(54) TRANSGENIC PIG MODEL OF CYSTIC FIBROSIS

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(63) Continuation-in-part of application No. 12/283,980, filed on Sep. 17, 2008, now Pat. No. 8,618,352, which is a continuation-in-part of application No. 12/074,632, filed on Mar. 5, 2008, now Pat. No. 7,989,675.
(60) Provisional application No. 60/966,971, filed on Aug. 30, 2007, provisional application No. 60/908,637, filed on Mar. 28, 2007.

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A01K 67/03 (2006.01)
A01K 67/027 (2006.01)

(52) U.S. Cl.
USPC ................................. 800/15; 800/13; 800/14

(58) Field of Classification Search
USPC ................................. 800/3, 17, 21, 24, 13-15 See application file for complete search history.

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(57) ABSTRACT
The present invention provides transgenic, large non-human animal models of diseases and conditions, as well as methods of making and using such animal models in the identification and characterization of therapies for the diseases and conditions.

10 Claims, 33 Drawing Sheets
(21 of 33 Drawing Sheet(s) Filed in Color)
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Figure 12

A

Pathology at birth

<table>
<thead>
<tr>
<th></th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>-/+</td>
<td>male infertility</td>
</tr>
<tr>
<td>+</td>
<td>respiratory disease</td>
</tr>
<tr>
<td>-</td>
<td>liver disease</td>
</tr>
<tr>
<td>-</td>
<td>distal intestinal obstructive syndrome</td>
</tr>
<tr>
<td>+</td>
<td>pancreatic insufficiency</td>
</tr>
<tr>
<td>+</td>
<td>meconium ileus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>birth</th>
<th>day</th>
<th>week</th>
<th>months</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

B

Body weight (kg)

Time following birth (h)

CFTR+/+

CFTR+/-

C

CFTR+/+

CFTR+/-

D

CFTR+/+

CFTR+/-

E

CFTR+/+

CFTR+/-
Figure 29
Figure 31

A. Vt (mV)

B. \( \Delta V_{\text{rest}} \) (mV)

C. \( \Delta V_{\text{dep}} \) (mV)

D. \( \Delta V_{\text{nap}} \) (mV)

E. Isc (\( \mu \text{A/cm}^2 \))

F. \( \Delta \text{Isc}_{\text{bas}} \) (\( \mu \text{A/cm}^2 \))

G. \( \Delta \text{Isc}_{\text{dep}} \) (\( \mu \text{A/cm}^2 \))

H. \( \Delta \text{Isc}_{\text{nap}} \) (\( \mu \text{A/cm}^2 \))

I. Gf (mS/cm²)

J. \( \Delta G_{\text{bas}} \) (mS/cm²)

K. \( \Delta G_{\text{dep}} \) (mS/cm²)

L. \( \Delta G_{\text{nap}} \) (mS/cm²)
Figure 32
TRANSGENIC PIG MODEL OF CYSTIC FIBROSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of, and claims priority from, U.S. Ser. No. 12/283,980, filed Sep. 17, 2008 now U.S. Pat. No. 8,618,352, which is a continuation-in-part of U.S. Ser. No. 12/074,632, filed Mar. 5, 2008 (now U.S. Pat. No. 7,898,675), which claims the benefit of the filing dates of U.S. Ser. No. 60/908,637, filed Mar. 28, 2007, and U.S. Ser. No. 60/966,971, filed Aug. 30, 2007. The contents of each of the prior applications are incorporated herein by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number HL51670 awarded by the National Heart Lung and Blood Institute, and grant number DK547759 awarded by the National Institute of Diabetes and Digestive and Kidney Diseases. The government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to transgenic, non-human animal models of disease, cells that can be used to make such animals, and methods of making and using these animals and cells.

BACKGROUND OF THE INVENTION

Many human diseases and conditions are caused by gene mutations. Substantial effort has been directed towards the creation of transgenic animal models of such diseases and conditions, to facilitate the testing of approaches to treatment, as well as to gain a better understanding of disease pathology. Early transgenic animal technology focused on the mouse, while more recent efforts, which have been bolstered by the development of somatic cell nuclear transfer, have included larger animals, including pigs, cows, and goats. This technology has resulted in the production of, for example, pigs in which the gene encoding α-1,3-galactosyltransferase has been knocked out, in efforts to generate organs that can be used in xenotransplantation (see, e.g., Lai et al., Science 295:1089-1092, 2002). Additional applications of this technology include the production of large quantities of human proteins (e.g., therapeutic antibodies; see, e.g., Grosse-Hovest et al., Proc. Natl. Acad. Sci. U.S.A. 101(18):6858-6863, 2004). Substantial benefits may be obtained by the use of somatic cell nuclear transfer technology in the production of large animal models of human disease.

An example of a disease caused by gene mutations is cystic fibrosis (CF), which is an inherited disease that affects many organs of the body, including the lungs, pancreas, sweat glands, liver, and organs of the reproductive tract. The disease is characterized by abnormalities in fluid secretion, which can lead to diverse physiological problems. For example, in the lungs of CF patients, secreted mucus is unusually heavy and sticky, and thus tends to clog small air passages, making it difficult for patients to breathe and leading to bacterial infection and inflammation. Repeated lung infections and blockages in CF patients can cause severe, permanent lung damage. Other features of CF arise from the clogging of ducts leading from the pancreas to the small intestine, which blocks the transport of critical digestive enzymes such as amylase, pro tease, and lipase. This can lead to problems including incomplete digestion, diarrhea, bowel blockage, and weight loss. Digestive complications of CF can also be caused by blockage of liver bile ducts. Due to these and other features of the disease, CF causes progressive disability in patients and ultimately leads to early death.

CF is caused by the presence of a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a chloride channel found in the membranes of epithelial cells lining passageways of the lungs, liver, pancreas, intestines, and digestive tract, and in the skin. The disease is autosomal recessive, and thus CF patients have mutations in both CFTR alleles, while asymptomatic CF carriers have mutations in only one allele. There are more than 1,200 different known mutations of the CFTR gene that can lead to cystic fibrosis in humans, with some mutations causing milder symptoms than others. However, about 70% of people with CF have the disease due to a particular gene mutation, a deletion of three nucleotides, leading to the loss of a phenylalanine that is normally present at position 508 of the CFTR protein. This form of the disease, often referred to as ΔF508 (CFTR-DelF508), is both the most common and the most severe form of the disease. The loss of phenylalanine at position 508 results in improper CFTR protein folding, which causes retention of the mutant protein in the ER and targets it for degradation before it even reaches the cell membrane. Additionally, this deletion alters channel gating, reducing the rate of channel opening.

There is no cure for CF. Current approaches to treatment include the use of mucous thinning drugs, digestive enzyme supplementation, bronchodilators, respiratory therapy, antibiotics, and lung transplantation. Even given the availability of these approaches to treatment, as the disease progresses, patients typically suffer from an increasingly poor quality of life. New approaches to treating diseases such as CF, which may be identified, for example, by the use of large animal models, are therefore needed for this and other devastating diseases.

SUMMARY OF THE INVENTION

The invention provides large, non-human animal models of human diseases or conditions, in which one or more genes associated with the diseases or conditions include one or more targeted mutations or are inactivated. The animals of the invention can be, for example, ungulates such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene (CFTR).

The animal models of the invention can include the mutation(s) in one or both alleles of the gene in the genome of the transgenic animal, and the mutation(s) can result in full or partial inactivation of the gene(s). In one example, the mutation includes an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence. In another example, the mutation substantially eliminates expression of a functional gene product of the targeted gene in cells in which such expression normally takes place, absent the mutation. In the case of an animal with a mutation or mutations in both alleles of a gene, the mutation or mutations in each allele can be identical to one another or can be different.

The animal models of the invention may optionally include a homologous transgenic copy of a wild-type or mutant gene from a different animal. The animal models may thus include, for example, in addition to a mutation/inactivation of an
endogenous gene, an inserted copy of a corresponding gene from another species. Thus, for example, an animal (such as a pig) in which an endogenous CFTR gene is mutated or inactivated may be modified to include a CFTR gene from another animal (such as a human), which may be wild-type or may include a mutation (e.g., CFTR-Δ508). The invention therefore provides transgenic, large non-human animal models of human diseases and conditions (e.g., pigs), in which one or more endogenous genes associated with the diseases or conditions are knocked-out (i.e., genetically altered in such way as to inhibit the production or function of the product of the gene) and replaced with a homologous wild-type or mutated gene derived from a different animal (e.g., a human).

In one example, a pig with its endogenous porcine CFTR knocked-out expresses a human transgene encoding a CFTR gene, such as the CFTR-Δ508 gene.

Examples of CFTR mutations that can be included in the animals (and cells) of the invention include (i) class I mutations, which result in little or no mRNA production, and thus little or no protein production (e.g., nonsense mutation (e.g., G542X), a frameshift mutation (e.g., 394delITT), a splice junction mutation (e.g., 1717-1G→A)), (ii) class II mutations, which result in a protein trafficking defect where CFTR is made, but fails to traffic to the cell membrane (e.g., F508del), (iii) class III mutations, which result in CFTR trafficking to the cell membrane, but failing to be properly regulated or responding to cAMP stimulation (e.g., G551D, which fails to respond to cAMP stimulation), (iv) class IV mutations, which result in a CFTR channel function defect (e.g., R117H), and (v) class V mutations, which cause CFTR synthesis defects, resulting in reduced synthesis or defective processing of normal CFTR (e.g., missense mutation (e.g., A455E), or a mutation introduced by alternative splicing (e.g., 3849+10 kbc→T). Additional mutations include 621+1→T, W1282X, R347P, S549L, N804→C, R553X, and N1300K.

In the case of animals having CFTR mutations, the animals may be characterized by one or more (e.g., 2, 3, 4, 5, or 6) phenotypic characteristics, such as the phenotypic characteristics of the CFTR→− pigs described below. Thus, for example, the animals may be characterized by one or more phenotypic characteristics selected from the group consisting of: (i) an electrophysiological phenotype similar to that of human cystic fibrosis, (ii) meconium ileus, (iii) exocrine pancreatic insufficiency or abnormalities, (iv) hepatic abnormalities, (v) gall bladder and/or bile duct abnormalities, and (vi) lack of abnormalities in vas deferens or lungs at birth.

The invention also provides isolated cells of transgenic, large non-human animal models of human diseases or conditions, in which one or more genes associated with the diseases or conditions include one or more targeted mutations. The animals can be, for example, ungulates, such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene.

The cells of the invention can include the mutation(s) in one or both alleles of the genes in the genomes of the cells, and the mutation(s) can result in full or partial inactivation of the gene(s). In one example, the mutation includes an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence. In another example, the mutation substantially eliminates expression of a functional gene product of the targeted gene in cells in which such expression normally takes place, absent the mutation. In the case of a cell with a mutation or mutations in both alleles of a gene, the mutation or mutations in each allele can be identical to one another or can be different. In one example, the cells are fetal cells, such as fetal fibroblasts. The cells may include a homologous transgenic copy of a wild-type or mutated gene from a different animal, such as a human, as described above. Additional examples of cell types included in the invention are provided below.

The invention further provides methods of making transgenic, large non-human animal models of diseases or conditions, as described above and elsewhere herein. The methods can include the steps of: (i) introducing one or more mutations into an allele of one or more genes associated with a disease or condition in a cell (e.g., a fetal fibroblast) to generate a donor cell; (ii) introducing the nucleus of the donor cell into a recipient cell (e.g., an enucleated oocyte) to generate an embryo; and (iii) transferring the embryo into a surrogate female to generate the transgenic, large non-human animal model. The animals can be, for example, ungulates, such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene.

In a variation of these methods, the donor cell includes one or more mutations in one allele of a gene, and the method is carried out to introduce one or more mutations into the other allele. In another example, the donor cell includes a homologous transgenic copy of a wild-type or mutated gene from a different animal (e.g., a human), as described above. In a further example, the methods further involve breeding an animal that is born from the surrogate female to obtain a homozygous mutant.

The invention also includes methods of identifying therapeutic agents that can be used in the treatment of diseases or conditions (e.g., cystic fibrosis). These methods involve administering one or more candidate therapeutic agents to a transgenic animal, as described herein, and monitoring the animal for one or more symptoms of the disease or condition (e.g., one or more phenotypic characteristics of CF models of the invention, as described herein). Detection of improvement in a symptom of the disease or condition indicates the identification of a compound that can be used in the treatment of the disease or condition.

The invention further provides methods of targeting the introduction of mutations into pig cells. These methods involve the steps of providing pig cells (e.g., fetal fibroblasts), using an adenovirus-associated viral vector to deliver a gene targeting construct to the isolated pig cells, in the absence of cell detachment and reattachment, and selecting gene-targeted clones. The cells are in culture for 30 days or less (e.g., 20 days or less; see below) during the targeting construct delivery and selection steps. These methods can be used, for example, for the introduction of a mutation into a cystic fibrosis transmembrane conductance regulator gene (e.g., the ΔF508 mutation) in the pig cell. Information concerning other examples of mutations that can be used in the invention, as well as the use of the present methods to inactivate or replace genes (e.g., to replace pig genes with human genes), is provided below.

In more specific examples, the invention includes a transgenic pig in which a first allele of a cystic fibrosis membrane transporter (CFTR) gene in the genome of the pig includes a mutation in sequences encoding phenylalanine at position 508 of the CFTR protein encoded by the allele (e.g., a deletion of the codon for phenylalanine at position 508 of the CFTR protein), and the mutation results in one or more phenotypic characteristics of cystic fibrosis in pigs in which the first allele and a second allele of the CFTR gene in the genome of the pig has the mutation. The second allele can optionally include the mutation or a null mutation. Further, the second allele can optionally include a class I, II, III, IV, or V mutation, as described above, or a mutation selected from a nonsense
mutation, a frameshift mutation, a splice junction mutation, a missense mutation, or a mutation introduced by alternative splicing. The mutation can be present in the first CFTR allele and not in the second CFTR allele, or can be present in both alleles. In other words, the pig can be homozygous or heterozygous for the mutation. The transgenic pig having a mutation in both alleles can have one or more phenotype characteristic of human cystic fibrosis present in a lung, the pancreas, the intestines, a sweat gland, the liver, the trachea, or a kidney of said pig. In more detail, the transgenic pig can have one or more condition selected from (a) meconium ileus, (b) hepatic abnormalities, (c) gall bladder abnormalities, (d) bile duct abnormalities, (e) pancreatic abnormalities, (f) lung abnormalities, (g) electrophysiological abnormalities, (h) exocrine pancreatic insufficiency, (i) a lack of abnormalities in vas deferens or lungs at birth, and (j) tracheal abnormalities of both the first CFTR allele and the CFTR and CFTR allele in the genome of the pig as a mutation as described herein. The invention also includes an isolated cell of a transgenic pig described above, such as a pig in which the mutation is present in one allele of the gene, or the mutation is present in both alleles of the gene.

By “donor cell” is meant a cell from which a nucleus or chromatin material is derived, for use in nuclear transfer. As is discussed elsewhere herein, nuclear transfer can involve transfer of a nucleus or chromatin only, as isolated from a donor cell, or transfer of an entire donor cell including such a nucleus or chromatin material.

By “genetic modification,” “mutation,” or “disruption” of a gene (e.g., a CFTR gene) is meant one or more alterations in gene sequences (including coding sequences and non-coding sequences, such as introns, promoter sequences, and 5’ and 3’-untranslated sequences) that alter the expression or activity of this gene by, for example, insertion of, e.g., heterologous sequences, such as selectable markers, and/or termination signals, deletion, frame shift mutation, silent mutation, nonsense mutation, missense mutation, point mutation, or combinations thereof. In one example, the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid altered as compared to a naturally occurring sequence. Examples of mutations include the insertion of a polynucleotide into a gene, the deletion of one or more nucleotides from a gene, and the introduction of one or more base substitutions into a gene. Preferred modifications of CFTR sequences are those that lead to one or more features of CF in transgenic animals including a mutation in, or disruption of, both CFTR alleles. As is discussed elsewhere herein, the modifications in the two CFTR alleles of such animals can be identical or different. Further, the modifications can result in a complete loss of functional CFTR activity (as in the human AF508 mutation), or can result in diminished functional CFTR production, as may be characteristic of less severe forms of the disease.

Examples of such mutations include but are not limited to: i) class I mutations, which result in little or no mRNA production, and thus little or no protein production (e.g., nonsense mutations, G542X; frameshift mutations, 394delT; and splice junction mutations, 1717-1G→A), ii) class II mutations, which result in a protein trafficking defect where CFTR is made, but fails to traffic to the cell membrane (e.g., F508del), iii) class III mutations, which are those in which CFTR traffics to the cell membrane, but fails to be properly regulated (e.g., G551D, which fails to respond to cAMP stimulation), iv) class IV mutations, which result in a CFTR channel function defect (e.g., R117H), and v) class V mutations, which cause CFTR synthesis defects, resulting in reduced synthesis or defective processing of normal CFTR (e.g., missense mutations, A455E; alternative splicing, 3849+10kbC→T).

In one example, a mutation is introduced by the insertion of a polynucleotide (e.g., a positive selection marker, such as an antibiotic resistance gene (e.g., a neomycin resistance gene)) into an endogenous gene. Optionally, a mutation that is introduced into such an endogenous gene reduces the expression of the gene. If desired, the polynucleotide may also contain recombinase sites flanking the positive selection marker, such as loxP sites, so that the positive selection marker may be removed by a recombinase (e.g., Cre recombinase).

By “homologous” genes is meant a pair of genes from two animal species that encode proteins having similar functional and physical properties. The proteins encoded by homologous genes are often very similar in structure and function (although not always), and typically have a common evolutionary origin. The sequence identity is typically equal to or greater than 80% between two gene homologs. One example of a homologous gene pair is the porcine CFTR and human CFTR gene loci.

By “homozygous knock-out non-human mammal” is meant a mammal other than a human in which the two alleles of an endogenous gene (such as the CFTR gene) have been genetically targeted, resulting in a marked reduction or elimination of expression of a functional gene product, which is achieved by gene deletion or disruption. According to this invention, the genetic targeting event at both alleles may or may not be the same. Thus, a non-human mammal, in which the two alleles of an endogenous gene (such as a CFTR gene) have been genetically targeted by two different targeting vectors resulting in the null expression of the gene, would be considered as being a homozygous knock-out non-human mammal. An example of a “knock-in mutation” is one resulting in the insertion of a mutation into an endogenous gene, for example, introducing the AF508 or another CF mutation into a CFTR gene.

By animal “knock-out” is meant an animal (e.g., a pig or mouse; also see other animals described herein) having a genome in which the function of a gene has been disrupted, or “knocked-out.” A common method of producing disabled genes using recombinant DNA technology involves inserting an antibiotic resistance gene into the normal DNA sequence of a clone of the gene of interest by homologous recombination. This disrupts the action of the gene, thereby preventing it from leading to the production of an active protein product. A cell (or cell nucleus) in which this transfer is successful can be injected into a recipient cell (e.g., an enucleated oocyte) to generate a transgenic animal by nuclear transfer. In another approach, the cell is injected into an animal embryo, producing a chimeric animal. These animals are bred to yield a strain in which all of the cells contain the knock-out gene.

By “recipient cell” is meant a cell into which a donor cell, a donor cell nucleus, or donor cell chromatin is introduced. Preferably, recipient cells are enucleated prior to nuclear transfer. Examples of recipient cells include oocytes, fertilized zygotes, and two-cell embryos.

By “transgenic, large non-human animal” is meant any non-human animal that includes a genetic modification, as defined herein. Examples of such animals include animals other than mice such as, for example, ungulates. Examples of ungulates that can be used in the invention include members of the orders Perissodactyla and Artiodactyla, such as any members of the family Suidae, and in particular any member of the genus Sus, such as Sus scrofa, which is also known as the domestic pig or a subspecies thereof (Sus scrofa domestica). In addition to porcine ungulates, additional ungulates
that can be used in the invention include bovine, ovine, and caprine ungulates. Thus, for example, the invention can include the use of cows (e.g., Bos taurus or Bos indicus), sheep, goats, buffaloes, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, and elephants.

The invention provides several advantages, as it provides large, non-human animal models that can be used in the identification and characterization of therapies for genetic diseases. One example of such a disease is cystic fibrosis which, as discussed above, is a devastating disease, leading to increased levels of disability and, eventually, early death. Despite progress in understanding and treating CF, the pathogenesis of the disease is not well understood and therapies remain inadequate. A major impediment to answering questions is the lack of an animal model that shows disease similar to that in humans. Availability of a CF pig will allow investigators to address key problems that have persisted unresolved for years. As a result, it will be possible to develop new treatments, therapies, and preventions.

Further, given the close physiological relationship between humans and large animals, such as pigs, there is an increased likelihood that results obtained using the animal models of the invention can be applied to humans, relative to other animal models (e.g., mice, which do not develop the airway and pancreatic disease typical of human CF). Specifically with respect to pigs, it is noted that pigs and humans have anatomical, histological, biochemical, and physiologic similarities. Further, pigs and humans possess similar abundance of submucosal glands and glycoprotein synthesis/secretion. In addition, pigs and humans have similar respiratory immune systems and pulmonary inflammatory responses, making the pig a particularly good model for CF disease of humans. Further, the use of human sequences in large animals such as pigs, as in some examples of the invention, provides additional benefits of providing a system that is very similar to that of humans. Indeed, the data described below show the close similarities between human CF and the pig CFTR−/− model of the invention. The invention thus can be used to provide substantial benefits in the treatment of diseases and conditions caused by or associated with gene mutations, such as cystic fibrosis.

Other features and advantages of the invention will be apparent from the drawings, the detailed description, the experimental examples, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The application file contains drawings executed in color (FIGS. 2, 7, 10, 12-17, 20, and 22). Copies of this patent or patent application with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 is a graph showing CFTR expression in pig fetal fibroblasts. Data are quantitative RT-PCR of pig CFTR mRNA relative to GAPDH in primary pig fetal fibroblasts, nasal epithelia, and rectal epithelia. Similar results were obtained on two other occasions.

FIG. 2 is a schematic representation of targeting constructs for homologous recombination for CFTR-null and CFTR-ΔF508. Exons 8-11 of pig CFTR are depicted in black boxes. NeoR contains a neomycin resistance cDNA driven by the PGK promoter and flanked by loxP sites. The engineered stop codon is indicated in the CFTR-null targeting vector. The positions of probes for NeoR and CFTR Southern blots are indicated. PCR screen primers are depicted as arrowheads.

FIG. 3 shows screening results from CFTR-null targeted pig fetal fibroblasts. A) Example of PCR results. Primers amplified a 2.0 kb product from the wild-type allele and 3.7 kb product from the CFTR-null allele. Lanes 5, 8, 9, 12, and 13 are examples of PCR-positive clones. B) Southern blot of the PCR gel using a NeoR-specific biotin-labeled oligonucleotide. This assay confirms that the 3.7 kb product contains the NeoR sequence. The weaker hybridization signal at 2.0 kb appears to be an artifact, with some of the targeted band co-migrating with the wild-type product. Note the differences in intensity of the two bands in panel A relative to panel B.

FIG. 4 is a genomic Southern blot of DNA from CFTR-null targeted pig fetal fibroblasts. A) BglIII-digested genomic DNA was hybridized with a probe that detects pig CFTR downstream of the targeting vector boundary. CFTR-null targeted allele yields a ~9.7 kb band and wild-type is ~7.9 kb. These bands also allow us to identify wells containing monoclonal colonies and those containing more than one type of G418R colony. For example, wells 3 and 11 appeared to have more intense signals in the wild-type band than the targeted band, indicating that those wells likely contained one targeted clone and one or more random integration events. B) The same digested DNAs were hybridized with a NeoR-specific probe. The CFTR-null-targeted band is at ~9.7 kb. Note that the band in lane 6 likely represents a random integration event, and lane 1 may have two random integration events. Wells 4, 5, 7, 8, 10, 12-14, 16, and 17 are examples of cells that may be ideal nuclear donors for generating a heterozygote animal.

FIG. 5 shows screening results from CFTR-ΔF508 targeted pig fetal fibroblasts. A) Example of PCR results. Primers amplified a 2.0 kb product from the wild-type allele and a 3.7 kb product from the CFTR-ΔF508 allele. B) Southern blot of the PCR gel using a ΔF508 allele-specific biotin-labeled oligonucleotide. This assay confirms some of the 3.7 kb products contained the ΔF508 mutation. Note that lanes 1, 2, and 4 contain clones that underwent homologous recombination but failed to carry the ΔF508 mutation. On the right, wells contained either wild-type CFTR or CFTR-ΔF508 plasmid DNA. This control is included to ensure that the assay Southern blot is specific to ΔF508.

FIG. 6 is a Southern blot of amplified genomic DNA from CFTR-ΔF508 targeted pig fetal fibroblasts. In contrast to our experience with the CFTR-null targeting, the CFTR-ΔF508 targeted cells failed to proliferate after transfer to larger dishes. As a result, we were unable to obtain sufficient quantities of genomic DNA for a genomic Southern blot. Therefore, we used the relatively small amount of DNA for whole genome amplification. A) BglIII-digested amplified genomic DNA was hybridized with a probe that detects pig CFTR downstream of the targeting vector boundary. The CFTR-ΔF508-targeted allele yields a ~9.7 kb band and the wild-type is ~7.9 kb. B) Digested DNAs from similar clones were hybridized with a NeoR-specific probe. The CFTR-ΔF508-targeted band is at ~9.7 kb. Note that all lanes in this Southern blot contain an intense band at ~5 kb. This band was also present in non-injected fibroblast control DNA wells. This probe is possibly hybridizing to the endogenous PGK promoter sequence, because the probe includes some PGK promoter sequence. Consistent with this, the NeoR-probed blot in FIG. 4A also contains a faint band at 5 kb in all samples if markedly overexposed.

FIG. 7 is a photograph of the first CFTR+/- piglet taken at one day of age.

FIG. 8 is a Southern blot of genomic DNA from CFTR-targeted pigs. BglIII-digested genomic DNA was hybridized with a probe that detects pig CFTR downstream of the targeting vector boundary, shown in FIG. 2. CFTR-null and CFTR-ΔF508-targeted alleles produced a ~9.7 kb band, and wild-
type is ~7.9 kb. A) CFTR-null. Lanes 1-11 contain DNA from individual cloned pigs. Note that pig 10 was wild-type. WT well contains DNA from a wild-type control. B) CFTR-Δ508. Lanes 1-5 contain DNA from individual cloned pigs. Note that pig 4 was wild-type. WT well contains DNA from a wild-type control.

FIG. 9 shows CFTR mRNA expression in CFTR+/− and CFTR+/Δ508 pigs. A) Quantitative RT-PCR was used to measure wild-type CFTR mRNA levels in rectal epithelial samples from CFTR+/− and wild-type pigs. B) Quantitative RT-PCR was used to measure Δ508-CFTR mRNA relative to wild-type mRNA levels in CFTR+/Δ508 and wild-type pigs. Error bars represent ±SD.

FIG. 10 shows that CFTR+/− piglets appear normal at birth. A) Upper panel depicts insertion into porcine CFTR exon 10 of a PGK promoter (yellow) driving a neomycin resistance cDNA (orange), and an engineered stop codon. Position of probe (green), PCR primers (arrowheads) and BglIII sites (B) are indicated. Second and third panels show genotyping by PCR and Southern blot of genomic DNA. Lanes C1, C2, and C3 contain controls of CFTR+/+, +/−, and −/− DNA. Fourth panel shows northern blot of fetal CFTR and GAPDH mRNA. Consistent with the northern blot, quantitative RT-PCR of exon 10, the targeted site, detected <0.1% of CFTR transcripts in CFTR+/− ileum relative to CFTR+/+. (n=6 and 4). Fifth panel shows immunoprecipitation and phosphorylation of CFTR plus recombinant CFTR in BHK cells. B) First litter containing piglets of all three genotypes. C) Birth weights. Means±SD of weights: 1.31±0.24 kg for CFTR+/+, 1.35±0.28 kg CFTR+/−, and 1.31±0.23 kg CFTR−/−. D) Immunocytochemistry of CFTR in airway epithelia (top) and ileum (bottom). Figures are differential interference contrast with staining for ZO-1 (a component of tight junctions, red), CFTR (green), and nuclei (DAPI, blue). See also FIG. 11. Bars. 10 μm. E) Tracings of in vivo nasal voltage (Vt) measured in newborn piglets. After baseline measurements, the following agents/solutions were sequentially added to the epithelial perfusate: amiloride (100 μM), CFTR-free solution, isoproterenol (10 μM), ATP (100 μM), and GlyH-101 (100 μM). F) Average nasal Vt measurements as indicated in panel E. Data from 4 CFTR+/+, 4 CFTR−/− piglets were not statistically different and were combined and compared to data from 5 CFTR−/− piglets. Values of baseline nasal Vt for CFTR−/− piglets differed from the controls, as did the changes in Vt induced by adding amiloride, a CFTR-free solution, and GlyH-101 (P<0.05). Data are means±SEM.

FIG. 11 is images showing staining for CFTR, ZO-1, and DAPI, plus differential interference contrast. Bars, 10 μm.

FIG. 12 shows that CFTR+/− piglets develop meconium ileus. A) Schematic shows some clinical and histopathological CF manifestations. Note that pathological abnormalities are present before clinical disease becomes apparent. B) Weight following birth. Animals were fed colostrum and milk-replacer. n=7 CFTR+/+ and 4 CFTR−/−. Data are means±SEM. *P<0.05. C) Gross appearance of gastrointestinal tract. Piglets were fed colostrum and milk-replacer for 30-40 h and then euthanized. Stomach (black *), small intestine (arrowheads), pancreas (white arrow), rectum (white *), and spiral colon (black arrow). Of 16 CFTR−/− piglets, the obstruction occurred in small intestine in 7 and spiral colon in 9. D, E) Microscopic appearance of the ileum (D) and colon (E). H&E stain. Bars, 1 mm. Images are representative of severe meconium ileus occurring in 16 of 16 CFTR−/− piglets.

FIG. 13 shows that CFTR+/− piglets have exocrine pancreatic destruction and liver and gallbladder abnormalities. A) Gross appearance of pancreas. Bar, 0.5 cm. B) Loss of parenchyma in the CFTR−/− pancreas. H&E stain. Bars, 500 μm. C) Pancreatic ducts and islets of Langerhans (arrowheads). Bars, 100 μm. D) CFTR−/− ductules and acini dilated by eosinophilic inspissated material that formed concentrically lamellar concretions (arrows and insert). H&E stain. Bars, 50 μm. E) Ducts within the CFTR−/− pancreas. H&E stain, left; PAS stain, right. Bars, 50 μm. F) Microscopic appearance of liver. H&E stain. Arrows indicate focal expansion of portal areas by chronic cellular inflammation. Bars, 100 μm. G) Gross appearance of gallbladder. When the CFTR+/+ gallbladder was sectioned, bile drained away rapidly with collapse of the mucosal wall. CFTR−/− bile was congealed (arrow) and retained in the lumen of a smaller gallbladder. Bar, 0.5 cm. H) Microscopic appearance of gallbladder. CFTR−/− gallbladders had congealed, inspissated bile with variable mucous production (arrows, H&E stain) highlighted as a magenta color in periodic acid-Schiff (PAS) stained tissue. Bars, 500 μm. Images are representative of severe pancreatic lesions (15/15 CFTR−/− piglets), mild to moderate liver lesions (3/15), and mild to severe gall bladder duct lesions (15/15).

FIG. 14 shows that the lungs of newborn CFTR+/− and CFTR+/Δ508 piglets appear normal. A) Microscopic appearance of lung from piglets <12 hours old. H&E staining. Bars, 1 mm (left) and 50 μm (right). B) Bronchial epithelia and submucosal glands. H&E staining. Bars, 50 μm. Images are representative of lesions in 15 of 15 CFTR−/−.

FIG. 15 shows results from bacterial culture of bronchoalveolar lavage fluid obtained from piglets between 6 and 12 hours after birth.

FIG. 16 shows results from bronchoalveolar lavage (BAL) on unfed piglets <12 hours old. Data are total numbers of cells in the lavage, percentages of macrophages and neutrophils, and levels of IL-8. Data are from 2 CFTR+/+, 2 CFTR+/−, and 5 CFTR−/− piglets. Values were not statistically different, P=0.1.

FIG. 17 is an amino acid sequence alignment of human, pig, and mouse CFTR. Transmembrane domains (TM), nucleotide-binding domains (NBD), and the R domain are boxed and labeled. Walker A and B motifs, signature motifs (SM), and F508 are shaded. The alignment was generated using ClustalW. The NBD boundaries are based on the NBD1 crystal with the NBD2 boundaries based on amino acids counting up from Walker A and down from Walker B.

FIG. 18 shows that pig and mouse CFTR-Δ508 produce some mature band C protein. The images show immunoprecipitated and in vitro phosphorylated wild-type and Δ508 CFTR of human, pig, and mouse. A) Constructs were expressed for 24, 48, and 72 hours in COS7 cells. B and C. Constructs were expressed for 48 hours in NIH-3T3 (B) and LLC-PK1 (C) cell lines. H: human; P: pig; M: mouse. Bands B and C are indicated by arrows.

FIG. 19 shows that fully glycosylated pig and mouse Δ508 are not endoglycosidase-H sensitive. The images show immunoprecipitated and in vitro phosphorylated human, pig, and mouse wild-type and Δ508 CFTR incubated in the presence (+) or absence (−) of 10 μM of endoglycosidase H. Human CFTR was from electroporated COS7 cells; we expressed pig and mouse CFTR using adenoviral vectors. The last 2 lanes are COS7 cells infected with Ad-GFP. Bands A, B, and C are indicated by arrows.

FIG. 20 shows that human, pig, and mouse wild-type CFTR and pig and mouse CFTR-Δ508 are expressed on the apical surface of differentiated airway epithelia. Immunostaining of differentiated human CF airway epithelia expressing human, pig, and mouse wild-type and Δ508 CFTR. Data are X-Y (A,B,E,F,I,J) and X-Z (C,D,G,H,K,L).
confocal images. CFTR immunostaining is in green and ZO-1 (tight junction) in red. Apical membrane is shown by arrow and filter (at the basal membrane) is indicated by dotted line. In panel B, faint staining of CFTR-AF508 is visible beneath the apical surface. Bar indicates 10 μm.

FIG. 21A shows single-channel currents from human, pig, and mouse wild-type and AF508 CFTR. Representative current traces from excised, inside-out patches of HEKa cells containing single channels of human, pig, and mouse wild-type and ΔF508 CFTR. Holding voltages were human at −80 mV, pig at −100 mV, mouse wild-type at −50 mV, and mouse ΔF508 at −80 mV. Human tracings were from cells incubated at reduced temperature and then studied at 37°C and are taken from Teem et al. (Receptors Channels 4:63-72, 1996); pig and mouse tracings were from cells incubated at 37°C and studied at −25°C. Expanded tracings on bottom show trace conductance in human wild-type and ΔF508 CFTR. FIG. 21B shows the properties of wild-type and ΔF508-CFTR. Data are mean ± S.E.M. for single-channel conductance (g), open state probability (Po), burst duration (BD), and inter-burst interval (IB). n = number of membrane patches for each. Asterisks indicate p < 0.05 compared to wild-type CFTR using Mann-Whitney Rank Sum test. Note that values for human CFTR and CFTR-AF508 were taken from Teem et al. (Receptors Channels 4:63-72, 1996).

FIG. 22 shows transcellular currents in human CF airway epithelium expressing human pig, and mouse CFTR and CFTR-AF508. Examples of current traces of human, pig, and mouse wild-type CFTR and CFTR-AF508 expressed in differentiated human CF airway. Agents were present during times indicated by bars.

FIG. 23 shows the bumetanide-sensitive eAMP-stimulated current in differentiated CF airway epithelia. A. Currents in human and mouse airway epithelial cells. B. CFTR-AF508. Examples of current traces of human, pig, and mouse wild-type CFTR and CFTR-AF508 after subtraction of currents from GFP-expressing control epithelia. Bumetanide-inhibited current in CF epithelia expressing CFTR-AF508 as a percentage of bumetanide-inhibited current in CF epithelia expressing wild-type CFTR of each species. FIG. 24 shows the pathology of newborn CFTR-AF508/AF508 pigs. A. Location (in cm) of meconium ileus obstruction in CFTR-AF508/AF508 (n=10) and CFTR-AF508–/– (n=9) pigs. B. CFTR-AF508/AF508 ileum distal to the obstruction had a small caliber and was heterogeneously filled with mucocellular debris (arrows). B. Pancreatic expression of CFTR-AF508/AF508 pigs had increased connective tissue (asterisks) and destruction compared to CFTR+–. Histopathological changes in CFTR-AF508/AF508 pancreas were slightly less severe than in CFTR–/–. C. Pancreas from CFTR-AF508/AF508 pigs had increased connective tissue (asterisks). C. Pancreas from CFTR-AF508/AF508 pigs had increased connective tissue (asterisks) and destruction compared to CFTR+–. Histopathological changes in CFTR-AF508/AF508 pancreas were slightly less severe than in CFTR–/–. He stain. Bar = 457 μm. D. Lobular parenchyma in CFTR-AF508/AF508 pigs (n=17) was reduced compared to CFTR+– (n=9, * P < 0.001) and greater than in CFTR–/– (n=19, # P < 0.05, Dunn’s post-test). Data from CFTR+– and CFTR–/– pigs were previously published (Meyerholz et al., Am. J. Pathol. 176(3):1377-1389, 2010). E. Liver from newborn CFTR-AF508/AF508 and CFTR–/– pigs showed portal areas that were focally expanded (arrows) by inflammation, duct proliferation and connective tissue. He stain. Bar = 46 μm. F. CFTR-AF508/AF508 pigs had microglandular bladder variably filled by mucus and bile. He stain. Bars = 928 μm (++) and 463 μm (ΔF508/AF508). G. Lung from newborn CFTR-AF508/AF508 pigs lacked mucus accumulation or inflammatory changes. He stain. Bar = 93 μm. H. Bronchoalveolar lavage liquid analyses from newborn pigs, including total cell counts (H), neutrophil percentages (I), and IL-8 concentrations (J) revealed no statistically significant differences between genotypes. Lines are medians. CFTR+– (n=5) combined with CFTR–/– (n=4); CFTR-AF508/AF508 (n=11). Compared to an earlier study that showed undetectable IL-8 for many lung lavage samples (Stoitz et al., Science Translational Medicine 2(29):29ra31, 2010), the current IL-8 assay protocol was more sensitive.

FIG. 25 reveals the morphometry of newborn CFTR-AF508/AF508 trachea. A. Cross section of trachea. MT stain. Bars = 1 mm. Images from CFTR+– and CFTR–/– are from reference (Meyerholz et al., Am. J. Respir. Crit. Care Med. 182:1251-1261, 2010). B. Tracheal morphometry in CFTR+– (n=20), CFTR-AF508/AF508 (n=19), and CFTR–/– (n=18) newborn pigs. * indicates different from CFTR+– and # indicates different from CFTR–/– (* P < 0.05 vs. CFTR+–, and # P < 0.05 vs. CFTR–/–, 1-way ANOVA with Bonferroni’s post test). B. Tracheal lumens cross-sectional area. C. Tracheal circumference. D. Submucosal gland area normalized to tracheal lumens circumference. E. Smooth muscle area normalized to tracheal lumens area.

FIG. 26 shows disease progression in pigs 2-6 weeks of age and older. A. Pancreas from a 77-day-old CFTR-AF508/AF508 pig and 69-day-old CFTR+– pig for comparison. Islands of degenerative, fibrotic and inflamed CFTR-AF508/AF508 pancreas were surrounded by abundant adipose tissue (asterisk). HE stain. Bar = 75 μm. B-D. Porcine liver. MT stain. Bars = 570 μm. E. Liver from a 136-day-old CFTR+– pig. C. Diffuse zone 1 steatosis (black arrows) in a 77-day-old CFTR-AF508/AF508 pig. D. A 62-day-old CFTR-AF508/AF508 pig had focal bridging expansion (black arrow) of triads by fibrosis, duct proliferation and inflammation. E-M. Histopathological examination of CFTR-AF508/AF508 lungs. F-I are HE stain and I-M are PAS stain. E. Lung from a 69-day-old CFTR+– pig; changes like those in panels F-H were not observed in wild-type pigs. F-H. Lung from 13-day-old CFTR-AF508/AF508 pig. I. Lung shows mucopurulent airway obstruction (arrow) and adjacent atelectasis (asterisks). Bar = 757 μm. G. Affected airway lumens often contained a heterogeneous mixture of mucopurulent debris obstructing the airway (arrows) and adjacent atelectasis (asterisks). H. Airways sometimes showed nominal inflammatory changes in the wall (asterisks) adjacent to luminal neutrophils (arrows) suggesting the dispersion of the luminal mucocellular debris from more severely affected airways. Bar = 38 μm. J. Lung from 87-day-old CFTR-AF508/AF508 pig. L. Some airways showed focal airway mucus obstruction (arrow). Bar = 162 μm. J. The surface epithelium showed focal goblet cell hyperplasia (black arrows) and inflammation in the airway wall around submucosal glands (arrowheads). Bar = 81 μm. Insets: magnified PAS-stained images of airway epithelium of 4.5-mo CFTR+– (top) and 87-day-old CFTR-AF508/AF508 (bottom) pigs. K-L. Lung from 62-day-old CFTR-AF508/AF508 pig. K. Lungs showed mucopurulent inflammation associated with focal dilated submucosal glands and ducts (arrow). Bar = 40 μm. L. Airways lumens showed mucopurulent material in lumen (arrow) with epithelial proliferation and wall inflammation. Bar = 40 μm. M. Lung from 77-day-old pig showed lesions included complete lobular atelectasis (arrows), although in this image airway obstruction was not present. Bar = 378 μm.

FIG. 27 shows mRNA and protein expression in intestine and airway. A. Quantitative RT-PCR of CFTR mRNA in CFTR-AF508/AF508 and CFTR+– pigs. Data are from triplicate assays repeated on multiple days. For each tissue, amounts of CFTR mRNA were normalized to β-actin mRNA. These normalized values were then expressed relative to that in wild-type duodenum. Data are mean ± S.E. from intestinal tis-
sues from 6 CFTR** and 6 CFTR** piglets, and from cultured nasal epithelium from 1 CFTR** piglet (n=3) and 1 CFTR** piglet (n=4). B. Northern blot analysis of duodenal CFTR mRNA, indicated by arrow. C. Immunoprecipitated and in vitro phosphorylated CFTR isolated from intestine: rec (lanes 1, 8, 13) indicates recombinant protein. Lanes 2-7, proximal intestine, CFTR** and CFTR** 750 µg. Lanes marked with * show enhanced exposure. Lanes 6 and 7 are same as lanes 4 and 5. Lanes 9-12, distal intestine. CFTR** 200 µg and CFTR** 1000 µg. Lanes 11 and 12 are same as 9 and 10. Lanes 2 and 3 are from a different gel than lanes 4-7. D. Immunoprecipitated and in vitro phosphorylated CFTR isolated from airway epithelium. Recombinant protein, lanes 1, 6, 7. Lanes 2-5, trachea; CFTR** 623 µg and CFTR** 1208 µg. Lanes 4 and 5 are same as lanes 2 and 3. Lanes 8-10, cultured bronchial epithelia; each lane 750 µg.

FIG. 28 shows immunocytochemical localization of CFTR in intestinal and airway epithelia of newborn pigs. Data are from 100. A. Sections of intestine from newborn pigs. Third panel (asterisk) shows an electronically enhanced image of second panel. CFTR is green, ZO-1 is red, and nuclei are blue. Non-specific staining was occasionally found in some CFTR** and CFTR** crypts in areas of extensive mucus. B. Sections of trachea. C. Images of cultured airway epithelia. Top panels are enface images, and bottom panels are single vertical sections. Images from CFTR** differ from CFTR** epitopes. There is no evidence (*) to show CFTR. Cell size heterogeneity was observed with all genotypes.

FIG. 29 shows electrophysiological properties of freshly excised porcine tracheal epithelia. Data are from CFTR** (23 tissues), CFTR** (19 tissues, 17 pigs), and CFTR** (16 tissues, 14 pigs) epithelia. Data from CFTR** and most CFTR pigs were previously reported (Chen et al., Cell 143:911-923, 2010). * indicates CFTR** differs from CFTR**, and † indicates CFTR** differs from CFTR** at p < 0.01 by unpaired t-test with Welch's correction. A) Transmembrane voltage (Vt) and response to sequential apical addition of 100 µM amiloride, 100 µM DIDS, 10 µM forskolin and 100 µM IBMX, and 100 µM GlyH-101. B) ABlum indicates change in Vt with addition of amiloride. C) ABlum indicates change in Vt with addition of forskolin and IBMX. D) ABlum indicates change in Vt with addition of GlyH-101. E-H) Short-circuit current (Isc) measurements corresponding to Vt measurements in panels A-D. I) Transmephalic conductance (Gt) measurements corresponding to Vt measurements in panels A-D. Changes in Vt, Isc, and Gt with DIDS were small and did not differ by genotype.

FIG. 30 shows electrophysiological properties of freshly excised porcine nasal epithelia. See description of FIG. 29, above, for further details.

FIG. 31 shows electrophysiological properties of differentiated primary cultures of porcine nasal epithelia. See description of FIG. 29, above, for further details.

FIG. 32 shows electrophysiological properties of differentiated primary cultures of porcine tracheal epithelia. See description of FIG. 29, above, for further details.

FIG. 33 shows HCO\textsuperscript{3-} transport, apical CT currents, and effect of increasing cAMP-dependent stimulation. A-B Changes in Isc and Gt in tracheal epithelium bathed in CT-free solution containing 25 mM HCO\textsuperscript{3-}. Change in Isc (A) and Gt (B) stimulated by forskolin (10 µM) and IBMX (100 µM) (T&I) and inhibited by GlyH-101 (100 µM, apical). * indicates p<0.05, unpaired t-test. C-E Changes in T current after permeabilization of basolateral membrane. N=7 CFTR** and 7 CFTR**. C) Current traces in response to indicated agents in CFTR**, CFTR**, and CFTR** epithelia. Concentrations are those indicated in FIG. 6 legend; nystatin was 0.36 µg/ml. D) Change in current in response to nystatin plus forskolin and IBMX (10 nM, 100 µM, 100 µM). E) Change in current in response to GlyH-101 (100 µM, 100 nM). F) Examples of Isc current traces following addition of increasing forskolin and IBMX concentrations. For concentrations, see panel G. G) Changes in Isc with increasing forskolin and IBMX concentrations. N=7 CFTR** and 6 CFTR**. H) Examples of Isc current traces following addition of increasing cAMP concentrations. For concentrations, see panel I. I) Changes in Isc with increasing cAMP concentrations. N=6 CFTR** and 7 CFTR**.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides animal models of human diseases (e.g., cystic fibrosis (CF)) and conditions, which can be used in methods including the identification and characterization of approaches for treating the diseases and conditions. As is discussed further below, the animal models of the invention are large, non-human animals, such as pigs, which have been genetically modified to include one or more mutations in a gene associated with a particular disease or condition (e.g., the cystic fibrosis transmembrane regulator (CFTR) gene in CF). The genetic modifications can result in the animals having one or more symptoms characteristic of the disease or condition. Animals exhibiting such symptoms are particularly advantageous in the development of therapeutic approaches, as candidate drugs and other approaches to treatment can be evaluated for effects on the symptoms in such animals. Thus, in addition to the animal models themselves, the invention also provides methods of using the animals for identifying and characterizing treatments. Further, the invention includes methods of making transgenic, large non-human animal models and cells that can be used in these methods. The animal models systems, methods, and cells of the invention are described further below.

In addition to animals including knock-outs or mutations in endogenous genes, the invention also includes transgenic, large non-human animal models of human diseases and conditions (e.g., pigs), in which one or more endogenous genes associated with the diseases or conditions are knocked-out (i.e., genetically altered in such a way as to inhibit the production or function of the products of these genes) and replaced with a comparable wild-type or mutated gene derived from a different animal (e.g., a human). In one example, a pig with its endogenous porcine CFTR knocked-out expresses a human transgene encoding a mutated CFTR protein, such as the CFTR-A508 gene (i.e., a CFTR-A508), hCFTR-A508 pig). Alternatively, the human transgene may encode a normal, wild-type copy of a gene of interest (e.g., CFTR). These embodiments of the invention are especially useful for the generation of non-human animal models of human diseases and conditions that can be used to test existing and potential therapeutics that may only (or may preferentially) modulate or treat the disease when contacting, or being in the presence of, human copies of the disease gene or protein in question.

The invention is described herein in reference to animal models of CF, which are generated by mutation, deletion, or replacement of the CFTR gene. However, the methods of the invention are also applicable to the development of animal models of additional diseases and conditions, examples of which are provided below.
The transgenic animals of the invention can be made using the following general strategy. Briefly, the genome of a cell (e.g., a fetal fibroblast) from an animal of interest, such as a pig, is genetically modified or replaced by, for example, gene targeting by homologous recombination, to create a “donor cell.” According to the methods of the invention, the genetic modification results in at least partial inactivation of an endogenous gene associated with a particular disease or condition (e.g., a CFTR gene in CF), as will be described in further detail below. The nucleus of such a genetically modified donor cell (or the entire donor cell, including the nucleus) is then transferred into a so-called “recipient cell,” such as an enucleated oocyte. After activation and, typically, a brief period of in vitro culture, the resulting embryo is implanted into a surrogate female in which development of the embryo proceeds. Typically, the donor cell, oocyte, and surrogate female are of the same species, but the sources can be different species, as is known in the art.


The transgenic animals of the invention can be any non-human mammals, including, for example, ungulates. Examples of ungulates that can be used in the invention include members of the orders Perissodactyla and Artiodactyla, such as any members of the family Suidae, and in particular any member of the genus Sus, such as Sus scrofa, which is also known as the domestic pig or a subspecies thereof (Sus scrofa domestica). In one specific example, the animal is a miniature swine that is a descendent from the miniature swine described by Sachs et al., Transplantation 22:559, 1976. In addition to porcine ungulates, additional ungulates that can be used in the invention include bovine, ovine, and caprine ungulates. Thus, for example, the invention can include the use of cows (e.g., Bos taurus or Bos indicus), sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llamas, alpacas, and elephants.

The invention includes animals in which only one allele of a targeted gene (e.g., CFTR) is disrupted, mutated, or replaced with the other allele remaining unaffected. These animals, which are referred to herein as “heterozygous” or “hemizygous” animals, can be used, for example, in breeding approaches to generate homozygous mutants, if desired, for example, in the case of diseases caused by homozygous recessive mutations. These animals can also be used as animal models themselves, in the case of diseases caused by autosomal dominant mutations.

Also included in the invention are homozygous mutant animals, in which both alleles of a target gene (e.g., CFTR) are disrupted or mutated, by the same or different mutations (or replaced with the same or different gene(s), optionally with the same or different mutations). In addition to being obtainable by breeding approaches involving hemizygous animals, homozygous mutant animals can also be obtained using an approach in which a cell (e.g., a fetal fibroblast) including a mutation in one allele, such as a cell obtained from an animal produced using the method summarized above, is subjected to gene targeting by homologous recombination to achieve modification of the remaining allele. The resulting donor cell can then be used as a source of a modified nucleus for nuclear transfer into a recipient cell, such as an enucleated oocyte, leading to the formation of a homozygous mutant embryo which, when implanted into a surrogate female, develops into a homozygous mutant animal.

A target gene (e.g., a CFTR gene) can be subject to genetic modification in any appropriate cell type of a species for which it is desired to create an animal model of a disease associated with mutation of the gene, according to the invention. As is understood in the art, it is necessary to be able to culture and carry out homologous recombination in a cell that is to be used as a donor cell. A particular example of such a cell, which is described in more detail below in connection with pigs, in the experimental examples, is the fetal fibroblast. These cells can be obtained using, for example, the approach described in U.S. Patent Application Publication 2005/0120400 and other references cited herein.

The invention also includes the use of other cell types that may be present in the cell preparations obtained using the method described in U.S. Patent Application Publication 2005/0120400. Additional examples of cells that can be used as donor cells in making the transgenic animals of the invention include other fetal cells, placental cells, or adult cells. Specific examples of such cells for gene targeting include differentiated cells such as fibroblasts, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, placental, and muscle cells.

If a cell to be genetically altered is derived from an embryo or a fetus, the cell (e.g., a fetal cell or placental cell) can be isolated at any time during the gestation period until the birth of the animal, which may or may not be itself genetically altered. In the case of a pig, such cells can be obtained, for example, between 20 to 90 days of gestation, between 25 to 60 days of gestation, between 30 to 45 days of gestation, or between 35 to 40 (e.g., at 35 days) of gestation. The time periods for obtaining cells from other animals is known in the art (see, e.g., WO 2005/104835).

Gene targeting carried out to make the cells and animals of the invention can result in gene inactivation by disruption, removal, modification, or replacement of target gene sequences. For example, inactivation can take place by the insertion of a heterologous sequence and/or a stop codon into a target gene. A specific example of this type of inactivation, in the context of a CFTR gene, is described in the experimental examples, below. As is known in the art, inserted sequences can replace previously existing sequences in a gene or can be added to such sequences, depending on the design of the targeting construct. Also as is known in the art, the design of targeting constructs can be altered, depending upon whether it is desired to completely knock out the function of a gene or to maintain some level of reduced function. In the case of CFTR, for example, complete knock out of function is consistent with the most common form of CF (F508; see above), but other, less dramatic changes may be desirable for the generation of models of disease maintaining some CFTR function. Such changes may be achieved by, for example, replacement with sequences that are identical to wild-type sequences, except for the presence of specific
mutations giving rise to features of the target disease. In other approaches, coding sequences are not altered or are minimally altered and, rather, sequences impacting expression of a target gene, such as promoter sequences, are targeted. In any case, selectable marker insertion is often desirable to facilitate identification of cells in which targeting has occurred. If desired, such markers or other inserted sequences can later be removed by, e.g., cre-10c or similar systems.

A CFTR-Δ508 (i.e., knock-out), hCFTR-Δ508 pig can be made numerous ways, including, but not limited to: i) introducing a human CFTR-Δ508 cDNA, partial human CFTR-Δ508 gene, or entire human CFTR-Δ508 gene into pig CFTR-Δ508 cells, selecting for human CFTR-Δ508 expression, and using these cells as nuclear donors in somatic cell nuclear transfer, and ii) introducing a human CFTR-Δ508 cDNA, partial human CFTR-Δ508 gene, or entire human CFTR-Δ508 gene into pig CFTR-Δ508 into mature oocytes, fertilizing, then transferring to a recipient female. The human CFTR sequence is described, for example, by Riordan et al., Science 245(4922):1066-1073, 1989 (erratum in Science 245(4925):1437, 1989)). Human, pig, and mouse CFTR sequences are also provided in SEQ ID NO:1-6.

As is known in the art, targeted gene modification requires the use of nucleic acid molecule constructs having regions of homology with a targeted gene (or flanking regions), such that integration of the construct into the genome alters expression of the gene, either by changing the sequence of the gene and/or the levels of expression of the gene. Thus, to alter a gene, a targeting construct is generally designed to contain three main regions: (i) a first region that is homologous to the locus to be targeted (e.g., the CFTR gene or a flanking sequence), (ii) a second region that is a heterologous poly-nucleotide sequence (e.g., encoding a selectable marker, such as an antibiotic resistance protein) that is to specifically replace a portion of the targeted locus or is inserted into the targeted locus, and (iii) a third region that, like the first region, is homologous to the targeted locus, but typically is not contiguous with the first region of the genome. Homologous recombination between the targeting construct and the targeted wild-type locus results in deletion of all such sequences between the two regions of homology represented in the targeting vector and replacement of that sequence with, or insertion into that sequence of, a heterologous sequence that, for example, encodes a selectable marker. In the case of targeting transcriptionally inactive genes, such as, for example, the CFTR gene in fibroblasts, or a gene having only very low levels of transcription, the constructs of the invention can include a promoter, such as a PGK promoter, which drives expression of the selectable marker (e.g., Neo). Use of such promoters may not be required in cases in which transcriptionally active genes are targeted, if the design of the construct results in the marker being transcribed as directed by an endogenous promoter. Exemplary constructs and vectors for carrying out such targeted modification are described herein.

In order to facilitate homologous recombination, the first and third regions of the targeting vectors (see above) include sequences that exhibit substantial identity to the genes to be targeted (or flanking regions). By “substantially identical” is meant having a sequence that is at least 80%, 90%, 95%, 98%, or 100% identical to that of another sequence. Sequence identity is typically measured using BLAST® (Basic Local Alignment Search Tool) or BLAST® 2 with the default parameters specified therein (see, Altschul et al., J. Mol. Biol. 215:403-410, 1990; Titiana et al., FEMS Microbiol. Lett. 174:247-250, 1999). These software programs match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Thus, sequences having at least 80%, 90%, 98%, or even 100% sequence identity with the targeted gene loci can be used in the invention to facilitate homologous recombination.

The total size of the two regions of homology (i.e., the first and third regions noted above) can be, for example, approximately 2-25 kilobases (e.g., 4-20, 5-15, or 6-10 kilobases), and the size of the second region that replaces a portion of the targeted locus can be, for example, approximately 0.5-5 kilobases (e.g., 1-4 or 3-4 kilobases). A specific example of such a construct is described below, in the experimental examples.

The targeting constructs can be included within any appropriate vectors, such as plasmid or viral vectors (e.g., adenovirus or adeno-associated virus vectors), which can be introduced into cells using standard methods including, for example, viral transduction, electroporation, or microinjection. One example employs an adeno-associated viral vector (AAV) (e.g., rAAV2, which can be made by standard methods using a pAV2 plasmid (ATCC 37216), rAAV1, and rAAV5).

The use of AAV to deliver the targeting construct offers many benefits. First, AAV1 (and other AAV serotypes) infects pig fetal fibroblasts with 95-100% efficiency. Second, AAV infection of pig fetal fibroblasts results in little or no cell toxicity. Third, AAV infection results in the delivery of a single-stranded gene targeting construct directly to the nucleus. Single-stranded gene targeting vectors are thought to yield more efficient gene targeting and result in a more favorable homologous recombination to non-homologous recombination ratio (Eliandre and Russell, Molecular Therapy 12(1):9-17, 2005).

The methods of the invention, employing AAV vectors, resulted in high levels of gene targeting efficiency in these somatic cells, as compared to prior methods. Central to the methods of the invention is the fact that the entire procedure was performed in a time-sensitive manner, because excessive cell culture time (more than 30 days) negatively impacts nuclear transfer efficiency (Li et al., Cloning and Stem Cells 5(4):233-241, 2003). In one example, following fibroblast harvest from day 35 fetuses, the fetal fibroblast cells were frozen within 48 hours. The use of an AAV vector to deliver the gene targeting construct allowed targeting to begin 24 hours after thawing cells and required no cell detachment and re-attachment, which is required in other methods. Multiple cell detachment and re-attachment events (trypsinization) are thought to decrease the ability of a cell to serve as a nuclear donor in nuclear transfer. Further, G418 selection in 48 96-well plates prevents the need for the more conventional, time-consuming isolation of resistant clones with cloning rings. The screen for gene-targeted clones was designed such that all positive clones could be identified and frozen within a 3-5 day period. All clones were frozen by day 18, therefore the cells have been in culture approximately 20 days since being harvested from the fetus. This is an important aspect of the invention, because reduction of the time in culture increases the likelihood that cells used as nuclear donors are viable, normal, and euploid.

Accordingly, the invention provides a method of gene-targeting cells, such as pig cells (e.g., pig fetal fibroblasts), in which the number of days in culture (during which targeting and selection takes place) is less than 30 days, e.g., 25-29, 20-24, 19, 18, 17, 16, 15, or fewer days. To facilitate this method, the selection can take place in multi-well plates, as described further below. Further, the cells may be frozen shortly after harvest (e.g., within 24, 48, or 96 hours). After cell thawing (or after harvest, if the cells are not previously
frozen), gene targeting with an AAV vector can be carried out within, for example, 12, 24, 36, or 48 hours, without the use of multiple detachment/re-attachment events, and selection can proceed in an expedited manner, such as by use of multiwell plates (e.g., 96-well plates), prior to freezing.

Other types of vectors, or more specifically, other types of targeting construct delivery methods, are available, and were used during initial attempts to disrupt the pig CFTR gene. Cell transfection methods, including calcium phosphate, lipofection, electroporation, and nuclear injection can be used to deliver the targeting construct, though the disadvantages of inefficient transfection efficiency, cell toxicity, requirement of a pure (clean) targeting construct DNA sample, and poor ratio of homologous recombination to non-homologous recombination far outweigh the benefit of ease. If the gene is transcriptionally active in the cell being used, then a promotorless selectable marker strategy can be employed, so that antibiotic resistance will only be found in cell that have had a recombination event within a transcribed unit.

Genetically targeted cells are typically identified using a selectable marker, such as neomycin. If a cell already contains a selectable marker, however, a new targeting construct containing a different selectable marker can be used. Alternatively, if the same selectable marker is employed, cells can be selected in the second targeting round by raising the drug concentration (for example, by doubling the drug concentration), as is known in the art. As is noted above, targeting constructs can include selectable markers flanked by sites facilitating excision of the marker sequences. In one example, constructs can include loxP sites to facilitate the efficient deletion of the marker using the cre/lox system. Use of such systems is well known in the art, and a specific example of use of this system is provided below, in the experimental examples.

Upon obtaining cells in which a target gene (e.g., a CFTR gene) has been targeted (one or both alleles, as described above), nuclear transfer can be carried out. Optionally, the genetically modified nuclear donor cells can be frozen prior to nuclear transfer. Recipient cells that can be used in the invention are typically oocytes, fertilized zygotes, or two-cell embryos, all of which may or may not have been enucleated. Typically, the donor and the recipient cells are derived from the same species. However, it is possible to obtain development from embryos reconstructed using donor and recipient cells from different species.

Recipient oocytes can be obtained using methods that are known in the art or can be purchased from commercial sources (e.g., BoMed Inc., Madison, Wis.). As is known in the art, the donor nucleus or the donor cell itself can be injected into the recipient cell or injected into the perivitelline space, adjacent to the oocyte membrane. The nuclear transfer complex formed in this manner can be activated by standard methods, which may involve electrical fusion/activation or electrical fusion/chemical activation, as is described further below. Further processing of the nuclear transfer complex, including implantation of the complexes into surrogate mothers, is described further below.

The transgenic animals of the invention can be used in the identification and characterization of drug and other treatment methods for the disease or condition associated with mutation of the gene targeted according to the invention. In these methods, for example, a candidate therapeutic agent can be administered to an animal and the impact of the agent on a feature of the disease exhibited by the animal can be monitored. Optionally, the methods can also involve exposure of the animals to environmental or other conditions known to contribute to or exacerbate the disease or condition. For example, in the case of CF animal models having impaired respiratory function, the effect of the drug on such function can be assessed by measurement of standard respiratory parameters. In another example, in the case of animals exhibiting impaired digestion, due to blockage of pancreatic and/or liver ducts, the effect of a treatment on digestion can be determined.

With the porcine model of the invention, it is possible to test hypotheses that lead to new treatments and to evaluate potential therapies for CF lung disease. The porcine model also makes it possible to assess electrolyte transport by porcine airway epithelia in vitro and in vivo, the volume of airway surface liquid in vitro and in vivo, the ion composition of airway surface liquid in vitro and in vivo, the airway surface liquid pH in the airway, and electrolyte transport in the small airways. It is also possible to measure respiratory mucociliary transport in vitro and in vivo. For assessing inflammation, several tests and assays can be carried out, including (but not limited to) assays of key markers of inflammation in amniotic fluid, fetal lung liquid, and bronchoalveolar lavage by using lung tissue histochemistry, large-scale gene expression profiling of pulmonary tissues, cytokine and cell assays, and proteomics. It is also possible to raise CF and non-CF piglets in isolators under completely germ free conditions and to test for the development of pulmonary inflammation, and then selectively expose the piglets to inflammatory stimuli including bacteria and viruses. In addition, investigators can test how loss of CFTR function in airway epithelia results in altered NFkB signaling, the function of secreted epithelial antimicrobials/host defense proteins, and the consequences of loss of CFTR function in macrophages or neutrophils. The availability of the porcine CF model allows tests of the early manifestations of the CF, an important question that remains unanswered. The natural history of pulmonary infections in CF pigs can also be monitored, leading to a determination of whether the airway epithelia of CF pigs can be colonized by CF or porcine pathogens and/or non-pathogenic opportunistic organisms.

Although lung disease is the current main cause of mortality, patients suffer from CF disease in many other organs. Availability of a CF model allows new investigations and tests of therapeutics in the pancreas, intestine, sweat gland, liver, vas deferens, kidney, and other organs affected primarily or secondarily by CF. The screening methods of the invention can be carried out to test the efficacy of new compounds, combinations of new and old compounds, non-pharmaceutical treatments, and combinations of pharmaceutical and non-pharmaceutical treatments.

As described further below, CFTR−/− and CFTRΔF508/ΔF508 pigs of the invention have been generated and extensively characterized with respect to genotype of phenotypic characteristics. The CFTR−/− pigs have been found to share phenotypic characteristics with human CF, many of which are not also shared by murine CF models. These differences highlight the importance of the animal models of the invention in the development of therapeutics for human CF. Animal models of the invention thus include large non-human animals, such as pigs, having any one or more of the following clinical, electrophysiological, or pathological characteristics, relative to corresponding wild-type animals: (i) electrophysiological features similar to CF humans including, e.g., any one or more of (a) hyperpolarized baseline Vt, (b) reduction of Vt by amiloride, and (c) no CFTR or other CF channel activity (as measured by, e.g., perfusion of apical surface with CT-free solution and addition of isopro-
enol; perfusion with ATP to activate P2Y2 receptors and Ca++-activated CF channels; and perfusion with the CFTR inhibitor GlyH-101; (ii) meconium ileus, as characterized by, e.g., one or more of (a) obstruction in the small intestine and/or colon (e.g., near the ileocecal junction), (b) the appearance of microcolon distal to the obstruction, (c) intestinal perforation and/or peritonitis, (d) failure to pass feces or gain weight, (e) failure to eat, (f) abdominal distension, (g) bile-stained emesis, (h) proximal dilation of the small intestine, (i) meconium distension of the intestine, (j) degenerated and atrophied villi adjacent to the meconium, (k) reduced luminal diameter distal to the meconium, (l) mucinous hyperplasia (including mucoid luminal plugs) distal to the meconium, and (m) dilated intestinal obstruction syndrome (DIOS); (iii) extrinsic pancreatic insufficiency or abnormalities, as characterized by, e.g., one or more of (a) decreased size, (b) degenerative lesions with, e.g., increased loose adipose and myxomatous tissue, and scattered to moderate cellular inflammation, (c) diminished eosinophilic zymogen granules in residual acini, (d) variable dilution and obstruction of centroacinar spaces, ductules, and ducts with eosinophilic mural plus infrequent neutrophils and macrophages mixed with cellular debris, (e) foci of mucinous metaplasia in ducts and ductules, and (f) increased redness; (iv) hepatic abnormalities consist of focal biliary cirrhosis, as characterized by, e.g., any one or more of (a) mild to moderate hepatic lesions, (b) chronic cellular inflammation, (c) ductular hyperplasia, and (d) mild fibrosis; (v) gall bladder and/or bile duct abnormalities, as characterized by, e.g., any one or more of (a) gallstones, (b) reduced size, (c) congealed bile and mucus, and (d) epithelia with diffuse mucin increases in folds extending into the lumen; (vi) tracheal abnormalities characterized by, for example, altered lumen area, circumference, submucosal gland area, and smooth muscle area; (vii) characteristics of CF lung disease, for example, any one or more of obstruction of some airways with mucopurulent material, scattered mucopurulent debris in airway lumens with chronic purulent to lymphoid airway wall inflammation, surface epithelium with areas of goblet cell hyperplasia, mucocellular material in submucosal glands, lobular atelectasis, host defense defect as shown by detection of a variety of bacterial species in lung samples; and (viii) lack of abnormalities in vas deferens and lungs at birth.

The invention has been described above in reference to mutation of the CFTR gene to generate non-human animal models of cystic fibrosis. As is stated above, the invention can also be used in the generation of transgenic, non-human animal models of other diseases and conditions associated with gene mutations. There are innumerable examples of such diseases and conditions known in the art, which can be included in this invention. Some specific examples are listed in Table 1.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>p53, BRCA1 and 2</td>
<td>Levine et al., Cell, 88: 323-331, 1997</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>huntingtin</td>
<td>Gdunsmat and Ashworth, Oncogene. 25(43): 5864-5874, 2006</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>PKD1 and 2</td>
<td>Gattone V., Current Opinion in Pharmacology 5: 353-342, 2005</td>
</tr>
<tr>
<td>Ataxia-telangiectasia</td>
<td>ATM</td>
<td>Concannon and Gatti, Hum. Mutat. 10(2): 100-7, 1997</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>RB1</td>
<td>Lohmann, Hum. Mutat. 14(4): 283-8, 1999</td>
</tr>
</tbody>
</table>

Possible mutations to these disease genes include knock-outs (by, e.g., insertion of a selection cassette), knock-ins (e.g., by point mutations that correspond to human disease mutations), and, in the case of Huntington’s disease (and any other trinucleotide repeat expansion disorder family members), an expansion of the trinucleotide repeat to pathogenic sizes.

The following Examples are meant to illustrate the invention and are not meant to limit the scope of the invention in any way.

**EXPERIMENTAL EXAMPLES**

Pigs with two different alterations in their CFTR gene, a null allele and the ΔF508 mutation will provide useful animal models for the study of CF. A null allele would lack any CFTR function and should therefore provide a valuable model for assessing the porcine CF phenotype, for comparing the consequences of other CF-associated mutations, for exploring pathogenesis, and for evaluating many therapeutic strategies. The ΔF508 mutation deletes Phe508 and is the most common CF-associated mutation, accounting for ~70% of CF alleles (Zielenski et al., Am. Rev. Genet. 29:777-807, 1995). In humans, this mutation disrupts processing of the protein, so that nearly all CFTR-ΔF508 is retained in the endoplasmic reticulum (ER) and degraded, preventing maturation to the plasma membrane. In addition, this deletion reduces the activity of single CFTR channels and shortens their lifetime on the cell surface (Dalemans et al., Nature 354:526-528, 1991; Teen et al., Receptors Channels 4:63-72, 1996; Skoah, Kidney Int. 57:825-831, 2000). Earlier work showed that reducing the incubation temperature and other interventions allowed some of the mutant protein to escape the ER and traffic to the cell surface, where it retained significant activity (Denning et al., Nature 358:761-764, 1992). These findings and the prevalence of the ΔF508 mutation have driven efforts to correct the CFTR-ΔF508 defects (Lukacs et al., N. Engl. J. Med. 349:1401-1404, 2003; Verkman et al., Curr. Pharm. Des. 12:2235-2247, 2006). We have found that porcine
CFTR-ΔF508 showed at least partial processing in vitro (Ostedgaard et al., Proc. Natl. Acad. Sci. U.S.A. 104:15370-15375, 2007; also see below). A pig with the ΔF508 mutation could be of value for understanding the mechanisms responsible for the CFTR-ΔF508 biosynthetic defects in vivo and for developing pharmacological agents to correct the CFTR-ΔF508 biosynthetic defects. To begin developing these porcine models of CF, we combined gene targeting and SCNT.

The following experimental examples describe the generation of cystic fibrosis pig models (CFTR-null and CFTR-ΔF508 alleles), an interspecies analysis of the ΔF508 mutation, and approaches to making pigs expressing human CFTR sequences (e.g., human ΔF508 CFTR).

I. Cystic Fibrosis Pig—Generation of CFTR-/ ΔF508 Heterozygote Results

Fetal Pig Fibroblasts Express Little CFTR

We worked with fetal fibroblasts from domestic pigs (Sus scrofa) since they have been used successfully for transgenic SCNT (Park et al., Animal Biotechnol 12(2):173-181, 2001). Because a promoter-trap strategy was previously used in porcine fibroblasts (Lai et al., Science 295:1089-1092, 2002), we asked if CFTR is expressed in fetal fibroblasts. We used quantitative RT-PCR and compared the results to transcript levels in nasal and rectal epithelia, which are known to express CFTR at low levels (Trappnell et al., Proc. Natl. Acad. Sci. U.S.A. 88:6565-6569, 1991). Fig. 1 shows that the primary fibroblasts produced very little CFTR mRNA. This result prevented the use of a promoter-trap strategy as was done for the only other gene targeted in pigs (Lai et al., Science 295:1089-1092, 2002; Dai et al., Nat. Biotechnol. 20:251-255, 2002).

Developing Vectors to Target the Pig CFTR Gene

We designed a “null” targeting construct to disrupt CFTR exon 10 with a neomycin resistance cassette (NeoR) (FIG. 2). Because CFTR can exhibit some alternative splicing, we chose to disrupt exon 10, which encodes a portion of nucleotide binding domain 1; this exon is required for CFTR function. We also included an engineered stop codon at position 508. Therefore, F508X would be expected to trigger nonsense-mediated mRNA decay as well as prematurely interrupt any translation of CFTR. The ΔF508 targeting vector was designed to delete residue Phe508 (FIG. 2). We also inserted a NeoR in the intron downstream of exon 10 as a positive selection marker. In this vector, NeoR was flanked by loxp sites so that it could be removed at a later time if it was found to markedly reduce the level of the CFTR-ΔF508 mRNA, a situation encountered in some attempts to make a CFTR-ΔF508 mouse (Colledge et al., Nat. Genet. 10:445-452, 1995; Zeiler et al., J. Clin. Invest. 96:2051-5064, 1995).

We initially used nuclear microinjection and then electroporation to deliver the null targeting vector to fetal fibroblasts. However, we recovered no clones with homologous recombination. We then investigated AAV-mediated gene targeting, which has been used to deliver targeting vectors to cell lines and primary cells (Inoue et al., J. Virol. 73:7376-7380, 1999; Hirata et al., Nat. Biotechnol. 20:735-738, 2002; Porteous et al., Mol. Cell. Biol. 23:3558-3565, 2003; Russell et al., Nat. Genet. 18:325-330, 1998). Using an AAV vector that had the advantage that it delivers single-stranded DNA to the nucleus, the amount of DNA per cell is small, and it can infect many cell types (Hendrie et al., Mol. Ther. 12:9-17, 2005). To first determine which AAV serotypes can infect pig fibroblasts, we infected them with eGFP-expressing AAV1, 2, and 5 (each with AAV2 ITRs). Each AAV infected the cells with at least 50-80% efficiency, however, AAV1 appeared to infect nearly 100% of cells. Because of rAAV genome size constraints, the total length of the targeting vectors was limited to ~4.5 kb. NeoR was centrally located in both vectors (FIG. 2).

AAV Vectors Introduced the CFTR-Null and CFTR-ΔF508 Alleles

We obtained fetal fibroblasts from males so that all of our clones would be male, which would allow us to more rapidly expand the number of animals. Primary cultures of pig fetal fibroblasts were infected with AAV1 carrying the null targeting vector. After 24 hours, cells were transferred to a series of 96-well plates. Approximately two weeks later, cells in each well of the 96-well plates were “replicated” by splitting among three plates: 96-well culture plates for cell expansion, 96-well culture plates for potential cryopreservation, and 96-well PCR plates for cell lysis.

We screened cell lysates by PCR to identify wells containing gene-targeted clones (FIG. 3A) and then hybridized with a NeoR-specific probe to test for inclusion of this marker (FIG. 3B). We then froze positive clones; by that time, cells had been in culture 15-17 days. We also passaged positive clones from the “cell expansion” plates to generate DNA for genotyping determination. Southern blots with CFTR- and NeoR-specific probes identified clones with a targeted CFTR allele that were free of random integration (FIG. 4). On average, 75% of PCR-positive clones were also positive by Southern blot and were clonal.

We used identical procedures to introduce the CFTR-ΔF508 construct and screen for homologous recombinants. We identified numerous PCR-positive clones (FIG. 5A), that were confirmed by Southern blotting with a ΔF508 allele-specific probe (FIG. 5B). Eighteen of 25 (72%) PCR-positive clones contained the F508 deletion. The other 28% failed to contain ΔF508, suggesting that gene targeting had occurred, but crossing over was downstream of F508. Subsequent Southern blots revealed CFTR-ΔF508 targeted clones (FIGS. 5A and 5B).

Variability in Homologous Recombination Depended on the Donor

Over the course of these studies, we targeted the CFTR gene in fibroblasts derived from several fetuses. The fetuses were all siblings harvested from the same uterus at the same time. Yet, surprisingly, we saw a striking fetus-to-fetus variability in targeting frequency (Table 2, below). Even when fibroblasts from different fetuses were infected and screened at the same time, with the same reagents, and by the same people, pronounced differences occurred; an example is fetus 5 vs. fetus 7 in Table 2. These results suggest the difference was not due to experimental process.

<table>
<thead>
<tr>
<th>Donor</th>
<th>G418-resistant (%)</th>
<th>Targeted/G418-resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.13</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.13</td>
<td>0.07</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CFTR targeting data from donor cells derived from multiple fetuses. “Donor” refers to the number of the donor for the fibroblasts. The percentage of G418-resistant cells was determined by dividing the number of G418-resistant clones by the number of AAV-infected cells *100. Targeted clones are those that were PCR-positive for homologous recombination.

SCNT Produced Gene-Targeted Piglets

To produce heterozygote pigs, we used the CFTR-null targeted fetal fibroblasts as nuclear donors for transfer to enucleated oocytes. Then to each of eight surrogate females, we transferred between 94 and 144 SCNT embryos. At 117-118 days of gestation (full term), we delivered piglets by Cesarean section. Five surrogates produced ten males; three
surrogates did not produce offspring. FIG. 7 shows the first CFTR+/− piglet. Southern blots revealed that none of the ten offspring were CFTR-null heterozygotes, and one was wild-type (FIG. 8). The CFTR+/− males reached sexual maturity, and they sired numerous litters of heterozygote offspring, both males and females.

In addition, each of four surrogates received 103-185 CFTR-ΔF508 SCNT embryos. Five males were recovered from three surrogates on days 116-117. Southern blots revealed that four were CFTR-ΔF508 heterozygotes and one was a wild-type. The CFTR-ΔF508 males have not yet reached sexual maturity. All of the CFTR+/− and CFTR+/ΔF508 were phenotypically normal.

The ΔF508 Allele, but not the Null Allele, Generated mRNA

We asked whether the targeted alleles were transcriptionally active in an epithelium where CFTR is normally expressed. We biopsied rectal epithelium and measured CFTR mRNA using quantitative RT-PCR. In CFTR+/− animals, mRNA was present at ~50% of wild-type levels (FIG. 9A). We cannot be certain that the remaining mRNA arose from the non-targeted allele, however the result is consistent with disruption of one CFTR allele and nonsense-mediated mRNA decay.

To assess the influence of the Neo6 cassette that resides in the intron downstream of exon 10, we used probes specific for wild-type CFTR and CFTR-ΔF508. CFTR-ΔF508 mRNA was present at 65-70% of wild-type levels (FIG. 9B). This expression level suggests that the retained Neo6 cassette has only minimal effects on transcription. Moreover, this amount of transcript is likely sufficient to produce relatively normal amounts of CFTR-ΔF508 protein.

Materials of Methods

Fetal Fibroblasts

Fetal fibroblasts were isolated from day 35 fetuses as previously described (La et al., Cloning Stem Cells 5:233-241, 2003). Cells were grown at 39° C in F10 media (Invitrogen) containing 20% FCS and 30 μg/ml gentamicin. Fetus gender was determined by PCR amplification of the Y-chromosome-specific Sry gene (Pomp et al., J. Anim. Sci. 73:1408-1415, 1995).

Targeting Vector Construction

Genomic clone: Genomic DNA was isolated (Puregene, Gentra) from pig fetal fibroblasts. A 5683 bp PCR product including CFTR exon 10 and flanking intronic sequence was amplified from pig fetal fibroblast genomic DNA using primers GC1F and GC8R (for primer sequences see Table 3, below) and a high fidelity polymerase (PfuUltra, Stratagene). Primers were designed based on the domestic pig genomic sequence from the NIH Intramural Sequencing Center (NISC) Comparative Vertebrate Sequencing Project (Genbank: AC092478 and AC092497). This PCR product was subcloned into pCR-Blunt II-TOPO (Invitrogen), verified by sequencing (using primers GC1F-GC8R), and served as the template for PCR amplification of 5′ and 3′ targeting arms. This plasmid is referred to as pG16.

CFTR-KO construction: Using PCR, the 5′ and 3′ homologous recombination arms were amplified from pG16 and sequentially subcloned upstream and downstream of the Neo6 cassette in pPK1-Neo-1 (a generous gift from Tim Ley, Washington University; Genbank Accession Number AF335419) such that the Neo6 cassette is in the opposite orientation to the CFTR sequence. Primers: 5′ arm: G16-No5′ and G16-No5′R; 3′ arm: G16-No3′F and G16-No3′R. The Neo6 cassette consists of a Neo6 cDNA driven by the PGK promoter and is flanked by loxp sites. In the resulting construct, the Neo6 cassette disrupts CFTR exon 10 immediately after an in-frame stop codon that was introduced to follow isoleucine 507. Thymidine 1531 is effectively deleted, becoming the first nucleotide of the stop codon. This targeting construct is referred to as pG16-Neo.

CFTR-ΔF508 construction: The CFTR-ΔF508 targeting vector was constructed in a similar way using the following primers: 5′ arm: dl-No5′F-Xhol and dl-No5′R-EcoRV; 3′ arm: dl-No3′F-BamHI and dl-No3′R-HindIII. The nucleotides encoding F508 were subsequently deleted from exon 10 using PCR mutagenesis. This targeting construct is referred to as pdl-Neo.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC1F</td>
<td>TTTCCTCCTTGCCTATTTCCCT (SEQ ID NO: 7)</td>
</tr>
<tr>
<td>GC2R</td>
<td>AGAAAAACACTGAGAGATCGCT (SEQ ID NO: 8)</td>
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<td>GC8F</td>
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| Table 3 |
### Table 3-continued

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<tr>
<td>Ex10a5F</td>
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Quantitative RT-PCR primers and probes

Pig CFTR and GAPDH expression in fetal fibroblasts, nasal and rectal tissue (FIG. 1) and CFTR expression in CFTR +/− pigs (FIG. 9A).

<table>
<thead>
<tr>
<th>CFTR primers and probe</th>
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<tbody>
<tr>
<td>pCFTR-1019F (anneals within exon 18)</td>
<td>GAPDH</td>
<td>mRNA expression (FIG. 9B)</td>
</tr>
<tr>
<td>pCFTR-1019F (anneals within exon 19)</td>
<td>GAPDH</td>
<td>mRNA expression (FIG. 9B)</td>
</tr>
<tr>
<td>pCFTR-1019F (spans exon 18/19 junction)</td>
<td>GAPDH</td>
<td>mRNA expression (FIG. 9B)</td>
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<td>pGAPDH-TM-F (SEQ ID NO: 26)</td>
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<td>pGAPDH-TM probe (SEQ ID NO: 29)</td>
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Pig CFTR and AFS508-CFTR expression in CFTR +/AFS508 pigs (FIG. 9B). Primers are the same for both, probes are allele specific.

<table>
<thead>
<tr>
<th>CFTR primers and probe</th>
<th>Protein name</th>
<th>Function</th>
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<tbody>
<tr>
<td>pCFTR-TM-F (SEQ ID NO: 29)</td>
<td></td>
<td></td>
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<tr>
<td>pCFTR-TM-R (SEQ ID NO: 30)</td>
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<td>pCFTR-TM probe (SEQ ID NO: 31)</td>
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<table>
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<th>AFS508 primers and probes</th>
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<td>delF-TM-Forward (SEQ ID NO: 32)</td>
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<td>delF-TM-Reverse (SEQ ID NO: 33)</td>
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<td>delF-TM-Probe (SEQ ID NO: 34)</td>
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PCR Primers and Probes. All DNA sequences are 5′-3′. FAM: 6-carboxyfluorescein; NFQ: Non Fluorescent Quencher.

### AAV Production

The targeting vector sequences were amplified from pG16-Neo and pdf-Neo by PCR to include flanking SbfI sites and were subcloned into the pAAV proviral plasmid, pAV2 (ATCC 37216). Because of AAV genome size constraints, the total length of the targeting vectors is ~4.5 kb with the NeoR cassettes centrally located (G16-Neo: 5′ targeting arm=1510 bp; 3′ targeting arm=1274 bp; NeoR cassette=1706 bp. df-Neo: 5′ targeting arm=1475 bp; 3′ targeting arm=1296 bp; NeoR cassette=1706 bp). pAV2-G16-Neo was transfected in Sure2 cells (Stratagene) and purified via a CsCl2 method (Sambrook, Fritz, and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1989). AAV1 (with AAV2 ITRs) was also produced as previously described (Yan et al., J. Virol. 76:2043-2053, 2002). Helper-free virus stocks were treated with nuclelease and purified by high-performance liquid chromatography. Physical titers of AAV were determined by slot blot hybridization. These viruses are referred to as AAV-G16-Neo and AAV-df-Neo.

### Infection and Selection

1.5×10⁶ fetal fibroblasts were thawed and plated on a 100 mm collagen-coated culture dish. 24 hours later, cells were infected with AAV-G16-Neo or AAV-df-Neo (200 μl, 2.5×10¹² particles/ml). 24 hours later, cells were trypsinized and transferred to 48 96-well, collagen-coated plates (BD Biosciences). 48 hours later, G418 (100 μg/ml) was added to the cell media. 10 days later, each well was trypsinized (60 μl trypsin, 0.5% EDTA) and split among 3 different vessels. For cell freezing, ¼ of the cells were transferred to 96-well collagen-coated culture dishes and returned to the incubator for growth. For cell propagation, ¼ of the cells were transferred to 96-well collagen-coated culture dishes and returned to the incubator for growth. For PCR screening, ¼ of the cells were transferred to 96-well PCR plates. PCR Screen and PCR Southern Blot

Cells in the 96-well PCR plates were spun down and resuspended in lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl, pH 8.5, 0.5% Nonidet P-40, 0.5% Tween, 400 μg/ml Proteinase K) (McCreath et al., Nature 405:1066-1069, 2000). Most wells (~70%) contained only dead cell debris following selection, but all wells were processed to minimize human error. The cells were lysed for 30 min at 65°C, followed by 10 min at 95°C. 1 μl of lysate was used in a 50 μl PCR reaction. PCR conditions: 2 min at 95°C, 30 cycles of 95°C for 20 sec, 56°C for 20 sec, and 68°C for 4 min, then 68°C for 5 min. Primers Ex10a5F and GC7R are expected to amplify a 2.0 kb product from wild-type alleles and a 3.7 kb product from G16-Neo targeted alleles. PCR products were electrophoresed on 1.0% E-Gel 96 gels (Invitrogen). Positive PCR reactions were also electrophoresed on standard 1.0% agarose gels and transferred to a nylon membrane. The membranes were probed with biotin-labeled Neo-specific or ΔF508-allele-specific oligonucleotides and detected by chemiluminescence (NorthSouth, Pierce). Processing Screen-Positive Cells
Following identification of PCR-positive clones, the corresponding cells from the “freezing” plate were grown to confluency (~10,000 cells). Cells were detached with 60 µl trypsin and 20 µl of detached cells were placed into each of 3 cryovials. Three hundred µl freezing media was added to each cryovial and the vials were transferred to an isopropanol cryofreezing container at ~70°C. After 24 hours, the vials were transferred to liquid nitrogen. The corresponding cells from the propagation plate were transferred to 24-well plates, and subsequently to 6-well and 100 mm culture dishes. The sequential transfer to increasingly larger culture dishes was carried out to achieve consistent cell growth and viability. Southern Blotting

For CFTR-KO targeting, genomic DNA was isolated from 100 mm dishes (Genta) and 10 µg was digested with BglII overnight. For CFTR-Δ508 targeting, genomic DNA was isolated from 24-well dishes. Ten µl was used for whole genome amplification (Repli-G, Qiagen) and 25 µg amplified DNA was digested with BglII overnight. Genomic digests were electrophoresed on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Roche) by using an alkaline transfer procedure. Blots were pre-hybridized for 15 min at 65°C in Rapid-hyb buffer (Amersham). The blot was then hybridized in Rapid-hyb buffer with a 32P-labeled probe specific for a region of CFTR that is outside of the targeting vector boundaries. For Neo-specific probing, blot were either stripped, or, in most cases, the BglII digest and Southern blot procedure was repeated using a 32P-labeled Neo-specific probe.

Preparation of Donor Cells for SCNT

Frozen aliquots of CFTR-targeted cells were thawed at 37°C. and pre-warmed in F-10 medium (Invitrogen) with 20% fetal calf serum (FCS). The cells were washed twice by centrifugation and cultured (F-10, Invitrogen; 20% FCS, Hyclone; gentamicin, 2.5 µg/ml FGF and G418, Invitrogen) for 1-2 days in 24-well collagen-coated plates (35-4408, Bicocat cellware). Confluent cells were dispersed with 0.05% trypsin/EDTA for 3-5 min at 38.5°C. and 500 µl F-10 with 20% FBS, followed by centrifugation twice at 3000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in micromanipulation medium (25 mM HEPES, TCM199, Gibco; 0.3% BSA).

Oocyte Maturation and SCNT

Oocytes were received from BoMed, Inc (Madison, Wis.) ~24 hours after placing them into maturation medium, and were then transferred to a 4-well dish and cultured for a total maturation of 42-44 hours at 38.5°C. In a humidified atmosphere of 5% CO2 in air. After 42-44 h of in vitro maturation, oocytes were stripped of their cumulus cells by gentle vortexing in 0.5 mg/ml hyaluronidase. After removal of the cumulus cells, oocytes with good morphology and a visible polar body (metaphase II) were selected and kept in micromanipulation medium at 38.5°C until SCNT.

SCNT was performed essentially as previously described (Lai et al., Science 295:1089-1092, 2002; Lai et al., Nat. Biotechnol. 24:435-436, 2006) in micromanipulation medium supplemented with 7.5 µg/ml cytochalasin B. The metaphase II chromosomes and the polar body were aspirated by inserting a micropipette through the zona pellucida and aspirating the polar body and the adjacent cytoplasm into the pipette. Next a donor cell was aspirated into the same pipette, the pipette was inserted into the previously made hole in the zona pellucida, and the cell deposited under the zona pellucida. The nuclear transfer complex was fused in a medium with a low Ca2+ concentration (0.3 M mannitol, 0.1 mM CoCl2, 2H2O, 0.1 mM MgCl2, 8H2O, and 0.5 mM HEPES), activated with 200 µM thimerosal for 10 min in the dark, and then rinsed and treated with 8 mM diithiothreitol (DTT) for 30 min. Finally, the oocytes were rinsed to remove any traces of DTT (Lai et al., Nat. Biotechnol. 24:435-436, 2006). Following fusion/activation, oocytes were washed three times with PZM3 as previously described for 30 min (Im et al., Theriogenology 61:1125-1135, 2004). Those that had fused were cultured for 15-21 hours until surgical embryo transfer to a surrogate.

Surrogate Preparation and Embryo Transfer

The embryonic cleavage rate was examined before transferring the reconstructed embryos into recipients. The recipients were synchronized by administering 18-20 mg Recumi- mate and hCG as previously described (Lai et al., Cloning Stem Cells 5:233-241, 2003). Twelve surrogates on the first day of estrus (designated day 0) or the first day after standing estrus were used. Embryo transfer was performed surgically as previously described (Lai et al., Cloning Stem Cells 5:233-241, 2003) and 94 to 185 embryos were inserted into one oviduct through the ovarian fimbria. Surrogates were checked for pregnancy by abdominal ultrasound examination after day 21 and then checked weekly throughout gestation, and were allowed to go to term. A cesarean section was performed to recover the piglets on day 116-118. After delivery the piglets were provided medical care, fed colostrums, and initially raised on a commercial pig milk replacer until mature enough to be placed on standard pig diets.

Rectal Biopsy

Pigs were lightly anesthetized with ketamine (20 mg/kg) and acepromazine (0.2 mg/kg). A 10 cm anoscope was partially inserted in the rectum and rectal tissue was collected using gastrointestinal biopsy forceps (2.2 mm). Tissue samples were immediately placed in RNAlater (Ambion). Recovery from anesthesia was monitored continuously until the pigs returned to normal activity (2-4 hours).

Quantitative RT-PCR

Quantitative RT-PCR using TaqMan chemistry and an ABI 7500 Fast Real-time PCR System was used to measure pig CFTR mRNA. Briefly, total RNA was isolated from fibroblasts and nasal and rectal biopsy tissue (RNeasy, Qiagen). First-strand cDNA was synthesized with random primers (SuperScript III, Invitrogen). Sequence-specific primers and probes for pig CFTR and GAPDH were designed and ordered using Assays-by-design (Applied Biosystems). For measuring total CFTR mRNA primer/probe sets spanning exons 18 and 19 of CFTR and GAPDH were used in separate reactions. For measuring ΔF508 mRNA levels, one primer set and two probes (F508 and F508o) were used in separate reactions. Primer and probe sequences are included in Table 2. TaqMan Fast Universal PCR Master Mix was used for all reactions. The reaction volume was 20 µl (10 ml of 2x Master Mix without UNG, 1 µl of 20x target primer and probe, 8 µl of Nuclease-free water, and 1 µl of cDNA sample). The reaction plates were covered with optical film and centrifuged briefly. The thermocycler conditions were as follows: 20 seconds at 95°C, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All experiments were run in triplicate. Because the efficiencies of CFTR and GAPDH amplification were not equal, the relative quantification of transcript levels was performed using the standard curve method.

II. Cystic Fibrosis Pig—Generation of CFTR+/+ Homozygote

To develop a new CF model, we chose pigs because compared to mice their anatomy, biochemistry, physiology, size, lifespan, and genetics are more similar to those of humans (Ibrahim et al., Xenontransplantation 13:488, 2006; Rogers et al., Am. J. Physiol. Lung Cell Mol. Physiol. 295(2):F240-63, 2009). We used homologous recombination in fibroblasts of
outbred domestic pigs to disrupt the CFTR gene and somatic cell nuclear transfer to generate CFTR−/− pigs (see above and Rogers et al., J. Clin. Invest. 118(4):1571, 2006). At sexual maturity (6–7 months), female CFTR−/− pigs were bred to CFTR+/+ males. Six litters produced 64 piglets. Genotyping (FIG. 10A) revealed 18 CFTR+/+, 26 CFTR+−, and 20 CFTR−/− animals, a ratio not significantly different from the expected 1:2:1 (see below). FIG. 10B shows the first litter. Birth weights varied, but did not segregate by genotype (FIG. 10C). Piglets looked normal at birth, and genotype could not be discerned by appearance. A normal appearance is consistent with findings in humans.


In humans, we assessed CFTR function in vivo by measuring transepithelial voltage (VT) across nasal epithelia (Standaert et al., Pediatr. Pulmonol. 37:385, 2004) (FIG. 10E, F). Like humans with CF, baseline VT was hyperpolarized in CFTR−/− piglets. Amiloride, which inhibits ENaC Na+ channels, reduced VT in all genotypes. To test for CFTR channel activity, we perfused the apical surface with a CF−free solution and added isoproterenol to increase cellular levels of cAMP; these interventions hyperpolarized nasal VT in wild-type and heterozygous, but not CFTR−/− animals. Perfusion with ATP to activate P2Y2 receptors and Ca2+-activated Cl− channels (Standaert et al., Pediatr. Pulmonol. 37:385, 2004) further hyperpolarized VT, and the response did not differ significantly between genotypes. Perfusion with the CFTR inhibitor GlyH-101 (Muniprasat et al., J. Gen. Physiol. 124: 125, 2004) depolarized VT in controls, but not CFTR−/− piglets. These data reveal loss of CFTR CF channel activity in newborn CFTR−/− piglets. While lack of data from newborn humans precludes direct comparison, the data qualitatively match those from adults and children with CF (Standaert et al., Pediatr. Pulmonol. 37:385, 2004).


Following birth, CFTR−/− piglets failed to pass feces or gain weight (FIG. 12B). By 24–40 hours, they stopped eating, developed abdominal distention, and had bile-stained emesis. These are clinical signs of intestinal obstruction. We examined histopathology between birth and 12 hours in piglets that had not eaten and between 24 and 40 hours in pigs fed colostrum and milk replacer. Except as noted, the pathologic changes refer to the early time period. After 30–40 hours, CFTR−/− piglets had stomachs containing small amounts of green, bile-stained milk (FIG. 12C). The proximal small intestine was dilated by small amounts of milk and abundant gas. The site of obstruction ranged from mid-distal small intestine to proximal spiral colon, the anatomical equivalent of the human ascending colon. Perforation and peritonitis occurred in some piglets. Dark green meconium distended the CFTR−/− intestine and adjacent villi showed degeneration and atrophy, whereas CFTR+/+ ileum had long villi (FIG. 12D). Distal to the meconium, luminal diameter was reduced with mild to severe mucinous hyperplasia including mucoid luminal “plugs” (FIG. 12E). These changes replicate those in humans with CF (Welsh et al., in The Metabolic and Molecular Basis of Inherited Disease, C. R. Scriver et al., Eds. (McGraw-Hill, New York, 2001) pp. 5121-5189; Oppenheimer et al., Perspect. Pediatr. Pathol. 2:241, 1975).

The penetrance of meconium ileus was greater in newborn CFTR−/− piglets (100%) than in newborn humans with CF (~15%). There are several potential explanations for this difference. First, although our pigs were not inbred, they have a more uniform genetic background than humans with CF and hence may show less variability (Blackman et al., Gastroenterology 131:1030, 2006). Second, patients with two CFTR null mutations are very rare, and it is possible that a tiny amount of residual function from CFTR with common CF-associated mutations is sufficient to prevent a greater frequency of meconium ileus in humans. Third, the greater frequency in piglets might be due to anatomical or physiological differences between the species that increase susceptibility to obstruction.

CF mice also develop intestinal abnormalities, although their phenotype differs from the meconium ileus in newborn humans. Some mice die with intestinal disease a few days after birth, and others die around the time of weaning (Clark et al., Lab Anim. Sci. 46:612, 1996; Guibault et al., Am. J. Respir. Cell Mol. Biol. 36:1, 2007; Grubb et al., Physiol. Rev. 79:S193, 1999). When the intestines were examined before the onset of weight loss, it could appear similar in CFTR+/+ and CFTR−/− mice (Snoweart et al., Science 257:1083, 1992). In CF mice that developed an obstruction, the intestinal crypts were dilated, villi were atrophic and sometimes necrotic, and there was increased mucus. Thus, in humans, pigs and mice, the intestine is susceptible to loss of CFTR, although the manifestations can differ.

and mild fibrosis were typical of focal biliary cirrhosis. Gallbladder abnormalities, including gallstones, occur in 15-30% of patients, and a small gallbladder is a common autopsy finding (Welsh et al., in The Metabolic and Molecular Basis of Inherited Disease, C. R. Scriver et al., Eds. (McGraw-Hill, New York, 2001) pp. 5121-5189). Similarly, porcine CFTR-/- gallbladders were small and often filled with congealed bile and mucus (FIG. 13G, H). Epithelia showed diffuse mucinous changes with folds extending into the lumen.

Approximately 97% of males with CF are infertile (Welsh et al., in The Metabolic and Molecular Basis of Inherited Disease, C. R. Scriver et al., Eds. (McGraw-Hill, New York, 2001) pp. 5121-5189); the vas deferens is often normal at birth, and obstruction is thought to cause progressive deterioration. Although patients are commonly said to have a "congenital" absence of the vas deferens, the incidence of abnormal vas deferens is low in fetuses and young children, and mucinous obstruction is proposed to cause progressive degeneration (Oppenheimer et al., Perspect. Pediatr. Pathol. 2:241, 1975; Guillard et al., J. Urol. 158:1549, 1997; Oppenheimer et al., J. Pediatr. 75:806, 1969).

In all piglets the vas deferens appeared intact. Paraanasal sinus abnormalities occur in most children and adults with CF (Welsh et al., in The Metabolic and Molecular Basis of Inherited Disease, C. R. Scriver et al., Eds. (McGraw-Hill, New York, 2001) pp. 5121-5189). Although CFTR-/- porcine paraanasal sinuses showed no abnormalities, this negative result is difficult to interpret because it is unclear when sinus disease develops in humans. Salivary glands, nasal cavity, esophageal glands, kidney, heart, striated muscle, spleen, adrenals, eyes, brain, skin, and a few eccrine sweat glands on the snout revealed no abnormalities in CFTR-/- piglets. In all tissues, we observed no differences between wild-type and CFTR-/- animals.

Lung disease is currently the major cause of CF morbidity and mortality (Quinton, Physiol. Rev. 79:83, 1999; Welsh et al., in The Metabolic and Molecular Basis of Inherited Disease, C. R. Scriver et al., Eds. (McGraw-Hill, New York, 2001) pp. 5121-5189; Rowe et al., N. Engl. J. Med. 352:1992, 2005). The onset of clinical respiratory manifestations varies with some patients developing symptoms a few months after birth and others after several years. Eventually, most patients develop chronic airway infection and inflammation that destroy the lung. The lungs of neonatal CFTR-/- piglets appeared the same as their littermates. CFTR-/- lacked evidence of cellular inflammation in airways or parenchyma (FIG. 14A). Airway epithelia and submucosal glands appeared similar in all three genotypes, and we found no evidence of dilated or plugged submucosal gland ducts (FIG. 14B). Bronchoalveolar lavage 6-12 hours after birth showed no evidence of infection, and there were no significant differences between cell counts or levels of IL-8 across genotypes (FIGS. 15 and 16).

In neonates with CF, the lung parenchyma and airways appear histologically normal at birth. However, the submucosal glands have sometimes been reported to be abnormal in neonates with CF. A report from Oppenheimer (Hum. Pathol. 12:36, 1981) studied samples from 90 infants and young children with CF and 90 age matched controls. She concluded that there was no difference in tracheobronchial submucosal glands. Two studies have investigated this issue quantitatively. Chow et al. (Eur. J. Ped. 139:240, 1982) examined autopsy material from 21 patients who died within the first 3 weeks of life from meconium ileus. They quantitatively measured submucosal gland size and acinar diameter, and found no difference between CF and non-CF glands. Sturgess and Irri (Am. J. Pathol. 106:303, 1982) quantitatively examined...
submucosal glands from patients who had died between birth and 4 months of age. They found no difference between non-CF and CF in multiple measures of submucosal gland size and acinar diameter. However in CF, they reported an increase in the lumen fraction (lumen volume/acinar cell volume + lumen volume), an index of dilatation of acini. This increase could be attributed to patients who had lung infection. If only patients who had meconium ileus without obvious pulmonary infection were considered, then there was no difference between the groups. Thus, the evidence suggests a lack of difference between CF and non-CF lungs, including submucosal glands, at the time of birth.

The “chicken and egg” conundrum about whether CF airway inflammation occurs before infection or whether infection precedes inflammation remains a persistent question. Studies of bronchoalveolar lavage in infants and young children have both supported and argued against the presence of inflammation (increased IL-8 and neutrophilia) without infection (Khan et al., Am. J. Respir. Crit. Care Med. 151: 1075, 1995; Armstrong et al., Pediatr. Pulmonol. 40:500, 2005). In vitro airway epithelial models have also given conflicting results (Steenko et al., Inflammation 25:145, 2001; Aldallal et al., Am. J. Respir. Crit. Care Med. 166, 1248, 2002). Studies of human fetal trachea transplanted into mice suggested inflammation might occur in developing CF airways (Tirouvanziam et al., Am. J. Respir. Cell Mol. Biol. 23, 121, 2000). While our data do not resolve this controversy, we had the advantage of studying lungs between birth and 12 hours, and we found no evidence of abnormal infection or inflammation. Tracking the lungs as CFTR-/- piglets are exposed to additional environmental challenges should inform understanding of how respiratory disease develops during childhood and in young adults.

The clinical, electrophysiological, and pathological findings in newborn CFTR-/- pigs were remarkably similar to those in human neonates with CF (Table 4). Abdominal lesions dominate the initial presentation in both, with identical appearance of meconium ileus and exocrine pancreas destruction. In addition, both have hepatic changes consistent with early focal biliary cirrhosis and abnormalities of the gallbladder and bile ducts. The lack of abnormalities in vas deferens and lungs at birth is another similarity. Finding that the phenotype of newborn CFTR-/- piglets copies that of human newborns with CF suggests this model should provide investigators with new opportunities to understand the disease and develop novel prevention and treatment strategies. In addition, there are many other human diseases in which knock-out and knock-in mice fail to reproduce typical human phenotypes and fail to predict responses to therapeutics. This development of a gene-targeted, mammalian disease model, other than a mouse, also suggests strategies to circumvent research bottlenecks presented by limitations of mice.

### Table 4

<table>
<thead>
<tr>
<th>Organ</th>
<th>Tissue</th>
<th>Lesions in newborn CFTR-/- piglets</th>
<th>Detectable in human neonates with CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>Exocrine tissue</td>
<td>Reduced amounts of exocrine tissue with increased adipose and connective tissue filling the void</td>
<td>Yes</td>
</tr>
<tr>
<td>Ducts/ductules</td>
<td>PAS+ eosinophilic inspissated material and mucus filling lumen, ductular hyperplasia</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Acini</td>
<td>Eosinophilic material with rare mucus, atrophic and degenerated acinar cells; reduced zymogen granules</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>None to moderate lymphoid and eosinophilic infiltrate; scattered neutrophils and macrophages in dilated acini and ducts</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>Brunner glands dilated by inspissated mucus</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>Meconium ileus/obstruction</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Proximal to proximal colon</td>
<td>Dilated intestine filled with sticky, viscous meconium</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Distal to obstruction</td>
<td>Small intestine/colon small, stenotic; variably filled with mucus</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Complications</td>
<td>In utero perforation, chronic peritonitis, atresia</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Liver</td>
<td>Portal regions</td>
<td>Focal biliary cirrhosis; variable ductular hyperplasia, fibrosis, and inflammation</td>
<td>Yes</td>
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<tr>
<td>Biliary ducts</td>
<td>Eosinophilic material/mucus in lumen; scattered neutrophils</td>
<td>Yes, rare</td>
<td></td>
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<tr>
<td>Gallbladder</td>
<td>Small and variably filled with inspissated bile and mucus</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Cystic duct</td>
<td>Obstructed by mucus and eosinophilic material</td>
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<tr>
<td>Vas deferens</td>
<td>Duct</td>
<td>None</td>
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TABLE 4—continued

<table>
<thead>
<tr>
<th>Organ</th>
<th>Tissue</th>
<th>Lesions in newborn CFTR−/− piglets</th>
<th>Detectable in human necroaetes with CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Parenchyma</td>
<td>None; no inflammation or infection</td>
<td>No</td>
</tr>
<tr>
<td>Airways</td>
<td>None; no inflammation or infection</td>
<td>No</td>
<td></td>
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</table>


Materials and Methods

Animals

We previously reported production of CFTR−/− male and female pigs (Rogers et al., J. Clin. Invest. 118(4):1571, 2008). For this study, they were crossed and the progeny studied. Standard procedures for animal husbandry were used throughout. The Institutional Animal Care and Use Committees of the University of Iowa and the University of Missouri approved all animal experiments. We studied two groups of animals. One group was studied between birth and 12 hours and were not fed. The other group was fed colostrum and milk replacer and studied between 24 and 40 hours.

Genotyping

Genotyping was performed using genomic DNA isolated from fresh umbilical cord. Tissue was lysed for 15 min at 55°C in lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-CI, pH 8.5, 0.5% Nonidet P-40, 0.5% Tween, 400 μg/ml Proteinase K) (McCreadh et al., Nature 405:1066, 2000). Two μl of lysate were used in each PCR reaction (Platinum Taq HF, Invitrogen). PCR conditions: 2 minutes at 95°C, 30 cycles of 95°C for 20 seconds, 56°C for 20 seconds, and 68°C for 4 minutes, then 68°C for 5 minutes. Primers Ex10α5F (AGAAATTCATCTGCTTCAGT; SEQ ID NO:35) and GC7R (GAAGACCTTACCTCCCTCAG; SEQ ID NO:36) amplify a 2.0 kb product from wild-type CFTR alleles and a 3.7 kb product from gene-targeted CFTR alleles. PCR products were electrophoresed on a 0.7% agarose gel. For Southern blotting, 10 μg of genomic DNA was digested with BglII overnight. Genomic digests were electrophoresed on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Roche) using an alkaline transfer procedure. Blots were pre-hybridized for 15 min at 65°C in Rapidhyb buffer (Amersham). The blot was then hybridized in Rapid-hyb buffer with a 32P-labeled probe specific for a region of CFTR that is outside of the targeting vector boundaries. The blot was washed once in 2xSSC/0.1% SDS for 20 minutes at room temperature followed by two washes in 0.1xSSC/0.1% SDS for 15 minutes at 65°C. Signal was detected using a phosphorimager. The CFTR probe hybridizes at 7.9 kb for wild-type CFTR alleles and 9.7 kb for gene-targeted CFTR alleles.

Northern Blot

Northern blot was performed using total RNA isolated from ileum (RNasy, Qiagen). Total RNA was electrophoresed on a denaturing gel and transferred to a positively-charged membrane (NorthernMax, Ambion). The membrane was hybridized with 32P-labelled DNA probes corresponding to nucleotides 1-1000 of the porcine CFTR cDNA or 1-1002 of porcine GAPDH cDNA. Signal was detected using a Fuji FLA7000 phosphorimager. Porcine CFTR mRNA was detected at ~6.5 kb and GAPDH mRNA at ~1.5 kb.

Quantitative RT-PCR

Quantitative RT-PCR using TaqMan chemistry and an ABI 7500 Fast Real-time PCR System was used to measure porcine CFTR mRNA. Briefly, total RNA was isolated from ileum (RNeasy, Qiagen). First-strand cDNA was synthesized with oligo-dT primers (SuperScript II, Invitrogen). Sequence-specific primers and probes for porcine CFTR and GAPDH were designed and ordered using Assay-by-design (Applied Biosystems). For measuring CFTR mRNA, primer/probe sets annealing to exon 10 of CFTR and GAPDH were used in separate reactions (CFTR: Forward: TCATGC-CGGGCACCTATAAA; Reverse: CGTTTGTAGACACTCCTGTACTA; SEQ ID NO:37, Probe: FAM-ACACCAAAGATGATGTTCCT; SEQ ID NO:39; GAPDH, Forward: AAGCTATTCTCCTGATCAGAAT; SEQ ID NO:40, Reverse: GGAGGCCTATGGAACCAT; SEQ ID NO:41, Probe: FAM-TCCACACCCCTGTGTG; SEQ ID NO:42). TaqMan Fast Universal PCR Master Mix was used for all reactions. The reaction volume was 20 μl (10 μl of 2× Master Mix without UNG, 1 μl of 20× target primer and probe, 8 μl of Nuclease-free water, and 1 μl of cDNA sample). The reaction plates were covered with optical film and centrifuged briefly. The thermocycler conditions were as follows: 20 seconds at 95°C, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All experiments were run in triplicate. Because the efficiencies of CFTR and GAPDH amplification were not equal, the relative quantification of transcript levels was performed using the standard curve method.

Immunoprecipitation and Phosphorylation

We used lysis buffer with 1% NP-40 and centrifugation as previously described (Ostgaard et al., Proc. Natl. Acad. Sci. U.S.A. 104:15370, 2007) to prepare soluble protein from cultured airway epithelial cells. Equal amounts (750 μg) of soluble protein were immunoprecipitated with M3A7 antibody (Upstate Technology) and then in vitro phosphorylated as described previously (Ostgaard et al., Proc. Natl. Acad. Sci. U.S.A. 104:15370, 2007). Immunoprecipitated, phosphorylated proteins were electrophoresed on 6% PAGE, the gels dried and then exposed to phosphor screen before scanning on Fuji FLA7000 (Fuji Corp.). BHK cells stably expressing wild-type CFTR (a gift from Dr. Gergely Lukacs) were used as controls (7.5 μg) and were treated similarly.

Histopathology

Tissues were collected at necropsy and immediately placed in a bath of 10% neutral buffered formalin and gently agitated for a minimum of 72 hours. Tissues were then processed, paraffin-embedded, sectioned (4–5 μm) and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Histopathological examination was performed by a veterinary pathologist blinded to pig genotype. This allowed for unbiased pathological assessment of tissues ranging from mild/moderate multifocal lesions (e.g., liver parenchyma) to severe lesions with 100% penetrance (e.g., pancreas).
Measurement of Nasal \( V_t \)

The transepithelial voltage (\( V_t \)) across the nasal epithelium was measured using previously described methods (Zabner et al., J. Clin. Invest. 97:1504, 1996; Standaert et al., Pediatr. Pulmonol. 37:385, 2004) under propofol anesthesia with the animal spontaneously breathing. The reference electrode was a small Ringer’s solution-filled catheter inserted into the leg muscle (25 g needle). The exploring electrode was a 6 French foley catheter filled with Ringer’s solution (148 mM NaCl, 2.4 mM KH\(_2\)PO\(_4\), 0.4 mM K\(_2\)PO\(_4\), 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\)) inserted 4 cm into the nasal cavity, and the nasal mucosa was perfused at a rate of 5 ml/min using Ringer’s solution to obtain baseline \( V_t \). Subsequent perfusion solutions included: Ringer’s solution containing 100 \( \mu \)M amiloride; CT-free Ringer’s solution containing substituted for CF plus amiloride; CT-free Ringer’s solution containing amiloride plus 10 \( \mu \)M isoproterenol; CT-free Ringer’s solution containing amiloride, isoproterenol, plus 100 \( \mu \)M ATP; CT-free Ringer’s solution containing amiloride, isoproterenol, ATP, plus 100 \( \mu \)M GlyH-101. Voltage was measured with a voltmeter connected to a strip chart recorder. Each solution was perfused for 3-5 minutes. Following completion of the measurements, the epithelium was disrupted by brushing and \( V_t \) measured again to determine the zero value of \( V_t \).

Immunocytochemistry

Heal tracheal tissues were excised from newborn piglets, immediately placed in ice-cold 30% sucrose, and quick frozen in OCT. Tissues were cryosectioned at 7 \( \mu \)m thick onto polylysine-coated microscope slides, fixed in 100% MeOH at \( -20^\circ \)C for 15 min, permeabilized in 0.2% TX-100 (Thermo Scientific) in PBS, and blocked in Super-Block (Thermo Scientific) with 5% normal goat serum (Jackson Immunoresearch). Tissue sections were incubated overnight at 4\(^\circ\)C in anti-CFTR antibodies MM13-4, M3A7 (Chemicon), and 24-1 (R&D Systems) and polyclonal antibody to ZO-1 (Zymed) (all at 1:100 dilution), followed by secondary antibodies (goat-anti-mouse A488 and goat-anti-rabbit A568 (Molecular Probes) (1:1000 dilution). Sections were mounted with Vectashield containing DAPI (Vector Labs) to visualize nuclei. Images were acquired on an Olympus Fluoview FV 1000 confocal microscope with a UPLSAPO 60x oil lens, 1.35 NA. Images were scanned sequentially at 2 \( \mu \)s/ pixel. Images were processed identically using Fluoview FV10-ASW 1.6 confocal software. RGB images were converted to white images for green and red channels and overlayed on a single Z-section of DIC with DAPI for merged image.

Bronchialveolar Lavage (BAL) Fluid Collection and Analysis

Animals were studied between 6 and 12 hours after euthanasia. BAL was performed immediately following euthanasia. We instilled 5 ml of normal saline through an intratracheal catheter three times. The total number of recovered cells was quantified with a hemacytometer and morphologic differentiation of cells was performed on cytospin preparations that were stained with Diff-Quick Stain kit (Baxter). BAL levels of IL-8 were determined on recovered supernatant after centrifugation (1600g for 10 min). IL-8 levels were measured by antibody capture assay (Thermo Scientific). Standard quantitative microbiologic techniques were used to identify and quantify the bacteria in BAL.

Statistical Analysis

Data are presented as either mean±standard error of the mean (SEM) or individual data points. Statistically significant differences between genotype groups were determined using either the Student’s t-test or one-way ANOVA and the Student-Newman-Keuls test to determine group differences. Differences were considered statistically significant for \( P<0.05 \).

The AF508 Mutation—an Interspecies Analysis

The AF508 mutation confers at least three defects on human CFTR: it reduces channel activity, it impairs processing, and it reduces the protein’s stability at the cell surface. The AF508 mutation inhibits gating of CFTR channels from three species (i.e., mouse, pig, and human) studied by the same mechanism, a reduced opening rate. In contrast, the characteristic processing defect observed in human CFTR-AF508 is less severe in pig and mouse proteins. This conclusion is supported by our finding that the mouse and pig proteins showed complex glycosylation, were readily excised for patch-clamp experiments at 37\(^\circ\)C, immunocytochemistry localized some of the protein to the apical membrane of airway epithelia, and corrected the CF transport defect in CF airway epithelia more than did human CFTR-AF508. This shows that there is a gradient in the severity of the AF508 processing defect, with human worse than pig, and pig somewhat worse than mouse. Additionally, this also shows that the processing defect and the functional defect in CFTR-AF508 arise from different causes.

Vectors and Expression

Human, pig, and mouse CFTR cDNA (SEQ ID Nos: 1, 3, and 5) were amplified from Homo sapiens, domestic pig (Sus scrofa), and domestic mouse (mus musculus). We cloned all three CFTR cDNAs into pcDNA3.1 (Invitrogen) and recombinant adenoviruses. For recombinant adenovirus of mouse CFTR, we had to remove intron 11 which had been inserted previously to stabilize the vector.

For protein expression studies, COS7 cells were electroporated; 3T3 and LLC-PK1 cells were transfected with plasmid and Lipofectamine 2000. For deglycosylation studies, COS7 cells were electroporated with human CFTR or infected with adenovirus encoding pig or mouse CFTR. For patch-clamp studies, HEK293 cells were infected with adenovirus encoding mouse CFTR or transfected using a hybrid vaccinia virus system encoding pig CFTR. Expression in human and mouse airway epithelium was with recombinant adenoviruses. Murine tracheal epithelia were cultured from AF508/AF508 transgenic mice (CFTR\(^{AF508/AF508}\)) or CFTR null mice expressing the intestinal FABP-CFTR (CFTR\(^{AF508/AF508;FABP-CFTR}\)). In the absence of gene transfer, there were no cAMP-stimulated CFTR currents in either mouse genotype.

Biochemical Studies

COS7 cells were solubilized in lysis buffer with 1% TX-100 and protease inhibitors. CFTR was immunoprecipitated with M3A7 antibody (Upstate Technology) and then in vitro phosphorylated as described previously. Note that the consensus phosphorylation sites and N-glycosylation sites are conserved in all three species (FIG. 17). Processing studies in NIH-3T3 and LLC-PK1 cells were carried out similarly to those in COS7 cells. For deglycosylation studies, membranes were isolated from COS7 cells and solubilized in LB plus 1% NP40 (Pierce). Supernatant were divided, immunoprecipitated and resuspended with or without endoglycosidase H. Following incubation, precipitates were in vitro phosphorylated.
Immunochemistry

Three days following gene transfer, epithelia were fixed, permeabilized, and incubated with a mixture of anti-CFTR antibodies (M3A7, MM13-4 (Upstate Biotechnology) and 13-1 (R&D Systems) and anti-ZO-1 (Zymed) primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and examined by confocal laser scanning microscopy.

Electrophysiology

For Ussing chamber studies, transepithelial current was measured 3 days following gene transfer using a Cl- concentration gradient as previously described. For patch-clamp studies CFTR currents were studied in excised, inside-out membrane patches of HeLa cells as previously described. Channels were activated with the catalytic subunit of PKA and Mg-ATP; PKA was present in all cytosolic solutions that contained ATP. Holding voltage was -50 to -100 mV. Experiments were performed at 23-26°C. Data acquisition, processing, and analysis were performed as previously described. Data are mean±SEM unless otherwise stated. P<0.05 was considered statistically significant.

Sequence of Pig CFTR

We cloned the pig CFTR cDNA and used it to predict the amino acid sequence (Fig. 17). The pig CFTR amino acid sequence is nearly 93% identical to that of human CFTR. For comparison, mouse CFTR shows 78% identity to human CFTR. The region immediately surrounding F508 is highly conserved.

Glycosylation of Human, Pig, and Mouse CFTR-

The pattern of human CFTR glycosylation changes as the protein migrates from the ER to the Golgi complex. The nascent protein lacking glycosylation is called “band A.” In the ER, CFTR undergoes core glycosylation and migrates more slowly during electrophoresis as “band B.” In the Golgi complex, more extensive glycosylation occurs, which further slows and broadens the electrophoretic migration of the “band C” form. Differences in glycosylation do not appear to affect function, but do provide a convenient way to assess the biosynthetic processing of CFTR. When we expressed wild-type human, pig, and mouse CFTR in the monkey kidney cell line COS7, we observed the typical appearance of bands B and C (Fig. 18A). Human CFTR-ΔF508 produced band B, but not band C, consistent with defective exit from the ER. This result agrees with many previous reports in several different cell lines. Surprisingly, in addition to band B, mouse CFTR-ΔF508 generated a significant proportion of band C protein. Pig CFTR-ΔF508 also produced a small amount of band C. These results suggested that some mouse and pig mutant protein may have trafficked to the Golgi complex.

To learn whether the differences between the three species of CFTR-ΔF508 depended on the primate COS7 cell line, we expressed the constructs in the mouse NIH-3T3 fibroblast line and the pig LLC-PK1 kidney cell line (Figs. 18B and C), as well as human HEK-293T cells (not shown). In each of these cell lines, human CFTR-ΔF508 generated only the band B form, whereas pig and mouse CFTR-ΔF508 produced both band B and some fully glycosylated protein, consistent with our studies in COS7 cells. We also noted that some of the wild-type and ΔF508 pig CFTR migrated slightly more rapidly than band B of either human or mouse.

To confirm that the high molecular mass C forms of pig and mouse CFTR-ΔF508 were due to complex glycosylation, we used endoglycosidase H digestion. Endoglycosidase H removes carbohydrate from proteins that contain only the sugar added in the ER, but it does not delete complex glycosylation added in the Golgi complex. Endoglycosidase H treatment shifted the migration of the band B form of all the proteins to the unglycosylated form (Fig. 19). However, like the band C form of the wild-type CFTRs, the fully glycosylated mouse and pig CFTR-ΔF508 were resistant to endoglycosidase H, confirming that these proteins were glycosylated in the Golgi complex.

Expression of Human, Pig, and Mouse CFTR-ΔF508 at the Cell Surface

To determine if the human, pig and mouse CFTR-ΔF508 were localized at the apical membrane of airway, we expressed the proteins in well-differentiated human CF airway epithelia and examined them with confocal immunoassay. Consistent with earlier studies, wild-type human CFTR localized at the apical membrane and human CFTR-ΔF508 appeared to be expressed differently throughout the cell (Fig. 20). As expected from the biochemical studies, both pig and mouse wild-type were localized to the apical membrane. However, in contrast to human CFTR-ΔF508, both the pig and mouse mutants were also present in the apical portion of the airway cells.

Single Channel Gating of Human, Pig, and Mouse CFTR-

Most, although not all studies indicate that human CFTR-ΔF508 manifests a channel gating defect that reduces activity. To learn whether the ΔF508 mutation compromises the channel activity in pig and mouse CFTR, we studied excised, inside-out patches of membrane containing CFTR channels. We readily detected channels in patches taken from cells expressing pig and mouse CFTR-ΔF508 grown at 37°C, consistent with the conclusion that pig and mouse CFTR-ΔF508 are able to reach the cell membrane under physiological conditions. This contrasts with human CFTR-ΔF508, which must be incubated at lowered temperatures to produce significant amounts of cell surface protein. Phosphorylation by the catalytic domain of cAMP-dependent protein kinase (PKA) and cytosolic ATP were required for activity of all versions studied. The single channel conductances for the wild-type channels were human (8.3 pS>pig (6.7 pS)>mouse (4.3 pS), and they were not significantly altered by the ΔF508 mutation (Figs. 21A and 21B). Lansell et al. (J. Physiol. 508:379-392, 1998) reported that heavily filtering currents recorded from mouse CFTR revealed a subconduction state that was ~10% of the amplitude of the full conductance. With heavy filtering, we also observed the subconduction in both wild-type and ΔF508 channels (Fig. 21A).

In the presence of PKA and 1 mM ATP, the open state probability (Po) of wild-type CFTR varied in the order of pig (0.39)>human (0.37)>mouse (0.08) (Fig. 21B); the values for human were taken from our earlier study. In assessing mouse Po, we did not take into account the subconduction state; as reported by Lansell et al., it was very difficult to study due to its small single-channel conductance. The ΔF508 mutation reduced the Po of human CFTR to 27%, pig CFTR to 46%, and mouse CFTR to 50% of the corresponding wild-type channel (Figs. 21A and 21B). The cause of the reduced Po was a decrease in the rate of channel opening without a significant alteration of burst duration (Fig. 21B). Thus, in all three species, the ΔF508 mutation altered gating by a similar mechanism.

Transepithelial Cl- Current Generated by Human, Mouse, and Pig CFTR-ΔF508

Because both pig and mouse CFTR-ΔF508 were partially processed through the Golgi complex and likely targeted to
the apical membrane, and because they both retained partial CF channel activity, we predicted they would generate trans-
sepithelial Cl⁻ current when expressed in well-differentiated CF airway epithelia. To assay transsepithelial CF₆ transport, we mounted epithelia in modified Ussing chambers and measured
transsepithelial CF₆ current. We first inhibited Na⁺ current with amiloride, which hyperpolarizes the apical mem-
brane voltage, increasing the driving force for secretion through CFTR. Then, we increased CFTR activity by elevat-
ing cellular levels of cAMP with forskolin and IBMX. Finally, we reduced transsepithelial CF₆ current by inhibiting the Na⁺—K⁺—Cl⁻ cotransporter with basolateral bumet-
amide; the resulting change in current provides a good mea-
sure of the CF₆ current. We chose bumetamide rather than CFTR inhibitors, because they can have different efficacy on CFTR from different species.

Expressing wild-type human CFTR produced significant transsepithelial Cl⁻ current (FIGS. 22 and 23), as previously described. The same was true for wild-type pig and mouse CFTR. As expected, human CFTR-ΔF508 failed to generate much current. However, relative to the wild-type current of each species, both pig and mouse CFTR-ΔF508 produced substantial transsepithelial Cl⁻ currents (FIG. 23B). To deter-
mine if these results were limited to expression in human epithelia, we repeated the study using airway epithelia
derived from CF mice; the results were qualitatively similar (FIGS. 23A and 23B). Thus, pig and mouse CFTR-ΔF508
generated transsepithelial Cl⁻ currents in CF airway epithelia from two different species. These results indicate that some pig and mouse CFTR-ΔF508 was present and active in the apical membrane of airway epithelia.

IV. A Transgenic, Non-Human Animal Model of Cystic Fibrosis Using Transgenic Human CFTR-ΔF508

The above-mentioned defects between mutant mouse, pig, and human CFTR illustrate the need for the generation of a transgenic non-human animal model of CF that contains a CFTR-ΔF508 mutation that closely mimics the functional and processing characteristics of the human CFTR-ΔF508.

The generation of a large animal, such as a pig, in which the endogenous CFTR gene is knocked-out and a human CFTR-ΔF508 transgene is introduced allows for the study of the causative factor of human CF without suffering from experimental artifacts introduced by the different physical and func-
tional characteristics of the endogenous CFTR of the animal. Such models also facilitate the identification, characteriza-
tion, and development of approaches (e.g., small molecule-

Based on the above, the most common CF-associated mutation is ΔF508, which deletes a phenylalanine in position 508. In vitro studies indicate that the resultant protein, CFTR-ΔF508, is misprocessed. To better understand the effects of the ΔF508 mutation in vivo, we produced CFTRΔF508/ΔF508 pigs. Our biochemical, immunocytochemical, and electrophysiological data on CFTR-ΔF508 in newborn pigs indicated that CFTRΔF508/ΔF508 airway epithe-
ia retain a small residual CFTR conductance, with maximal stimulation producing 6% of wild-type function. cAMP ago-
nists were less potent at stimulating current in CFTRΔF508/ΔF508 epithelia, suggesting that quantitative tests of maximal anion current may over-

CFTRΔF508/ΔF508 pigs developed lung disease strikingly similar to human CF. These results show that this limited CFTR activity is insufficient to prevent lung or gastrointestinal disease in CF pigs.

Results

CFTRΔF508/ΔF508 Pigs were Generated

We previously generated male CFTRΔF508/ΔF508 pigs using somatic cell nuclear transfer and embryo transfer (Rogers et al., J. Clin. Invest. 118 (4):1571-1577, 2008; also see Example 1, above). The nucleotide sequence...
did not differ statistically (chi-squared test) from the predicted Mendelian ratio of 1:2:1. Newborn CFTR<sup>M508A/Δ508</sup> pigs have pathology like that of CFTR<sup>+/+</sup> pigs.

Like CFTR<sup>−/−</sup> and CFTR<sup>Δ508/Δ508</sup> piglets (Meyerholz et al., Am. J. Pathol. 176(3):1377-1389, 2010; Stoltz et al., Science Translational Medicine 2(29):29na31, 2010; also see above), CFTR<sup>Δ508/Δ508</sup> pigs had meconium ileus with 100% penetrance. The site of obstruction varied, but was generally near the ileocecal junction (FIG. 24A). Distal to the obstruction, the intestine was of small caliber and variably filled with mucocellular debris (FIG. 24B). We did not discern differences between the intestinal pathology or meconium ileus of CFTR<sup>M508A/Δ508</sup> and CFTR<sup>+/+</sup> pigs (Rogers et al., Science 321 (5897):1837-1841, 2008; Meyerholz et al., Am. J. Pathol. 176(3):1377-1389, 2010).

In the pancreas, lobular parenchyma was decreased in CFTR<sup>M508A/Δ508</sup> pigs (FIG. 24C). Pancreatic acini and ducts were often dilated by zymogen concretions with scattered neutrophils, macrophages, and mucus, like that found in newborn CFTR<sup>−/−</sup> pigs (Meyerholz et al., Am. J. Pathol. 176(3):1377-1389, 2010). CFTR<sup>Δ508/Δ508</sup> pancreata had reduced parenchyma compared to CFTR<sup>+/+</sup>, but the destruction was slightly less severe than in CFTR<sup>−/−</sup> (FIG. 24D).

The CFTR<sup>M508A/Δ508</sup> liver had focal portal areas expanded by bile duct proliferation, inflammation, and/or increased connective tissue, changes characteristic of early focal biliary cirrhosis (FIG. 24E). The frequency and severity of changes were similar to those we observed in CFTR<sup>−/−</sup> pigs (Meyerholz et al., Am. J. Pathol. 176(3):1377-1389, 2010). Likewise, the microgallbladder and mucinous changes in gallbladder epithelium observed in CFTR<sup>−/−</sup> were ubiquitous in CFTR<sup>M508A/Δ508</sup> animals (FIG. 24F).

Airway epithelia of newborn CFTR<sup>M508A/Δ508</sup> pigs were normal in appearance and lacked evidence of mucus accumulation. Like CFTR<sup>−/−</sup> piglets, on histopathological examination CFTR<sup>M508A/Δ508</sup> Airways lacked inflammatory cells (FIG. 24G) (Stoltz et al., Science Translational Medicine 2(29):29na31, 2010). The alveolar and airway epithelia were indistinguishable in CFTR<sup>+/+</sup>, CFTR<sup>Δ508/Δ508</sup>, and CFTR<sup>−/−</sup> pigs. Analysis of bronchoalveolar lavage of newborn pigs revealed no statistically significant differences in total cell counts, differential cell counts, or IL-8 concentrations between CFTR<sup>+/+</sup> and CFTR<sup>M508A/Δ508</sup> pigs (FIG. 24H-J).

Tracheal abnormalities occur in humans with CF as well as in CFTR<sup>−/−</sup> mice and mice (Bonvi et al., J. Physiol. 586(13): 3231-3245, 2008; Meyerholz et al., Am. J. Respir. Crit. Care Med. 182:1251-1261, 2010). Compared to wild-type trachea, CFTR<sup>M508A/Δ508</sup> trachea had an altered lumen area, circumference, submucosal gland area, and smooth muscle area (FIG. 25A-E). However, changes in smooth muscle area were not as severe as those in CFTR<sup>−/−</sup> pigs.

Thus, newborn CFTR<sup>M508A/Δ508</sup> pigs are remarkably similar to their CFTR<sup>−/−</sup> counterparts with the exception of slightly less severe abnormalities in pancreas and tracheal smooth muscle.

CFTR<sup>M508A/Δ508</sup> Lungs Develop Disease with Time

Meconium ileus would prevent survival of all CFTR<sup>M508A/Δ508</sup> pigs. Therefore, to learn whether disease in CFTR<sup>M508A/Δ508</sup> pigs would progress after birth, surgical intervention was used to bypass the intestinal obstruction. Therefore, we placed a ileostomy or cecostomy in the pigs within 15 hours after birth. The procedures and treatments were the same as previously described for CFTR<sup>−/−</sup> pigs (Stoltz et al., Science Translational Medicine 2(29):29na31, 2010).

We examined the histopathology of four CFTR<sup>M508A/Δ508</sup> pigs ranging in age from 13 to 87 days at time of euthanasia (Table 5). Over time, CFTR<sup>M508A/Δ508</sup> pigs lost pancreatic parenchyma, which was replaced with fatty and fibrous tissue (FIG. 26A). In the liver, changes varied from minimal to diffuse stenosis (FIG. 26B, C). One animal (Case 2) had portal areas with focal to bridging fibrosis, duct proliferation, and inflammation; these changes are typical of progressive focal biliary cirrhosis (Meyerholz et al., Am. J. Pathol. 176 (3):1377-1389, 2010) (FIG. 26D).

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Genotype</th>
<th>Type of Surgery</th>
<th>Age for Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>AF508/AF508</td>
<td>loop ileostomy</td>
<td>13 d. prolapsed oesomy</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>AF508/AF508</td>
<td>cecostomy</td>
<td>62 d. gastric ulcer*</td>
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<tr>
<td>3</td>
<td>F</td>
<td>AF508/AF508</td>
<td>cecostomy</td>
<td>77 d. poor oral intake, weight loss**</td>
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<tr>
<td>4</td>
<td>F</td>
<td>AF508/AF508</td>
<td>cecostomy</td>
<td>87 d. prolapsed oesomy</td>
</tr>
</tbody>
</table>

*Gastric ulcer has previously been reported to occur in both non-CF and CF pigs (Stoltz et al., Science Translational Medicine 2(29):29na31, 2010)
**Edema of poor oral intake and weight loss was not identified. Pig was hypocholesteremic prior to euthanasia and on post-mortem examination the stomach and proximal small intestine had a large quantity of luminal material suggestive of an ileus. Clinically, the animal appeared septic, but bacterial cultures were negative.

Like CFTR<sup>−/−</sup> and CFTR<sup>Δ508/Δ508</sup> piglets (Stoltz et al., Science Translational Medicine 2(29):29na31, 2010), all the CFTR<sup>M508A/Δ508</sup> pigs showed changes consistent with CF lung disease. Disease severity varied from animal to animal, and changes within lungs of individual pigs were heterogeneous such that some areas of lung showed no abnormality. As early as two weeks of age (Case 1), CFTR<sup>M508A/Δ508</sup> lung showed mucopurulent material obstructing some airways with areas of adjacent atelectasis (FIG. 26C-H). In cases 2 and 4 (62 and 87 days old), lung changes included scattered mucopurulent debris in airway lumens with chronic purulent to lymphoid airway wall inflammation (FIG. 26I-L). The surface epithelium showed areas of goblet cell hyperplasia, and mucocellular material was detected in some submucosal glands. In case 3 (77 days old), the lungs showed a range of severity from normal mucinous changes to lobular atelectasis consistent with airway obstruction (FIG. 26M).

At the time of necropsy, lung samples were aseptically removed for bacterial culture from three of the four animals. Bacteria were present in the cultures, but in relatively low numbers ranging from 10-1650 cfu/g lung tissue (Table 6). As in CFTR<sup>−/−</sup> pigs, a variety of bacterial species were isolated. This result suggests a host-defense defect for many bacterial species and is consistent with data from humans with early CF lung disease (Stoltz et al., Science Translational Medicine 2(29):29na31, 2010). In contrast, no bacteria were isolated from lungs of three of the four control pigs, and in the fourth only 10 cfu/g were cultured. In addition, CFTR<sup>M508A/Δ508</sup> pigs, but not CFTR<sup>+/+</sup> pigs, received some systemic antibiotics (see Materials and Methods; below), which may have suppressed bacterial recovery and minimized differences between the two groups.

These results show that CFTR<sup>M508A/Δ508</sup> pigs spontaneously develop lung disease that resembles that in CFTR<sup>−/−</sup> pigs and humans homozygous for the AF508 mutation.
TABLE 6

Microbiology of CFTR<sup>ΔF508/AF508</sup> Mice

<table>
<thead>
<tr>
<th>Case #</th>
<th>(avg CFU/g) (range)</th>
<th>Cultured species</th>
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</thead>
<tbody>
<tr>
<td>AFS08/AF508</td>
<td>1</td>
<td>-</td>
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<tr>
<td></td>
<td>2</td>
<td>10</td>
</tr>
<tr>
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<td>4</td>
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* samples were not collected for microbiology culture.

** n = 1.

CFTR<sup>ΔF508/AF508</sup> Mice Produce CFTR<sup>ΔF508</sup> mRNA

Because the phenotype of CFTR<sup>ΔF508/AF508</sup> mice was like that of CFTR<sup>ΔF508</sup> mice, we asked whether newborn CFTR<sup>ΔF508/AF508</sup> mice produced CFTR transcripts. We assessed expression from the AF508 allele using quantitative RT-PCR. Amounts of wild-type CFTR mRNA decreased from proximal to distal intestine and were lower in cultured airway epithelia than in intestine (Fig. 27A). In CFTR<sup>ΔF508/AF508</sup> mouse intestine, CFTR transcripts followed a similar axial pattern and did not statistically differ from those in CFTR<sup>ΔF508</sup> mice. Northern blots of CFTR<sup>ΔF508/AF508</sup> duodenal RNA were consistent with the RT-PCR data (Fig. 27B). In cultured airway epithelia, CFTR<sup>ΔF508</sup> and CFTR<sup>ΔF508/AF508</sup> had the same abundance of CFTR transcripts. These data suggest that the Neo<sup>6</sup> cassette in intron 10 has relatively minor effects on transcription from the CFTR<sup>ΔF508</sup> allele. These results are also consistent with our estimate that CFTR<sup>ΔF508</sup> mRNA was present at ~70% of the wild-type amount (Rogers et al., J. Clin. Invest. 118(4):1571-1577, 2008). The Amount of CFTR<sup>ΔF508</sup> is Reduced Compared to Wild-Type CFTR


In proximal small intestine from wild-type mice, we detected band C and very little band B CFTR (Fig. 27C). This result suggests that most wild-type protein was processed to the mature form, consistent with maturation of endogenous wild-type human CFTR to band C (Varga et al., J. Biol. Chem. 279(21):22578-22584, 2004). Thus, presence of band B in vitro may be due to overexpression of recombinant protein. The amount of CFTR recovered from proximal CFTR<sup>ΔF508/AF508</sup> intestine was markedly reduced compared to CFTR<sup>ΔF508</sup> intestine, and we had to increase both the amount of protein studied and enhance the exposure to detect the mutant protein (Fig. 27C, lanes 2-7). Distal small intestine yielded similar results (Fig. 27C, lanes 9-12). In both cases, CFTR<sup>AF508</sup> was present in the mature band C and immature band B forms.

Because the intestine is affected by meconium ileus, we also assessed airway epithelia, which do not show secondary changes from the disease at birth. The data paralleled results from the intestine. First, we detected little band B in either excised trachea (Fig. 27D, lanes 2-5) or differentiated cultures (lanes 8-10) of wild-type nasal epithelia; the preponderance of CFTR was in band C. Migration of band C protein was slightly slower than recombinant wild-type CFTR, suggesting some differences in glycosylation of CFTR in vivo compared to recombinant CFTR. Second, we detected both band B and band C forms of CFTR<sup>AF508</sup>. Third, the amount of CFTR<sup>AF508</sup> protein was decreased compared to wild-type CFTR, although the reduction was less marked in cultured than excised epithelia.

These results agree with our in vitro studies of recombinant porcine wild-type and AF508 CFTR (Ostergaard et al., Proc. Natl. Acad. Sci. U.S.A. 104(39):15370-15375, 2007). They suggest that porcine CFTR<sup>AF508</sup> has a biosynthetic defect. However, they also indicate that a fraction of the mutant protein is processed to the mature form.

Immunostaining Reveals a Reduced Amount of CFTR<sup>ΔF508</sup>

We used immunocytochemistry as an additional way to evaluate CFTR<sup>ΔF508</sup>. In small intestine, we detected wild-type CFTR in the apical membrane of crypt, but not villus cells (Fig. 28A). In CFTR<sup>ΔF508/AF508</sup> intestine, we detected some immunostaining throughout the small intestine and the spiral colon. However, the signal was very weak and not uniformly detectable; the third panel of Fig. 28A shows an example in which we have electronically enhanced the CFTR (green) fluorescence so that the staining could be appreciated. CFTR<sup>ΔF508</sup> intestine had no immunostaining.

In excised trachea and differentiated primary cultures of nasal epithelia, wild-type CFTR localized almost exclusively at the apical membrane (Fig. 28B, C). In CFTR<sup>ΔF508</sup>
tracheal tissue and cultures, immunostaining was barely detectable. In Fig. 28B, we show a rare example from CFTR<sup>A508</sup>/Af508 trachea where we detected CFTR and found it localized similarly to that in CFTR<sup></sup>- trachea. For cultured CFTR<sup>A508</sup>/Af508 tracheal epithelia, we electronically amplified the signal post-collection to detect CFTR immunostaining that differed from that in wild-type cultured epithelia; staining extended from the apical membrane into the cytoplasm (Fig. 28C). We detected no CFTR immunostaining in excised or cultured CFTR<sup>-/-</sup> tracheal epithelia.

The marked decrease in immunostaining in CFTR<sup>A508</sup>/Af508 intestinal crypts and airway epithelia is consistent with the greatly reduced amounts of CFTR recovered from these tissues. These data suggested that ion transport by CFTR<sup>A508</sup>/Af508 epithelia may be abnormal.

CFTR<sup>A508</sup>/Af508 Airway Epithelia Show Reduced but not Absent CFT<sup>+</sup> Transport

We designed electrolyte transport studies to answer two questions. First, does transepithelial ion transport in newborn CFTR<sup>A508</sup>/Af508 airway epithelia differ from that in wild-type epithelia? Second, is CFT transport in CFTR<sup>A508</sup>/Af508 airway epithelia greater than that in CFTR<sup>-/-</sup> epithelia? We studied airway epithelia so that we could compare data to results from CFTR<sup>-/-</sup> pigs (Chen et al., Cell 143:911-923, 2010). We examined both nasal epithelia, which are often used to evaluate CF ion transport, and tracheal/bronchial epithelia because of their potential contribution to disease. We studied excised tissues as well as primary cultures of differentiated airway epithelia. We show data for excised tracheal epithelia in Fig. 29-32.

Baseline V<sub>t</sub> and I<sub>sc</sub> did not differ between excised trachea from CFTR<sup>A508</sup>/Af508 and CFTR<sup>-/-</sup> pigs (Fig. 29). An inhibitor of epithelial Na<sup>+</sup> channels (100 µM apical amiloride) reduced V<sub>t</sub> (ΔV<sub>t</sub><sub>amil</sub>) and I<sub>sc</sub> (ΔI<sub>sc</sub><sub>amil</sub>) in CFTR<sup>A508</sup>/Af508 more than in wild-type epithelia. Our earlier work indicates that the greater ΔV<sub>t</sub><sub>amil</sub> and ΔI<sub>sc</sub><sub>amil</sub> in CF epithelia is due to reduced CFTR anion conductance rather than greater Na<sup>+</sup> channel activity, and these data are consistent with that earlier study (Chen et al., Cell 143:911-923, 2010). Values of transepithelial electrical conductance (G<sub>t</sub>) were large, probably because of “edge damage” associated with clamping epithelia in Ussing chambers (Helman et al., Am. J. Physiol. Cell Physiol. 225(4):972-977, 1973) (compare with cultured epithelia in Fig. 31, 32). Amiloride reduced G<sub>t</sub> (ΔG<sub>t</sub><sub>amil</sub>) to a similar extent in CFTR<sup>A508</sup>/Af508 and CFTR<sup>-/-</sup> epithelia.

In nasal epithelia, compared to wild-type, CFTR<sup>A508</sup>/Af508 epithelia had a greater basal V<sub>t</sub> and ΔV<sub>t</sub><sub>amil</sub> in culture and a greater basal I<sub>sc</sub> and ΔI<sub>sc</sub><sub>amil</sub> in excised epithelia (Fig. 31, 32). These differences between CF and non-CF epithelia at the two locations are the result of differences between basal CF Cl<sup>-</sup> channel activity and other epithelial properties rather than differences in rates of Na<sup>+</sup> transport (Chen et al., Cell 143:911-923, 2010). ΔG<sub>t</sub><sub>amil</sub> in CFTR<sup>A508</sup>/Af508 nasal epithelia was less than or the same as that in wild-type epithelia consistent with the conclusion that Na<sup>+</sup> conductance is not greater in CF than non-CF epithelia (Chen et al., Cell 143:911-923, 2010). In excised and cultured nasal and tracheal/bronchial epithelia, these electrophysiological properties in CFTR<sup>A508</sup>/Af508 epithelia (Fig. 29-32) were approximately the same as those in CFTR<sup>-/-</sup> epithelia.

To assess CFTF function, we added 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) to block non-CFT CF channels, followed by forskolin and IBMX to increase cellular concentrations of cAMP and phosphorylate and activate CFTF (Sheppard et al., Physiol. Rev. 79(1):S23-S45, 1999).

In both tracheal/bronchial and nasal epithelia and in both excised tissue and cultured epithelia, the forskolin and IBMX-induced changes in V<sub>t</sub> (ΔV<sub>t</sub><sub>AMP</sub>) or ΔI<sub>sc</sub><sub>AMP</sub>, and G<sub>t</sub> (ΔG<sub>t</sub><sub>AMP</sub>) were markedly reduced in CFTR<sup>A508</sup>/Af508 compared to CFTR<sup>-/-</sup> epithelia (Fig. 29-32). Interestingly, for most of the electrophysiological measurements, there was either a statistialy significant difference or a non-significant trend for CFTR<sup>A508</sup>/Af508 epithelia to show more cAMP-stimulated Cl<sup>-</sup> conductance and/or Cl<sup>-</sup> transport than CFTR<sup>-/-</sup> epithelia. As an additional way of assessing CFTF-mediated Cl<sup>-</sup> transport, after adding forskolin and IBMX, we applied Gly-H+101, which inhibits CFTR Cl<sup>-</sup> channels (Sheppard et al., Physiol. Rev. 79(1):S23-S45, 1999). The results paralleled what we found with cAMP-dependent stimulation; the response was markedly attenuated in CFTR<sup>A508</sup>/Af508 compared to wild-type epithelia, but often greater than in CFTR<sup>-/-</sup> epithelia.

In addition to Cl<sup>-</sup>, CFTF also transports HCO<sub>3</sub> (Poulsen et al., Proc. Natl. Acad. Sci. U.S.A. 91(12):5340-5344, 1994; Smith et al., J. Clin. Invest. 89:1148-1153, 1992), and it has been proposed that defective HCO<sub>3</sub> transport may be critical for CF pathogenesis (Quinton, Lancet 372(9636):415-417, 2008). Therefore, we also examined changes in I<sub>sc</sub> and G<sub>t</sub> when tracheal epithelia were bathed in a Cl<sup>-</sup>-free HCO<sub>3</sub> solution. Like the reduction in Cl<sup>-</sup> conductance, HCO<sub>3</sub> conductance was markedly reduced in CFTR<sup>A508</sup>/Af508 (trachea) (Fig. 33A, B).

Thus, the ΔF<sub>S</sub> allele greatly decreased both HCO<sub>3</sub> conductances, consistent with a substantial loss of CFTF. However, compared to CFTF<sup>-/-</sup>, CFTF<sup>A508</sup>/Af508 epithelia retained some apical CFTF function. CFTF<sup>A508</sup>/Af508 Epithelia have Residual CFTF Function

The finding that CFTF<sup>A508</sup>/Af508 pigs develop lung disease and yet have some CFTF anion conductance provided us with an opportunity to begin to address the question of how much CFTF function is sufficient to prevent lung disease. As one assessment of residual CFTF function, we compared the forskolin and IBMX-induced increases in I<sub>sc</sub> (ΔI<sub>sc</sub><sub>AMP</sub>) and G<sub>t</sub> (ΔG<sub>t</sub><sub>AMP</sub>) in the presence of amiloride and under short-circuit conditions (i.e., Vi clamped to zero and symmetrical solutions) (Fig. 29-32). In CFTR<sup>A508</sup>/Af508 excised and cultured nasal and tracheal/bronchial epithelia, the ΔI<sub>sc</sub><sub>AMP</sub> was 9-15% of wild-type values (Table 7). Edge damage effects prevented accurate assessments of ΔG<sub>t</sub><sub>AMP</sub> in excised epithelia, but in cultured epithelia, the CFTF<sup>A508</sup>/Af508 ΔG<sub>t</sub><sub>AMP</sub> was 6-16% of CFTF<sup>-/-</sup> values. Although Gly-H+101 can have effects in addition to inhibiting CFTF (Kelly et al., J. Pharmacol. Exp. Ther. 333(1):60-69, 2010; Caputo et al., Science 322(5901):590-594, 2008), we also calculated Gly-H+101-induced changes (ΔI<sub>sc</sub><sub>Gly</sub> or ΔG<sub>t</sub><sub>Gly</sub>) and found that they varied from 3-32% in CFTF<sup>A508</sup>/Af508 compared to CFTF<sup>-/-</sup> epithelia. The mean of all these ΔI<sub>sc</sub> and ΔG<sub>t</sub> measurements was ~13.6% of values in CFTF<sup>-/-</sup> epithelia.

### Table 7

Changes in current and conductance in CFTF<sup>A508</sup>/Af508 epithelia as a percentage of changes in CFTF<sup>-/-</sup> epithelia.

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<th>Condition</th>
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<th>Excised tracheal</th>
<th>Cultured nasal</th>
<th>Cultured tracheal</th>
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<td>18.2</td>
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<td>15.1</td>
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<tr>
<td>ΔG&lt;sub&gt;t&lt;/sub&gt;&lt;sub&gt;AMP&lt;/sub&gt;</td>
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<td>15.7</td>
<td>5.6</td>
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</tr>
<tr>
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<td>4.3</td>
<td>7.6</td>
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</table>
TABLE 7-continued

| Changes in current and conductance in CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> epithelia as a percentage of changes in CFTR<sup>++</sup> epithelia. |
|-----------------------------------|-----------------------------------|-----------------|-----------------|
| CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> (% CFTR<sup>++</sup>) | Excised nasal | Excised tracheal | Cultured nasal | Cultured tracheal |
| | | | | |
| ΔG<sub>G<sub>max</sub>off</sub> | 13.1 | 4.2 |
| ΔG<sub>G<sub>max</sub>AMP</sub> | 7.7 | 6.6 |
| ΔG<sub>G<sub>off</sub></sub> | 8.3 | 2.1 |

Data are changes in Isc in response to forskolin and IBMX and GlyH-101 (Ala<sub>7</sub>gly→Ala<sub>9</sub>g90, Ala<sub>9</sub>g90→Gly<sub>101</sub>) of the corresponding changes in Gt (ΔG<sub>G<sub>off</sub></sub>, ΔG<sub>G<sub>max</sub>AMP</sub>, and ΔG<sub>G<sub>max</sub>off</sub>) in apical current induced by adding basolateral nystatin and apical forskolin and IBMX (ΔG<sub>G<sub>max</sub>off</sub>); in the presence of a C<sub>T</sub>-concentration gradient, and changes induced (by the subsequent addition of GlyH-101 (ΔG<sub>G<sub>max</sub>off</sub>)). To correct for any changes in the absence of CFTR, we subtracted values obtained in CFTR<sup>++</sup> epithelia and data were calculated from mean values of individual measurements in CFTR<sup>++</sup> (CFTR<sup>++</sup>→CFTR<sup>++</sup>). The average of the ΔG<sub>G<sub>off</sub></sub>,ΔG<sub>G<sub>max</sub>AMP</sub> and ΔG<sub>G<sub>max</sub>off</sub> values is 13.6%, and the average of the ΔG<sub>G<sub>max</sub>AMP</sub> and ΔG<sub>G<sub>off</sub></sub> values is 6.2%.

In addition to apical CFTR conductance, I<sub>sc</sub> and Gt are affected by other apical ion channels and basolateral mem-brane transport, and CFTR may be partially active before cAMP elevation. In addition, the relationship between CFTR conductance and I<sub>sc</sub> is not linear, and the percentage increase in I<sub>sc</sub> overestimates the amount of CFTR protein (Farman et al., Am. J. Physiol. Lung Cell Mol. Physiol. 280:L1123-L1130, 2005; Johnson et al., Nat. Genet. 2:21-25, 1992). Therefore, we imposed a transepithelial C<sub>T</sub>-concentration gradient and measured the current response to basolateral membrane permeabilization with nystatin and addition of forskolin and IBMX (FIG. 3C,D). CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> nasal and tracheal epithelia generated 7-8% as much current as wild-type controls (Table 7). Subsequent addition of GlyH-101 produced a current change in CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> epithelia that was 2-8% of that in CFTR<sup>++</sup> epithelia (FIG. 3C,E). The mean of the changes in current was 4% of values in CFTR<sup>++</sup> epithelia (Table 7).

These studies were done under conditions of maximal CFTR stimulation. Therefore, we also examined the response to increasing concentrations of forskolin and IBMX (at a fixed ratio of forskolin:IBMX of 1:10) (FIG. 3F,G). The EC50 for CFTR<sup>++</sup> epithelia was 0.07±0.01 forskolin. In contrast, the EC50 for forskolin in CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> epithelia was 0.60±0.19.

Because forskolin and IBMX might generate different cellular cAMP concentrations in CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> and CFTR<sup>++</sup> epithelia, we repeated the experiments with 8-CPT-cAMP, a membrane-permeable cAMP analog (FIG. 3H,I). The results were similar in that the EC50 in wild-type epithelia was 8.0±1.3 µM and in CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> it was 65.2±17.3 µM.

These results suggest that CFTR<sup>APSF<sub>8</sub></sup><sup>APSF<sub>8</sub></sup> epithelia have a reduced sensitivity to cAMP-dependent stimulation of CFTR transport.

Materials and Methods

Animals

We previously reported generation of CFTR<sup>APSF<sub>8</sub>APSF<sub>8</sub></sup> and CFTR<sup>++</sup> pigs (Rogers et al., J. Clin. Invest. 118 (4):1571-1577, 2008). Animals were produced by mating CFTR<sup>APSF<sub>8</sub>APSF<sub>8</sub></sup> male and female pigs or CFTR<sup>++</sup> male and female pigs. Newborn CF pigs and littersmates were obtained from Exemplar Genetics. The University of Iowa Animal Care and Use Committee approved all animal studies.

For studies of newborn animals, pigs were euthanized 8-15 hr after birth (Euthasol, Virbac). This time minimizes changes from infection, inflammation, feeding, and exposure to environmental influences; it ensures that the animals are all of approximately the same age; and it provides time for geno-
Tracheal cells were isolated by enzymatic digestion of the entire tracheas in HBSS (without CaCl₂ or MgCl₂), 1 EGTÁ, 20 EDTA, 0.05% collagenase, 1, 500 U/50 ml of DNase, and the protease inhibitors with shaking at 4°C for 2 hrs. Cells were filtered, centrifuged and dispersed as above.

Production of Recombinant CFTR
293T cells were transfected with pcDNA3 vectors encoding porcine wild-type CFTR or CFTR-ΔF508 as previously described (6). Cells were lysed 48 hrs after transfection, solubilized in lysis buffer (LB) [50 mM Tris (pH 7.4) 50 mM NaCl. 1% Triton X-100 and protease inhibitors (PI)], 2 μg/ml aprotinin, 7 μg/ml benzamide-HCl, 1 μg/ml pepstatin A and 2 μg/ml leupeptin], and centrifuged at 14,000g for 15 min at 4°C to separate soluble from insoluble pellet. Protein was measured using the BCA assay (Thermo-Fisher), 10-25 mg of protein was used per lane for electrophoresis of intestinal and tracheal samples.

Immunoprecipitation and Phosphorylation
Protein assays were performed using the BCA assay (Thermo-Fisher). Indicated amounts of intestinal tissue or cells from 1-2 tracheas were homogenized in the Tris-mannitol buffer on ice with 20 strokes of the loose-fitting and 15 strokes of the tight-fitting pestle of a Potter-Elvejhem homogenizer. Membrane pellets were isolated by centrifugation at 200,000g at 4°C for 30 min. The pellets were solubilized in a commercial detergent mix, Membrane Solution 2 (ProFind, Ca). Soluble proteins were separated from insoluble pellets by centrifuging at 200,000×g for 20 min. CFTR was immunoprecipitated from the supernatant of soluble proteins with anti-CFTR antibodies M537 and MM13-14 (Upstate Biotechnology) and in vitro phosphorylated with 32P-ATP and the catalytic subunit of PKA (Promega) (Westeergaard et al., Proc. Natl. Acad. Sci. U.S.A. 104(39):15370-15375, 2007). Washed precipitates were electrophoresed on 6% SDS-PAGE. Gels were stained, destained, dried and exposed to phosphorimages on a Fuji FLA7000 imager (General Electric).

Immunocytochemistry
Ileal and tracheal tissues were excised from newborn piglets, immediately placed in ice-cold 30% sucrose, and quick-frozen in OCT with liquid N₂. Tissue segments were kept at −80°C. Tissues were cryosectioned into 7 μm sections, fixed in 100% MeOH at −20°C for 10 min, permeabilized in 0.2% TX-100 (Thermo-Fisher) in PBS, and blocked in Super-Block (Thermo-Fisher) with 5% normal goat serum (Jackson ImmunoResearch). Tissue sections were incubated for 2 hrs at 37°C in anti-CFTR antibodies MM13-4, M537 (Chemicon), and polyclonal antibody to the tight junction protein ZO-1 (Zymed) (all at 1:100 dilution), followed by secondary antibodies (goat-anti-mouse Alexa-Fluor488 and goat anti-rabbit Alexa-Fluor568; Molecular Probes/Invitrogen) (1:1000 dilution). Sections were mounted with Vectashield (Hard-set) containing DAPI (Vector Labs) to visualize nuclei. Images were acquired with a Hamamatsu fluorescent microscope with a UPLSAPO 60x oil lens. Images were scanned sequentially at 2 pixel/pixel. Post collection enhancements were done identically, except where indicated in the figure legends to amplify CFTR-ΔF508 signal.

Electrophysiological Measurements of Freshly Excised and Cultured Epithelia
Epithelial tissues were excised from the nasal turbinate and septum, and from trachea through 2nd generation bronchi immediately after animals were euthanized. Tissues and cul-
tured epithelia were studied in modified Ussing chambers. Epithelia were bathed on both surfaces with solution containing (in mM): 135 NaCl, 2.4 K,HPO₄, 0.6 KH₂PO₄, 1.2 CaCl₂, 1.2 MgCl₂, 10 dextrose, 5 HEPES, pH 7.4, at 37° C, and gassed with compressed air. Na-glucanate was substituted for NaCl in low Cl⁻ bath solutions. Transepithelial voltage (VT) was maintained at 0 mV to measure short-circuit current (ISC). Transepithelial electrical conductance (Gt) was measured by intermittently clamping VT to ±5 and/or ±5 mV.

For studies of HCO₃⁻ transport, we bathed the epithelia in a solution of (in mM): 118.9 Na-glucanate, 25 NaHCO₃, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 5 Ca-glucanate, 1 Mg-glucanate and 5 dextrose, bubbled with 5% CO₂/95% air.

A standard protocol was the following: 1) Measurements under basal conditions. 2) 100 μM apical amiloride to inhibit ENaC Na⁺ channels. 3) 100 μM apical DIDS (4,4-diisothiocyanato stilbene-2,2-disulfonic acid) to inhibit most anion channels other than CFTR. 4) 10 μM forskolin and 100 μM IBMX (3-isobutyl-1-methylxanthine) to increase cellular levels of cAMP leading to phosphorylation and activation of CFTR. 5) 100 μM apical GlyH-101 to inhibit CFTR. 6) 100 μM bumetanide to inhibit basolateral Na-K-2Cl transporter in cultured epithelia.

To directly measure apical CFTR-mediated Cl⁻ transport, we pretreated cultured tracheal epithelia apically with amiloride (100 μM) and DIDS (100 μM) in symmetrical bath solutions (139.8 mM Cl⁻). We then imposed a large Cl⁻ concentration gradient across the epithelia by replacing NaCl in the apical solution with Na-glucanate (final Cl⁻ concentration 4.8 mM) containing the same amount of amiloride and DIDS. To assess apical CFTR Cl⁻ currents, we permeabilized the basolateral membrane with nystatin (0.36 mg/ml) and activated CFTR by adding 10 μM forskolin and 100 μM IBMX apically. After currents reached a plateau, we added 100 μM GlyH-101 apically to inhibit CFTR.

Statistical Analysis

Data are presented as means±SEM. Differences were considered statistically significant at P<0.05. Statistical analysis of morphometric data was performed with a 1 way ANOVA and Bonferroni post-test. For electrophysiological assays involving three comparisons (CFTR⁻/⁻, CFTR⁻/+; and CFTR⁺/⁺), the variances were often significantly unequal. We therefore used Welch’s unpaired t tests (Ostergaard et al., Proc. Natl. Acad. Sci. U.S.A. 104(39):15370-15375, 2007), and considered differences to be statistically significant if P<0.05/3 (i.e., P<0.017) (Fisher, The Design of Experiments (Oliver & Boyd, Edinburgh, 1935)).

Other Embodiments

All publications, patents, and other citations noted in this specification are incorporated herein by reference as if each individual publication, patent, or other citation were specifically and individually indicated to be incorporated by reference. Although the invention has been described above in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Use in the claims and elsewhere herein of singular forms, such as “a” and “the,” does not exclude indication of the corresponding plural form, unless the context indicates to the contrary. Thus, for example, if a claim indicates the presence of “a” mutation in “a” gene, it can be interpreted as covering one or more mutations, in one or more genes, unless otherwise indicated. Further, the term “or” as used herein is intended to be interpreted as both optional (i.e., one or the other and not both of multiple options) and inclusive (i.e., and/or)

Other embodiments are within the scope of the claims.
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What is claimed is:

1. A transgenic pig whose genome comprises a homozygous deletion of the codon for phenylalanine at position 508 of the CFTR protein of said pig, wherein said pig has a symptom of cystic fibrosis in its pancreas, intestine, liver, or gall bladder, and does not express wild-type CFTR.

2. An isolated cell of the transgenic pig of claim 1.

3. A method of identifying a therapeutic agent that can be used in the treatment of cystic fibrosis, the method comprising administering a compound to the pig of claim 1, wherein the pig has a symptom of cystic fibrosis in its pancreas, intestine, liver, or gall bladder, and monitoring the pig for said symptom of cystic fibrosis, wherein detection of improvement in said symptom of cystic fibrosis indicates that the compound can be used as a therapeutic agent in the treatment of cystic fibrosis.

4. The method of claim 3, wherein the symptom of cystic fibrosis is monitored in the pancreas, intestine, or liver of the pig.

5. The transgenic pig of claim 1, wherein the pig has a symptom of cystic fibrosis in its pancreas.

6. The transgenic pig of claim 1, wherein the pig has a symptom of cystic fibrosis in its intestine.

7. The transgenic pig of claim 1, wherein the pig has a symptom of cystic fibrosis in its liver.

8. The transgenic pig of claim 1, wherein the pig has a symptom of cystic fibrosis in its gallbladder.

9. A method of making the transgenic pig of claim 1 comprising:
   i) introducing a deletion into the codon for phenylalanine at position 508 of pig CFTR into a fetal pig fibroblast;
   ii) introducing the nucleus of the fetal pig fibroblast into an enucleated pig oocyte to generate a pig embryo;
   iii) transferring the pig embryo into a recipient female such that a transgenic pig whose genome comprises said deletion is obtained; and
   iv) breeding the pig obtained in iii) such that the transgenic pig of claim 1 is obtained.

10. A transgenic pig whose genome comprises a heterozygous deletion of the codon for phenylalanine at position 508 of the CFTR protein of said pig, wherein said pig, when crossed with another transgenic pig whose genome has a
heterozygous deletion of the codon for phenylalanine at position 508 of the CFTR protein of the pig, generates the transgenic pig of claim 1.
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Please replace the paragraph under STATEMENT AS TO FEDERALLY SPONSORED RESEARCH at column 1, lines 19-23, with the following:

--This invention was made with government support under grant number HL51670 and grant number DK547759 awarded by National Institutes of Health. The Government has certain rights in the invention.--

Signed and Sealed this
Twenty-first Day of April, 2015

Michelle K. Lee
Director of the United States Patent and Trademark Office