Culture Systems of Dissociated Mouse and Human Pluripotent Stem Cell–Derived Retinal Ganglion Cells Purified by Two-Step Immunopanning

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PURPOSE. We aimed to establish purification and culture systems for retinal ganglion cells (RGCs) differentiated from mouse and human pluripotent stem cells (PSC) for in vitro and regenerative medicine studies.

METHODS. We used a two-step immunopanning method to purify RGCs from mouse and human PSC-derived three-dimensional (3D) retinal organoids. To assess the method, we purified RGCs from 3D retinal organoids derived from embryonic stem cells (ESCs) generated from Thy1-EGFP transgenic (TG) mice. In addition, 3D retinal organoids differentiated from human induced PSCs (iPSCs) were cultured for up to differentiation day (DD) 120, and RGCs were purified by immunopanning. RGC marker expressions were confirmed by immunostaining and reverse transcription–quantitative PCR. The purified RGCs were cultured, and neurite outgrowth was measured and analyzed using an IncuCyte Zoom system.

RESULTS. Mouse RGCs purified from Thy1-EGFP TG mouse retinas and the ESC-derived 3D retinas could be maintained for approximately 2 to 3 weeks, expressing the markers BRN3B and SMI-312. Purified RGCs from human iPSC-derived retinal organoids expressed RGC markers and could be maintained for up to 4 weeks. The RGCs collected at DD 90 to 110 extended longer neurites than those collected at younger stages.

CONCLUSIONS. We successfully purified RGCs from mouse and human PSC-derived 3D retinal organoids cultured for approximately 120 days. RGCs from older retinal organoids would be useful for neurite tracking. This method would be effective not only for studying the pathology of human RGC diseases but also for therapeutic drug studies and RGC transplantation.

Keywords: pluripotent stem cell, retinal ganglion cell, 3D retinal organoid, immunopanning, neurite outgrowth

Retinal ganglion cells (RGCs) play important roles in transmitting visual information from photoreceptor cells to the visual brain area.1 Because the dysfunction and impairment of RGCs in syndromes such as glaucoma and optic neuropathy lead to vision loss, the pathology and etiology of RGC-linked diseases have been investigated widely. For example, glaucoma is the second most common cause of blindness worldwide2,3 and is characterized by RGC death caused by axonal degeneration. High intraocular pressure is a major risk factor for glaucoma4 and deterioration of optic nerve blood flow has been shown to be associated with RGC degeneration.5 The aberrant expressions of some genes have been reported to be associated with glaucoma by genome-wide studies.6,7 However, the pathogenesis and pathophysiology leading to glaucoma remain poorly understood. Sampling RGCs from human patients with glaucoma is not possible due to ethical issues as well as serious risks. Previously, human RGCs were purified from the retinas of aborted fetuses by an immunopanning method, and neurite outgrowth was analyzed. However, this is a rare situation and difficult to reproduce.8

A breakthrough was the use of three-dimensional (3D) self-organizing optic vesicles generated from mouse and human pluripotent stem cells (PSCs) by a serum-free floating culture of embryoid body–like aggregates with quick reaggregation (SFEBq) system.9,10 Also, Kuwahara et al.11 reported highly selective self-formation of the neural retina with bone morphogenetic protein (BMP) treatment. These reports confirm BRN3 expression, which is a pan-differentiation RGC marker associated with dendritic stratification, maintenance, and projection.12,13 Recently, RGCs were isolated from human 3D retinas by magnetic-activated cell sorting (MACS) using magnetic beads conjugated to antibodies against Thy1, a cell surface glycoprotein expressed in mature RGCs.14

Here, we tested a two-step immunopanning method to purify RGCs from three-dimensional (3D) retinal organoids, which have often been used in studies on rodent retinas.15,16 In the first step, dissociated retinal cells are incubated on the dish
coated with an antimacrophage antibody to selectively deplete macrophages and endothelial cells, and then RGCs are purified by an antibody against Thy1.15 We applied this method to RGCs differentiated from mouse and human PSCs. In the mouse system, we used 3D retinas differentiated from embryonic stem cells (ESCs) from Thy1-EGFP transgenic (TG) mice, in which enhanced green fluorescent protein (EGFP) is expressed from the Thy1 promoter,17 enabling live imaging of RGC survival using a scanning laser ophthalmoscope in vivo.18 In the human system, we purified RGCs from 3D retinal organoids differentiated from induced PSCs (iPSCs) and were able to culture them for approximately 120 days with growing neurites. This technique will facilitate pathophysiological and pharmacologic studies of human RGCs. Moreover, it would be useful for transplantation treatment with human RGCs to determine the optimal conditions for neurite outgrowth.

METHODS

Animals

All animal experiments were conducted with the approval of the RIKEN Center for Developmental Biology Ethics Committee and were performed by the guidelines for animal experiments of RIKEN Center for Developmental Biology and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Thy1-EGFP TG mice, as reported previously,17 were provided from the animal facility of Niigata University. Genotypes were confirmed by PCR amplification.19

Differentiation of 3D Retinal Organoids Derived From Thy1-EGFP TG Mouse ES Cells

Thy1-EGFP mouse ESCs were generated20 and maintained as described,21 and 3D retinal organoids were differentiated using the SFEBq protocol9,22 with minor modifications. Briefly, embryoid bodies forming optic vesicle-like structures at differentiation day (DD) 9 were transferred to 10-cm culture dishes and maintained in culture. The medium was supplemented with 0.5 mM retinoic acid (RA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1 mM taurine (Wako Pure Chemical Industries, Ltd.) on DD 13. Three-dimensional retinal organoids at DD 18 were transferred to six-well plates when RA was removed and then cultured further until DD 22.

Differentiation of 3D Retinal Organoids Derived From Human iPSCs

This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of RIKEN Center for Developmental Biology. Human iPSCs (201B7 cells), provided by the Center for iPSC Cell Research and Application, Kyoto University, were maintained and differentiated into 3D retinal organoids as described.11 Briefly, iPSCs were dissociated into single cells by a recombinant enzyme (TrypLE Select; Gibco BRL Life Technologies, Grand Island, MI, USA) with 0.05 mg/mL endonuclease (DNase I; Roche Diagnostics, Mannheim, Germany) and 20 mM Rock inhibitor (Y-27632; Wako Pure Chemical Industries, Ltd.), and 12,000 single iPSCs per well were quickly reaggregated using low cell-adhesion 96-well V-bottomed conical well plates (Sumitomo Bakelite, Tokyo, Japan) in growth factor–free chemically defined medium. This comprised Iscove’s modified Dulbecco’s medium and F12 medium (1:1; Life Technologies, Carlsbad, CA, USA), 1X lipid emulsion (Chemically Defined Lipid Concentrate; Life Technologies), and 450 mM mono-thioglycerol (Sigma-Aldrich Corp., St. Louis, MO, USA), supplemented with 10% knockout serum replacement (KSR). At DD 6, recombinant human BMP 4 (1.5 nM; R&D Systems, Minneapolis, MN, USA) was added to the medium, and half of the medium was exchanged at DD 9, DD 12, and DD 15. At DD 18, the retinal organoids were displaced to low-adhesion 10-cm dishes (Sumitomo Bakelite) in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life Technologies) containing 1% N2 supplement (Life Technologies), 3 mM GSK3-3 inhibitor (CHRIR9021; Stemgent, Cambridge, MA, USA), and a VEGFR/FGFR inhibitor (SU5402; Sigma-Aldrich Corp.) from DD 18 to DD 21. The medium was changed at DD 21 to Maturation Medium (DMEM/F12 containing 10% fetal bovine serum and 1% N2 supplement).

Two-Step Immunopanning and Culture of RGCs

Mouse and human RGCs were purified using a two-step immunopanning method as described,17 with minor modifications. The reported purification rate is over 99.5%.15 Briefly, the retinas were dissociated with papain (165 U/mL) and triturated with a rabbit anti-rat macrophage antibody (Accurate Chemical, Westbury, NY, USA). The cell suspension was first plated on a 150-mm petri dish (BD Falcon Labware, Franklin Lakes, NJ, USA) coated with goat anti-rat IgG (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was then repurified on 10-cm petri dishes (Thermo Fisher Scientific) coated with goat anti-mouse IgM (Thermo Fisher Scientific) and mouse anti-Thy1 antibodies derived from T11D7e2 cells (American Type Culture Collection, Manassas, VA, USA). The plate was washed with PBS, and the RGCs were recovered by trypsin treatment (0.125%; Sigma-Aldrich Corp.). The purified RGCs were cultured in defined RGC medium containing serum-free Neurobasal medium (Invitrogen Life Sciences, Invitrogen Life Sciences, Carlsbad, CA, USA), brain-derived neurotrophic factor (50 ng/mL; PeproTech, Rocky Hill, NJ, USA), ciliary neurotrophic factor (50 ng/mL; PeproTech), basic fibroblast growth factor (50 ng/mL; PeproTech), forskolin (10 μM; Sigma-Aldrich Corp.), B27 supplement (2%; Invitrogen Life Sciences), glutamine (1 mM; Gibco BRL Life Technologies), insulin (5 μg/mL; Sigma-Aldrich Corp.), sodium pyruvate (40 ng/mL), progesterone (62 ng/mL), putrescine (16 μg/mL), sodium selenite (40 ng/mL), triiodothyronine (40 ng/mL), and α-acetylcysteine (60 μg/mL). The 35-mm culture dishes were coated with poly-o-lysine (Sigma-Aldrich Corp.) and laminin (Sigma-Aldrich Corp.), and the RGCs were plated at a density of 50,000 cells per dish and cultured for approximately 3 to 4 weeks. Half of the medium was replaced with fresh RGC medium every 5 days.

Western Blotting

Seven 3D retinal organoids collected at DD 40, DD 50, DD 60, DD 80, and DD 100 were sonicated with buffer (Laemmli Sample Buffer; BioRad, Hercules, CA, USA). The cell lysates containing equal amounts of protein were heated at 95°C for 5 minutes and separated by SDS-PAGE. The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, and nonspecific binding was blocked with solution (Blocking One; Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature. The PVDF membranes were incubated overnight with primary antibodies against rat Thy1 (1:5000; Cederlane Labs, Burlington, ON, Canada) or rabbit GAPDH (1:5000; Cell Signaling Technology, Danvers, MA, USA) antibodies followed by the horseradish peroxidase–conjugated secondary antibodies. The membranes were scanned using software (Image Quant LAS 4000 mini; GE Healthcare, Piscataway, NJ, USA) with Chemi-Lumi One (Nacalai Tesque).
FIGURE 1. RGC-restricted EGFP expression in the Thy1-EGFP mice used in the study. (A) Heidelberg retinal angiography (HRA) images in Thy1-EGFP mouse retinas at 4 weeks (4W) of age; disc (left) and peripheral (middle) images taken using the fluorescein angiography mode. (Right) Retinal flat mount around the disc area of Thy1-EGFP mouse retinas at age 4W. Optic discs are indicated by white arrowheads. Scale bar: 500 μm. (B–E) Immunohistochemistry of sections double-stained using anti-GFP and -BRN3B antibodies applied to Thy1-EGFP TG mouse retinas at embryonic day 16 (E16), postnatal day 1 (P1), and at 4W and 1 year (1Y) of age. Cell nuclei were counterstained with DAPI. ONBL, outer
FIGURE 2. Immunopanning purification of RGCs from P2 Thy1-EGFP mouse retinas. (A) Schematic diagram of the two-step immunopanning method. An antimacrophage antibody was added to retinal cell suspensions, and attached cells were removed using an anti-IgG antibody in the first step. Nonadherent cells were treated with anti-Thy1 antigen in the second step. The adhered cells were collected as RGCs. (B) Time course of the culture of RGCs purified from Thy1-EGFP mouse retinas after immunopanning. Images taken at 1, 6, 12, and 15 days post immunopanning day (PID) are shown as higher magnification images (inset) to indicate neurite outgrowths from the RGCs. Scale bars: 200 μm. (C) Immunofluorescence analyses of expression of EGFP and BRN3B in purified RGCs at P2 + PID15 on the 35-mm dish. Cell nuclei were counterstained with DAPI. Scale bar: 200 μm.
FIGURE 3. Immunopanning purification and culture of RGCs from 3D retinal organoids differentiated from Thy1-EGFP mouse ESCs. (A) Schematic diagram of the differentiation of 3D retinal organoids from Thy1-EGFP mouse ESCs. The maintenance medium included Glasgow Modified Essential Medium (GMEM), 5% KSR, and AGN. Retinal Maturation Medium 1 included DMEM/F12, N2 supplement, and penicillin/streptomycin. Retinal Maturation Medium 2 included DMEM/F12, FBS, N2 supplement, and penicillin/streptomycin. AGN, RA receptor antagonist AGN193109. Optical micrographs of 3D retinal organoids at DD 1, DD 3, DD 9, DD 13, DD 18, and DD 22 are also shown. Scale bars: 200 μm (DD 1, DD 3, DD 9, and DD
Reverse Transcription–Quantitative PCR (RT-qPCR)

Expression of the mRNA levels for ATOH7, BRN3B, ISL1, RBPMS, and THY1.1 (RGC markers), GAD1 (developing RGC and amacrine marker), CRX (photoreceptor marker), PRKCA (bipolar cell marker), RPE65 (retinal pigment epithelium marker), GS (glutamine synthetase Müller glial cell marker), and GFAp (Müller glial cell and astrocyte marker) were assessed by RT-qPCR. Total RNA was isolated from human 3D retinal organoids on DD 40, DD 60, DD 80, DD 100, and DD 120, and from RGCs purified by immunopanning from DD 50 to 70, DD 70 to 90, and DD 90 to 110 human 3D retinal organoids. After synthesizing cDNA using reverse transcriptase (Super Script IV, Thermo Fisher Scientific), the expression of various molecules and GAPDH in triplicate samples were analyzed by RT-qPCR (LightCycler model 480; Roche Diagnostics), using a reagent (qPCR Mastermix; Roche Diagnostics) and highly specific Universal Probe Library assays (Roche Diagnostics). The tested primers and the Universal Probe Library are described in Supplementary Table S1. The RT-qPCR was performed as described.23 Briefly, cycles involved denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 15 seconds. Relative cDNA amounts were calculated with software (Relative Quantification; Roche Diagnostics) using an efficiency-corrected algorithm with standard curves and reference gene normalization against that of GAPDH (delta-delta cycle threshold \( \Delta \Delta C_t \)). Results are indicated as the relative expression of the molecules \( 2^{-\Delta \Delta C_t} \) values to control cells (where \( GAPDH = 1 \)).

Immunohistochemistry and Immunocytochemistry

Immunohistochemistry was performed as described.11 Three-dimensional retinal organoids were fixed for 30 minutes in 4% paraformaldehyde and then soaked in 30% sucrose/PBS overnight and imbedded in optimal cutting temperature (OCT) compound (Sakura FineTek, Tokyo, Japan). Immunocytochemistry was performed as described,24 with minor modifications. Purified RGCs plated on dishes were fixed with 4% paraformaldehyde for 30 minutes at 4°C and washed three times with Tris-buffered saline with Tween 20. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes, blocked using 5% horse serum in PBS for 1 hour at room temperature, and incubated with the primary antibody overnight at 4°C. RGCs were incubated with species-specific corresponding secondary antibodies for 1 hour at room temperature. The primary antibodies used in this study are described in Supplementary Table S2. Secondary antibodies used include Alexa Fluor 488, 546, and 647-conjugated antibodies (1:1000; Life Technologies), and nuclear staining was performed with 4,6-diamidino-2-phenylindole (DAPI; Nacalai Tesque). Images were obtained using a confocal microscope (LSM700; Carl Zeiss, Jena, Germany).

Time-Lapse Imaging of Neurite Outgrowth

Neurite outgrowth was observed using an IncuCyte Zoom system (Essen BioScience, Ann Arbor, MI, USA). The lengths of neurites and numbers of attached cells were analyzed using the software (Basic Analyzer and NeuroTrack) of the IncuCyte Zoom system. Briefly, 144 bright-field images (each image 1.8 × 1.8 mm) were taken every 6 hours, and total neurite lengths and cell body numbers were collected.

Statistical Analysis

Student’s t-test for two-group comparisons and the Mann-Whitney nonparametric U test with Bonferroni correction for multigroup comparisons were applied using statistical software (PRISM; GraphPad Software, San Diego, CA, USA); \( P < 0.05 \) was considered statistically significant.

RESULTS

Mouse RGCs Could Be Purified by Two-Step Immunopanning From 3D Retinal Organoids Differentiated From Thy1-EGFP TG Mouse ESCs

In Thy1-EGFP TG mice, green EGFP fluorescence could be observed in live animals using Heidelberg retinal angiography and in retinal whole mounts (Fig. 1A). During mouse development, there was little EGFP expression in the retinas at embryonic (E) day 16 (Fig. 1B). EGFP localization at the RGC layer was observed at postnatal (P) day 1, adult (P 4 weeks), and aged (P 1 year) Thy1-EGFP TG mouse retinas and confirmed for colocalization with BRN3B (Figs. 1C–E). The fraction of EGFP-positive cells increased from P 1 day to adulthood, indicating maturation of RGCs during development (Fig. 1F). We purified RGCs from Thy1-EGFP TG mouse retinas at P 2 by two-step immunopanning as described previously (Fig. 2A).15 Purified cells (50,000) were plated on a 35-mm coated dish and cultured with RGC culture medium. They were able to live for approximately 2 weeks (Fig. 2B) and expressed EGFP, BRN3B, and SMI-312 as shown by immunohistochemistry (Fig. 2C).

Next, we generated ESCs from the Thy1-EGFP TG mice, and 3D retinal organoids were differentiated in our modified SFEBq culture protocol (Fig. 3A).25 Retinal optic vesicle-like structures with clear neural epithelial layers were observed from around DD 7 to 10 and expanded gradually until DD 20 (Fig. 3A). BRN3B-positive cells were located in the basal half of the neural epithelium of the DD 21 retinal organoids, and CRX-positive cells were located on the apical side (Fig. 3B). There were BRN3B/EGFP double-positive cells present, but there were fewer EGFP-positive than BRN3B-positive cells. Next, we purified RGCs from DD 22 3D retinal organoids derived from Thy1-EGFP TG mouse ESCs by immunopanning. Purified cells (50,000) were plated on 35-mm coated dishes. The cells extended their neurites, could be maintained for approximately 2 to 3 weeks, and were positive for EGFP, BRN3B, and SMI-312. Thus, we successfully purified mouse RGCs not only from Thy1-EGFP TG mouse retinas but also 3D retinal organoids derived from Thy1-EGFP TG mouse ESCs.

Human RGCs Could Be Purified by Two-Step Immunopanning From 3D Retinal Organoids Derived From Human iPSCs

Human 3D retinal organoids were differentiated from 201B7 iPSCs by the SFEBq method, as reported (Fig. 4A).10 Clear

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FIGURE 4. Developmental RGC marker expression in the 3D retinal organoids differentiated from human iPSCs. (A) A schematic diagram of the procedure for differentiating these organoids from human iPSCs. Optical micrographs of organoids at DD 1, DD 6, DD 18, and DD 21 are also shown. Scale bar: 200 μm. (B) Time course of cultured 3D retinal organoids at DD 40, DD 60, DD 80, DD 100, and DD 120. Bright-field images of whole organoids (upper panels) and immunohistochemistry of sections treated with anti-BRN3B and -CRX antibodies are shown (lower three panels). Scale bars: 500 μm (bright field) and 200 μm (immunohistochemistry). (C) The cell density of BRN3B-positive cells and CRX-positive cells
neural epithelial layers of 3D retinal organoids were observed from around DD 10 to 13 (data not shown) and expanded gradually until DD 18. After the induction of differentiation reversal culture from DD 18 to DD 21, the culture system was changed to the maturation medium. We performed immunohistochemistry on sections of the 3D retinas collected from DD 40 to DD 120 and confirmed that BRN3B-positive cells were localized at the apical side of 3D retinal organoids (Fig. 4B). CRX-positive cells were localized at both apical and basal sides at DD 40, and almost all CRX-positive cells were localized at the basal side at DD 60 to 120 (Fig. 4B). The numbers of BRN3B-positive cells peaked at DD 60 and then decreased gradually. The numbers of CRX-positive cells increased during development, indicating proliferation and maturation of photoreceptor cells (Fig. 4C). We also carried out RT-qPCR to confirm the RGC markers (Fig. 4D). At DD 40, DD 60, DD 80, DD 100, and DD 120, typical RGC markers (ATOH7, BRN3B, ISL1, RBPMS, and THY1) were expressed, together with a developing RGC and amacrine cell marker (GAD1), a photoreceptor marker (CRX), a bipolar cell marker (PRKCA), and Müller glial cells (GFAP), a Müller glial cell and astrocyte marker (GFAP, DD 80–120), and a Müller glial cell marker (GS), which were observed inside the BRN3B-positive RGC layer of 3D retinal organoids (Supplementary Fig. S1A).

Next, we tested the two-step immunopanning method on human iPSC-derived 3D retinal organoids. We first confirmed the temporal expression of Thy1 by Western blotting for the crude lysates collected at DD 40 to 100, indicating the existence of Thy1-expressing cells (Fig. 5A). We then performed immunopanning using human iPSC-3D retinal organoids collected from three developmental periods (DD 50–70, DD 70–90, and DD 90–100). The RT-qPCR analyses showed that the purified cells expressed typical RGC markers (ATOH7, BRN3B, ISL1, RBPMS, and THY1) and a developing RGC and amacrine cell marker (GAD1) and that they did not express other cell-type markers such as CRX, PRKCA, RPE65, GFAP, or GS (Fig. 5B). For DD 90 to 110 3D retinas, we also confirmed nondetectable expression of markers for PSCs (NANOG) and photoreceptor cells (RCRYN) (data not shown). We also performed RT-qPCR using cell fractions that were not collected by immunopanning and observed expression of all retinal cell types, including THY1 (Supplementary Fig. S1B).

To examine the morphology and neurogenesis of the purified human iPSC-derived RGCs, we plated 50,000 cells on 35-mm coated dishes and cultured them with RGC culture medium for 3 to 4 weeks (Fig. 5C). All human iPSC-derived RGCs collected from DD 50 to 70, DD 70 to 90, and DD 90 to 110 attached to the dish within 24 hours, with neurite extension. More RGCs collected at DD 90 to 110 remained on the dish for longer periods (Fig. 5C) than those collected at earlier developmental stages. Most purified RGCs were BRN3B-positive, and the neurites could be visualized by the pan-axonal antibody SMI-312 (Fig. 5D).

Because we observed differences in survival and neurite extension of RGCs depending on the day of differentiation, we analyzed neurite outgrowth and the numbers of RGCs using the IncuCyte Zoom sensitive imaging system and original analyzing software (Fig. 6A). We started observations from 6 hours after cell plating and collected images until 18 days after plating (450 hours). The original analyzing software (NeuroTrack and Basic Analyzer) were used to analyze total neurite length (Fig. 6B) and cell body numbers (Fig. 6C) in the collected area, depending on user-defined parameters. Until 90 hours after plating, neurites of RGCs purified from DD 70 to 90 were larger than at DD 50 to 70 and DD 90 to 110. After 90 hours, the RGCs from DD 90 to 110 extended neurites and maintained them until the end of the observation period, while RGCs collected at younger ages showed shrinkage of neurites (Fig. 6B). Consistent with the observation shown in Figure 5C, there were more RGCs purified from DD 90 to 110 than from DD 50 to 70 or DD 70 to 90 (Fig. 6C). To analyze the neurite extension of individual RGCs, we divided the neurite length by the cell body numbers. The replotted data (Fig. 6D) indicated that single RGCs purified from DD 70 to 90 extended longer neurites than those from DD 50 to 70 and DD 90 to 110, until 180 hours after plating. However, after that time, the RGCs from DD 90 to 110 extended more neurites than those from DD 70 to 90. We were able to maintain them for up to 28 days (data not shown).

**Discussion**

In this study, we successfully purified human RGCs from 3D retinal organoids by a two-step immunopanning method. Because we could not obtain human retina samples for ethical reasons, it has been difficult to study human RGCs. As an alternative, we have been using experimental animals, although there are interspecific morphologic differences in RGC subtypes. Recent progress in stem cell research has enabled the generation of various tissues differentiated from PSC aggregates by maintaining them in media with defined factors. Irikaku et al.9 reported the formation of 3D retinal organoids from mouse ESC aggregates by the SFEBq method. Subsequently, Nakano et al.10 reported the production of self-forming 3D retinal organoids from human ESC aggregates by an improved SFEBq method. These findings have enabled the study of human retinal cells instead of using animal models, and experimental procedures designed for mouse models have been applied to human ones. We have previously reported an explant culture procedure in which human ESC-derived retinal organoids exhibit significant neurite outgrowth.25

Following this trend, we applied an immunopanning method designed for both rodent and human systems. We applied this to mouse retinas and mouse ESC-derived retinal organoids and compared the morphologic and immunohistochemical features of the purified cells. We selected an appropriate Thy1-EGFP TG mouse strain, as reported by Sakai et al.,19 in which EGFP is specifically expressed in RGCs because some of them express this in non-RGCs.17 Immunohistochemistry of the TG mouse tissues showed that the Thy1- and BRN3B-positive cells almost overlapped in aged mice, although there were BRN3B-negative RGC subtypes. This observation suggests that the Thy1-EGFP TG mice preferentially express EGFP in BRN3B-positive RGCs and that there are differences in Thy1 expression levels among RGC subtypes. We successfully purified Thy1-expressing cells by immunopanning from the 3D retinal organoids differentiated from Thy1-EGFP mouse ESCs; we observed neurite outgrowth and expression of BRN3B and SMI312, indicating that ESC-derived RGCs could be purified, as demonstrated in mouse retinas. Remarkably, most purified ESC-
**Figure 5.** Immunopanning purification and culture of RGCs from 3D retinal organoids differentiated from human iPSCs. (A) Western blotting analysis for the expression of THY1 protein used to perform the immunopanning. The expression of GAPDH was used as a control. (B) RT-qPCR analyses of the purified RGCs at DD 50 to 70, DD 70 to 90, and DD 90 to 110. ND, not detected. Results indicate the expression levels of these molecules relative to GAPDH. Error ranges are presented as the mean ± SD (n = 5). (C) Time course of the neurite outgrowth of RGCs collected from DD 50 to 70, DD 70 to 90, and DD 90 to 110 at PID 1, PID 6, PID 12, and PID 18. Scale bar: 200 μm. (D) Immunofluorescence analyses of the expressions of BRN3B and SMI-312 at DD 90 to 110 + PID18. The right panel shows a lower-magnification image indicating lengthy neurite extensions from purified RGCs. Scale bar: 200 μm.
FIGURE 6. Quantification of the cell number and neurite length of RGCs analyzed using the IncuCyte Zoom semi-live imaging system. (A) The system (left) was set inside the incubator, and 144 bright-field images (each image 1.8 × 1.8 mm) were taken every 6 hours. Total neurite lengths and cell body numbers were collected from these images. The left two images show representative quantification processes for total neurite length (B) and cell body (C). Scale bar: 100 μm. (B, C) Time courses of total neurite lengths (B) and cell body numbers (C) of RGCs collected at DD 50 to 70, DD 70 to 90, and DD 90 to 110. The values at 90, 180, 270, and 360 hours after plating are shown as bar graphs (right panels). (D) Time course of the number of RGC neurites per RGC. The nonparametric Mann-Whitney U test with Bonferroni correction was applied (n = 4, *P < 0.05). Error ranges are presented as the mean ± SD.
derived RGCs were EGFP- and BRN3B-positive, consistent with the results from native TG mice retinas.

Next, we were able to culture human RGCs for longer periods than mouse RGCs purified from both human and mouse ESC-derived 3D retinal organoids. It is remarkable that all purified RGCs were BRN3B positive, similar to the mouse experiments. Taken together with the RT-qPCR results showing that Thy1 was expressed in a certain fraction of the unpurified cells, our immunopanning approach selected RGCs with high levels of Thy1 expression.

The neurite outgrowths of human iPSC-derived RGCs were also longer and more widely spread than those from mouse ESC-derived RGCs. In 3D retinal organoids derived from human iPSCs, the numbers of BRN3B-positive cells peaked at around DD 60, and the numbers of CRX-positive cells peaked at around DD 100. These results are consistent with a previous report despite the different method used.30 RGCs in 3D retinal organoids decreased gradually after DD 60; however, the purified RGCs during DD 90 to 110 were more vigorous when considering the neurite lengths and cell numbers. It is well known that RGCs decrease in numbers during maturation,27 and our results suggest that human RGCs in 3D retinal organoids mature along with differentiation. Human retinal development is poorly understood compared with rodents; however, retinogenesis begins at approximately week 5 of gestation.3 Based on comparisons with other species, it has been predicted that human ganglion cells are generated shortly after this date, peaking at approximately fetal week 8 and ending at approximately week 12.29 Our neurite outgrowth analyses are consistent with those reports.

Purified RGC culture is helpful for investigating primary RGC responses in certain circumstances. Although there are several reports on the function and neurite outgrowth of human iPSC-RGCs differentiated by an organotypic culture system,25,30,51 little has been reported on purified RGCs from 3D retinal organoids.24 It is difficult to identify RGCs induced by direct genomic reprogramming completely as they are restricted to the eye.52 To avoid this problem, we purified RGCs from 3D retinal organoids already confirmed as being of retinal origin. There are several methods of cell separation from tissues, such as immunopanning, MACS, and fluorescence-activated cell sorting (FACS). The FACS method is known as a good choice for these types of experiments because the protocol is rapid and easy;35 however, it would be a relatively crude method of purification, affecting cell viability especially in fragile cells such as neurons. The complicated processes and mechanical separation used in FACS can weaken the RGCs and affect their yield.34 Although the MACS method is also simple and has a stable yield, the purity of RGCs isolated in this way is lower than that of RGCs isolated by two-step immunopanning,35 which is complicated and requires many chemical reagents.15 The yield of separation varies, and it takes a long time (4–5 hours) to purify RGCs. Our RT-qPCR showed that the fraction of CRX-positive cells increases until DD 120, although the BRN3B-positive cells decrease in number. To obtain more RGCs with longer neurite outgrowths, organoids with a better retinal layer morphology should be collected. In addition, it is well known that RGCs have several subtypes. We would not be able to access such subtypes because all purified RGCs are BRN3B-positive.

Here, we were able to obtain RGCs from 3D retinal organoids derived from mouse and human iPSCs. RGCs isolated using this approach will be useful in studies aimed at elucidating mechanisms underlying the pathology of glaucoma and related optic neuropathies. Moreover, we might be able to confirm the effects of neuroprotective drugs or neurotrophic factors in vitro. Although various eyes drops for glaucoma have been shown to have neuroprotective effects in animal or in vitro studies,37 it is difficult to confirm these effects using human RGCs. This study would help in screening of new therapeutic drugs and could lead to personalized medicine by using patient-specific iPSCs derived from patients with glaucoma. Furthermore, RGCs of high purity might be useful for transplantation therapy to compensate for losses.

CONCLUSIONS

Mouse and human RGCs have been purified successfully from iPSC-derived 3D retinal organoids. We might be able to apply supplemental purified RGC therapy for diseases involving loss of RGCs. Moreover, this model will be useful for studying the pathogenesis of RGC death-related diseases such as glaucoma and in drug screening.

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