MicroRNA-146a Inhibits Glioma Development by Targeting Notch1

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MicroRNA-146a Inhibits Glioma Development by Targeting Notch1

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Dysregulated epidermal growth factor receptor (EGFR) signaling through either genomic amplification or dominant-active mutation (EGFRvIII), in combination with the 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, overexpression of miR-146a attenuates the proliferation, migration, and tumorigenic potential of Ink4a/Arf−/− Pten−/− EgfrvIII murine astrocytes. Its ectopic expression also inhibits the glioma development of a human glioblastoma cell line in an orthotopic xenograft model. Such an inhibitory function of miR-146a on gliomas is largely through downregulation 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 migration and glioma 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 for treating brain tumors.

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. Epidermal growth factor receptor (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 (4). 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 gliogenic cells have not been fully elucidated.

MicroRNAs are 20- to 22-nucleotide noncoding RNA molecules that have emerged as key players in controlling NSC self-renewal and differentiation (11, 57). Aberrant expression of microRNAs, 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 the 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 (3, 10, 23). Upon binding to its ligand, Delta, the Notch intracellular

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gion-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 qRT-PCR. We extracted total RNAs from Ink4a/Arf−/− (I), Ink4a/Arf−/− EgfrvIII (IE), or Ink4a/Arf−/− Pten−/− EgfrvIII (IPE) astrocytes using the miReasy minikit (Qiagen). Biological replicates were collected. RNA quality was examined by Bioanalyzer (Illumina). Portions (5 μg) of each RNA sample were 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 VAMPIR software to identify statistically significant differences in gene expression between sample groups (http://genome.ucsd.edu/microarray). For quantitative reverse transcription-PCR (qRT-PCR), 1 μg of total RNA was reverse transcribed using by Ncode miRNA first-strand synthesis and qRT-PCR kits (Invitrogen). qRT-PCR 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 15 s at 95°C and 60 s at 58°C. Primer efficiency was monitored by determining the green fluorescent protein (GFP) expression. All experiments were performed using stably transfected cells within six passages.

Cell migration assay. Migration of malignant astrocytes in culture was determined by the “scratch” assay. For this, cells were seeded into a six-well tissue culture dish and allowed to grow to 90% confluence 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 24 h. The migratory distance was measured under a microscope equipped with a camera. A Transwell migration assay was conducted essentially as previously described (38, 48). The transfection efficiency was monitored by determining the green fluorescent protein (GFP) expression. All experiments were performed using stably transfected cells within six passages.

Luciferase reporter assay. The 3′ untranslated region (3′ UTR) of the Notch1 gene, which contains one putative miR-146a targeting site, was amplified by PCR into pTomo vector (provided by 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 titers, and transduced cultured cells according to previously described methods (38, 48). The transfection efficiency was monitored by determining the green fluorescent protein (GFP) expression. All experiments were performed using stably transfected cells within six passages.

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 V1007D 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−/− EgfrvIII, or Ink4a/Arf−/− Pten−/− EgfrvIII 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 micro-RNAs in Ink4a/Arf−/−, Ink4a/Arf−/− EgfrvIII, or Ink4a/Arf−/− Pten−/− EgfrvIII 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 downregulated in gliomas (32, 46). In contrast to the unchanged expression of miR-146b, miR-146a was significantly induced by EGFR V1007D 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
FIG. 1. Induction of miR-146a expression by EGFR and PTEN signaling in immortalized Ink4a/Arf−/− astrocytes. (A and B) Proliferation of Ink4a/Arf−/− (I), Ink4a/Arf−/− EgfrvIII (IE), or Ink4a/Arf−/− Pten−/− EgfrvIII (IPE) astrocytes, as determined by MTT assay (A) or by cell counting (B) (n = 3). (C) Heat map representation of differentially expressed miRNAs identified by microarray analysis. (D) qRT-PCR analysis was performed to confirm miR-146a expression induced by EGFR and PTEN signaling using independent RNA samples. (E) Effects of acute Pten loss on the expression of miR-146a in Ink4a/Arf−/− Ptenf/f (IPf/f) or Ink4a/Arf−/− Pten−/− EgfrvIII (IPvE) astrocytes. These cells were transduced with adenoviruses expressing either GFP or GFP-Cre. (F) Acute deletion of Pten in Ink4a/Arf−/− Ptenf/f and Ink4a/Arf−/− Pten−/− EgfrvIII 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-Cre-transduced astrocytes. The expression of Hprt was used as a loading control. Each experiment was performed in triplicate, except for microarrays that were performed in duplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

miR-146a inhibits the 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 the extremely low efficiency of transient transfections in these astrocytes, we used a lentivirus-mediated expression system, in which the cytomegalovirus (CMV) promoter drives the 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. qRT-PCR 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 and D), indicating that this miRNA 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 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 (5 × 105) 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 posttransplantation 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 the 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...
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miR-146a on the migration of malignant astrocytes (n/H11003
Nestin and Sox2 (Fig. 4A to D). In addition, these neuro-
readily formed large free-floating spheres, robustly expressing
of FGF. After 7 days in culture, control-virus-transduced cells
rum-free conditions in the presence of 20 ng of EGF and 20 ng

Growth curves of
duced astrocytes. Experiments were performed in triplicate. (C and D)

miR-146a mutant (Ctrl). The seeding sequence of miR-146a is replaced with a NotI digestion
site. (B) qRT-PCR analysis of miR-146a expression in lentivirus-trans-

miR-146a groups, respectively; miR-146a/**0.002 [log-rank test].

FIG. 3. miR-146a decreases the tumor burden induced by intracra-
nial transplantation of Ink4a/Arf−/−Pten−/−EgrfIII murine astrocytes. (A) Representative histological analysis of brain gliomas in NOD/SCID mice 3 weeks posttransplantation. 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−/−EgrfIII astrocytes (n = 11 and 8 for the control and miR-146a groups, respectively; P = 0.002 [log-rank test]).

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−/−EgrfIII astrocytes by culturing them under se-
rum-free conditions in the presence of 20 ng of EGF and 20 ng
of FGF. After 7 days in culture, control-virus-transduced cells
readily formed large free-floating spheres, robustly expressing
Nestin and Sox2 (Fig. 4A to D). In addition, these neuro-
spheres were able to differentiate into GFAP-positive astro-
cytes and TuJ1-positive neurons in response to 1% FBS and 5
µM FSK treatment, respectively (Fig. 4C).

How does miR-146a impose an inhibitory function on glioma stem-like cells and tumorigen-

miR-146a targets Notch1. How does miR-146a impose an inhibitory function on glioma stem-like cells and tumorigen-

FIG. 2. miR-146a inhibits the cellular transformation and migra-
tion of malignant astrocytes. (A) Schema of miR-146a mutant (Ctrl). (B) qRT-PCR analysis of miR-146a expression in lentivirus-trans-
duced astrocytes. Experiments were performed in triplicate. (C and D)

FIG. 4. miR-146a inhibits the cellular transformation and migra-
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ity of Ink4a/Arf−/−Pten−/−EgrfIII astrocytes to form neuro-
spheres, as indicated by a 40% reduction of sphere size and a
>30% decline in sphere number (Fig. 4A). miR-146a also
impaired the self-renewal ability of these neurospheres, dem-
ostated 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 migra-
tion 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 astro-
cytes and plated them onto laminin- and polyornithine-coated
chamber slides. After 48 h, control-virus-transduced cells rapidly
migrated from the edge of the attached spheres and spread
out onto the coated culture surfaces. In contrast, ectopic ex-
pression 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.
FIG. 4. miR-146a reduces the formation of glioma stem-like cells and their migration. (A) Glioma stem-like cells were established by culturing 5,000 Ink4a/Arf<sup>−/−</sup> Pten<sup>−/−</sup> Egfr<sup>VIII</sup> astrocytes in NSC medium. The number of spheres and their diameters 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<sup>−/−</sup> Pten<sup>−/−</sup> Egfr<sup>VIII</sup> murine astrocytes in NSC medium (n = 5). (C) In the absence of growth factor, neurospheres (derived from Ink4a/Arf<sup>−/−</sup> Pten<sup>−/−</sup> Egfr<sup>VIII</sup> astrocytes) were differentiated into GFAP-positive astrocytes and Tuj1-positive neurons in response to 1% FBS and 5 μM forskolin (FSK) treatments, respectively. (D) Ectopic expression of miR-146a induces the glial marker GFAP and suppresses their migration. Distance was measured from the edge of the sphere to the periphery of the migrating cells (n = 3). *, P < 0.05; **, P < 0.01; ***, 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 were presented as the ratio to that of empty-vector-transfected cells. (C) miR-146a blocks the protein expression of Notch1 and its downstream target, pAKT, in Ink4a/Arf<sup>−/−</sup> Pten<sup>−/−</sup> Egfr<sup>VIII</sup> astrocytes. Protein loading was monitored by β-actin. The 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, as determined by qRT-PCR. (F) NICD rescues the inhibitory effect of miR-146a on the formation of glioma stem-like cells from Ink4a/Arf<sup>−/−</sup> Pten<sup>−/−</sup> Egfr<sup>VIII</sup> astrocytes. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Notch1 to the firefly luciferase gene. The luciferase activity in COS7 cells was significantly reduced (by 34 to 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. 5E). 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 the respective 50 and 60% increase in the number and size of neurospheres 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 downregulation of miR-146a expression (data not shown).

miR-146a regulates NSC proliferation and differentiation.

Notch signaling plays an essential role in maintaining NSCs (1, 23). Its downregulation promotes neurogenesis during development or in the adult stage (40, 55). Our finding that miR-
miR-146a targets Notch1 expression suggests that miR-146a may regulate NSC behavior. Indeed, qRT-PCR analysis showed >12-fold induction of miR-146a 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 controls (Fig. 6B and C). Exogenous miR-146a also inhibited NSC proliferation, as demonstrated by a 50% reduction in either BrdU+ or Ki67+ cells over the total number of BrdU+ cells after 2 h of BrdU pulse (Fig. 6D). In contrast, miR-146a had no effect on apoptosis, since an equal number of active caspase 3-positive cells was observed (Fig. 6D). Together, these results indicate that miR-146a potentiates neuronal differentiation of NSCs by promoting cell cycle exit.

**miR-146a potentiates neuronal differentiation of NSCs.**

**miR-146a targets Notch1 expression.** Western blotting showed that ectopic miR-146a markedly reduced the level of both the full-length and the active forms (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 50% decline in sphere number, a 31% reduction in sphere size (Fig. 7C), and a nearly 40% decrease in 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 the tumor burden and significantly extended the survival of tumor-bearing mice (Fig. 7D and E). Together, these data suggest that miR-146a potentiates neuronal differentiation of NSCs by promoting cell cycle exit.

**miR-146a regulates normal NSCs.**

**miR-146a prolongs the survival of mice bearing human glioblastoma cells.**

miR-146a promotes the survival of mice transplanted with 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 showed that ectopic miR-146a markedly reduced the level of both the full-length and the active forms (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 >50% decline in sphere number, a 31% reduction in sphere size (Fig. 7C), and a nearly 40% decrease in 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 the tumor burden and significantly extended the survival of tumor-bearing mice (Fig. 7D and E). Together, these data suggest that miR-146a potentiates neuronal differentiation of NSCs by promoting cell cycle exit.
that miR-146a function is evolutionarily conserved from mice to humans and is able to modulate the behavior of glioma cells both in vitro and in vivo.

Downregulation 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 EGFRIII and Pten−/− loss (Fig. 8D). These data indicate that the use of a miRNA sponge is an effective and specific way to downregulate 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 downregulation promoted efficient formation of glioma stem-like cells by significantly increasing the number and the size of neurospheres (Fig. 8E and F). When neurospheres (2 × 10^5 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 an 80% increase in tumor size by 28 days posttransplantation (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

We reveal here that constitutively active EGFRvIII cooperates with the loss of Pten to synergistically induce expression of miR-146a in Ink4a/Arf−/− astrocytes. Counterintuitively, up-regulation of miR-146a inhibits tumor growth and the formation and migration of glioma stem-like cells by both malignant
murine \textit{Ink4a/Arf}^{-/-} \textit{Pten}^{-/-} \textit{Egfr}^{III} 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-κB 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-κB 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-κB activity in gliomas is the phosphatidylinositol 3-kinase (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 phosphoinositide 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 IκK, thus promoting NF-κB activation. The synergistic induction of miR-146a by constitutively active EGFR \textsuperscript{III} and the inactivation of \textit{Pten} in \textit{Ink4a/Arf}^{-/-} 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{Egrf} through p53 (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 present 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, and miR-7) function as tumor suppressors (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 (20, 21). miR-146a is unique in that it is significantly enriched in the human tissues of skin (melanoma), cervical, breast, pancreas, and prostate cancers compared to the same noncancerous tissues (42, 51, 53). Similarly, miR-146a was also upregulated in human glioblastoma tissues and in both human and mouse primary glioma cell lines (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 (8, 26, 33). A recent report also showed a positive correlation of miR-146a expression with the survival time of gastric cancer patients (25). Furthermore, the overexpression of miR-146a inhibits the proliferation and survival of breast, prostate, and pancreatic cancer cells through the downregulation 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). These data clearly indicate that miR-146a serves as a native molecular brake for oncogenesis (2).

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 \textit{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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