• Basic Research •

Organoid-derived human retinal progenitor cells promote early dedifferentiation of Müller glia in Royal College of Surgeons rats

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Abstract

● **AIM:** To explore whether the subretinal transplantation of retinal progenitor cells from human embryonic stem cell-derived retinal organoid (hERO-RPCs) could promote Müller glia dedifferentiation and transdifferentiation, thus improving visual function and delaying retinal degenerative progression.

● **METHODS:** hERO-RPCs were subretinally transplanted into Royal College of Surgeons (RCS) rats. Electroretinography (ERG) recording was performed at 4 and 8wk postoperation to assess retinal function. Using immunofluorescence, the changes in outer nuclear layer (ONL) thickness and retinal Müller glia were explored at 2, 4, and 8wk postoperation. To verify the effect of hERO-RPCs on Müller glia *in vitro*, we cocultured hERO-RPCs with Müller glia with a Transwell system. After coculture, Ki67 staining and quantitative polymerase chain reaction (qPCR) were performed to measure the proliferation and mRNA levels of Müller glia respectively. Cell migration experiment was used to detect the effect of hERO-RPCs on Müller glial migration. Comparisons between two groups were performed by the unpaired Student's *t*-test, and comparisons among multiple groups were made with one-way ANOVA followed by Tukey's multiple comparison test.

● **RESULTS:** The visual function and ONL thickness of RCS rats were significantly improved by transplantation of hERO-RPCs at 4 and 8wk postoperation. In addition to inhibiting gliosis at 4 and 8wk postoperation, hERO-RPCs significantly increased the expression of dedifferentiation-associated transcriptional factor in Müller glia and promoted the migration at 2, 4 and 8wk postoperation, but not the transdifferentiation of these cells in RCS rats. *In vitro*, using the Transwell system, we found that hERO-RPCs promoted the proliferation and migration of primary rat Müller glia and induced their dedifferentiation at the mRNA level.

● **CONCLUSION:** These results show that hERO-RPCs might promote early dedifferentiation of Müller glia, which may provide novel insights into the mechanisms of stem cell therapy and Müller glial reprogramming, contributing to the development of novel therapies for retinal degeneration disorders.

● **KEYWORDS:** retinal degeneration; retinal organoid; retinal progenitor cells; subretinal transplantation; Müller glia; dedifferentiation

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INTRODUCTION

Retinal degeneration disorders (RDD) refer to a group of diseases that can lead to visual impairment and blindness. Retinitis pigmentosa (RP) is one of the most common diseases involving RDD and is characterized by the gradual degeneration of photoreceptors[1-3]. RP patients usually begin with loss of night vision, peripheral vision, and then central vision[4-5]. Unfortunately, RP cannot be effectively treated now. Stem cell transplantation is considered a potential strategy to replace retinal neurons and restore vision[6-8]. Several clinical trials have demonstrated that stem cells are effective in treating RDD[9-11]. However, the underlying mechanism of stem cell therapy remains to be uncovered.

Müller glia are the major macroglia in the retina, extending through the whole retina, accounting for 90% of all glial cells in the retina[12-14]. As the supporting cells of the retina, they have important biological roles, such as maintaining the

stability and integrity of the retina, regulating the blood-retinal barrier, neuronal nutrient transport and metabolism, retinal angiogenesis and blood flow regulation and phagocytosis[15-18]. In mammals, Müller glia respond to retinal damage in various ways. Reactive Müller glia can protect tissue function by releasing neurotrophic factors and antioxidants. Nevertheless, Müller glial gliosis can also lead to neurodegeneration and hinder regeneration process of retinal tissue by forming glial scar as well[19-20]. Under special circumstances, Müller glia can proliferate and produce new neurons, but these reactions are weak and insufficient to repair the damaged retina[21-22]. In contrast, in some lower-order vertebrates such as zebrafish, after the retina is damaged, Müller glia can re-enter the cell cycle, proliferate and dedifferentiate, transdifferentiate into neurons and repair the damaged retina[23-24]. Therefore, Müller glia are considered endogenous retinal stem cells that can regenerate the damaged retina *in situ*. Identification of a strategy for promoting Müller glial reprogramming and regenerating the retina is the major challenge to be solved[25-27]. Müller glial reprogramming can be initiated by genetic strategies such as overexpression of the Ascl1 transcription factor and gene editing of transcription factors (Otx2, Crx and Nrl) in Müller glia[28-31]. Moreover, injection of exogenous factors, such as epidermal growth factor (EGF), fibroblast growth factor 1 (FGF1), FGF2 and ciliary neurotrophic factor (CNTF), was shown to promote Müller glial reprogramming and differentiation into retinal neurons after dedifferentiation[32-37]. Notably, our group and other groups previously demonstrated that exogenous stem cell transplantation might be able to promote the dedifferentiation/reprogramming of Müller glia as endogenous retinal stem cells to repair degenerated retina[38-41]. However, the dedifferentiation/reprogramming process of Müller glia triggered by stem cell transplantation needs to be further elucidated.

Our previous study showed that retinal progenitor cells from human embryonic stem cell-derived retinal organoid (hERO-RPCs) through cell surface markers (C-Kit$^+$/SSEA4$^-$) sorting readily eliminated tumorigenic embryonic cells and played an important role in the suppression of gliosis and the rescue of retinal degeneration[42]. While reversing the fate of gliosis, whether hERO-RPCs can promote Müller glial dedifferentiation/reprogramming is unknown. In this study, we aimed to explore whether the subretinal space transplantation of hERO-RPCs, from the early stage of transplantation to the middle and late stages, could stimulate Müller glia dedifferentiation and transdifferentiation to improve visual function and delay RP progression. Here, we found hERO-RPCs subretinal transplantation significantly improved visual function and protected photoreceptor structure in Royal College of Surgeons (RCS) rats. While inhibiting of gliosis, hERO-RPCs also increased the expression of dedifferentiation-associated transcriptional factor and promoted the migration of Müller glia but did not promote transdifferentiation. *In vitro*, we found that hERO-RPCs can also promote the proliferation and migration of Müller glia and induce the dedifferentiation of Müller glia at the mRNA level. Overall, these results demonstrated that hERO-RPCs might promote early dedifferentiation of Müller glia, which may provide a new treatment strategy for RDD.

MATERIALS AND METHODS

Ethical Approval RCS rats (a model of RDD)[43-44] at postnatal day (PND) 21 and Long-Evans (LE) rats at PND 7 were used in this study. The rats were raised in a pathogen-free room under a 12-hour light-dark cycle of the Experimental Animal Center of the Third Military Medical University (Army Medical University). All animal experimental procedures were formally approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (Army Medical University; AMUWEC20232718). All tissue collection and experimental procedures were conducted in accordance with protocol approved by the Institutional Review Board of the Third Military Medical University (Army Medical University) and performed to guidelines of the National Institutes of Health (NIH) on the ethical use of animals and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation and Culture of hERO-RPCs According to published methods[42], 30-day human embryonic stem cell-derived retinal organoid (hERO)[45] were dissociated into a single-cell suspension through TrypLE Express (Gibco), with a cell concentration of 1×10^6 cells/100 μL (a test). The anti-human C-Kit antibody conjugated with allophycocyanin (APC; Biolegend, 5 μL/test) and anti-human SSEA4 antibody conjugated with fluorescein isothiocyanate (FITC; BD Biosciences, 20 μL/test) were simultaneously added to the sample tube for 30min at 4℃. Afterward, 7-ADD dye was used and the sample was sent to a BD flow cytometer for analysis and sorting.

The sorted hERO-RPCs were inoculated into a VTN coated culture plate and cultured with serum-free medium (Lonza, Switzerland) with 20 ng/mL human epidermal growth factor (EGF, Peprotech, USA), 20 ng/mL human basic fibroblast growth factor (bFGF, Peprotech, USA), 1% GlutaMAX supplement (Gibco, USA), 1% N2 (Gibco, USA), and 2% B27 (Gibco, USA). The next day, after the cells adhered to the culture plate, the fresh medium was changed, and then the medium was replaced every 2d. Cell passage was carried out after the cell density was close to 80%-90%.

Subretinal Transplantation According to published protocol[42,46], P3 hERO-RPCs were infected with lentivirus

carrying EGFP driven by the CMV promoter. P3 EGFP-labeled hERO-RPCs were digested with TrypLE Express (Gibco, USA) and resuspended in sterile phosphate buffered saline (PBS) with DNase I (0.005%, Roche) at a density of 1×10^5 cells/μL. One day before transplantation, the RCS rats at PND 21 received oral cyclosporine A (210 mg/L, Huadong Medicine, China) dissolved in drinking water. The pupil was dilated with a 1% tropicamide eyedrop. After an incision was made in the temporal sclera, 2 μL of cell suspension was slowly injected into the subretinal space of the temporal retina through the incision using a 10 μL microsyringe. All rats received oral cyclosporine A until 2wk after transplantation.

Electroretinogram Recording Electroretinography (ERG) recording was performed at 4 and 8wk postoperation to assess retinal functional changes. According to our previous protocol[47], RCS rats should undergo at least 12h of dark adaptation. The pupil was dilated with a 1% tropicamide eyedrop. Two gold ring contact electrodes were positioned on each cornea as recording electrodes. The reference electrodes were placed into the rat's mouth and the ground electrodes clamped the rat's tail. We applied light stimulation at dark-adapted combined rod-cone response 3.0 cd·s/cm^2. The a-wave and b-wave amplitudes were recorded and processed by an ERG device (MAYO, Japan). All operations were performed with dim red safety light in a dark room. Five eyes in each group at 2 time points were included for statistical analysis.

Frozen Sections Preparation of Retina After enucleation, the eyeballs were fixed in 4% paraformaldehyde (PFA). Thirty minutes later, the cornea, lens and vitreous body were removed. Then, the eye cups were fixed in 4% PFA for 1.5h sequentially. The eye cups were removed from 4% PFA and incubated in 30% glucose solution overnight. The eye cups were placed on a freezing microtome with embedding medium. Then, 12-μm serial frozen sections were made.

Immunofluorescence Immunofluorescence was performed as previously described[48]. For immunofluorescence of retina or cell, sections or the cell slides were washed with PBS 3 times. The sections or the cell slides were perforated in 0.3% Triton X-100 for 10min and blocked in PBS with 3% albumin from bovine serum (BSA) at 37℃ for 30min. The sections or the cell slides were incubated with primary antibody (Table 1) in 1% BSA at 4℃ for one night. On the second day, the sections were incubated with secondary antibodies at 37℃ for 1h. After incubation with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) for 10min, the sections or the cell slides were washed with PBS 3 times. Finally, the sections or the cell slides were photographed through a confocal laser scanning microscope (Zeiss, Germany).

Primary Müller Glia Culture Primary Müller glia culture was performed as previously reported[49]. Eyes from Long-Evans (LE) rats at PND 7 were enucleated and incubated overnight in Dulbecco's modified Eagle's medium (DMEM). The eyes were subsequently transferred to dissociation solution (0.1% collagenase IV and 0.1% trypsin) and incubated at 37℃ for 30min. The eyes were washed with DMEM containing 10% fetal bovine serum (FBS) and a 1% penicillin-streptomycin solution. Retinas were dissected carefully and cultured in DMEM containing 20% FBS and 1% penicillin-streptomycin solution in a 37°C incubator for 7d. A week later, the floating retinal tissue aggregates and debris were removed, leaving Müller glia attached to the bottom of the cell culture flask. The cells were cultured in DMEM containing 20% FBS for 5d to further purify Müller glia were identified by immunofluorescence staining for Vimentin, GS, GFAP and Sox9. The cells from P3 to P5 were used for the following experiments.

Table 1 Primary antibodies used for immunofluorescence

Antibody	Manufacturer's catalog or lot number	Dilution
Mouse anti-MTCO2	Abcam, ab110258	1:200
Mouse anti-HuNu	Abcam, ab191181	1:200
Rabbit anti-GFAP	Abcam, ab7260	1:500
Mouse anti-Vimentin	Santa Cruz, sc-6260	1:200
Rabbit anti-Vimentin	ABclonal, A19607	1:200
Rabbit anti-Sox9	Abcam, ab185966	1:400
Mouse anti-Chx10	Santa Cruz, sc-373151	1:200
Rabbit anti-Ki67	Abcam, ab66155	1:500
Mouse anti-RAX	Santa Cruz, sc-271889	1:300
Rabbit anti-Sox2	Abcam, ab97959	1:400
Rabbit anti-Pax6	Abcam, ab195045	1:400
Mouse anti-Rhodopsin	Abcam, ab98887	1:500
Mouse anti-Crx	Santa Cruz, sc-377138	1:200
Mouse anti-Brn3a	Santa Cruz, sc-8429	1:500
Mouse anti-HuC/D	Santa Cruz, sc-515624	1:500
Rabbit anti-GS	Zen bio, R22765	1:100

MTCO2: Mitochondrially encoded cytochrome c oxidase II; HuNu: Human nuclei; GFAP: Glial fibrillary acidic protein; Sox9: SRY-box transcription factor 9; Chx10 (alias Vsx2): Visual system homeobox 2; RAX: Retina and anterior neural fold homeobox; Sox2: SRY-box transcription factor 2; Pax6: Paired box 6; Crx: Cone-rod homeobox; GS: Glutamine synthesis.

Coculture of hERO-RPCs and Müller Glia To test the proliferative effects of hERO-RPCs on Müller glia, we used 12-well Transwell plates with 0.4-μm aperture membranes (Corning, USA) and placed the cell slides in the lower chamber. hERO-RPCs were inoculated in the upper chamber 1×10^5 cells/well and Müller glia were inoculated in the lower chamber 1×10^4 cells/well. To test the effect of hERO-RPCs on Müller glia mRNA, we used 6-well Transwell plates with 0.4-μm aperture membranes. hERO-RPCs were inoculated in the upper chamber 2×10^5 cells/well and Müller glia were inoculated in the lower chamber 2×10^4 cells/well. After culture for 1, 3 and 5d, the cell slides were fixed in 4% PFA and the cells of Müller glia were isolated with TRIzol Reagent (Life Technologies, USA). The experiments were repeated 3 times.

Table 2 Real-time quantitative PCR primers

Genes (rat)	Forward primer	Reverse primer
GAPDH	GCCCATCACCATCTTCCAGGAG	GAAGGGGCGGAGATGATGAC
GFAP	CCACCAGTAACATGCAAGAAAC	TTGGCGGCGATAGTCATTAG
Vimentin	CAAAGCAGGAGTCAAACGAAT	GCCATCTTTACATTGAGCAGGT
Sox9	CACATCAAGACGGAGCAACTGAG	TGTAGGTGAAGGTGGAGTAGAGCC
Chx10	CTGCCAGAGTCCATCCTCAAGT	CTGTTCTCCCTCAGTTCTTCCTG

PCR: Polymerase chain reaction; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Cell Migration Assay Cell migration assays were performed as previously described[50]. Before coculturing, we treated Müller glia with 10 μg/mL mitomycin C (Biotopped, China) for 2h to prevent them from proliferating. To test the effects of hERO-RPCs on Müller glial migration, we used 24-well Transwell plate with 8-μm aperture membranes. hERO-RPCs were inoculated in the lower chamber 1×10^5 cells/well and Müller glia were inoculated in the upper chamber 1×10^4 cells/well. After culture for 24h, cells remaining on the upper surface of the chamber were removed using a cotton swab. Then, the Transwell membranes of the cocultured group and control group were fixed for 10min by 4% PFA and kept in PBS. Standard photomicrographs were obtained in the area of membranes that were stained with crystal violet dye (Saimike, China). The photographs of the membranes taken under a 200× microscope were manually counted and averaged. The experiments were repeated 3 times. Each experiment had 3 wells in each group, and 3 photos were selected in each well.

RNA Isolation and Real-Time Quantitative PCR On the basis of published protocol[51], total RNA was extracted from Müller glia using TRIzol reagent (Life Technologies, USA) followed by chloroform extraction. With a PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa Bio, Japan), total RNA was reverse transcribed into cDNA following the manufacturer's protocol. A SYBR Green qPCR Mix (TaKaRa Bio, Japan) was used to perform quantitative PCR with a CFX96 Real-Time PCR System (Bio-Rad, USA). The relative expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All primers were provided by Sangon Biotech (China; Table 2).

Quantitative Histological Analysis For the thickness of the outer nuclear layer (ONL) and distance of Sox9 distribution analysis, 3 sections stained with DAPI or Sox9 across the optic disk were chosen from each eye, and 3 eyes were included in each group. For each section, 3 (400×) visual fields were taken for imaging. For the line chart, the optic nerve head was defined as the original location and recorded as 0. The data were measured every 0.5 mm intervals from the optic nerve head. For each section, (100×) visual fields were taken for whole retina imaging. Photographs of the retina were taken under a Zeiss confocal microscope, and the data were measured by Image J (NIH, USA). For semiquantitative analysis of GFAP expression, 3 sections after GFAP staining were chosen from each eye, and 3 eyes were included in each of the groups. For each section, 3 (400×) visual fields were taken for imaging, and the average fluorescent density of GFAP was recorded by Image J (NIH, USA). For the number analysis of Sox9- and Chx10-positive cells, the imaging rules were the same as above, and the photograph was manually counted and averaged. For quantitative analysis of the number of Vimentin-Chx10 and Sox9-Chx10 double-positive cells, the imaging rules were the same as above. The photograph was manually counted, and values of 9 visual fields for each eye were summed. For quantitative analysis of the number and percentage of Ki67-positive Müller glia and the number of migrating cells, 3 cell slides were included from each experimental batch of each group, and the experiment was repeated 3 times. Three (200×) visual fields of the cell slide were taken.

Statistical Analysis The results were produced from at least 3 biological samples. Statistical analysis was conducted with GraphPad Prism version 6.02 for Windows (GraphPad Software, USA). Comparisons between 2 groups were performed by the unpaired Student's *t*-test, and comparisons among multiple groups were made with one-way ANOVA followed by Tukey's multiple comparison test. The data are presented as the mean±standard deviation (SD), and $P<0.05$ was considered a significant difference.

RESULTS

hERO-RPCs Improved the Visual Function in RCS Rats We sorted C-Kit$^+$/SSEA4$^-$ RPCs from 30-day hERO (Supplementary online Figure S1A-S1D) as previously reported[42]. The hERO-RPCs expressed the RPC markers RAX, Sox2, and Pax6 (Supplementary online Figure S1E-S1M) and the proliferation marker Ki67 (Supplementary online Figure S1N-S1P).

EGFP-labeled hERO-RPCs were transplanted into the subretinal space in the temporal eyes of RCS rats at PND 21. We detected the visual function of the RCS rats by ERG recording at 4 and 8wk postoperation (Figure 1A). The ERG test showed that transplantation of hERO-RPCs resulted in a significant improvement in the amplitudes of a wave at 4wk postoperation compared to those in the blank group and PBS group (Figure 1B-1E). The amplitudes of the b-wave were significantly higher than those in the blank group and PBS

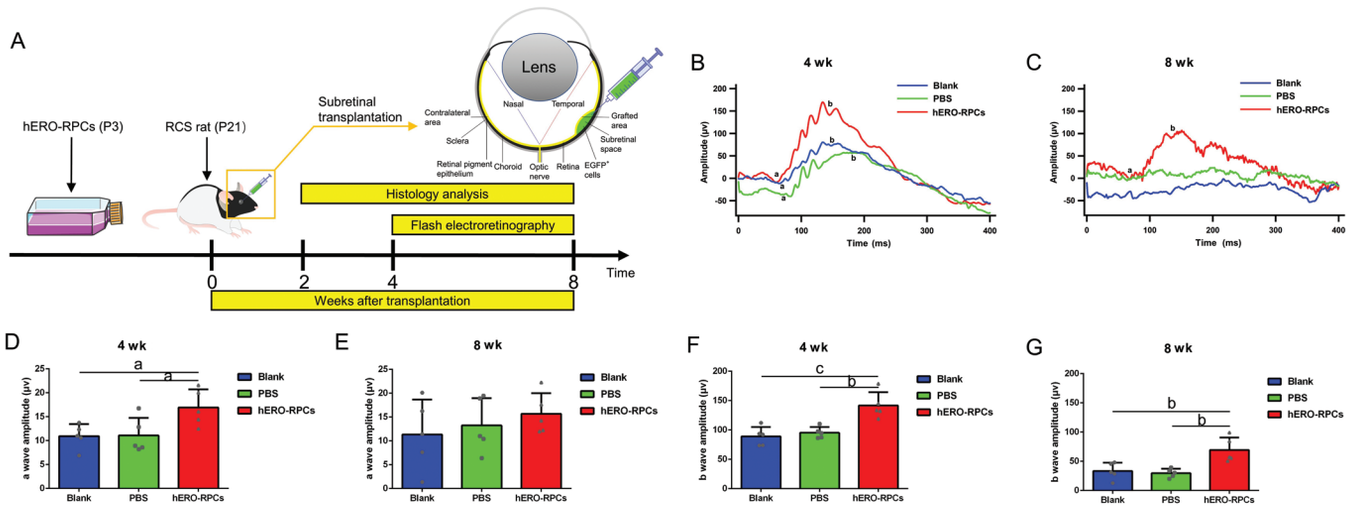

Figure 1 Visual functional improvement after transplantation of hERO-RPCs in RCS rats A: Diagram illustrating subretinal transplantation. Scheme of time points for hERO-RPCs transplantation, functional testing and histological analysis; B, C: Representative ERG results in the blank, PBS and hERO-RPCs groups at 4 and 8wk postoperation; D, E: Statistical analysis of the amplitudes of ERG a-waves in all 3 groups at 2 time points; F, G: Statistical analysis of the amplitudes of ERG b-waves in all 3 groups at 2 time points. The results are presented as the mean±SD, n=5 eyes/group, ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001. hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; EGFP: Enhanced green fluorescent protein; ERG: Electroretinography; PBS: Phosphate buffered saline; SD: Standard deviation.

group at 4 and 8wk postoperation (Figure 1B, 1C, 1F, 1G). Taken together, these results indicated that the visual function of RCS rats was significantly improved by transplantation of hERO-RPCs.

Transplantation of hERO-RPCs Protected the ONL in RCS Rats The expression of human mitochondria (MTCO2) and human nuclei (HuNu) in EGFP^{+} cells confirmed the origin of hERO-RPCs at 8wk postoperation (Figure 2A, 2B). In addition, hERO-RPCs showed excellent survival and migration in the retinas of RCS rats. We measured the thickness of the ONL to evaluate the protective effect of transplanted hERO-RPCs on the retinal structure of RCS rats (Figure 1A). As the disease progressed in RCS rats, the ONL thickness of retinas decreased continuously. Morphologically, the results showed that the ONL thickness of the grafted area group was significantly thicker than those in the blank group and PBS group at 2, 4 and 8wk postoperation. Inspiringly, the ONL thickness of the contralateral area group were also thicker than those in the blank group and PBS group at 2 and 4wk postoperation (Figure 2C-2R). In summary, hERO-RPCs transplantation significantly prevented photoreceptor loss and thus effectively protected the structure of the retina.

Suppression of Gliosis after Transplantation of hERO-RPCs With the progression of RD, the expression of GFAP, a marker of retinal gliosis, increased gradually in the retina. To analyze the gliosis of Müller glia, we performed GFAP immunofluorescence and detected the average fluorescent density of GFAP at 2, 4 and 8wk postoperation (Figure 1A). The results showed that at 2wk postoperation, the grafted area group and PBS group showed increased GFAP expression compared with the blank group and contralateral area group (Figure 3A-3D, 3P). Nevertheless, the gliosis of Müller glia was markedly suppressed in the grafted area group at 4 and 8wk postoperation. The gliosis of Müller glia was obviously reduced in the grafted area group but increased in the blank group, PBS group and contralateral area group and the difference was statistically significant (Figure 3E-3L, 3P). Moreover, we found differently in the expression of GFAP in the grafted area group and contralateral area group of the same retina of RCS rat at 8wk postoperation (Figure 3M-3O). Altogether, the results confirmed that hERO-RPCs transplantation is an effective method to delay the gliosis of Müller glia.

Transplantation of hERO-RPCs Increased the Numbers and Distribution Distance of Müller Glia in RCS Rats To determine whether hERO-RPCs transplantation had an effect on Müller glia, we labeled retinas with Sox9 (Figure 1A), a marker of Müller glia nucleus that is expressed in the inner nuclear layer (INL) of retina. The results showed that the number of Sox9-positive cells in the grafted area group and contralateral area group significantly increased compared with those in the blank group and PBS group at all time points (Figure 4A-4L, 4N). In addition, we found that most Sox9-positive Müller glia were in the superior layer of the INL in the blank group and PBS group. In the grafted group, Sox9-positive Müller glia migrated to the ONL direction, and a small population of cells entered the ONL. The distribution distance of Sox9-positive cells in the grafted area group and contralateral area group was wider than that in the control group (Figure 4A-4L, 4O-4R). We showed this phenomenon

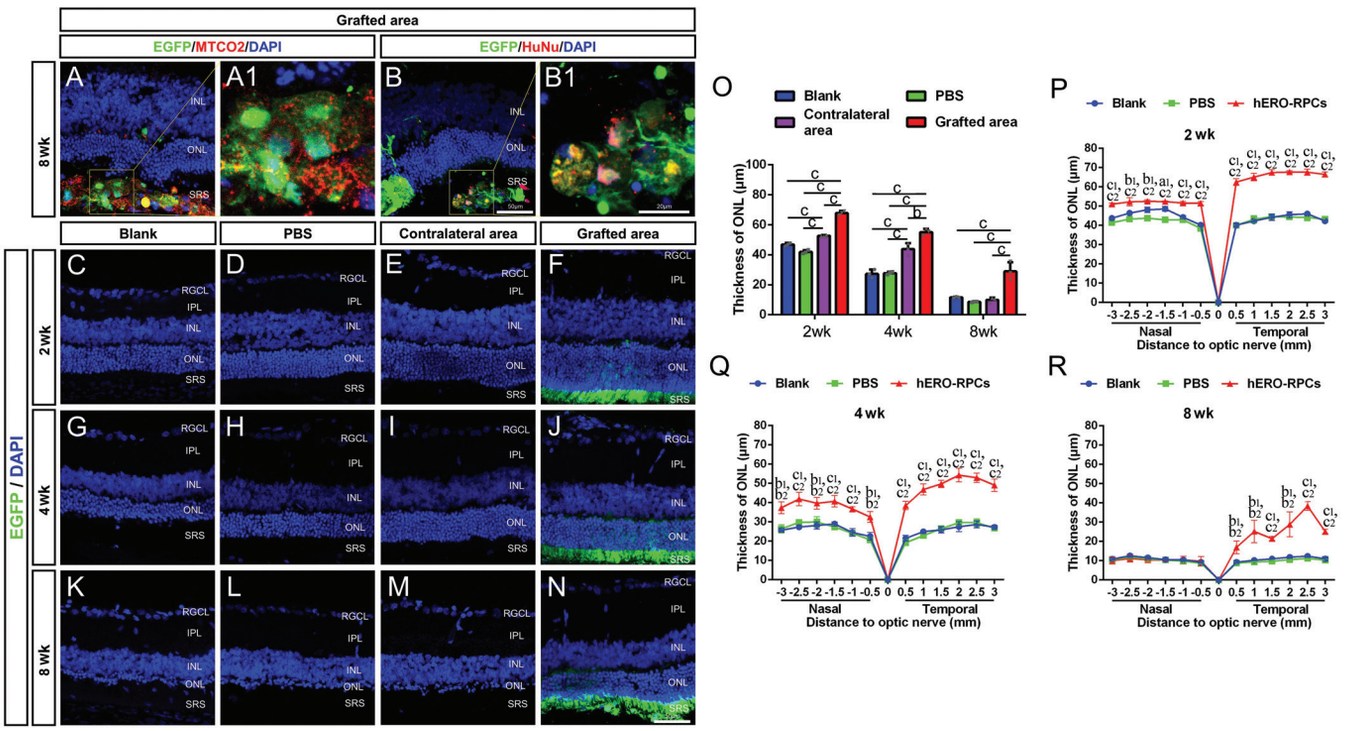

Figure 2 Protection of the ONL after transplantation of hERO-RPCs in rats A, A1: EGFP$^+$ hERO-RPCs costained with a human-specific MTCO2 mitochondrial antibody; B, B1: EGFP$^+$ hERO-RPCs costained with a human-specific HuNu nuclear antibody; C-N: Representative images of the ONL in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; O: Statistical analysis of ONL thickness in all 4 groups at 3 time points. P-R: ONL thickness in the nasal and temporal regions of the retina in each group at 3 time points. The results are presented as the mean±SD, *n*=3 eyes/group. Scale bar: 50 μm (A-N), 20 μm (A1, B1). [1]hERO-RPCs group *vs* blank group; [2]hERO-RPCs group *vs* PBS group. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. SRS: Subretinal space; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; MTCO2: Mitochondrially encoded cytochrome c oxidase II; HuNu: Human nuclei; SD: Standard deviation.

through the schematic (Figure 4M). These results suggested that hERO-RPCs transplantation could affect the number and migration of Müller glia, a potential sign of early dedifferentiation of Müller glia.

Increased Expression of Chx10 Partially Derived from Müller Glia after hERO-RPCs Transplantation To investigate whether hERO-RPCs transplantation could promote dedifferentiation of Müller glia, we labeled retinas with Chx10 (Figure 1A), a marker of bipolar cells in mature retina and progenitor cells in develop retina[52-53]. Chx10 was also considered as an upregulated transcriptional factor closely associated with adult Müller glia dedifferentiation[39,54-58]. The number of Chx10-positive cells in the grafted area group significantly increased compared with those in the blank group and PBS group at all time points. In addition, we found that the number of Chx10-positive cells in the contralateral area was also higher than that in the control group but lower than that in the grafted area (Figure 5A-5P). These results suggested that hERO-RPCs transplantation could increase the expression of Chx10 and lasted until at least 8wk postoperation.

To investigate whether the increased Chx10 expression were derived from Müller glia, we costained for the Müller glia markers Vimentin and Sox9 with Chx10 (Figure 1A). We showed representative colocated cells with Vimentin and Chx10 (Figure 6A-6D), and Sox9 and Chx10 (Figure 7A-7D). The results showed that the number of Vimentin-Chx10 double-positive cells and Sox9-Chx10 double-positive cells in the grafted area group was significantly greater than that in the blank group and PBS group at all time points. We also found that the number of double-positive cells in the contralateral area was higher than that in the control group (Figures 6E-6Q, 7E-7Q). These results suggested that the increased Chx10-positive cells were partly derived from Müller glia.

To explore whether Müller glia can transdifferentiate into retinal neurons after hERO-RPCs transplantation, we immunolabeled retinal sections for photoreceptors (Rhodopsin), photoreceptor precursors (Crx), ganglion cells (Brn3a), amacrine and ganglion cells (HuC/D). We did not find any Sox9-positive cells that coexpressed neuronal markers at 8wk postoperation (Supplementary online Figure S2A-S2D), indicating that hEROs-RPCs transplantation might not trigger the transdifferentiation of Müller glia.

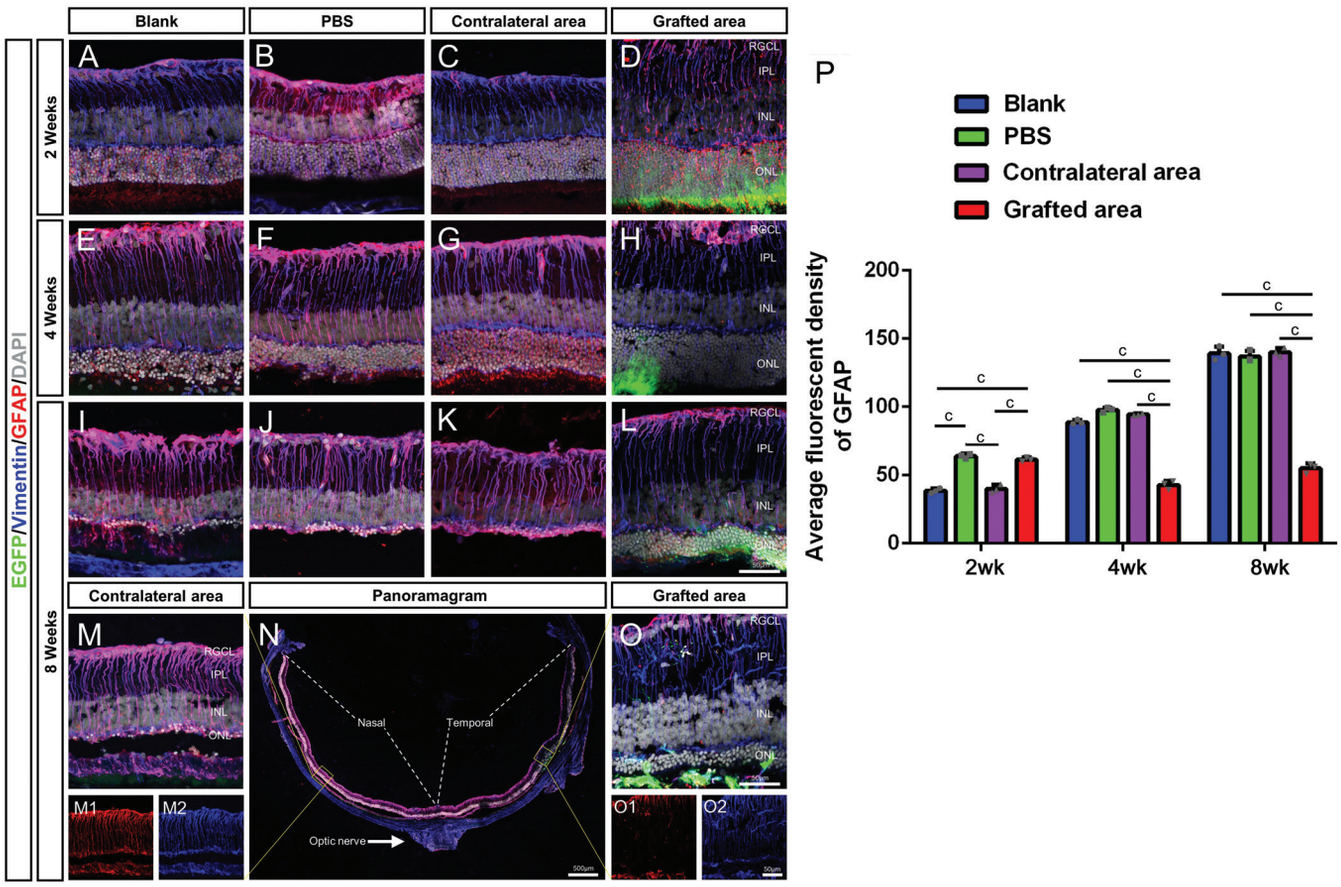

Figure 3 Suppression of reactive gliosis after transplantation of hERO-RPCs in RCS rats A-L: Costaining of Vimentin and GFAP in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; M-O: Whole retina section image and enlarged images to show the contralateral area (Nasal) and grafted area (Temporal) costained with Vimentin and GFAP at 8wk postoperation; P: Statistical analysis of the light density of GFAP expression in all 4 groups at 3 time points. The results are presented as the mean±SD, n=3 eyes/group, ^{c}P<0.001. Scale bar: 50 μm (A–L, M, O), 500 μm (N). ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; GFAP: Glial fibrillary acidic protein; SD: Standard deviation.

Effect of hERO-RPCs on Müller Glia *in Vitro* We isolated and purified retinal Müller glia from PND 7 LE rats. After 3 passages, the cell morphology of Müller glia changed into a large cell body and spindle-like morphology, indicating that the Müller glia became mature and purified (Supplementary online Figure S3A, S3B). The P3 Müller glia expressed the Müller glial markers Vimentin, GS, GFAP and Sox9 (Supplementary online Figure S3C-S3N) and the proliferation marker Ki67 (Supplementary online Figure S3O-S3R).

To verify the effect of hERO-RPCs on Müller glia *in vitro*, we cocultured hERO-RPCs with Müller glia with a Transwell system (Figure 8A). We used Ki67 staining to measure the proliferation of Müller glia. The results showed that the number of Müller glia and the percentage of Ki67-positive cells in the coculture group were significantly higher than those in the control group at 1, 3 and 5d after coculture (Figure 8B-8O). Moreover, we detected changes in Müller glia mRNA after coculture by qPCR. The results showed that the mRNA expression levels of GFAP and Vimentin significantly decreased and mRNA expression levels of Chx10 significantly increased after cocultured with hERO-RPCs at 1 and 3d (Figure 8P, 8Q, 8S). The mRNA expression levels of Sox9 also significantly increased at 1, 3 and 5d (Figure 8R). We tried to verify the effect of hERO-RPCs on Müller glial migration through cell migration experiments. The results showed that the number of migrating cells in the coculture group was significantly greater than that in the control group (Figure 8T-8V). In all, hERO-RPCs enhanced the proliferation and migration of Müller glia, suppressed the gliosis of Müller glia and promoted the dedifferentiation of Müller glia *in vitro*.

DISCUSSION

Müller glia, as the main glial cells in the retina, support the structure of the retina and have various functions in maintaining retinal homeostasis[15,59]. Gliosis and reprogramming are two distinct fates of Müller glia[60-61]. Under retinal injury, Müller glia in lower-order vertebrates can undergo dedifferentiation and transdifferentiation into retinal neurons to repair damaged retinas, while in mammals, Müller glia continuously activate

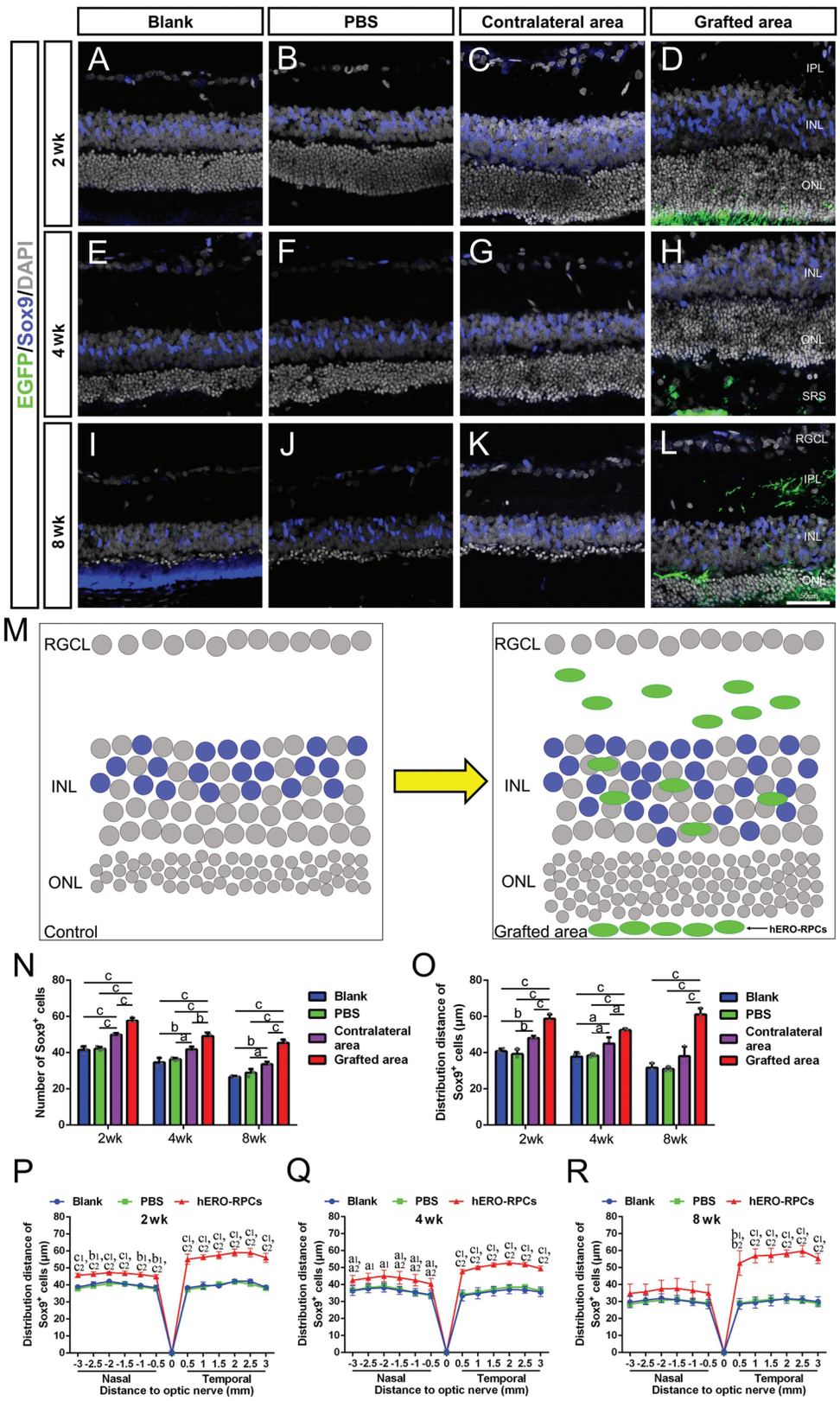

Figure 4 Increased number and migration of Müller glia after transplantation of hERO-RPCs in RCS rats A-L: Müller glia with Sox9 staining in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; M: Schematic of Sox9 positive cells in the control and grafted area groups; N: Statistical analysis of the number of Sox9-positive cells in all 4 groups at 3 time points. O: Statistical analysis of the distribution distance of Sox9-positive cells the in all 4 groups at 3 time points. P–R: Distribution distance of Sox9-positive cells in the nasal and temporal regions of whole retina sections in each group at 3 time points. [1]hERO-RPCs group *vs* blank group; [2]hERO-RPCs group *vs* PBS group. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. The results are presented as the mean±SD, *n*=3 eyes/group. Scale bar: 50 μm (A–L). SRS: Subretinal space; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; Sox9: SRY-box transcription factor 9; SD: Standard deviation.

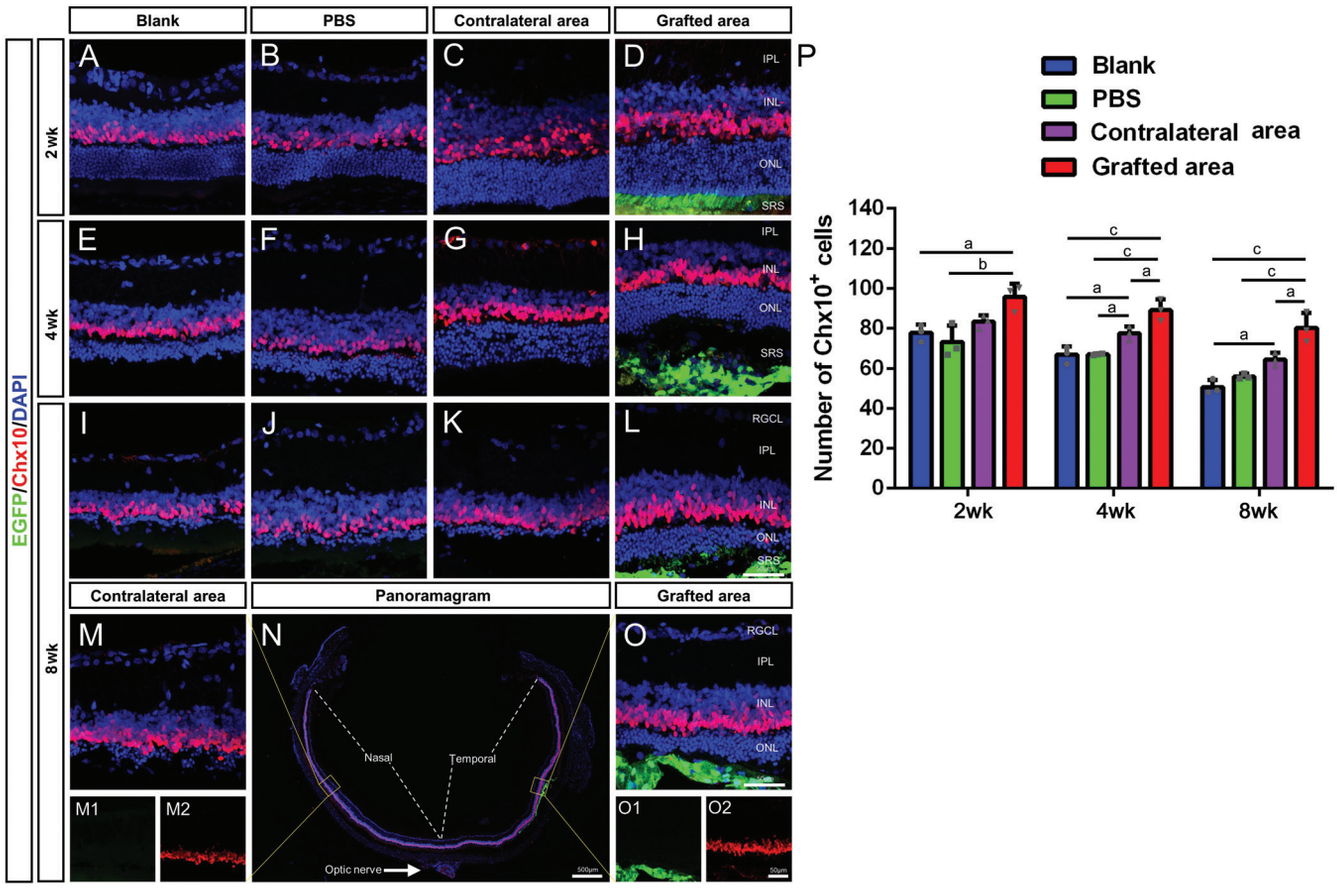

Figure 5 Increased numbers of Chx10-positive cells after transplantation of hERO-RPCs in RCS rats A-L: Immunofluorescence of Chx10 in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; M-O: Whole retina section image and enlarged images showing the contralateral area (nasal) and grafted area (temporal) stained with Chx10 at 8wk postoperation; P: Statistical analysis of the number of Chx10-positive cells in all 4 groups at 3 time points. The results are presented as the mean±SD, n=3 eyes/group, ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001. Scale bar: 50 μm (A–L, M, O), 500 μm (N). SRS: Subretinal space; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; Chx10 (alias Vsx2): Visual system homeobox 2; SD: Standard deviation.

and form glial scars, which accelerate retinal degeneration[25]. Stimulating endogenous stem cells to repair damaged retinas in mammals, especially in humans, is not ethically controversial and without the oncogenic incidence. Thus, the promotion of Müller glia reprogramming is highly promising for treating RDD. It has been revealed that transplanting exogenous stem cells into the subretinal space can promote dedifferentiation/reprogramming of Müller glia in the retina[38-41]. We previously showed that hERO-RPCs, a novel stem cell source established, can inhibit gliosis and delay retinal degeneration[42]. However, it is not known whether hERO-derived RPCs can also promote the Müller glia dedifferentiation/reprogramming and repair damaged retinas. This is the first study to show the effect of hERO-derived RPCs on the dedifferentiation and transdifferentiation of Müller glia in RDD rats, which will further elucidate the mechanism of stem cell therapy and promote the development of RDD treatment.

Stem cell transplantation can prevent photoreceptor loss through several mechanisms. Among them, cell replacement, including stem cell differentiation or Müller glia transdifferentiation into photoreceptors, holds promise for retinal regeneration, especially in the late stage of retinal degeneration, when almost no photoreceptor cells remain[39-40,62-63]. However, several reports have shown that only a small population of transplanted stem cells can integrate into the host retina, while Müller glial transdifferentiation also appears at a lower frequency[38,64-66]. On the other hand, other mechanisms, such as stem cell mediated paracrine effects, play a critical role in delaying photoreceptor loss, particularly in the early stage of degeneration, such as umbilical cord stem cells[67-68], bone marrow stem cells[69], bone marrow mesenchymal stem cells[40] and neural stem cells[70]. In our previous study, hERO-RPCs rescued retinal degeneration through a variety of mechanisms, such as material exchange, cell replacement, suppression of microglial activation and gliosis[42]. Here, we showed that hERO-RPCs transplantation promoted early dedifferentiation of Müller glia

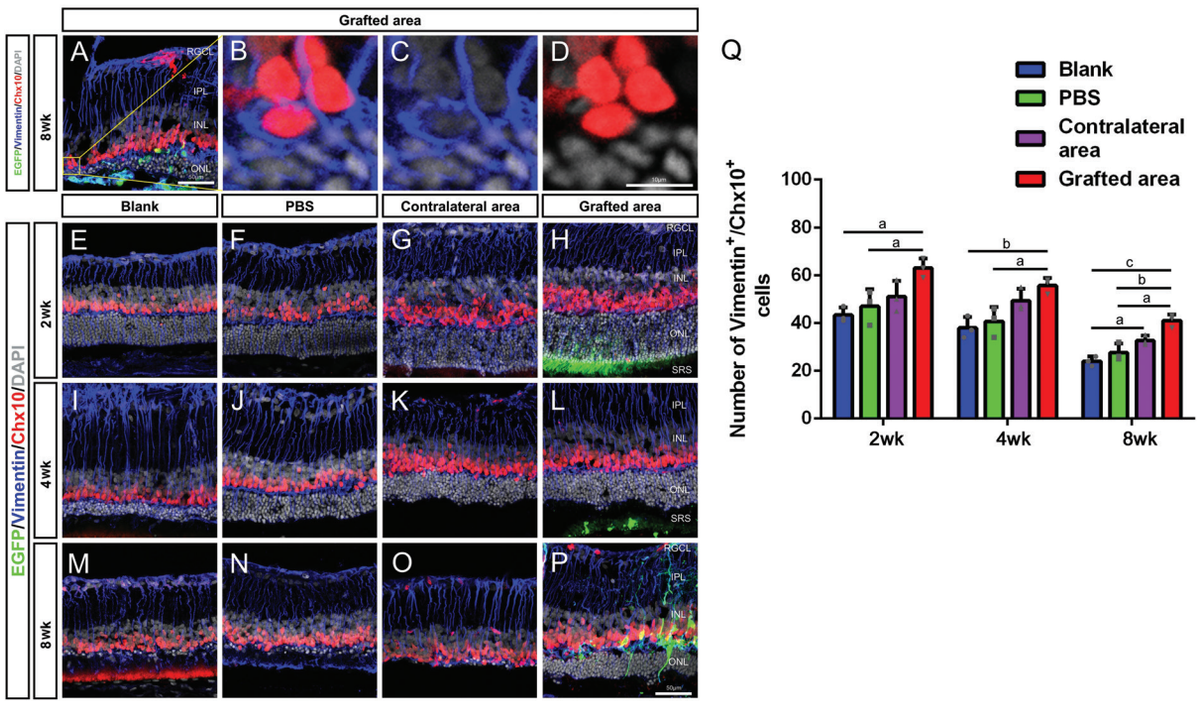

Figure 6 Increased numbers of Vimentin and Chx10 double-positive cells after transplantation of hERO-RPCs in RCS rats A-D: Representative images of cells costained with Vimentin and Chx10 in the grafted area groups at 8wk postoperation; E-P: Costaining of Vimentin and Chx10 in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; Q: Statistical analysis of the number of Vimentin and Chx10 double-positive cells in all 4 groups at 3 time points. The results are presented as the mean±SD, n=3 eyes/group, ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001. Scale bar: 50 μm (A–P). SRS: Subretinal space; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL: Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; Chx10 (alias Vsx2): Visual system homeobox 2; SD: Standard deviation.

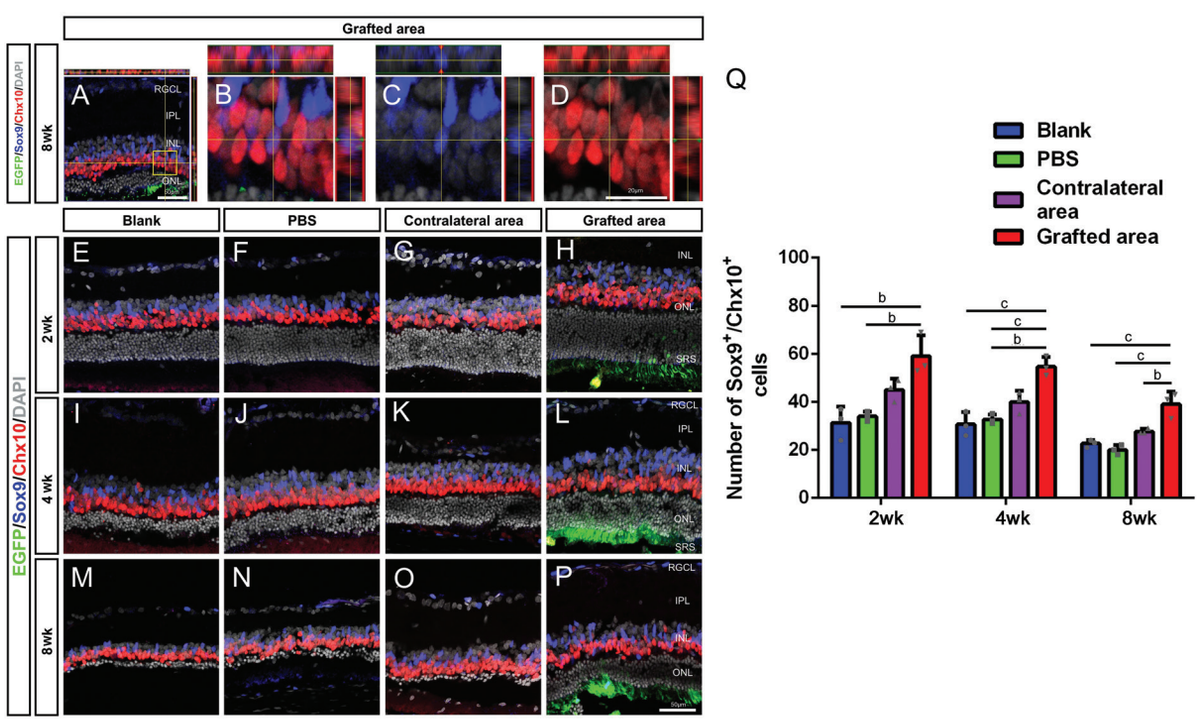

Figure 7 Increased numbers of Sox9 and Chx10 double-positive cells after transplantation of hERO-RPCs in RCS rats A-D: Representative images of cells costained with Sox9 and Chx10 in the grafted area groups at 8wk postoperation; E-P: Costaining of Sox9 and Chx10 in the blank, PBS, contralateral area and grafted area groups at 2, 4 and 8wk postoperation; Q: Statistical analysis of the number of Sox9 and Chx10 double-positive cells in all 4 groups at 3 time points. The results are presented as the mean±SD, n=3 eyes/group, ^{b}P<0.01, ^{c}P<0.001. Scale bar: 50 μm (A–P). SRS: Subretinal space; ONL: Outer nuclear layer; INL: Inner nuclear layer; IPL, Internal plexiform layer; RGCL: Retinal ganglion cell layer; hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; RCS: Royal College of Surgeons; PBS: Phosphate buffered saline; EGFP: Enhanced green fluorescent protein; Sox9: SRY-box transcription factor 9; Chx10 (alias Vsx2): Visual system homeobox 2; SD: Standard deviation.

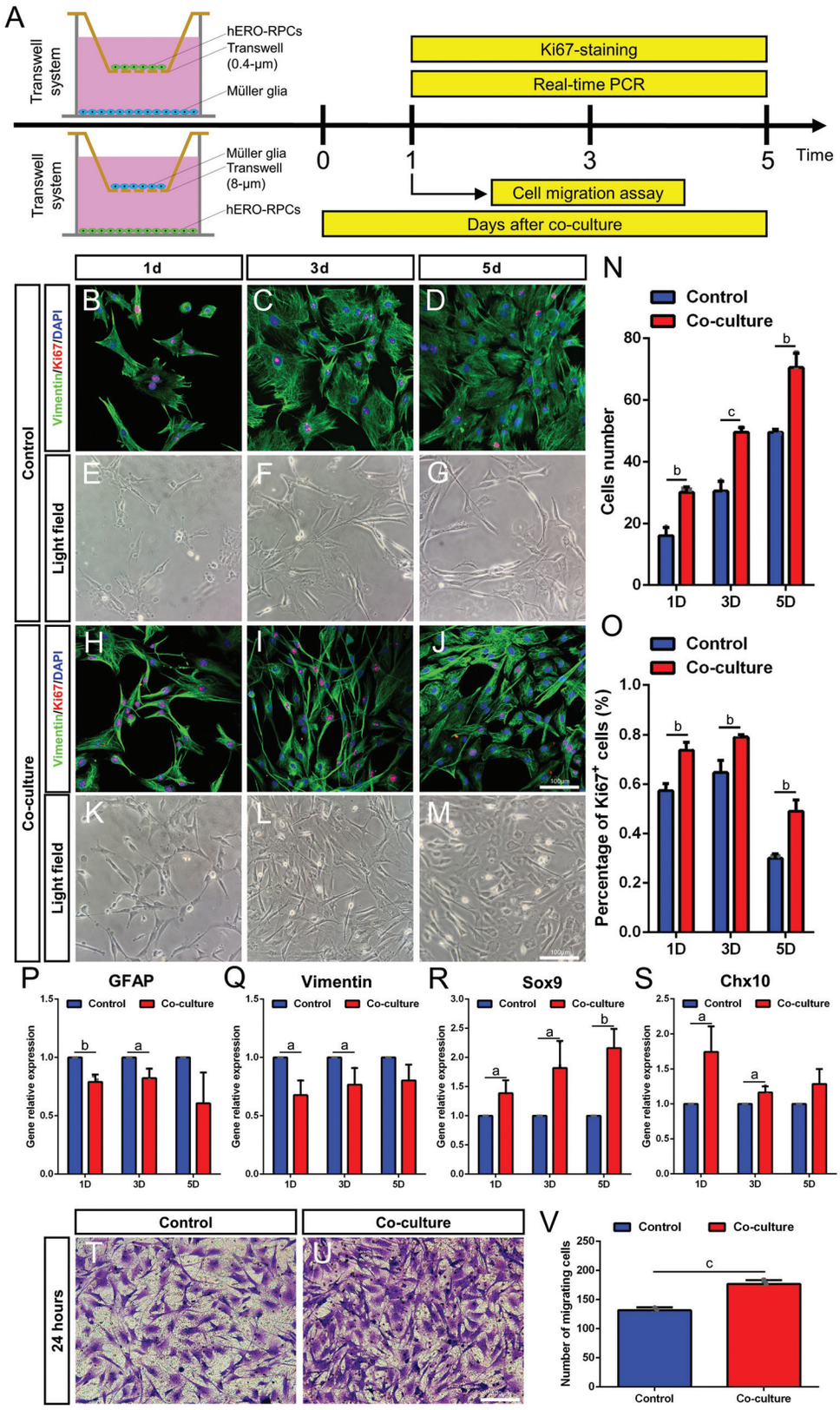

Figure 8 The effect on Müller glia after coculture with hERO-RPCs A: Diagram illustrating the Transwell system. Scheme of time points for Transwell coculture, Ki67 staining, real-time quantitative PCR and crystal violet staining; B-M: Light field and immunofluorescence staining of Ki67-positive Müller glia in the control and cocultured groups at 3 time points; N: Statistical analysis of the number of Müller glia in the control and cocultured groups at 3 time points; O: Statistical analysis of the percentage of Ki67-positive cells in the control and cocultured groups at 3 time points; P-S: Statistical analysis of the mRNA levels of GFAP, Vimentin, Sox9 and Chx10 in the control and cocultured groups at 3 time points; T, U: Crystal violet staining photos of Müller glia in the control and cocultured groups at 24h; V: Statistical analysis of the number of Müller glia that migrated in the control and cocultured groups at 24h. The results are presented as the mean±SD, n=3/group. ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001. Scale bar: 100 μm (B-M, T, U). hERO-RPCs: Retinal progenitor cells from human embryonic stem cell-derived retinal organoid; PCR: Polymerase chain reaction; GFAP: Glial fibrillary acidic protein; Sox9: SRY-box transcription factor 9; Chx10 (alias Vsx2): Visual system homeobox 2; SD: Standard deviation.

and did not observe transdifferentiation of Müller glia into photoreceptors or other neurons, probably due to the short observation time. Since gliosis and dedifferentiation are two fates of Müller glia, we speculate that hERO-RPCs may also be able to inhibit gliosis and delay degeneration by promoting cell dedifferentiation, possibly through paracrine mechanisms. Whether stem cells can reverse the overreactive state of Müller glia into dedifferentiation through extracellular vesicles or other molecules needs to be further explored.

Although reactive Müller glial activation has a protective effect on retinal neurons in the very early stage of retinal damage, the continuous activation and gliosis of Müller glia leads to structural reconstruction, proinflammatory cytokine secretion, abnormal proliferation and glial scar formation[60-61]. In addition, many studies have shown that a major determinant of the success of retinal transplantation is the degree of reactive gliosis in the recipient retina, which is the chemical and physical barrier of migrating cells[71-72]. This study found that transplantation of hERO-RPCs was able to decrease the gliosis levels marked by GFAP at approximately 4-8wk postoperation. However, 2wk after the operation, the degree of gliosis in the grafted area group was equivalent to that in the PBS group, which was higher than that in the blank group. We speculate that this finding is due to surgical response. The nerve layer and pigment layer of the retina are slightly separated during cell transplantation[73-74]. In order to place grafted cells near to the photoreceptor layer, the classical stem cell therapy for photoreceptor degeneration is mostly through subretinal transplantation, which leads to the separation of retinal pigment epithelium (RPE) and photoreceptor layer. However, it has been previously reported in our and other studies that the surgery-related retinal detachment will recovery and is well tolerated[9,75]. Therefore, most subretinal cell transplantation have observed significant therapeutic effects[9,76-78]. Notably, it previously showed that intravitreal injections may lead to severe vitreous cavity proliferation and the formation of an epiretinal membrane, which draws our attention[79-81]. After subretinal transplantation, the transplanted cells located near the photoreceptors and RPE so that they can differentiate into corresponding cells. For another, stem cells may promote photoreceptor survival by exchanging material with photoreceptors, or nourishing photoreceptors[9,76-78,82]. In our results, 4wk after the operation, the injury at the transplantation site was recovered, and the inhibitory effect of RPC on gliosis was more significant and lasted until 8wk postoperation. Meanwhile, this study also focused on the contralateral area of transplantation to show the range of therapeutic areas. Compared with the blank and PBS groups, the ONL thickness of the contralateral area also presented an increasing trend at 2 and 4wk postoperation, but gliosis in the contralateral area was not inhibited at 4wk postoperation, which is consistent with our previous studies showing that grafted cells might inhibit gliosis by interacting directly with Müller glia and inhibiting the Notch signaling pathway[42,83]. Notably, we cannot rule out the role of stem cell paracrine signaling since the secretory mediators might not reach the distant contralateral region of transplantation.

To our knowledge, Sox9 has been demonstrated to be expressed in multipotent progenitor cells during retinal development, but it is expressed only in adult Müller glia, astrocyte and RPE cells[55,84]. As a high mobility family box transcription factor, Sox9 is essential for the establishment and maintenance of neural stem cells in the embryonic and adult central nervous systems[85-86]. Our study found that the number and the distribution distance of Sox9 positive cells in the grafted area group both increased. In the control group, Sox9 was expressed in the INL near the ganglion cell layer (GCL). After hERO-RPCs transplantation, Sox9-positive cells moved toward the ONL and increased in number. Moreover, we found that hERO-RPCs could promote the proliferation and migration of Müller glia *in vitro*. Several studies have shown that Müller glia of the zebrafish retina are activated after damage, migrate to the ONL, undergo asymmetric cell division and regenerate neurons[87-88]. From this, we reasoned that hERO-RPCs might activate retinal Müller glia and regulate them to enter the early stage of dedifferentiation.

We found that the number of Chx10 positive cells increased significantly after hERO-RPCs transplantation, and we confirmed that some of the increased Chx10-positive cells were derived from Müller glia by double labeling Chx10 with the Müller glial markers Vimentin and Sox9. Consistent with our findings, previous studies showed that rat retinal stem cells (rSCs) transplantation could increase the number and expression of Chx10-positive cells, and this phenomenon was also found on the opposite side of the transplantation area[39]. Based on the distribution of Müller glia in the whole retina, we speculate that the gap junctions between Müller glia and Müller glia may provide a pathway for the direct intercellular exchange of ions and small signaling molecules, resulting in an increase in Chx10-positive cells on the side far from the transplantation area[12,89-90]. We believe that stem cell transplantation may promote functional recovery by regulating Müller glia, which may have therapeutic effects as potential stem cells in the mammalian retina.

Although we observed significant changes in Müller glia after hERO-RPCs transplantation, our transplantation observation time was only 8wk at most, and we did not explore the changes in Müller glia at a later stage. In addition, the underlying molecular mechanisms relating to dedifferentiation of Müller glia after hERO-RPCs transplantation were not explored in

the current study, which is a problem worth exploring in the future. Since hERO-RPCs transplantation can improve the microenvironment and promote early dedifferentiation of Müller glia, this therapeutic method is expected to benefit RDD, which needs further research in the future.

In summary, we isolated C-Kit$^+$/SSEA4$^-$RPCs from hERO as therapeutic cells and studied the effect of hERO-RPCs on Müller glia in degenerated rat retinas and through subretinal space transplantation *in vivo* and Transwell coculture systems *in vitro*. This is the first study to show that hERO-RPCs inhibited gliosis, increased the expression of dedifferentiation-associated transcriptional factor and potentially promoted the proliferation and migration of Müller glia, suggesting that Müller glia could promote the early dedifferentiation of Müller glia. These results also provide a new view for optimizing stem cell transplantation in the treatment of retinal degenerative diseases.

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Authors' contribution: Yin ZQ, Zou T and Guo Q designed the experiments. Guo Q, Zeng YX and Huang SD performed the experiments. Guo Q analyzed the data. Guo Q, Zou T and Yin ZQ wrote the manuscript. All authors reviewed and approved the final manuscript for submission.

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Conflicts of Interest: Guo Q, None; **Zeng YX,** None; **Huang SD,** None; **Zou T,** None; **Yin ZQ,** None.

REFERENCES

1 Ashok A, Singh N, Chaudhary S, Bellamkonda V, Kritikos AE, Wise AS, Rana N, McDonald D, Ayyagari R. Retinal degeneration and Alzheimer's disease: an evolving link. *Int J Mol Sci* 2020;21(19):7290.

2 Verbakel SK, van Huet RAC, Boon CJF, den Hollander AI, Collin RWJ, Klaver CCW, Hoyng CB, Roepman R, Klevering BJ. Non-syndromic retinitis pigmentosa. *Prog Retin Eye Res* 2018;66:157-186.

3 Liu W, Liu S, Li P, Yao K. Retinitis Pigmentosa: Progress in molecular pathology and biotherapeutical strategies. *Int J Mol Sci* 2022; 23(9):4883.

4 Adilovic M, Ignjatic E, Cabric A. Optical coherence tomography (OCT) diagnostic of retinitis pigmentosa - case study. *Acta Inform Med* 2022;30(4):329-333.

5 Vezinaw CM, Fishman GA, McAnany JJ. Visual impairment in retinitis pigmentosa. *Retina* 2020;40(8):1630-1633.

6 Fahim A. Retinitis pigmentosa: recent advances and future directions in diagnosis and management. *Curr Opin Pediatr* 2018;30(6):725-733.

7 Gagliardi G, Ben M'Barek K, Goureau O. Photoreceptor cell replacement in macular degeneration and retinitis pigmentosa: a pluripotent stem cell-based approach. *Prog Retin Eye Res* 2019;71:1-25.

8 Gasparini SJ, Llonch S, Borsch O, Ader M. Transplantation of photoreceptors into the degenerative retina: current state and future perspectives. *Prog Retin Eye Res* 2019;69:1-37.

9 Liu Y, Chen SJ, Li SY, Qu LH, Meng XH, Wang Y, Xu HW, Liang ZQ, Yin ZQ. Long-term safety of human retinal progenitor cell transplantation in retinitis pigmentosa patients. *Stem Cell Res Ther* 2017;8(1):209.

10 Tuekprakhon A, Sangkitporn S, Trinavarat A, Pawestri AR, Vamvanij V, Ruangchainikom M, Luksanapruksa P, Pongpaksupasin P, Khorchai A, Dambua A, Boonchu P, Yodtup C, Uiprasertkul M, Sangkitporn S, Atchaneeyasakul LO. Intravitreal autologous mesenchymal stem cell transplantation: a non-randomized phase I clinical trial in patients with retinitis pigmentosa. *Stem Cell Res Ther* 2021;12(1):52.

11 Zhao T, Liang Q, Meng X, Duan P, Wang F, Li S, Liu Y, Yin ZQ. Intravenous infusion of umbilical cord mesenchymal stem cells maintains and partially improves visual function in patients with advanced retinitis pigmentosa. *Stem Cells Dev* 2020;29(16):1029-1037.

12 Wang J, O'Sullivan ML, Mukherjee D, Puñal VM, Farsiu S, Kay JN. Anatomy and spatial organization of Müller glia in mouse retina. *J Comp Neurol* 2017;525(8):1759-1777.

13 Behar-Cohen F, Gelizé E, Jonet L, Lassiaz P. Anatomy of the retina. *Med Sci (Paris)* 2020;36(6-7):594-599.

14 Devoldere J, Peynshaert K, De Smedt SC, Remaut K. Müller cells as a target for retinal therapy. *Drug Discov Today* 2019;24(8):1483-1498.

15 Reichenbach A, Bringmann A. New functions of Müller cells. *Glia* 2013;61(5):651-678.

16 Tworig JM, Feller MB. Müller glia in retinal development: from specification to circuit integration. *Front Neural Circuits* 2021; 15:815923.

17 Eastlake K, Luis J, Limb GA. Potential of Müller glia for retina neuroprotection. *Curr Eye Res* 2020;45(3):339-348.

18 Chen Y, Xia Q, Zeng Y, Zhang Y, Zhang M. Regulations of retinal inflammation: focusing on Müller glia. *Front Cell Dev Biol* 2022; 10:898652.

19 Graca AB, Hippert C, Pearson RA. Müller glia reactivity and development of gliosis in response to pathological conditions. *Adv Exp Med Biol* 2018;1074:303-308.

20 Palko SI, Saba NJ, Mullane E, Nicholas BD, Nagasaka Y, Ambati J, Gelfand BD, Ishigami A, Bargagna-Mohan P, Mohan R. Compartmentalized citrullination in Muller glial endfeet during retinal degeneration. *Proc Natl Acad Sci U S A* 2022;119(9):e2121875119.

21 Goldman D. Müller glial cell reprogramming and retina regeneration. *Nat Rev Neurosci* 2014;15(7):431-442.

22 Rueda EM, Hall BM, Hill MC, Swinton PG, Tong X, Martin JF, Poché RA. The hippo pathway blocks mammalian retinal Müller glial cell reprogramming. *Cell Rep* 2019;27(6):1637-1649.e1636.

23 Wan J, Goldman D. Retina regeneration in zebrafish. *Curr Opin Genet Dev* 2016;40:41-47.

24 Martins RR, Zamzam M, Tracey-White D, Moosajee M, Thummel R, Henriques CM, MacDonald RB. Müller glia maintain their regenerative potential despite degeneration in the aged zebrafish retina. *Aging Cell* 2022;21(4):e13597.

25 Lahne M, Nagashima M, Hyde DR, Hitchcock PF. Reprogramming Müller glia to regenerate retinal neurons. *Annu Rev Vis Sci* 2020; 6:171-193.

26 Chohan A, Singh U, Kumar A, Kaur J. Müller stem cell dependent retinal regeneration. *Clin Chim Acta* 2017;464:160-164.

27 Salman A, McClements ME, MacLaren RE. Insights on the regeneration potential of Müller glia in the mammalian retina. *Cells* 2021;10(8):1957.

28 Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA. Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. *Proc Natl Acad Sci U S A* 2015; 112(44):13717-13722.

29 Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, Wong RO, Rieke F, Reh TA. Stimulation of functional neuronal regeneration from Müller glia in adult mice. *Nature* 2017;548(7665):103-107.

30 Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, Liu X, Chang B, Zenisek D, Crair MC, Demb JB, Chen B. Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. *Nature* 2018;560(7719):484-488.

31 Todd L, Hooper MJ, Haugan AK, Finkbeiner C, Jorstad N, Radulovich N, Wong CK, Donaldson PC, Jenkins W, Chen Q, Rieke F, Reh TA. Efficient stimulation of retinal regeneration from Müller glia in adult mice using combinations of proneural bHLH transcription factors. *Cell Rep* 2021;37(3):109857.

32 Fischer AJ, Omar G, Eubanks J, McGuire CR, Dierks BD, Reh TA. Different aspects of gliosis in retinal Muller glia can be induced by CNTF, insulin, and FGF2 in the absence of damage. *Mol Vis* 2004;10:973-986.

33 Kassen SC, Thummel R, Campochiaro LA, Harding MJ, Bennett NA, Hyde DR. CNTF induces photoreceptor neuroprotection and Müller glial cell proliferation through two different signaling pathways in the adult zebrafish retina. *Exp Eye Res* 2009;88(6):1051-1064.

34 Wan J, Zhao XF, Vojtek A, Goldman D. Retinal injury, growth factors, and cytokines converge on β-catenin and pStat3 signaling to stimulate retina regeneration. *Cell Rep* 2014;9(1):285-297.

35 Fischer AJ, Scott MA, Ritchey ER, Sherwood P. Mitogen-activated protein kinase-signaling regulates the ability of Müller glia to proliferate and protect retinal neurons against excitotoxicity. *Glia* 2009;57(14):1538-1552.

36 Beach KM, Wang J, Otteson DC. Regulation of stem cell properties of Müller glia by JAK/STAT and MAPK signaling in the mammalian retina. *Stem Cells Int* 2017;2017:1610691.

37 Gu D, Wang S, Zhang S, Zhang P, Zhou G. Directed transdifferentiation of Müller glial cells to photoreceptors using the sonic hedgehog signaling pathway agonist purmorphamine. *Mol Med Rep* 2017;16(6):7993-8002.

38 Sanges D, Simonte G, Di Vicino U, Romo N, Pinilla I, Nicolás M, Cosma MP. Reprogramming Müller glia via in vivo cell fusion regenerates murine photoreceptors. *J Clin Invest* 2016;126(8):3104-3116.

39 Tian C, Zhao T, Zeng Y, Yin ZQ. Increased Müller cell de-differentiation after grafting of retinal stem cell in the sub-retinal space of Royal College of Surgeons rats. *Tissue Eng Part A* 2011;17(19-20): 2523-2532.

40 Jian Q, Li Y, Yin ZQ. Rat BMSCs initiate retinal endogenous repair through NGF/TrkA signaling. *Exp Eye Res* 2015;132:34-47.

41 Zhai W, Gao L, Qu L, Li Y, Zeng Y, Li Q, Xu H, Yin ZQ. Combined transplantation of olfactory ensheathing cells with rat neural stem cells enhanced the therapeutic effect in the retina of RCS rats. *Front Cell Neurosci* 2020;14:52.

42 Zou T, Gao L, Zeng Y, Li Q, Li Y, Chen S, Hu X, Chen X, Fu C, Xu H, Yin ZQ. Organoid-derived C-Kit(+)/SSEA4(-) human retinal progenitor cells promote a protective retinal microenvironment during transplantation in rodents. *Nat Commun* 2019;10(1):1205.

43 Greferath U, Huynh M, Jobling AI, Vessey KA, Venables G, Surrao D, O'Neill HC, Limnios IJ, Fletcher EL. Dorsal-Ventral differences in retinal structure in the pigmented Royal College of Surgeons model of retinal degeneration. *Front Cell Neurosci* 2020;14:553708.

44 Rocco ML, Calzà L, Aloe L. NGF and retinitis pigmentosa: structural and molecular studies. *Adv Exp Med Biol* 2021;1331:255-263.

45 Zeng Y, Li M, Zou T, Chen X, Li Q, Li Y, Ge L, Chen S, Xu H. The impact of particulate matter (PM2.5) on human retinal development in hESC-derived retinal organoids. *Front Cell Dev Biol* 2021;9:607341.

46 He XY, Zhao CJ, Xu H, Chen K, Bian BS, Gong Y, Weng CH, Zeng YX, Fu Y, Liu Y, Yin ZQ. Synaptic repair and vision restoration in advanced degenerating eyes by transplantation of retinal progenitor cells. *Stem Cell Reports* 2021;16(7):1805-1817.

47 Liang Q, Li Q, Ren B, Yin ZQ. Intravenous infusion of small umbilical cord mesenchymal stem cells could enhance safety and delay retinal degeneration in RCS rats. *BMC Ophthalmol* 2022;22(1):67.

48 Xie J, Li Y, Dai J, He Y, Sun D, Dai C, Xu H, Yin ZQ. Olfactory ensheathing cells grafted into the retina of RCS rats suppress inflammation by down-regulating the JAK/STAT pathway. *Front Cell Neurosci* 2019;13:341.

49 Zhao C, Tao Z, Xue L, Zeng Y, Wang Y, Xu H, Yin ZQ. Lin28b stimulates the reprogramming of rat Müller glia to retinal progenitors. *Exp Cell Res* 2017;352(1):164-174.

50 Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, Dolznig H. In vitro cell migration and invasion assays. *Mutat Res* 2013;752(1):10-24.

51 Bian B, Zhao C, He X, Gong Y, Ren C, Ge L, Zeng Y, Li Q, Chen M, Weng C, He J, Fang Y, Xu H, Yin ZQ. Exosomes derived from neural progenitor cells preserve photoreceptors during retinal degeneration by inactivating microglia. *J Extracell Vesicles* 2020;9(1):1748931.

52 Gilhooley MJ, Hickey DG, Lindner M, Palumaa T, Hughes S, Peirson SN, MacLaren RE, Hankins MW. ON-bipolar cell gene expression during retinal degeneration: implications for optogenetic visual restoration. *Exp Eye Res* 2021;207:108553.

53 Lawrence JM, Singhal S, Bhatia B, Keegan DJ, Reh TA, Luthert PJ, Khaw PT, Limb GA. MIO-M1 cells and similar muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. *Stem Cells* 2007;25(8):2033-2043.

54 Fischer AJ, Reh TA. Müller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nat Neurosci* 2001; 4(3):247-252.

55 Goel M, Dhingra NK. bFGF and insulin lead to migration of Müller glia to photoreceptor layer in rd1 mouse retina. *Neurosci Lett* 2021; 755:135936.

56 Ke Y, Fan X, Hao R, Dong L, Xue M, Tan L, Yang C, Li X, Ren X. Human embryonic stem cell-derived extracellular vesicles alleviate retinal degeneration by upregulating Oct4 to promote retinal Müller cell retrodifferentiation via HSP90. *Stem Cell Res Ther* 2021;12(1):21.

57 Löffler K, Schäfer P, Völkner M, Holdt T, Karl MO. Age-dependent Müller glia neurogenic competence in the mouse retina. *Glia* 2015; 63(10):1809-1824.

58 Suga A, Sadamoto K, Fujii M, Mandai M, Takahashi M. Proliferation potential of Müller glia after retinal damage varies between mouse strains. *PLoS One* 2014;9(4):e94556.

59 Reichenbach A, Bringmann A. Glia of the human retina. *Glia* 2020; 68(4):768-796.

60 Bringmann A, Wiedemann P. Müller glial cells in retinal disease. *Ophthalmologica* 2012;227(1):1-19.

61 Bringmann A, Iandiev I, Pannicke T, Wurm A, Hollborn M, Wiedemann P, Osborne NN, Reichenbach A. Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. *Prog Retin Eye Res* 2009;28(6):423-451.

62 Jayakody SA, Gonzalez-Cordero A, Ali RR, Pearson RA. Cellular strategies for retinal repair by photoreceptor replacement. *Prog Retin Eye Res* 2015;46:31-66.

63 Singh MS, Charbel Issa P, Butler R, Martin C, Lipinski DM, Sekaran S, Barnard AR, MacLaren RE. Reversal of end-stage retinal degeneration and restoration of visual function by photoreceptor transplantation. *Proc Natl Acad Sci U S A* 2013;110(3):1101-1106.

64 Pearson RA, Gonzalez-Cordero A, West EL, Ribeiro JR, Aghaizu N, Goh D, Sampson RD, Georgiadis A, Waldron PV, Duran Y, Naeem A, Kloc M, Cristante E, Kruczek K, Warre-Cornish K, Sowden JC, Smith AJ, Ali RR. Donor and host photoreceptors engage in material transfer following transplantation of post-mitotic photoreceptor precursors. *Nat Commun* 2016;7:13029.

65 Santos-Ferreira T, Llonch S, Borsch O, Postel K, Haas J, Ader M. Retinal transplantation of photoreceptors results in donor-host cytoplasmic exchange. *Nat Commun* 2016;7:13028.

66 Singh MS, Balmer J, Barnard AR, Aslam SA, Moralli D, Green CM, Barnea-Cramer A, Duncan I, MacLaren RE. Transplanted photoreceptor precursors transfer proteins to host photoreceptors by a mechanism of cytoplasmic fusion. *Nat Commun* 2016;7:13537.

67 Cao J, Murat C, An W, Yao X, Lee J, Santulli-Marotto S, Harris IR, Inana G. Human umbilical tissue-derived cells rescue retinal pigment epithelium dysfunction in retinal degeneration. *Stem Cells* 2016;34(2):367-379.

68 Bammidi S, Bali P, Kalra J, Anand A. Transplantation efficacy of human ciliary epithelium cells from fetal eye and Lin-ve stem cells from umbilical cord blood in the murine retinal degeneration model of laser injury. *Cell Transplant* 2020;29:963689720946031.

69 Liu X, Hou M, Zhang S, Zhao Y, Wang Q, Jiang M, Du M, Shao Z, Yuan H. Neuroprotective effects of bone marrow Sca-1(+) cells against age-related retinal degeneration in OPTN E50K mice. *Cell Death Dis* 2021;12(6):613.

70 Li Z, Zeng Y, Chen X, Li Q, Wu W, Xue L, Xu H, Yin ZQ. Neural stem cells transplanted to the subretinal space of rd1 mice delay retinal degeneration by suppressing microglia activation. *Cytotherapy* 2016;18(6):771-784.

71 Hippert C, Graca AB, Pearson RA. Gliosis can impede integration following photoreceptor transplantation into the diseased retina. *Adv Exp Med Biol* 2016;854:579-585.

72 Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A. Müller cells in the healthy and diseased retina. *Prog Retin Eye Res* 2006;25(4):397-424.

73 Lee SH, Park YS, Paik SS, Kim IB. Differential response of Müller cells and microglia in a mouse retinal detachment model and its implications in detached and non-detached regions. *Cells* 2021;10(8):1972.

74 Sørensen NB, Christiansen AT, Kjaer TW, Klemp K, Cour M, Heegaard S, Warfvinge K, Kiilgaard JF. Loss of retinal tension and permanent decrease in retinal function: a new porcine model of rhegmatogenous retinal detachment. *Acta Ophthalmol* 2020;98(2):145-152.

75 Sørensen NB. Subretinal surgery: functional and histological consequences of entry into the subretinal space. *Acta Ophthalmol* 2019;97 Suppl A114:1-23.

76 Mahato B, Kaya KD, Fan Y, Sumien N, Shetty RA, Zhang W, Davis D, Mock T, Batabyal S, Ni A, Mohanty S, Han Z, Farjo R, Forster MJ, Swaroop A, Chavala SH. Pharmacologic fibroblast reprogramming into photoreceptors restores vision. *Nature* 2020;581(7806):83-88.

77 da Cruz L, Fynes K, Georgiadis O, *et al*. Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. *Nat Biotechnol* 2018;36(4):328-337.

78 Pearson RA, Barber AC, Rizzi M, Hippert C, Xue T, West EL, Duran Y, Smith AJ, Chuang JZ, Azam SA, Luhmann UF, Benucci A, Sung CH, Bainbridge JW, Carandini M, Yau KW, Sowden JC, Ali RR. Restoration of vision after transplantation of photoreceptors. *Nature* 2012;485(7396):99-103.

79 Hu C, La H, Wei X, Zhou Y, Ou Q, Chen Z, Zhu X, Xu JY, Jin C, Gao F, Wang J, Zhang J, Zhang J, Lu L, Xu GT, Tian H. Transplantation Site Affects the Outcomes of Adipose-Derived Stem Cell-Based Therapy for Retinal Degeneration. *Stem Cells Int* 2020;2020:9625798.

80 Kuriyan AE, Albini TA, Townsend JH, Rodriguez M, Pandya HK, Leonard RE, 2nd, Parrott MB, Rosenfeld PJ, Flynn HW, Jr., Goldberg JL. Vision Loss after Intravitreal Injection of Autologous "Stem Cells" for AMD. *N Engl J Med* 2017;376(11):1047-1053.

81 Saraf SS, Cunningham MA, Kuriyan AE, Read SP, Rosenfeld PJ, Flynn HW Jr., Albini TA. Bilateral Retinal Detachments After Intravitreal Injection of Adipose-Derived 'Stem Cells' in a Patient With Exudative Macular Degeneration. *Ophthalmic Surg Lasers Imaging Retina* 2017;48(9):772-775.

82 Li T, Lewallen M, Chen S, Yu W, Zhang N, Xie T. Multipotent stem cells isolated from the adult mouse retina are capable of producing functional photoreceptor cells. *Cell Res* 2013;23(6):788-802.

83 Xie J, Huo S, Li Y, Dai J, Xu H, Yin ZQ. Olfactory ensheathing cells inhibit gliosis in retinal degeneration by downregulation of the Müller cell notch signaling pathway. *Cell Transplant* 2017;26(6):967-982.

84 Poché RA, Furuta Y, Chaboissier MC, Schedl A, Behringer RR. Sox9 is expressed in mouse multipotent retinal progenitor cells and functions in Müller glial cell development. *J Comp Neurol* 2008;510(3):237-250.

85 Scott CE, Wynn SL, Sesay A, Cruz C, Cheung M, Gomez Gaviro MV, Booth S, Gao B, Cheah KS, Lovell-Badge R, Briscoe J. SOX9 induces and maintains neural stem cells. *Nat Neurosci* 2010;13(10):1181-1189.

86 Sanhueza Salas LF, García-Venzor A, Beltramone N, Capurro C, Toiber D, Silberman DM. Metabolic imbalance effect on retinal Müller glial cells reprogramming capacity: involvement of histone deacetylase SIRT6. *Front Genet* 2021;12:769723.

87 Nagashima M, Barthel LK, Raymond PA. A self-renewing division of zebrafish Müller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. *Development* 2013;140(22):4510-4521.

88 Lahne M, Li J, Marton RM, Hyde DR. Actin-cytoskeleton- and rock-mediated INM are required for photoreceptor regeneration in the adult zebrafish retina. *J Neurosci* 2015;35(47):15612-15634.

89 Kihara AH, Mantovani de Castro L, Belmonte MA, Yan CY, Moriscot AS, Hamassaki DE. Expression of connexins 36, 43, and 45 during postnatal development of the mouse retina. *J Neurobiol* 2006;66(13):1397-1410.

90 Zahs KR, Ceelen PW. Gap junctional coupling and connexin immunoreactivity in rabbit retinal glia. *Vis Neurosci* 2006;23(1):1-10.