MicroRNA-146a Inhibits Glioma Development by Targeting Notch1

Running title: miR-146a restricts gliomagenesis

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ABSTRACT

Dysregulated EGFR signaling through either genomic amplification or dominant active mutation (EGFRvIII), in combination with dual inactivation of INK4A/ARF and PTEN, is a leading cause of gliomagenesis. Our global expression analysis for microRNAs revealed that EGFR activation induces miR-146a expression, which is further potentiated by inactivation of PTEN. Unexpectedly, over-expression of miR-146a attenuates proliferation, migration and tumorigenic potential of Ink4a/Arf−/−Pten−/−EgfrvIII murine astrocytes. Its ectopic expression also inhibits glioma development of a human glioblastoma cell line in an orthotopic xenograft model. Such inhibitory function of miR-146a on gliomas is largely through down-regulation of Notch1, which plays a key role in neural stem cell maintenance and is a direct target of miR-146a. Accordingly, miR-146a modulates neural stem cell proliferation and differentiation, and reduces the formation and migration of glioma stem-like cells. Conversely, knockdown of miR-146a by microRNA sponge upregulates Notch1 and promotes tumorigenesis of malignant astrocytes. These findings indicate that, in response to oncogenic cues, miR-146a is induced as a negative feedback mechanism to restrict tumor growth by repressing Notch1. Our results provide novel insights into the signaling pathways that link neural stem cells to gliomagenesis and may lead to new strategies treating brain tumors.

INTRODUCTION

Gliomas are the most frequently observed brain tumors, with glioblastoma multiforme (GBM) being the most common and aggressive form in adults (35). Despite major therapeutic improvements made by combining neurosurgery, chemotherapy and
radiotherapy, the prognosis and survival rate for patients with GBM is still extremely poor (7). The deadly nature of GBM originates from explosive growth and invasive behavior, which are fueled by dysregulation of multiple signaling pathways. EGFR activation, in cooperation with loss of tumor suppressor functions, such as mutations in Ink4a/Arf and Pten genes, constitutes a lesion signature for GBM (1). Such dysregulated genetic pathways are sufficient to transform neural stem cells (NSCs) or astrocytes into cancer stem-like cells. This gives rise to high-grade malignant gliomas with a pathological phenotype resembling human GBM (5, 59). However, the downstream events underlying these genetic dysregulations in gliomagenic cells have not been fully elucidated.

MicroRNAs are 20-22 nucleotide non-coding RNA molecules that have emerged as key players in controlling NSC self-renewal and differentiation (11, 57). Aberrant expression of miRNAs, such as miR-21, miR-124 and miR-137, is linked to glioma formation (49). miR-199b-5p and miR-34a impair cancer stem-like cells through negative regulation of several components of the Notch pathway in brain tumors (18, 21). The Notch pathway is an evolutionarily conserved signaling pathway that plays an important role in neurogenesis (4, 10, 23). Upon binding to its ligand, Delta, the Notch intracellular domain (NICD, the activated form of Notch) is released from the membrane by presenilin/γ-secretase-mediated cleavage and translocates to the nucleus. In the nucleus, NICD forms a complex with Rbpj and activates the expression of several transcriptional repressors, such as Hes1 and Hes5, which inhibit neurogenesis (15, 27, 29). Thus, activation of the Notch pathway is essential to maintain both developing and
adult NSCs (36). This property of the Notch pathway enables it to promote glioma growth (30), and its inhibition by drugs could abolish glioma stem-like cells and reduce tumorigenesis (17).

In this study, we show that miR-146a is specifically induced as a converging downstream target of EGFR and PTEN signaling in immortalized Ink4a/Arf⁻/⁻ astrocytes. We further demonstrate that miR-146a acts as a native safeguarding mechanism to restrict the formation of glioma stem-like cells and glioma growth by directly controlling the expression of Notch1.

MATERIALS AND METHODS

Cell culture, MTT and anchorage-independent growth assays. We isolated primary NSCs by mechanical dissociation using 1 ml pipettes from embryonic day 14.5 (E14.5) mouse forebrains in growth medium consisting of DMEM/F12, 1 mM L-glutamine, N2 (Invitrogen), 20 ng/ml EGF and 20 ng/ml FGF2 (Peprotech). These cells were cultured as free-floating neurospheres under 37°C and 5% CO₂. For differentiation, we exposed NSCs to DMEM/F12 media with N2 supplement, further supplemented with 5 μM forskolin and 0.5% FBS (FSK), 1 μM retinoic acid and 0.5% (v/v) FBS (RA), or 0.5% (v/v) FBS (-GF). This was done in 60-mm dishes or 8-well chamber slides coated with laminin (5 μg/ml) and poly-L-ornithine (10 μg/ml). The Ink4a/Arf⁻/⁻, Ink4a/Arf⁻/⁻EGFR⁺/+ and Ink4a/Arf⁻/⁻Pten⁻/⁻EGFR⁺/+ astrocytes, or human U87 glioma cells were cultured in DMEM containing 10% FBS. Glioma stem-like cells from malignant astrocytes or U87 cells were enriched by culturing in NSC medium with growth factors. Cell growth was
determined by the CellTiter 96® nonradioactive cell proliferation assay kit (MTT assay, Promega) according to the manufacturer’s instructions. For anchorage-independent growth by soft agar assay, 2,000 Ink4a/Arf−/−Pten−/−EGFRIII astrocytes transduced with either wild-type or seed-region-mutated miR-146a were plated and cultured in 12-well plates, as described previously (47). Five weeks later, cell colonies in the plates were stained with 0.5 ml of 0.005% crystal violet and counted under an inverted microscope. We conducted each experiment in triplicate.

**miRNA microarray and quantitative RT-PCR (qPCR).** We extracted total RNAs from Ink4a/Arf−/− (I), Ink4a/Arf−/−EGFRIII (IE), or Ink4a/Arf−/−Pten−/−EGFRIII (IPE) astrocytes using the miReasy mini kit (Qiagen). Biological replicates were collected. RNA quality was examined by Bioanalyzer (Illumina). Five μg of each RNA sample was processed for labeling and hybridization to GeneChip miRNA arrays (Affymetrix) by the Microarray Core Facility at UT Southwestern Medical Center. After scanning and normalization to control probes, the array data was analyzed by VAMPIRE software to identify statistically significant differences in gene expression between sample groups (http://genome.ucsd.edu/microarray). For qPCR, 1 μg of total RNA was reverse transcribed using NCode miRNA First-Strand Synthesis and qRT-PCR Kits (Invitrogen). qPCR reactions were performed in a 384-well plate using an ABI 7900HT instrument. The PCR program consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 60s at 58°C. Primer quality was analyzed by dissociation curves. The expression of miR-146a and Notch1 was normalized to that of U6 and Hprt, respectively.
Lentivirus production and transduction. A genomic fragment encompassing the miR-125a coding region or a seed-region-mutated version was cloned by PCR into pTomo vector (provided by Dr. Inder Verma at Salk Institute). To construct the miRNA sponge, we inserted the synthesized DNA fragment into the BamHI site of a pCSC-SP-PW-IRES/GFP lentiviral vector, and the empty vector as the control. We produced the lentiviruses, determined their titer, and transduced cultured cells using previously described methods (38, 48). Transduction efficiency was monitored by GFP expression. All experiments were performed using stably transduced cells within 6 passages.

Cell migration assay. Migration of malignant astrocytes in culture was determined by the ‘scratch’ assay. For this, cells were seeded into a 6-well tissue culture dish and allowed to grow to 90% confluency in complete medium with 10% FBS. Cells in monolayers were scratched in a single straight line using a pipette tip (1 mm in diameter). Wounded monolayers were washed three times with culture medium to remove cell debris and then incubated for another 24h. Migratory distance was measured under a microscope equipped with a camera. Transwell migration assay was conducted essentially as previously described (43). Invasive behavior of glioma stem-like cells in vitro was examined by measuring migration ability of cultured neurospheres. Neurospheres with similar diameter were selected and plated onto 6-well culture plates coated with laminin (5 μg/ml) and poly-L-ornithine (10 μg/ml) and cultured for 48h in NSC growth medium. Cell migration and spreading was quantified by measuring the
distance between the edge of the neurosphere and the periphery of radially migrating cells.

**Luciferase reporter assay.** The 3’UTR of the *Notch1* gene, which contains one putative miR-146a targeting site, was amplified by PCR and inserted into the SacI and Pmel sites of *pMIR-REPORT* vector (Ambion). To express miR-146a, a 610-bp genomic fragment encompassing the coding region was cloned by PCR and inserted into the HindIII and BamHI sites of *pCMV6* vector. A seed-sequence-mutated version of miR-146a was used as a control for all of the experiments. To create this mutant (*pCMV6-miR-146a-mt*), we replaced the seed sequence **TGAGAAT** with **GCGGCCGC** through site-directed mutagenesis using the QuickChange kit (Stratagene). Similarly, the binding site (**AGTTCT**) for miR-146a within the 3’UTR of Notch 1 gene was replaced with **ACGCGT** to generate a control for the luciferase reporter assays. HEK293 cells were transiently transfected using Fugene 6 reagent (Roche) in 48-well plates. The following plasmids were used: *pMIR-REPORT* with either *pCMV6-miR-146a* or its mutant, *pCMV6-miR-146a-mt*. *pCMV-LacZ* was used as a control to monitor transfection efficiency. Total plasmid amount (200 ng) was kept constant for each transfection with empty *pCMV6* vector. Forty-eight hours later, cells were lysed and assayed for luciferase and β-galactosidase activity using the Dual-Light system (Applied Biosystems). Relative reporter activities were determined by normalizing luminescence units to β-galactosidase expression.
Western blotting and immunocytochemistry. Protein expression was examined by Western blotting, according to a standard procedure. Antibodies against the following proteins were used: β-Actin (Sigma, 1:8000), GFAP and p-AKT-T308 (Cell Signaling Technology, 1:1,000), Sox2 (Chemicon, 1:1,000), Nestin (Aves, 1:10,000), Notch1 (Santa Cruz, 1:1,000). To partially block processing of Notch1 protein by γ-secretase, cells were treated with 10 μM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Sigma) for 36 h. Blots were scanned and quantified by NIH software ImageJ. To examine proliferation and cell cycle exit, cultured cells were pulse-labeled with BrdU (10 μM) for 2 h and then fixed and processed for immunostaining, as previously described (56). Antibodies against the following proteins were used: BrdU (BD phamingen; 1:1,000), Ki67 (Novocastra; 1:500), cleaved caspase 3 (Cell Signaling Technology; 1:300) and Tuj1 (Covance; 1:500).

Intracranial and subcutaneous cell transplantation model for tumorigenesis. NOD/SCID and nude mice (nu/nu) were purchased from Harlan Laboratories. They were housed in a pathogen-free facility under standard 12h light/dark cycles, controlled temperature conditions and had free access to food and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at UT Southwestern Medical Center. Astrocytes (5X10^5 cells) or neurospheres (2X10^5 cells) were respectively implanted into the right caudoputamen of the brain (for orthotopic transplantation) or subcutaneous tissues of 5 to 6 weeks old NOD/SCID or nude mice using a 25 gauge-needle. The injection sites were monitored frequently. Mice transplanted with neurospheres were euthanized 3 weeks later. Tumors were finely
dissected out, imaged, and measured according to the following formula: Volume of
tumor (mm$^3$) = $d^2\times D/2$, where $d$ and $D$ are the shortest and the longest diameters, respectively. For survival analysis, the mice were euthanized when they demonstrated severe health deterioration according to IACUC guidelines.

Statistical analysis. Data are expressed as mean ± SD. Except for the survival studies, all data were analyzed by two-tailed Student $t$ tests. The Kaplan-Meier survival curves were determined by using Graphpad Prism v5.0 (Graphpad Software, Inc.) and the Log-rank test. A $p$ value of < 0.05 was considered significant.

RESULTS

EGFR activation and PTEN inactivation synergistically induce expression of miR-146a. Loss of Ink4a/Arf results in immortal growth of primary murine astrocytes. Subsequent activation of EGFR signaling through expression of a constitutively active EGFR$^{vIII}$ mutant transforms these astrocytes into tumorigenic and neurosphere-forming glioma stem-like cells when cultured in defined medium for NSCs (5, 24). In vivo, concomitant activation of EGFR and inactivation of Ink4a/Arf and Pten produce rapid-onset and fully penetrant high-grade gliomas that resemble GBM in humans (60), suggesting cooperative actions of these signaling pathways on cell proliferation. To confirm this, we performed MTT assays and direct cell counts to assess the growth rate of Ink4a/Arf$^+$, Ink4a/Arf$^+$EGFR$^{vIII}$, or Ink4a/Arf$^+$Pten$^+$EGFR$^{vIII}$ astrocytes. Indeed, constitutive activation of EGFR confers Ink4a/Arf$^+$ astrocytes with a growth advantage that is further enhanced by ablation of Pten (Fig. 1A and B).
To understand the downstream molecular events of this genetic cooperation and because of the emerging role of microRNA in cancers, we performed global expression analysis of microRNAs in Ink4a/Arf\(^{-/-}\), Ink4a/Arf\(^{-/-}\)EGFR\(^{vIII}\) or Ink4a/Arf\(^{-/-}\)Pten\(^{-/-}\)EGFR\(^{vIII}\) astrocytes. We identified a total of 19 unique miRNAs whose expression is altered by activation of EGFR alone or in combination with loss of Pten (Fig. 1C). Among these miRNAs, miR-146a, miR-182, miR-183 and miR-199a-3p are shown to be enriched in diverse cancers, whereas miR-127 and miR-140 are down-regulated in gliomas (32, 46). In contrast to unchanged expression of miR-146b, miR-146a was significantly induced by EGFR\(^{vIII}\) alone or in combination with ablation of Pten (Fig. 1C). The change in expression was subsequently confirmed by qPCR using independent RNA samples (Fig. 1D). We also examined the expression of miR-146a after acute deletion of Pten through Cre-mediated recombination of floxed Pten alleles in immortalized astrocytes (Fig. 1E and F). Pten loss resulted in more than a 7-fold induction of miR-146a expression. This induction was further increased to 9-fold after activation of EGFR. These data indicate a cooperative nature of EGFR, PTEN and INK4A/ARF signaling on cell proliferation and miR-146a expression.

**miR-146a inhibits proliferation and oncogenic potential of malignant astrocytes.** The induction of miR-146a by EGFR and/or PTEN signaling in Ink4a/Arf\(^{-/-}\) astrocytes suggests that miR-146a may act as an onco-miR by transducing oncogenic signals to control cell behavior. To test this hypothesis, we performed overexpression experiments to examine whether exogenous miR-146a could further enhance proliferation and...
tumorigenesis of malignant astrocytes. Due to extremely low efficiency of transient
transfections in these astrocytes, we employed a lentivirus-mediated expression system,
in which the CMV promoter drives expression of both miR-146a and GFP. As a control,
we mutated eight nucleotides within the seed region of miR-146a (Ctrl, Fig. 2A). Such a
mutation presumably abolishes the interactions of miR-146a with its targets.

Examination of GFP expression showed that nearly 100% of the cells were transduced
by lentivirus. qPCR analysis demonstrated a 26-fold increase of wild-type miR-146a in
Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes after lentiviral transduction (Fig. 2B). Since miR-
146a had no effect on apoptosis (Fig. 2E), we examined cell growth by MTT assays and
direct cell counting. Interestingly, overexpression of miR-146a inhibited proliferation of
Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes (Fig. 2C-D), indicating that this microRNA may
instead function as a tumor suppressor. Indeed, overexpression of miR-146a resulted in
a 25% reduction in the diameter (Fig. 2F) and a 35% decrease in the number (Fig. 2G)
of colonies in an anchorage-independent growth assay, which is an *in vitro* model for
cellular transformation.

High-grade gliomas exhibit aggressive behavior, which is manifested by rapid cellular
migration under culture conditions *in vitro* (12, 50). Using a standard ‘scratch’ assay
after the cells become confluent in culture dishes, we found that enhanced expression
of miR-146a significantly reduced the rate of migration of Ink4a/Arf−/−Pten−/−EGFRvIII
astrocytes when compared to the same cells transduced with a control lentivirus (Fig.
2H). This observation was further confirmed by a Matrigel transwell migration assay,
which showed a 50% reduction of migrating malignant astrocytes upon ectopic
expression of miR-146a (Fig. 2I). Finally, we examined the in vivo function of miR-146a during glioma development after cell transplantation into the right caudoputamen of NOD/SCID mice. For this, we grafted an equal number (5X10^5) of Ink4a/Arf⁻/⁻Pten⁻/⁻ EGFRvIII astrocytes that were transduced with lentiviruses expressing either wild-type miR-146a or seed-region-mutated miR-146a (Ctrl). Mice were monitored daily for morbidity. A subset of mice was also sacrificed around 3 weeks post transplantation to examine tumor burden by histology (Fig. 3A). Ectopic expression of miR-146a but not the Ctrl significantly reduced the tumor burden and prolonged the survival of mice that were transplanted with Ink4a/Arf⁻/⁻Pten⁻/⁻EGFRvIII astrocytes (Fig. 3B). Together, these data suggest that miR-146a acts as a tumor suppressor of glioma development.

miR-146a inhibits formation of glioma stem-like cells from malignant astrocytes.

Accumulating evidence suggests that self-renewable stem-like cells within the bulk of brain tumors are the driving force for initiation and maintenance of aggressive gliomas (13). These cells share common features with NSCs, including the ability to form neurospheres and the expression of stem cell markers, such as Sox2 and Nestin (5, 21). In fact, neurosphere formation is routinely used to enrich glioma stem-like cells from primary human brain tumors (31, 41). The finding that miR-146a inhibits tumorigenesis raised that possibility that it may negatively control the behavior of glioma stem-like cells. We enriched these cells from Ink4a/Arf⁻/⁻Pten⁻/⁻EGFRvIII astrocytes by culturing them under serum-free conditions in the presence of 20 ng EGF and 20 ng FGF. After 7 days in culture, control-virus-transduced cells readily formed large free-floating spheres, robustly expressing Nestin and Sox2 (Fig. 4A-D). Additionally, these neurospheres were
able to differentiate into GFAP-positive astrocytes and Tuj1-positive neurons in response to 1% fetal bovine serum and 5 μM forskolin treatment, respectively (Fig. 4C). In sharp contrast, ectopic expression of miR-146a markedly diminished the ability of Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes to form neurospheres, as indicated by a 40% reduction of sphere size and more than a 30% decline in sphere number (Fig. 4A). miR-146a also impaired the self-renewal ability of these neurospheres, demonstrated by a significant reduction of secondary and tertiary sphere formation upon serial passages (Fig. 4B). Furthermore, exogenous miR-146a led to a considerable decrease in the expression of Nestin and Sox2. This was accompanied by a sharp increase in the level of GFAP expression, indicating enhanced glial differentiation (Fig. 4D). The invasive behavior of glioma stem-like cells is evidenced by rapid cellular migration from neurospheres when attached to the coated plates in culture. We selected neurospheres with similar diameters from either control or miR-146a virus-transduced malignant astrocytes and plated them onto laminin- and polyornithine-coated chamber slides. Forty-eight hours later, control-virus-transduced cells rapidly migrated from the edge of the attached spheres and spread out onto the coated culture surfaces. In contrast, ectopic expression of miR-146a caused a 38% reduction of migratory distance from the edge of the spheres (Fig. 4E). These data indicate that miR-146a controls not only the number of glioma stem-like cells but also their invasive behavior in vitro.

miR-146a targets Notch1. How does miR-146a impose an inhibitory function on glioma stem-like cells and tumorigenesis? Using the Targetscan bioinformatics algorithm, we searched for direct targets of miR-146a with an emphasis on those genes that were
shown to play a role in cancer stem cells, tumorigenesis or NSCs. Among several potential candidates, Notch1 is most prominent due to its established function in maintaining NSCs (23, 27) (Fig. 5A). Notch1 also interacts with the EGFR and AKT pathways to positively regulate the proliferation of glioma stem-like cells and tumorigenesis (54). To confirm the regulation of Notch1 by miR-146a, we first performed a luciferase reporter assay by linking the 3’UTR of Notch1 to the firefly luciferase gene. Luciferase activity in COS7 cells was significantly reduced (by 34-57%) when the reporter was cotransfected with increasing amount of plasmids expressing wild-type miR-146a. Such reduction is specific since a control plasmid expressing seed-region-mutated miR-146a (Ctrl) has little effect on reporter activity. Moreover, wild type miR-146a did not change the activity of a luciferase reporter when the binding site for miR-146a in 3’UTR of Notch1 was mutated (Fig. 5B). Western blot analysis further showed that ectopic miR-146a in malignant astrocytes induced a dramatic reduction of Notch1 protein, especially the processed intracellular NICD, which is the most predominant form in these glia cells (Fig. 5C). When these cells were treated with DAPT, an inhibitor for γ-secretase, to partially block Notch1 processing, miR-146a significantly reduced the appearance of both full-length Notch1 and NICD (Fig. 5D). The inhibitory role of miR-146a on Notch1 is mainly through posttranscriptional control since it does not change the mRNA level of Notch1 (Fig. 5D). Furthermore, phosphorylated AKT, a known downstream target of Notch1 signaling (22, 58), was also markedly reduced (Fig. 5C). It is known that individual microRNAs can target numerous mRNAs (6), raising the possibility that Notch1 may not be the major target of miR-146a in glioma stem-like cells. We examined this possibility by performing a rescue experiment. Interestingly, ectopic
expression of NICD completely reversed the inhibitory effect of miR-146a on the formation of glioma stem-like cells. This was indicated by a respective 50% and 60% increase in the number and size of neurospheres when compared to miR-146a-expressing cells (Fig. 5F). Importantly, overexpressing NICD did not change the level of miR-146a, suggesting that the rescue was not due to down-regulation of miR-146a expression (data not shown).

**miR-146a regulates NSC proliferation and differentiation.** Notch signaling plays an essential role in maintaining NSCs (2, 23). Its down-regulation promotes neurogenesis during development or in the adult stage (40, 55). Our finding that miR-146a targets Notch1 expression suggests that miR-146a may regulate NSC behavior. Indeed, qPCR analysis showed over 12-fold induction of miR-146a expression after 4 days of culturing primary mouse E14.5 NSCs under differentiation conditions (Fig. 6A). Ectopic expression of miR-146a through lentiviral transduction enhanced neuronal differentiation, indicated by a 5-fold increase of Tuj1+ cells compared to control-virus-transduced NSCs under low EGF and FGF concentrations (1 ng/ml) (Fig. 6B and C). Exogenous miR-146a also inhibited NSC proliferation, demonstrated by a 50% reduction of either BrdU+ or Ki67+ cells (Fig. 6D and E). Such reduction of proliferation was accompanied by increased cell cycle exit, which was shown by a higher ratio of BrdU+Ki67− cells over the total number of BrdU+ cells after 2 hr of BrdU pulse (Fig. 6F). In contrast, miR-146a had no effect on apoptosis, since an equal number of active caspase 3-positive cells were observed (Fig. 6D). Together, these results indicate that miR-146a potentiates neuronal differentiation of NSCs by promoting cell cycle exit.
miR-146a prolongs the survival of mice bearing human glioblastoma cells. We next examined whether the biological role of miR-146a is evolutionarily conserved in humans. Similar to murine Notch1 gene, the 3’UTR of its human ortholog also harbors a potential binding site for miR-146a, albeit not a perfect match (Fig. 7A). Western blotting analysis showed that ectopic miR-146a markedly reduced the level of both full-length and the active form (NICD) of human Notch1 protein in U87 glioblastoma cells (Fig. 7B). miR-146a also diminished the ability of U87 cells to form glioma stem-like cells. This was indicated by a more than 50% decline in sphere number, a 31% reduction of sphere size (Fig. 7C), and a near 40% decrease on the appearance of secondary and tertiary spheres (data not shown). When U87 glioblastoma cells were transplanted into the caudoputamen of NOD/SCID mice, miR-146a reduced tumor burden and significantly extended the survival of tumor-bearing mice (Fig. 7D-E). Together, these data suggest that miR-146a function is evolutionarily conserved from mice to human and is able to modulate the behavior of glioma cells both in vitro and in vivo.

Down-regulation of miR-146a function enhances tumorigenesis. miR-146a is induced by oncogenic EGFR and PTEN signaling (Fig. 1). However, its overexpression inhibits the behavior of glioma stem-like cells and tumorigenesis by targeting Notch1. These seemingly paradoxical data suggest that miR-146a may act as a feedback mechanism to restrict tumorigenesis (see below). To vigorously test this hypothesis, we used an ‘miRNA sponge’ (14) to knock down miR-146a function in Ink4a/Arf−/−Pten−/− EGFRvIII astrocytes. This sponge was designed to have an imperfect binding site near
the miR-146a seed region – with a bulge from positions 10 to 13 (Fig. 8A). This design is presumed to be more effective and stable for inhibiting miR-146a function. We first evaluated its efficacy by using a Notch1 3'UTR-containing luciferase reporter and found that the sponge completely reversed the inhibitory effect of miR-146a on this reporter (Fig. 8B). We also examined this miRNA sponge on the expression of Notch1 through Western blotting. As expected, transduction of Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes with sponge-expressing lentiviruses totally rescued Notch1 protein level that was inhibited by ectopic miR-146a (Fig. 8C). Moreover, this sponge also enhanced the expression of endogenous Notch1, most likely by releasing the inhibition of miR-146a that is induced by EGFRvIII and Pten-loss (Fig. 8D). These data indicate that the use of a miRNA sponge is an effective and specific way to down-regulate miR-146a function. We next examined the impact of knocking down miR-146a on the behavior of glioma stem-like cells and tumorigenesis. As expected if miR-146a has an inhibitory role, its down-regulation promoted efficient formation of glioma stem-like cells from Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes by significantly increasing the number and the size of neurospheres (Fig. 8E and F). When neurospheres (2X10^5 cells) from lentivirus-transduced malignant astrocytes were grafted into the flanks of female nude mice, knocking-down miR-146a function using a miRNA sponge caused more than an 80% increase in tumor size 28 days post transplantation (Fig. 8G). Therefore, these results indicate that miR-146a is induced as a feedback mechanism to restrict the oncogenic potential of EGFR and PTEN signaling.

DISCUSSION
Here, we reveal that constitutively active EGFR\textsuperscript{vIII} cooperates with the loss of Pten to synergistically induce expression of miR-146a in Ink4a/Arf\textsuperscript{-/-} astrocytes. Counterintuitively, upregulation of miR-146a inhibits tumor growth and the formation and migration of glioma stem-like cells by both malignant murine Ink4a/Arf\textsuperscript{-/-}/Pten\textsuperscript{-/-}/EGFR\textsuperscript{vIII} astrocytes and human glioblastoma cells. We further show for the first time that miR-146a directly downregulates Notch1 and potentiates differentiation of normal NSCs. Knocking down miR-146a function enhances glioma stem-like cell formation and exacerbates tumor burden. These data suggest that miR-146a integrates oncogenic cues to restrict tumor development as a feedback mechanism (Fig. 8H).

Previously, miR-146a was shown to be induced by endotoxin (lipopolysaccharide) through two consensus NF-\kappa B binding sites in the promoter region (9). This region is highly homologous between human and mouse, suggesting an evolutionarily conserved regulatory mechanism for controlling miR-146a expression. NF-\kappa B is constitutively activated in glioma and many other cancer cells, in which it promotes survival and metastatic potential of these cells as well as tumorigenesis (28, 52). One of the key pathways that controls NF-\kappa B activity in gliomas is the PI3K pathway (16, 39), which is a converging point of EGFR- and PTEN-dependent signal transduction. Activation of receptor tyrosine kinase EGFR leads to recruitment and activation of PI-3 kinase, which phosphorylates phosphoinositol lipids to generate phosphatidylinositol-3-phosphates. These lipids in turn, recruit and activate AKT in the plasma membrane through PDK1. The function of PI3K is antagonized by PTEN, a lipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate. Activated AKT further...
phosphorylates IKK, thus promoting NF-κB activation. The synergistic induction of miR-146a by constitutively active EGFR\textsuperscript{vIII} and inactivation of \textit{Pten} in \textit{Ink4a/Arf}\textsuperscript{-/-} astrocytes may reflect a convergence of these two signaling pathways on NF-κB activity. Activated AKT also promotes Notch1 expression, which subsequently modulates transcription of \textit{Egfr} through p53 \textsuperscript{(44, 45)}. These data indicate that miR-146a belongs to an integrated genetic circuit consisting of the PTEN, EGFR, NF-κB and Notch pathways. The results from our current study reveal that miR-146a regulates the activity of this circuit by targeting Notch1 expression.

Recent studies indicate that certain miRNAs (such as miR-124, miR-137, miR-128, miR-7) function as tumor suppressors \textsuperscript{(19, 49)}. These miRNAs are rarely expressed in gliomas; however, their overexpression restrains proliferation and self-renewal of glioma stem-like cells by promoting neural differentiation \textsuperscript{(20, 21)}. miR-146a is unique in that it is significantly enriched in human tissues of skin (melanoma), cervical, breast, pancreas and prostate cancers, compared to the same non-cancerous tissues \textsuperscript{(42, 51, 53)}. Similarly, miR-146a was also up-regulated in human glioblastoma tissues and in both human and mouse primary glioma cell lines \textsuperscript{(32)}. In this regard, increased expression of miR-146a can be viewed as a biomarker for cancers. Unexpectedly, our study reveals that, instead of promoting gliomagenesis through its upregulation, miR-146a rather plays an inhibitory role in restricting the formation of glioma stem-like cells and tumor burden. This result is consistent with a demonstrated role of miR-146a in other cancers, such as pancreatic and breast cancers, where it inhibits cancer progression and invasion \textsuperscript{(8, 26, 33)}. A most recent report also showed a positive correlation of miR-
Furthermore, overexpression of miR-146a inhibits proliferation and survival of breast, prostate and pancreatic cancer cells through down-regulation of other targets in these cells, including ROCK1, EGFR, and MTA-2 (8, 33, 34). Our study adds Notch1 as a major target of miR-146a in glioma cells. Supporting these cell culture models of tumorigenesis, it was recently reported that mice with a deletion of miR-146a spontaneously developed subcutaneous flank tumors (37). This data clearly indicates that miR-146a serves as a native molecular brake for oncogenesis (3).

In summary, our current results and other emerging data indicate that miR-146a constitutes an endogenous feedback system to counteract the oncogenic potential of dysregulated signaling pathways, such as activation of EGFR and inactivation of Pten in gliomas. By regulating multiple targets, including key neural stem cell factor Notch1, a miR-146a-mediated innate regulatory mechanism provides an opportunity to devise novel therapeutic strategies against aggressive and deadly brain tumors.

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REFERENCES


FIGURE LEGENDS

**FIG. 1.** Induction of miR-146a expression by EGFR and PTEN signaling in immortalized Ink4a/Arf⁻/⁻ astrocytes. (A-B) Proliferation of Ink4a/Arf⁻/⁻ (I), Ink4a/Arf⁻/⁻EGFR⁻/⁻ (IE), or Ink4a/Arf⁻/⁻Pten⁻/⁻EGFR⁻/⁻ (IPE) astrocytes determined by MTT assay (A) or by cell counting (B) (n=3). (B) Heat map representation of differentially expressed miRNAs identified by microarray analysis. (C) qPCR analysis to confirm miR-146a expression induced by EGFR and PTEN signaling using independent RNA samples. (D) Effects of acute Pten loss on expression of miR-146a in Ink4a/Arf⁻/⁻ Pten⁻/⁻ (IP⁻/+ and IP⁻/⁻) astrocytes. These cells were transduced with adenoviruses expressing either GFP or GFP-Cre. (F) Acute deletion of Pten in Ink4a/Arf⁻/⁻ Pten⁻/⁻ and Ink4a/Arf⁻/⁻Pten⁻/⁻EGFR⁻/⁻ astrocytes. Astrocytes were transduced with adenoviruses expressing GFP (Ad-GFP) or GFP-Cre (Ad-GFP-Cre). Using primers located in the floxed exon 5 of the Pten gene, a 157-bp PCR product can be detected in Ad-GFP-transduced but not Ad-GFP-Cretransduced astrocytes. Expression of Hprt was used as a loading control. Each experiment was performed in triplicate, except for microarrays that were in duplicate (*p < 0.05, **p < 0.01, and ***p < 0.001).
FIG. 2. miR-146a inhibits cellular transformation and migration of malignant astrocytes.

(A) Schema of miR-146a mutant (Ctrl). The seeding sequence of miR-146a is replaced with a Not1 digestion site. (B) qPCR analysis of miR-146a expression in lentivirus-transduced astrocytes. Experiments were performed in triplicate. (C-D) Growth curves of Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes transduced with lentiviruses expressing either miR-146a or its mutant (Ctrl) by MTT assay (C) or by cell counting (D) (n=3). (E) Apoptotic cells were detected by an antibody recognizing cleaved caspase 3 and nuclei were shown by DAPI-staining. (F-G) Ectopic expression of miR-146a restrains clonogenic growth of Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes in a soft-agar assay (n=3). Representative images (100× magnification) are shown. (H) A scratch assay to examine the effect of miR-146a on migration of malignant astrocytes (n=3). Representative images (40× magnification) at 0 and 24 hours are shown. (I) Transwell migration assay of Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes transduced with lentiviruses expressing either miR-146a or its mutant (Ctrl) (n=3) (*p < 0.05, **p < 0.01, and ***p < 0.001).

FIG. 3. miR-146a decreases tumor burden induced by intracranial transplantation of Ink4a/Arf−/−Pten−/−EGFRvIII murine astrocytes. (A) Representative histological analysis of brain gliomas in NOD/SCID mice 3 weeks post transplantation. In each experiment, a seed region-mutated version of miR-146a was used as a control (Ctrl). (B) Kaplan-Meier survival curve of mice transplanted with Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes (n=11 and 8 for the control and miR-146a group, respectively; p=0.002 by Log-rank Test).
FIG. 4. miR-146a reduces formation of glioma stem-like cells and their migration. (A) Glioma stem-like cells were established by culturing 5,000 Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes in NSC medium. The number of spheres and their diameter were determined 7 days later. A representative morphology of the spheres is shown in the left panels at ×100 magnification (n=3). (B) Number counts of primary, secondary and tertiary neurospheres established by culturing 2,500 Ink4a/Arf−/−Pten−/−EGFRvIII murine astrocytes in NSC medium (n=3). (C) In the absence of growth factor, neurospheres (derived from Ink4a/Arf−/−Pten−/−EGFRvIII astrocytes) were differentiated into GFAP-positive astrocytes and Tuj1-positive neurons in response to 1% fetal bovine serum and 5 μM forskolin (FSK) treatment, respectively. (D) Ectopic expression of miR-146a induces glial marker GFAP and suppresses expression of stem cell markers Sox2 and Nestin. Actin was used as a loading control for Western blotting. Relative protein level is shown. (E) miR-146a inhibits spreading and migration of glioma stem-like spheres. Representative photographs were taken at ×100 magnification. Distance was measured from the edge of the sphere to the periphery of the migrating cells (n=3; *p < 0.05, **p < 0.01 and ***p < 0.001).

FIG. 5. Notch1 is a direct target of miR-146a. (A) Sequence alignments between 3’UTR of mouse Notch1 and miR-146a. (B) miR-146a suppresses the activity of a luciferase reporter that is linked to the 3’UTR of Notch1, but not mutant 3’UTR of Notch1. After normalization to β-galactosidase expression, which was used as a control for transfection efficiency, the data was presented as the ratio to that of empty-vector–transfected cells. (C) miR-146a blocks protein expression of Notch1 and its downstream
target, pAKT, in Ink4a/Arf−/−Pten−/−EGFRVIII astrocytes. Protein loading was monitored by β-actin. Relative protein level is indicated. (D) Blocking Notch1 processing by DAPT, a γ-secretase inhibitor, reveals the inhibitory effect of miR-146a on full-length Notch1 protein in murine astrocytes. (E) miR-146a has no effect on the expression of Notch1 transcripts, determined by qPCR. (F) NICD rescues the inhibitory effect of miR-146a on the formation of glioma stem-like cells from Ink4a/Arf−/−Pten−/−EGFRVIII astrocytes (*p < 0.05 and ***p < 0.001).

FIG. 6. miR-146a regulates normal NSCs. (A) Induction of miR-146a after culturing NSCs under differentiation conditions for 4 days. GF, growth factor bFGF and EGF; RA, retinoic acid; FSK, forskolin. (B) and (C) Promoting neuronal differentiation of normal NSCs by miR-146a. Neurons were stained with an antibody against Tuj1 and nuclei were shown by DAPI staining. Representative photographs were taken at ×400 magnification. (D) - (F) miR-146a inhibits NSC proliferation by inducing cell cycle exit. (D) Proliferating cells were examined by BrdU incorporation and Ki67 staining, which are shown in merged panels with phase-contrast images. Apoptotic cells were stained with an antibody for cleaved caspase 3 (Casp3). Representative photographs were taken at ×200 magnification. (E) Quantification of proliferating NSCs (BrdU+ or Ki67+). (F) miR-146a enhances cell cycle exit, which was measured by the ratio of BrdU+Ki67− cells to total number of BrdU+ cells. Experiments were performed in triplicate (**p < 0.01 and ***p < 0.001).
FIG. 7. miR-146a promotes survival of mice transplanted with human glioblastoma cells. (A) A recognition site for miR-146a in the 3’UTR of human Notch1 gene. (B) miR-146a targets human Notch1. Protein levels were examined by Western blotting analysis of lysates from human U87 glioblastoma cells. Protein loading was monitored by β-actin. Relative protein level is indicated. (C) miR-146a inhibits the formation of glioma stem-like cells from U87. These cells were enriched in NSC medium. The number of spheres and their diameter were measured 7 days later. (D) and (E) miR-146a promotes survival of tumor-bearing mice by reducing glioma burden. (D) Histological analysis of brain tumors 3 weeks post intracranial transplantation. (E) Kaplan-Meier survival curve of mice transplanted with human U87 glioblastoma cells (n=6 for each group; p=0.006 by Log-rank Test).

FIG. 8. Knocking down miR-146a function promotes tumorigenesis. (A) Design of miRNA sponge. (B) Sponge suppresses miR-146a’s function on activity of a luciferase reporter that is linked to the 3’UTR of mouse Notch1 gene. The data was presented as the ratio to that of miR-146a mutant–transduced cells. (C) miRNA sponge reverses miR-146a-mediated down-regulation of Notch1 expression. Relative protein level is indicated. (D) Enhancing endogenous Notch1 expression by miRNA sponge in Ink4a/Arf−/Pten−/EGFRVIII astrocytes. (E) and (F) miRNA sponge against miR-146a promotes formation of glioma stem-like cells from malignant astrocytes (n=3). (G) Knocking down miR-146a function by sponge exacerbates tumor burden. The volume of subcutaneous tumors was measured after microdissection (n=5; *p < 0.05, **p < 0.01, and ***p < 0.001). (H) A diagram showing miR-146a-involved signaling network.
**A**

Seed
GCAAGAGT-5'  miR-146a
TTAA CAGTCCTCA-3'

5'--AACCAATGG
3'--TTGGGTACC
Sponge

**B**

Luciferase activity

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**E**

Neurosphere number

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**F**

Diameter (μm)

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**G**

Tumor volume (mm³)

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**H**

Gliogenesis

- EGFRvIII
- Notch1
- Other targets

Gliogenesis