Omentin-1 promotes the growth of neural stem cells via activation of Akt signaling

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Abstract. Omentin is a novel adipokine, which is expressed in and released from omental adipose tissue. In the present study, the effect of omentin on neural stem cells (NSCs) was investigated. NSCs are a subtype of stem cell in the nervous system, which are able to self-renew and generate neurons and glia for repairing neural lesions. Mouse NSCs were isolated and cultured *in vitro*. Treatment with recombinant omentin for 3 and 5 days significantly increased the size of NSC neurospheres ($P<0.01$) and enhanced NSC cell viability in normal conditions. In addition, omentin protected against the decrease in cell viability induced by the pro-inflammatory cytokine tumor necrosis factor-α. In the NSCs, incubation of omentin for 2, 4, 6, 8 and 16 h enhanced the phosphorylation of Akt at the Thr308 site and of AS160 at the Ser318 site, peaking 6 h after treatment. Additionally, treatment with LY294002 (10 μM), a specific inhibitor of phosphatidylinositol 3-kinase/Akt signaling, eliminated the omentin-induced increase in neurosphere size and cell viability. Overall, the present study provided the first evidence, to the best of our knowledge, that omentin promotes the growth and survival of NSCs *in vitro* through activation of the Akt signaling pathway. These results may contribute to the understanding of the role of omentin in the nervous system.

Introduction

Neural stem cells (NSCs) are a subtype of stem cells in the nervous system that are able to self-renew and generate neurons and glia (1). Although the location of NSCs in the adult brain and the brain regions to which their progeny migrate in order to differentiate remain to be fully elucidated, it is hypothesized that the majority of NSCs are located in the subventricular zone of the forebrain and the subgranular layer of the hippocampal dentate gyrus in the adult mammalian brain (2). Under certain pathophysiological conditions, NSCs are activated to generate progenitor cells, which then migrate to the subventricular zone where they differentiate and replace degenerate neural cells (1,2). Although the therapeutic application of NSCs is limited due to the relatively low regenerative capacity of the adult central nervous system and the difficulties associated with isolating patient NSCs, NSC-based approaches have been considered for the potential treatment of neurodegenerative disorders, ischemic stroke and cerebral traumatic injury. Therefore, identifying efficient endogenous or exogenous factors, which enhance the survival and growth of NSCs may be important for NSC-based therapeutic approaches.

Previous studies have demonstrated that adipose tissue is not only a depot of lipid, but is also an active endocrine organ, producing biologically active substances termed 'adipokines', including leptin, adiponectin, resistin and angiotensin (3-7). Omentin is a novel adipokine, which is expressed in and released from omental adipose tissue (8,9). Using a large-scale *in situ* hybridization screening method, the omentin gene has been cloned in small intestinal paneth cells in mice (10). Notably, this gene was found to regulate insulin-stimulated glucose uptake in human adipocytes (8-9). Omentin can be detected in human blood with a physiological concentration ranging between 300 and 600 ng/ml (11,12). In patients with obesity (12) or obesity-linked disorders, including type 2 diabetes (13), endothelial dysfunction (14), carotid atherosclerosis (15) and coronary artery disease (16), the blood omentin levels are decreased. However, few studies have investigated the role of omentin in the nervous system. Brunetti *et al* demonstrated that injection of omentin into the arcuate nucleus of the hypothalamus did not modify hypothalamic feeding behavior-associated peptide gene expression (17). In the present study, the effect of omentin on NSCs was investigated.

Materials and methods

Animals. Pregnant C57BL/6J mice were purchased from Vital River Laboratories Animals Technology Co., Ltd. (Beijing,

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China). All experiments were performed according to the guidelines of the Experimental Animal Care Committee of Jilin University (Changchun, China).

Reagents. Recombinant human omentin-1 and LY294002 were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Mouse monoclonal antibody against nestin, rabbit monoclonal antibody against vimentin and rabbit polyclonal antibody against SIRT1 were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody against phospho-Akt^{Thr308}, rabbit polyclonal antibody against total-Akt (t-Akt), rabbit monoclonal antibody against phospho-AS160^{Ser318} and rabbit polyclonal antibody total-AS160 (t-AS160) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal antibody against tubulin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Tumor necrosis factor (TNF)-α, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). The Cell Counting kit (CCK)-8 viability assay was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). DAPI was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence for western blot analysis was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA).

NSC isolation and culture. NSC isolation and culture was performed, as described previously (18). Briefly, the cortex was dissected and triturated until a single cell suspension was obtained. The cells were then grown in non-adherent conditions in NeuroCult® NSC Basal Medium (mouse; Stem Cell Technologies, Vancouver, Canada) with 20 ng/ml fibroblast growth factor 2 and 20 ng/ml epidermal growth factor (PeproTech, Inc.) for 7 days to enable the formation of neurospheres. Neurospheres were passaged every 5 days. For subcloning, the neurospheres were collected and dissociated using papain for 20 min at 37˚C by gentle agitation. The cells were replated at the same cell density ($5x10^5$/l) for each condition. At the time points indicated in the Figures, the number and size of the neurospheres and the total number of cells were analyzed.

Immunochemistry. Immunochemistry was performed, as described previously (19). Briefly, the neurospheres were seeded onto poly-l-lysine-coated coverslips (Sigma-Aldrich) for 5 days and then fixed using 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature prior to immunostaining (20). The cells were treated with 0.1% Triton X-100 (Sigma-Aldrich) at room temperature for 20 min, followed by incubation with an antibody against nestin or vimentin at 37˚C for 2 h and incubation with Alexa Fluor 488 and Alexa Fluor 555-conjugated secondary antibodies at room temperature for 1 h (21). The cell nuclei were counterstained using DAPI and visualized under a fluorescent microscope (Olympus IX71; Olympus, Tokyo, Japan).

Assessment of NSC survival. The survival of NSCs was evaluated using a non-radioactive CCK-8 assay, as described previously (22,23). The neurospheres were treated with omentin (100 ng/ml or 1 μg/ml), TNF-α (100 ng/ml) or LY294002 (10 μM) (24) for the indicated time periods. The medium was then discarded and the cells were incubated with 10 μl CCK-8 solution for 1 h at 37˚C. The optical density at 450 nm was analyzed using a microplate reader (Infinite 200; Tecan, Männedorf, Switzerland). The experiments were performed in duplicate.

Western blotting. Western blotting was performed, as described previously (25,26). Briefly, the cells were lysed using lysis buffer (50 mM Tris; pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanate, 1 mM sodium fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin A and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) (27). The cell lysates were centrifuged at 10,000 x g for 10 min and the supernatants were collected. The samples were then boiled for 10 min. The protein concentration was determined using a bicinchoninic acid assay (28). Equal quantities of the proteins were separated using 10% SDS-PAGE and transferred onto nitrocellulose membranes using standard procedures (29). The membranes were incubated overnight at 4˚C with antibodies against p-Akt (1:200), t-Akt (1:500), SIRT1 (1:250) or tubulin (1:1,000). Following incubation with the corresponding secondary antibodies (1:5,000), the membranes were washed three times and the bands were detected using the enhanced chemiluminescence kit (30).

Statistical analysis. All statistical analyses were performed using the GraphPad Prism 5 software program (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean and were compared using analysis of variance with Tukey's correction test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Omentin promotes NSC growth in a dose-dependent manner. In the present study, the neurospheres were initially stained using two well-accepted NSC markers, nestin and vimentin. As shown in Fig. 1A, the neurospheres were positive for nestin and vimentin staining, matching the NSC phenotype. Notably, treatment with omentin (100 ng/ml) for 3 and 5 days significantly increased the size of the NSC neurospheres ($P<0.01$; Fig. 1B). The proliferative effects on the NSCs were also compared between omentin and two other neurotrophic factors, BDNF and GDNF. The proliferative effect of omentin on the NSCs was weaker compared with those of BDNF and GDNF (Fig. 1B). The effect of different concentrations of omentin (100 and 500 ng/ml and 1 μg/ml) on neurosphere size was also investigated. All three concentrations of omentin increased neurosphere size (Fig. 2A and B), however, the proliferative effect of omentin was most significant at the highest concentration (1 μg/ml). These results indicated that omentin promoted NSC growth in a dose-dependent manner.

Omentin increases NSC viability in normal conditions and conditions of stress. The present study then investigated the effect of omentin on NSC viability. Two concentrations of omentin (100 ng/ml and 1 μg/ml) were supplemented

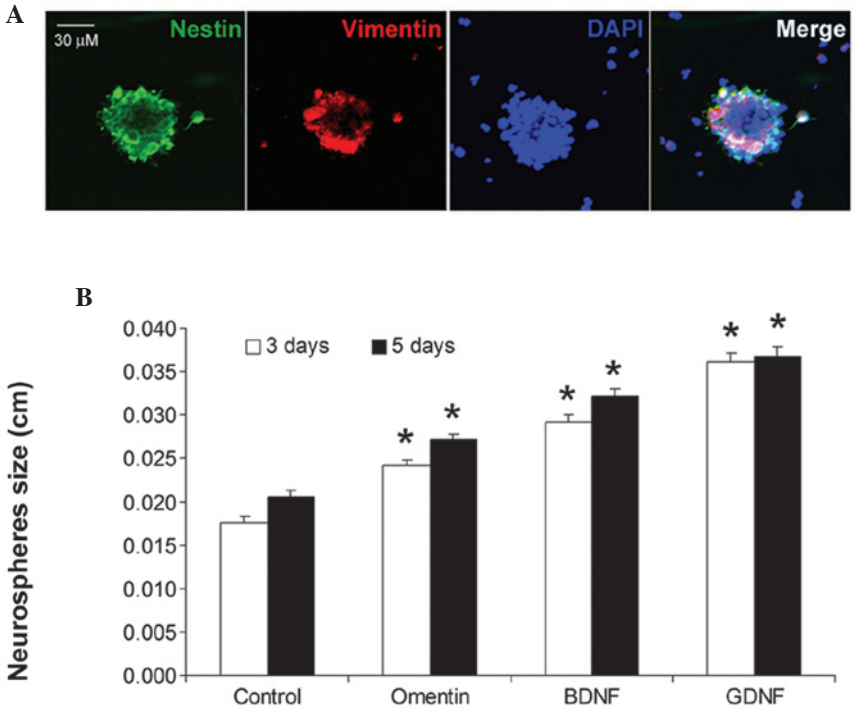

Figure 1. Isolation/culture of NSCs and the effect of omentin on neurosphere size. (A) NSCs were isolated and cultured *in vitro*. The neurospheres were stained using nestin and vimentin, two NSC markers. DAPI was used to stain the nuclei. (B) Effects of omentin (100 ng/ml) on neurosphere size. Omentin was added to the medium for 3 and 5 days. BDNF and GDNF were used as positive controls. *P<0.05, vs. control (n=8). NSCs, neural stem cells; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor.

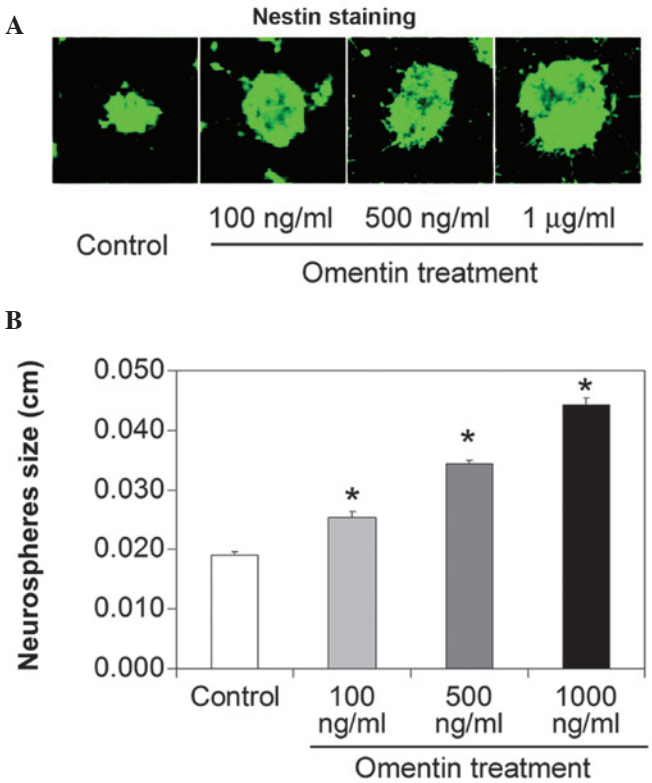

Figure 2. Omentin promotes neural stem cell growth in a dose-dependent manner. (A) Representative images of nestin immunofluorescence on neurospheres treated with different concentrations of omentin for 5 days. (B) Quantitative analysis of neurosphere size. *P<0.05, vs. control (n=8).

for 3 and 5 days. The viability of the NSCs was significantly increased (P<0.01) at these concentrations of omentin (Fig. 3A). The ability of omentin to protect NSCs under conditions of stress was also investigated. TNF-α, a pro-inflammatory cytokine, caused a marked decrease in cell viability, which was partly inhibited by omentin (Fig. 3B). These results indicate

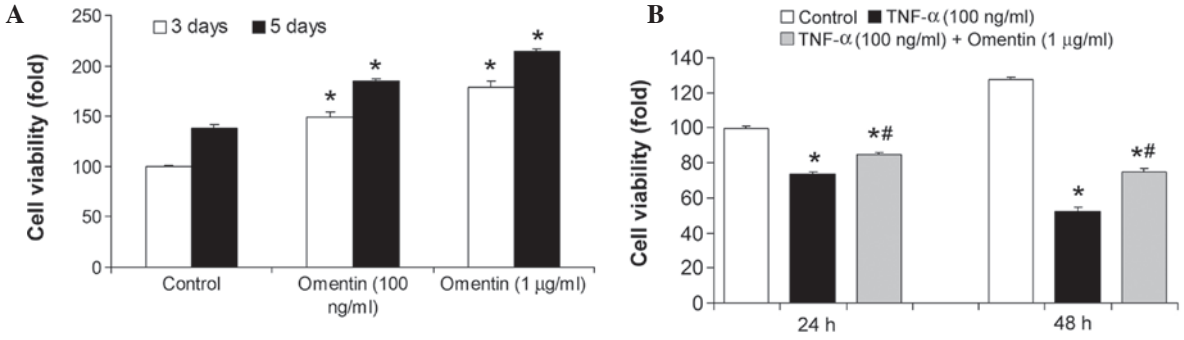

Figure 3. Omentin increases the viability of NSCs in normal and stress conditions. (A) NSCs were treated with omentin (100 ng/ml and 1 μg/ml) for 3 and 5 days and the cell viability was then measured. *P<0.05, vs. control (n=6). (B) NSCs were treated with TNF-α (100 ng/ml) or TNF-α (100 ng/ml) plus omentin (1 μg/ml). *P<0.05, vs. control; #P<0.05, vs. TNF-α treatment (n=6). NSCs, neural stem cells; TNF-α, tumor necrosis factor-α.

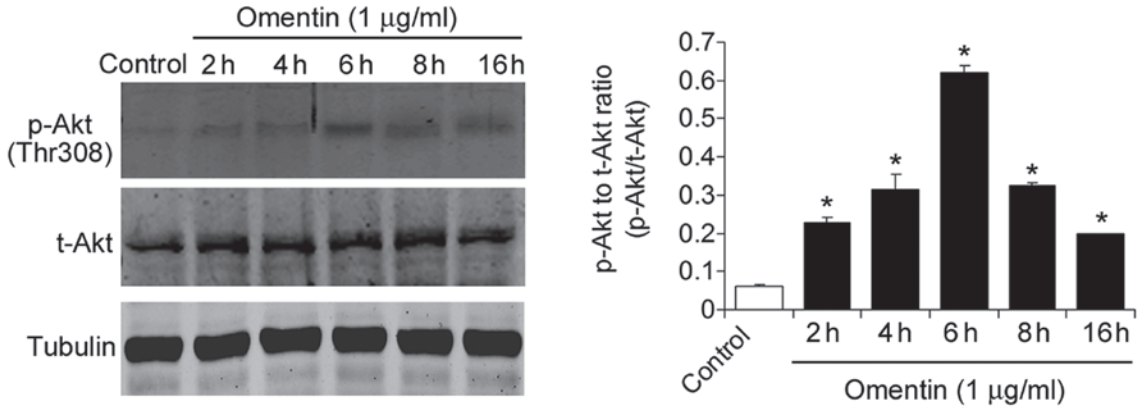

Figure 4. Omentin increases the phosphorylation of Akt in NSCs. Representative images and quantitative analysis of the phosphorylation of Akt (ratio of p-Akt^{Thr308} to t-Akt) in NSCs treated with omentin (1 μg/ml) for 2, 4, 6, 8 and 16 h. *P<0.05, vs. control (n=5). NSCs, neural stem cells; pAkt, phosphorylated Akt; t-Akt, total Akt.

that omentin increased NSC viability in normal conditions and conditions of stress.

Omentin activates Akt signaling in NSCs. Since Akt is a serine/threonine protein kinase, which is an important regulator of cell survival and proliferation (31), the present study investigated the effect of omentin on Akt signaling. The NSCs were treated with omentin (1 μg/ml) for 2, 4, 6, 8 and 16 h. Omentin treatment significantly increased the level of phospho-Akt^{Thr308}, peaking at 6 h (Fig. 4). Furthermore, the phosphorylation of AS160, the substrate of phosphorylated Akt was measured. Treatment with omentin also significantly increased the level of phospho-AS160^{Ser318}, peaking at 6 h (Fig. 5). These results suggested that omentin activated Akt signaling in the NSCs.

Activation of Akt signaling by omentin is required for its pro-survival effect on NSCs. To evaluate the importance of Akt signaling by omentin, LY294002, a specific inhibitor of Akt signaling, was used. Inhibition of Akt signaling by treatment with LY294002 (10 μM for 24 and 48 h) markedly suppressed the increase in neurosphere size promoted by omentin (Fig. 6A). Similarly, LY294002 eradicated the promoting effect of omentin on NSC viability (Fig. 6B). These results indicated that the activation of Akt signaling by omentin was required for its pro-survival effect on NSCs.

Discussion

The present study demonstrated that omentin promoted the growth of NSCs and protected them against inflammatory factor-induced damage. Additional investigation revealed that omentin markedly increased Akt phosphorylation (Thr308) and AS160 phosphorylation (Ser318) in NSCs. Treatment with the PI3K/Akt inhibitor, LY294002, in NSCs eliminated the promoting effect of omentin.

To the best of our knowledge, the present study is the first to provide evidence that omentin promotes NSC growth. As an adipokine released by adipose tissue, omentin has been observed to exhibit proliferating and anti-proliferative effects in certain types of tissues/cells (9). Omentin is present in the fetus and neonate, the concentration of which is higher than that in maternal serum (32), suggesting it may be crucial to enhance a growth-promoting effect. Xie *et al* demonstrated that omentin (25-200 ng/ml) stimulates proliferation and inhibits differentiation in primary mouse osteoblasts (33). In addition, omentin was demonstrated to inhibit matrix mineralization in osteoblasts (33) and 25-200 ng/ml omentin has been found to promote the proliferation of human osteoblasts through the PI3K/Akt signaling pathway (34). By contrast, Zhang and Zhou provided evidence that omentin upregulates p53 to induce apoptosis in human hepatocellular carcinoma

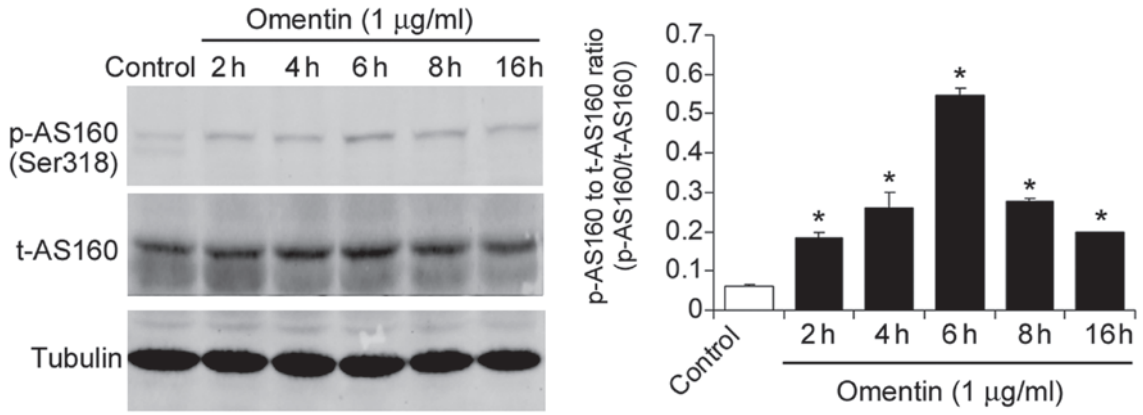

Figure 5. Omentin increases AS160 phosphorylation in NSCs. Representative images and quantitative analysis of the phosphorylation of AS160 (ratio of p-AS160^{Ser318} to t-AS160) in NSCs treated with omentin (1 ng/ml) for 2, 4, 6, 8 and 16 h. *P<0.05, vs. control (n=5). NSCs, neural stem cells.

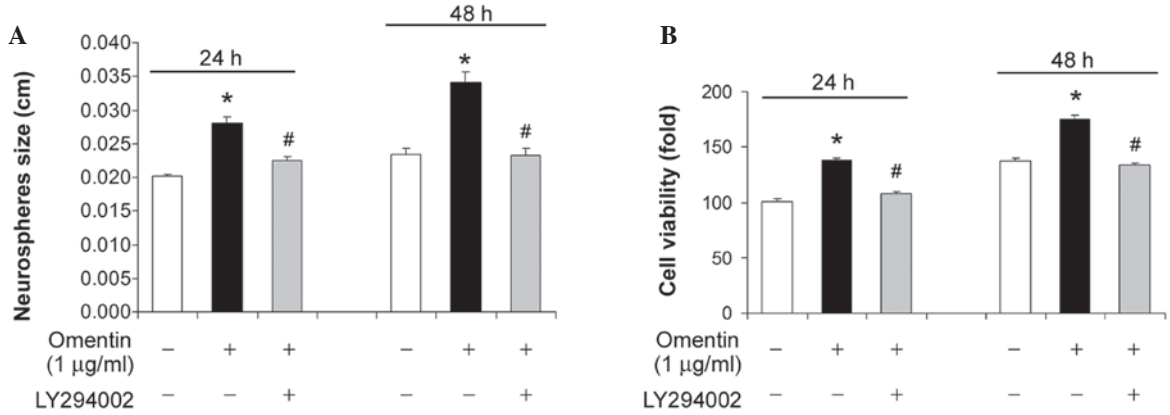

Figure 6. Phosphatidylinositol 3-kinase/Akt signaling pathway inhibitor LY294002 inhibits the promoting effect of omentin on neural stem cells. (A) Size of neurospheres treated with omentin or omentin with LY294002 (10 μM) for 24 and 48 h. *P<0.05, vs. control; #P<0.05, vs. omentin (n=8). (B) Cell viability of neurospheres treated with omentin or omentin with LY294002 (10 μM) for 24 and 48 h. *P<0.05, vs. control; #P<0.05, vs. omentin (n=8).

cells (35). In the present study, the effects of three different concentrations of omentin (100 and 500 ng/ml and 1 μg/ml) in NSCs were assessed. All three concentrations of omentin had a significant promoting effect on the growth of the NSCs *in vitro*. Of note, these concentrations were similar to the physiological concentration (300-600 ng/ml) (11,12). Therefore, the neurotrophic effect of omentin under physiological conditions may be important in the development and maintenance of NSCs. In addition, these results provide new information regarding the potential inherent connection between adipose and brain tissues.

The present study also identified that the activation of Akt signaling by omentin underlies the molecular mechanism of the promoting effect of omentin on NSCs. Previously, it has been found that omentin is able to trigger intracellular signaling pathways, among which the Akt signaling pathway has been extensively investigated (34,36). In human adipocytes, omentin increases Akt phosphorylation in the absence and presence of insulin (9). Omentin inhibits the osteoblastic differentiation of calcifying vascular smooth muscle cells and attenuates the arterial calcification and loss of bone observed in osteoprotegerin-deficient mice through the PI3K/Akt pathway (33,36). Additionally, omentin stimulates endothelial cell function and ischemia-induced revascularization through its ability to stimulate the Akt-endothelial nitric oxide synthase (eNOS) signaling pathway (37). A previous study reported that omentin did not induce Akt phosphorylation in endothelial cells (38). The present study observed significant increases in the expression of Akt and its downstream factor AS160 in NSCs. Inhibition of PI3K/Akt signaling by LY294002 eliminated the promoting effect of omentin on NSCs, suggesting that the activation of Akt signaling is essential for its promoting effect on NSCs. In addition, these results support the hypothesis that Akt mediates the pro-survival effect of omentin in NSCs. Previous studies have demonstrated that omentin activates other signaling pathways, including the extracellular signal-regulated kinase (39), AMP-activated protein kinase (40), c-Jun N-terminal kinase (40) and p38 (41) signaling pathways. These findings indicate that omentin may have multiple receptors to induce diverse intracellular signaling cascades, for which further investigation is warranted.

In conclusion, the present study demonstrated for the first time, to the best of our knowledge, that omentin promotes the growth and survival of NSCs *in vitro* through activation of the Akt signaling pathway. These results may improve current understanding on the role of omentin in the nervous system.

Acknowledgements

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