Conditional Expression of Genes in the Respiratory Epithelium in Transgenic Mice

Cautionary Notes and Toward Building a Better Mouse Trap

In vitro and *in vivo* studies have repeatedly demonstrated the limitations and potential toxicity of various genes (proteins) used for both labeling cells (e.g., with green fluorescent protein [GFP], β -galactosidase, and luciferase) or for the deletion/addition of mutation of genes (e.g., reverse tetracycline transactivator protein [rtTA], tetracycline transactivator protein [tTA], Cre-recombinase [Cre], or Cre^{ER}). High levels of the introduced protein can cause endoplasmic reticulum stress, genetic instability, immunologic recognition, and/or disrupt cellular homeostasis. Resultant cell injury, death, or other off-target effects on gene expression may be caused by expression of the transgene. A number of systems for gene addition and deletion in the respiratory epithelium have been developed and widely used for the study of gene function, lung morphogenesis, and function. The *Scgblal* (Clara Cell Secretory Protein or CCSP) and *SFTPC* (Surfactant Protein C or SP-C) promoters have been used by our laboratory and others (1, 2). These promoters are highly cell specific and generate robust levels of gene expression. To conditionally express genes in the lung, transgenic mice were produced expressing the reverse tetracycline transactivator that is active when doxycycline is provided to the mouse (3, 4). To target proximal airways, the 2.3-kb rat *Scgblal* promoter was used to drive the reverse tetracycline activator (CCSP-rtTA transgenic mice). To target distal lung structures, we used the 3.7-kb human *SFTPC* promoter (SP-C-rtTA transgenic mice). Airspace enlargement unrelated to the effects of the transgene were observed in various mouse strains bearing the CCSP-rtTA transgene (line 1) (5–7). The potential for toxicity related to the reverse tetracycline activator, doxycycline, and Cre-recombinase was reviewed previously (7). Such overt rtTA toxicity was not seen in many experiments with the initial 3.7-kb human SP-C-rtTA transgenic mice that have been used in numerous studies.

Subsequently, both CCSP-rtTA and SP-C-rtTA mice were bred to a line of (otet)₇CMV-Cre mice that enables doxycycline-regulated expression of Cre-recombinase in the respiratory epithelium *in vivo* (6, 8). When mated to mice bearing floxed alleles, the addition of doxycycline to chow or drinking water (3) causes excision and recombination of DNA sequences located between engineered loxP recognition sites, causing deletion, mutation, or activation of the appropriately engineered floxed gene in the mouse lung (6–8). This system has been useful in lineage analysis, the study of gene function, and lung development. Initial studies failed to reveal significant misexpression of Cre-recombinase or overt histologic or biological toxicity.

As our laboratory has bred the SP-C-rtTA, (otet)₇CMV-Cre mice (primarily maintained in FVBN strain) with other strains bearing floxed alleles and other mouse genetic backgrounds, we have observed off-target effects influencing both lung morphogenesis and perinatal survival. In generating mice in which GP130 receptor, mediating the activation of STAT3, was deleted (9),

initial F₁ mice were bred, in line, with resultant production of two distinct strains that (1) lacked discernable phenotype, unless injured (9), or (2) died of severe lung pathology at birth. Severe morphologic effects were observed when doxycycline was provided to the dam from approximately Embryonic Day (E)6 to birth. Toxicity was not dependent on the presence of the floxed allele, but on the presence of both SP-C-rtTA and (otet)₇CMV-Cre, indicating that toxicity was likely dependent on the expression of Cre-recombinase or the combined expression of rtTA and Cre-recombinase in this strain. Changes in lung morphogenesis and perinatal survival have not been seen if doxycycline was limited to E6.5–14.5 in a number of experiments with SP-C-rtTA, (otet)₇CMV-Cre mice bred to a number of mouse strains in our laboratory. The distinct mouse lines produced from the initial SP-C-rtTA, (otet)₇CMV-Cre, *GP130* loxP founders maintained these distinct features, indicating the potential for the presence of inherited genes that modify susceptibility or resistance to this pulmonary toxicity. As previously noted (4), SP-C-rtTA mice (line 1) cannot be maintained in homozygous state, indicating the potential for a gene dose effect on survival. SP-C-rtTA line 1 mice express high levels of rtTA, and the activity increases in the perinatal period, consistent with the expression of endogenous *Sftpc* gene expression in the lung (3). Taken together, these observations demonstrate the potential for variable and strain-dependent off-target toxicity of rtTA, Cre-recombinase, and doxycycline. Because doxycycline is stored in tissues, prolonged exposure to doxycycline can result in its continued release from tissue pools. To avoid repeated or prolonged doxycycline exposure to the dams or pups, we now routinely limit the period of doxycycline treatment. The timing and duration of treatment needed to target subsets of epithelial cells have been described (6, 8). It is sufficient to expose dams to doxycycline from E8.5 to E14.5 (SP-C-rtTA mice) and E14.5 to E18.5 (CCSP-rtTA mice) to permanently target floxed genes. To control for phenotypes not related to the activation/inactivation of the gene of interest, compound mutant mice should be tested in the absence of doxycycline. Similarly, heterozygous compound mutant mice bearing the floxed allele should be tested after doxycycline treatment. Littermate controls are used when possible to minimize strain or age related variability.

BUILDING A BETTER MOUSE TRAP: NEW SP-C-rtTA AND CCSP-rtTA TRANSGENIC LINES

SP-C-rtTA (Line 2)

Because of high levels of non-doxycycline-dependent expression (3) and Cre-related toxicity seen when double transgenic SP-C-rtTA/otet-Cre mice were exposed to doxycycline in late gestation, we have produced a new SP-C-rtTA line (SP-C-rtTA [line 2]) and bred it to (otet)₇CMV-Cre mice. These mice can be made homozygous for the SP-C-rtTA transgene, and are doxycycline responsive with less non-doxycycline-dependent expression (“leak”) (10). When crossed into the (otet)₇CMV-Cre line, Cre is expressed throughout the alveolar region and peripheral bronchioles at lower levels per cell than in SP-C-

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Figure 1. Immunohistochemistry for Cre protein expression is shown after 48 hours of doxycycline treatment of adult transgenic mice. SP-C-rtTA (line 1) and SP-C-rtTA (line 2) otet-Cre mice express Cre in conducting airways (open arrowheads) and alveolar type II cells (arrowheads in A and B). (C) CCSP-rtTA (line 1) mice express Cre at high levels in a subset of nonciliated bronchiolar and alveolar cells. (D) With the CCSP-rtTA (line 2) mice, Cre was readily detected at high levels in most non-ciliated bronchiolar epithelial cells and staining was rarely seen in alveolar type II cells.

rtTA/(otet)-CMV-Cre (line 1) (Figure 1). We have not seen lung pathology or dysfunction in experiments when used to delete several genes with these mice, but our experience is not as extensive as with SP-C-rtTA (line 1). Gene deletion in the respiratory epithelium was widespread and perinatal toxicity in the absence of floxed alleles has not been seen to date. Thus, this new SP-C-rtTA (line 2) may have features that have advantages compared with SP-C-rtTA (line 1).

CCSP-rtTA (Line 2)

CCSP-rtTA mice (line 1) commonly develop postnatal airspace enlargement (5, 6, 11, 12). The presence and severity of emphysema varies with mouse strain, occurs postnatally, and is not dependent upon doxycycline. In CCSP-rtTA (line 1), rtTA expression is directed to subsets of conducting airway epithelial cells, as well as to a subset of alveolar type II cells, consistent with the site of expression of the endogenous CCSP in the rat lung (1, 6). Because of these limitations, we have generated a new doxycycline-inducible Clara cell-specific rtTA transgenic mouse line (CCSP-rtTA [line 2]) by oocyte injection of the initial 2.3-kb rat *Scgb1a1*-rtTA construct. Seventeen new CCSP-rtTA transgenic founder lines were produced. The extent and specificity of Clara cell targeting was verified by screening for doxycycline-inducible Cre expression. In the new line, CCSP-rtTA (line 2), conditional gene expression was doxycycline dependent and specific to conducting airway cells, with little expression in the alveolus. Minimal focal airspace enlargement was observed in the adult mice. When bred to the (otet)-Cre mice, Cre was conditionally expressed when mice were exposed to doxycycline from E14.5-birth, as well after short-term (48 h) exposure to doxycycline in adults. Expression of Cre-recombinase in the absence of doxycycline was not detected in the conducting airways. After 48 to 72 hours of doxycycline treatment, Cre expression was detected in most nonciliated respiratory epithelial cells and rarely in alveolar type II cells. When mated to ZEG mice (bearing a lox-stop construct expressing GFP after recombination) and exposed to doxycycline from E14.5 to birth, extensive GFP expression was noted in the adult lung, indicating the long-term survival of cells expressing GFP in this model. Thus, this new transgenic line (CCSP-rtTA [line 2]) has advantages of specificity and lack of overt off-target toxicity compared with the previous mouse (CCSP-rtTA [line 1]). Sites

and intensity of Cre expression in the new and previous mouse lines are compared in Figure 1 (6, 8, 10). It should be noted that both the SP-C-rtTA and the CCSP-rtTA promoters may be influenced by the genes being conditionally regulated, thereby influencing the sites of transgene expression.

SUGGESTED GUIDELINES FOR USE OF SP-C-ttTA/tetO-CRE AND CCSP-rtTA/tetO-CRE MICE

Many systems for conditional gene addition, mutation, and deletion have been developed, and all have potential problems. Nevertheless, these systems are useful for the study of gene function and are applicable to many areas of lung biology. These mice have been widely provided to the research community and experience with this system has increased. We can suggest some guidelines for their use that include breeding strategies, limitations of the timing and duration of exposure to doxycycline, and the distinct features of the mouse lines that we have produced to date. Critical to the avoidance or minimization of off-target effects are the recognition that rtTA, Cre-recombinase, and doxycycline are potentially toxic, and that toxicity may be combinatorial and may vary among various mouse strains. Considerations for timing of doxycycline to target gene expression and recombination to specific subsets of cells have been described previously (6, 8). We hope that this information will minimize off-target errors and conclusions derived from the use of previous and new transgenic lines that we have made widely available to the research community.

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