Generation of Retinal Ganglion–like Cells from Reprogrammed Mouse Fibroblasts

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PURPOSE. Somatic cells can be reprogrammed into an embryonic stem cell–like pluripotent state by Oct-3/4, Sox2, c-Myc, and Klf4. Sox2 as an essential reprogramming factor also contributes to the development of the eye and the retina. This study was conducted to determine whether induced pluripotent stem (iPS) cells express retinal progenitor cell (RPC)-related genes and whether iPS cells can directly differentiate into retinal ganglion cells (RGCs).

METHODS. Mouse iPS cells were induced by the ectopically expressed four factors in tail-tip fibroblasts (TTFs). The expression of RPC-related genes in iPS cells was analyzed by RT-PCR and immunofluorescence. iPS cells were induced to differentiate into RGCs by the addition of Dkk1 + Noggin (DN) + DAPT and overexpression of Math5. iPS-derived retinal ganglion (RG)-like cells were injected into the retina, and the eyes were analyzed by immunohistochemistry.

RESULTS. iPS cells inherently express RPC-related genes such as Pax6, Rx, Otx2, Lhx2, and Nestin. Overexpression of Math5 and addition of DN can directly differentiate iPS into retinal ganglion-like cells. These iPS-derived RG-like cells display long synapses and gene expression patterns, including Math5, Bm3b, Islet-1, and Thy1.2. Furthermore, inhibiting Hes1 by DAPT increases the expression of RGC marker genes. In addition, iPS-derived RG-like cells were able to survive but were unable to be integrated into the normal retina after transplantation.

CONCLUSIONS. The four factor iPS cell inherently expressed RPC-related genes, and the iPS cell could be further turned into RG-like cells by the regulation of transcription factor expression. These findings demonstrate that iPS cells are valuable for regeneration research into retinal degeneration.

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Sox2 is necessary for maintaining the properties of neural progenitor cells. Conditional ablation of Sox2 in the retina causes the complete loss of neural progenitor competence to differentiate.\textsuperscript{37} Interestingly, recent evidence has indicated that overexpression of Sox2 in the developing chick eye and RPE cell results in differentiation toward retinal neurons.\textsuperscript{38} It has also been shown that Sox2 associates with Pax6 and Otx2, is expressed in the neural plate, and cooperatively controls eye development.\textsuperscript{56} Sox2 autoregulates or cross-regulates with Pax6, and the forced expression of Sox2 activates the Pax6 promoter in Y79 cells.\textsuperscript{59} In addition, Sox2 and Otx2 proteins are direct upstream regulators and coregulate expression of the retinal homeobox gene Rx.\textsuperscript{40} All these studies suggested that reprogrammed pluripotent cells generated by transcription factors containing Sox2 may have the capacity to differentiate into retinal neurons.

The purpose of this study, therefore, was to explore whether iPSC cells express retinal progenitor marker genes and whether iPSC cells can be directly differentiated into RG-like cells. Our results demonstrated that iPSC cells intrinsically express retinal progenitor cell–related genes, including Pax6, Rx, Otx2, Hox2, and Nestin. Overexpression of Math5 and addition of DN can directly differentiate iPSC into RG-like cells. The iPSC-derived RG-like cells display long synapses and gene expression patterns, including Math5, Brn3b, Islet-1, and Thy1.2. Furthermore, iPSC-derived RG-like cells were able to survive but rarely engraft into the normal host retina after transplanting into the vitreous cavity. Our findings demonstrated that iPSC cells are valuable for research into retinal degeneration diseases and illuminate the potential to generate patient-tailored RG-like cells.

Materials and Methods

Generation and Culture of Mouse iPSC Cells

Mouse iPSC cells were induced from tail-tip fibroblasts (TTFs) by the four factors according to previous reports.\textsuperscript{8–10} Briefly, Oct3/4, Sox2, Klf4, and c-Myc (Addgene) were introduced into cells (EcoPack2–293; Clontech, Mountain View, CA) using a transfection reagent (Fugene6; Roche, Indianapolis, IN). TTFs were transduced with the four transcription factors by retrovirus-mediated transient gene transfer. Twelve to 14 days after infection, iPSC colonies were picked up and transferred onto mitomycin C-inactivated MEF feeder cells in ES–Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), L-glutamine, 10 ng/mL bFGF, 0.1 mM β-mercaptoethanol, cell growth medium (GlutaMAX; Invitrogen), 1% penicillin, and 0.2 mg/mL streptomycin. Two iPSC cell lines, including iPSC clone 6 (iPS-C6) and GFP-iPSC clone 5 (GFP-IPS-C5), were used in this study.

Fluorescence-Activated Cell Sorter Analysis

iPSCs were dissociated into a single-cell suspension with 0.125% trypsin/EDTA, washed with PBS, resuspended in fluorescence-activated cell sorter analysis (FACS) buffer (PBS + 5% FBS), and returned to the incubator for antigen recovery for 1 hour. Cells (1 × 10^6) were stained with 10 µL phycoerythrin (anti-stage-specific embryonic antigen [SSEA]–1) 480; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature in the dark, washed twice with PBS, and resuspended in FACS buffer for analysis on a cell sorter (FACS Aria; BD Biosciences, Franklin Lakes, NJ).

DL- and DN-Induced Differentiation from iPSC Cells

To differentiate iPSC cells into retinal neurons, the ES differentiation protocol was adopted according to previous reports\textsuperscript{8–10} with some modifications. In brief, iPS-C6 cells were cultured without feeders for two passages, and 4 × 10^6 cells were subsequently plated in bacterial dishes for embryoid body (EB) formation in EB medium (containing DMEM/F12, 15% FBS, 10 ng/mL bFGF, 0.1 mM β-mercaptoethanol, 1 × 10^-4 M nonessential amino acids, cell growth medium [GlutaMAX, Invitrogen], 50 U/mL penicillin, and 50 µg/mL streptomycin). Recombinant proteins Dkk1 (100 ng/mL; R&D Systems, Minneapolis, MN), Lefty A (100 ng/mL; R&D Systems), and Noggin (100 ng/mL; R&D Systems) were added to the EB medium after suspension in culture for 1 day. EBs were dissociated using 0.05% trypsin on day 3, and 0.1 to 0.2 × 10^6 cells were seeded onto a 0.1% gelatin-coated six-well plate in N2 medium containing 3% FBS, 10 ng/mL bFGF, 1 × 10^-4 M nonessential amino acids, cell growth medium (GlutaMAX, Invitrogen), supplement (N2; Invitrogen), and serum-free supplement (B-27, Invitrogen). Recombinant proteins were added as EB medium. After 2 days, N2 medium was replaced with neuronal medium (containing Neurobasal; Invitrogen), 3% FBS, 1 × 10^-4 M nonessential amino acids, supplement (N-2), serum-free supplement (B-27), and cell growth medium (GlutaMAX, Invitrogen). Recombinant proteins were supplemented to continue differentiation for 5 to 6 days.

Generation of RG-like Cells by Overexpression of Math5

iPS-C6 and GFP-iPSC-C5 cells (0.1–0.2 × 10^6) were passaged onto 0.1% gelatin-coated six-well plates in N2 medium, supplemented with Dkk1 (100 ng/mL) + Noggin (100 ng/mL) (DN), and 10 µM DAPT (N-[3,5-difluorophenacetyl]-L-alanyl-S-phenylglycine t-butyl ester, y-secretase inhibitor; Calbiochem). After 2 to 3 days, pRK5KS-Math5 expression plasmids (a gift from Mengqing Xiang, UMDNJ-Robert Wood Johnson Medical School) were transfected into iPSC cells. Briefly, the medium was replaced with DMEM that did not contain antibiotics 3 hours before transfection. Reagent (30 µL; Fugene6; Roche) was added to 570 µL serum-free DMEM (Invitrogen). After incubation for 5 minutes, 10 µg Math5 expression plasmid DNA was added and incubated for at least 15 minutes. The DNA/reagent mixture (100 µL/well) was added dropwise to iPSC cells, and the medium was changed with fresh neuronal medium 8 to 10 hours after transfection. Dkk1, Noggin, and DAPT were added as supplements to continue differentiation for 3 days.

Cell Transplantation

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four- to 5-week-old BALB/c mice were used in the study of transplantation. Differentiated GFP-iPSC cells (treated by DN + DAPT and Math5 overexpression) were transplanted into the vitreous chamber as previously described.\textsuperscript{13,14} Briefly, cells were dissociated with 0.05% trypsin into a single-cell suspension (1 × 10^7/mL). Animals were deeply anesthetized by intramuscular injection of ketamine hydrochloride (50 mg/kg) and chlorpromazine hydrochloride (0.5 mg/kg). Before transplantation, anterior chamber paracentesis was performed under ophthalmic microscopic observation, and 1- to 2-µL cell suspension (1–2 × 10^5 cells) was subsequently injected into the vitreous cavity. Two to 4 weeks after transplantation, animals were killed and their eyes were removed. After fixing in 4% paraformaldehyde/PBS for 30 minutes, eye samples were embedded with OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA). Sections measuring 7 to 10 µm were obtained with a freezing microtome (Leica, Wetzlar, Germany) and were stored at −20°C until immunofluorescence analysis.

RT-PCR and Real-Time PCR

Total RNA was extracted using reagent (TRI; Ambion, Austin, TX) and then treated with DNase I (Sigma, St. Louis, MO) to remove the likelihood of genomic DNA contamination. A first-strand cDNA synthesis kit (RevertAid; Fermentas, Glen Burnie, MD) was used to synthesize complementary DNA from 2 µg total RNA. For nonquantitative RT-PCR analysis, 100 to 200 ng cDNA was amplified with master mix (GoTaq...
Green Master Mix; Promega, Madison, WI) and a PCR system (GeneAmp 9700; Applied Biosystems, Foster City, CA). For quantitative analysis, cDNA samples (≥100 ng) were amplified (ABI PRISM 7000; Applied Biosystems) with an RT-PCR kit (SYBR PrimeScript; Takara, Shiga, Japan). Each PCR analysis was performed in 33 cycles; primer sequences are shown in Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/11/5970/DC1. The mRNA expression level of each gene relative to GAPDH (housekeeping gene) was calculated as previously described.45

Immunofluorescence

For immunofluorescence staining, cells on coverslips and tissue sections were fixed in 4% paraformaldehyde/PBS for 10 to 15 minutes and rinsed with PBS, then permeabilized with 0.1% Triton X-100/PBS for 10 minutes and blocked in 5% BSA for 30 minutes. Coverslips or sections were incubated with primary antibodies for 1 hour at room temperature (to facilitate location in nucleus staining of cells on coverslips, primary antibodies were diluted in 0.2% Triton X-100/PBS and incubated overnight at 4°C), washed three times with PBS, and incubated with secondary antibody for 1 hour. The following primary antibodies were used: rabbit anti-Sox2 (1:100 dilution; Millipore), rabbit anti-Nestin (1:100 dilution; Millipore), mouse anti-Pax6 (1:200 dilution; Millipore), mouse anti–microtubule associated protein 2 (MAP2; 1:300 dilution; Abcam), mouse anti-synaptophysin (1:100 dilution; Boster Biological Technology, Wuhan, China), rabbit anti-GFP (1:100 dilution; Beyotime Institute of Biotechnology, Haimen, China), rabbit anti-Math5 (1:500 dilution; Abcam, Boston, MA), goat anti–Islet-1 (1:100 dilution; Santa Cruz Biotechnology), goat anti–Brn3b (1:200 dilution; Santa Cruz Biotechnology), and rat anti–Thy1.2 (1:100 dilution; Abcam). Coverslips or eye sections were subsequently incubated with FITC, Alexa Fluor 488, or Cy3-labeled secondary antibodies for 1 hour at room temperature in the dark. After washing three times with PBS for 5 minutes each, samples were counterstained with DAPI (Invitrogen). Negative controls were stained without primary antibodies. Confocal fluorescence images were acquired with laser scanning microscope (LSM 510; Zeiss, Thornwood, NY).

To examine the efficiency of transfection and differentiation, iPS-C6 cells (0.1 × 10⁶/well) were plated on 0.1% gelatin-coated slides and differentiated by DN + DAPT and Math5 overexpression for 3 to 4 days. Counting was based on respective antibody and DAPI labeling. Positive cells were counted from a total of at least 200 cells per well (n = 3) in randomly selected fields, as described.46

Statistical Analysis

All data were obtained from at least three independent experiments and were represented as mean ± SD. Statistical analysis of the data was performed (SPSS 13.0), and significance of the differences was examined with the Student’s t-test. P < 0.05 was considered significant.

RESULTS

iPS Cells Induced by Four Factors Inherently Express RPC-Related Genes

Mouse iPS cells were induced by the transduction of Oct-3/4, Sox2, c-Myc, and Klf4. Undifferentiated iPS-C6 cells displayed ES-like morphology when cultured on MEF (Fig. 1A) and expressed endogenous Sox2, as we described previously.43 Approximately 20% iPS cells were positive for SSEA-1, a typical surface marker of ES cells (Fig. 1B). Other studies have shown a similar rate of SSEA-1+ in mouse iPS cells.47

![Figure 1](https://www.iovs.org/cgi/content/full/51/11/5970/DC1)
Given that the reprogramming factor Sox2 participates in eye development and cross-regulates with Pax6 and Otx2, we first analyzed whether undifferentiated iPS cells express RPC-related marker genes. Mouse brain tissue RNA was isolated on E11.5 and served as a positive control. Given the variability among iPS cell lines, two iPS cell lines were analyzed by RT-PCR. Interestingly, the results revealed that both undifferentiated iPS-C6 and GFP-iPS-C5 cells expressed RPC-related genes, including Pax6, Rx, Otx2, Lhx2, and Nestin, but not Six6 (Fig. 1C), Chx10 (Fig. 1C), or Six3 (data not shown). All these markers were not observed in TTFs and ES (data not shown). Immunofluorescence analysis further confirmed that undifferentiated iPS-C6 cells were positive for Sox2, Pax6, Nestin, Otx2, Lhx2, and Rx (Figs. 1D, 1E). Nestin, Pax6, Otx2, and Lhx2 were expressed in almost all iPS clones of both the iPS-C6 and the GFP-iPS-C5 (Supplementary Fig. S1A, http://www.iovs.org/cgi/content/full/51/11/5970/DC1) cell lines. These results demonstrated that four factor iPS cells inherently express RPC-related genes and present potential differentiation into retinal neurons.

**DL or DN Upregulated Pax6 and iPS Cell Differentiation into Neuron-like Cells**

To cause iPS cells to differentiate toward RGCs, previous protocols described for ES cell differentiation were used in our initial study (Fig. 2G). First, mouse iPS-C6 cells were expanded on MEF feeders (Fig. 2A), and EBs were generated by suspension-cultured iPS cells in EB medium for 2 to 3 days (Fig. 2B). Recombinant proteins Dkk1 (Wnt antagonist), Lefty A (Nodal antagonist), and Noggin (BMP inhibitor) were added to the medium for differentiation. After dissociating and culturing in N2 medium, the cells showed differentiation morphology on day 4 (Fig. 2C). After treatment with DL (Dkk1 + Lefty A) or DN (Dkk1 + Noggin), many cells displayed neuronal morphology on day 8 (Figs. 2E, 2F). In contrast, most cells presented random differentiation with a few neuron-like cells and no factor control (Fig. 2D).

Given that the persistent expression of pluripotent genes would prevent iPS cell differentiation, we subsequently detected the expression of Oct3/4 and Nanog (pluripotency-related genes). The results showed that undifferentiated iPS cells expressed Oct3/4 and Nanog, and the expression of total Oct3/4, endogenous Oct3/4, and Nanog decreased after the withdrawal of LIF from control. A more obvious decrease of Oct3/4 (both total and endogenous) was found after the addition of DL or DN but was still detectable on day 8, whereas the expression of Nanog decreased to an undetectable level on day 8 (Fig. 2H). These results are consistent with previous reports that BMP4 and Wnt4 sustain self-renewal and preserve multilineage differentiation of ES cells. Our results indicated that the expression of pluripotent-related genes in iPS cells were LIF dependent and could be induced by DL or DN downregulation.

We next examined the expression of RPC-related genes after differentiation. RT-PCR analysis showed that Lhx2, Otx2, Rax, and Nestin were decreased after DL- or DN-induced differentiation, and similar decreases were found in
control (Fig. 2). Our data also showed that the expression of Pax6 was upregulated in DL and DN groups when compared with undifferentiated iPS-C6. Real-time PCR demonstrated that there was a 4× or 16× upregulation of Pax6 after the addition of DL or DN, respectively, but both were lower than that on E11.5 (Fig. 2J).

By immunofluorescence assay, we further confirmed that the neuron-like cells induced by DL or DN were positive for GFAP (a marker of glia cells) and MAP2 (Fig. 2K). Although Pax6 acts as a Math5 upstream gene and directly activates Math5, the expression of RGC markers, including Brn3b, Islet-1, and Thy1.2, was not detected in this study. These results indicated that DL or DN upregulated Pax6 but did not sufficiently differentiate iPS toward RGCs.

**Overexpression of Math5 Activates RGC-Related Genes in iPS Cells**

RGCs are the first cell type to be specified during vertebrate retinogenesis, and the specification and differentiation of the RGC lineage is a stepwise process involving a hierarchical gene regulatory network.

Math5 plays a key role in the gene regulation of RGC fate determination. Pax6 directly activates Math5, the expression of RGC markers, including Brn3b, Islet-1, and Thy1.2, was not detected in this study. These results indicated that DL or DN upregulated Pax6 but did not sufficiently differentiate iPS toward RGCs.

To directly differentiate iPS cells into RGCs, therefore, we upregulated Pax6 by DN, overexpressed Math5, and further inhibited Hes1 with DAPT (γ-secretase inhibitor) in iPS cells. Figure 3A shows a schematic diagram of our differentiation methods. First iPS-C6 cells were cultured without feeder cells (Fig. 3B) for two passages, and then they were treated with DN and DAPT for 3 days. After the transfection of Math5 for 72 hours, many iPS-C6 cells displayed RGC morphology with long synapse-like structures (Fig. 3C). Figure 3D shows the typical shape of RGC-like cells. These RGC-like cells also emerged in GFP-iPS-C5 cells after differentiation by this protocol (Supplementary Fig. S1B, http://www.iovs.org/cgi/content/full/51/11/5970/DC1). RT-PCR analysis confirmed the expression of exogenous Math5 in iPS-C6 cells. (F) Real-time PCR showed that exogenous Math5 markedly increased 96 hours after transfection. (G) DAPT inhibited Hes1 expression in iPS-C6 cells. (H) Real-time PCR revealed an obvious decrease of Hes1 compared with control. (I) Differentiated iPS-C6 cells initiated the expression of RGCs markers, including Brn3b, Islet-1, and Thy1.2. These genes were not detected in control (DN treated only). M5, transient transfection of Math5 expression plasmid; M5+DA, transfection of Math5 and addition of DAPT. (J, K) Real-time PCR showed the expression levels of Brn3b (J) and Thy1.2 (K) were further increased by supplementation with DAPT. Graphs show mean ± SD (n = 3). Scale bars: 100 μm (B, C); 50 μm (D).
entiation by the overexpression of Math5 and the addition of DN + DAPT.

**Limited Engraftment of Transplanted Cells**
We next transplanted the RG-like cells derived from GFP-iPS cells into the vitreous chamber of normal mice after differentiation by DN + DAPT and Math5 transfection. After 2 weeks, most GFP-expressing cells were concentrated near the injection site and rarely engrafted into the host retina (Fig. 4C). The results suggested that differentiated RG-like cells can survive but integrate with difficulty in the normal host retina. This poor integration can be explained by the fact that the intact normal retinal environment prevents a barrier to graft integration and the transplantation of differentiated iPS cells into the injured retina of the disease model may form extensive integration.

We also found teratoma formation in both eyes of one mouse (12 in total mice) after transplantation of the differentiated iPS cells (induced differentiation at passage 9). However, teratoma did not emerge when using the postnatal day (P) 20 cells (data not shown). These results suggested the pluripotency of iPS cells remained in early passages even after transient differentiation and the gradual loss of pluripotency when passage numbers grew. Whether teratoma formation was related to pluripotent genes that did not silence completely or to exogenous genes reexpressed in early passages of iPS cells was unclear. These results indicated the risk for tumorigenesis after the transplantation of virus-induced iPS cells into the retina.

**DISCUSSION**

**Undifferentiated iPS Cells Have Retinal Neuron Differentiation Potency**
Pluripotent stem cells can be generated by reprogramming patient somatic cells, thereby avoiding immune rejection and ethical issues, and patient-tailed iPS cells will be valuable for regeneration medicine.17,18,54 However, several obstacles must be overcome before the therapeutic application of iPS cells to human diseases. How to directly differentiate iPS cells into specific donor cells is one of the issues that must be resolved.

As mentioned, Sox2 is necessary and sufficient for maintaining the pan-neural properties of neural progenitor cells, and the constitutive expression of Sox2 inhibits neuronal differentiation and maintains progenitor characteristics.55 Sox2 levels in the retina regulate the choice between maintenance of the progenitor state and differentiation, and downregulated expression of Sox2 triggers aberrant retinal progenitor differentiation.7 It has also been reported that Sox2 associates with Pax6 and that Otx2 cooperatively controls eye development.56

Therefore, Sox2, as a necessary reprogramming factor, may contribute to neuron differentiation potency and may assign the gene expression pattern of RPCs to iPS cells. In this report, we demonstrated that undifferentiated iPS cells inherently expressed RPC marker genes such as Pax6, Rx, Otx2, Lbx2, and Nestin. Recently, it was also reported that the overexpression of Sox2 activates Pax6.59 However, other RPC markers, such as Six3, Six6, and Chx10, were not expressed in undifferentiated iPS cells, indicating that the iPS cells have only partial RPC potency. These findings also suggested that Sox2 may contribute to activate RPCs markers and endow iPS cell with the competence to differentiate into retinal neurons. Although Sox2 was expressed in undifferentiated ES cells, we did not find that RPC-specific genes were activated, suggesting that the transcription regulatory network of ES cells turns off expression of these genes.

**iPS Cell Differentiation Induced by DL or DN**
Wnt and BMP signals contribute to retinal neurogenesis in development,56,57 and inhibition of Nodal signaling in ES promotes their specification into neural progenitors.58 Previous reports have revealed that Dkk1, Lefty A, and Noggin upregulated Pax6 in ES cells.8–10 Inhibition of BMP signaling by Noggin is sufficient for the derivation of neural progenitors from human ES.59,60 Moreover, the synergistic action of Noggin and SB431542 induced Pax6 expression and neural conversion from ES and iPS cells.19 More recently, another study also showed that treating iPS cells with Dkk1 and Lefty A induced RPC marker expression and generated RPE cells.26 However, the expression of RPC genes in undifferentiated iPS cells were not revealed in these reports.

Our results confirmed that iPS cells inherently expressed Pax6 and, consistent with previous reports,19,26 that Pax6 expression was further increased in DL- and DN-treated cultures and DN induced a more significant increase of Pax6. However, other RPC markers, such as Rx, Otx2, Lbx2, and Nestin, were decreased in differentiation conditions, including no factor control. These results suggested that DL or DN can upregulate Pax6 but not other genes and that the expression of
RPC-related genes in iPS cells tends to vanish after departing from the ES medium. In this study, DL- or DN-treated cultures failed to differentiate iPS into Brn3b, Islet-1, and Thy1.2-positive RGCs, suggesting that the inhibition of Wnt, BMP, and Nodal signaling did not sufficiently differentiate iPS cells into RGCs.

On the other hand, although Nanog was silent after DL and DN treatment, the sustained expression of the pluripotent gene Oct3/4 could delay iPS cell uniform differentiation. Whether a longer period of differentiation induced by DL or DN could promote Oct3/4 silent is unclear. Similar results have been reported in differentiation cardiomyocytes from mouse iPS cells.\(^{24}\)

**Overexpression of Math5 Initiates iPS Cells to Differentiate toward RGCs**

RGCs are the final output neurons of the vertebrate retina. They collect the messages from bipolar cells and amacrine cells and represent the ultimate signal to the vision center in the brain. RGCs are multipolar neurons with long axons; they are bigger on average than most retinal neurons and have large-diameter axons capable of passing the electrical signal. Primary cultured RGCs generally exhibit two to four dendrites and one axon per cell.\(^{61}\)

Math5 is a proneural gene essential for RGC specification.\(^{62}\) Math5 expression was initiated at E11 before the onset of RGC differentiation, and the targeted deletion of Math5 blocks the initial differentiation of RGCs.\(^ {63}\) Transient transfection of a Math5 expression plasmid with the reporter construct in 293T and ND7 cells, respectively, resulted in 10- and 8-fold increases in luciferase activity, suggesting that the expression of Brn3b can be activated directly by Math5.\(^ {64}\) Math5 expression is required to activate a comprehensive transcription network of RGC differentiation, and other factors are required for RGC fate determination.\(^ {65}\)

**Hes1** and **Hes5** act as essential effectors for Notch signaling, and both are directly regulated by Notch activity during retinal development.\(^ {52}\) The expression of Hes1 and Hes5 promotes the maintenance of progenitors in the embryonal retina.\(^ {66}\) Hes1 and Hes5 inhibit Math5, and Notch activity is downregulated before RGC differentiation.\(^ {52}\) Furthermore, Notch signaling appears to temporarily prevent the production of ganglion cells; reducing Notch expression with antisense oligonucleotide increases ganglion cell genesis.\(^ {67}\)

We therefore investigated whether the overexpression of Math5 initiates RGC-related gene expression in iPS cells. Gene expression analysis revealed that the overexpression of Math5 is sufficient to activate RGC markers, including Brn3b, Islet-1, and Thy1.2, and the addition of DAPT further increased the expression of these genes. Other studies have reported that Brn3b is essential for the later events of RGC differentiation, including axonal growth, pathfinding, and survival of developing RGCs, but not for the initial commitment of RGC fate.\(^ {68}\) In this study, although we found approximately 10% iPS cells positive for Brn3b after Math5 transfection, <5% eventually expressed Thy1.2. One likely explanation for the limited differentiation is the low transfection rate of Math5 mediated by plasmids. Our findings are consistent with the recent report that Math5 upregulates RGC expression patterns in RPCs and changes the expression of Math5-associated genes.\(^ {69}\)

**Transplanted Cells Limitedly Engraft into Host Retina**

To explore the possibility of neuronal replacement in mammalian retina, we next assessed the transplantation potential of iPS after differentiation. We observed that GFP-labeled cells concentrated and survived near the site of injection after 2 weeks but that almost no cells migrated into the inner retina. One possible explanation is that the normal retinal environment presents a barrier to graft integration and to the formation of functional synapses.

Transplanted cells have a higher probability of integration into the adult or degenerating retina if recipient and donor retinas are at equivalent stages of development.\(^ {1}\) Similarly, the retinas of mice deficient in GFAP and vimentin provided a permissive environment for transplanted cells to survive, migrate, and form neuritis.\(^ {40}\) Other studies showed that neural progenitor cells transplanted to the subretinal space readily engraft into a host retina that has undergone ischemic injury.\(^ {41}\) These studies suggested that deficient or degenerating retinas seemed to facilitate the migration of transplanted cells. Further studies of transplantation-differentiated iPS cells into a mouse model of glaucoma would improve the efficiency of integration.

In conclusion, our research revealed that iPS cells induced by the four factors inherently express RPC-related genes, which will be a valuable source of cells for retina regeneration research. Furthermore, the overexpression of Math5, in combination with DN and DAPT, activated RGC downstream genes and directly differentiated iPS cells into RG-like cells. Our study, reprogramming somatic cells to pluripotent stem cells and then directly differentiating them into RG-like cells, showed a strategy of artificial change of cell fate by transcription factors.

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