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Original Article

MicroRNA-765 regulates neural stem cell proliferation and differentiation by modulating Hes1 expression

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Abstract: Neural stem cells (NSCs) are multipotent, self-renewing and undifferentiated cells that have the ability to differentiate to both glial and neuronal lineages. miRNAs act a key role in regulating neuronal fate and self-renewal of NSCs. In this study, we found that ectopic expression of miR-765 promoted NSCs proliferation. Moreover, miR-765 overexpression increased the ki-67 and β-tubulin-III expression inNSCs. Overexpression of miR-765 inhibited the expression of GFAP in NSCs. Furthermore, Hes1 was identified as a direct target gene of miR-765 in NSCs. Overexpression of Hes1 decreased miR-765-induced proliferation of NSCs and inhibited NSCs differentiation to neurons in miR-765-treated NSCs. These results demonstrated that miR-765 acted a crucial role in NSCs differentiation and proliferation by inhibiting Hes1 expression.

Keywords: Neural stem cells, MicroRNAs, miR-765, Hes1, differentiation

Introduction

Neural stem cells (NSCs) are multipotent, self-renewing and undifferentiated cells that have the ability to differentiate to both glial (oligodendrocytes and astrocytes) and neuronal lineages [1-4]. Recent findings have demonstrated that NSCs could act as cell therapies for many neurological disorders including spinal cord injuries (SCI), Alzheimer's disease, Huntington's disease and Parkinson's disease [5-8]. However, there is still a long way before the clinical use of NSCs. More studies are needed to investigatethe mechanism inNSCs differentiation and proliferation.

MicroRNAs (miRNAs) area class of small, non-coding (20-22 nucleotides) and endogenous RNAs that act as important regulators ingene expression [9-13]. MiRNAs are involved in various cell processes including cell differentiation, proliferation, migration and apoptosis [11, 14-17]. Deregulation of miRNA is associated with tumor development, in which theycan act as tumor suppressor or oncogene [18-21]. Furthermore, recent studies have showed that miRNAs also play a key role in regulating neuronal fate and self-renewal of NSCs [8, 22-26].

In this study, we showed that ectopic expression of miR-765 promoted NSCs proliferation. Moreover, miR-765 overexpression increased the ki-67 expression. In addition, overexpression of miR-765 could promote the β-tubulin-IIIexpression in NSCs and this effect was confirmed by immunofluorescence. Overexpression of miR-765 inhibited the GFAP expression in NSCs. Furthermore, Hes1 was identified as a direct target gene of miR-765 in NSCs.

Materials and methods

Ethics statement

This study was approved by the ethical board of the Institute of Mudanjiang Medical University and complied with Declaration of Helsinki.

Cell culture and transfection

NSCs were cultured from embryos (13.5 days) of rats (Wistar) and kept in medium supplement with the 20 ng/ml human EGF (R&D), 10 ng/ml bFGF (R&D), and 1% N2 (Gibco). Neurospheres were digested by using trypsin to get clone neurosphere. miR-765 mimics and scramble mimic were buy from Ambion. MiR-765 mimics (20 ng/

Table 1. Primer sequence

Name	Sequence (5'-3')
Hes1	TGAAGGATTCCAAAAATAAAATTCTCTGGG
	CGCCTCTTCTCCATGATAGGCTTTGATGAC
β-tubulin III	AGCAAGGTGCGTGAGGAGTA
	AAGCCGGGCATGAAGAAGT
GAPDH	AATGGGCAGCCGTTAGGAAA
	TGAAGGGGTCATTGATGGCA
Nestin	GATCTAAACAGGAAGGAAATCCAGG
	TCTAGTGTCTCATGGCTCTGGTTTT
GFAP	CAACGTTAAGCTAGCCCTGGACAT
	CTCACCATCCCGCATCTCCACAGT

ml) and scramble was transfected to NSCs by Lipofectamine 2000 according to manufacturer's information.

Immunocytochemistry

Cell was fixed in paraformaldehyde and then blocked with Triton X-100 and serum. The cell was incubated with following primary antibodies: (Nestin, GFAP, β-tubulin III, GDPDH and Hes1, Sigma) overnight. After washed with PBS for three times, the incubated with secondary antibodies for 1 hour and Nuclei was stained with DAPI (Sigma, USA).

Cell proliferation

Counting kit 8 (CCK8) kit (Dojindo, Japan) was performed to measure cell proliferation according to production's information. Proliferation rate was detected every 1 day after treatment. Optical density (OD) was measure at 450 nm wavelength.

QRT-PCR

Total RNA from samples was isolated using TRIzol reagent (Invitrogen). The mRNA and miRNA expression was measured using qRT-PCR following to the previous protocol. The primer sequence was shown in **Table 1**. GAPDH was used as control for mRNA expression and U6 snRNA was used as control for miRNA expression.

Western blot

Total proteins were exacted from cells and separated using electrophoresis on 12% SDS-PAGE gels and then transferred into membranes (Amersham, UK). The following primary antibody was used as following: Nestin, GFAP, β-tubulin III, GDPDH and Hes1 (Sigma, USA). GAPDH was used as loading control.

Luciferase reporter assay

To build the luciferase reporter plasmid, wild type (WT) 3'UTR fragment of Hes1 having binding sites of miR-765 was amplified. Cells were cultured in plate and then transfected with miR-765 mimic or scramble and luciferase activity was measured using luciferase activity kit (Promega) according the manufacturer's protocol.

Statistical analysis

Statistical analyses was performed by using by SPSS (17.0). All data was shown as mean ± SD and $p<0.05$ was considered as significant. Student's t-test was performed to detect differences between two groups and one-way analysis of variance (ANOVA) was used to measure the differences between more than two groups.

Results

NSCs could proliferate and differentiate into neurons and astrocytes

The isolated cells could form neurospheres (**Figure 1A**) and express NSCs marker Nestin (**Figure 1B**), suggesting that the cells were NSCs. After with draw of EGF and bFGF, these neurospheres cells can differentiate into neurons (**Figure 1C-E**) and astrocytes (**Figure 1F**), thus further confirming these cells are NSCs.

MiR-765 promoted NSCs proliferation

The expression of miR-765 was increased after treated by miR-765 mimics (**Figure 2A**). CCk-8 analysis showed that ectopic expression of miR-765 promoted NSCs proliferation (**Figure 2B**). In line with this, miR-765 overexpression increased the ki-67 mRNA (**Figure 2C**) and protein expression (**Figure 2D**). MiR-765 overexpression increased the nestin expression (**Figure 2E**) and neurospheres formation (**Figure 2F**).

MiR-765 regulated NSCs differentiation

Overexpression of miR-765 could promote the β-tubulin-III expression in NSCs (**Figure 3A**).

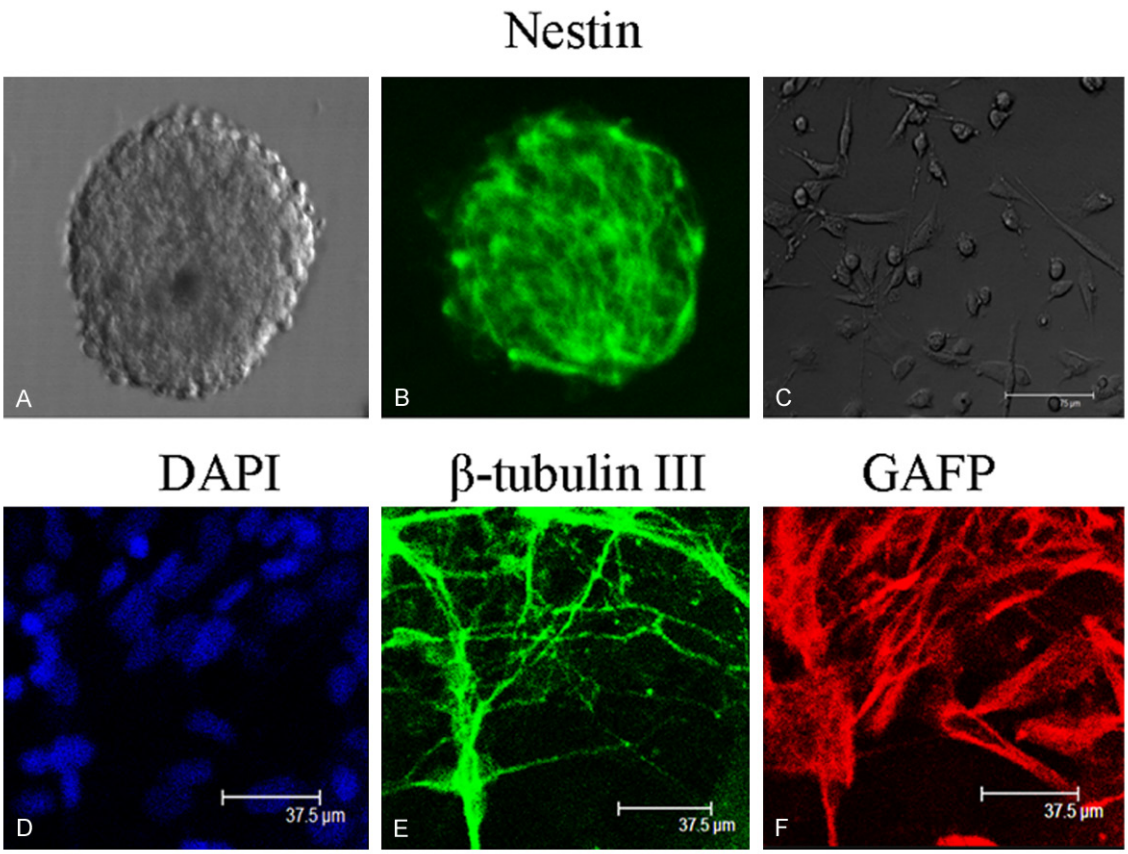

Figure 1. NSCs could proliferate and differentiate into neurons and astrocytes. A. Representative neurospheres photomicrograph in culture. B. Immunocytochemical staining of purified NSCs with Nestin. C. Representative differentiated cells from NSCs photomicrograph in culture. D. Nucleus staining of differentiated cells from NSCs with DAPI. E. Immunocytochemical staining of purified neurons with β-tubulin-III. F. Immunocytochemical staining of purified protoplasmic astrocytes with GFAP.

Moreover, miR-765 overexpression also increased β-tubulin-III protein expression in NSCs (**Figure 3B**). This effect also was also confirmed by immunofluorescence analysis (**Figure 3C**). In addition, overexpression of miR-765 inhibited the GFAP mRNA expression in NSCs (**Figure 3D**). Western blot assay showed that miR-765 overexpression suppressed the GFAP protein expression in the NSCs (**Figure 3E**). This effect also was confirmed by immunofluorescence analysis (**Figure 3F**).

Hes1 was the direct target of miR-765 in NSCs

There was a miR-765 targeting sequence in the 3'-UTR (3'-untranslated region) of Hes1 (**Figure 4A**). Dual-luciferase reporter data showed that miR-765 inhibited the luciferase activity of WT (wild type) Hes1 3'UTR vector compared with mutant Hes1 3'UTR vector (**Figure 4B**). MiR-765 overexpression decreased the Hes1 mRNAexpression in NSCs (**Figure 4C**). Meanwhile, ectopic expression of miR-765 suppressed the protein expression of Hes1 in the NSCs (**Figure 4D**).

MiR-765 regulated NSCs proliferation and differentiation through targeting Hes1

Western blot analysis proved that miR-765 overexpression enhanced the Hes1 protein expression (**Figure 5A**). Furthermore, Hes1 overexpression inhibited miR-765-induced proliferation in NSCs (**Figure 5B**). Moreover, overexpression of Hes1 repressed the mRNA expression of β-tubulin-III expression (**Figure 5C**) and this effect was confirmed by immunofluorescence analysis (**Figure 5D**). Overexpression of Hes1 promoted the mRNA expression of GFAP expression (**Figure 5E**) and this effect was confirmed by immunofluorescence analysis (**Figure 5F**).

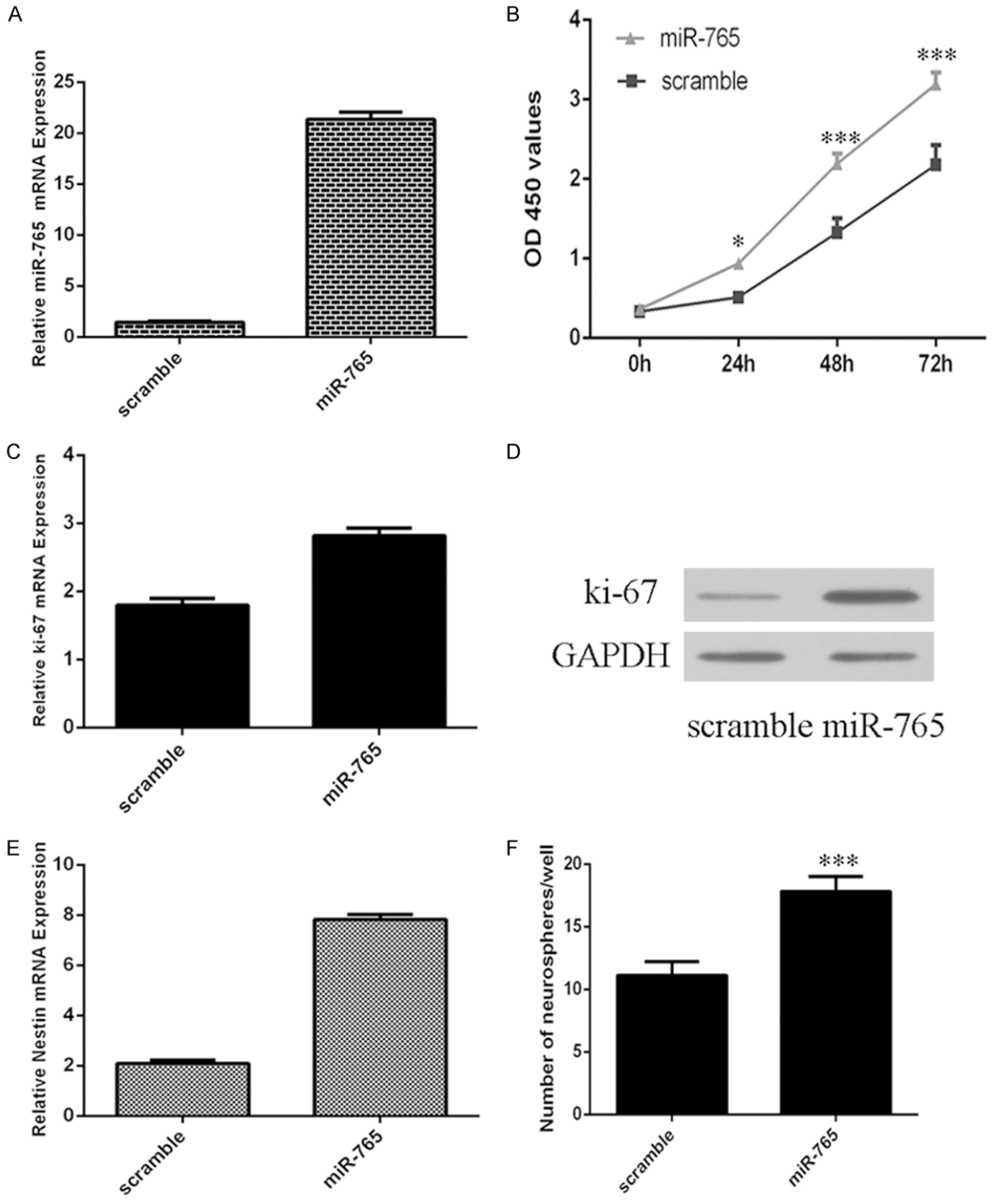

Figure 2. MiR-765 promoted NSCs proliferation. A. The expression of miR-765 was measured by qRT-PCR. B. Overexpression of miR-765 promoted the NSCs proliferation. C. The mRNA expression of ki-67 was detected by qRT-PCR. D. The protein expression of ki-67 was measured by Western blot. E. Overexpression of miR-765 promoted the thenestin mRNA expression in NSCs. F. miR-765 promoted neurospheres formation. *p<0.05 and ***p<0.001.

Discussion

In our study, we showed that ectopic expression of miR-765 promoted NSCs proliferation. Moreover, miR-765 overexpression increased the ki-67 and β-tubulin-III expression in NSCs. Overexpression of miR-765 inhibited the GFAP in NSCs. Furthermore, Hes1 was identified as a

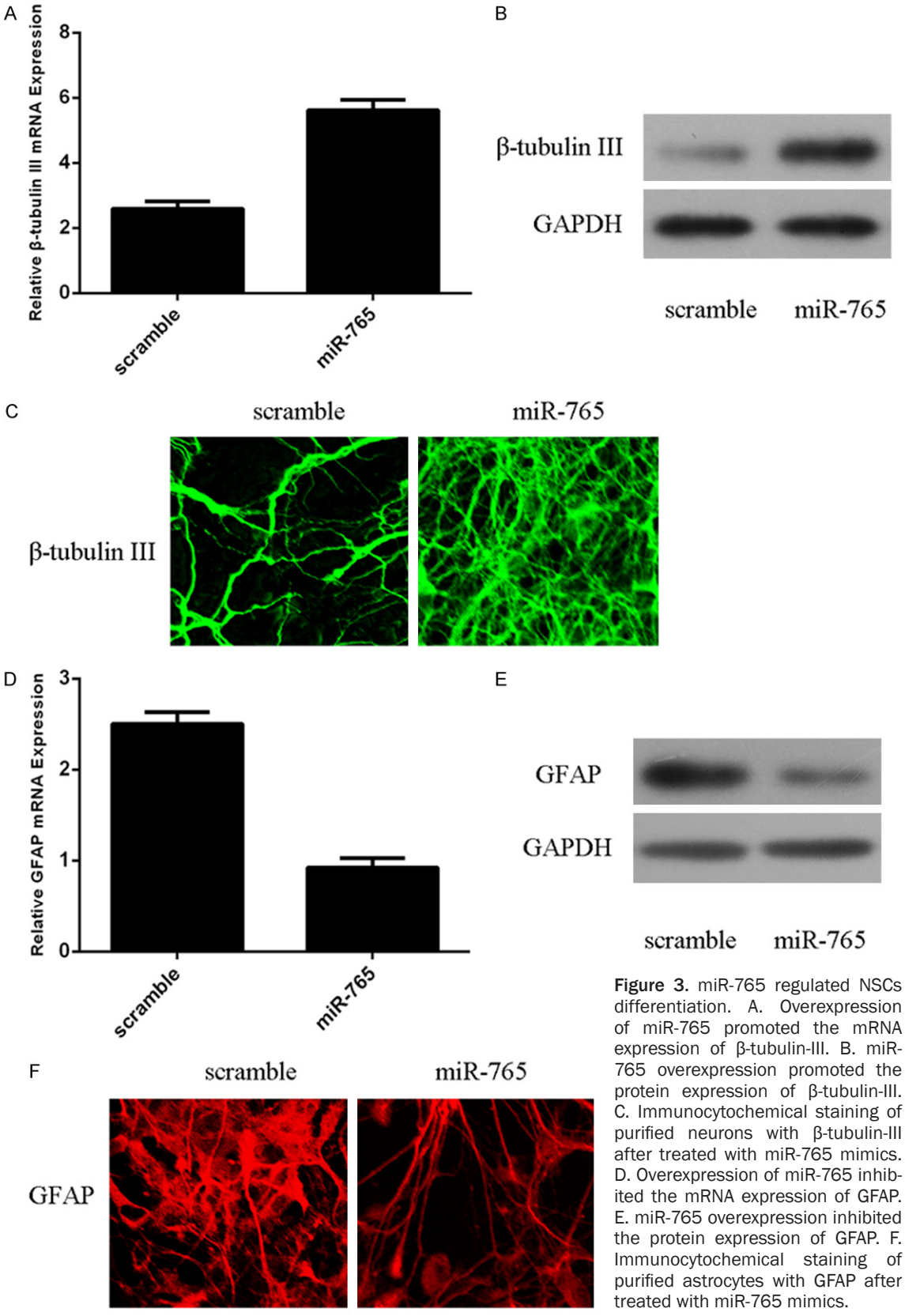

Figure 3. miR-765 regulated NSCs differentiation. A. Overexpression of miR-765 promoted the mRNA expression of β-tubulin-III. B. miR-765 overexpression promoted the protein expression of β-tubulin-III. C. Immunocytochemical staining of purified neurons with β-tubulin-III after treated with miR-765 mimics. D. Overexpression of miR-765 inhibited the mRNA expression of GFAP. E. miR-765 overexpression inhibited the protein expression of GFAP. F. Immunocytochemical staining of purified astrocytes with GFAP after treated with miR-765 mimics.

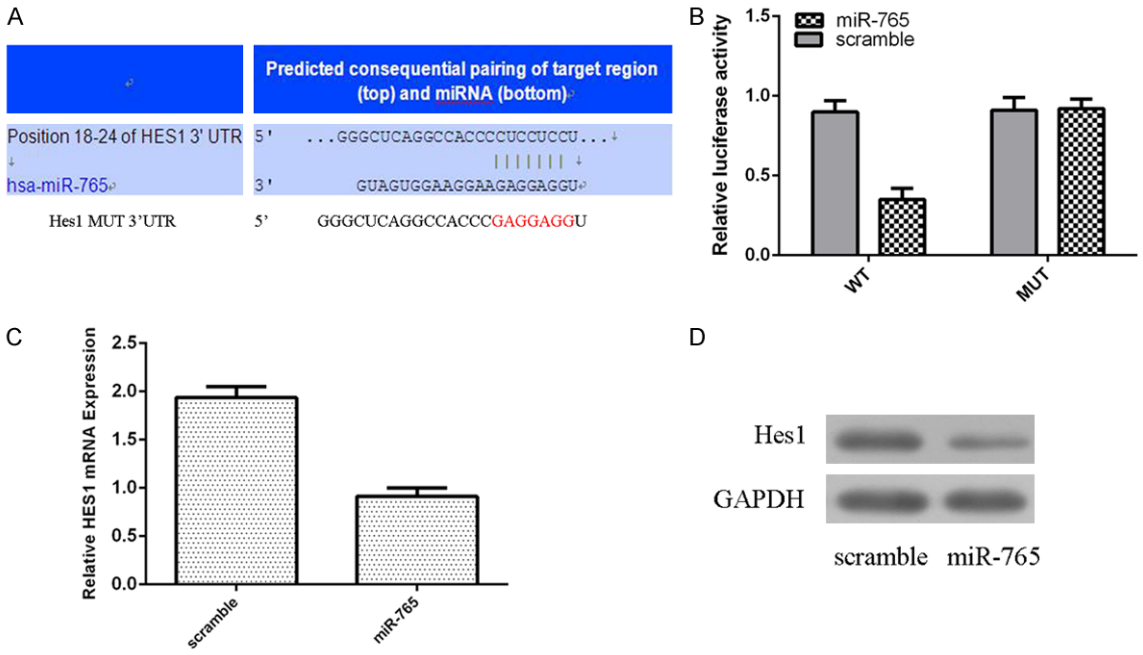

Figure 4. Hes1 was the direct target of miR-765 in NSCs. A. Hes1 was predicted to be target gene of miR-765 by TargetScan. B. Luciferase reporter assay was done to confirm the predictions in NSCs. C. The mRNA expression of Hes1 was detected by qRT-PCR in NSCs. D. The protein expression of Hes1 was measured by Western blot in NSCs.

direct target gene of miR-765 in NSCs. Overexpression of Hes1 decreased miR-765-induced proliferation of NSCs and inhibited NSCs differentiation to neurons in miR-765-treated NSCs. These results demonstrated that miR-765 acted a crucial role in NSCs differentiation and proliferation.

Previous studies demonstrated that miR-765 played important roles in a number of diseases including prostate cancer, infection of Hepatitis C virus, coronary artery disease and failing hearts [27-30]. For example, Leung et al. showed that miR-765 acted as a tumor suppressor gene in repressing proliferation, invasion and migration of fulvestrant-treated PC (prostate cancer) [27]. Liao et al. demonstrated that miR-765 regulated arterial stiffness by targeting apelin expression [29]. Cai et al. showed that miR-765 was upregulated in failing hearts accompanying with inhibitor-1 downregulation [31]. Overexpression of miR-765 inhibited cardiac function by reducing inhibitor-1 expression and promoted PP-1 activity. However, the role of miR-765 in NSCs function is still uncovered. In this study, we showed that ectopic expression of miR-765 promoted NSCs proliferation. Moreover, miR-765 overexpression increased ki-67 and β-tubulin-III expression in the NSCs. Overexpression of miR-765 could inhibit the GFAP in NSCs. These results proved that miR-765 played an important role in NSCs development.

Hes gene is mammalian homologues of Drosophila hairy that can encode basic helix-loop-helix (bHLH) transcriptional modulators [32, 33]. Hes1 is a downstream modulator of Notch pathway and immensely expressed in the CNS (central nervous system) [34, 35]. A number of references showed that Hes1 acted a crucial role in the CNS growth and it can regulate NSCs proliferation and differentiation [36-38]. Tan et al. demonstrated that miR-9 could promote NSCs differentiation to neurons through modulating Hes1 expression [23]. Recent study also found that miR-381 modulated NSCs differentiation and proliferation through inhibiting Hes1 expression [39]. In this study, we identified Hes1 was a direct target gene of miR-765 in NSCs. There was a miR-765 targeting sequence in the 3'-UTR of Hes1. Dual-luciferase reporter data showed that miR-765 inhibited the luciferase activity of WT Hes1 3'UTR vector compared with mutant Hes1 3'UTR vector. MiR-765 overexpression de-

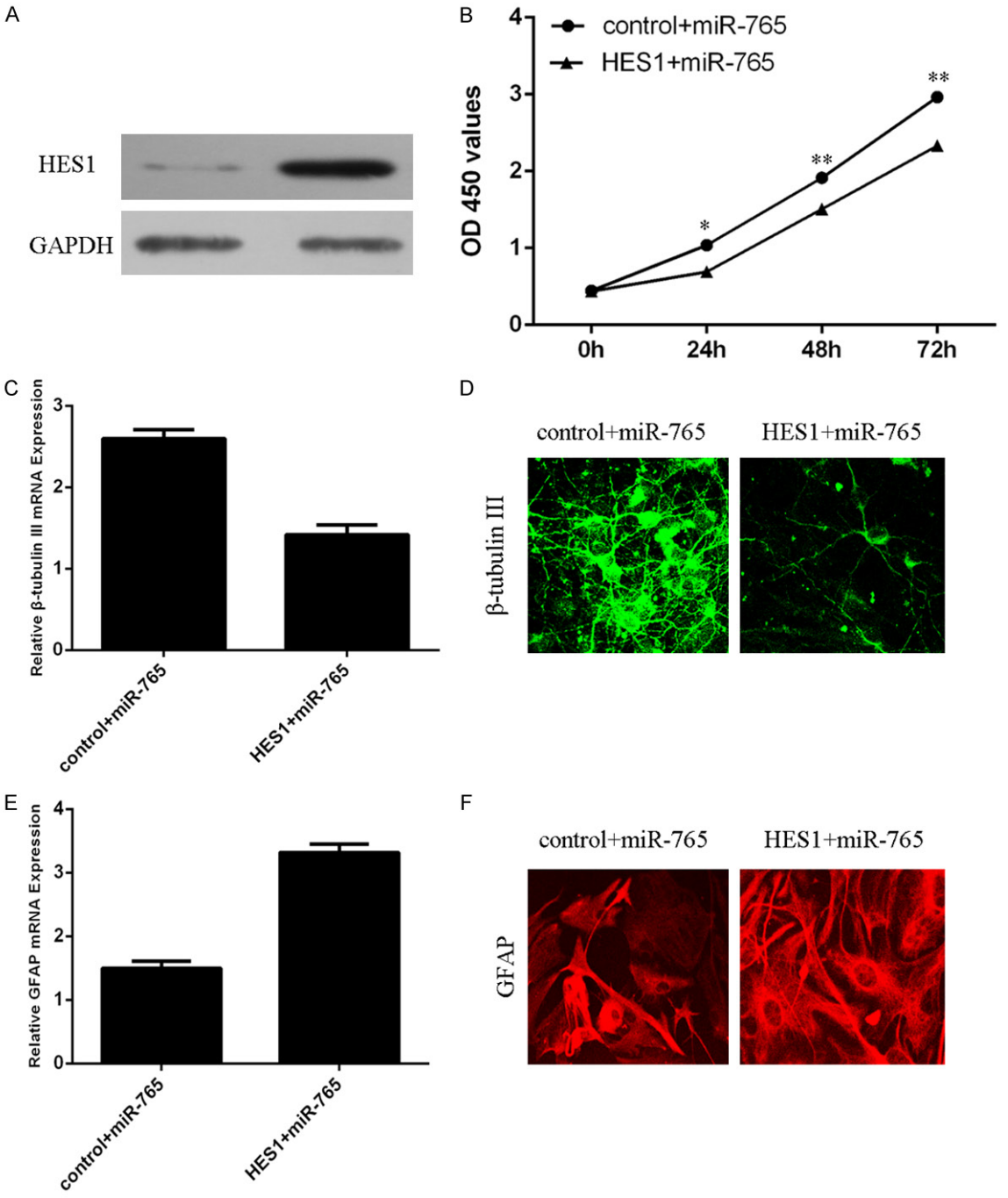

Figure 5. miR-765 regulated NSCs proliferation and differentiation through targeting Hes1. A. The protein expression of Hes1 was measured by Western blot in NSCs. B. CCK-8 was performed to detect the NSCs proliferation. C. The mRNA expression of β-tubulin-III was measured by qRT-PCR in neural stem cells. D. Immunocytochemical staining of purified neurons with β-tubulin-III. E. The mRNA expression of GFAP was measured by qRT-PCR in neural stem cells. F. Immunocytochemical staining of purified astrocytes with GFAP.

creased the Hes1 expression in NSCs. Moreover, Overexpression of Hes1 decreased miR-765-induced proliferation of NSCs and inhibited NSCs differentiation to neurons in miR-765-treated NSCs. Our results demonstrated miR-765 regulated NSCs proliferation and differentiation partly by repressing Hes1 expression.

In conclusion, our results also demonstrated that miR-765 modulated the NSCs proliferation and differentiation through inhibiting Hes1 expression.

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