Identification and isolation of mouse type II cells on the basis of intrinsic expression of enhanced green fluorescent protein

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Roper, Jason M., Rhonda J. Staversky, Jacob N. Finkelstein, Peter C. Keng, and Michael A. O’Reilly. Identification and isolation of mouse type II cells on the basis of intrinsic expression of enhanced green fluorescent protein. Am J Physiol Lung Cell Mol Physiol 285: L691–L700, 2003.—The unique morphology and cell-specific expression of surfactant genes have been used to identify and isolate alveolar type II epithelial cells. Because these attributes can change during lung injury, a novel method was developed for detecting and isolating mouse type II cells on the basis of transgenic expression of enhanced green fluorescence protein (EGFP). A line of transgenic mice was created in which EGFP was targeted to type II cells under control of the human surfactant protein (SP)-C promoter. Green fluorescent cells that colocalized by immunostaining with endogenous pro-SP-C were scattered throughout the parenchyma. EGFP was not detected in Clara cell secretory protein-expressing airway epithelial cells or other nonlung tissues. Pro-SP-C immunostaining diminished in lungs exposed to hyperoxia, consistent with decreased expression and secretion of intracellular precursor protein. In contrast, type II cells could still be identified by their intrinsic green fluorescence, because EGFP is not secreted. Type II cells could also be purified from single-cell suspensions of lung homogenates using fluorescence-activated cell sorting. Less than 1% of presorted cells exhibited green fluorescence compared with >95% of the sorted population. As expected for type II cells, ultrastructural analysis revealed that the sorted cells contained numerous lamellar bodies. SP-A, SP-B, and SP-C mRNAs were detected in the sorted population, but T1α and CD31 (platelet endothelial cell adhesion molecule) were not, indicating enrichment of type II epithelial cells. This method will be invaluable for detecting and isolating mouse type II cells under a variety of experimental conditions.

ALVEOLAR TYPE II epithelial cells are critical for normal lung development, homeostasis, and repair after injury. Type II cells produce pulmonary surfactant lipids and proteins required for reducing alveolar surface tension (10, 29, 30). As essential progenitors for type I epithelial cells, they are also critical for normal alveolar development and tissue remodeling after injury (1, 2). Type II cell hypertrophy and hyperplasia are often associated with chronic lung disease (17). Because type II cells express vascular endothelial growth factor, they may also regulate vascularization (21). Given their importance, it is no surprise that a large number of different animal models have been used to study the type II cell phenotype in vivo and ex vivo. The ability to investigate organogenesis and disease progression by overexpressing and deleting genes in mice, particularly genes expressed by type II cells, has recently favored the use of mice in pulmonary research (27).

Although mice are advantageous for manipulating genes, they have not been useful for isolating type II cells for ex vivo study. In contrast, rat and rabbit type II cells have successfully been isolated using velocity centrifugation through a gradient of albumin (8, 10). Isolation of mouse type II cells by this method has been less successful, because airway Clara cells, which are extremely abundant in mice, frequently contaminate the preparations (7, 18). Relatively pure populations of mouse type II cells have been obtained by dispase with agarose instillation and laser-flow cytometry, but it was necessary to label cells with lipid-soluble phosphine fluorescent dye (14). High yields have also been obtained when flow sorting was replaced with panning on plates containing anti-CD32 and anti-CD45 (5, 29). Although epithelial cells are readily obtained with this method, we found that type II cell purity varied between preparations (unpublished observations). Given that the strength of the mouse model is its ability to be genetically manipulated, it would be advantageous to develop new methods by which type II cells could be routinely identified and isolated from genetically defined strains under different experimental conditions.

Green fluorescent protein (GFP) from the jellyfish Aequorea victoria fluoresces brightly when exposed to ultraviolet or blue light (34). The active chromophore is encoded within the primary translation product and does not require enzymatic digestion for activity. A mutant form of GFP has been generated with a red-shifted peak that fluoresces 35 times more intensely than wild-type GFP. This enhanced GFP (EGFP) has been introduced into mammalian cells in vitro and in vivo and has proven useful in detecting transgene expression and isolating cells with specific phenotypes (11, 12).
vivo (9, 13, 24, 32, 37). Fluorescence microscopy and fluorescence-activated cell sorting (FACS) have been used to visualize and purify EGFP-expressing cells from transgenic mouse thymus, bone marrow, and cardiac myocytes (9, 24, 37). On the basis of these studies, we generated a line of mice in which EGFP was expressed in type II epithelial cells under control of the human surfactant protein (SP)-C promoter. This promoter targets genes specifically to the respiratory epithelium (11, 12). Using these mice, we demonstrate that type II cells may be visualized in real time and isolated on the basis of their endogenous green fluorescence.

MATERIALS AND METHODS

Generation and identification of transgenic mice. The EGFP open-reading frame was excised from pEGFP plasmid (Clontech, Palo Alto, CA) using SstI and EcoRI enzymes and ligated directly into a pUC18 vector containing the 3.7-kb human SP-C gene promoter kindly provided by Dr. Jeffrey Whitsett (35). The promoter sequences comprised −3683 to +21 of the human SP-C gene. The EGFP cDNA was followed by a simian virus 40 (SV40) small t intron and a 0.4-kb sequence containing a poly(A) addition signal with stop codons in all reading frames. The SV40 sequence does not encode viral proteins. The expression cassette of 4.8 kb was ligated directly into a pUC18 vector containing the 3.7-kb human SP-C promoter kindly provided by Dr. Jeffrey Whitsett (35). The expression cassette was digested with restriction enzymes EcoRI and EcoRV and transferred into diethylaminoethyl membrane. Membranes were blocked overnight at 4°C with 5% nonfat dry milk and stored at −20°C before they were frozen in liquid nitrogen. Frozen sections (4-μm thick) were immersed in 4°C phosphate-buffered saline (PBS) and 0.006% sodium bicarbonate in Hanks balanced salt solution (1× HBS). Sections for CCSP immunostaining were subjected to antigen retrieval (AR) by boiling in 50 mM Tris, pH 9.5, incubated overnight in primary antibody, subjected to tyramide signal amplification (TSA) using a TSA-biotin system, and detected with streptavidin-Texas red (New England Nuclear). EGFP was detected with anti-EGFP serum (Clontech) or anti-SP-C serum (Chemicon International, Temecula, CA). Paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through graded ethanol and water. Sections for CCSP immunostaining were incubated overnight in primary antibody and detected with Texas red dye-conjugated donkey anti-rabbit IgG (Jackson Labs, West Grove, PA). Sections for pro-SP-C immunostaining were subjected to antigen retrieval (AR) by boiling in 50 mM Tris, pH 9.5, incubated overnight in primary antibody, subjected to tyramide signal amplification (TSA) using a TSA-biotin system, and detected with streptavidin-Texas red (New England Nuclear). EGFP was detected with anti-EGFP serum (Clontech) and 3,3′-diaminobenzidine precipitation using previously described methods (25) or in sections subjected to AR with an FITC-conjugated goat anti-EGFP antibody (Novus Biologicals, Littleton, CO). Sections were immersed in 4′,6-diamidino-2-phenylindole, and fluorescence was visualized with a fluorescence microscope (model E800, Nikon, Melville, NY). Images were captured with a digital camera (SPOT-RT, Diagnostic Instruments, Sterling Heights, MI).

Protein expression was also determined by Western blot analysis. Tissues were homogenized in lysis buffer containing protease inhibitors (25). Proteins were separated on 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were dehydrated in graded ethanol and water. Sections for CCSP immunostaining were subjected to antigen retrieval (AR) by boiling in 50 mM Tris, pH 9.5, incubated overnight in primary antibody, subjected to tyramide signal amplification (TSA) using a TSA-biotin system, and detected with streptavidin-Texas red (New England Nuclear). EGFP was detected with anti-EGFP serum (Clontech) or β-actin serum (Sigma Chemical, St. Louis, MO). Immune complexes were detected by chemiluminescence (Amersham, Arlington Heights, IL) and visualized by exposure to Kodak Bio-Max film.

Isolation of type II cells by FACS. Mice were euthanized by injection with pentobarbital sodium (100 mg/kg ip). The renal vessels of the left kidney were isolated and lacerated to reduce blood volume. The pulmonary capillaries were perfused with 10 ml of sterile saline to remove erythrocytes. The lungs were dissected free of the thoracic cavity and rinsed in sterile saline. Two different methods were used to dissociate epithelial cells from the lungs. One method involved dissociating the lungs with surgical scissors and chopping them into a fine paste using an automatic tissue chopper. Minced tissue was incubated in 10 ml of protease cocktail containing 0.5 mg/ml collagenase, 0.5 mg/ml pronase, 0.4 mg/ml DNase, and 0.006% sodium bicarbonate in Hanks’ balanced salt solution for 1 h at 37°C. Dissociated tissue was then filtered through a sterile 100-μm nylon cell strainer, and the single-cell suspension was pelleted at 300 g and 4°C. Pellets were resuspended in a suitable volume of PBS, and cell counts were obtained using a Coulter counter. A second method involved instillation of perfused lungs with dispase and then with low-melt agarose before preparation of single-cell suspensions (5). Cell suspensions were successively filtered through 100- and 40-μm cell strainers and finally through 25-μm nylon gauze. Single-cell suspensions were then pelleted by centrifugation at 300 g for 5 min at 4°C. Cells obtained from transgenic mice (10 per experiment) were pooled and resuspended in 10 ml of DMEM with 0.5% FBS and 25 mM HEPES. Cells obtained from nontransgenic mice (1 per experiment) were resuspended in 2 ml of medium. Presort cell counts were obtained using a Coulter counter.

Green fluorescent (EGFP) type II cells from dissociated lung tissues were isolated using a cell sorter (B-D FACSVantage SE, Becton Dickinson Immunocytometer Systems, Palo Alto, CA). A two-step sorting procedure was used in our study to isolate the small population of EGFP-expressing cells with high purity and yield. Single-cell suspensions were sorted first with the "enrich" mode to collect every EGFP-positive cell. This procedure resulted in enrichment of the EGFP-positive cells from ~1% to 26%. The enriched cell populations were sorted again with the “normal-R” mode to achieve final purity of ~95%. Cellular EGFP was excited by an argon ion laser.
laser emitted at the wavelength of 488 nm, and the fluorescence was collected after a 530 ± 30 nm band-pass filter. A two-parameter sorting window (forward light scattering and EGFP fluorescent intensity) was used to define the EGFP-positive cell populations. The cells were sorted through a flow chamber with an 80-μm nozzle tip under 12 psi sheath fluid pressure. The sorted cells were collected into 15-ml conical tubes filled with sterile media for morphological and biochemical assays. On average, ~2.5 h were required to prepare a single-cell suspension of 1.5 × 10^6 cells from 10 mice. The enrich-mode sort was carried out at a rate of 10,000 cells/s, and completion requires ~4.5 h. The normal-R mode sort occurred at a rate of 1,000 cells/s, and completion requires ~1 h.

Isolated cells were cytospun onto glass slides, fixed in a 2.5% phosphate-buffered glutaraldehyde, pH 7.4, and postfixed in a 1.0% phosphate-buffered osmium tetroxide. The slides were passed through a graded series of ethanol to 100% and infiltrated with liquid Spurr epoxy resin and embedded on the glass surface with inverted capsular molds containing fresh resin. After polymerization at 70°C, the hardened capsules were then popped off the glass slide by dipping into liquid nitrogen. The hardened capsules were then popped off the glass slide by dipping into liquid nitrogen. The grids were contrasted with aqueous 100% and in 2.5% phosphate-buffered glutaraldehyde, pH 7.4, and postfixed with 3% osmium tetroxide. The grids were contrasted with aqueous uranyl acetate for 10 min and lead citrate for 15 min, examined, and photographed with a transmission electron microscope (model T100, Hitachi).

**Genotypic analysis by RT-PCR.** Gene expression was determined by RT-PCR using total RNA isolated by phase-lock gel microcentrifuge tubes (5 Prime-3 Prime, Boulder, CO) (25). cDNA templates were synthesized at 42°C for 1 min, 51°C (SP-C), 57°C (SP-A) and 72°C for 1 min. Primers for EGFP were 5′-ATGGTGAGCAAGGGCCAGGAGCTG-3′ and 5′-CTTGTACAGCTCGTCCATGCCGAG-3′, which amplified a 716-bp product. Primers for SP-A were 5′-AAAGGGGCTTCCAGGTCCAGC-3′ and 5′-ATTCCTGGGGGCAATGTGG-3′, which amplified a 224-bp product. Primers for SP-B were 5′-TGTCTACTGCCCCTGGTTATG-3′ and 5′-GATCCTCAGCTGGAAA CATCGG-3′, which amplified a 471-bp product. Primers for SP-C were 5′-CATGAAAGATGGCTCCAGA-3′ and 5′-TTTTGGA TTAGATGTCGC-3′, which amplified a 400-bp product. Primers for aquaporin-5 were 5′-GGAGGTGTGTTCAGTT -GATAGGATCCCCC-3′, which amplified a 400-bp product. Primers for surfactant protein-C (hSP-C) promoter from SstI sites, purified, and injected. Founder 67 transmitted the transgene in ~50% of his progeny, with typical Mendelian inheritance by the second generation. Recently, homozygous (transgenic on both chromosomes) mice have been obtained. Transgenic haplozygous (transgenic on one chromosome with no complementary allele) progeny contained approximately three to five copies of the transgene integrated as head-tail con-

**Statistical analysis.** Where appropriate, values are means ± SD. Group means were compared by ANOVA using Fisher’s procedure post hoc analysis, and P < 0.05 was considered significant.

**RESULTS**

**Identification and characterization of transgenic mice.** The EGFP cDNA was inserted into unique SalI and EcoRI sites located between the 3.7-kb human SP-C gene promoter and the 0.4-kb simian virus small intron and poly(A) signal (Fig. 1A). The small t intron and poly(A) signal enhance RNA stability and do not encode viral oncogenic proteins. The 4.8-kb transgene was excised free of plasmid sequences using SstI and injected into C57BL/6J pronuclei. Three founder mice were identified by Southern blot analysis from a total of 76 mice screened. Two male and one female founder mice were identified with 3–40 copies of the transgene integrated. All three mice were independently mated to establish germ-line transmission. Although plugs were observed when founder 9 was mated, females never became pregnant. After unsuccessfully breeding for >1 yr, founder 9 unexpectedly died and was destroyed before transgene expression could be ascertainment. Germ-line transmission of the transgene was successful in the remaining two founders. The transgene was detected in ~20% of progeny from founder 28a, indicating that her germ cells were mosaic for the transgene. Her F1 progeny transmitted the transgene in typical Mendelian fashion. Unfortunately, EGFP mRNA and protein were not detected, so this line was not examined further. Founder 67 transmitted the transgene in ~50% of his progeny, with typical Mendelian inheritance by the second generation. Recently, homozygous (transgenic on both chromosomes) mice have been obtained. Transgenic haplozygous (transgenic on one chromosome with no complementary allele) progeny contained approximately three to five copies of the transgene integrated as head-tail con-
catamers (Fig. 1B). Because EGFP mRNA was detected in their lungs (data not shown), these haplozygous mice were studied further.

EGFP expression in transgenic mice did not affect fecundity, Mendelian inheritance, or health of the mice. Nor have signs of respiratory distress or abnormal histopathology been observed in older mice. EGFP expression was assessed in a number of tissues by Western analysis. EGFP was readily detected in lungs of transgenic, but not nontransgenic, littermates (Fig. 2). As predicted for the lung-specific expression of the SP-C promoter, EGFP was also not detected in heart, intestine, kidney, liver, or brain of transgenic mice. The expression of β-actin confirmed that all lanes contained protein.

Several methods were used to detect cells expressing EGFP. Immunohistochemistry was initially used to localize EGFP expression in transgenic and nontransgenic lungs (Fig. 3, A and B). EGFP-positive cells were restricted to cells in corners of alveoli, rather than along alveolar walls. Under higher-power resolution, they exhibited cuboidal and granular morphology, consistent with the type II cell phenotype (inset, Fig. 3A). Staining was not detected in airway epithelium, fibroblasts underlying airway, endothelial cells surrounding blood vessels, or any cells of the nontransgenic lung. This pattern of expression was confirmed by visualization of intrinsic green fluorescence in frozen sections prepared from lungs of transgenic and nontransgenic mice (Fig. 3, C and D). To confirm EGFP expression in type II cells, tissue sections were immunostained with anti-pro-SP-C and visualized using TSA and streptavidin-Texas red conjugate. Sections were then stained with FITC-conjugated anti-EGFP serum, because endogenous EGFP fluorescence is extinguished by the AR method required for pro-SP-C staining (Fig. 3, E and F). Cells with green and red fluorescence were readily detected, indicating that EGFP-expressing cells also expressed pro-SP-C. Although all EGFP-positive cells expressed pro-SP-C, all type II cells did not express detectable levels of EGFP. EGFP cells also colocalized with pro-SP-B-expressing cells (data not shown). Because the human SP-C promoter can target transgenes to airway Clara cells, EGFP expression was also compared with CCSP expression. Intrinsic green fluorescence was used instead of EGFP immunostaining, because, unlike pro-SP-C, CCSP staining does not require AR. Red CCSP fluorescence was observed in terminal airway epithelial cells that did not overlap with intrinsic green fluorescence (Fig. 3, G and H). These findings indicate that, in this particular line of mice, EGFP expression is restricted to type II epithelial cells with little to no expression in bronchiolar epithelium.

**EGFP fluorescence is retained during lung injury.** SP expression is markedly altered when lungs are injured. For example, hyperoxia (>95% oxygen) stimulates SP-B mRNA and protein expression in bronchiolar epithelium while diminishing expression in alveolar type II cells (16, 36). Although the mechanism is unknown, decreased protein expression in type II cells is associated with mRNA loss and, presumably, failure to maintain intracellular levels of precursor protein that is secreted along with surfactant lipids. Because EGFP is not secreted, we hypothesized that it would be retained during hyperoxia, thereby permitting identification of type II cells when SP expression is lost. Transgenic mice were exposed to room air or >95% oxygen for 72 h, and pro-SP-C and EGFP immunostaining were compared (Fig. 4). Green EGFP-positive type II cells were detected in lungs exposed to room air as a yellow-green color because of their colocalization with red pro-SP-C immunostaining. Although EGFP-positive cells were readily detected in hyperoxic lungs, red pro-SP-C immunostaining was markedly diminished throughout the parenchyma. Compared with room air-exposed tissues, the EGFP-positive cells exhibited a bright green fluorescence due to the lack of red pro-SP-C staining. Thus EGFP protein expression is retained during oxygen-induced lung injury and can be used to identify type II cells that have diminished SP expression.

**Isolation of type II epithelial cells on the basis of green fluorescence.** Previous studies have shown that intact cells expressing EGFP can be isolated by FACS when excited under ultraviolet light (9, 24, 37). Two different protease cocktails were used to prepare single-cell suspensions that were visualized by fluorescence microscopy. A rare fluorescent cell was detected when lungs were dissociated with pronase and collagenase (data not shown). This suggested that these proteases did not effectively remove type II cells from their matrix. In contrast, brightly fluorescent cells were frequently seen when dispase and agarose were instilled. This confirmed previous studies showing that this method efficiently dissociates epithelial cells from lung tissue (5, 14). Because all cells have low intrinsic fluorescence, especially conducting airway epithelial and red blood cells, the overall fluorescence was initially determined by FACS using dissociated cells from a nontransgenic lung (Fig. 5). After we set an upper-limit gate that considered 0.053 ± 0.075% (M2 region in Fig. 5) of the cells highly fluorescent, cells from a transgenic lung were sorted using the normal-R mode and a 1.5-drop sort envelope. We discovered that a long sorting time was required and very few EGFP-positive cells
were collected. Furthermore, overall purity varied between experiments because of contaminating red blood cells. To improve the purity and recovery of sorted cells, a two-step sorting procedure was established. Cells were first sorted using the enrich mode to ensure that every EGFP-positive cell was collected. Assessment of the fluorescence intensity showed that the first-step sorting increased the percentage of EGFP-positive cells from $1.12 \pm 0.32\%$ (M2 region in Fig. 5B) to $26.55 \pm 1.07\%$ (M2 region in Fig. 5C). The smaller fluorescent peaks in Fig. 5C comprised mostly red blood cells, which were discarded. Enriched EGFP-positive cells were resorted using the normal-R mode and a three-drop sort envelope under more stringent conditions ($M3 = 20.79 \pm 1.83\%$). As expected for the heterocellular nature of lung tissue, forward light scatter revealed that the presorted population exhibited a wide variation in cell size (Fig. 6). Although multiple cell sizes were present in the discarded EGFP-negative population, the EGFP-positive population exhibited a uniform cell size consistent with enrichment of a single cell type.

Pre- and postsorted cells were visualized by fluorescence microscopy. An occasional brightly fluorescent
green cell was observed in the presorted population (Fig. 7). Dispase and agarose were used to obtain ~2,000,000 cells per lung, of which 2.5 ± 0.71% (n = 3) were highly fluorescent. The higher percentage of fluorescent cells counted by eye than gated by FACS (~1%, region M2 in Fig. 5B) represents the more stringent criteria used by the laser in the cell sorter. In contrast, 94 ± 6.8% (n = 3) of the postsorted cells were highly fluorescent as assessed by fluorescence microscopy. Approximately 50–100,000 fluorescent cells were obtained per lung, which could vary depending on the rate at which cells passed through the FACS. Ultrastructural analysis of sorted cells revealed that they contained lamellar bodies, characteristic of type II epithelial cells (Fig. 7, C and D).

RT-PCR was used to confirm enrichment of type II epithelial cells in the sorted population. With the exception of EGFP, primers were designed to bridge introns so that amplification of genomic DNA could be distinguished from RNA. Amplification of RNA-derived...
products was also confirmed by failure to amplify EGFP when RT was omitted from the reaction (Fig. 8). SP-A, SP-B, SP-C, aquaporin-5, T1α, CD31, and CCSP were detected in RNA isolated from whole lung. As expected, SP-A, SP-B, and SP-C were detected in the sorted cells, consistent with their expression by type II cells. The type I epithelial cell gene T1α and the endothelial cell gene CD31 (platelet endothelial cell adhesion molecule) were not detected in the sorted cells. Interestingly, aquaporin-5 was detected in some experiments, but only when most of the RT-PCR product was loaded onto the gel (not shown). Because aquaporin-5 was not always detected, it is likely to be due to the presence of a contaminating cell type or occasionally expressed by type II cells. Clara cells are most likely the predominant contaminant, because CCSP was faintly detected in the sorted population, even though EGFP did not colocalize with cells expressing CCSP.

DISCUSSION

This study established that transgenic expression of EGFP could be used to identify or isolate alveolar type II epithelial cells from mice. In one example of its utility, type II cells were identified in hyperoxic lungs by their green fluorescence, even though endogenous SP expression had diminished. In another example, type II cells were purified on the basis of their intrinsic green fluorescence. Purity exceeded 90% and could be increased by narrowing the gate used to select the cells or slowing the rate at which cells passed through the sorter. Each mouse lung yielded ~2,000,000 cells from which 50–100,000 fluorescent type II cells were obtained. Although transgene expression was not ascertained in the discarded cells, nearly all EGFP-expressing cells are calculated to have been positively selected for. Higher yields per mouse will require the generation of additional lines with a greater percentage of type II cells expressing EGFP. Even with these limitations, this study established that type II cells can be tracked during lung injury or isolated by FACS simply on the basis of transgenic expression of EGFP.

The human SP-C/EGFP mice were developed with the intention that the intrinsic nature of EGFP-mediated fluorescence might allow one to observe and isolate type II cells in real time. Taking advantage of the targeting capabilities of the human SP-C promoter and the knowledge that other lines of mice have been generated that express EGFP, we successfully generated a line of mice from which fluorescent type II cells can be identified and isolated. Surprisingly, EGFP expression recapitulated endogenous SP-C expression, in that it was restricted to the parenchyma. Previous studies revealed that the human promoter drives ectopic expression of chloramphenicol acetyltransferase (CAT) in bronchiolar epithelium and in alveolar type II cells (12). A detailed deletion analysis of the promoter revealed that ectopic bronchiolar expression was dependent on sequences between −1910 and −215, with alveolar expression being dependent on more proximal sequences (11). Although the proximal region provided appropriate expression to type II cells, the proportion of type II cells expressing CAT varied between individual lines of mice. As pointed out by Glasser et al. (11), variation in transgene expression has also been reported for the β-globin promoter. On the basis of studies with the β-globin promoter, Glasser et al. speculated that the chromatin environment around the integration site influences the proportion of type II cells that express the SP-C/CAT transgene. Such a scenario would be consistent with the relatively low proportion of type II cells that express EGFP in our line of mice. In the same manner, the integration site may have blocked activity of the distal elements responsible for
the ectopic expression typically seen in bronchiolar epithelium. The Southern blot analysis in Fig. 1 indicates that the SP-C/EGFP transgene did not undergo a gross deletion or inversion, which might explain why bronchiolar expression is not detected. In some ways, the lack of bronchiolar expression in this line of mice is a benefit for those interested in exclusively tracking type II cells.

Even though high levels of EGFP can be toxic, it is unlikely to explain why only some type II cells express the protein. First, lung pathology is normal, and the mice appear to be unaffected by the transgene. Second, propidium iodide exclusion revealed that viability of the isolated cells was 94.2 ± 0.9% (n = 3). Third, transgenic mice have been created in which EGFP is expressed in the lung and other tissues under control of the chicken β-actin promoter and cytomegalovirus enhancer (24). Furthermore, EGFP has recently been expressed throughout the lung or in specific epithelial populations depending on when Cre-mediated recombination occurred during embryogenesis (26). Although it is highly speculative, EGFP may be expressed only in a subpopulation of type II cells that are distinguished by factors other than expression of surfactant genes. Evidence that supports this hypothesis is as follows: weak expression of aquaporin-5 was detected in some experiments, even though T1α was not. Interestingly, aquaporin-5 was faintly detected in one of the sorting experiments where 98% of the sorted cells expressed EGFP. The fact that T1α has never been detected in the sorted population suggests that type I cells were

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**Fig. 7.** Fluorescence-activated cell sorting of cells expressing EGFP. Cytospin of presorted (A) and sorted (B) cells stained with 4',6-diamidino-2-phenylindole display green EGFP fluorescence and blue nuclei. Arrow in B points to a contaminating nonfluorescent cell in the sorted population. This photograph was derived from an experiment in which >98% of the isolated cells were fluorescent. Ultrastructural analyses confirmed that the sorted cells exhibited morphological characteristics of alveolar type II cells, including the presence of lamellar bodies (C and D). Magnification: ×1,500 (C) and ×6,000 (D).

**Fig. 8.** Sorted cells express surfactant genes. Total RNA was prepared from intact lung (top) or sorted cells (bottom) and analyzed by RT-PCR for expression of EGFP, SP-A, SP-B, SP-C, aquaporin-5 (AQ-5), T1α, CD31, and Clara cell secretory protein (CCSP). Reverse transcriptase was omitted (−) in some samples to ensure that products were amplified from RNA. PECAM, platelet endothelial cell adhesion molecule.
successfully excluded. Normally, alveolar type II cells do not express aquaporin-5 (22). However, aquaporin-5 has been observed in hyperplastic type II cells as a result of conditional expression of fibroblast growth factor-7, indicating that type II cells have the capacity to express this gene (33). The finding that aquaporin-5 is occasionally detected in the sorted cells suggests that some or all type II cells may express low levels of this gene. Genotypic analysis of these cells using microarray technology may someday reveal whether subpopulations of type II cells exist.

A number of different methods have been used previously to isolate type II cells. One method involved purification by velocity sedimentation (8, 10, 18). Although type II cell purity approximated 80% in rats and rabbits, yields from mice rarely reach 65%. Alternatively, type II cells have been isolated by flow cytometry on the basis of their size, shape, and lipid content using phosphine fluorescence (14, 20). Purity was improved by dissociating lungs with dispase and gating against macrophages labeled with fluorescent lectins. With these methods, approximately 1,000,000–2,000,000 type II cells per mouse were obtained at >90% purity. The present study also used dispase and FACS to purify mouse type II cells. Unlike previous flow studies that used fluorescent dyes to label lipids, type II cells were selected on the basis of their expression of EGFP. Even though yields were considerably lower than those from other published methods, type II cell purity was extremely high. Indeed, only 1 of ~100 cells examined by electron microscopy did not contain lamellar bodies, a key characteristic of type II cells. Moreover, RNA was purified and used successfully to genotype enrichment of genes expressed by type II cells. Expression of the CCSP message in the sorted population indicates that Clara cells, which did not express EGFP, may contaminate the sorted population. Increasing the sorting stringency will reduce this population, albeit at the expense of reducing the yield. To our knowledge, this is the first time that type II cells were not isolated on the basis of predetermined characteristics of cell shape, size, or lipid content. As such, a uniform population of type II cells was purified solely on the basis of their ability to express a single gene.

In addition to isolating cells, this line of mice can also be used to follow type II cells in real time. As shown in Fig. 4, EGFP may be used to identify type II cells during hypoxia when SP expression diminishes. We have recently been able to isolate fluorescent type II cells from oxygen-exposed lungs for studies on molecular oxidative damage. Thus these isolation methods will be an invaluable new tool for distinguishing and isolating type II cells from animals exposed to a number of different inhaled pollutants. EGFP expression may also be followed in real time as cultured type II cells differentiate into type I cells (3, 4). This process is thought to mimic the normal differentiation events that occur during embryogenesis or remodeling of the injured lung (1, 2). It will be interesting to determine whether EGFP fluorescence can be maintained or restored by altering culturing conditions that allow differentiation of type I-like cells to a type II phenotype (6, 30, 31). Branching morphogenesis has been studied in explant cultures. This process is thought to involve inductive interactions between mesenchyme and epithelium (15). A previous study followed ureteric bud development in three dimensions using EGFP-mediated fluorescence and confocal microscopy (32). Similar studies may now be applied to lung branching morphogenesis. EGFP fluorescence may also be used to follow SP-C transcriptional activity under various experimental conditions, such as during organogenesis or type II cell injury (21, 23). By first backcrossing the present line of mice onto genetically modified strains, one can also study the role of specific genes in type II cell function. Clearly, the use of EGFP as a noninvasive marker will enhance our ability to study type II cells under a variety of experimental conditions.

In summary, we have shown that mouse type II cells can be identified and isolated on the basis of transgenic expression of EGFP. As we identify new cell type-specific promoters and develop new isoforms of GFP that fluoresce at different wavelengths, our ability to track multiple cell types at the same time should increase. Indeed, the present method for isolating EGFP-expressing type II cells should be amenable to isolating other cell types as new lines of intrinsically fluorescent mice become available. Until that time, the present isolation procedures used with this line of mice provides an exciting new opportunity to investigate mouse type II epithelial cells.

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DISCLOSURES

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