

# The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo

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Activated oncogenic signaling is central to the development of nearly all forms of cancer, including the most common class of primary brain tumor, glioma. Research over the last two decades has revealed the particular importance of the Akt pathway, and its molecular antagonist PTEN (phosphatase and tensin homolog), in the process of gliomagenesis. Recent studies have also demonstrated that microRNAs (miRNAs) may be responsible for the modulation of cancer-implicated genes in tumors. Here we report the identification miR-26a as a direct regulator of PTEN expression. We also show that miR-26a is frequently amplified at the DNA level in human glioma, most often in association with monoallelic *PTEN* loss. Finally, we demonstrate that miR-26a-mediated PTEN repression in a murine glioma model both enhances de novo tumor formation and precludes loss of heterozygosity and the *PTEN* locus. Our results document a new epigenetic mechanism for PTEN regulation in glioma and further highlight dysregulation of Akt signaling as crucial to the development of these tumors.

[*Keywords:* microRNA; miR-26a; PTEN; glioma; Akt]

Supplemental material is available at <http://www.genesdev.org>.

Received December 31, 2008; revised version accepted April 21, 2009.

Glioma represents the most prevalent diagnostic category of primary brain tumor in the adult population. Many of its variants, including its most malignant form, glioblastoma multiforme (GBM), demonstrate striking refractiveness to even aggressive treatment regimens. Recent advances have implicated a defined set of oncogenic pathways in the underlying biology of this tumor group (Baehring 2005; Funari et al. 2007). Among these crucial signaling networks, the Akt pathway and its negative regulator PTEN (phosphatase and tensin homolog) have emerged as being particularly important in glioma pathogenesis. Mutation or deletion of *PTEN*, often by way of complete loss of its locus on chromosome 10q, is found in a large percentage of GBM (James 2005; The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008) and is correlated with poor prognosis in multiple glioma subtypes (Sasaki et al. 2001; Smith et al. 2001). Furthermore, *PTEN* loss dramatically enhan-

ces gliomagenesis in a number of murine model systems (Hu et al. 2005; Wei et al. 2006; Kwon et al. 2008). Given the central role of PTEN in glioma biology, the identification of alternative mechanisms for PTEN regulation in this tumor entity would be of considerable interest.

MicroRNAs (miRNAs) are a recently discovered class of small (~22-nucleotide) noncoding RNAs that, in mammals, regulate genes at the translational level by binding loosely complimentary sequences in the 3'-untranslated regions (UTRs) of target mRNAs (Zamore and Haley 2005). The relatively low stringency requirements for functional miRNA/mRNA interactions give each miRNA the capacity to regulate numerous mRNAs. Additionally, the 3'-UTRs of regulated mRNAs frequently contain numerous miRNA recognition sites, further increasing the potential complexities of this regulatory mechanism. Several miRNAs and polycistronic miRNA clusters have been implicated in human cancer (Dalmay 2008; Fabbri et al. 2008), and a smaller group has been specifically linked to glioma biology. miR-221, for instance, has been found to be up-regulated in GBM and directly targets the tumor suppressor p27 (Ciafre et al.

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1777409>.

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2005; le Sage et al. 2007). Conversely, miR-7, whose confirmed targets include the epidermal growth factor receptor (EGFR), frequently demonstrates decreased levels in GBM, and its forced expression reduces proliferation, survival, and invasiveness in cultured glioma cells (Kefas et al. 2008). miR-124 and miR-137, both down-regulated in high-grade glial neoplasms, also inhibit glioma cell proliferation in vitro and induce neuronal differentiation (Silber et al. 2008). miR-21 is almost invariably overexpressed in GBM along with a number of other tumor types (Calin and Croce 2006), and targets a wide variety of genes involved in cell motility, migration, and apoptosis (Chan et al. 2005; Corsten et al. 2007; Zhu et al. 2007, 2008; Gabriely et al. 2008). Antisense knockdown of miR-21 in glioma cell lines leads to reduced viability in murine xenografts (Corsten et al. 2007). Interestingly, miR-21 appears to directly regulate PTEN expression in hepatocellular carcinoma (HCC) (Meng et al. 2007), although the importance of this interaction in gliomagenesis has not been confirmed. On a similar note, miR-19a and miR-214 have also been shown to target PTEN (Pezzolesi et al. 2008; Yang et al. 2008), but neither appears to be up-regulated in glioma. No miRNA has been definitively shown to either enhance or suppress the process of glioma formation in vivo.

We were initially interested in miRNAs with potential effects on glioma development, specifically those that might regulate relevant tumor suppressor genes like *PTEN*. In this report, we show that the miRNA miR-26a is overexpressed in a subset of high-grade gliomas and directly targets the *PTEN* transcript. We also demonstrate that overexpression of miR-26a in glioma primarily results from amplification at the *miR-26a-2* locus, a genomic event strongly associated with monoallelic *PTEN* loss. Finally, we show that miR-26a reduces *PTEN* levels and facilitates glioma formation in a well-characterized murine model system, and that overexpression of the miRNA can functionally substitute for loss of heterozygosity at the *PTEN* locus.

## Results

### *miR-26a is overexpressed in high-grade glioma and directly targets PTEN*

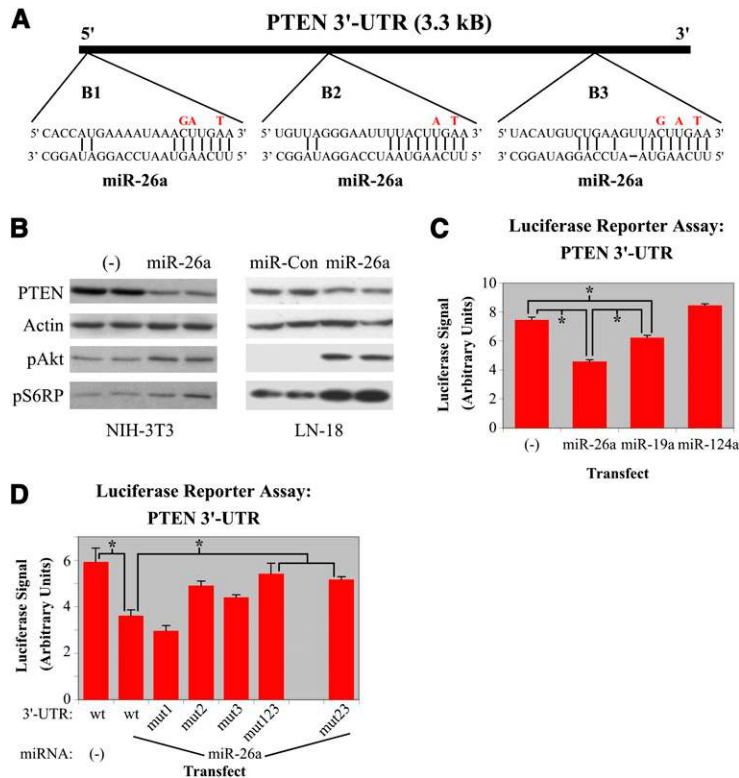
To identify miRNAs potentially involved in glioma pathogenesis, we extracted total RNA from three human GBM samples and performed microarray analysis. Two RNA extracts derived from unaffected human cerebral cortex served as negative controls. After further limiting our study to miRNAs whose expression was elevated by a minimum of twofold in at least two of three GBM samples relative to both normal controls, we were left with 29 miRNAs exhibiting some degree of overlap with those identified in previous reports (Table 1; Chan et al. 2005; Ciafre et al. 2005). Using published results and the Targetscan bioinformatics algorithm (Lewis et al. 2003, 2005), we were then able to assess which glioma-implicated tumor suppressor genes (*PTEN*, *NF1*, *RBI*,

**Table 1.** miRNAs consistently overexpressed in human GBM

miRNA	GBM/Norm	<i>PTEN</i>	<i>NF1</i>	<i>P53</i>	<i>CDKN2A</i>	<i>P27</i>	<i>APC</i>	<i>RBI</i>	<i>TSC1</i>
hsa-miR-10b	208.98								
hsa-miR-182	91.71	1							
hsa-miR-10a	73.35	1							
hsa-miR-183	24.64								1
hsa-miR-21	23.45	1							
hsa-miR-148a	12.03	2			1				
hsa-miR-503	9.77								
hsa-miR-505	9.55								
hsa-miR-451	8.72								
hsa-miR-144	7.72	1							
hsa-miR-542-5p	6.83								
hsa-miR-542-3p	6.53								
hsa-miR-26a	6.00	3						1	
hsa-miR-142-3p	5.51					1			
hsa-miR-199b	5.46								
hsa-miR-15b	5.37								1
hsa-miR-550	5.13								
hsa-miR-25	4.65	1							
hsa-miR-9*	4.53								
hsa-miR-92b	4.19	1							1
hsa-miR-142-5p	4.02								
hsa-miR-27a	3.71		1				1		1
hsa-miR-23a	3.59	2							
hsa-miR-486	3.12	1	1						
hsa-miR-362	3.00								
hsa-miR-532	2.95								
hsa-miR-93	2.86	1						1	
hsa-miR-106b	2.49	1						1	
hsa-miR-223	2.09								

The table shows miRNAs that are elevated by a minimum of twofold in at least two of three sampled GBMs relative to two normal control brains. GBM/Norm reflects the average ratio of microarray signal intensities between GBM and normal brain for each miRNA. The numbers of predicted binding sites of each miRNA (<http://www.targetscan.org>) in the 3'-UTRs of several tumor suppressor mRNAs (*PTEN*, *NF1*, *p53*, *CDKN2A*, *p27*, *APC*, *RBI*, and *TSC1*) are shown in the last eight columns. The three sites on the *PTEN* 3'-UTR predicted to be targeted by miR-26a are highlighted in bold.

*p27*, *APC*, *TSC1*, *CDKN2A*, and *P53*) harbored predicted binding sites for the individual miRNAs on our list. Notably, the *PTEN* 3'-UTR was found to contain potential binding sites for no less than 10 of the miRNAs elevated in GBM, more than twice as many as any other analyzed tumor suppressor (Table 1, *PTEN*). We then chose to narrow our focus to miRNA/mRNA pairings characterized by multiple 3'-UTR-binding sites. This criterion, which has emerged as an independent factor in the accurate prediction of functional miRNA/mRNA interactions (Bartel 2009), directed our attention to three *PTEN*-targeting miRNAs (Table 1). Interestingly, one member of this group, miR-26a, was found to have three potential binding sites in the *PTEN* 3'-UTR (Fig. 1A, B1–B3), making it a particularly attractive target for further investigation.



**Figure 1.** miR-26a directly targets PTEN. (A) Schematic of the PTEN 3'-UTR showing binding sites for miR-26a. Nucleotide changes for binding site mutants are indicated in red. (B) NIH-3T3 or human glioblastoma (LN-18) cells were retrovirally transduced with empty vector (-), miR-26a, or control miRNA (miR-Con). Western blots for PTEN, pAkt, pS6RP, and actin are shown. (C) miR-26a targets PTEN directly as measured by luciferase reporter assay. A luciferase reporter plasmid containing the PTEN 3'-UTR was transfected into HEK 293 cells along with miRNA-expressing constructs (transfected miR;  $n = 3$  for each). Cells were lysed 24–48 h later, and luciferase activity was quantified. (D) Mutagenesis of the three potential miR-26a-binding sites in the PTEN 3'-UTR either singly or in combination (M1, M2, M3, M123, M23) in the context of this assay demonstrated that sites B2 and B3 are functional. (\*)  $P < 0.01$ .

miR-26a was found to significantly repress PTEN in cell culture, an effect that, notably, was not seen with any other miRNA predicted to interact directly with the tumor suppressor, whether through one or two potential binding sites (data not shown). Western blotting demonstrated that miR-26a overexpression achieved a ~50% knockdown of PTEN protein in both NIH-3T3 cells and LN-18 human glioblastoma cells (Fig. 1B). The observed decrease in PTEN expression was accompanied by enhanced Akt pathway signaling, as was evident by concomitant increases in pAkt and pS6RP protein levels in both cell types (Fig. 1B). As a side note, we were also able to confirm in glioblastoma cells the miR-26a-mediated knockdown of EZH2 and SMAD1, both of which are known targets of the miRNA (Supplemental Fig. 1a; see the Discussion).

We then assessed whether miR-26a directly targets the PTEN mRNA transcript using a reporter construct harboring the entire murine PTEN 3'-UTR downstream from a luciferase gene. Transfection of this reporter into HEK-293 cells led to robust expression of luciferase whose activity could be readily quantified in cell lysates. Subsequent cotransfection with miR-26a resulted in a 39% reduction in luciferase levels, exceeding the knockdown achieved with the known PTEN regulator miR-19a (Pezzolesi et al. 2008). No drop in luciferase activity was seen with coexpression of miR-124a, which does not target PTEN (Fig. 1C). To further confirm that miR-26a binds the PTEN 3'-UTR, we generated luciferase reporters with mutated miR-26a-binding sites both singly and in combination (Fig. 1A). While disrupting the B1 site

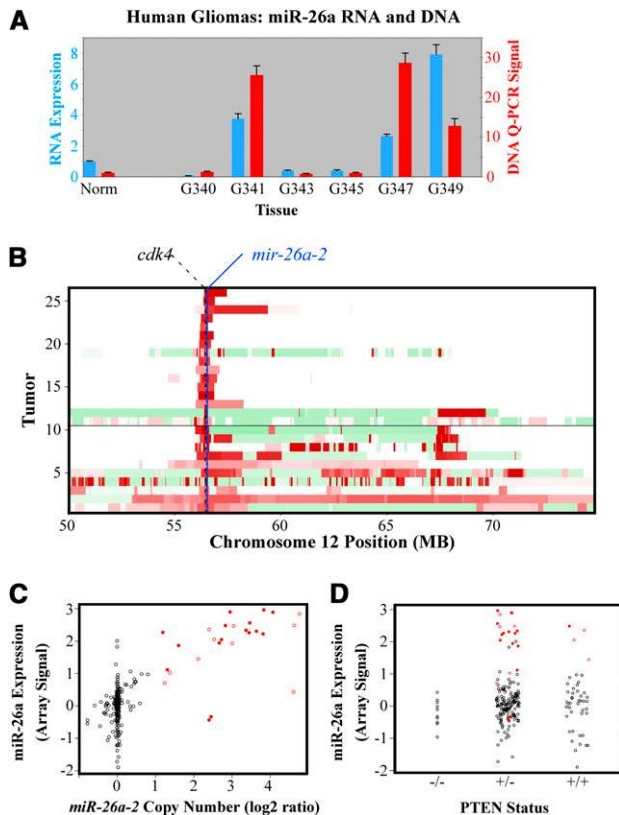
alone failed to restore any luciferase activity, mutating either the B2 or B3 site yielded partial rescue (Fig. 1D, mut1, mut2, and mut3). Additionally, mutating all three binding sites or only the B2 and B3 sites in combination led to an almost complete restoration of luciferase activity (Fig. 1D, mut123 and mut23). These findings indicated that the glioma-implicated miRNA miR-26a directly binds to the B2 and B3 sites in the 3'-UTR of PTEN, mediating translational repression and reduced steady-state levels of the protein.

*miR-26a is amplified in a subset of high-grade gliomas, and its overexpression is strongly associated with monoallelic PTEN loss*

To ascertain the consistency with which miR-26a is up-regulated in high-grade gliomas, we performed quantitative RT-PCR (QRT-PCR) for the miRNA on a larger cohort of six tumor samples. This analysis revealed miR-26a to be significantly overexpressed in only a subset of high-grade gliomas (Fig. 2A). Given that GBM-associated oncogenes, such as EGFR, are frequently amplified (Wong et al. 1992; The Cancer Genome Atlas Research Network 2008; Parsons et al. 2008), we next determined whether either of the two *miR-26a* loci present in the human genome (*mir-26a-1* and *miR-26a-2*) exhibited elevated copy number in our tumor set. Q-PCR performed on extracted genomic DNA demonstrated amplification of *miR-26a-2* on chromosome 12q in the same tumors where miR-26a itself was up-regulated (Fig. 2A), suggesting that, in high-grade gliomas, miR-26a overexpression



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**Figure 2.** miR-26a is amplified in glioma. (A) QRT-PCR and Q-PCR performed on total RNA and DNA extracted from human high-grade gliomas (G340–G349) and normal cerebral cortex (Norm). RNA (blue) and DNA (red) levels are expressed as fold increase relative to the normal control. (B) Schematic of TCGA data showing GBMs with amplification of the *miR-26a-2* locus on chromosome 12q ( $n = 25$  of 233). Each tumor corresponds to a horizontal bar across the chart. Red and green indicate copy number increases and decreases, respectively. The locations of *miR-26a-2* and *CDK4* are indicated by the blue line and the dashed black line, respectively. Tumors with chromosome 12 amplicons containing <30 genes (defined as focal in this study) are presented above the solid black line. (C) Schematic of TCGA data demonstrating that miR-26a overexpression (Y-axis) is almost invariably associated with *miR-26a-2* amplification (X-axis). Array signal intensity for miR-26a is normalized to the mean of all samples (0 on Y-axis). (D) Schematic of TCGA data demonstrating the association of miR-26a overexpression (Y-axis) with PTEN status (X-axis). (C,D) Amplified tumors are indicated in red with focal amplifications indicated as solid red circles.

is primarily mediated by copy number increases at the genomic level.

We then sought to expand our analysis of miR-26a amplification in GBM using the cancer genome atlas (TCGA), a publicly available repository that has accumulated comparative genomic hybridization (CGH), gene expression, and DNA methylation analyses for ~200 GBM samples to date (for complete results, see Supplemental Table 1; The Cancer Genome Atlas Research Network 2008). Using this database, we were able to confirm amplification of *miR-26a-2* in a subset of GBMs

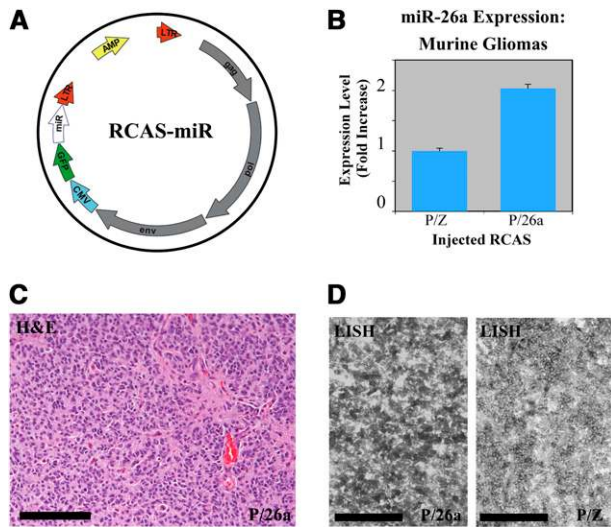
(11%) that also tended to exhibit elevated miR-26a (Fig. 2B,C). No amplification events were identified at the *miR-26a-1* locus. Additionally, we found that *miR-26a-2* is coamplified in all cases with a defined set of genes, including that of the oncoprotein *CDK4*. Indeed, only ~80 kb separate the *CDK4* and *miR-26a-2* genes (Fig. 2B). *CDK4* amplification has been repeatedly associated with a significant minority of GBMs and is thought to contribute to oncogenesis via its inhibitory effects on the RB tumor suppressor (Henson et al. 1994; Funari et al. 2007).

The presence of *miR-26a-2* within a previously characterized oncogenic amplicon raised the possibility that miR-26a overexpression in GBM simply represents a functionally insignificant epiphenomenon associated with *CDK4* amplification. To address this question, we correlated TCGA data for both miR-26a and PTEN and found, importantly, that miR-26a overexpression was absent from all tumors characterized by homozygous *PTEN* deletion. Additionally, amplification of the miRNA segregated, for the most part, with tumors harboring monoallelic deletion at the *PTEN* locus (Fig. 2D). When nonfocal amplicons—those containing >30 genes—were excluded from our analysis, this correlation became even stronger, with only one exception falling into the *PTEN*<sup>+/+</sup> subgroup.

The association of miR-26a overexpression with monoallelic *PTEN* loss suggests that amplification of the miRNA serves to silence residual *PTEN* transcript in *PTEN*<sup>+/-</sup> tumors, analogous to a loss of heterozygosity event. Our data indicate that 12% of *PTEN*<sup>+/-</sup> GBMs (21 out of 170) are characterized by miR-26a amplification. Interestingly, we also found that the vast majority (25 out of 28) of GBMs pairing monoallelic deletion with inactivating mutations at their *PTEN* loci—a group that accounts for 26% of *PTEN*<sup>+/-</sup> gliomas with available sequencing data (28 out of 109)—do not exhibit miR-26a amplification (Supplemental Table 1). This suggests that miR-26a amplification and *PTEN* mutation represent alternative *PTEN* silencing mechanisms that operate, for the most part, in nonoverlapping sets of monoallelic tumors. Furthermore, while *PTEN* mutation appears to be more frequent (26% vs. 12%) than miR-26a amplification in *PTEN*<sup>+/-</sup> tumors, both occur at considerably higher rates than deletion of the remaining *PTEN* allele, given that only 4% of TCGA GBM samples (10 out of 233) exhibit homozygous *PTEN* deletion (Supplemental Table 1). Taken together, these bioinformatics correlations are consistent with a functional role for the miR-26a/*PTEN* interaction in glioma biology regardless of the miRNA's linkage with *CDK4*.

#### *miR-26a* enhances tumorigenesis in a murine glioma model

To further demonstrate the physiological relevance of miR-26a in glioma pathogenesis, we incorporated miRNA expression into the RCAS/tv-a system. RCAS/tv-a technology uses a specific avian leukemia virus (RCAS) to mediate somatic gene transfer into cells transgenic for its receptor (tv-a) (Holland and Varmus 1998;



**Figure 3.** miR-26a-expressing murine gliomas (P/26a). (A) Schematic of RCAS-miR constructs; CMV denotes CMV promoter, GFP denotes GFP cassette, and miR denotes miRNA domain. (B) Overexpression of miR-26a was verified in lysates of crudely dissected tumors by QRT-PCR. (C,D) A grade III murine glioma with forced miR-26a expression. H&E staining (C) and LNA-in situ hybridization (LISH; D) for miR-26a are shown. (D) For the latter, a grade III tumor with no forced miR-26a expression (P/Z) is also shown (bars, 100 μm).

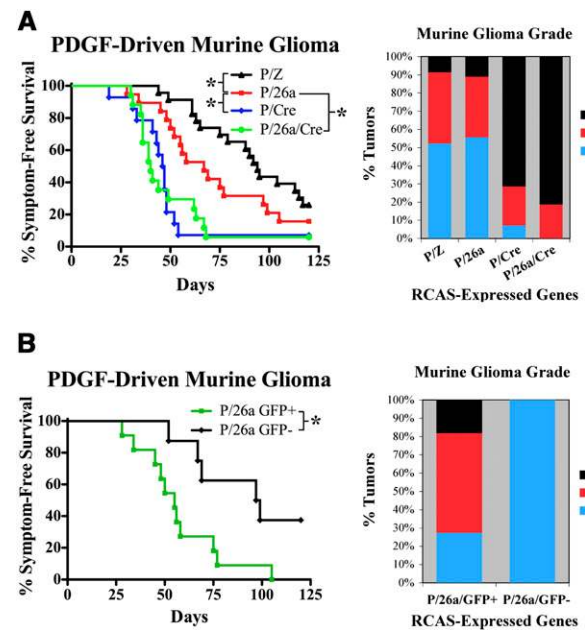
Holland et al. 1998). We showed previously that localized delivery of RCAS retrovirus encoding the human *PDGF-B* gene (RCAS-PDGf) into the brains of newborn mice expressing a nestin-driven tv-a retroviral receptor (*Ntv-a*) yields gliomas by 3–4 mo with high penetrance (Dai et al. 2001; Shih et al. 2004). Elevated PDGF signaling and/or PDGF receptor amplification has been found in a variety of low- and high-grade glioma subtypes (Funari et al. 2007). Of the 24 GBMs in the TCGA study group harboring PDGFR amplification, 25% (six out of 24) and 79% (19 out of 24) are characterized by miR-26a amplification and PTEN loss, respectively. Additionally, 15 of 26 miR-26a-amplified tumors fall within the ~39% of TCGA GBMs (91 out of 233) whose transcriptional profiles cluster with PDGFR-amplified tumors (CW Brennan, H Momota, D Hambarzumyan, T Ozawa, A Tandon, A Pedraza, and EC Holland, in prep.), an enrichment ( $P = 0.053$ ) that further underscores the relevance of PDGF-driven gliomagenesis in the present study.

We developed a new series of RCAS vectors expressing single miRNA constructs downstream from a GFP reporter (Fig. 3A). As a negative control, one such construct was designed bearing a short hairpin sequence targeting the Lac-Z transcript (miR-Z). Additionally we used a murine *Ntv-a* strain containing homozygous floxed *PTEN* alleles (*Ntv-a/PTEN<sup>fl/fl</sup>*) (Hu et al. 2005), such that the RCAS-mediated delivery of cre-recombinase (RCAS-cre) in these mice results in complete loss of PTEN expression in nestin-positive cells.

Coinjection of RCAS-PDGf and RCAS-miR-26a (P/26a) into *Ntv-a/PTEN<sup>fl/fl</sup>* mice led to a mixture of low- and high-grade gliomas over the course of 4 mo that

were morphologically indistinguishable from those generated previously using RCAS-PDGf as the primary oncogenic driver (Fig. 4A; Dai et al. 2001; Shih et al. 2004). Retrovirally mediated miR-26a overexpression was documented in these tumors by either QRT-PCR, immunohistochemistry for GFP, or miR-26a-specific LNA in situ hybridization (LISH) (Fig. 3B–D; Supplemental Fig. 1b). P/26a mice were then compared with analogous cohorts injected with either RCAS-PDGf and RCAS-miR-Z (P/Z) or RCAS-PDGf and RCAS-cre (P/Cre). While the deletion *PTEN* in P/Cre mice led to dramatically reduced symptom-free survival, consistent with previous reports (Hu et al. 2005; Wei et al. 2006; Kwon et al. 2008), P/26a mice exhibited an intermediate degree of disease-free latency relative to both the P/Z and P/Cre cohorts (Fig. 4A). The employment of two independent vectors in the generation of P/26a mice resulted in a mixed population of tumors, with only some actually infected by RCAS-miR-26a virus. Using detectable GFP immunostaining as a marker, we were able to extract this group and compare them with GFP-nonexpressing counterparts. Subsequent Kaplan-Meier analysis revealed that symptom-free survival was more strongly reduced in mice with confirmed miR-26a expression (Fig. 4B).

Histological grades were also assigned to the generated tumors across all three murine genotypes. While our initial compilation demonstrated similar grading profiles



**Figure 4.** miR-26a facilitates gliomagenesis in vivo. (A) Kaplan-Meier curves demonstrating symptom-free survival for P/Z, P/26a, P/Cre, and P/26a/Cre mice ( $n = 23, 19, 14,$  and  $17$  respectively; [\*]  $P < 0.05$ ). The percentages of tumors exhibiting WHO grade II, grade III, and grade IV histological features are also shown for each genotype. (B) Kaplan-Meier curves demonstrating symptom-free survival for P/26a mice with and without detectable immunostaining for GFP reporter ( $n = 11$  and  $8$  respectively; [\*]  $P < 0.05$ ). Histological grades for tumors in the P/26a GFP<sup>+</sup> and P/26a GFP<sup>-</sup> groups are also shown.

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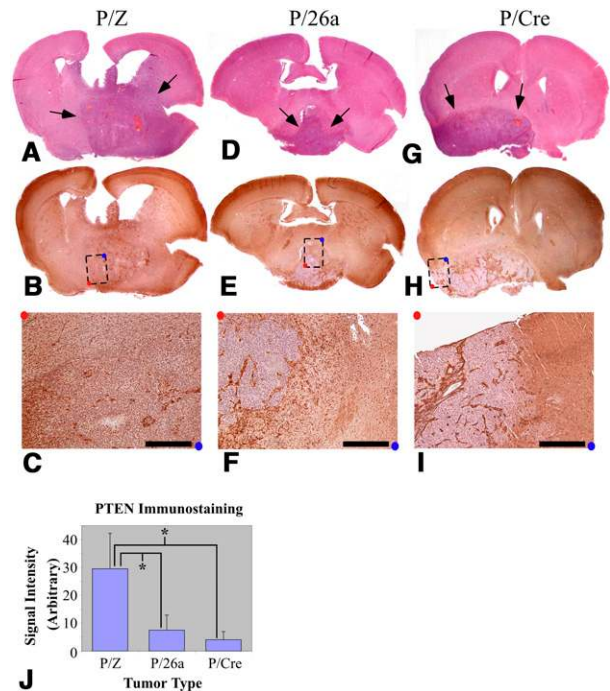
between the P/Z and P/26a cohorts, with both exhibiting fewer floridly high-grade features than P/Cre tumors, examination of the P/26a group after GFP stratification revealed a plurality of high-grade lesions in GFP<sup>+</sup> mice and only grade II tumors in GFP<sup>-</sup> mice (Fig. 4A,B). Taken together, these findings demonstrate that miR-26a contributes to gliomagenesis *in vivo*. Furthermore, the less malignant phenotype exhibited by P/26a mice relative to their P/Cre counterparts is consistent with the notion that PTEN activity, while not entirely lacking in these mice, is sufficiently reduced so as to facilitate oncogenesis.

In order to assess whether down-regulation of PTEN represented the primary tumorigenic mechanism of miR-26a in our murine model system, we generated gliomas using RCAS-PDGF, RCAS-miR-26a, and RCAS-cre (P/26a/Cre). We reasoned that miR-26a-mediated repression of additional non-PTEN mRNA targets could augment gliomagenesis even in the absence of *PTEN* provided those interactions were consequential to the oncogenic process. However, we observed a phenotypic enhancement that was minimal at best when measured by either disease-free latency or tumor grade (Fig. 4A). These data, while not formally excluding the existence of other pathologically relevant miR-26a–target interactions in glioma biology, are consistent with the miR-26a/PTEN repression event being the predominant effect of the miRNA in this tumor type. Furthermore, we confirmed that forced miR-26a expression actually reduced PTEN levels in our murine gliomas by immunostaining multiple grade IV tumors derived from P/Z, P/26a, and P/Cre mice for PTEN protein. We found minimal PTEN labeling in P/26a gliomas, whose staining pattern was strikingly similar to that seen in P/Cre tumors (Fig. 5D–J). This effect notably exceeded the more modest (~50%) reduction in PTEN expression achieved *in vitro* (Fig. 1B). Furthermore, PTEN expression in P/Z tumors was relatively retained (Fig. 5A–C,J). In all cases, nonneoplastic tumor vasculature stained strongly for PTEN serving as an excellent internal control for signal quantification. These findings demonstrate that, in addition to enhancing tumorigenesis, miR-26a effectively represses endogenous PTEN protein in a relevant glioma model system.

As an aside, it bears mentioning that expression of several other glioma and cancer-implicated miRNAs in this experimental paradigm, including miR-21, miR-10b, and miR-19a, yielded no significant effects on gliomagenesis (Supplemental Fig. 1c). Additionally, the invariable coamplification of miR-26a with CDK4 in human GBM prompted us to assess the functional impact of CDK4 in our murine model system. Surprisingly, however, RCAS-mediated CDK4 expression in PDGF-driven gliomas failed to increase either tumor grade or the rate of tumor formation (Supplemental Fig. 2).

#### *miR-26a overexpression functionally substitutes for loss of heterozygosity at the PTEN locus*

As stated above, our analysis of TCGA data suggests a role for miR-26a in the epigenetic silencing of residual PTEN protein following monoallelic loss at the genomic

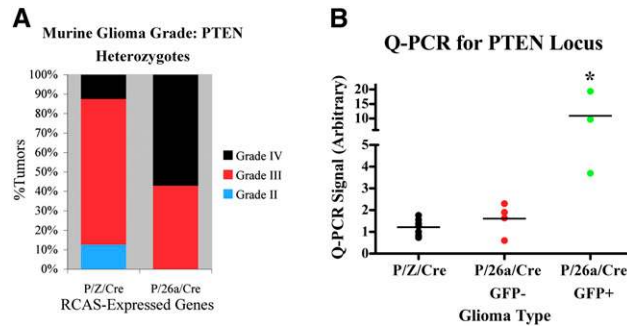


**Figure 5.** miR-26a represses PTEN expression in murine gliomas. Grade IV tumors from P/Z (A–C), P/26a (D–F), and P/Cre (G–I) mice. H&E staining is shown in A, D, and G; immunostaining for PTEN is shown in B, C, E, F, H, and I. C, F, and I are enlargements at 5× magnification of the regions designated in by dashed boxes in B, E, and H, respectively (bars, 400 μm). Arrows indicate tumors on H&E staining. Red and blue dots are orientation guides. (J) Quantification of PTEN immunostaining for two tumors in each genotype (see the Materials and Methods); units are arbitrary. (\*)  $P < 0.05$ .

level. To address this possibility experimentally, we bred Ntv-a/*PTEN*<sup>fl/fl</sup> mice with Ntv-a/*PTEN*<sup>+/+</sup> counterparts to generate Ntv-a/*PTEN*<sup>+/fl</sup> heterozygotes and coinjected them either with RCAS-PDGF, RCAS-cre, and RCAS-miR-Z (P/Z/Cre), or with RCAS-PDGF, RCAS-cre, and RCAS-miR-26a (P/26a/Cre). We reasoned that if miR-26a served to functionally eliminate residual PTEN activity in heterozygous mice, its overexpression in this context might result in enhanced gliomagenesis. Furthermore, given that PDGF-driven *PTEN*<sup>+/-</sup> gliomas are prone to genomic loss of their remaining *PTEN* allele (E Fomchenko and EC Holland, in prep.), we anticipated that miR-26a-expressing tumors, as a result of robust miRNA-induced PTEN silencing, would exhibit a decreased tendency to develop loss of heterozygosity at the *PTEN* locus, and that such an effect would ultimately be reflected in the relative retention of *PTEN* at the genomic level in P/26a/Cre tumors.

We generated equal numbers of P/Z/Cre and P/26a/Cre gliomas in Ntv-a/*PTEN*<sup>+/fl</sup> mice and found a somewhat higher percentage of grade IV tumors in the P/26a/Cre group (Fig. 6A), although no effect on symptom-free survival was observed (data not shown). More importantly, however, Q-PCR analysis of genomic DNA from





**Figure 6.** miR-26a overexpression precludes *PTEN* loss of heterozygosity in PDGF-driven *PTEN*<sup>+/-</sup> murine gliomas. P/Z/Cre and P/Z/26a gliomas were generated in Ntv-a/*PTEN*<sup>+fl</sup> mice ( $n = 7$  and  $7$ , respectively). (A) P/Z/26a Ntv-a/*PTEN*<sup>+fl</sup> mice exhibited more grade IV gliomas. (B) Additionally, genomic DNA was extracted from paraffin sections of all *PTEN*<sup>+/-</sup> tumors and analyzed for *PTEN* by Q-PCR. GFP<sup>+</sup> P/26a/Cre tumors exhibited a significantly higher level of retained *PTEN* than GFP<sup>-</sup> P/26a/Cre and P/Z/Cre tumors. (\*)  $P < 0.05$ . Means are designated by horizontal lines.

each glioma revealed that P/26a/Cre *PTEN*<sup>+/-</sup> tumors exhibited a relative maintenance of their remaining *PTEN* allele compared with P/Z/Cre *PTEN*<sup>+/-</sup> counterparts, and that this effect closely mirrored miR-26a expression as assessed by GFP staining (Fig. 6B, GFP<sup>+</sup> and GFP<sup>-</sup>). These findings indicate that miR-26a overexpression can functionally substitute for and, consequently, preclude *PTEN* loss of heterozygosity in the context of gliomagenesis in vivo, and thus correlate well with our bioinformatics associations between miR-26a amplification and monoallelic *PTEN* loss in GBM.

## Discussion

Since the discovery of miRNAs as a new class of gene regulators, numerous studies have emerged correlating altered expression levels of particular miRNAs with the neoplastic phenotype (Iorio et al. 2005, 2007; Lu et al. 2005; Volinia et al. 2006; Dalmay 2008; Fabbri et al. 2008). Precise classification of specific miRNAs as oncogenes or tumor suppressors, however, necessitates a deeper analysis of their functional roles in vivo. In the present study, we identified miR-26a as a negative regulator of *PTEN*, documented its overexpression in human glioma, and demonstrated its oncogenic potential in a relevant murine model system. We initially found miR-26a to be overexpressed in high-grade gliomas using miRNA microarrays. A similar array-based analysis did not reveal miR-26a to be consistently up-regulated in either GBM samples or glioblastoma cell lines (Ciafre et al. 2005). This discrepancy most likely reflects the stochastic nature of miR-26a overexpression in high-grade glioma, which seems to depend heavily on amplification at the *miR-26a-2* locus.

miR-26a has been sparsely studied to date, and its relevance to the oncogenic process is only beginning to be elucidated. We found miR-26a to repress *PTEN* in cell

culture both by Western blot and luciferase reporter assay. As mentioned briefly above, *PTEN* is a known target of multiple miRNA species including, most notably, the glioma-implicated miR-21 (Meng et al. 2007; Pezzolesi et al. 2008; Yang et al. 2008). Despite repeated efforts, however, we were unable to demonstrate that overexpression of miR-21 reduces *PTEN* levels in vitro (data not shown). Our findings may simply reflect differences in cell type; we note that the prior study linking miR-21 with *PTEN* used primarily hepatocytes and HCC-derived cell lines (Meng et al. 2007). This discrepancy also raises the possibility that the reported oncogenic effects of miR-21 in glioma cell lines may result from the targeting of other proteins (Chan et al. 2005; Corsten et al. 2007; Zhu et al. 2007, 2008; Gabriely et al. 2008; Papagiannakopoulos et al. 2008). As indicated in the main text, incorporation of miR-21 into our RCAS/tv-a model system did not significantly enhance gliomagenesis. However, miR-21 levels were found to be significantly up-regulated in our PDGF-driven murine gliomas even without RCAS-mediated miRNA overexpression (data not shown), potentially implying a saturation of relevant mRNA interactions prior to the forced addition of the miR-21. Alternatively, miR-21 might only enhance gliomagenesis in the context of different oncogenic drivers (i.e., not PDGF).

The physiologic relevance of miR-26a in gliomagenesis was demonstrated using a well-established murine model system. Coexpression of miR-26a with the oncogenic driver PDGF impacted both disease-free latency and tumor grade, although these effects were not as dramatic as those seen with cre-mediated *PTEN* deletion. These findings correlated well with our in vitro data demonstrating partial, but not complete, knockdown of *PTEN* protein with miR-26a overexpression. Additionally, we were able to document *PTEN* repression within P/26a murine gliomas. It remains quite possible that miR-26a mediates its tumorigenic effects by regulating a number of targets not limited to *PTEN*; a potential interaction with the *RB1* transcript is particularly intriguing, though as of yet unverified in our hands (Table 1; data not shown). Indeed, two large recent studies have further solidified the notion that individual miRNAs impact cellular processes by modestly repressing the levels of numerous proteins (Baek et al. 2008; Selbach et al. 2008). However, the failure of miR-26a to significantly enhance murine gliomagenesis in the absence of *PTEN* strongly underscores the particular importance of the miR-26a/*PTEN* interaction in this process, a conclusion that is further supported by both our bioinformatics analysis and the ability of miR-26a overexpression to preclude *PTEN* loss of heterozygosity in vivo (see below).

On a related note, miR-26a has been shown to directly target both the *SMAD1* transcription factor and the histone methyltransferase *EZH2* (Luzi et al. 2007; Wong and Tellam 2008), with the latter of these interactions actually appearing to have tumor-suppressive effects in both lymphoma and rhabdomyosarcoma (Sander et al. 2008; Ciarapica et al. 2009). Coupled with our own findings, these reports suggest that the pro-oncogenic

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properties of miR-26a in glioma may largely reflect the cellular context of the miRNA's overexpression and the array of potential mRNA targets, including PTEN, at its disposal. Our studies indicate that miR-26a overexpression in LN-18 cells does decrease EZH2 and SMAD1 levels, implying that the transcripts of both proteins could be effectively targeted by the miRNA during gliomagenesis. Whether such repression is functionally relevant in this tumor type, however, remains unclear. The fact that miR-26a overexpression enhances gliomagenesis in vivo argues that the miRNA's effects on EZH2 and SMAD1 are either irrelevant in this tumor type or minor enough to be overcome by the physiologic impact of substantial PTEN repression. Interestingly, one recent study has directly linked EZH2 to glioma, specifically regarding a potential role for the protein in the maintenance of the tumor initiating phenotype in cells derived from human GBM (Lee et al. 2008). These findings, however, were documented in a single glioblastoma cell line (out of four tested) that itself was not characterized by miR-26a amplification, implying a fundamental difference in the biology of this particular glioma subset and further underscoring, perhaps, the diversity of oncogenic mechanisms at work in the disease entity as a whole.

We investigated the scope of miR-26a overexpression and amplification in high-grade glioma using the extensive resources of the TCGA. Analysis of their data set revealed that miR-26a amplification is a relatively frequent occurrence in human GBM and is correlated with monoallelic *PTEN* deletion. In contrast, no association was seen between miR-26a up-regulation and either *PTEN*<sup>+/+</sup> or *PTEN*<sup>-/-</sup> tumors. These correlations not only support a functional, tumor-promoting role for the miR-26a/PTEN interaction but also suggest a temporal sequence to the molecular evolution of miR-26a-amplified gliomas, with *PTEN* loss most likely preceding *miR-26a-2* copy number gain. Subsequent repression of the remaining *PTEN* allele by miR-26a would then, presumably, eliminate the driving force for formal loss of heterozygosity. The additional finding that miR-26a amplification tends not to occur in the setting of coupled *PTEN* deletion and mutation provides further support for such an oncogenic mechanism. More significantly, our in vivo glioma modeling has effectively recapitulated this sequence of events, with miR-26a expression in genetically engineered *PTEN*<sup>+/-</sup> mice precluding loss of their remaining *PTEN* allele.

Our bioinformatics studies also revealed the invariable coamplification of the *mir-26-2* locus with the known oncogene *CDK4*. Such clustering of cancer-related genes is hardly unprecedented, with the *CDKN2A* locus, containing the *INK4A*, *INK4B*, and *ARF* tumor suppressors, being a prime example (Gil and Peters 2006). The reciprocal relationship between miR-26a and PTEN discussed above, coupled with the very tangible impact of expressing the miRNA both in vitro and in vivo, demonstrates that increased copy number at the *miR-26a-2* locus, in all likelihood, carries with it a biological significance independent of its proximity to *CDK4*, a conclu-

sion that is all the more compelling in light of the failure of *CDK4* overexpression to significantly alter PDGF-driven gliomagenesis in mice. Nevertheless, the close physical association of *miR-26a-2* and *CDK4* does imply a cooperative relationship between the two genes, and additional in vivo studies should provide further insight into the functional significance of this association. The distinct lack of amplification at either the *miR-26a-1* locus or highly homologous *miR-26b* locus in glioma might be seen as additional evidence of a synergistic link between miR-26a and *CDK4*. However, this discrepancy could simply reflect basic differences in genomic fragility at the respective chromosomal positions, the determinants of which are still very much unclear (Glover et al. 2005).

Akt pathway activation has been shown to occur in a plurality of GBMs, most frequently resulting from modulation of its primary negative regulator PTEN (The Cancer Genome Atlas Research Network 2008). In this report, we document a novel epigenetic mechanism by which PTEN activity is regulated in glioma. While deletion and mutation represent the two most common avenues to PTEN loss, alternative means do exist. Promoter methylation has been documented as a cause for reduced PTEN expression in a variety of glioma subtypes (Baeza et al. 2003; Wiencke et al. 2007). Much like miR-26a expression, *PTEN* promoter methylation is, for the most part, mutually exclusive with *PTEN* mutation/deletion and, interestingly, tends to occur in lower-grade gliomas that subsequently evolve in to GBMs (so-called secondary GBMs) (Wiencke et al. 2007). This latter finding is reminiscent of our mouse modeling studies showing that miR-26a expression yields lower grade tumors than those associated with complete *PTEN* loss, perhaps reflecting a more modest degree of PTEN knockdown.

Epigenetic mechanisms for PTEN repression, such as promoter methylation and miR-26a amplification, enrich the spectrum of molecular abnormalities with the potential to drive glioma progression. Our data suggest that approximately one-eighth of GBMs already lacking one *PTEN* allele achieve silencing of their remaining copy through overexpression of miR-26a. While *PTEN* mutation or deletion appears to serve the same functional purpose in an additional 26% and ~4% of *PTEN*<sup>+/-</sup> tumors, respectively, it remains to be seen what portions of this glioma subgroup employ *PTEN* promoter methylation or other still undiscovered epigenetic means to mediate similar effects. High-quality promoter methylation data for GBMs has yet to be released by TCGA on a large scale. Regardless, it appears almost certain that human gliomas use a variety of strategies to functionally eliminate PTEN and, in this way, activate oncogenic Akt signaling.

In conclusion, we identified the first miRNA species that facilitates the process of glioma formation in vivo, along with a mechanism for its overexpression in human tumors. Furthermore, the repression of PTEN by miR-26a provides an intriguing link to an oncogenic pathway whose role in glioma biology is already well established,



and, in this way, strengthens this case for therapeutic inhibition of Akt signaling. Additional characterization of this and other glioma-implicated miRNAs, along with their mRNA targets, should greatly enhance the understanding of this novel epigenetic regulatory mechanism as it relates to cancer biology in general and gliomagenesis in particular.

## Materials and methods

### *Cell culture, transfections, and Western blots*

Cells were cultured, lysed, and processed for Western blotting by standard methods. Transfections were performed using the Fugene 6 reagent. The following primary antibodies were used for Western blots: PTEN (Cascade Bioscience); Actin (Santa Cruz Biotechnologies); pAkt, pS6RP, and EZH2 (Cell Signaling Technology); and SMAD1 (Abcam). Western blots were developed using ECL Detection Reagents (GE Healthcare), and bands were quantified by densitometry with ImageJ 1.40 (NIH).

### *Retroviral transduction*

miR-Vec constructs were generated as described previously (Voorhoeve et al. 2006). Retrovirus was produced in the Plat-E packaging cell line (Cell Biolabs) and harvested 48 h after miR-Vec transfection. NIH-3T3 cells were infected using virus-containing supernatant supplemented with 5  $\mu$ g/mL polybrene and selected with blasticidin. RCAS-PDGf and RCAS-Cre constructs were generated as described previously (Shih et al. 2004; Hu et al. 2005). RCAS-miR constructs were initially assembled in the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen) by inserting PCR-amplified human or murine miR sequences (250–500 base pairs [bp]) downstream from the EmGFP expression cassette. A larger sequence including the CMV promoter, EmGFP cassette, and miRNA domain was then PCR-amplified and cloned into the RCAS plasmid as between the NotI and ClaI restriction sites. RCAS virus was produced in DF-1 packaging cells (American Type Culture Collection) as described previously (Holland and Varmus 1998; Holland et al. 1998).

### *Luciferase reporter assay*

The PTEN 3'-UTR luciferase reporter construct (PCPTEN) was generated by PCR-amplifying the murine PTEN 3'-UTR from genomic DNA. The fragment was then subcloned into pYAP6 to obtain appropriate restriction sites before insertion into the psiCHECK-2 vector downstream from the renilla luciferase cassette. Predicted miR-26a-binding sites were mutated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). HEK-293 cells were transfected with PCPTEN and RCAS-miR constructs, lysed 24–48 h later, and processed for luciferase assay using the Dual-Luciferase Reporter Assay System (Promega). Assays were read in a Veritas Microplate Luminometer (Turner Biosystems) normalizing renilla luciferase signal to firefly luciferase signal.

### *Q-PCR and QRT-PCR*

DNA was extracted from human brain tissue using the DNeasy Blood and Tissue Kit and from paraffin sections of murine tumors using the QIAamp DNA FFPE Tissue Kit (both from Qiagen). Total RNA was extracted from cells and/or tissue using the Trizol reagent and protocol (Invitrogen). Q-PCR and QRT-PCR was performed using miRNA-specific primer sets, reagents, and

protocols from Applied Biosystems in a 7900 HT Fast Real-Time PCR System (Applied Biosystems).

### *Microarray analysis*

Total RNA extracted from brain tumors and normal controls was enriched for miRNA and labeled by the Agilent protocol followed by hybridization to the human mRNA version 1.0 array (Agilent Technologies). Raw data were normalized to total signal intensity prior to comparative analysis.

### *Analysis of TCGA data*

miRNA expression, gene expression, array CGH, and mutational analysis data from GBM samples were collected as previously described (The Cancer Genome Atlas Research Network 2008). Processed data sets were obtained directly from the public access data portal (<http://cancergenome.nih.gov/dataportal/data/about>; Supplemental Material). Data sets were cross-referenced using tumor-specific identification numbers: 233 samples had miRNA expression, gene expression, and array-CGH. Post-segmentation array-CGH data were used for local copy number estimation. Amplification of mir-26a was defined by regional log<sub>2</sub> ratio >1.0. PTEN locus was classified by minimal log<sub>2</sub> ratio (log<sub>2</sub>R) across the gene as follows: “euploid” =  $-0.2 < \log_2R < 0.4$ ; “single copy loss” =  $-1.0 < \log_2R < -0.2$ ; “homozygous deletion” =  $\log_2R < -1.5$ ; and “intermediate” = intermediate log<sub>2</sub>R. PTEN mutations were derived from the TCGA data as described in the Supplemental Material. Tumor IDs, source samples, and derived data values are given in Supplemental Table 1.

### *Generation of murine gliomas*

Ntv-a/*PTEN*<sup>fl/fl</sup> mice, Ntv-a/*PTEN*<sup>+/-</sup> mice, and procedures for RCAS-mediated gliomagenesis have been described previously (Holland and Varmus 1998; Holland et al. 1998; Hu et al. 2005). After injection of relevant RCAS viruses during the newborn period, mice were aged until they developed symptoms of disease (lethargy, poor grooming, weight loss, macrocephaly).

### *Statistics*

Error bars in all cases indicate standard deviation. Kaplan-Meier analysis of symptom-free survival in murine gliomas was performed using the Gehan-Breslow-Wilcoxon test in Prism 5 software (GraphPad). Luciferase reporter assays and Q-PCR data for *PTEN* were analyzed by two-tailed *t*-test in either Excel 2004 (Microsoft) or Prism 5, respectively. Correlations between tumor groups identified in TCGA data were analyzed by Fisher's exact test (R package:stats, <http://www.cran.org>).

### *Histology and immunohistochemistry*

Mouse brains were prepared for either paraffin or frozen sectioning and stained with hematoxylin and eosin as described previously (Hu et al. 2005; Becher et al. 2008). Tumor grade was established using World Health Organization (WHO) criteria (Louis et al. 2007). Briefly, grade II tumors exhibited increased cellularity with predominantly monomorphic cellular features and minimal mitotic activity; grade III tumors exhibited more marked cellular atypia, increased mitoses, and mild microvascular proliferation; and grade IV tumors exhibited florid microvascular proliferation and foci of pseudopalisading necrosis. Immunohistochemical staining was performed as previously described (Hambarzumyan et al. 2008). The following antibodies

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were used: GFP (Invitrogen) at 1:200; PTEN (Cell Signaling Technology) at 1:100. Immunohistochemical staining for PTEN was quantified using Metamorph software (Molecular Devices). Two tumors from each genotype (P/Z, P/26a, and P/Cre) were used for the analysis. Eight 20× images from each tumor were loaded into Metamorph (Molecular Devices). Threshold signal intensity was established in each case using staining from non-neoplastic tumor vasculature. The amount of signal reaching threshold was then quantified in identically sized rectangles placed over solid areas of tumor cells in each micrograph.

### LISH

In situ hybridization was carried out using a DIG-labeled locked nucleic acid probe directed against miR-26a ([A][G]CC[T]A[T]C C[T]GG[A]TT[A]C[T]T[G][A]A, locked residues marked with brackets) as described previously (Supplemental Material; Pena et al. 2009).

### Acknowledgments

We thank Yevgeniy Romin and Katia Manova for their assistance in the quantification of immunohistochemical staining, Agnes Viale for running the miRNA microarrays, and Robert "Jim" Finney for technical support. We also thank Massimo Squatrito, Nduka Amankulor, Elena Fomchenko, Sohail Tavazoie, and Anne-Marie Bleau for their helpful discussions, and Andrea Ventura for his critical reading of the manuscript. This work was supported by a STARR Cancer Consortium Award (to T.T. and E.C.H.) and the David Tetenbaum Hope American Brain Tumor Association Research Fellowship (to J.T.H.).

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*Genes Dev.* 2009, **23**:

Access the most recent version at doi:[10.1101/gad.1777409](https://doi.org/10.1101/gad.1777409)

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