miR-124 Inhibits STAT3 Signaling to Enhance T Cell–Mediated Immune Clearance of Glioma

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Abstract
miRNAs (miR) have been shown to modulate critical gene transcripts involved in tumorigenesis, but their role in tumor-mediated immunosuppression is largely unknown. On the basis of miRNA gene expression in gliomas using tissue microarrays, in situ hybridization, and molecular modeling, miR-124 was identified as a lead candidate for modulating STAT3 signaling, a key pathway mediating immunosuppression in the tumor microenvironment. miR-124 is absent in all grades and pathologic types of gliomas. Upon upregulating miR-124 in glioma cancer stem cells (gCSC), the STAT3 pathway was inhibited, and miR-124 reversed gCSC-mediated immunosuppression of T-cell proliferation and induction of forkhead box P3 (Foxp3)+ regulatory T cells (Treg). Treatment of T cells from immunosuppressed glioblastoma patients with miR-124 induced marked effector response including upregulation of interleukin (IL)-2, IFN-γ, and TNF-α. Both systemic administration of miR-124 or adoptive miR-124–transfected T-cell transfers exerted potent anti-glioma therapeutic effects in clonotypic and genetically engineered murine models of glioblastoma and enhanced effector responses in the local tumor microenvironment. These therapeutic effects were ablated in both CD4+ and CD8+–depleted mice and nude mouse systems, indicating that the therapeutic effect of miR-124 depends on the presence of a T-cell–mediated antitumor immune response. Our findings highlight the potential application of miR-124 as a novel immunotherapeutic agent for neoplasms and serve as a model for identifying miRNAs that can be exploited as immunotherapeutics. Cancer Res; 73(13); 3913–26. ©2013 AACR.

Introduction
Glioblastoma multiforme (GBM) is one of the most aggressive primary brain tumors because of its rapid cell growth and immunosuppressive capabilities. Because of the ineffectiveness of many chemotherapy agents and drug treatments, immunotherapeutic strategies are an appealing approach; however, they are limited by the profound immunosuppression that these tumors mediate. Increased antitumor immune responses have been linked to enhanced survival in many cancers, including GBM (1–7). STAT3 is a transcription factor that is a potent regulator of tumorigenesis and immunosuppression (8, 9). STAT3 is upregulated in many cancers, including gliomas (10) and promotes tumorigenesis by preventing apoptosis and enhancing proliferation, angiogenesis, invasion, and metastasis (11, 12). The STAT3 pathway can also become active in tumor-infiltrating immune cells, markedly impairing their antitumor effector responses (9), whereas enhancing the functional activity of immunosuppressive cells (13, 14). Glioma cancer stem cells (gCSC) show activation of the STAT3 pathway (12), which has been shown to modulate their profound immunosuppressive properties (13, 14).

Recent studies have shown that the levels of distinct miRNAs (miR) in the glioma environment differ from those in peritumor tissue. These miRs are noncoding molecules involved in posttranscriptional gene regulation, which have been shown to modulate tumor cell proliferation and apoptosis and to act as oncogenes or tumor-suppressor genes (15–18). Although miRs have been linked to tumor progression, the connection between tumor-mediated immunosuppression and miRs has yet to be explored. It is plausible that they control the STAT3 pathway or are themselves regulated by STAT3, such as miR-21 (19). In addition, oncogenic miR inhibition in murine glioma models has resulted in in vivo growth inhibition (16).

miR-124, which is highly expressed in the central nervous system (CNS), including the cerebellum (20), plays a role in neurogenesis (21), and stimulates neuronal differentiation by antagonizing the transcriptional repressor element 1 silencing transcription factor (REST), which maintains embryonic stem...
cells' self-renewal abilities and pluripotency (21, 22). In high-grade malignant gliomas and astrocytes, miR-124 is scarcely expressed or is absent (23, 24). Furthermore, the loss of miR-124 enhances stem-like traits and increases the invasion of glioma cells (25), whereas miR-124 is strongly induced during neural differentiation of embryonic stem cells (24, 26). miR-124 has been shown to inhibit GBM and medulloblastoma cell proliferation and differentiation (27, 28), and the expression of miR-124 in GBM cell lines results in decreased migration and invasion (23). Here, we hypothesized that miR-124, by interacting with the STAT3 pathway, regulates the immunosuppressive properties of glioma cells and that miR-124 upregulation or administration in vivo will exert potent antitumor immune effects. To determine whether miRs can be exploited as immunotherapeutics, we conducted a tissue microarray (TMA) analysis to detect miRs preferentially absent in gliomas relative to miR expression in normal brain, selected lead candidates that could bind to key immunosuppressive pathways, and then evaluated the therapeutic efficacy of these candidates in multiple murine glioma models.

Materials and Methods

miR comparison of GBM with normal brain tissue

This study was approved by the Institutional Review Board at MD Anderson (Houston, TX) and conducted according to protocol #LAB03-0687. Tumors were pathologically confirmed as GBM (World Health Organization grade IV) by a board-certified neuropathologist. Tumors were washed in RPMI-1640 medium and dissected to remove blood products and surrounding nontumor brain tissue. The total tissue was broken down into smaller pieces and digested in digesting buffer from the Cancer Cell Isolation Kit (Panomics) for 2 hours. The cells were suspended in RNAlater solution (Ambion) in RNAse-free tubes and stored at 4°C overnight; after 24 hours, they were transferred to −80°C until needed for total RNA extraction. Extraction was conducted using the mirVana kit (Ambion). Once extracted, RNA levels were analyzed for concentrations and purity using UV/Vis spectroscopy at 230, 260, and 280 nm.

Total RNA extracted from patients was sent to Phalanx Biotech Group for miR and mRNA-gene expression analyses. Total RNA from normal brain tissues was obtained from Biochain. The results of the GBM human miRNA OneArray Microarray v2 analysis were used to determine which miRs had significant differences in expression compared with normalonor miRs. Expressional differences in terms of multiples (-fold differences) were calculated with Microsoft Excel, and miRs with the most significant differences in expression levels were chosen for the miR target analysis using TargetScan (Release 5.1; ref. 29). miRs of interest were selected on the basis of putative targets and the degree of deviation from normal brain.

Real-time PCR to confirm relative miR expression levels

Total RNA extracted from GBM cells or gCSCs was used as the template for reverse transcription using the TaqMan Reverse Transcription Kit (Applied Biosystems) in a thermocycler, as per the manufacturer's instructions. Primers for reverse transcription PCR (RT-PCR) were purchased for human miR-124, miR-21, U6, and U18 snRNAs (Applied Biosystems). U6 and U18 were used as endogenous controls. cDNA was used as the template for real-time PCR. U18 and miR-124 amplifications were run in triplicate using the TaqMan Real-Time PCR Kit (Applied Biosystems) in the 7500 real-time PCR system (Applied Biosystems). Further reactions, substituting water for the cDNA template, were used as additional controls. Excel was used to calculate the mean levels of each miR and the U18 internal control. The relative expression levels of miR-124 were compared with those of the internal controls, and a bar graph was generated.

Glioma tissue microarray and in situ hybridization

See Supplementary Information for details.

miR-124 transfection in gCSCs, astrocytes, and T cells

The precursor form of miR-124 (30 nmol/L) and the scramble negative control were used to transfect gCSCs and T cells using the siPORT NeoFX transfection agent (Applied Biosystems) or Nucleofector transfection kit (Lonza). Cells were incubated for 72 hours at 37°C to determine cell surface marker expression and collect secreted cytokines. miR-124 expression was verified via RT-PCR after transfection. The morphologic characteristics of the gCSCs were documented at 48 hours after the transfection. A rescue experiment of miR-124 inhibition was accomplished by cotransfection with a plasmid expressing wild-type, constitutively active STAT3 without a miR-124 binding 3′-untranslated region (UTR) site (kindly provided by Dr. Jinbo Yang, Lerner Research Institute of Cleveland Clinic).

In vivo experiments

The miR-124 duplex that mimics pre-miR-124a (sense: 5′-UAAGGCACCGGGUAGAAUGCCA-3′; antisense: 3′-UAAUUCG-GUGCACCUACUCAGC-5′) and the scramble control miRNA duplex (sense: 5′-AGUAUACCUACGAAUCCGGTT-3′; antisense: 3′-TTUCAGCGAUGACUGC-5′) were synthesized (SynGen). The sequence of murine miR-124 is identical to human miR-124 on the basis of National Center for Biotechnology Information (NCBI) blast data (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The treatment cohorts consisted of 20 μg of the miR-124 duplex or scramble control in 10 μL of PBS mixed with the vehicle (80 μL PBS + 10 μL Lipofectamine 2000; Invitrogen) or the vehicle control (90 μL PBS + 10 μL Lipofectamine 2000). The dosing was identical for intratumoral delivery or intravenous infusion. Mice were maintained in the MD Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards and handled according to the approved protocol 08-06-11831.

See supporting information for syngeneic subcutaneous, intracranial, and genetically engineered murine glioma models.

Statistical analysis

The distribution of each continuous variable was summarized by its mean, SD, and range. The distribution of each categorical variable was summarized in terms of its frequencies and percentages. Continuous variables were compared...
between treatment groups by a two-sample t test. In the case of comparing 2 paired groups, a paired t test is conducted. Kaplan–Meier curves were used to estimate unadjusted time to event variables. Log-rank tests were used to compare each time-to-event variable between groups. P values of less than 0.05 (two-sided) were considered statistically significant. All statistical analyses were conducted using the Statistical Package for the Social Sciences v.12.0.0 (SPSS) and SAS v. 9.1 (SAS Institute). Error bars represent SD.

Results

miR-124 expression in gliomas

To determine the pattern of miR expression in GBM relative to normal brain tissue, we used the Human miR OneArray Microarray v2. miR-124 emerged as a leading candidate, with a mean 24.6-fold decrease in expression from that seen in normal brain tissue (Supplementary Table S1). A subsequent analysis using RT-PCR confirmed that miR-124 was absent or minimally expressed in GBM specimens (n = 4), glioma cell lines (n = 2), and gCSCs (n = 4) compared with normal brain tissues (n = 3; Fig. 1A). GBM-associated microglia/macrophages also have low or undetectable expression of miR-124 (Supplementary Fig. S1). When the gCSCs were placed under neural differentiation conditions, miR-124 expression levels were increased (Supplementary Fig. S1). Because miR-124 was a leading candidate downregulated in GBM, as observed by ourselves and others (27, 30), we determined whether it was decreased in other types of gliomas. Using a glioma TMA and in situ hybridization, we found that all glioma grades and types lacked miR-124 expression (Fig. 1B and Table 1). All cortex-containing neurons showed positive expression of miR-124 (n = 19). No differences in survival time among patients with GBM were found on the basis of the relative but negligible expression of miR-124 in The Cancer Genome Atlas dataset (http://cancergenome.nih.gov).

miR-124 interacts with the STAT3 pathway

To determine which miRs interact with STAT3, we used TargetScan to identify a group of miRs with conserved target sites in the STAT3 3′-UTR. Theoretically, these miRs can inhibit...
mimic-124 reverses gCSC-mediated immunosuppression

To determine whether miR-124 reverses gCSC-mediated immunosuppression, we transiently transfected human immunosuppressive gliomas (13) with precursor miRNAs and confirmed the upregulation of miR-124 by RT-PCR. The miR-124 expression was increased in the range of 5- to 20,000-fold among different gCSCs. After 24 hours, gCSCs showed increased adherence to the bottom of the plate, which was more pronounced after 48 hours. Specifically, the typical neurosphere morphology of the gCSCs was altered to become Petri dish-attached with an elongated configuration and with contact inhibition (Fig. 2A). In contrast, transfection of astrocytes with miR-124 did not alter morphology, proliferation, apoptosis, or cell-cycle status (data not shown). To characterize their immunologic phenotype, gCSCs were assessed for their expression of MHC I, MHC II, CD40, CD80, CD86, and B7-H1, by RT-PCR and flow cytometry after transfection with miR-124. No changes were found in MHC I, MHC II, CD40, CD80, B7-H1, or CD86 mRNA and protein expression levels (data not shown). To determine what immunosuppressive soluble factors are affected by miR-124, we analyzed the conditioned medium of miR-124- or scramble (control)-transfected gCSCs using ELISAs and cytokine and chemokine arrays. Here, we found lower levels of IL-8 (scramble: 5,844 ± 72 pg/mL; miR-124: 4 pg/mL; n = 4; P < 0.05), galectin-3 (scramble: 933 ± 214 pg/mL vs. miR-124: 4 ± 1 pg/mL; n = 4; P < 0.01), and MIC-1 (scramble: 13 ± 4 pg/mL vs. miR-124: 4 ± 1 pg/mL; n = 4; P < 0.05; Supplementary Fig. S1) but not of VEGF. Cytokine and chemokine array data revealed a modest decrease in levels of TGF-β2, macrophage migration inhibitory factor, Serpin E1, CX3CL1, CXCL10, CXCL16, and chemokine C–C motif-2, when miR-124 was overexpressed in gCSCs, but these findings were not statistically significant.

To determine whether miR-124 transfection renews the functional gCSC-mediated immune inhibition of T cells, we activated with anti-CD3/CD28 naive CD4+ T cells from healthy donors’ peripheral blood mononuclear cells (PBMC) in the presence of gCSC medium, 3-day gCSC-conditioned medium from gCSCs transfected with scramble control, miR-124, and miR-124 plus STAT3. The medium from scrambled miRNA-transfected gCSCs inhibited T-cell proliferation by 63.5% ± 13.8% versus 33.0% ± 10.1% in miR-124–transfected gCSCs (n = 4; P = 0.023; Fig. 2B). Moreover, fewer apoptotic T cells were induced from naive CD4+ T cells, mediated by gCSC-conditioned medium. Indeed, the medium from miR-124–transfected gCSCs led to decreased FoxP3+ T-cell generation compared with scrambled miRNA-transfected gCSCs (Fig. 2D).
Moreover, these were functional Tregs, as assessed by autologous CD4\(^+\) T-cell proliferation in coculture assays (Fig. 2E). Furthermore, all the effects mediated by miR-124 were reversed by cotransfection of wild-type, constitutively active STAT3 lacking a miR-124-sensitive 3′-UTR fragment (Fig. 2B–E). In contrast, miR-21 enhanced gCSC-mediated immunosuppression as assessed by suppression of T-cell proliferation (Supplementary Fig. S3).

Because miR-124 can modify the immunosuppressive function of gCSCs, we determined whether it could exert a direct effect on the immune effector function in immunosuppressed patients with GBM. PBMCs were obtained from patients newly diagnosed with GBM during tumor resection. The baseline miR-124 expression in GBM patient’s T cells (n = 4) and normal donors (n = 4) is undetectable when determined by RT-PCR (data not shown). The T cells were stimulated and simultaneously transfected with the scrambled control oligonucleotides or with miR-124. Levels of IL-2, TNF-\(\alpha\), and IFN-\(\gamma\) were significantly increased in miR-124–transfected CD4\(^+\) T cells and CD8\(^+\) T cells (Fig. 3). In parallel, we also observed that miR-124 overexpression in healthy donor peripheral blood T cells enhances production of effector cytokines, such as IFN-\(\gamma\), TNF-\(\alpha\), and IL-12, from CD4\(^+\) and CD8\(^+\) T cells (data not shown).

miR-124 inhibits in vivo glioma growth

Given miR-124’s role in modulating the STAT3 pathway and immune responses, we next determined whether miR-124 could exert a therapeutic effect in vivo. To assess the in vivo antitumor efficacy of miR-124, we implanted GL261 murine glioma cells into immune-competent C57BL/6 mice...
and treated them with miR-124 or scramble control (n = 10/group). After the subcutaneous GL261 tumors had grown to a palpable size, miR-124 duplex or scramble control was administered. Subcutaneous tumor growth progressed in all the C57BL/6j mice treated with the scramble control. In contrast, in the miR-124–treated group, the tumor volume was markedly suppressed (P = 0.01; Fig. 4A). Gliomas started to shrink as soon as miR-124 was administered; moreover, the tumors continued to regress even after miR-124 treatment was discontinued. In contrast, tumors kept growing aggressively in scramble miRNA-treated and untreated tumor-bearing mice groups. An immunohistochemical analysis revealed that p-STAT3 glioma expression levels were markedly inhibited in the miR-124–treated cohort (P = 0.0039; Fig. 4B).

To determine whether enhanced immunologic tumor cytotoxicity was correlated with miR-124’s efficacy in vivo, we evaluated the immune cytotoxic responses directed toward GL261 glioma cells. Splenocytes from tumor-bearing mice treated with miR-124 duplex or scramble miRNA were isolated and cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled GL261 target cells for 48 hours. The immune cells from the tumor-bearing mice treated with miR-124 increased the cytotoxic clearance of the GL261 target cells relative to that in scramble-treated mice (P < 0.05; Fig. 4C and Supplementary Fig. S4). We next analyzed ex vivo GL261 tumor tissues from miR-124– or scramble miRNA-treated tumor-bearing mice and found that the percentage of FoxP3+ Tregs in the tumor microenvironment was reduced to 19.0% (Fig. 4D). We observed no significant decrease in the number of FoxP3+ Tregs in the spleen or lymph nodes of miR-124–treated tumor-bearing mice relative to control-treated mice (data not shown), indicating that miR-124’s Treg modulatory effects were confined to the tumor. To determine whether miR-124 mediates an enhanced immune activation of effector T cells in the tumor microenvironment, we determined the production of effector cytokines such as IFN-γ and TNF-α in tumor-infiltrating T cells. Consistent with the enhanced antitumor activity in the miR-124–treated group, a marked increase in effector cells (i.e., producing IFN-γ or TNF-α) was found in the glioma microenvironment, including CD4+ T cells (Fig. 4E; IFN-γ: from 7.7% ± 2.0% to 21.6% ± 3.3%, P = 0.0032; TNF-α: from 6.4% ± 1.7% to 29.1% ± 7.4%, P = 0.0066) and CD8+ T cells (Fig. 4F; IFN-γ: from 10.9% ± 3.3% to 26.0% ± 4.0%, P = 0.007; TNF-α: from 6.4% ± 1.7% to 16.4% ± 1.7%, P = 0.0019).

The therapeutic effect of miR-124 is immune mediated

Although we found that miR-124 had a therapeutic effect when injected directly into the tumor, this is unlikely to be a viable therapeutic approach for patients. Therefore, we tested intravenous miR-124 administration in established murine glioma models. Confirming the results of the direct delivery approach, intravenous administration of miR-124 led to marked inhibition of glioma growth in vivo (Fig. 5A). To determine whether this therapeutic effect was secondarily mediated by the immune system, we implanted GL261 murine glioma cells in immune-incompetent (nude) mice and treated them with miR-124 or scramble control. Intratumoral treatment was initiated when the tumors grew to a palpable size. In the immune-incompetent animal background, miR-124 failed to exert a therapeutic effect, indicating that miR-124 mediates
in vivo activity via the immune system (Fig. 5B). To determine whether treatment with miR-124 was effective against established intracerebral tumors, we administered miR-124 to C57BL/6J mice with intracerebral tumors from GL261 cells, starting after tumor cell implantation. The median survival duration for the scramble control group was 32 days. For mice treated with miR-124, the median survival duration was 32 days (P = 0.02; Fig. 5C). When the experiment was repeated
in an immune-incompetent model system, therapeutic efficacy was once again lost (Fig. 5D).

**The immunotherapeutic efficacy of miR-124 depends on T cells**

To further investigate which T-cell compartment mediates miR-124’s in vivo antitumor activity, we depleted CD4⁺ or CD8⁺ T cells in GL261 tumor-bearing mice with neutralizing antibodies while treating those mice with miR-124 or scramble RNA oligonucleotides. We found that depletion of both CD4⁺ T cells and CD8⁺ T cells completely abrogated the anti-glioma efficacy of miR-124 (Fig. 6A), indicating that CD4⁺ and CD8⁺ T cells are critical immune cell components mediating miR-124 therapeutic efficacy in vivo. To determine whether CD3⁺ T cells are directly targeted during intravenous administration of miR-124, we isolated CD3⁺ T cells from the peripheral blood, spleens, and GL261 tumors and measured the expression of miR-124 by quantitative RT-PCR. There is minimal baseline expression of miR-124 in the T cells from nontumor-bearing mice (Supplementary Fig. S5). After in vivo miR-124 treatment, there is an increase in the miR-124 expression levels in both the peripheral blood T cells and within the glioma-infiltrating T cells. This coincided with decreased intracellular pSTAT3 expression (Supplementary Fig. S5).

Next, we isolated CD3⁺ T cells, transfected them with miR-124 or scramble control, and expanded their numbers in vitro...
for 48 hours before adoptively transferring these cells into GL261 tumor-bearing mice. This miR-124 transfection inhibited pSTAT3 activity in the adoptively transferred T cells (Fig. 6B). Consistent with miR-124–enhanced T-cell effector function (as shown in Fig. 3) and the miR-124 therapeutic effects relying on T cells (as shown in Fig. 6A), we found that GL261 gliomas regressed upon adoptive transfer of miR-124–transfected T cells but did not with control scramble-transfected T cells (Fig. 6C), further showing the pivotal role of the immune system in miR-124–mediated antitumor effects. To investigate the in vivo cellular mechanisms of adoptively miR-124–transfected T-cell treatment, we determined the percentage of infiltrating CD4+ T cells, CD8+ T cells, and FoxP3+ Tregs in the GL261 tumors 6 days after treatment with the miR-transfected CD3+ T cells. Within the glioma microenvironment, there was an increase in the CD4+ T-cell infiltration from 2.6% ± 0.9% in the scramble-control-transfected CD3+ T-cell-treated group to 7.4% ± 1.9% in the miR-124–transfected CD3+ T-cell-treated group (P = 0.04; n = 3/group), a decrease in FoxP3+ Tregs from 26.9% ± 5.9% to 7.0% ± 0.3% in the respective groups (P = 0.014), but no change in the absolute numbers of CD8+ T-cell infiltration. Similar to the findings in Fig. 4E and F, there was a marked increase in immune effector cells within the glioma microenvironment after treatment with the miR-124–transfected T cells; specifically, in the CD4+ T-cell compartment (IFN-γ; from 3.7% ± 2.2% in the scramble control–transfected CD3+ T cells to 22.5% ± 6.2% in the miR-124–transfected CD3+ T cells, P = 0.023; TNF-α: 4.1% ± 1.9% to 17.2% ± 2.6%, P = 0.0076). Although there was no increase in the absolute number of CD8+ T cells, the effector status of the CD8+ T cells within the glioma microenvironment was enhanced (IFN-γ: from 1.4% ± 0.7% to 7.3% ± 1.8%, P = 0.0018; TNF-α: 5.2% ± 0.8% to 15% ± 4.4%, P = 0.043).
miR-124 modulates T-helper cell differentiation

To further investigate whether T-helper cell (TH)1 and TH17 differentiation are responsive to modulation with miR-124, we activated CD4⁺CD45RA⁺CD45RO-naive T cells with plate-bound anti-CD3 and soluble anti-CD28 under TH1, TH17, and inducible Treg polarization conditions before miR-124 transfection. IL-17A⁺ TH17 cells and FoxP3⁺ Treg induction was inhibited when miR-124 was overexpressed, whereas miR-124 promoted differentiation of IFN-γ⁺ TH1 cells (Supplementary Fig. S6).

miR-124 exerts a therapeutic effect in STAT3-expressing genetically engineered murine models

The limitation of evaluating therapeutic strategies in clonotypic models has been previously noted (31); we created a genetically engineered murine model that expresses STAT3 (11). We injected newborn Ntv-a mice with RCAS-STAT3 and RCAS-PDGFB vectors to reproducibly and consistently obtain high-grade gliomas, with the defining histologic features of microvascular proliferation, necrosis, and invasion (Fig. 7A) and lacking miR-124 expression (Fig. 7B). Similar to the

Figure 7. miR-124 exerts a therapeutic effect in Ntv-a mice. A, representative hematoxylin and eosin staining of a high-grade glioma induced in Ntv-a mice transfected with RCAS-PDGFB and RCAS-STAT3 transgenes shows neovascular proliferation (arrow) and pseudopalisading necrosis (arrowhead) at ×100 magnification. B, representative specimen from the brain of an Ntv-a mouse transfected with the RCAS-PDGFB and RCAS-STAT3 transgenes shows miR-124 expression by in situ hybridization in neurons surrounding a glioma devoid of miR-124 expression (arrow) at ×400 magnification. C, treatment schema and graph of the Kaplan–Meier estimate shows improved survival in miR-124–treated Ntv-a mice transfected with the RCAS-PDGFB and RCAS-STAT3 transgenes (n = 9/group) compared with scramble control and untreated mice (Lipofectamine 2000 vehicle only). *, P = 0.04. D, summary graph shows the incidence of high- and low-grade gliomas on the basis of hematoxylin and eosin staining features of necrosis and neovascular proliferation in miR-124–treated Ntv-a mice transfected with RCAS-PDGFB and RCAS-STAT3 transgenes (n = 7) compared with scrambled controls (n = 8) and untreated mice (n = 7; P < 0.0001). E, an ex vivo immunohistochemical analysis of gliomas, untreated (n = 7) or treated with a scramble control (n = 6) or miR-124 (n = 7), shows a marked decrease in pSTAT3 expression in the local tumor microenvironment of the miR-124–treated group (scramble vs. miR-124: P = 0.003; untreated vs miR-124: P = 0.007; untreated vs. scramble: P = 0.87). Quantification of pSTAT3 expression was obtained by averaging the number of nuclear positive pSTAT3 cells by immunohistochemistry from 10 nonoverlapping high-power microscopic fields (magnification, ×400) of the gliomas obtained from either untreated RCAS-PDGFB + RCAS-STAT3 mice or mice treated with the scramble control or miR-124. Each dot represents the analysis of one mouse glioma.
findings in patients with glioma, miR-124 expression in these induced gliomas was also markedly diminished. To determine whether treatment with miR-124 was also efficacious in this model system, we treated Ntv-a mice with miR-124, starting on day 21 after tumor induction. No behavioral or neurologic abnormalities of the mice were noted during treatment. The median survival duration in the control group was 26 days. In mice treated with miR-124, the median survival duration was 39 days ($P = 0.04$; Fig. 7C). Necropsies of glioma-bearing Ntv-a mice revealed that the miR-124–treated cohort had a lower incidence of high-grade gliomas, as determined by the study neuropathologist, on the basis of the characteristic features of necrosis and neovascular proliferation (Fig. 7D). Furthermore, there was no evidence of demyelination, macrophage infiltration, or lymphocytic infiltration in the nontumor bearing areas of the CNS that would indicate the induction of autoimmunity (data not shown). Systemic administration of miR-124 resulted in lower pSTAT3 expression in the gliomas than in scrambled miRNA and untreated controls (Fig. 7E).

**Discussion**

To our knowledge, this is the first study to show that miRNA approaches can be exploited for immunotherapeutic purposes against malignancies. A significant confounding factor in the translational implementation of miR-based approaches has been adequate delivery to the target tumor cells. To circumvent this, we selected a miR that could reverse tumor-mediated immunosuppression—specifically, a key molecular hub, STAT3—resulting in immunomodulation and clearance of the malignancy. We have also provided a strategy for identifying potential miR immunotherapeutics that may be applicable to other types of malignancies by sequentially: (i) screening for downregulated miRNAs using tumor microarrays; (ii) determining the scope of potential use in humans by in situ hybridization of TMAs; (iii) screening and selecting the miR candidates that target immunosuppressive pathways and/or mechanisms; and (iv) evaluating mechanism and therapeutic effect within immune-competent model systems. Although we used the STAT3 target as proof-of-principal, other immunosuppressive targets such as CTLA-4, PD-1, and TGF-β could be used. Several other candidate miRNAs identified in the human glioblastoma miR microarray expression library likely target several of these as well and are also being evaluated for their potential as therapeutic agents in a complementary or alternative fashion with miR-124.

Our findings support the immune modulatory effects of miR-124. First, in vivo therapeutic efficacy was ablated in immune-competent murine model systems. Second, miR-124 transfection reduced the immunosuppressive properties in gCSCs, including inhibiting secretion of immunosuppressive cytokines such as galectin-3 [which is downstream from the STAT3 pathway (32) and induces T-cell apoptosis, promotes tumor growth, and induces Tregs], MIC-1 (which inhibits macrophage production of antitumor TNF-α), and IL-8 (which induces immune chemotaxis and is a potent promoter of angiogenesis). Furthermore, inhibition of T-cell proliferative responses and effector functions by the gCSCs was reversed upon transfection with miR-124. The restoration of T-cell TNF-α effector functions with miR-124 is consistent with a previous report that STAT3 negatively regulates TNF-α (33). Third, miR-124 treatment of T cells from immunosuppressed patients with GBM induced potent effector responses, including IL-2 and IFN-γ production. Fourth, the immune responses in the glioma microenvironment in miR-124–treated murine models showed an enhancement of proinflammatory effector CD4 and CD8 T cells, with diminished Treg intratumoral trafficking. Finally, ex vivo glioma cytotoxicity assays from miR-124–treated mice showed enhanced glioma killing. Cumulatively, these data are consistent with those of previous studies that showed that modulating the STAT3 pathway in the immune cell population is sufficient to mediate efficacious antitumor immune responses (34).

STAT3 signaling has been shown to be a key regulator of microglia/macrophage–mediated immunosuppression (14). miR-124 is low or undetectable in these cells; thus miR-124 administration may abolish or reverse their immunosuppression by downregulating STAT3 activity. Although this study focused on adaptive antitumor immune responses, we cannot exclude that part of the therapeutic effect was mediated via innate immunity. Other investigators have shown that the peripheral administration of miR-124 in an experimental murine autoimmune encephalomyelitis model caused deactivation of macrophages, reduced activation of myelin-specific T cells, and markedly suppressed the disease (35). This discrepancy can be explained by the contextual target – that is, miR-124 targets overactive C/EBP-α–PU.1 signaling in the context of induced autoimmune versus STAT3 signaling in the glioma microenvironment with the resulting contrasting immune functional differences. Alternatively, one could hypothesize that the GBM is negatively regulating the expression of miR-124 in the surrounding microglia/macrophage population.

When data from The Cancer Genome Atlas were used to compare miR-124 expression and survival in patients with GBM, no differences in patient outcome were identified; however, the miR-124 expression levels were negligible in these patients, and the marginal differences are probably attributable to the submitted specimens containing intervening miR-124–expressing infiltrating neurons. Given miR-124’s role in neuronal development, we were not surprised to find that it was expressed in the normal CNS as assessed by in situ hybridization. miR-124 expression was lost across all grades and types of gliomas, suggesting not only that this loss is an early event in glioma initiation and development but also that miR-124 therapeutic approaches will be useful in a variety of gliomas.

On the basis of multiple predictive binding algorithms, luciferase expression assays, and mutational analyses, miR-124 seems to downregulate the expression of STAT3, including the activated form, pSTAT3. This finding was further supported by the results of in vitro studies that showed pSTAT3 inhibition in human gCSCs and immune cells and in vivo in the local glioma microenvironment. These data are also consistent with a recent publication showing that miR-124 binds to the STAT3 3'–UTR in the rat cardiomyocyte (36). However, miR-124
also targets other components of the STAT3 signaling pathway such as Shc1. Although IL-6Rα has been proved to be a target of miR-124 in hepatocarcinoma cell lines (37), this was not the case in any of the gCSCs, indicating that miR-124 has differential targets in various cells or tissues. Shc1 is not present in normal brain but is expressed in all grades of gliomas (38). In patients with glioma, the loss of miR-124 may result in the expression of Shc1, which assembles the EGFR/MAPK1/3 signaling complex, thereby enhancing the activation of this signaling pathway. Because Shc1 is upstream of MAPK1/3 in the EGFR/MAPK1/3 signaling pathway, the reduced p-MAPK1/3 level might be due to the downregulation of Shc1 by miR-124. However, downmodulation of Shc1 in most of the gCSCs did not correlate with downmodulation of p-MAPK1/3. In the one gCSC that showed reduced p-MAPK1/3 expression, the IL-6Rα expression was absent, indicating a potential greater reliance on the EGFR/MAPK1/3 signaling pathway and illustrates that although miR-124 inhibits p-STAT3, inhibition of other components of the signaling axis are contextual and hierarchical.

Other than by immune regulation, miR-124 may also reduce gliomagenesis via multiple mechanisms, including inducing gCSC differentiation, targeting multiple oncogenic signaling pathways (such as NFATc and PIK3CA), and repressing tumor cell proliferation (39), if sufficient levels of miR-124 are able to enter the CNS. Our data in the miR-124-treated Ntv-a glioma model showed a decreased incidence of high-grade glioma, probably secondary to the diminished pSTAT3 expression in the local tumor microenvironment. This finding confirms those of previous studies that have linked miR-124 to gliomagenesis.

An advantage of intravenous administration of miR-124 is the ease of translational implementation as opposed to siRNA approaches that have required ex vivo transduction of the cancer cells (40), direct tumor delivery (41), knockout in the hematopoietic cell population (34), or conjugation to CpG to target the immune population (42). Moreover, it is possible that the physiologic expression of miR-124 in normal brain tissues confers tolerance to exogenous administration of this miR, thus minimizing toxicity. Indeed, we did not observe any evidence of CNS toxicity or induced autoimmunity in treated mice. Alternatively, because miR targets "networks" as opposed to a singular target, as is the case with siRNA, other unidentified therapeutic targets may be contributing to the beneficial in vivo effects observed with the miR-124. Specifically, miR-124 has been previously shown to target a variety of mRNAs (43) and we found it can also target miR-21, which is regulated by STAT3 (19). miR-21 has been shown to be significant in GBMs and can regulate multiple genes associated with preventing glioma cell apoptosis (44) and enhancing migration and invasion (15). miR-21 inhibition can inhibit the growth of GBM cells in vitro (18) and in vivo (16, 45). Thus, a component of the observed in vivo therapeutic effect could be secondary to the modulation of miR-21 by miR-124.

In summary, these findings provide proof-of-concept support for the systemic delivery of immune modulatory miRs as a powerful and specific anticancer therapeutic modality. In the future, immune modulatory miRs could be used in combination and delivered in the context of nanoparticles, liposomes, or exosomes or used to modify cellular vaccine strategies. Because the STAT3 pathway has been shown to mediate resistance to chemotherapeutics by modulating miR-17 (46), miR-124 may also have a therapeutic role in the setting of treatment failure. Screening miR expression in tumors could ultimately lead to a personalized medicine approach. Ultimately, this novel immunotherapeutic approach has the potential to not only overcome immune quiescence and resistance but also to overcome the vexing issue of miR delivery by exploiting the immune system as an antitumor "Trojan horse."

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Drs. Patrick Hwu and Elizabeth Grimm for insightful commentary, Dr. Jun Yao for helping to generate heatmaps, and Audria Patrick, Ann Sutton, and Dr. David M. Wildrick for editorial assistance.

Grant Support
These studies were supported by the Anthony Bullock III Foundation (A.B. Heimberger), Cynthia and George Mitchell Foundation (A.B. Heimberger), the Dr. Silverman Foundation (A.B. Heimberger), the Vauhkh Foundation (A.B. Heimberger), and the NIH CA120813-01, P50 CA127001, P50 CA094549 (A.B. Heimberger), MD Anderson Cancer Center (MDACC) Brain Specialized Program of Research Excellence (SPORE) Career Developmental Grant (J. Wei) and K08 NS070928 (G. Rao).

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Received November 27, 2012; revised April 3, 2013; accepted April 3, 2013; published OnlineFirst May 1, 2013.

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*Cancer Res* 2013;73:3913-3926. Published OnlineFirst May 1, 2013.

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