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Chapter

Targeting MYC and HDAC8 with a Combination of siRNAs Inhibits Neuroblastoma Cells Proliferation In Vitro and In Vivo Xenograft Tumor Growth

Nagindra Prashad

Abstract

HDAC8, c MYC and MYCN are involved in the tumorigenesis of neuroblastoma. A mouse Neuroblastoma (NB) tumor model was used to understand the role of miRNA, miR-665 in NB tumorigenesis and cellular differentiation. During cellular differentiation of NB cells there is an up regulated miRNA-665. We found that HDAC 8, c MYC and MYCN are the direct targets of mimic miR-665 which was validated by luciferase reporter plasmid with 3' UTR and ELISA. Mimic miR-665 inhibited cell proliferation, arrested cells in G1 stage and decreased S Phase in cell cycle. miR-665 increased the acetylation of histones and activated Caspase 3. This is the first report to recognize miRNA 665 as a suppressor miRNA of NB. The effects of miR-665 were confirmed with the transfection of siRNA for HDAC8 and siRNA for MYC. Individual siRNA- HDAC8 or siRNA-MYC inhibited 40–50% of cell proliferation in vitro, however, the treatment with the combination of both siRNA-MYC + siRNA- HDAC8 inhibited 86% of cell proliferation. Indicating that both the targets c MYC and HDAC 8 should be reduced to obtain a significant inhibition of cell proliferation. Intratumoral treatment of xenograft tumors in mice with the combination of siRNA-MYC + siRNA- HDAC8 reduced the levels of target c-MYC protein by 64% and target HDAC 8 protein by 85% and the average tumor growth reduced by 80% compared to control tumors treated with NC-siRNA. Our results suggest the potential therapeutic effect of suppressor miR-665 and the combination of siRNA-MYC + siRNA-HDAC8 for neuroblastoma treatment.

Keywords: neuroblastoma, miRNA, siRNA, MYC, HDAC8

1. Introduction

Neuroblastoma is the most frequently diagnosed extracranial solid tumor in children. About 90% of cases occur in children less than 5 years old and it is rare in adults. Of cancer deaths in children, about 15% are due to neuroblastoma [1]. Chances of long-term survival, however, are less than 40% despite aggressive treatment [2].

MYC is an oncogenic transcription factor that is overexpressed in many types of cancer. MYC has been shown to directly upregulate a protumorigenic group of miRNAs and represses several suppressor miRNAs, thus contributing to tumorigenesis [3]. For example, MYC overexpression can upregulate the oncogenic miR-17-92 cluster, that are directly activated in lymphoma [4], and can also repress several suppressor miRNAs [3]. The MYC gene is amplified in various human cancers, including in lung carcinoma, breast carcinoma, and colon carcinoma [5].

Histone deacetylases affect gene expression by altering the histone acetylation status, and that as a consequence, HDAC overexpression contribute to tumorigenesis by affecting the expression of key mRNAs and miRNAs. HDACs are overexpressed in most cancers, leading to histone deacetylation, inhibition of growth- suppressive genes, and increased cell proliferation [6]. HDAC8 overexpression correlates with advanced neuroblastoma in patient tumor samples, and HDAC8 inhibition reduced cell proliferation and induced neuroblastoma cell differentiation [7]. HDAC inhibitors reduced the proliferation and induced the apoptosis of neuroblastoma cells in vitro and in vivo in mice [8, 9].

Given that both MYC and HDACs play an important role in the maintenance of the normal cellular physiological functions and that their overexpression is linked to neuroblastoma tumorigenesis, we asked whether the levels of both MYC and HDAC8 should be reduced to obtain significant inhibition of cell proliferation.

Our results demonstrate that miR-665 targets c-MYC and HDAC8 m RNA, miR-665 treatment also increased the percentage of cells in G1 phase and reduced the percentage of cells in S phase of the cell cycle. This is the first report to show that miR-665 is a suppressor miRNA directly targeting the 3'-UTR of c-MYC and HDAC8 in neuroblastoma [10].

We investigated the effects of small interfering RNAs (siRNAs) targeting HDAC8 and MYC in murine neuroblastoma cells. RNA interference is a process of posttranscriptional gene silencing in which a double stranded RNA inhibits gene expression in a sequence-dependent manner via degradation of the corresponding mRNA. siRNAs can be used as potent and specific tools for gene knockdown. Several laboratories have reported siRNA targeting of gene expression in cancer cells and the inhibition of cell proliferation *in vitro* and tumor growth *in vivo* [11–14].

We reported that *in vitro*, single-agent siRNA HDAC8 or siRNA-MYC inhibited cell proliferation by 40–50%; however, treatment with the combination of siRNA MYC + siRNA-HDAC8 inhibited cell proliferation by 86% [10]. To further confirm these findings in an animal model, we set out to verify if tumor growth can be inhibited in a neuroblastoma xenograft mouse model when tumors are treated with a combination of siRNA-MYC and siRNA HDAC8. Our findings from this study show that the tumor growth was reduced by 80% following intratumoral delivery of a combination of siRNAs targeting both MYC and HDAC8 simultaneously [15].

2. Materials and methods

2.1 Reagents

Cell culture media, DMEM with high glucose (D6429), essential and non-essential amino acids (M5550, m7145), Bt2c AMP (D0627), the colorimetric Caspase 3 kit (Code CASP-3-C), and propidium iodide (P4170) were purchased from Sigma Aldrich, St. Louis. Fetal bovine serum (FBS) was purchased from Phenix Research Products, Candler, NC, USA. BD-Falcon tissue culture 96-well plates (353072) were purchased from BD Biosciences. The RNA extraction miR-Neasy kit (Cat No. 217084) was purchased from Qiagen, Germantown, MD, USA.

The MTS Cell. Titer 96 Aqueous One Solution (Cat # G3580) cell proliferation assay was purchased from Promega Biotechnology, Madison,WI, USA. The HDAC Kit (#K331–100) was purchased from BioVision, Inc. Co rning. 96-well EIA/RIA plates (CLS3369) were used for ELISA. Antibodies for HDAC 8, H-145 (sc11405), C MYC, C-19 (SC-786), acetylated Histones, Ac- H2B, Lys 5/12/15/20 (SC-8652), Ac-H3, lys9 (sc-8655), Ac-H4, lys16 (sc-8662), and siRNA for c-MYC (pool of 4 different siRNA duplexes, sc-29227) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Negative control #2 siRNA (#4390846), siRNA-HDAC 8 (S88696) and Lipofectamine RNAi Max (#13778075) were purchased from Life Technologies/ Ambion/ Invitrogen. Negative control miRNA Cel-miR-67 (#CN-001000) sequences based on *C. elegans* miRNA, mimic hsa-miR-665 (#C 301246–01), and transfection reagent Dharmafect Duo (#T2010–01) were purchased from Dharmacon. Luciferase expression plasmids with the 3'-UTR for HDAC8 (#S804229), C-MYC (#S804638), MYCN (Product No S807230), or empty vector without 3'-UTR (#S890005), and the LightSwitch luciferase assay kit (#32031, LS010) were purchased from Active Motif, CA, USA.

2.2 Cells and cell culture

Mouse neuroblastoma cholinergic clonal cells (S20) were obtained from Dr. Marshall Nirenberg of The US National Institutes of Health (NIH). Cells were grown in monolayers in DMEM supplemented with essential and nonessential amino acids, penicillin/streptomycin, and 10% FBS at 37°C with 5% CO_2 and humidity.

Neuroblastoma cells were plated in 96-well plates at 12×10^3 cells per well and After 48–72 h, cell viability was measured colorimetrically using the MTS Cell Titer 96 Aqueous One Solution. Samples were incubated at 37°C for 3–4 h and samples were read at 490 nm in a plate reader according to the manufacturer's instructions.

For cell cycle analysis, 1×10^6 cells were plated in T25 flasks. After 48 h, cells were trypsinized, treated with 75% ethanol and 100ug/ml RNAse A, and then stained with propodium iodide (PI). Untreated and (20,000 cells/ sample) were analyzed for cell cycle distribution via flow cytometry at the Core lab of Children's Cancer Center Hospital, Houston, TX, USA.

2.3 Transfections

The effects of miR-665 or siRNA on cell proliferation were determined using reverse transfection. First, 100 nM negative control miRNA, miR-665, negative control siRNA, C-MYC siRNA, or HDAC8 siRNA was mixed with Lipofectamine RNAimax. This mixture was added to 12×10^3 cells, which were then plated in 96-well plates. After 48–72 h, cell viability was measured using MTS Cell Titer 96 Aqueous One Solution and incubated at 37°C for 3–4 h. Samples were read at 490 nm according to manufacturer's instructions.

miRNA effects on the cell cycle were assessed using reverse transfection of cells with 100 nM negative control miRNA or miR-665 mimic plus Lipofectamine RNAimax. The transfection mixture was added to 1×10^6 cells, which were then plated in a T25 flask. After 48 h, cells were trypsinized, treated with 75% ethanol and 100ug/ml RNAse A, and then stained with PI. For cell cycle analysis, 20,000 cells/sample were analyzed via flow cytometry in the Core lab of Children's Cancer Center Hospital, Houston, TX.

2.4 Whole cell extracts

Cell extracts were prepared from untreated,, and miRNA transfected cells for target assays. miRNA-transfected cells were reverse transfected with 100 nM

negative control miRNA, miR-665, negative control siRNA, c-MYC siRNA, or HDAC8 siRNA plus Lipofectamine RNAimax. Transfected cells were plated in T25 flasks. After 48–72 h, cell extracts were prepared in assay buffer as described by Khandelia, *et al.* [16]. Assay buffer consisted of 20 mM Tris–HCL pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P40, and protease inhibitor cocktail from Sigma (P8340). Protein concentrations were determined using Pierce's BCA Assay as per the manufacturer's instructions.

miR-665 inhibit cell growth compared to untreated cells and cells treated with negative control miRNA. Assays were normalized using equal concentrations of protein (50–100 ug) from untreated, negative control miRNA-, and miR-665-treated cells in assessing total HDAC and Caspase 3 activity, and HDAC8 and c-MYC levels via ELISA.

2.5 Quantitation of miR-665 in transfected cells

Mouse neuroblastoma cells were transfected with 100 nM miR-665 mimic and negative control cel-miR-67. 48 h post-transfection, total RNA was extracted from three biological replicates per treatment using the Qiagen RNEasy mini kit. miR-665 was quantitated via realtime qPCR by Arraystar, Inc. (Rockville, MD, USA).

Real-time PCR was performed for each RNA sample to quantify miR-665 and the housekeeping gene, U6. According to the standard curve, mRNA concentrations in each sample are determined directly using Rotor-Gene Real-Time Analysis software v.6.0 and the $2^{\Delta\Delta Ct}$ method.

2.6 Total HDAC activity

Total HDAC activity was measured in 50–75ug of protein from cell extracts prepared from untreated, or negative control miRNA- or miR 665-transfected cells using the Biovision kit (#K331–100). Acetylated HDAC substrate and other reagents were added according to the manufacturer's instructions and the final deacetylated product was read at 405 nm in a plate reader.

2.7 HDAC8 and c-MYC protein quantitaion via ELISA

HDAC8 and c-MYC proteins were quantitated using cell extracts prepared from untreated or 1 mM Bt2cAMP treated cells, or negative control miRNA- or miR-665- transfected cells via ELISA. 100ug protein per sample was mixed with 0.02 M carbonate coating buffer (pH 9.5) and added to 96-well BD-Falcon ELISA plates.

Samples were incubated at 4°C for 15 h. Wells were blocked with 10% FBS in PBS, treated with antibodies (diluted 1:30) specific for HDAC8 (SC11405) or c-MYC (SC-798), and incubated at 37°C for 2 h. Samples were washed with PBS + 0.05% Tween, treated with goat anti-rabbit IgG.

HRP secondary antibody (diluted 1:500), and incubated at 37°C for 1 h. Wells were washed and treated with substrate TMB and incubated at room temperature for 30 min, and then the reaction was stopped with 2 N H_2SO_4. Samples were read at 450 nm in a plate reader.

2.8 Caspase 3 activity

Caspase 3 activity was measured in 50ug protein from untreated, Bt2cAMP-treated, or miR-665-transfected cells using Sigma Aldrich's colorimeter kit (Code

CASP3-C). 50ug protein was mixed with the peptide substrate, Ac-DEVD-pNA (p-nitroanilide), in the presence of 10 mM DTT. Caspase 3 hydrolyzes the substrate, releasing p-nitroaniline, which is read at 405 nm. The specificity of caspase 3 activity was determined in the presence of the inhibitor, Ac-DEVD- CHO.

2.9 Target validation using luciferase expression plasmids

HepG2 cells were used for miR-665 target validation, because miR-665 does not inhibit the growth of these cells. When mouse neuroblastoma cells were used for target validation, the negative control luciferase vector plasmid without any target 3'-UTR showed a 50% decrease in luciferase activity when co-transfected with miR-665 compared to negative control miRNA. This decrease in luciferase activity was non-specific and was caused by cell growth inhibition due to miR-665 transfection.

To validate miR-665 targets, HepG2 cells were grown for 24 h in a 96-well plate. Cells were then co transfected with 100 ng luciferase expression plasmids containing the 3'-UTR for HDAC8, c-MYC, or MYCN, or the empty vector without any target 3'UTR, plus 100 nM negative control miRNA or miR-665 with Dharmafect Duo transfection agent. After 48 h of cotransfection, luciferase activity was measured using the Active Motifs LightSwitch luciferase assay kit. Luminescence was read on a Molecular Devices Soft Max Pro5 luminometer.

2.10 Histone acetylation

Histone acetylation was quantified via ELISA in cell extracts prepared from cells transfected with negative control miRNA, miR-665, negative control siRNA, or HDAC8 siRNA. 100ug protein was mixed with 0.02 M carbonate coating buffer (pH 9.5), added to 96-well BD Falcon ELISA plates, and incubated at 4°C for 15 h. Wells were blocked with 10% FBS in PBS, treated with acetylated antibodies (diluted 1:30) for Ac H2B (Lys 5/12/15/20), Ac-H3 (lys9), or Ac-H4 (lys16), and incubated at 37°C for 2 h. Samples were washed with PBS + 0.05% Tween, treated with an appropriate HRP-conjugated secondary antibody (diluted 1:500), and incubated at 37°C for 1 h. Wells were washed and treated with substrate TMB and incubated at room temperature for 30 min, and then the reaction was stopped using 2 N H_2SO_4. Samples were read at 450 nm in a plate reader.

2.11 Neuroblastoma tumor model

Mice experiments were performed with the approval of the institutional Animal Care and Use Committee, IACUC at Nanospectra Biosciences Inc. Houston, Texas.

A/J female mice six weeks old were purchased from Jackson Laboratory, Bar Harbor, Maine, USA. Murine neuroblastoma cells, 1x106 cells in DMEM media with 50% matrigel in 100 ul without fetal bovine serum and without antibiotics were subcutaneously injected on the right flanks. After 12 days, tumor growth can be seen and tumors were measured with a caliper.

When tumors reached 100 mm3 in size, mice were divided into two groups with 8–10 mice in each group.

Intratumoral delivery of siRNA.

siRNA-HDAC8 (S88696), Sense Sequence: (5′----3′).

CGACGGAAAUUUGACCGUAtt.

Antisense Sequence:

UACGGUCAAAUUUCCGUCGca.

siRNA-MYC (S70224), Sense Sequence: (5′------3′).

AGGUAGUGAUCCUCAAAAAtt.

Antisense Sequence: UUUUUGAGGAUCACUACCUtg.

Negative control #2 siRNA (#4390846), and Lipofectamine RNAi Max (#13778075) were purchased from Life Technologies/Ambion.

A total of 3 nmol Negative control siRNA or 3 nmol combinations of siRNA-MYC + siRNA-HDAC8 were mixed with Lipofectamine RNAi max (Liposome) in DMEM media without fetal bovine serum and without antibiotic. siRNA complexed with Lipofectamine in a volume of 30 ul was delivered into tumors by intratumoral injection every third day. Tumors were measured every second day with a caliper and mice were weighed every third day. Tumor volume was calculator with a formula, $V = Length \times width2/2$. Experiment was stopped when the control tumors treated with negative control siRNA reached a tumor burden volume of 1200 mm3. Mice were euthanized by CO_2 inhalation 2 days after last treatment with siRNA. Tumors were removed and weighed. Tumors were frozen in liquid nitrogen and stored at -80o C freezers until used for preparation of tumor extracts for ELISA.

2.12 Tumor extract preparation

Tumors treated with NC-siRNA or with combined siRNA-HDAC8 + siRNA-MYC were cut into small pieces and homogenized in assay buffer in a glass homogenizer. Assay buffer as described by Khandelia et al. [16], consisted of 20 mM Tris–HCL pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P40, and protease inhibitor cocktail from Sigma (P8340). Protein concentrations were determined using Pierce's BCA Assay as per the manufacturer's instructions.

2.13 Statistical analysis

Error bars represent standard error of the mean (SEM) from 2 to 3 biological replicates from 3 to 5 independent experiments. P-values were calculated using T.Test (2 tailed, 3 samples, unequal variance) and $p < 0.05$ was considered statistically significant.

3. Results

3.1 miR-665 inhibits cell proliferation

NB cells transfected with miR-665 show changes in cell morphology, lost the normal spindle shape and cells grew in clumps without processes compared to cells transfected with negative control miRNA (**Figure 1A** and **B**). Cell cycle analysis results show that miR-665 treated cells show an increase of 16% of cells in G1 phase of cell cycle and the cell number decreased by 18% in S phase compared to negative control miRNA transfected cells. miR-665 treatment did not affect the cells in G2 phase (**Figure 1C**). Cell viability decreased proportionally with the increasing concentration of miR-665, represented by black bars (**Figure 1D**).

3.2 miR-665 targets HDAC 8, c MYC and MYCN

Computational algorithm prediction site TargetScan and miRanda (microRNA.org) predicts mRNA targets for Mirna. *miranda* predicted hsa-miR-665 targets 3' UTR of HDAC 8 and the sequence alignment is presented in **Figure 2A**. *miranda*, also predicts that hsa-miR-665 targets MYCN 3' UTR and the sequence alignment is presented in **Figure 2B.**
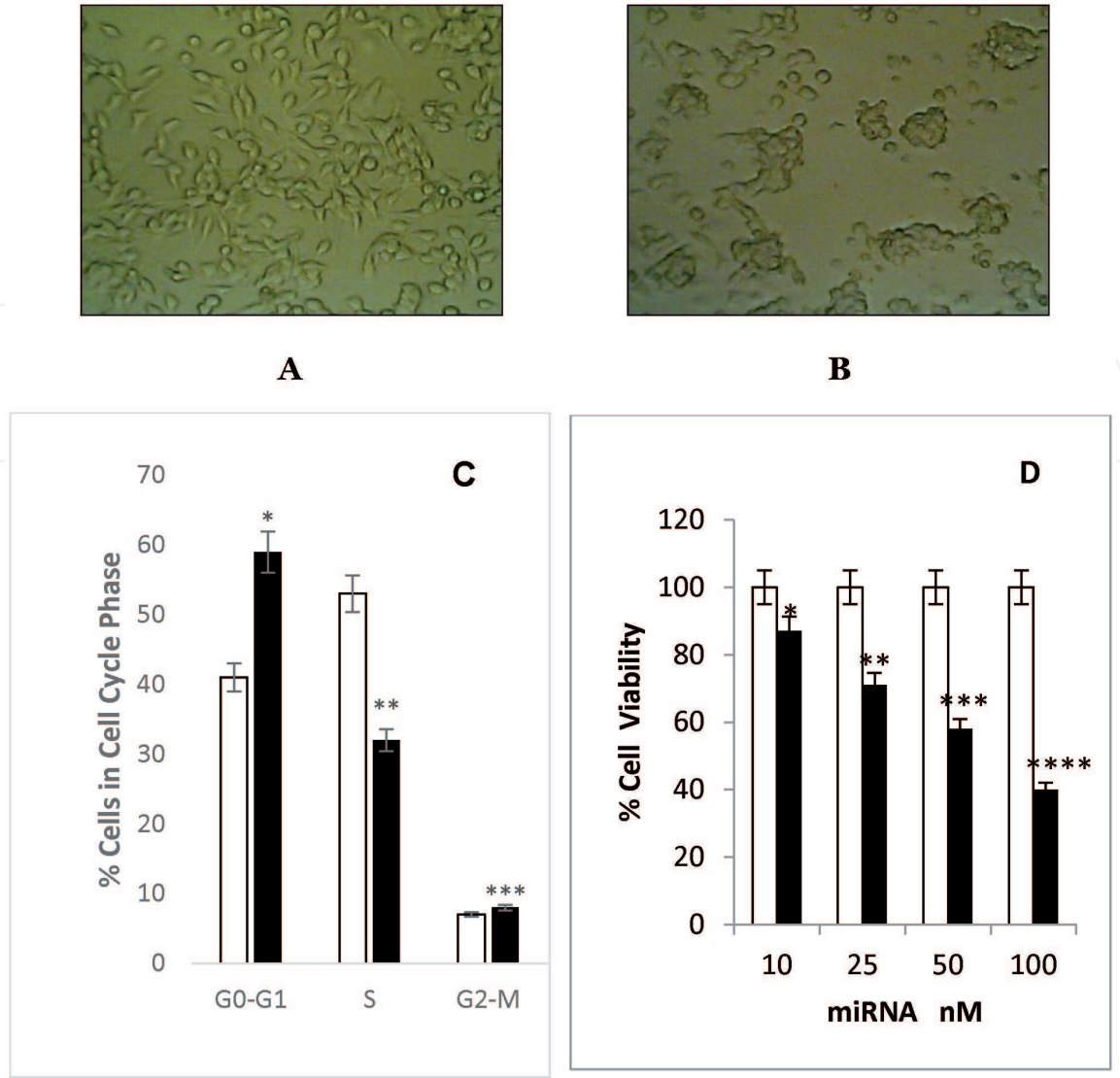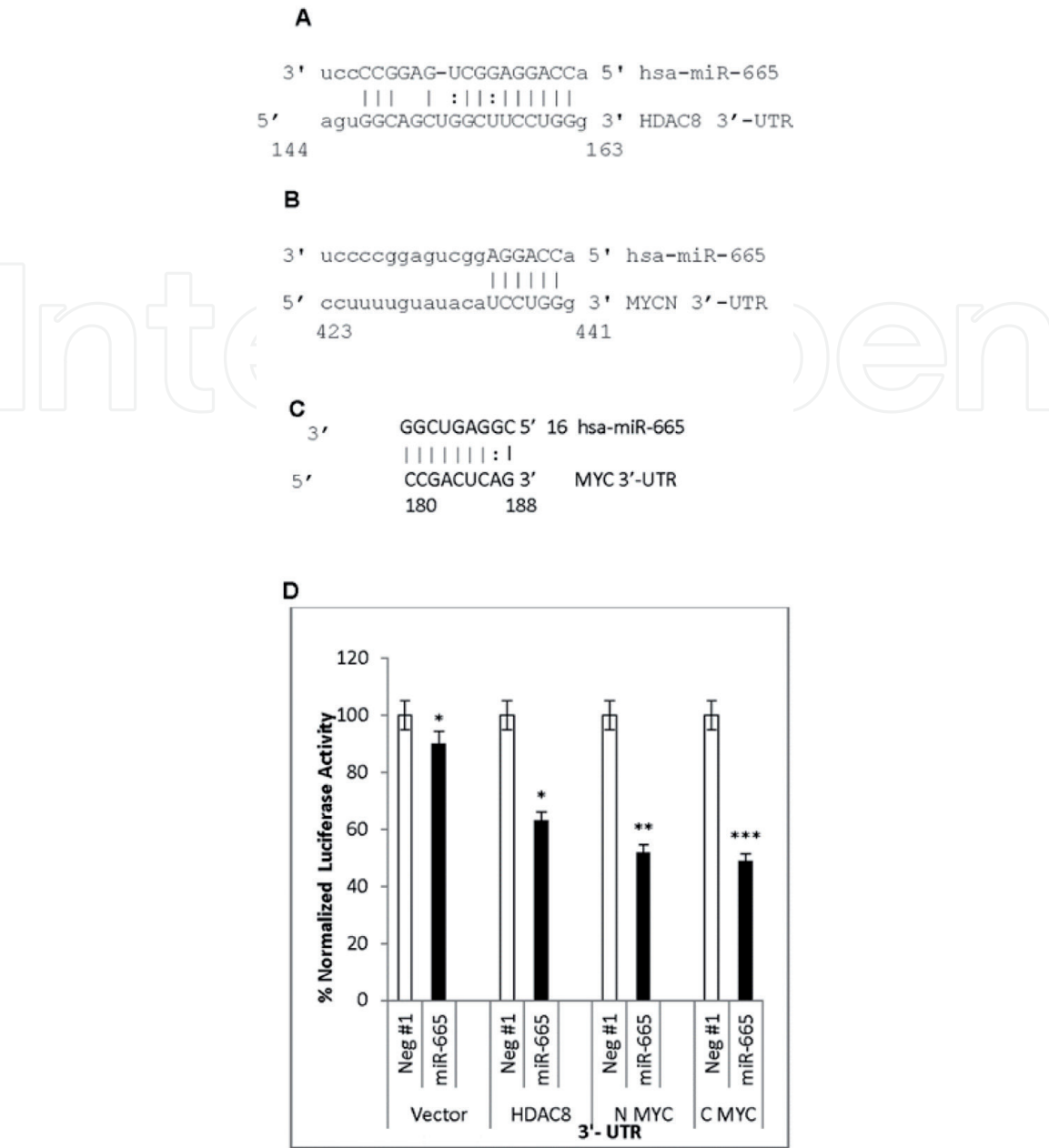
Figure 1.
miR-665 effects on cell proliferation (A) cells treated with 100 nM negative control miRNA and miR-665 (B) for 72 hr. were prepared for cell cycle distribution analysis. Propidium iodide stained cells were analyzed by FLOW cytometry (C) Several concentration of miR-665 effect on cell viability (D) STDEV was used for +/− standard error bar; data is from 2 independent experiments with 3 biological replicates for each experiment was used. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

MYC is overexpressed in 30% of all human cancers and frequently predicts for a poor clinical outcome, and deregulated expression of MYC is a hallmark feature of cancer [3] miRanda and Targetscan did not include 3' UTR of C MYC as miR-665 target. Therefore, Complimentary sequences between miR-665 and 3' UTR of C MYC were compared at online pairwise sequence alignment site www.ebi.ac.uk. Results show two complementary binding sites for miR-665 in the 3' UTR of c MYC (**Figure 2C**).

First we measured total HDAC activity in the cell extracts prepared from negative control miRNA and miR-665 transfected cells in the presence of acetylated HDAC substrate Ac-Lys (Ac)-p NA and deacetylated end product was measured colorimetrically. HDAC 8 and c MYC proteins were measured with antibody in ELISA. The results show that total Pan HDAC activity was decreased by 40%, and HDAC 8 and c MYC proteins were decreased by 40% in miR-665 transfected cells compared to negative control miRNA treated cells (**Figure 3A–C**).

Next, we tested whether HDAC 8, c MYC and MYCN genes are the direct target of miR-665. In these experiments HepG2 cells were used because miR-665 does not affect the growth of these cells (our unpublished results). Luciferase reporter

A

```
3' uccCCGGAG-UCGGAGGACCa 5' hsa-miR-665
       |||  |  :||:||||||
5'   aguGGCAGCUGGCUUCCUGGg 3' HDAC8 3'-UTR
     144                   163
```

B

```
3' uccccggagucggAGGACCa 5' hsa-miR-665
               ||||||
5' ccuuuuguauacaUCCUGGg 3' MYCN 3'-UTR
   423                 441
```

C

```
3'      GGCUGAGGC 5' 16 hsa-miR-665
        ||||||||:|
5'      CCGACUCAG 3'    MYC 3'-UTR
        180      188
```

D

Figure 2.
Predicted binding sites for miR-665 in targets HDAC 8, c MYC and MYCN 3' UTR. Computational prediction site miRanda (microRNA.org) predicted hsa-miR-665 targets 3' UTR of HDAC 8 and the sequence alignment is presented in (A). miranda, also predicts that hsa-miR-665 targets MYCN 3' UTR and the sequence alignment is presented in (B). miranda and Targetscan did not include 3' UTR of C MYC as miR-665 target. Therefore, complimentary sequences between miR-665 and 3' UTR of C MYC were compared at online pairwise sequence alignment site www.ebi.ac.uk. Sequence alignment is presented in (C). (D) miR-665 targets were validated by co transfection of 100 ng luciferase expression plasmids with 3'-UTR and 100 nM negative control miRNA and miR-665 into HepG2 cells. Empty vector without 3' UTR was used as a control. After 48 hr., luciferase activity was measured and normalized luciferase activity is presented. Data is presented from 2 independent experiments with 3 biological replicates were used. STDEV was used for +/− standard error bar. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

plasmids with 3' UTR were cotransfected with negative control miRNA and miR-665 in HepG2 cells and after 48 hr. luciferase activity were measured in cell extracts. Results show a 40% decrease in luciferase activity of HDAC 8 3' UTR, a 51% decrease in luciferase activity of c MYC 3' UTR and a 50% decrease in luciferase activity of MYCN 3'UTR from the cells co transfected with miR-665 compared to the co transfection with negative control miRNA (**Figure 2D**). These results validate that m RNAs of HDAC 8, c MYC and MYCN are the direct targets of suppressor miR-665.
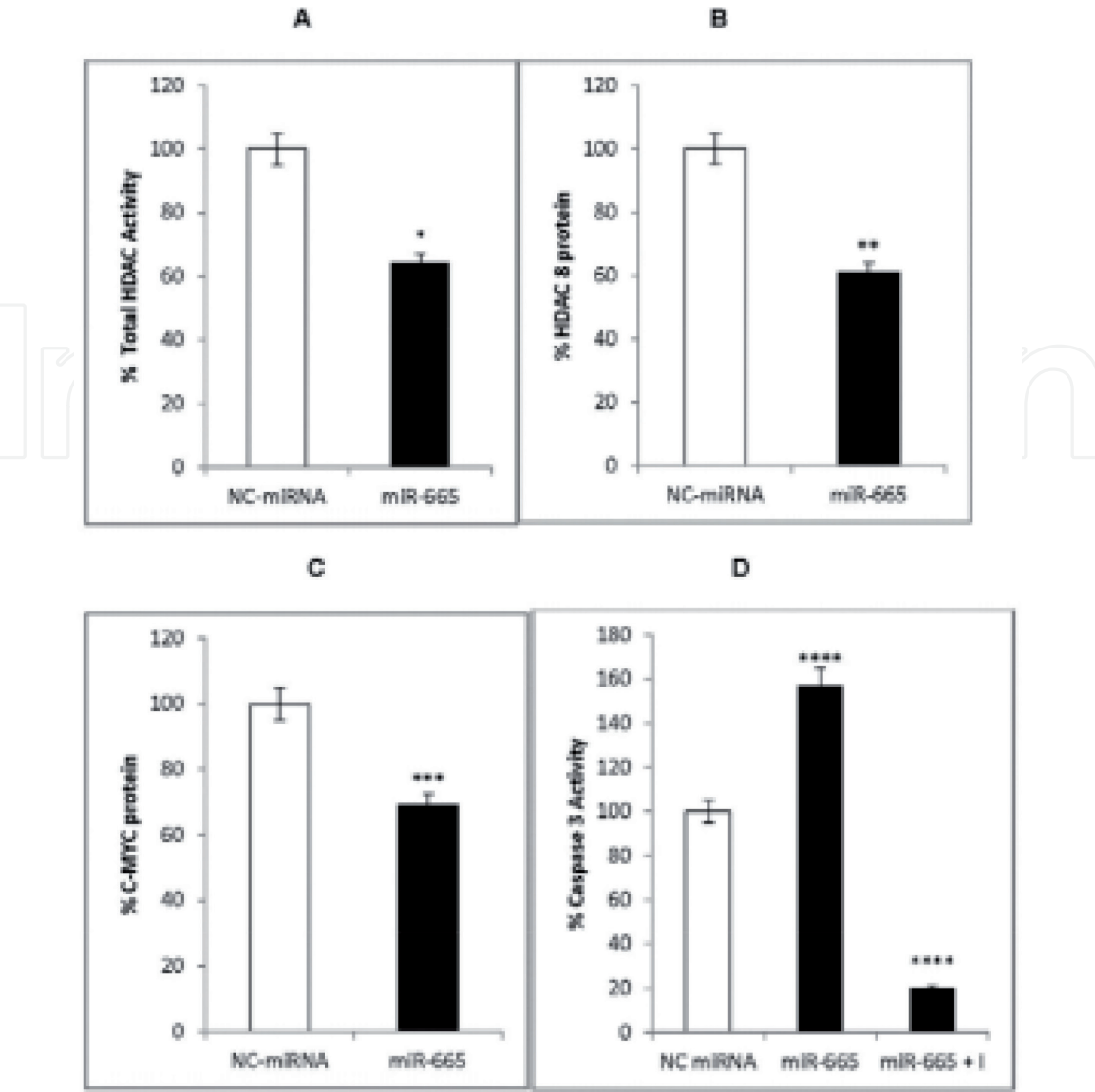
Figure 3.
miR-665 effect on target HDAC 8, c MYC and MYCN cell extracts from100nM negative control miRNA and miR-665 transfected cells were used for the quantitation of; (A) total HDAC activity, (B) HDAC8 protein by ELISA, (C) c MYC protein by ELISA and (D) caspase 3 activity and specificity of caspase 3 enzyme activity was determined in the presence of inhibitor. Data is presented from 2 independent experiments with 3 biological replicates were used. STDEV was used for +/− standard error bar. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

3.3 MiR-665 induced activation of caspase 3

HDAC inhibitors induce the caspase 3-dependent apoptosis [8] Suppressor miR-34a increased the activation of caspase 3 and caused caspase dependent apoptosis in neuroblastoma cells [17, 18]. Caspase 3 is a critical part of apoptosis, and is required for the DNA fragmentation and for the typical morphological changes of cells undergoing apoptosis. We investigated the effect of miR-665 on the activation of caspase 3 and activity was measured by the hydrolysis of the peptide substrate attached to p-nitroanilid. Caspase 3 activity was measured in cell extracts prepared from cells transfected with negative control miRNA and miR-665. Results show that caspase 3 activity was increased by 2.5-fold in miR-665 transfected cells compared to negative control miRNA (**Figure 3D**). Specificity of the caspase 3 was determined by the addition of caspase 3 inhibitor TSA before the addition of substrate in the assay. The results show that inhibitor binds to caspase 3 and inhibited 90% of miR-665 activated caspase 3 activity (**Figure 3D**). These

results show that mimic miR-665 activated caspase 3 in neuroblastoma cells, suggesting that miR-665 can inhibit cell growth and reduced viable cells by caspase 3 dependent apoptosis.

3.4 miR-665 levels following transfection

miR-665 levles were quantitated in neuroblastoma cells transfected with negative control miRNA and miR 665 using real time qPCR. Mouse neuroblastoma cells have very low levels of endogenous miR-665 **(Figure 4A)**; however, miR-665 expression increased 848-fold in cells transfected with mimic miR-665 compared to cells transfected with the negative control miRNA, cel miR-67 **(Figure 4A** and **B)**. miRNA levels reportedly increased by over 1000-fold in cells transfected with miR 200a [19]. Our results strongly indicate that miR-665 upregulation decreased MYC and HDAC8 expression, thus inhibiting proliferation and inducing apoptosis in mouse neuroblastoma cells.
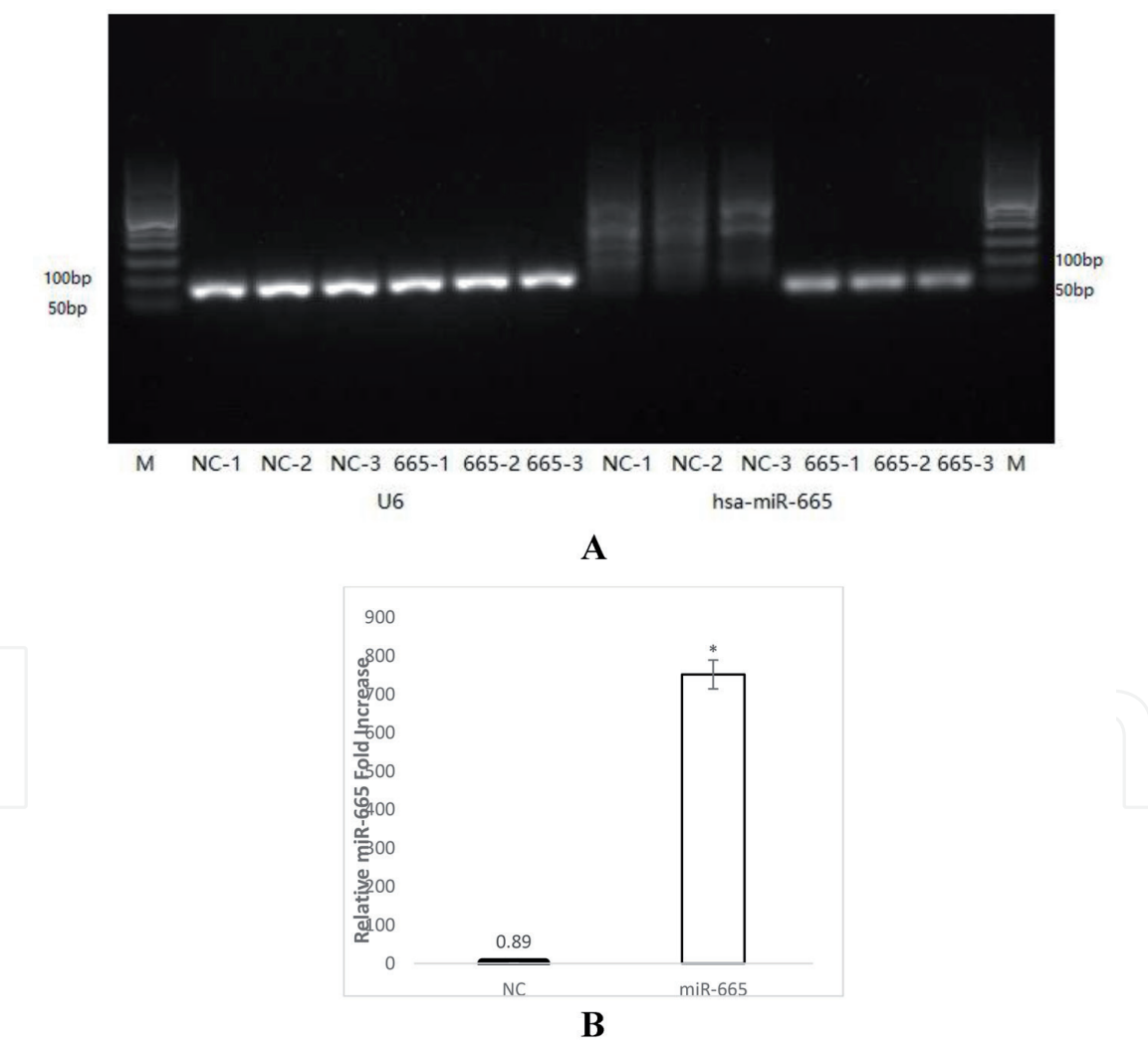
Figure 4.
Quantitation of miR-665 in transfected cells. miR-665 was quantitated via real-time qPCR normalized to the U6 gene from three biological replicates 48 h after transfection with negative control miRNA (cel-miR-67) or miR-665. From left, lane 1 and 14 (M), show DNA molecular weight ladder (A) lanes 2–7 (NC-1–NC3 and 665–1–665-3) show the U6 gene. Lanes 8–10 (NC-1–NC3) show miR-665 levels from cells transfected with negative control miRNA. Lanes 11–13 (665–1–665-3) show miR-665 levels from cells transfected with miR-665. The miR-665 fold increase in miR-665-transfected cells was quantitated using the 2-ΔΔCt method (B) miR665 levels are shown in cells transfected with negative control miRNA (black bar) and in miR-665-transfected cells (white). Error bars were calculated from the standard deviation from three biological replicates. *P < 0.54x10–6. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

3.5 siRNA effect on mouse neuroblastoma cells

miRNA targets hundreds of m RNAs and suppresses their expression, however, siRNA targets a specific m RNA. In these experiments, siRNA for HDAC 8 (siRNA-HDAC 8) and siRNA for MYC (siRNA-c MYC) were used to substantiate the effects of miR-665 on neuroblastoma cells.

3.6 SiRNA effect on cell proliferation

NB cells were transfected with negative control siRNA, siRNA-HDAC 8, siRNA-c MYC and the combination of siRNA-HDAC 8 + siRNA-c MYC. After 48 hr. of growth, cell viability was determined with CellTiter assay (Promega). SiRNA-HDAC8 inhibited 42% and siRNA-c MYC inhibited 55% of cell proliferation, however, the combination of both siRNAs inhibited 86% of the growth of the cells (**Figure 5A**). Therefore, the combination of siRNA-HDAC8 plus siRNA-c MYC was more targeted towards mRNA of HDAC8 and c MYC and caused more effective apoptosis and loss of cells. These results show that HDAC 8 and MYC are critical targets and inhibition of both targets is required for the inhibition of neuroblastoma.

3.7 SiRNA effect on HDAC 8 and C MYC

HDAC 8 and c MYC proteins were quantitated by antibody in ELISA in cell extracts prepared from the cells transfected with negative control siRNA and siRNA-HDAC 8. The results show that siRNA-HDAC 8 transfection inhibited 40% of HDAC 8 proteins (**Figure 5B**) as well as inhibited 35% of MYC protein. Inhibition of MYC may be indirect effect of HDAC 8 inhibition. HDAC8 inhibition increases acetylation of histones and alters gene expression, thus decreasing MYC expression. Therefore, miR-665 represses the expression of c MYC both at the transcription and at the post transcription levels. siRNA-HDAC 8 and siRNA-c MYC substantiated the effects of miR-665 on neuroblastoma cells.

### 3.8 siRNA activates caspase 3

Caspase 3 activity was measured in the cell extracts prepared from cells transfected with negative control siRNA, siRNA-HDAC8 and siRNA-c MYC. The results show that siRNA-HADC8 increased the activity of caspase 3 by 1.8-fold and siRNA –c MYC increased the activity of caspase 3 by 2.5-fold compared to negative control siRNA (**Figure 5C**). Therefore, the results of siRNA effects substantiate the effects of miR-665 on the activation of caspase 3.

3.9 Effect of miR-665 and siRNAs on histone acetylation

Our results show that miR-665 and siRNA-HADC8, decreased total HDAC activity and decrease HDAC 8 protein, therefore, we measured the acetylation of histones in the cell extracts and the results were compared among all treatments. MiR-665 transfected cells show increases in the acetylihtion of histones Ac-H2B by 25%, Ac-H3 by 40% and Ac-H4 by 50% compared to negative control miRNA transfected cells (**Figure 6A**). miR-665 acetylates predominantly H3 and H4 histones.

Likewise, Si RNA-HDAC8 treated cells also show increases in the acetylation of histones Ac-H2B by 38%, acetylation of Ac-H3 by 58% and show higher acetylation of Ac-H4 by 2-fold (200%) compared to negative control siRNA treated cells
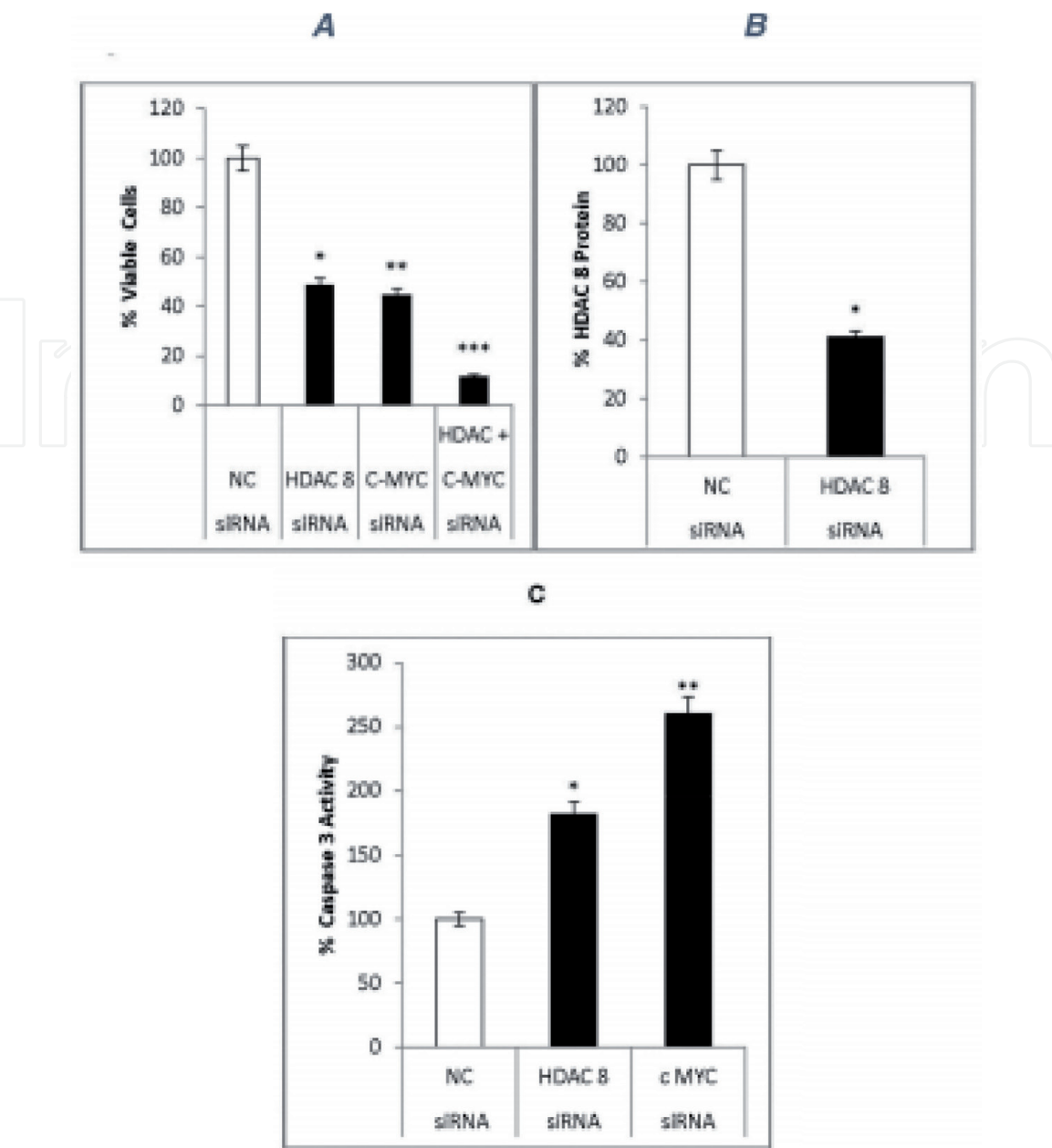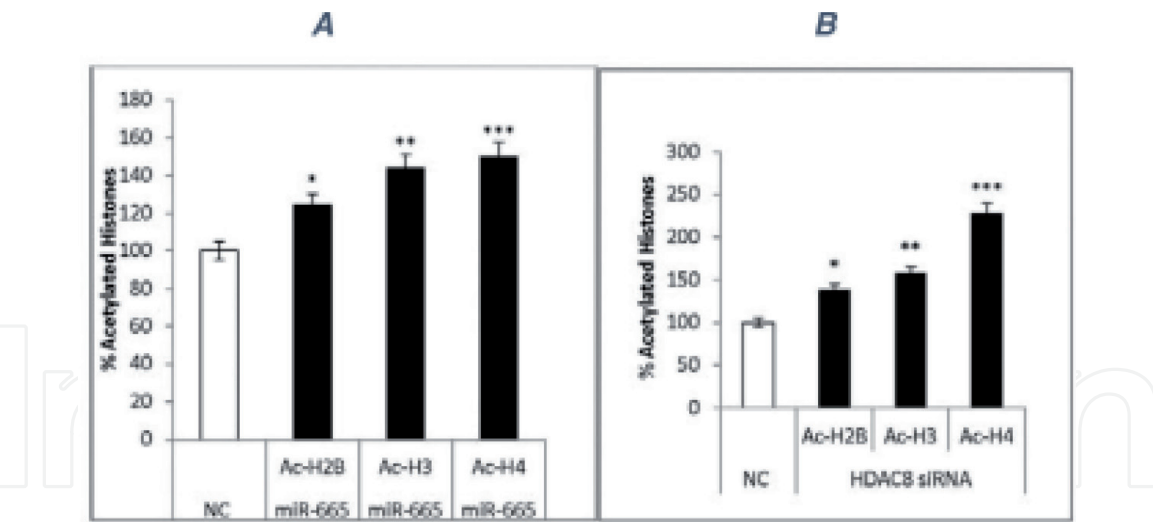
Figure 5.
siRNA effects on neuroblastoma cells.siRNA specific for HDAC8 (siRNA-HDAC8) or c-MYC (siRNA-c-MYC, a mixture of 4 siRNAs) were used to confirm the effects of miR665 in neuroblastoma cells. (A) siRNA effect on cell proliferation. Neuroblastoma cells were transfected with 50 nM siRNA-HDAC8, 100 nM siRNA-c-MYC, or both siRNAs together. Cell viability was measured via MTS assay. SEM bars represent the standard deviation from two independent experiments with three biological replicates each. *P < 0.005, **P < 0.001, ***P = 6.8x10–5. (B) Cell extracts from negative control siRNA- or siRNA-HDAC8-treated cells were used to quantify HDAC8 levels via ELISA. HDAC8 was down regulated in HDAC8-siRNA-transfected cells *P < 0.04. (C) Caspase 3 activity was quantified in cell extracts via Casp-3 kit. Caspase 3 activity increased in siRNA-HDAC8- and siRNA-c-MYC-transfected cells. SEM bars represent the standard deviation from two independent experiments with two biological replicates each. *P < 0.01, **P < 0.004. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

(**Figure 6B**). siRNA-HDAC8 acetylated predominately AC-H4 and correlate with the results of miR-665.

3.10 miR-665 targets c-MYC and HDAC8

Taken together, our results indicate that miR-665 targets c-MYC and HDAC8, decreasing their expression, increasing histone acetylation, and modulating expression of cell proliferation related genes. We propose a model (**Figure** 7)

Figure 6.
Histone acetylation. Cell extracts from negative control miRNA-, miR-665-, negative control siRNA-, or siRNA-HDAC8 treated cells were used to quantitate histone acetylation via ELISA. Data represent standard deviations from two independent experiments. Negative control miRNA (white) and miR-665 transfection increased histone acetylation (black) (A) *P < 0.01, **P < 0.01, ***P < 0.04. Negative control siRNA (white) and siRNA-HDAC8 increased histone acetylation (black) (B) *P < 0.008, **P < 0.04, ***P < 0.001.
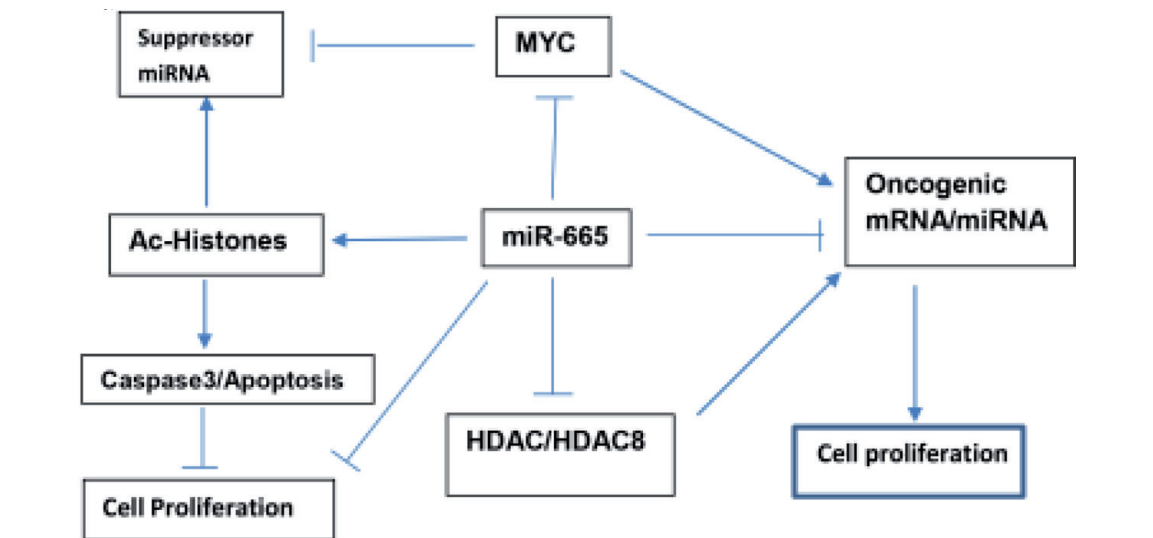
Figure 7.
Proposed model illustrating how suppressor miR-665 targets c-MYC and HDAC8 to inhibit neuroblastoma cell proliferation and maintain cellular homeostasis. (figures were printed from published article in "Oncotarget", N.Prashad Vol 9, 33186–33201, 2018).

illustrating suppressor miR-665 involvement in the inhibition of neuroblastoma cell proliferation [10].

3.11 Effects of combination of siRNA-HDAC 8 ± siRNA-MYC on neuroblastoma cells in vitro

When cells were treated with the combination of siRNA HDAC 8 + siRNA-MYC, cell proliferation was inhibited by 86% [10]. Therefore, HDAC 8 and MYC are critical targets and effective blockade of both targets is required to ensure a maximum inhibition of neuroblastoma cell proliferation.

On the basis of these results, we hypothesized that neuroblastoma tumor xenograft growth in mice can be inhibited when treated with the combination of siRNA-MYC + siRNA- HDAC8.

3.12 Neuroblastoma tumor treatment with the combination of siRNA-HDAC8 + siRNA-MYC

We explored the therapeutic effect of the combination of siRNA-HDAC8 + siRNA-MYC treatment on neuroblastoma tumor xenograft in mice. A total of 1x106 mouse neuroblastoma cells in 50% Matrigel were inoculated sub-cutaneously in 6 weeks old female A/J mice. Tumors were formed with an average
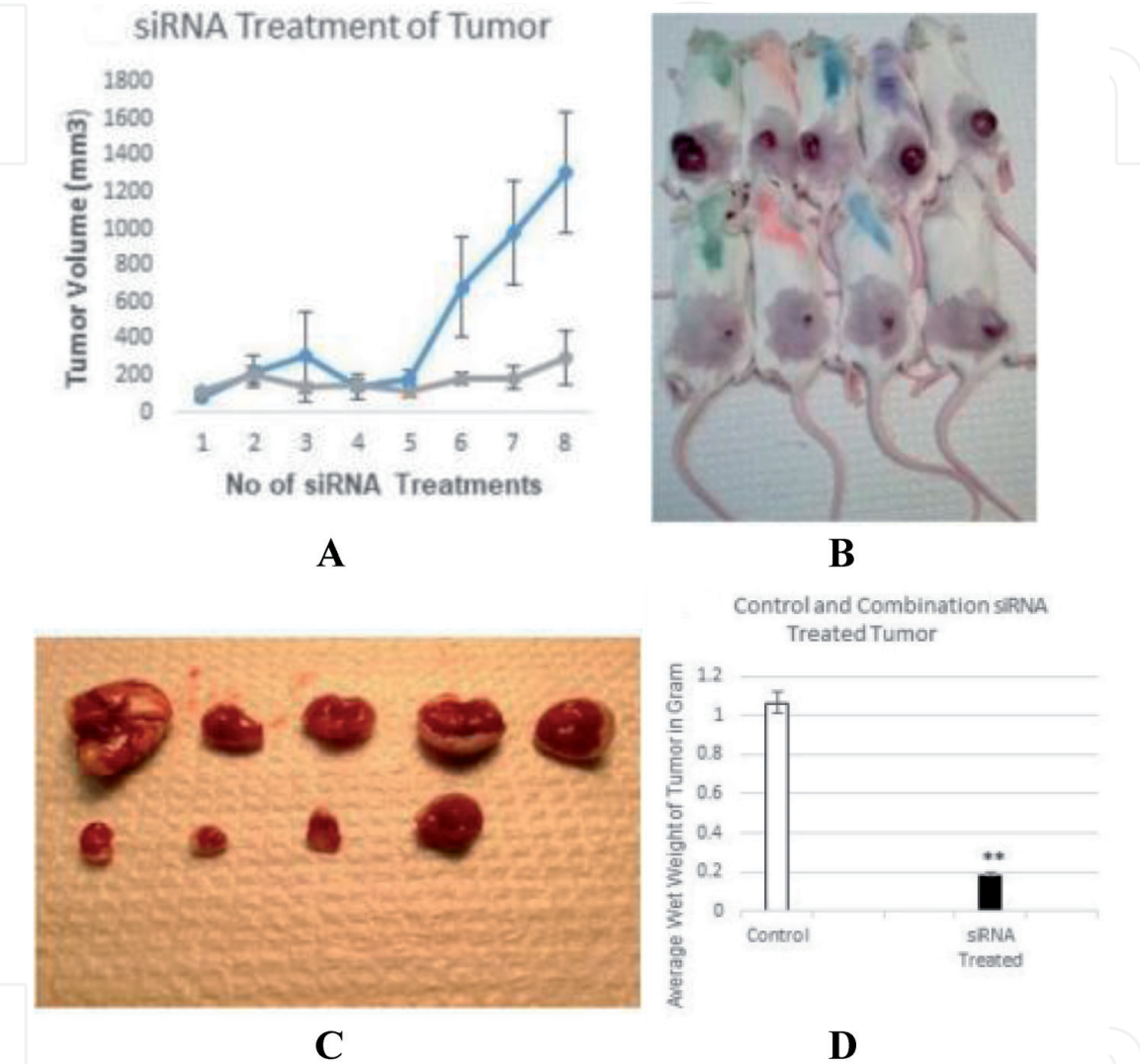
Figure 8.
(A) Effect of combination of siRNA on the growth of neuroblastoma tumor. Murine neuroblastoma cells, 1 × 106 cells in DMEM media with 50% matrigel in 100 ul were subcutaneously injected on the right flanks. After 12 days, tumor growth can be seen and tumors were measured with a caliper. When tumors reached 100 mm3 in size, mice were divided into two groups with 8 mice in each group. Negative control siRNA or combination of siRNA-MYC + siRNA-HDAC8 was mixed with Lipofectamine RNAi max (liposome) in DMEM media without fetal bovine serum and without antibiotic. siRNA complexed with Lipofectamine in a volume of 30 ul was delivered into tumors by intratumoral injection every third day. Tumors were measured every second day with a caliper and mice were weighed every third day. Tumor volume was calculator with a formula, V = length × width 2 /2. The numbers on X-axis show the number of siRNA treatments. Control tumor growth is shown by diamond (^) markers and the growth of tumors treated with siRNAs is shown by round (o) markers. SEM bars represent the standard deviation from 8 mice at each point P* < 0.031. (figure is printed from published article in "J. Cancer Biology and Therapeutics" N. Prashad V 6: 301–307 2020). (B) Mice with control and combination of siRNA treated tumors. Top row of mice with control tumors treated with NC siRNA and the bottom row of mice with tumors treated with the combination of siRNA-MYC + siRNA-HDAC. (C) Tumors from control and combination of siRNA treated mice. Top row representative control tumors treated with NC-siRNA and the bottom row representative tumors treated with the combination of siRNA-MYC + siRNA-HDAC8. (figure is printed from published article in "J. Cancer biology and therapeutics" N. Prashad V 6: 301–307 2020). (D) Weights of control and combination of siRNA treated tumors. Average of 8 control tumors was 1 gram and average of 8 tumors treated with combination of siRNA MYC + siRNA-HDAC8 was 0.186 gram. SEM bars represent the standard deviation from 8 tumors P** < 0.004.

volume of 100 mm3, 12 days after cells were inoculated. A 3 nmol negative control siRNA (NC-siRNA) or a 3 nmol combination of siRNA-HDAC + siRNA-MYC complexed with Lipofectamine RNAi max (Invitrogen) were inoculated into 10 tumors each by intratumoral injections every 3rd day. Tumor growth was measured every 2 days with a caliper and volume was calculated with the formula, length X width2/2. The growth of control tumors treated with NC-siRNA increased, however, the tumors treated with the combination of siRNA-HDAC8 + siRNA-MYC show inhibition of the growth of tumors (**Figure 8A**). The rates of tumor growth were significantly decreased when treated with combined siRNA-MYC + siRNA-HDAC8 compared to tumors treated with control negative siRNA.

All mice experiments were performed under IACUC approved animal study protocol.

Experiment was stopped when the control tumors reached an average volume of over 1200 mm3, then mice were euthanized by CO_2 and tumors were removed and weighed. Pictures of mice with tumors were taken before tumors were removed (**Figure 8B**) and pictures of tumors removed and weighed are shown in (**Figure 8C**). The average wet weight of 8 control tumors treated with NC-siRNA and tumors treated with combination of siRNA-HDAC + siRNA-MYC is presented in **Figure 8D**. The average weight of tumors treated with the combination of siRNA-HDAC8 + siRNA-MYC was decreased by 5-fold [0.186 g] compared to average weight of control tumors [1 g] treated with NC-siRNA. Tumor xenograft experiment was repeated twice with 10 mice treated with NC-siRNA and 10 mice treated with a combination of siRNA-HDAC8 + siRNA-MYC.

3.13 The quantitation of targets HDAC 8 and MYC in tumors

Tumor targets HDAC 8 and MYC proteins were quantitated by ELISA in extracts prepared from tumors treated with negative control siRNA and combination of siRNA-HDAC 8 + siRNA-MYC treated tumors. The results indicate that targets HDAC 8 and MYC were decreased by 85% and 65% in tumors treated with the
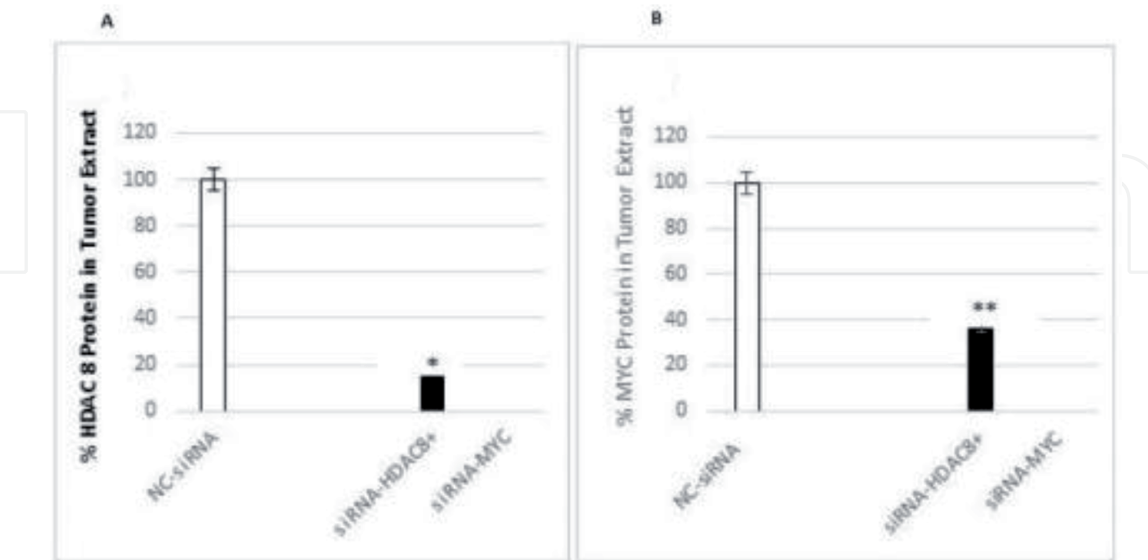
Figure 9.
(A, B) Quantitation of Myc and Hdac8 proteins from control and combination of siRNA treated tumors. Tumor targets Hdac8 and Myc proteins were quantitated by ELISA in extracts prepared from 3 tumors treated with negative control siRNA and 3 tumors treated with the combination of siRNA-HDAC 8 + siRNA-MYC. Average targets Hdac8 (A) and Myc (B) proteins were decreased by 85% and 65% in tumors treated with the combination of siRNA-HDAC8 + siRNA-MYC compared to tumors treated with NC-siRNA. SEM bars represent the standard deviation from 3 tumors ($P^* < 0.030$, $P^{**} < 0.01$). (figures printed from the article published in "J Cancer Biol Therap," N. Prashad 6(1): 301–307 (2020)).

combination of siRNA-HDAC8 + siRNA-MYC compared to tumors treated with NC-siRNA (**Figure 9A** and **B**).

The results indicate that a decrease in the tumor targets HDAC 8 and MYC caused the inhibition of the growth of tumors.

4. Discussion

miRNAs are both oncogenic and tumor suppressors. In normal cells homeostasis is maintained by keeping equilibrium between oncogenic and suppressor miRNA. If this equilibrium is disrupted that can cause dysfunction with increases in oncogenic miRNA and decreases in suppressor miRNA. A decrease in a specific suppressor miRNA can cause overexpression of HDCAs, c MYC and MYCN which can alter gene expression and cause cancer. However, when suppressor miRNAs are added exogenously to these cells then these cells restore normal properties and show growth arrest and apoptosis Therefore, suppressor miRNAs seem to be critical in the maintenance of cellular homeostasis.

A decrease in suppressor miRNA can over express genes like c MYC, MYCN and HDACs and cause cancers. Over expression of c MYC and MYCN cause the down regulation of suppressor miRNAs. HDACs indirectly effect gene expression by the deacetylation of histones, therefore, this process can also effect the expression of miRNA.

Transfection of miR-665 into murine NB cells caused growth inhibition, cell cycle arrest, decreased total HDAC activity, decreased HDAC8 and MYC protein expression, activated caspase 3 and increased the acetylation of histones. miR-665 targets HDAC8, c MYC and MYCN oncogene and decreases their expression. These targets are validated by the co- transfection of luciferase reporter with target 3' UTR and miRNA-665. Therefore, miRNA 665 directly targets HDAC 8, MYCN and c MYC in the inhibition of mouse neuroblastoma cells. This is the first report to show that miR-665 is a suppressor miRNA of mouse neuroblastoma.

In targeted therapy of cancer, critical genes and proteins involved in the tumorigenesis are identified and therapeutic agents' miRNA and siRNA are used to inhibit the expression of target genes to inhibit the growth of cells in vitro and in vivo. SiRNA-mediated gene knockdown is much more potent and specific with only one mRNA target, whereas miRNA has multiple mRNA targets. siRNA therapeutic approach was used in gene targeting overexpressed cancer proteins in inhibiting cancer cell growth in vitro and inhibited tumor growth in vivo in the following mouse models: breast cancer mouse model, Glioma cells tumor and colon cancer tumor. MYCN, c-MYC, and HDAC8 may each contribute to neuroblastoma tumorigenesis. We reported that transfection of mimic suppressor miR-665 inhibited the expression of c-MYC and HDAC 8 and increased caspase 3 involved in apoptosis and inhibited the growth of neuroblastoma cells in vitro [10].

Our data also indicate that both c-MYC and HDAC 8 are critical targets and targeting these two targets with siRNA inhibited cell growth by 86% in vitro. The combination of siRNAs inhibited tumor growth in vivo by 80%, therefore, inhibiting more than one target is critical for the successful treatment of tumors in vivo.

5. Conclusion

Neuroblastoma is the most frequently diagnosed extracranial solid tumor in children. These tumors account for 15% of childhood deaths from cancer. Survival in one- year-old children is <30% despite aggressive therapies.

We used mouse neuroblastoma tumor model and identified MYC and HDAC8 are the critical targets in neuroblastoma tumorigenesis. Treatment of the tumors in mice with the combination of siRNA-MYC + siRNA-HDAC8 inhibited both the targets MYC and HDAC8 simultaneously and inhibited the growth of tumor by 80%. Therefore, inhibiting more than one target is critical for the successful treatment of tumors in vivo.

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Conflicts of interest

The author declares that they have no conflicts of interest.

Declarations

Ethics approval: Mice experiments were performed with the approval of the institutional Animal Care and Use Committee, IACUC at Nanospectra Biosciences Inc. Houston, Texas.

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