FEBS Letters 587 (2013) 3729–3737

FEBS Letters

journal homepage: www.FEBSLetters.org

miR-338-3p suppresses neuroblastoma proliferation, invasion and migration through targeting PREX2a

Xin Chen [a,1], Min Pan [b,1], Lulu Han [a], Hongting Lu [a], Xiwei Hao [a], Qian Dong [a,*]

[a] Department of Pediatric Surgery, The Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shandong, China
[b] Normal College, Qingdao University, Qingdao, Shandong, China

ARTICLE INFO

Article history:
Received 18 July 2013
Revised 30 August 2013
Accepted 13 September 2013
Available online 15 October 2013

Edited by Tamas Dalmay

Keywords:
miR-338-3p
PREX2a
PTEN
AKT
Neuroblastoma
Metastasis
Microarray

ABSTRACT

MicroRNAs (miRNA) can regulate cancer cell proliferation and metastasis. Here, we show that miR-338-3p is down-regulated in metastatic tumor tissues compared to primary tumors, and that that miR-338-3p can inhibit cell proliferation by inducing cell cycle arrest, as well as restrain cell migration and invasion. PREX2a is confirmed as a direct target of miR-338-3p. Knockdown of PREX2a inhibits cell proliferation, migration and invasion through the PTEN/Akt pathway. miR-338-3p-dependent inhibition of proliferation and invasion can be rescued by PREXa. Overall, this study demonstrates that miR-338-3p affects the PTEN/Akt pathway by down-regulating PREX2a. This newly identified function of miR-338-3p provides novel insights into neuroblastoma and may foster therapeutic applications.

1. Introduction

Neuroblastoma (NB), one of the most common forms of cancer in young children, originates from precursor cells of the sympathetic nervous system, accounting for 7% of childhood malignancies and 15% of pediatric cancer-related deaths [1]. It is a heterogenous disease, ranging from spontaneous regression without treatment to rapid disease progression and mortality. Despite advances in treatment and disease management, the overall 5-year survival rates remain poor in high-risk disease (25–40%). Seventy-three percent of patients with NB have already developed malignant lesions outside the primary tumor at the time of diagnosis [2]. Although a number of genetic and molecular lesions have been correlated with NB tumorigenesis, the molecular basis of this tumor is still far away from being completely understood. Elucidating the mechanism will potentially contribute to a greater understanding of response to therapy, leading to the identification of suitable therapeutic agents.

To clarify the mechanism, a recently discovered class of genes transcribing small non-coding RNAs, namely microRNAs, come into focus, which could be potential serving as new therapeutic strategies for cancers. MicroRNAs are 20–22 nucleotide molecules which can regulate gene expression through RNA interference effecter complex (RISC) mediated mRNA degradation or translational suppression via complimentary pairing predominantly to the 3′-untranslated region (3′-UTR) of their targeted messenger RNAs [3]. Recently, an increasing number of studies have demonstrated the misexpression patterns of miRNAs in many human cancers participating in critical pathways linked to proliferation, cell cycle progression and metastasis [4]. Several miRNAs have been linked to MYCN, a risk factor of NB. For example, Swarbrick, et al. found miR-380-3p increased tumor growth by repressing p53 and was associated with poor outcome in MYCN-amplified neuroblastoma [5]. Recently, researchers identified that over-expression of miR-497 resulted in significantly cell cycle arrest at G0/G1 and G2/M phase in MYCN-amplified neuroblastoma Kelly cells. Further analysis revealed miR-497 functioned through targeting 3UTR of WEE1, a tyrosine kinase regulator of the cell cycle [6]. Furthermore, cytofluorimetric analysis confirmed that an accumulation of a G0/G1 phase population was observed in miR-214 down-regulated in neuroblastoma SK-N-SH cells [7]. A study conducted by Zhang, et al. demonstrated that miR-9 inhibited invasion and angiogenesis in NB cells through targeting matrix metalloproteinase (MMP)-14 [8].

In our previous study, we showed a widespread alteration of 54 miRNAs in metastatic NB compared with primary NB, Among

* Corresponding author. Address: 16th Jiangsu Road, Shinan District, Qingdao, Shandong 266003, China.
E-mail addresses: brabus-cx@163.com (X. Chen), 0532pm@163.com (M. Pan), dong.qian@sohu.com (Q. Dong).
[1] These authors contributed equally to this work.

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.09.044

which, miR-338-3p exhibited a potential role in regulating NB metastasis, because it was significantly down-regulated in metastatic NB compared to primary NB in animal models [2]. PREX2a is a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase (GTPase) [9]. The PREX2a gene is located on chromosome 8q13, a region linked to frequent amplification and aggressive cancer phenotypes in breast, prostate, and colorectal cancers [10]. In this report, we identify the important molecular mechanism by which miR-338-3p exerts its negative effects on neuroblastoma cell proliferation, migration and invasion in human neuroblastoma cells, which involves the direct targeting the 3′UTR of PREX2a mRNA and affects the PTEN/Akt pathway.

2. Materials and methods

2.1. Human tissue samples

Eighteen paired neuroblastoma tissue sections from patients with metastasis and primary tissues were obtained from the Tumor Bank Facility of the Affiliated Hospital of Medical College, Qingdao University, China. Detailed pathologic and clinical data were collected for all samples including Edmondson tumor grade, invasion and metastasis. The diagnoses of these samples were verified by pathologists. The collection of human tissue samples was approved and supervised by the Ethics Committee of Qingdao University.

2.2. Cell culture and transfection

For the culture of cell lines used in this study, the human SH-SY5Y neuroblastoma cells were cultivated in DMEM containing 10% fetal bovine serum plus 2 mM L-Glutamine. The GI-Li-N cell was grown in 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-Glutamine. All cells were splitted before confluence and incubated at 37 °C in a humidified incubator with 5% CO_2. MiR-338-3p mimics and miR-338-3p ASO were purchased from Shanghai GenePharma (Shanghai, China). Transfections of miRNA mimics and ASO were carried out using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.3. RNA preparation and quantitative PCR

RNA was extracted from cells or tissue samples using the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. Small RNA fraction (smaller than 200 nt) were separated and purified according to this procedure. cDNA was obtained using M-MLV (Promega, USA) and 1 μg of obtained RNA. The relative level of miR-338-3p was detected by stem-loop RT-PCR with following conditions: denaturing the DNA at 94 °C for 4 min, followed by 40 cycles of amplification: 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s for data collection. U6 snRNA was used as an endogenous control. Quantitative PCR was performed on an ABI 7500 thermocycler (Applied Biosystems) using SYBR® Premix Ex TaqTM (Perfect Real Time) Kits (TaKaRa, Japan) according to the manufacturer's instructions. The primers used are listed in Table 2.

2.4. Microarray analysis

miRNA microarray chips were conducted as previously described [11]. Briefly, 50 μg total RNA was purified and labeled with fluorescein, and hybridization was carried out on miRNA microarray chip (CapitalBio Corp., Beijing, China) containing 508 probes in triplicate, corresponding to predicted miR-338-3p targets. Finally, hybridization signals were detected and scanner images were quantified.

2.5. Detection of cell proliferation capacity

To determine the cell proliferation capacity, cells were examined with cell growth curve and colony formation assay. Cells were seeded in triplicate in 24-well plates with 1×10^4 SH-SY5Y and GI-Li-N cells per well and were counted over an 6-day period since the second day. Cells were harvested by trypsinization and counted for four times per well. The growth curves were drawn for 6 days according to the mean values of the three wells. The number of viable cell colonies were determined after 14 days of inoculation 100 SH-SY5Y or GI-Li-N cells per well in 12-well plates during colony formation assay. Colony formation ratio was calculated with the following equation: colony formation ratio (%) = (number of colonies/number of seeded cells) × 100.

2.6. Cell invasion and wound healing assays

A total of 5×10^4 SH-SY5Y and 1×10^5 GI-Li-N cells (in 0.2 ml RPMI 1640 with 5% FBS) were seeded into the upper part of a Transwell chamber (Corning, USA), which was pre-coated with 1 mg/ml Matrigel (Growth Factor Reduced BD MatrigelTM Matrix) for 2 h. In the lower part of the chamber, 0.6 ml RPMI 1640 with 20% FBS was added. After incubating for 30 h, chambers were disassembled and the membranes were stained with 2% crystal violet for 10 min and placed on a glass slide. Then cells invasing across the membrane were counted in 5 random visual fields under a light microscope. All assays were performed in triplicate and independently performed three times. For the wound healing assay, 5×10^4 SH-SY5Y and 1×10^5 GI-Li-N cells were seeded into 24-well plates and cultured until confluent. A P200 pipette tip was used to make a straight line simulation "wound". Then cells were washed several times with appropriate culture medium to remove cell debris. The extent of wound closure was monitored at 24 h.

2.7. Fluorescent reporter assays

The human PREX2a 3′UTR harboring three miR-338-3p potential target-binding sequences was synthesized by GenPharm (Shanghai, China). Luciferase constructs were made by ligating the synthesized 3′UTR as well as the seed-sequence mutated version after the luc ORF in the pMIR-REPORT luciferase vector (Ambion). For the fluorescent reporter assay, cells were seeded in a 48-well plate the day before transfection. The cells were co-transfected with miR-338-3p mimics or ASO as well as the controls and PREX2a-3UTR or PREX2a-3UTRmut. The cells were lyzed 48 h later and the intensity of luciferase was detected.

2.8. Western blotting

Western blotting was performed to determine protein expression of PREX2a and Akt. Total protein from cells was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and cells were lyzed by RIPA buffer. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as the endogenous normalizer. The polyclonal rabbit anti-human PREX2a, GAPDH (Saierbio, China) and Phospho-Akt (Thr308, Ser473), Akt (cell signaling technology, New England Biolabs) were used.

2.9. Knockdown of PREX2a by siRNA

The siRNA targeting PREX2a (region 153–177, GGAGTTCCTGGT GTCGGCATTCTTA) was designed and synthesized by GenPharm (Shanghai, China). An unrelated sequence was used as a negative control (provided by GenPharm).

2.10. Cell cycle assay

Transfected SH-SY5Y or GI-Li-N cells were seeded into 6-well plates for 24 h in complete medium before cells were deprived of serum for 48 h and then returned to complete medium for an additional 24 h. All cells were collected by centrifugation, fixed in 95% ethanol, incubated at −20 °C overnight and washed with phosphate buffered saline (PBS). Then, cells were resuspended in 1 ml FACS solution (PBS, 0.1% TritonX-100, 60 ug/ml propidium iodide (PI), 0.1 mg/ml DNase free RNase, and 0.1% trisodium citrate). After a final incubation on ice for 30 min, cells were analyzed using a FACS Calibur flow cytometer (Beckman Coulter). A total of 10 000 events were counted for each sample.

2.11. Quantification of PI(4,5)P2 for determination activity of PTEN

For PI(4,5)P2 quantification, SH-SY5Y and GI-Li-N cells were plated in 6-well plates and transfected in three parallels with PREX2a siRNA and control for 48 h. PI(4,5)P2 was quantified using the PI(4,5)P2 Mass ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA) following the manufacturer's instructions. Briefly, cells were incubated with cold 0.5 M TCA for 5 min, centrifuged and resuspended in 5% TCA/1 mM EDTA. After centrifugation, neutral lipids were extracted with methanol:chloroform (2:1) and acidic lipids with methanol:chloroform:12 M HCl (80:40:1). The extracts were centrifuged and chloroform plus 0.1 M HCl were added to the supernatant followed by centrifugation to separate organic and aqueous phases. The organic phase was dried in a vacuum dryer. Then, extracted lipids were resuspended in PBS 0.25% protein stabilizer, incubated with a PI(4,5)P2 detector protein for 1 h at room temperature and then added to a 96-well PI(4,5)P2-coated microplate. A peroxidase-linked secondary detection reagent and a colorimetric substrate were added to detect the PI(4,5)P2 detector protein binding to the plate. The colorimetric signals were measured at 450 nm using a Tecan M1000 instrument. The data represent PI(4,5)P2 levels in pmol/mg cell lysate calculated from the standard curve generated according to the manufacturer's instructions.

2.12. Statistical analysis

A Student's test was performed to analyze the significance of differences between the sample means obtained from three independent experiments. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Down-regulation of miR-338-3p in metastatic NB compared to primary NB

Previously, we established a heterotopic transplant mice model of NB and performed a microarray-based analysis identifying expression of miR-338-3p was decreased (a 34% reduction) in six metastatic tumor tissues compared with paired primary tumor tissues [2]. To investigate the potential involvement of the miR-338-3p in the process of human NB metastasis, we measured the expression levels of miR-338-3p in eighteen pairs of human tissues. As shown in Fig. 1, we found that miR-338-3p expression levels had an average of 36% reduction in metastatic tumor tissues compared with paired primary tumor tissues, which was consistent with the results of animal models.

3.2. miR-338-3p suppresses NB cell growth in vitro

Although the anti-oncogenic contribution of miR-338-3p has been described in liver cancer [12] and colorectal carcinoma [13], the biological significance of miR-338-3p in NB has not been investigated in detail. Gain/loss-of-function experiments are widely used for functional studies of miRNAs. To investigate the role of miR-338-3p in NB cell growth, miR-338-3p mimics and antisense oligonucleotide (ASO) were synthesized, and the transfection efficiency was confirmed by qRT-PCR, which indicated a 12/15-fold increase and 70/65% reduction in SH-SY5Y and GI-Li-N cells (Fig. 2A). To characterize the effects of miR-338-3p on cell growth, cells were transfected with miR-338-3p mimics or ASO, and growth was monitored using growth curve (Fig. 2B, C) and colony formation assays (Fig. 2D), which indicated that overexpression of miR-338-3p inhibited SH-SY5Y and GI-Li-N cells growth, while miR-338-3p ASO exhibited opposite effect, indicating that miR-338-3p suppressed NB cell viability and its long-term proliferative capacity.

To follow up the finding that miR-338-3p could decrease growth of NB cells, FACS was used to analyze whether miR338-3p can affect cell cycle. As shown in Fig. 2E, overexpression of miR-338-3p resulted in a significant increase of cells in G1 phase (from 55% to 79% in SH-SY5Y cells and from 61% to 84% in GI-Li-N cells), an obvious decrease of cells in S phase (from 37% to 16% in SH-SY5Y cells and from 28% to 17% in GI-Li-N cells), compared to the control. The results demonstrated that miR-338-3p can serve as a tumor suppressor in neuroblastoma.

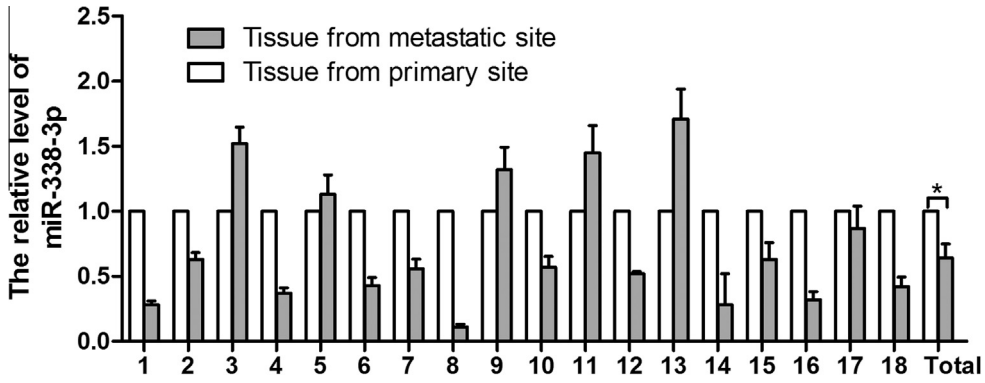

Fig. 1. Identification of the differential expression of miR-338-3p in neuroblastoma tissues. The expression level of miR-338-3p in eighteen pairs of tissues from metastasis site and primary site were detected by quantitative RT-PCR. GAPDH was used as endogenous normalizer. The relative expression level of miR-338-3p in the eighteen pairs of neuroblastoma tissues as well as the combined result (mean ± S.D.) is shown. $^{*}p < 0.05$.

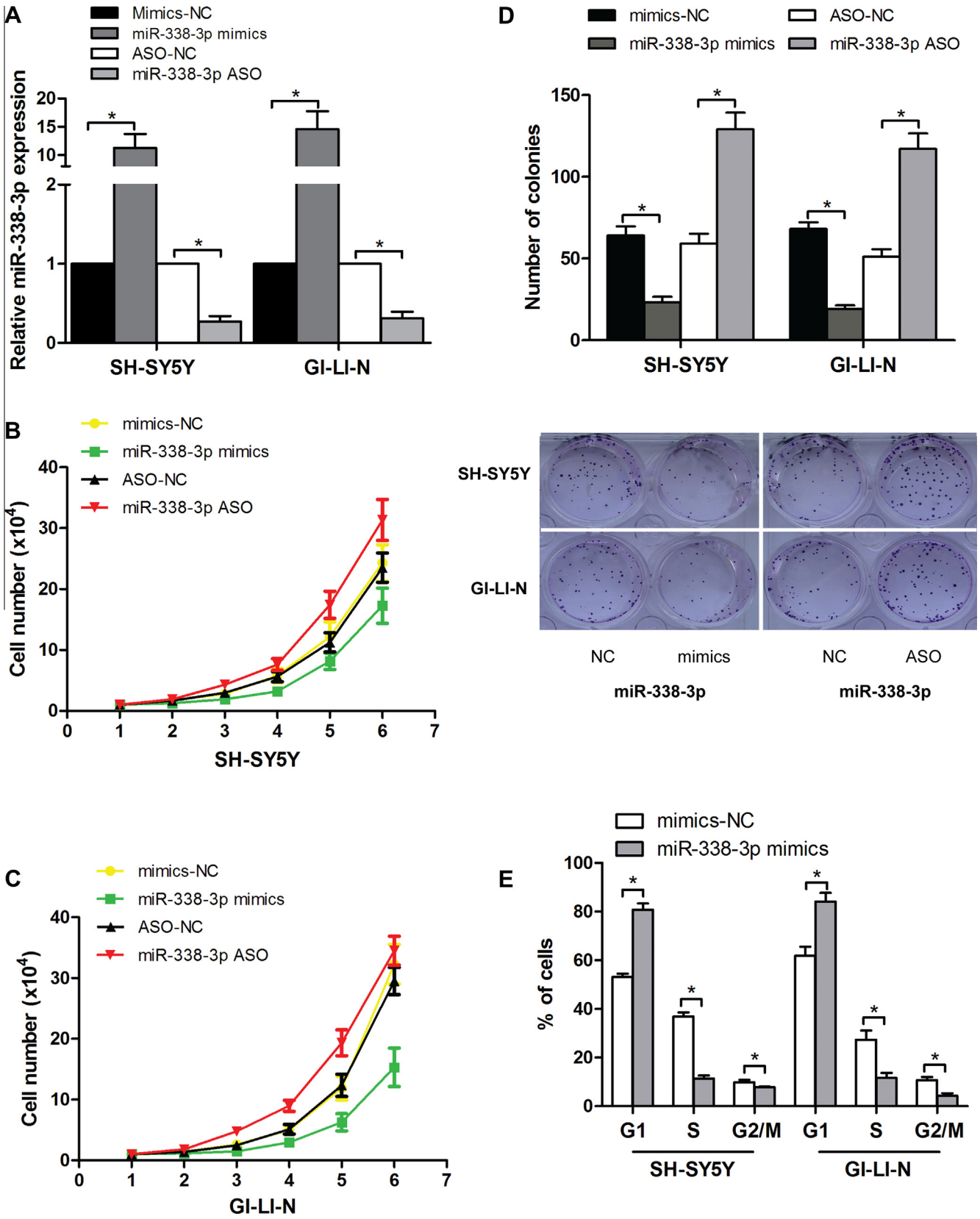

Fig. 2. The effect of miR-338-3p on SH-SY5Y and GI-Li-N cell proliferation. (A) SH-SY5Y and GI-Li-N cells were transfected with miR-338-3p mimics or ASO. The expression level of miR-338-3p was detected by qRT-PCR. (B and C) Cell growth curve assay was performed to investigate the effect of miR-338-3p on the proliferation of SH-SY5Y and GI-Li-N cells at indicated time points. (D) Colony formation assay of SH-SY5Y and GI-Li-N cells. (E) The effect of miR-338-3p on cell cycle progression. The indicated percentages are the average of three independent experiments, $^{*}p < 0.05$.

3.3. miR-338-3p inhibits migration and invasion of NB cells in vitro

NB has an unexplained metastasis tendency to bone marrow, liver and non-contiguous lymph nodes [14]. Children with bone metastasis have a mortality rate greater than 90% [15]. Thus, understanding the nature of metastatic NB is of great importance. Our result showed that metastatic tumor tissues had significant decrease of miR-338-3p, implying its potential role in regulating the NB metastasis. Therefore, we conducted invasion and migration assays. Transwell assay demonstrated that overexpression of miR-338-3p reduced the invasion of SH-SY5Y cells by 47% and GI-Li-N cells by 40% when compared to the control cells. In contrast, miR-338-3p ASO increased the invasion of SH-SY5Y cells by 2.1-fold and GI-Li-N cells by 1.4-fold (Fig. 3A) when compared with ASO-NC. Wounding healing assays further confirmed that miR-338-3p suppressed NB cell migration (Fig. 3B).

3.4. PREX2a serves as a target of miR-338-3p for post-transcriptional repression

To investigate the mechanisms through which miR-338-3p induces growth arrest and represses invasion, we examined predicted overlapping target sets using four algorithms: Target-Scan Human 5.1, PicTar, mirna.org, and mirdb by microarray assay. Several interesting genes were predicted to be targets of miR-338-3p, many of which are involved in neural or cancer-associated processes (Table 1). One gene in particular, PREX2a, scored well in all algorithms used and was selected for further analysis. Inspection of the 3′-UTR of PREX2a revealed three potential miR-338-3p target sites that was closely positioned (Fig. 4A). To demonstrate that miR-338-3p directly regulates PREX2a expression, we transfected a PREX2a 3′-UTR luciferase reporter construct together with miR-338-3p mimics or ASO into SH-SY5Y cells and noticed an

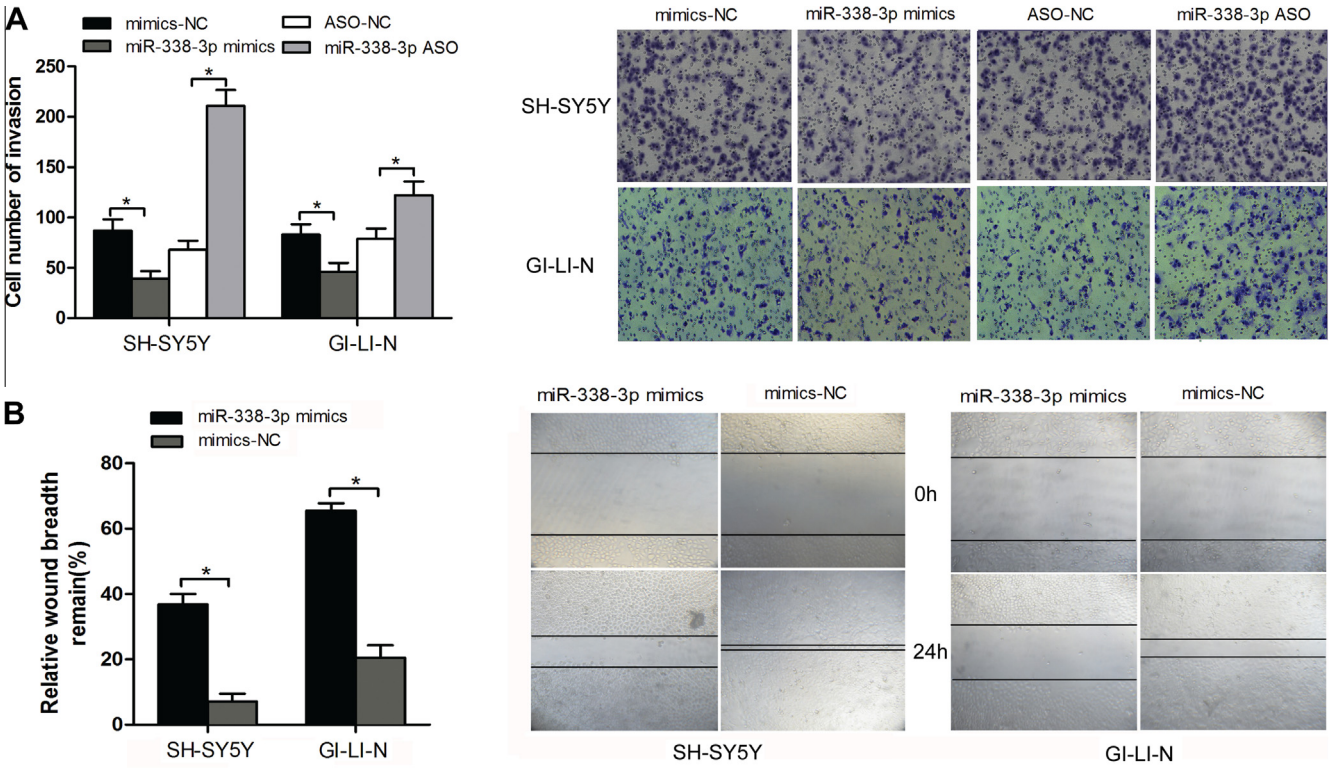

Fig. 3. Influences of miR-338-3p on migration and invasion ability in neuroblastoma cells. The changes of cell migration and invasion ability affected by transfected with miR-338-3p mimics or ASO were determined by transwell assay (A) and wound healing assay; (B) $^{*}p < 0.05$.

Table 1
mRNA expression profile detected by cDNA chip when miR-338-3p was transfected.

Gene symbol	Gene name	Accession number	SH-SY5Y	IMR-32	Target Scan	Microrna	Pictar	Mirdb
UBE2Q	Ubiquitin-conjugating enzyme E2Q family member 1	NM_017582	0.58	0.36	–	+	+	–
ZNF470	Zinc finger protein 470	NM_001001668	0.64	0.71	–	+	–	+
PREX2	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	NM_024870	0.32	0.44	+	+	–	+
STXBP1	Syntaxin binding protein 1	NM_003165	0.71	0.47	–	–	+	–
CCND1[a]	Cyclin D1	NM_053056	0.63	0.72	+	+	+	–
NR3C2	Nuclear receptor subfamily 3, group C, member 2	NM_000901	0.29	0.74	–	–	+	–
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	NM_021627	0.61	0.78	–	+	–	+
EPAS1	Endothelial PAS domain protein 1	NM_001430	0.65	0.49	–	–	–	+
KIAA1456	Putative methyltransferase KIAA1456	NM_001099677	0.39	0.68	+	–	–	+

[a] It has been identified as miR-338-3p target by Fu X et al. [22].

Table 2
The oligonucleotides used in this work.

miR-338-3p	
RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCAACAAAA
Forward	TGCGGTCCAGCATCAGTGAT
Reverse	CCAGTGCAGGGTCCGAGGT
U6	
RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG
U6 Forward	TGCGGGTGCTCGCTTCGGCAGC
U6 reverse	CCAGTGCAGGGTCCGAGGT

60% reduction and 1.8-fold increase in luciferase activity compared to cells transfected with the control. Conversely, no reduction or increase in luciferase activity was detected with the miR-338-3p seed-mutant PREX2a 3′-UTR reporter construct (Fig. 4B), demonstrating that the introduced target site mutations abolished the ability of miR-338-3p to regulate the PREX2a 3′-UTR in these settings. Taken together, these data suggests that PREX2a expression may be negatively regulated via miR-338-3p 3′-UTR miRNA binding sites. To assess miR-338-3p-mediated repression of endogenous PREX2a, we overexpressed miR-338-3p using mimics in both SH-SY5Y and GI-Li-N cells. Ectopic expression of miR-338-3p resulted in a substantial down-regulation (70% in SH-SY5Y and 65% in GI-Li-N cells) of PREX2a protein levels, while miR-338-3p ASO lead to an increase of the PREX2 protein levels (2.4-fold and 1.7-fold, seperatively), we also confirmed the results in another cell line IMR-32 (40% decrease and 2.1-fold increase) (Fig. 4C), indicating that miR-338-3p can directly down-regulate the PREX2a expression.

3.5. Knockdown of PREX2a represses proliferation, migration and invasion of NB cells

It has been demonstrated that PREX2a can decrease PTEN lipid phosphatase activity, as a consequence, leading to an increase activity of the kinase AKT to promote cellular growth, thereby contributing to oncogenesis. However, the mechanism has not been identified in neuroblastoma cells. Therefore, specific siRNA targeting PREX2a was transfected into SH-SY5Y and GI-Li-N cells and Western blotting showed PREX2a siRNA caused a significant reduction of PREX2a protein levels in SH-SY5Y and GI-Li-N cells (Fig. 5A). Cells with reduced levels of PREX2a had a significant increase of PTEN activity and decrease on the phosphorylation of AKT (Fig. 5A and B), indicating PREX2a can affect NB cells function through PREX2a/PTEN/AKT pathway. In order to further shed light on the function of PREX2a in vitro, we undertook colony formation, cell invasion and wound healing assays. Cells with reduced levels of PREX2a had a decreased rate in colony formation rate compared to the negative control cells (Fig. 5C). Furthermore, cells expressing reduced PREX2a significantly decreased invasion ability of tumor cells through Matrigel (Fig. 5D) and the rate of migration of cells into a denuded area of confluent monolayer (Fig. 5E). All these findings showed that knockdown of PREX2a inhibited NB cell growth, migration and invasion.

3.6. Role of miR-338-3p in PREX2a-mediated NB cell growth

We have demonstrated that miR-338-3p can directly and negatively regulate PREX2a expression, therefore, we aimed to explore whether miR-338-3p repressed NB cell growth, invasion was

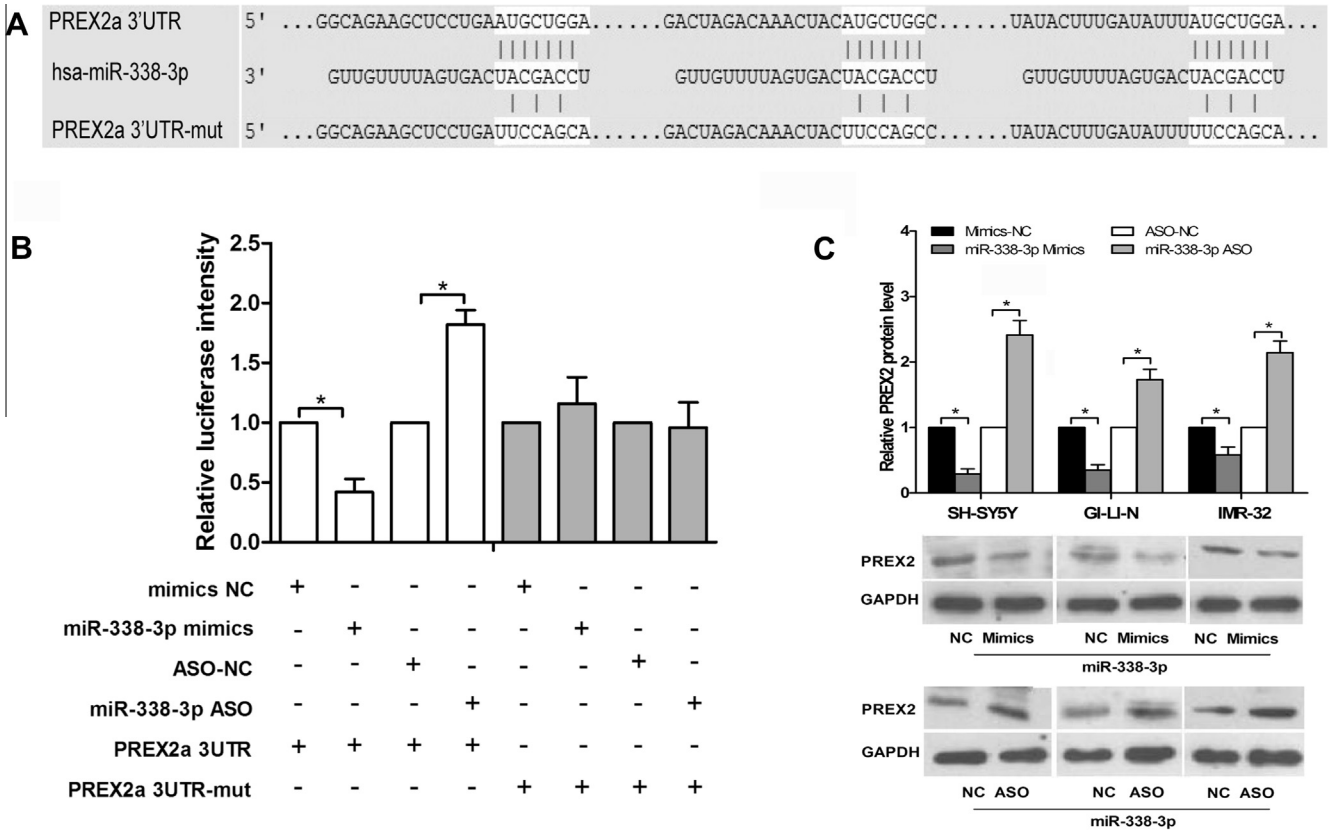

Fig. 4. miR-338-3p directly targets PREX2a and inhibits its expression. (A) A schematic of the bioinformatics predicted seed region in the 3′UTR of PREX2a as well as mutated 3′UTR used in this study is shown above. (B) Effect of miR-338-3p on the luciferase activity of PREX2a-3′UTR and PREX2a-3′UTR-mut. For 3UTR reporter assays, cells were co-transfected with miR-338-3p mimics or ASO and PREX2a-3UTR or 3UTR-mut, then harvested for lysis of cells 48 h after transfection. (C) Western blot was used to detect the PREX2a protein.

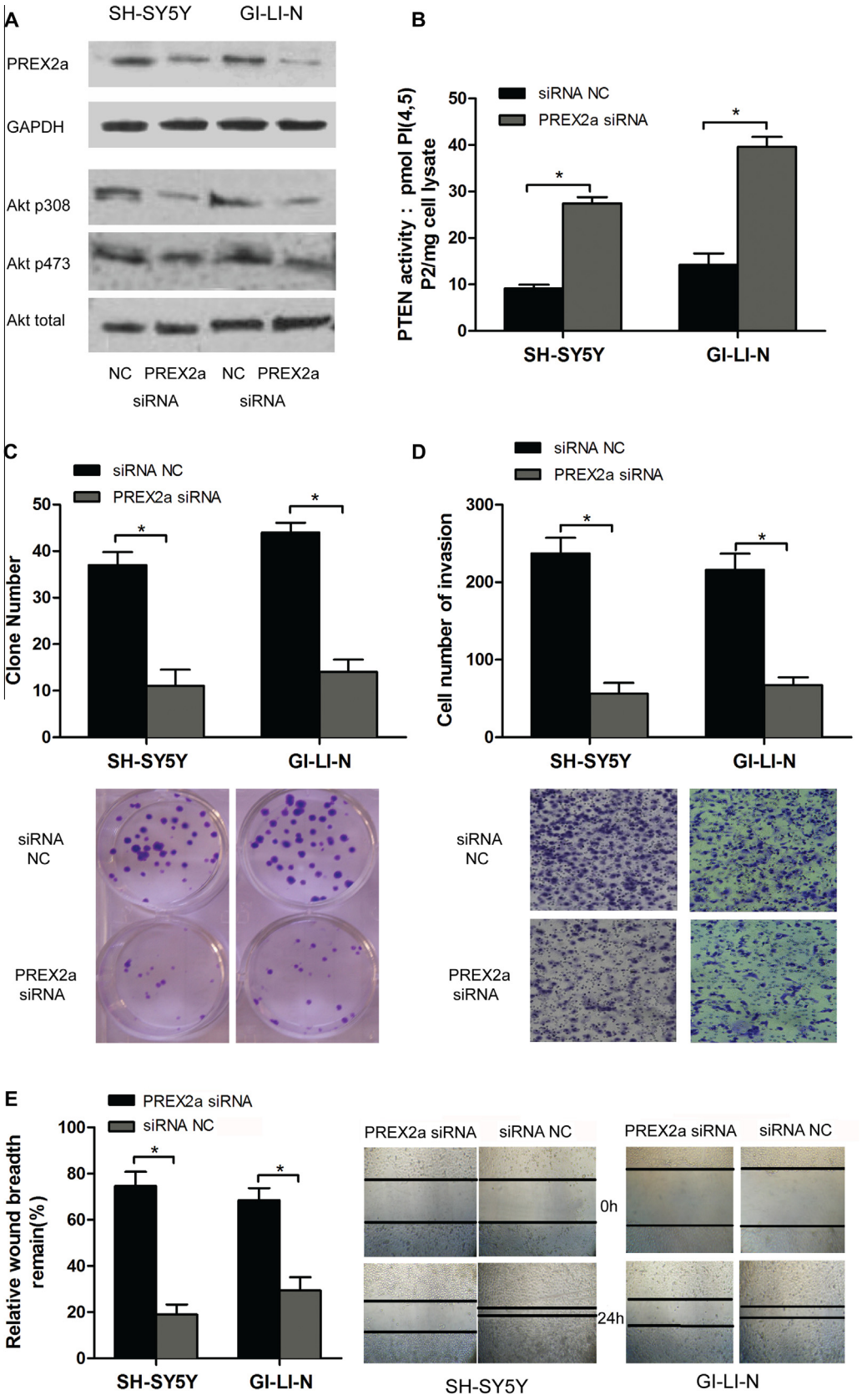

Fig. 5. Knockdown of PREX2a on NB cells proliferation, invasion and migration. (A) Western blot was used to detect the effect of PREX2a siRNA plasmid on PREX2a protein level. (B) The activity of PTEN was evaluated by ELISA. Knockdown the expression of PREX2a suppressed the proliferation; (C) invasion; (D) and migration; (E) of indicated cells.

mediated by PREX2a. We co-transfected miR-338-3p ASO and PREX2a siRNA into SH-SY5Y and GI-Li-N cells and evaluated whether the effects caused by blocking miR-338-3p can be aborted by the knockdown of PREX2a. As shown in Fig. 6A, the induction of PREX2a expression caused by miR-338-3p ASO can be partly rescued by the reduction of PREX2a by transfection of PREX2a siRNA. The colony formation assay indicated that inhibition of PREX2a abrogated miR-338-3p ASO mediated SH-SY5Y and GI-Li-N cells growth (Fig. 6B). We further examined the cell metastasis alteration in this process and found that transfection of SH-SY5Y and GI-Li-N cells with PREX2a siRNA, but not siRNA NC, ablated the miR-338-3p ASO induced cell invasion (Fig. 6C). These results suggested that miR-338-3p-repressed NB cell growth, invasion was mediated by down-regulation of PREX2a.

4. Discussion

Over the past decades, researchers have indentified numerous genes contribute to NB growth, invasion and migration. The dysregulated expression of miRNAs have been found to correlate with these phenotypes, thus reinforcing the importance of miRNA biology in NB-associated tumorigenesis. Researchers found miR-34a induced cell cycle arrest and subsequent apoptosis activation in NB cells [16]. The overexpression of miR-128 in SH-SY5Y cells can increase cell number, proliferation, with a remarkable upregulation of Bcl2, an important anti-apoptosis protein that inhibits caspase activity [17]. Metastasis is a major cause of NB-related death and patients with NB have a tendency to develop malignant lesions outside the primary tumor. It has been identified that miR-29-a/b was overexpressed in metastatic neuroblastoma and two of this miRNAs predicted targets were CASP8 and integrins, the absence of which have been implicated in neuroblastoma metastasis in vivo [18]. Other related miRNAs associated with NB metastasis include mir-10b, miR-335 and let-7 miRNA family [19,20], etc.

Published data have indicated that the proliferative potential of colorectal carcinoma cells was suppressed after miR-338-3p overexpression, due to miR-338-3p suppressed cell cycle at G0/G1 phase and induced apoptosis [21]. In this study, we identified miR-338-3p could suppress NB cells (SH-SY5Y and GI-Li-N) growth, invasion and migration using cell growth curve, colony formation, FACS, as well as transwell and wound healing assays, further demonstrating the tumor suppressor role of miR-338-3p.

To date, the mechanism of miR-338-3p function in neuroblastoma has not been investigated, although several targets have been identified including CyclinD1, Runx2, smoothened with a key role affecting cell cycle, differentiation and invasion [12,22,23]. Here, We identified PREX2a as a novel target of miR-338-3p. PREX2a can directly interact with PTEN to inactivate its lipid phosphatase activity, leading to accumulation of PIP3 and as a consequence increasing phosphorylation of threonine-308 AKT and serine-473 AKT, which promotes cellular survival, cell cycle progression, and growth, thereby contributing to oncogenesis. To test whether endogenous PREX2a influenced the PI3K pathway in NB cells, we transfected SH-SY5Y and GI-Li-N cells with PREX2a siRNA and displayed increased activity of PTEN and decreased amounts of pAKT. It also reduced cell proliferation, invasion and migration after introduction of PREX2a siRNAs.

As previously indicated miR-338-3p is transcribed from the intron 8 of apoptosis-associated tyrosine kinase (AATK) gene, located on chromosome 17q25, playing a critical role in promoting cell death, neuronal differentiation and neurite extension [24,25]. A point that deserves further investigation is the potential contribution of miR-338-p host gene ATKK in the regulation of neuroblastoma. Cécile Jacovetti [26] found that during gestation in rat islets under insulin resistance conditions, the decrease in AATK levels parallels that of miR-338-3p, indicating a cooperative action of miR-338-3p and its hosting gene. Since Cdk5 activator p35 was found to bind and phosphorylate AATK [27], and Cdk5 has been identified exerting a key role in promoting neuronal survival by regulating Akt activity through neuregulin/PI3K signalling pathway [28], therefore we speculate that ATKK may function through Cdk5/ATKK/Akt pathway to cooperate with miR-338-3p exerting its regulation effect in neuroblastoma. However, the potential mechanism needs to be further investigated.

In conclusion, this present study first identified PREX2a was directly and negatively regulated by miR-338-3p, which was downregulated in NB metastasis tumor tissues and function as tumor suppressor, as we found miR-338-3p can inhibit SH-SY5Y and GI-Li-N cells proliferation, migration and invasion. Further we identified that PREX2a exerted its function through PTEN/Akt pathway,

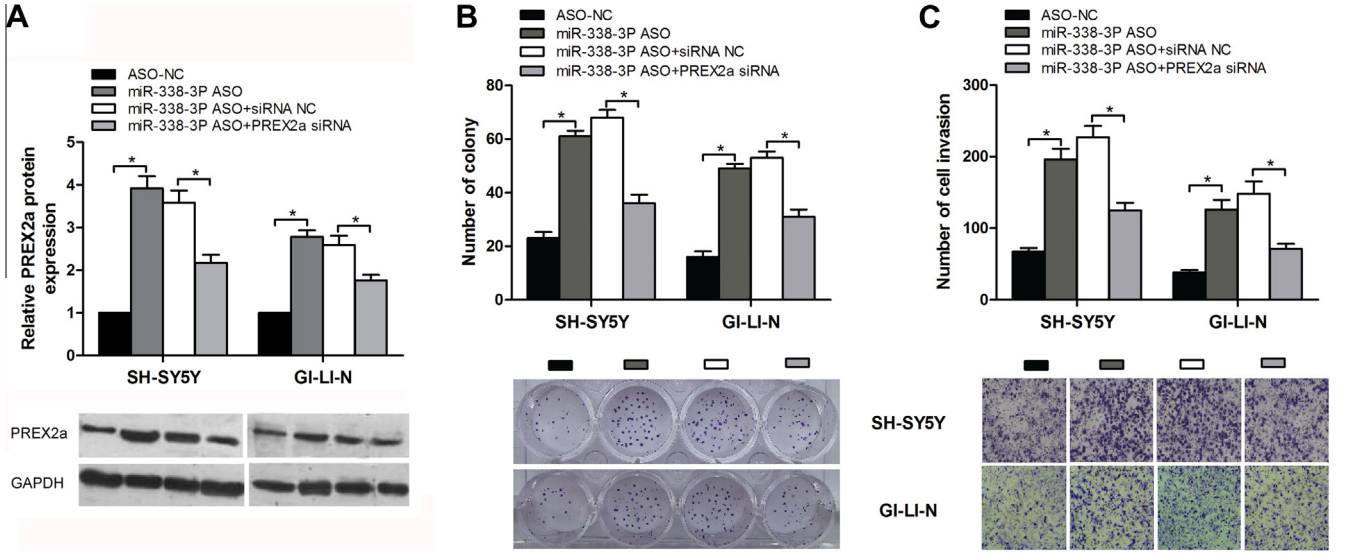

Fig. 6. MiR-338-3p induced NB cell growth and invasion was mediated by PREX2a. SH-SY5Y and GI-Li-N cells were transfected with ASO-NC, miR-338-3p ASO, or cotransfected with miR-338-3p ASO and siRNA NC, or miR-338-3p ASO and PREX2a siRNA. PREX2a protein level was detectd by western blot analysis; (A) Colony formation assay was used to detected cell proliferation activity; (B) Transwell assay was used to detected the cells invasion ability; (C) $^{*}p < 0.05$.

therefore miR-338-3p may regulate PTEN/Akt pathway by suppressing PREX2a expression. These findings may have potentially relevant therapeutic implications in NB diagnosis.

Acknowledgements

This work was supported by National Natural Science Foundation of China (General Program, No. 81272986) and Natural Science Foundation of Shangdong Province of China (Key Program, No. ZR2011HZ002).

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