Bone Marrow Mesenchymal Stem Cells Stimulate Proliferation and Neuronal Differentiation of Retinal Progenitor Cells

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Abstract

During retina development, retinal progenitor cell (RPC) proliferation and differentiation are regulated by complex inter- and intracellular interactions. Bone marrow mesenchymal stem cells (BMSCs) are reported to express a variety of cytokines and neurotrophic factors, which have powerful trophic and protective functions for neural tissue-derived cells. Here, we show that the expanded RPC cultures treated with BMSC-derived conditioned medium (CM) which was substantially enriched for bFGF and CNTF, expressed clearly increased levels of nuclear receptor TLX, an essential regulator of neural stem cell (NSC) self-renewal, as well as betacellulin (BTC), an EGF-like protein described as supporting NSC expansion. The BMSC CM- or bFGF-treated RPCs also displayed an obviously enhanced proliferation capability, while BMSC CM-derived bFGF knocked down by anti-bFGF, the effect of BMSC CM on enhancing RPC proliferation was partly reversed. Under differentiation conditions, treatment with BMSC CM or CNTF markedly favoured RPC differentiation towards retinal neurons, including Brn3a-positive retinal ganglion cells (RGCs) and rhodopsin-positive photoreceptors, and clearly diminished retinal glial cell differentiation. These findings demonstrate that BMSCs supported RPC proliferation and neuronal differentiation which may be partly mediated by BMSC CM-derived bFGF and CNTF, reveal potential limitations of RPC culture systems, and suggest a means for optimizing RPC cell fate determination in vitro.

Introduction

Visual impairment, including retinitis pigmentosa, age-related macular degeneration, glaucoma and diabetic retinopathy, severely affects the quality of life of patients and their families. These retinal disorders are all characterised by the dysfunction and loss of retinal neurons, leading to an irreversible decline in visual function, and there are at present no effective restorative therapies available for these diseases [1,2]. Cell replacement therapy is a promising therapeutic approach to restoring visual function to the abnormal retina and has become an important strategy in retinal regeneration research.

Retinal progenitor cells (RPCs) are a subset of undifferentiated cells that have the ability to self-renew and the potential to differentiate into various retinal neurons [3]. They are capable of cytoarchitectural integration and differentiation towards cells expressing characteristic markers of retinal neurons, thereby improving visual function in the host [3,4]. These findings suggest that RPCs may be able to replace degenerating retinal cells. Although these studies described promising therapeutic applications of RPCs, there are numerous related issues and concerns, including the improvement of proliferation capacity and the preferential differentiation into specific neurons but not glial cells. These concerns must be addressed for the successful use of RPCs in cell replacement therapy in the future. One way to explore the promise of RPCs is to modify culture conditions in an attempt to improve the potential of RPC proliferation and differentiation. Among the most accessible strategies is an attractive strategy of co-culture that has been considered to improve the proliferation and differentiation of progenitor cells [5-7].
Bone marrow mesenchymal stem cells (BMSCs) have attracted much attention because they can be readily obtained through a well-established procedure and are relatively simple to isolate and expand in vitro. A previous series of reports indicated that BMSCs have been shown to provide a powerful neuroprotection in degenerative disorders of the central nervous system. BMSCs were able to secrete neurotrophic factors and anti-inflammatory cytokines, which are potentially therapeutic in models of Huntington's disease, Parkinson's disease, glaucoma and light-damaged retina [8-10]. In addition, previous studies also indicated that BMSCs can provide instructive signals to direct the differentiation of neural stem cells (NSC) and promote axonal development when BMSCs and NSCs are co-cultured in vitro [11].

Here, we investigated the communication between BMSC-derived CM and RPCs, and demonstrate that compared to untreated RPCs, BMSC CM-treated RPCs displayed enhanced expression of nuclear receptor TLX (an essential regulator of NSC self-renewal) and betacellulin (BTC, an EGF-like protein reported to support NSC proliferation and enhance neurogenesis), and exhibited a large capacity to stimulate RPC proliferation and enhance RPC neuronal differentiation in vitro.

**Experimental Procedures**

**Experimental animals**

All animal procedures used in the present study were performed according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated with the Shanghai Jiao tong University School of Medicine.

**Isolation and culture of retinal progenitor cells**

RPCs were isolated from the neural retina of postnatal day 1 GFP transgenic C57BL/6 mice [12,13]. Briefly, retinas were harvested and subjected to several cycles of 0.1% type I collagenase (Invitrogen, Carlsbad, CA) digestion. The cell suspensions were then forced through a nylon mesh with 100-μm pores, centrifuged, and resuspended in standard culture medium (SM), containing advanced DMEM/F12 (Invitrogen), 1% N2 neural supplement (Invitrogen), 100 U/ml penicillin-streptomycin (Invitrogen), and 10% FBS. These cells were then incubated at 37 °C with 5% CO₂. After seeding for 12 h, the non-adherent cells were removed by replacing the medium. The medium was added and replaced every 3 or 4 days. When the cells grew to confluence, they were harvested with 0.25% trypsin and were replated on 25-cm² plastic flasks, again cultured to confluence and harvested. All of the experiments described below were performed using cells from the third to the fifth passage.

**Neural stem cell (NSC) culture**

Mouse NSCs were kindly provided by Dr T. Q. Wen and were cultured in DMEM with 10% FBS, 5% horse serum (Invitrogen), 1 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, as previously described [15].

**Lens epithelial cell culture**

Lens epithelial cells were isolated from the eye of the mature rabbit [16]. The anterior lens capsule was torn with tweezers and scissors and was incubated in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) at 37 °C with 5% humidified CO₂ for 24 h. Then, the capsules were digested with 0.25% trypsin for 10 min, centrifuged, and resuspended in complete medium. After seeding for 48 h, the non-adherent cells were removed by replacing the medium. The medium was added and replaced every 3 or 4 days. When the cells grew to confluence, they were harvested with 0.25% trypsin and were replated on plastic flasks.

**Preparation of conditioned media and ELISA analysis**

When the BMSCs, lens epithelial cells, RPCs and NSCs were grown to 70-80% confluence in 25-cm² plastic flasks, the culture media were replaced with SM (but not including 20 ng/ml EGF). After 12 h, the conditioned media were collected and sterile-filtered through a 0.2-μm membrane and conditioned samples were stored at -80 °C before use. For ELISA analysis, ELISA was performed using a Mouse bFGF ELISA kit (abcam INC., Cambridge, MA) and a Rat CNTF ELISA kit (abcam INC.), and their protocols. As a reference for quantification, a standard curve was established by a serial dilution for bFGF protein (18.8 pg/ml-2.0 ng/ml) and CNTF protein (8.23pg/ml-2.0 ng/ml).

**Proliferation and differentiation studies of RPCs**

For the proliferation experiments, the RPCs were seeded in flasks at a density of 2×10³ cells/ml under different conditions: CM from BMSCs, lens or NSCs (all supplemented with 20 ng/ml EGF), or the CM with or without 1.2 ng/ml bFGF (Invitrogen) or 1.2 ng/ml CNTF (Chemicon, Temecula, CA), or BMSC CM plus 2.5 ug/ml anti-bFGF (Millipore, Temecula, CA) or 2.5 ug/ml anti-CNTF (R & D Systems, Minneapolis, MN). For the differentiation experiments, the RPCs were cultured with the standard differentiation medium (SDM) consisting of advanced DMEM/F12, 1% N2 neural supplement, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin and 5% FBS, or SDM with 1.2 ng/ml bFGF or 1.2 ng/ml CNTF, or the CM from

bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin. These cells were then incubated at 37 °C with 5% CO₂. After seeding for 12 h, the non-adherent cells were removed by replacing the medium. The medium was added and replaced every 3 or 4 days. When the cells grew to confluence, they were harvested with 0.25% trypsin and were replated on 25-cm² plastic flasks, again cultured to confluence and harvested. All of the experiments described below were performed using cells from the third to the fifth passage.
were incubated for another 3 h at 37 °C according to the reagent instructions, the absorbance at 490 nm was measured with an ELISA microplate reader (ELX800, BioTeK, USA). The efficiency of the reaction was measured with primers using an RT-PCR kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) [17]. Briefly, RPCs were suspended at a final concentration of 1×10^4 cells/well and 5% FBS, or BMSC plus 2.5 ug/ml anti-bFGF or 2.5 ug/ml anti-CNTF were half changed daily.

### BMSCs, lens or NSCs supplemented with 5% FBS, or BMSC CM plus 2.5 ug/ml anti-bFGF or 2.5 ug/ml anti-CNTF containing 5% FBS. For all of the culture conditions, the media were half changed daily.

#### Cell viability

The effect of CM from the BMSCs, NSCs and lens epithelial cells on RPC proliferation was assessed using the cell counting properties are classified and listed in Table 2. After washing with PBS solution and incubated in fluorescent secondary antibodies (Alexa Fluor488 goat anti-mouse or goat anti-rabbit, 1:800 in PBS, BD) for 1 h at room temperature. The cells were then washed with PBS and blocked for 1 h in a blocking solution (PBS containing 10% (v/v) normal goat serum (Invitrogen)), 0.3% Triton X-100 (Sigma-Aldrich) and 0.1% NaN3 (Sigma-Aldrich). The samples were then incubated in the primary antibodies (Table 2) at 4 °C overnight. All of the identified protein markers and their properties are classified and listed in Table 2. After washing with PBS solution and incubated in fluorescent secondary antibodies (Alexa Fluor488 goat anti-mouse or goat anti-rabbit, 1:800 in PBS, BD) for 1 h at room temperature. The cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Media Cybernetics, Roper, GA). The quantification of cells was based on counting the number of DAPI-stained nuclei and immunoreactive cells in at least 5 independent fields, for a total of at least five hundred to one thousand cells.

| Genes | Accession no. | Forward (5’–3’) | Reverse (5’–3’) | Annealing temperature (°C) | Product size (base pairs) |
|-------|---------------|-----------------|-----------------|-----------------------------|--------------------------|
| Pax6  | NM_0136277    | agtgaagcttcggttagatgc | acggcttgagaatctggtgacaggga | 60                          | 123                      |
| Mash1 | NM_005855     | tctcctggaagggactttcg | ggcttggggtctggtggtggtg | 60                          | 142                      |
| Nestin| NM_016701     | aacagcactcaagatgttc | tcaagggattaggaagggga | 60                          | 235                      |
| Vimentin | NM_011701 | tgtgctcaccacctcaaaaaaatattcagatatg | gctcttgctctgcaagttttg | 60                          | 269                      |
| Ki-67 | X82786        | cagatctggaatgagccaa | cacagctgcagttttgagctag | 60                          | 100                      |
| Beta2m | NM_007568 | cacaggatccaccctcagca | ccdttccacagatgctgagagag | 60                          | 135                      |
| TLR  | NM_152222.2   | gattagaccccctactga | ggtatctggtatgaatgtagc | 60                          | 166                      |
| Map2  | NM_001039934  | agaaatggctagatgaagctdgg | acaagctgtggtgaaggtactgg | 60                          | 122                      |
| β3-tubulin | NM_023279 | cgagacactcgtcgcagcaga | cttctgcctctctgtcctgtg | 60                          | 208                      |
| PKC-α | NM_011101     | ccctctccagagagattgta | tctctgctctgtcctgtg | 60                          | 212                      |
| AP2u | NM_001122948.1 | gctgctcctcactgcagga | gatgctgctgctgctgcctg | 60                          | 208                      |
| Bm3a | NM_011144.4   | gctctgcacaacacatga | ttctctgccggccttgga | 60                          | 121                      |
| GFAP | NM_010277     | agaaacgcagctcaccactc | tcacatcacaagttctgtag | 60                          | 184                      |
| Rhodopsin | NM_145383 | tcacacccacccccctcaaca | tgtctccagggagaacaccac | 60                          | 216                      |
| β-actin | NM_0073933   | acggctgctgtggtggtgaa | cttctccagctgctggggtgtgaa | 60                          | 152                      |

The proliferation and differentiation of RPCs were studied under the four culture conditions described above. RPCs were cultured on glass coverslips (VWR, West Chester, PA) coated with laminin (Sigma-Aldrich, Saint Louis, MO) and were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH2PO4, 135.60 mM NaCl and 8.10 mM Na2HPO4) for 15 min at room temperature. The cells were then washed with PBS and blocked for 1 h in a blocking solution (PBS containing 10% (v/v) normal goat serum (Invitrogen)). 0.3% Triton X-100 (Sigma-Aldrich) and 0.1% Na3 (Sigma-Aldrich). The samples were then incubated in the primary antibodies (Table 2) at 4 °C overnight. All of the identified protein markers and their properties are classified and listed in Table 2. After washing with PBS solution and incubated in fluorescent secondary antibodies (Alexa Fluor488 goat anti-mouse or goat anti-rabbit, 1:800 in PBS, BD) for 1 h at room temperature. The cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Immunopositive cells were detected using a fluorescent microscope (Olympus BX51, Japan). The control samples were processed using the same protocol but with the omission of the primary antibody.

### Cell counts

The cell counts were performed using a fluorescent microscope (Olympus BX51, Japan) and Image pro plus 6.0 (Media Cybernetics, Roper, GA). The quantification of cells was based on counting the number of DAPI-stained nuclei and immunoreactive cells in at least 5 independent fields, for a total of at least five hundred to one thousand cells.

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**Table 1. Primers used for quantitative RT-PCR.**

| Genes   | Accession no. | Forward (5’–3’) | Reverse (5’–3’) | Annealing temperature (°C) | Product size (base pairs) |
|---------|---------------|-----------------|-----------------|-----------------------------|--------------------------|
| Pax6    | NM_0136277    | agtgaagcttcggttagatgc | acggcttgagaatctggtgacaggga | 60                          | 123                      |
| Mash1   | NM_005855     | tctcctggaagggactttcg | ggcttggggtctggtggtggtg | 60                          | 142                      |
| Nestin  | NM_016701     | aacagcactcaagatgttc | tcaagggattaggaagggga | 60                          | 235                      |
| Vimentin | NM_011701 | tgtgctcaccacctcaaaaaaatattcagatatg | gctcttgctctgcaagttttg | 60                          | 269                      |
| Ki-67   | X82786        | cagatctggaatgagccaa | cacagctgcagttttgagctag | 60                          | 100                      |
| Beta2m  | NM_007568     | cacaggatccaccctcagca | ccdttccacagatgctgagagag | 60                          | 135                      |
| TLR     | NM_152222.2   | gattagaccccctactga | ggtatctggtatgaatgtagc | 60                          | 166                      |
| Map2    | NM_001039934  | agaaatggctagatgaagctdgg | acaagctgtggtgaaggtactgg | 60                          | 122                      |
| β3-tubulin | NM_023279 | cgagacactcgtcgcagcaga | cttctgcctctctgtcctgtg | 60                          | 208                      |
| PKC-α   | NM_011101     | ccctctccagagagattgta | tctctgctctgtcctgtg | 60                          | 212                      |
| AP2u    | NM_001122948.1 | gctgctcctcactgcagga | gatgctgctgctgctgcctg | 60                          | 208                      |
| Bm3a    | NM_011144.4   | gctctgcacaacacatga | ttctctgccggccttgga | 60                          | 121                      |
| GFAP    | NM_010277     | agaaacgcagctcaccactc | tcacatcacaagttctgtag | 60                          | 184                      |
| Rhodopsin | NM_145383 | tcacacccacccccctcaaca | tgtctccagggagaacaccac | 60                          | 216                      |
| β-actin | NM_0073933   | acggctgctgtggtggtgaa | cttctccagctgctggggtgtgaa | 60                          | 152                      |

The data are expressed as fold change relative to untreated controls after normalizing to β-actin.
Results of bFGF in SM, RPCs displayed enhanced proliferation.

bFGF, the effect of BMSC CM on enhancing RPC proliferation
cells compared with the cultures in BMSC CM (Figure 1B, F, J). In the presence of BMSC or
In response to NSC CM (Figure 1C, G, K) or SM (Figure 1D, H, L), most RPCs grew as spherical clusters that adhered to the
Lens CM, the RPCs grew as an adherent monolayer of cells or
extended from a few cells were observed by day 1, more cells
extended processes with time, and their were fewer adherent
cells (data not shown). These data suggest that the
undifferentiated state of RPCs was unaffected by exposure to
BMSC CM and that the BMSC CM can stimulate RPC proliferation.

Statistical analysis
The results represent the average of three experiments, and the data are presented as the mean ± SD. Each experiment
was performed at least three times unless otherwise specified. Statistical significance was determined using unpaired
Student’s t-test, and a value of *P<0.05 was considered to be statistically significant.

Results

Morphology and expansion potential of CM-treated RPCs
Under proliferation conditions, the RPCs were cultured in
BMSC, lens and NSC CM, and morphology images were taken
on days 1, 4 and 7 (Figure 1). In the presence of BMSC or
Lens CM, the RPCs grew as an adherent monolayer of cells or
cellular clusters. In BMSC CM, most of the cells extended short
processes by the first day; with time, most cells exhibited two
or more long processes that formed a network between cells by
day 7 (Figure 1A, E, I). Under the Lens CM, short processes
extended from a few cells were observed by day 1, more cells
extended processes with time, and their were fewer adherent
cells compared with the cultures in BMSC CM (Figure 1B, F, J). In response to NSC CM (Figure 1C, G, K) or SM (Figure 1D, H, L), most RPCs grew as spherical clusters that adhered to the
uncoated flask or floated in the culture medium. The spherical
clusters grew with time, but the average spherical cluster size
in NSC CM was larger than that observed in SM.

To investigate the effect of CM on RPC proliferation, the
proliferation capacity of the cells exposed to CM was evaluated
by cell counting kit-8 (CCK-8) analysis. In comparison with the control, RPCs cultured in the presence of CM, especially
BMSC CM, exhibited obvious increases in expansion capacity (Figure 1M).

In addition, our ELISA analysis exhibited that the BMSC CM
was substantially enriched for bFGF and CNTF compared to
RPC-cultured SM (Figure S1). When cultured in the presence of bFGF in SM, RPCs displayed enhanced proliferation
capacity (Figure S2A), while the obvious effect of the addition
of CNTF to SM on RPC expansion was not detected (Figure
S2B). After BMSC CM-derived bFGF knocked down by anti-
bFGF, the effect of BMSC CM on enhancing RPC proliferation
was partly reversed (Figure S2). These results suggest that the
effect of BMSC CM on RPC expansion may be partly mediated
by BMSC CM–derived bFGF.

Quantitative evaluation of the effect of CM on progenitor and proliferation marker expression in proliferating RPCs

qPCR was used to determine the expression of critical retinal
progenitor-related markers, including nestin, vimentin, PAX6
and Mash1, which play important roles in retinal development,
in CM-treated RPCs. The qPCR results showed that the levels
of vimentin, PAX6 and Mash1 were significantly or marginally
higher in CM-treated RPC cultures than in SM cultures (Figure
2A). No obvious change was detected in the expression of
nestin between different groups. Meanwhile, the expression
levels of the cell proliferation marker Ki-67 were slightly
upregulated in the CM-treated cells, especially in the BMSC
CM-treated RPC cultures (Figure 2B). In response to CM
treatment, especially with BMSC-CM, we also found high
expression levels of TLX and BTC, which have reported to play
important roles in neural stem cell proliferation (Figure 2C).
These results indicated that the proliferation of RPCs can be
enhanced by BMSC CM treatment.

The expression levels of progenitor- and proliferation-
related markers by immunocytochemistry analysis

Immunocytochemistry was performed to evaluate the
expression of progenitor- and proliferation-associated markers (Figure 3). Our data showed that there were no significant
differences in the proportions of nestin-positive cells between
different groups, while the percentages of Ki-67-positive cells
were high in the RPC cultures treated with CM, especially
those treated with BMSC CM (69.1 ± 4.1%), when compared
with the controls (58.9 ± 3.5%) (Figure 3A-I). Even in RPCs
treated with CM for up to 3 passages, the percentages of
nestin- and Ki-67-positive cells were sustained. In addition,
cells that were immunoreactive for the differentiated cell
markers β3-tubulin, MAP-2, protein kinase C alpha (PKC-α),
rhodopsin and glial fibrillary acidic protein (GFAP) were not
detected in either the CM-treated RPC cultures or the control
cells (data not shown). These data suggest that the
undifferentiated state of RPCs was unaffected by exposure to
BMSC CM and that the BMSC CM can stimulate RPC proliferation.

Effect of CM on RPC morphology and gene expression under differentiation conditions

In the differentiation conditions, the RPCs in all of the groups
differentiated into cells with divergent morphologies and
neurite-like processes (Figure 4A-L). The cells in differentiation
medium without CM only occasionally extended short
processes, while most of the RPCs extended short processes
in CM-treated cultures within the first day of culture in the
differentiation conditions (Figure 4A-D). The cultured cells
typically exhibited increasing neurite-like cellular processes that
formed an intercellular network over time; however, the cellular
processes of RPC cultures treated with CM, especially BMSC

Table 2. Primary antibodies used for immunocytochemistry.

| Antibodies | Type | Specificity in retina | Source | Dilution |
|------------|------|----------------------|--------|----------|
| Nestin     | Mouse monoclonal | Progenitors reactive glia | BD      | 1:200    |
| Ki-67      | Mouse monoclonal | Proliferating cells | BD      | 1:200    |
| Map2       | Rabbit monoclonal | Neurons | Epitomics | 1:200    |
| β3-tubulin | Mouse monoclonal | Neurons | Chemicon | 1:100    |
| AP2a       | Mouse monoclonal | Amacrine cells | DSHB    | 1:600    |
| Bm3a       | Rabbit polyclonal | Ganglion cells | Millipore | 1:500    |
| PKC-α      | Mouse monoclonal | Bipolar cells | BD      | 1:200    |
| GFAP       | Mouse monoclonal | Glia | Chemicon | 1:200    |
| Rhodopin   | Mouse monoclonal | Photoreceptors (rods) | Chemicon | 1:100    |

*P<0.05 was considered to be statistically significant.
CM, appeared longer and more numerous than those of RPCs cultured without CM (Figure 4E-L).

qPCR analysis was carried out to investigate the fate potential of RPCs exposed to CM. A notable upregulation in the expression of β3-tubulin, activator protein 2 alpha (Ap2α, an amacrine cell marker) and Brn3a (a ganglion cell marker) was detected in the BMSC CM-treated RPC cultures (Figure 4M). Neuronal markers MAP-2 and PKC-α (a marker for bipolar cells) were significantly higher in BMSC CM- and Lens CM-treated RPC cultures compared with the control. The expression levels of rhodopsin (a photoreceptor cell marker), which was of the most interest to us, were significantly raised in the BMSC CM- and Lens CM-treated RPC cultures compared with the control. The expression levels of rhodopsin (a photoreceptor cell marker), which was of the most interest to us, were significantly raised in the BMSC CM- and Lens CM-treated RPC cultures compared with the control. The expression levels of rhodopsin (a photoreceptor cell marker), which was of the most interest to us, were significantly raised in the BMSC CM- and L

In addition, when BMSC CM-derived CNTF knocked down by anti-CNTF, the effect of BMSC CM on enhancing RPC differentiation towards above retinal neurons were slightly inhibited; While the addition of CNTF to the RPC cultures in SDM, retinal neuronal marker expression levels of MAP-2, AP2α, Brn3a and rhodopsin were obviously upregulated (Figure S3). The effect of BMSC CM-derived bFGF on RPC differentiation was not detected (Data not shown), indicating the effect of BMSC CM on RPC differentiation may be at least partly mediated by BMSC CM-derived CNTF.

Multipotentiality of CM-treated RPCs

The expression of several essential markers involved in the differentiation of RPCs was also evaluated using immunocytochemistry analysis. The percentages of nestin- and Ki-67-positive cells were markedly decreased (less than 15% for both), with no significant difference between different group cultures under the differentiation conditions (Figure 5A-I) when compared with those in the proliferation conditions (more than...
85% and 58% for nestin- and Ki-67-positive cells, respectively) (Figure 3I). This result indicates that the proliferation capacity of RPCs decreased and that most RPCs differentiated into their daughter cells under the differentiation conditions. Our study also showed that the BMSC CM-treated RPC cultures displayed more β3-tubulin-, AP2- and Brn3a-immunoreactive cells than the other groups (36 ± 1.73%, 16.3 ± 2.8% and 19.3 ± 2.1% in BMSC CM; 32.6 ± 1.05%, 10.2 ± 1.5% and 10.4 ± 1.3% in lens CM; 31.7 ± 3.5%, 10.1 ± 1.6% and 12.6 ± 1.9% in NSC CM; 27.3 ± 1.5%, 8.4 ± 1.8% and 9.5 ± 2.1% in SDM) (Figure 6A-D, I-Q). For MAP-2 (Figure 6E-H, Q) and PKC-α (Figure 7E-H, M), the immunoreactive cell ratios were significantly higher in lens and BMSC CM-treated cultures. The proportions of rhodopsin-positive cells were significantly higher in lens and BMSC CM-treated cultures. The proportions of rhodopsin-positive cells were significantly higher in NSC and BMSC CM-treated cultures than in the control cells (Figure 7A-D, M). In contrast, the ratio of GFAP-labelled cells was clearly decreased in the BMSC CM-treated cultures compared with the control cells (16.7 ± 2.2% and 29.0 ± 1.9%, respectively) (Figure 7I-J, M). These findings are consistent with the qPCR results.

Taken together, these data demonstrated that the bone marrow mesenchymal stem cell-derived conditioned medium (BMSC CM) can enhance the proliferation and neuronal differentiation of RPCs, which may be partly mediated by BMSC CM-derived bFGF and CNTF.

Discussion

Signals from adjacent differentiated cells and extracellular matrix molecules play an important role in the properties of self-renewal and multilineage differentiation of stem cells [4,19,20]. In the present study, we investigated the effect of BMSC-derived conditioned medium on the expansion and differentiation of RPCs in vitro.

The present study investigated the expression of critical retinal progenitor-related markers, including nestin and Pax6. The transcription factors nestin and Pax6 have been reported to mediate the full retinogenic potential of RPCs and to be required for the maintenance of RPCs in the undifferentiated state [21]. Our present data showed that the expression levels of nestin and Pax6 in the BMSC CM-treated RPC cultures were significantly higher than in the other groups (Figure 3A-D, M). These findings are consistent with the qPCR results.

The Effect of BMSCs on RPC Growth

85% and 58% for nestin- and Ki-67-positive cells, respectively) (Figure 3I). This result indicates that the proliferation capacity of RPCs decreased and that most RPCs differentiated into their daughter cells under the differentiation conditions. Our study also showed that the BMSC CM-treated RPC cultures displayed more β3-tubulin-, AP2- and Brn3a-immunoreactive cells than the other groups (36 ± 1.73%, 16.3 ± 2.8% and 19.3 ± 2.1% in BMSC CM; 32.6 ± 1.05%, 10.2 ± 1.5% and 10.4 ± 1.3% in lens CM; 31.7 ± 3.5%, 10.1 ± 1.6% and 12.6 ± 1.9% in NSC CM; 27.3 ± 1.5%, 8.4 ± 1.8% and 9.5 ± 2.1% in SDM) (Figure 6A-D, I-Q). For MAP-2 (Figure 6E-H, Q) and PKC-α (Figure 7E-H, M), the immunoreactive cell ratios were significantly higher in lens and BMSC CM-treated cultures. The proportions of rhodopsin-positive cells were significantly higher in NSC and BMSC CM-treated cultures than in the control cultures (Figure 7A-D, M). In contrast, the ratio of GFAP-labelled cells was clearly decreased in the BMSC CM-treated cultures compared with the control cells (16.7 ± 2.2% and 29.0 ± 1.9%, respectively) (Figure 7I-J, M). These findings are consistent with the qPCR results.

Taken together, these data demonstrated that the bone marrow mesenchymal stem cell-derived conditioned medium (BMSC CM) can enhance the proliferation and neuronal differentiation of RPCs, which may be partly mediated by BMSC CM-derived bFGF and CNTF.
RPCs display a dramatic growth promotion when exposed to BMSC-derived conditioned medium during the expansion period. In addition, in the BMSC CM-treated RPC cultures, the expression of TLX and betacellulin (BTC) were clearly upregulated when compared with the controls. Previous studies indicated that TLX and BTC play important roles in promoting neural stem cell proliferation [22,23].

The observed robust growth of the BMSC CM-treated RPCs may have contributions from multiple soluble factors, including BMSC CM-derived mitogenic factor bFGF as shown in present study. Several published reports have indicated that the effect of BMSCs on neurons may be mediated by the supply of cell protective and mitogenic factors in a paracrine manner [24,25]. The other factor likely responsible for this finding may be that the BMSC CM-treated RPCs grow as adherent cells rather than as the spherical clusters in the control cultures. This hypothesis is supported by our previous result that the expansion capacity of RPCs grown in adherent conditions was better than that of cells grown as spherical clusters [26]. Thus, the BMSC CM may stimulate RPC growth through a combination of the supply of cell protective and mitogenic factors and by promoting adherence.

For cell replacement therapy in retinal neurodegenerative diseases, the optimal target is to induce stem/progenitor cells to produce a low proportion of glial cells and a high proportion of retinal neurons. Previous studies have reported that retinal stem/progenitor cells are more inclined to generate glia [4,27]. The present study demonstrated that PRCs exposed to BMSC CM differentiated less often into GFAP-positive glia and were more likely to generate retinal neurons. Our data revealed that the retina-specific interneuronal markers PKC-α and AP2α were highly expressed in BMSC CM-treated RPC cultures. Meanwhile, the retinal ganglion cell (RGC) marker Bm3α and photoreceptor cell marker rhodopsin also showed significantly increased expression levels in the RPC cultures, indicating that the BMSCs can stimulate RPC differentiation towards retinal interneurons, photoreceptors and ganglion cells. These observations are consistent with previous studies showing that BMSCs provide instructive signals that can direct the neuronal differentiation of neural stem cells (NSCs) and promote axonal growth when BMSCs and NSCs are co-cultured in vitro [11,28]. In addition, our present study showed that CNTF was enriched about 3 fold in conditioned media from BMSCs compared to RPCs. An upregulation in the expression of MAP-2, AP2α, Bm3α and rhodopsin was detected in the CNTF-treated RPC cultures under differentiation conditions, which was supported with a previous report [4], indicating that the effect of BMSC CM on RPC differentiation may be partly mediated by BMSC CM-derived CNTF.

In summary, our findings demonstrate that BMSCs supported RPC neuronal differentiation, indicating a means for optimizing RPC cell fate determination and allowing potential efficient production of specific retinal neurons for use in future neuroretinal cell replacement therapies.

Figure 3. Immunostaining analysis of the expression of progenitor- and proliferation-related markers. After four days of culture in proliferation conditions, the cells were fixed and immunostained with antibodies against nestin (A-D) and ki-67 (E-H). The ratio of the nestin-positive is similar between different groups, while the percentages of ki-67-positive cells were high in the RPC cultures in BMSC CM, Lens CM and NSC CM, when compared with the cultures in SM condition (I). The percentage of positive cells was determined by dividing the number of immunopositive cells by the number of nuclei stained with DAPI. Five hundred to one thousand cells for each RPC subgroup and each culture were counted in random fields. Scale bars: 50 µm.

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The present study investigated the effect of BMSC CM on the proliferation and multipotentiality of RPCs. Our data demonstrated that BMSC CM can enhance RPC proliferation and keep RPCs in an undifferentiated state. More interestingly, treatment with BMSC CM favoured RPC differentiation towards retinal neurons, including photoreceptors and RGCs. These findings suggest that the BMSC CM can profoundly influence the proliferation and differentiation of RPCs, which may be partly mediated by BMSC CM-derived bFGF and CNTF. However, further investigation is needed to determine how BMSCs promote RPC proliferation and stimulate RPC neuronal differentiation.

**Figure 4. Morphology and gene expression levels of RPCs under differentiation conditions.** One day after the cells were cultured in the differentiation conditions, the cells in the differentiation medium without CM only occasionally extended short processes (D), whereas most of the cells extended short processes in the CM-treated cultures (A, B, C). Under differentiation conditions, RPCs treated with BMSC CM (A, E, I), lens CM (B, F, J), NSC CM (C, G, K) and SDM (D, H, L) typically exhibited increasing neurite-like cellular processes and formed a network among the cells with time. However, the cellular processes of RPC cultures treated with CM, especially with BMSC CM, were longer and appeared more numerous than in the control cultures (without CM treatment). In the qPCR analysis (M), a notable up-regulation in the expression of β3-tubulin, activator protein 2 alpha (Ap2α, an amacrine cell marker) and Brn3a (a ganglion cell marker) was detected in the BMSC CM-treated RPC cultures. The levels of the neuronal markers MAP2 and PKC-α (a marker for bipolar cells) were significantly higher in the BMSC CM- and lens CM-treated RPC cultures compared with the control. The expression levels of rhodopsin (a photoreceptor marker) were higher in RPC cultures treated with BMSC CM or NSC CM. In addition, low expression levels of the glial marker GFAP were found in the RPC cultures treated with CM. Scale bars: 100 µm.

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**Conclusion**

The present study investigated the effect of BMSC CM on the proliferation and multipotentiality of RPCs. Our data demonstrated that BMSC CM can enhance RPC proliferation and keep RPCs in an undifferentiated state. More interestingly, treatment with BMSC CM favoured RPC differentiation towards retinal neurons, including photoreceptors and RGCs. These findings suggest that the BMSC CM can profoundly influence the proliferation and differentiation of RPCs, which may be partly mediated by BMSC CM-derived bFGF and CNTF. However, further investigation is needed to determine how BMSCs promote RPC proliferation and stimulate RPC neuronal differentiation.
Figure 5. Progenitor and proliferation marker expression of RPC cultures during differentiation. After seven days in the differentiation medium, the cells were fixed and immunostained with antibodies against nestin (A-D) and ki-67 (E-H). The proportion of nestin- and ki-67-positive cells showed no significant difference between different groups and was less than 15% (I). The quantification of immunoreactive cells was performed as described in Figure 3-I. *P<0.05. Scale bars: 50 µm.

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Figure 6. Potential of RPC differentiation towards neurons after exposure to CM. After RPCs were cultured in the differentiation condition for 7 days, the cells were immunolabelled for anti-β3-tubulin (A-D), -Map2 (E-H), -AP2α (I-L) and -Brn3a (M-P). The proportion of β3-tubulin, AP2α and Brn3a-positive cells was highest in BMSC CM-treated RPCs (Q). The percentage of MAP2-immunoreactive cells was significantly higher in lens and BMSC CM cultures (Q). The quantification of immunoreactive cells was performed as described in Figure 3-I. *P<0.05. Scale bars: 50 µm.

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Figure 7. Potential of RPC differentiation towards neuronal and glial cells after exposure to CM. After RPCs were cultured in differentiation medium for seven days, the cells were fixed and immunostained with antibodies against rhodopsin (A-D), PKC-α (E-H) and GFAP (I-L). The percentages of rhodopsin-positive cells were higher in NSC and BMSC CM-treated RPC cultures than in other groups, and PKC-α immunoreactive cells were detected more in CM treated RPCs. However, the ratio of GFAP-positive cells was decreased in the BMSC and NSC CM-treated cultures compared with the controls (M). Quantification of immunoreactive cells was performed as described in Figure 3-I. *P<0.05. Scale bars: 50 µm.
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Supporting Information

Figure S1. ELISA analysis of BMSC CM-derived bFGF and CNTF. The conditioned medium from BMSCs was substantially enriched for bFGF and CNTF compared to the RPCs-cultured medium. (TIF)

Figure S2. The effect of bFGF or CNTF on RPC proliferation capacity. Under proliferation conditions, the expansion capacity of the cells was evaluated by CCK-8 analysis. In comparison with the cells treated with BMSC CM, the expansion capacity of the RPCs cultured in the presence of BMSC CM plus anti-bFGF was partly inhibited. Addition of bFGF to the RPC cultures in SM, RPCs exhibited obvious increase in expansion capacity as compared with the cells in SM (without bFGF) (A). In addition, the obvious effect of CNTF or anti-CNTF on RPC expansion capacity was not detected (B). (TIF)

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