Central nervous system (CNS) neuron loss is observed in many neurodegenerative diseases such as glaucoma, and those neurons are generally not replaced from endogenous repair. Neuron transplantation is a promising approach to restore neurons lost in degenerative diseases in the CNS. Our previous studies demonstrated that retinal ganglion cells (RGCs) could be successfully transplanted onto an adult retina. Transplanted RGCs survived and integrated into the host retina, displaying both morphologically and electrophysiologically mature phenotypes, with some extent of axonal and dendritic regeneration in vivo. However, RGC transplant efficiency was low, possibly because most transplanted cells remained in the vitreous cavity, an environment low in neurotrophic survival signals.

Recently, various stem cells have been proposed as sources of neuroprotection of endogenous host neurons in degenerative disease. For example, mesenchymal stem cells (MSCs) protect neurons throughout the CNS from numerous types of injuries including traumatic, ischemic, and inflammatory. In particular, MSCs promoted endogenous RGC survival in an experimental glaucoma model. Like MSCs, induced pluripotent stem cells (iPSCs) are multipotent, proliferative, and capable of differentiating into many cell types; however, their neuroprotective abilities have not been well studied. Could stem cells provide neuroprotective signaling to mature neurons undergoing transplant into the adult CNS? The present study was aimed to assess whether iPSCs promote RGC survival and neurite growth after transplant to the adult retina.

**Materials and Methods**

**Animals and Stem Cells**

All use of animals conformed to the ARVO Statement for the Use of Animals in Research and was approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee of Stanford University. GFP mice, both male and female, were bred from the C57BL/6-Tg(CAG-EGFP)1Osb/J strain obtained from Jackson Laboratories (Bar Harbor, ME, USA). Ten- to 12-week-old Sprague-Dawley (SD) rats, both male and female, were obtained from Charles River (Portage, MI, USA). Human iPSC line 297 (iPSC/297), derived from blood cells with Sendai virus transfection (Cytonute 2.0 kit; Life Technologies Inc., Gaithersburg, MD, USA) were kindly provided by the Stem Cell Core, Stanford University Department of Genetics (Stanford, CA, USA).

**RGC Purification**

RGCs were purified from 2- to 4-day postnatal GFP mice by sequential immunopanning with the CD90 (Thy1.2; AbD Serotec, Düsseldorf, Germany) antibody as previously described, yielding 99.5% pure RGCs. Freshly extracted retinas were dissociated with papain (65 units; Worthington, Freehold, NJ, USA), followed by removal of contaminating macrophages and endothelial cells by immunopanning using an anti-macrophage antibody (A1A31240; Accurate Chemical and Scientific Corporation, Westbury, NY, USA). RGCs were
selected from the macrophage-depleted cell suspension immunologically, using an anti-CD90 antibody.

**RGC-iPSC Coculture**

iPSCs were maintained on reconstituted basement membrane matrix (Matrigel; Corning, Kennebunk, ME, USA) coated on six-well tissue culture plates in serum-free human iPSC growth medium (StemMACS iPSC-Brew XF human; Miltenyi Biotec, Bergisch Gladbach, Germany) containing 1X of antibiotic-antimycotic (Life Technologies; 15240062). After the wells were at least 80% confluent, cells were detached using 0.02% EDTA (Sigma-Aldrich Corp., St. Louis, MO, USA) and resuspended with 2 mL iPS growth medium. Volumes from 10 to 40 μL of the suspension were taken out for coculture.

RGCs for culture were stained with a fluorescent tracking dye (CellTracker Deep Red Dye, 1 μM working concentration; Life Technologies) for 30 minutes at the end of the immunopanning procedure. Immediately after the purification, RGCs were plated on Matrigel-coated 24-well tissue culture plates (Falcon; Corning, Acton, MA, USA) at a concentration of 20,000 cells per well with or without iPSCs in 400 μL serum-free RGC growth medium plus 200 μL iPSC growth medium. For direct-indirect culture comparison, RGCs were plated on plates with hanging inserts (Costar; Corning), and iPSCs were plated either directly with RGCs (direct coculture) or on Matrigel-coated hanging inserts (6.5 mm diameter, 0.4 μm pore size; indirect coculture). RGC growth medium was prepared with Neurobasal (Life Technologies) supplemented with insulin (5 mg/mL; Sigma-Aldrich Corp.), sodium pyruvate (1 mM; Sigma-Aldrich Corp.), t-glutamine (1 mM; Sigma-Aldrich Corp.), triiodothyronine (T3; 40 ng/mL; Sigma-Aldrich Corp.), N-acetyl cysteine (5 mg/mL; Sigma-Aldrich Corp.), GS21 (1:100; GlobalStem, Gaithersburg, MD, USA), brain-derived neurotrophic factor (BDNF; 50 ng/mL; Peprotech, Rocky Hill, NJ, USA), ciliary neurotrophic factor (CNTF; 10 ng/mL; Peprotech), and forskolin (5 mM; Sigma-Aldrich Corp.) as described.9,9 RGCs were cultured or cocultured for 7 days before proceeding to viability tests.

**In Vitro RGC Viability Assays**

RGC viability was measured with Hoechst 33342 (4 μM)/calcein acetoxyethyl (AM; 2 μM) and SYTOX orange (1 μM) combination staining. A 3 × 3-tile 5× microscopy image was captured for each well using an inverted fluorescent microscope (Zeiss Germany, Oberkochen, Germany) 10 minutes after staining. All Hoechst+/Calcein-/SYTOX−/CellTracker− cells were counted as live RGCs, and all Hoechst+/SYTOX+/CellTracker− cells were counted as dead RGCs. Cell numbers were calculated using the automated counting function on FIJI (NIH, Bethesda, MD, USA). In addition, to verify iPSC growth in the indirect coculture, cells in all hanging inserts were also stained with the same protocol and imaged under a Stereo and Zoom Microscope (Zeiss Germany).

**Ex Vivo RGC-iPSC Cotransplantation**

Culture of retinal explants was performed following our previous protocol.5,10 Briefly, fresh retinas were dissected from SD rats and plated on Millicell chamber filters (Millipore, Billerica, MA, USA; diameter, 30 mm; pore size, 0.4 μm), keeping the ganglion cell layer upward. GFP+ RGCs (40,000 cells per explant in 40 μL RGC medium), with or without human iPSCs (40 μL/retina out of 4 mL suspension), were directly transplanted on the top of the retinal explants and allowed to settle on the inner retinal surface by gravity. Twenty minutes after transplantation, the chamber was transferred to a six-well culture plate (Falcon; VWR, Radnor, PA, USA) containing 1.2 mL RGC medium plus 0.6 mL iPSC medium as above. The explants were cultured for 7 days before fixation.

**In Vivo RGC-iPSC Cotransplantation**

GFP+ RGCs, with or without iPSCs, were transplanted intravitreally into rats, as previously described.3,4 Both retinas from a given rat were used, one assigned to the transplant and the other to the cotransplant group. For cotransplantation, after iPSCs were detached and resuspended, they were centrifuged at 300g for 5 minutes and again resuspended with 100 μL iPSC medium. After that, 2 μL from the 100 μL cell suspension or vehicle (stem cell medium) was mixed with 40,000 RGCs in 2 μL RGC medium. The 4-μL mixture was then injected into the vitreous of anaesthetized rats using a 33-gauge Hamilton syringe. The rats were placed on a heating pad until awake and were killed 7 days after transplantation.

**Immunostaining and Imaging**

The retinas from ex vivo transplantation were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich Corp.) for 3 hours and then placed on glass slides. After in vivo transplantation, rats were killed and perfused with PBS followed by 4% PFA. Eyes were dissected and fixed in 4% PFA for 3 hours, and then retinas were dissected and flat-mounted on glass slides. The flat mounted samples, either from the ex vivo or in vivo procedure, were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich Corp.) for 20 minutes, blocked with 5% normal goat serum (Invitrogen, San Diego, CA, USA) in PBS for 1 hour, and then incubated with a mouse monoclonal anti-human nucleus primary antibody (1:200; Millipore, Schwalbach, Germany) overnight at 4°C. Retina samples were rinsed three times with PBS and incubated with rabbit polyclonal Alexa Fluor 488-tagged anti-GFP antibody (1:500; Life Technologies) and Alexa Fluor 647-conjugated secondary antibody (1:500; Life Technologies) overnight. The explants were then rinsed twice, stained with Hoechst (1:500 in PBS) for 15 minutes, rinsed twice again, and sealed under 1.5-mm coverslips with anti-fade mounting medium (ProLong Gold; Life Technologies) before imaging via confocal microscopy.

**Neurite Length Measurement**

Neurites from GFP+ RGCs on retinal explants were measured using the Simple Neurite Tracer plugin in FIJI. Average neurite length was calculated as overall neurite length divided by live RGC number on the explant.

**Statistical Analysis**

Paired t-tests were conducted to identify RGC survival rate differences among control and transplanted groups in vitro, ex vivo, and in vivo, as well as the average neurite length differences between control and variable groups in vivo. If more than two variants were compared, we performed repeated-measures ANOVA, followed by Bonferroni-corrected paired t-tests. P < 0.05 was considered significant.

**RESULTS**

**Human iPSCs Enhance RGC Viability In Vitro**

We first asked whether coculture with iPSCs increased RGC survival in vitro, using a combination of staining for Hoechst, Calcein, SYTOX, and CellTracker (Fig. 1a). After one week of

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**RESULTS**

**Human iPSCs Enhance RGC Viability In Vitro**

We first asked whether coculture with iPSCs increased RGC survival in vitro, using a combination of staining for Hoechst, Calcein, SYTOX, and CellTracker (Fig. 1a). After one week of
RGC-iPSC direct culture, a significant iPSC dose-dependent increase in RGC viability was observed (Fig. 1b; \( P < 0.05; N = 3 \) experiments; \( n > 1000 \) cells/experiment). The coculture with highest iPSC dose demonstrated almost 2.5 times the RGC survival rate as the control.

We next examined whether indirect coculture, in which the iPSCs are kept in hanging inserts away from direct RGC contact (Fig. 2a), would have a similar effect. Indirect RGC-iPSC cocultures also displayed a similar dose-dependent increase in RGC survival, but to a lesser extent than the direct coculture, as shown by live/dead assays (Fig. 2b; \( P < 0.05; N = 3 \) experiments; \( n > 1000 \) cells/experiment). Thus, RGC-iPSC indirect coculture also promotes RGC survival, but less than observed in direct coculture, suggesting the hypothesis that at least part of the survival effect is mediated by soluble factors released by the iPSCs. In addition, we found that RGCs showed slightly poorer survival in culture plates with hanging inserts, even if no cells were added into the inserts. This finding was further validated through head-to-head tests (results not shown).

### iPSCs Promote Transplanted RGC Survival After Ex Vivo and In Vivo Transplant to Adult Retina

We next used an ex vivo retinal explant model of RGC transplant to examine whether iPSCs would influence RGC survival after transplant. Transplanted RGCs were identified by GFP expression; transplanted iPSCs were identified by expression of a human nuclear antigen detected by immunofluorescence staining (Figs. 3a, 3b). Most live iPSCs were detected in oval-shaped or irregular colonies, whereas others were widely scattered; most live transplanted RGCs, as well as their neurites, were found in close proximity to iPSCs (Figs. 3a, 3b). We quantified transplanted RGC survival with and without cotransplanted iPSCs and found significantly improved transplanted RGC survival after RGC-iPSC cotransplant to adult retinas ex vivo (Fig. 3c, \( P < 0.001; N = 3 \) experiments; \( n = 40,000 \) cells/experiments).

We also asked whether the GFP \(^+\) RGCs were “real” transplanted cells, because of increasing concerns about cell-cell cytoplasmic exchange with which the results would be illusory. To address whether cytoplasmic exchange occurred between the two types of transplanted cells, we screened all the retinal explants under high magnification (40×) for the presence of GFP\(^+\)/human nucleus\(^+\) (i.e., double-positive) cells but did not find any (e.g., inset magnified in Fig. 3b).

Finally, we asked whether cotransplant of iPSCs would increase RGC survival after transplant in vivo, injecting transplanted cells intravitreally and then harvesting retinas for immunostaining and quantification 7 days later. Twenty percent of these transplantation experiments showed retinal engraftment after intravitreal delivery, similar to our prior in vivo transplant experiments, and in the successful 20%, transplanted cells typically distributed on approximately one-fifth to one-third of the host retina. In contrast to the explanted retina model, transplanted RGCs found at the retina after in vivo transplant were still close to but not restricted to the proximity of iPSCs, which themselves were less likely to grow in colonies after in vivo transplant (Figs. 4a, 4b). Again, no cytoplasmic exchange between two transplanted cells was observed (Fig. 4b). Similar to our ex vivo transplantation results, RGCs transplanted with iPSCs in vivo demonstrated a significantly higher survival (Fig. 4c; \( P < 0.001; N = 3 \) experiments; \( n = 40,000 \) cells/experiments).
experiments; \( n = 40,000 \text{ cells/experiment} \). Cotransplantation also promoted a significantly higher average neurite length of transplanted RGCs compared with RGC-only transplantation (Fig. 4d; \( P < 0.001; N = 3 \text{ experiments}; n > 26 \text{ cells/experiment} \)). Thus, RGC-iPSC cotransplantation promotes transplanted RGC survival and neurite extension in vivo.

**DISCUSSION**

The present study demonstrates that human iPSCs possess a potent neuroprotective capacity capable of enhancing mouse donor RGC survival on adult rat retinas after transplant ex vivo and in vivo, and this may be attributable at least in part to iPSCs’ secretion of one or more neuroprotective compounds. These data extend previous findings on stem cell transplantation for endogenous (host) RGC neuroprotection. For example, MSCs have been shown to promote endogenous RGC survival in experimental glaucoma models,\(^5,7\) and a similar RGC neuroprotection in a rat optic nerve crush model was observed with human umbilical cord blood stem cells transplanted intravitreally.\(^11\) Our study indicates that iPSCs also can confer neuroprotection for RGCs in vitro and in vivo, although future experiments could directly compare iPSCs’ and MSCs’ or other transplanted cells’ capacity in animal models of glaucoma or trauma.

The survival rates of transplanted RGCs in this study were lower than in some of our previous data,\(^3\) potentially due to the differences between the cell medium, syringes and needles being used. We made these modifications in this study, as in our recent publication,\(^4\) because we found the current protocol enhanced the proportion of successful transplants into the retina, although the survival and neurite growth for example may have suffered in return. Also, the mixture of the RGC- and stem cell–optimized media is hypothesized to improve the injection milieu for both RGCs and iPSCs, although it might affect RGC survival and integration on the retina after injection.

Recently, cell-to-cell cytoplasmic exchange has been widely discussed in interpreting cell transplant experiments,\(^12,15\) as it could confound interpretation of what is a transplanted cell. Here we studied this question by looking for coimmunostaining of GFP marking RGCs and human nuclear antigen marking
iPSCs both ex vivo and in vivo and found no evidence for cytoplasmic exchange. Although we did not specifically stain for cytoplasmic exchange between donor RGCs and recipient host cells, we did not find any GFP⁺ RGCs that resembled typical native RGCs in terms of small round-shaped cell morphology or axon targeting to the optic nerve head. Although this could have occurred in rare instances, such cytoplasmic exchange has not yet been documented between postmitotic neurons. Another interesting finding is that, although most transplanted iPSCs retained typical stem cell-like morphologies, some of them, especially those that were dissociated, transformed into different morphologies, suggesting at least some measure of differentiation after transplant. Whether and into what those iPSCs differentiated presents another question to be studied in future experiments, ideally using lines that express cell type-specific markers after differentiation.¹⁴

These data also suggest that iPSCs may promote transplanted RGC survival through secretion of neuroprotective compounds, consistent with previous MSC studies.₅ ₇ MSCs secrete a number of neuroprotective proteins, with platelet-derived factor regarded as the strongest one.¹⁵ Other studies have found that some stem cells, including MSCs and neural stem cells, secrete exosomes that carry neuroprotective compounds.¹⁶ However, the mechanisms of iPSC’s neuroprotective capacity are still not clear. In addition, our data showed that, although RGC-iPSC indirect coculture enhanced RGC survival, it was less potent than direct coculture, suggesting that cell-cell interaction might also confer additional promotion of RGC survival.

To our knowledge, this study is the first cell-level approach for protecting primary grafted CNS neurons. Transplanted RGCs, unlike endogenous RGCs, are exposed in the vitreous to a hostile environment and receive no trophic support from...
other retinal cells before they integrate into host retina, and
perhaps form synapses with host cells. Limited survival rates
likely limit retinal integration after transplant. We also noted
that the transplanted RGCs showed much less axon targeting
reaching the optic nerve head by 1 week, in contrast to a few
RGCs that successfully grew axons to the optic nerve head at
longer survival times in our previous findings, highlighting the
need in future work to extend these time courses. Here our
study demonstrates that human iPSCs enhance donor RGC
survival as well as neurite growth of donor RGCs after
transplant. On the other hand, many more questions need to
be addressed with regard to potential therapeutic application (e.g., whether cotransplanted iPSCs enhance other key
measures of RGC including integration synapse formation,
electrophysiological response to light, neurite growth and
orientation toward the optic nerve, and what level of
transplant efficiency is needed to protect or restore function
in normal and disease model recipient retinas), which should
be examined in future studies.

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