

Targeting of the Bmi-1 Oncogene/Stem Cell Renewal Factor by MicroRNA-128 Inhibits Glioma Proliferation and Self-Renewal

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Abstract

MicroRNAs (miR) show characteristic expression signatures in various cancers and can profoundly affect cancer cell behavior. We carried out miR expression profiling of human glioblastoma specimens versus adjacent brain devoid of tumor. This revealed several significant alterations, including a pronounced reduction of miR-128 in tumor samples. miR-128 expression significantly reduced glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo*. miR-128 caused a striking decrease in expression of the Bmi-1 oncogene, by direct regulation of the Bmi-1 mRNA 3'-untranslated region, through a single miR-128 binding site. In a panel of patient glioblastoma specimens, Bmi-1 expression was significantly up-regulated and miR-128 was down-regulated compared with normal brain. Bmi-1 functions in epigenetic silencing of certain genes through epigenetic chromatin modification. We found that miR-128 expression caused a decrease in histone methylation (H3K27me³) and Akt phosphorylation, and up-regulation of p21^{CIP1} levels, consistent with Bmi-1 down-regulation. Bmi-1 has also been shown to promote stem cell self-renewal; therefore, we investigated the effects of miR-128 overexpression in human glioma neurosphere cultures, possessing features of glioma "stem-like" cells. This showed that miR-128 specifically blocked glioma self-renewal consistent with Bmi-1 down-regulation. This is the first example of specific regulation by a miR of a neural stem cell self-renewal factor, implicating miRs that may normally regulate brain development as important biological and therapeutic targets against the "stem cell-like" characteristics of glioma. [Cancer Res 2008;68(22):9125–30]

Introduction

Approximately 20,000 new patients are diagnosed in the United States every year with glioma, and for the most malignant form (glioblastoma), median survival is approximately 14 months, even with aggressive surgery, radiation, and chemotherapy (1). Recent studies have shown that gliomas retain many features of neuronal progenitor cells, including the ability to grow as neurospheres in

culture, self-renew, and migrate in the brain (2–4). Profiling studies have shown that gliomas express many genes known to play a role in neural progenitor cells (5). These observations have created novel opportunities for developing therapeutics based on differentiation or targeting of self-renewal features of glioma (6).

MicroRNAs (miRs) play important roles in diverse biological processes, including development, differentiation, stem cell maintenance, and cell identity (7). miRs also play a role in cancer by controlling the expression of certain oncogenes and tumor suppressor genes (8). miR profiling has revealed distinct expression signatures in various human cancers, including glioma (9). The functional significance of most of these alterations remains unclear. In this study, we report that miR-128 is down-regulated in gliomas, that its expression reduces glioma cell proliferation, and that Bmi-1 (B lymphoma mouse Moloney leukemia virus insertion region) is a direct target of miR-128. Bmi-1 is up-regulated in several cancer types and is a positive regulator of stem cell renewal (10–12), and studies in transgenic mice revealed a critical role for Bmi-1 in driving glioma growth (13). Our results show that the effects of miR-128 on glioma cells are consistent with Bmi-1 down-regulation, including a reduction in glioma stem cell self-renewal, suggesting that miR-128 may be a candidate for the therapeutic targeting of glioma stem cells.

Materials and Methods

Cell culture. U87MG, U251MG, and U373MG glioma cells were obtained from the American Type Culture Collection and cultured in DMEM (Invitrogen) containing 10% FCS (Sigma). Primary human glioma OHG02 cells were derived from freshly resected human glioma specimens and grown as tumor spheres as previously reported (4). Tumors were dissociated using TrypLE Express (Invitrogen) and cultured in Neurobasal medium (Invitrogen) with B27 (Invitrogen), human recombinant leukemia inhibitory factor (10 ng/mL; Chemicon International), basic fibroblast growth factor (50 ng/mL; PeproTech), epidermal growth factor (50 ng/mL; PeproTech), penicillin (100 units/mL), streptomycin (100 µg/mL), and L-glutamine (2 mmol/L; Invitrogen). OHG02 cells possessed many of the characteristics that distinguish the glioma initiator or "stem-like" cell subpopulation (data not shown), including self-renewal, CD133 expression (4), and appropriate lineage markers (nestin, β-tubulin, and GFAP) when differentiated. When implanted intracranially in athymic mice, OHG02 cells formed invasive tumors, with phenotypic characteristics of human glioblastoma (data not shown). All tumor samples were obtained as approved by the Institutional Review Board at The Ohio State University. Surgery was performed by E.A. Chiocca. At the time of surgery, brain adjacent to tumor but pathologically devoid of gross tumor was also harvested.

Microarray profiling of miRs in glioma. Expression profiling was performed using total RNA with the human/mouse miR expression array (V 4.0) and analyzed as described (14). Microarray data were deposited in the ArrayExpress database according to MIAME recommendations (accession number E-MEXP-1796).

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Table 1. miR alterations in glioblastoma tissue compared with adjacent brain devoid of tumor from the same patients

miR	SAM score*	Fold change
Down-regulated in tumor		
<i>hsa-miR-124a</i>	-6.05	0.04
<i>hsa-miR-137</i>	-5.34	0.06
<i>hsa-miR-323</i>	-4.11	0.09
<i>hsa-miR-139</i>	-3.46	0.04
<i>hsa-miR-218</i>	-3.25	0.09
<i>hsa-miR-128-2</i>	-3.24	0.10
<i>hsa-miR-483</i>	-2.94	0.13
<i>hsa-miR-128-1</i>	-2.36	0.14
<i>hsa-miR-299</i>	-2.29	0.13
<i>hsa-miR-511-1</i>	-1.51	0.20
<i>hsa-miR-190</i>	-1.14	0.25
Up-regulated in tumor		
<i>hsa-miR-383</i>	2.64	4.53
<i>hsa-miR-519d</i>	2.59	5.90
<i>hsa-miR-21</i>	2.20	28.01
<i>hsa-miR-516-35p</i>	2.20	5.34
<i>hsa-miR-26a</i>	1.22	4.84
<i>hsa-miR-10b</i>	1.19	5.26
<i>hsa-miR-486</i>	1.12	12.03
<i>hsa-miR-451</i>	1.06	4.26

NOTE: A microarray experiment was carried out to compare glioblastoma with adjacent brain as described in Materials and Methods. Data show that all alterations shown are >4-fold detected in the experiment.

*SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific scores (i.e., paired *t* tests). Each gene is assigned a score based on its change in gene expression relative to the SD of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant.

Vector construction. A DNA fragment containing the *hsa-miR-128-1* locus situated at chromosome 2 (2q21.3) plus 300 bp upstream and 200 bp downstream from the 82-bp miR-128-1 precursor was amplified from U87MG genomic DNA and cloned into the pMIF-cGFP-Zeo lentiviral vector (System Biosciences).

A 3'-untranslated region (UTR) reporter vector was constructed by ligating a 338-bp fragment of the *Bmi-1* 3'-UTR encompassing the target sequence for miR-128 from U87MG genomic DNA into pGL3 control vector (Promega). This construct was mutated using the QuickChange kit (Stratagene), in which the predicted miR-128 target site cactgtga was substituted with a tgttgtga. Primer sets (5'-3') were as follows: pri-miR-128-1 cloning, TATAGGCGGCCACTGGAGTCAATGAAAGCAA and CCCCGCTAGCTAAGCAATAGCTTTCACAAATT; *Bmi-1* reporter construct, TATATCTAGATTCCTGTATTACGCTGTTTTG and AGATTCTAGAATGTCATATACCAATATGGC; mutagenic primers, TAATGCATTCTATGTAGC-CATGTTGTTGTGAATAACGATTTCTTGTCATATTTAG and TAAATATGCAAGAAATCGTTATTCACAACAACATGGCTACATAGAATG-CATTA; *Bmi-1* quantitative reverse transcription-PCR (qRT-PCR), GGAGACCAGCAAGTATGTCTATTT and CATTGCTGCTGCTGGG-CATCGTAAAG; and *18S rRNA* qRT-PCR, AACTTTCGATGGTAGTCGCCG and CCTTGGATGTGGTAGCCGTTT.

Real-time PCR. Total RNA was extracted using Trizol (Invitrogen) and treated with RNase-free DNase (Qiagen). Mature miR expression analysis was carried out using the miR real-time PCR detection kit (Applied Biosystems). qRT-PCR was performed using the Applied Biosystems

7500 Real Time PCR System, with human 18S rRNA as an endogenous control. cDNAs for RT-PCR were synthesized using iScript (Bio-Rad). mRNA expression analysis was carried out using Power SYBR Green (Applied Biosystems) with human *Bmi-1* and 18S rRNA primers.

Cell studies. Transfection of pre-miR-128 oligo (25–75 nmol/L; Ambion) and reporter plasmids was done with Lipofectamine 2000 (Invitrogen). The pri-miR-128-1 lentiviral construct was used for establishing U251MG, U87MG, and OHG02 cell lines constitutively overexpressing miR-128-1, according to the manufacturer's instructions (System Biosciences). Antibodies used were *Bmi-1*, H3K27me³ (Millipore), β -actin, α -tubulin (Sigma), p21^{Waf1/Cip1}, total Akt, phosphorylated Akt (Ser⁴⁷³), and phosphorylated Akt (Thr³⁰⁸; Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse IgG were used (Amersham). Cell viability was measured using Wst1 (Roche). Flow cytometry was carried out using the FACSCalibur machine (Becton Dickinson). For luciferase assays, the wild-type or mutated *Bmi-1* 3'-UTR reporter plasmid was cotransfected with pre-miR-128-1 or control into NIH3T3 cells. Targeting efficiency was determined by measuring luciferase levels (Promega) in a FLUOstar Optima plate reader (BMG Labtech) in triplicate.

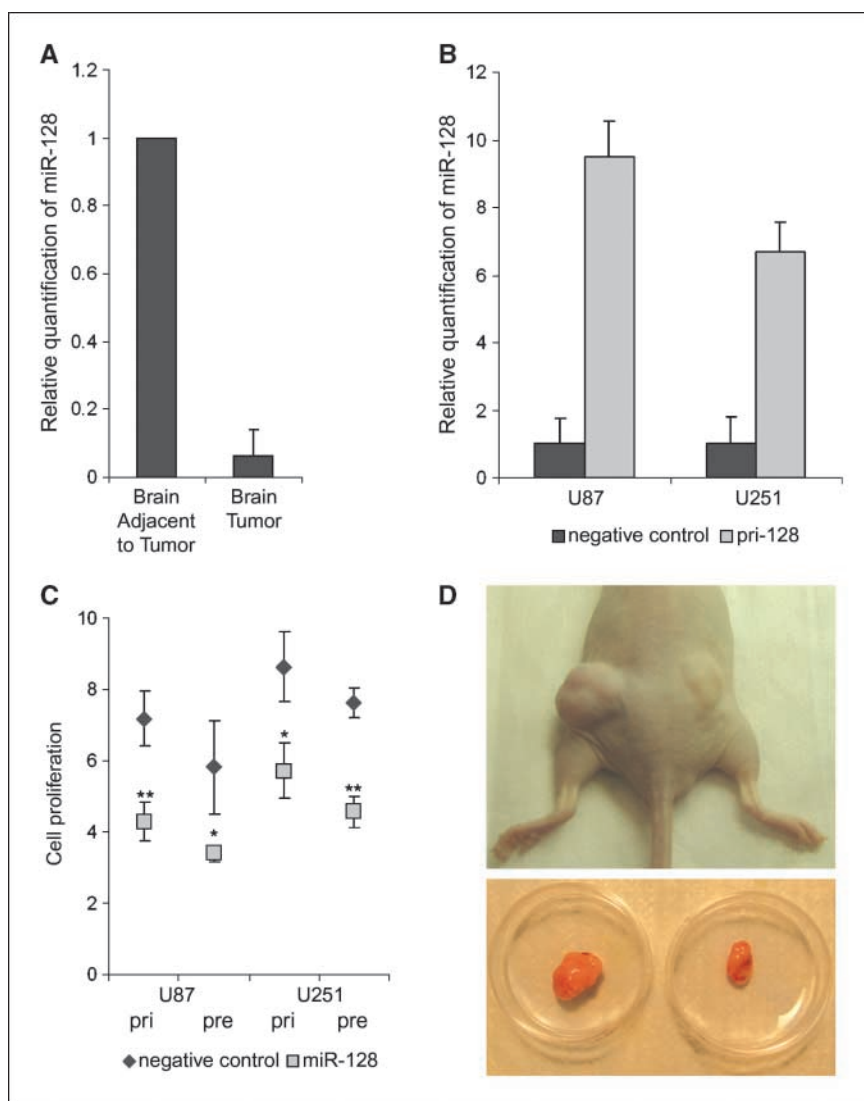
In situ hybridization and immunohistochemistry. Immunohistochemical testing was performed using the Ventana Benchmark System (Ventana Medical Systems). Expression of *Bmi-1* was optimized at a 1:500 dilution with the antigen retrieval CC1 for 30 min using the Ultraview Universal Red system; hematoxylin was the counterstain. Negative controls included omission of the primary antibody and use of tissues, such as normal brain, with very low *Bmi-1* expression. The protocol for detection of miRs by *in situ* hybridization has been previously published (15). The sequence of the probes (Exiqon) that contained the locked nucleic acid (LNA)-modified bases with digoxigenin conjugated to the 5' end was as follows: miR-128, AAAGAGACCGGTTCACTGTGA; miR-128scrambled, CGTATAGCCCCAAGAATTAGG.

Animal studies. U87 cells stably expressing miR-128 or empty vector controls were implanted s.c. in the flanks of athymic mice ($2.5 \times 10^5/200 \mu\text{L}$ Matrigel; BD Biosciences).

Results and Discussion

Expression of miR-128 reduces proliferation of glioma cells *in vitro* and *in vivo*. We first used a microarray to compare miR expression profiles in glioblastoma with adjacent normal human brain tissue. Among several differentially expressed miRs, we found that miR-128 was strongly down-regulated in glioblastomas (Table 1). This was verified by real-time PCR, which showed that mature miR-128 levels in human glioblastoma samples were strikingly and consistently reduced compared with those from adjacent brain devoid of gross tumor (Fig. 1A). miR-128 is encoded by two distinct genes, *miR-128-1* and *miR-128-2*, which are processed into an identical mature sequence. miR-128-1 and miR-128-2 are both intronic, embedded in the *R3HDM1* gene on chromosome 2q21.3 and *ARPP21* on chromosome 3p22, respectively. miR-128 is enriched in brain (16), but its function is not known. We initially determined whether overexpression of miR-128 would have an effect on the phenotype of cultured glioma cells. Stable transduction with a lentiviral vector containing the primary transcript of miR-128 (pri-miR-128-1) produced high levels of mature miR-128 in U87 and U251 glioma cells (Fig. 1B). Mir-128-transduced cells showed reduced proliferation compared with control cells, as measured by cell counting (Fig. 1C) or by Wst1 cell viability assays (data not shown). Similarly, a short hairpin oligonucleotide mimic of pre-miR-128-1 led to high levels of mature miR-128 expression and significantly reduced the proliferation of U87 and U251 glioma cell lines compared with a control oligonucleotide (Fig. 1C). The fold increase in miR-128 expression

Figure 1. miR-128 is down-regulated in human glioblastoma and its expression inhibits cell growth *in vitro* and *in vivo*. **A**, down-regulation of miR-128 in human glioblastoma versus paired adjacent brain ($n = 5$) devoid of gross tumor, as determined by quantitative RT-PCR. Columns, mean; bars, SE. **B**, expression of mature miR-128 was measured by quantitative RT-PCR in U87 and U251 glioma cells, stably transfected with pri-miR-128-1, shown as a fraction of cells transfected with control vector. **C**, cell counts were performed in triplicate 4 d after plating 20,000 cells. The fold growth of cells at day 4 over initial count at day 0 is shown in the graph. **D**, U87 cells stably expressing miR-128-1 formed visibly smaller tumors in flanks (on the right flank) of 11 nude mice compared with controls (on the left flank). A representative animal flank is shown both before (top row) and after (bottom row) excision from animal. *, $P < 0.05$; **, $P < 0.01$ (Student's t test).



in transfection experiments was similar to the difference in expression observed between patient tumor samples and surrounding normal brain, indicating that the effects are of physiologic significance. Flow cytometry revealed a significant decrease in the percentage of glioma cells in S phase after miR-128 transfection, with no significant alterations in the sub-G₀ apoptotic population (reduction in S phase in miR-128-transfected glioma cells compared with controls = 0.705 ± 0.053 ; $P = 0.005$).

When implanted in the flanks of athymic mice, the growth of U87 cells expressing pri-miR-128-1 was significantly decreased (Fig. 1D). Flank tumors expressing miR-128 were 50% smaller than controls ($n = 11$ animals per group; SD = 17.7%; $P = 1.42 \times 10^{-6}$). Thus, miR-128 expression significantly inhibited the proliferation of glioma cells both in culture and *in vivo*.

Bmi-1 is a direct target of miR-128. miR-128 has multiple predicted conserved targets in the human genome.⁴ Initially, we

selected a panel of predicted targets known to play a role in glioma biology or regulation of cell differentiation and self-renewal. In a RT-PCR-based screen, we observed a consistent 5-fold down-regulation in expression of the *Bmi-1* gene across three glioma cell lines transfected with pre-miR-128-1 oligonucleotide. This was also seen in the pri-miR-128-1 stably transfected cell lines and also by Western blotting (Fig. 2A). Results with other potential targets were neither as consistent nor as significant (data not shown). The *Bmi-1* gene acts as part of the polycomb repressor complex (PRC1) that plays a role in epigenetic gene silencing by chromatin modifications (10). *Bmi-1* also plays a role in stem cell renewal, a process that is also known to be important in glioma (11, 12). Indeed, *Bmi-1* has recently been shown to play an important oncogenic role in glioma formation in a mouse model (13). The TargetScan miR target prediction tool predicts a strong (8-mer overlap in the seed region) and highly conserved miR-128 target site in the 3'-UTR of *Bmi-1* at position 467-488 (Fig. 2B). A 338-bp fragment encompassing this region inserted 3' of a luciferase expression cassette significantly reduced luciferase expression after pre-miR-128-1 transfection in a dose-dependent

⁴ <http://www.targetscan.org>

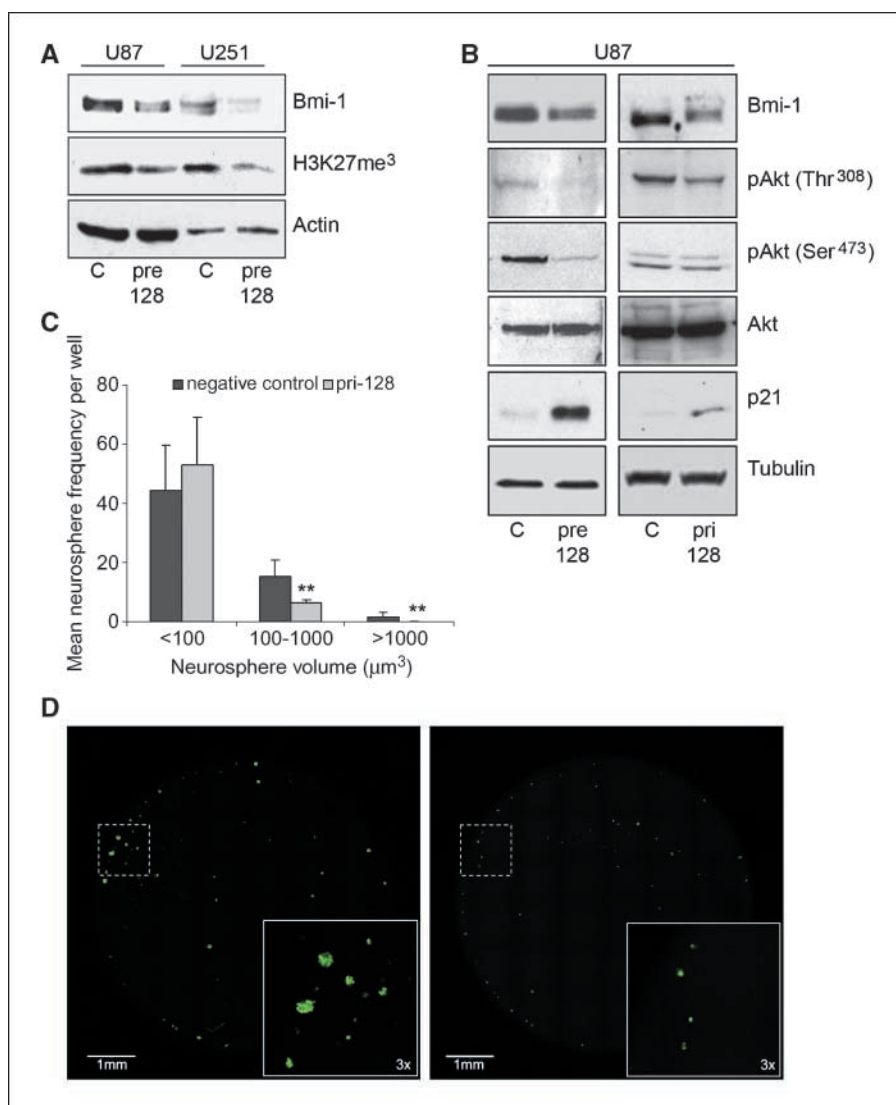


Figure 3. miR-128 affects cellular pathways regulated by Bmi-1 and blocks glioma stem-like neurosphere self-renewal. *A*, Western blotting revealed down-regulation of H3K27 methylation in pre-miR-128-1-transfected cells compared with control cells. *B*, Western blotting showed down-regulation of Akt phosphorylation and an increase in p21 levels in U87 cells overexpressing pre-miR-128-1 or pri-miR-128-1 compared with control cells. *C*, the glioma neurosphere line OHG02 was transduced with pri-miR-128-1, leading to a reduction in glioma neurosphere number and volume in defined medium. *D*, plates under low-power and high-power (*inset*) magnification. The picture is shown under fluorescence because OHG02 in this experiment was stably transduced with the pri-miR-128-1 or control vector, which coexpress GFP, allowing improved resolution for volume and number measurements. Scale bar, 1 mm. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

manner (Fig. 2*B*). Mutagenesis of the predicted miR-128 binding site blocked this effect (Fig. 2*B*). These findings indicate that miR-128 negatively regulates the expression of Bmi-1, a neural stem cell and glioma maintenance factor, directly through its predicted miR-128 binding site.

RT-PCR and staining studies carried out on a panel of glioblastoma and normal tissues confirmed the down-regulation

of miR-128 in tumor and the converse up-regulation of Bmi-1 (Fig. 2*C*). However, to determine whether there is a significant inverse correlation between miR-128 and Bmi-1 levels, it will be necessary to analyze a much larger sample set. *In situ* hybridization of patient glioblastoma sections and normal brain showed expression of miR-128 in brain, but not in glioma cells (Fig. 2*D*), whereas immunostaining for Bmi-1 showed its up-regulation with no

Figure 2. Direct targeting of Bmi-1 by miR-128. *A*, left, U87, U251, and U373 glioma cells were transfected with 25 nmol/L pre-miR-128-1 (precursor) or scrambled oligonucleotide as a negative control; right, U87 and U251 were stably transduced with a lentiviral vector expressing pri-miR-128-1 or control vector. Expression of Bmi-1 was measured by real-time PCR and shown as a fraction of control. Each experiment was performed in triplicate. Corresponding immunoblots are shown beneath the RT-PCR graphs. *B*, expression of luciferase with the putative miR-128-1 target site in wild-type (*wt*) or mutated 3'-UTR from *Bmi-1* was measured in a luminometer and shown as a fraction of control. **, $P < 0.01$ (Student's *t* test). The predicted site of miR-128 hybridization to the *Bmi-1* 3'-UTR is also shown. *C*, top, miR-128 and Bmi-1 mRNA levels are significantly different in human brain tumor versus brain adjacent to tumor as determined by RT-PCR. *P* values are shown underneath figure. Bottom, percentage of miR-128-expressing and Bmi-1-expressing cells in glioblastoma and normal brain as measured by *in situ* hybridization and immunohistochemistry, respectively. *D*, top, Bmi-1 protein expression was found by immunohistochemistry in many malignant cells (arrow) in human glioblastoma. Left, low magnification; right, high magnification. The positive cells are red and the counterstain is blue. Middle, in comparison, very few, if any, cells expressed Bmi-1 in brain adjacent to tumor. Left, low magnification; right, high magnification. Each panel shows no positive cells, which was indicative of most fields. Bottom, miR-128 expression was assessed with a LNA probe and *in situ* hybridization; the signal is blue and the counterstain is pink. Left, note that many of the cells in the normal brain expressed miR-128. Inset, a strip of positive cells in Ammon's horn of the hippocampus, indicating that many of the positive cells had the cytologic features of neurons. Right, in comparison, miR-128 was not detected in glioblastoma. Inset, the negative results in the normal brain using the scrambled probe for miR-128. Scale bars, 20 µm.

detectable expression in normal brain (Fig. 2D). This confirms Bmi-1 up-regulation in glioblastoma where there are also significantly reduced levels of miR-128 in contrast to normal brain, further supporting a model of Bmi-1 regulation by miR-128.

miR-128 causes changes in glioma cells consistent with the down-regulation of Bmi-1, including a reduction in self-renewal capacity. The main function of the Bmi-1-containing PRC1 complex is to maintain the silencing of specific genes through modifying chromatin structure (10). PRC1 recognizes trimethylated histone 3 (H3K27) and causes its effects through histone ubiquitylation and by promoting further H3K27 trimethylation (10). Because levels of H3K27me³ are reduced in *Bmi1*^{-/-} mice (17), we sought to determine if down-regulation of Bmi-1 by miR-128 led to a decrease in this marker. Figure 3A shows that glioma cells transfected with pre-miR-128-1 had reduced H3K27me³ levels compared with controls. We then investigated two more molecular readouts associated with reduced Bmi-1 levels. Recently, loss of Bmi-1-mediated self-renewal of neural stem cells was shown to be associated with up-regulation of p21^{CIP1} (12) and decreased Akt activation (18). Expression of miR-128 in glioma cells not only led to a decrease in Bmi-1 protein levels but also to a concomitant overexpression of p21^{CIP1} and a decrease in phosphorylated Akt (Fig. 3B). This does not discount the possibility that the effects of miR-128 may be due to its actions on targets in addition to Bmi-1; however, its effects on glioma cells are biochemically consistent with Bmi-1 reduction, leading to a decrease in H3K27 methylation and modulation of cellular pathways involved in cell cycle exit and cell survival (p21^{CIP1} and Akt).

Finally, we examined the effects of miR-128 on glioma self-renewal, a phenotype that is thought to be a characteristic of the glioma stem-like cell subpopulation and may be under the control of Bmi-1 (12). Cells from freshly excised surgical glioblastoma specimens were grown under conditions thought to maintain the glioblastoma stem-like cell population (2, 4). As described in Materials and Methods, these cells (OHG02) express the features that define "stem cell-like" (progenitor, initiator) characteristics. When miR-128 was overexpressed in these neurosphere cultures, Bmi-1 levels were reduced, consistent with the cell line data (data

not shown). In addition, we observed a significant decrease in the number and volume of OHG02 neurospheres stably expressing miR-128 compared with controls (Fig. 3C and D), suggesting that miR-128 is acting on glioma cells at least in part by blocking Bmi-1-mediated pathways involved in stem cell renewal.

Regulators of *Bmi-1* expression are likely to be critical determinants of normal stem cell maintenance and renewal, and increasingly, it seems that they may also dictate the ability of tumors, such as glioblastoma, to self-renew. In this study, we have been able to identify miR-128 as one such regulator. This will likely be of relevance not only for studies related to normal nervous system development and neural stem cell maintenance but also to glioma biology. Furthermore, therapeutic modalities using miR-128 gene delivery or miR-128-like nucleic acids may now be pursued in an effort to target the self-renewing glioma cell subpopulation. Physiologically, miR-128 doubtless has its effects through actions on multiple targets in concert with other miRs. For example, epidermal growth factor receptor is a target of miR-128 in lung cancer (19). However, in glioma, Bmi-1 is the most robustly and consistently down-regulated target in a panel of glioma cell lines, and our data are consistent with the idea that Bmi-1 is a major target of miR-128 in gliomas. Another down-regulated miR in glioblastoma (miR-124) plays a direct role in promoting neuronal differentiation (20), suggesting that the coordinated action of multiple miRs is vital in the maintenance of undifferentiated glioma stem-like cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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