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TITLE: Identifying "o lẹtqTPCu" that Regulate Neuroblastoma Cell Differentiation

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Identifying microRNAs that Regulate Neuroblastoma Cell Differentiation

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-449a mimics 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.

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

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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.

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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.
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-449a 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-449a-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


6.2. Abstracts


6.3. Presentations


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


11. Appendices

Please see the attached for the research paper that is in press in RNA Biology.
microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest

Zhenze Zhao, Xiuye Ma, Derek Sung, Monica Li, Adam Kosti, Gregory Lin, Yidong Chen, Alexander Pertsemlidis, Tzu-Hung Hsiao & Liqin Du

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

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

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

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

Department of Medical Research; Taichung Veterans General Hospital; Taichung, Taiwan

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

PLEASE SCROLL DOWN FOR ARTICLE
microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest

Zhenze Zhao¹, Xiuye Ma¹, Derek Sung¹, Monica Li¹, Adam Kosti¹, Gregory Lin¹, Yidong Chen¹,², Alexander Pertsemidis¹,³,⁴, Tzu-Hung Hsiao⁵, and Liqin Du¹,⁴,*

1Greehey Children’s Cancer Research Institute; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; ²Epidemiology and Biostatistics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; ³Pediatrics; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; ⁴Cellular and Structural Biology; The University of Texas Health Science Center at San Antonio; San Antonio, TX USA; ⁵Department 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; micro-RNA; Oligo; oligonucleotides; PKP4; plakophilin 4; STMN1; stathmin 1; TSEN15; tRNA splicing endonuclease 15 homolog; RA; 13-cis retinoic acid

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,⁷ 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
microRNAs (miRNAs) are endogenously expressed small RNAs that play a critical role in tumorigenesis.\textsuperscript{9,13} and miRNA-based anti-cancer therapeutics are under intensive investigation.\textsuperscript{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 endogenous miRNAs.\textsuperscript{18} These miRNA mimics have been used to effectively treat cancers in animal studies,\textsuperscript{14,15} showing the promise of developing them into anti-cancer agents.\textsuperscript{14-17} Recent studies have shown that miRNAs play an important role in regulating neuroblastoma cell differentiation,\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 BIII-tubulin, neuron specific enolase (NSE) and growth associated protein 43 (GAP43),\textsuperscript{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.
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 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-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 down-regulated 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. 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 vectors 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. 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 BE(2)-C.
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 caspase 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.

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 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.
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 assay46 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.

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.
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. 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.

**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 molecular 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. 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
The sensitive differentiation-inducing effect in KELLY cells. The sensitive differentiation-inducing effect in KELLY cells shows that another miRNA, miR-506–3p shows a strong differentiation path 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. 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, and each biological phenotype induced by a specific miRNA is likely achieved by concordantly down-regulating multiple targets. 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.
differentiation has also been indicated in previous studies. 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, 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. In addition, it has been demonstrated that miRNA may also target genes through non-canonical interactions. 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 Dharmaco and Sigma, respectively. pCMV-CDK6 expression vector was a gift from Sander van den Heuvel (Addgene plasmid #1868).

#### 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 Gleehey 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 G 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 Immunoblot 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. 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. Briefly 1 × 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.

Colonies 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. Total 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 mRNA 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 luciferase reported assay was performed as previously described. 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. 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 patient cohorts: the Versteeg cohort which includes 88 patients, and the Kocak cohort which includes 476 patients. 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.

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.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
12. Kopp F WE, Roisli A. The proto-oncogene KRAS is targeted by miR-200c. Oncotarget 2013; 5:185-95; PMID:24436861


n-105, target the differentiation inhibitor ID2. PLoS One 2012; 7(7):e40269; PMID:22488378; http://dx.doi.org/10.1371/journal.pone.0040269


27. Noonan EJ, Place RF, Basak S, Pookot D, Li LC. miR-449a and miR-449b are direct transcriptional targets of E2F1 and E2F2. Oncogene 2009; 28(39):4326-35; PMID:19252524; http://dx.doi.org/10.1074/1074.M109.027680


19. Noonan EJ, Place RF, Basak S, Dahiya R, Whi...


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