Am J Transl Res 2020;12(9):5752-5761
www.ajtr.org /ISSN:1943-8141/AJTR0110501

Original Article

Let-7f promotes the differentiation of neural stem cells in rats

Zhenhan Deng[1,2], Yujia Wei[1,3], Yue Yao[1], Shanshan Gao[4], Xia Wang[1]

[1]Department of Pediatrics, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China; [2]Department of Sports Medicine, Department of Sports Medicine, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University Health Science Center, Shenzhen 518035, Guangdong, China; [3]Department of Pediatrics, The First Affiliated Hospital of South China University, Hengyang 421001, Hunan, China; [4]Department of Cardiology, University of Colorado Anschutz Medical Campus, Aurora 800045, CO, USA

Received March 8, 2020; Accepted July 19, 2020; Epub September 15, 2020; Published September 30, 2020

Abstract: Hypoxic-ischemic brain damage (HIBD) is the major recognized perinatal cause of neurological morbidity in full-term new borns. Neural stem cells (NSCs) have been extensively studied because of their clinical applications in treating neuro degenerative diseases and brain injuries, including HIBD, while microRNAs (miRNAs) are deemed critical regulators of the proliferation and differentiation of NSCs. However, the role of let-7f in NSC differentiation remains unknown. Our study aims to investigate the role of let-7f in the differentiation of NSCs and brain development in rats and hence to explore the therapeutic potential of let-7f in the treatment of HIBD. The quantitative real-time polymerase chain reaction (qRT-PCR) was applied to assess the expressions of let-7f, and western blot was performed to detect GFAP, Tuj1 and Nestin in rat brains at postnatal day 1, 8 and 14 (n=12 per time point). The NSCs isolated from the brains of rat fetuses at gestational day 15 were transduced with lenti virus expressing let-7f or let-7f inhibitor so as to observe altered expressions of let-7f, GFAP, Tuj1 and Nestin. A gradually-increasing expression of let-7f was detected by qRT-PCR in rat brain tissues during postnatal brain development. Increased levels of GFAP and Tuj1, while a decreased level of Nestin, were detected by western blot in let-7f-overexpressing NSCs. In contrast, the cells expressing the let-7f inhibitor exhibited lower levels of GFAP and Tuj1, while a higher level of Nestin, compared with control cells. Therefore, let-7f is involved in brain development and promotes the differentiation of NSCs in rats.

Keywords: Hypoxic-ischemic brain damage, miRNA, let-7, neural stem cells, brain development

Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) is a leading cause of death and perpetual neurological dysfunction in neonates. Currently, there is no effective clinical treatment for HIBD yet [1, 2]. Meanwhile, neural injuries involved in stroke or traumatic brain injuriescan also result in brain cell death, and under certain circumstances, the newly-generated neurons and astrocytes are not sufficient to replenish the lost brain cells [3, 4].

Neural stem cells (NSCs), which are found in the neonatal brain, are characterized by extensive self-renewal and pluripotent differentiation. Therefore, NSCs may provide a potential possibility of self-repair after HIBD [5]. However, the transplantation of NSCs might involve surgical risk, allograft rejection, ethical problems, as well as other disadvantages [6, 7]. Accordingly, various strategies for enhancing the differentiation of endogenous NSCs have been developed, among which microRNAs (miRNAs) have drawn the most attention for their gene regulation ability and multiple *in vivo* delivery approaches available [8]. Specifically, several miRNAs have been identified as key mediators of NSC differentiation and neurogenesis. Shi, et al. revealed that miR-381 could promote the proliferation and differentiation of NSCs to neurons [9]. Jiao, et al. showed that miR-124, especially those expressed in adult brain, stimulated the proliferation and differentiation of NSCs by suppressing the Notch signaling pathway [10].

The lethal-7 (let-7) gene was first discovered in nematode as a critical regulator of development [11]. The family members of let-7 are involved in a wide range of physiological and pathological processes including cell cycle arrest, cancer development and aging [12-14]. Let-7 microRNAs are highly expressed during embyogenesis in the brain [15, 16]. Lin28, an RNA-binding protein that binds to let-7 pre-microRNA and hinders the production of mature let-7, has been found to control the neuronal cell fate determination in postnatal neurogenesis [17]. Let-7 promotes the differentiation of neurons and Müller glia [18]. Moreover, the expressions of Let-7 family members let-7a and let-7b are up-regulated during neuronal differentiation [19]. At present, most of the existing studies on let-7f focus on its regulatory role in cell proliferation, metastasis and chemotherapeutic resistance in cancer [20-22]. However, whether let-7f is involved in neurogenesis stays elusive, although accumulating evidence indicates that let-7f microRNA might exerta certain effect on the differentiation of NSCs.

In view of this, our study aims to determine whether let-7f plays a rolein the differentiation of NSCs, in order to offer new angles on the therapy development for neonatal HIBD.

Methods and materials

Animals

The animal experiment was carried out in accordance with the currently applicable guidelines and regulations, and was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University. Sprague-Dawley (SD) rats provided by the Experimental Animal Center at Central South University were used for all the animal tests. Brain tissues from SD rats of different ages including Postnatal Day 1 (P1), 8 (P8) and 14 (P14) were used for protein and RNA extraction (n=12 per time point). For the isolation of NSCs, brain tissues of rat embryos from 15-daypregnant female rats were used.

Isolation and culture of NSCs

The protocol described in some of our previous publications was followed [19, 23]. Rat embryos were extracted aseptically from 15-daypregnant female rats and washed with sterile phosphate buffered saline (PBS). The scalp and skull of the rat were cut open carefully to take the brain tissue. Meninges and blood vessels on the brain surface were removed first, followed by cutting the brain tissue in a sterile petri dish. The 0.25% Trysin/ethylene diamine tetraacetic acid (EDTA) solution was used for digestion at 37°C for 15 minutes. Then the DMEM/F12 medium (GE Healthcare, Chicago, IL) supplemented with 10% fetal bovine serum (FBS), was added to stop the digestion reaction. The solution was pipetted up and down to obtain single suspended cells. The cell suspension was filtered through a 200-mesh filter and washed twice with the sterile DMEM/F12 medium, and then was resuspended and cultured in the DMEM/F12 medium which was supplemented with 20 ng/mL basic fibroblast growth factor (bFGF, Changsha Lixin Biotechnology Co. Ltd., China), 20 ng/mL epidermal growth factor (EGF, PeproTech), and 2% B27 (Beijing Dingguo Biotechnology Co. Ltd., China). The culture medium was changed every 2-3 days. To induce the differentiation of NSCs, the neurospheres were pipetted into small clumps of cells or single-cell suspension in DMEM/F12 (not containing bFGF, EGF or B27) supplemented with 2% FBS and then transferred onto poly-L-lysine coated cell culture slides for an incubation of 7 days at 37°C.

Immunofluorescence

The neurospheres of NSCs that had grown to around 200 μm in diameter were broken into smaller cell clusters or single cell suspension by pipetting up and down, and then were transferred onto poly-L-lysine coated slides or culture slides. For immunofluorescence on undifferentiated NSCs, the cells were incubated for 12 h at 37°C to allow attachment to the slides. For immunofluorescence on differentiated NSCs, the cells were cultured on culture slides for 7 days at 37°C. Then NSCs were fixed with 4% paraformaldehyde at room temperature (RT) and permeabilized with 0.1 Triton X-100, followed by blocking with 5% FBS and incubation with the following primary antibodies: rabbit anti-Nestin antibody (Abcam, Cambridge, UK; dilution 1:100), mouse anti-Tuj1 antibody (Beyotime Biotechnology; dilution 1:100), and rabbit anti-GFAP antibody (Boster Biological Technology; dilution 1:100). The following sec-

ondary antibodies were also applied: anti-rabbit IgG-TRITC (Boster Biological Technology; dilution 1:100), anti-mouse IgG-FITC (Sigma-Aldrich, St.Louis, MO; dilution 1:100), and anti-rabbit IgG-TRITC (Boster Biological Technology; dilution 1:100). The slides were mounted with glycerol and observed under fluorescence microscope.

Design of lentiviral expression constructs

For this study, the miRBase database access number of rno-let-7f is MI0000833. Based on the miRBase database, the rno-let-7f inhibitor sequence (CCGGAACTATACAATCTACTACCTCATTTTTG) and rno-let-7f pri-miRNA sequence (AUCAGAGUGAGGUAGUAGAUUGUAUAGUUGUGGGGUAGUGAUUUUACCCUGUUUAGGAGAUAACUAUACAAUCUAUUGCCUUCCCUGAG) were acquired. The lentiviral vectors GV159 (H1-MCS-CMV-EGFP, Genechem, China) and GV217 (Ubi-EGFP-MCS, Genechem, China) were used for the let-7f overexpression construct and let-7f inhibitor construct respectively. Double stranded oligonucleotides as let-7f-1 inhibitor were synthesized in the following sequence: 5'-TGAGGTA GTAGATTGTATAGTT-3' and 5'-AACTATACAATCTACTACCTCA-3'. The let-7f-1 sequence was chemically synthesized according to the sequence in the database. Then the lentiviral expression vectors containing either let-7f-1 microRNA sequence or let-7f-1 inhibitor sequence were co-transfected into 293T cells with lentiviral packaging plasmids pHelper 1.0 (Genechem, China) and pHelper 2.0 (Genechem, China), using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The titer of lentivirus produced by the transfected 293T cells was determined by performing serial dilution of viral stock in a 96-well plate. The viral stock was stored under -80°C.

Lentiviral infection of NSCs

One group of NSCs was suspended in the complete culture medium first and then transferred to T25 cell culture flasks. 250 μL of lentiviral solution with a titration of 1×10^8 TU/mL was added onto the cells, and infected cells were examined by fluorescence microscope to observe any fluorescence before/after differentiation. Another group of NSCs was suspended with the DMEM/F12 medium supplemented with 2% FBS, and then transferred to the culture flasks coated with poly-L-lysine to induce differentiation; subsequently, the cells were collected 7 days after viral infection.

Quantitative real-time polymerase chain reaction

The total RNA was extracted with the miRVana-miRNA isolation kit (Ambion, Austin, TX), and cDNA synthesis was performed on 500 ng of RNA using the NCode Vilo miRNA cDNA synthesis kit (Invitrogrn, Carlsbad, CA). Then, qRT-PCR for rno-let-7f was performed with SYBR-GreenER (Invitrogen) using a CFX PCR detection system (Bio-Rad, Hercules, CA) and RNU44 as an internal control. The relative expression of rno-let-7f was calculated by the 2-DDCt method [24].

Western blotting

The proteins extracted from tissues and cells with RIPA buffer (Life Technology, Carlsbad, CA) were separated on polyacrylamide gels, and transferred to PVDF membranes (Millipore, Burlington, MA). The membranes were then blocked with 5% nonfat milk in TBS-T (Tris-buffered saline, 0.1% Tween 20) at room temperature for 1 h. To detect the protein expression, the antibodiesspecific for Nestin (Abcam, Cambridge, UK; dilution 1:2000), Tuj1 (Beyotime Biotechnology; dilution 1:1000), GFAP (Boster Biological Technology; dilution 1:2000), and β-actin (Abgent, San Diego, CA; dilution 1:5000) were used. Thereafter, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody, while signals were developed using the enhanced chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

Data was analyzed with SPSS 13.0 software and presented as mean ± SD. The Student's t-test was applied to perform statistical comparisons between the two groups. One-way analysis of variance was conducted to assess the differences between multiple groups. $P< 0.05$ was considered statistically significant.

Results

Characterization of NSCs in culture

The NSCs isolated from prenatal rat brains formed clonal structures *in vitro,* which are

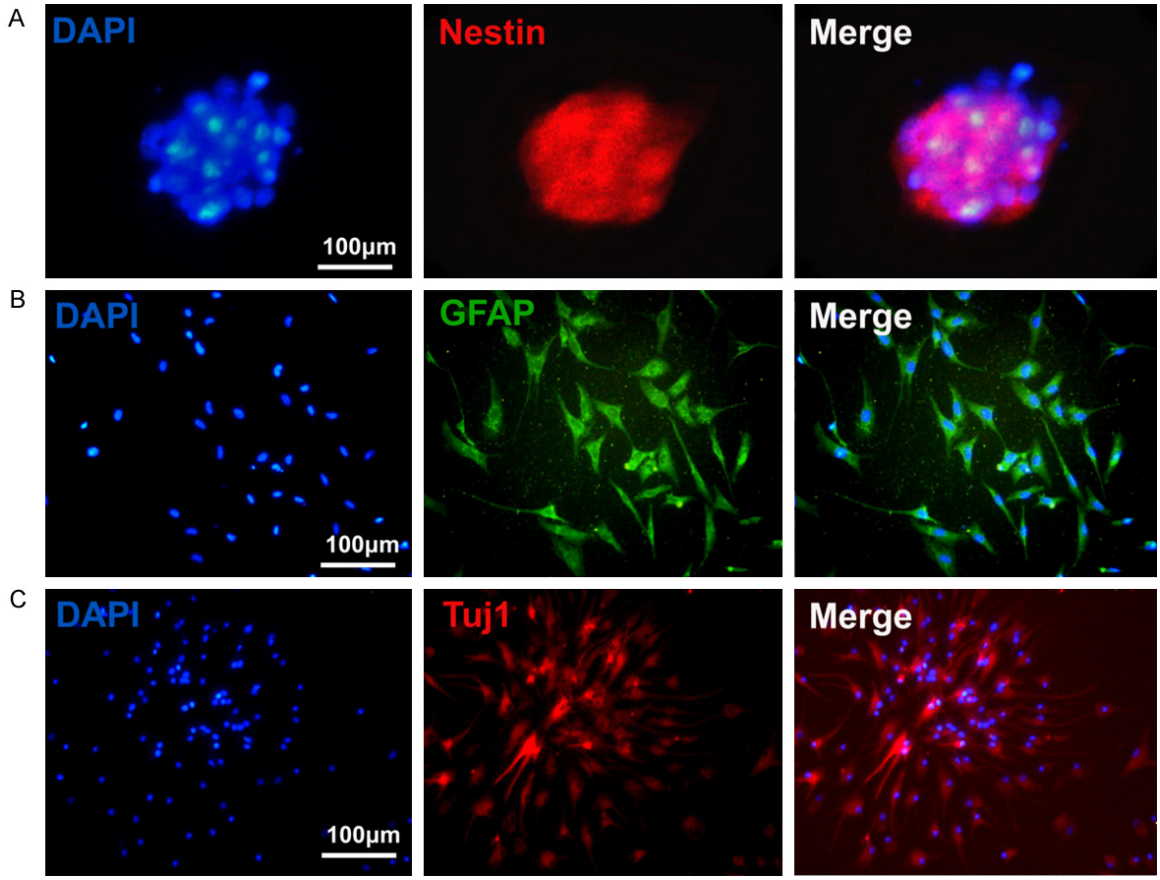

Figure 1. In vitro differentiation of NSCs. Immunofluorescence staining of adherent neurospheres showed positive staining for Nestin (A). Immunofluorescence staining of differentiated NSCs showed positive staining for astrocyte marker GFAP (B) and neuron marker Tuj1 (C). Cell nuclei were counterstained with DAPI (blue). DAPI, 4',6-diamidino-2-phenylindole.

known as neuroshperes. The immunofluorescence staining of undifferentiated neurospheres showed positive signal of Nestin, a neural stem/progenitor cell marker (**Figure 1A**). After 7 days of incubation in the differentiation medium, cells were stained positive for GFAP (**Figure 1B**) and Tuj1 (**Figure 1C**), suggesting that these NSCs possessed the capability to differentiate into both neural and glial lineage.

The expression level of let-7f is associated with the postnatal brain development in rats

Proteins and RNA were extracted from the brain tissues that were collected from P1, P8 and P14 animals. Then western blot was performed to detect the protein expressions of neural progenitor cell marker Nestin, astrocyte marker GFAP and neuron marker Tuj1. We observed a decrease in signal, which was indicated by the Nestin-specific antibody, with maturation. This suggests a reduced number of NSCs in postnatal brain development progresses. Additionally, we observed increased signals of GFAP and Tuj1, implying more astrocyte and neuron differentiation with maturation (**Figure 2A**). The expression of let-7f manifested by qRT-PCR was found to be upregulated during postnatal brain development (**Figure 2B**), indicating detectable expression of let-7f in the rat brain and the association of let-7f expression level with brain development.

The expression level of let-7 is related to the differentiation of NSCs

NSCs were isolated from rat embryos at embryonic day 15 (E15), and were cultured in the DMEM/F12 medium containing B27, bFGF, and EGF for 7 days to obtain neurospheres. Then the NSCs were plated onto poly-L-lysine coated flasks and cultured in the differentiation medi-

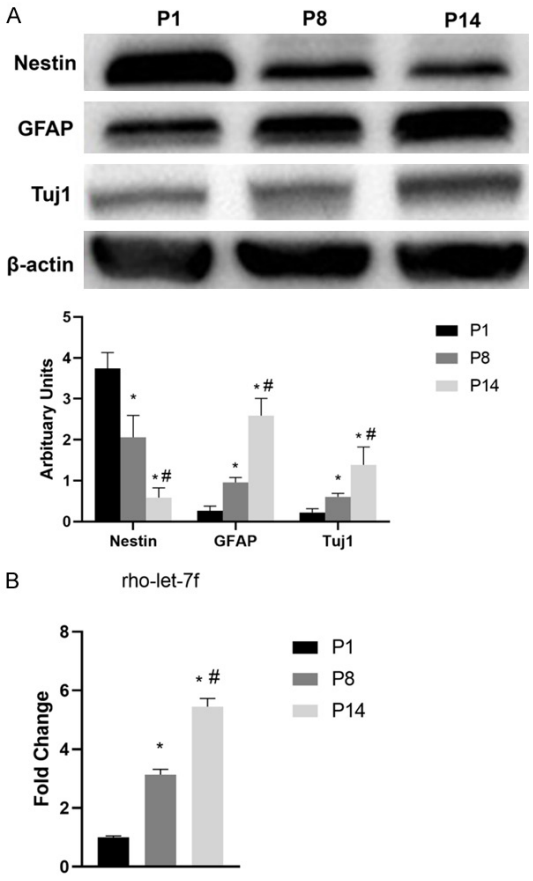

Figure 2. The increase of let-7f expression level is age-dependent. A. Representative western blotting showing the protein expressions of Nestin, GFAP and Tuj1 in brains of rats at three different ages (Postnatal Day 1 (P1), P8 and P14), with densitometric analysis reflecting the expression levels of Nestin, GFAP and Tuj1. B. Let-7f expression level detected by qRT-PCR in brains of P1, P8 and P14 rats. Data shown as mean ± SD. **P*<0.05 vs. P1; #*P*<0.05 vs. P8. Data representative of three independent experiments.

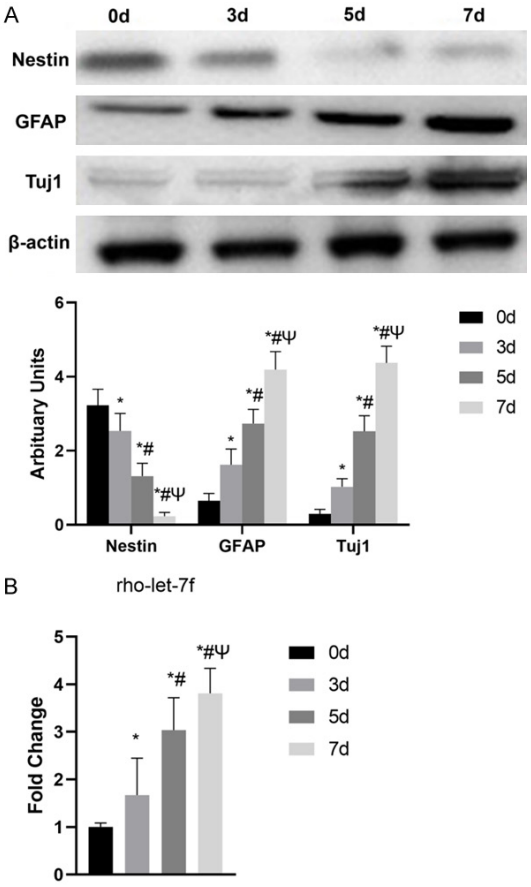

Figure 3. The increase of let-7f expression level is associated with differentiation of NSCs *in vitro*. A. Representative western blotting showing the protein expressions of Nestin, GFAP and Tuj1 following the induction of NSCs on days 0, 3, 5 and 7, with densitometric analysis reflecting the expression levels of Nestin, GFAP and Tuj1. B. Let-7f expression level detected in NSCs subject to differentiation on days 0, 3, 5 and 7 by qRT-PCR. Data shown as mean ± SD. **P*<0.05 vs. 0 d; #*P*<0.05 vs. 3 d; Ψ*P*<0.05 vs. 5 d. Data representative of three independent experiments.

um for up to 7 days to induce differentiation. The cells at day 0, 3, 5 and 7 of differentiation were collected for RNA and protein extraction. Western blot was applied to detect the protein expressions of Nestin, GFAP and Tuj. A gradually-decreasing signal of Nestin during differentiation was observed (**Figures 3A**, S1 and S2), suggesting the reduced population of NSCs in differentiation progresses. Moreover, stronger bands that are correlated with higher protein expression levels of GFAP and Tuj1 were also observed during differentiation (**Figure 3A**), indicating that an increasing population of cells has differentiated into astrocytes and neurons. The expression of let-7f manifested by qRT-PCR showed a significant increasing trend of let-7f level across ages (**Figures 3B**, S1-4), implying an association between let-7f expression and the differentiation of NSCs.

Leverage of let-7 expression level has an impact on the differentiation of NSCs

Lentiviral constructs harboring let-7f pre-miRNA or let-7f inhibbitor were sequenced to confirm successful cloning (Figure S5). For viral infection, NSCs were incubated with lentivirus carrying the empty GV159 vector, or with lentivirus containing either let-7f (let-7f mimic) or let-7f inhibitor in the complete medium for 3 days.

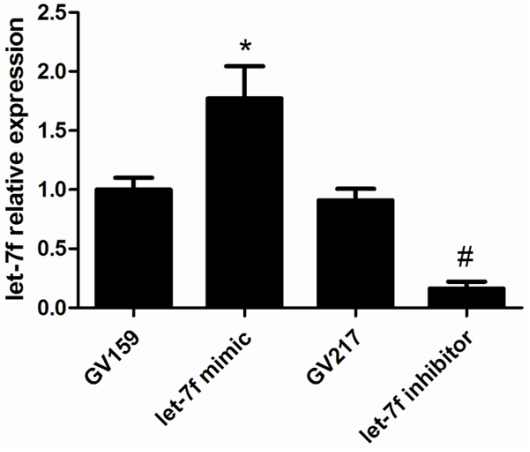

Figure 4. Detection of let-7f expression in NSCs. The expression level of let-7f in NSCs following lentiviral infection was evaluated with qRT-PCR. Data shown as mean ± SD. **P*<0.05 vs. GV159; #*P*<0.05 vs. GV217.

A positive signal of green fluorescence was observed in over 80% of cells following lentiviral infection, suggesting successful and efficient lentiviral infection of NSCs. The expression level of let-7f in NSCs transduced with lentivirus was detected by qRT-PCR 3 days after lentiviral infection. A significant increase of let-7f was observed in the let-7f mimic group of cells compared to the GV159 group, while drastic suppression was observed in the let-7f inhibitor group of cells compared to the GV217 group (**Figure 4**). The complete culture medium was then replaced with the differentiation medium so as to differentiate the NSCs for 7 days, with the fluorescence being detected under microscope 7 days post differentiation (**Figure 5A**). Cell lysis was obtained either from undifferentiated NSCs (for Nestin detection) or at the endpoint of differentiation (for GFAP and Tuj1 detection). Then western blot was performed to detect the protein expressions of Nestin, GFAP and Tuj1. A lower expression of Nestion was observed in the NSCs infected with let-7f mimic lentivirus compared to the other groups, indicating that the pluripotency or "stemness" of NSCs was decreased as a response to let-7f overexpression. On the contrary, the cells infected with lentivirus carrying let-7f inhibitor exhibited the lowest expressionof Nestin among all the groups (**Figures 5B**, S1 and S2). Hence, we speculate that the suppression of let-7f microRNA might enhance the pluripotency of NSCs. It was also noticed that the astrocyte marker GFAP and the neuron marker Tuj1 expressions were drastically elevated in the differentiated NSCs with let-7f overexpressed compared to the other groups. This implies that let-7f might potentially enhance the differentiation of NSCs into astrocytes and neurons. Inhibition of let-7f, conversely, lowered the expressions of GFAP and Tuj1 in the differentiated NSCs (**Figures 5B**, S1-4). These findings suggest that the astrocyte and neuron differentiations of NSCs are regulated by let-7f.

Discussion

Regeneration of the nervous system has been a research hotspot for the past few years. The exploration for new targets involved in nervous regeneration has led to the discovery of multiple novel potential therapies for nervous repair. In our study, we have identified let-7f as a novel microRNA regulator of NSC differentiation as well as brain development in rats. The results from our study may provide clues for future clinical intervention in terms of neural regeneration.

We observed a gradual increase of let-7f expression in the process of brain development in rats. Based on previous studies, several members from the let-7 microRNA family have been shown to be critical players in cell fate determination and development. For instance, let-7d induced by hypoxia is involved in the differentiation of microvascular pericytes [25]; let-7g is implicated in mammary cell differentiation in mice [26]; let-7f is upregulated during the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) and is shown to affect the expression levels of multiple osteogenic markers such as Runx2 and Osteocalcin [27]. In terms of neural differentiation and brain development, let-7a and let-7b can enhance the differentiation of NSCs [28, 29], but no study has yet explored the association between let-7f and brain development or NSC differentiation. In our experiment, the brain tissues from postnatal rats (P1, P8 and P14) were used for the detection of let-7f expression by considering the fact that the maturation of neurons continues postnatally and turns out to be the most pronounced between P7 and P14 in rats [30]. Additionally, Nixdorf-Bergweiler, et al. revealed the changes in the number and sizes of GFAP-positive cells in rat

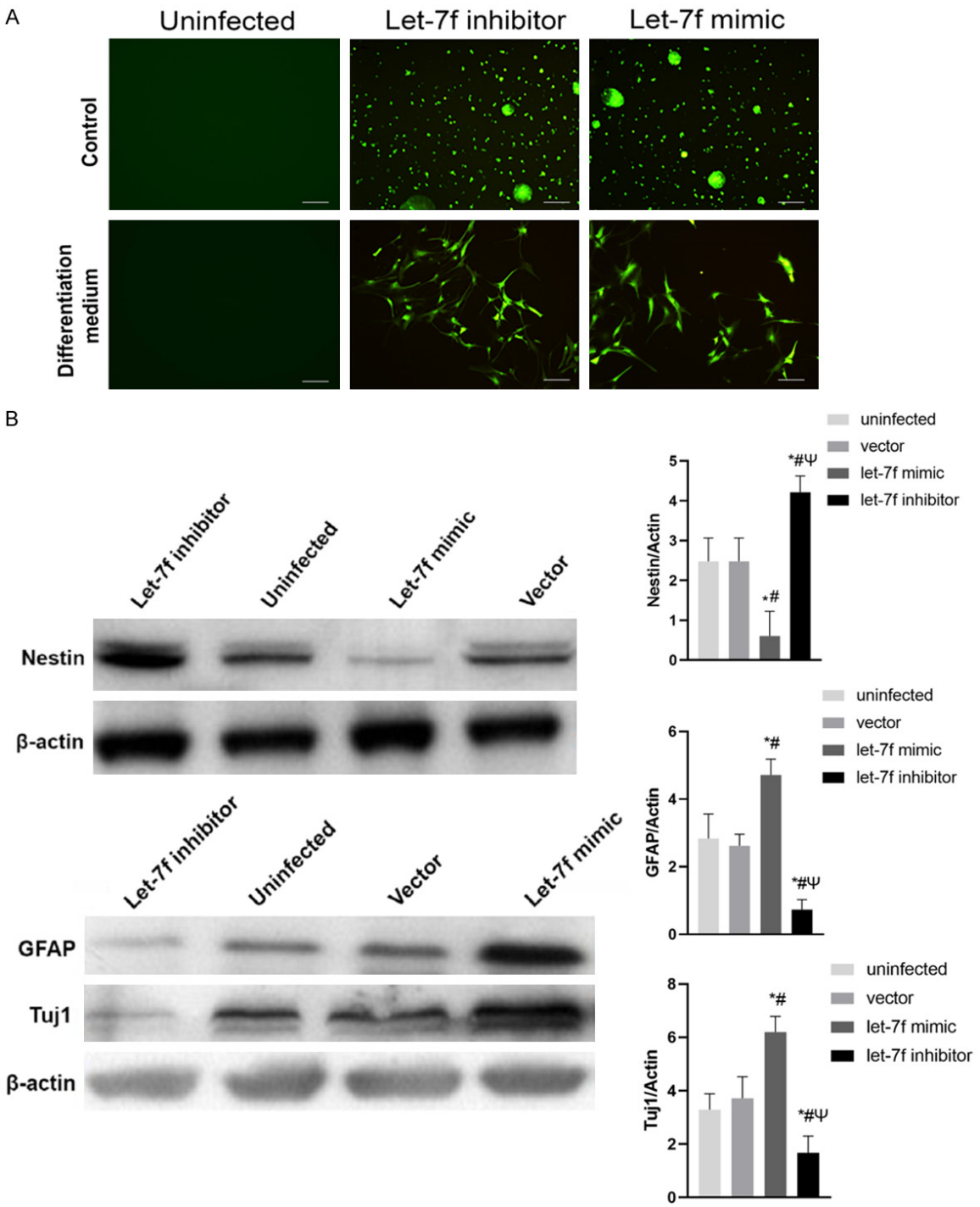

Figure 5. Leverage of let-7 expression level has an impact on the differentiation of NSCs. A. Expression of EGFP was observed in transduced cells under fluorescence microscope. Uninfected cells were used as controls. B. Representative western blotting showing the expressions of Nestin, GFAP and Tuj1 in uninfected and infected differentiated NSCs, with densitometric analysis reflecting the expression levels of Nestin, GFAP and Tuj1. Data shown as mean ± SD. *P<0.05 vs. uninfected; #P<0.05 vs. vector; ΨP<0.05 vs. let-7f mimic. Data representative of three independent experiments.

hippocampus between P8 and P16, indicating the postnatal maturation of astrocytes in the brain [31]. We detected elevated expressions of GFAP and Tuj1 in postnatal rat brains in an

age-dependent manner, which is consistent with previous findings. Furthermore, we also observed induced expression of let-7f during postnatal brain development, which has never been published before to our best knowledge. Several let-7 family members are undetectable in early embryos and embryonic stem cells, but are highly expressed in most adult organs, including brain [32].

In the following experiments, we differentiated rat-derived NSCs in culture based on a well-established protocol and observed enhanced expressions of GFAP, Tuj1 as well as let-7f in the differentiated NSCs compared to undifferentiated cells [33]. The knockdown of let-7f was shown to suppress the expressions of Tuj1 and GFAP, while the overexpression of let-7f upregulated the expressions of Tuj1 and GFAP during the induced differentiation of NSCs. We did not identify any let-7f targets that are involved in neurogenesis in our current study, but via the microRNA target prediction software miRDB, we managed to obtain a list of potential target genes of let-7f (data not shown). Among the potential let-7f targets, the RNA-binding protein LIN28 has been shown to promote the proliferation/self-renewal of neural progenitor cells (NPCs) [34]. The level of LIN28 decreases during neural differentiation and constitutive expression of LIN28 in progenitor cells residing in the subventricular zone (SVZ) of mice leads to reduction in numbers of neurons and astrocytes [17]. Coilin, another potential target of let-7, serves as a component of the Cajal bodies [35]. It is present in abundance in the adult brain and has an impact on neurite overgrowth as well as on neuronal differentiation [36]. Therefore, let-7f may regulate the differentiation of NSCs by targeting LIN28 or Coilin. Consequently, identification and confirmation of the specific targets of let-7f will be crucial to unraveling the underlying mechanisms of how let-7f enhances the differentiation of NSCs in the future.

In the present study, we observed that the overexpression of let-7f is conducive to the differentiation of NSCs into both neurons and astrocytes. However, it is noteworthy that increased differentiation of NSCs into astrocytes does not always facilitate the repair process of the nervous system, especially the central nervous system (CNS). The CNS, comprised of the brain and spinal cord, is generally considered incapable of extensive regeneration, partially due to the fact that glial scars are formed immediately following injury, creating a physical and chemical barrier against neuron regeneration [37, 38]. The glia in the CNS mainly consists of astrocytes, oligodendrocytes, microglia, and ependymal cells. The increased differentiation, proliferation, migration and phenotypic changes of astrocytes all contribute to the astrocyte accumulation in the damaged area of CNS. Therefore, the enhanced differentiation of NSCs into astrocytes, mediated by let-7f, may lead to faster glial scar formation in response to the injury, which can in turn inhibit the regeneration of neurons and eventually results in impaired recovery. Moreover, the complexity of CNS and the difficulty of replacing the injured or lost neurons with functionally identical cells also deserve particular consideration [39]. Although we have shown that let-7f promotes the neuronal differentiation in our study, we lack *in vivo* data to prove that the differentiated neurons obtained in culture are fully functional and able to at least partially restore the injured nervous system in animal models. In view of the concerns mentioned above, it appears critical to perform further research in order to decide the appropriate way and time to administrate let-7f *in vivo*. It is also important to consider combining other therapeutic applications with let-7f to optimize the healing effect on nervous damage.

Conclusions

Our results demonstrate that let-7f is associated with brain development and promotes the differentiation of NSCs into neurons and astrocytes in rats. This study unravels novel potential therapeutic targets involved in neural differentiation which may be applied clinically in the future. Nevertheless, thorough investigations are needed to elucidate the mechanisms underlying the regulation of neural differentiation by let-7f. Meanwhile, more *in vivo* data is also required to address the specific functions of let-7f in brain development and neural regeneration.

Acknowledgements

This research was funded by National Natural Science Foundation of China (30801246, 81-902303) and Guangdong Basic and Applied

Basic Research Foundation (2020A151501-048).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xia Wang, Department of Pediatrics, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China. Tel: +86-13574839428; E-mail: xiawang@csu.edu.cn

References

[1] Zheng XR, Zhang SS, Yin F, Tang JL, Yang YJ, Wang X and Zhong L. Neuroprotection of VEGF-expression neural stem cells in neonatal cerebral palsy rats. Behav Brain Res 2012; 230: 108-115.

[2] Zhang XY, Yang YJ, Xu PR, Zheng XR, Wang QH, Chen CF and Yao Y. The role of β-catenin signaling pathway on proliferation of rats neural stem cells after hyperbaric oxygen therapy in vitro. Cell Mol Neurobiol 2011; 31: 101-109.

[3] Puig B, Brenna S and Magnus T. Molecular communication of a dying neuron in stroke. Int J Mol Sci 2018; 19: 2834.

[4] Sato M, Chang E, Igarashi T and Noble LJ. Neuronal injury and loss after traumatic brain injury: time course and regional variability. Brain Res 2001; 917: 45-54.

[5] Yao Y, Zheng XR, Zhang SS, Wang X, Yu XH, Tan JL and Yang YJ. Transplantation of vascular endothelial growth factor-modified neural stem/progenitor cells promotes the recovery of neurological function following hypoxic-ischemic brain damage. Neural Regen Res 2016; 11: 1456-1463.

[6] Tang Y, Yu P and Cheng L. Current progress in the derivation and therapeutic application of neural stem cells. Cell Death Dis 2017; 8: e3108.

[7] Fainstein N and Ben-Hur T. Brain region-dependent rejection of neural precursor cell transplants. Front Mol Neurosci 2018; 11: 136.

[8] Stappert L, Roese-Koerner B and Brustle O. The role of microRNAs in human neural stem cells, neuronal differentiation and subtype specification. Cell Tissue Res 2015; 359: 47-64.

[9] Shi X, Yan C, Liu B, Yang C, Nie X, Wang X, Zheng J, Wang Y and Zhu Y. miR-381 regulates neural stem cell proliferation and differentiation via regulating hes1 expression. PLoS One 2015; 10: e0138973.

[10] Jiao S, Liu Y, Yao Y and Teng J. miR-124 promotes proliferation and differentiation of neuronal stem cells through inactivating Notch pathway. Cell Biosci 2017; 7: 68.

[11] Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB and Bartel DP. The microRNAs of caenorhabditis elegans. Genes Dev 2003; 17: 991-1008.

[12] Han Z, Li Q, Wang Y, Wang L, Li X, Ge N, Wang Y and Guo C. Niclosamide induces cell cycle arrest in G1 phase in head and neck squamous cell carcinoma through Let-7d/CDC34 axis. Front Pharmacol 2018; 9: 1544.

[13] Li XX, Di X, Cong S, Wang Y and Wang K. The role of let-7 and HMGA2 in the occurrence and development of lung cancer: a systematic review and meta-analysis. Eur Rev Med Pharmacol Sci 2018; 22: 8353-8366.

[14] Gendron CM and Pletcher SD. MicroRNAs mir-184 and let-7 alter Drosophila metabolism and longevity. Aging Cell 2017; 16: 1434-1438.

[15] Colas AR, McKeithan WL, Cunningham TJ, Bushway PJ, Garmire LX, Duester G, Subramaniam S and Mercola M. Whole-genome microRNA screening identifies let-7 and mir-18 as regulators of germ layer formation during early embryogenesis. Genes Dev 2012; 26: 2567-2579.

[16] Pena JT, Sohn-Lee C, Rouhanifard SH, Ludwig J, Hafner M, Mihailovic A, Lim C, Holoch D, Berninger P, Zavolan M and Tuschl T. miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. Nat Methods 2009; 6: 139-141.

[17] Romer-Seibert JS, Hartman NW and Moss EG. The RNA-binding protein LIN28 controls progenitor and neuronal cell fate during postnatal neurogenesis. FASEB J 2019; 33: 3291-3303.

[18] Xia X and Ahmad I. let-7 microRNA regulates neurogliogenesis in the mammalian retina through Hmga2. Dev Biol 2016; 410: 70-85.

[19] Morgado AL, Rodrigues CM and Solá S. MicroRNA-145 regulates neural stem cell differentiation through the Sox2-Lin28/let-7 signaling pathway. Stem Cells 2016; 34: 1386-1395.

[20] Tie Y, Chen C, Yang Y, Qian Z, Yuan H, Wang H, Tang H, Peng Y, Du X and Liu B. Upregulation of let-7f-5p promotes chemotherapeutic resistance in colorectal cancer by directly repressing several pro-apoptotic proteins. Oncol Lett 2018; 15: 8695-8702.

[21] Liang S, He L, Zhao X, Miao Y, Gu Y, Guo C, Xue Z, Dou W, Hu F, Wu K, Nie Y and Fan D. MicroRNA let-7f inhibits tumor invasion and metastasis by targeting MYH9 in human gastric cancer. PLoS One 2011; 6: e18409.

[22] Ghanbari R, Mosakhani N, Sarhadi VK, Armengol G, Nouraee N, Mohammadkhani A, Khorrami S, Arefian E, Paryan M, Malekzadeh R and Knuutila S. Simultaneous underexpression of let-7a-5p and let-7f-5p microRNAs in plasma and stool samples from early stage colorectal

carcinoma. Biomark Cancer 2016; 7 Suppl 1: 39-48.
[23] Tan J, Zheng X, Zhang S, Yang Y, Wang X, Yu X and Zhong L. Response of the sensorimotor cortex of cerebral palsy rats receiving transplantation of vascular endothelial growth factor 165-transfected neural stem cells. Neural Regen Res 2014; 9: 1763-1769.
[24] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.
[25] Esen N, Vejalla A, Sharma R, Treuttner JS and Dore-Duffy P. Hypoxia-induced Let-7d has a role in pericyte differentiation. Adv Exp Med Biol 2016; 923: 37-42.
[26] Tian L, Li Y, Wang C and Li Q. Let-7g-5p regulates mouse mammary cells differentiation and function by targeting PRKCA. J Cell Physiol 2019; 234: 10101-10110.
[27] Shen GY, Ren H, Shang Q, Zhao WH, Zhang ZD, Yu X, Huang JJ, Tang JJ, Yang ZD, Liang D and Jiang XB. Let-7f-5p regulates TGFBR1 in glucocorticoid-inhibited osteoblast differentiation and ameliorates glucocorticoid-induced bone loss. Int J Biol Sci 2019; 15: 2182-2197.
[28] Song J, Cho KJ, Oh Y and Lee JE. Let7a involves in neural stem cell differentiation relating with TLX level. Biochem Biophys Res Commun 2015; 462: 396-401.
[29] Zhao C, Sun G, Li S, Lang MF, Yang S, Li W and Shi Y. MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. Proc Natl Acad Sci U S A 2010; 107: 1876-1881.
[30] Radic T, Friess L, Vijikumar A, Jungenitz T, Deller T and Schwarzacher SW. Differential postnatal expression of neuronal maturation markers in the dentate gyrus of mice and rats. Front Neuroanat 2017; 11: 104.
[31] Nixdorf-Bergweiler BE, Albrecht D and Heinemann U. Developmental changes in the number, size, and orientation of GFAP-positive cells in the CA1 region of rat hippocampus. Glia 1994; 12: 180-195.
[32] Schulman BR, Esquela-Kerscher A and Slack FJ. Reciprocal expression of lin-41 and the microRNAs let-7 and mir-125 during mouse embryogenesis. Dev Dyn 2005; 234: 1046-1054.
[33] Chojnacki A and Weiss S. Production of neurons, astrocytes and oligodendrocytes from mammalian CNS stem cells. Nat Protoc 2008; 3: 935-940.
[34] Yang M, Yang SL, Herrlinger S, Liang C, Dzieciatkowska M, Hansen KC, Desai R, Nagy A, Niswander L, Moss EG and Chen JF. Lin28 promotes the proliferative capacity of neural progenitor cells in brain development. Development 2015; 142: 1616-1627.
[35] Hebert MD, Szymczyk PW, Shpargel KB and Matera AG. Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. Genes Dev 2001; 15: 2720-2729.
[36] Forthmann B, van Bergeijk J, Lee YW, Lubben V, Schill Y, Brinkmann H, Ratzka A, Stachowiak MK, Hebert M, Grothe C and Claus P. Regulation of neuronal differentiation by proteins associated with nuclear bodies. PLoS One 2013; 8: e82871.
[37] Fawcett JW and Asher RA. The glial scar and central nervous system repair. Brain Res Bull 1999; 49: 377-391.
[38] Yiu G and He Z. Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 2006; 7: 617-627.
[39] Llinas RR. Intrinsic electrical properties of mammalian neurons and CNS function: a historical perspective. Front Cell Neurosci 2014; 8: 320.

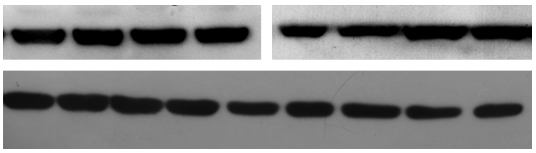

Figure S1. Representative original western images for β-actin including the whole membranes.

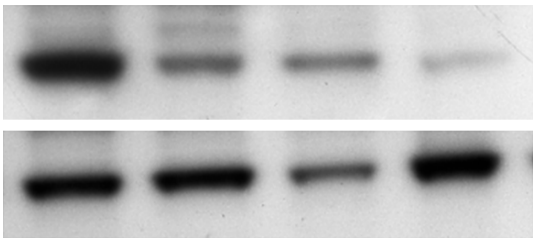

Figure S2. Representative original western images for Nestin including the whole membranes.

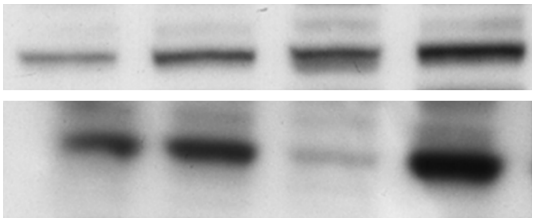

Figure S3. Representative original western images for GFAP including the whole membranes.

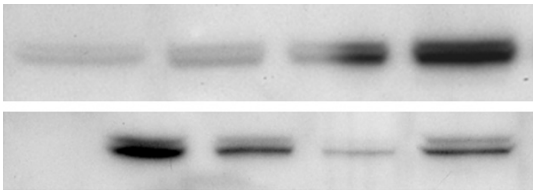

Figure S4. Representative original western images for Tuj1 including the whole membranes.

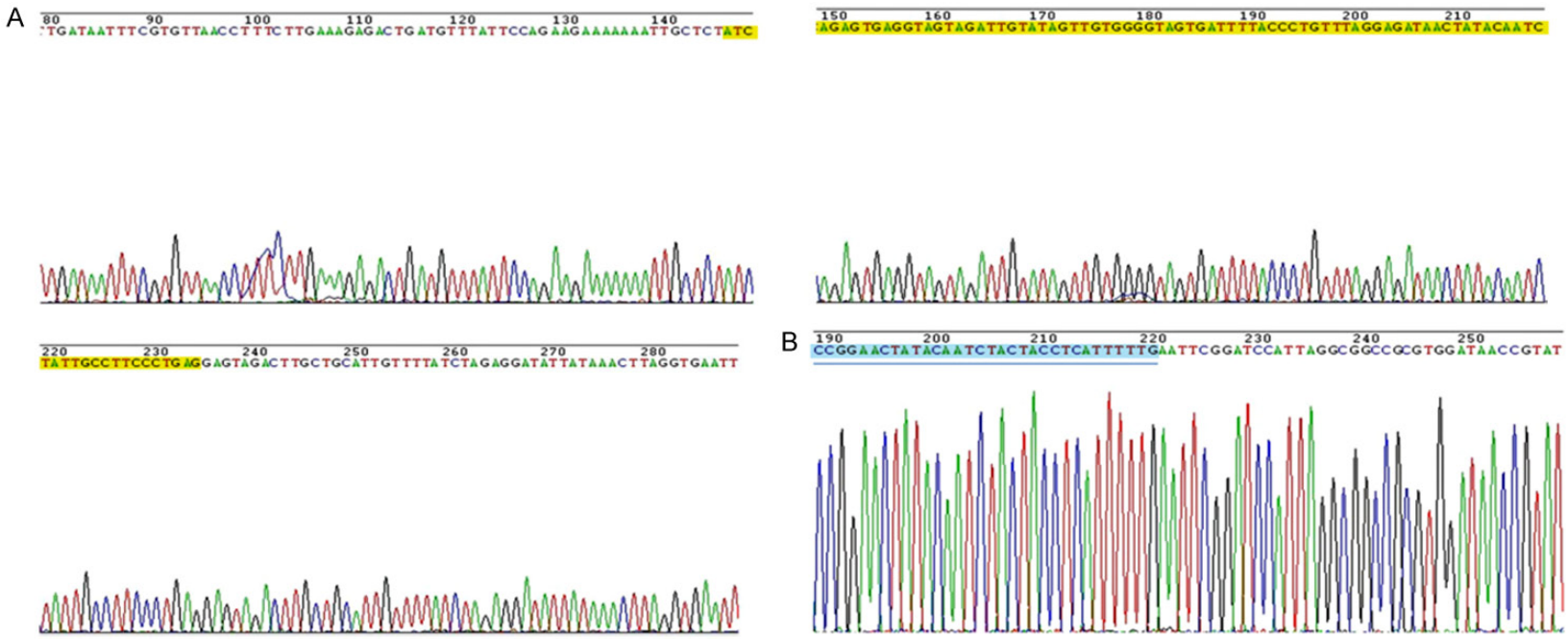

Figure S5. Sequence analysis of the lentiviral constructs carrying let-7f mimic or let-7f inhibitor. The sequence of let-7f pri-miRNA inserted into the vector GV129 is highlighted in yellow (A). The sequence of let-7f inhibitor inserted into the vector GV217 is highlighted in light blue (B).