Induction of Differentiation of Mesenchymal Stem Cells into Retinal Pigment Epithelial Cells for Retinal Regeneration by Using Ciliary Neurotrophic Factor in Diabetic Rats

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Summary: Diabetic retinopathy (DR) is a common cause of blindness all over the world. Bone marrow mesenchymal stem cells (BMSCs) have been considered as a promising strategy for retinal regeneration in the treatment of DR. However, the poor viability and low levels of BMSCs engraftment limit the therapeutic potential of BMSCs. The present study aimed to examine the direct induction of BMSCs differentiation into the cell types related to retinal regeneration by using soluble cytokine ciliary neurotrophic factor (CNTF). We observed remarkably increased expression of cellular retinaldehyde-binding protein (CRALBP) and retinoid isomerohydrolase (RPE65) in BMSCs treated with CNTF *in vitro*, indicating the directional differentiation of BMSCs into the retinal pigment epithelium (RPE) cells, which are crucial for retinal healing. *In vivo*, the diabetic rat model was established by use of streptozotocin (STZ), and animals treated with BMSCs+CNTF exhibited better viability and higher delivery efficiency of the transplanted cells than those treated with BMSCs injection alone. Similar to the *in-vitro* result, treatment with BMSCs and CNTF combined led to the differentiation of BMSCs into beneficial cells (RPE cells), and accelerated retinal healing characterized by the activation of rod photoreceptor cells and phagocytosis function of RPE cells. In conclusion, CNTF contributes to the differentiation of BMSCs into RPE cells, which may help overcome the current stem cell therapy limitations in the field of retinal regeneration.

Key words: mesenchymal stem cells; differentiation; ciliary neurotrophic factor; retinal regeneration; retinal pigment epithelium (RPE)

Retinal pigment epithelium (RPE) is located between the vessels of choriocapillaris and outer segments of photoreceptors with light-sensitive function, and it has been demonstrated to closely interact with photoreceptors for preserving the visual function[1]. RPE plays significant roles in physiological and biochemical processes, including the transportation of nutrient substances, the retinol circulation and the formation of pigment[2].

Although some animals such as amphibians are capable of accomplishing complete regeneration of retina[3], most mature mammalian eyes are believed to lack the retinal regenerative capacity. Therefore, novel therapies that could enhance the retinal regenerative capacity, especially for the regeneration of RPE cells, have always been desirable.

Stem cells are able to generate several types of daughter cells, and they are quite suitable for regeneration of damaged tissue. Stem cells such as embryonic stem cells, endothelial progenitor cells and mesenchymal stem cells have been proved effective for a variety of retinopathies[4, 5]. Mesenchymal stem cells (MSCs) have been found in many organs of the body and they can differentiate into integrated functional tissues, which is attributed to their powerful ability to produce cytokines and repair vascular system[4, 5]. Normally, MSCs can be isolated from Wharton's jelly, the placenta, bone marrow, teeth, and adipose tissue, making them favorable for autologous transplantation[4, 5]. As a powerful tool to enhance the regenerative function of tissue, bone marrow derived mesenchymal stem cells (BMSCs) have been widely applied in preclinical treatment studies focusing on tissue regeneration and engineering. It has been demonstrated that intravitreal injection of MSCs is effective in replacing pericytes[6], preserving

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blood retina barrier integrity and differentiating into indispensable cells such as photoreceptor cells in diabetic retinopathy models[7]. Functional vision could be improved after the migration and differentiation of retinal progenitor cells to mature retinal cells[8]. It can be created by integrating stem cells into the retina through forming new synapses[9].

BMSCs can be obtained by simple procedure of isolation and propagation, which makes them more suitable than retinal progenitor cells or induced pluripotent stem cells to be used for retinal regeneration as candidate cells. They have been proved to prevent apoptosis of photoreceptor and decrease retinal damage by expressing basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF)[10]. In another report, CNTF was found to be able to enhance the engraftment and further neuroglial differentiation of human embryonic stem cells into retinal progenitor cells in injured retina[11]. Few studies addressed the induction of BMSCs differentiation into the retinal regeneration related cell types such as RPE cells using soluble cytokine alone[12–14]. In this study, we intravitreally injected rat BMSCs and CNTF into a streptozotocin (STZ)-induced diabetic rats to examine whether BMSCs and CNTF combined could increase the viability of transplanted BMSCs and further drive their directional differentiation, thereby accelerating the RPE regeneration and retinal repair.

1 MATERIALS AND METHODS

1.1 BMSCs Isolation, Culture and Characterization

Briefly, 3-week old male Sprague-Dawley (SD) rats (Laboratory Animal Center of Wuhan University, China) were sacrificed, and the femoral and tibial bones were subsequently harvested aseptically. The bone marrow was obtained by flushing the marrow cavity with complete BMSCs culture medium containing low-glucose Dulbecco's modified Eagle's medium (L-DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Invitrogen, USA). After centrifugation, the cells were re-suspended in complete culture medium, and cultured at 37°C in 5% CO_2. The medium was refreshed 72 h post-isolation to remove non-adherent cells and changed every 2–3 days. Cells of 3–4 passages were used for the following experiments.

BMSCs were characterized by flow cytometry analysis at the third passage. The cells were detached, centrifuged, rinsed and re-suspended in PBS at a concentration of 10^5 cells/mL. Next, the cell suspension was incubated with 5 mL CD90 (Biolegend, USA), CD45 (Biolegend, USA), CD44 (Biolegend, USA), and CD31 (Affymetrix eBioscience, USA) in the dark at 4°C for 20 min. The analyses were performed using a flow cytometer (FACS Calibur, Becton Dickinson, Mountain View, USA).

1.2 Induction of BMSCs Differentiation Using CNTF

For the *in vitro* differentiation experiments, the protocol was modified according to a previous report[15]. Briefly, BMSCs were cultured with the standard differentiation medium (SDM) consisting of advanced DMEM/F12, 2 mmol/L L-glutamine, 100 U/mL penicillin-streptomycin and 5% FBS, supplemented with 1.2 ng/mL CNTF. The media were half changed daily.

1.3 Establishment of Diabetic Animal Model

Thirty-six male Sprague-Dawley rats (6–8 weeks old strain) weighing 200–250 g were purchased from the Laboratory Animal Center of Wuhan University (China) in accordance with the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (China). All the experimental procedures were performed in accordance with IACUC approval. One day before the operations, BMSCs were labeled with tetramethylindocarbocyanine perchlorate (CM-DiI, CellTracker™, Life Technologies, USA) for cells tracking according to the manufacturer's instruction. On day 0, rats were anesthetized with ketamine hydrochloride (60 mg/kg body weight). They were given an intraperitoneal injection of 100 mg/kg of 1% STZ (Sigma Aldrich Co., USA)[16]. The blood glucose levels were measured at 72 h after STZ injection by collecting the blood sample from the tail vein. The rats having a blood glucose level higher than 16.7 mmol/L (measurement was repeated with twice) were assigned to experimental group. Those with blood glucose levels less than 16.7 mmol/L were injected intraperitoneally again with 30 mg/kg STZ (1%) as the second dose.

After three months, all animals were divided into four groups: sham, BMSCs, CNTF, CNTF+BMSCs. For BMSCs injection group, 2 μL solution containing 2×10^5 CM-DiI labeled BMSCs were injected into the bilateral eyes of rats. For CNTF group, 0.5 μL CNTF (Gibco, USA) was injected into the bilateral eyes of rats. For CNTF+BMSCs group, 2 μL solution containing 2×10^5 CM-DiI labeled BMSCs and 0.5 μL CNTF were injected sequentially into the bilateral eyes of rats. For the shame group, the eyes of rats were injected with 2 μL phosphate-buffered saline (PBS, Euroclone, Italy). Intravitreal injections were performed by a skilled ophthalmologist under the binocular stereomicroscope (Tronic XTX 3C, China) through the cornea-scleral limbus with the needle's bevel up. When the needle reached the vitreous body, the solution was injected slowly while avoiding any contact with the lens.

The rats were deeply anesthetized with ketamine hydrochloride (100 mg/kg), and they were

intracardially perfused with 4% paraformaldehyde. Their eyes were enucleated and immersion-fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C. They were subsequently dehydrated over several hours and embedded in paraffin in transverse orientation. Particularly, samples were cross-sectioned at 5 μm and then deparaffinized and rehydrated for hematoxylin and eosin (H&E) staining, Masson's Trichrome staining and immunofluorescence analyses. Localization of CM-DiI labeled BMSCs was also analyzed with immunofluorescence.

1.4 Real-time Quantitative PCR

BMSCs were plated into 6-well plates either with standard medium or differentiation medium as described above. For *in vivo* RT-PCR, harvested tissues at different time points were stored at –80°C. Then, Total RNA was extracted via the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara Bio, Japan) following the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ Ⅱ First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Takara Bio, Japan). PCR conditions comprised an initial step of denaturation for 1 min at 95°C, followed by total 40 cycles of 15 s at 95°C, 20 s at 58°C and 20 s at 72°C. After normalization against the housekeeping gene GADPH, the expression of genes of interest (table 1) was measured using the $2^{-\Delta\Delta Ct}$ method.

1.5 Western Blot Analyses

Total protein was isolated from BMSCs cultured in 6-well plates either with standard medium or differentiation medium as described above, or from harvested tissues with a Total Protein Extraction Kit (Aspen, China). Equal amounts of protein from cell or tissue lysates were loaded onto a 5% SDS polyacrylamide gel (Aspen, China) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Membranes were blocked with 5% BSA in TBS and then incubated with primary antibodies against cellular retinaldehyde-binding protein (CRALBP, 1:500), retinoid isomerohydrolase (RPE65, 1:500), rhodopsin (1:300) and MER tyrosine kinase (METRK, 1:500) for overnight at 4°C. Next, an HRP-conjugated secondary antibody was applied (1:10 000) and detected with the Immobilon Western Chemiluminescent HRP Substrate system (Millipore, USA). All of the antibodies were purchased from Abcam Inc (UK).

1.6 Immunofluorescence

BMSCs cultured with standard medium or differentiation medium as described above, or the tissues were fixed in 4% paraformaldehyde for 20 min. For wound tissues, the samples were further embedded in paraffin and cross-sectioned at 5 μm. Then, the samples were stained with primary antibodies against CRALBP (1:500) or RPE65 (CR1:500) (Abcam, UK). After incubation with Cy3-conjugated secondary antibodies (1:50, Aspen, China), they were added with DAPI to stain the cell nuclei.

1.7 Assessment of BMSCs Localization and Differentiation within Wounds

For localization of CM-DiI-labeled cells, frozen tissue sections embedded in OCT compound were fixed in 4% paraformaldehyde for 10 min and then stained with DAPI for 5 min to visualize transplanted BMSCs and nuclei of tissue cells in wounds. Furthermore, to assess the fate and differentiation process of BMSCs within the wound bed, frozen sections were fixed in 4% paraformaldehyde for 10 min, and blocked with 5% BSA (Roche, Switzerland) followed by staining with primary antibodies against specific cell markers including CRALBP (1:500) or RPE65 (1:500) (Abcam, UK). Then, the samples were incubated with Cy3-conjugated secondary antibodies (1:50, Aspen, China) and DAPI was used to stain the nuclei. Finally, Image Pro Plus 6.0 was utilized to analyze the localization of CM-DiI labeled cells and co-staining of CM-DiI and specific cell markers.

1.8 Statistical Analysis

All of the values are expressed as mean±standard deviation (SD). Statistical significance was measured using the Student's unpaired *t* test (two-tailed). The difference among different groups was detected using one-way analysis of variance (ANOVA) test and multiple comparison between the groups was then performed using Student-Newman-Keuls (S-N-K) test. A P value <0.05 was considered statistically significant.

2 RESULTS

2.1 Induction of BMSCs Differentiation Using CNTF

BMSCs exhibited a spindle-shaped morphology at passage 3. They expressed CD44 (99.6%) and

Table 1 The primer sequences for each primer used in the real-time RT-PCR

Genes	Primer sequences (5′–3′)	Annealing temperature (°C)
β-Actin	Forward: CGTTGACATCCGTAAAGACCTC	58
	Reverse: TAGGAGCCAGGGCAGTAATCT	
RPE65	Forward: GCCCAGGAGCAGGACAAAAG	58
	Reverse: GCGCATCTGCAAGTTAAAACCA	
CRALBP	Forward: AGGTAGGTGGCCAACAGTA	58
	Reverse: TATGCCCAAAGAACCTCCCT	

RPE65: retinoid isomerohydrolase; CRALBP: cellular retinaldehyde-binding protein

CD90 (99.77%) and did not express CD31 (12.3%) and CD45 (5.95%). After 7-day culture with CNTF, BMSCs showed an increase in the expression of RPE-related genes including CRALBP (fold change, 3.904±0.131, P<0.001), and RPE65 (fold change, 2.295±0.286, P<0.001) compared with BMSCs in 2D culture without CNTF (fig. 1A). Similarly, Western blot analysis and immunofluorescence staining confirmed the same results as those observed in the RT-PCR (fig. 1B–1D).

2.2 Assessment of BMSCs Viability and Engraftment into the Retina

Of note, more CM-DiI labeled BMSCs were observed in retina tissues treated with BMSCs+CNTF than those treated with local BMSCs injection on days 14 and 21 (fig. 2A). Quantification of CM-DiI labeled BMSCs within vitreous body showed a significantly higher viable percentage of cells in rats treated with BMSCs+CNTF compared with local BMSCs injection (P<0.001, fig. 2B).

2.3 Differentiation of BMSCs within the Retina

To analyze the induction of BMSCs differentiation by adding CNTF for retinal regeneration, the co-localization of CM-DiI with several cell-specific markers was analyzed on days 14 and 21 post-injection between BMSCs+CNTF treated and BMSCs treated wounds (fig. 2C). In BMSCs+CNTF group on day 21, co-staining for CM-DiI and the RPE cells markers CRALBP, RPE65 and ZO-1 demonstrated that 87.1%±7.3% of BMSCs had differentiated into RPE cells. On the contrary, no obvious sign of BMSCs differentiation was observed in the BMSCs local injection group both on days 14 and 21 (fig. 2C). In addition, immunofluorescence staining reflected enhanced CRALBP and RPE65 mRNA levels in the group treated with BMSCs+CNTF compared with untreated controls (day 14, P<0.001) (fig. 3A). Real-time-PCR analysis also reflected the same tendency (fig. 3B and 3C).

2.4 Activation of Rod Photoreceptor Cells and Phagocytosis Function of RPE Cells

To evaluate the number and the activation of the rod photoreceptor cells and the phagocytosis function of RPE cells in the retina of all the groups, we further assessed the expression of rhodopsin and METRK. Western blot analyses reflected enhanced rhodopsin and METRK protein levels in day 14 and 21 in the retina treated with BMSCs+CNTF as compared with untreated controls (P<0.001) (fig. 4A–4D). Immunofluorescence staining of rhodopsin and METRK revealed similar results (fig. 4E).

3 DISCUSSION

The study demonstrated the enhancement of biological roles of BMSCs in the retina of the murine diabetic retinopathy model by adding CNTF *in vivo*, which led to the accelerated regeneration of RPE cells. The viability of engrafted BMSCs was improved, BMSCs were induced to differentiate into RPE cells

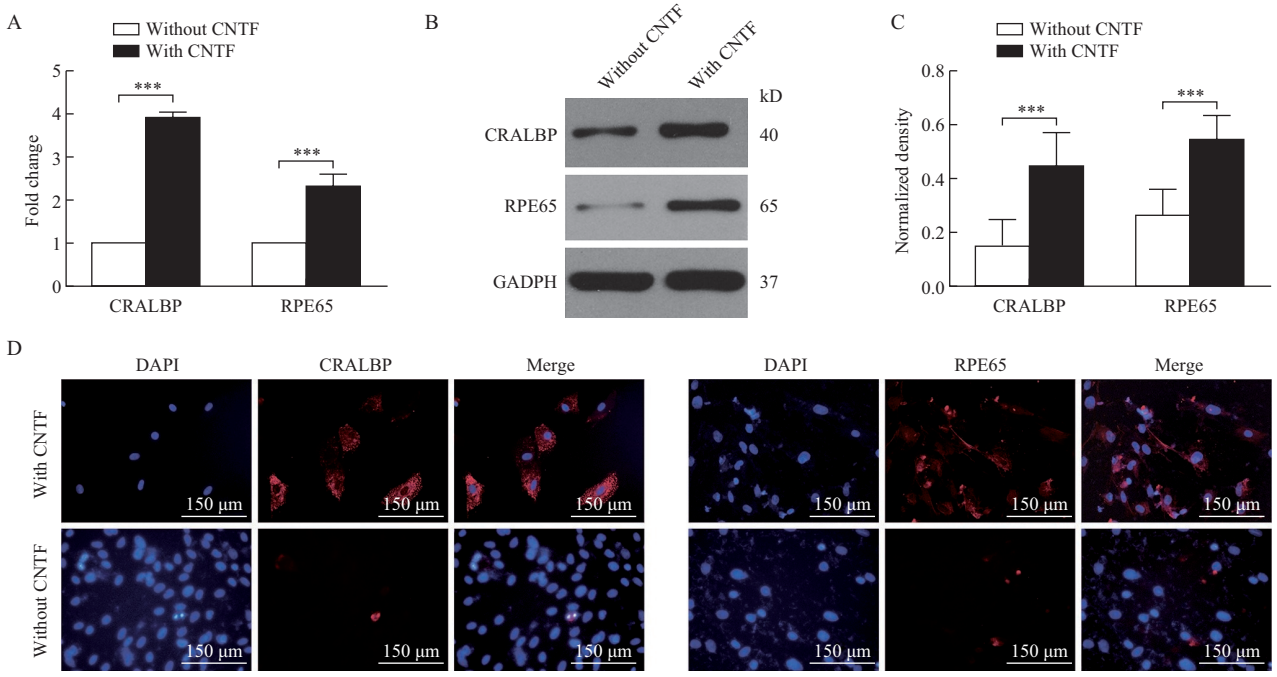

Fig. 1 Effect of CNTF culture on BMSCs differentiation *in vitro*

A: qRT-PCR analysis of CRALBP and RPE65 gene expression with or without CNTF. The data of without CNTF were considered as 1. B: Western blot analysis of CRALBP and RPE65; C: quantification of Western blotting data; D: Immunofluorescent staining demonstrated that BMSCs cultured with CNTF expressed increased levels of CRALBP and RPE65. Data are given as the mean±SD. $^{***}P$<0.001

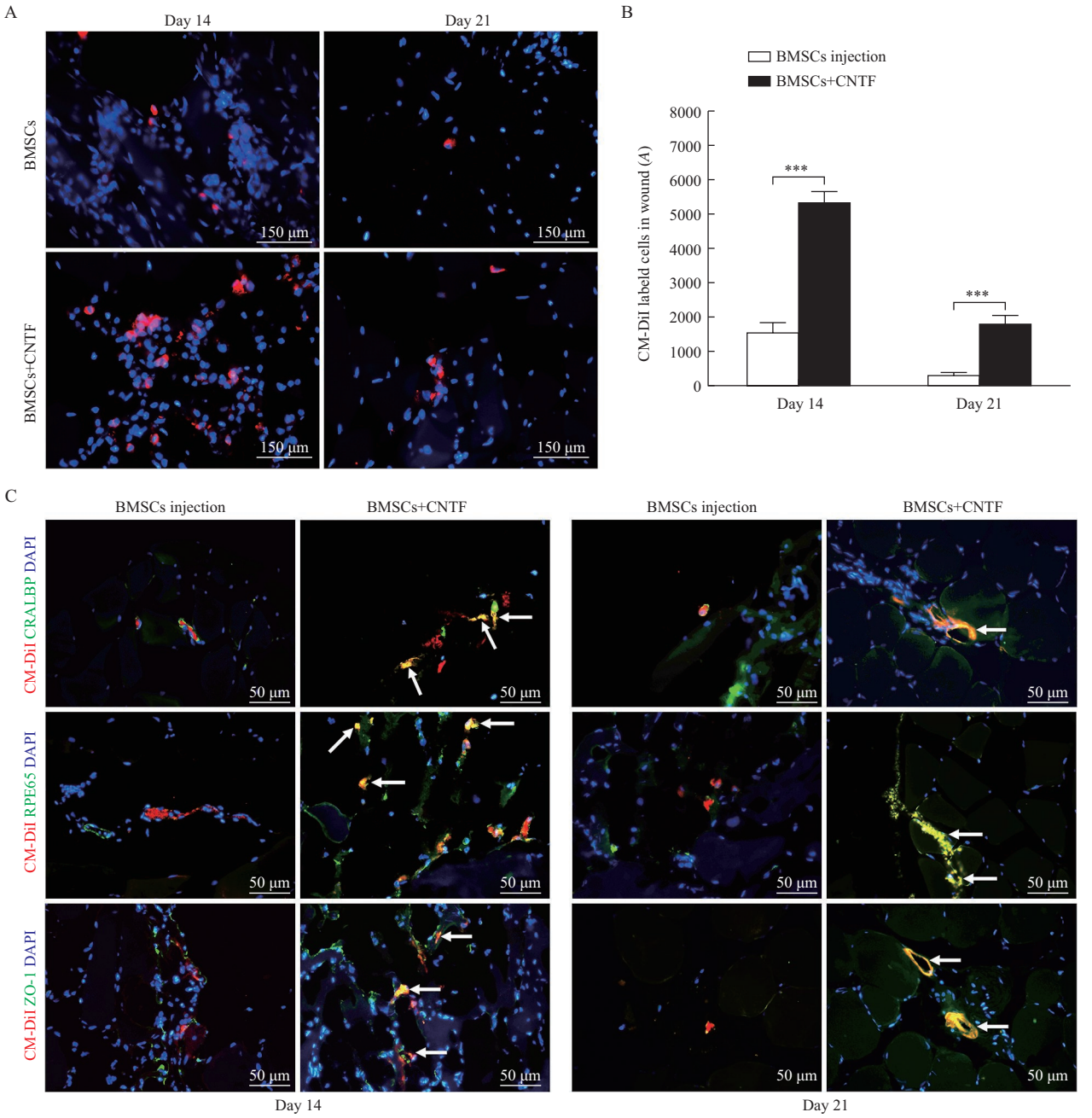

Fig. 2 Engraftment and differentiation of BMSCs in retina

A: images of CM-DiI labeled BMSCs (red) in retina on days 14 and 21 in BMSCs injection group and BMSCs+CNTF group; B: quantification of CM-DiI labeled cells in retina; C: co-localization of CM-DiI labeled BMSCs with cell-specific markers to determine BMSCs fate within wounds in BMSCs+CNTF group on days 14 and 21 . BMSCs were co-localized with CRALBP, RPE65, and ZO-1 (yellow, indicated with white arrowheads). Data are given as the mean ± SD. $^{***}P<0.001$

and the expression of fundamental proteins such as METRK and rhodopsin increased in the retina.

Stem cell-based therapy, particularly with BMSCs, has been demonstrated as a promising approach for the treatment of various types of retinopathies[4, 5]. However, the poor viability and low levels of BMSCs engraftment often limit the therapeutic potential of these cells[17]. Many studies have proved that transplantation of stem cells gives rise to local inflammation and macrophage infiltration[18]. As a result, BMSCs were likely to be eliminated by the detrimental microenvironment of early-stage wounds before they can effectively play a role. In our study, we utilized CNTF to enhance the viability of the engrafted BMSCs. We assume that CNTF might serve as an important factor to stimulate BMSCs to secrete more cytokines, thus promoting photoreceptor cell survival, which in turn improved the viability of the transplanted BMSCs[19].

In our study, the immunofluorescence analysis showed CM-DiI labelled BMSCs were detectable in the retina and were co-stained with specific

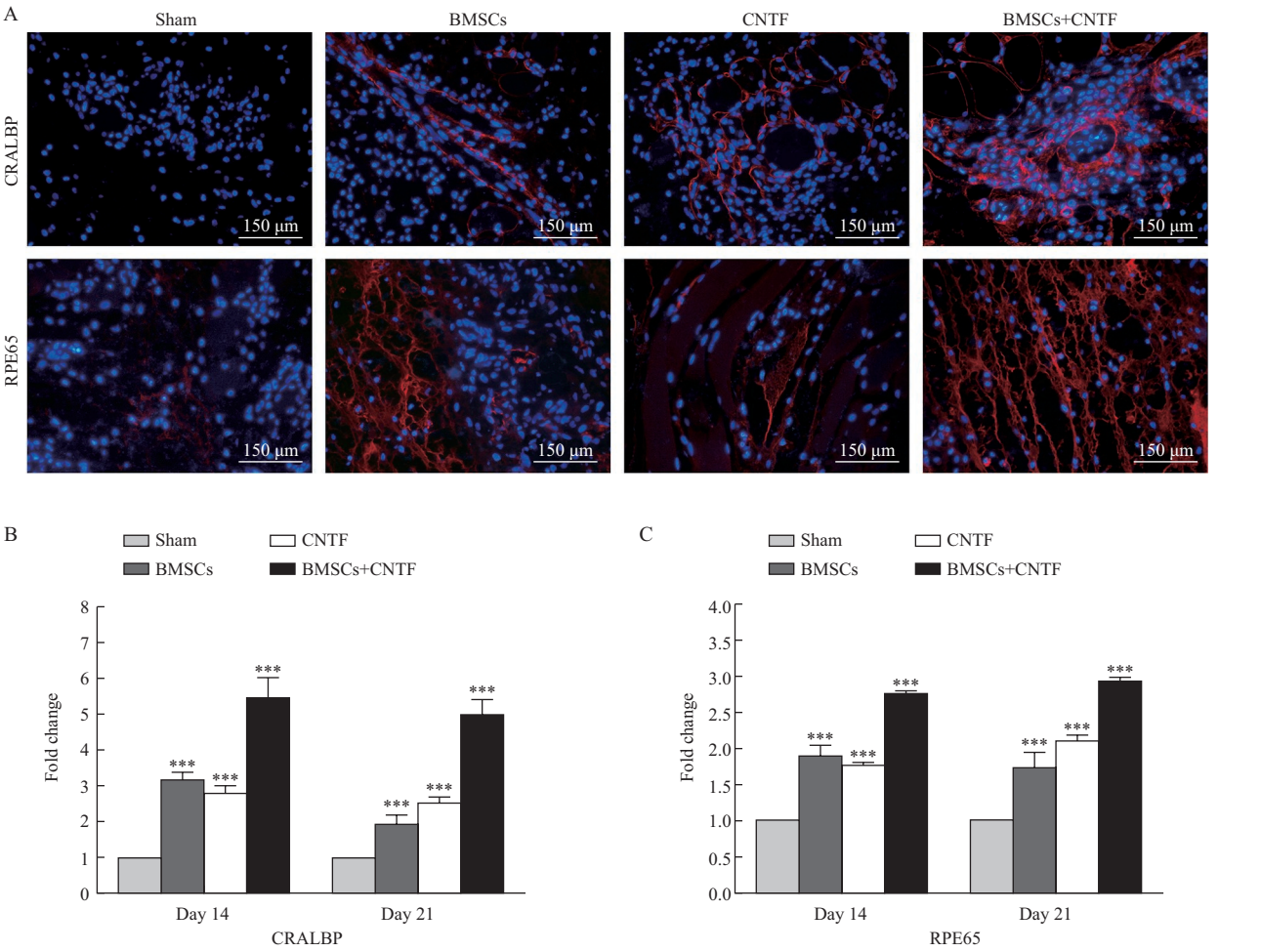

Fig. 3 Expression of CRALBP and RPE65 in RPE cells

A: immunofluorescence staining of CRALBP and RPE65 within retina among different groups on day 14; B, C: RT-PCR results revealed more expression of CRALBP and RPE65 in retina treated with BMSC+CNTF group compared to other three groups on days 14 and 21. Data are given as the mean±SD. $^{***}P<0.001$ *vs.* sham group

markers including CRALBP and RPE65, suggesting the differentiation of BMSCs into RPE cells[20, 21]. RPE cells play significant supporting roles in RPE-mediated retinal disorders, and contribute to retinal self-regeneration. Previously, Ezquer *et al*[22] evaluated the MSC administration in the vitreous body of mouse by a single intravitreal injection of MSCs and found that most donor cells could not differentiate into the neural or perivascular-like cells. The same results were observed in our study that there were no obvious differentiation detected in the single BMSCs injection group, implying that the extra inductive factors such as CNTF or drugs need to be used in the pretreatment when the stem cell therapy is applied[23]. Our findings further elucidated that the combined therapy using BMSCs and CNTF was effective for the differentiation of engrafted BMSCs and had important implications in the process of retinal regeneration after injury.

CNTF was proved to be closely linked with the neurotrophic activity of the eye[24]. It has been demonstrated to have a remarkable ability for neuro-protection of rod photoreceptors in a variety of retinal degeneration models in some species[25]. A previous study revealed that CNTF was able to induce the activation of JAK/STAT3 signaling pathway, thus leading to an increase in the survivability of RPE cells[26]. More importantly, CNTF was able to prevent the atrophy of the inner plexiform layer and cavity formation in the pigment epithelium in CNTF-administered diabetic rats, thus inhibiting the retinal degeneration[27]. Similarly, our study indicated that CNTF injection alone could protect the damage of the retina by stimulating the expression of rhodopsin both on days 14 and 21 post-injection. In addition, CNTF has been demonstrated to induce the directional differentiation of human MSCs to express retinal markers (rhodopsin) from rat retina[28]. Considering that rhodopsin is the key protein of RPE cells, we raised the hypothesis that CNTF may also drive the differentiation of BMSCs into the RPE cells, thus enhancing the regenerative process in diabetic retinopathy. Therefore, we explored the biological

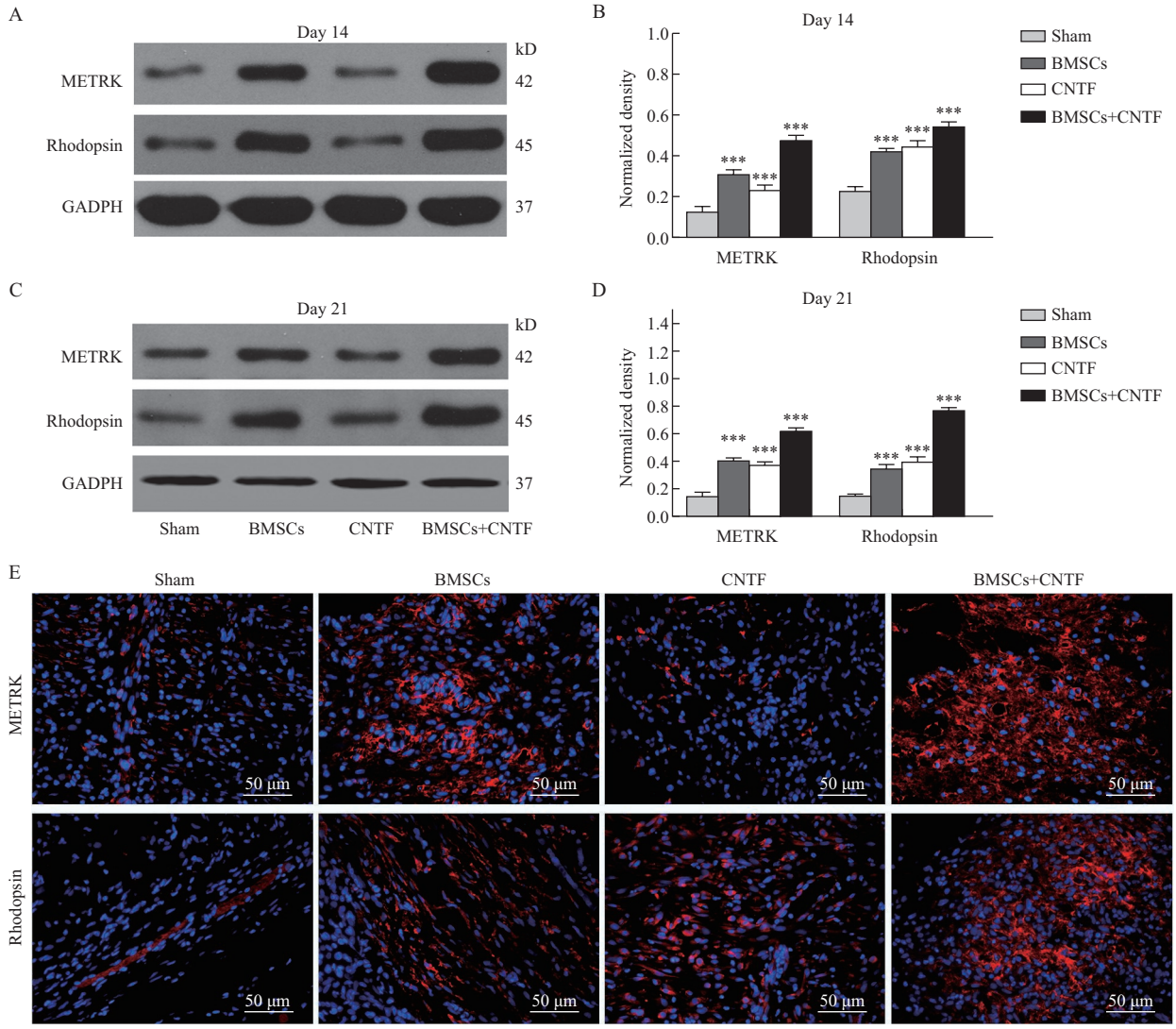

Fig. 4 Evaluation of activation of rod photoreceptor cell and phagocytosis function of RPE cells
A: Western blotting of METRK and rhodopsin protein expression within retina among different groups on day 14; B: quantification of Western blot results on day 14; C: Western blot analysis of METRK and rhodopsin protein expression within retina among different groups on day 21; D: quantification of Western blot results on day 21; E: immunofluorescence staining of METRK and rhodopsin within retina on day 21; Nuclei were stained with DAPI (blue). Data are given as the mean ± SD. $^{***}P<0.001$ *vs.* sham group

behavior of BMSCs by adding CNTF into the vitreous body and our results exhibited that BMSCs were shown to differentiate into RPE cells, which, to our knowledge, has not been documented before. This is a crucial finding considering that the microenvironment of the impaired retina in clinical cases are usually lack of sufficient stimulus for inducing exogenous stem cells to differentiate into the key cells that are indispensable for retinal regeneration[29].

Nevertheless, there are some limitations in our study. Due to the technical delivery problem and variability we encountered during the experiment, we did not test whether multiple injections of CNTF and BMSCs would be more effective or less. Additionally, we did not evaluate the relationship between the different concentrations of CNTF and the therapeutic effect on DR *in vivo*.

In conclusion, our findings demonstrated the capacity of CNTF to enhance BMSCs regenerative property for retinal healing potentially by preserving the viability of the cells, further inducing them to differentiate into the desired cells (RPE cells) that are beneficial for retinal healing. This combination strategy is superior to either cells transplantation or CNTF injection alone, leading to the accelerated retinal regeneration. Although further optimization of the applicable parameter of CNTF and BMSCs, such as the therapeutic effect of multiple injection, needs to be evaluated in the future, we believe that our findings, to some extent, may provide benefits to clinical application of stem cells for retinal regeneration.

Conflict of Interest Statement

The authors have declared that there is no conflict of interest.

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