Hybrid Nonviral/Viral Vector Systems for Improved *piggyBac* DNA Transposon *In Vivo* Delivery

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The DNA transposon *piggyBac* is a potential therapeutic agent for multiple genetic diseases such as cystic fibrosis (CF). Recombinant *piggyBac* transposon and transposase are typically codelivered by plasmid transfection; however, plasmid delivery is inefficient in somatic cells *in vivo* and is a barrier to the therapeutic application of transposon-based vector systems. Here, we investigate the potential for hybrid *piggyBac*/viral vectors to transduce cells and support transposase-mediated genomic integration of the transposon. We tested both adenovirus (Ad) and adeno-associated virus (AAV) as transposon delivery vehicles. An Ad vector expressing hyperactive insect *piggyBac* transposase (iPB7) was codelivered. We show transposase-dependent transposition activity and mapped integrations in mammalian cells *in vitro* and *in vivo* from each viral vector platform. We also demonstrate efficient and persistent transgene expression following nasal delivery of *piggyBac*/viral vectors to mice. Furthermore, using *piggyBac*/Ad expressing Cystic Fibrosis transmembrane Conductance Regulator (CFTR), we show persistent correction of chloride current in well-differentiated primary cultures of human airway epithelial cells derived from CF patients. Combining the emerging technologies of DNA transposon-based vectors with well-studied adenoviral and AAV delivery provides new tools for *in vivo* gene transfer and presents an exciting opportunity to increase the delivery efficiency for therapeutic genes such as CFTR.

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INTRODUCTION

Our goal for gene therapy vector development for life-long genetic diseases such as cystic fibrosis (CF) is to create a vehicle with the ability to efficiently, safely, and persistently express a transgene in the appropriate cell types.[1,2] There are multiple viral-based vectors for delivering genes to airway epithelia. Each system has its advantages and disadvantages. Nonviral vectors provide an expanded tool-set for gene transfer to cells. For example, research with the "cut-and-paste" DNA transposon *Sleeping Beauty* pioneered the use of a recombinant transposon and transposase to achieve genomic integration of a transgene.[3] *Sleeping Beauty*-mediated gene transfer resulted in functional correction of coagulation factor deficiencies,[4–6] lysosomal storage disease,[7] as well as in cancer therapeutics.[8]

PiggyBac is also a DNA transposon and a promising alternative to *Sleeping Beauty*. Similar to *Sleeping Beauty*, *piggyBac* is highly active when introduced into mammalian cells[9–11] mediates long-term expression *in vivo*[12,13] and is a potential therapeutic agent for multiple genetic diseases such as CF.[14,15] Recombinant *piggyBac* transposon and transposase are typically codelivered by plasmid transfection; however, the greatest barrier of delivering naked DNA is inefficient delivery to somatic cells *in vivo.*

Yant *et al.*[16] incorporated the *Sleeping Beauty* integration machinery into Ad vectors. They observed that the Ad genome needed to circularize before releasing the *Sleeping Beauty* transposon.[17,18] They employed a Flp recombination system to drive circularization of the Ad genome in target cells. Here, we explore the potential utility of adenoviral (Ad)- or adeno associated virus (AAV)-based vectors to deliver *piggyBac* components to airway epithelia without an additional recombination step. For these studies, a second adenoviral vector expressing hyperactive insect *piggyBac* transposase (iPB7) is codelivered to achieve transposase-mediated genomic integration of the transposon. These novel hybrid vector systems provide valuable additional tools for *in vivo* gene transfer.

RESULTS

DNA transposition from a *piggyBac*/AAV vector into the genome

Hybrid *piggyBac*/AAV vectors expressing the mCherry reporter and puromycin resistance genes were generated (shown schematically, **Figure 1a**; **Supplementary Figure S1**). For these studies, iPB7 was codelivered with an Ad5 vector (Ad-iPB7). To determine if iPB7 mobilized the DNA cargo from the *piggyBac*/AAV vector and catalyze integration into the genome, colony formation assays were performed in a mammalian cell line (**Figure 1b**). Colony formation assays are a widely used indirect measure of transposition efficiency. *PiggyBac*/AAV was delivered to HeLa cells at multiplicity of infections (MOIs) ranging from 100 to 100,000. Ad-iPB7 was simultaneously delivered at

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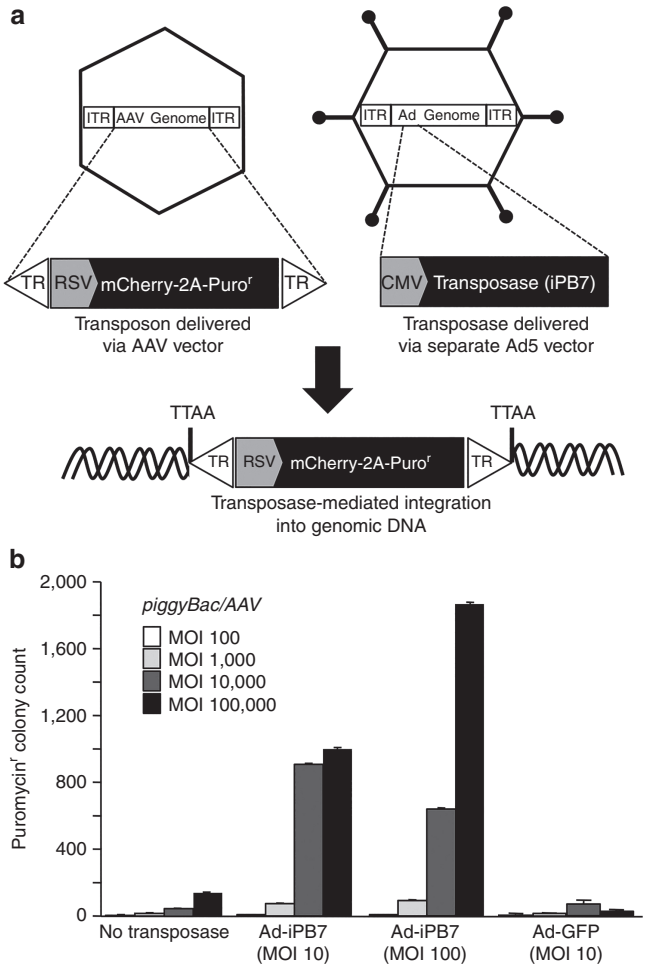

Figure 1 ***piggyBac*/AAV transduction and colony formation assays.** (**a**) Schematic representation of the *piggyBac* transposon expressing mCherry and the puromycin resistance gene, puromycin N-acetyltransferase (Puro^r), driven by an RSV promoter and delivered by AAV2/5. Hyperactive *piggyBac* transposase (iPB7) is delivered *in trans* by an Ad5 vector. ITR, inverted terminal repeat of AAV; TR, *piggyBac* terminal repeat. (**b**) HeLa cells were cotransduced with increasing vector multiplicity of infections as indicated. Cells were selected for 10–14 days with 0.5 µg/ml puromycin, stained with methylene blue, and the drug resistant colonies were counted.

MOIs ranging from 0 to 100. Following vector transduction, cells were selected for puromycin resistance for ~2 weeks and colonies counted. Puromycin-resistant colony formation is indicative of a successful integration event. In the absence of Ad-iPB7, we observed a dose-dependent increase in puromycin resistant colonies, suggesting that there is baseline level of integration from an AAV vector alone, consistent with previous observations.[19] However, in the presence of Ad-iPB7, the colony numbers increased dramatically.

As an additional control to confirm that the presence of Ad vector does not enhance *piggyBac*/AAV conferred colony formation in our assay, we substituted Ad-iPB7 with Ad-GFP (**Figure 1b**). In the presence of Ad-GFP, the number of puromycin-resistant colonies closely resembled "no transposase" group. These data suggest that iPB7 is necessary to achieve an increase in puromycin resistant colony formation.

In general, the levels of transposition were dose-dependent; however, the *piggyBac*/AAV (MOI 10,000)/Ad-iPB7 (MOI 100) condition (*ratio 100:1*) resulted in fewer colonies than the *piggyBac*/AAV (MOI 10,000)/Ad-PB7 (MOI 10) condition (*ratio 1000:1*). This result is perhaps counterintuitive because one would expect that if the transposon is constant and the transposase is increased, increased transposition should occur. Yet, we observed that a 1,000:1 ratio consistently resulted in the best fold-increase of colonies over background. This may be the result, in part, of cellular toxicity associated with high MOI delivery of adenoviral vector or overexpression inhibition.

To determine if high MOIs of adenoviral vector might lead to decreased colony counts, we delivered Ad-GFP to HeLa cells at MOIs of 0, 1, 2, 10, 20, 100, and 200 and used flow cytometry to quantify live cells 24 hours later. There was very little variation in the percentage of live cells in all groups. The range was from 94.8 to 97.5% (**Supplementary Figure S2**). Interestingly, the percentage of GFP-positive cells plateaued at an MOI of 20 (91.1%) and dropped off at MOI 200 (78.7%). These data suggest that the highest nontoxic MOI of adenoviral vector may not be the optimal dose for maximal gene expression.

To confirm transposase-mediated genomic integration, cellular DNA was purified and libraries were generated using LAM-PCR as described in *Materials and Methods*. The libraries were shotgun-cloned and Sanger sequenced. Of the 54 sequences, ~2/3 (37/54) were bona fide transposase-mediated genomic integrations and the remaining ~1/3 (17/54) were recovered vector. A transposase-mediated integration event is defined by the tell-tale sign of a precise junction between the transposon terminal repeat and genomic DNA occurring at a TTAA. A "no transposase control" was not included because not enough puromycin-resistant colonies could be recovered to yield enough DNA to generate the libraries. Based on the sequencing data, nontransposase-mediated genomic integrations cannot be distinguished from episomal vectors. All but three of the genomic integrations occurred at canonical TTAA sites. The remaining three integrations occurred at either CTAA (1 integration) or TTAG (2 integrations) sites. Low-level NTAA or TTAN integration is consistent with plasmid delivered *piggyBac* transposon.[15,20] These data strongly suggest that delivery with AAV allows for transposase-mediated integration of the *piggyBac* transposon.

Next, we deep sequenced the libraries using the Illumina HiSeq 2000 platform and recovered 19,059 reads from *piggyBac*/AAV-transduced HeLa cells and compared the results to 31,078 reads recovered from HeLa cells that were transfected with standard *piggyBac* transposon and iPB7 expression plasmids. For these analyses, we only included verified genomic integrations that included the TTAA junction between the *piggyBac* terminal repeat and mappable human genomic sequence. Multiple metrics were used to determine if the *piggyBac* integration patterns were altered following delivery by an AAV vector. Metrics included distance from transcription start sites, oncogenes, DNase hypersensitive sites, CpG islands, gene density, gene expression, and GC content (**Supplementary Figure S3**). We observed only subtle differences in the integration profile between AAV- and plasmid-delivered *piggyBac* transposons or computationally selected matched random controls. There was a very modest increase in

integrations near gene dense regions or CpG islands and *piggyBac*/AAV had a modest decrease in integrations near transcriptional start sites.

To further test the utility of this hybrid system *in vivo*, *piggyBac*/AAV vector expressing firefly luciferase was evaluated for its ability to deliver and transpose into the genome of murine conducting airways. A dose of 9×10^{10} vector genomes (vg) formulated with 1% methylcellulose[21] was delivered via nasal instillation to immunocompetent Balb/c mice. Ad-iPB7 was codelivered at a dose of 9×10^{7} pfu, achieving the 1,000:1 ratio as determined *in vitro*. These doses were chosen as a maximal titer that could be delivered in a 50 µl volume. Control mice were untreated (naive) or received Ad-Empty in place of Ad-iPB7. Beginning 4 days post-transduction, luciferase expression was measured at the indicated intervals using a Xenogen CCD camera imaging system (**Figure 2a**,**b**).

Both in the presence and absence of iPB7, a decline in nasal bioluminescence was observed between 1 week and 4 weeks postdelivery. The subsequent luciferase expression in animals that received iPB7 stabilized at ~30% initial levels, whereas, expression in animals without iPB7 stabilized at ~6% initial expression. After 12 weeks, the surface epithelial cells were ablated by two consecutive treatments with the detergent 2% polidocanol.[22] Monitoring of the bioluminescent expression resumed 2 days following the second polidocanol treatment. Ablation of the surface epithelia is followed by a rapid proliferation and repopulation phase. This procedure helps distinguish between stable integration events in progenitor cells from episomal expression. The luciferase expression in animals that received iPB7 restabilized at ~10% of starting levels and the expression in animals without iPB7 dropped to ~1% of the first timepoint (**Figure 2a**). These data suggest that the transposase catalyzed *piggyBac*/AAV integration into a progenitor population of nasal airway cells in mice.

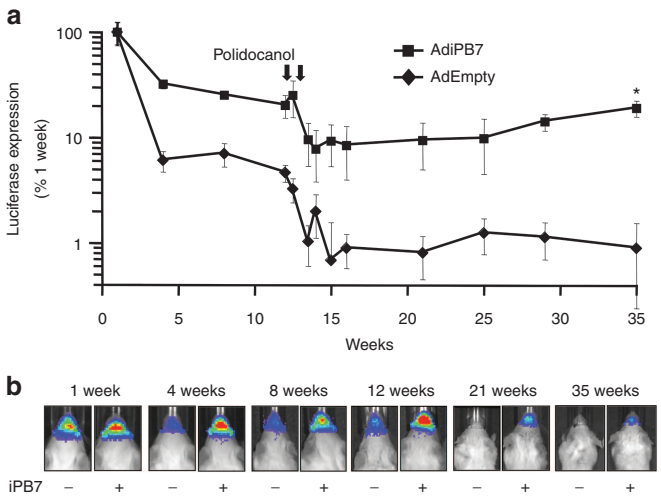

Figure 2 AAV delivered *piggyBac* transposon mediates persistent transgene expression in mouse airways. (**a**) *piggyBac*/AAV2/5 expressing firefly luciferase was co-delivered to the nasal airways with Ad-iPB7 or Ad-Empty formulated with 2% methylcellulose. At the indicated time points, mice were given luciferin via i.p. injection and luciferase expression was quantified by bioluminescent imaging. At 12 weeks postdelivery, mice received two doses of polidocanol via nasal delivery, as indicated by arrows and described in *Materials and Methods*. Luciferase expression was measured as photons/sec/cm^2 and is reported as a percentage of levels from week one. *$P = 0.002$, $n = 10$. (**b**) Representative *in vivo* imaging system images at selected time points are shown.

We isolated nasal septa from mice 12 months after being transduced with *piggyBac*/AAV and Ad-iPB7. Using a similar protocol as described for our *in vitro* integration library generation, we performed genomic DNA isolation followed by LAM-PCR. Using shot-gun cloning and Sanger sequencing, we mapped 47 *piggyBac* genomic integrations (**Supplementary Figure S4**, red arrows). Interestingly, we observed a much lower frequency of recovered vector (1 out of 48). This change in ratios may point to an *in vitro* sequencing artifact that may not be relevant *in vivo*.

DNA transposition from a *piggyBac*/Ad vector into the genome

To determine if an Ad5-based viral vector is a suitable delivery vehicle for *piggyBac* transposon, we inserted the transposon sequence into the E1 region of a first-generation Ad5 vector. Similar to the AAV vector, the transposon expressed mCherry and puromycin resistance genes separated by a T2A element (**Figure 3a**; **Supplementary Figure S1**). Transposase-mediated genomic integration was determined by colony formation assays (**Figure 3b**). Unlike *piggyBac*/AAV, very few puromycin-resistant colonies were observed for *piggyBac*/Ad in the absence of iPB7. Interestingly, a *piggyBac*/Ad MOI of 10 was optimal for each increasing dose of Ad-iPB7. Addition of Ad-iPB7 (MOI = 100) resulted in ~150-fold increase in colony count as compared to *piggyBac*/Ad without Ad-iPB7. The decreased colony formation counts in the high-dose groups may be the result, in part, of cellular toxicity associated with high MOI delivery of adenoviral vector.

Transposase-mediated genomic integration in HeLa cells was again verified by LAM-PCR, shotgun cloning, and Sanger sequencing. Of the 65 informative sequences, 23.1% (15/65) were recovered vector and 76.9% (50/65) were confirmed genomic integrations. All but four of the genomic integrations occurred at canonical TTAA sites. The remaining four integrations occurred at TTAG, CTAA (two integrations), or TCAA sites. While the sample size is too small to draw conclusions concerning integration patterns, these data suggest that, like AAV, Ad is a functional delivery vehicle for *piggyBac* transposon.

To quantify gene transfer efficiencies, *piggyBac*/Ad expressing firefly luciferase was delivered to SCID mouse airways via nasal instillation. The bioluminescent signal was quantified using a CCD camera 5 minutes following intraperitoneal (i.p.) luciferin delivery. As shown (**Figure 4a**), in the presence of iPB7, expression stabilized by 8 weeks postdelivery at ~20–30% of the initial time point. In the absence of iPB7, expression continued to decline to ~2% of the initial time point. These results suggest that iPB7 confers persistent expression from a *piggyBac*/Ad vector in the airways of immunodeficient mice *in vivo*. Replicate studies in immunocompetent Balb/c mice resulted in loss of expression to naive levels, regardless of the presence or absence of transposase, by 4 weeks postdelivery (**Supplementary Figure S5**).

To discern the cell types transduced *in vivo*, similar experiments were repeated using a *piggyBac*/Ad vector expressing the visual reporter gene mCherry. As before, *piggyBac*/Ad was

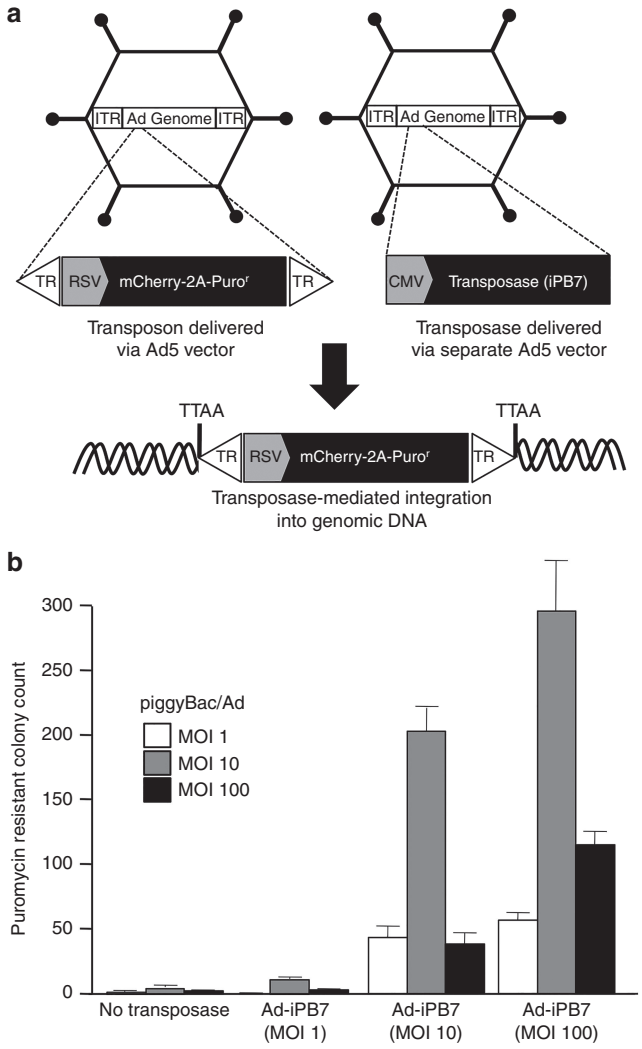

Figure 3 ***piggyBac*/Ad transduction and colony formation assays.** (**a**) Schematic representation of the *piggyBac* transposon expressing mCherry and the puromycin resistance gene, puromycin N-acetyl-transferase (Puro^r), driven by an RSV promoter and delivered by Ad5 vector. iPB7 is delivered *in trans* by an Ad5 vector. (**b**) HeLa cells were cotransduced with increasing multiplicity of infections of vector as indicated. Cells were selected for 10–14 days with 0.5 µg/ml puromycin, stained with methylene blue, and the drug resistant colonies were counted.

delivered via nasal instillation to SCID mice with either Ad-iPB7 or Ad-Empty. Reporter gene expression in fixed frozen tissue was examined at 1 week and 21 weeks postdelivery. At 1 week postdelivery of *piggyBac*/Ad and Ad-iPB7, we observed abundant mCherry expression that was restricted to the surface epithelia of the conducting airways (**Figure 4d–g**). At the 1 week time point, the pattern of mCherry expression was indistinguishable between the group receiving iPB7 and the group without iPB7 (data not shown). Furthermore, this pattern of expression is consistent with previous observations using Ad5 vector expressing β-galactosidase and formulated with methylcellulose.[21] Consistent with the luciferase reporter gene results (**Figure 4a**), abundant expression was observed in the conducting airways of mice collected at the 21-week time point from the Ad-iPB7 cohort (**Figure 4h**) but not the Ad-empty cohort (**Figure 4i**). As anticipated, no mCherry expression (**Figure 4b**) or evidence of inflammation (**Figure 4c**) was observed in control naive SCID mice.

We isolated lungs from SCID mice 12 months after being transduced with *piggyBac*/Ad and Ad-iPB7. Importantly, abundant mCherry expression was still observed in large airways (**Figure 4j,k**) at levels similar to 21 weeks postdelivery (**Figure 4h**). We performed genomic DNA isolation followed by LAM-PCR, using a protocol similar to discussed for *in vitro* transduced HeLa cells. Using shot-gun cloning and Sanger sequencing, we mapped 49 *piggyBac* genomic integrations (**Supplementary Figure S3**, blue arrows). Similar to the *piggyBac*/AAV mice, but unlike the *in vitro* shot-gun cloning, we observed a low frequency of recovered vector (2 out of 51). These data strongly support the notion that the observed *in vivo* persistence of transgene expression in the absence of selection is the result of transposase mediated *piggyBac* transposition from the Ad genome into the host genome.

piggyBac/Ad-mediated CFTR correction in CF primary airway cells

Potential advantages of the *piggyBac*/Ad vector include a large packaging capacity and preparations with high titers. Because the CFTR cassette flanked by piggyBac terminal repeats exceeded the packaging limit of AAV, we focused on *piggyBac*/Ad as a delivery vehicle. Specifically, we examined if a *piggyBac*/Ad-delivered *CFTR* cDNA can persistently rescue the anion transport defect in CF airway epithelial cells. Well-differentiated human primary CF tracheal epithelia were cultured at air-liquid interface[23] and co-transduced with basolateral application of *piggyBac*/Ad-CFTR (MOI = 50) with Ad-iPB7 or Ad-Empty. At progressive time points, chloride currents were measured in modified Ussing chambers as previously reported.[24] Upon addition of forskolin and 3-isobutyl-1-methylxanthine (IBMX), a significant increase in cAMP-stimulated chloride current was observed in epithelia treated with *piggyBac*/Ad-CFTR (**Figure 5a**). Changes in short circuit current are not observed in naive CF cultures (**Figure 5a**). Chloride current (I) increased by ~4 µA/cm^2 in these cultures. This increase in cAMP-stimulated chloride current was significant compared to the Ad-empty control-treated cultures (**Figure 5b**; $P < 0.001$). Further, these levels of correction are consistent with our previous experience using Ad5-CFTR.[22] This observation indicates that sufficient expression was achieved to functionally correct CF airway epithelia.

DISCUSSION

The choice of a vehicle to deliver therapeutic genes to specific target tissues is a vital consideration. Important features for a CFTR gene transfer vector for airway delivery include a large packaging capacity, efficient transduction, persistent expression, and the capacity to be concentrated and purified. There are multiple viral-based vectors for delivering genes to the airways, but none possess all of these attributes. Here, we codelivered *piggyBac*/Ad or *piggyBac*/AAV with Ad-transposase to cells *in vitro* and mouse airways *in vivo* and demonstrated efficient transduction, transposase-mediated integration, and persistent expression. Hybrid *piggyBac*/Ad and *piggyBac*/AAV vectors are valuable new tools for *in vivo* gene transfer.

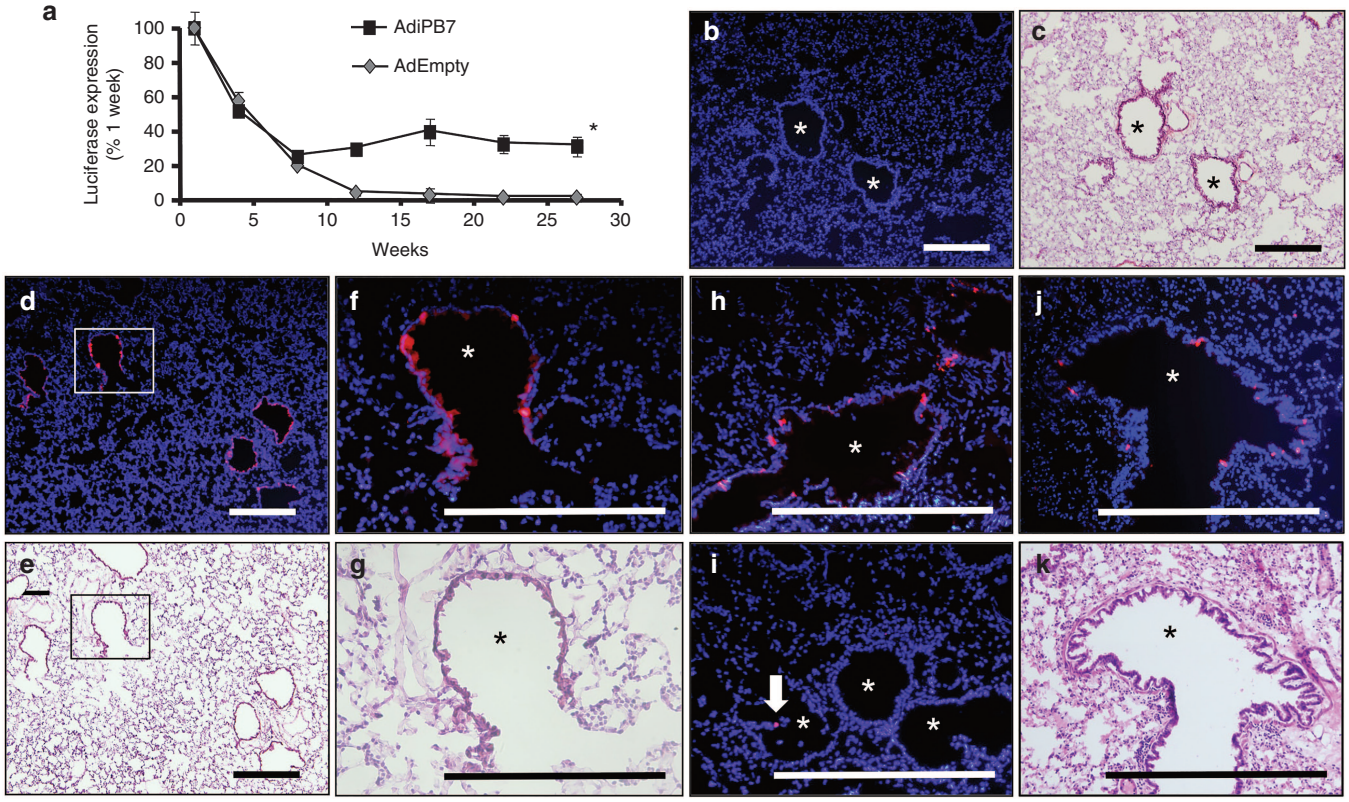

Figure 4 Ad delivered *piggyBac* transposon mediates persistent transgene expression in mouse airways. (**a**) *piggyBac*/Ad expressing firefly luciferase was codelivered to the nasal airways with Ad-iPB7 or Ad-Empty formulated with 2% methylcellulose. At the indicated time points, mice were given luciferin via i.p. injection and luciferase expression was quantified by bioluminescent imaging. Luciferase expression was measured as photons/sec/cm^2 and is reported as a percentage of levels from week one. *$P < 0.001$, $n = 10$. (**b–i**) Histological analysis of the airways was performed on SCID mice that received nasal delivery of *piggyBac*/Ad expressing mCherry. (**b**) Fluorescent expression and (**c**) hematoxylin and eosin (H&E) images of naïve mouse lung sections of age matched control mice. (**d,f**) Representative fluorescent expression and (**e,g**) H&E images of mice 1 week postdelivery of *piggyBac*/Ad-mCherry and Ad-iPB7. (**f,g**) Higher power images of boxed areas in **d** and **e**, respectively. (**h**) Mouse airways 21 weeks postdelivery of *piggyBac*/Ad-mCherry and Ad-iPB7. (**i**) Mouse airways 21 weeks postdelivery of *piggyBac*/Ad-mCherry and Ad-Empty. (**j**) Fluorescent expression and (**k**) hematoxylin and eosin (H&E) images of airways of mice 1 year postdelivery of *piggyBac*/Ad-mCherry and Ad-iPB7. Arrow indicates rare mCherry positive cell. Scale bar = 500 µm. Asterisks indicate large airways. A DAPI stain was used to label nuclei. Representative images from three mice/condition.

Our results indicate the *piggyBac*/Ad and *piggyBac*/AAV vectors transpose and express their transgenes in the host genome *in vitro* and *in vivo*. We observed transposase-dependent transposition activity in HeLa cells and in the airways of mice. These data suggest that these viral vectors can support *piggyBac* transposition. This important observation contrasts with previous reports using the *Sleeping Beauty* based DNA transposon and Ad vectors.[16] Yant *et al.* observed that the Ad genome required Flp-mediated recombination that resulted in circularization before releasing the *Sleeping Beauty* transposon. It is unclear why *piggyBac* does not require this circularization requirement. The unmethylated Ad and AAV genomes may be more suitable for *piggyBac* transposition as compared to *Sleeping Beauty*.[25] The ability of the *piggyBac*-based viral vectors to integrate without an intermediate recombination step greatly simplifies *in vivo* delivery and potentially increases integration efficiency.

A prerequisite to life-long expression from a gene therapy vector is genomic integration into progenitor cells; therefore, integrating vector systems may have the greatest potential for treating genetic diseases. There is inherent risk when introducing a transgene with integrating vectors. Insertional mutagenesis may disrupt normal cell functions by inactivating an essential host gene or inappropriately causing expression of an undesirable gene. However, the risk will vary depending on the vector used, the therapeutic gene, and the cell type targeted. In many cases, enhancer effects pose the greatest danger. Using deep sequencing, we mapped viral vector-delivered *piggyBac* integrations in HeLa cells. Previous reports show that *piggyBac* preferentially integrates near transcription start sites at a frequency of 16–20%, in a manner similar to murine moloney leukemia virus.[11,26–28] However, we did not observe this pattern of integration near transcription start sites in our studies (**Supplementary Figure S3**). In fact, we observed a slightly disfavoured integration pattern at transcription start sites as compared to a matched random control consistent with our previously published mapping data using a plasmid-based delivery system.[20] The reason for this discrepancy is unclear but may result from the transposon delivery method, the cell types transduced, or the library generation method. The integration profile of a high capacity Ad-delivered *Sleeping Beauty* vector was also recently evaluated using adapter mediated-PCR and a near random pattern was observed.[29] This finding was consistent with a

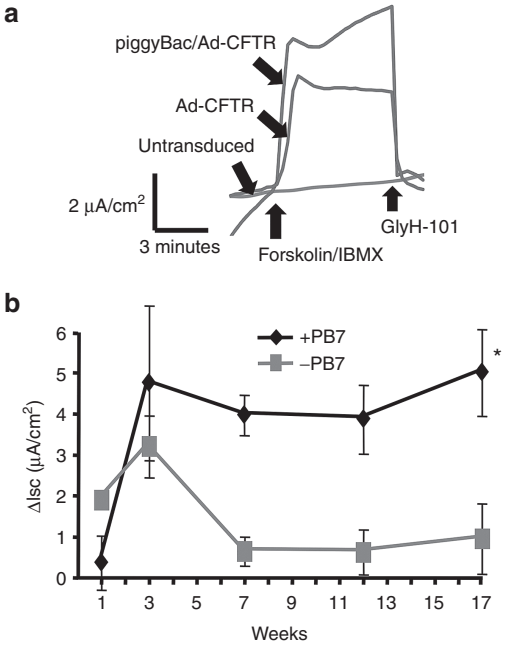

Figure 5 *piggyBac*/Ad-CFTR restores Cl^- transport in CF tracheal epithelia. (**a**) Ad-CFTR or *piggyBac*/Ad-CFTR were delivered to well-differentiated primary cultures of airway epithelia from CF human donors. Anion channel correction was measured by Cl^- current responses to Forskolin/IBMX and GlyH-101 in Ussing chambers. (**b**) Well-differentiated tracheal epithelia cultures were cotransduced with *piggyBac*/Ad-CFTR (multiplicity of infection (MOI) = 50) with Ad-iPB7 (MOI = 50) or Ad-Empty (MOI = 50). At the indicated time points, transepithelial Cl^- currents were measured in Ussing chambers. Bars represent means of change in Cl^- current upon addition of Forskolin (10 μmol/l) and IBMX (100 μmol/l). Error bars represent SEM. $n = 3$ epithelial cultures/treatment. $^*P < 0.001$.

lentiviral-*Sleeping Beauty* hybrid vector system or plasmid-based delivery.[30–32]

Several features of adenoviral vectors make them attractive vehicles for delivering therapeutic genes such as CFTR, including their large carrying capacity, efficient gene transfer capabilities, ability to transduce nondividing cells, the ability to be grown to high titer, and ease of purification. Indeed, Ad-based viral vectors were the first to be used for gene therapy trials in CF patients.[33] Therapeutic levels of CFTR mRNA were achieved in the airway epithelium of CF patients, but expression quickly waned and subsequent administrations were limited by humoral immunity against the vector.[34,35] Thus, the two greatest impediments to adenoviral vectors are robust immune responses and transient expression resulting from episomal expression. As demonstrated, the use of *piggyBac*/Ad can overcome the limitation of episomal expression; however, immune-deficient SCID mice were required to observe the effect.

Consistent with previous observations using first-generation Ad based vectors, we observed that *piggyBac*/Ad-mediated transgene expression was transient in immunocompetent mice.[36–38] This short-lived transgene expression is attributed to the induction of a cytotoxic T lymphocyte immune response against adenoviral antigens.[38] Immunogenic responses to adenoviral vectors preclude the potential for readministration. In addition, there are increased levels of inflammation and cytotoxicity associated with adenoviral infections.[39] In these studies, when both Ad-iPB7 and *piggyBac*/Ad were codelivered, the total viral load delivered was 7.5×10^8 pfu. This amount was chosen based on the following three considerations: (i) the optimal ratios determined *in vitro*, (ii) the titer of the vector preparations, and (iii) a maximal dose that could be delivered in a 25 μl volume. We chose Ad to deliver iPB7 because it efficiently and transiently produces a transgene product. Interestingly, in our Ad-iPB7 and *piggyBac*/AAV experiments, we only delivered a total dose of 9×10^7 pfu of Ad-based vector and observed persistent transposon expression in immunocompetent Balb/c mice. As before, this amount was chosen based on the same three considerations. A potential explanation for persistent expression in immunocompetent mice from the Ad/AAV combination and not the Ad/Ad combination could be a threshold of Ad (~10^8 pfu) that triggers a cytotoxic T lymphocyte immune response when delivered intranasally. Perhaps, de-escalation studies would reveal a dose that results in stable expression of the Ad/Ad combination in immunocompetent mice. Pulmonary immune responses in mice may not be predictive of humans. Our data serve as a proof of principle that *piggyBac*-mediated transgene delivery can lead to long-term transgene persistence *in vivo* but further testing in a large animal model is necessary.

As we turn our attention to the future, there are at least four strategies to consider when addressing the immune response. (i) Helper dependant (HD)-Ad vectors lack any viral encoded genes and have precedence for long-term expression *in vivo*.[40–42] Thus, helper-dependent (HD) *piggyBac*/Ad and HD-Ad-transposase vectors would likely attenuate a vector-mediated immune response. (ii) Transient immune suppression at the time of delivery could be considered. (iii) The vector load could be reduced by delivering both *piggyBac* and a self-inactivating transposase from the same Ad vector. Alternatively, the transposase could be supplied as RNA or with an AAV-based vector. (iv) Alternate vector delivery systems could also be considered. Indeed, integrase-deficient lentiviral vectors have been successfully used to deliver *Sleeping Beauty*.[30,32]

Our goal for CF gene therapy is to provide a life-long gene replacement strategy for the airways that would be efficacious regardless of the CFTR disease-causing mutation. Primary cultures of airway epithelia derived from humans with CF manifest defective CFTR-dependent anion transport. We determined that the hybrid *piggyBac*/Ad vector conferred CFTR expression in transduced cells and functionally corrected primary cultures of CF epithelial cells *in vitro.* We measured the persistence of cAMP-stimulated Cl^- dependent short circuit current across the epithelia for 17 weeks.[43,44] The demonstration of functional correction of CFTR in primary CF epithelia is a notable benchmark that provides an important foundation for future *in vivo* studies.

A gene transfer vector with the capacity to persistently express CFTR in airways *in vivo* may prevent or significantly slow CF disease progression. A high priority will be to demonstrate persistence and stable restoration of CFTR function in large-animal models, such as the CF pigs or ferrets.[45–48] Long-term expression following a single vector dose will require vector delivery to airway cells with progenitor capacity without causing toxicity. Access to potential airway progenitor cells, such as keratin 5-positive basal cells, will be assessed by screening *in vivo* vector delivery techniques. Transient disruption of tight junctions may help assure

vector access to the appropriate cellular compartments. Attaining long-term expression of CFTR and prevention of the progression of changes associated with lung disease *in vivo* would provide a powerful proof-of-principle for translational gene therapy.

MATERIALS AND METHODS

Constructs. The *piggyBac* transposon constructs in these studies expressed either an mCherry-T2A-puromycin N-acetyl-transferase (Puro^r) cassette or firefly luciferase for *in vitro* and *in vivo* studies, respectively. The mCherry-T2A-Puro^r cassette flanked by the *piggyBac* terminal repeats (TRs) was designed *in silico* and *in vitro* synthesized (GenScript, Piscataway, NJ). Gene cassettes within the transposons were driven by an RSV promoter and cloned from pUC57 into pAAV2 vector to create *piggyBac*/AAV. For these studies the RSV promoter was chosen because in our experience the RSV promoter is sufficient to drive life-long expression in mice. The AAV2 vector was pseudotyped with the AAV5 serotype capsid. The transposon inserts were separately cloned into the AAV vector using the restriction enzymes *Xho*I and *Not*I. The *piggyBac*/Ad construct was cloned with the same restriction enzymes and ligation protocols from pUC57 into the Ad5 vector. The Ad-iPB7 transposase was cloned by cutting the hyperactive insect *piggyBac* transposase[15] from pcDNA3.1/myc-HisA (Invitrogen, Grand Island, NY) to the Ad5 vector by restriction enzymes *EcoR*I and *Not*I. All clones were sequence confirmed. Adenoviral and Adenoviral-Associated Vector production was performed as a fee for service at the University of Iowa Viral Vector Core (http://www.uiowa.edu/~gene).

Colony formation assay. HeLa cells were transduced in a 24-well plate (5×10^4 cells/well) with *piggyBac*/AAV alone, *piggyBac*/Ad alone or in combination with Ad-iPB7 at the indicated MOIs in quadruplicate for 4 hours At 24 hours post-transduction, each well was trypsinized and expanded into a 100 mm plate and placed under puromycin selection (0.5 µg/ml). The cells were cultured under puromycin selection for two weeks and the selection media was changed three times per week. Following selection, puromycin-resistant colonies were fixed with 4% paraformaldehyde, stained with methylene blue and counted. Each assay was repeated at least three independent times.

Integration site recovery for Illumina HiSeq2000 sequencing. Integration sites were recovered as described previously.[15] Briefly, HeLa cells (5×10^6) were transduced with *piggyBac*/AAV (MOI = 10,000; 5×10^9 vector genomes (vg)) or *piggyBac*/Ad (MOI = 10; 5×10^7 plaque forming units (pfu)) in combination with Ad-iPB7 (MOI = 10) for 4 hours. Integrants were selected with puromycin (0.5 µg/ml) for 3 weeks. Genomic DNA from three separate transfections was extracted from the integration library using the DNeasy tissue kit (Qiagen, Valencia, CA). Pooled DNA (2 µg) was digested overnight with *Apo*I or *BstY*I at 50 and 60 °C, respectively; DNA was purified with the QIAquick PCR purification kit (Qiagen) and ligated to *Apo*I and *BstY*I linkers overnight at 16 °C. Nested PCR was carried out under stringent conditions using transposon end-specific primers AAACCTCGATATACAGACCGATAAAACACATGCGTCAATTTT ACGC (primary) and *AATGATACGGCGACCACCGAGATCTACACTC TTTCCCTACACGACGCTCTTCCGATCT*XXXXCGTACGTCACAA TATGATTATCTTTC (secondary; XXXX denotes bar code, underlined sequence indicates Illumina cluster-generation sequence) and linker-specific primers CGTAGGGAGCAAGCAGAAGACGG (primary) and CAAGC AGAAGACGGCATACGAGCTCTTCCGATCT (secondary). DNA barcodes were included in the second-round PCR primers in order to track sample origin. The PCR products were gel purified, pooled, and sequenced using the Illumina HiSeq2000 sequencing platform.

Ethics statement. All animal procedures were previously approved (Animal Protocol #1304995) by the Institutional Animal Care and Use Committee (IACUC) and in accordance with National Institutes of Health guidelines.

In vivo *delivery and bioluminescence.* All mice for this study were housed at the University of Iowa Animal Care Facilities. Female mice (6–8-week-old) were transduced intranasally with *piggyBac*/Ad or *piggyBac*/AAV vector plus Ad-iPB7 or Ad-empty formulated 1:1 with 2% methylcellulose (50 µl total volume) as previously described.[21] We observed no signs of visible toxicity or mortality as a result of viral delivery. Animals were imaged at indicated time points using the *in vivo* imaging system (Caliper Life Sciences, Hopkinton, MA). Luciferin substrate (200 µl, 15 mg/ml; Caliper Life Sciences) was administered via intraperitoneal injection and the mice were imaged for 5 minutes. Data were analyzed using Living Image software (Caliper Life Sciences). Polidocanol treatments were performed in mice that received *piggyBac*/AAV-luciferase. At 3 months postdelivery, 25 µl of 2% polidocanol (Sigma, St Louis, MO) was administered to mice intranasally. A second dose was delivered 48 hours later. Luciferase expression was quantified by bioluminescent imaging at indicated time points before and after polidocanol treatment.[22]

Primary epithelial cultures and electrophysiology studies. Tracheal epithelial cells from human CF lungs were isolated by enzymatic digestion, seeded onto permeable filters, and grown at air-liquid interface as previously described.[23] Cystic Fibrosis transmembrane Conductance Regulator (CFTR)-null porcine tracheal epithelia cultures were studied in modified Ussing chambers as previously described.[24] Briefly, epithelia were bathed on both surfaces with solution containing: 135 mmol/l NaCl, 2.4 mmol/l K_2HPO_4, 0.6 mmol/l KH_2PO_4, 1.2 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 10 mmol/l dextrose, 5 mmol/l HEPES (pH = 7.4) at 37 °C and gassed with compressed air. Baseline transepithelial currents were measured. After apical addition of 100 µmol/l amiloride (Amil) and 100 µmol/l 4,4′-diisothiocyanoto-stilbene-2,2′-disulfonic acid (DIDS), currents were allowed to stabilize and the apical solution was replaced with a 4.8 mmol/l Cl^- solution containing 135 mmol/l D-Gluconic Acid, 2.4 mmol/l K_2HPO_4, 0.6 mmol/l KH_2PO_4, 1.2 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 10 mmol/l dextrose, 5 mmol/l HEPES (pH = 7.4) at 37 °C and gassed with compressed air. cAMP-dependent Cl^- current was stimulated by apical addition of 10 µmol/l forskolin and 100 µmol/l 3-isobutyl-1-methylxanthine (IBMX), and CFTR-specific Cl^- transport was inhibited with 100 µmol/l GlyH-101. Transepithelial voltage (V_t) was maintained at 0 mV to measure transepithelial current (I). Transepithelial electrical conductance (G_t) was measured by intermittently clamping V_t to +5 and/or −5 mV. Spontaneous values of V_t were measured by transiently removing the voltage clamp.

Immunohistochemistry. Approximately 2.5×10^8 pfu of *piggyBac*/Ad plus Ad-iPB7 or Ad-empty vector in a 50 µl volume with 1% methylcellulose (1:1) was delivered via nasal instillation to the airways of 3-month-old male SCID/NCr mice (NCI, MD, Balb/c background, 01S11) mice under ketamine/xylazine (87.5 + 2.5 mg/kg) anesthesia. Fluorescence analyses of *in vivo* mCherry expression in mice lungs were examined as described in ref. [46]. Briefly, mice lungs were harvested from a subset of mice and fixed in 4% paraformaldehyde in PBS at 4 °C overnight. Prior to fixation, lungs were gently inflated with 15% sucrose via the trachea to maintain lung architecture. After fixation, lungs were submerged in 15% sucrose for 8 hours and then in 30% sucrose overnight. All steps were performed at 4 °C. Lungs were embedded in OCT media, frozen in liquid nitrogen, and 10 µm sections were obtained using a microtome at −26 °C. The serial sections were then stained with hematoxylin and eosin using standard techniques. Images were captured using an Olympus BX60 fluorescence microscope (Leeds Precision Instrument, Minneapolis, MN).

Statistics. All numerical data are represented as mean ± standard error. Standard one and two sample *t*-tests were performed using the statistical computer program R (www.r-project.org) with the lme4 package. Analysis of variance was performed using Prism software (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL

Figure S1. Detailed schematic of vector constructs used in this study.
Figure S2. Dose escalation of adenovirus expressing GFP was performed in HeLa cells.
Figure S3. The heat maps summarize *piggyBac* distributions to genomic features.
Figure S4. Mouse genomic DNA was isolated from mice 12 months following delivery of *piggyBac*/AAV or *piggyBac*/Ad with Ad-iPB7 to the mouse airways.
Figure S5. *piggyBac*/Ad expressing firefly luciferase was co-delivered to the nasal airways with Ad-iPB7 or Ad-Empty formulated with 2% methylcellulose.

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