REVIEW

Animal and Cell Culture Models for Cystic Fibrosis

Which Model Is Right for Your Application?

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Over the past 30 years, a range of cystic fibrosis (CF) animal models have been generated for research purposes. Different species, including mice, rats, ferrets, rabbits, pigs, sheep, zebrafish, and fruit flies, have all been used to model CF disease. While access to such a variety of animal models is a luxury for any research field, it also complicates the decision-making process when it comes to selecting the right model for an investigation. The purpose of this review is to provide a guide for selecting the most appropriate CF animal model for any given application. In this review, the characteristics and phenotypes of each animal model are described, along with a discussion of the key considerations that must be taken into account when choosing a suitable animal model. Available *in vitro* systems of CF are also described and can offer a useful alternative to using animal models. Finally, the future of CF animal model generation and its use in research are speculated upon. (*Am J Pathol* 2021, 191: 228–242; <https://doi.org/10.1016/j.ajpath.2020.10.017>)

Cystic fibrosis (CF) is a genetic disorder caused by mutations in the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) epithelial chloride channel.¹ CFTR protein is dysfunctional or absent in CF patients, resulting in multi-organ disease that affects the lungs, gastrointestinal tract, pancreas, liver, and reproductive tract. Progressive pulmonary disease is the major cause of morbidity and mortality among CF patients.² CF airways exhibit reduced chloride transport and hyperabsorption of sodium ions. This dysregulated ion transport causes water to be osmotically drawn into the epithelium, leading to dehydration of the airway surface liquid, mucus accumulation in the airways, impaired mucociliary clearance (MCC), and bacterial infections.³ In CF animal models, reduced CFTR-dependent bicarbonate secretion has been shown to lead to lower airway surface pH, impaired antimicrobial function of the airway surface liquid, and decreased bacterial killing;^{4,5} but the role of pH in human lung disease pathogenesis is less clear.⁶ Regardless of the pathophysiological basis, CF airways are highly susceptible to colonization by

opportunistic bacteria, with lung disease characterized by recurrent cycles of bacterial infections, inflammation, and mucus obstruction, ultimately leading to lung damage and failure.²

CF animal models have been generated by researchers to aid understanding of disease pathophysiology and to facilitate testing of potential therapeutics. CF mice have typically dominated research reports, but advancement in genetic engineering technologies has more recently enabled other species, including large mammals, to be utilized for modeling CF. Accordingly, a range of CF models are now available, including mouse strains,^{7–9} rats,^{10–13} ferrets,^{14,15} pigs,^{16,17} sheep,¹⁸ rabbits,^{19,20} zebrafish,²¹ and, most recently, *Drosophila*.²²

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Access to this plethora of animal models has facilitated research into many facets of CF. Studies have ranged from investigations into the mechanisms underlying CF lung disease pathogenesis²³ to trialing the effects of CFTR modulators *in utero*.¹⁵ However, with numerous animal models now available—and additional models likely to be generated in the near future—it has become increasingly challenging for researchers to determine which model they should employ for their investigations. This review provides an overview of existing animal models and addresses the factors that should be considered when selecting a model for any given research application. The use of *in vitro* systems as alternatives to animal models is addressed, along with the benefits and limitations of their use in research. Lastly, the future of CF animal-based research and model generation is also discussed.

CF Animal Models

Numerous animal models have been developed to facilitate CF-related research investigations. An important factor in selecting a suitable animal is having a general understanding of each of the animal models and their characteristics. In the following section, an overview of the available animal models is provided, including details of the advantages and limitations of each species and the associated CF phenotypes. The CF phenotypes of each animal model are also summarized in Table 1^{24–36} for quick reference.

Murine Models

Mice are often the go-to species for disease model development. They breed rapidly, are relatively inexpensive, and are easy to care for and house in standard research facilities. In addition, inbred mouse lines are available for model establishment, mouse-specific molecular reagents are readily available, and methods for their genetic modification are well advanced. Mice were initially considered suitable for modeling CF disease as they were found to express the *Cftr* gene and there was a reasonably high (78%) amino acid sequence identity with human CFTR (hCFTR).³⁷ A large number of CF mouse strains have since been documented and characterized in the literature. These mice can be broadly classified into three categories: i) *Cftr* null or knockout (KO) models,^{7,26} ii) strains with human CF-causing mutations introduced into the mouse *Cftr* gene (eg, Phe508del, Gly551Asp, and G542X),³⁸ and iii) transgenic models [eg, gut-corrected models, mice expressing human *CFTR*, and β -epithelial sodium channel (β -ENaC) mice].^{9,27,39}

Cftr Knockout and Mutant Models

Phenotype severity varies among CF mouse strains as a result of the method used to disrupt the *Cftr* gene, the type of mutation introduced, and the levels of *Cftr* mRNA produced.⁸ Despite these variations, all CF mouse models generally

recapitulate similar disease characteristics, including nasal electrophysiological defects, intestinal disease, reduced survival, impaired growth, and malformations of the male reproductive tract.^{7,8,25,26,40} Notably, however, CF mouse strains do not appear to develop key disease phenotypes, such as spontaneous lung disease or major pancreatic and liver histopathologies. More recent in-depth investigations reveal that CF mice have increased susceptibility to lung pathogens and some airway abnormalities,^{41–43} but these findings do not reflect the severe muco-obstructive phenotype observed in humans.

A lack of substantial lung disease in early mouse models drove researchers to explore alternative approaches to reproduce CF lung disease in mice. In 1997, Kent et al⁴⁴ postulated that the lack of lung disease development was due to the first strains being generated on a mixed genetic background and the influence of modifier genes partially correcting the ion transport abnormalities. Subsequently, a *Cftr* KO mouse model generated from a single genetic background (congenic) was found to develop aspects of lung disease, including impaired MCC, acinar and alveolar hyperinflation, parenchymal interstitial thickening, fibrosis, and inflammatory cell infiltration.⁴⁴

Shortly following the generation of CF KO mouse strains, their use was found to be significantly hindered by high mortality rates associated with intestinal obstructions. To overcome this limitation, transgenic CF KO mice were generated to express hCFTR cDNA in the intestinal tract under control of the rat fatty acid binding protein 2 gene promoter.³⁹ This modification rescues the CF gut phenotype, enabling adequate numbers of CF KO mice to be bred. This advancement resulted in fatty acid binding protein CF mice becoming one of the most widely used mouse models to date.

Strains carrying Phe508del (Δ F508) and Gly551Asp (G551D) *Cftr* mutations demonstrate phenotypes similar to KO models, although they tend to be less severe with a lower perinatal mortality. Although these strains were originally designed to test CFTR-modulating compounds, the structure, expression, biophysical properties, and function of the mouse *Cftr* channel were subsequently found to have distinct differences to those of humans.⁷ For example, previous studies indicate that the mouse Phe508del-*Cftr* processing defect is much less severe compared with humans, with more Phe508del-*Cftr* protein able to be delivered to the plasma membrane and little to no adverse effect on channel gating.⁴⁵ These CFTR species-dependent differences can strongly influence the response to drugs, particularly CFTR modulators. Correctors, such as lumacaftor, have been found to rescue mouse Phe508del-*Cftr*, whereas many potentiators, including ivacaftor, fail to augment mouse Gly551Asp-*Cftr* or Phe508del-*Cftr* function.^{45,46} While mutant strains can still provide value for testing experimental compounds,⁷ the differences between human and mouse CFTR protein must be taken into account. The more recently developed G542X mutant mouse

Table 1 Summary of Phenotypes Present in Available CF Animal Models

Human phenotype	Animal models of CF						
	Zebrafish	Mice	β -ENaC mice	Rats	Ferrets	Pigs	Sheep
Intestine	Unknown	Yes	No	Yes	Yes	Yes	Yes
Lung disease	N/A	No (except for congenic KO mouse strain)	Yes	No	Yes	Yes	No (further analysis needed)
Spontaneous lung infections	N/A	No	No	No	Yes	Yes	No (further analysis needed)
Exocrine pancreas	Yes	Yes, mild	No	No (US and French rats) Yes (Australian KO rats)	Yes	Yes	Yes
Endocrine pancreas	No	No	No	No	Yes	Yes	Yes
Liver	Unknown	No	No	No	Yes	Yes	Yes
Gallbladder	N/A	No	No	N/A	Yes	Yes	Yes
Male reproductive tract	Unknown	Yes	No	Yes	Yes	Yes	Yes
References	21,24	7,8,25,26	27–29	5,10–13	14,30–33	16,17,23,34–36	18

CF rabbits have also been generated, but published phenotype information is currently unavailable.
 β -ENaC; β -epithelial sodium channel; CF, cystic fibrosis; KO, knockout.

model demonstrates manifestations typical of CF mouse models. More importantly, G542X mice show responsiveness to pharmacologic read-through compounds, providing a useful tool for assessment of nonsense mutation therapies.³⁸

Humanized Mouse Models

Recently, a transgenic mouse model expressing *hCFTR* was developed. These mice demonstrate robust hCFTR protein function in tissues that typically express mouse *Cfr*, such as the intestinal mucosa and airways.⁹ By use of gene editing, the hCFTR mouse model is now being used to generate a range of mouse models that harbor human CF-causing mutations.⁴⁷ Although these models are still undergoing phenotype characterization (<http://www.cf-mice.org/cf-mouse-strains.html>, last accessed September 16, 2020), they have potential to provide great value for the CF research field, particularly for testing therapeutics that target CFTR.

β -ENaC Mouse Model

To mimic the increased ENaC activity observed in CF lungs, a transgenic mouse model was generated to overexpress the β -subunit of ENaC in the airways.²⁷ This β -ENaC mouse model displays key features of CF lung disease, including airway surface liquid volume depletion, reduced MCC, airway mucus obstruction, chronic neutrophilic airway inflammation, and mortality associated with pulmonary disease.^{27,28,48} Although spontaneous lung infections are not apparent, β -ENaC mice demonstrate an impaired ability to clear pathogens following intrapulmonary challenge. As the β -ENaC subunit is only overexpressed in the airways, the utility of this model is restricted to lung-related investigations. Moreover, the airways of β -ENaC mice do not have disrupted *Cfr* expression

or function, making them unsuitable for answering CFTR-specific research questions or for testing therapies that correct or potentiate CFTR.²⁸

Mouse Models with Short-Term Cfr Inhibition

Murine models have been generated to mimic CF disease by temporarily inhibiting Cfr function. Systemic delivery of Cfr antagonists, such as CFTR_{inh}-172 and GlyH-101, to mice has been shown to effectively block Cfr channel activity.^{49,50} The airways of these mouse models exhibit CF-like features, including reduced MCC in the trachea. This model has previously proved useful for *in situ* studies investigating the efficacy of compounds that have potential to improve airway mucus transport.⁴⁹ Unlike genetically modified CF mice that take time to breed to sufficient numbers for studies, Cfr inhibition mouse models can be rapidly generated using readily available inbred mouse strains. However, the duration of chemically induced Cfr inhibition is limited; therefore, these models are not well-suited for long-term investigations. The use of Cfr inhibitor compounds must also be optimized for each experiment to ensure effective and specific blocking of Cfr channel activity, which may include altering factors such as dose rate, timing, and the route of administration.^{49,50}

Rat Models

Rats offer a range of benefits for use in disease modeling. As with mice, rats are easy to rapidly rear in large numbers and are suited to housing in a research environment. When compared with mice, rats are appreciably bigger, which can enable improved exploratory studies and provide larger tissue specimens and blood samples for analyses. Rats have been used extensively in biomedical research for pharmacology and toxicology trials; therefore, their anatomy, physiology,

and pharmacokinetics are well characterized.¹⁰ In regard to modeling CF, unlike mice, rats have submucosal glands throughout the trachea,⁴⁷ an important factor given that these glands are implicated in human CF lung disease pathogenesis.⁵¹ The rat *Cftr* amino acid sequence has only 76% identity with human CFTR.⁵²

The first CF rat model to be developed was a *Cftr* KO strain.¹⁰ More recently, two additional CF KO models have been generated, along with two new Phe508del rat strains.^{11,12} These CF rat models demonstrate some phenotypic differences, and in general the Phe508del models show milder phenotypes when compared with their CF KO counterparts.^{11,12} All CF rat models appear to exhibit reduced survival, impaired weight gain, intestinal obstructions, a CF bioelectric profile in the nasal epithelium, and defects in the male reproductive tract anatomy. Hepatic disease is not present in any of the rat models, although exocrine pancreas histopathology is evident in a proportion of the Australian CF KO rats.¹² There has been no evidence of spontaneous lung disease or mucus obstruction in any of these CF rat models, although Birket et al⁵ (2018) have identified airway abnormalities in US CF KO rats, including reduced MCC and periciliary liquid, increased mucus viscosity, and a hyperacidic airway surface. Increased mucus in the nasal¹⁰ and tracheal airways^{10,12} has also been noted in CF KO rats.

Humanized Rat Model

Recently, a novel humanized CF rat model expressing *hCFTR* with the Gly551Asp (G551D) mutation was generated, denoted hG551D rats. This model recapitulates the same phenotype characteristics as the US-developed KO rat mentioned above. More important, the hG551D rat responds to the CFTR potentiator ivacaftor. Following ivacaftor treatment, hG551D rats demonstrated restored nasal and tracheal electrical currents and normalization of airway mucus defects, including restored MCC and reduced mucus viscosity.¹³

Rabbit Model

Rabbits have useful features for modeling CF disease. They are suited to rearing in a research environment and have a relatively low cost of maintenance. Rabbits have previously been used to study lung diseases and therapies as they have similarities to humans in terms of airway anatomy, cellular architecture, and inflammatory responses.⁵³ Interestingly, rabbits do not have submucosal glands present in the airways, which could prove beneficial for studying epithelial-specific contributions to airway disease pathogenesis.²⁰ Rabbits are phylogenetically closer to primates than rodents, and have a 92% CFTR amino acid identity with humans.^{20,37,53} Characterization of the recently developed *CFTR* KO rabbit model is currently ongoing and awaiting peer-reviewed publication, although initial investigations reveal presence of CF phenotypes, including bioelectric defects in the gastrointestinal tract, trachea, and nose, and intestinal disease.²⁰

Ferret Models

Domestic ferrets are used commonly for respiratory-related investigations as their lung cell biology is highly conserved with humans.⁵⁴ Like humans, ferrets express abundant submucosal glands throughout the cartilaginous airways, and the goblet cell is the major secretory cell type.⁴⁷ Ferret CFTR has a 92% amino acid identity with hCFTR,³⁷ and the airway expression patterns and bioelectric properties of ferret CFTR have been found to be similar to those of humans.^{55,56}

First developed over a decade ago,¹⁴ *CFTR* KO ferrets follow a course of disease similar to that in humans and accurately recapitulate several CF phenotypes. In early life, CF ferrets are susceptible to life-threatening bacterial lung infections and are reared on antibiotics to improve survival during this period. Once mature, the lung infections are less acute and pulmonary disease progresses slowly. Common CF airway disease features are observed in the KO ferrets, including mucus obstruction, impaired MCC, air trapping, atelectasis, and pneumonia.³²

Pancreatic disease in KO ferrets progresses in a manner similar to that in humans. Newborn CF ferrets exhibit mild exocrine pancreatic histopathology that advances with age and results in destruction of both exocrine and endocrine tissue. CF-related diabetes also develops, with glucose metabolism abnormalities and altered insulin secretion.^{31,33} Lethal meconium ileus is frequently observed in CF ferrets, with 75% of newborns affected. Intestinal histopathology includes villus blunting, mucus accumulation in the crypts, and inflammation. Other phenotypes, including hepatic and gallbladder disease, are apparent,³³ while male CF ferrets also demonstrate absence of the vas deferens at birth.³⁰

Severe gastrointestinal disease limits the utility of KO ferrets; therefore, a transgenic gut-corrected CF KO ferret was generated using a similar approach as described for the mouse model.³⁰ Another ferret model was recently generated to harbor the Gly551Asp (G551D) *CFTR* mutation. This model exhibits multi-organ disease and is responsive to the CFTR potentiator ivacaftor. The G551D ferret model has recently been employed to investigate the effects of *in utero* ivacaftor use on survival and pathology development, in the first study of its kind to date.¹⁵ As this animal model is responsive to CFTR modulator therapy, the ferrets can be reared on ivacaftor to prolong their survival for investigations. Modulator use can then be withdrawn to study disease aspects of interest.

Pig Models

Pigs are an attractive candidate for modeling diseases as they have a similar size, anatomy, genetics, and physiology to humans.⁵⁷ Because of these similarities, genetically modified porcine models have been generated for a range of human diseases. The human-like size and characteristics of pigs also make them valuable for translational research

studies and bridging the gap between proof-of-principle trials established in rodent models and human clinical trials. Specific to modeling CF, porcine CFTR has a 92% amino acid sequence identity with hCFTR.³⁷ Porcine lungs also have parallels to humans in regard to their morphologic, biochemical, and histologic features, including submucosal glands that extend throughout the cartilaginous airways. Pigs have previously been employed to study respiratory infections unrelated to CF, as their pulmonary inflammation and infection responses are comparable to those observed in humans.⁵⁷ Similarities to humans in regard to lung size, structure, volumes, airway dimensions, and cardiopulmonary rates make pigs particularly useful for performing lung medical imaging studies.^{57,58}

At least two types of CF porcine models have been developed, *CFTR* KO models^{16,17} and the Phe508del (F508del or Δ F508) pig.¹⁶ Arguably, CF pigs present the most CF-related phenotypes of all the characterized animal models; however, the manifestation of many of these traits is more severe when compared with the human equivalent.³⁵ At birth, CF KO and Phe508del pig lungs lack inflammation, but over time, inflammation, infection, mucus accumulation, and remodeling spontaneously develop. CF pig lungs also have an impaired ability to eradicate bacteria, consistent with that observed in CF patients with early lung disease.^{23,36} Notable morphologic and histologic abnormalities are also present in the tracheae of these CF pig models, similar to those found in patients with CF.^{17,34}

CFTR KO pigs are severely affected by intestinal disease. All newborn piglets experience meconium ileus, which is fatal unless corrective surgery is performed shortly after birth to relieve the obstruction.³⁵ Phe508del pigs are also affected, although the intestinal phenotype is slightly less severe.³⁶ To overcome meconium ileus, gut-corrected CF pigs were generated to express porcine *CFTR* in the intestines under the control of the fatty acid binding protein 2 promoter.⁵⁹

The exocrine pancreas of newborn CF pigs presents with severe destruction, inflammation, and fibrosis, consistent with the late-stage disease seen in CF patients,³¹ with this pathology slightly less severe in Phe508del pigs.³⁶ CF KO and Phe508del pigs also demonstrate pathology of the liver, which consists of focal biliary cirrhosis, the hallmark feature of CF liver disease. The gallbladder is also affected, presenting as a microgallbladder filled with mucus and bile, resembling that observed in humans.^{17,35}

Sheep Model

Sheep have long been considered a candidate species for modeling CF disease. Ovine lungs have similar anatomic, functional, histologic, and electrophysiological features to humans.⁶⁰ The comparative size and structure of sheep lungs to humans provides a useful platform for performing substance delivery (eg, fluid and aerosols) and sampling techniques (eg, bronchoalveolar lavage and lung tissue biopsy) as clinical equipment and methods can be employed.

Sheep have also been used to model a range of human respiratory diseases, including extensive studies into allergic asthma.⁶¹ CFTR is well-conserved between sheep and humans, with a 91% identity at the amino acid level.³⁷ The tissue-specific pattern of *CFTR* expression is also similar between sheep and humans during development and postnatally.⁶² Sheep have small litter sizes, a relatively long gestation period, and a large fetus size; therefore, a CF sheep model may prove useful for *in utero* investigations and development of perinatal treatments.⁶³

Although an ovine model of CF has long been considered, its successful generation has only recently come to fruition because of the availability of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing, which enabled the sheep *CFTR* locus to be targeted. Characterization of the *CFTR* KO sheep model revealed the presence of severe CF phenotypes. At birth, 100% of *CFTR* KO lambs present with life-threatening meconium ileus and euthanasia is necessary, as they are unable to survive. Lung histopathologies are not evident in newborn CF KO lambs; however, further investigation of older animals is required to determine whether pulmonary disease progresses with age. Other phenotypes include fibrosis of the pancreas, which, like CF pigs, is already advanced at birth. Progressed liver disease, apparent in nearly all CF newborn sheep, consists of periportal fibrosis, biliary hyperplasia, and intrahepatic cirrhosis. The liver disease is more severe than that observed in humans, where only a minority of patients are affected. Most CF KO sheep also present with a hypoplastic gallbladder, and the male lambs demonstrate aplasia or severe atrophy of the vas deferens.¹⁸ Currently, lack of survivability prevents the use of the sheep CF model in many research studies; however, the tissues of newborns can still provide value for pathology-based investigations. As with other CF animal models, it is possible that generation of a gut-corrected sheep model could improve survival and usability of the model.

Zebrafish Models

Zebrafish can be rapidly bred to large numbers, are inexpensive, have minimal housing and care requirements, and their genome can be easily manipulated.⁶⁴ For these reasons, zebrafish are increasingly being used in biomedical research to study embryology, development, human genetics, and diseases.⁶⁵ In terms of modeling CF disease, zebrafish Cfr shares a 55% amino acid identity with human CFTR.³⁷ Despite this, Cfr is found to play a role in the regulation of fluid secretion in the zebrafish,²⁴ and the Cfr protein responds to some of the same pharmacologic activators and inhibitors as human CFTR.⁶⁶ Interestingly, a recent study using a zebrafish with the Phe508del mutation found that the corrector lumacaftor failed to rescue zebrafish Cfr

	Animal models of CF						
	Zebrafish	Mice	β-ENaC mice	Rats	Ferrets	Pigs	Sheep
Longitudinal or long-term	High	High	High	High	Medium	Low	Low
Large statistically powered studies	High	High	High	High	Low	Low	Low
Translation to humans (physical size)	Low	Low	Low	Low	Medium	High	High
CFTR gene or protein expression	Medium	Medium	Low	Medium	High	High	High
CF electrophysiology	High	High	Medium	High	High	High	High
Lung disease pathology	Low	Low	High	Medium	High	High	Low
Lung structure and cellular architecture	Low	Low	Low	Medium	High	High	High

Figure 1 Suitability of available cystic fibrosis (CF) animal models for selected research applications. β-ENaC; β-epithelial sodium channel; CFTR, CF transmembrane conductance regulator.

misprocessing, but that channel activity could be potentiated by ivacaftor.⁶⁷

Cftr is expressed in the pancreatic duct of zebrafish; therefore, disruption of *Cftr* expression produces a pancreatic disease phenotype. The pancreatic disease manifests in a manner similar to that in humans with CF, consisting of destruction of the exocrine acinar tissue, mucus accumulation in the pancreatic ducts, and inflammation indicated by recruitment of neutrophils. Although the exocrine pancreas is affected, the endocrine portion (islets) appears normally developed.²¹ In addition to modeling pancreatic disease, CF zebrafish could also provide a useful *in vivo* model for screening compounds that modulate human CFTR. The Kupffer vesicle is a ciliated organ transiently present during zebrafish embryogenesis that is inflated by *Cftr*-dependent fluid secretion, thereby providing a rapid assay of *Cftr* activity.^{21,68} Pulmonary ionocytes have recently been identified in humans and are a major source of *CFTR* expression in the airway epithelium.^{69,70} Gene expression profile of zebrafish ionocytes resembles that of human pulmonary ionocytes,⁶⁹ and thus CF zebrafish could prove useful in investigating the role of ionocytes in disease physiology and pathogenesis, as well as in strategies for targeting these cells in therapies.

Drosophila Model

The common fruit fly, *Drosophila melanogaster*, can be used to model human diseases because of its highly conserved disease pathways. *Drosophila* can also provide an inexpensive, high-throughput model for screening treatment compounds and identifying targets in early drug discovery phases.⁷¹ Currently, the use of flies does not require animal ethics clearance; therefore, research studies can be rapidly

arranged. A *Drosophila* model of CF gastrointestinal disease has recently been generated for research purposes. An equivalent protein with the similar structural and functional properties as human CFTR has been identified in *Drosophila*. Knockdown of this gene, denoted *Dmel\CFTR*, results in a CF gastrointestinal phenotype that consists of disrupted intestinal osmosis, stem cell hyperplasia, and increased expression of mucin gene, *Muc68D*. The *Dmel\CFTR* *Drosophila* also exhibits increased susceptibility to *Pseudomonas aeruginosa* infection, evidenced by decreased viability of the flies following infection. Additionally, expression of wild-type *hCFTR* appears to fully rescue the CF-like phenotypes in these mutant flies.²² Although still in its early phase, this *Drosophila* model of CF provides researchers with yet another option for investigations.

Selecting the Right Animal Model

Determining which model is appropriate for a research investigation is complicated by the already large and ever-increasing range of animal models available to choose from. There are several factors to consider when selecting a suitable model. The following section will guide readers through the considerations that should be made when choosing an appropriate animal model for their research investigation. Figure 1 summarizes the suitability of available animal models for a selection of research applications.

Logistics

Animal housing, and the costs and labor associated with husbandry can be major limiting factors. Species, such as rodents, fish, and flies, can be housed in standard research

facilities and husbandry costs are relatively low, although CF mice and rats have additional dietary requirements^{10,26} and increased health monitoring is necessary. Larger animals, such as ferrets, pigs, and sheep, require specialized accommodation and trained animal handlers. CF ferret and pig models also have severe disease that requires close monitoring and ongoing management with costly medications.^{30,36}

Model and Species Characteristics

The lifespan and disease severity of the animal model will significantly influence study duration. Caring for models with severe disease, such as CF pigs and ferrets, requires labor-intensive and costly husbandry to keep the animals alive for long durations, so they tend to be better suited to short-term studies that use young animals. CF sheep have not been continued past the newborn stage because of poor health and are therefore only suited to studies involving post-mortem analyses. Milder disease phenotypes present in mouse and rat models enable longitudinal studies and repeated measures to be performed; however, the experiment cannot exceed the expected approximate 2-year lifespan of a rodent.⁷² Size of the species is also a factor. In general, rodents tend to be useful for proof-of-concept investigations, whereas larger species, such as pigs and sheep, are suited to translational studies as clinical equipment and techniques can be used.^{57,61}

The reproductive parameters of the species and disease-associated mortality rates are also important, as this will dictate how many animals can be bred for any given study. Generally speaking, rodents, fish, and *Drosophila* models are better suited to statistically powered investigations as they can be bred rapidly to high numbers. Ferret and pig models are recommended for exploratory studies because of their smaller litter sizes, more severe disease phenotypes, and larger physical size, which all contribute to making studies with high subject numbers practically challenging and cost prohibitive. For instance, performing an airway gene therapy study using a viral vector in pigs would require large volumes to effectively dose a single pig lung. Not only would this be practically challenging and costly, but it is likely that many hundreds of rodents could be tested with the same quantity of gene vector.

Recapitulation of human-like immune responses is imperative when validating the safety of therapies or investigating the molecular mechanisms of disease pathogenesis. It is important to consider species-dependent differences in immune responses, as the structure and function of the immune system can vary significantly. Only vertebrates express both innate and adaptive immune systems. Within the vertebrate models, rodents can provide value for immunologic investigations, but there are notable differences to humans in regard to immune system development, activation, and response to challenge.⁷³ Higher-order species, particularly nonhuman primates (NHPs), tend to

provide greater value for immunologic investigations as they most closely emulate the human immune system.⁷⁴ Immune and inflammatory responses, particularly neutrophils and their products, play a central role in CF lung disease pathogenesis. Accordingly, it is important that researchers understand which animal models are suitable for studies involving infection and inflammation. Among the available CF models, pigs and ferrets are considered the most physiologically relevant models for lung-based immune research.⁷⁵ β -ENaC mice may also provide value for such studies as they demonstrate a neutrophil-dominated immune response in the lungs.²⁹

Desired Genotype and Phenotype

Presence of the desired phenotype(s) in the chosen animal model is crucial. As described above, disease manifestations vary significantly between models; therefore, it is important to select the animal that recapitulates the phenotype of interest. For instance, only a handful of animal models exhibit lung disease; therefore, the researcher is already limited for choice for lung-related investigations. Another consideration is whether *CFTR* expression is disrupted, or the desired mutation is present in the animal model. Some models, such as the β -ENaC mouse, manifest CF phenotypes but have normal *Cftr* expression and are therefore unsuitable for investigations related to CFTR, or for testing therapies that target CFTR.²⁷ Moreover, most CF animal models have *CFTR* completely knocked out, which does not reflect the human disease, where patients display a large variation in the levels and function of the CFTR protein, on the basis of the presence of a combination of *CFTR* mutations.⁷⁶ In addition, only a handful of animal models harbor specific human *CFTR* mutations; therefore, investigation and treatment development for less common variants are not possible without further animal model generation.

Regulation and Restriction of Animal Use

Accessibility of the model is another critical factor as quarantine and licensing restrictions may hinder use of the desired animal model. For example, countries such as Australia have strict quarantine legislation that prevents the import of many species (including CF pigs from the United States or Europe), with the exception of laboratory animals. Furthermore, within the United States, the use of certain animal species is regulated by the US Department of Agriculture, so their use in research is subject to further requirements. Some animal models—particularly commercially developed genetically modified models—may also have licensing agreements that prevent their use or restrict the types of research that can be performed using them. It is important to understand any potential regulations or restrictions before committing to a particular animal model.

In Vitro Models of CF

From an ethical perspective at least, it is important to consider the use of *in vitro* models as an alternative to animal models. Additionally, there are often circumstances where *in vitro* systems are better suited to answering a particular research question. It is widely known that mice are limited in their predictive value for preclinical studies, with many drugs failing in clinical trials as a result.⁷⁷ For instance, mouse *Cftr* has been found to be unresponsive to some CFTR modulators and previous studies trialing potentiator drugs in mouse models have yielded conflicting results,⁴⁵ making their usefulness for testing the efficacy of modulator therapies questionable. An array of *in vitro* CF models has been established for research purposes. Broadly speaking, *in vitro* models can be separated into two categories: two-dimensional and three-dimensional (3D) culture systems. Here, the common CF *in vitro* systems are briefly reviewed to provide the reader with an understanding of alternative options to animal models. Comprehensive reviews of *in vitro* systems for CF can be found elsewhere.^{78,79}

Two-Dimensional Culture Systems

The availability of immortalized cell lines has played a significant role in advancing CF research, particularly via the identification of novel CFTR-modulating compounds. Cell lines are valuable in situations where there is a low abundance of patient tissue or primary cells available for research purposes.⁸⁰ Immortalized cell lines have the advantage of easy culturing and expansion, making them suitable for use in high-throughput drug screening. *CFTR* variants can be readily introduced into cell lines for either transient or stable expression, which is much faster than the rate at which animal models can be generated and their phenotypes characterized. This means they can be particularly useful for assessing the responsiveness of ultrarare *CFTR* mutations, where animal models do not exist, or where primary cells cannot be obtained. Fischer rat thyroid cells stably expressing wild-type *CFTR* or various mutants have been used extensively in the CF field for drug discovery purposes.⁷⁹ Notably, Fischer rat thyroid cells were used in the identification of the CFTR modulator ivacaftor.⁸¹ Other commonly used immortalized lines include CF (CFBE41o-) and wild-type (16HBE14o-) human bronchial epithelial cells.⁸²

It is important to understand that cell line-based systems have considerable limitations. Cell lines are commonly cancer derived or are immortalized by conventional methods, such as transformation with viral oncogenes, processes that can result in genome instability, karyotypic changes, and alterations in gene expression.^{83,84} Immortalized cell lines also lack biochemical and mechanical cues and can lose expression of differentiation features (eg, immortal lung cell lines do not have polarity, tight junctions, mucus secretion, and cilia formation),^{80,82} and as a

consequence they poorly represent the *in vivo* situation.⁸⁵ Immortalized cells are typically used in monocultures, which does not reflect the heterogeneity of *in vivo* tissues, where many different cells interact with one another. To improve recapitulation of the tissue microenvironment, different cell types can be cultured together to study cell-to-cell interactions (eg, a lung system could be generated by co-culturing respiratory epithelial cells and immune cells).^{86,87}

Three-Dimensional Culture Systems

Recent advances in cell biology and technology have enabled the development of a wide range of 3D cell culture technologies.⁸⁵ 3D systems tend to be favored over the two-dimensional cultures, as they more closely resemble *in vivo* morphology, cell interactions, and function. The most valuable *in vitro* models for CF research include primary human airway epithelial cells (HAECs) cultured in air-liquid interface (ALI) systems and intestinal organoids,⁷⁶ and thus will be the focus of this section.

Primary HAECs can be obtained from a range of patient sources, including the nasal turbinates, nasal polyps, trachea, and bronchi.⁸⁶ Although primary HAECs can be cultured in two-dimensional planar systems, established protocols also allow them to be used for the generation of a differentiated cell culture under ALI conditions.^{88,89} The result of ALI conditions is a 3D culture that strongly resembles the human airway, consisting of a polarized, pseudostratified epithelium with mucociliary differentiation. Gene expression patterns of ALI-cultured epithelia are found to closely resemble *in vivo* respiratory epithelium.⁹⁰ As ALI systems faithfully recapture many *in vivo* characteristics of the airway epithelium, they provide ideal platforms for performing functional assays and histologic assessments. ALI cultures have proved useful for trialing a range of candidate treatments, including gene therapies and compounds, such as mucolytics and antimicrobials.^{91–93}

Up until recently, widespread use of ALI cultures was hindered by the limited ability to expand primary HAECs in culture due to rapid onset of replicative senescence. This has now been overcome by improvements in cell expansion techniques, such as conditional reprogramming, which allow cells to be passaged numerous times while still maintaining a normal cellular phenotype, morphology, and disease-specific functional characteristics upon ALI generation.^{84,94} Furthermore, normal and CF airway epithelial cells can now be readily purchased from commercial suppliers or obtained from biobank repositories, avoiding the arduous process of gaining ethical clearances and performing patient tissue collection.

Organoids (or miniorgans) have become an extremely useful tool in CF research. Organoids are derived from stem cells that, under the right culturing conditions, spontaneously form *in vivo*-like organs with differentiated, functioning cells and a distinct internal lumen.⁹⁵ Organoids

retain the genotype and phenotype of the paternal tissue, allowing measures such as swelling assays to be used for rapid and accurate assessments of CFTR activity.^{78,79,96} Non-CF organoids with functional CFTR exhibit a rapid swelling in response to cAMP-inducing agents, such as forskolin, whereas CF-derived organoids have a significantly reduced or absent swelling.⁹⁷

CF- and non-CF-derived respiratory and intestinal organoids are of particular interest for research and can be generated from patient samples. Established airway organoids have been found to replicate an *in vivo* airway, with a polarized, pseudostratified epithelium consisting of ciliated, basal, goblet, and club cells.⁹⁸ Colorectal biopsies are used to generate intestinal organoids that differentiate into multicellular structures that contain all the distinct cell types present *in vivo*. These intestinal organoids can also be readily expanded and maintained in culture long-term.⁷⁸

As mentioned above, 3D culture systems are typically derived from tissue-resident adult stem cells obtained from the relevant patient material. However, recent technological advances now allow for induced pluripotent stem cells to be used as an alternative. Induced pluripotent stem cells can be generated from CF patient cells, such as fibroblasts or blood cells, by introducing specific transcription factors that reprogram the cells to become pluripotent.^{79,99} Protocols are then used to differentiate the induced pluripotent stem cells into CF-relevant epithelia, which can subsequently be used to generate ALI or organoid cultures.^{100,101} Additionally, CFTR mutant and gene-corrected induced pluripotent stem cell lines can be used in a range of applications, including patient phenotype analysis and drug discovery.⁹⁹

When Should *in Vitro* Models of CF Be Employed?

In vitro models are increasingly being employed in different aspects of CF-related research and offer benefits for investigators. Well-powered and high-throughput studies can be performed using *in vitro* systems, as large numbers of replicates can be rapidly produced. Compared with animal studies, the costs associated with performing *in vitro* studies are substantially reduced, and widely established protocols are available for investigators to perform the techniques locally. As patient tissue and cell material are used to generate the *in vitro* systems, less common CFTR variants and compound heterozygotes can also be captured, which is not possible in animal studies. Patient-derived cultures can also be expanded and cryopreserved, enabling generation of biobanks that can be accessed by researchers who do not have the resources or patient availability to perform biopsy collection. Access to biobanks also allows new therapies to be rapidly tested as they become available, thus reducing the time taken for effective treatments to reach patients. To facilitate *in vitro* studies, a range of assays have been developed to rapidly and accurately measure CFTR activity and modulation by therapies.^{97,102–104} *In vitro* systems can also be generated

using tissues acquired from CF animal models to generate data to accompany *in vivo* studies.

Patient-derived *in vitro* systems are proving valuable for developing personalized medicine approaches for CF and predicting which patients will or will not respond to a particular medical therapy, a novel concept termed theratyping.^{76,79,105} Applications include the following: i) improving the understanding of complex or rare CFTR mutations, ii) testing the efficacy and responsiveness of approved modulator therapies on rare CFTR variants to support label expansion to those patients who are not represented in traditional clinical trials (so-called N-of-1 trials), and iii) improving clinical outcomes for patients who already have existing modulators available by determining the optimal combination of CFTR-restoring drugs.^{76,78,79}

Drug discovery programs are also taking advantage of *in vitro* research tools to develop high-throughput screening systems for identifying and testing novel compounds.^{85,96} Although most CF patients now have access to highly effective modulator therapies, around 7% of individuals have CFTR variants that do not produce CFTR protein and therefore cannot be treated by pharmacologic rescue approaches (<https://www.cff.org/Research/Research-Into-the-Disease/Restore-CFTR-Function/Exploring-Treatments-for-Nonsense-and-Rare-Mutations>, last accessed September 16, 2020). The lack of treatments for these patients has intensified the development of corrective strategies, such as gene therapy, gene editing, and RNA therapy. Once promising therapies have been identified, preclinical trials in animal models can be performed to complement *in vitro* data.

Although *in vitro* systems are becoming increasingly useful in CF research, it is important to recognize their limitations. First and foremost, no *in vitro* system is capable of modeling all of the pathophysiological processes that are observed in a whole-body disease, such as CF. Cell cultures are only capable of representing a single tissue or organ; therefore, disease attributes and treatment responses can only be assessed in an independent manner. Furthermore, although ALI cultures and organoids have come close to replicating *in vivo* tissues, they still lack important features. For example, ALI systems do not develop the glandular structures that naturally exist in the airways and are known to play a role in CF pathogenesis. Also, *in vitro* systems may not always accurately predict clinical benefit in CF patients.⁷⁹ Another major limitation of *in vitro* systems is the lack of an immune system. For this reason, investigations of the safety and longevity of novel therapeutics cannot be addressed using cell cultures, and testing with an appropriate *in vivo* model that recapitulates the relevant key features of the human immune response is necessary before proceeding with clinical trials.

There can be logistical limitations to using *in vitro* approaches. First, employing ALI cultures and organoids requires expensive culture media, reagents, and plasticware.

Obtaining patient material to establish cultures necessitates human ethics approval, access to consenting patients, and training in the sample collection protocols. These processes are both time-consuming and location-dependent. Functional assays can also have high levels of variability and be challenging to master, particularly electrophysiological measures that often require specialized equipment and extensive user training. Finally, establishing *in vitro* systems for therapeutics testing requires standardization, validation, and studies to correlate *in vitro* data to clinical response in CF patients.⁷⁹

Future Directions for Research with CF Animal Models

Animal models of CF will remain pertinent to progressing research in both basic and translational sectors for the foreseeable future. Looking forward, there are important questions to consider in relation to the use of CF animal models. First, one should evaluate the costs and benefits of generating CF animal models, and question whether researchers should continue to develop new models. It may also be valuable to assess alternative approaches for improving the accuracy of disease models (eg, NHPs could potentially produce a better model for advancing CF disease understanding and therapeutics). Future CF research is expected to answer these questions.

Are New Animal Models of CF Needed?

It has become apparent over decades of generating CF animals that none of the existing models perfectly recapitulates human CF disease characteristics. In addition, testing therapeutic strategies in animal models, in particular those that target CFTR, can be challenging because of the limited range of models with human CF mutations. Given this, it is likely that the focus on generating CF animal models will shift from using new species to expanding the range of *CFTR* mutations present in currently available models. Considering the high priority to identify and develop effective treatments for patients with nonsense mutations, it is expected that animal models with these types of *CFTR* variants are likely to become more common. The generation of humanized CF models is also an area that is gaining momentum with the successful development of *hCFTR*-expressing mice⁹ and recent generation of a rat model with the hG551D mutation.¹³ Because this approach provides benefits for testing human-specific mechanisms of gene function, regulation, and response to therapeutics in an *in vivo* system,⁴⁷ humanization of other CF models is predicted to occur in the near future.

The cost and time required to generate animal models has reduced significantly with the invention of efficient gene editing technologies, and the ease with which genetic modifications can be made is likely to continue to improve into the future. However, for any animal model to be useful

for researchers, it must first undergo extensive phenotype characterization. Characterizing the disease of an animal model can be time-consuming and resource draining, often taking years to complete. The process of generating and characterizing the model is made more challenging when using species with small litter sizes, seasonal breeding, and long gestations, often leading to a bias toward using species that are easier and cheaper to breed, particularly rodents. Obtaining adequate numbers of animals at the appropriate ages for disease characterization is also difficult if the model demonstrates early mortality, which is often the case for many CF animal models.

Conversely, each of the existing animal models has significantly contributed to improving knowledge and understanding of CF. Although the models differ significantly in their phenotypes, species-dependent differences in disease development can help to elucidate the mechanisms that underlie CF pathogenesis, as well as the factors that are disease-protective.¹⁰⁶ Generation of novel animal models will further improve the CF knowledge base, as it has done in the past. Moreover, the availability of a wide variety of CF animal models provides great value as each species has advantages to offer and researchers can select and utilize models with appropriate characteristics for their applications. Ultimately, it is important to determine if the research question could be addressed with an existing model and to consider whether the benefits of generating a new model outweigh time and financial costs.

Is a Nonhuman Primate Model of CF Needed?

Existing animal models do not and cannot perfectly recapitulate human CF disease attributes. The value of these models in CF research is also dependent on the research question, thereby limiting translatability to humans for some applications. Great apes such as the chimpanzee, old world primates such as macaques and baboons, and new world primates such as marmosets, could together be considered the gold standard for disease modeling as they are the most closely related species to humans and thus have significant physiological, immunologic, anatomic, and genetic similarities.¹⁰⁷ This means that studies employing a nonhuman primate (NHP) model of CF should provide more informative outcomes and better translational capacity for human application. In addition, development of an NHP CF model could lead to the use of fewer animals overall because of improved relevance of scientific investigations.¹⁰⁸

Renewed interest in the use of NHPs for modeling human diseases has resulted from significant advances in genetic engineering technologies that now allow for the genomes of large mammals to be precisely modified. Although technically feasible, gene editing NHPs has additional challenges when compared with that in rodents. The costs are increased and the allowable tolerance for error is lower as their unfavorable reproductive characteristics means the time required to produce genetically modified NHPs is much greater.^{108–110}

Given their close similarities to humans, it is highly likely that an NHP model will provide a more accurate model of CF disease.¹¹¹ Unsurprisingly, NHPs have the highest degree of CFTR homology with humans. For example, rhesus macaques, one of the most widely used NHPs in biomedical research,¹⁰⁷ have a 98% identity with human CFTR at the amino acid level.¹¹¹ This is particularly valuable for reproducing causative *CFTR* mutations as the molecular defect and subsequent phenotypes are more likely to accurately reflect the human situation. Primates also have longer life-spans, which may allow for a more human-like disease course for studying pathogenesis and treatment responses longitudinally.

In regard to lung disease development, NHPs have similar lung cellular architecture, airway branching, and immunologic structures to humans. For these reasons, NHPs have been used to model inflammatory lung diseases as well as respiratory viral and bacterial infections. Useful techniques have also been established in NHPs to enable lung-related investigations, including endotracheal intubation, bronchoscopy, and bronchoalveolar lavage.¹¹² Because of their advantages for lung-based studies, the rhesus macaques, common marmosets, and chimpanzees have previously been used to trial the efficacy and safety of candidate CF airway gene therapies.^{113–115} Some early studies of viral gene therapies in rodents were found to poorly predict the effectiveness of gene transfer to human airway epithelial cells and the magnitude of the immune response to the vector; therefore, it is reasonable to suggest that NHPs would serve as a superior model for determining the behavior of viral-mediated gene transfer in humans.¹¹⁵

Despite their potential superiority for modeling diseases, the use of NHPs in biomedicine prompts significant considerations. NHPs have special requirements, and considerable expertise and resources are needed to ensure their appropriate care. For housing, NHPs have strict minimum space requirements and require environmental enrichment and social grouping. In addition, the availability of the species, high cost, and difficulty breeding large numbers could be significant limiting factors.¹⁰⁷ The increased life-span of NHPs also means they may take longer to exhibit the desired disease state.¹⁰⁸ A CF NHP is likely to exhibit meconium ileus, and like CF pig and ferret models, they are expected to require expensive and ongoing medical care to manage life-threatening disease aspects. However, certain disease traits can be well managed or altogether mitigated (eg, fatal gut obstruction could be transgenically corrected by expressing *CFTR* in the intestines, as has been done with other CF models).

Most importantly, because of their elevated perceptive and cognitive characteristics, using NHPs in biomedical research is rightly a contentious issue that raises serious ethical questions, particularly when modeling human diseases. CF is a chronic and painful condition in humans, often requiring substantial treatment to prevent rapid health decline, and has a large impact on quality of life. An NHP

model of CF is likely to result in a high welfare burden to the animals. If a CF NHP was to be developed, careful consideration of experiments would be necessary in addition to a health monitoring program to assess the psychological and physiological effects of the disease state, along with appropriate steps to mitigate associated pain, discomfort, or physical and emotional distress.¹⁰⁷

Logistical factors, ethics arguments, and welfare impact must be considered when employing NHPs in research. Ultimately, though, scientific reasoning should be used to justify whether a CF NHP should be generated and if it would be the most appropriate model for research. It is likely that an NHP model of CF would more closely recapitulate human disease and could improve understanding of CF pathophysiology and facilitate treatment development. However, the CF field has proved that research can successfully advance with currently available *in vivo* and *in vitro* models (eg, highly effective CFTR modulator therapies were largely developed in cell culture systems). Given that research will undoubtedly progress without the availability of a CF NHP model, it is unlikely that an NHP model would provide enough benefit to warrant its generation.

Conclusion

Animal models have significantly advanced knowledge of CF, enabling us to better understand disease pathogenesis and to accelerate research into novel concepts and therapies. Numerous CF animal models and *in vitro* systems have been developed for research purposes, which can make it challenging for researchers to determine which model to select. It is important to acknowledge there is no perfect model of CF disease when it comes to either *in vivo* or *in vitro* systems. Each model or tool has limitations that users must recognize to draw accurate conclusions from their research study.

The generation of novel CF animal models is likely to continue because of widespread availability of rapid and cost-efficient gene editing technologies. Although new models will surely contribute to a growing body of knowledge, generating and characterizing a CF animal model is no small feat and consideration should be made as to whether the research question could be answered with the currently available *in vivo* and *in vitro* models. Acknowledging that no perfect model currently exists, the question of generating an NHP CF model naturally arises. A CF NHP could offer a more accurate model of disease and provide better translatability to humans, but at this stage the costs of producing such a model do not appear to outweigh the benefits. In addition, significant innovation in the field can still progress with the use of currently existing models. Ultimately, selection of the right research model, animal or *in vitro*, should be at the forefront of every investigation.

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