[9] Heuman DM, Abou-Assi SG, Habib A, Williams LM, Stravitz RT, Sanyal AJ, et al. Persistent ascites and low serum sodium identify patients with cirrhosis and low MELD scores who are high risk for early death. Hepatology 2004;40:802–810.
[10] Ruf AE, Kremers WK, Chavez LL, Descalzi VI, Podesta LG, Villamil FG. Addition of serum sodium into the MELD score predicts waiting list mortality better than MELD alone. Liver Transpl 2005;11:336–343.
[11] Arroyo V, Rodes J, Guttieres-Lizarraga MA, Revert L. Prognostic value of spontaneous hyponatremia in cirrhosis with ascites. Am J Dig Dis 1976;21:249–256.
[12] Kim R, Biggins SW, Kremers WK, Wiesner RH, Kamath PS, Benson JT, et al. Hyponatremia and mortality among patients on the liver transplant waiting list. New Engl J Med 2008;359:1018–1026.

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Are pigs more human than mice? ☆

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Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ.

Almost two decades after CFTR was identified as the gene responsible for cystic fibrosis (CF), we still lack answers to many questions about the pathogenesis of the disease, and it remains incurable. Mice with a disrupted CFTR gene have greatly facilitated CF studies, but the mutant mice do not develop the characteristic manifestations of human CF, including abnormalities of the pancreas, lung, intestine, liver, and other organs. Because pigs share many anatomical and physiological features with humans, we generated pigs with a targeted disruption of both CFTR alleles. Newborn pigs lacking CFTR exhibited defective chloride transport and developed meconium ileus, exocrine pancreatic destruction, and focal biliary cirrhosis, replicating abnormalities seen in newborn humans with CF. The pig model may provide opportunities to address persistent questions about CF pathogenesis and accelerate discovery of strategies for prevention and treatment.

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In the era of molecular genetics we tend to think that once we have discovered the gene that is responsible for a monogenetic disease, we will understand the pathogenetic mechanisms of this disease. For this purpose, we "simply" make a knockout mouse for this gene, which we believe will always solve the problem. Alas, in many cases this is far from true. Just as an example: Niemann-Pick C disease is an inherited storage disorder characterized by cholesterol accumulation in the lysosomes; the gene (*NPC1*) mutated in this disease was identified in 1997 [1] and mice with a defect in the *Npc1* gene were available at that time. In spite of intense research for two decades by many groups, the function of this gene is still unsolved.

The situation is only slightly better for cystic fibrosis. The responsible gene, the cystic fibrosis transmembrane regulator (CFTR), was identified in 1989 [2] and knockout mice have been available since 1993 [3]. CFTR is known as a cAMP-regulated chloride channel, but various other functions have been attributed to this protein as well. Cystic fibrosis as a disease has many appearances and few of them are well understood. A complicating factor is that the $Cftr^{-/-}$ mouse as a disease model is far from a phenocopy of this multi-organ disease.

Cystic fibrosis is a common, potentially fatal genetic disorder affecting about 1 in 3000 live births [4]. Seventy percent of the patients carry the ΔF508 mutation in which the amino acid phenylalanine at position 508 is deleted. This mutant protein is not properly folded and largely broken down in the endoplasmatic reticulum. The high frequency of this genetic disorder suggests that heterozygotes for *CFTR* mutations may have some selective advantage. It has been proposed that carriers are more resistant towards cholera toxin-induced diarrhoea. Cystic fibrosis is a devastating disease that affects many tissues, including lungs, pancreas, intestine and liver. Thickened mucus is the hallmark of this disease leading to secretory failure of these organs. Meconium

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ileus after birth, pulmonary infections and exocrine pancreatic insufficiency may lead to death at an early age. In patients surviving all these complications in early life, the liver may increasingly come into focus with a cholestatic, frequently progressive liver damage associated with multifocal fibrosis.

The pathogenesis of cystic fibrosis-associated liver disease (CFALD) is not properly understood. making the development of adequate therapeutic strategies difficult. Hence, it is of utmost importance that an animal model is available to study the mechanism of pathology in various organs. Because of the available stem cell technology the mouse usually represents the animal model of choice for introduction of gene mutations and deletions. Indeed, a large collection of mouse strains with different mutations and deletions, including complete knockout mice ($Cftr^{-/-}$ mice) has been produced and tested against different genetic backgrounds (for review see [5]). The bottom line of these studies is that mice develop only limited pathology. All strains developed an intestinal phenotype with different degrees of abnormalities depending on genetic background and the nature of the mutation, leading to premature death in a wide range from 5% to 95%. Using $Cftr^{+/-}$ mice it could be demonstrated that impaired Cftr function provides a resistance against the effects of cholera toxin [6], providing evidence for the theory that carriers of *CFTR* mutations have a selective advantage during an epidemic of cholera. In contrast, none of these mouse strains developed spontaneous lung disease or pancreatic pathology. More important in the present context was the finding that liver pathology was also absent or minimal. Some focal biliary fibrosis, inflammation and steatosis was seen in $Cftr^{-/-}$ mice against C57Bl/6 background but this was mild and only seen after extended periods (>1 year of life) [7]. Conversely, $Cftr^{-/-}$ mice have a very marked defect in enamel deposition on the growing incisors, whereas CF patients have minimal defects in enamel deposition [8]. Enamel deposition requires the joint action of CFTR and the chloride bicarbonate exchanger AE2 in ameloblasts [9]. This striking different phenotype may be caused by the fact that, in contrast to the situation in humans, rodent incisors continuously grow which may require much larger chloride and bicarbonate fluxes.

The conclusion of these extensive studies must be that mice are not the most suitable animal model to study the disease mechanism of CF, particularly not if one is interested in CF-related liver disease.

In a recent issue of *Science* the group of Michael Welsh report on a pig model for cystic fibrosis [10]. The authors disrupted the *Cftr* gene by homologous recombination in fibroblasts from fetal pigs. The nuclei from these fibroblasts were transferred to porcine oocytes, which were subsequently used to generate embryos. The authors decided to undertake this ambitious project because mouse models are clearly insufficient and because pigs are "in terms of anatomy, biochemistry, physiology, size, life span and genetics, more similar to humans than mice". The latter argument appears to be more intuitive than based on scientific data and the fact that the same group was also involved in genetic disruption of the ferret *Cftr* gene [11] demonstrates that they did not put their money on a single horse (or pig?). Fortunately, their investment paid off and the produced $Cftr^{-/-}$ pigs displayed more pathology than the corresponding mice. The animals developed meconium ileus, destruction of the exocrine pancreas, as well as male infertility (the latter also being a typical phenotype of human cystic fibrosis). In addition, these pigs developed liver disease that resembles human pathology: histology revealed cellular inflammation, ductular hyperplasia and mild fibrosis, typical for focal biliary cirrhosis. Similar to CF patients, the $Cftr^{-/-}$ pigs had small gallbladders filled with congealed bile and mucus.

These observations raise the question which differences between pigs and mice cause liver disease in the first and not in the latter. The histopathology in pigs and humans suggests a problem concerning bile formation. Indeed, the only cell type in the liver that normally expresses CFTR is the cholangiocyte and the protein is localized in the apical membrane of this cell, where it most likely mediates chloride conductance. Bile formation is an osmotic process driven by active excretion of solutes. Bile acids are the main solute and generate "bile acid dependent bile flow". The two main other solutes, generating "bile acid independent bile flow", are glutathione and bicarbonate. Bicarbonate secretion occurs across the apical membrane of cholangiocytes and is, therefore, generated in the ducts whereas bile acids and glutathione are excreted by the hepatocytes [12]. It is assumed that this mechanism of ductular flow involves chloride secretion via CFTR and subsequent chloride/bicarbonate exchange (possibly via the anion exchanger AE2 that has been localized in the apical membrane of the cholangiocyte [13]). More recently two papers in *Gastroenterology* modified this model and provided evidence to suggest that Cftr mediates release of ATP which triggers apical purinergic receptors in an autocrine fashion, giving rise to stimulation of apical chloride channel activity and subsequent chloride/bicarbonate exchange [14,15]. In the latter mechanism Cftr represents a regulator of bicarbonate secretion rather than a driving force. In addition, there is evidence to suggest that bicarbonate enters bile via a mechanism that is independent of chloride secretion [5]. In other words the mechanism of bile ductular bicarbonate secretion is not firmly established. Whatever the mechanism may be, it is clear that the contribution of ductular flow to overall bile flow is very different from species to species. Mice have a relatively small "bile acid independent

bile flow" (about 25% of total bile flow [16], which largely consists of ductular flow) whereas in humans the contribution may be as high as 40% [17]. In pigs, the contribution is also considerable (about 50%, [18]). Hence, impaired ductular flow, caused by defective CFTR function may have a bigger impact in humans and pigs than in rodents.

A next level of potential differences between mice and pigs may be the bile acid composition of the two species. In a situation of impaired flow, bile salt toxicity may represent a more important parameter than under conditions of normal, high flow as is also the case in other cholestatic conditions such as primary biliary cirrhosis. Indeed, in both diseases replacement of the bile acid pool by administration of ursodeoxycholate has proven to alleviate the pathology by increasing bile acid dependent and bile acid-independent flow as well as by reducing the cytotoxicity of the produced bile [19]. Due to its physicochemical properties UDCA has an extremely low cytotoxicity. If this represents an important determinant in CF liver pathology, the cytotoxicity of the endogenous bile acid pool may determine the extent of liver pathology in various species as well. The cytotoxicity of bile acids is determined for a large part by their detergent capacity (i.e. their capacity to extract phospholipids from membranes [20]). It is well known that the detergent capacity of murine bile acids is low. This is because mice mainly produce trihydroxy bile salts muricholate (detergent capacity even less than UDCA) and cholate (higher than UDCA). Moreover, mice are very efficient in rehydroxylating secondary bile acids, such as deoxycholate, back to muricholate and cholate after reabsorption in the intestine. Humans have a much lower rehydroxylation capacity and this is why the human bile salt pool contains substantial amounts of deoxycholate (much stronger detergent capacity). In this respect pigs are a particular species, because they synthesize mainly hyocholate, a trihydroxy bile acid with comparable detergent capacity to UDCA. However, the secondary bile acid hyodeoxycholate (with stronger detergent capacity than cholate) is not rehydroxylated by the pig. Hence, in terms of bile acid cytotoxicity, the pig is intermediate between mice and man.

Obviously, the "genetic make up" of pigs differs from mice in many more aspects. It may for example be that pigs have much less capacity than mice to compensate for the loss of Cftr in cholangiocytes by overexpression of other chloride channels. The strong influence of genetic background on the CF phenotype in mice demonstrates that even within one species the genetic environment is of great importance. The same holds for humans; studies in mono- and dizygotic twins have shown that modifier genes play a role in the development of CF symptoms. It is interesting though that so far only a very limited number of genes have proven to be true modifiers of CF [21].

All in all it can be concluded that the CF pig is an attractive animal model to study CF pathology. Nevertheless, the burden to perform studies with pigs is enormous in various terms. Hence, it will remain attractive to use the information derived from pig studies and translate them back to mice, so that an improved mouse CF model can be generated in the future which finally may help to develop more efficient therapeutic strategies for patients with CF.

References

[1] Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, et al. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science 1997;277:228–231.
[2] Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066–1073.
[3] Gabriel SE, Clarke LL, Boucher RC, Stutts MJ. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. Nature 1993;363:263–266.
[4] Feranchak AP, Sokol RJ. Cholangiocyte biology and cystic fibrosis liver disease. Semin Liver Dis 2001;21:471–488.
[5] Guilbault C, Saeed Z, Downey GP, Radzioch D. Cystic fibrosis mouse models. Am J Respir Cell Mol Biol 2007;36:1–7.
[6] Gabriel SE, Brigman KN, Koller BH, Boucher RC, Stutts MJ. Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. Science 1994;266:107–109.
[7] Durie PR, Kent G, Phillips MJ, Ackerley CA. Characteristic multiorgan pathology of cystic fibrosis in a long-living cystic fibrosis transmembrane regulator knockout murine model. Am J Pathol 2004;164:1481–1493.
[8] Arquitt CK, Boyd C, Wright JT. Cystic fibrosis transmembrane regulator gene (CFTR) is associated with abnormal enamel formation. J Dent Res 2002;81:492–496.
[9] Lyaruu DM, Bronckers AL, Mulder L, Mardones P, Medina JF, Kellokumpu S, et al. The anion exchanger Ae2 is required for enamel maturation in mouse teeth. Matrix Biol 2008;27:119–127.
[10] Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 2008;321:1837–1841.
[11] Sun X, Yan Z, Yi Y, Li Z, Lei D, Rogers CS, et al. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. J Clin Invest 2008;118:1578–1583.
[12] Anwer MS. Cellular regulation of hepatic bile acid transport in health and cholestasis. Hepatology 2004;39:581–590.
[13] Martinez-Anso E, Castillo JE, Diez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. Hepatology 1994;19:1400–1406.
[14] Fiorotto R, Spirli C, Fabris L, Cadamuro M, Okolicsanyi L, Strazzabosco M. Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. Gastroenterology 2007;133:1603–1613.
[15] Minagawa N, Nagata J, Shibao K, Masyuk AI, Gomes DA, Rodrigues MA, et al. Cyclic AMP regulates bicarbonate secretion in cholangiocytes through release of ATP into bile. Gastroenterology 2007;133:1592–1602.
[16] Oude Elferink RP, Ottenhoff R, van WM, Smit JJ, Schinkel AH, Groen AK. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J Clin Invest 1995;95:31–38.
[17] Nathanson MH, Boyer JL. Mechanism and regulation of bile secretion. Hepatology 1991;14:551–566.

[18] Duerksen DR, Van Aerde JE, Gramlich L, Meddings JB, Chan G, Thomson AB, et al. Intravenous ursodeoxycholic acid reduces cholestasis in parenterally fed newborn piglets. Gastroenterology 1996;111:1111–1117.
[19] Beuers U. Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006;3:318–328.
[20] Cohen DE, Leonard MR. Immobilized artificial membrane chromatography: a rapid and accurate HPLC method for predicting bile salt-membrane interactions. J Lipid Res 1995;36:2251–2260.
[21] Collaco JM, Cutting GR. Update on gene modifiers in cystic fibrosis. Curr Opin Pulm Med 2008;14:559–566.

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