

Retinal Pigment Epithelium Extracellular Vesicles Induce Microglia Polarization in *MERTK*-Associated Retinal Degeneration

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PURPOSE. Retinitis pigmentosa (RP) is a hereditary retinal disease. *MERTK*-associated RP is characterized by earlier onset and rapid progression, featuring retinal pigment epithelium (RPE) inflammation and microglial activation, yet the underlying mechanisms remain incompletely understood. The study aimed to elucidate the intrinsic interactions between RPE inflammation and microglial activation mediated by RPE-derived extracellular vesicles (EVs).

METHODS. Induced pluripotent stem cell (iPSC)-derived RPE models from *MERTK* mutant patients and healthy controls were established and characterized by transcriptomic analysis. RPE-derived EVs were isolated, and their RNA and protein cargo were systematically profiled using transcriptomic and proteomic data, revealing a potential role in retinal inflammation regulation. The effects of RPE-EVs on microglial activation were confirmed through in vitro co-culture and in vivo animal experiments.

RESULTS. Transcriptomic analysis revealed that differentially expressed genes between *MERTK* mut-RPE (M-RPE) and control RPE (C-RPE) were enriched in inflammatory signaling pathways and EV-related terms. Multi-omics data further indicated that the altered RNA and protein cargo of M-RPE-derived EVs were closely associated with inflammation and immune regulation. In vitro co-culture confirmed that M-EVs could rapidly activate microglia and upregulate pro-inflammatory factors. In vivo experiments indicated that microglia phagocytosed M-EVs exhibited more pronounced M1 polarization and migratory changes.

CONCLUSIONS. RPE-derived EVs act as critical drivers of microglial M1 polarization in *MERTK*-associated RP. Our study revealed their pivotal role in the progression of early-onset severe RP, providing theoretical support for the potential of targeting EVs to modulate the retinal immune microenvironment and intervene in the progression of retinal degeneration.

Keywords: extracellular vesicle, retinal degeneration, retinal pigment epithelium, inflammation, microglia

Retinitis pigmentosa (RP) is an inherited retinal degenerative disease with an incidence of 1 in 3500 to 1 in 5000 worldwide.¹ To date, more than 100 distinct gene mutations have been identified as causative of non-syndromic RP (RetNet, <https://retnet.org/>), and most of these genes encode proteins that are essential for various functions in photoreceptor or retinal pigment epithelium (RPE) homeostasis.² RP is primarily characterized by night blindness, ultimately resulting in complete vision loss.³ *MERTK* encodes a receptor tyrosine kinase that is highly expressed on the apical membrane of RPE cells. Compared with typical RP cases, *MERTK* mutations are associated with a more severe disease progression. These patients often experience night vision impairment as early as 3 years old and exhibit severe

retinal dysfunction and early macular involvement.⁴ Previous studies have suggested that defective phagocytosis of photoreceptor outer segments (POSs) by RPE cells with *MERTK* mutations leads to photoreceptor cell degeneration.¹ However, this mechanism alone cannot adequately explain the early onset and rapid progression of *MERTK*-associated RP, and further exploration is needed.

Inflammatory activation is a prominent characteristic of RP, characterized by activation of the innate immune system, including dysfunction of immune barrier function, activation and infiltration of immune cells, and upregulation of local and peripheral inflammatory factors.⁵ Microglia, the resident immune cells of the retina, typically maintain a “resting” state under physiological conditions and are predom-

inantly localized in the retinal ganglion cell layer (GCL), inner plexiform layer (IPL), and outer plexiform layer (OPL). These cells display a highly active ramified morphology, with long and dynamic processes that continuously monitor the ocular microenvironment.⁶ In response to stimuli such as injury, infection, or widespread hypoxia, they transform into an amoeboid morphology (M1 type), rapidly retracting their highly branched processes and migrating toward the affected area. They become active phagocytes and release pro-inflammatory cytokines, such as IL-1 α , IL-6, IL-12, IL-23, TNF- α , and IL-1 β .^{7,8} Remarkably, in *MERTK*-deficient retinas, activation and redistribution of microglia can be observed, even before the RPE cells are fully matured and when RPE phagocytic activity remains at a very low level.

Given the irreversibility of retinal degeneration and the early onset of *MERTK*-associated degeneration, investigating the early pathological changes of this disease may help identify potential strategies for early intervention. Severe early-onset photoreceptor degeneration associated with *MERTK* mutations displayed early RPE inflammation and subsequent microglial activation.⁴ However, the potential association between RPE inflammation and microglial activation remains to be elucidated. Given the distinct stratification and non-adjacent positioning of RPE and microglia in the retina, it is likely that inflamed RPE cells with *MERTK* mutations communicate with distant microglia through the release of vesicle cargoes.

Extracellular vesicles (EVs) serve multiple biological functions, including intercellular communication, immune regulation, waste management, and modulation of the extracellular microenvironment.⁹ EVs derived from RPE are enriched in proteins associated with oxidative stress, complement activation, and inflammation and play a significant role in the initiation and progression of inflammation in various ocular diseases.¹⁰ However, their role in RP remains unclear, partly due to the lack of research on the contents of RPE-derived EVs and comprehensive omics analyses.

RPE forms a monolayer of cuboidal epithelial cells situated between the neural retina and the choroid.^{11,12} Comprehensive analysis of the contents and functional studies of RPE-derived EVs using well-established RP disease models is essential for elucidating their role in the onset and progression of RP. However, human RPE research lacks reliable disease models, as obtaining sufficient primary RPE cells from patients is challenging. Stem cell reprogramming techniques enable the differentiation and cultivation of patient-derived autologous induced pluripotent stem cell-derived RPE (iPSC-RPE) in vitro.^{12–14}

In this study, we employed stem cell reprogramming technology to develop a patient-specific RPE model to investigate changes in RPE-derived EV secretion in *MERTK*-associated retinal degeneration. Both in vivo and in vitro validations demonstrated that RPE-derived EVs play a critical “bridging” role between early RPE inflammation and microglial activation in *MERTK*-associated retinal degeneration. These findings highlighted the potential of targeting RPE EVs as a therapeutic strategy for severe early-onset retinal degeneration associated with *MERTK* mutations.

MATERIALS AND METHODS

Study Subjects

This study included two patients with autosomal recessive RP carrying *MERTK* mutations and two healthy controls.

The initial clinical manifestations of the patients primarily included progressive visual impairment, visual field defects, and night blindness. All procedures were conducted in accordance with the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University. Informed patient consent was obtained from all subjects in advance of sample collection. For the sequencing samples, we used iPSC lines from one *MERTK*-mutant patient and two healthy controls, each undergoing three (Control 1 and Patient 1) or one (Control 2) independent differentiation batches. Subsequent in vivo and in vitro experiments used RPE-derived EVs obtained from both patients and both controls. All in vitro and in vivo experiments were independently repeated by two trained technicians to ensure the reliability and reproducibility of the results.

Cell Cultures

Generation of iPSCs. Human iPSCs were generated from peripheral blood mononuclear cells (PBMCs) obtained from two *MERTK*-associated RP patients and two healthy donors (Fig. 1A). PBMC collection was approved by the Institutional Ethics Committee with informed consent. PBMCs were transfected with episomal plasmids expressing reprogramming factors Oct4, Sox2, Myc, Nanog, Lin28, and Klf4.^{12,15} iPSC lines were successfully generated, with normal karyotype, pluripotent morphology, and expression of typical markers. Sanger sequencing confirmed that patient iPSCs carried *MERTK* mutations. The iPSCs were cultured on vitronectin (Nuwacell Biotechnologies, Anhui, China)-coated plates in nEpic hPSC medium (Nuwacell Biotechnologies), passaged using 0.5-mM EDTA solution (Thermo Fisher Scientific, Waltham, MA, USA), and maintained at 37°C in 5% CO₂.

Generation of RPE. For RPE differentiation, we optimized a protocol based on previous methods.^{16,17} iPSCs were dissociated with accurate solution (Innovative Cell Technologies, San Diego, CA, USA) and plated onto Matrigel (Corning, Inc., Corning, NY, USA)-coated culture plates (NEST Scientific, Woodbridge, NJ, USA) at 5.0×10^5 cells/well. After 3 days, when confluence reached 85% to 90%, nEpic hPSC medium was replaced with RPE priming medium. This medium consisted of Gibco Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific) supplemented with 15% Gibco Knock-Out Serum Replacement (Thermo Fisher Scientific), 0.2-mM Gibco GlutaMAX (Thermo Fisher Scientific), 1 $\times$ MEM Non-Essential Amino Acids Solution (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL Gibco penicillin–streptomycin (Thermo Fisher Scientific), and 0.1-mM Gibco β -mercaptoethanol (Thermo Fisher Scientific). After 24 hours, when confluence exceeded 95%, the medium was changed to RPE priming medium with 10-mM nicotinamide (Sigma-Aldrich) and 50-nM chetomin (MedChemExpress, Monmouth Junction, NJ, USA), with daily feeding. For the first 7 days, suspended cells were observed daily, but the induction continued if the cells remained tightly packed. After 13 days, the medium was changed to RPE priming medium with 10-mM nicotinamide only and changed daily for 7 days, then every 2 days until day 28 (D28). On D28, cells were treated with Accutase (Thermo Fisher Scientific) for 15 minutes, replated onto new Matrigel-coated plates at a 1:3 ratio after passing through a 70- μ m strainer (Thermo Fisher Scientific), and fed with RPE maturation medium consisting of DMEM/F-12 basic (7:3)

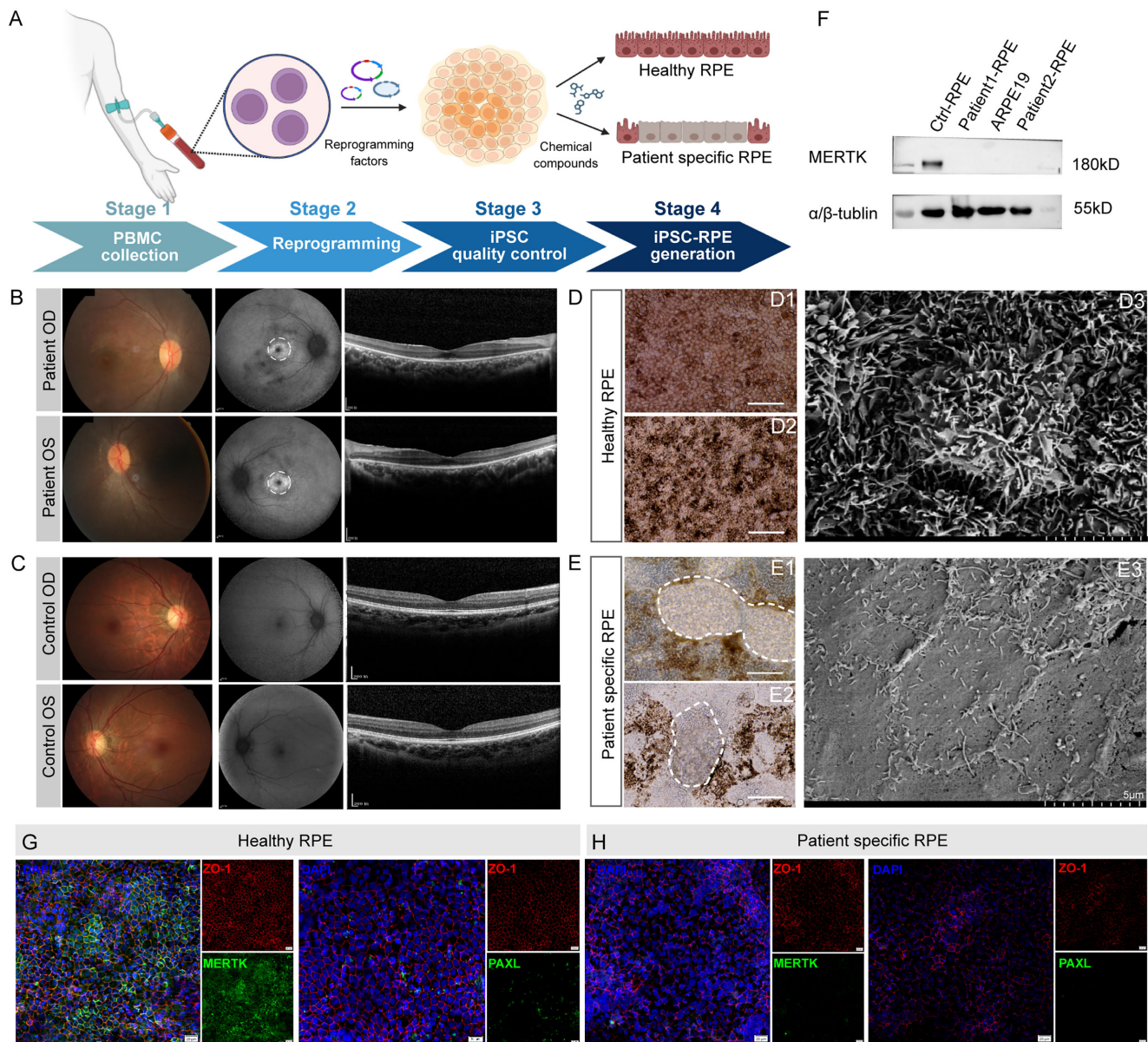


FIGURE 1. Establishment and characterization of in vitro RPE research model with RP-associated *MERTK* gene mutation. **(A)** Workflow of patient-specific iPSC and iPSC-RPE generation. **(B)** Fundus photography, fundus autofluorescence imaging, and OCT of the RP patient with the *MERTK* mutation. **(C)** Fundus photography, fundus autofluorescence imaging, and OCT of a healthy control. **(D)** Phase-contrast images (left) and SEM imaging (right) of iPSC-RPE from the control. Scale bars: 100 μ m (**D1**, **D2**), 5 μ m (**D3**). **(E)** Phase-contrast images (left) and SEM imaging (right) of iPSC-RPE from an RP patient. Patient iPSC-RPE cells also formed a tightly packed monolayer across the culture plates, except for local rosette-like cell clusters. Scale bars: 100 μ m (**E1**, **E2**), 5 μ m (**E3**). **(F)** Western blot analysis of MERTK protein expression in the ARPE19 line, control, and patient in the in vitro RPE model. **(G, H)** Immunofluorescence staining of MERTK and its phosphorylated protein PAXL in control iPSC-RPE (**G**) and patient iPSC-RPE (**H**). Scale bars: 20 μ m.

supplemented with 100 U/mL penicillin–streptomycin and 2% Gibco B27 supplement (Thermo Fisher Scientific). Fresh media was changed every 3 days, and 10- μ M ROCK inhibitor Y-27632 was added for 3 days after each passage. On D56, mature iPSC-RPE cells were harvested or passed again.

Differentiation of Microglia In Vitro. The iPSC line with luciferase/green fluorescent protein (GFP)-labeled nucleus was obtained from Nuwacell Biotechnologies, and the differentiation of iPSC-derived microglia was conducted as previously described.¹⁸ Briefly, when iPSCs reached 80% confluence, embryoid bodies were formed in iPS base medium. Cells were cultured for 4 weeks in an induc-

tion medium supplemented with 25 ng/mL hIL-3 (R&D Systems, Minneapolis, MN, USA) and 50 ng/mL hM-CSF (R&D Systems) to generate myeloid precursors. These precursors were induced into mature microglia using the microglia mature medium supplemented with 100 ng/mL hIL-34 (PeproTech, Cranbury, NJ, USA) and hM-CSF in suspension culture dishes.

Animals

This investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The

C57BL/6 mice and CX3CR1-GFP mice (The Jackson Laboratory, Bar Harbor, ME, USA) were bred in a specific-pathogen-free (SPF) animal facility under controlled temperature, humidity, and a 12-hour light/dark cycle. This study received approval from the Institutional Animal Care and Use Committee of Beijing Tongren Hospital.

EV Isolation

The cell supernatant was collected and centrifuged at 300g for 10 minutes, then at 3000g for 20 minutes at 4°C, and stored at -80°C. After thawing at 4°C, it was centrifuged at 12,000g for 30 minutes at 4°C and filtered through a 0.22-μm filter. EVs were isolated using the exosome detection via the ultrafast-isolation system (EXODUS),^{19,20} resuspended in 1× Gibco Dulbecco's Phosphate-Buffered Saline (DPBS; Thermo Fisher Scientific) to 200 μL, and stored at -80°C. EXODUS is an efficient platform that enables automatic label-free purification of EVs from various biofluids. The system achieves ultrahigh efficiency in EV purification through negative pressure oscillation and dual-coupled harmonic oscillator-enabled membrane vibration, with superior speed, yield, and purity compared with traditional separation techniques.

Nanoparticle Tracking Analysis

The concentration and size distribution of small EVs were assessed using a NanoSight NS300 (Malvern Panalytical, Worcestershire, UK) with a 488-nm laser and scientific complementary metal-oxide semiconductor (sCMOS) camera. Each EV sample purified by EXODUS was diluted 1000- to 2000-fold in 1× DPBS. Triplicate dilutions were analyzed, and each diluted sample was measured three times for 30 seconds. Consistent settings were used for all nanoparticle tracking analysis (NTA) measurements.

Quantitative Real-Time PCR Analysis

Cell RNA was isolated using an RNeasy Kit (QIAGEN, Hilden, Germany). Total RNA (300–1000 ng) was reverse-transcribed to cDNA with the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). SYBR Green-based quantitative PCR (qPCR) was performed on a Rotor-Gene 6000 system (QIAGEN). Data were analyzed using Prism 8.3 (GraphPad Software, Boston, MA, USA), with relative gene expression fold changes calculated by the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH.

Western Blot

Cells or EVs were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Proteintech, Rosemont, IL, USA) supplemented with the serine protease inhibitor phenylmethylsulfonyl fluoride (Thermo Fisher Scientific) and EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). After 15 minutes on ice, lysates were centrifuged at 4°C and 13,000g for 15 minutes, and the supernatant was collected. The lysate was quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific), with absorbance measured at 562 nm using a spectrophotometer (Molecular Devices, San Jose, CA, USA). The samples were denatured by boiling for 10 minutes with loading buffer (0.05-M Tris-HCl at pH 6.8, 1% sodium dodecyl sulfate [SDS], 10% glycerol, and 0.1% 2-mercaptoethanol).

Under an electrical current, protein samples were resolved using 10% and 5% SDS-polyacrylamide gel electrophoresis (PAGE) gels and subsequently transferred onto nitrocellulose membranes. After the membranes were blocked with a Tris-buffered saline solution containing 5% skim milk and 0.1% Tween-20 for 1 hour, they were incubated overnight at 4°C with primary antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies and exposure to the chemiluminescent substrate, immunoreactive bands were visualized using a gel imaging system (VILBER, Collégien, France).

Immunofluorescent Staining

Cells or Cryosections. RPE cells on glass slides or retinal cryosections fixed with 4% paraformaldehyde (Solarbio, Norton, VA, USA) at room temperature were blocked with PBS solution containing 4% bovine serum albumin (BSA; Beyotime Biotechnology, Jiangsu, China) and 0.5% Triton X-100 (Beyotime Biotechnology) at room temperature for 1 hour. Subsequently, they were incubated overnight at 4°C with the primary antibodies in a 1× PBS dilution containing 0.5% Triton X-100 and 1% BSA. After washing with 1× PBS, staining was performed in the dark at room temperature (RT) for 1 hour using Alexa Fluor-labeled secondary antibodies (1:400 dilution; Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI; D1306; Invitrogen). The following primary antibodies were used: RPE65 (1:400, ab235950; Abcam, Cambridge, UK); ZO-1 (1:400, 33-9100 and 61-7300; Invitrogen); MiTF (1:400, ab3201; Abcam); MERTK (1:400, 4319; Cell Signaling Technology, Danvers, MA, USA); P-Axl/Mer/Tyro3 (1:400; 44463, Cell Signaling Technology); OCT4 (1:400, ab18976; Abcam); TYRP2 (1:400, ab74073; Abcam); BEST1 (1:400, NB300-164; Novus Biologicals, Centennial, CO, USA); and PMEL17 (1:400, 38815; Cell Signaling Technology). Images were captured using a confocal microscope (IXplore SpinSR10; Olympus, Tokyo, Japan).

Retinal Whole Mount. The eyeballs were immediately isolated after sacrifice. Following removal of the cornea and lens, the eyecups were fixed in 4% paraformaldehyde at RT for 20 minutes. The retinas were then gently peeled off. After the harvested retinas were rinsed with 1× PBS, they were blocked overnight at 4°C in PBS solution containing 8% BSA and 1% Triton X-100. The primary antibody dilution was incubated at 4°C for 24 hours, followed by incubation with the Alexa Fluor-labeled secondary antibody (1:400 dilution) and DAPI at 4°C for 12 hours. The primary antibody used was IBA1 (1:400, 019-19741; Wako Chemicals, Osaka, Japan). Images were observed using an Olympus confocal microscope.

Scanning Electron Microscopy

The scanning electron microscopy (SEM) observation was performed following previously reported procedures.²¹ Briefly, RPE cells on slides were fixed overnight at 4°C in 2.5% glutaraldehyde, followed by 1-hour fixation at 4°C in 1% osmium tetroxide. After they were washed with 1× PBS, the samples were dehydrated with varying concentrations of alcohol, dried, and gold-coated after drying. Images were captured using an SU8000 scanning electron microscope (Hitachi, Tokyo, Japan).

Transmission Electron Microscopy

For transmission electron microscopy (TEM) observation, the EVs (10 μ L) were placed onto a carbon-coated copper grid for 10 minutes, excess droplets were removed by blotting with filter paper, and negative staining was performed with 2% uranylacetate for 1 minute. After excess staining solution was blotted away with filter paper and air-drying for 10 minutes, samples were observed using a Tecnai G2 12 transmission electron microscope (FEI Company, Hillsboro, OR, USA).

Nanoparticle Flow Cytometry

The EVs separated by the EXODUS were incubated with fluorescent-labeled antibodies for 30 minutes in the dark. After resuspension in 1 $\times$ PBS, they were subjected to centrifugation at 4°C and 110,000g for 70 minutes twice to remove excess dye. The supernatant was removed, and the samples were analyzed using an N30E nanoparticle flow cytometer (NanoFCM, Xiamen, China) after they were resuspended in 1 $\times$ PBS. The following antibodies were used: FITC Mouse Anti-Human CD9, CD63, and CD81 (all from Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

EV Transplantation

EVs secreted by RPE were stained with a cell plasma membrane staining kit (Beyotime Biotechnology) according to the manufacturer's instructions. Wild-type mice at post-natal day 30 (P30) were anesthetized with pentobarbital sodium and ketamine. Subretinal transplantation of 1 μ L (2×10^6 particles) of EVs was performed using a blunt 5- μ L Hamilton syringe, with subretinal injection of DPBS as a control. Levofloxacin eye ointment was used in the first 3 days post-surgery to prevent intraocular infection.

RNA Sequencing

Previously published procedures were employed to study RPE cells.¹⁸ Briefly, the cells were lysed using TRIzol Reagent (Invitrogen), and total RNA was extracted and assessed for purity and integrity. A total of 1 μ g of RNA was used to prepare sequencing libraries using the NEBNext Ultra II RNA Library Prep Kit and NEBNext Multiplex Small RNA Library Prep Set (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. High-throughput paired-end sequencing was performed using the PE150 platform (Illumina, San Diego, CA, USA). Data were mapped to human genome version hg38 by HISAT2 7.5.0, and differential expression analysis was performed using the DESeq2 1.26 package in R 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria). Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and tissue expression enrichment analyses on the differentially expressed genes (DEGs) were performed with DAVID (<https://davidbioinformatics.nih.gov/>).

For EV RNA sequencing, the cell supernatant was collected and confirmed to be free of mycoplasma contamination. EV total RNA was extracted using the exoRNeasy Serum/Plasma Maxi Kit (QIAGEN). Long-chain RNA and small RNA libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit and NEBNext Multiplex Small RNA Library Prep Set, respectively. The libraries were sequenced

using the Illumina PE150 platform and aligned to the human reference genome. The microRNA (miRNA) target genes were predicted using the TargetScan and miRanda databases. Differential expression analysis was performed for messenger RNA (mRNA), long noncoding RNA (lncRNA), and miRNA. The KEGG, REACTOME, and GO enrichment analyses were performed with DAVID. Gene Set Enrichment Analysis (GSEA) was used for normalized counts of all genes from various samples.

Proteomics

Total protein was extracted from collected RPE-derived EVs, and the concentration was quantified by the Bradford method. Subsequently, the samples were separated using 12% SDS-PAGE. Mass spectrometry was employed for protein detection, following pre-separation of the digested proteins through the timsTOF Pro 2 platform. Raw files were filtered and then processed for protein identification using Proteome Discoverer 2.2 (Thermo Fisher Scientific) by searching against the human SWISS-PRO database. The differential protein expression analysis and visualization were performed using R 4.3.0. The PROGENy 1.16.0 package in R was used to compute activity scores for 14 well-characterized signaling pathways across all samples.²²

Statistical Analysis

Statistical analysis was performed using Prism 8.3 and R Studio 4.3. All data are presented as mean $\pm$ SEM. A two-tailed unpaired Student's *t*-test was used to assess differences between the two groups. Differences were considered significant when **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

RPE Developmental Abnormality and Dysfunction in MERTK-Associated Retinal Degeneration

A 14-year-old male patient (Patient 1) was diagnosed with RP. He had experienced night blindness for 1 year, along with decreased visual acuity, with a best-corrected visual acuity of 0.5/0.6. Fundus photography revealed abnormal pigmentation in the macular area of both eyes and a general cyan-gray appearance of the peripheral retina. Fundus autofluorescence imaging exhibited focal hyperautofluorescence in the central macular areas measuring 1 papilla diameter in size (Fig. 1B). Optical coherence tomography (OCT) revealed severe loss of the outer retinal layer compared with healthy volunteers (Figs. 1B, 1C). An electroretinogram indicated complete impairment of visual function. Screening of 164 known retinal degeneration genes through whole-exome sequencing identified a homozygous mutation (c.296_297delCA, p.T99Sfs*8) in the *MERTK* gene,²³ which was confirmed by Sanger sequencing. A young female patient (Patient 2) with similar but milder clinical manifestations was found to have a compound heterozygous mutation in the *MERTK* gene (c.291_292delAC, p.T99Sfs*8; c.1296+1G>A).

Because the lack of *MERTK* protein expression was observed in the human RPE cell line ARPE19 obtained from the Procell Life Science & Technology Co., Ltd. (Wuhan, China), we utilized iPSC technology to establish a stable patient-specific in vitro research model of RPE. Using a non-viral, non-integrating episomal reprogramming plasmid

system, iPSCs were generated from *MERTK* patients and unaffected control individuals and further differentiated into iPSC-RPE (Fig. 1A, Supplementary Fig. S1A). Both iPSC-RPE expressed RPE-specific markers RPE65, BEST1, TYRP2, and PMEL17 (Supplementary Figs. S1B, S1C). Under an optical inverted microscope, control iPSC-RPE displayed a tightly connected cobblestone-like arrangement with noticeable pigmentation (Fig. 1D), whereas patient iPSC-RPE showed a disordered cell arrangement, reduced pigmentation, and rosette-shaped protrusions in local areas (Fig. 1E). Patient 1 cells exhibited merged bulges, and the lesions in Patient 2 cells appeared milder, with more retained pigmentation and fewer rosette-like cell clusters, which did not yet contact each other (Fig. 1E). SEM also showed abundant microvilli structures on the apical surface of control cells (Fig. 1D), whereas the microvilli structures on the apical surface of rosette-like cell clusters from the patient cells appeared noticeably shorter and fewer (Fig. 1E). Immunofluorescence analysis showed that control iPSC-RPE (also referred to as C-RPE or control-RPE) expressed *MERTK* protein and its phosphorylated protein PAXL, but patient iPSC-RPE (also referred to as M-RPE or *MERTK* mut-RPE) did not express these proteins. Western blot (WB) corroborated these findings (Figs. 1F, 1G, 1H).

***MERTK* Mutations Caused Abnormal RPE-Derived EV Secretion**

We conducted a transcriptomic analysis to investigate the development of the in vitro model of RPE. Principal component analysis (PCA) was performed on mRNA expression profiles of control iPSC-RPE at several time points to depict the developmental dynamics of RPE cells over the course of differentiation (Fig. 2A). During the initial 56 days, cells exhibited considerable transcriptional heterogeneity, as indicated by the dispersed distribution of samples across time points, suggesting a phase of rapid development. Beyond 56 days, the samples clustered closely, suggesting transcriptional stabilization and indicating that RPE cells had reached a preliminary maturation by D56. Additionally, the gene expression profiles of the two groups were similar at the onset of development (D0), but their transcriptional trajectories progressively diverged as the induction proceeded, with a clear separation observed upon reaching maturity at D56 (Fig. 2B).

At D56, we identified a total of 4052 DEGs between the two groups (Fig. 2C). Tissue enrichment analysis indicated that these DEGs exhibited significant expression in the retina, followed by the brain and hippocampus, suggesting that *MERTK* mutation primarily impacted the development of the central nervous system, including the retina (Fig. 2D). GO and KEGG analyses revealed that these DEGs were associated with visual perception, nervous system development, synapse, and inflammatory pathways, as well as significantly regulated cell surface and extracellular components, particularly EVs (Figs. 2E, 2F). These analyses suggest that *MERTK* mutation might lead to abnormal EVs secretion from RPE cells and intercellular communication in retina.

EVs From M-RPE Exhibited Inflammation-Related Transcriptomics Alteration

EVs were isolated from mature iPSC-RPE cell supernatants, and we characterized their morphology, particle concen-

tration, size, and surface markers²⁴ to further explore the changes of content components in EVs (Fig. 3A). TEM showed that the EVs exhibited a cup-shaped morphology, and NTA revealed a size distribution of 50 to 200 nm for the majority of vesicles (Fig. 3B). Nanoflow cytometry confirmed the expression of the surface markers CD9, CD63, and CD81 on the isolated EVs (Supplementary Fig. S2).

Temporal profiling revealed pronounced stage-specific alterations in EV RNA content. RNA abundance was lowest at baseline (D0), peaked at the critical differentiation stages (D14 and D28; >1.5-fold vs. D0), and subsequently declined to a stable yet elevated level at D56 (Fig. 3C). Comparative profiling further revealed dynamic changes in EV RNA composition. Fewer than 1000 RNAs differed between C-EV and M-EV at D0, whereas the number of differentially represented RNAs increased by >1.5-fold at D14 and D28 (Fig. 3C). Database alignment revealed that protein-coding RNAs constituted ~50% of the EV transcriptome, with known non-coding RNA accounting for approximately 25% to 30% and the remaining being unknown RNA. However, for differentially expressed RNAs, the proportion of protein-coding RNAs decreased as the RPE matured, and the proportion of non-coding RNA gradually increased (Fig. 3C). The analysis indicated that protein-coding RNA was the predominant species in EVs, with mRNAs comprising the majority of differentially expressed transcripts. This suggests substantial reprogramming of mRNA cargo during RPE development and that RPE cells might selectively regulate gene expression and protein translation in recipient cells through this mechanism. However, non-coding RNA may have a more significant impact on the RP pathogenesis with *MERTK* mutation. Further analyses were focused on the RNA cargo of EVs isolated from early maturity RPE cells at D56. Among the representative 20 up- and downregulated genes, *OTX2* and *MITF*, which are associated with RPE development, maturation, and photoreceptor survival,²⁴ were found to be downregulated in M-EVs, whereas the photoreceptor-related genes *RCVRN* and *RPGR* were upregulated in M-EVs (Fig. 3D). This result suggests that RPE cells with *MERTK* mutation might tend to develop into photoreceptors and secrete insufficient factors to protect and maintain photoreceptor cells, which would be detrimental to the healthy survival of photoreceptors. GSEA indicated that the inflammation pathways, such as the carboxyl-terminal Src kinase (CSK) pathway, IL-12/signal transducer and activator of transcription 4 (STAT4) pathway, insulin like growth factor 1 (IGF-1)/mammalian target of rapamycin (mTOR) pathway, and immunoregulatory-related genes, were significantly upregulated in M-EVs compared with control EVs (Fig. 3E). This analysis indicated that mature M-RPE at D56 secreted inflammatory EVs and might potentially regulate the retinal microenvironment by transmitting inflammatory signals to retinal cells through EVs.

Given the obviously differential expression of non-coding genes in mature M-RPE at D56 (Fig. 3C), we also focused on differentially expressed lncRNAs and miRNAs and predicted their target gene functions. Enrichment analysis suggested that these target genes not only regulate phagocytosis and retinoid metabolism (processes closely related to the primary physiological functions of RPE), but, more importantly, were also extensively involved in inflammatory responses and immune regulation (Figs. 3F, 3G). This finding suggests that early-stage M-RPE cells with *MERTK* mutations may exhibit a subtle inflammatory state, which

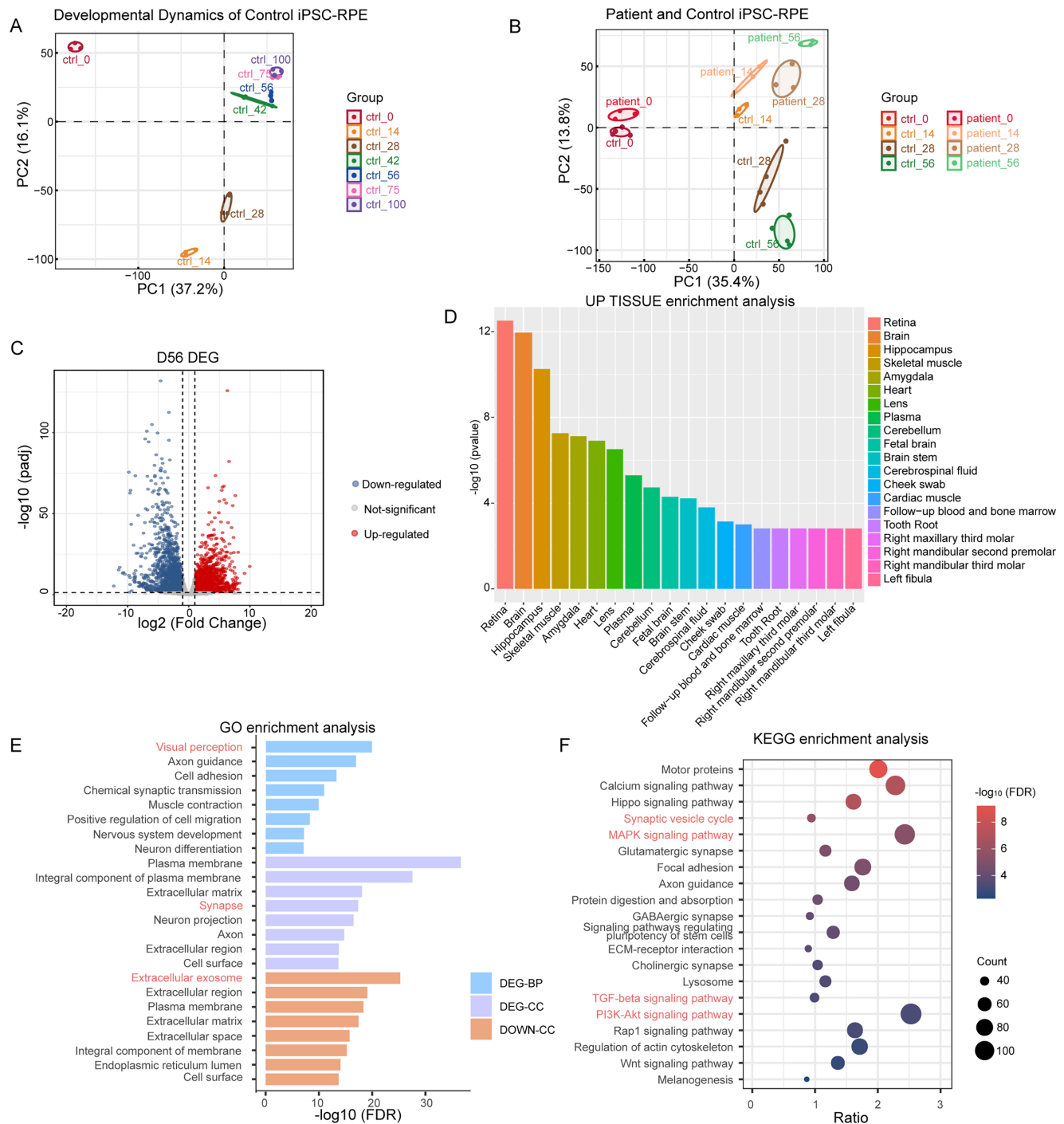


FIGURE 2. Transcriptome analysis indicated that *MERTK* mutations cause abnormal EV secretion from RPE cells. **(A)** PCA of mRNA expression in control iPSC-RPE at multiple time points to capture developmental changes in gene expression. Sample labels indicate the day of differentiation: ctrl_0, control iPSC-RPE at day 0; ctrl_14, at day 14. **(B)** PCA of mRNA expression in patient and control iPSC-RPE across developmental stages. Sample labels indicate the day of differentiation: ctrl_0, control iPSC-RPE at day 0; patient_0, patient iPSC-RPE at day 0. **(C)** Analysis of DEGs between patient and control at D56. **(D)** DAVID UP TISSUE enrichment analysis of D56 DEGs. **(E)** GO enrichment analysis of D56 DEGs. **(F)** KEGG enrichment analysis of D56 DEGs. GO and KEGG analysis of DEGs at D56 showed significant enrichment in visual perception, inflammatory pathways, and extracellular exosome terms.

might or might not be detectable through inflammatory markers but was subtly magnified through small vesicles. This widespread effect influenced the physiological functions of other retinal cells and might potentially lead to a cascading immune response.

Protein Cargo From M-RPE EVs Participated in Inflammation Pathway Alterations

In the process of RPE development, the protein components in EVs were very similar at the initial stage, but, as

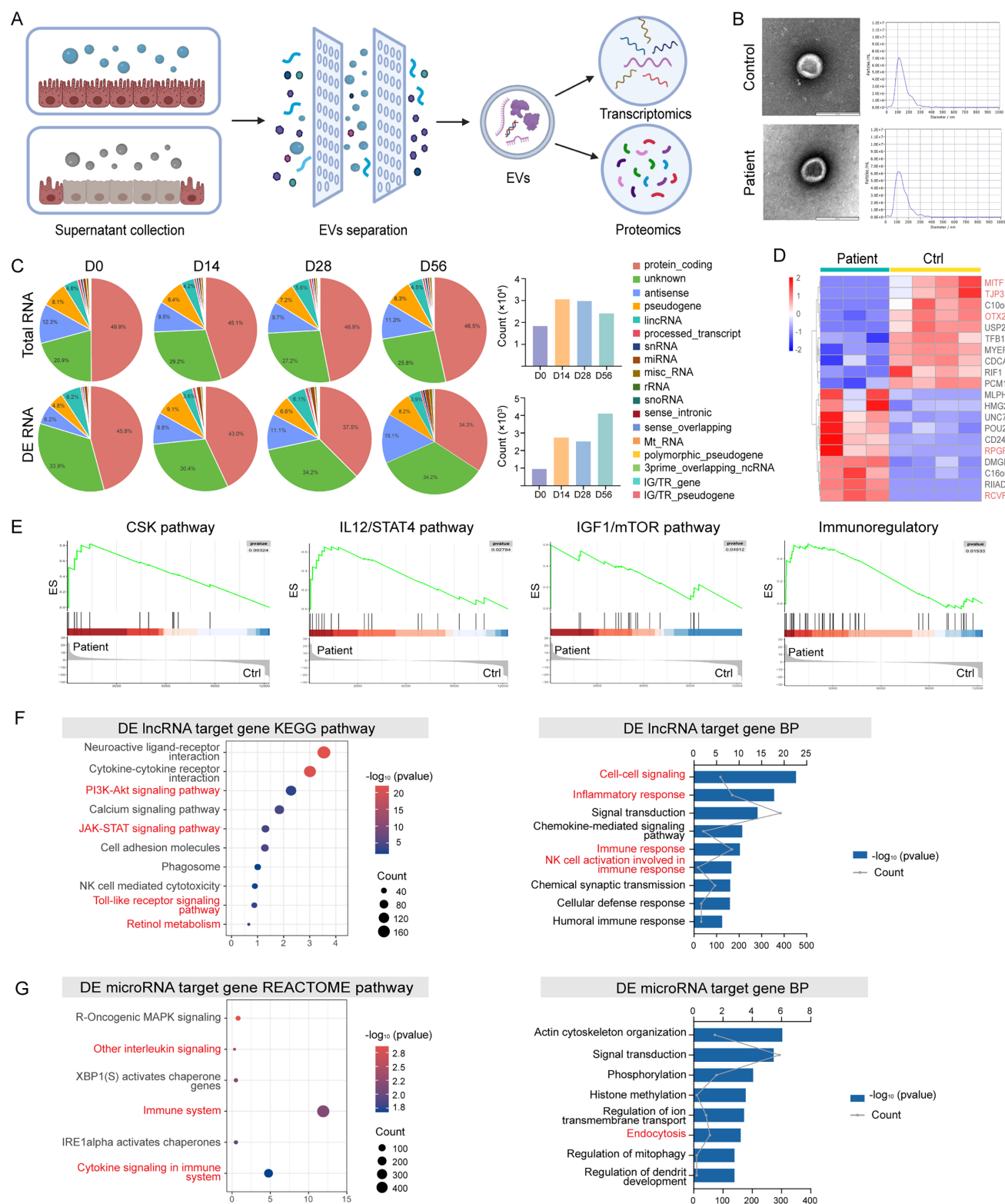


FIGURE 3. Systematic mRNA profiles of human RPE-derived EVs. **(A)** Workflow of transcriptomics and proteomics from RPE-derived EVs. **(B)** TEM images exhibited a cup-shaped morphology of EVs (*left*). Scale bars: 200 nm. NTA showed that most of the vesicles had a diameter of less than 200 nm (*right*). **(C)** The pie charts for total RNA in both EVs (*top*) and differentially expressed RNA in M-EV (*bottom*) display the proportions of various types of RNA at four stages. **(D)** Heat map of representative 20 upregulated and downregulated genes of patient RPE-derived EVs at D56. **(E)** GSEA indicated that D56 M-EVs participated in inflammation-related pathways significantly. **(F)** KEGG and GO analysis of differentially expressed lncRNA target genes at D56. **(G)** REACTOME and GO analysis of differentially expressed miRNA target genes at D56. BP, biological process.

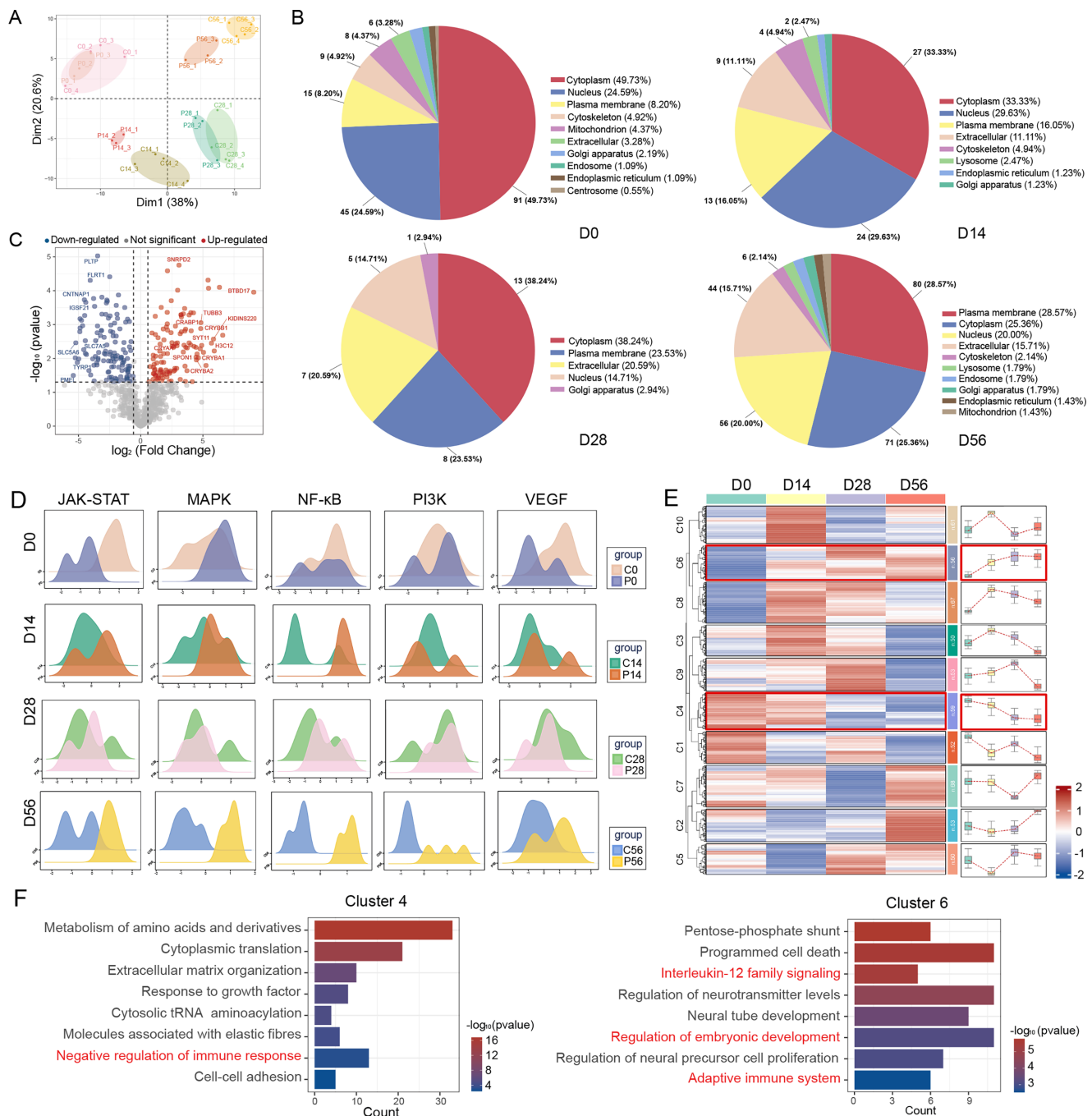


FIGURE 4. Proteome profiles of human RPE-derived EVs. **(A)** PCA analysis of protein expressions of RPE-derived EVs at different development stages. **(B)** Subcellular localization of DEPs between the two groups EVs. **(C)** DEPs of EVs at D56. **(D)** EVs from mature M-RPE (D56) promoted typical inflammation pathways upregulation, whereas immature M-RPE (D0, D14, D28) did not display this characteristic. **(E)** Cluster analysis of protein expression changes of patient RPE-derived EVs compared with control. **(F)** Enrichment analysis of representative clusters.

the RPE matured at D56, the protein cargoes in the EVs of the two groups differed significantly (Fig. 4A). The differentially expressed proteins (DEPs) in vesicles come from various sources, primarily from the nucleus, cytoplasm, and cell membrane, as well as from intracellular organelles such as mitochondria, Golgi apparatus, and lysosomes. Unlike at D0, D14, and D28, when the DEPs originated from the cytoplasm, the DEPs at D56 primarily originated from the plasma membrane (Fig. 4B). In addition, the quantity of DEPs was the largest at D56 (Fig. 4B). DEP analysis of

EVs at D56 showed an upregulation of lens-related proteins in patient EVs, including CRYBA1, CRYBA2, CRYBB1, and CRYBP1 (Fig. 4C). This finding was consistent with previous reports that RP often is associated with posterior subcapsular cataracts,²⁵ suggesting that M-RPE might regulate lens development through vesicle secretion. As RPE matured, the protein cargoes in the patient vesicles notably contributed to the promotion of an inflammatory response (Fig. 4D). Protein expressions of patient EVs displayed different trends as RPE matured (Fig. 4E). Compared with the control, the

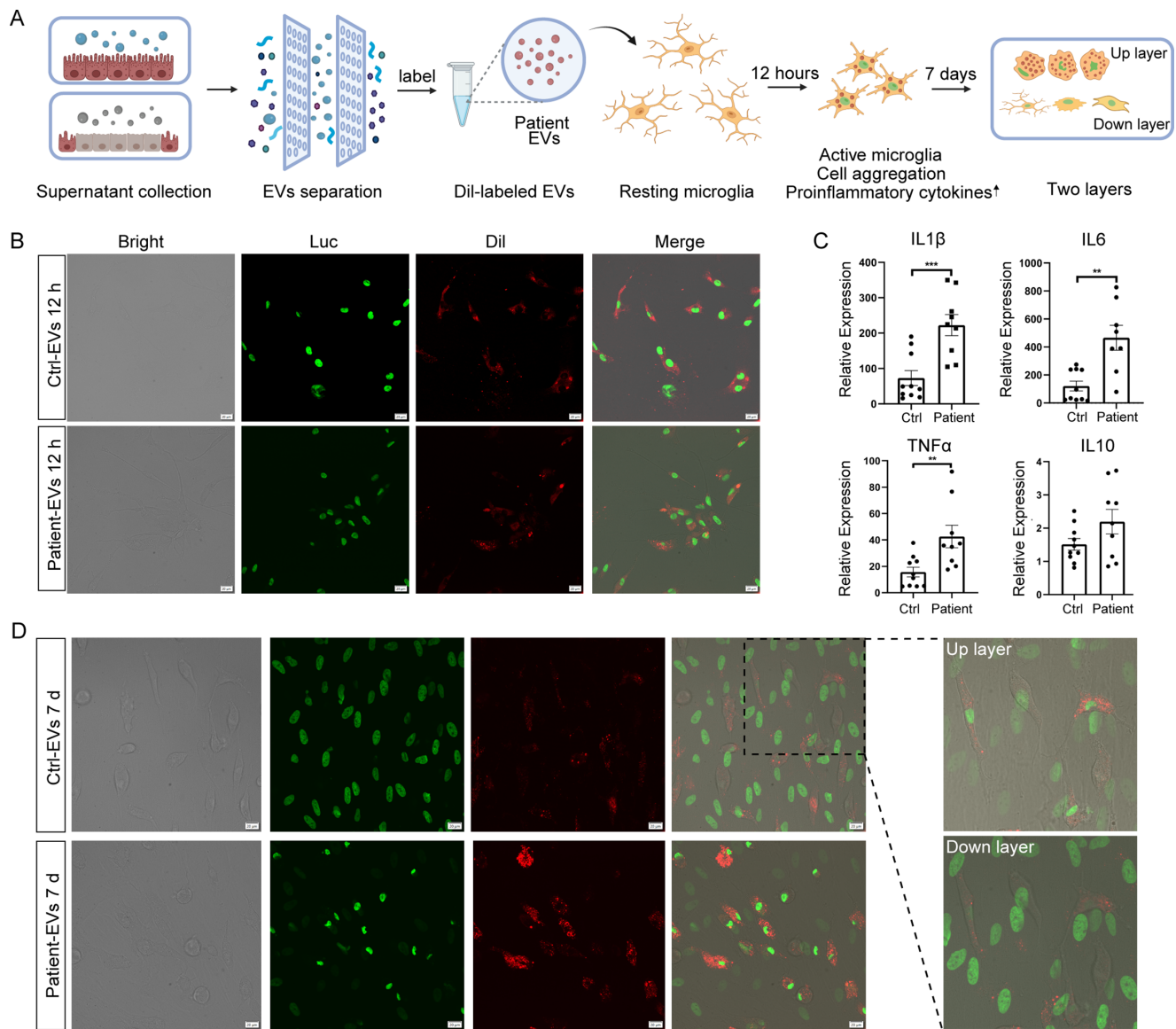


FIGURE 5. Coculture of microglia and RPE-derived EVs. **(A)** Workflow of co-culture. **(B)** Confocal imaging of microglia and EVs co-cultured for 12 hours. **(C)** qPCR analysis of inflammatory factors expression in microglia co-cultured for 12 hours. **(D)** Confocal imaging of microglia and EVs co-cultured for 7 days. *Green* indicates GFP labeled microglia nucleus; *red*, Dil-labeled EVs. Scale bars: 20 μm (**B**, **D**).

proteins with consistently increased expression (cluster 6) were associated with pro-inflammatory factor IL-12 family signaling and adaptive immune response, whereas proteins with consistently decreased expression (cluster 4) were related to negative regulation of the immune response and cell-cell adhesion (Fig. 4F).

EVs From M-RPE Promoted M1 Microglial Polarization

To observe the pro-inflammatory effects, RPE-derived EVs were co-cultured with microglia, the primary resident immune cells in the retina. EVs labeled with Dil were utilized for co-culture with iPSC-derived microglia over both the short term (12 hours) and longer term (7 days) (Fig. 5A). Upon the addition of vesicles, quiescent microglia detected changes in the culture environment, resulting in

cell migration and gradual stabilization. After 12 hours of co-culture, the addition of M-RPE vesicles led to a more pronounced aggregation of microglia compared to control vesicles (Fig. 5B). Additionally, the expression of inflammatory factors was detected in microglia after 12 hours of co-culture. The results demonstrated a significant upregulation of pro-inflammatory factors IL-6, IL-1β, and TNF-α in microglia upon exposure to EVs from M-RPE (Fig. 5C). Continuing the culture for 1 week, a distinct stratification of cells was obvious. The microglia, initially adherent to the plate, gradually separated into upper and lower layers. The upper layer cells exhibited increased engulfment of fluorescently labeled vesicles, accompanied by nuclear pyknosis, but the nucleus of the lower layer cells appeared normal. Compared to the control group, the experimental group displayed more pronounced nuclear pyknosis, enhanced fluorescence, and a higher density of engulfed vesicles (Fig. 5D). These findings revealed that M-RPE-derived EVs

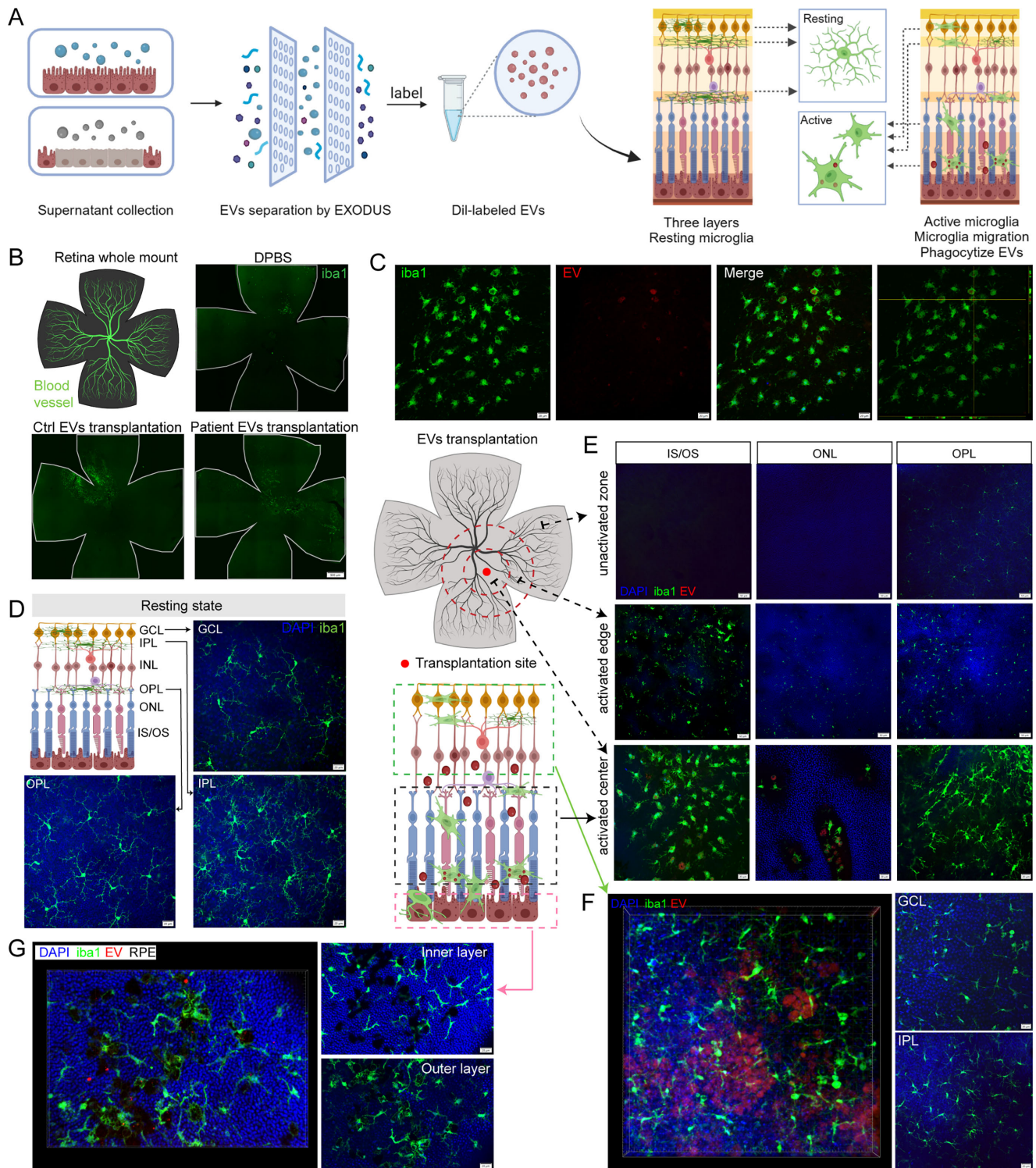


FIGURE 6. Subretinal transplantation of RPE-derived EVs promoted microglial activation and migration in the retina. **(A)** Workflow of EV transplantation. **(B)** Whole diagram of retina in the vascular layer (*top, left*). *Green* represents blood vessels in the schematic diagram. Whole-mount images of retinas approximately 40 μm outside the ONL after transplantation. *Green* indicates migrated IBA1⁺ microglia. *Scale bar*: 500 μm . **(C)** Amplified images of Dil-labeled activated and migrated microglia. *Green* indicates IBA1⁺ microglia; *red*, Dil-labeled EVs. **(D)** Distribution and morphology of microglia in the retina under physiological conditions. **(E)** Microglia morphology and distribution of OPL, ONL, and subretinal in the activated central zone, quiescent zone, and transition zone for the same retina after transplanting EVs. **(F)** Microglia morphology and distribution of IPL and GCL in the retinal activation area. **(G)** Morphology and distribution of microglia/macrophages near RPE in the retinal activation area. *Scale bars*: 20 μm (C–G).

rapidly activated microglia, and prolonged co-culture led to microglial death. The aberrantly dead microglia typically required the migration of surrounding immune cells for further phagocytosis and digestion of the debris. This inflammatory response would further exacerbate the inflammatory environment in the retina.

Microglia Were Activated After Phagocytosis of RPE EVs

Dil-labeled EVs were transplanted into the subretinal space of 4-week-old wild-type or CX3CR1-GFP mice, and the whole retina was observed after 10 days (Fig. 6A). In the retina injected with blank DPBS, local IBA1⁺ microglia were only observed near the injection site, whereas mice injected with vesicles released by RPE exhibited a larger area of microglia distribution in the subretinal space. Mice transplanted with vesicles released by patient RPE displayed even greater subretinal migration of microglia (Fig. 6B). Further observation of migrating microglia revealed the engulfment of Dil-labeled EVs (Fig. 6C).

In the physiological state, microglia were usually distributed in three layers of the mouse retina: the GCL, IPL, and OPL.²⁶ Microglia extended their branches to monitor the whole retina (Fig. 6D). However, following vesicle transplantation, a disorganized arrangement of microglia in the OPL was observed in the prominently activated areas, where some ameboid-like microglia cell bodies migrated through the dense outer nuclear layer (ONL) to the outer segment area, approximately 30 to 50 μm away from the ONL (Supplementary Fig. S4). In the quiescent zone, the microglia

distribution resembled that observed under physiological conditions. In the transition zone, the branches of microglia in the OPL shortened, and the cell bodies aggregated (Fig. 6E). No distinct microglia cell bodies were observed in the ONL, but clear microglia branches were distributed. Microglia branching distribution was observed beneath the retina, with localized microglia cell bodies visible (Fig. 6E). In the activated central zone, numerous EV aggregates were observed, and disrupted microglia with contracted branches were also present in the inner IPL and GCL (Fig. 6F). Near the RPE, microglia/macrophages were divided into two layers, with fewer branches in the inner layer but a noticeable cell distribution. The outer microglia/macrophages surrounded the RPE, and labeled EVs were also observed near the RPE (Fig. 6G).

DISCUSSION

In this study, we established a stable human RPE cell model and, for the first time, to our knowledge, revealed the critical role of RPE-derived EVs in RP. Our findings demonstrate that aberrant expression of the *MERTK* protein directly contributes to the abnormal secretion of EVs during early RPE development. Mutant RPE cells release EVs enriched with inflammatory factors, which induce M1-type polarization of microglia, thereby driving retinal inflammation and disease progression (Fig. 7). This finding highlights the significant role of EVs in the early stages of RP.

Through integrating multi-omics data with cellular and animal validation experiments, we systematically elucidated the mechanism by which *MERTK* mutations lead to dysreg-

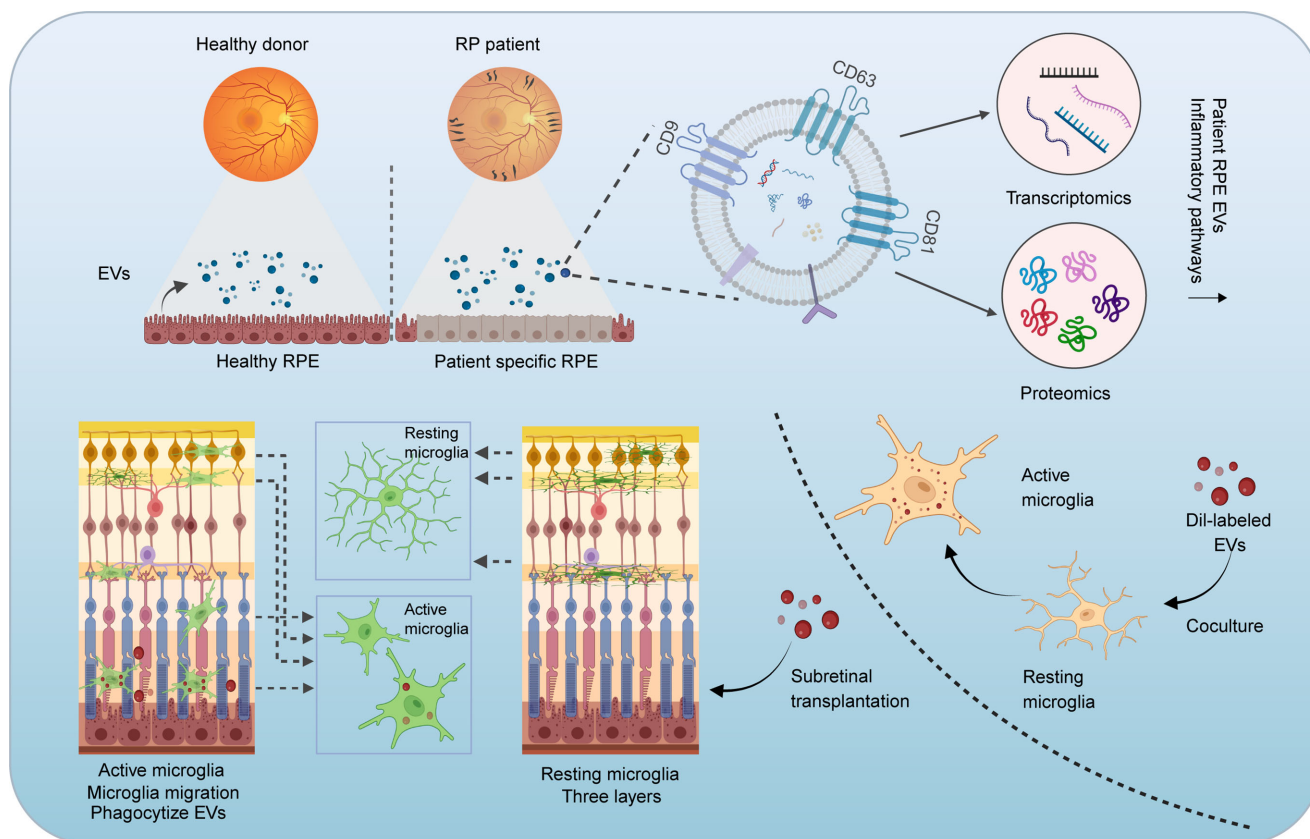


FIGURE 7. Schematic graphic of research on *MERTK*-mutated RPE-derived EVs promoting retinal inflammation.

ulation of the retinal immune microenvironment. Transcriptomic analysis revealed that *MERTK*-mutant iPSC-RPE cells not only exhibited abnormal expression of genes associated with visual perception and inflammatory signaling pathways, such as mitogen-activated protein kinase (MAPK), TGF- β , and phosphoinositide 3-kinase (PI3K)/Akt, but, more strikingly, also showed significant alterations in pathways related to extracellular exosomes (Fig. 2). These findings suggest that *MERTK*-deficient RPE cells may possess an intrinsic abnormal EV secretion. Further analyses of EV cargo confirmed this hypothesis. The proteomic and transcriptomic profiling identified that EVs derived from *MERTK*-mutant RPE displayed pronounced inflammatory traits, indicating an active and selective packaging of a “pro-inflammatory signal set” into EVs rather than a mere passive leakage of intracellular contents (Figs. 3, 4). We demonstrated through in vitro co-culture experiments that these pathogenic *MERTK*-derived EVs were efficiently internalized by microglia and triggered their activation, as evidenced by the upregulation of pro-inflammatory cytokines and morphological transformation toward an amoeboid-like phenotype (Fig. 5). In vivo experiments confirmed the pathogenic cascade. Subretinal injection of *MERTK*-derived EVs resulted in extensive uptake of these vesicles by retinal microglia/macrophages, accompanied by enhanced M1 polarization and increased migration into the subretinal space (Fig. 6). This established a key early pathological feature in patients with *MERTK*-associated RP.

Previous studies have reported that *MERTK* mutation impairs the ability of RPE cells to phagocytose and clear shed POS, a critical physiological process required to maintain photoreceptor health and metabolism. Due to this phagocytic dysfunction, apoptotic debris and degenerated outer segments abnormally accumulate in the subretinal space, which not only disrupts the normal retinal architecture but also exerts continuous toxic effects on photoreceptors.²⁷ Over time, this persistent phagocytic impairment leads to progressive apoptosis and death of photoreceptors, forming the pathological basis of vision loss in patients with RP.⁴ Moreover, the present study reveals the importance of a non-cell-autonomous mechanism, the regulation of immune cells via EVs, thereby expanding our understanding of the disease mechanism.

EVs, complex membrane-bound vesicles filled with an array of bioactive molecules, are instrumental in mediating intercellular signaling and modulating cellular functions.^{28,29} Neuron-derived EVs mediate neurotoxicity by transferring lncRNA and promoting M1 polarization of microglia,³⁰ and RPE EVs have been indicated to regulate the retinal microenvironment in proliferative vitreoretinopathy by fostering a beneficial RPE epithelial-mesenchymal transition (EMT).³¹ In age-related macular degeneration, RPE EVs were shown to be unable to prevent angiogenesis from leakage, and they helped increase autophagy in neighboring cells, facilitating the development of drusen.³² However, the role of RPE EVs in RP has yet to be thoroughly investigated. An ideal in vitro RPE model is essential for the isolation of RPE-derived EVs. In this study, we utilized reprogramming techniques to generate patient-specific RPE in vitro, which offers a robust platform for examining the changes in EV cargo related to *MERTK*-associated RP and an abundant cellular resource for exploring the roles of RPE-derived EVs. Traditionally, research has relied on primary RPE cells and the immortalized ARPE19 cell line to model RPE behavior. Nonetheless, due to differences in gene expression, difficulties in prolif-

eration, susceptibility to EMT, and functional discrepancies, both the ARPE19 cell line and primary RPE cells are limited in their ability to stably recapitulate the authentic characteristics of in vivo RPE.³³ Additionally, only 631 proteins were detected in EVs from primary cultured pig RPE,³⁴ whereas over 3000 proteins were detected in EVs from iPSC-RPE cells in our study. This disparity underscores the strategic importance of patient-specific iPSC-RPE models in RPE research.³⁵

Inflammation has emerged as a crucial mechanism in the progression and deterioration of neurodegenerative diseases.^{36–38} During the thinning of the photoreceptor layer, microglial infiltration has been observed at the degenerative sites, where microglia contribute to retinal inflammation through infiltration, phagocytosis, and secretion of pro-inflammatory mediators.³⁹ Related clinical studies have also shown elevated levels of inflammatory factors in the serum, vitreous, and aqueous humor of RP patients, suggesting an active intraocular inflammatory response.^{40,41} Clinically, synthetic corticosteroids, which are known for their strong anti-inflammatory and immunosuppressive properties, are used to treat RP-associated cystoid macular edema.⁴² A previous animal study reported that RP with *MERTK* mutations was associated with incipient RPE inflammation and microglial activation, with RPE inflammation preceding microglial activation.⁴ Our study further revealed that RPE EVs could serve as media for inflammatory signaling from RPE to retinal immune cells, particularly microglia. In cases where microglia are unable to effectively clear excess inflammatory EVs, they may activate a self-protection program, leading to their destruction along with the indigestible inflammatory EVs, which are then cleared by surrounding healthy microglia. However, when the balance between pro-inflammatory and anti-inflammatory responses is disrupted, coupled with the continuous generation of inflammatory EVs from mutated RPE, the RP retina may eventually develop a chronic inflammatory microenvironment.³⁶ Such an environment could significantly affect the performance of all retinal cell types, including photoreceptors.

This study pioneers the identification of a connection between RPE-derived EVs and RP, proposing a novel mechanism underlying the progression of *MERTK*-associated RP. The findings highlight the central role of RPE in retinal immune regulation. Beyond serving as a maintainer of immune privilege, RPE can transform into an initiator of immune-driven inflammation under certain genetic mutations. This deepens our understanding of the dual immunoregulatory functions of RPE. Furthermore, by elucidating how *MERTK*-associated RP manifests earlier onset and rapid progression from the perspective of immune microenvironment remodeling, the study provides a plausible explanation for disease heterogeneity and offers important molecular insights into the diverse clinical phenotypes of inherited retinal disorders.

In terms of clinical translation, this study shows promising potential. First, it provided a possible biomarker, as RPE-derived EVs or their associated inflammatory factor profiles may serve as noninvasive diagnostic tools for assessing disease activity and predicting the rate of progression. Second, the study identified new therapeutic targets. By focusing on the pathogenic pathway of RPE–EVs–microglia, it provides possibilities for developing drugs that specifically modulate EV biogenesis, release, or pro-inflammatory cargo. Such approaches may offer innovative therapeutic strategies to delay or halt disease progression and ultimately contribute to the advancement of personalized treatment

options. Third, the study developed a novel paradigm for combination therapy. In the future, modulating the immune microenvironment could be integrated with conventional gene therapy or cell replacement therapy. By combining multiple intervention strategies, this approach has the potential to achieve synergistic therapeutic effects.

This study also has some limitations. First, although the two-dimensional iPSC-RPE offers human-derived cells, it lacks the three-dimensional microenvironment of the in vivo retina, such as the choriocapillaris and other structural components, which may limit its ability to fully recapitulate the complex intercellular interactions. Second, due to the lack of confidence in the diagnosis and treatment of this disease among patients with early-onset severe RP and their families, we were able to recruit only two patients carrying *MERTK* monogenic mutations, one of whom had a typical adolescent-onset history. Patient 1, who carried a homozygous mutation in exon 2, exhibited an earlier onset and more severe visual impairment. Based on the microscopic morphology of iPSC-RPE, this patient and two healthy controls were used for transcriptomics and proteomics analysis. However, we used EVs from both patients for subsequent molecular experiments and animal studies. In this study, RPE-EVs from two patients carrying distinct *MERTK* mutations were pooled for analysis, which neglected potential genotype-specific phenotypic differences between homozygous and compound heterozygous mutations. Additionally, due to the random diffusion of liquid near the injection site after transplantation, it is challenging to precisely quantify the migration or activation status of microglia in vivo. Therefore, further assessment methods should be explored. Moreover, RPE cells with *MERTK* mutations exhibit diminished adhesion on the Transwell, preventing the isolated collection of apical and basal EVs.

In summary, we delineated a clear pathogenic RPE-EVs-microglia axis in *MERTK*-associated RP. Our findings provide crucial evidence that EVs derived from *MERTK*-mutated RPE cells play a significant role in amplifying the early inflammatory processes and modulating the retinal microenvironment in early-onset severe RP. This study provides a novel understanding of the early onset and rapid progression of *MERTK*-associated RP and establishes a foundation for the development of immune microenvironment-targeted therapeutic strategies.

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Data Availability Statements: All data needed to evaluate the conclusions in the paper are present in the paper and the supplementary materials. The RNA-seq data in this study have been deposited in the GSA database with the accession

code HRA006740. The proteomics data are being prepared for uploading to the database.

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