**Effect of miR-142-3p on the M2 Macrophage and Therapeutic Efficacy Against Murine Glioblastoma**

Shuo Xu, Jun Wei, Fei Wang, Ling-Yuan Kong, Xiao-Yang Ling, Edjah Nduom, Konrad Gabrusiewicz, Tiffany Doucette, Yuhui Yang, Nasser K.Yaghi, Virginia Fajt, Jonathan M. Levine, Wei Qiao, Xin-Gang Li, Frederick F. Lang, Ganesh Rao, Gregory N. Fuller, George A. Calin, Amy B. Heimberger

Manuscript received April 29, 2013; revised April 8, 2014; accepted May 13, 2014.

**Correspondence to:** Amy B. Heimberger, MD, Department of Neurosurgery, University of Texas M. D. Anderson Cancer Center, Unit 442, Houston, TX 77030-1402 (e-mail: aheimber@mdanderson.org).

**Background**

The immune therapeutic potential of microRNAs (miRNAs) in the context of tumor-mediated immune suppression has not been previously described for monocyte-derived glioma-associated macrophages, which are the largest infiltrating immune cell population in glioblastomas and facilitate gliomagenesis.

**Methods**

An miRNA microarray was used to compare expression profiles between human glioblastoma-infiltrating macrophages and matched peripheral monocytes. The effects of miR-142-3p on phenotype and function of proinflammatory M1 and immunosuppressive M2 macrophages were determined. The therapeutic effect of miR-142-3p was ascertained in immune-competent C57BL/6J mice harboring intracerebral GL261 gliomas and in genetically engineered Ntv-a mice bearing high-grade gliomas. Student t test was used to evaluate the differences between ex vivo datasets. Survival was analyzed with the log-rank test and tumor sizes with linear mixed models and F test. All statistical tests were two-sided.

**Results**

miR-142-3p was the most downregulated miRNA (approximately 4.95-fold) in glioblastoma-infiltrating macrophages. M2 macrophages had lower miR-142-3p expression relative to M1 macrophages (P = .03). Overexpression of miR-142-3p in M2 macrophages induced selective modulation of transforming growth factor beta receptor 1, which led to subsequent preferential apoptosis in the M2 subset (P = .01). In vivo miR-142-3p administration resulted in glioma growth inhibition (P = .03, n = 5) and extended median survival (miR-142-3p–treated C57BL/6J mice vs scramble control: 31 days vs 23.5 days, P = .03, n = 10; miR-142-3p treated Ntv-a mice vs scramble control: 32 days vs 24 days, P = .03, n = 9), with an associated decrease in infiltrating macrophages (R² = .303).

**Conclusions**

These data indicate a unique role of miR-142-3p in glioma immunity by modulating M2 macrophages through the transforming growth factor beta signaling pathway.


Profound immune suppression exists in the glioblastoma microenvironment that promotes glioma invasion and progression and systemically inhibits antitumor immunity. MicroRNAs (miRNAs) are noncoding molecules involved in post-transcriptional gene regulation that have been shown to modulate tumor cell proliferation and apoptosis and to act as oncogenes or tumor-suppressor genes. miR-142-3p has been shown to be a useful biomarker and potential therapeutic target in a variety of malignancies. Although most studies on miR-142-3p focus on expression frequency or prognostic impact, emerging data indicate that miR-142-3p is involved in immunological reactivity and activation, including T-cell immune suppression. Moreover, miR142-3p may influence the differentiation state of immune cell populations.

Tumor-associated macrophages, the largest infiltrating immune cell population in glioblastomas, originate from the peripheral blood monocytes and are recruited by a variety of tumor-derived signals. Glioblastomas actively recruit circulating macrophages to the tumor site and induce them to adopt a tumor-supportive M2 phenotype capable of mediating immune suppression and promoting invasion. This shift away from the desired proinflammatory/antitumor M1 phenotype by cytokines such as transforming growth factor beta (TGF-β) may be regulated by miR142-3p. Although the immune suppressive properties of glioblastoma-infiltrating macrophages have been investigated, the relationship to miRNA dysregulation has not been evaluated to date. Therefore, we hypothesized that miR-142-3p, by interacting with the TGF-β pathway, regulates the tumor-supportive properties of glioma-associated macrophages and potentiates antitumor immune effects by manipulating miR-142-3p levels in vivo.
Methods

Isolation of Human Glioblastoma-Infiltrating Macrophages and CD14+ Monocytes
This study was approved by the institutional review board of the University of Texas M. D. Anderson Cancer Center and conducted under protocol LAB03-0687. Informed consent was obtained from each subject. Tumors were confirmed as glioblastoma based on the 2007 World Health Organization classification of tumors of the central nervous system (20) by a board-certified neuropathologist. Further details are provided in the Supplementary Methods (available online).

Real-Time Polymerase Chain Reaction to Confirm Relative miR-142 Expression Levels
Total RNA extracted was used as the template for reverse transcription using the TaqMan real-time polymerase chain reaction (PCR) kit (Applied Biosystems, Carlsbad, CA) in the 7500 real-time PCR system (Applied Biosystems). Primers for reverse transcription were purchased for human miR-142-3p (PN4427975, assay ID: 000464; Life Technologies, Grand Island, NY) and U18 (PN4427975, assay ID: 001204; Life Technologies). Further details are provided in the Supplementary Methods (available online).

In Vivo Experiments
The miR-142-3p duplex that mimics pre-miR-142-3p (sense: 5′-UGUAAGUGUUCUACUUAUGAU-3′, antisense: 5′-CGCAGUGUAGAUCUAAA-3′) and the scramble control miRNA duplex (sense: 5′-AGUACUGCUACAGUGTT-3′, antisense: 5′-CCGUAUGCUAGCAGCUTT-3′) were synthesized (Avetra Bioscience, San Carlos, CA). The sequence of murine miR-142-3p is identical to human miR-142-3p on the basis of National Center for Biotechnology Information blast data (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The treatment regimens consisted of 2 L of miR-142-3p or scramble control (10 μg/mL) + 48 mL of phosphate-buffered saline (PBS) mixed with the vehicle (40 mL PBS + 10 mL lipofectamine 2000; Life Technologies) or the vehicle control (90 mL PBS + 10 mL lipofectamine 2000). Preliminary pharmacokinetic data indicate that the elimination half-life of miRNA administered in this manner is 6 hours in serum and 12 hours within the peripheral blood immune cells. Mice were maintained in the M. D. Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards and handled according to the approved protocol 08-06-11831.

Syngeneic Subcutaneous Model
The murine glioma GL261 cell line was obtained from the National Cancer Institute–Frederick Cancer Research Tumor Repository and cultured in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C in Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin/L-glutamine (Mediatech, Manassas, VA). The cells were kept in ~80°C and used within 15 passages after thawing. To induce subcutaneous tumors, logarithmically growing GL261 cells were injected into the right hind flanks of 6-week-old C57BL/6j female mice or nude mice at a dose of 1 × 10^6 cells suspended in 100 μL of matrigel basement membrane matrix (BD Biosciences, San Jose, CA). When palpable tumors formed that were approximately 0.5 cm in diameter, the mice were treated by local tumor injection in blinding and randomization (n = 5 per group). Tumors were measured twice per week. Tumor volume was calculated with slide calipers using the following formula: V = (L x W x H)/2, where V is volume (mm³), L is the long diameter, W is the short diameter, and H is the height.

Syngeneic Intracranial Clonotypic Glioma Model
To induce intracerebral tumors in C57BL/6j mice, GL261 cells were collected in logarithmic growth phase and mixed with an equal volume of 10% methyl cellulose in improved modified Eagle medium (zing option; Life Technologies), and loaded into a 250-μL syringe (Hamilton, Reno, NV) with an attached 25-gauge needle. The mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments, Tujunga, CA), as previously described (21). The intracerebral tumorigenic dose for GL261 cells was 5 × 10^6 in a total volume of 5 μL. Mice were randomly assigned to scramble control or the miR-142-3p treatment group (n = 10 per group) and observed and weighed three times per week. When they showed signs of neurological symptoms, they were compassionately killed by prolonged carbon dioxide inhalation. Their brains were removed, placed in 4% paraformaldehyde, and embedded in paraffin.

Pharmacokinetic Study
Non-tumor-bearing C57BL/6j mice (n = 3 per time point) were administered miRNA + lipofectamine 2000 intravenously once and subsequently killed at 0, 15 minutes, 1, 4, 8 and 24 hours. The liver, peripheral blood mononuclear cells, and serum were subsequently analyzed for miRNA expression by quantitative PCR. Further details are provided in the Supplementary Methods (available online).

Genetically Engineered Murine Model of Glioma
The use of immune-competent Ntv-a mice with RCAS-platelet-derived growth factor B + RCAS-B-cell lymphoma 2 induced high-grade gliomas for testing immune therapeutics has been previously described (22). To transfer genes by RCAS vectors, DF-1 producer cells transfected with a particular RCAS vector (1 × 10^6 DF-1 cells in 1–2 μL of PBS) were injected on postnatal day 1 or 2 into the frontal lobes of Ntv-a mice at the coronal suture. Twenty-one days after introducing the glioma-inducing transgenes, the mice were randomly assigned to the treatment or control group (n = 9 per group). Mice were treated intravenously on Monday, Wednesday, and Friday for 3 weeks. The mice were killed 90 days after injection or sooner if they demonstrated morbidity related to tumor burden.

Statistical Analysis
All quantified data represent a mean of triplicate samples ± standard deviation (SD) or as indicated. Statistical significance was determined by Student’s t test or as indicated. P values less than .05 were considered statistically significant. Outliers were eliminated by the Grubbs test. All of the statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, La Jolla, CA), except for

2 of 11 Article | JNCI Vol. 106, Issue 8 | dju162 | August 13, 2014
Figure 1. miR-142-3p expression in gliomas. A) Heatmaps demonstrating the microRNA (miRNA) expression pattern in glioblastoma-infiltrating macrophages compared with matched peripheral blood monocytes (n = 4) using the Human miRNA OneArray Microarray v2. With a mean 4.95-fold decrease in level relative to matched peripheral blood monocytes, miR-142-3p emerged as a leading downregulated candidate. B) Total RNA was extracted from a validating set of glioma cancer stem cells (gCSCs; round dots; n = 5), glioma cell lines (cubes; n = 2), glioblastoma tumor tissues (triangles; n = 4), healthy donor peripheral blood CD14+ monocytes (inverted triangles; n = 3), glioblastoma patient peripheral blood CD14+ monocytes (diamonds; n = 6), and glioblastoma infiltrating CD11b+ macrophages (empty circles; n = 3). Analysis by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) demonstrated the various miR-142-3p expressions among different samples. Of note, miR-142-3p is downregulated within the glioblastoma-infiltrating macrophages relative to peripheral blood monocytes. An unpaired t test was used to calculate the two-sided P values. Central horizontal lines are means, and error bars represent standard deviations. *P < .001.
the survival comparison with log-rank test performed by SAS version 9.3 (SAS Institute, Cary, NC) and TIBCO Spotfire S+ version 8.2 (Somerville, MA). For the subcutaneous tumor measurement, linear mixed models and F test were fit to assess tumor growth after adjusting for treatment effect and taking into account the associations among repeated measures within each subject. All statistical tests were two-sided.

Results

miR-142-3p Expression in Monocyte-Derived Glioblastoma-Associated Macrophages

Using the Human miRNA OneArray Microarray v2 to assess miRNA expression profiling, the miRNA expression profile in glioblastoma-infiltrating macrophages was matched to that of monocytes from the peripheral blood. With a mean 4.95-fold decrease relative to the level in matched peripheral monocytes, miR-142-3p emerged as a leading candidate (Figure 1A; Table 1). Meanwhile, miR-142-3p downregulation was not detected in glioblastoma tissue relative to normal brain tissue by total tissue miRNA microarray (Supplementary Table 1, available online). Subsequent real-time PCR analysis revealed that, although miR-142-3p expression was detected in total glioblastoma tumor tissues, glioblastoma cell lines or glioma cancer stem cells seldom express this miRNA. Rather it is the monocytes that are the main source of miR-142-3p. Intriguingly, miR-142-3p is downregulated within the glioblastoma-infiltrating macrophages relative to peripheral blood monocytes (mean relative expression of infiltrating CD11b+ macrophages = 2.03, SD = 1.17; mean relative expression of peripheral CD14+ monocytes = 9.45, SD = 0.69; P < .001) (Figure 1B).

Table 1. MicroRNA (miRNA) expression in glioblastoma-infiltrating macrophages relative to that in peripheral blood monocytes. The most upregulated/downregulated miRNAs and fold change of expression in glioblastoma-infiltrating macrophages relative to that of matched peripheral blood monocytes (n = 4) are listed.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Relative downregulation</th>
<th>miRNA</th>
<th>Relative upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-142-3p</td>
<td>5.0</td>
<td>hsa-miR-4792</td>
<td>8.6</td>
</tr>
<tr>
<td>hsa-miR-92a</td>
<td>4.9</td>
<td>hsa-miR-634-5p</td>
<td>6.4</td>
</tr>
<tr>
<td>hsa-miR-15a</td>
<td>4.4</td>
<td>hsa-miR-34a</td>
<td>6.4</td>
</tr>
<tr>
<td>hsa-miR-92b</td>
<td>3.8</td>
<td>hsa-miR-4290</td>
<td>6.0</td>
</tr>
<tr>
<td>hsa-miR-378</td>
<td>3.8</td>
<td>hsa-miR-3149</td>
<td>5.7</td>
</tr>
<tr>
<td>hsa-miR-16</td>
<td>3.7</td>
<td>hsa-miR-4455</td>
<td>5.6</td>
</tr>
<tr>
<td>hsa-miR-636</td>
<td>3.7</td>
<td>hsa-miR-32*</td>
<td>5.6</td>
</tr>
<tr>
<td>hsa-miR-20b</td>
<td>3.6</td>
<td>hsa-miR-1273f</td>
<td>4.7</td>
</tr>
<tr>
<td>hsa-miR-378i</td>
<td>3.6</td>
<td>hsa-miR-4634</td>
<td>4.6</td>
</tr>
<tr>
<td>hsa-miR-106a</td>
<td>3.6</td>
<td>hsa-miR-3653</td>
<td>4.1</td>
</tr>
<tr>
<td>hsa-miR-142-5p</td>
<td>3.6</td>
<td>hsa-miR-634</td>
<td>4.1</td>
</tr>
<tr>
<td>hsa-miR-20a</td>
<td>3.5</td>
<td>hsa-miR-2116*</td>
<td>3.9</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
<td>3.4</td>
<td>hsa-miR-4323</td>
<td>3.9</td>
</tr>
<tr>
<td>hsa-miR-18a</td>
<td>3.3</td>
<td>hsa-miR-1273e</td>
<td>3.8</td>
</tr>
<tr>
<td>hsa-miR-18b</td>
<td>3.3</td>
<td>hsa-miR-532-3p</td>
<td>3.7</td>
</tr>
<tr>
<td>hsa-miR-93</td>
<td>3.3</td>
<td>hsa-miR-4508</td>
<td>3.6</td>
</tr>
<tr>
<td>hsa-miR-17</td>
<td>3.3</td>
<td>hsa-miR-92b*</td>
<td>3.4</td>
</tr>
<tr>
<td>hsa-miR-19a</td>
<td>3.3</td>
<td>hsa-miR-4713-5p</td>
<td>3.3</td>
</tr>
<tr>
<td>hsa-miR-425</td>
<td>3.3</td>
<td>hsa-miR-30c-1*</td>
<td>3.3</td>
</tr>
<tr>
<td>hsa-miR-223*</td>
<td>3.2</td>
<td>hsa-miR-4492/4508</td>
<td>3.3</td>
</tr>
</tbody>
</table>

expression in peripheral monocytes from patients with glioblastoma compared with healthy donors.

miR-142-3p Expression in M1 vs M2 Macrophages

Based on the observation of less miR-142-3p expression in the glioblastoma-infiltrating macrophages relative to its monocyte precursor, we further clarified the miR-142-3p expression in the proinflammatory M1 subset and immunosuppressive M2 subset. These subsets were induced ex vivo and characterized based on their morphological, phenotypic, and phagocytic characteristics. Specifically, the M1 macrophages appeared more round, whereas the M2 macrophages assumed a more elongated phenotype, as previously described (Figure 2A) (23). Moreover, the M2 macrophages expressed lower levels of CD11b, CD86, and major histocompatibility complex II than the M1 macrophages but higher levels of CD163 (Figure 2B) and exhibited more robust phagocytosis (mean fluorescence intensity of M1 macrophages: 1.01, SD = 0.05; mean fluorescence intensity of M2 macrophages = 2.25, SD = 0.50; P < .001; n = 6) (Figure 2C), consistent with prior reports (24,25). Quantitative reverse-transcription PCR confirmed the downregulation of miR-142-3p during macrophage differentiation; however, immune-suppressive M2 macrophages were found to have lower miR-142-3p expression. Specifically, the mean expression ± standard deviation of miR-142-3p in M1 macrophages relative to matched M2 macrophages was 1.38 ± 0.36 vs 1.00 ± 0.08 (P = .03; n = 6) (Figure 2D).

miR-142-3p Interacts With the TGF-β Pathway

Using multiple bioinformatics tools (TargetScan 6.2, PicTar, miRanda, and miRDB), potential target genes of miR-142-3p were identified. A cluster of TGF-β pathway genes with conserved target sites in their 3’-untranslated regions (UTRs) were identified, including transforming growth factor beta receptor 1 (TGFBR1) and transforming growth factor beta 2 (TGFBR2). The TGF-β pathway is well known for its potent role in immune suppression and tumorigenesis, including polarization of tumor-promoting M2 macrophages (26). Thus, we investigated the effect of miR-142-3p overexpression on the TGF-β pathway in the monocyte-derived M1 and M2 subsets (Supplementary Figure 1A, available online). In the miR-142-3p overexpressing cells, the TGFBR1 mRNA levels remained unchanged (Supplementary Figure 1B, available online), whereas the TGFBR1 protein levels were repressed in the M2 macrophages (Figure 3, A and B), which indicates that miR-142-3p mediates its effect on TGFBR1 by post-transcriptional regulation rather than by targeting mRNA degradation. TGF-β2 cytokine secretion was not inhibited by miR-142-3p in either the M1 or M2 macrophages (Supplementary Figure 1C, available online). Anti-miR-142-3p treatment resulted in increased TGFBR1 protein levels only in the M2 macrophages (Figure 3A), signifying that TGF-β pathway regulation by miR-142-3p is contextual and cell specific. Other predicted targets of miR-142-3p such as integrin beta 8 (ITGB8), integrin alpha V (ITGAV), SMAD4, NF-kB, and TGFBR2 were not found to be inhibited in either the M1 or M2 macrophage populations (Figure 3A). Furthermore, we detected the activation of the TGFBR1 downstream protein, SMAD2, after stimulation of M1 and M2 cells with TGF-β1. As expected, p-SMAD2 was inhibited by miR-142-3p only in M2 macrophages.
but not in the M1 macrophages (Figure 3B). TGFBR1 SMAD-independent targets such as p-AKT and RhoA were not appreciably altered by miR-142-3p. p-TAK1 was not expressed in either M1 or M2 populations (data not shown).

To further prove that miR-142-3p binds to TGFBR1 mRNA, a luciferase assay was conducted by mutating the predicted miR-142-3p TGFBR1 3’-UTR-luciferase reporter binding site (Figure 3C). In cotransfected HeLa cells, TGFBR1 luciferase activity was substantially inhibited by miR-142-3p compared with a parental luciferase vector control, whereas mutational alteration of the miR-142-3p TGFBR1 3’-UTR binding site resulted in full abolishment of the inhibition (Figure 3D).

**Overexpression of miR-142-3p Does Not Alter Macrophage Phenotype**

We next assessed whether miR-142-3p could shift macrophage differentiation or phenotype based on the expression of the general macrophage marker CD68 and the M2 specific marker CD163 (27–29). After transfection with either miR-142-3p or an miR-142-3p inhibitor, CD68 expression levels in the M1 and M2 macrophages were unchanged (mean CD68 expression percentage of scramble control group = 87.8%, SD = 14.4%; mean CD68 expression percentage of miR-142-3p = 87.8%, SD = 14.9%; \( P = .98 \); \( n = 8 \)) (Supplementary Figure 2, A and C, available online). CD163 expression was occasionally downregulated by over-expression of miR-142-3p (mean CD163 expression percentage of scramble control group = 74.6%, SD = 7.95%; mean CD163 expression percentage of miR-142-3p = 63.9%, \( P = .02 \); \( n = 8 \)) (Supplementary Figure 2, B and C, available online), but this was inconsistently observed.

**miR-142-3p Overexpression Induces M2 Macrophage Apoptosis**

During ex vivo culture of miR-142-3p overexpressing macrophages, we observed a significant decrease in the number of M2 macrophages. Further evaluation revealed that miR-142-3p
transfection induced more early (Annexin V+ 7-AAD−) and late apoptosis/cellular death (Annexin V+ 7-AAD+) in M2-committed macrophages (mean fold relative to control after excluding one outlier = 2.12; SD = 1.01; n = 8) compared with M1-committed macrophages (mean fold relative to control after excluding one outlier = 1.17; SD = 0.34; n = 8) at 48 hours (P = .01 after excluding the outliers by Grubbs test) (Figure 4A). Because TGF-β has been shown to have a role in autocrine growth of cells (30), we next investigated whether TGFBR1 blockade in the M2 cells, which may have autocrine dependency on this pathway, would

Figure 3. miR-142-3p interacts with the transforming growth factor beta receptor 1 (TGFBR1) pathway. A) Western blot analyses of the predicted miR-142-3p targets indicated by bioinformatics tools in M1 and M2 macrophages untreated (blank), transfected with scramble control (ctrl), transfected with miR-142-3p (miR), or transfected with anti-miR-142-3p (anti). The predicted targets include integrin beta 8 (ITGB8), integrin alpha V (ITGAV), transforming growth factor beta 2 (TGFβ2), SMAD4, NF-κB, and TGFBR1. Similar results were observed in 3 replicates. B) miR-142-3p overexpression inhibits the downstream target, p-SMAD2, in M2 macrophages. The M1 and M2 macrophages were treated with transforming growth factor-beta (TGF-β1), transfected with scramble control (ctrl) or miR-142-3p (miR), and then measured for p-SMAD2. Similar results were observed in 3 replicates. C) Sequences of the predicted miR-142-3p binding site on the wild-type TGFBR1 3′-untranslated region (3′-UTR; upper) and the mutated TGFBR1 3′-UTR sequence (lower), which potentially disrupts miR-142-3p binding (middle). D) The relative luciferase activity in HeLa cells after transfection with miR-142-3p in conjunction with the parental luciferase vector (Vector), the wild-type (WT) TGFBR1 3′-UTR, or the miR-142-3p binding site mutant reporter construct (Mut). Luciferase activity is shown relative to the parental luciferase vector, and error bars represent standard deviations. Similar results were observed in 3 replicates.
induce selective apoptosis. As expected, more apoptosis was observed within the M2-committed macrophages treated with either SB431542 (mean fold relative to control of M2-committed macrophages = 2.38, SD = 1.04; mean fold relative to control of M1-committed macrophages = 1.23, SD = 0.26; \( P = .04 \); n = 6) or LY364947 (mean fold relative to control of M2-committed macrophages = 2.29, SD = 0.35; mean fold relative to control of M1-committed macrophages = 1.28, SD = 0.37; \( P = .001 \); n = 6) compared with the M1-committed macrophages at 48 hours (Figure 4B). Identical results were obtained with specific TGFBR1 small interfering RNA (Supplementary Figure 3, A and B, available online), and this appeared to be cell-specific because miR-142-3p overexpression did not induce apoptosis in glioma cancer stem cells (Supplementary Figure 3, C and D, available online).

miR-142-3p Inhibits Glioma Growth

We next investigated whether miR-142-3p could exert an antiglioma effect in vivo (Figure 5A). Aggressive subcutaneous GL261 glioma growth was observed in the control group of syngeneic C57BL/6J mice, whereas glioma growth was markedly inhibited during the 3-week course of miR-142-3p local treatment (\( P = .03 \); n = 5 per group) (Figure 5B). To determine whether miR-142-3p treatment can exert a similar effect against intracerebral glioma, syngeneic C57BL/6J mice harboring GL261 were treated with miR-142-3p intravenously for 3 weeks (Figure 5C). The median survival duration for the scramble control group was 23.5 days, which was extended to 31 days for mice treated with miR-142-3p (\( P = .03 \); n = 10 per group) (Figure 5D). During the treatment course, no behavioral or neurological abnormalities were observed in the mice. Necropsies of the brains of miR-142-3p–treated mice demonstrated no evidence of demyelination, macrophage infiltration, or lymphocytic infiltration in the non-tumor-bearing areas that would indicate the induction of autoimmunity. Furthermore, administration of miR-142-3p did not induce a peripheral lymphocytosis or monocytosis (Supplementary Figure 4, available online).

miR-142-3p Inhibits Glioma-Infiltrating Macrophages

In a genetically engineered Ntv-a murine model system of high-grade glioma, miR-142-3p–treated mice also had extended survival (median survival: 32 days vs 24 days; \( P = .03 \); n = 9) (Figure 6, A and B). This model system has robust glioma-infiltrating macrophages (22), and treatment with miR-142-3p resulted in lower F4/80+ macrophage infiltration compared with the control group (F4/80 positive percentage in control mice = 19.1%, n = 8. * \( P = .01 \). B) Representative flow cytometry analyses and summarized dataset of blockade of TGFBR1 by the antagonists SB431542 (left two columns; n = 6) and LY364947 (right two columns; n = 6) demonstrating induced preferential M2 macrophage apoptosis. The medium dimethyl sulfoxide (DMSO) was examined as control. Round dots are M1-committed monocytes, and empty circles are M2-committed monocytes. A paired \( t \) test was used to calculate the two-sided \( P \) values. Error bars represent standard deviations. * \( P = .04 \) for SB431542; ** \( P = .001 \) for LY364947.
SD = 10.2%; F4/80 positive percentage in miR-142-3p–treated mice = 5.35%, SD = 2.23%; \( P = .009; n = 8 \) (Figure 6C). A negative linear correlation was established between the infiltrating F4/80+ macrophages and survival in the miR-142-3p–treated mice \( (R^2 = .303) \) (Figure 6D), indicating that miR-142-3p is exerting a therapeutic effect by modulating glioma-infiltrating macrophages. miR-142-3p suppressed CD11b+ macrophages elaborating interleukin 6 (IL-6) consistent with M2 skewed cells by 29% (mean percentage of CD11b+ IL-6+ M2 macrophages in controls = 71.8%, SD = 6.5%; mean percentage of CD11b+ IL-6+ M2 macrophages in miR-142-3p = 51.1%, SD = 5.2%; \( P = .01; n = 3 \)) and enhanced CD11b+ macrophages elaborating interferon gamma (IFN-\( \gamma \)) consistent with M1 skewed cells by 83% (mean percentage of CD11b+ IFN-\( \gamma \)+ M1 macrophages in controls = 43.4%, SD = 11.9%; mean percentage of CD11b+ IFN-\( \gamma \)+ M1 macrophages in miR-142-3p = 79.6%, SD = 16.9%; \( P = .04; n = 3 \)) within the spleen. In contrast, miR142-3p did not affect the functional M1 and M2 composition in blood and lymph nodes (Supplementary Figure 5A, available online). However, there was no direct effect of miR-142-3p on dendritic cell expansion, CD8+ effector responses as reflected by the production of effector cytokines such as IFN-\( \gamma \), tumor necrosis factor alpha, and interleukin 2, or T-cell–mediated tumor cytotoxicity (Supplementary Figure 5, B–D, available online).

**Discussion**

The objective of this study was to identify potential therapeutic immune modulatory miRNAs. As such, we cataloged downregulated miRNAs using a novel subtractive approach to identify the preferential expression profile of miRNAs within immune-suppressive (M2) glioblastoma-infiltrating macrophages relative to the monocyte precursor. This screening strategy was intended to identify those miRNAs that are downregulated by the tumor microenvironment that could have biological roles in the monocyte-to-macrophage differentiation state, skewing to or maintenance of the M2 phenotype, or mediating M2 immune suppression. We then selected and prioritized potential candidates on the basis of binding to immunosuppressive pathways or mechanisms. Although several alternative candidates identified in the human miRNA microarray expression library may have roles in modulating regulatory T-cell induction pathways, miR-142-3p was predicted to interfere with immune suppressive TGF-\( \beta \) signaling, which is known to influence the M2 phenotype \( (31,32) \). Other candidates such as miR-378 have been shown to inhibit macrophage proliferation \( (33) \); however, preferential inhibition of only the M2 phenotype would be desirable from a cancer therapeutic perspective. As additional mechanisms and pathways of glioblastoma-mediated immune suppression become elucidated, additional miRNAs are likely to be categorized as potential therapeutic immune modulatory miRNAs.
and these could ultimately be used in a complementary or alternative fashion with miR-142-3p.

The biological role of miR-142-3p in the tumor-associated M2 macrophage has not been previously described. Overexpression of miR-142-3p induces selective apoptosis in the M2 macrophage population due to the inhibition of autocrine-dependent TGFBR1. The specificity of miR-142-3p for TGFBR1 was predicted by multiple binding algorithms and confirmed by luciferase reporting assays and mutational analyses. Furthermore, despite the off-target effect of LY364947 on vascular endothelial growth factor (34), which can induce macrophage to M2 skewing under selective conditions (35), the more specific TGFBR1 blockade data (ie, SB431542 and small interfering RNA) indicate that the induced M2 macrophage apoptosis is secondary to inhibition of its dependent TGFBR1 pathway. Besides the autocrine growth dependence on TGF-β reported in cancer cells (30), we now extend these observations to the immune-suppressive M2 macrophage. However, we have not examined the exact biochemical mechanisms for cell death induced by TGFBR1 blockade.

We have previously demonstrated that glioma cancer stem cells can induce M2 macrophages (16) and that the M2 population is a negative prognosticator in murine models of high-grade gliomas (22). The specific targeting of the M2 immune population while sparing the M1 population in vivo for therapeutic intent is desirable. Prior indirect macrophage targeting strategies have included inhibiting macrophage differentiation and cytokine production (36) or trafficking to the tumor microenvironment (37). We are now showing that targeting of TGFBR1 with miR-142-3p, likely in the circulating monocytes as they become differentiated to the M2, can exert a therapeutic effect against malignant gliomas in vivo.

The exploitation of the immune system to mediate the therapeutic effects of miRNA, such as miR-142-3p, can circumvent previous limitations of miRNA delivery, including getting past the blood–brain barrier. Furthermore, circulating immune cells are the first point of contact to administrated miRNAs, affording an opportunity to directly modulate their functional activity. Despite a therapeutic effect of miR-142-3p against intracerebral gliomas, therapeutic “cures” were not observed, especially in the heterogeneous, genetically engineered murine models. The heterogeneity of immune suppressive mechanisms and pathways within malignant gliomas is widely acknowledged and documented; however, patients that have glioblastomas enriched with M2 macrophages may be particularly responsive to treatment with miR-142-3p, especially because the therapeutic effect of miR-142-3p in vivo was associated with a decreased glioma macrophage infiltration. A greater understanding of the compartmentalization and the

Figure 6. miR-142-3p inhibits glioma-infiltrating macrophages. The intravenous (i.v.) treatment schema (A) and graph of the Kaplan–Meier estimate of survival time (B) demonstrating improved survival in miR-142-3p–treated (miR) Ntv-a mice transfected with the intracerebral (i.c.) RCAS-platelet-derived growth factor B (PDGFB) + RCAS-B-cell lymphoma 2 (Bcl-2) transgenes compared with scramble control (ctrl). Empty triangles are scramble controls, and round dots are miR-142-3p–treated mice. Log-rank test was used to compare overall survival between groups. n = 9 per group. *P = .03. C) Immunohistochemistry demonstrating staining with anti-F4/80 antibodies to identify glioma-infiltrating macrophages. Left panel: representative images of mice treated with scramble control (ctrl) and miR-142-3p (miR), respectively. Scale bar = 50 µm. Right panel: quantification of glioma-infiltrating macrophage and comparison between the two groups. Triangles are scramble controls, and round dots are miR-142-3p–treated mice. A paired t test was used to calculate the two-sided P values. n = 8 per group. *P = .009. D) The correlation between the percentage of glioma-infiltrating F4/80+ macrophages and the survival duration of miR-142-3p treated mice. n = 8. R² = .303.
pharmacokinetics of in vivo miRNA administration is needed, especially in the context of more stable and efficient vectors before clinical trial implementation.

This study did have some limitations in that we have not examined the exact biochemical mechanisms for cell death induced by TGFBR1 blockade. Furthermore, our therapeutic approach is not scalable or appropriate for use in human subjects and will require further refinement of formulation, likely nanoparticles. However, miR-142-3p treatment was well tolerated, and there was no evidence of central nervous system toxicity or induced autoimmunity.

Although miR-142-3p induces preferential apoptosis in the M2 population, the peripheral monocyte counts of miR-142-3p–treated mice were not affected, probably secondary to their pre-existing expression of miR-142-3p and their lack of dependency on the TGFBR1 pathway. Moreover, an oncogenic effect of miR-142-3p in human T-cell acute lymphoblastic leukemia has been reported (38); however, we did not observe any statistically significant differences in peripheral blood lymphocyte counts between the miR-142-3p–treated and control mice. Reconciliation of these results suggests that miR-142-3p plays differential roles (oncopromoter vs oncosuppressor) in different malignancies.

References


**Funding**

This work was supported by the Anthony Bullock III Foundation (to ABH), Cynthia and George Mitchell Foundation (to ABH), the Dr. Marnie Rose Foundation (to ABH), the Vaughn Foundation (to ABH), and the National Institutes of Health (CA120813-01 and P50 CA127001 to ABH; MDACC Brain SPORE Career Developmental Grant to JW; and K08 NS070928 to GR).

**Notes**

The authors were fully responsible for the design of the study, analysis and interpretation of results, the decision to submit the manuscript, and the writing of the manuscript.

We thank Dr Jun Yao for generating the heatmaps and Audria Patrick and Dr David M. Wildrick for editorial assistance.

The contents of this article were a platform presentation at the annual meeting of the Society for Immunotherapy of Cancer in Bethesda, Maryland, October 2012.

**Affiliations of authors:** Department of Neurosurgery, Qilu Hospital of Shandong University, Jinan, China (SX, X-GL), Department of Neurosurgery (SX, JW, FW, L-YK, X-YL, EN, KG, TD, YY, FFL, GR, ABH), Department of Biostatistics (WQ), Department of Pathology (GNF), and Department of Experimental Therapeutics (GAC), University of Texas M. D. Anderson Cancer Center, Houston, TX; Baylor College of Medicine, Houston, TX (NKY); Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX (VF).