

# MicroRNA-26-5p Functions As a New Inhibitor of Hepatoblastoma by Repressing Lin-28 Homolog B and Aurora Kinase A Expression

Yutong Zhang,<sup>1,2</sup> Yulan Zhao,<sup>1</sup> Jianguo Wu,<sup>1</sup> Suthat Liangpunsakul,<sup>3-5</sup> Junqi Niu,<sup>6</sup> and Li Wang<sup>1,7,8</sup>

Hepatoblastoma (HB) is the most common liver tumor in children. Despite recent improvements in treatment strategies, the survival of children with hepatoblastoma remains poor. In this study, we identified a novel role of microRNA-26a-5p (miR-26a-5p), lin-28 homolog B (LIN28B), Ras-related nuclear protein (RAN), and aurora kinase A (AURKA) in HB. The expression of LIN28B, RAN, and AURKA was significantly up-regulated in human HB livers and cell lines. Knock-down of LIN28B and RAN by small interfering RNAs inhibited HB tumor cell proliferation and foci formation. We also elucidated miR-26a-5p-mediated translational inhibition of LIN28B and AURKA in HB. Overexpression of miR-26a-5p markedly decreased LIN28B and AURKA 3'-untranslated region activities and protein expression and repressed HB cell proliferation and colony formation. In contrast, re-expression of LIN28B and AURKA rescued miR-26a-5p-mediated suppression of HB cell growth and clonality. Importantly, a decreased miR-26a-5p expression correlated with the poor outcome of patients with HB. **Conclusion:** miR-26a-5p is a newly identified repressor of HB growth through its inhibition of the oncogenic LIN28B-RAN-AURKA pathway. (*Hepatology Communications* 2018;2:861-871)

## Introduction

**H**epatoblastoma (HB) is the most common liver tumor with an estimated incidence of 1 per 100,000 children. HB originates from liver progenitor cells that acquire malignant transformation during embryogenesis.<sup>(1)</sup> Certain inherited conditions as well as congenital anomalies and low birth weight are believed to increase the risk of HB; however, the etiology of HB remains elusive due to the extreme rarity of this disease.<sup>(2)</sup> Somatic mutations in genes encoding components of the Wnt/ $\beta$ -catenin

signaling pathway are prevalent in sporadic HB cases. Recently, the interplay of Wnt/ $\beta$ -catenin and Myc signaling has been shown to play a critical role in poorly differentiated aggressive HB.<sup>(3)</sup> Overexpression of mutant  $\beta$ -catenin in mice induces HBs that express high levels of c-Myc.<sup>(4)</sup> These findings suggest Myc as a potential therapeutic target for HB. However, MYC does not harbor any cavities into which small molecules can easily bind, indicating Myc oncoproteins are undruggable.<sup>(5)</sup> In addition, small molecule drugs in combination with cytotoxic chemotherapy have not been developed for patients with HB. Because the

*Abbreviations:* 3'-UTR, 3'-untranslated region; AURKA, aurora kinase A; FFPE, formalin-fixed paraffin-embedded; HB, hepatoblastoma; LIN28B, lin-28 homolog B; miRNA, miR, microRNA; mRNA, messenger RNA; RAN, Ras-related nuclear protein; siRNA, small interfering RNA.

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event-free survival rate is less than 50% in children with high-risk HB<sup>(6)</sup> and pediatric liver transplantation is not easily accessible, current major research efforts are directed toward identifying new regulators of HB.

MicroRNAs (miRNAs) are small, endogenous, noncoding RNAs that regulate gene expression by specifically binding to the 3'-untranslated region (3'-UTR) of target gene messenger RNAs (mRNAs).<sup>(7)</sup> miRNAs are involved in many important processes, including development, proliferation, differentiation, apoptosis, and stress response.<sup>(8-10)</sup> Because miRNAs regulate diverse physiological and developmental processes,<sup>(11)</sup> their own expression and processing must be tightly controlled.<sup>(12-14)</sup> Due to the rarity of HB and the limited availability of fresh-frozen tumor samples, analyzing miRNA expression in HB has mostly been achieved from formalin-fixed paraffin-embedded (FFPE) samples. Several miRNAs have been reported aberrantly expressed in HB, including miRNA-21 (miR-21) and miR-221.<sup>(15)</sup> Despite these studies, how miRNAs control the development of HB remains little known.

Lin-28 homolog B (LIN28B) is a highly conserved RNA binding protein that can regulate cell fate to control embryonic development.<sup>(16)</sup> It sustains the proliferative and metabolic capacities of pluripotent stem cells and facilitates the transition from naive to primed pluripotency. Aberrantly expressed LIN28B plays an oncogenic role in various human cancers. Aurora kinase A (AURKA) is a serine-threonine mitotic kinase that plays an important role in regulating mitosis, cell division, and cell cycle progression.<sup>(17)</sup> Overexpression of AURKA promotes cell cycle progression by disrupting cell cycle checkpoints, leading to aneuploidy and genomic instability; the latter is a hallmark of malignant transformation. Emerging results suggest that inhibiting AURKA may be a novel strategy for

reducing the stability of Myc protein that has not been previously targetable. Ras-related nuclear protein (RAN) is a well-known nuclear trafficking protein that is a member of the Ras family.<sup>(18)</sup> RAN is overexpressed in diverse malignancies and promotes AURKA phosphorylation. Recently, a study demonstrated that LIN28B-RAN-AURKA signaling drives neuroblastoma oncogenesis, providing a pathway that may be amenable to therapeutic targeting.<sup>(19)</sup>

In this study, we aimed to understand the function of LIN28B and AURKA in HB. We demonstrate that both genes are direct targets of miR-26a-5p. LIN28B and AURKA are up-regulated in HB, and knockdown of both genes by small interfering RNAs (siRNAs) or by miR-26a-5p inhibits HB tumor cell proliferation and foci formation. Our results suggest that miR-26a-5p plays an important regulatory role in HB, providing new insights into developing treatment strategies for HB.

## Materials and Methods

### HB FFPE SAMPLES

Patient HB FFPE samples were collected retrospectively from the First Hospital of Jilin University, China. The study was approved by the institutional review board at the First Hospital of Jilin University.

### CELL CULTURE

Human hepatocyte cell line HC-04, human hepatoblastoma cell line HepG2 (generously provided by Dr. Yuzuru Shiio and Dr. Hongbing Wang<sup>(20,21)</sup>), and cell line HUH6 (a generous gift from Dr. Thomas Pietschmann<sup>(22)</sup>) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

### ARTICLE INFORMATION:

From the <sup>1</sup>Department of Physiology and Neurobiology and Institute for Systems Genomics, University of Connecticut, Storrs, CT; <sup>2</sup>Department of Pediatric Oncology, The First Hospital of Jilin University, Changchun, China; <sup>3</sup>Division of Gastroenterology and Hepatology, Department of Medicine and <sup>4</sup>Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN; <sup>5</sup>Roudebush Veterans Administration Medical Center, Indianapolis, IN; <sup>6</sup>Department of Hepatology, The First Hospital of Jilin University, Changchun, China; <sup>7</sup>Department of Internal Medicine, Section of Digestive Diseases, Yale University, New Haven, CT; <sup>8</sup>Veterans Affairs Connecticut Healthcare System, West Haven, CT.

### ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Li Wang, Ph.D.  
75 North Eagleville Road U3156  
Storrs, CT 06269

E-mail: li.wang@uconn.edu  
Tel: +1-860-486-0857

Hep293TT cells (a generous gift from Dr. Yuzuru Shiio) were cultured in Roswell Park Memorial Institute 1640 supplemented with 10% fetal calf serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, and 1 mM sodium pyruvate.<sup>(20)</sup>

## OLIGONUCLEOTIDES, PLASMIDS, AND siRNAS

MiR-26a-5p mimics, miR-29a-3p mimics, and negative control oligonucleotides (miR-CON) were purchased from Sigma (#HMI0415, #HMC0434, #HMC0002, respectively). Small interfering pool RNA of Lin28B, RAN, and control RNA (siLin28B, siRAN, siCon; five siRNAs per pool) were purchased from Santa Cruz Biotechnology (#SC-105614, #SC-36382, #SC-37007, respectively). LIN28B and AURKA plasmids were purchased from Addgene (#51375 and #20427, respectively). Luciferase reporter gene plasmids, including wild-type AURKA 3'-UTR, wild-type LIN28B 3'-UTR, and their seed mutants, were synthesized and subcloned into pmirGLO vectors, which were confirmed by sequencing.

## CELL TRANSFECTION

Cells were seeded in six-well plates the day before the experiment and transfected using RNAiMAX transfection reagent (#13778075; Thermo Fisher Scientific), with microRNA mimics (miR-26a-5p or miR-29a-3p)/negative control at a final concentration of 50 nM or with siRNAs (siLin28B or siRAN)/negative control at a final concentration of 50  $\mu$ M. After 48 hours, cells were harvested for RNA and protein isolation,<sup>(23)</sup> respectively.

## 3'-UTR LUCIFERASE REPORTER ASSAYS

HUH6 and HepG2 cells were seeded in 24-well plates at a density of  $5 \times 10^4$  cells/well. After 24 hours, wild-type AURKA 3'-UTR and LIN28B 3'-UTR or their seed mutant luciferase reporter vectors with miR-26a-5p mimic or miR-29a-3p mimic were transfected into the cells using RNAiMAX transfection reagent. The dual-luciferase reporter assay system (Promega, Madison, WI) was applied 48 hours after transfection to determine changes in relative luciferase units as described.<sup>(24)</sup> All assays were performed in triplicate, and all values were normalized for transfection efficiency against *Renilla* luciferase activities.

## CELL PROLIFERATION ASSAY

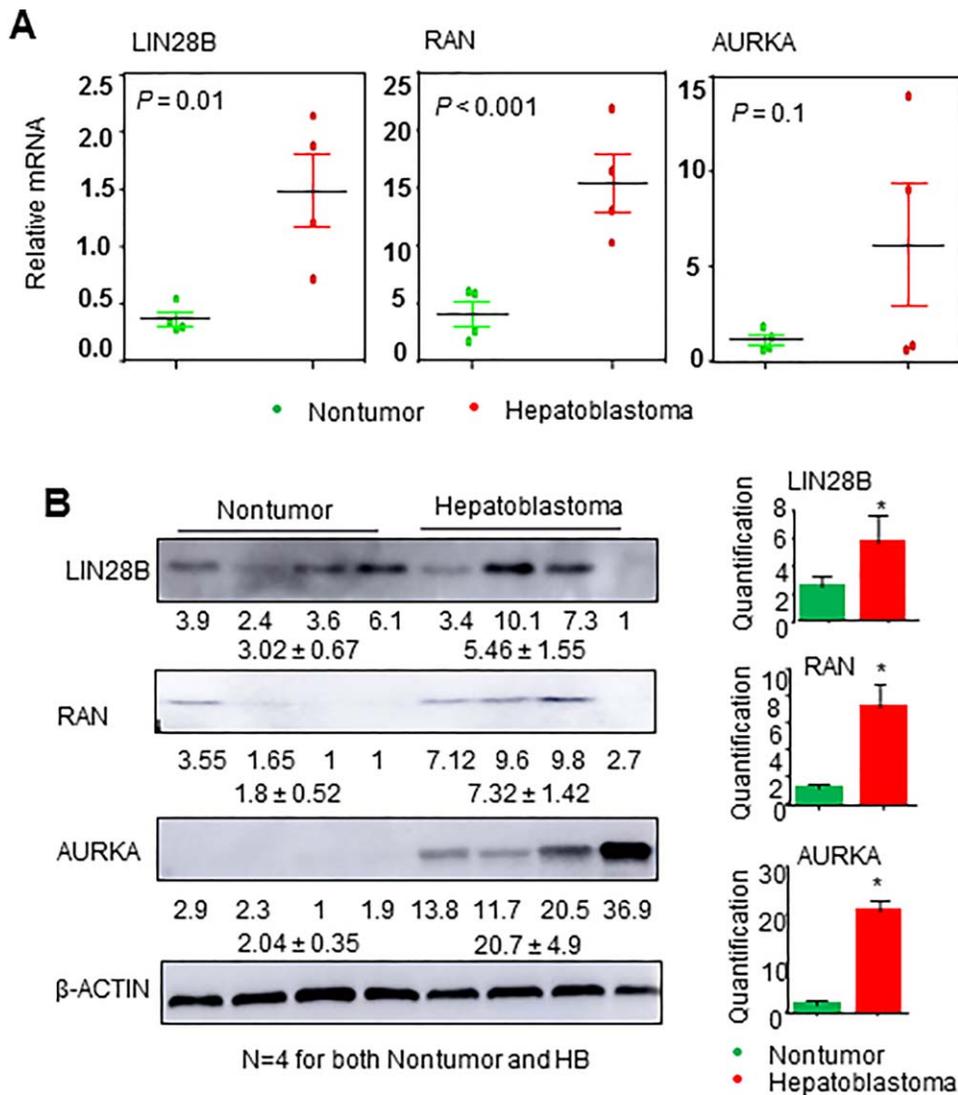
The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed to measure the proliferative ability of HUH6, Hep293TT, and HepG2 cells as described.<sup>(25)</sup> In brief,  $5 \times 10^3$  cells per well were seeded into 96-well plates containing complete Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium (100  $\mu$ L) in triplicate for each condition and were maintained in an incubator at 37°C and 5% CO<sub>2</sub>. MTS solution (20  $\mu$ L) was added to each well and incubated for 2 hours. Absorbance at 490 nM (A490) was read on a microplate reader (168-1000 Model 680; Bio-Rad Laboratories), and proliferation curves were plotted. The colony formation assay was conducted as described.<sup>(8)</sup>

## QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from tissues or cells with TRIzol reagent (#15596018; Thermo Fisher Scientific).<sup>(26)</sup> Real-time quantification of mRNA levels of the genes of interest was performed using Brilliant II SYBR Green QPCR Master Mix (#1725274; Bio-Rad Laboratories, Munich, Germany) as described.<sup>(27)</sup> Normalized cycle threshold (Ct) values were subjected to statistical analysis, and the fold difference was calculated by the  $\Delta\Delta$  Ct method. Human  $\beta$ -actin and U6 mRNAs were also measured as internal controls for normalization. Specific primers for quantitative polymerase chain reaction are shown in [Supporting Table S1](#).

## WESTERN BLOTTING

Tissue or cell lysate was harvested by radio immunoprecipitation assay lysis buffer with protease inhibitors (#78410; Thermo Fisher Scientific). Proteins (30  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes according to standard procedures.<sup>(28)</sup> Membranes were blocked, immunostained with primary antibodies, and finally detected by horseradish peroxidase-conjugated corresponding secondary antibody. Signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (#34080; Thermo Fisher Scientific) according to the manufacturer's protocol. Equal loading of protein was verified with  $\beta$ -actin.



**FIG. 1.** Lin28B, RAN, and AURKA are highly expressed in human HB. (A) Quantitative polymerase chain reaction analysis of LIN28B, RAN, and AURKA expression in nontumor livers and HB. (B) Western blot analysis of LIN28B, RAN, and AURKA protein expression in nontumor livers and HB, with  $\beta$ -actin as a loading control (n = 4 samples/group). Quantification of the western blot analysis is presented on the right. Data are shown as mean  $\pm$  SEM (triplicate assays). \* $P < 0.05$  versus nontumor sample.

## RNA EXTRACTION FROM FFPE SAMPLES

Total RNAs from 14 FFPE tissues blocks were isolated using the commercially available miRNeasy FFPE kits according to the respective manufacturers' instructions (#217504; QIAGEN, Hilden, Germany).

## STATISTICAL ANALYSIS

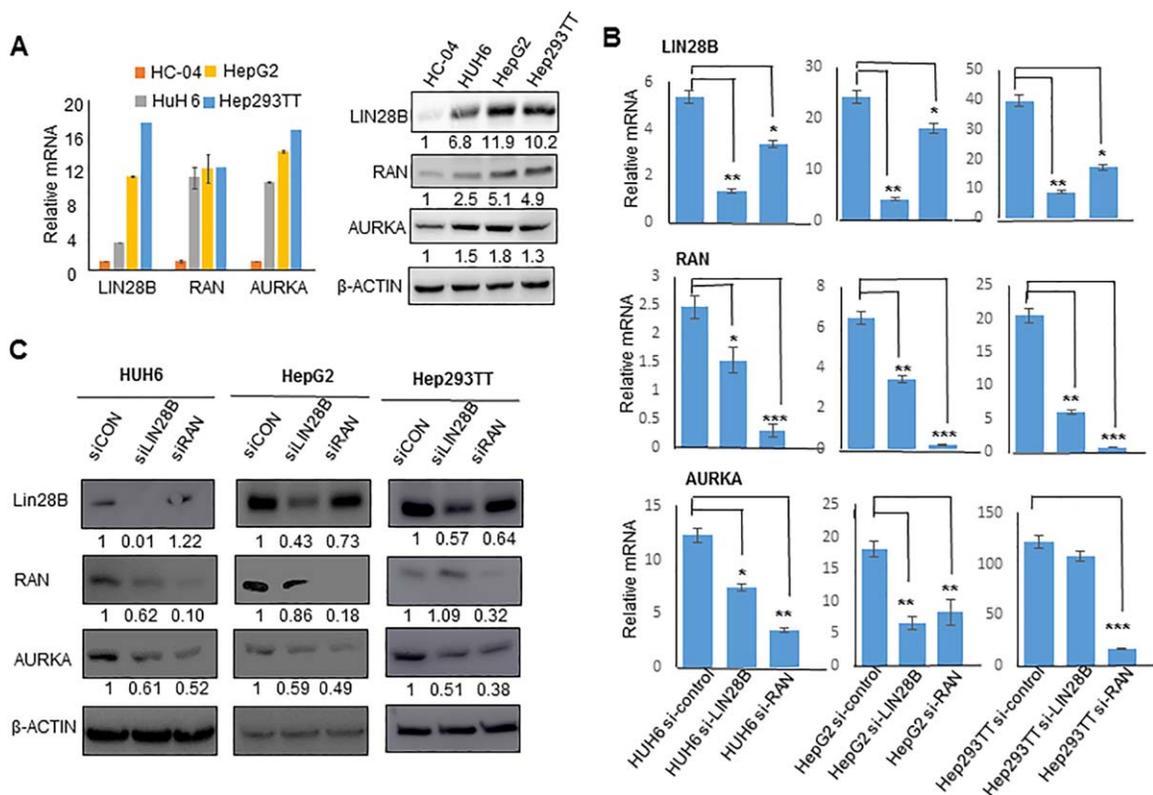
Statistical analysis and plots were performed and generated using GraphPad Prism software (version 6.0; GraphPad Software). Data are presented as mean values with SEM, and  $P < 0.05$  was considered statistically significant.<sup>(29)</sup> All experiments were performed

independently at least 3 times. Statistical significance between two groups was measured using the Student  $t$  test.

## Results

### EXPRESSION OF LIN28B, RAN, AND AURKA IS UP-REGULATED IN HUMAN HB LIVER

Hepatoblastoma results from the failure of cells to differentiate properly into their final cell types during fetal or postnatal development. Recently, LIN28B was identified as an oncogenic driver in high-risk

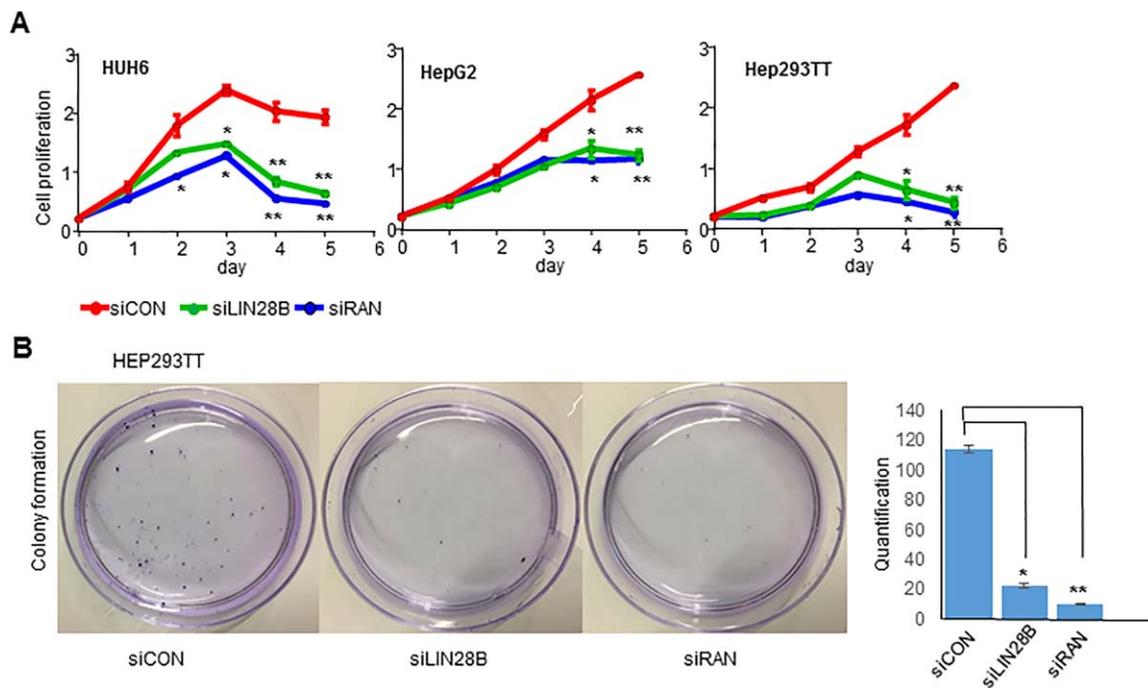


**FIG. 2.** Lin28B directly regulates AURKA through RAN. (A) qPCR (left) and western blot (right) analysis of Lin28B, RAN, and AURKA expression in HB cell lines HUH6, HepG2, and Hep293TT and normal hepatocyte cell line HC-04. (B) qPCR analysis of LIN28B, RAN, and AURKA mRNA levels in HUH6, HepG2, and Hep293TT cells transfected with control, siLIN28B, or siRAN after 48 hours. LIN28B, RAN, and AURKA mRNA levels are normalized to  $\beta$ -actin levels. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data are shown as mean  $\pm$  SEM (triplicate assays). (C) Western blot analysis of LIN28B, RAN, and AURKA protein expression in HUH6, HepG2, and Hep293TT cells transfected with control, siLIN28B, or siRAN for 2 days, with  $\beta$ -actin serving as a loading control. Pooled samples ( $n = 3$  per group) were run in a single lane. Numbers indicate density relative to siCon. Abbreviations: qPCR, quantitative polymerase chain reaction; siCon, siControl.

neuroblastoma.<sup>(30)</sup> LIN28B–RAN–AURKA signaling was further revealed to drive neuroblastoma oncogenesis.<sup>(19)</sup> We were interested in determining whether LIN28, RAN, and AURKA act as oncogenes in hepatoblastoma. We first examined their expression in human tissue biopsies from patients with HB by quantitative polymerase chain reaction and western blot. The mRNA levels of LIN28B and RAN were significantly higher in HB than in normal liver, whereas AURKA mRNAs showed greater variations among the samples (Fig. 1A). Interestingly, LIN28B protein exhibited larger variations among the samples, whereas RAN and AURKA proteins were notably up-regulated in HB versus nontumors (Fig. 1B). The inconsistency between mRNA and protein expression of LIN28B and AURKA in HB suggests that both transcriptional and translational mechanisms may be involved.

## A MOLECULAR LINK EXISTS BETWEEN LIN28B, RAN, AND AURKA

We next assessed the expression levels of LIN28B, RAN, and AURKA in a panel of human HB cell lines versus normal hepatocyte cell line HC-04. All three genes were highly expressed in HUH6, HepG2, and Hep293TT cells but with a low expression in HC-04 (Fig. 2A). To further investigate the molecular link between LIN28B, RAN, and AURKA, we knocked down LIN28B using siLIN28B and examined mRNA expression of RAN and AURKA in all three HB cell lines. LIN28B depletion led to decreased RAN mRNA expression in HUH6, HepG2, and Hep293TT cells, whereas it decreased AURKA mRNA expression in HUH6 and HepG2 but not in



**FIG. 3.** Knockdown of LIN28B and RAN inhibits HB cell growth. (A) Growth curves of control and HUH6 (left), HepG2 (middle), and Hep293TT (right) cells transfected with siLIN28B and siRAN for 5 days. Data are shown as mean  $\pm$  SEM (triplicate assays). \* $P < 0.05$ , \*\* $P < 0.01$ . (B) Colony formation assay of Hep293TT cells transfected with siControl, siLIN28B, or siRNA for 14 days. Quantification of colony numbers is presented on the right. Abbreviation: siCon, siControl.

Hep293TT cells (Fig. 2B). Interestingly, knockdown of RAN by siRNA significantly decreased AURKA mRNA but also had a modest effect in reducing LIN28B mRNA in all three HB cell lines. At the protein level, siLIN28B decreased RAN protein expression in HUH6 and HepG2 but not in Hep293TT cells, whereas RAN-siRNA decreased AURKA protein in all three lines but had a moderate effect on LIN28B protein expression (Fig. 2C). Taken together, the results suggest that LIN28B may act upstream of RAN and AURKA whereas RAN may be an activator of AURKA expression but may also regulate LIN28B in a feedback fashion. In addition, posttranscriptional regulation may be a mechanism contributing to the differential expression pattern between mRNA and protein.

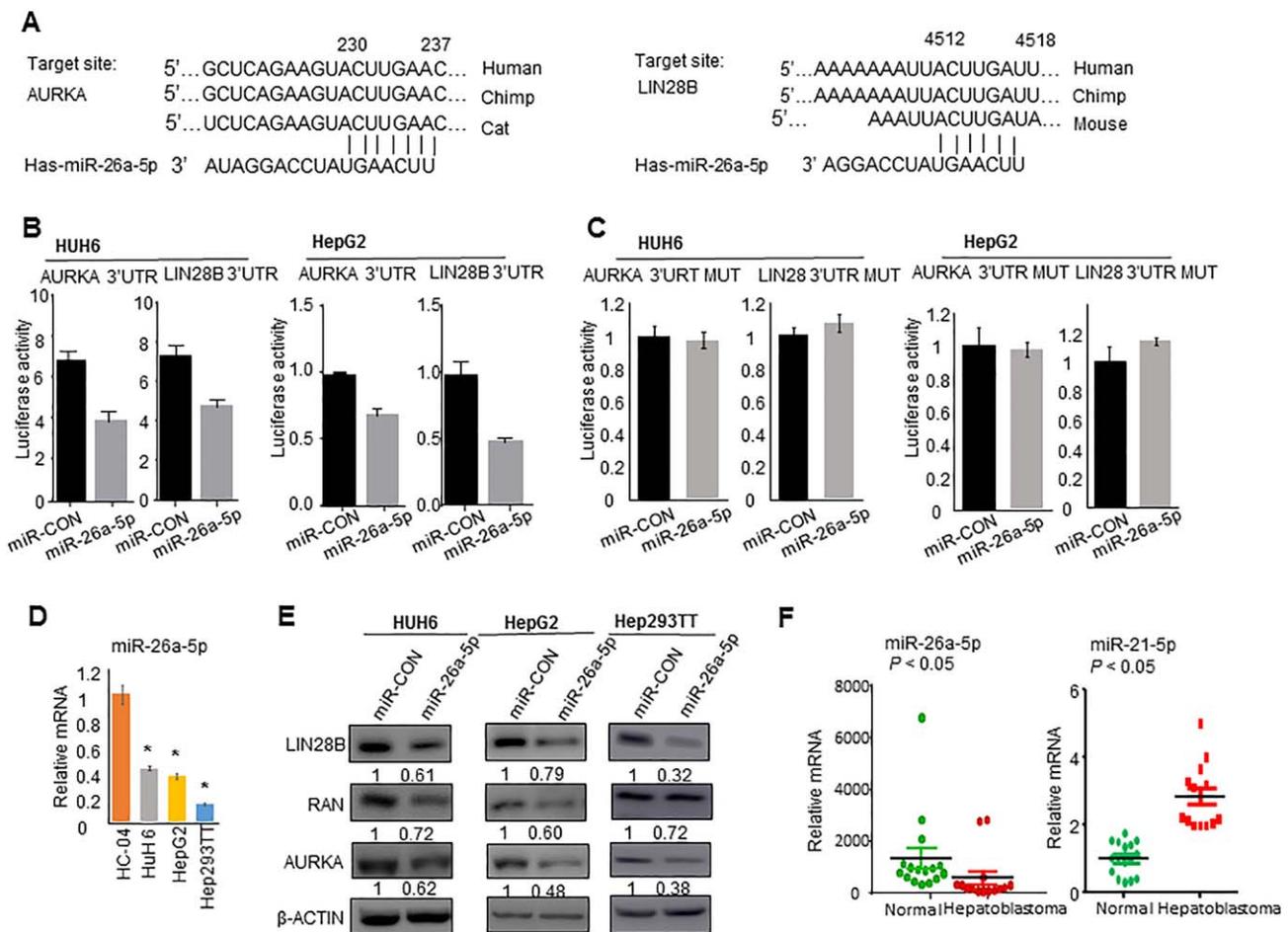
### KNOCKDOWN OF LIN28B AND RAN SUPPRESSES HB CELL PROLIFERATION AND COLONY FORMATION

To determine the functional roles of LIN28B and RAN in HB, we depleted LIN28B and RAN using

their respective siRNAs in HUH6, HepG2, and Hep293TT cells. Knockdown of LIN28B or RAN markedly diminished HB cell proliferation (Fig. 3A). In addition, siLIN28B and siRAN notably suppressed colony formation of HB cells (Fig. 3B). The results suggest an oncogenic effect of LIN28B and RAN in HB.

### LIN28B AND AURKA ARE DIRECT TARGET GENES OF miR-26a-5p IN HB

The prediction of miRNA target regions by TargetScan indicated that both LIN28B 3'-UTR and AURKA 3'-UTR contain a binding site for miR-26a-5p (Fig. 4A). Both target sequences are conserved in several different species. To further investigate whether LIN28B and AURKA were directly regulated by miR-26a-5p, luciferase reporter assays were performed in HUH6 and HepG2 cells. Cotransfection of miR-26a-5p mimics markedly decreased luciferase reporter activities of LIN28B 3'-UTR and AURKA 3'-UTR (Fig. 4B), which were relieved when the miR-26a-5p seed



**FIG. 4.** Lin28B and AURKA are novel target genes of miR-26a-5p. (A) Target prediction for miR-26a-5p using Targetscan identified miR-26a-5p binding sequences within LIN28B 3'-UTR and AURKA 3'-UTR. (B) Luciferase reporter assays of LIN28B 3'-UTR and AURKA 3'-UTR in HUH6 and HepG2 cells transfected with 100 nmol miR-26a-5p. (C) Luciferase reporter assays of LIN28B 3'-UTR mutant and AURKA 3'-UTR mutant in HUH6 and HepG2 cells transfected with 100 nmol miR-26a-5p. (D) qPCR analysis of miR-26a-5p expression in normal hepatocyte cells HC-04 and HB cell lines HUH6, HepG2, and Hep293TT. (E) Western blot analysis of LIN28B, RAN, and AURKA protein expression in the indicated cells transfected with mimic control and miR-26a-5p mimic for 48 hours, with  $\beta$ -actin serving as a loading control. Numbers indicate density relative to control. (F) qPCR analysis of miR-26a-5p and miR-21-5p expression in normal liver ( $n = 16$ ) and FFPE HB tissues ( $n = 14$ ). All data are shown as mean  $\pm$  SEM (triplicate assays). \* $P < 0.05$  versus HC-04. Abbreviations: CON, control; MUT, mutant; qPCR, quantitative polymerase chain reaction.

regions were mutated (Fig. 4C). MiR-26a-5p was significantly suppressed in HB cell lines HUH6, HepG2, and Hep293TT compared to HC-04 cells (Fig. 4D). In addition, western blot showed decreased LIN28B and AURKA protein expression after transfection with miR-26a-5p mimics in HB cells (Fig. 4E). Interestingly, RAN protein was also decreased by miR-26a-5p mimics in HUH6 and HepG2 cells. We examined the microRNA target prediction databases [Targetscan](#) and [MicroRNA.org](#) but found no miR-26a-5p binding sites within RAN 3'-UTR, which supports that RAN

is not a canonical miR-26a-5p target. The results suggest that RAN down-regulation likely resulted from LIN28B knockdown.

## DOWN-REGULATION OF miR-26a-5p IS ASSOCIATED WITH POOR PATIENT OUTCOMES IN HB

To establish the clinical relevance of miR-26a-5p in HB, we determined its expression in 14 paraffin HB tissues (Table 1) and compared this with normal

TABLE 1. CHARACTERISTICS OF FFPE HB SAMPLES AND THE RELATIVE EXPRESSION OF MIR-26A-5P

	Sex	Age	Histologic Type	PRETEXT Stage*	Outcome	Expression of miR-26a-5p
1	Male	4 months	fetal	I	Alive	2,754.47
2	Male	1 year	fetal	I	Alive	2,798.26
3	Male	1 year	embryonal	III	Alive	327.425
4	Male	20 months	embryonal	III	Alive	68.3725
5	Male	7 months	embryonal	IV	Alive	576.186
6	Male	17 months	fetal	IV	Alive	136.56
7	Male	2 years	fetal	IV	Alive	167.30
8	Female	1 year	mixed	IV	Alive	118.61
9	Male	2 months	mixed	IV	Alive	276.85
10	Male	6 years	fetal	IV	Alive	284.91
11	Male	3 years	embryonal	IV	Relapsed	175.98
12	Male	10 years	unclear	IV	DOD	49.46
13	Female	8 years	unclear	IV	DOD	53.43
14	Male	15 months	mixed	IV	DOD	53.79

\*PRETEXT = 2005 PRETEXT staging system.  
Abbreviation: DOD, death of disease.

children liver specimens (Fig. 4F). Overall, the expression level of miR-26a-5p was much lower in HB tissues versus normal tissues. miR-26a-5p expression in stage one disease was compatible with normal liver; however, its level decreased drastically in stage four disease (Table 1). In addition, the expression level of miR-26a-5p in patients with poor outcomes (i.e., death of disease) was the lowest compared to other stages ( $P < 0.01$ ). In contrast, miR-21-5p was expressed at higher levels in HB versus normal tissues.

### miR-26a-5p INHIBITS HB CELL PROLIFERATION AND COLONY FORMATION

To further investigate the potential function of miR-26a-5p in HB, we transfected miR-26a-5p mimics in Hep293TT, HUH6, and HepG2 cells. miR-26a-5p mimics but not the control mimics or miR-29a-3p mimics drastically attenuated the proliferation of these cells compared to the control cells (Fig. 5A). Because miR-26a-5p showed a similar effect on Hep293TT, HUH6, and HepG2 cells, we next examined the ability of miR-26a-5p to inhibit foci formation using Hep293TT cells. Overexpression of miR-26a-5p mimics also dramatically decreased colony formation (Fig. 5B); however, miR-26a-5p inhibitor increased colony formation (Fig. 5C). In addition, the inhibitory effect of miR-26a-5p on Hep293TT proliferation was rescued by miR-26a-5p inhibitor (Fig. 5C). The results suggest a tumor suppressor function of miR-26a-5p in HB.

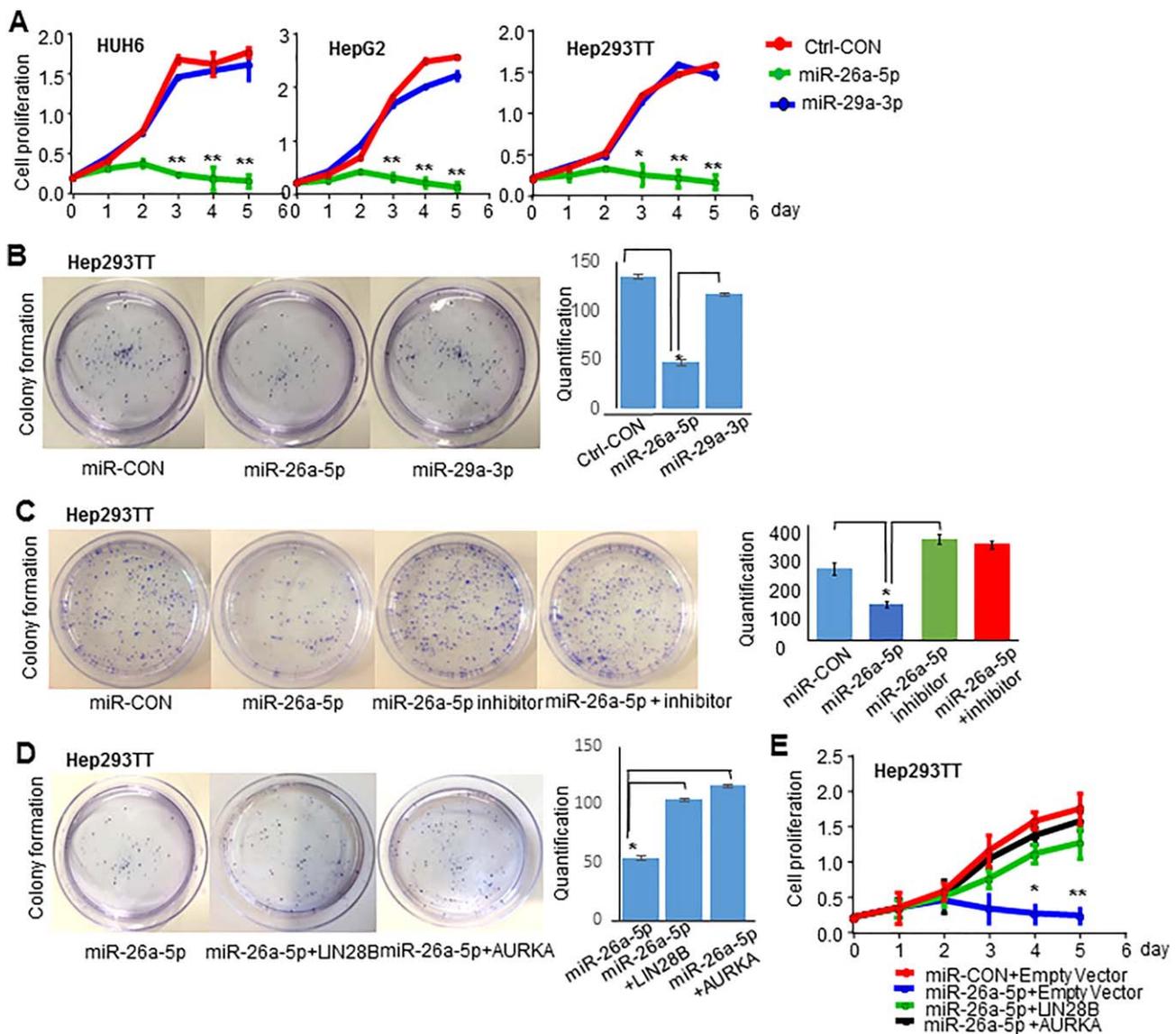
### LIN28B AND AURKA BLOCK THE INHIBITORY EFFECT OF miR-26a-5p ON HB CELL GROWTH

Because LIN28B and AURKA are downstream targets of miR-26a-5p, as shown in Fig. 4, we conducted rescue experiments. As expected, re-expression of LIN28B and AURKA blocked the effect of miR-26a-5p on the repression of colony formation (Fig. 5D) and cellular growth (Fig. 5E) of Hep293TT cells, suggesting that the effect of miR-26a-5p is mediated through both genes.

## Discussion

Our work was motivated by a report that LIN28B and AURKA expressions were up-regulated in a transcriptomic and genomic analysis of human HB.<sup>(31)</sup> In addition, neuroblastoma tumorigenesis can be promoted by the LIN28B–RAN–AURKA signaling network. We propose a central regulatory role for LIN28B in HB by its effect on RAN and AURKA.

Our results show that malignant tissues have higher LIN28B, RAN, and AURKA expression than normal liver samples. LIN28B, RAN, and AURKA are also up-regulated in a panel of malignant HB cells. We demonstrate RAN to be a key downstream component of LIN28B signaling, with LIN28B depletion decreasing RAN expression. As such, AURKA is a key downstream component of RAN signaling, with RAN depletion decreasing AURKA expression. We further show that inhibiting LIN28B by siRNAs significantly

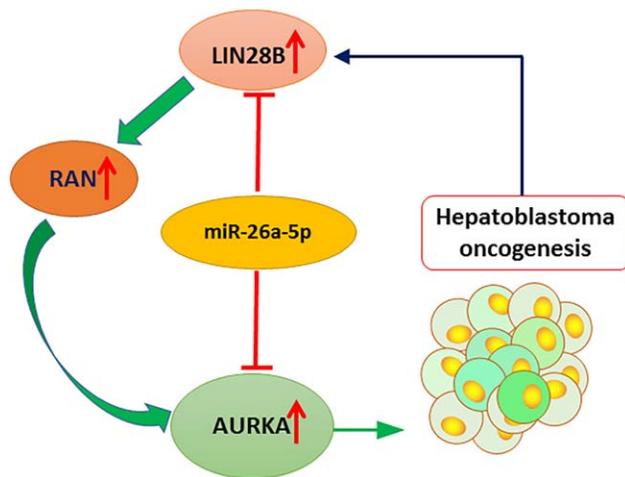


**FIG. 5.** Re-expression LIN28B and AURKA promotes HB cell growth. (A) Growth curves of control and HUH6 (left), HepG2 (middle), and Hep293TT (right) cells transfected with mimic control, 100 nmol miR-26a-5p mimic, and 100 nmol miR-29a-3p mimic. (B) Colony formation assay of Hep293TT cells transfected with mimic control, miR-26a-5p mimic, and miR-29a-3p mimic. (C) Colony formation assay of Hep293TT cells transfected with mimic control, miR-26a-5p mimic, miR-26a-5p inhibitor, and miR-26a-5p mimic with its inhibitor. (D) Colony formation assay of Hep293TT cells transfected with control, miR-26a-5p mimic, miR-26a-5p mimic plus LIN28B plasmid, and miR-26a-5p mimic plus AURKA plasmid. (E) Hep293TT cells were transfected with mimic control plus 2  $\mu$ g empty vector control, miR-26a-5p mimic plus 2  $\mu$ g empty vector control, miR-26a-5p mimic plus 2  $\mu$ g LIN28B plasmid, and miR-26a-5p mimic plus 2  $\mu$ g AURKA plasmid. The cell proliferation assay was performed from day 0 to day 5. All data are shown as mean  $\pm$  SEM (triplicate assays). Abbreviation: CON, control.

decreases HB cell proliferation and colony formation, suggesting that the LIN28B–RAN–AURKA pathway may play a critical role in HB development. Interestingly, knockdown of RAN expression using siRANs decreases LIN28B expression. Because RAN is a downstream signaling molecule of LIN28B, we

hypothesize that there is a feedback regulatory loop between RAN and LIN28B.

Overexpression of LIN28B and AURKA has been observed in a variety of tumors. However, the mechanisms that control their expression in HB remain unknown. Recent emerging evidence suggests that



**FIG. 6.** Working model of miR-26a-5p as a novel HB repressor. The LIN28B–RAN–AURKA signaling pathway plays a crucial role in hepatoblastoma oncogenesis. MiR-26a-5p translationally inhibits LIN28B and AURKA expression by targeting their 3'-UTR, thereby suppressing HB cell growth.

miRNAs impact malignant behaviors of HB. Previous studies provided evidence for several prognostic miRNAs associated with HB, such as miR-492<sup>(32)</sup> and miR-21.<sup>(33)</sup> Here, we demonstrate that miR-26a-5p inhibits LIN28B and AURKA expression by binding to their 3'-UTR regions. Interestingly, LIN28B regulates AURKA levels through RAN and both are target genes of miR-26-5p. Because HB is a rare disease, our research can only rely on FFPE samples. Recent research has demonstrated that miRNA expression is relatively stable and well preserved in FFPE samples.<sup>(34)</sup> Accordingly, we extracted RNAs from 14 FFPE samples and obtained high-quality miRNAs from these samples.

The miR-26a-5p level is significantly lower in HB tissues compared with normal liver. Although the sample size was relatively small, it is evident that miR-26a-5p expression shows a negative correlation with disease stage and prognosis. Stage one diseases have much higher miR-26a-5p expression and a better outcome compared to stage four diseases. Importantly, miR-26a-5p exhibits a remarkable ability to suppress HB cell proliferation and clone formation. Notably, miR-26a-5p-mediated suppression of these events is subsequently impaired by re-expression of LIN28B and AURKA, indicating that miR-26a-5p regulates HB development at least in part by restraining the LIN28B–RAN–AURKA pathway.

Taken together, our study established for the first time a crucial role of the LIN28B–RAN–AURKA signaling pathway in HB oncogenesis (Fig. 6). In addition, we revealed miR-26a-5p as a new repressor of LIN28B and AURKA expression. Further investigation of the potential of miR-26a-5p as a disease marker and therapeutic target for HB is warranted.

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Author names in bold designate shared co-first authorship.

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