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ARTICLE

In Utero AAV-Mediated Gene Transfer to Rabbit Pulmonary Epithelium

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In utero **intra-amniotic administration of adeno-associated virus (AAV) for treatment of cystic fibrosis (CF) has the potential to be an efficient way to target the rapidly dividing undifferentiated cells of the fetal pulmonary epithelium, while simultaneously treating other tissues involved in CF (such as the intestines), but has never before been studied. Intra-amniotic administration of 1×10^{12} particles of AAV-luciferase vector to 110 fetal rabbits at 24–25 days gestation resulted in transgene expression in amniotic membranes, trachea, and pulmonary epithelium. The highest level of transgene expression was found in amniotic membranes. Transgene expression peaked in the lungs 10 days after vector delivery, decreased at day 17, and was no longer detectable after 24 days. The number of pulmonary cells transduced was approximately 1 in 500 and immunohistochemical analysis showed expression in varying cell types, including alveolar cells. Transgene expression was not detected in fetal rabbit intestines, skin or liver, nor in maternal ovaries or liver. Intra-amniotic administration of AAV does not result in the tissue inflammation and fetal loss previously documented with *in utero* adenoviral administration, and results in high levels of transgene expression in amniotic membranes with lower levels in fetal pulmonary epithelium.**

Key Words: adeno-associated virus (AAV), *in utero*, gene therapy, luciferase, cystic fibrosis

INTRODUCTION

Despite substantial progress in the development of gene replacement therapy for treatment of cystic fibrosis (CF) and other genetic disorders, difficulty with vector entry and transduction of pulmonary epithelia [1], low level and short duration of transgene expression, and immunologic response to dosing remain persistent obstacles [2]. In CF other barriers have also been identified: both mucus [1] and the inflammatory mediators present in CF bronchial washings [3] decrease vector efficacy. Numerous strategies have been proposed to address these obstacles, but one relatively new approach is the use of *in utero* gene replacement to potentially increase efficacy and duration of transgene expression. Successful *in utero* gene transfer has been demonstrated in several settings, although its effect on duration of transgene expression and degree of immunologic response is not well established. CF is a particularly inviting target disease for the development of *in utero* gene therapy because amniotic fluid circulation provides vector exposure to pulmonary [4], gastrointestinal [5], and sinus epithelia, all primary sites of CF pathology. Previous work [6–8] demonstrated that vector introduced into amniotic fluid of mice, rats, and sheep results in reporter gene expression in both pulmonary and gastrointestinal epithelia. Transgene expression was also demonstrated in pulmonary epithelia after intratracheal instillation of vector *in utero* [9–12]. Persistence of transgene expression in the lung ranged from 14 to 30 days postinfection. Adenoviral vectors have been used in most *in utero* investigations, except those using retroviral vectors [6,11]. Although having the advantage of being able to be concentrated in high titers and providing efficient gene transfer, adenoviral vectors may lead to substantial inflammation and even fetal loss [8–11,13]. Retroviral vector use in *in utero* applications has been limited because retroviral infectivity is reduced by amniotic fluid [14].

Adeno-associated virus (AAV), a replication-deficient, nonpathogenic parvovirus, generates little inflammatory response, does not require cell division, and has the potential for long-term transgene expression through stable integration and episomal persistence [15]. Previous work, however, suggests that AAV gene transfer to respiratory epithelia is limited by vector entry and postentry interactions of cellular components with vector DNA [16]. In particular, well-differentiated pulmonary epithelial cells may lack the factors necessary for efficient AAV-mediated gene

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expression [1]. AAV-mediated gene expression is much more efficient in undifferentiated pulmonary cells [1]. These observations suggest that one strategy for generating higher degrees of vector entry and gene expression in AAV may be targeting rapidly dividing undifferentiated epithelial cells with an *in utero* or neonatal approach. The neonatal approach has been studied [17,18], but studies of *in utero* administration of AAV are limited to recent investigations of the effect of intraperitoneal and intramuscular delivery in a mouse model [19,20]. This work revealed the potential for long-term AAV-mediated transgene expression after *in utero* delivery, demonstrating persistence of transgene 18 months after intraperitoneal injection [19] and 3 months after intramuscular injection [20]. Intra-amniotic administration of AAV *in utero* for pulmonary applications has not been investigated.

The two primary goals of our study were to investigate for the first time the efficacy of intra-amniotic *in utero* AAV-mediated gene therapy and to identify potential advantages and disadvantages of *in utero* administration of AAV. Our chief hypothesis was that the efficacy of AAV gene transfer and the duration of transgene expression would be increased by targeting the undifferentiated, rapidly dividing cells of the fetal pulmonary epithelia.

RESULTS

Intra-amniotic Dye Study

Previous work has demonstrated that particles injected into amniotic fluid will be deposited in fetal pulmonary and gastrointestinal epithelium [4]. Before proceeding to vector instillation, we documented our ability to safely access amniotic fluid of individual fetal rabbits and expose their lungs and gastrointestinal tract to instilled material. Two pregnant rabbits at 24 days gestation underwent surgery and each individual fetus was injected intra-amniotically with 200 μl of 1% Fast Green in sterile normal saline (Fig. 1A). The litters were harvested two hours later and the fetuses examined. Within two hours of injection, the Fast Green had filled the amniotic sac and was present in the lungs (Figs. 1B and 1C).

Experimental Design, Size, and Fetal Mortality

A total of 15 litters and 110 fetal rabbits were treated with intra-amniotic AAV-luc vector or vehicle alone at 24 or 25 days gestation, and harvested 3, 10, 17, or 24 days later (Table 1). Two litters were spontaneously aborted within hours of surgery due to surgical complications (one control and one vector) and one litter was lost after the mother failed to build a nest (vector). Among all other litters, 70 of 88 fetuses survived until the scheduled time of harvest, with there being no significant difference in mortality between the control group (6/35, 17%) and the vector group (12/53, 22%; $\chi^2 = 0.53$). None of the animals demonstrated evidence of respiratory compromise in the perinatal period or later.

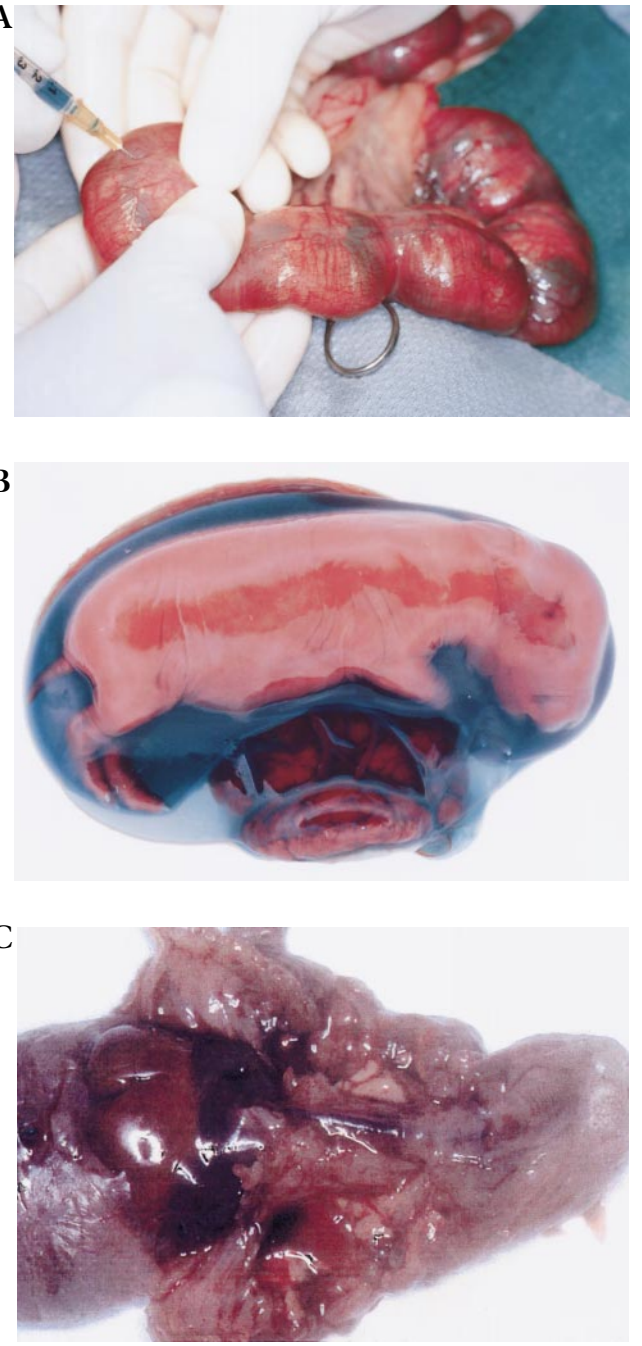

FIG. 1. Delivery *in utero* into amniotic fluid. (A) Rabbit litters accessed by elevation through midline incision on gestational day 25 and dye-marked vector or vehicle administered intra-amniotically to each individual fetus with 25-gauge needle. (B) Dye is clearly visible in the amniotic sac 2 h after injection. (C) Subsequent dissection demonstrated the presence of dye-marked instillate in trachea and lungs.

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Extent and Duration of Expression of AAV-luc in Fetal Trachea and Lungs

Luciferase expression was detectable by luminometry in lung and tracheal tissue homogenates of fetal rabbits receiving AAV-luc. The levels detected were significantly higher than those in fetuses treated with control solution alone (Figs. 2A and 2B). At three days after vector instillation, the lungs revealed a low level of transgene expression, with the vector group having 0.57 ± 0.15 pg luciferase/mg (n = 10) and the control lungs having 0.02 ± 0.02 pg luciferase/mg (n = 7; P = 0.003). Tracheal expression of luciferase after three days was threefold higher than in lung tissue (1.68 ± 0.5 pg luciferase/mg; n = 14). Luciferase expression peaked in both trachea and lung tissue at day 10 after vector administration. The tracheas receiving vector had 3.40 ± 1.08 pg luciferase/mg (n = 8), whereas those receiving vehicle alone had 0.14 ± 0.05 pg luciferase/mg (n = 5). The lungs receiving vector averaged 1.63 ± 0.50 pg luciferase/mg of lung (n = 8) and control lungs averaged 0.29 ± 0.09 pg luciferase/mg (n = 7; P = 0.02). Luciferase expression decreased at day 17 and, by day 24, luciferase expression in vector-treated tissues did not differ significantly from levels seen in control tissues (Fig. 2).

Immunohistochemistry confirmed the presence of luciferase expression in lungs and trachea at 10 days. Even at peak expression, only approximately 1 in 500 cells expressed luciferase, with much of the expression in lungs occurring in alveoli (Figs. 3A and 3B). Luciferase expression was not detected by immunohistochemistry in trachea or lungs at days 17 and 24, suggesting more sensitivity by enzymatic detection (luminometry) than by immunolocalization. PCR with luciferase-specific primers demonstrated vector DNA presence in both lungs and trachea at days 3 and 10 (Fig. 4). By day 24, vector DNA was not detectable in either trachea or lungs by PCR (data not shown).

Table 1: Harvest times for *in utero* AAV-luciferase and control

Harvest time[a]	Control	Vector
3 days post delivery	10 fetuses (2 litters)	14 fetuses (2 litters)
10 days post delivery	11 rabbits (2 litters)	17 rabbits (2 litters)
17 days post delivery	4 rabbits (1 litter)	4 rabbits (1 litter)
24 days post delivery	4 rabbits (1 litter)	6 rabbits (1 litter)
Totals[b]	29 fetuses (6 litters)	41 fetuses (6 litters)

[a]All fetuses received vector or control solution on day 24 or 25 of the 31-day gestation.
[b]Three other litters were lost: two secondary to surgical complications and one not nursed by the mother.

Extent and Duration of AAV-luc Expression in Other Tissues

The tissues with highest expression three days after vector instillation were amniotic membranes. The membranes exposed to vector had on average 5.80 ± 0.82 pg luciferase/mg (n = 13) at day 3, with two of the membranes having the highest expression of luciferase of any tissue at any time point (11.2 and 7.9 pg luciferase/mg). None of the control membranes demonstrated significant luciferase activity (0.04 ± 0.03 pg luciferase/mg, n = 3). Amniotic membranes were not available for testing at day 10 because they were eaten by the dam at the time of delivery (delivery usually occurred approximately 5 to 6

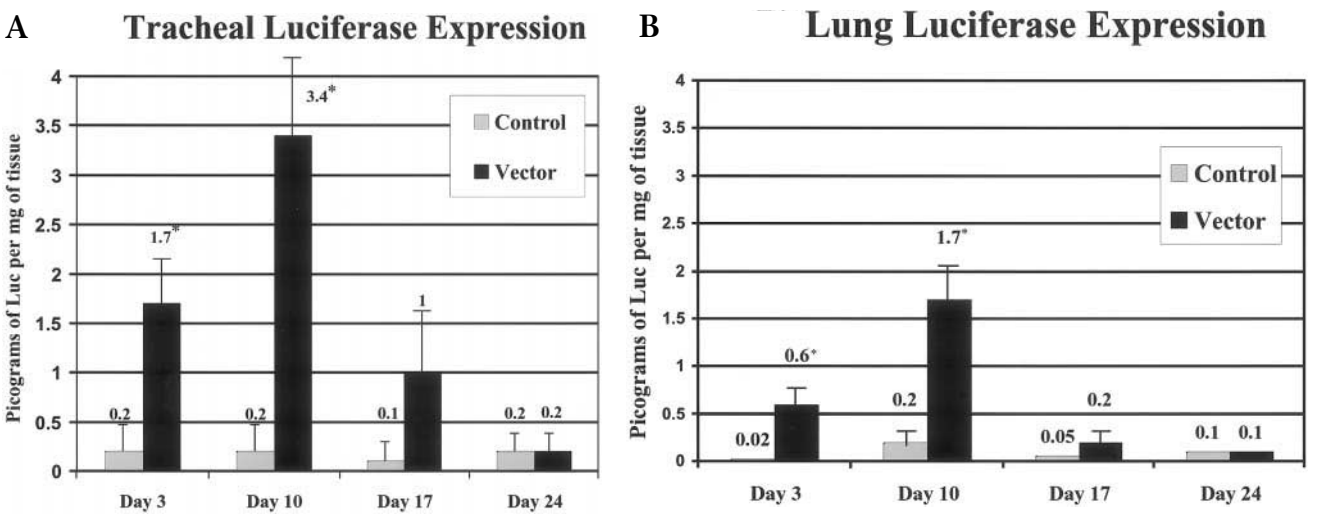

FIG. 2. Time course of luciferase expression in trachea and lungs. (A and B) Tracheal and lung luciferase expression peaked at 10 days after vector administration. Absolute amount of luciferase per mg of tissue was calculated using a standard curve from recombinant luciferase to convert luminometry light units to pg of luciferase. *Significantly different from control, $P < 0.05$.

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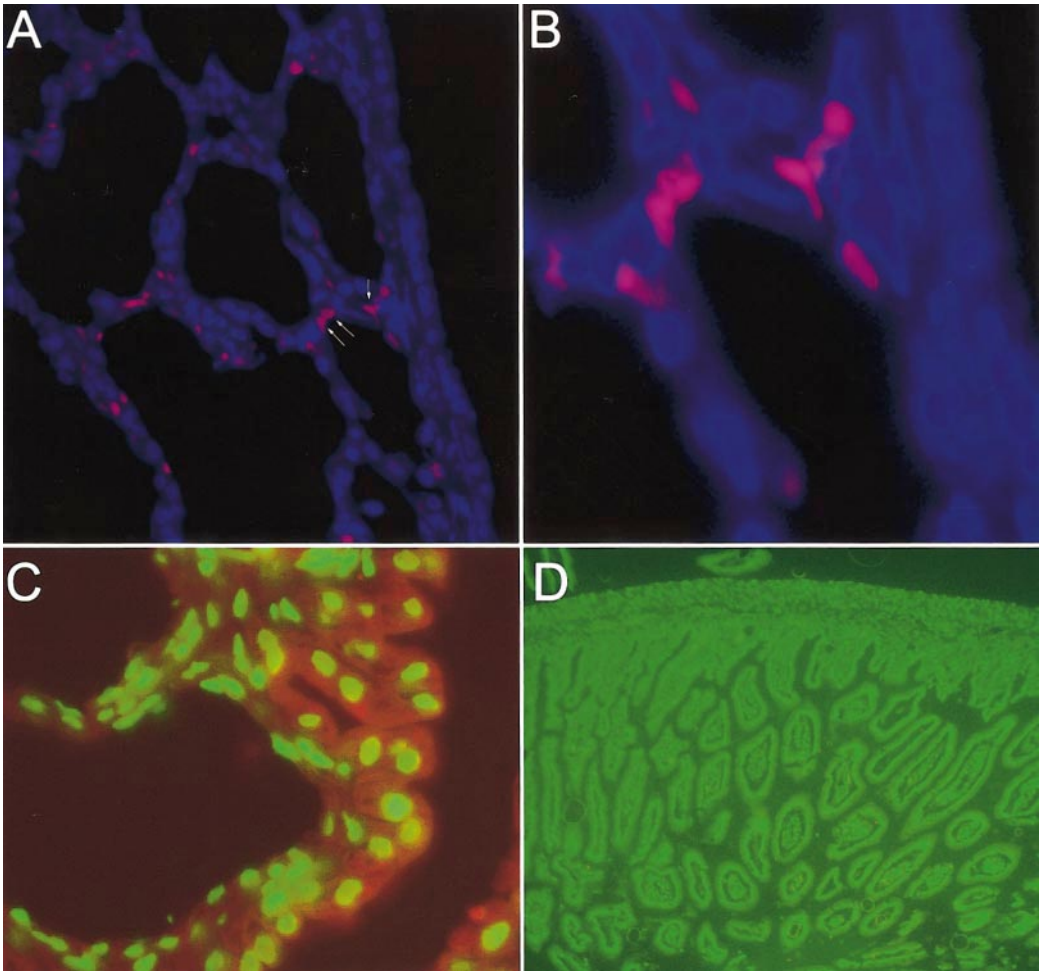

FIG. 3. Immunohistochemistry: Cy3 secondary antibody detection of luciferase expression. DAPI stain was used for nuclear counterstain. (A) Fetal rabbit lung 10 days after administration of vector demonstrating alveolar luciferase expression (magnification, ×400). (B) Fetal rabbit lung 10 days after administration of vector (magnification, ×1000). (C) Amniotic membrane three days after administration of vector demonstrating luciferase expression. (D) Fetal rabbit intestine 10 days after vector administration demonstrating no evidence of luciferase expression.

days after vector delivery). Immunohistochemistry confirmed the high level of transgene expression in amniotic membranes (Fig. 3C).

Despite evidence of the Fast Green marker in the stomach and distal intestines of rabbits at the time of harvest, no luciferase expression was detected by luminometry or immunohistochemistry at any time point in fetal rabbit small intestines (Fig. 3D). PCR of proximal intestinal tissue at days 3 and 10 also failed to reveal any evidence of vector (data not shown). Evaluation of fetal rabbit skin and liver by luminometry and immunohistochemistry also failed to demonstrate luciferase expression at any time point.

Vector Effect on Maternal Ovaries and Liver

There was no evidence of vector DNA presence by PCR in homogenates of maternal livers or ovaries (data not shown). There was also no evidence of transgene expression by luminometry analysis of maternal ovary and liver homogenates. Immunohistochemistry of these tissues was not performed.

DISCUSSION

Because of the obstacles to efficient transgene expression in mature pulmonary epithelium, there is currently a need for new strategies to improve both vector entry and transgene expression. In pulmonary gene replacement intended for treatment of CF, strategies to avoid the barriers to gene transfer of mucus and inflammation are also needed. An approach which would improve vector efficacy by targeting the rapidly dividing undifferentiated cells of the fetal pulmonary epithelium before the onset of infection and inflammation would be an attractive one. *In utero* intra-amniotic administration of vector for treatment of CF has the potential to improve vector efficacy by both of these strategies while simultaneously targeting other tissues which demonstrate pathology secondary to CFTR dysfunction: intestines and nasal epithelium. Use of AAV in this setting may be particularly advantageous because of its safety profile and tropism for respiratory and gastrointestinal epithelia.

Our initial experiments demonstrated the ability to successfully access lungs and intestines by intra-amniotic

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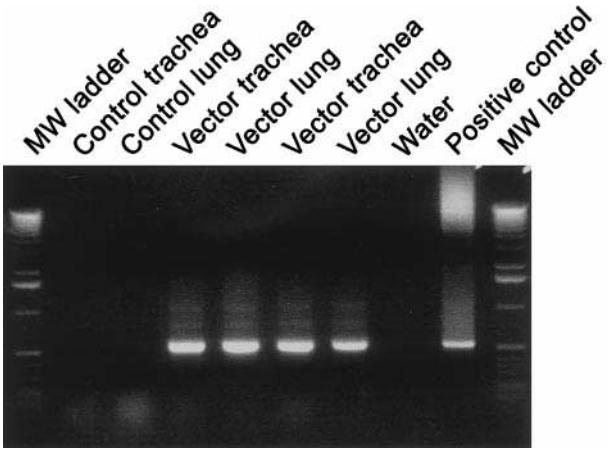

FIG. 4. PCR detection of luciferase DNA. Trachea and lung had detectable levels of luciferase DNA at 10 days after vector administration. Both trachea and lung were tested from two different vector-treated animals. PCR was performed using unique luciferase primers for 30 cycles with B-actin control for loading. At day 24, vector DNA was no longer detectable (data not shown).

innocula. We chose the lagomorph species to test this delivery of intra-amniotic AAV-luciferase partly because the size allowed for easier surgery, but also because it would allow comparison with our previous results with neonatal intra-tracheal AAV delivery [17].

Delivery of high titer AAV (1×10^{12} AAV particles) into amniotic fluid of individual fetal rabbits resulted in detectable transgene expression at three days in amniotic membranes, trachea, and pulmonary epithelia. This tracheal and pulmonary expression peaked at day 10, declined at day 17, and was no longer detectable by day 24. Transfection was visible in approximately 1 in 500 cells, with the regions of greatest transfection percentage occurring in the alveoli, including type II pneumocytes. Transgene expression was not detectable in skin, gastrointestinal, or liver tissue at any time point. Although one frequent concern with *in utero* delivery of gene replacement therapy has been the potential for vector leakage into the maternal circulation, this investigation demonstrated no evidence of transmission of vector DNA to maternal ovaries or liver.

Although transgene expression can be generated in pulmonary epithelium after intra-amniotic administration of AAV, the late gestational *in utero* administration of AAV does not seem to offer a significant advantage over AAV administration in the neonatal period. The percentage transfection observed in this experiment is lower than that seen in our experience after intratracheal delivery of 10^{10} particles of AAV (10% of the current dose) in the neonatal period in the same rabbit model [17]. Duration of detectable transgene expression was similar to the 21 days seen after intratracheal neonatal delivery [17]. In our experiment we did not see evidence for AAV-mediated transgene expression in another key site of CF pathology, the intestines. Intra-amniotic administration of AAV seems to provide greatest levels of transgene expression in amniotic membranes, with lower levels in the trachea and pulmonary epithelia and little, if any, in intestines, liver, and skin.

An important observation is that alveolar cell expression accounts for a significant amount of the transgene expression seen after late gestational intra-amniotic delivery of AAV, including type II pneumocytes. Type II pneumocytes are the one pulmonary cell type still undergoing differentiation during the saccular phase of lung development (the period during which this vector was delivered). This result is consistent with the observation that cells still undergoing differentiation are more susceptible to vector entry and transgene expression, and suggests that proximal airway cells may be more efficiently targeted earlier in gestation during their differentiation in the canalicular and pseudoglandular periods of lung development.

The percentage of pulmonary cells transfected and the duration of transgene expression observed in this experiment suggest that, although fetal pulmonary epithelium is an inviting target, late gestation intra-amniotic delivery of vector cannot be considered a particularly efficient method for AAV-mediated transgene delivery to the lungs or intestines. This low efficiency of intra-amniotic delivery of AAV is in contrast to the long-term gene transfer after intraperitoneal and intramuscular *in utero* injections of AAV (18 months and 3 months, respectively [19,20]). While some of the observed differences in expression may be due to promoter influences and tissue-specific ubiquitination of viral capsid proteins, the differences also provide support for the observation of a significantly lower transduction efficiency of rAAV at the apical surface of airway epithelial cells [1,16,21].

So while some known barriers in CF to vector entry may be avoided by the intra-amniotic approach, there are other potential barriers which may contribute to the decreased vector efficacy seen with intra-amniotic delivery of AAV. First is the resistance of well-differentiated airway epithelia to rAAV infection from the apical surface. Our finding of transgene expression in only the still-differentiating airway cells (type II pneumocytes) indicates that this resistance of well-differentiated cells may have a role. Administration of intra-amniotic AAV earlier in gestation may be a strategy to increase AAV efficacy. Second is the potential role of amniotic fluid as a barrier. Previous work has demonstrated that amniotic fluid reduces the infectivity of retroviral vectors [22]. Amniotic fluid effect on AAV has not been investigated. While the degree of transgene expression demonstrated in the amniotic membranes suggest that amniotic fluid is not an insurmountable barrier, identification of constituents in amniotic fluid that might inhibit vector infectivity might lead to interventions designed to limit amniotic fluid's effect on AAV. A third potential barrier to the success of intra-amniotic administration of vector is the dilutional effect. From our

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initial experiments, we found that administration of more than 200 μl of volume into the rabbit amniotic sac increased the risk of fetal compromise. Because of the current titer limitations of AAV, the total particle dose per fetus was limited to 1×10^{12} total particles. These particles were further diluted by the over 1 ml of amniotic fluid volume in each fetus. Continued improvements in titer concentrations may allow this dilutional obstacle to be overcome in the future. Fetoscopic techniques designed to deliver vector directly into fetal lung may overcome some of the dilutional obstacle, but would remove the potential advantages of exposure of gastrointestinal and sinus epithelia to vector. Lastly, the expanding population of cells of the pulmonary epithelia during fetal development may result in cellular dilution of transduced cells and loss of episomal AAV genomes when there is failure to stably tranduct pulmonary stem cells. This loss of vector DNA in rapidly expanding cell populations was observed in an *in utero* investigation [19] and was thought to contribute to the failure to detect persistence of vector DNA in hematopoetic cells. This may also be the explanation for our inability to detect AAV vector DNA beyond 24 days. Identification of the time period during fetal lung development in which pulmonary stem cells are most susceptible to stable transduction may help circumvent this problem. Almost without exception, *in utero* gene replacement investigations so far have only looked at vector administered at 80% of gestation (because size limitations have made successful delivery earlier in gestation difficult). Vector administration in earlier time points in gestation may be required to best target stem cells.

Our investigation of intra-amniotic delivery of an adeno-associated viral vector demonstrates the potential for pulmonary epithelial expression after *in utero* delivery. Intra-amniotic AAV administration does not result in the tissue inflammation and fetal loss previously documented with *in utero* adenoviral administration, and mediates highest levels of transgene expression in amniotic membranes. Transgene expression evident in pulmonary epithelia after intra-amniotic AAV administration is encouraging, but this animal model suggests that late gestational *in utero* administration of AAV does not seem to offer a significant expression advantage over administration in the neonatal period. Further improvement in AAV efficacy after intra-amniotic delivery is required for it to become a viable treatment strategy for congenital lung disease.

MATERIALS AND METHODS

Construction of recombinant AAV vector. We selected luciferase as our reporter gene to enable quantitation of protein expression and provide unambiguous immunocytochemical localization. A series of constructs were made by inserting small promoters 5′ to the luciferase reporter gene and subcloning into AAV vector pTR-UF5. These constructs were pGL3+ (SV40 promoter alone), pGL3+E (SV40 promoter along with an enhancer region), p237CLC2-luc (a *CLCN2* promoter fragment found to mediate high-level reporter gene expression in pulmonary epithelial cell lines [23]), and pGL3– (promoterless control). Because pGL3+E consistently directed the highest level of luciferase expression after lipofectamine introduction into pulmonary epithelial cell lines FHTE (a fetal human tracheal epithelial cell line), IB3-1 (a human CF bronchial epithelial cell line), and preII-19 (a rat fetal type II cell line), we selected it for packaging in AAV using a cotransfection technique [24]. We infected 293 cells with adenovirus type 5 and then cotransfected them with vector plasmid and packaging pAAV/Ad. Cells were grown for 3–4 d, collected, lysed, and then purified using CsCl density gradient ultracentrifugation. Fractions were dialyzed and heat-treated to inactivate any possible residual adenovirus. Titer was determined by PCR, with vector particle titers ranging from approximately 5×10^{12} to 1×10^{13} DNase-resistant particles per ml. After packaging, AAV-pGL3+E (AAV-luc) efficacy was demonstrated by luciferase expression in infected cell lines by both luminometry and immunohistochemistry (data not shown).

In utero administration of vector in fetal rabbits. New Zealand white rabbits 24 d into the 31-d gestational period were anesthetized with ketamine (35 mg/kg) and acepromazine (0.75 mg/kg), then intubated and mechanically ventilated with a mixture of oxygen, halothane, and nitrous oxide. Under sterile conditions, the uterus of each animal was exposed with a midline abdominal incision and the fetuses gently raised out of the abdominal cavity so that each individual amniotic sac could be accessed with a 25-gauge needle (Fig. 1A). Each individual fetus then received 200 μl of either control solution (normal saline/1% Fast Green FCF Stain) or vector (10^{12} AAV-luc particles in normal saline/1% Fast Green). Correct placement of each injection was confirmed visually by the dispersion of green fluid in the amniotic sac. Fetuses were then returned to the abdominal cavity and the mother's abdominal incision sutured. Each mother received one SC injection of the analgesic buprenorphine (0.05 mg/kg) and a second dose 12 h post-surgery. The mother was returned to her cage and the litters allowed to grow until their assigned harvest dates. At the time of harvest, both mother and fetuses were sacrificed with a euthanizing dose of sodium pentobarbital (100 mg/kg) and their lungs, trachea, intestines, skin, and liver collected. Maternal ovaries and liver were also harvested as well as amniotic membranes from those fetuses harvested before delivery. All procedures were in compliance with applicable regulations and guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee.

PCR for vector DNA presence. Tissues were quick-frozen in liquid nitrogen, then pulverized by mortar and pestle. A DNeasy Tissue kit (Qiagen) was used to prepare genomic DNA following the manufacturer's protocol for animal tissues. PCR was then carried out using unique luciferase primers (5′, 5′-GAAAACTCTCTTCAATTCTTTATGCCG-3′; 3′, 5′-CTCGAAATCCA-CATATCAAATATCCG-3′) and *Taq* DNA polymerase with *Taq* Start Antibody (Clontech) for 40 cycles (95°C for 1.5 min, 58°C for 1 min, 72°C for 1 min). PCR products were identified both by size and by sequencing.

Luminometry and immunohistochemistry for tissue luciferase expression. Tissues were removed from each rabbit, quick-frozen in liquid nitrogen, and assayed for luciferase activity by luminometry after pulverization and resuspension in lysis buffer (Promega). Absolute amount of luciferase per mg of tissue was calculated by using a standard curve from recombinant luciferase to convert luminometry light units to pg of luciferase. A Biorad protein assay was used to calculate the protein content of each tissue sample. For immunohistochemistry, each rabbit was euthanized and then underwent perfusion of lungs and intestines with ice-cold 4% paraformaldehyde followed by fixation overnight in the same solution. Tissues were then passed through successive solutions of PBS, saline, and 70% ethanol before being paraffin-embedded and sectioned into 5 μm thick sections for slides. Paraffin was removed from slides with two 10-min rinses in xylene followed by a 2-min rinse in 100% ethanol. Slides were rehydrated by successive washes in 100%, 100%, 95%, 85%, 75%, 50%, and 30% ethanol for 2 min each, then fixed in 4% paraformaldehyde for 20 min at 37°C. Tissue was permeabilized with Triton X (0.2×) for 10 min and then blocked with 5% mouse serum for 30 min. Each specimen was treated with goat anti-luciferase antibody (Cortex Biochem) at 1:100 concentration, followed by incubation in Cy3-conjugated secondary antibody mouse anti-goat IgG (Jackson Immunoresearch) at 1:200 concentration. A DAPI nuclear

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stain was also applied at a concentration of 0.3 mM. Tissues were then examined with an immunofluoresence microscope fitted with a far-red detector, after excitation with a Krypton/Argon laser at 570 nm.

Statistical analysis. For luminometry assessment of luciferase expression, mean scores were calculated for each group and reported as mean ± standard error. Comparison of luminometry values between control and vector groups was made by unpaired *t*-test. Mortality comparisons were made by χ^2 analysis.

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REFERENCES

1. Bals, R., *et al.* (1999). Transduction of well-differentiated airway epithelium by recombinant adeno-associated virus is limited by vector entry. *J. Virol.* **73:** 6085–6088.
2. Brigham, K. L. (1997). *Gene Therapy for Diseases of the Lung.* Marcel Dekker, Inc., NY, NY.
3. Flotte, T. R., and Poirer, A. W. J. (1998). Protease activity in cystic fibrosis BAL fluid inhibits RAAV transduction and renders RAAV DNase sensitive. *Pediatr. Pulmonol.* **26:** 208.
4. Badalian, S. S., Chao, C. R., Fox, H. E., and Timor, T. I. (1993). Fetal breathing-related nasal fluid flow velocity in uncomplicated pregnancies. *Am. J. Obstet. Gynecol.* **169:** 563–567.
5. Brace, R. A. (1995). Current topic: progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membrane. *Placenta* **16:** 1–18.
6. Douar, A. M., *et al.* (1997). Foetal gene delivery in mice by intra-amniotic administration of retroviral producer cells and adenovirus. *Gene Ther.* **4:** 883–890.
7. Sekhon, H. S., and Larson, J. E. (1995). In utero gene transfer into the pulmonary epithelium. *Nat. Med.* **1:** 1201–1203.
8. Holzinger, A., Trapnell, B. C., Weaver, T. E., Whitsett, J. A., and Iwamoto, H. S. (1995). Intraamniotic administration of an adenoviral vector for gene transfer to fetal sheep and mouse tissues. *Pediatr. Res.* **38:** 844–850.
9. Yang, E. Y., *et al.* (1999). Persistent postnatal transgene expression in both muscle and liver after fetal injection of recombinant adenovirus. *J. Pediatr. Surg.* **34:** 766–772.
10. McCray, P. B., Jr., *et al.* (1995). Adenoviral-mediated gene transfer to fetal pulmonary epithelia in vitro and in vivo. *J. Clin. Invest.* **95:** 2620–2632.
11. Pitt, B. R., *et al.* (1995). Retrovirus-mediated gene transfer in lungs of living fetal sheep. *Gene Ther.* **2:** 344–350.
12. Sylvester, K. G., Yang, E. Y., Cass, D. L., Crombleholme, T. M., and Adzick, N. S. (1997). Fetoscopic gene therapy for congenital lung disease. *J. Pediatr. Surg.* **32:** 964–969.
13. Iwamoto, H. S., Trapnell, B. C., McConnell, C. J., Daugherty, C., and Whitsett, J. A. (1999). Pulmonary inflammation associated with repeated, prenatal exposure to an E1, E3-deleted adenoviral vector in sheep. *Gene Ther.* **6:** 98–106.
14. Douar, A. M., Themis, M., and Coutelle, C. (1996). Fetal somatic gene therapy. *Mol. Hum. Reprod.* **2:** 633–641.
15. Zeitlin, P. L. (1997). Adeno-associated virus-based delivery systems. In *Gene Therapy for Diseases of the Lung* (K. L. Brigham, Ed.), pp. 53–81. Marcel Dekker, NY, NY.
16. Duan, D., Yue, Y., Yan, Z., McCray, P. B., Jr., and Engelhardt, J. F. (1998). Polarity influences the efficiency of recombinant adenoassociated virus infection in differentiated airway epithelia. *Hum. Gene Ther.* **9:** 2761–2776.
17. Rubenstein, R. C., McVeigh, U., Flotte, T. R., Guggino, W. B., and Zeitlin, P. L. (1997). CFTR gene transduction in neonatal rabbits using an adeno-associated virus (AAV) vector. *Gene Ther.* **4:** 384–392.
18. Zeitlin, P. L., *et al.* (1995). Alveolar stem cell transduction by an adeno-associated viral vector. *Gene Ther.* **2:** 623–631.
19. Lipshutz, G. S., *et al.* (2001). *In utero* delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression. *Mol. Ther.* **3:** 284–292.
20. Mitchell, M., Jerebstova, M., Batshaw, M. L., Newman, K., and Ye, X. (2000). Long-term transfer to mouse fetuses with recombinant adenovirus and adeno-associated virus (AAV) vectors. *Gene Ther.* **7:** 1986–1992.
21. Duan, D., Yongping, Y., Ziying, Y., Yang, J., and Engelhardt, J. F. (2000). Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J. Clin. Invest.* **105:** 1573–1587.
22. Douar, A. M., Themis, M., Sandig, V., Friedmann, T., and Coutelle, C. (1996). Effect of amniotic fluid on cationic lipid mediated transfection and retroviral infection. *Gene Ther.* **3:** 789–796.
23. Boyle, M. P., Chu, C. S., Rubenstein, R. C., and Zeitlin, P. L. (1998). Truncated CLC-2 promoter mediates high level reporter gene expression in normal and cystic fibrosis pulmonary epithelial cell lines. *Pediatr. Pulmonol.* **26:** 284.
24. Flotte, T. R., *et al.* (1992). Gene expression from adeno-associated virus vectors in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **7:** 349–356.

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