• Basic Research •

miRNA-451 regulates rhesus choroid-retinal endothelial cell function and proteome profile

Hong-Lian Wu[1], Yan Shao[1], Zhen-Na Chen[2], Hui Zhang[1], Xiao-Min Zhang[1], Xiao-Rong Li[1]

[1]Tianjin Key Laboratory of Retinal Functions and Diseases; Tianjin International Joint Research and Development Centre of Ophthalmology and Vision Science; Eye Institute and School of Optometry, Tianjin Medical University Eye Hospital, Tianjin 300384, China

[2]Department of Ophthalmology, Affiliated Hospital of Weifang Medical University, Weifang 261031, Shandong Province, China

Co-first authors: Hong-Lian Wu and Yan Shao

Correspondence to: Xiao-Rong Li. Tianjin Key Laboratory of Retinal Functions and Diseases; Tianjin International Joint Research and Development Centre of Ophthalmology and Vision Science; Eye Institute and School of Optometry, Tianjin Medical University Eye Hospital, No.251, Fukang Road, Nankai District, Tianjin 300384, China. xiaorli@163.com

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Abstract

● **AIM:** To evaluate the effect of miRNA-451 on rhesus macaque choroid-retinal endothelial (RF/6A) cell function and proteome profile.

● **METHODS:** The RF/6A cells were transfected with miRNA-451 mimic and inhibitor. The role of miRNA-451 on proliferation ability was evaluated by CCK-8 assay. Furthermore, iTRAQ quantitative proteomic analysis was applied to comprehensively illuminate the change of cellular proteins and biological function between different groups.

● **RESULTS:** In miRNA-451 overexpression group, cell proliferation of RF/6A decreased both at 24h and 48h; while in miRNA-451 inhibition group, on the contrary, RF/6A cell proliferation was increased at 48h. Based on iTRAQ quantitative proteomic analysis, 23 differentially expressed proteins (DEPs) were detected in the comparison of miRNA-451 mimic and mimic control-transfected RF/6A cells, and 30 DEPs were identified in the comparison of RF/6A cells transfected with miRNA-451 inhibitor and inhibitor control. DEPs such as GORASP2, KRT1, SLC7A2, RIC8A, DDX42, CAP1, PCBP2 might be closely related to the inhibitory effect of miRNA-451 on RF/6A cell proliferation, while PCYT1A, MGAT1, TUBB, MCU, SIL1, BID, MSH6 might account for the positive effect of miRNA-451 inhibitor on RF/6A cell growth. PTPN1, as the only protein exhibiting an opposite trend between miRNA-451 mimic and inhibitor-transfected cells, was most likely accountable for the inhibition of miRNA-451 mimic on RF/6A cell growth, and the promotion of miRNA-451 inhibitor on RF/6A cell proliferation.

● **CONCLUSION:** miRNA-451 overexpression can suppress the growth of RF/6A cells while knockdown of miRNA-451 can promote RF/6A cell viability. Among all DEPs, increased PTPN1 is most likely to account for the negative regulation of miRNA-451 on RF/6A proliferation. miRNA-451 can be a protective factor for neovascular disease of fundus *via* regulating choroid retinal endothelial cell function.

● **KEYWORDS:** miRNA-451; RF/6A; retinal endothelial cells; iTRAQ quantitative proteomics; proteins; PTPN1

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INTRODUCTION

Abnormal neovascularization is the common pathological process of neovascular diseases of fundus, such as diabetic retinopathy (DR) and age-related macular degeneration (AMD). DR is a common microvascular lesion of diabetes mellitus (DM), accounting for the majority of acquired blindness among the working-age population[1]. Chronic hyperglycemia could damage the basement membrane of endothelial cells, promote the pathological proliferation of endothelial cells, and eventually cause the formation of retinal neovascularization[2]. The emergence of retinal neovascularization is the hallmark of proliferative diabetic retinopathy (PDR), and it's also an important indication of the rapid progress of the patient's fundus pathology and the sharp decline of visual acuity. Similarly, in the evolutionary stage of wet AMD (nAMD), neovascularization is the major feature that could result in vascular leakage or retinal hemorrhagic detachment, and eventually cause the sharp decrease of vision[3]. Since anti-angiogenesis therapy is a critical aspect in the treatment of these diseases, research targeting the

intervention of abnormal proliferation of retinal endothelial cells is undoubtedly valuable.

miRNA-451 (miR-451), a small non-coding RNA molecule, is involved in the regulation of cell proliferation[4]. Based on our previous data, the expression of miR-451a was down-regulated both in the retina of *Akita* mice and 4-hydroxynonenal (4-HNE)-treated RPE cells[5]. We further verified that miR-451a inhibited the proliferation and migration of retinal pigment epithelium (RPE) *in vitro* and showed a protective effect on mitochondria function of RPE in diabetic conditions[5]. Studies on patients' blood have demonstrated that miR-451 could become a prognostic biomarker in DR since its expression level gradually decreased with the progression of the disease[5]. In fact, miR-451 is expected to be a therapeutic target for different cancers since it could suppress tumor proliferation, invasion, and metastasis[4]. For example, Nan *et al*[6] revealed that miR-451 could suppress glioma cell growth and invasion through mTOR/HIF-1α/VEGF pathway, and repressed the epithelial-mesenchymal transition (EMT) and metastasis of cancer *via* PI3K/Akt/Snail pathway[7]. Liu *et al*[8] also exemplified that the proliferation and invasion of hepatocellular carcinoma, a highly vascularized tumor, could be inhibited by miR-451 *via* IL-6R-STAT3 pathway or caspase-3- and MMP9-dependent pathway[9]. Thus, we expected that miR-451 might also play a therapeutic role in fundus neovascular disease by regulating the function of retinal endothelial cells.

In fact, the biological function and regulation of miR-451 on different cells is extremely intricate. Despite increasing studies illustrated that miR-451 primarily repressed cell growth in various cancers, there were still a small portion of research that reported miR-451 could promote the proliferation and migration of tumor cells[10]. Proteomics offers an effective method for the large-scale and in-depth analysis of the intracellular protein composition; hopefully, the new protein targets which are critical for cellular interaction can be discovered and quantified. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) is a powerful proteomic technique that combines the separation capabilities of liquid chromatography with the high sensitivity and selective quality analysis capabilities of triple quadrupole mass spectrometry. isobaric tags for relative and absolute quantification (iTRAQ) is a newly established method to analyze proteins using mass spectrometry. By specially attach 4 or 8 isotope tags to the amino group of polypeptides, iTRAQ can simultaneously compare the relative or absolute content of proteins in 4 or 8 different samples. In this study, we intend to explore the potential effect of miR-451 on the function and proteomic profile of rhesus macaque choroid-retinal endothelial (RF/6A) cell, a choroid retinal endothelial cell line. iTRAQ coupled with LC-MS/MS was used to identify and quantify the differentially expressed proteins (DEPs) in RF/6A cells after miR-451 overexpression or inhibition. To elucidate the altered cellular function and proteins' interactions, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein-protein interaction (PPI) web analysis were performed.

MATERIALS AND METHODS

Cell Culture RF/6A cell line was purchased from Chinese Academy of Science (Shanghai, China) and has been characterized previously[11]. Cells were maintained in MEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37℃ in the cell incubator with 5% CO_2 and 95% air.

Transfection miR-451 mimic, mimic control, inhibitor, and inhibitor control were purchased from Genepharma (Suzhou, China). The coding sequences were as below.

miR-451 mimic: 5'-AAACCGUUACCAUUACUGAGU U-3'; miR-451 mimic control: 5'- UUCUCCGAACGUGU CACGUTT-3'; miR-451 inhibitor: 5'- AACUCAGUAA UGGUAACGGUUU-3'; miR-451 inhibitor control: 5'- UUGUACUACAAAAGUACUG-3'.

Cells were inoculated on a 24-well plate at an appropriate density (1×10^5) and were transfected at about 70% confluence. Lipofectamine 2000 (Life Technologies, USA) was used during cell transfection according to manufacturer's protocol. RF/6A cells were transfected by miR-451 mimic, miR-451 mimic control, miR-451 inhibitor, and miR-451 inhibitor control separately.

Cell Counting Kit-8 Assay The standard growth curve of RF/6A cells was prepared according to the manufacturer's protocol (CCK-8; Beyotime, Shanghai, China). RF/6A cells in the logarithmic growth phase were inoculated into 96-well plates with 5×10^3 cells/well in 100 μL complete medium then incubated with 5% CO_2 at 37℃ in humidified 95% room air overnight. About 70% confluent RF/6A cells were transfected with miR-451 mimic, miR-451 mimic control, miR-451 inhibitor, and miR-451 inhibitor control for 24 and 48h respectively. Then, after aspirating out the previous medium, 90 μL fresh serum-free medium with 10 μL CCK-8 solution was added into each well. Cells were incubated at 37℃ for 4h, and the absorbance of each well was measured at 450 nm using a microplate reader.

Protein Extraction, Digestion and Labeled with iTRAQ Reagents Totally 12 samples consisting of three biological replicates of 4 groups including miR-451 mimic, miR-451 mimic control, miR-451 inhibitor, miR-451 inhibitor control transfected RF/6A cells were prepared. Proteins were extracted with lysis buffer containing 8 mol/L urea, 50 mmol/L NH_4HCO_3

and 0.2% SDS and followed by 5min of ultrasonication on ice. The lysate was centrifuged at 15 000× g for 15min at 4℃, and the supernatant was moved to a clean tube. Samples were further reduced with 10 mmol/L DTT for 1h, then alkylated with 40 mmol/L IAM for 1h at room temperature in the dark. Samples mixed with pre-cold acetone were incubated at -20℃ for 2h. After that, according to the manufacture protocol, proteins were dissolved, denatured, and blocked on cysteines. The protein solution (100 μg) was then diluted with 100 mmol/L TEAB, digested with trypsin Gold, and desalted with a Strata X C18 column. Peptide labeling was performed with iTRAQ Reagent 8-plex kit following the manufacturer's instruction.

LC-MS/MS Analysis The combined peptide mixture was fractionated with a C18 trap column using an Eksigent nanoLC system (SCIEX, Framingham, MA, USA) at a flow rate of 10 μL/min, and was eluted from the trap column by the gradient solvent B. The peptides were subjected to MS/MS analysis with a Triple TOF 6600 System (SCIEX, Framingham, MA, USA). Data-dependent acquisition mode as below was applied for data collection.

Protein Identification and Quantification Raw data from iTRAQ LC-MS/MS was analyzed by Proteome Discoverer Software 1.4 (Thermo Fisher Scientific, San Jose, CA, USA), and was searched against the UniProt Macaca mulatta database (https://www.uniprot.org/proteomes/UP000006718). The confidently identified protein requires at least one unique peptide with FDR <1%. IQuant software was used for quantitative analysis[12]. We defined proteins with fold change >1.2 or <0.83 and P<0.05 as DEPs.

Bioinformatic Analysis DEPs were submitted to the Gene Ontology database (http://www.geneontology.org/) to interpret their annotations in biological process (BP), cellular component (CC), and molecular function (MF). The potential pathway analysis of DEPs was performed by the KEGG (http://www.genome.jp/kegg/mapper.html). And the PPI network was carried out by STRING database (https://string-db.org/). Cytoscape software 3.8.2 was used for the visualization of GO enrichment results.

Statistical Analysis Each experiment at least comprised three independent repeats. All the quantitative value was presented as mean±standard deviation (SD) and Student's t-test was performed to analyze the differences among groups. Prism software v. 8.4.0 (GraphPad Prism, San Diego, CA, USA) was used for the statistical analyses. P-value <0.05 was considered statistically significant.

RESULTS

Transfection of miR-451 Mimic and Inhibitor into RF/6A Cells and Proliferation Assay To investigate the biological characteristic changes of endothelial cells in the presence of miR-451, RF/6A cells were transfected with miR-451 mimic and inhibitor respectively (Figure 1A). CCK-8 test was performed at different co-culture times. Compared with miR-451 mimic control, OD450 value of RF/6A cells transfected miR-451 mimic was lower both at 24h and 48h, indicating the decreased proliferation ability induced by miR-451 on RF/6A cells (Figure 1B). On the contrary, in comparison to the inhibitor control, miR-451 inhibitor promoted RF/6A cell proliferation after 48h of transfection despite it showed no effect at 24h (Figure 1B).

iTRAQ LC-MS/MS Analysis of RF/6A Cells Transfected with miR-451 Mimic Versus Mimic Control

Differentially expressed proteins To explore the DEPs following miR-451 overexpression, total cell lysates of RF/6A transfected with miR-451 mimic and mimic control were collected and analyzed by iTRAQ and LC-MS/MS (Figure 2A). A total of 2343 proteins were detected and quantified in both mimic-transfected and mimic control-transfected RF/6A cells. We considered proteins with fold change over 1.2 or less than 0.83 and P-value <0.05 as DEPs. Therefore, in the comparison of miR-451 mimic and mimic control, 23 DEPs were identified, with 9 up-regulated proteins and 14 down-regulated proteins as volcano plot (Figure 2B), fold change of DEPs (Figure 2C) and heatmap (Figure 2D) exhibited. Among these DEPs, up-regulated proteins were golgi reassembly stacking protein 2 (GORASP2), cytokeratin-1 (KRT1), [solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 (SLC7A2)], engulfment and cell motility protein 2 (ELMO2), tropomyosin 4 (TPM4), and the five most down-regulated proteins were RIC8 guanine nucleotide exchange factor A (RIC8A), ATPase H+ transporting V1 subunit F (ATP6V1F), ATP-dependent RNA helicase DDX42 (DDX42), Adenylyl cyclase-associated protein (CAP1), [poly(rC) binding protein 2 (PCBP2)].

Gene ontology, encyclopedia of genes and genomes, and protein-protein interaction web analysis To illuminate the biological characterization of the DEPs, both up-regulated and down-regulated proteins were subjected to GO (Figure 3A) and KEGG (Figure 3B) enrichment analysis. The BP enrichment analysis revealed that the DEPs mainly participated in the cytoskeleton organization, endoplasmic reticulum stress, intrinsic apoptotic signaling pathway, JUN kinase stress, peptide cross-linking, and L-alpha-amino acid transmembrane transport. The MF enrichment analysis exhibited that the DEPs were mainly associated with receptor tyrosine kinase binding, protein tyrosine kinase binding, ephrin receptor binding, Rac GTPase binding, aminopeptidase activity, L-amino acid transmembrane transport activity, *etc*. The CC enrichment analysis showed that the DEPs were mainly involved in the actin cytoskeleton, COP9 signalosome, keratin filament, polymetric cytoskeletal fiber, growth cone, site of polarized

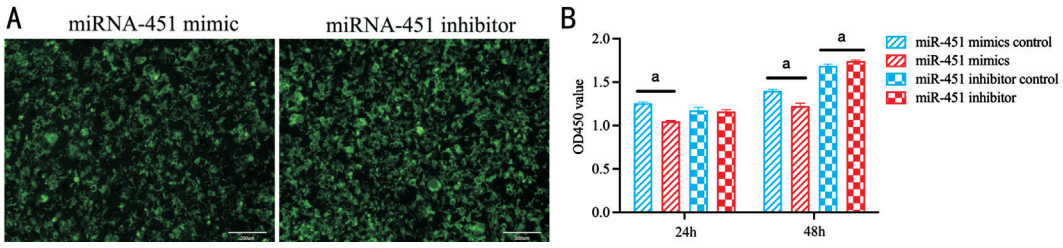

Figure 1 Effect of miR-451 on the proliferation ability of RF/6A cells A: Representative images of RF/6A cells transfected with miR-451 mimic and inhibitor (scale bar=200 μm); B: Cell counting kit-8 assay of RF/6A cells transfected with miR-451 mimic and inhibitor at 24h and 48h by measuring the absorbance at 450 nm. Data presented as means±SD of three independent experiments. [a]P<0.05.

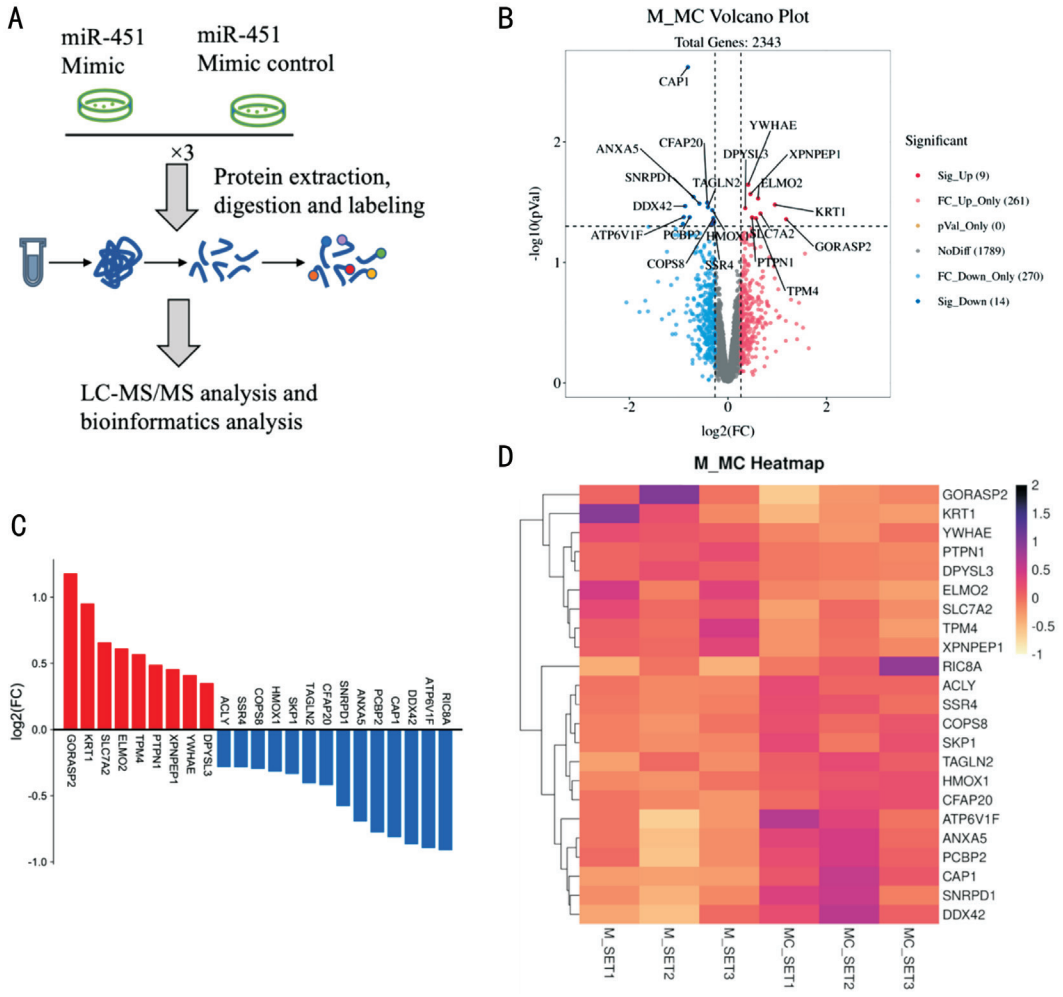

Figure 2 Analysis of the response to miR-451 overexpression in RF/6A cells by iTRAQ LC-MS/MS A: Experimental workflow of proteomic analysis; B: Volcano plot of DEPs in miR-451 mimic- versus mimic control-transfected RF/6A cells; C: Fold change of DEPs; D: Heatmap of DEPs.

growth, *etc*. The KEGG pathways involved by the DEPs included oocyte meiosis, cell cycle, spliceosome, protein processing in endoplasmic reticulum, circadian rhythm, and collecting duct acid secretion. STRING database was applied to investigate the interaction between these DEPs (Figure 3C). There were two groups of strongly interacted proteins including YWHAE-DPYSL3-CAP1-TPM4-TAGLN2-ANXA5-HMOX1-PCBP2-DDX42-SNRPD1, and SLC7A2-XPNPEP1-ACLY. PCBP2 and XPNPEP1 act as hub proteins in their respective groups.

iTRAQ LC-MS/MS Analysis of RF/6A Cells Transfected with miR-451 Inhibitor Versus Inhibitor Control

Differentially expressed proteins iTRAQ LC-MS/MS was also applied to analyze the proteome profile of RF/6A cells after miR-451 knockdown. According to the criteria of fold change >1.2 or <0.83 and P-value <0.05, we identified 30 DEPs, including 13 up-regulated and 17 down-regulated proteins (Figure 4A-4D). The top five up-regulated proteins were phosphate cytidylyltransferase 1, choline, alpha (PCYT1A), alpha-1,3-mannosyl-glycoprotein 2-beta-

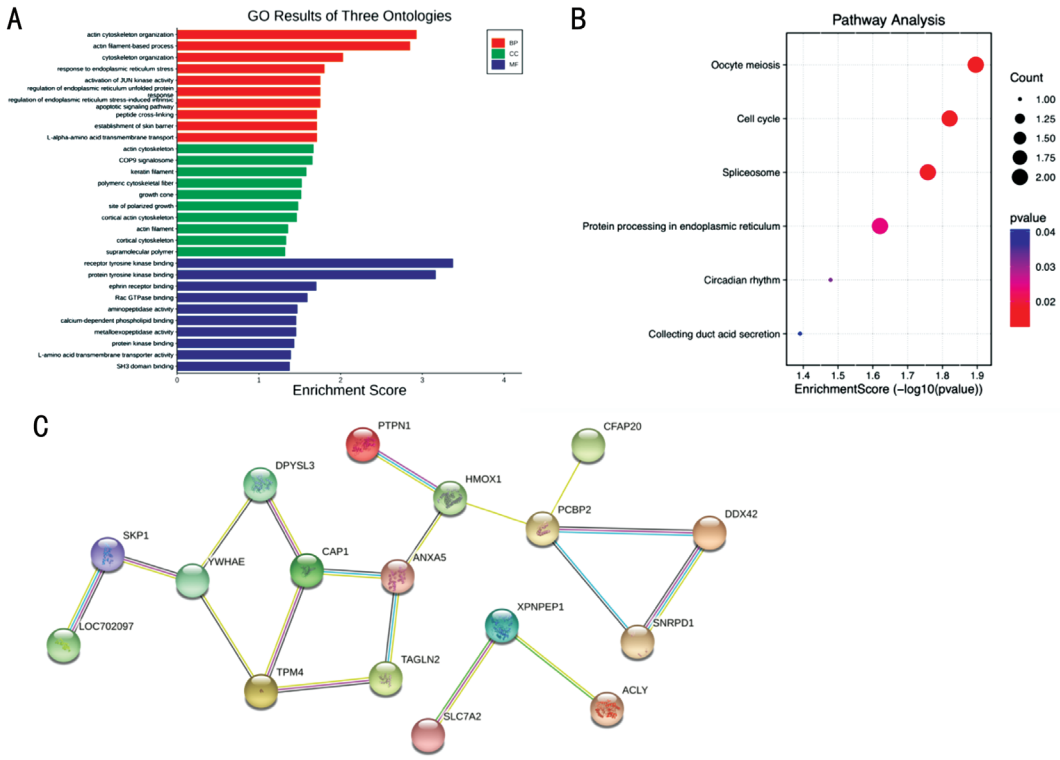

Figure 3 Functional analysis of DEPs identified in RF/6A with miR-451 mimic transfection A: GO annotation analysis of DEPs; B: KEGG pathway analysis of DEPs; C: PPI analysis of DEPs.

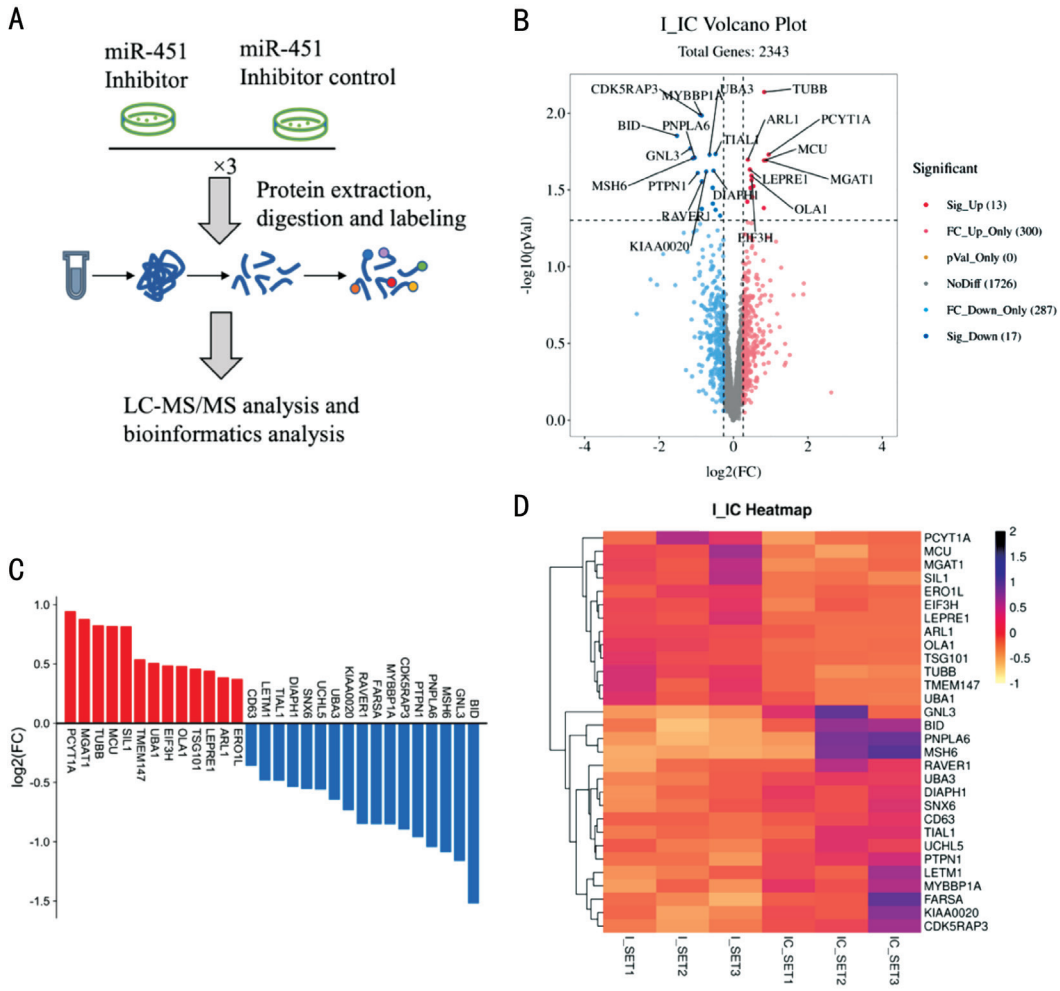

Figure 4 Proteomic analysis of miR-451 knockdown in RF/6A A: Experimental design of study; B: Volcano plot of DEPs in RF/6A transfected with miR-451 inhibitor versus inhibitor control; C: Fold change of DEPs; D: Heatmap of DEPs.

N-acetylglucosaminyltransferase (MGAT1), tubulin beta chain (TUBB), mitochondrial calcium uniporter (MCU), and Nucleotide exchange factor SIL1 (SIL1), and the top five down-regulated proteins were (BH3 interacting domain death agonist (BID), [guanine nucleotide binding protein-like 3 (nucleolar) (GNL3)], mutS homolog 6 (MSH6), patatin-like phospholipase domain containing 6 (PNPLA6), protein tyrosine phosphatase non-receptor type 1 (PTPN1).

Gene ontology, encyclopedia of genes and genomes, and protein-protein interaction web analysis DEPs were analyzed in GO, KEGG, and STRING database for functional annotation (Figure 5A-5C). The BP participated by DEPs included stem cell division, actin cytoskeleton reorganization, regulation of endocytosis, regulation of hydrolase and catalytic activity, receptor metabolic process, and regulation of protein localization, *etc*. The MF included ligase activity (forming carbon-sulfur bonds), ribosome binding, GTP binding, nucleoside binding, and protein-containing complex binding, *etc*. The CC included eukaryotic 43S and 48S preinitiation complex, DNA repair complex, multivesicular body, lno80 complex, DNA helicase complex, polysomal ribosome, cytoplasmic stress granule. The KEGG pathway enrichment analysis revealed that the DEPs mainly took part in the regulation of ubiquitin mediated proteolysis. STRING database revealed that there was one major protein interaction network composing of the most DEPs, of which GNL3 acts as a hub protein in the network.

Protein with Opposite Expression Levels Between miR-451 Mimic- and Inhibitor-Transfected RF/6A Cells-PTPN1 miR-451 mimic and inhibitor were supposed to play an opposite role in regulating cell function. So, protein, whose fold change presented an opposite expression level between mimic *vs* mimic control and inhibitor *vs* inhibitor control, might exert an essential effect in cell regulation. By comparing the significantly increased and reduced proteins among the four groups, PTPN1 was the only protein exhibiting an opposite expression tend (up-regulated when miR-451 mimic versus mimic control and down-regulated when miR-451 inhibitor versus inhibitor control; Figure 6A-6B). According to the STRING database, the most intensively correlated proteins with PTPN1 in human were SRC, BCAR1, GRB2, IRS1, EGFR, JAK2, IGF1R, INSR, CDH2, and RMDN3 (Figure 6C). These proteins were involved in the insulin receptor signaling pathway, platelet-derived growth factor receptor signaling pathway, peptidyl-tyrosine autophosphorylation, ERBB2 signaling pathway, and positive regulation of glucose metabolic process (Figure 6D).

DISCUSSION

As one of the small non-coding RNA molecules, miR-451 has participated in the various pathophysiological process[13-14] and is expected to be a promising therapeutic target in different diseases[15-16]. In fundus oculi disease, endothelial cell dysfunction induced by hypoxia, ischemia, or hyperglycemia is responsible for retinal neovascularization[17]. Previous studies revealed that miR-451 has a therapeutic effect in suppressing angiogenesis in hepatocellular carcinoma[8] and other vascular diseases[18]. The latest research by Trotta *et al*[19] found that circulating miR-451a was gradually reduced as DR progressed and could be a promising biomarker for DR. And our research of miR-451a on RPE found that miR-451a inhibited the proliferation and migration of RPE cells, exhibiting the potential effect to inhibit the formation of the epiretinal membrane which was a critical feature of the severe stage of PDR[5]. However, whether miR-451 could regulate the biological function of retinal endothelial cells and play a protective role in neovascular disease of the fundus is still unknown. Therefore, we intend to reveal the potential role of miR-451 in regulating the function and proteome profile of RF/6A, to explore whether miR-451 could be a therapeutic target for neovascular diseases of the fundus.

In this experiment, we found that up-regulation of miR-451 could inhibit RF/6A cell proliferation, and the inhibitory effect could last from 24h to 48h after transfection, suggesting that miR-451 itself played an inhibitory effect on cell proliferation. In addition, the downregulation of miR-451 level did not affect RF/6A cell proliferation at 24h but promoted cell proliferation at 48h. We believe that this phenomenon was probably related to the specific mechanism of miR-451 mimic and inhibitor function. miR-451 mimic can act directly on the target mRNA such as inhibiting mRNA translation after entering RF/6A cells. However, miR-451 inhibitors may need to combine with miR-451 pieces before playing the inhibiting role, leading to the occurrence of functional delay. On the other hand, downregulation of miR-451 may be insufficient for the inhibition of RF/6A cell proliferation before causing cell biology behavior change.

DEPs detected by iTRAQ LC-MS/MS in RF/6A after miR-451 overexpression included 9 up-regulated proteins (GORASP2, KRT1, SLC7A2, ELMO2, TPM4, *etc*.), and 14 down-regulated proteins (RIC8A, ATP6V1F, DDX42, CAP1, PCBP2, *etc*.). GORASP2 also known as GRASP55, is a Golgi stacking protein involved in autophagy, protein and lipid trafficking, sorting, processing, and modification[20]. Ahat *et al*[21] found that depletion of GRASP increased the growth of HeLa cells though exact mechanism has not been revealed. In our research, the upregulation of GORASP2 might be partly accountable for the inhibitory effect of miR-451 on RF/6A cell proliferation. KRT1, a member of the keratin gene family, constituted the skin epidermis structure. Blanckaert *et al*[22] revealed that increased KRT1 was related

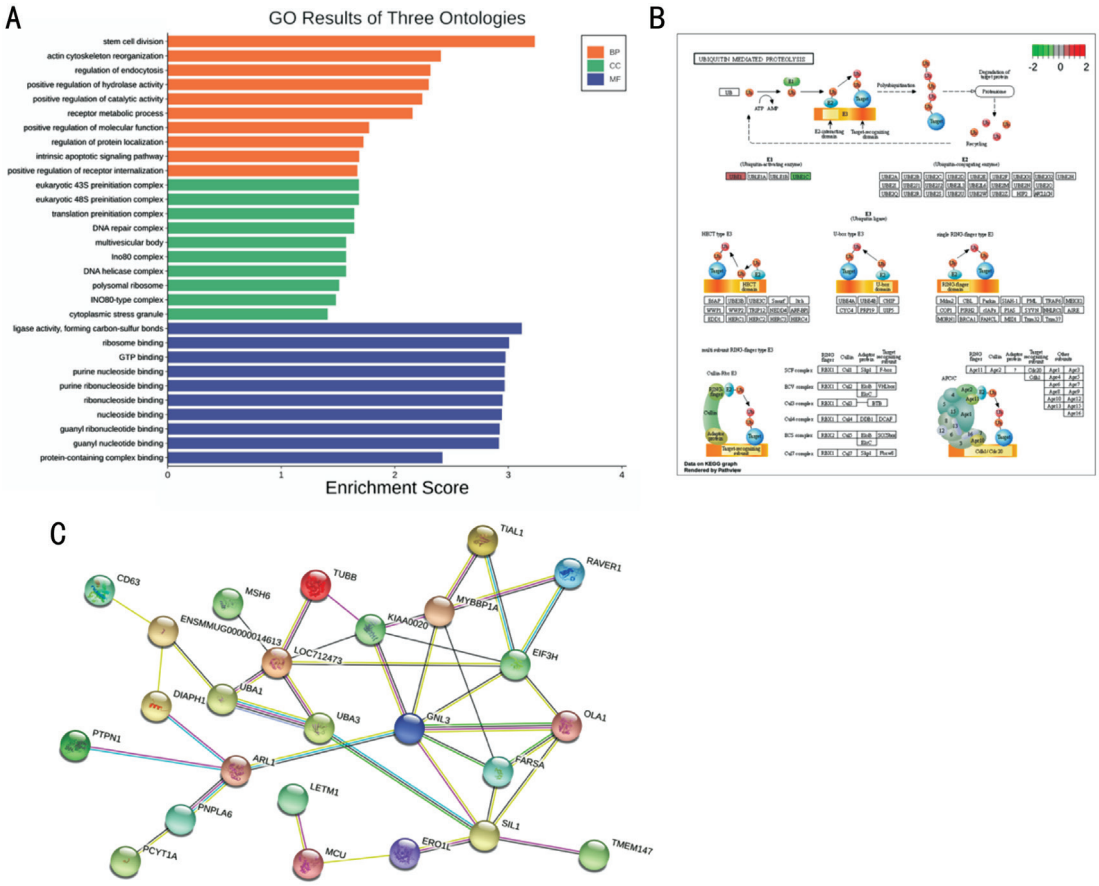

Figure 5 Functional analysis of DEPs identified in RF/6A with miR-451 knockdown A: GO annotation analysis of DEPs; B: KEGG pathway analysis of DEPs; C: PPI analysis of DEPs.

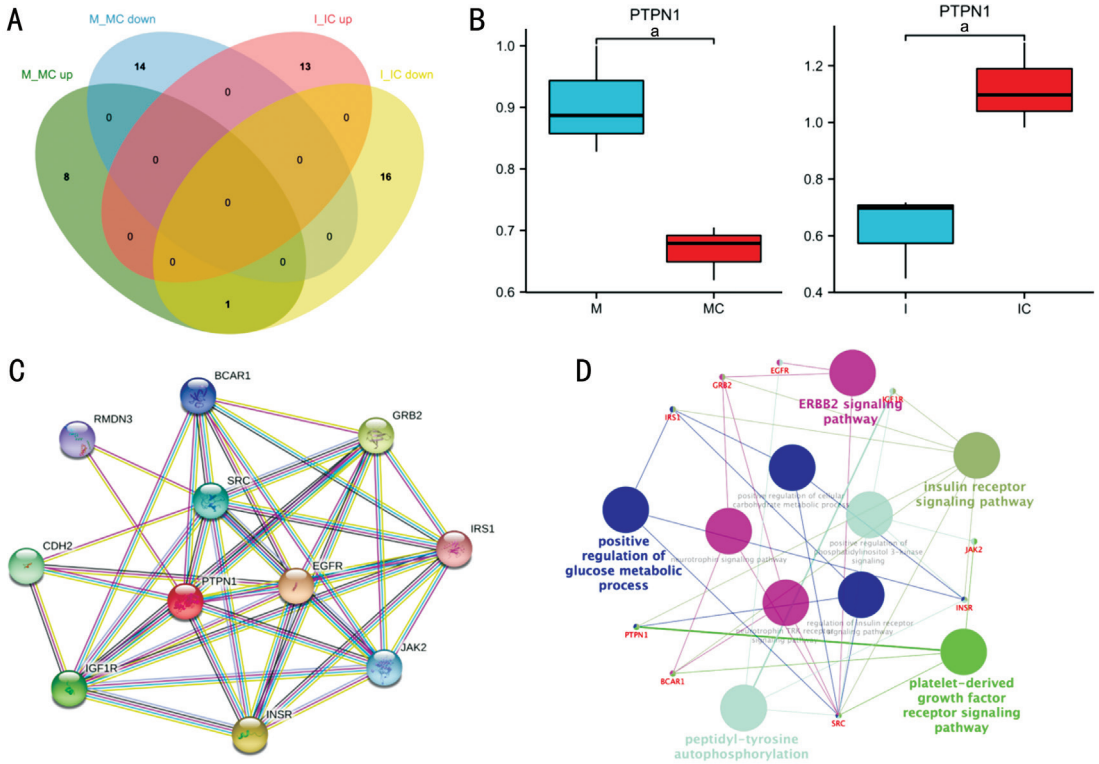

Figure 6 Protein exhibiting opposite expression level in RF/6A transfected with miR-451 mimic and inhibitor-PTPN1 A: Venn diagram of the up-regulated and down-regulated proteins between miR-451 mimic versus mimic control and inhibitor versus inhibitor control; B: Boxplot of PTPN1 expression level in RF/6A cells transfected with miR-451 mimic, mimic control, inhibitor, and inhibitor control, $^{a}P<0.05$; C: PPI of PTPN1 in human; D: Biological process and molecular process of PTPN1 and its related proteins in human.

to the inhibition of docosahexaenoic acid (DHA) on the invasion of MDA-MB-231 breast cancer cells. SLC7A2, a member of the solute carrier family, was related to the amino acid transmembrane transporter activity. A latest research by Xia *et al*[23], demonstrated that overexpression of SLC7A2 suppressed the invasion and metastasis of hepatocellular carcinoma by regulating myeloid-derived suppressors cell recruitment. However, increased ELMO2[24] and TPM4[25] were related to the proliferation and metastasis of different cancers respectively, suggesting that they might contributed little to the regulation of miR-451 on RF/6A cell proliferation.

RIC8A, was involved in the regulation of cell adhesion and migration. Researchers found that knockdown of RIC8A gene inhibited the tumorigenesis in a mouse model of melanoma[26]. Therefore, we supposed that miR-451 probably inhibited the proliferation of RF/6A cells through down-regulating RIC8A expression. ATP6V1F, participating in the transport of hydrogen ions, was confirmed to be associated with the prognosis of rectal cancer[27] and renal clear cell carcinoma[28]. However, there was no study that verified its role in the regulation of cell proliferation. DDX42, participated in the protein localization and regulation of apoptotic process, *etc*. Sohn and Chay[29], found that DDX42 inhibited the apoptosis of Ba/F3 cells (a mouse pro-B cell line). Besides, CAP1, involved in the regulation of the actin cytoskeleton, was extensively studied in various cancers. The upregulation of CAP1 was responsible for the proliferation, migration and invasion of lung cancer[30], gastric cancer[31], and breast cancer[32]. Moreover, increase of PCBP2, one of the major cellular poly(rC)-binding proteins, significantly promoted cell viability, metastasis and invasion in hepatocellular carcinoma[33]. Thus, these down-regulated DEPs were very likely to take part in the regulation of miR-451 on RF/6A cell function.

Of all the DEPs in RF/6A transfected with miR-451 inhibitor compared with inhibitor control, 13 proteins (PCYT1A, MGAT1, TUBB, MCU, SIL1, *etc*.) are up-regulated, and 17 proteins (BID, GNL3, MSH6, PNPLA6, PTPN1 *etc*.) are down-regulated. PCYT1A is a rate-limiting enzyme that participated in the phosphatidylcholine biosynthesis. Xiong *et al*[34] revealed that expression silencing of PCYT1A led to the remarkable proliferation suppression of B-lymphoma cells. However, another research found that PCYT1A knockdown promoted the proliferation and migration of lung adenocarcinoma cells[35]. These seemingly controversial results might be due to the heterogeneous response of different cells to PCYT1A. MGAT1 promoted glioma cell proliferation and migration[36], and inhibition of MGAT1 tended to suppress breast cancer metastasis[37]. Higher expression level of TUBB, the major constituent of microtubules, was correlated with the worse prognosis of lung adenocarcinoma[38]. In addition, MCU, locating on the mitochondrion inner membrane and mediating calcium uptake into mitochondria, enhanced cell growth remarkably and played a critical role in the progression of various cancers such as colorectal cancer[39], esophageal cancer[40] and gastric cancer[41]. MCU also contributed to the angiogenesis of breast cancer *via* negatively regulating the secretion of miR-4488 into extracellular vesicles (EVs); that is, increased MCU led to the lower level of miR-4488 in the EVs, thus resulted in the angiogenesis in the metastatic niche[42]. Furthermore, SIL1, a regulator of endoplasmic reticulum function, promoted the progression of breast cancer *via* regulating cell proliferation, migration and invasion[43]. These up-regulated DEPs explained the positive effect of miR-451 mimic on RF/6A cells to some extent.

For the down-regulated DEPs, different proteins also exemplified various effect on cell viability. BID is related to the cellular apoptotic process. Bi and Wang[44] recently exemplified that LINC00472, a tumor inhibitor, could increase apoptosis and repress the proliferation of pancreatic cancer cells by promoting BID expression. Since BID ranked first among the down-regulated proteins, it might exert critical effect in the regulation RF/6A cell viability. However, GNL3, participated in GTP binding and mRNA 5'-UTR binding, was up-regulated in tumor progression. Li *et al*[45] confirmed that knockdown of GNL3 inhibited the growth, migration and invasion of osteosarcoma cell lines. MSH6 (DNA mismatch repair protein) is one of the components of the post-replicate DNA mismatch repair system (MMR). Palassin *et al*[46] found that NRIP1 (nuclear receptor interacting protein 1) mutant participated in the progression of colorectal cancer *via* down-regulating MSH6 expression. Therefore, the decrease of MSH6 expression might partly be responsible for the proliferation of RF/6A. Besides, PNPLA6, was closely related to the normal development of nervous system. Although the mutant of PNPLA6 was verified in different neurological syndrome[47-48], there was no research on its potential role in cell proliferation and migration.

Among all the DEPs, PTPN1 was the only protein that showed an opposite trend between miR-451 mimic and inhibitor group. As a member of the protein tyrosine phosphatase (PTP) family, PTPN1 specifically catalyzes the dephosphorylation of tyrosine residues, involved in the cell growth, differentiation, mitotic cycle, and oncogenic transformation[49]. The role of PTPN1 in the regulation of cell growth was inconsistent referring to different cancer cells. For colon cancer, Radhakrishnan *et al*[50] reported that curcumin increased the activity of PTPN1, resulting in the reduced migration of HCT116 and SW480 cells *via* dephosphorylating pTyr(421)-cortactin. Nunes-Xavier *et al*[51] also found that in neuroblastoma, PTPN1 deficiency led to an increase of SH-SY5Y cell proliferation. In lung

adenocarcinoma, PTPN1 inhibited cell growth and metastasis through the dephosphorylation of c-Met and PIK3R2[52]. The latest research on bladder cancer by Monoe *et al*[53] revealed that PTPN1 knockdown resulted in the increase of cancer cell proliferation and migration, and miR-130-targeted LNA (locked nucleic acid) increased PTPN1 and exhibited as a promising therapeutic agent. However, PTPN1 was also found to be positively correlated with the progression of melanoma[54] and glioma[55]. These seemingly controversial results might partly be due to the heterogeneity of different cells. Therefore, we suppose that miR-451 mimic probably inhibited RF/6A cell growth through up-regulation of PTPN1, while miR-451 inhibitor promoted cell proliferation *via* down-regulating PTPN1.

In conclusion, our study revealed that when miR-451 was overexpressed, the proliferation ability of RF/6A cells was inhibited; on the contrary, miR-451 inhibitor enhanced cell growth of RF/6A. Data obtained from iTRAQ LC-MS/MS showed that a variety of proteins were involved in RF/6A cell growth and angiogenesis. GO and KEGG pathway analysis of DEPs depicted the cellular function influenced by miR-451. PTPN1, as the unique protein showing opposite expression trend between miR-451 mimic and inhibitor, was most likely to be the target protein of miR-451 regulating the proliferation of RF/6A. We suppose that miR-451 inhibited RF/6A cell growth *via* up-regulating the expression level of PTPN1, and miR-451 might be a promising therapeutic target for retinal neovascularization. However, further research is required to exemplify the exact protective role of miR-451 in neovascular disease of fundus based on the animal model.

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