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Cystic fibrosis

Ferreting with fibroblasts for cystic fibrosis

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Murine models of human genetic diseases have been created using gene-targeted embryonic stem cells for over a decade, however, for many diseases, such as cystic fibrosis (CF), mice have failed to replicate crucial aspects of the human phenotype. In two recent back-to-back publications by Sun *et al*.[1] and Rogers *et al*.,[2] pig and ferret models for CF have been created for the first time.

The embryonic stem cell-based cloning strategies developed for mice had never worked for other species, but 10 years ago somatic cell nuclear transfer came to the rescue and paved the way for the creation of Dolly the sheep. Nuclei isolated from somatic cells (fetal fibroblasts) were transferred into enucleated donor oocytes, which were subsequently implanted into pseudopregnant sheep, thereby opening the possibility of manipulating the genome of the somatic cells before transfer. Gene targeting of somatic cells suitable for somatic cell nuclear transfer has been challenging, however, and only a few gene-targeted non-rodent models have been generated to date. These include pigs for organ transplantation, and sheep for the production of secreted protein. The two recent papers by Sun *et al*.[1] and Rogers *et al*.[2] employ somatic cell nuclear transfer of gene-targeted fetal fibroblasts to generate pig and ferret models of genetic disease. The authors have targeted the CF gene, but the technology used will be widely applicable for other genetic diseases as well.

During the course of their research the authors, many based at the University of Iowa, had to overcome several important hurdles. The first was that classical homologous recombination used to 'knockout' specific genes is extremely inefficient in somatic cells, compared with murine embryonic stem cells. Most gene-targeting strategies to date have employed promoter-trap strategies, which involve the insertion of a targeting construct carrying a promoterless selectable marker gene (such as antibiotic resistance) close to the promoter of a transcribed gene ('promoter-trap'). This leads to expression of the selectable marker and enrichment of those cells in which recombination events have occurred when exposed to the relevant antibiotic. Promoter-traps require the target gene to be expressed but, unfortunately, the CF gene (*CFTR*) is not expressed in fetal fibroblasts so alternative strategies had to be sought. The selection of adeno-associated virus to deliver the targeting vector was an inspired decision. Various adeno-associated virus serotypes efficiently transduce fetal fibroblasts and deliver single-stranded DNA to the nucleus, avoiding the need for large quantities of DNA to be delivered by other means and reducing the risk of random, non-homologous recombination. The targeting frequency was approximately 0.01% in pig and 0.5–2% in ferret fibroblasts.

The second hurdle was that fetal fibroblasts have a short life span and undergo rapid senescence when in culture, which proved a particular problem in the ferret. This was overcome by performing an additional round of nuclear transfer ('rejuvenation'). Following the initial PCR screening, positive but senescing clones were transferred into recipient oocytes and re-implanted into pseudopregnant ferrets. Three-week-old fetuses were subsequently harvested to collect fetal fibroblasts for additional analysis by southern blotting. Porcine fetal fibroblasts were less prone to senescence, and the additional 'rejuvenation' step was not required.

The gene-targeted fetal fibroblasts were transferred into oocytes, which were re-implanted into pseudopregnant females and a total of 13 healthy heterozygote pigs and 8 healthy heterozygote ferrets were born (see also Table 1). All heterozygote animals were males, because the fetal fibroblasts were derived from male animals, allowing for rapid expansion of animal numbers and generation of heterozygote females, which is currently ongoing. The selection of pigs and ferrets as potential models for CF was an obvious choice as lung architecture, CFTR expression and the pharmacological properties of CFTR are similar in both species to humans. The authors opted to generate heterozygote pigs and ferrets by disrupting exon 10 of the *CFTR* gene (null mutation), a strategy that was moderately successful in knocking out murine *CFTR*. In addition, pig heterozygotes were produced with a phenylalanine deletion (ΔF508), which is the most common mutation in humans.

Although CF is a multiorgan disorder involving the intestine and pancreas, most morbidity and mortality is caused by progressive pulmonary infections and inflammation ultimately leading to respiratory failure. To aid understanding of pathophysiology, and help with the development of new treatments, a good animal model should recapitulate CF lung disease as closely as possible. The CF knockout mouse model failed at this hurdle, developing the characteristic CF ion transport abnormalities (the CF gene encodes a chloride channel) only in the nasal epithelium. Although animals show extensive CF intestinal pathology, they do not develop CF lung disease. The exact reason for this is unknown but it has been suggested that expression of alternative chloride channels in the murine lung,[3] as well as the short life expectancy of mice (approximately 2 years), may explain the findings. Pigs have a life expectancy of 10–15 years and so may help to disentangle these factors. Interestingly, Liu *et al*.[4] have recently shown that the porcine ΔF508 mutation has a much milder phenotype (processing defect) when assessed *in vitro*, which could potentially affect the phenotype in ΔF508 pigs. These findings further highlight the importance of species-specific differences and underline

Table 1 Cloning of *CFTR*-targeted pigs and ferrets by SCNT

	Mutation	*Pseudopregnant females*	*Embryos implanted per female*	*Pups born*	*Pups heterozygote for CFTR*
Pig	Null	8	94–144	10	9
	ΔF508	4	103–185	5	4
Ferret	Null	11	35–60	14	8

Abbreviation: SCNT, somatic cell nuclear transfer.
Table adapted from Sun *et al*., 2008 and Rogers *et al*., 2008.

that success cannot be taken for granted.

Chris Rogers, of the University of Iowa, reported at the American Society for Gene Therapy meeting in Boston this year that the first 20 CF piglets had been born. All had abnormal ion transport in the nose and showed blockage of the distal small intestine (meconium ileus) and severe pancreatic pathology perinatally. Unsurprisingly at this early stage, there was no evidence of lung disease but the gut and pancreatic pathologies were much more pronounced in the pigs than is seen in humans, and the reasons for this are currently unclear. The next challenges will be to overcome the perinatal intestinal disease and to assess the ion transport properties of the porcine lung, as an early indicator of potential CF lung disease. The first CF ferrets are eagerly awaited.

We have no doubt that CF pigs and ferrets will significantly catalyze CF research if the animals recapitulate human lung disease. However, it is important to note that husbandry and breeding will not be straightforward. Pigs reach sexual maturity at 5–6 months of age and have comparatively large litters (>10 piglets), but the size and weight of pigs (up to 120 kg) will make it difficult to handle and house them in many university facilities and may restrict research to a few centers. Longer term cross breeding with mini pigs may be beneficial. Although the smaller size of ferrets (0.5–2.5 kg) may help with housing, these animals are more difficult to breed. In addition, they only reach sexual maturity at the age of 9–12 months. If pigs and ferrets recapitulate the human phenotype, males will be infertile and breeding from heterozygote parents will be necessary. If this is the case, only 1–2 offspring per litter will be CF. Thus, it may take many years to establish colonies with sufficient output for meaningful research.

Irrespective of whether the CF animal models recapitulate the human lung phenotype, these studies are a milestone in the development of non-rodent genetic disease models. The observation, that gene targeting frequency in fetal fibroblasts is donor-dependent, may allow further improvement of targeting frequency. We applaud the findings and especially the 'ferret team' for their stamina, given that all aspects of ferret somatic cell nuclear transfer and oocyte transfer have been developed and optimized from scratch. CF pathophysiology has not yielded its secrets easily, but we hope that one or both of these animal models will provide an important step forward in our understanding. ■

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