ORIGINAL ARTICLE

MicroRNA-26a Promotes Tumor Growth and Angiogenesis in Glioma by Directly Targeting Prohibitin

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SUMMARY

Backgrounds and Aims: Glioma accounts for the majority of primary malignant brain tumors in adults. Upregulation of microRNA-26a (miR-26a) has been observed in glioma. However, the biological function and molecular mechanism of miR-26a in glioma remain to be elucidated. **Methods:** Glioma cells stably overexpressing or down-expressing miR-26a were analyzed for both *in vitro* and *in vivo* biological functions. Novel target of miR-26a was identified by bioinformatics searching and molecular biological assays. Glioma specimens and normal brain tissues were analyzed for both expression levels of miR-26a and its target. **Results:** Forced expression of miR-26a in glioma cells significantly increased both growth rate and colony formation *in vitro* and tumor growth and angiogenesis *in vivo*, while reduced expression of miR-26a played opposite roles. MiR-26a directly targeted prohibitin (PHB) whose expression levels were downregulated in glioma specimens. The levels of miR-26a were inversely correlated with PHB expression levels in glioma samples and strongly correlated with clinical WHO grades of glioma. **Conclusion:** These results reveal that miR-26a regulates PHB and promotes glioma progression both *in vitro* and *in vivo* and that miR-26a and its target PHB are associated with glioma development, which can be helpful in developing microRNA-based treatment for glioma in the future.

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Introduction

Glioma is the most prevalent type of primary brain tumors in adult population [1]. Despite modern surgical and medical treatments, the median survival of glioblastoma patients remains only 14.6 months [2]. Therefore, further understanding of molecular mechanism and the finding of novel biomarkers and improved therapies of glioma are needed. MicroRNAs (miRNAs) are a class of small (~22 nucleotides) nonprotein-coding RNAs that regulate a wide variety of genes at the post-transcriptional level by binding loosely complementary sequences of 3'-untranslated regions (3'-UTR) of target mRNAs [3,4]. There are about 1000 miRNAs in

human genomes, which are believed to regulate more than one-third of human mRNA transcripts [5], thus participating in a variety of cellular functions including cell apoptosis, cell proliferation, neural development, and stem cell differentiation [6,7]. Growing evidence has shown that a large amount of human miRNAs are located in cancer-related genomic regions and function as tumor promoters or tumor suppressors depending on their targets [8–10], indicating the importance of discovering targets of miRNAs in cancer development. A recent study showed that miR-26a is amplified in high-grade glioma tissues and promotes tumorigenesis of glioma in a murine mode system by targeting PTEN [11]. Meanwhile, Kim et al. also demonstrated that miR-26a promoted

glioblastoma cell growth *in vitro* by decreasing PTEN, RB1, and MAP3K2/MEKK2 protein expression [12]. These data suggested that miR-26a may play as a tumor-promoting gene in glioma. However, it has been demonstrated that miR-26a served as tumor suppressor in other kinds of cancers such as liver cancer, breast cancer, and nasopharyngeal cancer [13–16]. These results suggest that miR-26a can be a glioma-specific upregulated miRNA and a potential target for treatment distinguished from other kinds of cancers. However, the role and mechanism of miR-26a in regulating glioma tumorigenesis and angiogenesis remains to be elucidated.

Prohibitin (PHB), a potential tumor suppressor, is a highly conserved and ubiquitously expressed protein, which displays diverse cellular functions [17,18]. PHB is localized to cell membrane and mitochondrial inner membrane, as well as cytoplasm or nucleus depending upon the cell types and context [19–22]. Previous studies have demonstrated that PHB plays an active role in modulating gene transcription [18]. PHB colocalizes with E2F1, retinoblastoma protein (Rb) and p53 in the nucleus and regulates E2F1- and p53-mediated transcriptional activities [22,23]. Recent discoveries have shown that PHB is involved in phosphatidylinositol-3 kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) signaling cascades, the versatile signaling processes in modulating cell metabolism, proliferation, and development [24,25]. It has been observed that PHB is downregulated in glioma [26], but how PHB is involved in glioma progression still needs to be clarified.

The aim of this study is to determine the novel target gene of miR-26a for further understanding the role and mechanism of miR-26a in human glioma. We examined the expression of miR-26a in human glioma tissues and tested its effect on tumor growth and angiogenesis. We validated *PHB* as a novel target gene of miR-26a and found the inverse correlation between miR-26a and PHB in glioma specimens. The possible mechanism and relative signaling molecules in miR-26a-regulating glioma were also addressed. Our findings will be useful to better understand the mechanism of human glioma development and identify miR-26a as glioma-specific miRNA and a potential target for glioma treatment.

Methods

Clinical Specimens

Human glioma specimens ($n = 27$) and normal brain tissues ($n = 9$) were obtained from patients undergoing standard surgical procedure in the Department of Neurosurgery of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. Samples were collected with informed consents of patients and frozen in liquid nitrogen until the analysis. All experiments were approved by the Ethics Committee of Nanjing Medical University.

Cell Culture and Antibodies

Human U87 and HEK-293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Cells were cultured in humidified 5% CO₂ incubator at 37°C. Antibodies against PHB, p-

AKT (Ser473), total AKT, p-ERK1/2, and total-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against HIF-1 α was purchased from BD Biosciences (Franklin Lakes, NJ, USA), and antibodies against β -actin and GAPDH were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, California USA), respectively.

Lentiviral Packaging and Stable Cell Line Establishment

Lentiviruses carrying miR-26a, miRNA-negative control (miR-NC), miR-26a inhibitor (miR-26a-I) or miRNA inhibitor-negative control (miR-NC-I) were packaged using lentiviral packaging kit in HEK-293T cells following the manufacturer's instructions (Open Biosystems, AL, USA). Stable cell lines, U87/miR-26a, U87/miR-NC, U87/miR-26a-I, and U87/miR-NC-I, were established by infecting U87 cells with the virus soups and screened by puromycin (Sigma, MI, USA).

Cell Proliferation and Anchorage-independent Colony Formation assay

For cell proliferation, cells were seeded in 96-well plates at confluence of 1000 cells/well overnight and measured using a CCK8 kit (Dojindo Laboratories, Kumamoto, Japan) at different indicated time points according to the manufacturer's instruction. Data were from three separate experiments with six replications each time. For anchorage-independent colony formation assay, 1 mL of 0.8% solidified SeaPlaque agarose (BMA, ME, USA) was added to each well of 6-well plates; 5000 cells were mixed with 1 mL of 0.4% SeaPlaque and added onto the top of the well. After about 2 weeks, colonies were fixed with methanol for 15 min and stained with 0.1% crystal violet. Colonies with diameter more than 1.5 mm were counted. Experiments were performed in triplicate for three times.

Dual-luciferase Reporter Assay

For dual-luciferase assay, PHB 3'-UTR containing predicted miR-26a seed-matching sites (wide type, WT) and corresponding mutant sites (mut) were amplified by PCR from cDNA of U87 cells and inserted into pMIR-REPORTER vector (Ambion, CA, USA). Primers used for reporter constructions were shown in Supplemental Table S1. Wild-type and mutant constructs were confirmed by sequencing. U87 cells were seeded in a 24-well plate and cotransfected with either wild-type (WT) or mutant-type (mut) luciferase reporter plasmids containing PHB-3'UTR, pGL4.74 vector (Ambion), and equal amounts of pre-miR-26a, pre-miR-NC, pre-miR-26a inhibitor, or pre-miR-NC inhibitor (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. To determine the effects of miR-26a on transcriptional activation of VEGF, VEGF reporter plasmid pMAP11-WT or pMAP11-mut [27] was cotransfected into U87 cells with pGL4.74 vector and equal amounts of pre-miR-26a, pre-miR-NC, pre-miR-26a inhibitor, or pre-miR-NC inhibitor. Luciferase activities were measured 24 h after transfection using a dual luciferase assay kit (Promega, WI, USA).

Experiments were performed in triplicate with three independent replicates.

RNA Isolation and Quantitative Real-time PCR (qPCR)

Total RNAs of cells or human tissues were extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. To determine the mRNA levels of VEGF, total RNAs were reversely transcribed by oligo dT primer using PrimeScript RT Reagent Kit (Takara, Dalian, China). Housekeeping gene GAPDH was used as an internal control. To measure the expression levels of miR-26a, stem-loop-specific primer method was used as described before [28,29]. The expression of U6 was used as an endogenous control. The cDNAs were amplified by qPCR using SYBR Premix DimerEraser (Takara, Dalian, China) on a 7900HT system. Relative fold changes in the expression of the target gene transcripts were determined using the comparative cycle threshold method ($2^{-\Delta CT}$). Primers used for qPCR were shown in Supplemental Table S1.

Protein Extraction and Immunoblotting

Cells or grounded tissues were lysed on ice for 30 min in RIPA buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and 2 mM pepstatin A. The lysates were centrifuged at 12,000 rpm 4°C for 15 min, and the supernatants were collected and protein concentration was determined using BCA assay (Beyotime Institute of Biotechnology, Jiangsu, China). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes in transfer buffer (20 mM Tris, 150 mM glycine, and 20% (v/v) methanol). Membranes were blocked with 5% nonfat dry milk and incubated with antibodies against PHB, phospho-AKT (p-AKT, Ser-473), phospho-ERK1/2 (p-ERK1/2), total AKT, total ERK, HIF-1 α , GAPDH, or β -actin. The protein bands were probed with secondary antibody IgG conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate Kits (Thermo Scientific, MA, USA).

PHB siRNA constructs

2'-O-methylation modified and highly purified siRNA duplexes against PHB were used as previously described [30,31] and obtained from GenePharma (Shanghai, China). The VEGF levels influenced by PHB siRNA were assayed by RT-PCR as described previously [32]. Primers used for RT-PCR were seen in Supplemental Table S1.

The siRNA sequences were as follows:

PHB siRNA-1 (Si-1): 5'-GCGACGACCCUACAGAGCG-3';

PHB siRNA-2 (Si-2): 5'-GUUUGGCCUGGCCUAGCU-3'.

Negative control sequence (SiNC): 5'-UUCUCCGAACGUGU-CACGU-3'.

Animal Studies

Male BALB/c nude mice aged from 4 to 5 weeks were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China) and maintained in special pathogen-free (SPF) condition for 1 week. Animal handling and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Experimental Ethics Committee of Nanjing Medical University. U87/miR-NC (negative control), U87/miR-26a, and U87/miR-26a-I cells at 4×10^6 were suspended in 150 μ L of FBS-free DMEM medium and subcutaneously injected into each side of the posterior flanks nude mice. For each group, four mice were used to inoculate eight xenografts. Tumor sizes were measured using vernier caliper every 2 days when the tumors were apparently seen and tumor volume was calculated according to the following formula: volume = $0.5 \times \text{Length} \times \text{Width}^2$. Mice were killed and tumors were dissected 24 days after implantation. Total proteins and RNAs were extracted for immunoblotting and mRNA expression assays, respectively. Tumors were formalin-fixed, paraffin-embedded, and sectioned at 5 μ m for CD31 (Abcam, Cambridge, UK) immunohistochemical staining under the standard procedure as described before [33].

Statistical Analysis

Data in this study were represented as means $\pm$ SE from at least three independent experiments unless indicated. Student's unpaired *t*-test was used for comparison between the two groups. For human tissue samples, miR-26a and PHB expression levels in normal brain and glioma tissues at different WHO grade were explored by Kruskal-Wallis test, respectively. Protein levels of PHB between normal brain and glioma tissues were conducted by Mann-Whitney *U* test. The correlation between miR-26a expression and PHB levels in glioma tissues was analyzed using Spearman's rank test. Values were considered significantly different when $P < 0.05$.

Results

MiR-26a Expression is Upregulated in Glioma and Correlates with Clinical WHO Grades

To determine miR-26a expression levels in glioma specimens, qPCR based on stem-loop primer method was used to detect miR-26a levels in a cohort of brain tissues including 27 glioma specimens (10 WHO Grade II-III and 17 WHO Grade III-IV) and 9 normal brain tissues from noncancerous patients. The results showed that the expression levels of miR-26a were overall increased in cancers than those in noncancerous tissues (Figure 1A). Moreover, miR-26a was found to be much more upregulated in WHO Grade III-IV than that in WHO Grade II-III, indicating that miR-26a expression was significantly correlated with the malignant degree of glioma (Figure 1B). The trend of miR-26a up-expression in human primary glioma tissues indicates the possibility to develop miR-26a as a biomarker for glioma diagnosis and treatment.

Figure 1 MiR-26a was upregulated in human glioma samples and correlated with WHO grade of glioma. (A) Relative expression levels of miR-26a in 27 glioma specimens and 9 normal brain tissues were analyzed by qPCR using stem-loop primer method. Abundance of miRNAs was normalized by the expression levels of U6. (B) Relationship between relative expression levels of miR-26a and clinical grade of glioma. Kruskal–Wallis test showed that miR-26a expression levels were correlated with clinical WHO grade of glioma ($P < 0.0001$).

MiR-26a Promotes Glioma Cell Proliferation and Colony Formation

To investigate the biological function of miR-26a in glioma cells, we established stable cell lines overexpressing or suppressing miR-26a. RT-PCR assay showed that the mature miR-26a expression was increased in U87/miR-26a cells and decreased in U87/miR-26a-I cells, confirming that stable cell lines were successfully established (data not shown). Overexpression of miR-26a significantly enhanced the cell growth rate of U87 cells, while inhibition of miR-26a markedly reduced it when compared to miR-NC or miR-NC-I control cells (Figure 2A). To illustrate the effect of miR-26a on the tumorigenic capacity *in vitro*, anchorage-independent colony formation assay was performed. Because the cell growth rate was similar in U87/miR-NC and U87/miR-NC-I, U87/miR-NC was used as a control alone. MiR-26a extremely increased the number of colonies by 50%, while miR-26a inhibitor reduced the number to less than 50% compared to the control (Figure 2B), demonstrating that miR-26a plays a vital role in cancer cell growth *in vitro*.

PHB is a Direct Target of MiR-26a and Inversely Correlates with MiR-26a Expression in Glioma

To predict the target genes of miR-26, we searched TargetSearch, a self-designed bioinformatics algorithm [34]. We found that PHB, a tumor suppressor gene, could be a potential target of miR-26a. The putative binding sites between miR-26a and 3'-UTR of PHB were totally conserved in human, chimpanzee, rhesus, mouse, dog, and cow (Table 1). To explore whether miR-26a directly targets the 3'-UTR of PHB, we constructed luciferase reporter plasmids containing the putative wild-type binding sites (WT) and seed sequence mutant sites (mut) at 3'-UTR of PHB (Figure 3A, top) and verified by sequencing. Luciferase assay showed that the luciferase activity of wild-type PHB 3'-UTR reporter was inhibited by 30% in U87 cells overexpressing miR-26a. On the contrary, inhibition of miR-26a by its inhibitor increased the luciferase activity of wild-type reporter by nearly 40% due to the reduction of the endogenous level of miR-26a in U87 cells (Figure 3A, bottom). Neither miR-26a nor miR-26a inhibitor regulated the luciferase activities of mutant reporters. This result suggested that miR-26a regulates PHB transcriptional

Figure 2 MiR-26a increased glioma cell proliferation and anchorage-independent colony formation. (A) Cell proliferation assay of U87 cells overexpressing miRNA-negative control of precursor (miR-NC), miR-26a precursor, negative control of miRNA inhibitor (miR-NC-I), and miR-26a inhibitor using a CCK-8 kit. Data were presented as means $\pm$ SE from three independent experiments performed in sextuple. (B) Anchorage-independent colony formation assay of U87 cells overexpressing miR-NC, miR-26a, and miR-26a-I. * and ** indicate significant difference between miR-26a and miR-NC group ($P < 0.05$ and $P < 0.001$); # and ## indicate significant differences between miR-26a-I and control group ($P < 0.05$ and $P < 0.001$).

Table 1 Conserved seed-matching sequence between miR-26a and 3'-UTR of PHB in different species

Species	3'-UTR of PHB
Chimpanzee	5'-CCCAGUGGAAUCCA ACUUGA AGGAUUG-3'
Rhesus	5'-CCCAGUGGAAUCCAG CUUGA AGGAUUG-3'
Mouse	5'-CCCAUUGGAAUCCAG CUUGA AGGAUUG-3'
Dog	5'-CCCACGGAAUCCAG CUUGA AGGAUUG-3'
Cow	5'-CCCGCGGAUCCAG CUUGA AGGAUUG-3'
Human	5'-CCCAGUGGAAUCCA ACUUGA AGGAUUG-3'
has-miR-26a	3'-UCGGAUAGGACCUAA UGAACUU -5'

activity by binding the seed sequence at its 3'-UTR. Further study by immunoblotting assay showed that forced expression of miR-26a tremendously inhibited the expression levels of PHB by 70%, while blockade of endogenous expression of miR-26a adversely upregulated PHB levels to 4-fold of control (Figure 3B). These

results indicated that miR-26a directly targets PHB by binding to its seed sequence at 3'-UTR.

To determine the expression levels of PHB in clinical glioma specimens and normal brain tissues, immunoblotting assay was performed to detect PHB expression in tissues, then analyzed by densitometric measurement and normalized to the β -actin expression levels. Compared to the normal brain tissues ($n = 9$), glioma tissues ($n = 27$) showed lower protein levels of PHB ($P = 0.001$, Figure 3C). And for the first time, we found that PHB expression levels were correlated with WHO grades of glioma, in which PHB expression was much lower in WHO Grade III-IV than that in WHO Grade II-III ($P = 0.0006$, Figures 3D,E). Furthermore, Spearman's rank correlation analysis showed an inverse correlation between the expression levels of PHB and miR-26a in human glioma specimens ($r = -0.439$, $P = 0.022$, Figure 3F). These results confirm the negative correlation between the expression levels of miR-26 and its target PHB in human glioma tissues.

Figure 3 Prohibitin (PHB) was a direct target of miR-26a associated with glioma development. (A) Scheme of construction of reporter plasmids containing the putative seed-matching sites (wild type, bold italic) and mutant sites (bold italic, with underline) of miR-26a on human PHB mRNA (up panel). Constructs of wild-type and mutant PHB 3'-UTR reporters were verified by sequencing. Relative luciferase activities of PHB 3'-UTR reporters by overexpressing or inhibiting miR-26a levels were determined by dual-luciferase reporter assay. Data were presented as means $\pm$ SE from three independent experiments with triple experiments. * indicates significant difference between the groups of miR-26a and miR-NC ($P < 0.05$); # indicates significant difference between the groups of miR-26a-I and miR-NC-I ($P < 0.05$). (B) MiR-26a suppressed PHB protein expression. Whole protein extracts were subjected to immunoblotting analysis for detecting the levels of PHB protein (note: the exposure times of two panels are different, and the signals between them cannot be used for comparison). The expression levels of GAPDH were used as internal control. (C) Relative expression levels of PHB in normal and glioma tissues. The relationship of PHB expression levels between glioma and normal brain tissues was analyzed by Mann-Whitney test ($P = 0.0001$). (D) Correlation between PHB expression levels and WHO grades of glioma. The relationship was analyzed by Kruskal-Wallis test ($P = 0.0006$). (E) PHB protein levels were downregulated in glioma specimens compared to normal brain tissues in representative tissues. (F) The reverse correlation of miR-26a and PHB levels in glioma specimens. Scatter plots and Spearman analysis showed that PHB expression levels were inversely correlated with miR-26a expression levels in glioma tissues (Spearman $r = -0.439$, $P = 0.022$).

MiR-26a Activates AKT and ERK1/2 Signaling Pathways and Regulates HIF-1 and VEGF Expression via PHB

As already known, the PI3K/AKT and MAPK/ERK signaling cascades are frequently hyperactivated and play critical roles in controlling cell proliferation, angiogenesis, and development. PHB is a highly conserved and widely expressed protein that is involved in PI3K/AKT and MAPK/ERK signaling pathways [24]. To explore the effect of PHB on downstream signaling molecules in glioma, PHB was knocked down by transfecting U87 cells with PHB siRNA duplexes (Si-1 and Si-2) or negative control siRNA (SiNC) for 48 h. Knockdown of PHB subsequently increased the phosphory-

lation of AKT and ERK1/2 and expression of HIF-1 α (Figure 4A). Vascular endothelial growth factor (VEGF) is one of the most important factors in regulating tumorigenesis and angiogenesis. Inhibition of PHB also increased VEGF expression at both mRNA and protein levels (Figures 4B,C).

Given the fact that PHB is the target of miR-26a, we hypothesize that miR-26a regulates AKT and ERK1/2 pathways by targeting PHB. As expected, forced expression of miR-26a induced AKT and ERK1/2 activation and increased the expression of HIF-1 α , an important downstream regulator of PI3K/AKT and MAPK/ERK pathways in modulating tumorigenesis and angiogenesis (Figure 4D). On the other hand, reduction of endogenous levels of miR-26a in U87 cells inhibited AKT and ERK1/2 activation and

Figure 4 MiR-26a regulated AKT and ERK1/2 signaling pathways and the expression of HIF-1 α and VEGF via PHB. **(A, B)** RNAi-mediated knockdown of PHB activated AKT and ERK1/2 signaling pathways and promoted the expression of HIF-1 α and VEGF. **(A)** Cells were transfected with PHB siRNAs (Si-1 and Si-2) for 72 h. Proteins from control (siNC) and PHB knockdown cells were analyzed by immunoblotting to detect the levels of PHB, phospho-AKT (p-AKT), total AKT (t-AKT), phospho-ERK1/2 (p-ERK1/2), total ERK1/2 (t-ERK1/2), HIF-1 α , and β -actin. **(B)** Semiquantitative RT-PCR analysis of VEGF expression levels in control and PHB knockdown cells. **(C)** ELISA analysis of VEGF expression in control and PHB knockdown cells. ** indicates significant difference between control (siNC) and PHB siRNA groups ($P < 0.001$). **(D)** Cells were transfected with miR-NC, miR-26a, miR-NC-I, and miR-26a-I as above. The expression levels of p-AKT, t-AKT, p-ERK1/2, t-ERK1/2, HIF-1 α , and β -actin were tested by immunoblotting. **(E)** Cells were treated as above. Total RNAs were subjected to qRT-PCR to determine the VEGF mRNA levels. The relative VEGF expression levels were normalized to that of GAPDH. **(F)** MiR-26a modulated VEGF expression through HIF-1 α . Reporter plasmids containing a fragment of VEGF promoter with HIF-1 α modulating regions (WT) or mutant sites (mut) were cotransfected with miR-NC, miR-26a, or miR-NC-I, miR-26a-I, to perform reporter assay. * indicates significant difference between miR-26a and miR-NC group ($P < 0.05$); # indicates significant difference between miR-26a-I and miR-NC-I group ($P < 0.05$).

HIF-1 α expression (Figure 4D), indicating that miR-26 induces AKT and ERK1/2 activation, thus upregulating HIF-1 α expression. VEGF is one of the downstream molecules of HIF-1 in regulating angiogenesis. Here, we observed elevated mRNA level of VEGF in response to overexpression of miR-26a and decreased VEGF mRNA level in response to miR-26a inhibitor in U87 cells (Figure 4E), indicating that miR-26a plays important role in regulating VEGF expression. To examine whether HIF-1 α is required for miR-26a-regulated VEGF expression, U87 cells were cotransfected with pre-miR-26a or pre-miR-NC, pMAP11-WT containing a functional promoter fragment with HIF-1 α binding sites and pGL4.74 plasmids. Luciferase activities showed that miR-26a markedly enhanced VEGF transcriptional activation, while blockade of endogenous miR-26a expression by its inhibitor decreased VEGF transcriptional activation (Figure 4F). To determine whether HIF-1 α is necessary in miR-26a-induced VEGF transcriptional activation, we employed pMAP11-mut VEGF promoter reporter containing a 3-bp mutation at the HIF-1 α binding site and luciferase activities were performed as above. As expected, neither miR-26a nor miR-26a inhibitor regulated the luciferase activities of pMAP11-mut, further demonstrating that miR-26a

regulates VEGF transcriptional activation through HIF-1 α expression.

MiR-26a Increases Tumor Growth and Angiogenesis

To further investigate the role of miR-26a in tumor growth and angiogenesis *in vivo*, ectopic transplantation model of human glioma in nude mice was employed. Stable cell lines, U87/miR-NC, U87/miR-26a, and U87/miR-26a-I cells, were collected and subcutaneously injected into both posterior blanks of male BALB/c nude mice, respectively. Each group includes four mice. Because there is no difference between miR-NC and miR-NC-I treatment in cell proliferation, VEGF transcriptional activation, and relative expression of VEGF, U87/miR-NC was used as the only control for *in vivo* study. Tumor volumes were monitored every 2 days during the tumor inoculation period. Compared to the control group, the tumor size of miR-26a group was significantly increased by Day 20 ($P < 0.05$) and grew more and more quickly, while the tumor size of miR-26a-I group was still small after 24 days of inoculation (Figure 5A). Nude mice were killed 24 days after implantation

Figure 5 MiR-26a induced tumor growth *in vivo*. U87 cells stably expressing miR-NC, miR-26a, or miR-26a-I were collected. Cells were counted, suspended in FBS-free DMEM medium, and subcutaneously injected into each side of posterior flanks of nude mice ($n = 4$). (A) Growth curve of *in vivo* tumors. (B) Twenty-four days after implantation, tumors were harvested and photographed. Representative pictures from each group were shown (top, Bar = 2 mm) and tumors were weighted (bottom). (C) Total proteins were assayed by Western blotting to determine the levels of PHB, HIF-1 α , p-AKT, t-AKT, p-ERK1/2, and t-ERK1/2, respectively. Levels of GAPDH were used as internal control. (D) Total RNAs were isolated and assayed by qRT-PCR to determine the VEGF expression in tumors. (E) CD31 staining of tumor sections. Tumor sections were trimmed at 5 μ m sections and processed for immunohistochemical staining using monoclonal antibody against CD31, a specific marker of human endothelial cells. Top, representative pictures of CD31 staining. Magnification, $\times 100$ (upper) and $\times 400$ (lower). Bars, 200 and 50 μ m, respectively. Bottom, CD31-positive blood vessels were counted from 5 replicate sections of $\times 400$ magnification. Data were presented as means $\pm$ SE. * and ** indicate significant difference between miR-26a and miR-NC group ($P < 0.05$ and $P < 0.001$). # and ## indicate significant difference between miR-26a-I and miR-NC-I group ($P < 0.05$ and $P < 0.001$).

and xenografts were trimmed out. The tumor size of miR-26a group was larger than that of the control group, while the tumors from miR-26a-I group were much smaller (Figure 5B, top). Consistent with tumor size, the tumor weight of miR-26a group was 2-fold of control group, and tumors from miR-26a-I group were decreased by 90% when compared to control group (Figure 5B, bottom). In agreement with the results *in vitro*, overexpression of miR-26a suppressed PHB expression, while miR-26a inhibitor enhanced PHB expression in tumor tissues. At the same time, miR-26a activated AKT and ERK1/2 and increased the expression of HIF-1 α , while miR-26a inhibitor (miR-26a-I) played oppositely (Figure 5C). The expression levels of VEGF were increased by 35% in xenografts from miR-26a group and decreased by 40% in xenografts from miR-26a-I group, respectively (Figure 5D). Moreover, tumors from U87/miR-26a group showed an increased number of microvessels with CD31-positive staining by 50%, while the tumors from U87/miR-26a-I group showed decreased microvessels by 60% when compared to control group (Figure 5E). These results suggest that miR-26a enhances tumorigenesis and angiogenesis of human glioma in nude mice.

Discussion

Glioma is the most common primary brain tumor that diffusely infiltrates adjacent brain tissues, rendering it hardly curable [1,35]. The median survival time for patients with glioma is only beyond 1 year [2,36–38]. It has been identified that human glioma exhibits an aberrant expression profiles of miRNAs [39–42], and biological functions of many of these miRNAs in glioma have been well documented. For example, miR-7 has been shown to regulate the EGFR and AKT pathways, and its forced expression reduces proliferation and invasiveness in glioma cell [43]. MiR-10b was upregulated in glioma, and its inhibition resulted in the reduction of glioma growth *in vitro* and *in vivo* [44]. In addition, miR-21, a universally upregulated miRNA in many tumor types, was overexpressed in glioma and modulated hTERT in a STAT3-dependent manner to control glioma cell growth [45]. Preliminary studies showed that miR-26a was upregulated in glioma tissues, but the function and the molecular mechanism of miR-26a in gliomas still remain to be elucidated. Our results demonstrated an upregulation of miR-26a expression in human glioma specimens, which was related to the grade of malignancy. Moreover, miR-26a markedly increased cell growth and anchorage-independent colony formation and promoted *in vivo* tumorigenesis and angiogenesis in nude mice, whereas inhibition of miR-26a suppressed these processes, suggesting that miR-26a is a potential tumor promoter in human glioma. However, these findings were controversial with the findings in liver cancer, nasopharyngeal cancer, pancreatic cancer, or lymphoma, in which miR-26a expression levels were downregulated and acted as a tumor suppressor [46–49]. These controversies indicate that the function of miR-26a is tumor-type specific and miR-26a is a potential glioma-specific biomarker and target for glioma treatment. Some recent studies have

indicated the possible mechanism of this miR-26a expression controversy between glioma and other kinds of cancers. It was found that miR-26a expression was induced in glioma because this miRNA was localized within an amplicon at 12q which was mostly common amplified in glioma [11,12], while in other types of cancers, miR-26a was usually diminished due to hypermethylation or repressed by Myc oncogene [50,51].

Prohibitin (PHB) is a highly evolutionary conserved and widely expressed protein, which is believed to be involved in cell signaling pathways including PI3K/AKT and MAPK/ERK cascades [52–56], thus playing diverse functions such as cell cycle, senescence, and apoptosis of cancer cells [24,57]. Growing lines of evidence have unveiled PHB as a potential tumor suppressor in many kinds of cancers, for example, in liver cancer and gastric adenocarcinoma [58,59]. A two-dimensional electrophoresis (2-DE) analysis found PHB levels were downregulated in Grade III glioma tissues [26], but the function of PHB in glioma has not been clearly documented yet. For the first time, we discovered that PHB is a novel target of miR-26a by binding to the seed sequence of its 3'-UTR. In glioma cells, forced expression of miR-26a or knockdown of PHB showed similar results to activate AKT and ERK1/2 signaling pathways and increase the expression of their downstream molecules HIF-1 α and VEGF, the well-known pivotal modulators of tumorigenesis and angiogenesis [25,33,34,60]. In this study, we also found that PHB protein levels were inversely correlated with miR-26a expression in glioma specimens, suggesting that the expression balances of miR-26a and PHB might play important roles in human glioma progression. Owing to the specific expression profile of miR-26a in glioma that is different from other kinds of human cancers [15,16,46,48,49] and the low expression of PHB that is strongly associated with malignant glioblastoma, we believe that miR-26a and PHB are helpful for the diagnostic and clinical treatment of glioma.

Taken together, our results suggest that miR-26a functions as a tumor promoter of human glioma by negatively regulating PHB expression at the post-transcriptional level via directly binding to the specific motif of 3'-UTR. Furthermore, miR-26a is overexpressed in glioma tissues and plays a vital role in promoting glioma tumor growth and angiogenesis by suppressing PHB and downstream AKT and ERK pathways, suggesting that miR-26a can serve as a specific biomarker of glioma and that microRNA-based therapeutic strategies could be a promising approach to cancer treatment in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Primers used in this study.