Genetic Modification of Airway Progenitors after Lentiviral Gene Delivery to the Amniotic Fluid of Murine Fetuses

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Lentiviral vectors with the firefly luciferase or enhanced green fluorescent protein (EGFP) transgenes were delivered to the amniotic fluid of murine fetuses at Embryonic Day (E) 14.5 or E16.5. Whole-body imaging of luciferase recipients after birth demonstrated transgene expression in the peritoneal and thoracic regions. Organ imaging showed luciferase expression in lung, skin, stomach, and/or intestine. Histological immunofluorescence analysis of EGFP recipients demonstrated that small clusters ($\leq$ three cells) of EGFP-positive epithelial cells were present in the large and small airways of recipients at up to 7 months ($n = 11$). There was no difference in the frequency of transgene expression in mice injected at E14.5 or E16.5 in respiratory or nonrespiratory organs. Analysis of the bronchoalveolar duct junctions on tissue sections of recipient mice identified multiple EGFP-positive epithelial cells. Cells coexpressing EGFP, Clara cell 10-kd protein, and surfactant protein C (SPC) were also found in lungs, consistent with the transduction of bronchoalveolar stem cells. Next, naphthalene lung injury in both luciferase and EGFP recipients was performed to determine whether transduced cells could contribute to tissue repair. In luciferase recipients, the whole-body luciferase signal increased 2- to 20-fold at 2 weeks after naphthalene treatment. Remarkably, immunohistological analysis of the lungs of EGFP recipients after lung injury repair demonstrated repopulation of airways with long stretches of EGFP-positive epithelial cells ($n = 4$). Collectively, these data demonstrate that lentiviral gene delivery to the amniotic fluid of murine fetuses genetically modifies long-lived epithelial progenitors capable of contributing to lung injury repair.

Keywords: lentiviral vector; *in utero*; gene therapy; lung stem cell; lung progenitor cell

In utero gene delivery has been investigated for the treatment of single-gene inherited diseases (1–5). An advantage of *in utero* gene delivery is the correction of the genetic defect either before, or early in, the development of the disease. Delivery of the gene transfer vector to the amniotic fluid during fetal life allows for exposure of the vector to the respiratory and di-

CLINICAL RELEVANCE

In utero gene therapy is being developed to prevent or ameliorate genetic disorders with early disease onset. Our study is the first to demonstrate that long-lived respiratory epithelial progenitors with the ability to repair lung injury can be transduced after amniotic fluid lentiviral gene delivery *in utero*. This approach has potential translational significance in the treatment of diseases affecting the airway epithelium, including cystic fibrosis.

gestive organs due to the normal developmental breathing and swallowing motions of the fetuses (6–9). Other advantages of *in utero* lentiviral gene delivery for genetic diseases include intervention before the onset of tissue pathology and, possibly, a reduction in immune response to the viral proteins and transgene products due to immaturity of the fetal immune system. Finally, stem and progenitor cells of developing organs may be more accessible for transduction than cells in adult organs, and thus may result in a long-term therapy for genetic lung diseases (10, 11).

In this study, we evaluated lentiviral vectors for gene delivery as they stably integrate the transgene cassette into the target cell genome, resulting in long-lasting transgene expression. In addition, lentiviral vectors have a large enough carrying capacity for most therapeutic cDNAs, including the 5.8-kb cystic fibrosis (CF) transmembrane regulator gene, which is defective in CF (12, 13), and a candidate for *in utero* gene therapy (11, 14). In theory, integrating the therapeutic gene into the genome of epithelial stem or progenitor cells will produce genetically corrected progeny cells for long periods of time, possibly the lifespan of the individual.

Studies have demonstrated lentiviral gene delivery to the lungs of fetal rats after direct intrapulmonary injection (15, 16). This resulted in transduction of the lung parenchyma, although the airway epithelium was not transduced in this approach. In contrast, a number of rodent and large animal studies have demonstrated the transduction of respiratory epithelium after lentiviral supernatant gene delivery to the amniotic fluid (15–19). In this strategy, normal fetuses inhale and swallow the amniotic fluid, providing the vector access to the airways and digestive system. In general, these studies identified transgene expression in the lungs of recipients for weeks or months, suggesting that the amniotic fluid lentiviral gene delivery resulted in transduction of long-lived cells in the respiratory epithelium. Furthermore, the studies evaluating which cell lineages were transduced determined that the predominant cell type in the lung is the epithelium (16–19). However, none of these studies determined whether stem or progenitor cells capable of contributing to tissue injury repair were transduced.

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Our study has evaluated the frequency and lineage profile of transgene expression in the respiratory and digestive organs after amniotic fluid lentiviral gene delivery to fetal murine recipients at different gestational time points. Our results demonstrate that amniotic fluid delivery of lentiviral supernatant at both Embryonic Day (E) 14.5 and E16.5 can transduce the respiratory epithelium with transgene expression at levels predicted to be therapeutic for some diseases (20, 21). We further demonstrate that amniotic fluid gene transfer results in genetic modification of respiratory epithelial progenitors and stem cells capable of contributing to lung injury repair.

MATERIALS AND METHODS

Animal Husbandry and Surgery

All mice used in this study were housed in the animal care facility of Children's Hospital Los Angeles. All animal studies were approved by the institutional animal care and use committee. Male and female CF-1 mice were purchased from Charles River Laboratories (Wilmington, MA) to maintain a breeding colony. Timed matings were set up in the afternoon and the morning of plug detection, recorded as E0.5. Timed-pregnant females at E12.5, E14.5, and E16.5 were used for the delivery of lentiviral vectors *in utero*, as detailed in the online supplement. Pups were killed at various time points, and their organs harvested for immunofluorescence microscopic analysis.

Naphthalene Lung Injury

Naphthalene (Fluka, St. Louis, MO) was dissolved in corn oil (30 mg/ml) and injected intraperitoneally into mice (200 mg/kg). Mice were monitored until full recovery; they were weighed 3 days after injury, and the percent body weight loss was recorded.

Lentiviral Vectors and Supernatant Production

Self-inactivating lentiviral vectors were used for these studies. The enhanced green fluorescent protein (EGFP) vectors were based on the pCCL series from Dr. Luigi Naldini (37), in which a 178-bp fragment of the core central polypurine tract was added upstream of the transcriptional unit. The phosphoglycerate kinase (PGK)-EGFP had a bicistronic design, with the EGFP gene expressed from the murine PGK promoter. The firefly luciferase vector backbone was based on the SMPU vector from Dr. Paula Cannon with a firefly luciferase gene expressed from the constitutive MND promoter (38).

Vesicular stomatitis virus G protein-pseudotyped lentiviral vector supernatants were produced, concentrated, and titered as previously described (39). Concentrated supernatant was stored in aliquots at -80°C and thawed rapidly at 37°C before use.

In Vivo Bioluminescence Imaging

Mice were imaged longitudinally and bioluminescent signals determined as previously described (40). Mice were individually marked and imaged longitudinally. Briefly, mice were anesthetized using isoflurane inhalation and injected with luciferin potassium sulfate (Promega, Madison, WI) at 60 mg/kg body weight for the first experiment. For the injury experiments, luciferin was administered in a split dose intraperitoneally (30–300 μl) and intranasally (10–50 μl volume), to a final dose of 180 mg/kg.

Acquisition time was 3 minutes for mice, except when a saturated signal appeared at 3 minutes, in which case those mice were imaged for shorter time periods to obtain unsaturated signals that could then be used for quantification (*see* the online supplement). For individual organ imaging, mice were injected with luciferin via the tail vein, and maintained under isoflurane anesthesia. After 15 minutes, the inferior vena cava was cut, and the mouse perfused with 10 ml PBS using a 25-gauge needle inserted into the left ventricle of the mouse's heart. Candidate organs were harvested and placed in six-well plates for imaging.

Immunofluorescence Analysis

Mice were killed with intraperitoneal injection of pentobarbital (150 mg/kg) and perfused as described previously here. Organs

(abdominal skin and muscle, heart, thymus, esophagus (in 28-d-old mice), lung, trachea, stomach, intestine, kidney, spleen, pancreas, and liver) were excised and processed for immunofluorescence, as detailed in the online supplement.

RESULTS

In Utero Surgery and Survival

Intra-amniotic injections were performed on a total of 156 fetuses from 15 timed-pregnant females at 3 different gestational ages: E12.5, E14.5, and E16.5. At E12.5, *in utero* surgery was performed on a total of 21 fetuses from 2 pregnant females, with only 1 fetus surviving to birth. The remaining fetuses injected at E12.5 miscarried or were stillborn (*see* Table E1 in the online supplement). The females had fully recovered from the surgery and were otherwise well, suggesting that the high rate of fetal loss associated with injections at E12.5 resulted from the gestational time of the injection rather than the health of the mothers or any adverse effects from surgery. However, given the small sample size ($n = 2$), we are unable to draw conclusions about fetal survival after E12.5 delivery. Given the high mortality of pups associated with manipulation at early gestational periods in our study, all subsequent experiments focused on injections at E14.5 and E16.5, which had a higher survival rate than that associated with injections at E12.5. To further increase the survival of the E14.5- and E16.5-injected pups, we compared survival after natural birth versus Cesarean section delivery and fostering, as outlined in the online supplement.

Luciferase Expression Was Detected by Whole-Body Imaging in Recipients

In the first series of experiments, the luciferase lentiviral vector supernatant was used to evaluate transgene expression with whole-body imaging of experimental pups over time. In two independent experiments, 10 μl of luciferase lentiviral supernatant containing 4×10^7 transduction units (tu) was injected into the amniotic fluid of individual E16.5 fetuses. After birth, three surviving pups from each experiment (total of six) were imaged longitudinally for 4 weeks. After intraperitoneal injection of luciferin into each pup, total bioluminescence signal was quantified weekly in all pups from postnatal day (P)7 through P28 (Figure 1A). The bioluminescent signal from a representative experimental mouse over 1 month is shown in Figure 1A, and shows higher signals during the first 2 weeks, with a reduction in signal thereafter. Although the total amount of bioluminescent signal varied among the six mice at any given time point, the signal was consistently present in the thoracic and upper abdominal regions of all mice, and decreased over time.

After the whole-body imaging analysis at P28, bioluminescence was observed in the skin, lung, stomach, and intestine, but not in muscle, spleen, pancreas, ribcage, nor diaphragm (Figure 1B). These studies demonstrate that amniotic fluid delivery of lentiviral supernatant to E16.5 murine fetuses resulted in transduction of respiratory and digestive organs. Furthermore, luciferase expression was detected in organs at 1 month after birth, despite the barely detectable whole-body luminescent signal present in some of the recipients at that time.

Transgene Expression in the Lung and Trachea of Recipients of PGK-EGFP Lentiviral Supernatant

To identify the specific cell types expressing the transgene in each organ, we injected lentiviral vector supernatant with the cellular PGK promoter expressing the EGFP (PGK-EGFP)

Figure 1. Bioluminescence imaging of luciferase lentiviral supernatant injected mice *in utero* (Embryonic Day [E] 16.5). (A) Whole-body images of a representative mouse showing luciferase signal weekly over 1 month compared with an uninjected control mouse. (B) Imaging of specific organs taken from the same mouse at 31 days of age. Skin samples were taken either from the thoracic (thor) or peritoneal (perit) regions. Bioluminescence was observed in the skin, lung, stomach, and intestine.

into the amniotic fluid of murine fetuses at E14.5 and E16.5. For these experiments, 5 μ l of supernatant containing 5×10^7 tu were injected into the amniotic fluid of each fetus. Various organs were harvested at P0, P7, and P28 and prepared for transgene expression. Two animals were evaluated at each time point for each gestational age of injection, except for P28 in the E16.5 group, which had three animals.

Tissue sections from the trachea and lung of each animal were double immunostained with antibodies against EGFP and an epithelial-specific marker. Tracheal epithelial cells were identified with a pan-cytokeratin antibody (Figures 2A and 2B). Airway epithelial cells in the lung were identified with an antibody against Clara cell 10-kd protein (CC10; Figures 2C–2E). These studies identified EGFP-positive epithelial cells in the large and small airways of supernatant recipients, as shown in Figure 2, with some airways containing small clusters of EGFP-positive cells. We confirmed the specificity of EGFP detection with the EGFP antibody used in all our immunofluorescence analyses, as there was no EGFP staining observed in tissue samples from an *in utero* saline-injected mouse (Figure E1).

Half of the mice (6 of 13) had EGFP-positive epithelial cells in the lungs and trachea, with up to 68 double-immunostained sections screened per organ per mouse. Figure E2A shows a tiled reconstruction of 25 adjacent sections of a lung lobe from

a mouse analyzed by confocal microscopy, showing a moderate level of transgene expression. Furthermore, a three-dimensional reconstruction of 81 optical sections (0.78 μ m each) through a 60- μ m section of lung tissue is shown in Figure E2B. The arrowheads in this figure identify clusters of EGFP and CC10 double-positive epithelial cells along the airways. A movie of this three-dimensional reconstruction of this lung section demonstrating multiple airways positive for EGFP expression is presented in Figure E2C. The images and movie in Figure E2 provide an overview of the spatial relationship and relative levels of gene expression among the airways in a large area of tissue for this mouse with a moderate level of transgene expression.

To determine the proportion of epithelial cells expressing the EGFP transgene, the total number of airway epithelial cells (i.e., pan-cytokeratin-positive tracheal or CC10-positive airway epithelial cells) and the EGFP-positive epithelial cells were counted in immunostained sections from the tracheas and lungs of the four mice with the highest proportion of transgene-expressing cells (Table 1). In mice 1 and 2, with the highest level of EGFP-positive cells, EGFP transgene expression was restricted to epithelial cells, evidenced by costaining of EGFP and either pan-cytokeratin or CC10 in cells lining the tracheas and airways, respectively (Figure 2). From a total of 6,520 epithelial cells in the trachea and airway counted in these two

Figure 2. Double immunofluorescence analyses of trachea and lung showing epithelial-restricted enhanced green fluorescent protein (EGFP) expression at 1 and 7 months. Shown are representative *photomicrographs* of trachea (A and B) and lung (C–F) sections from three different mice at 1 month (A–E) and 7 months of age (F). The antibodies used for the analyses are labeled on each picture. The gestational ages for lentiviral gene delivery were E14.5 (A–D) or E16.5 for (E and F). The magnifications were 200 $\times$, except for (B) and (D), which were 400 $\times$. Mounting media containing the nuclear stain, 4'-6-diamidino-2-phenylindole (DAPI) (blue), and antibodies against EGFP (green) and pan-cytokeratin (PCK) or Clara cell-specific protein Clara cell 10-kd protein (CC10) (red) were used. Green cells identify transduced cells expressing GFP, and red cells identify pan-cytokeratin or CC10-positive epithelial cells lining the trachea and lungs. In the merged images, yellow cells indicate pan-cytokeratin or CC10-positive epithelial cells expressing EGFP.

mice, 378 cells were also EGFP positive (Table 1). Specifically, an average of 6% of the trachea and airway epithelial cells expressed EGFP, with a range of 1–14% EGFP-positive airway epithelial cells on each section (data not shown). For mice 3 and 4, with a medium level of EGFP expression, a total of 2,997 pan-cytokeratin and/or CC10-positive epithelial cells were counted, with 52 cells positive for EGFP (Table 1). This corresponds to an average of 2% of the epithelial cells positive for EGFP, with a range of 1–4% on each section. EGFP-positive cells were not detected in the trachea of mouse 3. Interestingly, EGFP-positive cells were commonly found in clusters (3–5 cells) in these mice (Figure 2). The two mice with the lowest level of EGFP expression had rare EGFP-positive epithelial cells in the trachea and lung, which were not quantified (data not shown). The quantitative analysis demonstrates that the highest level of transgene expression was in the airway epithelium. Of note, we also observed rare EGFP-positive nonairway cells morphologically similar to macrophages at 1 month; however, their identity was not confirmed

by immunostaining. There was no difference in the proportion of EGFP-positive cells between the E14.5 and E16.5 ages of delivery.

To determine whether genetically modified cells persisted in the lungs of recipient mice in the long term, we analyzed tissues from recipients of the PGK-EGFP lentiviral supernatant at 7 months of age after amniotic fluid delivery at E16.5. Similar to the animals evaluated at up to 1 month of age, there were clusters of EGFP-immunopositive epithelial cells (CC10 and EGFP double positive) in the airways of three of the four recipients evaluated (Figure 2F). We also observed cells within the alveolus that were morphologically similar to type II alveolar epithelial cells (AECII). Immunostaining confirmed that these EGFP positive cells were AECII, as they costained with the specific markers *Nkx2-1* and prosurfactant protein C (SPC) (data not shown). These data demonstrate that long-lived EGFP-positive epithelial cells persist in the lungs of recipients, consistent with the transduction of a long-lived cell or progenitor.

TABLE 1. QUANTITATION OF ENHANCED GREEN FLUORESCENT PROTEIN-EXPRESSING RESPIRATORY EPITHELIAL CELLS

Animal	Day of Lentivirus Delivery	Day of Analysis	Organs	No. of Sections Positive/ No. of Screened	EGFP-Positive Cells/ Epithelial Cells (%)
1	E16.5	P7	Lung	15/15	68/1,501 (5)
			Trachea	15/15	84/1,156 (7)
2	E14.5	P28	Lung	15/15	123/1,733 (7)
			Trachea	15/15	103/2,130 (5)
3	E16.5	P7	Lung	14/68	39/2,031 (2)
4	E14.5	P7	Lung	4/66	6/408 (2)
			Trachea	5/66	7/558 (1)

Definition of abbreviations: E, Embryonic Day; EGFP, enhanced green fluorescent protein; P, postnatal day.

Transgene Expression in the Nonrespiratory Organs of Recipients of PGK-EGFP Lentiviral Supernatant

Transgene expression was evaluated in nonrespiratory organs, including skin, liver, esophagus, pancreas, stomach, and intestine in PGK-EGFP recipients after birth. EGFP expression was detected in the skin and liver of 31% of the mice (4 of 13 mice for each tissue), and in the stomach and intestine of 8% of the mice (1 of 13 mice for each tissue; Figures E3 and E4) up to 1 month of age. EGFP expression was not detected in any of the 13 esophagi or pancreases.

The EGFP-immunopositive cells were further characterized with different cellular markers to identify their cell lineage(s). With the exception of the stomach, the EGFP-immunopositive cells in the skin, liver, and intestine did not costain with pan-cytokeratin, indicating that they were nonepithelial cells (Figures E3 and E4). Furthermore, in one highly transduced skin sample, EGFP-positive cells were either immunoreactive for macrophage (F4/80) or fibroblast (vimentin) markers (data not shown). In liver samples, EGFP immunostaining was observed in cells positive for Kupffer cell (F4/80) or hepatocyte (hepatocyte nuclear factor4 α) markers (Figure E4). Collectively, these results demonstrate that, in contrast to the respiratory organs, transgene expression in nonrespiratory organs was present at a lower frequency, and was not restricted to epithelial cell types.

Amniotic Fluid Lentiviral Gene Delivery Transduces Long-Lived Respiratory Epithelial Cells and Progenitors

For the development of long-term stem cell gene therapy for CF or other lung diseases, it is important to transduce respiratory progenitor cells capable of contributing to tissue repair. In the next series of experiments, we evaluated whether respiratory stem or progenitor cells were transduced in our amniotic fluid gene transfer protocol.

A number of different progenitor or stem cell populations have been identified in the lungs of mice, including the bronchoalveolar stem cells (BASC) found at the bronchoalveolar duct junction, which can give rise to both alveolar and airway epithelial cell progeny, at least *in vitro* (22). As shown in Figure 3, we observed EGFP-positive cells in the bronchoalveolar duct junctions of mice at 1 and 7 months. In addition to their morphological location, BASC are identified by their coexpression of airway (CC10) and alveolar (SPC) genes. We next triple stained tissue sections with antibodies against CC10, pro-SPC, and EGFP to determine whether we had transduced cells with the BASC phenotype. Cells co-triple stained for CC10, pro-SPC, and EGFP were observed, suggesting that amniotic fluid gene delivery can transduce BASC cells (Figure E5, arrows). Although, BASC have been defined by their expression profile and *in vitro* differentiative capacity (22) to produce both airway and alveolar epithelial cells, it has not been clearly determined whether they can contribute to lung homeostasis and injury

repair *in vivo* (23). Thus, in the next series of experiments, we investigated whether epithelial progenitor cells capable of contributing to injury repair were genetically modified after amniotic fluid lentiviral gene delivery.

Amniotic Fluid Lentiviral Gene Delivery Transduces Respiratory Epithelial Progenitors that Contribute to Tissue Repair after Injury

We next evaluated whether genetically modified cells could be expanded after lung injury repair using luciferase recipients.

Figure 3. Double immunofluorescence analysis of lung tissue sections of phosphoglycerate kinase (PGK)-EGFP recipients showing the bronchoalveolar duct junction (BADJ). Shown are merged and separated images of EGFP and CC10 immunostained lung sections from two different mice at 1 month (A) and 7 months (B) of age at 200 $\times$ magnification. The gestational ages for lentiviral gene delivery were E16.5 for (A) or E14.5 for (B). Mounting media containing the nuclear stain, DAPI (blue), and antibodies against EGFP (green) and Clara cell-specific protein, CC10 (red), were used. Arrows point to yellow cells at the BADJ expressing both EGFP and CC10.

Pups from three pregnant dams were each injected with luciferase lentiviral supernatant at E16.5, as described previously here, although the supernatant titer was higher (4×10^{10}) and each pup received 10 μ l with 4×10^8 tu, which was approximately 1 log higher than the first luciferase experiment presented in Figure 1. A total of 18 pups survived and were available for whole-body imaging analysis at weekly intervals for the first month, and monthly thereafter. In this experiment, luciferin was delivered both intraperitoneally and intranasally to mice. All mice in this cohort of animals were positive for bioluminescence on at least one time point during the first month. In three of the mice with the highest intensity, the signal persisted to 4 months, whereas, in the rest of the mice, the signals decreased to background levels by 1–2 months.

Six mice were killed and subjected to organ imaging at 2, 5, or 8 weeks of age. All animals had positive lungs, regardless of whether they had a signal by whole-body imaging ($n = 4$) or not ($n = 2$). There was no consistent difference between the signal intensity on the specific organs from the animals at these different time points. However, as the organ analyses were performed at the time of death, longitudinal imaging on organs was not possible to determine changes over time.

At 4 months of age, all remaining luciferase recipients (12) and control animals (2) were imaged, and then injected with naphthalene to induce airway epithelial damage. Injury was confirmed by an average weight loss of 6% (range, 2–14%) in all animals at 3 days. The nine animals that were negative by whole-body imaging before injury continued to be negative 2 weeks after injury, and were killed for organ imaging analysis. The lungs of two animals, the nasal regions of two other animals, and the skin from yet another animal were positive by organ imaging. All other organs were negative (data not shown). However, because we do not have organ images before injury, it is unclear whether the naphthalene injury increased the signal in any of these mice or not.

In the three animals that were positive for bioluminescence before injury, the average bioluminescent signal in the head and thoracic regions had increased 2- to 20-fold at 2 weeks, and was maintained at 4 weeks (Figure 4). The increased luciferase signal in the thoracic regions of mice after naphthalene-mediated lung injury provides strong evidence that cells transduced using the amniotic fluid lentiviral gene delivery can contribute to airway epithelial injury repair by expansion of luciferase-positive airway cells. However, as discussed previously here, although the luciferase experiments provide invaluable information on the kinetics of cells expressing the transgene, they do not provide information on which cell types are expressing the transgene.

In the next experiments, we evaluated the contribution of genetically modified cells to the repopulation of airways after naphthalene injury. In these experiments, fetal mice were injected at E16.5 with 5×10^7 tu of the PGK-EGFP lentiviral supernatant, as described previously here. Four EGFP recipients were injured with naphthalene at 9 weeks of age, as described previously here, and killed for analysis at 1 month after injury (Figure 5). Double immunofluorescence analysis of tissue sections from all four mice showed multiple airways with long stretches with ≥ 20 EGFP-positive cells (Figure 5). The frequency of EGFP-positive cells was in stark contrast to the original set of PGK-EGFP mice analyzed as discussed previously here that were not injured and typically showed small clusters of contiguous EGFP-positive cells (Figure 2). As an example, we determined the proportion of EGFP-positive cells within individual airways of mice at 1 and 7 months of age shown in Figure 2, and after naphthalene injury repair in Figure 5. In the uninjured animals, 9% of the airway epithelial cells were EGFP positive at 1 month (10 EGFP⁺ of 110 airway

Figure 4. Bioluminescent signal is increased after naphthalene injury. Each line in the graph represents the bioluminescent signal from a region of interest drawn around the head and thoracic regions of a single mouse before and after intraperitoneal injection of naphthalene.

epithelial cells) and 7 months (7 EGFP positive of 75 airway epithelial cells) of age. This is in contrast to the after-injury sample shown in Figure 5 at 4 months of age with 41% (30 EGFP positive cells of 73) and 33% (38 EGFP positive cells of 114) of the airway epithelial cells expressing EGFP in Figures 5A and 5B, respectively. Collectively, these data demonstrate that the amniotic fluid lentiviral gene transfer protocol genetically modifies long-lived airway progenitor cells capable of contributing to lung injury repair.

DISCUSSION

Our study determined that lentiviral gene delivery to amniotic fluid transduces epithelial cells in the respiratory and digestive organs. Furthermore, we clearly demonstrate that amniotic fluid gene delivery results in the transduction of long-lived airway epithelial progenitors that are capable of contributing to lung injury repair. This finding has potential translational significance in the correction of genetic or developmental airway diseases.

In these studies, we compared survival and gene delivery to the respiratory epithelium at three delivery time points during mouse gestation to identify the optimal transduction of epithelial stem or progenitor cells in the respiratory and digestive organs. Similar to other studies, we found high mortality associated with intra-amniotic injections performed on E12.5 fetuses (16, 24). The survival rates of fetuses injected at E14.5 and E16.5 were higher, with up to 100% of the fetuses present at injection surviving to delivery. Furthermore, delivery of lentiviral supernatant to the amniotic fluid did not affect the survival rate, as we observed similar survival rates between fetuses receiving saline and those receiving vector. Based on this finding, subsequent studies focused on evaluating gene transfer in E14.5 and E16.5 recipients after birth.

We first evaluated transgene expression after lentiviral delivery of the luciferase gene by longitudinal whole-body imaging and specific organ imaging at the time of death. Luciferase expression was detected in the thoracic and/or upper abdominal cavities of all mice through P21, after which it declined to an average level similar to control mice in most recipients. However, organ imaging revealed the persistence of luciferase signal in lungs, stomach, and/or intestine in mice, with little, or no, whole-body signal. This demonstrated that transgene-expressing cells were present despite the minimal signal detected by whole-body imaging. This discrepancy in the detection of luciferase signal is likely due to the differing sensitivity or

Figure 5. Double immunofluorescence analysis on lung sections from an adult EGFP recipient 1 month after naphthalene injury. Mounting media containing the nuclear stain, DAPI (blue), and antibodies against EGFP (green) and Clara cell-specific protein, CC10 (red), were used. Green cells identify transduced cells expressing EGFP, and red cells identify CC10-positive epithelial cells lining the lung. In the merged images, note the long stretches of EGFP and CC10 double-positive epithelial cells in the airways (shown in yellow). The magnifications were 100 $\times$ (A) and 200 $\times$ (B).

threshold of detection achievable in the whole-body versus organ imaging. This may be the consequence of the increased size and volume of the animals, and corresponding depth of tissue that the light travels through to reach the detector in the larger adult animals. Furthermore, it was recently reported that the intraperitoneal route of luciferin results in suboptimal distribution of the substrate to all tissues, with intranasal delivery of luciferin providing significantly higher levels of bioluminescence detection in the respiratory tissues (25). Based on this report, subsequent luciferase experiments used both intraperitoneal and intranasal luciferin delivery to achieve maximal luminescent detection. However, given that the set of luciferase mice also received a higher dose of lentiviral particles, we cannot directly compare the results of these two studies.

Other factors that may have contributed to the decreased luciferin signal are either a reduction in the number of transgene expressing cells due to normal cell turnover, and/or a decrease in transgene expression as a consequence of vector silencing, as has been observed with other vectors (26), as well as lentiviral vectors in other cell types (27). There are several studies demonstrating that SIN lentiviral vectors (similar to ours) can be silenced in human and murine embryonic, hematopoietic, and neural stem cells (reviewed in Ref. 27), and that, to some extent, this can be minimized by multiple integrations. However, there are no studies evaluating lentiviral vector silencing within lung cell types, although other studies suggest that lentiviral vector silencing

can occur in epidermal cells after a similar *in utero* gene delivery approach (10). Further analysis would be required to specifically determine the parameters contributing to the reduction in bioluminescent signal observed over time in our studies.

Having established that amniotic fluid lentiviral gene delivery results in transgene expression in respiratory organs from half of the mice, we next evaluated the specific lineages expressing the transgene in these tissues. These studies used an EGFP transgene to identify cell lineages by double immunofluorescence analysis in tissues. Interestingly, transgene expression was detected in the airway epithelium of lungs and tracheas of mice at up to 7 months. In some mice, the proportion of epithelial cells expressing the transgene were at levels predicted to be therapeutic for diseases such as CF (28, 29), with up to 14% of airway epithelial cells on specific tissue sections expressing the transgene. However, despite the high level of transgene-positive cells in some recipients, there was variability in the overall proportion of transgene-expressing cells among all recipients. This variability has also been reported in other studies (16, 30, 31). One possible source of variability is the proximity of the injection site to the oral cavity being inconsistent between individual fetuses, producing a variable local concentration of virus being inhaled or swallowed. Indeed, ultrasound guidance has been used advantageously to instill viral vectors in close proximity to the oral/nasal cavities within the amniotic fluid of rodents (16) and nonhuman primates (32). There are also likely to be individual differences in the intensity, frequency, and rate of the breathing and swallowing movements among the fetuses contributing to the variability in transgene exposure among different organs. In turn, this would affect the transduction and transgene expression in specific organs and animals after birth.

We also evaluated the digestive organs for transgene expression. We observed transgene expression in the stomach epithelium and liver hepatocytes in several recipients. In the intestine, transgene expression was not observed in the epithelium, confirming that, although the vector physically entered the intestine, it did not transduce intestinal epithelial progenitors. One possible reason for this is that the epithelial progenitor cells in the intestine may not have been available and accessible at the time of supernatant delivery at E14.5 or E16.5. In support of this theory, a recent paper by Endo and colleagues (10) demonstrates that the developmental stage at the time of vector delivery determines the specific cell populations transduced. Thus, it is likely that the absence of EGFP expression in the intestinal epithelium was because intestinal progenitors were not available or accessible at the time of lentiviral vector delivery.

Our analysis determined that the transgene-positive airway epithelial cells were often in small clusters, suggesting that the vector was able to transduce airway epithelial cells with the capacity for cell division, and possibly progenitor cells. To determine whether amniotic fluid lentiviral gene delivery genetically modified respiratory epithelial progenitor or stem cells, we performed two types of studies. In the first, we determined that cells consistent with a BASC phenotype (22) also expressed the EGFP transgene, suggesting that we had transduced some BASC. However, because it is unclear whether BASC contribute to lung development or repair *in vivo* (23), we also investigated the function of the genetically modified cells. In our study, mice were injured with naphthalene, which severely damages the Clara cells in the airway, leading to a dramatic denuding of the airway epithelium at 2–3 days (33). Repair is completed by 1 month. This injury model has been used in a number of studies to identify the airway epithelial progenitor cell populations (reviewed in Ref. 34). We first evaluated the change in whole-body luminescent signal after naphthalene injury repair in luciferase recipients. This study demonstrated

that the whole-body bioluminescence signal doubled after lung repair, specifically in the nasal and thoracic regions of the animals, suggesting that there were more transgene-positive cells after injury. In the next set of experiments, we evaluated the lineages of the transgene-positive cells by immunofluorescence microscopic analysis of EGFP recipients. Interestingly, these data clearly demonstrated the repopulation of long stretches of airway epithelium with transgene-positive cells, consistent with the presence of transduced airway epithelial progenitors. Of interest, transduction of epithelial progenitors that contribute to naphthalene injury repair was recently described using a recombinant adeno-associated viral vector (35). However, given their small carrying capacity of roughly 4.7 kb, and inefficient rate of integration into the target cell genome (36), this approach will be most effective for gene therapies where long-term transgene expression is not required, the endogenous gene can be corrected after transient expression (such as zinc fingers), and/or where the therapeutic expression cassette is less than 4.7 kb in size.

Our study is the first demonstration that amniotic fluid lentiviral gene delivery can genetically modify long-lived respiratory epithelial progenitors capable of contributing to lung injury repair. These findings strongly support the further investigation of amniotic fluid lentiviral gene delivery to achieve long-lasting gene therapy in murine models of diseases affecting the airway epithelium.

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