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ARTICLE

Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss

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Leber hereditary optic neuropathy is due to mitochondrial DNA mutations; in ~70% of all cases, a point mutation in the mitochondrial NADH dehydrogenase subunit 4, ND4, gene leads to central vision loss. We optimized allotypic expression (nuclear transcription of a gene that is normally transcribed inside the mitochondria) aimed at designing a gene therapy for ND4; its coding sequence was associated with the cis-acting elements of the human COX10 mRNA to allow the efficient mitochondrial delivery of the protein. After ocular administration to adult rats of a recombinant adeno-associated viral vector containing the human ND4 gene, we demonstrated that: (i) the sustained expression of human ND4 did not lead to harmful effects, instead the human protein is efficiently imported inside the mitochondria and assembled in respiratory chain complex I; (ii) the presence of the human protein in the experimental model of Leber hereditary optic neuropathy significantly prevents retinal ganglion cell degeneration and preserves both complex I function in optic nerves and visual function. Hence, the use of optimized allotypic expression is relevant for treating mitochondrial disorders due to mutations in the organelle genome.

INTRODUCTION

Leber hereditary optic neuropathy (LHON, MIM535000) is the most common illness caused by mitochondrial DNA (mtDNA) mutations, with an estimated prevalence of 1 in 40,000 in Europe.1 In 95% of LHON cases, the disease is due to point mutations in the ND1 (G3460A), ND4 (G11778A), or ND6 (T14484C) genes which encode proteins of the respiratory chain complex I (CI); about 70% of patients harbor the ND4 mutation.2 CI defect is the direct cause of retinal ganglion cell (RGC) loss and optic atrophy.3 There is currently no treatment for LHON; even though recent reports described that idebenone (an analog of Coenzyme Q10) or EPI-743 (a para-benzoquinone) administration partially ameliorate the visual outcome of LHON patients.4–6 LHON offers a unique opportunity for gene therapy setting for three reasons: (i) vision loss often occurs in a bilateral sequential fashion, the second eye becoming involved after the first with a median delay of 2 months,3 thus, a prospect exists for a therapeutic intervention after vision loss in the first eye before second eye involvement; (ii) drugs or gene therapy vectors may be easily and directly delivered to RGCs, by injection into the vitreous body.4,6 Thus, we intended to develop a strategy which could be translated to clinic research for LHON. Though, the techniques required for introducing genes directly into mitochondria are scarce yet, consequently, direct targeted repair or replacement of mutated mtDNA genes is not easy to perform except in yeast using bioinformatics strategies.7 Progress could be obtained with the use of modified AAV vectors which could deliver DNA to the organelle as recently reported.8,9 We choose to circumvent this barrier by using allotypic expression which is defined as a wild-type version of defective mitochondrion-encoded gene is delivered via a suitable vector to the nucleus of recipient cells. The corresponding mRNA is translated in the cytosol and the protein imported into the mitochondrion, where it should complement the genetic defect. However, because of the highly hydrophobic nature of mitochondrion-encoded proteins,10 several teams were unable of fully rescuing respiratory chain dysfunction in cultured cells with mtDNA mutations;10–13 despite some reports described satisfactory results.13,14 We did benefit from the knowledge, in Saccharomyces cerevisiae, on mitochondrial biogenesis and proteome.15 Reports on electron microscopy described the presence in the mitochondrial outer membrane of specifically attached 80S ribosomes.16 Biochemical purifications of these ribosomes confirmed that they contained mRNAs encoding mitochondrial proteins.17 Moreover, biochemical studies established that about 60% of the overall mRNA population encoding mitochondrial proteins specifically localized to the mitochondrial surface18 and that cis-acting elements in the 5′ and 3′ of these genes are involved in this specific localization.19 Hence, to optimize allotypic expression and guaranty the mitochondrial protein uptake, we built vectors in which the mtDNA sequences were combined with the Mitochondrial Targeting Sequence (MTS) in 5′ and

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the 3′UTR (UnTranslated Region) of the nuclear COX10 gene. COX10 mRNA, encoding an inner mitochondrial membrane protein involved in heme biosynthesis, is enriched at the surface of the mitochondria in human cells. Consequently, the hybrid mRNA should localize to the mitochondrial surface where the translation and the transport are tightly coupled.\textsuperscript{25,26} The beneficial effect of the procedure was demonstrated by the rescue of respiratory chain dysfunction in fibroblasts harboring mutations in ATP6, ND1, or ND4 genes.\textsuperscript{27,28} The extent of complex I or V activity restoration was higher when the COX10-3′UTR was used in the constructions instead of the poly adenylation signal of SV40; this is why we consider the procedure as “optimized” relative to the absence of this 3′UTR signal.\textsuperscript{27} Next, mitochondrial function modulation through optimized allotopic expression was confirmed \textit{in vivo} since a rat model of ND4 dysfunction in RGCs was generated by electroporation (ELP) of mutant ND4.\textsuperscript{29} We report here data from experiments conducted in three complementary axes aimed at translating this procedure to the clinic: (i) wild-type human ND4 expression driven by an adenov-associated virus type 2 (AAV2/2) vector does not cause rat retinal injury, though the lifetime of gene expression was up to 12 months; (ii) human ND4 protein produced from the vector is assembled in C; (iii) human ND4 protein is able to prevent CI deficiency and optic atrophy in a LHON rat model.

\section*{RESULTS}

Optimized allotopic expression of the human wild-type ND4 gene leads to sustained mRNA and protein accumulation in rat retinas without adverse effects on retinal integrity or function

A recombinant AAV2/2 vector harboring the human open reading frame of the mitochondrial ND4 gene and the signals allowing the efficient mitochondrial translocation of the protein (MTS and 3′UTR of the COX10 gene) was obtained (AAV2/2-ND4) (Figure 1a). It has been reported the long-term ocular gene transfer without adverse effects in mammals (mouse, rat, dog, nonhuman primates) when AAV2 vectors were used.\textsuperscript{30} In addition, the serotype 2 is highly efficient on transducing inner retina, primarily RGCs.\textsuperscript{31} The question arises whether AAV2/2-ND4 administration in the vitreous body of rat eyes is able to efficiently transduce RGCs without compromising retinal function. AAV2/2-ND4 vector was administered by a single injection in the vitreous body of adult rat eyes at different doses (Supplementary Table S1; Supplementary Material). First, we evaluated the transduction efficiency by determining the number of RGCs which expressed the GFP (gene included in the vector) in retinal sections from rats euthanized up to 12 months after vector administration. A recent report concluded that BRN3A antibody staining of radial retinal sections is a preferred method for estimating RGC frequency when compared to techniques using retinal whole mounts in rodent models of ocular disease.\textsuperscript{32}

When nine independent retinas subjected to AAV2/2-ND4 administration \textit{via} intravitreal injection were evaluated 6 months postinjection, we found that about 75% of RGCs expressed the transgene indicating both efficient and stable transduction with AAV2/2-ND4 (Supplementary Figure S1). Next, to determine the kinetics of human ND4 mRNA appearance, semiquantitative real-time PCR of reverse-transcribed mRNAs (RT-qPCR) were performed with RNAs purified from retinas isolated from eyes subjected to vector administration or contralateral untreated eyes (animals were euthanized between 2 to 16 weeks postinjection). Messenger RNA steady-state levels of the mitochondrial ATP6 gene was the most stable in the 60 independent samples evaluated. Therefore, it was chosen to normalize the mRNA species and to estimate their relative abundance. Human ND4 mRNA was detected 2 weeks after vector administration and its amount remained stable 14 weeks later (Figure 1b). This result may be considered as weak when compared to endogenous ATP6 mRNA; however the signal obtained by RT-qPCR for each mRNA molecule reflected their level in all the retinal cells (~10 millions) while human ND4 mRNA accumulated only in transduced RGCs representing ~1% of the overall retinal cell population.\textsuperscript{33} The human ND4 transcript was 15.5-fold (Figure 1c) and 331-fold less abundant than BRN3A and SNCG mRNAs respectively which encode known RGC markers.\textsuperscript{34,35} The sustained expression of human ND4 did not modify the relative abundance of the two transcripts (Figure 1d); indeed mRNA levels in treated eyes relative to untreated ones were not significantly different (P values: 0.26 for BRN3A and 0.88 for SNCG). Protein extractions from single retinas were subjected to Western blotting analyses to determine the steady-state levels of the human ND4 protein in retinas from four treated eyes (6 months after treatment), and compared with the abundance of two CI subunits (NDUFA9 and NDUF8B) and one complex V subunit (ATP synthase α). The antibody against HA1 epitope (three HA1 epitopes are appended at the C-terminal of the human ND4 protein) revealed a signal with an apparent molecular mass of ~54kDa only in retinas from treated eyes (Figure 1e); the predicted size of the human ND4 protein, after the cleavage of the COX10 MTS, is about 54 kDa: 51.7kDa for the 459 amino acids of ND4 + 2.7kDa for the 27 amino acids of the 3 HA1 epitopes. The mitochondrial proteins NDUFA9, NDUF8B and ATP synthase α were detected as single bands of approximately 39, 19, and 60 kDa (Figure 1e).

To establish whether human ND4 expression could be detrimental for retinal function or morphology two parameters were evaluated: eye fundus using confocal Scanning Laser Ophthalmoscopy (cSLO)\textsuperscript{36} and optomotor head-tracking responses.\textsuperscript{37} SLO evaluations allow obtaining images of the nerve fiber layer in which the disposition of the nerve bundles in a single plane is well resolved because of their cylindrical shape over the pigmented background, thus distinguishing individual bundles of RGC axons becomes possible.\textsuperscript{36,38} Evaluations were performed in both eyes from 60 animals subjected to intravitreal administration of AAV2/2-ND4 (Supplementary Table S1) at four points’ postinjection (1, 3, 6, and 9 months). Representative examples of two of animals injected with 10\textsuperscript{10} or 10\textsuperscript{9} VG in their left eyes are shown in Figure 2a. Images obtained 3 and 6 months postinjection did not show any difference of nerve fiber densities, when compared to those of eyes before vector administration, indicating that wild-type ND4 expression did not lead to noticeable RGC axon disappearance. Next, optomotor head-tracking responses were recorded in 30 rats 6 months after AAV2/2-ND4 administration and in 24-week-old control rats (Figure 2b). Three grating frequencies were tested in clockwise and counterclockwise directions of motion and responses scored for left eyes and right eyes respectively were similar for all of them; thus we illustrated those of the 0.25 cycle per degree frequency (Figure 2b). Control rats mostly presented head tracking responses of similar magnitude for the clockwise (LE) or counterclockwise (RE) drum rotations (P = 0.58). Scores recorded in rats subjected to AAV2/2-ND4 administration were similar to control rat responses (P = 0.76 and 0.79 for clockwise motion and 10° and 10° VG respectively). Therefore, long-term human ND4 expression did not lead to deleterious effects on optic fiber densities or visual function.

Human ND4 protein localizes to rat RGC mitochondria and interacts with respiratory chain complex I proteins

The subcellular distribution of the human ND4 protein was examined by immunohistochemistry using antibodies against BRN3A
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and the HA1 epitope in retinal sections from six rats euthanized 3–6 months after AAV2/2-ND4 administration. We observed RGC-positive signal for the anti-HA1 antibody; furthermore, the immunoreactivity was often revealed as a punctuate distribution of fluorescent dots excluded from the nuclei which could represent mitochondria (Figure 3a). To confirm the localization of the human ND4 in the mitochondrial compartment, antibodies against the mitochondrial protein ND6 (a CI subunit) and the HA1 epitope were combined (Figure 3b). Several cells within the ganglion cell layer (GCL) were intensely labeled in the cytoplasm, signals appeared...
as punctuate fluorescent dots excluded from the nuclei with both antibodies indicating some extent of colocalization between the two proteins. In Figure 3b, the merge of the images illustrates yellow-orange pixels corresponding to the regions in which both proteins appear to be apposed (white arrowheads) within the same cells. This suggests that human ND4 proteins synthesized from the AAV2/2-ND4 vector localize to the mitochondria.

To determine whether the human ND4 protein is assembled in respiratory chain CI, three pairs of independent retinal sections from eyes transduced with AAV2/2-ND4 and their untreated counterparts were subjected to in situ Proximity Ligation Assay (PLA), which was reported as efficient to prove that two proteins are positioned within close proximity (<40 nm) of each other allows visualization of protein interactions in fixed tissues. Moreover, the reliability of the technique for demonstrating protein interactions was proven in rodent retinal sections. For the assay, antibodies against different doses (1 × 10^9 and 1 × 10^10 VG) of each other allows visualization of protein interactions in fixed tissues. Moreover, the reliability of the technique for demonstrating protein interactions was proven in rodent retinal sections.

Figure 2  Morphological and functional in vivo evaluation of adult rats after ocular administration of AAV2/2-ND4. (a) Eight-week-old rats were subjected to injection with different doses of the AAV2/2-ND4 vector (Supplementary Table S1) in the vitreous body of their left eyes. Twelve animals were evaluated for each dose using a cSLO at different points post-injection. Images shown were taken 3 and 6 months after vector administration; eyes before the intervention are also shown (left panel). No change was noticed in nerve fiber density after AAV2/2-ND4 administration (1 × 10^10 or 1 × 10^10 VG). (b) Three different groups of rats were evaluated for head tracking movements in photopic conditions. No significant difference was observed in any of the 30 animals examined in both clockwise (LE) and counterclockwise (RE) directions of motion when the AAV2/2-ND4 was administrated at two different doses (1 × 10^9 and 1 × 10^10 VG/eye). Their optomotor behavior was identical to the one elicited in control rats.

of closely apposed binding of the antibodies were observed; the overall responses for the HA1 antibody were comparable to those obtained for NDUFB6/N6 and NDUFB6/NDUFS1 antibody combinations (Figure 4a). Fluorescent signals were particularly apparent in the GCL and also in the inner plexiform layer (IPL) which contains RGC dendrites and bipolar cell axons. Very weak signal was detected when anti-NDUFB6 or anti-hA1 antibodies were combined with anti-VDAC or anti-OPA1 antibodies; these results are indicators for a non-existing interaction between: NDUFB6 and VDAC, NDUFB6 and OPA1, ND4-HA1 and VDAC, ND4-HA1 and OPA1. In contrast, abundant fluorescent spots were detected when the assay was performed with anti-OPA1 and anti-SDHA1 antibodies (Figure 4a,b) confirming that these two proteins are located in close proximity in vivo as reported in human fibroblasts in which OPA1 was shown to physically interacts with the 70-kDa (FP) subunit of complex II (i.e., SDHA1) when immunoprecipitation experiments were performed. Importantly, when retinal sections from untreated eyes were assessed using the anti-HA1 antibody, no staining was detected supporting the specificity of the signal in retinal sections.

Figure 4  Protein interaction analysis of the human ND4 proteins with ND6 and NDUFS1. (a) Blue Native-PolyAcrylamide Gel Electrophoresis (BN-PAGE) which results in the optimal separation of mitochondrial respiratory chain complexes in their native form, thus allowing to demonstrate whether a protein resides within one
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Figure 3  Expression of human ND4 protein in rat retinas. Retinal sections from six independent eyes transduced with AAV2/2-ND4 (5 × 10⁹ VG/eye) were obtained 3–6 months postinjection. Nuclei were stained with DAPI (blue); GCL represents the ganglion cell layer and scale bars are 20 µm for all panels shown. (a) Immunostaining of retinal sections from eyes transduced with AAV2/2-ND4 (5 × 10⁹ VG/eye) was performed with antibodies against the nuclear factor BRN3A (green) and the HA1 epitope (red); the contralateral untreated eye was used as negative control. (b) Retinal sections were double labeled for the mitochondrial ND6 protein (green) and the anti-HA1 epitope (red); images for two treated-eyes and one untreated-eye are illustrated. The yellow-orange pixels (ND6 + ND4-HA1 panel) show that ND6 and ND4-HA1 were in close apposition in some cells (white arrowheads).

Therefore, the human ND4 protein comigrates with three CI subunits reinforcing the idea that the human ND4 protein is correctly assembled in CI.

Long-lasting expression of the human ND4 gene in retinas from rats mimicking LHON

The ocular administration of the human ND4 gene harboring the G11778A mutation via in vivo electroporation (ELP) leads to RGC loss, optic atrophy and visual function impairment about 30 days after the intervention in adult rats.²⁹ To demonstrate that AAV2/2-ND4 is efficient on preventing RGC dysfunction in vivo, we first chose the procedure which better resembles the clinical situation. About 70% of LHON patients bear the G11778A mutation (ND4 gene) in all the mtDNA molecules of the body.² At one moment of patient’s life mitochondrial impairment reaches a threshold in which RGCs, unable to ensure their function, degenerate leading to central vision loss.²² In the LHON model we previously described, the coexistence of human ND4 protein with the R340H substitution,
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Figure 4  In situ PLA analysis of retinal sections from eyes expressing human ND4. Retinal sections from three independent eyes treated with AAV2/2-ND4 or their partner untreated eyes were subjected to in situ PLA analysis; the human ND4 protein was revealed using a monoclonal antibody against the HA1 epitope. PLA signals are red and the nuclei stained with DAPI are blue; scale bars correspond to 25 µm. GCL, ganglion cell layer; INL, inner nuclear layer. (a) Several combinations of antibodies were tested in retinal sections from eyes subjected to AAV2/2-ND4 administration. The antibody against HA1 epitope (ND4-HA1) gave strong positive signals when combined with antibodies against ND6 and NDUFS1. No interaction between ND4-HA1 with VDAC or OPA1 protein was inferred by the assay while a strong signal was noticed when antibodies against OPA1 and SDHA1 (70-kDa subunit of complex II) were combined. (b) Retinal sections from the untreated eyes were also subjected to the assay. Very weak fluorescence was noticed when the anti-HA1 antibody was used confirming that in treated eyes, signal evidenced corresponded to the human ND4 protein. For all other antibody combinations the results were similar to those obtained in treated eyes.

Figure 5  Effect of AAV2/2-ND4 treatment on RGC and optic fiber integrity in the LHON model
Eye fundus was performed using cSLO to follow optic fiber integrity in treated eyes. Representative examples are shown in Figure 7a; the eye electroporated with mutant ND4 and 10 days later transduced with AAV2/2-GFP shows a severe loss of optic fibers which corresponds to almost half of the examined area (Figure 7a).

a

| ND4-HA1 + ND6 | ND4-HA1 + VDAC | ND4-HA1 + OPA1 | ND4-HA1 + NDUFS1 | SDHA1 + OPA1 |
|--------------|---------------|---------------|-----------------|-------------|

| NDUF6 + ND6  | NDUF6 + VDAC  | NDUF6 + OPA1  | NDUF6 + NDUFS1  | TOMM20 + NDUFS1 |

b

| ND4-HA1 + ND6 | ND4-HA1 + NDUFS1 | ND4-HA1 + OPA1 | ND4-HA1 + VDAC |
|---------------|------------------|---------------|---------------|

| NDUF6 + ND6  | NDUF6 + NDUFS1  | NDUF6 + OPA1  | SDHA + OPA1   |

Eye treated with AAV2/2-ND4 (5 × 10^9 VG)

Control eye

encoded by the nuclear version of the human ND4 gene carrying the G11778A mutation, and the endogenous ND4 protein did not engender deleterious effects on RGCs within the first 14 days after in vivo ELP. Therefore, 8-week-old rats were subjected to ELP with mutant ND4 and ten days later, a single intravitreal injection in the electroporated eye was performed with AAV2/2-ND4 or AAV2/2-GFP. Evidently, AAV2/2-GFP administration is not expected to counteract the mutant ND4 effect, neither to induce in vivo toxicity. This protocol should allow to seek after AAV2/2-ND4 ability to protect RGCs when vector is administrated, mutant ND4 expression driven by ELP was already started and human ND4 mRNA accumulates in transduced RGCs about 2 weeks after vector administration (Figure 1b). First, we determined the AAV2/2 transduction efficiency when vector is administrated, mutant ND4 mRNA levels were measured when RNA preparations from retinas of 12 eyes transduced with mutant ND4 via ELP and subjected 10 days later to an intravitreal injection of AAV2/2-GFP were evaluated. A 15% reduction in SNCG and BRN3A mRNA steady-state levels was measured when RNA preparations from untreated and treated retinas were compared (Figure 6c; P = 0.0011 and 0.0003 respectively). This diminution reveals AAV2/2-GFP inability to counteract the deleterious effect of mutant ND4 expression on RGC integrity. Conversely, the relative abundance of BRN3A and SNCG mRNAs was not significantly different between untreated eyes and eyes from the LHON model expressing the human ND4 gene; P values were 0.54 and 0.7 respectively (Figure 6d). Therefore, AAV2/2-ND4 administration hindered RGC loss induced by mutant ND4 expression.
The disappearance was generally noticed about 6 weeks after ELP; two times post-AAV2/2 injection are illustrated (3 and 6 months). Out of the 18 eyes evaluated in this group, significant reduction in optic fiber density was evidenced in 6 eyes; by contrast, no RGC axons loss was observed in 52/54 of the animal eyes which were injected with different doses of AAV2/2-ND4 (Supplementary Table S1). The imaging of one of those animals eye fundus showed that the tracks of RGC axons were well preserved 3 and 6 months after vector administration (Figure 7a). To further confirm these results transversal optic nerves (ONs) sections from the two animals shown in Figure 7a, euthanized 6 months after vector administration, were subjected to immunohistochemistry for the heavy chain subunit of neurofilaments (NF200) to detect RGC axonal profiles. Figure 7b illustrates a recognizable reduction of immunopositive dots in the ON from the animal in which the eye was treated with mutant ND4 and AAV2/2-GFP relative to one ON isolated from an age-matched control rat or to the ON from the rat illustrated in Figure 7a in which the eye was treated with mutant ND4 and AAV2/2-ND4. Since RGC axon number was diminished in eyes electroporated with mutant ND4 and treated with AAV2/2-GFP, we next estimated RGC number in retinal flat mounts immunostained for BRN3A. Figure 7c shows retinas from untreated eyes and eyes transduced with mutant ND4 and subsequently treated with AAV2/2-GFP or AAV2/2-ND4. Mean RGC count per mm² ± SEM for 26 retinal flat mounts isolated from untreated eyes was 1796 ± 26.8. These values are similar to those previously described. In contrast, average RGC densities for eight eyes expressing mutant ND4 and subjected to AAV2/2-GFP injection was 1213 ± 35.7 (Figure 7d); a 33% reduction relative to control retinas. RGC count difference between these two groups was significant (P < 0.0001). Conversely, when retinas from eyes expressing mutant ND4 and transduced with AAV2/2-ND4 were evaluated a significant protection of the overall RGC density was observed (Figure 7d). The mean of RGC counts per mm² was 1741 ± 51.8 (97% of the control value), this number was not significantly different to the one measured in control retinas (P = 0.24). Hence, RGC degeneration due to mutant ND4 expression protein is efficiently prevented by AAV2/2-ND4 administration.
Impairment of visual function in the LHON model is prevented by AAV2/2-ND4 treatment

To examine the impact of human ND4 expression in the LHON rat model at the functional level, we performed head-tracking experiments to discriminate sensitivities of right eyes or left eyes in the LHON model. We evaluated rats transduced with mutant ND4 in their left eyes and 10 days later subjected to a single intravitreal injection, in the same eye, with AAV2/2-GFP or AAV2/2-ND4. Optomotor responses of these groups were compared to head tracking scores for the left or right eyes of 24-week-old control rats (Figure 2). Left eye scores were significantly reduced in animals treated with AAV2/2-GFP (40% of right eye responses); they were statistically different to responses recorded for the left eyes in control rats (P < 0.0001) indicating that visual performance of eyes expressing mutant ND4 was compromised. On the other hand, animals which received AAV2/2-ND4 mostly presented head tracking scores of similar magnitude for each of their eyes (P = 0.4). The scores recorded for the left eyes, in the 30 rats evaluated, were similar to control rats (P = 0.8). Hence, the salutary impact of AAV2/2-ND4 administration on nerve fiber integrity (Figure 7a,b) was confirmed by the sustained visual function protection evidenced in the AAV2/2-ND4 treated eyes.

Respiratory chain complex I activity was preserved in optic nerves from LHON rats when eyes were treated with AAV2/2-ND4.

To demonstrate that “optimized allotopic expression” of ND4 is efficient in vivo besides the strong indication on the presence of the human ND4 protein within CI (Figures 4 and 5), it is also required setting a robust and reliable assay for CI enzymatic activity. We exploit a spectrophotometric method which allows the sequential evaluation of the enzymatic activities of CI (NADH decyubiquinone reductase) and CV (ATP hydrolase) on a single rodent ON.16 The sequential evaluation of CI and CV enzymatic activities allowed also CI/CV ratio determination; an accurate parameter for detecting an impairment of respiratory chain activity.17 Animals were euthanized between 8 and 12 months after AAV2/2 injection. CI and CV activities were assessed in four groups: (i) 84 ONs from untreated eyes; (ii) 30 ONs from eyes subjected to a single AAV2/2-ND4 intravitreal injection; (iii) 12 ONs from animals injected with AAV2/2-GFP (3 × 10^11 VG) after mutant ND4 transduction via ELP; (iv) 26 ONs from animals injected with AAV2/2-ND4 after mutant ND4 transduction via ELP. CV activity did not change in any of the groups evaluated when compared to values assessed in control ONs (Figure 8b). The long-term expression of human wild-type ND4 gene driven by a single AAV2/2-
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Figure 7  Effect of AAV2/2-ND4 on RGC and optic fiber integrity in the LHON model. (a) cSLO fundus imaging of two rat eyes: one subjected to ELP (mutant ND4) + AAV2/2-GFP (3 × 10⁹ VG, left panel) and one subjected to ELP (mutant ND4) + AAV2/2-ND4 (5 × 10⁹ VG, right panel) as well as the partner untreated-eyes. Images were taken 3 and 6 months after vector administration; yellow discontinued lines show the region with nerve fiber loss in the eye treated with AAV2/2-GFP. (b) Proximal ON transversal sections were immunostained with an antibody against NF200 (red); they were obtained from: four rats subjected to intravitreal injection with mutant ND4-DNA followed by ELP and 10 days later to another intravitreal injection with AAV2/2-GFP (3 × 10⁹ VG) in the same eye; four rats subjected to intravitreal injection with mutant ND4-DNA followed by ELP and 10 days later to another intravitreal injection with AAV2/2-ND4 (5 × 10⁹ VG in the same eye) and four age-matched controls. Images shown correspond to ONs from the two animals from which eye fundus imaging were illustrated in panel a. Two different magnifications are shown; scale bars represent 200 µm (left panel) and 25 µm (right panel). The reduction in optic fiber density was evidenced in all the four ON sections evaluated out of the six animals in which eye fundus imaging revealed optic fiber loss. In the contrary, the four ON sections evaluated from eyes treated with AAV2/2-ND4 gave results similar to those observed in ONs from age-matched controls. (c) Retinal flat mounts obtained from animals sacrificed 6 months after vector administration were immunolabeled for BRN3A antibody (red). Images of two distinct retinal regions are illustrated for: (i) an untreated retina; (ii) an eye subjected to ELP (mutant ND4) + AAV2/2-GFP, and (iii) an eye subjected to ELP (mutant ND4) + AAV2/2-ND4; the scale bar corresponds to 25 µm. (d) Histogram shows the means of RGC number/mm² ± SEM obtained after all the BRN3A-positive cells were counted in the three groups evaluated.
intravitreal injection was safe for CI function (Figure 8c,d); since no significant difference was observed when CI or CI/CV values were compared to those measured in control ONs (P value was 0.92 or 0.25 respectively). On the contrary, mutant ND4 expression led to CI defect in ONs from eyes treated with AAV2/2-GFP after in vivo ELP with mutant ND4 (Figure 8c,d); the reduction of about 40% relative to untreated eyes was significant (P < 0.0001 for either specific CI activity or CI/CV ratio). Furthermore, AAV2/2-ND4 was efficient on protecting CI function; indeed CI activity or CI/CV ratios was not statistically different from values obtained in ONs from untreated eyes: P values were 0.7 and 0.98 respectively (Figure 8c,d). Thus, at the molecular level the human ND4 protein largely hindered the deleterious effect of mutant ND4 expression by preserving CI activity.

**DISCUSSION**

The first evidence that mitochondrial impairment yields to human disease was described in 1962. In 1988, it was demonstrated that mtDNA deletions were present in the muscle of people suffering from mitochondrial myopathy, and that a mtDNA mutation was responsible for LHON. Up to date molecular defects observed in both mtDNA-encoded and nuclear DNA-encoded genes of mitochondrial proteins are associated with a wide spectrum of clinical problems including myopathy, encephalomyopathy, gastrointestinal syndromes, dystonia, diabetes, blindness, deafness, and cardiomyopathy. Therefore, mitochondrial disorders represent a common cause of chronic morbidity and are more prevalent than previously thought; indeed they affect at least 1 in 5,000 individuals. Nevertheless, mitochondrial medicine only made slow progress.
progress towards effective treatment of these devastating diseases; a recent review encompassing 1,335 reports and 12 randomized controlled treatment trials in mitochondrial diseases, did not find a clear evidence supporting the efficacy of any of these interventions.13 Hence, our main objective was to develop a gene therapy that could prevent optic atrophy due to mitochondrial dysfunction. In this pursuit, we have focused our interest on the LHON disease due to the G11778A mutation on the mitochondrial ND4 gene, since its accounts for 60-90% of LHON cases worldwide with less than 20% chance of recovery.1 Clinical manifestations in LHON patients are very peculiar; indeed about 75% of them present sequential visual loss in each eye, with the interval between the first and second eye involvement ranging from weeks to 2–4 months.7 This opportunity along with the evidence that gene therapy is well tolerated and efficient in Leber Congenital Auromosis56 has great potential for a generalization to mitochondrial disorders specially devastating neurodegenerative diseases.53

However, an array of obstacles needs to be overcome before being able to offer a clinical protocol safe and beneficial to patients harboring mtDNA mutations. The greatest difficulties are: (i) the scarcity of reliable experimental models of the disease required for the validation of gene therapy or pharmacological approaches in vivo. In this respect, a report of Dr. Wallace and colleagues is very promising since it describes the generation of a mouse model with the G13997A mutation in the ND6 gene, leading to an amino acid substitution (P25L), reported in an optic atrophy and Leigh syndrome family57; the mice exhibit some of the LHON hallmarks, especially optic atrophy;16 (ii) inserting genetic material directly into mtDNA is up to date a difficult task; the reports of J. Guy and colleagues in which the modification of an AAV vector leads to the expression of the transgene inside the mitochondria could allow progress in this pathway10,11; (iii) the efficacy of allotopic expression in vivo is still under debate. Several reports since 2002 provided controversial results in vitro, in which authors indicated that mitochondria behavior in cultured cells can give a false impression on allotopic expression achievement.14–16 For instance, Perales-Clemente’s et al.15 claimed a potential generalization of their data on unsuccessful allotopic expression, because in a mouse cell line, in which wild-type mtDNA was <10%, respiratory chain dysfunction rescue was due to the artefactual selection of revertants from the mutated gene. However authors did not prove this statement; indeed these cells when transduced with any kind of vector should be equally able to drive Cl assembly. Moreover, independent groups worldwide did show unambiguously that allotopic expression results on the functionality of the protein within the mitochondria in mice and rats,29,59–63 and in cancer cells.64 Additionally, it was reported the generation of transgenic mice for wild-type or a mutated version of the mitochondrial ATP6 gene via allotopic expression. The approach was successful in the mitochondrial delivery of ATP6 derived from the nuclear transcripts. Besides, transgenic mice expressing the mutated gene (single nucleotide change equivalent to the human mutation responsible for Neuropathy, Ataxia and Retinitis Pigmentosa) displayed performance inferior to control mice in four out of seven neuromuscular and motor assays. Though the characterization and comparison of the two transgenic mice should be completed, these experiments reinforce the notion that allotopic expression is efficient in vivo.45

Therefore, our aim was to make the undeniable proof-of-principle that: (i) in vivo allotopic expression of human ND4 leads to the efficient mitochondrial import and assembly of the protein in CI without affecting its activity; (ii) its expression, driven by an AAV2 vector, impedes vision loss in a LHON rat model by fully preventing CI defect due to mutant ND4 expression. We first demonstrated that the human ND4 protein, which shares 67% of identity and 84% of similarity with the rat protein, can efficiently compete with this latter and assemble into a functional rat CI by using two independent protocols: (i) The PLA assay46 corroborated in situ interaction of human ND4 with ND6 or NDUF51, two CI proteins. (ii) BN-PAGE experiments63 showed that human ND4 (molecular mass of ~54 kDa) migrated similarly than NDUF9, NDUF8B, and NDUF51, three sub-units of CI indicating its presence within assembled CI. Importantly, human ND4 expression driven by AAV2/2 was sustained for up to 12 months in about 75% of the total RGC population with no change in cell viability or function. This was supported by: (i) eye fundus imaging; (ii) optomotor behavior; (iii) RGC number estimation; (iv) CI activity assessment in ONs.

The next step was to confirm the effectiveness of AAV2/2-ND4 treatment in a reliable animal model of LHON. It is well known that in some mtDNA diseases, not all mtDNA molecules are affected and the extent of heteroplasmy is accountable of symptom severity.66 In LHON patients, the ND4 mutation is generally homoplasmic but some rare cases of heteroplasmy were described; for instance in a clinically unaffected member from the fifth generation of an Australian pedigree57 and in pedigrees of the G11778A LHON mutation from Thailand in which the proportion of mutated mtDNA correlated with visual loss.68 Hence to better mimic what happens in LHON patients and permit the use of AAV2/2-ND4 to assess its ability to impede RGC loss we designed an improved protocol of eye intervention different to the one we previously reported:29 an intravitreal injection of a plasmid carrying mutant ND4 followed by in vivo ELP and 10 days later the same eye was subjected to an intravitreal injection of either AAV2/2-ND4 or AAV2/2-GFP. Then, it becomes feasible to compare the effect of GFP or human ND4 protein accumulation in eyes that express mutant ND4. The benefit of AAV2/2-ND4 for RGC integrity was proven by the fact that the ~33% RGC number reduction in eyes expressing mutant ND4 was prevented in eyes subjected to AAV2/2-ND4 injection. This data was in agreement with eyes fundus visualizations and ON immunostaining for NF200 which clearly showed that nerve fiber preservation was only noticed when rat eyes were treated with AAV2/2-ND4. As expected, rats in which eyes possessed optic fiber density comparable to controls responded normally to the optomotor test. In contrast, animals transduced with GFP consistently showed an approximate 40% reduction in their treated eye scores. Unquestionably, the final demonstration of AAV2/2-ND4 effectiveness in a mouse model was reinforced because the protein in the LHON model was expressed in CI with similar efficacy to human wild-type ND4 promoting a genuine genetic model for LHON, we demonstrated that mutant ND4 expression driven by AAV2/2-ND4 administration undeniably showed the preservation of CI function. Even though we did not circumvent the lack of a genuine genetic model for LHON, we demonstrated that mutant ND4 injury in the model used for testing AAV2/2-ND4 effect was identical to the one evidenced previously using in vivo ELP.29 This emphasizes the authenticity of mutant ND4 detrimental effect on RGC viability. Accordingly, the benefit of human wild-type ND4 protein in the LHON model was reinforced because the protein was capable to protect RGC function durably and safely.
In conclusion, we provide converging lines of evidence that optimized allotypic expression is safe and efficient in vivo, therefore, the clinical trial for LHON patients bearing the G11778A mutation using AAV2/2-ND4 was initiated (NCT02064569). The follow-up of enrolled subjects will have a strong accent on evaluating safety (lack of adverse events related to vector injection) but it can also begin to address the potential for benefit in terms of visual function. Clinically meaningful improvement including patient’s quality of life will depend on RGC functional plasticity. Hypothetically, residual RGCs in treated LHON patients could optimize their connectivity leading to structural and functional reorganization close to the macula. This region is responsible for color and high acuity vision due to the largest concentration of cones. A putative rewiring inside the retina may work in concert with a cortical plasticity in the visual cortex, recently described in asymptomatic LHON carriers. At last, if the clinical trial is safe and efficacious, the strategy could be extended to other devastating neurodegenerative disorders with mitochondrial etiology from which few efficient treatments are available up to date. \[11\] MATERIALS AND METHODS Adeno-associated viral vectors The vector used for gene therapy is a recombinant AAV2/2 containing the human NADH dehydrogenase 4 (ND4) gene under the control of the cytomegalovirus early promoter in an intron-containing expression cassette (β globin intron, HB2B), flanked by the viral inverted terminal repeats of the pAAV- IRES-hrGFP vector (Agilent Technologies, Les Ulis, France). The human open reading frame (ORF) of ND4 was recoded for the 34 non universal codons and optimized to achieve high-level expression in mammalian cells; in vivo with the AAV-ND4 codon was appended B base pairs encoding the first 28 amino acids of COX10 corresponding to the mitochondrial targeting sequence (MTS). The human growth hormone polyA signal originally included in the vector (478 nucleotides) was replaced by 603 nucleotides of the human COX10 3’ UTR instead of the full-length 3’UTR previously described. Indeed, pAAV-IRES-hrGFP can only accommodate inserts ≤1.7 kb. The vector leads to the synthesis of the human ND4 protein in which three HA1 epitopes are appended at its C-terminal immediately followed by two stop codons; the whole ORF was synthesized by GenScript. As control, the pAAV-IRES-hrGFP was used without ORF but the GFP. The AAV2/2-ND4 was manufactured either in the Vector Core of Genethon (http://www.genethon.fr/en/); its titer is 1.6 × 10^12 VG/ml or the “Centre de Production de Vecteurs et des INSERM UMR1089, Nantes” which titer was 1.6 × 10^10 VG/ml and 8.3 × 10^10 VG/ml respectively. Animals Male Long Evans rats were used at 8-week old (Janvier, Le Genest Saint Isle, France); they were housed two per cage in a temperature-controlled environment and 12 hour light/dark cycle. All animal studies were conducted in accordance with the guidelines issued by the French Ministry of Agriculture and the Veterinarian Department of Paris (Permit number D/E/F, 2010, PA1000298), the French Ministry of Research (Approval number S575) and UPMC/INSERM ethics committees (Authorization number 75–1712). In vivo electroporation and intravitreal injection of AAV2/2 vectors The ELP procedure was performed in one eye as previously described. After dilution of the pupil with topical 1% tropicamide (CibaVision) and under anesthesia with isoflurane (40/65 mg/kg body weight), the protocol was initiated with the injection in the vitreous body of 20 µg (5 µl) of pAAV- IRES-hrGFP plasmid containing the human ND4 gene harboring the G11778A mutation and 3 Flag epitopes appended to the last amino acid codon. Next, contact-lens-type and needle electrodes were positioned, and square wave pulses were delivered by a Square Wave Electroporation System (ECM 830; BTX, Harvard Apparatus, Les Ulis, France) as previously described. For AAV2/2 administration, intravitreal injections were performed with 5 µl of vector suspension using an ophthalmic surgical microscope. Supplementary Table S1 recapitulates the eyes subjected to AAV2/2 administration, vectors, and the doses used. Fundus imaging by confocal scanning laser ophthalmoscopy (cSLO) The Heidelberg Retina Angiograph (Heidelberg Engineering, Dossenheim, Germany) is a scanning laser ophthalmoscope (cSLO) which allows imaging of eye fundus. To adapt the commercial system to the optics of rodent eyes, the 40 mm focal lens of the front objective was replaced by a 25 mm focal lens (Linos Optics, Milford, MA). The system allows the examination of nerve fiber layer in each cardinal area of rat eyes before treatment and different times after vector administration. Images were obtained using the 30° field of view and the automatic real-time mode at a sensitivity setting that maximized the signal/noise ratio; all the cardinal areas of the eye fundus were observed, thus, obtaining fiber bundle distribution in the whole retina. The built-in software was used for postprocessing the images, including alignment, adjustment of contrast, construction of a mean image and/or of a composite image, as previously described. Optomotor tests The head-tracking method is based on an optomotor test frequently used in rodents. The protocol yields independent measures of right and left eye acuities based on their unequal sensitivities to pattern rotation: right and left eyes are most sensitive to counterclockwise and clockwise rotations, respectively. Long Evans rats were placed individually on an elevated horizontal platform surrounded by a motorized drum (12%‘s in photopic conditions). Vertical black-and-white lines of three varying widths, subtending 0.125, 0.25, and 0.5 cycle/degree (cyc/deg) were presented to the animal and rotated alternatively clockwise and counterclockwise, each for 60 seconds. Head movements were recorded with a video camera mounted above the apparatus; light levels were kept at 240 lux. Animals were scored only when the speed of the head turn corresponded to the rotation speed of the stripes. Assessments were performed before ocular treatment and monthly after the ocular intervention; experimenters were blind to previously recorded results, and thresholds were regularly confirmed by more than one observer. Retinal and optic nerve histology Retinas or optic nerves were fixed in PBS + 4% paraformaldehyde at 4 °C and then cryoprotected by overnight incubation in PBS containing 30% sucrose at 4 °C. Retinas were embedded in optimal cutting temperature compound (Neg 50, Richard-Allan Scientific, Villebon-Sur-Yvette, France) and frozen in liquid nitrogen. Optic nerves were embedded in a solution of PBS + 7.5% gelatin from porcine skin Type A (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 10% sucrose and frozen in a 2-methyl-butane solution at –45 °C. Ten micrometer-thick cryosections of retinas and optic nerves were cut on a cryostat (Microm HM550 ThermoScientific, Villebon-Sur-Yvette, France). For immunohistochemistry, retinal sections were rinsed with PBS and treated for 10 minutes with PBS + 0.1% Triton and then for 1 hour with 1% BSA, 0.1% Triton, and 0.05% Tween 20. Next, they were incubated with primary antibody overnight at 4 °C. Sections were washed in PBS and incubated with appropriate secondary antibodies and DAPI (Sigma-Aldrich) for 2 hours at room temperature. Primary and secondary antibodies used are shown in Supplementary Table S2. For retinal flat mounts, the protocol previously described was followed. In situ proximity ligation assay Duolink Kit (Olink Bioscience, Uppsala, Sweden) is based on in situ PLA, proximity ligation assay technology which allows visualization of protein interactions in fixed tissues. In the assay, oligonucleotide-tagged secondary antibodies are linked with circle-forming oligonucleotides. If two antigens, detected by primary antibodies derived from different species, are in close proximity (<40 nm) to each other, after the ligation step of the two linker oligonucleotides, the rolling circle amplification using complementary fluorophore-tagged oligonucleotide probes results in fluorescent puncta at the site of interaction. The technique has been reported as an effective demonstrating protein interactions in rodent retinal sections. Assays were performed with retinal sections from 4 independent eyes treated with AAV2/2-ND4 (5 × 10^10 VG) obtained 3–6 months postinjection or their fellow untreated eyes. The manufacturer’s instructions were essentially followed using the different antibodies listed in Supplementary Table S2. Briefly, different mouse antibodies against: HA1 epitope, NDUF6 complex I subunit, SDHAI (the 70-kDa (F1) subunit of complex II) or TOMM20 (mitochondrial translocase of the outer membrane) in combination with rabbit antibodies against: ND6 (complex I subunit), NDUF51 (complex I subunit), VDAC (outer mitochondrial membrane protein), or OPA1 (inner mitochondrial membrane) were used.
Microscopic observations
Fluorescence labeling was monitored at the Cellular Imaging Facility of the Vienna Institute with a confocal laser scanning microscope FV1000 (Olympus) and a fluorescence microscope (Leica DMS000 B). Image acquisition was conducted with Olympus Fluoview software version 3.1 and the MetaVue software. Finally, all the images were analyzed with Photoshop and Image J softwares.

RNA extraction and RT-qPCR assay
Total RNA from rat retinas were extracted using RNeasy Plus Mini kit from Qiagen (Courtaboeuf, France). To ensure the absence of DNA, a treatment with RNase-free DNase (Qiagen) and a subsequent cleanup with the RNeasy MiniElute cleanup kit (Qiagen) were performed. This was confirmed by subjecting 10ng of each RNA preparation to qPCR with human ND4, endogenous ATP6 or BRR2A primers. One microgram of total RNA was reverse transcribed with oligo-dT using Superscript II Reverse Transcriptase (Life Technologies, Saint-Aubin, France). Quantitative PCR reactions were performed using ABI 7500 Fast (Applied Biosystems, Saint Aubin, France) and the specific primers (Life Technologies) listed in Supplementary Table S3. The equivalent of 10 and 2ng of cDNAs were used per gene as template for qPCR reactions with Power Sybr green PCR Master Mix (Applied Biosystems). Each biological sample was subjected to the assay in triplicates per gene. Ct values (number of cycles required for the fluorescent signal to cross the background threshold) were obtained with the ABI 7500 software (v.2.0.4). To determine the relative mRNA amount of each studied gene we used the comparative ∆∆Ct method and the mitochondrial ATP6 gene as normalizing gene since its mRNA steady-state levels remained almost unchanged in all the samples evaluated.

Western blotting analysis and blue native polyacrylamide gel electrophoresis
For whole proteins extracts, single retinas were homogenized in 50 µl of 20 mmol/l HEPES and 60 mmol/l mannitol (pH 7.2) using a 200 µl micro-hand-driven glass–glass potter at 4 °C. Large cellular debris was spun down by a low speed centrifugation (1,000 g for 5 minutes at 4 °C) and supernatants were subjected to protein quantification (Bradford reagent, Bio-Rad). Fifteen and 30 µg of protein extracts from: four control retinas and four retinas which received AAV2/2-ND4 intravitreally were denatured at 94 °C for 15 minutes, resolved in 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with antibodies against HA1 epitope, NDUF9, NDUF8B, ATP synthase subunit α and β-actin (Supplementary Table S2). Immunoreactive bands were visualized with appropriate secondary antibodies coupled to horseradish peroxidase (0.1 µg/ml) (Supplementary Table S2) followed by detection with Pierce ECL Plus Western Blotting Substrate (Pierce, ThermoScientific, Villebon-Sur-Yvette, France). Apparent molecular mass of each protein was estimated by comparing the electrophoretic properties of each specific signal in the immunoblots with the “PageRuler Plus Prestained Protein Ladder” (Pierce Protein Biology products, ThermoScientific).

For blue native polyacrylamide gel electrophoresis (BN-PAGE) mitoplasts isolation was adapted from the report of Calvaruso et al. Briefly, after careful dissection, retinas were kept frozen and samples were prepared at ice-melting temperature by homogenization of 8 retinas using a 1 ml hand-driven glass–glass potter in 1 ml of ACBT buffer (aminocaproic acid 1.5 mol/l; Bis-Tris 75 mmol/l; pH 7). 100 µl of 1% lauryl-maltoside 20% (Biorad, Marnes-la-Coquette, France) were added to allow the isolation of respiratory chain complexes. The mixture was kept on ice for 10 minutes and then centrifuged for 30 minutes at 14,000 rpm. The protein content of the supernatant was measured with the BCA protein assay kit (Pierce ThermoScientific) and then kept at −20 °C until use. BN-PAGE 5–15% gradient gels were casted and then loaded with 200 µg of proteins. To perform two-dimensional blue native/SDS electrophoresis, lanes were cut out of the first dimension and incubated with 1% SDS and 1% β-mercaptoethanol for one hour at room temperature. The gel strip was placed at the top of glass plates in order to poor the SDS gel (4% for stacking and 10% for separation). Next, standard western blotting protocols for SDS gels were applied.46 Immunodetection was performed using primary antibodies against proteins of the respiratory chain (Supplementary Table S2). To reuse membranes, the antibody was removed with the Re-Blot Plus Western Blot Strong Antibody Stripping Solution (Millipore, Molsheim, France).

Tissue homogenate preparation and respiratory chain enzyme assays
Optic nerves were rapidly collected and kept frozen (~80 °C). Cl and CV activities were measured using a Cary 50 UV-Vis spectrophotometer (Agilent Technologies) as described for retinas and ONs from rodents.48 Cl activity values were either normalized by CV or converted to specific activities (expressed as nanomoles of oxidized NADH/minute/mg protein) after protein quantification by the Bradford method. All chemicals were of the highest grade from Sigma-Aldrich.

Statistical analyses
Values are expressed as means ± standard error of the mean. Statistical analyses were performed with the GraphPad Prism 6.0 software assuming a confidence interval of 95%. Data collected for all the independent observations were compared using the non parametric significance test of Mann–Whitney (*p≤0.05, **p≤0.01, and ***p≤0.005).

CONFLICT OF INTEREST
J.-A.S. is one of the cofounders of GenSight Biologics, a privately owned biopharmaceutical company, dedicated to the development and commercialization of gene therapy based treatments of retinal degenerative diseases. The company obtained the patent-licensing arrangement for the clinical development of AAV2/2-ND4 (GS010). The clinical trial started in February 2014 (NCT02064569). There are no other patents, products in development or marketed products to declare.

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Supplementary Information accompanies this paper on the Molecular Therapy—Methods & Clinical Development website (http://www.nature.com/mtm)