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ORIGINAL ARTICLE

# Dysregulation of the circRNA_0087207/miR-548c-3p/PLSR1-TGFB2 axis in Leber hereditary optic neuropathy in vitro

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## Abstract

**Background:** Leber hereditary optic neuropathy (LHON) is mainly the degeneration of retinal ganglion cells (RGCs) associated with high apoptosis and reactive oxygen species (ROS) levels, which is accepted to be caused by the mutations in the subunits of complex I of the mitochondrial electron transport chain. The treatment is still infant while efforts of correcting genes or using antioxidants do not bring good and consistent results. Unaffected carrier carries LHON mutation but shows normal phenotype, suggesting that the disease's pathogenesis is complex, in which secondary factors exist and cooperate with the primary complex I dysfunction.

**Methods:** Using LHON patient-specific induced pluripotent stem cells (iPSCs) as the in vitro disease model, we previously demonstrated that circRNA_0087207 had the most significantly higher expression level in the LHON patient-iPSC-derived RGCs compared with the unaffected carrier-iPSC-derived RGCs. To elaborate the underlying pathologies regulated by circRNA_008720 mechanistically, bioinformatics analysis was conducted and elucidated that circRNA_0087207 could act as a sponge of miR-548c-3p and modulate PLSCR1/TGFB2 levels in ND4 mutation-carrying LHON patient-iPSC-derived RGCs.

**Results:** Using LHON iPSC-derived RGCs as the disease-based platform, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on targeted mRNA of miR-548c-3p showed the connection with apoptosis, suggesting downregulation of miR548c-3p contributes to the apoptosis of LHON patient RGCs.

**Conclusion:** We showed that the downregulation of miR548c-3p plays a critical role in modulating cellular dysfunction and the apoptotic program of RGCs in LHON.

**Keywords:** Apoptosis; Electron transport; Mutations; Reactive oxygen species; Retinal ganglion cells

## 1. INTRODUCTION

Leber hereditary optic neuropathy (LHON) is the first to be attributed to a maternally inherited disease named after Dr. Theodore Leber who depicted this disease in 15 patients in four families.[1] As one of the most common inherited optic neuropathies, the prevalence of LHON varies from 1:50 000 to 1:25 000 in Europe, primarily affecting males (80%-90% cases).[2] LHON caused by a point mutation in complex I of the mitochondrial electron transport chain leads to selective degeneration and death of retina ganglion cells (RGCs), which transfer visual signals from the eyes to the brain, resulting in vision loss.[3] According to statistics, three well-known mutations (m.3460G > A in the MT-ND1 gene, m.11778G > A in the MT-ND4 gene, and m.14484T > C in the MT-ND6 gene) are account for more than 95% of cases of LHON, especially m.11778G > A with 70% of all cases.[4] The symptoms of LHON usually present as painless, subacute, central visual loss in one eye, and 6 to 8 weeks later, the second eye is involved.[5] Another noticeable side, the symptoms mostly started between 20 and 30 years old although there has been reported LHON onset from 2 to 87 years.[6] In clinical, LHON patients can have optic disc hyperemia, peripapillary telangiectatic blood vessels, vascular tortuosity, and swelling of the retinal nerve fiber layer around the optic disc in the acute stage. Visual evoked potentials (VEPs) and electroretinograms (ERGs) are often abnormal in these people.

Circular RNAs (circRNAs) are a subclass of non-coding RNA whose polynucleotides form a circular shape. CircRNA was first found at the beginning of the 1970s in viruses[7] and then in mammals.[8] With the development of RNA sequencing, more and more circRNAs were detected in humans but for a long time, it was just considered an irregular product of RNA splicing.

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Now, more than 100 000 circRNAs have been discovered in humans[9] and a subset of them was demonstrated to have a role in many biological processes. Besides being implicated in diabetes mellitus, neurological disorders, cardiovascular diseases, chronic inflammatory diseases, and cancer,[10] the functions and mechanisms of circRNA actions in neural development have been indicated.[11] In the previous study, we demonstrated that the circRNA_0087207 was significantly upregulated in patient RGC compared to normal and carrier. This circRNA_0087207 may participate in the distinguishment of disease manifestations between LHON patients and unaffected carriers. However, the downstream regulation of microRNA (miRNA) targets and responsible functions has not been fully uncovered. In this study, using next-generation sequencing (NGS) technology, we further investigate the potential pathogenic-molecular mechanisms and miRNA-targeted pathways in LHON patient-specific induced pluripotent stem cells (iPSCs) and LHON-specific iPSC differentiated into retinal ganglion cells (iPSC-RGCs) with bioinformatic transcriptome analysis.

## 2. METHODS

### 2.1. 2iPSCs culture and RGCs differentiation

iPSCs are grown by Stemflex medium (Gibco, USA) after being coated with Geltrex (Gibco) until 80% confluent then be passaged or differentiated into RGCs. RGCs differentiation follows this protocol: iPSCs, after detached (day 1) by Versene (Gibco), were cultured by neural induction medium (NIM) containing DMEM/F12 (1:1; Life Technologies, USA), 1% N2 supplement, 1% MEM non-essential amino acids solution (NEAAs), and 2 μg/mL heparin (Sigma-aldrich, Saint Louis, MO, USA) and Stemflex in next 6 days with the ratio 1:3 (days 1 to 2), 1:1 (days 3 to 4), 3:1 (days 5 to 6) and became embryonic body (EB). On day 7, EBs were seeded on a 10 cm dish containing NIM with 10% fetal bovine serum (FBS) at an approximate density of 100 to 120 EBs/dish. Every 2 days, the medium was renewed by NIM medium. On day 16, cells were cultured in retinal differentiation medium (RDM) containing DMEM/F12 (1:1, Life Technologies), 1% B27 supplement without vitamin A (Life Technologies), 1% NEAA, and 1% penicillin/streptomycin then became optic vesicle (OV). Around days 18 to 22, the nice morphology OVs were picked up by pipetting and cultured in RDM to form three-dimensional optic cup structures. On day 25, cells were verified into RGC single cells.

### 2.2. RNA isolation and real-time quantitative polymerase chain reaction

Total RNAs were isolated using the TRIzol reagent (Introvigen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of RNA was detected by a spectrophotometer at 260 nm. Reverse transcription was conducted with 5 μg of total RNA using the superscript first-strand synthesis system into cDNA. Real-time quantitative polymerase chain reaction (RT q-PCR) was conducted using q-PCR Master Mix Plus for SYBR Green (T Eurogentec, Seraing, Belgium) on QuantStudio 3 real-time PCR system. Each sample was detected in triplicate which contained 50 ng RNA. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression. All data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and shown as the fold increase compared with the control group.

### 2.3. Cell transfection

Knockdown or overexpression of circRNA_0087207 in RGCs was performed using Lipofectamine 3000 (ThermoFisher Scientific, Vilnius, Lithuania) 1 day after culture into single cells according to the manufacturer's protocols. In brief, 5 μL P3000™ Reagent, 7.5 μL Lipofectamine™ 3000 Reagent with 250 μL Opti-MEM (Gibco), and 2.5 μg of knockdown/overexpress plasmid or control plasmid were diluted in RDM medium for one of six-wells plate. The cells were harvested after 48 hours for analysis.

### 2.4. RNA library preparation and data analysis

To understand the role of circRNA, miRNA, and mRNA in LHON pathogenesis, we performed NGSon normal RGCs, carrier RGCs, and patient RGCs, respectively. Total RNA was extracted with TRIzol (Invitrogen) and followed the manufacturer's protocol to construct the library with the NEBNext Ultra RNA library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). The library product was sequenced on HiSeq2500. The thresholds $|\log_2(\text{Fold-change})| > 1$ and $p < 0.05$ were used for calculating the differential expression of miRNA and mRNA. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was utilized to analyze the biological function.

## 3. RESULTS

### 3.1. CircRNA_0087207 functions as a sponge for miR-548c-3p

We previously found that circRNA_0087207 had the most significantly higher expression level in the patient iPSC-derived RGCs compared with the unaffected carrier-iPSC-derived RGCs. Prediction software revealed that circRNA_0087207 could function as a miRNAs sponge, so we primarily focus on this pathway to dig deep into the mechanism of LHON disease. For circRNA_0087207, the circRNA-miRNA-mRNA interaction was predicted by bioinformatics prediction analysis and shown by Cytoscape software (Fig. 1A). Of those miRNAs, miR-548c-3p is the only one that has two binding sites with circRNA_0087207 (Fig. 1B). Moreover, KEGG pathway analysis on targeted mRNA of miR-548c-3p showed a connection with apoptosis, one of the representative hallmarks of LHON RGC disease phenotype (Fig. 1C). This evidence indicates that miR-548c-3p could be the most potential candidate for our study. To confirm the interaction between circRNA_0087207 and miR-548c-3p, RT q-PCR was performed on three lines of LHON RGCs, that is, circRNA_0087207-overexpressing carrier RGCs and circRNA_0087207-silencing patient iPSC-derived RGCs. The results showed that miR-548c-3p level fell off when overexpressing circRNA_0087207 in carrier iPSC-derived RGCs (Fig. 1D) and upregulated when knockdown circRNA_0087207 in patient iPSC-derived RGCs (Fig. 1E). Moreover, the miR-548c-3p levels significantly decreased in the patient iPSC-derived RGCs compared to normal and carrier iPSC-derived RGCs (Fig. 1F). Thus, there is a negative correlation between circRNA_0087207 and miR-548c-3p. Overall, our results showed that circRNA_0087207 serves as the sponge for miR-548c-3p.

### 3.2. CircRNA_0087207 regulate PLSCR1 and TGFB2 via miR-548c-3p

Based on the prediction and NGS data for mRNA in LHON-derived RGCs, PLSCR1, TGFB2, SKIL, THBS1, and STK3 are the most potential targets for miR-548c-3p (Fig. 2A). Therefore, we evaluated the expression levels of these mRNA in LHON patient iPSC-derived RGCs and also LHON iPSC-derived RGCs with either overexpression or knockdown of circRNA_0087207. The RT q-PCR results exhibited a robust upregulation of PLSCR1 and TGFB2 when circRNA_0087207 was overexpressed (Fig. 2B) and significant downregulation after circRNA_0087207 silencing (Fig. 2C). Similar outcomes were indicated in which two mRNAs increased in patient RGCs which have a high level of circRNA_0087207 and a low level of miR-548c-3p (Fig. 2D). Taken together, these results demonstrate that PLSCR1 and TGFB2 can be modulated by circRNA_0087207 through miR-548c-3p.

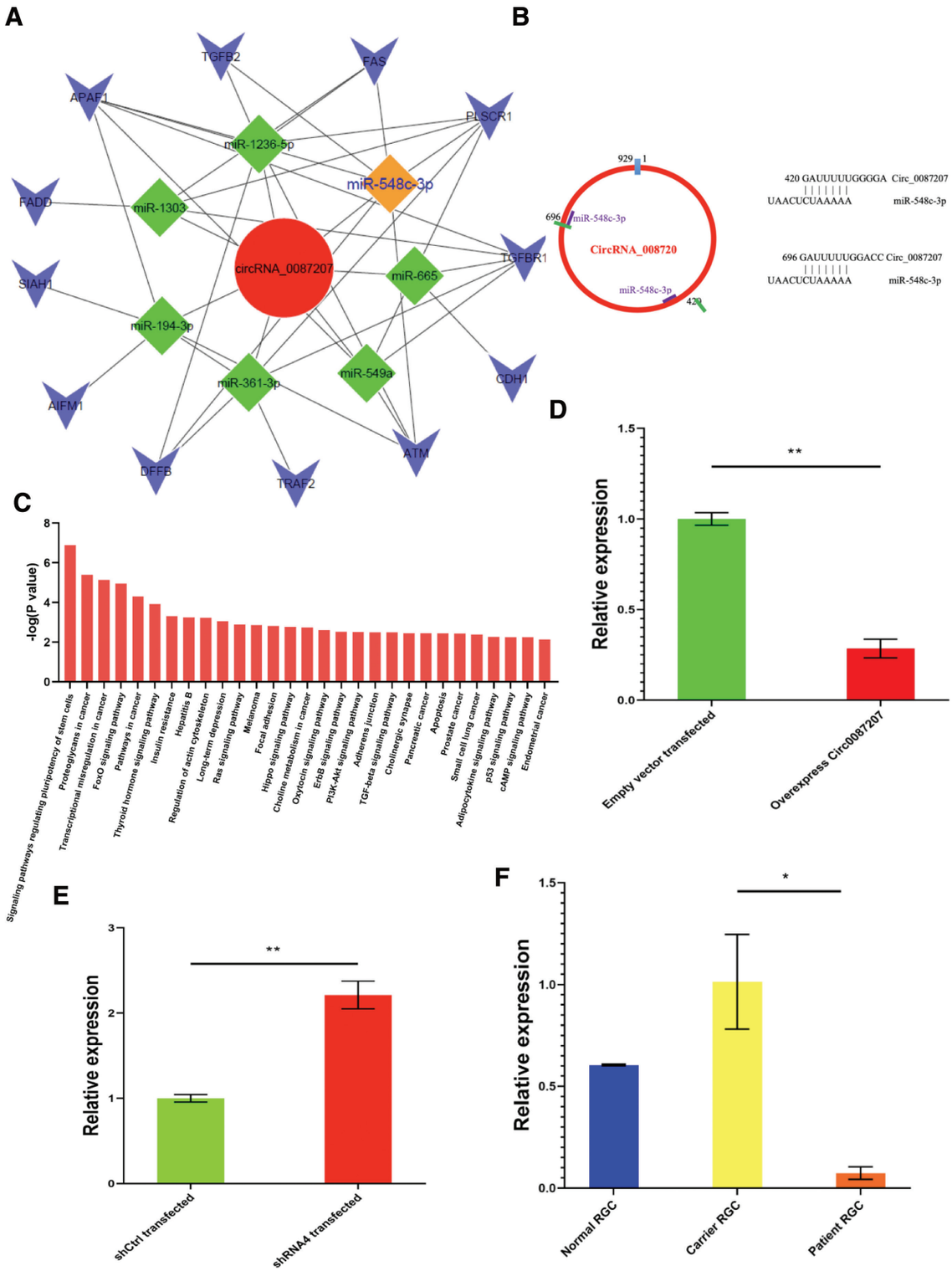


**Fig. 1** CircRNA_0087207 acts as a sponge of miR-548c-3p in LHON-derived RGCs. A, The circRNA-miRNA-mRNA interaction based on circRNA_0087207 was demonstrated by prediction and bioinformatics analysis using Cytoscape software. B, The diagram displays predicted binding sites of miR-548c-3p on circRNA_0087207. C, Top 30 biological pathways targeted by miR-548c-3p using KEGG analysis. D-E, miR-548c-3p level significantly decreased when circRNA_0087207 was overexpressed (D) and statically increased when circRNA_0087207 was silenced (E) (n = 3, $p < 0.01$). F, miR-548c-3p was significantly downregulated in patient RGCs compared to carrier RGCs (n = 3, $p < 0.05$). KEGG = Kyoto Encyclopedia of Genes and Genomes; LHON = Leber hereditary optic neuropathy; RGC = retinal ganglion cell.

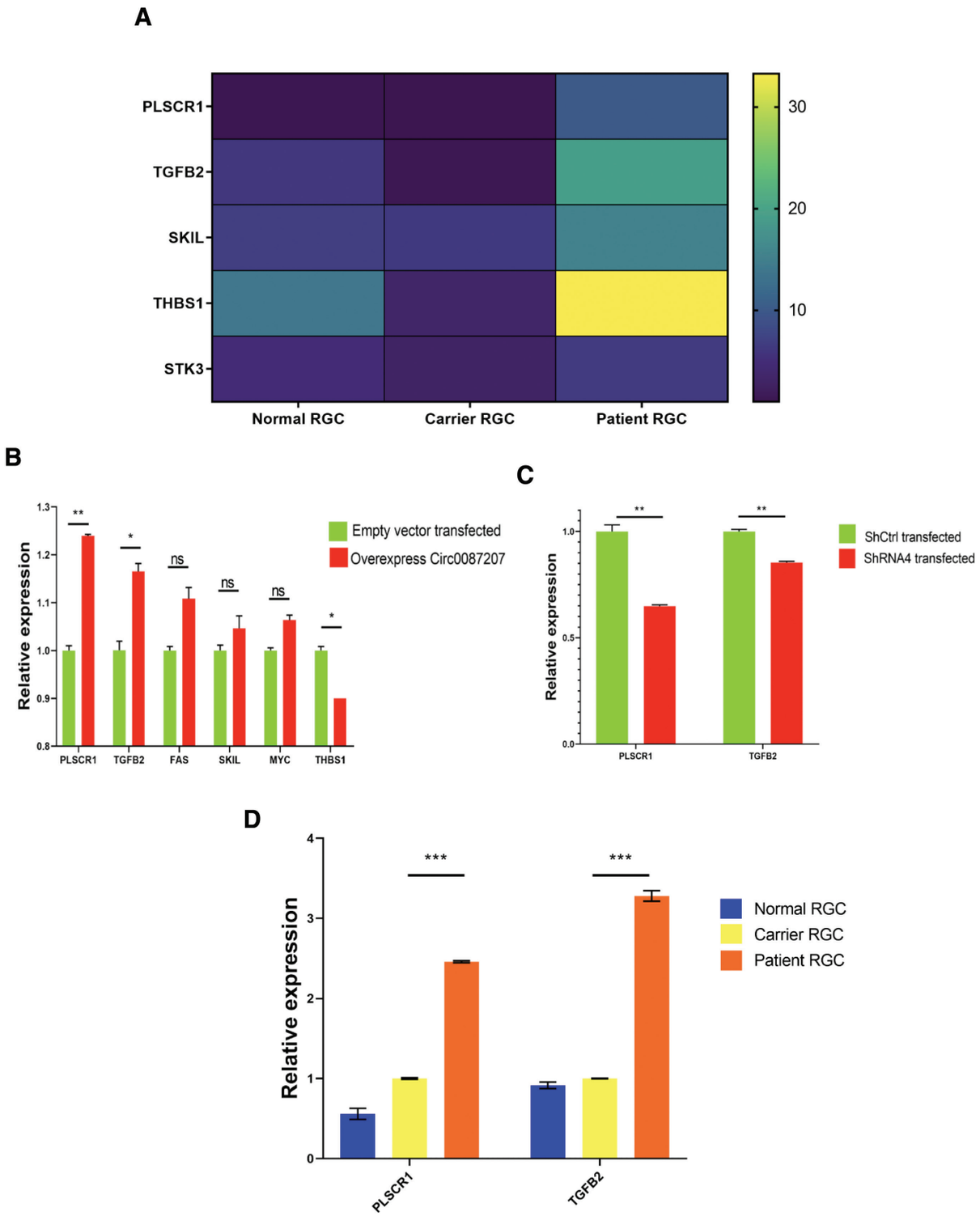


**Fig. 2** PLCSR1 and TGFB2 mRNA show a positive correlation with circRNA_0087207 and negative collaboration with miR-548c-3p in LHON-derived RGCs. A, The heatmap shows potential targeted mRNAs of miR-548c-3p level in NGS data in LHON-derived RGCs samples. B, The expression level of targeted mRNA candidates was performed by RT q-PCR in carrier RGCs after overexpressing circRNA_0087207 (*$p < 0.05$, **$p < 0.01$ vs empty vector transfected group). C, PLSCR1 and TGFB2 mRNA were confirmed after knocking down circRNA_0087207 in patient RGCs (**$p < 0.01$). D, RT q-PCR was conducted to detect the expression level of PLSCR1 and TGFB2 in three kinds of LHON-derived RGCs (***$p < 0.001$). LHON = Leber hereditary optic neuropathy; NGS = next-generation sequencing; ns = not significant; RGC = retinal ganglion cell; RT q-PCR = real-time quantitative polymerase chain reaction.

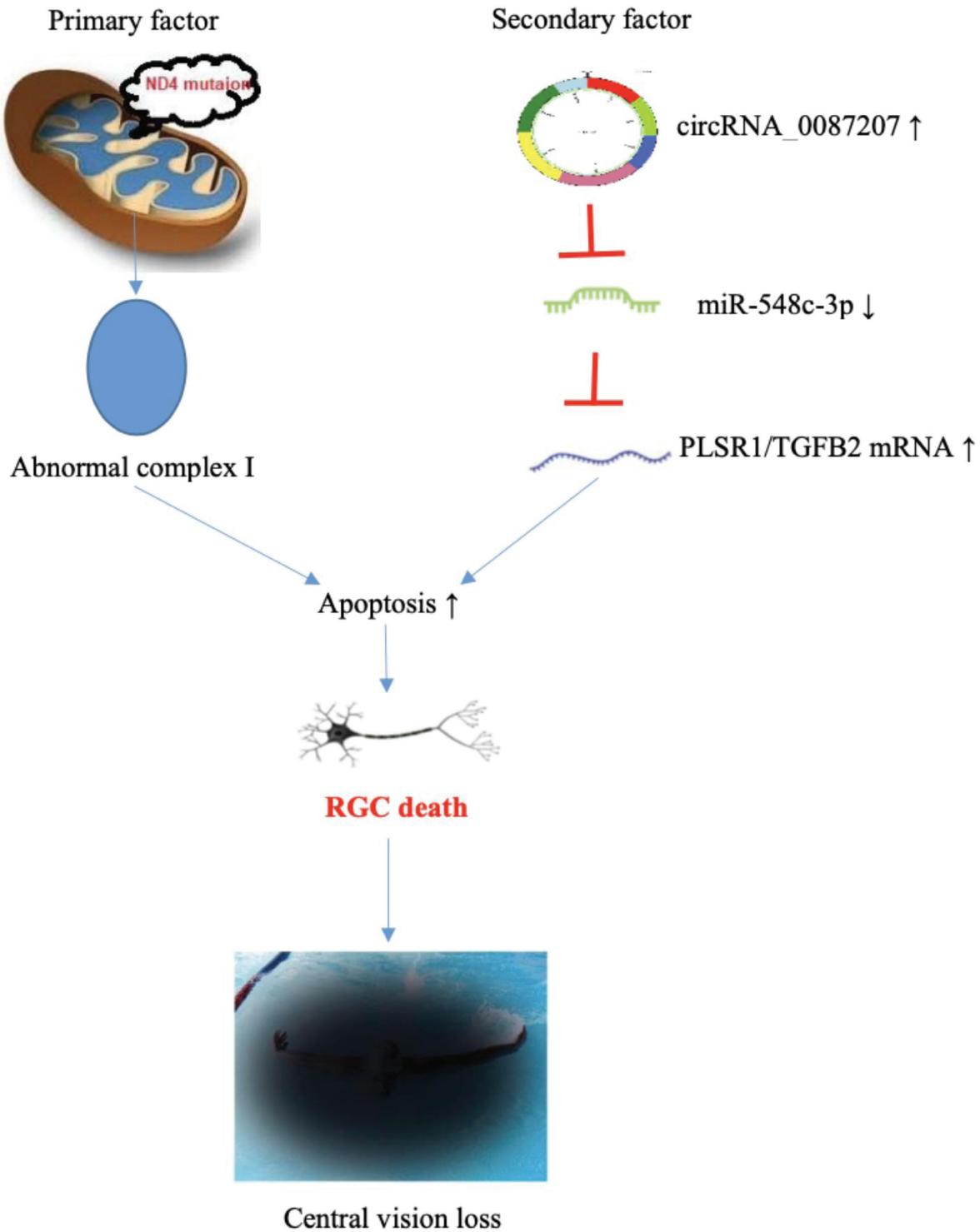


**Fig. 3** Upregulation of circRNA_0087207 as a plausible biomarker for LHON disease. The circRNA_0087207/miR-548c-3p/PLSR1-TGFB2 axis could cooperate with ND4-LHON mutation to regulate apoptosis level in LHON-derived RGCs, revealing a new approach for LHON pathogenesis and predictive marker for this disease. LHON = Leber hereditary optic neuropathy; RGC = retinal ganglion cell.

## 4. DISCUSSION

LHON is a rare hereditary disease caused by a point mutation in complex I of mitochondria. Previous studies revealed that RGCs death, related to upregulating ROS and apoptosis level, is the reason for blindness in LHON patients. Carrier phenotype—carrying LHON mutation but not present optic neuropathy symptoms—suggests that this disease has complicated pathogenesis in which other factors combine with mitochondrial mutation to cause vision loss. Smoking, alcohol, and reproductive hormones are secondary factors mentioned in LHON research. Other elements referred to that could be secondary factors in LHON mechanism like nuclear factors. Because the mechanism of RGCs degeneration is unclear, there is no effective treatment approved by the U.S. Food and Drug Administration till now. Although Idebenone treatment showed support in some studies and gene therapy is a promising strategy, new approaches should be applied to fulfill LHON pathogenesis and open new methods for this disease. CircRNA, which is mentioned in many physiology and pathology pathways, is a nuclear factor. Regulating genes via miRNAs is a foundation function of circRNA; this pathway was shown in neural degeneration in some research before.

We previously found that circRNA_0087207 was highly expressed in patient iPSC-derived RGCs but not in the carrier iPSC-derived RGCs and normal iPSC-derived RGCs, suggesting a link of this mystic circRNA to LHON disease manifestation in affected RGCs. To identify the downstream miRNA-mRNA for circRNA_0087207, KEGG pathway, and Ontology database analysis were executed, and then miR-548c-3p was chosen for potential candidates. Our results showed this miRNA had a negative relationship with circRNA_0087207 in LHON-derived RGCs. Thus, circRNA_0087207 can serve as a sponge for miR-548c-3p. Although we previously found that miR-665 may also have a high affinity to circRNA_0087207. In the present study, our validation study using the transfected cells showed that PLSCR1 and TGFB2 are potential downstream targets of miR-548c-3p. Remarkably, these two mRNAs are upregulated in circRNA_0087207-overexpression RGCs and downregulated in circRNA_0087207-knockdown RGCs.

PLSCR1, a $Ca2^{+}$-sensitive protein located on the cell membrane, can trigger the apoptosis pathway by phosphatidylserine exposure.[12] The role of PLSCR1 in the apoptotic process of neural degeneration was also indicated.[13] Upregulation of PLSCR1 in neuron cells leads to redistribution of phosphatidylserine. In particular, phosphatidylserine tends to move to the extracellular space, which changes the phospholipid compositions and results in the disruption of plasma membrane asymmetry.[14] TGFB2 is mentioned to be involved in the apoptosis pathway of neuron diseases such as vitreoretinopathy or rat retinal ganglion cell line.[15,16] Based on the above-mentioned evidence, we know that PLSCR1 and TGFB2 could take part in apoptosis activation in LHON-derived RGCs. CircRNA_0087207 overexpression sponges and inhibits miR-548c-3p function, then releases the high level of PLSCR1 and TGFB2. This pathway collaborates with ND4 mutation to increase apoptosis levels in LHON-derived RGCs, leading to the number of RGCs death overcoming the threshold, and then presenting vision loss. Moreover, we first demonstrated that circRNA_0087207/miR-548c-3p/PLSR1-TGFB2 axis could cooperate with the putative ND4 mutation to regulate apoptosis level in LHON-derived RGCs, revealing a novel mechanism of this disease (Fig. 3). This study demonstrated a new approach for LHON pathogenesis and a predictive marker for this disease.

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