miR-182 integrates apoptosis, growth, and differentiation programs in glioblastoma

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Glioblastoma multiforme (GBM) is a lethal, therapy-resistant brain cancer consisting of numerous tumor cell subpopulations, including stem-like glioma-initiating cells (GICs), which contribute to tumor recurrence following initial response to therapy. Here, we identified miR-182 as a regulator of apoptosis, growth, and differentiation programs whose expression level is correlated with GBM patient survival. Repression of Bcl2-like12 (Bcl2L12), c-Met, and hypoxia-inducible factor 2α (HIF2A) is of central importance to miR-182 anti-tumor activity, as it results in enhanced therapy susceptibility, decreased GIC sphere size, expansion, and stemness in vitro. To evaluate the tumor-suppressive function of miR-182 in vivo, we synthesized miR-182-based spherical nucleic acids (182-SNAs); i.e., gold nanoparticles covalently functionalized with mature miR-182 duplexes. Intravenously administered 182-SNAs penetrated the blood–brain/blood–tumor barriers (BBB/BTB) in orthotopic GBM xenografts and selectively disseminated throughout extravascular glioma parenchyma, causing reduced tumor burden and increased animal survival. Our results indicate that harnessing the anti-tumor activities of miR-182 via safe and robust delivery of 182-SNAs represents a novel strategy for therapeutic intervention in GBM.

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Patients diagnosed with the most common and aggressive manifestation of malignant glioma, glioblastoma multiforme (GBM), have a median survival of well under 2 years (Dunn et al. 2012). The current standard of treatment entails surgical resection followed by radiation therapy combined with administration of the DNA-alkylating agent temozolomide (TMZ), which increases median survival by only 2.4 mo (Stupp et al. 2009). Continued advances in surgical practices, the advent of new radiotherapy techniques (Crowley et al. 2006), and the testing of targeted therapies have failed to improve this outcome.

Recently, we identified the atypical Bcl-2 family protein Bcl2-like12 [Bcl2L12] as a potent oncogene and therapeutic RNAi target in GBM that blocked therapy-induced apoptosis and promoted gliomagenesis in vivo by inhibiting effector caspase and p53 activation [Stegh et al. 2007, 2008a,b, 2010; Stegh and DePinho 2011; Jensen et al. 2013]. While Bcl2L12 is highly expressed in almost all primary GBM specimens, levels of overexpression vary by GBM subtype. Specifically, in silico oncogenic analyses revealed robust genomic amplification (nonfocal 19q...
miR-182 has anti-tumor activity in GBM

Results

Identification of miR-182 as a p53-controlled proapoptotic factor in GBM that targets Bcl2L12

Bcl2L12 is a critical inhibitor of therapy-induced apoptosis with overexpression in nearly all GBM [Stegh et al. 2007, 2008a,b, 2010; Stegh and DePinho 2011]. We aimed to identify miRNAs that control the expression of Bcl2L12 in GBM, as such miRNAs represent potent tumor suppressors with chemosensitizing activity and are thus ideal candidates for subsequent design and preclinical evaluation of SNA therapeutics.

As a first step, in silico studies of GBM samples of the multidimensional Cancer Genome Atlas (TCGA) data set [http://cancergenome.nih.gov/dataportal; Cancer Genome Atlas Research Network 2008] were conducted to discover miRNAs with expression levels that negatively correlate with Bcl2L12 mRNA abundance. This oncogenic analysis identified miR-182 that negatively correlated with Bcl2L12 mRNA, especially in proneural tumor specimens \[R = -0.32, P = 0.01\] [Supplemental Fig. S1A], which are characterized by frequent p53 mutation [Verhaak et al. 2010]. Negative correlation between Bcl2L12 and miR-182 expression levels were subsequently confirmed by RT-qPCR in a collection of frozen primary GBM specimens \[n = 45, R = -0.61, P = 8.65 \times 10^{-6}\] [Supplemental Fig. S1B]. Here, GBM tumors carrying p53 mutations showed elevated levels of miR-182 compared with those with wild-type p53 [Supplemental Fig. S2A].

Next, we determined molecular mechanisms that regulate miR-182 expression. In silico promoter analysis [Transcriptional Regulatory Element Database (TRED)] identified a total of five putative p53-binding sites, three of which are located in the promoter proximal to the miR-182 transcriptional start site [termed proximal p53 response elements [p53REproximal], and two found in an up-stream promoter region that controls the expression of the entire miR-183-96-182 cluster [p53REdistal] [Supplemental Fig. S2B]. Luciferase assays revealed that wild-type but not dominant-negative p53 repressed reporter activity of both proximal and distal promoters [Supplemental Fig. S2C,D]. Induction of a luciferase reporter containing multimerized p53 consensus sites is shown as a positive control to confirm that ectopically expressed p53 is functional [Supplemental Fig. S2E]. Similarly, endogenous miR-182 levels decreased upon treatment of U87MG and transformed astrocytes with doxorubicin, a well-described DNA damage-inducing agent and p53 activator [Supplemental Fig. S2F]. Together, these data suggest that miR-182 expression in GBM is controlled by p53, and that
elevated miR-182 levels in proneural GBM tumors negatively correlate with Bcl2L12 mRNA abundance.

To functionally validate these in silico analysis results and further characterize a potential Bcl2L12–miR-182 axis, we engineered a luciferase reporter construct harboring the full-length (167-base-pair [bp]) human Bcl2L12 3′ untranslated region [UTR] [Fig. 1A], including an evolutionarily conserved miR-182-binding site identified by TargetScan (position 117–124 of the Bcl2L12 3′ UTR) [Fig. 1B,C]. Cotransfection with synthetic miR-182 but not a nontargeting control miR (Co-miR) resulted in 50% reduction in luciferase levels [Fig. 1D]. miR-96 and miR-183, members of the 183-96-182 miR cluster, failed to repress Bcl2L12 3′ UTR reporter activity [Fig. 1E]. To further confirm that miR-182 binds the Bcl2L12 3′ UTR, we generated a luciferase reporter with a mutated miR-182-binding site. Disrupting the binding site completely prevented repression by the synthetic miR-182 [Fig. 1D]. Subsequently, Co-miR or miR-182 sequences were ectopically expressed in three glioma cell lines [i.e., U87MG, LNZ308, and SF767] (see Supplemental Table S1 for p53 mutational status of glioma cell lines used in this study). Western blot and RT-qPCR analyses confirmed robust down-regulation of Bcl2L12 on mRNA and protein levels upon miR-182 expression and also showed increased Bcl2L12 protein and mRNA levels upon expression of a miR-182 inhibitor [anti-miR-182] [Fig. 1F,G]. These studies indicate that miR-182, but not other members of the miR-183-96-182 cluster transcribed from the same polycistronic transcript, directly binds to the 3′ UTR of Bcl2L12 and represses its mRNA and protein expression.

miR-182 sensitizes glioma cells toward chemotherapy via down-regulation of Bcl2L12

Next, we determined whether miR-182 regulates oncogenic properties of Bcl2L12, in particular its ability to neutralize effector caspases in cells and tumors. To assess the functional consequences of miR-182-mediated knockdown of Bcl2L12, we examined effector caspase activation,
miR-182 controls a stem cell-associated signature in GBM

Consensus clustering of miRNA expression profiles in 261 TCGA high-grade gliomas identified five clinically and genetically distinct subclasses of GBM resembling...
major stages of neural cell development; i.e., radial glia, oligoneural, neural, neuroepithelial/neural crest, and astrocyte precursors [Kim et al. 2011]. miR-182 is expressed in the oligoneural GBM subtype, which is characterized by reduced mRNA expression of embryonic and neural stem cell signatures and is associated with significantly increased patient survival [Kim et al. 2011]. In support of a role for miR-182 in regulating growth and differentiation processes, expression of miR-182 in GICs decreased neurosphere size and expansion upon low-density seeding (Supplemental Fig. S3). In addition, miR-182 reduced cellular growth in response to mitogenic factors, such as transforming growth factor \( \beta_1 \) (TGF\( \beta_1 \)) (Supplemental Fig. S4), a critical driver of GBM progression with pleiotropic effects on tumor cell growth, invasion, self-renewal, and neo-angiogenesis [Golestaneh and Mishra 2005]. Furthermore, miR-182 expression was decreased in CD133-positive, patient-derived GICs in comparison with CD133-negative GICs [Fig. 3A]. Upon ectopic expression, miR-182 promoted a more differentiated phenotype, as evidenced by decreased expression of stem cells markers (i.e., Oct4, CD44, Nanog, and Sox2) [Fig. 3B–D], increased numbers of adherent cells (Supplemental Fig. S3C), and elevated levels of MAP2 and GFAP cell positivity [Fig. 3E,F].

To investigate the molecular basis of miR-182-dependent growth and differentiation, we subjected patient-derived GICs, following modification with lentivirus for expression of miR-182 or Co-miR, to whole-genome expression profiling [a heat map and a complete list of significantly affected genes are shown in Supplemental Fig. S5; Supplemental Table S2]. A comparative analysis of the transcripts repressed by miR-182 and mRNA signatures up-regulated in seven stages of neural cell differentiation [i.e., mRNA expression profiles derived from differentiated neural cells [astrocytes, oligodendrocytes, and neurons] and stem cells [embryonic, hematopoietic, and neural stem cells]] revealed that, similar to miR-182-expressing oligoneural GBM tumors, the miR-182-repressed transcriptome in GICs is enriched for stem cell-related genes.
miR-182 has anti-tumor activity in GBM

miR-182 controls HIF2A-dependent cell dedifferentiation in GBM

Next, we determined molecular mechanisms of miR-182-enhanced cell differentiation. We found that miR-182 recognized an evolutionarily conserved site within the 3′ UTR of HIF2A (Supplemental Fig. S9A). UTR binding was confirmed by reporter assay demonstrating significant reduction in luciferase activity in the presence of miR-182 as compared with Co-miR [Fig. 3J]. Furthermore, miR-182 could reduce HIF2A mRNA and protein levels under normoxic and hypoxic conditions [Fig. 3H,I]. As HIF2A is a well-annotated mediator of hypoxic stress in the cellular environment that is coexpressed with cancer stem cell markers and promotes self-renewal, cell survival, and the tumor-initiating potential of GICs [Li et al. 2009], we determined whether miR-182, by down-regulating HIF2A, impacts stem cell marker expression. Anti-miR-182 expression triggered an increase in stem cell and proliferation marker expression (e.g., Nestin, Sox2, CD44, and CCNB1), which was negated by concomitant siRNA-mediated silencing of HIF2A, indicating that HIF2A knockdown contributes to the regulation of stem cell and proliferation marker expression in response to miR-182 expression [Fig. 3K, see L for densitometric quantification of Western blot data].

miR-182 regulates c-Met-controlled glioma cell growth

Besides hypoxia-driven, HIF2A-controlled differentiation processes, we found that miR-182 also regulates glioma cell growth by targeting the RTK c-Met, which represents a functional glioma stem cell marker and promoter of GIC self-renewal [Boccaccio and Comoglio 2013]. A survey of the TCGA data set showed a prominent negative correlation between miR-182 and c-Met mRNA expression, predominantly in classical and proneural GBM tumors [Fig. 4A]. A series of RT-qPCR [Fig. 4B], Western blot [Fig. 4C], and luciferase reporter assays [Fig. 4D] in GICs and transformed glioma cell lines revealed that miR-182, by binding to an evolutionarily conserved 8-mer 3′ UTR sequence (Supplemental Fig. S9B), down-regulates c-Met mRNA and protein. c-Met knockdown contributes to the cell cycle inhibitory activity of miR-182 [Fig. 4E; Supplemental Fig. S4], as transfection of anti-miR-182 sequences provoked a decrease in G1-phase content, which was antagonized by c-Met-targeting siRNAs [Fig. 4E]. Similarly, analyses of cell morphology and cell cycle marker expression revealed that c-Met knockdown mediated a morphological transition into a flattened cell shape and antagonized anti-miR-182-driven Akt activation and CCNB1 induction [Fig. 4F,G]. Thus, c-Met and HIF2A are critical miR-182 target genes that regulate GIC growth and differentiation in GBM.

miR-182-based SNAs reduce expression of miR-182 target genes and show anti-tumor activity in vitro

Inherent barriers of miRNA-based functional studies and miRNA-directed cancer therapy include the lack of delivery tools that enable robust, nontoxic, nonimmunogenic penetration of miRNAs into tumor cells and tissue. Delivery is in particularly challenging for brain tumors, as miRNA–carrier complexes have to cross the BBB and BTB and distribute throughout extravascular areas of the glioma parenchyma. Here, we designed a series of functional and efficacy studies to test the hypothesis that conjugation of gold nanoparticles bearing a monolayer of alkylthiol-modified miR-182 duplexes [Fig. 5A–C] could provide an approach for delivering proapoptotic and anti-proliferative miR-182 into GBM cells and tumors. First, we investigated the uptake of Cy5.5-labelsted 182-SNAs into glioma lineages. In contrast to nonconjugated linear Cy5.5-miR-182 oligoucleotides [Fig. 5D, inset], 182-SNAs robustly penetrated >90% of glioma cells and patient-derived GICs [Fig. 5D], significantly decreased Bcl2L12 and c-Met protein levels compared with Co-SNA-treated cultures [Fig. 5E], increased apoptosis in association with STS treatment [Fig. 5F], and diminished proliferation of glioma cells, as indicated by reduced BrdU incorporation [Fig. 5G].

miR-182 displays significant organ retention and a low-toxicity profile in vivo

To evaluate the miR-SNA platform in vivo, we assessed 182-SNA pharmacokinetics, distribution, toxicity, cytokine induction, and intratumoral accumulation upon intravenous administration. Pharmacokinetics of 182-SNAs follow a two-compartment model, as shown by nonlinear regression analysis, suggesting that transport between the central and peripheral compartments and the elimination of SNA conjugates follow first-order
kinetics (Fig. 6A). 182-SNA half-life for distribution (\(\alpha t_{1/2}\)) was \(~27\) min (Fig. 6A), and SNA retention was significant, as indicated by a slow elimination phase (half-life for elimination, \(\beta t_{1/2}\), \(~12.6\) h) (Fig. 6B) and low levels of SNAs in the blood throughout the 72-h course of the study. Tissue distribution in healthy animals was primarily in the liver and spleen, with small accumulations in the lungs, kidneys, heart, brain, and intestines (Supplemental Fig. S10).

To determine potential adverse side effects of SNAs, 182-SNAs (10 mg of RNA per kilogram of animal weight) or an equivalent volume of saline were administered as a single dose via intravenous injection into Sprague-Dawley rats, with the effects on body weight, blood chemistry, blood counts, and major organ histopathology determined. Importantly, we did not observe 182-SNA-related clinical signs or difference in body or organ weight at 1 or 14 d after SNA administration. Complete blood counts (CBCs/hematology) and blood chemistry panels were within normal range (Supplemental Table S4), confirming the absence of early or late toxic responses at therapeutic dosages. Histopathologic evaluation of major organs failed to detect malignant lesions or signs of abnormal processes and indicated organ structures that were consistent with clinically normal, age- and strain-matched rats (Fig. 6C).

To address whether there was any indication of induced immune response, a screen for inflammatory cytokine induction was performed using blood samples from SCID mice inoculated with U87MG cells and treated with Co-SNAs, 182-SNAs, or saline every other day. After receiving seven treatments and \(~10\) mg/kg SNAs, no significant differences were noticed between the groups that would support an inflammatory response (Supplemental Table S5).

We next determined intraglioma SNA accumulation upon systemic administration to elucidate whether 182-SNAs crossed the BBB/BTB. In orthotopic GIC-derived GBM xenografts systemically injected with Cy5.5-labeled 182-SNAs, we demonstrated by in vivo imaging system (IVIS) (Fig. 6D), inductively coupled plasma mass spectrometry (ICP-MS) (Fig. 6E), and silver staining of coronal sections (Fig. 6F,G) that 182-SNAs penetrated glioma cell line and GIC-derived tumor grafts, with robust accumulation of Co-SNAs and 182-SNAs in glioma-associated vasculature and parenchyma (Fig. 6F,G).

**Figure 4.** miR-182 regulates cell cycle progression through c-Met. [(A)] In silico correlation analysis of c-Met and miR-182 mRNA levels using the TCGA data set. [(B)] RT-qPCR analysis of c-Met mRNA levels in different GICs overexpressing Co-miR or miR-182, shown as \(\log_2\) fold change ± SD. [(C)] Western blot analysis of c-Met protein levels in GICs and adherent cell lines overexpressing Co-miR or miR-182. Hsp70 served as loading control. [(D)] Luciferase activity measured in 293T cells 24–48 h after transfection of c-Met-3′ UTR-pGL3 reporter vectors in combination with miR-182 or Co-miR sequences at a concentration of 200 nM. [(E)] Cell cycle analysis of U87MG cells transfected with 100 nM siCo, 100 nM sic-Met, 100 nM anti-miR-182, or a combination of sic-Met and anti-miR-182 for 48 h. [(F)] Western blot analysis of c-Met downstream targets in U87MG and SF767 cells transfected with siCo, sic-Met, or anti-miR-182 as single agents or in combination for 48 h. [(G)] Bright-field images of U87MG cells transfected with 100 nM siCo, 100 nM sic-Met, or 100 nM anti-miR-182 alone or in combination with 100 nM sic-Met for 48 h. Bar, 60 μm.
miR-182 is associated with increased survival and a less aggressive glioma phenotype in vivo

To further evaluate the tumor-suppressive functions of miR-182 and 182-SNAs, we analyzed TCGA patient survival as a function of miR-182 expression levels. miR-182 expression levels in TCGA GBM tumors, in particular in the proneural subclass, positively correlated with patient survival (Supplemental Fig. S11; Celiku et al. 2014). Studies in orthotopic xenograft models using transformed glioma cells and GICs ectopically expressing miR-182 showed that the presence of miR-182 increased glioma-free survival (Fig. 7A) and reduced tumor burden, as determined by measurement of tumor weight (Fig. 7B) and bioluminescence imaging of luciferase-labeled GICs (Fig. 7C,D). Histopathological analysis of Co-miR-expressing and miR-182-expressing tumors revealed that the presence of miR-182 decreased the number of proliferating and increased the number of apoptotic tumor cells, as determined by Ki67 and caspase-3 immunohistochemistry (IHC), respectively (Fig. 7E,F).

To assess the impact of 182-SNAs on GBM progression in vivo, we administered 182-SNAs and Co-SNAs through tail vein injection to SCID mice that were inoculated with U87MG cells and GIC-20. We found that robust BBB/BTB penetration and intratumoral accumulation of 182-SNAs resulted in significant reduction in tumor burden, as assessed by weighing of tumor tissue (Fig. 7G), bioluminescence imaging of xenografts from luciferase-labeled GICs (Fig. 7H,I), and prolonged animal subject survival (Fig. 7J,K). Thus, efficient BBB/BTB penetration and robust intraglial dissemination of 182-SNAs upon systemic administration resulted in decreased tumor burden and increased xenograft survival in the absence of adverse side effects.

Discussion

In this study, we report the identification and functional characterization of miR-182 as a regulator of growth,
differentiation, chemotherapeutic susceptibility, and tumor progression in GBM by targeting an oncogenic signature that includes Bcl2L12, c-Met, and HIF2A via direct binding to evolutionarily conserved, 3′ UTR-localized sequences. Using miR-182-functionalized SNAs, we demonstrate that miR-182 sequences effectively reduce tumor burden, thereby providing the first proof of concept that systemically administered miRNAs can cross the BBB/BTB, infiltrate glioma parenchyma, and inhibit GBM progression. miR-182 has been the topic of extensive research in developmental and cancer biology. Together with its family members, miR-96 and miR-183, miR-182 was first described in mouse neurosensory cells, specifically in the retina, inner ear, and dorsal root ganglia (Kloosterman and Plasterk 2006; Xu et al. 2007; Weston et al. 2011). During the embryonic stages of development, miR-182 levels are low but significantly increase postnatally, suggesting a role for miR-182 in terminal differentiation of retinal progenitor cells and maintenance of mature retinal neuron function [Xu et al. 2007]. Several other recent studies have suggested that miR-182 promotes cellular differentiation by regulating the expression of the transcription factor SNAI2 and induces mesenchymal-to-epithelial transition [Qu et al. 2013]. Consistent with these findings, we demonstrate that miR-182 overexpression in GICs reduces sphere size and expansion and promotes differentiation along neuronal and astroglial axes at least in part via targeting of c-Met and HIF2A signaling. We further show that the miR-182-repressed transcriptome of GICs was enriched for stem cell signatures. The miR-182 target gene c-Met is hyperactivated in GBM (Huang et al. 2007; Stommel et al. 2007; Cancer Genome Atlas Research Network 2008); represents a critical driver of GBM growth, invasion, hypoxia-induced dedifferentiation, and tumor progression (Boccaccio and Comoglio 2006, 2013); and is critical for adult stem cells to transition between a quiescent G0 and an "alert" G phase. Such transition primes stem cells for cell cycle entry and enables them to effectively mobilize their tissue-regenerative function [Rodgers et al. 2014]. Similarly, HIF2A is an important effector of hypoxia-driven stem cell maintenance that promotes GIC self-renewal, growth, and...
tumorigenicity, and its expression negatively correlates with glioma patient survival (Li et al. 2009).

Further demonstrating prodifferentiation activity of miR-182, GBM tumors, according to TCGA-based miRNA clustering, are classified into five clinically and genetically distinct groups that each relates to a different neural precursor cell type (Kim et al. 2011). In particular, the oligoneural tumors, which are characterized by elevated miR-182 levels, exhibit significantly longer survival than patients with radial glial, neural, astrocytic, or neuromesenchymal tumors. On a genetic level, oligoneural tumors show repression of neural and embryonic stem cell-related mRNA signatures, suggesting a more differentiated, less stem-like state.

We propose that miR-182 expression in GICs promotes a more differentiated, less aggressive phenotype and that this activity is at least in part due to transcriptional repression of c-Met and HIF2A. While our mRNA, protein, and 3' UTR reporter studies point to direct transcriptional regulation of c-Met and HIF2A by miR-182, only c-Met but not HIF2A mRNA expression is negatively correlated with miR-182 levels in TCGA GBM. This suggests that HIF2A is regulated through additional mechanisms in GBM tumors besides transcriptional repression, including repression of mRNA translation or modulation of HIF2A protein expression/stability.

In addition to the regulation of GIC and glioma cell plasticity, we identified miR-182 as a chemosensitizer that promotes apoptosis induced by conventional and targeted therapies through down-regulation of Bcl2L12, a validated oncogene with high-level expression in GBM tumors (Stegh et al. 2007; Jensen et al. 2013). Bcl2L12 is significantly expressed in the majority of primary GBM tumor specimens and is distantly related to canonical Bcl-2 proteins. Mechanistically, our previous studies determined that, unlike prototypic Bcl-2 family members, Bcl2L12 did not safeguard mitochondrial membrane integrity but instead potently inhibited apoptosis at the level of post-mitochondrial effector caspase-3 and caspase-7 activation. A combination of enforced expression, RNAi-mediated extinction, colocalization, and protein-interaction studies revealed that Bcl2L12 inhibits caspase-3 and caspase-7 via distinct mechanisms. Direct physical interaction underlies Bcl2L12’s inhibition of caspase-7.
SNAs exhibit a number of unique properties (Allhenn et al. 2012). In a recent study, we characterized parenchyma beyond perivascular regions (Blakeley 2008; Blakeley et al. 2009; Blakeley et al. 2011). By down-regulating BclL2L2, miR-182 sensitizes cells toward extant therapies primarily by increasing effector caspase activity.

Similar to other miRNAs, miR-182 has been reported to have cancer-promoting or inhibitory activity, which largely depends on cancer type and genetic context. For instance, it has been shown that miR-182 has oncogenic properties in medulloblastoma and cervical, breast, colon, prostate, and bladder cancer and tumor-suppressive activity in lung cancer, gastric adenocarcinoma, oropharyngeal carcinoma, and uveal melanoma by regulating oncogenes such as Bcl-2, Cyclin D2, MITF, and, via indirect mechanisms, c-Met. In addition, miR-182 acts as a tumor suppressor in renal cell carcinoma by targeting the FLOT1/AKT/FOXO3/CyclinD1 pathway (Sarver et al. 2009; Segura et al. 2009; Cekaite et al. 2012; Hirata et al. 2012, 2013; Kong et al. 2012; Liu et al. 2012, 2013; Poell et al. 2012; Weeraratne et al. 2012; Yan et al. 2012; Hui et al. 2013; Li et al. 2014; Tang et al. 2014; Xu et al. 2014; Yang et al. 2014). This is particularly relevant to GBM, as a recent study suggested that miR-182 can trigger TGF-β1-dependent NF-κB induction in glioma cells and provoke a more aggressive glioma phenotype by targeting the deubiquitinase CYTLB (Song et al. 2012). These data suggest that miR-182 activity is highly context-dependent, pointing to important roles of the tumor microenvironment and the cooperating cancer (subtype)-specific genetic landscape in shaping miR-182 responses.

With regard to molecular mechanisms controlling miR-182 expression, we propose that p53 negatively regulates miR-182 transcription. p53 is an important regulator of cancer stem cell differentiation, as loss of p53 promotes neurosphere formation and a stem cell-like phenotype (Puzzio-Kuter and Levine 2009). Thus, we hypothesize that in tumors with compromised p53 function, miR-182 levels are elevated and compensate for p53 loss of function by promoting a more differentiated GIC state.

Drug delivery to intracranial tumor sites represents a major challenge for the development of GBM therapeutics. In particular, the BBB/BTB represents a major physiological barrier that often prevents the accumulation of therapeutic drug concentrations within intracerebral lesions. Delivery of RNA-based therapeutics is particularly challenging, since RNA can be unstable, often entrapped in endosomes, and incapable of infiltrating tumor parenchyma beyond perivascular regions (Blakeley 2008; Allhenn et al. 2012). In a recent study, we characterized SNAs as a novel RNAi-based nanoconjugate using siRNAs designed to target BclL2L2 (Jensen et al. 2013). SNAs exhibit a number of unique properties—i.e., high cellular uptake (>90% of cell populations), including patient-derived GICs without the use of toxic, auxiliary transfection agents or viral delivery platforms; extraordinary stability in physiological environments; robust resistance to nuclease degradation (Giljohann et al. 2009; Seferos et al. 2009); no acute or long-term toxicity at high and therapeutic doses in animal models; and low activation of the innate immune response (Massich et al. 2009). siRNA-based SNAs crossed the BBB/BTB and displayed preferential accumulation within intracerebral glioma due to the enhanced permeability and retention (EPR) effect, i.e., the increased accumulation of nanomaterials in tumors due to the abnormal form and architecture of tumor blood vessels. Thus, SNAs represent a powerful platform for RNAi-mediated biotherapeutic gene silencing, allowing us to combine potent knockdown of GBM oncogenes with efficient BBB/BTB penetration and robust intragliaoma dissemination upon systemic administration. Here, we developed and characterized a novel construct of SNAs functionalized with mature miR-182 sequences to harness the tumor-suppressive activities of miR-182. 182-SNAs effectively crossed the BBB/BTB upon systemic intravenous administration and disseminated throughout glioma parenchyma, as evidenced by IVIS-based imaging, ICP-MS, and silver staining on serial brain sections. SNAs therefore have potential as a tractable, nontoxic platform for biotherapeutic gene silencing via passive tumor targeting driven by EPR of the tumor-associated vasculature. While SNA conjugates were readily detectable in non-tumor-bearing mice by IVIS, intracranial levels were significantly higher in glioma-bearing mice, suggesting that a compromised BBB/BTB together with EPR significantly contributes to accumulation of 182-SNAs in tumor versus normal brain elements. Validation experiments in glioma cells revealed robust cellular uptake of 182-SNAs that resulted in potent down-regulation of BclL2L2 and c-Met proteins, sensitization toward drug-induced apoptosis, and reduction in cellular proliferation. Mirroring proapoptotic and growth inhibitory effects in cells in vitro, systemic delivery of 182-SNAs reduced tumor burden in an orthotopic GBM xenograft model. Together with toxicity studies confirming the absence of significant adverse side effects and inflammatory responses associated with systemic introduction of miR-182, we propose that 182-SNAs represent a tool for discovery science to characterize miR function in cells and animals in the absence of viral or lipoplex delivery agents and a novel therapeutic paradigm for the treatment of GBM and possibly other genetic malignancies and disorders of the CNS. Thus, the potent tumor-suppressive activity of miR-182 resulting from repression of critical GBM oncogenes—primarily BclL2L2, c-Met, and HIF2A—together with the capacity of the SNA platform to effectively infiltrate GBM tumors points to SNA-mediated miR-182 transfer as an attractive strategy to target multiple oncogenes.

Materials and methods

Reagents
The pan-specific protein kinase inhibitor STS (0.5 μM) (Sigma) and the following RTK inhibitors at 5 μM each were used to treat glioma cell lines: EGFR inhibitor erlotinib (Sigma), c-Met inhibitor SU11274 (Sigma), PDGFR inhibitor imatinib mesylate (Selleck), insulin-like growth factor-1, and receptor inhibitor AG1024 (Calbiochem-EMD). The alkylating agent TMZ (Sigma) was used at a final concentration of 100 μM.
Cell culture and human patient samples

Glioma cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco). Patient-derived tumor neurospheres (GIC) were a gift from Dr. Kenneth Aldap (University of Texas M.D. Anderson Cancer Center) and were cultivated under neurosphere conditions according to established protocols [Bao et al. 2006]. Cells were transduced with lentivirus stably expressing firefly luciferase to assess xenograft tumor growth in vivo by bioluminescence imaging [IVIS Spectrum, PerkinElmer] [Dinca et al. 2007]. Tumor tissue from GBM patients (n = 45) was obtained after surgery at Northwestern Memorial Hospital in compliance with the Northwestern Memorial Hospital Institutional Review Board.

Cell differentiation

GICs were plated at 50,000 cells per milliliter on poly-D-lysine/laminin-coated coverslips in GIC medium with 1 ng/mL EGF and bFGF [Invitrogen]. Immunocytochemistry was performed as previously described [Srikant et al. 2013] using the following antibodies: rabbit anti-GFAP [1:1000, DakoCytomation] and mouse anti-Map2 [1:500, BD Pharmingen]. Cells were imaged using a Leica SP-5 confocal microscope, and quantification was performed using NIH ImageJ software.

Cell cycle analysis

U87MG cells were plated in six-well plates and, at ~50% confluency, were transfected with different siRNA or miRNA constructs for 24 h at low serum. The next day, media were changed to 10% FBS for 48 h. Next, the cells were starved for 3 h and lysed by Nicolletti buffer containing propidium iodide [Stegh et al. 2007]. Cells were analyzed by flow cytometry (30,000 counts, LSRFortessa).

Comparative in silico analysis using TCGA

Pearson’s correlation coefficient [PCC] was used to measure the correlation between miR-182 and Bcl2L12 mRNA levels in TCGA GBM samples. PCCs were calculated for correlations in mesenchymal, classical, proneural, and neural samples individually and all tumor samples collectively.

SNA synthesis

Gold nanoparticles [13 nm ± 1 nm] were prepared using the Frens method [Frens 1973]. The particles were treated with DEPC (0.1% v/v) and autoclaved before being functionalized with miRNA duplexes. The miRNA-conjugated SNAs were purified by three successive centrifugations steps, supernatant removal, and addition of PBS at 4°C or by tangential flow filtration. Each 182-SNA contained ~30 antisense strands per 13-nm gold nanoparticle. For the generation of fluorochrome-labeled SNAs, Cy5.5 phosphoramidite (Glen Research) was manually coupled to the 5’ end of either Co-SNA or 182-SNA sense strands prior to hybridization. For further information on the functionalization and characterization of 182-SNA and Co-SNA, see the Supplemental Material.

Tumor xenograft model and in vivo SNA administration

All animals used were under an approved protocol of the Institutional Animal Care and Use Committee of Northwestern University. Female SCID mice (6–8 wk) were intracranially injected with U87MG or GIC-20 cells to form tumors according to published protocols [Jensen et al. 2013]. To demonstrate the therapeutic efficacy of the 182-SNAs, 500 nM 182-SNAs or Co-SNAs was administered via the tail vein at a dose of 1.4 mg of RNA per kilogram of mouse weight per injection, with injections performed every other day. The mice received seven injections of SNAs.

Statistical analysis

All experimental data are presented as mean ± SD (n ≥ 3) unless otherwise stated. Tumor weights were analyzed by Mantel-Cox and Gehan-Breslow-Wilcoxon test. Control and treatment groups in in vitro experiments were compared by two-tailed Student’s t-test. A P value of ≤0.05 was considered statistically significant.

Competing interest statement

D.A.G., C.A.M., W.L.D., and A.H.S. have interest in AuraSense Therapeutics, which develops spherical nucleic acid-based technologies.

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**Supplemental Material**

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