In Utero Lung Gene Transfer Using Adeno-Associated Viral and Lentiviral Vectors in Mice

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Abstract

Virus-mediated gene transfer to the fetal lung epithelium holds considerable promise for the therapeutic management of prenatally diagnosed, potentially life-threatening inherited lung diseases. In this study we hypothesized that efficient and life-long lung transduction can be achieved by *in utero* gene therapy, using viral vectors. To facilitate diffuse entry into the lung, viral vector was injected into the amniotic sac of C57BL/6 mice on embryonic day 16 (term, ~20 days) in a volume of 10 μ l. Vectors investigated included those based on adeno-associated virus (AAV) (serotypes 5, 6.2, 9, rh.64R1) and vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped HIV-1-based lentivirus (LV). All vectors expressed green fluorescent protein (GFP) under the transcriptional control of various promoters including chicken β -actin (CB) or cytomegalovirus (CMV) for AAV and CMV or MND (myeloproliferative sarcoma virus enhancer, negative control region deleted) for LV. Pulmonary GFP gene expression was detected by fluorescence stereoscopic microscopy and immunohistochemistry for up to 9 months after birth. At equivalent vector doses (mean, 12×10^{10} genome copies per fetus) three AAV vectors resulted in long-term (up to 9 months) pulmonary epithelium transduction. AAV2/6.2 transduced predominantly cells of the conducting airway epithelium, although transduction decreased 2 months after vector delivery. AAV2/9-transduced cells of the alveolar epithelium with a type 1 pneumocyte phenotype for up to 6 months. Although minimal levels of GFP expression were observed with AAV2/5 up to 9 months, the transduced cells immunostained positive for F480 and were retrievable by bronchoalveolar lavage, confirming an alveolar macrophage phenotype. No GFP expression was observed in lung epithelial cells after AAV2/rh.64R1 and VSV-G-LV vector-mediated gene transfer. We conclude that these experiments demonstrate that prenatal lung gene transfer with AAV vectors engineered to target pulmonary epithelial cells may provide sustained long-term levels of transgene expression, supporting the therapeutic potential of prenatal gene transfer for the treatment of congenital lung diseases.

Introduction

THE ABILITY TO TRANSFER and express therapeutic genes in the lung holds considerable potential for treatment of lethal inherited diseases including surfactant protein B deficiency, cystic fibrosis, and α_1 -antitrypsin deficiency. There are also abnormalities of lung growth (e.g., congenital diaphragmatic hernia) or lung maturation (e.g., respiratory distress syndrome of prematurity) that may benefit from strategies that achieve transient gene expression in specific pulmonary cell populations (Albelda *et al.*, 2000). Despite much effort, postnatal lung gene transfer has achieved limited

experimental and clinical success (Flotte *et al.*, 2007), primarily due to the physical and immunological barriers to lung epithelial gene transfer (Flotte *et al.*, 2007; Vareille *et al.*, 2011).

The fetal environment provides a unique opportunity to effectively transfer genes to the pulmonary epithelium while avoiding many of the obstacles of postnatal lung gene transfer. Specifically, in contrast to the postnatal lung, the early and mid-gestation fetal lung is predominantly non-ciliated (Alcorn *et al.*, 1981) and devoid of airway mucus (Olver *et al.*, 1981) and macrophages (Higashi *et al.*, 1992), and the glycocalyx has yet to develop (Faraggiana *et al.*,

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1986; Cerna *et al.*, 2002) and contains a higher proportion of stem/progenitor cells (Sekhon and Larson, 1995; Waddington *et al.*, 2003; David and Peebles, 2008). Further, the fetus is immunologically naive (Sekhon and Larson, 1995; Tran *et al.*, 2000), which may facilitate immune tolerance for transgene-encoded exogenous proteins and allow for long-term transgene expression (Tran *et al.*, 2001; Waddington *et al.*, 2003; Sabatino *et al.*, 2007; David and Peebles, 2008; Endo *et al.*, 2010). Finally, prenatal gene therapy has the potential to treat disorders before the onset of clinical manifestation.

To date, lung gene transfer in postnatal mice has been achieved with adeno-associated viral (AAV) vectors (Zabner *et al.*, 2000; Halbert *et al.*, 2001, 2007; Auricchio *et al.*, 2002; Limberis and Wilson, 2006) and lentiviral (LV) vectors (Kremer *et al.*, 2007; Buckley *et al.*, 2008). AAV vectors can target specific compartments of the lung, that is, conducting airway epithelium for AAV2/6.2 (Limberis *et al.*, 2009) and alveolar epithelium for AAV2/5 and AAV2/9 (Limberis and Wilson, 2006). More recently, fetal intratracheal, intra-amniotic (IA), and intrapulmonary injections were demonstrated to result in efficient gene transfer to airway epithelium, respiratory epithelium, and lung interstitium, respectively (Henriques-Coelho *et al.*, 2007; Buckley *et al.*, 2008; Carlon *et al.*, 2010). One limitation of these experimental studies is the relatively short follow-up postnatally (e.g., less than 1 month; Carlon *et al.*, 2010). For monogenetic lung diseases, such as cystic fibrosis, life-long lung expression is required to maintain effectiveness of *in utero* lung gene transfer.

Therefore the primary goal of this current study was to assess long-term transgene expression in mice that had received a single fetal IA injection from a panel of viral vectors with known pulmonary tropism. These included AAV vector serotypes 5, 6.2, and 9 (Zabner *et al.*, 2000; Halbert *et al.*, 2001, 2007; Auricchio *et al.*, 2002; Limberis and Wilson, 2006) and vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped LV vectors (Skarsgard *et al.*, 2005; Kremer *et al.*, 2007; Toelen *et al.*, 2007; Yu *et al.*, 2007; Buckley *et al.*, 2008), all expressing green fluorescent protein (GFP).

Materials and Methods

AAV vectors: DNA constructs and vector production

Recombinant AAV vector serotypes 5, 6.2, 9 (Zabner *et al.*, 2000; Halbert *et al.*, 2001, 2007; Auricchio *et al.*, 2002; Limberis and Wilson, 2006), or rh.64R1 (Vandenberghe *et al.*, 2009), namely, AAV2/5, AAV2/6.2, AAV2/9, and AAV2/rh64.R1 vectors, flanked with AAV2 inverted terminal repeats (ITRs) carried the enhanced GFP gene under the transcriptional control of the cytomegalovirus (CMV) or CMV-enhanced chicken β -actin (CB) promoter (Auricchio *et al.*, 2002). The AAV vectors were produced by triple transfection of HEK 293 cells with *cis*-plasmid, adenoviral helper plasmid, and a chimeric packaging construct in which the AAV2 *rep* gene is fused with *cap* genes of novel AAV serotypes (Gao *et al.*, 2002). These HEK 293 cells are of unknown origin, probably from an induced abortion (Wong, 2006). AAV vectors were produced as previously described (Gao *et al.*, 2002; Wang *et al.*, 2005; Limberis *et al.*, 2009).

Lentiviral vectors: DNA constructs and vector production

HIV-1-based LV vectors were pseudotyped with VSV-G. Vector was produced as previously described (Endo *et al.*, 2007) with some minor modifications. Modifications of the CS-CG vector included deletion of the remaining right U3 region except for 23 nucleotides downstream of the 3' polypurine tract (PPT), deletion of the residual envelope and ancillary *gag/pol* sequences, insertion of the central DNA flap, insertion of the Rev response element, and insertion of the woodchuck hepatitis virus posttranscriptional regulatory element, which is modified by eliminating the initiation codon for the woodchuck hepatitis X protein (Sena-Esteves *et al.*, 2004; Endo *et al.*, 2007).

The GFP (Clontech Laboratories/Takara Bio, Mountain View, CA) was expressed from an internal downstream-located MND2.1 (myeloproliferative sarcoma virus enhancer, negative control region deleted, *dl587rev* primer-binding site substituted) promoter or a human CMV immediate-early promoter. To the CMV-promoted vector was specifically added the transgene TaV3-nlsLacZ located downstream of the GFP gene. The LV vector constructs were named VSV-G.HIV.CMV and VSV-G.HIV.MND.

Animal models

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and breeder pairs were set up in house to achieve accurate time-dated pregnancies. Female mice were inspected daily and the day of appearance of the vaginal plug was taken as embryonic day 0, that is, day 0 postcoitum (E0). Animals were housed in the Laboratory Animal Facility of the Abramson Pediatric Research Center at the Children's Hospital of Philadelphia (Philadelphia, PA) and were maintained in standard cages and given standard laboratory chow and tap water *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Joseph Stokes, Jr. Research Institute at the Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (protocol no. 748). Of note, *E* abbreviates here the embryonic days of gestation, that is, days postcoitum, and *P* the postnatal period in days.

Intra-amniotic gene transfer on E16

This strategy of injection into the amniotic sac of pregnant mice takes advantage of the fetal breathing movements to facilitate entry of vector into the lung, enabling transduction of airway epithelial cells (Endo *et al.*, 2010). We used an automated microinjection system (IM 300; Narishige, Tokyo, Japan) for IA vector injection of an average of 10 μ l on E16. Under isoflurane anesthesia, the uterus was exposed through a full-depth 2-cm-long midline laparotomy and a uterine horn was exteriorized. Vector administration was performed by transuterine injection of vector, using a hand-pulled and beveled glass micropipette connected to the microinjection system. To stimulate the fetal breathing movements of mice on E16 and to allow increased vector transfer into the fetal lungs, a combination of IA administration of theophylline (1.6 mg/ml) and postoperative exposure of

TABLE 1. LEVELS OF FLUORESCENCE ON *D/I* SCALE

Level of fluorescence (<i>D/I</i> scale)	Density (number of fluorescent spots)	Intensity (exposure time <i>E</i> [msec])
0	No fluorescent spots	Absence: $E > 4000$
1	Non-symmetrical spots	Minimal: $E = 2000-4000$
2	Symmetrical spots = all lobes	Moderate: $E = 500-2000$
3	Symmetrical spots, diffuse and dense	Strong: $E < 500$

D, density; *I*, intensity.

the pregnant mice to elevated 10% CO₂ levels for 1 hr was used (Buckley *et al.*, 2005) for intra-amniotic gene transfer (IAGT) of AAV2/9 vector.

The laparotomy was closed in two layers, using interrupted 4-0 Vicryl sutures, and the pregnant dams were allowed to recover under a radiant warmer as previously described (Endo *et al.*, 2007, 2008).

Assessment of *in situ* gene expression by stereoscopic fluorescence microscopy analysis

We used stereoscopic fluorescence microscopy (MZ16FA; Leica Microsystems, Heerburg, Switzerland) to analyze areas of GFP expression in various organs of vector-injected mice (Endo *et al.*, 2007). In this study lungs and liver were initially examined *in situ* at serial time points after birth: (1) newborn period (P1–P7), (2) neonatal period (P8–P30), and (3) adult period ($\geq$ P30). To allow comparison of the fluorescence intensity and density, we used our laboratory semi-quantitative density/intensity (*D/I*) scale defining four levels of fluorescence from 0 to 3 (Table 1).

Immunohistochemistry analysis of gene expression

Slides of 4- to 5- μ m sections, from formalin-fixed paraffin-embedded tissue, were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed in a microwave (Ted Pella, Redding, CA) in target retrieval buffer (pH 6.0) (cat.

no. S699; Dako, Carpinteria, CA) followed by a 30-min cool-down period. Slides were placed and rinsed in TBST buffer (cat. no. 999 TT; Thermo Shandon, Fremont, CA) at room temperature for 10–20 min. After rinsing, non-specific protein binding was blocked with SuperBlock (cat. no. 375452Z; Pierce Biotechnology, Rockford, IL) for 30 min at room temperature. Immunofluorescence staining was used to evaluate and localize fluorescent GFP protein in the harvested tissues. Slides were incubated overnight at 4°C with a rabbit anti-GFP antibody (cat. no. A11122; Invitrogen, Carlsbad, CA) at a dilution of 1:400. The next day, to quench autofluorescence caused by free aldehydes, the slides were washed in 1% phosphate-buffered saline (PBS) for 10–20 min. They were then incubated with Alexa Fluor 488 FluoroNanogold Fab' fragment of goat anti-rabbit IgG (Invitrogen) for 30 min at room temperature and rinsed again in PBS for 10 min.

Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, a biological fluorescent nuclear stain; Invitrogen), dehydrated in graded alcohols, cleared in xylene, and mounted with Permount medium (Fisher Scientific, Pittsburgh, PA).

Slide sections were evaluated with a Leica DM-RE stereoscopic immunofluorescence microscope (Leica Microsystems) and images were captured with a Hamamatsu digital camera and analyzed with Openlab 4.0 software (Improvision, Boston, MA).

On each slide, bright green cells are GFP positive; blue characterizes the nuclei; and dull green, dull red, or dull yellow corresponds to cellular non-specific autofluorescence, respectively, under green, red, or combined channels of the immunofluorescence stereomicroscope.

Results

Neonatal survival rate after IAGT on E16

We injected a total of 65 pregnant C57BL/6 female mice and 486 fetal (male and female) mice on E16. The overall mean survival rate was 37% (median, 35%), which was comparable to similar studies, that is, between 40.4 and 51.9% on E16 (Endo *et al.*, 2007, 2008, 2010) but lower than the 100% survival rate of historical controls after IA injections of PBS (Mitchell *et al.*, 2000). The specific mean survival rates after each vector administration are shown in

TABLE 2. MEAN SURVIVAL RATE FOR EACH GROUP OF VECTOR-INJECTED MICE

Groups of virus-injected mice	Pregnant mice (no.) ^a	Injected fetuses (no.)	Mean viral titer injected per fetus ^b	Injection volume (μ l)	Survivors (no.)	Survival rate (%)
VSV-G.HIV.CMV (LV)	3	27	10.5×10^5	10	12	44
VSV-G.HIV.MND (LV)	3	21	5×10^5	10	7	33
AAV2/rh.64R1	5	38	14.5×10^{10} (12.5–17.5 $\times 10^{10}$)	10	9	24
AAV2/5	10	75	8.9×10^{10} (7.35–9.28 $\times 10^{10}$)	10	28	37
AAV2/6.2	15	116	8.45×10^{10} (5.9–9.09 $\times 10^{10}$)	10 (10–15)	35	30
AAV2/9	28	199	15×10^{10} (5.7–22.7 $\times 10^{10}$)	10 (5–20)	86	43
Total	64	476	—	—	177	Average: 37% Median: 35%

^aLentivirus (LV): Only three mice were injected because no lung or liver fluorescence expression was seen.

^bMean viral titers in transducing units (TU) for LV and genome copy (GC) number for AAV injected per fetus.

FIG. 1. Distribution and frequency of *in situ* intensity (*I*) of GFP expression by fluorescence stereomicroscopic analysis in lung tissue after intra-amniotic gene transfer (IAGT) on embryonic day 16 (E16). w, weeks; M, months.

Table 2. As previously reported, surviving mice appeared healthy with no clinical effects related to IAGT (Mitchell *et al.*, 2000; Endo *et al.*, 2007, 2008, 2010).

Long-term AAV2/5-mediated gene expression of alveolar macrophages

When 10 μ l of AAV2/5 (mean titer, 8.9×10^{10} GC/fetus) was injected, *in situ* lung GFP transduction was diffuse and homogeneous and persisted until 9 months postnatally, despite decreasing intensity (Figs. 1, 2, and 3A–C). Liver GFP transduction was not observed.

GFP immunohistochemistry demonstrated lung transduction that was restricted predominantly to circular interstitial alveolar cells, that is, alveolar macrophages (Fig. 3a–c). These transduced cells immunostained positive for F480 and were retrievable by bronchoalveolar lavage, confirming an alveolar macrophage phenotype and eliminating a type II pneumocyte phenotype. A few cells with alveolar type I morphology, that is, elongated cells located on the apical surface of distal airspaces, were also transduced, albeit at low intensity (Fig. 3b and c).

Mid-term AAV2/6.2-mediated gene expression of airway epithelial cells and alveolar macrophages

After IA injections of 10 μ l of AAV2/6.2 vector (mean titer, 8.45×10^{10} GC/fetus), *in situ* lung transduction was homogeneous but diffuse (Fig. 4A and B), and persisted

until 2 months of age at moderate intensity ($I=2$; Fig. 4C). The intensity of gene expression resulted in a descending curve: strong ($I=3$) plateauing until 1 month, and decreasing to moderate ($I=2$) at 2 months, the end of the experiment (Figs. 1 and 2). GFP expression was not detected in the liver at any time point examined.

GFP immunohistochemistry confirmed the transduction profile of AAV2/6.2 vector, with tropism for alveolar macrophages as well as bronchial epithelial cells of ciliated morphology (Fig. 4a–c). The distal bronchi were transduced, showing diffuse but relatively persistent gene expression for up to 8 weeks (Fig. 4c). Because of the tropism of this vector for alveolar macrophages from birth and because our specific research interest is directed at transduction of cells of the respiratory tract, we did not perform analysis beyond 2 months of age.

Long-term AAV2/9-mediated gene expression of alveolar type I-like pneumocytes

IAGT of 10 μ l of AAV2/9 vector (mean viral titer, 15×10^{10} GC/fetus) was performed. *In situ* lung transduction was even and homogeneous (density $D=2$; Figs. 2 and 5A and B), and persisted for at least 6 months with moderate intensity ($I=2$; Figs. 1, 2, and 5A–D). Analysis of the intensity of gene expression revealed a bell-shaped curve: ascending to reach a plateau of moderate intensity ($I=2$) from 2 to 4 weeks, and then descending to minimal intensity

FIG. 2. Distribution and frequency of *in situ* density (*D*) of GFP expression by fluorescence stereomicroscopic analysis in lung tissue after IAGT on E16.

($I=1$) at 6 months (Fig. 1). No liver fluorescence expression was observed at any time point examined.

GFP immunohistochemistry confirmed lung transduction for up to 6 months and revealed a specific tropism of AAV2/9 for alveolar type I-like pneumocytes at minimal intensity and density (Fig. 5a–d).

AAV2/rh.64R1 does not transduce lung and liver tissue

IA injections of $10\mu\text{l}$ of AAV2/rh.64R1 (mean titer, 14.5×10^{10} GC/fetus) were performed ($n=38$ fetuses). At 1 month postnatally no gene expression *in situ* was observed in the lungs or liver of any animals (Figs. 1 and 2).

VSV-G-pseudotyped LV vector does not transduce lung and liver tissue

IAGT of $10\mu\text{l}$ of VSV-G.HIV.CMV.GFP vector and VSV-G.HIV.MND.GFP vector was performed, using mean titers of 1.05×10^6 and 5×10^5 transducing units (TU)/fetus, respectively (Table 2). No lung or liver GFP expression was seen in either group at 1 week postnatally (Figs. 1 and 2).

Discussion

We examined the durability of GFP transgene expression in murine lung after IAGT of four AAV vectors with airway tropism (AAVs 5, 6.2, rh.64R1, and 9) and two VSV-G.HIV LV vectors. We found that AAVs 5, 6.2, and 9 can transduce cells of the fetal murine lung whereas neither AAV2/rh.64R1 nor the VSV-G.HIV.CMV and VSV-G.HIV.MND LV vectors transduced fetal lung. Our results suggest that despite GFP expression declining shortly after birth, sustained transgene expression was achieved postnatally by IAGT on E16, using AAV vectors. In particular, the AAV2/5 vector resulted in sustained transgene expression that was maintained for up to 9 months postnatally. These results are in accordance with previous studies demonstrating that the IA injection on E15/16 of AAV2 encoding GFP results in stable gene expression during the first year of life (Garrett *et al.*, 2003). The other AAV serotypes studied here also exhibited distinct tropism profiles for different lung compartments: AAV5 for alveolar macrophages up to 9 months, AAV6.2 for airway epithelial cells and alveolar macrophages up to 2 months, and AAV9 for alveolar type I-like pneumocytes up to 6 months.

Several routes of prenatal vector administration have been used to achieve fetal gene transfer to the lung including systemic (Schachtner *et al.*, 1999; Waddington *et al.*, 2003), intratracheal (Skarsgard *et al.*, 2005; Carlon *et al.*, 2010), and intrapulmonary (Boyle *et al.*, 2001; Tarantal *et al.*, 2001) delivery. Although less specific than the intratracheal or intrapulmonary route, the ability to achieve long-term gene transfer by a simple fetal IA injection provides an important tool for the exploration of potential therapeutic strategies for lung disorders as well as for biological studies exploring the mechanism of action of specific genes on development. The obvious advantages of IAGT include ease of manipulation, minimal expense, and the availability of well-characterized murine models of human lung disease. Nevertheless, one major limitation of IAGT on E16 is the relatively high fetal loss of more than

50% in mice (Endo *et al.*, 2007, 2008, 2010). The low survival rate after IAGT appears to be a result of the rupture of the amnion during the surgical procedure caused by the high volume ($10\mu\text{l}$) injected rather than vector toxicity (Endo *et al.*, 2007, 2008), theophylline injection, or CO_2 exposure. In fact, vectors that did not show GFP expression had the same survival rate as vectors that showed expression. Moreover, IA theophylline injection was performed only for AAV2/9 vector and did not modify the survival rate.

The gradual loss of AAV lung transgene expression during the follow-up evaluation period may be explained at least in part by innate and adapted immunity (for AAV2/5 and AAV2/6.2), and the turnover of transduced airway or alveolar epithelium (for AAV2/6.2 and AAV2/9, respectively). Of note, murine ciliated airway epithelial cells have an average half-life of 6 months in the trachea and 17 months in the lungs (Rawlins and Hogan, 2008). The variable duration and level of lung gene expression after fetal injection of AAV depends on several factors that include serotype, nature of the transgene product, vector dose, route of administration, kinetics of expression, host species, and preexisting immunity to AAV (Vandenberghe and Wilson, 2007). The gradual loss of gene expression over time in our study appears paradoxical given that (1) AAV vectors can, at least in nondividing cells, generate long-term transgene expression because of their ability to express themselves throughout the full life cycle of a cell by remaining episomal and rarely integrating into host cell DNA (Garrett *et al.*, 2004); and (2) they display relatively low immunogenicity (Rapti *et al.*, 2012). The absence of AAV genome integration in the lung, which has high fetal cell turnover at the transition from the canalicular to the terminal saccular stage of development (De Paepe *et al.*, 2000) and medium postnatal cell turnover lasting several days to weeks when injured (Crosby and Waters, 2010), may impact on long-term transduction. Moreover, adapted immunity may effectively clear AAV-transduced cells from the lung. Rapti and colleagues have demonstrated that even naive C57BL/6 mice purchased from commercial vendors harbored neutralizing antibodies against AAV1 and AAV6 serotypes (Rapti *et al.*, 2012). Notwithstanding, modest inhibition of AAV2 transduction by individual murine sera was the result of unidentified serum factors and was not due to preexisting neutralizing antibodies (Rapti *et al.*, 2012). In our study, AAV2/5- and AAV2/6.2-mediated expression could have resulted from postnatal phagocytosis of transduced cells by macrophages.

We demonstrated that VSV-G.HIV-mediated gene transfer in lung via IA delivery did not result in transduction. Previous studies in adult mice concluded that a single dose of VSV-G.HIV vector, expressing the cystic fibrosis transmembrane conductance regulator, delivered intranasally produced immediate as well as lifetime airway expression (Kremer *et al.*, 2007; Stocker *et al.*, 2009). Similarly, fetal rat studies demonstrated long-term (7 months) transduction of the lung parenchyma only after intrapulmonary injection of the VSV-G.HIV vector (Toelen *et al.*, 2007). Mid-gestation fetal lambs exhibited low transduction of the more distal bronchiolar airway epithelium after intratracheal injection of VSV-G.HIV vector (Yu *et al.*, 2007). In fetal rhesus monkeys, intrapulmonary

administration of VSV-G.HIV vector resulted in transfection of pulmonary epithelium at birth (Tarantal *et al.*, 2001). In contrast, after IA injections of VSV-G.HIV vector in fetal mice no lung transduction was observed (Buckley *et al.*, 2008), while long-term (~ 1 year) airway transduction was demonstrated when using the baculovirus gp64-pseudotyped LV vector (Buckley *et al.*, 2008), and in fetal rabbit lung transduction of tracheobronchial epithelium was seen within 4 days of delivery until 1 month (Skarsgard *et al.*, 2005). Mishra and colleagues demonstrated that IAGT of VSV-G-pseudotyped LV vector on E14.5 or E16.5 resulted in long-term (7 months) transduction of epithelial cells of the small and large airways as well as transduction of long-lived respiratory epithelial progenitors with the ability to repair lung injury, suggesting a fetal development lung tropism for this vector (Mishra *et al.*, 2011).

Although our results support the notion of IAGT, using lung-targeting AAV vectors to achieve moderate levels of long-lasting pulmonary gene expression, several limitations need to be acknowledged. The main aim of the present study was to qualitatively assess the durability of GFP transgene expression; hence we did not quantitatively assess GFP expression. Further, as predicted from the stage of murine immune system development on E16, we saw no overt inflammatory response in the lung by histological analysis. However, as the purpose of this study was not to induce immune tolerance, we did not perform a detailed analysis of immune response to the AAV vector products or transgene. Moreover, our data are in agreement with other published reports (Buckley *et al.*, 2005) suggesting that efficient transduction of pulmonary epithelium after IA delivery of vector is limited to a narrow gestational win-

dow related to the onset of fetal respiratory movements. Human fetal breathing movements can be observed from 10 weeks of gestation onward, with an increase in incidence thereafter (de Vries *et al.*, 1985; Cosmi *et al.*, 2003). From a therapeutic perspective, IAGT of lung-targeting vectors could therefore be used to induce immunological tolerance to vector/transgene before the development of a potential immune response at approximately 14 weeks of gestation. Finally, while dilution of the vector in the large volume of amniotic fluid in the human is a potential hurdle, the volume at 10 weeks of gestation is approximately 25 ml and increases to 100 ml at 13 weeks of gestation (Weissman *et al.*, 1996), making delivery of AAV vector practically feasible.

Many challenging issues, including vector safety and complex ethical issues, need to be addressed before a vectorized approach is considered for therapeutic treatment. AAV-based vectors have a number of advantages including high production yields, transduction of dividing and non-dividing cells, and absence of integration into the host genome and therefore less concern about germ-line transduction/insertional mutagenesis/bystander developmental effects. Our experiments provide proof-of-concept that *in utero* lung gene therapy via a simple IA approach, using lung-targeting AAV, can achieve efficient and durable transgene expression. Provided that a similar AAV9 vector transduction profile applies to human airway cells and safe delivery techniques are developed, it might become possible to offer early fetal gene therapy in conjunction with prenatal diagnosis as an alternative preventive therapy for fetuses with a risk of lung immaturity, bronchopulmonary dysplasia, α_1 -antitrypsin deficiency, and cystic fibrosis.

FIG. 4. AAV2/6.2 lung gene expression up to 2 months postnatally. (A–C) Fluorescence stereomicroscopic images of lungs showing diffuse transduction of lung parenchyma (A) and trachea (B and B') at 1 month at strong intensity and of lung parenchyma at 2 months at moderate intensity (C). Original magnification: (A–B') 8-, 20-, and 20-fold, respectively; (C) 20-fold. (a–c) Immunohistochemical GFP analysis demonstrating tropism for alveolar macrophages and bronchial epithelial cells that express GFP at 1 month (a and b) and 2 months (c). Original magnification: (a and b) terminal bronchioles at 400 and 1000-fold, respectively; (c) terminal bronchioles at 200-fold. On each slide, bright green cells are GFP positive; blue characterizes the nuclei; and dull green, dull red, or dull yellow corresponds to cellular non-specific autofluorescence.

FIG. 5. AAV2/9 lung gene expression up to 6 months postnatally. (A–D) Fluorescence stereomicroscopic images showing diffuse lung transduction at 2 weeks at moderate intensity and density (A and B), 1 month at moderate intensity and density (C), and 6 months at minimal intensity and moderate density (D). Original magnification: (A and B) 7- and 33-fold, respectively; (C) 33-fold; (D) 50-fold. (a–d) Immunohistochemical GFP analysis of lungs demonstrating tropism for alveolar type I-like pneumocytes that express GFP at 2 weeks at moderate intensity and density (a and b), 1 month at moderate intensity and density (c), and 6 months at minimal intensity and density (d). Original magnification: (a and b) 50- and 100-fold, respectively; (c) 400-fold; and (d) 400-fold. On each slide, bright green cells are GFP positive; blue characterizes the nuclei; and dull green, dull red, or dull yellow corresponds to cellular non-specific autofluorescence.

FIG. 3. AAV2/5 lung gene expression up to 9 months postnatally. (A–C) Fluorescence stereomicroscopy images of lungs showing diffuse transduction at 1 month at moderate intensity (A and B) and at 9 months at minimal intensity (C). Original magnification: (A and B) 7- and 24-fold, respectively; (C) 32-fold. (a–c) Immunohistochemical GFP analysis of bronchioles and alveoli, demonstrating tropism for alveolar macrophages that express GFP at 1 month (a and b) and 9 months (c). Original magnification: (a and b) terminal bronchioles at 200-fold; (c) alveoli at 400-fold. These transduced circular interstitial alveolar cells immunostained positive for F480 and were retrievable by bronchoalveolar lavage, confirming an alveolar macrophage phenotype and eliminating a type II pneumocyte phenotype. On each slide, bright green cells are GFP positive; blue characterizes the nuclei; and dull green, dull red, or dull yellow corresponds to cellular non-specific autofluorescence.

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