

see page 806

Adenovirus-mediated *In Utero* Expression of CFTR Does Not Improve Survival of CFTR Knockout Mice

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Gene therapy is being investigated in the treatment of lung-related aspects of the genetic disease, Cystic fibrosis (CF). Clinical studies have demonstrated CF transmembrane conductance regulator (CFTR) expression in the airways of adults with CF using a variety of gene transfer agents. *In utero* gene therapy is an alternative approach that facilitates vector transduction of rapidly expanding populations of target cells while avoiding immune recognition of the vector. In CF, *in utero* gene transfer could potentially delay the onset of disease symptoms in childhood and compensate for the role, if any, that CFTR plays in the developing organs. Previously published studies have suggested that transient expression of CFTR *in utero* was sufficient to rescue the fatal intestinal defect in S489X *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc} knockout mice. We replicated these studies using an identical CFTR-expressing adenoviral vector and CF mouse strain in sufficiently large numbers to provide robust Kaplan–Meier survival data. Although each step of the procedure was carefully controlled and vector-specific CFTR expression was confirmed in the fetal organs after treatment, there was statistically no significant improvement in the survival of mice treated *in utero* with AdCFTR, compared with contemporaneous control animals.

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INTRODUCTION

Since the discovery of the gene responsible for Cystic fibrosis (CF), gene therapy strategies have been investigated to treat the progressive lung disease associated with this condition.¹ *In utero* treatment, a strategy whereby gene transfer agents are delivered to the cells of the developing fetus, has been suggested as an alternative method. The potential advantages of *in utero* delivery include a reduction in tissue and immunological barriers to gene transfer, and increased access to expanding populations of progenitor or stem cells.² *In utero* gene transfer has been demonstrated in several animal models using a range of delivery strategies³ but intra-amniotic injection appears to be the most straightforward. In the mouse, gene transfer following intra-amniotic injection of recombinant adenovirus is

dependent on the developmental stage of the fetus at the time of injection. After vector delivery at 16 days of gestation (equivalent to a 15- to 20-week gestation period in humans), transgene expression is observed in the lung, stomach and gut and is highly correlated with increased fetal breathing movements.⁴

Several CF transgenic mouse strains have been generated in an attempt to model and evaluate potential treatment strategies, but although these mouse models display many of the CF-related ion-transport bioelectric defects found in human CF tissues, lung pathology is not recapitulated.⁵ However, some abnormalities of the CF gastrointestinal tract such as intestinal obstruction are observed in mouse models, where they can be very severe, leading to poor survival.⁶ The CF knockout mouse strain S489X carrying the *Cftr*^{tm1Unc} mutation develops intestinal obstruction leading to intestinal perforation, peritonitis and death, resulting in only 5% survival to adulthood,⁷ although these mice can survive given the appropriate dietary intervention.⁸ Survival of these mice was also reported to increase dramatically when recombinant adenovirus expressing human CF transmembrane conductance regulator (hCFTR) was delivered via intra-amniotic injection to fetal mice *in utero*.⁹ The improved survival was observed even though the adenovirus-mediated CFTR expression was transient, implying that continuous expression of CFTR was not required, therefore challenging the widely held view that it is the absence of the CFTR chloride channel that leads to CF disease in the adult. Thus, it was claimed that by some undefined mechanism, transient CFTR expression during fetal development was sufficient to reverse the lethal intestinal disease permanently. Further studies by the same group reported that overexpression of CFTR during development of wild-type +/+ mice was also lethal¹⁰ and that phenotypic changes in the *Cftr* -/- intestines and lungs were partially corrected in the rescued knockout mice.¹¹ These were important observations, not only in the support they lent to the promise of *in utero* gene therapy for CF, but also because the corollary of this observation is that appropriate CFTR expression *in utero* is crucial for normal development of fully functional organs at birth. Thus if CFTR plays an essential role in normal development, not only could *in utero* gene therapy improve the efficiency of gene transfer, but it could also potentially delay the onset of disease symptoms in childhood and compensate for the role, if any, that CFTR plays in the developing organs. Furthermore, if CF is a developmental

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disease, these findings have major implications for the success, or otherwise, of adult CF gene therapy.

The original study demonstrating *in utero* correction of the CF mouse intestinal defect⁹ raised concerns,^{12,13} including questions regarding the small size of the experimental groups leading to the studies being underpowered. The studies presented here were designed to replicate and to control for each aspect of the original study, whilst using statistically rigorous experimental group sizes, in an attempt to independently verify the findings. Generously, the authors of the original study offered their full support. We have confirmed that time-sensitive, intra-amniotic delivery of adenoviral vectors to the murine fetus is straightforward, reproducible and can result in robust transgene expression in the amnion, lungs, and intestines of the developing fetus. However, we were unable to detect a statistically significant improvement in the survival of CFTR treated mice compared with contemporaneous control animals.

RESULTS

Bio-distribution of reagents following intra-amniotic injection

The first step of the *in utero* gene transfer protocol that we aimed to replicate,⁹ was to confirm the bio-distribution of gene transfer reagents after delivery to the developing mouse fetus. Preliminary studies were performed using intra-amniotic injection to deliver 0.1 μm red fluorescent microspheres to time-mated C57BL/6 mice

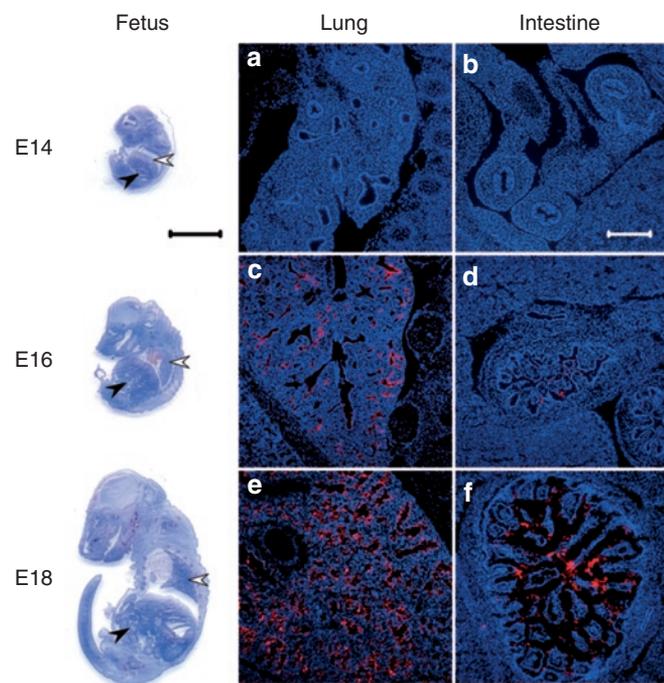


Figure 1 Distribution of fluorescent beads following intra-amniotic injection. Red fluorescent beads (0.1 μm ; 20 μl) were injected into the amniotic fluid of developing C57BL/6 mice at gestational days (a,b) E14, (c,d) E16, and (e,f) E18. Penetration of beads into the (a,c,e) lungs and (b,d,f) intestines 24 hours later was assessed by fluorescence microscopy. Beads are shown in red and nuclei were stained blue with 4',6-diamidino-2-phenylindole dihydrochloride. Scale bar represents 200 μm . Cryosections of fetuses at E14, E16, and E18 were stained with hematoxylin to demonstrate relative size and position of lungs (white arrows) and intestines (black arrows). Scale bar represents 5 mm.

at days 14, 16, and 18 of gestation (E14, E16, E18, respectively). Mice were sacrificed 24 hours after injection and the distribution of beads in each pup examined using fluorescence microscopy on cryosections. Intra-amniotic injection at E14 resulted in the detection of substantial quantities of fluorescent beads within the amniotic fluid, but not within the organs themselves ($n = 7$; **Figure 1a** and **b**). By contrast, delivery at E16 resulted in high levels of fluorescence being detected in the nasal and oral cavities (data not shown) and in both the lungs (7/8) and the intestines (5/8) (**Figure 1c** and **d**). The highest levels of lung (7/7) and intestinal (7/7) fluorescence were observed in fetuses treated at E18 (**Figure 1e** and **f**). No fluorescence was observed in other tissues from treated fetuses, or in any tissues from untreated control fetuses at similar gestational stages. These data confirmed that the gestational timing of injection was important and that intra-amniotic injection at E16 of gestation could deliver to the lungs and intestines, the target organs associated with CF disease. Although delivery at E18 led to increased delivery of fluorescent beads, it also resulted in a higher rate of spontaneous abortion (data not shown). In all subsequent experiments, we utilized E16 as the time of intra-amniotic delivery in line with the original studies.^{9,10}

Gene expression following intra-amniotic delivery of adenoviral vectors

The next step was to quantify reporter gene expression following injection of the recombinant adenoviral vector AdLuc [10^6 to 10^8 plaque forming units (pfu)/fetus] into the amniotic fluid of fetuses from time-mated C57BL/6 mice. After 48 hours, dose-related luciferase expression was detected in all tissues examined (**Figure 2**), although considerable variation between individual animals was observed. The highest levels of reporter gene expression were observed in the amnion, where luciferase expression was 3.9×10^6 -fold above background at a dose of 10^8 pfu/fetus. By contrast, luciferase activity in the lungs and intestines was 6.1×10^4 -fold and 1.5×10^4 -fold above background, respectively. No luciferase expression above background levels was detected in any tissue harvested from control animals that did not receive the AdLuc vector.

After confirming that recombinant AdCFTR could express mature, functional hCFTR in cell culture, by western blotting

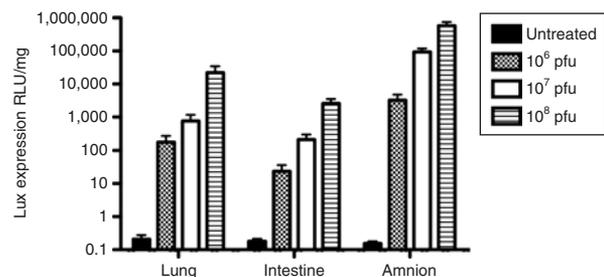


Figure 2 Reporter gene expression following intra-amniotic injection of adenoviral vectors. Dose-dependent luciferase expression: fetal mice at E16 gestation were treated with between 10^6 and 10^8 plaque forming units (pfu) of the luciferase expression vector AdLuc via intra-amniotic injection. Lungs, intestines, and amnion of each fetus were assayed for luciferase expression 48 hours post-treatment and compared with expression in organs from untreated fetuses at similar gestational age. Data represent mean \pm SEM with $n = 9$ –15 individuals per treatment group. RLU, relative light unit.

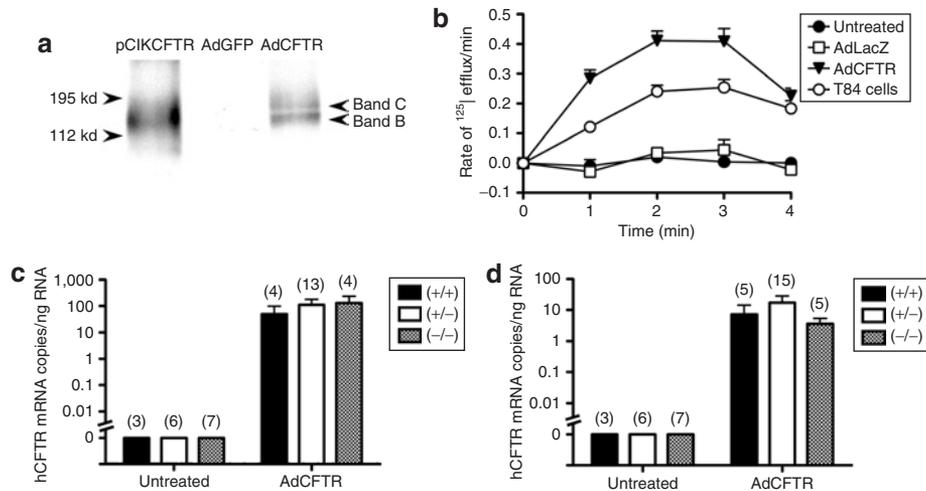


Figure 3 Human cystic fibrosis transmembrane conductance regulator (hCFTR) can be detected in lungs and intestines of S489X *Cftr*^{tm1Unc} mice following intra-amniotic injection of AdCFTR. AdCFTR was used to transduce HEK293T cells: (a) mature hCFTR band C and immature band B are visualized following western blotting with MA37 antibody. Cells transfected with plasmid pCIKCFTR expressing hCFTR, or transduced with AdGFP, acted as positive and negative controls, respectively. (b) Functional hCFTR was assayed by forskolin/3-isobutyl-1-methylxanthine-stimulated ¹²⁵I efflux. Untreated HEK293 cells acted as a negative control and T84 colonocytes that naturally express hCFTR were included as a positive control for CFTR function. Data represents mean ± SEM for six replicates in all groups. Expression of hCFTR mRNA was confirmed *in vivo* following intra-amniotic injection of 10⁸ plaque forming units (pfu)/fetus of AdCFTR at E16 to litters resulting from heterozygote matings of S489X *Cftr*^{tm1Unc} mice. (c) Lungs and (d) intestines from treated fetuses and untreated control litters after 48 hours were assayed for mRNA using TaqMan quantitative RT-PCR. All fetuses were genotyped and mRNA expression presented within fetal genotype groups. Data represents mean ± SEM for all groups. The number of animals per group is indicated in parentheses.

(Figure 3a) and iodide-efflux assay (Figure 3b), the AdCFTR vector was delivered to fetuses from time-mated S489X *Cftr*^{tm1Unc} mice at E16 (10⁸ pfu/fetus) and the level of hCFTR-specific mRNA quantified in the lungs and intestines 48 hours after injection. As all treated fetuses resulted from heterozygote matings, the genotype of each treated fetus was also determined. We showed that the resultant level of hCFTR mRNA was similar for all genotypes in both the lungs (Figure 3c) and intestines (Figure 3d) (*P* > 0.157, Mann-Whitney *U*). Importantly, no hCFTR-specific mRNA expression was detected in the lungs or intestines of any pups of any genotype from control S489X *Cftr*^{tm1Unc} litters that were untreated.

Effect of hCFTR expression on survival of *cftr* -/- mice

Any conclusion regarding the effectiveness of *in utero* hCFTR expression in correcting the lethal intestinal defect in S489X *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc} mice will be wholly dependent on the number of *Cftr* -/- mice surviving after injection of AdCFTR compared with injection of control AdLacZ vector. Therefore each step in the procedure was controlled to ensure that the survival of each mouse was due only to the treatment, rather than any confounding factor in the husbandry or genotyping of the mice. First we improved the published genotyping assay,¹⁴ which tended to have poor discriminatory power in our hands, by developing a multiplex, real-time TaqMan PCR assay that reliably determined genotypes even when using small parts of semi-cannibalized pups as source material (data not shown). Importantly, rather than relying on historical values, we assessed the survival of untreated litters in parallel to provide contemporaneous survival data in our facility. We observed that ~20% of *Cftr* -/- mice routinely survived into adulthood (70 days) even in the absence of the low-residue liquid diet often used to increase *Cftr* -/- survival.⁸ This 20% level

Table 1 *In utero* injection and survival of S489X *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc} mice

	Untreated	AdLacZ 10 ⁷ pfu	AdCFTR 10 ⁷ pfu	AdCFTR 10 ⁸ pfu
Litters	21	17	15	13
Injected	0	142	114	102
Lost	NA	11	14	6
Born	156	131	100	96
Day 0				
-/-	25	20	20	22
+/-	87	54	45	48
+/+	42	48	30	25
Unknown	2	9	5	1
Day 100				
-/-	4	2	2	3
+/-	70	28	39	29
+/+	33	28	26	13
Unknown	0	0	0	0

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; pfu, plaque forming units; NA, not applicable. Litters from heterozygote S489X matings were injected with AdLacZ or AdCFTR at 16 days of gestation. The total number of litters treated, the number of individual fetuses injected and the eventual number of pups detected at birth were recorded for each treatment group. Data for “lost” animals represent fetuses that were resorbed before birth, or cannibalized at birth before litter size was determined. Tissue samples from surviving mice or dead pups were used to determine the number of mice of each genotype at birth (Day 0) and the number of mice of each genotype surviving at 100 days after birth. Unknown genotypes represent pups that were born but could not be genotyped due to maternal cannibalism within the first few days of life.

of survival contrasts with published reports of only 5%,¹⁴ but was consistent over a period of 2–3 years and remained so at the time of the study (see survival of untreated mice in Table 1).

Finally, to assess the impact of *in utero* delivery of AdCFTR on the survival of *Cftr* -/- mice, S489X litters were injected at E16

of gestation with AdCFTR (10^7 or 10^8 pfu/fetus), or the control vector AdLacZ (10^7 pfu/fetus) and survival compared with litters that received no surgical intervention. A similar number of fetuses were injected for each of the three treated groups [142, 114, and 102 for AdLacZ (10^7), AdCFTR (10^7), and AdCFTR (10^8), respectively; **Table 1**]. The number of pups counted at full term was less than the number of fetuses injected for each of the three treated groups [131/142, 100/114, and 96/102 for AdLacZ (10^7), AdCFTR (10^7) and AdCFTR (10^8), respectively; **Table 1**; $P = 0.047$ paired *t*-test] and reflects fetuses that were either resorbed before birth, or cannibalized on the day of birth before litter size was determined. The frequency of such “lost” or missing fetuses did not appear to be related to treatment type as a similar percentage was noted for each of the three treated groups [7.8, 12.3, and 5.9% for the AdLacZ (10^7), AdCFTR (10^7), and AdCFTR (10^8), respectively; **Table 1**] and was also similar in other mouse strains such as C57BL/6 (data not shown). Mothers and offspring were monitored daily with any deaths recorded and tissue samples removed from deceased animals for genotyping wherever possible. It was not possible to determine the genotype of a small number of pups due to complete maternal cannibalism, but this did not appear to be related to treatment as it occurred with a similar frequency in all four experimental groups [1.3, 6.9, 5.0, and 1.0% for untreated, AdLacZ (10^7), AdCFTR (10^7), and AdCFTR (10^8), respectively; **Table 1**]. At 21 days, surviving offspring were weaned from their mother and tissue samples taken to determine the *Cftr* genotype and surviving offspring were monitored up to 100 days after birth.

In the untreated control group, high levels of mortality were observed for all genotypes shortly after birth, with 33% of *Cftr* $-/-$ pups dying within 3 days compared with 19% of $+/-$ and 22% of $+/+$ pups (**Figure 4a**). Following this initial critical period, few additional deaths were observed with 80% of $+/-$ and 79%

of $+/+$ mice surviving beyond the end of the study at 100 days. By contrast, only 16% of $-/-$ pups survived beyond the end of the study (**Figure 4a**) with 63% dying before weaning at 21 days. Crucially, *in utero* administration of adenoviral vectors expressing hCFTR resulted in no significant improvement in survival of *Cftr* $-/-$ mice to 100 days (**Figure 4b**). Kaplan–Meier estimates of the median survival age for 10^7 or 10^8 pfu/fetus AdCFTR-treated groups and the AdLacZ control group, were 9 ± 7.8 , 2 ± 2.3 , and 7 ± 6.7 days, respectively, similar to the 13 ± 2.5 days noted for the untreated group. In all four experimental groups the majority of *Cftr* $-/-$ deaths (~80–85%) occurred in the first 4 weeks after birth, although a modest number (~10–15%) in each treatment group survived beyond the end of the study at 100 days. Surviving $-/-$ animals demonstrated the “white teeth” phenotype commonly observed in homozygous CF mice¹⁵ and were much smaller than surviving $+/-$ and $+/+$ littermates ($P = 0.0005$; analysis of variance) with a mean weight (at day 70) of 21.0 ± 1.0 g compared with 25.4 ± 0.3 g and 25.0 ± 0.4 g in $+/-$ and $+/+$ mice (data from all groups combined). Crucially, the log rank survival test revealed no significant difference in survival between the four experimental groups of *Cftr* $-/-$ animals ($P = 0.71$). Therefore, in this study *in utero* treatment with adenoviral vectors expressing hCFTR did not enhance survival of CF animals compared with contemporaneous control animals.

DISCUSSION

We have confirmed that intra-amniotic injection at E16 of gestation allows access to the developing organs of the murine fetus (**Figure 1**) and that delivery of recombinant adenoviral vectors can lead to expression of luciferase reporter gene (**Figure 2**) and hCFTR mRNA (**Figure 3**) in fetal lungs and intestines. However, there was statistically no significant improvement in the survival of *Cftr* $-/-$ mice after AdCFTR treatment, compared with contemporaneous control animals (**Figure 4**). Therefore, despite careful replication, we were unable to verify the observation that expression of hCFTR in the fetal intestine could correct the lethal intestinal defect in the CF knockout mouse as described in an earlier study.⁹

Inspection of the ratio of *Cftr* $-/-$, $+/-$, and $+/+$ genotypes observed in the various treatment groups at birth (**Table 1**), revealed a departure from the expected 1:2:1 Mendelian distribution in both the untreated and the AdLacZ-treated groups ($P = 0.034$ and 0.0004 , respectively, χ^2). By contrast, no deviation from a Mendelian genotype distribution was observed in the AdCFTR (10^7) or AdCFTR (10^8) treated groups. One interpretation is that treatment with AdCFTR led to increased *in utero* survival of *Cftr* $-/-$ fetuses as argued previously.¹⁰ However other plausible explanations exist. The observed non-Mendelian distribution of genotypes between groups might be explained by pups lost to genotyping after complete maternal cannibalism, and/or by the fact that genotype distributions of the experimental groups at the time of treatment may not have been equivalent, or by chance alone. Support for these alternative explanations comes from the observation that the genotype distributions observed in the AdCFTR (10^7) and AdCFTR (10^8) treated groups were not significantly different from those observed in the untreated control group ($P = 0.151$ and 0.137 , respectively, χ^2).

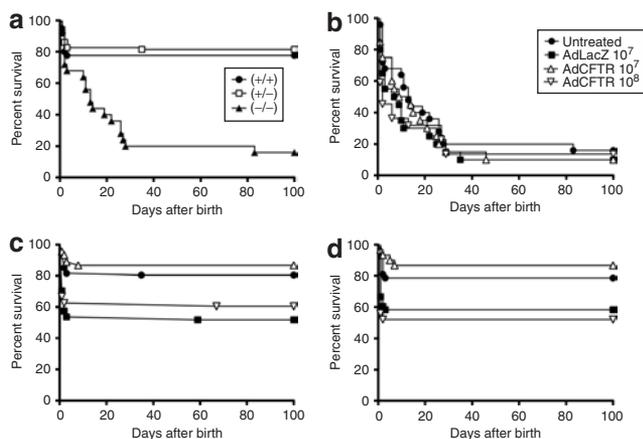


Figure 4 Survival of S489X *Cftr*^{tm1Unc} mice following expression of human cystic fibrosis transmembrane conductance regulator (hCFTR) from AdCFTR. Kaplan–Meier plots of S489X *Cftr*^{tm1Unc} survival following intra-amniotic injections. Fetuses from heterozygote matings were injected at E16 with 10^7 plaque forming units (pfu) of AdLacZ, 10^7 pfu AdCFTR or 10^8 pfu AdCFTR. Genotype-specific survival was monitored up to 100 days after birth and compared to survival rates seen in (a) contemporaneous untreated control litters. Survival rates were assessed separately for (b) $-/-$ mice, (c) $+/-$ mice and (d) $+/+$ mice. The numbers of animals are shown in **Table 1**.

Larson and colleagues also reported treatment-dependent alterations in the expected Mendelian ratio of *Cftr* +/- and +/- mice.¹⁰ In their untreated population, they observed an excess of *Cftr* +/- progeny (from untreated heterozygote matings) that survived >75 days, such that the ratio was closer to 1:1 (*Cftr* +/- to +/-) rather than 2:1 as expected from Mendelian inheritance.¹⁰ A similar 1:1 ratio was also observed after AdLacZ treatment, though the numbers of animals reported were too low to be statistically powerful.¹⁰ However, after AdCFTR treatment, the number of *Cftr* +/- and +/- animals surviving >75 days was closer to 10:1. The authors' interpretation was that *in utero* AdCFTR treatment had two distinct effects: firstly, the correction of the lethal intestinal phenotype in *Cftr* -/- mice, and secondly, a decreased survival of *Cftr* +/- mice due to a hyperplastic lung proliferative disorder.¹⁰ While we did not measure the lung pathology of the *Cftr* +/- mice in our treatment groups, our survival data (Table 1 and Figure 4) did not reproduce the AdCFTR dependent changes in *Cftr* +/- to +/- ratio that they observed.

It was unfortunate that the S489X *Cftr*^{tm1Unc} mouse strain demonstrated such high levels of maternal cannibalism and neglect leading to a high mortality of newborn pups of all genotypes, especially following surgical interventions. Losses were reduced by transferring mice onto a high protein breeders' diet, but to minimize the impact of nonspecific deaths upon the survival study, a series of exclusion criteria were incorporated into the study design such that data from the entire litter were excluded if no pups were born, or the entire litter was eaten, or if the entire litter died within the first week of life. Thus large numbers of litters were excluded from both control and treatment groups, but sufficient litters were treated to allow statistical analysis of survival with enough power to identify any treatment-associated increase in survival (Table 1).

It is formally possible that minor technical differences between our study and that of Larson *et al.*⁹ could explain the difference in results obtained. For example, the accurate calculation of gestational age can be difficult to confirm and small differences in the timing of the intra-amniotic injection could be crucial to subsequent intestinal development. It is also possible that there were inconsistencies in the calculation of the adenoviral vector titre causing an under- or overestimation of the final dose administered. In the original study 10⁸ pfu/fetus of AdCFTR were delivered,⁹ but later publications from the same group suggested that a dose of 10⁷ pfu/fetus was also sufficient¹⁰; we observed no difference in survival outcome following the delivery of 10⁷ or 10⁸ pfu AdCFTR and neither did the authors of the accompanying paper when an injected dose of 10⁸ or 2 × 10⁹ pfu/fetus was used.¹⁶ Although methods for calculating the potency (pfu/ml) of a viral preparation may vary between laboratories, the range of doses investigated here seems likely to be sufficient to reveal any dose-dependent differences.

An important difference is the ~20% survival of the *Cftr* -/- mice observed in our facility compared with a previously reported 5%.¹⁴ The intestinal pathology and mortality in CF transgenic mouse models can vary dependent on the precise *Cftr* knockout mutation, environmental influences and the independent segregation of modifier genes.^{6,17} The survival chances of one CF mouse model *Cftr*^{tm1G551D}/*Cftr*^{tm1G551D} were shown to vary between 27 and 67% depending on undetermined environmental factors in the

animal breeding facility.¹⁸ Interestingly, when we transferred a subset of our S489X *Cftr* -/- mice to an alternative animal facility they died of intestinal obstruction within a few days (data not shown). Together these observations suggest that survival is a problematic end point to utilize reliably in CF mice.

An additional and potentially confounding issue is the genetic heterogeneity in the transgenic mouse strain. Unavoidably, our studies used S489X *Cftr*^{tm1Unc} CF knockout mice as a 10th generation backcross onto to C57BL/6 background, as opposed to a 5th generation backcross.¹⁰ In the accompanying paper, Buckley *et al.*¹⁶ attempted independent verification of the original study, using the same adenoviral vector, but using an alternative knockout CF mouse allele (*Cftr*^{tm1Cam}/*Cftr*^{tm1Cam}) on an inbred genetic background (129 S6Sv/Ev) to minimize genetic heterogeneity and the effects of potential modifier genes. However, the authors were also unable to demonstrate improved survival of *Cftr* -/- mice following intra-amniotic injection of AdCFTR.

We have, to the best of our ability, replicated the original *in utero* gene transfer study of Larson and Cohen. We used identical surgical methods and adenoviral vectors, along with a population of CF mice that was as similar as possible; we also secured the support of the original authors for our approach. We have demonstrated the feasibility and success of each stage in the procedure, but were still unable to confirm that transient *in utero* expression of CFTR in the developing intestines of the CF mouse was sufficient for improved survival. This could be due to problems with the design of the original study, including the use of death as an end-point in a mouse strain where survival is susceptible to genetic heterogeneity and environmental factors. Finally, although adenoviral vectors appear to transduce a range of tissues after *in utero* delivery, in the absence of integration, these vectors will ultimately be diluted out following successive rounds of cell proliferation in the developing fetus. Thus alternative vectors capable of integration and/or persistent gene expression will be required if *in utero* gene therapy is to be investigated further for correction of genetic diseases.

MATERIALS AND METHODS

Animal model. The *Cftr*-mutant UNC mouse strain, S489X (*Cftr*^{tm1UNC}/*Cftr*^{tm1UNC}) was obtained as a 10th generation backcross on a C57BL/6 background (Dr Julia Dorin, MRC Human Genetics Unit, Edinburgh, UK) and was maintained via heterozygote matings in open cages with hardwood sawdust and R&M3 breeders' diet (Special Diet Services, Witham, UK) with weaning at 21 days. No additional precautions were taken to protect animals from exposure to pathogens.

Genotyping. Genomic DNA was extracted from tail biopsies at weaning, or from tissue taken from animals that died before weaning, using Qiagen DNeasy kit (Qiagen, Crawley, UK). Genotyping was performed using an ABI PRISM 7700 Sequence Detector (TaqMan) (Applied Biosystems, Warrington, UK) utilizing primer and fluorogenic probe combinations specific to murine *Cftr* exon 10 and to the inserted neomycin gene contained in the mutant allele. Genomic DNA (~250 ng) was analyzed in a 25 µl multiplex reaction containing 1 × Universal PCR Master Mix (Applied Biosystems) and primer/probe combinations for the simultaneous detection of both alleles. Detection of the wild-type allele utilized mCFTR exon 10-1283F 5'-TTGGTGTTCCTATGATGAGTACAGAT-3' (300 nmol/l) and mCFTR exon 10-1381R 5'-AGCTAACACAATCAGCATTTTTCATAA-3' (300 nmol/l) primers and the mCFTR exon 10-1317B probe VIC-5' TACCTGCTGTAGTTGGCAAGCTTTGACAACA 3'-TAMRA

(100 nmol/l). Detection of the mutant allele utilized UNCmut256F 5'-AGA GGAATTATTAAGCACAGTGGAA-3' (300 nmol/l) and UNCmut484R 5'-CATCTGCGTGTTCGAATTCG-3' (300 nmol/l) primers and the UNCmut433T fluorogenic probe FAM-5'-CGAGCAAACCCCGCCCA GC-3'-TAMRA (100 nmol/l). Reactions were incubated at 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were assigned as positive for each respective allele where amplification occurred before reaching a threshold cycle (C_t) of 33. Samples were assigned as mutant homozygous (-/-), wild-type homozygous (+/+), or heterozygous (+/-) based on whether positive amplification of mutant, wild type or both alleles were detected, respectively.

Adenoviral vectors. First generation E1/E3 deleted adenoviral vectors were used: AdLacZ (Ad5CMVLacZ) expressing nuclear-localized β -galactosidase from the human cytomegalovirus promoter; AdCFTR (Av1CF2) expressing hCFTR from the rous sarcoma virus promoter, and AdLuc expressing firefly luciferase from the human cytomegalovirus promoter (all provided by Dr Janet Larson, Stony Brook Health Center, Stony Brook, NY). Purified virus was prepared by CsCl banding by Qbiogene. (Montreal, Canada). Viral particles were determined by OD₂₆₀¹⁹ and pfu were determined using a plaque/particle ratio of 1/100 and confirmed by the TCID₅₀ method.²⁰ Viral stocks were stored at -80°C in 20 mmol/l Tris pH8.0, 25 mmol/l NaCl, 2.5% glycerol.

In utero administration of vectors. All animal procedures adhered to the UK Home Office Animals (Scientific Procedures) Act 1986. To generate time-mated pregnancies, heterozygote S489X *Cftr*^{AmiUnc} females were introduced to cages containing heterozygote males overnight with the following day regarded as day 1 of gestation (E1) in resulting pregnancies. Pregnant females at day 16 of gestation (E16) were anesthetized using 3% isoflurane (Abbott Laboratories, Queensborough, UK) and a midline laparotomy performed to expose the uterine horns. Using a 31G needle, and a 200- μ l precision syringe (Hamilton Company, Reno, NV), 20 μ l of adenoviral solution or fluorescent beads diluted in Dulbecco's modified Eagle's medium were injected through the uterine wall and into the amniotic fluid of each fetus. Following injection, the uterine horns were replaced within the abdomen and the abdominal wall sutured in two layers using 6/0 polydioxanone absorbable sutures (Ethicon, Ascot, UK). Treated females recovered in a heated cabinet and were subsequently housed individually with appropriate nesting material. Unless otherwise stated, pregnancies were allowed to run to term with the treated female caring for the litter until weaning at 21 days.

Fluorescence microscopy. Twenty-four hours after *in utero* delivery of 20 μ l 0.1 μ m red fluorescent beads (FluoSpheres; Molecular Probes, Leiden, Netherlands) individual fetuses were fixed in 4% paraformaldehyde at 4°C overnight. Whole fetuses were cryopreserved in 30% sucrose (wt/vol) in phosphate-buffered saline for 3 days at 4°C and embedded in OCT embedding matrix (Cellpath, Newtown, UK) before 8 μ m cryosections were prepared using a Leica CM3050 S cryostat (Leica Instruments, Nussloch, Germany). Visualization of fluorescent beads was achieved using a Nikon Optiphot 2 epifluorescence microscope (Nikon UK, Kingston on Thames, UK) equipped with a standard Texas red filter set. Nuclei were stained using 4',6-diamidino-2'-phenylindole dihydrochloride (Roche Diagnostics, Mannheim, Germany) applied directly into the mounting media at 5 μ g/ml.

Reporter gene detection. Fetal organs were homogenized in 100 μ l of Reporter Lysis Buffer (Promega, Southampton, UK) using disposable pellet pestles (Anachem, Luton, UK) and cleared lysates tested using the Luciferase assay system (Promega) in conjunction with a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). Total protein was determined using the DC-protein assay (BioRad, Hemel Hempstead, UK) and luciferase activity in relative light units was normalized for protein content before graphing. For comparative purposes, under the assay conditions utilized 100 relative light units corresponds to 4 pg recombinant Luciferase (Promega).

Detection of functional hCFTR protein. Expression of mature hCFTR protein was confirmed in HEK293 cells²¹ following transduction with 1×10^6 pfu/well of AdCFTR, or control AdGFP (Genethon, Evry, France) (multiplicity of infection of 200). Cells were lysed 48 hours after transduction using RIPA buffer (Upstate, Lake Placid, NY) and western blotting performed as described²² using mouse M3A7 anti-CFTR primary antibody (Upstate) (1:1,000 dilution) and a sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Little Chalfont, UK) (1:1,500 dilution). Cells transfected with the plasmid pCIKCFTR expressing hCFTR under control of the human cytomegalovirus promoter²³ were included as a positive control. CFTR chloride channel activity was assayed in HEK293 cells following 24 hours incubation with 2×10^6 pfu of AdCFTR, or control AdLacZ (multiplicity of infection of 20). The combined forskolin (30 μ mol/l) and 3-isobutyl-1-methylxanthine (100 μ mol/l) stimulated rate of cellular ¹²⁵I efflux was measured 24 hours later as described previously.²⁴ T84 colonocytes (American Type Culture Collection, LGC Promochem, London, UK) that naturally express CFTR²⁵ were included as a positive control.

Detection of hCFTR mRNA expression in fetal tissue. Fetal organs were homogenized using disposable pellet pestles (Anachem) in 300 μ l of RLT buffer containing 1% β -mercaptoethanol (Qiagen). Lysates were dispersed using QiaShredder columns (Qiagen) and total RNA extracted using RNeasy Mini kit (Qiagen) with on-column DNase digestion. All reagents were supplied by Applied Biosystems except where stated. Total RNA (125 ng) was reverse transcribed in a 20 μ l complementary DNA (cDNA) synthesis reaction using 400 nmol/l of reverse primer hCFTR-229R 5'-CCAGGCGCTGTCTGTATCCT-3' with or without reverse transcriptase (RT+ or RT-). Comparator samples included a no template control and synthetic human CFTR mRNA ranging from 12.5 to 195312.5 copies/reaction. Each complementary DNA reaction (5 μ l) was added to a 25 μ l TaqMan RT-PCR (1 \times TaqMan Universal Mastermix, 300 nmol/l hCFTR-150F 5'-GGAAAAGGCCAGCGTTGTC-3' primer, 300 nmol/l hCFTR-229R 5'-CCAGGCGCTGTCTGTATCCT-3' and 100 nmol/l hCFTR-170T fluorogenic probe FAM-5'-CCAAACTTTTTTTCAGCTGGACCAGAC CAA-3'-TAMRA). Reactions resulting from the RT+ were performed in triplicate and a single TaqMan reaction was performed from each RT- reaction. PCR and fluorescence detection were performed using a TaqMan 7900 Real-Time PCR System using standard PCR cycling conditions and results analyzed using Sequence Detection System Software Version 2.2.2 (Applied Biosystems). Samples where the RT- reaction was within 3 C_t of the mean RT+ C_t value were excluded from the analysis. Data were plotted as number of hCFTR copies/ng of total RNA within each reaction.

Statistical analysis. Kaplan-Meier survival analysis was used to predict median survival times. Survival times were censored at 100 days. Median survival times are reported \pm SEM. Differences between survival curves were determined using the Cox-Mantel log-rank test. Differences in luciferase expression and mouse weights were determined using analysis of variance on log-transformed data where appropriate, combined with protected least significance difference *post-hoc* tests. Levels of hCFTR expression that could not be log-transformed to normality were determined using Kruskal-Wallis combined with Bonferroni corrected Mann-Whitney *post-hoc* tests. Differences in treatment group sizes were determined using paired *t*-tests. Differences in genotype ratios were determined using χ^2 tests with two degrees of freedom. Statistical significance in all tests was accepted at $P < 0.05$. Appropriate group sizes were selected with the assistance of statistical power analysis. In all instances, statistical power for observed statistical differences exceeded 0.8.

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