Current Progress on Understanding MicroRNAs in Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme (GBM) is an aggressive grade IV astrocytoma with a 1-year median survival rate despite current treatment modalities. A thorough understanding of the vast genetic aberrations and signaling pathways involved in gliomagenesis as well as heterogeneous clinicopathological presentation remains elusive. The recent discovery of microRNAs (miRs) and their capability of simultaneously regulating multiple downstream genes may play a key role in explaining the complex mechanisms underlying GBM formation. miRs are 19 to 25 nucleotide non-protein-coding small RNA molecules involved in the suppression of mRNA translation. This review will summarize and discuss the most recent findings regarding miRs in GBM including downstream targets, functional effects, and therapeutic potentials. Specifically discussed miRs include miR-7, miR-9/miR-9*, miR-10a/miR-10a*/miR-10b, miR-15b, miR-17-92, miR-21, miR-26a, miR-34a, miR-93, miR-101, miR-124, miR-125a, miR-125b, miR-128, miR-137, miR-146b-5p, miR-153, miR-181a/miR-181b, miR-196a/miR-196b, miR-218, miR-221/miR-222, miR-296, miR-302-367, miR-326, miR-381, miR-451, and let-7a. In addition to gene regulatory roles, miRs have demonstrated significant diagnostic, prognostic, and therapeutic potential. These small molecules may both help in the understanding of GBM and in developing new therapeutic options.

Keywords

miR, GBM, glioma, microarray, review

Introduction

Despite significant advancement in the understanding of glioblastoma multiforme (GBM) and its treatment modalities, the median survival rate remains 1 year for this devastating disease.^{1,2} GBM is categorized as primary when arising *de novo* or secondary when developing from lower grade gliomas. Each type is characterized by specific clinical and mutational attributes. Recent studies have demonstrated 4 subtypes of GBM based on genomic and proteomic characterization.³ Complete understanding of the regulation of a vast number of genes and signaling pathways as well as the clinicopathological heterogeneity in GBM remains elusive. The recent discovery of microRNAs (miRs) and their simultaneous regulation of multiple genes alludes to potential key mechanisms of gliomagenesis. Indeed, onco-miRs, miRs abnormally expressed in various cancers, may have critical roles in regulating tumor development.

miR Processing and GBM

miRs are 19 to 25 nucleotide non-protein-coding small RNA molecules that silence mRNA translation.⁴ First described in *Caenorhabditis elegans*, over 1,500 human miRs are currently annotated, which may regulate approximately 30% of total genomic mRNA.⁵ Through the ability to regulate a large number of genes, miRs have generated interest in explaining the control of diverse oncogenic signaling pathways including the ability to regulate multiple critical functions, such as cell proliferation as well as migration. Onco-miRs, aberrantly expressed miRs promoting

tumorigenesis, have been demonstrated in many different cancers including GBM.⁴ Furthermore, miRs may play an important role in regulating suspected cancer stem cells (CSCs) in GBM, which have been suggested as a mechanism for resistance to therapeutic interventions.^{6,7}

A multistep process is involved in the generation of mature miRs (Fig. 1).⁴ Encoded within unique transcriptional units or as clusters, miRs are located at gene introns or intergenic regions. Primary miRs (pri-miRs) are transcribed by RNA polymerase II and form a hairpin stem-loop structure. Subsequent processing is performed in the nucleus by the RNase III endonuclease Drosha, which is associated with the double-stranded RNA binding domain protein (DGCR8) known as Pasha, to form preliminary miR (pre-miR). Pre-miR is exported out of the nucleus by Exportin-5, where terminal processing by the RNase III domain-containing nuclease Dicer generates a mature

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Figure 1. MicroRNA (miR) processing. A multistep process is involved in miR generation. Primary miRs (pri-miRs) are transcribed in the nucleus by RNA polymerase II and form hairpin stem-loop structures. Further processing in the nucleus by the endonuclease Drosha forms preliminary miR (pre-miR). Pre-miR is exported from the nucleus by Exportin-5, where cytosolic processing by Dicer generates mature 21- to 23-nucleotide miR duplexes. Duplexed miR is unwound with the guide or passenger strand loaded into RISC composed of Dicer, TRBP, and Ago2. Full complementarity of the miR to mRNA results in mRNA degradation, while partial complementarity results in transcriptional repression by mRNA sequestration in the cytoplasm. DGCR8/Pasha = double-stranded RNA binding domain protein; RISC = RNA-induced silencing complex; TRBP = HIV-1 TAR RNA binding protein.

21- to 23-nucleotide miR. Duplexed miR is unwound and loaded into the RNA-induced silencing complex (RISC) composed of Dicer, HIV-1 TAR RNA binding protein (TRBP), and Ago2, a member of the Argonaute protein family. While the guide miR strand is predominantly loaded into the RISC complex, the 3' passenger strand can also be loaded to target distinct mRNAs and is designated with an asterisk (e.g., miR-9*). miRs with complete complementarity to mRNA are involved with degradation of mRNA via the RISC complex, whereas miRs with partial complementarity can be involved in cytoplasm sequestration of mRNA.

Significant work has been performed in understanding the alterations of miR expression, their impact on gliomagenesis and patient prognosis, as well as methods for potential therapy. In addition, microarray technology has accelerated research in miRs and rapidly generated targets for further investigation (Suppl. Table S1). This review will outline the current field of knowledge regarding miRs studied in GBM and evaluated for functional effects *in vitro* and *in vivo* (Table 1). Discussed miRs are roughly divided into categories in which investigation has yielded significant insight (i.e., CSCs, cell cycle, proliferation/apoptosis, neoangiogenesis); however, each miR can regularly be seen to control multiple genes and show complex functional effects. Despite these complexities, relevant genetic signaling pathways and potential implications on GBM treatment are discussed.

miR Regulation of CSCs in GBM

miR-7. Epidermal growth factor receptor (EGFR) is a mediator of the phosphatase and tensin homolog (PTEN) signaling pathway, where downstream AKT and mammalian target of rapamycin (mTOR) have been implicated in a variety of oncogenic effects in GBM.⁸ Kefas *et al.* showed that miR-7 is a tumor suppressor that downregulated EGFR and subsequently the downstream mitogenic AKT signaling pathway.⁹ In addition, this study showed that miR-7 independently downregulated IRS-1 and IRS-2. IRS-1/IRS-2 are also key modulators of the AKT pathway previously shown to mediate therapeutic resistance to rapamycin and other inhibitors of mTOR.⁸ These results suggest a role of miR-7 in regulating mitogenic signaling via the simultaneous regulation of multiple proteins

Table 1. Summary of Function, Genetic Regulation, and Expression of MicroRNAs (miRs) in Glioblastoma Multiforme (GBM)

miR	Functions	Targets shown in GBM ^a	Expression pattern in GBM	Authors
miR-7	Differentiation, invasion, proliferation	EGFR, IRS1, IRS2	Down	Kefas et al. ⁹
miR-9/miR-9*	Proliferation, self-renewal	SOX2		Jeon et al., ¹⁰ Ben-Hamo and Efroni ¹²
miR-10a/miR-10a*/miR-10b	Apoptosis, autophagy, chemoresistance, invasion, prognosis, proliferation, senescence, tumor growth	BCL2L1/Bim, TFAP2C/AP-2g, CDKN1A/p21, CDKN2A/p16, HOXD10	Up	Sasayama et al., ¹³ Sun et al., ¹⁴ Gabriely et al. ¹⁵
miR-15b	Cell cycle, proliferation	CCNE1	Heterogeneous	Xia et al. ²⁹
miR-17-92	Cell cycle, proliferation	CDKN1A/p21, E2F1, PTEN, CTGF	Up, down in CD4 ⁺ T-cells from GBM patients	Ernst et al. ¹⁷
miR-21	Apoptosis, chemoresistance, invasion, proliferation, tumor growth	PTEN, RECK, PDCD4, TIMP3, BCL2, SPRY2, TIMP3, PDCD4, MTAP, SOX5, JMY, TGFRBR2, TGFRBR3, TP73L, APAF1, BMPR2, TOPORS, DAXX, TP53BP2, PPIF	Up	Chan et al., ⁴⁸ Corsten et al., ⁴⁹ Papagiannakopoulos et al., ⁵⁰ Zhou et al., ⁵¹ Kwak et al., ⁵² Chen et al., ⁵³ Li et al., ⁵⁴ Gabriely et al., ⁵⁷ Zhou et al., ⁵⁸ Ren et al. ⁵⁹
miR-26a/miR-26b	Apoptosis, proliferation, tumor growth, vasculogenic mimicry	PTEN, MAP3K2, IFNB, EphA2, Rb1	Up (genetic amplification)	Huse et al., ⁶⁰ Kim et al., ⁶¹ Wu et al. ⁶²
miR-29b	Apoptosis, invasion, proliferation	PDPN	Low	Cortez et al. ²¹
miR-34a	Apoptosis, cell cycle, differentiation, invasion, proliferation, tumor growth	MET, NOTCH1, NOTCH2, CDK6, MYC, SIRT1	Down (reduced by loss of p53)	Li et al., ³¹ Guessous et al., ³³ Luan et al., ³⁴ Wei et al. ³⁵
miR-93	Neoangiogenesis, tumor growth	Integrin- β 8	Unknown	Fang et al. ⁸⁶
miR-101	Invasion, neoangiogenesis, proliferation, tumor growth	EZH2	Down	Smits et al. ⁶⁴
miR-124a	Cell cycle, differentiation, invasion, proliferation	CDK6, SCP1, PTBPI, ITGB1, LAMC1, IQGAP1, pRB	Low	Silber et al., ¹⁹ Fowler et al. ²⁰
miR-125a/miR-125b	Apoptosis, invasion, proliferation	PDPN, BMF	Low (miR-125a)	Cortez et al., ²¹ Xia et al., ²³ Shi et al. ²⁴
miR-128	Proliferation, self-renewal, tumor growth	EGFR, BMI1, E2F3A, ARP5	Low	Cui et al., ⁶⁵ Zhang et al., ⁶⁶ Godlewski et al., ⁶⁷ Wu et al. ⁶⁸
miR-137	Cell cycle, differentiation, proliferation	CDK6, MTF, EZH2	Low (methylated)	Silber et al. ¹⁹
miR-146b-5p	Invasion, migration	EGFR	Low	Katakowski et al. ⁶⁹
miR-153	Apoptosis, proliferation	Bcl-2, Mcl-1	Low	Xu et al. ⁷¹
miR-181a/miR-181b/miR-181c	Apoptosis, colony formation, invasion, proliferation, radiosensitivity	Bcl-2	Low	Shi et al., ⁷² Chen et al., ⁷³ Slaby et al. ⁷⁴
miR-196a/miR-196b	Survival		High	Guan et al., ⁷⁵ Dou et al. ⁷⁶
miR-218	Migration	IKK- β	Low	Song et al. ⁷⁷
miR-221/miR-222	Apoptosis, cell cycle, CTL-mediated tumor lysis, invasion, proliferation	NIAP, p27/KIP1, p57/KIP2, STAT1, STAT2, PUMA, ICAM1	High	Gillies and Lorimer, ³⁷ Lu et al., ³⁸ Zhang et al., ⁴⁰ Medina et al., ⁴¹ Zhang et al., ⁴³ Zhang et al., ⁴⁴ Ueda et al., ⁴⁵ Lukiw ⁴⁶

(continued)

Table 1. (continued)

miR	Functions	Targets shown in GBM ^a	Expression pattern in GBM	Authors
miR-296 miR-302-367 cluster	Neoangiogenesis Differentiation, invasion, proliferation, self-renewal, stemness	HGS CXCR4/SDF1	High (endothelial cells) High (serum-mediated differentiation)	Würdinger <i>et al.</i> ⁶³ Fareh <i>et al.</i> ²⁵
miR-326	Apoptosis, invasion, metabolism, proliferation, tumor growth	NOTCH1, NOTCH2, PKM2	Low (reduced by notch signaling)	Kefas <i>et al.</i> , ²⁶ Kefas <i>et al.</i> ²⁸
miR-339	CTL-mediated tumor lysis	ICAM1	High	Ueda <i>et al.</i> ⁴⁵
miR-381 miR-451	Proliferation Apoptosis, cell cycle, chemoresistance, invasion, proliferation, proliferation/migration regulation	LRRC4 CAB39, MIF, UBE2H, ARPP19, GATA2, ABCC1, AKT1, CCND1, MMP-2, MMP-9, Bcl-2, SMAD3, SMAD4	High High (glucose regulated)	Tang <i>et al.</i> ⁷⁸ Nan <i>et al.</i> , ⁷⁹ Gal <i>et al.</i> , ⁸⁰ Godlewski <i>et al.</i> ⁸¹
let-7a	Migration, proliferation	Pan-RAS, N-RAS, K-RAS	Low	Lee <i>et al.</i> ⁸³

^aThis table shows miRs where functional and target-specific data are available. Not shown are additional targets of specific miRs that have been demonstrated by many microarray studies.

within the EGFR pathway. Further investigation demonstrated selectively reduced processing of pri-miR-7 to pre-miR-7 in GBM tumors.⁹ In addition, transfection of miR-7 in GBM promoted generation of a primary CSC line as well as inhibition of *in vitro* proliferation and invasion in established GBM cell lines. These results implicated the potential for miR-7 to be a key molecule and viable target in GBM.

miR-9/miR-9*. miR-9, the 5' read of pri-miR-9, and its 3' counterpart, miR-9*, may be important GBM mediators via regulation of multiple distinct signaling pathways and chemotherapeutic resistance. miR-9* plays an integral role in the inhibitor of differentiation 4 (ID4)/miR-9*/SRY (sex determining region Y)-box 2 (SOX2) signaling pathway.¹⁰ In previous studies by Jeon *et al.*, induced ID4 expression dedifferentiated human glioma cells and p16/INK4A-ADP ribosylation factor (ARF^{-/-}) mouse astrocytes to glioma CSCs, which demonstrated expression of stem cell markers nestin and CD133, induced pluripotent stem cell factor SOX2 and cell cycle regulator cyclin E (CCNE1), and upregulated Jagged-1-Notch1 signaling, involved in neural stem cell differentiation.¹¹ Upregulation of ID4 induced chemoresistance in GBM by suppressing miR-9* levels and leading to expression of the drug efflux proteins ATP binding cassette transporters 3 (ABCC3) and 6 (ABCC6).¹⁰ Furthermore, this mechanism was directed in a SOX2-dependent manner. Expression of ID4 or SOX2 significantly accelerated tumor growth and chemotherapeutic resistance *in vivo*. In contrast to miR-9*, a recent network-based analysis demonstrated that miR-9 may regulate the

p38 mitogen-activated protein kinase signaling network, important in regulating cell stress and differentiation as well as correlating with patient prognosis.¹² These results suggest miR-9/miR-9* may regulate a variety of tumorigenic processes and serve as a potential target in GBM therapy.

miR-10a/miR-10a*/miR-10b. Among miR-10, miR-10b has been extensively studied and shown to regulate glioma CSCs and proliferation. Analysis of miR-10b expression in human glioma tumors and glioma cell lines showed correlation with increasing glioma grade and tumor multifocality identified on magnetic resonance imaging.^{13,14} Furthermore, Sasayama *et al.* showed in human tumors that miR-10b expression correlated with changes in immunohistochemical expression of the G-protein RhoC and the urokinase receptor uPAR, which have been implicated in cell migration and invasion.¹³ Although this study did not utilize transfected *in vitro* vectors for confirmation of miR-10b targets, miR-10b was suspected to regulate RhoC and uPAR via the transcription factor HOXD10. In a study by Gabriely *et al.*, heterogeneous miR-10b overexpression was seen in 261 human GBM samples compared to baseline expression in 10 normal brain samples obtained from The Cancer Genome Atlas (TCGA).¹⁵ These researchers also showed that miR-10b was expressed in low- and high-grade glioma as well as glioma cell lines and tumorigenic CSC lines. Inhibition of miR-10b reduced cell proliferation and cell cycle as well as induced senescence, apoptosis, and autophagy. Also, suppression of miR-10b did not affect

proliferation of normal primary neuroglia while reducing *in vivo* tumor burden, suggesting that it could be a specific target for GBM and avoid normal brain tissue. miR-10b regulated Bim, a proapoptotic protein, and transcription factor AP-2 γ (TFAP2C), involved in cancer cell death, growth, and invasion. In addition, miR-10b targeted cell cycle regulators p16/CDKN2A and p21/CDKN1A. Surprisingly, the transcription factor homeobox D10 (HOXD10), previously shown to be regulated by miR-10b in studies of invasion with breast cancer cell lines, was unaffected by miR-10b alterations in this study of GBM. However, other studies have shown that HOXD10 is an important regulator downstream of miR-10b, where it can induce GBM invasion by upregulating matrix metalloproteinase 14 (MMP-14) and uPAR.¹⁴ Using the TCGA data, high expression of miR-10, but not miR-10a or miR-10b alone, correlated to poorer patient survival in a statistically significant manner.¹⁵ However, miR-10a*, the 3' read of the pri-miR-10 gene, along with miR-195 and miR-455-4p, has been implicated in mediating resistance to temozolomide in GBM.¹⁶ While open areas of inquiry remain in the study of miR-10b, evidence seems to continue supporting an important role for this miR in GBM.

miR-17-92. The miR-17-92 cluster, widely shown to be important in tumor regulation, has been demonstrated by a variety of studies to be significant in GBM. miR-17-92 showed elevated expression in a sample of primary and secondary GBMs.¹⁷ This study showed amplification of the miR-17-92 cluster by comparative genomic hybridization and fluorescence *in situ* hybridization (FISH) analyses. Furthermore, miR-17-92 expression directly correlated with increasing glioma tumor grade, showing an increase in expression in grade IV versus grade II glioma. Among miRs encoded in the miR-17-92 cluster, miR-17-3p was significantly upregulated in both primary and secondary GBM. Interestingly, miR-17-5p, miR-92a-1, and miR-106b were uniquely upregulated in secondary GBM but not primary GBM when compared to expression in a normal brain. The reason for these changes is currently unknown. In a study by Sasaki *et al.*, peripheral CD4⁺ T-cells derived from patients with GBM demonstrated decreased miR-17-5p expression compared to healthy controls.¹⁸ These results suggest a role for the miR-17-92 cluster in gliomagenesis and GBM immunoregulation.

A neurosphere model of stem cell proliferation, clonogenicity, and other stem cell attributes was also used to evaluate the effect of GBM differentiation on miR expression patterns.¹⁷ Neurosphere treatments with the differentiation compound all-trans retinoic acid (ATRA) downregulated expression of 28 miRs and upregulated 8 miRs. ATRA treatment induced downregulation of 5 miRs from the miR-17-92 cluster and 2 from paralog clusters, namely

miR-106b-25 and miR-106a-363. Furthermore, targeted inhibition of miR-17-92 *in vitro* induced apoptosis and decreased cell proliferation in GBM neurospheres. Downregulation of the miR-17-92 cluster resulted in increased expression of cell cycle regulators CDKN1A/p21 and E2F1 as well as PTEN and connective tissue growth factor (CTGF). These results support the potential role of miR-17-92 in GBM CSC regulation.

miR-124a/miR-137. A miR microarray study by Silber *et al.* showed that miR-124a and miR-137 were significantly downregulated in World Health Organization (WHO) grade III and grade IV astrocytomas relative to normal brain.¹⁹ Moreover, this study showed that miR-124 and miR-137 were upregulated during differentiation of murine astrocytic neural stem cells. Transfection of miR-124a or miR-137 in murine astrocytic neural stem cells, murine oligodendrogloma-derived S100 β -v-*erbB*⁺ stem cells, human GBM CD133⁺ CSCs, and GBM cell lines was performed, which resulted in increased neuronal differentiation of all examined stem cells. Furthermore, miR-124a and miR-137 co-transfection in GBM resulted in G0/G1 cell cycle arrest as well as decreased expression of cell cycle proteins CDK6 and phosphorylated Rb.

A variety of targets are regulated by miR-124a. A study by Fowler *et al.* showed expression of miR-124a was downregulated in 80% of 119 retrospectively reviewed immunohistochemical GBM samples as compared to normal brain.²⁰ Furthermore, downregulation of miR-124a correlated with poorer survival. Targets of miR-124a, namely Ras GTPase activating protein 1 (IQGAP1), and the cytoskeletal proteins laminin c1 (LAMC1) and integrin b1 (ITGB1) were upregulated in GBM samples and thus inversely correlated with miR-124a expression. Transfection of miR-124a in GBM cell lines resulted in diminished cell migration and invasion as well as downregulated IQGAP1, LAMC1, and ITGB1. These results suggested that altered expression of miR-124a in GBM may correlate with this tumor's propensity to disseminate.

miR-125a/miR-125b. miR-125a has been implicated in regulating GBM migration, proliferation, and CSCs. A recent study showed that the podoplanin membrane sialo-glycoprotein (PDPN) was regulated by miR-29b and miR-125a.²¹ PDPN, a transmembrane protein, has been previously shown to regulate glioma invasion and is expressed in a variety of central nervous system tumors.²² A study by Cortez *et al.* showed that miR-29b and miR-125a were downregulated in CD133⁺ GBM CSCs.²¹ This study showed that transfection of miR-29b or miR-125a downregulated PDPN expression, impaired GBM invasion, reduced proliferation, and increased GBM apoptosis. In contrast to miR-125a, miR-29b interestingly reduced proliferation and

induced apoptosis in wild-type p53 but not mutant p53-containing cells, therefore indicating an important p53-dependent aspect to miR functionality.

Similarly to miR-125a, miR-125b has been shown to play a crucial role in the regulation of GBM cell growth and CSC regulation. Treatment of GBM cell lines with ATRA resulted in decreased miR-125b expression.²³ During ATRA treatment, transfection of miR-125b stimulated GBM proliferation and inhibited cell apoptosis. This mechanism may have been explained by how upregulation of miR-125b decreased expression of proapoptotic proteins Bax and Bcl-2 modifying factor (Bmf) along with increasing expression of antiapoptotic protein Bcl-2. In a study by Shi *et al.*, miR-125b was downregulated in CD133⁺ glioma stem cells compared to CD133⁻ cells.²⁴ This study showed that upregulation of miR-125b reduced CD133⁺ cell proliferation as well as increased the percentage of cells in G0/G1 phase. Furthermore, these effects were dependent on cell cycle-regulating proteins CDK6 and CDC25A. CSC regulation and cell cycle control were thus partially explained by miR-125b.

miR-302-367. The miR-302-367 cluster, consisting of miR-302a, miR-302b, miR-302c, miR-302d, and miR-367, has been shown to play a role in GBM. miR-302-367 was upregulated in GBM during serum-mediated differentiation of CSCs.²⁵ Transfection of miR-302-367 promoted a loss of stem cell markers, upregulated expression of glial markers, inhibited self-renewal, reduced cell invasion, and inhibited *in vivo* tumor formation. Furthermore, induced expression of the miR-302-367 cluster altered the neurogenesis signaling molecule SHH, supporting early tumor lineage commitment. miR-302-367 also downregulated CXC chemokine receptor type 4 (CXCR4) and its ligand, stromal-derived factor 1 (SDF1), which were involved in clonal proliferation and regulation of the SHH/GLI1/NANOG network in GBM cells. These results implicated miR-302-367 in the regulation of a critical pathway involved in neurogenesis and CSCs.

miR-326. miR-326 is downregulated in GBM compared to normal brain in a study by Kefas *et al.*²⁶ Previously validated GBM tumor-derived CSCs²⁷ were transfected with miR-326 and demonstrated reduced viability, invasiveness, and clonogenicity as well as increased apoptosis. miR-326 regulated Notch-1 expression directly and was also itself regulated by a feedback loop. Furthermore, miR-326 transfection reduced glioma growth *in vivo*. In another study by Kefas *et al.*, regulation of pyruvate kinase type M2 (PKM2) was seen by miR-326.²⁸ PKM2 knockdown altered GBM cell line and CSC growth, invasion, and metabolic activity. In addition, PKM2 inhibition resulted in phosphorylation of AMP-activated protein kinase (AMPK), a key metabolic

regulator in cancer. High levels of PKM2 protein in human GBM tumors but not a normal human brain correlated with downregulation of miR-326. These results suggested that miR-326 plays an important role in stem cell regulation as well as cancer metabolism and may serve as a GBM-specific target for therapy.

miR Regulation of Cell Cycle in GBM

miR-15b. Limited data exist on the role of miR-15b in gliomagenesis, but a recent study showed this miR to be of significance.²⁹ This study showed heterogeneous expression of miR-15b in different GBM cell lines. Overexpression of miR-15b was found to arrest the cell cycle at G0/G1 and inhibit cell proliferation. Furthermore, miR-15b was found to directly regulate cyclin E1 but not cyclin D1 in mediating effects on cell cycle regulation. miR-15b may be important in understanding GBM cell turnover.

miR-34a. Expression of miR-34a is regulated by p53 in a variety of tumors including colon cancer, leukemia, hepatocellular carcinoma, non-small cell lung cancer, and glioma.^{30,31} Furthermore, miR-34a is a transcriptional target of p53. A recent study showed that miR-34a was downregulated in glioma compared to normal brain as well as glioma with mutant p53 compared to glioma with wild-type p53.³¹ Upregulation of miR-34a was shown to reduce cell proliferation, increase the percentage of cells in G0/G1 phase, induce apoptosis, limit invasion, and limit *in vivo* tumor growth without adversely affecting normal astrocytes. Furthermore, miR-34a was able to downregulate expression of the proto-oncogene c-Met, neurogenesis regulators Notch-1 and Notch-2, as well as cell cycle regulator CDK6. Forced upregulation of c-Met or Notch-1/Notch-2 partially reversed these effects during miR-34a expression in glioma cells and stem cells. Notch-1 targeting by miR-34a during regulation of GBM proliferation and colony formation also correlated with findings by other researchers.³² When transfected with miR-34a, tumor CSCs selected in EGF- and FGF-supplemented media showed reduced cell proliferation, induced G0/G1 cell cycle arrest, increased apoptosis, and decreased scratch migration.³³ Furthermore, this study showed that miR-34a transfection resulted in reduced expression of stem cell markers CD133 and nestin along with increased expression of terminal differentiation markers of astrocytes (GFAP), neurons (TUJ-1), and oligodendrocytes (Claudin-11). A study by Luan *et al.* showed that transfection of miR-34a in glioma also indicated the ability to downregulate sirtuin 1 (SIRT1), implicated in regulating aging.³⁴ Furthermore, this study showed that upregulation of miR-34a inhibited cell proliferation, arrested cells in G0/G1, reduced migration and invasion, and induced apoptosis. Various other targets have been verified for miR-34a in GBM,

including the oncogene MYC, but have thus far not been functionally evaluated.³⁵ miR-34a may play a critical role in GBM due to its critical regulation of p53, CSCs, and other aspects of gliomagenesis.

miR-221/miR-222. Multiple studies have shown a role for miR-221/miR-222 in GBM. miR-221/miR-222 expression directly correlated with glioma grade and were prevalent in grade IV astrocytoma, where they targeted an important cell cycle regulator, namely p27/KIP1.³⁶⁻⁴⁰ In a study by Zhang *et al.*, suppression of miR-221/miR-222 upregulated p27/KIP1, increased the number of cells in G1 stage, and reduced G1/S progression.⁴⁰ Furthermore, these researchers showed that suppression of miR-221/miR-222 reduced tumor volume *in vivo* and upregulated p27/KIP1 expression in tumors. In immunohistochemical analysis of tumor sections, suppressed miR-221/miR-222 correlated with a diminished percentage of cells staining with the proliferation marker Ki-67. Another study by Medina *et al.* showed that miR-221/miR-222 were upregulated during the G1/S transition and also regulated expression of p27/KIP1 as well as p57/KIP2.⁴¹ Furthermore, this study showed that miR-221/miR-222 were expressed during S phase re-entry from quiescence due to serum deprivation. Inhibition of miR-221/miR-222 resulted in premature entry into S phase, resulting in cell death upon serum stimulation. Others have confirmed that suppression of miR-221/miR-222 increased the number of cells in G0/G1 phase, upregulated p27/KIP1, and induced apoptosis.⁴⁰ These data support a critical role in the regulation of the cell cycle, proliferation, and invasion by miR-221/miR-222.

Multiple genes and signaling pathways are regulated by miR-221/miR-222. Microarray analysis of gene expression after inhibition of miR-221/miR-222 in GBM showed 158 differentially expressed genes involved in cell metabolism, cytoskeletal organization, and molecular signaling.⁴² This study showed that miR-221/miR-222 modulated the IFN- α signaling pathway including regulation of STAT1/STAT2 phosphorylation and nuclear localization. In a separate bioinformatics analysis, miR-221/miR-222 regulated about 70 common target genes.⁴³ Among these targets, regulation of 16 gene products was involved either directly or indirectly with AKT signaling. Another study showed that an inverse relationship between proapoptotic gene PUMA and miR-221/miR-222 expression has been demonstrated in glioma tissue, where upregulation of miR-221/miR-222 induced cell survival by targeting PUMA in human glioma cells.⁴⁴ In contrast, inhibition of miR-221/miR-222 induced PUMA expression and cell apoptosis in an *in vivo* xenograft tumor model. miR-222, as well as miR-339, has also been shown to target intracellular adhesion molecule 1 (ICAM-1) and regulate targeting of GBM tumors by cytotoxic T lymphocytes.⁴⁵ Furthermore, expression of Dicer, miR-222, and

miR-339 was inversely correlated to ICAM-1 expression in GBM tumors. Suppression of miR-221/miR-222 decreased transwell cell invasion and *in vivo* xenograft tumor growth.^{40,43} Upregulation of miR-221 has also been correlated with downregulation of one of its targets, surviving-1 homolog BIRC1 (NAIP), involved in neurodegeneration and apoptosis regulation.⁴⁶ These various targets explain the widespread regulation of GBM function by miR-221/miR-222.

miR Regulation of Proliferation and Apoptosis in GBM

miR-21. The role of miR-21 has been widely implicated in the regulation of GBM and as a potential target.⁴⁷ Initial studies by Chan *et al.* demonstrated that miR-21 was elevated in a variety of glioma cell lines and human tumors, where downregulation of miR-21 could inhibit cell survival by inducing apoptosis via caspase 3/7.⁴⁸ Furthermore, Corsten *et al.* showed that miR-21 suppression in neural progenitor cells constitutively expressing tumor necrosis factor-related apoptosis-inducing ligand (S-TRAIL) showed a synergistic decrease in cell viability, a lower *in vivo* tumor burden, and an increase in caspase 3/7 activity.⁴⁹

A variety of targets for miR-21 have been identified in regulating GBM proliferation. miR-21 has been shown to target multiple proteins of the p53, transforming growth factor- β (TGF- β), and mitochondrial apoptosis pathways.⁵⁰ This study also showed that miR-21 downregulation increased GBM apoptosis, reduced chemotherapeutic resistance to doxorubicin, decreased cell proliferation, and increased G0/G1 cell cycle arrest. Furthermore, knockdown of heterogeneous nuclear ribonucleoprotein K (HNRPK) and the tumor suppressor p63 blunted these effects, thereby playing critical roles in mediating the effects of miR-21. In a study by Zhou *et al.*, miR-21 was found overexpressed in GBM, where it downregulated the mTOR pathway regulator PTEN.⁵¹ This study also showed that inhibition of miR-21 resulted in decreased expression of EGFR, activation of AKT, as well as activation of cell cycle regulator cyclin D1, apoptosis inhibitor Bcl-2, and signal transducer and activator of transcription 3 (STAT3). miR-21 inhibition increased cellular apoptosis, halted cell cycle in G1/S phase, and diminished tumor growth. However, this study also showed that inhibited cell growth from miR-21 suppression was only partially dependent on PTEN status where the expression of 169 genes in 9 distinct cell signaling pathways seen with miR-21 suppression, suggesting a complex mechanism of GBM regulation. In a study by Kwak *et al.*, treatment of PTEN-deficient GBM cells with hyaluronic acid, an extracellular matrix glycosaminoglycan, induced GBM invasion, which depended on miR-21 and MMP-9 secretion.⁵² This effect was partially mediated by the Ras

regulator Spry2. In addition, Spry2 and miR-21 levels inversely correlated in human tumors, where high-grade glioma showed upregulated miR-21 expression. These results suggest the Ras/ERK1/2 pathway is a critical mediator of GBM invasion and dependent on miR-21. In another study, miR-21 was seen to downregulate the apoptosis-regulating protein programmed cell death 4 (PDCD4), S-methyl-5'-thioadenosine phosphorylase (MTAP), and transcription factor SOX5.⁵³ Furthermore, PDCD4 was critical in mediating GBM apoptosis. These results support miR-21 as a critical regulator of multiple downstream genes and signaling pathways involved in gliomagenesis.

miR-21 has also been shown to regulate GBM chemotherapeutic resistance, invasion, and apoptosis. Resistance to VM-26 (teniposide), a topoisomerase II inhibitor, as well as temozolomide (Temodar), an alkylating agent and first-line therapy, in GBM was mediated by miR-21.^{54,55} Chemotherapeutic resistance mediated by miR-21 was shown in part due to regulated expression of leucine-rich repeating flightless-interacting protein 1 (LRRFIP1), a transcriptional repressor and indirect inhibitor of NF- κ B signaling.⁵⁴ A study by Shi *et al.* showed upregulated miR-21 levels induced resistance through alteration of the ratio between apoptosis-regulating proteins Bax and Bcl-2.⁵⁵ miR-21 inhibition synergistically combined with taxol in inducing cell apoptosis and inhibiting cell invasion.⁵⁶ In controlling invasion, miR-21 influenced MMP levels via genetic regulation of reverse-inducing cysteine-rich protein with kazal motifs (RECK), a membrane-bound MMP inhibitor, and TIMP3, a tissue inhibitor of MMP3.^{57,58} Furthermore, inhibition of miR-21 in xenografted GBM tumors downregulated the activity of various MMPs (3, 9, 7, 12, and 13), reduced *in vitro* motility, and diminished invasion compared to sham miR inhibition.⁵⁷ A promising study by Ren *et al.* showed that combined treatment of GBM with 100 nm poly-amidoamine dendrimer (PAMAM) nanoparticles, comprised of antisense miR-21 and 5-fluorouracil (5-FU), was able to suppress cell proliferation and invasion as well as induce apoptosis.⁵⁹ These results suggest a method for miR delivery in human subjects and that miR-21 could serve as a potential target. Regulation of critical proteins involved in apoptosis and invasion was seen by miR-21, thereby suggesting a rationale for targeting this miR in therapy.

miR-26a. A number of recent studies have shown miR-26a to be upregulated in GBM.^{60,61} Monoallelic loss of PTEN correlated with amplified miR-26a, where miR-26a was found to target PTEN and, in effect, generate biallelic PTEN loss of heterozygosity.⁶⁰ This study also observed that PTEN could be targeted by at least 10 miRs, of which miR-26a was the most expressed in high-grade glioma. Furthermore, miR-26a was highly expressed due to copy number amplification in approximately 12% of analyzed GBM

samples. Co-injection of RCAS-PDGF and RCAS-miR-26a into Ntv-a/PTEN^{fl/fl} mice, where PDGF and miR-26a were overexpressed in PTEN-containing cells, demonstrated a mix of low- and high-grade glioma. Furthermore, RCAS-PDGF, RCAS-miR-26a, and RCAS-Cre, where PDGF and miR-26a expression was combined with PTEN loss, generated mice with predominantly high-grade glioma development, suggesting potent interaction between miR-26a expression and PTEN loss in gliomagenesis. Kim *et al.* showed by systems-based analysis that miR-26a was amplified as part of an onco-miR/oncogene DAN cluster including cell cycle protein CDK4 and neuronal antiapoptotic protein centaurin γ 1 (CENTG1).⁶¹ This study showed that miR-26a regulated expression of critical signaling molecules PTEN, RB1, and MAP3K2/MEKK2. Furthermore, this study demonstrated that overexpression of miR-26a promoted *in vivo* tumor growth in spite of PTEN status as well as inhibited JNK-dependent apoptosis. In a study by Wu *et al.*, expression of miR-26b was also upregulated in higher grade glioma and repressed erythropoietin-producing hepatocellular receptor A2 (EphA2), part of a family of receptor tyrosine kinases involved in neural development, proliferation, migration, and differentiation.⁶² This study also showed that EphA2 regulated GBM proliferation, migration, and invasion. Interestingly, vasculogenic mimicry, the formation of vascular-like networks by tumor cells, was seen by GBM cells and also regulated by miR-26b.

miR-101. miR-101 was shown to be downregulated in GBM, where its downstream target, histone-lysine N-methyltransferase EZH2, was upregulated.⁶⁴ EZH2 is a member of the Polycomb group family and involved in transcriptional regulation. In this study, EZH2 regulated GBM cell growth, migration/invasion, tumor-mediated neoangiogenesis, and *in vivo* tumor growth.

miR-128. miR-128 may play an important role in regulating cell cycle and CSC proliferation in GBM. miR-128 was downregulated in GBM and also inversely correlated with WHO glioma tumor grade.^{65,66} Targets of miR-128 upregulated in GBM included angiopoietin-related growth factor protein 5 (ARP5), the oncogene Bmi-1, and the transcription factor E2F3a.⁶⁵⁻⁶⁷ These targets were downregulated during miR-128 expression and modulated a decrease in cell proliferation during miR-128 overexpression studies. miR-128 expression has been shown to reduce GBM cell proliferation in xenograft models.⁶⁷ This study showed that expression of miR-128 decreased histone methylation (H3K27me³), AKT phosphorylation, and Bmi-1 levels and increased p21/WAF1 levels. miR-128 was also capable of blocking GBM self-renewal assessed by neurosphere culture, which correlated with changes in Bmi-1 expression. Interestingly, ginsenoside Rh2, a compound derived from

the medicinal plant ginseng, was shown to upregulate miR-128 in glioma.⁶⁸ Furthermore, miR-128 inhibition reduced apoptosis and E2F3a expression mediated by ginsenoside Rh2. These results indicated that miR-128 regulates a variety of targets in GBM and that induced expression may be found by targeted treatments. Regulation of other miRs by targeted therapies, such as ginsenoside, remains to be further explored.

miR-146b-5p. miR-146b-5p may be a potential regulator of GBM proliferation and migration. miR-146b-5p was downregulated in GBM and distinctly regulated the expression of EGFR.⁶⁹ miR-146b-5p was located on chromosome 10q24.3, a region commonly lost in glioma. This study also showed that upregulation of miR-146b-5p decreased cell invasion and migration. Xenograft-inoculated tumors showed that expression of miR-146b-5p was higher in the tumor core compared to areas >100 µm distal from the core as well as compared to disseminated tumor cells surrounded by brain parenchyma. miR-146b-5p expression also inversely correlated with AKT phosphorylation. In a different study, miR-146 upregulation reduced GBM migration and invasion as well as regulated expression of downstream MMP16.⁷⁰

miR-153. While few studies are available discussing the role of miR-153 in GBM, this miR has been shown to regulate gliomagenesis. miR-153, a downregulated miR in GBM, modulated cell proliferation in GBM.⁷¹ This study showed that *in vitro* transfection of miR-153 decreased cell proliferation and increased apoptosis. Furthermore, anti-apoptotic protein Bcl-2 and induced myeloid leukemia cell differentiation protein (Mcl-1) were downregulated by miR-153 upregulation.

miR-181a/miR-181b. miR-181a and miR-181b have demonstrated broad roles in the control of gliomagenesis. miR-181a and miR-181b expression were downregulated in GBM, where they indirectly correlated with glioma grade with the lowest expression seen in WHO grade IV astrocytoma and GBM cell lines.⁷² This study showed that transfection of miR-181a or miR-181b decreased GBM cell proliferation, colony formation, and GBM invasion as well as increased cell apoptosis. In a study by Chen *et al.* evaluating the effect of radiation on miR expression, miR-181a was downregulated in response to radiation.⁷³ Furthermore, transfection of miR-181a increased radiosensitivity and resulted in greater inhibition of cell proliferation and colony formation. Downregulation of Bcl-2 was seen with miR-181a transfection. Interestingly, miR-181b and miR-181c, but not other well-established miRs in GBM (e.g., miR-221/miR-222, miR-128a, miR-21), were downregulated in GBM patients with radiographic response to concomitant radiotherapy and temozolomide treatment.⁷⁴

These studies suggested the potential for miR-181a and miR-181b to serve as both therapeutic targets and unique prognostic tools.

miR-196a/miR-196b. miR-196a/miR-196b may be dysregulated in GBM and may predict prognosis. A miR microarray analysis of anaplastic astrocytoma versus GBM demonstrated 16 altered miR targets, including miR-196a and miR-196b that were significantly upregulated.⁷⁵ miR-196a and miR-196b showed increased expression in GBM compared to anaplastic astrocytoma and a normal brain. Furthermore, these researchers showed that high levels of miR-196, including miR-196a and miR-196b expression, significantly correlated with poorer survival and served as an independent prognostic factor. Dou *et al.* showed that the CC polymorphism of miR-196a has been associated with a decreased risk of glioma in the Chinese population compared to the T, C, TC, and TT haplotypes.⁷⁶ Moreover, the CC haplotype of miR-196a has been shown to confer a reduced risk within subgroups of adults and males as well as GBM compared to lower grade glioma.

miR-218. Expression of miR-218 reduced GBM invasion and migration. miR-218 was downregulated in GBM cells and tumors.⁷⁷ Upregulation of miR-218 suppressed GBM invasion and spheroid formation. Furthermore, miR-218 altered expression of the invasion-regulating protein MMP-9 and transcription factor NF-κB by targeting IκB kinase β (IKK-β). Exploration of other NF-κB-regulated genes in GBM by miR-218 may be of interest due to this potent transcription factor's known roles in modulating tumorigenesis.

miR-381. miR-381 has been implicated as an important regulator of GBM proliferation. In one study, miR-381 was upregulated in GBM compared to normal brain.⁷⁸ Overexpression of miR-381 increased *in vitro* proliferation and *in vivo* tumor growth. Furthermore, miR-381 regulated leucine-rich repeat containing 4 (LRRC4) directly, and LRRC4 regulated miR-381 levels via a feedback loop. Upregulated LRRC4 levels correlated with smaller *in vivo* tumor volume, better differentiated tumors, and reduced expression of miR-381. This feedback regulation was suggested to involve the mitogenic MEK/ERK/Ets-1 pathway; however, further study of potential signaling networks regulated by miR-381 is needed.

miR-451. Multiple studies have supported the role of miR-451 in the regulation of GBM via a variety of downstream targets. miR-451 showed diminished expression in GBM compared to normal brain.⁷⁹ Transfection of miR-451 resulted in reduced cell growth, G0/G1 arrest, increased apoptosis, and reduced invasion. Furthermore, miR-451 upregulation decreased expression of AKT1, cell cycle

protein cyclin D1, invasion regulators MMP-2 and MMP-9, and antiapoptotic protein Bcl-2, while increasing expression of cell cycle regulator p27/KIP1. Although CD133⁺ cells have been suggested as the key CSC population in GBM, a study of miR profiles in CD133⁺ and CD133⁻ GBM cells showed that miR-451 along with 6 other miRs were upregulated in CD133⁻ cells.⁸⁰ miR-451 transfection inhibited neurosphere formation and cell proliferation in addition to synergistically combining with imatinib mesylate (Gleevec), a tyrosine kinase inhibitor used to treat chronic myelogenous leukemia, in dispersing neurospheres. Furthermore, miR-451 regulated SMAD3/SMAD4, which have been previously implicated in oncogenesis.

miR-451 may also play a role in GBM migration, prognosis, and cancer metabolism. Migration assays demonstrated a variety of miRs were downregulated in GBM, including miR-123, miR-126, miR-223, miR-224, and miR-451, while miR-504 was upregulated.⁸¹ Evaluation of miR-451 using the TCGA database demonstrated a significant elevation of miR-451 in a subgroup of patients with poor survival compared to a subgroup with improves survival (median survival = 280 v. 480 days). Furthermore, transfection with miR-451 inhibited cell migration and promoted cell proliferation, which was mediated by one of its targets, CAB39, a binding protein for LKB1. These researchers showed that LKB1 could also regulate miR-451 levels via a potential feedback loop and that miR-451 was a critical regulator during glucose deprivation by allowing proper activation of LKB1 and downstream AMPK. Additional research has suggested a mathematical model between miR-451 and AMPK expression levels in regulating cell migration and proliferation during response to glucose levels.⁸² These results suggested miR-451 is a critical regulator of growth invasion cycling in GBM and may have an important role in regulating GBM metabolism.

let-7a. let-7a has been widely implicated in cancer regulation and has been recently shown to modulate gliomagenesis. Transfection of let-7a has been shown to inhibit GBM proliferation and migration.⁸³ Furthermore, transfection of let-7a reduced expression of pan-RAS, N-Ras, and K-RAS along with reducing sizes of *in vivo* xenograft-generated tumors. let-7a transfection did not alter normal human astrocyte cells, suggesting it may be a GBM-specific therapeutic target.

miR Regulation of Neoangiogenesis in GBM

miR-93. miR-93, a part of the miR-106b-25 cluster, may play a role in regulating GBM. The miR-106b-25 cluster is a paralog to the miR-17-92 cluster and consists of pre-miR-25, pre-miR-93, and pre-miR-106b.^{84,85} While the role of the miR-106b-25 cluster has been evaluated in neural

stem/progenitor cells, evaluation of the miR-106b-25 cluster in GBM has been limited. Transfection of miR-93 in GBM augmented neoangiogenesis of co-cultured Ypen endothelial cells, characterized by endothelial cell spread, growth, and tube formation.⁸⁶ Furthermore, miR-93 transfection induced *in vivo* tumor vascularization, which increased tumor growth and decreased overall survival in animals with xenografted tumors. miR-93 transfection promoted tumor cell spheroid growth and colony formation. Inhibition of a target of miR-93, integrin- β 8, partially mediated the effect of miR-93 on tumor growth and neoangiogenesis. In GBM samples, integrin- β 8 co-expressed with areas of cell death examined by TUNEL staining, areas of blood vessels stained by CD34, and normal brain adjacent to the tumor. These results support an important role for miR-93 in GBM neoangiogenesis.

miR-296. miR-296 has shown a potent role in the regulation of neoangiogenesis in GBM. Human microvascular endothelial cells from a normal human brain (HBMVECs) co-cultured with glioma cells showed alterations in miR expression induced by tumor cells.⁶³ Upregulated miR-296 induced neoendothelialization as assessed by endothelial tubule formation and HBMVEC migration. miR-296 targeted hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), which is involved in endosomal sorting and regulation of growth factor receptor expression levels. Angiogenesis modulated by HGS and induced by GBM promoted *in vitro* endothelial tube formation as well as *in vivo* tumor vascularization. Human glioma tumors showed elevated miR-296 expression in tumor blood vessels, which correlated with downregulation of HGS. Furthermore, increased levels of proteins downstream from HGS, including platelet-derived growth factor receptor β (PDGFR β) and vascular endothelial growth factor receptor 2 (VEGFR2), were also observed in GBM tumors.

Conclusion

Recent advances in targeting miRs within *in vivo* systems have promoted the evaluation of miRs in cancer treatment. However, significant challenges remain in the application of miR regulation to patient therapy. Proposed strategies to inhibit miRs have included small molecule inhibitors to regulate miR processing, antisense oligonucleotides to target mature miRs, synthetic miR mask constructs to compete with endogenous miRs for mRNA binding sites, and miR sponge constructs to “soak up” endogenous miRs.⁸⁷ Methods to induce miRs have included miR mimetic molecules to upregulate miR downstream activity and adenovirus-associated vectors upregulating miR expression. One novel method that has been evaluated in animal tumor models includes antagomirs, single-stranded RNA analogs with

2'-O-methylated backbones, a phosphorothioate linkage, and conjugation to cholesterol, which can be injected intravenously to downregulate miRs.⁸⁸ Another method includes locked nucleic acid (LNA) constructs, consisting of nucleic acids with methylene bridges between the 2'-O and 4'-C atoms in the ribose ring, and have been used to knock down miR expression.⁸⁹ Despite these advances, challenges in the delivery of miRs to target sites as well as regulation of undesired activity persist. The use of chemical modification, liposomes, polymers, hydrogels, and nanoparticles has been investigated in miR delivery.⁸⁷ However, few trials have reached beyond phase I with miR targeting in cancer, and none have been performed with brain malignancies, suggesting that significant progress yet remains to be made.

Investigation into the roles of miRs in GBM has yielded significant insight into these complex molecules. miRs have shown clear diagnostic, prognostic, and therapeutic potential. A recent study demonstrated that combined use of miRs predicted prognosis in GBM, where miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221, and miR-222 as well as MGMT methylation status were analyzed.⁹⁰ miR-195 and miR-196b positively correlated with overall survival, while miR-181c and miR-21 predicted 6-month progression with high sensitivity (92%) and specificity (81%). The studies in this review have demonstrated that miRs can modulate simultaneous and distinct functions such as tumor growth, invasion, and angiogenesis in GBM. Important advances in microarray technology such as standardized arrays and improved resolution of known miRs have also facilitated the study of miRs in GBM. Moreover, microarray technology will continue to resolve additional miRs for functional analysis, which can further support a rational basis for therapy. miRs may serve as an important part of understanding GBM as well as opening avenues for alternative targeted treatments.

Declaration of Conflicting Interests

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