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Wnt/PCP pathway regulates the migration and neural differentiation of mesenchymal stem cells *in vitro*

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Abstract

Introduction. Mesenchymal stem cells (MSCs) are an excellent donor graft source due to their potential for self-renewal and multidirectional differentiation. However, the potential mechanisms involved in MSC homing and neural differentiation are still unclear. The purpose of this study was to explore the effects of a chemokine, SDF-1α, and Wnt3a ligand on rat MSCs' migration and β-mercaptoethanol (BME)-induced neural differentiation of MSCs.

Materials and methods. MSCs were isolated from rat bone marrow and cultured *in vitro* to passage 3. Scratch tests and transwell assays were used to estimate the effects of SDF-1α (25 ng/mL) and Wnt3a (10 ng/mL) on the migration of MSCs. The expression of Wnt/PCP pathway proteins RhoA, c-Jun, ATF2, and Wnt3a were assessed by Western blot. The 5 mM BME-induced neural differentiation of MSCs was determined by immunofluorescence to detect neuron- and astrocyte-specific markers such as nestin, GFAP, and Olig2.

Results. Wnt3a promoted the migration ability of MSCs and regulated the expression of RhoA, c-Jun, and ATF2 proteins. MSCs could differentiate into neural stem cells and astrocytes. Wnt3a enhanced BME-induced neurogenesis in MSCs by increasing the protein expression of RhoA, c-Jun, and Wnt3a.

Conclusions. The present study demonstrated that the Wnt/PCP pathway promotes migration and neural differentiation of rat MSC. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 1, 44–54)*

Key words: mesenchymal stem cells; SDF-1α; migration; neural differentiation; Wnt3a; Wnt/PCP signaling

Introduction

Mesenchymal stem cells (MSCs) are multilineage cells with the ability to self-renew and differentiate into a variety of cell types. In addition, MSCs can be easily isolated from several adult tissues, readily expanded in vitro, and exhibit robust immunomodulatory properties. All these highly desirable attributes make MSCs an important source for cell therapy in regenerative medicine [1, 2]. MSCs have a homing ability, meaning that they can migrate into injured sites, and they possess the capacity to differentiate into local components of injured sites and the ability to secrete chemokines, cytokines, and growth factors that help in tissue regeneration [3, 4].

Administration of MSCs has been successfully tested in the treatment of chronic degenerative diseases since beneficial effects have been described in several preclinical investigations. In particular, better outcomes have been reported when transplanted MSCs were previously induced toward a neural differentiation [5]. Studies have shown that MSCs can be horizontally differentiated into non-mesoderm derived cells, such as glial cells and neurons [6, 7], which provide a new strategy for the clinical treatment of neurodegenerative diseases.

The directional migration of stem cells is precisely regulated by homing factors released from injury sites.

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Chemokine, stromal-derived factor-1 alpha (SDF--1α) [8], also known as CXCL12, is a critical factor in physiological and pathological processes, including embryogenesis, hematopoiesis, angiogenesis and inflammation, because it activates and/or induces migration of hematopoietic progenitor and stem cells, endothelial cells and most leukocytes [9]. Growing evidence indicates that SDF-1α and its cellular receptor, CXC chemokine receptor 4 (CXCR4), direct the migration of stem cells associated with injury repair in many species and tissue types [10–12]. However, the mechanism behind the directional migration of MSCs due to chemokines has not been clearly elucidated *in vivo* or in conventional in vitro experimental systems.

Wnt signaling can regulate several cellular functions, including proliferation, differentiation, migration, apoptosis, and migration [13]. Canonical Wnt was shown to augment the migration and differentiation of MSCs [14, 15]. Our previous research used β-mercaptoethanol (BME) to induce neural differentiation of MSCs, and the results suggested that Wnt/β-catenin signaling may play a pivotal role in neural differentiation of MSCs [16].

In addition to canonical β-catenin-mediated transcriptional effects, Wnt signaling can also affect cell morphology, migration, and adhesion in a more direct way *via* the non-canonical Wnt/planar cell polarity (PCP) pathway. In the Wnt/PCP pathway recruitment of Dvl results in activation of the small GTPases RhoA, Rac, and Cdc42, which actively rearrange the cell's cytoskeleton and control cell polarity and motility. Additionally, RhoA and Rac are capable of activating c-Jun N-terminal kinase (JNK), which can induce activation of AP1- and NFAT-mediated transcriptional programs [17, 18]. It was shown that Wnt/PCP pathway plays a major role in neural crest migration and MSC osteogenic differentiation [19, 20]. However, much less is known about whether Wnt/PCP signaling controls MSC migration and neural differentiation.

The previous study has shown that Wnt3a could activate both canonical and non-canonical Wnt signaling pathways [21]. Here, on the one hand, Wnt3a was used to activate the Wnt/PCP pathway. On the other hand, SDF-1α was used to promote the migration of MSCs, and β-mercaptoethanol (BME) was used to induce differentiation of MSCs into nerve cells. We, therefore, examined the effects of Wnt/PCP pathway on MSC migration and neural differentiation in vitro.

Material and methods

Generation and culture of MSCs. Male Sprague-Dawley (SD) rats (80–120 g) were purchased from Zhejiang Chinese Medical University Animal Center (Laboratory Animal Certificate: SYXK 2018-0006). All animal investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH and approved by the Institutional Animal Care Committee of Zhejiang Chinese Medical University. MSCs were isolated from rats and cultured in a sterile environment, the detailed characteristics for the identification of MSCs were confirmed in our previous study [22]. The bone marrow of the bilateral femur was flushed out with phosphate-buffered saline (PBS, BOSTER, China). The obtained bone marrow solution was centrifuged at 1,000 rpm for 5 min, the supernatant liquid was removed, and the cell pellets were resuspended in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). After 24 h, the culture medium was removed to discard the nonadherent cells. Then, the culture medium was replaced every 2 days, and the cells were passaged at 80% confluency. Passage 3 cells were used for the following experiments.

Cell treatment and experimental groups. To explore the role of the Wnt/PCP pathway in MSC migration, cells were randomly assigned to three groups: control, SDF-1α, and Wnt3a + SDF-1α. The cells in the SDF-1α group were cultured with a medium containing 25 ng/mL SDF-1α. The concentration of SDF-1α was chosen on the basis of preliminary data presented in Suppl. Fig. 1. For the Wnt3a + SDF-1α group, cells were cultured with a medium containing 10 ng/mL Wnt3a (PeproTech Inc., USA) dissolved in PBS and 25 ng/mL SDF-1α. For the control group, cells were treated with the same amount of PBS. The treated cells were used to determine migration ability, and after 48 h, the cells were collected for protein detection.

To explore the role of the Wnt/PCP pathway in MSC differentiation, cells were randomly assigned to three groups: control, BME, and Wnt3a + BME. In the Wnt3a + BME group, 10 ng/mL Wnt3a was added to the induction medium that contained 5 mM BME for induction of neural differentiation. The same volume of PBS was added to the control cells which were cultured without induction.

Scratch test. The migration of MSCs was analyzed using a scratch wound-healing assay. Briefly, 1×10^5 cells were plated in the culture dish and cultured until they reached 80% confluency. Next, the cells were scratched from the plate using a plastic tip to create the "wound". Then, the cells were washed with PBS, and the cells were observed by a fluorescence microscope (Nikon, Eclipse Ts2-FL, Japan). After 24 h, cells were fixed with 4% paraformaldehyde for

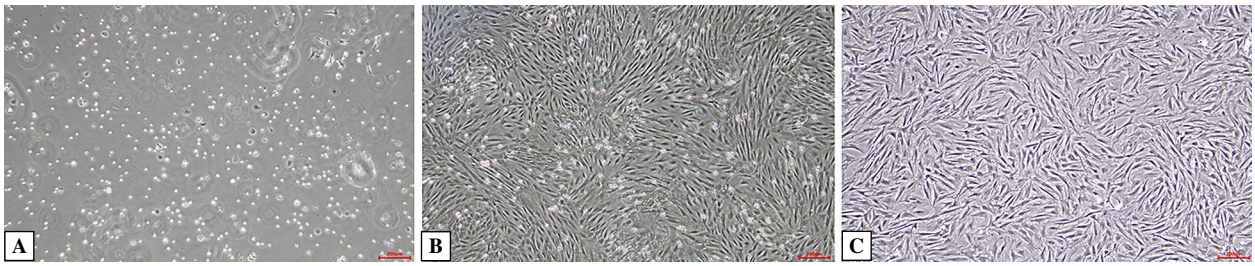

Figure 1. Morphology of rat mesenchymal stem cells (MSCs). **A.** Rat bone marrow-derived MSCs were cultured in an adherent flask for 24 h as described in Methods. **B, C.** MSCs in passages 1 and 3, respectively. Scale bar: 200 μm.

20 min and stained with hematoxylin to observe the ability of the cells to refill the created gap.

Transwell assay. MSC migration ability could also be tested by transwell plates (24-well plate, 8-μm pore size, Corning, NY, USA). Cells were suspended in DMEM/F12 containing 2% FBS. Then, 150 μL (1×10^5 cells/mL) of cell suspension was added to the upper chamber of the migration well. In contrast, 500 μL of complete medium (or containing 25 ng/mL SDF-1α or 10 ng/mL Wnt3a with 25 ng/mL SDF-1α) was added to the lower chamber. After 24 h of incubation at 37°C, the cells from the top of the filter were excluded with a cotton swab, and filters were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet solution for 10 min. The number of migrated cells was determined by averaging six random fields per well.

Neural induction in MSCs. MSCs were pre-induced for 24h in the pre-induction medium consisting of DMEM-F12/, 20% FBS, and 1 mM BME, and then washed with PBS and switched to the induction medium with the addition of DMEM-F12 and 5 mM BME for 1, 2, 3, 4, 5 and 6 h. Morphological changes in MSCs during neural differentiation were observed under a fluorescence microscope (Nikon, Eclipse Ts2-FL, Japan).

Immunofluorescence. For immunofluorescence analysis, the cells were first fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 0.5% Triton X-100 in TBS at room temperature for 10 min followed by incubation in blocking buffer (10% goat serum, 1% bovine serum albumin in TBS) at room temperature for 1 h. Then cells were incubated with rabbit anti-Nestin antibody (1:1,000, GeneTex), rabbit anti-GFAP antibody (1:1,000, Abcam) and rabbit anti-Olig2 antibody (1:1,000, GeneTex) overnight. After three washes with PBS, cells were incubated for 1 h at room temperature with the appropriate secondary antibody of Alexa Flour 488 goat anti-rabbit IgG (Abcam). Nuclear counterstaining was performed with DAPI. Finally, immunofluorescence was detected using a fluorescent microscope.

Western blotting. Total proteins from MSCs were separated on sodium dodecyl sulfate-polyacrylamide gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The blocked membranes were incubated with rabbit anti-beta catenin antibody (1:1000, Abcam, UK), Wnt3a antibody (1:1000, Abcam), RhoA antibody (1:5,000, Abcam), c-Jun antibody (1:1000, Abcam) and ATF2 antibody (1:1000, Abcam) overnight at 4°C. Membranes were then washed 4 times and incubated with secondary antibodies for 2 h at room temperature. The relative expression of proteins was analyzed by ImageJ analysis software (National Institutes of Health, USA) after exposure and imaging with an ECL chemiluminescence kit (BOSTER, China).

Statistical analysis. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). All data are presented as three independent experiments with the mean ± standard deviation (SD). Statistical differences between the groups were analyzed by one-way analysis of variance (ANOVA). Comparisons between two groups were performed using Dunnett's t-test. $p < 0.05$ was considered statistically significant.

Results

Isolation and expansion of MSCs

As shown in Fig. 1A, cells were isolated from rat bone marrow after 24 h. Primary MSCs were round, spindle, or polygonal. The first passage is shown in Fig. 1B. The adherent cells had a spindle shape, were densely distributed, and presented a "whirlpool-like" growth. By passage 3, cells were arranged closely with clear boundaries, good refraction, and higher purity as compared to passage 1 (Fig. 1C).

Wnt3a enhanced the migration ability of MSCs induced by SDF-1α

Scratch tests and transwell migration assays were used to observe the effects of SDF-1α on MSC migration. The results showed that the migration ability of MSCs was found to be significantly increased, especially after incubation with 25 ng/mL SDF-1α (Suppl. Fig. 1 A, B, C).

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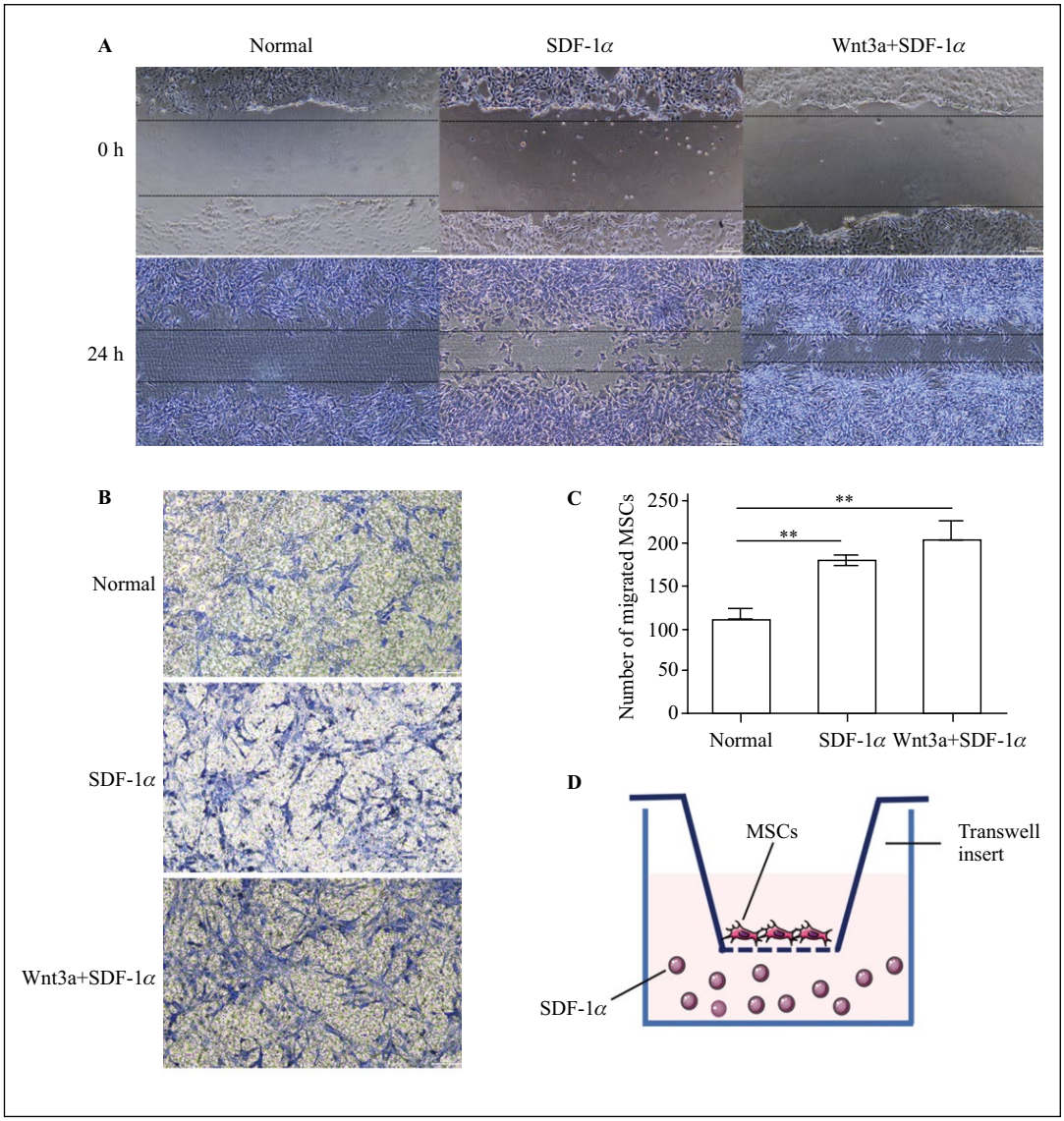

Figure 2. Wnt3a improved the migration ability of rat MSCs. 25 ng/mL SDF-1α was used to promote the migration of MSCs. **A.** The effect of 10 ng/mL Wnt3a on MSC migration in the scratch test at 0 h and 24 h. Scale bar: 100 μm. **B.** The effect of 10 ng/mL Wnt3a on MSC transwell migration capacity. Scale bar: 200 μm. **C.** Number of migrated MSCs. $^{**}p < 0.01$. **D.** A diagram of the transwell plate. All data are presented as mean ± SD (n = 3).

To investigate the role of Wnt/PCP pathway in MSC migration induced by SDF-1α, recombinant Wnt3a was added to the culture medium at the concentration of 10 ng/mL. As shown in Fig. 2A, 24 h after scratching in each group, the "wound" narrowed to some extent both in control and SDF-1α exposed cells compared with that at 0 h, indicating that MSCs had migration ability. The scratch tests showed that the combination of Wnt3a and SDF-1α had a stronger effect on MSC migration than the SDF-1α alone. Furthermore, transwell migration assays showed that MSCs migrated to the lower chamber more rapidly than control cells after being treated with SDF-1α or Wnt3a + SDF-1α (Fig. 2B, C). Such findings indicated that Wnt3a might provide benefit for the migration ability of MSCs compared with the control group.

Wnt3a activated the Wnt/PCP pathway in MSC migration

To further explore the mechanism involved in the Wnt/PCP pathway in the SDF-1α effects on MSC migration, Western blotting was used to detect the protein expression levels of RhoA, c-Jun, and ATF2 (Fig. 3A). We first characterized the effects of different concentrations of SDF-1α on the Wnt/PCP pathway in MSCs (Suppl. Fig. 2A). As shown

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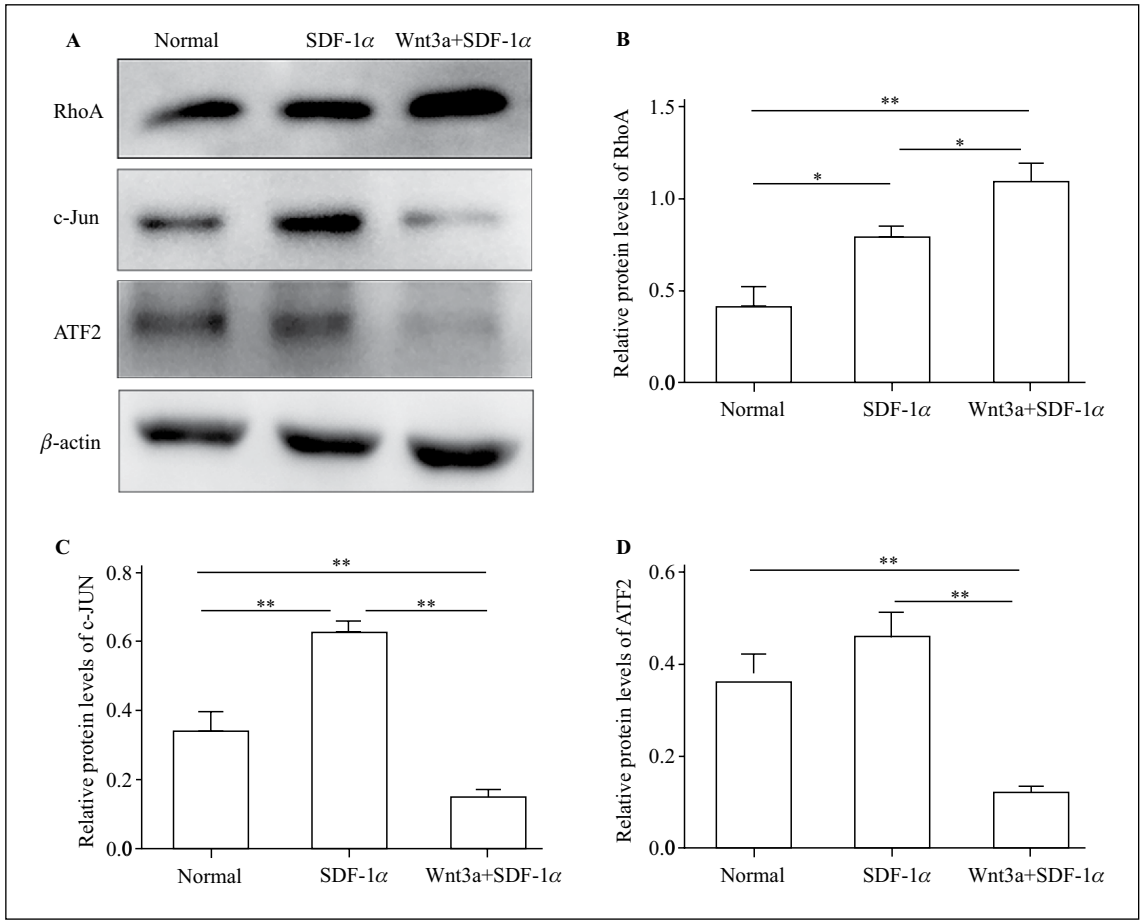

Figure 3. The effect of Wnt3a treatment of MSCs on RhoA, c-Jun, and ATF2 expression. Passage 3 cells were treated with 10 ng/mL Wnt3a or with PBS. **A.** Representative Western blots of RhoA, c-Jun, and ATF2 proteins. **B–D.** Expression levels of RhoA, c-Jun, and ATF2. $^{*}p < 0.05$, $^{**}p < 0.01$. All data are presented as mean ± SD (n = 3).

in Suppl. Fig. 2B, C, and D, RhoA, c-Jun, and ATF2 were significantly upregulated by 25 ng/mL SDF-1α. Fig. 3B, C, and D revealed a significant increase in RhoA and c-Jun levels in the MSCs of the SDF-1α group. Although the protein expression of ATF2 was not statistically significant ($P = 0.058$), there was a trend toward increased expression. Interestingly, Wnt3a treatment exacerbated the increase in RhoA but decreased the expression of c-Jun and ATF2 proteins.

MSCs can differentiate into neural stem cells and astrocytes

BME (1 mM) was used for pre-neural induction of MSCs. Twenty-four hours after pre-induction, there was no significant change in morphology compared with the control group (Fig. 4A). Within 3 hours (Fig. 4B, C) of neural induction (5 mM BME), the morphological changes became evident with a number of cells showing signs of cytoplasmic retraction toward the nucleus of the cell. The elongated membrane of 3 hours of induction produced a firm and contracted bipolar or multi-polar configuration. The cell bodies appear condensed and light refractive compared to the control group. However, cells were fell off and floated after 6 h of induction. Therefore, we chose 3-hour-long BME induction for the studies of neurally-differentiating MSCs.

To determine the ability of MSCs differentiated into neural cells, we evaluated a marker of neural stem cells, nestin, and markers of glial cells, GFAP, and Olig2. Immunofluorescence detection showed positive expression of nestin and GFAP, while negative expression of Olig2 after BME + Wnt3a induction (Fig. 5A, B, C). Our results revealed that MSCs can differentiate into neural stem cells and astrocytes and Wnt3a enhances BME-induced neurogenesis of MSCs.

Wnt3a regulates Wnt/PCP signaling pathway in MSC neural differentiation

To investigate the mechanism of Wnt3a on the neurogenesis of MSCs, we added human recombinant

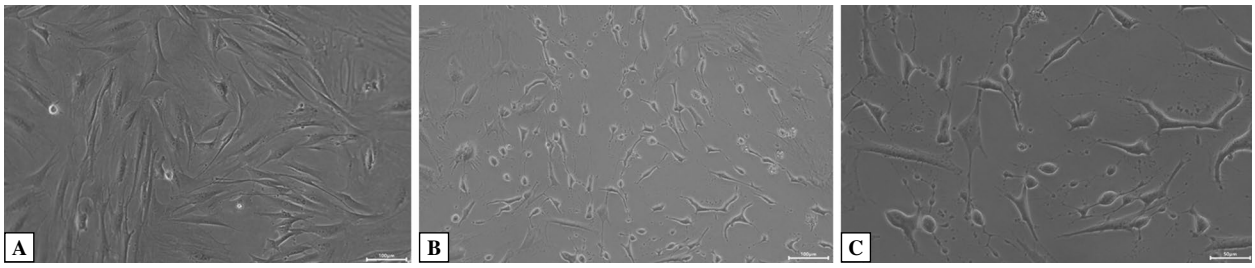

Figure 4. Morphology of MSCs after neural induction by β-mercaptoethanol (BME). **A.** Morphology of MSCs after 24 h of pre-induction. Scale bar: 100 μm. **B, C.** Morphology of MSCs after 3 h of BME-induction (5 mM). Scale bars:= 100 μm and 50 μm, respectively.

Wnt3a to BME-induced MSCs and subsequently analyzed the relative expression levels of Wnt/PCP pathway's proteins (Fig. 6A). The results of Western blotting analysis (Fig. 6B, C, D) showed that the expression of RhoA, c-Jun, and Wnt3a was significantly increased in the BME group compared with the control group. Moreover, Wnt3a upregulation of Wnt/PCP signaling pathway promoted the expression of all three proteins compared with the BME group. These results indicated that Wnt3a could activate Wnt/PCP pathway in MSC differentiation.

Discussion

MSCs are promising cell sources for tissue repair and regeneration because of their capacity of target homing, self-renewal, multipotent differentiation, and paracrine productions. MSC homing to damaged tissue is a key process for treatment. Despite the reported success, the amount of engrafted MSCs homing to target tissues is detected at only low frequencies [23, 24]. Besides, the differentiation of MSCs into neural cells makes them interesting and potentially useful for neural reconstitution in neurodegenerative diseases, stroke, and spinal cord injuries. Previous reports have indicated that the Wnt/β-catenin signaling pathway is involved in regulating MSC differentiation into neuron-like cells [16, 25]. However, the relationship between Wnt/PCP signaling pathway and neurogenesis in MSCs is unclear. Therefore, it is of great clinical significance to study the mechanisms of MSC migration and neural differentiation.

Trafficking MSCs from their niche to target tissues is a complex process that is driven by chemokines. SDF-1α is a small molecule cytokine that is a member of the chemokine protein family. It is an endogenous ligand for the chemokine receptor CXCR4. The commonly used methods to study MSC migration in vitro are transwell and scratch wound assays [26, 27]. Our data showed that SDF-1α enhanced the migration ability of MSCs (Suppl. Fig. 1). On the basis of these results, we explored the mechanisms of MSC migration induced by SDF-1α.

Previous work performed in mice and zebrafish has shown that the non-canonical Wnt/PCP pathway plays a major role in neural crest migration [28]. The Wnt/PCP pathway controls tissue polarity and cell movement partly through the activation of RhoA and c-Jun N-terminal kinase signaling cascades [29, 30]. Rho GTPase family includes small GTP binding proteins that act as a molecular switch in eukaryotic cells and control a wide range of signal transduction pathways [31]. Luo *et al.* found that RhoA was required for Jurkat cells (which belong to the human acute T lymphocyte leukemia cell line) chemotaxis in response to SDF-1 [32]. In addition, several studies have shown that CXCR4/SDF-1α axis could activate members of the FAK family of protein tyrosine kinases and further upregulate RhoA, which promotes cell contractility and migration in cancer cells [33, 34]. ATF2 and c-Jun are key components of activating protein-1 (AP-1) and function as homodimers or heterodimers [35]. Activation of AP-1 could induce cancer cell invasion [36]. Amin *et al*. suggested that IL-18 induced SDF-1α/CXCL12 up-regulation in rheumatoid arthritis synovial tissue fibroblasts could activate phosphorylation of ATF2 [37]. Another research demonstrated that SDF-1α activated the Rac/ERK and JNK signaling pathways in human lung fibroblasts, which in turn-initiated c-Jun phosphorylation and then promoted the expression of c-Jun [38]. Our findings of increased expression of RhoA, c-Jun, and ATF2 proteins in MSCs incubated with SDF-1α suggested that the Wnt/PCP signaling pathway may play an important role in the migration of MSCs triggered by SDF-1α.

Wnt3a is implicated in several key cellular processes, and its expression has been reported in different cell types. Our results are supported by the data shown by Shang *et al*. who indicated that Wnt3a signaling is also involved in the regulation of the proliferation and migration of MSCs [39]. Collectively, our data demonstrated a favorable promotional effect of SDF-1α

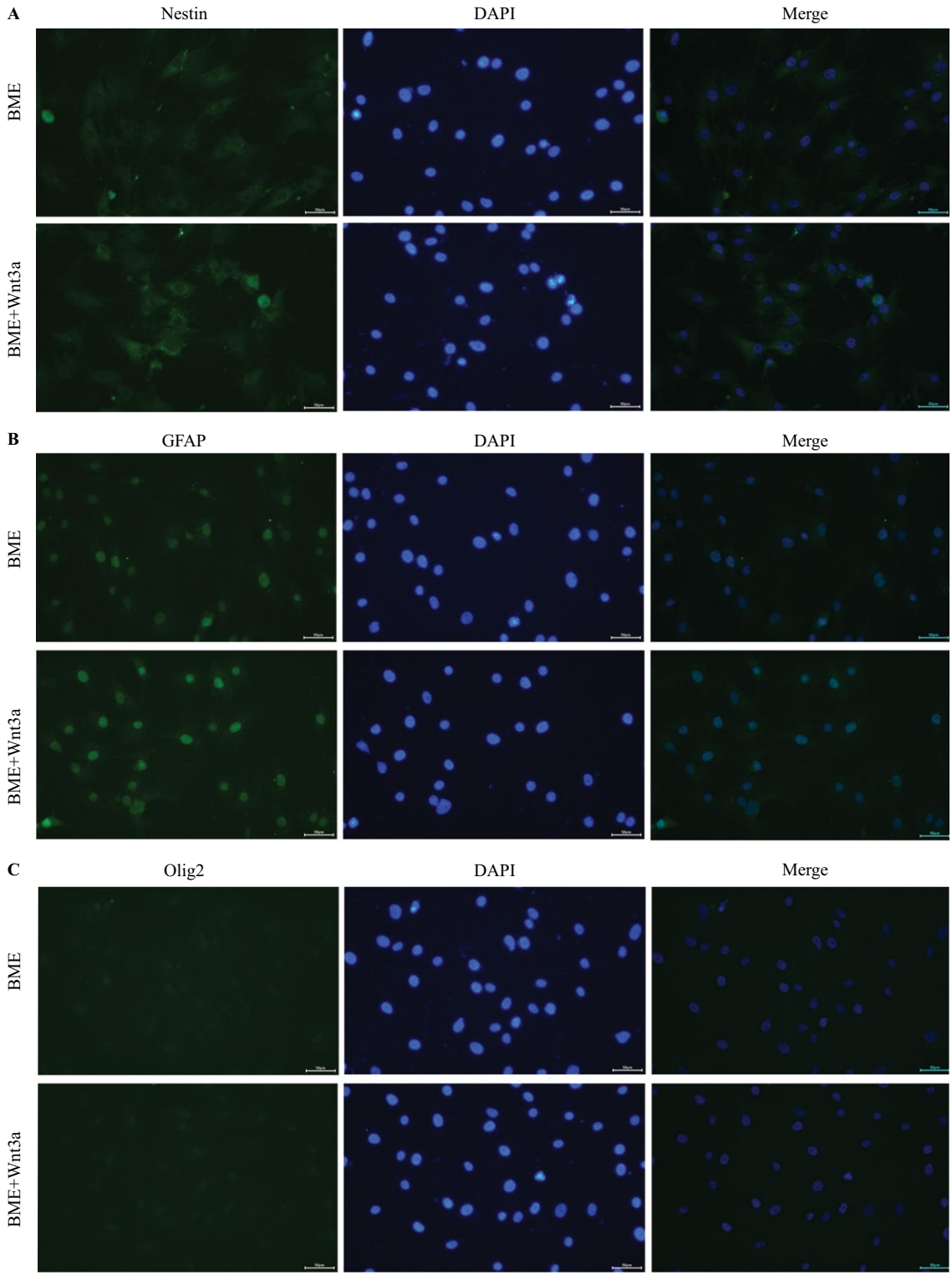

Figure 5. Neural differentiation of MSCs. **A.** Neural stem cells were identified by immunofluorescence staining for Nestin (green). **B.** Glial cells were identified by immunofluorescence staining for GFAP (green). **C.** Glial cells were identified by immunofluorescence staining for Olig2 (green). Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

on MSC migration. Growing evidence indicates that Wnt3a treatment or overexpression of Wnt3a in MSCs activated both the canonical and non-canonical Wnt pathways [40–42]. In the non-canonical signaling pathway, Wnt was known to activate Rho GTPases and induce cell migration [43]. Wnt3a has also been shown

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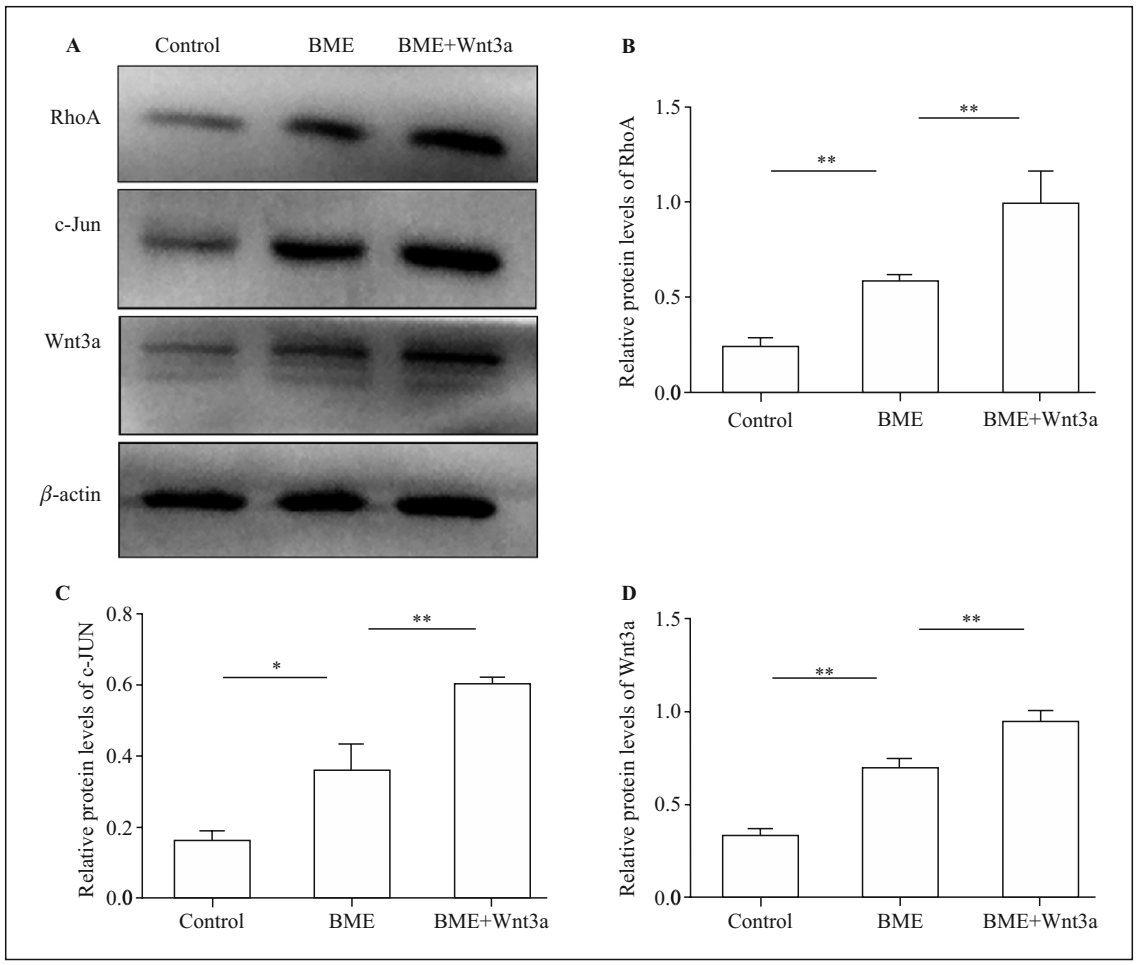

Figure 6. Wnt3a regulates Wnt/PCP signaling pathway in neurally differentiated MSCs. **A.** Representative Western blots of RhoA, c-Jun, and Wnt3a proteins. **B–D.** Expression levels of RhoA, c-Jun, and Wnt3a proteins. $^{*}p < 0.05$, $^{**}p < 0.01$. All data are presented as mean ± SD (n = 3).

to stimulate RhoA GTPase [44]. In the present study we demonstrated that in MSCs treated with Wnt3a RhoA protein level was increased whereas c-Jun and ATF2 protein levels were decreased. Together, these observations suggest that Wnt/PCP pathway promotes MSC migration in a signaling pathway involving RhoA, c-Jun, and ATF2.

Neuronal differentiation of MSCs has been achieved through a wide range of approaches, including chemical induction [45], gene transfection [46, 47], and the use of conditioned media from rodent brains [47]. Neuronal differentiation of MSCs by chemical induction is typically promoted using antioxidants including β-mercaptoethanol, dimethyl sulfoxide, and butylated hydroxyanisole [48]. In the present study, BME was used to induce differentiation of MSCs into nerve cells. Neuron- and astrocyte-specific markers, including Nestin, GFAP, and Olig2 were used to confirm the differentiation of neuronal lineages. Nestin is classically considered as a specific marker of neural stem cells. GFAP and Olig2 are glia markers. MSCs could express neural markers such as nestin or GFAP, associated with remarkable morphological modifications.

Wnt signaling not only regulates embryonic development and adult homeostasis but also controls several processes in adult stem cells. Several previous studies have focused on regulatory mechanisms among Wnts and osteogenesis [49], chondrogenesis [50], adipogenesis [51], and myogenesis. Canonical Wnt/β-catenin signaling system is an important pathway in the development of the central nervous system [52]. Since research on non-canonical *Wnt* signaling pathway in MSC neural differentiation are quite rare, in the present study we investigated the role of the Wnt/PCP pathway in BME-induced differentiation of MSCs into neural cells. Because we found that the relative protein levels of RhoA, c-Jun, and Wnt3a were activated after BME induction, we

examined whether Wnt3a-upregulated neurogenic differentiation involves non-canonical Wnt signaling. Our demonstration that Wnt3a markedly increased RhoA, c-Jun, and Wnt3a protein expression in the process of BME-induced MSCs indicates that Wnt3a facilitates neural differentiation in MSCs.

There are several limitations to this study. First, a shorter treatment of BME may initiate the induction toward a neurogenic differentiation of MSCs, while exposure of more than 6 h to the BME-containing media might be a stress-inducing environment to MSCs since we found cells to fell off and float. Second, we did not examine whether MSCs were differentiated into neurons. Furthermore, additional experiments are needed to examine whether the Wnt/PCP pathway was involved in MSCs migration and neural differentiation in proper animal models. Nevertheless, the present results add many other reports that encourage the potential future use of MSCs as a cellular therapy.

In summary, this study demonstrated that Wnt3a promoted MSC migration and neural differentiation through Wnt/PCP pathway. Our data broaden the knowledge of molecular mechanisms involved in the migration and neural differentiation of MSCs and may be of help to improve the therapeutic effect of MSCs in the future.

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Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

Authors' contributions

QY conceived the idea and designed the experiments. LPZ, PPY and LJZ performed the experiments and wrote the manuscript. XYZ and LLW performed the western blotting assay. JXL, YYC and HMS collected and analyzed the data. All authors read and approved the final manuscript.

Conflicts of interest

The authors report no conflicts of interest.

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