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ORIGINAL RESEARCH

Efficiency and Specificity of Gene Deletion in Lung Epithelial Doxycycline-Inducible Cre Mice

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Abstract

The transgenic mouse strains surfactant protein C–reverse tetracycline transactivator (SP-C-rtTA), club cell secretory protein (CCSP)-rtTA, and tetracycline operator (TetO)-Cre have been invaluable for spatiotemporally regulating gene deletion in the pulmonary epithelium. In this study, we measured the efficiency and specificity of gene deletion that can be achieved in these mice using the Rosa26-eYFP reporter. Triple-transgenic mice (tTg or rtTA/TetO-Cre/Rosa-eYFP) were bred and treated with various doxycycline (dox) regimens to induce gene deletion, which was then quantified in various cell populations by flow cytometry. In these crosses, we found that the TetO-Cre transgene must be transmitted through the female parent to avoid germline gene deletion. With dox exposure during lung development, SP-C-tTg mice deleted in ∼65–75% of alveolar epithelial type II (ATII) cells, but in only ∼45–50% of the integrin $\beta4^+$ population, which consisted of club cells and distal lung progenitor cells. In contrast, CCSP-tTg mice deleted in ∼50% of ATII cells and ∼80% of integrin $\beta4^+$ cells. Upon dox treatment of adults, deletion in ATII cells and integrin $\beta4^+$ cells in SP-C-tTg mice dropped significantly to ∼20% and ∼6%, respectively, whereas CCSP-tTg mice deleted in ∼57% of ATII and ∼40% of integrin $\beta4^+$ cells. Interestingly, untreated CCSP-tTg mice also deleted in ∼40% of integrin $\beta4^+$ cells, indicating significant leakiness of CCSP-tTg in $\beta4^+$ cells. In all mouse groups, minimal deletion occurred in mouse tracheal epithelial cells or in mesenchymal or hematopoietic cells. These data provide the first quantitative, side-by-side comparison of the deletion efficiency for these widely used transgenic mouse strains.

Keywords: lung epithelial cells; gene deletion; doxycycline; TetO-Cre; rtTA

Clinical Relevance

The study of lung epithelial biology has benefited from the use of transgenic mouse strains that allow inducible cell-specific deletion. In this study, we accurately quantified the efficiency, specificity, and inducibility of gene deletion that is achievable with these widely used strains. As such, this report serves as a guide for using and interpreting studies that utilize these transgenic mouse strains.

Lung epithelial cells play important roles in gas exchange, maintaining the fluid balance in the distal airways, and coordinating the tissue response to immune challenges. In the conducting airways, club cells are responsible for generation of the mucus layer as well as production of antimicrobial peptides, both of which provide the first line of defense against pathogen infection (1, 2). In the alveoli, alveolar epithelial type II (ATII) cells produce surfactants and are the major source of chemokines and chemoattractants for the coordination of pulmonary immune responses (3). The study of any given protein or functional response of club and ATII cells has been dramatically enhanced by the ability to direct specific genetic mutations to these cell types in mouse models.

To target genes specifically in lung epithelial cells *in vivo*, surfactant protein C–reverse tetracycline transactivator (SP-C-rtTA), club cell secretory protein (CCSP)-rtTA, and tetracycline operator (TetO)-Cre mice were generated ∼15 years ago (4, 5). The SP-C-rtTA transgene drives

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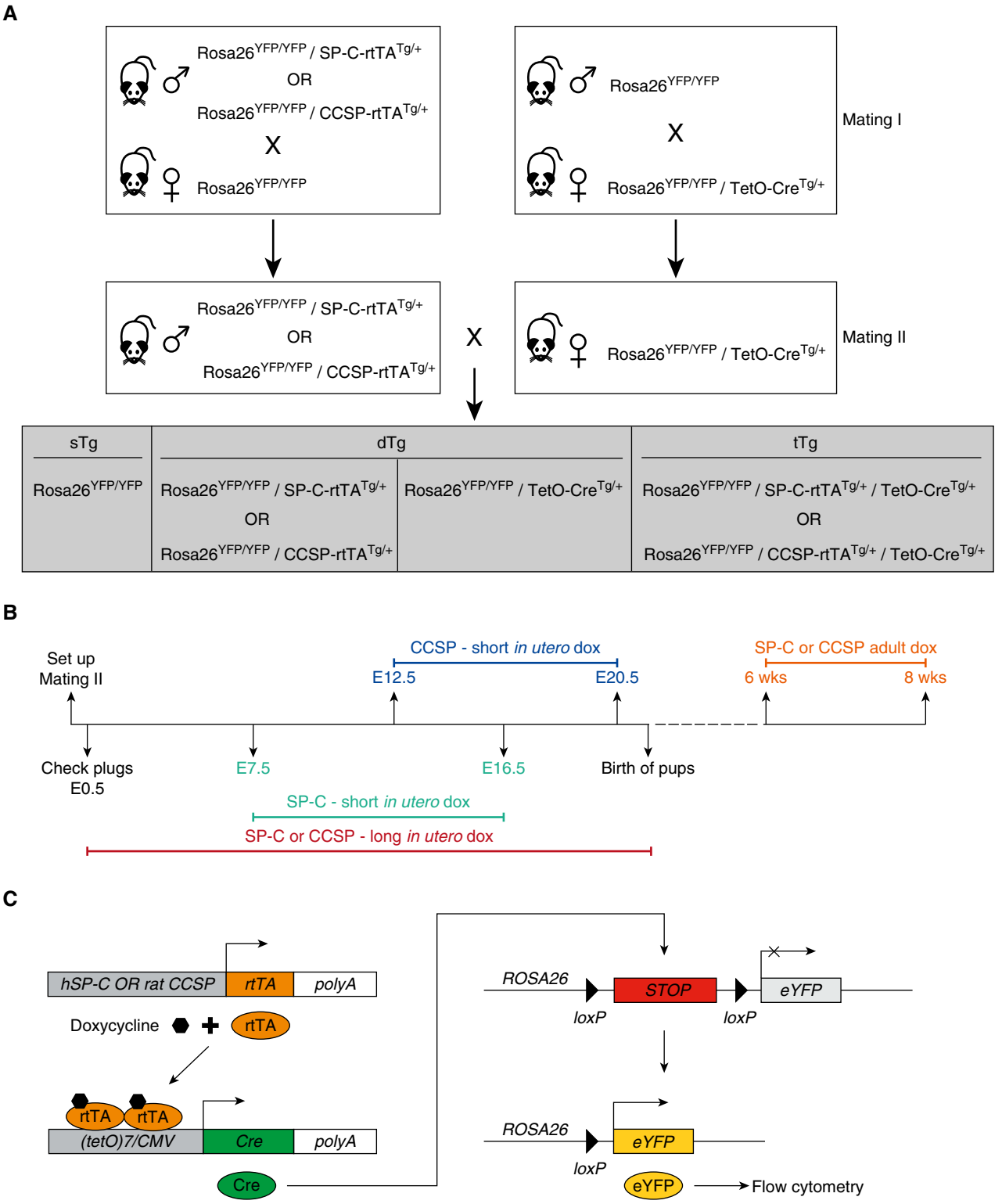

Figure 1. Breeding scheme, doxycycline (dox) treatment regimen, and mechanism of gene deletion in lung epithelial cell–specific, dox-inducible Cre deleter mice. Surfactant protein C–reverse tetracycline transactivator (SP-C-rtTA), club cell secretory protein (CCSP)-rtTA, and tetracycline operator (TetO)-Cre mice were crossed to Rosa26$^{YFP/YFP}$ single-transgenic (sTg) mice to generate Rosa26$^{YFP/YFP}$/SP-C-rtTA$^{Tg/+}$, Rosa26$^{YFP/YFP}$/CCSP-rtTA$^{Tg/+}$, and Rosa26$^{YFP/YFP}$/TetO-Cre$^{Tg/+}$ double-transgenic (dTg) mice, respectively. To avoid widespread dox-independent gene deletion, these dTg mouse strains were maintained by using a breeding scheme (*A*, mating I) in which the rtTA was always transmitted by the male and the TetO-Cre was of female origin. To generate triple-transgenic (tTg) mice, dTg mice were bred together (*A*, mating II), again making sure that the TetO-Cre was present in the female

expression of the rtTA from a 3.7 kb fragment of the human SP-C gene promoter. When bred to the TetO-Cre transgene–carrying mouse strain and treated with doxycycline (dox), *loxP* flanked ("floxed") genes of interest are deleted during lung development *in utero* and in adult mice (6, 7). The CCSP-rtTA line drives rtTA expression from a 2.3 kb fragment of the rat CCSP gene promoter, which allows deletion in club cells as well as in ATII cells (4). These mice have been used extensively by the pulmonary sciences community, mostly in applications involving developmental biology, cancer research, immunology, and inflammation. Because these transgenic mice allow for spatiotemporal gene regulation in lung epithelial cells, they have proven especially useful in scenarios where whole-body gene knockout leads to lethality or complex systemic phenotypes. However, the use of these strains is not without accompanying complications, which include leaky (i.e., non–dox-induced) expression of Cre and issues with rtTA toxicity or off-target dox toxicity (8–10). Perhaps more limiting is the efficiency of deletion for any given floxed gene using either of these strains. Optimizing the duration of dox exposure during lung development has been suggested to help decrease potential toxicity as well as improve gene deletion frequency (11).

Although much is known about the characteristics, advantages, and limitations of the SP-C-rtTA, CCSP-rtTA, and TetO-Cre systems, no reports have directly compared the efficiency and specificity of gene deletion, using a quantitative method, that can be achieved with these systems. Thus, in the course of our studies of immune signaling molecule expression in lung epithelium (12), we realized that we needed a better assessment of the utility of these Cre deleter strains to help with interpretation of our findings. To facilitate that, we crossed SP-C-rtTA, CCSP-rtTA, and TetO-Cre mice to the Rosa26-enhanced yellow fluorescent protein (eYFP) reporter strain, which is one of the mostly widely used reporter strains for lineage-marking studies (13). In these mice, cells and their descendants are permanently marked eYFP positive after Cre activity. Here, we report the deletion efficiency, specificity, and dox inducibility achieved in these triple-transgenic (tTg) mice during lung development and in adulthood using different dox treatment regimens.

Materials and Methods

Mice and Genotyping

Rosa26-eYFP (Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$) mice (14) (referred to as Rosa26$^{YFP/YFP}$ from here on) were obtained from The Jackson Laboratory (Sacramento, CA). SP-C-rtTA (line 2), CCSP-rtTA (line 2), and TetO-Cre mice were received from Dr. Jeffrey Whitsett. All mouse strains were backcrossed 15 generations to the C57BL/6 background, maintained in a specific pathogen-free facility at the University of California, San Francisco (UCSF), and used according to protocols approved by the UCSF Institutional Animal Care and Use Committee.

Genotyping for human SP-C-rtTA, rat CCSP-rtTA, and TetO-Cre transgenes (4, 5), as well as the Rosa26$^{YFP/YFP}$ locus (15), was performed as described earlier. Please refer to the online supplement for the reagents and antibodies used.

Mouse Breeding and Dox Treatment

SP-C-rtTA, CCSP-rtTA, and TetO-Cre mice were bred with Rosa26$^{YFP/YFP}$ single-transgenic (sTg) mice to generate Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA and Rosa26$^{YFP/YFP}$/TetO-Cre double-transgenic (dTg) mice (Figure 1A, mating I). Thereafter, Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA male mice were crossed to Rosa26$^{YFP/YFP}$/TetO-Cre female dTg mice to generate Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA/TetO-Cre tTg mice (Figure 1A, mating II). To avoid germ-line deletions, TetO-Cre was maintained in the female for all of the matings (I and II). The mating strategy also kept the transgene copy numbers the same across breedings.

To induce gene deletion during lung development, pregnant females (mating II) were dox treated from embryonic day 0.5 (E0.5) until the birth of pups (long *in utero* dox) or for shorter time durations (short *in utero* dox, Figure 1B) based on the SP-C and CCSP promoter activities described previously (4).

For adult gene deletion, mice were dox treated from 6 to 8 wk of age (adult dox, Figure 1B). To determine dox-independent gene deletion, i.e., promoter leakiness, mice were not treated. Mice were killed between 10 and 12 wk for eYFP expression analyses.

Dox (Alfa Aesar, Haverhill, MA) was reconstituted in cell-culture–grade DMSO (Sigma-Aldrich, St. Louis, MO) at 100 mg/ml, aliquoted, and stored at −20°C. Dox was diluted to 1 mg/ml in autoclaved 5% sucrose-supplemented drinking water, administered to mice in foil-wrapped water bottles, and changed every other day.

Dispase Digestion of Lungs and Trachea

Lungs and trachea from each mouse were digested into a single-cell suspension using dispase and DNase as described previously (12, 16). More details are provided in the online supplement.

Flow-Cytometric Staining and Analysis

For lung and tracheal cells, lineage markers were stained using a cocktail of biotinylated antibodies (anti-CD45, anti-CD16/32, anti-Ter119, and anti-CD31), which included anti-integrin β4 only for lung cells, followed by staining with streptavidin-Pacific Orange. All epithelial cells were marked with anti-epithelial cell adhesion molecule (EpCAM)-allophycocyanin (APC) (12, 16). Blood, spleen, and bone marrow single cells were Fc

Figure 1. (Continued). breeder mouse. (*B*) To induce gene deletion in tTg mice during embryonic development, pregnant females were treated with 1 mg/ml dox in drinking water either for the entire duration of the pregnancy (long *in utero* dox) or for a shorter time (short *in utero*) during which the SP-C or CCSP promoters are known to be most active. In some experiments, gene deletion was induced in adult tTg mice by treating them with 1 mg/ml dox in drinking water from 6 to 8 wk of age (*B*). To assess dox-independent gene deletion (leak), some mice were not treated with dox at all. (*C*) In tTg mice, the rtTA protein, the expression of which is driven by either human SP-C or rat CCSP promoters, binds the (tetO)7/CMV promoter in the presence of dox. This in turn drives expression of the Cre recombinase protein, which deletes the *lox P* flanked STOP sequence upstream of the eYFP gene, leading to stable expression of eYFP protein, which is then detected and quantitated by flow cytometry to assess deletion efficiency and specificity.

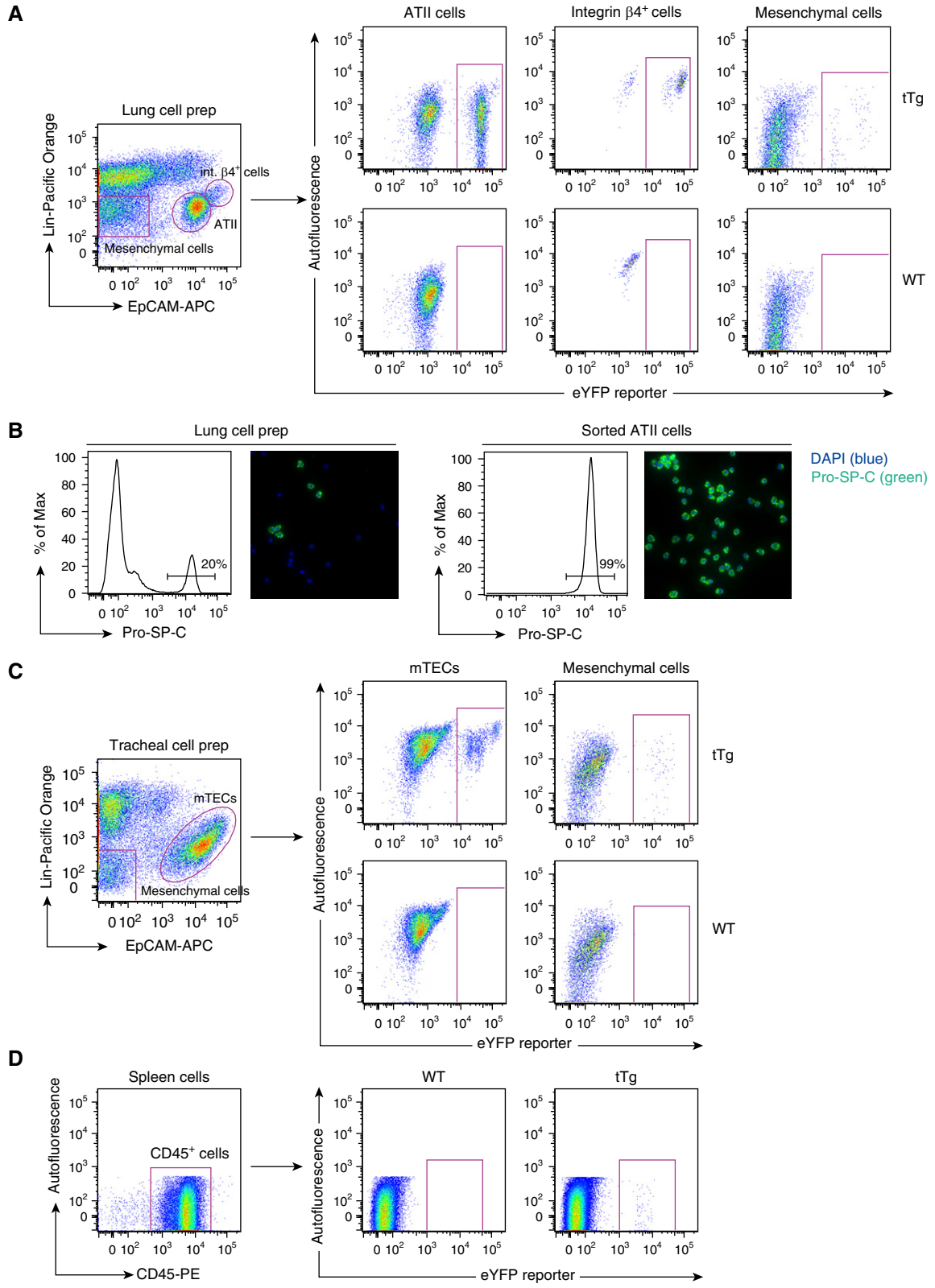

Figure 2. Flow cytometric cell gating strategy for analysis of gene deletion efficiency and specificity. Triple-transgenic mice (tTg or Rosa26$^{YFP/YFP}$/ SP-C-(or CCSP)-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$) were bred and treated with dox as outlined in Materials and Methods and in Fig. 1. Dispase-digested single-cell suspensions of lungs (*A*) and trachea (*C*) from wild-type (WT) or dox-treated tTg mice were stained for EpCAM, a pan-epithelial marker, along with several lineage markers. The lineage markers CD45 and CD16/32 were used for hematopoietic cells, CD31 for endothelial cells, and Ter119 was used for erythroid

blocked and then stained using anti-mouse CD45-PE. Dead cells were excluded by propidium iodide. Data were acquired using an LSRFortessa (BD Biosciences) with FACSDiva software (BD Biosciences) and analyzed by FlowJo (TreeStar, Ashland, OR).

Statistical Analysis

Data are displayed as mean ± SD. ANOVA with Tukey's *post hoc* test was performed using Prism software, Windows version 6.07 (GraphPad, La Jolla, CA), to statistically analyze the significance of the difference between multiple groups. $P < 0.05$ (*) was considered significant. $P < 0.01$ is represented as (**), $P < 0.001$ is represented as (***), and not significant is shown as (ns).

Results

Breeding Scheme, Dox Treatment Regimen, and Mechanism of Gene Deletion

To quantify the gene deletion efficiency that can be achieved in lung epithelial cells using lung epithelial cell–specific, dox-inducible Cre deleter mice, both the activator (SP-C-rtTA and CCSP-rtTA) and operator (TetO-Cre) sTg lines were crossed to Rosa26$^{YFP/YFP}$ reporter sTg mice to generate dTg mice as shown in mating I, Figure 1A. To generate tTg mice, dTg mice were bred as shown in mating II, Figure 1A. This resulted in the tTg mice Rosa26$^{YFP/YFP}$/SP-C-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (referred to as SP-C-tTg) and Rosa26$^{YFP/YFP}$/CCSP-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (referred to as CCSP-tTg). Importantly, because we noticed a very high frequency of litters that were reporter positive (with 98–99% peripheral blood leukocytes being eYFP fluorescent; data not shown) in a dox- and genotype-independent manner when the TetO-Cre gene was transmitted via the male, we routinely maintained the TetO-Cre transgene in the female parent in all breedings. In addition, the activator and operator lines were never bred to homozygosity.

To delete genes during lung development, pregnant female mice were treated either from E0.5, which was determined by checking for vaginal plugs, until the birth of pups (long *in utero* dox for both SP-C and CCSP mice) or from E7.5 to E16.5 (short *in utero* dox for SP-C mice) or from E12.5 to E20.5 (short *in utero* dox for CCSP mice) (Figure 1B). The shorter dox treatment timings were based on the transgenic SP-C and CCSP promoter activities reported by Perl and colleagues (4). To delete genes in adult mice, dox was administered to mice in drinking water from 6 to 8 wk of age. Additionally, to determine the levels of dox-independent gene deletion and promoter leakiness, some mice were left untreated.

The dox-inducible mechanism of gene deletion in these mice (Figure 1C) involves the binding of rtTA, which is produced constitutively under lung-specific promoters, to the *(TetO)7/CMV* promoter in the presence of dox to drive downstream expression of Cre recombinase, which then deletes the floxed transcription *STOP* sequence to permanently turn on eYFP reporter expression. However, this mechanism can exhibit "leakiness," i.e., low-level expression of Cre recombinase and gene deletion in the absence of dox treatment.

Flow Cytometric Gating Scheme

Single-cell suspensions of lung digests were stained for lineage (Lin) markers (CD45, C16/32, CD31, Ter119, and integrin β4) and EpCAM, a pan-epithelial marker (Figure 2A). As previously shown (12, 16), cells in the Lin$^-$ EpCAM$^+$ (or ATII) gate were routinely ∼99% positive for pro-SP-C, a specific marker for ATII cells (Figure 2B). Whereas the whole-lung, single-cell digest contained ∼20% pro-SP-C$^+$ cells (Figure 2B, *left panel*), cells sorted from the Lin$^-$ EpCAM$^+$ gate very reproducibly consisted of ∼99% pro-SP-C$^+$ cells (Figure 2B, *right panel*). Events in the integrin β4$^+$ gate, which were also high for EpCAM expression (EpCAM^{hi}), consisted of club cells and distal lung progenitor cells (12), whereas the Lin$^-$ EpCAM$^-$ gate consisted of mesenchymal cells (Figure 2A). Tracheal cell suspensions were stained similarly, except that they were not stained for integrin β4 (Figure 2C). The Lin$^-$ EpCAM$^+$ gate consisted of mouse tracheal epithelial cells (mTECs), whereas the Lin$^-$ EpCAM$^-$ dual-negative population consisted of tracheal mesenchymal cells. Single-cell suspensions from blood, spleen, and bone marrow were stained for CD45 and then gated on all CD45$^+$ cells to identify all hematopoietic cells (Figure 2D). To accurately record the deletion frequency in all populations, true eYFP fluorescence was distinguished from autofluorescence.

Deletion Efficiency in Lung Epithelial Cell Populations

For gene deletion in ATII cells, the highest efficiency, ∼74%, was observed in SP-C-tTg mice treated with dox *in utero* for the long duration, i.e., E0.5 to the birth of pups (Figure 3A). SP-C-tTg mice treated with dox *in utero* for the short duration deleted in ∼65% ATII cells (Figure 3A). In contrast, CCSP-tTg mice deleted in a significantly lower percentage of ATII cells, ∼52%, under both short and long dox treatment conditions (Figure 3A).

When dox was administered to adult mice, gene deletion in ATII cells significantly dropped to ∼20% in SP-C-tTg mice (Figure 3A). Interestingly, however, similarly treated CCSP-tTg mice did not display such a reduction in gene deletion efficiency in ATII cells, with ∼57% ATII cells being eYFP reporter positive. Untreated SP-C-tTg mice deleted in ∼5% ATII cells and untreated CCSP-tTg mice deleted in ∼2% ATII cells, indicating that neither promoter was significantly leaky in ATII cells. Further, no deletion was observed in ATII cells from any of the groups when the mice were sTg or dTg and lacked either one or both of

Figure 2. (Continued). cells. In lung-cell preparations, but not tracheal preparations, anti-integrin β4 antibody was also included in the lineage antibody cocktail to mark club cells and distal lung progenitor cells. (*B*) To confirm that the ATII gating strategy for lung cells as shown in (*A*) was reliable, lung single-cell preparations and cells sorted using the ATII gating strategy were stained intracellularly for pro-SP-C, a specific marker for ATII cells. Shown are histograms and cytospin pro-SP-C stains of the same. All hematopoietic cells were marked with CD45 staining in single-cell preparations from spleen (*D*) and blood and bone marrow (not shown). Events displayed in the dot plots are gated on live, singlet events. APC, allophycocyanin; ATII, alveolar epithelial type II; EpCAM, epithelial cell adhesion molecule; mTEC, mouse tracheal epithelial cells.

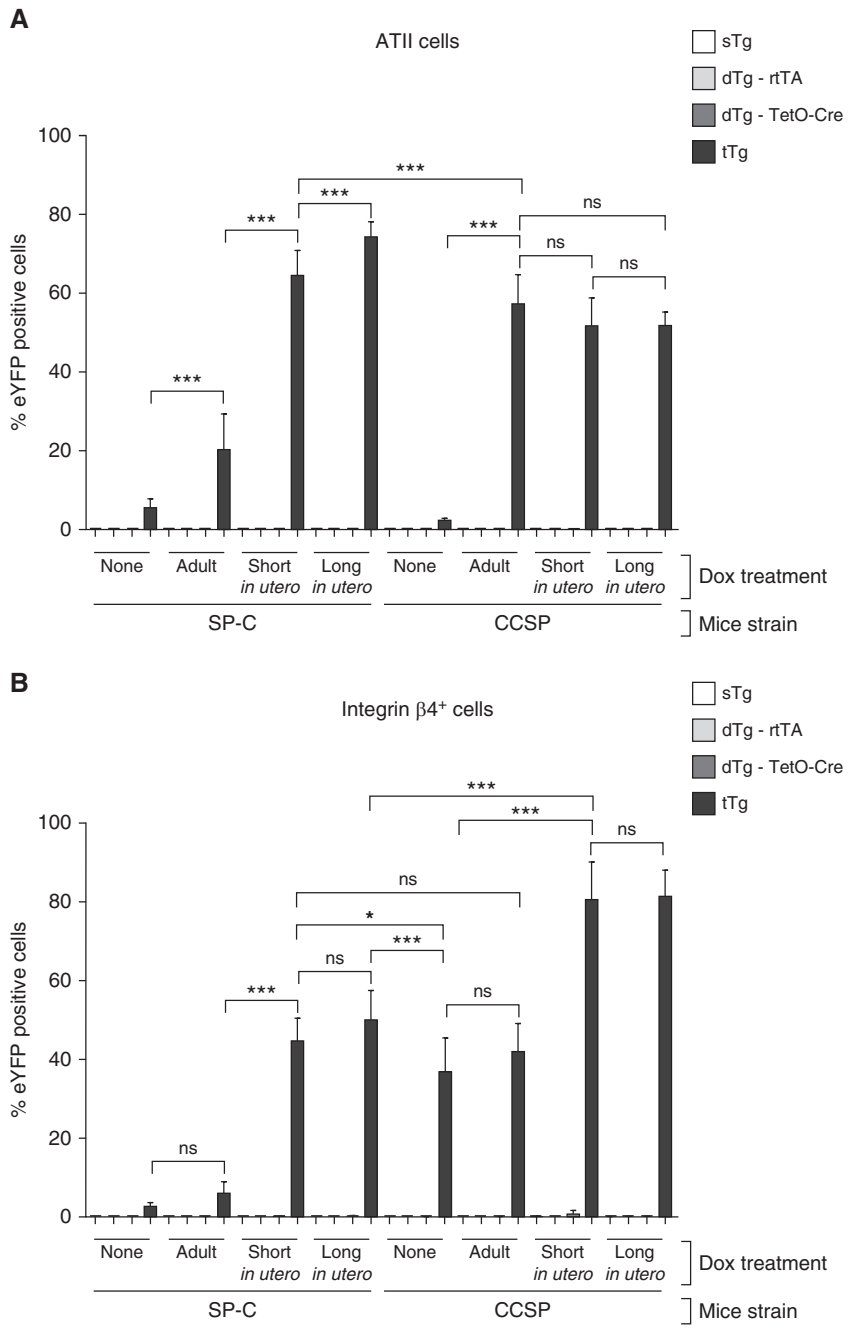

Figure 3. Efficiency of dox-induced gene deletion in lung epithelial cells. Shown are the percentages of eYFP reporter fluorescence–positive cells within populations of ATII cells (*A*) and integrin $\beta4^+$ EpCAM^{hi} epithelial cells (*B*) from dispase-digested lung single-cell preparations. The graphs represent data pooled from dox-treated or untreated mice generated in three to five separate breedings, as outlined in Materials and Methods, with each bar representing the mean ± SD of 5–13 mice. The genotypes of the mouse groups were as follows: Rosa26$^{YFP/YFP}$ (sTg), Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$ (dTg - rtTA), Rosa26$^{YFP/YFP}$/TetO-Cre$^{Tg/+}$ (dTg – TetO-Cre), and Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (tTg). *$P < 0.05$, ***$P < 0.001$; ns, not significant.

the activator (rtTA) and operator (TetO-Cre) elements (Figure 3A).

The deletion frequency in the EpCAM^{hi} integrin $\beta4^+$ gate, consisting of club cells and distal lung progenitor cells, was also quantified in the same mouse groups. A trend opposite to that seen with the ATII cells was observed, with CCSP-tTg mice deleting the most in the integrin $\beta4^+$ cell population, ~81%, when treated with dox *in utero* regardless of the treatment duration (Figure 3B). In SP-C-tTg mice, the deletion frequency in integrin $\beta4^+$ cell population was significantly lower, being ~45% and ~50% with short and long dox treatment, respectively. Upon treatment of adult mice with dox, SP-C-tTg mice deleted in only ~6% of EpCAM^{hi} integrin $\beta4^+$ cells, whereas CCSP-tTg mice deleted in ~42% of the same cell population. Interestingly, however, in both SP-C-tTg and CCSP-tTg mice, the adult dox deletion frequency was similar to that observed when the mice were not treated with dox at all (~3% for SP-C-tTg and ~37% for CCSP-tTg mice), indicating that the deletion observed in adults in the EpCAM^{hi} integrin $\beta4^+$ cells was due to promoter leakiness rather than dox treatment (Figure 3B). No deletion was observed in EpCAM^{hi} integrin $\beta4^+$ in any of the sTg or dTg mice from any of the groups (Figure 3B). Thus, SP-C-tTg mice are better suited to delete genes in ATII cells during *in utero* lung development, and CCSP-tTg mice are apt for deleting genes in ATII cells in adult mice and also in club cells and distal lung progenitor cells. However, at least half of the CCSP-tTg–driven *in utero* gene deletion in club and progenitor cells was constitutive (not regulated by dox).

Deletion Efficiency in mTECs

Deletion in mTECs was also assessed and was found to be significantly lower than that observed for either ATII cells or integrin $\beta4^+$ cells, regardless of the strain or dox treatment group (Figure 4). A deletion frequency of only ~2–3% was observed in SP-C-tTg mice that were either untreated or treated with dox as adults. When SP-C-tTg mice were treated with dox *in utero* for either the short or long duration, a higher deletion frequency of ~11% was observed. In the CCSP-tTg mice, deletion in mTECs was higher, being ~17–19% with or without dox treatment in any regimen. Regardless of the mouse group and dox treatment, a deletion frequency of ~2–3% was observed in dTg mice that were positive for the TetO-Cre transgene but negative for the rtTA transgene. This shows a small degree of leakiness of the

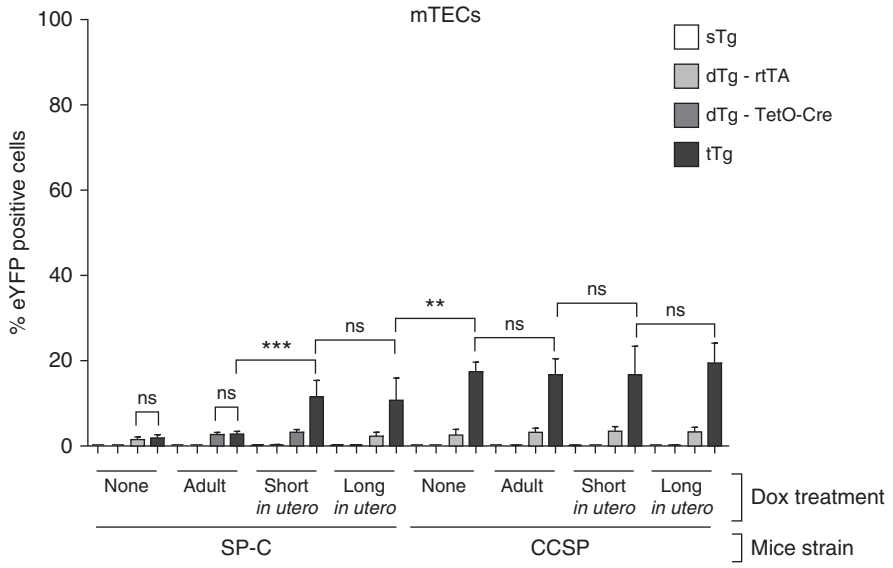

Figure 4. Efficiency of dox-induced gene deletion in tracheal epithelial cells. Shown are the percentages of eYFP reporter fluorescence–positive cells within mTECs from dispase-digested tracheal single-cell preparations. The graphs represent data pooled from dox-treated or untreated mice generated in three to five separate breedings, as outlined in MATERIALS AND METHODS, with each bar representing the mean ± SD of 5–13 mice. The genotypes of the mouse groups were as follows: Rosa26$^{YFP/YFP}$ (sTg), Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$ (dTg-rtTA), Rosa26$^{YFP/YFP}$/TetO-Cre$^{Tg/+}$ (dTg-TetO-Cre), and Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (tTg). $^{**}P < 0.01$, $^{***}P < 0.001$.

TetO-Cre gene in mTECs, which was not observed in lung epithelial cells. No deletion was observed in sTg mice from any of the groups. In summary, the overall deletion frequency in mTECs was small and was not inducible in CCSP-tTg mice.

Deletion in Nonepithelial Cells

Overall, a minimal deletion frequency was observed in mesenchymal cells from lungs (< 5%) and trachea (< 3%) (Figures 5A and 5B). Similarly, a very low deletion frequency (< 5%) was observed in hematopoietic cells (< 5%) from blood, spleen, and bone marrow (Figures 6A–6C) in all mouse groups with or without dox treatments. This shows that the deletion is highly specific to lung epithelial cells in dox-treated SP-C-tTg and CCSP-tTg mice.

Discussion

In this study, we report the efficiency and specificity of deletion in the most widely used lung epithelial cell–specific, dox-inducible Cre deleter mice. Deletion during lung development, induced by dox treatment of pregnant females, was most efficient in ATII cells in the Rosa26$^{YFP/YFP}$/SP-C-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (SP-C-tTg) mice compared with the Rosa26$^{YFP/YFP}$/CCSP-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (CCSP-tTg) mice. By contrast, deletion in ATII cells induced by treatment of adults was significantly better in CCSP-tTg mice compared with SP-C-tTg mice. Similarly, deletion in the EpCAM^{hi} integrin β4^{+} population, consisting of club cells and distal lung progenitor cells, induced during lung development was significantly better in CCSP-tTg mice compared with SP-C-tTg mice. In adults, CCSP-tTg mice also showed a significant deletion in EpCAM^{hi} integrin β4^{+} cells, whereas the SP-C-tTg mice were minimally active. To our surprise, however, approximately half of the deletion in this cell lineage during lung development and almost all of it in adult CCSP-tTg mice was due to CCSP-rtTA transgene leakiness, i.e., it was not regulated by dox. The difference between SP-C-tTg and CCSP-tTg mice is expected based on the described activity of the transgenic human SP-C and rat CCSP promoters (4, 11). There is no report on the deletion that can be achieved in tracheal epithelial cells using these mice. Our study using the Rosa26-eYFP reporter showed low levels of deletion in mTECs in all groups of mice under all conditions examined, thus demonstrating the unsuitability of these strains for targeting genes in the tracheal epithelium. Interestingly, all deletions observed in the mTECs in CCSP-tTg mice were dox independent (i.e., due to leaky Cre expression). Finally, deletion in mesenchymal hematopoietic cells in all groups was minimal (< 5%).

In the majority of studies using SP-C-rtTA and CCSP-rtTA transgenic mice, deletion has been induced *in utero* during lung development. Such approaches are useful, of course, to study the effects of any particular gene on lung development, but may not be feasible for studying gene function in adults (due to potential lethality caused by lung dysfunction at birth). To study the role of any gene in adults (for example, in immune responses to a pathogen or ischemic challenge), treatment of adult mice with dox has been used (17), although it is generally recognized that this is less efficient because both of these transgenes tend to be downregulated in mature epithelium and are not as dox inducible as they are before birth (4). Our study shows that the CCSP-rtTA transgene is more for efficient for deletion in adults, but is also quite leaky (i.e., not regulated by dox) in club cells.

To potentially rectify these issues, Duerr and colleagues (18) generated a second-generation CCSP-rtTA2^{s}-M2 transgene, using a modified version of the rtTA that has reduced basal activity (i.e., leakiness) and increased dox sensitivity (19). When expressed with the LC-1 reporter strain, which drives both luciferase and Cre recombinase under an rtTA-responsive, bidirectional promoter (20), this improved CCSP-rtTA2^{s}-M2 transgene drove highly regulated luciferase expression (i.e., with no leakiness) when mice were treated with dox. The promoter was ∼10-fold more active when mice were treated *in utero* versus as adults, based on total luciferase expression in lung homogenates. The efficiency of gene deletion was estimated by immunohistochemistry for Cre protein expression. Approximately 80% of

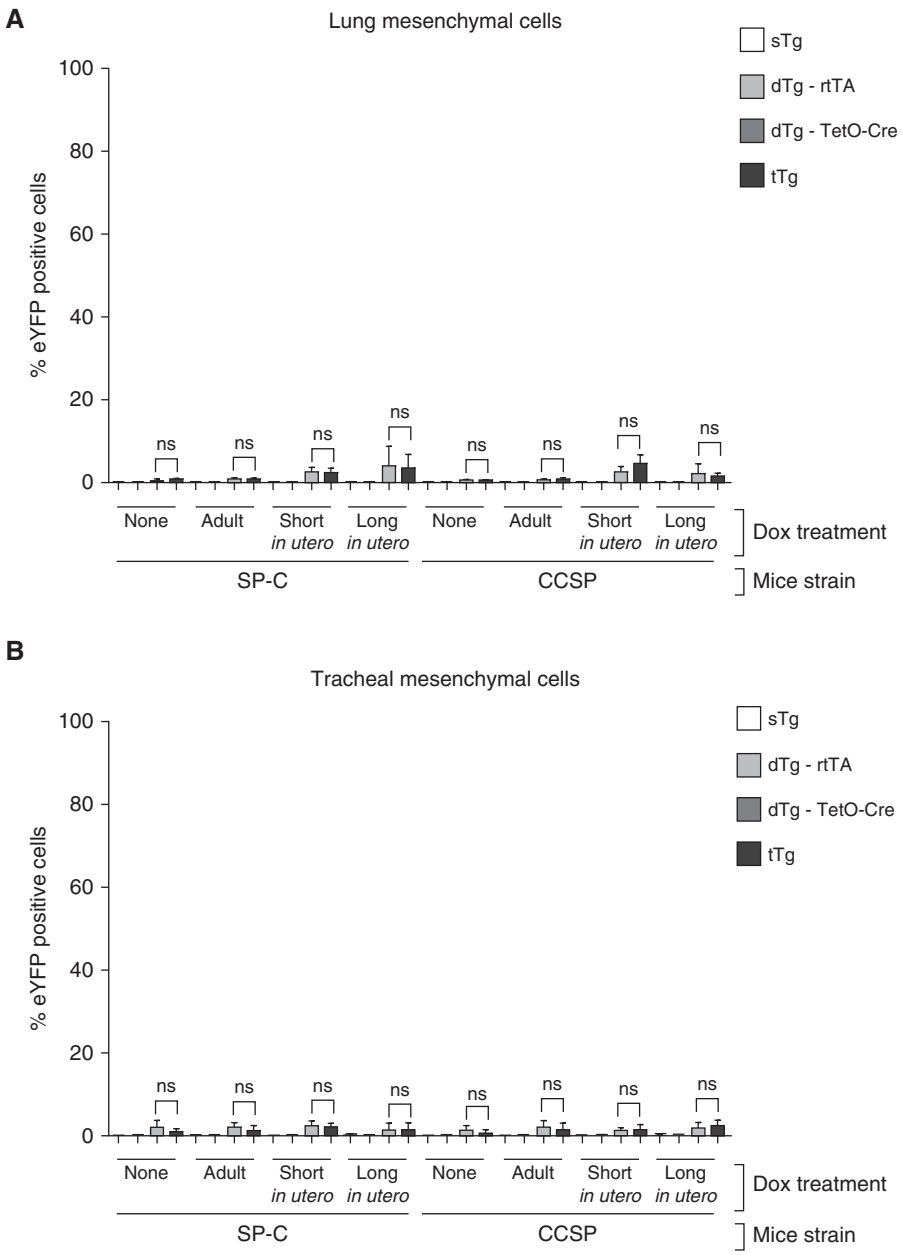

Figure 5. Gene deletion in lung and tracheal mesenchymal cells from dox-treated or untreated lung epithelial cell–specific Cre deleter mice. Shown are the percentages of eYFP reporter fluorescence–positive cells within (*A*) lung mesenchymal cells and (*B*) tracheal mesenchymal cells from single-cell lung and tracheal preparations, respectively. The graphs represent data pooled from dox-treated or untreated mice generated in three to five separate breedings, as outlined in MATERIALS AND METHODS, with each bar representing the mean ± SD of 5–13 mice. The genotypes of the mouse groups were as follows: Rosa26$^{YFP/YFP}$ (sTg), Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$ (dTg-rtTA), Rosa26$^{YFP/YFP}$/TetO-Cre$^{Tg/+}$ (dTg-TetO-Cre), and Rosa26$^{YFP/YFP}$/SP-C-(or CCSP)-rtTA$^{Tg/+}$/TetO-Cre$^{Tg/+}$ (tTg).

SP-C–positive ATII cells expressed Cre protein using the most active CCSP-rtTA2^{s}-M2 transgene. Hence, this improved CCSP transgene clearly reduces the leaky expression observed in the traditional CCSP system, which has been previously reported (5) and which we formally document here.

Since the Rosa locus is robustly and ubiquitously expressed (21), and, unlike the Z/AP and Z/EG reporters, is not silenced in adults (22, 23), we have likely quantitated the highest deletion efficiencies achievable with SP-C-tTg and CCSP-tTg mice. However, the deletion efficiency varies with the gene being targeted and must be determined for each. It is widely recognized that different floxed genes in the murine genome are rearranged with different efficiencies by the same Cre deleting strain (24). Moreover, the deletion frequency of any given floxed gene may also vary depending on ongoing diseases in the target tissue. For example, a variable frequency of deletion of the IL-4α receptor gene by the LysM-Cre deleting strain was reported in mice infected with schistosomiasis (25). Additionally, our study was limited to the use of mice on the C57BL/6J genetic background. Different gene deletion frequencies may be observed in different genetic backgrounds, especially during disease states, given the well-appreciated differences in immune responses between different inbred strains of mice. Therefore, although we provide a quantitative Rosa26-eYFP reporter-based comparison of deletion frequency with SP-C-rtTA and CCSP-rtTA systems, these results can only serve to guide the use of these deleting strains to target other genes in the murine lung epithelium in various disease models on other genetic backgrounds.

Because the various lung epithelial cell populations can be identified by flow cytometry, one could perform surface or intracellular staining for the protein of interest to look for deletion efficiency quantitatively, depending on the availability of high-quality antibodies. In the absence of that, lung epithelial populations can be sorted out by using fluorescence-activated cell sorting (12, 16) and then interrogated by PCR or quantitative real-time reverse-transcription PCR to quantitate the deletion efficiencies. This method would yield superior lung epithelial cell–specific quantitation compared with the more traditional methods, such as whole-lung Western blotting, reverse-transcription PCR, and lung tissue immunostaining. We believe that a quantitative assessment of deletion efficiency and specificity is crucial for interpretation of data.

Finally, it is not clear why ~20–30% of the ATII cells could not be targeted with either transgene system. It is likely that lower expression of the SP-C or CCSP promoters, as heterologous transgenes

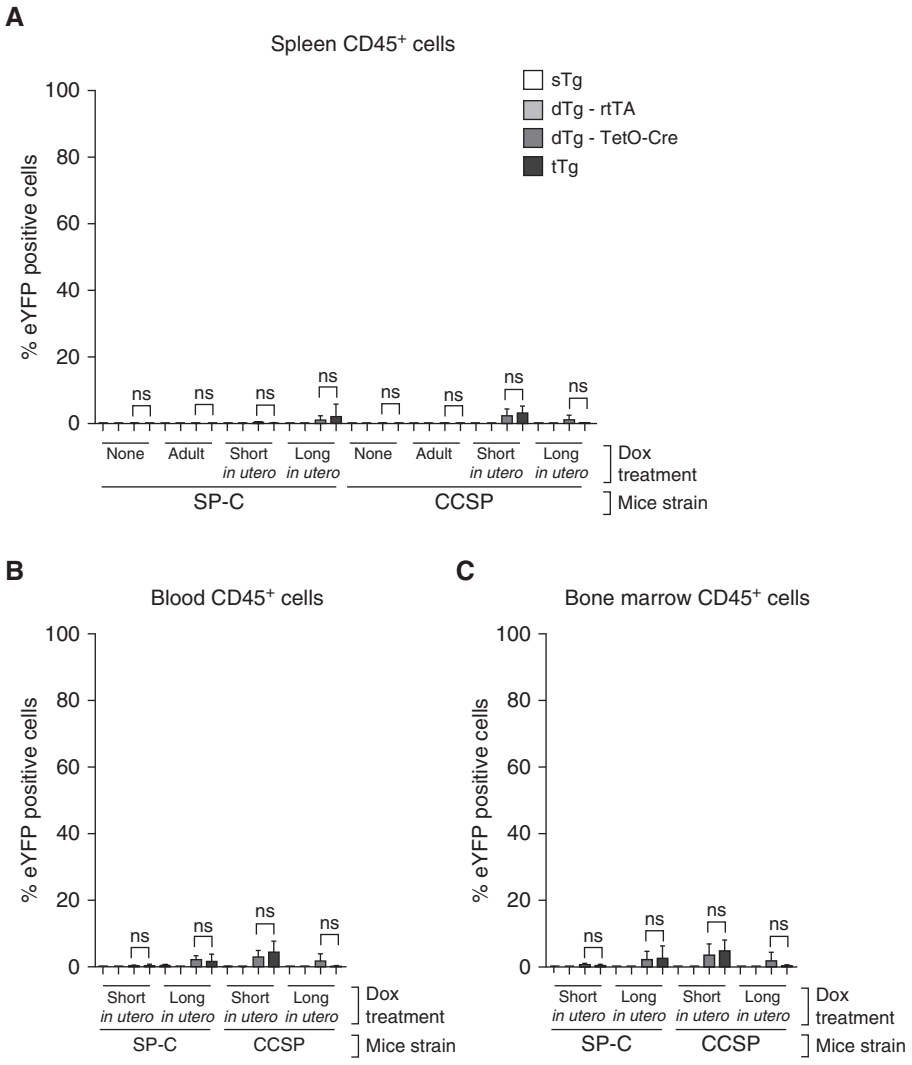

Figure 6. Gene deletion in hematopoietic cells in dox-treated or untreated lung epithelial cell–specific Cre deleter mice. Shown are the percentages of eYFP reporter fluorescence–positive cells within $CD45^+$ hematopoietic cells from (*A*) blood, (*B*) spleen, and (*C*) bone marrow single-cell preparations. The graphs represent data pooled from dox-treated or untreated mice generated in three to five separate breedings, as outlined in MATERIALS AND METHODS, with each bar representing the mean ± SD of 5–13 mice. The genotypes of the mouse groups were as follows: $Rosa26^{YFP/YFP}$ (sTg), $Rosa26^{YFP/YFP}$/SP-C-(or CCSP)-$rtTA^{Tg/+}$ (dTg - rtTA), $Rosa26^{YFP/YFP}$/TetO-$Cre^{Tg/+}$ (dTg – TetO-Cre), and $Rosa26^{YFP/YFP}$/SP-C-(or CCSP)-$rtTA^{Tg/+}$/TetO-$Cre^{Tg/+}$ (tTg).

(i.e., human and rat gene promoters in mice) is an issue. Nearby endogenous genetic elements, such as enhancers or silencers, may affect the transgenic promoters, especially during epithelial disease states. In addition, the low deletion efficiency we observed in ATII cells in SP-C-tTg mice treated with dox as adults was similar to that reported in a previous study using the same human SP-C transgenic promoter construct but coupled downstream to a tamoxifen-inducible Cre-ER^{T2} (26). In contrast, higher adult deletion efficiencies in ATII cells might be achievable in endogenous SP-C promoter Cre-ER^{T2} knock-in mice (27, 28). However, even with these newer-generation knock-in mice, the adult deletion in ATII cells may not be complete, given that the SP-C promoter is active in ~63% of adult ATII cells, as determined by means of a constitutive nuclear green fluorescent protein reporter downstream of the endogenous SP-C promoter (29). Further, they may not be suited for deletion *in utero* given the reproductive toxicity of tamoxifen exposure.

It should also be noted that the breeding schemes in most papers are not clearly outlined. We found that transmission of the TetO-Cre transgene via the male breeder mouse led to a high frequency of litters in which all the pups were reporter positive or gene deleted in a genotype- and dox-independent manner. Thus, in all of our breedings, we consistently maintained the TetO-Cre gene in the female breeders. Also, with the breeding scheme we followed, we were able to control for the copy number of each transgene. None of the transgenes, except the Rosa-eYFP reporter, were bred to homozygosity. This is important in terms of reducing potential off-target and toxic effects of the protein products of the transgenes.

In summary, our study provides a quantitative assessment of the deletion efficiency, specificity, and dox-inducibility of the SP-C-$rtTA^{Tg/+}$/TetO-$Cre^{Tg/+}$, and CCSP-$rtTA^{Tg/+}$/TetO-$Cre^{Tg/+}$ mouse strains using the ROSA26-eYFP reporter. Because these deleter strains have been used by many investigators, this study will assist in interpreting past studies and guiding future ones. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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