Retinal ganglion cell-conditioned medium and surrounding pressure alters gene expression and differentiation of rat retinal progenitor cells

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Abstract. Loss of retinal ganglion cells is implicated in glaucoma and high intraocular pressure. Factors that affect the differentiation of retinal progenitor cells into retinal ganglion cells remain unclear. The present study aimed to investigate the effects of retinal ganglion cell-conditioned medium on gene expression and differentiation in retinal progenitor cells, and the effects of surrounding pressure on the survival and differentiation of retinal progenitor cells. Retinal progenitor cells and retinal ganglion cells were isolated from rats. Immunofluorescence staining of Nestin and Thy1 was performed to identify rat retinal progenitor cells and retinal ganglion cells, respectively. Retinal progenitor cells and ganglion cells were cultured for 48 h under surrounding pressure of 0, 20, 40, 60 and 80 mmHg. Cellular apoptosis was detected using a caspase-3 assay kit. In addition, the culture supernatant of rat retinal ganglion cells was collected. Retinal progenitor cells were cultured in the presence or absence of retinal ganglion-conditioned medium for 72 h under normal pressure. Gene expression of Nestin, paired box protein 6 (PAX6), Thy1 and brain-specific homeobox/POU domain protein 3 (Brn-3) in retinal progenitor cells was detected by reverse transcription-quantitative polymerase chain reaction. Retinal progenitor cells were cultured in retinal ganglion-conditioned medium for 72 h under surrounding pressure of 0 and 40 mmHg, respectively, and flow cytometry was utilized to evaluate the effects of pressure on the differentiation of retinal progenitor cells into retinal ganglion cells. The results demonstrated that isolated retinal progenitor cells were Nestin-positive and retinal ganglion cells were Thy1-positive, suggesting successful isolation. The activity of caspase-3 increased in retinal progenitor cells and retinal ganglion cells in a pressure-dependent manner. When the surrounding pressure reached 40, 60 and 80 mmHg, the activity of caspase-3 in retinal progenitor cells and ganglion cells increased significantly compared with cells that were not under pressure. Compared with retinal progenitor cells cultured without ganglion-conditioned medium, those cultured with ganglion-conditioned medium had significantly decreased expression levels of Nestin and PAX6, and increased expression levels of Thy1 and Brn3. Compared with 0 mmHg pressure, retinal progenitor cells cultured in ganglion-conditioned medium under 40 mmHg pressure had increased percentages of Thy1-positive cells. In conclusion, the apoptosis of rat retinal progenitor cells and retinal ganglion cells was pressure-dependent. Retinal ganglion cell-conditioned medium increased the differentiation of retinal progenitor cells into retinal ganglion-like cells, and the differentiation increased as surrounding pressure increased. Current study provides insights that may contribute to the efforts of developing a treatment for glaucoma.

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Introduction

Glaucoma is the second-leading cause of blindness globally, following cataracts. There were 44.7 million people in the world with open angle glaucoma as of 2010 (1). Glaucoma is a group of eye diseases that result in damage to the optic nerve and vision loss. Increased intraocular pressure is the most important risk factor in the majority of glaucoma cases (2).

Retinal ganglion cells transmit visual information from the retina in the form of action potentials to the thalamus, hypothalamus and midbrain. They have a long axon that extends into the brain, forming the optic nerve, optic chiasm and optic tract. Loss of retinal ganglion cells has been implicated in a wide range of glaucoma stages, from preperimetric to advanced (3). An observational cohort study that examined 116 eyes of 62 glaucoma patients revealed that the rate of retinal ganglion cell loss resulted in improved detection of glaucoma progression,

compared with either optical coherence tomography or standard automated perimetry (4). In addition, transplanted stem cells were reported to migrate into and integrate in different layers of the retina (5). Therefore, in order to pave the foundation for retinal ganglion cell therapy for glaucoma, it is of importance to investigate the factors that may affect differentiation of retinal progenitor cells into retinal ganglion cells.

Fibroblast growth factor 2 was revealed to induce embryonic stem cell-derived neural progenitors to generate retinal ganglion cell-like cells *in vitro* (6). The combination of retinal pigment epithelial cell-conditioned medium and photoreceptor outer segments stimulated mesenchymal stem cell differentiation toward retinal pigment epithelial cell phenotype (7). However, the effects of retinal ganglion cell-conditioned medium on the gene expression and differentiation of retinal progenitor cells and the effects of surrounding pressure on the survival and differentiation of retinal progenitor cells remain unclear.

Nestin is a neuroectodermal stem cell marker, and is expressed in retinal progenitor cells (8). Upon differentiation, Nestin becomes down-regulated. Paired box protein (PAX)6 is a key regulatory gene of eye development (9). Retinal progenitor cell clones were established by transfection of the paired box protein 6 (PAX6) gene into mouse induced pluripotent stem cells (10). Thy1 is a surface glycoprotein uniquely expressed in retinal ganglion cells in the retina (11). Brain-specific homeobox/POU domain protein 3 (Brn3) is involved in the regulation of differentiation, dendritic stratification and axonal projection of retinal ganglion cells during development (12). Therefore, Nestin and PAX6 were utilized to identify retinal progenitor cells, and Thy1 and Brn3 were used to identify retinal ganglion cells. The retinal ganglia are a type of neuron near the inner surface of the retina. They transmit image-forming and non-image forming visual information from the retina to the thalamus, hypothalamus, mesencephalon and midbrain in the form of action potentials. Examining the differentiation of retinal progenitor cells into retinal ganglion cells may provide insights into vision restoration following injury in glaucoma. Therefore, the present study aimed to investigate the effects of retinal ganglion cell-conditioned medium on gene expression and differentiation in retinal progenitor cells, and the effects of surrounding pressure on the survival and differentiation of retinal progenitor cells.

Materials and methods

Reagents and equipment. Dulbecco's modified Eagle's medium (DMEM)/F12, B27, N2, heparin and glutamine were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Trypsin (Invitrogen; Thermo Fisher Scientific, Inc.), bicinchoninic acid assay kit, caspase-3 assay kit (Sigma-Aldrich; Merck KGaA), PBS (Sigma-Aldrich; Merck KGaA), were used in the present study. Anti-Nestin antibody, anti-Thy1 antibody and secondary antibody were purchased from Abcam (Cambridge, UK). Secondary antibodies included goat anti-rabbit immunoglobulin (Ig)G H&L (Alexa Fluor® 488; cat. no. ab150077; Abcam, Cambridge, UK), and donkey anti-rabbit IgG H&L (Alexa Fluor® 555; cat. no. ab150074; Abcam). Primers and probes, TRIzol reagent, SuperScript III Reverse Transcriptase, SYBR-Green I and DEPC H_2O were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). RNase inhibitor was purchased from Fermentas (Thermo Fisher Scientific, Inc.). Platinum Taq DNA polymerase, oligo dT/primer and 100 mM dNTPs were purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

The following equipment was used: Cell incubator (Thermo Fisher Scientific, Inc.), light microscope (Olympus Corporation, Tokyo, Japan), CFX96 Touch™ Real-Time polymerase chain reaction (PCR) Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), table-type refrigerated centrifuge, plate reader (Zhengzhou Nanbei Instrument Equipment, Inc., Hefei, China), LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software 7.6.2 (FlowJo LLC, Ashland, OR, USA).

Isolation and culture of rat retinal progenitor cells. A total of 20 Sprague Dawley® (SD) rats (male; 17 days old; 35-55 g; Shanghai SLAC Laboratory Animal, Inc., Shanghai, China) were sacrificed. Animals were raised at 25˚C with 65% humidity in normal atmosphere. Animals had free access to food and water and were housed under 12 h light/dark cycle.

Retinal pigment tissue at the ciliary margin zone of embryos was isolated under a microscope, and placed into cold PBS. The tissue was cut into small sections, and digested with 0.1% trypsin at 37˚C for 10 min. DMEM/F12 supplemented with 20% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) was added to stop digestion by trypsin. Trypsin (0.01%; Gibco; Thermo Fisher Scientific, Inc.) was added at room temperature for 20 min, and a single cell suspension was prepared by pipetting gently. The cell suspension was centrifuged at 500 x g for 5 min at room temperature, and cell pellet was resuspended in DMEM/F12 supplemented with 2% B27, 1% N2, heparin (90 μg/ml), glutamine (2 mmol/l), EGF (20 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml). The cell suspension was filtered (0.22 μm pores) and cultured in plates (25 cm^2) at 37˚C in 5% CO_2 and 95% air. Cell culture medium was replaced every 3 days. The current study was approved by the Animal Research Board of Second People's Hospital and Yan'an Hospital (Kunming, China). All efforts were made to reduce animal suffering.

Isolation and culture of rat retinal ganglion cells. A total of 20 SD rats (7 days old; male; 15-20 g; Shanghai SLAC Laboratory Animal, Inc.) were sacrificed. Animals were raised at 25˚C with 65% humidity in normal atmosphere. Animals had free access to food and water and were housed under 12 h light/dark cycle. Eyeballs were removed using aseptic technique, and washed with PBS three times. The cornea was removed followed by the limbus corneae, lens and vitreous body. The neuronal layer of the retinal tissue was isolated, washed with PBS supplemented with 1% penicillin-streptomycin three times and digested with 0.05% trypsin at 37˚C for 30 min. The digestion was terminated by adding DMEM containing 10% FBS. The cell suspension was filtered with a 40 μm filter, and centrifuged at 500 x g at room temperature for 5 min. The cell pellet was resuspended with DMEM and cultured in plates at 37˚C in 5% CO_2 and 95% air.

Pressure treatment. Cells ($1x10^5$) were plated in a 3.5 cm culture dish 1 day prior to the experiments. The bottom of a bag

containing normal saline (Fig. 1A) was cut open with scissors, and the normal saline was poured out. The culture dish was placed into the empty bag, and the bottom was sealed using a plastic envelope machine. Sphygmomanometer (Jiangsu Yuyue Medical Equipment & Supply Co., Inc., Nanjing, China; Fig. 1B) was utilized to pump air into the bag, and the pressure was measured using the instrument. Cells in the different experimental groups were treated with various pressures at room temperature for 48 h using this method.

Immunofluorescence. Rat retinal progenitor cells and ganglion cells were isolated, and cultured in 24-well plates with cover slips ($3x10^4$ cells/cm^2; 50% confluence). Cells adhered to cover slips were then fixed in 4% paraformaldehyde at room temperature for 10 min, and blocked with 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Rat retinal progenitor cells were incubated with a primary antibody against Nestin (1:500; cat. no. ab92391), and retinal ganglion cells were incubated with a primary antibody against Thy1 (1:500; cat. no. ab133350) at 4˚C overnight. Following overnight incubation, cover slips were washed with PBS, and incubated in the dark with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:1,000) at room temperature for 1 h. Cover slips were washed with PBS and stained with DAPI at room temperature for 5 min. Slides were prepared using an anti-quenching mounting medium. Slides were observed using a fluorescence microscope (magnification, x100).

Apoptosis assay. Retinal progenitor cells and ganglion cells were cultured for 48 h under surrounding pressures of 0, 20, 40, 60 and 80 mmHg, respectively. Cell apoptosis was detected using a caspase-3 assay kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol. Proteins from retinal stem cells and ganglion cells were extracted using cell lysis buffer from the caspase-3 assay kit, and the protein concentration was measured using a bicinchoninic acid assay kit. Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA; included in the caspase-3 assay kit), the substrate that is hydrolyzed by caspase-3, was mixed with cell proteins at 37˚C for 2 h, and the optical density values were measured at an absorbance of 405 nm using a plate reader. Experiments were repeated three times.

Induction of retinal progenitor cell differentiation by retinal ganglion cell-conditioned medium. Rat retinal ganglion cells were cultured to 80% confluence, and the cell culture medium was replaced with DMEM/F12 medium without serum. Retinal ganglion cells were cultured for a further 24 h, and the culture supernatant was collected. Retinal progenitor cells were cultured in retinal ganglion cell-conditioned medium for 72 h under normal pressure, or under 40 mmHg pressure. Retinal progenitor cells that were cultured without retinal ganglion cell-conditioned medium served as control (no treatment). Retinal progenitor cells were collected. Gene expression levels of Nestin, PAX6, Thy1 and Brn-3 were detected by reverse transcription-quantitative PCR (RT-qPCR), and flow cytometry was utilized to evaluate the effects of pressure on the differentiation of retinal progenitor cells into retinal ganglion cells.

RT-qPCR. Following induction by retinal ganglion cell-conditioned medium, retinal progenitor cells under normal pressure were collected. Total RNA was extracted using TRIzol reagent, following the manufacturer's protocol. A universal cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized for reverse transcription. Each reaction contained 0.5 μl random primers (0.2 μg/μl) and 1 μl SuperScript III reverse transcriptase (200 U/μl). The specific primer for Nestin was forward, CTGGAAGGTGGGCAGCAACT and reverse, TCT CAAGGGTATTAGGCAAGGG; the primer for PAX6 was forward, CTGGAGTGTCAGTTCCCGTC and reverse, ATA CCGTGCCTTCTGTACGC; the primer for Thy1 was forward, CAAAACGCGGGGAGAAATGG and reverse, CTGGTG TTCCATCGGGTCTC; and the primer for Brn-3 was forward, TTTCCCCCTTTGTTCCGCTT and reverse, GCCTAATGA CGCCTAGCCAA. PCR was performed by utilizing a SYBR qPCR mix kit (Invitrogen; Thermo Fisher Scientific, Inc.). PCR conditions were as follows: Predenaturation at 95˚C for 2 min, 40 cycles of denaturation at 95˚C for 10 sec and annealing and polymerization at 60˚C for 30 sec and 70˚C for 45 sec. PCR was performed using a CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Gene expression was determined and normalized to β-actin. The following rat β-actin primers were used: Forward, 5'AGGGAAATCGTG CGTGAC3' and reverse, 5'CGCTCATTGCCGATAGTG3'. The $2^{-\Delta\Delta Cq}$ method was utilized to measure PCR results (13).

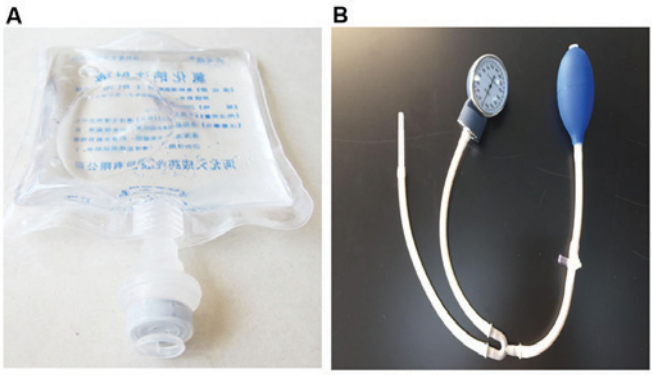

Figure 1. Pressure treatment of cells. (A) A normal saline bag was used to contain the culture dish. (B) Sphygmomanometer. Cells ($1x10^5$) were plated in a 3.5 cm culture dish 1 day prior to experiments. The bottom of a bag containing normal saline was cut open with scissors and the contents was poured out. The culture dish was placed into the empty bag, and the bottom was sealed with a plastic-envelope machine. A pressure instrument was utilized to pump air into the bag, and the pressure was measured using the instrument. Cells in the different experimental groups were treated with various pressures using this method.

Flow cytometry. Following induction with retinal ganglion cell-conditioned medium, retinal progenitor cells were cultured under surrounding pressure of 0 and 40 mmHg (50% confluence) at 37˚C for 48 h. Cells were washed with PBS twice, and incubated with trypsin at 37˚C for 1 min. Following digestion, the cell suspension was centrifuged at 400 x g at room temperature for 5 min. The cell pellet was resuspended with PBS and the centrifugation and resuspension steps were repeated a further two times. Cells were fixed in 4% paraformaldehyde at room temperature for 10 min, and blocked with 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Anti-Thy1 antibody (10 μl; 1:200) was added to 100 μl cell suspension, and incubated at 4˚C for

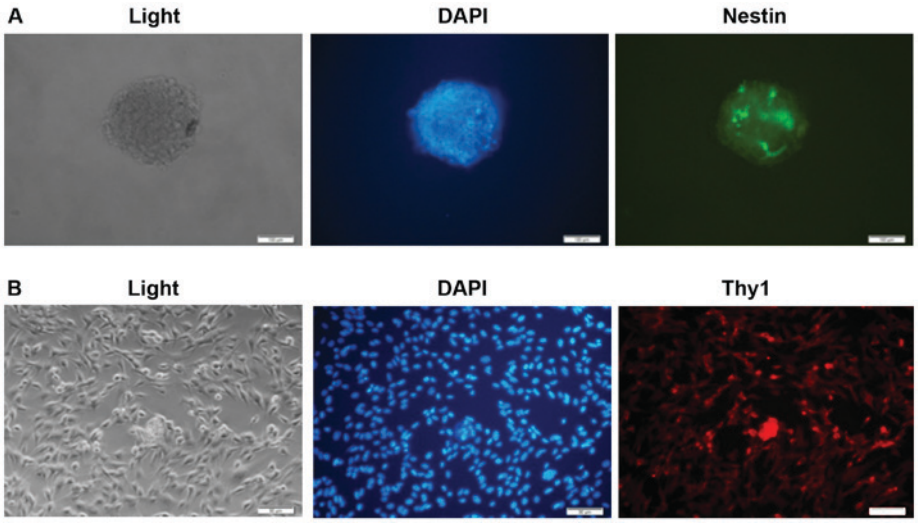

Figure 2. Identification of rat retinal progenitor cells and retinal ganglion cells. (A) Retinal progenitor cells and (B) retinal ganglion cells. Retinal progenitor cells and retinal ganglion cells were isolated from rats. Immunofluorescence was utilized to identify these cells. Rat retinal progenitor cells were stained with a primary antibody against Nestin, and retinal ganglion cells were stained with a primary antibody against Thy1. Isolated retinal progenitor cells were Nestin-positive (green), and retinal ganglion cells were Thy1-positive (red), which suggested a successful isolation. The cell nucleus was stained with DAPI (blue). Cell images captured under a light microscope are depicted in the leftmost panels. Scale bar, 100 μm.

30 min. Cells were centrifuged at 400 x g at room temperature for 5 min, and resuspended with PBS three times. Secondary green fluorescent protein-labeled goat anti-rabbit IgG H&L antibody (Alexa Fluor® 488; cat. no. ab150077; 1:2,000; Abcam) was added into the cell suspension, and incubated at 4˚C for 30 min. Cells were washed with PBS three times. Cells were resuspended in 500 μl PBS, and detected by flow cytometry. Data was acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software. Experiments were repeated three times.

Statistical analysis. Statistical data was analyzed by GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as mean ± standard error. Differences among ≥3 groups were compared by one-way analysis of variance followed by the Bonferroni post hoc test. Differences between 2 groups were compared by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Derivation and identification of rat retinal progenitor cells and retinal ganglion cells. Retinal progenitor cells and ganglion cells were isolated from rats as described in the methods. Immunofluorescence was utilized to identify these cells. Rat retinal progenitor cells were stained with a primary antibody against Nestin, and retinal ganglion cells were stained with a primary antibody against Thy1. Slides were observed with a fluorescence microscope. It was demonstrated that the isolated retinal progenitor cells were Nestin-positive, and retinal ganglion cells were Thy1-positive, suggesting the success of the isolation (Fig. 2A and B). From the immunofluorescence data, ~40 to 50% isolated cells were Nestin$^+$ and Thy1$^+$, respectively. Some non-specific positive staining may be observed.

Surrounding pressure induces apoptosis in retinal progenitor cells in a pressure-dependent manner. The retinal progenitor cell mixture was cultured for 48 h under surrounding pressures of 0, 20, 40, 60 and 80 mmHg. Cellular apoptosis was detected using a caspase-3 assay kit. The activity of caspase-3 increased in the retinal progenitor cell mixture in a pressure-dependent manner. When the surrounding pressure reached 40, 60 and 80 mmHg, the activity of caspase-3 in the retinal progenitor cell mixture increased significantly compared with cells that were not under pressure (0 mmHg; $P<0.001$; Fig. 3). As 40-50% of the cell mixture constituted retinal progenitor cells, and retinal progenitor cells are more susceptible to increased pressure compared with connective tissue cells or the epithelium, the increase in apoptosis in the cell mixture suggested that this was due to the presence of retinal progenitor cells. These

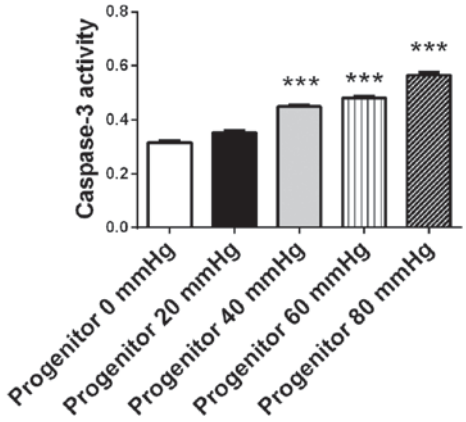

Figure 3. Surrounding pressure induces apoptosis in retinal progenitor cells in a pressure-dependent manner. The retinal progenitor cell mixture was cultured for 48 h under surrounding pressures of 0, 20, 40, 60 and 80 mmHg. Cellular apoptosis was detected using a caspase-3 assay kit. The activity of caspase-3 increased in the retinal progenitor cell mixture in a pressure-dependent manner. When the surrounding pressure reached 40, 60 and 80 mmHg, the activity of caspase-3 in retinal progenitor cells increased significantly compared with cells that were not under pressure (mean ± standard error; n=3 per group). Experiments were repeated three times. ***$P<0.001$ vs. 0 mmHg group.

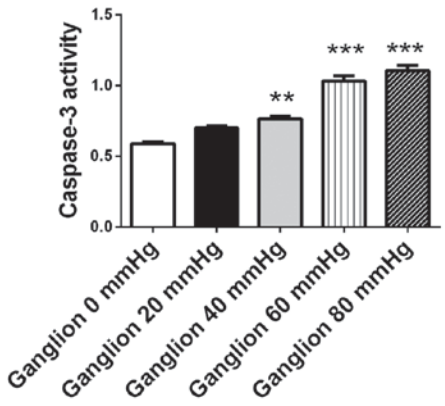

Figure 4. Surrounding pressure induces apoptosis in retinal ganglion cells in a pressure-dependent manner. Retinal ganglion cells were cultured for 48 h under surrounding pressures of 0, 20, 40, 60 and 80 mmHg. Cellular apoptosis was detected with a caspase-3 assay kit. The activity of caspase-3 increased in retinal ganglion cells in a pressure-dependent manner. When the surrounding pressure reached 40, 60 and 80 mmHg, the activity of caspase-3 in retinal progenitor cells significantly increased compared with cells that were not under pressure (mean ± standard error; n=3 per group). Experiments were repeated three times. **P<0.01 and ***P<0.001 vs. 0 mmHg group.

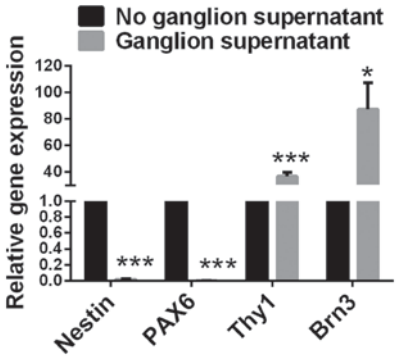

Figure 5. Expression levels of Nestin and PAX6 significantly decrease, and the expression of Thy1 and Brn3 significantly increases in retinal progenitor cells cultured with retinal ganglion cell-conditioned medium. The culture supernatant of rat retinal ganglion cells was collected. Retinal progenitor cells were cultured in retinal ganglion cell-conditioned medium for 72 h under normal pressure. Retinal progenitor cells that were cultured without retinal ganglion cell-conditioned medium served as a control. The mRNA expression levels of Nestin, PAX6, Thy1 and Brn-3 in retinal progenitor cells was measured by reverse transcription-quantitative polymerase chain reaction. Compared with retinal progenitor cells cultured without ganglion cell-conditioned medium, cells cultured with ganglion cell-conditioned medium exhibited significantly decreased expression levels of Nestin and PAX6, and increased expression levels of Thy1 and Brn3 (mean ± standard error; n=3 per group). Experiments were repeated three times. *P<0.05 and ***P<0.001 vs. retinal progenitor cells cultured without retinal ganglion cell-conditioned medium. PAX6, paired box protein 6; Brn3, brain-specific homeobox/POU domain protein 3.

results suggested that surrounding pressure induced apoptosis in retinal progenitor cells in a pressure-dependent manner.

Surrounding pressure induces apoptosis in retinal ganglion cells in a pressure-dependent manner. Retinal ganglion cells were cultured for 48 h under surrounding pressure of 0, 20, 40, 60 and 80 mmHg. Cellular apoptosis was detected using a caspase-3 assay kit. The activity of caspase-3 increased in retinal ganglion cells in a pressure-dependent manner. When the surrounding pressure reached 40, 60 and 80 mmHg, the activity of caspase-3

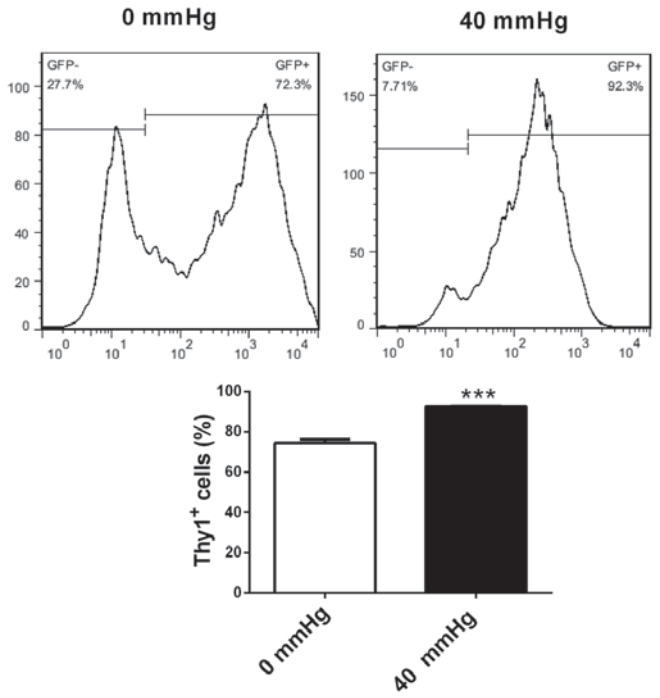

Figure 6. Increased surrounding pressure stimulates differentiation of retinal progenitor cells into retinal ganglion-like cells. Retinal progenitor cells were cultured in retinal ganglion cell-conditioned medium for 72 h under surrounding pressures of 0 and 40 mmHg. Flow cytometry was utilized to evaluate the effects of pressure on the differentiation of retinal progenitor cells into retinal ganglion cells. Retinal ganglion-like cells ($Thy1^+$) were stained with GFP. Compared with cells subjected to 0 mmHg pressure, retinal progenitor cells cultured in ganglion cell-conditioned medium under 40 mmHg pressure had increased percentages of Thy1-positive cells (mean ± standard error; n=4 per group). Experiments were repeated four times. ***P<0.001 vs. retinal progenitor cells that were cultured under 0 mmHg. GFP, green fluorescent protein.

in retinal progenitor cells increased compared with cells that were not under pressure (P<0.01 at 40 mmHg; P<0.001 at 60 and 80 mmHg; Fig. 4). The results demonstrated that surrounding pressure may induce apoptosis in retinal ganglion cells in a pressure-dependent manner.

Expression of Nestin and PAX6 significantly decreases, and expression of Thy1 and Brn3 increases in retinal progenitor cells cultured with retinal ganglion cell-conditioned medium. The culture supernatant of rat retinal ganglion cells was collected. Retinal progenitor cells were cultured in retinal ganglion cell-conditioned medium for 72 h under normal pressure. Retinal progenitor cells that were cultured without retinal ganglion cell-conditioned medium served as a control. Gene expression levels of Nestin, PAX6, Thy1 and Brn-3 in retinal progenitor cells were detected by RT-qPCR. Compared with retinal progenitor cells cultured without ganglion cell-conditioned medium, cells cultured with ganglion cell-conditioned medium had significantly decreased expression levels of Nestin and PAX6 (P<0.001), and significantly increased expression levels of Thy1 (P<0.001; Fig. 5) and Brn3 (P<0.05; Fig. 5).

Increased surrounding pressure stimulates differentiation of retinal progenitor cells into retinal ganglion-like cells. Retinal progenitor cells were cultured in retinal ganglion cell-conditioned medium for 72 h under surrounding

pressures of 0 and 40 mmHg. Flow cytometry was utilized to evaluate the effects of pressure on the differentiation of retinal progenitor cells into retinal ganglion cells. Compared with 0 mmHg pressure, retinal progenitor cells cultured in ganglion cell-conditioned medium under 40 mmHg pressure had increased percentages of Thy1-positive cells ($P<0.001$; Fig. 6). This suggested that increased surrounding pressure stimulated the differentiation of retinal progenitor cells into retinal ganglion-like cells.

Discussion

The present study demonstrated that apoptosis in rat retinal progenitor cells and retinal ganglion cells was pressure-dependent. Retinal ganglion cell-conditioned medium increased the differentiation of retinal progenitor cells into retinal ganglion-like cells, and the differentiation increased as the surrounding pressure increased.

Various animal models of different species have been used to study glaucoma, including monkeys, dogs, cats, pigs and rodents (14-18). Glaucoma in these animals was either spontaneous or induced. These models have provided valuable information about glaucoma. However, as the molecular mechanism of glaucoma differs among animal species, data obtained from a particular model may not be generalized to all species. Previously, *in vitro* and *ex vivo* glaucoma models have been developed to improve the accuracy and repeatability of experimental conditions (19,20). Hydrostatic pressure was applied to cells cultured *in vitro* and *ex vivo*. In addition, transgenic mouse glaucoma models that were modified by the introduction of a foreign DNA sequence into a mouse egg, have emerged (21). In the present study, an *in vitro* glaucoma model was utilized to ensure accuracy and repeatability.

It was revealed that apoptosis in rat ganglion cells was pressure-dependent. In a rat model of glaucoma, elevation of phosphorylated N-methyl-D-aspartate receptor 2A by cyclin dependent kinase (cdk)5/p35 was revealed to cause apoptosis in retinal ganglion cells. Apoptosis was ameliorated by inhibiting cdk5/p35 (22). Reactivated Muller cells were demonstrated to release excessive adenosine triphosphate, causing apoptosis in retinal ganglion cells via activation of purinergic receptor P2X 7 receptors (23). The proliferation and apoptosis of retinal ganglion cells was additionally reported to be mediated by the microRNA-187/mothers against decapentaplegic homolog 7 axis. A decrease in miR-187 induced apoptosis and inhibited proliferation in retinal ganglion cells (24). In addition, the apoptosis of retinal ganglion cells has been observed in other retinal diseases. In a rat model of light-induced retinal damage, transcription factor FOS-related antigen 1 was observed to be associated with apoptosis in retinal ganglion cells following light exposure, regulated by p38 mitogen-activated protein kinase (MAPK) through a cell cycle re-entry mechanism (25). Palmitic acid induced apoptosis in retinal ganglion cells through the protein kinase B/forkhead box protein O1 signaling pathway (26). The gene expression levels and signaling pathways in retinal ganglion cells that are directly affected by increased surrounding pressure, which may trigger cellular apoptosis, require further investigation.

It was additionally demonstrated in the present study that apoptosis in rat retinal progenitor cells was pressure-dependent. Various factors may impact on the proliferation of retinal progenitor cells. Activation of the type 5 metabotropic glutamate receptor promoted the proliferation of rat retinal progenitor cells through activation of the phosphatidylinositol 3-kinase and MAPK signaling pathways (27). Mutual antagonism of the paired-type homeobox genes, visual system homeobox 2 and diencephalon/mesencephalon homeobox 1, was demonstrated to regulate retinal progenitor cell cycle exit upstream of cyclin D1 expression (28). Toll-like receptor and MyD88-dependent and -independent pathways were revealed to be negative regulators of proliferation in retinal progenitor cells (29). SUMOylation controlled retinal progenitor proliferation by repressing cell cycle exit in *Xenopus laevis* (30). Tropomyosin receptor kinase C signaling was additionally required for retinal progenitor cell proliferation (31). The present study revealed for the first time that increased surrounding pressure induced apoptosis in retinal progenitor cells in a pressure-dependent manner. Increased research efforts are required to elucidate the possible underlying molecular mechanisms.

In addition, the present study revealed that retinal ganglion cell-conditioned medium increased the differentiation of retinal progenitor cells into retinal ganglion-like cells, and differentiation increased as surrounding pressure increased. The differentiation of retinal progenitor cells may be affected by many factors. The yes-associated protein gene was revealed to be essential for the cell cycle progression of retinal progenitor cells, and differentiation towards retinal pigment epithelium in the developing mouse eye (32). Perturbations during the early proliferative stages of retinal progenitor cells fated to be rods and bipolar cells altered the coordinated time-dependent progression of differentiation, and synaptic development (33). Activin/nodal signaling was reported to support the differentiation of retinal progenitor cells in a narrow time window during pluripotent stem cell neutralization (34). The Hippo signaling pathway controlled a switch between retinal progenitor cell proliferation and photoreceptor cell differentiation in zebrafish (35). Vascular endothelial growth factor was reported to activate divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation (36). In the present study, it was demonstrated that retinal ganglion cell-conditioned medium induced the differentiation of retinal progenitor cells into ganglion-like cells. It is likely that various growth factors were secreted by retinal ganglion cells into the culture medium, which stimulated the differentiation of retinal progenitor cells towards a ganglion direction. Increased surrounding pressure, as a stress factor, may activate the differentiation signaling of retinal progenitor cells towards ganglion regeneration. The specific growth factors or mediators involved, and the differentiation signaling pathway that is switched on in retinal progenitor cells, require further investigation.

In conclusion, the present study demonstrated that apoptosis in rat retinal progenitor cells and retinal ganglion cells was pressure-dependent, and retinal ganglion cell-conditioned medium increased the differentiation of retinal progenitor cells into ganglion-like cells. In addition, differentiation increased as surrounding pressure increased. Although further investigation is required, these results pave the foundation for possible cell therapy for glaucoma.

Acknowledgements

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