Targeted exon skipping of a CEP290 mutation rescues Joubert syndrome phenotypes in vitro and in a murine model

Simon A. Ramsbottom,a,1 Elisa Molinari,a,1 Shalabh Srivastava,a, b,1 Flora Silberman,a Charlie Henry,a Sumaya Alkanderi,a, Laura A. Devlin,a, Kathryn White,d David H. Steel,a,b,2 Sophie Saunier,a, Colin G. Miles,a,b,2 and John A. Sawyer,a,b,1,2

aInstitute of Genetic Medicine, Newcastle University, International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, United Kingdom; bRenal Services, The Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne NE7 7DN, United Kingdom; cLaboratory of Hereditary Kidney Diseases, INSERM UMR 1163, Sorbonne Paris Cité University, Imagine Institute, 75015 Paris, France; dElectron Microscopy Research Services, Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom; eSunderland Eye Infirmary, Sunderland SR2 9HP, United Kingdom; and fNational Institute for Health Research Newcastle Biomedical Research Centre, Newcastle upon Tyne NE4 5PL, United Kingdom

Edited by Martin R. Pollak, Harvard Medical School, Beth Israel Deaconess Medical Center, Brookline, MA, and approved October 19, 2018 (received for review June 1, 2018)

Genetic treatments of renal ciliopathies leading to cystic kidney disease would provide a real advance in current therapies. Mutations in CEP290 underlie a ciliopathy called Joubert syndrome (JBTS). Human disease phenotypes include cerebral, retinal, and renal disease, which typically progresses to end stage renal failure (ESRF) within the first two decades of life. While currently incurable, there is often a period of years between diagnosis and ESRF that provides a potential window for therapeutic intervention. By studying patient biopsies, patient-derived kidney cells, and a mouse model, we identify abnormal elongation of primary cilia as a key pathophysiological feature of CEP290-associated JBTS and show that antisense oligonucleotide (ASO)-induced splicing of the mutated exon (41, G1890+1) restores protein expression in patient cells. We demonstrate that ASO-induced splicing leading to exon skipping is tolerated, resulting in correct localization of CEP290 protein to the ciliary transition zone, and restoration of normal cilia length in patient kidney cells. Using a gene trap Cep290 mouse model of JBTS, we show that systemic ASO treatment can reduce the cystic burden of diseased kidneys in vivo. These findings indicate that ASO treatment may represent a promising therapeutic approach for kidney disease in CEP290-associated ciliopathy syndromes.

Significance

The treatment of genetic kidney disease is challenging, as this requires both the correction of the underlying gene defect and the delivery of the treatment. Here we show that by using antisense oligonucleotides, we can induce exon skipping of a mutated exon in CEP290, within renal epithelial cells derived from a patient with a ciliopathy syndrome called Joubert syndrome. This treatment rescues the truncated CEP290 protein to a near full-length protein and restores the ciliary phenotype. In a Cep290 murine model of Joubert syndrome, exon skipping is achievable with systemic treatment of an antisense oligonucleotide, which rescues both the ciliary and kidney disease phenotypes. This work paves the way toward personalized genetic therapies in patients with inherited kidney diseases.


The authors declare no conflict of interest.

Recent studies have suggested alternative therapeutic strategies for ciliopathies that directly correct the genetic lesion, in particular targeting CEP290 mutations in LCA (10). The most common cause of LCA is an intronic mutation that creates a splice donor site and a pseudoexon, disrupting the CEP290 transcript (11). Treatment with a splice-blocking antisense oligonucleotide (ASO) was able to restore the normal transcript in both patient cell lines and a mouse model of LCA (10). The majority of CEP290 mutations causing JBTS are within the coding sequence, which is more suited to gene replacement therapy. However, the size of the gene (54 exons) and its protein product (290 kDa) pose a considerable challenge for conventional, viral-based, gene replacement therapies, although lentiviral vector delivery of full-length CEP290 has been used successfully in patient fibroblast cells (12). Following a report of a patient with a mild LCA phenotype associated with nonsense-mediated alternative splicing of CEP290 (11), an extensive study of endogenous (wild-type) splicing revealed widespread, low-level alternative splicing that...

www.pnas.org/cgi/doi/10.1073/pnas.1809432115
could be modeled to predict genetic pleiotropy associated with CEP290 mutations (13). Indeed, confirming this hypothesis, endogenous basal exon skipping and nonsense-associated altered splicing has been documented in patient fibroblasts with nonsense mutations in CEP290 with mild retinal phenotypes (14). These observations are reminiscent of the Duchenne/Becker muscular dystrophy paradigm (15), which responds to targeted exon skipping therapies (16, 17). Given that most of the CEP290 protein consists of repeated coiled-coil domains, often encoded by a single exon, CEP290 seems an ideal candidate for ASO-mediated exon skipping therapy.

Results

Clinical and Genetic Investigations. Here we describe a 14 y old boy (JBTS-AA) from consanguineous parents (Fig. 1A) affected with JBTS. He initially presented with congenital ptosis and visual

![Figure 1](https://www.pnas.org/ cgi/doi/10.1073/pnas.1809432115)

**Fig. 1.** Clinical phenotype, molecular genetics, and primary cilia defects in human urine-derived renal epithelial cells from a Joubert syndrome patient. (A) Pedigree diagram showing single affected male, JBTS-AA (squares, males; circles, females). (B) Renal ultrasound scan of JBTS-AA showing minor cystic change within the kidney (arrowed) and loss of corticomedullary differentiation consistent with a diagnosis of nephronophthisis. (C) MRI scan of JBTS-AA showing a molar tooth sign (arrowed) with cerebellar vermis aplasia and elongated superior cerebellar peduncles. (D) Sequence chromatograms showing homozygous change in CEP290 c.5668G>T; p.G1890* segregating from parental DNA (father’s chromatogram is shown). (E) SMART Domain structure of WT CEP290 protein (2,479 amino acids) predicts multiple coiled-coil domains (show as blue bars). The truncated protein CEP290 G1890* results in loss of C-terminal coiled-coil domains. (F) Western blotting of protein from human urine-derived renal epithelial cells showing loss of full-length CEP290 protein in JBTS-AA. Blots were probed with a CEP290 antibody directed at the C terminus of CEP290, with GAPDH shown as a loading control. (G) Wild-type (WT) and (H) patient (JBTS-AA) hURECs imaged under high-power immunofluorescence using anti-ARL13B (green) to identify ciliary membrane. (Scale bar: 5 μm.) (I) Quantification of ciliary length in cells grown in serum-free medium for 48 h, measured by immunofluorescence imaging (WT, n = 55, JBTS-AA, n = 222; ***P < 0.0001, unpaired t test). (J) Wild-type (WT) and (K) patient (JBTS-AA) hURECs seen under scanning EM reveals abnormally long cilia in patient cells after 48 h serum starvation (Scale bar: 2 μm.). (L) Quantification of increase in cilia length (WT, n = 16, JBTS-AA, n = 10; *P < 0.05, unpaired t test).
failure secondary to an early onset retinal degeneration (SI Appendix, Fig. S1) and subsequently developed ataxia and significant renal impairment (Table 1). Clinical investigations revealed hyperechogenic kidneys showing cystic change and loss of corticomedullary differentiation (Fig. 1B), consistent with a diagnosis of nephronophthisis, and a “molar tooth” sign on brain MRI (Fig. 1C) which is due to cerebellar vermis aplasia and is the defining feature of JBTS. Molecular genetic investigations in JBTS-AA confirmed a homozygous nucleotide substitution leading to a stop codon (c.5668G>T; p.G1890*) in exon 41 of CEP290 (Fig. 1D). This mutation is predicted to lead to a truncated CEP290 protein lacking numerous C-terminal coiled-coil domains (Fig. 1E).

Phenotyping Using Primary Renal Epithelial Cells. To characterize the cellular and molecular consequences of this mutation, we derived primary, nontransformed human urine-derived renal epithelial cells (hURECs) from patient JBTS-AA and age/sex matched controls (WT). Western blotting, using a CEP290 C-terminal antibody, confirmed an almost complete absence of full-length CEP290 protein (Fig. 1F) from patient hURECs. Immunofluorescence analysis of cilia structure revealed elongated primary cilia on patient hURECs (JBTS-AA) compared with control (WT) cells (Fig. 1G–I). This was confirmed by scanning electron microscopy (Fig. 1J–L). In terms of percentage ciliation rates in hURECs, there were no differences between wild-type and JBTS-AA cells (SI Appendix, Fig. S2). We have previously described an elongated cilia phenotype in hURECs derived from an unrelated JBTS patient harboring CEP290 mutations (9), suggesting abnormally long primary cilia may be a common, renal feature of JBTS. To investigate this further, we carried out immunofluorescence analysis of cilia structure in two further (unrelated) JBTS patients with CEP290 mutations and kidney failure (SI Appendix, Table S1). Immunofluorescence staining of renal biopsies for acetylated tubulin and ARL13B revealed the presence of elongated and tortuous primary cilia in both patients compared with normal kidney (SI Appendix, Fig. S3) confirming that the elongated primary cilia found on patient hURECs accurately reflect the phenotype observed in vivo. It is intriguing to note that elongated cilia have also been found in a wide range of other ciliopathy models including: Bbs-4 null mice (18), jck mice (19), Meckel syndrome (20), Kif7 mutated cells (21), and JBTS secondary to KIAA0556 mutations (22), suggesting elongated primary cilia may be a widespread feature of ciliopathies in general and that correction of this phenotype attenuates cyspic kidney disease (23).
we wished to determine whether a similar strategy could be applied in vivo. The most faithful model of JBTS described to date is the Cep290(Gt(CC0582)Wtsi)Wbal mouse line (which we will refer to as Cep290(Gt), previously reported as Cep290p.Aac) that presents with a renal-retinal-brain phenotype caused by insertion of a “gene trap” within Cep290 after exon 25 (8). As the Cep290(Gt) mice were produced from embryonic stem (ES) cells containing an intronic splice acceptor/β-galactosidase/neomycin phosphotransferase (SA-IREs-βGEO) “gene trap” cassette (3,975 bp), we designed a new oligonucleotide to block the splice acceptor of the gene trap cassette (vivo-ASO) to restore full-length Cep290 transcript and protein (SI Appendix, Fig. S7). As proof of concept, treatment of immortalized kidney cells derived from homozygous Cep290p.Aac mice with vivo-ASO resulted in a modest restoration of correct splicing between exons 25 and 26, with a concomitant decrease in exon 25 splicing into SA-IRES-βGEO, and a return of a full-length Cep290 protein detected by Western blotting using a C-terminal Cep290 antibody (SI Appendix, Fig. S7).

In contrast to ASO treatment of JBTS-AA hURECs, wild-type levels of transcript/protein were not achieved in the transformed mouse cell line following vivo-ASO treatment. RT-PCR analysis with a panel of primers located across the SA-IREs-βGEO cassette suggests that we do not see complete restoration of wild-type transcript levels (SI Appendix, Fig. S7), possibly due to the presence of cryptic splice sites within the SA-IREs-βGEO cassette (24, 25). However, given that Cep290 is not haploinsufficient and it has been reported that small fragments of CEP290 can ameliorate LCA (26), we reasoned that even subwild-type levels of protein may be beneficial. Therefore, we proceeded to investigate the potential effects of a modest restoration of full-length protein in vivo, in Cep290p.Aac mice. Systemic administration of vivo-ASO was carried out via a series of i.v. injections over 10 d. RT-PCR analysis and sequencing of whole kidney RNA revealed correctly spliced transcripts (Fig. 3 and SI Appendix, Fig. S8), consistent with splicing from exon 25 to exon 26, skipping SA-IREs-βGEO. Full-length Cep290 protein was detected by a Western blot of whole kidney extracts using C-terminal Cep290 antibody (Fig. 3B) indicating that vivo-ASO was capable of inducing splicing similar to that observed in vitro (Fig. 3C).
Kidneys had increased Shh signaling, as demonstrated by treatment, similar to the 24 h ASO treatment. Remarkably, vivo-ASO resulted in a striking and significant improvement in cystic index (Fig. 3 E and F) of 37%, indicating that measurable therapeutic benefit can be achieved in vivo by relatively modest levels of ASO-induced splice blocking.

Discussion

We have shown that in human renal epithelial cells from a patient with JBTS secondary to a nonsense mutation in exon 41 of CEP290, ASO-mediated exon skipping can ameliorate the ciliary phenotype and restore localization of a near full-length CEP290 protein to the base of the cilium. We have then validated this approach in vivo, by using a different ASO targeting strategy to promote alternate splicing of a murine gene trap insertion within intron 25 of Cep290, thereby rescuing, in part, full-length Cep290 transcript and protein to achieve a phenotypic rescue of renal ciliary length in vivo as well as the cystic kidney disease. The observed phenotypic changes in murine and human cells, as well as in mouse kidney tissue following treatment with two completely different ASOs indicate that the observed effects are specific, and result from the restoration of CEP290/Cep290.

Overall, these data have several important implications. Firstly, systemic delivery of ASOs can ameliorate kidney pathology in a whole animal model. In addition, ASOs can rescue disease phenotypes after disease has become established in both JBTS patient-derived cells and an animal model, confirming the indications from pharmacological treatments that the cystic kidney disease is reversible. Finally, ASO-mediated exon skipping is tolerated at the level of protein localization, as the removal of a coiled-coil
domain does not result in the mislocalization of the CEP290 protein. Given that many CEP290 mutations cluster in the C-terminal coiled-coil-rich region and are potentially skippable (SI Appendix, Fig. S9), it is tempting to speculate that these mutations may also be amenable to ASO-mediated therapy. One limitation of this study is that despite G1890* being one of the most common mutations, we have not extended our human studies to other skippable exons in CEP290. Here true personalized medicine approaches, using patient-derived cells, such as we have used with patient JBTSS-1A, will be required to determine if exon skipping of alternate exons, which may contain more crucial functional domains than exon 41, are possible. The direct consequences of skipping exons within the CEP290 gene require testing in both ex vivo and in vivo systems. A Cep290 mouse model, mimicking the human G1890* mutation, would be required to determine fully the effects of ASO-mediated exon skipping in both kidney and extrarenal tissues. Given the ease of access for subretinal injections, to date there has been a focus on ASO-based therapies for human retinal disease, including CEP290-associated LCA (10, 27). As our work has shown, systemic delivery of ASOs is possible and seems to ameliorate kidney disease; this needs to be assessed more fully in renal tissues as well as retinal tissues for dosing regimens and timing of therapies. However, given the efficiency of ASO-mediated exon skipping observed in patient kidney cells, where the next available splice acceptor is used almost exclusively, this is likely to confirm that ASO treatments should be considered as a promising therapeutic approach. It seems likely, following on from the success of ASO-based therapy for targeting CEP290 retinal disease, that ASO-based therapy for CEP290-associated renal disease has true translational promise and may provide a genetic rescuing therapy for this severe ciliopathy syndrome.

In conclusion, we show that using ASO-mediated exon skipping rescues a ciliary defect in patient renal epithelial cells and can be used systemically to target CEP290 transcripts within kidney tissues, offering a therapeutic approach to treating inherited renal ciliopathies.

**Materials and Methods**

**Study Approval.** Ethical approval was obtained from the National Research Ethics Service Committee North East (14/NE/1076), United Kingdom. All animal experiments were conducted according to protocols approved by the Animal Ethics Committee of Newcastle University and the Home Office, United Kingdom.

**Statistics.** All data are shown as the mean ± SEM, unless otherwise stated and unpaired Student’s t test or one-way ANOVA followed by a Bonferroni corrected post hoc test when comparing two or more groups. A P value of less than 0.05 was considered statistically significant.

**Clinical and DNA Sequencing.** Following informed and written consent, urine and blood samples were obtained from a 14-y-old boy with clinical JBTSS with a renal, retinal, and cerebellar phenotype and a healthy gender- and age-matched control. All methods were performed in accordance with the relevant ethical guidelines and regulations. Sequencing of Joubert syndrome genes was performed by UKGTN using an 18 Gene Panel (Oxford Regional Genetic Laboratory Service). Confirmation of mutations and segregation analysis was performed on other family members. Following informed consent, using Sanger sequencing, using exon specific primers.

In **Vitro Studies Using Human Kidney and hURECs.** Immunostaining of human kidney tissues from patients with CEP290 mutations was performed in vitro studies following HUREC isolation (28), culture, and ASO treatments were performed, including Western immunostaining, Western blotting, and reverse transcription-PCR and quantitative RT-PCR. Electron microscopy studies were performed on hURECs. Please see SI Appendix for detailed materials and methods.

In **Vivo ASO Treatment of Cep290** Animals. In vivo studies were performed on 28-d-old Cep290 animals. Renal tissues were harvested and used for immunofluorescence studies, Western blotting, RNA extraction, and reverse transcription-PCR and quantitative RT-PCR. Please see SI Appendix for detailed materials and methods.

**ACKNOWLEDGMENTS.** Thank you to the patient and family members who contributed to this study. This work is funded by The Medical Research Council (Award MIR/M012212/1), a Kidney Research UK Post-doctoral fellowship (to S.A.R.) (Award PDF_003_20151124), and Northern Counties Kidney Research Fund. L.D. is funded by The Medical Research Council Discovery Medicine North Training Partnership.