ARTICLE
Journal of Cellular Biochemistry 116:943–953 (2015)

Journal of Cellular Biochemistry

MicroRNA-124 Regulates Neuronal Differentiation of Mesenchymal Stem Cells by Targeting Sp1 mRNA

Mahdieh Mondanizadeh,[1] Ehsan Arefian,[2] Ghasem Mosayebi,[3] Massoud Saidijam,[1]* Behzad Khansarinejad,[3] and Seyed Mahmoud Hashemi[4]

[1]*Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
[2]*Department of Microbiology, School of Biology, College of Science, University of Tehran, 1417614411, Tehran, Iran*
[3]*Department of Microbiology and Immunology, Arak University of Medical Sciences, Arak, Iran*
[4]*Department of Immunology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

ABSTRACT

MicroRNAs play an important role in neuronal development and function. miR-124 is the most abundantly expressed miRNA in the nervous system. Several different mRNA targets have been proposed for miR-124, but the precise function of endogenous miR-124 and its mRNA targets remain to be further elucidated. Specificity protein 1 (Sp1) is a transcription factor that plays key roles in many cell processes including cell cycle. However, this transcription factor is nearly absent in differentiated neurons and a remarkable suppression of Sp1 expression was shown after neurogenesis. Since miR-124 is expressed abundantly in neurons and because Sp1 levels decrease during neurogenesis, it is possible that miR-124 could regulate the expression of Sp1 during neuronal development. Therefore, the aim of the present study was to evaluate the putative targeting of Sp1 by miR-124. Overexpression of miR-124 using a plasmid coding for pri-miR-124 in HEK293 cells decreased the expression of Sp1 mRNA. The results of dual-luciferase reporter assay demonstrated that miR-124 directly targeted the 3′-untranslated regions of Sp1 mRNA. To evaluate whether Sp1 expression was regulated by miR-124 during the process of neuronal differentiation, Adipose-derived mesenchymal stem cells (A-MSCs) were differentiated into neuron-like cells. The results of qPCR analysis showed that with the gradual increase of miR-124 expression during neurogenesis, the expression of Sp1 mRNA decreased accordingly. In summary, this study demonstrated for the first time that miR-124 is able to suppress Sp1 expression, which in turn affected the neuronal differentiation of mesenchymal stem cells. J. Cell. Biochem. 116: 943–953, 2015.

KEY WORDS: MicroRNAs; miR-124; NEUROGENESIS; SPECIFIC PROTEIN 1; CELL DIFFERENTIATION

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level through sequence-specific base pairing to target specific mRNAs leading to mRNA cleavage or translational repression [Lee, 2004; Kuss and Chen, 2008]. Since the first miRNA was discovered [Lee, 1993], hundreds of miRNAs have been identified that play key regulatory roles in almost every aspects of biology and cellular processes, including etiology and progression of several diseases, developmental timing, immunity, neurodegeneration, and neuronal development [Gao, 2010; Meza-Sosa, 2012; Finch, 2014].

Neurogenesis is a highly orchestrated process that requires precise and exquisitely regulated gene expression patterns [Cao, 2007; Gao, 2010]. It is now understood that miRNAs have the capacity to provide such fine regulation in neuronal development or function [Schratt and Greenberg, 2008; Gao, 2010]. MicroRNA-124 (also known as miR-124) is the most abundantly expressed miRNA in the nervous system [Smirnova, 2005; Nelson, 2006; Yu, 2008; Liu and Xu, 2011]. This highly conserved and tissue specific miRNA is found in the nervous system of all animals studied to date from *Caenorhabditis elegans* to mammals [Clark, 2010]. The progressive increase of miR-124 expression during development of central

Conflict of interest: None.
Grant sponsor: Research Deputy of Hamadan University of Medical Sciences.
*Correspondence to: Massoud Saidijam, Research Center For Molecular Medicine, Hamadan University of Medical Sciences, Hamadan 6517838736, Iran.
E-mail: sjam110@yahoo.com
Manuscript Received: 30 July 2014; Manuscript Accepted: 16 December 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 January 2015
DOI 10.1002/jcb.25045 • © 2015 Wiley Periodicals, Inc.

nervous system raises the possibility that this miRNA may play unique functions during this process. However, in the most cases the role of miR-124 combines with other factors including some growth factors and transcription factors, but only a limited number of genes and proteins involved in this process has been known [Lagos-Quintana, 2002; Krichevsky, 2003; Sempere, 2004; Maiorano and Mallamaci, 2010]. A few studies have aimed to understand the molecular mechanism of miR-124 in neuronal development. It seems a primary function of miR-124 is to maintain neuronal state by downregulating non-neuronal mRNAs. Ectopic expression of miR-124 in HeLa cells, neuroblastoma cell lines, and embryonic stem cells represses the expression of non-neuronal transcripts and shifts the gene expression profile towards neuronal state [Lim, 2005; Krichevsky, 2006; Makeyev, 2007]. It has been reported that neurite outgrowth is promoted by overexpressing miR-124 in mouse P19 cells, whereas its downregulation delays neurite outgrowth and reduces the amount of acetylated α-tubulin [Yu, 2008]. However, the precise function of endogenous miR-124 and its mRNA targets, which are involved in neurogenesis, remain to be further elucidated.

One of the classical approaches to understand miRNAs' functions is to identify direct targets for them. The human specificity protein 1 (Sp1) is a transcription factor with 778 amino acid that recognizes GC-rich sequences in some eukaryotic promoters. Sp1 is involved in many cellular processes including cell cycle progression, angiogenesis, and cell migration and invasion [Black, 1999]. Sp1 can regulate hundreds of genes, such as Vascular endothelial growth factor (VEGF), Cyclin A2, Cyclin D1, E-cadherin, Phosphatase 2 A (PP2A), and Matrix metalloproteinase (MMP) [Qiu, 2014]. Sp1 also plays a key role in cell cycle and reduction in the level or activity of this protein will block mitosis [Abdelrahim, 2002]. It is known that "regulation of Sp1-dependent transcription could be affected by changes in Sp1 abundance and its DNA binding activity" [Black, 1999]. On the other hand, Sp1 is nearly absent in differentiated neurons [Mao, 2007]and a remarkable inhibition of Sp1 expression was shown after neuronal differentiation of NTera2 cells [Mao, 2009]. Since miR-124 is expressed abundantly in differentiating and differentiated neurons and because Sp1 levels decrease during neurogenesis, it is possible that miR-124 could regulate the expression of Sp1 in neuronal development. Therefore, the purpose of this study was to experimentally evaluate the possible implication of miR-124-mediated Sp1 suppression. The effect of miR-124 on Sp1 mRNA expression was also evaluated during in-vitro neuronal differentiation of mesenchymal stem cells.

MATERIALS AND METHODS

CELL CULTURE AND NEURONAL DIFFERENTIATION

The human embryonic kidney cell line HEK293 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma–Aldrich, St. Louis, MO). All transfections were in an antibiotic-free media using Lipofectamine™ 2000 (Invitrogen, CA), according to manufacturer's guidelines. Human adipose-derived mesenchymal stem cells (A-MSCs) were purchased from Stem Cell Technology Research Center, Tehran, Iran. A two-step protocol was used for neuronal differentiation of A-MSCs. Briefly, at 30% confluence, the cells were exposed for 5 days to Step-1 induction medium consisting of IMDM (Invitrogen) supplemented with 20% FBS, 5 μg/ml insulin, 5 ng/ml bFGF-2, 0.5 μM retinoic acid and 15 ng/ml EGF, followed by 5 days in Step-2 induction medium consisting of IMDM supplemented with 100 μM cAMP, phosphodiesterases 3-isobutyl-1-methylxanthine (IBMX; 500 μM nerve growth factor (NGF; 100 ng/ml), and 10 μM forskolin. All the above induction factors were purchased from Sigma (Sigma–Aldrich, St. Louis, MO).

CLONING OF MIR-124

Plasmids were constructed using standard restriction-based method. For cloning of human miR-124 gene (miRBase accession number: MI0000443), a fragment containing the pri-miR-124 (-212 to +160 bps relative to the 5′ end of pre-miR-124), was inserted into the pLenti-III-GFP vector (ABM, Canada). The sequence of forward and reverse primers used for miR-124 amplification was 5′-CTCCCTGAGTCTGTTTGCA-3′ and 5′-AACAAAGAGCCTTTG-GAAG-3′, respectively; and they contained the restriction sites for EcoRI and XhoI (ThermoScientific, Waltham, MA). Successful cloning was verified by both restriction endonuclease digestion and DNA sequence analysis.

LUCIFERASE REPORTER ASSAY

The three prime untranslated region (3′-UTR) of the human Sp1 gene was PCR amplified using the specific primers: forward, 5′-ATCAGTGGCAATGGCTTCTG-3′ and reverse, 5′-AGGAGCAGGAA-GAAACTTAC-3′, to produce a PCR product of 5,073 bp (position 2435–2454 to 7487–7507, refers to the NCBI's reference sequences accession number: NM_138473.2). Purified amplicons were digested using XhoI and NotI restriction enzymes and cloned downstream of the firefly luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI). HEK293T cells were plated in a 96-well plate at 5,000 cells/well. Following an overnight incubation, the cells were treated with a transfection mixture consisting of 25 μl of serum-free medium, 0.5 μl of Lipofectamine™ 2000, 0.1 μg of psiCHECK-2 vector, including the 3′-UTR of Sp1, and 0.05 μg of pLenti-III-miR-124 vector. At 48 h post-transfection, luciferase assays were performed using a Dual-Luciferase® Reporter assay system (Promega) on a Sirius luminometer (Titertek-Berthold, Pforzheim, Germany) according to manufacturer's instructions. Relative light units were calculated as the ratio of Renilla to firefly luciferase activity, and the reporters were normalized to the control psiCHECK-2 plasmid that carried the 3′-UTR region of Sp1 gene cloned in inverse orientation, to correct for nonspecific effects. Three technical replicates were performed for each condition.

IMMUNOCYTOCHEMICAL STAINING

Fluorescent staining of antigens β-tubulin III and microtubules associated protein (MAP2) was performed to confirm neuronal differentiation. The cells were fixed in 4% cold paraformaldehyde for 20 min at 4 °C and then 5 min at room temperature (RT). The cells were washed three times with PBS and permeabilized by Triton-X-100 (0.04%) for 20 min and blocked by 5% goat serum

for 45 min. The cells were incubated overnight at 4 °C with mouse polyclonal primary antibodies (diluted in BSA/PBS 0.2%) against β-tubulin III (polyclonal mouse IgG, Sigma,) and MAP-2 (polyclonal mouse IgG, Sigma) at 1:200 dilution. The plates were washed three times with PBS-Tween-20 (0.1%) and were incubated 1 h at RT with anti mouse polyclonal secondary antibodies (anti-ms IgG-FITC, Sigma). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1%) for 5 min and were examined on a fluorescent microscope.

REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR (RT-QPCR) ANALYSIS

Total RNA was extracted from 5×10^6 to 10^7 harvested cells using TRIzol reagent (Life Technologies, USA) according to manufacturer instructions and ~ 1 μg of RNA were reverse-transcribed using the M-MLV enzyme (Vivantis, Malaysia). Expression of SNORD47 (U47) RNA was used as reference for normalization of miR-124 expression and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene for normalization of mRNA expression levels. Random hexamer primers were used to prime the cDNA synthesis for all mRNAs and the stem-loop gene-specific reverse transcription primers (miR-124, 5′-GTCGTATCGAGAGCAGGGTCC-GAGGTATTCGCACTCGATACGACGGCATT-3′; U47, 5′-GTCGTAT GCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGA-CAACCTC-3′) were used to specifically reverse transcribe the mature forms of miR-124 and U47 molecules [Guttilla and White, 2009]. RT-qPCR was then performed on cDNAs with sequence-specific forward primers (miR-124, 5′-GCTAAGG-CAAGCGGTG-3′; U47, 5′-ATCACTGTAAAACCGTTCCA-3′) and a common reverse (GAGCAGGGTCCGAGGT). For analysis of gene expressions, exon-junction primer sets were designed using AlleleID7.0 software, (Premier Biosoft International, CA) for analysis of gene expression (Table I). RT-qPCR reactions were performed in a Rotor-Gene Q cycler (Qiagen, Hilden, Germany) using the SYBR® Premix Ex Taq ™ II (Takara Bio Inc, Otsu, Japan). The amplification profile consisted of a single cycle of enzyme activation at 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 5 s, annealing/extension at 60 °C for 30 s, with a single fluorescence acquisition at channel green. Amplification was followed by melting curve analysis with a ramp rate of 0.4 °C/second to 95 °C with continuous fluorescence acquisition. The expression analyses were measured in triplicate for three technical replicates and the relative changes were calculated by the comparative Cq method using the relative expression software tool (REST) [Pfaffl, 2002].

LENTIVIRAL VECTOR PRODUCTION AND TRANSDUCTION

Construction and titration of the lentiviral vector encoding miR-124 was performed based on an established method with modifications [Zufferey and Trono, 2001]. Briefly, the plasmid pLenti-III-miR-124 was transfected into HEK293 cells along with packaging plasmid psPAX2 (Addgene, Cambridge, MA) and envelope plasmid pMD2.G (Addgene). The medium was removed and replaced with fresh medium, 16 h post-transduction. Cell supernatants were collected 24 h, 48 h and 72 h post-transduction. The supernatant pool was filtered (0.45 μm) to remove cellular debris and concentrated by ultracentrifugation at 50,000 g for 2 h at 4 °C. Lentiviral concentrates were titrated in HEK293 cells as described previously [Zufferey and Trono, 2001]. For lentiviral transduction, A-MSCs (4×10^4) were seeded in 24-well plates and, at 30% confluency, the lentiviral supernatant at a multiplicity of infection (MOI) of 15. Forty-eight hours after transduction, the efficiency of transduction was evaluated using flow cytometry and direct observation under an invert fluorescence microscope. The growth of transducted cells was supported by adding puromycin (2 μg/ml), in order to kill non-transduced cells.

STATISTICAL ANALYSIS

Relative RT-qPCR data were calculated by REST 2009. The data in graphs are expressed as the mean ± SE. The difference between two or more groups was compared by Student's *t*-test or analysis of variance (ANOVA), respectively. The correlation between miR-124 and Sp1 expression levels was calculated using the Pearson correlation test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

THE TRANSCRIPTION LEVEL OF SP1 IS DECLINED IN HEK293 CELLS AFTER ECTOPIC OVEREXPRESSION OF MIR-124

HEK293 cells, grown on 12-well plates, were transfected with 2 μg/0.1 ml of either pLenti-III-miR-124 or control plasmid (empty pLenti-III). Transfection efficiency was confirmed after 24 h by detecting green fluorescent protein (GFP) positive cells using both flow cytometry and direct observation under an invert fluorescence microscope. The wells with transfection rate of more than 60% were chosen for gene expression analysis. RT-qPCR analysis showed that miR-124 was dramatically overexpressed in comparison to empty vector trasfected groups on day 1 (24-fold), day 2 (32.55-fold), and day 3 (52.8-fold) post-trasfection. Accordingly, the mRNA level of Sp1 was significantly reduced in the miR-124-groups (Fig. 1).

TABLE I. Primers Used for the qPCR Assay

Gene	Primer sequence (5′-3′)	Amplicon size (bp)
Sp1	F: GGCTGTGGGAAAGTGTATGG R: GGCAAATTTCTTCTCACCTGTG	168
GAPDH	F: CCTCAAGATCATCAGCAATG R: CATCACGCCACAGTTTCC	167
β-tubulin III	F: GATCGGAGCCAAGTTCTG R: GTCCATCGTCCCAGGTTC	178
MAP2	F: AGTTCCAGCAGCGTGATG R: CATTCTCTCTTCAGCCTTCTC	95
NSE	F: GGAGAACAGTGAAGCCTTGG R: GGTCAAATGGGTCCTCAATG	239
Nestin	F: GAAGGTGAAGGGCAAATCTG R: CCTCTTCTTCCCATATTTCCTG	97
NF	F: TCAACGTCAAGATGGCTCTG R: TGTGTTGGACCTTAAGCTTGG	189

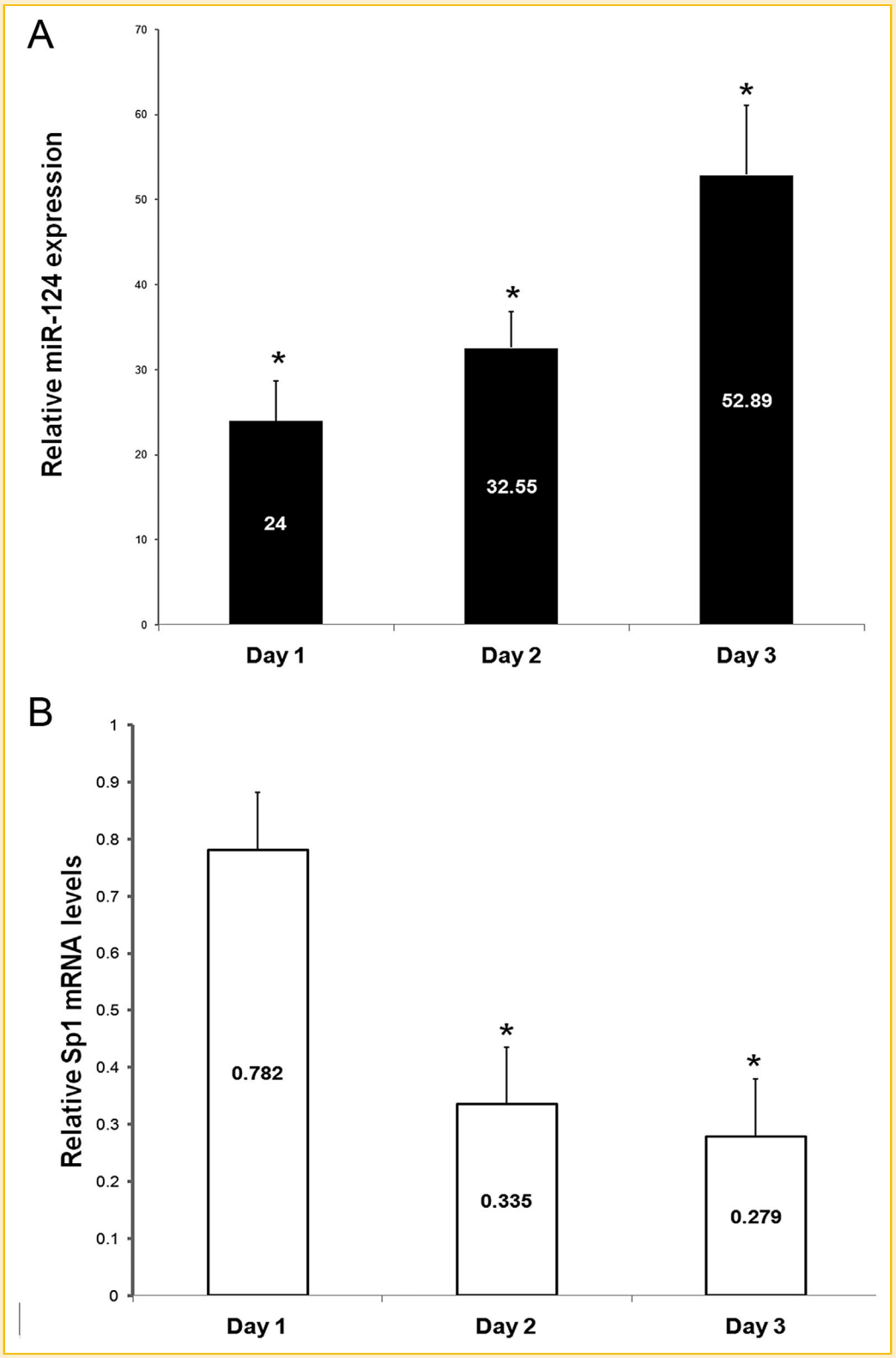

Fig. 1. Expression levels of miR-124 and Sp1 transcripts in HEK293 cells. Plasmid encoding miR-124 (pLenti-III-miR-124) or empty vector (pLenti-III) was transfected into the cells. (A) Expression levels of miR-124 measured by RT-qPCR assay on three successive days after transfection. (B) The levels of Sp1 mRNA measured at the same time points. Error bars indicate the standard error of the mean. $^{*}P < 0.05$, significantly different from control group.

MIR-124 TARGETS SP1 BY BINDING TO THE 3′-UTR REGION OF SP1 MRNA

Using two online miRNA target prediction algorithms, Targetscan and Pictar, it was shown that the 3′-UTR sequence of Sp1 mRNA contains two putative targets of miR-124 seed region (Fig. 2A). To verify the in silico prediction experimentally, a dual luciferase reporter assay using the psiCHECK-2 vector was applied. The reporter plasmid and pLenti-III-miR-124 were cotransfected into HEK293 cells. As it is shown in Fig. 2B, transfection of miR-124 caused a significant decrease in luciferase activity on the luciferase

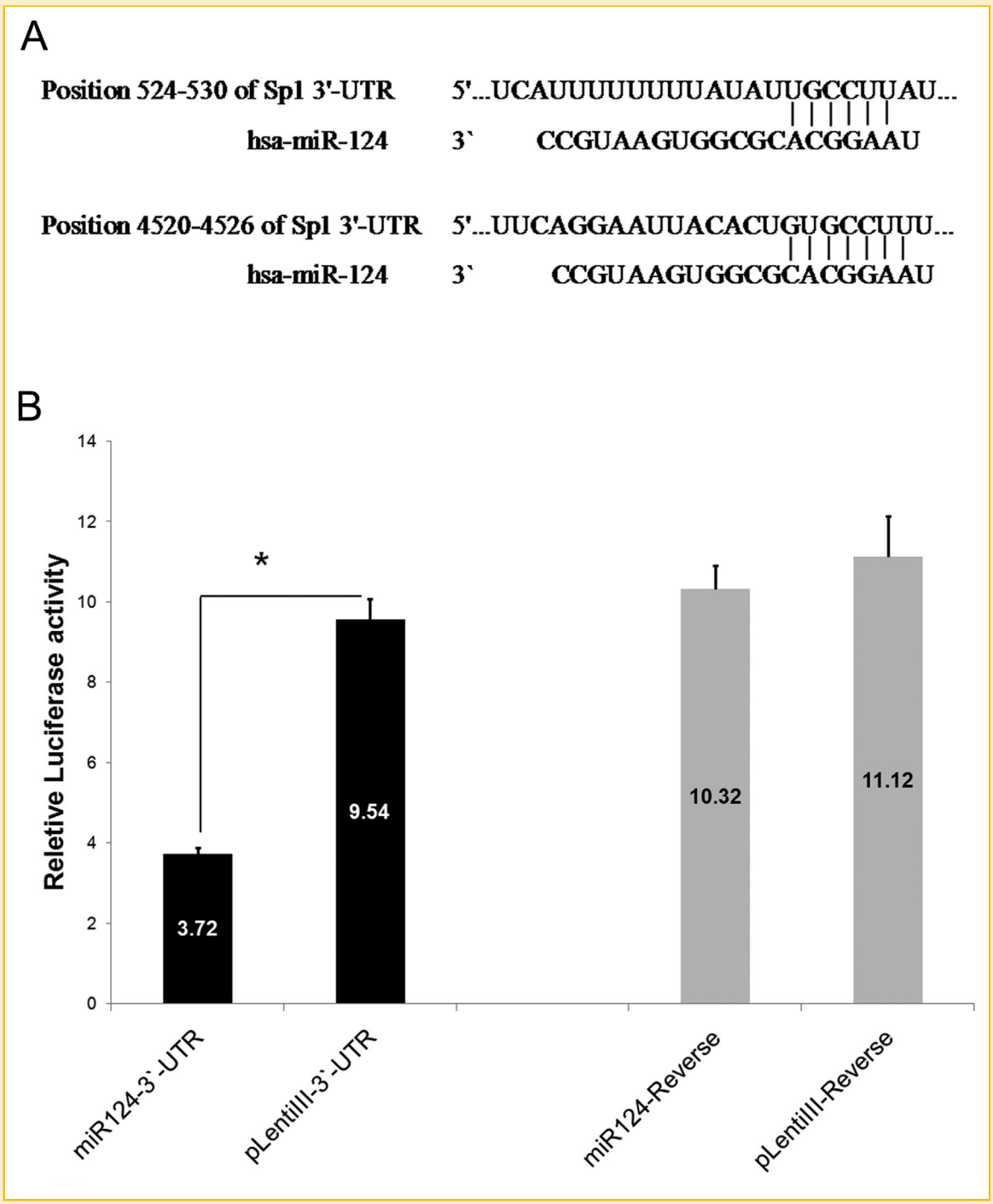

Fig. 2. miR-124 targets 3'-URT of Sp1 mRNA. (A) A schematic shows that the seed region of miR-124 contains two complementary sites within the 3'-UTR of Sp1 mRNA. (B) pLenti-III-miR-124 vector and its negative control (empty pLenti-III) was co-transfected with either psiCHECK-Sp1–3'-UTR or psiCHECK-Sp1-reverse 3'-UTR plasmid into HEK293 cells. Forty-eight hours posttransfection Renilla and firefly luciferase activities were measured and normalized to firefly luciferase data. The data represent the mean of eight wells plus or minus the standard deviation. $^{*}P < 0.05$, significantly different from control.

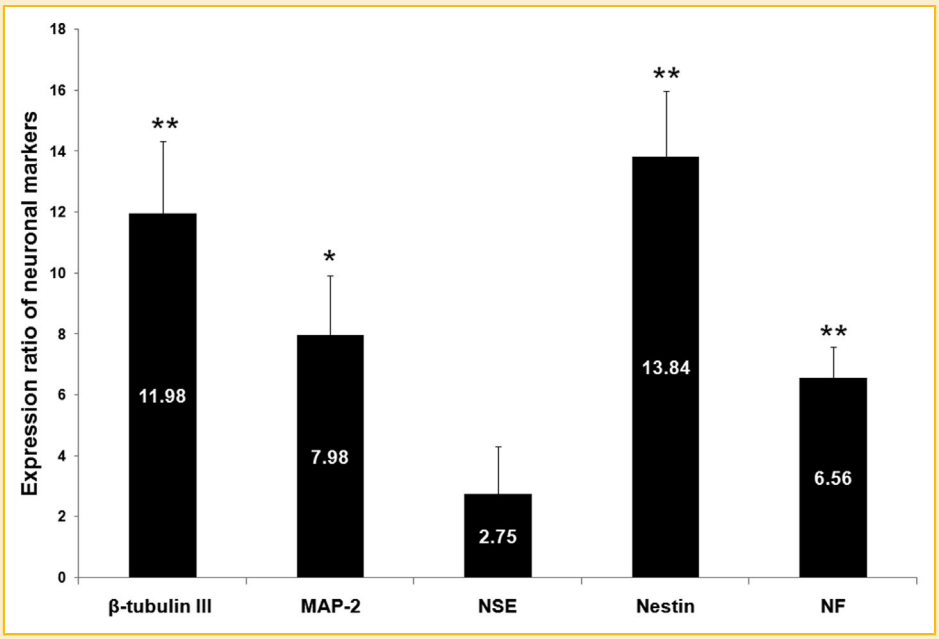

Fig. 3. Relative transcription of neuron-specific markers in differentiated A-MSCs. The box plots show the expression levels of the genes which were measured by RT-qPCR and compared to undifferentiated cells. Error bars indicate the standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from undifferentiated A-MSCs.

expression constructs carrying the target fragment. Furthermore, the negative control construct, containing the inverse copy of the 3′-UTR, did not affect the luciferase activity. These data indicate that the miR-124 can suppress expression of Sp1 transcripts through interaction with their 3′-UTR region.

MIR-124 INHIBITS SP1 EXPRESSION DURING NEURONAL DIFFERENTIATION

In order to evaluate whether Sp1 expression was regulated by miR-124 during the process of neuronal differentiation, A-MSCs were differentiated into neuron-like cells. To confirm neuronal differentiation, the expression level of neuron-specific markers including microtubule-associated protein 2 (MAP-2), β-tubulin III, neuron specific enolase (NSE), neurofilament (NF), and Nestin was detected using RT-qPCR assay. Moreover, antibodies against neuron-specific proteins MAP2 and β-tubulin III were also used for immunocytochemistry staining of the cells. As it is shown in Fig. 3, the results of RT-qPCR analyses indicated that the mRNA levels of the neuronal markers β-tubulin III (11.97-fold, $P = 0.002$), MAP-2 (7.98-fold, $P = 0.013$), Nestin (13.83-fold, $P = 0.002$), and NF (6.56-fold, $P = 0.005$) were significantly higher compared with undifferentiated A-MSCs. However, although the expression level of NSE (2.7-fold, $P = 0.143$) was increased in differentiated cells, this result was not statistically significant (Fig. 3).

Differentiated cells were first morphologically evaluated using phase contrast microscopy. Immunofluorescence staining was also confirmed the presence of both β-tubulin III and MAP-2 proteins in differentiated cells (Fig. 4). It was shown that after 10 days in induction media, many cells expressed neuronal differentiation key markers. Finally, the expression levels of miR-124 and Sp1 RNAs were quantified and compared relative to each other at two different time points. RT-qPCR results on days 5 and 10 post-induction demonstrated that the miR-124 expression levels were increased gradually (Fig. 5A), while those of Sp1 expressions were decreased accordingly (Fig. 5B).

To evaluate correlation between miR-124 and Sp1 expressions, twenty separate wells of differentiated cells were analyzed on days 5 and 10 post-induction. An inverse correlation between miR-124 and Sp1 RNA levels was observed ($r = -0.446$, $P = 0.049$ by Pearson correlation test). The results showed that the higher the expression level of miR-124 RNA, the lower level of Sp1 mRNA could be detected (Fig. 6).

OVEREXPRESSION OF MIR-124 IN MESENCHYMAL STEM CELLS

In order to assess whether the overexpression of miR-124 itself could promote neuronal differentiation, A-MSCs were transducted with retroviral constructs carrying miR-124 shRNA. To evaluate neuronal differentiation, mRNA expression analysis for neuron-specific markers and immunocytochemistry staining for MAP2 and β-tubulin III proteins were applied on day 10, post- transduction. Immunofluorescence staining demonstrated that in comparison to control group, transduced A-MSCs were positive for β-tubulin III, but immunoreactivity was not observed for MAP2 protein (Fig 7A). According to the results of RT-qPCR, both the β-tubulin III and MAP2 showed mRNA overexpression of 4.83 ($P < 0.001$) and 2.34 ($P < 0.001$), respectively. However, the mRNA level of other neuron-specific markers showed no significant overexpression (Fig 7B). The mRNA expression of Sp1 was also evaluated in transduced cells and it was observed that the expression level of Sp1 was significantly reduced as well (0.619-fold, $P < 0.01$, Fig 7C).

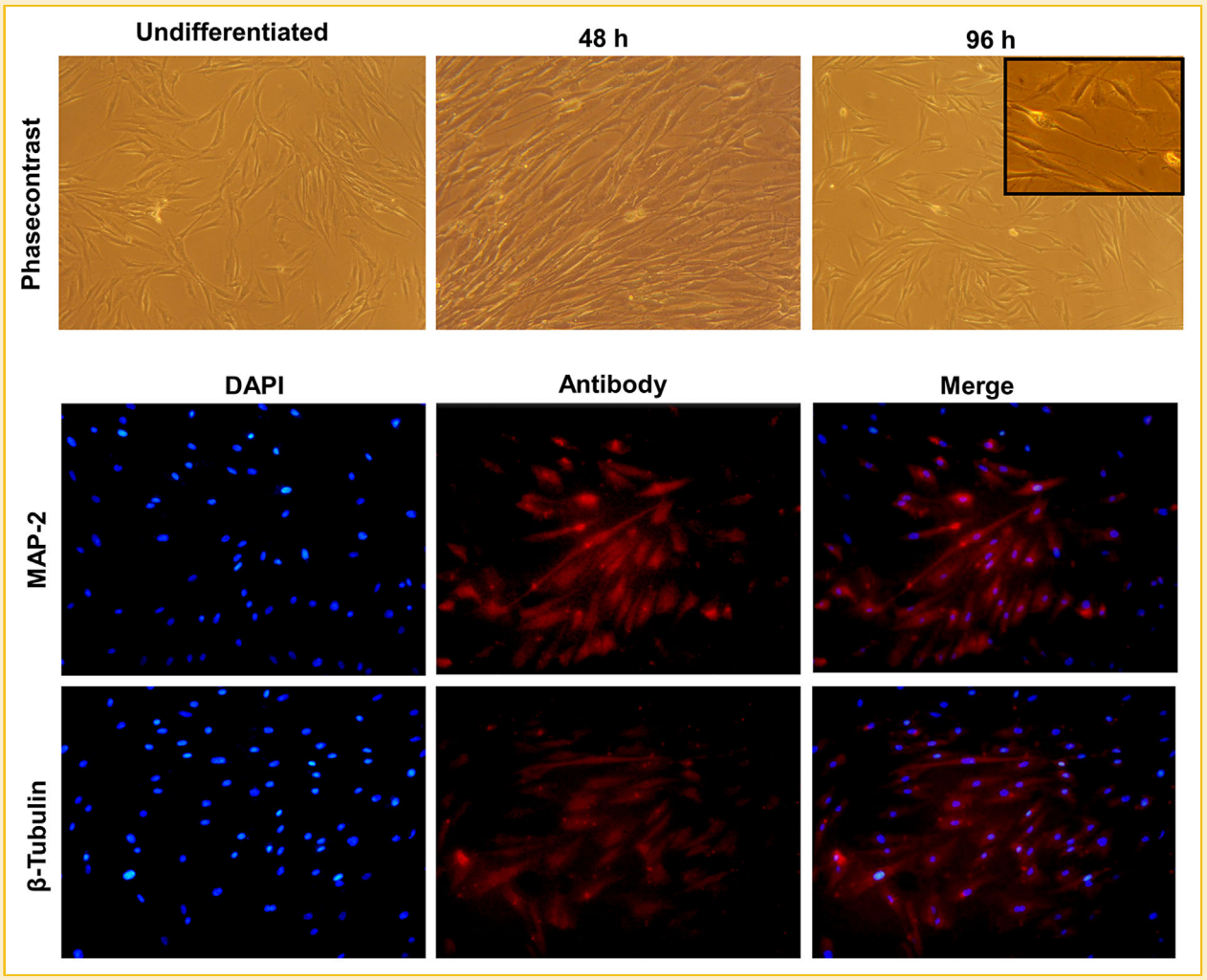

Fig. 4. Neuronal like differentiation of A-MSCs. (A–C) Phase contrast morphology of undifferentiated and differentiated cells on days 3 and 6 after induction (B and C). (D–I) Immunofluorescence detection of MAP-2 (D–F) and β-tubulin III (G–I) proteins in differentiated cells on day 10 post-induction.

DISCUSSION

Once a cell commits itself to adopt neuronal phenotype, it has to change its gene expression pattern both by activating the expression of neuron-specific genes and by repressing the expression of genes that are not compatible with the neuronal state. microRNA-dependent regulation of gene expression plays an important role in neuronal differentiation [Schratt and Greenberg, 2008]. miR-124 down-regulates a large number of non-neuronal transcripts thereby promote neuronal fate to the cells [Lim, 2005]. It has been shown that miR-124 directly targets the mRNA of polypyrimidine tract-binding protein 1 (PTBP1) and results in a more neuron-specific alternative splicing pattern [Makeyev, 2007]. miR-124 can bind to the 3′-UTR of Krüppel-like factor 6 (KLF6) and STAT3 molecules, suggesting that this miRNA could be involved in appropriate nerve regeneration [Nagata, 2014]. It is suggested that miR-124 acts as a suppressor for the ras homology growth-related (RhoG) protein and results in axonal and dendritic branching in hippocampal neurons [Franke, 2012]. miR-124 has been also shown to suppress the expression of genes with anti-neuronal activities, including Sox9 [Cheng, 2009], repressor-element-1-silencing transcription factor (REST), and SCP1 [Conaco, 2006; Visvanathan, 2007] Finally, a recent study showed that miR-124 directly down-regulates the Histone-lysine N-methyltransferas]. Ezh2 expression and promote neuronal differentiation [Neo, 2014].

In the present study, it was shown that miR-124 is able to target Sp1 mRNA and downregulate its expression during neurogenesis. The study was performed in four stages. In the first stage, miR-124 was overexpressed in HEK293 cells. These cells were chosen because they present a normal Sp1 function but express very few level of miR-124 RNA. It was observed that with the increase of miR-124 expression, there was a significant reduction in the expression of Sp1 mRNA. MicroRNAs normally induce mRNA cleavage or inhibit mRNA translation by base pairing to the 3′-UTR region of target genes. The bioinformatics analyses showed that the seed region of miR-124 could potentially bind 3′-UTR of Sp1 mRNA in two separate regions. Therefore, in the second stage, a luciferase reporter

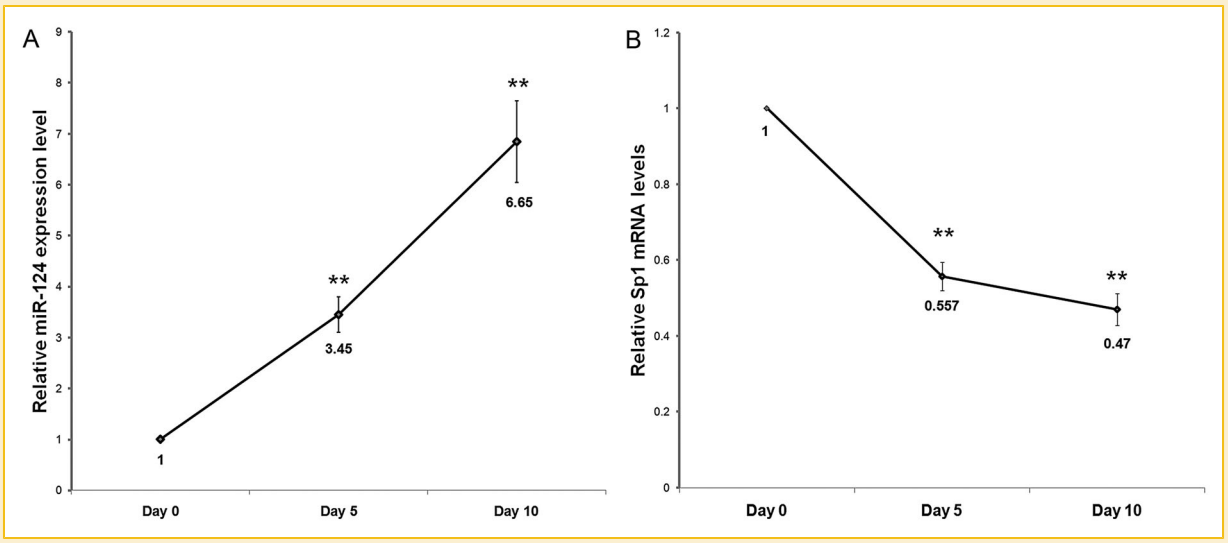

Fig. 5. Differential expression of miR-124 and Sp1 during neuronal differentiation of A-MSCs. (A) Expression levels of miR-124 RNA was identified by RT-qPCR in differentiated cells at two different stages of neuronal differentiation (day 5 and day 10). (B) Expression ration of Sp1 mRNA. The relative expression of untreated A-MSCs (Day 0) was set to one, as control. Error bars indicate the standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control group.

construct containing the 3′-UTR of Sp1 was generated. Sp1 mRNA has a very long 3′-UTR region (~5 kb), presumably containing multiple regulatory elements that are necessary for its biological function [Liang and Wang, 2012]. Since the two potential binding sites of miR-124 were far from each other (positions 524–530 and 4520–4526) and to try to maintain the natural secondary structure of the untranslated region, the whole 3‘-UTR sequence was cloned into the vector. Based on the results of luciferase reporter assay it was identified that miR-124 regulates Sp1 expression through interaction with the 3′-UTR region of its mRNA.

In order to evaluate whether Sp1 expression was regulated by miR-124 during the process of neuronal differentiation,

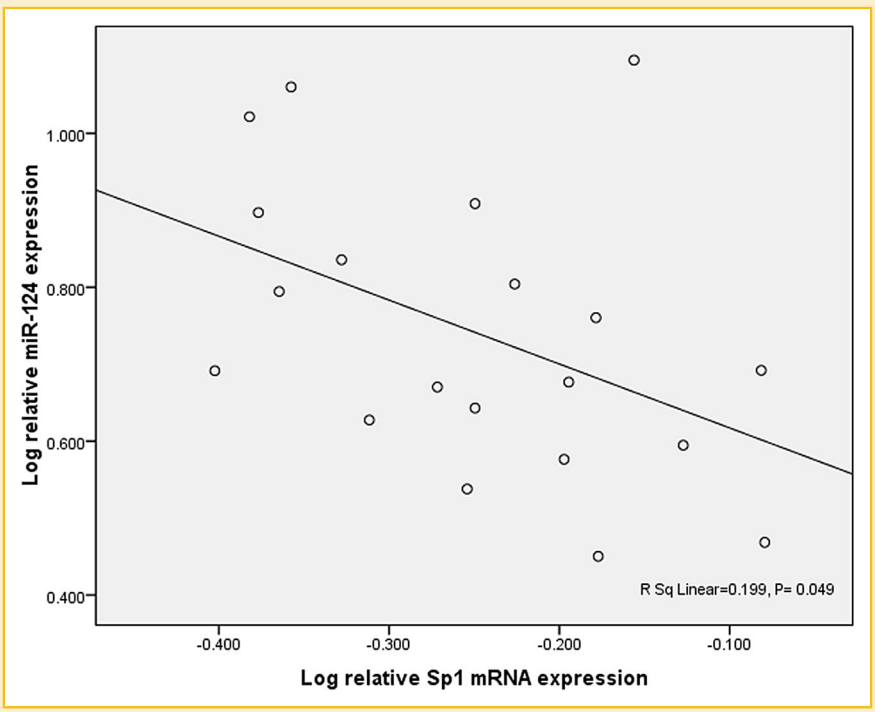

Fig. 6. Correlation analysis of miR-124 and Sp1 expression in neuronal differentiated A-MSCs. The R^2 value and *P*-value are shown in the lower right corner of the Figure.

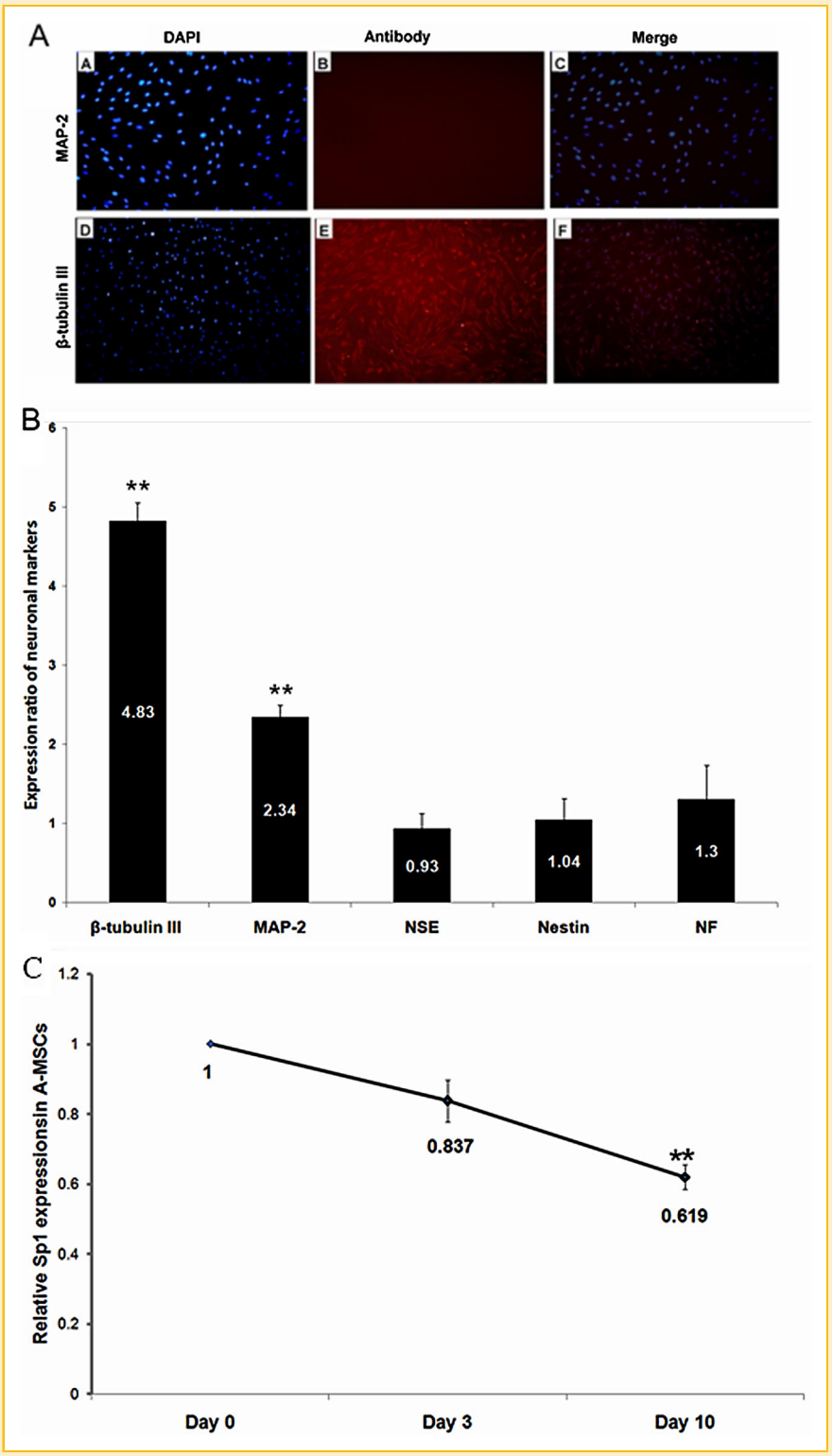

Fig. 7. Expression of neuron-specific markers in lentiviral transduced cells. (A) Immunofluorescence staining of MAP-2 (A–C) and β-tubulin III (D–F) proteins in A-MSCs on day 10 post- transduction. (B) RR-qPCR results of neuron-specific markers expressions. Error bars indicate the standard error of the mean. $^{**}P < 0.01$, significantly different from undifferentiated A-MSCs. (C) Relative expression of Sp1 mRNA during miR-124-induced neuronal differentiation of A-MSCs. $^{**}P < 0.01$, significantly different from control group.

Adipose-derived mesenchymal stem cells were differentiated into neuron-like cells, in the third stage of the study. Overexpression of some neuron-specific markers in mRNA and protein levels confirmed neuron-like differentiation of the cells. The results of RT-qPCR analysis showed that with gradual increasing of miR-124 expression during neurogenesis, the expression of Sp1 mRNA decreased accordingly. This finding suggests that miR-124 acts as endogenous attenuators of Sp1 expression during neurogenesis. Finally in the fourth stage, a retroviral constructs carrying miR-124 shRNA was transducted into the A-MSCs, In order to evaluate whether overexpression of miR-124 could promote neuronal differentiation. The RT-qPCR results showed that among all neuron-specific markers analyzed in this study, the mRNA levels of β-tubulin III and MAP2 were significantly increased. On the other hand, Immunofluorescence staining was positive only for β-tubulin III. Since β-tubulin III, as well as MAP2, are known as the neuronal early markers, the finding suggests overexpression of miR-124 is able to promote the early stages of neuronal differentiation not a complete differentiation toward neuronal cells. This finding is in agreement with a recent study that showed microRNA-124 promotes the neuronal differentiation of bone marrow-derived mesenchymal stem cells [Zou, 2014]. Our study was also shown that the mRNA level of Sp1 decreased in A-MSCs that received miR-124 via transduction. Sp1 is a transcription factor that regulates the expression of a plethora of mammalian genes and plays an important role in many cellular activities, especially cell proliferation. Sp1 expression is suppressed in differentiated neurons [Mao, 2009], a feature that is in agreement with non-replicating nature of neuronal cells. Some previous studies have reported that certain microRNAs including miR-128 and miR-337 could target Sp1 mRNA and inhibit glioblastoma cells [Dong, 2014; Zhang, 2014], but our study is the first which experimentally showed miR-124 could directly targets Sp1 mRNA.

In conclusion, the current study for the first time uncovered that miR-124 was able to target the 3′-UTR of Sp1, which in turn affected the neuronal differentiation of mesenchymal stem cells. This finding may provide novel insights into our understanding of the function of miR-124 and molecular mechanisms underlying differentiation into neuronal cells and maintaining neuronal state.

ACKNOWLEDGEMENTS

This study was supported by the Research Deputy of Hamedan University of Medical Sciences and was a part of PhD thesis of M. Mondanizadeh. The authors thank Dr. Mahmood Naderi, Dr. Mahdi Paryan, Dr. Samira Mohammadi-Yeganeh, and Samaneh Sharifi for their assistance in this research.

REFERENCES

Abdelrahim M,Samudio I, Smith R 3rd, Burghardt R, Safe S. 2002. Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. J Biol Chem 277:28815–28822.

Black AR, Jensen D, Lin SY, Azizkhan JC. 1999. Growth/cell cycle regulation of Sp1 phosphorylation. J Biol Chem 274:1207–1215.

Cao X, Pfaff SL, Gage FH. 2007. A functional study of miR-124 in the developing neural tube. Genes Dev 21:531–536.

Cheng LC, Pastrana E, Tavazoie M, Doetsch F. 2009. MiR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat Neurosci 12:399–408.

Clark AM, Goldstein LD, Tevlin M, Tavare S, Shaham S, Miska EA. 2010. The microRNA miR-124 controls gene expression in the sensory nervous system of Caenorhabditis elegans. Nucleic Acids Res 38:3780–3793.

Conaco C, Otto S, Han JJ, Mandel G. 2006. Reciprocal actions of REST and a microRNA promote neuronal identity. Proc Natl Acad Sci USA 103: 2422–2427.

Dong Q, Cai N, Tao T, Zhang R, Yan W, Li R, Zhang J, Luo H, Shi Y, Luan W, Zhang Y, You Y, Wang Y, Liu N. 2014. An Axis Involving SNAI1, microRNA-128 and SP1 Modulates Glioma Progression. PLoS ONE 9:e98651.

Finch ML, Marquardt JU, Yeoh GC, Callus BA. 2014. Regulation of microRNAs and their role in liver development, regeneration and disease. Int J Biochem Cell Biol.

Franke K, Otto W, Johannes S, Baumgart J, Nitsch R, Schumacher S. 2012. MiR-124-regulated RhoG reduces neuronal process complexity via ELMO/Dock180/Rac1 and Cdc42 signalling. EMBO J 31:2908–2921.

Gao FB. 2010. Context-dependent functions of specific microRNAs in neuronal development. Neural Dev 5:25.

Guttilla IK, White BA. 2009. Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. J Biol Chem 284:23204–23216.

Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. 2003. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 9:1274–1281.

Krichevsky AM, Sonntag KC, Isacson O, Kosik KS. 2006. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. Stem Cells 24:857–864.

Kuss AW, Chen W. 2008. MicroRNAs in brain function and disease. Curr Neurol Neurosci Rep 8:190–197.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. Curr Biol 12:735–739.

Lee RC, Feinbaum RL, Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854.

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23:4051–4060.

Liang R, Wang E. 2012. Full-length 3′-untranslated region reporter construction with recombineering. Anal Biochem 424:162–167.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769–773.

Liu NK, Xu XM. 2011. MicroRNA in central nervous system trauma and degenerative disorders. Physiol Genomics 43:571–580.

Maiorano NA, Mallamaci A. 2010. The pro-differentiating role of miR-124: Indicating the road to become a neuron. RNA Biol 7:528–533.

Makeyev EV, Zhang J, Carrasco MA, Maniatis T. 2007. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol Cell 27:435–448.

Mao XR, Moerman-Herzog AM, Chen Y, Barger SW. 2009. Unique aspects of transcriptional regulation in neurons–nuances in NFkappaB and Sp1-related factors. J Neuroinflammation 6:16.

Mao X, Yang SH, Simpkins JW, Barger SW. 2007. Glutamate receptor activation evokes calpain-mediated degradation of Sp3 and Sp4, the prominent Sp-family transcription factors in neurons. J Neurochem 100:1300–1314.

Meza-Sosa KF, Valle-Garcia D, Pedraza-Alva G, Perez-Martinez L. 2012. Role of microRNAs in central nervous system development and pathology. J Neurosci Res 90:1–12.

Nagata K, Hama I, Kiryu-Seo S, Kiyama H. 2014. MicroRNA-124 is down regulated in nerve-injured motor neurons and it potentially targets mRNAs for KLF6 and STAT3. Neuroscience 256:426–432.

Nelson PT, Baldwin DA, Kloosterman WP, Kauppinen S, Plasterk RH, Mourelatos Z. 2006. RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. RNA 12:187–191.

Neo WH, Yap K, Lee SH, Looi LS, Khandelia P, Neo SX, Makeyev EV, Su IH. 2014. MicroRNA miR-124 controls the choice between neuronal and astrocyte differentiation by fine-tuning Ezh2 expression. J Biol Chem 289:20788–20801.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36.

Qiu T, Zhou X, Wang J, Du Y, Xu J, Huang Z, Zhu W, Shu Y, Liu P. 2014. MiR-145, miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. FEBS Lett 588:1168–1177.

Schratt G, Greenberg M. 2008. MicroRNA function in the nervous system. In: Appasani K, editor. MicroRNAs: From basic science to disease biology. New York: Cambridge University Press.

Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 5:R13.

Smirnova L, Grafe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. 2005. Regulation of miRNA expression during neural cell specification. Eur J Neurosci 21:1469–1477.

Visvanathan J, Lee S, Lee B, Lee JW, Lee SK. 2007. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. Genes Dev 21:744–749.

Yu JY, Chung KH, Deo M, Thompson RC, Turner DL. 2008. MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation. Exp Cell Res 314:2618–2633.

Zhang R, Luo H, Wang S, Chen W, Chen Z, Wang HW, Chen Y, Yang J, Zhang X, Wu W, Zhang SY, Shen S, Dong Q, Zhang Y, Jiang T, Lu D, Zhao S, You Y, Liu N, Wang H. 2014. MicroRNA-377 inhibited proliferation and invasion of human glioblastoma cells by directly targeting specificity protein 1. Neuro Oncol.

Zou D, Chen Y, Han Y, Lv C, Tu G. 2014. Overexpression of microRNA-124 promotes the neuronal differentiation of bone marrow-derived mesenchymal stem cells. Neural Regen Res 9:1241–1248.

Zufferey R, Trono D. 2001. Production of high-titer lentiviral vectorseditor, Current protocols in human genetics. John Wiley & Sons, Inc.