Supplementary Methods

Immunohistochemistry

RPE spheroids were collected and fixed with 4% paraformaldehyde (PFA) for 30 min on ice. The tissues were prepared for OCT embedding and frozen section as reported,\textsuperscript{1,2} or embedded in 4-5% agarose for vibratome sections and cut in 50-70 µm thickness.\textsuperscript{3} Cells on coverslips or in chambers were fixed with 4%PFA for 5 min. After block and permeabilization, slides or coverslips were incubated overnight at 4 °C with primary antibodies (Supplementary Table S1). Next day, samples were washed and incubated for 1 hr at RT with the corresponding secondary antibody conjugated to Alexa 488 or Alexa 555 or Alexa 647 (Life Technologies, CA, USA), and nuclei are counterstained with DAPI (C1005; Beyotime) for 10 min. For vibratome sections, all procedures were done with the double incubation time. Then cells were washed and mounted using mounting medium (S302380; DAKO, Beijing, China). Images were taken by an Olympus fluorescence microscope (BX53F; Olympus) or a Zeiss confocal microscope (LSM 510; Carl Zeiss Meditec, Inc.).

RT-PCR and qRT-PCR

Samples from hiPSCs, hRPE and hiPSCs-derived RPE cells at various timepoints after passage were collected. RNA extraction was done with TRIzol Reagent (15596026; Invitrogen, CA, USA) following the manufacturer's protocol. RNA quality was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription was performed with EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (AE311; TransGen Biotech, China). Reactions without reverse transcriptase enzyme were used as control. PCR was done with
Premix Taq (RR902A; Takara, Japan) on a Thermal Cycler (070-851; Biometra). The PCR conditions consisted of 98°C for 2 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were visualized on 2% agarose gels containing 0.1% ethidium bromide.

qPCR (40 cycles) was performed with QuantiNova SYBR Green PCR Kit (208054; QIAGEN, Germany) in Step One Plus (Applied Biosystems) following the manufacturer’s instructions. Sequences of PCR primers for genes of interest were from literature 1, 4-9 or qPCR primer database (http://biodb.swu.edu.cn/qprimerdb/), 10 and listed in Supplementary Table S2. The specificity and efficiency of these primers amplifying target genes were validated by gel electrophoresis, melting curves and standard curves. 11 $2^{\Delta\Delta Ct}$ method was used to analyze data. GAPDH served as an internal control and hiPSCs as a reference. Three independent experiments were performed.

Phagocytosis Assay

Swine retina was dissected from freshly slaughtered porcine eyes in dark room. Photoreceptor outer segments (POS) were prepared with a protocol published, 12 and labeled with CM-Dil (C7001; Invitrogen, CA, USA) following the instructions. The phagocytosis assay was performed as previously described. 13-15 Briefly, expanded monolayers of RPE on D28-42 after passage were exposed to CM-Dil labeled POS (>30 POS per cell) at 37 °C or 4 °C for the desired time. Subsequently, the cells were washed thoroughly, and 0.20% Trypan Blue was used to quench external fluorescence for 10 min. The cells were then fixed and immunostained with an apical side marker ZO-1. Z-stack images were taken with a Zeiss LSM 510 confocal microscope.
(Carl Zeiss Meditec, Inc.) to determine whether POS were internalized. For quantitative calculation, POS with a minimum diameter of 0.5 μm were counted using imageJ software, and 3 random fields of view were photographed per group. Three independent experiments were conducted.

Reference: