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ORIGINAL RESEARCH

Stichopus japonicus Polysaccharide, Fucoidan, or Heparin Enhanced the SDF-1α/CXCR4 Axis and Promoted NSC Migration via Activation of the PI3K/Akt/FOXO3a Signaling Pathway

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Abstract *Stichopus japonicus* Polysaccharide (SJP) is a sulfated polysaccharide from the body wall of the sea cucumber, *Stichopus japonicus*. Fucoidan is a heparinoid compound that belongs to a family of sulfated polyfucose polysaccharides. Heparin is a glycosaminoglycan. SJP, fucoidan, and heparin profoundly promoted stromal cell-derived factor 1 alpha (SDF-1α)-induced neural stem cell (NSC) migration in a concentration-dependent manner. In addition, the basal migration capacity of cells was significantly promoted after incubation with SJP, fucoidan, or heparin. Interaction of SJP, fucoidan, or heparin with SDF-1α efficiently showed additive effects on the promotion of cell migration from the neurosphere. SJP, fucoidan, or heparin interaction with SDF-1α treatment could increase Nestin expression. SDF-1α modulated by SJP, fucoidan, or heparin activated the CXCR4 receptor and directed cellular migration via the activation of the PI3K/Akt/FOXO3a signaling pathway. Moreover, interaction of SJP, fucoidan, or heparin with SDF-1α effectively promoted NSC migration and induced SDF-1α and CXCR4 expressions. Results suggested that SJP, fucoidan, and heparin might be good candidates for alleviating injury-initiated signals to which NSCs respond.

Keywords NSC · SJP · Fucoidan · Heparin · SDF-1α · CXCR4

Introduction

Neurological disability remains a challenge in cerebrovascular disease treatment despite significant advances in our understanding of stroke pathophysiology (Corbett et al. 2014). A growing number of studies highlight the potential of stem cell transplantation as a novel therapeutic approach for stroke (Bliss et al. 2007). Neural stem cells (NSCs) that possess multiple differentiation potentials and self-renewal ability can differentiate into mature neurons and glial cell lines in the injury region and organically integrate with the host's nervous system. Thus, NSCs can effectively treat the subacute phase and chronic injury of nerve function (Pluchino et al. 2005). Undifferentiated NSCs can lead to regenerative neurogenesis in various central nervous system (CNS) diseases (Teng et al. 2002). Cell therapy is safe and feasible, but the mechanisms underlying functional regeneration remain elusive (Horner and Gage 2000).

The therapeutic benefit of cell therapy critically depends on the appropriate migration and homing of the injected cells. Migration is a critical process required for stem cell recruitment to the target area. The chemokine stromal cell-derived factor 1 alpha (SDF-1α, CXCL12) and its receptor CXCR4 play important roles in brain and CNS development, and can possibly regulate migration during neurogenesis repair (Peng et al. 2007). SDF-1α promotes neural precursor cell (NPC) migration both in vitro (Liu et al. 2008) and in vivo to sites of focal cerebral ischemia (Robin et al. 2006), to areas damaged after a traumatic brain injury (Itoh et al. 2009), or to sites of acute neuroinflammatory disease (Carbajal et al. 2010). SDF-1α/CXCR4 axis can help promote NSC migration in neural regeneration.

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SJP is a sulfated polysaccharide isolated from the sea cucumber *Stichopus japonicus*. SJP promotes neurosphere migration in a dose-dependent manner (Sheng et al. 2012). Fucoidan, a heparinoid vegetal compound extracted from the cell wall of brown algae, belongs to a family of sulfated polyfucose polysaccharides (Huang and Liu 2012). Fucoidan enhances the effect of released SDF-1α on the mobilization of hemopoietic stem/progenitor cell (Sweeney and Papayannopoulou 2001). Fucoidan can mobilize SDF-1α, thereby promoting therapeutic revascularization (Luyt et al. 2003). Heparin inhibits the formation of tumor cell lines by disrupting the interaction of SDF-1α and CXCR4 (Harvey et al. 2007; Ma et al. 2012). Heparin can interact with specific cell-surface receptors to direct cellular responses of NPCs (Brickman et al. 1995). However, the influence of SJP, fucoidan, and heparin on the migration and the homing function of cells used for NSC cell therapy are unknown.

Phosphatidylinositol 3-kinase (PI3K) is widely involved in SDF-1α/CXCR4-mediated chemotaxis and plays crucial roles in the regulation of multiple stem cell migration (Yu et al. 2010; Zheng et al. 2007). PI3K functions early in intracellular signal transduction pathways and affects many biological functions. PI3K signaling contributes to cell migration, and coordinates the localization and function of effector lipids through pleckstrin homology (PH) domains, such as protein kinase Akt. Phosphorylation of Thr^{308} and Ser^{473} is sufficient to fully activate Akt (Downward 1998). FOXO3a is one of the FOXO subclass of Forkhead transcription factors, and it is a downstream signaling molecule of Akt, which is involved in cell migration. The activation of the PI3K/Akt/FOXO3a signaling pathway occurs due to the presence of the G protein-coupled receptor. CXCR4 is a G protein-coupled receptor. SDF-1α is modulated by polysaccharides, activates CXCR4 receptor, and directs cellular migration. However, whether or not PI3K/Akt/FOXO3a pathway is involved in NSC migration mediated by SJP, fucoidan, or heparin and in the SDF-1α/CXCR4 axis is unclear. In this report, we focused on the regulatory activity of molecular regulators of SDF-1α/CXCR4 on NSC migration. The biochemical mechanisms underlying the activities of SJP, fucoidan, and heparin were also investigated.

Experimental Procedures

Source of SJP, Fucoidan, and Heparin

SJP was isolated from the sea cucumber *Stichopus japonicus* from Weihai Marine Aquaculture WulingDe Holy Margin Co. at Weihai in the Shandong Province in China, as described previously (Sheng et al. 2012). The weight-average molecular weight of SJP was 1.79×10^5 Da as determined by HPSEC analysis. Fucoidan and heparin were purchased from Sigma (USA).

Animals

Pregnant Wistar rats were purchased from the Animal Center of Shandong University (Jinan, China). The animals were housed under controlled temperature (22 ± 1 °C) and a 12 h/12 h light/dark cycle with food and water available. The use of experimental animals in this study was conducted in accordance with the ethical guidelines of the Shandong University animal experimentation committee.

Neural Stem Cells Isolation, Culture, and Differentiation

NSCs were obtained from the cerebral cortex of 14-day-embryonic Wistar rats. The cerebral cortex was carefully isolated from adjacent tissues by syringes, and collected in cold, serum-free medium consisting of DMEM/F-12 nutrient (1:1; Invitrogen, USA). The tissue was digested with 0.125 % trypsin (1:250; Invitrogen) for 20 min, and mechanically dissociated using a fire-polished pipette. The dissociated cells were suspended with basal medium (DMEM/F-12 plus B27; Invitrogen) supplemented with 20 ng/ml basal fibroblast growth factor (bFGF, Invitrogen) and 20 ng/ml epidermal growth factor (EGF, Sigma) and cultured in uncoated dishes without serum. NSCs were examined by immunocytochemical staining. Over 90 % of cells expressed the neural stem cell marker, Nestin. Less than 6 % of cells expressed the neuronal marker, β-III-tubulin (TUJ-1); less than 3 % of cells expressed the astrocyte marker, glial fibrillary acidic protein (GFAP). For neuronal differentiation, dissociated NSCs were plated on poly-L-lysine-coated cell culture dishes (Costar, USA) with basal medium supplemented with 20 ng/ml bFGF and 20 ng/ml EGF. For astrocyte differentiation, dissociated cells were cultured in basal medium supplemented with 20 ng/ml bFGF and 20 ng/ml EGF and 10 % fetal bovine serum (FBS). Cells were grown in differentiation media for 1–10 days before analysis.

Quantitative Cell Migration Assay

The migration response of 1×10^5 NSCs to different experimental groups was evaluated using a modified 24-well microchemotaxis transwell system. For some experiments, 100 ng/mL SDF-1α was added to the lower chamber. After 24 h of incubation at 37 °C, transmigrated cells were counted. The experiments were done in triplicate. The number of cells actively crossing the membrane is interpreted to represent their migratory capacity.

Cell Viability Assay

Cell viability assay was performed according to the manufacturer's instruction of CCK-8 kit (Dojindo). NSCs were plated in 96-well plates at a density of 5000 cells/well. When cells were grown to 70–80 % confluence, SJP, fucoidan, and heparin was added. After 24 h of incubation at 37 °C, CCK-8 solution at a dilution of 1/10 culture medium was added to each well and the cells were incubated for 4 h at 37 °C. Absorbance at 450 nm was measured with a microplate reader (Biotek, MQX200).

$$\text{Cell viability } (\%) = \left(\text{OD}_{\text{treatment group}} - \text{OD}_{\text{blank}}\right) / \times \left(\text{OD}_{\text{control group}} - \text{OD}_{\text{blank}}\right) \times 100$$

Hoechst33342/PI Assay

Cell apoptosis was analyzed by staining with Hoechst33342 and propidium iodide (PI) staining. NSCs cells were collected, washed in PBS and resuspended in 0.8–1 ml of cell staining buffer containing 5 μl of Hoechst33342 and 5 μl of PI incubated for 20–30 min at 4 °C in the dark, washed in PBS and smeared. The red fluorescence and the blue fluorescence were observed under the fluorescence microscope.

Neurosphere Culture and Treatment

Neural stem cells proliferate to form neurosphere after 7 days. Then, neurosphere was seeded onto poly-L-lysine-coated cell culture dishes (Costar, USA) in basal medium. Neurosphere was treated with 20 μM LY294002 (Abcam, England) for 1 h before exposed to 100 ng/ml SDF-1α and 80 μg/ml SJP, 10 μg/ml fucoidan, or 10 μg/ml heparin for 24 h. The migrating distance of the cells was measured from the edge of the sphere, using the NIH image J software.

Immunofluorescence Staining

Plated cells were fixed with pre-cooled methanol/acetone (v/v, 1:1) at room temperature for 5 min and rinsed in PBS. Fixed cells were blocked in 10 % goat serum (Boster, China) with 0.1 % Triton X-100 (Aplichem, Germany) for 30 min, then incubated with primary antibodies overnight at 4 °C. Primary antibodies included anti-Nestin (1:400, milipore, USA), anti-beta III Tubulin (1:500, Abcam, England), anti-GFAP (1:400, milipore, USA), anti-BrdU (1:500, Abcam, England), anti-SDF-1α (1:500, Abcam, England) and anti-CXCR4 (1:400, Abcam, England). After three washes in PBS, cells were incubated with fluorescein-labeled (FITC) or rhodamine-labeled (TRITC) antibody (1:50, KPL, USA) for 1 h at room temperature. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Applichem, Germany). Samples were observed using a fluorescence microscope with a high-resolution digital camera (DMRA, Q-fish system, Leica, Germany). Negative controls were incubated with non-immune serum and with an appropriate secondary antibody.

Isolation of RNA and Reverse Transcription-PCR

Total RNA was extracted from NSCs by using TRIZOL Reagent (Invitrogen, USA). RNA was reversely transcribed into cDNA using the RevertAid^{TM} First strand cDNA Synthesis Kit (Fermentas, Canada). Briefly, 4 μl of total RNA was added as template for cDNA synthesis in the presence of 1 μl M-MuLV reverse transcriptase (200 U), 1 μl of oligo $(dT)_{18}$ primer, 4 μl of 5 × reaction buffer, 1 μl RiboLock^{TM} RNase Inhibitor (20 U), 2 μl of dNTP mix (10 mM each), and 7 μl of nuclease-free water. After incubation for 60 min at 42 °C, the reverse transcriptase was inactivated at 70 °C for 5 min, and cDNAs were stored at −40 °C until further analysis. The sequences of primers, annealing temperature and size of PCR product were on the Table 1.

Table 1 Primers for RT-PCR

Molecules	MW	Locus	Primer Sequence	Tm (°C)
Nestin	220	NM_012987.1	F 5′-CAACCACAGGAGTGGGAACT-3′	58
			R 5′-TCTGGCATTGACTGAGCAAC-3′	
SDF-1α	240	NM_022177.3	F 5′-TCTTTGGCCTCCTGTAGAATGG-3′	50
			R 5′-TCACGGCAAGATTCTGGCTTA-3′	
CXCR4	241	NM_022205.3	F 5′-CGTGAATGAGTGTCTAGGCAGG-3′	51
			R 5′-GGCTTTGGTTTTAAGTGCCATC-3′	
β-actin	254	NM_031144.3	F 5′-AGACCTTCAACACCCCAG-3′	56
			R 5′-CACGATTTCCCTCTCAGC-3′	

PCR products were subjected to 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. The images were digitally captured (Gel Doc 2000; Bio-Rad, USA)

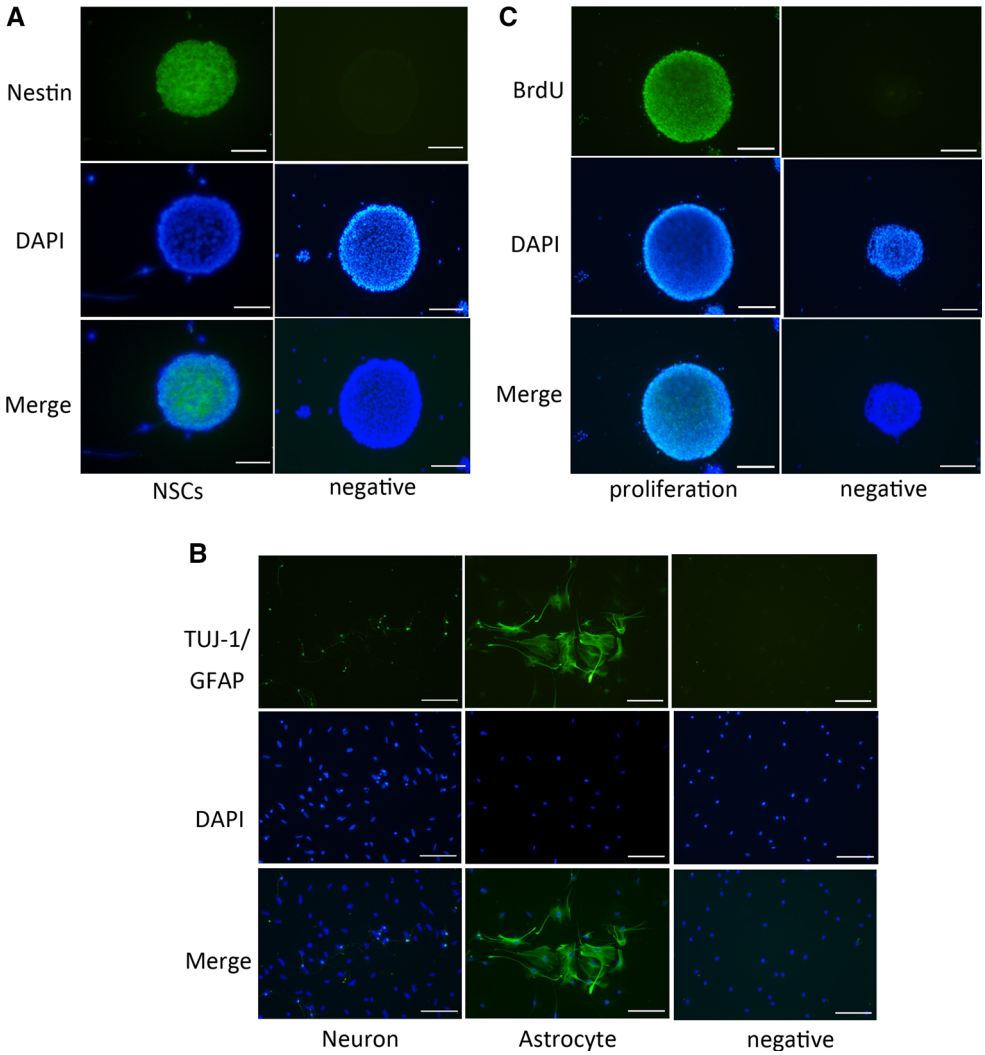


Fig. 1 NSC identification. **a** NSCs were stained with Nestin (*green*), and cell nucleus was stained with DAPI (*blue*). Negative controls were incubated with non-immune serum and with the appropriate secondary antibody. *Scale bar* = 100 μm. **b** NSCs were induced to differentiate into neurons or astrocytes by culturing in DMEM/F-12 supplemented with bFGF and EGF or in DMEM/F-12 supplemented with bFGF and EGF and 10 % FBS, respectively, for 7 d. The phenotypes of the differentiated cultures were verified by immunocytochemistry. Cells were stained with the neuronal precursor marker β-tubulin III (*green*) and the astrocyte specific maker GFAP (*green*). Cell nucleus was stained with DAPI (*blue*). Negative controls were incubated with non-immune serum and with the appropriate secondary antibody. *Scale bar* = 100 μm. **c** NSCs were stained with BrdU (*green*), and cell nucleus was stained with DAPI (*blue*). Immunofluorescence staining was used to analyze NSC proliferation. Negative controls were incubated with non-immune serum and with the appropriate secondary antibody. *Scale bar* = 100 μm (Color figure online)

Western Blot Analysis

Cells were homogenized in RIPA lysis buffer containing protease inhibitor PMSF. Total protein was measured by BCA (Pierce) and size separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted to polyvinylidene difluoride membranes (Amersham Biosciences). Blots were incubated with antibodies against Nestin (1:500, milipore, USA), SDF-1α (1:1000, abcam), CXCR4 (1:1000, abcam), phosphorylated FOXO3a (1: 500, Cell Signaling, USA), FOXO3a (1:500, Cell Signaling, USA), phosphorylated Akt (ser473, 1:500, Cell Signaling, USA), Akt (1:500, Cell Signaling, USA), and β-actin (1:1000, abcam). Goat anti-rabbit IgG and goat anti-mouse IgG (abcam) were added and the blots were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Statistical Analyses

All results are expressed as mean ± SEM. For multiple comparisons, the statistical analysis was performed by using one-way ANOVA followed by the Tukey's multiple comparison tests. Results were considered significant when $P < 0.05$.

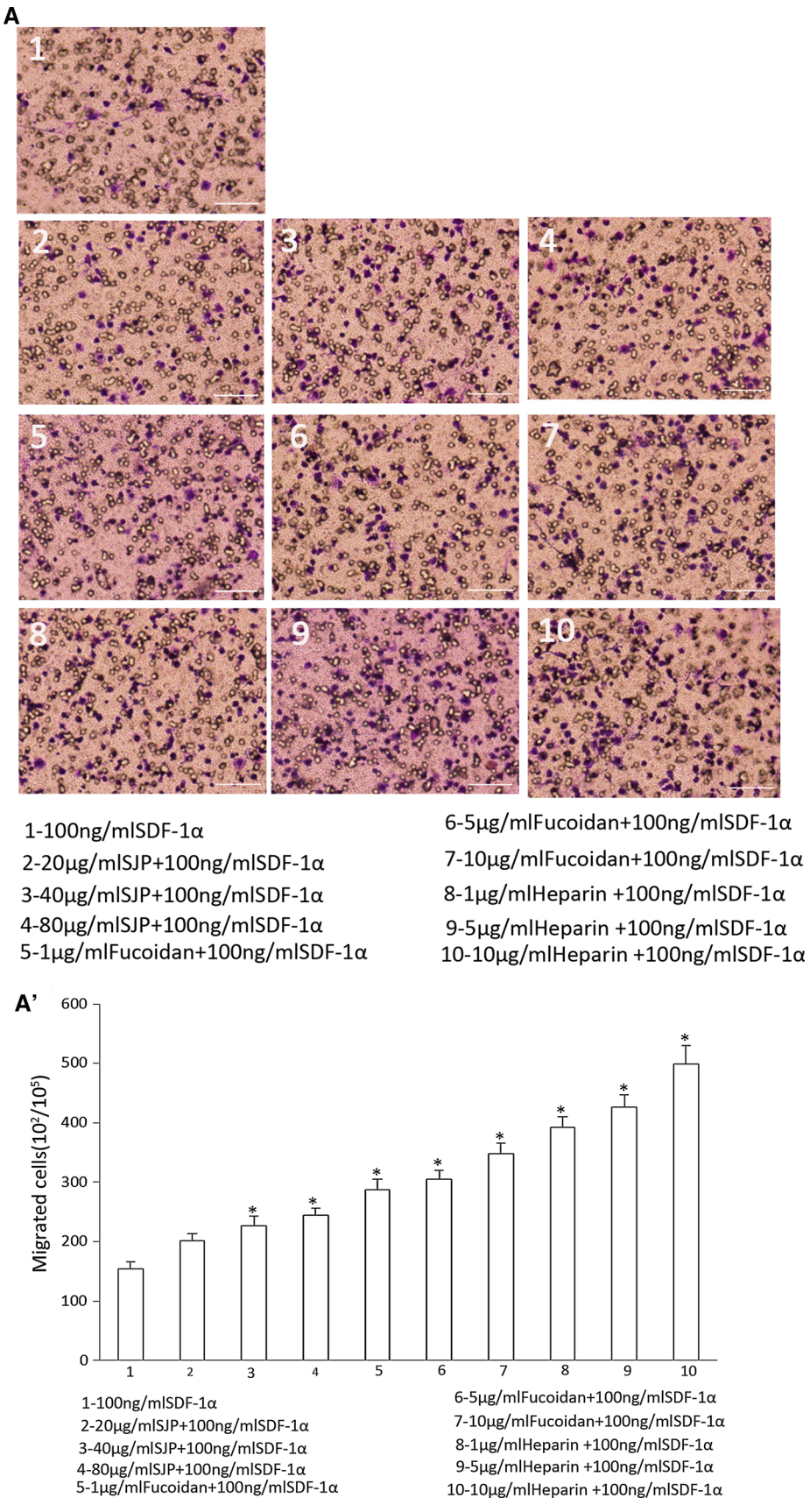
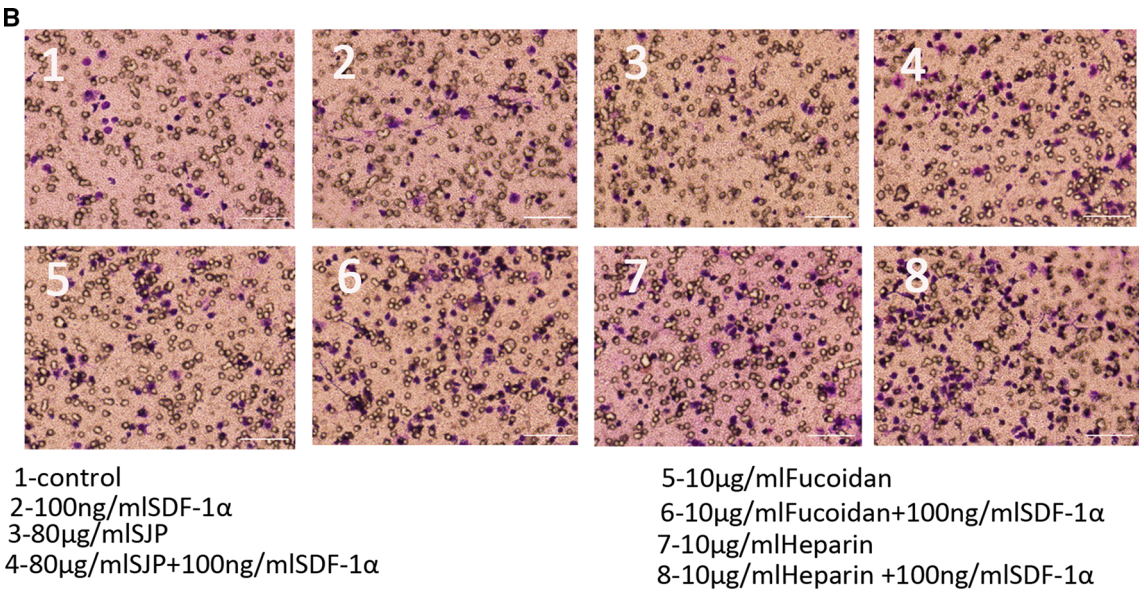
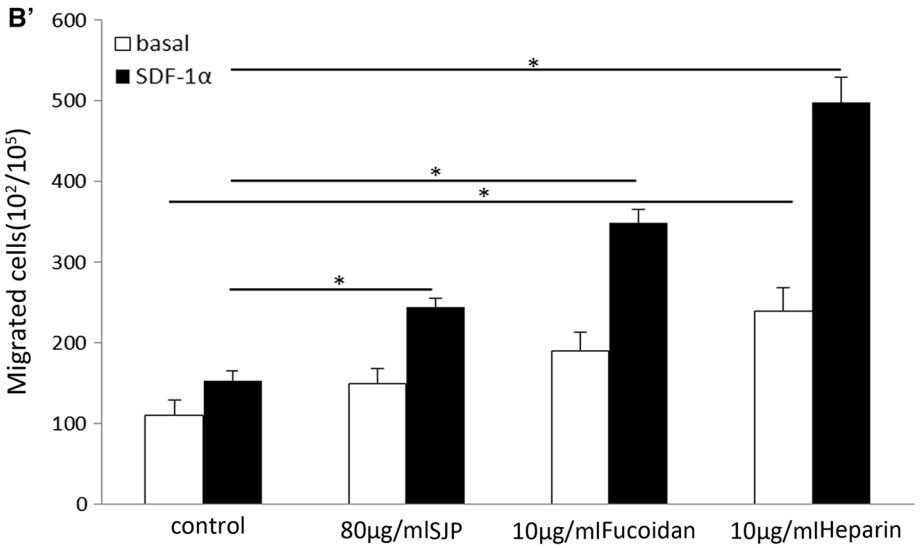
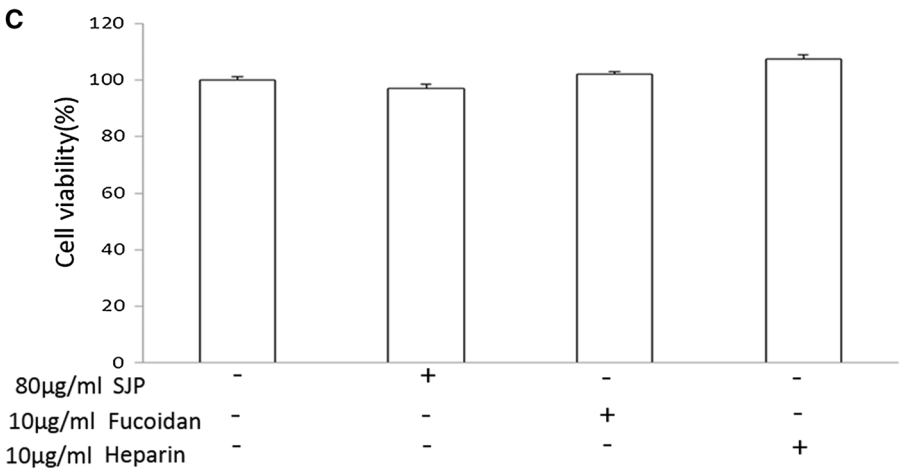


Fig. 2 Interaction of SJP, fucoidan, or heparin with the SDF-1α/CXCR4 axis promoted the functional capacity of NSCs. **a** SDF-1α-stimulated migration capacity of 1×10^5 total NSCs. NSCs were pre-incubated with SJP, fucoidan, or heparin at the specified concentrations. *Scale bar* = 50 μm. **a′** Quantification of migration capacity of NSCs. $n = 3$. $^*P < 0.05$ versus SDF-1α group. **b** Basal and SDF-1α-stimulated migration capacity of 1×10^5 total NSCs. NSCs were pre-incubated with 80 μg/ml SJP, 10 μg/ml fucoidan, or 10 μg/ml heparin. *Scale bar* = 50 μm. **b′** Quantification of Quantification of migration capacity of NSCs. $n = 3$. $^*P < 0.05$. **c** NSCs were incubated with 80 μg/ml SJP, 10 μg/ml fucoidan, or 10 μg/ml heparin for 24 h. Cell viability was examined by CCK-8. Data are presented as mean ± SEM. $n = 3$. **d** NSCs were incubated with 80 μg/ml SJP, 10 μg/ml fucoidan, or 10 μg/ml heparin for 24 h. Cells were stained with Hoechst33342/propidium iodide (PI), and analyzed by fluorescence microscope. *Scale bar* = 100 μm. **d′** Quantification of NSC cell apoptosis. Representative percentages of PI^+ are shown. Data are presented as mean ± SEM of at least 5 views. $n = 3$







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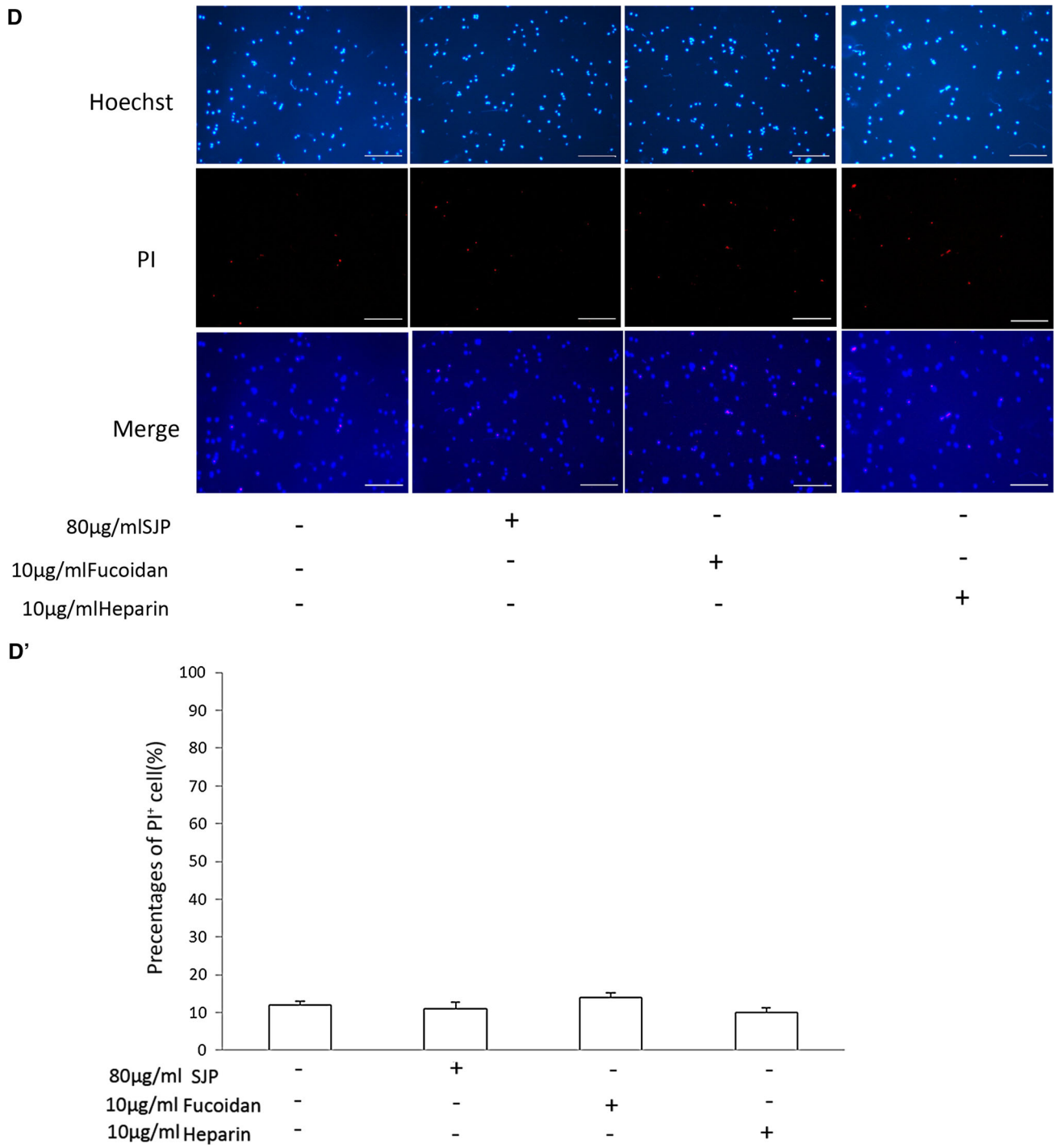


Fig. 2 continued

Results

Characterization of NSCs

NSCs can be homogenously expanded while maintaining high expression of pan-neural markers, such as Nestin (Brafman 2014). These cells differentiate to the majority of β-tubulin III (TUJ-1) positive neuronal population in DMEM/F-12 supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Alternatively, they differentiate to the majority of GFAP astrocyte population in DMEM/F-12 supplemented with bFGF and EGF and 10 % FBS. The Nestin of NSCs (Fig. 1a), TUJ-1, and GFAP (Fig. 1b) were verified by

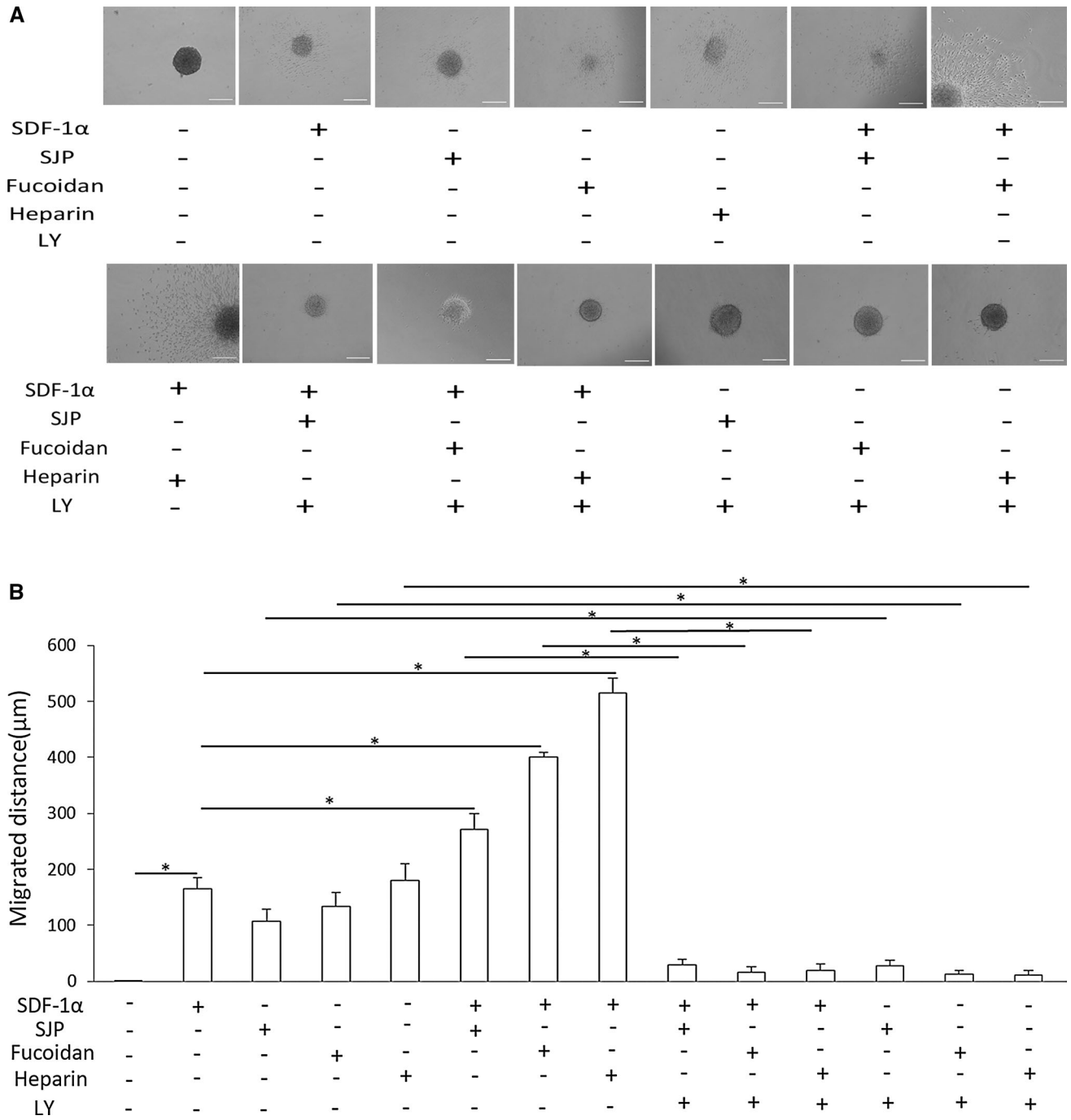


Fig. 3 Effects of SJP, fucoidan, or heparin and SDF-1α treatment on neurosphere migration. **a** Representative photomicrographs of the neurosphere migration are shown. *Scale bar* = 100 μm. **b** Migration distance from the rim of the sphere to the farthest migrated cell was quantified. Data are presented as mean ± SEM of at least 10 neurosphere. $n = 3$. $^*P < 0.05$

immunocytochemistry. Bromodeoxyuridine (BrdU) is a thymidine analog that combines with DNA during the mitotic phase. Thus, NSC proliferation is demonstrated. Immunocytochemistry results showed that BrdU was positive, and that NSCs could proliferate (Fig. 1c). Cells were obtained from the cerebls showed NSC characteristics.

Interaction of SJP, Fucoidan, or Heparin with the SDF-1α/CXCR4 Axis Promoted Migration and Homing of NSCs

NSCs were incubated with three different pharmacologically relevant concentrations of SJP, fucoidan, or heparin

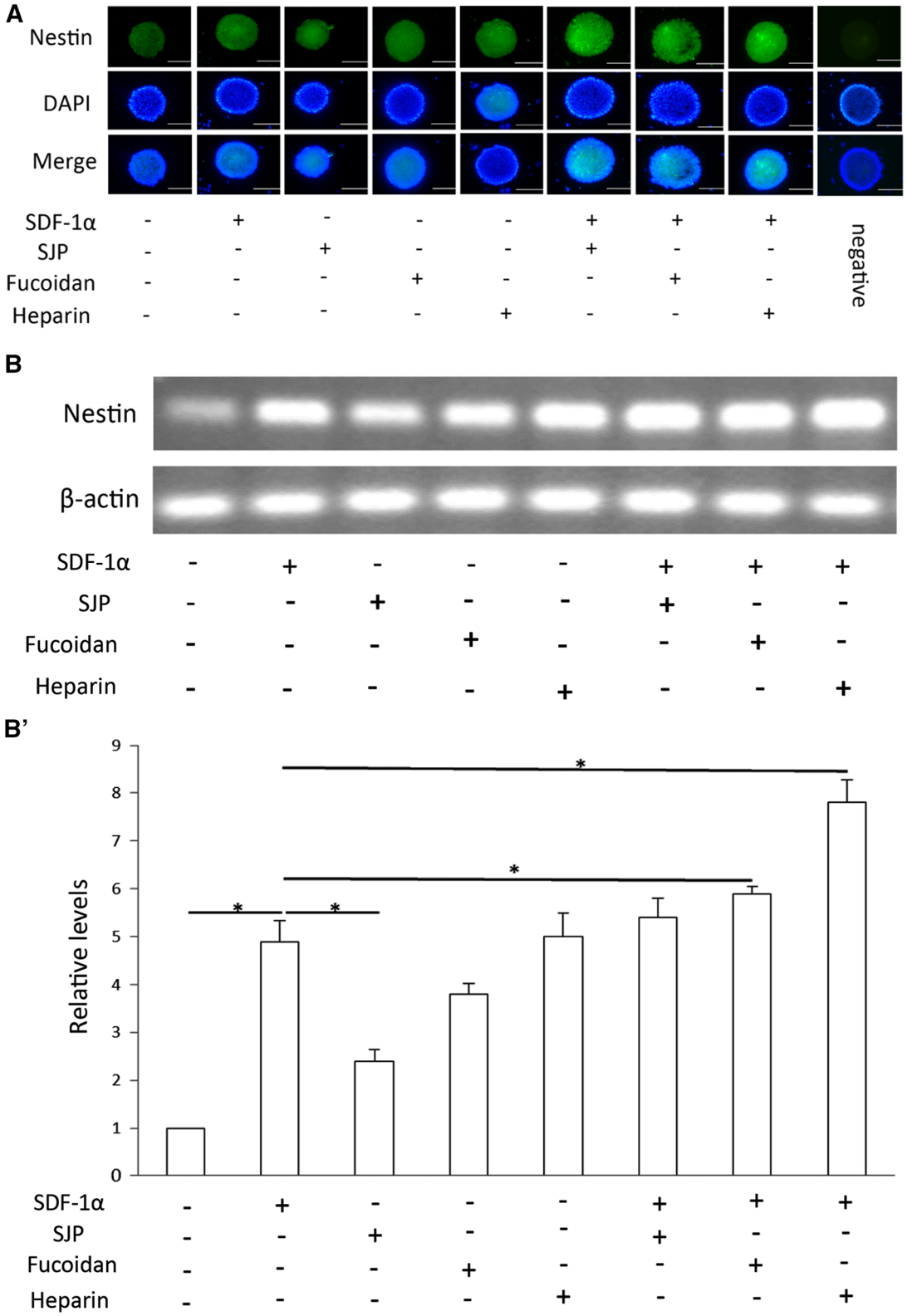


Fig. 4 SJP, fucoidan, or heparin increased Nestin expression. **a** Representative photographs were obtained from neurosphere immunostained by an anti-Nestin antibody. Immunocytochemical analysis of neurosphere was performed by using Nestin (*green*). Nuclei were counterstained with DAPI (*blue*). Data are presented as mean ± SEM of at least 10 neurosphere. $n = 3$. *Scale bar* = 100 μm. **b** RT-PCR was performed using total RNA to analyze mRNA level of Nestin. **b′** Quantitative analysis of mRNA level of Nestin in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$. **c** Western blot analysis of Nestin is shown. **c′** Quantitative analysis of protein levels in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$ (Color figure online)

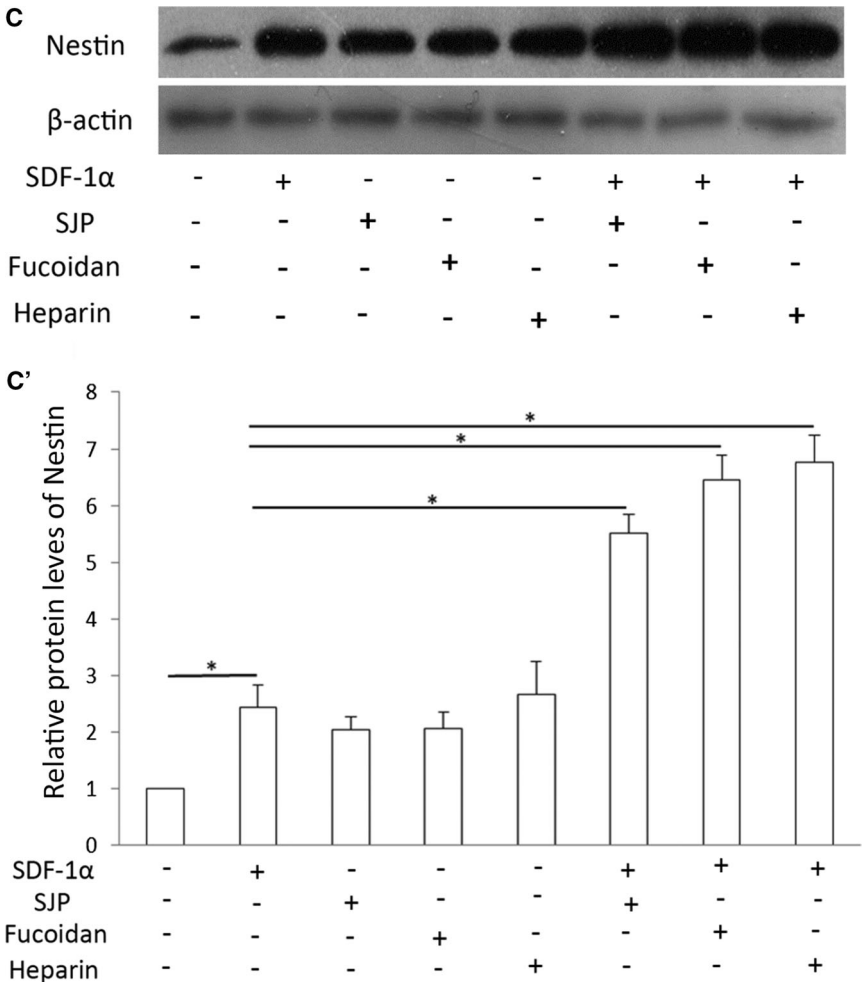


Fig. 4 continued

and 100 ng/ml SDF-1α. Chemotaxis assays were performed using transwell cell chambers to detect NSC migration. SJP, fucoidan, and heparin profoundly promoted SDF-1α-induced NSC migration in a concentration-dependent manner (Fig. 2a, a′). In addition, the basal migration capacity of cells was significantly promoted after incubation with SJP, fucoidan, or heparin (Fig. 2b, b′). To exclude proliferation effects, cell viability was examined by CCK-8. No significant difference in proliferation was observed between NSCs pre-incubated with SJP, fucoidan, or heparin and untreated control cells (Fig. 2c). To eliminate toxic drugs, we measured the cell apoptotic rate by fluorescence microscopy and staining with Hoechst33342 and PI. No significant difference in apoptosis and necrosis was observed between NSCs pre-incubated with SJP, fucoidan, or heparin and NSCs (Fig. 2d, d′).

Interaction of SJP, Fucoidan, or Heparin with the SDF-1α Induced Migration of Neurosphere

NSCs grown as neurosphere normally undergo a unique form of tangential migration (known as chain migration) from the spheres during CNS development, and the migrating cells showed a simple unipolar or bipolar morphology (Jacques et al. 1998). Exposure of SDF-1α to NSCs promoted cell migration. Whole neurosphere was isolated, plated on poly-L-lysine-coated cell culture dishes, and cultured in basal medium with SJP, fucoidan, or heparin and 100 ng/ml SDF-1α. The untreated group failed to attach to the bottom of the plate and failed to display cell migration. SDF-1α alone was sufficient to increase neurosphere migratory distance. However, SJP, fucoidan, or heparin interaction with SDF-1α treatment

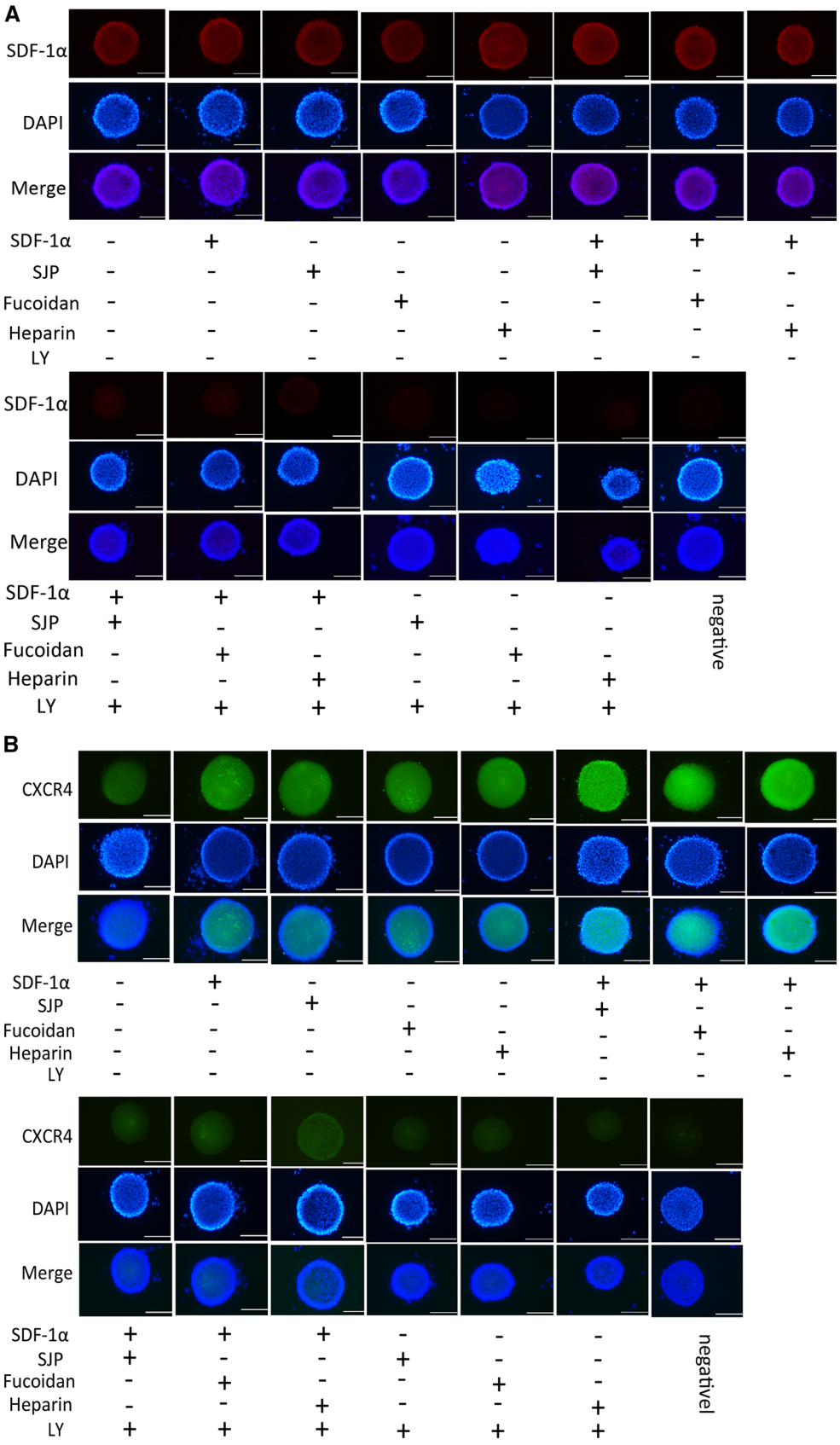


Fig. 5 SJP, fucoidan, or heparin increased SDF-1α and CXCR4 expressions by immunocytochemical analysis. **a** Representative photographs were obtained from neurosphere immunostained by an anti-SDF-1α antibody. Immunocytochemical analysis of neurosphere was performed using SDF-1α (*red*). Nuclei were counterstained with DAPI (*blue*). Data are presented as mean ± SEM of at least 10 neurosphere. $n = 3$. *Scale bar* = 100 μm. **b** Representative photographs were obtained from neurosphere immunostained by an anti-CXCR4 antibody. Immunocytochemical analysis of neurosphere was performed by using CXCR4 (*green*). Nuclei were counterstained with DAPI (*blue*). Data are presented as mean ± SEM of at least 10 neurosphere. $n = 3$. *Scale bar* = 100 μm (Color figure online)

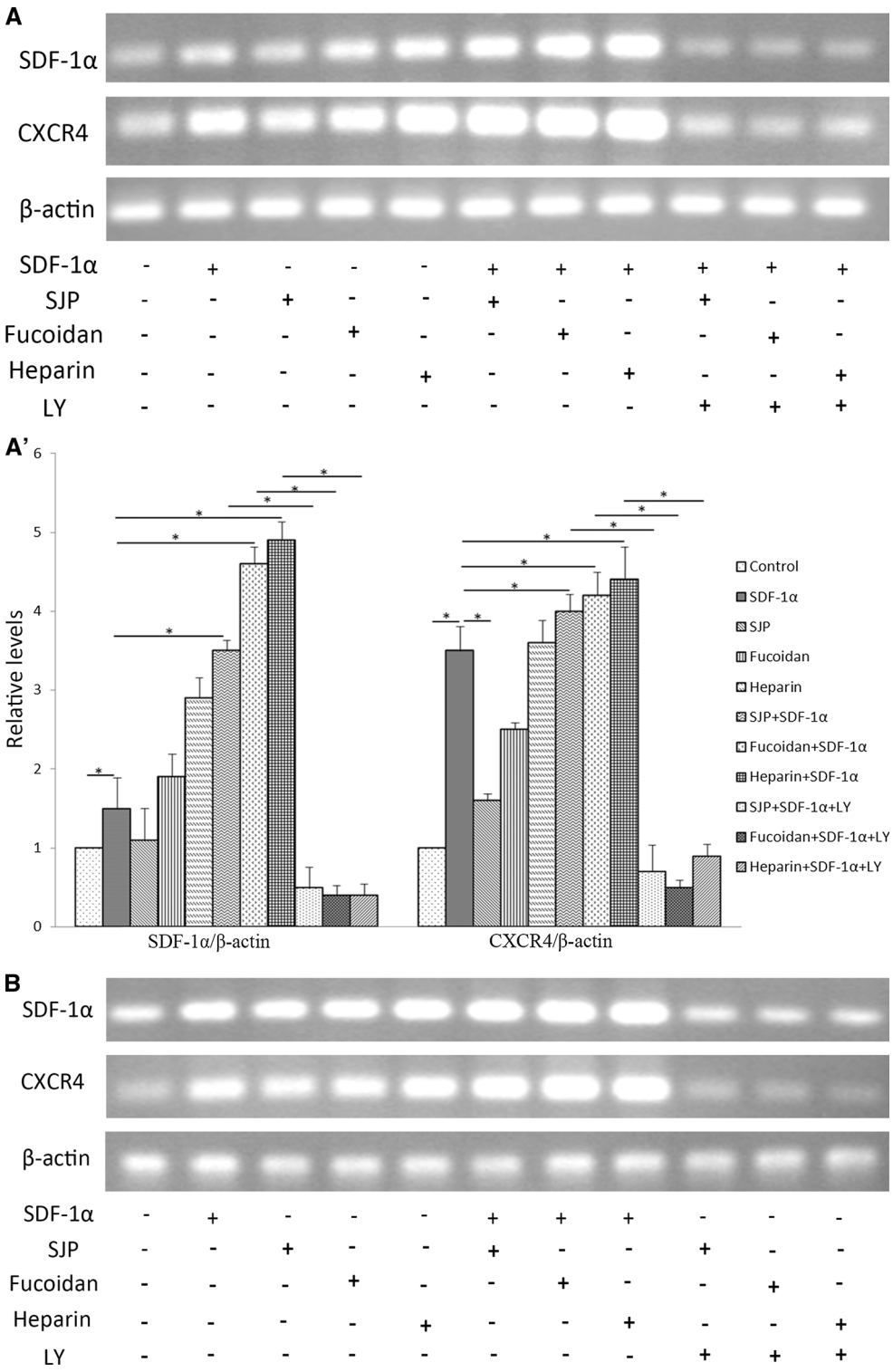
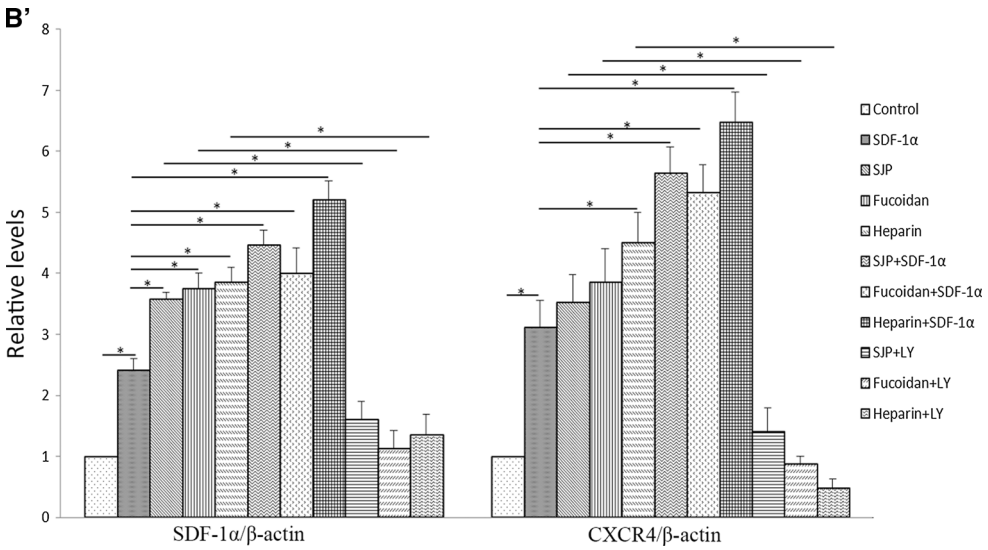


Fig. 6 SJP, fucoidan, or heparin increased SDF-1α and CXCR4 expressions by RT-PCR. **a** RT-PCR was performed using total RNA to analyze mRNA levels of SDF-1α and CXCR4. **a′** Quantitative analysis of mRNA levels of SDF-1α and CXCR4 in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$. **b** Effect of Akt inhibitor LY294002 on separate treatment alone with SJP, fucoidan, or heparin by RT-PCR. **b′** Quantitative analysis of mRNA levels of SDF-1α and CXCR4 in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$

led to the attachment of neurosphere onto the plate surface and induced cell migration and neurite outgrowth better than SDF-1α, SJP, fucoidan, or heparin alone (Fig. 3a, b). SJP, fucoidan, or heparin interacting with SDF-1α showed additive effects on the promotion of cell migration from neurosphere with an efficacy that was superior to the effects of SJP, fucoidan or heparin applied alone. However, this process was significantly

Fig. 6 continued



inhibited by the PI3K inhibitor, LY294002. The enhanced neurosphere migration effect of SJP, fucoidan, or heparin was also blocked by LY294002.

SJP, Fucoidan, or Heparin Increased Nestin Expression

Nestin was described as a NSC marker during CNS development. Expression of Nestin correlates with NSC migration. SDF-1α alone was sufficient to increase Nestin expression. SJP, fucoidan, or heparin can also increase Nestin expression. However, SJP, fucoidan, or heparin combined with SDF-1α could increase the expression of Nestin in neurosphere better than SDF-1α, SJP, fucoidan, or heparin alone. To examine the migration of NSCs, we determined that the expressions of NSC marker Nestin were examined using immunocytochemistry (Fig. 4a), RT-PCR (Fig. 4b, b′), and western blot (Fig. 4c, c′) analysis. These data showed that SJP, fucoidan, or heparin interaction with SDF-1α treatment could increase Nestin expression. Therefore, Nestin may play an important role in NSC migration.

SJP, Fucoidan, or Heparin Increased SDF-1α and CXCR4 Expressions

The SDF-1α/CXCR4 axis is important in NSC migration. Chemokine SDF-1α regulates survival and migration of neural precursors (Dziembowska et al. 2005). CXCR4 chemokine receptor is of great importance in directing the migration of NSCs in the CNS (Tran and Miller 2003). NSCs express CXCR4, the cognate receptor for SDF-1α. Therefore, SDF-1α and CXCR4 expression was measured by immunocytochemistry. As shown in Fig. 5a, b, SDF-1α and CXCR4 signals were significantly stronger after SDF-1α stimulation in SJP, fucoidan, or heparin-treated NSCs. The results demonstrated that SJP, fucoidan, or heparin can increased SDF-1α and CXCR4 expressions after SDF-1α stimulation for 24 h. However, this effect was greatly reduced by LY294002. In the presence of LY294002, SDF-1α and CXCR4 signals were also essentially abolished following SJP, fucoidan, or heparin stimulation.

SDF-1α chemokine and its receptor CXCR4 are major players in the interactions mediating NSC migration (Adler and Rogers 2005). Therefore, we determined that the expressions of SDF-1α and CXCR4 were detected by RT-PCR. These findings showed that SDF-1α and CXCR4 levels increased after the interaction of SJP, fucoidan, or heparin with the SDF-1α/CXCR4 axis. The pharmacological inhibitor of PI3K, LY294002, inhibited SDF-1α and CXCR4 levels (Fig. 6a, a′). Furthermore, LY294002 also inhibited SJP, fucoidan, or heparin's effect (Fig. 6b, b′). These data indicated that PI3K/Akt modulated the activity of SDF-1α and CXCR4 to promote NSC migration. This finding was consistent with immunocytochemistry observations, thereby indicating that SJP, fucoidan, or heparin with SDF-1α/CXCR4 axis-induced NSC migration could increase the expression levels of SDF-1α and CXCR4.

To determine if the increase in mRNA correlated with the increase in protein production, the amounts of SDF-1α and CXCR4 secreted were measured by Western blot. The results of Western blot analysis revealed that SJP, fucoidan, or heparin with SDF-1α/CXCR4 axis-induced NSC migration could increase the expression levels of SDF-1α and CXCR4. As observed with mRNA levels, SDF-1α and CXCR4 protein levels were also increased by SJP, fucoidan, or heparin with the SDF-1α/CXCR4 axis and LY294002 blocked the expression levels of SDF-1α and CXCR4 (Fig. 7a, a′). The data showed that LY294002 could also attenuate the effect of SJP, fucoidan, or heparin on the expression levels of SDF-1α and CXCR4 (Fig. 7b, b′).

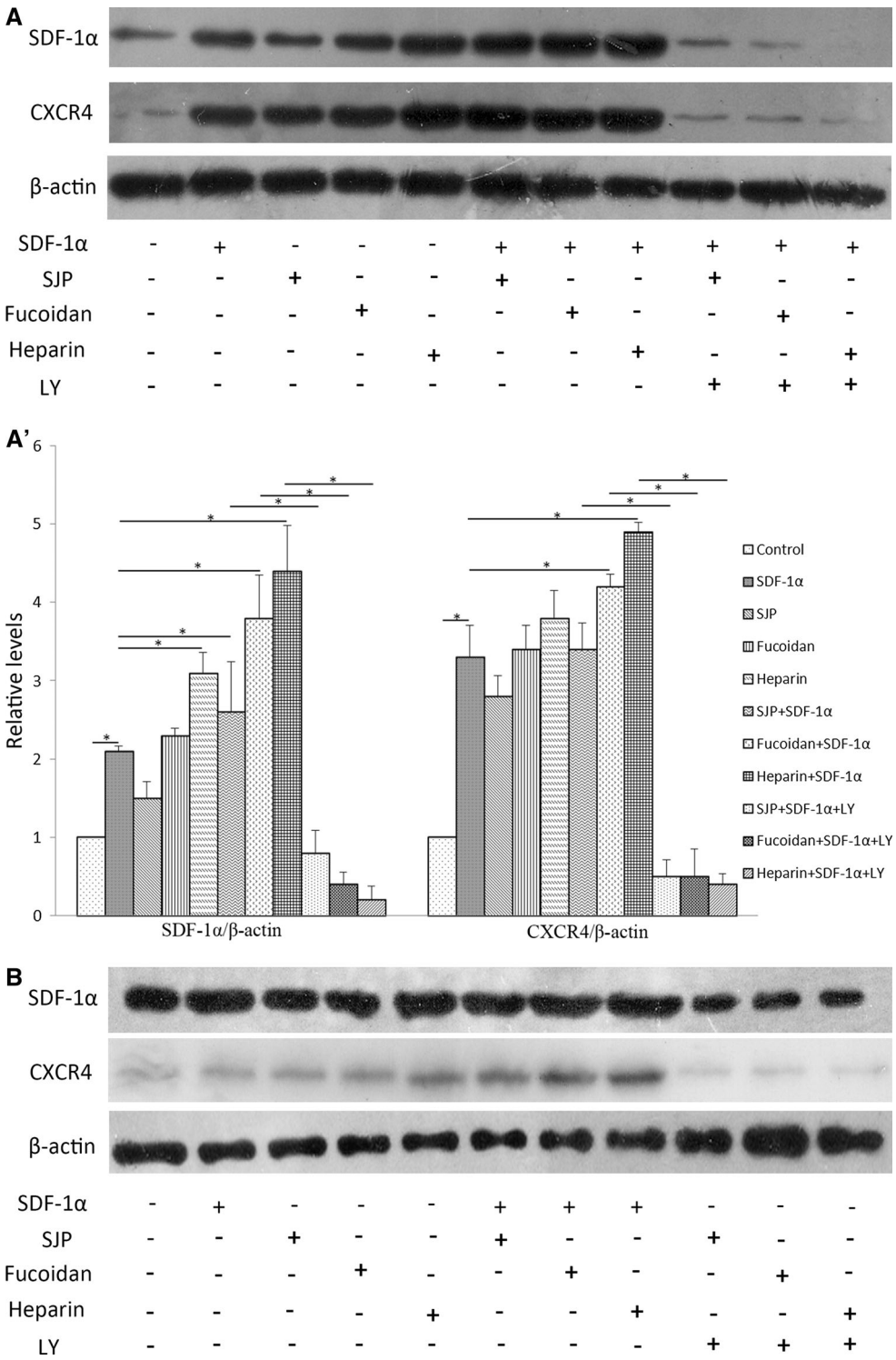
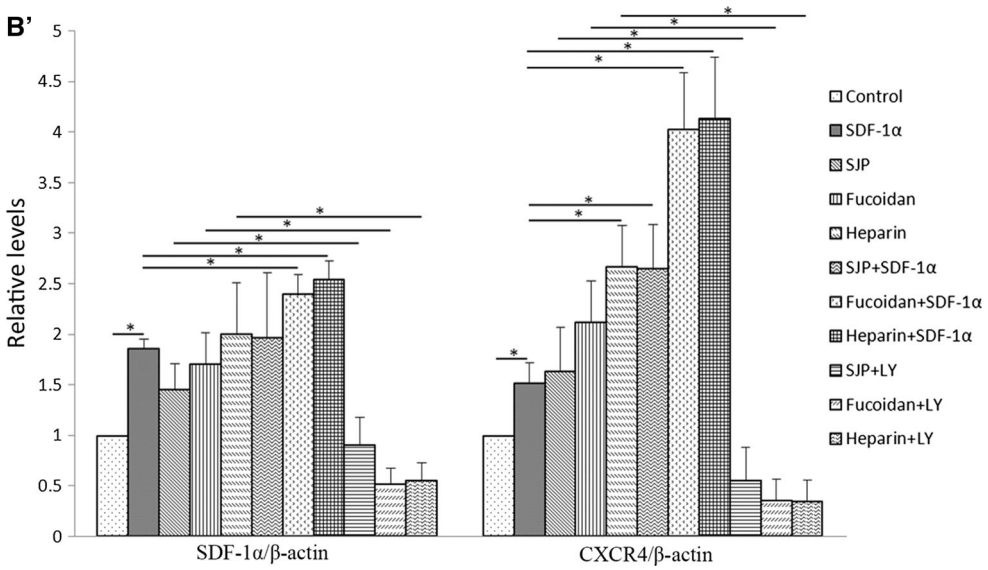


Fig. 7 SJP, fucoidan, or heparin increased SDF-1α and CXCR4 expressions by Western blot. **a** Western blot analysis of SDF-1α and CXCR4 are shown. **a′** Quantitative analysis of protein levels in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$. **b** Effect of Akt inhibitor LY294002 on separate treatment alone with SJP, fucoidan, or heparin by Western blot. **b′** Quantitative analysis of protein levels of SDF-1α and CXCR4 in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$

SJP, Fucoidan, or Heparin Promoted the SDF-1α/CXCR4 Axis Through the PI3K/Akt/FOXO3a Pathway

PI3K inhibitor, LY294002, inhibited the up-regulation of SDF-1α and CXCR4. CXCR4 stimulation by SDF-1α activated the PI3K/Akt pathway in NPCs (Wu et al. 2009). To verify whether the PI3K/Akt/FOXO3a pathway was involved in the interaction of SJP, fucoidan, or heparin with SDF-1α/CXCR4 axis-induced NSCs migration, we measured the activation of Akt, a known downstream signal of the CXCR4 receptor (Bonavia et al. 2003). The transcription factor FOXO3a is a downstream target of Akt. SJP, fucoidan, or heparin significantly increased SDF-1α-

Fig. 7 continued



induced Akt and FOXO3a phosphorylation, thereby indicating the promotion of the SDF-1α/CXCR4 signaling axis by SJP, fucoidan, and heparin. Pretreatment of NSCs with the PI3K inhibitor, LY294002 inhibited the Akt-increased serine 473 and FOXO3a phosphorylation under the conditions of SJP, fucoidan, or heparin and SDF-1α. This effect was diminished by LY294002 (Fig. 8a, a′). LY294002 could also significantly decrease the phosphorylation of Akt and FOXO3a caused by SJP, fucoidan, or heparin (Fig. 8b, b′). It has been shown that the CXCR4/G protein/PI3K-Akt pathways are responsible for SJP, fucoidan, or heparin with SDF-1α-mediated NSC migration, through the phosphorylation of Akt and FOXO3a. Altogether, these data indicated that SJP, fucoidan, or heparin with SDF-1α/CXCR4 axis induced SDF-1α and CXCR4 expressions by activating the PI3K/Akt/FOXO3a signaling pathway.

Discussion

In this study, we determined that SJP, fucoidan, or heparin with SDF-1α/CXCR4 axis induced NSC migration. SJP is from the body wall of the sea cucumber *S. japonicus*. Fucoidan is a heparinoid compound that belongs to a family of sulfated polyfucose polysaccharides. Heparin is a glycosaminoglycan. SJP, fucoidan, or heparin interacted with SDF-1α/CXCR4 signaling to promote migration and homing of NSCs by activating the PI3K/Akt/FOXO3a signaling pathway. Therefore, SJP, fucoidan, and heparin can potentially be used for NSC cell therapy.

NPCs can migrate directly to damaged adult CNS, thereby indicating their potential use in the treatment of human neurodegenerative disorders (Fallon et al. 2000). The chemokine SDF-1α and its G-protein-coupled receptor CXCR4 represent an important pathway that regulates homing and maintenance of NSCs for initiating new therapies of cell repair (Li et al. 2012). SDF-1α is an important mediator in the pathogenesis of many human diseases and physiological conditions (Bonecchi et al. 2009). CXCR4 chemokine receptor is important in regulating the migration toward regions of brain injury during cell therapy for potential self-repair (Tran et al. 2007). We studied the expressions of SDF-1α and CXCR4 in NSCs. The increase of CXCR4 receptor expression correlated with the increase in functional sensitivity to SDF-1α stimulation. Increased expression of CXCR4 may facilitate an increase in chemotactic response of NSCs to SDF-1α. SDF-1α is involved in the migration of NSCs. CXCR4 signaling appears to be a hallmark of the neurosphere and triggers migration in NSCs. This observation provides insights into the migration of NSCs to neurodegeneration sites. Thus, the regulation of SDF-1α and CXCR4 expression results in NSC migration, which should be investigated further. Our data suggested that SJP, fucoidan, or heparin interacted with SDF-1α/CXCR4 axis. Thus, NSC migration was promoted by SDF-1α and CXCR4.

NSC marker Nestin overexpression was observed after SJP, fucoidan, or heparin interaction with SDF-1α treatment. Besides, the expressions of SDF-1α and CXCR4 were also significantly increased. Results showed that SJP, fucoidan, or heparin can interact with SDF-1α, which significantly enhanced the expressions of Nestin, SDF-1α, and CXCR4 and NSC migration. Our results suggested that the migration of NSCs was activated by chemotactic effect of SJP, fucoidan, or heparin and SDF-1α. Taken together, these results demonstrated that SJP, fucoidan, or heparin enhanced the SDF-1α/CXCR4 axis and promoted NSC migration.

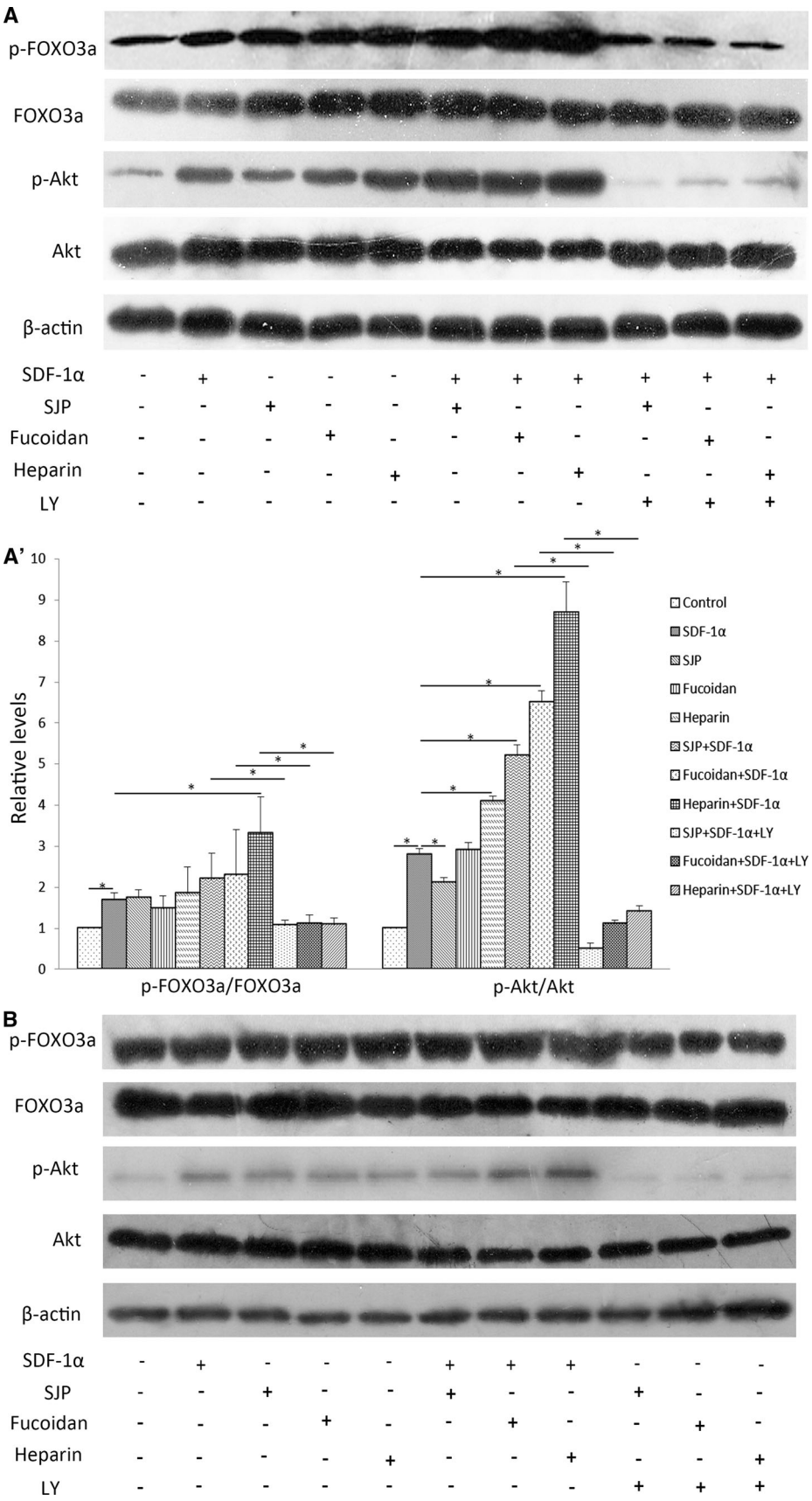
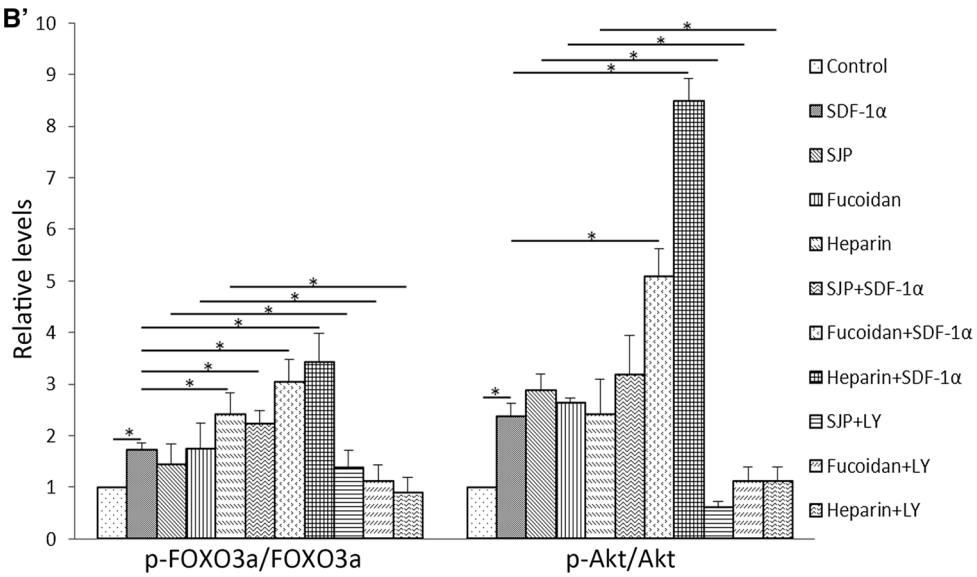


Fig. 8 Interaction of SJP, fucoidan, or heparin with the SDF-1α promotes NSC migration via the PI3K/Akt/FOXO3a signaling pathway. **a** Western blot analysis of p-FOXO3a, FOXO3a, p-Akt, and Akt are shown. **a′** Quantitative analysis of protein levels in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$. **b** Effect of Akt inhibitor LY294002 on separate treatment alone with SJP, fucoidan, or heparin by Western blot. **b′** Quantitative analysis of protein levels of p-FOXO3a, FOXO3a, p-Akt, and Akt in each group. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$

Fig. 8 continued



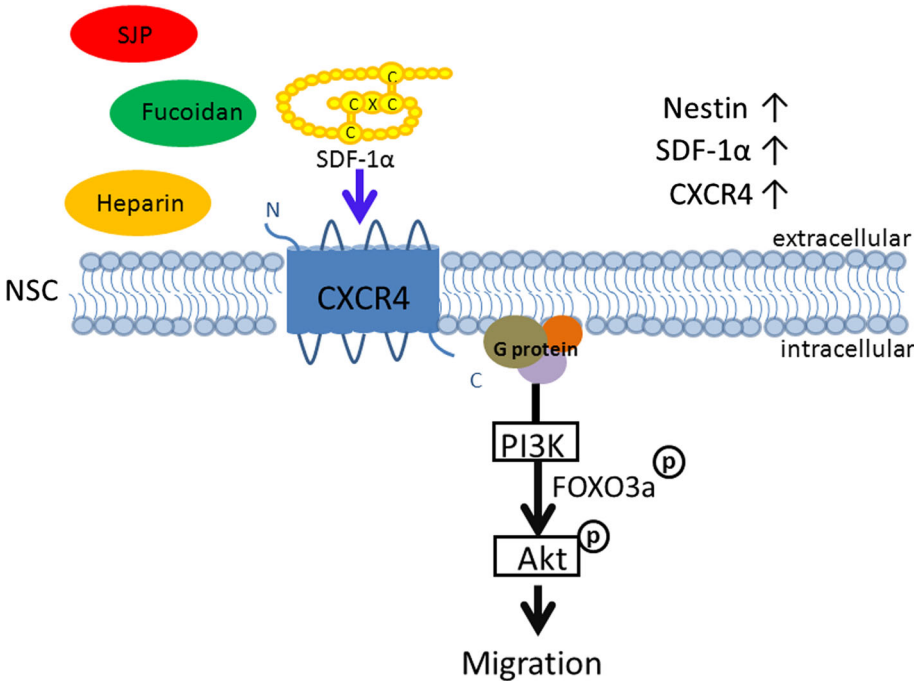


Fig. 9 SJP, fucoidan, or heparin enhanced the SDF-1α/CXCR4 axis and promoted NSC migration via activation of the PI3K/Akt/FOXO3a signaling pathway. SJP, fucoidan, or heparin interaction with SDF-1α treatment could increase Nestin, SDF-1α, and CXCR4 expressions. SDF-1α modulated by SJP, fucoidan, or heparin activated the CXCR4 receptor and directed cellular migration via activation of the PI3K/Akt/FOXO3a signaling pathway

SDF-1α is a potent chemoattractant for NPCs to regulate migration selectively (Stumm et al. 2003). Glycosaminoglycans (GAGs) are polysaccharides with a high negative charge. The negative charge is due to the presence of sulfate and carboxyl groups. GAGs attach to core proteins to form proteoglycans (Middleton et al. 2002). The biological properties of SDF-1α are the major reasons for its involvement in the binding of cellular or extracellular matrix GAGs (Amara et al. 1999). The basic amino acid residues in the loop structures away from the C-terminal helix of SDF-1α are important for GAG-binding motif (Johnson et al. 2005). Our data reinforced the function of SJP, fucoidan, or heparin, which is to interact with SDF-1α to promote effective NSC migration.

SDF-1α/CXCR4 axis is important in inducing NSC migration. Exposure of NSCs to SDF-1α and the consequent induction of CXCR4-mediated intracellular signaling cascade trigger cell migration. SDF-1α/CXCR4 axis mediates chemotactic migration of NSCs through PI3K/Akt/FOXO3a signaling pathway in the CNS. SDF-1α activates protein kinase Akt, which is a downstream

effector of PI3K (Li et al. 2010). CXCR4 is a G-protein-coupled cell-surface receptor that initiates PI3K/Akt signaling pathways by activating the associated G protein (Bokoch 1995). FOXO3a is a major player in the effects of SDF-1α on NSC migration. Activation of the PI3K/Akt pathway is coupled to transcription factor FOXO3a via phosphorylation. We examined the relationship between the migration ability, which was improved by the interaction of SJP, fucoidan, or heparin, and SDF-1α and phosphorylation of Akt and FOXO3a activation. The interaction of SJP, fucoidan, or heparin with SDF-1α increased p-Akt, p-FOXO3a, and NSC migration. However, NSC migration ability significantly decreased in the presence of PI3K/Akt inhibitor (LY294002). Our previous study has also demonstrated that SJP significantly promoted NSC migration via the PI3K/Akt signaling pathway (Sheng et al. 2012). In the present study, our data suggest that PI3K/Akt/FOXO3a pathway might be critical in NSC migration after the interaction of SJP, fucoidan, or heparin with SDF-1α, which is consistent with previous findings. PI3K/Akt/FOXO3a activation resulted in increased SDF-1α and CXCR4 expressions. PI3K/Akt inhibitor abrogated phosphorylation of both Akt and FOXO3a, suggesting these two critical proteins are the downstream effectors of SDF-1α/CXCR4 (Fig. 9). The present study suggests that SJP, fucoidan, or heparin and SDF-1α are capable of stimulating the phosphorylation of the Forkhead transcription factor FOXO3a via the PI3K/Akt kinase pathway in NSC. Nevertheless, the important link between SJP, fucoidan, or heparin and SDF-1α-mediated NSC migration and FOXO3a phosphorylation suggests an essential role of FOXO3a in the regulation of NSC migration.

Our study has elucidated the effects of SJP, fucoidan, and heparin in SDF-1α-induced NSC migration. Further studies are needed to identify the underlying mechanisms of migration of NSCs mediated by SJP, fucoidan, or heparin interacted with SDF-1α/CXCR4 axis both in vitro and in vivo. The use of animal models may better help to elucidate some mechanisms in future studies. The understanding of molecular mechanisms on the interaction of SJP, fucoidan, or heparin and SDF-1α in NSC migration could contribute to develop a clinically viable platform for cell therapy.

In summary, our results provide evidence that SJP, fucoidan, or heparin enhanced the SDF-1α/CXCR4 axis and promoted NSC migration via activation of the PI3K/Akt/FOXO3a signaling pathway. SJP, fucoidan, and heparin can potentially be used for brain injury via cell therapy.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest for all authors.

Ethical approval The study complies with current ethical consideration.

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