Stem Cell Reports, Volume *8*

Supplemental Information

iPSC-Derived Retina Transplants Improve Vision in *rd1* End-Stage Retinal-Degeneration Mice

Michiko Mandai, Momo Fujii, Tomoyo Hashiguchi, Genshiro A. Sunagawa, Shinichiro Ito, Jianan Sun, Jun Kaneko, Junki Sho, Chikako Yamada, and Masayo Takahashi

Supplemental Experimental Procedures

Generation of Nrl-GFP::pNrl-CtBP2-tdTomato lines

The mouse *Nrl* promoter was obtained from an Nrl-L-EGFP construct provided by Dr. Akimoto (Akimoto et al., 2006). CtBP2 cDNA was amplified from pCMV-SPROT6-mouseCtbp2 (GRAS, MCG clone, IMAGE: 4511826) without the last 3-bp stop codon. The tdTomato sequence was cloned into a pCAT3 reporter vector (Promega) and cut out together with SV40 late poly A to fuse to Ctbp2 in-frame. Next, the *Nrl* promoter and the Ctbp2 -tdTomato-SV40 late poly A were cloned into the targeting vector of the ROSA26 locus provided by LARGE CDB, in the reverse direction (Abe et al., 2011). The vector was then linearized and transfected into *Nrl*-GFP iPS cells (5 μg/ 2.5 x 10^6 cells) with a Nucleofector (Lonza, Japan) using the A-13 or A-30 program. The cells were selected with G418 (500 μg/ml), and 55 colonies were screened for the presence of gene insertion by homologous recombination using a primer pair, one in the Neomycin-resistance gene and the other outside the targeting arm on the genome (indicated as arrows in Figure S1A; 5’-GTACTCGGATGGAAGCCGGTCTTGTC, and 5’-TTGACTCCTAGACTTGTGACCCAGC). Four of the positive clones by the first PCR screening were tested by Southern blotting. DNAs were digested with Pac1, run on 0.7% agarose gel (Nacalai Tesque, #01163-76), capillary-transferred to a Hybond-N+ membrane (GE Healthcare, RPN203B), UV-crosslinked, and hybridized at 40 °C overnight with a probe designed outside the short targeting arm (red bars on Figure S1A). The probe was synthesized by amplifying the sequence from genomic DNA with the primers 5’-GTCAGTAGTCTTAAGTGGTCTTTATTGGCC and 5’-GACTTTAAGAGCCATGGCAATGTTCAAG using the DIG Probe Synthesis Kit (Roche, #11636090910) according to the manufacturer’s protocol. The band was then visualized with CDP-Star Detection Reagent (GE Healthcare, #RPN3682), and the images were captured using an ImageQuant LAS4000 (GE Healthcare). Three iPSC lines with wild-type and targeted allele bands were then used for retinal differentiation.

Differentiation of miPSC-retina and its subretinal transplantation

Nrl-GFP transgenic miPSCs were generated from Nrl-eGFP mice (Akimoto et al., 2006; Homma et al., 2013), and the genetically modified lines were maintained and differentiated as already described (Assawachananont et al., 2014). Optic vesicle structures were cut out on the day of transplantation at differentiation day (DD) 11-17 and were cut into small pieces (around 0.5 mm × 2 mm) in preparation for transplantation (Assawachananont et al., 2014). The miPSC-retinas were transplanted into six- to nine-week-old *rd1-2J*, *rd1-2J*/L7-GF*P*, or *rd1/B6* mice as described in each experiment. The graft piece was inserted subretinally into the eye of the mouse using a glass micropipette with a tip diameter of approximately 500 μm, as previously described (Assawachananont et al., 2014). Indomethacin (10 mg/l) was added to the drinking water of all transplanted mice starting on the day of transplantation

MEA recordings

MEA was performed as previously described to record mERGs and multiunit responses from RGCs using the MED64 system (Alphamed Scientific Inc., Osaka, Japan) (Fujii et al., 2016). The signals were amplified and filtered between 1 Hz and 10 kHz for the mERGs and 100 Hz and 10 kHz for the RGC multiunit responses. We used light stimuli of 10 ms to elicit the mERGs and 1 s to elicit the RGC responses. The animals were dark-adapted overnight, and the isolated retina with the grafted region was placed ganglion-cell-side down on the 64 electrodes and perfused with 6 nM 9-*cis* retinal/0.01% BSA in bicarbonate-based Ames' medium for 20 min, and then with 100 μM 9-*cis* retinal/0.01% BSA/Ames' medium for 10 min to recover light-responsive opsins. The mERGs and RGC responses were obtained with 0.45 log cd/m^2 light stimuli before treatment, again after the retina was treated with the mGluR6 inhibitor L-2-amino-phosphonoburyric acid (L-AP4; WAKO, Osaka, Japan) for 20 min, and finally after a 30-min washout. Next, mERGs and RGC responses were recorded with 3.01 log cd/m^2 light stimuli to confirm the presence of light responses on the same channel. For further analysis, RGC responses were separated into individual cell sources by spike sorting, as previously described (Fujii et al., 2016)

Immunostaining for 3D image reconstruction

Three-dimensional (3D) synaptic observation was performed as previously described with a slight modification (Assawachananont et al., 2014). Eyes were fixed with 4% paraformaldehyde (PFA) after perfusion fixation, then cryoprotected in 10% sucrose in PBS for 1 h and 30% sucrose in PBS overnight at 4 °C. Then the samples were embedded in OCT compound (Tissue Tec; Sakura Finetechnical Co) and frozen at -180 °C, followed by sectioning with a cryostat (HM560; Thermo) at a 50-µm thickness. For immunostaining, the sections were blocked overnight with 3% TritonX-100/ 5% goat serum at 4 °C, incubated with primary and secondary antibodies in 3% TritonX-100/ 5% goat serum at 4 °C for 2 nights and overnight respectively, and mounted with 60% 2,2'-thiodiethanol (Sigma). The antibodies used were mouse anti-CtBP2 (612044, BD biosciences, Franklin Lakes, NJ, USA), mouse anti-rhodopsin antibody RET-P1 (Abcam, Cambridge, UK), rabbit anti-protein kinase Cα antibody (P4334, Sigma-Aldrich, St Louis, MO, OSA), mouse anti-CACNA1s antibody (MAB427, Millipore, CA, USA)

Images were acquired with a Leica-TCS SP8, and a series of XY optical sections at system-optimized intervals was taken throughout the 50-µm sections and reconstructed to obtain 3-dimentional sectional images using Imaris software (Bitplane). For the whole-mount retinal immunostaining, images of the whole graft area were obtained using the Z-stack and tiling programs of the Leica-TCS SP8.

For rosette area analysis, the rosettes were marked on the sectional plane close to (approximately within 10 µm) the margin of host INL and calculated as the percentage to the graft area using ImageJ.

Supplemental Text Legends

Data S1

A raster plot of light responses with manually annotated clusters (the data are summarized in Figure S5A).

Data S2

A table of manually annotated clusters with the cluster identifier. Each column represents the following information:

- spike.id: a unique identifier for each light response.
- cluster: a cluster number (See Figure S5A).
- line.no: a unique integer for each light response.

Data S3

Source code to train the deep-learning model for clustering the light responses. Download sample data from https://goo.gl/JtQqIF (training_data.xdr; a binary data for light response saved in an xdr format) and place Text S3 in the same working directory and run Text_S3_training.txt in R. The H2O package is also required to run the code. The code will produce trained models named ‘model1_128x4_yyyymmddhhmmss’ and ‘model2_128x5_yyyymmddhhmmss’ in the model folder; ‘yyyymmddhhmmss’ is the time stamp showing when the model is produced.

Supplemental Movie Legends

Movie S1

A mouse with a retinal transplant (M10 in Figures 3G and 3H) during an SAS test. This mouse could predict and avoid the electric shock by the light signal before the shock, and was thus light-responsive.

Movie S2

A mouse with a retinal transplant during an SAS test (M8 in Figures 3G and 3H). This mouse was not able to avoid the electric shock by responding to the light signal in advance of the shock; also note that this mouse often moves before light signal, thereby increasing a chance to avoid the shock by chance; the mouse was judged as unresponsive to light.

Supplemental References

Abe, T., Kiyonari, H., Shioi, G., Inoue, K.-I., Nakao, K., Aizawa, S., Fujimori, T., 2011. Establishment of conditional reporter mouse lines at ROSA26 locus for live cell imaging. Genesis 49, 579–90. doi:10.1002/dvg.20753

Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J.A., Khanna, R., Filippova, E., Oh, E.C.T., Jing, Y., Linares, J.-L., Brooks, M., Zareparsi, S., Mears, A.J., Hero, A., Glaser, T., Swaroop, A., 2006. Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc. Natl. Acad. Sci. U. S. A. 103, 3890–5. doi:10.1073/pnas.0508214103

Assawachananont, J., Mandai, M., Okamoto, S., Yamada, C., Eiraku, M., Yonemura, S., Sasai, Y., Takahashi, M., 2014. Transplantation of embryonic and induced pluripotent stem cell-derived 3D retinal sheets into retinal degenerative mice. Stem Cell Reports 2, 662–674. doi:10.1016/j.stemcr.2014.03.011

Fujii, M., Sunagawa, G.A., Kondo, M., Takahashi, M., Mandai, M., 2016. Evaluation of micro Electroretinograms Recorded with Multiple Electrode Array to Assess Focal Retinal Function. Sci. Rep. 6, 30719. doi:10.1038/srep30719

Homma, K., Okamoto, S., Mandai, M., Gotoh, N., Rajasimha, H.K., Chang, Y.S., Chen, S., Li, W., Cogliati, T., Swaroop, A., Takahashi, M., 2013. Developing rods transplanted into the degenerating retina of Crx-knockout mice exhibit neural activity similar to native photoreceptors. Stem Cells 31, 1149–1159. doi:10.1002/stem.1372