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ORIGINAL ARTICLE

Gene editing of *DNAH11* restores normal cilia motility in primary ciliary dyskinesia

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ABSTRACT

Background Primary ciliary dyskinesia (PCD) is a rare autosomal recessive genetic disorder characterised by dysfunction of motile cilia. Ciliary dysmotility causes poor mucociliary clearance and leads to impairment of pulmonary function and severe respiratory infections. PCD has no specific therapy. With the aim to permanently restore gene function and normalise ciliary motility, we used gene editing to replace mutated with wild-type sequence in defective cells.

Methods The target gene was dynein heavy chain 11 (*DNAH11*), an essential component of ciliary structure. Airway ciliated cells were collected from two patients with PCD with *DNAH11* nonsense mutations and altered ciliary beating and pattern. Repair of the genetic defect was performed ex vivo by site-specific recombination using transcription activator-like effector nucleases (TALENs).

Results In an epithelial cell line engineered to contain the *DNAH11* target site, TALENs cleaved over 80% of the mutated *DNAH11* sequence and replaced the mutated sequence with wild-type sequence in about 50% of cells. In airway ciliated cells of patients with PCD, site-specific recombination and normalisation of ciliary beating and pattern occurred in 33% and 29% of cells, respectively.

Conclusion This study demonstrates that gene editing can rescue ciliary beating ex vivo, opening up new avenues for treating PCD.

INTRODUCTION

Primary ciliary dyskinesia (PCD) (OMIM No 244400) is a rare genetic disorder of motile cilia resulting in neonatal respiratory distress, recurrent or chronic otosinopulmonary infections, male infertility, and laterality defects in ~50% of cases.[1–3] PCD is usually diagnosed by the combination of clinical features using transmission electron microscopy and ciliary beat frequency (CBF) and ciliary motion pattern (CMP) analysis of respiratory cilia obtained by nasal or bronchial brush biopsy.[4] In addition to these tests, ciliogenesis in cultures of respiratory ciliated cells can be helpful for diagnosing PCD in the presence of conflicting results, particularly when ultrastructural changes are too subtle to be identified by transmission electron microscopy.[5–7]

Several hundred genes are involved in ciliary structure, motility and beating; as a result, many genes have been linked to PCD.[8–10] Identification of causative mutations and lack of specific therapies have stimulated gene therapy research for the treatment of PCD.

Ciliary function can be rescued by transferring a wild-type gene copy in defective airway epithelial cells.[11 12] Unfortunately, this approach is unsuitable for quite a few genes linked to PCD, as they are too big to be transported by current delivery systems. One of these is *DNAH11* (OMIM No 603339) which encodes dynein heavy chain 11, is 353 kb in size, and comprises 82 exons yielding a 14 kb mRNA, also too big for most delivery systems.[13 14] Mutations in *DNAH11* are responsible for 6% of PCD cases, but this percentage is probably underestimated, since there is no alteration of axoneme ultrastructure.[2 7 9]

Gene therapy through site-specific recombination (gene editing) replaces mutated with wild-type sequence and is not affected by gene size.[15] Site-specific recombination occurs by means of proteins that bind to and cleave the mutated region. The DNA repair machinery then repairs the break by either joining the DNA extremities (non-homologous end joining) or homologous recombination (HR) in the presence of the homologous DNA segment.[16] HR results in the permanent correction of mutated sequence and recovery of gene function.

In this study we repaired *DNAH11* and corrected abnormal ciliary function by HR using transcription activator-like effector nucleases (TALENs) which, when the study started and compared with zinc finger nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats (CRISPRs), exhibited the lowest unspecific DNA binding and cleavage (off-target activity).[17 18]

METHODS

Subjects

The study was performed in two monochorionic bi-amniotic male twins, 17 years of age, with PCD diagnosis based on cilia with non-specific ultrastructural abnormalities, non-flexible beating pattern (up to 56.5% of microscopic fields) and hyperkinetic beat (up to 22 Hz) (see online supplementary methods for full clinical and diagnostic details). Both were *DNAH11* compound heterozygotes (p.R2250*/p.Q3604*).[19] The study was approved by Pisa University Hospital Ethics Committee (No 3344/40956/2011).

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Cultivation and analysis of ciliated cells

Ciliated cells were collected by brushing lower nasal turbinates and cultured as described previously.[5] In culture, ciliated epithelial cells form spheroids consisting of layers of respiratory epithelial cells with ciliated apical membranes orientated outward which, in the presence of normal ciliary motility, but not in patients with PCD, move and rotate in the medium. Spheroids maintain their cilia for 14–20 days of cultivation and are usually identifiable after 24–48 h of culture.[6 20 21] It has been speculated that, after correction of a congenital defect or as observed in secondary ciliary dyskinesia, restoration of normal ciliary activity is rapid and does not require formation of new cilia, which takes 2–3 weeks in vitro.[6 12 20]

Spheroids at ×60 magnification were analysed for CBF and CMP at −4, −2, 0, 2, 4 and 8 days post-transduction (pt) by means of a high-frequency video (55 MHz or more) acquired with a digital video camera and a computer-assisted microscope (full description in the online supplementary methods). Videos before and after cell manipulation were acquired at 37°C by positioning the well culture plates on a heated plate under the microscope; analyses were performed by two investigators (MPif and MPir, who were blinded to cell manipulation) as described previously.[6] Approximately 120 spheroids were treated from each patient.

Transduction of ciliated cells

Spheroids were transduced at day 6–8 of cultivation with the lentiviral vectors (below) and using a multiplicity of infection (MOI; vector/target cell ratio) of 30 (vectors added at 15 MOI twice, 6 h apart).

TALEN design and construction

TALENs were designed to target the *DNAH11* nonsense mutation p.R2250* (position g.172381). A full description is given in online supplementary methods.

Lentiviral vectors and plasmid constructs

Non-integrating vectors were derived from feline (FIV) and human (HIV) immunodeficiency viruses. LAW34-GFP and LAW34E-GFP carry enhanced green fluorescent protein (GFP) (figure 1A,B).[22 23] Left (L-TALEN) and right (R-TALEN) TALENs were cloned into LAW34E (figure 1C). CL-TBS-GFP contains a GFP expression cassette flanked by two repeats of TALEN-binding sites (TBSs) (figure 1D). HIV vector HR-TBS-GFP carries haemagglutinin epitope (HA), *mut* (ie, *DNAH11* TBS and nonsense mutation and poly-A signal), $DNAH11_{693–704}$ epitope, internal ribosome entry site (IRES) and GFP (figure 1E); HIV-GFP delivers GFP. LAW34E-Rec provided *DNAH11* wild-type sequence (figure 1F). Construction and generation of vectors are described in full in online supplementary methods.

Other methods

Flow cytometry, western blot, droplet digital PCR (ddPCR), immunofluorescence and immunohistochemical analyses, pyrosequencing, and statistical analyses are described in online supplementary methods.

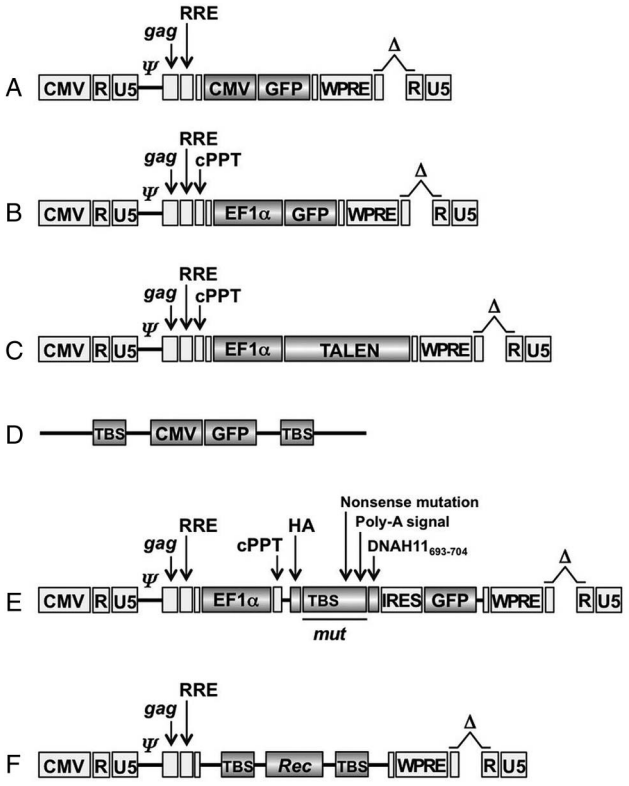

Figure 1 Genomic maps of vectors and plasmids used in the study. (A) LAW34-GFP was produced by cloning green fluorescent protein (GFP) into LAW34, a minimal self-inactivating feline immunodeficiency virus (FIV) vector with both U3 long terminal repeat (LTR) domains deleted and devoid of all accessory and structural proteins.[50] ψ is the packaging site and contains the domains important for encapsidation; RRE is the Rev-responsive element and is important for exportation of vector mRNAs from the nucleus to the cytoplasm; CMV is the cytomegalovirus promoter; WPRE is the woodchuck post-transcription regulatory element and increases stability of vector mRNAs. (B) LAW34E-GFP was derived from LAW34 by adding the central polypurine tract domain (cPPT) of FIV, reported to facilitate nuclear importation of lentiviral vector genome,[23] and by replacing the CMV promoter with the human elongation factor-1 α (EF-1 α) promoter. (C) L- and R-transcription activator-like effector nucleases (TALENs) were produced by cloning left- and right-TALENs, respectively, in LAW34E. Three sets of vectors were produced, one for each TALEN-binding site (TBS) found within a 1 kbp DNAH11 sequence encompassing the stop codon. (D) CL-TBS-GFP plasmid was used to measure cleavage efficiency of TALENs in 293T cells and was derived from pcDNA3 by cloning two TBS repeats between a CMV promoter-GFP expression cassette. Three plasmids were produced, one for each TBS. (E) Homologous recombination (HR)-TBS-GFP was used to test HR in 293T cells of the TALEN binding in the intron upstream exon 42 and was constructed using LVXII Ef1α, a HIV-1 vector produced in our laboratory, as backbone. HR-TBS-GFP delivered a 500 bp DNA fragment encoding haemagglutinin (HA) epitope, *DNAH11* TBS-stop codon segment, $DNAH11_{693-704}$ epitope from *DNAH11* internal region, internal ribosome entry site (IRES) of encephalomyocarditis virus, and GFP. (F) LAW34E-Rec provided Rec, a 350 bp wild-type *DNAH11* fragment. Rec was used for HR in 293T and ex vivo ciliated cells and was obtained by amplification from blood cell DNA. Rec was cloned into LAW34E devoid of EF1α promoter.

RESULTS

Selection of FIV vector and optimisation of ciliated cell transduction

This task was carried out as described in online supplementary data and using unwanted ciliated cell cultures from nasal brushing of subjects who underwent PCD diagnostic testing. Briefly, LAW34E-GFP performed better than LAW34-GFP and was therefore selected for the study and used to test three transduction protocols. No matter the method used, fluorescent cells were localised mainly externally plus in some scattered internal

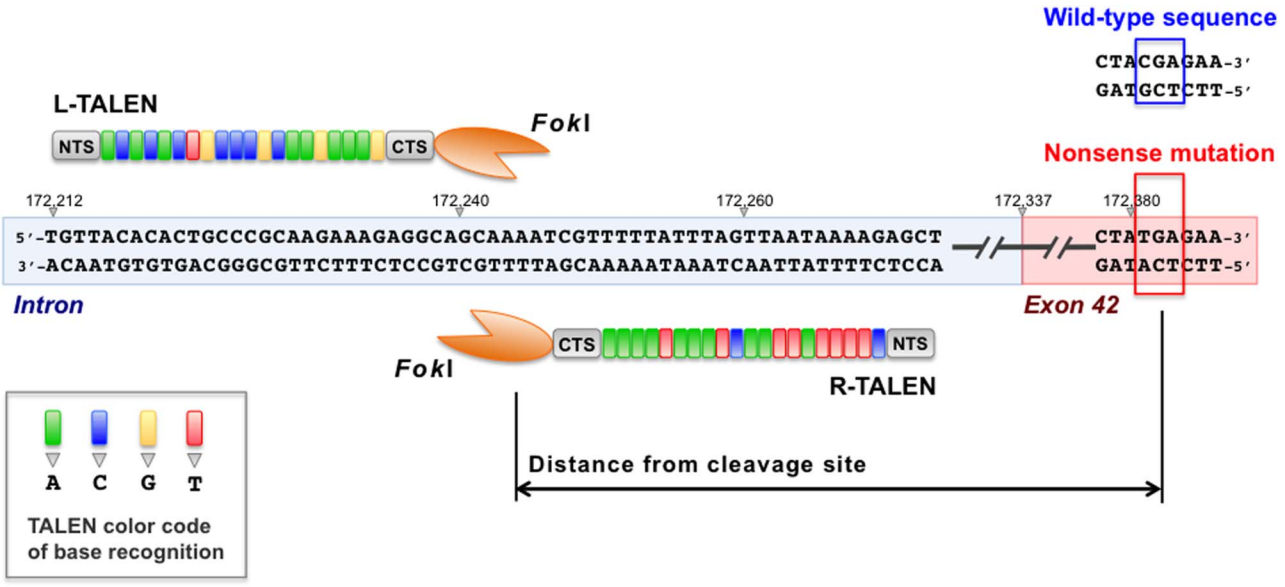

Figure 2 Schematic of architecture and localisation of L- and R- transcription activator-like effector nucleases (TALENs) compared with the stop codon. Each TALEN is composed of an N-terminal segment (NTS, grey box), a central repeat domain, a C-terminal segment (CTS, grey box), and a *Fok*I catalytic domain (orange oval). The central repeat domain comprises a series of repeat units that are responsible for specific recognition of thymine (red boxes), adenine (green boxes), cytosine (blue boxes) and guanine (yellow boxes). The formation of a heterodimer by two TALENs in a tail-to-tail orientation at the target site cleaves the DNA in a specific site. The cleavage site is located in the intron upstream exon 42 and approximately 140 nt upstream of the stop codon. This nonsense mutation is a consequence of a C>T transition which changes the wild-type triplet CGA (Arg) to TGA (stop) and causes premature arrest of translation.

areas of the spheroids (see online supplementary figures S1 and S2), suggesting that transduction occurred preferentially, if not solely, in the external ciliated cells. The method that worked best in our hands was a double transduction with 15 MOI LAWE-GFP added twice 6 h apart. This condition produced nearly 80% fluorescent spheroids at day 2; as expected from non-integrating vectors and transient transduction,[13] fluorescence progressively declined thereafter and averaged 10% at day 15 pt when cultures were terminated (see online supplementary figure S1).

Selection of TALENs and analysis of off-target activity

Selection of TALENs is detailed in online supplementary methods. Briefly, computing analysis of a 4 kb *DNAH11* fragment encompassing the targeted mutation identified about 100 TALEN pairs and relative TBSs. After several sequential screenings to eliminate combinations with potential off-target activity and low binding affinity to the *DNAH11* sequence, we selected three TALEN pairs: two with TBSs located in exon 42 and 500 nucleotides downstream of the targeted mutation and one with TBS in the intron upstream of exon 42 and 150 nucleotides from the targeted mutation (see figure 2 and online supplementary table S1). The three TALENs were first examined for off-target activity in spheroid cultures and then for cleavage and HR. Off-target activity was tested in three leftover ciliated cell cultures of normal subjects that were split into five aliquots (about 20 spheroids/aliquot); three aliquots were transduced with the TALEN pairs, one with LAWE-GFP and one sham at day 6 of culture. Spheroids were monitored for viability, CBF and CMP for 2 weeks pt. Efficiency of transduction, as determined by GFP-positive spheroid count at day 3 pt, ranged from 65% to 85%. No differences in viability or changes in ciliary activity were observed compared with the sham control, suggesting that neither TALEN nor vector transduction perturbed spheroid functions (data not shown).

Measurement of cleavage and HR activity

To minimise nasal brushings, cleavage and HR were analysed in 293T cells engineered to contain the *DNAH11* target site. For cleavage analysis, 293T cells were transfected with CL-TBS-GFP (293T/CL-TBS-GFP) and examined for GFP production, which occurs only with intact CL-TBS-GFP. For each of the three TBSs, we constructed specific CL-TBS-GFP and TALEN pairs. 293T/CL-TBS-GFP cells were transduced with: empty LAW34E (baseline GFP expression); L-TALEN alone (to measure interference of GFP mRNA transcription); L- and R-TALENs (cleavage activity). 293T cells transduced with L- and R-TALENs were used to check for cell damage (figure 3A). Reduction of fluorescence (RF) relative to baseline was measured at day 3 pt. For all TBSs, RF in 293T/CL-TBS-GFP transduced with L-TALEN was 6–12%, suggesting marginal interference with transcription; in 293T/CL-TBS-GFP transduced with L- and R-TALENs, RF was over 80% for the TBS located in the intron and about 60% for the TBSs of exon 42 (figure 3A). Fluorescence rapidly declined to 3% at day 5 pt, suggesting that fluorescence at day 3 pt was mostly due to GFP produced before TALEN transduction. This hypothesis is supported by the GFP half-life[24] and western blotting at day 5 pt (figure 3B). TALENs alone did not alter 293T cell viability (figure 3A). From these results we selected the TBS located in the intron.

HR was evaluated with HR-TBS-GFP which, as it stands, encodes HA only. Once *mut* recombines and the nonsense mutation and poly-A signal are replaced with *DNAH11* wild-type sequence (provided by Rec), translation continues and GFP is expressed. HR-TBS-GFP also allowed detection of frameshifts generated by HR: $DNAH11_{693–703}$, cloned in-frame and downstream of *mut*, is expressed only if the open reading frame is maintained (figure 1E). 293T cells were transduced with HR-TBS-GFP (293T/HR-TBS-GFP) and, 3 days later, transduced again with: L- and R-TALEN; Rec alone; L- and R-TALENs and Rec. Minimal fluorescence was observed in 293T/HR-TBS-GFP

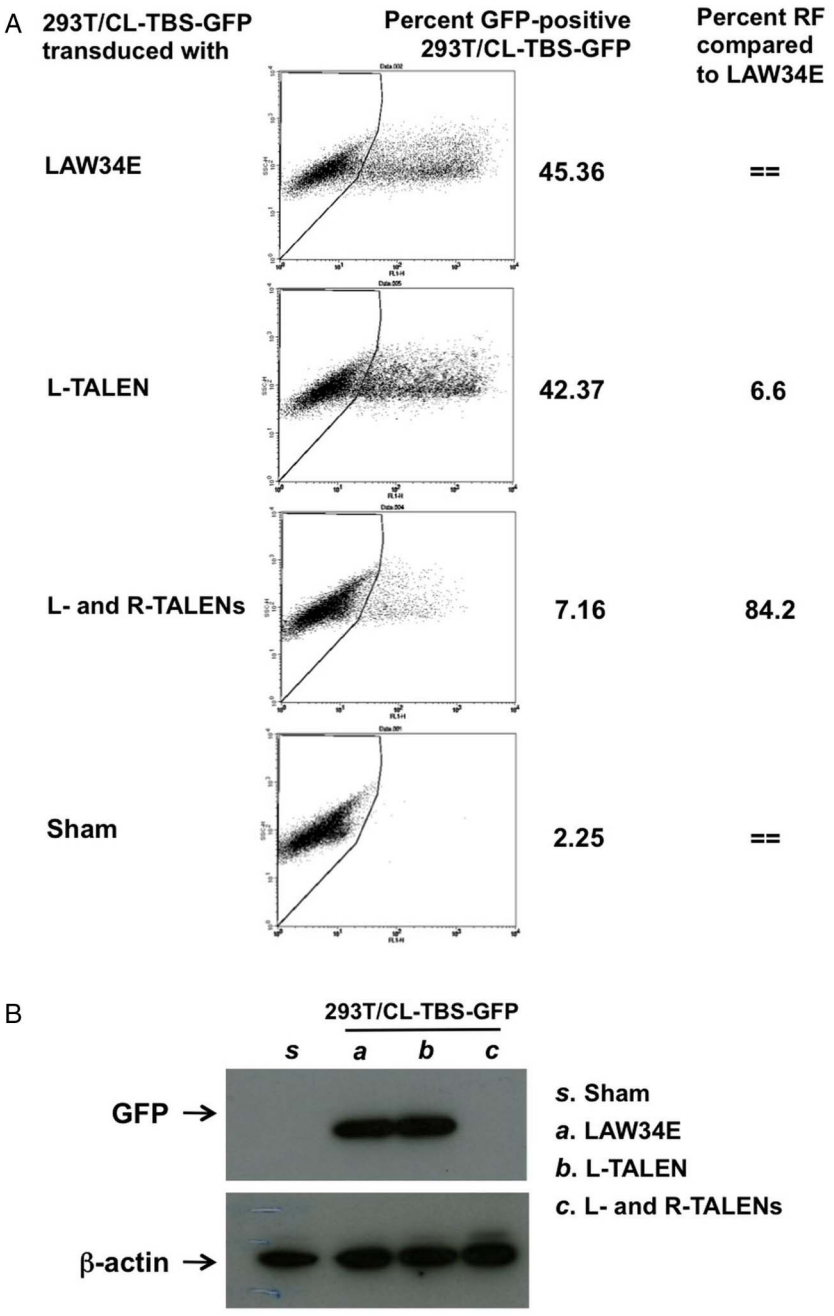

Figure 3 Efficiency of transcription activator-like effector nuclease (TALEN) cleavage. This analysis was performed on 293T cells transfected with CL–TALEN-binding site (TBS)–green fluorescent protein (GFP) and transduced, 2 days later, with TALENs and Rec in various combinations. Intact CL-TBS-GFP expressed GFP and cells fluoresced, but once targeted and cleaved by TALENs, CL-TBS-GFP no longer expressed GFP and fluorescence was reduced. This figure shows a representative example of three independent experiments. (A) CL-TBS-GFP-transfected cells were transduced as indicated on the left and analysed for GFP expression by flow cytometry 3 days later. Transduction with empty LAW34E provides GFP expression at baseline; L-TALEN alone measures interference of GFP mRNA transcription by L-TALEN binding; L- and R-TALENs provide cleavage efficiency. Sham are naive 293T cells transduced with L- and R-TALENs monitored to determine changes in viability and kinetics of replication. Reduction of fluorescence relative to baseline was calculated by arbitrarily setting the fluorescence in the LAW34E sample to 100%. (B) At day 5 post-transduction, these cells were collected, and protein was extracted and examined for GFP and β-actin content by western blot (WB). Whereas GFP is produced at similar levels in cells transduced with LAW34E or L-TALEN alone, no GFP was observed in cells transduced with L- and R-TALENs, indicating that all or nearly all CL-TBS-GFP molecules were cleaved by the TALENs binding the intron upstream of exon 42.

transduced with TALENs or Rec alone as opposed to combined transduction, which yielded 30% fluorescence (figure 4A). Since parallel transduction with HIV-GFP yielded 58% fluorescence (data not shown), we may infer that HR occurred in about 50% of instances. No frameshifts were introduced, as demonstrated by $DNAH11_{693–704}$ production and DNA sequencing (figure 4B).

Ex vivo editing of *DNAH11* in ciliated cells of patients with PCD

As detailed in online supplementary information, spheroids of patients A and B were obtained from ciliated cells collected by nasal brushing; they did not rotate in culture (table 1). At day 4 of culture, spheroids were split into five aliquots (about 25 spheroids/aliquot) and, 2 days later, transduced with: sham (one aliquot); L- and R-TALENs (one aliquot); L- and R-TALENs and Rec (two aliquots); FIV34E-GFP and Rec (one aliquot). As a whole, transduction with L- and R-TALENs and Rec normalised CBF and CMP in 24% and 15% of spheroids of patients A and B, respectively, at day 2 pt, which increased to 29% and 20% at day 4 pt—that is, day 14 of cultivation. All other combinations neither rescued CBF and CMP nor affected the viability of spheroids compared with untreated cells (see figure 5, table 1 and online supplementary video clips). The difference between normal and abnormal ciliary beat phenotypes of L- and R-TALENs and Rec versus negative controls (L- and R-TALENs alone and FIV34E-GFP and Rec) reached significance by day 2 as determined with Fisher's exact test (see online supplementary table S2).

To confirm that correction of CMP and CBF was indeed due to HR, genomic DNA of spheroids was analysed by ddPCR, one of the most sensitive methods for detecting minute variations in template copy numbers[25] and for pyrosequencing. The latter was performed using a procedure widely used to quantify single-nucleotide polymorphisms[26] (full description in online

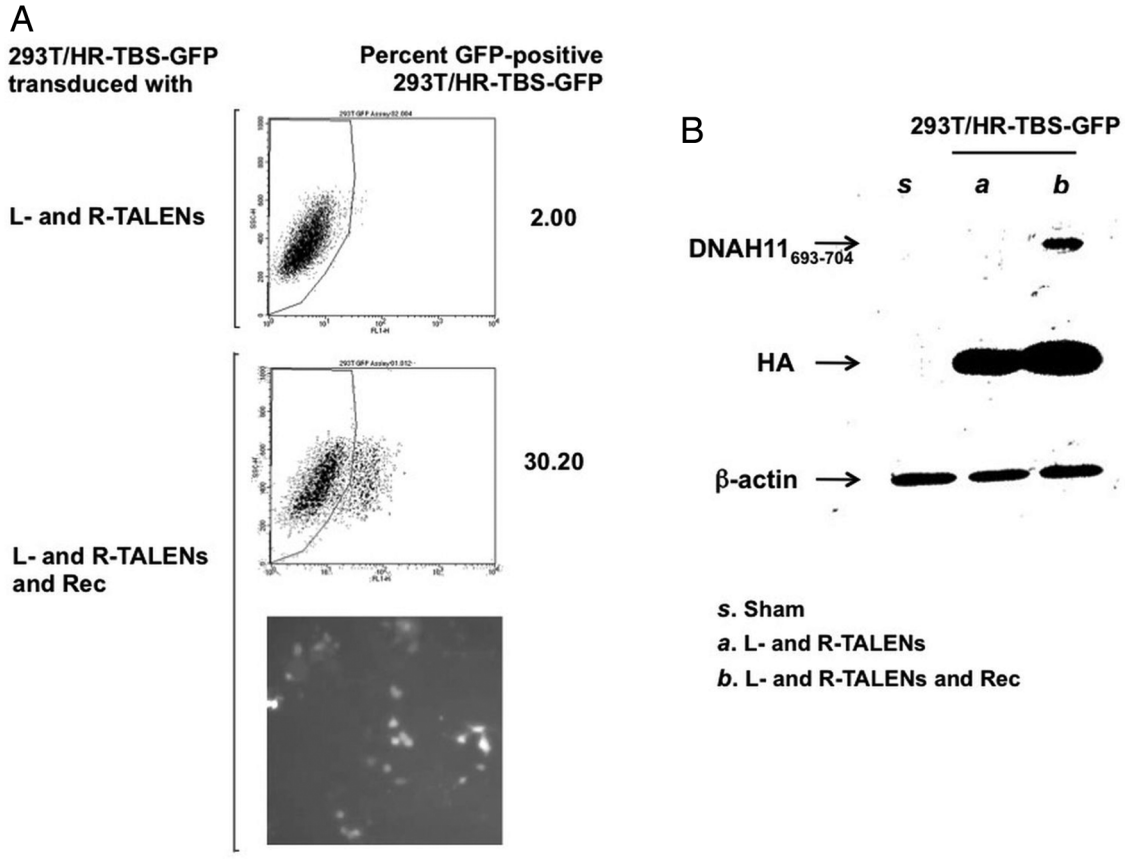

Figure 4 Analysis of homologous recombination (HR) efficiency and accuracy. This test was performed on 293T cells transduced with HR–TALEN-binding site (TBS)– green fluorescent protein (GFP) (293T/HR-TBS-GFP) and, 3 days later, transduced again with: L- and R-transcription activator-like effector nucleases (TALEN); Rec alone; L- and R-TALENs and Rec. HR-TBS-GFP contained the *DNAH11* TBS-stop codon fragment and GFP separated by an internal ribosome entry site. *DNAH11* fragment and GFP were expressed as bicistronic mRNA but, because of the stop codon, GFP was not produced. Once the stop codon was replaced with wild-type sequence, translation continued and cells became fluorescent. (A) Analysis of HR efficiency. Since 293T/HR-TBS-GFP cells were transduced with L-and R-TALENs and Rec, the *DNAH11* wild-type fragment was not provided, HR did not occur, and there was no fluorescence (upper panel); 293T/HR-TBS-GFP cells were transduced with L-and R-TALENs and Rec. Here HR occurred and GFP was expressed as determined by flow cytometry (lower panel) and fluorescence microscopy (image) analyses. (B) Analysis of HR accuracy. This test was performed to ascertain whether HR accidentally introduced some mutations and altered the reading frame. This occurrence was investigated by placing haemagglutinin (HA) and $DNAH11_{693\text{-}703}$ epitopes upstream and downstream of the TBS-stop codon fragment. HA was expressed no matter whether and how HR occurred; $DNAH11_{693\text{-}703}$ was in-frame with the TBS-stop codon and expressed only if replacement of the stop codon with wild-type sequence occurred without altering the reading frame. As shown by western blot (WB), the HA epitope was present in 293T/HR-TBS-GFP cells transduced with L- and R-TALENs with or without Rec, and $DNAH11_{693\text{-}703}$ was present only in 293T/HR-TBS-GFP cells transduced with L- and R-TALENs and Rec.

supplementary information). As regards ddPCR, control tests with 293T cells transduced with HR-TBS-GFP and Rec demonstrated that ddPCR detected up to 0.1 wild-type molecule among 1000 mutated molecules and vice versa (data not shown). As expected for compound mutations, mock controls and spheroids before treatment had similar numbers of wild-type and mutated alleles (figure 5). In contrast, on transduction with L- and R-TALENs and Rec, wild-type alleles increased 32.7% (30.2% and 35.3% per aliquot) and 26.6% (24.0% and 29.2%) in patients A and B, respectively (figure 5 and data not shown). Both increments were statistically significant ($p<0.01$) and in line with the number of spheroids with normalised CMP and CBF (table 1). Percentage correction of the mutated allele was confirmed by pyrosequencing analysis of the DNA extracted from spheroids at day 8 (see online supplementary figure S3).

To obtain final and definitive proof of corrected translation of coding DNA, we attempted to localise the mutated and wild-type (either naïve or corrected) proteins in cultured spheroids. Since western blotting on cultured spheroids was not practicable for the very large size of DNAH11 protein and insufficient amount of cells, we performed immunofluorescence and immunohistochemical analyses on fixed spheroids from normal and PCD patients. As described in online supplementary results, immunohistochemical analysis with a DNAH11 antibody recognising the $DNAH11_{963\text{–}1060}$ epitope located in exon 15 showed that cilia of spheroids treated with L- and R-TALENs and Rec were similar in shape and length compared with before treatment when they appeared stiffer and slightly shorter (see online supplementary figure S4).

Thus, molecular analysis, evaluation of ciliary beat phenotype, and visual examination of cells treated for immunohistochemistry confirmed that the nonsense mutation was replaced by the wild-type sequence in a significant proportion of ex vivo primary ciliated cells.

DISCUSSION

Treatment of PCD is currently directed at the downstream consequences of ciliary dysmotility, such as chronic infection and

Table 1 Results of qualitative evaluation of ciliary motion waveform and efficiency in two ciliated cell cultures (about 25 spheroids/culture) of PCD patient A transduced with L- and R-TALENs and Rec

	Days post-transduction			
Parameter monitored	**0**	**2**	**4**	**8**
Rotation of spheroids, n/N (%)	0/51 (0)	2/51 (4)	4/51 (8)	0/45 (0)
Absence of spheroid rotation, n/N (%)	51/51 (100)	49/51 (96)	47/51 (92)	45/45 (100)
Normal ciliary beat pattern, n/N (%)	0/51 (0)	12/51 (24)	15/51 (29)	9/45 (20)
Abnormal non-flexible beat pattern with hyperkinetic beat, n/N (%)	51/51 (100)	39/51 (76)	36/51 (71)	36/45 (80)

Spheroids were divided into two aliquots (25 spheroids/aliquot) and transduced at day 6 of cultivation. Results are expressed as total number of spheroids.
PCD, primary ciliary dyskinesia; TALEN, transcription activator-like effector nuclease.

inflammation. Most recommendations are based on expert opinion or extrapolated from available evidence for cystic fibrosis, despite the differences in pathophysiology between the two diseases.[3 8 10] However, rapid DNA sequencing methods have greatly accelerated the discovery of genes involved in the disease and opened up new perspectives for personalised treatment approaches to PCD, as is now increasingly being seen for cystic fibrosis.

Three published studies have shown that gene therapy is applicable to PCD and ciliopathies. In the first study, ex vivo human airway epithelial cells with mutated dynein axonemal intermediate chain type 1 (*DNAI1*) gene and immotile cilia were transduced with whole *DNAI1* and recovered CBF, CMP and ciliary structure.[11] The second study worked on *DNAI1* mouse orthologous *Dnaic1*. Here, transfer of the whole gene restored ciliary activity, ex vivo and in vivo, of mouse tracheal cells bearing a targeted deletion in *Dnaic1*.[12] In the third study, gene therapy re-established ciliary beating and olfactory function in mice.[27]

The present study applied, for the first time in PCD, a novel approach called 'gene editing' and was designed to restore gene function by replacing the inactivating mutation(s) with wild-type sequence in the diseased cell. Conventional gene therapy—that is, transfer of the full-length wild-type gene—is exploitable only for genes transportable by current delivery systems; in fact, a number of genes exceed vector capacity. Gene length is irrelevant for gene editing. Further, transferred and resident cellular genes are driven by different promoters, implying that

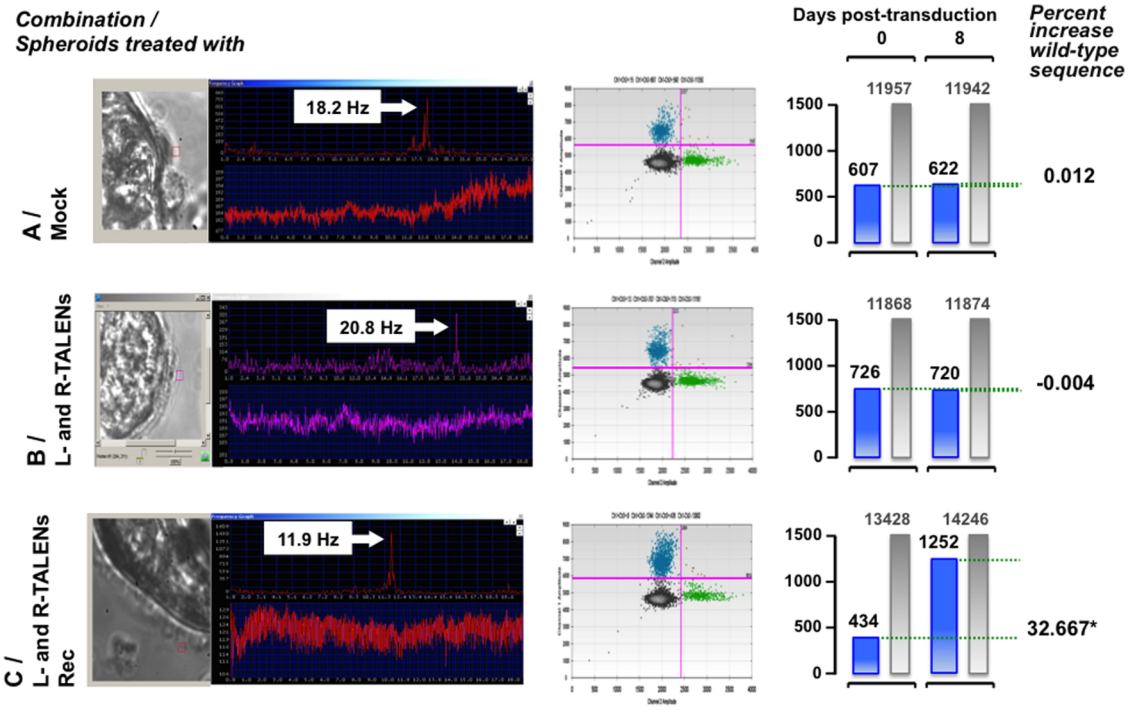

Figure 5 Analysis of ciliary motility and number of wild-type alleles at day 0 and 8 post-transduction. Spheroids from patient A were transduced with the vectors indicated on the left and monitored every 2 days for ciliary beating pattern and frequency. Panels show the ciliary beat frequency measured at day 5 post-transduction for untreated cells (upper panel), cells treated with L- and R- transcription activator-like effector nucleases (TALENs) (middle panel), and L- and R-TALENs and Rec (lower panel). The latter treatment reduced beating frequency to normal levels (12–14 Hz). Normalisation lasted throughout the observation treatment. At day 0 and 8 post-treatment, spheroids were collected in part and processed to extract the genomic DNA. Extracted nucleic acids were analysed by droplet digital PCR (ddPCR) to count the number of molecules containing the wild-type sequence at nucleotide position 172 381. Centre grey panels show, spatially separated, the wild-type sequence molecules in the top left quadrant (blue dots), mutated sequence molecules in lower right quadrant (green dots) and irrelevant molecules in lower left quadrant (grey dots) counted by ddPCR. The histograms on the right show the number of wild-type sequence molecules counted in spheroids at day 0 and 8 post-transduction and the percentage increment in wild-type molecules between the examined times. Spheroids treated with L- and R-TALENs and Rec show >30% increase in wild-type molecules. This increment reached statistical significance (*) as determined by the sequential probability ratio test (α value 0.01).

expression of a transferred gene may differ and be physiologically unrelated; again this does not apply to gene editing, which repairs the native cellular gene. Finally, gene therapy can be transient or permanent. The latter is achieved by integrating the transferred gene into the host cell genome, which causes serious concerns.[15] Gene editing does not require integration and permanently corrects the genetic defect. Gene editing also has its own drawbacks: the editing machinery must be tailored and designed for a specific mutation (or more mutations if in close vicinity to the TBS) and it may show off-target activity. The latter guided us in selecting the gene editing system. When this study started, over 2 years ago, ZFNs and, at that time, novel CRISPRs exhibited higher off-target activity and, according to the software available, fewer cleavage sites for efficient *DNAH11* sequence correction compared with TALENs.[28 29] Constant software and technology updates progressively reduced off-target risks to the extent that the technically simplest and most error-prone CRISPR boasts an ever-growing number of applications in vivo,[29 30] and a clinical trial in which human haematopoietic stem cells were ZFN-engineered to increase resistance to HIV infection showed only one serious adverse effect—attributed to transfusion reaction—after several months.[31] As a result, gene editing is applied in an ever-growing number of fields.[32–35] Within the limits of the study, our results suggest that off-target activity is not a threat, and gene editing is well tolerated.

Here we focused on *DNAH11*, mutations of which account for 6% of all PCDs, and 22% of those with normal ultrastructure.[9 10 19] This gene was chosen, as we have a relatively high number of PCD patients with *DNAH11* mutations referred to our paediatric unit and it is non-transportable by available delivery systems.[13] The outcome of this study could thus pave the way for the many ciliopathy genes for which conventional gene therapy is precluded. We chose to target the nonsense mutation of exon 42 because its replacement allows translation of a full-length protein, which is readily detectable compared with a single amino acid change, and there were two patients with the same mutation,[19] allowing us to test the approach in two patients simultaneously. To avoid repeated collection of ciliated cells from the two patients and other technicalities, we tested TALEN cleavage and HR in a surrogate system using GFP as a handy readout. In parallel, leftovers of ciliated cell cultures were used to evaluate off-target activity and optimise the efficiency of the transduction by an FIV vector safe in vitro and in vivo and known to transduce ciliated cells.[22 36–39]

The strengths of this study include the following. (1) Selected TALENs bind the intron upstream of the mutation, as was our deliberate intention. HR in an intron is advantageous because mutations, either natural or artificially introduced, have no or marginal effects on coding sequence and translation.[40] If HR disrupts the TBS, TALENs cannot cut the sequence again. Just a few mismatches are sufficient to abolish site recognition.[35 41] (2) HR did not introduce frameshifts, which can occur and create aberrant proteins.[42] (3) CBF and CMP were normalised in 28% and 20% of spheroids of patients A and B, respectively. While it is not possible to establish if this translates to clinical benefit, accruing evidence suggests that this degree of correction is sufficient to improve clinical conditions in humans and experimental models.[17 27–29]

There are unanswered questions that require more research. (1) Spheroids from ciliated cells were highly susceptible to transduction and showed minimal inter-patient variation, but transduction mostly occurred in external, most-vector-accessible, ciliated cells. Here, LVs were pseudotyped with vesicular stomatitis virus glycoprotein G, which allows penetration of respiratory cells via the basolateral surface[36] and confers broad cell tropism, but makes the vector prone to inactivation by complement and other host defence mechanisms.[43] Pseudotyping vectors with filovirus, baculovirus and influenza coat proteins and pretreating the respiratory mucosa with clinically approved mucolytic agents[44–46] should overcome local barriers and allow meaningful HR levels to be obtained in vivo. (2) Spheroids were transduced at day 6 post-collection, and normalisation of ciliary motility was observed by 2 days later, at a time when spheroids still retain the original cilia.[6] Although little is known about the time required for assembly or turnover of axonemal proteins in mammalian systems,[12] the observation that the original cilia are maintained in culture for about 2 weeks and are then progressively replaced by new cilia[6 20] suggests that, in the present work, normalisation of ciliary motility occurred by replacement of defective with normal DNAH11 protein. This may be facilitated by the fact that mutations of *DNAH11* do not overtly alter axoneme structure. Any effects of gene repair on new cilia formation remain to be established. (3) Transduction was achieved with three vectors entering the same cell. This is very difficult to achieve in vivo, but second-generation TALENs can be delivered by a single vector.[47] (4) Apical ciliated cells are terminally differentiated and thus, for long-lasting benefits, the genetic defect must be corrected in dividing progenitor cells residing in epithelial basal layers. There are valuable tools—for example, three-dimensional respiratory epithelial cell cultures—and recent studies[28 48 49] to assist in the design of strategies to target basal cells.

In summary, this study demonstrates that ciliary beating and motility in primary ciliated cells can be rescued by gene editing. Further studies are warranted to assess the clinical benefit of this approach. If successful, this study will open up new ways to treat PCD.

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Contributors ML designed and constructed the gene editing system; MPif and MPis designed the study, evaluated the experimental data, wrote the manuscript, and approved the version to be submitted; ABu and ABo contributed to data evaluation and revised the manuscript; MPif, MPir and MDC collected and cultured airway ciliated cells and performed cilia analyses; AM, SF and PS performed genetic analyses; ADG, PQ, CC, ET and MCM performed in vitro tests; GS helped to draft the manuscript.

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Gene editing of *DNAH11* restores normal cilia motility in primary ciliary dyskinesia

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