Award Number: W81XWH-13-1-0241

TITLE: Identifying microRNAs that Regulate Neuroblastoma Cell Differentiation

PRINCIPAL INVESTIGATOR: Dr. Liqin Du

CONTRACTING ORGANIZATION: University of Texas Health Science Center
San Antonio, TX 78229-3901

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| October 2015 | Annual | 1 Sep 2014 – 31 Aug 2015 |

**4. TITLE AND SUBTITLE**

Identifying microRNAs that Regulate Neuroblastoma Cell Differentiation

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-13-1-0241

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
**Liqin Du, PhD; Zhenze Zhao, phD; Alexander Pertsemlidis, PhD; Xiuye Ma**

E-Mail: dul2@uthscsa.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

**UT Health Science Center at San Antonio**
7703 Floyd Curl Drive.
San Antonio, TX 78229

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

In this reporting period, we mainly focused on characterizing another novel differentiation-inducing miRNA, miR-449a. We examined the differentiation-inducing function of miR-449a in multiple neuroblastoma cell lines. We have demonstrated that miR-449a functions as an inducer of cell differentiation in neuroblastoma cell lines with distinct genetic backgrounds, including the MYCN amplification status. We further show that miR-449s mimic induces cell cycle exit, induces cell apoptosis, and decreases the capacity of the neuroblastoma cells to form colonies. Our results support the strong tumor suppressive function of miR-449a in neuroblastoma cells, providing the experimental evidence for developing miR-499a-based differentiation therapy for neuroblastoma.

**15. SUBJECT TERMS**

Neuroblastoma, miRNA, miRNA mimic, high-content screen (HCS), neurite outgrowth, cell differentiation

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 27 | 19b. TELEPHONE NUMBER *(include area code)* |

**Progress Report**
(09/01/2014-08/31/2015)

## 1. Introduction

*The objective of this study* is to use a functional high-content screening **(**HCS) approach to directly and comprehensively identify microRNAs (miRNAs) that induce neuroblastoma cell differentiation by applying a library of miRNA mimics used to raise the intracellular levels of miRNAs. *Two specific aims* are proposed: **Aim 1,** identify miRNAs that induce neuroblastoma cell differentiation by HCS; **Aim 2,** Validate the function of the identified miRNAs to induce cell differentiation and cell growth arrest in neuroblastoma cells**.**

## 2. Keywords

Neuroblastoma, miRNA, miRNA mimic, miR-449a, neurite outgrowth, differentiation, differentiation marker, cell cycle exit, MYCN amplification

## 3. Overall Project Summary

In this reporting period, we mainly focused on characterizing another novel differentiation-inducing miRNA, miR-449a. We demonstrated the differentiation-inducing function of miR-449a in MYCN-nonamplified and MYCN-amplified neuroblastoma cell lines. This fulfills the objective of **Aim 1**. We further validated the effect of miR-449a on the expression of molecular differentiation markers, on cell cycle distribution, and on cell survival and proliferation. This fulfills the task proposed in **Aim 2.**

## 4. Key Research Accomplishments

### Specific Aim 1: Identify miRNAs that induce neuroblastoma cell differentiation by HCS.

The objective of this Aim is to identify miRNA mimics that potentially have general differentiation-inducing effect in neuroblastoma cell lines regardless of the genetic backgrounds of the cell lines. We have completed the screen in the MYCN-amplified cell line BE(2)-C cells, and we have identified a group of novel miRNA mimics that induce BE(2)-C cell differentiation. Although we have not completed the screen in the MYCN-nonamplified cells, we did demonstrate that several differentiation-inducing miRNAs identified in BE(2)-C cells also induce differentiation in MYCN-nonamplified cell lines, and we have reported these miRNAs in the Progress Report in the previous grant period.

Here, we report an additional miRNA, miR-449a that have general differentiation-inducing effect in neuroblastoma cell lines with distinct genetic backgrounds, including the MYCN amplification status.

### 4.1. miR-449a overexpression induces cells differentiation in neuroblastoma cells with different genetic backgrounds.

In our HCS, we identified that miR-449a mimic significantly induces neurite outgrowth in BE(2)-C cells at 25nM (1). To further characterize the differentiation-inducing function of miR-449a, we examined the time-dependent and dose-dependent effect of miR-449a mimic on neurite outgrowth in BE(2)-C cells. As shown in **Figure 1A-B,** miR-449a mimic induces neurite elongation in both time- and dose-dependent manners, and the effect is significant at very low concentrations of miR-449a mimic (as low as 0.5 nM). We further confirmed the effect of miR-449a on neurite outgrowth using a miR-449a precursor mimic. A miRNA precursor mimic is a partially double-stranded RNA designed to mimic the endogenous miRNA precursor, in contrast to the fully complementary double-stranded design of a miRNA mimic that recapitulates the mature miRNA. **Figure 1C-D** shows that the effect of the miR-449a precursor mimic on neurite outgrowth is comparable to that of the miR-449a mimic, indicating that the differentiation-inducing function of miR-449a mimic is unlikely to be caused by off-target effects.

In order to examine whether the differentiation-inducing effect of miR-449a mimic in neuroblastoma is universal, we further investigated the effect of miR-449a mimic on cell differentiation in multiple neuroblastoma cell lines with different genetic backgrounds (**Table 1**). As shown in **Figure 1E-F**, miR-449a mimic significantly induces neurite outgrowth in all of these neuroblastoma cells as compared to control,

although the potency of its effect distinctly varies among cell lines. These results support the general differentiation-inducing function of miR-449a in neuroblastoma cells.

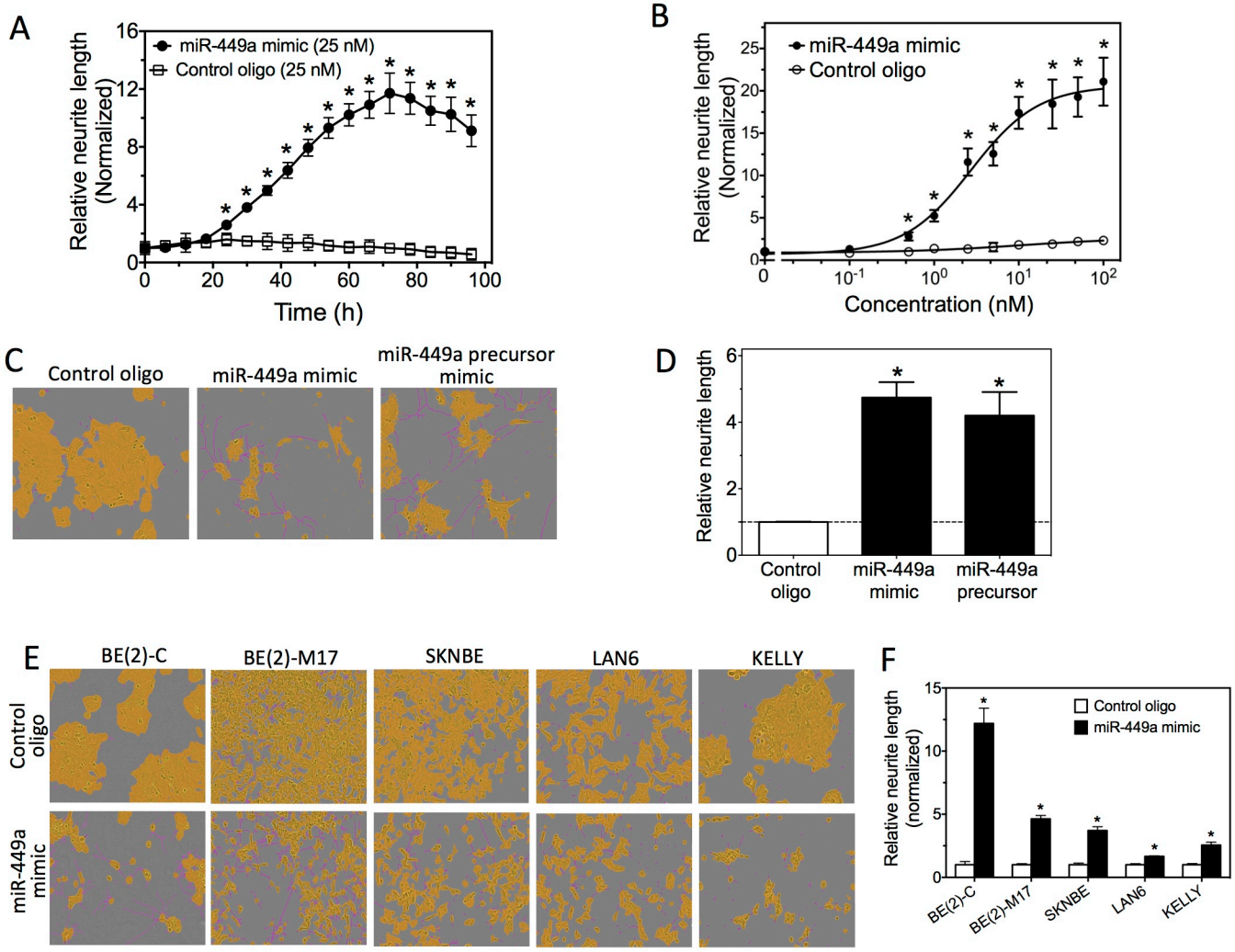



**Figure 1. miR-449a functions as an inducer of cell differentiation in neuroblastoma cells. (A-B),** Time- (**A**) and Dose- (**B**) dependent effect of miR-449a mimic on neurite outgrowth in BE(2)-C cells. 2500 cells were transfected with different concentrations of miR-449a mimic or negative control oligo in triplicates in 96-well plates, and neurite outgrowth was measured every 6 hours for 4 days. Relative neurite length is defined as neurite length per cell body area. Shown are the normalized relative neurite length, presented as average values of the three replicates. *, $p<0.05$, comparing to the negative control oligo at the corresponding time or dose points. (**C-D**), Effect of miR-449a mimic and precursor mimic on neurite outgrowth in BE(2)-C cells. Cells were transfected with 25 nM control oligo, miR-449a mimic or precursor mimic in triplicates, with neurite lengths measured as above after 4 days. Shown are the representative cell images (**C**) analyzed to define neurites (pink) and cell body areas (yellow), and the quantification of neurite length (**D**). *, $p<0.05$, comparing to control. (**E-F),** Effect of miR-449a mimic on neurite outgrowth in multiple neuroblastoma cell lines. Cells were transfected with 25 nM miR-449a mimic or control oligo in triplicates. Neurite lengths were measured as above. **E,** Representative cell images analyzed to define neurites (pink) and cell body areas (yellow) after 4 days of transfection. **F**, Quantification of neurite length. *, $p<0.05$, comparing to control.

| Cell line | Age | Gender | Stage | Chr 1p alteration | Chr 17 alteration | MYCN ampl. |
|---|---|---|---|---|---|---|
| BE(2)-C | 2 | male | 4 | Y | N | Y |
| SK-N-BE | 1.8 | male | 4 | Y | N | Y |
| LAN6 | 5.8 | male | 4 | N | unk | N |
| BE(2)-M17 | 2 | male | 4 | Y | N | Y |
| KELLY | unk | unk | unk | unk | unk | Y |

**Table 1. Genetic backgrounds of neuroblastoma cell lines used in the study.** Shown are the name of the cell line, age and gender of the patient, stage of the tumor from which the cell lines are derived, chromosome 1p and 17 alterations, and MYCN gene amplification status. *unk, unknown; Chr, Chromosome; ampl, amplification.

**Specific Aim 2. Validate the function of the identified miRNAs in inducing cell differentiation and cell growth arrest in neuroblastoma cell lines**.

The goal of this Aim is to use 2nd and 3rd tier analyses to validate the candidate miRNA mimics identified from the HCS, by examining the effects of the miRNA mimics on inducing neuroblastoma cell differentiation and cell growth arrest. Focusing on miR-449a, we validated its tumor suppressive function by examining its effect on expression of molecular differentiation markers, on cell survival and proliferation, and on cell cycle distribution.

**Aim 2A. Validate the function of the identified miRNAs in inducing cell differentiation by examining the expression of molecular differentiation markers.**

**4.2. miR-449a mimic induces expression of molecular differentiation markers in multiple neuroblastoma cell lines.**

In order to further validate the differentiation-inducing function of miR-449a, we examined the effect of miR-449a mimic on the expression of molecular differentiation markers in multiple neuroblastoma cell lines. Consistent with its effect on neurite outgrowth, as shown in **Figure 2,** miR-449a mimic significantly increases the expression of neuroblastoma cell differentiation markers, including βIII–tubulin, neuron specific enolase (NSE) and growth associated protein 43 (GAP43) (2-4), in all the five cell lines tested.

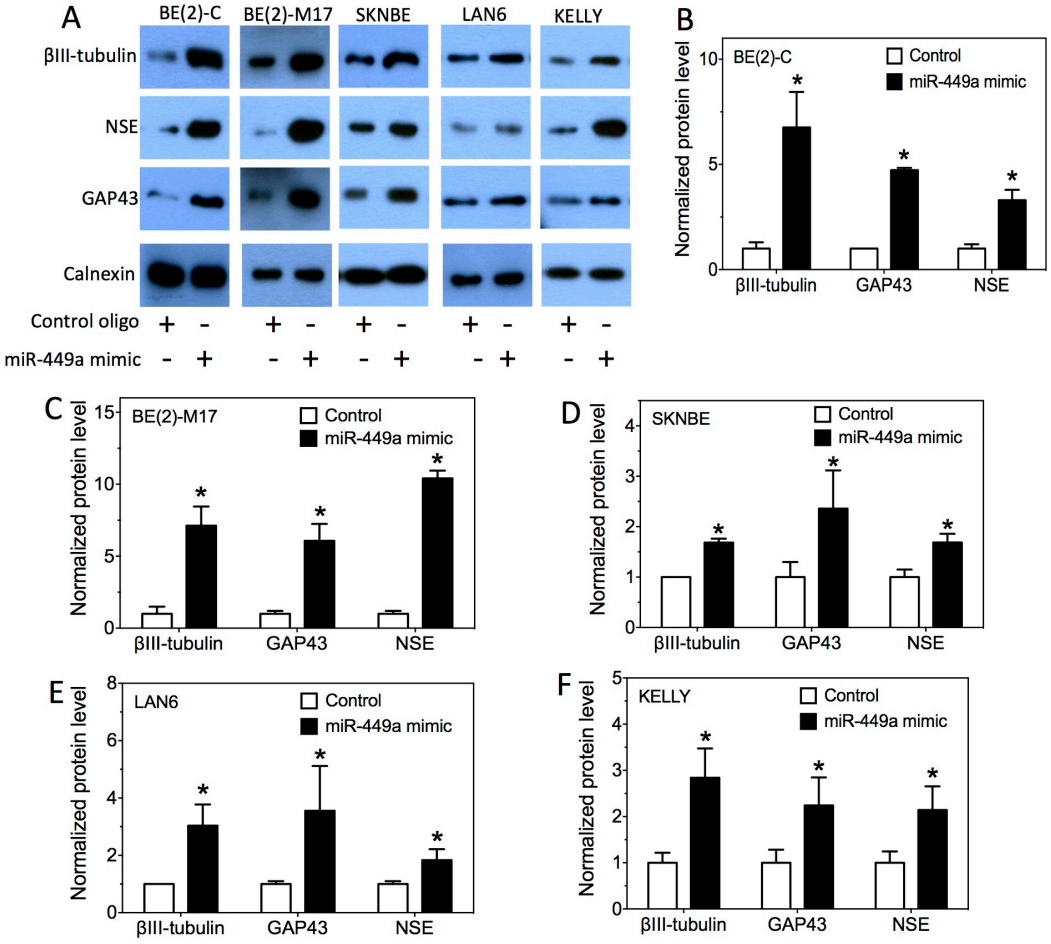



**Figure 2. Effect of miR-449a mimic on protein expression levels of differentiation markers in multiple neuroblastoma cell lines.** Cells were treated with 25 nM miR-449a mimic or control oligos (Control) for 4 days. Expression of differentiation markers βIII-tubulin, GAP43 and NSE were determined by Western blots with calnexin protein levels used as a loading control. Shown are (**A**) the images of the Western blots as well as (**B-F**) the quantification of the band intensities using Image J. The band intensities associated with the differentiation markers were normalized to those associated with calnexin to allow comparison between samples. Shown are the average results from three independent experiments. *, $p<0.05$, comparing to Control.

**Aim 2b. Validate the function of the identified miRNA mimics in inducing cell growth arrest of neuroblastoma cells by cell cycle analysis.**

**4.3. Overexpression of miR-449a induces G0/G1 arrest and cell cycle exit in neuroblastoma cells.**

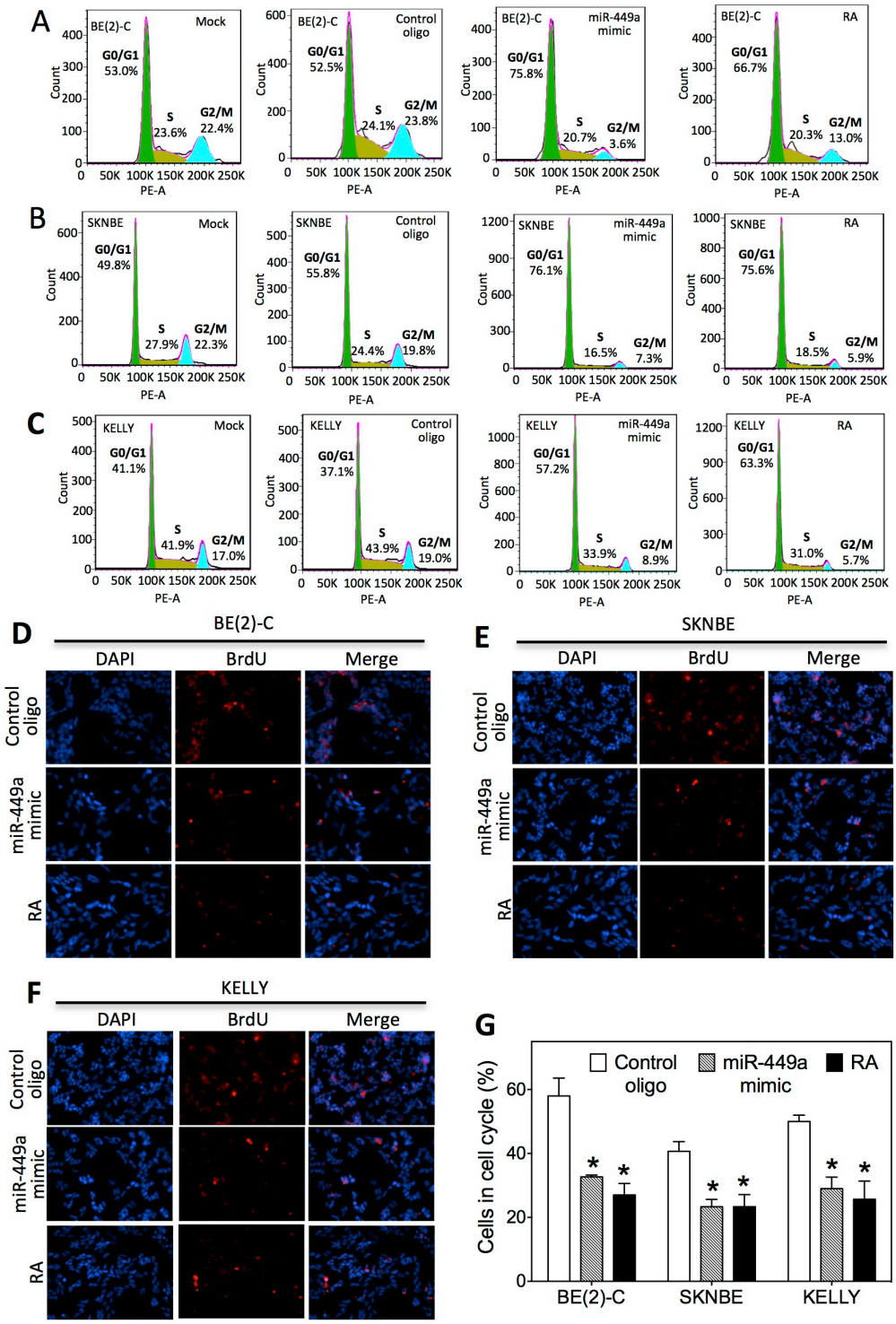



**Figure 3. Effect of miR-449a on cell cycle progression in neuroblastoma cells. A-C,** Effect of miR-449a overexpression on cell cycle distribution in (**A**) BE(2)-C, (**B**) SKNBE and (**C**) KELLY cells. Cells were transfected with 25 nM miR-449a mimic or control oligo, or treated with 5 μM RA. Mock transfected cells were used as an additional negative control. After 3 days, cells were collected and stained with propidium iodide for cell cycle analysis. The fraction of cells in G0/G1, S and G2 phases was quantified using the Jean-Jett-Fox model. Similar results were obtained from three independent experiments. **D-G,** Effect of miR-449a and RA on DNA synthesis as measured by BrDU incorporation in BE(2)-C (**D**), SKNBE (**E**) and KELLY (**F**) cells. Cells were transfected with miR-449a mimic or control oligos (25 nM), or treated with RA (5 μM) for 4 days. Cells were incubated with BrdU during the final 2 h of culture. Cells were then stained for BrDU incorporation (red) as described in the Materials and Methods. Cell nuclei were identified by nuclear staining with DAPI (blue). **G,** Quantification of percentage of cells positive for BrDU staining. *, $p<0.05$, comparing to control.

Cell cycle arrest is another marker that indicates the differentiation of neuroblastoma cells (5). We therefore further examined the effect of miR-449a mimic on cell cycle distribution and on cell cycle exit. As shown in **Figure 3A-C**, comparing to the control cells, miR-449a overexpression leads to marked increase of cell numbers in G0/G1 phase and dramatic decrease of cells numbers in S- and G2/M phases, suggesting that miR-449a blocks cell cycle progression through arresting cells in G0/G1 phase. We further show that the effect of miR-449a on cell cycle distribution is comparable to that induced by RA, which is a differentiation agent that is currently used as standard of care for high-neuroblastoma therapy. It has been known that RA treatment of neuroblastoma cells lead to terminal cell differentiation, which is accompanied with cell cycle exit (6). We therefore examined whether miR-449a overexpression has similar effect in neuroblastoma cells by examining the BrDU incorporation into DNA in the nucleus of individual cells using immunofluorescence staining. As shown in **Figure 3D-G**, comparing to the control cells, more cells treated with miR-449a mimic or RA are negative for BrDU staining, which indicates that these cells failed to enter S-phase. These results indicate that induction of cell cycle exit is achieved by miR-449a overexpression in neuroblastoma cells, and the effect of miR-449a in inducing cell cycle exit is comparable to RA.

### 4.4. miR-449a reduces neuroblastoma cell survival and proliferation.

To further characterize the potential tumor suppressive function of miR-449a in neuroblastoma, we examined whether the induced cell differentiation by miR-449a is coupled with reduced neuroblastoma cell survival. As show in **Figure 4A**, miR-449a mimic decreases cell viability in a dose-dependent manner in all the examined neuroblastoma cell lines. We further investigated the mechanisms underlying the reduced cell survival induced by miR-449a. **Figure 4B** shows that miR-449a mimic activates the apoptotic pathway in neuroblastoma cells, as measured by increased levels of cleaved caspase 3. To further demonstrate its tumor suppressive function, we next examined the impact of the miR-449a mimic on the tumorigenic potential of neuroblastoma cells by colony formation assay. As shown in **Figure 4C–D**, miR-449a mimic significantly decreases the capacity of the neuroblastoma cells to form colonies, indicating that miR-449a reduces the tumorigenicity of neuroblastoma cells. Altogether, our results support that miR-449a functions as a strong suppressor of tumorigenicity in neuroblastoma, and this is achieved by inducing cell differentiation, reducing cell proliferation and activating the cell apoptotic pathway.

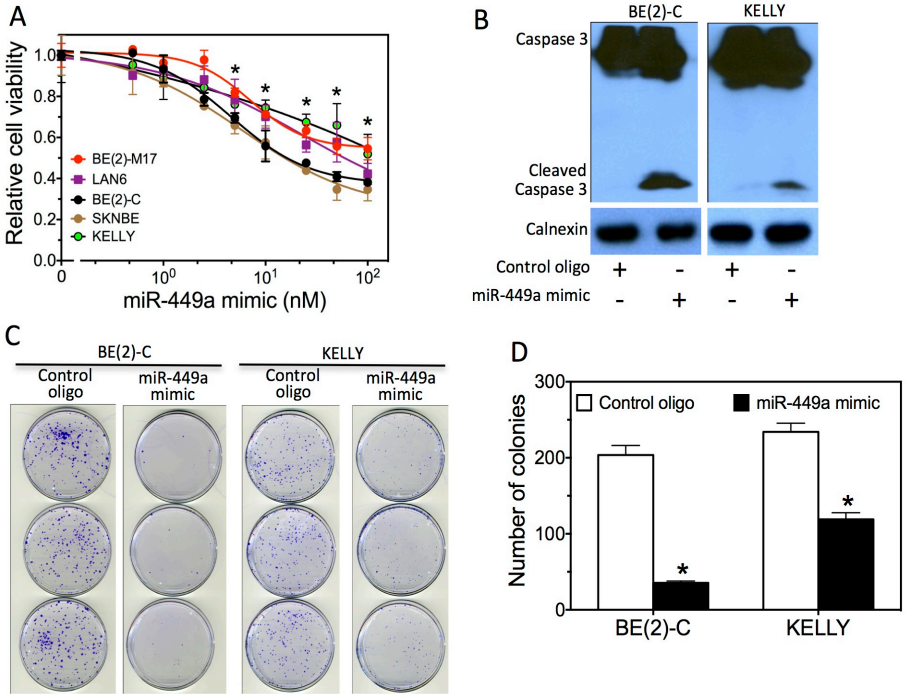



**Figure 4. Effect of miR-449a mimic on neuroblastoma cell survival and proliferation. A**, Dose-dependent effect of miR-449a mimic on cell viability in multiple neuroblastoma cell lines. Cells were transfected with different concentrations of miR-449a mimic in triplicates in 96-well plates. After 4 days, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay. Shown are the relative cell viabilities, presented as averages of the three replicates, normalized to the viabilities of cells at the 0 nM dose point (mock transfected cells). *, $p<0.05$, comparing to the 0 nM dose point. **B,** Western blot analysis of Caspase-3 activation. Cells were transfected with 25 nM miR-449a mimic or control oligos for 72 h, after which cell lysates were harvested. Intact and cleave caspase-3 was detected using an anti-caspase-3 antibody that recognizes both the intact and the cleaved caspase-3. Calnexin levels were measured as a loading control. **(C-D)** Colony formation assay as a function of miR-449a mimic. Treatments were conducted in five replicates. Show are the representative images (**C**) and quantification of the number of colonies (**D**). * $p<0.05$, comparing to Control.

## 5. Conclusion and future plans

In summary, we have demonstrated that miR-449a functions as an inducer of cell differentiation in neuroblastoma cell lines with distinct genetic backgrounds, including the MYCN amplification status. We further show that miR-449s mimic induces cell cycle arrest and cell cycle exit, induces cell apoptosis, and decreases the capacity of the neuroblastoma cells to form colonies. All these results support the strong tumor suppressive function of miR-449a in neuroblastoma cells, providing the experimental evidence for developing miR-499a-based differentiation therapy for neuroblastoma.

In the next grant period, we will (1) complete the HCS screen in MYCN-nonamplified cells and identify additional miRNAs that potentially have general effect on cell differentiation in neuroblastoma, and (2) validate the rest of the identified differentiation-inducing miRNAs by examining expression of the molecular differentiation markers, cell survival and cell cycle distribution.

## 6. Publications, Abstracts and Presentations

### 6.1. Publications

Zhao Z, Ma X, Sung D, Li M, Kosti A, Lin G, Chen Y, Pertsemlidis A, Hsiao TH and ***Du L**. microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest. *RNA Biology*. 2015; 12(5): 538-554. ***, Corresponding author.**

### 6.2. Abstracts

1. Ma X, Li M, Zhao Z, Pertsemlidis A, Sung D and **Du L**. Crosstalk between MYCN and differentiation-inducing microRNAs in neuroblastoma. *Greehey Children's Cancer Research Institute 2014 Symposium. University of Texas Health Science Center at San Antonio*. San Antonio, TX. November 21, 2014.
2. Zhao Z, Ma X, Li M, Kosti A, Lin G, Chen Y, Pertsemlidis A, Hsiao TH and **Du L**. microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest. *Greehey Children's Cancer Research Institute 2014 Symposium. University of Texas Health Science Center at San Antonio*. San Antonio, TX. November 21, 2014.

### 6.3. Presentations

1. Identifying novel differentiation agents for neuroblastoma therapy. Innovative Drug Discovery & Nanotechnology session. 2015 Drug Discovery and Therapy World Congress. *Boston, USA*. July 22-25$^{th}$, 2015.
2. Using high-content screening to identify novel differentiation agents for neuroblastoma. Session 603: Lead Discovery and Optimization. 2015 BIT's 8th Annual World Cancer Congress. *Beijing, China*. May 15-17th, 2015.
3. Targeting the differentiation pathway in neuroblastoma differentiation therapy. *Department of Chemistry and Biochemistry. Texas State University.* San Marcos, TX. December 04, 2014.
4. Differentiation therapy in neuroblastoma. 2014 EDT Program Retreat. *Cancer Therapy and Research Center, University of Texas Health Science Center at San Antonio.* San Antonio, TX. November 7, 2014.

## 7. Inventions, Patents and Licenses

None in this grant period.

## 8. Reportable Outcome

**8.1.** We demonstrated that our identified novel differentiation-inducing miRNA-449a has general effect in inducing cell differentiation in multiple neuroblastoma cell lines with distinct genetic backgrounds, including MYCN-nonamplified and MYCN-amplified neuroblastoma cells.

**8.2.** We demonstrated the miR-449a mimic induces neuroblastoma cell cycle exit, reduces cell viability and reduces the capacity of neuroblastoma cells to form colonies, suggesting that miR-449a suppresses

tumorigenecity in neuroblastoma. This evidence strongly supports the therapeutic potential of miR-449a mimic in neuroblastoma.

## 9. Other Achievements

Based on the work supported by this award, I submitted the following grant applications:

1) **NIH/NCI** (1R01CA194182-01A1) **(R01) (L. Du)** 12/01/2015-11/30/2020 4.8 Cal. mos. ($1250,000 direct/$1853,900 total)
**Therapeutic mechanisms of differentiation-inducing microRNAs in neuroblastoma**
**Goal:** To define the role of microRNAs in regulating neuroblastoma cell differentiation, and to develop miRNA-based differentiation agents for treating neuroblastoma.
**Role:** Principal Investigator
**Status:** pending

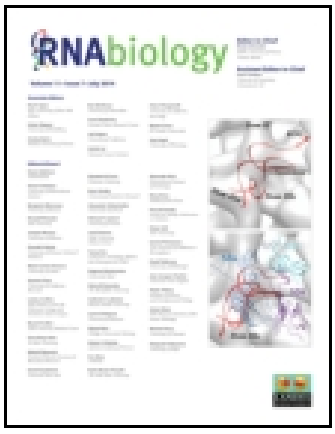
2) **Pew Charitable Trust (L. Du)** 07/01/2015-06/30/2019 2.4 Cal. mos. ($240,000 direct)
**Differentiation-inducing microRNAs in neuroblastoma therapeutics**
**Goal:** To develop novel microRNA-based differentiation therapies for treating neuroblastoma.
**Role:** Principal Investigator
**Status:** pending

## 10. References


1. Zhao Z, Ma X, Hsiao TH, Lin G, Kosti A, Yu X, et al. A high-content morphological screen identifies novel microRNAs that regulate neuroblastoma cell differentiation. Oncotarget. 2014;5(9):2499-512.
2. Radio NM, Breier JM, Shafer TJ, Mundy WR. Assessment of chemical effects on neurite outgrowth in PC12 cells using high content screening. Toxicol Sci. 2008;105(1):106-18.
3. Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 2009;30(1):127-35.
4. Mao L, Ding J, Zha Y, Yang L, McCarthy BA, King W, et al. HOXC9 links cell-cycle exit and neuronal differentiation and is a prognostic marker in neuroblastoma. Cancer Res. 2011;71(12):4314-24.
5. Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature. 1985;313(6001):404-6.
6. Feliciano DM, Edelman AM. Repression of Ca2+/calmodulin-dependent protein kinase IV signaling accelerates retinoic acid-induced differentiation of human neuroblastoma cells. J Biol Chem. 2009;284(39):26466-81.


## 11. Appendices

Please see the attached for the research paper that is in press in *RNA Biology*.





RNA Biology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/krnb20

# microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest

Zhenze Zhao[a], Xiuye Ma[a], Derek Sung[a], Monica Li[a], Adam Kosti[a], Gregory Lin[a], Yidong Chen[ab], Alexander Pertsemlidis[acd], Tzu-Hung Hsiao[e] & Liqin Du[ad]

[a] Greehey Children's Cancer Research Institute; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA

[b] Epidemiology and Biostatistics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA

[c] Pediatrics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA

[d] Cellular and Structural Biology; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA

[e] Department of Medical Research; Taichung Veterans General Hospital; Taichung, Taiwan

Accepted author version posted online: 11 Mar 2015.

To cite this article: Zhenze Zhao, Xiuye Ma, Derek Sung, Monica Li, Adam Kosti, Gregory Lin, Yidong Chen, Alexander Pertsemlidis, Tzu-Hung Hsiao & Liqin Du (2015) microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest, RNA Biology, 12:5, 538-554, DOI: 10.1080/15476286.2015.1023495

To link to this article: http://dx.doi.org/10.1080/15476286.2015.1023495

RESEARCH PAPER

RNA Biology 12:5, 538–554; May 2015; © 2015 Taylor and Francis Group, LLC

# microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest

Zhenze Zhao[1], Xiuye Ma[1], Derek Sung[1], Monica Li[1], Adam Kosti[1], Gregory Lin[1], Yidong Chen[1,2], Alexander Pertsemlidis[1,3,4], Tzu-Hung Hsiao[5], and Liqin Du[1,4,*]

[1]Greehey Children's Cancer Research Institute; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; [2]Epidemiology and Biostatistics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; [3]Pediatrics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; [4]Cellular and Structural Biology; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; [5]Department of Medical Research; Taichung Veterans General Hospital; Taichung, Taiwan

**Keywords:** cell cycle arrest, differentiation, differentiation therapy, microRNA, neuroblastoma

**Abbreviations:** Bio-control; biotinylated control oligonucleotides; Bio-miR-449a; biotinylated miR-449a mimic; CASP2; Caspase 2; CCNE2; Cyclin E2; CDC25A; cell division cycle 25A; CDK6; cyclin-dependent kinase 6; FDR; false discovery rate; FLOT2; Flotillin 2; GAP43; growth associated protein 43; GAPDH; glyceraldehyde-3-phosphate dehydrogenase; HDAC1; histone deacetylase 1; LEF1; lymphoid enhancer-binding factor 1; MFAP4; microfibril-associated protein 4; miR-449a; microRNA-449a; miRNA; microRNA; Oligo; oligonucleotides; PKP4; plakophilin 4; STMN1; stathmin 1; TSEN15; tRNA splicing endonuclease 15 homolog; RA; 13-*cis* retinoic acid

microRNA-449a (miR-449a) has been identified to function as a tumor suppressor in several types of cancers. However, the role of miR-449a in neuroblastoma has not been intensively investigated. We recently found that the overexpression of miR-449a significantly induces neuroblastoma cell differentiation, suggesting its potential tumor suppressor function in neuroblastoma. In this study, we further investigated the mechanisms underlying the tumor suppressive function of miR-449a in neuroblastoma. We observed that miR-449a inhibits neuroblastoma cell survival and growth through 2 mechanisms—inducing cell differentiation and cell cycle arrest. Our comprehensive investigations on the dissection of the target genes of miR-449a revealed that 3 novel targets- MFAP4, PKP4 and TSEN15 -play important roles in mediating its differentiation-inducing function. In addition, we further found that its function in inducing cell cycle arrest involves down-regulating its direct targets CDK6 and LEF1. To determine the clinical significance of the miR-449a-mediated tumor suppressive mechanism, we examined the correlation between the expression of these 5 target genes in neuroblastoma tumor specimens and the survival of neuroblastoma patients. Remarkably, we noted that high tumor expression levels of all the 3 miR-449a target genes involved in regulating cell differentiation, but not the target genes involved in regulating cell cycle, are significantly correlated with poor survival of neuroblastoma patients. These results suggest the critical role of the differentiation-inducing function of miR-449a in determining neuroblastoma progression. Overall, our study provides the first comprehensive characterization of the tumor-suppressive function of miR-449a in neuroblastoma, and reveals the potential clinical significance of the miR-449a-mediated tumor suppressive pathway in neuroblastoma prognosis.

## Introduction

Neuroblastoma is one of the most common solid cancers of childhood. High-risk neuroblastoma is one of the leading causes of cancer-related deaths in childhood,[1,2] because only a few high-risk neuroblastoma patients become long-term survivors with currently available therapeutic agents for treating neuroblastoma. Differentiation therapy was developed based on the knowledge that neuroblastoma arises from the neural crest cell precursors that fail to complete the differentiation process.[2,3] It is an approach to induce malignant cells to differentiate into mature cells and thereby leading to tumor growth arrest.[2,4-6] Currently, the differentiation agent 13-*cis*-retinoic acid (RA) is used as the standard of care for post-remission maintenance therapy in high-risk neuroblastoma,[2] highlighting the critical role of differentiation therapy in neuroblastoma treatment. However, resistance to RA treatment is common, and more than 50% of patients treated with RA still subsequently develop recurrent neuroblastoma.[7,8] The development of new differentiation agents has been greatly hampered due to the poor understanding of the mechanisms that control

*Correspondence to: Liqin Du; Email: dul2@uthscsa.edu
Submitted: 11/13/2014; Revised: 02/19/2015; Accepted: 02/20/2015
http://dx.doi.org/10.1080/15476286.2015.1023495

neuroblastoma cell differentiation. This highlights the need to further investigate the mechanisms that regulate neuroblastoma cell differentiation in order to identify novel therapeutic targets for developing new and more effective differentiation therapies.

microRNAs (miRNAs) are endogenously expressed small RNAs that play a critical role in tumorigenesis,[9-13] and miRNA-based anti-cancer therapeutics are under intensive investigation.[14-17] One of the most promising approaches that have been developed is called miRNA replacement therapy, in which the intracellular levels of tumor suppressive miRNAs are raised using miRNA mimics, synthetic oligonucleotides that mimic the sequences and function of the endogenous miRNAs.[18] These miRNA mimics have been used to effectively treat cancers in animal studies,[14,15] showing the promise of developing them into anti-cancer agents.[14-17] Recent studies have shown that miRNAs play an important role in regulating neuroblastoma cell differentiation,[19-25] thus suggesting the potential application of miRNA-based therapeutics in neuroblastoma differentiation therapy.

To this end, we recently identified a group of novel miRNAs that function as potent inducers of neuroblastoma cell differentiation,[20] which provides candidates for developing novel miRNA replacement therapies in neuroblastoma. One of the most potent differentiation-inducing miRNAs that we identified was miR-449a, which has been demonstrated to function as an important tumor suppressor in several types of cancers.[26-33] This evidence highlights the universal tumor suppressive function of miR-449a in cancers and supports the potential application of miR-449a replacement therapy in cancer treatment. In neuroblastoma, miR-449a has been shown to target the 3′ untranslated region (3′UTR) of the MYCN mRNA.[34] However, the tumor suppressive function of miR-449a in neuroblastoma has not been fully evaluated, and the molecular mechanisms underlying its tumor suppressive function as well as the clinical relevance of this tumor suppressive mechanism is not defined. In the current study, we aim to further define the tumor suppressive function of miR-449a in neuroblastoma, by elucidating the underlying mechanisms for such function and evaluating the potential clinical significance of the miR-449a-mediated tumor suppressive pathway in neuroblastoma development.

## Results

### miR-449a overexpression induces cells differentiation in neuroblastoma cells with different genetic backgrounds

We previously found that increasing intracellular levels of miR-449a with a miR-449a mimic significantly induces cell differentiation in a neuroblastoma cell line BE(2)-C.[20] Here, to further characterize the differentiation-inducing function of miR-449a in neuroblastoma, we first examined the time-dependent and dose-dependent effect of miR-449a mimic on neurite outgrowth in BE(2)-C cells. As shown in **Figure 1A–B**, miR-449a mimic induces neurite elongation in both time- and dose-dependent manners, and the effect is significant at very low concentrations of miR-449a mimic (as low as 0.5 nM). We further confirmed the effect of miR-449a on neurite outgrowth using a miR-449a precursor mimic. A miRNA precursor mimic is a partially double-stranded RNA designed to mimic the endogenous miRNA precursor, in contrast to the fully complementary double-stranded design of a miRNA mimic that recapitulates the mature miRNA. **Figure 1C–D** shows that the effect of the miR-449a precursor mimic on neurite outgrowth is comparable to that of the miR-449a mimic, indicating that the differentiation-inducing function of miR-449a mimic is unlikely to be caused by off-target effects. We further investigated the effect of miR-449a mimic on cell differentiation in multiple neuroblastoma cell lines with different genetic backgrounds (**Table S1**). As shown in **Figure 1E–F**, miR-449a mimic significantly induces neurite outgrowth in all of these neuroblastoma cells as compared to control, although the potency of its effect distinctly varies among cell lines. Consistent with its effect on neurite outgrowth, as shown in **Figure 1G** and **Supplementary Figure 1**, miR-449a mimic significantly increases the expression of neuroblastoma cell differentiation markers, including βIII–tubulin, neuron specific enolase (NSE) and growth associated protein 43 (GAP43),[35-37] in all the 5 cell lines tested.

Next we were interested in examining whether the endogenous expression of miR-449a is regulated during neuroblastoma cell differentiation. We measured the endogenous miR-449a levels in BE(2)-C cells that were induced into differentiation by RA (**Fig. S2**). As shown in **Figure 1H**, the endogenous expression of miR-449a in differentiated BE(2)-C cells is significantly increased comparing to the undifferentiated (Control) cells. These results strongly suggest that the endogenous expression of miR-449a is suppressed in undifferentiated neuroblastoma cells.

### miR-449a overexpression reduces cell proliferation and induces apoptosis in neuroblastoma cells

To further characterize the potential tumor suppressive function of miR-449a in neuroblastoma, we examined whether the induced cell differentiation by miR-449a is coupled with reduced neuroblastoma cell survival. As shown in **Figure 2A**, miR-449a mimic decreases cell viability in a dose-dependent manner in all the examined neuroblastoma cell lines. We further investigated the mechanisms underlying the reduced cell survival induced by miR-449a. As shown in **Figure 2B**, miR-449a overexpression significantly decreases DNA synthesis in the cells, as measured by the reduced bromodeoxyuridine (BrdU) incorporation into cell DNA, indicating its direct effect in inhibiting cell proliferation. **Figure 2C** shows that miR-449a mimic also activates the apoptotic pathway in neuroblastoma cells, as measured by increased levels of cleaved caspase 3. To further demonstrate its tumor suppressive function, we next examined the impact of the miR-449a mimic on the tumorigenic potential of neuroblastoma cells by colony formation assay. As shown in **Figure 2D–E**, miR-449a mimic significantly decreases the capacity of the neuroblastoma cells to form colonies, indicating that miR-449a reduces the tumorigenicity of neuroblastoma cells.

Altogether, our results support that miR-449a functions as a strong suppressor of tumorigenicity in neuroblastoma, and this is achieved by inducing cell differentiation, reducing cell proliferation and activating the cell apoptotic pathway.

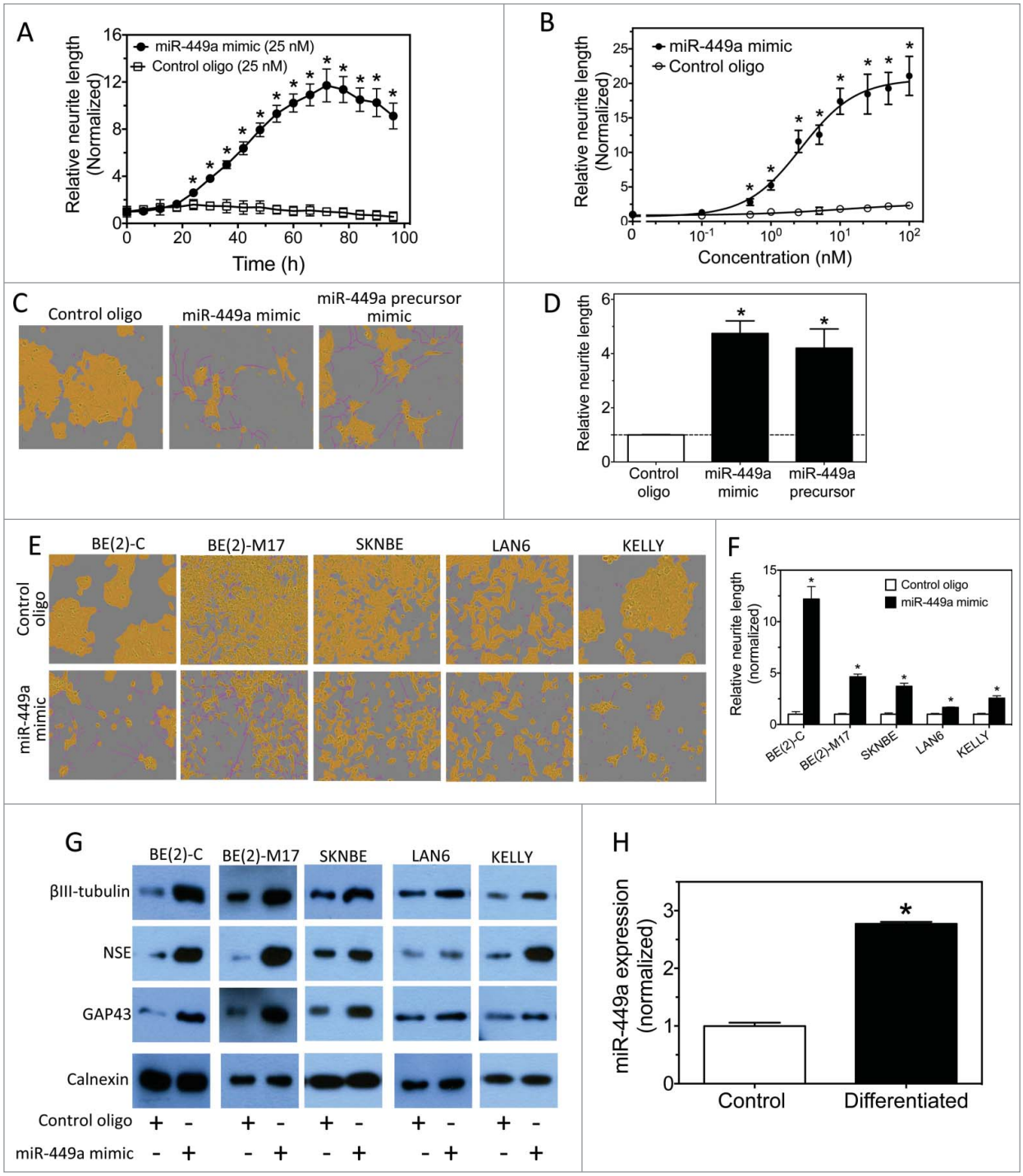



**Figure 1. miR-449a functions as an inducer of cell differentiation in neuroblastoma cells. (A–B)**, Time- (**A**) and Dose- (**B**) dependent effect of miR-449a mimic on neurite outgrowth in BE(2)-C cells. 2500 cells were transfected with different concentrations of miR-449a mimic or negative control oligo in triplicates in 96-well plates, and neurite outgrowth was measured every 6 h for 4 d. Relative neurite length is defined as neurite length per cell body area. Shown are the normalized relative neurite lengths, presented as average values of the 3 replicates. *, $P < 0.05$, comparing to the negative control oligo at the corresponding time or dose points. **(C–D)**, Effect of miR-449a mimic and precursor mimic on neurite outgrowth in BE(2)-C cells. Cells were transfected with 25 nM control oligo, miR-449a mimic or precursor mimic in triplicates, with neurite lengths measured as above after 4 d. Shown are the representative cell images (**C**) analyzed to define neurites (pink) and cell body areas (yellow), and the quantification of neurite length (**D**). *, $P < 0.05$, comparing to control. **(E–F)**, Effect of miR-449a mimic on neurite outgrowth in multiple neuroblastoma cell lines. Cells were transfected with 25 nM miR-449a mimic or control oligo in triplicates. Neurite lengths were measured as above. (**E**) Representative cell images analyzed to define neurites (pink) and cell body areas (yellow) after 4 d of transfection. (**F**) Quantification of neurite lengths. *, $P < 0.05$, comparing to control. **G**, Effect of miR-449a overexpression on the protein expression levels of cell differentiation markers βIII-tubulin, NSE and GAP43 with calnexin protein levels used as a loading control. Cells were transfected with 25 nM miR-449a mimic or control oligo, and protein levels were determined by Western blot after 4 d. (**H**) Endogenous expression levels of miR-449a in undifferentiated and differentiated BE(2)-C cells. BE(2)-C cells were treated with 10 μM of RA or the carrier DMSO (Control) for 5 d to induce cell differentiation. RNA was then isolated, and expression of miR-449a in cells were measured by qPCR with levels of 18s rRNA as a loading control. *, $P < 0.05$, comparing to control.

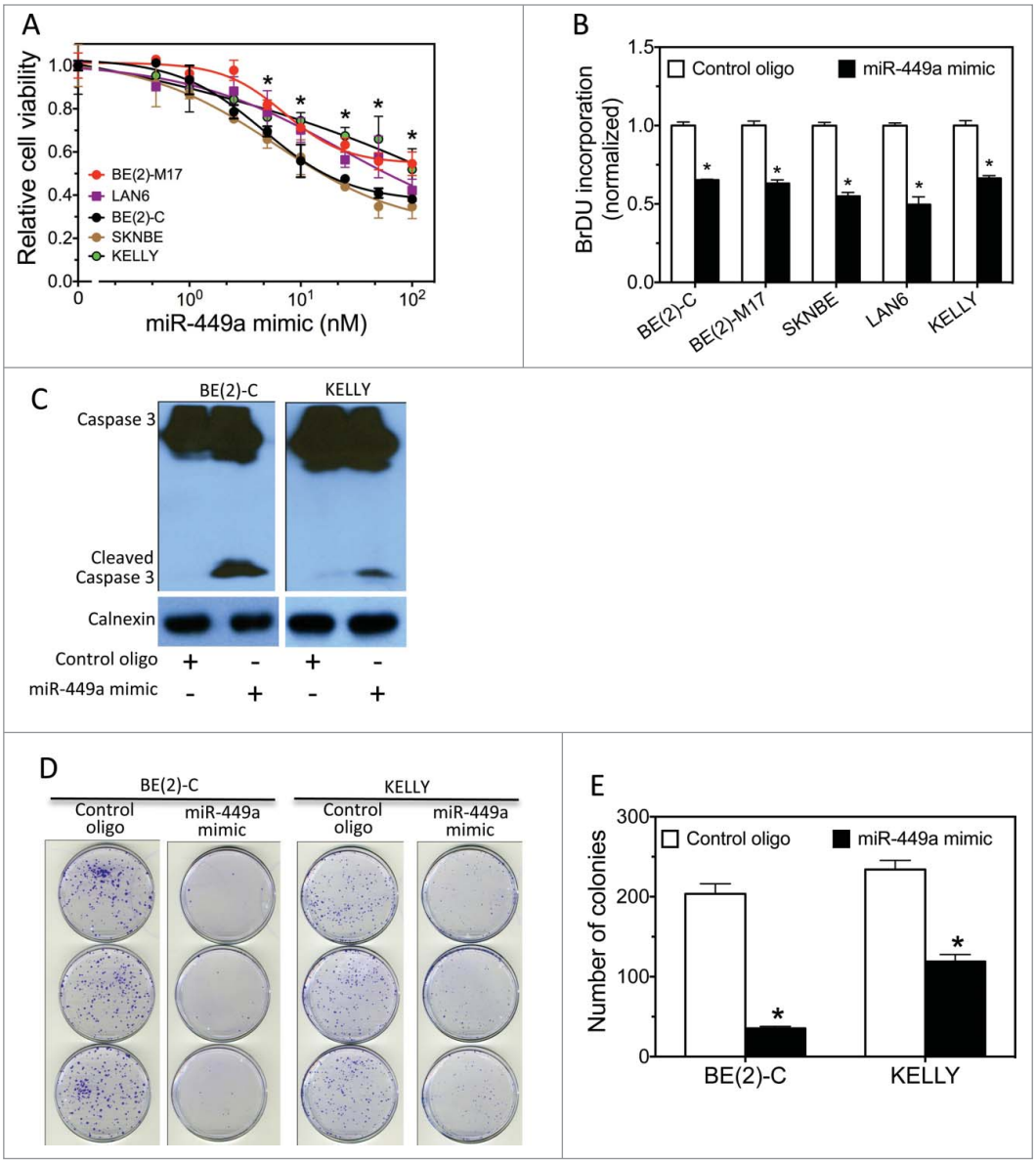



**Figure 2. Effect of miR-449a mimic on neuroblastoma cell survival and proliferation.** (**A**) Dose-dependent effect of miR-449a mimic on cell viability in multiple neuroblastoma cell lines. Cells were transfected with different concentrations of miR-449a mimic in triplicates in 96-well plates. After 4 d, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay. Shown are the relative cell viabilities, presented as averages of the 3 replicates, normalized to the viabilities of cells at the 0 nM dose point (mock transfected cells). *, $P < 0.05$, comparing to the 0 nM dose point. (**B**) BrdU incorporation as a function of miR-449a overexpression. Cells were transfected with 25 nM miR-449a mimic or control oligos (Control). After 4 d, BrdU incorporation was determined as described in the Materials and Methods. Shown are the relative BrDU incorporation levels with the BrDU levels associated with cells treated with miR-449a normalized to those treated with control oligos for each of the cell lines. * $P < 0.05$, comparing to control. (**C**) Western blot analysis of Caspase-3 activation. Cells were transfected with 25 nM miR-449a mimic or control oligos for 72 h, after which cell lysates were harvested. Intact and cleave caspase-3 was detected using an anti-caspase-3 antibody that recognizes both the intact and the cleaved caspase-3. Calnexin levels were measured as a loading control. (**D–E**) Colony formation assay as a function of miR-449a mimic. Treatments were conducted in 5 replicates. Show are the representative images (**D**) and quantification of the number of colonies (**E**). * $P < 0.05$, comparing to Control.

### The differentiation-inducing function of miR-449a is mediated by down-regulating multiple direct targets of miR-449a

miRNAs regulate gene expression largely by decreasing their target mRNA levels[38,39] via binding to specific target sites in the 3'UTRs. In order to comprehensively identify the targets that mediate the differentiation-inducing function of miR-449a, we first exploited microarray analysis to identify the predicted miR-449a targets that are down-regulated by miR-449a overexpression in BE(2)-C cells. **Figure 3A** shows the extent of miR-449a overexpression following transfection with miR-449a mimic for 24 h. As shown in **Figure 3B**, much more of the predicted miR-449a targets decrease in expression than increase. In contrast, as shown in **Figure 3C**, the changes in mRNAs not predicted as miR-449a targets follow a normal distribution pattern—roughly equal numbers of mRNAs not-predicted as miR-449a targets decrease and increase in expression. The empirical density curves in **Figure 3D** further show the difference in the 2 distributions, and the difference is statistically significant ($p = 2.21e\text{-}30$). These results indicate that a significant portion of the miR-449a predicted targets are true miR-449a targets. The predicted miR-449a targets that are downregulated by miR-449a overexpression potentially play roles in mediating the differentiation-inducing function of miR-449a. In this study, we focused on investigating the top 25 predicted targets that are down-regulated by >40% by miR-449a mimic (**Table S2**), since this level of down-regulation of a functional gene typically leads to a dramatic, biologically meaningful phenotype.[20,40-42] We confirmed the downregulation of the 25 target genes by miR-449a mimic using qPCR (**Fig. S3**). By exploiting a set of siRNAs against the 25 genes, we examined whether

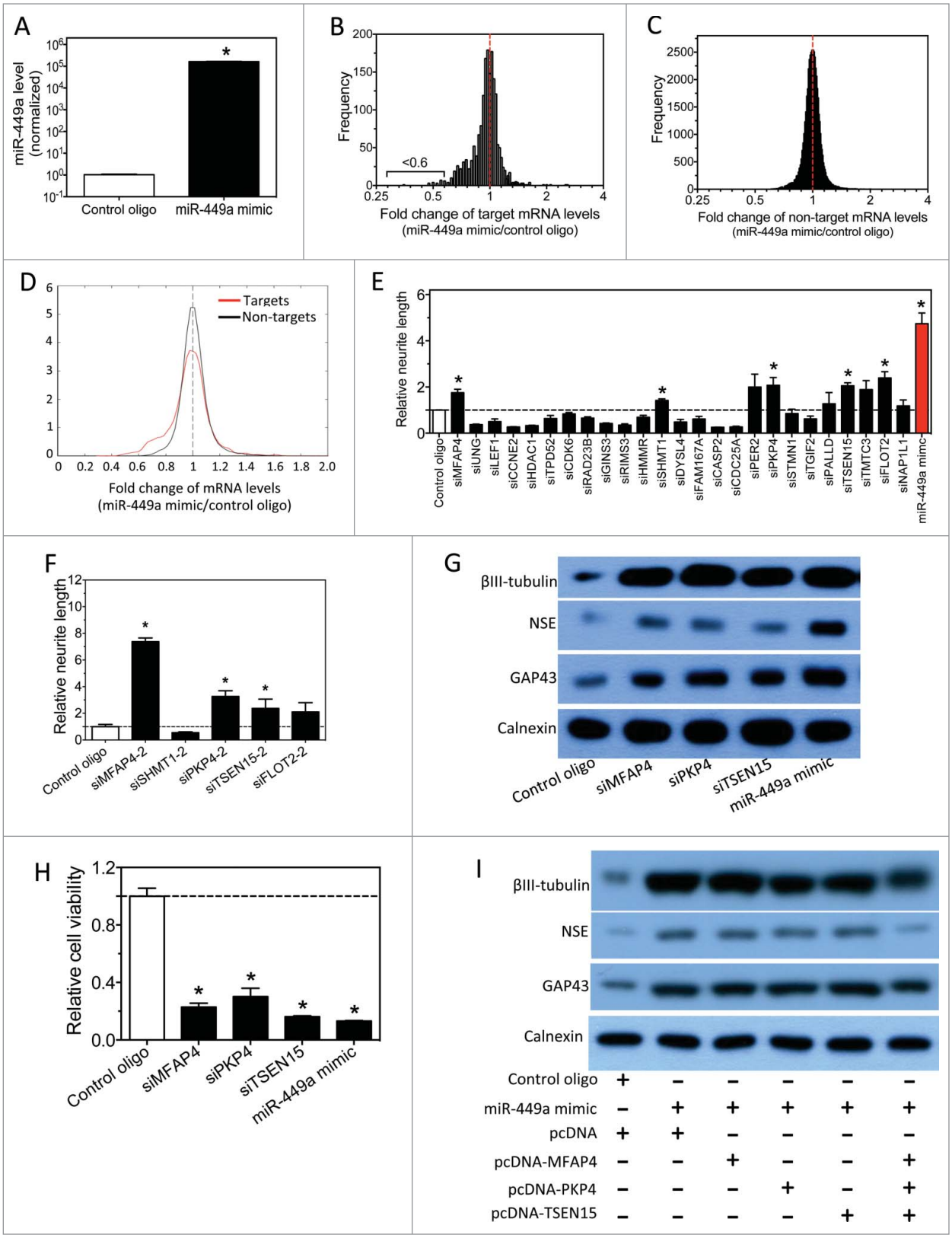



**Figure 3.** For figure legend, see page 543.

knocking down each of the 25 target gene expression induced cell differentiation in BE(2)-C cells. The depletions of the gene expression by the corresponding siRNAs were confirmed by qPCR (**Fig. S4**). As shown in **Figure 3E**, siRNAs against 5 of the 25 target genes significantly induced neurite outgrowth. To exclude the possibility that the neurite outgrowth induced by the siRNAs is due to the off-targets effect of the synthetic oligos, we used a second set of siRNAs against these 5 genes to validate their function. As shown in **Figure 3F and Supplementary Figure 5**, knockdown of only 3 genes, microfibril-associated protein 4 (MFAP4), Plakophilin 4 (PKP4) and TRNA Splicing Endonuclease 15 Homolog (TSEN15), was confirmed to induce neurite outgrowth. In addition, we further show that knockdown of the 3 genes dramatically induces expression of the cell differentiation markers at protein levels (**Fig. 3G**) and significantly reduces cell viability (**Fig. 3H**), further demonstrating that true cell differentiation is induced by depletion of these 3 genes.

To further characterize the role of the 3 genes in mediating the differentiation-inducing function of miR-449a, we investigated whether overexpression of these genes block the differentiation effect of miR-449a in BE(2)-C cells. We used expression constructs expressing only the protein-coding regions of the genes (i.e., lacking the 3'UTR regions) to avoid the down-regulation of the genes by miR-449a. The protein overexpression levels of the 3 genes by their corresponding expression vectors were confirmed by Western blots, as shown in **Supplementary Figure 6**. As shown **Figure 3I**, overexpression of each of the genes individually does not reduce the differentiation-inducing effect of miR-449a, as measured by expression of differentiation markers. However, co-overexpression of the 3 genes dramatically inhibits the effect of miR-449a on the expression of the differentiation markers. The results suggest that the differentiation-inducing effect of miR-449a is mediated by coordinative downregulation of the 3 genes.

### Overexpression of miR-449a induces G0/G1 arrest by directly targeting CDK6 and LEF1

Previous studies have demonstrated that miR-449a inhibits cell growth and reduces cell viability through arresting cells in G0/G1 phase in other cancer type.[28] We therefore examined whether miR-449a has a similar function in neuroblastoma cells. As shown in **Figure 4A-C**, comparing to the control cells, miR-449a overexpression leads to marked increases of cell numbers in G0/G1 phase and dramatic decreases of cells numbers in S and G2/M phases, suggesting that miR-449a blocks cell cycle progression through arresting cells in G0/G1 phase. We further show that the effect of miR-449a on cell cycle distribution is comparable to that induced by RA. It has been known that RA treatment of neuroblastoma cells lead to terminal cell differentiation, which is accompanied with cell cycle exit.[43] We therefore examined whether miR-449a overexpression has similar effect in neuroblastoma cells by examining the BrDU incorporation into DNA in the nucleus of individual cells using immunofluorescence staining. As shown in **Figure 4D–G**, comparing to the control cells, more cells treated with miR-449a mimic or RA are negative for BrDU staining, which indicates that these cells failed to enter S-phase. These results indicate that induction of cell cycle exit is achieved by miR-449a overexpression in neuroblastoma cells, and the effect of miR-449a in inducing cell cycle exit is comparable to RA.

We further intended to identify the targets that mediate the effect of miR-449a on cell cycle arrest. Among the 25 targets that were downregulated greater than 40% by miR-449a mimic, 7 genes, including CCNE2, CDC25A, CDK6, LEF1, STMN1, CASP2 and HDAC1, are identified by Ingenuity Pathway Analysis (IPA, www.ingenuity.com) analysis as involved in regulating cell cycle progression. We first examined whether individual knockdown of these 7 genes can exert an effect on cell viability. Surprisingly, as shown in **Figure 5A–B**, only the knockdown of CDK6 and LEF1 significantly reduces cell viability in BE(2)-C and SKNBE cells. The depletions of the gene expression by the corresponding siRNAs in BE(2)-C cells were confirmed by qPCR (**Fig. S4**). This effect of CDK6 and LEF1 knockdown on cell survival was further confirmed using a 2nd set of siRNAs in BE(2)-C and SKNBE cells (**Fig. 5C–D**), and was validated in KELLY cells using 2 sets of siRNAs (**Fig. 5E**). Further examination of cell cycle distributions indicated that knocking down CDK6 and LEF1 indeed leads to increased accumulation of cells in G0/G1 phase as compared with control oligo (**Fig. 5F–H**) in all the tree cell lines BE(2)-C, SKNBE and

**Figure 3 (See previous page). The systematic identification of miR-449a targets that regulate neuroblastoma cell differentiation**. (**A**) Expression level of miR-449a in BE(2)-C cells after transfected with miR-449a (25 nM) mimic for 24 h, as measured by qPCR. (**B-C**) Histogram of changes in mRNA expression levels of genes (**B**) predicted as miR-449a targets and (**C**) not predicated as miR-449a targets induced by miR-449a overexpression. The mRNA expression levels were determined by gene expression microarray. Shown is the relative frequency of observed log2 fold changes for the predicted miR-449a targets. (**D**) The empirical density curves generated based on the above frequency distributions by Gaussian Kernel Smoothing. (**E**) Effect of knocking down the top 25 target genes on neurite outgrowth in BE(2)-C cells. Cells were transfected with 25 nM of the indicated siRNAs or negative control oligos (Control) in triplicated for 4 d. Neurite outgrowth was measured as above. *, $P < 0.05$, comparing to Control. (**F**) Validation of the effect of knocking down the 5 candidate targets on neurite outgrowth using the 2nd set siRNAs. BE(2)-C cells were transfected and neurite outgrowth were measured as above. *, $P < 0.05$, comparing to control. (**G**) Effect of gene knockdown on expression of differentiation markers in BE(2)-C cells. Cells were transfected with the indicated siRNAs or control oligo (25 nM). Protein levels were measured by Western blot as above. (**H**) Effect of gene knockdown on cell viability in BE(2)-C cells. Cells were transfected with the indicated siRNAs or control oligo (25 nM) in triplicates in 96-well plates. After 4 d, cell viability was measured as above. Shown are the relative cell viabilities, presented as averages of the 3 replicates, normalized to the control oligos. *, $P < 0.05$, comparing to the control. (**I**) Effect of gene overexpression on expression of differentiation markers. BE(2)-C cells were co-transfected with the indicated expression vectors and oligos. After 4 d, protein levels of expression markers were measured as above. *, $P < 0.05$, comparing to Control.

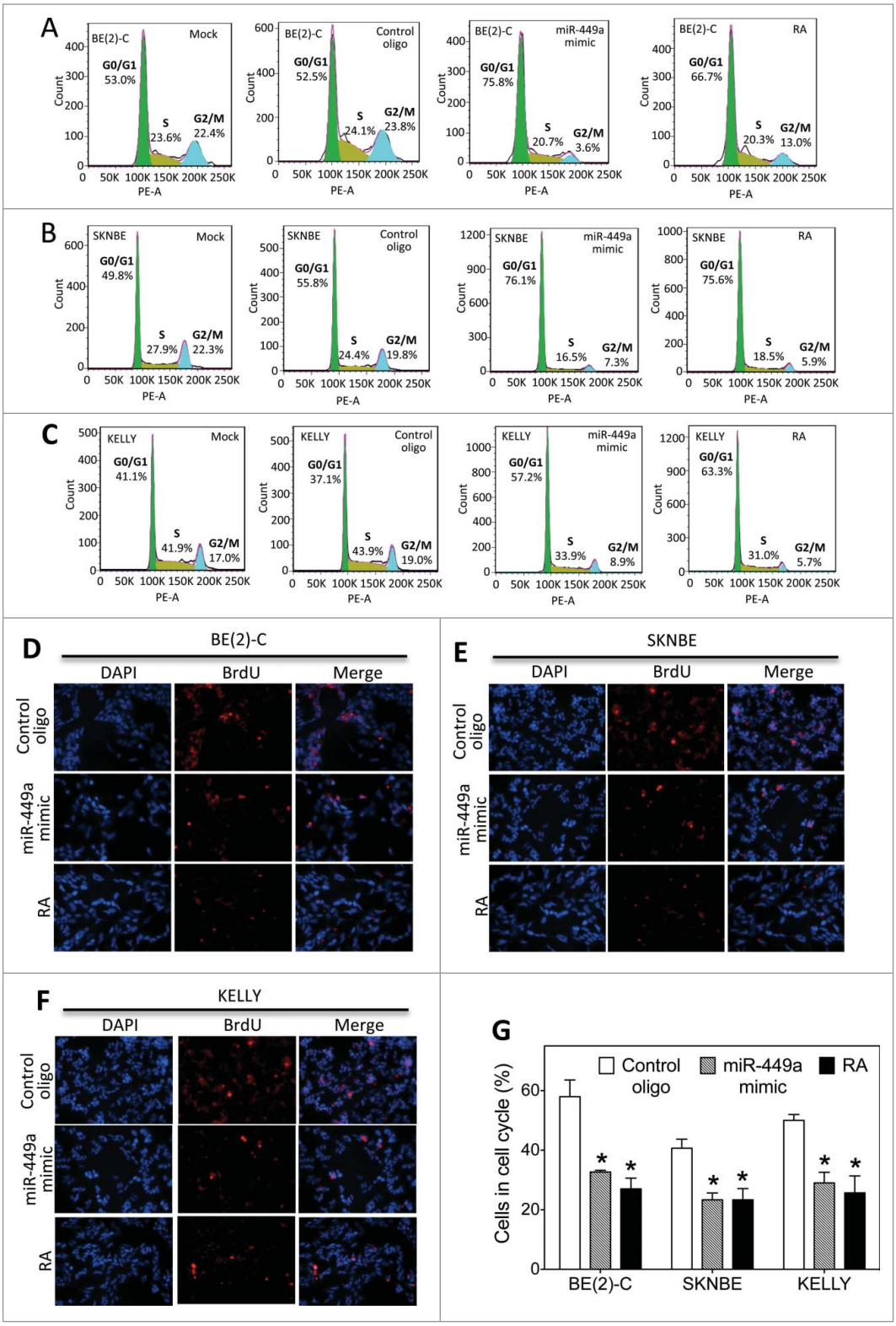



**Figure 4. Effect of miR-449a on cell cycle progression in neuroblastoma cells**. (**A–C**) Effect of miR-449a overexpression on cell cycle distribution in (**A**) BE(2)-C, (**B**) SKNBE and (**C**) KELLY cells. Cells were transfected with 25 nM miR-449a mimic or control oligo, or treated with 5 μM RA. Mock transfected cells were used as an additional negative control. After 3 d, cells were collected and stained with propidium iodide for cell cycle analysis. The fraction of cells in G0/G1, S and G2 phases was quantified using the Jean-Jett-Fox model. Similar results were obtained from 3 independent experiments. (**D–G**) Effect of miR-449a and RA on DNA synthesis as measured by BrDU incorporation in BE(2)-C (**D**), SKNBE (**E**) and KELLY (**F**) cells. Cells were transfected with miR-449a mimic or control oligos (25 nM), or treated with RA (5 μM) for 4 d. Cells were incubated with BrdU during the final 2 h of culture. Cells were then stained for BrDU incorporation (red) as described in the Materials and Methods. Cell nuclei were identified by nuclear staining with DAPI (blue). (**G**) Quantification of percentage of cells positive for BrDU staining. *, $P < 0.05$, comparing to control.

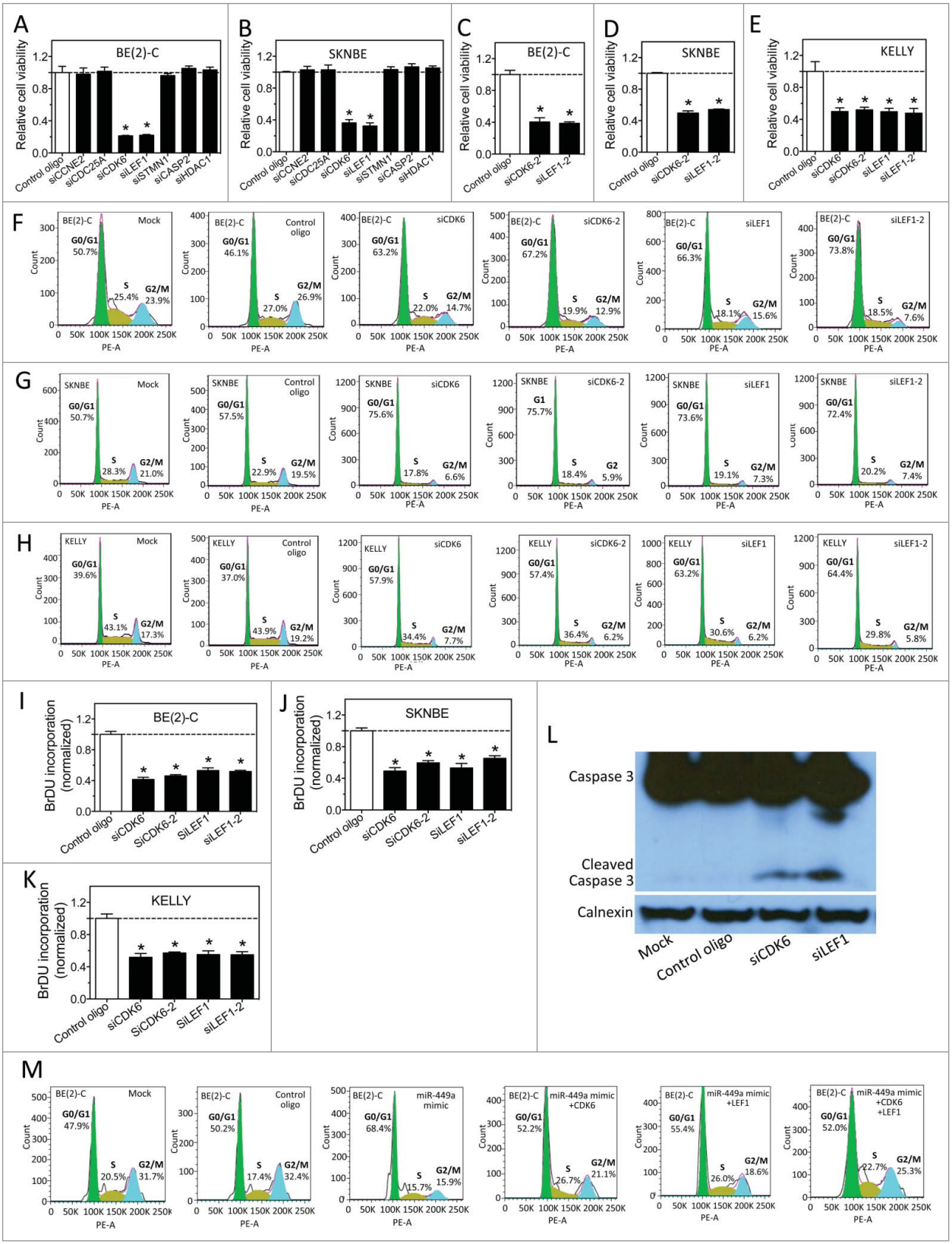



**Figure 5. Function of the predicted miR-449a cell cycle-regulating targets in regulating cell cycle progression and cell survival.** (**A–E**), Effect of knocking down the predicted miR-449a targets on cell viability in BE(2)-C, SKNBE and KELLY cells using 2 sets of siRNAs for the indicated genes. Cells were transfected with 25 nM of the indicated siRNAs or control oligo (Control). After 4 d, cell viability was measured as above. *, $P < 0.05$, comparing to Control. (**F–H**), Effect of knocking down CDK6 and LEF1 on cell cycle distribution in (**F**) BE(2)-C, (**G**) SKNBE and (**H**) KELLY cells. Cells were transfected with 25 nM of the indicated siRNAs or control oligo (Control). Mock-transfected cells were used as an additional negative control. After 3 d, cell cycle distributions were measured as above. (**I–K**) Effect of knocking down CDK6 and LEF1 on BrDU incorporation in (**I**) BE(2)-C, (**J**) SKNBE and (**K**) KELLY cells. Cells were transfected as above, and the level of BrDU incorporation was measured as above. (**L**) Western blot analysis of Caspase-3 activation. BE(2)-C cells were transfected with 25 nM of the indicated oligos for 3 d, and caspase-3 levels were measured as above. (**M**) Effect of gene overexpression on cell cycle distribution. BE(2)-C cells were transfected with the indicated expression vectors and oligos. After 3 d, cell cycle distributions were analyzed as above.

KELLY. Correspondingly, CDK6 and LEF1 knockdown leads to reduced cell proliferation, as measured by BrDU incorporation assay (**Fig. 5I–K**). In addition, we show that CDK6 and LEF1 knockdown also leads to the activation of the apoptotic pathway as indicated by the increased levels of cleaved capase 3 (**Fig. 5L**), consistent with the effect of miR-449a overexpression. We further examined the role of CDK6 and LEF1 in mediating the cell cycle-regulating function of miR-449a by overexpressing these 2 genes in BE(2)-C cells. The protein overexpression levels of the 2 genes by their corresponding expression vectors were confirmed by Western blots (**Fig. S6**). As shown in **Figure 5M**, overexpression of CDK6 and LEF1 individually decrease the effect of miR-449a on G0/G1 arrest, and co-overexpression of CDK6 and LEF1 shows enhanced effect comparing to individual gene overexpression. These results indicates the function of miR-449a in regulating cell cycle distribution is mediated by coordinately down-regulating expression of CDK6 and LEF1.

Overall, our results suggest that, among the 7 target genes involved in cell cycle progression, CDK6 and LEF1 play dominant roles in mediating the effect of miR-449a on cell cycle progression.

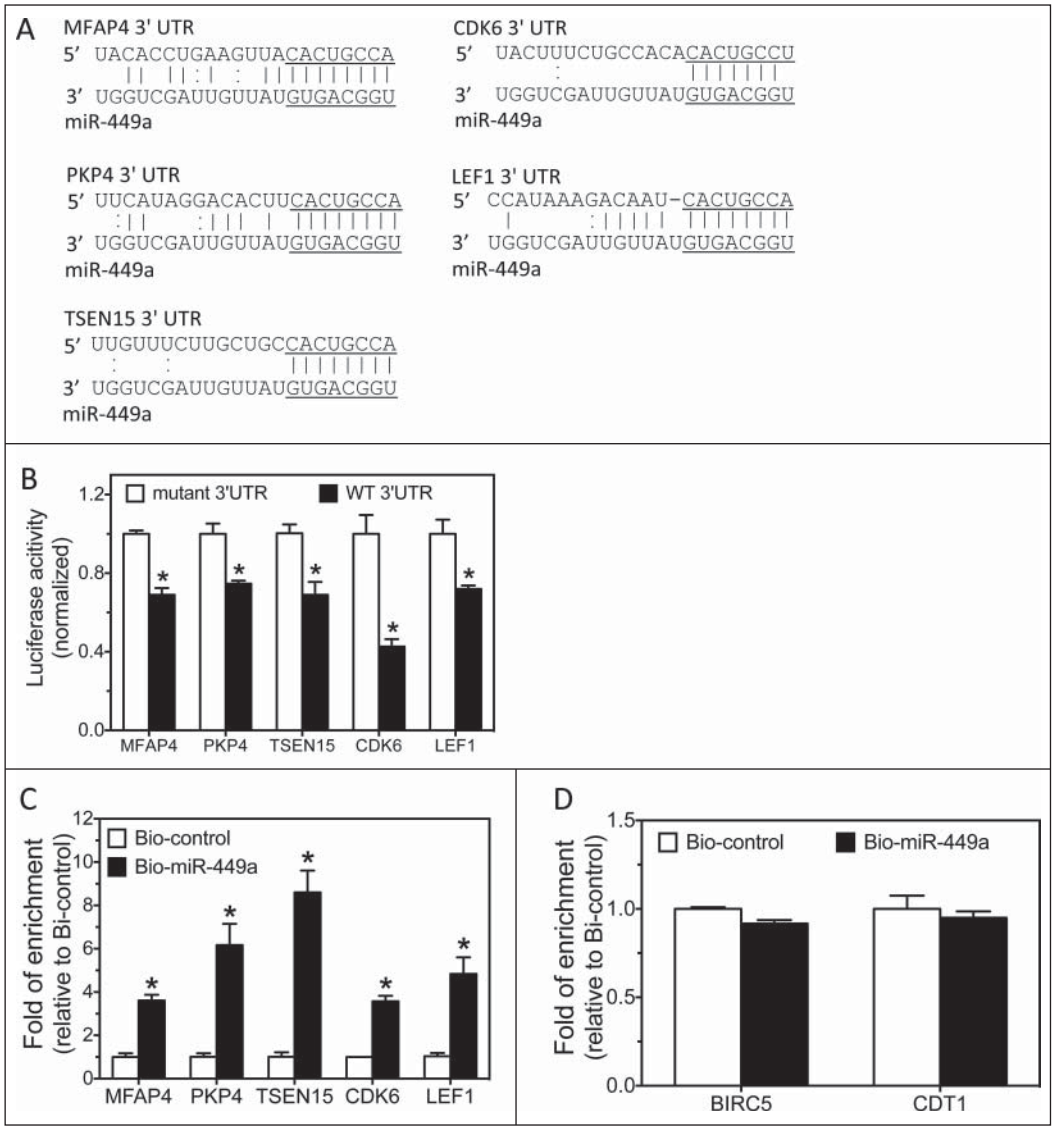



**Figure 6. Validation of the predicted direct targets of miR-449a**. (**A**) The predicted interactions between miR-449a and the target sites in the 3′UTRs of the indicated mRNAs. The seed sequences are underlined. (**B**) Validation of the target sites of miR-449a by luciferase reporter assay. BE(2)-C cells were co-transfected with the indicated vectors and miR-449a mimic. After 3 d of transfection, cells were lysed and luciferase activity was measured. Shown are normalized luciferase activities with the luciferase activity associated with wildtype (WT) 3′UTRs normalized to those associated with the corresponding mutant 3′UTRs. *, $P < 0.05$, comparing to mutant 3′UTRs. (**C**) Validation of the direct interactions of miR-449a with the target genes by Bio-miR-449a pulldown assay. BE(2)-C cells were transfected with the 25 nM of the Bio-miR-449a or control oligo (Bio-Control). The pulldown assay was performed following the protocol described in the Materials and Methods. Shown are fold of enrichment of the indicated mRNAs by Bio-miR-449a, calculated by normalizing the levels of mRNAs associated with Bio-miR-449a to those associated with the Bio-control. *, $P < 0.05$, comparing to the Bio-control. (**D**) BIRC5 and CDT1 mRNA levels in the Biotin pulldown products were measured by qPCR. *, $P < 0.05$, comparing to the Bio-control.

**miR-449a directly targets the 3′ UTRs of MFAP4, PKP4, TSEN15, CDK6 and LEF1**

The above results identified 3 direct targets of miR-449a that mediate its effect on cell differentiation and 2 targets that mediate its effect on cell cycle arrest. The predicted target sites of miR-449a in the mRNA 3′UTRs are shown in **Figure 6A**. We used a luciferase reporter assay to validate the predicted target sites. As shown in **Figure 6B**, miR-449a over-expression significantly decreased the luciferase activity in cells expressing the wildtype 3′UTRs as compared with mutated 3′UTRs for all of the 5 target genes. For each of the targets, the luciferase activity for wildtype 3′UTRs were decreased by >25 % comparing to the mutated

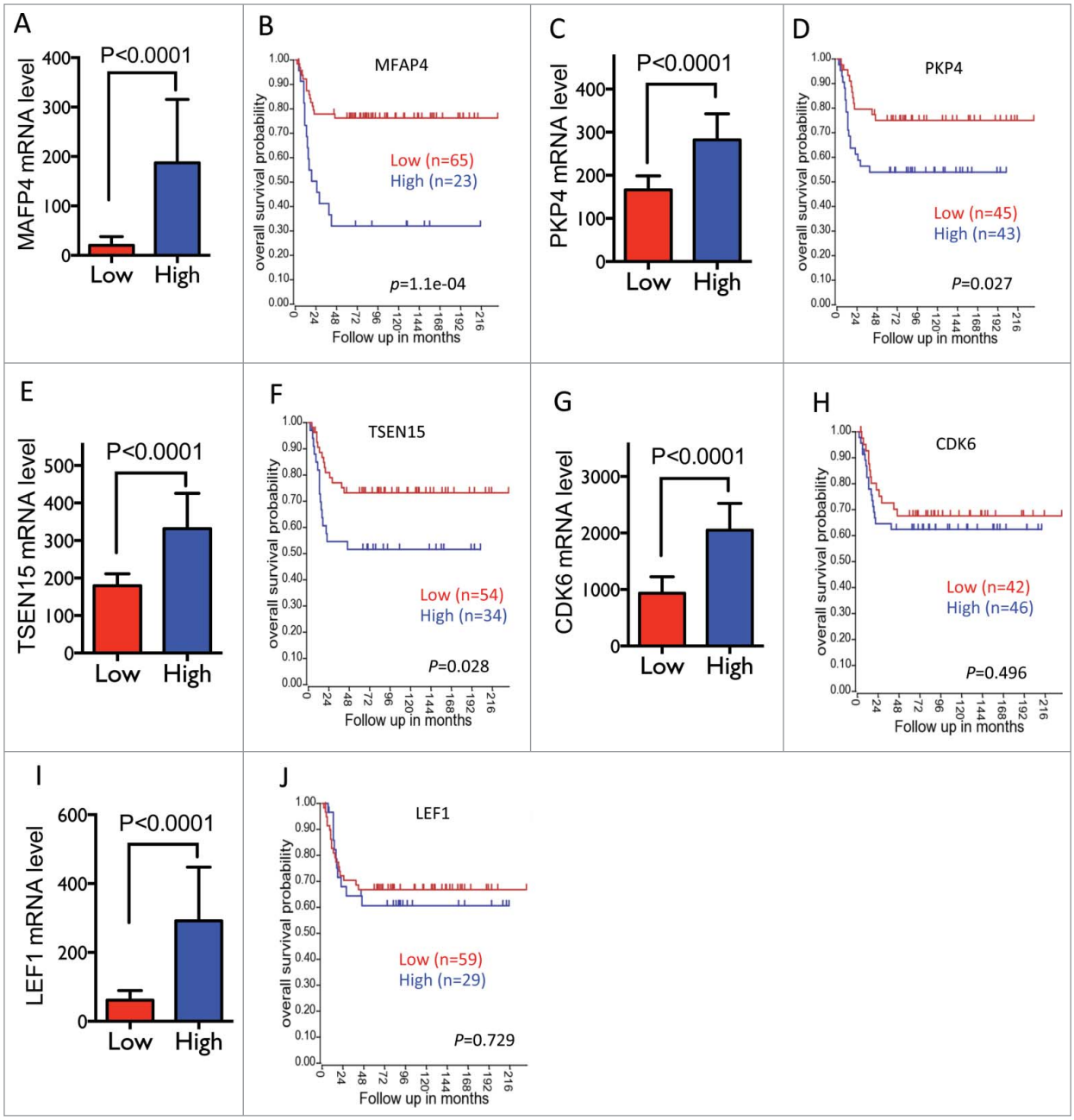



**Figure 7. Correlation of tumor mRNA expression levels with survival of neuroblastoma patients in the Vertseeg patient cohort**. For each of the indicated gene, the 88 neuroblastoma patients were divided into high and low groups as described in the Materials and Methods. Shown are the mRNA expression levels (**A, C, E, G, I**) and the Kaplan-Meier overall survival curves (**B, D, F, H, J**) in the low and high expression groups for each of the indicated genes. The mRNA expression levels were presented as average levels of the normalized signal intensities in each group.

3′UTRs. This magnitude of repression is consistent with that observed in previous studies of miRNA regulation using luciferase reporter vectors.[44,45] These results demonstrate that the predicted target sites are true target sites of miR-449a. In order to further demonstrate the physical interactions of these mRNAs with miR-449a, we performed biotinylated-miRNA pulldown assay[46] in BE(2)-C cells transfected with biotinylated miR-449a mimic (Bio-miR-449a). As shown in **Figure 6C**, for all the 5 target genes examined, their mRNA levels are significantly enriched by Bi-miR-449a pull-down relative to the biotinylated control oligo (Bio-control). In contrast, the mRNA levels of BIRC5 (Baculoviral IAP Repeat Containing 5) and CDT1 (Chromatin Licensing and DNA Replication Factor 1), which are not targets of miR-449a, are not enriched by Bio-miR-449a pulldown (**Fig. 6D**), further supporting that Bio-miR-449a pulldown product is specifically enriched with mRNAs that are miR-449a targets. Overall, our studies demonstrate the physical interactions of the 5 target mRNAs with miR-449a.

**High tumor expression levels of MFAP4, PKP4 and TSEN15, the differentiation-regulating targets of miR-449a, are correlated with poor survival of neuroblastoma patients**

The above results suggest that miR-449a functions as a tumor suppressor in neuroblastoma through at least 2 mechanisms—inducing cell differentiation and arresting cells in G0/G1 phase. In order to further understand the clinical relevance of the miR-449a-mediated tumor suppressive pathway in neuroblastoma development, we assessed the correlation of the expression of the identified target genes in neuroblastoma tumor specimens with patient survival in 2 published neuroblastoma patient cohorts: the Versteeg and the Kocak cohorts.[47,48] As shown in **Figure 7A–F and Figure 8A–F**, Kaplan-Meier survival analysis indicates that, in both patient cohorts, patients with high tumor MFAP4, PKP4 or TSEN15 mRNA levels have significantly lower overall survival rates comparing to patients with low expression levels of these genes. However, as shown in **Figure 7G–J** in the Versteeg cohort, tumor expression levels of CDK6 and LEF1 are not significantly correlated with patient survival. We observe similar results for CDK6 in the Kocak cohort (**Fig. 8G–H**). Paradoxically, in the Kocak cohort, the low levels of LEF1 in tumors are correlated with poor survival (**Fig. 8I-J**), which contradicts the tumor suppressive function of miR-449a. Overall, our comprehensive analyses suggest that expression levels of the identified differentiation-regulating targets of miR-449a, but not the cell cycle-regulating targets, are clinically relevant in determining the prognosis of the neuroblastoma patients.

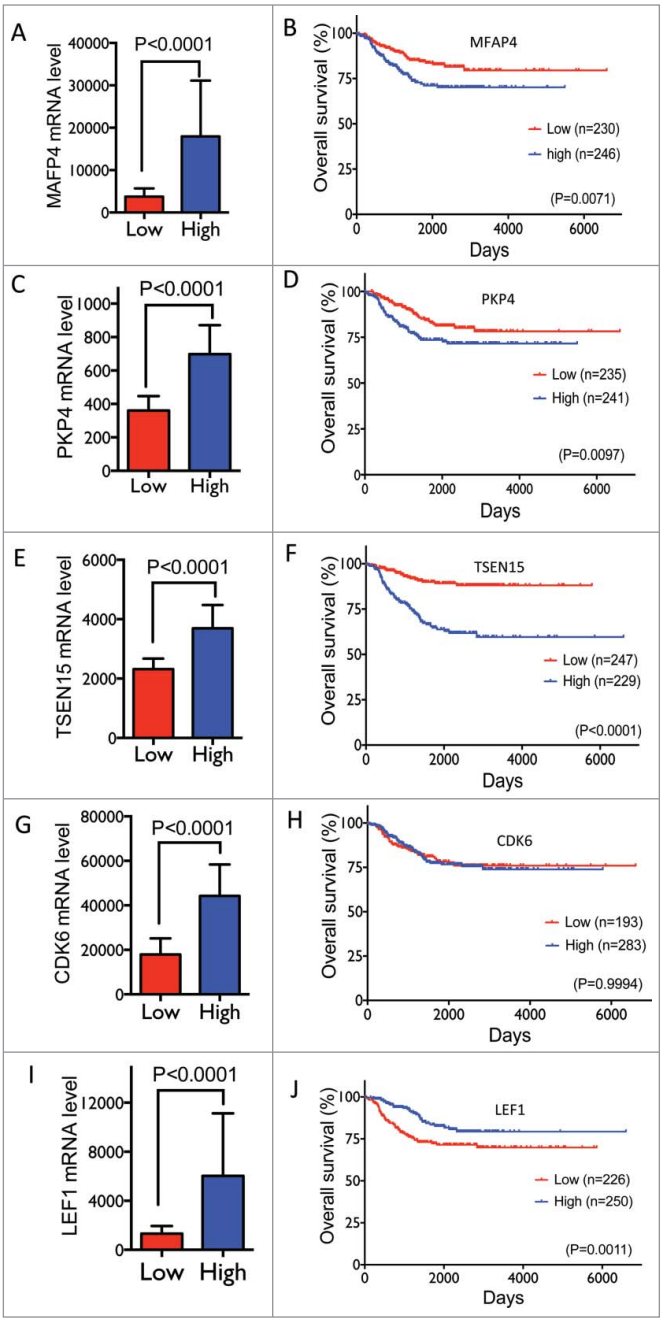



**Figure 8. Correlation of tumor mRNA expression levels with survival of neuroblastoma patients in the Kocak patient cohort.** The 476 patients were divided into high and low groups as above. Shown are the mRNA expression levels (**A, C, E, G, I**) and the Kaplan-Meier overall survival curves (**B, D, F, H, J**) in the low and high expression groups for each of the indicated genes. The mRNA expression levels were presented as average levels of the normalized signal intensities in each group.

## Discussion

Our study identifies a novel tumor suppressive mechanism in neuroblastoma mediated by miR-449a. Our results clearly indicate that the function of miR-449a in reducing neuroblastoma cell survival involves 3 molcular mechanisms: inducing cell cycle arrest and cell differentiation, inducing cell apoptosis, and reducing cell proliferation. Previous studies have demonstrated that miR-449a functions as a tumor suppressor through blocking cell cycle progression in several types of cancers, including lung cancer, prostate cancer, gastric cancer and retinoblastoma.[26-29,32] However, whether miR-449a regulates cell cycle progression in neuroblastoma has not been investigated. Consistent with previous studies in other cancer types, we show that miR-449a also induces G0/G1 phase arrest in neuroblastoma cells. Aside from its function in regulating cell cycle, we show here that miR-449a functions as a tumor suppressor through an additional mechanism—inducing neuroblastoma cell differentiation. In addition, we identified novel target genes of miR-449a that mediate its function in regulating cell differentiation and cell cycle progression. It is possible that the reduced cell proliferation and induced cell apoptosis by miR-449a overexpression are just the subsequent consequences of cell differentiation and cell cycle arrest. However, it is possible that miR-449a also targets genes in the cell proliferation and apoptosis pathways that are independent of the cell cycle/differentiation-regulating pathways. This is certainly interesting and warrants further investigations in the future. Overall, our study characterizes for the first time the underlying the molecular mechanisms that mediate the tumor suppressive function of miR-449a in neuroblastoma cells, as illustrated in **Figure 9**. Our in vitro investigations certainly suggest that this tumor suppressive mechanism mediated miR-449a may

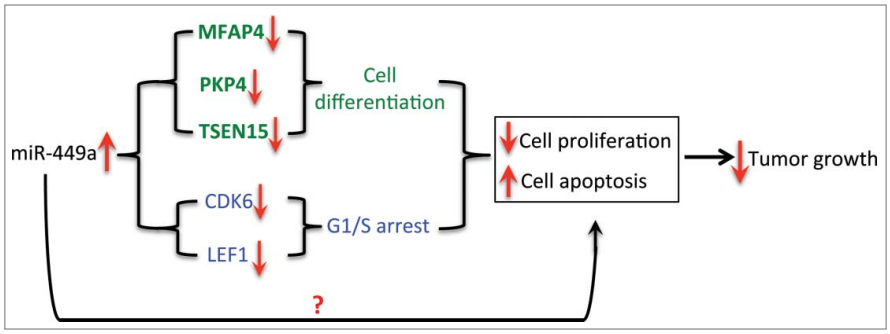



**Figure 9. The mechanisms of the miR-449a tumor suppressive function in neuroblastoma cells**. Our results support that miR-449a functions as a tumor suppressor in neuroblastoma through 2 molcular pathways: The cell differentiation pathway, which is mediated by down-regulating its direct targets MFAP4, PKP4 and TSEN15; The cell cycle pathway, which is mediated by down-regulating its direct targets CDK6 and LEF1. The induced cell differentiation and cell cycle arrest will result in reduced cell proliferation and increased cell apoptosis, which will lead to tumor growth arrest. However, it is possible that miR-449a also targets genes in the cell proliferation and apoptosis pathways that are independent of the cell cycle/differentiation-regulating pathways—this potential mechanism needs to be further investigated in the future.

play a critical role in neuroblastoma tumorigenicity. However, future in vivo investigations in neuroblastoma animal models are clearly needed in order to fully define the role of this miR-449a-mediated molecular pathway in determining neuroblastoma initiation and progression.

In our investigation of multiple neuroblastoma cell lines with different genetic backgrounds, we found that although miR-449a shows a general effect on cell differentiation, the sensitivity of different cell lines to miR-449a-induced differentiation varies greatly—it only induce cell differentiation to a minor extent in some cell lines (LAN6 and KELLY cells). Our previous work shows that another miRNA, miR-506–3p shows a strong differentiation-inducing effect in KELLY cells.[20] The sensitive response of KELLY cells to the miR-506–3p-mediated differentiation-inducing pathway indicates that this cell line does not lack intrinsic differentiation capacity, suggesting that its lack of response to miR-449a is a miR-449a-specific effect. The observed distinct response of a particular neuroblastoma cell line to different differentiation-inducing miRNAs is certainly interesting and potentially has great clinical significance. It not only indicates that different differentiation-inducing miRNAs functions through distinct molecular pathways, but also suggest that neuroblastoma cell differentiation can be induced through different molecular pathways by differentiation agents that act through distinct molecular mechanisms. For neuroblastoma that is not responsive to one type of differentiation agents, it is possible to use differentiation agents of different mechanisms to successfully treat it.

Interestingly, despite their distinct responses to the differentiation pathway, all the cell lines respond well to miR-449a-induced cell growth arrest. These results suggest that, when one tumor suppressive pathway mediated by miR-449a is inactive, the other can overcome the silencing of this pathway to achieve its strong tumor suppressive function—this dual tumor suppressive mechanisms make miR-449a-based therapeutics a promising approach to treat neuroblastoma with a wide spectrum of genetic backgrounds.

Our study identifies novel targets of miR-449a that mediate its tumor suppressive function. It is well known that each miRNA has hundreds of direct targets,[49] and each biological phenotype induced by a specific miRNA is likely achieved by concordantly down-regulating multiple targets.[50-52] Multiple miR-449a targets that are involved in mediating its tumor suppressor function have been identified and validated in other cancer types. However, our investigation shows that only 8 of the 20 targets validated in other cancer cell types are down-regulated in neuroblastoma cells (**Table S3**). This suggests that the function of miR-449a in regulating its target expression is cell-context specific. More importantly, we identified novel targets of miR-449a, including MFAP4, PKP4 and TSEN15, that play roles in regulating neuroblastoma cell differentiation. The molecular functions of these 3 genes have been defined in previous studies.[53-57] However, the role of these genes in tumorigenesis as well as in regulating cell differentiation has not been intensively explored previously—we are the first to identify their potential oncogenic functions in neuroblastoma by participating the cell differentiation process. More remarkably, we found that all the 3 differentiation-regulating targets of miR-449a, MFAP4, PKP4 and TSEN15, are significantly correlated with neuroblastoma patient survival, with high mRNA expression levels of these genes in neuroblastoma tumor specimens correlated with poor patient survival. These findings strongly suggest their critical role in determining the prognosis of neuroblastoma patients. Further studies are warranted to characterize the molecular mechanisms by which these genes regulate neuroblastoma cell differentiation, to elucidate how they fit into the known signaling network that control neuroblastoma cell differentiation, and to investigate whether specific differentiation agents targeting these genes can be developed.

Of note, in this study, we found that the expression levels of CDK6 in neuroblastoma are not significantly correlated with patient survival, although we observed its role in regulating cell cycle progression *in vitro* (**Fig. 5**) and their oncogenic functions have been well demonstrated previously.[58,59] One possible explanation is that CDK6 may need to coordinate with additional oncogenic pathways in neuroblastoma cells in order to reach a clinical significant impact on patient survival. This is certainly an interesting possibility and worth to be further pursued. The oncogenic function of LEF1 has also been reported previously.[60-62] However, we observed contradictive results in 2 neuroblastoma patient cohorts, which leaves the association of LEF1 expression with neuroblastoma patient prognosis undefined in our study. Future studies are certainly needed to clearly define the function of LEF1 in neuroblastoma development. In addition, the role of CDK6 and LEF1 in regulating cell

differentiation has also been indicated in previous studies.[63,64] However, we failed to observe the function of these 2 genes in regulating neuroblastoma cell differentiation. These results further demonstrate the complexity and the cell-context specificity of the cell differentiation pathways—it is reasonable to believe that there are alternative, cell type-specific signaling pathways in neuroblastoma cells to control the cell differentiation process that are normally controlled by CDK6 and LEF1 in other cell types, which makes CDK6 and LEF1 not essential to determine the differentiation fate of neuroblastoma cells.

Another finding in our study is that co-overexpression of the 3 differentiation-regulating targets MFAP4, PKP4 and TSEN15 only partially inhibited the differentiation-inducing effect of miR-449a. Likewise, co-overexpression of CDK6 and LEF1 only partially inhibited the effect of miR-449a on cell cycle distribution. These results suggest that there are additional targets playing an important role in its differentiation-inducing and cell cycle-regulating functions. In this study, we only investigated the genes that are predicted as miR-449a targets using the canonical miRNA target prediction approach, which is based on the interactions of seed region, a 6–8 nucleotides at the 5′ end of the miRNA, with the target sites in the mRNA 3′ UTR,[65-67] and we only investigated the predicted targets genes that are downregulated by >40% at mRNA levels by miR-449a. Although miRNAs down-regulate the majority of their targets at mRNA levels, it is known miRNAs can also regulate its target gene expression through translational repression, leading to decreased protein expression of the target genes without affecting the mRNA levels.[68] In addition, it has been demonstrated that miRNA may also target genes through non-canonical interactions.[69–72] It is highly possible that there are additional miR-449a targets that not investigated in this study playing important roles in mediating its differentiation-inducing and cell cycle-regulating functions. Future studies are certainly warranted to investigate the role of these targets in order to completely elucidate the tumor suppressive mechanisms of miR-449a in neuroblastoma cells.

Due to the limited resources for investigating the miR-449a expression in neuroblastoma tumor specimens, we were unable to examine the miR-449a endogenous expression levels in neuroblastoma primary tumors and therefore unable to characterize the correlation of miR-449a levels with neuroblastoma patient survival in the present study. However, we show that the endogenous expression of miR-449a in differentiated neuroblastoma cells is significantly increased comparing to the undifferentiated cells. These results strongly suggest miR-449a is endogenously expressed in normal differentiated neural crest cells, but its expression is significantly suppressed in undifferentiated neuroblastoma cells—this raises the possibility to activate its endogenous expression as a possible approach to differentiation therapy. The mechanisms that control its endogenous expression certainly warrant further investigation in order to develop therapeutic strategies to activate its endogenous differentiation-inducing function.

To summarize, we have identified a novel miR-449a-mediated tumor suppressive mechanism in neuroblastoma. The clinically observed significant correlation of the differentiation-regulating targets of miR-449a with neuroblastoma patient survival suggests the potential clinical significance of the dysregulation of miR-449a expression in neuroblastoma tumorigenesis — loss of miR-449a expression in neuroblastoma cells may play a critical role in neuroblastoma development by inducing aberrant expression of MFAP4, PKP4 and TSEN15. Further studies are definitely warranted to investigate the correlation of tumor miR-449a expression with patient prognosis.

## Materials and Methods

### Materials

miR-449a mimic and negative control oligos were obtained from Thermo Fisher Scientific. miR-449a precursors were purchased from Ambion. Rabbit anti-GAP43, anti-NSE and anti-βIII-tubulin antibodies were obtained from Abcam. Rabbit anti-calnexin, and goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-human caspase-3, CDK6 and LEF1 antibodies were from Cell Signaling Technology. Rabbit anti-human MFAP4 and PKP4 antibodies were from Thermo Fisher Scientific, Inc. Rabbit anti-human TSEN15 antibody was from Aviva Systems Biology. Bio-miR-449a and Bio-control oligo were purchased from Sigma. siRNAs and the negative control oligos were purchased from Dharmacon and Sigma, respectively. pCMV-CDK6 expression vector was a gift from Sander van den Heuvel (Addgene plasmid #1868).[73]

### Cell lines

BE(2)-C, SKNBE and BE(2)-M17 cells were purchased from the American Type Culture Collection (ATCC). LAN6 cells were obtained from Children's Oncology Group. KELLY cells were obtained from the cell line repository at the Greehey Children's Cancer Research Institute, University of Texas Health Science Center at San Antonio. Cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum.

### Detection and quantification of neurite outgrowth

Cells were plated and treated in 96-well plates. For measuring neurite outgrowth, cells were placed into IncuCyte ZOOM Live Cell Imaging System (Essen BioScience), and images were taken under 20X magnification. For measuring neurite outgrowth in a time-dependent manner, cell images were taken every 6 hours. The neurite lengths associated with each treatment were calculated using the neurite definition determined for each specific cell line using the NeuroTrack system (Essen BioScience).

### Cell cycle analysis

Cells were detached by scrapping, and cell suspension was pipetted for 10 times to separate the cell clumps into single cells. Cells were then collected by centrifuging at 1,000 rpm for 5 min. The cells were washed once with 1X PBS. To fix cells, ice-cold 1X PBS containing 1 mM EDTA and 85% ethanol was added to cells drop-wise while vortexing, in order to prevent cell aggregation. After 1 hour, the cells were harvested by centrifugation at 1,400 rpm for 5 minutes at 4°C, and cells were re-suspended in 1X PBS, and treated with 50 μg/ml Propidium iodide

and 100 μg/ml RNase A for 30 minutes at 37°C. Cells were then passed through 271 /2G needle for 5 times and filtered through 35 μM strainers. Cell cycle data were collected on a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed using FlowJo version 7.6.5 (TreeStar, Ashland, OR).

### Western blots

Cell lysates were prepared using RIPA buffer. Protein concentration was determined using the Pierce BCA assay (Thermo Fisher Scientific). For electrophoresis, equal amounts of cell lysates were resolved by SDS-PAGE and transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories). Membranes were blocked and probed with primary antibodies to specific proteins. Bound antibodies were detected with secondary antibodies conjugated with horseradish peroxidase (HRP) and visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

### Cell viability assay

Cell viability was measured as previously described.[41] Briefly, cells were plated in 96-well format and treated as specified. After culturing for 4–5 d, cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

### BrdU incorporation assay

Cells were seeded in 96-well plates and treated as indicated. After culturing for 3 d, cells were incubated with 10 μM BrdU (Roche, Indianapolis, IN, USA), and then fixed with FixDenat solution. Anti-BrdU-POD was added to fixed cells, and substrate solution containing tetramethylbenzidine was used to detect and quantify the amount of BrdU incorporation.

### Immunofluorescence detection of DNA synthesis by BrdU labeling

We used a protocol described by Feliciano DM.[43] Briefly $1 \times 10^4$ BE(2)-C cells were plated on coverslips in 12-well plates, and were transfected with miR-449a mimic (25 nM) or control oligos (25 nM), or treated with RA (5 μM) for 4 d. Cells were then incubated with 10 μM BrdU for 2 h. Cell were fixed with 1 ml of 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were then washed with PBS and incubated in 2 N HCl at 37°C for 15 min to denature chromatin. After washing with PBS, cells were incubated with 2% goat serum at 4°C for 18 h to block nonspecific binding. Cells were then labeled by incubating with mouse BrdU monoclonal antibody (Cell Signaling) for 2 h, followed by incubating with goat anti-mouse Alexa 555 (Molecular Probes). DAPI (Life Technologies) was used to stain cell nucleus.

### Colony formation assay

Cells were transfected with miR-449a mimic or control oligos (25 nM) for 24 h. 1000 cells were then re-seeded on each 10 cm dish. After 14 d, colonies were visualized by staining with 1% crystal violet. Colonies were counted using ImageJ (NIH), and differences were assessed by 2-tailed t-test.

### Construction of the expression vectors

The protein-coding regions of the MFAP4, PKP4, TSEN15 and LEF1 genes (without the 3'UTRs, to avoid the down-regulation by miR-449a) were amplified by PCR from human cDNA clones (GE Dharmacon) of the corresponding genes. The amplified MFAP4, TSEN15 and LEF1 sequences were inserted into the EcoRI and XhoI restriction sites of the multiple cloning site of expression vector pcDNA3.1+. The amplified PKP4 was inserted into the HindIII and NotI restriction sites of pcDNA3.1+. All the inserted sequences were verified by sequencing.

### miRNA target prediction

miR-449a target sites in 3′UTRs of the mRNAs were identified based on seed sequence complementarity using IPA, which identifies any 7-nucleotide region (3′- 5′) in a given 3′UTR completely complementary to the seed sequence of a miRNA (nucleotides 2nd–8th or 1st – 7th, 5′- 3′) as a potential target site.

### mRNA and miRNA expression

Total RNA was isolated as previously described.[74] mRNA expression profiling was performed using the Illumina mRNA WG-6 v3 microarray platform. qPCR measurement of mRNA expression was done using Taqman Gene Expression Assay (Life Technologies) with 18s rRNA expression as a control for normalizing RNA loading. miRNA expression was measured by qPCR using Taqman microRNA Assays (Life Technologies) with expression of RNU44 RNA used as a loading control.

### Luciferase reporter assay

The luciferease reported assay was performed as previously described.[42] Briefly, the segments of the wildtype 3′UTRs containing the predicted target sites of miR-449a were cloned from human genomic DNA. Mutant constructs were generated with the seed target sequences mutated to CAGACGGA/U. The 3′UTRs were cloned downstream of the firefly luciferase coding sequences into the pmirGLO dual-luciferase reporter (Promega), a vector containing both firefly and Renilla luciferase cDNAs under the control of separate promoter/terminator systems. The firefly luciferase was used as the primary reporter for miRNA regulation of the 3'UTR. The Renilla luciferase is an internal control for normalization. BE(2)-C cells were co-transfected with luciferase reporters (0.8 ng/ul) and miR-449a mimic or control oligo (25 nM). Luciferase activities were measured after 3 d using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity to evaluate the effect of the miRNAs.

### Biotinylated-miR-449a pulldown assay

The pulldown was performed using a protocol based on Lal A, et al.[46] The protocol and the calculation for the target enrichment by Bio-miR-449a pulldown is illustrated in **Suppl. Figure 7**. Briefly, cells were transfected with either Bio-miR-449a or control oligo (Bio-Control) (25 nM). After 24 h, cells were lysed, and cell lysate was separated into 2 parts: 95% was subject to biotin pull-down with streptavidin-conjugated beads, and RNA was then isolated using the RNeasy Kit (Qiagen); 5% was

used to isolate RNA as an input control. The RNA samples were analyzed by qPCR for the levels of individual target mRNAs with GAPDH mRNA, which is not a target of miR-449a, as an internal control for normalizing the target mRNA levels in each sample. The mRNA enrichment by Bio-miR-449a or Bio-Control pulldown was then determined by the ratio of mRNA levels associated with the pulldown RNA versus the input RNA.

### Neuroblastoma patient survival analysis

Survival analyses were based on 2 published neuroblastoma patient cohorts: the Versteeg cohort which includes 88 patients, and the Kocak cohort which includes 476 patients.[47,48] The microarray gene expression and the patient survival data for the Versteeg cohort were directly obtained from http://r2.amc.nl. The microarray gene expression data for the Kocak cohort were downloaded from http://r2.amc.nl, and patient prognosis data for the Kocak cohort were downloaded from the authors' previous publication.[48] For patient survival studies, overall survival was defined as the time from diagnosis until death or until last contact. For the analysis of genes in each of the patient cohort, the patients were divided into 2 groups based on whether the tumor gene expression level of the patient is lower (low group) or higher (high group) than the average gene expression of the patient cohort. Survival analyses were performed using the Kaplan and Meier method, and significance of the survival difference between the 2 groups was determined using 2-sided log-rank test.

### Statistical analysis

The statistical significance of the difference in frequency distribution between the expression of the predicted miR-449a targets and non-targets were determined by 2-sample Kolmogorov-Smirnov test, with $P < 0.05$ considered as significant. For evaluating effect of the panel of 25 siRNAs on neurite outgrowth, the *p*-values for neurite lengths associated with siRNAs was determined by comparison with neurite length associated with the negative control oligo using multiple-sample t-test. We consider a siRNA with False Discovery Rate (FDR) <0.01 as significantly inducing neurite outgrowth. For all other conditions, the statistical significance for each treatment was determined by *t*-test by comparing the treatment group with control, with $P < 0.05$ considered statistically significant.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


### Acknowledgments

We thank Dr. Subrata Haldar for critical reading of the manuscript and providing constructive suggestions.

### Funding

This work was supported by PRMRP Discovery Award PR121532 from the Department of Defense (to L. Du), NIH Cancer Center Support Grants P30 CA054174-17, and NIH CTSA 1UL1RR025767-01 (to Y. Chen).


### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

### References


1. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. Lancet 2007; 369(9579):2106-20; PMID: 17586306
2. Park JR, Eggert A, Caron H. Neuroblastoma: biology, prognosis, and treatment. Hematol Oncol Clin North Am 2010; 24(1):65-86; PMID:20113896; http://dx.doi.org/10.1016/j.hoc.2009.11.011
3. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003; 3(3):203-16; PMID:12612655; http://dx.doi.org/10.1038/nrc1014
4. Reynolds CP. Differentiating agents in pediatric malignancies: retinoids in neuroblastoma. Curr Oncol Rep 2000; 2(6):511-8; PMID:11122886; http://dx.doi.org/10.1007/s11912-000-0104-y
5. Cruz FD, Matushansky I. Solid tumor differentiation therapy – is it possible? Oncotarget 2012; 3(5):559-67; PMID:22643847
6. Nowak D, Stewart D, Koeffler HP. Differentiation therapy of leukemia: 3 decades of development. Blood 2009; 113(16):3655-65; PMID:19221035; http://dx.doi.org/10.1182/blood-2009-01-198911
7. Matthay KK, Reynolds CP, Seeger RC, Shimada H, Adkins ES, Haas-Kogan D, Gerbing RB, London WB, Villablanca JG. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. J Clin Oncol 2009; 27(7):1007-13; PMID:19171716; http://dx.doi.org/10.1200/JCO.2007.13.8925
8. Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 1999; 341(16):1165-73; PMID:10519894; http://dx.doi.org/10.1056/NEJM199910143411601
9. Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev 2009; 28(3–4):369-78; PMID:20012925; http://dx.doi.org/10.1007/s10555-009-9188-5
10. Li M, Li J, Ding X, He M, Cheng SY. microRNA and cancer. Aaps J 2010; 12(3):309-17; PMID:20422339; http://dx.doi.org/10.1208/s12248-010-9194-0
11. Kinoshita T, Hanazawa T, Nohata N, Kikkawa N, Enokida H, Yoshino H, Yamasaki T, Hidaka H, Nakagawa M, Okamoto Y, et al. Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma. Oncotarget 2012; 3(11):1386-400; PMID:23159910
12. Kopp F WE, Roidl A. The proto-oncogene KRAS is targeted by miR-200c. Oncotarget 2013; 5:185-95; PMID:24368337
13. Bier A, Giladi N, Kronfeld N, Lee HK, Cazacu S, Finniss S, Xiang C, Poisson L, de Carvalho AC, Slavin S, et al. MicroRNA-137 is downregulated in glioblastoma and inhibits the stemness of glioma stem cells by targeting RTVP-1. Oncotarget 2013; 4(5):665-76; PMID:23714687
14. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, Weidhaas JB, Bader AG, Slack FJ. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Mol Ther: J Am Soc Gene Ther 2011; 19(6):1116-22; PMID:21427705; http://dx.doi.org/10.1038/mt.2011.48
15. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 2009; 137(6):1005-17; PMID:19524505; http://dx.doi.org/10.1016/j.cell.2009.04.021
16. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. Nature 2005; 438(7068):685-9; PMID:16258535; http://dx.doi.org/10.1038/nature04303
17. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, Weidhaas JB, Bader AG, Slack FJ. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Mol Ther 2011; 19(6):1116-22; PMID:21427705; http://dx.doi.org/10.1038/mt.2011.48
18. Bader AG, Brown D, Winkler M. The promise of microRNA replacement therapy. Cancer Res 2010; 70(18):7027-30; PMID:20807816; http://dx.doi.org/10.1158/0008-5472.CAN-10-2010
19. Mishra PJ, Merlino G. MicroRNA reexpression as differentiation therapy in cancer. J Clin Invest 2009; 119(8):2119-23; PMID:19620782
20. Zhao Z, Ma X, Hsiao TH, Lin G, Kosti A, Yu X, Suresh U, Chen Y, Tomlinson GE, Pertsemlidis A, et al. A high-content morphological screen identifies novel microRNAs that regulate neuroblastoma cell differentiation. Oncotarget 2014; 5(9):2499-512; PMID:24811707
21. Makeyev EV, Zhang J, Carrasco MA, Maniatis T. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol Cell 2007; 27(3):435-48; PMID:17679093; http://dx.doi.org/10.1016/j.molcel.2007.07.015

22. Annibali D, Gioia U, Savino M, Laneve P, Caffarelli E, Nasi S. A new module in neural differentiation control: two microRNAs upregulated by retinoic acid, miR-9 and -103, target the differentiation inhibitor ID2. PLoS One 2012; 7(7):e40269; PMID:22848373; http://dx.doi.org/10.1371/journal.pone.0040269
23. Le MT, Xie H, Zhou B, Chia PH, Rizk P, Um M, Udolph G, Yang H, Lim B, Lodish HF. MicroRNA-125b promotes neuronal differentiation in human cells by repressing multiple targets. Mol Cell Biol 2009; 29 (19):5290-305; PMID:19635812; http://dx.doi.org/10.1128/MCB.01694-08
24. Foley NH, Bray I, Watters KM, Das S, Bryan K, Bernas T, Prehn JH, Stallings RL. MicroRNAs 10a and 10b are potent inducers of neuroblastoma cell differentiation through targeting of nuclear receptor corepressor 2. Cell Death Differ 2011; 18(7):1089-98; PMID:21212796; http://dx.doi.org/10.1038/cdd.2010.172
25. Swarbrick A, Woods SL, Shaw A, Balakrishnan A, Phua Y, Nguyen A, Chanthery Y, Lim L, Ashton LJ, Judson RL, et al. miR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma. Nat Med 2010; 16 (10):1134-40; PMID:20871609; http://dx.doi.org/10.1038/nm.2227
26. Martin A, Jones A, Bryar PJ, Mets M, Weinstein J, Zhang G, Laurie NA. MicroRNAs-449a and -449b exhibit tumor suppressive effects in retinoblastoma. Biochem Biophys Res Commun 2013; 440(4):599-603; PMID:24120948; http://dx.doi.org/10.1016/j.bbrc.2013.09.117
27. Luo W, Huang B, Li Z, Li H, Sun L, Zhang Q, Qiu X, Wang E. MicroRNA-449a is downregulated in non-small cell lung cancer and inhibits migration and invasion by targeting c-Met. PLoS One 2013; 8(5):e64759; PMID:23734217; http://dx.doi.org/10.1371/journal.pone.0064759
28. Noonan EJ, Place RF, Basak S, Pookot D, Li LC. miR-449a causes Rb-dependent cell cycle arrest and senescence in prostate cancer cells. Oncotarget 2010; 1 (5):349-58; PMID:20948989
29. Noonan EJ, Place RF, Pookot D, Basak S, Whitson JM, Hirata H, Giardina C, Dahiya R. miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 2009; 28(14):1714-24; PMID:19252524; http://dx.doi.org/10.1038/onc.2009.19
30. Lize M, Pilarski S, Dobbelstein M. E2F1-inducible microRNA 449a/b suppresses cell proliferation and promotes apoptosis. Cell Death Differ 2010; 17 (3):452-8; PMID:19960022; http://dx.doi.org/10.1038/cdd.2009.188
31. Yang X, Feng M, Jiang X, Wu Z, Li Z, Aau M, Yu Q. miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. Genes Dev 2009; 23(20):2388-93; PMID:19833767; http://dx.doi.org/10.1101/gad.1819009
32. Wei B, Song Y, Zhang Y, Hu M. microRNA-449a functions as a tumor-suppressor in gastric adenocarcinoma by targeting Bcl-2. Oncol Lett 2013; 6(6):1713-8; PMID:24260067
33. Chen S, Dai Y, Zhang X, Jin D, Li X, Zhang Y. Increased miR-449a expression in colorectal carcinoma tissues is inversely correlated with serum carcinoembryonic antigen. Oncol Lett 2014; 7(2):568-72; PMID:24396489
34. Buechner J, Tomte E, Haug BH, Henriksen JR, Lokke C, Flaegstad T, Einvik C. Tumour-suppressor microRNAs let-7 and mir-101 target the proto-oncogene MYCN and inhibit cell proliferation in MYCN-amplified neuroblastoma. Br J Cancer 2011; 105(2):296-303; PMID:21654684; http://dx.doi.org/10.1038/bjc.2011.220
35. Radio NM, Breier JM, Shafer TJ, Mundy WR. Assessment of chemical effects on neurite outgrowth in PC12 cells using high content screening. Toxicol Sci 2008; 105(1):106-18; PMID:18539913; http://dx.doi.org/10.1093/toxsci/kfn114
36. Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 2009; 30 (1):127-35; PMID:19056420; http://dx.doi.org/10.1016/j.neuro.2008.11.001
37. Mao L, Ding J, Zha Y, Yang L, McCarthy BA, King W, Cui H, Ding HF. HOXC9 links cell-cycle exit and neuronal differentiation and is a prognostic marker in neuroblastoma. Cancer Res 2011; 71(12):4314-24; PMID:21507931; http://dx.doi.org/10.1158/0008-5472.CAN-11-0051
38. Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 2006; 103(11):4034-9; PMID:16495412; http://dx.doi.org/10.1073/pnas.0510928103
39. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 2010; 466(7308):835–40; PMID:20703300; http://dx.doi.org/10.1038/nature09267
40. Du L, Schageman JJ, Subauste MC, Saber B, Hammond SM, Prudkin L, Wistuba, II, Ji L, Roth JA, Minna JD, et al. miR-93, miR-98, and miR-197 regulate expression of tumor suppressor gene FUS1. Mol Cancer Res: MCR 2009; 7(8):1234-43; PMID:19671678; http://dx.doi.org/10.1158/1541-7786.MCR-08-0507
41. Du L, Subauste MC, DeSevo C, Zhao Z, Baker M, Borkowski R, Schageman JJ, Greer R, Yang CR, Suraokar M, et al. miR-337-3p and its targets STAT3 and RAP1A modulate taxane sensitivity in non-small cell lung cancers. PLoS One 2012; 7(6):e39167; PMID:22723956; http://dx.doi.org/10.1371/journal.pone.0039167
42. Du L, Zhao Z, Ma X, Hsiao TH, Chen Y, Young E, Suraokar M, Wistuba I, Minna JD, Pertsemlidis A. miR-93-directed downregulation of DAB2 defines a novel oncogenic pathway in lung cancer. Oncogene 2013; 33:4307-15; PMID:24037530; http://dx.doi.org/10.1038/onc.2013.381
43. Feliciano DM, Edelman AM. Repression of Ca2+/calmodulin-dependent protein kinase IV signaling accelerates retinoic acid-induced differentiation of human neuroblastoma cells. J Biol Chem 2009; 284 (39):26466-81; PMID:19633294; http://dx.doi.org/10.1074/1074.M109.027680
44. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, et al. Combinatorial microRNA target predictions. Nat Genet 2005; 37(5):495-500; PMID:15806104; http://dx.doi.org/10.1038/10381536
45. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007; 282(19):14328-36; PMID:17363372; http://dx.doi.org/10.1074/1074.M611393200
46. Lal A, Thomas MP, Altschuler G, Navarro F, O'Day E, Li XL, Concepcion C, Han YC, Thiery J, Rajani DK, et al. Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. PLoS Genet 2011; 7(11):e1002363; PMID:22102825; http://dx.doi.org/10.1371/1371.pgen.1002363
47. Kocak H, Ackermann S, Hero B, Kahlert Y, Oberthuer A, Juraeva D, Roels F, Theissen J, Westermann F, Deubzer H, et al. Hox-C9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma. Cell Death Dis 2013; 4: e586; PMID:23579273; http://dx.doi.org/10.1038/1038.2013.84
48. Oberthuer A, Juraeva D, Li L, Kahlert Y, Westermann F, Eils R, Berthold F, Shi L, Wolfinger RD, Fischer M, et al. Comparison of performance of one-color and two-color gene-expression analyses in predicting clinical endpoints of neuroblastoma patients. Pharmacogenomics J 2010; 10(4):258-66; PMID:20676065; http://dx.doi.org/10.1038/1038.2010.53
49. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136(2):215-33; PMID:19167326; http://dx.doi.org/10.1016/1016.cell.2009.01.002
50. Leucht C, Stigloher C, Wizenmann A, Klafke R, Folchert A, Bally-Cuif L. MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. Nat Neurosci 2008; 11(6):641-8; PMID:18454145; http://dx.doi.org/10.1038/1038.2115
51. Hudson RS, Yi M, Esposito D, Watkins SK, Hurwitz AA, Yfantis HG, Lee DH, Borin JF, Naslund MJ, Alexander RB, et al, MicroRNA-1 is a candidate tumor suppressor and prognostic marker in human prostate cancer. Nucleic Acids Res 2012; 40(8):3689-703; PMID:22210864; http://dx.doi.org/10.1093/1093/gkr1222
52. Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D, Bader AG. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. Cancer Res 2010; 70(14):5923-30; PMID:20570894; http://dx.doi.org/10.1158/0008-5472.CAN-10-0655
53. Schlosser A, Thomsen T, Shipley JM, Hein PW, Brasch F, Tornoe I, Nielsen O, Skjodt K, Palaniyar N, Steinhilber W, et al. Microfibril-associated protein 4 binds to surfactant protein A (SP-A) and colocalizes with SP-A in the extracellular matrix of the lung. Scand J Immunol 2006; 64(2):104-16; PMID:16867155; http://dx.doi.org/10.1111/1111.1365-3083.2006.01778.x
54. Kasamatsu S, Hachiya A, Fujimura T, Sriwiriyanont P, Haketa K, Visscher MO, Kitzmiller WJ, Bello A, Kitahara T, Kobinger GP, et al. Essential role of microfibrillar-associated protein 4 in human cutaneous homeostasis and in its photoprotection. Sci Rep 2011; 1:164; PMID:22355679; http://dx.doi.org/10.1038/103800164
55. Keil R, Schulz J, Hatzfeld M. p0071/00714, a multifunctional protein coordinating cell adhesion with cytoskeletal organization. Biol Chem 2013; 394(8):1005-17; PMID:23640939; http://dx.doi.org/10.1515/1515-2013-0114
56. Wolf A, Keil R, Gotzl O, Mun A, Schwarze K, Lederer M, Huttelmaier S, Hatzfeld M. The armadillo protein p0071 regulates Rho signalling during cytokinesis. Nat Cell Biol 2006; 8(12):1432-40; PMID:17115030; http://dx.doi.org/10.1038/10381504
57. Dhungel N, Hopper AK. Beyond tRNA cleavage: novel essential function for yeast tRNA splicing endonuclease unrelated to tRNA processing. Genes Dev 2012; 26 (5):503-14; PMID:22391451; http://dx.doi.org/10.1101/1101.183004.111
58. Bullrich F, MacLachlan TK, Sang N, Druck T, Veronese ML, Allen SL, Chiorazzi N, Koff A, Heubner K, Croce CM, et al. Chromosomal mapping of members of the cdc2 family of protein kinases, cdk3, cdk6, PISSLRE, and PITALRE, and a cdk inhibitor, p27Kip1, to regions involved in human cancer. Cancer Res 1995; 55(6):1199-205; PMID:7882308
59. Lu X, Fang Y, Wang Z, Xie J, Zhan Q, Deng X, Chen H, Jin J, Peng C, Li H, et al. Downregulation of gas5 increases pancreatic cancer cell proliferation by regulating CDK6. Cell Tissue Res 2013; 354(3):891-6; PMID:24026436; http://dx.doi.org/10.1007/100700441-013-1711-x
60. Li Y, Wang L, Zhang M, Melamed J, Liu X, Reiter R, Wei J, Peng Y, Zou X, Pellicer A, et al. LEF1 in androgen-independent prostate cancer: regulation of androgen receptor expression, prostate cancer growth, and invasion. Cancer Res 2009; 69(8):3332-8; PMID:19351848; http://dx.doi.org/10.1158/0008-5472.CAN-08-3380
61. Wang WJ, Yao Y, Jiang LL, Hu TH, Ma JQ, Ruan ZP, Tian T, Guo H, Wang SH, Nan KJ. Increased LEF1 expression and decreased Notch2 expression are strong predictors of poor outcomes in colorectal cancer patients.

Dis Markers 2013; 35(5):395-405; PMID:24223455; http://dx.doi.org/10.1155/2013/983981
62. de la Roche M, Ibrahim AE, Mieszczanek J, Bienz M. LEF1 and B9L shield beta-catenin from inactivation by Axin, desensitizing colorectal cancer cells to tankyrase inhibitors. Cancer Res 2014; 74(5):1495-505; PMID:24419084; http://dx.doi.org/10.1158/0008-5472.CAN-13-2682
63. Ogasawara T, Kawaguchi H, Jinno S, Hoshi K, Itaka K, Takato T, Nakamura K, Okayama H. Bone morphogenetic protein 2-induced osteoblast differentiation requires Smad-mediated down-regulation of Cdk6. Mol Cell Biol 2004; 24(15):6560-8; PMID:15254224; http://dx.doi.org/10.1128/1128.24.15.6560-6568.2004
64. Valdivia LE, Young RM, Hawkins TA, Stickney HL, Cavodeassi F, Schwarz Q, Pullin LM, Villegas R, Moro E, Argenton F, et al. Lef1-dependent Wnt/beta-catenin signalling drives the proliferative engine that maintains tissue homeostasis during lateral line development. Development 2011; 138(18):3931-41; PMID:21862557; http://dx.doi.org/10.1242/1242.062695
65. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003; 115(7):787-98; PMID:14697198; http://dx.doi.org/10.1016/10160092-8674(03)01018-3
66. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, et al. Combinatorial microRNA target predictions. Nat Genet 2005; 37(5):495-500; PMID:15806104; http://dx.doi.org/10.1038/10381536
67. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120(1):15-20; PMID:15652477; http://dx.doi.org/10.1016/1016.cell.2004.12.035
68. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116(2):281-97; PMID:14744438; http://dx.doi.org/10.1016/10160092-8674(04)00045-5
69. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell 2013; 153(3):654-65; PMID:23622248; http://dx.doi.org/10.1016/1016.cell.2013.03.043
70. Chi SW, Hannon GJ, Darnell RB. An alternative mode of microRNA target recognition. Nat Struct Mol Biol 2012; 19(3):321-7; PMID:22343717; http://dx.doi.org/10.1038/1038.2230
71. Reczko M, Maragkakis M, Alexiou P, Grosse I, Hatzigeorgiou AG. Functional microRNA targets in protein coding sequences. Bioinformatics 2012; 28(6):771-6; PMID:22285563; http://dx.doi.org/10.1093/1093/bts043
72. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, Rothballer A, Ascano M Jr, Jungkamp AC, Munschauer M, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 2010; 141(1):129-41; PMID:20371350; http://dx.doi.org/10.1016/1016.cell.2010.03.009
73. van den Heuvel S, Harlow E. Distinct roles for cyclin-dependent kinases in cell cycle control. Science 1993; 262(5142):2050-4; PMID:8266103; http://dx.doi.org/10.1126/1126.8266103
74. Du L, Schageman JJ, Subauste MC, Saber B, Hammond SM, Prudkin L, Wistuba, II, Ji L, Roth JA, Minna JD, et al. miR-93, miR-98, and miR-197 regulate expression of tumor suppressor gene FUS1. Mol Cancer Res 2009; 7(8):1234-43; PMID:19671678; http://dx.doi.org/10.1158/1541-7786.MCR-08-0507