Experimental Cell Research 363 (2018) 299–309

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Bioactivity and gene expression profiles of hiPSC-generated retinal ganglion cells in *MT-ND4* mutated Leber's hereditary optic neuropathy

You-Ren Wu[a], An-Guor Wang[b,c], Yan-Ting Chen[d,e,f], Aliaksandr A. Yarmishyn[g], Waradee Buddhakosai[g], Tien-Chun Yang[g], De-Kuang Hwang[b,c], Yi-Ping Yang[c,h], Chia-Ning Shen[i], Hsin-Chen Lee[a], Shih-Hwa Chiou[a,g,i], Chi-Hsien Peng[j,*], Shih-Jen Chen[b,c,**]

[a] Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan
[b] Department of Ophthalmology, Taipei Veterans General Hospital, Taipei, Taiwan
[c] School of Medicine, National Yang-Ming University, Taipei, Taiwan
[d] Department of Ophthalmology, Changhua Christian Hospital, Changhua, Taiwan
[e] Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan
[f] Department of Optometry, Central Taiwan University of Science and Technology, Taichung, Taiwan
[g] Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan
[h] Department of Neurosurgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[i] Genomic Research Center, Academia Sinica, Taipei, Taiwan
[j] Department of Ophthalmology, Shin Kong Wu Ho-Su Memorial Hospital and Fu-Jen Catholic University, Taipei, Taiwan

ARTICLE INFO

Keywords:
Leber's hereditary optic neuropathy
Induced pluripotent stem cells
Mitochondrial DNA
Retinal ganglion cells
MT-ND4
Microarray

ABSTRACT

Leber's hereditary optic neuropathy (LHON) is the maternally inherited mitochondrial disease caused by homoplasmic mutations in mitochondrial electron transport chain Complex I subunit genes. The mechanism of its incomplete penetrance is still largely unclear. In this study, we created the patient-specific human induced pluripotent stem cells (hiPSCs) from *MT-ND4* mutated LHON-affected patient, asymptomatic mutation carrier and healthy control, and differentiated them into retinal ganglion cells (RGCs). We found the defective neurite outgrowth in affected RGCs, but not in the carrier RGCs which had significant expression of *SNCG* gene. We observed enhanced mitochondrial biogenesis in affected and carrier derived RGCs. Surprisingly, we observed increased NADH dehydrogenase enzymatic activity of Complex I in hiPSC-derived RGCs of asymptomatic carrier, but not of the affected patient. LHON mutation substantially decreased basal respiration in both affected and unaffected carrier hiPSCs, and had the same effect on spare respiratory capacity, which ensures normal function of mitochondria in conditions of increased energy demand or environmental stress. The expression of anti-oxidant enzyme catalase was decreased in affected and carrier patient hiPSC-derived RGCs as compared to the healthy control, which might indicate to higher oxidative stress-enriched environment in the LHON-specific RGCs. Microarray profiling demonstrated enhanced expression of cell cycle machinery and downregulation of neuronal specific genes.

1. Introduction

Leber's hereditary optic neuropathy (LHON) is an important maternally inherited optic neuropathy associated with mitochondrial dysfunction. The clinical signs of LHON are characterized by subacute central scotoma, painless and progressive visual disturbance in young adults. This visual problem affects both eyes simultaneously or sequentially with short interval. The main lesions at acute phase include edema of nerve fiber layer, optic disc hyperemia, telangiectasia and tortuosity of retinal vasculature [1]. The swelling of nerve fiber layer is followed by irreversible atrophy of optic nerve and permanent visual loss. Histopathology of LHON patients demonstrates severe loss of RGCs in temporal and central fibers and relatively moderate loss in nasal peripheral fibers and large axons [2–4]. The degeneration of axons persists long after the onset of LHON [3].

Three most common pathogenic mutations recognized as a cause of LHON were identified in three genes, encoding subunits of NADH dehydrogenase (mitochondrial Complex I): *MT-ND4* (m.11778G > A) [5], *MT-ND1* (m.3460G > A) [6,7] and *MT-ND6* (m.14484T > C) [8]. The mutations cause Complex I dysfunction, which ultimately leads to

* Corresponding author.
** Corresponding author at: Department of Ophthalmology, Taipei Veterans General Hospital, Taipei, Taiwan.
E-mail addresses: chpeng1008@gmail.com (C.-H. Peng), sjchen@vghtpe.gov.tw (S.-J. Chen).

https://doi.org/10.1016/j.yexcr.2018.01.020

apoptosis of retinal ganglion cells (RGCs), associated with cytochrome c release [9], Fas-induced apoptosis [10] or caspase-independent apoptosis driven by energetic failure [11]. Unlike other mitochondrial disorders, whose phenotypes are often correlated with different degree of heteroplasmy, LHON pathogenic mutations are usually homoplasmic [3]. Although the causative mutations are documented, little is known about the actual factors that potentiate LHON special clinical features, such as incomplete penetrance, gender bias towards males and specificity in affecting RGCs, but not other cell types [12]. This implies that the mitochondrial DNA (mtDNA) mutations are necessary, but not sufficient to cause LHON [13] and there might be a special mechanism underlying the pathogenesis of LHON.

Several factors are thought to be related to the phenotypic expression of homoplasmic mitochondrial diseases: environmental factors, mtDNA haplotype, nuclear DNA background, and tissue-specific expression of interacting genes [14,15]. The expression of mammalian nuclear genes may regulate the mitochondrial function and activity through some transcription factors [16]. For example, transcription factors can indirectly regulate the nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which would alter the expression of nuclear-encoded mitochondrial respiratory chain components [17]. Some transcription factors can even be imported into mitochondria and directly regulate the expression of mitochondrial genes [16]. The regulation of these transcription factors could be different in each individual and in each specific organ or tissue, so they could be the key factors related to LHON penetrance and its characteristic tissue-specific expression.

The mitochondrial biogenesis could be another key factor in the pathogenesis of LHON. The elevation of mitochondrial biogenesis is a common response to the stimulation, such as cellular stress or damage. For example, mtDNA copy number was elevated in people with higher oxidative stress [18], while the decrease of mtDNA copy number was found to be associated with poorer long-term outcome in cardiac dysfunction [19]. This compensatory process might be a factor related to low penetrance of LHON [20]. Several studies revealed that subjects carrying the LHON-related mutation showed higher mitochondrial content and DNA copy number than normal control [21], and that the copy number was higher in unaffected carriers than affected subjects [20,22,23].

The discovery of the factors that regulate pathogenesis of LHON can be facilitated by disease-specific in vitro cell model. Previously, cybrid cell models carrying *MT-ND4* mutation with decreased cell growth rate and oxygen consumption were utilized for LHON research [24]. However, these cybrid cells are not neuronal cells, such as RGCs, which are selectively impaired in LHON patients [24–27] and, therefore, do not represent the actual disease environment. Another method, adeno-associated virus (AAV) transfection, was introduced recently to transport the mutated mtDNA into the cells [28–30]. Using this technique, RGCs carrying mutated mitochondria could be created for LHON study. However, these AAV-based studies cannot generate a homoplasmic LHON mitochondrial mutation conditions due to limited efficiency of transfection.

Induced pluripotent stem cell (iPSC) technology is another promising method for generating disease-specific cell models. In a recent study, LHON patient-specific iPSCs, containing double mutation in the genes *MT-ND1* (m.4160T > C) and *MT-ND6* (m.14484T > C) were utilized to model LHON [31]. In this study, we generated the iPSC-derived RGCs harboring the most frequently occurring mutation in *MT-ND4* (m.11778G > A) from LHON-affected patient and unaffected mutation carrier by using specific three-dimensional culture conditions [32]. We then comparatively observed their different pathogenesis, biogenesis and gene profiles in order to elucidate the underlying factors that trigger the pathogenic phenotype of this homoplasmic mutation. Our report aims to provide the optimized model for further pathogenetic research and therapeutic development of LHON.

2. Materials and methods

2.1. Generation of human induced pluripotent stem cells (hiPSCs)

Sendai reprogramming vectors (SeV) reprogramming was performed using the CytoTuneTM-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) following the manufacturer's protocol. Briefly, 5×10^5 of peripheral blood mononuclear cells (PBMCs) were plated to the middle section of a 24-well plate in complete PBMC medium and were transduced with a mix of four SeV vectors carrying OCT3/4, SOX2, KLF4 and c-MYC at a multiplicity of infection (MOI) of 3. The medium was changed every other day and on the day 7 post transduction, 1.25×10^5 cells were plated onto a 10-cm dish previously coated with mouse embryonic fibroblasts (MEFs) feeder layer. The day after, the medium was switched to hES medium and the cells were fed every other day for a week before switching to the daily feeding. Once the colonies emerged, they were picked by mechanical dissection and transferred to a fresh feeder.

2.2. Differentiation of hiPSCs into RGCs

The procedure to induce early stages of retinal differentiation was based on a previously described protocol with several modifications [33–35]. Briefly, on day 0 of differentiation, hiPSCs were enzymatically detached by dispase treatment, dissociated into small clumps and cultured in suspension with mTeSR1 medium (STEMCELL Technologies) to induce embryoid bodies (EBs) formation. The EBs were gradually transitioned into neural induction medium (NIM) containing DMEM/F12 (1:1), 1% N2 supplement (Invitrogen), non-essential amino acids (NEAAs), 2 mg/ml heparin (Sigma), by replacing the medium with a 3:1 ratio of mTeSR1/NIM on day 0, 1:1 on day 2, 1:3 on day 4 and 100% NIM on day 6. On day 7, aggregates were seeded onto 6-well plates containing NIM at approximate density of 20 aggregates per cm2 and neural rosettes appeared (NRs) after several days. The NRs were mechanically lifted and switched to retinal differentiation medium (RDM) containing DMEM/F12 (3:1) supplemented with 2% B27 (without vitamin A, Invitrogen), NEAA and antibiotic on D16. Thereafter, the medium was changed every 2–3 days to allow the formation of neurospheres. The neurospheres were then transferred to poly-D-lysine/laminin-coated plates. The medium and fresh DAPT were renewed every other day to promote RGC axons development.

2.3. Quantification of m.G11778A mutation content by pyrosequencing

Pyrosequencing was performed by Mission Biotech Corporation (Taipei) according to the protocol by White et al. [36]. In brief, the biotin-labeled PCR products were captured by Streptavidin-Sepharose HP (Amersham Pharmacia). The PCR products bound on the beads were purified and made single stranded using the Pyrosequencing Vacuum Prep Tool. The sequencing primers were annealed to the single-stranded PCR product and pyrosequencing was performed using the PyroMark Q24 system (Qiagen). Quantitation of cytosine methylation was done using the PyroMark Q24 software. The following primers were used: PCR amplification primer pair ATCGCCCACGGGCTTACAT, biotin-GGAGAACGTGGTTACTAGCACAGA and sequencing primer CGAACGCACTCACAG.

2.4. Alkaline phosphatase staining and immunofluorescence staining

Alkaline phosphatase (AP) staining was performed using the Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories) following the manufacturer's instructions. Immunofluorescence staining was performed with the indicated antibodies. Briefly, cells were sub-cultured in 4-well chamber-slides (Millipore). The cells were fixed in 1% (w/v) paraformaldehyde for 10 min at room temperature and washed thrice with phosphate-buffered saline (PBS). An additional permeabilization