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REVIEW

Impact of gene editing on the study of cystic fibrosis

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Abstract Cystic fibrosis (CF) is a chronic and progressive autosomal recessive disorder of secretory epithelial cells, which causes obstructions in the lung airways and pancreatic ducts of 70,000 people worldwide (for recent review see Cutting Nat Rev Genet 16(1):45–56, 2015). The finding that mutations in the CFTR gene cause CF (Kerem et al. Science 245(4922):1073–1080, 1989; Riordan et al. Science 245(4922):1066–1073, 1989; Rommens et al. Science 245(4922):1059–1065, 1989), was hailed as the very happy middle of a story whose end is a cure for a fatal disease (Koshland Science 245(4922):1029, 1989). However, despite two licensed drugs (Ramsey et al. N Engl J Med 365(18):1663–1672, 2011; Wainwright et al. N Engl J Med 373(3):220–231, 2015), and a formal demonstration that repeated administration of CFTR cDNA to patients is safe and effects a modest but significant stabilisation of disease (Alton et al. Lancet Respir Med 3(9):684–691, 2015), we are still a long way from a cure, with many patients taking over 100 tablets per day, and a mean age at death of 28 years. The aim of this review is to discuss the impact on the study of CF of gene-editing techniques as they have developed over the last 30 years, up to and including the possibility of editing as a therapeutic approach.

Introduction

Precise editing of the mammalian genome, the ability to manipulate the DNA sequence at the level of single base-pair resolution in cells, requires homologous recombination (HR) between the target region of the genome and a donor template molecule with the desired sequence, but typically occurs in less than 1:1000 treated cells (Smithies et al. 1985; Thomas et al. 1986). Nonetheless, the development of powerful selection strategies to isolate edited cells, and techniques to generate mice from embryonic stem (ES) cells, enabled successful germline editing to create gene-targeted mice (Thompson et al. 1989) which opened up a new field of in vivo modelling of genetic disorders. The first impact of gene editing on the study of CF was the development of knockout mice in which a whole exon was removed (Snouwaert et al. 1992; Dorin et al. 1992; Ratcliff et al. 1993), or precisely-edited with a 3-bp deletion equivalent to the most common CF-causing mutation, ΔF508 (Colledge et al. 1995; van Doorninck et al. 1995; Zeiher et al. 1995). As discussed in detail below, these models recapitulated many features of this human disease; a notable exception was that these mice have a very mild lung phenotype, a stark contrast to the progressive deterioration of lung function which is the cause of death in the vast majority of CF individuals. The exact cause of this difference was not known, and an inability to derive ES cell lines from other mammalian species effectively blocked the development of other models to study lung function for over a decade.

One small step—shorter templates for editing

A considerable limitation to widespread use of gene-editing techniques at the time was the need to create very large (>10 kb) and complicated template molecules or targeting constructs (Koller et al. 1989; Zijlstra et al. 1989). A significant breakthrough in editing for CF was the demonstration that short (≪1 kb) DNA fragments (SDFs) could precisely

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modify the human CFTR gene in both transformed and non-transformed primary normal airway epithelial cells (Kunzelmann et al. 1996; Goncz et al. 1998), proving the principle that isogenic human cells could be created. The small fragment homologous replacement (SFHR) approach was also used to stably introduce a 3-bp deletion (equivalent to ΔF508) in the Cftr gene of mouse embryonic stem (ES) cells (Sangiuolo et al. 2008). However, it was the fusion of two different techniques exploiting the recombinogenic properties of adeno-associated virus (AAV) template molecules (Cathomen 2004) and somatic cell nuclear transfer (Campbell et al. 1996), that facilitated the development of CF-null ferrets (Sun et al. 2008) and CF-null and ΔF508 pigs (Rogers et al. 2008).

A key feature of CF pigs and ferrets was the development of lung disease similar to that seen in CF patients, a pathological feature that had not been observed in any of the CF mouse models. The availability of three gene-edited species placed CF researchers in a unique position for a genetic disease to undertake comparative pathophysiological studies which have revealed a number of less well-characterised features of the disease, such as abnormalities in alveolar macrophages, bone, and cartilage (reviewed by Wilke et al. 2011; Keiser and Engelhardt 2011). These models will also be critical to optimization of delivery of editing machinery (Cao et al. 2013, 2016) and/or edited cells (Butler et al. 2016).

A giant leap in editing efficiency—targeted double-stranded breaks by programmable and RNA-guided nucleases

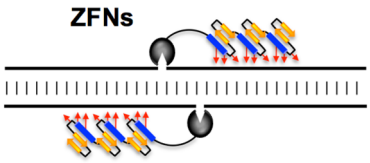
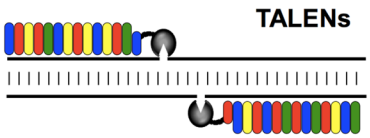
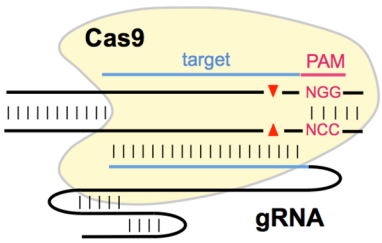
For nearly 20 years, gene editing relied solely upon the donor to mediate changes to the genome, even though early plasmid recombination studies indicated that a targeted double-stranded break (DSB) in the genomic DNA close to the site to be edited would boost repair efficiency by at least one order of magnitude (Kucherlapati et al. 1984). Proof of principle for this idea was established with the intron-encoded meganuclease I-*Sce*I (Rouet et al. 1994), but to make DSBs at custom sites in the genome, an endonuclease with a programmable recognition site of at least 16 bp would be required (see Table 1). The first step towards this was the creation of zinc finger nucleases (ZFNs), obligate dimers of concatemerised zinc finger transcription factor domains fused to the nuclease domain of *Fok*I which together recognised and cut a unique 18-bp sequence (Kim et al. 1996). The second step was a decade-long search to determine the rules to program ZFNs to bind any desired target site in the genome, culminating in the use of ZFNs to boost the efficiency of template-mediated editing to as many as 1 in 5 transfected mammalian cells (Urnov et al. 2005). With a relatively simple set of rules in place, the ability to make targeted DSBs at any point in the genome marked the start of a revolution in gene editing with the demonstration that ZFNs could be used to edit fertilised eggs and, thus, create models of disease in a broad range of species other than mice, and with the speed of transgenic techniques (Geurts et al. 2009). The therapeutic potential for in vivo editing was elegantly demonstrated by delivering ZFNs and donor using virus vectors to effectively cure haemophilia A in a mouse model (Li et al. 2011).

The widespread uptake of ZFN editing was relatively slow, partly due to the finding that robust programming of ZFNs was harder than originally anticipated, with the first CFTR-specific designer nucleases only capable of DSB formation in 1.2 % of transfected cells (Maeder et al. 2008). Using different design rules (Dreier et al. 2001; Liu et al. 2002), we created a ZFN pair based on the original nuclease scaffold (Kim et al. 1996) to successfully target a different site in the human CFTR gene and edit and repair the ΔF508 mutation (Lee et al. 2012) in patient-derived tracheal epithelial cells (Kunzelmann et al. 1993). Around this time, a second programmable nuclease system, TAL-effector nucleases (TALENs), was described, with human CFTR-specific TALENs reported to show good activity in a yeast-based assay, but not tested in human cells (Cermak et al. 2011). However, the breakthrough that brought gene editing to the public attention and to thousands of labs around the world was CRISPR Cas9/gRNA (Jinek et al. 2012). This RNA-guided DNA-specific nuclease system was quickly shown to be capable of doing essentially everything that ZFNs and TALENs could do, but via much simpler, quicker and cheaper protocols (Cong et al. 2013; Mali et al. 2013).

Isogenic models to study CF

The first published report of Cas9/gRNA to study CF was conducted in the gut stem cell organoid model (Sato et al. 2009); gut stem cells from a patient with the ΔF508 mutation were repaired using Cas9/gRNA and a donor plasmid containing a selectable marker. Correctly edited cells were selected and subsequently expanded in culture to form organoids; when exposed to cyclic AMP, a CFTR agonist, the gene-edited organoids rapidly swelled confirming that genetic correction results in a concomitant functional correction of the CFTR ion channel (Schwank et al. 2013). A host of other strategies to generate isogenic human cell models quickly followed, the first of which used ZFNs to integrate a CFTR transcription unit into a potential safe-harbour locus within inducible pluripotent stem (iPS) cells (Ramalingam et al. 2013). Direct repair of the ΔF508 mutation in iPS cells using ZFNs (Crane et al. 2014),

Table 1 Gene editing with ZFNs, TALENs and Cas9—comparison of the three technologies

Schematic representation	Features
	• Synthetic nuclease made of zinc finger (ZF) domains fused to *Fok*I nuclease domain • Each ZF domain binds 3 bp, total recognition site 18–24 bp, depending on number of ZF domains in each ZFN • Cuts DNA as obligate dimer with 5′ overhang • High level of specificity, easy to assemble • Easy to deliver with range of non-viral and viral vectors
	• Synthetic nuclease made of repeat variable di-residue (RVD) domains fused to *Fok*I nuclease domain • Each RVD domain binds 1 bp, total recognition site of ≥30 bp, depending on number of RVDs in each TALEN • Cuts DNA as obligate dimer with 5′ overhang • Very high level of specificity, reasonably easy to assemble • Easy to deliver with non-viral and viral vectors, but cDNA encoding TALENs exceeds packaging limit for AAV vectors, and highly repetitive nature of RVDs complicates use with lentivirus vectors
	• Bacterial RNA-guided nuclease with 20-bp recognition site modified to work in eukaryotic cells • Cuts as monomer with blunt end double-stranded break • Early versions of Cas9 showed high level of off-target effects, but modified versions available with almost undetectable levels of off-target binding • Requires a short PAM sequence which varies depending on source of Cas9—synthetic variants available which recognise different PAM sequences • Very easy to target with single guide RNA • Easy to deliver as DNA (viral or non-viral vectors), mRNA/gRNA or protein/gRNA complex • Cas9 variants available that lack nuclease activity but retain DNA-binding activity which enables them to act as targeted activators or repressors of gene expression

Schematic representations are not to scale

followed by differentiation of corrected cells into epithelia, resulted in the full restoration of Cl^- channel function, though the excision of the selection marker to enrich edited cells left a single 34-bp loxP site in the genome. Truly scarless gene editing of CFTR in iPS cells was achieved using Cas9/gRNA and a selection marker flanked by PiggyBac transposon sequences (Firth et al. 2015), whereas a cyclic enrichment strategy involving allele-specific PCR enabled the isolation of edited cells generated using SDFs with TALENs with significantly fewer genetic manipulations (Suzuki et al. 2016). Upon differentiation, these cells also showed fully restored CFTR function as assessed by several different assays.

Study of CF with isogenic models

These isogenic models provide the opportunity for a systematic evaluation of the 2000 mutations in the CFTR2.org database, only 127 of which have been definitively shown to cause CF (Castellani and CFTR2 team 2013); the disease-causing status is of particular relevance where pregnant women and their partners are offered testing for mutations in CFTR and one or both shown to carry a mutation of unknown status (see Pearson 2009). These models may also uncover novel targets for drug development (Farinha and Matos 2016), and could also be adapted for high throughput screening (Verkman et al. 2015) to identify lead compounds for clinical evaluation, analogous to the strategy (van Goor et al. 2009) which gave rise to Ivacaftor (Kalydeco), a drug that has radically improved lung function and quality of life for CF patients with the G551D mutation (Ramsey et al. 2011; Harrison et al. 2013). The iPSc models may also be useful in identifying other mutations which may respond to existing medications, an approach which has already been successful with the stem cell organoid model (Mini guts for Cystic Fibrosis). The therapeutic potential of corrected iPS cells is explored below.

Third generation CF animal models—rats, rabbits and more mice

As mentioned above, the demonstration that the programmable nucleases or RNA-guided nucleases can be used to edit the nuclei of a fertilised egg, radically altered the way in which germline editing can be used to make gene-modified animal models. One of the first examples was the development of CF knockout rats using ZFNs (Tuggle et al. 2014). These animals showed many of the features seen in CF patients including abnormalities in the ileum and airway surface liquid height. A robust system to generate knockout rabbits from Cas9/gRNA-edited pronuclear-stage rabbit embryos has also been described, and the generation

of *Cftr*-null embryos (Yang et al. 2014) should provide a rabbit model of CF disease.

Whilst existing mouse models continue to give mechanistic insight into the various aspects of CF disease such as mucoviscidosis, the production of viscous mucus in the glands and ducts of affected organs (Liu et al. 2015), the next generation of models, a suite of humanised CF mice in development by this group where the endogenous Cftr gene is replaced with the complete human CFTR gene, will be particularly useful for the evaluation of small molecule drugs and gene-editing therapies that are being developed in human isogenic cell models.

Editing as a therapeutic approach

Within a year of cloning the CFTR gene, a number of studies demonstrated that the CFTR cDNA could functionally complement the genetic defect (Drumm et al. 1990; Gregory et al. 1990; Rich et al. 1990) and served as proof of concept for cDNA addition as a therapeutic approach to treat CF. After more than 20 clinical trials, the safety of DNA delivery to the CF lung is now well established, but the best clinical outcome so far is a small but significant stabilisation of lung function during a multiple-dose protocol (Alton et al. 2015).

So, given that the editing machinery can be delivered by similar size DNA molecules to those used in the complementation trials, is it now appropriate to try editing as a therapeutic approach? The feasibility of site-specific lung editing was originally demonstrated using SFHR (Goncz et al. 2001), and whilst programmable and/or RNA-guided nucleases in combination with template molecules or SFHR have not yet been reported in vivo for CF, two alternative approaches have demonstrated editing in the CF lung cells in situ.

Using triplex-forming peptide nucleic acids and donor DNA delivered by nanoparticles, efficient correction of the ΔF508 mutation with concomitant restoration of Cl^- efflux was observed in up to 25 % of human cells in vitro (McNeer et al. 2015). When the same triplex-forming peptide nucleic acids were used with a different donor (due to sequence differences between human CFTR and mouse Cftr genes), deep sequencing analysis revealed correct editing of the ΔF508 in ~1 % of mouse lung cells in vivo (McNeer et al. 2015).

The development of strategies to correct the ΔF508 CFTR mutation at the RNA level has also been reported. Proof of principle for mRNA editing was established using a complex of two modified RNA molecules to insert the three missing bases, possibly via an RNaseH-dependent process, resulting in restored ion channel activity in a CF cell line (Zamecnik et al. 2004). Following on from this work, two clinical trials are now in progress (ProQR clinical trials), evaluating the ability of a single antisense oligonucleotide to correct the ΔF508 mRNA. The mechanism of repair is not clear, although the use of a single oligonucleotide appears to rule out a role for RNaseH in the repair process (De Boer and Ritsema, 2014). Identity across a 35-bp region centred almost exactly on the ΔF508 mutation in the human and mouse genes means that the same editing tools can be used provided they bind within this region. However, until humanised animal models of CF become available, the correction of mutations outside this region will require the creation of species-specific reagents.

Alternatives to editing—characterisation and alteration of CFTR gene expression

The regulation of the CFTR gene is critical to understanding the pathophysiology of CF, and studies of gene expression using Cas9/gRNA to remove control elements have already revealed much about the role of cis-acting elements in terms of CFTR expression, its chromatin landscape, and higher order organisation (Yang et al. 2016). Further studies should also reveal the identity of proteins involved in tissue-specific levels of CFTR expression and post-transcriptional regulation by microRNAs (Gosalia and Harris 2015). Such studies could lead to the use of editing to increase expression levels sufficient to address the pathophysiological effects of a small subset of specific CF-causing mutations, for example, the premature stop codon (PTC) class of CF-causing mutants which generally show reduced mRNA levels due to nonsense-mediated decay (NMD), so a strategy to increase the mRNA levels in combination with PTC suppression strategies may provide a critical breakthrough for this group of mutations (see Cutting 2015). The option also exists to use non-editing CRISPRa (activation) strategies to regulate levels of gene expression (Dominguez et al. 2016). In the case discussed here, gRNA could be used to guide dCas9 variants fused to transcriptional activators such as VP64 to boost gene expression. Alternatively, one could evaluate the CRISPRi (interference) approach which used gRNA to guide dCas9 variants fused to transcriptional repressors such as KRAB to block expression of, for example, ATP12A, in an attempt to ameliorate lung disease. For cell models, inducible regulation of gene regulation via the doxycycline-inducible system is also possible (Mandegar et al. 2016).

Outstanding questions on the road to a cure

Are there still better ways to edit?

A concern about off-target effects of gene editing, that is the creation of DSBs at other regions of the genome which may have deleterious effects, has been assuaged by a number of developments that have gradually increased nuclease specificity. Shortly after the first mammalian use of ZFNs for gene correction, two new scaffolds with substantial reductions in off-target effects were described (Miller et al. 2007; Szczepek et al. 2007), and TALENs have an inherently high level of specificity as elegantly demonstrated by their ability to discern between two closely related human genes, CCR2 and CCR5, at a site which differs by a single base pair (Mussolino et al. 2011). There have been numerous advances to increase the specificity of Cas9/gRNA, most recently the rational design of enhanced-specificity Cas9 (Slaymaker et al. 2016) and Cas9-high fidelity (Kleinstiver et al. 2016) which show almost undetectable levels of off-target DSB formation, even with exquisitely sensitive detection assays. Small-scale clinical studies using cells edited by ZFNs (Tebas et al. 2014) and TALENs (Poirot et al. 2015) have shown no serious adverse reactions, and it is likely that Cas9/gRNA will be evaluated in humans in the near future. The use of asymmetric donors (Richardson et al. 2016) and careful choice of target locus, nuclease, and cell type (Miyaoka et al. 2016) can substantially improve the efficiency of on-target editing by increasing the ratio of precision repair events by template-directed HR relative to imperfect non-homologous end joining (NHEJ) events. One other challenge is to conquer the low efficiency of HR-dependent editing in cells that are terminally differentiated or divide slowly, with obligate ligation-gated recombination (ObLiGaRe) currently offering the most promise (Maresca et al. 2013). Though feasible with ZFNs and TALENs, the blunt end DSB generated by Cas9/gRNA has meant this strategy has not found widespread use. However, the discovery that CRISPR Cpf1/gRNA generates a DSB with a 5′ overhang suggests that Obligare may be feasible using this newly identified RNA-guided endonuclease (Zetsche et al. 2015). The use of microhomology-mediated end-joining (MMEJ) techniques such as PITCh may also be feasible with TALENs and Cas9/gRNA (Sakuma et al. 2016).

Why don't mice get lung disease?

As mentioned above, a significant obstacle in CF research for many years was the lack of an animal model for lung disease, solved only with the availability of pig and ferret models. Possibly the most exciting finding from comparative studies is the recent discovery that humans and pigs express a H^+/K^+-ATPase (ATP12A) in the lung, such that when CFTR is absent, the ensuing loss of bicarbonate secretions [CFTR is a bicarbonate and Cl^- channel (Gray et al. 1993; Poulsen et al. 1994; Quinton 2001)] leads to an unchecked H^+ secretion resulting in acidified airway surface liquid which subsequently impairs airway defence mechanisms. In stark contrast, mouse airways express very low levels of ATP12A, and so, there is minimal H^+ secretion even when CFTR was absent, thus, the airway surface liquid in CF and wild-type mice has similar pH (Shah et al. 2016). When CF mice were analysed 3 days after infection with an adenovirus vector expressing ATP12A, their airways had become acidified which impaired defences and increased bacterial load in the lungs. These findings suggest that CF mice engineered to upregulate or overexpress ATP12A would most likely have a significant lung phenotype. If combined with the humanisation strategy described above, this could create a very powerful in vivo model to study CF.

How many cells need to be edited to correct disease?

Two key studies from the last century suggested that only a relatively small number of cells would need to be corrected to restore, at least in part, lung function. The first study established that the overall Cl^- transport properties of an epithelial layer comprising mainly homozygous ΔF508 cells with just 6–10 % functionally corrected cells were essentially indistinguishable from an epithelial layer where all the homozygous ΔF508 cells had been functionally corrected (Johnson et al. 1992). The second, a study of healthy non-CF volunteers, showed that as little as 8 % of the normal level of bronchial CFTR transcripts are needed to maintain normal airway function (Chu et al. 1992). With the increase in editing technology, it should be possible to revisit this problem in a number of novel ways, particularly if a mouse model with significant lung disease becomes available (see above). One option could be to mirror the recent approach used to study Duchenne muscular dystrophy (DMD), an X-linked progressive muscle disorder caused by mutations in the gene encoding dystrophin, by simply using Cas9/gRNA to edit single cell mouse embryos. The F1 generation of animals produced were genetically mosaic containing 2–100 % correction of the DMD gene, allowing a correlation between genetic correction and disease severity to be robustly established (Long et al. 2014). A similar approach could shed new light on the number of cells required to cause, or prevent CF, depending on whether the starting cells are wild-type or CF, respectively.

Functional studies of modifier genes

A long-standing and open question is which genes contribute to the variability of disease symptoms and drug responsiveness amongst different CF individuals. A recent genome-wide association analysis of over 6000 CF patients identified five loci that display significant association with variation in lung disease (Corvol et al. 2015). Given that Cas9/gRNA tools are now available to simultaneously edit multiple genes in cells or in vivo (Wang et al. 2013), it is now feasible to generate biological models to test these predictions.

Delivery challenges for therapeutic application

For therapeutic use, essentially two options exist: direct editing of lung cells in vivo, or adminstration of gene-modified cells. The repeated delivery of plasmids expressing the CFTR cDNA to stablise lung function in patients (Alton et al. 2015) suggests that plasmids of a similar size could be used to deliver gene-editing nucleases, and clinical trials to assess the safety and efficacy of direct RNA-editing delivered by non-viral vectors are underway (ProQR clinical trials). Nanocomplex formulation of lipids and peptides to efficiently deliver minicircle DNA-encoding reporter genes to mouse lung also provides proof of principle for delivery of the cDNAs encoding gene-editing nucleases (Munye et al. 2016). Adeno-associated virus (AAV) has been used to deliver cDNA encoding ZFNs (Li et al. 2011) and Cas9 derived from *S. aureus* (Ran et al. 2015), and adenovirus has been used to deliver cDNA encoding TALENs (Holkers et al. 2014). Lentivirus vectors have even been used to deliver the ZFN or TALEN proteins and edit cells (Cai et al. 2014), and more recently, direct delivery of Cas9/gRNA ribonucleoprotein complexes has been described (Choi et al. 2016). Of particular interest for CF, the combination of non-viral and viral delivery, specifically the use of chemically modified mRNA encoding site-specific nucleases delivered with chitosan nanoparticles, and AAV to deliver the donor, resulted in precise editing in the lungs, within a notable phenotypic change in a well-established transgenic mouse model of surfactant protein B deficiency (Mahiny et al. 2015). The availability of large animal CF models has also established the feasibility of delivery of aerosolised virus vectors intratracheally into pigs under bronchoscopic guidance (Cao et al. 2013; Yan et al. 2015). With regard to cell-based therapies for CF, there are less data at present, but a recent proof-of-concept study in mice showed successful lung reconstitution by canicular-stage lung cells (Rosen et al. 2016) raising the possibility of testing some of the recently described gene-edited iPSc models in conjunction with CF animal models as an alternative to direct editing of the lung.

In conclusion

Gene editing has had a long-standing impact on the study of CF, and as new techniques have been developed, many by CF researchers, they have been fully exploited to increase our understanding of the disease and develop new treatments. The CF field now has a surfeit of human and animal models at its disposal which will only add to the growing number of mutation-specific therapeutic approaches already available. CF is already a shining example of personalised medicine (Corvol et al. 2016), and the transition from classifying DNA variants by disease-causing properties to theratypes, a classification according to the molecular-based treatment to which they respond (Cutting 2015) is now well under way.

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