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Supplemental Information

Single-cell transcriptome analysis of xenotransplanted human retinal organoids defines two migratory cell populations of nonretinal origin

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Supplementary Materials

Single-cell transcriptome analysis of xenotransplanted human retinal organoids defines two migratory cell populations of nonretinal origin

Ying V. Liu, *et al*.

Supplemental Figure 1

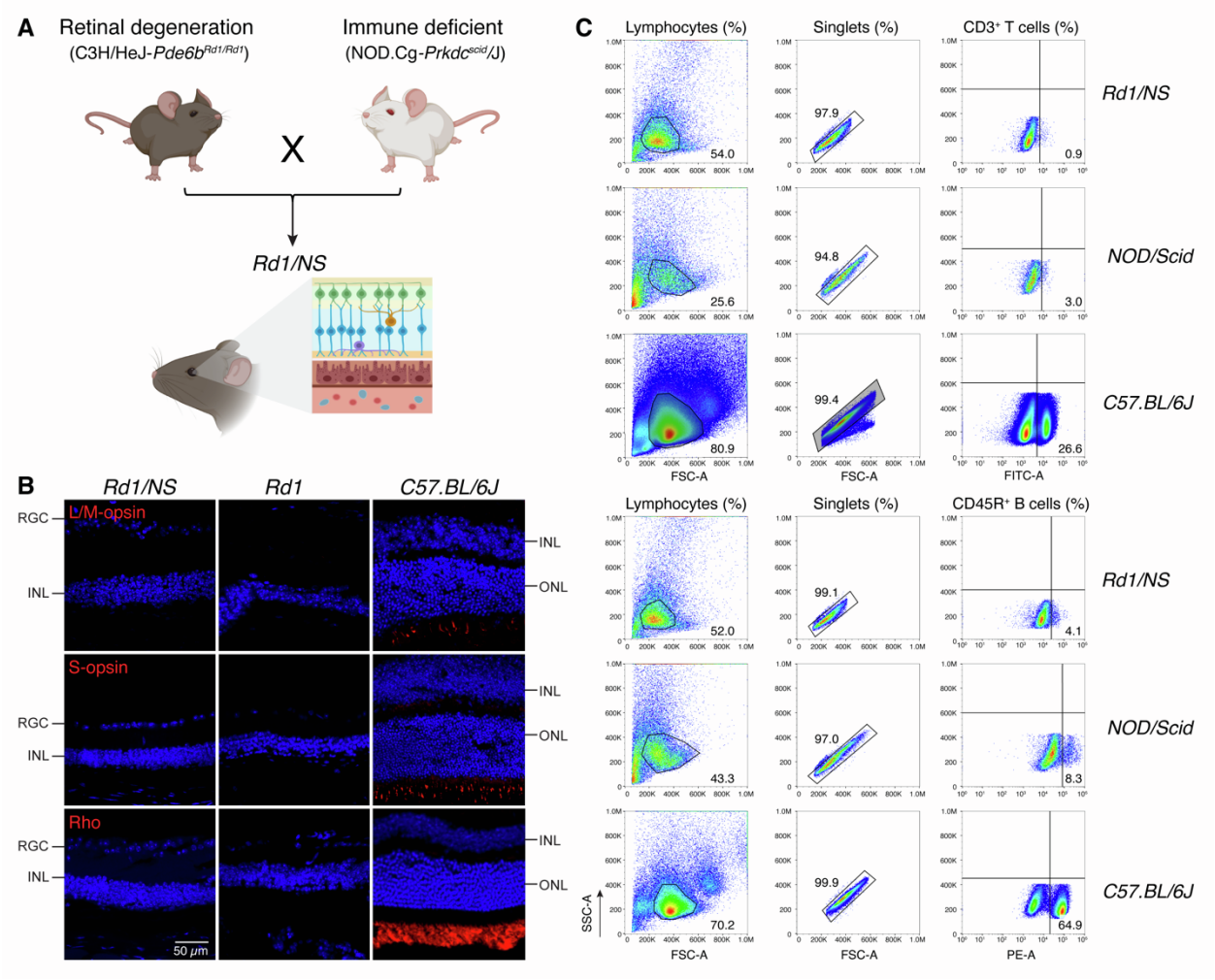

Fig. S1. Breeding and phenotyping of the recipient *Rd1/NS* mice. (A) Schematic showed the recipient *Rd1/NS* mice were generated by crossbreeding C3H/HeJ-*Pde6b*$^{Rd/Rd11}$ (*Rd1)* and NOD.Cg-*Prkdc*^{scid}/J (*NOD/Scid*) mice. **(B)** IHC staining showed fully degenerated photoreceptor cells and negative expression of L/M-opsin, S-opsin, and Rhodopsin (Rho) in adult *Rd1/NS* mice

and *Rd1* mice. *C57.BL/6J* mice served as *wild-type* controls. **(C)** Flow cytometry analysis showed the deficiency of $CD3^+$ T cells and $CD45R^+$ B cells in *Rd1/NS* mice, corresponding to the immune deficient phenotype of *NOD/Scid* mice. *C57.BL/6J* mice served as *wild-type* controls. N= 4 eyes per group.

Supplemental Figure 2

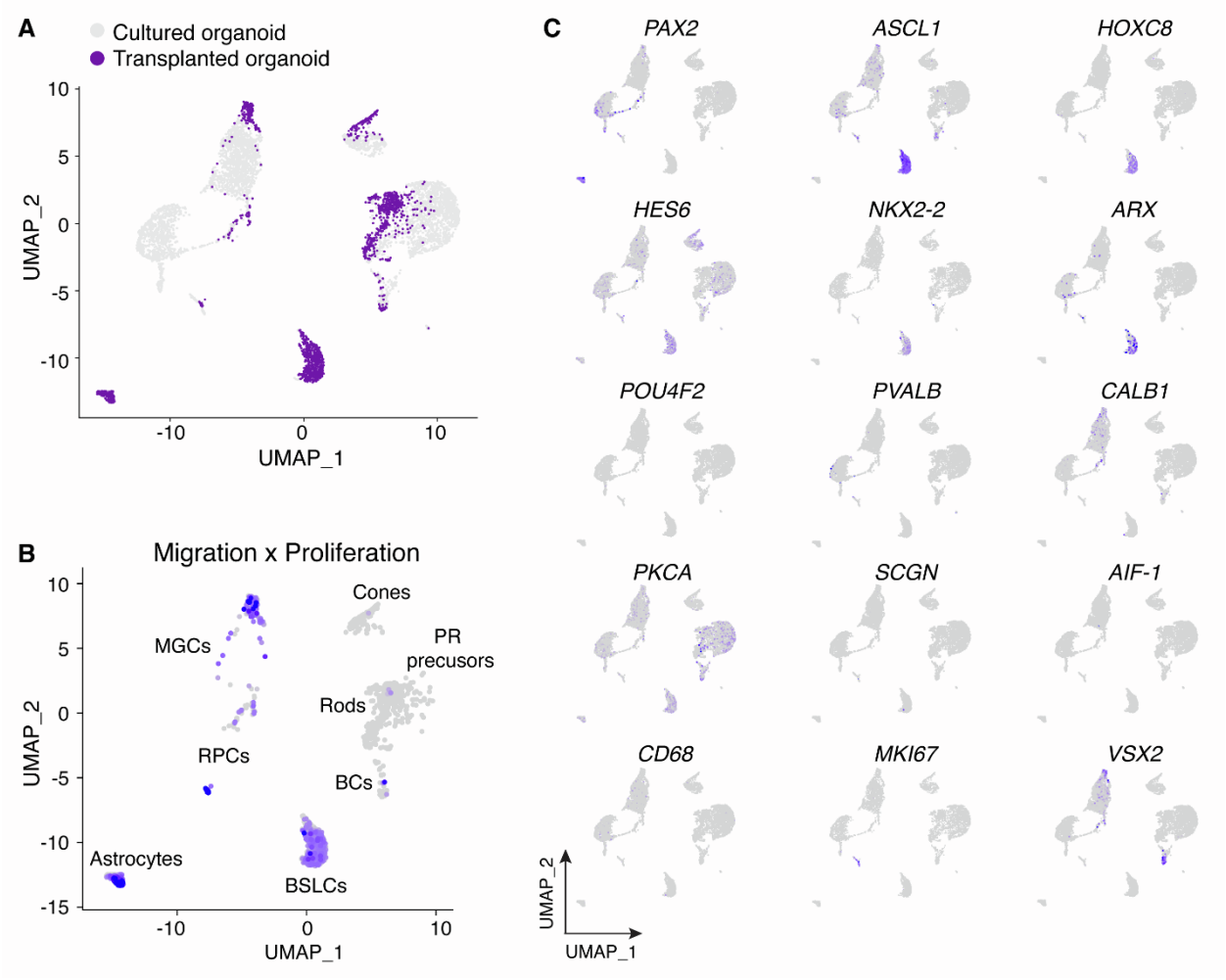

Fig. S2. UMAP plots of migration and proliferation cell clusters. (A) UMAP plot colored cell clusters of cultured (grey, n=2 organoids from one batch) and transplanted retinal organoids (purple, n=3 transplanted eyes). **(B)** UMAP plot colored cell clusters sharing transcriptomic characteristics of migration and proliferation. **(C)** UMAP plots displayed the expression of marker genes in cell clusters of transplanted and cultured retinal organoids.

Supplemental Figure 3

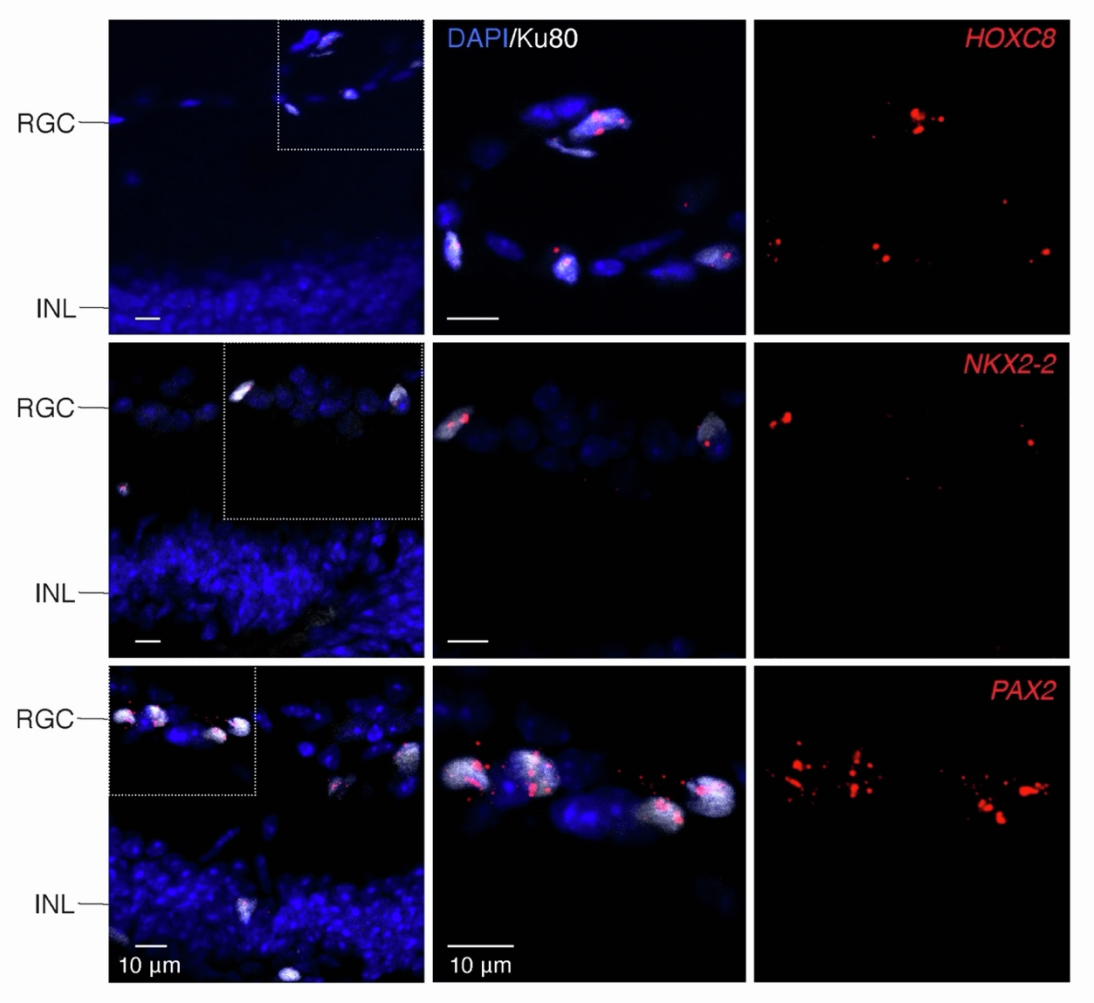

Fig. S3. Human iPSC donor-derived migratory cells include astrocytes and brain/spinal cord-like neural precursors. Migratory cells from human iPSC-derived retinal organoid grafts (n=3 organoids from one iPSC batch, distinct from the H9 ESC-derived organoids used in Fig. 1-7, Fig. S1-S2, and Fig. S5-S6) were detected in the RGC layer and INL of recipient mouse retinae by human nuclear specific antibody Ku80 staining. RNAscope staining showed migratory cells expressing markers of BSL cells *(HOXC8, NKX2-2*) and astrocytes (*PAX2*), consistent with the characteristics of those migratory cells in Crx:tdTomato^{+} hESC-derived retinal organoid grafts. *Abbreviation: RGC: retinal gaglion cell layer; INL: inner nuclear layer.*

Supplemental Figure 4

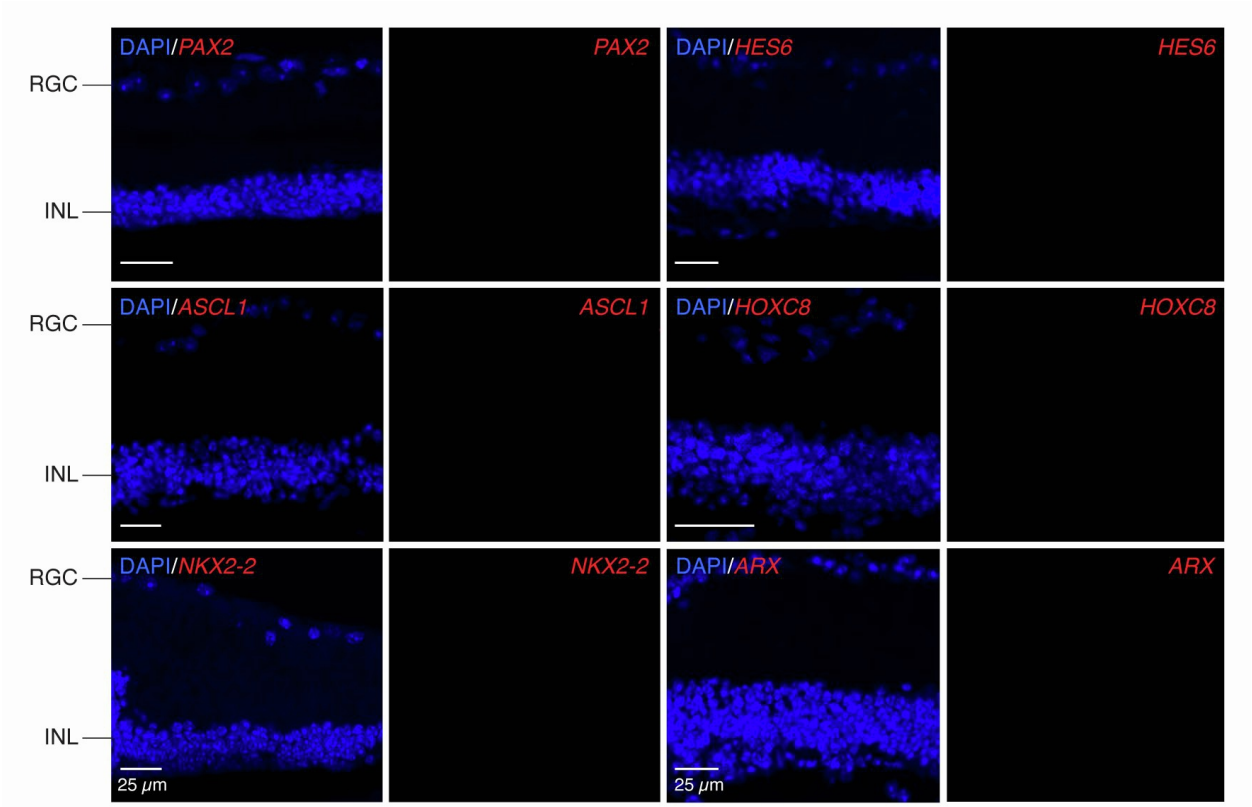

Fig. S4. RNAscope staining of human astrocyte and BSL on non-transplanted *Rd1/NS* control mice. Cryosections of non-transplanted *Rd1/NS* mice were stained with human probes of astrocyte (*PAX2*) and BSL cells (*HES6*, *ASCL1*, *HOXC8*, *NKX2-2*, *ARX*). None of these human genes were detected in non-transplanted *Rd1/NS* mice retinae. *Abbreviation: RGC: retinal ganglion cell layer; INL: inner nuclear layer.*

Supplemental Figure 5

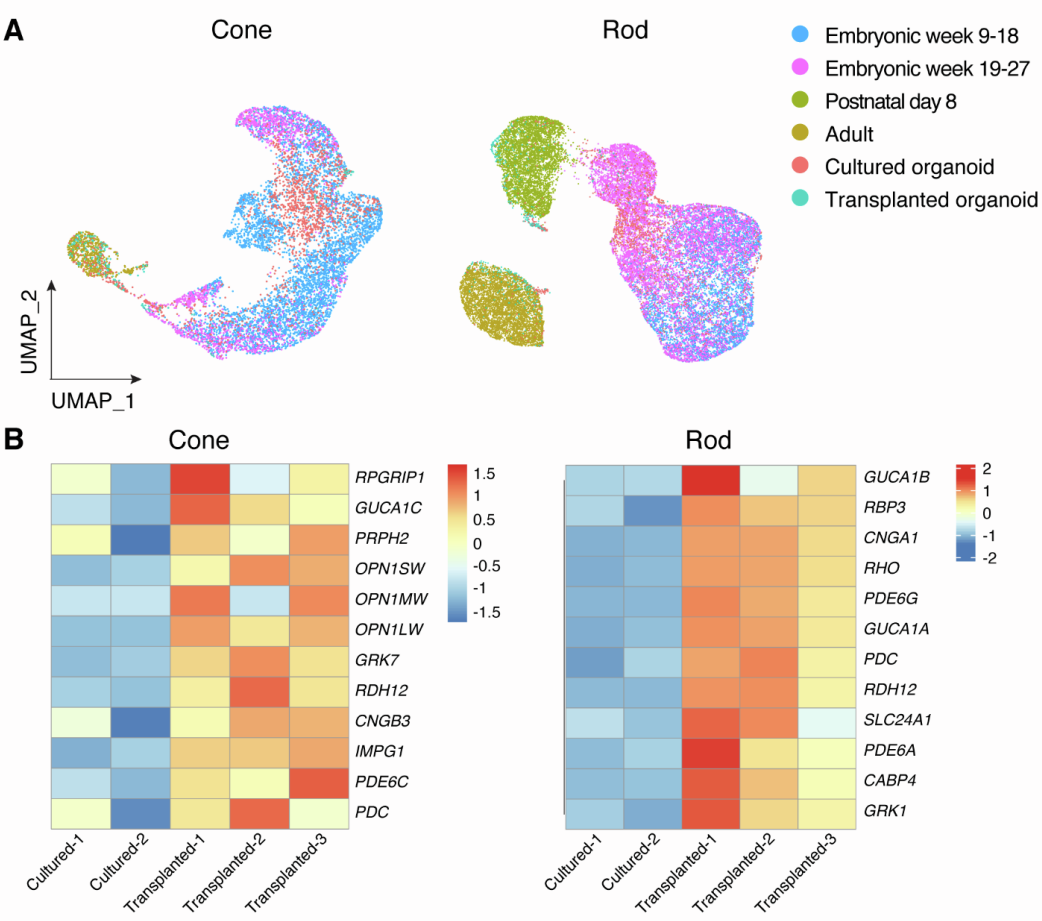

Fig. S5. Upregulation of cone and rod marker genes in transplanted retinal organoids. (A) UMAP plots showed cells colored by sample libraries, including human retina developmental datasets (cone: n = 7,654 cells, rod: n = 25,186 cells), cultured retinal organoids (cone: n =1,639 cells, rod: n=1,469 cells, collecting from two organoids in one batch), and transplanted retinal organoids (cone: n=210 cells, rod: n =504 cells, collecting from three transplanted eyes). **(B)** Heatmaps demonstrated the upregulation of marker genes specific for cone and rod photoreceptors in transplanted retinal organoids (including three independent replicates "Transplanted-1, Transplanted-2, Transplanted-3"), compared to cultured retinal organoids (including two independent replicates "Cultured-1, Cultured-2" from one batch).

Supplemental Figure 6

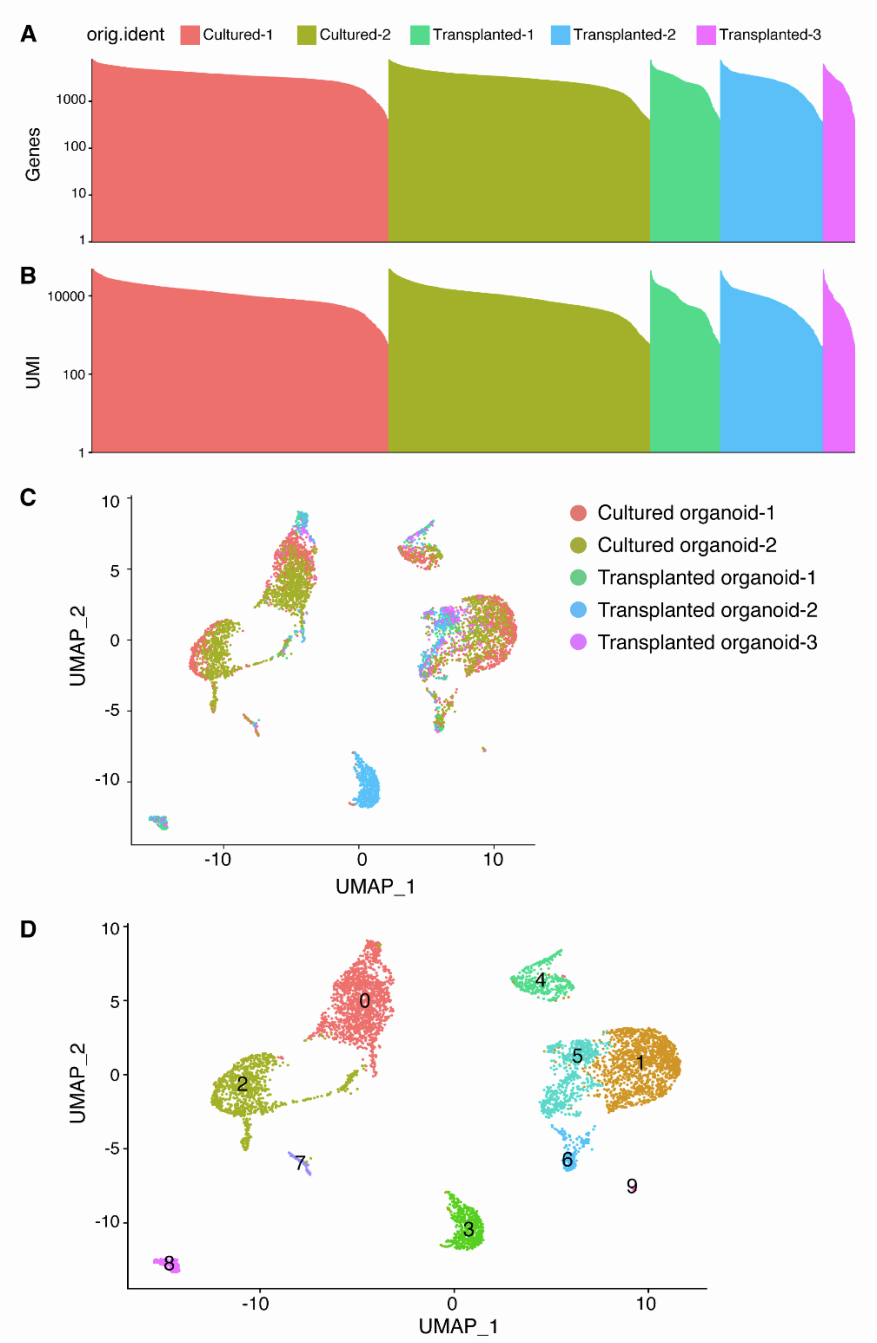

Fig. S6. Quality control of scRNA-seq data. (A) Number of genes and **(B)** unique molecular identifiers (UMI) per cell. Each bar is a cell and is colored by the sample library and ordered along the x-axis in descending order. **(C)** UMAP plot showing cells colored by sample library. **(D)** UMAP plot showing 10 (0-9) transcriptionally distinct cell clusters.

Supplemental Figure 7

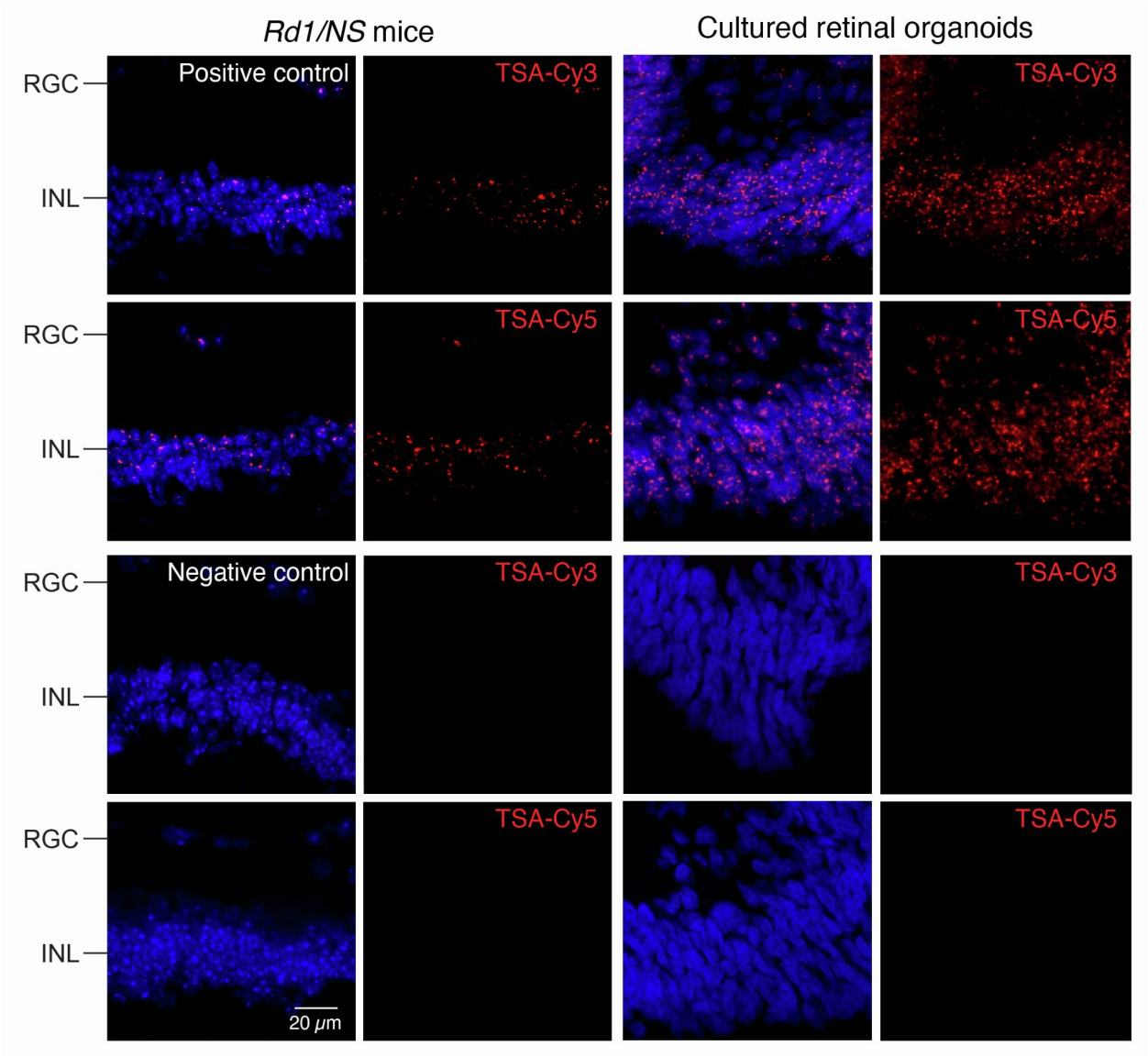

Fig. S7. RNAscope staining of positive and negative control probes. Cryosections of non-transplanted *Rd1/NS* mice and cultured retinal organoids were stained with 3-plex positive and negative control probes in combination with TSA-Cy3 or TSA-Cy5 fluorophores. Positive probes target common housekeeping genes *PPIB* (Cy3) and *POLR2A* (Cy5). Negative probe targets the bacterial dapB gene.

Table S1. BE6.2 media and long-term retina media (LTR) for Crx:tdTomato^{+} retinal organoids.

Reagent	Concentration	Source	Catalog Number
BE6.2 media			
DMEM	—	Gibco	11885084
B27 minus vitamin A	2%	Gibco	12587010
Glutamax	1%	Gibco	35050061
NEAA	1%	Gibco	11140050
Sodium pyruvate	1mM	Gibco	11360070
NaCl	0.87 mg/mL	Sigma-Aldrich	S9888
E6 supplement	**2.5%**		
Insulin	970 ug/mL	Roche	11376497001
Holo-transferrin	535 ug/mL	Sigma-Aldrich	T0665
L-ascorbic acid	3.20 mg/mL	Sigma-Aldrich	A8960
Sodium selenite	0.7 ug/mL	Sigma-Aldrich	S5261
long-term retina (LTR) media			
DMEM	—	Gibco	11885084
F12	25%	Gibco	11765062
B27	2%	Gibco	17504044
NEAA	1%	Gibco	11140050
Fetal bovine serum	10%	Gibco	16140071
Sodium pyruvate	1mM	Gibco	11360070
Glutamax	1%	Gibco	35050061
Taurine	1 mM	Sigma-Aldrich	T-8691

Table S2. Retinal organoid culturing media using hiPS cell line.

Reagent	Concentration	Source	Catalog Number
Neural Induction Media (NIM) (Day 3-Day 15)			
DMEM/F12 (1:1)	—	Life Technologies	11330-057
100x N2 Supplement	1% (v/v)	Life Technologies	17502-048
HEPARIN (stock as 1mg/mL in DMEM, 0.1%)	2 ug/mL	Sigma	H3149-100
100x MEM-NEAA	0.01	Life Technologies	11140050
Retinal Differentiation Media (RDM) (Day 16-Day 30)			
DMEM	—	Life Technologies	11330-057
F12	—	Life Technologies	11965
50x B27 (without Vit A)	1x	Life Technologies	11765
100x Antibiotic and Antimycotic	1x	Life Technologies	12587-010
100x MEM-NEAA	1x	Life Technologies	15240
RC2 (Day 30-Day 91)			
DMEM	----	Life Technologies	11965
F12	----	Life Technologies	11765
50x B27 (without Vit A)	1x	Life Technologies	12587010
100x Antibiotic and Antimycotic	1x	Life Technologies	15240
100x MEM-NEAA	1x	Life Technologies	11140050
FBS	10%	Gibco or Atlanta Biologicals	S11150
100x Glutamax	1x	Life Technologies	35050061
1000x Taurine (100mM)	100 uM	Sigma	T0625

Retinoic Acid (directly add RA to cells when changing media)	0.5-1 uM	Sigma-Aldrich	R2625
RC1 (>Day 91)			
DMEM/F12-Glutamax	----	Life Technologies	10565-018
100x N2 Supplement	1% (v/v)	Life Technologies	17502-048
100x Antibiotic and Antimycotic	1x	Life Technologies	15240
100x NEAA	1x	Life Technologies	11140050
FBS	10%	Atlanta Biologics	S11150
1000x Taurine (100mM)	100 uM	Sigma	T0625
Retinoic Acid (directly add RA to cells when changing media)	0.5 uM	Sigma-Aldrich	R2625

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Table S3. Forward and reverse primer sequences used for mice genotyping.

Gene	F primer (5' to 3')	R primer (5' to 3')
Rd1 Wild type	ACTCTGTGGCCTCAAAGATACATC	TGCAGGTCACAGAATCATCATAACA
Rd1 Mutant	GGGTCTCCTCAGATTGATTGACTAC	GTCACTCTGTGGCCTCAAAGAT
NOD/Scid	TGTAACGGAAAAGAATTGGTATCCACA	GTTGGCCCCTGCTAACTTTCT

Table S4. Reagents used for RNAscope staining.

Reagents	Dilution	Source	Catalog Number
RNA probes			
PAX2-Hs	No dilution	Advanced Cell Diagnostics	442541
HES6-Hs	1:50	Advanced Cell Diagnostics	521301-C2
ASCL1-Hs	1:50	Advanced Cell Diagnostics	459721-C2
NKX2-2-Hs	No dilution	Advanced Cell Diagnostics	821401
HOXC8-Hs	No dilution	Advanced Cell Diagnostics	506531
ARX-Hs	1:50	Advanced Cell Diagnostics	486711-C2
VSX2-Hs	1:50	Advanced Cell Diagnostics	493031-C2
3-plex positive control-Mm	No dilution	Advanced Cell Diagnostics	320881
3-plex positive control-Hs	No dilution	Advanced Cell Diagnostics	320861
3-plex negative control	No dilution	Advanced Cell Diagnostics	320871
TSA-fluorophores			
TSA plus-Cy3	1:1500	AKOYA Biosciences	NEL744001KT
TSA plus-Cy5	1:1500	AKOYA Biosciences	NEL745001KT

Table S5. Antibodies used for RNAscope counterstaining and immunohistochemistry staining.

Reagents	Dilution	Source	Catalog Number
Primary antibodies			
Rabbit anti-RBPMS	1:500	MilliporeSigma	ABN1362
Mouse anti-NeuN	1:500	MilliporeSigma	MAB377
Mouse anti-Calbindin	1:500	Sigma-Aldrich	C9848
Goat anti-PKCα	1:200	R&D Systems	AF5340
Goat anti-SCGN	1:200	Thermo Fisher Scientific	PA5-47664
Rabbit anti-IBA1	1:250	Abcam	Ab178680
Rabbit anti-CD68	1:100	Abcam	Ab125212
Rabbit anti-Ki67	1:500	Thermo Fisher Scientific	MA5-14520
Rabbit anti-L/M-opsin	1:500	Kerafast	EDK101
Rabbit anti-S-opsin	1:500	MilliporeSigma	Ab5407
Rabbit anti-Rhodopsin	1:500	Abcam	Ab3424
Mouse anti-human nuclear antibody (HNA)	1:1000	MilliporeSigma	Mab1281
Sheep anti-Ki67	1:40	R&D Systems	AF7617
Rabbit anti-Ku80	1:50	Thermo Fisher Scientific	MA5-32212
Secondary antibodies			
Goat anti-Rabbit 488	1:500	Abcam	Ab150077
Goat anti-Mouse 488	1:500	Thermo Fisher Scientific	A-11001
Goat anti-Mouse 647	1:500	Thermo Fisher Scientific	A32728
Donkey anti-Goat 488	1:500	Abcam	Ab150129
Donkey anti-Sheep 647	1:200	Thermo Fisher Scientific	A-21448
Donkey anti-Rabbit 488	1:200	Thermo Fisher Scientific	A21206

Supplemental experimental procedures

Cell Lines

The use of human stem cells was approved by the Johns Hopkins ISCRO (ISCRO00000249). The H9 CRX:tdTomato human embryonic stem cell line (hESCs) was a kind gift from Dr. David M. Gamm (University of Wisconsin Hospitals, USA) and Dr. Donald J. Zack (Johns Hopkins University, USA). The hiPSC line derived from CD34+ cord blood is a commercially available cell line (A18945, Thermo Fisher Scientific)(Burridge et al., 2011). The use of human iPSCs for generation of retinal organoids in this study conforms to the University of Colorado Office of Regulatory Compliance. Stem cells were maintained in mTeSR1 (Stem Cell Technologies, Cambridge, MA, USA) on 1% (vol/vol) Matrigel-GFRTM (BD Biosciences, USA, No. 354230,) coated dishes and grown in a 37°C HERAcell 150i incubator at 10%CO2 and 5% O2 incubator (Thermo Fisher Scientific, MA, USA). Cells were passaged upon confluence (every 3-6 days) using Accutase (Sigma-Aldrich, MO, USA, No. SCR005) for 7–10 minutes, and dissociated into single cells. Cells in Accutase were added 1:2 to mTeSR1 plus 5 μM Blebbistatin (Bleb; B0560, Sigma), pelleted at 700 g for 5 minutes, and suspended in mTeSR1 plus Bleb and plated at 5,000 cells per well in a six-well plate. After 48 hours, cells were fed with mTeSR1 (without Bleb) every 24 hours until the next passage. To minimize cell stress, no antibiotics were used in RPMI (Gibco, USA) and supplement media (10% fetal bovine serum (FBS), 2.5% penicillin). Cells were maintained at 37°C and 5% CO2 and passaged every 3-4 days at ~1 x $10^{\wedge 5}$ – 2 x $10^{\wedge 6}$ cells/ml in uncoated flasks. Cells were routinely tested for mycoplasma using MycoAlert (Lonza, Switzerland, No. LT07).

Retinal organoid culturing

For H9 CRX:tdtomato^{+} retinal organoid culturing, the hESCs were dissociated in Accutase at 37°C for 12 min and seeded in 50 µl of mTeSR1 at 3,000 cells/well into 96-well ultra-low adhesion round bottom Lipidure coated plates (AMSBIO, MA, USA, No.51011610). Cells were placed in hypoxic conditions (10% CO2 and 5% O2) for 24 hours to enhance survival. Cells naturally aggregated by gravity over 24 hours. On day 1, cells were moved to normoxic conditions (5% CO2). On days 1- 3, 50 µl of BE6.2 media, **Table S1**) containing 3 µM Wnt inhibitor (IWR1e, EMD Millipore, MA, USA, No. 681669,) and 1% (v/v) Matrigel were added to each well. On days 4-9, 100 µl of media were removed from each well, and 100 µl of media were added. On days 4-5, BE6.2 media containing 3 µM Wnt inhibitor and 1% Matrigel was added. On days 6-7, BE6.2 media containing 1% Matrigel was added. On days 8-9, BE6.2 media containing 1% Matrigel and 100 nM Smoothened agonist (SAG, EMD Millipore, No. 566660) was added. On day 10, aggregates were transferred to 15 mL tubes, rinsed 3X in DMEM (Gibco, No. 11885084), and resuspended in BE6.2 with 100 nM SAG in untreated 10 cm polystyrene petri dishes. From this point on, media was changed every other day. Aggregates were monitored and manually separated if stuck together or to the bottom of the plate. On day 11, retinal vesicles were manually dissected using sharpened tungsten needles. After dissection, cells were transferred into 15 mL tubes and washed 2X with 5 mLs of DMEM. On days 14-17, long-term retina (LTR, **Table S1**) media with 100 nM SAG was added. On days 18-21, cells were maintained in LTR and washed 2X with 5 mLs of DMEM, before being transferred to new plates to wash off dead cells. To increase survival and differentiation, 1 µM all-trans retinoic acid (ATRA; R2625; Sigma) was added to LTR medium from days 22-138. 10 µM Gammasecretase inhibitor (DAPT, EMD Millipore, No. 565770) was added to LTR from days 28-42. Retinal organoids were grown at low density (10-20 per 10 cm dish) to reduce aggregation.

For the generation of retinal organoids from human iPS cells, a human induced pluripotent stem cell (hiPSC) line derived from $CD34^+$ cord blood was used for all experiments in this study (A18945, ThermoFisher Scientific) (Burridge *et al.*, 2011). Cell culture, retinal differentiation, and human retinal organoid formation were conducted as previously described (Zhong et al., 2014). A more detailed protocol of the methodology for generating retinal organoids was recently described (Aparicio-Domingo et al., 2023). Briefly, hiPSCs were maintained on Matrigel (growth-factor-reduced; BD Biosciences) coated plates. After 6 days in culture, hiPSC colonies were lifted and cultured as free-floating neural aggregates (NAs); this was established as Day 0 (D0) of differentiation. On D7, NAs were seeded onto Matrigel (growth-factor-reduced; BD Biosciences) coated dishes, and individual mechanical detachment of the NR and RPE domains was performed between D21 and D23. The culture media and reagents are listed in **Table S2.** Undifferentiated hiPSCs and derived retinal organoids were routinely tested for Mycoplasma contamination by PCR.

Animals

All animal experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee (approval M016M17). The C3H/HeJ-*Pde6*$^{Rd1/Rd1}$ (referred to as *Rd1*), and NOD.Cg-*Prkdc*^{scid}/J (referred to as *NOD/Scid*) mice of either gender (aged 6 to 8 weeks) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in cages under a 12:12-hour light-dark cycle with water and food provided *ad libitum*.

Recipient mice

We created a recipient mouse model with immune-deficiency and retinal degeneration (referred to as *Rd1/NS*) by crossbreeding *Rd1* mice and *NOD/Scid* mice (aged 8 weeks). The breeding strategy was performed as previously reported (Wenzel et al., 2007). Genomic DNA of the third-generation

offspring was extracted from ear biopsies and genotyped by Transnetyx Tag Center (Cordova,TN, USA). Primers were listed in **Table S3**. Eyes of adult *Rd1/NS* mice (n=3) were collected to characterize photoreceptor degeneration using immunohistochemistry (IHC) staining, as previously reported (Liu et al., 2021). Flow cytometry was performed using spleen biopsies of adult *Rd1/NS* mice (n=3) to confirm the deficiency of T cells and B cells, as previously described (Hensel et al., 2019). Phenotyping data of *Rd1/NS* mice are shown in **Fig. S1**.

Preparation of donor cells

Donor retinal organoid cells (harvested as micro-dissected multilayered retinal fragments) were obtained from CRX:tdTomato^{+} hESC-derived retinal organoids (aged 134 days, n=4) and hiPSC derived retinal organoids (aged 150 days, n=3). The cultured human retinal organoids were imaged using a fluorescent microscope (Carl Zeiss, Jena, Germany). The images were used as a reference to isolate the retinal cluster from the donor retinal organoids. The isolated retinal organoids clusters were then cut into 1 × 1 mm$^{\wedge 2}$ or 1 × 2 mm$^{\wedge 2}$ microdissected fragments using a 27-gauge horizontal curved scissors (VitreQ, Kingston, NH, USA) under a dissection microscope. Donor cells were transplanted within two hours of isolation.

Transplantation of donor cells

Donor retinal organoid fragments were transplanted into the subretinal space of *Rd1/NS* mice (aged 6 to 8 weeks, n=16 eyes for hESC-derived retinal organoids, n=5 eyes for iPS-derived retinal organoids), as previously reported (Liu et al., 2020). Briefly, recipient mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (20 mg/kg body weight). Mouse pupils were dilated with 1% (wt/vol) tropicamide (Bausch & Lomb, Rochester, NY, USA). Mouse corneas were covered with Sodium Hyaluronate (Healon GV, Abbott Medical Optics Inc. CA, USA) and cover glasses (Deckglaser, USA) to facilitate

transpupillary visualization. The donor retinal organoid fragments were loaded into the bevel of a 26G microneedle with the photoreceptor side facing down, gently aspirated into the attached micro-syringe (Hamilton, Reno, NV, USA), then tangentially injected into the subretinal space through the sclera of the recipient mice. Successful injection was verified by direct visualization through the dilated pupil of the recipient under the surgical microscope (Leica, Wetzlar, Germany).

Single cell RNA sequencing

Single cell RNA sequencing (scRNA-seq) was performed on dissociated cells from transplanted and cultured CRX:tdTomato^{+} retinal organoids using the Chromium platform (10X Genomics). Briefly, retinal organoid cells were dissociated into a single cell suspension using the Papain Dissociation System (Worthington) for 60 minutes at 37°C, with gentle mixing every 5 minutes, before stopping the reaction using ovomucoid protease inhibitors. Cells were centrifuged and resuspended in ice-cold PBS containing 0.04% bovine serum albumin (BSA) and 0.5 U/µl RNase inhibitor and were filtered through a 40-µm Flowmi cell strainer (Bel-Art SP). Cell counts and viability were assessed by Trypan blue staining before loading 6000 cells on a Chromium Single Cell system using Next GEM 3' reagent v3.1 kits. Libraries were pooled and sequenced on Illumina NextSeq 500 with ~50,000 reads per cell. The Cell Ranger 4 (10X Genomics) pipeline was used to process the raw sequencing reads for demultiplexing. Since the starting material used to generate the library consisted of human and mouse cells, reads were aligned to a hybrid GRCm38 mouse and GRCh38 human reference genome. Cell barcodes that had most of their reads mapped to GRCm38 mouse genes were considered to be of mouse origin and excluded. The remaining cellular barcode-associated reads were re-mapped to the GRCh38 human reference genome and a cell-by-gene count matrix was generated for downstream analysis. In this study, only the human cells were ultimately analyzed. The generated cell-by-gene count matrices were

analyzed using the Seurat ver3 R package (Stuart et al., 2019). We filtered out cells that had UMIs less than 300 or greater than 50000 and with a mitochondrial fraction of greater than 20%. Doublets were identified and removed using the DoubletFinder R package (McGinnis et al., 2019). Log-normalization, scaling, UMAP dimensional reduction and clustering were performed using the standard Seurat pipeline. Quality control of scRNA-seq data were shown in **Fig. S6**. Major retinal cell types were identified using previously identified cell type markers (Hoang et al., 2020). Enriched genes from the brain/spinal-like cell cluster were compared to the ASCOT (Ling et al., 2020) gene expression summaries of public RNA-Seq data to determine its classification. Differential gene tests were performed by Seurat's *FindMarkers* function using the Wilcoxon rank sum test with default parameters (Stuart *et al.*, 2019). Hierarchical clustering was used to group the differentially expressed genes. The UCell R package (Andreatta and Carmona, 2021) was used to calculate the migration potential score or the proliferation score (**data files S1, S2**). The gene sets were constructed by identifying enriched genes within the gene ontology terms cell migration and cell motility for the migration potential score and cell division for the proliferation score respectively. The Seurat integration functions (*SelectIntegrationFeatures*, *FindIntegrationAnchors* and *IntegrateData*) were used to integrate the organoid data onto the human retinal developmental dataset (Lu et al., 2020). Monocle 3 (Cao et al., 2019) was used to perform pseudotime analysis and identify trajectory routes within the data.

Histology

Four and a half months post-transplantation, the recipient mice were sacrificed with over-dose anesthesia and pre-fixed by heart-perfusion with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS. Eyes were gently removed, post-fixed in 4% PFA/PBS for one hour at room temperature (RT), and dehydrated in a sucrose gradient (10%, 20%,

30%), then blocked in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Cultured retinal organoids were fixed in 4% PFA at RT for 15 minutes (min), dehydrated in gradient sucrose (10%, 20%, 30%), and blocked in the OCT compound. OCT-blocked recipient mouse eyes and cultured retinal organoids were cut into 7-10 μm thick cryosections using a microtome (CM 1850; Leica) for histological staining.

RNAscope and IHC counter-staining was performed according to the manufacturer's protocol (Advanced Cell Diagnostics (ACD), see Protocol #MK 51-150, Appendix D.). Briefly, cryosections of recipient mice eyes and cultured retinal organoids were rinsed with PBS, baked in a HybEZTM oven (ACD, USA) for 30 min at 60°C, and post-fixed in pre-chilled 4% PFA in PBS for 15 min at 4°C. Slides were dehydrated in gradient ethanol (50%, 70%, 100%), treated with hydrogen peroxide (10 min at RT), then subjected to target retrieval using the Co-detection Target Retrieval solution (ACD, Cat. No. 323180) at 98-102°C for 5 min. After rinsing in distilled water (2 min x 2) and PBS-T (5 min x 1), the slides were incubated with diluted primary antibody at 4°C overnight. On day 2, slides were post-fixed with 4% PFA for 30 min at RT, treated with protease III at 40°C for 30min, and subjected to RNAscope staining using the RNAscope Multiplex Fluorescent V2 assay according to the manufacturer's protocol (ACD, RNAscope USM-323100, see "fixed-frozen tissue sample protocol"). Briefly, RNA probe hybridization was performed with the HybEZTM oven for two hours at 40°C. Slides were then assigned for three series of amplification, fluorochromes combination, and HRP blocking. After the RNAscope procedure, slides were incubated with secondary antibody at RT for one hour, counter stained with DAPI, and mounted with Prolong Diamond (Life Technology, Carlsbad, CA, USA). The RNA probes, fluorophores used were listed in **Table S4**. The primary and secondary antibodies used for IHC

counter staining were listed in **Table S5**. Negative and positive multiplex control probes staining were run in parallel with the target probes following the same protocol (data shown in **Fig. S7**).

IHC staining was performed as previously described (Liu *et al.*, 2020). Briefly, cryosections of transplanted *Rd1/NS* mice and cultured retinal organoids were rinsed with PBS (5 min x 1), permeabilized, and blocked with a mixture of 0.1% Triton-X100 and 5% goat serum in PBS for one hour at RT. The slides were rinsed in PBS (5 min x 3), incubated with primary antibodies at 4°C overnight, incubated with secondary antibodies at RT for one hour, then counter stained with DAPI and mounted using ProLong Diamond mounting media. The primary antibodies and secondary antibodies used here were listed in **Table S5**.

Quantification of donor cell migration of recipient retina

For migratory distance quantification of transplanted retinal organoid cells, retinal sections from recipient mice were stained with human nuclear specific antibodies HNA (Sigma-Aldrich, MO, USA) or Ku80 (Thermo Fisher Scientific, MA, USA). Tile scan images were collected using Confocal LSM 880 (Zeiss, Oberkochen, Germany) for distance quantification. The migratory distance of transplanted retinal organoids was defined as the shortest distance between the migratory cells and the nearest graft edge (i.e., the graft-left migratory cells to the left endpoint of the graft; the graft-right migratory cells to the right endpoint of the graft). We used a mathematical method to facilitate distance quantification. Specifically, the graft edge was defined as a "starting point" and the migratory cells in different retinal laminae (RGC, IPL, INL, RPE/C) were manually targeted, both processed with the "Cell Counter" plugin in ImageJ. The cell coordinates were automatically collected to quantify the X and Y axial distances of individual cells by the Cell Counter. The axial distance of the graft edge (starting point) was referred to as "X_{start}" and "Y_{start}". The axial distance of the migratory cells was referred to as "$X_{migratory}$" and "$Y_{migratory}$". The

migratory distance was computed in R platform (see supplementary **coding file S1**) following the formula:

$$Migrating\ distance\ = \sqrt{(X_{start} - X_{migratory})^2\ +\ (Y_{start} - Y_{migratory})^2}$$

The unit of the migrating distance was converted from pixel to micron according to the image scale.

For cell quantification, the number of positively stained cells was manually counted using the "Cell Counter" plugin in ImageJ. The representative pre-synapse graphs of the transplanted and cultured retinal organoids were drawn by Imaris software (Version 9.5.0, Bitplane AG, Zurich, Switzerland).

Electrophysiology

The electrophysiological recording was performed on the transplanted CRX:tdTomato$^+$ photoreceptors eight months post-transplantation to measure their physiological properties. We were able to test only one recipient mouse (the second recipient mouse died before the assay during the long-term observation). The recipient's eyes were gently pulled out from the recipient mouse and put in Ames' medium (Sigma No. A1420). Retinas with transplanted retinal organoids were dissected by removing the corneas and lens under infrared light, attached to a piece of filter, sectioned into 200μm slices, and transferred to a recording chamber. The CRX:tdTomato$^+$ photoreceptors of the transplanted retinal organoids were targeted under an epifluorescence microscope for consequent whole-cell patch-clamp recording. Fluorescent signal was imaged by a Nikon CCD camera with data acquisition synchronized with a 20-ms flash of epi-fluorescence excitation light. The total exposure time to excitation light before recording was <500 ms. During recording, retina was perfused with Ames' medium bubbled with 95% O_2/5% CO_2. Patch electrodes (5-7 MΩ) were pulled from borosilicate capillaries (GC150-10, Harvard Apparatus) and

filled with an internal solution containing typically (in mM): 120 K-gluconate, 5 NaCl, 4 KCl, 10 HEPES, 2 EGTA, 4 ATP-Mg, 0.3 GTP-Na_2, and 7 Phosphocreatine-Tris, with pH adjusted to 7.3 with KOH. Whole-cell patch-clamp recording was made at 30–32^{o}C with an Axon Instruments Multiclamp 700B amplifier. Series resistance of patch electrodes was 10–30 MΩ. Liquid-junction potential (measured to be -13 mV) has been corrected. In voltage-clamp mode, recorded cells were held at -40 mV, followed by voltage steps of 100-ms (-70 mV to -10 mV). All procedures were carried out in the darkroom to avoid photoreceptor bleaching.

Statistical analysis

Quantitative histology data were analyzed using two-way ANOVA. Sidak's test or Tukey's test was adopted for multiple comparisons (two-tailed). Independent T-test or Mann-Whitney U test was used for two variants comparison. Statistical analysis was carried out using SPSS software (version 25, IL, USA). $p < 0.05$ was taken to be significant. Statistical data were presented as mean ± SD. Graphs were drawn with GraphPad Prism software (version 8, CA, USA). Schematics were created with BioRender.com (agreement number QH23QWJX12, KY23QWKEPB).

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Supplemental coding file S1

R coding algorithms for migrating distance quantification

```
library(data.table)
library(plyr)

##############################################################################
#
# Extract the X,Y coordinate of migrated cells, and generate subsequent distance data presented in
Table 1
#
##############################################################################

#---> Extracting data exported from ImageJ

common_path = "~/Desktop/cell invasion/Processed_Image"

files_to_read = list.files(
  path = common_path,        # directory to search within
  pattern = ".*(Rd1-NS).*csv$", # regex pattern
  recursive = TRUE,          # search subdirectories
  full.names = TRUE          # return the full path
)

#---> Hypothetical Researcher has 37 retina slides to quantify
#     and wants to localize 5 cell types per retina slides. So that's
#     37 rows and 6 columns (including cell id) in the data list

data_lst = lapply(files_to_read, read.csv)  # read all the matching files
celltype_summary = data.frame(matrix(ncol = 6, nrow = 37))
colnames (celltype_summary) <- c("File_name", "Type_1", "Type_2", "Type_3", "Type_4",
"Type_5")

#---> Calculating the distance from starting point for each cell
#     categorized by cell types

for (i in 1:length(data_lst)){
  data_lst[[i]]$Address <- rep(files_to_read[i],nrow(data_lst[[i]]))
```

```
  File_Name <-  files_to_read[i]
  Type_1 <- sum(data_lst[[i]]$Type == 1)
  Type_2 <- sum(data_lst[[i]]$Type == 2)
  Type_3 <- sum(data_lst[[i]]$Type == 3)
  Type_4 <- sum(data_lst[[i]]$Type == 4)
  Type_5 <- sum(data_lst[[i]]$Type == 5)
  celltype_summary[i,1:6] = c(File_Name, Type_1, Type_2, Type_3, Type_4, Type_5)
  print(c(File_Name, Type_1, Type_2, Type_3, Type_4, Type_5))
  for (j in 1:nrow(data_lst[[i]])){
  data_lst[[i]][j,"Z.µm."]=sqrt(( data_lst[[i]][j,"X.µm."]- data_lst[[i]][1,"X.µm."])^2 +
                                ( data_lst[[i]][j,"Y.µm."]- data_lst[[i]][1,"Y.µm."])^2) #distance calculation
  }
}

#---> Exporting analyzed invasion distance summary

dat1<-ldply(data_lst)

write.table(as.data.frame(dat1),file="Detialed_Result.csv", quote=F,sep=",",row.names=F)
write.table(as.data.frame(celltype_summary),file="Celltype_Summary.csv",
quote=F,sep=",",row.names=F)
```