Role of MicroRNA-26b in Glioma Development and Its Mediated Regulation on EphA2

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Abstract

Background: MicroRNAs (miRNAs) are short, non-coding RNAs that regulate the expression of multiple target genes. Deregulation of miRNAs is common in human tumorigenesis. Low level expression of miR-26b has been found in glioma cells. However, its underlying mechanism of action has not been determined.

Methodology/Principal Findings: Real-time PCR was employed to measure the expression level of miR-26b in glioma patients and cells. The level of miR-26b was inversely correlated with the grade of glioma. Ectopic expression of miR-26b inhibited the proliferation, migration and invasion of human glioma cells. A binding site for miR-26b was identified in the 3' UTR of EphA2. Over-expression of miR-26b in glioma cells repressed the endogenous level of EphA2 protein. Vasculogenic mimicry (VM) experiments were performed to further confirm the effects of miR-26b on the regulation of EphA2, and the results showed that miR-26b inhibited the VM processes which regulated by EphA2.

Significance: This study demonstrated that miR-26b may act as a tumor suppressor in glioma and it directly regulates EphA2 expression. EphA2 is a direct target of miR-26b, and the down-regulation of EphA2 mediated by miR-26b is dependent on the binding of miR-26b to a specific response element of microRNA in the 3' UTR region of EphA2 mRNA.

Introduction

MicroRNAs (miRNAs) are short single stranded RNA molecules, which serve as master regulators of gene expression. miRNAs regulate gene expression in a sequence-specific fashion; miRNAs bind to 3' untranslated regions (UTRs) of mRNAs and then affect the translation and/or stability of that mRNA, leading to a reduction in protein levels. Tumors analyzed by miRNA profiling have exhibited significantly distinct miRNA signatures compared to normal cells from the same tissue [1,2]. The abnormal levels of miRNAs in tumors have important pathogenetic consequences [3]. Some miRNAs are over-expressed in tumors and act as oncogenes, promoting tumor aggression by down-regulating tumor suppressors [4]. For example, the miR-17-miR-92 cluster in T-cell acute lymphoblastic leukemia reduces the level of the transcription factor E2F1 [5,6]; miR-21 down-regulates the tumor-inhibiting factor PTEN in lung cancer cells; and miR-125b is an important repressor of p53 and inhibits p53-induced apoptosis in human neuroblastoma cells [7]. On the other hand, tumors lost miRNAs generally participate in oncogene over-expression. For example, the let-7 family represses Ras and Myc oncogenes in cancers [8,9], and the miR-13-miR-16-1 cluster down-regulates Bel-2 and induces apoptosis in a leukemic cell line model [10].

miR-26b is one of the miRNAs involving in the response to hypoxia, a well documented tumor microenvironment factor [11]. Recent study confirmed that the expression of miR-26b was changed in several human cancer cell lines including glioma cells, [12], miRNA profile analyses revealed that miR-26b was one of the significantly decreased miRNAs in glioma cells compared to normal brain tissues [12]. However, the role of miR-26b in glioma development has not been well documented and little is known about its target genes. Additionally, the effect of abnormal expression of miR-26b on tumor grade needs to be addressed.

Erythropoietin-producing hepatocellular (EPH) receptors and their Ephrin ligands constitute the largest sub-family of receptor tyrosine kinases (RTKs), which are involved in many biological processes and play important roles in disease and development [13]. To date, 14 Eph receptors have been found in mammals. They were divided into two distinct classes, A and B, based on the sequence homology of their extracellular domains. More recently, EphA receptors and their corresponding ligands have been implicated in numerous malignancies [14]. Among them, EphA2 and ephrinA1 are the most widely studied with respect to development, tumorigenesis, angiogenesis, and metastasis and they may represent as the potential therapeutic targets because of their diverse functions in several types of cancer. Studies have shown that activation of the EphA2 receptor tyrosine kinase by...
**Results**

The expression of miR-26b in tissues of glioma patients has not been well documented. In order to determine the relationship between miR-26b expression and glioma grades, the expression of miR-26b in normal brain tissues, glioma tumors and glioma cell lines was analyzed by real-time stem-loop RT-PCR. The results showed that in normal brain tissues, miR-26b exhibited a relatively high level expression, whereas the expression of miR-26b was significantly (p<0.01) down-regulated in glioma samples (WHO I, WHO II, WHO III and WHO IV). The expression of miR-26b becomes lower with increasing grades of glioma. The expression of miR-26b was also down-regulated in the three tested glioma cell lines, U251, U87 MG and C6 (Fig. 1).

We next determined the effect of miR-26b on the proliferation of glioma cells. The growth ability of glioma cells was determined by MTT assay. As shown in Fig. 2A, over-expression of miR-26b resulted in the growth inhibition of both U251 human glioma cells and C6 rat glioma cells. However, the growth inhibition induced by miR-26b was abolished when an antisense of miR-26b (26b-AS) was introduced into the cells (Fig. 2A). In contrast, substituting the 26b-AS with a negative control single strand RNA (NC-AS), similar inhibition effect on cell proliferation was found as the transfected 26b-DP alone (Fig. 2A). We examined the inhibitory effect of miR-26b on glioma cells at different time points and found that the maximum inhibition was at the 48 h (Fig. 2B). The inhibition rates were 34.28% and 29.547% in U251 and C6 cells transfected with 26b-DP, respectively (Fig. 2B). These results suggest that miR-26b plays a key role in the proliferation of some glioma cells, and might function as a tumor suppressor in glioma cell lines.

To evaluate the role of miR-26b in glioma cell migration, a wound healing assay was performed. An artificial wound was made 24 h after transfection with 26b-DP, and the cells migrating into the wound were measured after culturing for another 24 h. As shown in Fig. 3A, the migration was significantly decreased in U251 and C6 cells transfected with 26b-DP alone (Fig. 3A). To determine the invasion ability of glioma cells, an in vitro Matrigel invasion assay was employed. As shown in Fig. 3B, cell invasion was markedly reduced in the cells transfected with 26b-DP, exhibiting a 67.98% and 51.78% in reduction of invasion with a statistically significant

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**Figure 1. The expression of miR-26b in glioma samples and U251, U87 MG and C6 cells.** The grade of glioma was evaluated according to WHO criteria as described in Materials and Methods. Samples ID 1–5 are from normal brain tissues; ID 6–10 from pilocytic astrocytomas classified to WHO I; ID 11–15 from astrocytoma classified to WHO II; ID 16–20 from anaplastic astrocytomas classified to WHO III; and ID 21–25 from Glioblastoma Multiforme classified to WHO IV. Each sample was divided by a dashed line. Total RNA was isolated from the glioma tissues and glioma cells of U251, U87 MG and C6 and real-time PCR was performed to analyze the expression of miR-26b as described in Materials and Methods. The relative expression of miR-26b was expressed as the ratio of the expression level of U6. **P<0.01, as compared to Normal brain tissues group.**

doi:10.1371/journal.pone.0016264.g001
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Figure 2. Ectopic expression of miR-26b inhibits glioma cell proliferation in vitro. (A) The proliferation of glioma cell lines, U251 and C6. Cells were first transfected with miR-26b duplex, negative control RNA duplex, co-transfected with 26b-DP and miR-26b specific antisense oligonucleotides (26b-AS) or negative antisense oligonucleotides (NC-AS). After incubation for 48 h, cell proliferation rates were evaluated by MTT assay at 12, 24, 36, 48 and 72 h. The inhibitory rates of cells were the percentage of the ratio of cells transfected with miR-26b to that transfected with NC-DP. doi:10.1371/journal.pone.0016264.g002

We confirmed the binding of miR-26b to the 3’UTR of human EphA2 using a luciferase reporter assay (Fig. 5A). Ectopic expression of miR-26b significantly suppressed the luciferase activity in HEK-293 cells co-transfected with miR-26b duplex (26b-DP) and Luc+miR-26b MRE, which contained the miR-26b response element (MRE) region in the 3’UTR of human EphA2 (Fig. 5B). Similarly, the activity of luciferase in the cells co-transfected with miR-26b and the construct Luc-ΔEphA2 3’UTR, which contains the entire 3’UTR region of human EphA2, was suppressed by 60%–70% (P<0.01) (Fig. 5B). In contrast, suppression of luciferase activity was almost abolished when the miR-26b MRE was deleted from the 3’UTR of EphA2 (Fig. 5B). The inhibition of luciferase activity was also greatly decreased when introducing a 3-base mismatch mutation into the seed region of the MRE in the 3’UTR of EphA2 (Fig. 5A and B). These data indicate that the predicted MRE is critical for the direct and specific binding of miR-26b to EphA2 mRNA.

To study the regulation of endogenous EphA2 by miR-26b, 26b-DP was transfected into U251 and C6 cells, both of which express high levels of EphA2. An EphA2 specific siRNA [29] was transfected as a positive control. When the EphA2 siRNAs were transfected into U251 or C6 cells, the expression level of endogenous EphA2 proteins was inhibited nearly 90% (Fig. 5C and D). Over-expression of miR-26b in U251 cell reduced the level of EphA2 protein by ~60% (P<0.01) (Fig. 5C and D). The expression of EphA2 protein was reduced ~70% in C6 cells transfected with miR-26b (Fig. 5C and D). These results indicate that miR-26b is a regulator of EphA2 at the protein level.

In order to further confirm the function of miR-26b as an EphA2 regulator, we next studied the effect of miR-26b in U87 MG glioma cells expressing only low levels of endogenous EphA2 [27] (Fig. 4B). Compared to U251 and C6 which expressed high levels of EphA2 (Fig. 4A and B), the cell proliferation reduced by miR-26b were not significant (p>0.05) in the U87 MG cells (Fig. 4A). The growth ability of glioma cells in U87 MG, U251, and C6 cells transfected with 26b-DP was reduced with inhibition rates of 10.11% (Fig. 6A), 34.28% and 29.54% (Fig. 2B), respectively, suggesting that the inhibition rates of 26b-DP transfectants were related to endogenous EphA2 levels in glioma cells. Similarly, the reduction of migration activity in U87 MG cells transfected with 26b-DP was also not notable (Fig. 6B) compared with that in U251 and C6 glioma cells (Fig. 3A). Additionally, as shown in Fig. 3B and 3C, transfection with 26b-DP greatly reduced the ability of U251 and C6 cells to invade Matrigel, exhibiting about 67.98% and 51.78% in reduction of invasion, respectively. However, in U87 MG, only 11.73% reduction was found compared with the cells transfected with scrambled oligonucleotides (NC-DP) (Fig. 6C and D). These results provide solid evidence that miR-26b suppresses glioma cell proliferation, migration and invasion activity in a manner dependent on EphA2 expression level.

Glioma is an extremely invasive, well-vascularized tumor. It has been reported that vasculogenic mimicry (VM) exists in glioma [30] and EphA2 is an important regulator for VM formation. In order to evaluate the effects of miR-26b and EphA2 on VM
formation in glioma cells, we performed VM network formation experiments in U87 MG, U251 and C6 cells. As shown in Fig. 7A, U251 and C6 glioma cells, which expressed high levels of EphA2, could develop VM networks when cultured in Matrigel for 24 h (Fig. 7 I and III) and the VM network showed a positive reaction with periodic acid-Shiff (PAS) (Fig. 7 II and IV). In contrast, VM network was not found in U87 MG cells which expressed low level of EphA2 (data not shown). Our primary finding suggested that VM network formation was dependent, at least in part, on the expression of EphA2. Therefore, we transfected the miR-26b duplex (26b-DP) into glioma cells to determine if down-regulation of EphA2 could affect the VM formation. As predicted, our results showed that the VM network could not be formed in either U251 or C6 cells (Fig. 7 V and VII). However, we did find that over-expression of EphA2 fully rescued from the effects of miR-26b when a EphA2 expression vector lacking the 3’UTR (pCMV6-XL6-EphA2) was delivered into the miR-26b over-expressed U251 and U6 cells (Fig. 7 VI and VIII). These results suggested that EphA2 plays a key role in the VM formation, and miR-26b affects the VM formation by down-regulation of EphA2 expression in glioma cells.

**Discussion**

In the present report we detected the miR-26b expression level in human glioma samples and found that the decreased expression...
The level of miR-26b was negatively correlated with the increased malignancy of glioma. Transfection of miR-26b duplex decreased the aggressive feature of glioma cells, suggesting that miR-26b plays a critical role in glioma development, and it may act as an anti-tumor factor in glioma cells.

Our studies indicate that EphA2 is a novel target gene of miR-26b, and the direct interaction between miR-26b and EphA2 mRNA is supported by several lines of evidence: (1) the 3’ UTR of both human and murine EphA2 mRNAs contain a putative binding site (the MRE) for miR-26b with significant seed match; (2) miR-26b suppresses the activity of a luciferase reporter fused with the 3’ UTR of EphA2 mRNA in an MRE dependent manner; (3) miR-26b represses the endogenous expression of human/murine EphA2 at both the mRNA and protein level; (4) A previous study has shown that EphA2 gene knockdown by siRNAs resulted in failure of VM formation [29]. Similar results were found in our present study, when miR-26b was over-expressed in U251 and C6 cells, the VM process was impaired, suggesting that miR-26b affects VM formation of glioma cells by down-regulating EphA2. This study is the first to identify a miRNA that directly regulates EphA2.

EphA2 expression is frequently elevated in cancers and is associated with poor prognosis [17–19,24,31]. High levels of EphA2 have been reported in diverse cell lines and clinical specimens, including breast, colon, prostate, non-small cell lung cancers, aggressive melanomas and glioblastoma [14,18]. However, EphA2 does not appear to function simply only as a biomarker [27] but also actively participated in malignant progression [17,20,28]. For example, ectopic expression of EphA2 in non-transformed mammary epithelial cells is sufficient to promote a malignant phenotype as defined using in vitro and in vivo methods [19]. It has been reported that knockdown of EphA2 in cancer cells

Figure 4. EphA2 is a predicted target of miR-26b. (A) The mRNA expression level of EphA2 in glioma samples and glioma cell lines. The mRNA expression level of EphA2 was detected by real-time PCR. The grades of glioma were classified in Materials and Methods. The experiments were performed more than three times. The relative expression of EphA2 was the ratio of the expression level of to that of β-actin. **P < 0.01, as compared to Normal brain tissues group. (B) The protein expression level of EphA2 in U251, U87 MG and C6 cells. Western blotting analysis was performed to evaluate the expression level of EphA2 in different glioma cells. β-actin was used as a loading control. (C) The shaded region represents the MRE sequences of miR-26b in the 3’ UTR of human EphA2 mRNA as predicted by TargetScan 5.1, miRBase and Pictar. (D) The predicted miR-26b binding site in EphA2 3’ UTR is highly conserved in mammals. The miR-26b seed sequences and their predicted binding sites in the EphA2 3’ UTR are shown underlined.
inhibited cell malignancy and invasion [32]. In our present study, we showed that high level expression of EphA2 was found in high grade glioma samples. Our study is consistent with other studies that high level expression of EphA2 plays a critical role in cell malignancy. Additionally, we further confirmed that miR-26b is an important regulator of EphA2, and there was an interaction between miR-26b and EphA2 in glioma cells. The inhibition induced by miR-26b in glioma cells is partly dependent on the expression of EphA2. This finding increases our understanding of EphA2 function and regulation in glioma cells.

Vasculogenic mimicry (VM) was first described in highly aggressive uveal melanomas which formed vasculogenic networks with tumor cells instead of endothelial cells [33]. VM is associated with tumor blood supply and tumor metastasis. In recent years, VM has been seen in several types of malignant tumors such as breast cancer [34], liver cancer, glioma, ovarian cancer [35], melanoma [33], prostate cancer [36], colorectal cancer [37] and some other bidirectional differentiated malignant tumors [38]. It has been reported that VM also exists in glioma cells [30]; high aggressive glioma cells can form VM in three dimension culture medium [39]. EphA2 is known to be an important regulator during VM formation [14,29,38,40]. Knockdown of EphA2 in malignant melanoma cells impaired formation of the VM network [29]. EphA2 expression was found in both highly aggressive uveal and cutaneous melanoma cells, but not in poorly aggressive melanoma cells. Moreover, down-regulation of EphA2 expression using a specific small interference RNA (siRNA) inhibits VM formation in aggressive melanoma cells [40]. In the present work, U251 and C6 glioma cells, which express...
high levels of EphA2, formed classical VM networks on Matrigel. In contrast, the VM networks could not be formed in U87 MG cells, which have low levels of EphA2. The result further suggests that EphA2 plays a critical role in VM formation in glioma cells. We further demonstrated that the function of EphA2 is regulated by miR-26b; transfection of miR-26b duplex into U251 and C6 cells inhibited their ability to form VM networks. However, when we transfected these cells with EphA2 expression vector without the 3’UTR regain, the VM destroyed by over-expression of miR-26b were fully restored. The formation of vasculogenic like networks has not been well understood; the processes involve several signaling molecules, including vascular endothelial (VE)-cadherin, erythropoietin-producing hepatocellular carcinoma-A2 (EphA2), phosphatidylinositol 3-kinase, focal adhesion kinase, matrix metalloproteinases and laminin 5 γ2-chain [38]. Here we found that miR-26b is a negative regulator of EphA2 in VM formation. This finding provides insight into the function of miRNAs and EphA2 in regulating VM processes.

In conclusion, this study demonstrates that miR-26b plays a key role in the malignancy of glioma cells by directly regulating EphA2 expression, which affects cell proliferation, migration and invasion. VM formation was also affected by ectopic expression of miR-26b. This study helps us to better understand of the function of miR-26b and its regulation of EphA2 in glioma cells. However, further study is needed to determine if EphA2 activity is affected by miR-26b in other cancers with high EphA2 expression, like breast cancer, colon cancer, and prostate cancer; and whether miR-26b-EphA2 dysregulation represents a new mechanism for cellular transformation.

Materials and Methods
Cell lines and tumor tissues
Human HEK-293T cells, human glioma U251 and U87 MG cells and rat glioma C6 cells were purchased from the American Type Culture Collection (ATCC, MD). All cell lines were
The U251 and C6 cells transfected with 26b-DP first, then seeded on the coated coverlips after 24 h incubation. The VM formation was assessed using an inverted microscope after growth for another 24 h. U251 and C6 network formation stained by PAS, respectively. U251 (V) and C6 (VI) cells were transfected with 26b-DP, the transfected cells were then seeded on the coated coverlips. The VM formation was assessed using an inverted microscope after growth for 24 h. II and IV represent the collected and stored at University Associate Hospital (Qingdao, China). Samples were obtained from the Brain Institute, Qingdao Medical Institute, Affiliated Hospital of Qingdao Medical University reviewed by the neuropathologist Dr. W.C Yao in the Brain information are shown in Table S1. All samples were thoroughly passaged at 2 to 3 day intervals.

Tissue samples from human glioma and normal brain tissues were obtained from the Brain Institute, Qingdao Medical University Associate Hospital (Qingdao, China). Samples were collected and stored at −80°C. The histopathologic diagnoses were determined using WHO criteria and evaluated by the hospital’s pathologist using both morphologic criteria and immunocytochemistry. Written consent of tissue donation for research purposes was obtained from the patients before tissue collection and the protocol was approved by the Institutional Review Board of the Affiliated Hospital of Qingdao Medical University. Twenty-five samples were used for this study with 5 samples for each group, including primary grade pilocytic astrocytomas (WHO I), grade II astrocytoma (WHO II), grade III anaplastic astrocytomas (WHO III), grade IV Glioblastoma Multiforme (WHO IV) and normal brain tissues derived from the temporal lobes and saddle area of the patients with arachnoid cyst (AC) after surgery. The clinical data and patient information are shown in Table S1. All samples were thoroughly reviewed by the neuropathologist Dr. W.C Yao in the Brain Institute, Affiliated Hospital of Qingdao Medical University (Qingdao, China).

RNA isolation
Total RNA was extracted from the frozen tissue samples or cultured cells using the TRIzol kit (Invitrogen, CA) following the manufacturer’s protocol. Briefly, tissue samples were homogenized in TRIzol reagent using an Omni-Mixer Homogenizer (Omni International, CA). The cells were collected from a culture flask into RNase free tubes, and TRIzol solution (Invitrogen, CA) was added. RNA quantity was determined by UV measurement of OD 260/280 nm using the NanoDrop 2000 instrument (Thermo Scientific, FL).

Real-time quantitative RT-PCR
To quantitate the expression level of mature miR-26b (MIMAT0000083), the isolated RNA was reverse transcribed and amplified by a two-step quantitative RT-PCR method using the Hairpin-it TM miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China) according to the manufacturer’s protocol. miR-26b specific reverse primers and the sequence-specific primers for mature miR-26b were designed according to Chen et al. [41] and their sequences are listed in Table 1. PCR reactions were performed using an ABI 7300 System (Bio-Rad, CA) with the following conditions: 95°C, 10 min for 1 cycle, then 95°C, 15 s, 60°C, 1 min for 40 cycles. Signals were detected at the end of each cycle. The U6 small nuclear RNA was amplified as a loading control. The primers for this U6 internal control were provided by GenePharma (Shanghai, China). The relative expression level of mature miR-26b from each sample was determined using the 2(−ΔΔC(T) Method [42].

Real-time PCR for EphA2 was performed using the ABI 7300 System (Bio-Rad, CA) with the QuantiTect SYBR Green PCR mixture (Invitrogen, CA). β-actin was used as control. Primers used for detecting gene expression are listed in Table 1. Expression of EphA2 was determined using the 2(−ΔΔC(T) Method. Amplification conditions were as follows: 95°C, 3 min, 95°C, 30 s, 60°C, 30s, 72°C, 40s, for 40 cycles, and 72°C, 8 min for extension.

Transfection
The two miRNA mimics used in the experiments were purchased from Dharmacon company (Dharmacon, CO), and the sequence of NC-DP microRNA, as a negative control, is based on cel-miR-67(MIMAT0000039) and it has been confirmed to have minimal sequence identity with miRNAs in human, mouse and rat. Another microRNA mimics is 26b-DP, which is a duplex of miR-26b. The antisense of two microRNAs, negative antisense of negative control microRNA, cel-miR-67 (NC-AS) and miR-26b antisense (26b-AS) were also provided by Dharmacon. Certain amount of miRNA duplexes (NC-DP and 26b-DP) and antisense
oligonucleotides (NC-AS and 26b-AS) were transfected into U87 MG, U251 or C6 cells, respectively. EphA2 expression vector (pCMV6-AC-GFP-EphA2) which contains the full length human EphA2 gene ORF sequence without the 3' UTR region of EphA2 was purchased from OriGene Technologies (OriGene Technologies, MD). The pCMV6-AC-GFP-EphA2 vector was transfected into U251 or C6 cells with 20 nm/L. Specific siRNAs targeting EphA2 (GenePharma, Shanghai, China) were transfected at 100 nm/L. Lipofection 2000 (Invitrogen, CA) was used as the transfection reagent following the manufacturer’s protocol.

Table 1. Primer sequences.

| Primer name   | Type                      | Sequence 5' to 3'             |
|--------------|---------------------------|-------------------------------|
| β-actin -F   | RT PCR forward primer     | GTTGGGTATTACCCCTTCTTG         |
| β-actin -R   | RT PCR reverse primer     | GTACCCTCATCCGCAGTCTCA         |
| EphA2-F      | RT PCR forward primer     | CACTTACGCAAGAAGGAGAA          |
| EphA2-R      | RT PCR reverse primer     | ACAGCCAGCAGCGCAAATCA          |
| miR-26b-RT   | miR-26b RT primer         | GTGTATCCAGGTTGGCCGAGGATTTAGCTGAGGATCATT |
| miR-26b-f    | RT PCR forward primer     | CGCCGCTTCAAGATTTCAAGGAT       |
| miR-26b-R    | RT PCR reverse primer     | GTGCCGTTGCGGAGG               |
| EphA2-MRE-F  | MRE top strand            | tccagCATCGCGCAAGAATACTTTGAAgc |
| EphA2-MRE-R  | MRE bottom strand         | gggcgcTCTAGATTTCTGGGCGATGcGc |
| EphA2-UTR-F  | PCR forward primer        | CCGGccgTCCTGGAGCCCCATGCGGCCGAAAT |
| EphA2-UTR-R  | PCR reverse primer        | TTCTTTCTggtgccgcAGAGTGGCTCTGTGCCTCAT |
| EphA2-UTR-NC | MRE delete primer         | TTCTTTCTggtgccgcAGAGCAGAAGAATAGACTATTTC |
| EphA2-UTR-R  | PCR reverse primer        | TTCTTTCTggtgccgcAGAGCAGAAGAATAGACTATTTC |
| EphA2-UTR-M  | Mutagenesis primer        | GGAGGCCCATGGCGCAAGAATGTTGACCAAGG |
| EphA2-UTR-S  | Mutagenesis primer        | AACACACCGTCCGTCTGAGCGGCGCCG |
| EphA2-siRNA  | EphA2 siRNA forward strand| CCGGAGTCCGTCCGTCTGCCTGGCCCG |
| EphA2-siRNA  | EphA2 siRNA forward strand| CCGGAGTCCGTCCGTCTGCCTGGCCCG |

Lower case text indicates restriction enzyme sites; Lower case with underline indicates mismatched mutations in miR-26b seed region.

doi:10.1371/journal.pone.0016264.t001

Synthesis of luciferase reporter constructs

To construct the luciferase reporter vectors, the predicted miR-26b binding site (miRNA response element, MRE) on the EphA2 3’UTR or the whole 3’UTR of EphA2 were inserted into the Xhol and NotI sites of a psiCheck2 vector (Promega, WI) immediate downstream of the Renilla luciferase gene. The sense and antisense sequences of the MRE were synthesized, annealed, and ligated into the psiCheck2 vector to construct a miR-26b MRE luciferase reporter, Luc+miR-26bMRE. The full length 3’UTR of human EphA2 was amplified from the total cDNA of U251 cells and inserted into TOPO PCR2.1 (Invitrogen, CA) and then ligated into the psiCheck2 vector to synthesize the luciferase report constructs Luc+wt EphA2 3’UTR. Another construct, Luc+MRE deleted 3’UTR, in which the miR-26b MRE region of EphA2 3’UTR was deleted was also constructed with a similar approach. A mutation in the seed region of miR-26b MRE of the EphA2 3’UTR was made using the Quick change sit-Directed mutagenesis kit (Stratagene, CA) according to the manufacturer’s instructions. The construct, Luc+MRE mutated 3’UTR, was synthesized by ligating the mutation fragment into the psiCheck2 vector. The sequences of MRE and all primers are listed in Table 1.

Luciferase reporter assay

miRNA duplexes, including the negative control duplex (NC-DP) and miR-26b duplex (26b-DP), and miRNA antisense oligonucleotide, including the negative control antisense (NC-AS) and miR-26b antisense (26b-AS) were purchased from Dharmacon and dissolved in ddH2O. Each luciferase reporter construct, including Luc+miR-26bMRE, Luc+wt EphA2 3’UTR, Luc+MRE deleted 3’UTR and Luc+MRE mutated 3’UTR was co-transfected with 50 nm/L of miRNA duplexes or 100 nm/L miRNA antisense into HEK-293T cells in a 24-well plate using Lipofectamin-2000 (Invitrogen, CA). After incubation for 48 h, cells were harvested by centrifugation at 1000 g for 10 min and lysed using lysis buffer. Firefly and Renilla luciferase activities were determined with the Dual-Luciferase reporter system (Promega, WI) following the manufacturer’s protocol.

Western blotting analysis

U251 or C6 cells were transfected with Oligonucleotides (26b-DP or NC-DP, or 26b-AS or EphA2 siRNAs). After incubation for 72 h, the transfected cells were harvested by centrifugation at 1000 g for 10 min and lysed using ice-cold RIPA buffer (50 mM Tris–HCl, 1% NP40, 0.25% Na-deoxycholine, 150 mM NaCl, 1 mM EDTA). PMSF was added at a final concentration of 1 mM/mL. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, FL). Whole-cell lysates (50 μg protein) were resolved by 9% SDS-PAGE and electroblotted onto nitrocellulose membranes using the Idea electrophoresis system (Idea scientific, MN). Membranes were incubated in blocking solution (1× PBS, 0.1% Tween-20, and 5% nonfat dry milk powder) for 2 h at room temperature, and then incubated for 1 h with primary antibodies at the following dilutions: Rabbit monoclonal antibodies against human EphA2 with 1:500 dilutions (H-20, sc-48789, Santa Cruz Biotechnology, CA) or mouse polyclonal antibody against β-actin diluted 1:1000 (E-5, sc-47778, Santa Cruz Biotechnology, CA). After washing 10 minutes with 1× PBS and 0.1% Tween-20 for three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (IgG goat anti-rabbit or anti-mouse; 1:2000; Bio-Rad, CA) for 1 h at room temperature. After an additional three 10 minute washes with 0.1% Tween-20 in PBS buffer, the
chemiluminescence method was employed to detect the signals using Super Signal West Dura (Thermo Scientific, FL) and protein bands were visualized by autoradiography. Quantitation of signal intensities was performed using densitometry on a Hewlett-Packard ScanJet 5370C (Hewlett-Packard, CA) with NIH image 1.62 software.

**MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bro-121 mide] assay**

U251 or C6 cells (5 x 10^5) were plated onto 96-well plates and allowed to adhere overnight. The cells were transfected with 26b-DP at the final concentration of 50 nm or 26b-AS at the final concentration of 100 nm using Lipofectation 2000 reagent (Invitrogen, CA). At certain time points (12, 24, 36, 48 and 72 h) after transfection, MTT dye (30 μL, 5 mg/ml, Sigma-Aldrich, MO) was added to the culture medium. After incubation at 37°C for 4 h, the MTT solution was removed and 150 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO) was added to dissolve the formazan crystals. Spectrometric absorbance at 490 nm was measured by Multiskan EX microplate photometer (Thermo Scientific, FL).

**Wound healing assay**

Cell culture and transfection conditions were optimized to ensure a homogeneous and viable cell monolayer prior to wounding. One day before transfection, U251 (3.5 x 10^5), U87 MG (3.5 x 10^5) or C6 (5 x 10^5) cells were seeded onto 6-well plates. Cells were then transfected with 50 nm/L 26b-DP or NC-DP using the Lipofectation 2000 transfection reagent. When the cell confluence reached about 90%, at 48 h post-transfection, an artificial homogenous wound was made onto the monolayer with a sterile plastic 200 μL micropipette tip. After wounding, cell debris was removed by washing the cells with serum-free medium. After incubation for another 24 h, the cells that migrated into the wounded area or with extended protrusion from the border of wound were photographed using an inverted microscope (40 x magnifications) (CKX41, LYLMPAS, Japan).

**In vitro invasion assay**

U251, U87 MG or C6 cells were plated in 6-well plates and transfected with final concentration of 50 nm 26b-DP or 100 nm 26b-AS as mentioned above. After incubation for 48 h, the cells were treated with 0.25% trypsin-EDTA (Hyclone, Japan), collected by centrifugation at 1000g, and re-suspended into serum-free medium. Transfected cells (5 x 10^5) were reseeded into the upper chamber of transwells. 20% FBS was added to the medium in the lower chamber. After incubation for another 24 h, non-invading cells on the upper surface of the membrane were scrubbed gently with a cotton-tipped swab. The invasive cells on the lower surface of the membrane were fixed with 95% methanol and stained with 0.1% Crystal Violet (Sigma-Aldrich, MO). The stained invasive cells were photographed under an inverted light microscope (100 x magnification) and quantified by manual counting in three randomly selected areas. The experiments were performed in triplicate in three independent experiments.

**Vasculogenic mimicry formation assay**

Vasculogenic mimicry (VM) formation experiments were performed as previously described [33] with a little modification. Briefly, 50 μL ECM Matrigel (Sigma-Aldrich, CA) was dropped onto 18-mm glass coverslip in 6-well tissue culture plates and then incubated at 37°C for 30 min. U251 and C6 cells (5 x 10^5) were transfected with 26b-DP, 26b-AS or contransfected with 26b-DP and EphA2 expression vector (pCMV6-XL6-EphA2) a day before seeded onto the coated coverslip. After growth for 24 or 48 h on the coverslip, VM formation was assessed using an inverted microscope (CKX41, LYLMPAS, Japan). Additionally, the periodic acid-Shift’s Reaction (PAS) staining of VM formation was performed as described by Maniotis et al. [33]. Briefly, cells cultured on coverslip were fixed with 95% ethanol for 5 min, oxidized in 0.5% periodic acid solution for 5 min, rinsed using distilled water 3 times and placed in Schiff reagent for 15 min, and then the coverslips were immediately picked out and washed with lukewarm tap water for 5 min. The coverslips were dried at room temperature and the PAS signal was photographed using an inverted light microscope at 40 x magnifications.

**Statistics**

Statistical analyses were performed using the software from SPSS for Windows 13.0 (SPSS Inc., IL). All data were described as mean ± SEM. To analyze the data statistically, we performed Student’s t-test for the analysis. Differences were considered significant when P < 0.05.

**Supporting Information**

Table S1 Characteristics of patient tissues used in this study. Twenty five patient tissues were used in this study and the patient tissue information was described.

**Author Contributions**

Conceived and designed the experiments: NW XL. Performed the experiments: NW XZ ML HL YZ. Analyzed the data: NW XZ ML XL. Contributed reagents/materials/analysis tools: WY. Wrote the paper: NW XL SC.

**References**

1. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, et al. (2007) MicroRNA signatures in human ovarian cancer. Cancer Res 67: 8699–8707.
2. Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6: 582–583.
3. Hammond SM (2007) MicroRNAs as tumor suppressors. Nat Genet 39: 582–583.
4. Nicoloso MS, Spizzo R, Shimizu M, Rossi S, Calin GA (2009) MicroRNAs–the micro-steering wheel of tumour metastases. Nat Rev Cancer 9: 293–302.
5. Nagel S, Vennari L, Przybilsky GK, Grabarzczuk P, Schmidt CA, et al. (2009) Activation of miR-17-92 by NK-like homedomain protein suppresses apoptosis via reduction of E2F3 in T-cell acute lymphoblastic leukemia. Leuk Lymphoma 50: 101–108.
6. Hyun S, Lee JH, Jin H, Nam J, Namkoong B, et al. (2009) Conserved MicroRNA miR-8/miR-200 and its target USH1/OAG2 control growth by regulating PTEN. Cell 139: 1096–1108.
7. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, et al. (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635–647.
8. Kim HH, Kiscsan Y, Srikant S, Lee EK, Marrinade JL, et al. (2009) HuR recruits let-7/RISC to repress c-Myc expression. Genes Dev 23: 1743–1748.
9. Ciannino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, et al. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102: 13944–13949.
10. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, et al. (2007) Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res 67: 2456–2460.
13. Dodelet VC, Pasqualle EB (2000) Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. Oncogene 19: 5614–5619.

14. Walker-Daniels J, Hess AR, Hendrix MJ, Kinch MS (2003) Differential regulation of EphA2 in normal and malignant cells. Am J Pathol 162: 1037–1042.

15. Salata K, Nair PM, Petit RS, Neve RM, Das D, et al. Restricted of receptor movement alters cellular response: physical force sensing by EphA2. Science 327: 1380–1385.

16. Ory B, Pasquale R, Lindberg RA, Kain R, Freeman AL, et al. (2000) The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 19: 6043–6052.

17. Lu C, Shahzad MM, Wang H, Landen CN, Kim SW, et al. (2008) EphA2 overexpression promotes ovarian cancer growth. Cancer Biol Ther 7: 1098–1103.

18. Wykosky J, Debinski W (2008) The EphA2 receptor and ephrin-A1 ligand in solid tumors: function and therapeutic targeting. Mol Cancer Res 6: 1795–1806.

19. Zelinski DP, Zantek ND, Stewart JC, Frizzary AR, Kinch MS (2001) EphA2 overexpression causes tumorigenesis of mammary epithelial cells. Cancer Res 61: 2301–2306.

20. Thaker PH, Deavers M, Celestino J, Thornton A, Flecher MS, et al. (2004) EphA2 expression is associated with aggressive features in ovarian carcinoma. Clin Cancer Res 10: 5145–5150.

21. Walker-Daniels J, Coffman K, Azimi M, Rhim JS, Bostwick DG, et al. (1999) Overexpression of the EphA2 tyrosine kinase in prostate cancer. Prostate 41: 275–280.

22. Easty DJ, Bennett DC (2000) Protein tyrosine kinases in malignant melanoma. Melanoma Res 10: 401–411.

23. Nemoto T, Ohashi K, Akashi T, Johnson JD, Hirokawa K (1997) Overexpression of protein tyrosine kinases in human esophageal cancer. Pathobiology 65: 195–203.

24. Miyazaki T, Kato H, Fukuchi M, Nakajima M, Kuvano H (2005) EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 105: 637–663.

25. Xu F, Zhong W, Li J, Shanshen Z, Cui J, et al. (2005) Predictive value of EphA2 and Ephrin-A1 expression in oesophageal squamous cell carcinoma. Anticancer Res 25: 2943–2950.

26. Cercone MA, Schroder W, Schomberg S, Carpenter TC (2009) EphA2 receptor mediates increased vascular permeability in lung injury due to viral infection and hypoxia. Am J Physiol Lung Cell Mol Physiol 297: L568–863.

27. Wykosky J, Gibo DM, Stanton C, Debinski W (2005) EphA2 as a novel molecular marker and target in glioblastoma multiforme. Mol Cancer Res 3: 541–551.

28. Li X, Wang Y, Wang Y, Zhen H, Yang H, et al. (2007) Expression of EphA2 in human astrocytic tumors: correlation with pathologic grade, proliferation and apoptosis. Tumour Biol 28: 165–172.

29. Hess AR, Seifor EA, Gardor LM, Carles-Kinch K, Schneider GB, et al. (2001) Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2). Cancer Res 61: 3236–3255.

30. Yue WY, Chen ZP (2003) Does vasculogenic mimicry exist in astrocytoma? J Histochem Cytochem 51: 997–1002.

31. Kamat AA, Coffley D, Merritt WM, Nagent E, Urbauer D, et al. (2009) EphA2 overexpression is associated with lack of hormone receptor expression and poor outcome in endometrial cancer. Cancer 115: 2604–2609.

32. Zhou Z, Yuan X, Li Z, Tu H, Li D, et al. (2008) RNA interference targeting EphA2 inhibits proliferation, induces apoptosis, and cooperates with cytotoxic drugs in human glioma cells. Surg Neurol 70: 562–568; discussion 568–569.

33. Maniotis AJ, Folkberg R, Hess A, Seifor EA, Gardor LM, et al. (1999) Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. Am J Pathol 155: 739–752.

34. Shirakawa K, Kobayashi H, Heike Y, Kawamoto S, Brechbiel MW, et al. (2002) Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. Cancer Res 62: 560–566.

35. Wang JY, Sun T, Zhao XL, Zhang SW, Zhang DF, et al. (2008) Functional significance of VEGF-A in human ovarian carcinoma: role in vasculogenic mimicry. Cancer Biol Ther 7: 758–766.

36. Sharma N, Seifor RE, Seifor EA, Gruman LM, Heidger PM, Jr., et al. (2002) Prostatic tumor cell plasticity involves cooperative interactions of distinct phenotypic subpopulations: role in vasculogenic mimicry. Prostate 50: 189–201.

37. Barten CI, Hilleen F, Pauwel P, de Briaine AP, Barten CG (2009) Prognostic role of vascular mimicry in colorectal cancer. Dis Colon Rectum 52: 2020–2035.

38. Zhang S, Zhang D, Sun B (2007) Vasculogenic mimicry: current status and future prospects. Cancer Lett 234: 137–146.

39. Le Mercier M, Fortin S, Mathieu V, Roland I, Spiegler-Kreinecker S, et al. (2009) Galectin 1 proangiogenic and promigratory effects in the Hu683 oligodendroglialoma model are partly mediated through the control of BEX2 expression. Neoplasia 11: 485–496.

40. Hess AR, Margaryan NV, Seifor EA, Hendrix MJ (2007) Deciphering the signaling events that promote melanoma tumor cell vasculogenic mimicry and their link to embryonic vasculogenesis: role of the Eph receptors. Dev Dyn 236: 3203–3206.

41. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33: e175.

42. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔC(T) Method. Methods 25: 402–408.