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miR-362-5p inhibits proliferation and migration of neuroblastoma cells by targeting phosphatidylinositol 3-kinase-C2β

Kai Wu [1], Liucheng Yang [1], Jianfeng Chen, Haijun Zhao, Jianjun Wang, Shuai Xu, Zonghai Huang *

Department of General Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282, China

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ABSTRACT

miR-362-5p is down-regulated in high-risk neuroblastoma and can function as a tumor suppressor. However, its role remains poorly understood. We show that miR-362-5p is down-regulated in metastatic neuroblastoma compared with primary neuroblastoma. Overexpression of miR-362-5p inhibits cell proliferation, migration and invasion of neuroblastoma cells in vitro and suppresses tumor growth of neuroblastoma in vivo. Phosphatidylinositol 3-kinase (PI3K)-C2β is a target of miR-362-5p. Knockdown of PI3K-C2β by siRNA had a similar effect to overexpression of miR-362-5p on SH-SY5Y cells. Overexpression of PI3K-C2β partially reversed tumor-suppressive effects of miR-362-5p. We suggest that miR-362-5p suppresses neuroblastoma cell growth and motility, partially by targeting PI3K-C2β.

1. Introduction

Neuroblastoma is one of the most common solid tumors in childhood, and accounts for 7% of childhood malignancies and about 15% of all childhood cancer deaths [1]. Although recent multimodal treatments, such as surgery, chemoradiotherapy, stem cell transplantation, and immunotherapy, have improved the survival rate of neuroblastoma in the post 10 years [2], the overall 5-year survival rate of high-risk neuroblastoma is still very low and its treatment remains challenging. Elucidating the molecular mechanism will help in identifying new treatments for neuroblastoma.

MicroRNAs (miRNAs) are endogenous small 22–25 nucleotide non-coding RNAs. miRNA can bind to the 3′-untranslated region (3′-UTR) of target mRNA and lead to translational repression or degradation of mRNA. Single miRNA can regulate multiple mRNAs and single mRNA can combine with multiple miRNAs. Numerous studies suggest that miRNAs play an important role in regulating various biological and pathologic processes, such as cell proliferation, apoptosis, metastasis and differentiation [3,4]. Moreover, the development and progression of tumors are also associated with abnormal expression of specific miRNAs [5,6]. miRNAs might serve as a new therapeutic strategy for cancer, including neuroblastoma, by targeting oncogenes or serving as tumor suppressors.

Many miRNAs were identified as down-regulated miRNAs in high-risk neuroblastoma, and loss of their expression was associated with unfavorable prognosis [7]. Many misexpressed miRNAs, such as miR-432, miR-124, miR-329, have been reported to play an important role in tumor development and progression [8–10]. miR-362-5p was identified by Bentwich et al. and played an important role in tumor growth and metastasis [11]. Bienertova-Vasku et al. discovered that miR-362-5p expression levels were significantly down-regulated in metastatic neuroblastoma tissues compared with primary neuroblastoma tissues [7]. It has also been demonstrated that miR-362-5p plays an important role in tumor cell proliferation, apoptosis, and metastasis [12–14].

In this study, we confirmed that miR-362-5p is down-regulated in metastatic neuroblastoma tissues compared with primary neuroblastoma tissues. In addition, we have identified the important molecular mechanism by which miR-362-5p exerts its tumor-suppressive effects on neuroblastoma cell proliferation,

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* Corresponding author at: Department of Surgery, Zhujiang Hospital, Southern Medical University, 253 Middle of Gongye Avenue, Haizhu District, Guangzhou 510282, China. Fax: +86 20 62782395.

E-mail addresses: aibeike@126.com (K. Wu), sdylc@aliyun.com (L. Yang), 742932527@qq.com (H. Zhao), xushuai523@126.com (S. Xu), zjhzh_1954@126.com (Z. Huang).

[1] Two authors contributed equally to this study.

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migration and invasion, which involves direct targeting of the 3′-UTR of phosphatidylinositol 3-kinase (PI3K)-C2β mRNA.

2. Materials and methods

2.1. Tissue samples

After receiving approval from the institutional review board of Zhujiang Hospital of Southern Medical University, 12 metastatic and 12 primary neuroblastoma tissues were obtained from our department. All the patients underwent Computed Tomography, Magnetic Resonance Imaging, Bone Marrow Examination and careful physical examination to distinguish whether the tumor metastasis had occured. The diagnosis of these tissue samples was verified pathologically and immunohistochemically. Pathologic and clinic data, including tumor grade, invasion, and metastasis, were collected. All the tissue samples were either immediately snap-frozen in liquid nitrogen or stored at −80 °C.

2.2. Cell culture and transfection

Human neuroblastoma cell lines SH-SY5Y and IMR-32 and human embryonic kidney cell line HEK-293 were obtained from the animal experimental center of Sun Yet-sen University (Guangzhou, China). Cells were cultivated in DMEM with 10% FBS (Gibco, NY, USA). All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The SH-SY5Y cell line was a subclone of the parental SK-N-SH cell line. The primary site was thorax and the metastatic site was bone marrow. And IMR-32 cells were primary neuroblastoma cells which primary site was abdominal and had 1p alternation [15].

Mature miR-362-5p mimics (~20 nucleotide), miR-362-5p inhibitors and negative control which was tested to be non-functional gene were purchased from Shanghai GenePharma (Shanghai, China). Transfection of miRNA mimics and inhibitors was performed using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. RNA preparation and quantitative real time (qRT)-PCR

RNA was extracted from cells or tissue samples using a mirVana miRNA isolation kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. Small RNA fractions were separated and purified. cDNA was obtained using M-MLV (Promega, Madison, WI, USA). The PCR reaction conditions were: denaturing the DNA at 94 °C for 5 min, followed by 40 cycles of amplification: 94 °C for 60 s, 60 °C for 60 s, 72 °C for 60 s for data collection. The relative expression level of miR-362-5p was detected using SYBR® Premix Ex Taq™Kits (TaKaRa, Tokyo, Japan) on an ABI 7500 thermocycler (AppliedBiosystems, FosterCity, CA, USA), with U6 small nuclear RNA (Applied Biosystems) as an endogenous control. Primers were purchased from Beijing Genomics institution (Beijing, China). The primers were as follows: miR-362-5p RT primers 5′-GTCGTATCC AGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACACTCAC-3′; PCR primers: forward 5′-GTCACGAAATCCTTGGAA CCTAG-3′, reverse 5′-TATGGTTGTTCTCGTCTCCTTCTC-3′.

2.4. Plasmids

The small interfering RNA (siRNA) plasmid targeting PI3K-C2β and PI3K-C2β expression plasmids was obtained from GenePharma (Shanghai, China). The siRNA sequences for targeting PI3K-C2β were 5′-CTCAAGAGCTCTGGCCGAATC-3′ (siRNA-1), 5′-A TGCTGAGACCCTGCGTAAGA-3′ (siRNA-2). Scrambled sequences from GenePharma which was confirmed not to interact with any mRNA sequence else was used as a negative control.

2.5. Cell proliferation assays

Cell proliferation was measured using a cell counting kit (CCK-8) (Beyotime Institute of Biotechnology, Jiangsu, China). Cells were seeded in 96-well plates (4×10^3 cells per well) and cultured for 48 h. The viability of SH-SY5Y cells transfected with miR-362-5p mimics or negative control mimics was detected every 24 h after transfection. 10 μl CCK-8 was added to each well of the 96-well plates and incubated for 4 h. The optical density was detected at 490 nm using a Bio-Rad 2550 EIA Reader (Bio-Rad, Hercules, CA, USA).

2.6. Flow cytometry analysis

To determine the effect of miR-362-5p mimics on apoptosis of SH-SY5Y cells, AnnexinV-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) were used for flow cytometry analysis. Cells were treated with miR-362-5p mimics and negative control for 24 h. The cells were then harvested, washed twice with PBS, and adjusted to a concentration of 5×10^5 cells/mL with PBS. Suspensions (200 μl) were added to each labeled tube, 5 μl of Annexin V-FITC and 10 μl propidium iodide were added and the tubes were incubated for at least 10 min at room temperature in the dark. Cells were assayed by flow cytometry (BD Biosciences, USA).

2.7. Tumor cell xenograft

A genomic sequence encoding miRNA-362-5p and negative control were amplified and then cloned into pLenti6.3/V5-DEST Gateway Vector (Invitrogen, USA). After confirmed by gene sequencing, the lentiviruses infected to SH-SY5Y cells.

All experimental procedures involving animals were approved by the Animal Care and Use Committee of Southern Medical University, China. 20 male nude mice (4–6 weeks old, 18–20 g) were purchased from the Laboratory Animal Center of Sun Yet-Sen University (Guangdong, China). SH-SY5Y cell suspensions (either stably transfected with miR-362-5p or negative control) were injected subcutaneously (5×10^6 cells in 200 μl). Mice were observed daily to ensure that the injection sites were healthy. Animals were sacrificed 20 days after injection, the relative expression of miR-362-5p was detected using qRT-PCR, and the final tumor volumes were determined. The tumor volume was calculated using the formula: tumor volume = 1/2 (length × width^2).

2.8. Cell migration and invasion assays

Cell migration and invasion assays were carried out using a BD BioCoat™ Growth Factor Reduced Matrigel™ Invasion Chamber according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). First, cells were harvested and resuspended in serum-free medium after pretreatment for 24 h with miR-362-5p or negative control. Then, cells were seeded in the upper compartments of an 8-μm Boyden chamber. Migration and invasion assays were carried out with coated Matrigel (invasion) or uncoated Matrigel (migration). After incubation for 24 h, cells migrated and invaded through the lower chamber were fixed with methanol, stained with 0.5% crystal violet, and counted using a microscope.

2.9. Fluorescent reporter assays

Human PI3K-C2β 3′-UTR harboring miR-362-5p potential target-binding sequences and mutated PI3K-C2β 3′-UTR, which was not paired with miR-362-5p, was synthesized by GenePharma (Shanghai, China). Luciferase vectors were made between the Hind III and MluI sites. The luciferase activity was

measured using a Dual-Luciferase Reporter Assay System (Promega, Beijing, China). Luciferase activities were expressed as the ratio between firefly luciferase and *Renilla* luciferase activities.

2.10. Western blot

Treated cells were washed twice with ice-cold PBS. Total proteins from cells were lyzed with RIPA buffer. Equal amounts of protein were separated using 10% SDS–PAGE and transferred to polyvinylidene fluoride membranes. Following transfer, the membranes were blocked for 1 h at room temperature and blotted with an appropriate primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Beyotime Institute of Biotechnology, Jiangsu, China) overnight at 4 °C. Horseradish-peroxidase-labeled secondary antibodies (1:1500) were added and incubated for 1 h at room temperature. Blots were visualized using ECL detection reagents (Amersham Biosciences, Uppsala, Sweden). β-Actin (Beyotime Institute of Biotechnology, Jiangsu, China) served as a loading control. Bands were quantified using Image J Software.

2.11. Statistical analysis

All data are expressed as mean ± standard deviation. The differences between groups were analyzed using Student's *t* test when only two groups were present, or assessed by one-way analysis of variance (ANOVA) when more than two groups were compared. All data were analyzed using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 5.0. In this study, $P < 0.05$ is considered statistically significant.

3. Results

3.1. Association of miR-362-5p expression levels with neuroblastoma risk factor

To investigate potential involvement of the miR-362-5p in the process of human neuroblastoma metastasis, 12 human primary and 12 metastatic neuroblastoma tissues was included in this study. The clinic pathological features of neuroblastoma patients were shown in Supplementary Table S1. The miR-362-5p expression was investigated by qRT-PCR. As shown in Fig. 1A, we found that miR-362-5p was down-regulated in metastatic neuroblastoma tissues compared with primary neuroblastoma tissues ($P < 0.01$). Lower miR-362-5p expression was also highly associated with patients who were diagnosed >1.5 years (1.54-fold; $P < 0.05$) (Fig. 1B). Compared with stage 1, 2, 3 or 4s neuroblastoma, the stage 4 neuroblastoma tissues had lower miR-362-5p expression ($P < 0.01$) (Fig. 1C). As shown in Fig. 1D, the miR-362-5p expression in the metastatic neuroblastoma SH-SY5Y cell line was lower than that in the primary neuroblastoma IMR-32 cells.

3.2. miR-362-5p inhibits proliferation and promotes apoptosis of SH-SY5Y and IMR-32 cells in vitro

To investigate the effect of miR-362-5p on neuroblastoma cell growth, SH-SY5Y and IMR-32 cells were transfected with miR-362-5p mimics, miR-362-5p inhibitors or negative control. The transfection efficiency was confirmed by qRT-PCR. In both cell lines, we found that the miR-362-5p expression in the miR-362-5p mimics group was higher than that in the negative control group

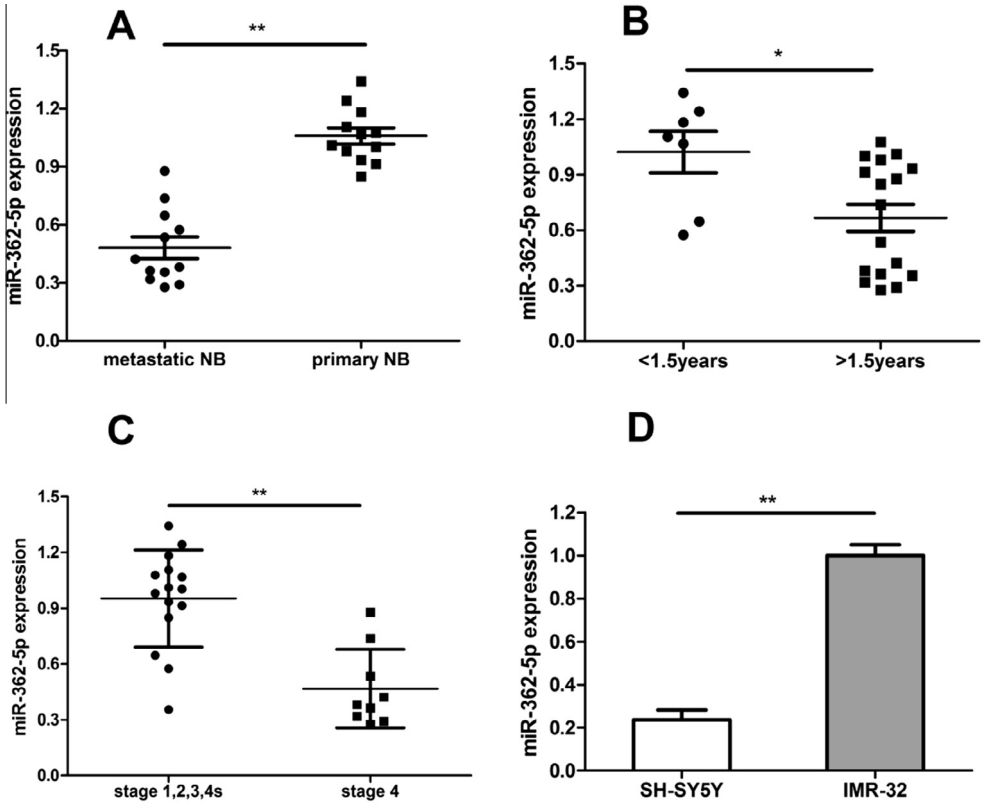

Fig. 1. Expression level of miR-362-5p in neuroblastoma tissues and cells. NB, neuroblastoma. (A) miR-362-5p expression was determined by qRT-PCR in human metastatic neuroblastoma tissues and primary neuroblastoma tissues. The expression of miR-362-5p in (B) different diagnosis ages; (C) different stages of tumor. (D) miR-362-5p expression was detected by qRT-PCR in different neuroblastoma cells. The relative expression of miR-362-5p was normalized to U6. $^{**}P < 0.01$, $^{*}P < 0.05$.

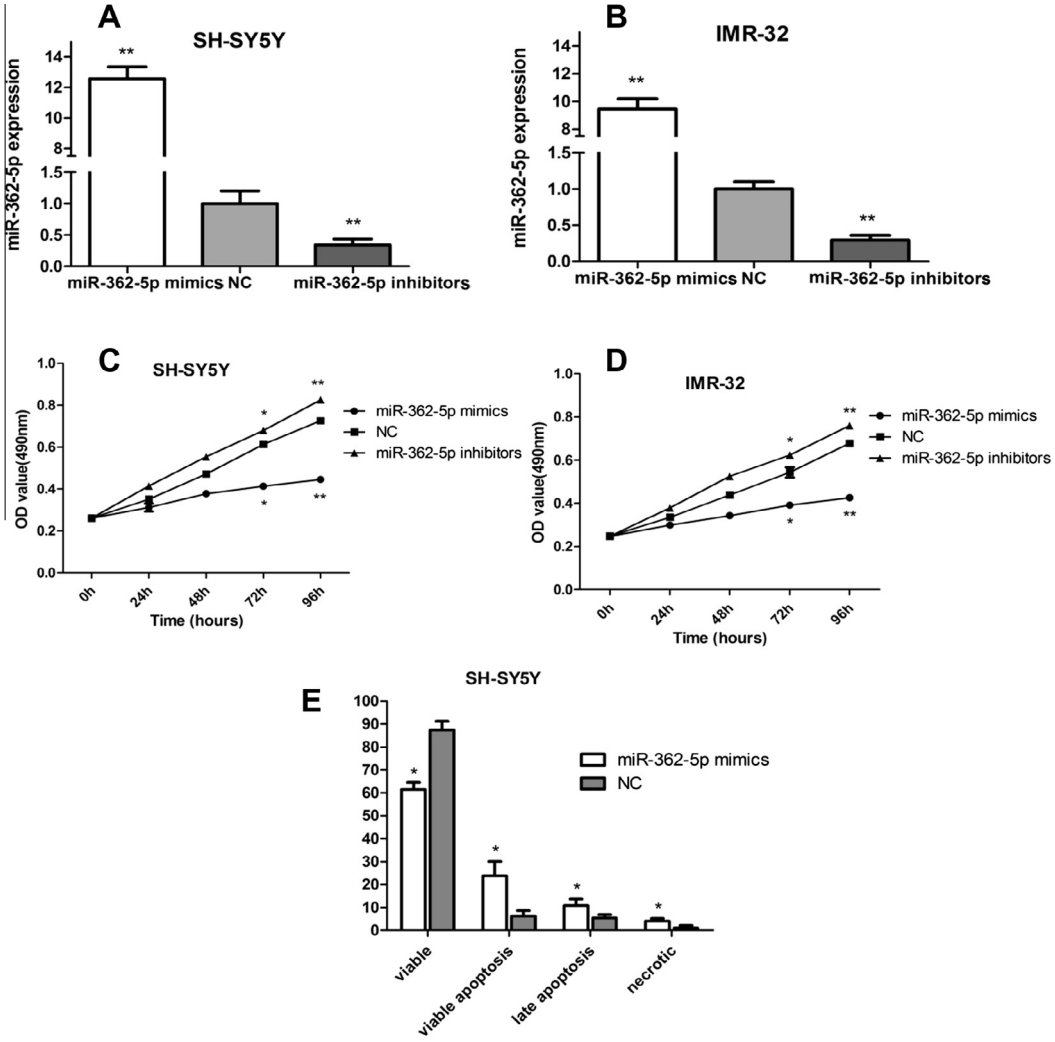

Fig. 2. Overexpression of miR-362-5p inhibits proliferation and promotes apoptosis of SH-SY5Y and IMR-32 cells in vitro. OD, optical density. (A) SH-SY5Y and (B) IMR-32 cells were transfected with miR-362-5p mimics, miR-362-5p inhibitors or negative control. The expression level of miR-362-5p was detected by qRT-PCR. Cell Counting Kit (CCK-8) assay was performed to investigate the effect of miR-362-5p on the proliferation of SH-SY5Y (C) and IMR-32 cells (D) at different time points. (E) The effect of miR-362-5p mimics on apoptosis of SH-SY5Y was detected by flow cytometry analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with NC.

(Fig. 2A), while miR-362-5p inhibitors suppressed the miR-362-5p expression (Fig. 2B). The CCK-8 assay showed that miR-362-5p mimics significantly inhibited the proliferation of SH-SY5Y cell lines, while miR-362-5p inhibitors increased the cell viability (Fig. 2C). miR-362-5p had similar effect on IMR-32 cells viability (Fig. 2D). Moreover, flow cytometry revealed that the proportion of apoptotic and necrotic cells in the miR-362-5p mimic group was significant larger than the negative control group ($P < 0.05$) (Fig. 2E). In other words, the miR-362-5p promoted apoptosis of SH-SY5Y cells in vitro.

3.3. miR-362-5p inhibits tumor growth of neuroblastoma in vivo

Because our in vitro studies indicated that miR-362-5p probably acts as a tumor suppressor in neuroblastoma cells, we investigated whether miR-362-5p could inhibit tumor growth of neuroblastoma in vivo. At 20 days after injection, the relative expression of miR-362-5p was detected by qRT-PCR. The expression level of miR-362-5p in tumors stably transfected with miR-362-5p is significantly higher than negative control ($P < 0.01$) (Fig. 3A). As shown in Fig. 3B, tumor cells transfected with miR-362-5p mimics formed smaller tumors than did the cells transfected with the negative control. The tumors were significantly smaller in the miR-362-5p transfected group than in the negative control transfected group ($P < 0.01$) (Fig. 3C).

3.4. miR-362-5p inhibits the migratory and invasive behavior of SH-SY5Y and IMR-32 cells

As our results indicated, the expression of miR-362-5p in metastatic tumor tissues is significant lower than in primary neuroblastoma tissue. miR-362-5p may play a role in regulating neuroblastoma metastasis. Therefore, in vitro invasion and migration assays were conducted. We found that miR-362-5p mimics reduced the migration and invasion abilities of SH-SY5Y and IMR-32 cells compared with negative control, while miR-362-5p inhibitors had the opposite effect in both SH-SY5Y and IMR-32 cells (Fig. 4).

3.5. PI3K-C2β is target of miR-362-5p in neuroblastoma cells

To screen the function target of miR-362-5p in neuroblastoma cells, Bioinformatics software TargetScan 6.2 (http://www.targetscan.org/) was used to screen the target gene of miR-362-5p. Considering the potential roles of candidate genes in neuroblastoma, PI3K-C2β, which plays an important role in signaling

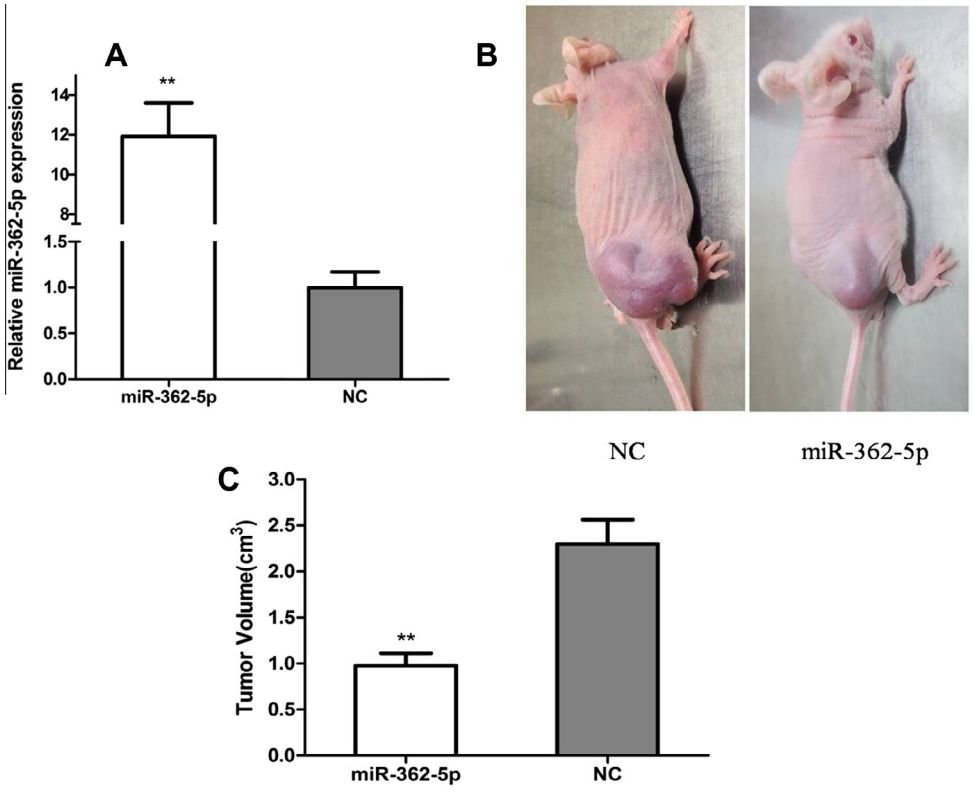

Fig. 3. miR-362-5p reduces tumor growth of neuroblastoma in vivo. (A) The expression level of miR-362-5p in xenograft tumors was detected by qRT-PCR. SH-SY5Y cells stably infected with miR-362-5p or negative control were injected subcutaneously into nude mice. (B) Representative images of xenograft tumors. (C) Tumor volumes formed by miR-362-5p or negative control infected SH-SY5Y cell lines were measured at the end of the experiment (20 days after injection). $^{**}P < 0.01$ compared with NC.

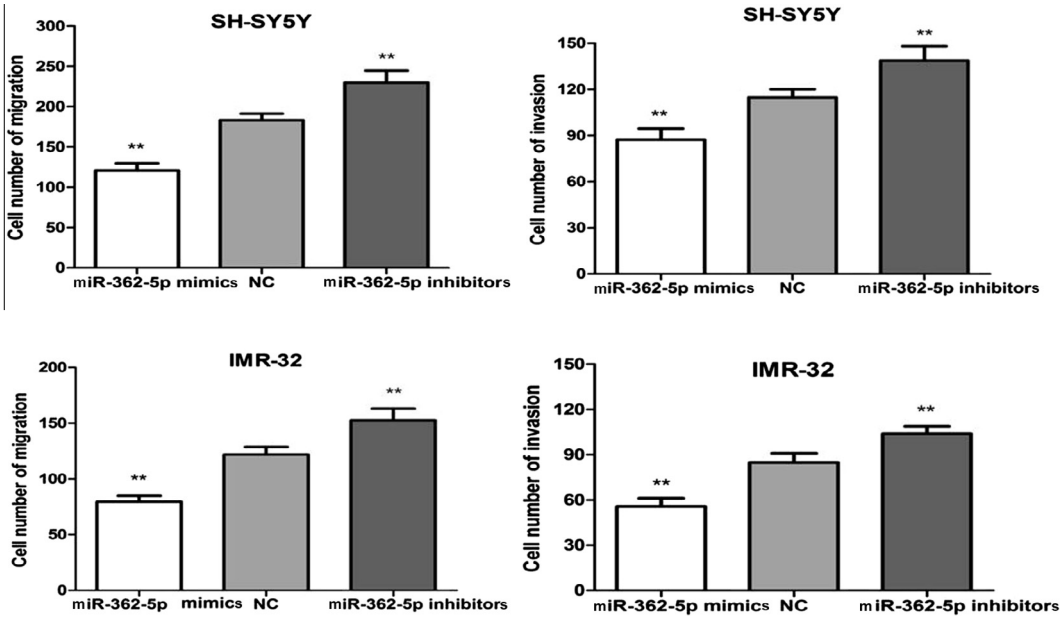

Fig. 4. Effect of miR-362-5p on SH-SY5Y and IMR-32 cells motility. Transwell assay was used to evaluate migration and invasion of SH-SY5Y cells transfected with miR-362-5p mimics, miR-362-5p inhibitors and negative control mimics. (A) Migration; (B) invasion. (C and D) The graph represented the number of migratory or invasive IMR-32 cells per field. $^{**}P < 0.05$ compared with NC.

pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking, was selected as our candidate target. Inspection of the 3′-UTR of PI3K-C2β revealed only one potential miR-362-5p target site that was closely positioned (Fig. 5A).

A luciferase activity assay was used to determine the potential interaction between miR-362-5p and PI3K-C2β. It was found that miR-362-5p significantly suppressed activity of wild-type 3′-UTR but not the mutant 3′-UTR of PI3K-C2β in SH-SY5Y cells (Fig. 5B). In addition, Western blot analysis showed that miR-362-5p mimics

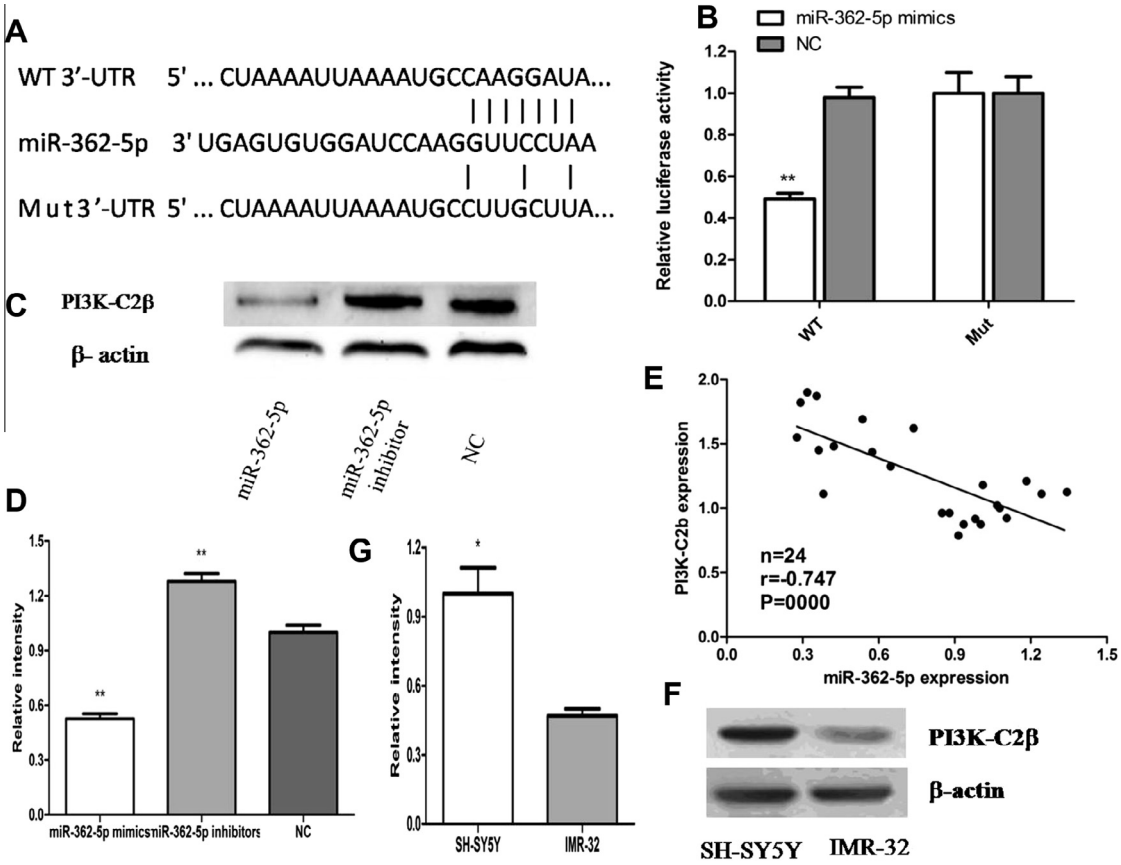

Fig. 5. PI3K-C2β is a target of miR-362-5p in SH-SY5Y cells. (A) A schematic illustration of predicted seed region in the wild type (WT) PI3K-C2β 3′-UTR as well as mutated (Mut) 3′-UTR used in this study. (B) Fluorescent reporter assays in SH-SY5Y cells. Cells were transfected with wild-type or mutant 3′-UTR-reporter constructs together with miR-362-5p or negative control. (C) Protein level of PI3K-C2β was detected by Western blot in SH-SY5Y cells transfected with miR-362-5p mimics, miR-362-5p inhibitors or negative control. β-Actin was served as a loading control. (D) Intensity of PI3K-C2β protein compared to β-actin ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with NC. (E) Pearson's correlation analysis was carried out to evaluate the relationship between PI3K-C2β and miR-362-5p. The miR-362-5p expression were detected by qRT-PCR and normalized to U6. (F and G) Protein level of PI3K-C2β in different neuroblastoma cells was tested by Western blot ($n = 5$). The PI3K-C2β protein levels were evaluated by Western blot and normalized to β-actin.

reduced PI3K-C2β protein levels of SH-SY5Y cells compared with negative control (Fig. 5C and D).

Since PI3K-C2β was one of the targets of miR-362-5p in vitro, we next evaluated the association between miR-362-5p and PI3K-C2β from neuroblastoma tissues and cells. Western blot found a significantly reverse correlation between miR-362-5p and PI3K-C2β in the neuroblastoma tissues (Fig. 5E, Pearson's correlation coefficient = −0.747; $P = 0.0000$). The results showed that the miR-362-5p expression and PI3K-C2β expression were complementary clinically and suggested that miR-362-5p might negatively regulate the expression of PI3K-C2β in vivo. To further investigate the relationship between the miR-362-5p expression and PI3K-C2β expression, the PI3K-C2β protein levels of the primary and metastatic neuroblastoma cell lines were detected by Western blot. As shown in Fig. 5F and G, the PI3K-C2β expression in the metastatic neuroblastoma SH-SY5Y cells was significant higher than that in the primary neuroblastoma IMR-32 cells ($P < 0.05$), while the miR-362-5p expression in the SH-SY5Y cells were lower than that in the IMR-32 cells.

3.6. Inhibition of PI3K-C2β shows similar effects to miR-362-5p overexpression

To study the effect of PI3K-C2β on neuroblastoma cell growth, SH-SY5Y cells were transfected with PI3K-C2β siRNA or negative control siRNA. Western blot results showed that both PI3K-C2β siRNA (number 1 or number 2) decreased the protein level of PI3K-C2β compared with negative control siRNA ($P < 0.05$) (Fig. 6A and B). However, PI3K-C2β-siRNA-2 has a stronger inhibitory effect than PI3K-C2β-siRNA-1 ($P < 0.05$). PI3K-C2β-siRNA-2 significantly suppressed the growth, migration and invasion of SH-SY5Y cells and showed a similar effect to miR-362-5p overexpression (Fig. 6C–E).

3.7. PI3K-C2β overexpression attenuates the suppressive effect of miR-362-5p

Finally, we investigated whether overexpression of PI3K-C2β could reverse the effect of miR-362-5p. The overexpression vector pcDNA3/PI3K-C2β (without PI3K-C2β 3′-UTR) tagged by GFP was synthesized by GenePharma (Shanghai, China) and the empty vector (pcDNA3) was served as negative control. First, the transfection efficiency was determined by fluorescence microscopy and the effect of PI3K-C2β overexpression plasmid on SH-SY5Y cells was confirmed by Western blot. As shown in Fig. 7A and B, the PI3K-C2β overexpression plasmid significantly increased the PI3K-C2β protein level in SH-SY5Y cells compared to the empty vector (pcDNA3) ($P < 0.01$). As shown in Fig. 7C and D, transfection of PI3K-C2β expression plasmids increased its protein levels, which were down-regulated by miR-362-5p. A CCK-8 assay (Fig. 7E), and migration and invasion assays (Fig. 7F and G) showed that overexpression of PI3K-C2β

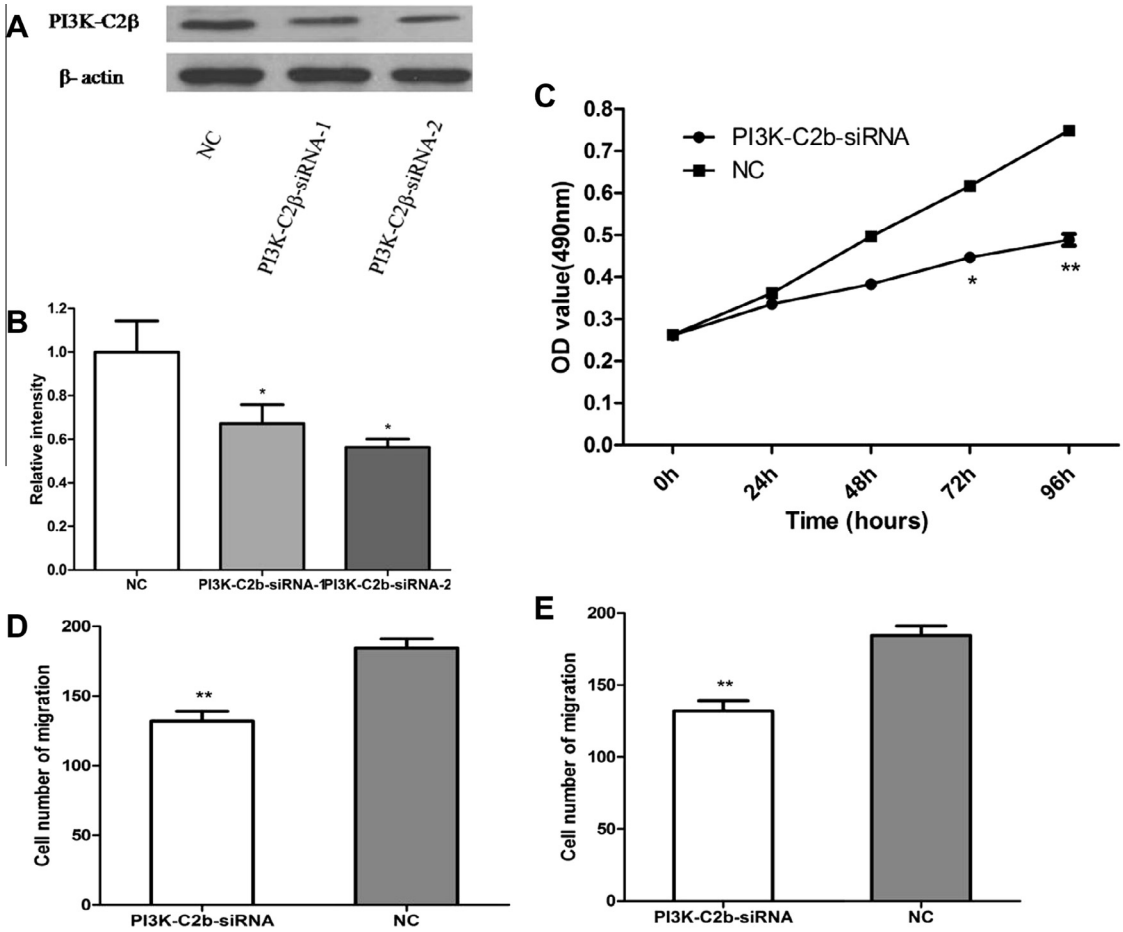

Fig. 6. Inhibition of PI3K-C2β shows similar effects to miR-362-5p overexpression in SH-SY5Y cells. OD, optical density. (A) Protein level of PI3K-C2β was detected by Western blot in SH-SY5Y cells transfected with PI3K-C2β or the negative control siRNA. (B) Intensity of PI3K-C2β protein in SH-SY5Y cells transfected with PI3K-C2β siRNA or negative control siRNA ($n = 5$). (C) CCK-8 assay was used to determine the effect of PI3K-C2β siRNA on the proliferation of SH-SY5Y. Knockdown the expression of PI3K-C2β by siRNA suppressed the (D) migration and (E) invasion of SH-SY5Y cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with NC. NC, negative control siRNA.

could significantly reverse the suppressive effect of miR-362-5p on SH-SY5Y cells.

4. Discussion

In past decades, numerous genes were identified to relate with neuroblastoma growth, invasion, and migration. miRNAs, which are non-coding short-stranded RNAs, have been demonstrated to play important roles in multiple aspects of tumor biology, such as proliferation, invasion, and angiogenesis [16]. miRNAs have been revealed as 'onco(genic)-miRs' or 'tumor-suppressor miRs' in neuroblastoma. Chen et al. reported that miR-338-3p suppresses neuroblastoma proliferation, invasion and migration by targeting PREX2a, which would affect the PTEN/Akt pathway [17]. Yang et al. reported that miR-329 suppressed the growth and motility of neuroblastoma by targeting KDM1A, which contributes to tumorigenesis via activation of the Wnt/β-catenin signaling pathway [8].

Many miRNAs are found to be up- or down-regulated in cancer samples relative to their normal tissue counterparts, including neuroblastoma. Human tissue miRNA microarray analysis showed that numerous miRNAs are differentially expressed in high-risk neuroblastoma compared with low-risk neuroblastoma. miR-30b, miR-146a, miR-190, miR-204, miR-215, miR-299-5p, etc. [7,18] were down-regulated in high-risk neuroblastoma. miR-362-5p was also found to be aberrantly down-regulated in high-risk neuroblastoma tissues [7]. In this study, we confirmed that miR-362-5p was down-regulated in metastatic neuroblastoma (high-risk neuroblastoma) compared with primary neuroblastoma (low-risk neuroblastoma) by qRT-PCR. Several mechanisms caused miRNA dys-expression in cancer, such as Chromosomal abnormalities [19], structural genetic alterations and Drosha or Dicer activity alterations. miRNA can also be affected by other miRNAs or other mRNAs. Despite the advances in our understanding of the mechanisms causing miRNA deregulation, the daunting task still remains the elucidation of the biological role of miRNAs in the initiation and in the development of cancer.

miRNA play an important role in tumor biology. Xia et al. reported that miR-362 induces cell proliferation and apoptosis resistance in gastric cancer by activation of NF-κB signaling pathway [13]. Fang et al. discovered that miR-362-5p was significantly up-regulated in hepatocellular carcinoma (HCC) and associated with HCC progression. Inhibition of miR-362-5p in HCC cells dramatically decreased cell proliferation, clonogenicity, migration and invasion in vitro as well as tumor growth and metastasis in vivo by targeting the CYLD gene [20]. In our study, we demonstrated that miR-362-5p decreased cell viability, proliferation, migration, and invasion of neuroblastoma cells in vitro, and inhibited tumor growth in a neuroblastoma nude mouse model in vivo. The different roles of miRNA in cancers may mostly depend on the roles of their target genes. Different cancer cells may have different genetic backgrounds and miRNA expression profiles. There were different gene phenotypes between neuroblastoma and HCC, and miR-362-5p may play a different role by targeting different gene.

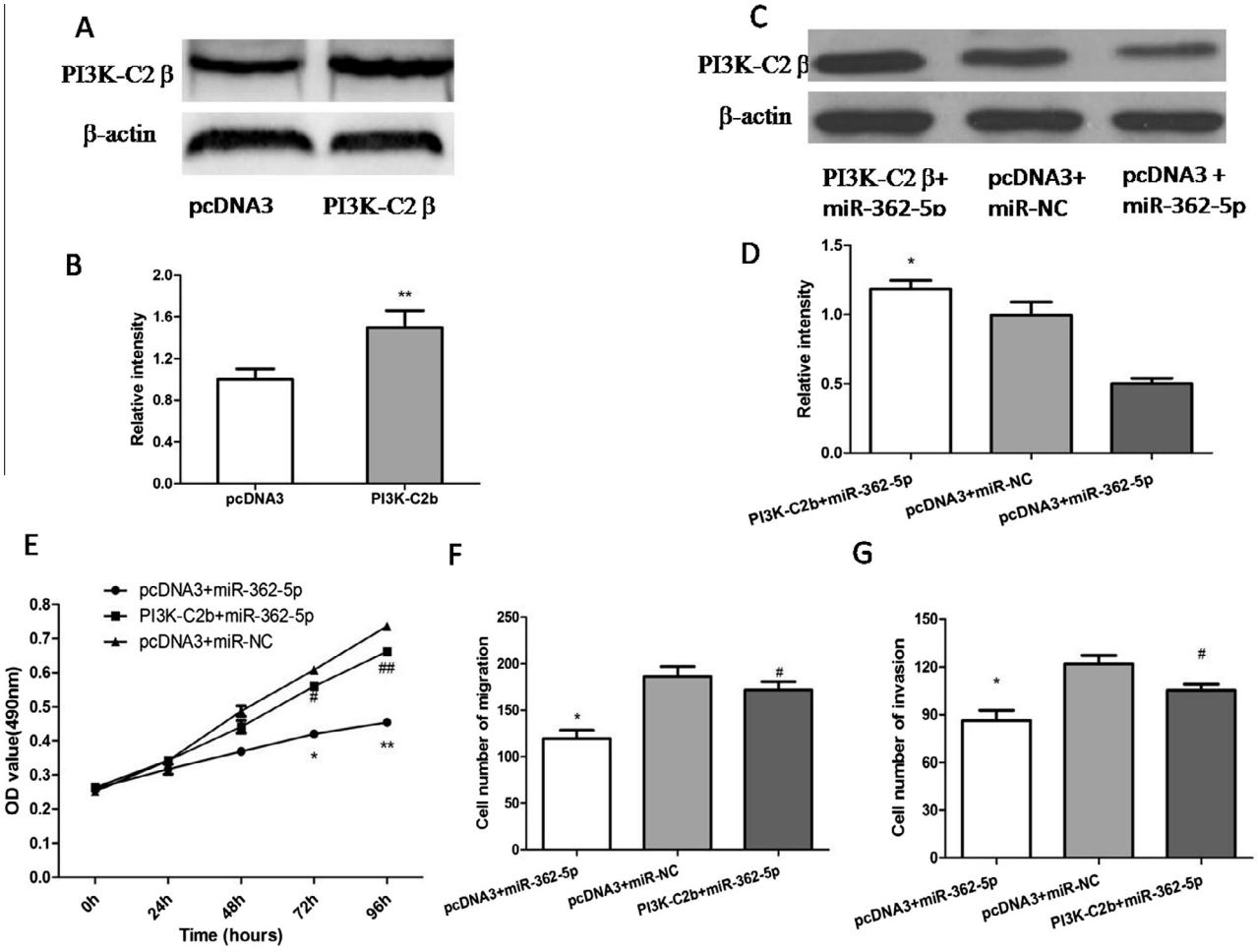

Fig. 7. PI3K-C2β overexpression reversed the suppressive effect of miR-362-5p in SH-SY5Y cells. OD, optical density. (A) Expression of PI3K-C2β was detected by Western blot in SH-SY5Y cells transfected with PI3K-C2β plasmid or pcDNA3 (the empty vector). (B) Intensity of PI3K-C2β protein in SH-SY5Y cells transfected with PI3K-C2β plasmid or pcDNA3 ($n = 5$). (C–G) The SH-SY5Y cells were transfected with miR-362-5p plus pcDNA3, miR-362-5p plus PI3K-C2β expression plasmid or miR-NC plus pcDNA3. (C and D) Protein level of PI3K-C2β was detected by Western blot ($n = 5$). The proliferation ability (E), migration (F) and invasion ability (G) were determined in SHSY5Y cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with miR-NC plus pcDNA3. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with miR-362-5p plus pcDNA3 group.

To date, the mechanism of the miR-362-5p function in neuroblastoma has not been investigated. Several targets have been identified by bioinformatics analysis. Here, we identified PI3K-C2β as a novel target of miR-362-5p, and verified that miR-362-5p decreased the PI3K-C2β protein level. PI3K generates 3′-phosphorylated phosphoinositides as intracellular second messengers and is a hallmark of many signaling pathways [21]; it controls multiple cellular responses, including proliferation, growth and invasion. PI3K-C2β has also been reported to contribute to the tumorigenesis of multiple cancers. Liu et al. reported that PI3K-C2β is associated with oesophageal squamous cell carcinoma metastasis and PI3K-C2β knockout enhanced cisplatin-induced apoptosis, and inhibits metastasis of oesophageal squamous cell carcinoma cells. Moreover, they demonstrated that PI3K-C2β, via the Akt signaling pathway, might play a key role in tumorigenesis [22]. PI3K-C2β has been identified as a potential target for neuroblastoma tumor treatment [23]. Russo et al. discovered that Intersectin 1-PI3K-C2β was an important pathway in neuroblastoma tumorigenesis [24]. In this study, we also found that inhibition of PI3K-C2β remarkably suppressed the growth and motility of neuroblastoma cells, with similar effects to miR-362-5p, while its overexpression successfully reversed the effects of miR-362-5p. However, a miRNA may target many genes and a single gene may be a target of many microRNA. Whether other targets of miR-362-5p are involved in the regulation of neuroblastoma remains to be elucidated. Our results also showed that miR-362-5p reduces the expression of PI3K-C2β to about 50% and such small changes alone may not show any cellular phenotype. However miR-362-5p has powerful cell inhibition function on neuroblastoma cells. It could be that miR-362-5p may target many other genes in the same pathway or influence the other biological processes or pathways.

In conclusion, we found that miR-362-5p can inhibit SH-SY5Y cell proliferation, migration, and invasion in vitro and in vivo. Our study identified a functional link between miR-362-5p and PI3K-C2β expression in neuroblastoma, and also demonstrated that PI3K-C2β was directly target of miR-362-5p. miRNA may represent a promising treatment strategy for neuroblastoma.

Disclosure

All the authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.056.

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