Original Article
Angiopoietin-2 induces the neuronal differentiation of mouse embryonic NSCs via phosphatidylinositol 3 kinase-Akt pathway-mediated phosphorylation of mTOR

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Abstract: The fate of neural stem cells (NSCs) is decided by numerous growth factors. Among these factors, the well-known angiogenic factor angiopoietin-2 (Ang-2) has been revealed to participate in neurogenesis separate from its role in angiogenesis. However, the effect of Ang-2 on the fate determination of mouse embryonic NSCs and the underlying mechanism remain unclear. This result of this study indicated that treatment of mouse embryonic NSCs with 200 ng/ml Ang-2 significantly promoted neuronal differentiation without affecting glial differentiation, and mammalian target of rapamycin (mTOR) was phosphorylated in a phosphatidylinositol 3-kinase (PI3K)/Akt-dependent manner during this process. Rapamycin, a specific mTOR inhibitor, suppressed the increase in neuronal differentiation stimulated by Ang-2, and this suppression did not result from an effect of Ang-2 or rapamycin on the apoptosis of differentiated NSCs. Collectively, our research demonstrates that PI3K/Akt pathway-mediated mTOR phosphorylation plays an important role in the Ang-2-enhanced neuronal differentiation of mouse embryonic NSCs.

Keywords: Neural stem cell, neuronal differentiation, Ang-2, mTOR, rapamycin

Introduction

In light of the potential of neural stem cells (NSCs) to generate new neurons to compensate for loss and to reconstruct damaged neuronal circuitry, NSC-based therapies have shown great promise in treating numerous central nervous system (CNS) injuries and neurological diseases, such as Parkinson's disease, ischemic stroke, traumatic brain injury (TBI), and spinal cord injury (SCI) [1-4]. Therefore, strategies to promote the neuronal differentiation of NSCs are attracting considerable investment worldwide [1-4]. Accumulating evidence supports the idea that neurogenesis is linked to angiogenesis by many growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor and angiogenic factors [5-7]. Among these factors, Ang-2, which was originally identified as a vascular-specific growth factor that affects vascular formation and function, has been revealed to also have a regulatory effect on neurogenesis [8-10]. Ang-2 is expressed in endothelial cells, neurons and neural progenitor cells in the embryonic cerebral cortex and adult subventricular zone (SVZ) [8-10]. Ang-2 expression is mainly increased in perivascular cells and nonvascular glial cells, and the level of Ang-2 upregulation was related to better spontaneous functional recovery after SCI [11]. In particular, Liu et al. [10] found that Ang-2 enhanced the migration of neural

progenitor cells (NPCs) and stimulated the neuronal differentiation of NPCs in a dose-dependent manner. However, neither the effects of Ang-2 on the differentiation of mouse embryonic NSCs nor the underlying signaling mechanisms are fully understood.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is of particular interest due to its involvement in the proliferation, differentiation, survival, and migration of NSCs [12-14]. This pathway is involved in the neuroprotective effect against apoptotic stress induced by Ang-1 [15] and participates in Ang-2-induced chemotaxis [16]. Mammalian target of rapamycin (mTOR), an important downstream target of PI3K/Akt, is implicated in the differentiation of multiple cell types and the development of embryos [17, 18]. mTOR plays an important role in the insulin-stimulated neuronal differentiation of NPCs derived from the telencephalon [17] and enhances the neuronal differentiation of SVZ cells [18]. However, little is known about the role of the PI3K/Akt/mTOR pathway in mouse embryonic NSCs. Therefore, the aims of the present study were to investigate the effect of Ang-2 on mouse embryonic NSC differentiation and to ascertain whether the PI3K/Akt/mTOR signaling pathway mediates the process, with a particular focus on the role of mTOR.

Materials and methods

NSC culture

All animal procedures were approved by the Ethics Committee of Tianjin Medical University and were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. NSCs were obtained from the embryonic cortex of specific pathogen-free (SPF) C57BL/6J mice (E13.5) as described previously [19, 20]. Briefly, cerebral hemispheres were dissected, minced and incubated with a mixture of Accutase (Invitrogen, Carlsbad, CA, USA) and 20 units/ml deoxyribonuclease I (DNase I; Worthington, NJ, USA). After centrifugation, the pellets were resuspended in freshly prepared serum-free Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12; Invitrogen) containing 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF) (Pepro-Tech, Rocky Hill, NJ, USA); 2.5 µg/ml heparin

(Tocris Bioscience, Minneapolis, MN, USA); and 2% B-27 supplement, 1 mM L-glutamine and 1% penicillin/streptomycin (P/S; Invitrogen). The cells were cultured in a humidified incubator at 37°C with 5% CO_2. Half of the growth medium was replaced every three days, and the cells were subcultured every seven days by treatment with Accutase for 10 min. Then, third-passage neurospheres were collected and seeded on plates precoated with poly-L-lysine (PLL) (0.1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 24 hours, followed by immunostaining for nestin (a specific marker of NSCs).

NSC differentiation assay and treatment

To assess the multipotent differentiation capability of NSCs, we collected third-passage neurospheres, dissociated them, and resuspended them in differentiation medium (DMEM/F-12 medium supplemented with 2% B-27 and 1% fetal bovine serum; Invitrogen). The cells were then plated at 5×10^4 cells/cm² onto PLL-coated culture plates and allowed to differentiate for 7 days.

To investigate the exact effects of Ang-2 on the fate determination of mouse embryonic NSCs, 200 ng/ml recombinant human Ang-2 (R&D Systems, Minnesota, USA) was added to the differentiation medium for 7 days. Then, immunocytochemistry and Western blot analysis were performed.

To identify the involvement of the PI3K/Akt pathway in the response to Ang-2 stimulation, we added specific inhibitors of PI3K (LY294002, 50 µM; Cell Signaling Technology, Danvers, MA, USA) and Akt (rapamycin, 10 nM; Cell Signaling Technology) to the differentiation medium for a 2-hour pretreatment.

Immunocytochemistry

Immunocytochemistry procedures for cultured cells were performed as described previously. Cells (neurospheres and differentiated NSCs) were fixed in 4% paraformaldehyde and then washed with phosphate-buffered saline (PBS). After being permeabilized for 10 min with 0.3% Triton X-100 (Sigma-Aldrich), the cells were incubated in 10% normal goat serum (NGS; Invitrogen) for one hour to block nonspecific bin-

ding sites. Then, the cells were incubated overnight at 4°C with primary antibodies against class III beta-tubulin (β-III-tubulin, rabbit monoclonal, 1:500; Covance, Princeton, NJ) and microtubule-associated protein-2 (MAP-2, rabbit monoclonal, 1:200; Millipore, Billerica, MA, USA) to identify neurons; glial fibrillary acidic protein (GFAP, rabbit monoclonal, 1:1000; Abcam, Cambridge, UK) to identify astrocytes; and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, mouse monoclonal, 1:200; Abcam) to identify oligodendrocytes. The cells were then washed with PBS and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibody (1:100; ZSGB-BIO, Beijing, China) or goat anti-mouse secondary antibody (1:100; ZSGB-BIO). 4',6-Diamidino-2-phenylindole (DAPI; 1 µg/ml; Beijing Solarbio Science & Technology Co., Beijing, China) was applied to the cells for five minutes to stain nuclei, and fluorescence was detected using a fluorescence microscope (IX71, Olympus, Tokyo, Japan). Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Cells in ten random fields were counted in each of three independent experiments (n = 10). Percentages of differentiated cells under different treatment conditions were calculated by dividing the number of marker-positive cells by the total number of DAPI-stained cells.

Western blot analysis

Cells were lysed in RIPA buffer (Santa Cruz Biotechnology, CA, USA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and a phosphatase inhibitor cocktail (Roche). Protein concentrations were assessed by using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein extracts (20 µg/lane) were separated by 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Nonspecific binding sites were blocked with 5% bovine serum albumin (Sigma-Aldrich), and the membranes were subsequently incubated overnight at 4°C with primary antibodies against β-III tubulin (rabbit monoclonal, 1:2000; Covance); MAP-2 (rabbit monoclonal, 1:2000; Millipore); GFAP (rabbit monoclonal, 1:1000; Abcam); CNPase (mouse monoclonal 1:500; Abcam); p-Akt, Akt, p-mTOR, mTOR, p-p70S6K (Thr^{421}/Ser^{424}), p-

p70S6K (Thr^{389}), and p70S6K (all rabbit monoclonal, 1:1000; Cell Signaling Technology); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit monoclonal, 1:1000; ZS-GB-BIO). Then, the membranes were incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (1:5000; ZSGB-BIO) at room temperature for one hour and subsequently with enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA). Western blots were quantified with ImageJ software, and target protein band intensities were normalized to the GAPDH band intensity.

Apoptosis analysis

Apoptosis was assessed using an annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Pharmingen, San Diego, CA). Briefly, differentiated NSCs in each group were washed twice with ice-cold PBS, digested in Accutase and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/ml after centrifugation. Subsequently, 5 µl of annexin V-FITC and 5 µl of PI were added to 100 µl of the cell suspension. The mixtures were gently vortexed and then incubated for 15 min in the dark. The samples were analyzed using the Accuri™ C6 personal flow cytometer (BD, Oxford, UK) immediately after the addition of 400 µl of 1 × binding buffer. Early apoptotic (annexin V^+ and PI^-), late apoptotic (annexin V^+ and PI^+), necrotic (annexin V^- and PI^+), and live cells (annexin V^- and PI^-) were counted.

Statistical analysis

All data were analyzed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA), and the values are presented as the mean ± standard error of the mean (SEM); each value represents the average of three independent experiments. Statistical significance was determined by the independent sample t-test when two groups were being compared, by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis for multiple comparisons when three or more groups were being compared, and by the chi-squared (χ^2) test when enumeration data were being compared. Differences were considered significant at $P < 0.05$.
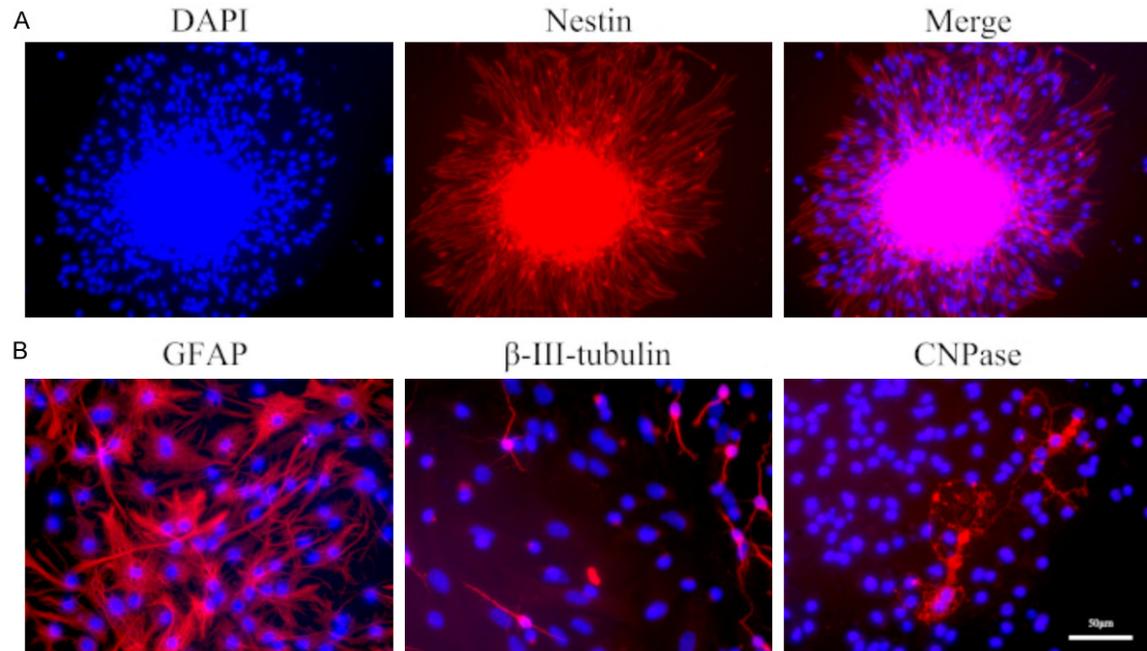
Figure 1. Identification of mouse embryonic NSCs. A. Immunocytochemical staining of third-passage neurospheres revealed that approximately two-thirds of the cells in the outer zone were nestin positive. B. After third-passage neurospheres were dissociated and cultured in differentiation medium, immunocytochemical staining of differentiated NSCs showed the presence of cells positive for β-III tubulin, GFAP, and CNPase.

Results

Identification of mouse embryonic NSCs

Mouse embryonic NSCs proliferate in the form of floating neurospheres on the second day after primary culture. Immunocytochemical staining of third-passage neurospheres revealed that approximately two-thirds of the cells in the outer zone were nestin positive (**Figure 1A**). After third-passage neurospheres were dissociated and cultured in differentiation medium, immunocytochemical staining of differentiated NSCs showed the presence of cells positive for β-III tubulin, GFAP, and CNPase (**Figure 1B**). These results demonstrated that mouse embryonic NSCs able to differentiate into neurons, astrocytes and oligodendrocytes were successfully isolated and cultured.

Ang-2 promotes the neuronal differentiation of embryonic NSCs without affecting glial differentiation

To examine the effect of Ang-2 on NSC fate determination in vitro, the experiment described in **Figure 2A** was performed. The cells were cultured in differentiation medium containing Ang-2 for 7 days and then immunostained for β-III tubulin, MAP-2, GFAP, and CNPase. The data revealed that in the differentiation medium, the proportion of β-III tubulin-positive neurons was significantly higher in the Ang-2-treated group than in the control group (27.46 ± 1.98% versus 16.53 ± 2.42%, $P < 0.05$, **Figure 2B**, **2C**). The induction of neuronal differentiation was validated using MAP-2, another neuron-specific marker (**Figure 2B**, **2C**). Consistent with the β-III tubulin results, the MAP-2-positive fraction increased from 11.06 ± 1.30% to 23.18 ± 2.06% with the addition of Ang-2 ($P < 0.05$, **Figure 2B**, **2C**), demonstrating that Ang-2 promotes the neuronal differentiation of mouse embryonic NSCs. In contrast, treatment with Ang-2 did not alter the percentage of GFAP-positive cells or CNPase-positive cells ($P > 0.05$, **Figure 2B**, **2C**). These results suggested that Ang-2 strongly promoted neuronal differentiation without affecting the glial differentiation potential of mouse embryonic NSCs.

In agreement with the immunocytochemical results, the changes in the protein expression levels of β-III tubulin, MAP-2, GFAP and CNPase in each group were confirmed by Western blot (**Figure 2D**). Compared to those in the control group, β-III tubulin and MAP-2 expression levels in the Ang-2 treatment group were approx-
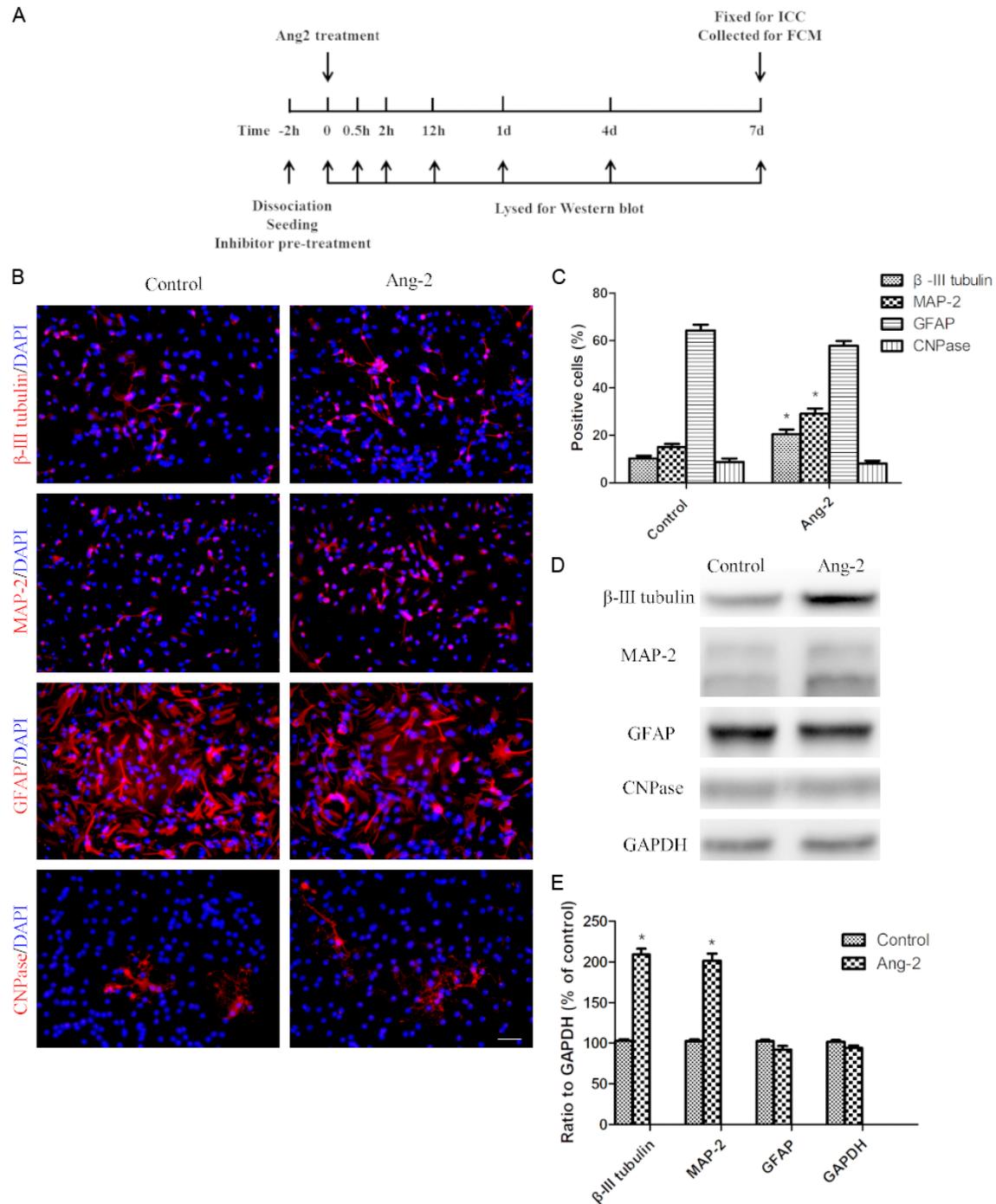
Figure 2. Ang-2 induced the neuronal differentiation of mouse embryonic NSCs. A. A schematic of the experimental process. B. Representative immunostaining of differentiated mouse embryonic NSCs with anti-β-III tubulin, anti-GFAP and anti-CNPase antibodies after 7 days of culture in differentiation medium. C. The numbers of differentiated neurons, astrocytes, and oligodendrocytes expressed as the percentages of cells positive for β-III tubulin, GFAP and CNPase among all DAPI-positive cells. D, E. The protein expression levels of β-III tubulin, GFAP and CNPase as analyzed by Western blot. Data are shown as the mean ± SEM of three independent experiments. Scale bar = 50 μm. *P < 0.05 compared with the control cultures.

imately 1.70-fold and 2.10-fold higher, respectively (P < 0.05, **Figure 2E**). Meanwhile, GFAP and CNPase protein expression levels were not significantly different between the two groups (P > 0.05, **Figure 2E**). These Western blot results validated the finding that Ang-2 promotes
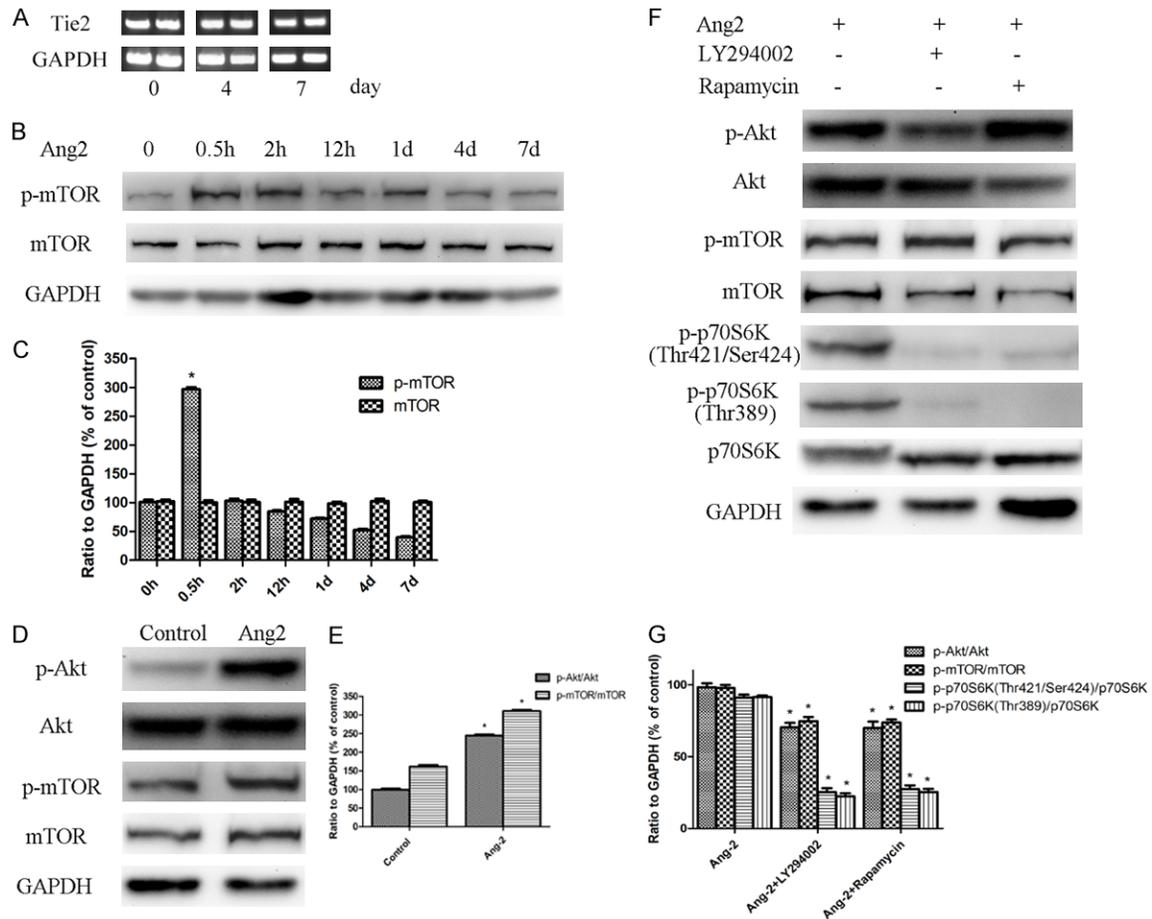
Figure 3. Activation of mTOR by Ang-2 was mediated by the PI3K/Akt pathway. A. RT-PCR analysis of Tie-2 expression in differentiated NSCs was performed at different time points. B, C. The protein expression levels of p-mTOR and mTOR at different time points over 7 days of Ang-2-mediated neuronal differentiation of NSCs. D, E. The protein expression levels of p-Akt, Akt, p-mTOR and mTOR after treatment with Ang-2 for 7 days. F, G. The dissociated neurospheres were pretreated with LY294002 or rapamycin for 2 hours before Ang-2 treatment. The protein expression levels of p-Akt, Akt, p-mTOR, mTOR, p-p70S6K (Thr^{421}/Ser^{424} and Thr^{389}), and p70S6K were evaluated by Western blot analysis.

neuronal induction but has no significant influence on the glial differentiation potential of mouse embryonic NSCs.

Tie-2 is expressed in differentiated embryonic NSCs

RT-PCR analysis of Tie-2 expression in differentiated NSCs was performed at different time points. We found that Tie-2 was expressed in all cells in the control group and in the Ang-2-treated group on days 4 and 7 (**Figure 3A**).

mTOR is activated by Ang-2 via the PI3K/Akt pathway in embryonic NSCs

Mochizuki et al. [16] reported that Ang-2 regulates chemotaxis via PI3K/Akt signaling.

However, the signaling pathway through which Ang-2 induces the neuronal differentiation of mouse embryonic NSCs remains to be elucidated. Here, we monitored p-mTOR at different time points over 7 days of Ang-2-mediated neuronal differentiation of NSCs. p-mTOR levels peaked at 30 min and then declined markedly with time, but total mTOR levels were not affected (**Figure 3B**, **3C**). Rapamycin (10 nM) mostly inhibited mTOR activity induced by Ang-2 (**Figure 3D**, **3E**). Moreover, Ang-2 caused a significant elevation in phosphorylated, but not total, Akt levels (**Figure 3D**, **3E**), and this increase was significantly suppressed by the PI3K inhibitor LY294002 (**Figure 3F**, **3G**). Once the Ang-2-mediated activation of Akt was inhibited, p-mTOR levels were also suppressed (**Figure**
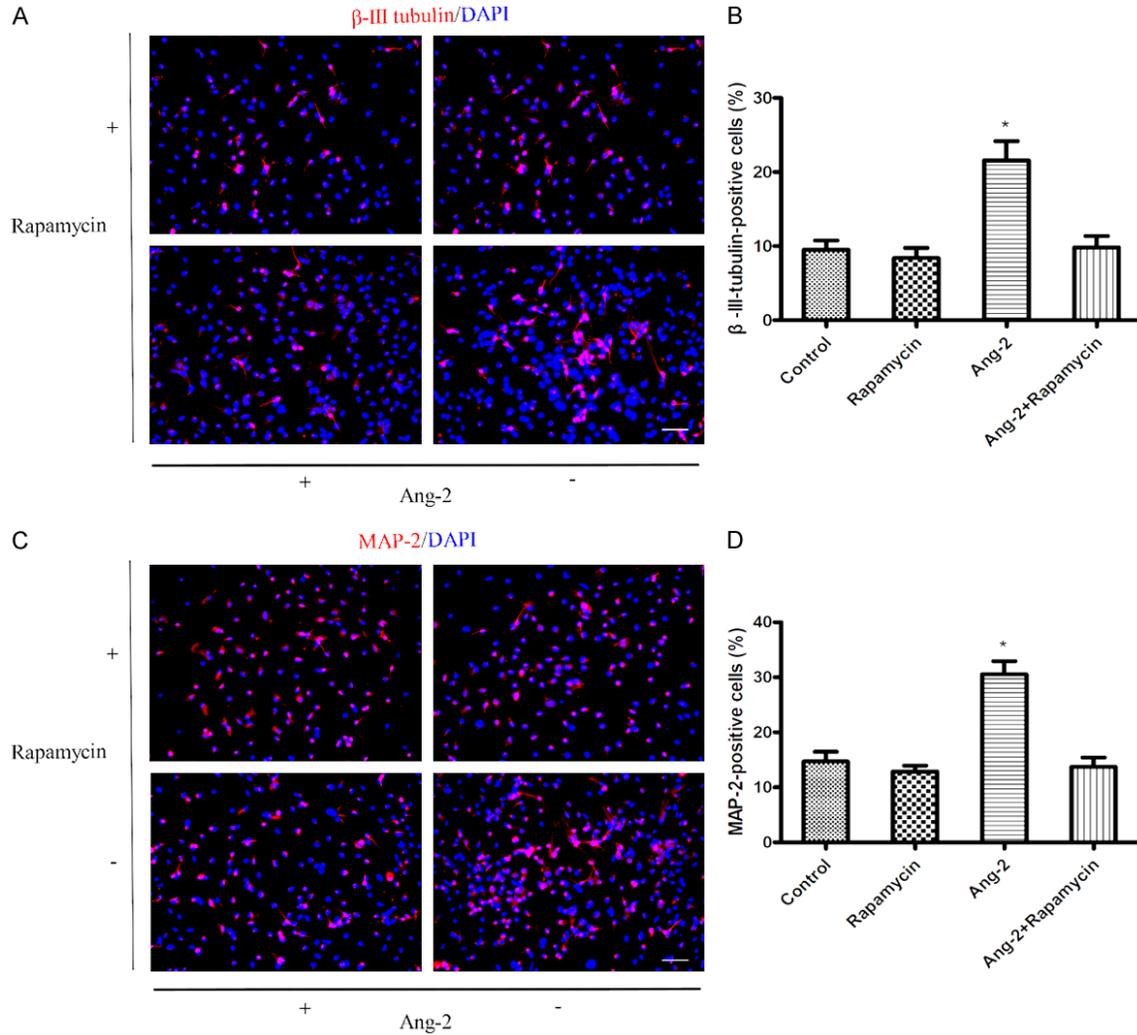
Figure 4. mTOR was involved in the promotion of mouse embryonic NSC neuronal differentiation by Ang-2. A. Dissociated neurospheres were pretreated with rapamycin for 2 hours before Ang-2 treatment and subsequently grown on PLL-coated plates. Representative immunostaining of mouse embryonic NSCs for β-III tubulin. B. The percentage of β-III tubulin-positive neurons was calculated. C. Representative immunostaining of mouse embryonic NSCs for MAP-2. D. The percentage of MAP-2-positive neurons was calculated. Scale bar = 50 μm. *$P < 0.05$ compared with the control cultures.

3F, **3G**). When rapamycin was applied, the induction of p-Akt by Ang-2 was not affected, but that of p-mTOR and p-p70S6K (Thr^{421}/Ser^{424} and Thr^{389}) was repressed (**Figure 3F**, **3G**). These results demonstrated that mTOR was activated by Ang-2 via the PI3K/Akt pathway in embryonic NSCs.

Ang-2-induced neuronal differentiation is dependent on mTOR

Our results suggest that mTOR is activated by PI3K/Akt signaling in Ang-2-treated embryonic NSCs. Consequently, we wondered whether mT-OR is involved in the Ang-2-induced neuronal differentiation of mouse embryonic NSCs. Therefore, we cultured embryonic NSCs on PLL-treated dishes in Ang-2-free or Ang-2-containing differentiation medium in the presence or absence of rapamycin for 7 days. In the absence of rapamycin, there was a significant difference in the percentage of β-III tubulin-positive neurons between the Ang-2-treated group and the control group ($P < 0.05$), as shown in **Figure 4A**, **4B**. However, when rapamycin was added to the Ang-2-containing medium, the percentage of β-III tubulin-positive cells decreased to 15.32 ± 1.63% ($P < 0.05$, **Figure**
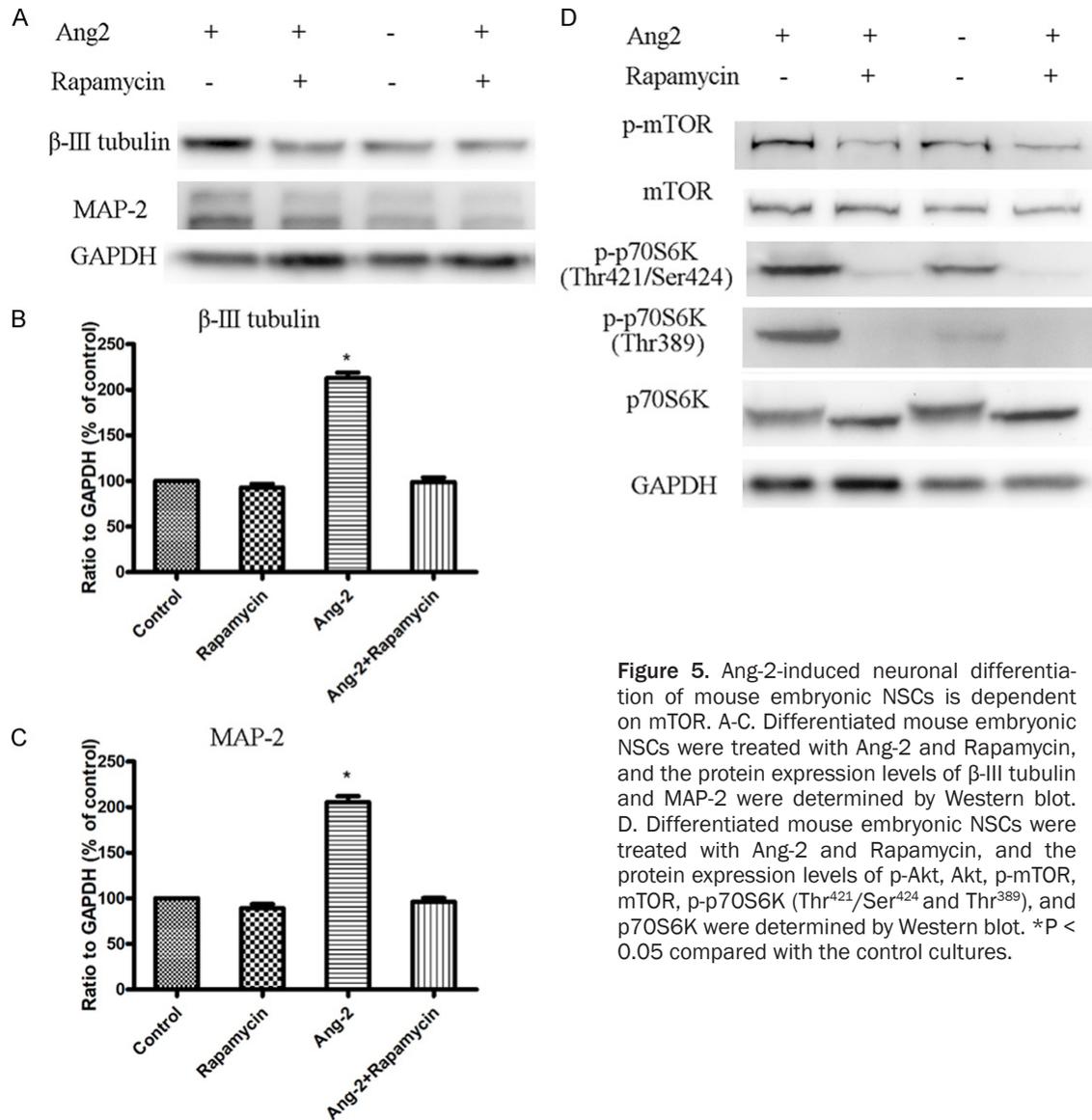
Figure 5. Ang-2-induced neuronal differentiation of mouse embryonic NSCs is dependent on mTOR. A-C. Differentiated mouse embryonic NSCs were treated with Ang-2 and Rapamycin, and the protein expression levels of β-III tubulin and MAP-2 were determined by Western blot. D. Differentiated mouse embryonic NSCs were treated with Ang-2 and Rapamycin, and the protein expression levels of p-Akt, Akt, p-mTOR, mTOR, p-p70S6K (Thr^{421}/Ser^{424} and Thr^{389}), and p70S6K were determined by Western blot. *P < 0.05 compared with the control cultures.

4A, 4B), but no significant difference was observed between the control group and the rapamycin-only treatment group (P > 0.05, **Figure 4A, 4B**). Similar data were recorded for MAP-2 immunostaining (**Figure 4C, 4D**), confirming that rapamycin could repress the Ang-2-enhanced neuronal differentiation of mouse embryonic NSCs. Western blotting further demonstrated that the expression levels of the neuronal markers β-III tubulin and MAP-2 were significantly upregulated after Ang-2 treatment (P < 0.05, **Figure 5A, 5B**; P < 0.05, **Figure 5A, 5C**), while the levels of both markers returned to control levels in the presence of rapamycin (P < 0.05, **Figure 5A, 5B**; P < 0.05, **Figure 5A, 5C**). As

shown in **Figure 5D**, the levels of p-mTOR and p-p70S6K (Thr^{421}/Ser^{424} and Thr^{389}) were significantly increased in Ang-2-treated NSCs but decreased markedly to control levels upon the addition of rapamycin. These results suggested that rapamycin repressed the neuronal differentiation of NSCs induced by Ang-2 and that mTOR was necessary for Ang-2 to promote the neuronal differentiation of embryonic NSCs.

Neither Ang-2 nor rapamycin induces apoptosis

Combined annexin V-FITC and PI staining was applied to rule out the possibility that the al-
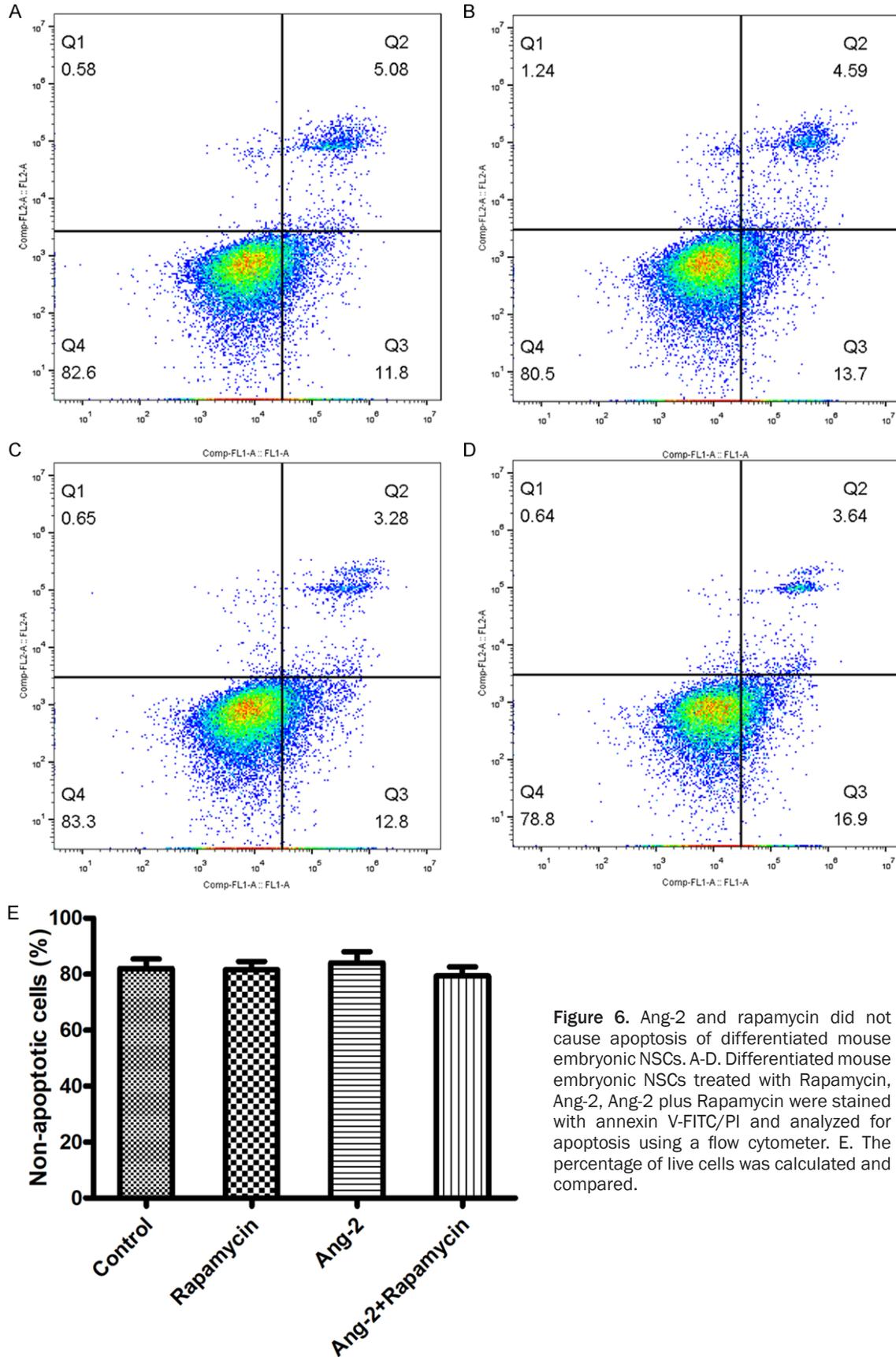
Figure 6. Ang-2 and rapamycin did not cause apoptosis of differentiated mouse embryonic NSCs. A-D. Differentiated mouse embryonic NSCs treated with Rapamycin, Ang-2, Ang-2 plus Rapamycin were stained with annexin V-FITC/PI and analyzed for apoptosis using a flow cytometer. E. The percentage of live cells was calculated and compared.

tered neuronal ratio upon treatment with Ang-2 and rapamycin was due to NSC apoptosis during the 7-day treatment period. As shown in **Figure 6A-D**, the percentage of live (annexin V$^-$ and PI$^-$) cells was greater than 80% in the Ang-2-treated, rapamycin-treated and control groups, and neither the Ang-2-treated group nor the rapamycin-treated group showed any significant difference compared to the control group (P > 0.05; **Figure 6E**), demonstrating that Ang-2 and rapamycin have no effect on apoptosis during NSC differentiation.

Discussion

In the present study, we found that Ang-2, originally identified as a vascular regulatory molecule, played a neurogenic role in mouse embryonic NSCs in vitro by stimulating neuronal differentiation without affecting glial differentiation. Moreover, our data showed that mTOR activation mediated by the PI3K/Akt signaling pathway was necessary for Ang-2 to promote neuronal differentiation, as indicated by our finding that repression of this pathway with the mTOR inhibitor rapamycin suppressed the Ang-2-mediated increase in mouse embryonic NSC neuronal differentiation.

A number of reports have described the crosstalk between the nervous and vascular systems [11, 21-24]. At the anatomical level, arteries are aligned with peripheral nerves and follow their branching pattern [21, 22]; neurogenesis has been observed in close proximity to blood vessels [21, 22, 24], which play an important role in the suitable patterning of neurogenesis [21, 22]. Neurovascular pathology has been observed in many CNS diseases, such as ischemic stroke, TBI, and SCI [21, 25]. In addition, neurogenesis and angiogenesis are linked via some vascular growth factors, such as VEGF, Ang-1, and Ang-2 [24, 26], and the functions of these growth factors in neurogenesis, independent of their role in angiogenesis, have been intensively investigated. VEGF promotes neurogenesis by augmenting the proliferation and neuronal differentiation of NPCs [5, 27]. Ang-1 promotes SVZ neurogenesis, elicits neurite outgrowth in PC12 cells, and plays a role in the neuronal differentiation and neurite outgrowth of mouse embryonic cortical and dorsal root ganglion cells [6, 7, 12, 28]. Despite the well-described involvement of Ang-2 in angiogen-

esis as an important and complex vascular growth factor, its effect on neurogenesis is not clear. More recently, roles for Ang-2 in increased proliferation in the rat SVZ [29] and in the neuronal differentiation and migration of SVZ progenitor cells [10] have been revealed. In addition, Marteau et al. [9] reported that Ang-2 plays an important role in embryonic cortical neurogenesis and especially in the radial migration of projection neurons. Based on these findings, we hypothesized that this angiogenic factor is a potent inducer of neurogenesis and can increase neuronal differentiation in mouse embryonic NSC cultures. In this study, we treated mouse embryonic NSCs with Ang-2 at a concentration of 200 ng/ml, which was reported to be the most effective dose for promoting differentiation without affecting cell proliferation or death [10]. Consistent with the results of a study by Liu et al. [10], the immunocytochemistry and Western blot results in this study demonstrated that Ang-2 increased the ratio of neurons and the expression levels of β-III tubulin and MAP-2. These results emphasize the essential role of Ang-2 in promoting the differentiation of mouse embryonic NSCs into neurons. Notably, neither the astrocyte or oligodendrocyte ratio nor GFAP or CNPase expression levels were significantly affected. Further, our study demonstrated that the effect of Ang-2 was not mediated by apoptosis. A possible explanation for this finding is an increase in the number of undifferentiated NSCs or other neurons, astrocytes, or oligodendrocytes not expressing β-III tubulin, GFAP, or CNPase, respectively.

Although the neurogenic effects of Ang-2 on mouse embryonic NSCs were revealed, the underlying mechanism remains unknown. PI3K/Akt signaling plays a crucial role in neurogenesis by stimulating the survival, proliferation, migration, and differentiation of NSCs [13, 30-32]. Activation of PI3K/Akt signaling is required for the neuroprotective effect of Ang-1 [15], as well as for regulating the proliferation and neuronal and astrocytic differentiation of olfactory bulb stem cells [30], and Ang-1 enhances neurogenesis in NPCs [6]. Moreover, Mochizuki et al. [16] reported that PI3K is activated by Ang-2 and is a prerequisite for Ang-2-induced chemotaxis. PI3K/Akt signaling can activate various downstream target molecules, such as nuclear factor kappa-light-chain-enh-

ancer of activated B cells (NF-κB), mouse double minute 2 homolog (MDM2), endothelial nitric oxide synthase (eNOS), mTOR, and S6 kinase [33]. Importantly, among these numerous target molecules, mTOR is one of the most widely explored; Bateman et al. [34] first demonstrated the necessity of mTOR for neuronal differentiation in the fly retina, and mTOR plays a role in insulin-induced neuronal differentiation in telencephalon-derived progenitors in vitro [17]. In addition, mTOR is involved in the initiation of neuronal differentiation and facilitates the coordination of differentiation programs and the cell cycle [17]. Therefore, based on previous research, we focused our attention on mTOR. Our data showed that the PI3K/Akt/mTOR pathway was activated by Ang-2 in an LY294002- and rapamycin-sensitive manner, suggesting that the PI3K/Akt pathway transduces the Ang-2 signal to mTOR to promote the neuronal differentiation of mouse embryonic NSCs. However, Chan et al. [35] reported that the PI3K signaling pathway is not required for the differentiation of NSCs; this discrepancy may be attributable to differences in cell source or experimental conditions. Meanwhile, we observed no significant neuronal differentiation in the rapamycin-only treatment group, perhaps because NSC differentiation status is maintained by several signaling pathways, some of which are not affected by rapamycin. Further, we demonstrated that mTOR suppression by rapamycin did not cause apoptosis during the induction of neuronal differentiation.

NSCs are regarded as a potential therapeutic tool for SCI as they could replace lost neurons and reconstruct the disrupted neuronal circuitry. Recently, a beneficial function of Ang-2 in SCI repair has been revealed; Durham-Lee demonstrated that high levels and persistent expression of Ang-2 are linked to successful locomotor recovery after SCI in a rat contusion model [11]. Therefore, the ability of Ang-2 to stimulate the neuronal differentiation of NSCs may suggest new strategies for SCI repair. Furthermore, the effect of combination therapy with Ang-2 and NSC transplantation for SCI will be investigated. In addition, there are several lines of evidence that the effects of Ang-2 are mainly mediated by binding to the tyrosine kinase receptor Tie2 [23], and we found that differentiated NSCs express Tie2. Th-

erefore, transduction of the Ang-2 neuronal differentiation stimulation signal to the PI3K/Akt/mTOR pathway via Tie2 remains to be explored.

Our results suggest that Ang-2, previously identified as an angiogenic factor, promotes the neuronal differentiation of mouse embryonic NSCs in vitro with no significant effect on glial differentiation. Notably, mTOR plays vital roles in the neuronal development of embryos, and mTOR knockout causes deficient embryonic development or even death at midgestation [36], making it difficult to investigate the neurogenic roles of mTOR in vivo. By applying specific inhibitors of PI3K and mTOR in vitro, this study extended the understanding of the functions of mTOR by revealing its determinant role as a downstream effector of the activated PI3K/Akt pathway in transducing the Ang-2 signal to stimulate the neuronal differentiation of mouse embryonic NSCs.

Disclosure of conflict of interest

None.

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References

[1] Huang H, Mao G, Chen L, Liu A. Progress and challenges with clinical cell therapy in neuro-restoratology. J Neurorestoratol 2015; 3: 91-99.

[2] Zhang BY, Liu CM, Wang RY, Zhu Q, Jiao Z, Zhou FQ. Akt-independent GSK3 inactivation downstream of PI3K signaling regulates mammalian axon regeneration. Biochem Biophys Res Commun 2014; 443: 743-748.

[3] Yu S, Yao S, Wen Y, Wang Y, Wang H, Xu Q. Angiogenic microspheres promote neural regeneration and motor function recovery after spinal cord injury in rats. Sci Rep 2016; 6: 33428.

[4] Ottoboni L, Merlini A, Martino G. Neural stem cell plasticity: advantages in therapy for the injured central nervous system. Front Cell Dev Biol 2017; 5: 52.

[5] Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 2002; 99: 11946-11950.

[6] Bai Y, Cui M, Meng Z, Shen L, He Q, Zhang X, Chen F, Xiao J. Ectopic expression of angiopoietin-1 promotes neuronal differentiation in neural progenitor cells through the Akt pathway. Biochem Biophys Res Commun 2009; 378: 296-301.

[7] Ward NL, Putoczki T, Mearow K, Ivanco TL, Dumont DJ. Vascular-specific growth factor angiopoietin 1 is involved in the organization of neuronal processes. J Comp Neurol 2005; 482: 244-256.

[8] Hegen A, Koidl S, Weindel K, Marme D, Augustin HG, Fiedler U. Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements. Arterioscler Thromb Vasc Biol 2004; 24: 1803-1809.

[9] Marteau L, Pacary E, Valable S, Bernaudin M, Guillemot F, Petit E. Angiopoietin-2 regulates cortical neurogenesis in the developing telencephalon. Cereb Cortex 2011; 21: 1695-1702.

[10] Liu XS, Chopp M, Zhang RL, Hozeska-Solgot A, Gregg SC, Buller B, Lu M, Zhang ZG. Angiopoietin 2 mediates the differentiation and migration of neural progenitor cells in the subventricular zone after stroke. J Biol Chem 2009; 284: 22680-22689.

[11] Durham-Lee JC, Wu Y, Mokkapati VU, Paulucci-Holthauzen AA, Nesic O. Induction of angiopoietin-2 after spinal cord injury. Neuroscience 2012; 202: 454-464.

[12] Chen X, Fu W, Tung CE, Ward NL. Angiopoietin-1 induces neurite outgrowth of PC12 cells in a Tie2-independent, β1-integrin-dependent manner. Neurosci Res 2009; 64: 348-354.

[13] Koh SH, Lo EH. The role of the PI3K pathway in the regeneration of the damaged brain by neural stem cells after cerebral infarction. J Clin Neurol 2015; 11: 297-304.

[14] Peltier J, O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. Dev Neurobiol 2007; 67: 1348-1361.

[15] Valable S, Bellail A, Lesne S, Liot G, Mackenzie ET, Vivien D, Bernaudin M, Petit E. Angiopoietin-1-induced PI3-kinase activation prevents neuronal apoptosis. FASEB J 2003; 17: 443-445.

[16] Mochizuki Y, Nakamura T, Kanetake H, Kanda S. Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. J Cell Sci 2002; 115: 175-183.

[17] Han J, Wang B, Xiao Z, Gao Y, Zhao Y, Zhang J, Chen B, Wang X, Dai J. Mammalian target of rapamycin (mTOR) is involved in the neuronal differentiation of neural progenitors induced by insulin. Mol Cell Biol 2008; 39: 118-124.

[18] Rosa AI, Goncalves J, Cortes L, Bernardino L, Malva JO, Agasse F. The angiogenic factor Angiopoietin-1 is a proneurogenic peptide on subventricular zone stem/progenitor cells. J Neurosci 2010; 30: 4573-4584.

[19] Chu T, Zhou H, Wang T, Lu L, Li F, Liu B, Kong X, Feng S. In vitro characteristics of Valproic acid and all-trans-retinoic acid and their combined use in promoting neuronal differentiation while suppressing astrocytic differentiation in neural stem cells. Brain Res 2015; 1596: 31-47.

[20] Lu L, Zhou H, Pan B, Li X, Fu Z, Liu J, Shi Z, Chu T, Wei Z, Ning G, Feng S. c-Jun amino-terminal kinase is involved in valproic acid-mediated neuronal differentiation of mouse embryonic NSCs and neurite outgrowth of NSC-derived neurons. Neurochem Res 2017; 42: 1254-1266.

[21] Park JA, Choi KS, Kim SY, Kim KW. Coordinated interaction of the vascular and nervous systems: from molecule- to cell-based approaches. Biochem Biophys Res Commun 2003; 311: 247-253.

[22] Raab S, Plate KH. Different networks, common growth factors: shared growth factors and receptors of the vascular and the nervous system. Acta Neuropathol 2007; 113: 607-626.

[23] Masjkur J, Rueger MA, Bornstein SR, McKay R, Androutsellis-Theotokis A. Neurovascular signals suggest a propagation mechanism for endogenous stem cell activation along blood ves-

sels. CNS Neurol Disord Drug Targets 2012; 11: 805-817.

[24] Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 2000; 425: 479-494.

[25] Machein MR, Plate KH. Role of VEGF in developmental angiogenesis and in tumor angiogenesis in the brain. In: Kirsch M, Black PM, editors. Angiogenesis in Brain Tumors. Boston, MA: Springer; 2004. pp. 191-218.

[26] Ward NL, Lamanna JC. The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol Res 2004; 26: 870-883.

[27] Sun Y, Jin K, Childs JT, Xie L, Mao XO, Greenberg DA. Vascular endothelial growth factor-B (VEGFB) stimulates neurogenesis: evidence from knockout mice and growth factor administration. Dev Biol 2006; 289: 329-335.

[28] Kosacka J, Figiel M, Engele J, Hilbig H, Majewski M, Spanel-Borowski K. Angiopoietin-1 promotes neurite outgrowth from dorsal root ganglion cells positive for Tie-2 receptor. Cell Tissue Res 2005; 320: 11-19.

[29] Androutsellis-Theotokis A, Rueger MA, Park DM, Mkhikian H, Korb E, Poser SW, Walbridge S, Munasinghe J, Koretsky AP, Lonser RR, McKay RD. Targeting neural precursors in the adult brain rescues injured dopamine neurons. Proc Natl Acad Sci U S A 2009; 106: 13570-13575.

[30] Otaegi G, Yusta-Boyo MJ, Vergano-Vera E, Mendez-Gomez HR, Carrera AC, Abad JL, Gonzalez M, de la Rosa EJ, Vicario-Abejon C, de Pablo F. Modulation of the PI 3-kinase-Akt signalling pathway by IGF-I and PTEN regulates the differentiation of neural stem/precursor cells. J Cell Sci 2006; 119: 2739-2748.

[31] Wahane SD, Hellbach N, Prentzell MT, Weise SC, Vezzali R, Kreutz C, Timmer J, Krieglstein K, Thedieck K, Vogel T. PI3K-p110-alpha-subtype signalling mediates survival, proliferation and neurogenesis of cortical progenitor cells via activation of mTORC2. J Neurochem 2014; 130: 255-267.

[32] Sato A, Sunayama J, Matsuda KI, Tachibana K, Sakurada K, Tomiyama A, Kayama T, Kitanaka C. Regulation of neural stem/progenitor cell maintenance by PI3K and mTOR. Neurosci Lett 2010; 470: 115-120.

[33] Marteau L, Valable S, Divoux D, Roussel SA, Touzani O, MacKenzie ET, Bernaudin M, Petit E. Angiopoietin-2 is vasoprotective in the acute phase of cerebral ischemia. J Cereb Blood Flow Metab 2013; 33: 389-395.

[34] Bateman JM, McNeill H. Temporal control of differentiation by the insulin receptor/tor pathway in Drosophila. Cell 2004; 119: 87-96.

[35] Chan WS, Sideris A, Sutachan JJ, Montoya G JV, Blanck TJ, Recio-Pinto E. Differential regulation of proliferation and neuronal differentiation in adult rat spinal cord neural stem/progenitors by ERK1/2, Akt, and PLCγ. Front Mol Neurosci 2013; 6: 23.

[36] Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol Cell Biol 2004; 24: 9508-9516.