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**The optimized allotopic expression of *ND1* or *ND4* genes
restores respiratory chain complex I activity in fibroblasts
harboring mutations in these genes**

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Running title: Allotopic expression of *ND1* or *ND4* genes in fibroblasts from LHON patients

Summary

Leber's Hereditary Optic Neuropathy (LHON) was the first maternally inherited mitochondrial disease identified and is now considered the most prevalent mitochondrial disorder. LHON patients harbor mutations in mitochondrial DNA (mtDNA). In about 90% of cases, genes involved encode proteins of the respiratory chain complex I. Even though the molecular bases are known since 20 years almost all remains to be done regarding physiopathology and therapy. In this study, we report a severe decrease of complex I activity in cultured skin fibroblasts isolated from two LHON patients harboring mutations in *ND4* or *ND1* genes. Most importantly, we were able to restore sustainably (a) the ability to grow on galactose, (b) the ATP synthesis rate and (c) the complex I activity initially impaired in these cells. Our strategy consisted to force mRNAs from nuclearly-encoded *ND1* and *ND4* genes to localize to the mitochondrial surface. The rescue of respiratory chain defect observed was possible by discreet amounts of hybrid mRNAs and fusion proteins demonstrating the efficiency of their mitochondrial import. Hence, we confirmed here for two mitochondrial genes located in the organelle that the optimized allotopic expression approach represents a powerful tool that could ultimately be applied in human therapy for LHON.

1. Introduction

Leber Hereditary Optic Neuropathy (LHON) is a maternally inherited neurodegenerative disorder, recognized as the most frequently occurring mitochondrial disease [1]. The pathology is due to point mutations in the mitochondrial genome. The three most common pathogenic mutations found in about 90% of LHON's patients are located in *ND1* (G3460A), *ND4* (G11778A) or *ND6* (T14484C) genes. They encoded subunits of the respiratory chain complex I and the mutations have the double effect of lowering ATP synthesis and increasing oxidative stress chronically. The pathology is characterized by selective death of retinal ganglion cells and optic nerve atrophy, prevalently in young males leading to irreversible visual failure [2]. Since the discovery in 1988 of the deleterious nucleotide substitution G11778A in the *ND4* gene [3] and thereafter the mutations in *ND1* [4] and *ND6* [5] genes, the pathogenesis of LHON is poorly understood. One hypothesis to explain complex I dysfunction on LHON patients underlies on the abnormal mitochondrial distribution along the optic nerve head due to an incorrect mitochondrial recruitment to appropriate regions and an inefficient mitochondrial axonal transport. This would constitute the first step of a vicious event cycle that further compromises cell respiration which eventually lead to profound energy depletion, increased production of reactive oxygen species (ROS) and neuronal cell death through apoptosis [6, 7]. Besides, perturbations of the respiratory chain function due to LHON mutations have been very complex to evidence. Biochemical studies in patient-derived tissues and cellular experimental models have produced conflicting results regarding the extent of respiratory chain dysfunction associated with the disease [8, 9]. The 3460/*ND1* mutation induced a severe defect of complex I activity in mitochondria from platelets and lymphoblasts while 11778/*ND4* and 14484/*ND6* mutations are noticeably less severe [10-13]. Moreover, the *ND4*/G11778A mutation, found in 50 to 70 % of patients, is associated to an

increased resistance to rotenone [14], a classical inhibitor of complex I. Accordingly, the electron transfer through complex I is altered in cybrid cells lines harboring LHON mutations [15-19]. A conformational modification of complex I structure may lead to an increased production of ROS [20]. Indeed, it has been shown that, while mitochondria represent the major intracellular source of ROS, most of the superoxide radicals are produced at complex I in brain mitochondria [21]. Another study has demonstrated an induction of apoptosis in LHON patients due to oxidative stress [22], possibly associated with a decrease in antioxidant defences [23]. Finally, a recent report suggests that in primary retinal cultures glutamate toxicity resulting from impaired glutamate transport may play a role in the pathogenic process of LHON [24]. The many unanswered questions that remain about the disease molecular pathogenesis hampers devising appropriate therapeutic strategies. Several trials with vitamins or idebenone (an oxygene radical scavenger) were performend, however no evidence to date supports its use and prevention of vision loss in patients [25]. Thus, allotopic expression (expression of mitochondrial genes transferred to the nucleus) of some of mtDNA genes has been tried in cybrid cells harboring deleterious mtDNA mutations as a possible therapeutic option to cure LHON. In 2002, two laboratories reported improvements in oxydative phosphorylation (OXPHOS) function when allotopic expression was used as strategy in cybrid cells harboring mutations in the mitochondrial *ND4* and *ATP6* genes [26, 27]. However, several attempts failed to confirm these data and to obtain a complete and long-lasting rescue of the mitochondrial defect *ex vivo* [28, 29]. Probably, the highly hydrophobic nature of proteins encoded by the mitochondrial genome represents a physical impediment to mitochondrial import of the allotopically expressed protein [30]. This strongly limited the potentiality of allotopic expression as a therapeutic approach for mtDNA-related diseases [31].

In a recent work, we reported the incapacity to grow in galactose and ATP synthesis rate impairment of fibroblasts harboring the G11778A *ND4* mutation. The optimized allotropic expression of the engineered nucleus-localized wild-type *ND4* gene rescued their inability to grow in galactose and restore a normal rate of ATP synthesis *in vitro* [32]. Our approach has consisted to maximize the positive effect on mitochondrial function by the sorting of the *ND4* mRNA to the mitochondrial surface, thus ensuring the mitochondrial translocation of the corresponding polypeptide. The specific subcellular transport of the hybrid mRNA was achieved by the generation of an expression vector in which the engineered *ND4* gene was combined with the mRNA cis-acting elements of the *COX10* gene. Indeed, *COX10* transcript localizes exclusively to the mitochondrial surface in HeLa cells [33].

In the present study, we examined human cultured skin fibroblasts from LHON patients carrying either the *ND4*/G11778A mutation or the *ND1*/G3460A mutation and their counterparts allotopically expressing nuclear recoded *ND4* or *ND1* wild-type genes. Mitochondrial function in these cells was compared according to three different criteria : growth capacity in galactose medium, ATP synthesis rate and complex I activity. We observed in LHON fibroblasts a severe decrease in complex I activity, *i. e.* 60% and 80% for *ND4* and *ND1* mutations respectively. Moreover, we demonstrated the presence of hybrid mRNAs and their corresponding proteins in fibroblasts stably expressing our vectors of optimized allotropic expression. When the three criteria of mitochondrial function were assessed in these cells, we observed a significant restoration of OXPHOS function. In fibroblasts bearing the *ND1* mutation, the optimized allotropic expression vector led to an increase of approximately 1.8-fold in the overall amount of ND1 protein, this increase appeared to be sufficient for salvaging OXPHOS in these cells. Hence, our approach for the mitochondrial *ND1*, and *ND4* genes ensures the efficient mitochondrial translocation of the corresponding precursors. The rescue of the OXPHOS defect, without observable deleterious

consequences for cell fitness, suggested that the processed polypeptides were functional within their respective respiratory chain complexes and, therefore, able to compensate for the endogenous defective proteins.

Materials and methods

1.1. Plasmid construction

The construct directing the synthesis of the nuclear version of the *ND4* gene, namely *COX10*^{MTS}*ND4-3'UTR*^{*COX10*}, was obtained as previously described [32].

The mitochondrial *ND1* sequence was purchased from Genscript Corp. (Piscataway, NJ USA). The *ND1* ORF of 954 nucleotides (318 amino acids) was recoded for the 22 non-universal codons and optimized to achieve a high-level expression in human cells. The *COX10*^{MTS}*ND1-3'UTR*^{*COX10*} construct was obtained according to the methodology previously described for the *COX10*^{MTS}*ND4-3'UTR*^{*COX10*} plasmid [32]. Final constructs were entirely sequenced (Genome Express, France) to confirm accuracy of the *COX10* MTS-*ND1* ORF and the *COX10* 3'UTR sequences within the *pCMV-Tag 4A* vector (Stratagene, La Jolla, CA).

1.2. Cell culture and transfection

Cultured skin fibroblasts were derived from forearm biopsies of a healthy subject (15-month-old), a 18-year-old man who carries the G11778A mutation in the *ND4* gene (LHON#1) and a 20-year-old man carrying the LHON *ND1* G3460A (LHON#2). In both LHON patient's fibroblasts, the pathogenic mtDNA mutation was found homoplasmic (all mtDNA molecules were mutated). Fibroblasts were grown under standard conditions in DMEM-Glucose medium (Gibco, Invitrogen) supplemented with 12% fetal bovine serum (Gibco, Invitrogen), HEPES (10 mM), uridine (2.25 mM), 10 µg/ml gentamicin and 2 mM glutamine. About one half million LHON fibroblasts (80000 per well) were seeded in 6-well plates (approximately 70% of confluency). 24 hours later they were transfected with FuGENE 6 transfection reagent as recommended by the manufacturer (Roche Biochemicals, Indianapolis). The selection was performed 68h later with 0.15 mg/ml of G418 (Roche Biochemicals, Indianapolis) added to

the medium, which has been changed each other day. 11 days after the selection, approximately 100,000 cells were counted using a Malassez plate after trypsination of the whole 6-well plates. We estimated the transfection yield to around 25% (number of cells collected after 11 days of G418 selection as compared to the total amount of cells initially seeded for the transfection). However this value would decrease if the number of passages in culture of the recipient cells is higher than 6. After trypsination, cells were seeded in the absence of G418 for 48h. Thereafter stable clones were expanded for 3 additional weeks in the presence of G418. After each trypsination, 20% of the cells were conserved (liquid nitrogen), the others were used for mitochondrial function evaluation. 36 h before each evaluation the G418 was withdrawn from the medium. In our experience, control or untransfected fibroblasts as well as stably transfected cells cannot be monitored rigorously after their 12th-16th passage in culture.

1.3. Glucose/Galactose assay

To determine cell ability to grow in galactose medium immediately after the transfection, 1500 fibroblasts from LHON#2 per well were seeded in two 24-well plates. Transfections were performed 24h later using either the *COX10*^{MTS}*NDI-3'UTR*^{COX10} or the *pCMV-Tag 4A* vectors. After 68h, the cells were washed three times with a DMEM medium lacking glucose. Plates were incubated in glucose (25 mM)- or galactose (10 mM)-containing DMEM medium lacking glucose (Gibco, Invitrogen) supplemented with 12% fetal bovine serum (Gibco, Invitrogen), Hepes (10 mM), uridine (2.25 mM), gentamicin (10 µg/ml), sodium pyruvate (2 mM), and glutamine (2 mM). The cells were then cultured for 10 days in the presence of 0.15 mg/ml G418. The medium was renewed every two days. At 5 and 10 days, cells were trypsinized and counted, with Malassez plates to compare the amount of viable cells under each condition. In parallel, the ability of control fibroblasts to grow in either glucose or galactose media was assessed.

The capacity to grow in galactose medium of stably transfected cells was also compared to control fibroblasts. Fibroblasts stably transfected with either the *COX10*^{MTS}*NDI-3'UTR*^{COX10} or the *pCMV-Tag 4A* vectors were maintained for 3 days in a glucose-containing medium lacking G418. After trypsination, 1500 of each transfected cells and control fibroblasts were seeded per well into two 24-well plates. The next day, one of the plates was extensively washed with a medium lacking glucose before the galactose-containing medium was added. The cells were cultured for 14 days, the medium of each plate was changed every two days. The number of viable cells was determined after 14 days of growth in either glucose- or galactose-containing media.

1.4. Immunocytochemistry

Immunochemistry was performed using either the mouse monoclonal anti-Flag M2 (Sigma, St. Louis, MO) or anti-ATP synthase α -subunit (Molecular Probes, Invitrogen) antibodies, as previously described [32, 34]. The membrane potential-dependent probe MitoTracker Red CMXRos (MT) was obtained from Molecular Probes (Invitrogen) and used at 10 nM as recommended by the manufacturer. Fluorescence was observed using a Leica DM 5000 B Digital Microscope to evaluate the subcellular distribution of the allotopically expressed polypeptides and their possible colocalization with the endogenous ATP synthase α -subunit or with the fluorescent dye MitoTracker. The digital images were acquired and processed with the MetaVue imaging system software.

1.5. RNA extraction, RT-PCR analyses

RNAs from untransfected and transfected fibroblasts were obtained using RNeasy Protect Mini kit (QIAGEN). RT-PCR analyses were performed with the Superscript III one step RT-PCR Platinum Taq kit (Invitrogen). Table 1 shows the specific couple of primers used for

each gene examined, the expected sizes of the PCR products, the quantity of RNA used for reverse-transcription, and the number of PCR cycles performed.

1.6. Protein extract and Western Blot analysis

Protein extraction was essentially performed according to Nijtmans *et al.* [35]. Briefly, one 75-cm² flask of fibroblasts was trypsinized (about 2-4 millions of cells depending of the confluency) and washed twice with cold phosphate-buffered saline (PBS). Cells were incubated 10 min on ice with cold digitonin (8 mg/ml in PBS, v/v) to dissolve membranes. Digitonin was diluted by addition of cold PBS. Cells were spun for 5 min, at 10,000g at 4°C. The pellet was washed once more in 1ml cold PBS. To solubilize, the pellet was vigorously pipetted in buffer A containing 1.5M aminocaproic acid, 50mM Bis-Tris/HCl, pH 7.0. Next, cells were incubated at 0°C for 5 min by addition of dodecylmaltoside (10% w/v). Cells were centrifuged at 20,000g for 30 min at 4°C. Pellet was resuspended in buffer A. The protein concentration was estimated by the Bradford protein assay (Sigma), with BSA as standard. After denaturation at 70°C, 8 µg proteins were separated by SDS-PAGE, using a 4-12% polyacrylamide gel (NuPAGE® system, Invitrogen) and transferred to a nitrocellulose membrane (GE Healthcare). Membranes were probed with mouse monoclonal antibodies anti-Flag M2 (3.5 µg/mL) or anti-ATP synthase subunit α (0.5 mg/mL) and with a polyclonal anti-ND1 subunit antibody kindly provided by Dr. A. Lombes (1:800). Immunoreactive bands were visualized with an anti-mouse antibody coupled to horseradish peroxidase (0.1 µg/mL) followed by ECL Plus detection (GE Healthcare).

2.7. Biochemical assays

Rotenone-sensitive complex I, oligomycin-sensitive complex V activities and ATP synthesis rate were assayed as recently described [32, 36]. Antimycin-sensitive quinol cytochrome *c* reductase (complex III activity) was measured as previously described [37]. The quinone derivative used to measure complex III was decylubiquinol [37]. Complex IV activity is triggered by the addition of reduced cytochrome *c* [36]. Note that when half the cytochrome *c* has been oxidized, the rate of cytochrome *c* oxidation is reduced by about half. Measurement of the three respiratory chain complexes was achieved from a single culture of skin fibroblasts transfected or not. Each assay was performed with 2×10^5 cells. Protein concentration was measured with the Bradford assay. All chemicals were of the highest grade from Sigma (St. Louis, MO).

2.8. Statistics

Differences between control fibroblasts, untransfected and transfected LHON-fibroblasts were assessed using the paired Student's *t* Test with a minimal significance of $p < 0.05$.

Results

3.1 Comparison between endogenous and hybrid mRNAs from the ND4 and ND1 genes

To estimate the efficiency of our constructions on directing the expression of nuclearly-encoded *ND4* and *ND1* genes, we analyzed in transfected cells the amount of hybrid mRNAs and compared it to endogenous *ND4* and *ND1* mRNAs by RT-PCR (Fig. 1A). The integrity of each RNA preparation was assessed by electrophoresis; (Fig. 1A upper-left panel) no difference in 28S and 18S ribosomal RNA bands from the different RNA samples examined was observed confirming the integrity of the preparations. Moreover, the amount of the endogenous mitochondrial *ATP6* mRNA was similar in transfected and untransfected LHON fibroblasts (Fig. 1A, upper-right panel). Hybrid mRNAs produced from both *COX10*^{MTS}*ND4*-3'UTR^{COX10} and *COX10*^{MTS}*ND1*-3'UTR^{COX10} vectors were only detected in LHON#1 + *WT ND4* and LHON#2 + *WT ND1* fibroblasts respectively (Fig. 1A bottom panel). In spite of the strong CMV promoter, these cells did not accumulate very high amounts of hybrid *ND4* and *ND1* mRNAs as compared to endogenous mRNAs; indeed as shown in Table I, 35 cycles of amplification and 300 ng of RNA extraction were required to obtain an amount of PCR product for hybrid mRNAs comparable to the one detected from the endogenous mRNAs, which needed only 50 ng of RNAs and 25 cycles of amplification.

3.2. Steady-state levels of the fusion ND4 and ND1 proteins in stably transfected LHON fibroblasts

To determine whether hybrid mRNAs were translated in detectable levels of fusion wild-type proteins ND4 and ND1, protein extracts from digitonin-permeabilized fibroblasts were subjected to Western-blotting analyses. Immunoblots were first performed using anti-ATP synthase α antibody. This naturally imported mitochondrial protein of respiratory complex V

was present, as a single band of approximately 65 kDa, at similar extent in all cells tested (Fig. 1B). The anti-Flag antibody was used to detect the fusion proteins, since a flag epitope was appended in frame to the C-terminus of each protein. The antibody revealed one band of approximate 53 kDa in fibroblasts transfected with the *COX10*^{MTS}*ND4-3'UTR*^{COX10} vector, and two forms of about 38 and 33 kDa in cells expressing the *COX10*^{MTS}*ND1-3'UTR*^{COX10} gene, as implied by the molecular weight markers. The larger band could represent the precursor form of the fusion ND1 protein; indeed its signal was always weaker as compared to the one detected for the form of about 33 kDa. Both ND4 and ND1 fusion proteins detected by the anti-Flag antibody presented electrophoretical properties similar to the ones expected from their theoretical molecular weights of approximate 51.69 kDa and 35.66 kDa respectively (<http://www.mitomap.org/>). No signal was detected with the anti-Flag antibody in the other samples examined. The amount of ND1 protein was compared in each protein-extract using a polyclonal anti-ND1 subunit antibody (Fig. 1B). Noticeably, the amount of the endogenous ND1 protein was particularly low in fibroblasts bearing the 3460/*ND1* mutation. After normalization with the amount of ATP synthase α , a 2.7 or 2.1-fold reduction in the amount of ND1 protein in these cells was observed when compared to control fibroblasts and fibroblasts harboring the 11778/*ND4* mutation respectively. In fibroblasts expressing the *COX10*^{MTS}*ND1-3'UTR*^{COX10} gene, the anti-ND1 antibody was able to recognize both the endogenous and the mature form of the fusion protein; indeed the difference between these fibroblasts and controls or fibroblasts harboring the the 11778/*ND4* mutation became of only 1.46 and 1.3-fold respectively (Fig. 1B, right panel). These results indicate that optimized allotypic expression led to the production of detectable amounts of the fusion ND4 and ND1 proteins. Interestingly in LHON # 2 fibroblasts, we observed that both the endogenous *ND1* gene and the one synthesized from our vector directed the synthesis of similar amounts of

proteins. The question arises whether the fusion ND4 and ND1 proteins localized to mitochondria and were able to compensate the defective endogenous protein in these cells.

3.3 Mitochondrial subcellular distribution of allotopic ND1 and ND4 gene products in human fibroblasts

First, we examined the mitochondrial morphology in fibroblasts from two patients harboring the G11778A *ND4* (LHON#1) and G3460A *ND1* (LHON#2) substitutions. We used the membrane potential-dependent probe MitoTracker Red CMXRos as mitochondrial marker as well as a monoclonal antibody directed against α -subunit of mitochondrial ATP synthase. The two labelings are perfectly superimposable (see Fig.2A, merged panel). In about 90% of LHON#1 fibroblasts both the anti-ATP synthase α -subunit monoclonal antibody and the MitoTracker probe revealed a punctuate distribution of fluorescent dots, as recently described [32] (Fig. 2A). This mitochondrial fragmented appearance may indicate a perturbation in the balance between the organelle fusion and fission in these cells. The remaining cells showed long and tiny filaments around the nuclear envelop. By contrast, in 90% of LHON#2 fibroblasts, mitochondria appeared more elongated and with a shape more complex than in LHON#1 fibroblasts (Fig. 2A). The mitochondrial morphology in the two patient fibroblasts was undeniably different. Additionally, fibroblasts from a healthy control subject and from the LHON#2 patient displayed a similar mitochondrial morphology (Fig. 2A). This observation suggests that in the cells examined the *ND4* mutation reduced mitochondrial shape complexity at a higher extent than the *ND1* mutation.

Fibroblasts from the two patients were subsequently stably transfected with constructions leading to the expression of engineered nucleus-localized wild-type *ND4* or *ND1* genes, respectively. To increase the probability for the hydrophobic ND1 or ND4 precursors to be efficiently translocated inside mitochondria, we combined to the *ND4* and *ND1* ORFs the

sequence encoding the MTS and the 3'UTR from the *COX10* gene, as we recently reported [32]. Then, we examined by indirect immunofluorescence the ability of the proteins synthesized from these plasmids to localize to mitochondria using the anti-Flag antibody. There was a colocalization of the anti-Flag and the ATP synthase- α subunit antibodies for each transfected fibroblasts examined (Fig. 2B). The overlay of the two specific antibody labelings indicates that allotopically expressed proteins and the endogenous mitochondrial ATP synthase α -protein were distributed in the same subcellular compartment *in vivo*. Moreover, in LHON#1 fibroblasts allotopically expressing the *COX10*^{MTS}*ND4-3'UTR*^{COX10} vector we consistently observed a less dotted appearance of their mitochondria as compared to untransfected fibroblasts (Fig. 2A) or fibroblasts transfected with the *pCMV-Tag 4A* vector (unpublished data). Mitochondria appeared more elongated and networks were visualized in approximately 40% of cells, indicating that the mitochondrial shape in LHON#1 after transfection became more comparable to the one observed in both control and LHON#2 fibroblasts. Thus, the optimized allotopic expression of the wild-type *ND4* gene in fibroblasts carrying the *ND4* mutation led to an increased mitochondrial morphology complexity.

3. 4. The ability to grow in galactose medium is improved in LHON fibroblasts carrying the G3460A substitution by the allotopic expression of ND1 gene

It has been well documented that when galactose replaces glucose as carbon source in the medium, cells deficient in mitochondrial function are greatly curtailed in their growth rate [15, 38]. We have recently described a 3.5-fold decrease in the growth rate of LHON#1 fibroblasts compared to control cells after 8 days of growth in galactose medium. This decrease was compensated when LHON#1 fibroblasts allotopically expressed the *COX10*^{MTS}*ND4-3'UTR*^{COX10} vector [32]. To determine whether LHON#2 fibroblasts presented also a curtailed growth rate in galactose and to evaluate the impact of *ND1* allotopic

expression, the overall number of cells transfected with either the *pCMV-Tag 4A* or the *COX10^{MTS}NDI-3'UTR^{COX10}* vectors was estimated after 5 or 10 days of culture in either glucose or galactose media; immediately after transfection. Figure 3A shows a striking negative impact on cell survival after 10 days of culture in galactose medium in LHON#2 untransfected and fibroblasts transfected with the *pCMV-Tag 4A* plasmid as compared to fibroblasts allotopically expressing the wild-type gene. Fibroblasts from a healthy subject did not show differences in their growth rates when glucose- or galactose-containing medium were measured. Indeed, after 10 days of growth in galactose the overall amount of cells was $92.96\% \pm 8.4$ compared to the cells grown in glucose (Fig. 3A; $p = 0.20$, $n = 6$). We observed an approximately 7-fold decrease in the growth rate of LHON#2 fibroblasts transfected with the *pCMV-Tag 4A* compared to control cells after 10 days of growth in galactose medium (Fig. 3B). LHON#2 fibroblasts expressing the *COX10^{MTS}NDI-3'UTR^{COX10}* vector showed a 5.1- and a 5.9-fold improvement ($p < 0.0001$, $n = 6$) after 5 and 10 days in their ability to grow in galactose compared to LHON#2 fibroblasts transfected with the *pCMV-Tag 4A* plasmid (Fig. 3A).

To further evaluate the impact of *NDI* allotopic expression on the growth rate in galactose medium the number of stably transfected cells with either the *pCMV-Tag 4A* or the *COX10^{MTS}NDI-3'UTR^{COX10}* vectors were evaluated after 14 days of growth in either glucose or galactose media and compared to the overall number of viable cells for control fibroblasts under the same conditions (Table 2). The survival rate on galactose medium was improved of about 5.1 fold ($p = 0.0016$, $n = 4$) in cells expressing the recoded *NDI* gene, strengthening the data obtained immediately after transfection. Moreover, cell growth rates in galactose were not statistically different between control cells and fibroblasts allotopically expressing the *NDI* gene after 14 days of selection ($p = 0.28$, $n = 4$).

Thus, as we have recently reported for cells carrying the *ND4* G11778A substitution [37], the allotropic expression of the engineered nucleus-localized *NDI* gene led to a significant benefit for mitochondrial function measured by the ability to growth in galactose of cells harboring the G3460A substitution.

3.5 Decrease of ATP synthesis in LHON#2 fibroblasts was rescued by the allotropic expression of NDI

The impairment of energy metabolism due to mutations in mtDNA encoded genes has been frequently correlated with a reduced ability of cells harboring these mutations to synthesize ATP *in vitro* from ADP and Pi through the action of respiratory chain complex V [39]. Therefore, to further confirm that allotopically expressed *NDI* gene product was functional within complex I, oligomycin-sensitive ATP production by digitonin permeabilized fibroblasts was measured using complex I-linked (malate/pyruvate) substrates (Table 3). For LHON#2 fibroblasts, we evidenced a 75% reduction in the rate of complex I-dependent ATP synthesis when compared to controls ($p = 0.0004$, $n = 3$). When LHON#2 fibroblasts were transfected with the mock vector, we showed a decrease of approximately 70% in ATP synthesis statistically significant for complex I-linked substrates ($p < 0.0001$, $n = 3$ for pyruvate/malate). ATP synthesis measurement in LHON fibroblasts allotopically expressing the engineered *NDI* gene (LHON#2 + *COX10*^{MTS}*NDI*-3'*UTR*^{*COX10*}) indicated an almost complete recovery in the rate of complex I- dependent ATP production (80% relative to control). A statistical significant difference was reached between LHON#2 fibroblasts and cells allotopically expressing the *NDI* gene: $p = 0.0018$, $n = 3$ for pyruvate/ malate (Table 3). The difference between cells expressing *NDI* and mock-transfected fibroblasts was also significant ($p = 0.003$, $n = 3$ for pyruvate/malate). These data establish that the allotropic

expression of *ND1* corrected the energy metabolism impairment of fibroblasts harboring the G3460A *ND1* substitution.

3.6 The allotopic expression of ND4 or ND1 genes leads to restoration of complex I activity in LHON fibroblasts harboring these genes mutated

Since, both *in vivo* and *in vitro* biochemical studies in different patient-derived tissues and cellular experimental models have produced conflicting results regarding the extent of respiratory chain dysfunction in LHON [8, 9], we decided to perform spectrophotometric assays [36] to measure the activity of respiratory complexes I, III, IV and V in LHON fibroblasts and their counterparts stably transfected with either *ND4* or *ND1* genes. In these experiments, we also used NARP fibroblasts allotopically expressing *ATP6* gene as an internal control of fully respiratory chain function recovery [37] (Fig. 4). These measurements allow to estimate the severity of respiratory chain defects in these fibroblasts and to further validate the rescue by the optimized allotopic expression approach. We consistently observed differences in the rotenone-sensitive complex I activity (I) between control fibroblasts and LHON fibroblasts (Fig. 4C, compare (a), (b) and (d)). By contrast, we did not observe significant differences between control, LHON#2 + $COX10^{MTS}ND1-3'UTR^{COX10}$, LHON#1 + $COX10^{MTS}ND4-3'UTR^{COX10}$ and NARP + $SOD2^{MTS}ATP6-3'UTR^{SOD2}$ kinetics (Fig. 3C, compare (a), (c), (e) and (f)). We compared the three enzymatic activity ratios (IV/I), (V/I) and (III/IV). First, no significant difference were observed in the III/IV ratio between control, LHON#1 and LHON#2 fibroblasts (Fig 4D). Second, when we measured the two enzymatic activity ratios (IV/I) and (V/I) we noticed an increase in the two ratios in LHON fibroblast cells, indicating that they presented a marked defect in complex I activity. Indeed, we observed a 1.7- and 2.4-fold increase ($p < 0.0001$) of LHON#1 fibroblasts and a 2.6- and 4-

fold of LHON#2 fibroblasts in the IV/I and V/I ratios, respectively (Fig. 4D). Interestingly, fibroblasts allotopically expressing *ND4* and control fibroblasts revealed almost identical IV/I and V/I activity ratios. A statistical difference was reached between LHON#1 untransfected fibroblasts and their counterparts allotopically expressing the *ND4* gene ($p < 0.0001$, $n = 12$ for the IV/I ratio and $p < 0.0001$, $n = 12$ for the V/I ratio) (Fig. 4D). In LHON fibroblasts expressing the engineered *ND1* gene, we obtained a partial restoration of the complex I activity (60% for the V/I ratio). The difference between LHON#2 fibroblasts and cells expressing *ND1* was significant for this ratio ($p = 0.0005$, $n = 3$). Hence, the spectrometrically assay of complex I enzymatic activity in LHON fibroblasts harboring either the *ND4* or the *ND1* mutations was effective in the detection of a severe defect on these cells as compared to controls. Moreover, the measurements of complex III, IV and V activities clearly demonstrated that OXPHOS defect in these fibroblasts is restricted to complex I. Since a significant improvement of its activity was consistently noticed in stably transfected cells, fusion ND4 and ND1 proteins synthesized from our vectors appear to be functional within the respiratory chain complex I and able to compensate for the endogenous mutated gene products.

Discussion

Mitochondrial disorders can not be ignored anymore in most medical areas. They include specific and widespread organ involvement, with tissue degeneration or tumor formation. Primary or secondary actors, mitochondrial dysfunctions are also playing a role in the ageing process [40, 41]. Research dealing with mitochondrial physiology and pathology has almost 20 years of history all over the world. Despite the progresses made in the identification of disease molecular bases, nearly all remains to be done regarding therapy [25]. The Leber Hereditary Optic Neuropathy (LHON), a mitochondrial genetic disease, represents the main cause of male adolescent blindness. Its prevalence has been estimated of ~1 over 25,000 in the north east of England. Few data exist on the absolute prevalence of LHON for other populations, excepted in Australia where LHON is the cause of blindness of 2% from all registered blind persons [42]. Unfortunately since pathogenic mechanisms of LHON are not yet understood there are no efficient therapies for its treatment. Quantifications of complex I activity were controversial in cybrids which were used as cellular models to evaluate mitochondrial complex I defects in patients harboring LHON mutations [15, 19]. Cybrid cells are valuable in mitochondrial research to better understand mechanisms of several pathologies with mitochondrial implications, but they remain an “artificial” system contrarily to fibroblasts which can be supposed to reproduce more accurately natural physiological responses. Additionally studies performed with muscle tissues [10] or lymphocytes [11] led also to contentious results. For example, Majander *et al.* did not observe any decrease in complex I activity in lymphoblasts harboring the *ND4* mutation whereas Larsson *et al.* observed a 50% reduction. Nonetheless, it has been well established that LHON patients with the *ND1* mutation are more affected than patients with the *ND4* mutation. Compilation of experimental data showed a 60 to 80% decrease in complex I activity in cells harboring the

ND1 mutation whereas only a 0 to 50% reduction was detected in cells bearing the *ND4* mutation [42]. Up until today, we were able to find only one report on LHON fibroblasts OXPHOS studies *ex vivo*. Cock *et al.* have reported an approximate 60% defect in mitochondrial NADH CoQ1 reductase activity in cultured fibroblasts bearing the G3460A *ND1* mutation. However, complex I-linked ATP synthesis was found to be normal in these fibroblasts [13]. We reported here using spectrophotometric measurements that skin fibroblasts carrying the *ND1* (LHON #2) and *ND4* (LHON #1) mutations presented consistent 80% and 60% decreases in complex I activity respectively. This is probably due to the high sensitivity and robustness of the spectrometric assay performed. Indeed, the sequential determinations of complex activities on the same sample, allows the determination of enzyme activity ratios. This latter parameter has been previously shown to be the most efficient for detecting isolated and partial impairment of respiratory chain activity [43].

Notably, the cellular morphology of mitochondria in LHON#1 fibroblasts always showed punctuate dots suggesting an enhanced organelle fission in these cells in contrast to the LHON#2 fibroblasts or control fibroblasts which revealed more elongated and more branched mitochondria. Recently, Koopman and coworkers evidenced a relationship between complex I deficiency in fibroblasts obtained from patients carrying mutations in nuclear genes encoding complex I subunits and the mitochondrial shape. These authors claimed that changes in mitochondrial morphology ("mitochondrial complexity") are directly dependent on the severity of complex I deficiency. They distinguished two categories of fibroblasts: one presenting fragmented mitochondria, severe complex I amount and activity defects. The second group displayed a normal mitochondrial appearance and moderate complex I defects [44]. From our observations, LHON#1 fibroblasts would be categorized in the "fragmented mitochondria" group while LHON#2 cells would belong to the "normal appearance" group. However, the morphology difference do not correlated with the severity of complex I

deficiency extent, since LHON#2 cells with normal mitochondrial appearance presented the more severe complex I defect. Alternative explanation should therefore be envisaged. For example, differential effects on morphology might be associated with differences in the role of ND1 and ND4 in complex I assembly, and thus in mitochondrial organization [45, 46]. Interestingly, upon allotopic expression of *ND4* gene LHON#1 fibroblasts, the overall mitochondrial morphology became similar to the "normal" class and to both control and LHON#2 fibroblasts (Fig. 2B).

In a previous study, we measured two independent parameters of mitochondrial function in LHON fibroblasts harboring the *ND4* mutation (LHON#1) and their counterparts allotopically expressing the wild-type gene: (i) cells' ability to grow in a galactose-containing medium that relies on oxidative phosphorylation and (ii) cells' capacity to produce ATP *in vitro*. According to these two criteria, we have demonstrated that optimized allotopic expression of the wild-type *ND4* gene allowed a long-lasting benefit for mitochondrial function in cultured fibroblasts harboring the *ND4* mutation [32]. In the present study, we established unequivocally the complete restoration of OXPHOS function in these fibroblasts by measuring complex I, IV and V activities. Indeed, by analyzing two independent stably transfected fibroblasts with the *COX10*^{MTS}*ND4-3'UTR*^{COX10} vector, no significant difference was detected between these transfected cells and controls regarding complex I enzymatic activity. The increased number of experiments, compared to our previous work [32] allowed to statistically confirmed the restoration of complex I activity in stably transfected cells (V/I ratios of 1.66 ± 0.49 and 1.35 ± 0.5 in patient cells and controls respectively, Fig. 4). Subsequently, our goal was to alleviate mitochondrial dysfunction in fibroblasts carrying the G3460A mutation (LHON#2) using *ND1* optimized allotopic expression. These engineered fibroblasts retained a long-term expression of the *ND1* gene and were able to survive for up to 14 days in a medium in which glucose had been replaced by galactose. To further validate that

our approach conferred a real improvement of mitochondrial function in LHON#2 fibroblasts we measured the actual mitochondrial ATP synthesis capacity of cells allotopically expressing the wild-type gene. Stably transfected cells displayed a significantly improved ATP synthesis rate when complex I substrates were used. The overall ATP production rate of these cells became 80-82% relative to controls (Table 3).

Since we were aware that increased ATP synthesis cannot be viewed as unequivocal evidence of rescue of mitochondrial dysfunction we spectrometrically assessed complex I enzymatic activity in untransfected and transfected LHON#2 fibroblasts as we did for LHON#1 cells. For LHON#2 fibroblasts, our optimized allotopic approach led to a partial but significant rescue of complex I defect. Notably, the inactivation of complex I is more pronounced in LHON#2 (*ND1/3460*) than in LHON#1 (*ND4/11778*) fibroblasts (80% vs 60%). This can be explained by considering the central role of ND1 in the first step of complex I biogenesis. ND1 is found in a separate, smaller complex before its association with other ND subunits [45, 46]. The *ND1* mutation might be more detrimental to the complex I assembling than the *ND4* mutation, by consequence *ND1* allotopic expression can result in an only partial rescue of the mitochondrial function. Besides, we have measured the IV/I and III/I complex activity ratios in LHON fibroblasts to determine whether complex I defect in these cells is associated to either complex IV or complex III decreased activities. Both complex activities were similar in these cells to that of controls and confirmed that they presented a restricted complex I dysfunction which was significantly alleviated by using optimized allotopic expression. Our approach has led to the accumulation of sufficient amounts of functional proteins synthesized from our vectors (Fig. 1) which created a situation very similar to the heteroplasmy found in patients (coexistence of both wild-type and mutated mtDNA molecules). This heteroplasmy at the protein level drives to the restoration of complex I activity and the improvement of the overall mitochondrial function in these cells.

In conclusion, we are involved as many other laboratories worldwide in the challenge to find ways for fighting mitochondrial diseases. However, our main limitation is the absence of animal and cellular models required for both the understanding of the molecular mechanisms underlying the diseases and to evaluate therapeutic strategies, especially for mtDNA mutations. Last year, Qi and colleagues have shown that the nuclear version of the mutant ND4 subunit of complex I is translocated into the mitochondria of mouse RGCs and led to retinal and optic nerve degeneration. This important data represents a first step for the generation of a murine model resembling LHON [47]. Nevertheless, their approach encounters the limitation of the inefficient mitochondrial import of the protein since the corresponding mRNA cannot be sorted to the mitochondrial surface. Hence, we suspect that it is not a robust mouse model to evaluate putative treatments. Besides, their strategy has the potential to become toxic for mouse retina over time, indeed high levels of misfolded hydrophobic proteins in the cytosol have been shown to be deleterious *ex vivo* [29].

The data presented here and our previous work definitely confirmed for three mitochondrial genes (*ATP6*, *ND4* and *ND1*) that the optimized allotopic approach based on the mRNA sorting to the mitochondrial surface becomes the more suitable way for rescuing mitochondrial deficiencies caused by mutations in mtDNA genes. Our next challenge is to create an animal model of RGC degeneration due to LHON mutations and its cure using our optimized allotopic expression approach, if we succeed we will open the door to gene therapy for LHON.

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Legend to figures

Figure 1: *Endogenous and allotypic mRNA and gene products in transfected fibroblasts*

A: RT-PCR analysis to reveal amounts of hybrid and endogenous *ND1* and *ND4* mRNAs respectively. 300 ng of each RNAs preparation was electrophoresed on a formaldehyde-agarose gel to assess the integrity of the RNA. The amount of endogenous mitochondrial *ATP6* mRNA was used as internal control. The abundance of endogenous and hybrid *ND1* and *ND4* mRNAs was determined in each preparation using the conditions shown in Table 1.

1-3: LHON#1, 4-6: LHON#1 + *WT ND4*, 7-9: LHON#2, 10-12: LHON#2 + *WT ND1*. (1), (4), (7), (10): *ND1-1/ND1-2R*; (2), (5): *T3/hND4-1R*; (8), (11): *T3/hND1-1R*; (3), (6), (9), (12): *ND4-1/ND4-2R*

B: Recoded *ND1* protein is efficiently imported into mitochondria. Proteins extracted from untransfected and transfected cells were subjected to Western blot essays. Immunoblotting analysis was performed using anti-ATP synthase subunit α , anti-Flag M2 and anti-*ND1* antibodies.

Figure 2: *Mitochondrial morphology in control fibroblasts, untransfected and transfected LHON-fibroblasts*

A: Mitochondrial shape in fibroblasts from two LHON patients and from a healthy control subject were examined by indirect immunofluorescence using antibody against the ATP synthase α -subunit. The membrane potential-dependent probe MitoTracker Red CMXRos (MT) was used as a specific mitochondrial marker.

B: Subcellular localization of the allotypically expressed proteins and the endogenous ATP synthase α -subunit protein *ex vivo*. Stably transfected Leber hereditary optic neuropathy (LHON) cells with *COX10^{MTS}ND1-3'UTR^{COX10}* or *COX10^{MTS}ND4-3'UTR^{COX10}* plasmids were visualized by indirect immunofluorescence using antibodies against the Flag epitope or

against the ATP synthase α -subunit. A merged image of immunofluorescence revealed with each antibody is shown in the right panel. A significant colocalization of both COX10-ND1Flag, COX10-ND4Flag, and ATP synthase- α signals was observed in the cells examined. Fluorescence was inspected with a Leica DM 5000 B Digital microscope. The bar corresponds to 15 μm .

Figure 3: Fibroblast' ability to grow in galactose selective medium.

A: After 10 days of growth in galactose medium, LHON#2 untransfected fibroblasts and LHON#2 fibroblasts transfected with either the *pCMV-Tag 4A* vector or with the *COX10^{MTS}NDI-3'UTR^{COX10}* plasmid were observed by photonic microscope (10x, the bar represent 20 μm). Cells allotopically expressing the wild-type version of *NDI* exhibited a substantial increase in growth rate in galactose medium compared to untransfected and mock-transfected LHON#2 fibroblasts.

B: Cell survival after 5 or 10 days of galactose selection measured in LHON#2 cells transfected with the *pCMV-Tag 4A* vector (1) and LHON#2 fibroblasts transfected with the *COX10^{MTS}NDI-3'UTR^{COX10}* plasmid (2). The two transfected fibroblasts were compared to control fibroblasts (C) after 10 days of galactose selection. Bar graphs represented the cell growth capacity in galactose compared to that in glucose. One hundred percent represents the total number of cells that grew in glucose medium for each tested cells. Experiments were performed six times, Student's *t* test established that the abilities to grow in galactose were significantly improved in the transfected cells (*COX10^{MTS}NDI-3'UTR^{COX10}*) compared to either mock-transfected cells ($p < 0.0001$, $n = 6$).

Figure 4: Restoration of complex I activity in LHON-transfected fibroblasts. A: Illustrative curve for the successive measurement of rotenone-sensitive complex I and oligomycin-

sensitive complex V activities. B: Typical curve for the measurement of cytochrome c activity. C: Enzymatic activity measurement of rotenone-sensitive complex I and oligomycin-sensitive complex V activities for control fibroblasts (a), LHON#1 (b), LHON#1 + $COX10^{MTS}ND4-3'UTR^{COX10}$ (c), LHON#2 (d) LHON#2 + $COX10^{MTS}ND1-3'UTR^{COX10}$ (e) and NARP + $SOD2^{MTS}ATP6-3'UTR^{SOD2}$ (f). ATP, adenosine triphosphate; cyt, cytochrome; DQ, duroquinone (oxidized); KCN, potassium cyanide; LDH, lactate dehydrogenase; MgCl₂, magnesium chloride; NADH, reduced nicotinamide adenine dinucleotide; oligo, oligomycin; PEP, phosphoenol pyruvate; PK, pyruvate kinase; red, reduced. Numbers along the traces corresponds to activity expressed as nmol/min/mg protein. D: Table shows the enzyme activities ratio (IV/I),(V/I) and (III/IV) for control fibroblasts (Control), untransfected LHON cells (LHON#1 and LHON#2), LHON fibroblasts allotopically expressing *ND1* or *ND4* (LHON#1 + $COX10^{MTS}ND4-3'UTR^{COX10}$ and LHON#2 + $COX10^{MTS}ND1-3'UTR^{COX10}$) and NARP cells allotopically expressing *ATP6* used as control (NARP + $SOD2^{MTS}ATP6-3'UTR^{SOD2}$). Number of experiments was indicated in brackets. The difference between LHON#1 and LHON#1 + $COX10^{MTS}ND4-3'UTR^{COX10}$ was statistically significant ($p < 0.0001$, $n = 12$ for IV/I; $p < 0.0001$, $n = 9$ for V/I). For LHON #2, the ration of V/1 activity was also significantly different when the couple LHON#2 and LHON#2 + $COX10^{MTS}ND1-3'UTR^{COX10}$ was compared ($p = 0.0005$, $n = 3$). No signifant difference was observed between control fibroblasts and untransfected LHON cells (LHON#1 and LHON#2) in the enzyme activity ratio (III/IV).

Table 1: RT-PCR analyses

| mRNA | RT-PCR product length (pb) | Primers | | RT-PCR | |
|------------------------|----------------------------|-----------------------------|-----------------------------|---------------|---------------|
| | | 5' primer (5'-3') | 3' primer (5'-3') | Quantity (ng) | Cycle numbers |
| Endogenous <i>ND1</i> | 252 | ATACCCATGGCCAACCTCCTACTCCTC | GTTGGGTATGGGGAGGGGGTTCATAG | 50 | 25 |
| Hybrid <i>ND1</i> | 248 | T3 (AATTAACCCTCACTAAAGGG) | CAGTCAGCATGAGGAAGGCCATGG | 300 | 35 |
| Endogenous <i>ND4</i> | 227 | ATGCTAAAATAATCGTCCCAACAATT | ATAAGTGGCGTTGGCTTGCCATGATTG | 50 | 25 |
| Hybrid <i>ND4</i> | 330 | T3 (AATTAACCCTCACTAAAGGG) | CAGGAGAACAGGTTGTTGTTGATCTGG | 300 | 35 |
| Endogenous <i>ATP6</i> | 248 | ATGAACGAAAATCTGTTGCTTCATTC | GAGTCCGAGGAGGTTAGTTGTGGCAAT | 50 | 25 |

Table 2

Ability of LHON fibroblasts carrying the *NDI* mutation and their counterparts expressing the recoded *NDI* gene to grow in galactose medium

| | Number of cells/ml (glucose) | Number of cells/ml (galactose) | Survival rate % (galactose/glucose) |
|---|---------------------------------|-----------------------------------|--|
| LHON# 2 + <i>pCMV-Tag</i> <i>4A</i> | 31250 ± 957 | 4300 ± 600 | 13,76 |
| Control | 31075 ± 4728 | 28500 ± 4102 | 91.7 |
| LHON# 2 + <i>COX10</i> ^{MTS} <i>NDI-3'UTR</i> ^{<i>COX10</i>} | 34325 ± 4261 | 23950 ± 3879 | 69.8 |

The viability of stably transfected cells with either the *pCMV-Tag 4A* or the *COX10*^{MTS}*NDI-3'UTR*^{*COX10*} vectors was measured after 14 days of growth in either glucose- or galactose-containing media and compared to control fibroblasts. Four independent experiments were performed and the amount of viable cells under each condition was obtained by counting. The overall number of viable cells in galactose medium was significantly different according to the Student's *t* test when LHON# 2 + *COX10*^{MTS}*NDI-3'UTR*^{*COX10*} and LHON# 2 + *pCMV-Tag 4* fibroblasts were compared ($p = 0.0016$, $n = 4$) as well as for LHON# 2 + *pCMV-Tag 4* versus control fibroblasts ($p = 0.0014$, $n = 4$). In contrast, no significant difference was evidenced in the total number of cells after 14 days of growth in galactose between LHON# 2 + *COX10*^{MTS}*NDI-3'UTR*^{*COX10*} and control fibroblasts ($p = 0.28$, $n = 4$).

Table 3**Measurement of mitochondrial ATP synthesis in digitonin-permeabilized fibroblasts**

| | $\mu\text{M ATP/min}/10^6 \text{ cells/ mg protein}$ |
|--|--|
| | Pyruvate/malate |
| Control | 2423.1 ± 125.7 |
| LHON # 2 | 605.5 ± 72.8 |
| LHON + <i>pCMV-tag 4A</i> | 730.4 ± 76.5 |
| LHON + <i>COX10^{MTS}NDI-3'UTR^{COX10}</i> | 1979.0 ± 168.8 |

The rate of ATP synthesis, expressed in $\mu\text{M ATP/min}/10^6 \text{ cells/mg protein}$, was measured after 5 days of growth in glucose medium, in control fibroblasts, LHON#2 fibroblasts, LHON#2 fibroblasts stably transfected with the *pCMV-tag 4A* vector and LHON#2 fibroblasts stably transfected with *COX10^{MTS}NDI-3'UTR^{COX10}* plasmid. Three independent experiments were performed using pyruvate/malate (complex I-linked substrates). No significant difference in the ATP synthesis capacity was observed between control cells and fibroblasts allotopically expressing *NDI* for complex I -linked substrates, confirming the rescue of the respiratory chain defect.

ATP, adenosine triphosphate; LHON, Leber Hereditary Optic Neuropathy.