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Gene Therapy Using a miniCEP290 Fragment Delays Photoreceptor Degeneration in a Mouse Model of Leber Congenital Amaurosis

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Mutations in the cilia-centrosomal protein CEP290 are frequently observed in autosomal recessive childhood blindness disorder Leber congenital amaurosis (LCA). No treatment or cure currently exists for this disorder. The Cep290rd16 (retinal degeneration 16) mouse (a model of LCA) carries a mutation in the Cep290 gene. This mutation leads to shorter cilia formation and defective photoreceptor structure and function. A roadblock to developing a gene replacement strategy for CEP290 using conventional adenovirus (AAV) vectors is its large size. The identification and characterization is reported of a miniCEP290 gene that is amenable to AAV2/8-mediated delivery and delaying retinal degeneration in the Cep290rd16 mice. Using the ability of Cep290rd16 mouse embryonic fibroblasts to form shorter cilia as a platform, a human CEP290 domain encoded by amino acids 580–1180 (miniCEP290580-1180) was identified that can recover the cilia length in vitro. Furthermore, subretinal injection of AAV particles carrying the cDNA expressing miniCEP290580-1180 into neonatal Cep290rd16 mice resulted in significantly improved photoreceptor survival, morphology, and function compared to control injected mice. These studies show the potential of using a truncated CEP290 to treat this fast progressing and devastating disease.

Keywords: cilia, retina, photoreceptor, ciliopathies, gene therapy, LCA

INTRODUCTION

The cilium is an evolutionarily conserved sensory organelle involved in regulating development and homeostasis.1 In the light-sensing neurons called photoreceptors (PRs), the bridge-like “connecting cilium” links the light-sensing outer segment to the protein-synthesizing inner segment.2 Mutations in the genes encoding ciliary proteins result in disorders collectively termed “ciliopathies” that can affect the retina alone or as part of a syndromic disease.3 One of the most severe forms of retinal ciliopathies is Leber congenital amaurosis (LCA).4 This disorder results in early and profound PR dysfunction and degeneration, usually beginning at birth or by around 5 years of age. CEP290 mutations are also associated with syndromic ciliopathies, including Joubert syndrome, Meckel–Gruber Syndrome, Bardet–Biedl syndrome (BBS), and Senior–Løken syndrome.5–10 No treatment or cure currently exists for such diseases.4

Mutations in the cilia-centrosomal protein encoding gene CEP290 are the most frequent cause of LCA, accounting for >25% of LCA (LCA10; OMIM 611755).11 It has been demonstrated that CEP290 is involved in regulating the cilium assembly program. Mouse embryonic fibroblasts (MEFs) derived from a Cep290-mutant (Cep290rd16) mouse model of LCA-like PR degeneration12 have fewer ciliated cells and form shorter cilia.13 Moreover, the CEP290-associated disease is likely due to reduction or the loss of normal protein function.6,14–16 Although antisense oligonucleotides and genome editing have been used to correct a common intronic mutation in CEP290, both are mutation-dependent approaches.17–20 Lentivirus-mediated delivery of the full-length (FL) CEP290 into patient-derived fibroblasts was also shown to rescue the CEP290-associated defect in vitro.13

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However, its efficacy and long-term safety have not been demonstrated in vivo. Recent studies have reported the development and characterization of several vertebrate models of Cep290. Mice generated either using gene trap alleles or by creating a null allele within exons 1–4 or exons 36–37 of mouse Cep290 recapitulate the phenotypes associated with syndromic ciliopathies. However, they are not ideal model systems to investigate nonsyndromic LCA due to CEP290 mutations. Earlier studies also identified a naturally occurring model of retinal degeneration due to CEP290 mutation in exon 50. As predicted, this model undergoes relatively delayed and late onset form of retinal degeneration. The identification and characterization of a naturally occurring mouse mutant of Cep290, termed Cep290\textsuperscript{rd16}, was previously reported. This mutant carries an in-frame deletion within exons 37–41 of mouse Cep290, which encodes a part of the myosin-tail homology domain (deleted aa 1606–1904). The deletion results in the production of a truncated CEP290 variant, which is partially functional. The Cep290\textsuperscript{rd16} mouse exhibits non-syndromic retinal degeneration, which mimics the LCA onset and phenotype observed in patients. Moreover, the region of the human CEP290 gene that is homologous to the domain deleted in the Cep290\textsuperscript{rd16} mouse harbors LCA-causing mutations. Thus, the Cep290\textsuperscript{rd16} mice provide an excellent platform to initiate therapeutic investigations.

Adeno-associated virus (AAV) vector-mediated delivery of the transgene into the subretinal space is a safe and effective approach to treat PR degenerative diseases. However, a roadblock in AAV-mediated CEP290 delivery is the size of the CEP290 cDNA (~8 kb), which exceeds the transgene packaging capacity of the conventional AAV vectors (~4.0 kb). This study was undertaken to engineer AAV-amenable miniCEP290 genes encoding functionally optimized proteins, as described previously for muscular dystrophy causing gene dystrophin. The studies provide evidence for the suitability of using miniCEP290 as a readily translatable paradigm for CEP290-LCA.

**MATERIALS AND METHODS**

**Plasmid constructs and AAV production**

For in vitro experiments, FL or miniCEP290-expressing cDNAs were cloned into a pEGFP-C1 plasmid expressing GFP-tagged proteins under the control of a CMV promoter. For AAV production, the miniCEP290-encoding cDNAs were cloned into a pAAV2 vector plasmid between a CMV enhancer/CA (chicken β-actin) promoter upstream of the internal ribosome entry site (IRES) GFP and β-globin intron. This expression cassette was flanked with AAV2 inverted terminal repeats (ITRs). The recombinant AAV2 genomes were packaged with AAV8 capsid by HEK293-triple transfection method and purified by CsCl gradient centrifugation method, as previously described.

**Cell culture, transient transfection, and immunostaining**

MEFs derived from wild-type (WT) and Cep290\textsuperscript{rd16} mice were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Transient transfection with GFP-CEP290-FL or GFP-miniCEP290s was performed using Lipofectamine 2000 (Thermo Fisher). The transfected cells were then immunostained and imaged under a Leica microscope (DM5500). The cilia length was measured by using Image J. The pixel values of cilia length were transformed into actual length using the Leica Application Suite. The relationship between pixel length and actual length at 63× magnification is: 680 pixels = 100 μm.

**Subretinal injection**

All animal studies were approved by the Institutional Animal Care and Use Committee of the UMASS Medical School. The WT C57BL6/J mice were obtained from Jackson Laboratories. The Cep290\textsuperscript{rd16} mice have been described previously. The Cep290\textsuperscript{rd16} mouse pups (P0/P1) were sub-retinally injected unilaterally with 8 × 10⁹ vg/μL (total volume 1 μL) of the virus.

**Electroretinogram and immunofluorescence microscopy of the retina**

Scotopic and photopic electroretinograms (ERGs) were performed, as previously reported. For scotopic response, mice were dark adapted overnight, and all procedures were performed under dim red light. The dark-adapted ERG protocol consisted of five steps with increasing stimulus intensities from 0.009 to 100 cd.s/m², with a mixed white light (white 6,500 K; ColorDome; Diagnosys LLC). The flash frequency was 0.07 Hz for bright flashes and up to 0.5 Hz for dim flashes. Averages ranged from 10 trials for dim flashes to five trials for bright flashes. Light-adapted (photopic) ERGs were recorded after light adaptation with a background illumination of 30 cd/m² (white 6,500 K) for 8 min.

Immunofluorescence microscopy was performed essentially as described. In brief, retinal cryosec-
tions were stained with primary antibodies: rhodopsin (mouse monoclonal; EMD Millipore), M-opsin (rabbit polyclonal; EMD Millipore), and peripherin-retinal degeneration slow (RDS; mouse monoclonal; gift from Dr. Muna Naash), ARL13B (rabbit polyclonal; Abcam), GFP (chicken polyclonal; Abcam), and γ-tubulin (mouse monoclonal; Sigma–Aldrich). After washing with phosphate-buffered saline, Alexa-488 or Alexa-546 or Alexa-633-conjugated secondary antibodies were added, and the sections were further incubated for 1 h. After washing, nuclei were stained with DAPI, and cells were imaged using a Leica microscope (DM5500).

Statistical analysis
All data are presented as means ± standard error of the mean. Data groups were compared by Student’s t-tests using the SigmaPlot software. Differences between groups were considered statistically significant if p < 0.05. The statistical significance is denoted with asterisks (*p < 0.01–0.05; **p < 0.001–0.01; ***p < 0.0001–0.001).

RESULTS
Construction and in vitro functional potential of miniCEP290s
The human CEP290 gene consists of 52 exons, which encode a protein of ~290 kDa (2,479 amino acids). Based on the previous studies on the involvement of the different CEP290 domains in its interactions and ciliary localization,12,14,38–42 plasmids encoding GFP-fused human miniCEP290580–1695, miniCEP2901751–2050 and miniCEP2902037–2479 were constructed (Fig. 1A). To test the functional potential of the miniCEP290s, a surrogate assay system was
designed using Cep290<sup>rd16</sup> MEFs. First, the expression and localization of the different miniCEP290s in the Cep290<sup>rd16</sup> MEFs were investigated. The analysis revealed that the FL CEP290 and all CEP290 domains expressed at comparable levels in the MEFs (Fig. 1B). Additional bands observed in the immunoblot most likely represent degradation products of the fusion protein. In addition to GFP-CEP290-FL, miniCEP290<sup>580–1695</sup> showed a predominant localization to the base of cilia (co-localization with γ-tubulin) and proximal cilia (co-localization with ADP-Ribosylation Factor-Like 13B; ARL13B; ciliary marker; Fig. 1C). Other variants showed a relatively diffuse pattern of localization. Next, the ability of the miniCEP290s to modulate cilia length in the Cep290<sup>rd16</sup> MEFs was assessed. Cilia of Cep290<sup>rd16</sup> MEFs are around 1.7 μm in length compared to controls, which have around 2.7 μm-long cilia. Cilia length of the Cep290<sup>rd16</sup> MEFs was significantly increased when either FL GFP-CEP290 or GFP-miniCEP290<sup>580–1695</sup> was expressed. Other variants and the plasmid expressing only GFP did not change the cilia length. No effect on the cilia length of the WT MEFs was observed (Fig. 1D).

Next, it was asked whether further shortening of miniCEP290<sup>580–1180</sup> would also result in a cilia length rescue. To this end, plasmids encoding GFP-miniCEP290<sup>580–1180</sup> and miniCEP290<sup>1181–1695</sup> were constructed. Both variants exhibited optimal expression, as determined by immunoblotting using GFP antibody (Fig. 2A). Additional degraded products of the fusion protein were also detected using the GFP antibody. While GFP-miniCEP290<sup>580–1180</sup> localized predominantly to the cilia, miniCEP290<sup>1181–1695</sup> exhibited a relatively diffuse localization in the cytosol with some ciliary localization (Fig. 2B). Cilia rescue assay showed that expression of either variant resulted in a comparable and significant increase in the cilia length of the Cep290<sup>rd16</sup> MEFs (Fig. 2C). Based on these results, miniCEP290<sup>580–1180</sup> and miniCEP290<sup>1181–1695</sup> were selected for further studies.

**Effect of miniCEP290 injection on PR function**

We cloned miniCEP290<sup>580–1180</sup>, miniCEP290<sup>1181–1695</sup>, and miniCEP290<sup>2037–2479</sup> (as negative control) into AAV2 vector genome with ubiquitously expressed CMVenhancer/CBA (chicken β-actin) promoter containing the IRES between the gene of interest and GFP. The IRES permits translation of CEP290 and GFP from a single bicistronic mRNA, which assists in identifying the transduced cells using GFP antibody. To assess the effect of GFP expression, we also used AAV2-GFP expressing construct as control. The viruses were packaged in AAV8 capsid, which is shown to transduce PRs efficiently. The viruses were injected at 8·10<sup>9</sup> vg/eye into the subretinal space of Cep290<sup>rd16</sup> pups at P0/P1. Similar doses have been successfully used to treat retinal degeneration in mice. Injections at higher doses resulted in toxicity (data not shown).
Analysis of PR function by ERG at 3 weeks post injection revealed improvement (25–30%) in both scotopic (rod PR-mediated) and photopic (cone PR-mediated) responses of the miniCEP290<sup>580–1180</sup>-injected mice (Fig. 3A–C). The miniCEP290<sup>1181–1695</sup>-injected mice revealed a relatively mild improvement in the outer nuclear layer preservation (Supplementary Fig. S1A and B; Supplementary Data are available online at www.liebertpub.com/hum) and scotopic and photopic ERG amplitudes (Supplementary Fig. S1C and D). No improvement was detected using miniCEP290<sup>2037–2479</sup> or GFP (Fig. 3). Age-matched un.injected mice were also used as controls. Further analysis revealed that the improvement in the ERG was stable up to 4 weeks and exhibited significantly decreased amplitudes at 5 and 8 weeks post injection (Fig. 3D). Although in vitro cilia length rescue assay showed similar rescue effect with both miniCEP290<sup>580–1180</sup> and miniCEP290<sup>1181–1695</sup> minigenes, the miniCEP290<sup>580–1180</sup> is more potent in vivo. Such results show that in vivo experiments are important to validate the in vitro data, and indicate the existence of domain-specific functions of CEP290.

**Effect of miniCEP290<sup>580–1180</sup> on retinal morphology**

To assess the effect of miniCEP290 injections on retinal morphology, retinal cryosections were used in which the injected regions were identified by staining with GFP antibody. The number of layers of the outer nuclear layer correlates with PR survival. The following were found: around six or seven layers in the Cep290<sup>d16</sup> retinas injected with miniCEP290<sup>580–1180</sup> and two to three layers in retinas injected with miniCEP290<sup>2037–2472</sup> or GFP (equivalent to un injected Cep290<sup>d16</sup> at 3 weeks of age; Fig. 4A). The number of layers in the outer nuclear layer progressively declined with age (Fig. 4B). Morphological analysis using ultrathin sections of the Cep290<sup>d16</sup> retinas injected with miniCEP290<sup>580–1180</sup> exhibited significant preservation of the outer nuclear layer at 3 weeks of age (Fig. 4C).

Next, the structural preservation of the PR outer segment was examined in the miniCEP290<sup>580–1180</sup>-injected mice by staining with peripherin-RDS (PR outer segment marker<sup>45</sup>). RDS is a structural protein that specifically localizes to the outer segment discs and maintains the OS structure. The miniCEP290<sup>580–1180</sup>-injected Cep290<sup>d16</sup> mice exhibited improved RDS localization to the outer segment compared to undetectable RDS expression in the GFP-injected mice (Fig. 4D; red staining).

The expression of rhodopsin and cone opsins, two of the key phototransduction proteins, was also examined. As predicted, there was undetectable opsin expression in the miniCEP290<sup>2037–2479</sup>-injected retinas. However, the miniCEP290<sup>580–1180</sup>-injected retinas revealed detectable expression of rhodopsin and cone opsins in the outer segments (Fig. 5). Some staining of cone opsins in the inner segment and outer nuclear layer was also observed. Overall, the results suggest that the expression of miniCEP290<sup>580–1180</sup> can improve the function, morphology, and opsin trafficking of the Cep290<sup>d16</sup> retinas.

**DISCUSSION**

Recent reports on the success of clinical trials of gene therapy for RPE65-LCA and choroideremia have provided considerable excitement in the field of AAV-mediated gene delivery into the diseased retina.<sup>28,30,46</sup> However, progress in the development of mutation-independent gene replacement strategies for CEP290-LCA has been delayed largely because of unsuitability of the long CEP290 gene to be packaged into conventional AAV vector system for gene therapy. This study reports the generation and potential of a truncated CEP290 domain (miniCEP290<sup>580–1180</sup>) to mitigate associated retinal degeneration. The studies provide the necessary first step to initiate the development of miniCEP290 strategy for tackling associated retinal ciliopathies, as well as systemic ciliopathies due to CEP290 mutations.<sup>47</sup> Taken together, evidence is provided for slowing down of the severe retinal degeneration using AAV-mediated delivery of a CEP290 minigene.

Although the scotopic and photopic ERG amplitudes and PR survival improved in the miniCEP290<sup>580–1180</sup>-treated retinas, they did not reach the levels of the WT mice. Moreover, the improvement declined with age. Such observations suggest that although the use of miniCEP290<sup>580–1180</sup> can be useful, additional analyses are needed to generate a more potent miniCEP290 that can mitigate CEP290-associated retinal degeneration in a diseased retina.

While the mechanism of action of miniCEP290<sup>580–1180</sup> is currently unclear, the findings reveal the potential of the miniCEP290 to compensate partially for the hypomorphic ΔCEP290 in the Cep290<sup>d16</sup> mouse.<sup>12</sup> Although the ACEP290 protein carries the domain encompassing 580–1180, the conformation of the resultant protein may no longer be functionally active. Supplementing the Cep290<sup>d16</sup> retina with aa 580–1180 likely provides the functional
Figure 3. In vivo physiological rescue potential of miniCEP290^{590-1180}. Cep290^{rd16} mice subretinally injected at P0/P1 stage with indicated miniCEP290s or GFP were analyzed by (A) scotopic and (B) photopic electroretinogram (ERG) at 3 weeks post injection. Age-matched uninjected WT or Cep290^{rd16} (littermates) mice were used as controls for ERG. The ERG a-wave is represented by arrows, while b-wave is depicted using arrowheads. (C) Statistical analysis of the scotopic a- and b-waves and photopic b-wave amplitudes was performed using at least six mice per experiment. ***p<0.001. (D) Scotopic (a- and b-waves) and photopic b-wave analysis of the injected mice was performed at 4, 5, and 8 weeks post injection and compared to the ERG amplitudes at 3 weeks post injection. Age-matched uninjected WT, GFP-injected Cep290^{rd16}, and uninjected Cep290^{rd16} mice were used as controls. *p<0.05; **p<0.001. Color images available online at www.liebertpub.com/hum
complementation needed to overcome the ciliary defect. These observations suggest that the CEP290 protein is modular and demonstrates flexibility in the protein structure–function relationship. This is also evident from the associated diverse phenotypes associated with Cep290 mutant mice. While the Cep290rd16 mice exhibit predominantly retinal degenerative and other sensory defects, the Cep290gt and Cep290ko mice manifest systemic disorders. Additional investigations are necessary to facilitate the generation of miniCEP290s that can mitigate systemic CEP290-ciliopathies. Nonetheless, the generation of partly functional PR outer segments provides a stepping stone to develop long-term disease correction when the therapy is initiated after disease onset. Further studies will focus on improving the efficacy of the miniCEP290s to mitigate associated retinal ciliopathy.

This work provides a strategy to tackle the critical roadblock in designing a suitable gene therapeutic strategy for CEP290-LCA by developing a mutation-independent approach. The relative sparing of the central cone-rich region of the CEP290-LCA patient retinas,48 such as in NPHP5-LCA,48 suggests that gene therapy in this region can be a viable option. However, there is dramatically reduced cone function in patients. Further investigations are needed to test the effect of miniCEP290s in dysfunctional CEP290-mutant cones, likely using larger animal models (such as cat)24,49 and cone-rich models.50

The use of minigenes as a therapeutic strategy is a viable option for delivering large genes, as has been demonstrated for dystrophin.32 Although lentiviral and nanoparticle-based delivery strategies for large genes, including ABCA4 (Stargardt disease) and Usher Syndrome genes, are also being investigated,51,52 the minigene approach offers a relatively faster and translatable paradigm for tackling such disorders.

Figure 4. In vivo morphological rescue potential of miniCEP290580–1180. Cep290rd16 retinas injected with indicated miniCEP290, GFP, or uninjected Cep290rd16 were stained with DAPI (blue) (A). The outer nuclear layer (ONL) is marked with vertical lines. WT retinal section is shown for comparison. INL, inner nuclear layer. (B) Quantification of the ONL thickness by counting the number of layers of the vector transduced region of the injected Cep290rd16 mice compared to uninjected retinas is represented. There is initial preservation of the number of layers, but progressive decline is detected with age. ***p < 0.0001. (C) The injected Cep290rd16 mouse retinas were also examined by ultrathin sectioning to assess morphological preservation at 3 weeks post injection. The ONL is marked with vertical lines. The outer segment (OS) layer is clearly visible in the miniCEP290580–1180 injected retinas. (D) Improved expression of RDS (rod) is detected in the miniCEP290580–1180 injected Cep290rd16 mice. Staining with GFP (green) marked the injected regions. Color images available online at www.liebertpub.com/hum
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AUTHOR DISCLOSURE

G.G. is a co-founder of Voyager Therapeutics and holds equity in the company, and is an inventor listed on patents with potential royalties licensed to Voyager Therapeutics and other biopharmaceutical companies. The remaining authors have nothing to disclose.

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