Transgenic laboratory animals in cystic fibrosis research.

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Cystic fibrosis – genetics and pathophysiology

Cystic fibrosis (CF) is the most common fatal autosomal recessive genetic disorder in the Caucasian population (*Wood et al.* 1976). The incidence is approximately 1 out of 2500 to 4500 live births (*Kane* 1988), and about 50,000 CF patients exist in Europe, U.S.A. and Latin America. In Denmark, 300 CF patients are known; the carrier frequency is approximately 3% (*Nielsen et al.* 1988). The main characteristics of CF are malabsorption due to exocrine pancreatic insufficiency, chronic bacterial infections in the lower respiratory tract, increased salt loss in sweat, and male infertility due to absence or stenosis of the vas deferens (*Boat et al.* 1989).

The gene was found to be located on chromosome 7 in 1985 (*Schmiegelow, et al.* 1986) and in 1989 the CF gene was identified

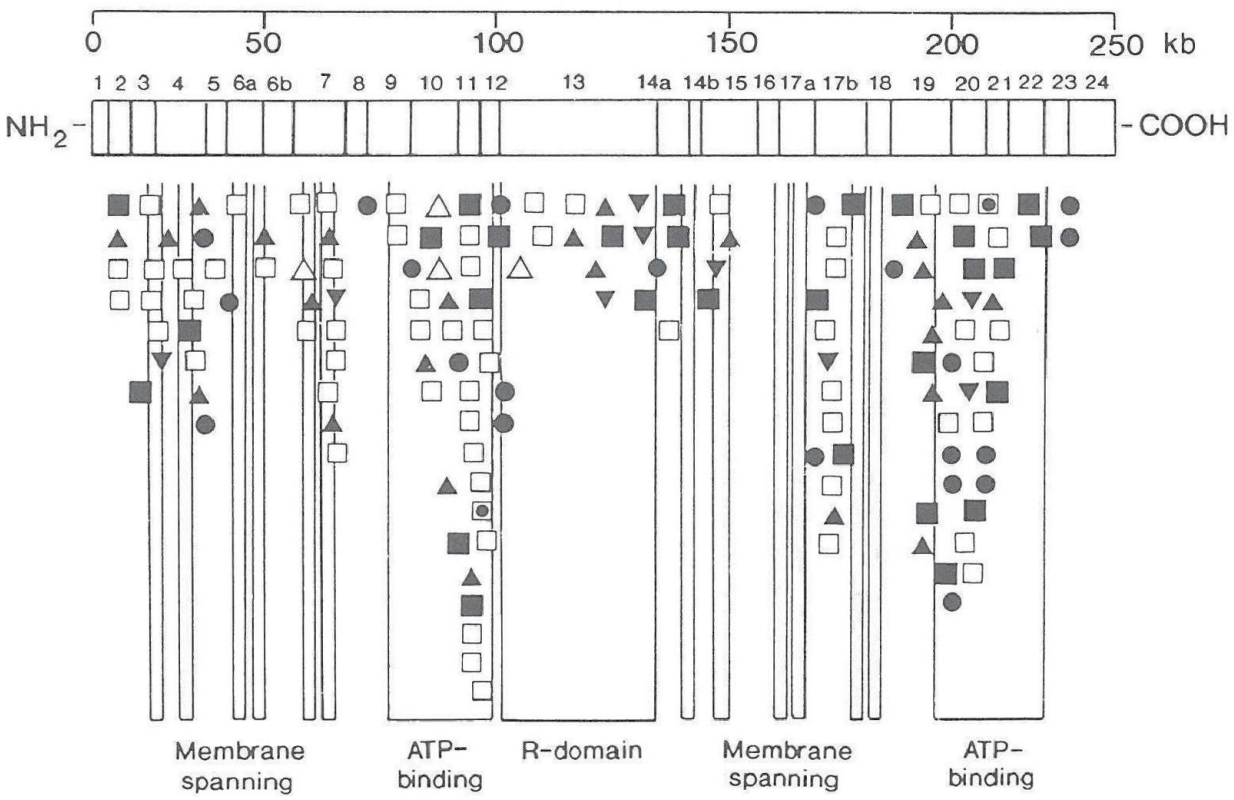

Figure 1
Schematic diagram showing the locations of known mutations in the CFTR gene. The exon-intron boundaries within the coding regions and the proposed membrane-spanning and ATP-binding domains are indicated. Symbols represent different types of mutations; missense (□): nonsense (■); frameshift deletion (▲); frameshift insertion (▼); in-frame deletion (∧); splice site (●) (Adapted from *Tsui et al.* 1993).

(*Rommens et al.* 1989, *Riordan et al.* 1989, *Kerem et al.* 1989) (Fig. 1). It comprises about 250,000 base pairs and encodes for a protein of 1,480 amino acids called the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Extensive population studies have indicated that the most common CF mutation, a deletion of the 3 nucleotides that give rise to a phenylalanine 508 (ΔF508), is present on approximately 70% of all CF chromosomes, but never on normal chromosomes (*Kerem et al.* 1989). The frequency of ΔF508 varies substantially among different populations, from 30% in Ashkenazi Israeli patients to 88% in Danish CF patients (*The Cystic Fibrosis Genotype-Phenotype Consortium* 1990, *Schwartz et al.* 1990). The second most frequent mutation, G542X, occurs on only 2.4% of the investigated CF chromosomes worldwide (43,849 chromosomes has been tested). Since the CFTR was cloned, more than 450 different mutations associated with CF have been detected (*Tsui* 1994). Many of the defects have been found in only single patients, however, (*Collins* 1992, *Cuthbert* 1994). The ΔF508 mutation has proved to be the most severe mutation. In a study from the CF Centre in Toronto it was (*Kerem et al.* 1990) found that the ΔF508 mutation lead to earlier diagnosis and more frequent and severe pancreatic insufficiency than other mutations. In Danish CF patients we found that patients who were homozygous for ΔF508 had significantly earlier onset of symptoms before 6 months of age, they were younger at time of diagnosis, required greater pancreatic enzyme substitution, and had poorer lung function. Further, the yearly incidence of chronic *Pseudomonas aeruginosa* infection and mortality rates were also greater in homozygotes than in patients heterozygous for the ΔF508 mutation (*Johansen et al.* 1991).

CFTR is a low conductance chloride channel, sensitive to cAMP, which is incorporated into the apical membranes of transporting epithelial cells (*Bear et al.* 1992, *Cuthbert* 1994). The CFTR protein consists of two transmembrane (anchoring) domains and two nucleotide binding domains joined at the Regulatory-domain which has multiple phosphorylation sites for protein kinases A and C (*Rommens et al.* 1989, *Riordan et al.* 1989, *Kerem et al.* 1989, *Collins* 1992) (Fig. 2). The mutation results in bronchial mucus which contains more potassium, less sodium and a reduced water content than normal mucus (*Boat et al.* 1989).

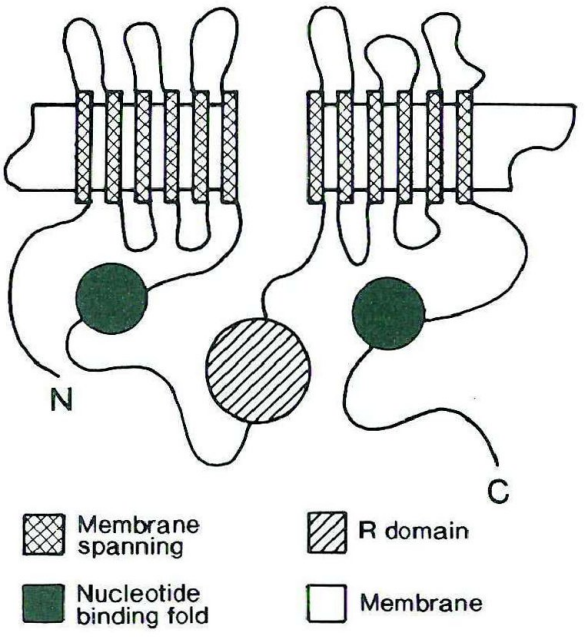

Figure 2
The possible organization of the CFTR protein in the lipid bilayer of the cell membrane. CFTR consists of two anchoring membrane-spanning domains, two nucleotide-binding folds and a regulatory R-domain (Adapted from *Cuthbert* 1994).

Lung infections

The altered secretions of the respiratory tract which leads to thick dehydrated mucus is thought to be the main reason why CF patients suffer from intermittent and chronic bacterial respiratory tract infections. Usually, infections with *Staphylococcus aureus* and *Haemophilus influenzae* predominate in younger children with CF, but with increasing age, these bacteria are surplanted by mucoid *P. aeruginosa*, and chronic endobronchial lung infection becomes the leading

cause of morbidity and mortality (*Pedersen* 1992). CF patients have no detectable immune deficiency and except for the respiratory tract they are not more susceptible to infections than normal children (*Høiby* 1977). Once the *P. aeruginosa* infection is chronically established in the lungs of CF patients, it is not possible to eradicate it with immune therapy (*Pennington et al.* 1975) or antibiotics (*Pedersen* 1992). Vaccination has no effect either (*Langford & Hiller* 1984). The main reason why *P. aeruginosa* persists in the respiratory tract is that it grows in microcolonies embedded in a biofilm of a heteropolysaccharide (alginate) (*Høiby & Koch* 1990, *Pedersen* 1992). The biofilm mode of growth protects the organism from host defence mechanisms and from antibiotics. When the chronic *P. aeruginosa* infection becomes established, the host responds with production of an abundance of specific antibodies and accumulation of inflammatory cells (mainly polymorphonuclear leukocytes), which cause immune-mediated inflammation (type III hypersensitivity reaction). This leads to lung tissue destruction, and irreversible pulmonary insufficiency (*Høiby et al.* 1986, *Berger* 1991, *Stiver et al.* 1988).

Clinical management

At the Danish CF Centre at Rigshospitalet, in Copenhagen, approximately 250 CF patients are seen monthly in the outpatient clinic (*Pedersen et al.* 1987). The visits include medical examination, measurement of height and weight, pulmonary function tests and microscopy and culture of sputum (*Høiby & Pedersen* 1989). The main principle for treatment of Danish CF patients is early and aggressive chemotherapy whenever pathogenic bacteria are isolated from the sputum (*Pedersen* 1992). In our centre, it has been possible to prevent or delay the onset of the chronic *P. aeruginosa* lung infection by early treatment of the intermittent colonization with colistin inhalation in combination with oral ciprofloxacin for 3 weeks periods (*Valerius et al.* 1991). Onset of chronic *P. aeruginosa* infection is defined as repeated positive culture of the bacteria from sputum for 6 months and/or an antibody response against *P. aeruginosa* standard antigens of 2 precipitins or more (*Høiby & Pedersen* 1989). Patients with chronic *P. aeruginosa* lung infection are hospitalized every third month for 2 weeks and treated with intravenous antipseudomonal chemotherapy (*Pedersen et al.* 1987).

Animal models

An experimental model of the chronic *P. aeruginosa* lung infection in animals was first described by Cash et al. (*Cash et al.* 1979). They embedded the bacteria in agar beads and installed the inoculum intratracheally to normal rats. Although the rats did not suffer from CF, the histopathologic changes mimicked the lesions seen in CF patients: acute inflammation, dominated by numerous polymorphonuclear leukocytes, surrounding the bacteria-containing beads (microcolonies). Since then, several models have been established in different species such as rats (*Boyd et al.* 1983, *Pedersen et al.* 1990, *Johansen et al.* 1993), mice (*Sordelli et al.* 1979, *Pier et al.* 1990), guinea pigs (*Pennington et al.* 1981), cats (*Winnie et al.* 1982), rabbits (*Wiener-Kronish et al.* 1993) and rhesus monkeys (*Cheung et al.* 1992). For many years these animal models were the only alternative to CF patients when studying the pulmonary pathophysiological events in CF.

Since the cloning of the CF gene, three groups have independently reported successful construction of CF transgenic mice. Two laboratories used replacement mutagenesis (*Snouwaert et al.* 1992, North Carolina, *Ratcliff et al.* 1993, Cambridge), the third insertional mutagenesis in exon 10 of the mouse CF gene (*Dorin et al.* 1992 a, Edinburgh).

The Edinburgh CF mouse

In our laboratory, in collaboration with Dr. Julia Dorin, Edinburgh, Scotland, we are breeding and subsequently establishing the chronic *P. aeruginosa* lung infection in the

Edinburgh mouse. This mouse was constructed by targeted insertional mutagenesis (Fig. 3). The cloning and sequencing of the murine homologous CFTR was described in 1991 (*Tata et al.* 1991, *Yorifuji et al.* 1991). The mouse CFTR protein is highly conserved, especially in exon 10 which harbours the most common CF mutation, ∧F508. An insertional targeting vector, pIV3.5H, was designed to disrupt the CFTR protein and create a »null« allele at the CFTR locus in cultured mouse embryonal stem cells by disrupting the gene within exon 10 upon homologous recombination (*Dorin et al.* 1992 a). The embryonal stem cell derived offspring carrying the targeted CFTR protein, were sib-mated and by day 17 the progeny was genotyped by Southern blot analysis of tail-tip DNA. DNAs were double digested with *Xba* I + *Sal* I and probed with the 1.2H genomic probe. In the blot a 5 kbp *Xba* I + *Sal* I fragment was diagnostic for the insertional mutation (homozygous for CFTR) whereas the 6 kbp *Xba* I fragment indicated the wild-type and carrier mice for CFTR (Fig. 3) (*Dorin et al.* 1992 a, *Dorin et al.* 1992 b, *Porteous* 1993).

In litter from crosses between heterozygotes, CFTR homozygous transgenic mice were born at the expected Mendelian frequency (wild-type:heterozygous:mutants 1:2:1), indicating little or no prenatal mortality. No significant differences in growth rate or survival of the mutants compared with heterozygotes

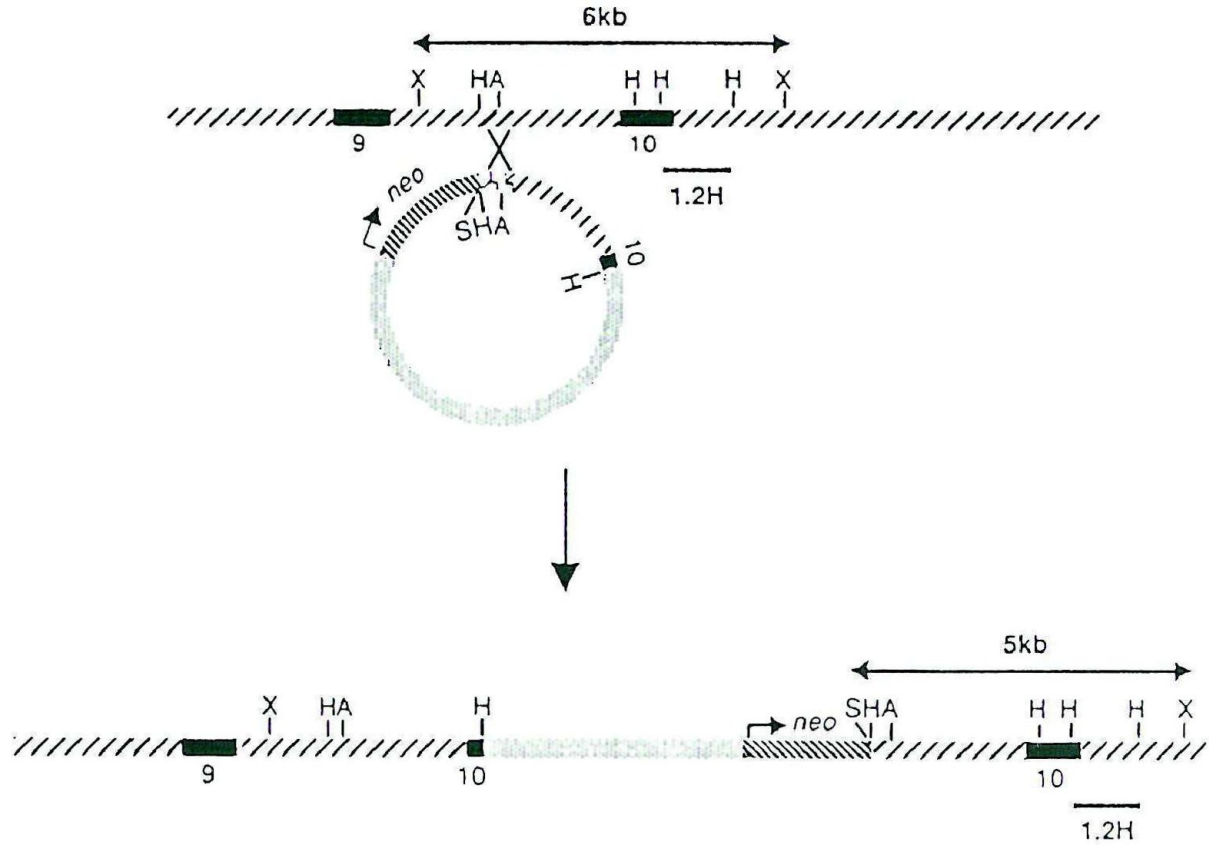

Figure 3
Insertional disruption of the murine CFTR gene. Targeting scheme. The figure illustrates the genomic structure, the point of insertion in intron 9 and the predicted gene disruption. Probe 1.2H is used to follow insertion events by Southern blot analysis of putative targeted cell lines and derived mice. The diagnostic restriction fragments are indicated. Abbreviations: S, *Sal* I, H, *Hind* III, X, *Xba* I; A, Asp718. The 6 kb fragment represents the wild-type whereas the 5 kb is diagnostic for the insertional mutation. (Kindly provided by J. Dorin and reproduced with permission from Nature 1992, 359, 211-215).

or wild-type offspring was observed between birth and sexual maturity (*Porteous* 1993). Epithelia from the gastrointestinal tract and from the airways were shown to possess abnormal cAMP mediated Cl^- transport similar to that observed in human CF tissue (*Clarke et al.* 1992, *Dorin et al.* 1992 a) and most mutant mice had intestinal pathological manifestations such as failure to thrive and meconium ileus, which is a blockage of the intestine observed in about 10% of all CF patients at birth (*Colledge et al.* 1992, *Snouwaert et al.* 1992).

Disease severity and tissue involvement were different for the three CF-mice which illustrates the potential importance of minor differences in experimental strategy and genetic background. The replacement targeted mutant created by Snouwaert et al. (*Snouwaert et al.* 1992) was crossed onto three inbred background mice (B6D2, C57BL/6 or BALB/c) whereas the Edinburgh mouse was outcrossed onto an outbred MF1 background (*Dorin et al.* 1992 a). The Cambridge and North Carolina mice die soon after birth as a consequence of intestinal blockage, and those who survive to later development stages show failure to thrive and loss of weight when compared with their litter mates. Very few of the mice survive beyond 30 days of age and by 40 days all have died (*Ratcliff et al.* 1993, *Snouwaert et al.* 1992). In contrast to the two other CF mice, the Edinburgh mouse does not seem to develop as serious intestinal problems, they have a longer life expectancy (>24 weeks; personal observation) and the specific alterations in lung pathology are consistent with the early stages of lung disease in CF patients (*Dorin et al.* 1992 a). These features include goblet cell hyperplasia, atelectasis, bronchiolar dilatation, bronchitis and/or bronchial pneumonia (*Porteous* 1993). The improved survival of the Edinburgh mouse may be explained, at least in part, by a low level of residual wild type message as a result of exon skipping and aberrant splicing. These levels are comparable to those measured in CF patients with mild to moderate disease (*Porteous* 1993).

Pathophysiological, clinical and therapeutic aspects of the CF mouse

The development of the CF mice will facilitate studies on the pathophysiology and cell biology of CF and the relationship between the defective CFTR and the clinical symptoms associated with CF. Although rodents have relatively fewer mucus generating cells than humans and the lung pathology of the CF mice reported so far has been less severe than that seen in the patients, the mouse seem to be a very good model for CF. The primary goal for CF gene therapy is to develop a carrying vector which is safe and practical to use and which efficiently transfers the gene into non-dividing epithelial cells. Furthermore, recombinant gene expression must be prolonged and must not elicit an immune response (*Wilson* 1993). Viral vectors have the advantage that they use natural infection mechanisms present in epithelial cells (*Cuthbert* 1994). Retroviral vectors requires actively dividing cells, but few cells in the airway surface are in that state. In contrast, recombinant adenoviruses do not require dividing cells and have a natural affinity to the respiratory tract. However, there are concerns about immunological reactions with repeated exposure (*Cuthbert* 1994). Another important problem was described in a clinical study on samples from non-CF lungs which demonstrated that the CFTR protein is not uniformly expressed throughout the lung. A minority of high expressing cells can be seen in the proximal bronchioles and alveoli but the highest levels of CFTR is present in the respiratory bronchioles (*Engelhardt et al.* 1994).

Gene transfer to the Edinburgh mouse by human CFTR cDNA-liposome complexes has been accomplished (*Alton et al.* 1993). After the gene complexes were nebulized into the airways, full restoration of cAMP re-

lated chloride responses could be demonstrated in most of the animals. The study suggested that non-invasive liposome mediated transfer of the CFTR gene to CF patients in vivo may be possible, but would also encounters difficulties, such as variation in aerosol deposition, transfection efficiency and differences in airway morphology. In addition there is a striking difference between mice and CF patients, since the bronchi of the mice are not filled with purulent secretions which is often the case in CF lungs (*Alton et al.* 1993). In another animal study performed by Hyde at al. (*Hyde et al.* 1993) using the Cambridge mouse, a plasmid expressing the CFTR protein was constructed in the vector pREP8. Liposomes were then used to deliver the CFTR expressing plasmid into the trachea of the mice. The mice transfected with the liposomes restored the cAMP-stimulated chloride secretion in the trachea to a level comparable with that of normal mice (*Hyde et al.* 1993).

In a clinical study (*Zabner et al.* 1993), recombinant adenovirus containing the normal CFTR protein was introduced into nasal airway epithelium of three CF patients. The nasal epithelium was used since that tissue has a morphology and function similar to the intrapulmonary airways and because it manifests the defective Cl^- transport (*Knowles et al.* 1983). The chloride transport, measured by transepithelial voltages, was corrected in all three patients and no evidence of viral replication or virus associated adverse effects was noted. However, the biological effect was transient and lasted for less than three weeks (*Zabner et al.* 1993).

Nearly all attempts at correcting the CF gene have been directed towards the airways, but in future clinical trials the pancreas and the alimentary tract present even greater challenges. The CF mouse represents a very important model since it makes in vivo experiments possible which are necessary before such clinical studies and trials can be designed.

Summary
Cystic fibrosis (CF) is the most common fatal autosomal recessive genetic disorder in Caucasian populations. The incidence in Denmark is approximately 1:4500 and about 50,000 CF patients are registred in Europe and the Americas. The disease is characterized by malabsorption due to exocrine panereatic insufficiency, chronic bacterial infections in the lower respiratory tract, increased salt loss in sweat, and male infertility due to absence or stenosis of the vas deferens. The CF gene was identified on chromosome 7 in 1989. More than 450 different mutations have been detected, the most common being the ΔF508. This gene encodes for the cystic fibrosis transmembrane regulator protein (CFTR) which is a Cl^- channel regulated by protein kinase C and ATP. It facilitates transport of Cl^- and other ions through the cell membrane. Two years ago, three different groups of scientists published articles describing three different variants of CF mice (»knock-out« mice). Two of these mice suffer from severe CF-like symptoms, especially in the intestine, and most of them die within 3 weeks. The last one has some residual CFTR activity and survives for several months. These mice allow investigations on gene therapy using different vectors and investigations on the pathogenesis of the chronic *Pseudomonas aeruginosa* lung infection to be carried out. Thus, the prospects for understanding CF seems promising.

Resumé
Cystisk fibrose (CF) er den hyppigste dødelige, autosomalt, recessivt, arvelige sygdom i den kaukasiske race. I Danmark har ca. 1 pr 4500 levende fødte børn CF, og der findes omkring 50.000 patienter i Europa og Amerika. Sygdommen er karakteriseret ved pancreasinsufficiens, tilbagevendende lungeinfektioner, forhøjet kloridindhold i sved og mandlig infertilitet. Genet som koder for CF blev lokaliseret til kromosom 7 i 1985 og endeligt klonet i 1989. Mere end 450 forskellige mutationer er siden blevet beskrevet; den hyppigste er ΔF508. Genet koder for et cystisk fibrose transmembrant regulator protein (CFTR), som er en Cl^- kanal, der reguleres af proteinkinase C og ATP. Kanalen faciliterer transporten af Cl^- og andre ioner gennem den apikale cellemembran. For 2 år siden blev konstruktionen af transgene mus med CF beskrevet af 3 uafhængige forskergrupper. To af musene havde svære CF lignende symptomer, specielt lokaliseret til mave-tarmkanalen, og de fleste af disse mus døde inden for 3 uger efter fødslen. Den sidste mus har formentlig en restfunktion af CFTR proteinet og kan overleve i adskillige uger. Ved hjælp af disse mus som model for CF kan forskellige vektorer for genterapi afprøves, og pathogenesen for den kroniske *Pseudomonas aeruginosa* lungeinfektion kan belyses. Konstruktionen af disse mus med CF vil formentlig komme til at bidrage væsentlig til vor forståelse af sygdommen og de patofysiologsike træk som er forbundet hermed.

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