

# Vector-Based let-7a miRNAs Function as Suppressors of Multiple Oncogenes in SK-N-MC and SHEP Neuroblastoma Cells

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## Abstract

**Objective:** This study was designed to determine the anti-tumor effect of vector-based let-7a miRNA in neuroblastoma cell lines.

**Methods:** A tetracycline (tet)-inducible let-7a expression vector was constructed and used to stably transfect SK-N-MC and SHEP neuroblastoma cells. The effects of let-7a overexpression induced by tetracycline on cell proliferation and adhesion were analyzed *in vitro* by MTT assays and adhesion assays. The mRNA expression of the let-7 target oncogenes NRAS, KRAS, c-myc, and HMGA2 was examined by quantitative real-time reverse transcription polymerase chain reaction. Protein expression of N-RAS, K-RAS, c-Myc, HMGA2, NeuN, and  $\beta$ -tubulin was analyzed using western blot and immunocytochemistry. Morphology of SK-N-MC and SHEP cells was also observed. Additionally, the let-7a-inducible vector-transfected SHEP cells were subcutaneously injected in nude mice, and tumor growth *in vivo* was also evaluated.

**Results:** Let-7a expression was significantly induced in neuroblastoma cells and inhibited cell proliferation and adhesion in both SK-N-MC and SHEP cells. Let-7a up-regulated  $\beta$ 3-tubulin and NeuN expression in SK-N-MC and SHEP cells. However, short neurite-like outgrowth was observed only in SK-N-MC cells, but not in SHEP cells. Let-7a overexpression decreased the expression of N-RAS and HMGA2 proteins in both SK-N-MC and SHEP cells. c-Myc expression also decreased in SK-N-MC cells. The tumor sizes of SHEP xenografts in the tet-inducible group were smaller than those in the mice without tetracycline induction.

**Conclusion:** Taken together, our data demonstrate the anti-tumor effects of vector-based let-7a miRNA overexpression in SK-N-MC and SHEP neuroblastoma cells against multioncogenes. Let-7a miRNA is a potential therapeutic molecule for the treatment of neuroblastoma.

**Keywords:** Cell differentiation; let-7a miRNA; N-RAS; c-Myc; HMGA2; Neuroblastoma; Proliferation

## Introduction

MicroRNAs (miRNAs) play critical roles in the regulation of cancer initiation and progression and act as oncogenes and tumor suppressors [1-3]. miRNAs now serve as new therapeutic molecules for the treatment of cancer. They are used to replace tumor suppressors or to silence oncogenes [4].

Let-7 miRNAs function as suppressors through regulation of downstream oncogenes such as KRAS, HMGA2, and c-myc in human cancers [5-7]. Let-7 miRNAs have emerged as a new class of potential therapeutic molecules that function as tumor suppressors [3,8]. Blocking the K-ras signaling pathway via let-7 miRNAs has been used as a therapeutic strategy for lung cancer and pancreatic carcinoma presenting with KRAS mutations and overexpression. Studies have confirmed that let-7 could inhibit lung carcinoma and pancreatic tumor growth by suppressing K-RAS and c-Myc [9,10]. However, NRAS mutations are rare in neuroblastomas, and RAS levels have been

reported to be relatively low [11-13]. Thus, blocking the ras pathway was not considered as a potential therapeutic strategy in neuroblastoma studies. Han et al. confirmed let-7e-mediated regulation of MYCN expression in a MYCN-amplified neuroblastoma cell line and that let-7e inhibited cell proliferation and growth when overexpressed in MNA neuroblastoma cell lines [14]. However, the effects of let-7 miRNAs via Ras and other let-7 targets in neuroblastoma remain unknown.

Recently, Han et al. reported that, although wild-type N-RAS protein is expressed at the same level in all 3 neuroblastoma cell phenotypes, the activated N-RAS-GTP level is significantly higher in I-type neuroblastoma cancer stem cells, and suggested that other pathways activate N-RAS signaling [15]. The highly conserved let-7 miRNAs have also been shown to have suppressive effects in cancers with overexpression of RAS proteins and RAS oncogenes with lesser mutations, such as hepatocellular carcinoma [4,16-18]. We previously verified the presence of let-7a target wild-type RAS genes in humans through luciferase activity. In addition, the results showed that KRAS caused a more than 30% decrease and wild-type NRAS provided a 50% decrease in luciferase activity relative to the control, while no such

decrease was observed in the case of wild-type HRAS (data not shown). Our previous studies confirmed that let-7a mimics exerted significant antitumor effects in hepatocellular carcinoma by downregulating all human RAS genes at both the transcriptional and translational levels and that, among the 3 human ras mRNAs, the expression of NRAS mRNA decreased the most [16]. A study conducted on *Caenorhabditis elegans* predicted that the 3'-untranslated regions (UTRs) of the human RAS genes contain multiple let-7 complementary sites (LCSs), which allows let-7 to regulate ras expression; the human NRAS, KRAS, and HRAS mRNA 3'-UTRs have 9, 8, and 3 potential LCSs, respectively [19]. These results suggest that let-7 is a potential suppressor of NRAS in neuroblastoma cells. In addition, other molecular target oncogenes for therapy of neuroblastoma, such as c-myc and HMGA2, are also let-7 miRNA downstream targets [20,21]. Thus, we hypothesize that let-7 miRNAs may be effective potential antitumor molecules in neuroblastoma against all these target oncogenes. We investigated the anti-tumor effects of vector-based let-7 miRNAs in SK-N-MC and SHEP neuroblastoma cells, in which let-7a expression was controlled by tetracycline (tet) via a vector-based shRNA system.

## Materials and Methods

### Let-7a-1 shRNA plasmid vector

The tetracycline-controlled (tet-controlled) short-hairpin RNA (shRNA) expression constructs of let-7a-1 (let-7a) were obtained by cloning the sequences into a pRNATin-H1.2/Neo vector (SD1223G418; GenScript Corporation, Piscataway, NJ, USA), in which the expression of the gene of interest is under the control of the RNA polymerase III H1-RNA gene promoter. This plasmid features a neomycin resistance gene that can be used to establish stable cell lines and a coral GFP (cGFP) marker that is under the control of a CMV promoter for tracking transfection efficiency.

The sequences of the let-7a for shRNAs (sense/antisense, GenScript, Guangzhou, China) were as follows:

Let-7a,5'  
TGAGGTAGTAGGTTGTATAGTTCTCACTATACAACCTACTACCT  
CAC-3'

and

5'-  
CACTCCATCATCCAACATATCACTCTTGATATGTTGGATGATG  
GAGTAAAA-3';

Ctrl, 5'-  
TCGCTGATTTGTGTAGTCGGAGACGACTACACAAATCAGCGA-  
3'

and

5'-  
TCGCTGATTTGTGTAGTCGTCTCCGACTACACAAATCAGCGA-  
3'

### Cell culture and stable cell lines

The human SK-N-MC (gift from Professor Dong Yi) and SHEP neuroblastoma cells were maintained in complete RPMI 1640 media and IMDM (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation) and tet (2.5 µg/mL) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

The tet-inducible constructs carrying an let-7a sequence via the shRNA system were transfected into SK-N-MC and SHEP cells by using Lipofectamine™ 2000 (Invitrogen Corporation). The cells were trypsinized 48 hr after transfection and seeded into a selective medium. Stable cells were established by neomycin selection (1000 µg/mL). The medium was changed every 3 days. Resistant colonies were trypsinized after 2 weeks, combined in pools, and cultured in the selection medium.

Stably constructs-transfected neuroblastoma cells were established for further study as control group, and produce tet-inducible let-7a expression by tet (2.5 µg/mL) as the let-7a-treated group. The final transfection efficiency was examined by western blot.

### Quantitative real-time RT-PCR

Human neuron total RNA (HN total RNA) was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Total cellular RNA samples were extracted from SK-N-MC and SHEP cells with or without vector-based let-7a expression using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare UK Limited, Amersham Place, Little Chalfont, UK). cDNA was synthesized using SuperScript™ III First-Strand Synthesis SuperMix for quantitative real-time reverse transcription (RT) PCR (qRT-PCR, Invitrogen Corporation). Real-time PCR was performed using the qRT-PCR SuperMix (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Quantitative values were obtained from the cycle number (Ct value) at which the increase in fluorescent signal associated with an exponential increase in PCR products could be detected by the laser detector of the ABI Prism 7500 Sequence Detection System (Perkin-Elmer Applied Biosystems) by using the Perkin-Elmer Biosystems analysis software. The procedures followed were as the manufacturer's instructions and have also been previously described [22].

To quantify let-7 gene expression in SK-N-MC and SHEP cells after tet-induction, the expression level of let-7a-1(let-7a) was calculated based on the amount of the target relative to the 5S rRNA control. Levels were examined by qRT-PCR and normalized to the initial input of total RNA. The primer pairs were (sense, antisense) as follows: Let-7a, 5'-CCTGGATGTTCTCTTCACTG-3' and

5'-GCCTGGATGCAGACTTTTCT-3';

5S RNA, 5'-TACGGCCATACCACCCTGAA-3', and

5'-TAACCAGGCCCGACCTGCT-3'

The mRNA expression levels of the NRAS, KRAS, and HMGA2 genes after let-7a expression in SK-N-MC and SHEP cells were calculated based on the amount of the target relative to the GAPDH control.

The primer pairs were (sense/antisense) as follows:

NRAS, 5'-AATCCAGCTAATCCAGAACC-3' and

5'-TGGTCTCTCATGGCACTGTA-3';

KRAS, 5'-CCAGGTGCGGGAGAGAG-3' and

5'-CCCTCATTGCACTGTACTCC-3';

HMGA2, 5'-GAAGCAGCAGCAAGAACCA-3'and

5'-TCTCCAGTGGCTTCTGCTTTC-3'; and

GADPH, 5'-GAAGGTGAAGGTCGGAGTC-3' and

5'-GAAGATGGTGATGGGATTTC-3'

### MTT-based cell proliferation assay

Cells ( $5 \times 10^3$ ) cultured in 200  $\mu$ L of RPMI 1640 or IMDM medium containing 10% FBS were harvested and seeded in the wells of 96-well flat-bottomed plates (200  $\mu$ L/well). After incubation for 1,2,3,4, and 5 days, the medium was removed and replaced with 125  $\mu$ L of complete medium containing 1 mg/mL of MTT (thiazolyl blue, Ameresco Inc. Solon, USA). Plates were incubated at 37°C for 4 hr. After incubation, cells were lysed in a buffer containing 20% (w/v) SDS and 50% (v/v) N,N-dimethylformamide adjusted to a pH of 4.5. Absorbance at 550 nm was determined for each well using a multiscanner (Clini Bio 128, Austria). The results are expressed as the mean of 10 wells. The experiments were repeated 2 times.

### Adhesion assay

For this assay, 96-well plates coated with the ECM molecules fibronectin or vitronectin (Fn and Vn, respectively; 25  $\mu$ g/mL) were purchased from Sigma (St. Louis, MO, USA). Then,  $15 \times 10^3$  cells, suspended in 100  $\mu$ L DMEM containing tet (2.5  $\mu$ g/mL) or not containing tet were seeded in each well and allowed to attach for 60 min. A MTT solution (20  $\mu$ L/well) was added for 5 hr of incubation. Then the supernatants were decanted, 150  $\mu$ L of 100% dimethyl sulfoxide (DMSO) added, and the cells were placed on a shaker for 10 min. Absorbance of the cells was measured at 485 nm and 520 nm with a multiscanner. The results are expressed as the mean of 10 wells. The experiments repeated 2 times.

### Western blot analysis

Proteins were extracted and examined using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology Inc., Rockford, IL, USA). Proteins (35  $\mu$ g) were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Pharmacia Products, PJKBio, Beijing China). The membranes were blocked in 5% nonfat milk and incubated with the primary antibody. Antibodies against the following proteins were used: N-RAS, K-RAS, c-Myc, and p-c-Myc (Santa Cruz Biotechnology, CA, USA); HMGGA2 (Abnova Corporation, Taiwan); cGFP (GenScript Corporation);  $\beta$ 3-tubulin, and NeuN (Chemicon International Company, Temecula, California, USA); and GAPDH (Proteintech, Chicago, IL, USA). After incubation with the primary antibody, the membranes were washed in PBS-T (PBS and 0.1% Tween-20) and incubated with the appropriate peroxidase-conjugated secondary antibody (DAKO, Carpinteria, CA, USA), followed by development with a chemiluminescent substrate (Pierce Biotechnology Inc.) and exposure to X-ray films (Kodak, Rochester, NY, USA).

### Immunocytochemistry

Cells ( $2-3 \times 10^4$ ) were seeded in 8-well BD Falcon™ and BD BioCoat™ CultureSlides (Becton Dickinson Labware, Franklin Lakes, NJ, USA). After 48 h, cells were fixed in 4% paraformaldehyde. After two washes with PBS-T, the cells were blocked with 5% FCS/TBS-T and exposed to primary antibodies (final concentration of 5  $\mu$ g/mL). Antibodies against the following proteins were used: N-RAS, K-RAS, c-Myc, and p-c-Myc (Santa Cruz Biotechnology); HMGGA2 (Abnova Corporation);  $\beta$ 3-tubulin, NeuN (Chemicon International Company); and cGFP (GenScript Corporation). The cells were then counterstained with PE-conjugated secondary antibodies (red) along with FITC (green)-conjugated secondary antibodies (Proteintech Group Inc, Chicago, IL, USA) diluted to 1:500. Cells were examined using an

Olympus BX51 upright microscope (Olympus, Tokyo, Japan) and all signals were quantified using VideoTest software (VideoTest, Petersburg, Russia). Images were captured under identical conditions (e.g., exposure time and image scaling) and a threshold intensity level was set by examining sections stained with the secondary antibody alone. The percentage of pixels reaching the threshold level was determined for each target and for 4',6-diamidino-2-phenylindole (DAPI) in each image. The signal intensity/pixel number for DAPI was used to normalize the specific signal for each target antibody. To compare the expression levels of N-RAS, K-RAS, c-Myc, HMGGA2,  $\beta$ 3-tubulin, and NeuN between cells, we used double-label immunofluorescence to examine p-c-Myc and the abovementioned proteins.

### Xenograft model

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication nos. 80-23, revised 1996) and with the experimental animal welfare ethics regulations of China, with the approval of the Institution Animal Care and Use Committee of Peking Union Medical College Hospital. All animal experiments were performed at the Centre for Experimental Animal Research (CEAR), Institute of Basic Medical Sciences (IBMS), CAMS & PUMC.

Female BALB/c nude mice (4 weeks old, 17g to 22 g) were obtained from the National Institutes for Food and Drug Control, China. The mice were housed in a pathogen-free facility in accordance with PUMC committee guidelines for animal care.

First,  $4 \times 10^6$  SHEP cells transfected with an inducible let-7a expression vector were transplanted into 8 female BALB/c nude mice subcutaneously at a single site on the back. One week later, 8 mice with successfully engrafted SHEP xenografts were randomized into 2 groups of 4 animals each. Four of the xenografted mice received tet (0.8 mg/mL) in the drinking water and tet (2.5  $\mu$ g/mL) were injected via tail vein to induce the expression of let-7a, these mice were considered as the let-7a-treated group (let-7a). Another 4 xenografted mice that did not receive tet for induction served as the control group (Ctrl).

Tumor size and blood vessels around the tumors during the experiment were confirmed by ultrasonography with a Vevo 2100 high-frequency ultrasound system (VisualSonics, Inc., Toronto, Ontario, Canada) with measurements in 3 orthogonal axes (a, b, and c). Tumor volumes were determined as  $V=(abc)/2[23]$ . The presence of tumors was confirmed via 2-dimensional vertical interfaces. Imaging was recorded using a Kodak FX Pro *in vivo* imaging system.

Tumor dimensions were measured weekly and tumor volumes were calculated respectively. Four weeks after implantation, the primary tumors were excised and measured. The xenograft tumor size of the 2 groups was based on the mean volume of 4 samples, and inhibition was calculated based on the volume at 4 weeks after treatment as follows:

Inhibitory rate (%) =  $1 - \frac{\text{mean volume of the treatment group}}{\text{mean volume (Ctrl)}} \times 100$

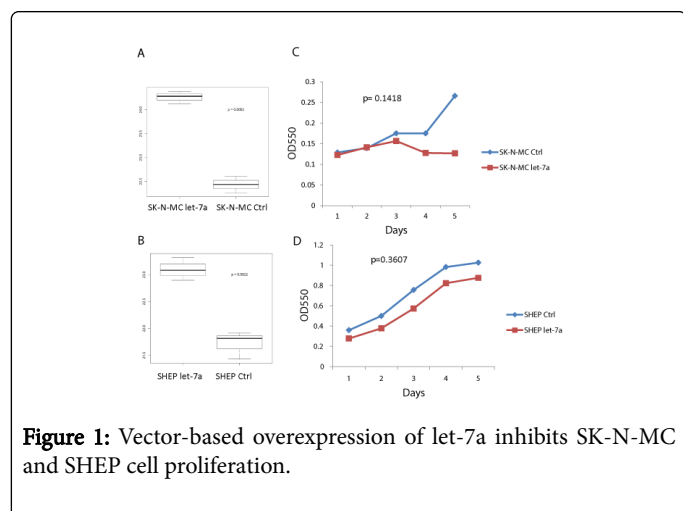
### Statistical analysis

Data are expressed as the mean  $\pm$  SEM. All data analyses were performed with SPSS 16.0 software (IBM, Inc., Armonk, NY, USA). Analysis of variance (ANOVA) and Student's t-test or Wilcoxon test were used for statistical comparisons between groups.  $p < 0.05$  was considered to be statistically significant.

## Results

### Let-7a inhibits cell proliferation

The let-7a tet-inducible expression constructs were transfected into SK-N-MC and SHEP cells, which were seeded into a selection medium to establish stable cell lines. The stable cell lines were grown in medium with tet to induce the expression of let-7a and were used as the let-7a-treated cells, while cells cultured in medium without tet were used as controls. We examined the let-7a levels of the SK-N-MC and SHEP cells with or without induction by tet by using qRT-PCR. Significant up-regulated let-7a expression was found in the tet-inducing neuroblastoma cells (SK-N-MC, let-7a vs Ctrl,  $p=0.0002$ ; SHEP, let-7a vs Ctrl,  $p=0.0022$ , Figure 1A, 1B). The relative levels of let-7a at 48 h after induction by tet were 7.45 and 5.21 times those in the original SK-N-MC and SHEP cells, respectively. Proliferation of SK-N-MC and SHEP cells was examined by MTT assays. Cell proliferation was inhibited and cell growth was slower but not significant in the let-7a up-expressed SK-N-MC and SHEP cells (SK-N-MC, let-7a vs Ctrl,  $p=0.1418$ ; SHEP, let-7a vs Ctrl,  $p=0.3607$ , Figure 1C and 1D). The let-7a inhibition effect was relative significant in SK-N-MC cells.



**Figure 1:** Vector-based overexpression of let-7a inhibits SK-N-MC and SHEP cell proliferation.

(A) Relative let-7a expression in SK-N-MC cells. Let-7a expression in SK-N-MC neuroblastoma cells, examined by qRT-PCR. Results examined at 48 hr after tet-induced expression.

(B) Relative let-7a expression in SHEP cells Let-7a expression in SHEP neuroblastoma cells, examined by qRT-PCR. Results examined at 48 h after tet-induced expression.

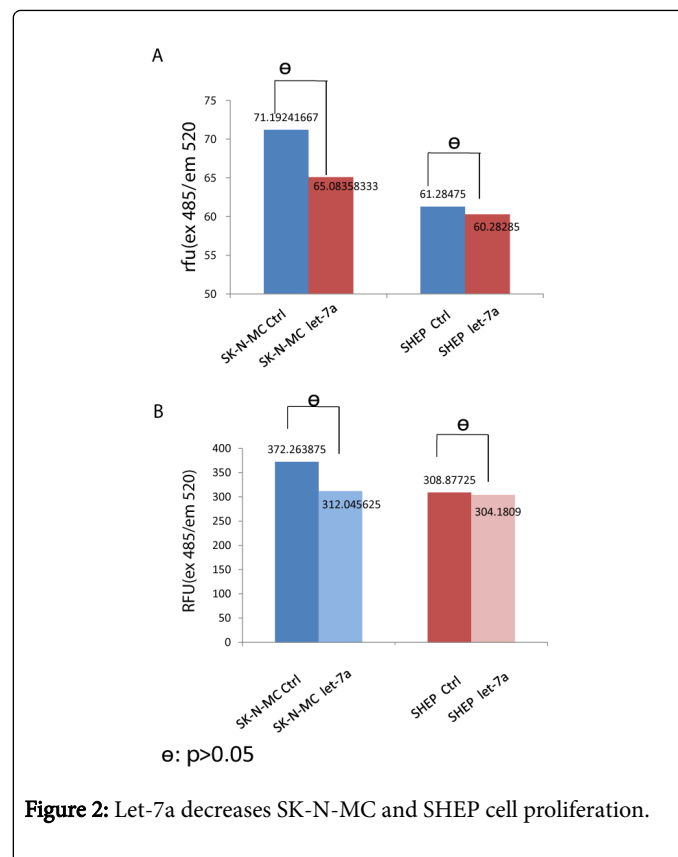
(C) Cell growth curve of SK-N-MC cells. SK-N-MC Ctrl: control cells without tet induction; SK-N-MC let-7a: SK-N-MC cells with let-7a induced by tet.

(D) Cell growth curve of SHEP cells. SHEP Ctrl: control cells without tet induction; SHEP let-7a: SHEP cells with let-7a induced by tet.

### Let-7a decreases cell adhesion *in vitro*

The adhesion ability of SK-N-MC and SHEP cells transfected with tet-inducible let-7a constructs was examined by Fn and Vn ECM Cell Adhesion Assays. The adhesion ability of both SK-N-MC and SHEP cells decreased in the tet-inducible let-7a expressed cells but not significant (Fn: SK-N-MC, let-7a vs Ctrl,  $p=0.5884$ ; SHEP, let-7a vs

Ctrl,  $p=0.1117$ ; Vn: SK-N-MC, let-7a vs Ctrl,  $p=0.0942$ ; SHEP, let-7a vs Ctrl,  $p=0.9065$ ; Figure 2). The adhesion ability of SHEP cells was lower than that of SK-N-MC cells before and after the induction of let-7a expression (Figure 2).



**Figure 2:** Let-7a decreases SK-N-MC and SHEP cell proliferation.

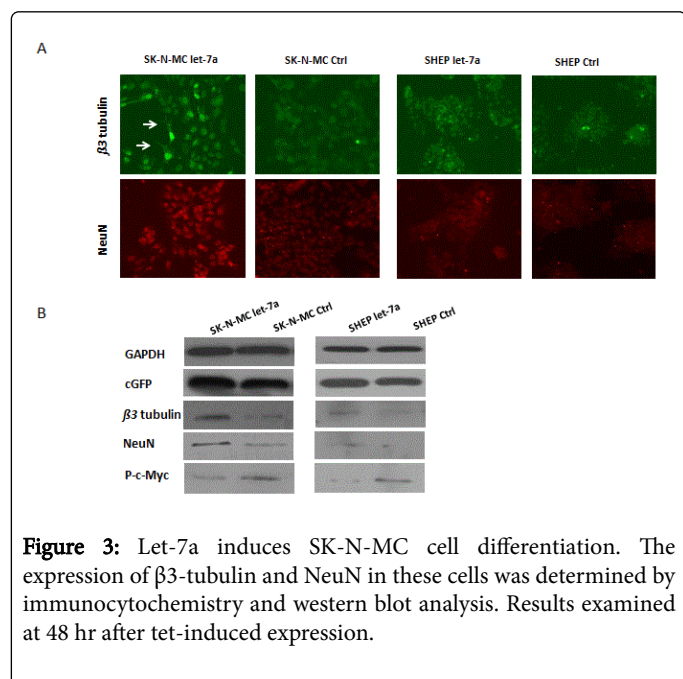
(A) Cell Adhesion Assay (Fn) of neuroblastoma cells The absorbance was measured at 485 nm and 520 nm , Here shows relative flurecences 485 nm and 520 nm. Data are presented as the mean  $\pm$  S.D. values from three separate experiments.

(B) Cell Adhesion Assay (Vn) of neuroblastoma cells The absorbance was measured at 485 nm and 520 nm, Here shows relative flurecences 485 nm and 520 nm. Data are presented as the mean  $\pm$  S.D. values from three separate experiments.

### Let-7a induces cell differentiation *in vitro*

To determine the effect of let-7a on cell differentiation in SK-N-MC and SHEP cells, the expression of  $\beta$ 3-tubulin and NeuN proteins was examined by immunocytochemistry (Figure 3A) and western blot (Figure 3B). GAPDH was used as a protein loading control in western blot and cGFP was used to assess the transfection efficiency. Upregulation of  $\beta$ 3-tubulin and NeuN expression was observed in tet-inducible let-7a-construct-transfected SK-N-MC and SHEP cells. Conversely, p-c-Myc expression was stronger in the control cells when compared with the let-7a treated cells, and was used as a control for  $\beta$ 3-tubulin and NeuN protein expression in immunocytochemistry staining. In addition, cell morphology analysis by immunocytochemistry indicated that tet-inducible let-7a-construct-transfected SK-N-MC neuroblastoma cells presented differentiated features; short neurite-like outgrowth feature was observed (Figure

3A). However, the construct-transfected SHEP cells did not show such features (Figure 3A).



(A) Immunocytochemistry. Short processes were also observed, extending from the cell aggregates and forming “neuronal-like” cells (arrows).

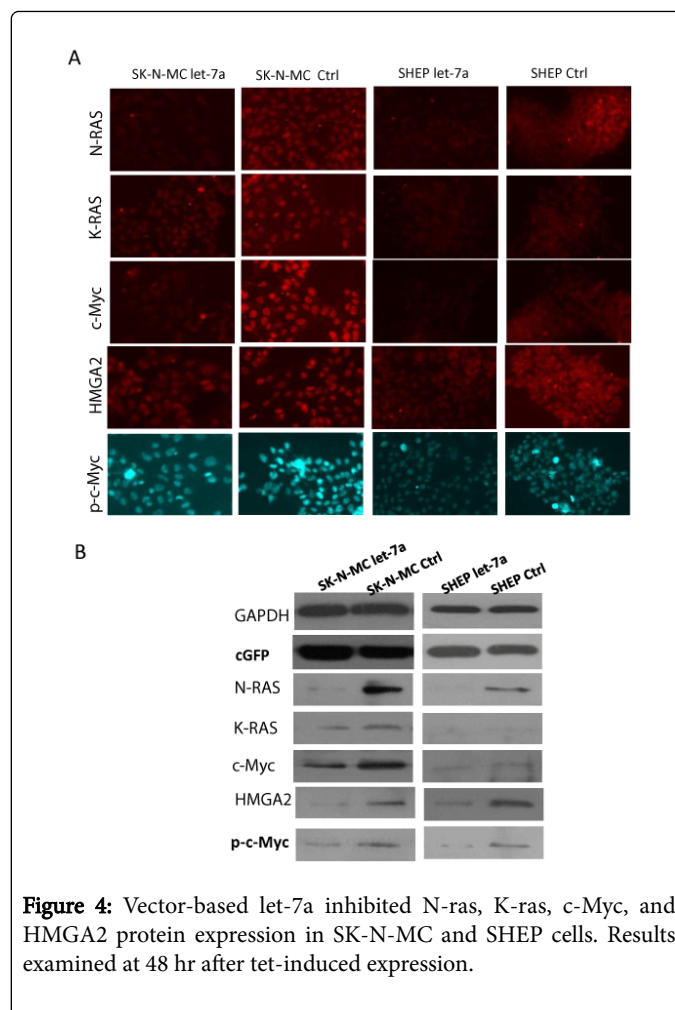
(B) Western blot.

### Let-7a decreases protein expression of multiple oncogenes

We determined the effects of let-7a on N-RAS and K-RAS, c-Myc, and HMGA2 in SK-N-MC and SHEP cells by immunocytochemistry (Figure 4A) and western blot (Figure 4B). N-RAS and HMGA2 protein levels were found to decrease in both SK-N-MC and SHEP cells. N-RAS, HMGA2, K-RAS, and c-Myc expression decreased in SK-N-MC cells (Figure 4). N-ras and HMGA2 protein levels also decreased in SHEP cells, whereas K-RAS and c-Myc expression was low and undetectable in the original SHEP cells (Figure 4).

### Let-7a did not decrease RAS and HMGA2 mRNA levels

We examined mRNA levels of NRAS, KRAS, and HMGA2. qRT-PCR results indicated that the mRNA levels of NRAS, KRAS, and HMGA2 in SK-N-MC and SHEP cells after induction of let-7a expression by tet were not lower than those in the control cells. Suppression was not observed for the expression of any of the mRNAs analyzed (data not shown).

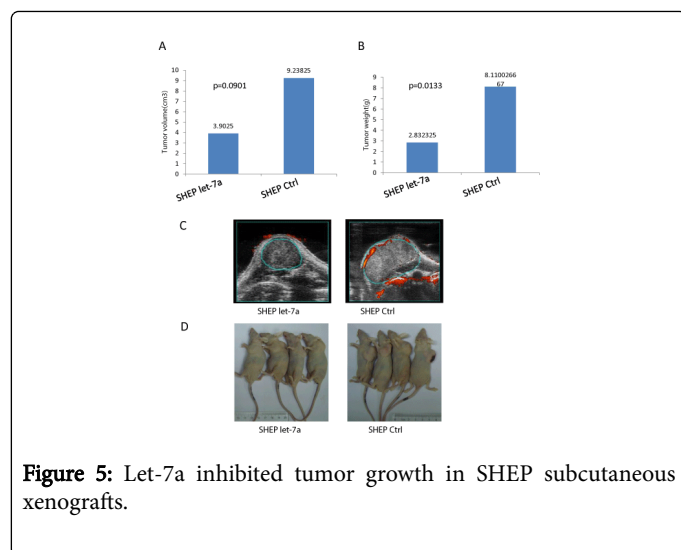


(A) Protein expression was determined by immunocytochemistry.

(B) Protein expression was determined by western blot; cGFP and GAPDH were used as controls.

### Let-7a inhibits tumor growth *in vivo*

To determine the long-term effects of let-7a on the growth of neuroblastomas, SHEP cells were injected subcutaneously into nude mice and xenografts were established. Tumor size and blood vessels around the tumors of the xenografts were confirmed by ultrasonography. Tumor sizes in the let-7a-treated xenografts were significantly lower than those in the control group (Figure 5A-D). The volumes of the tumors were  $3.903 \pm 2.031 \text{ cm}^3$  and  $9.238 \pm 1.693 \text{ cm}^3$  for the let-7a-treated and the control group, respectively ( $p=0.0901$ ). The inhibitory rate of the tumor volume was 57.75%. The tumor weights were  $2.832 \text{ g} \pm 1.689 \text{ g}$  and  $8.110 \text{ g} \pm 1.892 \text{ g}$ , respectively ( $p=0.0133$ ). These results showed that vector-based let-7a expression inhibited tumor growth of SHEP neuroblastoma after long-term induction via tet. In addition, ultrasonography showed a lesser number of blood vessels around the tumor in the let-7a-treated group as compared to those in the control group (Figure 5C).



**Figure 5:** Let-7a inhibited tumor growth in SHEP subcutaneous xenografts.

- (A) Tumor volume of SHEP; xenografts representative xenografts from all groups are illustrated (N=4 mice per cohort)
- (B) Tumor weight of SHEP xenografts.
- (C) SHEP xenografts examined by ultrasonography.
- (D) Macroscopic view of the tumor in nude mice.

## Discussion

In this study, we confirmed the let-7a mediated antitumor efficacy against multiple oncogenes. These results demonstrate the advantage of miRNAs as potential therapeutic molecules.

We confirmed that vector-based let-7a overexpression resulted in anti-tumor effects in SK-N-MC and SHEP neuroblastoma cells *in vitro*. *In vivo* experiments confirmed that let-7a inhibited tumor growth of SHEP xenografts. And downregulation of N-RAS and HMGA2 was observed in SK-N-MC and SHEP cells. These results confirmed our hypothesis that let-7a miRNAs are effective potential antitumor molecules in SK-N-MC and SHEP neuroblastoma against multiple target oncogenes. In addition, more decrease of N-RAS protein expression was observed than that of K-RAS in SK-N-MC cells. This result was also consistent with the predicted LCSs in human RAS mRNA 3'-UTRs reported previously.

Let-7a is encoded by 3 locations in the human genome. There are 3 homologues of let-7a: let-7a-1, let-7a-2, and let-7a-3 [24]. In this study, we chose let-7a-1, which showed a larger decrease in SK-N-MC and SHEP cells as compared to that in normal human neurons (data not shown). By designing shRNA and artificial miRNA to produce exactly the same products, shRNAs are expressed at considerably higher levels relative to those of artificial miRNAs [25,26]. Thus, we used a vector-based let-7a shRNA system to mediate more effective gene silencing. We chose SK-N-MC and SHEP neuroblastoma cells, all of which had N-RAS overexpression, but showed different amounts of KRAS and c-myc amplification and expression.

Previous studies confirmed the presence of different differentiation features among the three multipotent neuroblastoma cell types. S-type cells can differentiate into melanocytes, smooth muscle cells, or Schwann cells, instead of neuronal cells [27]. Different let-7a target oncogenes are expressed in SK-N-MC and SHEP cells. SHEP is a non-

neuronal, strongly substrate-adherent subclone of SK-N-SH neuroblastoma cells. MYCN- and c-myc oncogenes are not expressed in SHEP cells [28,29].

SK-N-MC is a peripheral neuroectodermal tumor cell line with strong c-Myc expression [30]. A previous study confirmed that c-myc is an effective antitumor target of siRNA in neuroblastoma. The anti-c-myc siRNA (siRNA-I) was shown to efficiently decrease c-myc mRNA expression in human epidermoid carcinoma KB-3-1 and neuroblastoma SK-N-MC cell lines. The reduction in the c-myc mRNA level is correlated with inhibition of cell proliferation [31]. Downregulation of c-myc oncogene expression was also noted in NGF-induced differentiated cells [32], whereas SHEP cells did not show differentiation features.

The results in this study showed the inhibition effects of let-7a were different in SK-N-MC and SHEP cells. Cell proliferation was less inhibited in SHEP cells than those in SK-N-MC cells *in vitro*. And let-7a had different effects on SK-N-MC and SHEP cell differentiation. Neurite outgrowth feature was only observed in SK-N-MC cells. And the adhesion ability of SK-N-MC cells was also higher than that of SHEP cells. This difference may be related to their cell type features with different genetic features. c-Myc downregulation was concomitant with the appearance of morphological differentiation in SK-N-MC cells, which is also consistent with previous reports [28].

Our results show that K-RAS and c-Myc were not overexpressed in SHEP cells. In SHEP cells, downregulation of only N-RAS and HMGA2 was observed, whereas in SK-N-MC cells, downregulation of N-RAS, K-RAS, c-Myc, and HMGA2 proteins was observed.

The differences in oncogene expression, especially the difference in c-Myc expression, may be one of the reasons for the stronger effect of let-7a inhibition seen in SK-N-MC cells than those in SHEP cells.

The c-Myc protein down-regulation in SK-N-MC cells may occur through a direct inhibition effect of let-7a and/or indirectly via the RAS-Myc pathway, in which c-Myc is located downstream of RAS, which we could not confirm in this study.

The shRNA vector used in this study, that is, the pRNATin-H1.2/Neo vector, is a kind of vector-based siRNA construct for producing dsRNAs (siRNAs) of interest via tet control. Using this vector system, we successfully increased let-7a levels and significantly decreased N-RAS, HMGA2, and c-Myc protein levels in SK-N-MC cells. However, N-ras, K-ras, and HMGA2 mRNAs in neuroblastoma cells were not downregulated by the overexpression of let-7a. These results suggest that the vector-based let-7a "siRNA" here mostly functions as an miRNA against multiple oncogenes, and not only as an siRNA against a special target oncogene. However, we cannot directly confirm the hypothesis based on the analyses performed in this study.

Taken together, we confirmed the anti-tumor effects of vector-based let-7a against multiple oncogenes in SK-N-MC and SHEP cells. Moreover, let-7a induced cell differentiation in SK-N-MC cells. Thus, let-7 miRNA is a potential therapeutic molecule for the treatment of neuroblastoma.

## Competing interests

The author declares that they have no competing interests.

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