c-Jun-mediated microRNA-302d-3p induces RPE dedifferentiation by targeting p21Waf1/Cip1

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Abstract
Dedifferentiation of retinal pigment epithelium (RPE) cells and choroidal neovascularization (CNV) contributes to the pathogenesis of age-related macular degeneration (AMD). MicroRNAs (miRNAs) have crucial roles in AMD onset and progression. We thus aim to investigate the effects of miRNAs on RPE dedifferentiation and endothelium cell (EC) behavior, and analyze its downstream pathways. We have previously identified miR-302d-3p as the most downregulated miRNA signature along with RPE differentiation. Herein, in vitro study supported that miR-302d-3p induces RPE dedifferentiation typified by reduction of RPE characteristic markers, interrupts its phagocytosis, and promotes its migration, proliferation, and cell-cycle progression. c-Jun was identified as a potential upstream transcript factor for MIR302D, which might modulate RPE function by regulating miR-302d-3p expression. P21Waf1/Cip1, a cyclin-dependent kinase inhibitor encoded by the CDKN1A gene, was identified as a downstream target of miR-302d-3p. Our data suggested that p21Waf1/Cip1 could promote RPE differentiation, and inhibit its proliferation, migration, and cell-cycle progression. We also demonstrated that miR-302d-3p suppresses RPE differentiation through directly targeting p21Waf1/Cip1. In addition, the miR-302d-3p/CDKN1A axis was also involved in regulating tube formation of ECs, indicating its potential involvement in CNV formation. Taken together, our study implies that miR-302d-3p, regulated by c-Jun, contributes to the pathogenesis of both atrophic and exudative AMD. MiR-302d-3p promotes RPE dedifferentiation, migration, proliferation and cell-cycle progression, inhibits RPE phagocytosis, and induces abnormal EC behavior by targeting p21Waf1/Cip1. Pharmacological miR-302d-3p inhibitors are prospective therapeutic options for prevention and treatment of AMD.

Introduction
Retinal pigment epithelium (RPE), located in the outer retina between photoreceptor outer segments and choroidal vessels, is a monolayer of pigmented cells essential for maintaining regular retinal functions. The post-mitotic RPE cells are required to cope with high metabolic rates and protein synthesis, digest toxic metabolite generated from phototransduction, and function under highly oxidizing conditions, all of which make RPE cells vulnerable to premature death. Abnormal RPE behaviors have been implicated in causing many retinal disorders, including age-related macular degeneration (AMD) and AMD is a leading cause for irreversible vision loss in people aged over 55, and can be further categorized into the atrophic and exudative forms. Other than abnormal RPE functions, exudative AMD is also typified by choroidal blood vessels growing through the Bruch’s membrane toward retina (choroidal neovascularization; CNV). Bleeding of these
vessels may cause acute vision loss\textsuperscript{5}. By far, no efficient treatment has been raised for atrophic AMD. Although therapies targeting neovascularization, like intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents and photodynamic therapy (PDT)\textsuperscript{6–8}, have been developed for AMD, treatment resistance, and CNV recurrence have been observed in a non-negligible fraction of patients\textsuperscript{9}. We have previously identified that RPE dedifferentiation, characterized by reduction of RPE specific proteins, is an early consequence of AMD\textsuperscript{12}. Thus, elucidation of early initiating events originating RPE abnormalities, especially RPE dedifferentiation, could allow the development of clinical prevention and interventions for AMD. However, the precise mechanism underlying RPE dedifferentiation is still poorly understood.

MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules ranging from 19 to 25 nucleotides. miRNAs usually regulate gene expressions by directly binding to particular sites in the 3′-untranslated region (3′-UTR) of targeted mRNAs\textsuperscript{13–15}. Other factors, including miRNA’s competition with other miRNAs, their interactions with transcriptional factors and long non-coding RNAs, and epigenetic modifications, like DNA methylation, would further confine a complete elucidation into their clear roles. By far, over 2000 human miRNAs have been identified, which regulate the expressions of almost 60% of protein-coding mRNAs including key factors involved in multiple signaling pathways, and stabilize gene networks against aberrant fluctuations\textsuperscript{16–18}.

MiRNAs are involved in many biological processes including development and differentiation\textsuperscript{19}. We have previously used a microarray to identify most differentially expressed miRNA signatures along with the differentiation from human-induced pluripotent stem cells (hiPSC) to RPE cells\textsuperscript{20}. Our array data suggested that miR-302d-3p is consistently downregulated along with the differentiation, which was further proved by real-time PCR\textsuperscript{20}. MiR-302d-3p is the mature miRNA encoded by the MIR302D (MIM: 614599) gene, which is located on 4q25 and belongs to the highly conserved miR-302 family. MiR-302 family has been revealed to target many biological pathways, including epigenetic regulation and cell-cycle progression\textsuperscript{21–23}. However, the role of miR-302s in RPE dedifferentiation and CNV formation is poorly understood. In the present study, we aim to reveal the effects of miR-302d-3p on RPE dedifferentiation and endothelium cell (EC) behavior, and analyze its downstream pathway, thus finding out potential therapeutic targets to interrupt this process.

**Results**

**MiR-302d-3p triggers RPE dedifferentiation**

To investigate the role of miR-302d-3p on RPE differentiation, two cell lines, including hiPSC-RPE cells at 30 days post differentiation (dpd) and adult retinal pigmented epithelium (ARPE-19) cells, were transfected with miR-302d-3p mimic or inhibitor to modulate its expression. MiR-302d-3p mimic is chemically synthesized oligonucleotides identical to endogenous miR-302d-3p sequence, which could be loaded into RNA-induced silencing complex (RISC) and silence target genes like endogenous miR-302d-3p\textsuperscript{24}. MiR-302d-3p inhibitors are antisense miR-302d-3p oligonucleotides, which could directly bind to the single strand mature miR-302d-3p to block its activity\textsuperscript{25}. According to our results, endogenous miR-302d-3p expression was remarkably reduced in hiPSC-RPE and ARPE-19 cells transfected with miR-302d-3p inhibitor (Fig. 1a, b).

We initially compared expressions of RPE characteristic markers at the mRNA and protein levels among different transfected groups. mRNA of retinoid isomerohydrolase (RPE65; NM_000329), retinaldehyde-binding protein 1 (RLBP1; NM_000326), tyrosine-protein kinase Mer (MERTK; NM_006343), bestrophin-1 (BEST1; NM_001139443), catenin beta-1 (CTNNB1; NM_001904), and tight junction protein ZO-1 (TJP1; NM_003257) were measured by real-time PCR. Protein levels for ZO-1 (NP_003248), MERTK (NP_006334), keratin type I cytoskeletal 18 (encoded by KRT18, NM_000224; NP_000215), and β-Catenin (NP_001895) were determined via immunoblotting. Our results revealed that ectopic miR-302d-3p overexpression suppressed both the mRNA and protein expressions of RPE characteristic markers in hiPSC-RPE cells (Fig. 1c, e, f), while endogenous miR-302d-3p insufficiency promoted their expression (Fig. 1d, g). We next conducted immunofluorescence staining to show the expression pattern of ZO-1 in hiPSC-RPE and ARPE-19 cells. Consistently, ZO-1 expression was enhanced in both hiPSC-RPE and ARPE-19 cells with miR-302d-3p knocked down, but decreased in miR-302d-3p mimic transfected cells (Fig. 1h, i).

We next compared the mRNA expressions of two pluripotency relevant genes, including homeobox protein NANOG (NANOG; NM_024865) and POU domain class 5 transcription factor 1 (POLISF1; NM_002701). Our data revealed that both NANOG and POLISF1 expression were elevated in hiPSC-RPE cells transfected with miR-302d-3p mimic (Fig. 1j), and were reduced in cells transfected with miR-302d-3p inhibitor (Fig. 1k). Taken together, our results indicated that miR-302d-3p induces RPE dedifferentiation.

**MiR-302d-3p inhibits RPE phagocytosis**

A crucial function of RPE is the phagocytosis of daily shed photoreceptor outer segments, which is essential to keep retinal homeostasis\textsuperscript{29}. Impairment of RPE phagocytic ability has an essential role in AMD pathogenesis\textsuperscript{27–29}. We next determined whether miR-302d-3p would disturb RPE phagocytosis. Consistent with above findings, phagocytic ability was suppressed in ARPE-19 cells transfected with miR-302d-3p mimic when compared to cells transfected with NC
mimic (Fig. 2a, b), but was promoted in cells transfected with miR-302d-3p inhibitor compared to the NC inhibitor transfected group (Fig. 2c, d). To further determine whether the effects of miR-302d-3p on RPE phagocytosis is independent of RPE cell death, we next measured the role of miR-302d-3p on RPE apoptosis using the Annexin-V-FITC/propidium iodide (PI) apoptosis assay. According to our results, miR-302d-3p showed no detectable effect on the apoptosis of ARPE-19 cells (Fig. 2e–h), further supporting its role in interrupting RPE phagocytosis. Taken together, our data implied that miR-302d-3p inhibits RPE phagocytosis, thus interrupting regular RPE functions.
Fig. 2 MiR-302d-3p disturbs RPE phagocytosis, promotes RPE proliferation, migration, and cell-cycle progression. a–d Phagocytosis ability was suppressed in ARPE-19 cells transfected with miR-302d-3p mimic compared to cells transfected with NC mimic (a, b), and was promoted in cells transfected with miR-302d-3p inhibitor compared to cells transfected with NC inhibitor (c, d). e–h No detectable change in apoptosis rate was revealed by Annexin-V-FITC/PI apoptosis assay in ARPE-19 cells transfected with miR-302d-3p mimic compared to cells transfected with NC mimic (e, f), nor in cells transfected with miR-302d-3p inhibitor compared to cells transfected with NC inhibitor (g, h). i–l Proliferative rates were elevated in ARPE-19 cells transfected with miR-302d-3p mimic compared to cells transfected with NC mimic (i), and were suppressed in cells transfected with miR-302d-3p inhibitor compared to cells transfected with NC inhibitor (j). Migration was promoted in ARPE-19 cells transfected with miR-302d-3p mimic compared to cells transfected with NC mimic (k), and was inhibited in cells transfected with miR-302d-3p inhibitor compared to cells transfected with NC inhibitor (l). m–p Cell-cycle progression was induced in ARPE-19 cells transfected with miR-302d-3p mimic compared to cells transfected with NC mimic (m, n), and was prohibited in cells transfected with miR-302d-3p inhibitor compared to cells transfected with NC inhibitor (o, p). Overall, 10,000 cells were analyzed for each sample. The values represented the percentage of cells in each phase of the cell cycle. *p < 0.05, **p < 0.01; ***p < 0.001. Scale bar = 20 μm.
MiR-302d-3p induces RPE migration, proliferation, and cell-cycle progression

Reportedly, dedifferentiation of post-mitotic tissues, including RPE, can be followed by cell proliferation and migration\(^{19,30}\). On the basis of our above findings, we hypothesized that miR-302d-3p might function in promoting RPE proliferation and migration. We continuously monitored cell proliferative and migratory rates up to 72 hours (h) post transfection to study the role of miR-302d-3p in ARPE-19 cells. Transforming growth factor beta-1 (TGF-β1) induces epithelial–mesenchymal transition in RPE cell, which could subsequently lead to its proliferation and migration\(^{31}\). Therefore, TGF-β1 treated ARPE-19 cells were taken as the positive control group, and untreated ARPE-19 cells were regarded as the negative control group. Both proliferation and migration were induced in cells overexpressing miR-302d-3p when compared to the negative control group and to cells transfected with NC mimic (Fig. 2i–k), and were inhibited in cells transfected with miR-302d-3p inhibitor when
compared to the positive control group and to cells transfected with NC inhibitor (Fig. 2j–l). We next tried to
determine the role of miR-302d-3p in cell cycle progression through DNA contents analysis. Our results
demonstrated that miR-302d-3p overexpression increased the relative fraction of ARPE-19 cells in S and G2/M
phases and decreased the fraction of ARPE-19 cells in G0/ G1 phase (Fig. 2m, n), while silencing of miR-302d-3p
showed opposite effects (Fig. 2o, p). Taken together, our findings suggested that miR-302d-3p could promote RPE
migration, proliferation, and cell-cycle progression.

c-Jun is a potential transcript factor of MiR302D
PROMO online program (http://alggen.lsi.upc.es/cgi-
bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was
applied to predict transcription factor binding sites
(TFBS) in the potential promoter region (1 to 2000 base
pairs [bp] upstream of the transcription start site) of
the MiR302D gene (NR_029859)32,33. c-Jun was identified as a
putative transcript factor of MiR302D with 8 potential
TFBS in MiR302D promoter (Fig. 3a). Dissimilarities for
the 8 TFBS were 10.15% (TFBS1), 5.78% (TFBS2), 7.54%
(TFBS3), 4.44% (TFBS4), 6.67% (TFBS5), 3.81% (TFBS6),
3.24% (TFBS7), and 8.81% (TFBS8). A sequence logo for
all 8 potential TFBS in MiR302D promoter was created by
WebLogo (Fig. 3b)34. To better illustrate the regulatory
role of c-Jun in MiR302D expression, we used SP600125
(Beyotime, Shanghai, China), an inhibitor of the c-Jun N-
terminal kinase (JNK), and anisomycin, a JNK activator, to
suppress or promote the JNK pathway and the activation
of c-Jun. On the basis of our findings, expression of miR-
302d-3p was remarkably reduced in ARPE-19 cells
incubated with SP600125 compared to cells treated with
dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA)
(Fig. 3c), while miR-302d-3p expression was elevated in
ARPE-19 cells treated with anisomycin compared to
DMSO treated cells (Fig. 3d). Thus, our study implies the
regulatory role of c-Jun on MiR302D expression.

CDKN1A is a direct target of miR-302d-3p in RPE cells
Cyclin-dependent kinase inhibitor 1 (CDKN1A; NM_000389) gene is previously found as a target of miR-
302d in human adipose tissue-derived mesenchymal stem
cells (hASCs)35. We thus aimed to investigate whether
CDKN1A is a target of miR-302d-3p in RPE cells. Initial
assessments indicated that expression level of CDKN1A is
inversely correlated with miR-302d-3p expression. In
ARPE-19 cells overexpressing miR-302d-3p, both mRNA
and protein levels of CDKN1A were decreased (Fig. 3e, g, h), while mRNA expression of CDKN1A were elevated in
ARPE-19 cells transfected with miR-302d-3p inhibitor
(Fig. 3f, g, i). Our data suggested that CDKN1A expression
was inhibited by miR-302d-3p in both mRNA and protein
levels. We next measured whether the effect of miR-302d-
3p on CDKN1A expression is a direct consequence of
miR-302d-3p binding CDKN1A 3′-UTR using luciferase
reporter assay. We transfected ARPE-19 cells with miR-
302d-3p mimic or NC mimic together with recombinant
plasmids CDKN1AWT and CDKN1AMU. CDKN1AWT
plasmid contains a 63 bp wild-type fragment of CDKN1A
3′-UTR covering binding sites with miR-302d-3p, while
11 nucleotides in the binding region were mutated in the
CDKN1AMU plasmid (Fig. 3j). According to our results,
Luciferase activity was significantly reduced in ARPE-19
cells co-transfected with CDKN1AWT and miR-302d-3p
mimic compared to cells co-transfected with CDKN1AWT
and NC mimic (Fig. 3k). Introduction of 11 nucleotides
mutation located in the core binding region of CDKN1A
abolished its ability to bind miR-302d-3p (Fig. 3k). Taken
together, our findings suggested that miR-302d-3p
directly targets CDKN1A 3′-UTR and suppresses
CDKN1A expression in RPE.

MiR-302d-3p modulates RPE differentiation by targeting
p21Waf1/Cip1
P21Waf1/Cip1, protein encoded by the CDKN1A gene, is
reported to prevent RPE cells from going through the G1/
S phase checkpoint and inhibit their proliferation36. Thus,
we hypothesized that miR-302d-3p modulates RPE differ-
entiation by targeting p21Waf1/Cip1. We next detected
whether p21Waf1/Cip1 could mediate the effects of miR-
302d-3p on RPE differentiation. Effectiveness of AcFlag-
CDKN1A and CDKN1A-small interfering RNA (siRNA)
in modulating CDKN1A mRNA and p21Waf1/Cip1 protein
expressions was initially confirmed in ARPE-19 cells
(Fig. 4a–e). Noteworthy, three pairs of siRNA oligos
targeting different regions of CDKN1A were designed and
synthesized (data not shown). Only siRNA with best
efficiency and stability was selected for further investiga-
tions. AcFlag-CDKN1A presented a transfection ef-
ciciency of over 60% in both ARPE-19 cells and human
umbilical vein endothelial cells (HUVECs). mRNA
expressions of several RPE characteristic markers,
including paired box protein pax-6 (PAX6; NM_00127612),
RLBP1, lecithin retinol acyltransferase (LRAT; NM_004744),
KRT18, and BEST1, were monitored in ARPE-19 cells from
different transfected groups to assess RPE status. We found that overexpression of p21Waf1/Cip1 could
medicate the negative effect of miR-302d-3p on RPE differentiation in a dose-dependent manner (Fig. 4f).
In addition, with sufficient p21Waf1/Cip1 dosage, the negative effect could be fully rescued (Fig. 4f).
Consistently, CDKN1A silencing was also found to sup-
press RPE differentiation in a dose-dependent manner
(Fig. 4g). Silencing of CDKN1A could also fully disturb
RPE differentiation (Fig. 4g). Collectively, our findings
suggest that miR-302d-3p inhibits RPE differentiation via
directly targeting p21Waf1/Cip1.

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P21Waf1/Cip1 promotes RPE differentiation, and inhibits RPE proliferation, migration, and cell-cycle progression

As miR-302d-3p regulates RPE differentiation by targeting p21Waf1/Cip1, we next determined whether p21Waf1/Cip1 could benefit the regular function of RPE. RPE differentiation relevant markers, including ZO-1, microphthalmia-associated transcription factor (MITF, NP_937802), MERTK, β-Catenin, and Keratin 18, were elevated in hiPSC-RPE cells at 30 dpd overexpressing endogenous p21Waf1/Cip1 (Fig. 5a, b). Concomitantly, those markers were reduced in cells transfected with CDKN1A-siRNA compared to scramble siRNA (Fig. 5a, c). Thus, our data suggested a promotive role of CDKN1A in RPE differentiation.

We also monitored impacts of p21Waf1/Cip1 on RPE proliferation, migration, and cell-cycle progression. As indicated by our findings, rates of both proliferation and migration were downregulated in ARPE-19 cells transfected with AcFlag-CDKN1A compared to empty vector (Fig. 5d, f), while were upregulated in cells transfected with CDKN1A-siRNA compared to scramble siRNA (Fig. 5e, g), supporting that p21Waf1/Cip1 inhibits RPE proliferation and migration. Furthermore, p21Waf1/Cip1 overexpression resulted in increased fraction of ARPE-19 cells in S and G2/M phases and decreased fraction in G0/G1 phases (Fig. 5h, i), while knock down of CDKN1A showed opposite effects by promoting cell-cycle progression (Fig. 5j, k). Altogether, our findings indicated that p21Waf1/Cip1 promotes RPE differentiation, and inhibits its proliferation, migration, and cell-cycle progression.
MiR-302d-3p induces VEGF-A secretion by RPE cells, and triggers tube formation of HUVECs through regulating of p21Waf1/Cip1

As RPE dysfunction or degeneration also contributes to the onset and progression of wet AMD, we therefore aimed to tell whether miR-302d-3p participates in CNV generation. Aberrant and redundant VEGF-A secretion in RPE cells triggers CNV formation, we thus first measured expressions of VEGF-A (NP_003367) protein secreted by RPE cells and VEGFA (NM_003376) mRNA expressed in RPE cells. Our data suggested that both secreted VEGF-A protein and expressed VEGFA mRNA were increased in ARPE-19 cells transfected with miR-302d-3p mimic when compared to cells transfected with NC mimic (Fig. 6a, c), and were decreased in cells transfected with miR-302d-3p inhibitor when compared to cells transfected with NC inhibitor (Fig. 6b, d). We next performed tube formation assay on HUVECs. We found that miR-302d-3p overexpression induces tube formation of HUVECs (Fig. 6e, f), while miR-302d-3p insufficiency suppresses HUVEC tube formation (Fig. 6e, g). Our data indicated the involvement of miR-302d-3p in triggering abnormal EC behaviors. We next detected whether miR-302d-3p regulates tube formation of HUVECs through p21Waf1/Cip1. Our findings suggested that p21Waf1/Cip1 overexpression could
medicate the promotive effect of miR-302d-3p mimic on tube formation; while silencing of CDKN1A suppressed the inhibitory role of miR-302d-3p inhibitor when compared to NC inhibitor (Fig. 6h–j). Further assessments suggested that tube formation of HUVECs was decreased in cells transfected with AcFlag-CDKN1A, and were increased in cells transfected with CDKN1A-siRNA (Fig. 6k–m). Collectively, these data implied that miR-302d-3p promotes VEGF-A secretion by RPE cells and tube formation of HUVECs by directly targeting p21Waf1/Cip1, implying its potential involvement in CNV generation.

**MiR-302d-3p promotes proliferation and migration of HUVECs**

To better understand the cellular effects of miR-302d-3p/CDKN1A axis on HUVEC function, we further assessed whether this axis will affect the proliferation and
mig**:ation** of HUVECs. According to our results, both proliferation and migration were promoted in HUVECs transfected with miR-302d-3p mimic (Fig. 7a, c), while were inhibited in cells transfected with miR-302d-3p inhibitor (Fig. 7b, d). In addition, proliferation and migration rates were reduced in HUVECs overexpressing p21Waf1/Cip1 (Fig. 7e, g), while were elevated in cells with p21Waf1/Cip1 insufficiency (Fig. 7f, h). No apoptosis was detected in HUVECs transfected with miR-302d-3p mimic or inhibitor when compared with the control group (Fig. 7i, j). Our data suggested that miR-302d-3p shows a promotive role in HUVEC proliferation and migration, further indicating its role in promoting tube formation of HUVEC.

**Discussion**

In mature organs and tissues, miRNAs have more essential roles in monitoring cellular stress than primary cellular functions. Essential roles of miRNAs and their processing factors in keeping the survival of RPE cells have been revealed. Thus, investigating the influence of miRNAs on retinal degenerative diseases and seeking for potential treatment are promising and expanding. We have previously identified that, miR-302 members including miR-302d-3p, miR-302a-5p, miR-302a-3p, and miR-302c-5p, were consistently downregulated along with the differentiation of RPE cells.

Herein, we select the most declined miR-302d-3p for further analysis, and revealed that miR-302d-3p triggers RPE dedifferentiation, inhibits RPE phagocytosis, and further induces abnormal EC behaviors by targeting p21Waf1/Cip1. Dedifferentiation of RPE contributes to the pathogenesis of atrophic AMD, and abnormal choroidal vascular functions associates with exudative AMD. Thus, our finding indicates that miR-302d-3p might have a role in the pathogenesis of both atrophic and exudative AMD. Compared to traditional therapeutic strategies, miRNA-based treatments have more advantages in drug efficiency and delivery. Gene therapies targeting miRNAs in treating CNV have also been well developed. MiR-302s have many cell-cycle targets, such as OCT4, SOX2, NR2F2, CCND1, CDK2, and CDKN1A. Previous studies have confirmed that miR-302s, including miR-302d-3p, could directly bind to the 3′-UTR of CDKN1A mRNA in various cell types. P21Waf1/Cip1, protein encoded by CDKN1A, is a cyclin-dependent kinase inhibitor that prevents cells from going through the G1/S phase checkpoint. Increased p21Waf1/Cip1 expression in RPE cells inhibits its proliferation and migration. In this study, our data support that miR-302d-3p modulates RPE function and EC behavior by targeting p21Waf1/Cip1. We also reveal that p21Waf1/Cip1 promotes RPE differentiation, inhibits RPE proliferation, migration, cell-cycle progression, and facilitates EC function.

In conclusion, our study implies that miR-302d-3p promotes RPE dedifferentiation and induces abnormal EC function by targeting p21Waf1/Cip1, which might contribute to the pathogenesis of both atrophic and exudative forms of AMD. MiR-302d-3p inhibitors are prospective therapeutic options for AMD treatment. However, in vivo animal studies are still warranted to better illustrate...
whether miR-302d-3p over-dosage could directly cause AMD and the underlying precise mechanism.

**Materials and methods**

**Mimics, inhibitors, siRNA, and plasmids**

Human miR-302d-3p mimic and inhibitor, together with negative control (NC) mimic and inhibitor, were purchased from GenePharma Co., Ltd (Shanghai, China). Scramble siRNA (NControl_0518) and CDKN1A-siRNA were purchased from Ribobio (Guangzhou, China). Sequences of mimics, inhibitors, and siRNAs were detailed in Supplementary Table S1. Open reading frame sequence of the CDKN1A gene was synthesized, amplified, and inserted into the expression vector pCMV-C-
Flag (Beyotime). BamHI and XhoI restriction sites were used to generate the AcFlg-CDKN1A plasmid with primers listed in Supplementary Table S2. A 63 bp fragment from the 3\'-UTR of the CDKN1A gene containing its binding region with miR-302d-3p was synthesized and cloned into the pGL3-Promoter Vector (Promega, Madison, WI, USA) using NheI and XhoI restriction sites to construct the recombinant plasmids CDKN1A\textsuperscript{WT} and CDKN1A\textsuperscript{MU}. The CDKN1A\textsuperscript{MU} plasmid covered 11 mutated nucleotides located in the core binding region of CDKN1A as shown in Fig. 3j. Constructed plasmids were sequenced and confirmed using Sanger sequencing.

Cell culture and transfection

HiPSC were cultured and differentiated into RPE cells as detailed previously\textsuperscript{20}. Both ARPE-19 cells and HUVECs were purchased from American Type Culture Collection (ATCC). ARPE-19 cells were maintained in Dulbecco\textquoteleft s Modified Eagle (DME)/F12 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 g/ml) at 37 °C, 5% CO\textsubscript{2}. HUVECs were cultured in F12 medium supplemented with 10% FBS, 0.05 mg/ml endothelial cell growth supplement (BD Biosciences, Palo Alto, CA, USA), penicillin (100 U/ml), and streptomycin (100 g/ml) at 37 °C, 5% CO\textsubscript{2}. Complete medium was short for supplemented culture medium in the following text. For transfection assay, hiPSC-RPE at 30 dpd, ARPE-19 cells and HUVECs were initially seeded into 6-well templates. Cells were transfected with 100 pmol mimic/inhibitor/siRNA, and/or 4 μg expression vector at 50–60% confluence using Lipofectamine\textsuperscript{TM} 2000 transfection reagent (Invitrogen) per the manufacturers\textquoteleft protocol.

RNA extraction, RT-PCR, and real-time PCR

Both hiPSC-RPE at 30 dpd and ARPE-19 cells were harvested at 48 h post transfection for RNA isolation. Total RNA was extracted with TRIzol reagent (Invitrogen) as indicated previously\textsuperscript{58,59}. RNA concentration and quality were measured using Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). cDNA was then synthesized for mRNA with a PrimeScript RT Kit (Takara, Otsu, Shiga, Japan), and was generated for miRNA by stem-loop reverse transcription (Applied Biosystems, Darmstadt, Germany). Primers were provided in Supplementary Table S2. Human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046) and U6 gene expressions were analyzed in parallel for normalization of mRNA and miRNA expressions, respectively.

Immunoblotting and immunofluorescence

ARPE-19 cells were planted into 6-well plates for immunoblotting, and were grown on 8-well chamber slides (Millipore, Billerica, MA, USA) for immunofluorescence. Cells were maintained in complete medium, and were harvested at 72 h post transfection. Immunoblotting and immunofluorescence were conducted using a previously described protocol\textsuperscript{60,61}. Antibodies were detailed in Supplementary Table S3. ImageJ software (https://imagej.nih.gov/ij/index.html) was applied to determine and quantify protein expressions.

Analysis of phagocytosis

Carboxylate-modified polystyrene latex beads (1 μm in diameter; Sigma) with yellow-green fluorescence (emission maximum: 515 nm) were used for phagocytosis analysis. ARPE-19 cells were grown and transfected on 8-well chamber slides (Millipore). At 48 h post transfection, cells were then incubated with phosphate buffered saline (PBS) diluted fluorescent beads (70 beads per cell) at 37 °C for 12 h. After the incubation, cells were washed with PBS for three times to stop the phagocytosis, treated with 0.2% trypan blue for 10 min (min) to quench extracellular fluorescence, and fixed in 4% paraformaldehyde (PFA) for 15 min. Cell nuclei were then counterstained by 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min. Images were collected with an Olympus IX70 confocal laser-scanning microscope (Olympus, Tokyo, Japan). ImageJ software was used to quantify fluorescence.

Apoptosis assay

ARPE-19 cells and HUVECs at 48 h post transfection were collected, washed with PBS, suspended in staining buffer, and incubated with Annexin-V-FITC (R&D, New Jersey, USA) and PI (R&D) per the manufacturers\textquoteleft protocol. Flow-cytometric analysis was subsequently performed to identify apoptotic cells with a gallios flow cytometry (Beckman Coulter, Brea, USA). A total of 10,000 living cells were collected for each sample. Three groups of untreated ARPE-19 cells and HUVECs were included for scatter gating: (1) Unstained cells for cell selection and adjustment of photomultiplier voltage; (2) Annexin-V-FITC stained only cells for adjustment of the FITC channel; (3) PI stained only cells for adjustment of the phycoerythin channel. Data were then displayed as two-color dot plot with Annexin-V-FITC (X axis) vs. PI (Y axis). Annexin-V positive cells were recognized as apoptotic cells.

Monitoring cell proliferation and migration

We used xCELLigence system E-Plate (Roche) to monitor the proliferation and migration rates of ARPE-19 cells and HUVECs according to the manufacturer\textquotesingle s protocol. To measure the proliferation rates, 5000 cells were
planted into each well of the E-Plate, and were transfected at 24 h post plantation. For migration analyses, 40,000 cells were seeded into each well right after transfection. All cells were maintained in complete medium. Impedance values were automatically monitored by the xCELLigence system and expressed as a cell index value.

Cell-cycle analysis
ARPE-19 cells from different transfected groups were harvested and fixed with 70% ethanol overnight. After extensive washing with PBS, cells were then suspended in PBS containing propidium iodide (Sigma), and incubated for 30 min before flow-cytometric analysis. Acquired data were then analyzed using the kaluz for gallios software (Beckman Coulter). A total of 10,000 cells from each sample were measured to modulate the cell-cycle.

Luciferase reporter assay
Luciferase reporter assay was performed according to a previously defined protocol. Briefly, cells were seeded into 24-well plates and transfected with 16 ng cytomegalovirus–Renilla (Promega), 20 pmol miR-302d mimic or NC mimic, and 800 ng cytomegalovirus–Renilla (Promega), 20 pmol miR-302d mimic or NC mimic, and 800 ng CDKN1A WT or CDKN1A WT or CDKN1A MU. Cells were collected 72 h post transfection for luciferase activities measurement using the dual luciferase system (Promega) with a GloMax-96 luminometer. Renilla luciferase activities were taken as internal standard indicators for transfection efficiency. Firefly luciferase activities were further normalized to Renilla luciferase activities.

Enzyme-linked immunosorbent assay (ELISA)
To determine the secretion of VEGF-A, ARPE-19 were planted onto 6-well transwell plates (Corning) precoated with matrigel (1:30 diluted in DMEM/F12 medium; BD Biosciences, San Jose, CA, USA). The culture medium was collected and changed every day post transfection. Collected medium was used to determine the expression level of VEGF-A using a commercial human VEGF-A ELISA kit (Beijing 4A Biotech Co., Ltd, Beijing, China) per the manufacturer’s protocol.

Tube formation assay
For detection of capillary-like structure formation, transfected HUVECs were planted onto growth factor-reduced matrigel (BD Biosciences) in a 24-well plate. Cells were observed at 24 h post plantation with a bright-filed microscope. ImageJ software was used to analyze and quantify tube number.

Statistics
We used GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA) for statistical analysis. Student’s t-test was applied for comparison between different groups. All experiments were performed in triplicates with data averaged. Data were presented as mean ± standard deviation (SD). p value < 0.05 was taken as statistically significant.

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Conflict of interest
The authors declare that they have no conflict of interest.

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