STAT3 combines with Rho-ROCK signaling pathway inhibitor, regulate axon growth of ganglion cells derived from retinal Müller cells

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Research

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Abstract

Background

Müller differentiated RGCs have potential therapeutic value for glaucoma. However, axonal regeneration of differentiated RGCs has been a difficult problem. Studies have confirmed that STAT3 and Y27632 play essential roles in regulating neuronal axon regeneration. Whether STAT3 and Y27632 can induce the Müller differentiated RGCs axon regeneration is still unknown.

Method

Retina Müller cells were isolated and purified from Day 21 SD rats’ retina and were differentiated into retinal stem cells. The stem cells were randomly divided into five groups (control group, AAV-STAT3 group, shSTAT3 group, Y27632 group and AAV-STAT3 + Y27632 group). The axon length in each group were measured by ImageJ. Immunofluorescence were used to label the RGCs. The mRNA level of pluripotent associated and differentiation-associated proteins was analysed by qRT-PCR. Stem cells in different groups were injected into mice model of glaucoma. Immunohistochemical, Immunohistochemistry and OCT were performed to access RGC layer thickness in glaucoma model. VEP was used to detect the optic nerve conduction function.

Results

In this study, we found that overexpression of STAT3 could promote the growth of RGCs axons generated by Müller cell differentiation. Combined with Y27632, axonal regeneration was significantly longer than that of the STAT3 group. However, after STAT3 was knocked out, axonal regeneration significantly decreased or even stopped. The mRNA levels of Esrrb, Prdm14, Sox2, and Rex1 in Müller differentiated RGCs after overexpression STAT3 combined with Y27632 were significantly increased, while the mRNA levels of Nestin, Eomes, Mixl1 and Gata4 were significantly decreased. The mRNA levels of Socs3, Pten, Klf9, and Mdm4 were significantly decreased, while the mRNA levels of Dclk2, Armcx1, C-MYC, and Nrn1 were significantly increased. The mRNA levels of differentiation and pluripotency marker genes showed opposite results after STAT3 deletion. After injecting Müller differentiated RGCs intervened by STAT3 combined with Y27632 into the eyes of the glaucoma model mice, the axon length, OCT displayed RGC layer thickness and the electrophysiology indicated by VEP were superior to those of the glaucoma model group.

Conclusions

These findings suggested that STAT3 combined with Y27632 can significantly improve the axonal growth level of RGCs, and reveal the potential mechanism to induce pluripotency of RGCs.

Introduction
Glaucoma is the first irreversible blindness eye disease in the world. In 2010, there were approximately 84 million glaucoma patients. It is estimated that by 2020, the number of glaucoma patients in the world will reach 796 million\(^1\,\,^2\). Glaucoma is a class of neurodegenerative diseases that can be generally classified as primary glaucoma and secondary glaucoma\(^3\). The selective and progressive death of retinal ganglion cells (RGCs) is the common pathway and final outcome of optic nerve damage in glaucoma\(^4\). The goal of glaucoma treatment is to prevent the progressive loss of neurons and protect the optic nerve, thereby preserving the patient’s visual function. Elevated pathological intraocular pressure is usually considered as the leading risk factor for optic nerve damage in glaucoma\(^5\). Thus, the current main treatment methods typically focus on the control of intraocular pressure. However, some patients did not receive good results through ocular hypotensive therapy, mainly due to the pathological changes in glaucoma are not only related to the changes in intraocular pressure\(^6\). Therefore, exploring the mechanisms of death of RGCs, seeking new protection to delay the death of RGCs, and repair or regenerate ganglion axons are fundamental strategies for the treatment of glaucoma. At present, the protection and treatment of the optic nerve of glaucoma mainly included: improvement of optic disc microcirculation, glutamate pathway inhibitors, neurotrophic factors, induction of heat shock protein expression, and anti-oxidation therapy\(^3\,\,^7\,\,^8\). However, these methods are still unable to prevent RGCs damage and protect the optic nerve effectively. Besides, these methods are not helpful for those patients who have lost the RGCs with advanced or absolute glaucoma.

In recent years, stem cell research has brought new hope for the alternative treatment of glaucoma RGCs. Many studies found that retinal Müller cells are a kind of abundant and self-derived potential retinal stem cells\(^9\,\,^{10}\). They provide nutrient and metabolic support for the retinal neurons and neurotransmitter cycle. Therefore, they may become essential cell sources for retinal neuron regeneration. In our previous studies, we have successfully purified retinal Müller cells in vitro and induced dedifferentiation into retinal stem cells\(^11\). Simultaneously, based on Müller cell-derived stem cells, it was also confirmed that the Atoh7 gene could promote a large number of RGCs directional differentiation, with a differentiation rate of 50.4\%\(^12\). However, in vivo studies showed that the differentiated RGCs did not appear axon growth and just expressed the RGCs specific markers Thy1.1 and Brn-3b, which lead to loss of the function of conducting signals. In addition, although it was found that in vitro RGCs differentiated with axons, their length was only about 600 µm. Therefore, how to regenerate the retinal Müller cell differentiated RGCs restart the axon growth, and guide them along the correct route to the brain projection zone has become a problematic point in current research.

Current research has confirmed that the reactivation of nerve growth ability is related to the transcriptional activator in nerve cells\(^13\). Among them, signal transducers and activators of transcription-3 (STAT3) play an essential role in the regeneration of RGCs\(^14\). Studies have shown that the expression of the endogenous gene STAT3 is low in normal retinas. It often exists in cells in a non-phosphorylated form when cytokines or growth factors are activated by binding to receptors, activated STAT3 is transferred from the cytoplasm into the nucleus and participated in regulating the gene expression\(^15\). Recent studies have also shown that Rho-associated coiled-coil-Containing protein kinase (Rho-ROCK) is negatively related to cell mitosis and nerve regeneration\(^16\). Rho/ROCK signaling...
pathway inhibitor Y27632 phosphorylates STAT3. The combination of Y27632 and STAT3 efficiently promotes neurite cell axon regeneration and significantly up-regulates its downstream genes p21, Irf1, and Spr1a(17). Therefore, this study was designed to investigate the effect of combination of Y27632 and STAT3 in regenerating ganglion cell axons derived from retinal Müller cells, and to explore its regulatory mechanisms, laying the foundation for the regeneration of glaucoma nerves.

**Materials And Methods**

**Ethics Statement**

The animals in this study were in accordance with the Guidelines for Animal Experiments of Central South University, Changsha, China.

**Animals**

C57BL6 mice, without specific pathogen grade, were obtained from Central South University Animal Experimental Department.

**Lentivirus PGC-FU-Atoh7-GFP Construction**

Atoh7 expression vector Lentivirus PGC-FU-Atoh7-IRES-GFP was constructed by GENEChem (Shanghai, China). The neurosphere cells were transfected with the lentivirus at the multiplicity of infection (MOI) of 10 and the efficiency of transfection was detected by FACS(12).

**Recombinant AAV-STAT3 Vectors Construction:**

AAV-STAT3 vectors were produced by the plasmid co-transfection method. Recombinant AAV-STAT3 was purified via iodixanol gradient ultracentrifugation. The 40% iodixanol fraction was buffer-exchanged with 0.001% Tween in phosphate buffered saline (PBS) and concentrated using 100K Amicon Ultra-15 centrifugal filter units. The final volume of the centrifugation filter unit is 200 ml. DNase-resistant viral genomes in the concentrated stock were then titered by quantitative PCR relative to standards. Vector concentrations were calculated in viral genomes/ml at 2–4 × 10^{13} vg/ml.

**Immunohistochemical Analysis**

Immunocytochemical analysis was performed for the detection of cell-specific markers. Briefly, cells fixed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) for 15 min at room temperature. The cells were permeabilized with 0.1% Triton X-100/PBS for 10 min, blocked with 5% goat serum for 1 h, and incubated with primary antibodies for 1 h at room temperature. For negative controls, 0.01 M PBS was used to replace primary antibodies. After extensive washing with PBS, the cells were blocked again with 5% goat serum for 20 min. Then the cells were incubated with Cy3 or Alexa Fluor 488-conjugated secondary antibodies (KPL, Rouses Point, NY, USA) for 1 h in the dark. Following extensive washing with PBS, 1 μg/μl DAPI (Sigma) was used to stain the nuclei. Images were taken by a fluorescence microscope or a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems GmbH, Wetzlar, Germany).
The positive cells were quantified in at least 10 fields across the coverslips from three independently dissociated cultures.

**FACS Analysis**

The purity of the enriched Müller cells was examined by fluorescence-activated cell sorting (FACS) analysis, as previously described(12). Briefly, the cells were separated into single cells by passage and centrifugation. Then the cells were fixed with 4% paraformaldehyde and blocked with a PBS containing 1% BSA and 0.1% TritonX-100 for 30 minutes at 4 °C, then incubated at 4 °C for 1 h with the primary antibodies(GS, Abcam) After incubation, the cells were incubated in a PBS-BSA solution containing the appropriate secondary antibodies linked to FITC at 4 °C for 1 h in the dark. The cells were washed with PBS and resuspended in PBS for FACS analysis.

**Edu Labeling Analysis**

To evaluate the proliferation of stem cells, neurospheres were incubated with 1:1,000 Edu (RiboBio) diluted in culture solution overnight at 37 °C. After several washes, the cells were fixed with 4% paraformaldehyde for 30 minutes. Fixed cells were incubated with an Apollo buffer (RiboBio) for 30 minutes at room temperature in the dark. The cells were washed with 0.5% TrixtonX-100 (diluted in PBS) for 10 minutes, followed by staining with Hoechst 33342 (RiboBio) at room temperature for 30 minutes in the dark. Images were captured using fluorescent microscopy.

**qRT-PCR Analysis**

Total RNA was isolated from cells using Trizol (Sigma, St. Louis, MO, USA) reagent according to the manufacturer’s protocol and reverse transcribed to cDNA. PCR reaction was performed in a 20 µl volume containing the following: 10 µl 2 × SYBR Green mix, 1 µl 10 µM forward primer and reverse primer, 2 µl diluted cDNA and 6 µl double-distilled H2O. Amplification conditions were as follows: 15 sec at 95 °C (one cycle); 5 sec at 95 °C, 5 sec at annealing temperature, and 30 sec at 72 °C (45 cycles). The RNA expression level was normalized to that of Gapdh. The corresponding quantitative RT-PCR primers were shown in Table S1.

**Western Blot Analysis**

Proteins were obtained from the cells using RIPA (RiboBio) buffer containing 1:100 protease inhibitor and 1:100 phosphatase inhibitor. The protein concentration was determined by using a microplate reader. Lysates were separated on SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in TBS plus 0.1% Tween (TBS-T) for 1 h, then incubated with primary antibodies for 1 h at room temperature. After several washes, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. Primary antibodies, including anti-STAT3 (Cell Signaling Technology), anti-p-STAT3 (Cell Signaling Technology), Tyrosinase (Santa Cruz), mGluR6 (Abcam), Atoh7 (Abcam), CD31 (Cell Signaling Technology), Rhodopsin (Cell Signaling Technology), β-tubulin ((Cell Signaling Technology), Iba1 (Abcam), Brn3b (Abcam), Pax6 (Abcam), HPC-1
(Abcam), Sprl1 (Santa Cruz), P21 (Santa Cruz), Irl (Santa Cruz), Musashi1 (Abcam), Gap43 (Abcam), Ki-67 (Abcam), Nestin (Abcam), and anti-β-actin (Santa Cruz), were used in this study.

**Establishment of Chronic Ocular Hypertension Glaucoma Model of Mice**

Ocular hypertension was induced using a method developed by Chiu et al(18). Briefly, mice were anesthetized with 10% chloral hydrate (0.4 mL/100 g; Sigma-Aldrich Inc., St. Louis, MO, USA) injected intraperitoneally and placed in front of a slit lamp equipped with a 532-nm diode laser that delivered 0.7W pulses for 0.6 s (Carl Zeiss, Germany). One drop of 1% proparacaine (Alcon-Pharm Inc., Texas, USA) was applied to the right eye (experimental eye) as a topical anesthetic before laser photocoagulation. Then, 50–60 laser pulses were directed to the trabecular meshwork 270° around the circumference of the aqueous outflow area and 15–20 laser spots on each episcleral aqueous humor drainage vein of the right eye. The left eye was the control eye without any treatment. IOP was measured bilaterally using a digital tonometer (Tonopen XL, Reichert, USA) at day 3, day 10, day 15, day 20, day 25, day 30, day 35, day 40, day 50, day 60 after laser photocoagulation.

**Stem Cells Transfection and Intraocular Injections**

Neurospheres dedifferentiated from Müller cells were transfected by lentivirus PGC-FU-Atoh7-GFP. After 24 h of transfection, the stem cells were divided into 5 groups: group A: PBS control group, PBS was added to medium; group B: STAT3 group, only added AAV-STAT3 to the medium; group C: shSTAT3 group, only added AAV-shSTAT3 to the medium; group D: Y27632 group, only add Y27632 to the medium; group E: STAT3 + Y27632 group, added both AAV-STAT3 and Y27632 to the medium. 24 hours later, the neurospheres were dissociated into single stem cells with accutase. The stem cells of each group were collected at a concentration of $1 \times 10^4$ cells/µL. The mice were anesthetized by inhalation of diethyl ether and i.p. injection of pentobarbital. The eyes were injected with 5 µL of stem cells, 5µL brain-derived neurotrophic factor (BDNF) (1 ng/mL) (Peprotech), 30 nmol BrdUrd, 100 ng RA (1 µM) (Sigma). Eyeball frozen sections were performed for Immunohistochemistry and immunofluorescence analysis.

**Eyeball Sections Immunohistochemistry Analysis**

Eyeball frozen sections and immunohistochemistry were performed as described(19, 20). Briefly, mice retinal tissue sections or retinal cells were incubated in PBS containing 3% bovine serum albumin (BSA), 5% goat serum, and 0.3% TritonX-100 at 37°C for 1 h, followed by incubation at 4°C overnight with the primary antibodies. Mice retinal tissue sections were then incubated in the dark at room temperature for 1 h with anti-rabbit IgG conjugated with FITC (1:100, Sigma), anti-mouse IgG conjugated with FITC (1:100, Sigma), anti-rabbit IgG conjugated with TRITC (1:100, Sigma), and anti-mouse IgG conjugated with TRITC (1:100, Sigma). Finally, the retinal tissue sections were incubated with 40,6-diamidino-2-phenylindole (DAPI) (Beyotime, Institute of Biotechnology, Wuhan, China) for 5 min and images were captured using fluorescent inverse microscopy (Leica DMI4000B).

**Statistical Analysis**
Data from at least three independently dissociated cultures, each measured in triplicate, were expressed as mean ± standard deviation. Statistical analysis was performed with one-way ANOVA and Student’s t-test using SPSS 18.0. The difference between the two samples was statistically significant when \( p < 0.05 \).

**Results**

**Müller Cell Culture and Identification**

We found that GS as Müller cell-specific marker was significantly high express in three generations cells dedifferentiated from mice retina (Fig. 1A). Results also showed that 99.8% of the third-generation purified cells were immunoreactive for GS by Flow cytometry (Fig. 1B). Western blot was used to access the protein expression of specific markers of other retinal cells in purified cells. The result showed that the protein expression of Atoh7, Rhodopsin, Pax6, β-tubulin, Bm-3b, HPC1, Tyrosinase, and mGluR6 were low. On the contrary, the protein expression of Iba1 and CD31 were significant in purified cells (Fig. 1C-D).

**Stem Cell Culture**

After cultured in DMEM/F12 dedifferentiation medium for 24 h, cells proliferate and differentiate into round shapes, and then some cells aggregate to form small cell spheres. After 48–72 h, the proliferation of cells was significantly accelerated, and the shape of single round cells was decreased compared with the former. The diameter of the cell spheres increased. After 5d, the number and the diameter of the cell sphere continued to increase, the boundary of the cell sphere was clearly defined, and the refractive index was strong. After 7 days, there was a slight increase in the volume of the cell sphere compared to before. At 10th day, the central refractivity of the visible neurosphere was reduced, showing that the cell bodies were atrophied and darkened (Fig. 2A). The cell proliferation was observed daily under a phase-contrast microscope and the diameter of the neurosphere was measured (Fig. 2B). When the cell diameter reached 150 µm, the cell sphere was digested with Accutase(Sigma) and passaged to obtain purified neurospheres. After three passages, the purified neurospheres were examined by immunofluorescence. Our results showed that the purified neurospheres are labeled with nestin, ki-67, pax6, and musashi1 antibodies, and the expression level of these four antibodies is approximately 90% (Fig. 3A). WB showed that the cell spheres purified after three passages highly expressed nestin, ki-67, pax6, and musashi-1 when compared with the purified Müller cells (Fig. 3B-D). These results indicated that the cells purified with three passages in the dedifferentiation medium are dedifferentiated into retinal stem cells. We then used Edu marker to detect the proliferation ability of these retinal stem cells. Our results showed that the proliferation ability of these purified retinal stem cells was very strong (Fig. 3E).

**Effect of STAT3 and Y27632 on Axonal Regeneration of Müller Cell Differentiated RGCs**

After the retinal stem cells were identified, they were transfected with the lentivirus PGC-FU-Atoh7-GFP. The cells were plated onto 0.01% poly-D-lysine (Sigma)-coated 24 mm coverslips (Corning) at a
concentration of $1 \times 10^4$ cells/well, and cultured in differentiation medium (1 ng/ml BDNF, 1 µM RA and 1% FBS). Three days later, stem cells were randomly divided into five groups: group A: PBS control group, PBS was added to medium; group B: AAV-STAT3 group, only added AAV-STAT3 to the medium; group C: AAV-shSTAT3 group, only added AAV-shSTAT3 to the medium; group D: Y27632 group, only add Y27632 to the medium; group E: AAV-STAT3 + Y27632 group, added both AAV-STAT3 and Y27632 to the medium. After 48 hours, the number of STAT3-labeled cells increased, the fluorescence intensity was enhanced, and the cytoplasm was uniformly distributed (Fig. 4A). The differentiation of RGCs in each group was observed every day.

With the prolongation of the differentiation time, the rounded stem cells gradually grow into a plurality of protrusions. And these cells also extended branches. The axon of the proximal cell body in these cells is thicker than that of the distal body. The axon walks more and more naturally. In the 12 days of differentiation, the length of axon in each group can be observed by IF staining (Fig. 4B).

In the control group, the axon length of ganglion cells was 384.3 µm. The axon length of the ganglion cells in AAV-STAT3 group and AAV-shSTAT3 was 956.3 µm and 278.2 µm respectively. In the Y27632 group, the axon length of ganglion cells was 1347.9 µm. In the AAV-STAT3 + Y27632 group, the axon length of ganglion cells was 1999.3 µm. The axon length of ganglion cells in the AAV-STAT3 group was longer than that in the control group. In the AAV-shSTA3 group, the axon length was shorter than other groups. The results showed that the axon length of RGCs in the AAV-STAT3 + Y27632 group was significantly longer than that in the other groups (Fig. 4C).

Western blot analysis was performed on STAT3 group and STAT3 + Y27632 group on day 12. The results showed that the protein expression levels of p-STAT3, p21, GAP43, Irfland, and Sprr1a1 in the STAT3 group were significantly higher than those in the control group. The protein expression levels of P-STAT3, P21, GAP43, Irfland, and Sprr1a1 in the STAT3 + Y27632 group were higher than those in the STAT3 group, and the differences were statistically significant (Fig. 4D-E). mRNA levels of axonal regeneration related proteins Socs3, Pten, Klf9, Mdm4, Dclk2, Armcx1, C-myc, and Nrn1 at D0, D3, D7, and D12 were also analyzed. The experimental results showed that the mRNA level of Socs3, Pten, Klf9 and Mdm4 in STAT3 + Y27632 group was significantly lower than that in STAT3 group, and the expression level was the lowest at D12. The mRNA levels of Dclk2, Armcx1, C-MYC and Nrm1 were significantly higher in STAT3 + Y27632 group than in STAT3 group. The mRNA level of Dclk2 was the highest at D7 and decreased at D12. The mRNA level of Nrm1 was the highest at D3, but decreased at D7 and D12. The mRNA level of Armcx1 and C-myc was highest at D12 (Fig. F-M).

The above results suggest that the use of STAT3 alone in vitro could promote the growth of axons. When combined with Y27632, the effect of promoting growth of axon was more substantial in RGCs.

**Mechanism of STAT3 Combined with Y27632 on the Regeneration of RGCs Differentiated by Müller Cell**
We used WB to detect the expression levels of STAT3, and p-STAT3 in each intervention groups of Müller cell differentiated RGCs (Fig. 5. A-C). The results showed that the expression levels of STAT3 and P-STAT3 proteins in the STAT3 group, Y27632 group and STAT3 + Y27632 group were all higher than those in the control group, while the expression levels of STAT3 and P-STAT3 proteins in the shSTAT3 group were lower. There was no significant difference in STAT3 and P-STAT3 protein expression levels between the STAT3 and Y27632 group. The expression levels of STAT3 and P-STAT3 proteins in the STAT3 + Y27632 group were significantly higher than those in the other groups. mRNA levels of pluripotent related genes (Esrrb, Prdm14, Sox2, and Rex1) and differentiated related genes (Nestin, Eomes, Mixl1, and Gata4) were also detected at D12 (Fig. 5D, E). The results showed that mRNA levels of pluripotent related genes (Esrrb, Prdm14, Sox2, and Rex1) in the STAT3, Y27632 and STAT3 + Y27632 groups were significantly increased, while mRNA levels in the shSTAT3 group were significantly decreased. The mRNA level of differentiated related genes in the STAT3 + Y27632 group was markedly lower than that in the other groups. We also detected the mRNA levels of axonal regeneration related proteins Socs3, Pten, Klf9, Mdm4, Dclk2, Armcx1, C-Myc, and Nr1 at D12. The mRNA levels of Socs3, Pten, Klf9, and Mdm4 in the shSTAT3 group were increased dramatically, while those in the STAT3, Y27632, and STAT3 + Y27632 groups were significantly decreased. mRNA levels of Dclk2, Armcx1, C-myc, and Nm1 were significantly increased in the STAT3 group, Y27632 group, and STAT3 + Y27632 group. mRNA levels of pluripotent related genes (Esrrb, Prdm14, Sox2, and Rex1) in the STAT3 and STAT3 + Y27632 groups were also detected at D0, D3, D7, and D12. mRNA levels of pluripotent related genes in the STAT3 group and the STAT3 + Y27632 group were higher than those in the control group at all time. The mRNA expression level of the STAT3 + Y27632 group was significantly higher than that of the STAT3 group and control group. mRNA levels of differentiated related genes (Nestin, Eomes, Mixl1, and Gata4) in the STAT3 group and the STAT3 + Y27632 group were also detected at D0, D3, D7, and D12. The results showed that the mRNA levels of differentiated genes in the STAT3 + Y27632 group and the STAT3 + Y27632 group were lower than those in the Control group at all time points, and the expression levels of mRNA in the STAT3 + Y27632 group were significantly lower than those in the STAT3 and control groups. The above results suggest that STAT3 combined with Y27632 may improve the pluripotency of MULLER differentiated RGCs cells to regulate the expression level of proteins related to axon regeneration and promote its axon regeneration.

Detection of Ganglion Cells in Mice Chronic Ocular Hypertension Glaucoma Model

To further verified the role of STAT3 and Y27632 in promoting the growth of RGCs axon, we established mice chronic ocular hypertension glaucoma model. IOP was measured bilaterally under anesthesia at day 3, day 10, day 15, day 20, day 25, day 30, day 35, day 40, day 50, day 60 after laser treatment using a
digital tonometer. Our result showed that the mean IOP of glaucomatous eyes was elevated significantly compared with those of contralateral eyes from day 3 to day 30. IOP was gradually increased with time and reached the maximum in 10–25 days. IOP began to decline at day 30 and reached a normal level about at 60 days (Fig. 5A).

HE stain was used to detect the numbers of retina ganglion cell nucleus in the glaucoma mice model (Fig. 5B). These data showed that the number of nuclei was gradually decreased with continuous high IOP. At day 60, IOP decreased to the normal level, but the number of RGCs was still decreasing (Fig. 5C). Moreover, to determine whether RGCs undergo apoptosis in glaucoma mice model, we performed TUNEL staining and found apoptosis of the RGCs in the retinal ganglion cell layer (Fig. 6). The result showed that the number of apoptotic nuclei gradually increased with the continuous increase in IOP. At day 3, RGCs was still regularly shaped and uniformly distributed in retina ganglion cell layer. At day 10, cytoplasm and nucleus of part of RGCs began to disintegrate. At day 30, more cell debris were detected in retina ganglion cell layer. At day 60, most of RGCs disappeared with a few debris left. The proportion of TUNEL positive cells were $6.5 \pm 2.1, 8.7 \pm 1.7, 13.5 \pm 3.6, 17.1 \pm 3.0\%$, respectively. These data showed that the number of apoptotic RGCs gradually increased with the continuous increased IOP. In the meantime, we found an increase in apoptosis of cells in outer nuclear layer.

**Growth of RGC Axon in Glaucoma Mice Model**

Stem cells dedifferentiated from Müller cells were transfected with lentivirus PGC-FU-Atoh7-GFP and cultured in differentiation medium. After 24 hours, these stem cells were randomly divided into four groups: group A: control group, PBS was added to medium; group B: AAV-STAT3 group, only added AAV-STAT3 to the medium; group C: Y27632 group, only add Y27632 to the medium; group D: AAV-STAT3 + Y27632 group, added both AAV-STAT3 and Y27632 to the medium. 24 hours later, we divided glaucoma mice models randomly into four groups as above and injected stem cells into the vitreous cavity in each group. IF staining of retinal tissue sections was performed to detect cell-specific markers GFP and ZO-1 (a kind of tight junction protein) after 12 days of retinal stem cell transplantation. The length of axons in each group can be observed by the chemical staining of immunofluorescence cells. In the control group, the axon length of RGCs was 360.5 µm. The axon length of RGCs in the AAV-STAT3 group was 877.3 µm. The axon length of RGCs in the Y27632 group was 1427.9 µm. In the AAV-STAT3 + Y27632 group, the axon length of ganglion cells was 1986.7 µm. The axon length of RGCs in the AAV-STAT3 group was longer than that in the PBS group. The axon length of RGCs in the AAV-STAT3 + Y27632 group was significantly longer than that in the other groups. We observed that the axons of RGCs were significantly longer than those of the glaucoma model group after transplantation of Müller cell differentiated RGCs after STAT3 + Y27632 intervention by retinal lamination. We observed that the axons of RGCs were significantly longer than those of the glaucoma model group after transplantation of Müller differentiated RGCs intervened with STAT3 + Y27632 by retinal lamination. We observed the thickness of RGCs layer in each group through OCT. The RGCs layer of the STAT3, Y27632, and STAT3 + Y27632 groups was significantly thicker than that of the glaucoma model group. In comparison the RGCs layer of the STAT3 + Y27632 group was significantly thicker than that of the other groups. Flash-VEP was used to detect
optic nerve conduction in each group. The optic nerve conduction function in the STAT3, Y27632, and STAT3 + Y27632 groups were significantly better than that of the glaucoma model group, and the optic nerve conduction function in the STAT3 + Y27632 group was significantly better than that of the other groups.

**Discussion**

Glaucoma is a main blinding disease in the world. It causes permanent damage of the optic nerve since it's impossible for mammalian nerves regenerated. Glaucoma is characterized by progressive loss of retinal ganglion cells along with their optic nerve axons. The current treatment for glaucoma is the reduction of intraocular pressure (IOP), nutrition of optic nerve, or the antioxidation treatment. These treatments postpone further death of the ganglion cell. However, these methods can not make the apoptosis RGCs regenerate. Therefore, for the patient with massive death of ganglion cells, we must find an effective therapy to activate the regeneration of ganglion cells to reestablish visual pathway and help these patients to regain their vision.

Atoh7 is a member of the bHLH family, and in our previous research, it had been verified that Atoh7 regulates Müller cell-derived stem cells differentiating into RGCs both in vitro and in vivo. This makes it possible for us to replace glaucoma-induced apoptosis of RGCs cells by transforming Müller cell-derived stem cells differentiating into RGCs. However, the axonal growth of the regenerated RGCs determines the reconstruction of the visual pathway. How to promote the growth of regenerated RGCs axons becomes our research focus. Studies have shown that STAT3 plays an important role in the axon growth of retinal ganglion cells. However, the specific mechanism of STAT3 promoting RGCs axon regeneration is still unknown.

STAT3 is a kind of bifunctional cytoplasmic protein coupling with tyrosine phosphorylation signal pathway in the cytoplasm (21). Normally STAT3 protein is in a non-phosphorylated form in cells, when cytokines or growth factors bind to the receptor, tyrosine kinases (JAK) that bind to receptors are activated. Activated JAK phosphorylates the 705th position of tyrosine residue (Tyr705) in the STAT3 cytoplasm. They combined to form the JAK/STAT3 homodimers. Then the homodimers entered into the nucleus, recognized specific DNA sequences and regulated the transcription of target genes. Therefore, the amount of nuclear-activated STAT3 represents the activation of the JAK/STAT3 pathway in the cell. Activated STAT3 will be dephosphorylated in the nucleus after transmitting signals and be restored to the monomeric form. And then, it will deactivate and return to the cytoplasm to participate in the next round of signal transduction. The activation degree of JAK/STAT3 in normal peripheral nerves is low. Only phosphorylated activated STAT3 may translocate into the nucleus to regulate the expression of certain genes to regenerate peripheral nerve. Our experimental results also confirmed that the axons of RGCs in the STAT3 overexpressed group were significantly longer than those in the STAT3 inhibited group. We hypothesized that STAT3 regulates the growth of RGCs axons through the above mechanisms.
Research showed that when adeno-associated virus vector containing STAT3 gene was injected into vitreous cavity of the optic nerve contusion model rats, they found that the number of STAT3 positive cells increased significantly as well as the expression of STAT3 protein and STAT3 mRNA increased by 4 to 6 fold after 3 weeks. And the expression of GAP-43 (growth-associated protein-43), a marker of retinal ganglion cell axon regeneration, significantly increased. The optic nerve 3D image analysis which used nerve fiber anterograde tracer CTb-594 showed that the axons of ganglion cells after STAT3 gene infected increased about 200 microns than the control group. But the axon density did not increase and formed many U-turn and right-angle turn. These results indicated that STAT3 signal transduction can increase the length of retinal ganglion cell axons and promote the extension of axonal growth. Another research showed that the use of the JAK2 inhibitor AG490 in vitro in RGCs inhibited CNTF-mediated axonal growth and significantly reduced the regenerative effect of RGCs in response to inflammatory stimuli in vivo. These results confirmed that the activation of JAK and STAT3 played an important role on the axis initial stage of outgrowth (22, 23). Studies have demonstrated that STAT3 signaling pathway plays an important role in the axon growth of RGCs(24, 25). The same results were obtained in our experiment. Deletion Pten, a negative regulator of the mammalian target of rapamycin (mTOR) pathway in adult RGCs, promotes robust axon regeneration after optic nerve injury (26). A high-throughput gene profiling study revealed that the deletion of Klf9 gene substantially promotes optic nerve regeneration in adults RGCs(27). Researches confirmed that a genetic deletion of PTEN, SOCS3, or PTEN/SOCS3 allows partial axon regeneration in the optic nerve after optic nerve crush(28, 29). Inhibition of Mdm4 in the eye and spinal cord promotes axonal regeneration and sprouting of the optic nerve after crush and of supraspinal tracts after spinal cord injury(30). Our research showed that the mRNA level of PTEN, SOCS3, Klf9, and Mdm4 decreased significantly in AAV-STAT3 group. Overexpression of Dclk2, Armcx1, c-myc, and Nrn1 are closely related to the regeneration of RGCs axon(31–34). The mRNA level of Dclk2, Armcx1, c-myc, and Nrn1 significantly increased in the AAV-STAT3 group. The inhibition of STAT3 expression inhibited the growth of the RGCs axon. The mRNA expression levels of RGCs axon-regenerated proteins were significantly opposite between the shSTAT3 group and the AAV-STAT3 group. All the above results confirmed that STAT3 overexpression could promote axonal regeneration of Müller differentiated RGCs.

In our study, it was found that Y27632 can also promote the axon growth of Müller cell differentiated RGCs. Recently years, studies have shown that Rho-associated coiled-coil-containing protein kinase (Rho-ROCK) is negatively correlated with cell mitosis and nerve regeneration. Rho is a small molecule homopolymer of the GTPases superfamily and is a mammalian gene homologue of the Ras superfamily. Its biological function is mainly through its downstream effector molecule ROCK. ROCK is a serine/threonine-protein kinase whose molecular structure includes an amino-terminal catalytic domain, an intermediate domain which combined with Rho's α-coiled-coil, a carboxy-terminal catalytic domain, and a Cys/His region. Activated Rho-GTP activates ROCK by binding to the alpha coiled-coil domain of ROCK and exposing the catalytic center of ROCK(35). Our results have shown that STAT3 could be phosphorylated by Y27632, which is an inhibitor of the Rho-ROCK signaling pathway. The Y27632/STAT3 combination can effectively promote axon regeneration of Müller cell differentiated RGCs. The axon length of RGCs in the AAV-STAT3 + Y27632 group was the longest, and the expression of GAP-43,
phosphorylated STAT3 and its downstream genes p21, Irf1 and Sprr1a was significantly increased. Inhibition of the Rho/ROCK signaling pathway not only reduced the U-turn of the axon and avoid navigation errors but also promote axon regeneration in RGCs. Our research is the first to confirmed that the combined treatment of STAT3/Y27632 on Müller-derived RGCs can improve the differentiation rate of RGC cells and significantly increase the length of axons, which is a significant improvement for RGCs regeneration. The mRNA levels of RGCs axon-growth-related proteins Pten, Socs3, Klf9, and Mdm4 in the STAT3 + Y27632 group were significantly lower than those in the STAT3 group, while the mRNA levels of Dclk2, Armcx1, C-myc, and Nrn1 were significantly higher than those in the STAT3 intervention group. In order to analyze the mechanism underlying the effect of STAT3 and Y27632 in promoting axon growth of Müller cells differentiated RGCs, we tested the mRNA level of pluripotency genes (Esrrb, Prdm14, Sox2, and Rex1) and differentiation genes (Nestin, Eomes, Milx1, and Gata4). In the STAT3 + Y27632 group, mRNA level of pluripotency genes (Esrrb, Prdm14, Sox2, and Rex1) compared with STAT3 group increased significantly, the mRNA level of differentiation genes (Nestin, Eomes Milx1, and Gata4) decreased obviously. We concluded that the reason for overexpression of STAT3 promote axon growth could be launched Müller cells differentiated RGCs pluripotency. The combination of STAT3 and Y27632 can promote the axon growth of Müller cells differentiated RGCs better than STAT3 alone, which is the same mechanism.

Furthermore, to verify whether this regulatory mechanism acts as the same in vivo, we established mice chronic ocular hypertension glaucoma model in this study. To verify whether STAT3 and Y27632 improve growth of RGCs differentiated from stem cells, the stem cells were transfected with lentivirus PGC-FU-Atoh7-GFP. The stem cells were transplanted into the vitreous cavity of the glaucoma mouse model. After 14 days, retina sections of glaucoma models were used to examine immunoreactivity for RGCs-specific marker GFP and ZO-1. The result showed that length of RGCs axons was significantly longer in the STAT3 + Y27632 group, which verified that combination STAT3 with Y27632 can promote the growth of RGCs axons. OCT results showed that the RGCs cell layer in the STAT3 + Y27632 group was significantly thicker than that in the other intervention groups. In the STAT3 + Y27632 group, the amplitude of VEP was reduced the least compared with other intervention groups.

In conclusion, STAT3 combined with Y27632 can significantly promote the axon growth of ganglion cells which was dedifferentiated from retina Müller cells either in vitro or in vivo.

**Abbreviations**

RGCs: retinal ganglion cells; VEP: Visual evoked potential; OCT: Optical coherence tomography; STAT3: signal transducer and activator of transcription 3; Rho-ROCK: Rho-associated coiled-coil-Containing protein kinase; MOI: multiplicity of infection; FACS: Flow cytometry; PBS: phosphate buffered saline; HE: Hematoxylin and eosin; qRT-PCR: Quantitative real time polymerase chain reaction; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IOP: intraocular pressure; BDNF: brain-derived neurotrophic factor.
Declarations

ACKNOWLEDGEMENTS
Not applicable

AUTHOR CONTRIBUTIONS

Weitao Song, Xuezhi Zhou: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. Yuejue Wang, Manjuan Peng, Jingjie Peng: Collection of data, data interpretation, manuscript writing. Ye He, Xuan Zhang, Chao Wang: Data interpretation, manuscript writing.

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AVAILABILITY OF DATA AND MATERIALS

All the data generated or analyzed during this study are included in this published article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The use of mouse Müller cells was in accordance with the relevant guidelines and regulations, and the experimental protocols were approved by the Medical Ethics Committee of the Xiangya Hospital of Central South University.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

All authors declare that they have no competing interests.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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Figures
Figure 1

Immunofluorescence staining showed that the three generations cells dedifferentiated from mice retina had a positive expression of Müller cell-specific marker GS (A), FACS showed that 99.8% of the third-generation purified cells were immunoreactive for GS (B). Western blot analysis showed that the purified cells rarely express the specific markers of other cells in each layer of the retina (C, D).
Figure 2

Cell proliferation was observed on 3,5,7,10, and 14 days by phase contrast microscopy (A). The scale bar was 100 μm. The diameters of the neurosphere were 11.55 μm, 29.64 μm, 61.82 μm, 88.36 μm and 177.73 μm at 3,5,7,10 and 14 days, respectively (B).
Immunofluorescence staining showed that the stem cells within the cell spheres had a positive expression of retinal stem cell-specific markers Nestin (90.60%), Ki67 (89.20%), Pax6 (91.00%), Musashi1 (89.90%) (A, B). Western blot analysis was used to detect the expression of retinal stem cells makers (C, D). Immunocytochemical analysis of Edu showed that newborn cell spheres had the capacity of effective proliferation (E).
Figure 4

Stem cells dedifferentiated from Müller cells were transfected with lentivirus AAV-STAT3 (A). Bar=100 mm. Immunocytochemical analysis showed that in the 12 days RGCs were stained with Tuj1 and Pax6 (B). Bar=200 mm. The length of axon in control group was 384.3 μm, in STAT3 group was 956.3 μm, in shSTAT3 group was 278.2 μm, in Y17632 group was 1347.9 μm, in STAT3 + Y27632 group was 1999.3 μm (C). Western blot analysis showed that the amount of protein and mRNA of GAP-43, phosphorylated...
STAT3 and its downstream genes p21, Irf1 and Sprr1a increased significantly in STAT3 and STAT3+Y27632 group (D, E). Quantitative RT-PCR assay to measure the mRNA level of Socs3, Pten, Klf9, Mdm4, Dclk2, Armcx1, c-myc, and Nrn1 in control group, STAT3 group and STAT3+Y27632 group (F-M).

Figure 5

The protein expression and phosphorylation levels of STAT3 in Müller differentiated RGCs in the STAT3 group, shSTAT3 group, Y27632 group and STAT3+Y27632 group (A-C). The mRNA level of axonal regeneration associated proteins Socs3, Pten, Klf9, Mdm4, Dclk2, Armcx1, c-myc, and Nrn1 in STAT3 group, shSTAT3 group, Y27632 group and STAT3+Y27632 group (D-G). Quantitative RT-PCR assay to measure the mRNA level of pluripotent associated proteins Esrrb, Prdm14, Sox2, and Rex1 in STAT3 group and STAT3+Y26632 group on D0, D3, D7, and D12 (H-K). Quantitative RT-PCR asssay to measure
the mRNA level of differentiation-associated proteins Nestin, Eomes, Mixl1, and Gata4 in the STAT3 group and STAT3+Y26632 group on D0, D3, D7, and D12 (L-O).

Figure 6

Ocular hypertension was induced using laser photocoagulation. The mean IOP level of glaucomatous eyes were detected by a digital tonometer from day 3 to day 60 (A). HE staining of RGCs in glaucoma mice model at day 15, day 30 and day 60. The number of RGCs was also calculated (B and C). TUNEL staining of RGCs in glaucoma mice model at day 3, day 10, day 30, and day 60. Scale bar=100 mm.
Figure 7

Differentiation and transplantation of retinal stem cells in the glaucoma mouse model. Immunofluorescence staining of retinal tissue sections showed that the length of GFP positive cells was 360.5 μm in group A, 877.3 μm in group B, 1427.9 μm in group C, and 1986.7 μm in group D. Group A: control group; group B: AAV-STAT3 group; group C: Y27532 group; group D: AAV-STAT3+ Y27532 group (A-B). IF staining of TUJ-1 protein in the retina of control group, Glaucoma group, and STAT3+Y27632 group (C). Flash-VEP 1.0 Hz (GF) graphs show the neural responses to visual stimuli in each group (D-E).
group mouse models (C). Fundus photography and OCT examination in the control group, glaucoma group, AAV-STAT3 group, Y27632 group, and AAV-STAT3+ Y27632 group mouse models (D). Flash-VEP examination in control group, glaucoma group, AAV-STAT3 group, Y27632 group, and AAV-STAT3+Y27632 group mouse models (E). Scale bar=100 μm.

Figure 8

STAT3 combined with Y27632 enhanced the pluripotency and inhibited the differentiation of Müller cell differentiated RGCs. By this mechanism, mRNA levels of axon growth-related proteins are regulated, and axon regeneration is promoted.

Supplementary Files

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- TableS1.docx