Conditional Recombination Reveals Distinct Subsets of Epithelial Cells in Trachea, Bronchi, and Alveoli

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To identify relationships amongst tracheal and alveolar epithelial cells during lung development, we used conditional systems controlled by the rat *CCSP* and human *SFTPC* gene promoters to express Cre-recombinase in the developing mouse lung, thereby permanently labeling cells by expression of alkaline phosphatase or green fluorescent protein. When controlled by the rat *CCSP* promoter, continuous exposure of the fetus to doxycycline caused widespread recombination in conducting airway epithelial cells, including cells of the trachea, bronchi, and bronchioles before birth, and in both conducting and peripheral airways after birth. Neuroepithelial cells, identified by CGRP staining, were never labeled. Recombination and permanent labeling were observed in both ciliated and nonciliated respiratory epithelial cells, demonstrating their derivation from common progenitor cells during lung morphogenesis. Remarkable dorsal–ventral and cephalo–caudal labeling patterns, established before birth, were identified by recombination controlled by the rat *CCSP* gene promoter. In the trachea, subsets of epithelial cells labeled by the *CCSP* promoter were organized horizontally along the dorsal–ventral axis of the trachea, where selective labeling of cells juxtaposed to tracheal and bronchial cartilage was observed. In sharp contrast, recombination controlled by the human *SFTPC* gene promoter identified related cells that were organized in linear patterns along the cephalo–caudal axis of the conducting airways. Conditional expression of Cre-recombinase in the respiratory epithelium provides a useful model for the study of gene expression and function in the mouse respiratory tract and in the lung.

Keywords: CCSP; conditional gene targeting; CRE-recombinase; trachea; patterning

The lung forms by evagination of endoderm-derived cells from the foregut epithelium, which invade the surrounding splanchnic mesenchyme. The primary conducting airways are established early in lung morphogenesis (embryonic day [E] 9.5–E 11.5). Thereafter, peripheral lung tubules are formed by further budding of the intrapulmonary conducting airways, which leads to formation of the peripheral gas exchange regions, or alveoli, in the postnatal lung (1). Epithelial cells lining the respiratory tract undergo differentiation to produce the numerous, distinct, epithelial cell types characteristic of the mammalian lung. The numbers and types of cells lining conducting and peripheral airways vary with species, developmental stage, and along the cephalo–caudal axis. Factors controlling epithelial cell differentiation at various sites along the respiratory tract are not fully known, but are likely to be influenced by autocrine and paracrine interac-

tions among epithelial cells, their precursors, and the underlying mesenchymally derived cells, including cartilage, stroma, smooth muscle, vascular, and marrow-derived cell types. Factors controlling the numbers and location of distinct epithelial cell types, including cells of the tracheal glands, ciliated, basal, intermediate, Clara, neuroepithelial, and goblet cells of the conducting airways, and squamous type I and cuboidal type II cells in the alveolar region, are poorly understood (2–4). Recent studies using gene addition and targeted deletion of genes in the respiratory epithelium of the mouse indicate that specification and differentiation of proximal and peripheral respiratory epithelial cells are influenced by multiple signaling pathways, including β -catenin (5), sonic hedgehog (6–8), BMP's (9, 10), Foxa2 (11), GATA-6 (12, 13), TTF-1 (14), Rb (15), and others.

In the present study, we used transgenic mice in which both rat *CCSP* and human *SFTPC* gene promoters were used to express the reverse tetracycline transactivator (rtTA) (16), thus placing the expression of Cre-recombinase (CRE) under conditional control of doxycycline during mouse lung morphogenesis. Expression of CRE was used to permanently activate alkaline phosphatase (AP) (17) or green fluorescent protein (GFP) (18) in subsets of respiratory epithelial cells in the conducting airways. Each promoter labeled respiratory epithelial cells in stereotypic patterns, either along the cephalo–caudal or dorsal–ventral axis or in relationship to tracheal-bronchial cartilage.

MATERIALS AND METHODS

Genotyping

Transgenic mice were identified using PCR primers specific for each transgene *CCSP-rtTA*: (5' *CCSP* promoter: 5'-ACT GCC CAT TGC CCA AAC AC-3' and the 3' primer in *rtTA* coding sequence (5'-AAA ATC TTG CCA GCT TTC CCC-3') forward *CRE* (5'-TGC CAC GAC CAA GTG ACA GCA ATG-3') and reverse *CRE* (5'-AGA GAC GGA AAT CCA TCG CTC G-3'). Amplification of PCR products was performed as follows: denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, followed by a 5-min extension at 72°C. ZAP and ZEG mice were genotyped by positive β -gal staining on tissue.

Animal Use and Doxycycline Administration

Animals were housed in pathogen-free conditions in accordance with institutional guidelines. Animals were mated, and gestational age was determined by detection of the vaginal plug and then correlated with length and weight of each pup at the time of killing. Dams bearing double and triple transgenic pups were maintained on doxycycline containing food (625 mg/kg; Harlan Teklad, Madison, WI) or drinking water (Sigma Chemical Co., St. Louis, MO) at 1 mg/ml for various time spans. The mice were killed by either placing them in a CO₂ chamber or by injection with 0.2–0.3 cc anesthetic (ketamine, xylazine, acepromazine). All experiments were performed with at least five triple transgenic mice from three independent litters.

Timing and Conditional Control of CRE Recombination

Doxycycline was administered to pregnant dams at E 0.5 and maintained until killing. E 0.5 was defined as 12 h after fertilization, as determined by the vaginal plug. To investigate recombination at different periods during embryonic development, doxycycline was administered for

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shorter time periods by providing the dam doxycycline food at the indicated time points and removing it after 48 h. For postnatal recombination, pregnant and nursing dams were placed on doxycycline food from E 18.5 until PN 9, and recombination was assessed in triple transgenic mice at 3 wk of age. AP staining or detection of fluorescent GFP expression was used to assess recombination. Each experiment represents a group of 3 to 4 pregnant females of a cross resulting in triple transgenic offspring (*CCSP-rtTA/tetOCre/ZAP* or *CCSP-rtTA/tetOCre/ZEG*).

Lung Histology and *In Situ* Hybridization

Timed matings were performed to produce litters with triple transgenic offspring. Pregnant dams were administered doxycycline food for times specified in each individual experiment. Before killing, mothers were injected with 0.2–0.3 cc anesthetic (ketamine, xylazine, acepromazine) to prevent pups from breathing. Genotyping was performed on tail DNA. The thorax of both triple transgenic mice and controls were immersion fixed overnight at 4°C with 4% paraformaldehyde in PBS. Lungs from PN5 to PN21 were inflation-fixed at 25 cm of pressure and fixed overnight at 4°C.

For detection of *rtTA* and *CCSP* mRNA by *in situ* hybridization, *CCSP-rtTA* transgenic pups were obtained from timed matings, lungs were isolated and fixed overnight in 4% paraformaldehyde at 4°C, washed with PBS, dehydrated through a graded series of ethanol solutions, and processed for paraffin embedding. Sections (5 µm) were loaded onto polysine slides. ³⁵S-UTP labeled sense and antisense riboprobes were generated from a pGEM3z-*rtTA* and a pGEM4Z-*CC10* DNA plasmid (Promega, Madison, WI) and transcribed *in vitro* with a riboprobe transcription kit (Promega). Conditions and solutions for hybridization were essentially as previously described (19). Hybridization was performed overnight at 55°C, and the sections were washed under highly stringent conditions. Slides were dipped in Kodak NTB2 emulsion (Fisher Scientific, Pittsburgh, PA), exposed for 7–10 d for detection of *rtTA* expression in the mouse lung and 10 wk for detection of *CCSP* expression in both mouse and rat lungs, and developed with Kodak D19.

For visualizing AP activity, samples were fixed in 0.2% glutaraldehyde in PBS, 0.02% Nonident P-40, and 0.01% SDS. Samples were washed with PBS, dehydrated through a graded series of ethanol solutions, and processed for paraffin embedding. Five-micron sections were loaded onto polysine slides. AP staining was performed on tissue sections as previously described (17). All sections were counterstained with nuclear fast red. Histology was documented with an Optronics digital camera (Optronics, Goleta, CA) attached to a Nikon Microphot FXA (Nikon, Tokyo, Japan). Images were processed with Optronics MagnaFIRE 1.1 and Adobe Photoshop software (Adobe Systems, San Jose, CA).

For studies of GFP expression and co-localization with other markers, samples were fixed in 4% paraformaldehyde in PBS. Images of whole mount trachea and lung parenchyma were acquired with an inverted Olympus microscope (Olympus, Lake Success, NY). For sections, samples were rinsed in PBS, cryoprotected in 30% sucrose in PBS, and infiltrated with a 2:1 mixture of 30% sucrose-OCT, before freezing in OCT. Cryosections were cut at 8 µm, loaded onto silanized slides, and dried at room temperature before storage at –80°C. Colocalization for GFP expression and specific cellular markers was performed by immunohistochemistry. GFP (green) and Alexa Fluor 568 (red) fluorescence were visualized with the appropriate filter sets, and images were acquired with a Zeiss Axioplan 2 Imaging Universal Microscope (Zeiss, Göttingen, Germany) and an Axiocam MRm black and white digital camera (Axiovision Release 4.3; Zeiss) and processed with an Apotome Slider (Zeiss) for pseudoconfocal imaging.

1. Ciliated cells were identified by labeling for β-tubulin IV (1:50, MU178-UC mouse monoclonal antibody OS1A6, Biogenex [San Ramon, CA]; 1:200, Alexa Fluor 568–conjugated donkey anti-mouse IgG1; Molecular Probes [Eugene, OR]).

2. Clara cells were identified by labeling for *CCSP* (1:500, affinity purified goat anti-rat polyclonal antibody, gift from Dr. Barry Stripp; 1:200, Alexa Fluor 568–conjugated donkey anti-goat IgG; Molecular Probes).

3. Type II cells were identified by labeling for pro-surfactant protein (SP)-C (1:1,000, R09337 mono-specific, rabbit anti-mouse polyclonal

antibody, in house; 1:200, Alexa Fluor 568–conjugated goat anti-rabbit IgG; Molecular Probes).

4. Neuroendocrine cells were identified by labeling for CGRP (1:500, C8198 polyclonal rabbit anti-rat antibody; Sigma; 1:1,000; 1:200, Alexa Fluor 568–conjugated goat anti rabbit IgG; Molecular Probes).

RESULTS

Expression of *rtTA* under Control of the Rat *CCSP* Promoter

Distinct lines of transgenic mice were produced that express *rtTA* under control of the rat *CCSP* and human *SFTPC* gene promoters (16, 20). These mice were bred to tetO-CRE mice and either ZEG or ZAP mice (17, 18), creating triple transgenic mice (herein designated as *CCSPrtTA/tetOCRE/ZEG*, *CCSPrtTA/tetOCRE/ZAP*, *SPCrtTA/tetOCRE/ZEG*, *SPCrtTA/tetOCRE/ZAP*), in which respiratory epithelial cells permanently expressed either AP or GFP after doxycycline-induced recombination (Figure 1). These mice have been bred and maintained for more than 4 yr, with normal lung function and longevity in the vivarium. Sites of CRE expression and recombination in the peripheral lung under control of the *SFTPC* promoter have been described previously (21).

To identify the sites of *rtTA* expression in the *CCSP-rtTA* mouse, *in situ* hybridization for the reverse tetracycline activator mRNA was performed in mouse lung from E 13.5 to Postnatal Day (PN) 21 (Figure 2). No *rtTA* mRNA was found in lung epithelium before E 14.5. At E 14.5 and E 16.5, *rtTA* mRNA was confined to respiratory epithelial cells in the conducting airways (trachea and bronchi) (Figures 2A and 2B) and bronchioles. From E 18.5 to PN 21 *rtTA* mRNA was found in the conducting airways (Figures 2C–2H) and in a subset of peripheral epithelial cells, consistent with previous findings demonstrating the sites of expression of the rat *CCSP* promoter in type II epithelial cells of the adult mouse lung (22, 23). In the adult, *rtTA* mRNA was detected in epithelial cells adjacent to tracheal cartilage rings on the ventral side of both trachea and bronchi. In the dorsal epithelium of the trachea, *rtTA* mRNA appeared to be random and less frequently expressed (Figure 2I). This pattern of *rtTA* expression, and of subsequent recombination

Figure 1. Permanent cell labeling by controlled expression of Cre recombinase. The *rtTA* gene is driven by a cell-type specific promoter (rat *CCSP*) and followed by a SV40 polyadenylation site. In the presence of doxycycline (Dox), the transactivator (*rtTA*) recognizes its specific DNA target sequence (tetO). Expression of CRE causes recombination at loxP sites. In Z/AP and Z/EG reporter mice β-gal expression changes to alkaline phosphatase (AP) or enhanced green fluorescent protein (GFP). Visualization of AP or GFP expression indicates doxycycline-induced *CCSP*-dependent recombination in triple transgenic *CCSPrtTA/tetOCre/ZAP* and *CCSPrtTA/tetOCre/ZEG* mice.

Figure 2. Localization of *rtTA* mRNA in *CCSPrtTA* transgenic mice. Radioactive *in situ* hybridization was performed on paraffin sections of lung from *CCSP-rtTA* transgenic mice killed at E 14.5 (A), E 16.5 (B), E 18.5 (C), PN 5 (D), PN 7 (E), PN 10 (F), PN 14 (G), and PN 21 (H, I). The *CCSP* promoter drives *rtTA* expression in the bronchus and bronchioles as early as E 14.5 and in the alveolar region as early as E 18.5. In the trachea of adult mice, *rtTA* expression was detected primarily in epithelial cells on the ventral side of the trachea. V, ventral; D, dorsal.

observed in the *CCSPrtTA/tetOCRE* mice, was distinct from that observed in *SPCrtTA/tetOCRE* mice, in which recombination was similar on the dorsal and ventral side of the trachea and increased along the proximal to distal axis (21). *In situ* hybridization for *CCSP* mRNA was performed on adult mouse and rat lung tissue to locate cellular expression of endogenous *CCSP*. In contrast to the mouse, where *CCSP* mRNA expression was restricted to the conducting airways (25), rat *CCSP* was expressed in a subset of alveolar type II cells, as well as in the conducting airways (Figure 3). This finding indicates that the rat *CCSP* promoter directs transgene expression to cells of both the conducting airways and the alveolar epithelium in transgenic mice (22). Therefore, *rtTA* mRNA expression in the mouse is consistent with endogenous rat *CCSP* gene expression.

Mild but consistent enlargement of alveoli was noted in the lungs of all *CCSPrtTA* mice in the presence or absence of doxycycline or additional transgenes.

Conditional Recombination in the Postnatal Lung Controlled by *CCSPrtTA*

Exposure of the adult triple transgenic *CCSPrtTA/tetOCRE/ZAP* mice to doxycycline for 3 wk caused expression of AP in

the conducting airways and in a subset of type II epithelial cells in the lung periphery (Figures 4A and 4B), demonstrating the sites and extent of CRE-mediated recombination. In the absence of doxycycline, very few cells in the bronchioles of adult and 3-wk-old mice were labeled (Figure 4A). Doxycycline-independent recombination was never found in the alveolar region (21) (Figures 4A, 4E, and 4F). AP staining was never found in double transgenic *tetOCRE/ZAP* mice (Figures 4C and 4D). When triple transgenic mice were exposed to doxycycline from E 18.5 to PN 9, clusters of AP-labeled epithelial cells were observed lining the bronchioles (Figure 4G). After this time of prolonged doxycycline exposure, a subset of type II cells was labeled (Figure 5H), consistent with the timing and extent of expression of the *rtTA* mRNA as detected by *in situ* hybridization (Figure 2). Labeling of alveolar type I cells was not observed in the *CCSPrtTA/tetOCRE/ZAP* mice. In contrast, both alveolar type I and type II cells were extensively labeled in *SPCrtTA/tetOCRE/ZAP* mice. This finding suggests that in the *CCSPrtTA* transgenic mice this subset AP labeled of type II cells does not differentiate into type I cells and, therefore, represents a distinct subset of type II cells from those targeted in *SPCrtTA* mice (21).

Timing of Conditional Recombination in the Embryonic Respiratory Epithelium with the Rat *CCSP* Promoter

When the *CCSPrtTA* dams were exposed to doxycycline from E 6.5 and thereafter, labeling of conducting airway epithelial cells was first detected at E 14.5 (Figure 5). The numbers of labeled epithelial cells in the conducting airways increased between E 15.5–16.5 and thereafter. Most bronchiolar cells were labeled during this period of doxycycline exposure. Alveolar cells were first labeled at E 16.5 and thereafter (Figure 5), in contrast to findings in the *SPCrtTA* mice, where labeling of peripheral progenitor cells occurred very early in lung morphogenesis (21). Endogenous *CCSP* mRNA expression was detected in mouse bronchioles at E 16.5 (22), and protein expression was detected by E 17.5 (24). E 16.5 defines the end of the pseudoglandular stage, a time when specific cellular markers of the proximal and peripheral epithelium are increasingly expressed. Dams were exposed to doxycycline for 48-h periods to label progenitor cells at precise intervals (Figure 6). Lungs of triple transgenic

Figure 3. Localization of endogenous *CCSP* mRNA in rat and mouse lung. Radioactive *in situ* hybridization was performed on paraffin sections of lung from adult rat (A, C) and mouse (B). Endogenous *CCSP* mRNA expression was detected in the bronchus, bronchioles, and a subset of alveolar type II cells in the rat (A). In the mouse, *CCSP* mRNA was detected in conducting airways but not in the alveoli (B). Hybridization with the sense RNA is shown on rat tissue (C). Size bars = 200 μ m.

Figure 4. CCSPrTA mediated recombination in adult and E 18.5 to PN 9 mouse lung. Lung sections of adult (A, B) and PN21 (C–H) double and triple transgenic mice were analyzed for AP expression. In the absence of doxycycline, rare recombination was observed in lung sections of adult (arrows in A) and PN 21 (arrowhead in E, F) triple transgenic CCSPrTA/tetOCRE/ZAP mice. No AP-positive cells were found in the lung periphery (A, F). No AP-positive cells were detected in double transgenic control lungs (C, D). After 3 wk of doxycycline treatment, recombination was found in ciliated cells, Clara cells (arrows in B), and a subset of type II cells (arrowheads in B) in triple transgenic adult CCSPrTA/tetOCRE/ZAP mouse lung. After doxycycline exposure from E 18.5 to PN 9, most nonciliated and ciliated cells in the bronchioles were positive for AP staining in triple transgenic CCSPrTA/tetOCRE/ZAP PN 18 lungs. Cil, ciliated cells; Cla, Clara cells. Size bars: A, B = 50 μ m; C–H = 10 μ m.

CCSPrTA/tetOCRE/ZAP offspring were analyzed at E18.5. When exposed to doxycycline from E 12.5–E 14.5 (Figures 6A, 6D, and 6G), infrequent tracheal and bronchial cells were labeled, but no cells in the bronchioles or peripheral saccules were labeled.

Figure 5. Recombination in embryonic lungs of CCSPrTA/tetOCRE/ZAP mice after continuous exposure to doxycycline. Dams were treated with doxycycline from E 6.5 until killing. Mice were killed on E 14.5 (A), E 15.5 (B), E 16.5 (C), and at birth (D). Rare AP-positive cells were detected in conducting airways as early as E 14.5 (arrowhead), and in the lung parenchyma as early as E 16.5 (arrowhead). Numbers of labeled epithelial cells increased from E 14.5 until birth. Size bar = 50 μ m.

Between E 14.5 and E 16.5 (Figures 6B, 6E, and 6H), extensive labeling was observed in trachea, bronchi and bronchioles, and frequently in the peripheral saccules. Between E 16.5 and E 18.5 (Figures 6C, 6F, and 6I), recombination was detected in virtually all cells lining the bronchioles, and in a subset of epithelial cells in the lung periphery (Figure 6). In contrast to SPCrTA mice (21), thyroid and thymus were never labeled in the CCSPrTA mice.

Distinct Patterns of Recombination in Conducting Airways

ZEG mice, expressing GFP following recombination, were used to more precisely image the spatial organization of labeled epithelial cells in the lung. Dams were provided doxycycline from conception throughout pregnancy. Triple transgenic CCSPrTA/tetOCRE/ZEG and SPCrTA/tetOCRE/ZEG mice were analyzed on PN 7 (Figure 7). Continuous exposure of the dams to doxycycline caused a stereotypic pattern of recombination in subsets of conducting airway cells. CCSPrTA/tetOCRE/ZEG, GFP expressing cells were observed in both cephalic and caudal regions of the trachea. GFP was expressed in a horizontal banding pattern along the ventral side of the trachea. Epithelial cells overlying the cartilage rings were labeled, while GFP labeling was rare in inter-cartilaginous regions (Figure 7A). This pattern of discontinuous labeling was observed from the larynx to the end of the cartilaginous region in the main stem and lobar bronchi. On the membranous, or dorsal, side of the trachea, GFP-labeled cells were distributed randomly (Figures 7C, 7E, 7G, 8A, and 8B). The same dorsal ventral pattern was found by *in situ* hybridization for *rtTA* mRNA in the trachea (Figure 21). The pattern of labeling in the CCSPrTA/tetOCRE/ZEG was distinct from that observed in SPCrTA/tetOCRE/ZEG mice, in which epithelial

Figure 6. Timing of recombination in the prenatal period. Dams were treated with doxycycline for 48-h periods. AP staining was assessed in triple transgenic *CCSPrtTA/tetOCRE/ZAP* lungs at E 18.5. When exposed to doxycycline from E 12.5–14.5, few cells were labeled in the bronchioles (A, D, G). After exposure from E 14.5–16.5, extensive labeling was observed in bronchioles and bronchiolar-alveolar portals, with a subset of cells labeled in peripheral saccules (B, E, H). After exposure from E 16.5–18.5, recombination was detected in most cells in the proximal and terminal bronchioles, but was rarely detected in peripheral lung saccules (C, F, I). Labeling was not detected in thymus or thyroid. Size bar = 100 μ m.

cells of the trachea were labeled with increasing frequency in a proximal to distal gradient. Distal trachea and mainstem bronchi of *SPCrtTA/tetOCRE/ZEG* mice contained labeled cells forming longitudinal stripes that were similar on both dorsal and ventral surfaces (Figures 7D, 7F, and 7H).

Conditional Labeling in the Peripheral Lung

After continuous doxycycline treatment during embryonic development, GFP-positive cells lined small airways and bronchioles of both *CCSPrtTA/tetOCRE/ZEG* and *SPCrtTA/tetOCRE/ZEG* lungs, and GFP-positive cells were found in the alveolar regions (Figures 7I, 7J, 9A, and 9B). GFP-positive alveolar type I and type II cells were found in *SPCrtTA/tetOCRE/ZEG* lungs. GFP-positive alveolar type II cells, but not type I cells, were found in the lungs of *CCSPrtTA/tetOCRE/ZEG* mice.

Correlations between Airway Epithelial Differentiation and Recombination

GFP expression in the tracheal epithelium of *CCSPrtTA/tetOCRE/ZEG* mice was correlated with sites of immunohistochemical staining for β -tubulin (ciliated cells), CCSP (Clara cells), pro-SP-C (alveolar type II cells), and CGRP (neuroepithelial cells). Ciliated cells overlying tracheal cartilage on the ventral surfaces of the trachea and bronchi expressed GFP. In contrast, ciliated cells on the dorsal side of the trachea or in the bronchioles were GFP-negative (Figures 8C, 8D, and 9C). Since the CCSP promoter is not active in ciliated cells, these findings suggest that expression of the rat *CCSPrtTA* transgene occurred in progenitor cells that were labeled before ciliated cell differentiation. Exposure of the dams to doxycycline for 48-h periods, starting at E 12.5, E 14.5, or E 16.5, revealed that recombination

in the tracheal epithelium occurred during doxycycline exposure from E 12.5 to E 14.5 and did not occur when the dam was exposed to doxycycline after E 16.5 (data not shown). Since targeting of tracheal epithelial cells occurred early in development, nontargeted ciliated cells are likely derived from progenitor cells that are distinct from those of GFP-positive ciliated cells on the ventral side of the trachea. Surprisingly, epithelial cells expressing endogenous mouse CCSP in the adult trachea did not express GFP. Thus, the rat *CCSP* promoter induced recombination in a subset of cells that have differentiated later and no longer express endogenous CCSP. Furthermore, differentiated CCSP-expressing cells in the trachea of postnatal mice are not susceptible to recombination.

Some, but not all bronchiolar epithelial cells co-expressed CCSP and GFP, indicating that the rat *CCSP* promoter targeted a subset of bronchiolar Clara cells in this transgenic mouse line (Figure 9D). In the peripheral lung, GFP was colocalized with pro-SP-C in a subset of alveolar type II cells (Figure 9E). This site of recombination is consistent with the expression of the *rtTA* mRNA under the control of the rat *CCSPrtTA* promoter and with expression of the endogenous *CCSP* gene in the rat. In both *CCSPrtTA* and *SPCrtTA* transgenic mice (21), GFP was never detected in CGRP-reactive cells, indicating that CCSP lineage did not overlap with that of the neuroepithelial cells identified by CGRP (Figure 9F). Cells of the tracheal glands (Figure 8B), which are located at the proximal end of the trachea, were not labeled by expression of doxycycline-induced CRE under the control of either the rat *CCSP* or human *SPC* promoter.

DISCUSSION

The *CCSPrtTA* and *SPCrtTA* transgenic lines have been used to conditionally express or delete expression of various genes from the developing and adult respiratory epithelium (5, 7, 11, 26, 27). By using either the *SPCrtTA* or *CCSPrtTA* transgenic mice, genes can be readily activated or inactivated at specific times during development. The sites and extent of gene activation or deletion can be influenced by the promoter, as well as timing of exposure to doxycycline. The pattern of *rtTA* expression controls time-dependent CRE activation and recombination that is useful for inducing gene addition or deletion in defined subsets of cells at specific times. The *CCSP rtTA* conditional system is particularly useful for alteration of gene expression in the conducting airways and for conditional regulation of genes that may be lethal if altered before birth. The present study compares the sites and timing of recombination, using the *CCSPrtTA* inducible system with previous studies using the *SPCrtTA*-inducible system (21). Because these mice are used by many investigators, knowledge of the utility and limitations of the models should be useful in the interpretation of experiments designed to discern gene function using this system.

Timing of Gene Expression and Recombination in *CCSPrtTA* versus *SPCrtTA*

CRE-mediated *SPCrtTA*-controlled recombination occurs before formation of definitive lung buds and mediates recombination throughout the intrapulmonary respiratory epithelium (21). In contrast, recombination with the *CCSPrtTA* transgene occurs after E 14.5 and targets subsets of epithelial cells, which are distinct from those targeted in the *SPCrtTA* mice. Since gene addition or deletion with the *SPCrtTA* line may occur throughout the epithelium, gene alterations may limit perinatal survival. The *CCSPrtTA* system targets subsets of epithelial cells later in lung morphogenesis and can be used to bypass potentially lethal gene effects. In the postnatal period, recombination with the

Figure 7. Pattern of recombination from the trachea to the alveolar epithelium. Dams were treated with doxycycline from E 6.5 to PN 7. Tracheas of triple transgenic *CCSPrtTA/tetOCRE/ZEG* (A, C, E, G, I, K) and *SPCrtTA/tetOCRE/ZEG* (B, D, F, H, J, L) mice were visualized using an inverted microscope with fluorescence optics. Whole mount of the proximal trachea is shown in A and B. The main stem bronchi are shown in C and D. Inserts in A and C demonstrate the ventral (A) and dorsal (C) aspects of the trachea at 4× higher magnification. In *CCSPrtTA/tetOCRE/ZEG* tracheas, labeled cells were present in a dorsal–ventral pattern with increased density of labeled cells overlaying the cartilage rings. In the *SPCrtTA/tetOCRE/ZEG* tracheas, labeled cells formed longitudinal stripes with increasing numbers of labeled cells observed from the proximal to distal region. In the mainstem bronchi (E, G), labeled cells were found in a random pattern in the lungs of *CCSPrtTA/tetOCRE/ZEG* mice. Longitudinal stripes were observed in the bronchi of *SPCrtTA/tetOCRE/ZEG* mice (F, H). Recombination was frequent in epithelial cells of the bronchioles, as well as in the alveoli of both transgenic lines (I–L). Size bars: A, B = 400 μm; E, F = 500 μm; G, H, I, J = 250 μm; K, L = 400 μm. Note: autofluorescence can be seen in all tissue. V, ventral; D, dorsal.

tetOCRE/ZEG tracheas, labeled cells formed longitudinal stripes with increasing numbers of labeled cells observed from the proximal to distal region. In the mainstem bronchi (E, G), labeled cells were found in a random pattern in the lungs of *CCSPrtTA/tetOCRE/ZEG* mice. Longitudinal stripes were observed in the bronchi of *SPCrtTA/tetOCRE/ZEG* mice (F, H). Recombination was frequent in epithelial cells of the bronchioles, as well as in the alveoli of both transgenic lines (I–L). Size bars: A, B = 400 μm; E, F = 500 μm; G, H, I, J = 250 μm; K, L = 400 μm. Note: autofluorescence can be seen in all tissue. V, ventral; D, dorsal.

CCSPrtTA line occurs more frequently than with the *SPCrtTA* mouse line (21), making the *CCSPrtTA* line more suitable for studies in which recombination is used to study the adult lung.

Cellular Localization of Recombination

Consistent and reproducible labeling patterns were found in the trachea and lung with both *SPCrtTA/tetOCRE* and *CCSPrtTA/tetOCRE* recombination systems. Analysis of both models indicated that extensive and widespread random migration or “shuffling” of cells does not occur during lung morphogenesis. In the *SPCrtTA* transgenic mice, exposure to doxycycline before E 9–10 targets only a few precursor cells that results in labeling of virtually all intrapulmonary epithelial cells (21). Thus, all intraparenchymal respiratory epithelial cells, except the neuroepithelial cells, are derived from a pool of common endodermal precursor cells identified by recombination with the SP-C promoter.

A rare subset of tracheal epithelial cells can be targeted with the *SPCrtTA* system in early lung development, providing a useful model for studying paracrine signaling between epithelial and mesenchymal cells of the trachea. For example, expression of *Fgf18* (28) or deletion of *Shh* (7) in the tracheal epithelium from E 11.5–12.5 using the *SPCrtTA* system, resulted in aberrant cartilage ring formation in the trachea close to the laryngeal region. Later in development, *SPCrtTA*-induced gene expression or deletion in the respiratory epithelium adjacent to cartilaginous structures had no effect on cartilage ring formation, indicating that tracheal epithelial cells influence the sites and extent of tracheal-bronchial cartilage formation only during early developmental stages. Likewise, the labeling pattern of distinct subsets of related cells identified by *CCSPrtTA*-induced recombination supports the concept that cartilage-related cells provide paracrine signals that influence cell type patterning along the ventral side of the trachea and bronchi. Most GFP-positive cells were found ventrally, overlying the cartilage rings. Previous scanning electron microscopy studies of the rat trachea demonstrate that

ciliated cells are concentrated in the tracheal ligament between cartilage rings, which may represent specialized regions of active mucous clearance (29). In the mouse, ciliated cells in the tracheal ligament were not extensively labeled using the *CCSPrtTA* mouse line, which suggests that the progenitor cells of these specialized ciliated cells were not targeted with the *CCSPrtTA* system. A subset of GFP-positive tracheal cells did not express the differentiation markers β -tubulin or CCSP, and may represent cells that have the potential for self renewal after injury, as described by Plopper and coworkers (30). In the bronchioles, a subset of CCSP-positive cells were also GFP-positive. Their random and scattered location, however, makes them distinct from the label retaining subset of Clara Cells found in association with neuroepithelial bodies in the airway (31). In addition, the rat CCSP promoter directed gene expression to a subset of Clara cells in contrast to the murine CCSP promoter, which labeled all of the Clara cells (31). Whether this subset of GFP-positive Clara Cells serves as a source of progenitor cells in the airway remains to be investigated.

Sites of *rtTA* Expression and Recombination Do Not Correlate in Adult Lung

In the rat, endogenous CCSP is expressed in a subset of alveolar type II cells, whereas in the mouse lung CCSP expression is restricted to Clara cells in the conducting airways. Transgenes driven by the rat *CCSP* promoter in the mouse were expressed in both conducting airways and in alveolar type II cells. In the postnatal period, *rtTA* expression directed by the rat *CCSP* promoter was detected at high frequency on the ventral side of the trachea. In the postnatal period recombination did not occur in Clara cells of the conducting airways, even though these cells do express endogenous CCSP. Thus, in the postnatal lung, rat *CCSP* promoter-driven *rtTA* expression and CRE-mediated recombination do not entirely overlap with sites and extent of endogenous mouse *CCSP* expression, indicating that either

Figure 8. Colocalization of GFP with β -tubulin and endogenous CCSP in the trachea. Dams were treated with doxycycline from E 6.5 to PN 7. Frozen sections of trachea (A, B) of triple transgenic *CCSPrtTA/tetOCRE/ZEG* mice were visualized with an upright microscope using fluorescence optics. Numerous GFP-positive cells were detected all along the ventral or cartilaginous side of the trachea (arrows in A) from the larynx to the mainstem bronchi (A, B). Tracheal glands lacked GFP staining (arrows in B). Frozen sections of *CCSPrtTA/tetOCRE/ZEG* trachea were stained for β -tubulin (C, D; red) or CCSP (E, F; red) and visualized for dual fluorescence with GFP (green). Ciliated cells on the dorsal side of the trachea were predominantly GFP-negative (C, arrows). The majority of ciliated cells (β -tubulin) on the ventral side of the trachea were labeled with GFP (D, arrow), although some ciliated cells were GFP-negative (C, D; arrowhead). CCSP did not colocalize with GFP anywhere in the trachea (E, F; arrowhead). Note: The yellow color on F results from close proximity of the red CCSP signal and the green GFP signal, but does not reflect colocalization. Arrows in all panels show GFP-negative cells that express differentiation markers; arrowheads demonstrate colocalization. Size bars: A, B = 500 μ m; C, E = 200 μ m; D, F = 50 μ m.

labeling occurs in precursor cells that differentiate into various cell types or that labeling occurs only in a subset of differentiated cells. Similarly in the alveolar region of the adult lung, only a subset of alveolar type II cells, which expresses CRE controlled by *CCSPrtTA* or *SPCrtTA*, undergoes recombination. In contrast to the paucity of doxycycline-induced recombination observed in type II cells in adult *SPCrtTA* mice, recombination occurred frequently in the adult *CCSPrtTA* mouse lung. Furthermore, labeling in alveolar type I cells was observed in *SPCrtTA* but not *CCSPrtTA* transgenic mice. Based on these results, we postulate that the *CCSPrtTA* and the *SPCrtTA* transgenes target distinct subsets of alveolar type II cells. The present *SPCrtTA* and *CCSPrtTA* systems are being widely used; therefore, characterization of the sites and timing of expression will be useful for design and interpretation of experiments. Because the sites and extent of gene expression can be controlled by the timing and duration of doxycycline treatment, the *SPCrtTA* and *CCSPrtTA*

Figure 9. Colocalization of GFP with epithelial cell markers in the peripheral lung. Dams were treated with doxycycline from E 6.5 to PN 7. Most bronchiolar cells (A) and some alveolar type II cells (B) expressed GFP. At PN 7 frozen sections of *CCSPrtTA/tetOCRE/ZEG* lungs were stained for β -tubulin, CCSP, pro-SP-C, or CGRP (red) and visualized for dual fluorescence with GFP (green). Some ciliated (β -tubulin-positive) cells expressed GFP (arrowhead in C). In the bronchioles, some Clara cells (CCSP-positive) expressed GFP (arrowhead in D), although not all nonciliated, GFP-expressing cells expressed CCSP (arrows in D). A subset of alveolar type II cells (pro-SP-C) expressed GFP (arrowhead in E). GFP was not detected in squamous type I epithelial cells (E). GFP expression (green) was not colocalized with CGRP (arrows in F). Note: arrows in all panels show GFP-negative cells that do express differentiation markers; arrowheads show colocalization. Size bars: A = 500 μ m; B = 125 μ m; C–F (left) = 50 μ m; C–F (right) = 10 μ m.

systems can be used to control differential gene-expression in subsets of lung epithelial cells. *CCSPrtTA* and *SPCrtTA*, when used with *tetOCRE* and other Tet operator-driven genes have been useful for conditional control of gene expression or for permanently altering gene expression in various subsets of respiratory epithelial cells in both fetal and postnatal mice. Moreover, *SPCrtTA* and *CCSPrtTA* transgenic mice can be used to manipulate gene expression during well-defined periods of development to target subsets of alveolar progenitor cells by the administration of doxycycline.

The present labeling experiments demonstrated an unexpected diversity of labeling and gene expression amongst morphologically indistinguishable cells. In the trachea, distinct dorsal-ventral and horizontal patterning of labeled cells was observed in the *CCSPrtTA* transgenic mouse line, while a proximal-distal gradient of longitudinal stripes was observed in the *SPCrtTA* transgenic mice (21). In the lung parenchyma, subpopulations of both ciliated and Clara cells were identified that were not directly lineage related. Likewise, sites and extent of cell types labeled with the *CCSPrtTA* or the *SPCrtTA* system were distinct. We

conclude that the *CCSPrtTA* system is most useful for targeting gene expression or deletion later in development and in the adult lung. The *SPCrtTA* system is well suited for alterations of gene expression during embryonic lung development.

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