

Promotion on the differentiation of retinal Müller cells into retinal ganglion cells by Brn-3b

Zhen-Kai Wu, Lan Cao, Xue-Yong Zhang, Wei-Tao Song, Xiao-Bo Xia

Department of Ophthalmology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China

Correspondence to: Wei-Tao Song; Xiao-Bo Xia.

Department of Ophthalmology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China.

wtsong1980@126.com; xbxia21@163.com

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Abstract

• **AIM:** To investigate the role of Brn-3b in differentiation process of stem cells derived from retinal Müller cells into the ganglion cell.

• **METHODS:** The passage culture method of Müller cells from retina of newborn Sprague Dawley rats was carried out by repeated incomplete pancreatic enzyme digestion method. The cells were detected by fluorescence-activated cell sorter (FACS), immunohistochemistry technology and reverse transcription-polymerase chain reaction (RT-PCR) to determine the purity. The third passage of cells was induced in the serum-free dedifferentiation medium. The expression of the specific markers Ki-67 and nestin of retinal stem cells was measured by RT-PCR and Western blot. The cell proliferation of retinal stem cells was detected by 5-Ethynyl-2'-deoxyuridine (Edu) staining. The cells were randomly divided into 5 groups as follows: group A: Brn-3bsiRNA group; group B: Brn-3b control siRNA group; group C: pGC-Brn-3b-green fluorescent protein (GFP) group; group D: pGC-GFP group; group E: control group (without any handling). The purified Müller cells were cultured for 3-7d, then, the percentage of ganglion cells was counted by immunofluorescence staining.

• **RESULTS:** FACS demonstrated the purity of retinal Müller cells was more 97.44%. A few spherical cell spheres appeared. Immunofluorescence staining showed that stem cells within the spheres were positive for retinal stem cell-specific markers nestin (red fluorescence, 92.94%±6.48%) and Ki-67 (green fluorescence, 85.96%±6.04%). Meanwhile, RT-PCR analysis showed cell spheres in the culture to have expressed a battery of transcripts characteristic of stem cells such as nestin and Ki-67, which were absent in the Müller cells. Western blot analysis further confirmed the expression of nestin and Ki-67 in the cell spheres but not in the Müller cells. Edu staining showed most of the nuclei within the

cell spheres were stained red (82.80%±6.65%), suggesting the new cell spheres had the capacity for effective proliferation. The statistics result showed the difference between Brn-3bsiRNA group and Brn-3b control siRNA group or the control group was significant ($F=15$, $P<0.05$), while the difference between Brn-3b control siRNA group or the control group was not statistically significant ($P>0.05$).

• **CONCLUSION:** The repeated incomplete pancreatic enzyme digestion method is an efficient and practical method to purify retinal Müller cells. Retinal stem cells were successfully cloned in the dedifferentiation medium. Retinal Müller cells are accessible sources of retinal stem cells. Brn-3b is an important regulatory gene in stem cells differentiated into retinal ganglion cell.

• **KEYWORDS:** Müller cells; retinal ganglion cells; Brn-3b; stem cells; differentiation

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INTRODUCTION

Glaucoma is a group of ocular disorders that can damage the eye's optic nerve and result in vision loss and blindness^[1]. These lective and progressive death of retinal ganglion cells (RGCs) is a final common feature of optic nerve damage of glaucoma^[2]. Present numerous approaches have been developed to provide protection to ganglion cells, but these approaches does not prevent the visual loss caused by the death of RGCs^[3-4]. In recent years, some preclinical studies started to find better treatments from induced differentiation of RGCs. However, stem cells derived from retina are limited in quantity, on the other hand, the use of other stem cells, such as embryonic stem cells and neural stem cells, is greatly restricted due to ethical issues, graft rejection and low efficiency of differentiation. Therefore, if some kind of cells which are abundant in quantity and also derived from the retinal cells can be found to be induces differentiation to RGCs, it will afford more effective cure for the clinical gene-therapy of glaucoma.

Recent studies refers that retinal Müller cells were an accessible source of retinal stem cells, which showed the same characteristics of RGCs in some particular situations^[5-6].

Müller cells derived from retinal cells were abundant in seed cells without the disadvantages of rejection reaction or causing ethical issue. The induction and differentiation of retinal stem cells are largely regulated by the some extracellular and intracellular factors. Recent studies [7] have shown that the Brn-3b transcription factor, a pit-Oct-unc (POU) IV-class protein, is a member of the proteins targeted by the notch pathway during RGCs development. This transcription factor plays a key role in RGC differentiation, survival, and axon outgrowth. Previous study [8] found that in the absence of Brn-3b, the affected ganglion cell precursors fail to appear during development and that the modest reduction in cell number in the inner and outer nuclear layers of the mature retina may follow as a secondary effect of reduced ganglion cell number. In this research, by purifying and cloning retinal Müller cells, inducing them to dedifferentiate into RGCs, we studied the regulatory influences of Brn-3b in the differentiation of retinal stem cells derived from Müller cells into RGCs.

MATERIALS AND METHODS

Experimental Animals Newborn Sprague Dawley (SD) rats of either gender. The experimental animals in this study was in accordance with the Guidelines for Animal Experiments of Central South University, Changsha, China. All animal experiments in this study were conducted with the approval of the Animal Research Committee, Xiangya School of Medicine, Central South University, Changsha, China (Permit No. SCXK 2006-0002).

Methods

Purification and culture of Müller cells The eye balls from a total of 10 newborn SD rats, were enucleated after sacrifice by soaking in 75% ethyl alcohol. Their retinas were removed carefully. The culture method of Müller cells was carried out by repeated incomplete pancreatic enzyme digestion method. The first, second, third passage of retinal Müller cells was detected respectively by fluorescence-activated cell sorter (FACS), reverse-transcription polymerase chain reaction (RT-PCR), Western blot and immunohistochemistry technology to determine the purity by testing the glutamine synthetase (GS), which is a specific marker to identify Müller cells.

Induced dedifferentiation and neurospheres generation of Müller cells After washing the third passage of retinal Müller cells isolated from the medium in phosphate buffer solution for three times, we dissociated the cells with 0.25% trypsin-EDTA in a 37°C tank for 3min. The digested retina was suspended in Dulbecco's modified Eagle's media: nutrient mixture F-12 (DMEM/F12) (1:1) (Grand Island Biological Company, GIBCO), 1 × N2 supplement (GIBCO), 2 × B27 supplement (GIBCO), 20 ng/mL epidermal growth factor (EGF, Peprotech), 10 ng/mL basic fibroblast growth factor (bFGF, Peprotech), 2 mmol/L glutamine, 100 U/mL penicillin

and 100 µg/mL streptomycin until the adherent cells became round under microscopy. The cell suspensions were collected in 15 mL centrifugal tube and the liquid was centrifuged for 3min at 800 r/min. We discarded the liquid supernatant and add dedifferentiated medium to dilute the cells at a density of 1×10^5 cells/cm². The cells were seeded into twelve-well culture plates, cultured at 37°C in a 5% CO₂ incubator. Half of the dedifferentiation media was changed every 3d. We observed the generation state of these cells and detected the specific marker of Müller cells in different time by FACS, RT-PCR, Western blot and 5-ethynyl-2'-deoxyuridine (Edu) staining to determine the multiplication capacity.

Differentiation of retinal stem cells The neurospheres suspension cells were collected and centrifuged them for 3min at 500 r/min. We discarded the liquid supernatant and add appropriate amount of Accutase to dissociate them to single cell. These cells were centrifuged again and resuspended with DMEM/F12 differentiation medium containing brain derived neurotrophic factor (BDNF) (1 ng/mL), retinoic acid (RA) (1 µmol/L) and 1% fetal bovine serum (FBS). The cells were randomly divided into 5 groups as follows: group A: Brn-3bsiRNA group (sc-38767, American Abcam Company); group B: Brn-3b control siRNA group (sc-37007, American Abcam Company); group C: pGC-Brn-3b-green fluorescent protein (GFP) group (Changsha Aijiabio technology Co. Limited); group D: pGC-GFP group (Changsha Aijiabio technology Co. Limited); group E: Control group (without any handling). The stem cells were intervened referred to laboratory manual and cultured in a 37°C incubator. RT-PCR, Western blot and immunohistochemistry technology were performed to detect the percentage of ganglion cells in total differentiated cells after 7d.

Cell Counting and Statistical Analysis Ten non-overlapping visual fields in each group were randomly selected under a fluorescence microscope (×10) to count the number of Müller cells. All data were expressed as the mean ± SD. Statistical analysis was performed with one-way ANOVA and Student's *t*-test in SPSS 13.0. The difference was statistically significant if *P* < 0.05.

RESULTS

Purity of Müller Cells FACS demonstrated that the purity of the first passage retinal Müller cells was 70.89% (Figure 1A), the second passage retinal Müller cells was 90.17% (Figure 1B), the third passage retinal Müller cells was more than 97.44% (Figure 1C).

Taking the first, second, third passage of Müller cells for immunofluorescence staining, we found that, as the cells proliferated, the amount of unwanted cells decreased, and the Müller cells tended to be in the same size and shape. Their cytoplasm became more abundant and the non-specific fluorescence decreased. The density of cultured cells increased and they were equally distributed (Figure 1D-1F).

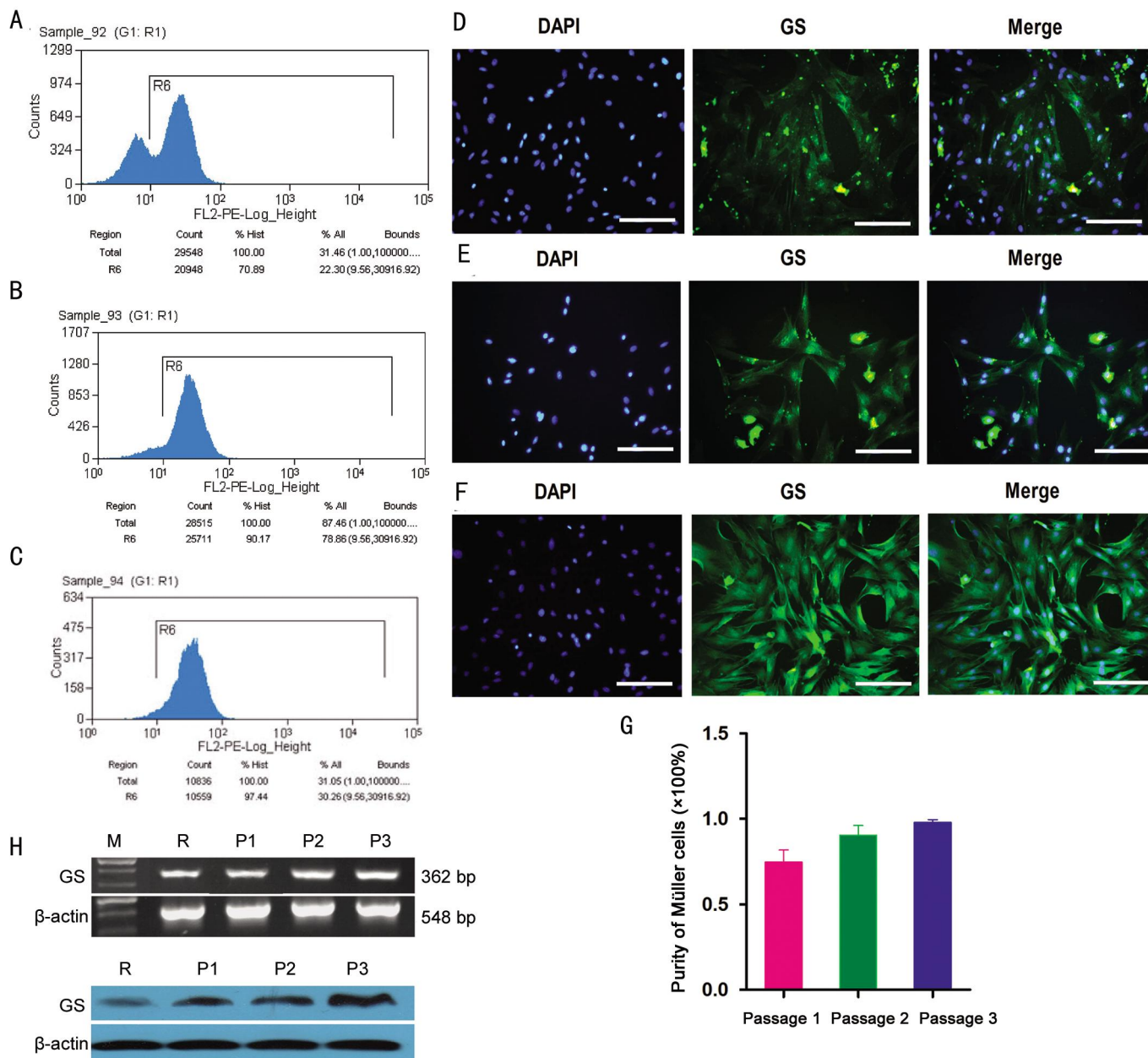


Figure 1 Purity of Müller Cells FACS demonstrated the purity of retinal Müller cells. A: The first passage was 70.89%; B: The second passage was 90.17%; C: The third passage was more than 97.44%; dual staining of primary culture of retinal Müller cells at different passage for 4,6-diamino-2-phenylindole (DAPI) and GS (×100); D: The first passage; E: The second passage; F: The third passage; the nuclei were stained with DAPI; G: As the cells proliferated, the density of cultured cells increased and they were equally distributed; the purity of the first, second, third passage of Müller cells were 73.45%±2.47%, 94.35%±1.26%, 98.50%±1.08% respectively; H: RT-PCR and Western blot analysis indicated that large amount of GS are expressed in both retinal tissues and purified Müller cells,while the GS amount of Müller cells were at a higher level; and as the cells proliferated, the GS amount rapidly increased.

Ten non-overlapping visual fields in each group were randomly selected under a microscope (×10) to count the immunoreactivity for GS. The purity of the first, second, third passage of Müller cells were 73.45%±2.47%, 94.35%±1.26%, 98.50%±1.08% respectively (Figure 1G).

To further determine the purity of cultured cells, we carried out RT-PCR and Western blot analysis to detect the specific markers GS, taking the retinal tissue as a control. The results indicated that large amount of GS are expressed in both retinal tissues and purified Müller cells, while the GS amount of Müller cells were at a higher level; and as the cells

proliferated, the GS amount rapidly increased (Figure 1H). **Characteristics of Müller Cells During Dedifferentiation and Proliferation**

Characteristics of Müller cells during induced dedifferentiation The purified Müller cells were cultured in stem-cell-conditioned medium for 3d. The cells became round and big, abundant of cytoplasm, with clear outlines and high refraction. The proliferation was clonal, and dozens of cells formed cell spheres suspending at the base of the medium, living in suspension (Figure 2A). We changed half of the dedifferentiation media carefully every 2d to avoid

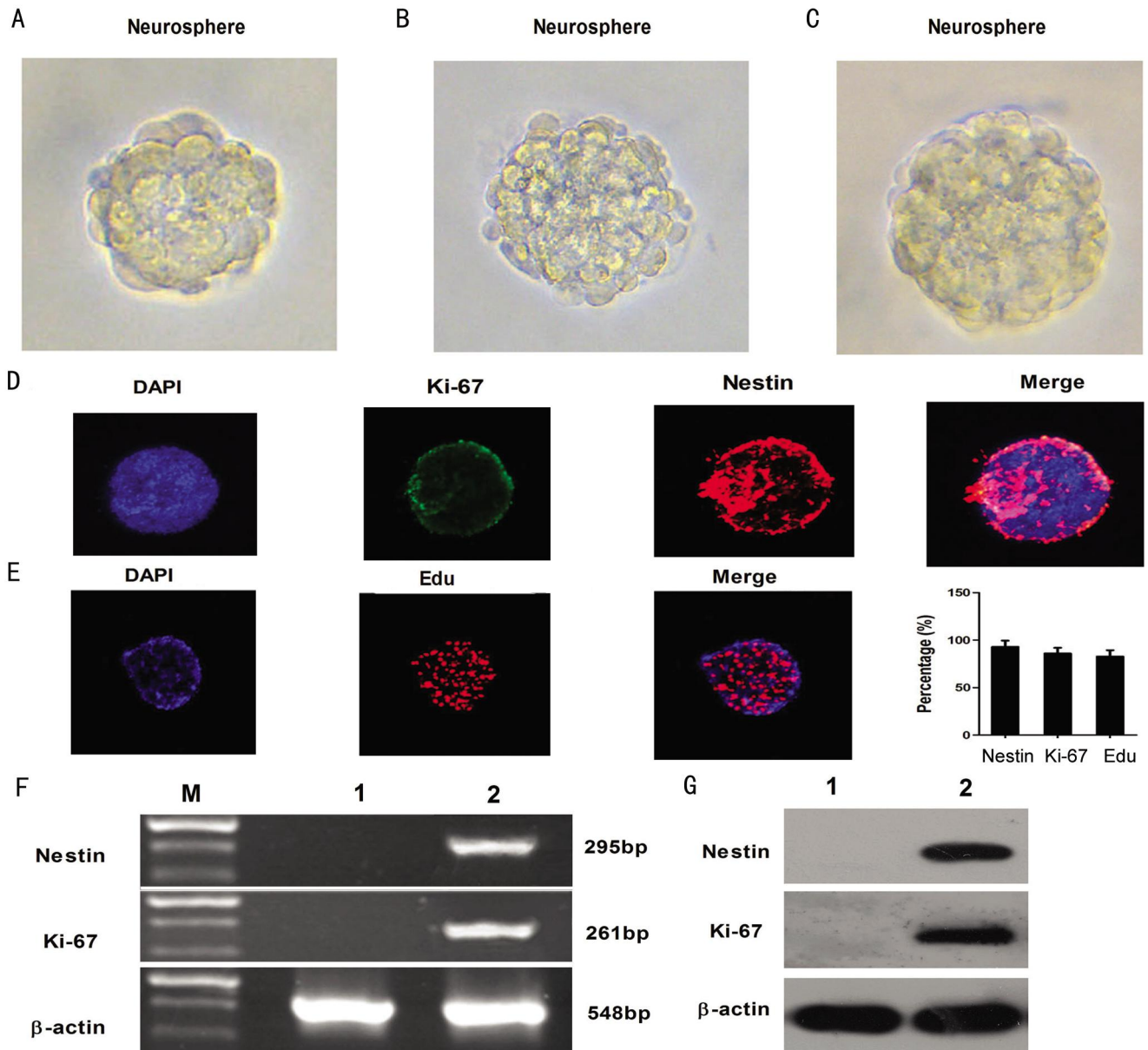


Figure 2 Cell spheres derived from Müller cells A: At the 3rd day of culture, the cells became round and big, abundant of cytoplasm, with clear outlines and high refraction. The proliferation was clonal, and dozens of cells formed cell spheres suspending at the base of the medium, living in suspension; B: At the 5th day of culture, the cell spheres increased in both number and size, and cells exhibited clear boundaries. At the edge of some cell spheres, we can see some short burrs-like protuberances with high refraction; C: At the 7th day of culture, the cell spheres showed no significant increase in number and size (bar=200 μ m); D: The results of immunofluorescence staining showed the cell spheres expressed a battery of Nestin (red fluorescence, 92.94% \pm 6.48%) and Ki-67 (green fluorescence, 85.96% \pm 6.04%); E: Edu staining showed that most of the nuclei within the cell spheres were positive and stained red (82.80% \pm 6.65%), suggesting that the cell spheres have the capacity for effective proliferation (bar=200 μ m); F: High expression of nestin, Ki-67 protein within cell spheres (1: purified Müller cells; 2: cell spheres); G: High expression of nestin, Ki-67mRNA within cell spheres (1: purified Müller cells; 2: cell spheres).

removing the cell spheres. At the 5th day of culture, the cell spheres increased in both number and size, and cells exhibited clear boundaries. At the edge of some cell spheres, we can see some short burrs-like protuberances with high refraction (Figure 2B). At the 7th day of culture, the cell spheres showed no significant increase in number and size (Figure 2C).

Detection of specific markers of cell spheres and the capability of proliferation Taking the cultured cell spheres of the 7th day for immunofluorescence staining, we detected

the specific markers, nestin and Ki-67. The result showed that the cell spheres expressed a battery of nestin (red fluorescence, 92.94% \pm 6.48%) and Ki-67 (green fluorescence, 85.96% \pm 6.04%), which are specific markers of neural stem cells (Figure 2D). Meanwhile, Edu staining showed that most of the nuclei within the cell spheres were positive and stained red (82.80% \pm 6.65%), suggesting that the cell spheres have the capacity for effective proliferation (Figure 2E).

Expression of specific markers and mRNA within cell spheres Taking the cultured cell spheres of the 7th day for

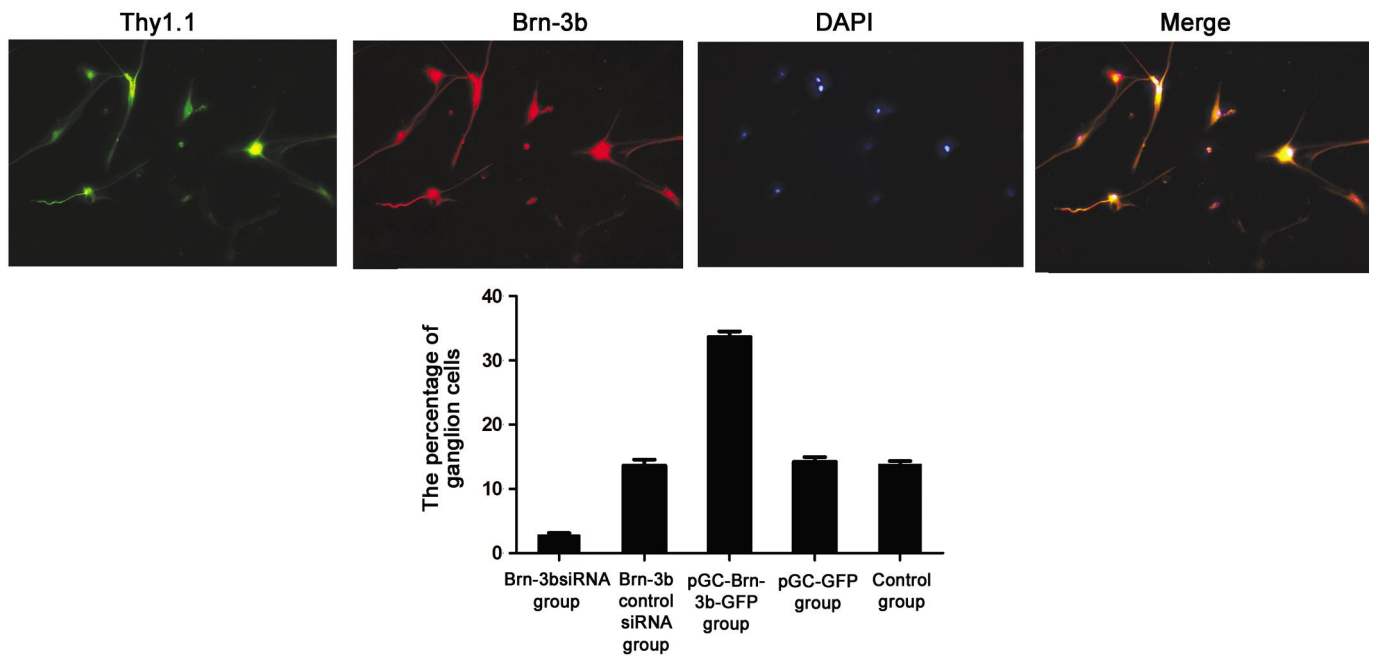


Figure 3 The percentage of ganglion cells differentiated from retinal stem cells The percentage of ganglion cells differentiated from retinal stem cells of Brn-3bsiRNA group: 2.79%±0.32%; control siRNA group: 13.64%±1.26%; pGC-Brn-3b-GFP group: 33.63%±4.27%; pGC-GFP group: 12.20%±1.32%; control group: 13.80%±1.04%. The statistics result showed the difference between Brn-3bsiRNA group and Brn-3b control siRNA group or the control group was significant ($F=15$, $P<0.05$), while the difference between Brn-3b control siRNA group or the control group was not statistically significant ($P>0.05$). The difference was significant between group C and A, B, D, E respectively ($F=34$, $P=0.001$).

RT-PCR and Western blot analysis, the high expression of nestin, Ki-67 and mRNA within cell spheres, compared them with the expression within purified Müller cells (Figure 2F, 2G).

Promotion on the differentiation of retinal Müller cells into retinal ganglion cells by Brn-3b After intervention with the 5 groups and cultured for 7d, immunocytochemical analysis was performed to calculate the percentage of ganglion cells differentiated from retinal stem cells derived from Müller cells in ten randomly selected non-overlapping visual fields (the percentage of ganglion cells differentiated from retinal stem cells=numbers of cells positive for Thy1.1 and Brn-3b/numbers of cell nucleus positive for DAPI×100%). The results showed that the percentage of ganglion cells differentiated from retinal stem cells of group A: 2.79%±0.32%, group B: 13.64%±1.26%, group C: 33.63%±4.27%, group D: 12.20%±1.32%, group E: 13.80%±1.04% (Figure 3). The statistics result showed the difference among group B, D, E was not statistically significant ($P>0.05$), suggesting that Brn-3b control siRNA and the control had no influence on the differentiation of retinal stem cells derived from Müller cells into RGCs; while the difference was significant between Group A and B, C, D, E respectively ($F=15$, $P=0.001$), the difference was significant between group C and A, B, D, E respectively ($F=34$, $P=0.001$), suggesting that Brn-3b acting as an important regulatory factor, promoted the differentiation of retinal stem cells derived from Müller cells into RGCs.

DISCUSSION

Retinal cells include six neurons and one neuroglial cell:

ganglion cells, cone cells, horizontal cells, amacrine cells, rod cells, bipolar cells and Müller cells. Müller cells are a special type of neuroglial cell. On the development of time, the first three cells developed earlier, and the last one is Müller cells. Müller cells and other six neurons share the same progenitor cell [9-10]. Müller cells are radial glial cells which span the entire depth of the neural retina. Radiating from the soma (in the inner nuclear layer) is an inwardly directed process that terminates in an expanded end foot at the inner border of the retina, adjacent to the vitreous humor. Also projecting from the soma is an outwardly directed process that ends in the photoreceptor layer. Microvilli project from this apical process into the subretinal space surrounding the photoreceptors [11-13]. In function, Müller cells have great influences in the following aspects: 1) Müller cells induce the transference, differentiation of retinal neurons and help to maintain their subsistence, promote the development of nervous process, build the retinal neural network during embryonic period; 2) Müller cells nourish retinal neurons and remove their metabolite; 3) Müller cells regulate the neuronal micro-environment including water, PH, K⁺ in the extracellular fluid bathing central nervous system cells; 4) Müller cells play an important role in removing neurotransmitters from extracellular space following their release from synaptic terminals; 5) by controlling the concentration of neuroactive substances in extracellular space, Müller cells can significantly modulate neuronal activity. Recent studies refers that Müller cells were an

accessible source of retinal stem cells, which showed the same characteristics of RGCs in some particular situations, there for Müller cells have become the focus in the generation of retinal neurons.

Retinal tissues contain many types of cell ingredients, we developed a modified approach called repeated incomplete pancreatic enzyme digestion to purify Müller cells. At present, the tissue culture and enzyme digestion methods are the main ways of purifying Müller cells^[14]. With the tissue culture method, retinal tissue is dissociated into small aggregates and the cells attached to the walls, which have been taken from tissues, are passaged. This method generally does not yield high-purity Müller cells, or if it does, the culturing time required is too long. In the enzyme digestion method, cells are passaged with pancreatic enzyme, which is able to detach cells from walls. Then cells are suspended digestion and centrifuged, cultured in medium. In the process of purifying retinal Müller cells from SD rats, the other retinal cells are removed after repeated light pancreatic enzyme digestion based on the characteristics of Müller cells that they can attach to walls quickly and firmly. Using this method, we were able to obtain plenty of high-purity Müller cells, which establish the foundation for further study on the Müller cells. In our study, the purity of the third passage retinal Müller cells was more than 95%, which showed that the repeated incomplete pancreatic enzyme digestion method is an efficient and practical method to purify retinal Müller cells.

GS is a key enzyme that transfers glutamic acid into glutamine and is only expressed in Müller cells^[15]. Therefore, we chose GS as the specific marker to identify Müller cells. Except GS, Müller cells also express vimentin, clusterin, however, both of the proteins are also expressed in other cells. Then we could also identify Müller cells by detecting the relatively specific markers. The RT-PCR results showed that retina Müller cells of SD rat expressed large amount of GS, vimentin, clusterin; in contrast, specific markers corresponding to other types of cells of retinal tissue, such as opsin (rod cells), mGluR6 (bipolar cells), syntaxin 1 (amacrine cells), Brn-3b (RGCs), tyrosinase (pigmented ciliary epithelium cells) and CD31 (endothelial cells) were not detected in purified Müller cells. By counting the immunoreactivity for GS, we detected the purity of the first, second, third passage of Müller cells were 73.45%±2.47%, 94.35%±1.26%, and 98.50%±1.08% respectively. These results demonstrate that our method of purifying Müller cells from retinal tissues is effective.

Purified Müller cells were cultured in a serum-free DMEM/F12 medium supplemented with EGF, bFGF and other nerve growth factor including N2 and B27 to induce the dedifferentiation of retinal Müller cells to neural-stem-cells-like cell spheres. A battery of nestin (green fluorescence,

92.94%±6.48%) and Ki-67 (red fluorescence, 85.96%±6.04%) of the cell spheres in immunofluorescence showed that the cells within the neuro spheres highly expressed retinal stem cell-specific markers nestin and Ki-67; Western blot and RT-PCR analysis showed that cell spheres in the culture expressed a battery of transcripts characteristic of stem cells such as nestin and Ki-67; Edu staining showed that nuclei within the cell spheres were stained strongly positively (82.80%±6.65%), suggesting that Müller cells can differentiate to retinal stem cells and have the capacity for effective proliferation.

EGF and bFGF are important mitogens in cell proliferation, regulating the activity of cell proliferation. Study *in vitro* showed that the amount of Edu-marked cells increased which cultured in medium with EGF. Retinal precursor cell of embryonic rat cultured with EGF could differentiate to retinal neural cells and gliocyte, indicating that EGF could regulate the proliferation of neural precursor cells^[16]. bFGF is a mitogen of neural precursor cells in rodent. These neural precursor cells are from embryonic retina, cortex, hippocampus, corpus striatum, midbrain, spinal cord and the subventricular zone of adult brain^[17]. bFGF, EGF and other cytokines together regulate the proliferation neural stem cells. Our results proved that purified Müller cells cultured in DMEM/F12 medium and supplemented with EGF, bFGF and other nerve growth factor including N2 and B27 can be effectively induced dedifferentiation to retinal stem cells, which establishes the foundation for further study on the differentiation of retinal stem cell.

Retinal stem cells differentiate naturally to any kind of retinal cells in medium for differentiation, which is regulated by several genes. Among these regulatory factors, Brn-3b can synergize to promote the growth and differentiation of rat RGCs during the embryo period, promoting the directed differentiation of embryonic stem cell into RGCs^[18-20]. To investigate whether Brn-3b has the same regulatory effects on retinal stem cells besides embryonic stem cells, we used Brn-3bsiRNA to knockout Brn-3b gene in Müller cells-derived stem cells. Brn-3bsiRNA targets Brn-3bmRNA in a RGC thus potentially killing the cells expressing it^[21]. The results showed that the differentiation rate of Müller cells-derived stem cells into RGCs decreased, confirming the crucial role of Brn-3b gene in promoting the directed differentiation of stem cells into ganglion cells. Our study demonstrates that we can use Brn-3b gene to induce the directed differentiation of retinal stem cells derived from Müller cells into RGCs efficiently, which offers a new method for gene replacement therapy and optic nerve regeneration in glaucoma. At the same time, the differentiation mechanism and regulatory network of stem cells are complicated, which is worth further investigating.

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