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研究課題名（和文）iPS細胞由来網膜移植にて視機能再建のための変性網膜の視覚回路の解析と最適化

研究課題名（英文）Optimizing the degenerated retinal circuitry for retinal organoid transplantation driven retinal circuitry reconstruction

研究代表者

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研究成果の概要（和文）：本研究は移植による再生治療の一環として、ホスト環境に着目し、視細胞シナプスの形成・機能に重要な水平細胞の関与を検討した。網膜オルガノイド移植後の視細胞シナプス再構築における水平細胞の役割を明らかにするため、タモキシフェンによる条件付き水平細胞をアブレーションモデルを網膜変性マウスバックグラウンドで確立した。水平細胞を除去した変性網膜に網膜オルガノイドを移植し、電気生理により移植網膜の光反応を評価した。移植前に変性網膜から水平細胞を除去することで、宿主の双極細胞と移植先の視細胞間の機能的なシナプス形成が促進されることを示唆している。

研究成果の学術的意義や社会的意義
我々はこれまでES/iPS細胞由来網膜組織を用いて、視細胞の変性疾患に対する移植治療の有効性を検証してきた。より良い視機能再建を目指して移植組織側の改良を行なってきたが、本研究では移植細胞を受け入る側が、より機能的に移植細胞を受け入れる環境に最適化する可能性を探るために、視細胞の神経接合に重要な水平細胞の関与について検討した。変性網膜に残存している水平細胞や移植組織中の水平細胞が移植網膜の機能的な生着に寄与するかどうかを組織学的に観察し、移植後の光に対する反応を解析することで、より移植に適した環境、及びその環境の最適化による視機能再建の可能性を提示している。

研究成果の概要（英文）：To clarify the role of horizontal cells in photoreceptor synapse reformation after retinal organoid transplantation, tamoxifen-induced conditional horizontal cell ablation model has been established in retinal degeneration mouse background. Mouse retinal organoids were transplanted to the horizontal cell-ablated degenerating retinas, and multielectrode array recording was conducted to assess the host-graft reconstruction. The host ganglion cells showed increased light responses with better signal-to-noise ratio compared to the regular transplantation preparation without horizontal cell disturbance. De novo synapses between host bipolar cells and graft photoreceptors were observed with the graft horizontal cell processes invaginated in the horizontal cell ablated retinas. These results indicate that the removal of horizontal cells from the degenerated retinas prior to transplantation facilitates the functional synapse formation between host bipolar cells and graft photoreceptors.

研究分野：Retinal neuroscience

キーワード：retinal degeneration　horizontal cell　multielectrode array　retinal organoid　transplantation

１．研究開始当初の背景

Our vision begins with photoreceptors sensing changes of ambient light, from where visual information is processed by the delicate retinal circuitry generalized to ON (depolarize when light gets brighter) and OFF (depolarize upon dimming) pathways. Via the very first visual synapses, bipolar cells (BCs) receive glutamatergic signals from photoreceptors and then transmit, directly or indirectly, to retinal ganglion cells as the final output of retina. This vertical signaling is further refined with lateral inhibition from horizontal cells (HCs) and amacrine cells (ACs).

While fundamental properties of the normal retinal wiring are largely revealed in the past several decades, other counterintuitive but intriguing properties have only been explored recently in diseased retinal models. In rd1 mice and many other animal models of retinitis pigmentosa, RGCs become hyperactive and exhibit robust spiking activities that act noisy to the visual brain. The loss of photoreceptors leaves ON BCs alone with no excitatory input driven by light, while inhibitory transmission from ACs and HCs stay active. Rod photoreceptor-driven BCs that presumably govern their downstream ACs by glutamate transmission are found inactivated in degenerated retinas and hence unleash the AC hyperactivity. In fact, the RGC hyperactivity has been shown partially resulting from the intrinsic bursting property of ACs and spreads across the whole retina via intensely coupled AC-BC gap junction network. Such cellular interaction of inner retinal neurons, masked by functional photoreceptor signaling in healthy retina, remained unknown until recently.

In addition, it is of great interest and importance to clarify if the inner retinal hyperactivity would affect the reconstruction of retinal circuitry when provided with healthy photoreceptors derived from ES/iPS cells. ES/iPS-retinal organoid transplantation aiming to cure retinal degeneration diseases has proven its potential to restore visual function at the retinal level, however, only partially. I have constantly observed 1) the graft-driven light responses dominated by ON but not OFF pathways, and 2) the same type of host BCs that are physically close to each other react oppositely to the graft photoreceptors. Such unbalanced synaptic reconstruction suggests that different types of BC may be entrained differently in the degenerated host environment, potentially by their active inhibitory neighbors. This study therefore aimed to dissect the cellular mechanisms underlying the potentiation and/or inactivation of bipolar cells initiated by horizontal, amacrine and even ganglion cells, respectively, by electrophysiology, pharmacology, and genetic manipulation.

２．研究の目的

This study aimed to facilitate the transplantation-initiated retinal circuitry reconstruction by studying the cellular mechanism underlying potentiation of host bipolar cells, the direct recipient of graft photoreceptors to form synapses and convey visual signals. Current finding shows a functional yet inefficient and unbalanced recovery of visual function in transplanted retinas, possibly due to the varying host bipolar cell states. To clarify the cellular interaction in the host retina that act on bipolar cell modulation, the players interact with bipolar cells, directly or indirectly, are targeted to be genetically and pharmacologically manipulated to isolate individual inputs to bipolar cells. Mouse models with tamoxifen-driven HC labeling/deletion were established and incorporated with retinal degeneration to study the intrinsic properties of HCs and their role in the reformation of bipolar cell synapses after retinal organoid transplantation.

３．研究の方法

Animals

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. Animal experiments were conducted with the approval of the Animal Research Committee at RIKEN Center for Developmental Biology (now Center for Biosystems Dynamics Research) and the Institute for Protein Research in Osaka University. Rd1-2J;L7-GFP Rd1-2J mice were crossed with Cx57-CreERT2 transgenic mice, NSE-DTA knock-in mice and/or Ai9-tdTomato mice sequentially to generate the end-stage retinal degeneration mouse model with GFP-expressing ON bipolar cells together with DTA expressions in HCs that can be induced with tamoxifen treatment. Mice were housed under the standard 12-hr light/dark cycle with free access to water and food.

Tamoxifen administration and retinal organoid transplantation

Mice were treated with intraperitoneal injection of tamoxifen (75-100 mg tamoxifen/kg body weight) or fed with tamoxifen containing food pellets (400 mg/kg) for 6 weeks until a week before retinal organoid transplantation. Procedures for maintenance, differentiation, and preparation of mouse ESC-derived retinal organoids (ESC-retinas) for transplantation were previously described (Matsuyama, et al., 2021). Briefly, the retinal organoids were differentiated from mouse ES cells and the optic vesicle structures (at differentiation day 13) were cut to small pieces (around 0.5 mm × 2 mm) on the day of transplantation. Mice were anesthetized with isoflurane using an inhalation anesthetic system (Narcobit-E type II, Natsume Seisakusho, Tokyo, Japan). Anesthesia was maintained 2.0-5.0% isoflurane at an O2 flow rate of 1.5 L/min using a modified nosecone for mice. Pupils were dilated with Mydrin-P® (0.5% phenylephrine + 0.5% tropicamide; Santen Pharm Co., Osaka, Japan). The prepared retinal sheets were inserted into the subretinal space of 3-month-old mice, along with 1 mM valproic acid using a glass micropipette with a tip diameter of approximately 500 μm. Indomethacin (10 mg/L) was added to the drinking water of all transplanted mice starting on the day of transplantation.

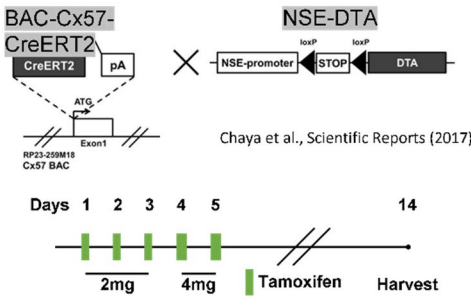

Figure 1 The strategy of horizontal cell conditional deletion induced by tamoxifen administration. BAC-Cx57-CreERT2 (transgenic) and NSD-DTA (knock-in) mice were established in Furukawa laboratory in the Institute for Protein Research, Osaka University, and the original intraperitoneal administration protocol of tamoxifen to conditionally ablate horizontal cell was reported in Chaya et al., 2017.

Multielectrode array (MEA) recording and data analysis

The MEA recording procedures and data analyses have been previously reported (Tu et al., 2018; Tu and Matsuyama, 2020; Matsuyama et al., 2021; Yamasaki et al., 2022). Mice transplanted with mouse ESC-retinas were used for MEA recording at around 4-5 weeks post-transplantation. Animals were dark-adapted for 1-3 days prior to MEA recording using the USB-MEA60-Up-System (MultiChannel Systems, Germany) with the standard 8x8 probe (60MEA200/30iR-Ti-gr)). Animals were deeply anesthetized with sevoflurane inhalation and sacrificed immediately by decerebration. Eyeballs were taken out by gently cutting off the optic nerves to avoid ganglion cell damages. Cornea and lens were removed first, and the remaining eyecups were then kept in constantly oxygenated (95% O2 and 5% CO2) Ames' medium (A1420, Sigma-Aldrich) at room temperature until use. Retinas were freshly isolated with vitreous body carefully removed for having good contact with MEA probe. The grafted areas were identified by their characteristic whitish, spotted looking, and mounted on the electrodes with the ganglion cell side down in assistance of a 0.5-g weighted anchor with mesh. The mounted retinas were constantly perfused at 3-3.5 mL/min with warmed (34 ± 0.5°C) Ames' medium during recording. Retinas were allowed to recover in the MEA chamber for at least 20 min before recording while the intrinsic hyperactive RGC firing was monitored as one of the degenerated retina features. During recording, L-AP4 (10 μM; 016-22083, Wako) was added to block the type 6 metabotropic glutamate receptor (mGluR6)-mediated transmission between photoreceptors and ON BCs. The procedures were conducted under dim red light with a peak wavelength at 700 nm. After recording, the IR and fluorescence images of the samples were taken. Retinas were then detached from the electrodes and fixed for 15 min with 4% PFA at room temperature for immunohistochemistry. The full-field light stimuli were generated using a white LED source with irradiance ranging from 10-13 log photons/cm2/s at the focal plane of electrodes, covering the scotopic/mesopic to mesopic/photopic range. Three repeats for each light intensity were recorded as a set before, during and after the application of L-AP4 to confirm the origin of light responses. Using different band pass filters, field potentials (microERG or mERG; 1-50 Hz Butterworth) and RGC action potentials (spikes; 200-2800 Hz Butterworth) were extracted for analysis. Candidate mERG a-waves were defined as local minima within 55 ms after the onset of 10-ms light pulse and b-waves as local maxima within 120 ms. Among these, the largest peaks within the time windows were then assigned as tentative a- and b-waves. The RGC spikes were sorted using Spike 2 Version 7.2 (Cambridge Electronic Design) to separate individual cells on the same electrodes based on spike amplitude and shape. By the tdTomato expression of the graft photoreceptors, the approximate graft location related to the MEA electrodes was manually mapped at a resolution of 552 x 552 pixels, and each pixel was defined as "on" or "off" the graft, or at the "edge" of the graft.

Immunohistochemistry and imaging

The flat-mounted retinas were immunostained for CtBP2, Calbindin at 4 °C for 4-7 days, followed by overnight incubation of the secondary antibody. Samples were mounted using VECTASHIELD Antifade Mounting Medium (H-1000, Vector) and kept at 4 °C for long-term preservation. Z-stack imaging was taken across the full sample depth and tiled automatically to cover the grafted and neighboring areas with a Leica-TCS SP8. Imaris software (Bitplane) was used to view and process the 3D images.

4．研究成果

At the photoreceptor-bipolar cell synaptic layer, HC neurites innervate into the ribbon synapses formed by photoreceptors and ON type BCs, constructing the synaptic triads and providing feedback/feedforward inhibition to these two cell types, respectively. The extremely dense gap junction network formed by HCs then laterally modulate the visual signaling at the very first synapses of vision. However, it remains unclear how the HCs behave after photoreceptor loss, and therefore how they affect the bipolar cell properties.

The tamoxifen administration protocol to generated the conditional HC ablation model was optimized with lighter dosages than the original tamoxifen administration previously reported, as the protocol proved to be highly toxic, causing lethality for the mice used in this study, potentially due to the different animal background. Conditional HC ablation model was first tested and confirmed in both normal (Figure 2) and degenerated (Figure 3) retinas, with the Calbindin-positive HC somata and proximal neurites disappeared after tamoxifen administration. Interestingly, the Calbindin-positive dendritic tips of HCs were found retained and colocalized with the CtBP2-positive ribbon synapses between photoreceptor and bipolar cells (Figure 3, upper panel), which were not observed in the mouse retinas with severe photoreceptor degeneration (Figure 3, lower panel). In contrast, in normal retina the ribbon synapses and bipolar dendritic extension were rather preserved even without healthy HCs (or vice versa), while in the degenerated retinas with HC abolishment there were almost no residual synapse or potential dendritic contact detected. Similar results were also confirmed with mice fed with tamoxifen containing chew during postnatal weeks (data not shown).

Following the establishment of mouse model for conditional ablation of HCs in photoreceptor degeneration, mouse retinal organoid transplantation and functional assay were conducted to probe the role of remaining HCs in the host-graft reconstruction in comparison with their littermate without HC deletion as the ideal control. De novo synapses between host bipolar cells and graft photoreceptors were observed with the graft HC processes invaginated in the HC-ablated retinas. As suggested by the MEA recording (Figure 4), the removal of HCs from the degenerated retinas prior to transplantation seemed to facilitate the functional synapse formation between host bipolar cells and graft photoreceptors. The host ganglion cells showed stronger light responses with better signal-to-noise ratio compared to the regular transplantation preparation without horizontal cell disturbance reported previously (Matsuyama et al., 2021). This preliminary observation indicates the residual horizontal cells may be short of the ability to form new synapses and in contrast physically block the interaction of host bipolar cells and graft photoreceptors upon transplantation. The clearance of these residual horizontal cell processes at the outer plexiform layer may hence allow the graft horizontal cells to be involved in the newly formed synapses. Further investigation with increased animal numbers is required

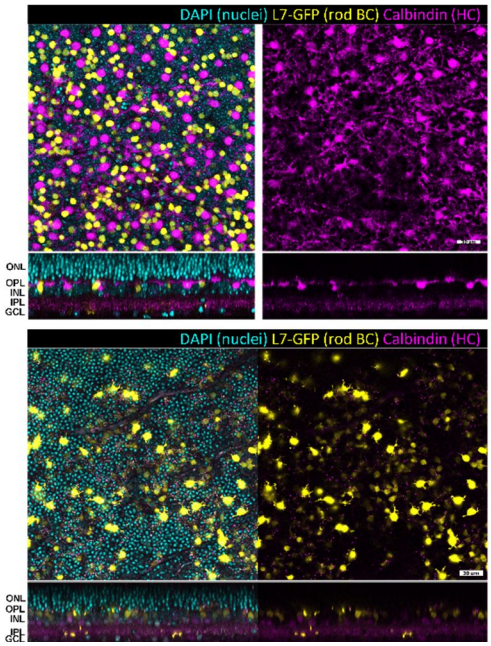

Figure 2 Conditional ablation of horizontal cells in L7-GFP mice. Upper panel, control retina without Cx57-CreERT2 induction. Lower panel, disappearance of Calbindin-positive horizontal cell bodies as well as neurites was confirmed in mouse retinas treated with tamoxifen to induce the Cx57-CreERT2 activity. Nuclei were counterstained with DAPI (cyan), rod bipolar cells were labeled with L7-GFP expression (yellow) and horizontal cells were immunostained against Calbindin (magenta).

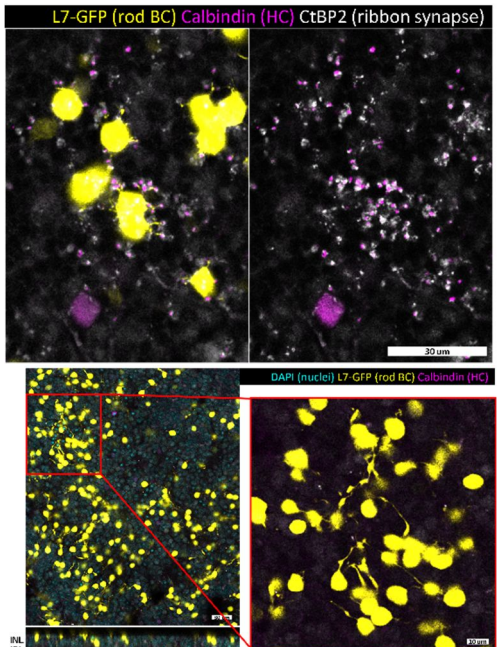

Figure 3 Conditional ablation of horizontal cells in degenerated retina. Upper panel, conditional deletion of horizontal cells in normal retina with photoreceptors led to Calbindin-positive puncta (magenta) remained within the L7-GFP expressing rod bipolar cell synapses marked by CtBP2 (white). Lower panel, complete clearance of horizontal cell dendritic tips was confirmed in mouse retinas with photoreceptor degeneration after tamoxifen administration. Nuclei were counterstained with DAPI (cyan).

to evaluate the effect of unused horizontal cell clearance in degenerated retinas prior to the retinal organoid transplantation.

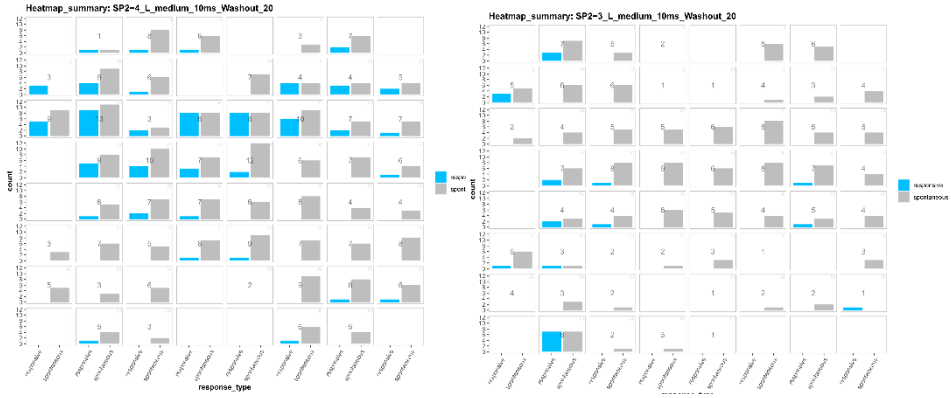

Figure 4 Increased signal-to-noise ratio of RGC light response after transplantation. Left panel, recording of host retinal ganglion cell light responses in transplanted retina with horizontal cell abolishment in advance. Right panel, recording in transplanted retina with host horizontal cells remaining. Numbers of light responsive cells were shown in blue, and spontaneously active cells in gray.

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　0件／うちオープンアクセス　0件）

１．著者名 Yamasaki S, Tu HY, Matsuyama T, Horiuchi M, Hashiguchi T, Sho J, Kuwahara A, Kishino A, Kimura T, Takahashi M, Mandai M.	４．巻 25
２．論文標題 A Genetic modification that reduces ON-bipolar cells in hESC-derived retinas enhances functional integration after transplantation	５．発行年 2022年
３．雑誌名 iScience	６．最初と最後の頁 103657～103657
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.isci.2021.103657	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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２．論文標題 Genetically engineered stem cell-derived retinal grafts for improved retinal reconstruction after transplantation	５．発行年 2021年
３．雑誌名 iScience	６．最初と最後の頁 102866～102866
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.isci.2021.102866	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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２．論文標題 Self-organization, quality control, and preclinical studies of human iPSC-derived retinal sheets for tissue-transplantation therapy	５．発行年 2023年
３．雑誌名 Communications Biology	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s42003-023-04543-5	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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２．論文標題 Competency of iPSC-derived retinas in MHC-mismatched transplantation in non-human primates	５．発行年 2022年
３．雑誌名 Stem Cell Reports	６．最初と最後の頁 2392～2408
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.stemcr.2022.09.014	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関