

Effect of Sonic hedgehog gene-modified bone marrow mesenchymal stem cells on graft-induced retinal gliosis and retinal ganglion cells survival in diabetic mice

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Abstract

• **AIM:** To investigate the effects of Sonic hedgehog (*Shh*) gene-modified bone marrow mesenchymal stem cells (MSCs) on graft-induced retinal gliosis and retinal ganglion cells (RGCs) survival in diabetic mice.

• **METHODS:** Bone marrow-derived MSCs were genetically modified with the *Shh* gene to generate a stably transfected cell line of *Shh*-modified MSCs (MSC-Shh). Intravitreal injections of MSC-Shh and green fluorescent protein-modified MSCs (MSC-Gfp; control) were administered in diabetic mice. After 4wk, the effects of MSC-Shh on retinal gliosis were evaluated using fundus photography, and markers of gliosis were examined by immunofluorescence and Western blotting. The neurotrophic factors expression and RGCs survival in the host retina were evaluated using Western blotting and immunofluorescence. The mechanisms underlying the effects of MSC-Shh was investigated.

• **RESULTS:** A significant reduction of proliferative vitreoretinopathy (PVR) was observed after intravitreal injection of MSC-Shh compared to MSC-Gfp. Significant downregulation of glial fibrillary acidic protein (GFAP) was demonstrated in the host retina after MSC-Shh administration compared to MSC-Gfp. The extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase B (AKT) and phosphatidylinositol-3-kinase (PI3K) pathways were significantly downregulated after MSC-Shh administration

compared to MSC-Gfp. Brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) levels were significantly increased in the host retina, and RGCs loss was significantly prevented after MSC-Shh administration.

• **CONCLUSION:** MSC-Shh administration reduces graft-induced reactive gliosis following intravitreal injection in diabetic mice. The ERK1/2, AKT and PI3K pathways are involved in this process. MSC-Shh also increases the levels of neurotrophic factors in the host retina and promoted RGCs survival in diabetic mice.

• **KEYWORDS:** mesenchymal stem cells; Sonic hedgehog signaling; reactive gliosis; diabetic retinopathy; retinal ganglion cells

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INTRODUCTION

Diabetic retinopathy (DR) is one of the most common complications of diabetes and remains the leading cause of blindness among working-age individuals in developed countries^[1]. Retinal neurodegeneration has been implicated in the pathogenesis of DR and is thought to contribute to the development of microvascular abnormalities^[2]. Loss of retinal ganglion cells (RGCs) due to apoptosis has been observed in diabetic eyes before the onset of retinal vascular endothelial abnormalities^[3]. Despite numerous attempts to delay or halt the neurodegeneration process in DR, therapeutic efficacy has been limited.

Stem cell transplantation is a promising therapeutic approach for degenerative eye diseases. Mesenchymal stem cells (MSCs) are considered an ideal candidate for cell therapy because they are multipotent, self-renewing cells that can be easily obtained and expanded *in vitro* for autologous applications. MSCs are known to produce abundant growth and neurotrophic factors and are proved to support the survival of retinal

cells. Many preclinical studies and early phase clinical trials have confirmed the neuroprotective effects of MSCs in the treatment of neurodegenerative eye diseases, including DR^[4]. Recently, intravitreal administration of MSCs has emerged as a promising intervention for neurodegenerative eye diseases. Ezquer *et al*^[5] demonstrated that intravitreal transplantation of MSCs effectively triggered a cytoprotective microenvironment in the host retina of diabetic mice. Early phase clinical trials have been initiated for the intravitreal administration of MSCs in neurodegenerative eye diseases such as retinitis pigmentosa, glaucoma, and DR^[3,6-7].

However, some limitations still need to be overcome. The most concerned problem is the graft-induced reactive gliosis that occurs in the host retina following MSCs administration. Reactive gliosis can lead to retinal folding, proliferative vitreoretinopathy (PVR), and even retinal detachment. Huang *et al*^[8] reported strong expression of glial fibrillary acidic protein (GFAP), a marker for glial activation, in retinal Müller cells in MSC-injected Sprague-Dawley rat eyes. Tassoni *et al*^[9] found that intravitreal transplantation of MSCs was associated with gliosis-mediated retinal folding and significant increased GFAP expression in Müller cells. These responses were accompanied by significant extracellular signal-regulated kinase 1/2 (ERK1/2) cascade activation in retinal Müller glia. They also demonstrated that the predominant barrier to retinal integration of intravitreally transplanted MSCs is glial cell reactivity^[10]. These researches indicated that the graft-induced reactive gliosis had become a critical issue that must be addressed to make this therapy safer and more effective, but current solutions to prevent or attenuate reactive gliosis of the host retina were still very limited.

Sonic hedgehog (Shh) is a member of the hedgehog family, widely expressed throughout the central nervous system, and plays an essential role in neural system development and damage repair^[11]. Modulation of the Shh signaling pathway is proved to be a potential neuroprotective treatment in animal models of central nervous system injury^[12]. Shh has been shown to reduce glial lesion formation and aid synapse growth in mice models of brain damage^[13]. Shh is also expressed in the retina and participates in retinal neuron damage repair. Our previous study found that Shh plays an important role in the regulation of gliosis in retinal Müller cells. We demonstrated that exogenous Shh significantly inhibits retinal Müller cell gliosis and promotes RGCs survival in a rat diabetes model. We also showed that exogenous Shh downregulates the ERK1/2 pathway and significantly inhibits gliosis in high-glucose cultured Müller cells^[14].

In this study, we aimed to find out whether modulation of the Shh signaling pathway could be used as a strategy to mitigate the reactive gliosis that occurs following transplantation of

bone marrow-derived MSCs (BMSCs) in a diabetic mice model. Specifically, we generated a stably transfected cell line of Shh-modified MSCs (MSC-Shh) and assessed the effect of intravitreal injection of these cells on retinal gliosis and RGCs survival in a diabetic mice model. We also explored the potential underlying molecular mechanisms that contribute to these effects. This study provides valuable insights into the potential of Shh-modified BMSCs as a promising therapeutic approach for the treatment of neurodegenerative eye diseases, particularly DR.

MATERIALS AND METHODS

Ethical Approval The Institutional Animal Care and Use Committee of State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University approved the animal experiments in this study (reference number 2020-153).

Culture and Characterization of Mesenchymal Stem Cells The mice bone marrow MSCs utilized in this study were procured from iCell (Shanghai, China). MSCs were cultured in a complete culture medium of mouse bone marrow MSCs (#PriMed-iCell-012, iCell, China) and identified using a primary antibody, anti-CD44 (#15675-1-AP, protein tech, USA).

Transfection of Mesenchymal Stem Cells Adeno-associated virus (AAV) overexpressing mice gene *Shh* and negative control AAV were developed by VectorBuilder (Website: <https://www.vectorbuilder.cn/vector/VB220221-1045cfw.html> and <https://www.vectorbuilder.cn/vector/VB010000-9394npt.html>). MSCs were inoculated in 6-well plates with 1.5×10^5 cells per well and incubated overnight. On the following day, MSCs were supplemented with 1 mL/well fresh culture medium containing AAV (MOI=20). After 72h of culture, the expression of *Shh* gene was observed by measuring the tagged protein green fluorescent protein and verified by Western blotting.

Establishment of Diabetic Retinopathy Mice Model Six-week-old C57BL/6 mice were housed in constant temperature and humidity, with a 12-hour light and dark cycle, and allowed free access to water and food. The mice were fasted overnight before intraperitoneal injection of 50 mg/kg 1% streptozotocin (STZ; #S0130, Sigma-Aldrich, USA). Blood glucose levels above 17.6 mmol/L in the treated mice were considered diabetic. Four weeks after diabetes induction, electroretinogram (ERG) was performed to confirm the establishment of DR mice model.

Electroretinogram Recording Electroretinography Technology (Roland consult® RETIanimal ERG, Germany) was used to carry out an ERG. Mice were dark adapted overnight, and anesthetized by intraperitoneal injection of 4% chloral hydrate (10 μ L/g). Their pupils were dilated by 1% tropicamide and

their corneas were lubricated by hypromellose. Then the corneal electrode was placed on the cornea, the reference electrode was inserted subcutaneously to the cheek, and the ground electrode was fixed near the tail. The mice were then placed in the center of the illuminating sphere, and exposed to light flashes of different intensities, and the resulting electrical signals were recorded and analyzed.

Intravitreal Injection of MSC-Shh and MSC-Gfp

Intravitreal injections of MSC-Shh and MSC-Gfp were performed on mice eight weeks after diabetes mellitus (DM) induction. Intravitreal injections were performed as described previously^[15]. The mice were first lightly anesthetized with 0.5% proparacaine (Alcon, Santiago Chile) topically applied. A cell suspension containing 1×10^5 MSC-Shh (MSC-Shh group) or MSC-Gfp (MSC-Gfp group) in 2 μ L saline, or 2 μ L saline (control group) was slowly injected into the vitreous cavity through the pars plana (1.5 mm from the limbus) using a 33-gauge microsyringe (Hamilton, USA). Eyes that showed signs of massive vitreous hemorrhaging after injection were excluded from the study.

Fundus Photography Color fundus photography was conducted using the Micron IV funduscopy system (Phoenix Research Laboratories, USA). Prior to imaging, the mice were anesthetized with an intraperitoneal injection of 2% tribromoethanol. Their pupils were dilated by 1% tropicamide and their corneas were lubricated by hypromellose. Then the mice were placed on the funduscopy system with their corneas contacted the objective. The photographs were saved in a digital format, and the stage of PVR was analyzed and scored. The PVR grade was assessed using a scoring system, with a score of 1 for PVR grade A, a score of 2 for PVR grade B, and so on.

Immunofluorescence Staining of Frozen Retinal Sections and Whole-Mount Retinas and RGCs Quantification

Immunofluorescent staining was performed with mouse monoclonal anti-GFAP antibody (1:100, Cell Signaling Technology, USA), mouse monoclonal anti-Brn-3a (1:500, Santa Cruz Biotechnology, USA), and secondary antibody conjugated to Alexa 488 (1:500; Invitrogen, USA). Cell nuclei were counterstained with DAPI (Sigma-Aldrich Corp, USA). The images were acquired with a laser confocal microscope (Carl Zeiss, Germany).

To quantify Brn-3a-labeled RGCs, retinas were divided into four eccentric zones concerning the optic nerve head. A total of 20 fields, five in each quadrant, were selected for counting at $200\times$ final magnification, starting at the optic disc and progressing to the border at 500- μ m intervals. Data were presented as the relative percentage of RGC loss (mean \pm SD) compared with that in the control group. In the case of sections, the number of RGCs in the ganglion cell layer was quantified by counting labeled cells in five consecutive sections, from

the temporal to the nasal ora serrata, using a laser confocal microscope (Carl Zeiss Axio Imager Z2, Germany). Data were expressed as the number of RGCs per 100 μ m of retina length.

Western Blot Analysis Retinas were dissected from all groups immediately after cervical dislocation and rapidly snap-frozen in liquid nitrogen. Total protein of retina was extracted using RIPA lysate (#P0013B, Beyotime, China) and quantified with a BCA protein assay kit (#KGP903, KeyGEN BioTECH, China) according to the manufacturer's instructions. Then 25 μ g of each protein sample was separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% skim milk solution (#FD0080, Fdbio Science, China) for 1–2h at room temperature, the membranes were incubated with primary antibodies against Shh (Affinity, BF0146), CD73 (Affinity, DF6763), CD105 (Affinity, DF7735), GFAP (Cell Signaling Technology, 3670S), vimentin (Cell Signaling Technology, 5741S), nestin (Novus, NBPI-02419), ciliary neurotrophic factor (CNTF, Affinity, DF6689), brain-derived neurotrophic factor (BDNF, Abcam, ab205067), nerve growth factor (NGF, Abcam, ab6199), ERK1/2 (Huabio, ET1601-29), pERK (Huabio, ET1608-22), protein kinase B (AKT, Huabio, ET1609-51), phospho-protein kinase B (p-AKT, Huabio, ET1607-73), phosphatidylinositol-3-kinase (PI3K, CST), phospho-phosphatidylinositol-3-kinase (p-PI3K, CST) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, 5174S) at 4°C overnight. Following five washes with TBST solution, the membranes were incubated with HRP-linked secondary antibodies for 1–2h at room temperature, followed by another five washes with TBST solution. The signals of specific antibodies were visualized using chemiluminescence fluid (#FD8030, Fdbio Science, China) according to the manufacturer's protocol.

Statistical Analysis Statistical analysis was conducted using the SPSS Statistics 20.0 software package (IBM, USA). Measurement data were presented as mean \pm standard error of the mean (SEM). The comparison of data between two and three groups was analyzed by two independent sample *t*-test or one-way analysis of variance, respectively. The statistical significance was set at a *P* value of less than 0.05.

RESULTS

Characterization of MSCs and MSC-Shh Construction

The morphology and immunophenotype of MSCs were assessed. MSCs displayed a uniform spindle or spiral-shaped morphology as a monolayer of large, flat cells. Immunofluorescence analysis revealed high expression of the CD44 marker, confirming their characteristic immunophenotype (Figure 1A). Lentiviral transduction was used to introduce the *Shh* gene into MSCs, which was confirmed by immunofluorescence staining of the tagged GFP protein (Figure 1A). Western blot analysis showed a significant

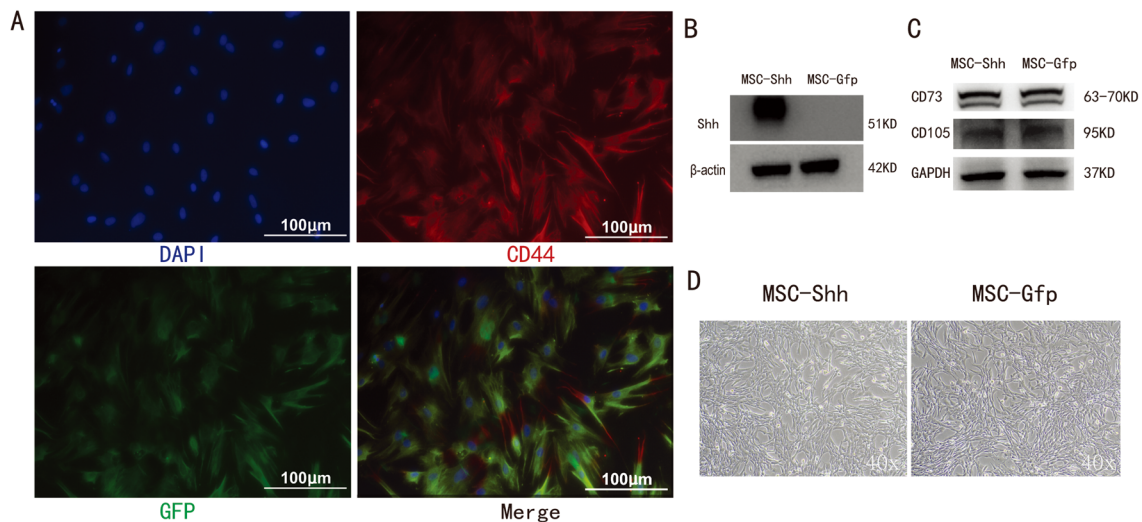


Figure 1 Characterization of MSCs and transduction of MSC-Shh Immunofluorescence indicated that MSCs exhibited a characteristic immunophenotype of high expressed CD44. The *Shh* gene expression was observed by measuring the tagged protein GFP with immunofluorescence (A). The Western blot assay showed that the protein level of Shh increased significantly in MSC-Shh compared with the control of MSC-Gfp (B). The Western blot assay showed the expression of CD73 and CD105 of MSC-Shh and the control MSC-Gfp (C). The morphological characteristics of MSC-Shh and MSC-Gfp was assessed (D). MSCs: Mesenchymal stem cells. MSC-Shh: *Shh*-modified MSCs; MSC-Gfp: Green fluorescent protein-modified MSCs.

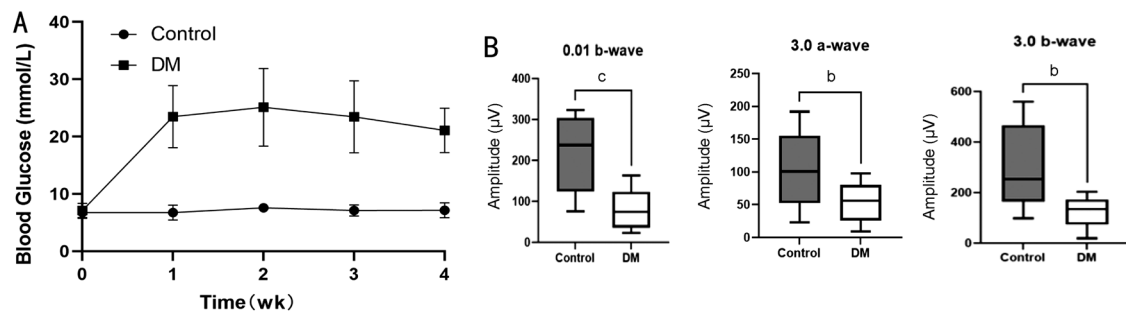


Figure 2 Diabetic retinopathy mice model establishment Blood glucose level was determined weekly and the blood glucose levels of the diabetic mice reached their highest concentration, three times higher than the control group (A). Electroretinogram data of scotopic 0.01 lx and 3.0 lx was quantified. Qualitative data are representative of eight eyes per group. Quantitative data correspond to mean±SEM of 10 eyes per group. Presence of donor cells in the eye was also corroborated by immunofluorescence. *Shh* was continuously expressed until 4wk (B). ^b $P<0.01$, ^c $P<0.001$. MSCs: Mesenchymal stem cells. MSC-Shh: *Shh*-modified MSCs; MSC-Gfp: Green fluorescent protein-modified MSCs; SEM: Mean±standard error of the mean.

increase in the protein level of Shh in MSC-Shh compared to the control MSC-Gfp ($P<0.05$; Figure 1B). No significant changes were observed in the expression of CD73 and CD105 (the marker of undifferentiated MSCs) between MSC-Shh and the control MSC-Gfp (Figure 1C). Additionally, there was no significant difference in the morphology between MSC-Shh and the control MSC-Gfp (Figure 1D).

Diabetic Retinopathy Mouse Model Establishment To induce diabetes, C57BL6 mice were treated with five doses of 50 mg/kg STZ. One week after STZ administration, the blood glucose level of the treated mice exceeded 17.6 mmol/L, and two weeks after administration, blood glucose levels reached their peak, which was three times higher than that of the control group (Figure 2A). Four weeks after DM induction, the diabetic mice presented a significant reduction in scotopic

a-wave amplitude under the stimulus of 3.0 lx compared to the control group ($P<0.01$), and the b-wave amplitude was significantly decreased in diabetic group compared to the control group under both the stimulus of 0.01 ($P<0.001$) and 3.0 lx ($P<0.01$; Figure 2B).

MSC-Shh Reduces Graft-Induced Gliosis and Down-Regulates ERK1/2, AKT/PI3K Pathways after Intravitreal Injection in Diabetic Mice Four weeks after intravitreal injection of MSCs (MSC-Shh or MSC-Gfp), we evaluated the gliosis change of the host retina using fundus photography. We found that the average level of PVR of the host retina was significantly lower after intravitreal injection of MSC-Shh compared to MSC-Gfp ($P<0.05$; Figure 3A, 3B). The markers of gliosis were also measured by immunofluorescence and Western blot assay. The immunofluorescence analysis

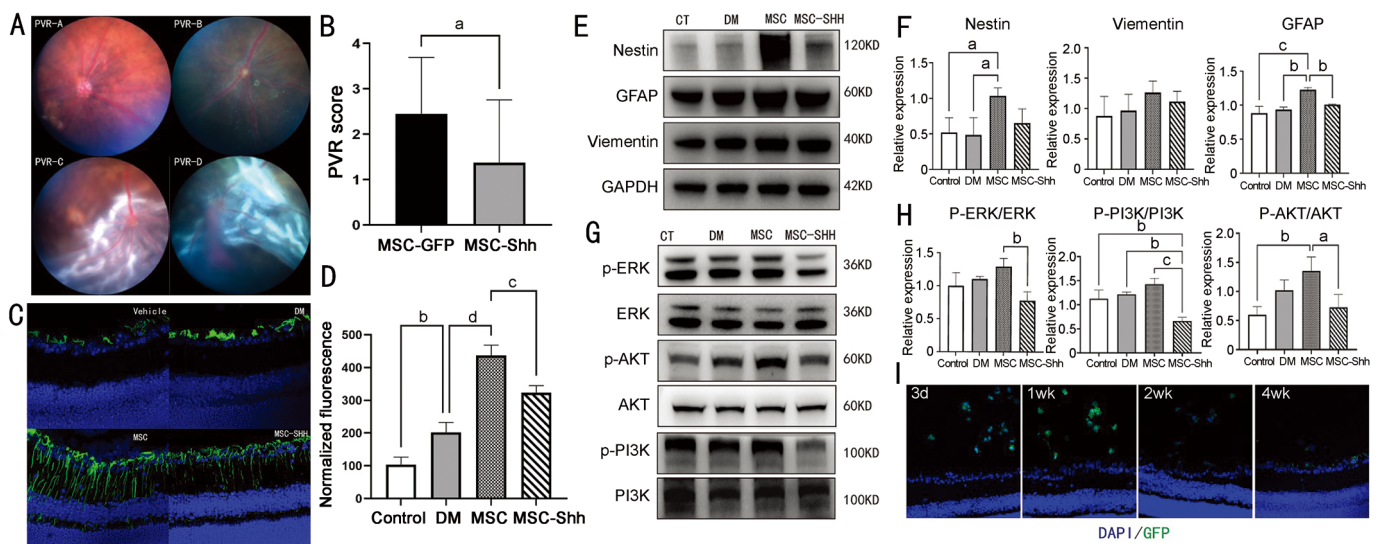


Figure 3 MSC-Shh attenuated the graft-induced gliosis and down regulated the ERK1/2, AKT, and PI3K expression after intravitreal injection in diabetic mice Representative mice fundus photography of PVR classification was showed (A). The average level of PVR of the host retina was significantly lower in the MSC-Shh group than in the MSC-Gfp group (B). Immunofluorescences showed that GFAP was significantly overexpressed in retinal Müller cells in diabetic mice. The intravitreal injection of MSC-Gfp resulted in a significant increase of GFAP expression in retinal Müller cells, and GFAP expression was significant lower after administration of MSC-Shh compared to MSC-Gfp (C, D). Western blot showed a significant increase of GFAP expression in the host retina following intravitreal injection of MSC-Gfp, and a significantly lower expression of GFAP was observed following the administration of MSC-Shh. The expression of nestin was also significantly increased after administration of MSC-Gfp (E, F). The p-ERK, p-AKT, and p-PI3K activation in the host retina was significantly inhibited after intravitreal injection of MSC-Shh compared to the injection of MSC-Gfp (G, H). The grafted MSCs were detectable in the vitreous cavity after administration (I). ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$. ERK1/2: Extracellular signal-regulated kinase 1/2; AKT: Protein kinase B; PI3K: Phosphatidylinositol-3-kinase; MSCs: Mesenchymal stem cells; MSC-Shh: Shh-modified MSCs; MSC-Gfp: Green fluorescent protein-modified MSCs; GFAP: Glial fibrillary acidic protein; p-ERK: Phospho-extracellular signal-regulated kinase; p-AKT: Phospho-protein kinase B; p-PI3K: Phospho-phosphatidylinositol-3-kinase.

showed that the GFAP expression in retinal Müller cells was significantly increased in diabetic mice ($P < 0.01$). The intravitreal injection of MSC-Gfp resulted in a significant upregulation in GFAP expression in Müller cells of the host retina ($P < 0.0001$), and the expression of GFAP was significantly lower after administration of MSC-Shh compared to MSC-Gfp ($P < 0.001$; Figure 3C, 3D). Western blot analysis also showed a significant increase in GFAP expression in the host retina following intravitreal injection of MSC-Gfp in diabetic mice ($P < 0.01$), and a significantly lower expression of GFAP in the host retina was observed following administration of MSC-Shh ($P < 0.01$). Along with GFAP, the expression of nestin in the retina was significantly increased after administration of MSC-Gfp in diabetic mice ($P < 0.05$). The expression of nestin showed a decreasing trend after administration of MSC-Shh, but without statistical significance ($P > 0.05$). The expression of vimentin was not significantly changed following the administration of either MSC-Gfp or MSC-Shh in diabetic mice ($P > 0.05$; Figure 3E, 3F). Grafted MSCs were still detectable four weeks after administration. Most donor cells remained in the vitreous cavity and did not integrate into the retina (Figure 3I).

We also evaluated the effect of MSC-Shh on the ERK1/2 and AKT/PI3K pathway in the host retina. Four weeks after intravitreal injection of MSCs (MSC-Shh or MSC-Gfp), Western blot analysis showed that phospho-extracellular signal-regulated kinase (p-ERK), p-AKT, and p-PI3K activation in the host retina was significantly inhibited after intravitreal injection of MSC-Shh compared to the injection of MSC-Gfp ($P < 0.01$; Figure 3G, 3H).

MSC-Shh Promoted Neurotrophic Factors Secretion and Prevented RGCs Loss in Diabetic Mice After intravitreal injection of MSCs (MSC-Shh or MSC-Gfp) in diabetic mice, the levels of neurotrophic factors in the host retina were measured four weeks later. Western blot analysis revealed that the expression of BDNF and CNTF significantly increased after the administration of MSC-Shh ($P < 0.05$), while no significant change was observed following MSC-Gfp injection ($P > 0.05$). NGF expression did not significantly change after either MSC-Gfp or MSC-Shh administration ($P > 0.05$; Figure 4A, 4B). Immunofluorescence staining of frozen retinal sections and RGCs quantification showed that diabetic mice exhibited a significant reduction in the number of RGCs in the retina ($P < 0.0001$). However, both MSC-Shh ($P < 0.0001$)

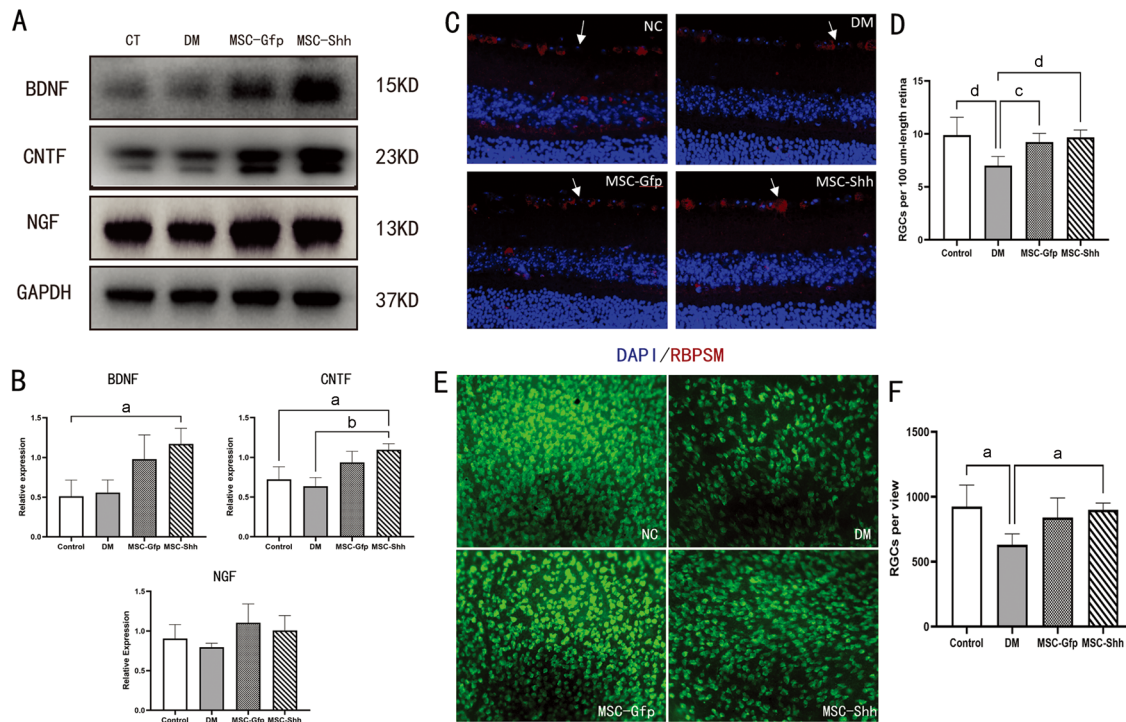


Figure 4 MSC-Shh triggered secretion of neurotrophic factors and prevented RGCs loss Western blot showed that the expression of BDNF and CNTF increased significantly after the administration of MSC-Shh, while the change of these factors was not significant after MSC-Gfp injection (A, B). Representative dissection of mice retina in each group were observed under confocal microscopy focusing on the ganglion cell layer of the retina (arrows; C). RGC quantification showed that the diabetic mice presented a significant reduction in the number of RGCs in the retina, and this reduction was significantly prevented after MSC-Shh and MSC-Gfp administration (D). Immunofluorescence staining of the whole-mount retina and RGC quantification showed that the surviving RGCs decreased significantly in the diabetic mice, and this reduction was significantly prevented after MSC-Shh administration (E, F). Quantitative data correspond to mean±SEM of four eyes per group and per analyzed time. ^a $P<0.05$, ^b $P<0.01$, ^c $P<0.001$, ^d $P<0.0001$. BDNF: Brain-derived neurotrophic factor; CNTF: Ciliary neurotrophic factor; RGC: Retinal ganglion cells; MSCs: Mesenchymal stem cells; MSC-Shh: *Shh*-modified MSCs; MSC-Gfp: Green fluorescent protein-modified MSCs.

and MSC-Gfp ($P<0.001$) administration significantly prevented this reduction (Figure 4C, 4D). Additionally, immunofluorescence staining of whole-mount retina and RGCs quantification showed that the surviving RGCs decreased significantly in diabetic mice ($P<0.05$), and this reduction was significantly prevented after MSC-Shh administration ($P<0.05$; Figure 4E, 4F).

DISCUSSION

There is mounting evidence to suggest that retinal neurodegeneration is an early and critical event in the pathogenesis of DR. Recent researches suggested that stem cell therapy using MSCs may offer a promising approach for treating retinal neurodegeneration including DR. Intravitreal injection of MSCs is one of the most common ways of intraocular delivery of MSCs, which has been shown to be more effective for treating retinal diseases^[16]. However, significant challenges remain to be addressed. Several studies had observed severe retinal folding following intravitreal injection of MSCs, accompanied by a significant overexpression of glial markers, which indicated an extensive reactive gliosis occurred in the host retina. Furthermore, PVR

and retinal detachment was also observed after intravitreal administration of MSCs^[17-18].

According to our previous study, Shh had been proved to inhibit retinal Müller cells gliosis and support RGCs survival^[14]. So we transfected bone marrow-derived MSCs with an Shh-overexpressing AAV and created a stable MSC-Shh cell line. The morphology and surface markers of MSC-Shh showed no significant differences compared to the control group MSC-Gfp, indicating that *Shh* gene transfection did not affect the characteristics and differentiation potential of MSCs. MSC-Shh administrated *via* intravitreal injected had the ability to continuously release Shh. The grafted cells were still detected four weeks after administration. Consistent with the results of previous studies, the donor cells persisted within the vitreous cavity and did not integrate into the retina, which suggested that the MSC-Shh primarily functioned through paracrine secretion.

In retina, the macroglial cells, including Müller cells and astrocytes, are known to undergo reactive gliosis in response to damage^[19]. Müller cells are the major component of retinal glia, and previous studies had proved that the intravitreal

injection of MSCs would trigger a significant reactive gliosis of Müller cells^[8-9]. The increase expression of intermediate filaments GFAP, vimentin and nestin is a crucial step in the gliotic response. GFAP upregulation is the most sensitive non-specific response in the pathologic retina. Initially recognized as a marker of neural stem cells, nestin has also been demonstrated to be upregulated in adult retinal tissue following injury, suggesting that glial cells may revert to a more developmentally immature state in response to damage. In the current study, we found significantly upregulated of both GFAP and nestin following the administration of MSC-Gfp. Additionally, GFAP expression was significantly downregulated in the host retina after intravitreal injection of MSC-Shh compared to MSC-Gfp, suggesting that MSC-Shh has the potential to inhibit retinal gliosis following intravitreal administration of MSCs. Immunofluorescence staining further confirmed the significantly decreased GFAP expression in retinal Müller cells.

Previous studies had showed that the graft-induced gliosis was associated with the significant ERK1/2 cascade activation in retinal Müller cells^[9]. Our results showed that ERK1/2, AKT and PI3K pathway was significantly downregulated after MSC-Shh administration, indicating that these pathways may be involved in the regulation of gliosis by MSC-Shh. Furthermore, researches had found that Müller cells might transdifferentiate into contractile myofibrocytes under the situation of proliferative gliosis. Studies had also suggested that the reactive gliosis of Müller cells might play a part in PVR formation^[18,20]. In the present study, we found a significant improvement in the occurrence of PVR after MSC-Shh injection compared to MSC-Gfp, which indicated that the inhibition of proliferative gliosis by MSC-Shh might also be profit to reduce PVR formation.

It is widely acknowledged that the neuroprotective mechanism of MSCs in retinal degeneration primarily involves a paracrine trophic effect, characterized by the secretion of neurotrophic factors. We have conducted cytokine profiling to assess the specific alterations in cytokine secretion following Shh overexpression. Our observations revealed a significant increase in the levels of BDNF and CNTF, along with a notable reduction in RGC loss in the host retina following MSC-Shh administration. These findings confirmed the neuroprotective effect of MSC-Shh and suggested that BDNF and CNTF might have a prominent role in this process. However, it should be noticed that no significant difference was found between the MSC-Shh group and MSC-Gfp group in terms of neurotrophic factor expression and RGCs loss, despite the MSC-Gfp group showing an increasing trend in both BDNF and CNTF levels, as well as the RGCs quantification of whole-mount retina. We hypothesized that the reason for this might be the large

variance caused by significant differences between samples, which was attributed to the higher frequency and severity of gliosis and PVR in this group. These results demonstrated that MSC-Shh had prominent neurotrophic function and was effective in reducing RGCs loss. Furthermore, MSC-Shh might provide the host retina a preferable microenvironment by attenuated gliosis and PVR, and might be preferable in supporting RGCs survival.

In conclusion, our study showed that MSC-Shh could reduce graft-induced reactive gliosis and PVR formation, increase the level of neurotrophic factors in the host retina, and promote RGCs survival in diabetic mice. We believe that these findings provide valuable evidence for reducing complications associated with intravitreal administration of MSCs through gene modification. Future studies should further investigate the mechanisms underlying the effects of MSC-Shh and explore its therapeutic potential for treating retinal degenerative diseases such as DR.

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