POLG p.A962T Mutation Leads to Neuronal Mitochondrial Dysfunction That is Restored After Mitochondrial Transplantation

Wei HU[1]*, Chun SHI[2]*, Han GUO[1]*, BenSi ZHANG[3]

** These authors contributed equally to this work.*

[1]Department of Pharmacy, Yiyang Medical College, Yiyang, China, [2]College of Dental Medicine, Western University of Health Sciences, Pomona, CA, USA, [3]Department of Anatomy, Dali University, Dali, Yunnan, China

Received January 2, 2024
Accepted May 14, 2024

Summary

Mutations in DNA polymerase gamma (POLG) are known as the predominant cause of inherited mitochondrial disorders. But how these POLG mutations disturb mitochondrial function remains to be determined. Furthermore, no effective therapy, to date, has been reported for POLG diseases. Using differentiated SH-SY5Y cells, a human neuronal model cell line, the current study investigated whether the novel POLG variant p.A962T impairs mitochondrial function. This involved quantifying mitochondrial DNA (mtDNA) content using PCR and assessing the expression levels of the subunits of complex IV (COXI-IV), a complex I subunit NDUFV1 and Cytochrome C (Cyto C) release using Western blotting. Activities of mitochondrial complex I, II, and IV were measured using colorimetric assays. Mitochondrial membrane potential (ΔΨm) and ATP were evaluated using fluorescence assays and luminescent assays, respectively. In addition, we investigated whether mitochondrial transplantation (MT) using Pep-1-conjugated mitochondria could compensate for mitochondrial defects caused by the variant in cells carrying mutant POLG. The results of this study showed that POLG p.A962T mutation resulted in mitochondrial defects, including mitochondrial DNA (mtDNA) depletion, membrane potential (ΔΨm) depolarization and adenosine triphosphate (ATP) reduction. Mechanistically, POLG mutation-caused mtDNA depletion led to the loss of mtDNA-encoded subunits of complex I and IV and thus compromised their activities. POLG p.A962T mutation is a pathogenic mutation leading to mitochondrial malfunction and mtDNA depletion in neurons. Cell-penetrating peptide Pep-1-mediated MT treatment compensated for mitochondrial defects induced by these POLG variants, suggesting the therapeutic application of this method in POLG diseases.

Key words

DNA polymerase gamma • Mitochondrial function • Mitochondrial DNA • Mitochondrial transplantation

Corresponding author

C. Shi, College of Dental Medicine, Western University of Health Sciences, Pomona, CA 91766-1854, USA. E-mail: Cshi@westernu.edu

Introduction

The replication and repair of mitochondrial DNA (mtDNA) depends on POLG [1-2]. POLG mutations are the predominant single-gene cause for inherited mitochondrial diseases. Patients with POLG disorders exhibit a wide spectrum of clinical manifestations with onset from birth to adulthood [1-2]. Mechanistically, POLG mutations cause mtDNA-maintenance defects, resulting in mitochondrial dysfunction [2]. Interestingly, POLG mutation-induced mtDNA defects are tissue-specific. For example, the mtDNA defects are displayed as multiple deletions in skeletal muscles and as depletion in neurons [2]. However, how POLG mutations influence neuronal mitochondrial bioenergetic function remains uncertain. Previous studies demonstrated that POLG mutation-caused mtDNA depletion and mitochondrial respiratory complex (MRC) deficiency accumulated in neurons with age [2]. In

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addition, these mitochondrial abnormalities primarily affected dopaminergic neurons of the substantia nigra [2].

To date, neither evidence-based therapies nor clinical trials have been reported for POLG disorders, and only symptomatic therapy has been applied [3]. Thus, an effective therapy for POLG disease is urgently needed [3]. Mitochondrial transplantation (MT), a therapy that replaces the compromised mitochondria with exogenous healthy mitochondria, has been reported to effectively improve mitochondrial disorders in clinical trials [3-7]. It would be interesting to elucidate whether MT rescues POLG mutation-induced mitochondrial defects.

A POLG point mutation p.A962T (c.2884G>A) has been observed in an individual affected with ataxia, muscular weakness and central hypoventilation [8]. This sequence change replaces alanine with threonine at codon 962 of the POLG protein. This variant is present in population databases (rs760305377, ExAC 0.03 %). Algorithms developed to predict the effect of missense changes on protein structure and function (SIFT, PolyPhen-2, Align-GVGD) all suggest that this variant is likely to be disruptive, but these predictions have not been confirmed by published functional studies. Using differentiated SH-SY5Y cell line, a human neuronal model cell line that can be converted to mature dopaminergic phenotypes [9,10], the current study elucidated how the novel POLG variant p.A962T influenced mitochondrial function, and whether MT rescued these POLG variants-induced mitochondrial defects.

Methods

Site-directed mutagenesis and cell transfection

Full-length POLG cDNA was subcloned into the lentiviral vector, pLV[Exp]-EGFP:T2A (VectorBuilder, Shenandoah, TX, USA). A site-directed mutant was generated using QuickMutation™ Site-Directed Mutagenesis Kit (Beyond, Shanghai, China). Primer for site-directed mutagenesis was forward 5′-CAACTTTGTACAAAAAAGCAGGCTGCCACCATGAGCCGCCTG CTCTGGAG-3′; reverse 5′-GCATTAGTAAGCGCTCAGTAAAGGGCTGCCCAGCACCATAG-3′. The primers designed for WT POLG based on the Coding DNA Sequence (CDS) of the human POLG gene obtained from the Consensus Coding Sequence Database under the National Center of Biotechnology Information. Additionally, following the naming rules of the Human Genome Variation Society, the variant NM_001126131.2 (POLG):c.2884G>A (p.Ala962Thr) represents the substitution of nucleotide G with nucleotide A at position 2884 of the CDS sequence of the POLG gene based on transcript number NM_001126131. This information was used to design primers for mutant POLG.

SH-SY5Y stable cells expressing WT or mutant POLG were created using the lentiviral vectors for WT and mutant POLG by a previously-available method [11]. Briefly, using molecular cloning methods, 3 lentiviral expression vectors, pLV[Exp]-EGFP:T2A:Puro-CMV>hPOLG[NM_001126131.1]/3xFLAG for WT POLG, pLV[Exp]-EGFP:T2A:Puro-CMV>{hPOLG [NM_001126131.1]*(G2884A)}/3xFLAG for mutant POLG and pLV[Exp]-EGFP:T2A:Puro for negative control, were constructed. HEK293T cells were then transfected with the constructed lentiviral expression vectors using Lipofectamine 2000 (Thermo Fisher, Waltham, Massachusetts, USA) for lentivirus packaging. SH-SY5Y cells exhibiting robust growth were infected with virus. Stable cell lines were then selected with 8 μg/ml puromycin (Thermo Fisher, Waltham, Massachusetts, USA). The three groups of stable cell lines were then differentiated into neurons using retinoic acid (RA, 10 μM) and 12-O-Tetradecanoylphorbol-13-acetate (TPA, 80 nM), as previously-described [12].

Pep-1-mediated MT and fluorescent microscopy

Mitochondria were extracted from normal SH-SY5Y cells using Mitochondria isolation kit (MITOISO2, Sigma, Burlington, MA, USA) and conjugated with Pep-1 (Abcam, Cambridge, UK) at a ratio of 1.75:1 (Mito/Pep-1) as previously described [13]. The SH-SY5Y cells harboring mutant or wt POLG were co-incubated with the Pep-1-Mito for 48 h at 1×10^7 Pep-1-mito/5000 cells. To harvest the host cells for Western blotting, the cells were washed 3 times with PBS, dissociated by trypsinization, and spun for 5 min at 600× g. Following this, the supernatant containing the extracellular mitochondria was removed and the pellet obtained were the host cells.

To visualize the incorporated mitochondria, mitochondria extracted from the donor cells pre-labelled with Mitotracker-red (100 nm, 40 min, Beyotime, Shanghai, China) were conjugated with Pep-1 and then added to the POLG-mutant cells grown in a 15-mm glass-bottom dish. Following 48-h co-culture, the recipient cells were observed under a fluorescent microscope (AXIOVERT, Carl Zeiss, Germany) equipped with MShot image software.

Real-time qPCR

Cells were collected, and intracellular DNA was isolated using a Genomic DNA Kit (Tiangen Biotech, Beijing, China), following the provided guidelines from the manufacturer. Analysis of mtDNA content was then evaluated using SYBR GREEN qPCR Super Mix (Thermo Fisher, Waltham, Massachusetts, USA) following the manufacturer's guidelines using ABI PRISM® 7500 Sequence Detection System (Thermo Fisher Scientific, UK). Each PCR reaction (20 µl total volume) contained 5 µl DNA, 1 µl of each primer (forward and reverse, totaling 0.5 µl each), 10 µl of 2× SYBR Green qPCR SuperMix, and 4 µl of water, with PCR conditions including initial denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 15 s, annealing at 60 °C for 32 s, and extension with plate reading for 40 cycles. The mtDNA primer designed for mitochondrial D-loop was forward 5′-CAAACCTAC-GCCAAAATCCA-3′ and reverse 5′-GAAATGAAT-GAGCCTACAGA-3′. Primer for human nuclear β-actin was forward 5′-CATGTACGTTGCTATCCAGGC-3′ and reverse 5′-CTCCTTAATGTCACGCACGAT-3′, as previously-described [15]. The $2^{-\Delta\Delta Ct}$ (mtDNA to β-actin) represents the relative mtDNA contents.

Mitochondrial functional analyses

Western blotting

Western blotting was conducted according to our published protocol [16-17] on whole-cell, cytoplasmic and mitochondrial proteins with 1:1000 specific antibodies against cytochrome c oxidase complex subunits I-IV (COXI-IV), Cyto C, porin, β-actin (Abcam, Cambridge, UK) and NDUFV1 (Proteintech, Beijing, China), followed by corresponding peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK). The protein bands were quantified by Amersham Imager 600 (Cytiva, Marlborough, MA, China).

Colorimetric assay

Activities of mitochondrial complex I, II and IV were assessed using colorimetric assay kits (BC0515, Solarbio, Beijing, China; A089-2-1 and A089-4-1, Nanjing Jiancheng, Nanjing, China), and standardized to corresponding mitochondrial protein concentration.

Fluorescence assay

Mitochondrial membrane potential (ΔΨm) Assay Kit II (Cell Signaling, Danvers, Massachusetts) and mitochondrial ROS detection kit (Caymen Chem., Ann Arbor, MI, USA) were applied to evaluate ΔΨm and mitochondrial ROS, respectively. ΔΨm and ROS were normalized to corresponding mitochondrial protein concentration.

Luminescent assay

Mitochondrial ATP was measured using a Luminescent detection kit (S0026, Beyotime, Shanghai, China), and standardized to corresponding mitochondrial protein concentration.

Statistical analysis

The data (Mean ± SD) were analyzed using SPSS 21.0 statistical software. Prior to analysis, normal distribution was assessed using the Kolmogorov-Smirnov test, and variance homogeneity was examined using Levene's test for Equal Variances. Subsequently, the data were subjected to either Student's *t*-test or One-way ANOVA followed by Tukey's *post hoc* tests.

Results

SH-SY5Y cells carrying POLG p.A962T mutation exhibit mitochondrial malfunction and mtDNA depletion

In this study, mitochondrial function was assessed in SH-SY5Y cells carrying POLG p.A962T mutation. We observed that POLG p.A962T mutation led to mitochondrial malfunction, including ΔΨm depolarization (Fig. 1A) and mitochondrial ATP reduction (Fig. 1C). The MRC is also known as an important site of reactive oxygen species (ROS) production. We, therefore, evaluated the levels of specific mitochondrial ROS, and observed that the novel POLG variant p.A962T induce statistically significant increase in mitochondrial ROS (Fig. 1B). In addition, this POLG variant did not trigger the release of mitochondrial cyto C (Fig. 1D), an indicator of cell apoptosis.

Next, we investigated mtDNA content in POLG-mutant cells and found a decreased mtDNA in POLG-mutant cells, compared to control cells (Fig. 1E). Taken together, these findings reveal that POLG p.A962T mutation has a detrimental impact on mitochondrial function and mtDNA content.

POLG-mutant cells display a loss of mtDNA-encoded subunits of complex I and IV that compromises their activities

The MRC complexes I and IV have been previously reported to be reduced in frontal and cerebellar neurons of patients with POLG diseases [18]. We,

therefore, assessed the levels of complex I subunit NDUFV1 and complex IV (COX) subunits I, II, III, and IV in POLG-mutant cells. Western blotting analysis showed a clear loss of NDUFV1, COXI, II, and III in POLG-mutant cells (Fig. 2A). In contrast, nuclear DNA (nDNA)-encoded COXIV was similar in POLG-mutant and control cells (Fig. 2A). To further verify if the loss of mtDNA-encoded subunits of complex I and IV affected their activities, their activities were also evaluated in POLG-mutant cells. As expected, POLG p.A962T mutation impaired the activities of complex I and IV (Fig. 2B). In contrast, POLG p.A962T mutation did not show any impact on the activity of the total nDNA-encoded complex II (Fig. 2B).

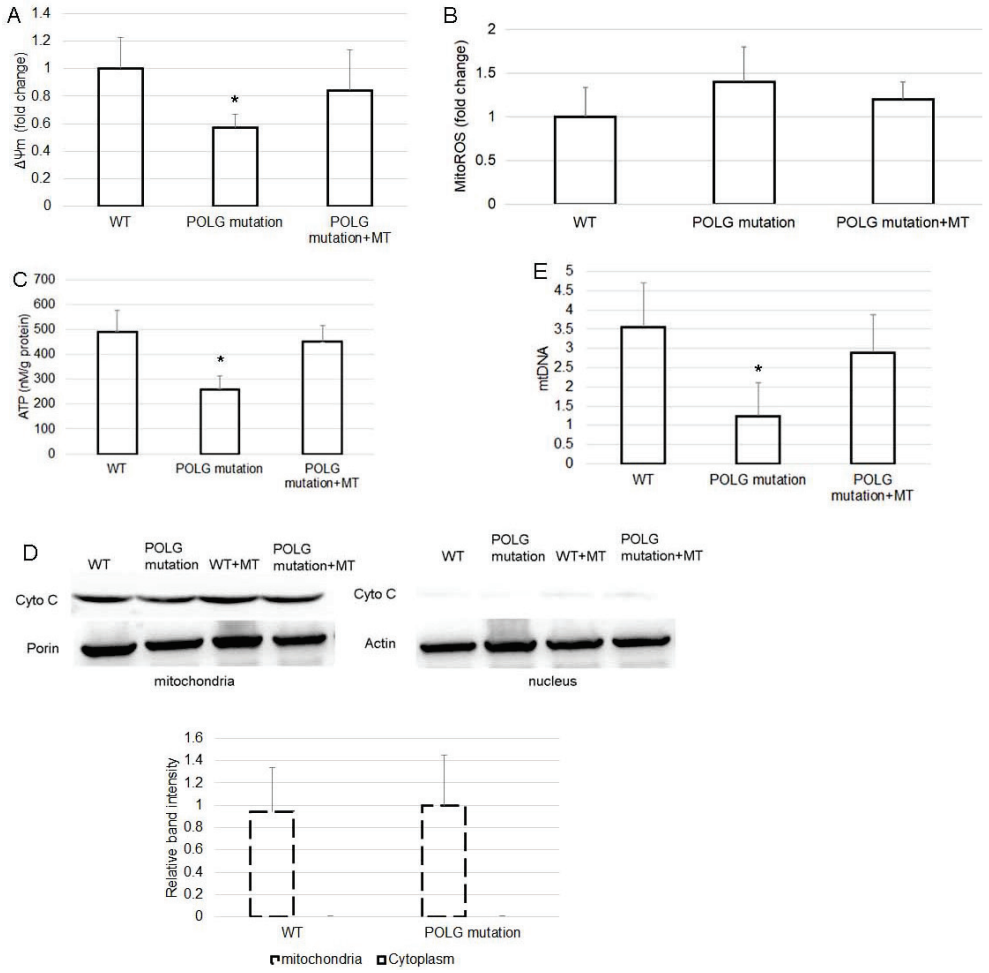

Fig. 1. SH-SY5Y cells carrying POLG p.A962T mutation manifest mitochondrial dysfunction and mtDNA depletion, which is rescued by Pep-1-mediated MT. (**A-B**) Mitochondrial ΔΨm and ROS of wt, POLG-mutant, and MT-treated POLG-mutant cells were assessed by fluorescence assay as specified in the Method. n=5. * *P*<0.05 vs. control. (**C**) Mitochondrial ATP of wt, POLG-mutant, and MT-treated POLG-mutant cells was determined by luminescent Assay as described in the Method. n=5. * *P*<0.05 vs. control. (**D**) Mitochondrial Cyto C leakage of wt and POLG-mutant, MT-treated wt, and MT-treated POLG-mutant cells was analyzed by Western blotting. The protein bands were quantified using the Amersham Imager 600. n=4. * *P*<0.05 vs. control. (**E**) mtDNA content of wt, POLG-mutant, and MT-treated POLG-mutant cells was evaluated by Real-time RT-PCR. n=5. * *P*<0.05 vs. control.

Our data indicate that the loss of MRC complexes I and IV is a primary mechanism responsible for POLG mutation-induced mitochondrial malfunction.

Pep-1-mediated MT restores mitochondrial function and mtDNA content in POLG-mutant cells

MT has been reported to effectively improve mitochondrial disorders in preclinical researches [4-7]. The current study elucidated whether Pep-1-mediated MT rescued this novel POLG variant-induced mitochondrial malfunction. To confirm the internalization of exogenous mitochondria into POLG-mutant cells, POLG-mutant cells were co-cultured with the exogenous mitochondria labeled with Mitotracker-red. Following Pep-1-mediated MT, the red fluorescence of these exogenous mitochondria was detected within the cells (Fig. 3A).

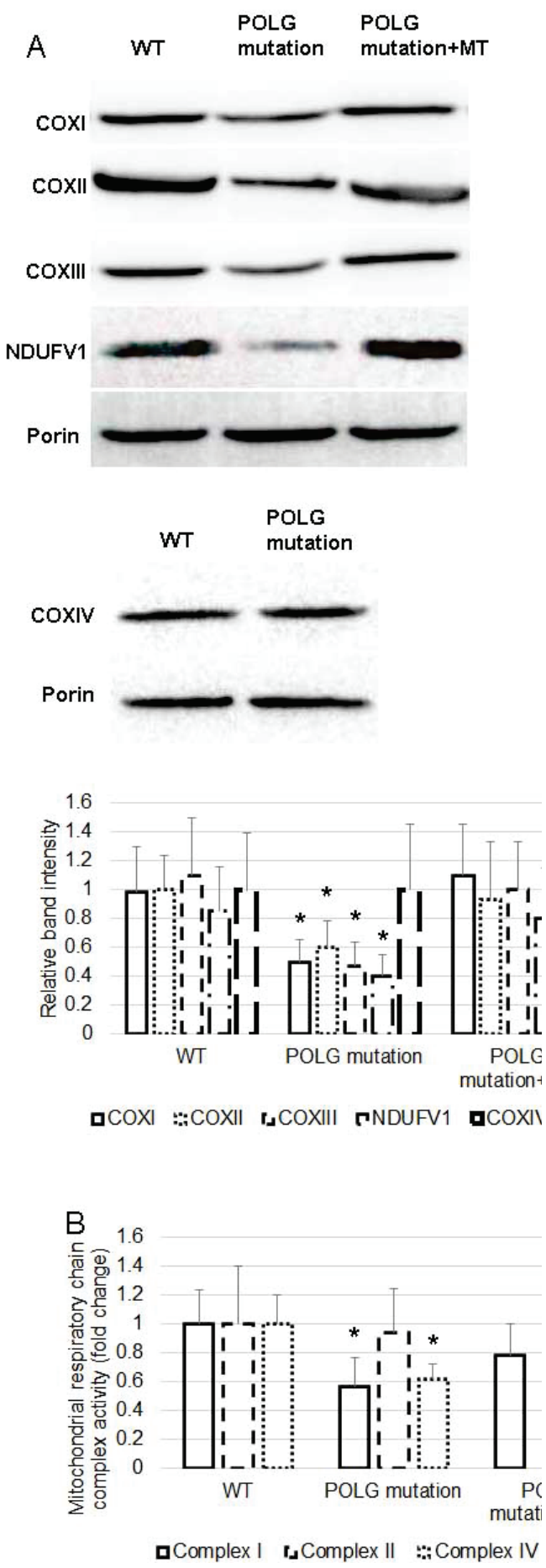

Fig. 2. POLG-mutant cells exhibit a loss of mtDNA-encoded complex I and IV subunits and compromised their activities, which is rescued by Pep-1-mediated MT. (**A**) Mitochondrial proteins were extracted from wt, POLG-mutant, and MT-treated POLG-mutant cells using the Mitochondria isolation kit. The expression of COX I, II, III, IV, and NDUFV1 in mitochondria was then analyzed by Western blotting. The protein bands were quantified using the Amersham Imager 600. n=5. * P<0.05 vs. control. (**B**) Activities of mitochondrial complex I, II, and IV were assessed in wt, POLG-mutant, and MT-treated POLG-mutant cells by colorimetric Assay as specified in the Method. n=5. * P<0.05 vs. control.

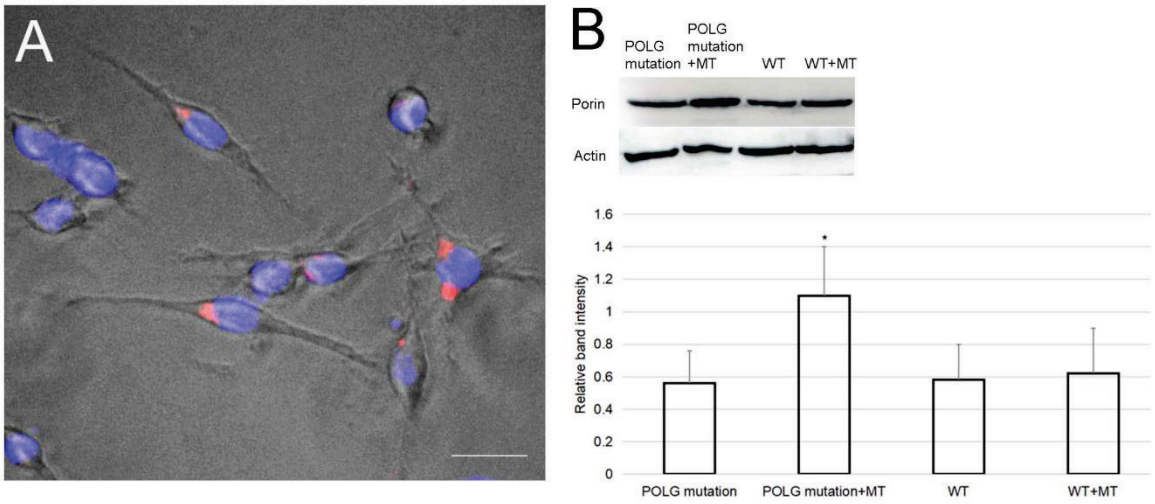

Fig. 3. Internalization of Pep-1-Mito by POLG-mutant SH-SY5Y cells. (**A**) Following MT, internalization of Pep-1-Mito by POLG-mutant cells was observed by fluorescent microscopy. Scale: 20 μm. Red (Mitotracker Red) and blue (DAPI) represent exogenous mitochondria and nuclei, respectively. (**B**) Following MT, porin levels were evaluated in the recipient cells by Western blotting. The protein bands were quantified using the Amersham Imager 600. n=5. * P<0.05 vs. control.

In addition, Pep-1-mediated MT induced an increased porin in the host cells (Fig. 3B), suggesting an increased mitochondrial density.

We then determined whether Pep-1-mediated MT afforded neuroprotection against POLG mutation-induced mitochondrial defect. The results showed that Pep-1-mediated MT restored ΔΨm (Fig. 1A), mitochondrial ATP levels (Fig. 1C), mtDNA content (Fig. 1E), and the activities of complex II and IV of POLG mutant cells, indicating the neuroprotective effect of this method.

Discussion

Mutations in POLG have been identified as the leading single-gene cause for inherited mitochondrial diseases in children and adults [2,4]. Neuronal POLG mutations are displayed as variable neurological manifestations, such as Parkinsonism [8]. Mechanistically, POLG mutations cause mtDNA defects in neurons, which are commonly presented as mtDNA depletion [2,4]. In addition, POLG mutation-triggered mtDNA depletion primarily affected dopamineergic neurons of the substantia nigra, and are associated with neurodegenerative disorders [2,4]. A novel POLG variant p.A962T in compound heterozygous with R964C has been reported previously in 2011 by Tang *et al.* [8] in US in a patient who displayed ataxia, muscular weakness, and central hypoventilation. But whether this novel variant has an impact on mtDNA and mitochondrial function has not been reported [8]. In the present study, mitochondrial defects including ΔΨm decrease, MRC complex deficiency, ATP reduction, and mtDNA depletion were observed in differentiated SH-SY5Y cells harboring POLG p.A962T mutation. Therefore, the POLG variant p.A962T might be a pathogenic mutation leading to mitochondrial malfunction and mtDNA depletion in neurons.

Loss of MRC complexes I and IV may contribute to the neurodegenerative process [19-22]. For example, the deficiencies of complex I and IV were reported in substantia nigra of patients with neurodegenerative disorders [19-20]. In POLG-related diseases, defects in complex I and IV have been demonstrated in various tissues [21-22]. In the current study, we have confirmed this finding by showing that POLG p.A962T mutation diminished mtDNA-encoded subunits of complex I and IV in neurons. Since POLG mutations resulted in mtDNA decrease, the reduction of complex I and IV subunits was probably a consequence of mtDNA depletion. Interestingly, complex I assembly depends on complex IV and is compromised by complex IV deficiency [23], which indicated that complex IV loss is a primary mechanism responsible for POLG mutation-induced mitochondrial malfunction. Complex IV is known to oxidize cytochrome c and to convert oxygen molecule to water. This process promotes the increase of proton electrochemical potential which drives the ATP synthase to generate ATP. In this study, POLG p.A962T mutation-induced complex IV subunit deficiency impaired its

activity, which might partially be responsible for ΔΨm depolarization and ATP reduction.

To date, no evidence-based therapy has been reported for POLG disorders, and only symptomatic treatments have been applied clinically [3]. Thus, the therapy for POLG diseases is urgently required. Intercellular transfer of mitochondria has been reported both *in vivo* and *in vitro* as a pro-survival phenomenon in response to external stress, since the incorporation of exogenous functional mitochondria can provide the host cells with mtDNA and protective mitochondrial proteins [13,24]. Therefore, transplantation of normal mitochondria into the cells with mitochondrial dysfunction may a potential therapeutic approach in mitigating mitochondrial diseases, and early clinical feasibility of this therapy has suggested the therapeutic effectiveness of this method on mitochondrial diseases [4-7]. Mitochondria have been reported to be taken up by cells via actin-dependent endocytosis [25], but the uptake ratio is low. Pep-1, a cell-penetrating amphiphilic peptide can insert into the membrane bilayer to facilitate membrane destabilization and mitochondrial internalization [13]. Pep-1-mediated MT has been proved to improve mitochondrial functions in cell models of inherited mitochondrial diseases [13,26]. In addition, injections of Pep-1-Mito into the medial forebrain bundle of a rat model of Parkinson's disease improved motor performance and maintained MRC levels in the substantia nigra [26]. In this study, the protective effect of Pep-1-mediated MT against POLG mutation-induced mitochondrial defect was observed *in vitro*, suggesting that this method may be applied for the treatment of POLG disorders. Despite that, future studies should be aimed at evaluating the potential efficacy of this therapy in animal models of POLG disorders.

In summary, using differentiated SH-SY5Y cell line, an *in vitro* model of human neuronal cells, the current study showed that the novel POLG variant p.A962T induced mitochondrial defects, including mtDNA depletion, ΔΨm depolarization, and ATP reduction. Mechanistically, POLG mutation-induced mtDNA depletion led to the loss of complex I and IV subunits, and thus compromised their activities. Therefore, POLG p.A962T mutation might be a pathogenic mutation leading to mitochondrial defects in neurons. Pep-1-mediated MT recued POLG p.A962T mutation-induced mitochondrial defects, suggesting the therapeutic application of this method in POLG diseases.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81960264) and the CDM funding of Western University of Health Sciences. Thanks to Dr. Guihua Li from the Neurology Department at Guangdong Second Provincial General Hospital for her guidance on designing the mutant and wild-type POLG primers.

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