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Subretinal Transplantation of Photoreceptor Precursors and Retinal Pigment Epithelium Derived from Human Embryonic Stem Cells in Retinal Degeneration Rats

망막변성쥐에서 인간배아줄기세포 유래 시각세포 전구체와 망막색소상피세포의 망막하 이식

2015년 8월

서울대학교 대학원
의학과 안과학 전공
박 정현
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지도교수 유형곤

이 논문을 의학박사학위논문으로 제출함

2015년 4월

서울대학교 대학원

의학과

박정현

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2015년 6월

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Abstract

Subretinal Transplantation of Photoreceptor Precursors and Retinal Pigment Epithelium Derived From Human Embryonic Stem Cells in Retinal Degeneration Rats

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Introduction: Degeneration and loss of photoreceptor or retinal pigment epithelium (RPE) is the major pathologic change in retinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration. The transplantation of RPE or photoreceptors derived from stem cells, has been attempted as a possible therapeutic method to regenerate retina and restore lost vision. Successful differentiation of RPE or photoreceptor precursors from human embryonic stem cells or induced pluripotent stem cells has been reported. However, obtaining a sufficient amount of cells still requires a great amount of time, and moreover, the efficacy of differentiation is still low. This study demonstrates a defined, effective method to differentiate photoreceptor precursors and RPEs from human embryonic stem cells with a relatively high efficiency and short incubation time. Furthermore, to determine a therapeutic potential of the derived cells, photoreceptor precursors
and RPEs were characterized and the anatomical and functional changes were evaluated after a subretinal transplantation.

**Methods:** Photoreceptor precursors and RPEs were differentiated from human embryonic stem cells (hESC) via the formation of cell clumps with neural structures, spherical neural masses (SNMs). It took four weeks to form SNM from hESC and additional two weeks to differentiate into photoreceptor precursors, or a week to differentiate into RPEs. The differentiated photoreceptor precursors and RPEs were characterized with immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR). The microstructure on electomicrography and the phagocytic function of RPEs were also evaluated.

After characterization of these hESC-derived retinal cells, the differentiated photoreceptor precursors or RPEs were transplanted into subretinal space of the retinal degeneration rats, Royal College Surgeon (RCS) rats. The RCS rats were divided into three groups, 1) as photoreceptor group of which the eyes were transplanted with photoreceptor precursors (n=25); 2) an RPE group of which the eyes were transplanted with RPEs (n=25); and 3) a control group that was injected only with culture media (n=26). The integration of transplanted cells and occurrences of the tumor were observed for up to 24 weeks after the transplantation. An electroretinogram (ERG) was recorded at 4, 12, and 24 weeks after transplantation and the amplitude of the b-wave was analyzed. A histologic examination was performed at 4 and 24 weeks after transplantation to analyze the thickness of the whole retina, inner nuclear layer, and outer nuclear layer.

**Results:** The differentiated photoreceptor precursors expressed photoreceptor-specific markers such as rhodopsin, recoverin and opsin as well as neural markers,
βIII-tubulin and nestin in immunocytochemistry and RT-PCR. The efficiency of differentiation of the photoreceptor precursors was highest at two weeks after culture of SNM, showing that about 80% of the cells expressed these photoreceptor specific markers.

The differentiated RPE cells showed the typical morphology of RPE, such as a polygonal shape and pigmentation. Transmission electron microscopy revealed apical microvilli, pigmented granules in the cytoplasm and tight junctions between cells. The RPEs also expressed molecular markers of RPE, including MITF, ZO-1, RPE65, and bestrophin. Over 90% of the cells expressed markers for RPE after a week of differentiation from SNM. They also showed phagocytosis of the bovine photoreceptor outer segment.

After subretinal transplantation of photoreceptor precursors or RPEs, the transplanted cells were integrated into the retina and there was no evidence of severe inflammation or tumor formation until 24 weeks of observation. The amplitude of b-wave in the photoreceptor (74.51 ± 27.78 uV) and RPE groups (37.48 ± 13.75 uV) were higher compared with the control group (9.28 ± 1.56 uV) after 4 weeks of transplantation (p=0.018 and 0.043, respectively). There was a tendency that the amplitude of b-wave in the photoreceptor group was higher, compared with the RPE group, although there was no statistical significance. After 12 weeks of transplantation, the amplitude of the b-wave on ERG was slightly decreased in all three groups, but the photoreceptor group and RPE group showed higher amplitude compared to the control group (56.47 ± 20.14 uV, 23±8.07 uV and 9.07 ± 2.10 uV, respectively). At 24 weeks after transplantation, the b-wave amplitude in the photoreceptor group was still higher, compared with the control group (50.33 ±
15.81 uV vs 5.63 ± 1.45 uV, p=0.0058). However, there was no statistical significant difference between the RPE group (22.80 ± 10.16 uV) and control group with the aspect to the b-wave amplitude (p=0.095).

The thickness of outer nuclear layer was also higher in the photoreceptor (14.03 ± 1.48 um, p=0.011) and the RPE groups (12.49 ± 1.20 um, p=0.048) compared with the control group (9.38 ± 0.92 um) at 4 weeks after transplantation. At 24 weeks after transplantation, the thickness of the outer nuclear layer was still higher in the photoreceptor (12.01 ± 1.48 um) or RPE group (9.85 ±1.23 um), compared with the control group (6.95 ± 0.68 um). The thickness of whole retina and inner nuclear layer were not different among groups at 4 weeks and 24 weeks after transplantation.

**Conclusions:** Photoreceptor precursors and RPE cells can be derived effectively in short time via SNMs from hESCs. Subretinal transplantation of photoreceptor precursors or RPE was well tolerated and delayed retinal degeneration, implying possibilities for cell therapy for retinal degenerative diseases.

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**Key words:** photoreceptor, retinal pigment epithelium, retinal degeneration, embryonic stem cell

**Student Number:** 2008-31010
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List of Abbreviations

DAPI : 4',6-diamidino-2-phenylindole
EB : embryoid body
ERG : electroretinogram
bFGF : basic fibroblast growth factor
FITC : fluorescein isothiocyanate
GAPDH : glyceraldehyde-3-phosphate dehydrogenase
hESC : human embryonic stem cells
H&E : Hematoxylin & eosin stain
INL : inner nuclear layer
iPSC : induced pluripotent stem cells
NP : neural progenitor
ONL : outer nuclear layer
PDE : Phosphodiesterase
PBS : phosphate-buffered saline
POS : photoreceptor outer segments
RCS rat : Royal College Surgeon rat
RPE : retinal pigment epithelium
RT-PCR : Reverse transcription-polymerase chain reaction
SNM : spherical neural mass
Introduction

The loss of photoreceptor and/or retinal pigment epithelium (RPE) in retinal degenerative diseases is the leading cause of blindness for which there is no fundamental treatment so far. The retina is a multi-layered innermost part of the eye that absorbs light and then transfers the light signal to brain via optic nerve. The photoreceptors layer is located in the outer retina, and plays a major part in light perception and the photo-transduction. The retinal pigment epithelium (RPE) is placed between the adjacent photoreceptors, and the underlying Bruch’s membrane. The RPE provides both metabolic and functional support of photoreceptors, forming a functional unit with photoreceptors.

Retinitis pigmentosa, the most common inherited retinal degenerative disease, results from primary defect of photoreceptors and then proceeds to the secondary degeneration of RPEs. Age-related macular degeneration, which is primarily attributed to the accumulation of reactive oxygen species and lipid peroxide, can evoke chronic inflammations in the retina and lead to apoptosis of the RPE cells, and finally damages the photoreceptors. In common with age-related macular degeneration, Stargardt’s disease is caused by genetic defect, which leads to disruption of RPEs and secondary dysfunction of photoreceptors. Consequently, in the late stage of retinal degeneration, whatever the cause may be, the degeneration of both photoreceptor and RPE occurs.

Several strategies have been attempted to replace photoreceptor and/or RPE
loss, including electronic artificial retina, gene therapy and stem cell replacement.\(^1\)\(^2\) Although significant developments have been made with electronic implants in recent years, there will still need to be an interface between the electronic hardware and biological tissues at some point along the visual pathway and this will be highly complex.\(^3\) Gene therapy has also achieved some visual improvement in animal models and in human subjects; however, it is confined to some specific inherited retinal degenerative diseases that are caused by single gene mutation.\(^4\)\(^5\)

The potential of cell-based therapy to rescue degenerated retinal cells has attracted considerable interest. Several studies have shown that stem cells, including embryonic or fetal retinal progenitors, neural stem cells and induced pluripotent stem cells (iPSCs), could be differentiated into precursors of specific types of retinal cells, such as photoreceptor precursors or RPEs.\(^6\)

In 2006, Banin et al. reported that hESC - derived neural precursors express markers of retinal progenitors and photoreceptor development. After transplantation into rat eyes, the neural progenitors survived for 16 weeks, migrated large distances, and integrated into the host retina without formation of teratomas. The expression of photoreceptor markers (e.g. rhodopsin, blue cone opsin, and neural retina leucine zipper transcription factor) was observed in subretinal grafts.\(^7\) Lamba et al. also differentiated the hESC into the retinal neurons using a combination of noggin, dkk1 and IGF-1 that promoted neural differentiation.\(^8\) However, the generation of photoreceptors from these progenitors
has remained inefficient unless the cells are co-cultured with retinal tissues. The retinal tissue provides favorable environment for stem cells to differentiate into retinal neurons. In contrast, Osakada et al. reported the induction of RPE and photoreceptors from ESCs in the absence of co-culture with retinal tissues, in a defined condition in 2008. Without co-culture with retinal tissues, there was no risk of transmittable diseases and no need for supply retinal tissues. However, this method required long differentiation time and was not efficient as expected. Several publications subsequently have shown that it is possible to derive hESCs and iPSCs toward photoreceptors using various approaches. However, photoreceptor differentiation protocols remain variable and require extensive culture periods.

Differentiation of RPE from hESC was first reported by Klimanskaya in 2004. In the same year, Haruta demonstrated the generation of RPE derived from primate ESC, indicating functional recovery and biological function of phagocytosis. The hESC-RPE not only displays consistent structure with adult RPE, but also exhibits resemblances, as the ability to phagocytose photoreceptor outer segments. The hESC-RPE in animals improved functional performance, after comparing with controls. Other investigators modified culture conditions to derive RPE thereafter. They also transplanted differentiated cells at different differentiating step to the animal models in order to confirm cell survival and RPE markers expression.

Several groups have transplanted photoreceptor precursors or RPEs into animal models of retinal degenerative diseases. The restorative potential of
RPEs derived from hESCs or iPSCs has demonstrated, and some early results of clinical trial have been reported. A few studies have shown that hESC- and iPSC-derived retinal cell grafts can express photoreceptor markers and integrate within the outer nuclear layer in intact newborn and adult retina. Recent advances on hESCs or iPSCs for differentiating RPE or photoreceptor precursors have increased the therapeutic possibilities for retinal degenerative diseases. However, obtaining a sufficient amount of cells still requires a great amount of time, and, the efficacy of differentiation is still low. Moreover, it still remains debatable whether transplantation of photoreceptor precursors or RPE into the degenerated retina can actually improve visual function.

This study demonstrates a defined, effective method to differentiate photoreceptor precursors and RPEs from human embryonic stem cells with a relatively high efficiency and short incubation time. Furthermore, to determine a therapeutic potential of the derived cells, photoreceptor precursors and RPEs were characterized and the anatomical and functional changes were evaluated after a subretinal transplantation.
Materials and Methods

Differentiation of photoreceptor precursors from human embryonic stem cells

The hESC line SNUhES3 was maintained as described previously. The procedure for photoreceptor precursors and RPE differentiation is illustrated in Fig. 1. For the embryoid body (EB) formation, undifferentiated hESC colonies were detached mechanically and cultured in a bacterial dish for 7 days. Clumps of neural progenitor cells, which is called spherical neural masses (SNMs), were formed from EBs, as described previously. In brief, EBs were cultured in neural progenitor (NP) selection media (modified EB medium containing a 0.5% N2 supplement (R&D systems, Minneapolis, MN, USA) without serum replacement) for 5 days, and the neural progenitors were then expanded by continuously culturing the cells for another 4 days in an expansion medium including 20 ng/ml bFGF (Invitrogen, Carlsbad, CA, USA) and a 1% N2 supplement. Neural rosettes and neural tube-like structures observed during the neural expansion culture were mechanically isolated and cultured onto bacterial culture dishes containing the NP expansion medium for another seven days to form clumps of neural progenitor cells, SNMs. For passaging, the SNMs were mechanically fragmented into four to six pieces and expanded for 7–10 days. During early passaging (passages 1–4) of the SNMs, some SNMs containing opened neural tube-like structures or cystic structures were removed. These
SNMs containing cystic structures were differentiated into RPE cells, which will be described later. The pure SNMs were used for differentiation into photoreceptor precursor cells. For the differentiation of pure SNMs into photoreceptor precursor cells, properly fragmented (~40-80 pieces) spheres were cultured onto Matrigel-coated (BD, Franklin Lakes, NJ, USA) culture dishes in a differentiation medium containing B27 supplement (Invitrogen) for 14 days.

**Differentiation of RPEs from human embryonic stem cells**

During early passaging (passages 1–4) of the SNMs, some neural tube-like structures form cystic portions, of which the outer part showed pigmentation after separation by mechanical dissection. These cystic structures were isolated mechanically and cultured on a monolayer on Matrigel coated culture dishes for 1 week to 4 weeks until the cells proliferate confluent monolayers. The basic fibroblast growth factor (bFGF) was replaced by B27 (2%) from the neural progenitor expansion medium for RPE differentiation and expansion. The techniques for differentiating photoreceptor precursors and RPEs are shown in Figure 1.
Figure 1. Schematic diagram of differentiation of hESCs into photoreceptor precursors and RPE cells. It usually takes about 14 days to generate photoreceptor precursors or RPE cells from SNMs. After serial differentiation of hESCs into SNMs, the cystic SNMs were selected and differentiated into RPE cells, whereas non-cystic SNMs differentiated into photoreceptor precursors. hESC, human embryonic stem cell; RPE, retinal pigment epithelium; and SNM, spherical neural masses.
Characterization of Photoreceptor Precursors

Immunocytochemistry

The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. After blocking overnight in 3% bovine serum albumin solution in PBS containing 0.1% Triton X100, the samples were incubated with primary antibodies overnight. The following primary antibodies were used for immunocytochemistry: anti-human rhodopsin (1:100), anti-recoverin (1:100), anti-Rx (1:100) and anti-Crx(1:100).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from photoreceptor precursors using TRIzol Reagent (Invitrogen), and cDNA synthesis was conducted using AMV reverse transcriptase (RT) and oligo-dT as a primer in accordance with the manufacturer's instructions (AccuPower RT PreMix; Bioneer, Daejeon, Korea).

PCR amplification was performed with Taq Polymerase (HiPi Plus 5× PCR Premix; Elpis Biotech, Daejeon, Korea) using a standard procedure. To analyze the relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The PCR conditions were optimized, and a linear amplification range was determined for each pair of primers by varying the annealing temperature and cycle numbers. The primer sets that were used can be seen in Table 1.
Table 1. Sequence of primers used in RT-PCR

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>forward</td>
<td>CAATGGAAATCCCATCACCA</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ATGATGT TTCTGGAGAGCCCC</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>forward</td>
<td>CACAGGATGCAATTTGGAGG</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ATCTTTCTCGGCTCGCTTTTC</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>forward</td>
<td>CCTTCACCCAAGTGTCGG</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>AGAGGGCTTCATTGTCGATG</td>
</tr>
<tr>
<td>Nestin</td>
<td>forward</td>
<td>CCAAGAGAAGCCTGGGAACT</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
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</tr>
<tr>
<td>PAX6</td>
<td>forward</td>
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<tr>
<td></td>
<td>reverse</td>
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</tr>
<tr>
<td>Mitf</td>
<td>forward</td>
<td>CCATAAACGTCAGTGTCGCC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ATCTGCATAACGGACGCTCG</td>
</tr>
<tr>
<td>RPE65</td>
<td>forward</td>
<td>TACAGAAAGCACTGAGTTGAGC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCATT TAGTAAAGTCCACATT CATTITCC</td>
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<tr>
<td>Bestrophin</td>
<td>forward</td>
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<tr>
<td></td>
<td>reverse</td>
<td>AGTGCCGTGTTCA GTTTCTAATC</td>
</tr>
</tbody>
</table>
**In vitro phagocytosis assays**

The phagocytosis of photoreceptor outer segments (POS) is one of the most important functions of RPE. The POS are digested by RPE and recycled and returned to photoreceptors to rebuild light-sensitive outer segments from the base of the photoreceptors. To investigate the ability of the phagocytosis of hESC derived RPE, the cells was incubated with FITC-labeled POS, which was isolated from bovine retina.

Phagocytosis assays were performed using bovine photoreceptor outer segments (POS). The retinas were removed from fresh bovine eyes that were obtained from a local slaughterhouse. Under dim red light, the retinas were placed in a 10 ml homogenization solution containing 20% (wt/vol) sucrose, 20 mM Tris HCl (pH 7.4), 10 mM glucose, 5 mM taurine, and 2 mM MgCl₂. The retina suspension was then layered onto a five-step sucrose gradient (27%–60% wt/vol) containing 20 mM Tris HCl, 10 mM glucose, and 10 mM taurine and then centrifuged at 25,000 rpm for 1 h at 4 °C with an ultracentrifuge (XL-90, Beckman, Palo Alto, CA). The purified bovine POS were labeled using the FluoReporter FITC protein labeling kit (Invitrogen) in accordance with the manufacturer's protocol. The FITC-labeled POS were washed, re-suspended in cell culture medium, and incubated with RPE cells at 37 °C in 5% CO₂. After a 4-hour incubation, surface-bound POS were removed by vigorous washing with PBS, and then Trypan Blue (0.4%) was added to quench extracellular fluorescence. After fixation and blocking, the cells were stained with an anti-ZO-
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Antibody (1:200). Alexa Fluor® 594 donkey anti-rabbit IgG was used to detect the primary antibody, and the cell nuclei were counterstained with DAPI. The assessment of intracellular POS uptake was performed with confocal microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany). To evaluate the internalization of POS, the cells were examined via a y-axis projection through the cells.

**Animals and Subretinal transplantation of differentiated retinal cells**

Royal College of Surgeons (RCS) rats at 21 days old were used in this study. Rats were supplied by the National BioResource Project of Japan. All procedures of the animal experiment were approved by the Institutional Review Board and Institutional Animal Care and Use Committee of Seoul National University Hospital Clinical Research Institute.

The Royal College of Surgeons (RCS) rat is a widely used animal model of inherited retinal degeneration. The genetic mutation in the *MERTK* gene results in defective function of cells in the retinal pigment epithelium (RPE), including the failure to phagocytize the rod outer segments. These rats experience progressive retinal degeneration and vision loss. The response of ERG and retinal morphology is nearly normal at birth and the changes of retinal morphology and visual function can be measured over time. Morphological changes in photoreceptor outer segments appear as early as postnatal day (P) 16, and only scattered photoreceptor cells remain by P105. These rats also experience
progressive decrease of retinal function, which can be measured by ERG. The ERG response is nearly normal at P21-25 in RCS rats; however, it shows no response or negative ERG at 12 weeks (P84) after birth.

Rats were randomly assigned into three groups: photoreceptor group (rats with transplantation of photoreceptor precursors, n=25), RPE group (rats with transplantation of differentiated RPE cells, n=25), and control group (rats with a subretinal injection of only culture media, n=26). In all groups, the right eyes (OD) were the treatment eyes, whereas the left eyes (OS) served as internal control and remained untreated.

Twenty-five rats that were successfully transplanted with photoreceptor precursors, of these, 22 rats were used to record the ERG response at 4 weeks after transplantation, whereas the ERG response could not be measured for three rats because of corneal opacity or noising during the ERG measurement. In the RPE or control groups, the ERG response could not be measured for two rats from each group at 4 weeks after transplantation. Consequently, the numbers of rats that were used for the ERG measurement at 4 weeks after transplantation were 22, 23, and 24 rats in the photoreceptor precursor group, RPE group and control group, respectively. At 4 weeks after transplantation, 12, 11, and 14 rats from each respective group (above ordinality) were selected randomly and sacrificed for histologic examination. At 12 weeks after transplantation, 13, 13 and 11 rats from each respective group (above) were available for ERG measurement except two rats (one from the RPE group and one from the control
group) that had corneal opacity. At the twenty-fourth week, the ERG response could not be measured for four rats from the photoreceptor group and three rats from the RPE group because of corneal opacity, thus, the rats, undergoing ERG at the twenty-fourth week, were 9, 10, and 11 in each respective group (above ordinality).

The rats were anesthetized with an intraperitoneal injection, with a mixture of Xylazine (10 mg/kg) and Zoletil (20 mg/kg). After the rats were anesthetized, their corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride (Alcaine; Alcon-Couvreur, Puurs, Belgium) and pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P, Santen, Osaka, Japan).

Before injection of cell suspension into subretinal space, the cornea was punctured to reduce the intraocular pressure and to limit the efflux of cells. After incision of conjunctiva at the superior temporal pole to expose the sclera, the sclera and choroid were penetrated by a 30-gauge syringe needle. A 2 μl cell suspension (approximately $1 \times 10^5$ cells) was slowly injected into the subretinal space using a stainless steel needle (33-gauge) connected to a Hamilton microsyringe (Hamilton, Reno, NV). The cell suspensions were injected slowly to produce a retinal detachment in the supero-temporal quadrant around the injection sites. The needle was allowed to remain in place for a few seconds and was then very slowly withdrawn to minimize efflux of the transplanted cell suspension. Immediately after injection, the fundus was examined to check for retinal damage or signs of vascular distress. Any animal that showed such
problems was removed from the study and is not included in the results.

To suppress immune activation, all animals were maintained on cyclosporine-A (Cipol N, Chong Kun Dang, Seoul, Korea) that was administered in the drinking water (210 mg/l) from 1 to 2 days prior to the cell injection until the animals were euthanized.\textsuperscript{25}

**Recording of electroretinogram (ERG)**

Full-field ERGs were recorded on both eyes for each rat at 4, 12 and 24 weeks after subretinal transplantation. Animals were dark-adapted over 6 hours and prepared for recording under dim red light. After being anesthetized, the eyes were dilated with tropicamide 5\% and topically anesthetized as the same manner as the transplantation.

The rat is laid prone with its face fully inserted into the light stimulus dome of a Handheld Multispecies electroretinogram unit (HMsERG system; OcuScience LLC, Rolla, MO, USA). A silver wire loop was placed directly on the center of the rat cornea after application of methylcellulose gel (GenTeal Gel, Excelvision AG, Hettlingen, Germany) and then contact lenses were placed on the cornea to prevent drying of cornea and the electrodes from moving or detaching. Subcutaneous needle electrodes in the forehead and thigh served as the reference and ground electrodes.
For later analysis, signals obtained from the corneal surface were amplified, digitized, averaged, and stored using commercial software (ERGView 4.380R; OcuScience LLC). A 50 Hz-filtering of the data was applied before evaluating the amplitude and latency of the a- and b-waves. The implicit times of the a- and b-waves were measured from the onset of stimuli to the peak of each wave. The a-wave amplitude was measured from the baseline to its trough, and the b-wave amplitude was determined from the trough of the a-wave to the peak of the b-wave.

**Histology of Retinal Cross-Sections**

Four weeks after transplantation, subsets of the each group were sacrificed and the eyes enucleated. The supero-temporal side of each eye was marked before enucleation. After removing the contents, including cornea, lens, and vitreous, the remaining parts of the eye were fixed in 4% paraformaldehyde in 100 mM PBS, pH 7.3, overnight at 4°C, before cryoprotection in sucrose and being mounted in an optimal counting temperature compound (Tissue-Tek® OCT; Sakura, Alphen aan den Rijn, The Netherlands). Retinal cross-sections were cut on a microtome (Thermo Fisher Scientific, Walldorf, Germany) in 4-μm thick sections.

To investigate structural changes as well as changes in the thickness of the retinal layers, hematoxylin & eosin stain (H&E) was used. Three H&E stained retina sections of each eye were photographed at a distance of 1000 μm dorsal
and ventral to the optic nerve with a microscope equipped with a charge-coupled device (CCD) camera (Axio Imager M1; Carl Zeiss Microscopy, Oberkochen, Germany). The images were analyzed with the measuring tool of image analysis software (ZEN 2011, v. 1.0.1.0.; Carl Zeiss Microscopy). There was a measurement of the thickness of the whole retina (excluding the outer segments), inner nuclear layer (INL), and outer nuclear layer (ONL). For each layer, an average of three measurements per photo was calculated.

**Statistical analysis**

Differences among the experimental groups were analyzed with a $t$-test, using GraphPad Prism version 6.0 software (GraphPad, SanDiego, CA).
Results

Characterization of differentiated photoreceptor progenitors

In an early stage, the cultured cells expressed antigens specific to photoreceptor progenitors (Rx and Crx). After two weeks, these cells expressed photoreceptor specific antigens such as rhodopsin, opsin and recoverin as well as retinal progenitor markers (PDE6-β), while also expressing antigens specific for neural precursors such as nestin and β3- tubulin (Figure 2). Over 80% of the cells expressed these photoreceptor markers after 2 weeks of culture of SNMs.

The mRNA expression of rhodopsin, which has a key role in phototransduction of photoreceptors, as well as nestin and beta-III tubulin, the specific markers of neural progenitor cells, were detected in hESC-derived photoreceptor precursors. The expression of rhodopsin was highest at 2 weeks after culture and decreased after 2 weeks (Figures 2 and 3).
Figure 2. Immunostaining of the hESC-derived photoreceptor precursors.

Expression of the molecular markers of photoreceptor or photoreceptor precursors. The expression of rhodopsin, βIII-tubulin, opsin, recoverin and PDE6-β in cultured cells after 2 weeks of culture from spherical neural masses.
Figure 3. RT-PCR of the hESC-derived photoreceptor precursors. The mRNA expression of rhodopsin, nestin and β-III tubulin was detected via RT-PCR in the cultured photoreceptor precursors. The expression of rhodopsin was highest at 2 weeks after culture and decreased after 2 weeks.
Characterization of differentiated RPEs

To confirm RPE differentiation, cultured monolayer cells from SNM-derived cystic vesicles were characterized. The expression of specific molecules associated with the cellular function of the mature RPE was investigated by RT-PCR and immunocytochemistry. The mRNA expression of Pax6, MITF, RPE65 and bestrophin were specifically detected in hESC-derived RPE cells. Immunostaining also showed the expression of the early RPE cell markers MITF as well as the mature RPE cell markers bestrophin and RPE65. Most of the cells (90%) of cystic vesicles expressed these RPE markers after monolayer culture for a week.

The electron micrographs of cells showed features characteristic of mature RPE. A transmission electron microscope showed pigmented cuboidal epithelial cells with apical microvilli, melanin granules, and tight junctions between cells in the apical portion of the cells (Figure 3).

The differentiated RPE cells expressed phagocytic activity. Confocal microscopy of cells immunostained for the RPE cell surface marker ZO-1 showed that differentiated RPE cells internalized the FITC-labeled POS (Figures 4 to 7).
Figure 4. Confirmation of the hESC-derived RPE cells with expression of the RPE-specific markers by immunocytochemistry.

Figure 5. Images of transmission electron microscope of RPE demonstrate cuboidal epithelial cells with melanin granules, apical microvilli, and tight junctions (arrow).
Figure 6. Phagocytosis of the differentiated RPE cells. A confocal microscopic exam showed the internalization of FITC-labeled photoreceptor outer segments.
Figure 7. RT-PCR of the RPE cells differentiated from hESC. The differentiated cells express the molecular markers of functional RPE cells.
Survival and localization of the photoreceptor progenitors after subretinal implantation

Grafts consisting of cultured retinal precursor cells can be detected by fluorescence in the subretinal space at 12 weeks after transplantation (Figure 8). The transplanted cells were well integrated into the subretinal space without disturbing the laminar organization of the host retina. At the end of the study, none of the retinal precursor cell-transplanted retinas showed evidence of uncontrolled cell proliferation and tumor formation. There was no evidence of severe inflammation or tumor formation until 6 months after transplantation.

Figure 8. Expression of human antigens in the retina 12 weeks after the transplantation of photoreceptor precursors. The transplanted cells were tagged with DAPI before transplantation and integrated in the retina.
Retinal precursor cells suppressed ONL thinning in RCS rats

To evaluate the effect of retinal precursor cells on RCS rats, the change of ONL thickness was measured in six areas on the 28th day (P49) after the subretinal transplantation. The RCS rats undergo progressive retinal degeneration in the early postnatal periods. The photoreceptor degeneration begins around P20, and by P60, there were few photoreceptor nuclei remaining in the outer nuclear layer.\textsuperscript{26}

The eyes in photoreceptor–transplanted or RPE-transplanted rats showed several layers of cells in the outer nuclear layer of retina (Figures 5A and B), whereas eyes in the control group had only 1-2 layers or sparsely distributed photoreceptor cell bodies in the outer nuclear layer at P49 (Figure 5C) appearing similar to the retinas of age-matched untreated RCS rats.

A statistical analysis of ONL thickness demonstrated that eyes with the transplantation of photoreceptor or RPE had significant preservation of the ONL, compared with the control group at P49. The thickness of the outer nuclear layer was also higher in the photoreceptor (14.03 ± 1.48 um, p=0.011) and RPE groups (12.49 ± 1.20 um, p=0.048) compared with the control group (9.38 ± 0.92 um) after 4 weeks. At 24 weeks after transplantation, the thickness of the outer nuclear layer was still higher in the photoreceptor or RPE group compared to the control group. (12.01 ± 1.48, 9.85 ±1.23, 6.95 ± 0.68, respectively, Table 2).
thickness of the whole retina and inner nuclear layer was not different between groups at 4 weeks and 24 weeks after transplantation.

Figure 9. Retinal tissue sections stained with hematoxylin and eosin (H&E) at 4 weeks after transplantation of photoreceptor precursors (A), retinal pigment epithelium (RPE) (B) and conditioned media only (C). After transplantation of photoreceptor precursors or RPE, the retinal degeneration of RCS rats was suppressed and the outer nuclear layer was relative preserved.
Table 2. Thickness of retinal layers at 4 and 24 weeks after subretinal transplantation

<table>
<thead>
<tr>
<th></th>
<th>Photoreceptor precursor</th>
<th>RPE</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole retina thickness (um)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>33.28 ± 1.73* (n=12)</td>
<td>31.64 ± 1.83 (n=11)</td>
<td>28.36 ± 0.94 (n=14)</td>
</tr>
<tr>
<td>24 weeks</td>
<td>30.04 ± 1.68, (n=10)</td>
<td>29.70 ± 1.61 (n=10)</td>
<td>27.33 ± 0.99 (n=12)</td>
</tr>
<tr>
<td><strong>INL thickness (um)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>18.33 ± 1.16 (n=12)</td>
<td>19.64 ± 1.11 (n=11)</td>
<td>18.93 ± 1.05 (n=14)</td>
</tr>
<tr>
<td>24 weeks</td>
<td>18.90 ± 1.32 (n=10)</td>
<td>19.90 ± 1.25 (n=10)</td>
<td>18.00 ± 0.90 (n=12)</td>
</tr>
<tr>
<td><strong>ONL thickness (um)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>14.03 ± 1.48* (n=12)</td>
<td>12.49 ± 1.20* (n=11)</td>
<td>9.38 ± 0.92 (n=14)</td>
</tr>
<tr>
<td>24 weeks</td>
<td>12.01 ± 1.48* (n=10)</td>
<td>9.850 ±1.23* (n=10)</td>
<td>6.95 ± 0.68 (n=12)</td>
</tr>
</tbody>
</table>

Abbreviations: RPE, retinal pigment epithelium; INL, inner nuclear layer; ONL, outer nuclear layer. Data presented as means±SEM. *P<0.05 when compared with the control group.
Figure 10. Changes of outer nuclear layer thickness at 4 and 24 weeks after subretinal transplantation of photoreceptor precursors or RPEs. Although the thickness of outer nuclear layer was decreased over time, the outer nuclear layer was thicker in photoreceptor precursors or RPE transplanted eyes than in control eyes. Data presented as means±SEM. *P<0.05 when compared with the control group.
Subretinal transplantation of photoreceptor precursors improved ERG in RCS rats

An electroretinogram was recorded at 4, 12 and 24 weeks after transplantation. The findings showed that subretinal transplantation of photoreceptor precursors or RPEs in RCS rats on the 28th day significantly minimized the reduction of the amplitudes of b-wave, compared with the control group and contralateral eyes in treated rats. The RCS rats showed significantly reduced b-wave and the responses are hardly detectable after P49 in the control group. The response of the electroretinogram on RCS rats was decreased and hardly detectable in the control group. In contrast, the amplitude of the b-wave in the photoreceptor (74.51 ± 27.78uV, p=0.018) and RPE groups (37.48 ± 13.75 uV, p=0.043) were higher, compared with the control group (9.28 ± 1.56 uV) after 4 weeks of transplantation. There was a tendency that the amplitude of the b-wave in the photoreceptor group was higher compared with the RPE group, although there was no statistical significance. After 12 weeks of transplantation, the amplitude of the b-wave on ERG was slightly decreased, but the photoreceptor group and RPE group showed higher amplitude, compared with the control group (56.47 ± 20.14, 3.23±8.07 and 9.07 ± 2.10, respectively). At 24 weeks after transplantation, the b-wave amplitude in the photoreceptor group was still higher, compared with the control group (50.33 ± 15.81 uV vs 5.63 ± 1.45 uV, p=0.0058). However, there was no statistical significant difference between the RPE group
(22.80 ± 10.16 uV) and control group in the b-wave amplitude (p=0.095).

No significant differences in the a-wave amplitude and implicit time of the a- or b-wave were found among the 4 groups.

Table 3. Amplitude of b-wave in electroretinogram

<table>
<thead>
<tr>
<th>b-wave amplitude (uV)</th>
<th>Photoreceptor precursor</th>
<th>RPE</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>74.51 ± 27.78*</td>
<td>37.48 ± 13.75*</td>
<td>9.28 ± 1.56,</td>
</tr>
<tr>
<td></td>
<td>(n=22)</td>
<td>(n=23)</td>
<td>(n=24)</td>
</tr>
<tr>
<td>12 weeks</td>
<td>56.47 ± 20.14*</td>
<td>30.23 ±8.07*</td>
<td>9.07 ± 2.10</td>
</tr>
<tr>
<td></td>
<td>(n=13)</td>
<td>(n=13)</td>
<td>(n=11)</td>
</tr>
<tr>
<td>24 weeks</td>
<td>50.33 ± 15.81*</td>
<td>22.80 ± 10.16</td>
<td>5.63 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td>(n=10)</td>
<td>(n=11)</td>
</tr>
</tbody>
</table>

Abbreviation: RPE, retinal pigment epithelium. Data presented as means±SEM, *P<0.05 when compared with the control group.
Figure 11. Changes of b-wave amplitude of ERG at 4, 12 and 24 weeks after the subretinal transplantation of photoreceptor precursors or RPEs. In the eyes transplanted with photoreceptor precursors or RPEs, the amplitude of b-wave was relatively preserved until 24 weeks compared with the eyes of the control group. Data presented as means±SEM. *P<0.05 when compared with the control group.
Discussions

This study showed that photoreceptor progenitors and RPEs can be efficiently derived from hESCs, and that visual function and retinal morphology were dramatically preserved up to 24 weeks following the subretinal transplantation of these derived retinal cells in the RCS rats. The preservation of visual function and retinal morphology was more definite following the transplantation of photoreceptor precursors than RPEs.

Retinal degenerations are one of the leading causes of blindness, and for which there is no effective treatment. The mammalian retina cannot be replaced following degeneration, like most other neural organs. The lack of regeneration in the retina has stimulated studies to explore the possibility of neuronal replacement with stem cells in order to restore retinal function following degeneration.

In the past several years, various potential sources for the replacement RPEs and photoreceptor cells have been analyzed. These include intact sheets of embryonic retina;\textsuperscript{27} dissociated retinal cells;\textsuperscript{28} multipotent retinal progenitor cells;\textsuperscript{29} bone marrow-derived cells;\textsuperscript{30} cells derived from the pigmented ciliary epithelium;\textsuperscript{31} hESCs,\textsuperscript{10} and iPSCs.\textsuperscript{32}

Human embryonic stem cells have been envisaged as an important source to generate RPE and photoreceptor precursors in sufficient amount for transplantation purposes. The induced pluripotent stem cells (iPSCs) also can
produce sufficient amount of cells. They have advantages in the aspect of that the somatic cells from the patients can be used and they have no ethical issues in their use. However, iPSCs may have mutations accumulated during the reprogramming process that will cause activation of oncogenes. Another disadvantage of iPSCs is that they may retain “epigenetic memories” of the tissue of origin that may influence differentiation of desired cells.  

Differentiation methods of RPE from human ESCs are based on either spontaneous or defined culture procedures. In order to induce spontaneous differentiation, the ESCs were grown to confluence in the absence of bFGF, and RPE appeared spontaneously if the cells were continuously grown for six to nine months. The ESCs were first grown as embryoid bodies and then plated on dishes coated either with laminin and fibronectin or with gelatin until they formed visible pigmented colonies. The pigmented cells expressed RPE-specific proteins, formed tight junctions and showed apical-basal polarity. Although researchers could derive RPE from hESC using these spontaneous differentiation processes, it was extremely inefficient and time-consuming, with an efficiency of ~1% after 1–2 months in culture. A defined differentiation method has been developed and resulted in more efficient differentiation of RPE. In 2008, Osakada et al. reported that with Wnt and Nodal antagonists, pigmented cells were shown within 38% of the hESC colonies after 8 weeks. Idelson et al. reported that directed differentiation under defined culture conditions, including
supplementation with nicotinamide and activin A, increase the efficiency of RPE differentiation to 51% of cell clusters contained pigmented areas after 6 weeks of differentiation. The highest reported efficiency of RPE generation was obtained by exposing pluripotent cells to bFGF, Noggin, retinoic acid, and Shh, which was upto 60% of Mitf+ cells after 60 days of differentiation. Recently, Leach et al derived RPE with an efficiency of 80% based on Pmel17 expression from hESC with the combined use of the retinal inducing factors as early as 14 days after differentiation. Induced pluripotent stem cells can also provide an unlimited source for RPE. Protocols for RPE differentiation of ESCs were also successfully applied to murine and human iPSCs, showing their equivalence to ESCs. However, these methods still needs complicate steps of applying various kinds of deriving factors at each differentiation stages. In the present study, the cells from the cystic portion of SNMs showed pigmentation one week after separation from SNMs (about 5 weeks from hESC) and over 90% of the cells expressed RPE markers. This method provides a faster and more simplified protocol for deriving RPE from easily storable and expandable cell mass, SNM.

In several pre-clinical studies, significant vision rescue was achieved in RCS rats after transplantation of RPEs derived from ESCs, iPSCs or other neural stem cells. Retinal pigment epithelial cells from hESCs are undergoing clinical trials in patients with age-related macular degeneration or Stargardt’s diseases, and there has been a report of visual improvement and no safety concerns for up to 22 months.
In contrast to RPEs, photoreceptor differentiation has shown low efficiency and required extensive culture periods in some cases. Lamba et al. has shown that the treatment of hESCs with B27, Dkk1, noggin, IGF-1 and subsequently with N2 and FGF can result in more than 80% of cells expressing retinal progenitor markers. However, although 12% of the population expressed Crx, less than 0.01% was found to be positive for mature photoreceptor markers. Osakada et al. achieved Crx expression in 11% of the population following 120 days of differentiation, which increased to around 20% by 170 days, with 5% of cells expressing rhodopsin by day 150. Mellough et al. generated 16% of Crx\textsuperscript{+} cells and 52% of cone-like photoreceptor cells within 45 days of differentiation.\textsuperscript{40} In 2013, Yanai et al. generate photoreceptor precursors expressing CRX\textsuperscript{+} with an efficiency of 78% 17 days after differentiation using size-controlled embryoid bodies and supplementation of T3 and taurine. This method showed more efficient and faster way for differentiating photoreceptor and comparable to the protocols in this study using SNM. The method demonstrated in this study showed that more than 80% of cells expressed photoreceptor precursor markers after 2 weeks of culture from SNMs (6 weeks from hESCs), which can induce photoreceptor precursor in a large amount in a relatively short time.

The previous studies which provided methods for direction differentiation of human pluripotent stem cells to RPE and photoreceptor precursors are described in Tables 5 and 6.
Recent success, with photoreceptor transplantation from neonatal mouse retina, highlights the fact that the mature retina can incorporate new photoreceptors into preexisting circuitry.\textsuperscript{41} Studies with photoreceptor precursors derived from fetal neural retina or hESCs are preparing for clinical trials.

The present study showed that the photoreceptor precursors and RPEs derived from hESCs demonstrated markers for retinal cells, hence suggesting a possibility to induce photoreceptors and RPEs in a defined condition. The photoreceptor precursors and RPEs were generated via clumps of cells with neural characteristics, called spherical neural masses (SNMs). Several unique procedural advantages are associated with these SNMs. First, the hESC-derived SNMs can be differentiated into photoreceptor precursors or RPEs at high efficiency. Second, the SNMs can be expandable for a long time (at least 4 months), while maintaining the same phenotype and capability to differentiate into neural progenitors or epithelial cells. Third, the expanded SNMs can be stored frozen and thawed at any time. Fourth, the generation of photoreceptor precursors or RPEs from SNMs takes a relatively short time (2 weeks). Finally, the feeder cells are not used in the expansion and differentiation of SNMs, which could save a lot of time and effort required for handling the feeder cells and reduce the risk of contamination from irrelevant cells during the transplantation.
Table 4. Methods for directing differentiation of human pluripotent stem cells to RPE

<table>
<thead>
<tr>
<th>Author</th>
<th>Source</th>
<th>Culture Method</th>
<th>Efficiency</th>
<th>Time</th>
<th>Quantification Method</th>
<th>Functional analysis or Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osakada, 2008</td>
<td>hESC</td>
<td>SFEB*/DL (Dkk-1 and Lefty A)</td>
<td>37.4%</td>
<td>60</td>
<td>Pigmented cells</td>
<td>Tight junction Electron micrograph phagocytosis</td>
</tr>
<tr>
<td>Hirami, 2009</td>
<td>iPSC</td>
<td>SFEB/DL</td>
<td>38.8%</td>
<td>day 40</td>
<td>pigmented cells</td>
<td>Tight junction</td>
</tr>
<tr>
<td>Indelson, 2009</td>
<td>hESC</td>
<td>Nicotinamide, Activin A</td>
<td>50.7%</td>
<td>6 weeks</td>
<td>pigmented cells</td>
<td>Transplantation to RCS rats</td>
</tr>
<tr>
<td>Zahabi, 2012</td>
<td>iPSC</td>
<td>Noggin or SB431542, bFGF (0–6 d); all-trans-retinoic acid, bFGF(6–12 d); Shh, bFGF(12–18 d)</td>
<td>57.4% 39.4%</td>
<td>60 days</td>
<td>MITF+ RPE65+ 1 cells</td>
<td>Disease-specific iPSC</td>
</tr>
</tbody>
</table>
Table 4. Methods for directing differentiation of human pluripotent stem cells to RPE (continued)

<table>
<thead>
<tr>
<th>Author</th>
<th>Source</th>
<th>Culture Method</th>
<th>Efficiency</th>
<th>Time</th>
<th>Quantification Method</th>
<th>Functional analysis or Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buchholz, 2013</td>
<td>hESC</td>
<td>Noggin, IGF1, Dkk1, NIC (0–2d) bFGF (2–4d)</td>
<td>63% (iPSC)</td>
<td>14 days</td>
<td>PMEL17+ cells</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td>iPSC</td>
<td>IGF1, Dkk1, activin A (4–6 d) activin A, VIP, SU5402 (6–14d)</td>
<td>79.8% (hESC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lane, 2014</td>
<td>hESC</td>
<td>Spontaneous differentiation method modified with dorsomorphin</td>
<td>17.7 (foci/m²)</td>
<td></td>
<td>Pigmented foci</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Noggin, IGF1, Dkk1, nicotinamide (day 0-2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leach, 2015</td>
<td>hESC</td>
<td>bFGF (day 2–4) IGF1, Dkk1, activin A (day 4–8) activin A, CHIR99021, SU5402 (day 8–14)</td>
<td>97.77%</td>
<td>14 days</td>
<td>PMEL17</td>
<td>Phagocytosis; PEDF/VEGF secretion</td>
</tr>
</tbody>
</table>

SFEB/DL: serum-free embryoid-body like culture/Dkk1 and Lefty A; PEDF: pigment epithelium-derived factor; VEGF: vascular endothelial growth factor
Table 5. Methods for directing differentiation of human pluripotent stem cells to photoreceptor precursors

<table>
<thead>
<tr>
<th>Author</th>
<th>Source</th>
<th>Culture Method</th>
<th>Efficiency</th>
<th>Time</th>
<th>Quantification Method</th>
<th>Functional analysis or Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamba, 2006^8</td>
<td>hESC</td>
<td>IGF-1, Dkk-1 and Noggin Coculture with mouse retina</td>
<td>82%</td>
<td>3 weeks</td>
<td>PAX6+</td>
<td>Markers of more differentiated cells were not tested.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>86%</td>
<td></td>
<td>Chx10+</td>
<td></td>
</tr>
<tr>
<td>Osakada, 2008^10</td>
<td>hESC</td>
<td>SFEB/DL conditions for 90-120 days Retinoic acid/taurine medium for 30 days</td>
<td>19.6%</td>
<td>170 days</td>
<td>CRx+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.5%</td>
<td>200 days</td>
<td>Rhodopsin+</td>
<td></td>
</tr>
<tr>
<td>Hirami, 2009^41</td>
<td>iPSC</td>
<td>SFEB /Dkk-1 and Lefty A Retinoic acid, taurine</td>
<td>13%</td>
<td>120 days</td>
<td>Recoverin+</td>
<td></td>
</tr>
<tr>
<td>Meyer, 2009^44</td>
<td>hESC</td>
<td>DMEM/F12 media with N2</td>
<td>26%</td>
<td>40 days</td>
<td>CRX10+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>iPSC</td>
<td>DMEM/F12 media with B27</td>
<td>12.9%</td>
<td></td>
<td>(hiPSC)</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Methods for directing differentiation of human pluripotent stem cells to photoreceptor precursors (continued)

<table>
<thead>
<tr>
<th>Author</th>
<th>Source</th>
<th>Culture Method</th>
<th>Efficiency</th>
<th>Quantification Method</th>
<th>Functional analysis or Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamba, 2010</td>
<td>iPSC</td>
<td>Noggin, Dkk1, IGF-1</td>
<td>70.22%</td>
<td>3 weeks</td>
<td>PAX+ SOX1+ SOX9+ CRX+ NRL+</td>
</tr>
<tr>
<td>Mellough, 2012</td>
<td>hESC/iPSC</td>
<td>3-step differentiation Dkk1 and Noggin-1 IGF-1 Lefty A, Shh, T3, taurin, retinoic acid B27 and/or N2</td>
<td>16%</td>
<td>45 days</td>
<td>Crx+ cone-like photoreceptor cells</td>
</tr>
<tr>
<td>Yanai, 2013</td>
<td>hESC</td>
<td>Size-controlled embryoid bodies triiodothyronine (T3) and taurine</td>
<td>78%</td>
<td>17 days</td>
<td>CRX+</td>
</tr>
</tbody>
</table>

SFEB/DL: serum-free embryoid-body like culture/Dkk1 and Lefty A; VIP: vasoactive intestinal peptide
The Royal College of Surgeons (RCS) rat is a widely used animal model for inherited retinal degeneration. These rats experience progressive vision loss after their birth. The genetic mutation in the MERTK gene results in defective function of the RPEs, including the failure to phagocytize rod outer segments. This defect causes degeneration of RPEs and photoreceptors and finally results in visual loss. Morphological changes in photoreceptor outer segments appear as early as postnatal day 16 (P16), and only scattered photoreceptor cells (cones) remain by P105. In this study, the subretinal transplantation of photoreceptor precursor cells or RPEs in RCS rats preserved visual response and retinal morphology for up to 24 weeks after the transplantation. Interestingly, the protective effect of the subretinal transplantation of retinal cells was more apparent after the transplantation of photoreceptor precursors than after transplantation of RPEs. The subretinal transplantation of RPEs also showed preservation of visual response and retinal morphology, however, not as good as the transplantation of photoreceptor precursors. As the role of RPEs in the retina is to support photoreceptors, the transplantation of RPEs may prevent photoreceptor degeneration via indirect mechanism, which results in less protective effects than the transplantation of photoreceptor precursors.
Conclusions

The strategy in this study using SNMs demonstrates a faster and efficient method to generate photoreceptor precursors and RPEs for retina regeneration cell therapy. The subretinal transplantation of photoreceptor precursors or RPE was well tolerated and delayed retinal degeneration, implying possibilities for cell therapy for retinal degenerative diseases.
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specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells. Stem Cells. 2012;30:673-86
국문 초록

서론 : 시각세포 혹은 망막색소상피세포의 변성 및 소실은 망막색소변성이나 나이관련황반변성과 같은 망막변성 질환의 중요한 병리학적변화 중 하나이다. 줄기세포유래 망막색소상피세포 또는 시각세포를 이식하는 것이 망막을 재생시키고 시력을 회복할 수 있는치료법으로 시도되고 있다. 인간배아줄기세포 또는 체세포유도만능줄기세포로부터 망막색소상피세포 또는 시각세포전구체를 성공적으로 분화하였다는 보고들이 많이 있다. 그러나 아직까지는 치료에 필요한충분한 세포를 얻기 위해서는 많은 시간이 필요하고, 분화 효율은 여전히 낮다. 이 연구에서는 일정한 조건하에 인간배아줄기세포로부터 시각세포전구체 또는 망막색소상피세포를 짧은 시간에 효율적으로 분화시킬수 있는 방법을 제시하였다. 또한 이렇게 분화한 세포가 잠재적인 치료 가능성이 있는지 알아보기 위해, 분화시킨 세포의특성을 분석하고 망막변성모델에 이식한 뒤 해부학적 및 기능적변화를 평가하였다.

방법 : 인간배아줄기세포로부터 구형의 신경세포덩어리인 신경전구체구를 유도하고, 이 신경전구체구를 분화하여 시각세포 전구체 또는 망막색소상피세포로 분화시켰다. 인간배아줄기세포에서 신경전구체구까지 분화되는 데는 약 4주가 소요되며, 신경전구체구로부터
각각 시각세포 전구체로 분화하기 까지는 2주, 망막색소상피세포로의 분화는 1주일 더 소요되었고. 분화된 시각세포 전구세포에서 변역세포염색 및 역전사 중합효소 연쇄반응으로 시각세포 특이 표지자인 rhodopsin, opsin, recoverin 및 PDE-6β 등의 발현을 확인하였다. 분화된 망막색소상피세포 역시 변역세포염색 및 역전사 중합효소 연쇄반응으로 RPE65, MITF, ZO-1, bestrophin 등의 발현을 확인하였으며 전자현미경으로 미세구조를 관찰하고 망막색소상피세포의 탐식능을 평가하였다.

분화된 세포의 특징을 평가한 뒤, 망막변성쥐인 Royal College Surgeon 쥐의 망막하공간에 시각세포 전구세포 또는 망막색소상피세포를 이식하였다. RCS 쥐는 시각세포 전구세포를 이식한 군 (n=25), 또는 망막색소상피세포를 이식한 군 (n=25), 배양액만을 이식한 대조군 (n=26)의 세 군으로 나누었다. 이식후에 망막안에서의 생착과 종양 발생에 대하여 24 주 까지 관찰 하였다. 망막 전위도 검사를 이식 후 4, 12, 24 주 후에 시행하였고 b-파의 진폭을 분석하였다. 이식 4주 및 24 주 후에 조직 검사를 시행하여 망막전체 두께, 내핵층 및 외핵층 두께를 각각 측정하여 분석하였다.

결과 : 변역세포염색 및 역전사 중합효소 연쇄반응을 통하여, 분화시킨 시각세포 전구세포가 시각세포 특이 단백질인 rhodopsin, recoverin, opsin과, 신경세포 표지자인 βIII-tubulin과 nestin을 발현함을 확인하였다. 시각세포 전구세포의 분화 효율은 신경전구체
구를 배양한지 2주째 가장 높아서 약 80% 의 세포가 시각세포 특이 단백질을 발현하였다. 분화된 망막색소상피세포는 망막색소상피세포의 특징적 모양인 다각형의 색소화 세포 모양을 보였으며 전자현미경상에서 세포질내 색소, 밀착연결, 미세융모 구조를 보였다. 또한 면역세포염색 및 역전사 중합효소 연쇄반응에서 망막색소상피세포의 표지자인 MITF, ZO-1, RPE65, bestrophin 을 발현함을 확인하였으며 시각세포 외질을 투시하는 탐식능을 보였다. 신경전구체구로부터 분화시킨지 1주째에 90% 이상의 세포가 망막색소상피세포의 단백질을 발현하였다.

시각세포전구세포 또는 망막색소상피세포를 망막하 이식하였을 때 이식된 세포는 망막층 내에 위치하였으며 24 주까지 관찰 기간 동안 종양 형성이나 심한 염증 반응은 없었다. 망막전위도에서 b-파형의 진폭은 이식 4주째에 시각세포 전구세포를 이식한 군이나 망막색소상피세포를 이식한 군에서 대조군에 비하여 높았으며 (74.51±27.78 uV, 37.48±13.75 uV, 9.28±1.56 uV, p=0.018, 0.043) 시각세포전구세포를 이식한 군에서 망막색소상피세포 이식군보다 높은 경향을 보였으나 통계적 의미는 없었다. 이식 후 12주째에는 망막전위도의 진폭이 약간 감소하였으나 이식군에서 대조군에 비하여 높은 b-파형 진폭을 보였다. 이식 후 24 주째에 시각세포전구세포를 이식한 군에서는 대조군에 비하여 높은 b-파 진폭을 보였으나 (50.33±15.81 uV vs 5.63±1.45 uV, p=0.0058)
망막색소상피세포를 이식한 군에서는 대조군과 통계적인 차이는 없었다.

망막의 외세포핵층의 두께는 이식 4주 후에 시각세포 전구세포 (14.03 ± 1.48 um, p=0.011) 또는 망막색소상피세포 (12.49 ± 1.20 um, p=0.048)를 이식한 군에서 대조군 (9.38 ± 0.92 um)에 비하여 높았으며 24 주에도 유사한 결과를 보였다 (12.01 ± 1.48 um, 9.85 ± 1.23 um, 6.95 ± 0.68 um). 내세포핵층 또는 망막 전체의 두께는 이식군과 대조군 사이에 4주, 24 주체에 모두 차이가 없었다.

결론: 시각세포 전구세포 또는 망막색소상피세포가 인간배아줄기세포로부터 신경전구체구를 거쳐 짧은 시간에 효율적으로 분화될 수 있으며, 이 세포들을 망막하 이식하였을 때 망막변성을 지연시킬 수 있었다. 이러한 방법은 향후 망막변성질환에서 세포 치료의 방법으로 쓰일 수 있을 것으로 기대된다.

주요어: 시각세포, 망막색소상피세포, 망막변성, 배아줄기세포

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