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Let7a involves in neural stem cell differentiation relating with TLX level

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ABSTRACT

Neural stem cells (NSCs) have the potential for differentiation into neurons known as a groundbreaking therapeutic solution for central nervous system (CNS) diseases. To resolve the therapeutic efficiency of NSCs, recent researchers have focused on the study on microRNA's role in CNS. Some micro RNAs have been reported significant functions in NSC self-renewal and differentiation through the post-transcriptional regulation of neurogenesis genes. MicroRNA-Let7a (Let7a) has known as the regulator of diverse cellular mechanisms including cell differentiation and proliferation. In present study, we investigated whether Let7a regulates NSC differentiation by targeting the nuclear receptor TLX, which is an essential regulator of NSC self-renewal, proliferation and differentiation. We performed the following experiments: western blot analysis, TaqMan assay, RT-PCR, and immunocytochemistry to confirm the alteration of NSCs. Our data showed that let7a play important roles in controlling NSC fate determination. Thus, manipulating Let-7A and TLX could be a novel strategy to enhance the efficiency of NSC's neuronal differentiation for CNS disorders.

1. Introduction

miRNAs as short noncoding RNAs that are approximately 22 nucleotides (nt) in length act as the regulator of the specific gene's expression [1,2]. Several researches reported that miRNAs could recognize a lot of targets, and could regulates many cellular processes [3], the cellular homeostasis [3] and could act as the mediation of cell fate decisions by controlling transcription regulators [4]. Numerous miRNAs expressed in the central nervous system (CNS) are dynamically controlled during neural differentiation of stem cells, indicating a considerable contribution to neural function [5,6]. Micro RNA Let-7A (Let7a) as a member of Let-7 miRNA family that consists of 12 different members [7] has been reported the highest expression in neural stem cells (NSCs) [8]. Let-7A induces neuronal differentiation in NSCs by targeting Lin28A and Lin28B [9–11] and contributes to the maintenance of the NSC self-renewal [12,13]. Specifically, Let-7 has been known to target TLX as a transcription factor that is expressed in early embryonic development and adult stem cell niches [14]. TLX is an important target in neural development by regulating cell cycle progression in NSCs [15] by recruiting histone deacetylases [16]. TLX maintains stem cell self-renewal and regulates the neurogenesis in the cortex [17]. In *in vivo* study using TLX transgenic mouse, the overexpression of TLX results in the increase of NSC self-renewal in the subventricular zone (SVZ) [18]. One study reported that TLX activates Wnt signaling to promote adult NSC proliferation and self-renewal [19]. Another study indicated that the TLX-positive cells in the hippocampal dentate gyrus are important in spatial learning and memory [20]. During the differentiation of adult NSCs, TLX has been reported that it is downregulated by several members of the Let-7 family such as Let-7b [21]. For CNS development, Let-7b also regulates target genes related to the nuclear receptor TLX as the cell cycle controller in NSCs [22]. Moreover, Let-7 suppresses the proliferation of NSCs through TLX inhibition and promotes neural and glial differentiation [22,23]. In present study, we focused on the mechanisms of action of Let7a functional implications during the transition from NSCs and neural progenitors to differentiated neurons. Especially, we investigated that Let-7a has the potential as TLX regulator resulting the neuronal differentiation and self-renewal of NSCs.

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2. Method and materials

2.1. NSC primary culture

Pregnant ICR mice were euthanized to obtain cortex NSC primary cultures according to the method described by Gritti et al. [24]. Embryonic pup brains were extracted from the ICR mice (E13.5) and placed in a Petri dish containing Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY, USA), and each cortex was dissected and washed 1–2 times with plain HBSS. After dissociating the tissues, supernatants were transferred to a fresh tube and centrifuged at 1000 g for 5min. Pellets were resuspended in NSC basal media with a proliferation supplement (Stem Cell Technologies, CA, USA), 20 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA, USA). The NSCs were plated on poly-D-lysine-treated plastic ware at density of 2.5×10^4 cells/mL. After 3 days of culture, the cells proliferated and formed primary neurospheres. The primary neurospheres were dissociated using Accumax (Sigma, St. Louis, MO, USA) into single cells. The single cells were seeded in culture plates precoated with 0.001% poly L ornithine (Sigma, St. Louis, MO, USA). In the differentiation group, NSCs were cultured in the differentiation media (Stem Cell Technologies, CA, USA), and were incubated for 5 days. NSCs of 2–3 passages were used for experiments [25].

2.2. miR-Let7A mimics and inhibitor treatment

Mimics and inhibitor of miRNA and siRNA were purchased from Ambion (Ambion, Austin, TX, USA) and they were negative control (cat #, 4464058), positive control (cat#, 4464062), mmu-let-7a-5p (cat#, 4464066), and Let7a inhibitor (cat#, 4464066; assay ID, MH10050). For the transfection of RNA duplexes, a 20 nM final concentration in Opti-MEM of Let-7A miRNA mimics and Let7a inhibitor, and each control for miRNA or siRNA was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in serum-free media and incubated at room temperature (RT) for 10 min. The mixture was then added at the time when the NSCs were subcultured into the next passage (third passage) and incubated for 72 h to form a neurosphere resulting from proliferation or to initiate differentiation. The cells were harvested to the transfection for total protein or RNA extraction.

2.3. Reverse transcription-PCR (RT-PCR)

To examine the expression of TLX, SOX2, and c-Myc in NSCs, RT-PCR was performed using each primer. Briefly, samples were lysed with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's protocol. cDNA synthesis from mRNA and sample normalization were performed. PCR was performed using the following thermal cycling conditions: 10 min at 95 °C; 35 cycles of denaturing at 95 °C for 15 s, annealing for 30 s at 70 °C, elongation at 72 °C for 30 s; final extension for 10 min at 72 °C, and held at 4 °C. PCR was performed using the following primers (5′ to 3′); TLX: forward (F): GGC TCT CTA CTT CCG TGG ACA, reverse (R): GTC AGT ATT CAT GCC AGA TAC AGC CAG TG, SOX2 (F): CCC CCG GCG GCA ATA GCA, (R) TCG GCG CCG GGG AGA TAC AT, c-Myc (F): TCA AGA GGC GAA CAC ACA AC, (R): GGC CTT TTC ATT GTT TTC CA, GAPDH (F): GGCATGGACTGTGGTCATGAG, (R): TGCACCACCAACTGCTTAGC. PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide.

2.4. Real Time qRT-PCR (SYBR and TaqMan assay)

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For quantitative analysis of miR-Let7-A, reverse transcription (RT) was first performed using TaqMan Micro RNA Reverse Transcription kit (Takara, Otsu, Shiga, Japan) according to manufacturer's instructions with total RNA at 10 ng. PCR reactions were then performed according to manufacturer's instructions to quantitate the expression levels of Let7a using TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems, Foster City, CA,USA), and the PCR amplification was performed in ABI 7500 Real Time PCR (Bio Rad, Philadelphia, PA, USA). The PCR forward and reverse primers for Let7a were 5′-GCGCCTGAGGTAGTA GGTTG-3′ and 5′-CAGT GCAGGGTCCGAGGT-3′. The PCR forward and reverse primers for U6 were 5′-CTCGCTTCGGCAGCACATAT ACT-3′ and 5′-ACGCTTCACGAATTTGCG TGTC-3′, respectively. The data were uniformly normalized to the internal control U6 and the relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method. All experiments were run in triplicate [26].

2.5. Western blot

The collected NSCs were homogenized in lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris, 1 mM Ethylene-diamine tetra acetic acid, 1 mM EGTA Ph 8.0. 0.2 mM sodium orthovandate, 0.2 mM phenulmethulsulfonyl fluoride, and protease inhibitor cocktail) and centrifuged (12,000 rpm at 4 °C) for 15 min. Equal amounts of protein (20 ug) from the supernants were separated on a 10% acrylamide gel ad proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking with Tris-buffered saline with Tween-20 and incubated with primary antibody at overnight. Primary antibodies used were anti-DCX (1:2000, Millipore, Massachusetts, MA, USA), and anti-NeuN (1:1000, Santa Cruz, Santa Cruz, CA, USA), β-actin (1:2000, Santa Cruz, Santa Cruz, CA, USA). The membranes were incubated with HRP-conjugated secondary antibodies. The blots were rinsed and protein bands were visualized using an enhanced chemiluminescence detection system (Amersham, Pittsburgh, PA, USA).

2.6. Immunocytochemistry

To confirm the differentiation of NSCs, NSCs after 7 DIV were washed three times with PBS for immunostaining and were blocked for 30 min. The NSCs were then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-SOX2 (1:200, Millipore, Massachusetts, MA, USA), anti-DCX (1:200, Millipore, Massachusetts, MA, USA), anti-GFAP (1:200, Millipore, Massachusetts, MA, USA). After incubating the NSCs with the primary antibodies, primary antibody was then removed, and cells were washed 3 times for 3 min each with PBS. Next, samples were incubated with Flurescein isothiocyanate-conjugated donkey anti-goat (1:200, Jackson Immunoresearch, West Grove, PA, USA), Rhodamine-conjugated goat anti-rabbit (1:200, Jackson Immunoresearch, West Grove, PA, USA), Textas red-conjugated donkey anti-mouse (1:200, Jackson Immunoresearch, West Grove, PA, USA) for 2 h at room temperature. The NSCs were washed again 3 times for 3 min each with PBS. The NSCs were then counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (1:100, Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature. Fixed samples were imaged using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

2.7. Statistical analysis

Statistical analyses were carried out using SPSS 18.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as mean ± S.E.M. Significant intergroup differences were determined by one-way analysis of variance followed by Bonferroni *post hoc*

multiple comparison test. Each experiment included three replicates per condition. Differences were considered significant at * $p < 0.05$, ** $p < 0.001$.

3. Results

To determine the alteration of proliferation and differentiation related microRNA on NSCs, we measured the mRNA level of miR-9 and Let7a known as the microRNA regarding the cellular proliferation and differentiation using TaqMan assay (Fig. 1A). The expression of miR-9 and Let7a were significantly decreased in the differentiation group which cultured in differentiation media comparing with that in normal NSC culture media with a proliferation supplement. The transcriptional level of c-Myc and N-Myc has been considered as the transcription factor regulating the cellular proliferation and differentiation. Fig. 1B showed the relative quantity of c-Myc and N-Myc mRNA during the differentiation of NSC. In differentiation group, c-Myc and N-Myc mRNA levels were significantly observed higher than the normal group (Fig. 1B). Fig. 1C showed that the alterations of neurospheres under the condition of Let7a overexpression and inhibition (Fig. 1C). The neurospheres more seem to maintain on the media in the Let7a mimic treatment group whereas the Let7a inhibitor group seems to sink into the dish's bottom to differentiate (Fig. 1C). In addition, to examine the expression of DCX and NeuN considered as the neuronal cell markers, we conducted western blot analysis using each antibody (Fig. 2A). In Let7a overexpression condition, we determined that the protein level of DCX and NeuN were significantly reduced compared to the normal control group (Fig. 2A). Also, we confirmed the immunoreactivity of SOX2, DCX (Fig. 2B), GFAP (Fig. 2C) on NSCs under Let7a overexpression condition (Fig. 2B and C). Our images showed that the NSCs in Let7a overexpression state were attenuated the expression of DCX as neuronal cell marker (Fig. 2B and C). To investigate the involvement between Let7a and TLX reported as the NSC proliferation related transcription factor, we measured the mRNA level of TLX in the Let7a over-expression group and Let7a inhibition group (Fig. 3A and B). By treating negative and positive miRNA mimic onto NSC, any alteration of TLX or SOX2 was not detected. In the Let7a overexpression group, TLX expression level was quite higher than the control (Fig. 3A), while TLX expression level was significantly reduced in the Let7a inhibitor treatment group (Fig. 3B). The expression of SOX2 which considered as the neural stem cell marker was indicated the opposite patterns between the Let7a over-expression group and the TLX inhibition group each. The expression of c-Myc on NSCs was observed considerably increased mRNA level than the other groups (Fig. 3C). The increased transcriptional expression of c-Myc in the Let7a over-expression group was counter-correlated in the Let7a inhibition group (Fig. 3C).

4. Discussion

In our study, we focused on the involvement of Let7a in NSC proliferation and differentiation, and some findings and suggestion are provided (/submitted) as follow: Let7a involves in mainly NSC differentiation rather than proliferation (Fig. 2). According to the Let7a level, expression level of TLX, SOX2, and c-Myc are positively regulated (Fig. 3). Let7a involves in neuronal differentiation relating with TLX at least partially. Multiple miRNAs have an important role in NSC proliferation and differentiation. Let-7a is one of the lethal-7 miRNA family members which have been identifying and characterizing [27,28]. In CNS, Let-7a has been elucidated to regulate the neuronal differentiation during brain development [27]. Among in various regulatory feedbacks between miRNA and its target genes, Let-7 family and miR-9 is reported to target TLX which plays a role in NSC self-renewal. For CNS development, Let-7b also regulates target genes related to the nuclear receptor TLX as the cell cycle controller in NSCs [22]. Moreover, Let-7 suppresses the proliferation of NSCs through TLX inhibition and promotes neural and glial differentiation [22,23]. While the TLX regulation relating with miRNAs has been reported in miR-9, miR-137, Let7b, and Let7d, there are little reports to elucidate the feedback between let7a and TLX in NSC proliferation and differentiation.

Besides of Let7a, we selected some miRNAs, TaqMan assay was performed with relatively well-known brain-specific miRNA, miR-9, miR-124, and miR-137. As previous mentioned reports, miR9 and Let7a level was significantly changed (Fig. 1). Let7a overexpression triggered increasing both of c-Myc and N-Myc transcriptional level. The c-Myc, oncoprotein, has been recently described to regulate stem cells by relating malignant cancer and

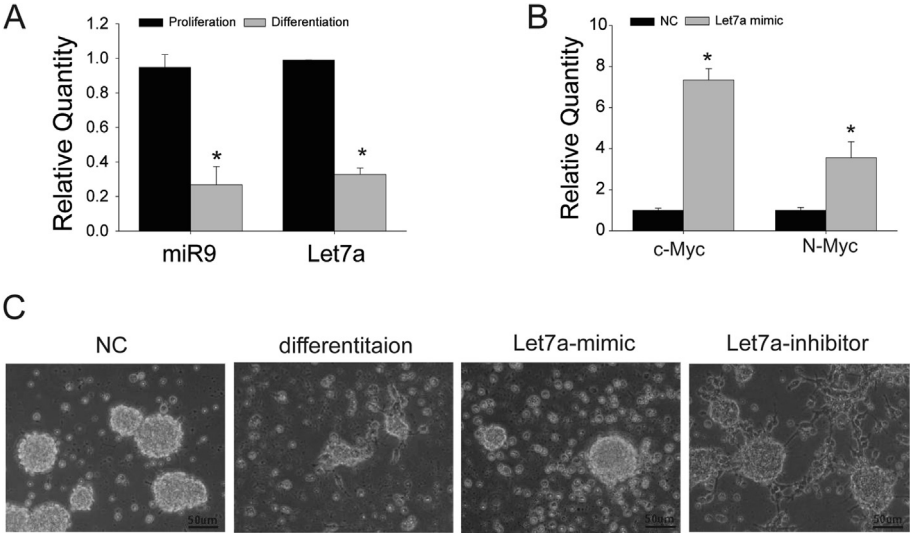

Fig. 1. The alteration of miRNAs level in neural stem cells and its effect (A) TaqMan assay was performed for Let7a and miR-9. **(B)** In differentiation media, the mRNA level of c-Myc was evidently increased in the Let7a overexpression group compared with the normal group. **(C)** The morphology of neurosphere in NSC was changed by differentiating condition, over-expressing Let7a, and under-expressing Let-7a under bright field microscope. **NC,** no treatment; **Let7A**, Let7A mimic; **Differentiation,** differentiation media.

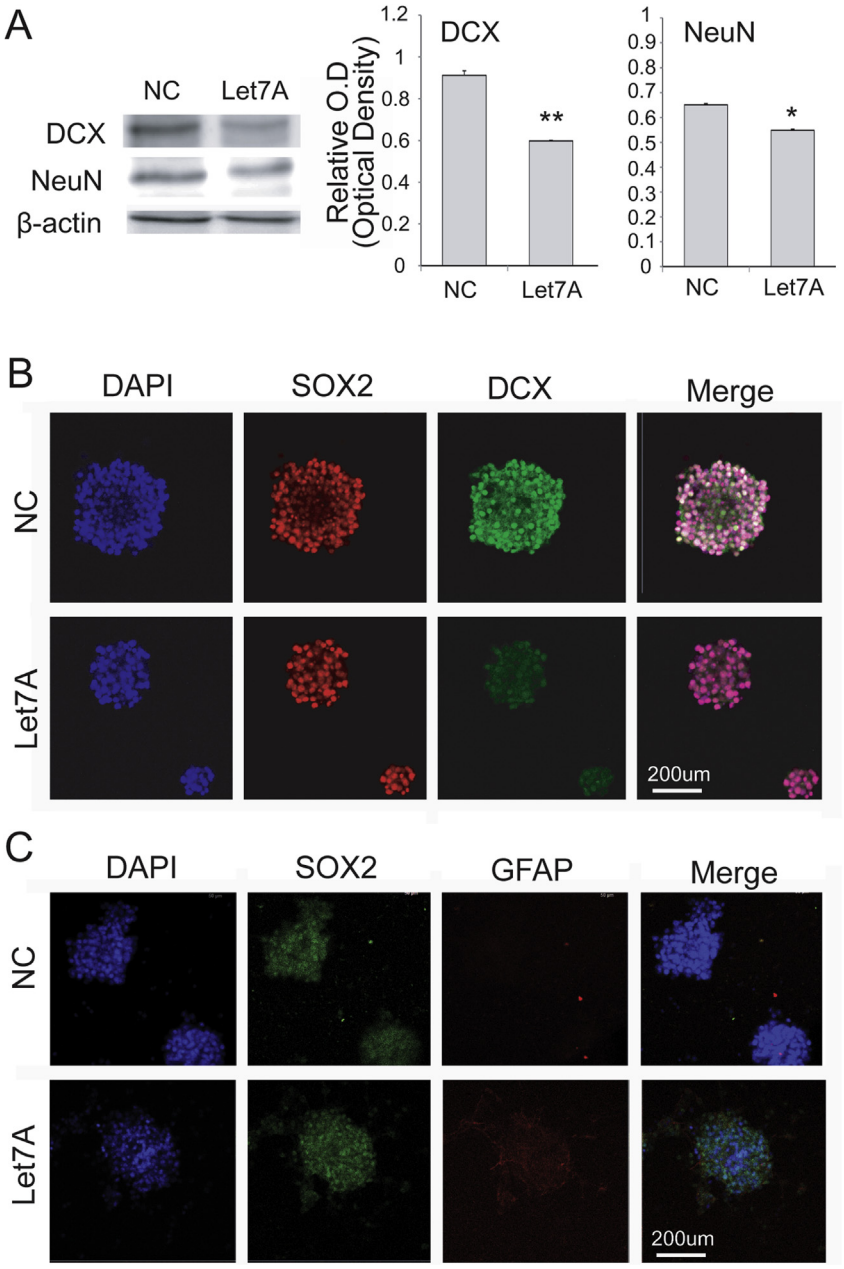

Fig. 2. The effect of Let-7a on Proliferation and differentiation of NSC (A) Western blotting showed that the relative protein level of DCX and NeuN considerably was reduced in the Let7A overexpression group compared to the normal control (NC) group. The bar graph shows the quantification of DCX and NeuN protein in all groups. **(B)** Immunostained images showed that SOX2-positive cells (red) were densely expressed in the Let7a overexpression group. In Let7a overexpression group, DCX expression (green) was a little bit expressed in NSCs compared with the normal group. **(C)** Immunochemical images showed that little SOX2-positive cells changed in two groups, whereas GFAP (red) expression was a little bit expressed in NSCs compared with the normal group in the Let7a overexpression group. $^{*}p < 0.05$, $^{**}p < 0.001$. **Scale bar** = 200 μm, **NC,** normal control; **Let7a,** Let7a mimic. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sustain stemness of stem cells [29]. Expression of c-Myc developed in mouse astroglia induces tumor, and the mass faster divides in c-Myc expression group than in the c-Myc down-expression group [30]. In agreement with previous reports, c-Myc as well as N-Myc mRNA showed much higher level in Let7a overexpression group. And it implicated that NSCs remained in proliferation level by Let7a over-expression, in coincidently differentiation of NSC was restrained. It was also detected the morphological change of neurosphere supposing the each NSC processing stage, which was affected by Let7a expression level. In the Let7a overexpression group, the size of neurospheres was reduced compared to the normal group (NC), but was not relatively small size of neurospheres compared to the differentiation group (Fig. 1). It could be confirmed by western blot and immunocytochemistry with neuronal marker and astrocyte marker (Fig. 2). The SOX2 stained images did not show difference between non-treated NSC (NC) and Let7a overexpressing NSC, which noted that manipulating Let7a level might be irrelevant to NSC proliferation. Rather, DCX-positive cells in Let7a overexpressing NSC showed apparent decrease contrast to NC. GFAP stained astrocyte was similar pattern of DCX staining result (Fig. 2). It is apparent that Let7a involved in NSC differentiation but it is unclear that Let7a regulate the mainly transition of NSC to neuron or to non-neuronal lineage in this study at least.

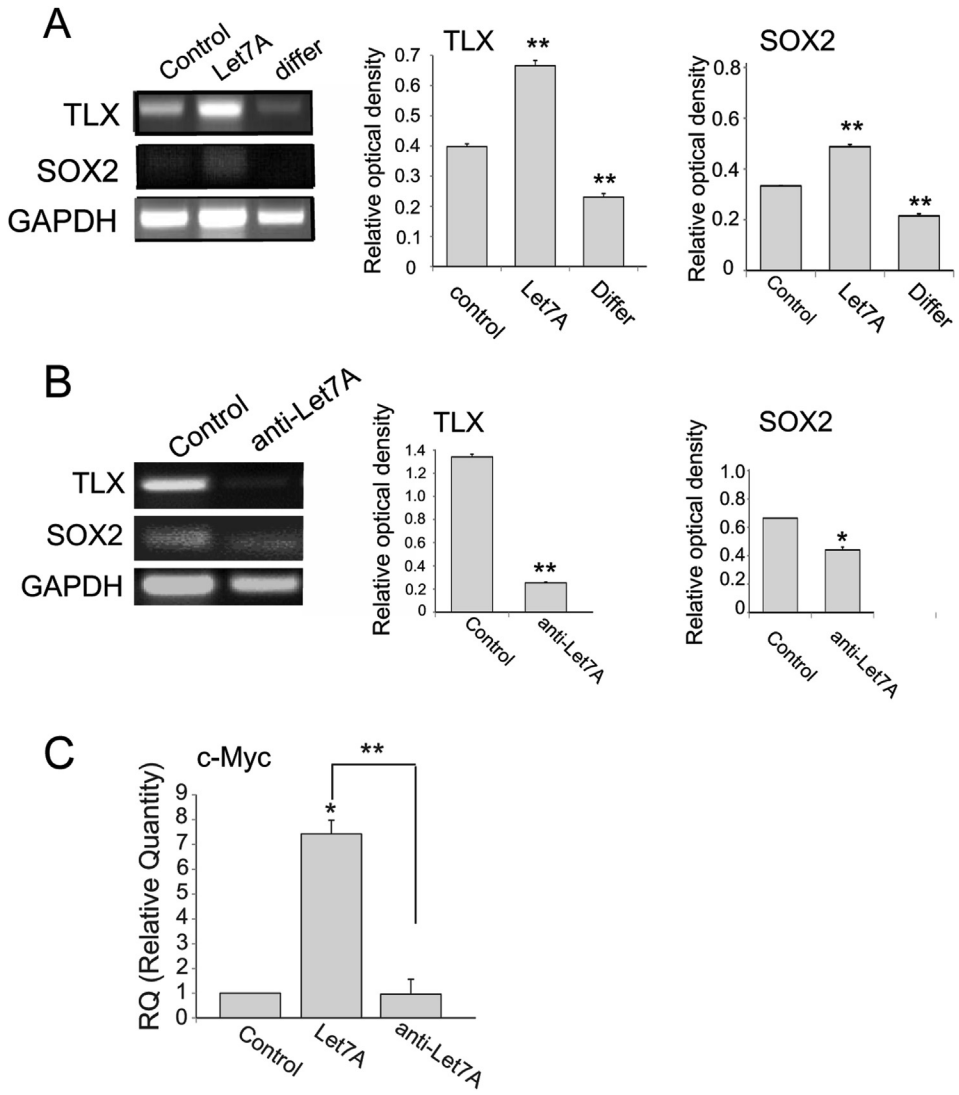

Fig. 3. The NSC differentiation under regulation of Let7a and the involvement of TLX in NSC differentiation (A) The mRNA level of TLX and SOX2 was increased in the Let7a over-expression group. **(B)** The mRNA level of TLX and SOX2 was decreased in the Let7a inhibition group compared to the normal control group. **(C)** The mRNA level of c-Myc was highly regulated by Let7a over-expressing, but its level in Let7a inhibition group was reduced comparing to the Let7a overexpression group. The bar graph shows the relative quantification of c-Myc mRNA in all groups. GAPDH was used as an internal control. Data are expressed as mean ± S.E.M. ($^{*}p < 0.05$, $^{**}p < 0.001$). **Control,** normal control; **Let7a,** Let7a mimic, **anti-Let7a**: Inhibition of Let7a.

In neuronal differentiation, let7a overexpression inhibits the differentiation of NSC vice versa let7a lowering NSC promotes neuronal differentiation. Generally miRNAs negatively regulate by binding the 3′ untranslated region (UTR) of target mRNAs [31]. TLX regulated by miRNA containing miR-9 plays an important role in NSC activation and proliferation. However, the decrease of miR-9 can be rescued by up-regulated TLX, and it was suggested the negative feedback regulation between miR-9 and TLX [32]. The changing level of TLX transcript in our study was examined and the TLX expression level was increased according to higher Let7a expression level. With reverse corresponding to it, Let7a inhibition was significantly reduced more under the control level. In addition, SOX2 expression was similar pattern with TLX that Let7a over-expression led SOX2 increase and Let7a inhibition led it decrease. On the other hand, c-Myc was one of genes targeted by Let7a some threshold level of c-Mcy is needed to sustain the immortality of cancer cells [33] or stem cells [29]. Recently c-Myc was reported to be controlled by Let7 family containing Let7a. When c-Myc was down-regulating, cellular proliferation was hindered. Recent report showed the relationship in bladder cancer that Let7a regulates c-Myc by participating Lin28/Let7a/c-Myc pathway in the cancer progression [34]. Although the regulating loop was elucidated and delineated in activating or maintain the cell proliferation, most of studies are in endowing the immortality on cancer cell. Most of studies focused on the cellular regulation under cancerous condition such as tumor proliferation cancer cell. This study however provides the regulating role of Le7a in NSC proliferation and differentiation mediating c-Myc along with TLX and SOX2 which are relatively often suggested. In conclusion, present study showed that neuronal differentiation of NSC is negatively regulated by over-expressed Let7a relating with TLX. It could suggest that manipulating Let7a may identify potential interventions with CNS diseases and also propose future therapeutic directions for CNS disorders.

Conflicts of interest

The authors declare no conflict of interest regarding the publication of this paper.

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