with the increase number of SOX2 cells based on the co-localization with GFP. One possible explanation is by increasing the number of SOX2 cells generates more neuroendocrine cells on top of the original cluster of neuroendocrine cells. Increasing the number of SOX2 cells did not change the salt and pepper pattern of conducting airway cells, but it did increase the number of neuroendocrine cells in a cluster. SOX2 is essential for club cell, ciliated cell, and basal cell differentiation regardless of the increase input of Sox2. Based on our gain of function experiments, SOX2 is an important component in the cell fate of conducting airway cells. SOX2 expression in the gas exchange region led to basal cell differentiation and club cell differentiation (Figures 4.8A, 4.8B, 4.12, 4.14, 4.19, and 4.20). We need to study SOX2 at a molecular level to determine how SOX2 is able to control cell differentiation in the conducting airways.

A



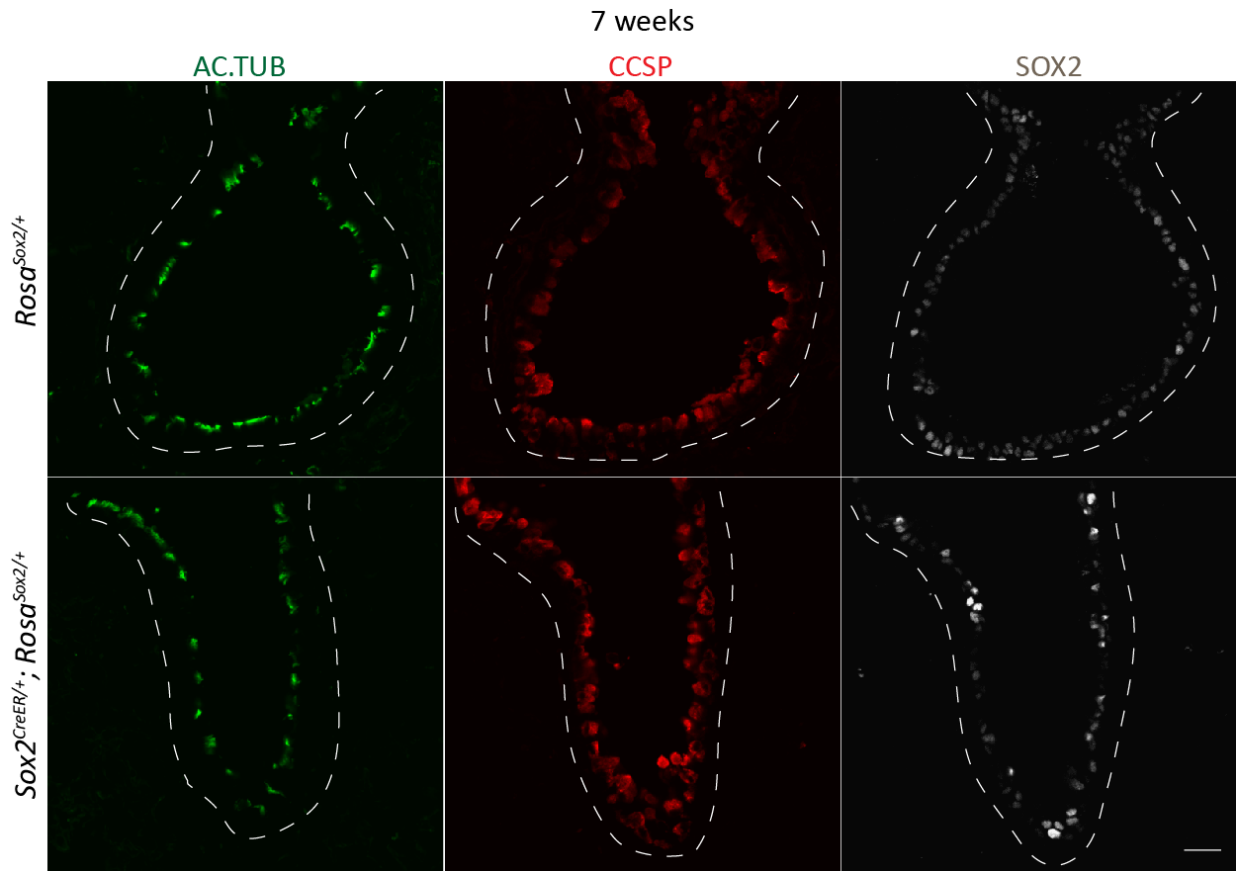
B



**Figure 4.22 The increase of SOX2 cells in the conducting airways did not increase basal cell formation.**

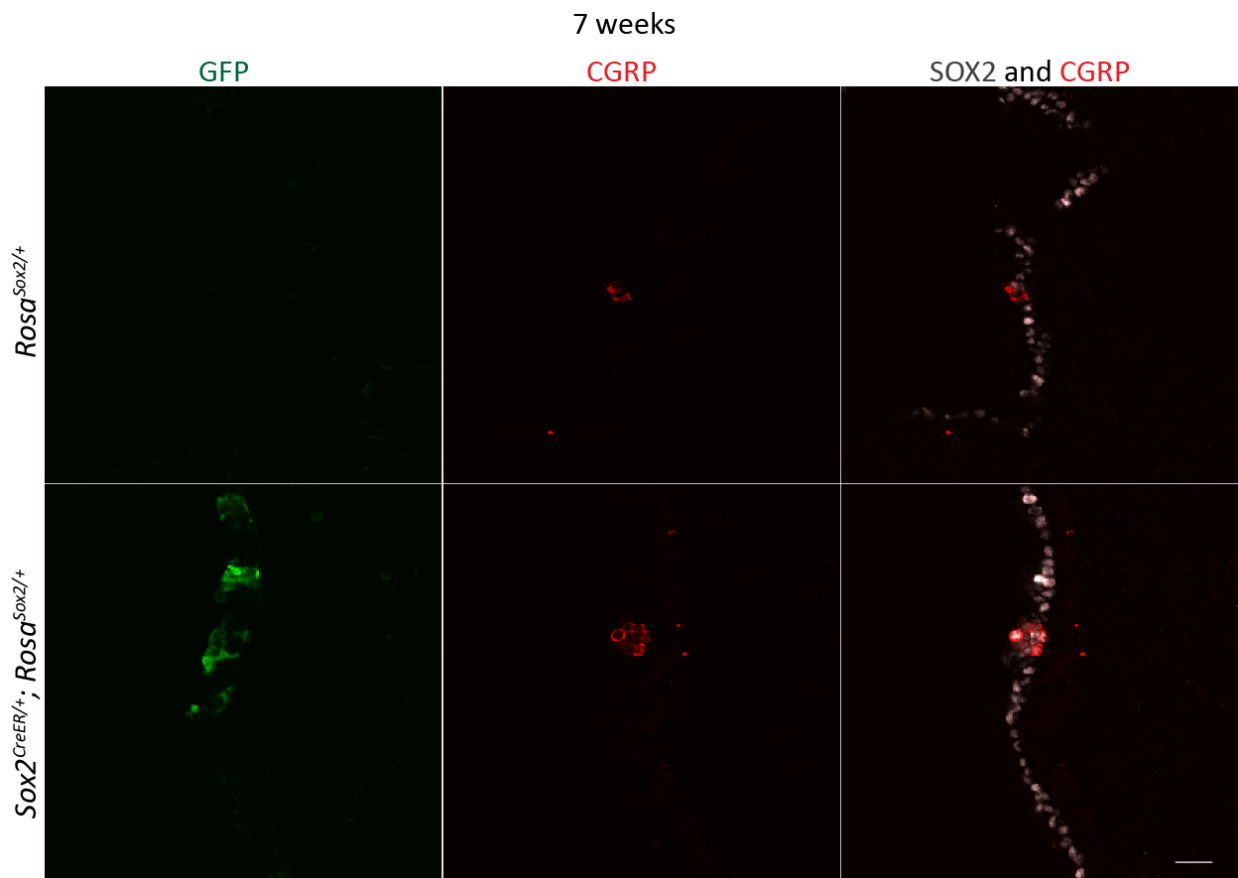
A) A schematic diagram of the *Sox2<sup>CreER/+</sup>* construct (50). B) Confocal images of immunostained 7 week old mice lung sections. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. SOX2 (labels) conducting airway cells and P63 (red) labels basal cells. The increase of SOX2 cells in the conducting airways did not generate more basal cells compared to the control lung. Scale bar: 20  $\mu\text{m}$ .





**Figure 4.23 Club cell and ciliated differentiation did not increase when SOX2 expression was increased in the conducting airways.**

Confocal images of immunostained 7 week old mice lung sections. The top panel is the SOX2 control lung and the bottom is the SOX2 mutant lung. AC.TUB (green) labels ciliated cells, CCSP (red) labels club cells, and SOX2 (gray) labels conducting airway cells. The expression level of club cells and ciliated did not increase with the increase of SOX2 expression. Scale bar: 20  $\mu$ m.



**Figure 4.24** The size of neuroendocrine clusters increased when SOX2 expression is increased in the conducting airways.

Confocal images of immunostained 7 week old mice lung sections. The top panel is the SOX2 control lung and the bottom panel is the SOX2 mutant lung. GFP (green) labels SOX2 cells from the *Rosa<sup>Sox2/Sox2</sup> IRES-GFP* allele, CGRP (red) labels neuroendocrine cells, and SOX2 (gray) labels conducting airway cells. The size of neuroendocrine clusters increased when SOX2 expression is increased in the conducting airways. Scale bar: 20  $\mu$ m.

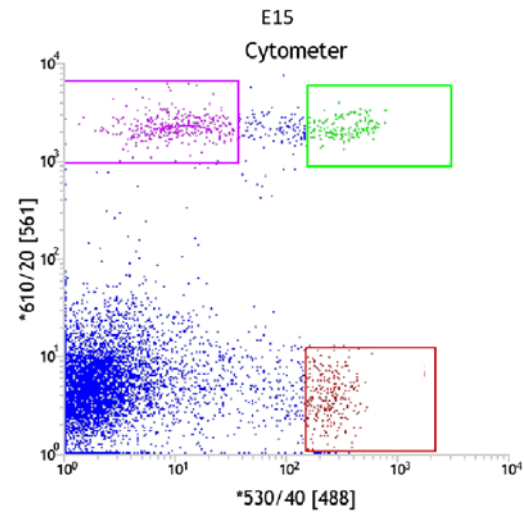
## 5. Results III: SOX21 has a unique expression pattern in the lung.

### 5.1 SOX21 is down regulated in the absence of SOX2.

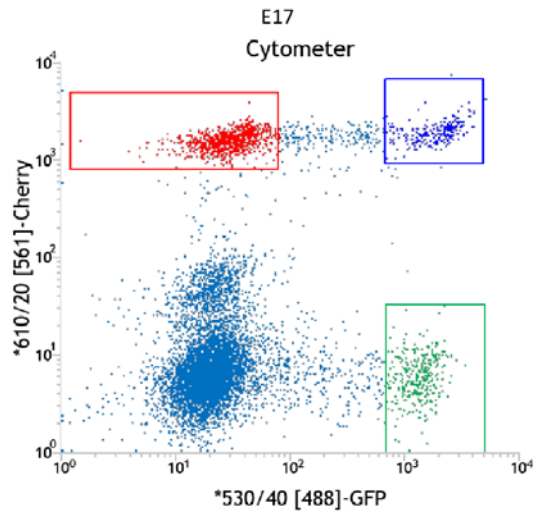
From our previous data, we show that SOX2 controls cell differentiation in the conducting airways. In the absence of SOX2, there is a decrease in club cell differentiation and absence of neuroendocrine cell differentiation. SOX2 is normally expressed in the conducting airways, but when SOX2 expressed in the gas exchange region there is basal cell differentiation and club cell differentiation. The next step is to investigate in a molecular level what gene is downstream of SOX2 gene and controls cell differentiation in the conducting airways. We seek to identify intermediate genes between SOX2 and cell differentiation. We used fluorescence activated cell sorting to isolate conducting airway cells. The breeding setup was: ♀ *Sox2*<sup>CKO/CKO</sup>; *Rosa*<sup>+/+</sup>; *Shh*<sup>+/+</sup> x ♂ *Sox2*<sup>EGFP/+</sup>; *Rosa*<sup>tdt/tdt</sup>; *Shh*<sup>Cre/+</sup>. The SOX2 mutant genotype is *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>Cre/+</sup> and the SOX2 control is *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>+/+</sup>. *Sox2*<sup>EGFP/+</sup> labels conducting airway cells. To delete SOX2 in the epithelium we used *Shh*<sup>Cre/+</sup>. *Rosa*<sup>tdt/tdt</sup> was used as a reporter to confirm Cre activity. The three embryonic stages for expression profiling were E15, E17, and E19 (Figure 5.1). We chose E15 because it is the stage after lung initiation. We chose E17 because it is the stage before cell differentiation. We chose E19 because it is the stage when cell differentiation occurs. A fluorescence microscope was used to screen mutant and control embryos. The control *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>+/+</sup> has green fluorescence. The mutant *Sox2*<sup>EGFP/CKO</sup>; *Rosa*<sup>tdt/+</sup>; *Shh*<sup>Cre/+</sup> has green and red fluorescence. We confirmed our fluorescence screening method by immunofluorescence staining of E13 lungs (Figure 3.1B). Dissociation of cells was done by using collagenase and DNase. After cells were sorted, we extracted total RNA. We used the Illumina TotalPrep RNA Amplification Kit to prepare libraries for microarray analysis. Two microarray analyses were done to identify genes down regulated in the absence of SOX2 (Figure 5.2). We screened for genes that had a large difference between SOX2 control and SOX2 mutant lungs. We chose candidate genes among the top 20 genes that are down regulated in the absence of SOX2. RSPH1, ASCL1, SOX21,

ALDHA1A1, SCGB1A1, and SOX2 were among the top genes that are highly down regulated in the absence of SOX2. Based on literature search, we decided to study the role of SOX21 in the conducting airways. SOX21 is a transcription factor and is part of the SOXB2 group (32). SOX21 is important in development of the central nervous system and hair follicle development (54, 55). In other lab studies, SOX21 upregulated when SOX2 expression is increased in mouse embryonic cells and glioblastoma (56,57). Our microarray analyses data indicates that SOX21 is highly downregulated in the absence of SOX2. To further address the downregulation of SOX21 in the conducting airways, we performed SOX21 antibody staining on E15 and E17 SOX2 control and mutant lungs. SOX21 is absent when SOX2 is deleted in the conducting airways (Figure 5.3A and 5.3B). Based on our immunofluorescence staining results, we saw a correlation between SOX21 and SOX2.

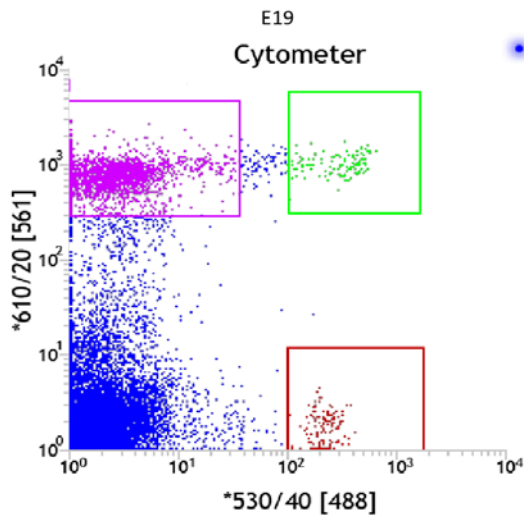
A



B



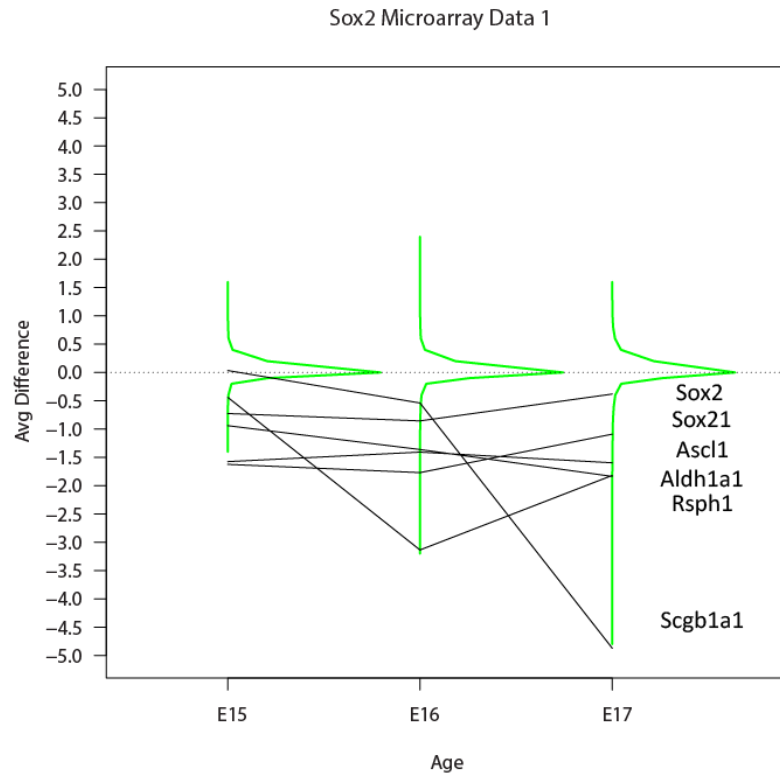
C



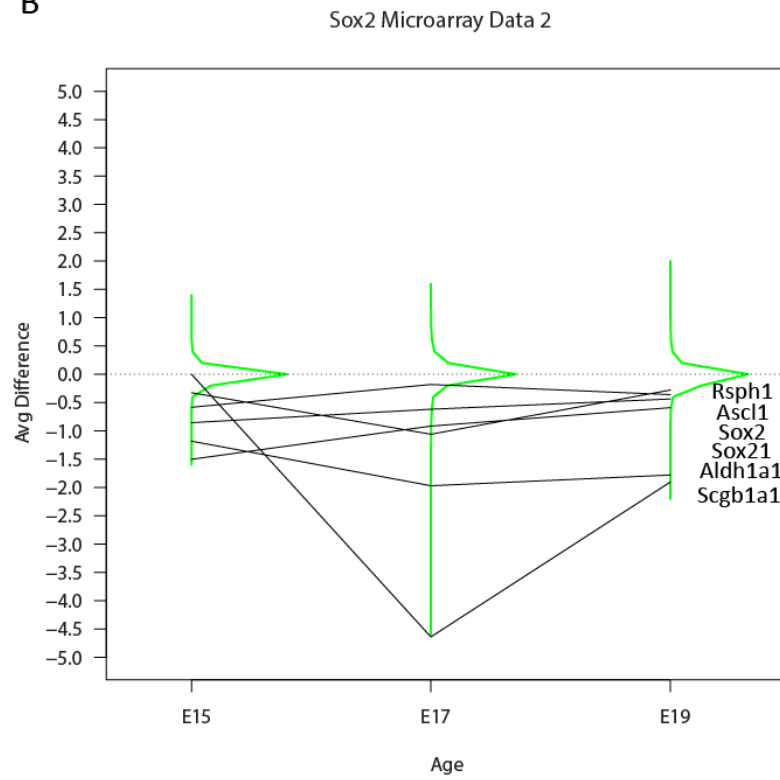
**Figure 5.1 Isolation of SOX2 epithelium cells for expression profiling of control and mutant lungs.**

(A-C) Fluorescence activated cell sorting graphs represent the two different populations of conducting airway cells that we collected from E15, E17, and E19 lungs. The control population of interest is GFP<sup>+</sup>, which labels SOX2<sup>+</sup>. The mutant population is double positive for both GFP<sup>+</sup> and tomato<sup>+</sup>, which labels SOX2<sup>-</sup> cells.

A



B



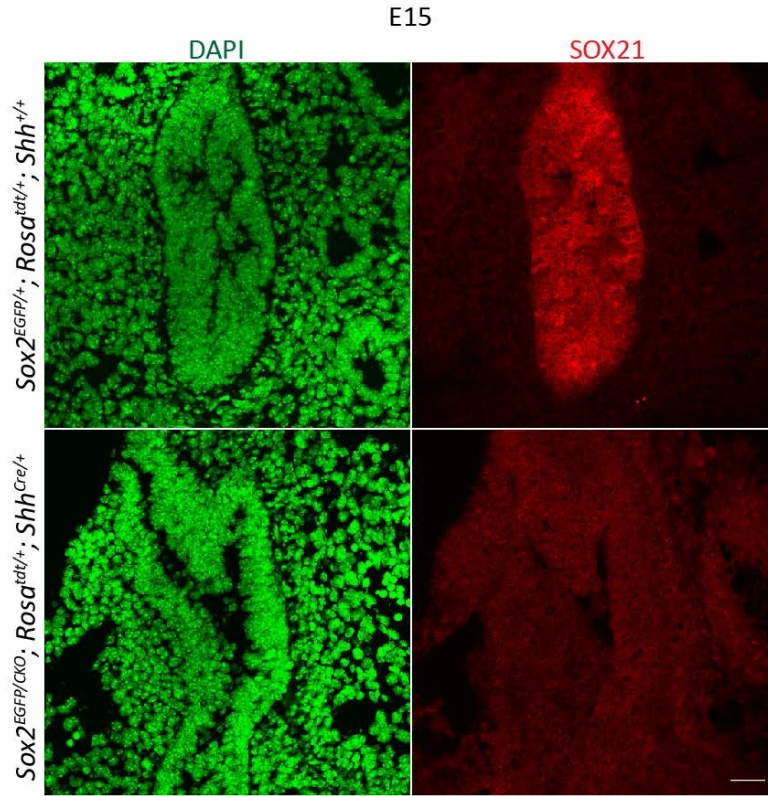
**Figure 5.2 SOX21 is down regulated in the absence of SOX2.**

A-B) The average difference represents the difference between the control and mutant for each embryonic stage. Age represents the embryonic stage. The genes listed are the top genes highly down regulated in the absence of SOX2. A) The first run of microarray analyses from E15, E16, and E17 lungs.

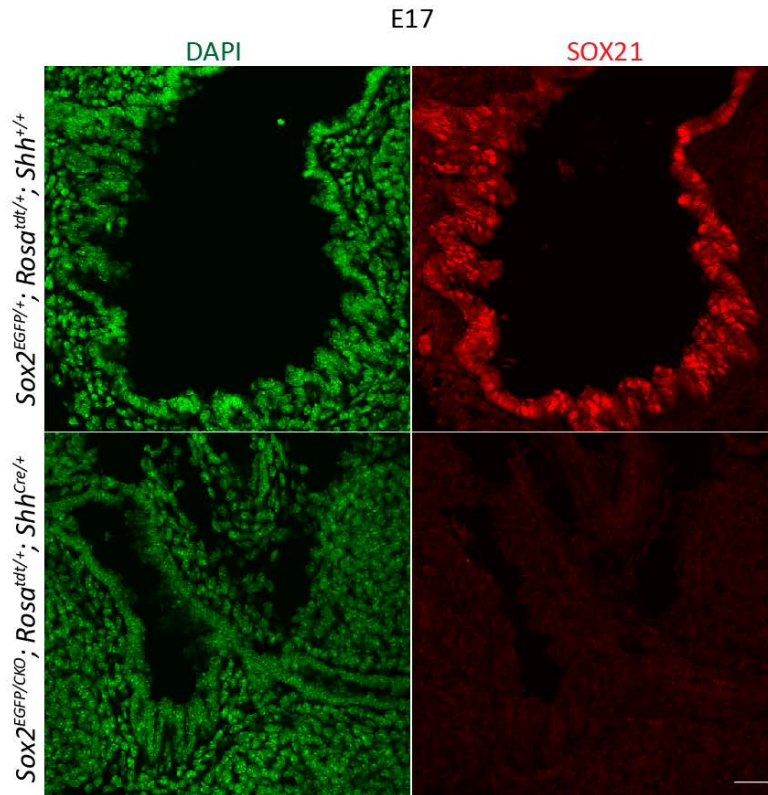
B) The second run of microarray analyses from E15, E17, and E19 lungs.



A



B



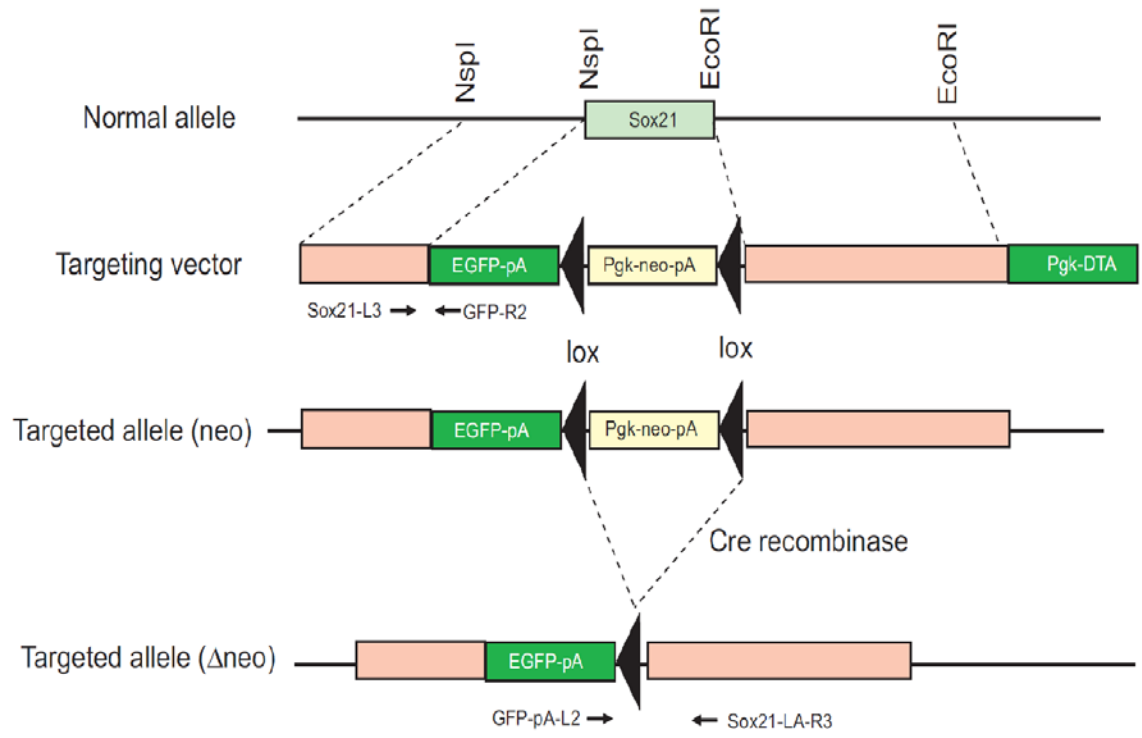
**Figure 5.3 SOX21 is absent in the conducting airways when SOX2 is absent.**

Confocal images of immunostained E15 and E17 lung sections. The top panels are the SOX2 control lungs and bottom panels are the SOX2 mutant lungs. DAPI (green) and SOX21 (red). A) SOX21 is absent in the E15 SOX2 mutant lung. B) SOX21 is absent in E17 SOX2 mutant lung. SOX21 is absent in the conducting airways when SOX2 is not present.

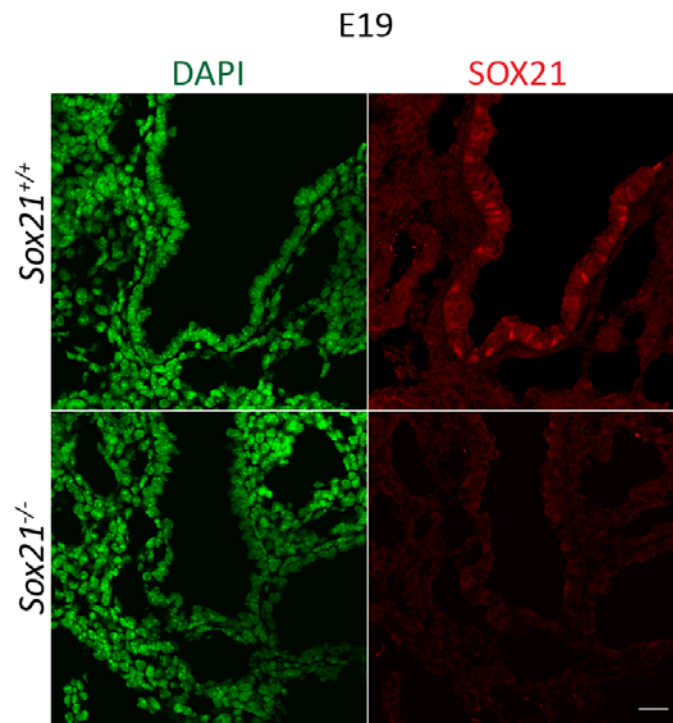
## **5.2 SOX21 has two expression patterns in the lung.**

The next question is when SOX21 is absent in the conducting airways what happens to cell differentiation? To address whether there is change in cell differentiation in the conducting airways when SOX21 is absent, we collaborated with Dr. Hideyuki Okano to obtain a SOX21 knock out mouse. The SOX21 knock out model is generated by replacing the SOX21 coding sequence with EGFP (Figure 5.4A, 55). We confirmed that SOX21 knock out mouse is a complete knock out by SOX21 antibody staining (Figure 5.4B). We took the left lobe of an E19 SOX21 heterozygous embryo for whole mount immunofluorescence staining and to determine a lung phenotype. The SOX21 heterozygous lung did not have abnormalities. Interestingly, the SOX21 heterozygous lung has two expression patterns (Figure 5.5). SOX21 expression is uniform in the proximal airways and sporadic in the distal airways. SOX21 has a unique expression pattern in the conducting airways. It is unknown if SOX21 controls cell differentiation in the conducting airways.

A



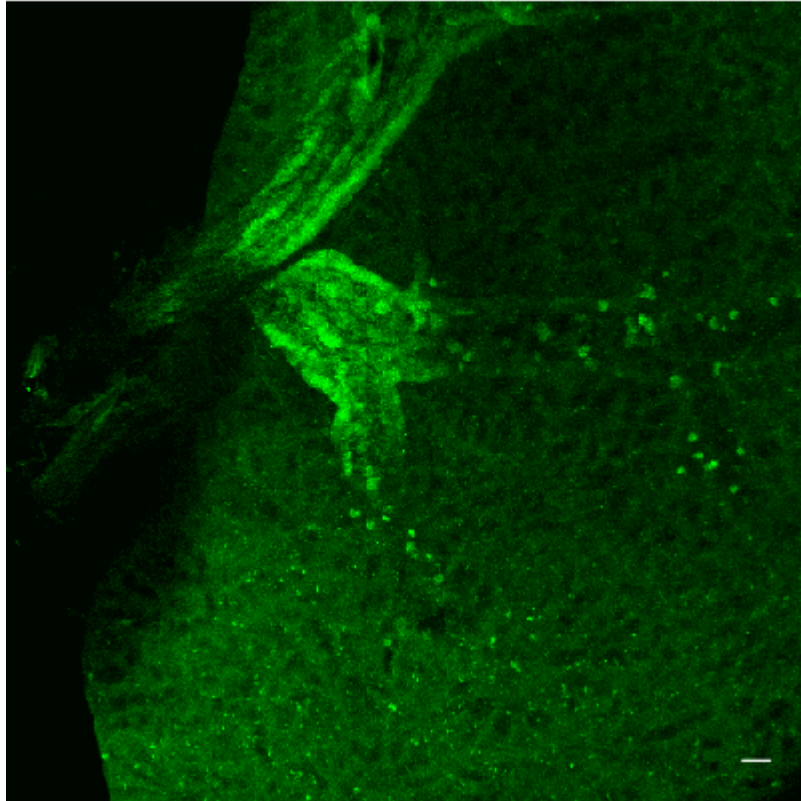
B



**Figure 5.4 SOX21 knockout mouse model is generated by replacing SOX21 with EGFP.**

A) A schematic diagram of the SOX21 knock out mouse construct (55). B) Confocal images of immunostained E19 lung sections of SOX21 control and knock out lungs. We confirmed SOX21 is absent in the SOX21 knock out lung. Scale bar: 20  $\mu\text{m}$ .

## *E19 Sox21 Heterzygous*

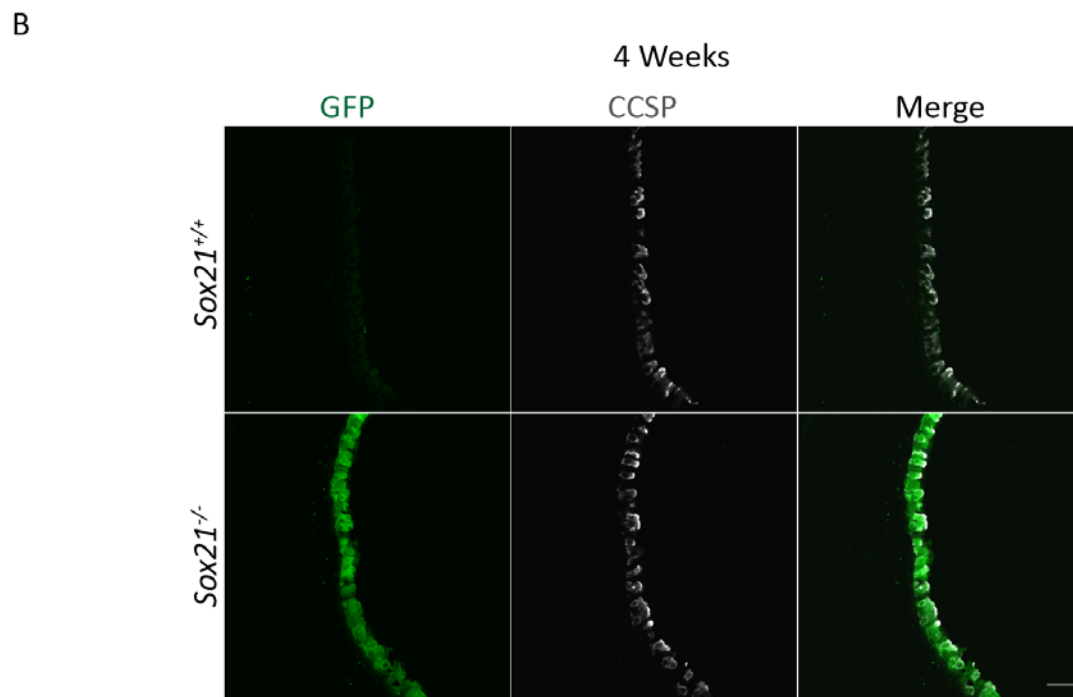
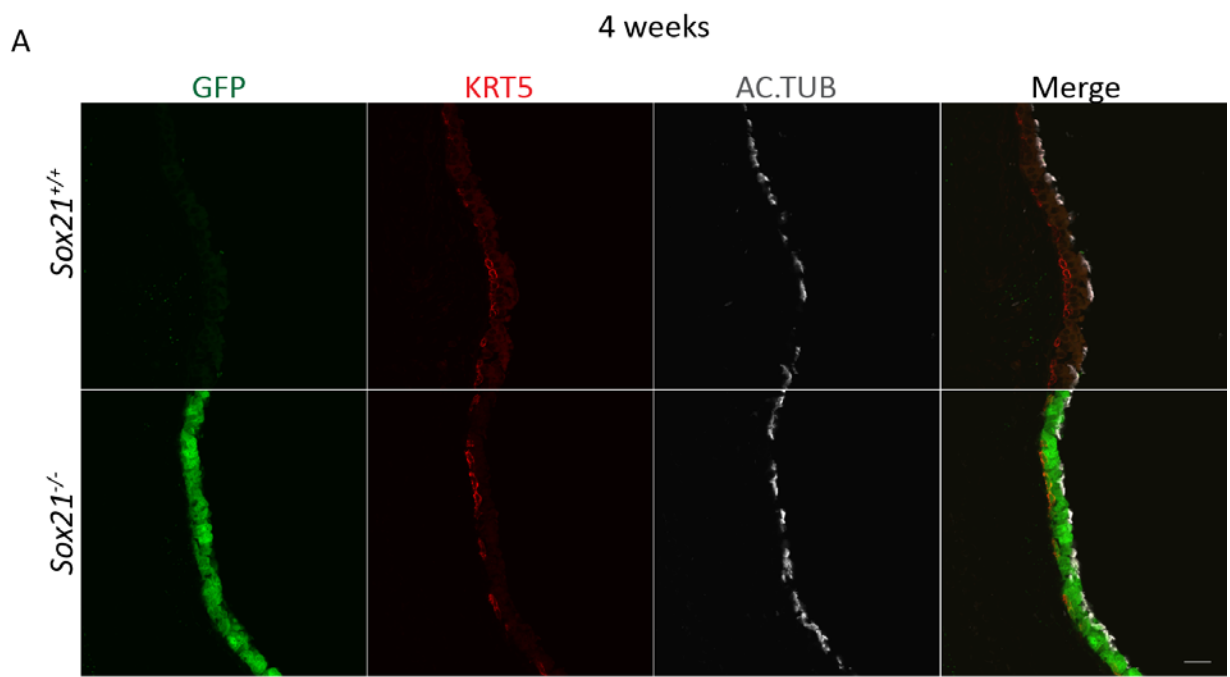


**Figure 5.5 SOX21 has two expression patterns in the lung.**

Confocal z-stack projection of immunostained E19 SOX21 heterozygous left lobe (z-stack of 31 images). After whole lung staining, the left lobe was cleared with BABB in order to image the conducting airways. We imaged the first lateral branch of the left lobe. GFP (green) labels the deletion of SOX21. We observed uniform GFP expression in proximal airways and sporadic GFP expression in the distal airways. Scale bar: 50  $\mu\text{m}$ .

### **5.3 Cell differentiation in the conducting airways is normal when SOX21 is absent.**

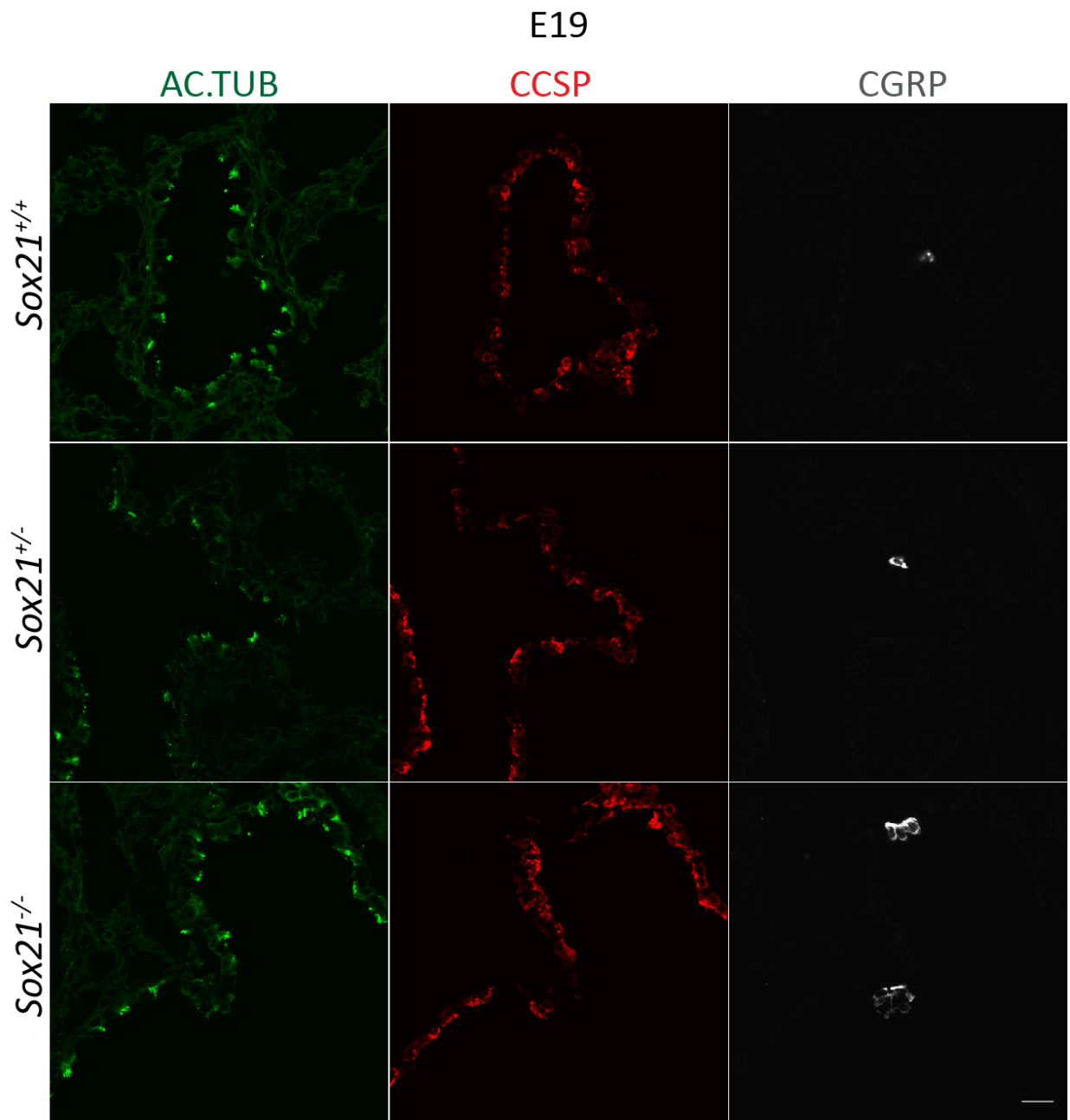
We examined the trachea region of 4 week old mice to determine if there is a change in cell differentiation when SOX21 is absent. We used immunofluorescence staining for different lung cell type markers to determine if there is change in cell differentiation when SOX21 is absent in the lung. In the trachea, cell differentiation is not affected by the absence of SOX21 (Figure 5.6A and 5.6B). We did not detect a difference in basal cell differentiation, club cell differentiation, and ciliated cell differentiation between the control and mutant trachea. We decided to investigate beyond the trachea by analyzing lobular bronchiole regions to determine if there is a change in cell differentiation when SOX21 is absent. We used confocal images of immunostained E19 lung sections to visualize cell differentiation in the conducting airways. Club cell differentiation, ciliated cell differentiation, and neuroendocrine cell differentiation occurs in a normal pattern by comparison of E19 control and mutant lungs (Figure 5.7). Based on our data, SOX21 deletion in the lung does not affect cell differentiation.





**Figure 5.6 Phenotypic Analysis of the SOX21 mutant trachea.**

A-B) Confocal images of immunostained 4 week old mice trachea sections. A) GFP (green) labels SOX21 deletion, KRT5 (red) labels basal cells, and AC.TUB (gray) labels ciliated cells. B) GFP (green) labels SOX21 deletion and CCSP (gray) labels club cells. Cell differentiation was not affected by the absence of SOX21 in the mutant trachea compared to the control trachea. Scale bar: 20  $\mu$ m.



**Figure 5.7 Phenotypic analysis of SOX21 mutant lung in the distal conducting airways.**

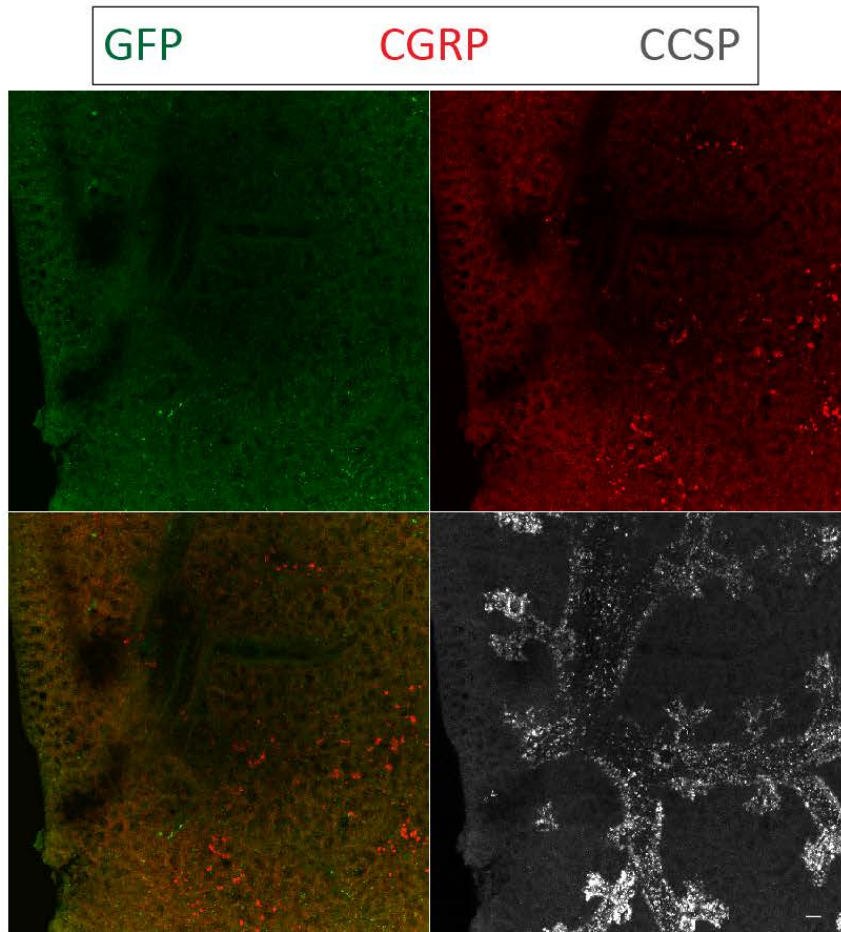
Confocal images of immunostained E19 lung sections. The top panel is the control lung, the middle panel is the heterozygous lung, and the bottom panel is the knock out lung. AC.TUB (green) labels ciliated cells, CCSP (red) labels club cells, and CGRP (gray) labels neuroendocrine cells. We did not detect abnormal differences in cell differentiation in the heterozygous and knock out lung when compared to the control lung. In the absence of SOX21, lung cell differentiation is unaffected.

#### **5.4 SOX21 does not affect the number of neuroendocrine cells in the lung.**

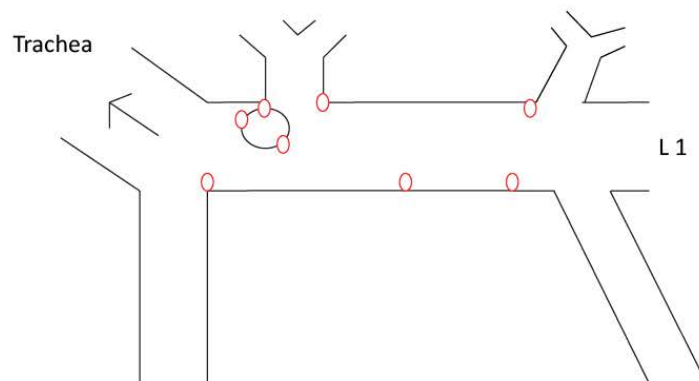
One of SOX21's expression patterns resembles the expression pattern of neuroendocrine cell differentiation. In the distal region of the lung, SOX21 has a sporadic expression pattern and is similar to the pattern of neuroendocrine cell differentiation. It is unknown if SOX21 controls neuroendocrine cell number in the lung. To determine if SOX21 controls neuroendocrine cell number, we analyzed E19 left lobes for immunofluorescence staining for CGRP. We used confocal images of E19 SOX21 knock out, heterozygous, and control left lobes. We imaged the first lateral branch 1 (L1) for all three E19 left lobes. We counted the number of neuroendocrine cells at L1 for E19 knock out, heterozygous, and control lungs. In the control lung we detected 8 neuroendocrine cells, in the heterozygous lung we detected 15 neuroendocrine cells, and in the knock out lung we detected 13 neuroendocrine cells. We suggest that SOX21 does not control the number of neuroendocrine cells in the lung based on the lung variation among the three left lobes. We have demonstrated that SOX21 does not control cell differentiation in the conducting airways. In the absence of SOX21, the lung undergoes normal development.

A

*Sox21 Control*



B



**Figure 5.8 Identifying the role of SOX21 in neuroendocrine cell number in the control lung.**

A) Confocal z-stack projection of immunostained E19 SOX21 control left lobe (z-stack of 36 images).

After whole lung staining, the left lobe was cleared with BABB in order to image the conducting

airways. We imaged the first lateral branch of the left lobe. GFP (green) labels SOX21 deletion in the

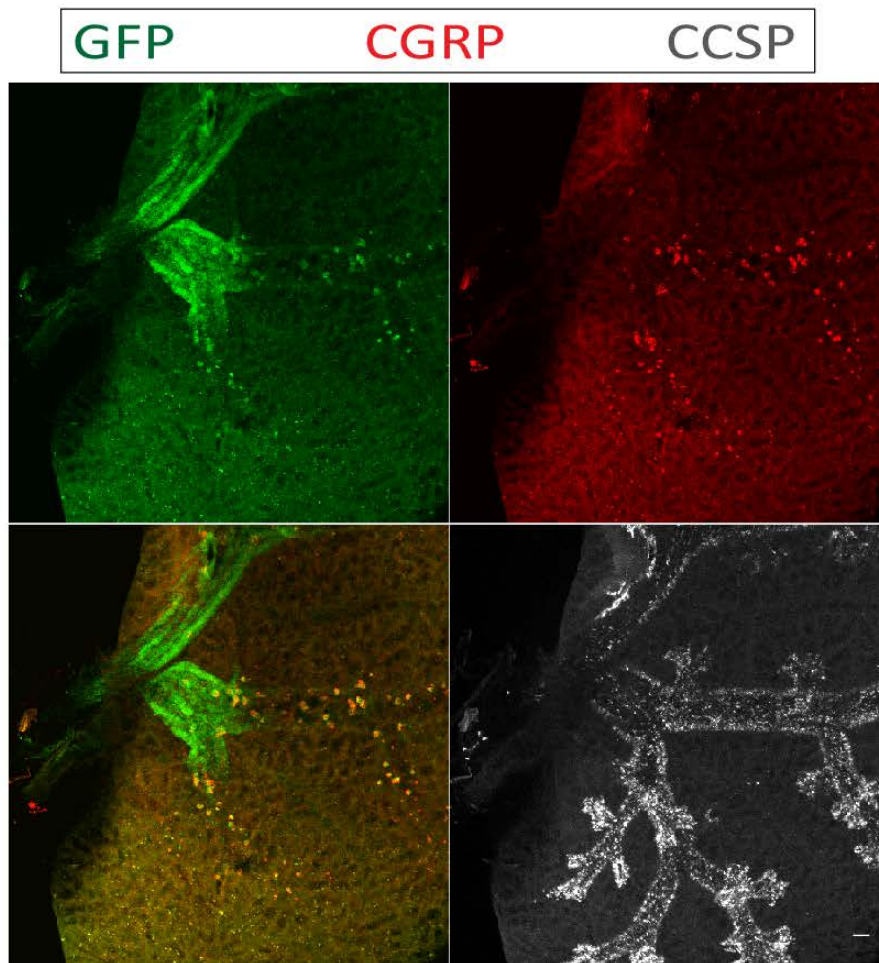
lung, CGRP (red) labels neuroendocrine cells, and CCSP (gray) labels club cells. B) A schematic diagram

of L1 from A. The red circles represent individual neuroendocrine cells. We counted 8 neuroendocrine

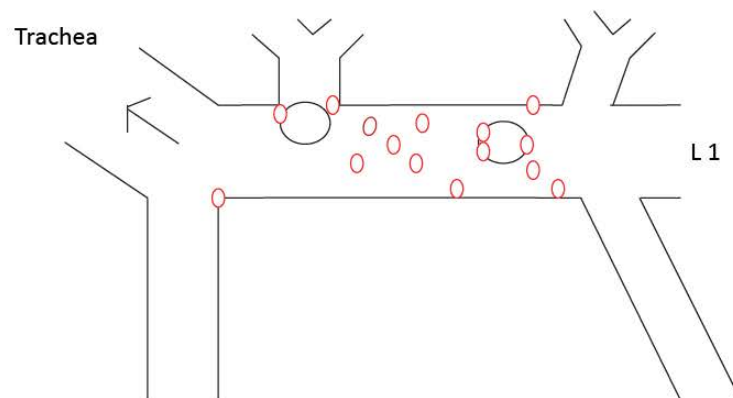
cells in the first lateral branch. Scale bar: 50  $\mu$ m.

A

*Sox21* Heterozygous



B



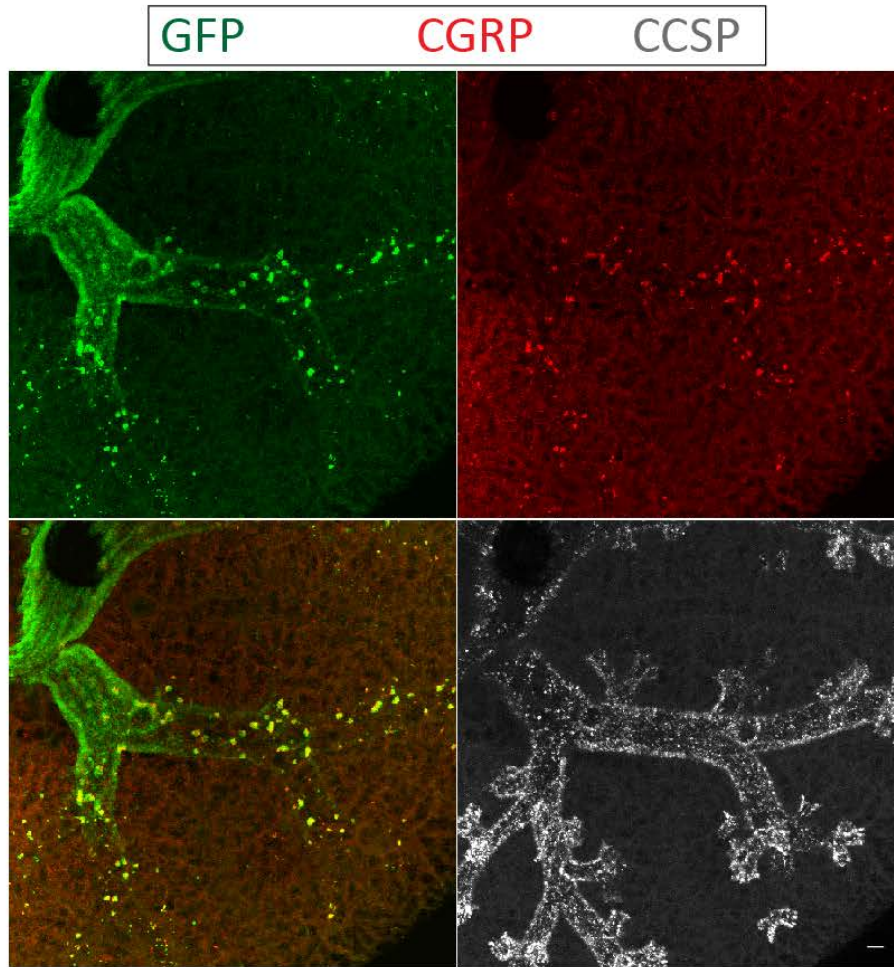
**Figure 5.9 Identifying the role of SOX21 in neuroendocrine cell number in the heterozygous lung.**

A) Confocal z-stack projection of immunostained E19 SOX21 heterozygous left lobe (z-stack of 31 images). After whole lung staining, the left lobe was cleared with BABB in order to image the conducting airways. We imaged the first lateral branch of the left lobe. GFP (green) labels SOX21 deletion in the lung, CGRP (red) labels neuroendocrine cells, and CCSP (gray) labels club cells. B) A schematic diagram of L1 from A. The red circles represent individual neuroendocrine cells. We counted 15 neuroendocrine cells in the first lateral branch. Scale bar: 50  $\mu\text{m}$ .

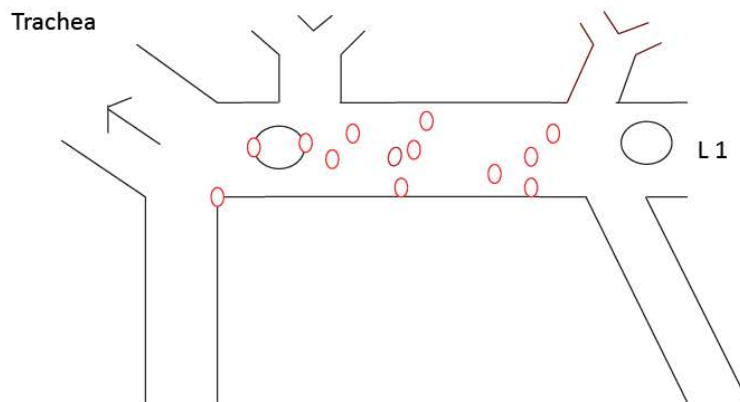


A

*Sox21 Knock Out*



B



**Figure 5.10 Identifying the role of SOX21 in neuroendocrine cell number in the knock out lung.**

A) Confocal z-stack projection of immunostained E19 SOX21 knock out left lobe (z-stack of 37 images). After whole lung staining, the left lobe was cleared with BABB in order to image the conducting airways. We imaged the first lateral branch of the left lobe. GFP (green) labels SOX21 deletion in the lung, CGRP (red) labels neuroendocrine cells, and CCSP (gray) labels club cells. B) A scheme diagram of L1 from A. The red circles represent individual neuroendocrine cells. We counted 13 neuroendocrine cells in the first lateral branch. Scale bar: 50  $\mu\text{m}$ .

## 5.5 Pathway Analysis of Microarray Data.

We chose SOX21 because it was one of the top genes highly down regulated in the absence of SOX2. Interestingly, the absence of SOX21 did not change cell differentiation in the conducting airways. We decided to run pathway analysis on previous microarray data to determine what pathways are involved when SOX2 is absent in the conducting airways. The software we used was Ingenuity Pathway Analysis (IPA). The top pathways retrieved from IPA are Aryl hydrocarbon receptor signaling, antiproliferative role of TOB in T cell signaling, oxidative ethanol degradation III, tight junction signaling, and ethanol degradation IV. Diseases associated with SOX2 deletion in the lung are cancer, dermatological diseases, gastrointestinal diseases, and hematological diseases. Based on IPA, SOX2 is involved in embryonic development, organ morphology, and renal system development.

## 6. Discussion and Future Directions

In this study, we show that SOX2 controls cell differentiation in the conducting airways. Based on our loss of function and gain of function SOX2 experiments, we are able to provide insight how SOX2 controls the cell fate of conducting airway cells. We used two mouse models to delete SOX2. The first mouse model involved deleted SOX2 in the lung epithelium by using *Sox2*<sup>CKO/CKO</sup> and *Shh*<sup>Cre/+</sup>. We analyzed E19 trachea and lobular bronchiole regions to identify which specialized lung cell type such as; club cells, ciliated cells, basal cells and neuroendocrine cells have a change in cell differentiation when SOX2 is absent. Neuroendocrine cell and basal cell differentiation is nearly completely absent in the SOX2 mutant based on immunostained lung sections and whole lung staining. Club cell differentiation is severely comprised in the SOX2 mutant. Ciliated cell differentiation is moderately affected in the SOX2 mutant. The four lung cell types in the conducting airways are affected by the absence of SOX2 at different levels. For the second mouse model, we used a mosaic model containing SOX2 positive and negative cells to identify which specialized lung cells require SOX2. We used *Sox*<sup>EGFP/+</sup> to label SOX2 cells. To delete SOX2, we used *Sox2*<sup>CKO/CKO</sup> and *Sox9*<sup>CreER/+</sup>. We analyzed E19 trachea and E19 left lobes to determine if SOX2 is cell autonomous in the conducting airways and identify which specialized lung cell type requires SOX2. The mosaic model data suggests that SOX2 is required cell autonomous for club cell differentiation. Based on the loss of function mouse models, ciliated cell differentiation occurs at low frequency in the absence of SOX2. A possible explanation why ciliated cell differentiation occurs at low frequency is that NOTCH is absent in the lung epithelium. NOTCH signaling is known to be involved in club cell and ciliated cell differentiation. In a normal lung setting, NOTCH inhibits ciliated cell differentiation and induces club cell differentiation, which yields the distribution of club cells and ciliated cells throughout the conducting airways (1, 58). One possible explanation for ciliated cell differentiation to be moderately affected in the SOX2 mutant is SOX2 and NOTCH work together to regulate club cell and ciliated cell differentiation. Without SOX2, NOTCH cannot inhibit ciliated cell differentiation and ciliated cells are being generated when there are no club cells present. We did not

study NOTCH activity in the lung epithelium. To address NOTCH activity in conducting airways, we could in the future perform immunofluorescence staining using a NOTCH antibody. Based on the literature, a decrease in NOTCH will explain why there are more ciliated cells than club cells in the SOX2 mutant. One possible result is that NOTCH is missing in the conducting airways and NOTCH is downstream of SOX2. A second possible result is that NOTCH is present in the SOX2 mutant lung. The presence of NOTCH can suggest that NOTCH and SOX2 are independent from one another. In another lung study, SOX2 was deleted using *Nkx2.5-Cre* and the result was a decrease in club cells, ciliated cells, and basal cells (42). In an injury lung model by Dr. Que using SO<sub>2</sub> and SOX2 deletion, there is a great decrease in the number of basal, ciliated, and club cells (42). Dr. Que's trachea experiments suggest that SOX2 is important for trachea cell regeneration and cell differentiation based on SO<sub>2</sub> injury and the absence of SOX2 in the conducting airways (42). Thompkins's work on deleting SOX2 in postnatal lungs using *Scgb1a1-Cre* suggests that SOX2 expression is necessary for differentiation and maintenance of club and ciliated cells in the conducting airways (43). In our lung studies, ciliated cell differentiation is not significantly reduced in SOX2 mutant lungs compared to Que's and Thompkin's work. We did detect a decrease in basal cell, club cell, ciliated cell and neuroendocrine cell differentiation in the SOX2 mutant compared to other SOX2 deletion studies in the lung. Our SOX2 deletion studies suggest that SOX2 is required for cell differentiation in the conducting airways.

The second part of this study focused on what determines the cell fate of conducting airway cells and why are specialized cells such as: basal cells, club cells, ciliated cells, and neuroendocrine cell restricted only in the conducting airways? Is SOX2 sufficient to induce airway differentiation? To address if SOX2 determines the cell fate of conducting airway cells, we used different mouse models to overexpress SOX2 and express SOX2 in the gas exchange region. SOX2 overexpression was done by using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/bih</sup>)*. The removal of the flanked loxP sites led to SOX2 expression and express green fluorescence (45). Our first SOX2 overexpression mouse model was overexpressing SOX2 in the lung epithelium by using *Shh<sup>Cre/+</sup>* at E19. SOX2 was overexpressed in the

conducting airways and expressed in the gas exchange region, where SOX2 is not normally present. In the gas exchange region, SOX2 inhibits alveolar type 1 cell and alveolar type 2 cell differentiation. Interestingly, basal cell differentiation occurred in the gas exchange region. 29% of SOX2 cells in the gas exchange region are able to generate basal-like cells and 4% of SOX2 cells are able to make mature basal cells. Double positive SOX2 and P63 cells are stuck as basal-like cells, and are not able to become mature basal cells. We perform immunofluorescence staining using only two basal cell markers P63 and KRT5. We need to use other basal cell markers other than P63 and KRT5 to be certain that the majority of P63 cells are not able to become mature cells. We determined that p63 $\Delta$ N is the type of isoform seen in the gas exchange region. We did not detect club cell, ciliated cell, and neuroendocrine cell differentiation in the gas exchange region. SOX2 overexpression did not lead to tumor formation. We detected abnormal branching defects when we overexpress SOX2 using *Shh*<sup>Cre/+</sup>. We used another mouse model to express SOX2 specifically in the alveoli and avoid branching defects. We used *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Sox9*<sup>CreER/+</sup> (49) to express SOX2 specifically in the gas exchange region without changing SOX2 expression in the conducting airways at E19. We did not observe abnormal branching defects and no tumors were present. SOX2 expression in SOX9 progenitor cells inhibits alveolar type 1 and alveolar type 2 cell differentiation. Our data suggests that SOX2 expression in the gas exchange region inhibits alveolar type 1 and alveolar type 2 cell differentiation. To determine the cell fate of SOX2 cells in the gas exchange region, we analyzed immunostained E19 lung sections. SOX2 expression in the gas exchange region induced basal cell differentiation. Club cell, ciliated cell, and neuroendocrine cell differentiation did not extend into the gas exchange region. SOX2 is not sufficient to induce club cell, ciliated cell, and neuroendocrine cell differentiation in the gas exchange region. There are other components along with SOX2 that are required to induce airway differentiation. SOX2 only makes up one part of the network to regulate airway differentiation. At E19, SOX2 changes the cell fate of alveoli cells based on the suppression of alveolar type 1 cell differentiation and alveolar type 2 cell differentiation. Our data shows that changing

the cell fate of alveoli cells through SOX2 during early embryonic development leads to have portion of SOX2 cells to induce basal cell differentiation. Thus far, we show conducting airway cell differentiation arises from SOX2. After mouse birth, we induced SOX2 specifically in alveolar type 1 and type 2 cells to determine if SOX2 can change the cell fate of alveoli cells. We induced SOX2 expression in alveolar type 1 and type 2 cells during early postnatal lung and late postnatal lung development. We expressed SOX2 in alveolar type 1 cells using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Hopx<sup>CreER/+</sup>*. Mutant alveolar type 1 cells expressing SOX2 were able to undergo cell division and change cell shape into a cuboidal shape. Alveolar type 1 cells are known not to proliferate and are squamous. Our data suggests that alveolar type 1 cells can undergo cell division with the help of SOX2. We collected two time points for early postnatal lung development at postnatal day 8 (P8) and postnatal day 21 (P21). We induced Sox2 in mice at postnatal day 4 (P4). At P8, we did not detect conducting airway cell differentiation in mutant alveolar type 1 cells expressing SOX2. At P21, which is 17 days after SOX2 induction, basal cell and club cell differentiation took place in mutant alveolar type 1 cells expressing SOX2. For late postnatal lung development, we induced SOX2 expression in mice at 4 weeks of age. We collected two time points for late postnatal lung development at 5 weeks and 7 weeks of age. In both time points, mutant alveolar type 1 cells expressing SOX2 were able to proliferate. We did not detect conducting airway cell differentiation in 5 week old and 7 week old mice. In early postnatal lung development, we induced SOX2 expression in committed alveolar type 1 cells. When SOX2 was induced at P4, committed alveolar type 1 cells became conducting airway cells by expressing basal cell and club cell markers. In late postnatal lung development, we induced SOX2 expression in mature alveolar type 1 cells. SOX2 induction at 4 weeks of age did not lead to conducting airway cell differentiation in mutant alveolar type 1 cells. SOX2 changes the cell fate of alveolar type 1 cells into conducting airway cells when SOX2 is induced during embryonic lung development and early postnatal lung development. The clusters of SOX2 positive at P21 and 7 weeks in the mutant alveolar type 1 cells could be tumor initiation even though we did not detect tumors in the mutant lungs. To address if there is tumor initiation in the

mutant lungs, we can use oncogenic markers that are known from the literature for immunofluorescence staining. If there is expression of oncogenes in the mutant lung, there is tumor initiation when SOX2 is induced alveolar type 1 cells. However, if there is no expression of oncogenes in the mutant lung, SOX2 induction leads to hyperplasia in the lung. We observed a low alveolar type 1 cells undergo cell recombination this could be due to the low efficiency of *Hopx*<sup>CreER/+</sup>. We injected mice with a single dose of tamoxifen which could have led to the low efficiency of recombination. We expressed SOX2 in alveolar type 2 cells using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Sftpc*<sup>CreER/+</sup> (52). We expressed SOX2 in alveolar type 2 cells during early and late postnatal lung development. In early and late postnatal lung development, mutant alveolar type 2 cells expressing SOX2 did not undergo alveolar type 2 cell differentiation. During early postnatal lung development, mutant alveolar type 2 cells expressing SOX2 induced basal cell and club cell differentiation. Ciliated cells and neuroendocrine cells did not arise from mutant alveolar type 2 cells expressing SOX2. Our data suggests that SOX2 controls conducting airway cell differentiation based on the results obtained from mutant alveolar type 1 and type 2 cells expressing SOX2. SOX2 is a key component that builds up the conducting airways. When SOX2 was expressed in different regions of the lung, conducting airway cell differentiation such as; club cells and basal cells occurred in the lung. Ciliated cell and neuroendocrine cell differentiation occurred in the conducting airways as long as SOX2 was present. SOX2 expression alone in the gas exchange region is not sufficient to induce cell differentiation of all four lung cell types. Interestingly, basal cell differentiation occurred in all SOX2 overexpression mouse models in this study. A possible explanation for basal cell differentiation to occur in the gas exchange region is that upon SOX2 expression a signal is triggered signaling that the conducting airways are beginning to form. Basal cells are found mainly in the trachea. Basal cells found in mutant SOX2 cells in the gas exchange resemble to be in form of the trachea, which throughout time can develop in full conducting airways. Our final SOX2 overexpression model was using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Sox2*<sup>CreER/+</sup> (50). We overexpressed SOX2 in the conducting airways during late postnatal lung



development. We induced SOX2 expression in mice at 4 weeks of age and harvest at 7 weeks of age. Basal cell, club cells, and ciliated cells did not increase when SOX2 expression was increased in the conducting airways. Interestingly, there were larger clusters of neuroendocrine cells in mutant conducting airways compared to the control. In other lab studies, SOX2 overexpression in the lung reprograms alveolar epithelial cells into cells with characteristics of the conducting airways (59). In SOX2 overexpression experiments done by Dr. Tompkins' lab, SOX2 overexpression was done using *rCCSP-rtTA<sup>tg/wt</sup>, (tetO)<sub>7</sub>CMVSox2<sup>tg/wt</sup>* (59). SOX2 was overexpressed in club cells, which led to ciliated cell, club cell, and basal cell differentiation in the gas exchange region. No tumors were present in the lung. In SOX2 ectopic expression experiments done by Dr. Gontan's lab, the SOX2 overexpression mouse model was done by using *SPC-rtTA*. SOX2 overexpression led to abnormal branching. Neuroendocrine cell and basal cell differentiation occurred in the gas exchange region. The basal cells in the gas exchange region were p63 $\Delta$ N. Both Gontan and Tompkins's work, suggest that SOX2 is a positive regulator of the conducting airways by being able to drive cell differentiation. We also show through our Sox2 overexpression mouse models, SOX2 is able to induce airway differentiation of basal cells and club cells.

We decided to isolate SOX2 cells from mouse embryonic lungs to study SOX2 in a molecular level to identify genes that are downstream of SOX2 and control airway differentiation. From fluorescence activated cell sorting (FACS) cells, we obtained two microarray analyses to identify genes that are highly down regulated in the absence of SOX2. We chose to study SOX21, a transcription factor highly down regulated in both microarray analyses. Our data shows that SOX21 is absent when SOX2 is absent in the conducting airways. Based on our SOX21 loss of function experiments, cell differentiation in the conducting airways was unaffected by the absence of SOX21. The lung undergoes normal development when SOX21 is absent. However, SOX21 has a unique expression pattern in the lung. In the proximal region of the lung, which includes the trachea and mainstem bronchi we observed uniform SOX21 expression. In the distal area of the conducting airways, which includes the lobular

bronchioles we observed sporadic expression of SOX21. Based on our experiments, the role of SOX21 in the lung is still unknown. SOX21 is part of the SOXB2 group along with SOX14. SOX14 is part of the same group of SOX21. We can use immunofluorescence staining to determine if SOX14 is absent in our SOX2 mutant lungs. We can study SOX14 to determine if there is a lung phenotype. We can use a SOX14 knock out mouse model to determine if there is abnormal branching or size change in the lung comparing it to a normal lung. We are specifically interested in the lung epithelium and conducting airways. If there is a lung phenotype such as defects in the conducting airways consisting of large or small airways, the phenotype suggests that SOX14 has an important role in airway differentiation. The change in airway size suggests that there is probably a change in airway differentiation. The absence of SOX14 may decrease basal cell, club cell, ciliated cell, and neuroendocrine cell differentiation. To address if SOX14 controls airway cell differentiation, we will use immunofluorescence staining to visualize the expression of conducting airway cells. If there is no abnormal lung phenotype present in the SOX14 knock out mouse, we will use immunofluorescence staining to determine if there is a small change in airway differentiation. One possible outcome could be that the lung is grossly normal and airway differentiation is normal by comparing to a normal lung. Normal lung development in the SOX14 knock out mouse suggests that SOX14 is not required or sufficient for airway differentiation. SOX14 is not a downstream target of SOX2 for airway cell differentiation. Another possible outcome is that in the SOX14 knock out mouse, there is a decrease in airway differentiation of the four lung cell types. A change in airway differentiation suggests SOX14 is important component to build SOX2 transcriptional control of airway differentiation. The usage of chromatin immunoprecipitation (ChIP) would have helped us identify a target gene downstream of SOX2. With ChIP results, we could have identified SOX2 interactions with other genes based on DNA binding. We could have been successful in identifying a target gene if we performed ChIP. Based on Chip results, we will obtain knock out mouse models based on the genes selected. We would perform immunofluorescence staining on genes we select to determine if there is a decrease in airway differentiation. We ran pathway analysis on the microarray

data we obtained. The pathway analysis did not give us a clear idea to identify a target gene downstream of SOX2. We chose a gene based on how significant downregulated it is in the absence of SOX2.

Future directions of this project are studying SOX2 expression further in alveolar type 1 and type 2 cells. In our study, we analyzed cell differentiation in early postnatal lung development when used *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Sftpc<sup>CreER/+</sup>*. We can analyze more time points in SOX2 expression in alveolar type 2 cells. We could induced SOX2 expression in alveolar type 2 cells by using *Rosa26R-lox-stop-loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* and *Sftpc<sup>CreER/+</sup>*. We can study conducting airway cell differentiation in late postnatal lung development by inducing SOX2 at 4 weeks of age and harvest mice 3 weeks later. Results from late postnatal lung development will help obtain a complete understanding if SOX2 can reprogram any cell into conducting airway cells regardless of the stage of development. The mutant alveolar type 2 cell experiments can help us determine to what extent SOX2 regulates airway cell differentiation and which lung cell types it is able to generate. Understanding SOX2 regulation in the conducting airways, will help us in the future how to approach cell therapy in the lung.

A future experiment can be RNA-seq analysis of SOX2 to identify genes that are downstream of SOX2. RNA-seq will help identify the gene that cooperates with SOX2 to drive cell differentiation in the conducting airways. RNA-seq can help us find new genes that were not detected in the microarray data. We can use *Sox2<sup>EGFP/+</sup>* to label conducting airways, *Sox2<sup>CKO/CKO</sup>* and *Shh<sup>Cre/+</sup>* to delete SOX2 in the lung epithelium, and *Rosa<sup>tdt/+</sup>* as a reporter. The cells that will be collected for RNA-Seq analysis can be the same cells that were collected in this study for microarray analysis. The SOX2 mutant *Sox2<sup>EGFP/CKO</sup>*; *Rosa<sup>tdt/+</sup>*; *Shh<sup>Cre/+</sup>* and the control *Sox2<sup>EGFP/CKO</sup>*; *Rosa<sup>tdt/+</sup>*; *Shh<sup>+/+</sup>*. Another RNA-Seq analysis can be done for mutant alveolar type 1 cells expressing SOX2. We can compare genes between normal and mutant alveolar type 1 cells. We can use *Scnn1a<sup>Cre/+</sup>* to label alveolar type 1 cells, *Rosa26R-lox-stop-*

*loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* to express SOX2 in alveolar type 1 cells, and *Rosa<sup>mT/mG</sup>* as a reporter.

The mutant embryo of interest will be *Rosa<sup>Sox2/+</sup>; Scnn1a<sup>Cre/+</sup>*. The mutant embryo will have SOX2 expression in alveolar type 1 cells. The control embryo of interest will be *Scnn1a<sup>Cre/+</sup>; Rosa<sup>+ /mG</sup>*. The control will have green fluorescence representing wild type alveolar type 1 cells. RNA-seq analysis from normal and mutant alveolar type 1 cells will help identify genes downstream of SOX2 that can induce conducting airway characteristics. Expression profiling of mutant and wild-type alveolar type 1 cells will help us determine how SOX2 at a molecular level is able to induce airway differentiation.

In the future, this project can study further alveolar type 1 cell fate when SOX2 is induced in alveolar type 1 cells. We can use *Hopx<sup>CreER/+</sup>* and *Scnn1a<sup>Cre/+</sup>* to label alveolar type 1 cells and *Rosa26R-lox-stop loxSox2(Gt(Rosa)26Sor<sup>tm1/Sox2/blh</sup>)* to express SOX2 in alveolar type 1 cells. We can use lung sections for immunofluorescence staining to stain for alveolar type 1 cell markers and SOX2. We can study early and postnatal lung tissue to determine how many alveolar type 1 cells express both SOX2 and alveolar type 1 cell markers. Immunostaining will help identify the mutant alveolar type 1 cells that retain their alveolar type 1 cell characteristics and possibly understand why not all mutant alveolar type 1 cells acquire conducting airway cell characteristics. We want to study further how mutant alveolar type 1 cells are able to generate basal-like cells. We would like to determine if cells that express both SOX2 and an alveolar type 1 cell marker are able to generate basal-like cells. To determine if basal-like cells arising from mutant alveolar type 1 cells lose completely alveolar type 1 cell fate.

In conclusion, SOX2 controls cell differentiation in the conducting airways. Based on our SOX2 overexpression mouse models, SOX2 has the capacity to reprogram cells into conducting airway cells and induce cell differentiation. In the absence of SOX2, there is a decrease of cell differentiation in the conducting airways. Basal cells, ciliated cells, club cells, and neuroendocrine cells require SOX2. Our data suggests that SOX2 is a positive regulator in the lung by direct differentiation from an immature

cell to a specialize cell. In the mouse lung, SOX2-dependent transcriptional network controls cell airway differentiation in the mouse lung.

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## **Vita**

Belinda Judith Hernandez was born in Houston, Texas on March 20, 1985. She graduated from Cypress Falls High School. She completed her Bachelor of Science degree with a major in Biology from the University of Houston-Main Campus in May 2008. After college, she worked as a research technician at Baylor College of Medicine in the Human Genome Sequencing Center. In August of 2012 she entered the master's program at The University of Texas Graduate School of Biomedical Sciences.