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Review

Gene editing & stem cells

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1. Introduction

Cystic Fibrosis (CF) occurs when both alleles of the *CFTR* gene contain a mutation which blocks the trafficking and/or function of the CFTR protein, and/or affects the integrity or stability of its mRNA. Five years ago it was shown that nuclease-dependent gene editing could correct the most common CF-causing mutation p.Phe508del (legacy name F508del) [1] and restore the function of the CFTR protein [2]. Subsequent studies have shown that CFTR mutations can also be corrected in primary human cells, lung cells in CF mice [3] and patient-derived inducible pluripotent stem (iPS) cells [4–7]. Together, these studies have driven interest in the use of gene editing as a potential therapeutic approach in vivo, and for in vitro gene editing of stem cells that can be applied in disease modelling or cellular therapies, but especially in drug screening.

Recently, a method to propagate intestinal organoids from individual CF patients was developed, which is now used as a novel, individualized screening platform for small molecules [8]. While this represents a major step forward, it has not yet been firmly shown that CFTR activity in intestinal organoids is an accurate predictor of CF lung disease, the most serious cause of morbidity and mortality in CF, or progressive pancreas and liver pathology caused by the plugging of pancreatic and biliary ducts, which also present serious and frequent complications and are untreatable using currently available interventions [9].

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With the major breakthrough presented by induced pluripotent stem cells (iPSCs) [10], another patient and disease-specific cell source for disease modelling and drug screening is now on hand, that can be easily obtained from any patient. Major advantages of iPSCs include the possibility to be genetically engineered on a clonal basis using novel genome engineering technologies, to be expanded to large cell numbers and to be specifically differentiated into different CF disease-relevant lineages, in particular respiratory epithelium [11].

To discuss some of the challenges involved in using stem cells for CF disease modelling and drug screening, in developing stem cell technology and gene editing for therapeutic use, and explore potential solutions, Patient Associations from CF Europe (CFE) in collaboration with ECFS organised a pre-conference symposium entitled "Gene editing & stem cells" at the 14th ECFS Basic Science Conference that took place in Albufeira, 29 March 2017. The purpose of the workshop was to have experts in the field introduce the latest methods in genome engineering and discuss the legal and ethical perspective of this technique. The discussions included the potential application of gene editing to CF in the context of transplantation of lung progenitor cells using mouse models of CF, the potential of gene edited inducible pluripotent stem cells for both modelling CF disease and drug screening, and the development and utilization of a mouse model engineered to express the human CFTR gene for testing gene editing therapies in vivo.

2. Gene editing: beginner's guide to state-of-the-art methods in genome engineering (Patrick Harrison)

To correct individual mutations in a cell just two reagents are needed. The first is a donor or template molecule with the desired DNA sequence flanked by homology arms. The second is a designer nuclease (ZFNs or TALENs) or an RNA-guided nuclease (CRISPR Cas9), to create a double-stranded break (DSB) at a unique site in the genome, very close to the DNA sequence to be edited. In the presence of the donor, the formation of the DSB by the nuclease triggers a cellular DNA response, known as homology-directed repair (HDR), which results in the removal of the mutant sequence and a "copy and paste" replacement with the desired sequence in the genome using the CRISPR Cas9/gRNA system (see Fig. 1).

Although HDR is the desired outcome of most gene editing strategies, DSBs are typically repaired by the default DNA repair pathway known as non-homologous end joining (NHEJ). This essentially joins the two free ends of DNA together, frequently with the creation of small insertions or deletions (indels), and this reduces the overall efficiency of precision gene editing. Moreover, if the nuclease target site is destroyed by indels before the HDR process occurs, this complicates subsequent attempts to edit the genome at that location. In spite of these potential difficulties, it may be possible to exploit some of advantages of NHEJ, namely its high efficiency and activity in non-dividing cells as shown later in this section.

Of the many advances in the rapidly changing field of gene editing, three recent studies are of particular interest in the context of developing this approach as a potential in vivo treatment. The first is an important proof-of-concept study which showed that many different CF-causing mutations can be repaired with a single nuclease and donor combination [12]. The donor in this case comprised CFTR exons 11–27 fused together as a single "superexon" preceded by a splice acceptor site and followed by a poly A site; the construct also contained a selectable marker to enable enrichment of edited cells and was flanked by homology arms to facilitate the HDR process (see Fig. 2A). When used with a nuclease which creates a DSB in intron 10 of the CFTR gene, the superexon was successfully incorporated into the genome resulting in a mRNA comprising exons 1 to 10 from the endogenous gene spliced seamlessly to the 11–27 of the superexon. Significant features of this approach are that the corrected mRNA is expressed under the control of the endogenous *CFTR* regulatory elements so should retain the correct temporal and spatial expression profile, and that ion channel function was restored when tested in cells homozygous for the c.1521_1523delCTT/p.Phe508del mutation. The major limitation for development of this approach for in vivo therapeutic application is the relatively low level efficiency of editing.

The second study of interest is the demonstration from our group that NHEJ editing can be used to repair at least three different CF-causing deep-intronic mutations at relatively high efficiency by using Cas9 with pairs of gRNAs [13]. By designing gRNAs either side of these mutations, and excising relatively short (141–209 bp) intronic regions, we showed very high levels of splicing restoration for the variants c.1679+1.6kbA>G (1811+1.6kbA>G), c.3140-26A>G (3272-26A>G) and c.3717+12191C>T (3849+10kbC>T). In addition to the higher efficiency, this approach has several advantages relative to the standard HDR approach. First, as a donor is not required, only Cas9 and the gRNAs need to be expressed within the cell which simplifies the gene editing cargo to be delivered and increases the options available. Thus, in addition to standard options of non-viral or viral delivery of DNA vectors encoding the Cas9 and gRNAs, the Cas9/gRNAs can be delivered directly as ribonucleoprotein (RNP) complexes, packaged as RNPs within lentivirus vectors [14] or other nanoparticle systems, or delivered as RNA only (Cas9 mRNA plus gRNAs). Also, as NHEJ-dependent editing will work in terminally differentiated cells in the lung epithelium this could be useful for in vivo therapeutic use if delivery to lung stem cells proves difficult.

Despite these advantages of the NHEJ-dependent described above, that particular approach is currently limited to the deep intronic mutations which are found in ~1.5% of individuals with CF. If gene editing is to be developed for in vivo therapeutic use, a high-efficiency technique that can correct all CF-causing mutations with a single donor/nuclease combination is highly desirable. One possible way to do this may be on the horizon by exploiting a new technique known as homology-independent targeted integration or HITI [15]. HITI enables relatively large segments of DNA, potentially a superexon as described above, to be integrated into the genome using a Cas9/gRNA NHEJ-based method with high efficiency. Whilst several other methods of NHEJ-mediated integration have recently been reported [16,17], the key advantages of HITI are its simplicity and efficiency as explained in Fig. 2b, using a CFTR superexon$^{11-27}$ construct as

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