Journal on Surgery

www.journalonsurgery.org

Research Article

Open Access, Volume 2

Bone Marrow Derived Cells Mediated Regulation of Schwann Cells Proliferation and Migration *in Vitro*

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Abstract

Schwann cells (SCs) are the unique glial cells in the peripheral nerve. After peripheral nerve injury, SCs change their morphology, function, and play a new role as repair cells, through this process, factors that enhance SCs proliferation and promote SCs migration will benefit nerve regeneration and functional recovery. Bone marrow-derived cells (BMDCs), which has been proved to offer several regenerative benefits for tissue and organ injuries. However, whether BMDCs support nerve regeneration by affect the proliferation and migration of SCs, remains unclear. Here, we taken BMDCs co-cultured with nerve segments, and then investigated the proliferation and migration of SCs in vitro. Results demonstrated that BMDCs can enhance the proliferation of SCs, and then promoted SCs migration, forming Bungner's brand-liked structure in vitro. These finding provide a potential new method for repair of injured peripheral nerves in clinical.

Keywords: Bone marrow derived cells; Schwann cells; Proliferation; Migration; Peripheral nerve injury; *In vitro.*

Introduction

Peripheral Nerve Injury (PNI) is occurred relatively commonly in the clinic, which often leads to sensory and motor dysfunction of limbs, and brings serious burden to family and society. Clinical and academic efforts are focusing on how to improve PNI repair. Schwann cells support nerve regeneration and motor function recovery by secreting a range of neurotrophic factors, cleaning damaged axons and myelin, and providing structural guidance, according to several studies [1,2], for these reasons, these cells are an ideal therapeutic target for future clinical strategies.

Under pathological conditions, SCs regain the function of promoting peripheral nerve regeneration and repair [3-5]. To understand the role of SCs in repair of PNI, it is important to understand the injury response during Wallerian degeneration. Through this process, SCs will become de-differentiated, similar to newborn SC progenitor cells, and will take on the identity of repair SCs [3, 6,7], which will proliferate and migrate to the wounded site [8]. There they aid in the creation of a permissive microenvironment for axon renewal and nerve regeneration [9-14]. Considering the critical roles played by SCs, treatments that might speed up the proliferation and migration of SCs could aid in the repair and regeneration of PNI.

Manuscript Information: *Received: Sep 22, 2022; Accepted: Oct 07, 2022; Published: Oct 14, 2022*

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Citation: *Wang xp, Zhao P-S, Chen X-T, Yan J, Wu M. Bone Marrow Derived Cells Mediated Regulation of Schwann Cells Proliferation and Migration in Vitro. J Surgery. 2022; 2(2): 1061.*

Previous studies have shown that Mesenchymal Stem Cell (MSC) implanted in vivo or cocultured with peripheral nerve extracts from the damaged sciatic nerve in vitro, can differentiate into SCs phenotype, providing necessary support and nourishment for axonal regeneration [15-18]. However, according to Sowa's research [19], transplanted MSCs, on the other hand, significantly enhance axonal outgrowth, myelin production, and the repair of denervated muscle atrophy, nevertheless, do not differentiate into SCs. This suggests that the therapeutic effect of transplanted MSCs is due to the indirect regeneration of endogenous SCs via a cellular paracrine mechanism rather than trans differentiation. Therefore, research into the effect of MSCs on SCs, particularly their proliferation and migration, is extremely important for therapeutic purposes.

As we known, bone marrow, as a systemic cell bank, contains various cell types, including MSC, macrophages, vascular endothelial cells and fibroblasts, here, be called bone marrow-derived cells (BMDCs), which has been proved to offer several regenerative benefits for tissue and organ injuries [20-23] also, for peripheral nerve injury [24-26]. However, the mechanism of BMDCs promoting nerve regeneration, by SCs or other means, is remain unknown, further more elevating affection of BMDCs on the proliferation and migration of SCs is not report. Therefore, in this study, we aim to understand the influence of BMDCs on the proliferation and migration SCs, providing a potential new method for repair of injured peripheral nerves in clinical.

Materials and methods

Animals

Shanghai SLAC Laboratory Animal Co., Ltd., China, provided 36 healthy male and female mature (6–8 weeks) C57Bl/6 mice weighing 22–26 g (license No. SYXK (Hu) 2012-0001). All mice were kept in the Central Laboratory of Bengbu Medical College in China, where they were kept in a 12-hour light/dark cycle at 22°C with a humidity of 40–67% and were free of particular pathogens. All operations were carried out in line with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Ministry of Science and Technology of China's Guidance Suggestions for the Care and Use of Laboratory Animals (2006). The Institutional Review Committee of China's Bengbu Medical College gave its approval to the animal trials.

By administering sodium pentobarbital (40 mg/kg) intraperitoneally, all mice were rendered completely unconscious, and then the proceed was doing as follow [27].

Isolation of mouse BMDCs

BMDCs were extracted from the bone marrow of the femur and tibia. The bone marrow was extracted in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin after being lysed with erythrocyte lysis buffer. After that, homogeneous cell suspensions were saved for co-culture with sciatic nerve segments.

Sciatic nerve tissue harvest

Sciatic nerve tissue was harvested according to an our previously reported methods [27]. In this experiment, the sciatic nerve was cut into 5 mm length segments, which will co-culture with BMDCs.

In vitro culture

In group 1, the nerve segments were placed in a 6-well plate with DMEM, 10% FBS, and 1% penicillin/streptomycin as the only growth media. In group 2, BMDC suspensions were seeded on a 6-well plate first, and then nerve segments were inserted directly in the plate for co-culturing with BMDCs. Every two days, the medium was replaced.

Separation and culture of SCs

SCs were isolated from cultured nerve segments. On 3, 7 days after in vitro culture, the nerve segments were rinsed with PBS and cut into smaller pieces. Then, the pieces were digested in dissociating enzyme solution which was prepared by dissolving collagenase NB4 (Serva, Heidelberg, Germany) in DMEM at a concentration of 0.2% (0.27 U/mL) at 37◦C, 5% CO_2 for 60 min, followed by 0.25% trypsin (Gibco) for 10 min. The mixture was centrifuged at 600 × g for 10 minutes. After removing the supernatant, the cell was re-suspended in SC culture medium consisting of DMEM supplemented with 10% FBS, 2 μM forskolin (Sigma, St. Louis, MO, USA), 10 ng/mL heregulin-β-1 (PeproTech, Rocky Hill, NJ, USA), and 50 ng/mL basic fibroblast growth factor (PeproTech). The cell suspension was seeded in a flask at and incubated at 37°C, 5% CO_2, and allowed to adhere overnight.

Western Blot

The proteins extracted from SCs using Radioimmunoprecipitation assay (RIPA) buffer were separated by SDS-PAGE, and then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was incubated with anti-$P75^{NTR}$primary antibodies overnight, 4°C.After washing with TBST, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and the immune complexes were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher).

SCs Proliferation Assay

Prepared SCs which obtained from nerve segments on3, 7days after in vitro culturing in two groups were seeded into 96-well plates at a density of 1×10^4 cells/well. MTT solution (Solarbio) was added into each well, followed by a 4h incubation. The absorbance, or optical density (OD), was measured at 570 nm with a microplate reader (TECAN).

Brdu stain

Prepared SCs were seeded into 24 well plates at a density of 5×10^4 cells/ml, and then grown for 48 H in SC culture medium. After 48 h, the SC culture medium was replaced with fresh SC culture medium and 10 μM of BrdU, and the culture was then incubated at 37°C for 24 h. The following day, SCs were fxed with 4% PFA, and then were incubated with 1N HCl on ice and then with 2N HCl at room temperature for DNA hydrolysis. To detect incorporated BrdU, samples were stained with BrdU (1:500; Thermo). The number of BrdU+ SCs was calculated from multiple fields of view under the microscope using Image (n=5, five random regions).

Tanswell experiment

Prepared SCs which obtained from nerve segments on 3,7 days after in vitro culturing in each group were suspended in serum-free medium and the cell density was adjusted to $5x10^4$ cells/mL. 24-well plates were used and 500 ul SC culture medium were added into each well, then the transwell chamber also were put into well, 200 ul SC suspensions were added into upper chamber and cultured for 48 h. Then the transwell chamber were taken out and washed with PBS. The cells in the upper chamber were wiped with a cotton swab, washed with PBS, fixed with 4% PFA for 20 min, stained with 0.5% crystal violet for 5 min, and washed with PBS. The number of migrated cells attached to the lower surface of the chamber was observed under a microscope, and 5 fields of view were randomly selected and averaged.

The Migration of SCs from nerve segment

On 3, 7 days after in vitro culture, the nerve segments in each group was observed by phase contrast microscopy (Olympus, Tokyo, Japan) at 100× magnification, and then the distance of SCs migration were record and compared(5 fields of view were randomly selected and averaged).

SCs co-cultured with PLA filament

Prepared SCs were seeded at a density of 5 × 10^4cells/ml in SC culture medium into 6-well plates, and then sterilized PLA filament were add in for co-culturing. After incubated at 37°C, 5% CO_2 for 48 h, under a microscope, SCs and PLA filament was observed, and which morphology and arrangement was described.

Statistical analysis

All data is presented as a mean standard deviation. The values were subjected to a two-sample t-test, and the least significant difference test using SPSS 22.0 software (SPSS, Chicago, IL, USA) for quantitative comparison and analysis. The threshold for statistical significance was fixed at P 0.05.

Result

BMDCs enhanced the proliferation of SCs

MTT assay and Brdu stain was used to detect the activity of Schwann cells in each group. Compared with group 1, BMDCs in group 2 induced a robustly higher proliferation rate at the 3rd ,7th day, indicating by MTT assay and which were statistically significant (P<0.05). Also, the Brdu positive SCs significantly increased in group 2 at the 7th day, but not at the 3rd day.

P75NTR, was a low-affinity receptor for multiple neurotrophins, was expressed in developing SCs, was involved in the proliferation and anti-apoptotic of SCs. So, we detect the difference of p75NTR expression between two groups. Compared with group 1, the expression of P75NTR in group 2 was significantly increased at the 3rd, 7th days (P<.05) (Figure 1).

BMDCs promoted the migration of SCs

Since the success of nerve repair is highly dependent on the ability of SCs to effectively migrate across the injury site, how to improving the migration of SCs is popular topic. In our experiment, SCs migrated from the nerve segments when cultured in vitro at the 3rd day, as time goes on, at the 7th day, a lot of SCs migrated, expanded and connected into sheets in all groups, observed by phase contrast microscopy. In addition, compared with group 1 (nerve segments cultured alone), the migration distance of SCs in group 2 (BMDCs co-cultured with never segments), was significantly longer at the 3rd and 7th day, and which were statistically significant (Figure 2).

Also, Transwell assay was used to detect the migration of SCs in each group. Compared with group 1, the migration of SCs in group 2 was significantly increased at the 7th day and were statistically significant (P<0.05). It can be seen, BMDCs promoted the migration of SCs (Figure 3).

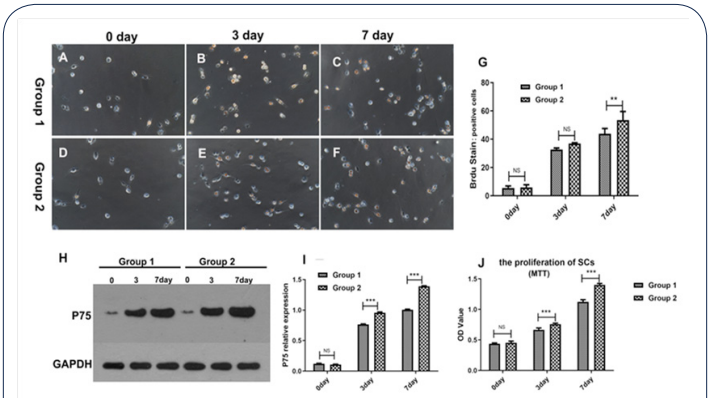

Figure 1: BMDCs affect the proliferation of Schwann cells: (A-F) Brdu stain was used to detect the proliferation of Schwann cells in two groups. Compared with group 1 **(A-C)**, BMDCs in group 2**(D-F)** induced a robustly higher proliferation rate at the 7th day **(G)** (The number of BrdU+ SCs was calculated from five random regions). Also, MTT assay indicated that BMDCs promote the proliferation of SCs at the 3rd ,7th day(J). **(H)** The expression of P75NTR between two groups detected by Western Blot, compared with group 1, the expression of P75NTR in group 2 was significantly increased at the 3rd, 7th days **(I)**. * $P < 0.05$.

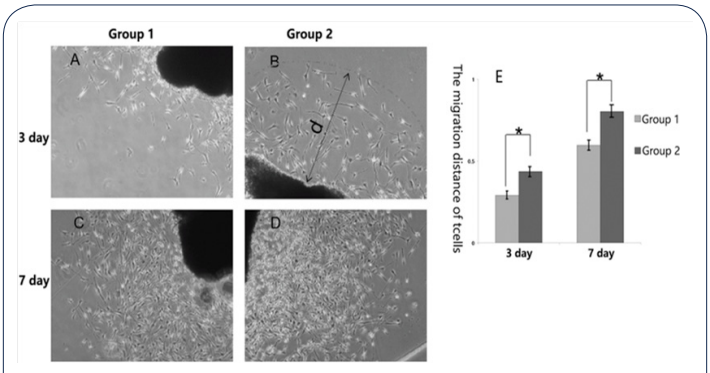

Figure 2: Schwann cells migrated from the nerve segments: We noticed that SCs migrated from the nerve segments when cultured in vitro at the 3rd day **(A,B)**, as time goes on, at the 7th day, a lot of SCs migrated, expanded and connected into sheets **(C,D)** in all groups. Compared with group 1 **(A,C)**, the migration distance of SCs in group 2(B, D), was significantly longer at the 3rd and 7th day, and which were statistically significant **(E)**. * $P < 0.05$.

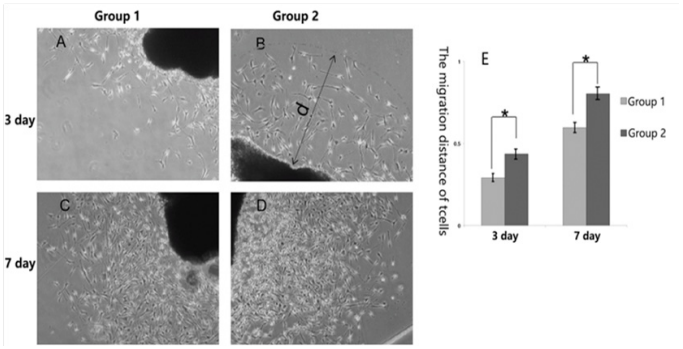

Figure 3: BMDCs promoted the migration of Schwann cells: (A-F) Transwell assay was used to detect the migration of Schwann cells in two groups. Compared with group 1(A-C), BMDCs in group 2(D-F) promoted the migration at the 7th day(G).

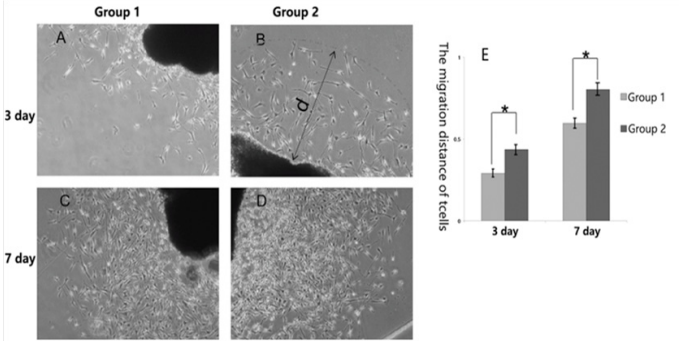

Figure 4: BMDCs promoted Schwann cells forming Bungner's brand-liked structure: When SCs and PLA filament co-cultured, SCs was orderly and arranged along the PLA filament forming Bungner's brand-liked structure at the 3rd,7th day in group 2(C, D), but not observed in group 1(A, B), Which SCs were disorderly and not arranged along the filament.

BMDCs promoted the SCs forming Bungner's brand-liked structure

In Wallerian degeneration, SCs form aligned elongated tubular structures called bands of Bungner that provide guidance to regrowing axons and support their linear regeneration. So, we detected SCs whether forming Bungner's brand-liked structure in vitro. In our experiment, when SCs obtained from group 2 were co-cultured with filament like structure made of PLA, the migration of SCs was orderly and arranged along the filament forming Bungner's brand-liked structure, however, in group 1, SCs were disorderly and not arranged along the filament (Figure 4).

Discussion

SCs are the unique glial cells in the peripheral nerve [28]. In the event of a peripheral nerve injury, SCs change their morphology, function, and play a new role as repair cells [3,6,7]. Through this process, a dynamic SCs reprogramming maybe briefly divide into two stages. At the early stage, SCs go through dedifferentiation, proliferation, myelin sheath clearance, this stage is characterized by proliferation of SCs [14,29]. Then at the later stage, repair SCs migrate into the lesion site, form bunger's band, which will create a permissive environment for nerve regeneration [30,31]. The change of SCs in two stages regulate by various cellular and molecular factors [14,32-34]. Factors that enhance SCs proliferation and promote SCs migration after peripheral nerve injury will benefit nerve regeneration and functional recovery.

In the current study, we examined the modulation of BMDCs for proliferation and migration of SCs by using western blot, cell proliferation assay, cell migration assay. In our results, BMDCs accelerate the proliferation of SCs, and the significant tendency was beginning on the 3rd day after BMDCs co-cultured with nerve segments, was peaking on the 7th day. Also, BMDCs promote the migration of SCs, nevertheless, the significant effectiveness was detected on the 7th, which was justly consistent with the character of SCs in Wallerian degeneration. We also find the migration of SCs was orderly and arranged along the filament forming bungner's brand-liked structure when co-cultured with filament.

Previous study has indicated numerous methods that could promote the proliferation and migration of SCs [35-37]. However, these methods frequently involve in complex, difficult to be popularized, and what is more, these factors cannot enhance SC proliferation in the early stage by one hand and further promote SC migration in the late stage by the other hand. In our research, we take BMDCs co-cultured with nerve segments, which is a simple, easy to implement, and both enhancing the proliferation in early stage and promoting the migration in late stage method, in addition, compared with previous reports, our method have other superiority, which can be list as follow:①As we prior study, taking BMDCs co-cultured with nerve segments is an effective way to generated a large of repair SCs in a short time frame, which is of the essence for clinical purposes. ②Based on "BMDCs promoting the proliferation and migration of SCs", when combination of BMDCs and SCs transplanted in artificial nerve conduits for nerve repair, the BMDCs will enhance the proliferation and sustain the survival of SCs in conduit site, and sustaining the survival of SCs was considered as a key factor for successfully repair in the fields of tissue engineering.

In recent studies, the importance of micro environment at the nerve lesion was recognized [10,38]. The dynamic biochemical changes in the micro environment ultimately improved the proliferation and migration of SCs. After nerve injury, beside SCs activated, other cell types contain macrophages, neutrophils, fibroblasts and endothelial cells, are also recruit and active at the site of lesion, all kinds of cells secrete various growth factors, cytokines, interleukins, ECM, and form a dynamic micro environment, in turn, these environmental cues promote the proliferation and migration of SCs by means of affecting multiple intracellular signaling pathways [10,34,39]. BMDCs are a mixed population of cells, including stem cells, mesenchymal cells, blood system cells, fibroblasts and vascular endothelial cells, and so on. In our trail, we take these cells co-cultured with nerve segments in vitro, and find that these cells infiltration in the inner of nerve segments, which is mimics the dynamic change of the micro environment in the site of nerve lesion in vivo and at last lead the proliferation and migration of SCs, we guess.

Taken together, this study provides a new method to improve the proliferation and migration of SCs in vitro and imply the potential role of the BMDCs and SCs in the treatment of peripheral nerve injury. However, there are still have some problems in our researcher that have not been solved, such as, further clarifying the mechanism and verifying the effect that combination of BMDCs and SCs transplanted in artificial nerve conduits for nerve repair.

Declarations

Ethics approval and consent to participate: All of the animal experiments in this study were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, the Ministry of Science and Technology of China's Guidance Suggestions for the Care and Use of Laboratory Animals (2006), and were approved by the laboratory animal ethical committee of Bengbu Medical College (Number: BYYFY-2021KY18). All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent to publish: Not applicable.

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

Competing interests: The authors declare that they have no competing interests.

Funding: This research support by the Key project of Natural Science Foundation of Bengbu Medical College (No. BYKY1837ZD and 2020byzd111); the Key University Natural Science Research Project of Anhui Province of China (No. KJ2021A0723). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contribution: Xiaopan Wang designed the study, conducted the experiments, analyzed the data, obtained the funding and wrote the paper. Min Wu obtained the funding and provided the critical revision of the paper. Jun Yan designed the study and provided the critical revision of the paper. Peishuai Zhao and Xiaotian Chen participated in experiments. All authors approved the final version of the paper.

Acknowledgements: Not applicable.

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