Transplantation of Wnt4-modified Neural Stem Cells Mediate M2 Polarization to Promote Spinal Cord Injury Repair

Xiang Li

Sun Yat-sen University First Affiliated Hospital

Lingli Long

Sun Yat-sen University First Affiliated Hospital

Yue Hu

Sun Yat-sen University First Affiliated Hospital

Wenwu Zhang

Sun Yat-sen University First Affiliated Hospital

Fangling Zhong

Sun Yat-sen University First Affiliated Hospital

Ying Tuo

Sun Yat-sen University First Affiliated Hospital

Xing Cheng

Sun Yat-sen University First Affiliated Hospital

Yong Wan (✉ wanyong@mail.sysu.edu.cn)

Sun Yat-sen University First Affiliated Hospital <https://orcid.org/0000-0002-1008-8072>

Research

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Abstract

Background: Neural stem cells (NSCs) transplantation has been considered as a potential strategy to reconnect the neural circuit after spinal cord injury (SCI) but the therapeutic effect was still unsatisfied because of the poor inflammatory microenvironment. Wnt4 has been considered to be neurogenesis and anti-inflammatory so that it would be an essential assistant agent for NSCs transplantation. To explore interaction between Wnt4-modified NSCs and macrophages; and the effect of Wnt4-modified NSCs on the inflammatory microenvironment of SCI is relevant for targeted and effective treatments that promote injured spinal cord repair.

Methods: *In vitro* NSCs-macrophages co-cultured system was established to unravel the interaction and involved mechanism between Wnt4-modified NSCs and macrophages. Wnt4-modified NSCs were transplanted into SCI model to confirm the effect of Wnt4-modified NSCs on modulation of inflammatory microenvironment of SCI and the therapeutic effect of Wnt4-modified NSCs on SCI.

Results: Wnt4-modified NSCs induce M2 polarization and inhibit M1 polarization of macrophages through suppress TLR4/NF- κ B signal pathway; furthermore, M2 cells promote neuronal differentiation of NSCs through MAPK/JNK signal pathway. *In vivo*, transplantation of Wnt4-modified NSCs improve inflammatory microenvironment through induce M2 polarization and inhibit M1 polarization of macrophages to promote axonal regeneration and tissue repair.

Conclusions: Transplantation of Wnt4-modified NSCs effectively improve the inflammatory microenvironment through inducing M2 polarization and suppressing M1 polarization of macrophages after SCI. Considering these positive therapeutic effects, Wnt4 may have remarkable potential to be optimal assistant agent in NSCs transplantation for SCI.

Introduction

Traumatic injury of the spinal cord disrupts the descending and ascending axonal tracts and interrupts the communication between the brain and body, leading to the loss of motor, sensory and autonomic functions [1–3]. Although the incidence of spinal cord injury (SCI) is relatively low, it often results in disastrous emotional, social and economic impacts on the injured person and their family, community, even society [3]. To date, there is still no sufficient therapies for SCI and the only therapeutic option for SCI patients is physical rehabilitation [4, 5]. Reconstruction of the damaged neural circuits was once considered to be hopeless as the adult mammalian central nervous system has very poor ability to regenerate [2, 6]. Cell transplantation has been considered as a potential strategy to reconnect the neural circuit after SCI [1, 2, 7]. Several studies have reported that Neural stem cells (NSCs) transplantation results in partial repairing due to the neuronal differentiation of NSCs [7]. However, most of exogenous NSCs at the lesion site differentiated into astrocytes, which are to the disadvantage of spinal cord repairing, rather than Neuron [6, 8]. Thus, the outcome of NSCs transplantation was not satisfied.

Following transplantation of NSCs, the transplanted cells were considered to mediate functional improvements following SCI through a variety of mechanisms, including neuroprotection and inflammatory immunomodulation [2]. Many repair mechanisms rely on beneficial aspects of inflammation [9–11], yet certain aspects of inflammation lead to damage to health tissue. Macrophages/microglia lineages play crucial roles in the pathophysiology of many repair including SCI [12, 13]. Macrophages/microglia after injury are organized on a continuum of phenotypes and these phenotypes have been defined as two major categories of proinflammatory M1 cells and anti-inflammatory M2 cells according to their primary functions [14, 15]. Both these cells represent the extreme activation states of monocyte and macrophages lineages [15]. M1 cells initiate the inflammation by releasing proinflammatory cytokines, nitric oxide and reactive oxygen species that lead to glial scar formation, neuronal cell apoptosis and causing activation of astrocytes. M2 response to promote tissue repair by secreting anti-inflammatory cytokines to enhance axonal growth and promote the proliferation and differentiation of oligodendrocyte progenitor cells [15–17]. In fact, both M1 and M2 are important in the whole inflammatory response respectively. However, in SCI, it has been demonstrated that the lesion site of spinal cord is continuously filled with M1 cells and these cells cannot switch to M2 cells that leading to irreversible tissue impairing [17]. Thus, it is important to elevate the ratio of M2 in the microenvironment to promote tissue repair in SCI treatment. Previous studies reported that cell transplantation can improve the immune response by increasing anti-inflammatory cytokines and reducing proinflammatory cytokines after SCI [12]. However, the involving mechanism is still unclear. Still, few studies investigated the direct interactions between transplanted cells and immune cells; instead, they measure global changes in cytokine release or the abundance of immune cells after SCI [18, 19].

Recently, lot of wingless-type MMTV integration site family (Wnt) proteins such as Wnt4, Wnt5a, Wnt7b, has gain growing attention as an attractive factor for neural differentiation and anti-inflammation [20]. Wnt4 has been considered to has dual role of neuronal differentiation and anti-inflammation and has been prove to be an optimal assistant agent in cell transplantation after SCI [21, 22]. Wnt4-modified NSCs has been considered to promote neuronal differentiation and functional recovery in SCI model according to our previous studies [22]. However, the effect and relative mechanism of Wnt4-modified NSCs on monocyte/macrophages niche and immune response in inflammatory microenvironment of SCI is still elusive.

In the current study, we investigated the effect and underlying mechanism of Wnt4-modified NSCs on macrophages in inflammatory microenvironment, the potential application of Wnt4-modified NSCs transplantation in SCI, that may provide useful information for translational application of NSCs transplantation.

Method And Material

NSCs isolation and culturing

NSCs cultures were obtained from the fetal brains of embryonic day 14 rats, which were extracted from pregnant Sprague-Dawley (SD) rats (Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) and identify as previously described [22, 23]. Briefly, the brain tissue was mechanically dissected and dissociated in Hanks Balanced Salt Solution, and the cell suspension was centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was diluted to a single-cell suspension. NSCs were plated on a T25 culture flask (Corning, Acton, MA) containing Dulbecco modified eagle medium (DMEM) /F-12 nutrient mixture, 2% B27, 1% penicillin/streptomycin, 1% l-glutamine (Gibco, Grand Island, NY), 20 ng/mL fibroblast growth factor-2 (FGF-2) and 20 ng/mL epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ). NSCs were cultured at 37°C in 5% CO₂ and were passaged via weekly digestion with accutase (Millipore, Bedford, MA) in the medium described above. All NSCs used in this study were between passages 2 and 4.

To induce neural differentiation, cells were plated at a density of 2×10^5 cells/well in 6- or 12-well tissue-culture plates and allowed to adhere for 24 h at 37°C, at which time cells were switch to neural differentiated medium consisting of basic medium supplemented with 2% B27, 1% penicillin/streptomycin, 1% l-glutamine. The medium was changed every 2-3 days.

Monocytes were isolated from the femurs of adult SD rats (Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) as previously described [24]. To induce M1 and M2 polarization of macrophage, cells were plated at a density of 2×10^5 cells/well in 6- or 12-well tissue-culture plates and allowed to adhere for 24 h at 37°C, at which time cells were switch to polarization medium consisting of basic medium supplemented with LPS (10µg/ml) for M1 and IL-4 (10ng/ml) for M2 polarization (Fig. S1a to c) [25, 26].

Lentiviral vector construction

The lentiviral vectors carrying green fluorescent protein (GFP) and a sequence that specifically overexpression the Wnt4. Lentiviruses carrying GFP and Wnt4 gene overexpression were constructed as previously described [22] (Fig. S2a). Briefly, the full-length Rat Wnt4 gene was encoded into vectors. The vectors and corresponding packaging plasmids were co-transfected into 293T cells using lipofectamine 2000 (Invitrogen, Camarillo, CA). The medium was changed with complete medium after 8 hours of incubation. After 48 hours, the supernatant was harvested from 293 T cells, filtered using a 0.45-mm pore size filter, and concentrated via ultracentrifugation at 96,500g for 2 hours at 4 °C. After resuspension, the serially diluted lentivirus was used to transduce 293T cells. Then, 4 days later, the labeled 293T cells were counted to calculate the viral titer, and high-titer recombinant lentiviral vectors carrying Wnt4 were harvested.

Surgical Procedures and Cell Transplantation

Adult female Sprague-Dawley (SD) rats (weighing 200-220 g, supplied by the Experimental Animal Center of Sun Yat-sen University, Guangzhou, China) were divided into four groups for this study: Sham group (n = 10), SCI group (n = 10), NSC^{vector} group (n = 10), and NSC^{Wnt4} group (n = 10). After 72 hours of

lentiviral transfection, NSCs were collected for transplantation. Briefly, animals were anesthetized with 1% pentobarbital sodium (40-45mg/kg), and the spinal cord was exposed at the 10th thoracic vertebral level (T10) via laminectomy. The animals in the sham group underwent spinal cord exposure but no injury. In the other three groups, the exposed spinal cord was contused with a weight-drop device by dropping a 10g rod from a precalibrated height of 12.5 mm [27]. Once the bleeding was stopped, 5µl NSCs were implanted at a density of 1×10^5 cells/µl to the rostral and caudal of injured site using microsyringe [28, 29]. The T8-T11 spinal cord segments were dissected at 8 weeks after SCI and NSCs transplantation.

Hindlimb locomotor scale

The 22-point (0-21) Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale was used to assess hindlimb locomotor function, including joint movements, stepping ability, coordination, and trunk stability. 21 score indicates unimpaired locomotion as observed in sham rats. All animals underwent behavioural testing, and the duration of each session was 5 minutes per rat. Finally, an overall score was calculated. The evaluation was performed by observers who were blinded to the treatment group of the tested animals [30].

Footprint analysis

Hindlimb behavior and motor coordination were assessed at 8 weeks post-injury. The fore limbs and hind limbs were coated with different colors dyes, and the rats of different groups were placed on a 10 cm x 100 cm runway covered with paper. The rats were encouraged to run straight so that the gait of the rats in different groups could be obtained and assessed. The footprint pattern was then digitalized and representative pictures were shown to assess coordination [31].

Spinal Cord–Evoked Potential (SCEP) Recording

At 8 weeks post-injury, total 20 rats (n = 5 in the sham group and n = 5 in each cell-transplanted group) were anesthetized with 1% pentobarbital sodium (40-45mg/kg) and stereotactically fixed. The T5-L1 vertebrae were completely exposed. Briefly, the stimulation electrode was inserted into the T5-T6 interspinous ligaments, and a pair of needle electrodes was inserted into the interspinous ligaments of T12-L1 for SCEP recording. Then, the electrodes were connected to a BL-420 Biological Function Experiment System (Taimeng, Chengdu, China). The variables of the SCEP signals were set according to previous reports as follows: gain of 2000 time constant of 0.01 seconds, and filtering at 300 Hz. To elicit a SCEP, a single pulse stimulation (50 ms in duration at a frequency of 5.1 Hz and a voltage increase of 1 mV) was transmitted through the electrodes until a mild twitch of the vertebral body of the animal was observed. One hundred SCEP responses were averaged for each rat to obtain high-quality waveforms for the SCEP signals [32].

Histological analysis

At 8 weeks after SCI, all rats were deeply anesthetized with an adequate dose of 10% chloral hydrate (5 mL/kg) and were transcranial perfused with 250 mL of 0.9% normal saline. Animals were perfused with 300 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS, pH 7.4). The T8-T11 cord segments were dissected based on the dorsal spinal root count, post fixed overnight in 4% PFA, and soaked at 4°C overnight in 10% sucrose followed by 30% sucrose. The specimens were embedded in optimal cutting temperature compound, frozen at -20°C and sliced at a thickness of 20 µm in the longitudinal or transverse plane.

To visualize the cavity area, animals (n = 5 per group) were killed for haematoxylin-eosin (HE) staining. The T8-T11 longitudinal spinal cord sections from each group were stained with HE according to standard protocols and observed under bright field microscope [22, 32].

For neuron counting, animals (n = 5 per group) were killed, and transverse sections of the injured spinal cord were used to stain neurons with Nissl. Sections at 2 mm rostral and caudal to the lesion epicentre were counted for each rat. The numbers of positively stained cells were counted and averaged per section in a blinded manner [22, 32].

For axonal tract tracing, dorsal laminectomy was performed at T12 and Fluorogold (FG, Biotium, Fremont, CA) was injected into the spinal cord at seven weeks after operation. One week after injection, the animals were perfused and T8 segment of the spinal cord were removed, cryopreserved, embedded in OCT compound, and sliced into 10µm frozen sections. A fluorescence microscope (Olympus, Tokyo, Japan) was used to detect FG-labeled neurons [28, 29].

Statistical analysis

Data obtained from experiments in triplicate and repeated at least three times was represented as mean ± SD. Statistical evaluations was analyzed via one-way analysis of variance (ANOVA) with Levene's homogeneity of variance test and, subsequently, Bonferroni's post hoc test or Dunnett's T3 post hoc test based on the comparison to be made and the statistical indication of each test. The analyses were performed using Statistical Package for Social Sciences, Version 16.0 for Windows (SPSS, Chicago, IL, USA). A statistical probability of $p < 0.05$ was considered significant.

Result

Wnt4-modified NSCs promote M2 polarization of macrophages

To investigate the interaction of NSCs and macrophages, macrophages were co-cultured with wild type NSCs or Wnt4-modified NSCs to establish the co-culture system. PCR and WB results showed that the M2 relative makers including CD163 and CD206 were increased in macrophages co-cultured with wild type NSCs and further increased in macrophages co-cultured with Wnt4-modified NSCs at mRNA and protein levels. In contrast, M1 makers CD68 were decreased in macrophages co-cultured with wild type NSCs or Wnt4-modified NSCs at protein levels (Fig. 1a to c). The results of FACS demonstrated

that macrophages pretended to polarize into M2 cells rather than M1 cells when co-cultured with Wnt4-modified NSCs (Fig. 1d and e). These results suggested that Wnt4-modified NSCs promote M2 polarization of macrophages.

Wnt4 induce NSCs to secrete multiple M2-polarization relative cytokines

Previous studies reported that different cytokines induced macrophages to polarize into different phenotypes. M1 cells are produced after the monocyte stimulated with tumor necrosis factor-alpha (TNF- α) or Interleukin (IL) -1 [33, 34], M2 cells are produced after the monocyte stimulated with IL-4 or IL-13 [33]. To investigate the mechanism of Wnt4-modified NSCs promote M2-polarization in macrophages, M1 and M2 related cytokines were detected in NSCs treated with Wnt4. The results showed that the expression of M2 related cytokines including IL-4 and IL-13 were increased in NSCs treated with Wnt4. In contrast, the expression of M1 related cytokines including IL-1 and TNF- α were decreased in NSCs treated with Wnt4 (Fig. S4a). ELISA results showed the concentration of IL-4 and IL-13 in the serum of NSCs treated with Wnt4 were significantly increased. In contrast, the concentration of IL-1 and TNF- α in the serum of NSCs treated with Wnt4 were significantly decreased (Fig. S4b). These results suggested that Wnt4 induce NSCs to produce multiple M2-polarization relative cytokines.

M2 cells promote NSCs differentiate into Neuron in vitro

The appropriated microenvironment can elevate the neuronal differentiation rate of transplanted NSCs which is the key point to therapeutic effect of NSCs transplantation. Macrophages is crucial for the inflammatory response in the spinal cord [35, 36]. The appropriate activation of macrophages can aid in the repair and regeneration of the injured spinal cord. Thus, we next investigated the effect of macrophages on neuronal differentiation of NSCs. The immunofluorescence results showed that β 3-tubulin and MAP2 positive cells were significantly decreased in NSCs co-cultured with M1 cells and increased in NSCs co-cultured with M2 cells (Fig. 2a and b, d and e). In contrast, GFAP positive cells were significantly increased in NSCs co-cultured with M1 cells and decreased in NSCs co-cultured with M2 cells (Fig. 2c to e). Similar results of RT-qPCR and WB analyses showed that neurogenic markers expression including β 3-tubulin, MAP2 and GFAP in mRNA and protein levels (Fig. 2f to h). These results suggested that M2 cells promote NSCs differentiate into neuron *in vitro*.

Wnt4-modified NSCs promote M2 polarization through suppressing of TLR4/NF- κ B signal pathway

Toll like receptor 4 (TLR4) /nuclear factor- κ B (NF- κ B) signaling was considered to a critical signaling pathway in neuroinflammation [37, 38] and M1 polarization of macrophages [38]. We further confirm whether the TLR4/NF- κ B signaling was involved in anti-inflammatory effect of Wnt4-modified NSCs. We first examined the mRNA and protein levels of TLR4. The results showed that the expression of TLR4 was significantly decreased in macrophages co-cultured with wild type NSCs and further decreased in macrophages co-cultured with Wnt4-modified NSCs (Fig. 3a to c). The immunofluorescence staining showed that the expression and nucleus translocation of p65 (NF- κ B) was significantly decreased in macrophages co-cultured with wild type NSCs and further decreased in macrophages co-cultured with

Wnt4-modified NSCs (Fig. 3d). Immunoblots of p65 in cytosolic extract (CE) and nuclear extract (NE) revealed that p65 nuclear translocation was significantly suppressed in macrophages co-culture with Wnt4-modified NSCs (Fig. 3e and f). These results suggested that Wnt4-modified NSCs promote M2 polarization through suppressing of TLR4/NF- κ B signal pathway.

M2 cells promote neuronal differentiation of NSCs through activation of MAPK/JNK signal pathway

MAPK/JNK is a crucial signal pathway to neuronal differentiation according to our previous study [22]. We next investigated whether MAPK/JNK was involved in promoting neuronal differentiation of NSCs by M2 cells. NSCs was pre-treated with JNK specific inhibitor (SP600125) in the NSCs-macrophages co-cultured cell system. The immunofluorescence results showed that after suppression of MAPK/JNK signal pathway in NSCs, β 3-tubulin and MAP2 positive cells were significantly decreased in NSCs co-cultured with M2 cells (Fig. 3g and h). Similar results of RT-qPCR and WB analyses showed that neurogenic markers expression including β 3-tubulin and MAP2 in mRNA and protein levels (Fig. 3i to k). These results suggested that M2 cells promote neuronal differentiation through activation of MAPK/JNK signal pathway.

Transplantation of Wnt4-modified NSCs mediate M2 polarization in inflammatory microenvironment after SCI

As mention above, the polarization of macrophages in inflammatory microenvironment is related to the therapeutic effect of NSCs transplantation because of inducing apoptosis and death of drafted cells and neuron by M1 cells [33]. Whether transplantation of Wnt4-modified NSCs mediate the polarization of macrophages is still elusive. Thus, we further investigated the effect of Wnt4-modified NSCs on polarization of macrophages in the inflammatory microenvironment of SCI. The immunofluorescence results showed that increasing M1 cells (iNOS⁺) were accumulated at the injured site of spinal cord. Interestingly, The M1 cells were significantly decreased and M2 cells (Arg1⁺) were significantly increased at the injured site of spinal cord after Wnt4-modified NSCs transplantation (Fig. 4a to c). PCR and WB results also showed that expression of iNOS was significantly increased in injured spinal cord after SCI. The expression of iNOS was significantly decreased and expression of Arg1 was significantly increased in injured spinal cord after Wnt4-modified NSCs transplantation (Fig. 4d to f). These results suggested that transplantation of Wnt4-modified NSCs induced M2 polarization of macrophages to improve inflammatory microenvironment after SCI.

Transplantation of Wnt4-modified NSCs improve inflammatory micro- environment to promote axonal regeneration and suppress tissue damaged

Furthermore, previous studies reported that increasing M2 cells is beneficial to axonal regeneration and tissue repair. Immunofluorescence results showed that increasing MAP2⁺ cells were along with increasing M2 cells at the injured site of spinal cord after transplantation of Wnt4-modified NSCs (Fig. 5a to c). In addition, c-caspase3⁺ cells were significantly increased at the injured site of spinal after SCI and decreased after transplantation of Wnt4-modified NSCs (Fig. 5d and e). WB analysis showed the similar

results of the expressions of MAP2 and c-caspase3 in the injured spinal cord (Fig. 5f and g). These results indicated that transplantation of Wnt4-modified NSCs improved the inflammatory microenvironment to promote axonal regeneration and suppress tissue damaged after SCI.

Wnt4-modified NSCs tended to differentiate into neuron to promote tissue repair and locomotor recovery

The appropriated microenvironment is to the benefit of neuronal differentiation of NSCs. We further investigated the differentiated status of the drafted cells. Immunofluorescence results showed that MAP2⁺ positive NSCs (GFP⁺) were significantly increased and GFAP⁺ positive NSCs (GFP⁺) were significantly decreased at the injured site of spinal cord after transplantation of Wnt4-modified NSCs (Fig. 6a to d). HE, Nissl and Flurogold staining indicated that the cavity was significantly smaller and there were more survival neuron at the injured site of spinal cord after transplantation of Wnt4-modified NSCs (Fig. 6e and f). Furthermore, behavioral test including BBB motor function scores, footprint analysis and SCEP result demonstrated that transplantation of Wnt4-modified NSCs improved the motor function of hindlimb in SCI rats (Fig. 7a to f). These results indicated that Wnt4-modified NSCs tended to differentiate into neuron to promote tissue repair and locomotor functional recovery.

Discussion

Macrophages act as important role in the inflammatory response of injured spinal cord. These cells have the ability to polarize into two phenotypes: M1 and M2 cells. The ratio of M1 and M2 cells determines the homeostasis of the local microenvironment [33]. M1 cells mainly modulate the innate immunity to clear foreign microbes and wound debris. M2 cells release various anti-inflammatory cytokines and growth factors to tune inflammatory response and promote tissue repair [16]. In general wound, M1 cells is mainly presented in the initiate stage of inflammation and switch to M2 to begin tissue repair in the latest stage. However, M1 cells is existed persistently in the microenvironment of SCI which lead to irreversible damage of spinal cord [17]. Thus, it is important to simultaneously achieve valid M2 ratio to promote tissue repair in the microenvironment for the treatment of SCI.

The microenvironment of the injured spinal cord is imbalance and complicated. The imbalance microenvironment is considered to be the main cause of the poor regeneration and recovery of SCI [13]. The cellular level imbalance in the microenvironment mainly involves the differentiation of stem cells and transformation phenotypes of microglia and macrophages [13]. After SCI, neuron, astrocytes and other neural cells can release lot of inflammatory cytokines such as IL-1 β , TNF- α , to induces the M1 activation of macrophages that cause the ratio of M1/M2 is persistently imbalance.

Cell transplantation has emerged as a potential strategy to promote tissue repair after SCI [2, 6]. The primary mechanism of cell transplantation mediate functional improvement and tissue repair is neuroprotection and immunomodulation [2]. Previous studies focus on global changes in various cytokines from different immune cells after SCI. Previous study also considered that NSPC transplantation improves hindlimb movement in association with increase of T cells and decrease in B

cells [39]. MSCs also were considered to modify the immune response by elevating of anti-inflammatory and reducing of pro-inflammatory cytokines after SCI [40, 41]. However, whether transplanted cells directly alter inflammation and the direct interactions between transplanted cells and immune cells are still unclear. In the current study, we provided first evidence to investigate the interactions between NSCs and macrophages in our co-cultured system. In one hand, Wnt4-modified NSCs induces M2 polarization of macrophages because of releasing of anti-inflammatory cytokines (IL-4 and IL-13) (Fig. 1, S4). On the other hand, M2 cells promote neuronal differentiation of NSCs through MAPK/JNK signal pathway (Fig. 2, 3).

Previous studies have reported that activation of NF- κ B/p65 signal pathway was induced in M1 polarization of macrophages [42, 43] and this activation was regulated by many other signal pathways, such as TLRs, AMPK [44]. TLR4 was considered to play important role in inflammation mediated by macrophages and promote the nuclear translocation of NF- κ B to induce the expressions of many inflammatory cytokines [45, 46]. Thus, we explored that whether TLR4 and NF- κ B/p65 signal pathways were involved in the interaction between macrophages and NSCs. Our results showed that the expression of TLR4 was decreased and NF- κ B/p65 signal pathway was suppressed in macrophages co-cultured with Wnt4-modified NSCs. These results indicated that the suppression of M1 polarization of macrophages was related to TLR4/NF- κ B signal pathway (Fig. 3).

Cell transplantation, especially NSCs transplantation, are considered to be potential therapeutics in SCI because NSCs can differentiate into neurons and oligodendrocytes to reconnect the neural recruit in the lesion [1, 2]. However, previous studies have reported that most NSCs transplanted into lesion have differentiated into astrocyte rather than neuron due to the poor inflammatory microenvironment of SCI [6, 8]. Thus, an assisting agent that sustains a sound rate of neuronal differentiation and improves the abominable inflammatory microenvironment is a necessity for the treatment of SCI. Wnt4, which is an important ligand of non-canonical Wnt signal pathway, have been considered to have neurogenesis and anti-inflammatory capacities [20–22, 47]. Previous studies reported that Wnt4 suppressed p65 translocation and NF- κ B activation through transforming TAK1 in macrophages [47]. Our previous research has reported that Wnt4 promotes neuronal differentiation through both Wnt/ β -catenin and MAPK/JNK signal pathways and Wnt4-modified NSCs transplanted into lesion have effectively differentiated into neuron and promote functional recovery after SCI [22]. In the current study, we also conducted a series of *in vivo* experiments to prove the beneficial therapeutic effect of Wnt4. Our results demonstrated that Wnt4-modified NSCs transplantation promoted M2 polarization and suppressed M1 polarization that cause macrophages tend to polarize into M2 cells rather than M1 cells at the injured site of spinal cord (Fig. 4). The increasing M2 cells improved the poor inflammatory microenvironment and provided an appropriated microenvironment which was to the benefit of axonal regeneration and suppressing of tissue damage to drafted NSCs (Fig. 5). Thus, the drafted NSCs tended to differentiate into neuron rather than astrocytes in this appropriated microenvironment after SCI (Fig. 6) and these effects may facilitate tissue repair and functional recovery after SCI (Figs. 6 and 7, Supplementary video 1) due to promotion of neurite growth and synaptic plasticity.

In summary, our finding first provide evidence to confirm the interaction between Wnt4-modified NSCs and macrophages and the involved downstream mechanism by using co-cultured system. Transplantation of Wnt4-modified NSCs effectively improve the inflammatory microenvironment through inducing M2 polarization and suppressing M1 polarization of macrophages after SCI (Fig. 8). Considering these positive therapeutic effects, Wnt4 may also have remarkable potential assistant agent in NSCs transplantation for SCI.

Abbreviations

SCI: Spinal cord injury, NSCs: Neural stem cells, Wnt: wingless-type MMTV integration site family, DMEM: Dulbecco modified eagle medium, FGF-2: Fibroblast growth factor-2, EGF: Epidermal growth factor, BBB: Basso, Beattie, and Bresnahan, SCEP: Spinal Cord-Evoked Potential, PFA: paraformaldehyde, FG: Fluorogold, TNF- α : tumor necrosis factor-alpha, IL: Interleukin, MAPK: Mitogen-activated protein kinase, NF- κ B: Nuclear factor kappa B, TLR: Toll like receptor

Declarations

Acknowledgement

Not Applicable

Author Contributions

XL and LL contributed equally to this work. Study design: YW and XL. Conducted the study: XL, LL and YW. Data collection: XL, LL, YH, FZ, YT and WZ. Data analysis: XL, LL, FZ, XC and YW. Data interpretation: XL, WZ and XC. Drafted the manuscript: XL and LL. Revised the manuscript content: XL. Approved the final version of the manuscript: YW. All authors take responsibility for the integrity of the data analysis.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethic approval and consent to participate

All procedures performed on experimental animals were approved by the Animal Care and Use Committee of Sun Yat-sen University and were conducted in accordance with the Guide to the Care and Use of Experimental Animals by the National Research Council (1996, United States).

Consent for publication

Not applicable

Conflict of Interest

None of the authors have conflicts of interest to disclose.

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Figures

Figure 1

Wnt4-modified NSCs promote M2 polarization of macrophages. (a) RT-qPCR analysis of expressions of CD163, CD206 and CD68 in macrophages co-cultured with NSCs, n=3. (b) Western Blot analysis of expressions of CD163, CD206 and CD68 in macrophages co-cultured with NSCs. (c) Quantification of western blot data in panel B, n=3. (d) Flow cytometric analyses of M1 cells (CD68+) in macrophages co-cultured with NSCs, n=3. (e) Flow cytometric analyses of M2 cells (CD163+ CD206+) in macrophages co-cultured with NSCs, n=3. (The data are presented as the means $\pm$ SD from one representative experiment of three independent experiments performed in triplicate. ** P < 0.01 compared between groups, * P < 0.05 compared between groups.)

Figure 2

M2 cells promote NSCs differentiate into Neuron in vitro. (a) Immunofluorescence analysis of β 3-tubulin+ cells in NSCs co-cultured with macrophages, n=3, bar: 20 μ m. (b) Immunofluorescence analysis of MAP2+ cells in NSCs co-cultured with macrophages, n=3, bar: 20 μ m. (c) Immunofluorescence analysis of GFAP+ cells in NSCs co-cultured with macrophages, n=3, bar: 20 μ m. (d) Quantification of immunofluorescence data in panel A to C, n=3. (e) RT-qPCR analysis of expressions of β 3-tubulin, MAP2 and GFAP in NSCs co-cultured with macrophages, n=3. (f) Western Blot analysis of expressions of β 3-tubulin, MAP2 and GFAP in NSCs co-cultured with macrophages. (g) Quantification of western blot data in panel F, n=3. (The data are presented as the means $\pm$ SD from one representative experiment of three independent experiments performed in triplicate. ** P < 0.01 compared between groups, * P < 0.05 compared between groups.)

Figure 3

TLR4/NF-κB and MAPK/JNK signal pathways were involved in the interaction between NSCs and macrophages. (a) RT-qPCR analysis of expressions of TLR4 in macrophages co-cultured with NSCs, n=3. (b) Western Blot analysis of expression of TLR4 in macrophages co-cultured with NSCs, n=3. (c) Quantification of western blot data in panel B, n=3. (d) Immunofluorescence analysis of expression of p65 in macrophages co-cultured with NSCs, n=3, bar: 10 μm. (e) Western blot analysis of expression of

p65 in nuclear extract (NE) and cytosolic extract (CE) of macrophages co-cultured with NSCs. (f) Quantification of western blot data in panel E, n=3. (g) Immunofluorescence analysis of $\beta 3$ -tubulin+ and MAP2+ cells in NSCs pre-treated with JNK specific inhibitor (SP) then co-cultured with macrophages, n=3, bar: 20 μ m. (h) Quantification of immunofluorescence data in panel I, n=3. (i) RT-qPCR analysis of expressions of $\beta 3$ -tubulin and MAP2 in NSCs pre-treated with SP then co-cultured with macrophages, n=3. (j) Western Blot analysis of expressions of $\beta 3$ -tubulin and MAP2 in NSCs pre-treated with SP then co-cultured with macrophages. (k) Quantification of western blot data in panel J, n=3. (The data are presented as the means $\pm$ SD from one representative experiment of three independent experiments performed in triplicate. ** P < 0.01 compared between groups, * P < 0.05 compared between groups.)

Figure 4

Transplantation of Wnt4-modified NSCs mediates M2 polarization of macrophages in the inflammatory microenvironment of SCI. (a) Immunofluorescence analysis of iNOS+ and Arg1+ cells (red) at the injured site of spinal cord in different groups, bar: 500 μ m in upper row, 100 μ m in lower row. (b and c) Quantification of immunofluorescence data in panel A, n=5. (d) RT-qPCR analysis of expressions of iNOS and Arg1 in the spinal cord tissue in the different groups, n=3. (e) Western Blot analysis of expressions of iNOS and Arg1 in the spinal cord tissue in the different groups, n=3. (f) Quantification of western blot

data in panel E, n=3. (The data are presented as the means $\pm$ SD. ** $P < 0.01$ compared between groups, * $P < 0.05$ compared between groups.)

Figure 5

Transplantation of Wnt4-modified NSCs improve inflammatory micro- environment to promote axonal regeneration and suppress tissue damaged. (a) Immunofluorescence analysis of Arg1+ (red) and MAP2+ (yellow) cells at the injured site of spinal cord in different groups, bar: 500μm in upper row, 100μm in lower row. (b and c) Quantification of immunofluorescence data in panel A, n=5. (d) Immunofluorescence analysis of c-caspase3+ (red) cells at the injured site of spinal cord in different groups, bar: 500μm in upper row, 100μm in lower row. (e) Quantification of immunofluorescence data in panel A, n=5. (f) Western Blot analysis of expressions of MAP2 and c-caspase3 in the spinal cord tissue in the different groups, n=3. (g) Quantification of western blot data in panel F, n=3. (The data are presented as the means $\pm$ SD. ** $P < 0.01$ compared between groups, * $P < 0.05$ compared between groups.)

Figure 6

Wnt4-modified NSCs tended to differentiate into neuron to promote tissue repair. (a) Immunofluorescence analysis of MAP2+ (red) and GFP+ (green) cells at the injured site of spinal cord in different groups, bar: 500µm in upper row, 100µm in lower row. (b) Quantification of immunofluorescence data in panel A, n=5. (c) Immunofluorescence analysis of GFAP+ (red) and GFP+ (green) cells at the injured site of spinal cord in different groups, bar: 500µm in upper row, 100µm in lower row. (d) Quantification of immunofluorescence data in panel C, n=5. (e) HE, Nissl and Fluorogold (FG) staining of spinal cord in different groups, bar: 500µm in upper row, 100µm in lower row. (f) Quantification of HE, Nissl and FG staining in different groups. (The data are presented as the means $\pm$ SD. ** P < 0.01 compared between groups, * P < 0.05 compared between groups.)

Figure 7

Transplantation of Wnt4-modified NSCs promote locomotor functional recovery after SCI. (a) Behavioral character images showing hindlimb movement in different groups. (b) Footprint analysis of different groups, blue is forelimb, red is hindlimb. (c) BBB scores in different groups. (d) Electrophysiological outcomes of spinal cord evoked potential (SCEP) in different groups. (e and f) Quantification of SCEP

latency and amplitude. (The data are presented as the means $\pm$ SD, n=5. ** P < 0.01 compared between groups.)

Figure 8

An schematic diagram of the current study. This study demonstrated that the interaction between transplanted cells (Wnt4-modified NSCs) and macrophages in microenvironment of SCI. Wnt4- modified NSCs improved the inflammatory microenvironment through increasing of M2 polarization in macrophages to facilitate axonal regeneration and tissue repair.

Supplementary Files

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