# Effect of leukocyte inhibitory factor on neuron differentiation from human induced pluripotent stem cell-derived neural precursor cells

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**Abstract.** Direct derivation of human induced pluripotent stem cells into neural precursor cells and differentiation of these into neurons holds great promise in the cell therapy of neurodegenerative diseases. However, the availability and survival rate of neurons requires improvement. In the present study, it was found that the addition of 5 ng/ml leukocyte inhibitory factor (LIF) during the process of differentiation significantly improved the expression of neuron-specific class III β-tubulin (TUJ1) and microtubule-associated protein 2 (MAP2), as detected by immunofluorescence and western blotting. In addition, LIF improved the cell viability, increased the expression of phosphorylated-protein kinase B (AKT), downregulated the expression of proinflammatory cytokines, including interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), and upregulated the expression of anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). After adding the phosphatidylinositol 3-kinase (PI3K)/AKT signaling inhibitor LY294002 or wortmannin to the LIF differentiation group, LIF-induced changes in the protein expression of TUJ1 and MAP2 were reversed, but this effect could not be prevented by rapamycin, a mechanistic target of rapamycin signaling inhibitor. The expression of cytokines associated with inflammation and cell viability was reversed by LY294002 and wortmannin, but not by rapamycin. In conclusion, LIF could improve neuronal differentiation and survival through the activation of PI3K/AKT signaling and the anti-inflammatory effect. The anti-inflammatory effect may be mediated by the activation of PI3K/AKT.

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## Introduction

Human induced pluripotent stem cells (hiPSCs) have broad prospects for application in the field of regenerative medicine due to their three germ layer differentiation ability and patient-specific cell source (1,2). Therefore, through differentiation into neural cells, hiPSCs can provide a good cell source for the cell therapy of neurodegenerative disease (3). It is known that, during the induction period, there is a precise molecular regulation mechanism, developmental sequence, overlap of time and overlap of space that should be followed (4,5). In the present study, the differentiation of mesodermal and endodermal cells was reduced through inhibition of bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signaling using the signaling pathway inhibitors LDN193189 and SB431542. In this manner, a large number of outer germ layer cells were obtained from which neural progenitors were derived. Efficient neuron differentiation from derived neural precursor cells (NPCs) in the cell treatment of neurodegenerative diseases is critical, but the low differentiation rate and poor survival state are still to be improved *in vitro* or *in vivo* (6,7). In previous studies, the addition of antioxidant and anti-inflammatory cytokines to the differentiation medium, and the combined use of neurotrophic factors during the differentiation period were able to improve the differentiation ratio and the survival state (8,9). However, the mechanism has not yet been clarified.

Leukocyte inhibitory factor (LIF) is a highly conserved gene of the interleukin (IL)-6 family (10). LIF has multiple functions, including the maintenance of the undifferentiated state of mouse embryonic stem cells (mESCs), the proliferation of primordial germ cells, and functions as a mediator in implantation and decidualization (11). It has been found that Janus kinase/signal transducer and activator of transcription (JAK/STAT3), protein kinase B (AKT), extracellular signal-regulated protein kinases 1/2 (ERK1/2) and mechanistic target of rapamycin (mTOR) signaling pathways are involved in the biological function of LIF (12-15). LIF could improve cell proliferation in fibroblasts

and cardiomyocytes through activating the PI3K-sensitive AKT kinase (16,17). Phosphatidylinositol 3-kinase (PI3K)/AKT signaling is involved in numerous events in neuronal proliferation and differentiation. Downregulating PI3K/AKT signaling could inhibit neuroendocrine differentiation. By contrast, upregulating of this signal could increase the neuronal differentiation from mouse cochlear neural stem cells (18,19). It has been reported that LIF has a similar function to neurotrophic factor in neuronal differentiation from the mouse neural crest. Moreover, LIF can improve the maturation of sensory neurons and maintain their morphological characteristics (20). In a study using mice with spinal cord injury, LIF-treated mice manifested greater recovery of locomotor behavior due to an increase in the number of neurons and NPCs in the brain (21). However, the mechanism of LIF in neuron development has not been elaborated.

Anti-inflammation is one of the strategies for the treatment of neurodegenerative diseases and for neuronal protection (22,23). Certain studies have demonstrated that methylprednisolone facilitates the survival of new neurons and improves the neurological deficit following transient cerebral ischemia through the suppression of inflammatory reactions (24). Anti-inflammatory treatment could protect the dopaminergic neurons in 6-hydroxydopamine-lesioned rats through targeting not only the microglia, but also the other immune cells, including cluster of differentiation 163-positive macrophages (25). Inflammation of the neurons causes the release of inflammatory cytokines, including IL-6, IL-5 and LIF (26). In addition, LIF serves an important role in the inflammatory responses. It has been reported that injecting lipopolysaccharide (LPS) into the trachea of rats could induce the expression and secretion of LIF in bronchoalveolar cells (27). Moreover, inflammatory cytokines, including IL-6 and tumor necrosis factor-α (TNF-α), could increase the mRNA or protein expression of LIF during cell culture (28,29). However, studies on the stimulation of an anti-inflammatory effect by LIF in *in vitro* neuronal differentiation are rare.

In the present study, we would use LIF to activate the PI3K/AKT signal and induce the anti-inflammatory effect during the neuron differentiation from hiPSCs derived NPCs. This effect might improve the neuron differentiation ratio and survival state.

## Materials and methods

*Culture of undifferentiated hiPSCs*. The hiPSC cell line and the H9 embryonic stem cell line (gifts from Professor Lan Feng, Beijing Anzhen Hospital Beijing Institute of Heart Lung and Blood Vessel Disease, Capital Medical University, Beijing, China) were maintained in essential 8 medium (Table I) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in Matrigel (B&D Technologies, Macon, GA, USA) pre-coated dishes, 37˚C, 5% $CO_2$. The hiPSCs were passaged every 4-7 days when the cells reached 80-90% confluence.

*Neural induction*. For neural induction, the hiPSCs were split using accutase (Thermo Fisher Scientific, Inc.) for 5 min and seeded in a Matrigel pre-coated 6-well plate at a density of 1.6-2x$10^4$ cells/$cm^2$ in the presence of 5 $\mu$M ϱ-associated, coiled-coil-containing protein kinase (ROCK) inhibitor (Selleck Chemicals, Houston, TX, USA). Following 1 day of incubation, medium containing ROCK inhibitor was aspirated, and N2/B27 basic medium (Table I) containing a mixture of 1:1 Dulbecco's modified Eagle's medium (DMEM)/F12 and neurobasal medium, supplemented with 1% N2 and 2% B27 (all Gibco; Thermo Fisher Scientific, Inc.) was added. SB431542 (10 $\mu$M) and 100 nM LDN193189 (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added to the basal medium to trigger the neural induction. The neural induction lasted 11 days. On day 5 of the induction, the SB431542 was removed. During the induction period, the culture medium was changed every day.

Table I. Culture media and reagents.

| Medium | Reagents |
|---|---|
| hiPSC | E8 and its supplements |
| NPC induction | DMEM/F12[a] |
| | Neurobasal medium[a] |
| | 1% N2[a] |
| | 2% B27[a] |
| | 10 $\mu$M SB431542[b] |
| | 100 nM LDN193189[b] |
| Neuron differentiation | DMEM/F12[a] |
| | 2% B27[a] |
| | 1% N2[a] |
| | 20 ng/ml BDNF[c] |
| | 20 ng/ml GDNF[c] |
| | 0.2 mM AA[b] |
| | 0.5 mM dibutyryl cAMP[b] |
| | 5 ng/ml of LIF[c] |
| | LY294002 (20 $\mu$M)[b] |

[a]Gibco; Thermo Fisher Scientific, Inc.; [b]Merck KGaA; [c]Peprotech, Inc. hiPSC, human induced pluripotent stem cells; NPC, neural precursor cell; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; AA, ascorbic acid; LIF, leukocyte inhibitory factor; cAMP, cyclic adenosine monophosphate.

*Neural differentiation*. On day 11 of induction, the induction medium was changed to differentiation medium (Table I) consisting of DMEM/F12, supplemented with 2% B27, 1% N2, 20 ng/ml brain-derived neurotrophic factor (Peprotech, Rocky Hill, NJ, USA), 0.2 mM ascorbic acid (Sigma-Aldrich; Merck KGaA), 20 ng/ml glial cell line-derived neurotrophic factor (Peprotech) and 0.5 mM dibutyryl cAMP (Sigma-Aldrich; Merck KGaA). To prepare the LIF-positive differentiation group, 5 ng/ml LIF (Peprotech) was added to the differentiation medium. To prepare the PI3K/AKT inhibition differentiation group, 20 $\mu$M PI3K/AKT inhibitor LY294002, or 50 nM wortmannin (both Sigma-Aldrich; Merck KGaA) combined with 5 ng/ml LIF was added to the differentiation medium. To prepare the mTOR inhibition differentiation group, 100 nM rapamycin (Sigma-Aldrich; Merck KGaA) combined with 5 ng/ml of LIF was added to the differentiation medium. Derived NPCs were differentiated for 9 days. On culture day 20 (counting the first culture day as the day of the initial derived NPC induction from hiPSCs), the cells were harvested and prepared for further experimentation.

Table II. Primers used in this study for reverse transcription-quantitative polymerase chain reaction.

| Name | Sense primers | Antisense primers |
|---|---|---|
| *GAPDH* | CCCATGTTCGTCATGGGTGT | GATGGCATGGACTGTGGTCA |
| *OCT4* | TGAGGCCCTGGAGAAAGAGT | TTGCTGGCCTGTCTTCTCTG |
| *NANOG* | GCAGGGATGCCTGGTGAAC | GGACTGTTCCAGGCCTGATT |
| *SOX2* | GGATAAGTACACGCTGCCC | ATGTGCGCGTAACTGTCCAT |
| *LIN28* | GGAAAGAGCATGCAGAAGCG | TGATGCTCTGGCAGAAGTGG |
| *PAX6* | TGAGGCCCTGGAGAAAGAGT | TTGCTGGCCTGTCTTCTCTG |
| *NES* | AGTGATGCCCCTTCACCTTG | GCTCGCTCTCTACTTTCCCC |
| *IL-10* | CGAGATGCCTTCAGCAGAGT | CGCCTTGATGTCTGGGTCTT |
| *TGF-β* | TTGACTTCCGCAAGGACCTC | CTCCAAATGTAGGGGCAGGG |
| *IL-1α* | CCTGAGCTCGCCAGTGAAAT | GGTGGTCGGAGATTCGTAGC |
| *TNF-α* | TCCTCTCTGCCATCAAGAGC | AGTAGACCTGCCCAGACTCG |
| *MAP2* | TCTGCACACTCACATCCACC | CTGAGGTCAGCTCTCCGTTG |
| *TUJ1* | GCTGGTGGAAAACACGGATG | GCCGATACCAGGTGGTTGAG |

*IL-10*, interleukin-10; *TGF-β*, transforming growth factor-β; *TNF-α*, tumor necrosis factor-α; *MAP2*, microtubule-associated protein 2; *TUJ1*, neuron-specific class III β-tubulin; *NES*, nestin; *PAX6*, paired box 6; *LIN28*, lin-28 homolog A; *SOX2*, SRY-box 2; *NANOG*, Nanog homeobox; *OCT4*, POU class 5 homeobox 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

*Immunofluorescence*. The immunofluorescence method was performed as previously described (30), with minor modifications. Briefly, the cells were washed with phosphate-buffered saline (PBS), fixed at room temperature for 20 min with 4% paraformaldehyde, washed again in PBS 3 times, for 5 min each time, and then permeabilized with 0.3 % Triton X-100/PBS for 10 min. Next, the cells were blocked with 5% bovine serum albumin (BSA)/PBS for 40 min. The cells were then exposed to primary antibodies diluted in PBS plus 1% BSA at 4˚C overnight, followed by incubation with corresponding secondary antibodies at room temperature for 2 h. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole for 15 min. Samples were analyzed using an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan) or a confocal Leica TCS SP8 (Leica Microsystems, Inc., Buffalo Grove, IL, USA). All images were acquired under identical settings using IPP7.0 Image Browser software (Olympus) or LAS AF Lite (Leica Microsystems, Inc.). The relative fluorescence intensity was qualified using ImageJ software. Primary antibodies used in this study were: POU domain class 5 transcription factor 1 (OCT4; sc-5279; 1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Nestin (A0484; 1:50 dilution; ABclonal Biotechnology Co., Ltd., Cambridge, MA, USA), paired box protein Pax-6 (PAX6; ab5790; 1:750 dilution; Abcam, Woburn, MA, USA), microtubule-associated protein 2 (MAP2; 4542; 1:200 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), neuron-specific class III β-tubulin (TUJ1; T2200; 1:200 dilution; Sigma-Aldrich; Merck KGaA), AKT (9272; 1:200 dilution) and phosphorylated (p)-AKT (4060; 1:200 dilution) (both Cell Signaling Technology, Inc.). Secondary antibodies used in this study were fluorescein isothiocyanate-conjugated goat anti-rabbit and Cy3-conjugated goat anti-rabbit antibodies (F-2765 and T-2767; 1:200 dilution; both Thermo Fisher Scientific, Inc.)

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA (hiPSCs, H9, hiPSCs-derived NPCs and neurons differentiated from hiPSCs-derived NPCs) used in the present study was isolated using TRIzol reagent (Takara Bio, Inc., Otsu, Japan). Isolated RNA (1 $\mu$g) was converted into cDNA using the SuperScript III First-Strand cDNA Synthesis kit (Takara Bio, Inc.). The RT-qPCR was performed as previously described (31) on an Applied Biosystems 7500/7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq™ (Takara Bio, Inc.) in a 20-$\mu$l reaction mixture containing 1 $\mu$l of each cDNA and 0.2 $\mu$M of each pair of primers. Samples were preheated at 95˚C for 30 sec. RT-PCR conditions consisted of 40 cycles of 5 sec at 95˚C and 34 sec at 60˚C. The dissociation stage consisted of 15 sec at 95˚C, 1 min at 60˚C and 15 sec at 95˚C. The pairs of primers are shown in Table II. GAPDH was used as an endogenous control. The thermocycling conditions and the method of quantification were performed as previously described (31).

*Western blotting*. Total proteins were extracted from different cell stages (day 11 or day 20 after induction) using radioimmunoprecipitation assay buffer and a cocktail mixture (Thermo Fisher Scientific, Inc.). The concentration of protein was measured using the Pierce Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.). Protein (30 $\mu$g) was subjected to a 10% resolving gel for electrophoresis. The SDS-polyacrylamide gel electrophoresis was performed at 80 V first for 20 min, and then at 100 V for 80 min. Next, the protein was transferred to polyvinylidene difluoride membranes at 276 mA for 150 min using a semi-dry transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blot membranes were washed in Tris-buffered saline with 0.1% Tween-20 (TBST) and blocked with 5% BSA in TBST for 1 h at room temperature. The membranes were then incubated with primary antibodies at 4˚C with agitation overnight. Following this, the membranes were washed in TBST three times for 5 min each time, incubated with their corresponding secondary antibodies for 2 h, and rinsed in TBST

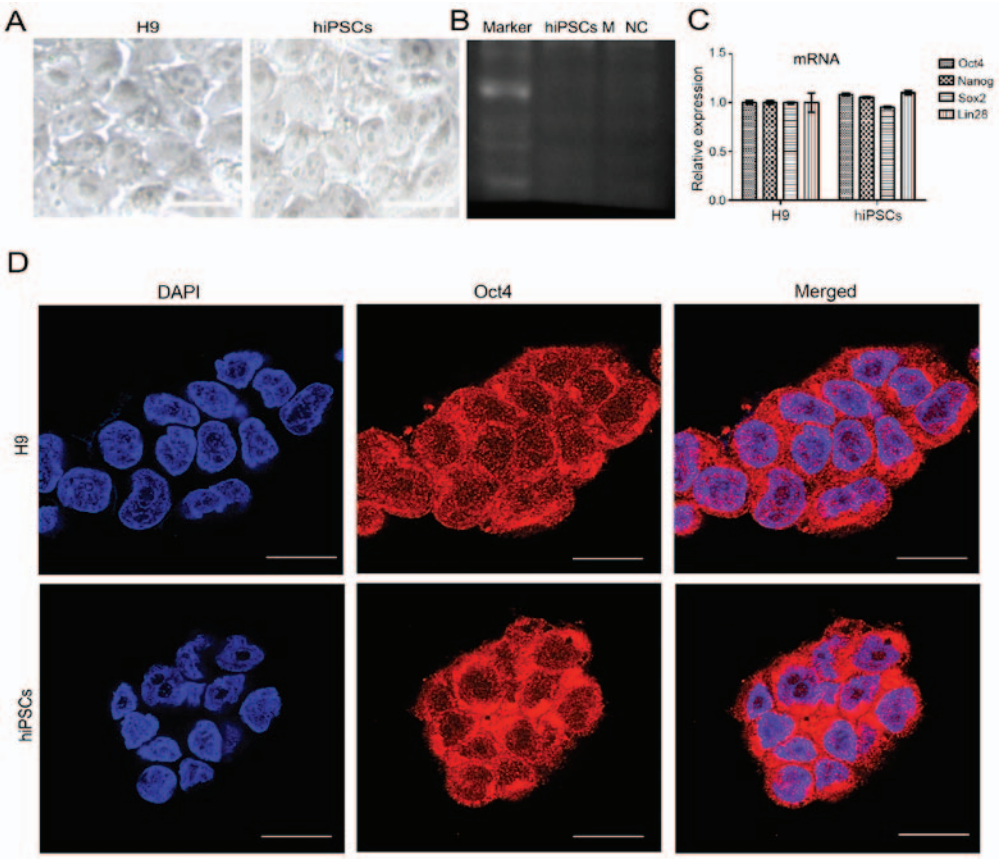


Figure 1. Pluripotency detection of hiPSCs. (A) The structure of hiPSCs and human H9 embryonic stem cell line under bright field microscopy Scale bar, 20 $\mu$m. (B) RT-PCR showing no mycoplasma contamination. (C) The mRNA expression of pluripotent genes *OCT4*, *NANOG*, *SOX2* and *LIN28* in hiPSCs were detected by RT-qPCR, with H9 used as a control. Results are presented as the mean ± standard deviation. (D) The protein expression of OCT4 in hiPSCs and the H9 cell line, observed using immunofluorescence. Scale bar, 20 $\mu$m. hiPSCs, human induced pluripotent stem cells; RT-PCR, reverse transcription-quantitative polymerase chain reaction; *NANOG*, Nanog homeobox; *OCT4*, POU class 5 homeobox 1; *LIN28*, lin-28 homolog A; *SOX2*, SRY-box 2; DAPI, 4',6-diamidino-2-phenylindole.

three times again. Enhanced chemiluminescence substrate was then applied (Thermo Fisher Scientific, Inc.). The value of the chemiluminescence was recorded and the band density was quantified using ImageJ software. Primary antibodies used in this study were MAP2 (4542; 1:1,000 dilution; Cell Signaling Technology, Inc.), TUJ1 (T2200; 1:1,000 dilution; Sigma-Aldrich; Merck KGaA), AKT (9272; 1:1,000 dilution) and p-AKT (4060; 1:1,000 dilution) (both Cell Signaling Technology, Inc.). Secondary antibodies used in this study were horseradish peroxidase-conjugated goat anti-rabbit antibody (7074; 1:5,000 dilution; Cell Signaling Technology, Inc.).

*Cell Counting kit-8 (CCK-8) assay*. The CCK-8 method was used to detect the viability of neurons differentiated from hiPSC-derived NPCs on culture day 20 in each differentiation group. Briefly, 20-day differentiated cells were seeded on 96-well plates at a density of 5,000/well, and then incubated with 10 $\mu$l CCK-8 (KeyGen Biotech Co., Ltd., Nanjing, China) in a total volume of 100 $\mu$l culture medium for 2 to 4 h at 37˚C. Next, the 96-well plate was placed on an enzyme standard instrument (ELx800; BioTek Instruments, Inc., Winooski, VT, USA). The plate was read at a wavelength of 450 nm. The neuron control differentiation medium was used as the control.

*Apoptosis and flow cytometry analyses*. Neurons differentiated from hiPSC-derived NPCs on culture day 20 in the control differentiation group and the LIF-positive differentiation group were subjected to standard procedure of cell apoptosis analysis. Cells ($1x10^5$) were fixed and labeled with Annexin V-FITC, 45 min at room temperature and propidium iodide using the Annexin V-FITC and propidium iodide using the Annexin-V-FLUOS staining kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols, followed by flow cytometry analysis. The Annexin V-positive cells were counted as apoptotic cells.

*Statistical analysis*. All the experiments were independently repeated at least three times, and statistical analysis was performed using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation. Pairwise comparisons between groups were performed using the independent sample t-test and one-way analysis of variance followed by pairwise t tests. The correction test we used here is Bonferroni's correction. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*hiPSCs exhibit similar characteristics to the H9 embryonic stem cell line*. hiPSCs exhibited the similar morphological characteristics as the H9 embryonic stem cell line (Fig. 1A). Mycoplasma contamination detection showed that the hiPSCs

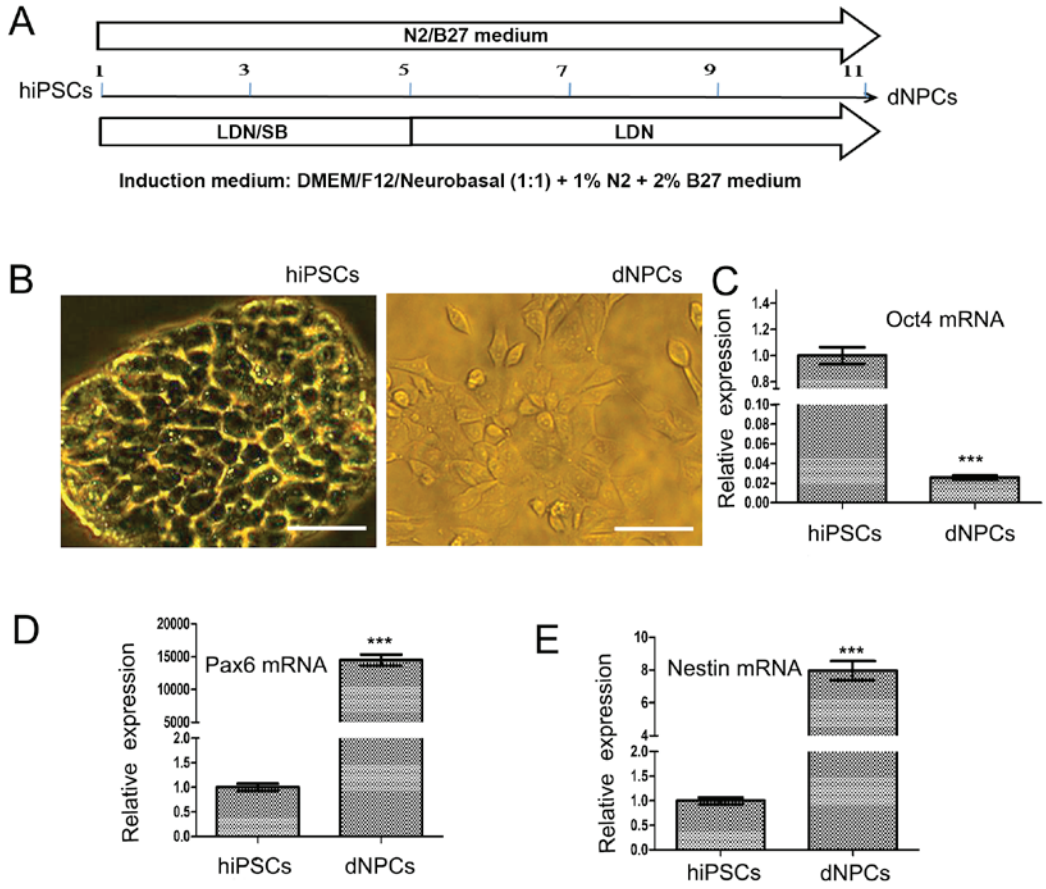


Figure 2. Generation of NPCs from hiPSCs. (A) The time course of NPC induction from hiPSCs. (B) The morphology of hiPSCs and hiPSC-derived NPCs on culture day 11 under bright field microscopy (scale bar, 20 $\mu$m). (C) The mRNA expression of *OCT4* in hiPSCs and derived NPCs on culture day 1 was detected by RT-qPCR. (D and E) The relative mRNA expression of *NES* and *PAX6* in derived NPCs and hiPSCs was detected by RT-qPCR on culture day 11. Results are presented as the mean ± standard deviation. ***P<0.001. NPCs, neural precursor cells; dNPCs, derived NPCs; hiPSCs, human induced pluripotent stem cells; RT-PCR, reverse transcription-quantitative polymerase chain reaction; DMEM, Dulbecco's modifed Eagle's medium; OCT4, POU class 5 homeobox 1; PAX6, paired box 6.

were not contaminated by mycoplasma (Fig. 1B). RT-qPCR analysis showed that the relative mRNA expression of the pluripotent markers in hiPSCs, including *OCT4*, Nanog homeobox, SRY-box 2 and lin-28 homolog A, showed no significant differences with the control H9 cell line (Fig. 1C). As with the H9 cells, the hiPSCs cells were also OCT4-positive, as shown in the immunofluorescence images (Fig. 1D).

*A large proportion of hiPSC-derived NPCs can be harvested after neural induction*. In the present study, hiPSCs were transformed into NPCs by adding BMP signaling inhibitor LDN193189 and TGF-β signaling inhibitor SB431542 into the N2/B27 basal culture medium. The transformation of NPCs was finished after 11 days of induction. The induction time course is shown in Fig. 2A. The derived cells have different morphological characteristics compared with the hiPSCs (Fig. 2B). The relative mRNA expression of pluripotent gene *OCT4* was significantly downregulated in the derived NPCs compared with that in the hiPSCs (Fig. 2C; P<0.001). By contrast, the mRNA expression of NPC markers, including nestin (*NES*) and *PAX6*, were significantly upregulated in the derived NPCs compared with that in the hiPSCs (Fig. 2D and E; both P<0.001). Furthermore, immunofluorescence data clearly showed that the Nestin and PAX6 proteins were highly expressed in the derived NPCs, but rarely expressed in the hiPSCs (Fig. 3A and B).

*LIF upregulates the expression of neuronal markers TUJ1 and MAP2 during the differentiation period*. Following the derivation of NPCs from hiPSCs, the derived NPCs were differentiated into neurons. The normal differentiation medium differentiation group was used as the control differentiation group. The differentiation time course is shown in Fig. 4A. The effect of LIF on neuronal differentiation was tested on culture day 20. The neuronal markers TUJ1 and MAP2 were detected by immunofluorescence methods (Figs. 4B and 5B). The relative fluorescence intensity analysis by ImageJ revealed that the expression of TUJ1 and MAP2 in the LIF (5 ng/ml) positive differentiation group was significantly higher than that of the control differentiation group (Figs. 4C and 5C; both P<0.01). The relative mRNA expression of *TUJ1* and *MAP2* in the LIF-positive differentiation group was significantly higher than that in the control differentiation group (Figs. 4D and 5D; both P<0.01). The protein expression of TUJ1 and MAP2 in the LIF-positive differentiation group was significantly higher than that in the control differentiation group too (Figs. 4E and F and 5E and F; both P<0.01). LIF concentration test experiments showed that adding 5 ng/ml LIF to the control

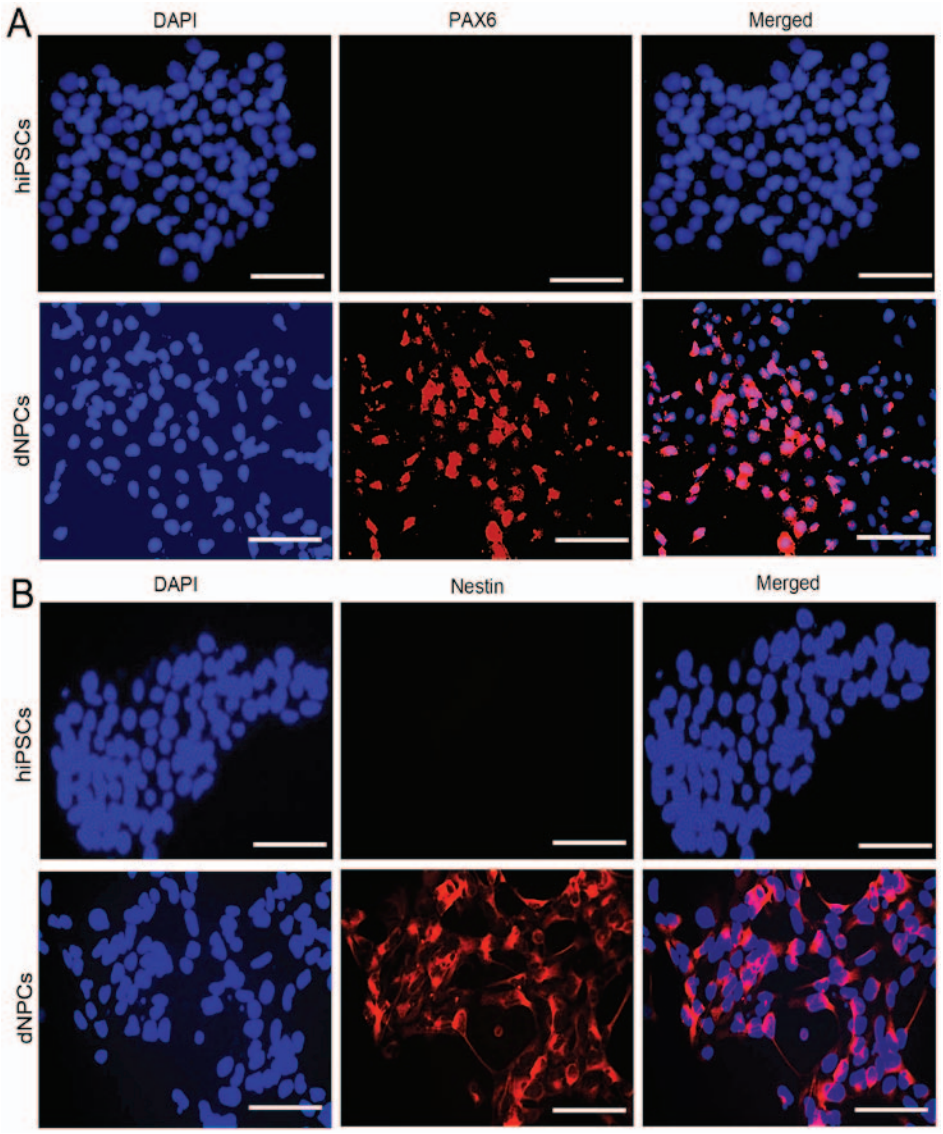


Figure 3. Detection of Nestin and PAX6 in derived NPCs and hiPSCs by immunofluorescence. (A) The expression of NPC marker PAX6 in the derived NPCs on culture day 11 and in hiPSCs was measured by immunofluorescence. (B) The expression of NPC marker Nestin in the derived NPCs on culture day 11 and in hiPSCs was measured byimmunofluorescence. Scale bar, 20 $\mu$m. DAPI, 4',6-diamidino-2-phenylindole; dNPCs, derived neural precursor cells; hiPSCs, human induced pluripotent stem cells; PAX6, paired box 6.

differentiation medium resulted in the strongest mRNA expression of *TUJ1* and *MAP2*, as detected by RT-qPCR on culture day 20 (Fig. 5A).

*LIF improves cell activity during differentiation*. On culture day 20, the cells were observed under light microscope and the cell state (structure under the optical microscope) was found to be better in the LIF-positive differentiation group than that in the control group (Fig. 6A). The number of differentiated neurons in the LIF-positive differentiation group was greater than that of the control group counted on culture day 20 (Fig. 6B; $P<0.05$). The viability of the cells was detected by CCK-8 method and it was found that the viability of neurons in the LIF-positive differentiation group was greater than that in the control group on culture day 20 (Fig. 6C; $P<0.01$). Apoptosis and flow cytometry analyses showed that there were significantly less apoptotic cells in the LIF differentiation group than in the control differentiation group (Fig. 6D; $P<0.05$).

*LIF influences the mRNA expression of inflammatory cytokines*. Inflammation is a primary pathological driving force of a number of neurodegenerative disorders (32); in the process of nervous system injury, pro-inflammatory cytokines (IL-1β and TNF-α) are robustly released, which may affect normal NPC differentiation, and lead to a vast number of astrocytes and a diminished neural population. In the present study, the expression of inflammation-related factors was checked by RT-qPCR on culture day 20 when neurons were differentiated from derived NPCs. The results showed that the expression of the pro-inflammatory cytokines, including IL-1α and TNF-α, was decreased, whilst the expression of anti-inflammatory cytokines, including IL-10 and TGF-β, was increased in the LIF-positive differentiation group compared with that in the control differentiation group (Fig. 6E and F; all $P<0.05$).

*LIF upregulates the expression of p-AKT*. A previous study showed that the inflammatory reaction could affect the

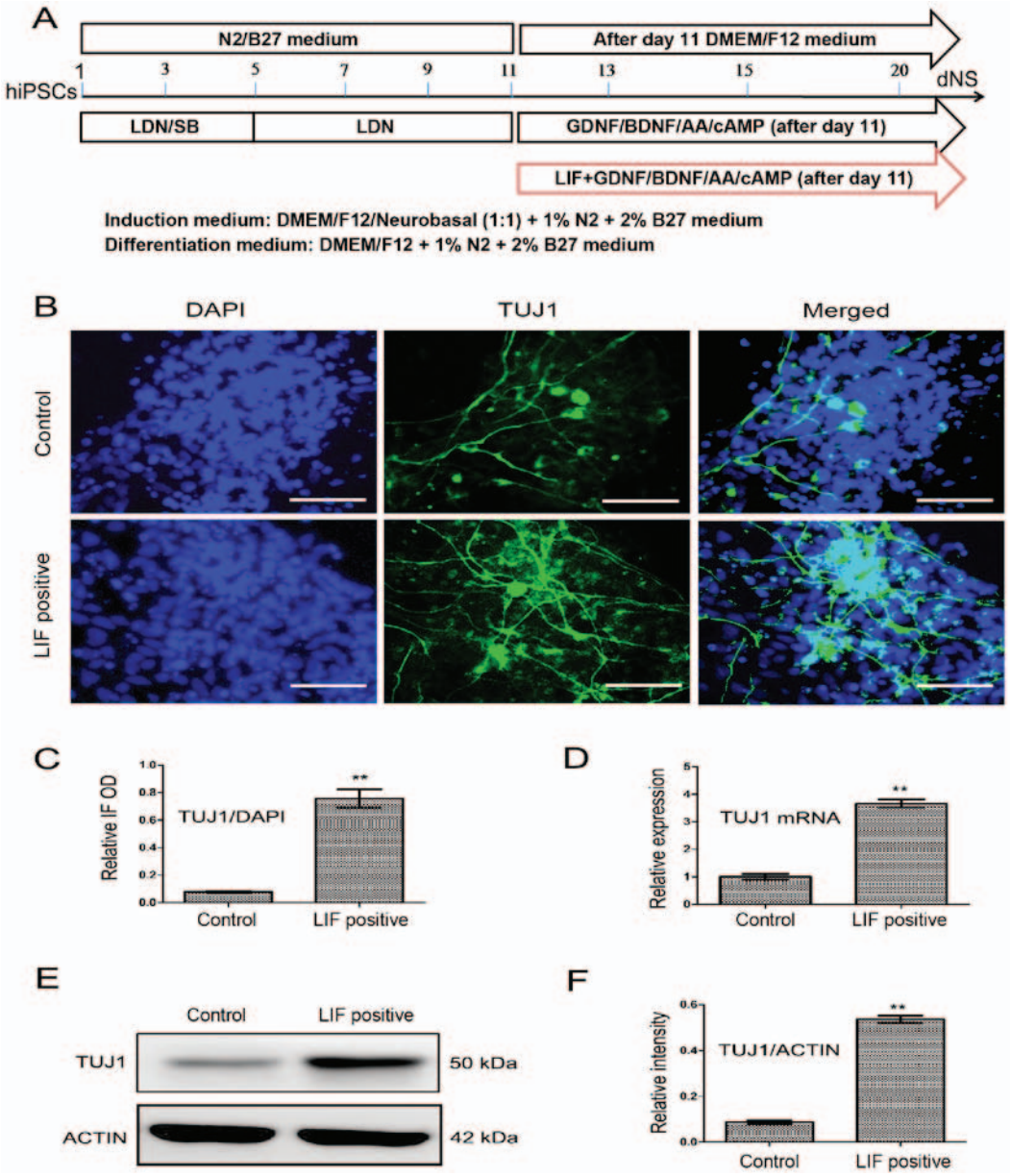


Figure 4. LIF improves the expression of TUJ1. (A) The time course of neuronal differentiation in the presence or absence of LIF. (B) The expression of TUJ1 (green) and DAPI (blue) was observed by immunofluorescence on culture day 20 in the LIF differentiation group and the control group. (C) The relative fluorescence intensity value of TUJ1 was qualified by ImageJ software and the data is expressed as the relative OD value (TUJ1/DAPI). (D) The relative mRNA expression of TUJ1 was detected by reverse transcription-quantitative polymerase chain reaction in the LIF differentiation and control groups. (E) The TUJ1 protein expression was detected by western blotting in the LIF differentiation and control groups. (F) The relative band intensity of TUJ1 was qualified by ImageJ software. Results are presented as the mean ± standard deviation. $^{**}$P<0.01. Scale bar, 20 $\mu$m. GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's modifed Eagle's medium; AA, ascorbic acid; cAMP, cyclic adenosine monophosphate; LIF, leukocyte inhibitory factor; DAPI, 4',6-diamidino-2-phenylindole; TUJ1, neuron-specific class III β-tubulin; IF, immunofluorescence; OD, optical density.

PI3K/AKT signaling pathway (33), and the activation of the PI3K/AKT signaling pathway could be involved in the inflammatory reaction. The present study found that in the LIF-positive differentiation group, p-AKT, the key composition factor of PI3K/AKT, was significantly increased (Fig. 6G and H; P<0.01). This finding revealed that the improved neuronal differentiation may be mediated by the PI3K/AKT signaling pathway.

*The effect of LIF could be prevented by PI3K/AKT inhibitor LY294002*. In order to confirm the effect of LIF on neuron differentiation was mediated by the activation of the PI3K/AKT signaling pathway. PI3K/AKT inhibitor LY294002 was used. Following the addition of LY294002 during the differentiation period, the protein expression of TUJ1 and MAP2 was downregulated compared with that in the LIF differentiation group (Fig. 7A and B; both P<0.05). Additionally, the expression of p-AKT was downregulated compared with that in the LIF-positive group (Fig. 7C and D; P<0.05). Furthermore, the mRNA expression of inflammatory cytokines IL-10, TGF-β, IL-1 and TNF-α was returned to a more normal level compared with that in the LIF-positive differentiation group (Fig. 7E and F; all P<0.01). The number and the viability of differentiated neurons decreased after the addition of

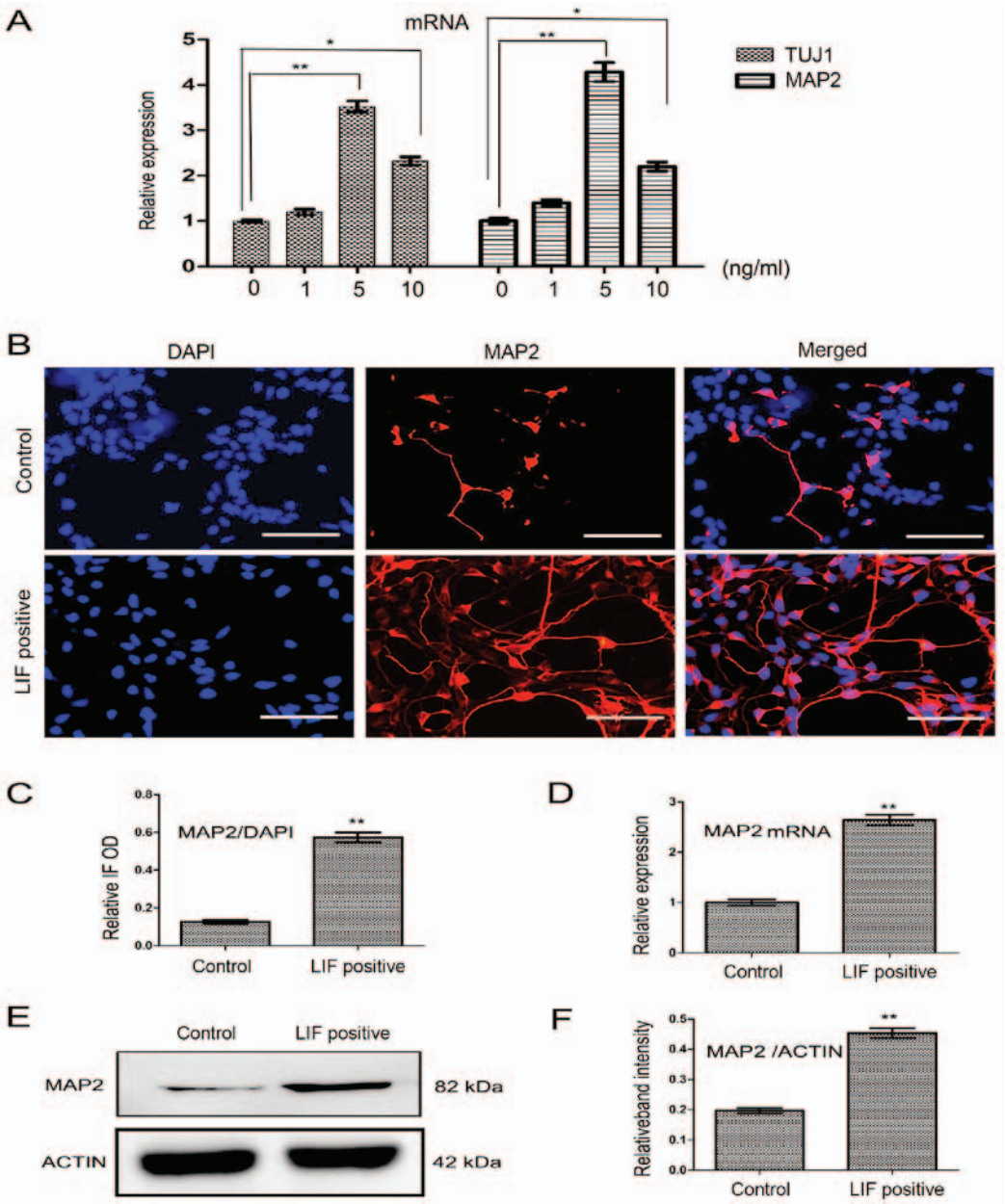


Figure 5. LIF enhances the expression of MAP2. (A) The relative mRNA expression of *TUJ1* and *MAP2* on culture day 20 in the different LIF concentration treated groups. Results showed that, in the 5 ng/ml LIF differentiation group, the mRNA expression of *TUJ1* and *MAP2* was strongest. (B) The protein expression of MAP2 (red) and DAPI (blue) on culture day 20 was observed by immunofluorescence. (C) The relative fluorescence intensity of MAP2 was qualified using ImageJ and the data are expressed as the relative OD value (MAP2/DAPI). (D) The relative mRNA expression of *MAP2* was detected by reverse transcription-quantitative polymerase chain reaction in the LIF differentiation group and the control group. (E) The MAP2 protein expression in the LIF differentiation group and the control group was detected by western blotting. (F) The relative band intensity of MAP2 protein was qualified by ImageJ software. Results are presented as the mean ± standard deviation. $^{*}$P<0.05 and $^{**}$P<0.01. Scale bar, 20 $\mu$m. MAP2, microtubule-associated protein 2; TUJ1, neuron-specific class III β-tubulin; DAPI, 4',6-diamidino-2-phenylindole; IF, immunofluorescence; OD, optical density; LIF, leukocyte inhibitory factor.

LY294002 compared with that in the LIF-positive differentiation group (Fig. 7G and H; both P<0.01).

*The effect of LIF could be prevented by PI3K/AKT inhibitor wortmannin but not rapamycin*. In order to confirm the effect of LIF on neuron differentiation was mediated by the PI3K/AKT signaling pathway. Another PI3K/AKT signaling pathway inhibitor, wortmannin, and an mTOR signaling inhibitor, rapamycin, were used here. Following the addition of wortmannin to the LIF-positive differentiation group, it was found that the changes in the protein expression of TUJ1 and MAP2 was similar to that found following the addition of LY294002 (Fig. 8A and B; P<0.05). However, this phenomenon was not found in the rapamycin-treated group (Fig. 8C and D; P<0.05). The changes in mRNA expression of inflammatory cytokines IL-10, TGF-B, IL-1 and TNF-α in the wortmannin-treated group were similar to those found in the LY294002-treated group, compared with the LIF-positive differentiation group (Fig. 8G and H; P<0.05). However, this phenomenon was not found in the rapamycin-treated differentiation group compared with the LIF-positive differentiation group (Fig. 8I and J).

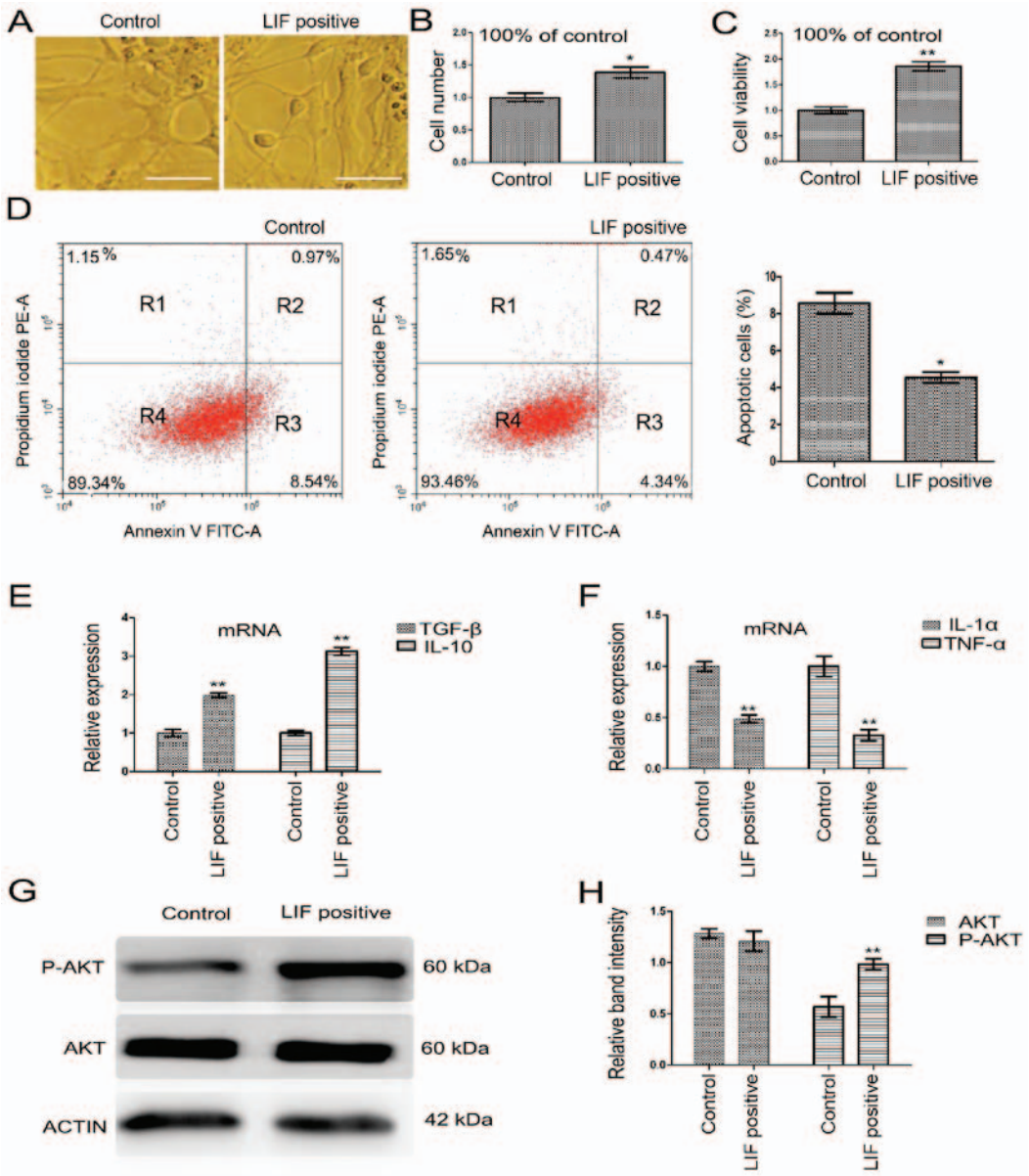


Figure 6. LIF affects the cell viability and the mRNA expression of inflammatory cytokines. (A) The neurons differentiated in LIF differentiation and control groups under bright field microscopy on culture day 20. (B) The cell number was counted on culture day 20. (C) The cell viability was measured by cell counting kit-8 method on culture day 20. (D) Cells on culture day 20 were labeled with Annexin V-FITC and PI. Cells in the R3 area were counted as apoptotic cells. (E) The relative mRNA expression of the anti-inflammatory cytokines TGF-β and IL-10 in LIF differentiation and control groups, and (F) the pro-inflammatory cytokines IL-1α and TNF-α on culture day 20 was detected by reverse transcription-quantitative polymerase chain reaction. (G) The protein expression of AKT and p-AKT was analyzed by western blotting. (H) The relative band intensity of AKT and p-AKT was qualified by ImageJ software. Results are presented as the mean ± standard deviation. $^{*}$P<0.05 and $^{**}$P<0.01. Scale bar, 20 $\mu$m. LIF, leukocyte inhibitory factor; FITC, fluorescein isothiocyanate; PI, propidium iodide; PE, phycoerythrin; TGF-β, transforming growth factor-β; IL, interleukin; TNF-α, tumor necrosis factor-α; p-AKT, phosphorylated protein kinase B.

## Discussion

The transformation of hiPSCs into neural cells provides one source of stem cells for the cell therapy of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (34). A high derivation quality and proportion of NPCs from hiPSCs is a crucial step in the whole cell therapy procedure. There are several induction methods to convert the pluripotent stem cells into NPCs, relying on co-culture with stromal cells or initiating by the formation of embryoid bodies (35,36). However, the low conversion efficiency, the contamination of miscellaneous cells and the complexity of experimental operation has made these methods impractical. In the present study, the dual inhibition method of neural induction used by Chambers *et al* (37) was meliorated using monolayer culture and N2/B27 medium to induce neuron differentiation. During the induction period, the BMP inhibitor LDN193189 and the TGF-β inhibitor SB431542 were used as the induction factor. Under these conditions, a high proportion of cells were converted into nerve cells, which were defined as NPCs, expressing Nestin and PAX6. This may be attributed to the induction inhibition of endodermal and mesodermal cells by LDN193189 and SB431542. Neuronal differentiation following successful induction of NPCs is another key step for

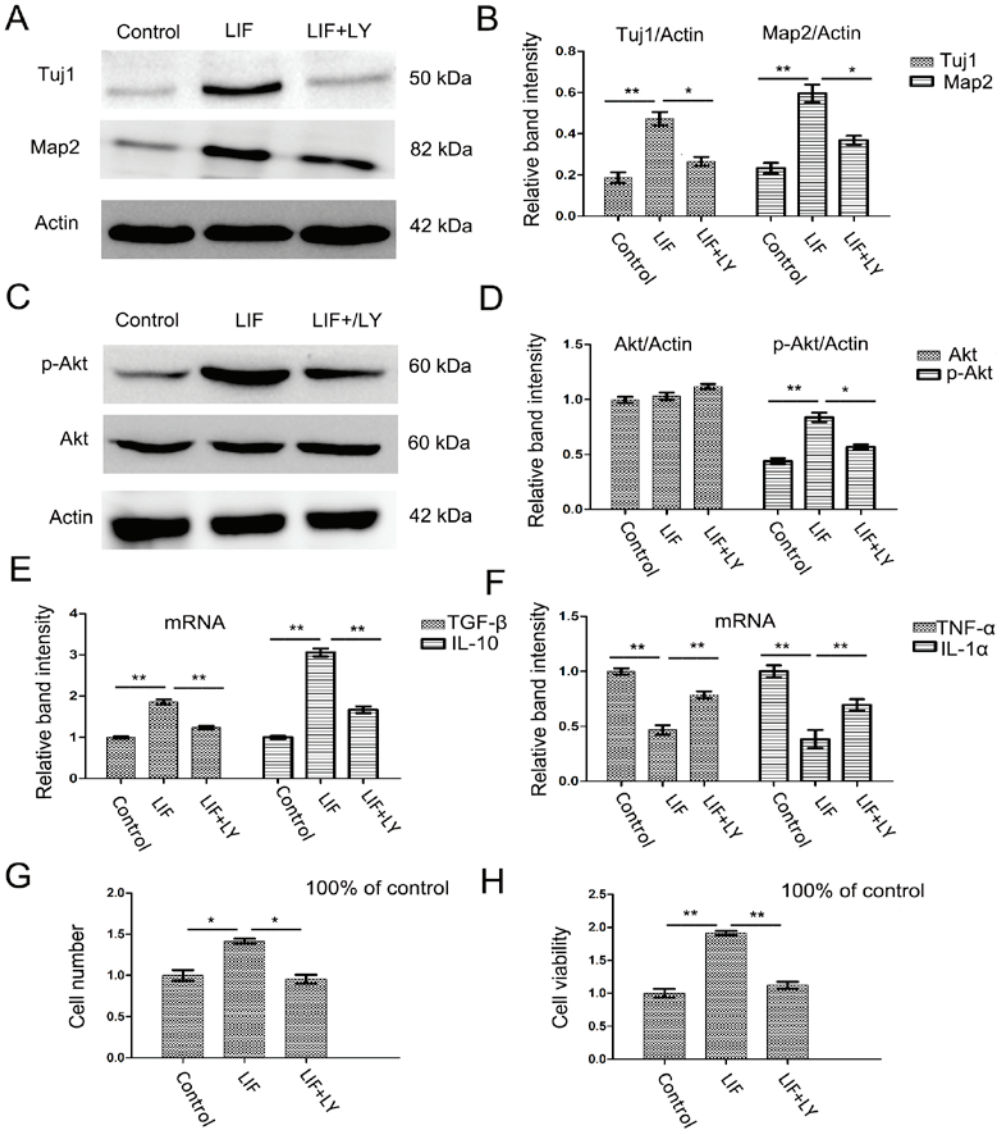


Figure 7. Inhibition of phosphatidylinositol 3-kinase/AKT by LY294002 prevents the LIF-induced changes in the expression of neuronal markers and converts the mRNA expression of inflammatory cytokines. (A) The protein expression of TUJ1 and MAP2 was examined on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells by western blotting. (B) The relative band intensity of (A) was qualified by ImageJ software. (C) The protein expression of AKT and p-AKT was detected on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells by WB. (D) The relative band intensity of (C) was qualified by ImageJ software. (E) The relative mRNA expression of anti-inflammatory factors TGF-β and IL-10 on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells by RT-qPCR. (F) The relative mRNA expression of pro-inflammatory factors TNF-α and IL-1α on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells were detected by RT-qPCR. (G) The number of neurons counted on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells. (H) The cell viability was detected on culture day 20 in control, LIF and LIF with LY294002 differentiation group cells by cell counting kit-8. Results are presented as the mean ± standard deviation. $^{*}$P<0.05 and $^{**}$P<0.01. p-AKT, phosphorylated protein kinase B; LY, LY294002; LIF, leukocyte inhibitory factor; MAP2, microtubule-associated protein 2; TUJ1, neuron-specific class III β-tubulin; TGF-β, transforming growth factor-β; IL, interleukin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α.

the cell treatment of diseases of the nervous system. However, the low differentiation ratio and poor survival state require improvement.

LIF, a member of the IL-6 superfamily, acts through binding to its specific receptor, LIFR. It conscribes gp130 and then an affinity receptor complex is formed, which can activate the downstream pathways, including the PI3K/AKT, ERK1/2, JAK/STAT3 and mTOR signaling pathways (12-15). LIF can regulate cell proliferation, differentiation and phenotype; its main function is inhibiting the differentiation of mESCs and promoting the proliferation of muscle cells. Under certain conditions, LIF can promote a neonatal rat dorsal root ganglion neuronal phenotype to change from the adrenergic type to the cholinergic type. Therefore, LIF is also known as cholinergic neuronal differentiation factor (20). In addition, LIF can promote the survival of cells differentiated from the transplanted neural crest (38). Laterza *et al* (39) revealed that transplanted mouse iPSC-derived NPCs exert neuroprotection through the secretion of LIF, which promotes the survival and differentiation capacity of oligodendrocytes. However, the *in vitro* effect of LIF on neuron differentiation is rare. In the present study, a concentration of 5 ng/ml LIF was added to the

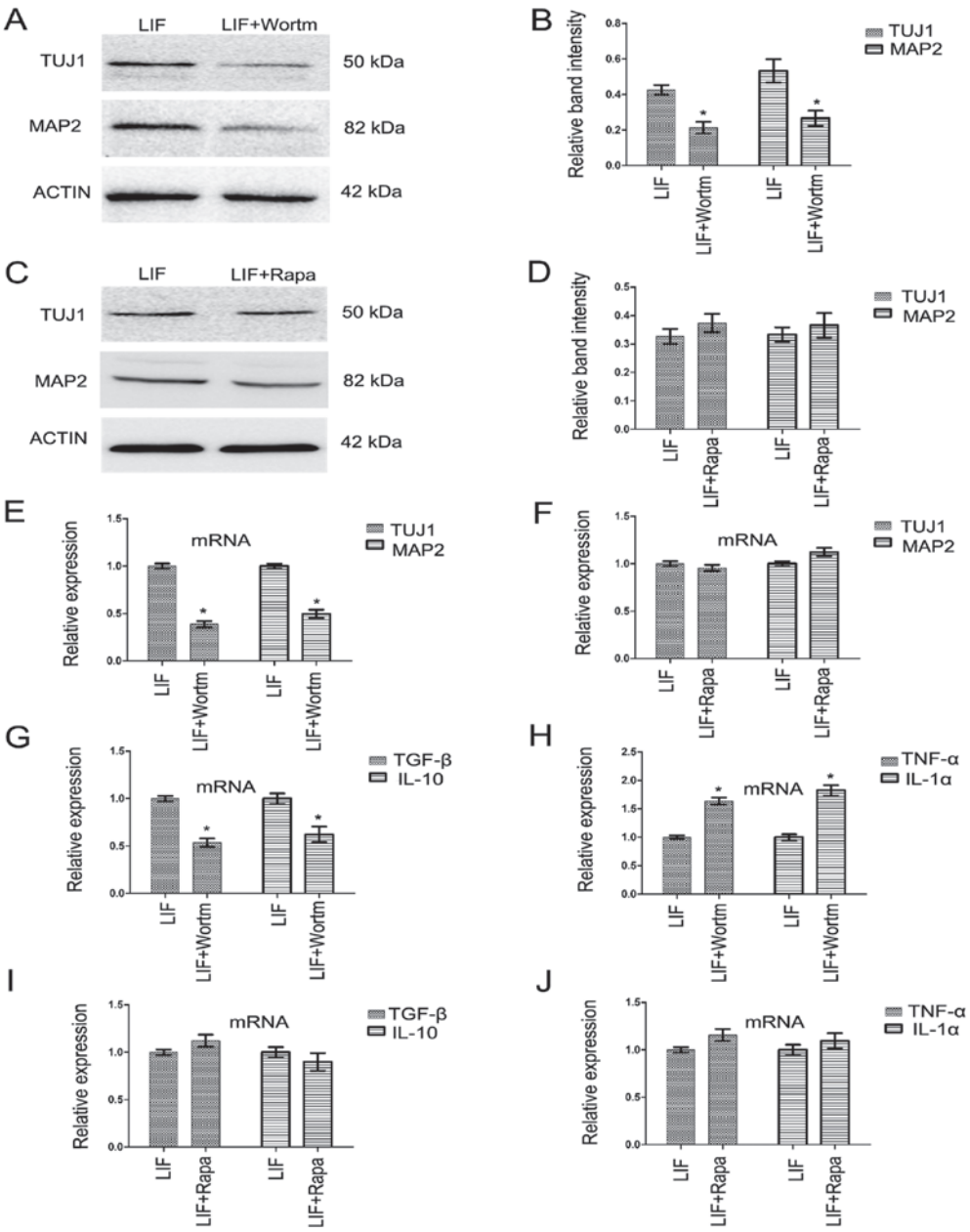


Figure 8. Inhibition of phosphatidylinositol 3-kinase/AKT by wortmannin could also prevent the LIF-induced changes in the expression of neuronal markers and the mRNA expression of inflammatory cytokines, but this effect was not found in the rapamycin differentiation group. (A and B) The protein expression of TUJ1 and MAP2 was examined by western blotting in LIF and LIF combined with wortmannin differentiation groups. The relative band intensity of TUJ1 and MAP2 was qualified by ImageJ software. (C and D) The protein expression of TUJ1 and MAP2 was detected by western blotting in LIF and LIF combined with rapamycin differentiation groups. The relative band intensity of TUJ1 and MAP2 was qualified by ImageJ software. (E) The relative mRNA expression of TUJ1 and MAP2 on culture day 20 was detected by RT-qPCR in LIF and LIF combined with wortmannin differentiation groups. (F) The relative mRNA expression of TUJ1 and MAP2 on culture day 20 was detected by RT-qPCR in LIF and LIF combined with rapamycin differentiation groups. (G and H) The relative mRNA expression of anti-inflammatory factors TGF-β and IL-10, and pro-inflammatory factors TNF-α and IL-1α on culture day 20 was detected by RT-qPCR in LIF and LIF combined with wortmannin differentiation groups. (I and J) The relative mRNA expression of anti-inflammatory factors TGF-β and IL-10, and pro-inflammatory factors TNF-α and IL-1α on culture day 20 was detected by RT-qPCR in LIF and LIF combined with rapamycin differentiation groups. Results are presented as the mean ± standard deviation. *P<0.05. AKT, protein kinase B; LY, LY294002; LIF, leukocyte inhibitory factor; MAP2, microtubule-associated protein 2; TUJ1, neuron-specific class III β-tubulin; TGF-β, transforming growth factor-β; IL, interleukin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α; wortm, wortmannin; Rapa, rapamycin.

differentiation medium, and it was found to strongly improve the expression of the neuronal markers TUJ1 and MAP2, and the expression of p-AKT. p-AKT is a key composition of the PI3K/AKT signaling pathway; it serves an important role in neuronal proliferation and differentiation (18). Upregulating p-AKT could increase the neuronal differentiation from mouse cochlear neural stem cells (19). In the present study, the PI3K/AKT signal was inhibited by its inhibitor LY294002 and wortmannin in the LIF-positive differentiation group, and it was found that the protein expression of neuron markers TUJ1 and MAP2 was returned to a similar level in the two inhibitor groups as the control group. However, the same phenomenon was not found in the in the rapamycin-treated group, This may suggest that the greater neuronal differentiation effect in the

LIF-positive differentiation group compared with that in the control differentiation group may occur through the activation of the PI3K/AKT signal, and that the mTOR signaling inhibition caused by LY294002 inhibitor or rapamycin may not participate in the greater neuronal differentiation and anti-inflammatory effect during the neuron differentiation from hiPSC-derived NPCs.

Anti-inflammation is one of the strategies to promote neuron protection and cell survival; it can reduce apoptosis and improve the survival of new derived neurons (25). The reaction of inflammation could regulate the expression of LIF. Injecting LPS into the trachea of a rat could induce the expression and secretion of LIF in bronchoalveolar cells (27). Meanwhile, inflammation-related cytokines such as IL-6 and TNF-α could increase the mRNA or protein expression of LIF during the cell culture (28,29). LIF upregulated the expression of anti-inflammatory cytokines, including IL-10 and TGF-β, and downregulated the expression of pro-inflammatory cytokines, including IL-1α and TNF-α, during the neuronal differentiation in the present study. Furthermore, LIF promoted the viability of the neurons during the differentiation. These data suggest the anti-inflammatory effect and cell survival effect of LIF on neuron differentiation. However, the expression of inflammatory cytokines and the cell viability were all reversed following the addition of PI3K/AKT inhibitor LY294002 and wortmannin to the LIF-positive differentiation group. Several studies revealed the involvement of PI3K/AKT signaling in metabolic dysfunction and inflammation. For example, furotrilliumoside prevented the LPS-induced upregulation of PI3K/AKT, as a result of which the expression of inflammatory cytokines TNF-α, IL-6 and IL-1β decreased (40). However, salvianolic acid A, a chemical type of caffeic acid trimer, blocks inflammatory responses through the activation of PI3K/AKT signaling (41). The present results suggested that the effect of LIF on inflammation and cell viability may be through the activation of PI3K/AKT signaling.

In conclusion, by use of a monolayer culture method, and N2/B27 basic medium combined with BMP inhibitor LDN193189 and TGF-β inhibitor SB431542 during neural induction, a large proportion of derived NPCs was harvested. During the neuronal differentiation period from the derived NPCs, LIF can improve the differentiation by upregulating the protein expression of MAP2 and TUJ1, and increasing the number of TUJ1 and MAP2-positive neurons, which may be through the activation of PI3K/AKT signaling. In addition to this, LIF could exert the anti-inflammatory effect and improve the cell viability through activation of PI3K/AKT during neuronal differentiation. These data suggest that LIF serves an important role in the differentiation of neurons *in vitro* and may have a prospective application in the stem cell treatment of central nervous system diseases.

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## Competing interests

The authors declare that they have no competing interests

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