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Lessons learned from the cystic fibrosis pig

David K. Meyerholz

Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242

Abstract

Deficient function in the anion channel cystic fibrosis transmembrane conductance regulator (CFTR) is the fundamental cause for cystic fibrosis (CF). This is a monogenic condition that causes lesions in several organs including the respiratory tract, pancreas, liver, intestines, and reproductive tract. Lung disease is most notable given it is the leading cause of morbidity and mortality in people with CF. Shortly after the identification of CFTR, CF mouse models were developed that did not show spontaneous lung disease as seen in humans and this spurred development of additional CF animal models. Pig models were considered a leading choice for several reasons including their similarity to humans in respiratory anatomy, physiology and in size for translational imaging. The first CF pig models were reported in 2008 and have been extremely valuable to help clarify persistent questions in the field and advance understanding of disease pathogenesis. Because CF pigs are susceptible to lung disease like humans, they have direct utility in translational research. In addition, CF pig models are useful to compare and contrast with current CF mouse models, human clinical studies and even newer CF animal models being characterized. This “triangulation” strategy could help identify genetic differences that underlie phenotypic variations, so as to focus and accelerate translational research.

Keywords

Animal model; Cystic fibrosis; CFTR; Lung disease; Pig model

1. Introduction

Cystic fibrosis (CF) is a recessive, monogenic disease caused by mutations in the gene encoding an anion channel, cystic fibrosis transmembrane conductance regulator (CFTR) [1]. Well over a thousand mutations have been reported, but the most common mutation is a deletion of phenylalanine in position 508 (F508) [2]. The F508 mutant protein has a processing defect permitting only a small portion of the CFTR to reach and function along the apical membrane [3–6]. Given the high incidence of this mutation and the recent advent of modulator therapies, there is scientific interest in the potential of using F508 models to study novel management strategies and therapies.

Contact information: David K Meyerholz, 500 Newton Road, 1165 Medical Laboratories, Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, ; Email: david-meyerholz@uiowa.edu

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Cystic fibrosis begins early in life and was considered a disease of young children, but thanks to decades of medical advances a newborn CF baby is now predicted to have a median survival of nearly 40 years (www.cff.org). The disease affects several organs including respiratory tract, pancreas, liver/gallbladder, intestines, reproductive tract and sweat glands [4, 7–9]. Respiratory disease is the most recognized clinical feature in CF because it is the leading cause of morbidity and mortality in CF patients. Lung disease is characterized by persistent airways infection, chronic inflammation and tissue remodeling [10] (Figure 1).

2. CF pig development

Clinical studies on humans are often limited by variations in treatment, secondary disease changes/remodeling, lack of adequate controls as well as appropriate ethical constraints. Thus, animal models serve as a useful surrogate for study in CF. Shortly after the *CFTR* gene was identified, several CF mouse models were developed; however, these models consistently lacked characteristic lesions including spontaneous respiratory disease as seen in humans. Why might CF mice have lacked disease? Several theories have been proposed [11]. Some of these include a short life span (~two years), small body size, lack of submucosal glands in lung airways, differences in modifier gene expression, etc. Since then, several other species have been targeted for study of CF including the pig [12], ferret [13], zebrafish [14], the rat [15] and others such as the sheep are being developed [16].

2.1 Pigs are an excellent candidate species for CF study

Pigs were considered a leading species candidate for genetic manipulation of *CFTR* because of several reasons [17, 18]. The pig has similar size, anatomy and physiology to humans. Pigs can live much longer (10 to 15+ years) than rodents with a generation interval of about 12 months and year-long breeding capacity. More specific to modeling CF lung disease [19], pigs have been used as models of pneumonia including viral [20] and bacterial [21] diseases, pigs have lung tissue markers that are similar to humans [22] and pigs are of similar size for translational lung imaging [19, 23]. Anatomically, pigs have submucosal glands, relevant target tissues for CF pathogenesis, which extend along cartilaginous airways into the pulmonary parenchyma [24, 25]. These features help exemplify why it would make a useful model species for CF lung research.

2.2 CF pig models

The first CF pigs were developed using a stop codon in exon ten of *CFTR*. Somatic cell nuclear transfer was used make heterozygous pigs and through subsequent heterozygous × heterozygous breeding, *CFTR*-null pigs were born [12]. At birth, CF pigs were characterized by severe intestinal obstruction (i.e. meconium ileus) that required surgical intervention, similar to about half of meconium ileus cases in CF infants [8, 26]. Another homozygous *CFTR*-null pig model was made using a STOP box that stopped message and protein synthesis in exon one and it displayed a similar phenotype [27]. The F508 mutation is common in people with CF and it confers partial *CFTR* function at the apical surface of cells, thus a F508 pig was developed with hopes that it might be able to partially overcome the meconium ileus phenotype seen in *CFTR*-null pigs. The F508-pig had a similar to

slightly less severe meconium ileus phenotype when compared to CF-null pigs [28]. Importantly, the porcine mutant F508 CFTR had a processing defect – similar to humans – but with only ~six percent CFTR activity compared to non-CF in airway epithelia and this was insufficient correction to prevent CF airway disease. With the advent of modulator therapeutics [29], the F508 pig could provide an attractive large animal model to test therapeutic strategies or drug combinations during various stages of lung disease.

To overcome the meconium ileus seen in newborn CF-null and CF- F508 pig models, a gut-corrected model was later generated using a transgene for porcine CFTR under the fatty acid binding protein 2 (FABP2) promotor. The results of this model suggested that raising the levels of intestinal CFTR message to as little as 20% of non-CF could mitigate severe meconium ileus at birth and importantly, these pigs retained lesions in other organs consistent with CF for use in postnatal studies [30].

2.3 CF pig phenotype

Phenotyping of CF pig models were needed to confirm that these animals mimicked the human condition. At birth, CF pigs had destruction of the exocrine pancreas, focal biliary cirrhosis, microgallbladder, segmental absence of the vas deferens, impaired glucose tolerance, deficient insulin-like growth factor-1 and meconium ileus obstruction. These features exemplify a broad scope of changes that are consistent with human CF. This emphasizes that CF pigs mimic the human condition to overcome several of the deficiencies found in CF mouse models [9, 12, 26, 28, 30–34].

While newborn pigs had severe changes in many organs, but it was surprising that the lungs of newborn CF pigs lacked evidence of inflammation. Even so, CF large airways had structural changes with reduced circularity, smaller caliber, hypoplastic submucosal glands and lesions in smooth muscle and cartilage rings [12, 28, 30, 35].

3. CF pigs and understanding of CF lung disease

Lung disease is the leading cause of morbidity and mortality in CF patients [10], therefore, the remainder of this review will emphasize how the CF pig model has helped to clarify our understanding of CF respiratory disease. While this targeted review will not necessarily discuss all the clinical features of CF lung disease (*Pseudomonas* infection, leukocytes, etc.), it will focus only on those that the CF pig model has significantly increased scientific understanding and perspective. For additional information on CF, including its clinical features, lesions, and animal models useful for translational studies, the reader is also encouraged to see recent publications [3, 7, 8, 10].

3.1 Which comes first: Infection or inflammation?

Recurrent to persistent airway inflammation and infection are cardinal features of CF lung disease [36, 37]; however, there had been enduring controversy as to whether inflammation or infection comes first. This has implications for clinical therapies and treatment of disease exacerbations. To address this “chicken or egg” type question, a model was required that faithfully recapitulated CF lung disease. At birth, CF pigs lacked evidence of inflammation in the lung, but in the ensuing postnatal period (i.e. weeks to months) spontaneously

developed disease characterized by inflammation, infection and remodeling of respiratory (lung and sinus) tissues [12, 26, 28, 30, 38]. While these data suggest that inflammation is not an innate phenotype in CF pig lungs, ongoing studies continue to evaluate the immune system for immunophenotypes.

3.2 CF lungs have defective bacterial clearance

To further investigate the origins of lung infection, CF pig lungs were evaluated for bacteria in the hours after birth. Lungs from CF pigs were less sterile and had higher bacterial counts than their non-CF littermates [26]. A wide breadth of environmental bacteria were isolated in these lungs suggesting this is an “equal opportunity” host defense defect [10]. Newborn pig airways were then challenged with aerosolized *Staphylococcus aureus* and after four hours CF pigs had more bacteria as they were less able to clear bacteria than non-CF lungs [26]. These combined data would suggest that newborn CF pig lungs lack inflammation, but already have a defect in bacterial clearance that increases susceptibility to lung infection. Together, these data would suggest that respiratory infection precedes inflammation in CF pig lung disease.

3.3 Anion transport and pH

It is known that CFTR is an anion channel and CF pig airway epithelia demonstrated significantly reduced chloride and bicarbonate transport compared to non-CF [39]. In contrast to some theories on CF pathogenesis, there was an absence of sodium or liquid hyperabsorption in CF epithelia, and these findings were consistent with subsequent studies on human tissues [39, 40]. As a consequence of the reduced anion transport seen in CF, one could speculate that the loss of bicarbonate transport (possibly along with other mediators) might contribute to reduced alkalization of the airways surface liquid (ASL). Newborn CF pig ASL was examined and found to have a lower pH compared to non-CF controls. But how might the lower pH influence host defense(s)?

3.4 Role of pH on endogenous antimicrobials

Normally, the lung secretes antimicrobial substances in the ASL that helps form a protective layer against inhaled microbial substances [41]. Evaluation of ASL did not show any differences in the quantity of several antimicrobial peptides and proteins (APPs) including lactoferrin and lysozyme; however, evaluation of the ability of these APPs to kill bacteria were reduced in a lower pH environment [42, 43]. Regardless of genotype, ASL pH could be manipulated higher for more effective bacteriocidal action and lower for less effective bacteriocidal action. These results suggest the reduced pH in CF airways make them prone to infection.

3.5 Airway structural defects in CF

In early autopsy studies, structural airway changes/remodeling (e.g. bronchiectasis) were recognized as features of CF disease [9, 36, 44]. Whether these structural changes were exclusively remodeling events secondary to postnatal disease, or if some of these changes were congenital in nature remained elusive. In 2008, CF mice were reported to have congenital tracheal defects [45], but at the time the translational significance of this finding

was not clear. At that same time, newborn CF pigs were observed to have small caliber tracheas with decreased circularity, and structural alterations in smooth muscle, cartilage and submucosal glands [35, 46, 47]. How might these structural changes clinical affect CF airways? Recently, a computerized model was used to test if airway structural defects could alter the fluid dynamics of airflow in CF airways [48]. Altered structure of CF airways caused increases in air flow velocity as well as resistance. Further, these airflow changes in the model predicted an enhanced and more irregular deposition of inhaled particulates in CF lungs. These suggest that CF airways might be prone to enhanced environmental contamination by microbes and pathogens [48]. In another study, newborn pigs were evaluated prior to postnatal accumulation of mucus or inflammation in airways, and showed increased evidence of air trapping and air flow obstruction by computer tomography [46]. Speculatively, these could have functional correlates with enhanced air flow resistance as predicted in the computerized model described above. A possible *in vivo* correlate supporting the idea for congenital structural changes in CF is that of the sinuses. In newborn CF pigs, ethmoid sinuses were hypoplastic suggesting a role for CFTR in fetal development [38]. This area of research is extremely limited in humans, so animal models that mimic CF lung disease must take the lead if we are to learn about CFTR's role during development.

Finding airway structural changes in CF pigs were compelling, but it remained unclear as to how this related, if at all, to human CF lung disease. Because pathological data of CF and non-CF control tracheas were lacking, historical published data was re-examined to identify autopsies from young infants. Even though CF infants were larger in size than their non-CF controls, they had small caliber tracheas - similar to that seen in the CF pig [35, 49]. Subsequent studies have added further evidence that CF tracheas are abnormal, even early in life, corroborating the data seen in the pig [47, 50]. It is inspiring to think that the CF pig model helped to identify a previously unrecognized clinical phenotype in CF infants. This finding has already helped to change medical paradigms on CF newborn care and treatments.

3.6 Nontraditional tissues in CF lung disease?

Historically, the sites of CFTR function had been mostly inferred to various epithelial tissues and organs, matching the distribution of major lesions. Studies in the CF pig have helped expand this definition to other nontraditional tissues types. For instance, recent work discovered CFTR is in Schwann cells, a glial cell of the peripheral nervous system that forms myelin sheaths around axons and contributes to effective nervous impulses. In CF pigs, the myelin sheaths of nerves had morphologic abnormalities along with expression of disease markers, physiologic changes and reduced conductance velocities – all features consistent with a neuropathy [51]. Besides the nervous system, other examples such as smooth muscle, cartilage, and the vasculature have shown abnormalities in the CF pig [35, 52, 53]. It will be interesting to see how the pig model can further define if these changes are primary - due to the loss of CFTR – and if these tissue changes contribute significantly to the development of CF disease.

3.7 Abnormal secretions in CF

Historically, CF has also been known by another name, “mucoviscidosis”. Farber, a physician, used this terminology in the mid twentieth century to describe the thick tenacious material often obstructing diseased CF lung and other tissues [54]. Similarly, newborn pigs have thick tenacious material obstructing organs such as the intestine (as part of meconium ileus) and the pancreas causing exocrine pancreas destruction [31]. Interestingly, when CF pigs develop sinus or airway disease, the obstructive material has similar tenacious properties to that seen in diseased airspaces of humans [26, 38, 55]. Thus, tenacious secretions, which are a consistent in CF pigs and humans, warrant further study to define what causes it and how to treat it more effectively.

3.8 Mucociliary Clearance

Submucosal glands contribute to fluid and mucus secretions for effective mucociliary clearance (MCC); an important host defense mechanism to physically move inhaled particulates and pathogens out of the lungs [56]. Study of the CF pig might identify lesions in submucosal glands contributing to CF disease. Newborn pig tracheas showed that CF submucosal glands were hypoplastic and had reduced secretion compared to non-CF controls [35, 57]. To further clarify the submucosal defects on MCC in trachea, radiodense microdisks were used to track MCC [58]. Under basal conditions, MCC showed no genotype differences, but under cholinergic stimulation to initiate mucus secretion, the microdisks became “stuck” in CF tracheas consistent with defective MCC. To exclude dehydration as a factor, excised tracheas were submerged under water and microdisks were again stuck following stimulation. Further analysis showed these microdisks were adhered to tethered mucus coming from submucosal gland ducts. These experiments suggest that submucosal gland mucus secretions fail to detach resulting in mucus tethered to airway walls and defective MCC. From these data, submucosal glands and its mucus secretions may be a therapeutic target to improve MCC.

4. Summary

This review paper has highlighted several areas that the CF pig has helped to clarify our understanding of CF, particularly in the pathogenesis of CF lung disease. These models have helped us learn more about proximate effects resulting from deficient CFTR, as well as how these can result in clinical effects that contribute to CF disease development. Some examples can be seen in Figure 2.

The CF pig is a leading example of how genetically modified pig models can significantly contribute to and advance understanding of human disease. These advances not only help clarify disease pathogenesis, but accelerate future research towards novel ideas and new targets for therapy [10, 59]. In CF, the pig model also provides an additional perspective to compare and contrast against traditional rodent studies, clinical human studies and newer CF animal models. This “triangulation” strategy could help identify genetic differences that underlie phenotypic variations so as to focus and accelerate translational research efforts.

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Figure 1. Human CF lung from an archival autopsy tissue block. Note the CF airway lumen is obstructed (arrows) by neutrophilic cellular inflammation and some mucus. Bar = 800 μ m.

Figure 2.

Proposed relationship(s) between deficient CFTR function and CF lung disease. Deficient CFTR function causes proximate effects that in turn create a local environment for development of clinical effects eventually manifesting as CF lung disease. While these relationships are still being clarified, the CF pig model has been instrumental to begin understanding these complex relationships.