Stem Cell Research & Therapy

RESEARCH

Open Access

Retinal ganglion cells induce stem cell-derived neuroprotection via IL-12 to SCGF-β crosstalk

Qing Xia[1], Kun-Che Chang[2,3], Yanan Sun[1], Michael Nahmou[2], Takahiko Noro[2,4], Yun Cheng[1], Xiangmei Kong[1], Xiaofen Mo[1*], Jeffrey L. Goldberg[2*] and Suqian Wu[1,2*]

Abstract

Background Stem cell-derived secreted factors could protect neurons in neurodegenerative disease or after injury. The exact neuroprotective components in the secretome remain challenging to discover. Here we developed a cell-to-cell interaction model to identify a retinal ganglion cell (RGC)-protective factor derived from induced pluripotent stem cells (iPSCs).

Methods Primary RGCs were co-cultured with iPSCs or treated with iPSC-conditioned media in vitro. Cell viability were assayed using live-cell staining, and culture supernatant were analyzed via multiplexed antibody-based assays and ELISA. In vivo tests were carried out under mouse optic nerve crush model and RGC transplantation study in rats. Paired *t*-tests were used for data analysis between two groups.

Results RGC viability was significantly enhanced when iPSCs were first stimulated with RGC-derived supernatant before iPSC-conditioned medium was collected and added into RGC culture. A significant increase of stem cell growth factor-beta (SCGF-β) concentration was detected in the latter conditioned medium. SCGF-β enhanced RGC survival in vitro and in vivo, and RGC-derived interleukin-12(p70) (IL-12[p70]) promotes secretion of iPSC-derived SCGF-β. Downstream of this IL-12(p70)-to-SCGF-β axis, ngn2 was significantly upregulated, and was found both necessary and sufficient for RGC survival.

Conclusion This study addresses a longstanding question of how neurons and stem cells interact to promote neuroprotection, and define a novel molecular interaction pathway whereby RGC's secretion of IL-12(p70) enhances iPSCs' secretion of SCGF-β, and SCGF-β protects RGCs via upregulating ngn2, suggesting that neurons may call on stem cells for their own protection.

*Correspondence:
Xiaofen Mo
xfmo@fudan.edu.cn
Jeffrey L. Goldberg
jlgoldbe@stanford.edu
Suqian Wu
sqwu15@fudan.edu.cn
[1] Shanghai Key Laboratory of Visual Impairment and Restoration, Department of Ophthalmology and Vision Science, Eye & ENT Hospital, Fudan University, Shanghai 200031, China
[2] Spencer Center for Vision Research, Byers Eye Institute, Stanford University School of Medicine, Palo Alto, CA 94304, USA
[3] Department of Ophthalmology and Neurobiology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA
[4] Department of Ophthalmology, Jikei University School of Medicine, Tokyo 105-8461, Japan

Introduction

Central nervous system (CNS) neuron degeneration occurs in many diseases such as glaucoma, and those neurons are generally not able to survive or regenerate after injury. Thus, there is an unmet need to develop neuroprotective strategies to prevent neuron loss in neurodegenerative diseases.

In the past two decades, many studies focused on cell-based approaches to aid endogenous CNS neuron loss, and various stem cell populations have been shown to promote neuroprotection via their secretomes (1, 2). Therefore, stems cells have now been regarded not only as an endless resource for cell replacement therapy but also as a pharmacological storehouse for bioactive molecule production and delivery (3, 4). For example, secretomes from mesenchymal stem cells (MSCs) have been shown great potentials for regenerative therapies in cardiovascular, genitourinary and oncologic disorders (5–7), and displayed neuroprotective ability throughout the CNS under a number of injuries including ischemic, traumatic and inflammatory (8). In particular, MSCs promoted host retinal ganglion cell (RGC) survival in an experimental glaucoma model by secreting neuroprotective factors such as platelet-derived growth factor (PDGF) (9).

Our previous study identified pluripotent stem cells (iPSCs) as another potent source of neuroprotection, at least partially through secreting as yet unidentified diffusible neuroprotective factors (10). Considering the translational challenges of stem cell transplant in vivo, discovering iPSC-derived neuroprotective factors and/or regulating the associated signaling pathways may be safer and more effective treatments for CNS neuron degeneration, but little is known about how injured or degenerating neurons might interact with donor stem cells to maximize their own survival. Here we uncovered an intercellular interaction-conferred induction of neuroprotective effect of iPSCs on RGCs, and identified key signaling molecules mediating neuron-induced, stem cell-derived neuroprotection.

Materials and methods

Animals, reagents, plasmids and viruses

All use of animals conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Research, adheres to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and was approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee of Fudan University. Wild-type C57BL/6 mice of varying ages and 10- to 12-week-old Sprague–Dawley (SD) rats, both male and female, were obtained from Charles River (Portage, MI, USA). GFP-tagged mice, both male and female, were bred from the C57BL/6-Tg(CAGEGFP)1Osb/J strain obtained from Jackson Laboratories (Bar Harbor, ME, USA). All animals were euthanized in accordance with the approved animal protocols at Fudan University. Postnatal mice were euthanized by guillotine decapitation, while adult mice and rats were euthanized by cervical dislocation under anesthesia detailed in the animal study methods sections.

Human iPSCs, Line 297, were kindly provided by Stem Cell Bank, Chinese Academy of Sciences, and cell Passage 16–18 were used in the study. Recombinant human stem cell growth factor-beta (SCGF-β) and mouse interleukin-12(p70) (IL-12[p70]) were purchased from Peprotech Inc. (Catalogue [Cat.] #100-22B and #210–12; Rocky Hill, NJ, USA).

Plasmids for neurogenin 2 (ngn2)-T2A-EGFP, ngn2-T2A-mCherry, ngn2 short hairpin RNA (shRNA)-mCherry, control EGFP, control mCherry, and scrambled control shRNA-mCherry were obtained and virus-prepared from Vector Builder (Guangzhou, China). All the plasmids with an mCherry label were lentivirus-packaged (11) for in vitro transfection, and those with an EGFP label were packaged with adeno-associated virus 2 (AAV2) (12) for in vivo transfection.

RGC purification, culture, and lentiviral transfection

RGCs were purified from 2- to 4-day postnatal mice by two-step immunopanning as previously described (13–15). Briefly, retinas were freshly extracted and dissociated with papain (65 units; Cat. #LS003124; Worthington, Freehold, NJ, USA), followed by removal of contaminating macrophages and endothelial cells by immunopanning using an anti-macrophage antibody (Cat. #AIAD31240; Accurate Chemical and Scientific Corporation, Westbury, NY, USA). RGCs were selected from the macrophage-depleted cell suspension immunologically, using an anti-CD90 antibody (Cat. #MCA02R; Bio-Rad Laboratories, Hercules, CA, USA). Purified RGCs were plated on reconstituted basement membrane matrix (Matrigel; Cat. #354,277; Corning, Kennebunk, ME, USA) -coated 24-well tissue culture plates (Falcon; Cat. #353,047; Corning, Acton, MA, USA) at a concentration of 30,000 cells per well in 500 μL serum free RGC growth medium, and lentiviruses were then immediately added at a concentration of 1:5000 and kept for 24 h. The cells were incubated in 37 °C and 10% CO_2. RGC growth medium was prepared with Neurobasal (Cat. #21,103–049; Life Technologies Inc., Gaithersburg, MD, USA) supplemented with sodium pyruvate (1mM; Cat. #11,360–070; Sigma), insulin (5 mg/mL; Cat. #91077C; Sigma), L-glutamine (1 mM; Cat. #25,030–081; Sigma), triiodothyronine (T3; 40 ng/mL; Cat. #T2877; Sigma), N-acetyl cysteine (5 mg/mL; Cat. #A8199; Sigma), B-27

Plus (1:100; Cat. #A3582801; Life Technologies), forskolin (5 mM; Cat. #F6886; Sigma), brain-derived neurotrophic factor (BDNF; 50 ng/mL; Cat. #450–02; Peprotech), ciliary neurotrophic factor (CNTF; 10 ng/mL; Cat. #450–13; Peprotech), and fibroblast growth factor (FGF; 20 ng/mL; Cat. #100-18B; Peprotech) as previously described (13, 14). For lentiviral transfection, lentivirus containing specific plasmids were added into the culture media at a final concentration of ~ 10^5 TU/mL on Day 0 of culture.

RGC-iPSC coculture

RGC-iPSC coculture was performed as previously described (10). Briefly, iPSCs were maintained on Matrigel-coated six-well tissue culture plates in serum-free human iPSC growth medium (mTeSR1, Cat. #85,850; STEMCELL Technologies, Vancouver, Canada). After the wells were at least 80% confluent, cells were detached using 0.02% EDTA (Cat. #E8008; Sigma) and resuspended with 2 mL iPSC growth medium. Volumes of 40 µL of the suspension were taken out for coculture. RGCs were seeded on wells with Matrigel-coated hanging inserts (6.5 mm diameter, 0.4 µm pore size; Transwell; Cat. #3470; Corning), and iPSCs were plated on the hanging inserts. For negative control, RGCs were cultured on the wells with the same Matrigel-coated hanging inserts with no cells plated on. The co-culture medium was a mixture of 400 µL serum-free RGC growth medium plus 200 µL iPSC growth medium. The culture supernatants illustrated in Fig. 2A were collected during every medium change post culturing and were then pooled for conducting subsequent assays. The components of all the media and supernatants used and produced in the study were shown in Table 1.

Culture supernatant assays

Culture supernatants were kept in − 80 °C before preceding to the subsequent tests. The supernatants were analyzed using multiplexed antibody-based cytokine assays (Cat. #12,007,283 and #M60009RDPD; Bio-Plex, Bio-Rad) according to the manufacturer's instructions. All the assays were analyzed using a Luminex 200 platform and data were collected using Bio-Plex Manager software. To further confirm the concentrations of certain proteins, ELISA kits (Cat. #EH11335 and #EM30277M; Biotech Well, Shanghai, China) were used according to manufacturer's recommendations. The assays were repeated three times using independent samples, and each sample was run in triplicate.

Generation of CLEC11A-knockout iPSCs

Crispr ribonucleoprotein (RNP) electroporation system was used in this study. Two guide RNAs (gRNAs) were designed to target exon 1 and 2 of *CLEC11A* (Table S1). 2×10^6 iPSCs were transfected with an RNP complex of 15 µg recombinant Cas9 protein (Cat. #A36499; Thermo Fisher, Waltham, Massachusetts, US) and 5 µg/µL of each gRNA in 100 µL Opti-MEM buffer (Cat. #31,985,062; Thermo Fisher), and the electroporation condition program was 700 V, 30 ms. After electroporation, iPSC clones were manually isolated, genomic DNA extracted, and PCR amplified with primers designed around the target site (Table S1). iPSC clones were analyzed by Sanger sequencing to confirm *CLEC11A* gene disruption. Off-Spotter (https://cm.jefferson.edu/Off-Spotter/) was used to predict off-targets for the selected gRNA and the top 10 off-targets were assessed with Sanger sequencing, which detected no changes (data available upon request).

In vitro *RGC viability assays*

Live RGCs were stained with calcein acetoxymethyl (Calcein AM, Calcein; 2 µM; Cat. #C3099; Life Technologies) and Hoechst 3334 (Hoechst, 4 µM; Cat. #H3570; Life Technologies). For RGCs without mCherry expression, the cells were also stained with SYTOX Orange Nucleic

Table 1 Terms and components of culture medium/supernatants used in the study

Term	Components	Color/illustration in Fig. 2
RGC medium	Neurobasal supplemented with sodium pyruvate, insulin, L-glutamine, triiodothyronine, N-acetyl cysteine, B-27 Plus, forskolin, BDNF, CNTF, and FGF	Sky blue
iPSC medium	mTeSR1 medium	Pink
iPSC-RGC coculture medium	Mix of 2/3 RGC medium and 1/3 iPSC medium	Pink background with sky blue slashes
RGC supernatant	The RGC medium cultured with RGCs for 24h	Navy blue
iPSC supernatant	The iPSC medium cultured with iPSCs for 24h	Dark red
iPSC-conditioned medium	The iPSC-RGC coculture medium cultured with iPSCs for 24h	Dark red background with sky blue slashes
RGC-iPSC-conditioned medium	Mix of 2/3 RGC supernatant and 1/3 iPSC medium, cultured with iPSCs for 24h	Dark red background with navy blue slashes

RGC retinal ganglion cell, *iPSC* induced pluripotent stem cell, *BDNF* brain-derived neurotrophic factor, *CNTF* ciliary neurotrophic factor, *FGF* fibroblast growth factor

Acid Dye (SYTOX, 1 μM; Cat. #S11368; Life Technologies) to validate dead population. Cell viability was measured as described in our previous study (10), with a few modifications. The stained cells were incubated under 37 °C and 10% CO_2 for 20min before proceeding to microscopy. A 3×3-tile 5×microscopy image was captured for each well using an inverted fluorescent microscope (Zeiss Germany, Oberkochen, Germany) 10 min after staining. All Calcein$^+$ cells with a blurry light blue Hoechst staining were counted as live RGCs, and all cells with a sharp dark blue Hoechst staining were counted as dead RGCs. Cell numbers were calculated using the automated counting function on FIJI ImageJ software (National Institutes of Health, Bethesda, MD, USA). A minimum of $N=3$ tests, each with $n>1000$ cells, were performed for each assay.

Quantitative real-time PCR

Total RNAs were isolated from cells in culture according to the manufacturer's protocol (RNeasy Microarray Tissue Micro Kit; Cat. #74,004; Qiagen, Hilden, Germany). 2 μg RNAs were reverse transcribed using First Strand cDNA synthesis Kit (Invitrogen). Primers were designed using NCBI Primer-BLAST, based on the sequences from the GenBank (www.ncbi.nlm.nih.gov/genbank/) (Table 2). QuantiNova SYBR Green PCR Kit (Cat. #208,054; Qiagen) were used according to the manufacturer's instruction. The thermocycler parameters were 95°C for 2 min, followed by 45 cycles of 95°C for 5s and 60°C for 10s. *GADPH* was used as internal control. All experiments were repeated at least three times for statistical comparison. The assays were repeated three times using independent samples, and each sample was run in triplicate.

Optic nerve crush and intravitreal injection

The right optic nerves of adult 5-week C57BL/6 mouse (either sex) were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg), and were exposed and crushed for 3 s with a Dumont #5 forceps (91,150–20, FST) ~1.5 mm behind the globe, keeping the blood supply intact. Mice with any significant postoperative complications (e.g., retinal ischemia, cataract) were excluded from further analyses. For SCGF-β injection, 10 ng of SCGF-β was dissolved in 2 μL RGC growth media and injected intravitreally into the mice on Day 0, 2, and 4 after the optic nerve crush. The injections were performed posterior to the pars plana with a 33-gauge needle connected to a 5 μL Hamilton syringe, and were repeated 2 days and after crush. The mice were placed on a heating pad until awake and were sacrificed under general anesthesia 5 days post crush and prepared for retinal flat-mounts. For AAV2-ngn2-EGFP (5×10^{12} TU/mL) intravitreal injection, the injections were performed 2 weeks before optic nerve crush, and the retinas were collected 2 weeks post crush.

Table 2 Primer sequences used for qPCR tests in this study

Species	Target Gene	Primer Sequence (5′ – 3′)	
		Forward	Reverse
Human	*CLEC11A*	gaggaagaggaggaggaagc	aagtgacgatgtcctcaggg
	GAPDH	gcgagatccctccaaaatcaa	gttcacacccatgacgaacat
Mouse	*Neurog2*	cttctccaccttcctcctgg	ctgtatggggacgtggagtt
	Clec11a	ctggggtgggaaatgaggat	agaagaaggtgctgtggtga
	GAPDH	aggtcggtgtgaacggatttg	tgtagaccatgtagttgaggtca

RGC transplantation

GFP-tagged mouse RGCs at Day 1 post culture and lentiviral infection of ngn2-mCherry were resuspended with 400 μL Accutase (Cat. #AT-104; Innovative Cell Technologies, San Diego, CA, USA), centrifuged at 700 rpm for 5 min, and resuspended in RGC medium at a concentration of 20,000 cells/μL for cell transplantation as previously described (10, 16). Note that the Accutase-treated cell detachment during cell preparation disrupted all RGC neurites according to our observation and could not be performed after 2 days in vitro with any surviving RGCs. Briefly, 2 μL from the cell suspension was injected into the vitreous of ketamine (80 mg/kg)/xylazine (10 mg/kg)-anaesthetized adult SD rats using a 33-gauge Hamilton syringe. We chose rats instead of mice because (1) the vitreous cavity of mouse is too small to keep the injection successful, and (2) it is an opportunity to test the results of cross-species transplantation which are often studied in retinal cell therapies. The rats were placed on a heating pad until awake and were killed under anesthesia 7 days after transplantation.

Immunostaining and imaging

The immunostaining and imaging were performed as previously described (10). In brief, after optic nerve crush or RGC transplantation, animals were sacrificed and perfused with PBS followed by 4% paraformaldehyde (PFA). Eyes were dissected and fixed in 4% PFA for 3 h, and then retinas were dissected and flat-mounted on glass slides. The flat mounted samples were permeabilized with 0.3% Triton X-100 (Cat. #T9284; Sigma-Aldrich) for 20 min, blocked with 5% normal goat serum (Cat. #16,210,064; Invitrogen, San Diego, CA, USA) in PBS for 1 h.

For the retinas from optic nerve crush, the samples were incubated with a rabbit polyclonal anti-RNA

Binding Protein, mRNA Processing Factor (RBPMS) antibody (1:500; Cat. #1832-RBPMS; PhosphoSolutions, Aurora, CO, USA) overnight at 4 °C, rinsed three times with PBS, and then incubated with Alexa Fluor 555-tagged secondary antibody (1:500; Cat. #A-21428; Life Technologies) overnight, again.

For the retinas from the transplantation, the explants were incubated with a mouse monoclonal anti-mCherry antibody (1:500; Cat. #ab125096; Abcam, Cambridge, MA, USA) overnight at 4 °C, rinsed three times with PBS, and then incubated with Alexa Fluor 555–tagged secondary antibody (1:500; Cat. #A-21422; Life Technologies) and Alexa Fluor 488–tagged anti-EGFP antibody (1:500; Cat. #A-21311; Life Technologies) overnight, again. The EGFP and mCherry antibodies were used to detect the entire transplanted and ngn2-overexpressing donor mouse RGCs, respectively. All the retinas from two overnight incubations were then rinsed twice, stained with Hoechst 3334 (1:500 in PBS) for 15 min, rinsed twice again, and sealed under 1.5-mm coverslips with anti-fade mounting medium (ProLong Gold; Cat. #P36930; Life Technologies) before imaging via confocal microscopy.

The RGC quantification for retinas from optic nerve crush was performed as previously described (17). Briefly, the retinas were divided into four quadrants, and one 200×200 μm micrograph was captured randomly from each of the four peripheral fields 3 mm from the optic nerve head. RBPMS-positive cells were counted manually in a masked fashion and analyzed as cells per micrograph.

Neurites from GFP^+ RGCs on retinas from in vivo transplant experiments were measured using the Simple Neurite Tracer plugin in FIJI as previously described (10). Average neurite length was calculated as sum of neurite length divided by the living RGC number on the explant. The assays were repeated three times using independent samples.

Statistical analyses

All animals were matched by gender, genotype and age. Mice and rats were randomly assigned to different experimental and control groups, as well as processing and analyses of the samples, were performed in blind. Paired *t*-tests were used to identify RGC survival rate differences among control and variable groups in vitro and in vivo, as well as the average neurite length differences between control and ngn2-overexpressing groups after transplant in vivo. Student *t*-tests were conducted to identify concentration differences of specific cytokines from ELISA, as well as the expression level differences of specific mRNAs in qRT-PCR tests. $P<0.05$ was considered significant.

Results

RGC-protective effect of iPSCs is induced by RGC coculture or RGC secreted signals

As non-proliferative cells, RGCs gradually die on their own once isolated in vivo, even under a delicate culture condition. We first asked whether iPSC supernatant directly promotes RGC survival in vitro, using a counterstaining for Calcein, SYTOX, and Hoechst (Fig. 1A). After 5 days of culture, no change in RGC viability in response to iPSC culture supernatant was observed (Fig. 1B; $P<0.05$). Consistent with our previous findings (10), iPSC-RGC indirect coculture significantly enhanced RGC viability. These results suggest that iPSC-RGC interaction, not iPSCs alone by default, promotes RGC survival.

We next asked if an RGC-derived secretome induced the iPSCs to protect RGCs. After 5 days of culture in a variety of specific conditioned media (Table 1 and Fig. 2A), a significant RGC viability increase was only observed in iPSC-derived supernatants in which iPSCs were stimulated by RGC conditioned medium (condition d in Fig. 2B; $P<0.05$). Note that a combination of RGC-conditioned media and iPSC-conditioned media without RGC-derived stimulation had no effect on RGC survival (condition b in Fig. 2B). Thus, iPSC's neuroprotective effect is induced by soluble signals from RGCs.

Identification of a novel iPSC-derived RGC protective factor

To identify candidate signals responsible for iPSC-derived RGC neuroprotection, we leveraged this finding that the neuroprotectant is induced in iPSCs after stimulation by RGC conditioned media. The concentrations of 47 cytokines in the RGC-iPSC-conditioned medium (d in Fig. 2B) and the iPSC-conditioned medium (c in Fig. 2B) were tested via multiplexed antibody-based assays. A significantly higher concentration of SCGF-β was observed (Fig. 3A; $P<0.05$). This finding was then validated through ELISA (Fig. 3B; $P<0.05$). Furthermore, CLEC11A (SCGF encoding gene) mRNA was not detected in RGCs using RT-qPCR (data available upon request), demonstrating that the detected SCGF-β was derived from the iPSCs.

We next asked whether human iPSC-derived SCGF-β is sufficient and necessary for iPSC-derived RGC neuroprotection. After 5 days of culture, a significant RGC viability increase was observed in RGCs cultured in medium containing 10 ng/mL human SCGF-β (Fig. 3C; $P<0.05$). In contrast, *CLEC11A*-KO iPSCs showed significantly decreased promotion of RGC survival compared with the parent iPSC line in the RGC coculture assay (Fig. 3D; $P<0.05$). Thus, SCGF KO depletes the neuroprotective capacity of iPSCs.

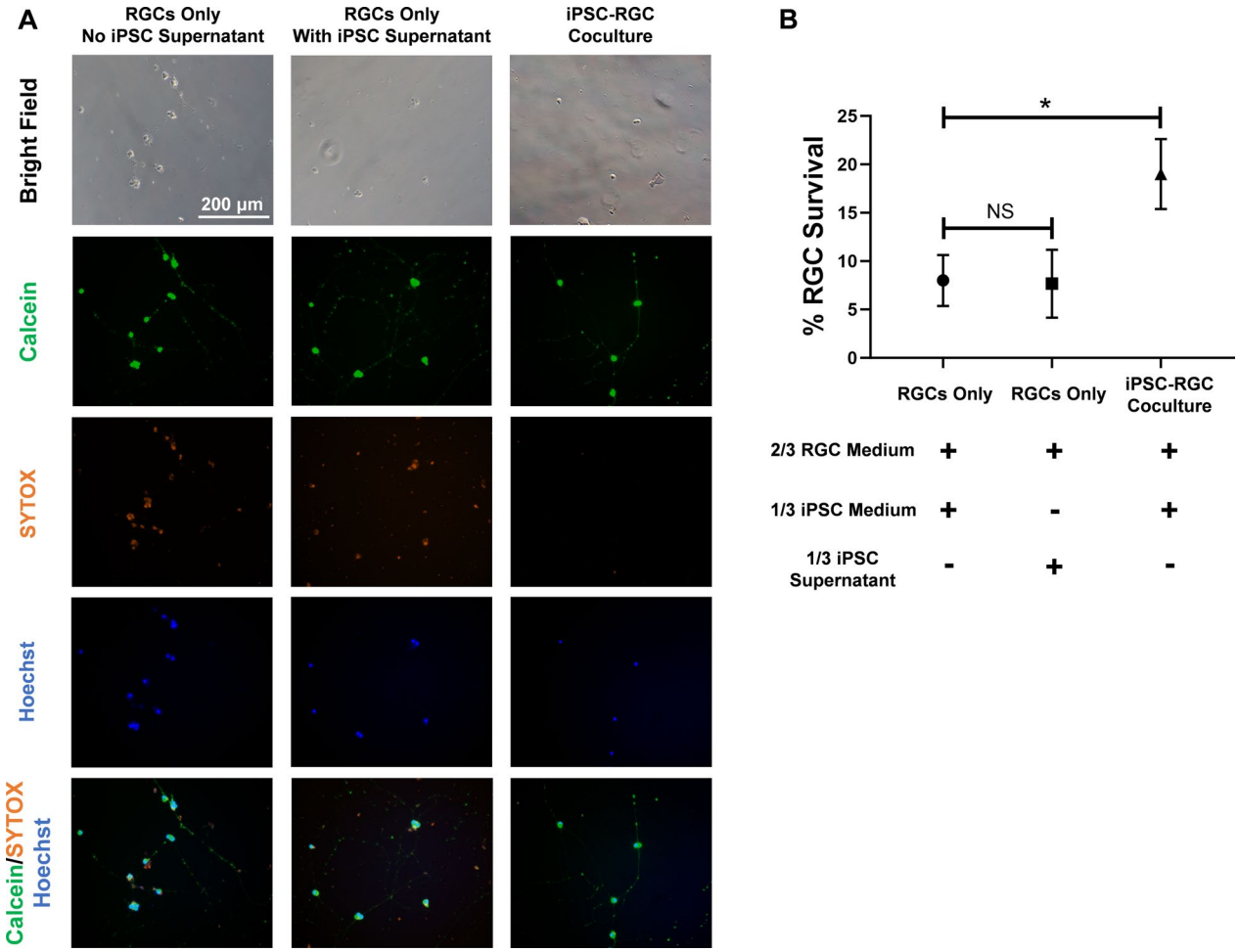

Fig. 1 iPSC-RGC coculture, but not iPSC-conditioned supernatant, promotes RGC survival. **A** Mouse RGCs were cultured alone or indirectly cocultured with iPSCs (plated in hanging inserts) in different medium described in (**B**), and after 5 days, the coculture promoted RGC survival, but iPSC-derived supernatant did not. All the Calcein$^{\pm}$/SYTOX^{+}/Hoechst^{+} dots are regarded as dead RGCs. All the culture media were replaced every 2 days. *$P < 0.05$. Error bar denotes SD. NS, nonsignificant

Finally, we asked whether SCGF-β promotes RGC survival in vivo after optic nerve crush, and found a significantly greater RBPMS^{+} RGC density 5 days post optic nerve crush in mice treated with SCGF-β (Fig. 4A-B; $P < 0.05$), demonstrating the in vivo neuroprotective capacity of SCGF-β.

RGCs enhance iPSC's SCGF-β release via *IL-12(p70)*

To explore the molecular basis for RGC-mediated induction of this neuroprotective iPSC phenotype and upregulation of iPSC's SCGF-β release, we again used a multiplexed antibody-based assay. One out of 23 mouse cytokines, IL-12(p70), was detected in RGC supernatant and not detected in RGC medium (Fig. 5A). This finding was further validated through ELISA (Fig. 5B). We found a dose-dependent induction of SCGF-β secretion in iPSC culture, with a peak at 5 ng/mL of IL-12(p70) (Fig. 5C; $P < 0.05$). RT-qPCR similarly showed significantly increased CLEC11A mRNA expression in iPSCs after IL-12(p70) treatment, with the same peak (Fig. 5D; $P < 0.05$). Importantly, IL-12(p70) did not directly promote RGC survival until concentrations 16-fold higher than those that induced iPSC secretion of SCGF-β (Fig. 5E; $P < 0.05$). Thus RGC-derived IL-12(p70) upregulates iPSC's SCGF-β expression and secretion.

iPSC-derived SCGF-β promotes RGC survival through upregulating ngn2

From our qRT-PCR results, we found that ngn2, a key neurogenesis regulator, was significantly upregulated in RGCs co-cultured with iPSCs as well as in those treated with SCGF-β (Fig. 6A, B; $P < 0.05$). These findings suggested the hypothesis that ngn2 is involved in the neuroprotective effect of iPSC-derived SCGF-β. With

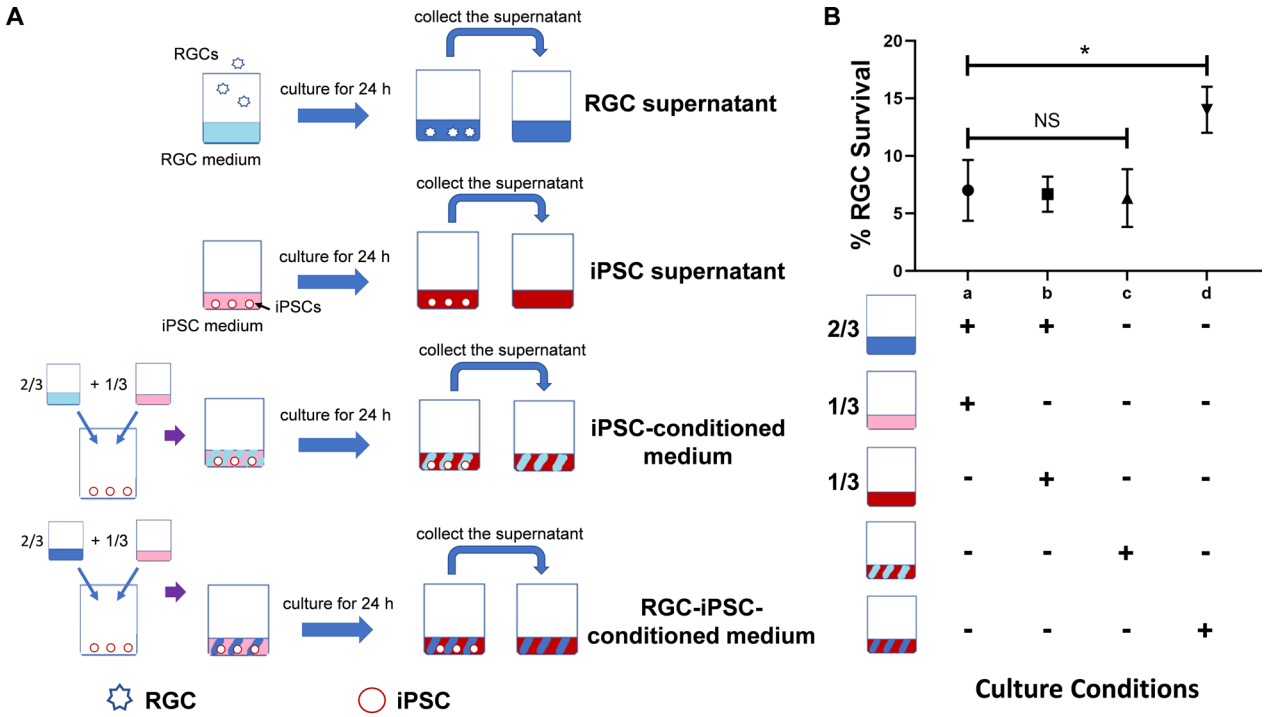

Fig. 2 RGC supernatant induces iPSC's neuroprotective effect. **A** Schematic procedure of preparing specific supernatants and conditioned media. RGCs are shown as blue stars and iPSCs were as red circles. The supernatants were collected every 24 h post seeding. **B** After 5-day culture, only RGCs in RGC-iPSC-conditioned medium yielded significantly greater viability than the control. *$P<0.05$. Error bar denotes SD

exogenous expression, ngn2 led to significantly greater RGC viability after 1 week in vitro (Fig. 6C, D; $P<0.05$). Conversely, the RGC-protective effect of SCGF-β was lost in ngn2-knockdown RGCs (Fig. 6E). These results demonstrate that ngn2 is necessary and sufficient for SCGF-β to promote RGC survival in vitro.

ngn2 overexpression protects endogenous and transplanted RGCs in vivo

We next asked whether ngn2 is sufficient to promote RGC neuroprotection in vivo. We observed a significantly greater RBPMS$^+$ RGC density 2 weeks after optic nerve crush in mice intravitreally injected with AAV2-ngn2 (Fig. 7A, B; $P<0.05$), indicating that ngn2 overexpression protects RGC survival after optic nerve injury.

Considering this neuroprotective effect on endogenous RGCs, we explored whether overexpressing ngn2 in RGCs transplanted intravitreally in adult rats would increase donor cell survival. After 7 days, 20% of these transplantation experiments showed retinal engraftment after intravitreal delivery of donor RGCs, with transplanted GFP$^+$ RGCs typically distributed on approximately one-fourth to one-third of the host retina, similar to our prior in vivo transplant experiments (10, 16). Many live RGCs were detected in irregular colonies, whereas others were widely scattered (Fig. 7C). Transplanted ngn2-overexpressing RGCs demonstrated a significantly higher survival (Fig. 7D; $P<0.05$). Overexpression of ngn2 also promoted a significantly longer average neurite length of transplanted RGCs (Fig. 7E; $P<0.05$). Thus, ngn2 overexpression promotes RGC survival in vivo, both of host RGCs after optic nerve injury, and of donor RGCs after retinal transplant.

Discussion

Stem cells are of broad interest for their ability to promote regeneration in many tissues, but it is often observed that their reparative capacity outstrips the number of persistent donor cells, suggesting that a significant component of stem cells' positive effects may be through signaling to local, host tissue. For example, considerable data identifies therapeutic effects of stem cell transplant after myocardial injuries, due in part to a stem cell-derived secretome. However, molecular understanding of iPSC's protective effect in neurodegeneration is still limited (18), especially compared with numerous reports characterizing neuroprotective capacities of MSC-derived extracellular vesicles (19, 20). Our previous study indicated the neuroprotective capacity of iPSCs for RGCs in vitro and in vivo, and suggested soluble signaling between RGCs and stem cells was required to maximally stimulate stem cell-mediated neuroprotection

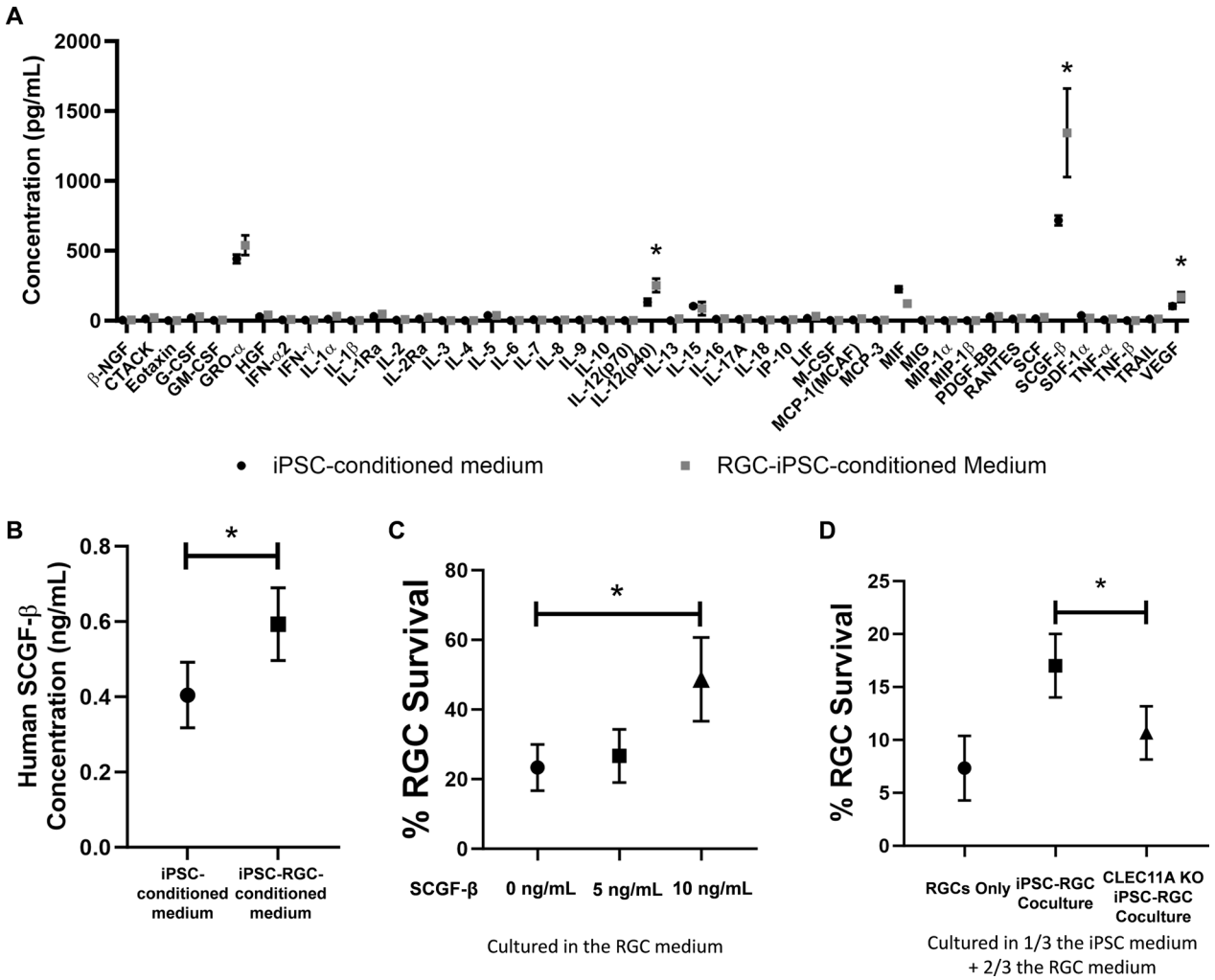

Fig. 3 Identification of a novel RGC-protective factor, SCGF-β. **A**-**B** A significantly enhanced concentration of SCGF-β was detected in the RGC-iPSC-conditioned medium via multiplexed antibody-based assays and confirmed by ELISA. **C** A significantly greater RGC viability was observed in RGCs treated with 10 ng/mL SCGF-β after 5 days in vitro. **D** When *CLEC11A* (SCGF encoding gene) was knocked out in iPSCs, the RGC survival-promoting effect of iPSC coculture was depleted. **P* < 0.05. Error bar denotes SD

(10). Here we have identified iPSC-derived SCGF-β and downstream induction of ngn2 in RGCs as critical for stem cells' neuroprotective effect, and furthermore demonstrate that degenerating RGCs themselves signal iPSCs through RGC-secreted IL-12(p70) to take on the neuroprotective phenotype (Fig. 8).

SCGF, also known as C-type lectin domain family 11 member A (Clec11a) or osteolectin, was initially identified as a growth factor for hematopoietic stem/progenitor cells (21). SCGF-β is the shorter isoform of full-length SCGF (also referred as SCGF-α), lacking 78 amino acids of the lectin domain at its C terminus. SCGF was reported to bind integrins and displayed protective effects against malarial anemia and age-related bone loss (22). To our knowledge, this paper is the first study showing that SCGF-β is also produced in iPSCs, is necessary for iPSC growth, and is neuroprotective to primary neurons in vitro and in vivo. Longer-term experiments using optic nerve injury models will be needed to assay RGC survival, axon regrowth and functional aspects.

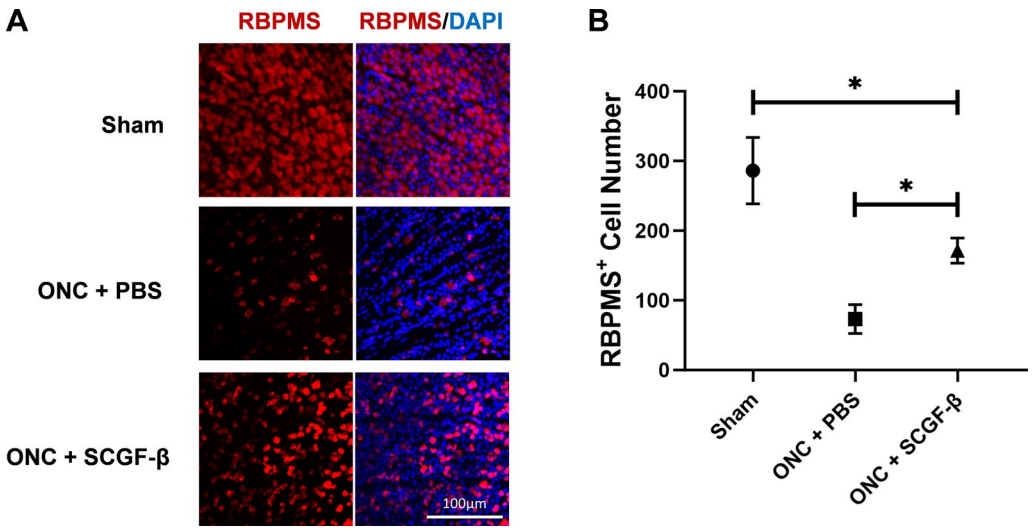

Fig. 4 Intravitreal injection of SCGF-β protects RGCs after optic nerve crush. **A** Human SCGF-β or PBS was intravitreally injected into the adult mouse eye right after optic nerve crush. The retina was explanted and immunostained 5 days post cush. **B** RBPMS$^+$ RGCs under SCGF-β administration showed significantly higher survival, compared with the PBS treatment. *$P < 0.05$. ONC, optic nerve crush. Error bar denotes SD

The present study demonstrated that 5 ng/mL of IL-12(p70) stimulated the highest concentration of SCGF-β in the iPSC supernatant. Furthermore, an increased dosage of IL-12(p70) was observed to downregulate SCGF-β expression. Given that SCGF and IL-12 are both regulators of stem cell growth (21, 23), it is possible that a higher dosage of IL-12 may induce the production of additional downstream pro-proliferation factors, which could potentially compete with SCGF for binding sites and thereby result in the observed decrease in SCGF-β expression. Additionally, it was observed that IL-12(p70) was unable to enhance RGC survival until the concentration reached 40 ng/mL, which was approximately 1000-fold the IL-12(p70) concentration detected in the RGC supernatant and eightfold the maximum concentration that upregulates SCGF-β expression in iPSCs. These findings indicate that the normal secretion of RGC-derived IL-12(p70) may be inadequate for promoting the survival of RGCs, and additional factors, such as SCGF-β, could play a role in supporting RGC survival.

The finding that RGC-derived IL-12(p70) amplified the secretion of the SCGF-β from iPSCs suggests a new model of stem cell-derived neuroprotection or possibly more broadly, tissue regeneration, in which damaged or degenerating cells signal donor stem cells to express regenerative phenotypes. Our results from this model demonstrate two key points. First, some stem cell-derived secretomes such as SCGF-β are unable to confer neuroprotective capacity unless triggered by exogenous signals. Second, CNS neurons, including RGCs, are capable of calling on untouched neighbor cells for their own protection. Furthermore, this model provides a novel pathway for new drug screening for neuroprotection whereby new bioactive molecules can be identified through the analysis of modulators between cell-to-cell interactions, thus providing novel, cell-free options treating neurodegenerative diseases. Interleukins have mainly been studied in the context of immune cell signaling, or for example derived from classic inflammatory microglia after injury in the CNS (24), but these data implicate IL-12 in signaling loops initiated from neurons. A similar expansion in our understanding of signaling proteins originally studied in the immune system is represented by the importance of complement proteins in neurodegeneration including in the retina (25–27). It is not clear what the endogenous target of RGC-derived IL-12 may be in vivo; in the future it will be interesting to study the specific contributions of RGC-derived IL-12(p70) to the degenerative or reparative process, or if these signaling pathways are activated in the peripheral nervous system after injury but there contribute to successful repair and regeneration.

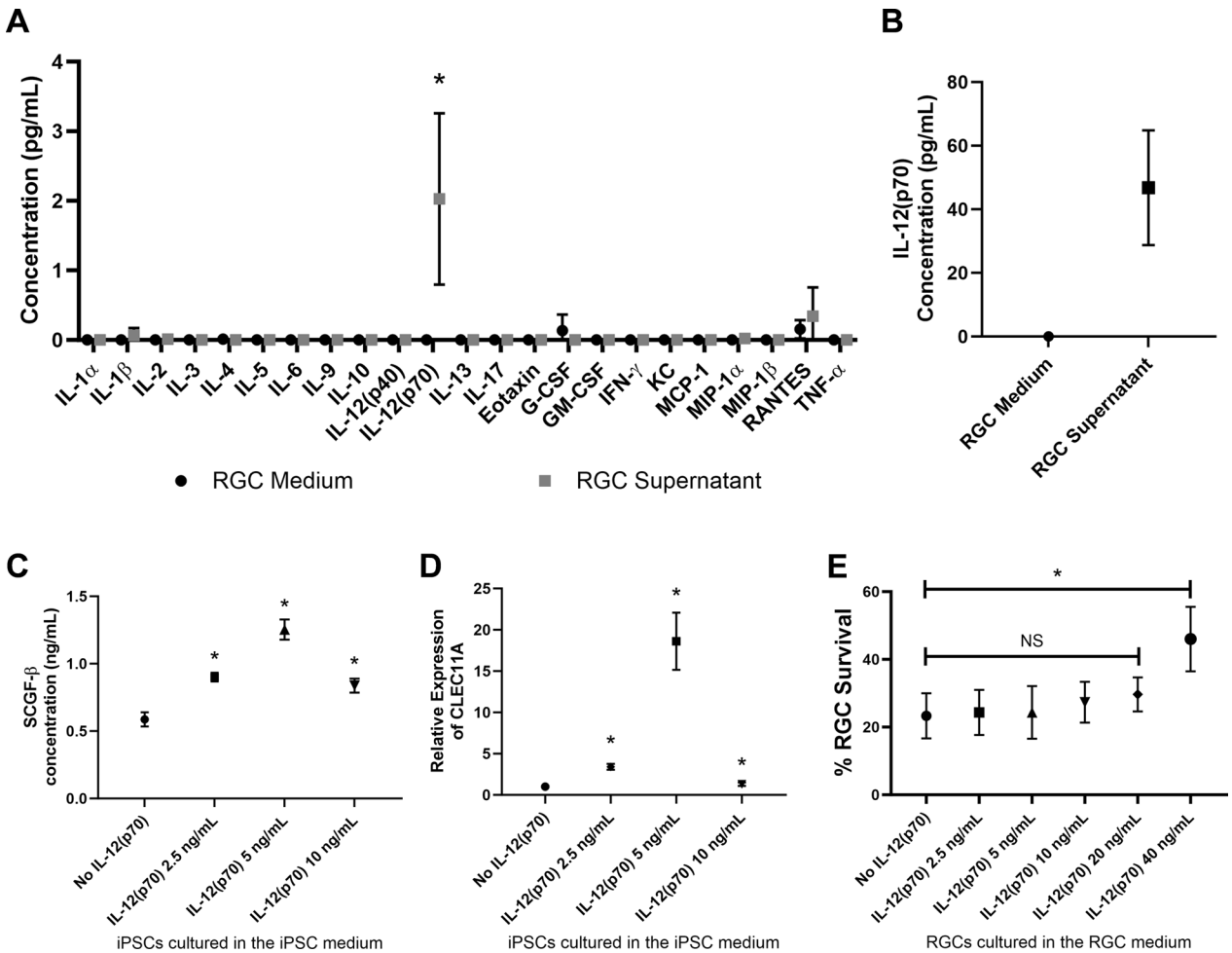

Fig. 5 RGCs enhance iPSC's SCGF-β release via IL-12(p70). **A**-**B** IL-12(p70) was detected in RGC supernatant, not in RGC medium, through multiplexed antibody-based assays and confirmed by ELISA. **C**-**D** Compared to the control ("No IL-12(p70)" group), significant upregulation of SCGF-β (via ELISA**)** and CLEC11A mRNA (via qRT-PCR**)** were observed in iPSCs treated with 2.5, 5, and 10 ng/mL of IL-12(p70). The expression peaked in the 5 ng/mL IL-12(p70)-treated group. **E** RGCs were cultured in the RGC medium with different dosages of IL-12(p70) administration. A significantly greater cell viability was not found until treated with 40 ng/mL of IL-12(p70). *$P < 0.05$. Error bar denotes SD. NS, nonsignificant

Our results indicate that SCGF-β protects RGCs through upregulating ngn2, a proneural basic helix-loop-helix (bHLH) transcription factor. Ngn2, also known as neurogenin 2 or neurog2, is a key regulator of neurogenesis and is essential for RGC differentiation in the developing retina and in human stem cell cultures (28–30). In retina it directly activates Atoh7, a critical inducer for RGC specification (31). Also, a recent study showed that ngn2 could be used to convert mouse Müller glia cells into RGCs (32). During mouse retinal development, expression of ngn2 peaks in retinal progenitor cells (RPCs), and then falls onto a fairly low level in matured RGCs (33). Our recent study reported that NGN2 induction in iPSCs or embryonic stem cells (ESCs) generated RGC-like cells that protected resident RGCs after transplant in the optic nerve crush model (28). The present study shows the protective effect of ngn2 on matured RGCs both in vivo after optic nerve

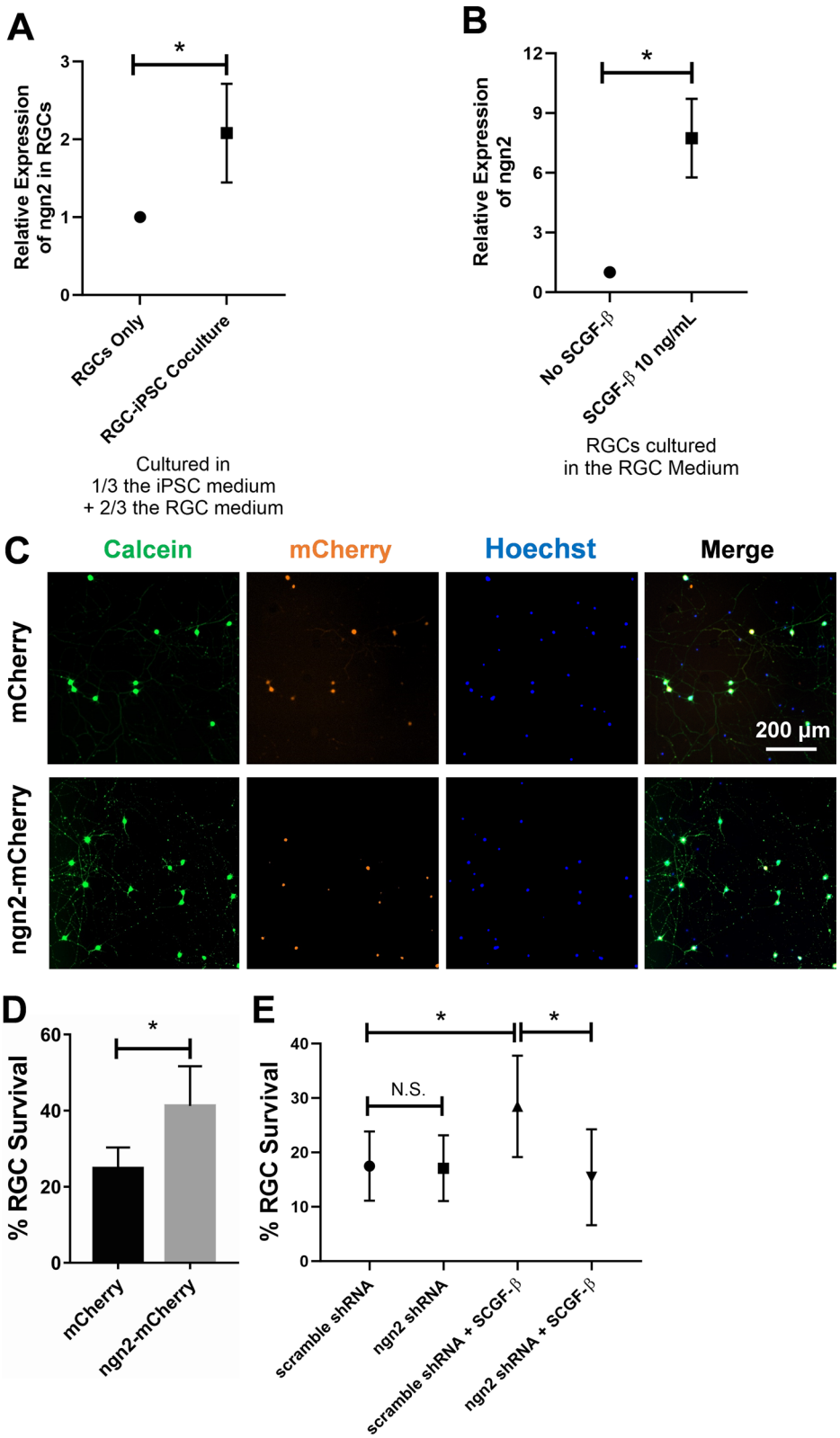

Fig. 6 iPSC-derived SCGF-β promotes RGC survival via upregulation of ngn2. **A**-**B** RT-qPCR results showed a significant increase of ngn2 mRNA in RGCs cocultured with iPSCs or treated with SCGF-β. **C**-**D** Overexpression of ngn2 in RGCs cultured in the RGC medium for 1 week significantly increased RGC viability. **E** Knockdown of ngn2 in RGCs cultured for 1 week does not significantly alter RGC survival, whether treated with SCGF-β or not. *$P < 0.05$. Error bar denotes SD

injury and in vivo of donor RGCs after transplant, indicating that some cell differentiation regulators are also capable of cell protection, and their low expression level after early development may still be critically functional, although the underlying mechanism needs further study.

Limitations to this study include: (1) Durations of all the SCGF-β treatments were no more than one week. To gain further insight into the iPSC-based protective effect on RGCs and to better understand axon growth, synapse formation, and functional changes of transplanted ngn2-overexpressing RGCs, longer-term experiments will be necessary. (2) The current study did not include functional assays of RGCs. Further studies would benefit from the inclusion of electrophysiological tests (34), such as electroretinograms (ERGs). In addition, the utilization of alternative survival assays, such as the Cell Counting Kit-8 (CCK8), will prove advantageous in augmenting the robustness of the in vitro data. Moreover, In order to gain further insight into the in vivo results, future work could also employ other anatomic measures, such as H&E staining, to characterize the outcomes in greater detail. (3) Further research is still required to improve cell survival and integration in retinal circuitry. One potential avenue for investigation is the role of non-coding RNAs which are also prominent components of stem cell-derived extracellular vesicles and have been shown to display neuroprotective potentials (1, 35). It will be advantageous to assess whether iPSC-derived non-coding RNAs enhance RGC survival and exert synergistic effects with SCGF-β.

Conclusion

Taking together, this study point to a novel signaling pathway whereby injured neurons interact with stem cells to call for their own neuroprotection. Also, a cell-to-cell interaction model like ours in the study can potentially be applied to the screening process of drug candidate.

(See figure on next page.)

Fig. 7 Overexpression of ngn2 protects endogenous and transplanted RGCs in vivo. **A** AAV2-ngn2-EGFP or AAV2-EGFP particles were intravitreally injected 2 weeks into adult mouse eyes 2 weeks before optic nerve crush. Retina explants were harvested and immunostained 2 weeks after the optic nerve crush. Surviving RGCs were labeled with RBPMS (red). **B** A significantly greater $RBPMS^+$ RGC number was found on retina explants from the AAV-ngn2 treated group. **C** The EGFP and mCherry were used to label the entire transplanted and ngn2-overexpressing donor mouse RGCs, respectively. Ngn2-mCherry-overexpressing mouse RGCs that were transplanted into adult rat eyes shows significantly higher survival rates, compared with the negative control RGC transplant 1 week after transplantation in vivo. **D** & **E** Difference between transplanted RGC survival rates and average neurite lengths with and without ngn2 overespression was quantified**.** Donor RGCs overexpressing ngn2-mCherry grew significantly longer neurites than those from negative control-RGC transplantation in vivo. *$P < 0.05$, paired t-test. ONC, optic nerve crush; NC, negative control; OE, overexpression. Error bar denotes SD

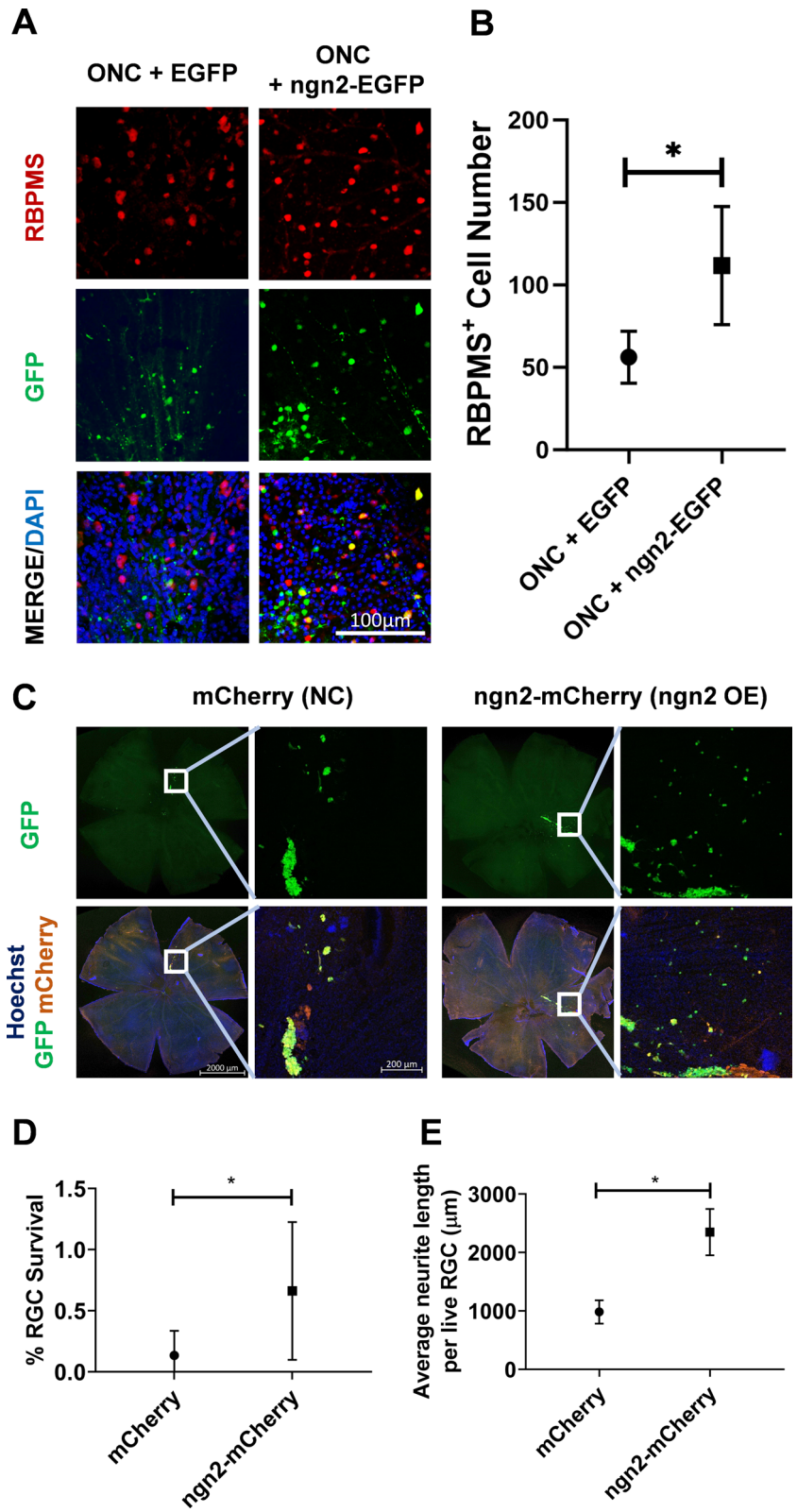

Fig. 7 (See legend on previous page.)

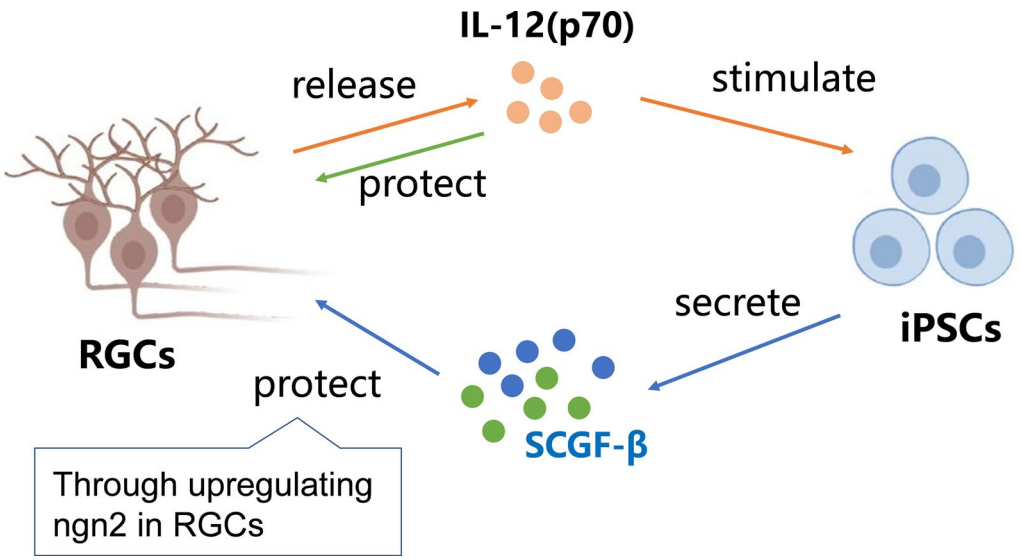

Fig. 8 RGC-derived IL-12(p70) activates iPSCs to secrete SCGF-β, which then mediates RGC neuroprotection via upregulating ngn2

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04198-5.

Supplementary file 1.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (82201175, 82070957), the China Scholarship Council (201606100222), the Shanghai Sailing Program (20YF1405100), the National Eye Institute (P30-EY026877, U24-EY029903), the Gilbert Family Foundation, and Research to Prevent Blindness, Inc.

Author contributions
Conceptualization, S.W., J.L.G., and X.M.; methodology, S.W., Q.X., K-C.C., and X.K.; formal analysis, S.W., Q.X., and K-C.C; investigation, S.W., Q.X., K-C.C., Y.S., M.N., T.N., and Y.C.; resources, S.W., Y.C., and X.K.; writing—original draft, S.W.; writing—review and draft, all authors; visualization, S.W., and J.L.G.; supervision, J.L.G. and X.M.; funding acquisition, S.W., X.K., J.L.G., and X.M.

Funding
National Natural Science Foundation of China, 82201175, Suqian Wu, 82070957, Xiangmei Kong, National Eye Institute, P30-EY026877, Jeffrey L. Goldberg, U24-EY029903, Jeffrey L. Goldberg, Gilbert Family Foundation, Research to Prevent Blindness.

Data availability
Data and materials are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee of Stanford University and Fudan University (Project Title: Retinal neuron protection, regeneration and transplantation strategies under cellular interactions; Approval Number: IACUC-DWZX-2023–046, granted on Dec. 22nd 2023). The iPSC source (Stem Cell Bank, part of Chinese National Collection of Authenticated Cell Cultures, https://cellbank.org.cn) has confirmed that there was initial ethical approval for the collection of human cells, and that the donor had signed informed consent.

Consent for publication
All authors given consent to publish the research data.

Competing interest
The authors declare no competing interests.

Received: 8 May 2024 Accepted: 30 January 2025
Published online: 25 February 2025

References

1. Sanie-Jahromi F, Mahmoudi A, Khalili MR, Nowroozzadeh MH. A review on the application of stem cell secretome in the protection and regeneration of retinal ganglion cells; a clinical prospect in the treatment of optic neuropathies. Curr Eye Res. 2022;47(11):1463–71.
2. Dali P, Shende P. Advances in stem cell therapy for brain diseases via the intranasal route. Curr Pharm Biotechnol. 2021;22(11):1466–81.
3. Daneshmandi L, Shah S, Jafari T, Bhattacharjee M, Momah D, Saveh-Shemshaki N, et al. Emergence of the stem cell secretome in regenerative engineering. Trends Biotechnol. 2020;38(12):1373–84.
4. Tran C, Damaser MS. Stem cells as drug delivery methods: application of stem cell secretome for regeneration. Adv Drug Deliv Rev. 2015;82–83:1–11.
5. Khanabdali R, Rosdah AA, Dusting GJ, Lim SY. Harnessing the secretome of cardiac stem cells as therapy for ischemic heart disease. Biochem Pharmacol. 2016;113:1–11.
6. Sun DZ, Abelson B, Babbar P, Damaser MS. Harnessing the mesenchymal stem cell secretome for regenerative urology. Nat Rev Urol. 2019;16(6):363–75.
7. Gemayel J, Chaker D, El Hachem G, Mhanna M, Salemeh R, Hanna C, et al. Mesenchymal stem cells-derived secretome and extracellular vesicles: perspective and challenges in cancer therapy and clinical applications. Clin Transl Oncol. 2023;25(7):2056–68.
8. Pearson C, Martin K. Stem cell approaches to glaucoma: from aqueous outflow modulation to retinal neuroprotection. Prog Brain Res. 2015;220:241–56.
9. Johnson TV, DeKorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B, et al. Identification of retinal ganglion cell neuroprotection conferred

by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain. 2014;137(Pt 2):503–19.
10. Wu S, Chang KC, Nahmou M, Goldberg JL. Induced pluripotent stem cells promote retinal ganglion cell survival after transplant. Invest Ophthalmol Vis Sci. 2018;59(3):1571–6.
11. Brown LY, Dong W, Kantor B. An improved protocol for the production of lentiviral vectors. STAR Protoc. 2020;1(3): 100152.
12. Challis RC, Ravindra Kumar S, Chan KY, Challis C, Beadle K, Jang MJ, et al. Systemic AAV vectors for widespread and targeted gene delivery in rodents. Nat Protoc. 2019;14(2):379–414.
13. Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron. 1995;15(4):805–19.
14. Barres BA, Silverstein BE, Corey DP, Chun LL. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. Neuron. 1988;1(9):791–803.
15. Winzeler A, Wang JT. Purification and culture of retinal ganglion cells from rodents. Cold Spring Harb Protoc. 2013;2013(7):643–52.
16. Venugopalan P, Wang Y, Nguyen T, Huang A, Muller KJ, Goldberg JL. Transplanted neurons integrate into adult retinas and respond to light. Nat Commun. 2016;7:10472.
17. Chang KC, Bian M, Xia X, Madaan A, Sun C, Wang Q, et al. Posttranslational modification of sox11 regulates rgc survival and axon regeneration. eNeuro. 2021. https://doi.org/10.1523/ENEURO.0358-20.2020.
18. Deng P, Wang L, Zhang Q, Chen S, Zhang Y, Xu H, et al. Therapeutic potential of a combination of electroacupuncture and human ipsc-derived small extracellular vesicles for ischemic stroke. Cells. 2022;11(5):820.
19. Meldolesi J. News about therapies of Alzheimer's disease: extracellular vesicles from stem cells exhibit advantages compared to other treatments. Biomedicines. 2022;10(1):105.
20. Teli P, Kale V, Vaidya A. Extracellular vesicles isolated from mesenchymal stromal cells primed with neurotrophic factors and signaling modifiers as potential therapeutics for neurodegenerative diseases. Curr Res Transl Med. 2021;69(2): 103286.
21. Mio H, Kagami N, Yokokawa S, Kawai H, Nakagawa S, Takeuchi K, et al. Isolation and characterization of a cDNA for human mouse, and rat full-length stem cell growth factor, a new member of C-type lectin superfamily. Biochem Biophys Res Commun. 1998;249(1):124–30.
22. Wang M, Guo J, Zhang L, Kuek V, Xu J, Zou J. Molecular structure, expression, and functional role of Clec11a in skeletal biology and cancers. J Cell Physiol. 2020;235(10):6357–65.
23. Jacobsen SE, Veiby OP, Smeland EB. Cytotoxic lymphocyte maturation factor (interleukin 12) is a synergistic growth factor for hematopoietic stem cells. J Exp Med. 1993;178(2):413–8.
24. Becher B, Prat A, Antel JP. Brain-immune connection: immuno-regulatory properties of CNS-resident cells. Glia. 2000;29(4):293–304.
25. Patel PN, Patel PA, Land MR, Bakerkhatib-Taha I, Ahmed H, Sheth V. Targeting the complement cascade for treatment of dry age-related macular degeneration. Biomedicines. 2022;10(8):1884.
26. Li S, Qiu Y, Yu J, Shao M, Li Y, Cao W, et al. Serum complement component 3, complement component 4 and complement component 1q levels predict progressive visual field loss in older women with primary angle closure glaucoma. Br J Ophthalmol. 2022;107:828.
27. Stasi K, Nagel D, Yang X, Wang RF, Ren L, Podos SM, et al. Complement component 1Q (C1Q) upregulation in retina of murine, primate, and human glaucomatous eyes. Invest Ophthalmol Vis Sci. 2006;47(3):1024–9.
28. Luo Z, Chang KC, Wu S, Sun C, Xia X, Nahmou M, et al. Directly induced human retinal ganglion cells mimic fetal RGCs and are neuroprotective after transplantation in vivo. Stem Cell Rep. 2022;17:2690.
29. Maurer KA, Kowalchuk A, Shoja-Taheri F, Brown NL. Integral bHLH factor regulation of cell cycle exit and RGC differentiation. Dev Dyn. 2018;247(8):965–75.
30. Meng F, Wang X, Gu P, Wang Z, Guo W. Induction of retinal ganglion-like cells from fibroblasts by adenoviral gene delivery. Neuroscience. 2013;250:381–93.
31. Hufnagel RB, Le TT, Riesenberg AL, Brown NL. Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev Biol. 2010;340(2):490–503.
32. Guimaraes RPM, Landeira BS, Coelho DM, Golbert DCF, Silveira MS, Linden R, et al. Evidence of muller glia conversion into retina ganglion cells using neurogenin2. Front Cell Neurosci. 2018;12:410.
33. Wu F, Bard JE, Kann J, Yergeau D, Sapkota D, Ge Y, et al. Single cell transcriptomics reveals lineage trajectory of retinal ganglion cells in wild-type and Atoh7-null retinas. Nat Commun. 2021;12(1):1465.
34. Porciatti V. Electrophysiological assessment of retinal ganglion cell function. Exp Eye Res. 2015;141:164–70.
35. Nasirishargh A, Kumar P, Ramasubramanian L, Clark K, Hao D, Lazar SV, et al. Exosomal microRNAs from mesenchymal stem/stromal cells: biology and applications in neuroprotection. World J Stem Cells. 2021;13(7):776–94.

Publisher's Note