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Supplemental Information

Synaptic repair and vision restoration in advanced degenerating eyes by transplantation of retinal progenitor cells

Xiang-Yu He, Cong-Jian Zhao, Haiwei Xu, Kang Chen, Bai-Shi-Jiao Bian, Yu Gong, Chuan-Huang Weng, Yu-Xiao Zeng, Yan Fu, Yong Liu, and Zheng-Qin Yin

Supplemental Information

Figure S1

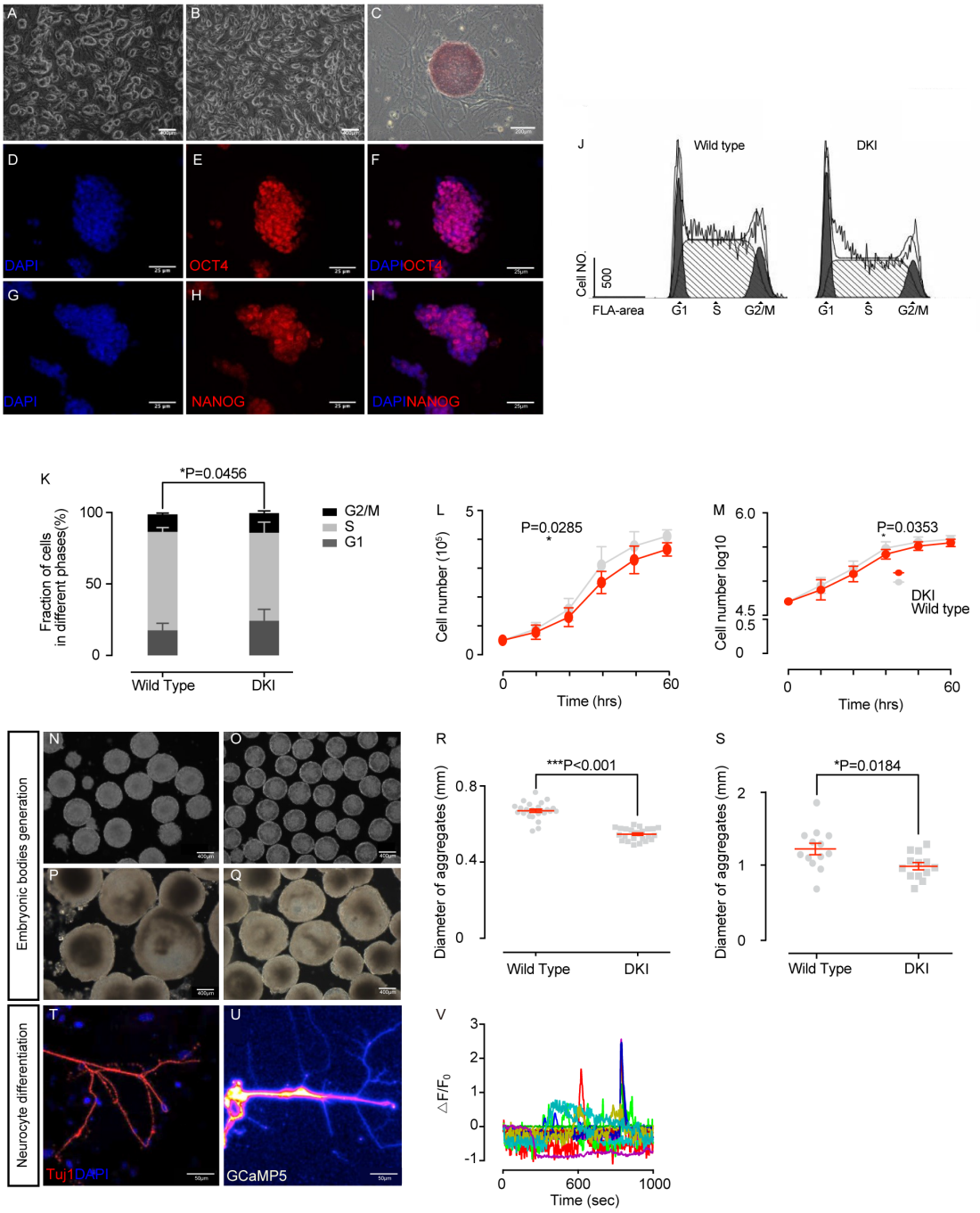


Figure S1 | *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESC line characterization and capability of differentiation in *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESCs, Related to Figure 1.

A, B, Image of mES cell colonies cultured in ES medium + LIF (2000 units/ml) on feeders (Mouse Embryonic Fibroblast, MEF); wild type mESCs (**A**), *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESC lines (**B**). **C,** ES cell colony stained for alkaline phosphatase (red). **D-I,** Presence of pluripotency genes OCT4, NANOG in the *C-kit-mCherry;*

Rosa26-lsl-GCaMP5 mESCs colonies at passage five. **J, K,** Cell cycle assay. After 24-h serum starvation in maintenance medium containing 0.5% fetal bovine serum (FBS), cells were planted in 24 wells plate in a density of 5*10^4 with medium containing 15% FBS. Cells were then incubated for a further 24h and implemented for cell cycle assay. 81.08 ± 1.79% of wild type mESCs were in the G2 and S phases. 75.36 ± 3.89% of *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESCs were in the G2 and S phases. Error bars, mean ± SEM (n = 4 independent experiments, FACS cell cycles were determined by BD Calibur and Igor Pro v. 6.10) *p <0.05, **p < 0.01 and ***p < 0.001, by paired t test. **L, M,** Growth curve of mESC at a total density of 5,000cells/cm^2, and the cell numbers were counted for further 60 hours. (n=4 of independent experiments, cell numbers were counted by Hemocytometer) *p <0.05, **p < 0.01 and ***p < 0.001, by paired t test.). **N-S,** Formation of embryoid bodies(EBs) by hanging drop method (300 cells per drop); Size comparison of EB formation of wild type mESC (Wild Type) (**N**) and EB formation of *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESC (DKI) (**O**) at incubation day 2 (**R**); Size comparison of EB formation of wild type mESC (**P**) and EB formation of *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESC (**Q**) at incubation day 4 (**S**). Error bars, mean ± SEM (n = 3 independent experiments with 75 aggregates counted per experiment). *p <0.05, **p < 0.01 and ***p < 0.001. **T-V,** Differentiation of *C-kit-mCherry; Rosa26-lsl-GCaMP5* mESC into neural cells; Immunofluorescence of neural specific microtubules proteins Tuj1 (T) and its autonomous calcium spikes by recording GCaMP5 fluorescence response (U, V). Scale bar: 400μm(A-C, N-Q), 50 μm (T, U) and 25μm(D-I).

Figure S2

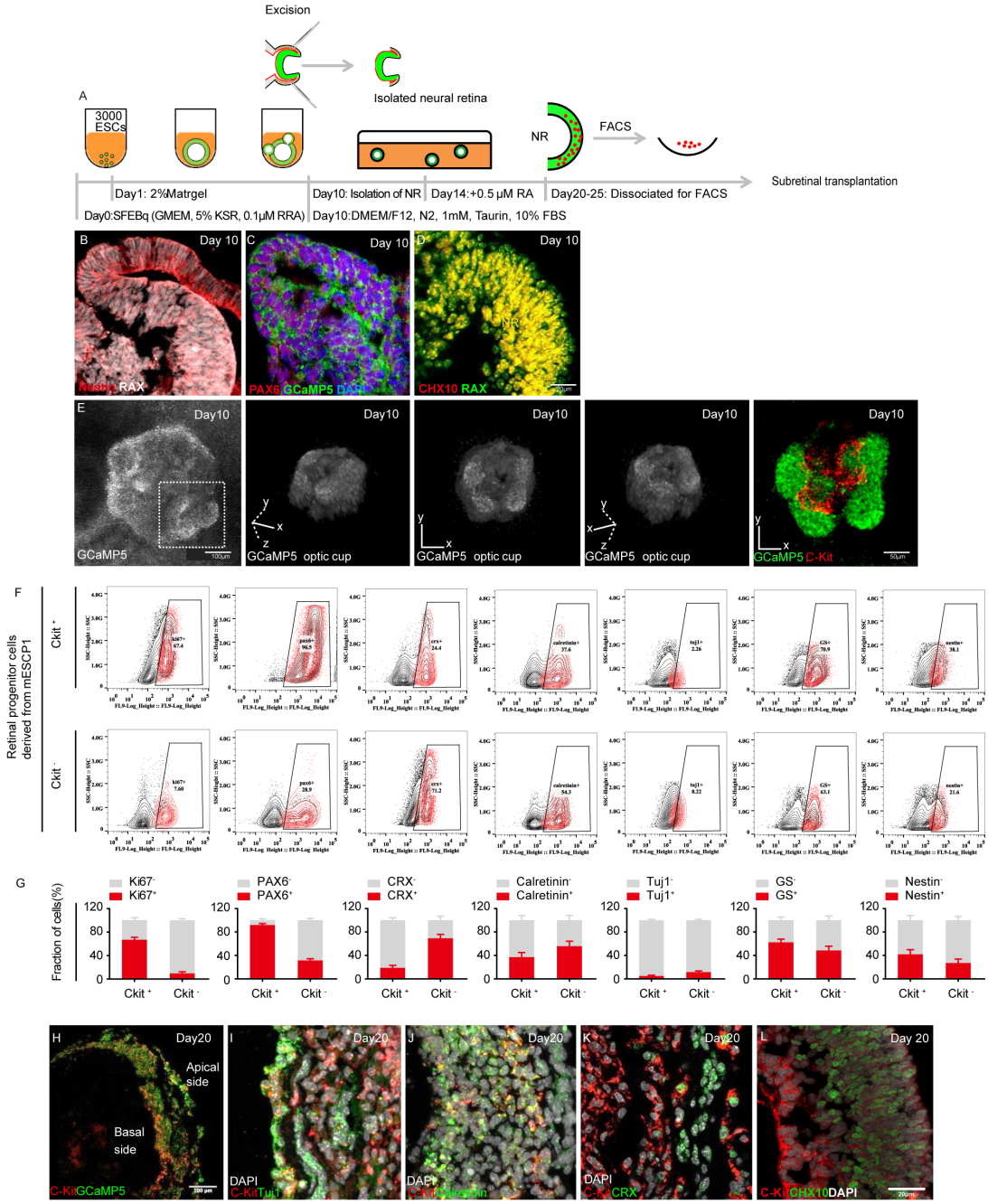


Figure S2| Three dimensional optic cup culture and FACS analysis of mEROs-derived C-kit^{+} retinal progenitor cell (RPC) co-express with retinal progenitor cell (RPC) markers, Related to Figure 1 and Figure 2.

A Schematic cartoon showing the process of retinal organoid differentiation in 3D culture system. Briefly, differentiation was initiated by the re-aggregation of dissociated DKI mESCs (3,000 cells) in a V-bottomed 96-well plate (Sumitomo Bakelite) filled with modified retinal differentiation medium (RDM) containing the retinoic acid receptor antagonist (RRA) AGN193109, and the knockout serum replacement (KSR) content was increased to 5% to

reflect a synergistic effect with Matrigel (BD Biosciences) to promote retinal anlage formation (Decembrini et al., 2014). Under these conditions, the mESC-derived continuous neuroepithelium generated four to eight optic vesicles that had evaginated from the main body; these vesicles gradually invaginated to form two-wall cup-like structures on day 10 of culture (Figure 1B-E, Figure S3E). Then, we mechanically isolated optic cups from the main aggregates at day 10. The 500 nM all-trans RA (Sigma-Aldrich) was supplemented between day 14 and 16 only. Normoxic conditions were used throughout the culture period.**B-D** Both of the inner portion (neural retina) and outer wall (pigment epithelium) expressed neural identify markers Nestin and PAX6 (**B, C**). Within optic cup-like structure, the neural retina strongly expressed retinal progenitor markers RAX and CHX10 (**B, D**) on differentiation day 10. **E** Surface-rendering of 3D-reconstructed images of GCaMP5 labelled optic cups. The right panel shows the C-kit-mCherry signal (red) was focused on the basal side of the NR. **F, G** To investing mEROs-derived C-kit^{+}/SSEA1^{-} co-express with retinal progenitor cell (RPC) markers, we examined the expression of various RPC markers PAX6 (for ganglion and amacrine cells), CRX (for photoreceptor cells), Calretinin (for amacrine cells), Tuj1 (for early stage of ganglion cells and amacrine cells), GS (for glia), Nestin (immature or glia cells) and that of the cell proliferation marker Ki67 in P1 C-kit^{+}/SSEA1^{-} cells. Among the RPC markers, Ki67, PAX6, GS and Nestin expression were detected in 66.800±4.225%, 91.833±2.685%, 62.467±5.575% and 41.711±8.088% of the cells in the C-kit^{+} cell population, respectively. CRX, Calretinin, and Tuj1 expression maintained in C-kit^{+}/SSEA1^{-} cells but was lower than in C-kit^{-}/SSEA1^{-} cells. **H-L** Immunofluorescence of 3D NR. C-kit signals were observed at the basal side of central NR (**H**) and were colocalized with retinal cell markers including Tuj1, Calretinin, CRX and CHX10 (**I-L**) post differentiation. Scale bar: 100μm (E left panel, H), 50 μm (E right panel) and 20μm (B-D, I-L).

Figure S3

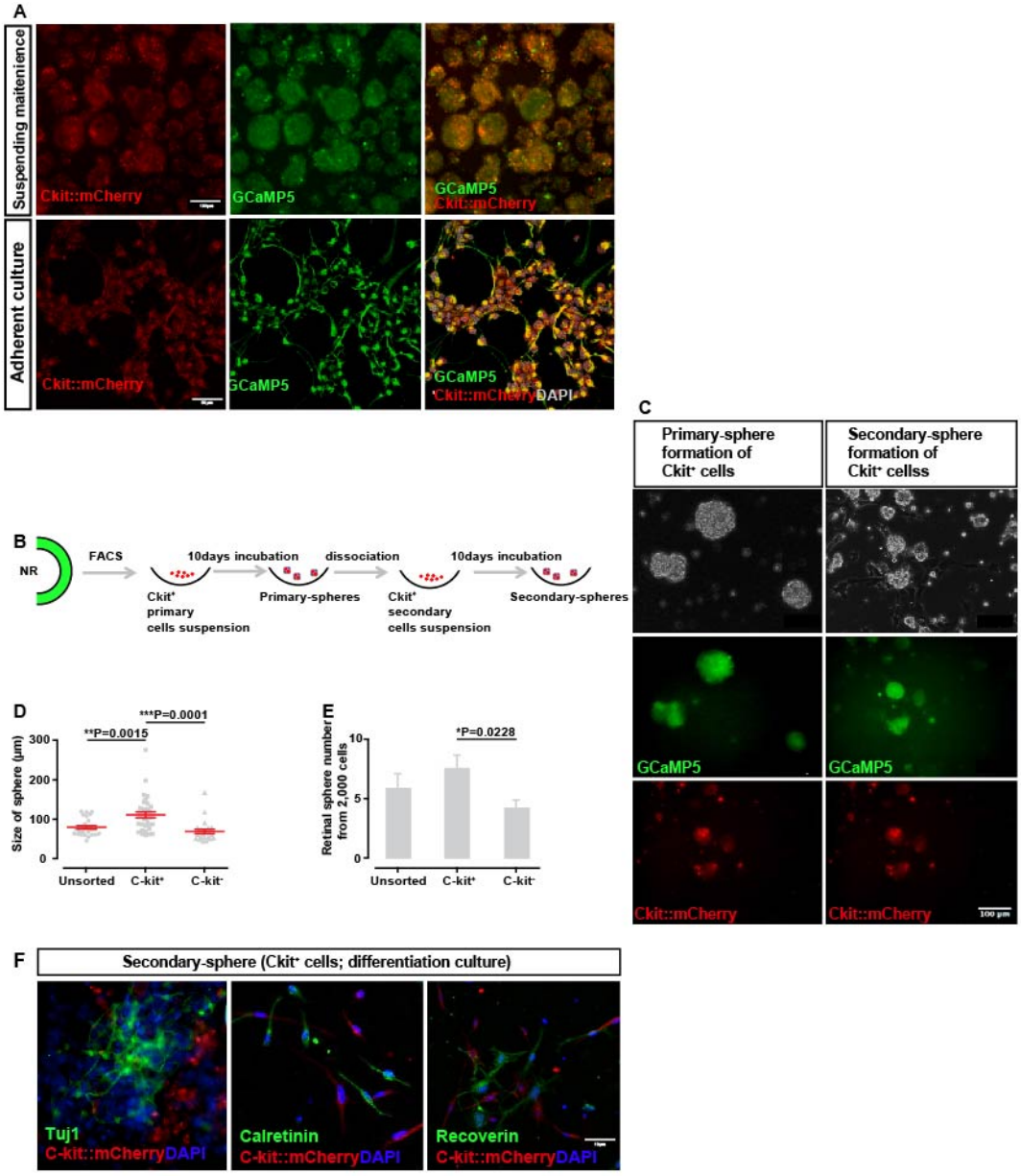


Figure S3 | Proliferation and differentiation of mEROs-derived C-kit^{+} retinal progenitor cell (RPC), Related to Figure 2.

A,B mEROs-derived C-kit^{+}/SSEA1^{-} cells were able to form colonies both in suspension and adherence conditions like fetal progenitors. **C** we confirmed the contribution of C-kit to colony-sphere formation. The cells were then subjected to culture with progenitor culture medium (1,000 cells per well). For comparison, we used unsorted SSEA1^{-} cells and C-kit^{-}/SSEA1^{-} cells from the same NR. The primary colony spheres were observed after 7-14 days of incubation. Cells that were subsequently dissociated from these colonies also showed

the same ability to form secondary spheres (1,000 cells per well). **D** The number of spheres formed by C-kit^{+}/SSEA1^{-} cells was significantly higher than the number spheres formed by C-kit^{-}/SSEA1^{-} cells. **E** C-kit^{+}/SSEA1^{-} cells formed larger spheres than C-kit^{-}/SSEA1^{-} cells. Although C-kit^{-} cells retained the capacity for sphere formation, C-kit^{+} cells play a major role in cell proliferation and colony-sphere formation at this stage of retinal differentiation and further verify the self-renewal capability of C-Kit^{+} cells. **F** To examine the differentiation ability of these cells, we applied a retinal specific differentiation protocol to P1 C-kit^{+}/SSEA1^{-} cells. After 2 weeks of incubation, the cells derived from spheres expressed fate-specific retinal cell markers, such as Tuj1 (ganglion and amacrine cells), Calretinin (amacrine cells), Recoverin (photoreceptors). This result indicates that mEROs-derived C-kit^{+}/SSEA1^{-} cells had the potency to differentiate into a subset of retinal neurons and gliacytes.

Figure S4

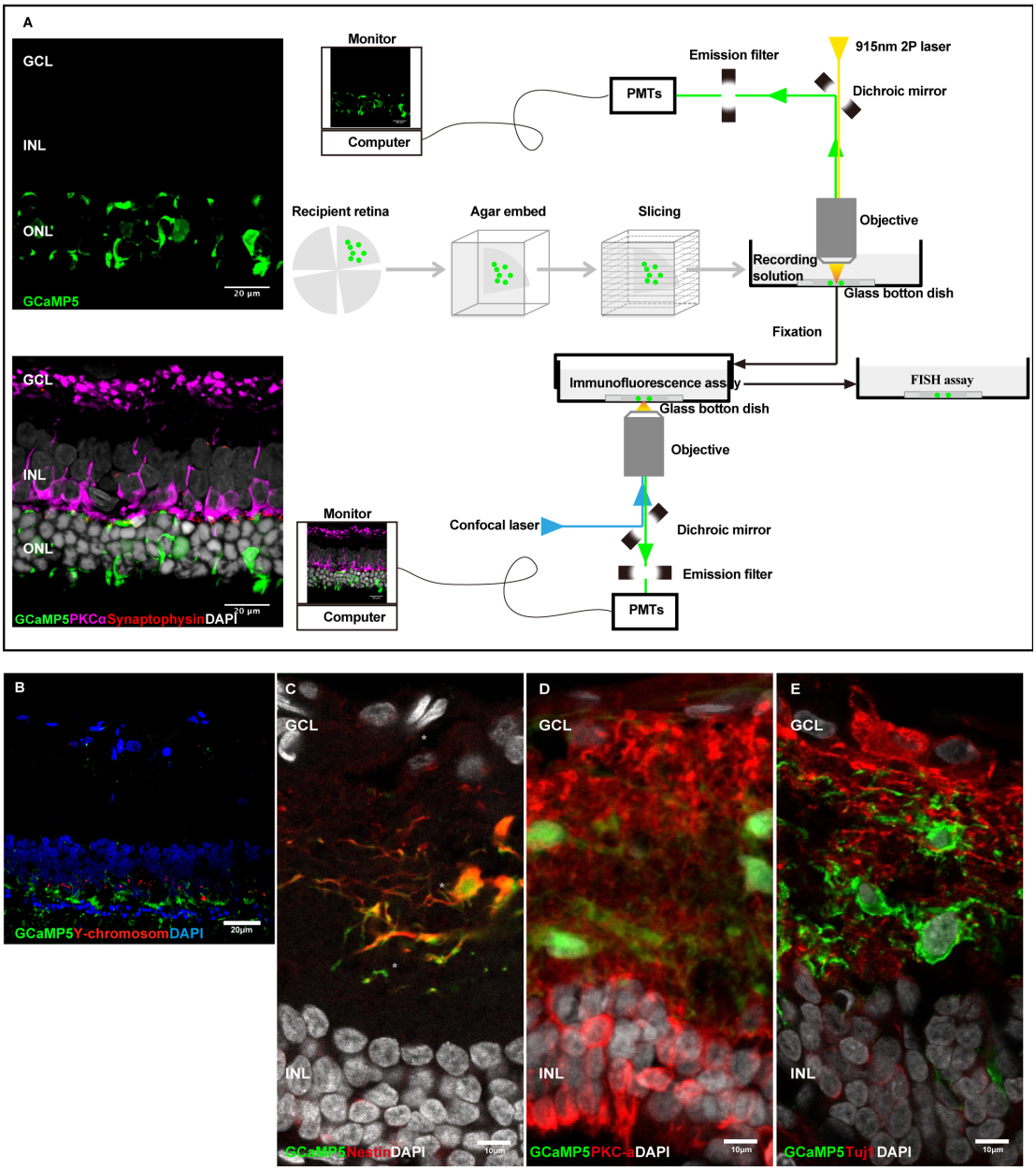
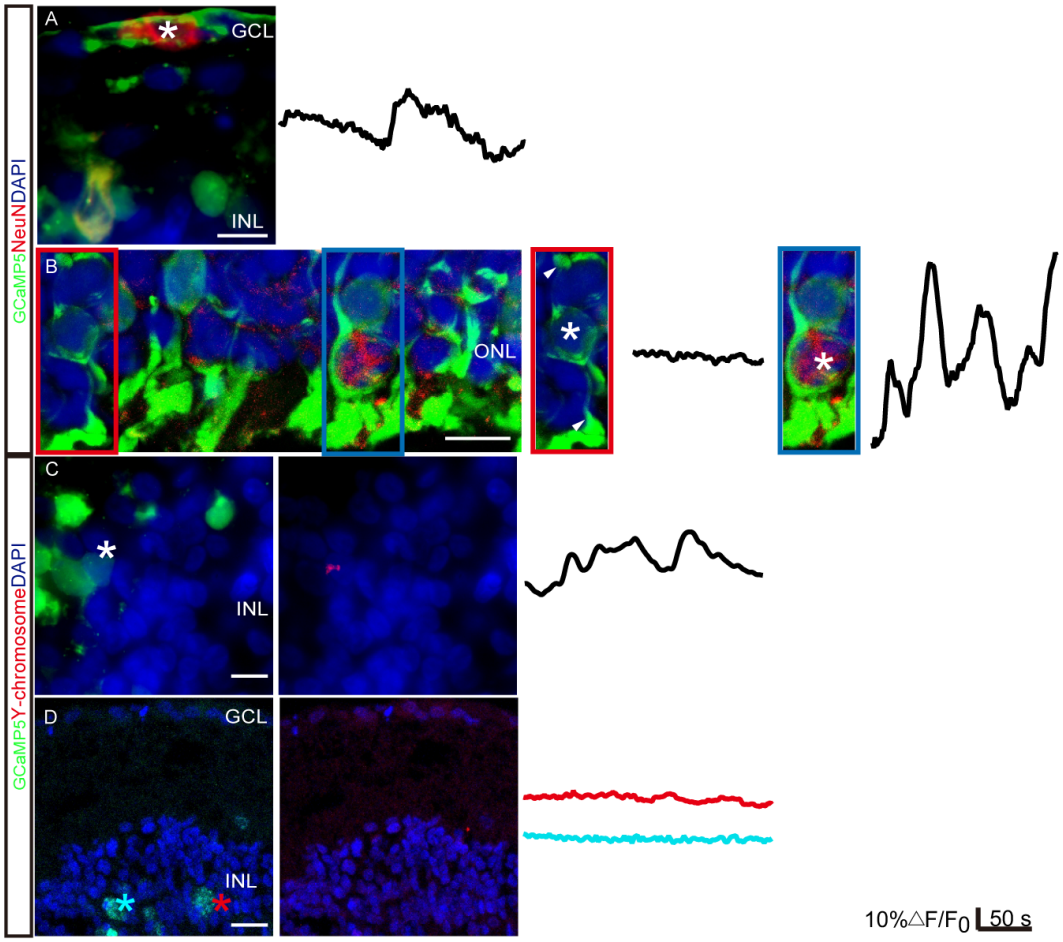


Figure S4 | Schematic of Two-photon laser-scanning on live retinal slices combined with immunofluorescence assay and confocal microscope imaging and immunofluorescence experiments on the migrated donor cells in the IPL layer, Related to Figure 3 and Figure 4.

A The upper panel presented preparation of low-gelling temperature agar-embedded live retina slice. During Two-photon laser scanning, light entering the “green” PMT was filtered with dichroic mirrors (Chroma Technology), with bandpasses set to collect GCaMP fluorescence. Then in site fixation of retinal slice was performed before immunofluorescence and FISH

assay. The lower panel presented that retinal slice was examined by confocal microscope scanning. **B** Examples of retinal sections co-stained for both GCaMP5 and Y chromosome following transplantation. **C-E** Representative images of the GCaMP5^{+} donors in the IPL layer Co-stained with markers nestin (precursor cells) (C), PKCα (bipolar cells) (D) and Tuj1 (ganglion cells) (E). Scale bar: 20μm (A, B) and 10 μm (C-E).

Figure S5



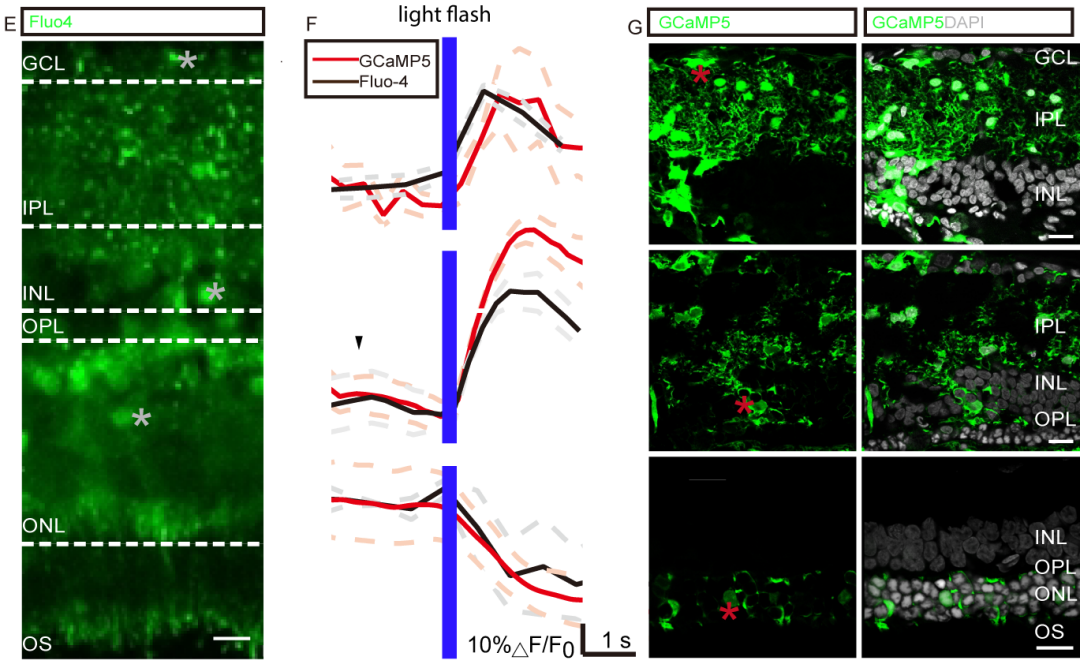


Figure S5 | Differences in morphologies and calcium signals arising from implanted cells versus those arising from material transfer and similar light stimulus-evoked

fluorescence changes were detected in both the donor cells implanted to the three retina layers (GCL, INL, ONL) and the retina neurons of the intact corresponding layers, Related to Figure 4 and Figure 5.

A, B The implanted GCaMP^{+} cells labeled with NeuN (NeuN^{+}) showed GCaMP fluorescence activity, while a proportion of cells that were not labeled with NeuN (NeuN^{-}) showed GCaMP fluorescence quiescence. **C, D** Quantification of Y^{+} nuclei in the same slice after two-photon laser scanning, data had shown that NeuN- cells with GCaMP fluorescence-active were immature in morphology and positive for Y-probe staining (n=116/116, N=4), whereas NeuN^{-} cells without GCaMP fluorescence were negative for Y-probe staining (n=372/372, N=4). **E** To test whether the implanted cells work like normal retina neurons, we compared Ca2^{+} activities of implanted cells with those of normal congenic rats. Live retinal slice of normal congenic rats (rdy^{+} rats) were labeled with calcium indicator Fluo-4 AM (green fluorescence). **F** Fluorescence response to a brief blue light flash (458 nm LED, 125 ms duration, 100 μW/cm2); The traces show fluorescence responses representative for the labeled neuron types shown in **E, G** (red and black solid lines; average ± SEM shown in dashed lines). The arrowhead indicates fluorescence response evoked by scan laser onset. **G** Fluorescence images of GCaMP-expressing donors implanting at the three retina layers (GCL, INL and ONL). Two-photon microscopy resolved all labeled structures. Images represent the average fluorescence image obtained from 3 to 10 no-flash trials. Scale bar: 10μm (A-C, E) and 20μm (D, G).

Supplemental Experimental Procedures

Animals

RCS rats and congenic control RCS-*rdy*$^{+}$ rats were maintained in the animal facilities maintained by the Institutional Animal Care and Use Committee of the Third Military Medical University. In the retinal degeneration recipient group, the adult rats were 21 days old at the time of cell injection. All treatments were conducted in accordance with NIH guidelines for the care of laboratory animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were housed under a standard 12-hr light-dark cycle. All visual function tests were conducted within the light phase. All recipients were restricted to drinking water containing 210 mg/l cyclosporine A starting from the day before transplantation to the day of euthanization.

mESC maintenance

The maintenance medium for ESC contained 500 ml of Glasgow minimum essential medium (GMEM, Gibco), 5.1 ml of 100× NEAA (Gibco), 5.1 ml of 100× pyruvate (Gibco), 0.91 ml of 55 mM β-mercaptoethanol, 58 ml of KSR (Gibco) and 58 ml of FBS (fetal bovine serum, Ausbian, serum batches should be screened for plating efficiency, colony morphology, and toxicity to mESC maintenance). A final concentration of 2,000 U ml^{-1} LIF (leukaemia inhibitory factor, Millipore) was freshly added to the maintenance medium prior to culture medium change.

mESCs were placed in 60 mm tissue culture plates (Thermo Fisher) containing radially inactivated mouse embryonic fibroblasts (MEFs). When the cell colonies had grown to 80% confluence, the cells were dissociated (Gibco, 0.05% trypsin) and resuspended. The cell suspension (5-10×10^{6} cells) was then added to a plate with a fresh 60 mm feeder (MEF) layer, containing 5 ml of maintenance medium.

Flow cytometry cell cycle assay

After 24-h serum starvation in maintenance medium containing 0.5% fetal bovine serum (FBS), cells were planted in 24 wells plate in a density of 5*10^4 with medium containing 15% FBS. Continue to incubate cells for a further 24h, the cells were fixed after cooling in 70% ethanol solution for 12 hours and were then stained using the Cycletest^{TM} Plus DNA Reagent Kit (BD Bioscience). DNA content was determined using a FACSCalibur Flow Cytometer, and the data were analyzed using the ModFit 2.0 software.

mEROs differentiation culture

For retinal differentiation, mESCs were treated with modified serum-free floating cultures of embryoid-body-like aggregates with quick re-aggregation (SFEBq)(Eiraku et al., 2011). Briefly, from differentiation day 0 to day 7, dissociated C-kit mCherry Rosa 26 GCaMP5 mESCs (3,000 cells) were reaggregated in V-bottomed 96-well plates (Sumitomo Bakelite). The retinoic acid receptor antagonist (RRA) AGN193109 (0.1 μM, Toronto Research Chemicals) and Matrigel (2% [vol/vol], Corning 354230, growth factor reduced, *LDEV-free) were added to RDM (retinal differentiation medium) containing 500 ml of GMEM (Gibco), 25 ml of KSR (Gibco), 5.1 ml of 100× NEAA (Gibco), 5.1 ml of 100× pyruvate (Gibco), and 0.91 ml of 55 mM β-mercaptoethanol. From day 8 to day 9, optic neural retinas were pinched off the RPE-like portion (Watchman's forceps; FST Biology no. 5, Dumont) and transferred to retinal maturation medium containing Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Gibco), with 1% N2 (Gibco), 10% FBS, 0.5 mM all-trans retinoic acid (Sigma), 1 mM L-taurine (Sigma), 0.25 mg ml^{-1} Fungizone (Gibco), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Gibco).

Dissociation of 3D neural retinas and FACS analysis

Neural retinas (day 8-35, from 96 aggregates per sample, n=3) containing C-kit: mCherry^{+} cells were gently dissociated into single cells via digestion with papain (papain-based kit, Sumitomo Bakelite) at 37°C for 30 min. For fluorescence-activated cell sorting (Beckman), dissociated single cells were incubated with fluorochrome-conjugated anti-SSEA1 antibody (5 μl for 1×10^{6} cells; BD Pharmingen^{TM} 562277). The details of the procedures were performed as previously described[12], and the cells were sorted by gating of GCaMP^{+}/C-kit: mCherry^{+} and SSEA1^{-}.

Sphere formation and differentiation of C-kit^{+} RPCs

After cell sorting, each type of cell (unsorted, C-kit^{+}, C-kit^{-}) was seeded into low-adherence 96-well plates at a density of 2,000 cells per well and cultured under 5% CO_2, 37°C for 10 days in sphere-formation medium containing DMEM/F12-Glutamax medium, 2% B27 (without vitamin A, Gibco), 5% FBS, 1% N2 (Gibco), 20 ng ml^{-1} human bFGF (PeproTech), 20 ng ml^{-1} human EGF (PeproTech), 5 μg ml^{-1} heparin (Sigma), 0.25 mg ml^{-1} Fungizone (Gibco), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Gibco).

For 3D NR-derived C-kit mCherry^{+} RPC differentiation, dissociated cells were plated in PDL (poly-D-lysine) and laminin-coated dishes and further feed with RPC differentiation medium which contained DMEM/F12-Glutamax medium, 1% N2 supplement (Gibco), 10% FBS, 0.5 mM all-trans retinoic acid (Sigma), 1 mM L-taurine (sigma), 0.25 mg ml^{-1} Fungizone (Gibco),

100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Gibco).

Retinal progenitor cell transplantation

C-kit^+ RPCs were prepared from C-kit mCherry Rosa 26 GCaMP5 mESC-derived nonpigmented 3D NR at differentiation day 20. Cells were manually dissociated using a papain-based kit (Worthington Biochemical). FACS-sorted C-kit^+ RPCs were collected and resuspended at a final concentration of 200,000 cells/µl in sterile ice-cold PBS (on ice) containing DNase (0.005%) prior to transplantation.

Surgery was performed under ophthalmoscopy (Leica). Recipient 63-day-old RCS rats were anaesthetized via intraperitoneal injection of pentobarbital sodium (1.5% [wt/vol], 0.2 ml/100 g). A Hamilton syringe with a 29-gauge needle (Hamilton, Reno, NV, USA) was tangentially inserted into the subretinal space through sclera puncture. Subsequently, 1 µl of pre-loaded cell suspension was gently injected into the subretinal space.

Optokinetic head-tracking tests

The visual acuities of both eyes were determined by quantitative measurements of the optomotor responses (head tracking) to rotating sinusoidal grating under scotopic conditions (<0.003 cds/m^2). The optomotor responses (head tracking) were driven by clockwise (left eye) - and counter-clockwise (right eye)-rotated gratings. The technician who performed the behavior tests were blinded. Each rat was placed in the center of four LCD monitors and was video recorded using an overhead camera. Once the rats were accustomed to the test environment, the trials were initiated by performing clockwise or counter-clockwise rotating sinusoidal grating with staircase-like eight spatial frequencies (0.05, 0.075, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 cycles/degree). As the frequency increases across experiments, the test becomes more difficult, as determined using MATLAB software.

Immunofluorescence and confocal microscopy imaging

For 3D differentiation experiments, aggregates were fixed with ice-cold 4% paraformaldehyde in PBS for 30 min. For transplantation experiments, animals were sacrificed using the same protocol described for retinal slice preparation. The eyes were quickly enucleated and hemisected in ice-cold 4% paraformaldehyde (PFA) in PBS and fixed for 60 min in PFA at 4°C. All the samples were cryoprotected with 30% (wt/vol) sucrose in PBS, embedded in O.C.T. (SAKURA Tissue-Tek O.C.T. Compound 4583) and cut into 10 µm-thick cryosections at -20°C. The retinal cryosections were then dried for 10 min at 37°C and washed with 0.1 M phosphate

buffer (pH 7.4) for 5 min 3 times. The sections were subsequently preblocked for non-specific sites using PBS solution containing 10% normal goat serum, 1% bovine serum albumin and 0.5% Triton X-100 for 1 h at room temperature before incubation with the primary antibodies (C-kit (1:500; Cell Signalling. 3074); SSEA1 (mouse, 1:200, Santa Cruz. SC21702); Nanog (rabbit, 1:500; Millipore. AB5731); Oct4 (rabbit, 1:500; Abcam, ab19857); Rx (rabbit, 1:500; Abcam, ab86210); MiTF (mouse, 1:1000; Abcam. Ab80651); PAX6 (rabbit, 1:500; BioLegend. 901301); Vsx2 (mouse, 1:200; Boster. BA1589); Nestin (mouse, 1:500; Millipore. MAB353); CRX (rabbit, 1: 500; Novus. NBP1-88059); Recoverin (rabbit, 1:2000; Chemicon. AB5585); Pkc-α (mouse, 1:500; Santa Cruz. sc8393); Calretinin (rabbit, 1:2000; Swant. 7697); Tuj1 (mouse, 1:500; Beyotime, AT809); CRALBP (mouse, 1:500; Abcam, Ab15051); Opsin (mouse, 1:2000; Sigma, O4886); Synaptophysin (rabbit, 1:500; Cell Signalling, 5461); Ctbp2 (goat, 1:500; Santa Cruz, sc5966); and Ki67 (rabbit, 1:400; Cell Signalling, 9129)) overnight (12 h) at 4°C. After rewarming for 1 h at room temperature, the sections were rinsed 5×5 min with PBS, prior to incubation with an appropriate fluorescently labeled secondary antibody (1:1000; Invitrogen) for 1 h at room temperature. After rising 5×5 min with PBS, the sections were stained with DAPI (Abcam, Ab104139). The primary antibody was omitted in the negative controls. All sections were observed using a confocal microscope (Zeiss LSM 880).

Supplemental Reference

Decembrini, S., Koch, U., Radtke, F., Moulin, A., and Arsenijevic, Y. (2014). Derivation of traceable and transplantable photoreceptors from mouse embryonic stem cells. Stem Cell Reports 2, 853-865.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., and Sasai, Y. (2011). Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472, 51-56.