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...
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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) (PeproTech, 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% CO2. 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/cm^2 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-
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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).

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 (Thr⁴⁴⁷/Ser⁴⁴⁹), p-p70S6K (Thr³⁸⁹), and p70S6K (all rabbit monoclonal, 1:1000; Cell Signaling Technology); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit monoclonal, 1:1000; ZSGB-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 × 10⁶ 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 (χ²) test when enumeration data were being compared. Differences were considered significant at P < 0.05.
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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...
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Ang-2 treatment:

Time -2h 0 2h 12h 1d 4d 7d

Fixed for ICC
Collected for FCM

Dissociation
Seeding
Inhibitor pre-treatment
Lysed for Western blot

A

B

Control
Ang-2

β-III tubulin
MAP-2
GFAP
CNPase
GAPDH

C

Positive cells (%)

0 20 40 60 80

Control
Ang-2

D

β-III tubulin
MAP-2
GFAP
CNPase
GAPDH

E

Ratio to GAPDH (% of control)

β-III tubulin
MAP-2
GFAP
GAPDH

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
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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 mTOR at different time points over 7 days of Ang-2-mediated neuronal differentiation of NSCs. 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, mTOR levels were also suppressed (Figure 3).

**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, mTOR, p-p70S6K (Thr421/Ser424 and Thr389), and p70S6K were evaluated by Western blot analysis.
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Our results suggest that mTOR is activated by PI3K/Akt signaling in Ang-2-treated embryonic NSCs. Consequently, we wondered whether mTOR 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 4C, 4D).

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 (Thr421/Ser424 and Thr389) was repressed (Figure 3F, 3G). These results demonstrated that mTOR was activated by Ang-2 via the PI3K/Akt pathway in embryonic NSCs.

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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 (Thr421/Ser424) and Thr389) 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-
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**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.
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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 cross-talk 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 angiogenesis 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-
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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.

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Disclosure of conflict of interest

None.

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