Protein phosphatase 2A regulates the p38 signaling pathway to affect the migration of astrocytes

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Abstract. The aim of the present study was to investigate the effect and mechanism of protein phosphatase 2A (PP2A) on the migration of astrocytes. The primary astrocytes of neonatal mice were isolated and cultured *in vitro*, and treated with the PP2A activator D-erythro-sphingosine (DES) (activated group) or inhibitor okadaic acid (inhibitory group). The control group was treated with equal amounts of dimethyl sulfoxide. The activity of PP2A in the cells was detected using a commercial kit and the migration of cells was investigated using a Transwell migration assay. The protein expression of p38, phosphorylated (p)-p38, matrix metalloproteinase (MMP)-2 and MMP-9 was detected by western blotting. Cell migration and the protein expression of p38, p-p38, MMP-2 and MMP-9 was also determined following treatment of astrocytes with the p38 signaling pathway inhibitor SB202190 with or without the PP2A activator DES. The results demonstrated that the activity of PP2A in the PP2A inhibitory group was significantly decreased compared with the control group, while that of the PP2A-activated cells was significantly increased compared with the control. The protein levels of MMP-2 and MMP-9 in the PP2A inhibitory group astrocytes were significantly decreased compared with the control group, while PP2A-activated astrocytes exhibited significantly increased levels of these proteins. By contrast, the p-p38 level in PP2A inhibitory group astrocytes was significantly increased compared with the control group, while astrocytes in the activated group exhibited significantly lower levels compared with the control group. Furthermore, the cell migration ability, and MMP-2 and MMP-9 protein levels, of astrocytes that received combined treatment with SB202190 and the PP2A activator DES were significantly increased compared with the levels in astrocytes treated with SB202190 alone. The results of the current study indicate that PP2A may negatively regulate the p38 signaling pathway to promote astrocyte migration.

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Introduction

Astrocytes, are the most abundant type of glial cell in the central nervous system (1). Astrocytes promote nerve repair and regeneration, and serve important roles in the maintenance of the blood-brain barrier, nervous system stability and nervous system damage (2-4). Any form of damage and lesions in the central nervous system can lead to the activation of astrocytes, which has been associated with astrocyte dysfunction and central nervous system disorders (5). A previous study demonstrated that the migration of astrocytes serves a key role in neurodegenerative diseases (6). Studies of patients with Parkinson's disease and animal models have revealed that the activation of astrocytes is accompanied with impaired mitochondrial function in astrocytes under pathological conditions (7,8). An important feature of astrocyte activation following nerve injury is the upregulation of glial fibrillary acidic protein (GFAP) expression. Inhibition of GFAP gene expression has been reported to delay the regeneration of nerve axons and functional recovery following nerve injury (9). Therefore, investigation of the migration of astrocytes is of great importance in the treatment of central nervous system diseases.

P38 signaling pathway is associated with the mitogen (MAPK) family of proteins, which serves an important role in cell apoptosis, cytokine production and transcriptional regulation (10). It has been reported that the activation of p38-MAPK serves an important role in the pathological reaction of Alzheimer's disease. A large number of phosphorylated p38-MAPK can be observed in the brain tissue of patients with early Alzheimer's disease (11). The activation of p38-MAPK has been associated with the damage of glial cells, which exhibit enhanced protective effects when p38 MAPK is inhibited. It also regulates the secretion of nerve growth factor and tumor necrosis factor-α and cell migration of glial cells (11). Protein phosphatase 2A (PP2A) is a serine/threonine protein phosphatase, with a total of 75 types of heterotrimers, that is involved in various cell biological functions (12). In neurodegenerative diseases, the main function of PP2A has been

proposed to involve the dephosphorylation of highly phosphorylated tau protein. During the development of the central nervous system, PP2A can dephosphorylate brain retardation response regulatory protein 2 and promote axonal polarization and growth (13,14). A recent study demonstrated that elevated levels of PP2A in astrocytes may alleviate neurotoxicity in APP/PS1 double transgenic mice (15). In a rat model of Alzheimer's disease (tg2576 rats), PP2A deficiency can lead to Aβ aggregation by inhibiting the migration of astrocytes (16). In the present study, astrocytes were isolated and cultured *in vitro* to investigate the effect of PP2A on their migration in order to provide a theoretical basis for the treatment of central nervous system diseases.

Materials and methods

Experimental animals. A total of 10 Sprague-Dawley rats of either gender, (aged 1-3 days and weighed ~200+20 g), were purchased from the Experimental Animal Center of Xinxiang Medical University (Xinxiang, China). All rats were housed in sterile conditions at 19-21˚C, with a relative humidity of 50-60%, *ad libitum* intake of water and food, under 12 h of light/dark cycle. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (16). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University.

Instruments and reagents. p38 (cat. no. 8690T), p-p38 (cat. no. 4511T), matrix metalloproteinase (MMP)-2 (cat. no. 40994S), MMP-9 (cat. no. 13667T) and β-actin (cat. no. 3700T) monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The inverted microscope was purchased from Olympus Corporation, (Tokyo, Japan). The CO_2 incubator was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The PP2A activity test kit was purchased from Promega Corporation (Madison, WI, USA). The BCA Protein Concentration assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Isolation and culture of astrocytes. Sprague-Dawley rats born within 48 h were sacrificed by cervical dislocation and disinfected with 75% alcohol. The neonatal brain tissue was removed and washed with PBS three times. When the washing liquid remained translucent, the cerebral cortex tissue was removed and cut with an ophthalmic scalpel, 0.125% trypsin was subsequently added at 37˚C for 15 min and agitated every 3 min. The digestion time of the tissue was different depending on the size of the tissue blocks. The tissue was observed until digested completely and Dulbecco's modified Eagle's medium/F12 cell culture medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin was added to terminate the digestion. Subsequently, the cell culture was sieved with 200-mesh sieve and centrifuged at 300 x g for 10 min at room temperature. The supernatant was discarded and the appropriate amount of cell culture medium was added to suspend the cells. Cells were subsequently seeded in the culture flask in a 37˚C and 5% CO_2 incubator. The culture medium was replaced every 3 days. After 9 days, the cell culture flask was sealed and placed on a shaker at 37˚C overnight at 100 rpm. The isolated, cultured cells expressed glial fibrillary acidic protein, by immunofluorescence and were therefore identified as astrocytes (17). Fresh culture medium was added and the cells were cultured until the second generation where they were subsequently employed in experiments. Briefly, the cells were fixed as follows: Cells were washed with PBS for 1-2 times; 4% polyformaldehyde was added for 15-20 min at room temperature. The cells were then rinsed with PBS for two times (5 min per wash) and 100 μl of 0.2% Triton X-100 was added to each hole to permeabilize for 15 min at room temperature. Subsequently, the cells were blocked for 30 min at room temperature by adding 100 μl of 3% bovine serum albumin (BSA) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A total of 100 μl of 1:2,000 1% BSA-diluted primary antibody against GFAP 1 (cat. no. D1F4Q, Cell Signaling Technology, Inc.) was added to the cells and incubated at 4˚C overnight. Following incubation, the cells were incubated for 1 h in the dark at room temperature with secondary antibody goat-anti-rabbit IgG H&L (1:1,000; ab150077, Abcam, Cambridge, MA, USA) diluted with PBS. After washing, a laser-scanning confocal microscopy (magnification, x40, TCS SP8 CARS, Leica Microsystems, Inc., Buffalo Grove, IL, USA) was used for analysis and to capture images (data not shown).

Experimental grouping. Astrocytes were divided into experimental groups: Control group, PP2A inhibitor group and PP2A activation group. Cells were seeded at concentration of $1x10^5$ cells/ml. A total of 10 μl dimethyl sulfoxide was added to the control cells, while 15 nM PP2A inhibitor, okadaic acid (OA) (Beyotime Institute of Biotechnology) was added to the inhibitor group and 15 nM PP2A activator, D-erythro-Sphingosine (DES) (Beyotime Institute of Biotechnology) was added to the activated group. Each group of the cells was treated for 48 h under normal cell culture conditions.

PP2A enzyme activity test. The cells in each group were cultured for 48 h following the aforementioned treatments, land subsequently lysed on ice for 20 min. The lysates were transferred to Eppendorf (EP) tubes and centrifuged at 15,000 x g for 10 min at 4˚C. The protein supernatant was extracted and transferred into EP tubes. A protein concentration detection kit was used to determine the concentration of the extracted protein. A 50 μg protein sample was used to detect the activity of PP2A in the sample according to the PP2A activity assay kit. Calculated the linear regression equation of the standard curve according to the concentration of the standard product, and the PP2A activities were calculated according to the OD value of the sample based on the regression equation.

Cell migration by Transwell assay. The second generation of astrocytes in each group was collected, digested by trypsin and the corresponding cell culture medium DMEM/F12 containing 1% FBS was added to resuspend cells. The cell concentration was adjusted to $1x10^5$ cells/ml. A total of 500 μl cell suspension was added to the upper chamber of the poly-D-lysine pre-coated Transwell chamber (Corning Incorporated, Corning, NY, USA) and 700 μl cell culture

medium DMEM/F12 containing 10% FBS was added to the lower chamber. The cells were placed on a 12-well plate and incubated for 8 h in a 5% CO_2 incubator at 37˚C, and the excess cells were removed using a cotton swab. The cells were fixed with methanol for 5 min at room temperature and stained with Giemsa dye for 15 min at room temperature. The number of cells that migrated after 8 h was observed under light microscope (magnification, x100). The total number of cells was the sum of the cells in the upper and the lower layer of the membrane. Cell mobility=Number of cells in the lower layer of the membrane/total cells.

Western blotting of p38, phosphorylated (p)-p38, MMP-2 and MMP-9 protein levels in astrocytes. Using radioimmunoprecipitation buffer (Sigma-Aldrich; Merck KGaA), total proteins were extracted from the cells of the respective groups after culture for 48 h. Following quantification with a Bicinchoninic Acid kit, 20 μg of each denatured protein sample was loaded into the SDS-PAGE gel wells with 6% stacking gel and 10% removal gel. When the bromophenol blue moved to the junction of the stacking and removing gels, electrophoresis with 100 V was performed for 90 min. The proteins were transferred to a nitrocellulose membrane with transferring current of 275 mA and transfer time of 60 min. The nitrocellulose membrane was blocked in 4% skimmed milk powder for 2 h at room temperature. Subsequently, membranes were incubated with the primary antibodies (1:800) at 4˚C overnight, followed by secondary antibody incubation (1:1,000) at 37˚C for 120 min. Membranes were then exposed after addition of the visualization enhanced chemiluminescent reagent (Beyotime Institute of Biotechnology) and analyzed via Tanon 4600 Chemiluminescence Image Analysis System version 3.0 (Tannon, Shanghai, China). The relative expression of proteins was calculated.

Effect of p38 signaling pathway inhibition on astrocyte migration. For a different group of experiments, second generation astrocytes were divided into untreated, SB202190 and DES + SB202190 groups. Cells were seeded at a density of $1x10^5$ cells/ml in DMEM/F12. Cells in the SB202190 group were incubated at 37˚C for 48 h with cell culture medium containing the p38 signal pathway inhibitor SB202190 (Cell Signaling Technology, Inc.) at a final concentration of 30 μmol/l. Cells in the DES + SB202190 group were treated with p38 signal pathway inhibitor SB202190 at a final concentration of 30 μmol/l and 15 nM PP2A activator DES at 37˚C for 48 h, while the untreated group received no treatment. The levels of p38, p-p38, MMP-2 and MMP-9 were detected by western blotting and migration was assessed by a Transwell assay.

Statistical analysis. The experimental data were analyzed by SPSS 22.0 statistical software (IBM, Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation of the mean. One-way analysis of variance was used to compare multiple groups. Multiple comparisons between the groups were performed using Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed at least three times and all samples were tested in triplicate.

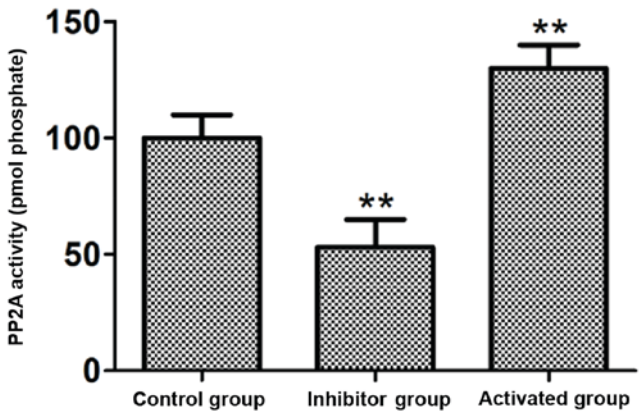

Figure 1. PP2A activity in astrocytes of the control, PP2A inhibitor and PP2A activation groups. **P<0.01 vs. control group. PP2A, protein phosphatase 2A.

Results

PP2A activity. A total of 15 nM PP2A inhibitor OA and 15 nM PP2A activator DES were used to treat the astrocytes, and the PP2A activity in cells was measured. The results demonstrated that the activity of PP2A in the cells treated with the PP2A inhibitor OA was significantly decreased compared with the control group (P<0.01; Fig. 1). The activity of PP2A in the cells treated with the PP2A activator DES was significantly increased compared with the control group (P<0.01; Fig. 1).

Effect of PP2A on cell migration. Following treatment of the astrocytes with 15 nM PP2A inhibitor OA and 15 nM PP2A activator DES, cell migration was measured using a Transwell chamber. The protein expression of MMP-2 and MMP-9 in the cells was also detected by western blotting. The results demonstrated that the cell migration ability, and the protein levels of MMP-2 and MMP-9, in astrocytes treated with OA were significantly decreased compared with the control group (P<0.01), while significant increases in migration and the expression of MMP-2 and MMP-9 were observed compared with the control group in those treated with DES (P<0.01; Fig. 2).

Effect of PP2A on p-p38 protein expression. Astrocytes were treated with 15 nM PP2A inhibitor OA and 15 nM PP2A activator DES, and the cell p-p38 level was detected by western blotting. The results demonstrated that the level of p-p38 protein in the cells treated with the PP2A inhibitor OA was significantly increased compared with the control group (P<0.01), while p-p38 protein levels in the cells treated with PP2A activator DES were significantly decreased compared with the control group (P<0.01; Fig. 3).

Effect of a p38 signal pathway inhibitor on p-p38 protein expression. Following treatment of astrocytes with 30 μmol/l p38 signal pathway inhibitor SB202190, the levels of p-p38 were measured by western blotting. The results demonstrated that the level of p-p38 protein following treatment with the p38 signal pathway inhibitor was significantly decreased compared with the untreated group (P<0.01; Fig. 4).

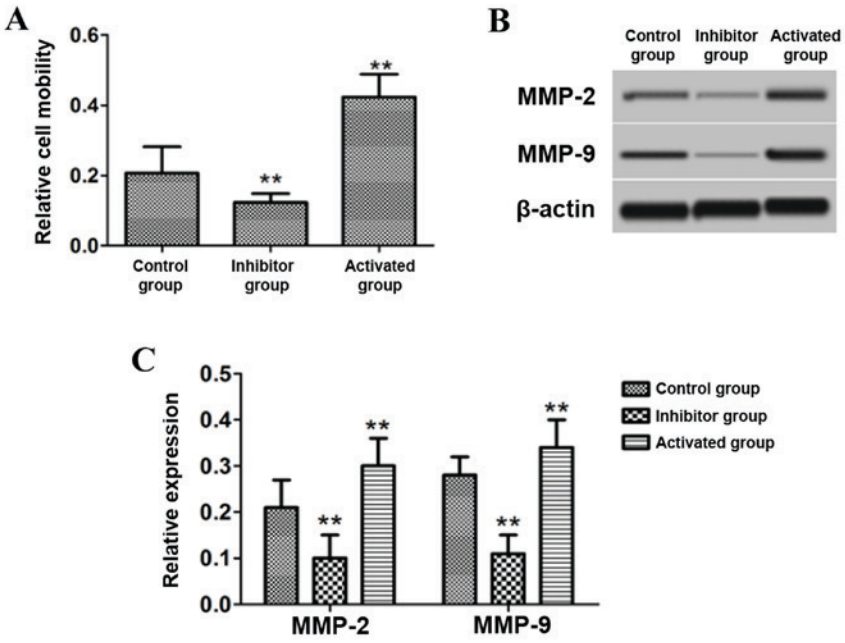

Figure 2. Effect of PP2A on cell migration and the protein expression of MMPs in astrocytes of the control, PP2A inhibitor and PP2A activation groups. (A) Cell migration was determined by a Transwell migration assay. (B) Representative western blot bands for the protein expression of MMP-2 and MMP-9 in control, PP2A inhibitor and PP2A activation groups. (C) Densitometric analysis was performed to obtain the relative expression level of target proteins with β-actin as the internal reference. **P<0.01 vs. control group. PP2A, protein phosphatase 2A; MMP, matrix metalloproteinase.

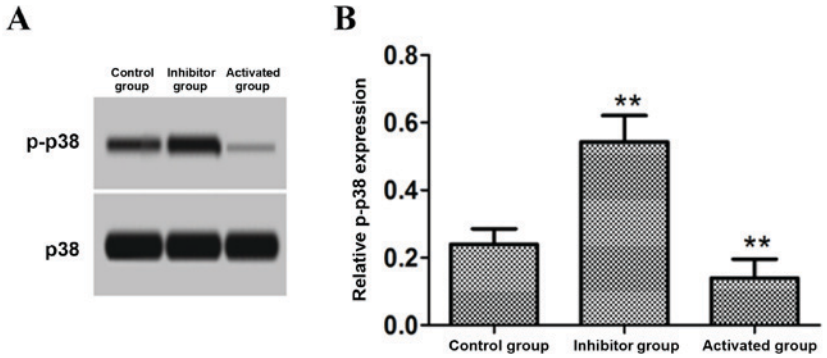

Figure 3. Effect of PP2A on p-p38 protein expression in astrocytes of the control, PP2A inhibitor and PP2A activation groups. (A) Representative western blot bands for the protein expression of p-p38 and p38 in control, PP2A inhibitor and PP2A activation groups. (B) Densitometric analysis was performed to obtain the relative expression level of p-p38 protein with p38 as the internal reference. **P<0.01 vs. control group. PP2A, protein phosphatase 2A; p-p38, phosphorylated-p38.

Effect of inhibition of p38 signaling pathway on cell migration. Following treatment of astrocytes with 30 μmol/l p38 signaling inhibitor SB202190 with or without 15 nM PP2A activator DES, the cell migration was detected using a Transwell chamber. The protein expression of MMP-2 and MMP-9 in the cells was detected by western blotting. The results demonstrated that the cell migratory ability and the protein levels of MMP-2 and MMP-9 in astrocytes following treatment with the p38 signal pathway inhibitor SB202190 were significantly increased compared with the untreated group (P<0.01; Fig. 5). Furthermore, the cell migratory ability and the protein levels of MMP-2 and MMP-9 in astrocytes that received combined treatment with the p38 signal pathway inhibitor SB202190 and the PP2A activator DES were significantly increased compared with those treated with SB202190 alone (P<0.01; Fig. 5).

Discussion

Astrocytes are a class of cells that are closely associated with the central nervous system. Astrocytes serve a supporting role in providing neurons with nutrition (18). Evidence has indicated that astrocytes exhibit a wide range of biological functions in the nervous system (19). Various effects have been reported for astrocytes within the central nervous system, including the transmission of neurotransmitters and the maintenance of synaptic biological functions (20-22). In addition, astrocytes have been reported to have an important role in central nervous system disorders. For example, a large number of astrocytes aggregated around the senile plaques in the brain of patients with Alzheimer's disease (AD) (23). Investigation of the migration of astrocytes may therefore be important to the pathogenesis of AD.

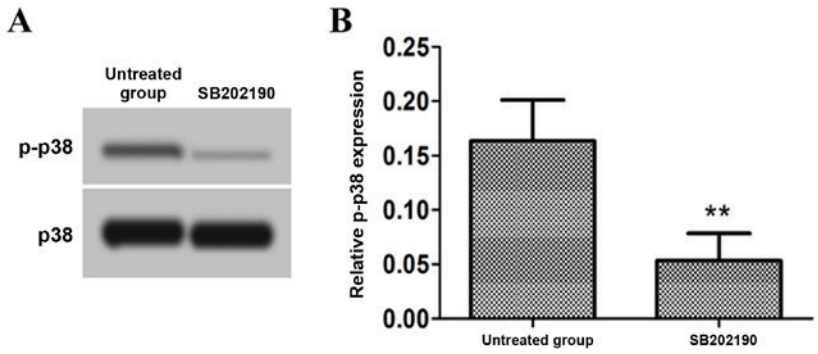

Figure 4. Effect of a p38 signaling pathway inhibitor on p-p38 protein expression in astrocytes. (A) Representative western blot bands for the protein expression of p-p38 and p38 in untreated astrocytes and astrocytes treated with the p38 signaling pathway inhibitor, SB202190. (B) Densitometric analysis was performed to obtain the relative expression level of p-p38 protein with p38 as the internal reference. **P<0.01 vs. untreated group. p-p38, phosphorylated-p38.

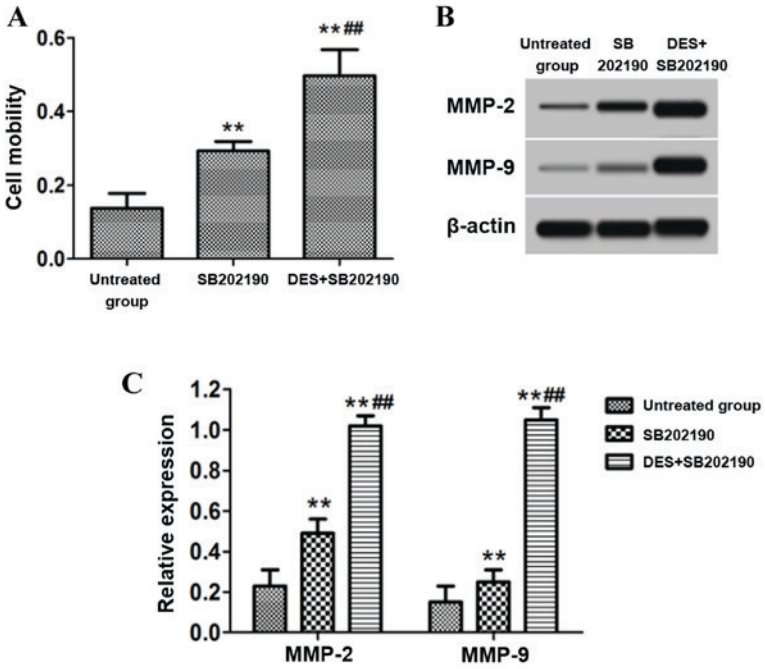

Figure 5. Effect of inhibition of the p38 signaling pathway on cell migration and the protein expression of MMPs in astrocytes of the untreated, SB202190 and (DES) + SB202190 groups. (A) Cell migration was determined by a Transwell migration assay. (B) Representative western blot bands for the protein expression of MMP-2 and MMP-9 in the untreated, SB202190 and DES + SB202190 groups. (C) Densitometric analysis was performed to obtain the relative expression level of target proteins with β-actin as the internal reference. SB202190 was employed as an inhibitor of the p38 signaling pathway, while DES was employed as an activator of protein phosphatase 2A. **P<0.01 vs. untreated group; ##P<0.01 vs. SB202190 group. DES, D-erythro-sphingosine; MMP, matrix metalloproteinase.

PP2A possesses A, B and C subunits that contain a large number of trimers, and these trimers affect intracellular proteins in various ways, therefore participating in a numerous biological function processes (24). Previous studies have demonstrated that PP2A may serve an important role in the central nervous system, and reduced PP2A activity was reported to disrupt the function of neuronal axons (25,26). PP2A activity in the brain of patients with AD has been reported to be reduced (27); whether PP2A can affect the migration of astrocytes is lack of sufficient evidence. In the present study, astrocytes were isolated and cultured *in vitro*, and treated with PP2A activators or inhibitors. The results demonstrated that PP2A activators and inhibitors effectively promoted PP2A activity and inhibited PP2A activity, respectively. There were no obvious morphological alterations following stimulation or inhibition of PP2A (data not shown). Further detection of cell migration demonstrated that PP2A activation had the ability to promote the migration of astrocytes, consistent with the findings in a rat model (15,27).

Cell migration is a form of cellular movement that serves an important role in angiogenesis, the immune response, embryonic development, cancer, wound healing and neurodegenerative diseases (28,29). Cell migration is a complex process and is strictly regulated by various genes. MMPs are the most investigated family of proteins associated with cell migration. MMP-2 and MMP-9 have been reported to have a key role in the process of cell migration (30). The MMP-2 and MMP-9 protein expression levels were analyzed in the present study and the results demonstrated that the PP2A activator

promoted the expression of the MMP-2 and MMP-9 proteins in astrocytes, while the PP2A inhibitor inhibited the expression of MMP-2 and MMP-9 in astrocytes. The results were consistent with that of the cell migration assay and observations that OA can activate MMP-9 (31).

p38 is a member of the MAPK family and serves a role in cellular signal transduction (32). Studies have demonstrated that p38 is involved in inflammatory processes, physiological stress, cell migration and neurological disorders (33,34). In the present study, the phosphorylation of p38 in cells treated with PP2A activators and inhibitors was measured. The results demonstrated that the PP2A activator attenuated the phosphorylation of p38, while the PP2A inhibitor enhanced the phosphorylation of p38. In order to investigate the effect of the p38 signaling pathway on astrocytes, a p38 signaling pathway-specific inhibitor was used to treat astrocytes, and the results demonstrated that the p38 signaling pathway-specific inhibitor enhanced the migratory ability of astrocytes and enhanced the ability of the PP2A activator to promote migration, which is consistent with reports in stem cells and tumor cells (35,36). These results indicated that PP2A activation may promote the migration of astrocytes through negative regulation of the p38 signaling pathway.

In conclusion, the current study demonstrated that PP2A activation promoted the migration of astrocytes and the underlying mechanism may be associated with the p38 signaling pathway. The results of the present study provide a basis for further investigation of the effect of PP2A on the biological activity of astrocytes and provide a theoretical basis for the treatment of neurodegenerative diseases and potential therapeutic targets. The heterogeneity of astrocytes in the CNS under normal and pathological conditions, however, requires further investigation. Therefore, relevant signaling pathways and possible treatment options also need to be performed verification and research in models *in vivo*.

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Availability of data and materials

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

Authors' contributions

SJ made substantial contributions to the design of the present study. LZ, LS, LW and JZ performed the experiments of enzyme activity test, Transwell assay and analyzed the data, and LZ was a major contributor in writing the manuscript. LZ, PM, QG and LM performed the analysis of astrocytes and western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal use protocol in the present study was reviewed and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University (Xinxiang, China). The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (16).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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