5-14-2023

Hematopoietic growth factors promote the proliferation and differentiation of oligodendrocyte progenitor cells

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ABSTRACT

Severe traumatic brain injury (TBI) causes severe damage in the white matter, leading to long-term disability and increased mortality. Demyelination, characterized by the damage of myelin sheaths and oligodendrocyte cell death, is a major pathological change in white matter injury after TBI, resulting in persistent neurological deficits. Previous studies have demonstrated that co-treatments with stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) (SCF+G-CSF) in the chronic phase of severe TBI improve neurological function recovery and enhance remyelination in the white matter. The SCF+G-CSF-enhanced remyelination is positively correlated with the proliferation of oligodendrocyte progenitor cells (OPCs) in the subventricular zone. However, it remains unclear whether SCF+G-CSF has a direct effect on OPCs to regulate OPC proliferation and differentiation. Using cultured primary OPCs, the findings of this thesis reveal that SCF+G-CSF can directly act on OPCs and promote the proliferation and differentiation of OPCs. The SCF+G-CSF-enhanced OPC proliferation is mediated by the NF-kB/cyclin D1 signaling pathway, and SCF+G-CSF promotes the differentiation of OPCs into oligodendrocytes in a time-dependent manner. These findings provide insights into SCF+G-CSF-enhanced remyelination during chronic TBI.

HEMATOPOIETIC GROWTH FACTORS PROMOTE THE PROLIFERATION AND DIFFERENTIATION OF OLIGODENDROCYTE PROGENITOR CELLS

by

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B.S., Harbin Normal University, 2019

Thesis
Submitted in partial fulfillment of the requirements for the degree of
Master of science in bioengineering.

Syracuse University
May 2023

ACKNOWLEDGEMENT

I would like to take this opportunity to express my sincere gratitude to the people who have helped me in completing my thesis.

First and foremost, I would like to thank my thesis supervisor, Dr. Li-Ru Zhao, for her unwavering support and guidance throughout my research in her lab. Her dedication, commitment, and encouragement have been integral to the successful completion of this thesis. Additionally, I am grateful for the financial support provided through her NIHR01 grant (R01NS118166), which allowed me to carry out all experiments for this thesis.

I am also deeply grateful to Michele Kyle, who provided me with her valuable feedback and support throughout all stages of my experiments.

Furthermore, I would like to recognize the contributions of Dr. Robert Gardner and Karen Hughes, whose support played an important role in enhancing the quality of my research in Dr. Zhao's lab.

I would also like to thank all the people who willingly took part in this research and provided me with their valuable insights and experiences.

Finally, I want to express my gratitude to my family, my boyfriend, and my dog for their constant love, companionship, and encouragement throughout my academic journey.

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CHAPTER 1

Introduction

Traumatic brain injury (TBI) is classified into mild, moderate, and severe TBI, depending on the severity of the injury caused by external force [1]. Globally, TBI is one of the leading causes of death; more than 10 million cases are reported each year, and that number is constantly rising [2]. TBI-related hospitalizations and deaths were 223,135 in 2019 and 64,362 in 2020 according to the data from the U.S Center for Disease Control and Prevention [3], of which 32 to 40% are classified as severe TBI [4]. Severe TBI causes lifelong disabilities, has a high rate of death, and increases the risk of developing dementia and neurodegenerative diseases [5], [6].

White matter is the area of the central nervous system (CNS) that is mainly made up of myelinated axons. White matter injury is the key pathology of TBI. The myelin sheath enwrapping axons is comprised of oligodendrocyte processes [7]. In rodent models of TBI, TBI-caused negative alterations in the CNS are associated with myelin loss and oligodendrocyte apoptosis [8]. One of the most common pathological changes in TBI patients is white matter degeneration which manifests as morphological changes in the white matter due to extensive axonal damage [9], [10]. It has been observed in rodent models of TBI that TBI-induced axonal injury and alterations in white matter structure include disorganization of myelin sheaths and reduction of myelin staining [11].

TBI-caused damages occur not only in the neurons but also happen in glial cells in the CNS, including microglia, astrocytes, oligodendrocytes, and oligodendrocyte progenitor cells (OPCs) [12]. OPCs are widespread in the brain parenchyma, both in the gray matter and white matter [13], [14]. The most distinctive function of OPCs is to proliferate and

differentiate into oligodendrocytes that generate myelin in the CNS [15]. Myelin is responsible for rapid impulse firing and axonal metabolic functions [16]. Loss of myelin can lead to axonal degeneration [17]. Severe TBI causes diffuse axonal injury and long-term neurological disability. TBI patients often process information slowly, resulting in delayed reaction times, poor attention and learning, and poor emotional adjustment [18]–[23]. Even though TBI causes catastrophic white matter damage, the repair of myelin damage due to remyelination by OPCs occurs spontaneously. Significantly, remyelination mitigates axonal lesions [24].

In humans, the renewal of oligodendrocytes and myelin continues for the entirety of adults' lives [25]–[27]. A small proportion of OPCs produced during development remains immature, and these OPCs maintain a slow proliferation rate in the adult CNS [28] and become activated in response to brain injury. In the setting of TBI, there is an active proliferation of OPCs in the corpus callosum. This process generates an increased number of OPCs recruited to the injured tissue where new axons require myelination [8]. In experimental TBI studies, it has been observed that the number of OPCs rose on days 2 and 7 but not on day 21 post-TBI [8], [29]. Thus, it is essential to develop treatments to promote the proliferation and differentiation of OPCs for enhancing remyelination and brain repair for severe TBI survivors.

Stem cell factor (SCF) is a potent costimulatory growth factor in hematopoiesis [30]–[32]. When combined with cytokines and other growth factors, SCF enhances the survival, proliferation, and differentiation of hematopoietic stem cells (HSCs) in a synergistic manner [33]–[37]. Known for being a powerful mobilizer of HSCs from the bone marrow into the blood and as a regulator of immune responses, granulocyte colony–stimulating factor (G-CSF) is a major extracellular regulator of hematopoiesis. In the immune system [38], [39], G-CSF is named for its relatively specific stimulation of the growth of neutrophil progeni-

tor cells; G-CSF influences the survival, proliferation, and differentiation of progenitor cells in the neutrophil lineage [40]. SCF and G-CSF have been demonstrated to be the essential hematopoietic growth factors that synergistically promote the proliferation and differentiation of HSCs and hematopoietic progenitor cells (HPCs) [41], [42]. Compared to G-CSF alone, the combination of SCF and G-CSF exhibits a synergistic effect in increasing the mobilization of HSCs/HPCs to peripheral blood [43]–[45]. Both SCF and G-CSF can pass through the blood-brain barrier in intact animals [46], [47], revealing the biological potential of SCF and G-CSF in regulating physiological function in the brain.

The evidence supporting the therapeutic potential of hematopoietic growth factors, SCF and G-CSF, in brain repair after TBI is mounting. It has been demonstrated that the combination of SCF and G-CSF (SCF+G-CSF) treatment enhances neurovascular regeneration and improve neurological function recovery in the chronic phase of experimental stroke [48]–[50]. In animal models of spinal cord injury, SCF+G-CSF treatment ameliorates white matter loss and increases the proliferation of OPCs after injury [51], [52]. In a mouse model of severe TBI, substantial evidence has revealed the efficacy of SCF+G-CSF treatment in enhancing brain repair. SCF+G-CSF treatment in the subacute phase of severe TBI promotes neural network remodeling, ameliorates post-traumatic anxiety and risk-taking behavior, attenuates TBI-induced neurodegeneration, and improves recovery of spatial learning and memory [53]. In the chronic phase of severe TBI, SCF+G-CSF treatment ameliorates the severe TBI-caused persistent impairments in spatial learning/memory and somatosensory-motor function, attenuates widespread neuropathology and microglial degeneration, and enhances regeneration of axons and dendrites [4], [54]. SCF+G-CSF treatment in the chronic phase of severe TBI also attenuates the TBI-caused persistent and progressive myelin loss and enhances remyelination in the white matter [4], [55]. The SCF+G-CSF-enhanced remyelination is positively correlated with the increased

proliferation of OPCs in the ipsilateral subventricular zone, suggesting a tight link between OPC proliferation and remyelination by SCF+G-CSF treatment in the chronic phase of severe TBI [55]. However, it remains unclear how SCF+G-CSF treatment regulates the proliferation and differentiation of OPCs.

The aim of this master thesis is to determine the efficacy and mechanism of SCF+G-CSF treatment in enhancing OPC proliferation and promoting the differentiation of OPCs into oligodendrocytes using primary culture of OPCs.

CHAPTER 2

Materials and Methods

2.1. Animals

All procedures of animal experiments were approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experimental design

To carry out experiments for determining the efficacy and mechanism of SCF+G-CSF on the proliferation and differentiation of OPCs, the isolated OPCs from the brains of postnatal mice were grown with OPC growth medium for 24h. Thereafter, the medium was completely changed (100% change) to either OPC growth medium for assessing OPC proliferation or OPC differentiation medium (i.e., without growth factors) for assessing OPC differentiation by SCF+G-CSF at different time points. SCF+G-CSF (20ng/ml each) (recombinant mouse SCF, Peprotech; recombinant human G-CSF, Cardinal Health) was added when the medium was completely changed. To determine the role of NF-κB signaling in mediating SCF+G-CSF-induced OPC proliferation, NF-κB signaling inhibitor (Bay11-7082, 2.5μM, Sigma-Aldrich) was added into the OPC growth medium 1 h before giving SCF+G-CSF. To examine the efficacy of SCF+G-CSF on OPC proliferation, 5-ethynyl-2'-deoxyuridine (EdU) (10ng/ml) (Thermo Fisher Scientific), a thymidine analogue for labeling proliferating cells, was added into the OPC growth medium 6h before ending the experiment (i.e., 18 h after giving SCF+G-CSF). To determine the efficacy of SCF+G-CSF on OPC differentiation, three experiments were carried out. The differentiated oligodendrocytes were assessed on day 3 (short-term study), day 7 (middle-term study), and day

11 (long-term study) after seeding OPCs.

2.3. The isolation and culture of oligodendrocyte progenitor cells

C57BL/6J mice (postnatal 5-7 days, The Jackson Laboratory) underwent cryoanesthesia and were decapitated. Their brains were removed quickly and placed in Hank's Balanced Salt Solution (Gibco). The brains were mechanically minced into 1-3mm pieces. The brain pieces were transferred to a C-tube containing an enzyme mixture (Neural Tissue dissociation kit, Miltenyi Biotec). The C-tubes were placed on aGentleMACS Octo Dissociator with Heaters (Miltenyi Biotec) for 20min. The cell suspension was filtered through a 70μM cell strainer and centrifuged at 300 x g for 10 min at 4 °C. To acquire purified OPCs, CD140a Microbeads (CD140a Microbeads Kit, Miltenyi Biotec) were used for magnetic-activated cell sorting according to the manufacturer's instructions. The sorted CD140a positive OPCs were flushed out of the column with D-PBS/BSA buffer (Miltenyi Biotec) and centrifuged at 300 x g for 10min at 4 °C. The CD140a positive cells were re-suspended in pre-warmed, CO_2 and O_2 pre-equilibrated OPC growth medium (i.e., OPC proliferation medium) containing DMEM high glucose medium (DMEM+ GlutaMAX, Gibco), 60µg/mL N-Acetyl cysteine (Sigma-Aldrich), 10μg/mL human recombinant insulin (Thermo Fisher Scientific), 1mM sodium pyruvate (Thermo Fisher Scientific), 50μg/mL apo-transferrin (Sigma-Aldrich), 16.1μg/mL putrescine (Sigma-Aldrich), 40ng/mL sodium selenite (Sigma-Aldrich), 60ng/mL progesterone (Sigma-Aldrich), 330μg/mL bovine serum albumin (Sigma-Aldrich) supplemented with platelet-derived growth factor (PDGF) (20ng/mL, Peprotech) and basic fibroblast growth factor (bFGF) (20ng/mL, Preprotech). The OPCs were seeded onto PDL-coated (100μg/ml, Sigma-Aldrich) Ibidi 8-well chamber μ-slides (ibidi) at 1x10^5 cells/well. The cell culture medium was completely exchanged (100%) 24 h after seeding OPCs, and the OPCs were then processed for the experiments to examine the efficacy and mechanism of SCF+G-CSF-induced OPC proliferation and differentiation.

To identify the purity of the isolated OPCs before culturing, the isolated OPCs were fixed with 4% paraformaldehyde in PBS (Biotium) for 15 min at room temperature. The OPCs were spun down for 30sec in a microfuge. Cell pellets were resuspended with 1ml of deionized water. After being spun down for 30sec in a microfuge, cell pellets were resuspended with 200μl of deionized water. The OPC suspension (5μl) was added to a Superfrost Plus Microscope Slide (3 spots/slide, Thermo Fisher Scientific) and smeared with the side of a pipette tip. After air-drying overnight at room temperature, the slides were processed for immunofluorescence staining (see the detailed methods in the next section). The quantification of NG2 (an OPC marker) positive cells in total isolated cells (DAPI positive cells) was used for determining the purity of isolated OPCs. In addition to the immunofluorescence staining, a flow cytometry method was also utilized to validate the purity of isolated OPCs. The isolated OPCs were washed in Dulbecco's Phosphate-Buffered Saline (D-PBS; Thermo Fisher Scientific) supplemented with bovine serum albumin (BSA; 1%, Miltenyi Biotec) and spun down at 300 x g for 10min at 4°C. The supernatant was aspirated, and the cell pellets were incubated with either isotype control PE-conjugated antibody (1:50, Miltenyi Biotec) or magnetic bead check PE-conjugated antibody (1:50, Miltenyi Biotec) diluted in 1% BSA in D-PBS for 15min at 4°C. After being washed 2 times with 1% BSA in D-PBS, the cell pellets were resuspended in 1% BSA in D-PBS, put into Fortessa cytometer (Becton Dickinson), and analyzed with FACSDiva software. Data processing was performed using Flow Jo v10. Single cells were selected for data analysis based on forward scatter area by forward scatter height. Isotype-stained controls were used for thresholding and identification of magnetically labeled OPCs. OPC purity was computed as the percentage of single cells positive for the magnetic label.

2.4. Proliferation and differentiation assays of oligodendrocyte progenitor cells

For proliferation assays, OPCs were cultured in the OPC growth medium with and without SCF+G-CSF (20ng/mL each) for 24 hours. EdU (10ng/mL), which labels the proliferating cells in the S phase of the cell cycle was added to the medium 6 hours before fixation. For immunofluorescence staining, the cultured OPCs were fixed with 4% paraformaldehyde in PBS (Biotium) for 15 min at room temperature. After washing with 3% BSA (IgG-free, protease-free, Jackson ImmunoResearch) in PBS, the cells were treated with 0.5% Triton X-100 permeabilization buffer (J.T. Baker Chemical Co.) for 20 min at room temperature. After rinsing with 3% BSA in PBS, the cells were treated with Reaction Cocktail (Click-Go EdU 488 Imaging Kit, Click Chemistry Tools) for 30 min in the dark at room temperature. After rinsing with 3% BSA in PBS, Wash Buffer, and PBS, the non-specific staining was blocked with 10% normal donkey serum (GeminiBio) diluted with 1% BSA and 0.25% Triton X-100 (J.T. Baker Chemical Co.) in PBS for 1 hour at room temperature. The OPCs were then incubated with primary antibody, rabbit anti-NG2 (1:300, Sigma-Aldrich), overnight at 4°C. The next day, the cells were rinsed with PBS 3 times and incubated with a secondary antibody, donkey anti-rabbit IgG conjugated with Alexa fluor 594 (1:400, Thermo Fisher Scientific), in the dark for 2 hours at room temperature. After rinsing with PBS, OPCs were stained with Hoechst 33342 (1:2000) in the dark for 30 min for DNA staining. Ki67 is a marker for cell proliferation as it is expressed throughout the active cell cycle. In addition to EdU chemical staining, OPCs were processed for immunofluorescence staining to detect Ki67 expression. After blockage of non-specific staining with 10% normal donkey serum, OPCs were incubated with mouse anti-Ki67 (1:300, Invitrogen) overnight at 4°C. The next day, OPCs were incubated with donkey anti-mouse IgG conjugated with Alexa fluor 488(1:400, Thermo Fisher Scientific). In the experiment

determining the molecular mechanism of SCF+G-CSF-regulated OPC proliferation, in addition to immunofluorescence staining for NG2, OPCs were also immunostained with primary antibody of mouse anti-cyclin D1(1:200, Invitrogen) followed by incubation with the secondary antibody of donkey anti-mouse IgG conjugated with Alexa fluor 488(1:400, Thermo Fisher Scientific). Except for EdU chemical staining, which required for Hoechst 33342 for DNA staining, DAPI (1:2000) staining was used for other immunofluorescence staining to visualize cell nuclei. OPCs were covered with Mounting Medium (ibidi) and imaged with an inverted confocal microscope (Leica SP8 STED) using a 40x objective lens. Images were captured 5 fields per well and analyzed with ImageJ software. The percentage of EdU/NG2 double positive cells, Ki67/NG2 double positive cells, and cyclin D1/NG2 double positive cells in total number of NG2 positive OPCs were quantified.

For differentiation assays, OPCs were cultured in the OPC differentiation medium which is similar to the OPC growth medium but without all growth factors. Every 4 days, 50% of well media was exchanged. OPCs were treated with medium alone as a control, triiodothyronine (T3, 40ng/mL, Sigma-Aldrich) as a positive control, or SCF+G-CSF (20ng/mL each) for 2, 6, and 10 days. At the end of the experiments, cells were fixed with 4% paraformaldehyde in PBS (Biotium) for 15 min at room temperature and rinsed with PBS three times. For blocking non-specific staining, cells were incubated with 10% normal donkey serum diluted with 1% BSA and 0.25% Triton X-100 in PBS for 1 hour at room temperature. The cells were then incubated with primary antibodies, rabbit anti-MBP (1:1000, Abcam), mouse anti-CC1 (1:500, Sigma-Aldrich), and goat anti-Olig2 (1:100, R&D Systems), overnight at 4°C. The next day, cells were rinsed with PBS and incubated with secondary antibodies, donkey anti-rabbit IgG conjugated with Alexa fluor 555 (1:400, Thermo Fisher Scientific), donkey anti-mouse IgG conjugated with Alexa fluor 488(1:400, Thermo Fisher Scientific), and donkey anti-goat IgG conjugated with Alexa fluor 647 (1:400, Thermo

Fisher Scientific), in the dark for 2 hours at room temperature. After rinsing with PBS, DAPI (1:2000) was added to the cells for nuclear counterstain. Cells in each well were covered with Mounting Medium (ibidi) and imaged with an inverted confocal microscope (Leica SP8 STED) using a 40x objective lens. Images were taken 5 fields per well and analyzed with ImageJ software. The percentage of CC1/Olig2 double positive oligodendrocytes and MBP/Olig2 double positive oligodendrocytes in the total number of Olig2 positive cells was quantified.

2.5. Statistical analysis

Data are presented as the mean± standard error of the mean (SEM). All statistical analyses were performed in GraphPad Prism (GraphPad Software, Inc.). Before performing data analysis, Shapiro-Wilk tests were performed to ensure the normality of the data. Unpaired Student's t-tests were used for two group comparisons. Two-tailed t-tests were used throughout, and statistical significance was defined as $p < 0.05$. Data of multiple group comparisons were analyzed using One-way ANOVA followed by Tukey's *post hoc* test and presented as significant differences when the p values reached statistical significance.

CHAPTER 3

Results

3.1. The purity of isolated primary OPCs

Prior to conducting the OPC experiments, the purity of the isolated OPCs was examined with two independent methods: immunofluorescence staining and flow cytometry. The experimental flowchart is presented in Figure 1A.

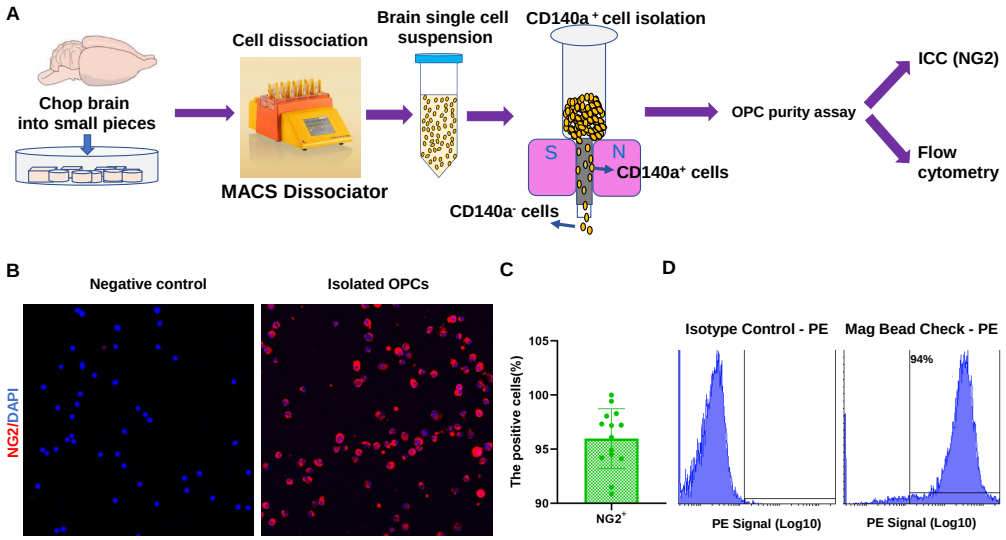

Figure 1: The purity of isolated oligodendrocyte progenitor cells (OPCs). (A) A schematic flowchart of OPC isolation and OPC purity assay. The brains of postnatal mice (postnatal days 5-7) were chopped into small pieces followed by dissociation of brain cells using a MACS dissociator. OPCs were isolated from the dissociated single cell suspension by magnetic-activated cell sorting using CD140a microbeads. CD140a-positive cells were retained and collected, while CD140a-negative cells passed through the magnetic device. The purity of OPCs was assessed using both immunofluorescence staining and flow cytometry. (B) Representative images show that the isolated OPCs express an OPC marker, NG2 (red). DAPI: nuclear counterstain. (C) The bar graph shows the percentage of $NG2^+$ cells in the isolated OPCs, which was 96%, indicating the high purity of the isolated OPCs. Sample size: n=8. (D) Flow cytometry data. Magnetic bead AB-labeled OPCs are 94% of the total of tested cells with clear separation from the isotype controls.

First, the purity of the isolated OPCs was examined with immunofluorescence staining us-

ing an OPC marker, NG2. As shown in Figure 1B and 1C, the percentage of NG2 positive cells was 96%, indicating the high purity of the isolated OPCs. To further validate the purity of the isolated OPCs, flow cytometry was utilized. The data of flow cytometry showed that magnetic bead AB-labeled OPCs were 94% of the total number of tested cells with clear separation from the isotype controls (Figure 1D), demonstrating the isolated primary OPCs with high purity.

The isolated OPCs with demonstrated high purity could be used for determining the efficacy and mechanism of SCF+G-CSF in governing the proliferation and differentiation of OPCs *in vitro*.

3.2. SCF+G-CSF promotes the proliferation of OPCs

To investigate the efficacy of SCF+G-CSF in promoting the proliferation of OPCs, OPCs were cultured in an OPC proliferation medium treated with or without SCF+G-CSF 24 hours (h) after seeding the isolated OPCs. The assessment of OPC proliferation was performed 24h after adding SCF+G-CSF into the medium. To detect proliferating OPCs, EdU was added 6h before the end of the experiment. The experimental flowchart is shown in Figure 2A.

The OPCs co-labeled with EdU and NG2 ($EdU^+/NG2^+$ cells) were considered proliferating OPCs. As shown in Figure 2B and C, the percentage of $EdU^+/NG2^+$ cells in the OPCs treated with SCF+G-CSF was significantly increased compared to the untreated OPCs ($p<0.01$), suggesting that SCF+G-CSF can promote the proliferation of OPCs.

To verify this finding, the proliferating OPCs were further identified using immunofluorescence double staining to detect Ki67 and NG2 ($Ki67^+/NG2^+$ cells) double positive OPCs (Figure 4A and 4B). Quantitative analysis of the $Ki67^+/NG2^+$ cells revealed a significant increase in the percentage of Ki67/NG2 double positive cells in the OPCs treated with

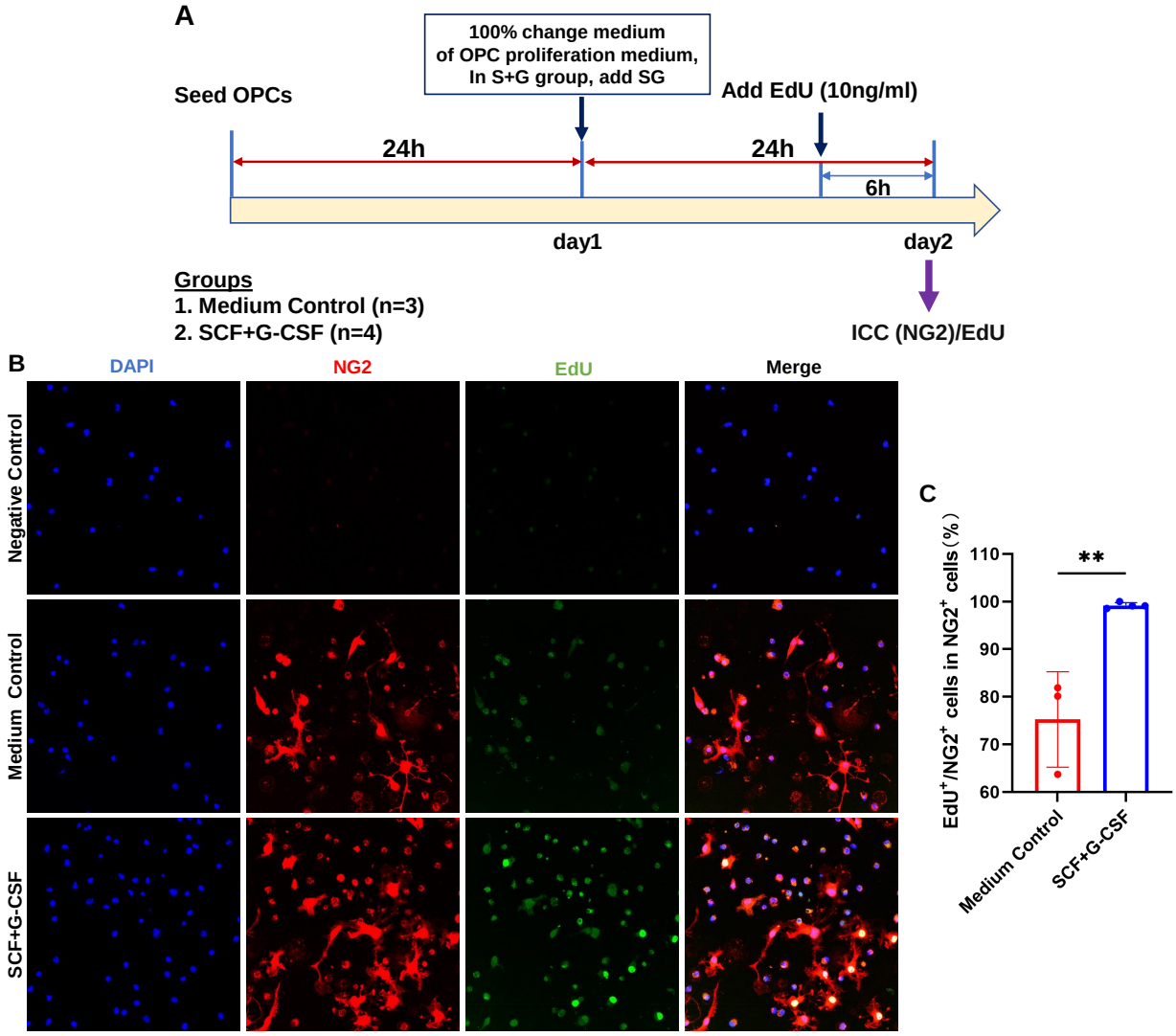

Figure 2: SCF+G-CSF treatment promotes the proliferation of oligodendrocyte progenitor cells (OPCs). (A) A schematic flowchart of the experimental design. The medium was 100% changed 24 hours (h) after seeding OPCs, and the OPCs in the SCF+G-CSF (S+G, SG) group were treated with SCF+G-CSF at the same time. EdU was added 6h before the end of the experiment. Immunocytochemistry (ICC) was performed at 24h post-treatment. (B) Representative confocal images of fluorescence chemistry for EdU and immunofluorescence staining for NG2 in cultured primary OPCs. (C) Quantitative data show that the percentage of proliferating OPCs ($EdU^+/NG2^+$ double positive cells) is increased by SCF+G-CSF. Unpaired Student's t-test. $^{**}p<0.01$, n=3-4.

SCF+G-CSF (SCF+G-CSF versus medium controls, $p<0.01$) (Figure 4C), indicating that SCF+G-CSF can enhance OPC proliferation.

NF-κB signaling plays a vital role in regulating the proliferation of OPCs [56]. To elucidate the molecular mechanism by which SCF+G-CSF promotes the proliferation of OPCs, the role of the NF-κB signaling pathway in SCF+G-CSF-enhanced OPC proliferation was determined through a series of experiments.

The design of the first experiment is shown in Figure 3A. In this experiment, OPCs were cultured in the OPC proliferation medium and treated with or without SCF+G-CSF. To block the NF-κB activation, Bay11-7082, an NF-κB signaling inhibitor, was added into the medium 1h before SCF+G-CSF. To label the proliferating OPCs, EdU was added into the medium 6h before ending the experiment. After being treated with/without SCF+G-CSF or SCF+G-CSF/NF-κB inhibitor for 24h, the proliferating OPCs were detected by the OPCs co-labeled with EdU and NG2 ($EdU^+/NG2^+$ cells) (Figure 3B). The quantification analysis revealed that the SCF+G-CSF-increased $EdU^+/NG2^+$ proliferating OPCs ($p<0.05$) were eliminated by the NF-κB inhibitor ($p<0.01$) (Figure 3C), suggesting that NF-κB activation is required for SCF+G-CSF-enhanced OPC proliferation.

To confirm these results, quantification of the $Ki67^+/NG2^+$ proliferating OPCs was performed after OPCs were treated with/without SCF+G-CSF or NF-κB inhibitor with SCF+G-CSF (Figure 4A). The findings were the same as observed in the experiment described above (Figure 3). OPCs treated with SCF+G-CSF exhibited significant increases in the percentage of $Ki67^+/NG2^+$ cells (Figure 4B and 4C) compared to those treated with both SCF+G-CSF and NF-κB inhibitor ($p<0.01$) as well as treated with the proliferation medium alone ($p<0.01$). These findings indicate that SCF+G-CSF enhances OPC proliferation through the activation of NF-κB signaling.

Cyclin D1 is a key regulator of cell proliferation. It has been proposed that OPC division is dependent on cyclin D1 [57], NF-κB is a direct transcriptional activator of cyclin D1, and NF-κB activation increases cyclin D1 expression to enhance cell proliferation [58]. To

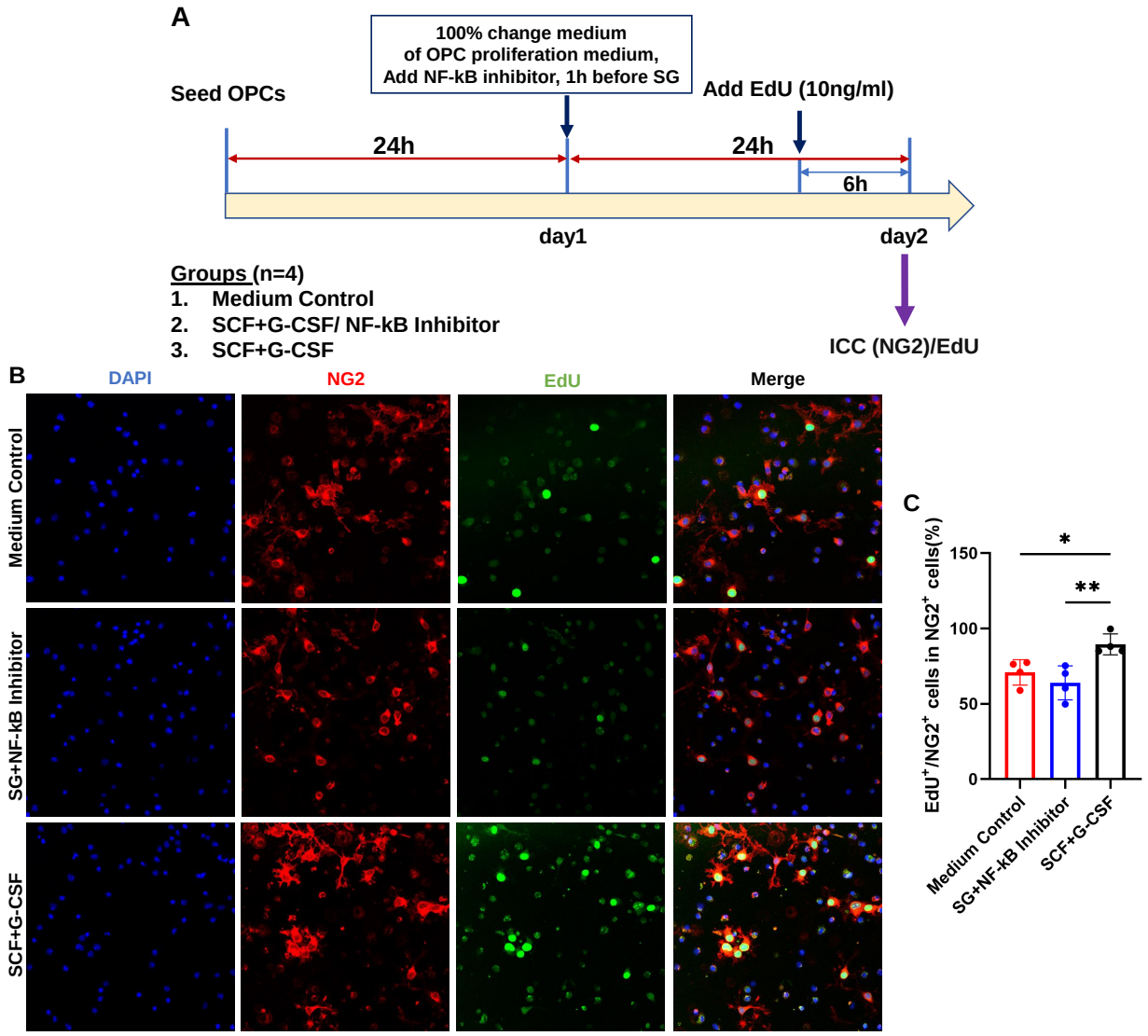

Figure 3: NF-κB inhibitor blocks the SCF+G-CSF-enhanced proliferation of oligodendrocyte progenitor cells (OPCs). (A) A schematic flowchart of the experimental design. The medium was 100% changed 24h after seeding OPCs. NF-κB inhibitor was added 1h before SCF+G-CSF (SG) treatment. EdU was added into the medium 6h before ending the experiment. Immunocytochemistry (ICC) was performed at the end of the experiment (i.e., 24h post-SG treatment). (B) Representative confocal images show the proliferating OPCs ($EdU^+/NG2^+$ double positive cells) in different experimental groups. (C) Quantitative data reveal that the SCF+G-CSF-increased $EdU^+/NG2^+$ proliferating OPCs are eliminated by NF-κB inhibitor. One-way ANOVA followed by Tukey *post hoc* test. *$p<0.05$, **$p<0.01$, n=4.

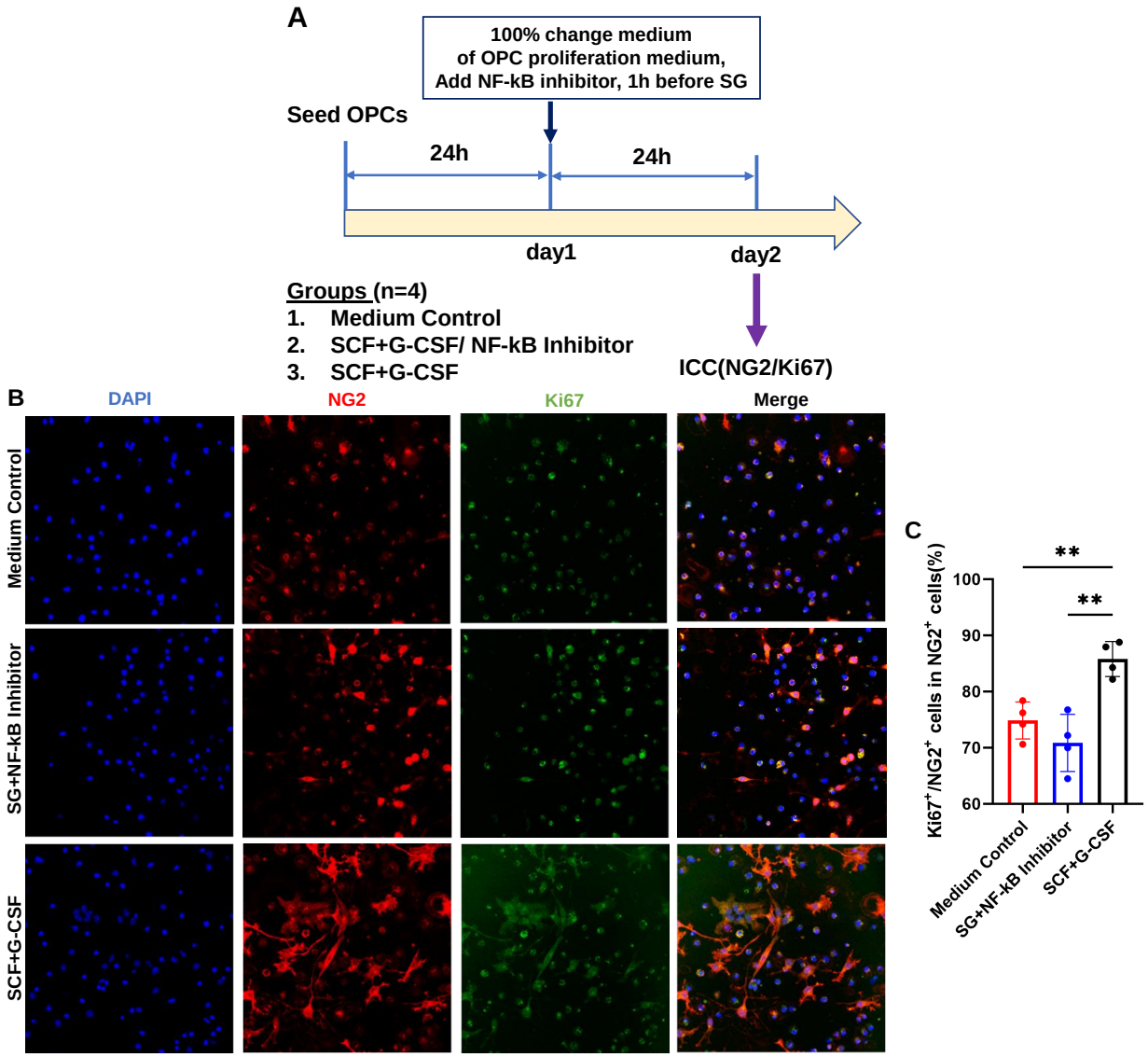

Figure 4: SCF+G-CSF promotes the proliferation of oligodendrocyte progenitor cells (OPCs) through NF-κB. (A) A schematic flowchart of the experimental design. When medium was 100% changed 24h after seeding OPCs, NF-κB inhibitor was added into the medium. The OPCs in SCF+G-CSF (SG) group were treated with SCF+G-CSF 1h after adding the NF-κB inhibitor, and immunocytochemistry (ICC) for detecting NG2 and Ki67 expression was performed 24h later. (B) Representative confocal images show the proliferating OPCs ($NG2^+/Ki67^+$ double positive cells) in different treatment groups. (C) Quantitative data display that the SCF+G-CSF-increased $Ki67^+/NG2^+$ proliferating OPCs are blocked by NF-κB inhibitor. One-way ANOVA followed by Tukey *post hoc* test. **$p<0.01$, n=4.

further determine whether cyclin D1 is the downstream regulator of NF-κB to mediate the SCF+G-CSF-enhanced OPC proliferation, similar to the other experiments shown in Figures 3A and 4A, NF-κB signaling inhibitor was added into the medium 1h before SCF+G-CSF, and cyclin D1 expression in NG2 positive OPCs was examined by immunofluorescence double staining (Figure 5A). The quantification analysis data uncovered that the OPCs treated with SCF+G-CSF showed significant increases in the percentage of cyclin $D1^+/NG2^+$ cells (Figure 5B and 5C) compared to the OPCs treated with both SCF+G-CSF and NF-κB inhibitor ($p<0.05$) as well as the OPCs treated with the proliferating medium alone ($p<0.01$). These findings suggest that NF-κB is the upstream regulator of cyclin D1 to mediate the SCF+G-CSF-enhanced OPC proliferation.

To further determine the efficacy and mechanism of SCF+G-CSF in supporting OPC growth, as in the other experiments shown in Figures 3A, 4A, and 5A, NF-κB inhibitor was added into the medium 1h before SCF+G-CSF, and NG2 expression area was assessed using immunofluorescence staining (Figure 6A). The quantification data showed that the percentage area of NG2 positive OPCs was significantly increased by SCF+G-CSF compared with the proliferating medium alone ($p<0.05$). NF-κB inhibitor completely blocked the SCF+G-CSF-increased NG2 expressing area ($p<0.01$). These findings indicate that SCF+G-CSF also promotes OPC growth through the activation of NF-κB signaling.

3.3. SCF+G-CSF promotes the differentiation of OPCs

A series of experiments were carried out to determine the efficacy of SCF+G-CSF in promoting the differentiation of OPCs. OPCs were cultured in OPC differentiation medium (i.e. OPC growth/proliferation medium without growth factors) with or without SCF+G-CSF. OPCs treated with T3 served as a positive control. After being treated with SCF+G-CSF or T3 for 2 days (the short-term experiment), 6 days (the middle-term experiment) and 10 days (the long-term experiment), differentiated oligodendrocytes were detected by

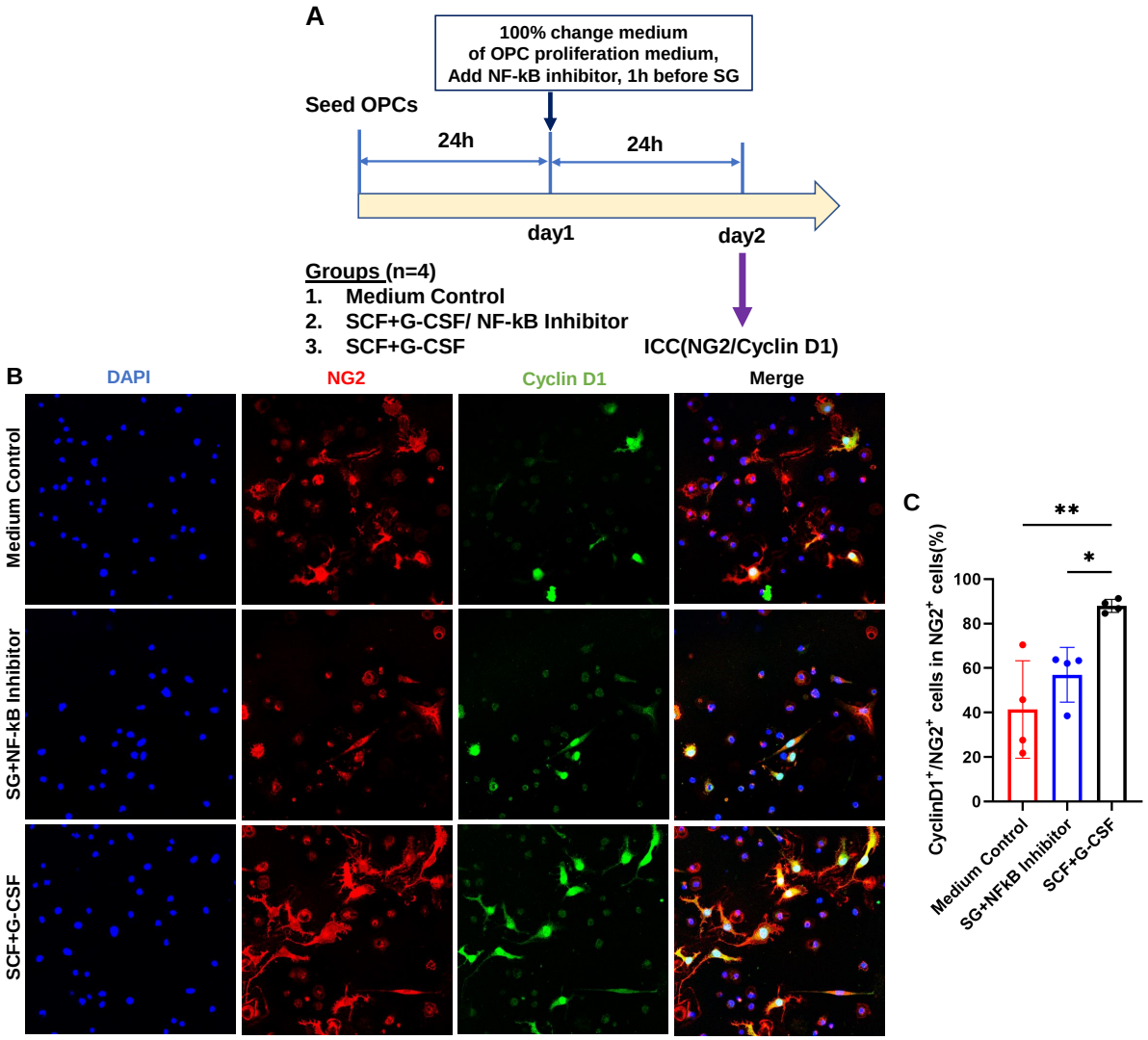

Figure 5: SCF+G-CSF-enhanced proliferation of oligodendrocyte progenitor cells (OPCs) is mediated by NF-κB/cyclin D1 pathway. (A) A schematic flowchart of the experimental design. When the medium was 100% changed 24h after seeding OPCs, NF-κB inhibitor was added into the medium. The OPCs in the SCF+G-CSF (SG) group were treated with SCF+G-CSF 1 h after adding the NF-κB inhibitor. Immunocytochemistry (ICC) for detecting cyclin D1 expression in NG2 positive OPCs was performed at 24h post-treatment. (B) Representative confocal images display the cyclin D1$^+$/NG2$^+$ double positive OPCs in different treatment groups. (C) Quantitative data reveal that SCF+G-CSF-increased cyclin D1$^+$/NG2$^+$ double-positive OPCs are eliminated by the NF-κB inhibitor. One-way ANOVA followed by Tukey *post hoc* test. *p$<$0.05, **p$<$0.01, n=4.

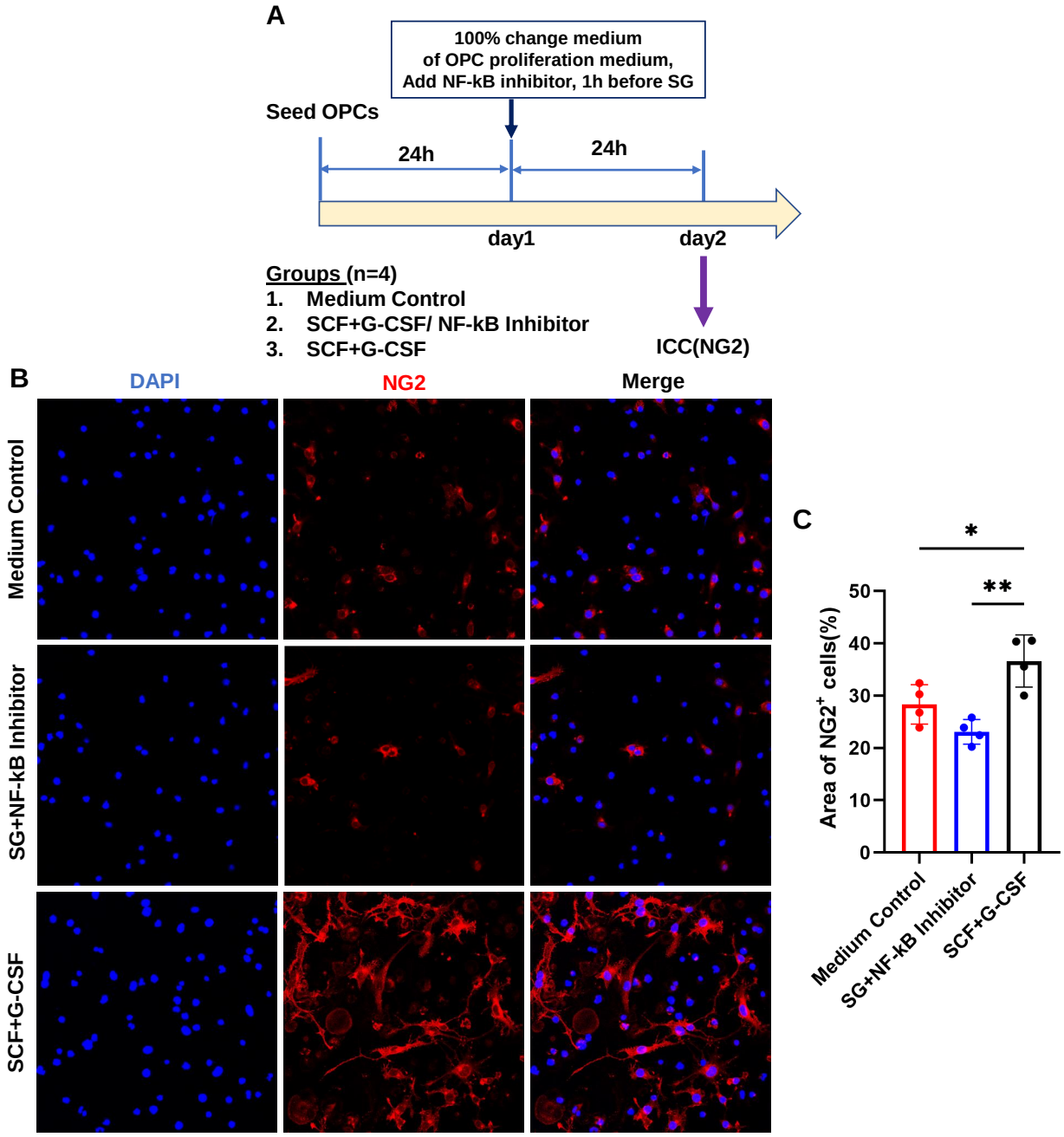

Figure 6: SCF+G-CSF enhances the growth of oligodendrocyte progenitor cells (OPCs) through the regulation of NF-κB. (A) A schematic flowchart of the experimental design. NF-κB inhibitor was added into the OPC proliferation medium when the medium was 100% changed 24h after seeding OPCs. The OPCs in the SCF+G-CSF (SG) group were treated with SCF+G-CSF 1h after adding NF-κB inhibitor. Immunocytochemistry (ICC) for detecting NG2 expression in cultured OPCs was performed at 24h post-treatment. (B) Representative confocal images show the expression of NG2 in OPCs in different treatment groups. (C) Quantitative data show that SCF+G-CSF-increased NG2 expression area in OPCs is blocked by NF-κB inhibitor. One-way ANOVA followed by Tukey *post hoc* test. *p$<$0.05, **p$<$0.01, n=4.

immunofluorescence staining. CC1 and MBP are the markers for oligodendrocytes while Olig2 is universally expressed in both oligodendrocytes and OPCs [59]. In the short-term, middle-term, and long-term experiments, the percentage of $CC1^+/Olig2^+$ and $MBP^+/Olig2^+$ oligodendrocytes in the total number of $Olig2^+$ cells was quantified at the end of each experiment.

In the short-term experiment (Figure 7A), after being treated with T3 or SCF+G-CSF for 2 days, only the T3-treated OPCs showed a significant increase in the percentage of $CC1^+/Olig2^+$ double positive oligodendrocytes compared to medium controls ($p<0.01$) and SCF+G-CSF treatment ($p<0.01$) (Figure 7B and 7C). This finding was further confirmed by analysis of $MBP^+/Olig2^+$ double positive oligodendrocytes. As expected, the percentage of $MBP^+/Olig2^+$ oligodendrocytes in T3-treated OPCs was significantly increased compared with medium controls ($p<0.05$) and SCF+G-CSF ($p<0.05$) (Figure 7D and 7E). These findings suggest that SCF+G-CSF does not affect OPC differentiation during this short-term treatment.

In the middle-term experiment (Figure 8A), following a 6-day treatment with T3 or SCF+G-CSF, the percentage of $CC1^+/Olig2^+$ oligodendrocytes in T3-treated OPCs was also significantly increased compared to medium controls ($p<0.001$). Additionally, SCF+G-CSF-treated OPCs showed a significant increase in the percentage of $CC1^+/Olig2^+$ oligodendrocytes compared to the medium controls ($p<0.05$) (Figure 8B and 8C). Consistent with this finding, the percentage of $MBP^+/Olig2^+$ oligodendrocytes in T3-treated OPCs was significantly increased compared with medium controls ($p<0.01$). The percentage of $MBP^+/Olig2^+$ oligodendrocytes was also significantly increased by SCF+G-CSF in comparison to the medium controls ($p<0.05$) (Figure 8D and 8E). These data suggest that SCF+G-CSF begins to promote the differentiation of OPCs into oligodendrocytes during the 6 days treatment.

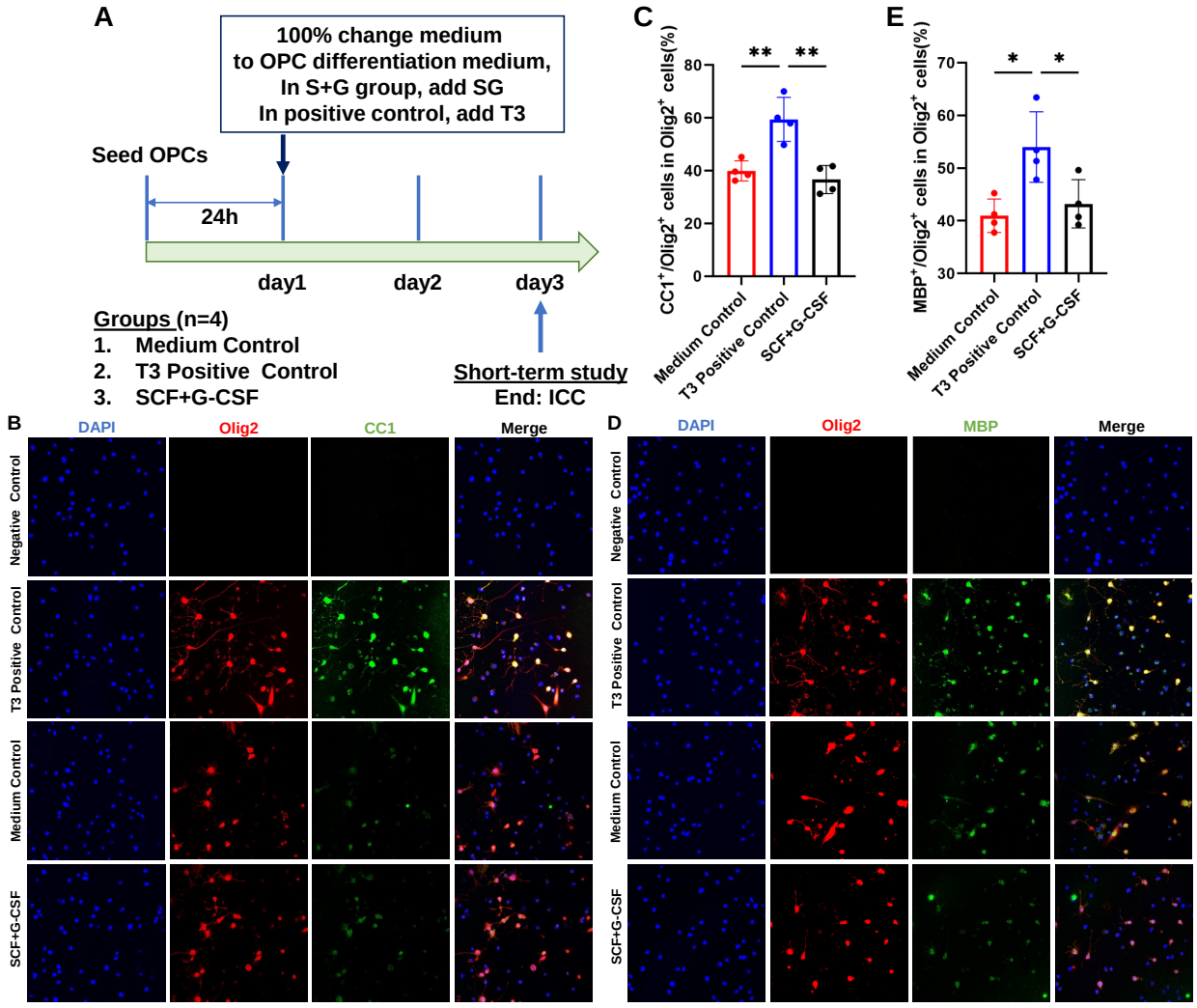

Figure 7: SCF+G-CSF has not promoted the differentiation of oligodendrocyte progenitor cells (OPCs) into oligodendrocytes in the short-term treatment. (A) A schematic flowchart of the experimental design for determining the effects of SCF+G-CSF in directing the differentiation of OPCs into oligodendrocytes in the short-term. The OPC growth medium was 100% changed to the OPC differentiation medium 24h after seeding OPCs. At this point, OPCs were treated with medium alone (control), T3 (positive control), and SCF+G-CSF (SG). Immunocytochemistry (ICC) staining for detecting OPC differentiation was performed at 2 days (short-term study) post-treatment. (B) Representative confocal images show the differentiated oligodendrocytes ($CC1^+/Olig2^+$ cells) 2 days after treatment. (C) Quantitative data show that only T3 (the positive control) but not SCF+G-CSF treatment increases $CC1^+/Olig2^+$ oligodendrocytes at 2 days post-treatment. (D) Representative confocal images show the differentiated oligodendrocytes co-expressing Olig2 and MBP at 2 days post-treatment. (E) Quantitative data reveal that OPCs treated with T3 but not SCF+G-CSF show increased $MBP^+/Olig2^+$ oligodendrocytes at 2 days post-treatment. One-way ANOVA followed by Tukey *post hoc* test. *$p<0.05$, **$p<0.01$, $n=4$

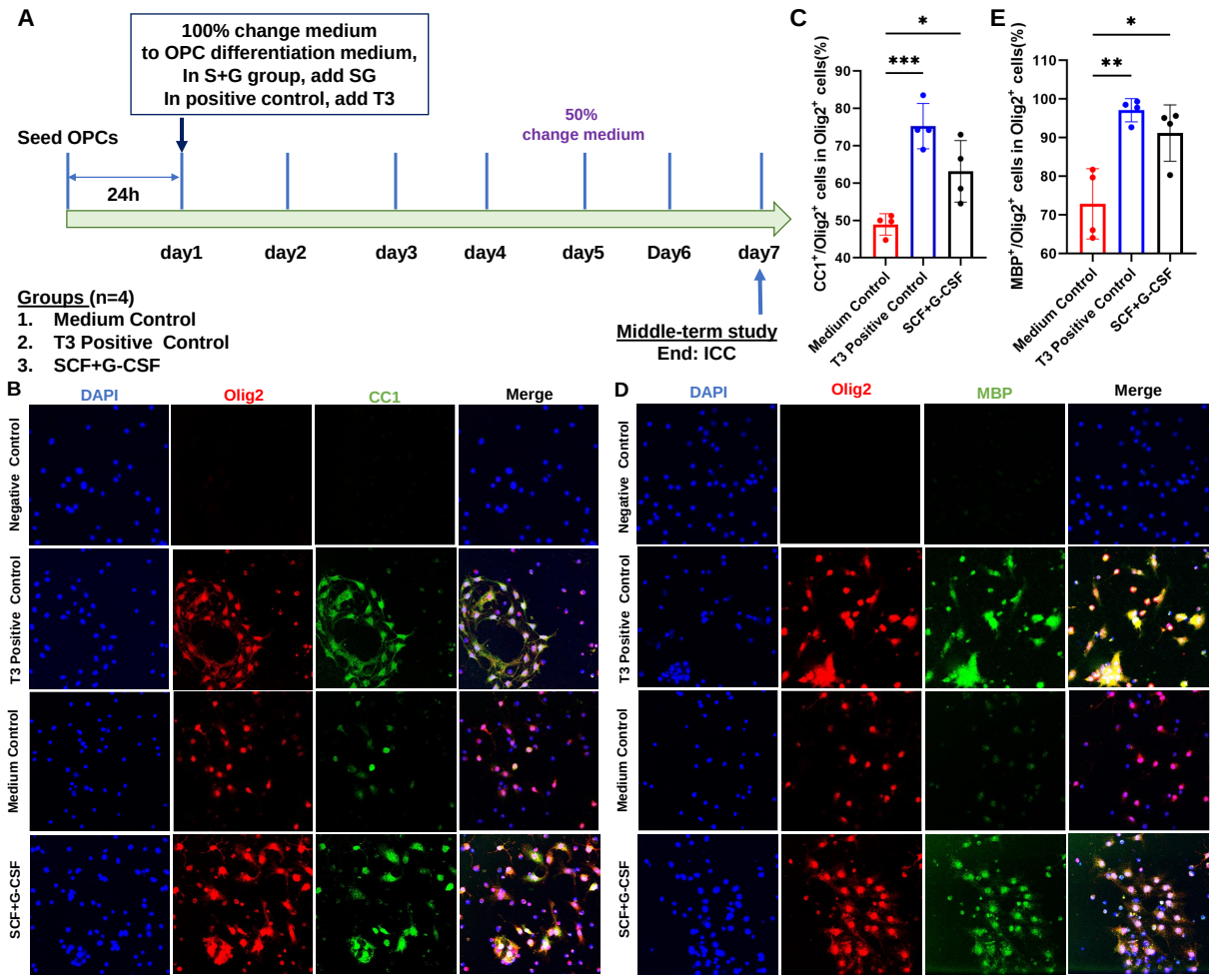

Figure 8: SCF+G-CSF shows increased differentiation of oligodendrocyte progenitor cells (OPCs) into oligodendrocytes in the middle-term treatment. (A) A schematic flowchart of the experimental design for determining the effects of SCF+G-CSF in directing the differentiation of OPCs into oligodendrocytes in the middle-term. The OPC growth medium was 100% changed to the OPC differentiation medium 24h after seeding OPCs. At this point, OPCs were treated with medium alone (control), T3 (positive control), and SCF+G-CSF (SG). Four days later, 50% of well media was exchanged. Immunocytochemistry (ICC) staining for detecting OPC differentiation was performed 6 days (middle-term study) after first treatment. (B) Representative confocal images display the differentiated oligodendrocytes ($CC1^+/Olig2^+$ cells) 6 days after first treatment. (C) Quantitative data reveal that OPCs treated with T3 as well as SCF+G-CSF show increased $CC1^+/Olig2^+$ oligodendrocytes at 6 days post-first treatment. (D) Representative confocal images show the differentiated oligodendrocytes ($MBP^+/Olig2^+$ cells) 6 days after first treatment. (E) Quantitative data of analyzing $MBP^+/Olig2^+$ oligodendrocytes confirm that both T3 and SCF+G-CSF increase the percentage of $MBP^+/Olig2^+$ oligodendrocytes 6 days after first treatment. One-way ANOVA followed by Tukey *post hoc* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, $n=4$.

In the long-term experiment (Figure 9A), after 10 days of treatments with T3 or SCF+G-CSF, the T3-treated OPCs still showed significant increases in the percentage of $CC1^+/Olig2^+$ oligodendrocytes compared to medium controls ($p<0.05$) (Figure 9B and 9C). Moreover, SCF+G-CSF also significantly increased the percentage of $CC1^+/Olig2^+$ oligodendrocytes compared to the medium controls ($p<0.05$) (Figure 9B and 9C). At this time point, $CC1^+/Olig2^+$ oligodendrocytes displayed more mature morphology with long and multiple processes in both T3 positive controls and SCF+G-CSF treatment (Figure 9B). Similar findings were also observed in the quantification of $MBP^+/Olig2^+$ oligodendrocytes. In T3 positive controls, the percentage of $MBP^+/Olig2^+$ oligodendrocytes was significantly increased compared to the medium controls ($p<0.01$) (Figure 9D and 9E). SCF+G-CSF also significantly increased the percentage of $MBP^+/Olig2^+$ oligodendrocytes compared to the medium controls ($p<0.05$) (Figure 9D and 9E). These results together with the findings of the short-term and middle-term experiments suggest that SCF+G-CSF promotes the differentiation of OPCs into oligodendrocytes in a time-dependent manner. The SCF+G-CSF-enhanced OPC differentiation primarily begins in the middle-term (i.e. 6 days of treatment) and continues during the long-term treatment (i.e.10 days of treatment).

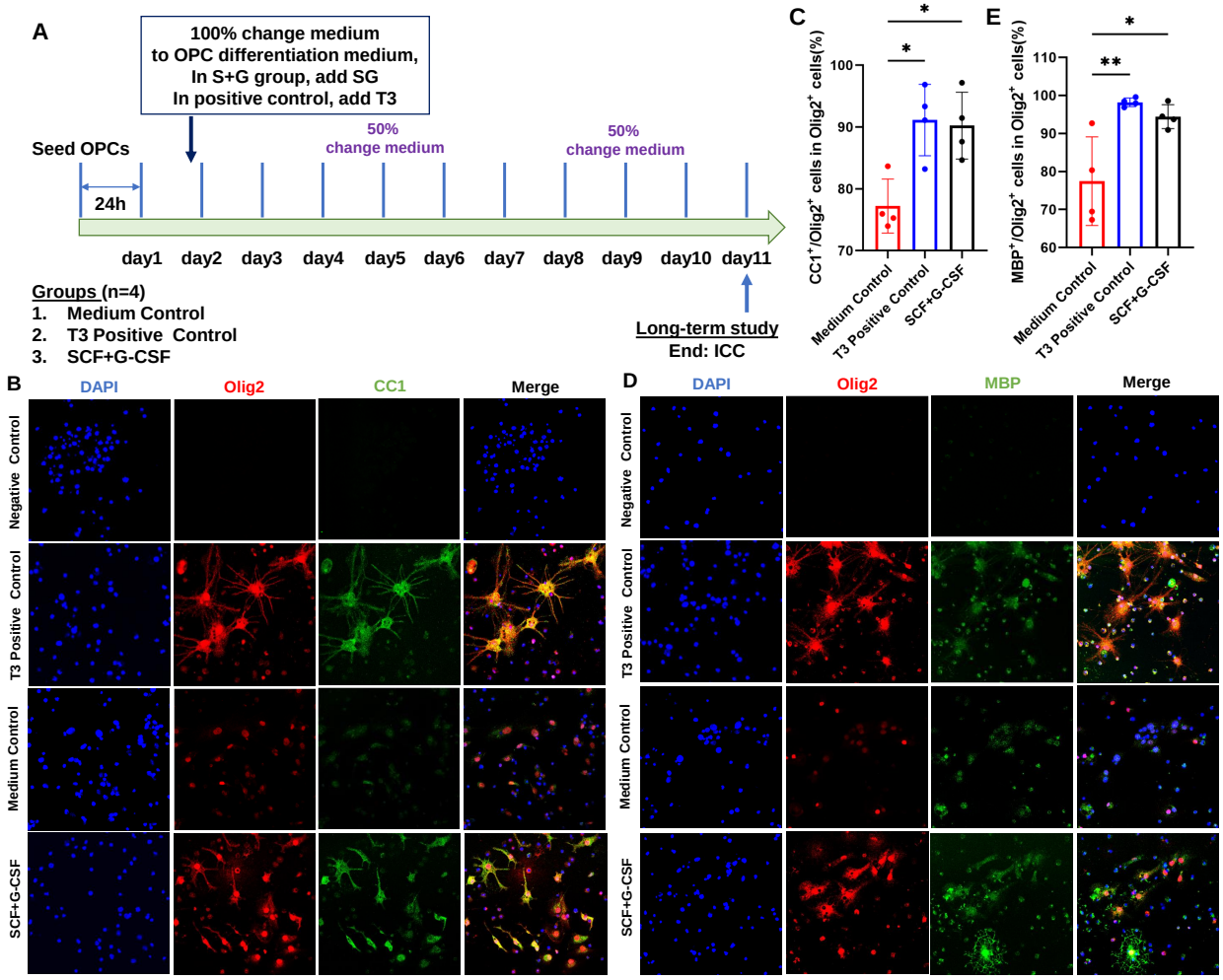

Figure 9: SCF+G-CSF robustly enhances the differentiation of oligodendrocyte progenitor cell (OPCs) into oligodendrocytes in the long-term treatment. (A) A schematic flowchart of the experimental design for determining the effects of SCF+G-CSF in directing the differentiation of OPCs into oligodendrocytes in the long-term. The OPC growth medium was 100% changed to the OPC differentiation medium 24h after seeding OPCs. At this point, OPCs were treated with medium alone (control), T3 (positive control), and SCF+G-CSF (SG). Every 4 days, 50% of well media was exchanged. Immunocytochemistry (ICC) staining for detecting OPC differentiation was performed at 10 days (long-term study) post-first treatment. (B) Representative confocal images show the differentiated oligodendrocytes ($CC1^+/Olig2^+$ cells)10 days after first treatment. (C) Quantitative data reveal that SCF+G-CSF robustly increases the percentage of $CC1^+/Olig2^+$ oligodendrocytes at 10 days post-first treatment, with no difference when compared to T3 treatment. The morphology of the differentiated oligodendrocytes becomes more mature with long and multiple processes at this timepoint. (D) Representative confocal images show the differentiated oligodendrocytes co-expressing MBP and Olig2 10 days after first treatment. (E) Quantitative data from the analysis of $MBP^+/Olig2^+$ oligodendrocytes in different treatment groups. Note that the highly increased $MBP^+/Olig2^+$ oligodendrocytes are seen in SCF+G-CSF-treated OPCs, which has no difference in comparison to the T3 treatment at 10 days post-first treatment. One-way ANOVA followed by Tukey *post hoc* test. *$p<0.05$, **$p<0.01$, $n=4$.

CHAPTER 4

Discussion

This study aimed to determine the efficacy and mechanism of SCF+G-CSF in promoting the proliferation and differentiation of oligodendrocyte progenitor cells (OPCs) *in vitro*. The findings of this study reveal that (1) SCF+G-CSF enhances OPC proliferation and growth, (2) NF-κB/cyclin D1 signaling pathway and NF-kB activation are required for the SCF+G-CSF-enhanced OPC proliferation and growth, respectively, and (3) SCF+G-CSF promotes the differentiation of OPCs into oligodendrocytes in a time-dependent manner. These data demonstrate a dual effect of SCF+G-CSF in governing both the proliferation and differentiation of OPCs.

SCF and G-CSF are essential hematopoietic growth factors, and SCF in combination with G-CSF (SCF+G-CSF) synergistically enhances the proliferation and differentiation of HSCs and HPCs [41], [42]. However, the regulative role of hematopoietic growth factors on OPCs remains poorly understood. The findings of this study, showing that SCF+G-CSF can direct the proliferation and differentiation of OPCs, provide important evidence supporting the new function of SCF+G-CSF in regulating OPC proliferation and differentiation.

White matter demyelination due to the loss of oligodendrocytes represents a crucial neuropathology of TBI. In a severe TBI mouse model, SCF+G-CSF treatment in the chronic phase of TBI enhances remyelination in the white matter of the hemisphere with injury [4], [55], increases OPC proliferation in the ipsilateral subventricular zone (SVZ) [55], and attenuates widespread neuropathology and microglial degeneration [4], [54]. Additionally, the SCF+G-CSF-enhanced remyelination shows a positive correlation with the increased

proliferation of OPCs in the ipsilateral SVZ. In addition to the findings of TBI studies, a study using a mouse model of spinal cord injury has also revealed that SCF+G-CSF treatment leads to active proliferation of intrinsic OPCs [51]. However, in these *in vivo* studies, it would be difficult to distinguish whether the SCF+G-CSF-increased OPC proliferation is the result of a direct effect of SCF+G-CSF on OPCs or an indirect effect of SCF+G-CSF-inducing a better microenvironment *in vivo*. The findings of this *in vitro* study provide the primary evidence to demonstrate that SCF+G-CSF can directly act on OPCs to enhance OPC proliferation and differentiation.

This study reveals that the SCF+G-CSF-enhanced OPC proliferation and growth is mediated by NF-κB. NF-κB is a transcription factor that plays a key role in the regulation of cell proliferation and growth [60]. It has been demonstrated that SCF+G-CSF synergistically increases NF-κB activation, which is required for SCF+G-CSF-enhanced neurite outgrowth [61]. The current study extends the knowledge by demonstrating the regulative role of NF-κB in mediating SCF+G-CSF-enhanced growth of OPCs. Cyclin D1 is a key regulator of cell proliferation. NF-κB has been shown to be a direct transcriptional activator of cyclin D1, and NF-κB activation increases cyclin D1 expression, resulting in G1-to S-phase transition and increased cell proliferation [58], [62]. The findings of the present study reveal that SCF+G-CSF increases cyclin D1 expression in OPCs, while NF-κB inhibitor eliminates the SCF+G-CSF-enhanced cyclin D1 expression in OPCs and blocks the SCF+G-CSF-enhanced OPC proliferation. These data indicate that cyclin D1 is the downstream regulator of NF-κB that mediates the SCF+G-CSF-enhanced OPC proliferation.

The precise mechanism by which SCF+G-CSF promotes OPC differentiation remains unknown. Previous studies have suggested that SCF+G-CSF may promote OPC differentiation through the activation of different signaling pathways. It has been revealed that

SCF+G-CSF promotes the differentiation of neural stem cells (NSCs) into neurons through the activity of neurogenin 1 [63]. *in vitro* studies have demonstrated that SCF+G-CSF synergistically enhance neurite extension through the PI3K/AKT/NF-κB pathway [64] as well as the MEK/ERK/p53 signaling pathway [65]. Both *in vivo* [66] and *in vitro* [67] studies have shown that the PI3K/AKT/mTOR pathway is involved in OPC differentiation and myelin formation [67]–[69]. In addition, it has been reported that ERK1/2 is a mediator of insulin-like growth factor 1-enhanced differentiation of NSCs into oligodendrocytes [70]. Together, these studies suggest that PI3K/AKT/NF-κB, PI3K/AKT/mTOR, and MEK/ERK/p53 pathways could be involved in the regulation of OPC differentiation by SCF+G-CSF. However, further studies are needed to identify and characterize these pathways in SCF+G-CSF-enhanced OPC differentiation.

Overall, this *in vitro* study provides primary evidence showing that SCF+G-CSF can directly act on OPCs and enhance OPC proliferation, growth, and differentiation into oligodendrocytes. NF-κB and cyclin D1 are involved in SCF+G-CSF-enhanced OPC proliferation and growth. These findings provide an insightful understanding of SCF+G-CSF-enhanced remyelination in chronic TBI. The findings of this study imply that SCF+G-CSF may have therapeutic potential for treating CNS demyelinating diseases such as multiple sclerosis, where enhancing remyelination is critical for improving recovery. Further studies are needed to fully elucidate the molecular mechanisms underlying SCF+G-CSF-enhanced OPC proliferation and differentiation and to examine the underlying mechanisms *in vivo*.

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