Published in final edited form as:

Clin Investig (Lond). 2011 December ; 1(12): 1637–1650. doi:10.4155/cli.11.159.

miRNA-mediated immune regulation and immunotherapeutic potential in glioblastoma

Krishan Jethwa, Jun Wei, Kayla McEnery, and Amy B Heimberger*

Department of Neurosurgery, The University of Texas MD Anderson Cancer Center, Unit 442, 1515 Holcombe Boulevard, Houston, TX 77030-4009, USA

Abstract

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.

Keywords

glioblastoma; immunosuppression; miRNA; STAT3

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

© 2011 Future Science Ltd

*Author for correspondence: Tel.: +1 713 792 2400, Fax: +1 713 794 4950, aheimber@mdanderson.org.

For reprint orders, please contact reprints@future-science.com

Financial & competing interests disclosure

This study was supported by NIH grants P50 CA093459, R01 CA120813, P50 CA127001 and R44-A1 077225, and the Cynthia and George Mitchell Foundation and the Dr. Marnie Rose Foundation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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 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 down-regulated 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* poly-comb 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, 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, over-expression 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 down-regulate 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 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 a Th2 response upon activation that is not believed to enhance antglioma 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 micro-environment 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.

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.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. Takane K, Fujishima K, Watanabe Y, et al. Computational prediction and experimental validation of evolutionarily conserved microRNA target genes in bilaterian animals. *BMC Genomics*. 2010; 11:101. [PubMed: 20144220]
2. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391(6669):806–811. [PubMed: 9486653]
3. Meltzer PS. Cancer genomics: small RNAs with big impacts. *Nature*. 2005; 435(7043):745–746. [PubMed: 15944682]
4. Sassen S, Miska EA, Caldas C. MicroRNA: implications for cancer. *Virchows Arch*. 2008; 452(1): 1–10. [PubMed: 18040713]
5. Asadi-Moghaddam K, Chiocca EA, Lawler SE. Potential role of miRNAs and their inhibitors in glioma treatment. *Expert Rev Anticancer Ther*. 2010; 10(11):1753–1762. [PubMed: 21080802]
6. Chen CZ. MicroRNAs as oncogenes and tumor suppressors. *N Engl J Med*. 2005; 353(17):1768–1771. [PubMed: 16251533]
7. Dong H, Siu H, Luo L, Fang X, Jin L, Xiong M. Investigation gene and microRNA expression in glioblastoma. *BMC Genomics*. 2010; 11(Suppl 3):S16. [PubMed: 21143783]
- 8•. Huse JT, Brennan C, Hambardzumyan D, et al. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis *in vivo*. *Genes Dev*. 2009; 23(11): 1327–1337. Reviews miRNA alterations and their roles in glioblastomas (GBs) and includes therapeutic strategies utilizing miRNAs or miRNA antagonists. [PubMed: 19487573]
9. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*. 2007; 133(2):647–658. [PubMed: 17681183]
- 10•. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an anti-apoptotic factor in human glioblastoma cells. *Cancer Res*. 2005; 65(14):6029–6033. First paper presenting miR-21 as an oncogenic miRNA in GB cells. [PubMed: 16024602]
11. Papagiannakopoulos T, Shapiro A, Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res*. 2008; 68(19):8164–8172. [PubMed: 18829576]
12. Chen Y, Liu W, Chao T, et al. MicroRNA-21 downregulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. *Cancer Lett*. 2008; 272(2):197–205. [PubMed: 19013014]
13. Sayed D, He M, Hong C, et al. MicroRNA-21 is a downstream effector of AKT that mediates its antiapoptotic effects via suppression of Fas ligand. *J Biol Chem*. 2010; 285(26):20281–20290. [PubMed: 20404348]
14. Gabrieli G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol Cell Biol*. 2008; 28(17):5369–5380. [PubMed: 18591254]
15. Zhang CZ, Zhang JX, Zhang AL, et al. MiR-221 and miR-222 target *PUMA* to induce cell survival in glioblastoma. *Mol Cancer*. 2010; 9:229. [PubMed: 20813046]
16. Zhang J, Han L, Ge Y, et al. miR-221/222 promote malignant progression of glioma through activation of the Akt pathway. *Int J Oncol*. 2010; 36(4):913–920. [PubMed: 20198336]
- 17•. Ueda R, Kohanbash G, Sasaki K, et al. Dicer-regulated microRNAs 222 and 339 promote resistance of cancer cells to cytotoxic T-lymphocytes by downregulation of ICAM-1. *Proc Natl Acad Sci USA*. 2009; 106(26):10746–10751. Demonstrates that Dicer regulates glioma cell susceptibility to immune surveillance through miRNA processing. [PubMed: 19520829]
18. Nan Y, Han L, Zhang A, et al. MiRNA-451 plays a role as tumor suppressor in human glioma cells. *Brain Res*. 2010; 1359:14–21. [PubMed: 20816946]
19. Fareh M, Turchi L, Virolle V, et al. The miR 302–367 cluster drastically affects self-renewal and infiltration properties of glioma-initiating cells through CXCR4 repression and consequent disruption of the SHH-GLI-NANOG network. *Cell Death Differ*. 2011 (Epub ahead of print). 10.1038/cdd.2011.89

20. Kefas B, Godlewski J, Comeau L, et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is downregulated in glioblastoma. *Cancer Res.* 2008; 68(10):3566–3572. [PubMed: 18483236]
21. Guessous F, Zhang Y, Kofman A, et al. microRNA-34a is tumor suppressive in brain tumors and glioma stem cells. *Cell Cycle.* 2010; 9(6):1031–1036. [PubMed: 20190569]
22. Li WB, Ma MW, Dong LJ, Wang F, Chen LX, Li XR. MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme. *Cancer Biol Ther.* 2011; 12(6):477–483. [PubMed: 21743299]
23. Li Y, Guessous F, Zhang Y, et al. MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. *Cancer Res.* 2009; 69(19):7569–7576. [PubMed: 19773441]
24. Cortez Ma; Nicoloso Ms; Shimizu M., et al. miR-29b and miR-125a regulate podoplanin and suppress invasion in glioblastoma. *Genes Chromosomes Cancer.* 2010; 49(11):981–990. [PubMed: 20665731]
25. Godlewski J, Nowicki MO, Bronisz A, et al. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res.* 2008; 68(22):9125–9130. [PubMed: 19010882]
26. Zhang Y, Chao T, Li R, et al. MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J Mol Med.* 2009; 87(1):43–51. [PubMed: 18810376]
27. Wuchty S, Arjona D, Li A, et al. Prediction of associations between microRNAs and gene expression in glioma biology. *PLoS One.* 2011; 6(2):e14681. [PubMed: 21358821]
28. Smirnova L, Grafe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. *Eur J Neurosci.* 2005; 21(6):1469–1477. [PubMed: 15845075]
29. Silber J, Lim Da, Petritsch C, et al. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med.* 2008; 6:14. [PubMed: 18577219]
30. Wei J, Barr J, Kong LY, et al. Glioma-associated cancer-initiating cells induce immunosuppression. *Clin Cancer Res.* 2010; 16(2):461–473. [PubMed: 20068105]
31. Jeon HM, Sohn YW, Oh SY, et al. ID4 imparts chemoresistance and cancer stemness to glioma cells by derepressing miR-9*-mediated suppression of SOX2. *Cancer Res.* 2011; 71(9):3410–3421. [PubMed: 21531766]
32. Ujifuku K, Mitsutake N, Takakura S, et al. miR-195, miR-455–3p and miR-10a are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. *Cancer Lett.* 2010; 296(2):241–248. [PubMed: 20444541]
33. Heimberger AB, Sampson JH. Immunotherapy coming of age: what will it take to make it standard of care for glioblastoma? *Neuro Oncol.* 2011; 13(1):3–13. [PubMed: 21149252]
34. Dey M, Hussain SF, Heimberger AB. The glioma microenvironment in immune modulation: potential targets for intervention. *Lett Drug Des Discov.* 2006; 3(7):443–451.
35. Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro Oncol.* 2006; 8(3):261–279. [PubMed: 16775224]
36. Calzascia T, Masson F, Di Berardino-Besson W, et al. Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. *Immunity.* 2005; 22(2):175–184. [PubMed: 15723806]
37. Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. *J Neurosci Res.* 1991; 28(2):254–260. [PubMed: 2033653]
38. Gabrilovich D, Ishida T, Oyama T, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages *in vivo*. *Blood.* 1998; 92(11):4150–4166. [PubMed: 9834220]
39. Platten M, Wick W, Weller M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech.* 2001; 52(4):401–410. [PubMed: 11170299]
40. Roszman T, Elliott L, Brooks W. Modulation of T-cell function by gliomas. *Immunol Today.* 1991; 12(10):370–374. [PubMed: 1958290]

41. Tada M, Suzuki K, Yamakawa Y, et al. Human glioblastoma cells produce 77 amino acid interleukin-8 (IL-8[77]). *J Neurooncol.* 1993; 16(1):25–34. [PubMed: 8410139]
42. Wu A, Wei J, Kong LY, et al. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* 2010; 12(11):1113–1125. [PubMed: 20667896]
43. Sakaguchi S. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol.* 2004; 22:531–562. [PubMed: 15032588]
44. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* 2004; 10(9):942–949. [PubMed: 15322536]
45. El Andaloussi A, Lesniak MS. CD4⁺ CD25⁺ FoxP3⁺ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas. *J Neurooncol.* 2007; 83(2):145–152. [PubMed: 17216339]
46. Fecci PE, Mitchell DA, Whitesides JF, et al. Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. *Cancer Res.* 2006; 66(6):3294–3302. [PubMed: 16540683]
47. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, et al. Incidence and prognostic impact of FoxP3⁺ regulatory T cells in human gliomas. *Clin Cancer Res.* 2008; 14(16):5166–5172. [PubMed: 18698034]
48. Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA. Inability of a fusion protein of IL-2 and diphtheria toxin (denileukin diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J Immunother.* 2005; 28(6):582–592. [PubMed: 16224276]
49. Berd D, Mastrangelo MJ. Effect of low dose cyclophosphamide on the immune system of cancer patients. reduction of T-suppressor function without depletion of the CD8⁺ subset. *Cancer Res.* 1987; 47(12):3317–3321. [PubMed: 2953413]
50. Foss F. Clinical experience with denileukin diftitox (ONTAK). *Semin Oncol.* 2006; 33(1 Suppl 3):S11–S16. [PubMed: 16516670]
51. Su YB, Sohn S, Krown SE, et al. Selective CD4⁺ lymphopenia in melanoma patients treated with temozolomide: a toxicity with therapeutic implications. *J Clin Oncol.* 2004; 22(4):610–616. [PubMed: 14726505]
52. Sica A, Schioppa T, Mantovani A, Allavena P. Tumor-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer.* 2006; 42(6):717–727. [PubMed: 16520032]
53. Matyszak MK, Denis-Donini S, Citterio S, Longhi R, Granucci F, Ricciardi-Castagnoli P. Microglia induce myelin basic protein-specific T cell anergy or T cell activation, according to their state of activation. *Eur J Immunol.* 1999; 29(10):3063–3076. [PubMed: 10540317]
54. Tirapu I, Huarte E, Guiducci C, et al. Low surface expression of B7–1 (CD80) is an immunoescape mechanism of colon carcinoma. *Cancer Res.* 2006; 66(4):2442–2450. [PubMed: 16489051]
55. Voigt H, Schrama D, Eggert AO, et al. CD28-mediated costimulation impacts on the differentiation of DC vaccination-induced T cell responses. *Clin Exp Immunol.* 2006; 143(1):93–102. [PubMed: 16367939]
56. Yi-Qun Z, Lorre K, De Boer M, Ceuppens JL. B7-blocking agents, alone or in combination with cyclosporin A, induce antigen-specific anergy of human memory T cells. *J Immunol.* 1997; 158(10):4734–4740. [PubMed: 9144487]
57. Parsa AT, Waldron JS, Panner A, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med.* 2007; 13(1):84–88. [PubMed: 17159987]
58. Boldin MP, Taganov KD, Rao DS, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation and cancer in mice. *J Exp Med.* 2011; 208(6):1189–1201. [PubMed: 21555486]
59. Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP- α -PU.1 pathway. *Nat Med.* 2011; 17(1):64–70. Demonstrates that miR-124 is a key regulator of microglia quiescence in CNS and is a modulator of monocyte and macrophage activation. [PubMed: 21131957]

60. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008; 132(5):875–886. [PubMed: 18329372]
61. Lawrie CH, Chi J, Taylor S, et al. Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. *J Cell Mol Med*. 2009; 13(7):1248–1260. [PubMed: 19413891]
62. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci USA*. 2005; 102(10):3627–3632. [PubMed: 15738415]
63. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA*. 2006; 103(18):7024–7029. [PubMed: 16641092]
64. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells. role of STAT3 in the tumour microenvironment. *Nat Rev Immunol*. 2007; 7(1):41–51. Describes how STAT3 is a molecular hub for tumorigenesis and tumor-mediated immune suppression. [PubMed: 17186030]
65. Humphries W, Wang Y, Qiao W, et al. Detecting the percent of peripheral blood mononuclear cells displaying p-STAT3 in malignant glioma patients. *J Transl Med*. 2009; 7:92. [PubMed: 19900287]
66. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003; 115(7):787–798. [PubMed: 14697198]
67. Lages E, Guttin A, El Atifi M, et al. MicroRNA and target protein patterns reveal physiopathological features of glioma subtypes. *PLoS One*. 2011; 6(5):e20600. [PubMed: 21655185]
68. Xu H, Cheung IY, Guo HF, Cheung NK. MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7-H3: potential implications for immune based therapy of human solid tumors. *Cancer Res*. 2009; 69(15):6275–6281. [PubMed: 19584290]
69. Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem*. 2011; 286(3):1786–1794. [PubMed: 21097505]
70. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*. 2005; 123(5):819–831. [PubMed: 16325577]
71. Fontana L, Pelosi E, Greco P, et al. MicroRNAs 17–5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol*. 2007; 9(7):775–787. [PubMed: 17589498]
72. Fukao T, Fukuda Y, Kiga K, et al. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell*. 2007; 129(3):617–631. [PubMed: 17482553]
73. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*. 2008; 451(7182):1125–1129. [PubMed: 18278031]
74. Tili E, Michaille JJ, Cimino A, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol*. 2007; 179(8):5082–5089. [PubMed: 17911593]
75. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA*. 2006; 103(33):12481–12486. [PubMed: 16885212]
76. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. *J Immunol*. 2008; 180(8):5689–5698. [PubMed: 18390754]
77. Monticelli S, Ansel KM, Xiao C, et al. MicroRNA profiling of the murine hematopoietic system. *Genome Biol*. 2005; 6(8):R71. [PubMed: 16086853]
78. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science*. 2007; 316(5824):604–608. [PubMed: 17463289]

79. Sonkoly E, Janson P, Majuri ML, et al. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. *J Allergy Clin Immunol.* 2010; 126(3):581–589. e1–e20. [PubMed: 20673989]
80. De Yebenes VG, Belver L, Pisano DG, et al. miR-181b negatively regulates activation-induced cytidine deaminase in B cells. *J Exp Med.* 2008; 205(10):2199–2206. [PubMed: 18762567]
81. Li QJ, Chau J, Ebert PJ, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell.* 2007; 129(1):147–161. [PubMed: 17382377]
82. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev.* 2007; 21(5):578–589. [PubMed: 17344418]
83. Sasaki K, Kohanbash G, Hoji A, et al. miR-17–92 expression in differentiated T cells – implications for cancer immunotherapy. *J Transl Med.* 2010; 8:17. [PubMed: 20167088]
84. Taguchi A, Yanagisawa K, Tanaka M, et al. Identification of hypoxia-inducible factor-1 alpha as a novel target for miR-17–92 microRNA cluster. *Cancer Res.* 2008; 68(14):5540–5545. [PubMed: 18632605]
85. Neumann AK, Yang J, Biju MP, et al. Hypoxia inducible factor 1 alpha regulates T cell receptor signal transduction. *Proc Natl Acad Sci USA.* 2005; 102(47):17071–17076. [PubMed: 16286658]
86. Sitkovsky M, Lukashev D. Regulation of immune cells by local-tissue oxygen tension. HIF1 alpha and adenosine receptors. *Nat Rev Immunol.* 2005; 5(9):712–721. [PubMed: 16110315]
87. Wei J, Wu A, Kong LY, et al. Hypoxia potentiates glioma-mediated immunosuppression. *PLoS One.* 2011; 6(1):e16195. [PubMed: 21283755]
88. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA.* 2006; 103(7):2257–2261. [PubMed: 16461460]
89. Rouas R, Fayyad-Kazan H, El Zein N, et al. Human natural Treg microRNA signature. role of microRNA-31 and microRNA-21 in FOXP3 expression. *Eur J Immunol.* 2009; 39(6):1608–1618. [PubMed: 19408243]
90. Lu LF, Boldin MP, Chaudhry A, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell.* 2010; 142(6):914–929. [PubMed: 20850013]
91. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge. the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol.* 2009; 182(5):2578–2582. [PubMed: 19234151]
92. Grange C, Tapparo M, Collino F, et al. Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. *Cancer Res.* 2011; 71(15):5346–5356. [PubMed: 21670082]
93. Kogure T, Lin WL, Yan IK, Braconi C, Patel T. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology.* 2011; 54(4):1237–1248. [PubMed: 21721029]
94. Ohshima K, Inoue K, Fujiwara A, et al. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. *PLoS One.* 2010; 5(10):e13247. [PubMed: 20949044]
95. Skog J, Wurdinger T, Van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2008; 10(12):1470–1476. [PubMed: 19011622]
96. Taylor DD, Atay S, Metzinger DS, Gercel-Taylor C. Characterization of humoral responses of ovarian cancer patients. antibody subclasses and antigenic components. *Gynecol Oncol.* 2010; 116(2):213–221. [PubMed: 19945743]
97. Yang M, Chen J, Su F, et al. Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells. *Mol Cancer.* 2011; 10:117. [PubMed: 21939504]
98. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol.* 2008; 10(5):619–624. [PubMed: 18425114]
99. Graner MW, Alzate O, Dechkovskaia AM, et al. Proteomic and immunologic analyses of brain tumor exosomes. *FASEB J.* 2009; 23(5):1541–1557. [PubMed: 19109410]

100. Turchinovich A, Weiz L, Langheinze A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 2011; 39(16):7223–7233. [PubMed: 21609964]
101. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci USA.* 2011; 108(12): 5003–5008. [PubMed: 21383194]
102. Katakowski M, Buller B, Wang X, Rogers T, Chopp M. Functional microRNA is transferred between glioma cells. *Cancer Res.* 2010; 70(21):8259–8263. [PubMed: 20841486]
103. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA.* 2008; 105(10):3903–3908. [PubMed: 18308936]
104. Trang P, Wiggins JF, Daige CL, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther.* 2011; 19(6):1116–1122. [PubMed: 21427705]
105. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell.* 2009; 137(6):1005–1017. [PubMed: 19524505]
106. Lawler SE, Peruzzi PP, Chiocca EA. Genetic strategies for brain tumor therapy. *Cancer Gene Ther.* 2006; 13(3):225–233. [PubMed: 16138122]
107. Kong LY, Wu AS, Doucette T, et al. Intratumoral mediated immunosuppression is prognostic in genetically engineered murine models of glioma and correlates to immunotherapeutic responses. *Clin Cancer Res.* 2010; 16(23):5722–5733. [PubMed: 20921210]
108. Grzelinski M, Urban-Klein B, Martens T, et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs *in vivo* exerts antitumoral effects in glioblastoma xenografts. *Hum Gene Ther.* 2006; 17(7):751–766. [PubMed: 16839274]
109. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov.* 2005; 4(2):145–160. [PubMed: 15688077]
110. Rust DM, Jameson G. The novel lipid delivery system of amphotericin B: drug profile and relevance to clinical practice. *Oncol Nurs Forum.* 1998; 25(1):35–48. [PubMed: 9460772]
111. Boussif O, Lezoualc'h F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA.* 1995; 92(16):7297–7301. [PubMed: 7638184]
112. Putnam D. Polymers for gene delivery across length scales. *Nat Mater.* 2006; 5(6):439–451. [PubMed: 16738681]
113. Heidel JD, Yu Z, Liu JY, et al. Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc Natl Acad Sci USA.* 2007; 104(14):5715–5721. [PubMed: 17379663]
114. Davidson BL, Harper SQ. Viral delivery of recombinant short hairpin RNAs. *Methods Enzymol.* 2005; 392:145–173. [PubMed: 15644180]
115. Robinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet.* 2003; 33(3):401–406. [PubMed: 12590264]
116. Heilbronn R, Weger S. Viral vectors for gene transfer: current status of gene therapeutics. *Handb Exp Pharmacol.* 2010; (197):143–170. [PubMed: 20217529]
117. Boudreau RL, Martins I, Davidson BL. Artificial microRNAs as siRNA shuttles. improved safety as compared to shRNAs *in vitro* and *in vivo*. *Mol Ther.* 2009; 17(1):169–175. [PubMed: 19002161]
118. Castanotto D, Sakurai K, Lingeman R, et al. Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res.* 2007; 35(15): 5154–5164. [PubMed: 17660190]
119. Grimm D, Streetz KL, Jopling CL, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature.* 2006; 441(7092):537–541. [PubMed: 16724069]

120. Gaziel-Sovran A, Segura MF, Di Micco R, et al. miR-30b/30d regulation of GalNAc transferases enhances invasion and immunosuppression during metastasis. *Cancer Cell*. 2011; 20(1):104–118. [PubMed: 21741600]
121. Watters JJ, Schartner JM, Badie B. Microglia function in brain tumors. *J Neurosci Res*. 2005; 81(3):447–455. [PubMed: 15959903]
122. Graeber MB, Scheithauer BW, Kreutzberg GW. Microglia in brain tumors. *Glia*. 2002; 40(2): 252–259. [PubMed: 12379912]
123. Pedersen I, David M. MicroRNAs in the immune response. *Cytokine*. 2008; 43(3):391–394. [PubMed: 18701320]
124. Plautz GE, Miller DW, Barnett GH, et al. T cell adoptive immunotherapy of newly diagnosed gliomas. *Clin Cancer Res*. 2000; 6(6):2209–2218. [PubMed: 10873070]
125. Yu J, Liu G, Ying H, Yong W, Black K, Wheeler CJ. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen-specific, cytotoxic T-cells in patients with malignant glioma. *Cancer Res*. 2004; 64(14):4973–4979. [PubMed: 15256471]
126. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007; 316(5824):608–611. [PubMed: 17463290]
127. Banerjee A, Schambach F, Dejong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN- γ signaling in CD4⁺ T cells. *Eur J Immunol*. 2010; 40(1):225–231. [PubMed: 19877012]
128. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell*. 2010; 39:493–506. [PubMed: 20797623]
129. Jiang S, Zhang HW, Lu MH, et al. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. *Cancer Res*. 2010; 70:3119–3127. [PubMed: 20354188]

Website

201. Santaris Pharma A/S. www.santaris.com

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 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.

Figure 1. Potential miRNAs' immune regulation in glioblastoma microenvironment
 GBM: Glioblastoma multiforme; NK: Natural killer; Th2: T-helper type-2; Tregs: Regulatory T cells.

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.

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.

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.