

miRNA-mediated immune regulation and immunotherapeutic potential in glioblastoma

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Glioblastoma (GB), the most common primary neoplasm of the CNS, remains universally fatal with standard therapies and has a mean overall survival time of only 14.6 months. Even in the most favorable situations most patients do not survive longer than 2 years. Another hallmark of GBs, apart from the poor control of proliferation, is an immune suppressed tumor microenvironment. miRNAs usually bind the 3' untranslated region of target mRNAs and direct their post-transcriptional repression. Certain miRNAs are known to have altered expression levels in GB tumors, and in many immune cell subtypes. miRNAs have been found to serve important roles in gene regulation and are implicated in many processes in oncogenesis and immune deregulation. In this article we focus on the miRNAs involved in gliomagenesis and in the regulation of the immune response. We also present current challenges and miRNA immunotherapeutic strategies that should be investigated further.

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The discovery of miRNAs & the elucidation of the mechanisms on regulation of gene & transcriptional networks

During the past few years, molecular biologists have discovered hundreds of genes that encode miRNA molecules, and many of these miRNAs have been conserved throughout evolution [1]. Fire *et al.* first characterized the role of miRNAs in 1998 in a process they termed RNA interference, based on the experimental introduction of miRNA into cells to interfere with the function of a mRNA transcript [2]. However, before miRNAs are able to exert regulatory effects they undergo a maturation process. This first involves the transcription of miRNA genes in the nucleus to form large capped and polyadenylated precursors, which are then processed to form a stem-loop structure by the RNase III enzyme Drosha and its cofactor DGCR8 resulting in a pre-miRNA that is then exported to the cytoplasm by exportin 5. Subsequent processing by RNase III protein, Dicer, creates a transient 21–25-nucleotide miRNA–miRNA duplex that is incorporated into the RNA-induced silencing complex (RISC), which contains argonaute proteins. However, only one mature single-stranded miRNA is preferentially retained in the complex. Base pairing between the miRNA and the 3' untranslated region of its target mRNA directs the RISC to either degrade the mRNA or impede its translation into protein [3].

The miRNAs can be involved in many gene regulatory pathways, and part of the reason that they are able to regulate so many different networks is because absolute complementation between a miRNA and the target mRNA is not necessary. Once the miRNA binds its target mRNA, the choice between translational inhibition and mRNA degradation is governed by the degree of mismatch between the miRNA and its target mRNA, with degradation being the outcome for best-matched targets [3]. As miRNA targeting can inhibit the translation of imperfectly matched targets

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by the RISC, miRNA can target multiple pathways and several miRNAs may regulate any given target.

Some of the first miRNAs identified were those that played key regulatory roles in cell growth and division. Thus, there was immediate interest in how these miRNAs related to cancer, and three important observations were made that suggested a role for miRNAs in human cancer. The first indirect piece of evidence was that miRNAs could control cell proliferation and apoptosis [4]. Second, the genes for many miRNAs are located at fragile sites in the genome or regions commonly amplified or deleted in cancer cell lines. For example, two miRNA genes, *mir-15* and *mir-16*, are found in the deleted region of chromosome 13 that is frequently associated with chronic lymphocytic leukemia [3]. Third, malignant tumors and tumor cell lines have been found to have widespread deregulation of miRNA expression compared with normal tissues, including a global decrease of mature miRNA expression [4]. This global repression of miRNAs may reflect the undifferentiated state of the tumors or may be causally contributing to malignant transformation. It is plausible that overexpression of a specific subset of miRNAs may increase susceptibility to malignancy.

miRNAs as mediators in gliomagenesis

Compelling data are emerging that link miRNAs to gliomagenesis [5–7]. Although the degree to which certain miRNAs are expressed in gliomas can vary among patients, there are several miRNAs associated with tumor proliferation and are frequently upregulated in gliomas. Decreased expression of the tumor suppressor phosphatase and tensin homolog (PTEN) in glioblastomas (GBs) has been found to be due to an increase in expression of miR-21 and miR-26a [8,9]. Specifically, miR-21 has been shown to be an oncogene [10] that targets TGF- β [11], programmed cell death 4 [12], Fas ligand [13], tumor-associated protein [11] and reversion-including-cysteine-rich protein with kazal motifs [14]. All of these targets exert tumor suppressor activities to inhibit glioma proliferation and invasion, and induce apoptosis. Antisense technology has effectively inhibited miR-21 both *in vitro* and in animal models, demonstrating the potential of anti-miR-21 oligonucleotides to inhibit tumor growth. Whereas both miR-21 and miR-26a are associated with the PTEN pathway, their potential link to one another has yet to be explored.

Other upregulated miRNAs in GBs include miR-221 and miR-222, which play roles in regulating the proliferation of GBs by inhibiting cell apoptosis via targeting the pro-apoptotic gene *PUMA* [15]. Knockdown of miR-221 and miR-222 was found to induce PUMA expression and cell apoptosis, whilst also decreasing tumor growth in a xenograft model. Later studies have suggested that miR-221 and miR-222 play roles in

enhancing the glioma malignant phenotype through activation of the AKT pathway [16]. miR-222 and miR-339 were found to have additional roles in suppressing intercellular adhesion molecule-1 (ICAM-1) expression on glioma cells, thereby decreasing their susceptibility to cytotoxic T-cell-mediated cytotoxicity [17], suggesting an immune regulatory role of miRNAs within GBs. Downregulation of miR-451 in GB produces results similar to increased miR-221 and miR-222 levels. Treatment of GB cell lines with miR-451 has been found to inhibit cell growth, induce G₀/G₁ phase arrest and increase cell apoptosis [18]. The invasiveness of the GBs was also reduced, potentially owing to a dose-dependent decrease in the expression of AKT1, CyclinD1, MMP-2, MMP-9, BCL-2 and increased expression of p27. Finally, the miR-302–367 cluster has been demonstrated to trigger a cascade of inhibitory events leading to the disruption of the stem cell-like and tumorigenic properties of GBs [19]. Cumulatively, these data demonstrate that the targeted delivery of several key miRNAs may be therapeutically efficacious for the treatment of gliomas.

Conversely, multiple key miRNAs are frequently downregulated in gliomas. These miRNAs have been shown to play key roles in the cell cycle, survival, migration, invasion and malignant properties of malignant gliomas (Table 1). For example, miR-7 has been shown to be downregulated in gliomas, which normally suppresses the AKT and EGFR glioma proliferative pathways [20]. miR-34 acts as a tumor suppressor [21] and directly targets c-Met, Notch-1, Notch-2 and CDK6 [21–23]. Transfection of miR-34 in glioma and medulloblastoma cells inhibits cell proliferation, G1/S cell cycle progression, cell survival, cell migration/invasion and induced glioma stem cell differentiation, whilst also inhibiting many of the malignant properties of GBs [21]. miR-29b and miR-125a have been found to be downregulated in GBs and the resultant overexpression of podoplanin has been linked with increased invasiveness, proliferation and decreased apoptosis [24].

One of the most studied miRNAs in GBs has been miR-128. Downregulation of miR-128 in GBs has been shown to be associated with increased GB proliferation and self-renewal [25]. miR-128 targets the *BM1* polycomb ring finger oncogene, which is a positive regulator of stem cell renewal, and its overexpression drives glioma growth. When miR-128 was overexpressed in glioma cancer cell lines, inhibition of tumor proliferation was observed, probably as a result of its association with E2F3, a known controller of cell cycle progression [26]. miR-128 can also associate with WEE1, a protein kinase that is involved in cell proliferation and survival [27]. By association with these known mechanisms of cell division, miR-128 seems to play an important role in several pathways of tumor cell growth. Furthermore,

Table 1. Key miRNAs involved in glioma pathogenesis.

miR	Status in GB	Role	Target	Ref.
7	Downregulated	Tumor proliferation	AKT, EGFR	[20]
9	Downregulated	Chemotherapeutic resistance of GB	SOX2, a transcription factor involved in regulating ABC-transporter expression	[31]
29b	Downregulated	Regulation of invasive properties, proliferation and apoptosis	Podoplanin	[24]
34	Downregulated	Regulation of cell proliferation, G1/S cell cycle progression, cell survival, migration and invasion	c-Met, Notch-1, Notch-2 and CDK6	[21–23]
124	Downregulated	Regulates differentiation and cell cycle	CDK6, phosphorylation of Rb	[29]
125a	Downregulated	Regulates cell growth, G ₀ /G ₁ transition, cell apoptosis and invasiveness	Podoplanin	[24]
128	Downregulated	Increased GB proliferation and self renewal and induction of cellular differentiation	BMI1 polycomb ring finger oncogene (BMI-1), E2F3 and WEE1	[25–28]
137	Downregulated	Regulates differentiation and cell cycle	CDK6, phosphorylation of Rb	[29]
451	Downregulated	Regulates cell growth, G ₀ /G ₁ transition, cell apoptosis and invasiveness	AKT1, CyclinD1, MMP-2, MMP-9, BCL-2 and p27	[18]
10a	Upregulated	Acquired temozolomide resistance		[32]
21	Upregulated	Gliomagenesis	PTEN and TGF- β	[9–11]
26a	Upregulated	Gliomagenesis	PTEN	[8]
195	Upregulated	Acquired temozolomide resistance		[32]
221	Upregulated	Glioma cell proliferation and apoptosis	PUMA and possibly AKT-dependent pathways	[15,16]
222	Upregulated	Glioma cell proliferation, apoptosis, and susceptibility to cytotoxic T-cell-mediated cytotoxicity	PUMA, AKT-dependent pathways and ICAM-1	[15–17]
302–367 cluster	Upregulated	GB stem-like and tumorigenic properties	Unknown	[19]
339	Upregulated	Susceptibility to cytotoxic T-cell-mediated cytotoxicity	ICAM-1	[17]
455–3p	Upregulated	Acquired temozolomide resistance		[32]

ABC: ATP-binding cassette; GB: Glioblastoma; ICAM: Intercellular adhesion molecule.

miR-128, which can induce cellular differentiation, has been found to be downregulated in all grades of glioma, including GBs [28].

Furthermore, miR-124 and miR-137 have also been found to be downregulated in various gliomas including GBs [29]. Transfection of human GB-derived stem cells with miR-124 or miR-137 induced differentiation of the stem cells and G1 cell cycle arrest, with an associated decrease in cyclin-dependent kinase 6 expression and phosphorylated retinoblastoma (pSer 807/811) proteins. The induction of a stem cell differentiation state may be a potential immune therapeutic strategy, because we have previously demonstrated that stem cell differentiation reduces glioma stem cell-mediated immunosuppression [30].

The therapeutic use of miRNAs may also be able to overcome chemotherapeutic resistance. Several miRNAs have been shown to contribute to the resistance of GBs to chemotherapy. For example, downregulation of

miR-9 in gliomas directly targets *SOX2*, a transcription factor involved in regulating the expression of ATP-binding cassette transporters [31]. These transporters play roles in eliminating chemotherapeutics from cells, and their increased expression in glioma contributes to their resistance to drugs. In addition, miR-455–3p, miR-10a and miR-195 each play a role in increasing the acquired temozolomide resistance in GBs [32]. Overall, the cumulative effect of miRNA dysregulation in gliomas is increased ‘stemness’ of the cells, the failure of proper cell cycle regulation and resistance to standard chemotherapeutic regimens.

Mechanisms of immunosuppression & deregulation in GB patients & the tumor microenvironment

Patients with cancer, and especially those with malignant gliomas, have a variety of mechanisms that contribute to their overall state of immunosuppression,

allowing the disease to progress without adequate anti-tumor responses by the immune system [33]. Generalized manifestations of immune impairment in these patients include low peripheral lymphocyte counts, reduced delayed-type hypersensitivity reactions to recall antigens, impaired mitogen-induced proliferation by peripheral blood mononuclear cells and increased numbers of regulatory T cells (Tregs) [34]. Adaptive immune responses are noticeably deficient, with diminished responsiveness of peripheral T cells associated with impaired signaling through the T-cell receptor–CD3 complex. In addition, reduced immunoglobulin synthesis by B cells *in vitro* from the peripheral blood of patients with intracranial tumors appears to be related to diminishment of T-helper (Th) cell activity. Finally, in addition to the overall low counts of peripheral lymphocytes, primed CD8⁺ cytotoxic T cells that do gain CNS access are functionally impaired [35–37].

Many cancers, including gliomas, secrete a variety of factors, such as prostaglandin E2, IL-10, VEGF and TGF- β . These are capable of suppressing cytotoxic responses of T cells against tumor targets, downregulating major histocompatibility complex expression, suppressing T-cell proliferation and inhibiting the maturation of dendritic cells [38–41]. At a molecular level, many of these alterations in cell secretions are correlated with alterations in signal transduction. For example, overexpression and enhanced activation of STAT3 has been found in GBs and STAT3 inhibition results in decreased immunosuppression [42]. In addition to secreted products, there appears to be a systemic increase, especially within the glioma microenvironment of immune suppressive cells. Data indicate that Tregs not only inhibit the initial systemic immune activation, but also suppress the tumor reactive effector T-cell responses in the tumor microenvironment [43]. In fact, CD4⁺CD25⁺FoxP3⁺ Treg-mediated suppression has been demonstrated in many human malignancies [44–47]. The elimination of Tregs (by many approaches) has successfully enhanced antitumor immunity [43,48–51]. In addition, immunosuppressive myeloid cells and M2 macrophages, otherwise known as tumor-associated macrophages, are able to suppress adaptive immunity, increase angiogenesis and promote tumor growth, survival, metastasis and invasion [52]. Furthermore, the absence or low expression of costimulatory molecules within the CNS provides an immune escape advantage to cancer cells since costimulatory signals are essential for differentiation of functional tumor-specific CD8⁺ T effector cells [35,53–56]. In fact, the expression of costimulatory inhibitory molecules (e.g., B7-H1) that are expressed in malignant gliomas (especially with PTEN gene loss), can further inhibit immune responses through interactions with programmed cell death receptors [57]. Cumulatively,

because there are multiple redundant mechanisms of glioma-mediated immunosuppression that are heterogeneous among patients' gliomas, it is unlikely that therapeutic targeting of a single mechanism will result in a long-term durable response.

It is possible that several key miRNAs may be regulating multiple mechanisms of glioma-mediated immunosuppression that could provide a potent immunotherapeutic strategy. Additionally, miRNAs may be identified that play a key role in both tumorigenesis and immunosuppression, providing a multipronged therapeutic approach. As an initial step in elucidating the biology of operational miRNAs in glioma-mediated immunosuppression, the various immune cell populations need to be isolated from GB patients, and the operational miRNAs identified that participate in tumor-mediated immunosuppression.

The role of miRNAs in innate & adaptive immune responses

Distinct miRNAs have been shown to interact on multiple levels within the immune system (Figure 1 & Table 2). For example, miR-146a has been shown to be upregulated during immune activation within T cells, macrophages and B cells [58]. Furthermore, miR-124 has been shown to promote microglia quiescence [59], and has been found to be significantly decreased within GB compared with normal brain tissue [5]. Thus, it is possible that the decreased miR-124 in macrophages/microglia in the glioma microenvironment induces the immunosuppressive M2 phenotype, but this awaits further study. Furthermore, the miRNA cluster miR-17–92 has been shown to associate with B-cell development among other functions [60], and its overexpression has been associated with various cancers, including B-cell lymphoma [61]. The upregulation of miR-155 has also been observed in malignancies such as lymphoma [62] and miR-155 also controls activation of B cells [63], thus demonstrating the ability of several of these miRNAs to not only act as possible oncogenes, but also as key regulators of immunosuppression within the tumor environment.

STAT3 has been shown to be a molecular hub for both tumorigenesis and tumor-mediated immunosuppression [64]. STAT3 has been shown to be elevated in the immune cells in malignant glioma patients [65]. Thus, it is plausible that a reduction in specific miRNAs in GB patients, specifically those that target STAT3, may be responsible for enhanced STAT3 levels (Figure 2). To identify a group of miRNAs that could interact with conserved target sites in the STAT3 3' untranslated region, TargetScan was employed to identify that miR-124, miR-125b and miR-17 could theoretically downregulate STAT3-mediated immunosuppression in GBs

[66]. miR-17 has been shown to interact with the STAT3 pathway [67], but its effect on immunosuppression has yet to be explored. Concordantly, miR-21, which is under transcriptional control by STAT3, has been found to be elevated in GB cells [14]. Cumulatively, these findings support the notion that the miRNA profile alterations may mediate generalized immunosuppression within the tumor microenvironment.

The innate immune system uses genetically expressed receptors to recognize a broad array of pathogens. The usual mediators of innate immune responses include natural killer (NK) cells, eosinophils, basophils, monocytes/macrophages/microglia and neutrophils. GB cells have been shown to be capable of inhibiting NK cell activity through an overexpression of B7-H3, a direct inhibitor of NK cells and T cells [68].

miRNA analysis of many solid tumors, including brain tumors, has revealed that the increased expression of B7-H3 correlates with underexpression of miR-29. Xu *et al.* specifically demonstrated that transfection with either miR-29 or antisense miR-29 was able to either suppress or induce B7-H3 expression, respectively [68]. In addition, miR-155 has been found to play a central role in the establishment of the proinflammatory, M1 macrophage phenotype through its regulation of the IL-13 receptor [69], which has been shown to aid glioma progression and invasion [42]. Apart from the regulation of macrophage phenotype, miR-17-5p, -20a, -106a and -223 have all been shown to inhibit myeloid progenitor proliferation [70–73], and several of these have been shown to stimulate differentiation and maturation of monocytes [71]. In contrast, myeloid-specific miR-223 was found to negatively regulate differentiation and activation through regulatory circuits involving NFI-A and C/EBP α [70,72,73].

In contradistinction, certain miRNAs have also been found to be involved in activation of the innate immune response. For example, miR-155 expression has been found to increase in response to the presence of lipopolysaccharide (LPS), whereas miR-125b expression was found to be decreased [74]. The oscillatory changes in miR-155 and -125b also correlated with changes in the expression of TNF- α . Tili *et al.* concluded that these miRNAs must, in some way, be under direct control of NF- κ B transcriptional activity, and be involved in modulation of the overall response to

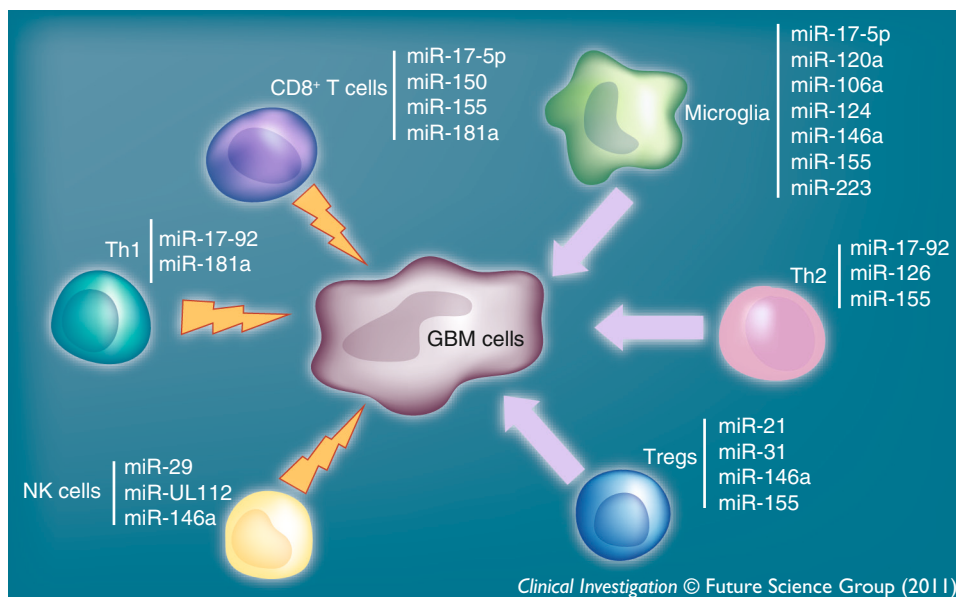


Figure 1. Potential miRNAs' immune regulation in glioblastoma microenvironment.

GBM: Glioblastoma multiforme; NK: Natural killer; Th2: T-helper type-2; Tregs: Regulatory T cells.

LPS stimulation [74]. By promotor analysis, miR-146a was found to be NF- κ B-dependent and involved in the control of TLR signaling, cytokine signaling through regulation of IL-1 receptor-associated kinase 1 and TNF-receptor-associated factor 6 protein levels [75]. Furthermore, miR-146a has been shown to play a role in myeloid cell proliferation, and have an anti-inflammatory role through negative regulation of IL-8 and chemokine ligand 5 [58,76]. Evidence that miR-146a may have a further role in preventing oncogenesis stemmed from the fact that miR-146a-null mice developed tumors [58].

The bridge between the innate and adaptive immune systems is characterized by the activation of T cells through antigen presentation. Depending on the means of antigen-presentation and the factors secreted by the antigen presenting cell and T cell, the type of adaptive immune response can be regulated. The major mediators of the adaptive immune responses are CD4⁺ and CD8⁺ T cells and B cells. As previously discussed, these cellular mediators of the adaptive immune response are also suppressed in GB patients. Probable candidates mediating the suppression of function in T cells include miR-150, -155, -181a and the miR-17–92 cluster. It has been suggested that miR-150 plays a role in maintaining the undifferentiated status of naive T cells, or in promoting early steps in T-cell differentiation [77], whereas miR-155 plays a critical role in the T-cell differentiation processes of the immune response [78]. T cells deficient in miR-155 have been found to produce more IL-4, IL-5 and IL-10, while producing less IFN- γ , demonstrating

Table 2. miRNAs involved in immune modulation.

miR	Role	Target	Ref.
17	Regulation of STAT3-mediated immunosuppression	STAT3	[67]
17–5p	Inhibits myeloid progenitor proliferation and stimulates differentiation and maturation of monocytes by blocking downstream expression of M-CSFR	AML-1	[71]
17–92 cluster	Regulation of Th1 versus Th2 immune response	HIF- α , TGF- β receptor	[60,61,80,83,84,88]
20a	Regulation of STAT3-mediated immunosuppression, inhibits myeloid progenitor proliferation and stimulates differentiation and maturation of monocytes by blocking downstream expression of M-CSFR	STAT3, AML-1	[67,71]
21	Regulated by STAT3, indirectly leads to increased expression of FoxP3, regulates T-cell-dependent immune responses	PDCD4	[14,89,128]
29	Regulates NK cell-mediated cytotoxicity	B7-H3	[68]
31	Regulation of Treg differentiation	FoxP3	[89]
106a	Inhibits myeloid progenitor proliferation and stimulates differentiation and maturation of monocytes by blocking downstream expression of M-CSFR	AML-1	[71]
124	Regulation of STAT3-mediated immunosuppression and microglia quiescence	STAT3, CCAAT/C/EBP- α , PUMA1	[59,66]
125b	Regulation of STAT3-mediated immunosuppression, under transcriptional control by NF- κ B, increased expression in response to LPS stimulation and regulates TNF- α expression	STAT3, TNF- α	[66,74]
146a	Control of TLR signaling, cytokine signaling, myeloid proliferation and prevention of oncogenesis, under transcriptional control by NF- κ B and FoxP3	IL-1 receptor-associated kinase, TNF receptor-associated factor 6, M-CSFR, IL-8, CCL5	[58,75,76,90]
150	Maintenance of undifferentiated status of naive T cells or in promoting early stages of T-cell differentiation	Notch3	[62,77,129]
155	Regulation of B-cell activation, under transcriptional control by NF- κ B and FoxP3, increased expression in response to LPS stimulation and upregulates TNF- α expression, role in establishment of M1 versus M2 phenotype through regulation of the IL-13 receptor Th1 versus Th2 differentiation and downregulation of CTLA-4	FADD, I κ B kinase epsilon, and the receptor (TNFR superfamily)-interacting serine–threonine kinase 1 (Ripk1)	[62,63,69,74,78,79,91]
181a	Differentiation and activation of T cells through regulation of Bcl-2, CD69 and T-cell receptor expression, regulation of mature T cell sensitivity to stimulation by peptide antigens	BCL-2, CD69 and T-cell receptors	[77,80–83]
223	Inhibit myeloid progenitor proliferation and granulocyte differentiation and maturation	Mef2c	[70,72,73]

LPS: Lipopolysaccharide; NK: Natural killer; Th1: T-helper type-1; Th2: T-helper type-2; Treg: Regulatory T cell.

a Th2 response upon activation that is not believed to enhance antitumor immune responses. Finally, miR-155 has been shown to downregulate cytotoxic T lymphocyte-associated antigen 4 – an inhibitor of T-cell activation [79].

Several roles of miR-181 have been shown in the differentiation and activation of T cells [80–82]. Impaired expression of miR-181a during maturation of a T cell impairs both the positive and negative selection process. During normal T-cell maturation, miR-181a expression

is increased at the CD4⁺/CD8⁺ (double positive) stage and exerts control during this process through regulation of BCL-2, CD69 and T-cell receptor expression [82,83]. The miR-181a also plays a role in regulating mature T cells by increasing sensitivity to peptide antigens [81].

The miR-17–92 cluster has also been shown to play a significant role in many aspects of the immune response and tumor survival [83]. Microarray analysis revealed that the miR-17–92 cluster is one of the most significantly overexpressed miRNAs in murine Th1 cells [83]. T cells

from tumor-bearing mice and GB patients have been shown to have decreased levels of miR-17-92 compared with T cells from nontumor-bearing counterparts. Transfection with miR-17-92 into these cells promotes a Th1 response marked by IFN- γ production and very late antigen expression. It is thought that the Th2-skewing tumor microenvironment induces the downregulation of miR-17-92 expression in T cells, thereby reducing tumor-specific immunity. miR-17-92 also targets HIF-1 α [84]. HIF-1 α contributes to the negative regulation of T-cell function [85–87]. As the miR-17-92 cluster is downregulated in the T cells of cancer patients, it is likely that HIF-1 α overexpression may be partly driving the Th2 response. HIF-1 α is under the transcriptional regulation of STAT3, which is known to play significant roles in mediating the immunosuppression in GB patients [64,87]. Furthermore, miR-17-92 targets the TGF- β receptor [88], which is a well delineated immune suppressive pathway in GB patients.

Finally, FoxP3, a specific transcriptional factor of Tregs, has been shown to correlate with cancer immunosuppression, especially within GB patients [47]. Several miRNAs have been shown to interact with FoxP3 [89], including miRNAs being regulated by it [90,91]. Many of the immune modulatory miRNAs have not yet been characterized in gliomas or may have been under appreciated, given the paucity of immune infiltration relative to the bulk of the tumor mass. Current investigation is being conducted to elucidate operational miRNAs within the immune populations of GB patients, including immune populations isolated from the glioma microenvironment, to determine new immune therapeutic targets. Common miRNAs mediating multiple mechanisms of immunosuppression expressed in a variety of immune cell populations would be attractive initial therapeutic candidates.

miRNAs exchange & transfer in glioma microenvironment

It is well established that the miRNA profiles of individual cells can have a significant impact on gene regulation and therefore the phenotype of those particular cells. However, an important question is whether one

affected cell has the capacity to alter the gene expression of neighboring cells through communication via miRNA transfer. It has been shown that such a process occurs in many forms of cancer, GB included [92–97]. miRNA transfer can occur through the secretion of exosomes containing miRNAs, through the release of argonaute2–miRNA complexes by lysed or damaged cells or through direct cell–cell communication via gap junctions. The miRNA profile within GB-derived microvesicles has demonstrated a correlation with the miRNA profile of the GB cells themselves [95]. These GB-secreted microvesicles and exosomes have been shown to be taken up by other cells, and then subsequently regulate angiogenesis, proliferation and immunosuppression [95,98,99]. Although miRNAs have been detected within exosomes and microvesicles in humans [100], the highest levels of miRNAs within human plasma are independent of vesicles, and circulate as stable argonaute2–miRNA complexes [101]. Regardless, targeting the argonaute2 complexes carrying circulating immune modulatory miRNAs is a novel therapeutic approach that has yet to be explored [102]. Ultimately, the selection of miRNAs for therapeutic targeting that are involved both in gliomagenesis and immunosuppression would provide a unique, multiprong approach.

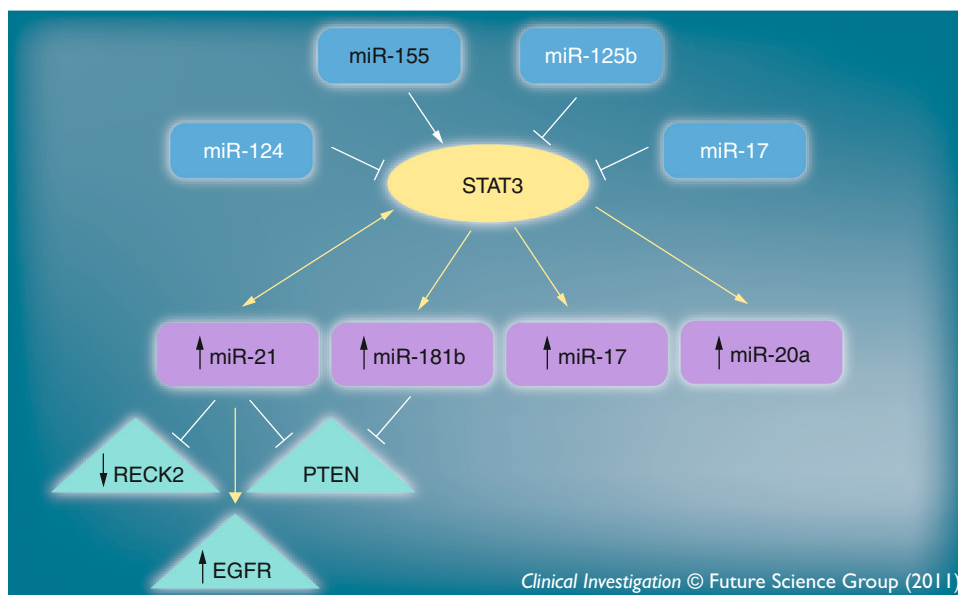


Figure 2. STAT3 has been shown to induce immune suppression and to upregulate certain miRNAs, including miR-21, miR-181 b, miR-17 and miR-20a [67, 128]. miR-21 has been shown to inhibit both PTEN and RECK2 as well as to upregulate EGFR in gliomas. These pathways are directly linked to tumor immune suppression. In breast cancer, miR-155 has been shown to induce the STAT3 pathway [129]; it has yet to be explored in gliomas. The miRNAs highlighted in white text have been predicted to interact with STAT3, but this has not yet been demonstrated.

Preclinical/translational studies of miRNAs as an anticancer approach

The safety of miRNA-based therapies and the potential for miRNA-specific resistance mechanisms has not yet been addressed. However, preclinical studies in syngeneic and immunocompetent murine models show promise. The first reported use of miRNAs as an *in vivo* therapeutic technique exploited the tumor-suppressive property of one miRNA, let-7, to prevent and treat lung tumors in mice [103,104]. A recent study showed that a therapeutic miR-26a delivered by adeno-associated virus (AAV) could suppress tumorigenesis very effectively in an autochthonous murine liver cancer model [105]. To date, a wide variety of therapeutic approaches are under development for GBs [106]. The most obvious candidates for therapeutic miRNA delivery based on current data would include miR-124, miR-7 and miR-128, all of which have effects on glioma tumorigenicity. Of note, from an immune therapeutic treatment perspective, these miRNAs have been tested *in vivo* in xenograft immune-deficient models. As preclinical translational studies move forward in evaluating specific miRNAs with immune modulatory properties, these will need to be conducted in syngeneic or genetically engineered murine models that are immune competent [107]. However, one theoretical distinct advantage of targeting miRNAs that mediate immunosuppression in GB patients is that the CNS penetration properties of the miRNA itself need not be a limitation, because the induction of potent antiglioma immune effector responses may be sufficient to result in tumor eradication and/or suppression.

The use of miRNAs in cancer therapy

Both a disadvantage and an advantage of miRNAs is their ability to over-reach targets within the tumor microenvironment and in normal tissue. First, a given miRNA can have hundreds of target mRNAs, and this may prove to be a disadvantage based on unknown effects of the miRNA on various pathways within the cell. Second, the expression of the target gene may be controlled by several different miRNAs, which may compromise the effects of single-miRNA-based treatment. Third, there is still a lack of a miRNA delivery system with sufficient specificity and efficacy. At the same time, by targeting miRNAs that have been associated with oncogenes, one can target many different pathways of tumor proliferation, angiogenesis and immunosuppression using the same miRNA. Finally, not all miRNAs are organ specific or even system specific, but by targeting a miRNA that is system specific, it may be possible for therapy to produce advantageous results.

Delivery

With the rapid increase in knowledge about miRNA expression levels in cancer patients, another question is brought to mind. How can we apply our knowledge of miRNAs to patient care? One of the greatest drawbacks of treatment in a living system is the short half-life of miRNA, owing to degradation by ribonucleases. Second, difficulties arise in delivering specific miRNAs to the targeted cells. The targeting of certain tissues may require local delivery, whereas others may be amenable to systemic delivery. For example, the eye, mucus membranes and cutaneous lesions could be treated via topical therapy; and lung lesions with intranasal or intratracheal administration. Another alternative is direct intratumoral injection of siRNA delivery complexes. siRNA complexed with the delivery agent polyethyleneimine has been shown to inhibit tumor growth upon intratumoral injection in mice bearing GB xenografts [108]. However, these model systems usually have very small tumors relative to human gliomas, and thus distribution and delivery with this approach will be a significant limitation.

In general, delivery vehicles are designed to facilitate uptake into the target and to protect miRNA payloads and inhibit nonspecific delivery. Delivery vehicles may include synthetic vehicles such as liposomes, polymers, nanoparticles (NPs) or viral agents. Liposomes form structures similar to micelles, in which a lipid bilayer forms a sphere with an aqueous core, and the nucleic acid siRNA can be stored within this hydrophilic core [109]. Liposomes show promise for future clinical use, and the US FDA has approved pegylated liposomes for delivery of doxorubicin and amphotericin B [110]. Cationic polymers with linear or branched structure have also been shown to be efficient transfection agents. These polymers are able to condense nucleic acids into stabilized NPs, and thereby increase their half-life [111,112]. Other polymers, such as cyclodextrin, have also been used as small molecular RNA delivery agents. For example, cyclodextrin-transferring NPs that target a subunit of ribonucleotide reductase were safely delivered via an intravenous route in nonhuman primates [113].

Viral transfection agents such as adenoviruses, AAVs, retroviruses and lentiviruses have also shown potential as delivery vehicles. An advantage of viral transfection vectors is that long-term gene suppression is attainable after a single injection of viruses engineered to express stem-loop RNAs [114,115]. As with other delivery methods, the ability of viruses to diffuse within tissue and transduce target cells limits their miRNA-delivery capacity. Lentiviral transfections and retroviruses are advantageous when transfecting proliferating cells, because the gene is incorporated

into the host genome and thus is maintained during cell division [115]. AAVs may be preferred for targeting nondividing cells because they do not integrate into the host genome, but persist episomally and are therefore less prone to causing insertional mutagenesis [116]. AAVs are particularly interesting for the delivery of miRNA expression cassettes, because the small size of miRNAs makes them amenable to AAV packaging. A great deal of *in vivo* studies have been conducted using AAV vectors that deliver miRNA cassettes to treat diseases, including human cancers, metabolic diseases, muscular dystrophies, cardiac diseases, retinal diseases, neurodegenerative disorders and infectious diseases.

Although there are quite a few agents that could potentially be used for delivery of miRNAs, more research is still needed to effectively translate this approach into clinical practice. Researchers must determine which combinations of delivery options will produce an appropriate miRNA dose to achieve sufficient therapeutic effects. High doses of agents may lead to multiple problems, including direct cellular toxicity by the transfection agents and problems with endogenous miRNA-mediated gene regulation [117–119]. In the case of miRNAs being utilized to reverse glioma-mediated immunosuppression, this approach may not require direct delivery to the tumor *per se*, since the resulting antitumor immune response would act as the therapeutic ‘Trojan horse’.

Current status of clinical studies & trials using miRNAs to treat cancer patients

Recently, Santaris (CA, USA) announced the launch of the first ever clinical trial for the evaluation of a miRNA-targeted drug. This was a successful Phase I trial of a locked nucleic acid-modified anti-miR-122 that has been shown to decrease hepatitis C virus (HCV) replication in liver cells and has paved the way for a Phase II study that will consist of weekly or bi-weekly subcutaneous injections in 55 patients with chronic HCV infection [201].

A few candidate miRNAs are emerging as potential therapeutics for malignancies, but the notion of treating cancer with miRNA replacement or miRNA-inhibitors is still in its infancy and will require more functional *in vivo* studies. Safety and delivery mechanisms will also need to be established. Due to the redundancy and complexity of signal pathways in GB that lead to relapse, even with combined targeted therapies, development of miRNA therapy, which employs the pleiotropic role of miRNA in gene regulation, has the potential to overcome the limitations of present GB therapies. It is plausible that miRNA-based drugs will be used for the clinical treatment of GBs, which may offer a

safe and cancer tissue-specific treatment modality, and could potentially aid in the detection and prognosis of the disease.

Future perspective

The miRNAs that regulate or are regulated by STAT3 are top candidates for therapeutic modulation because they may be involved in both gliomagenesis and glioma-mediated immunosuppression. In addition, other immune-related molecules have been identified as potential targets in which expression levels can be modulated by miRNAs, such as B7-H3 [68], ICAM-1 [17] and GalNAc transferase GALNT7 [120]. Their specific use as immunotherapeutics will need to include an analysis of how specific miRNAs are operating within subsets of immune cells and in the context of glioma biology. In each miRNA-based strategy, it is important to identify the target molecules of each specific miRNA, thereby gaining an accurate understanding of the impact of miRNA-engineering therapeutic approaches.

Microglia/macrophages constitute the most dominant tumor-infiltrating innate immune cells (5–30% in glioma cells) in the GB microenvironment and originate from peripheral monocytes. GB-infiltrating microglia/macrophages (GIMs) are immune quiescent [121], have known immunosuppressive properties [42] and promote glioma growth [122]. We found that GIMs expressed substantial levels of TLRs, but despite adequate LPS binding to TLR-4, the GIMs could not induce T-cell proliferation, which suggests a deregulation or breakdown of key signaling pathways of innate inflammatory stimulation [35]. Recent studies have revealed that miRNAs target multiple key components of the TLR signaling pathways and play a critical role in limiting potentially dangerous inflammation from spiraling out of control in macrophages [123]. This implies that there is altered expression of a group of miRNAs in GIMs responsible for their severely compromised phagocytic and antigen presentation functions. Therefore, screening and identification of GIM-specific miRNAs mediating immune quiescence would be a novel immunotherapeutic approach to overcome immune quiescence and resistance in GB patients.

Dendritic cells and T cells, essential players in adaptive immunity, have been used for GB immunotherapy [124,125]. However, their efficacy in clinical trials is limited, and it is necessary to develop new ways to augment this promising form of GB treatment. Certain miRNAs regulate expression of genes that are critically involved in immune responses mediated by dendritic cells and T cells, which represent a highly attractive target for miRNA gene therapy approaches.

For example, miR-155 appears to have a suppressive role in dendritic cells [126], probably through post-transcriptional silencing of critical mediators of the NF- κ B pathway such as IKK ϵ . miR-146a may also have similar functions in dendritic cells [75]. Thus, introduction of an inhibitor of miR-155 or miR-146a into dendritic cells *ex vivo* prior to their infusion back into the patient may represent an effective way to enhance the antigen presentation ability of these cells and augment GB-specific immunity in those clinical trials that use dendritic cells as an immune therapeutic.

One major barrier for successful T-cell-based GB immunotherapy is the low persistence of tumor antigen (TA)-specific T cells in tumor-bearing hosts. It would be feasible to generate genetically modified TA-specific T cells *ex vivo* that are resistant to tumor-mediated immunosuppression, promoting robust and long-lived anti-GB responses during adoptive T-cell immunotherapy. Specifically, miR-17–92 has been shown to promote Th1 cell skewing [83] and thereby could confer resistance to tumor-derived immunosuppressive factors and improve Th1 reactivity. Alternatively, other miRNAs, including miR-155 [127] and miR-181a [81],

can promote Th1 differentiation and reduce the T cell activation threshold in T cells that recognize normally weak TAs.

Recent advances in our understanding of the miRNA process, as well as in how the immune system is regulated, have created great opportunities in the field of GB immunotherapy. The development of synthetic and chemically modified miRNAs over the past few years has made it feasible to apply miRNAs to clinical treatment. However, numerous hurdles remain regarding their intracellular stability, tissue-specific delivery, penetration across the blood–brain barrier, and ability to sustain silencing. These issues must be addressed before this technology can be applied widely in the clinic. In addition, questions related to the nonspecific effects of miRNAs, as well as cellular resistance to them, must be addressed before miRNAs are used therapeutically. Nonetheless, as our understanding of the way in which miRNAs intricately regulate the innate, adaptive immune cells and GB tumor cells and their functions rapidly advances, we hope that the use of miRNAs in GB immunotherapy will soon become a reality that improves the lives of many GB patients worldwide.

Executive summary

Background

- Glioblastoma (GB) is the most malignant neoplasm of the CNS with a mean survival time of only 14.6 months.
- GB patients have multiple heterogeneous and redundant immune suppressive mechanisms.
- MiRNAs are naturally derived 21–25 nucleotide RNAs that usually bind the 3' untranslated region of target mRNAs and direct their post-transcriptional repression.
- miRNAs have widespread deregulation of expression within malignancies and are implicated in many processes of oncogenesis and immune dysregulation.
- miRNAs play key regulatory roles in glioma cell growth, division, proliferation and apoptosis.
- Modulation of specific miRNAs in GBs can inhibit/delay gliomagenesis.

Preclinical evidence for the immune regulation of miRNAs in GBs

- miRNAs are important regulators in the development and function of multiple types of immune cells, including macrophages/microglia and T cells.
- Some miRNAs act as key regulators of immune suppression within the tumor microenvironment.
- Signal transducer and activator of transcription 3 is a molecular hub for both GB tumorigenesis and for tumor-mediated immune suppression, and its activity is regulated by miRNAs.
- Decreased levels of specific miRNAs upregulate immune inhibitory molecules in GBs and render tumor cells more resistant to recognition and killing by effector immune cells.

Translational evidence for the immunotherapeutic potentials of miRNAs in GBs

- By knocking down specific overexpressing miRNAs, antisense oligonucleotides have effectively decreased tumor growth in GB murine models, demonstrating the potential of anti-miRNA oligonucleotides for immune therapeutic applications to treat GBs.
- Modulation of various miRNAs has been shown to regulate both innate and adaptive immunity.

Clinical evidence for the immunotherapeutic potentials of miRNAs

- Some miRNAs with immune regulatory properties such as let-7, miR-16 and miR-34a will soon be evaluated in Phase I clinical trials for breast, lung and prostate cancer patients.
- A successful Phase I trial of anti-miR-122 has been shown to decrease hepatitis C virus replication in liver cells, and therefore has the potential to treat liver cancer.
- Due to the redundancy and complexity of signal pathways in GB that lead to relapse, even with combined targeted therapies, development of miRNA therapy, which employs the pleiotropic role of miRNA in gene regulation, has the potential to overcome the limitations of present GB therapies.

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