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RESEARCH PAPER

miR-762 regulates the proliferation and differentiation of retinal progenitor cells by targeting NPDC1

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ABSTRACT

Retinal degenerations, which lead to irreversible decline in visual function, are still no effective recovery treatments. Currently, retinal progenitor cell (RPC) transplantation therapy is expected to provide a new approach to treat these diseases; however, the limited proliferation capacity and differentiation potential toward specific retinal neurons of RPCs hinder their potential clinical applications. microRNAs have been reported to serve as important regulators in the cell fate determination of stem/progenitor cells. In this study, our data demonstrated that miR-762 inhibited NPDC1 expression to positively regulate RPC proliferation and suppress RPC neuronal differentiation. Furthermore, the knockdown of miR-762 upregulated NPDC1 expression in RPCs, leading to the inhibition of RPC proliferation and the increase in neuronal differentiation. Moreover, NPDC1 could rescue anti-miR-762-induced RPC proliferation deficiency and the inhibitory effect of miR-762 on RPC differentiation. In conclusion, our study demonstrated that miR-762 plays a crucial role in regulating RPC proliferation and differentiation by directly targeting NPDC1, which is firstly reported that microRNAs positively regulate RPC proliferation and negatively regulate RPC differentiation, which provides a comprehensive understanding of the molecular mechanisms that dominate RPC proliferation and differentiation in vitro.

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Retinal progenitor cells; miR-762; proliferation; differentiation; NPDC1

1. Introduction

Retinal degenerations are a class of diseases characterized by progressive gradual loss of retinal neuronal cells leading to irreversible decline in vision and even blindness [1]. Although some treatments for these diseases are being developed, currently, there are still no effective recovery treatments. In recent years, stem cell transplantation has offered a promising alternative therapeutic approach for retinal diseases [2–6]. Stem cells, including retinal progenitor cells (RPCs), embryonic stem cells and induced pluripotent stem cells [7–9], have been suggested as potential seed cells for cell-based transplantation therapy. Compared with other seed cells, RPCs are free of ethical and biosafety concerns (such as tumourigenicity, mutations and epigenetic changes) [10] and are thus one of the most prospective candidates to replace degenerating retinal cells. However, their limited capacity to proliferate and differentiate toward specific retinal neurons in vitro impedes their future clinical applications [11–15]. Recently, a growing number of researches have demonstrated that miRNAs play vital roles in governing proliferation and differentiation of stem cells. [16,17].

miRNAs, which arise from longer precursors, are an extensive class of small noncoding RNAs with mature transcripts of 18 to 25 nt [18]. Mature animal miRNAs are bound by Argonaute protein to form RNA-induced silencing complexes that can recognize their messenger RNA (mRNA) targets through imprecise base-pairing to repress target gene expression [19,20]. To date, the area of miRNA biology has extended considerably since the first miRNA was discovered [21,22]. microRNAs (miRNAs) have been reported to

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Supplemental data of this article can be accessed here.

play a vital role in governing the proliferation and differentiation of stem cells, and such a role has been revealed in embryonic stem cells, germline stem cells and multifarious somatic tissue stem cells [23–25].

miRNAs dominate gene expression via binding to the 3 -untranslated region (3 -UTR) of their target mRNAs and triggering either mRNA degradation or translational repression [26,27]. Emerging evidence has shown that miRNAs are especially attractive candidates for regulating stem cell fate determination [28–31]. It is well known that some miRNAs can positively regulate stem cell proliferation and negatively regulate stem cell differentiation, while others may do the opposite [32]. For instance, miR-216a promotes bone marrow stromal cells to differentiate into osteoblast via targeting c-Cbl, while miR-31 has a negative effect on differentiation by silencing Satb2 [33,34]. In brain neural progenitor cells, miR-145 could inhibit cell proliferation and promote neuronal differentiation by repressing Sox2. In contrast, miR-137 increased cell proliferation, while decreased neuronal differentiation was observed by the negative regulation of EZH2 [35,36]. According to previous studies, only a few microRNAs have been reported to have effects on decreasing RPC proliferation and enhancing neuronal differentiation [37,38]. Whether there are microRNAs that can positively regulate RPC proliferation and/or negatively regulate the differentiation of RPCs remains unknown. In our research, considering that the retina is the extension of the central nervous system, 18 miRNAs (related to the brain neural progenitor cell proliferation and differentiation) were selected to analyze their expression in RPC cultures by a miRNA PCR array developed in our lab. The data demonstrated that miR-762 was abundantly expressed in proliferative RPCs and that it was one of the most sharply decreased miRNAs during the differentiation of RPCs, indicating its potential role in governing RPC fate. Herein, we demonstrated that miR-762 could positively regulate RPC proliferation and was negatively associated with RPC differentiation by directly targeting NPDC1 (neural proliferation, differentiation and control 1). Our data highlight a novel role for the miRNA regulatory network between RPC proliferation and differentiation.

2. Materials and methods

2.1. Retinal progenitor cell (RPC) isolation and culture

RPCs were obtained as previously described [39]. Briefly, the cells were obtained from fresh neural retina of postnatal day 1 GFP transgenic C57BL/6 mice (a kind gift from Dr. Masaru Okabe, University of Osaka, Japan). Then, RPCs were seeded on T25 flasks and cultured with proliferation medium containing advanced DMEM/F12 (Invitrogen), 1% N2 neural supplement (Invitrogen), 100 U mL-1 penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 20 ng ml-1 epidermal growth factor (Invitrogen). After two days, the proliferation medium was replaced and the cells were passaged at intervals of 3 days. RPCs were cultured under differentiation medium containing advanced DMEM/F1, 1% N2 neural supplement, 100 U mL-1 penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) without EGF and 10% fetal bovine serum (FBS, Invitrogen) for differentiation research.

All animal experiments performed in this study were based on the Association for Research in Vision and Ophthalmology (ARVO) and were approved by the Animal Research Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

2.2. RNA isolation, reverse transcription and quantitative polymerase chain reaction (qPCR analysis)

Total RNA was harvested from RPCs using TRIzol reagent (Invitrogen) based on the standard manufacturer's protocol. Then RNA was reverse transcribed utilizing the PrimeScript RT Reagent Kit with gDNA Eraser treatment (TaKaRa). qPCR analysis using Power SYBR Green PCR Master Mix was implemented using a QuantStudio 6Flex Real-Time PCR system (Applied Biosystems, Foster, CA). MicroRNA qPCR analysis was

handled with PrimeScript RT Reagent Kit and SYBR-Premix Ex Taq II (TaKaRa). After normalization to β-actin (for mRNA) or U6 (for miRNA) expression, the relative amounts of mRNA and mature miRNAs were presented as relative fold change to the controls (Supplementary Table1). The primers used in qPCR analysis are listed in Table 1 (mRNAs) and Supplementary Table 2 (miRNAs).

2.3. Western blotting

Protein lysates were obtained using the radio immunoprecipitation assay lysis buffer (Thermo Fisher Scientific), and the BCA protein assay (Thermo Fisher Scientific) was used to analyze the protein concentration. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate the proteins and after that, the proteins were transferred to 0.45-mm polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Then, membranes were blocked using 5% nonfat milk and they were incubated with mouse monoclonal anti-NPDC1 (Abcam; 1:1000), mouse monoclonal anti-Myod1 (BD; 1:1000), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (Chemicon; 1:1000), mouse monoclonal anti-PKC-a (BD; 1:1000), rabbit polyclonal anti-Recoverin (Millipore; 1:1000), mouse monoclonal anti-Rhodopsin (Chemicon; 1:1000), mouse monoclonal anti-β3-tubulin (Chemicon; 1:1000) and mouse anti-β-actin (Sigma, 1:1000) antibodies at 4°C for 8 h. Subsequently, the respective secondary antibodies (Sigma-Aldrich) were added to the membranes, and then protein expression was visualized by a Tanon image viewer system (Shanghai, China).

2.4. Immunocytochemistry

Immunocytochemistry was conducted in 24-well plates to investigate RPC differentiation (7 days) ability. The cells were rinsed with warm PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) and blocked for 1 h at room temperature using blocking solution including TBS with 10% normal goat serum (Sigma-Aldrich) and 0.3% Triton X-100. Afterward, the cells were incubated with various antibodies that included mouse monoclonal anti-NPDC1 (Abcam; 1:200), anti-glial fibrillary acidic protein (GFAP) (Chemicon; 1:200), rabbit polyclonal anti-Recoverin (Millipore; 1:200), mouse monoclonal anti-Rhodopsin (Chemicon; 1:200) at 4°C for 8 h and incubated with fluorescently conjugated secondary antibody (Alexa Fluor 546-conjugated goat anti-mouse/rabbit, (BD, 1:800)) in the dark for 1 h at room temperature. Cell nucleus were counterstained with diamidino-2-phenylindole (DAPI; Invitrogen) and viewed and imaged with a fluorescence microscope (Olympus BX51, Japan).

2.5. CCK8 analysis

A CCK8 kit was used to assess RPC proliferation (1 × 104 cells/well) in a 96-well plate using transfected cells. Afterward, the CCK8 solution (10 μL well-1) was into every well at days 0, 1, 2 and 3, and then the RPCs were incubated for 4 h at room temperature. The proliferation capability of RPCs was evaluated utilizing an ELISA microplate reader (ELX800, BioTeK, USA) by measuring optical density at 450 nm. The cell viability was evaluated as the A450 value because its viability was directly linked to the absorbance at 450 nm.

Table 1. Primer list for qRT-PCR.

Genes	Forward (5 -3)	Reverse (5 -3)	"Annealing temperature (°C)"	"Product size (base pairs)"
β3-tubulin	cgagacctactgcatcgaca	cattgagctgaccagggaat	60	152
Recoverin	atggggaatagcaagagcgg	gagtccgggaaaaacttggaata	60	179
Rhodopsin	tcaccaccaccctctacaca	tgatccaggtgaagaccaca	60	216
PKC-a	cccattccagaaggagatga	ttcctgtcagcaagcatcac	60	212
GFAP	agaaaaccgcatcaccattc	tcacatcaccacgtccttgt	60	184
β-actin	agccatgtacgtagccatcc	ctctcagctgtggtggtgaa	60	152
Ki-67	cagtactcggaatgcagcaa	cagtcttcaggggctctgtc	60	170
NPDC1	tgctacggctgctgctctcc	tccttctccttcagtgccagttcc	60	292
Myod1	cgggacatagacttgacaggc	tcgaaacacgggtcatcataga	60	83

2.6. Luciferase assay

The NPDC1 3 -UTR including the predicted miR-762 binding site (positions 28–34) was cloned downstream of the luciferase reporter sequences. Then, HEK293 cells were co-transfected with either the miR-762 mimic or control miRNA and the luciferase reporter construct. After 48 h, a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to assess the luciferase activity based on the manufacturer s protocol.

2.7. Edu assay

RPCs were seeded in 24-well plates with the addition of Edu reagent. After disposing of the Edu medium, the cells were fixed with 4% paraformaldehyde in room temperature for 30 min, recolored with Apollo color arrangement. Then the cells were incubated with Hoechst 33,342 for 30 min and fluorescence microscopy and laser filtering microscopy (Nikon) was used to take the images. The cell proliferation rate was decided by ImageJ with (Edu positive cells/Hoechst 33,342 recolored cells) × 100%.

2.8. Transfection

In order to allow the RPCs to attach, the cells were cultured under differentiation medium for 10 h before transfections. Based on the manufacturer's protocol, RPCs were treated with NPDC1 clone, siNPDC1, miR-762 mimics, miR-762 inhibitor and negative control (GenePharma, Shanghai, China) at a final concentration of 20 nM using Lipofectamine RNAiMAX (Invitrogen, cat. no. 13,778,030) and they were all synthesized by GenePharma Technology, Inc. (China). CMV-MCS-IRES-Cherry-SV40-Neomycinclone of NPDC1 was synthesized by GenePharma Technology, Inc. (China). RPCs were harvested 2 days after transfection. To research for a long time, siRNAs or the cDNA clones and miR-762 mimics or miR-762 inhibitors were repeatedly transfected at the interval of 3 days. The oligonucleotide sequences of miRNA mimic/miRNA inhibitor are listed in Supplementary Table 1. The oligonucleotide sequences for siNPDC1 were synthesized by GenePharma and were as follows:

siNPDC1-1, 5 -CCAAGGAAACACCUAUCUUTT-3 ;
siNPDC1-2, 5 GACCAUGCCUUCAGUCCUUTT3 ;
siNPDC1-3, 5 -GCAACCCACUGUUUGACCATT-3 ;
siNC, 5 -UUCUCCGAACGUGUCACGUTT-3

2.9. Bioinformatics analysis

To study miR-762 target genes in retinal progenitor cells, we selected candidate genes utilizing three miRNA target prediction databases: miRWalk (mirwalk.umm.uni-heidelberg.de), TargetScan (www.targetscan.org) and miRbase (www.mirbase.org).

2.10. Statistical analyzes

All of the experiments were repeated at least three times. The experimental results presented in this study are presented as the mean ± SD. Then statistical significance between the experimental and control groups was analyzed using Student's two-tailed t test (for comparisons between the means of two groups) or a one-way ANOVA (for comparisons between the means of more than two groups). $P < 0.05$ was considered statistically significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

3. Results

3.1. The endogenous expression level of miR-762 in RPC cultures during differentiation

To further investigate the effect of microRNAs in governing retinal progenitor cell (RPC) proliferation and differentiation, and because retina is the extension of the central nervous system, we selected 18 miRNAs related to the brain neural progenitor cell proliferation and differentiation and then analyzed their expression using a homemade miRNA PCR array during RPC differentiation (Figure 1a; Supplementary Table S1). Among the 18 miRNAs we tested, 7 and 6 were significantly upregulated and downregulated, respectively, during RPC differentiation (Figure

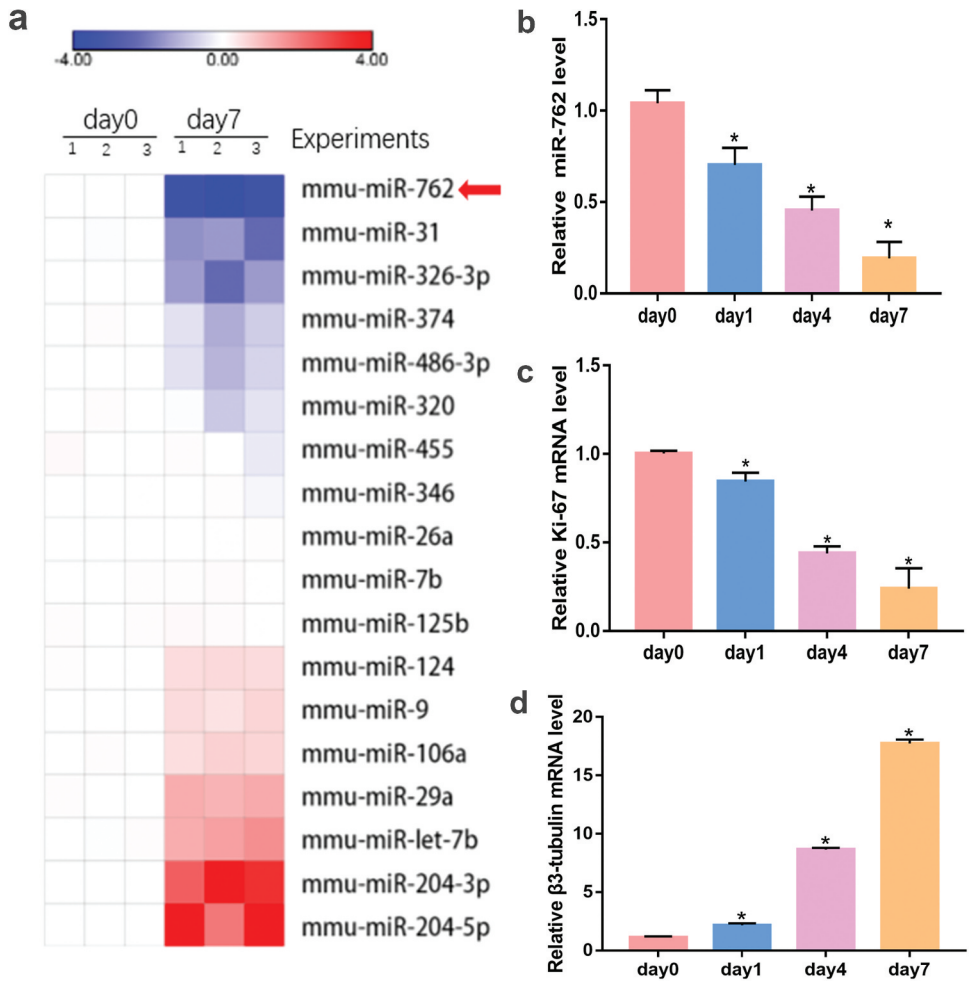

Figure 1. The endogenous expression level of miR-762 in RPC cultures during differentiation. (a) Eighteen miRNAs related to the proliferation and differentiation of brain neural progenitor cells were tested by a homemade miRNA PCR array during RPC differentiation. The relative expression levels of miRNAs in RPCs are presented in a heat map format and the data from three independent experiments are shown. A three-colored scale was used with blue, white and red, signifying low, intermediate and high expression, respectively. (b) qPCR analysis data revealed that miR-762 expression gradually reduced during the RPC differentiation. (c-d) Based on the qPCR analysis results, Ki-67 (a cell proliferation marker) expression decreased during RPC differentiation, while the RPC differentiation marker β3-tubulin (a pan-neuronal marker) showed the opposite trend. All experiments were repeated at least three times. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

1a; Supplementary Table S1). Of note, miR-762 was abundantly expressed in proliferating RPCs and was one of the most downregulated miRNAs (~10-fold) (Supplementary Table S1) during RPC differentiation, suggesting its potential role in determining RPC fate. Therefore, miR-762 was selected for further study. Besides, qPCR analysis showed that endogenous miR-762 expression decreased gradually during RPC differentiation (Figure 1b). Moreover, the cell proliferation marker Ki-67 and the retinal neuronal differentiation marker β3-tubulin were used to monitor the RPC differentiation process. Our data demonstrated that Ki-67 expression level gradually decreased, while the expression level of β3-tubulin was significantly upregulated during the differentiation of RPCs (Figure 1c-d), implying that the differentiation process was occurring. Together, these results demonstrated that miR-762 expression was negatively related to RPC differentiation, suggesting that miR-762 may regulate RPC proliferation and differentiation.

3.2. miR-762 promotes RPC proliferation and inhibits RPC differentiation

To investigate whether miR-762 plays a role in dominating RPC proliferation and differentiation,

RPCs were treated with miR-762 mimics (pre-miR -762 group) or inhibitors (anti-miR-762 group) to overexpress or silence miR-762 (Figure 2a). After RPCs were cultured for 3 days, the cells were used for qPCR analysis, CCK8 assay and Edu assay under proliferation conditions. qPCR analysis revealed a remarkably increase in the expression levels of Ki-67 upon miR-762 mimics treatment in the RPC cultures, while silencing of miR-762 had the opposite effect (Figure 2b). However, qPCR results showed that the expression levels of differentiation markers including GFAP (a glial cell

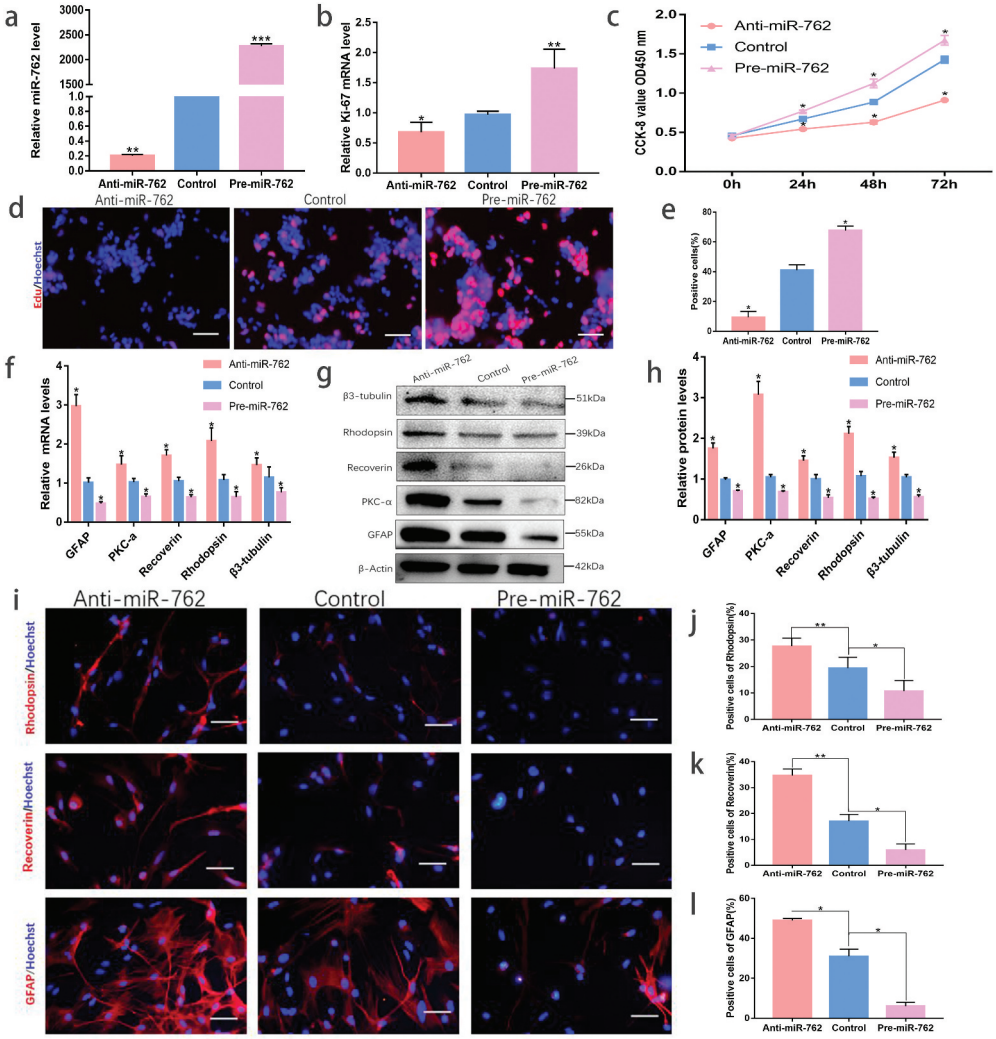

Figure 2. miR-762 promotes RPC proliferation and inhibits RPC differentiation. (a) The qPCR results showed that miR-762 expression in RPCs was sharply upregulated with pre-miR-762 treatment and significantly downregulated with anti-miR-762 treatment. (b) According to the qPCR results, Ki-67 expression increased under the transfected with pre-miR-762 RPCs whereas decreased when the cells were transfected with anti-miR-762 under proliferation conditions. (c) RPC proliferation ability was evaluated by CCK8 analysis. The proliferation ability of RPCs obviously enhanced when the cells were transfected with pre-miR-762, whereas it decreased after treatment with the miR-762 inhibitor under proliferation conditions. (d-e) Edu-positive cells were markedly increased in the RPC cultures treated with miR-762 mimics while dramatically decreased in the RPC cultures treated with miR-762 inhibitor compared to the control group. (f-h) Based on the qPCR and western blotting results, RPC differentiation-related markers (GFAP, PKC-α, Recoverin, Rhodopsin, and β3-tubulin) expression levels were repressed by the miR-762 mimic and were remarkably upregulated by the miR-762 inhibitor under differentiation conditions. (i-l) Compared with the control, immunocytochemistry demonstrated that the ratios of Rhodopsin-, Recoverin- and GFAP-positive cells were meaningfully increased with miR-762 inhibitor-treated RPC cultures, while they were reduced when RPCs were transfected with the miR-762 mimic under differentiation conditions. All experiments were repeated at least three times. Scale bars: 50 μm. β-Actin was used as a loading control. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

marker), PKC-α (a marker for retinal bipolar neurons), Recoverin (a marker for rod and corn photoreceptors), Rhodopsin (a marker for photoreceptors) and β3-tubulin (a pan-neuronal marker) have no obvious change, indicating that miR-762 has no effect on the differentiation of RPCs under proliferative culture conditions (Supplementary Figure 1). In addition, CCK8 analysis was applied to verify the impacts of miR-762 on the proliferation of RPCs. Pre-miR-762 treatment significantly promoted the proliferation of RPCs, whereas miR-762 knockdown elicited the opposite effect (Figure 2c). Besides, according to the Edu assay results, Edu-positive cells were increased in the miR-762 mimics-treated cultures (68.5 ± 4.17%) while Edu-positive cells were decreased in the miR-762 inhibitors-treated cultures (9.33 ± 3.78%) compared to control (41 ± 5.03%) (Figure 2d–e). Collectively, these results revealed that miR-762 could positively regulate RPC proliferation, which is important for obtaining a large number of cells.

RPCs transfected with miR-762 mimics or miR-762 inhibitors were cultured for 7 days under differentiation conditions and then cells were subjected to qPCR analysis, western blotting and immunocytochemistry. Knockdown of miR-762 was accompanied by an increase in the expression levels of RPC differentiation markers, including GFAP, PKC-α, Recoverin, Rhodopsin and β3-tubulin; however, pre-miR-762 decreased with the levels of these markers (figure 2f). Additionally, consistency with qPCR results, western blotting demonstrated that miR-762 played a notable role in the protein levels of the differentiation markers (Figure 2g-h). As revealed in the immunocytochemistry assay, the proportions of GFAP- (6.56% vs 31.01%, respectively), Recoverin- (5.87% vs 17.13%, respectively) and Rhodopsin-positive (10.67% vs 19.33%, respectively) cells were reduced in miR-762 mimic-treated RPCs compared with the control group. On the contrary, downregulation of miR-762 enhanced the percentage of GFAP- (49.01% vs 31.01%, respectively), Recoverin- (34.66% vs 17.13%, respectively) and Rhodopsin-positive cells (27.67% vs 19.33%, respectively) (Figure 2i-l). These results indicated that miR-762 negatively regulates RPC differentiation toward retinal neural cells.

In summary, these results revealed that miR-762 serves as a positive regulator in the proliferation of RPCs and negatively regulates RPC differentiation.

3.3. NPDC1 acts as a potential target of miR-762

miRWalk, TargetScan and miRbase [21,40,41] were utilized to predict potential targets of miR-762. As shown in Figure 3a, two potential targets, NPDC1 (neural proliferation, differentiation and control 1) and Myod1 (myogenic differentiation 1), which have been reported to have important effects on cell proliferation and differentiation, were identified. Based on our qPCR analysis and western blotting, NPDC1 expression level progressively enhanced during the differentiation of RPCs, whereas Myod1 expression had no distinct change at the same time. (Figure 3b-g), implying that only NPDC1 was the potential functional target of miR-762 in this process.

3.4. NPDC1 reduces RPC proliferation and enhances RPC differentiation

To investigate the role of NPDC1 in RPC fate determination, qPCR analysis, CCK8 analysis, western blotting and immunocytochemistry were performed in this study. As shown in Figure 4a, qPCR analysis displayed that the downregulation of NPDC1 by siNPDC1 and the upregulation of NPDC1 by NPDC1 clone were significant. Then, to evaluate the impact of NPDC1 on the proliferation of RPCs, NPDC1 siRNA or NPDC1 clone was transfected into RPCs for 3 days under proliferation conditions. Compared with the control cells, significantly upregulated and downregulated expressions of Ki-67 were observed after siNPDC1 and NPDC1 clone were transfected into RPCs, respectively (Figure 4b). In addition, CCK8 analysis revealed that the expansion capability of RPCs was enhanced in the siNPDC1-treated cells and inhibited in NPDC1 clone-treated cells (Figure 4c). These results indicate a crucial role for NPDC1 in negatively governing RPC proliferation.

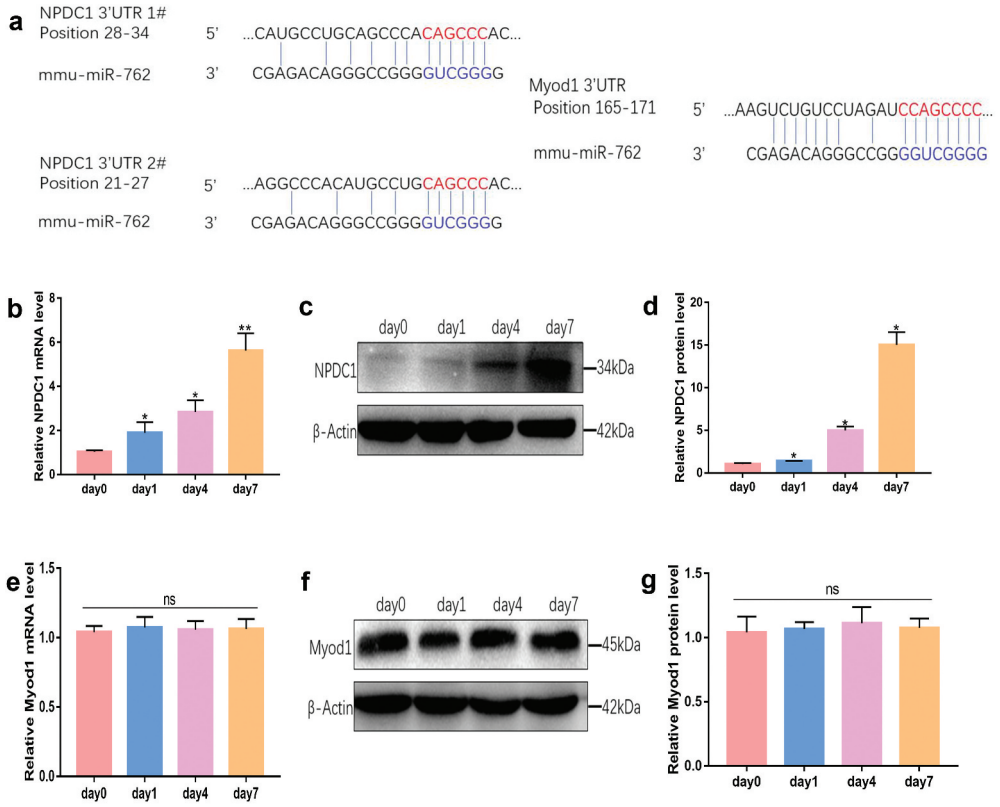

Figure 3. Potential target genes of miR-762. (a) miRWalk, TargetScan and miRbase predicted the 3 UTRs of NPDC1 (neural proliferation, differentiation and control 1) and Myod1 (myogenic differentiation 1) as potential targets of miR-762. The seed sequence is underlined. (b-g) qPCR analysis and western blotting revealed that NPDC1 expression was greatly upregulated, while no obvious change in Myod1 was observed during RPC differentiation. All experiments were repeated at least three times. β-Actin was used as a loading control. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

To evaluate the role of NPDC1 in the differentiation of RPCs, the cells were cultured in differentiation medium for 7 days with siNPDC1 (siNPDC1 group) or NPDC1 clone (NPDC1 group) treatment. The expression levels of GFAP, PKC-α, Recoverin, Rhodopsin and β3-tubulin in the RPC cultures were obviously decreased with siNPDC1-treated and markedly increased with NPDC1 clone treatment, based on the qPCR analysis and western blotting results (Figure 4d-f). Moreover, compared with the control, immunocytochemistry showed a decrease (GFAP 9.09% vs 28.87%, Recoverin 4.40% vs 14.93% and Rhodopsin 8.41% vs 18.20%, respectively) and increase (GFAP 48.12% vs 28.87%, Recoverin 36.74% vs 14.93% and Rhodopsin 32.69% vs 18.20%, respectively) in the positive percentages of above differentiation markers on siNPDC1 and NPDC1 clone treatment, respectively, suggesting that NPDC1 positively regulated RPC neural differentiation (Figure 4g-h).

In general, these data collectively demonstrated that NPDC1 plays an important role in negatively regulating the proliferation of RPCs and positively regulating RPC differentiation.

3.5. NPDC1 is a direct and functional target of miR-762 in RPC proliferation and differentiation

In Figures 1 and 3, the negative correlation of the expression patterns of miR-762 and NPDC1 during RPCs differentiation indicates that miR-762 might negatively govern NPDC1 to impact the determination of RPC cell fate. To demonstrate our assumption, the cells were treated with pre-miR-762 or anti-miR-762. As shown in Figure 5a, the mRNA level of NPDC1 was not significantly affected by either miR-762 knockdown or overexpression in RPC culture. Contrary to this result, western blotting results displayed that NPDC1 expression was markedly inhibited by miR-762 mimic while enhanced by the miR-762 inhibitor

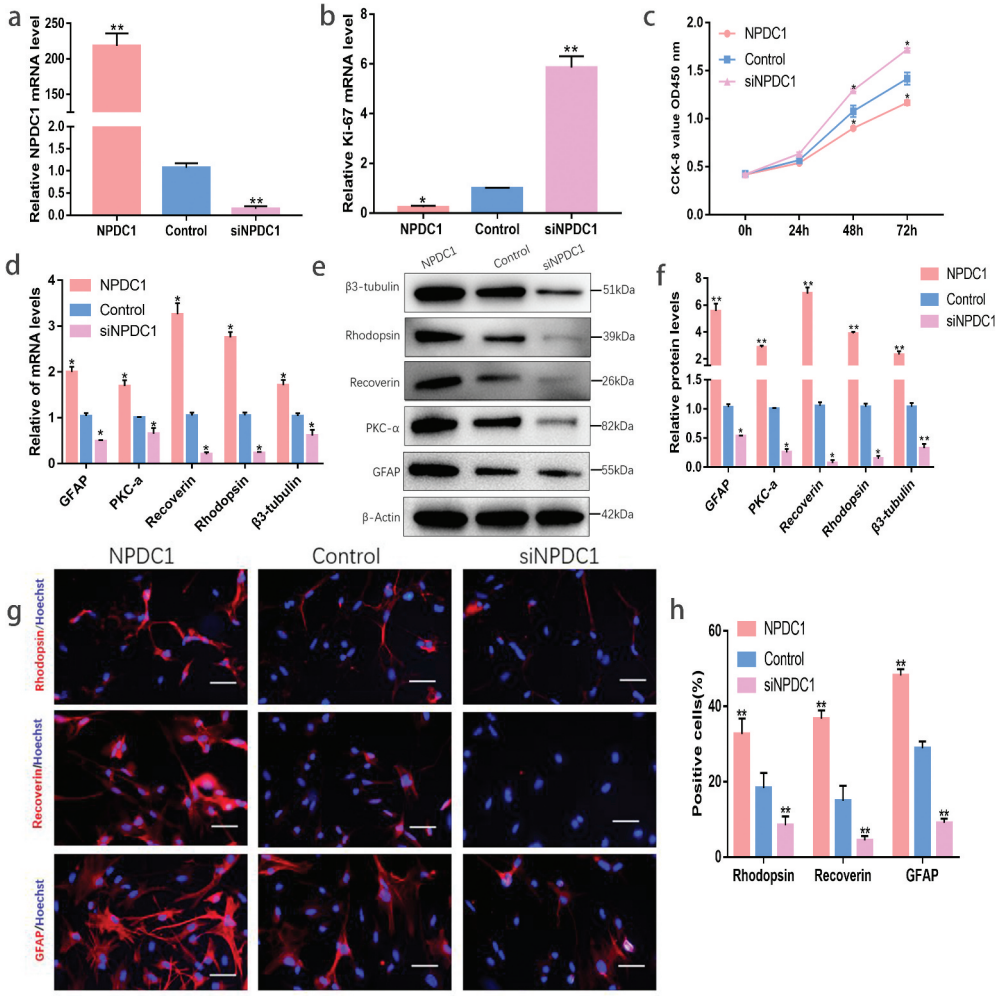

Figure 4. NPDC1 reduces RPC proliferation and enhances RPC differentiation. (a) Compared with the control, qPCR analysis showed that NPDC1 expression sharply reduced and enhanced treated with siNPDC1 and NPDC1 clone, respectively. (b) qPCR analysis demonstrated that the expression of Ki-67 increased sharply with siNPDC1 treatment and decreased with NPDC1 clone treatment under proliferation conditions. (c) After culture for 3 days, the proliferation ability of RPCs with siNPDC1 and NPDC1 clone treatment were increased and decreased, respectively, compared with other groups, as demonstrated by the CCK8 assay. (d-f) As shown in the qPCR analysis and western blotting, the RPC differentiation ability was markedly inhibited with the treatment of siNPDC1 and enhanced with NPDC1 clone treatment under differentiation conditions. (g-h) Immunocytochemistry with antibodies against Rhodopsin, Recoverin and GFAP suggested the effects of siNPDC1 and NPDC1 clone on RPC differentiation were similar to qPCR analysis and western blotting. All experiments were repeated at least three times. Scale bars: 50 μm. β-Actin was used as a loading control. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

compared to the control cells (Figure 5b-c). Based on the immunocytochemistry data, compared with the control group, the proportions of NPDC1-positive (4.85% vs 23.61%, respectively) cells were reduced in miR-762 mimic-treated RPCs while increased (57.57% vs 23.61%, respectively) in miR-762 inhibitor-treated RPCs (Figure 5d-e). The data indicate that NPDC1 may be a direct target of miR-762.

To further define whether miR-762 directly targets NPDC1, we carried out a luciferase-based assay to validate whether NPDC1 was directly regulated by miR-762. The sequences of the NPDC1 3 -UTR, which included the miR-762 binding site (NPDC1 3 -UTR-wt) and its mutant (NPDC1 3 -UTR-mut) at positions 28–34, were inserted immediately downstream of the firefly luciferase coding sequence in the GP-miRGLO plasmid. Compared to NPDC1 3 UTR-mut, co-transfection of NPDC1 3 UTR-wt with miR-762 effectively decreased luciferase activity, whereas reporter plasmids co-transfected with miR-NC had no effect on luciferase activity (Figure 5f-g). These data demonstrate that miR-762 dominates

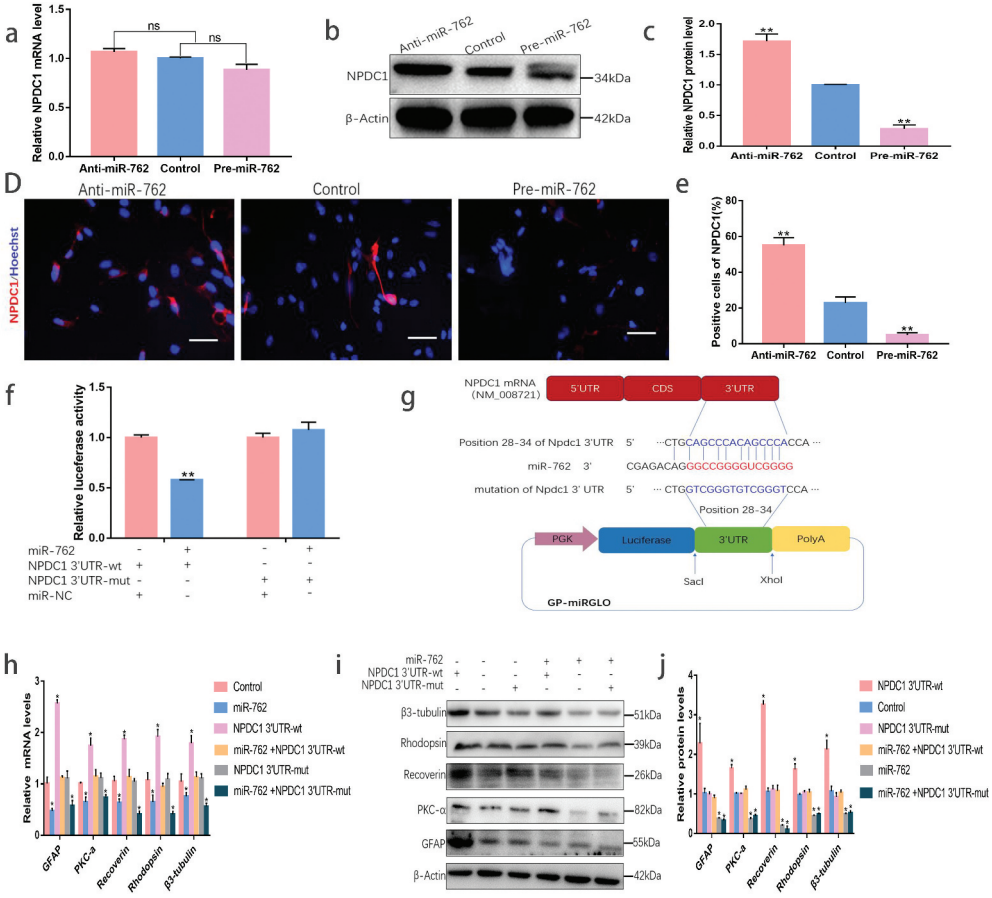

Figure 5. NPDC1 is a direct and functional target of miR-762 in RPC cultures. (a) qPCR analysis demonstrated that there were no significant differences in the levels of NPDC1 mRNA in response to transfection with miR-762 mimics or inhibitors. (b-e) Western blotting and immunocytochemistry indicated that NPDC1 expression was downregulated upon overexpression of miR-762 and upregulated with the treatment of miR-762 inhibitor. (f-g) Positions 28–34 of the wild-type NPDC1 mRNA 3 -UTR (NPDC1 3 UTR-wt) or a mutated 3 -UTR (NPDC1 3 UTR-mut) sequence were inserted into the GP-miRGLO plasmids. Compared with the other groups, co-transfection of the NPDC1 3 UTR-wt and miR-762 distinctly reduced the luciferase activity after normalization to Renilla luciferase activity as a control, implying that miR-762 bound to NPDC1 3 UTR-wt. (h-j) The impacts of miR-762 on the differentiation of RPCs could be antagonized by NPDC1 3 -UTR-wt overexpression, while the co-transfection of miR-762 and NPDC1 3 -UTR-mut had no obvious impact on RPC differentiation compared with transfection of miR-762 alone, as shown by qPCR analysis and western blotting. All experiments were repeated at least three times. Scale bars: 50 μm. β-Actin was used as a loading control. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

NPDC1 expression via direct binding to its 3 -UTR.

A "rescue" experiment was also carried out to determine whether miR-762 regulates RPC fate determination by targeting NPDC1. qPCR analysis and western blotting revealed that overexpression of NPDC1 3 UTR-wt rescued the inhibition of RPC differentiation induced by pre-miR-762 treatment. In addition, NPDC1 3 UTR-mut could not rescue the inhibitory effect of miR-762 on the differentiation of RPCs (Figure 5h-j).

Overall, as shown in the schematic model in Figure 6, our results convincingly demonstrated that miR-762 positively regulates the proliferation but negatively regulates the differentiation of RPCs by directly repressing its target NPDC1.

4. Discussion

Stem cell therapy has emerged as a promising strategy to treat many diseases [42]. Retinal degenerative diseases including age-related macular degeneration (AMD) and retinitis pigmentosa (RP) cause permanent visual loss and affect millions worldwide with no effective treatments to slow or reverse the progression of these diseases. Stem cells including retinal progenitor cells (RPCs), embryonic stem cells and induced

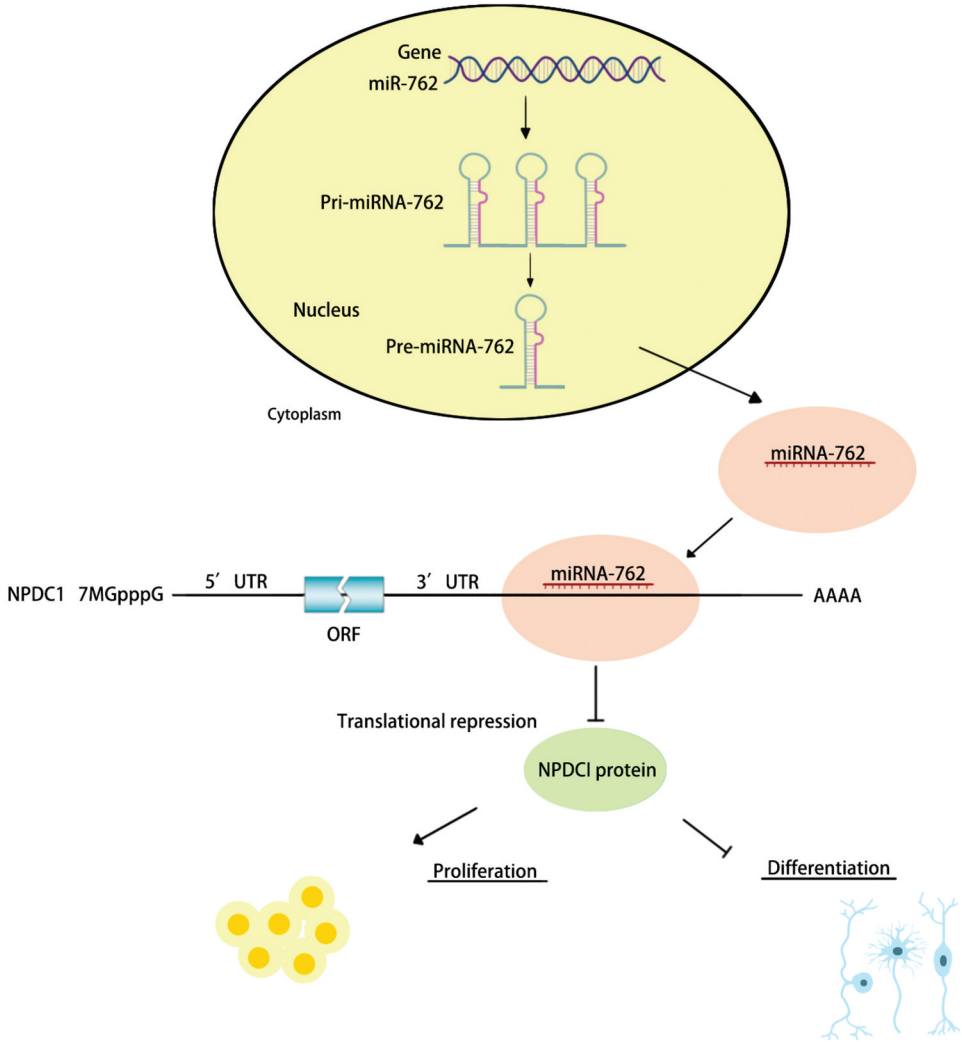

Figure 6. A schematic model of the interaction mechanism between miR-762 and NPDC1 in governing RPC proliferation and differentiation. miR-762 decreases the mRNA expression level of NPDC1 by binding to the NPDC1 3 -UTR and, as a result, positively regulates RPC proliferation while negatively governs the differentiation of RPCs.

pluripotent stem cells hold great promise for transplantation therapy to apply for retinal degenerative diseases. Among the three stem cell types, RPCs isolated from the retina are the preferred cell source for ophthalmic cell-based therapeutics without issues of tumourigenesis and immunological responses [43]. Retinal progenitors can integrate into the retina, differentiate into retinal neuronal cells, form synaptic connections and even improve visual function [44–46]. However, the limited proliferation and differentiation capacity toward specific retinal neurons of RPCs is a significant obstacle for their potential future clinical application. An increasing body of studies shows that microRNAs have a vital impact on stem cell fate determination [16,17]. In the present study, our data demonstrate that miR-762 plays an important role in governing the proliferation and differentiation of RPCs via directly targeting NPDC1.

In previous studies, a large group of microRNAs have been implicated to play important roles in promoting stem cell proliferation and/or inhibiting stem cell differentiation, whereas others have the opposite effect. For instance, miR-9 and miR-124 have the ability to induce bone marrow stromal cells trans-differentiation into neuron-like cells, whereas miR-128 negatively regulates the neuronal differentiation of these cells [47–49]. In neural progenitor cells, miR-124 and miR-200 can inhibit proliferation while enhancing neuronal differentiation [50,51], whereas other microRNAs do

the opposite, that is, enhance proliferation while inhibiting differentiation [52–54]. However, according to recent reports on retinal progenitors, only a few microRNAs have been shown to have a vital influence on the negative regulation of RPC proliferation and/or the positive regulation of RPC differentiation [37,38]. Whether microRNAs can enhance RPC proliferation and/or decrease RPC differentiation remains unclear. In this study, our data revealed that miR-762, abundantly expressed in proliferative RPCs, can markedly enhance RPC proliferation, providing a new way to obtain a sufficient number of RPC cells, which is very important for potential applications of RPCs in future cell transplantation therapy. Moreover, our data demonstrated that miR-762 could weaken RPC neuronal differentiation. To our knowledge, this is the first report that miRNAs (miR-762) can positively regulate RPC proliferation while negatively regulating RPC differentiation.

miRNAs are 18–25 nt long noncoding RNAs that bind target messenger RNAs (mRNAs), leading to destabilization and translational inhibition of the transcripts [18]. In our study, based on the predictions in miRWalk (mirwalk.umm.uni-heidelberg.de), TargetScan (www.targetscan.org) and miRbase (www.mirbase.org), two potential target genes of miR-762 were identified, namely, NPDC1 (neural proliferation, differentiation and control 1) and Myod1 (myogenic differentiation 1) [55–58]. Our further data revealed that only NPDC1 served as the direct and functional target of miR-762 in regulating RPC proliferation and differentiation. NPDC1 is highly expressed in the nervous system, which could inhibit brain neuronal progenitor cell proliferation while accelerating their neural differentiation [55,56]. Consistent with these findings, our data demonstrated that NPDC1 could decrease RPC proliferation and enhance RPC neural differentiation.

5. Conclusion

In conclusion, this is the first report that a microRNA (miR-762) is positively correlated with RPC proliferation and negatively regulates RPC differentiation by directly regulating its target (NPDC1) in vitro, which provides a better understanding of the molecular mechanism of microRNAs in governing RPC fate determination. We expect that further in vivo investigations of miR-762 and NPDC1 will shed light on future cell transplantation treatments for retinal degenerative diseases.

Disclosure statement

No potential conflict of interest was reported by the authors.

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