Abstract

**Background:** Cystic Fibrosis is a pleiotropic disease in humans with primary morbidity and mortality associated with a lung disease phenotype. However, knockout in the mouse of \textit{cftr}, the gene whose mutant alleles are responsible for cystic fibrosis, has previously failed to produce a readily quantifiable lung phenotype.

**Results:** Using measurements of pulmonary mechanics, a definitive lung phenotype was demonstrated in the \textit{cftr}-/- mouse. Lungs showed decreased compliance and increased airway resistance in young animals as compared to \textit{cftr}+/- littermates. These changes were noted in animals less than 60 days old, prior to any long term inflammatory effects that might occur, and are consistent with structural differences in the \textit{cftr}-/- lungs. Surprisingly, the \textit{cftr}+/- animals exhibited a lung phenotype distinct from either the homozygous normal or knockout genotypes. The heterozygous mice showed increased lung compliance and decreased airway resistance when compared to either homozygous phenotype, suggesting a heterozygous advantage that might explain the high frequency of this mutation in certain populations.

**Conclusions:** In the mouse the gene dosage of \textit{cftr} results in distinct differences in pulmonary mechanics of the adult. Distinct phenotypes were demonstrated in each genotype, \textit{cftr}-/-, \textit{cftr}+/-, and \textit{cftr}+/+. These results are consistent with a developmental role for CFTR in the lung.

**Background**

Cystic fibrosis (CF) is a progressive disease primarily affecting the intestines, lungs, and pancreas. The gene responsible for CF was identified in 1989 [1] as coding for the cystic fibrosis transmembrane conductance regulator (\textit{cftr}), a membrane chloride channel. CF is one of the most common autosomal recessive diseases in Caucasians with a carrier rate of 3–4% [2], and is characterized by recurrent infection and chronic inflammation. Recently it was found that infants with CF demonstrate changes in forced expiratory volume in 1 second (FEV\textsubscript{1}), functional residual capacity (FRC), and other parameters of lung function prior to the onset of recurrent infection [3-5].

Soon after the CF gene was discovered, a knockout mouse was developed. This mouse demonstrates subtle changes
in epithelial cell phenotype, including alterations in secretory glycoconjugates and changes in secretory vesicles [6]. Monocytic infiltrates and altered lung mechanics have also been found [7]. Unfortunately, the cftr knockout mouse does not develop overt lung disease, which has severely limited its usefulness. However, the availability of new methods for pulmonary testing in rodents [8,9] now presents the opportunity to re-examine the cftr knockout mouse for functional lung changes. In the present study, therefore, we examined pulmonary function in young adult cftr-/-, cftr +/-, and cftr+/+ S489X mice in an effort to establish a lung phenotype.

Results
Effect of cftr gene dosage on pressure-volume (PV) curves
Routine evaluation of dynamic lung function employs the stepwise variation in air volume on both the inflation and deflation phases of a single breath. Measurement of airway pressures at each step results in the classic pressure-volume (PV) curve which is dependent upon both lung structure and interfering pathology. PV curves were measured in triplicate, starting from positive end-expiratory pressure (PEEP) values of 0, 3, and 6 cmH2O in S489X mice at 30–60 days of age following genotyping for the normal and mutant cftr alleles. The 3 cmH2O PEEP curves obtained for each genotype are presented in Figure 1A. Note that these PV curves all begin at V = 0 ml, which is the FRC defined by the 3 cmH2O PEEP. The PV curves obtained at PEEP levels of 0 and 6 cmH2O were similar.

The static compliance (Cst) of the lungs, which reflects elastic recoil at a given pressure, was calculated from the slopes of the PV curves. As shown in Figure 1B, the static compliance of the homozygous knockout lung was significantly decreased compared to the homozygous normal lung (p < 0.01)). Furthermore, Cst was significantly reduced in both cftr+/+ (p < 0.001) and cftr-/- (p < 0.001) as compared to age-matched cftr+/- mice. Hysteresis was altered among the 3 genotypes (Figure 1C). A statistically significant increase in hysteresis was observed in both cftr+/- (P < 0.0001) and cftr+/- mice (p < 0.05) compared to cftr-/- mice. These data suggest the presence of a gene dosage effect in which an altered lung structure in the heterozygous animals leads to an elevated compliance relative to the two homozygous animals. Lung weights were measured and no statistically significant differences were observed among the three genotypes (Figure 1D).

Airway mechanics of cftr deficient lungs
We applied the forced oscillation method to the mice and determined respiratory mechanical input impedance [10,11]. We fit the constant-phase model of respiratory mechanics [12] to impedance (Zrs) and determined values for airway resistance (Raw), tissue damping (G), and tissue elastance (H). Figure 2 shows that Raw, G, and H were significantly reduced in the cftr+/+ mice as compared to both cftr+/- and cftr-/- animals (Panels A, C, & D) at all PEEP levels. Raw also decreased with PEEP in a similar fashion in all three genotypes. In contrast, Raw, H and G were significantly increased in the cftr-/- mice compared with cftr+/- and cftr+/-, and showed a greater dependence on PEEP (Panels A, C & D). The ratio G/H, termed hysteresivity (both the low and high frequency), was not significantly affected by either genotype or PEEP (Panel E). Thus, the absence of either 1 or 2 copies of the cftr gene had significantly different effects on the phenotype of the lung. Paradoxically, the absence of only one cftr copy resulted in a greater lung compliance (lower elastance) than if neither or both copies were present.

Discussion
The usefulness of the cftr knockout mouse as a model of cystic fibrosis has been severely limited by its failure to demonstrate readily measurable lung disease, the primary cause of morbidity and mortality in humans [13]. However, in the present study use of sophisticated measurements of lung function revealed a functional lung phenotype in the knockout mouse (Table 1); the complete absence of cftr in the lung of young adult animals resulted in decreased Cst and η and increased Raw, G and H as compared to normal littermate controls.

A particularly intriguing further observation was that Cst and hysteresis in cftr+/- mice was significantly higher than in cftr+/- animals while G and H were decreased. As this was not associated with any pathology such as emphysema, we conclude that it represents a functionally different lung from that of the cftr+/. Our data thus reveal a remarkable inverse correlation between the effect of one and two non-functional copies of the cftr gene.

What do these data mean in terms of lung structure? The knockout animal has cystic fibrosis by definition, and our data now show it to also have lung disease manifest as a reduced compliance and increased resistance. These changes could reflect changes in the intrinsic mechanical properties of the parenchyma, or simply a reduction in the biophysical properties of the air-liquid interface in the lungs, and would be expected to result in a change in η[14]. Indeed, because cftr is a chloride channel and is thought to be involved in water balance, a change in surface tension in the lung, and consequently in η, might be expected. However, as shown in Figure 2 and Table 1, although G and H both increase, they do so in the same proportion so there is no significant change in η between the three cftr genotypes. On the other hand, lung weights were not different among the different groups of mice, so the decreased compliance and increased resistance of the cftr-/- animals was not simply due to their having smaller
Figure 1
Relationship between cftr genotypes and PV curves. Littermates from 30–60 days of age were genotyped and individuals with cftr+/+ (Black), cftr+/- (Green), and cftr-/- (Red) were evaluated using pressure volume curve analysis at Peeps of 0, 3, and 6. A: PV curve at PEEP 3; B: Calculated Cst for all PV curves; C: Calculated hysteresis for PV curves in A. All measures were corrected individually for lung weight. Error bars are +/- standard deviation. *p < 0.05 when compared to cftr+/+ and **p < 0.05 when compared to cftr+/-.
lungs than control animals. This suggests that the parenchymal structure in the lungs of the homozygous and heterozygous animals were organized differently, in a manner that affected $G$ and $H$ similarly.

As documented in numerous publications, the mouse strain used in the present study does not develop chronic inflammatory disease up to the age (30–60 days) used in this study (for review see [15]). On the other hand,
Broaches-Carter et al. [16] have shown that cftr levels are highest in the developing lung and decrease 75-fold at birth. In utero over-expression of cftr has also been shown to affect lung growth and development [17], and the severity of disease in the knockout mouse has been shown to be influenced by genetic background [18]. These data thus suggest that cftr may affect the early development of the lung in a manner that is affected by the interaction of other genes.

Are there any functional consequences for increased lung compliance in the heterozygous cftr animals? Interestingly, there is no decrement in lung function in human heterozygotes [19-21]. Also, the heterozygote frequency for CF in humans is higher than expected, likely reflecting a selective advantage because there is no evidence for genetic drift [22,23]. Indeed, selective advantage in CF has been proposed to reflect resistance to tuberculosis, influenza and cholera [24]. When one looks in nature for other examples of heterozygous advantage, the sickle cell trait which confers resistance to malaria [25] is perhaps the only such recognized genetic trait in humans. In Norway rats, a single Mendelian gene controls resistance to Warfarin, an anticoagulant used to control rat populations; homozygous wild-type rats are killed by Warfarin and homozygous mutant allele animals are highly susceptible to vitamin K deficiency [26]. The results of the present study indicate that a similar selective advantage may pertain to cftr, something we term a "Goldilocks Effect". That is, while two defective copies of the gene are detrimental and two normal copies are satisfactory, one normal and one defective gene may results in an optimal dosage for lung development.

Further studies of pulmonary mechanics in cftr knockout mice should reveal additional genetic loci that modulate the influence of cftr on lung growth and development. Corresponding studies in humans should be useful in evaluating the effect of therapies on reversing altered pulmonary function in the CF patient.

Conclusions
Using sophisticated techniques to evaluate rodent pulmonary function; a distinct, readily quantifiable lung phenotype was identified in the cftr knockout mouse. In addition, the cftr+/- mouse had a distinguishable pulmonary function phenotype from that observed in either the homozygous normal or mutant genotype mice. These data are consistent with CFTR-dependent, physiologic changes in the structure and function of the lung.

Methods
Mouse strain
The S489X mouse 5th generation backcross to C57Bl/6 has been maintained by random mating for the past 8 years.

This colony has a 100% mortality rate among cftr knockouts by 45 days of age unless the animals are placed on an elemental liquid diet and corn cob bedding upon weaning [27]. Mice 30–60 days of age from our S489X mouse colony were genotyped for the normal and mutant cftr alleles. Age and litter matched cftr+/+ and cftr+-/ were used for each cftr-/- mouse examined. Six animals were included in each group. All experiments were approved by the animal care and use committee.

Pulmonary function tests
The mice were anesthetized with intra-peritoneal pentobarbital (90 mg/kg) and the trachea was dissected free of surrounding tissue and cannulated with a 20-gauge cannula. The animals were then connected to a small animal ventilator (flexiVent, SCIREQ Inc. Montreal, PQ, Canada) and ventilated with a tidal volume of 10 ml/kg; inspiratory-expiratory ratio of 66.67%, respiratory rate of 150 breaths/minute, and maximum pressure of 30 cmH2O. PEEP was controlled by submerging the expiratory limb from the ventilator into a water trap. Each animal was paralyzed with pancuronium bromide (0.5 mg/kg) and allowed to equilibrate on the ventilator until spontaneous breathing ceased (5 minutes).

Respiratory mechanics
To measure Zrs, mechanical ventilation was interrupted and the animal was allowed to expire against the set level of PEEP for 1 s. We then applied an 8 s broad-band volume perturbation signal to the lungs with the flexiVent, after which ventilated was resumed. This was repeated at PEEP levels of 0, 3 and 6 cmH2O. The volume perturbation signal consisted of the superposition of 18 sine waves having frequency spaced roughly evenly over the range 0.25 Hz to 19.625 Hz. Zrs was calculated from the displacement of the ventilator's piston and the pressure in its cylinder as described previously [10,11]. Correction for gas compressibility as well as resistive and accelerative losses in the flexiVent, connecting tubing and the tracheal cannula were performed as described previously [28] using dynamic calibration data obtained by applying volume perturbations through the tubing and tracheal cannula first when it was completely closed and then when it was open to the atmosphere.

We interpreted the measurement of Zrs in terms of the constant phase model [12] 

\[ Zrs(f) = Raw + i2\pi flaw + \frac{G - iH}{(2\pi f)^a} \]

where Raw is a frequency independent Newtonian resistance reflecting that of the conducting airways [29]. fraw is airflow gas inertance, G characterizes tissue damping, H characterizes tissue stiffness (elastance), i is the imaginary
unit, $\alpha$ links G and $H$, and f is frequency. We also calculated a quantity known as hysteresivity ($\eta = G/H$), which is believed to increase when regional heterogeneities develop in the lung [30]. Raw, G and $H$ were all normalized by multiplication by lung weight.

**PV curves**

Starting at the FRC defined by the PEEP, the flexiVent was programmed to deliver seven inspiratory volume steps for a total volume of 0.8 ml followed by seven expiratory steps, pausing at each step for 1 s. Plateau pressure ($P$) at each step was recorded and related to the total volume ($V$) delivered to produce a quasi-static PV curve. $Cst$ was calculated from the slope of each curve [31], and was normalized by division by lung weight.

$Zrs$ measurements at each PEEP level and PV curves were obtained in triplicate. Data were statistically evaluated using paired t-test with $p < 0.05$ being taken as significant.

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**References**

1. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenziger J, Lok S, Plasvick N, Chou JL, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989, 245:1066-1073.

2. Raman V, Clary R, Siegrist KL, Zehnbauer B, Chatila TA: Increased prevalence of mutations in the cystic fibrosis transmembrane conductance regulator in children with chronic rhinosinusitis. Pediatrics 2002, 109:e13.

3. Hart N, Polkey MI, Clement A, Boule M, Moxham J, Lofasso F, Fauroux B: Changes in pulmonary mechanics with increasing disease severity in children and young adults with cystic fibrosis. Am J Respir Crit Care Med 2002, 166:61-66.

4. Sharp JK: Monitoring early inflammation in CF. Infant pulmonary function testing. Clin Rev Allergy Immunol 2002, 23:59-76.

5. Tepper RS, Zander JE, Eigen H: Chronic respiratory problems in infancy. Curr Prog Pediatr 1986, 16:305-359.

6. Cohen JC, Morrow SL, Cork RJ, Delcarpio JB, Larson JE: Molecular pathophysiology of cystic fibrosis based on the rescued knockout mouse model. Mol Genet Metab 1998, 64:108-118.

7. Kent G, Oliver M, Fosket J, Frndova H, Durie P, Forstner J, Forstner GG, Riordan JR, Percy D, Buchwald M: Phenotypic abnormalities in long-term surviving cystic fibrosis mice. Pediatr Res 1996, 40:233-241.

8. Allen JT, Spiteri MA: Growth factors in idiopathic pulmonary fibrosis: relative roles. Respir Res 2002, 3:13.

9. Pillow JJ, Wilkinson MH, Neil HL, Ramsden CA: In vitro performance characteristics of high-frequency oscillatory ventilators. Am J Respir Crit Care Med 2001, 164:1019-1024.

10. Gomes RF, Shen X, Ramchandani R, Tepper RS, Bates JH: Comparative respiratory system mechanics in rodents. J Appl Physiol 2000, 89:908-916.

11. Hirai T, McKeown KA, Gomes RF, Bates JH: Effects of lung volume on lung and chest wall mechanics in rats. J Appl Physiol 1999, 86:16-21.

12. Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ: Input impedance and peripheral inhomogeneity of dog lungs. J Appl Physiol 2002, 92:168-178.

13. Geiser M, Zimmermann B, Baumann M, Cruz-Orive LM: Does lack of Cftf gene lead to developmental abnormalities in the lung? Exp Lung Res 2000, 26:551-564.

14. Fredberg JJ, Stamenovic D: On the imperfect elasticity of lung tissue. J Appl Physiol 1989, 67:2408-2419.

15. Staub D, Pridie BC: Mouse models of chronic lung infection with Pseudomonas aeruginosa: models for the study of cystic fibrosis. Pediatr Pulmonol 2000, 30:413-424.

16. Brookes-Carter FC, Mouchel N, Gill D, Hyde S, Bassett J, Harris A: Temporal regulation of CFTR expression during ovine lung development: implications for CF gene therapy. Hum Mol Genet 2002, 11:125-131.

17. Larson JE, Delcarpio JB, Farberman MM, Morrow SL, Cohen JC: CFTR modulates lung secretory cell proliferation and differentiation. Am J Physiol Lung Cell Mol Physiol 2000, 279:L333-41.

18. Hantos CK, McKeeler C, Newbigging S, Corey M, Rozmahel R, Tsai LC: Detection of modifier loci influencing the lung phenotype of cystic fibrosis knockout mice. Mamm Genome 2002, 13:605-613.

19. Halliet WY, Knudson A. G., Jr., Massey F. J., Jr.: Absence of detrimental effect of the carrier state for the cystic fibrosis gene. Am Rev Respir Dis 1965, 92:714-724.

20. Byard P, Davis PB: Pulmonary function in obligate heterozygotes for cystic fibrosis. Am Rev Respir Dis 1988, 138:312-316.

21. Davis PB, Byard PJ: Heterozygotes for cystic fibrosis: models for study of airway and autonomic reactivity. J Appl Physiol 1989, 66:2124-2128.

22. Pritchard DJ: Cystic fibrosis allele frequency, sex ratio anomalies and fertility: a new theory for the dissemination of mutant alleles. Hum Genet 1991, 87:671-676.

23. Romeo G, Devoto M, Galletta LJ: Why is the cystic fibrosis gene so frequent? Hum Genet 1989, 84:1-5.

24. Gabriel SE, Brignan KN, Koller BH, Boucher RC, Stutts MJ: Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. Science 1994, 264:107-109.

25. Bayoumi RA: The sickle-cell trait modifies the intensity and specificity of the immune response against P. falciparum malaria and leads to acquired protective immunity. Med Hypotheses 1987, 22:287-298.

26. Greeneves HJ, Ayres PB: Multiple allelism at the locus controlling warfarin resistance in the Norwegian rat. Genet Res 1982, 40:59-64.

27. Larson JE, Morrow SL, Happend L, Sharp JC, Cohen JC: Reversal of cystic fibrosis phenotype in mice by gene therapy in utero. Lancet 1997, 349:619-620.

28. Bates JH, Schuessler TF, Dolman C, Eidelman DH: Temporal dynamics of acute isovolume bronchoconstriction in the rat. J Appl Physiol 1997, 82:255-62.

29. Tomioka S, Bates JH, Irwin CG: Airway and tissue mechanics in a murine model of asthma: alveolar capsules vs. forced oscillations. J Appl Physiol 2002, 93:263-270.

30. Lutchen KR, Hantos Z, Petrak F, Adamicz A, Suki B: Airway inhomogeneities contribute to apparent lung tissue mechanics during constriction. J Appl Physiol 2000, 88:1841-1849.

31. Salazar E, Knowles JH: An Analysis of Pressure-Volume Characteristics of the Lungs. J Appl Physiol 1964, 19:97-104.