Embryonic stem cell–derived photoreceptor precursor cells differentiated by coculture with RPE cells

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Purpose: To describe the derivation of photoreceptor precursor cells from human embryonic stem cells by coculture with RPE cells.

Methods: Human embryonic stem cells were induced to differentiate into neural precursor cells and then cocultured with RPE cells to obtain cells showing retinal photoreceptor features. Immunofluorescent staining, reverse transcription–PCR (RT–PCR), and microarray analysis were performed to identify photoreceptor markers, and a cGMP assay was used for in vitro functional analysis. After subretinal injection in rat animal models, retinal function was determined with electroretinography and optokinetic response detection, and immunofluorescent staining was performed to assess the survival of the injected cells.

Results: Cocultured cells were positive for rhodopsin, red and blue opsin, recoverin, and phosphodiesterase 6 beta on immunofluorescent staining and RT–PCR. Serial detection of stem cell–, neural precursor–, and photoreceptor-specific markers was noted in each stage of differentiation with microarray analysis. Increased cGMP hydrolysis in light-exposed conditions compared to that in dark conditions was observed. After the subretinal injection in the rats, preservation of optokinetic responses was noted up to 20 weeks, while electroretinographic response decreased. Survival of the injected cells was confirmed with positive immunofluorescence staining of human markers at 8 weeks.

Conclusions: Cells showed photoreceptor-specific features when stem cell–derived neurogenic precursors were cocultured with RPE cells.

Photoreceptor cells absorb photons and transform light signals into biologic signals in the retina, which is essential for the formation of vision. Loss of these photoreceptors leads to the deterioration of vision, which is a common late-stage feature of many forms of retinal degeneration, such as retinitis pigmentosa, cone-rod dystrophy, and age-related macular degeneration, despite the various differences in the early pathophysiologic changes. As the damage occurring to photoreceptor cells is considered to be irreversible, there is no known effective treatment for these conditions, despite numerous efforts.

Embryonic stem (ES) cells have been considered a possible source of cell therapy for retinal degeneration. RPE cells, which are pigmented cells that form the outer blood–retinal barrier, play various roles in maintaining the retina. RPE cells have been considered target cells for cell replacement therapy for retinal degeneration, and various methods have been established to generate RPE cells from pluripotent stem cells. However, as retinal degeneration is characterized by photoreceptor dysfunction and death, causing eventual deterioration of vision, it may be theoretically most effective if photoreceptors can be replaced along with RPE cells. The development of photoreceptor cells from stem cells has been challenging due to disappointingly low yields and complicated processes, which have mostly involved various two- and three-dimensional (3D) culture systems [1-11] and supplementation with various exogenous inhibitors or growth factors, including Noggin, Dkk1, LeftyA, DAPT, insulin-like growth factor 1, acidic or basic fibroblast growth factor (FGF), triiodothyronine, sonic hedgehog, retinoic acid, and taurine [1-3,5,12-14]. Therefore, the key to clinical application of stem cell–derived cell therapy is to generate retinal photoreceptor cells by using a simpler, more controllable method without genetic modification using viral vectors.

The differentiation of retinal cells takes place in a microenvironment with many cell-to-cell interactions through direct contact or diffusible signaling molecules. As RPE cells are essential for the survival and maintenance

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of photoreceptors, they may play a role in the development of photoreceptors. Previous studies have shown that RPE–photoreceptor interactions by contact and secreted factors are important for photoreceptor differentiation and maturation. In previous studies using ES cells, mouse retinal explant coculture was used for retinal photoreceptor differentiation stimulation [12,13]. Recently, photoreceptors were derived from ES cells or induced pluripotent stem cells by the formation of 3D optic cup-like structures [5,7] with the development of the neural retina adjacent to the RPE cells, which provide RPE-derived factors. However, very little is known about how RPE cells assist in the derivation of photoreceptor cells.

We report our method for the differentiation of retinal photoreceptor precursors from human ES cells by coculture with RPE cells and the results of in vitro and in vivo functional evaluation studies of these cells. Subretinal transplantation was performed in Royal College of Surgeons (RCS) rats, a widely used retinal degeneration animal model, and the survival of these cells and their effect on retinal function were evaluated.

METHODS

Differentiation of RPE cells from human embryonic stem cell–derived SNMs and photoreceptor precursor cells by coculture with these RPE cells: As previously reported, human ES cells (SNUhES3 line, previously established and reported [15]) were induced to differentiate into neuroepithelial cells through the in vitro developmental process of the formation of the spherical neural mass (SNM) from the embryoid body (EB) [16-18]. Briefly, human ES cells were detached and transferred into culture dishes with essential 6 medium (GIBCO, Grand Island, NY) for 5 days to generate EBs, which were then attached onto CELLstart CTS (GIBCO)-coated dishes and cultured in neural precursor (NP) selection medium including 0.5% N2 supplement (GIBCO) for 5 days. Then, the medium was switched to NP expansion medium with 1% N2 supplement and 40 ng/ml basic fibroblast growth factor (bFGF; GIBCO). After 7 to 14 days, neural rosettes and neural tube structures were mechanically isolated and then cultured in suspension in NP expansion medium to form SNMs. When grown to approximately 0.5–1.00 mm in diameter, these SNMs can be passaged for use in further studies by mechanically cutting them into six to eight pieces and maintaining them in suspension culture for 5 to 7 days to allow them to grow. Some SNMs in early culture (passages 1 to 4) show optic vesicle-like structures that balloon outward, and these cystic portions were separated and fragmented with mechanical dissection under a stereomicroscope and cultured in a CELLstart-coated dish for 1 to 4 weeks in RPE medium

with 1% N2 supplement and 1% B-27 supplement (GIBCO), which resulted in the generation of cells showing typical RPE morphologies that were previously described as ES-derived RPE cells. Detailed characteristics of these ES-derived RPE cells can be found elsewhere [17] (Figure 1).

For the generation of photoreceptor precursor cells, a noncontact coculture system with transparent permeable cell culture inserts suspended in a six-well plate was used for the coculture of SNM-derived neural precursors and ES-derived RPE cells. The neural tube–like portions of the SNMs from passages 3 to 8 were mechanically cut into small pieces approximately 100–200 μm in diameter and enzymatically dissociated into single cells, after which they were transferred onto CELLstart-coated surfaces of culture dishes. These cells were cocultured with ES-derived RPE cells in medium supplemented with N2 and B-27 for up to 21 days to induce differentiation into photoreceptor precursor cells (Figure 1). To identify the optimal coculture method, alternative placement of SNM-derived single cells and ES-derived RPE cells was attempted by using the following combinations: 1) SNM-derived single cells in the insert and ES-derived RPE cells in the bottom of the well, 2) ES-derived RPE cells in the insert and SNM-derived single cells in the bottom of the well, and 3) SNM-derived single cells on the outer surface of the insert and ES-derived RPE cells in the bottom of the well (Figure 2). During the differentiation period, the medium was changed every other day.

Immunofluorescent staining of cultured cells for the identification of photoreceptor marker expression: Cultured cells were fixed with 4% paraformaldehyde. After blocking for 1 h in 3% bovine serum albumin (BSA; Sigma, St. Louis, MO) in PBS (1X; 155 mM NaCl, 1 mM KH_2PO_4, 3 mM Na_2HPO_4-$7H_2O$, pH 7.4; GIBCO) containing 0.3% Triton X-100 (Sigma), the samples were incubated overnight at 4 °C in 1% BSA in PBS containing 0.1% Tween-20 (Sigma) containing the primary antibodies. The primary antibodies are listed in Table 1. To detect the primary antibodies, Alexa Fluor® 488 donkey anti-mouse and anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR), Alexa Fluor® 594 donkey anti-mouse and anti-rabbit immunoglobulin G (IgG; 1:200, Molecular Probes), and Alexa Fluor® 594 donkey anti-goat IgG were used. The cell nuclei were counterstained with 4',6-diamidine-2-phenylindol (DAPI; Molecular Probes).

RT–PCR for the identification of photoreceptor marker expression: Reverse transcription–PCR (RT–PCR) was performed as described previously [17]. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from RNA using AMV reverse transcriptase (RT) and oligo-dT as a primer according to the

manufacturer's instructions (AccuPower RT PreMix; Bioneer, Taejeon, South Korea). PCR amplification was performed with Taq Polymerase (HiPi Plus 5x PCR Premix; Elpis Biotech, Taejeon, South Korea) using a standard procedure. The primer sequences are summarized in Table 2.

RNA extraction, cDNA synthesis, and microarray analysis: For the microarray analysis, total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA), purified using RNeasy columns (Qiagen, Valencia, CA), and then amplified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX) to yield biotinylated cRNA, which was then reverse-transcribed into cDNA using a T7 oligo(dT) primer. The second-strand cDNA was synthesized,

transcribed in vitro, and labeled with biotin-NTP, and the labeled samples were hybridized to each human HT-12 expression v.4 bead array for 16–18 h at 58 °C according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Detection of the array signal was performed using Amersham Fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK), and arrays were scanned with an Illumina bead array reader confocal scanner. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 and Gene Expression Module v1.9.0). Samples from each stage of differentiation with the same cell counts were used, and three biologic replicates were analyzed for each stage; the median values of the replicates were used for further analysis.
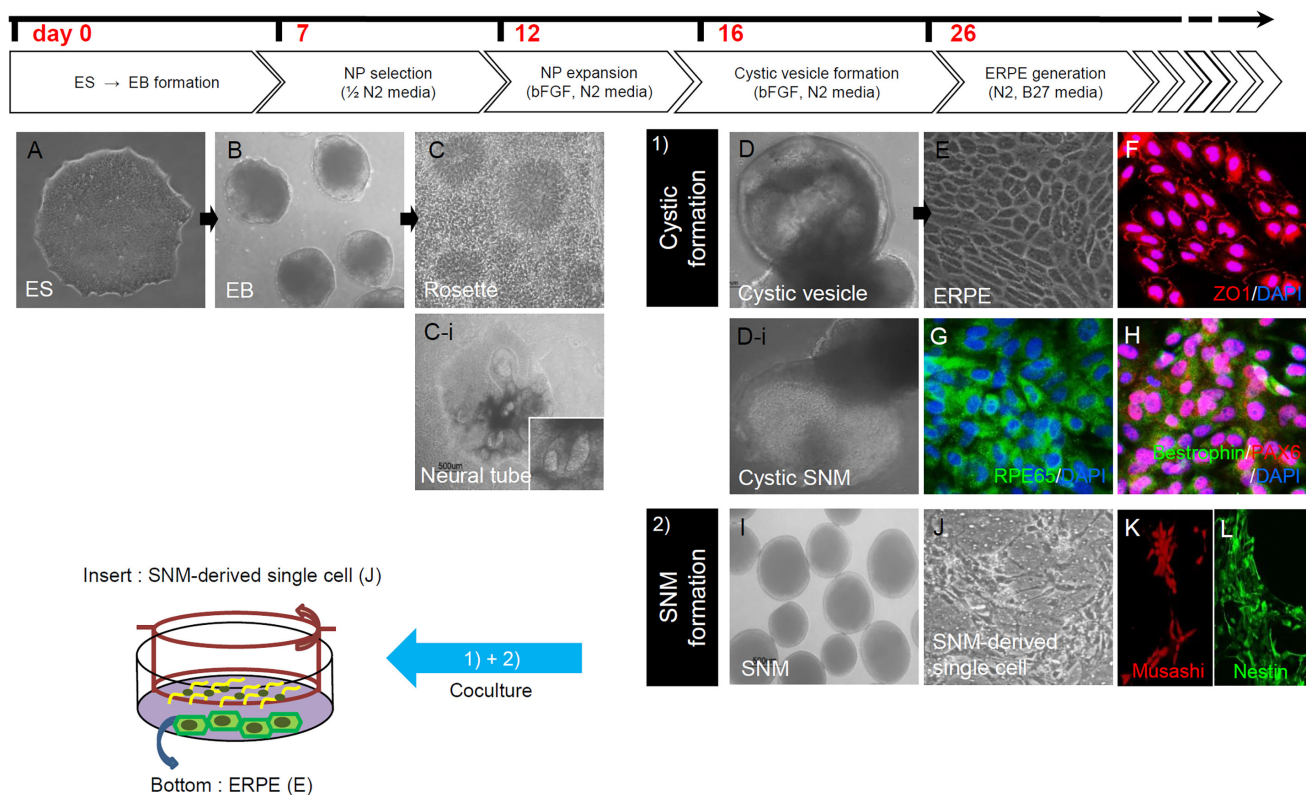

Figure 1. Figure 1. Differentiation of photoreceptor precursors from human embryonic stem (ES) cells via embryonic body (EB) and spherical neural masses (SNMs). Human ES cells (**A**) are detached to generate EBs (**B**), which were then attached and cultured in neural precursor (NP) selection medium including 0.5% N2 supplement for 5 days. Then, the medium was switched to NP expansion medium with 1% N2 supplement and basic fibroblast growth factor (bFGF). Neural rosettes (**C**) and neural tube structures (**C-I**) were mechanically isolated and then cultured in suspension to form SNMs. Some SNMs in early culture show cystic structures (**D**) that balloon outward, and these cystic SNMs (**D-I**) were separated and cystic portions were fragmented with mechanical dissection under a stereomicroscope and were attached and cultured in RPE (retinal pigment epithelium) generation medium with 1% N2 supplement and 1% B-27 supplement, to form ES-derived RPE cells (ERPE, **E**), which are positive for zonula occludens 1 (ZO1, **F**), retinal pigmented 486 epithelium-specific 65-kDa protein (RPE65, **G**), and bestrophin (**H**). Other non-cystic SNM (**I**) were passaged and mechanically cut and plated as SNM-derived single cells (**J**), positive for neural stem cell markers musashi (**K**) and nestin (**L**). SNM-derived single cells (**J**) were cocultured with ES-derived RPE cells (ERPE, **E**) to differentiate photoreceptor precursor cells.
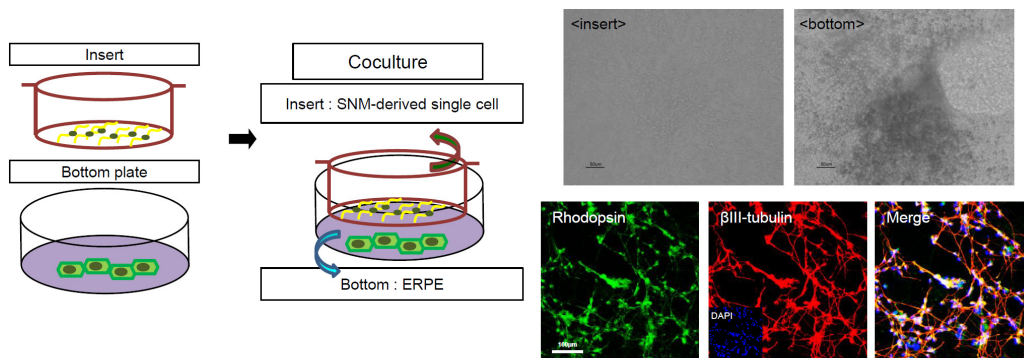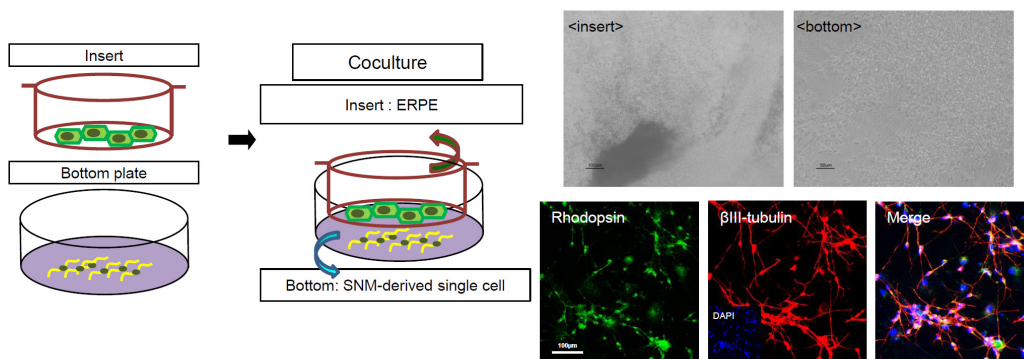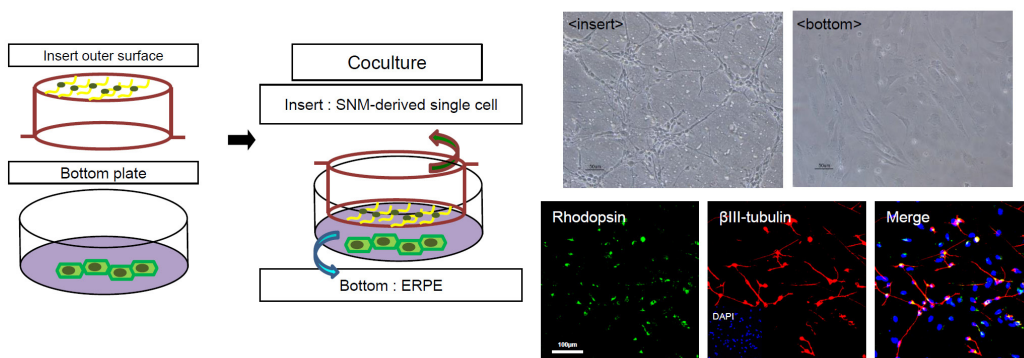
Figure 2. Alternative placement of human ES-derived RPE cells and cells from the neural tube–like portions of SNMs to identify the optimal coculture method for the differentiation of photoreceptor precursor cells. **A**: Spherical neural mass (SNM)-derived single cells in the insert and embryonic stem (ES)-derived RPE cells in the bottom of the well. **B**: ES-derived RPE cells (ERPE) in the insert and SNM-derived single cells in the bottom of the well. **C**: SNM-derived single cells on the outer surface of the insert and ES-derived RPE cells in the bottom of the well.

Antibody	Dilution	Source	Location
Polyclonal antibody (rabbit)			
β-Tubulin	1:1000	BioLegend	San Diego, CA
Opsin, blue	1:200	Chemicon	Temecula, CA
Opsin, red/green	1:100	Chemicon	Temecula, CA
Recoverin	1:100	Chemicon	Temecula, CA
PDE6β	1:50	abcam	Cambridge, UK
ZO1	1:50	ThermoFisher	Rockford, IL
RPE65	1:100	Novus biologicals	Littleton, CO
Bestrophin	1:100	Novus biologicals	Littleton, CO
PAX6	1:100	Novus biologicals	Littleton, CO
Rhodopsin	1:100	Millipore	Temecula, CA
Monoclonal antibody (mouse)			
β-Tubulin	1:500	BioLegend	San Diego, CA
Anti-human nuclei	1:50	Millipore	Temecula, CA
PAX6	1:100	Chemicon	Temecula, CA

TABLE 1. PRIMARY ANTIBODY INFORMATION.

PDE6β, phosphodiesterase 6 beta; ZO1, zonula occludens 1; RPE65, retinal pigmented epithelium-specific 65-kDa protein; PAX6, paired box 6.

In vitro functional phototransduction pathway characterization with a cGMP assay: In vitro functional evaluation of the phototransduction pathway was performed by measuring the level of cGMP with an enzyme immunoassay kit (Biotrack [EIA] System; GE Healthcare, Buckinghamshire, UK) as described previously [19,20]. The cGMP level was measured according to the manufacturer's protocol in ES-derived photoreceptor precursor cells (cocultured for 7 days) under dark conditions, and then the cells were maintained in ambient daylight for 24 h to evaluate whether the cGMP level was decreased under light-exposed conditions due to the increase in cGMP hydrolysis by functional phototransduction. A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 50 mM), was added 48 h before the determination of the cGMP levels to evaluate whether the observed decrease in the cGMP level under light-exposed conditions was inhibited.

Immunofluorescent staining and in vivo functional evaluation using electroretinography and optokinetic response measurement after subretinal injection of ES-derived retinal cells in a retinal degeneration animal model: The animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital. The 3-week-old RCS rats were sorted into five groups: group 1 with no injection, group 2 with media-only injection, group 3 with ES-derived RPE cell (10^5 cells/2 µl) injection, group 4 with ES-derived photoreceptor precursor cell (10^5 cells/2 µl) injection, and group 5 with an injection of a 1:1 mixture of ES-derived RPE cells and photoreceptor precursor cells (10^5 cells/2 µl), with five rats per group based on the resource equation method [21]. The RCS rats were anesthetized with an intraperitoneal injection of tiletamine/zolazepam (20-40 mg/kg) and xylazine (5-10 mg/kg). The pupils were dilated with eye drops containing 0.5% tropicamide and 2.5% phenylephrine hydrochloride

TABLE 2. GENE-SPECIFIC PRIMER SEQUENCES.

Gene	Forward sequence	Reverse sequence
Rhodopsin	CACAGGATGCAATTTGGAGG	CCTTCTGTGTGGTGGCTGAC
β-Tubulin	CAACAGCACGGCCATCCAGG	CTTGGGGCCCTGGGCCTCCGA
Nestin	CAGCTGGCGCACCTCAAGATG	AGGGAAGTTGGGCTCAGGACTGG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(HCl), and topical anesthetic eye drops with 0.5% proparacaine HCl were administered. Before implantation, the putative ES-derived RPE cells or photoreceptor precursor cells (cocultured for 7 days) were incubated with DAPI (10 µg/ml) for 30 min, washed several times to remove the DAPI in the media, and then dissociated with incubation for 5 min in 0.05% trypsin/0.1% EDTA at 37 °C. Under visualization with an operating microscope, injection of cells in a 2 µl volume into the dorsal subretinal space was performed via a transscleral approach using a 33-gauge needle attached to a Hamilton syringe. Immediately after injection, the fundus was examined, and any animals with massive subretinal hemorrhage or vitreous hemorrhage were removed from the study. Cyclosporin A (210 mg/l, Cipol-N; Chong Kun Dang, Seoul, South Korea) was administered in the drinking water starting 1 day before transplantation until enucleation was performed. Electroretinograms (ERGs) were measured at 4, 8, 12, and 20 weeks, and the amplitude of the dark-adapted ten ERG b-waves was assessed. The maximum cycle per degree of optokinetic response was also measured. The animals were euthanized (intraperitoneal injection of tiletamine/zolazepam [200 mg/kg] and xylazine [50 mg/kg]) at 1 week and 8 weeks after injection, and the enucleated eyes were rapidly frozen in embedding compound (FSC 22; Leica Microsystems, Richmond, IL). The cryosectioned eyes were used for immunofluorescent staining to evaluate the survival of the injected cells as described in the previous section.

Statistical analysis: Statistical analysis was performed using SPSS version 22.0 (IBM, Armonk, NY); p values of less than 0.05 were considered statistically significant. The Mann–Whitney U-test was used to examine the significance of comparisons among groups.

RESULTS

Identification of the most efficient coculture method for the differentiation of retinal photoreceptor precursor cells from ES cells: After coculture of SNM-derived single cells with ES-derived RPE cells, immunofluorescent staining revealed the presence of neuronal cells expressing rhodopsin. These cells showed a morphology with small nuclei and relatively short neurites. To identify the optimal coculture method, the alternative placement of the ES-derived RPE cells and the cells derived from the SNM neural tube–like portions was attempted, as shown in Figure 2. Coculture of SNM-derived single cells in the inserts with the ES-derived RPE cells in the bottom of the wells was the most efficient method, as coculture with ES-derived RPE cells in the inserts and SNM-derived single cells in the bottom of the wells or coculture

with SNM-derived single cells on the outer surface of the inserts and ES-derived RPE cells in the bottom of the wells resulted in fewer rhodopsin-positive cells.

In vitro expression of photoreceptor markers on immunofluorescence, PCR, and microarray analysis, and in vitro functional evaluation of phototransduction with a cGMP assay: On immunofluorescence staining, rhodopsin expression was markedly increased in cells cocultured with ES-derived RPE cells compared to that in cells that were not cocultured with RPE cells, as shown in Figure 3A. These cells were also positive for other photoreceptor markers, such as red and blue opsin, recoverin, and phosphodiesterase 6 beta on immunofluorescence assay (Figure 3B). We used PCR to evaluate the amount of rhodopsin expression according to the length of the coculture period, and the expression of rhodopsin was increased most after 7 days of coculture. The expression of the early neural marker nestin decreased over time (Figure 3C). Microarray analysis showed the increased expression of markers representative of stem cells, neural differentiation, and retinal photoreceptor cells at each stage of differentiation (Figure 3D). On in vitro functional evaluation of the phototransduction pathways that was performed by measuring the level of cGMP hydrolysis, a decrease in the cGMP level was observed when the ES-derived photoreceptor precursor cells were maintained under light compared to when they were kept in the dark ($p<0.05$). With the addition of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, the difference in cGMP levels under light and dark conditions was abolished (Figure 3E).

Subretinal transplantation of ES-derived cells in a retinal degeneration rat model: When visual function was assessed at 4, 8, 12, and 20 weeks after subretinal transplantation of ES-derived cells, decreased amplitudes of the ERG b-waves were observed in all five groups with no statistical significant difference (Figure 4A). The maximum cycle per degree in which the RCS rats showed an optokinetic response showed a gradual decreasing trend in control group 1, while group 2 showed a decreased response from 4 weeks and throughout, suggesting injection itself inducing some lesions. Response was relatively preserved in groups 3 to 5 compared to the decrease observed in groups 1 and 2 at 20 weeks (Figure 4B). After enucleation at week 1, immunofluorescence staining of the eyes of the RCS rats showed that cells positive for human markers were clumped in the subretinal space and were mixed with cells positive for the photoreceptor markers red and blue opsin. There were similar findings showing positive staining for the same markers in RCS rats euthanized at 8 weeks after injection, suggesting long-term survival of these cells (Figure 4C).
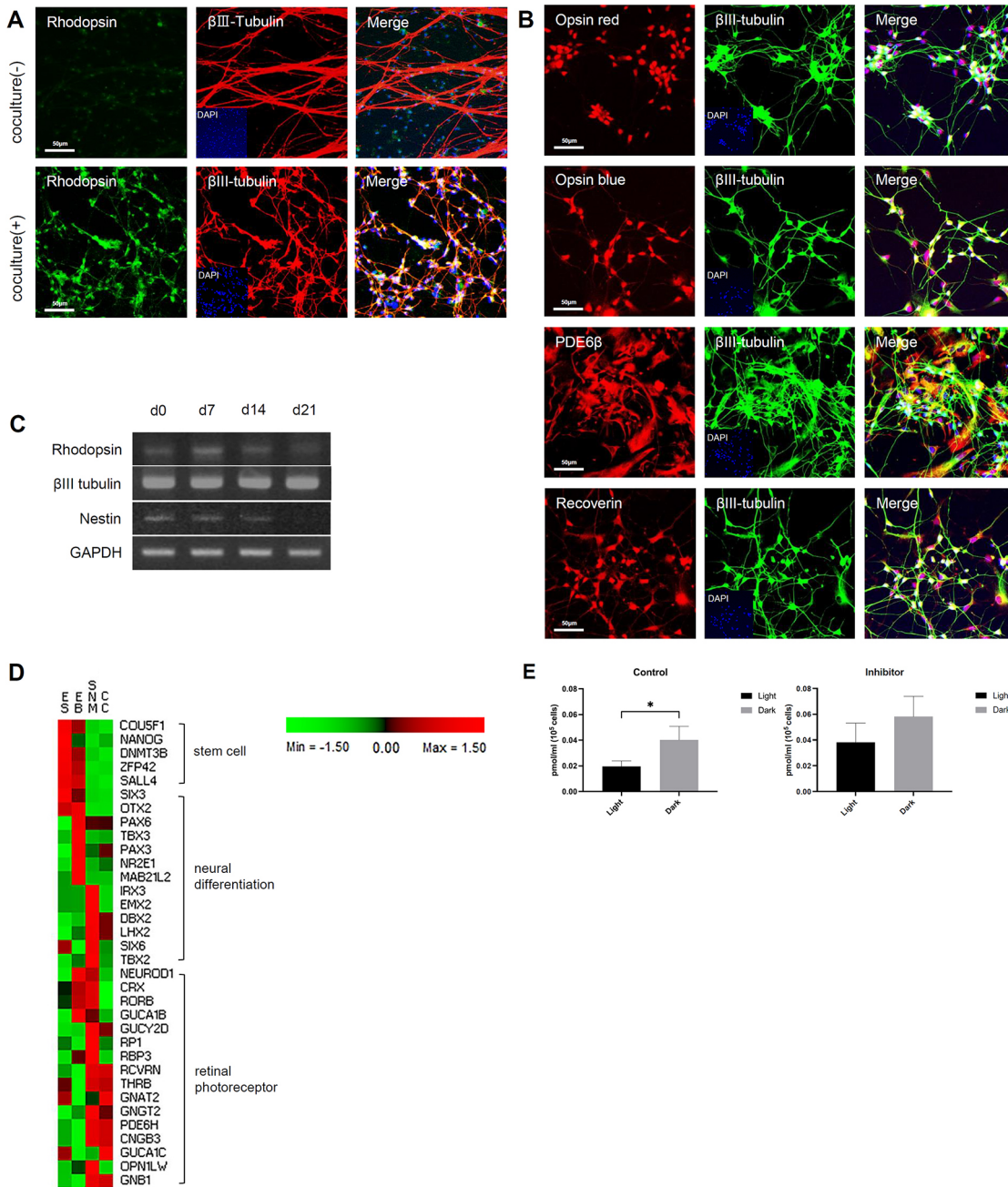
Figure 3. In vitro studies of cocultured cells demonstrating features of photoreceptor precursor cells. **A**: Immunofluorescence staining of human embryonic stem cell (ES)-derived photoreceptor precursor cells expressing rhodopsin (lower row) compared to that of spherical neural mass (SNM)-derived single cells that were not cocultured with human ES-derived RPE cells (upper row). **B**: Expression of red opsin, blue opsin, recoverin, and phosphodiesterase 6 beta (PDE 6B) in human embryonic stem cell–derived photoreceptor precursor cells. **C**: PCR results for the expression of rhodopsin to evaluate the amount of expression according to the coculture period. The expression of rhodopsin was most increased on day 7 of coculture, and the expression of the early neural marker nestin decreased over time. **D**: Microarray heatmap showing the expression of markers representative of different stages of retinal photoreceptor cell differentiation (ES, embryonic body (EB), SNM, and 3 weeks after coculture). **E**: In vitro functional evaluation by measuring the level of cGMP hydrolysis in ES-derived photoreceptor precursor cells kept in the dark or maintained in ambient daylight for 24 h. A decrease in the cGMP level was observed when ES-derived photoreceptor precursor cells were kept in the light condition compared to when they were kept in the dark condition (n=5 in each group, *p<0.05, Mann-Whitney U test). When a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (50 mM), was added 48 h before the determination of cGMP levels, the difference between the cGMP levels in the light and dark conditions was abolished.
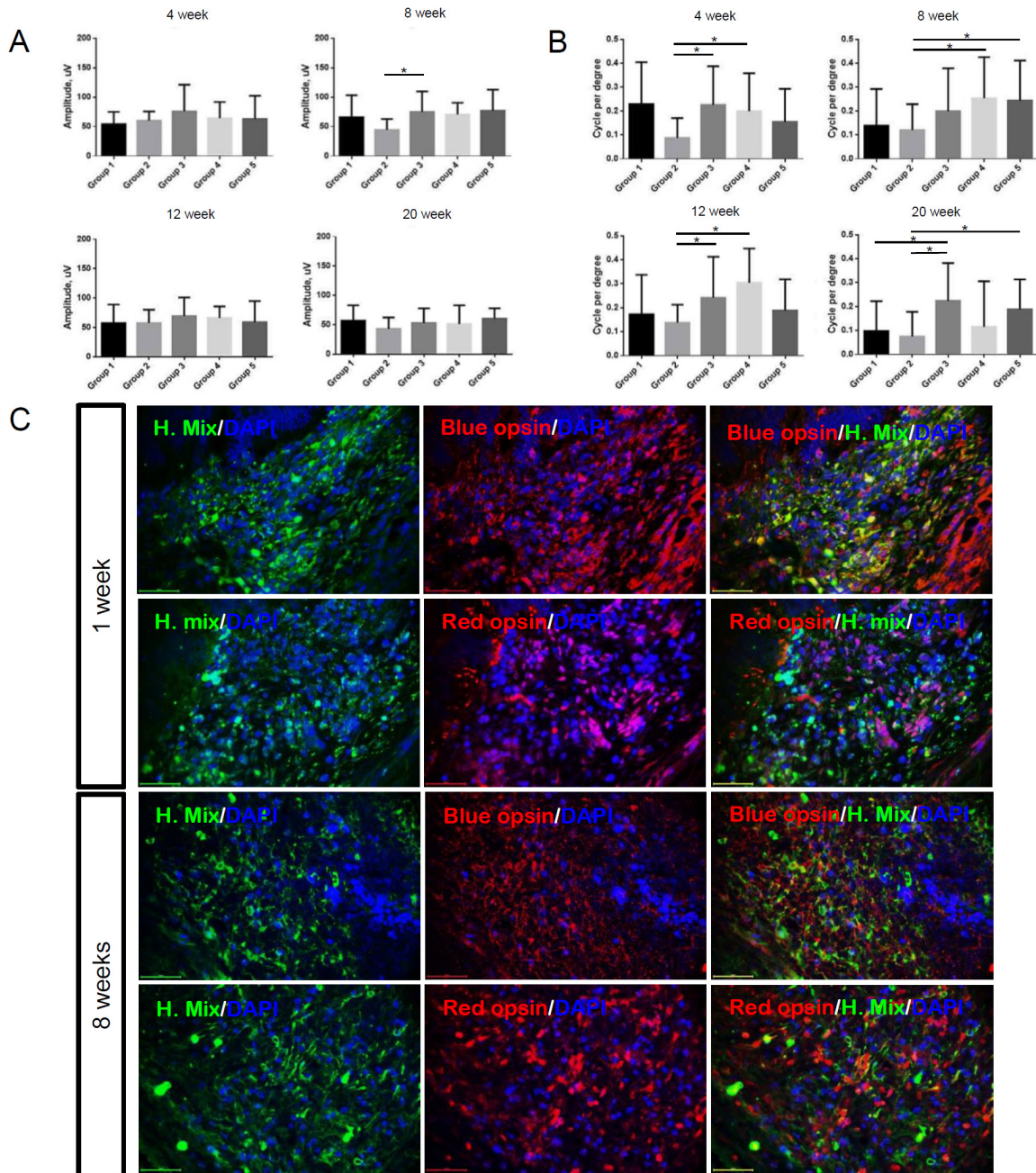
Figure 4. In vivo studies of subretinal injection of cocultured cells in RCS rat animal models. **A**: Amplitudes of dark-adapted ten electroretinogram b-waves from each group of Royal College of Surgeon (RCS) rats at 4, 8, 12, and 20 weeks are shown (n=5 per group). A gradual decrease in amplitude is observed in all groups with no statistically significant difference. Error bars indicate standard deviation, and asterisks (*) indicate statistical significance of p<0.05 (Mann–Whitney U test). **B**: Maximum cycle per degree, by which the RCS rats showed optokinetic responses at 4, 8, 12, and 20 weeks are shown (n=5 per group). Preservation of optokinetic response was observed in group 3 (embryonic-stem-cell (ES)-derived RPE cells), group 4 (ES-derived photoreceptor precursor cells), and group 5 (1:1 mixture of both cells), compared to groups 1 (no injection) and 2 (media only injection), with statistical significance in some groups. Error bars indicate standard deviation, and asterisks (*) indicate statistical significance of p<0.05 (Mann–Whitney U test). **C**: Immunofluorescent staining of the injected cells in the subretinal area in the RCS rats euthanized at 1 week and 8 weeks were positive for anti-human nuclei (H. Mix) and the photoreceptor markers red and blue opsin.

DISCUSSION

Cells with properties consistent with those of retinal photoreceptor precursors were derived from human ES cells using coculture with RPE cells. These cells showed positive staining of markers for photoreceptors, and increased cGMP hydrolysis under light-exposed conditions. When implanted in RCS rats, these cells survived for up to 8 weeks, with preservation of functional response or optokinetic response for up to 20 weeks.

The importance of the microenvironment for stem cell–derived photoreceptor differentiation has been demonstrated by various studies [5,12,13,22]. Direct physical contact between photoreceptors and RPE and RPE-derived secretory factors are considered important for photoreceptor development. Coculture methods for photoreceptor cell differentiation were reported more than a decade ago, while earlier studies used retinal explants [12,13] instead of RPE cells, which were used in the present method. In recent studies, the successful derivation of 3D retinal cups has been reported [7,11,22]. In these methods, RPE cells derived from stem cells also aid the differentiation of cells with photoreceptor properties in the neural retina. Zhong et al. [7] suggested that physical contact with the RPE may not be required, at least at the beginning of functional maturation. In the 3D retinal tissue cultures, RPE cells were clustered at the tips of the retinal cups far away from the photoreceptors, indicating that direct physical contact with the RPE was not required for photoreceptor differentiation. In contrast, Akhtar et al. [23] described the accelerated photoreceptor differentiation of human-induced pluripotent stem cell–derived retinal organoids by contact coculture with primary mouse RPE cells. Their results suggested that conditioned media derived from the RPE did not influence the expression of different photoreceptor markers, which indicated that direct contact between the RPE and retinal organoids was mandatory. In our method, we hypothesized that secretory factors derived from the RPE may induce the differentiation of photoreceptor cells, which we isolated in separate dishes for noncontact coculture. By isolating the cells rather than culturing them as a whole retinal 3D structure, maintenance of the culture was easier compared to that of 3D retinal cultures, which may sometimes grow to a larger size with increasing demand of exogenous factors. Additionally, our method of coculture using separate dishes is more convenient for generating a single cell suspension of each cell type separately for injection into retinal degeneration animal models and for the potential development as a cell therapy for clinical application. As photoreceptor cells are cells with polarity, it may be considered that culturing the whole retina in a 3D culture and implanting the cultured retina as a whole may be more effective; however, intact whole retinal structures may not be necessary to produce an effect on the host retina. The majority of previous studies that cultured the retina in a 3D structure dissociated the cells into a single cell suspension for injection into animal models.

The mechanism of RPE-induced photoreceptor differentiation remains to be clarified in future studies. RPE cells are known to secrete multiple pigment epithelium–derived factors and various other growth factors, such as vascular endothelial growth factor, which are known to play a role in the maintenance of retinal photoreceptors. However, very little is known about their mechanism of action in the differentiation of photoreceptors. We also evaluated RPE conditioned media to induce photoreceptor precursor cells, but these cells showed negative results for expression of photoreceptor markers. A sequential and continuous secretory interaction between the RPE and induced photoreceptor cells is suspected, but future studies are needed to evaluate the exact mechanism. Proteomic or transcriptomic analysis may help to identify the molecular mechanism of how RPE coculture enhances the differentiation and maturation of photoreceptor precursor cells while also contributing to the investigation of photoreceptor development and pathophysiology. Identification of the specific factor that most contributes to photoreceptor differentiation may lead to a further increase in the efficiency of the production of ES-derived photoreceptor precursor cells.

After subretinal injection in retinal degeneration animal models, the injected cells had no effect on preserving the electroretinographic response but had a relatively larger effect on preserving the optokinetic response. This may be due to various causes, including that the degeneration process may have caused diffuse degeneration throughout the retina with a decrease in the electroretinographic response, while focal residual areas adjacent to the injection site may have been positively affected by the injected cells, which contributed in preserving some optokinetic response, as previously reported [24]. Moreover, very little is known about how these cells affect the host retina when they are injected in animal models. There were no eminent interactions observed between the injected cells and the host retina in the histological studies. However, the injected human cells may have a neuroprotective effect rather than an effect by integration and new synaptic connection. In this study, ES-derived photoreceptor precursor cells were injected at only one time point in the RCS rats to evaluate whether the cells could aid in the survival of the retinal cells fated to degenerate. We did not evaluate the effect in the earliest stages or later stages, and further studies should investigate at which time point it may be most

effective to inject the cells in RCS rats. Further studies should be conducted with more sensitive functional study methods to confirm these findings in various other retinal degeneration animal models. Additionally, higher dosages of ES-derived photoreceptor precursor cells, a combination of photoreceptor precursor cells with ES-derived RPE cells at various ratios, or repeated injections may further improve outcomes in future studies.

Noncontact coculture with ES-derived RPE cells induced the differentiation of retinal photoreceptor precursor cells, which was performed with a simple method without any genetic modification or 3D culture. Moreover, minimal supplementation with various exogenous inhibitors or growth factors and the avoidance of transgenic approaches allow easier potential clinical application, as all the supplements used in this method can be modified to use agents free of xeno-derived material. This method also allows possible cryopreservation in certain stages of the culture process, such as the passages of SNMs or ES-derived RPE cells, which aids in scaling production to generate larger amounts for clinical usage. The RPE cells used for coculture can also be potentially substituted with other sources of cells with RPE features, such as adult RPE (ARPE) or cultured human primary RPE cells, to enhance availability and convenience and aid in the adaptation of the method for large-scale production as a cell therapeutic method. However, further studies evaluating such substitutions are required in the future before clinical application.

There are some limitations of this study. The differentiated photoreceptor precursor cells are not identical to adult photoreceptor cells, exhibiting all the features of a mature photoreceptor cell. The differentiated precursor cells comprise a heterogenic population with varying expression of photoreceptor markers, and it is not clear whether the photoreceptor precursor markers observed by immunofluorescent staining and microarray assays are colocalized within the same cells. However, the cells derived from SNMs showed neural differentiation by forming neurites, and no undifferentiated stem cell properties were observed during the culture process. Further in vivo studies are needed to evaluate safety before clinical application of these ES-derived cells for replacement therapy in retinal degenerative diseases.

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