A Novel Treatment for Glioblastoma: Mesenchymal Stem Cells as Natural Bio-Factories for Exosomes Carrying miR-124a

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ABSTRACT

Background: There is currently no effective treatment for glioblastoma, the most common and most deadly primary adult brain tumor. MicroRNAs (miRs), important post-transcriptional regulators, represent a new class of anti-glioma agents. However, major unanswered problems in glioma therapy are which miRs will be most effective against tumor-homing glioma sphere-forming cells (GSCs) and how these miRs will be delivered. Here, we build upon the recent observation that tumor-homing, bone marrow mesenchymal stem cells (MSCs) secrete exosomes, nano-sized vesicles that transport various cargoes, including miRs. We hypothesized that specific miRs can effectively treat GSCs and that these miRs can be delivered to glioblastomas using MSCs themselves or exosomes derived from ex vivo-cultured MSCs.
Methods: We first screened a panel of eight miRs for their anti-glioma effects against five GSCs. In vitro Transwell and exosome feeding experiments were used to assay the efficacy of each delivery strategy. Mechanistic studies were performed to understand how the miR-of-choice inhibited GSCs.
Results: Of the screened miRs, we identified miR-124a as an effective anti-glioma miR-of-choice. We then showed that MSCs engineered to overexpress miR-124a inhibit GSC growth. More importantly, we showed for the first time that MSCs can be engineered to package miR-124a into exosomes and that these exosomes can be isolated and used to inhibit GSC growth. We found this inhibition to be due, at least partly, to miR-124a-induced silencing of FOXA2, an important lipid metabolism regulator.
Conclusion: We demonstrate proof-of-principle for a novel glioma therapy – that MSCs can be used as “biofactories” for miR-124a-carrying exosomes.

INTRODUCTION

There is currently no effective treatment for glioblastoma (GBM), the most common primary brain tumor in adults. With a median survival of only 14.6 months after surgery, and concurrent radiation/chemotherapy, GBM remains the most deadly primary adult brain tumor (1). Recent well-conducted clinical trials have shown that altering the dose or schedule of standard cytotoxic chemotherapy, or inhibiting angiogenesis has little impact on patient survival (2, 3). This therapeutic failure is not only due to the complex molecular and cellular biology of GBMs, including the presence of Glioma Sphere-forming Cells (GSCs), which render GBMs resistant to most therapies, but also due to the inability to deliver therapeutic agents to the tumor because of the blood brain/tumor barrier (BBB/BTB) (4, 5). There is an urgent need to develop new therapeutic strategies that incorporate novel agents that kill GSCs with novel methods to deliver these agents.

Recent studies have suggested that microRNAs (miRs), which are small, non-coding RNAs that modulate post-transcriptional gene expression (6, 7), are dysregulated in cancer, and several tumor-suppressor miRs have been shown to be downregulated in GBMs (8-10). Because of their intrinsic ability to modulate cancer-promoting genes, miRs may represent a new class of anti-cancer biological agents. Specifically, restoration of down-regulated tumor suppressor miRs has been shown to inhibit GBM growth (11). Despite the therapeutic potential of miRs, it is not known which miRs will be most effective against GBMs, and determining these miRs is of critical importance.

In addition, it remains unclear how miRs will be packaged and delivered to tumors (12). There are many obstacles for the successful delivery of miRs to GBMs, including low cellular uptake of the RNA, endosomal escape, immunogenicity, and degradation in the bloodstream

(13). Moreover, the administered miRNA needs to be successfully routed to the target organ, enter the cell and reach its intracellular target in an active form (13). In order to exploit the promise of miRNA-mediated glioma therapy, there is an urgent need for efficient methods to deliver miRNAs to human gliomas. Ultimately, identifying effective anti-glioma miRs and addressing how these miRs will be delivered to GBMs could dramatically alter GBM therapy.

We and others have shown that bone marrow-derived human mesenchymal stem cells (MSCs) can be used as cellular vehicles to deliver biological therapies to gliomas (14-20). MSCs are naturally occurring stem cells that can be easily acquired from the bone marrow of patients with GBM, and are readily expanded and engineered ex vivo (21). Their safe clinical use has been demonstrated in clinical trials (22, 23). MSCs have been shown to migrate toward gliomas after intracranial injection and to home to gliomas after systemic administration (14, 19). This tropism has been demonstrated in a variety of glioma models, including genetically engineered mouse models (15) and xenograft models utilizing GSCs (17), which are currently thought to be the gold-standard models of human gliomas. Importantly, MSCs have been shown to be capable of delivering therapeutic proteins to gliomas (14, 17, 18, 20).

Recent evidence has also shown that MSCs are capable of secreting exosomes, which are physiological nanospheres (30-100 nm diameter) composed of bi-layer membranes derived from the originating cell (24, 25). In addition to using MSCs as delivery vehicles, MSC-derived exosomes could be an effective delivery mechanism to GBMs. Exosomes provide a form of communication with neighboring cells (paracrine) and with distant cells (endocrine) because they are stable in blood. Breakfield and co-workers were among the first to show that GBMs efficiently take up exosomes (26). Importantly, Gatti et al. showed that exosomes can be isolated from the supernatant of cultured MSCs and that these exosomes, much like the MSCs from

which they are derived, have a physiological tendency to home to inflammation and wounded tissue (27, 28). Indeed, we have shown that exosomes home to gliomas after systemic administration (data unpublished). Recent studies have also shown that exosomes from MSCs contain miRs, indicating that MSCs naturally package miRs into exosomes (27-31). This finding is consistent with reports showing that the RNA-Induced-Silencing-Complex (RISC), the major component of miR biogenesis, is physically associated with multivesicular endosomes, the primary sites of exosome biogenesis (32, 33).

The above lines of evidence suggest that GBMs can be effectively treated with miRs and that these miRs can be delivered to GBMs using MSCs or alternatively using MSC-derived exosomes. Although MSCs have been proposed as delivery vehicles of miRs, the use of MSC-derived exosomes to deliver miRs remains less explored in brain tumors (34). To demonstrate the feasibility of this approach, we first screened a panel of eight miRs against five GSCs and identified miR-124a as an effective anti-glioma "miR-of-choice" against most GSCs. Mechanistic studies showed that miR-124a acts by downregulating FOXA2, a known mediator of lipid metabolism, providing new evidence for a critical link between miR-124 and tumor metabolism. Then, using miR-124a in a series of proof-of-principle studies, we engineered MSCs to secrete exosomes containing miR-124a and showed that not only can these miR-124-engineered MSCs themselves kill GSCs, but that miR-124-containing exosomes isolated from the supernatant of ex vivo-cultured MSCs can also be used to treat GSCs. These studies suggest that MSCs can be used as natural "biofactories" for the ex vivo production of nano-sized exosomes containing anticancer miRs-of-choice that can be isolated and delivered to patients for the purpose of eradicating malignant gliomas.

MATERIALS AND METHODS

GSC profiling. Five GSCs were used in these studies (Table 1 and Supplementary Fig. 1). All GSCs were isolated from fresh surgical specimens on an IRB-approved protocol according to the methods of Singh et al. and as previously published (35). GSCs were fingerprinted using the Short Tandem Repeat method by the MDACC Characterized-Cell-Line Core Facility. Consistent with the definition of GSCs, all GSCs grow as spheroids in serum free medium in vitro, and could be serially passaged. GSCs were proven to initiate tumors after implantation of low cell numbers (1000-10,000 cells) into the brains of athymic mice *in vivo*, as previously described (35, 36), and re-implantation of cells from harvested tumors resulted in tumor formation. GSCs expressed known stem cells markers on their surface based on flow cytometry (Table 1), and all expressed known stem cell proteins Oct3/4 and SOX2 (Supplementary Fig. 1), consistent with the current definition of cancer stem cells. Expression profiling of GSCs using the Affymetrix platform showed that these GSCs represented TCGA-type GBMs (Table 1), and RNAseq analyses revealed that the 5 lines expressed a variety of molecular alterations commonly found in GBMs (Table 1). The survival of mice after implantation of 10^5 cells allowed for dichotomization into fast (survival <90 days) and slower (survival > 90 days) growing tumors (Table 1).

Cell Culture. GSCs were maintained in α-minimum essential medium (MEM) (Mediatech, Manassas, VA), B-27 (Sigma-Aldrich, St. Louis, Mo), penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD), epidermal growth factor (Sigma-Aldrich, St. Luis, Mo), and fibroblast growth factor (Sigma-Aldrich). MSCs were obtained from Lonza (Allendale, NJ) and maintained in MEM supplemented with 10% FBS (Life Technologies, Inc., Rockville,

MD), and penicillin-streptomycin mixture. MSCs were used at passage 3-4. Both GSCs and MSCs were maintained at 37ºC in a humidified atmosphere containing 5% CO_2/ 95% air.

Construction of Lentivirus. Lentivirus vectors were used to overexpress miRs-of-choice in GSCs and MSCs. Each lentivirus contained the cDNA precursor of the miR-of-choice and a green fluorescent protein (GFP) marker. Lentiviruses were made in human embryonic kidney (HEK) 293FT cells, transfected with packaging vectors and vectors containing the cDNA of each miR precursor (called lenti-miR-of-choice-GFP), or a scrambled miR precursor (lenti-miRControl-GFP) (GeneCopoeia Inc., Rockville, MD), using Lipofectamine 2000 (Invitrogen, Waltham, MA). To construct shRNA lentiviruses against FOXA2, HEK293FT cells were transfected with packaging vectors and GIPZ FOXA2 shRNA1/2/3 vectors (called shRNA1/2/3-FOXA2) (Dharmacon, Cat. #RHS4287-EG3170). To construct lentiviruses to overexpress FOXA2, HEK293FT cells were transfected with packaging vectors and FOXA2 Precision LentiORF vectors (Dharmacon, Clone ID: PLOHS_100010592). Lentiviruses were purified and virus titer was determined by measuring the percentage of GFP positive cells by flow cytometry after transduction of HEK293FT cells.

Cell Transduction with Lentivirus. To transduce cells (GSCs or MSCs) with lentivirus, the appropriate number of cells were plated and virus and polybrene (8 μg/mL) were added to the media at 3 viral particles per cell, i.e. multiplicity of infection 3 (MOI 3). For experiments involving direct cell count assays, 5 x 10^5 GSCs were plated in each well of 6-well plates. For experiments involving WST1 assays, 10^5 GSCs were plated in each well of 12-well plates. One day after plating, GSCs (which grow as floating spheroids) were centrifuged, the supernatant was removed to discard toxic free-floating viral particles, and the infected cells were reconstituted in

media. For MSCs, the supernatant was removed one day after viral transduction, cells were washed, and media was added to the culture.

WST1 assay, direct cell count, and neurosphere assay. Proliferation assays were performed at least one week after lentivirus transduction, so that there was sufficient time for the miR to be overexpressed. WST1 assays were performed per manufacturer's recommendations. GSCs to be tested were harvested after accutase, centrifuged, and reconstituted in 100 µl of media. Cells were then transferred to 96-well plates and 10 µl of the WST1 solution was added. After one to three hours, the plate was read using a spectrophotometer.

For direct cell count assays, GSCs were harvested after accutase treatment and isolated in 5 ml of media after centrifugation. 100 µl of media containing cells was added to 900 µl of PBS; this solution was then placed into the Vi-Cell Cell Viability Analyzer (Beckman Coulter, Inc, Fullerton, CA), and the number of viable cells was measured.

For the neurosphere assay, GSCs were plated and treated with exo-miRControl ($1x10^4$ exosomes/cell), exo-miR124 (10^4 exosomes/cell) or PBS (no exosomes) as a control for three consecutive days. Single-cell suspensions were generated for each GSC and a single GSC was seeded in 100 mL NSC media in each well of 96-well tissue culture plates. Cells were allowed to grow for three weeks, and, after this period, neurospheres were counted under microscope.

Transwell experiment. MSCs were transduced with lentivirus at MOI 3, and 48 hours after transduction, MSCs (10^5 cells/well in 24-well plates or 10^6 cells/10cm dish) were plated in the upper wells of Transwell plates (0.4-µm pores, Corning Inc., NY). GSCs ($2.5x10^4$ cells/well in 24-well plates or $5x10^5$ cells/10cm dish) were plated in the lower wells. After one week, GSC

proliferation was measured with the WST1 assay for cells plated in 24-well plates or direct cell count for cells plated in 10cm dishes.

Exosome isolation and treatment of GSCs. Exosomes were isolated from the supernatant of MSCs using ultracentrifugation as previously described (37). The supernatant, which contained exosomes, of cultured MSCs was centrifuged at 1000x g to remove cell debris and then passed through a 0.2 μm filter to remove large, non-exosome microvesicles. A subsequent ultra-centrifugation at 100,000x g for one hour yielded a raw exosome extract, which was washed with PBS once, followed by another ultracentrifugation at 100,000x g for 1 hour. The resulting precipitate contained pure exosomes, which were suspended in an appropriate volume of PBS.

Isolated vesicles were proven to be exosomes based on electron microscopy and western blotting using antibodies to CD63, CD9, CD81 and cytochrome c. Exosome concentration and size were quantified using Nanosight™ technology. To assess the anti-glioma effects of exosomes carrying miRs, $2.5x10^4$ GSCs were cultured in 24-well plates. Exosomes ($1x10^4$ particles/cell) were added to each well daily for three days. One week after plating, GSC proliferation was measured by WST1 assay.

Quantitative Real-Time PCR (qRT-PCR). qRT-PCR was used to analyze the expression level of miR-124a in GSCs and MSC-derived exosomes using miR-16 as the internal control. MSC-derived exosomes or GSCs were isolated and exposed to RNase for 15 minutes to eliminate free-floating extra-vesicular RNA elements. Remaining RNase was removed by PBS wash and subsequent centrifugation. Total RNA was then extracted using the mirVana miRNA isolation kit (Ambion, Green Island, NY). The assay was based on stem-loop reverse transcription (RT)-based TaqMan® MicroRNA quantification (Life Technologies Corp,

Carlsbad, CA). Specifically, reverse transcriptase reactions were performed using 10 ng total RNA from exosomes, 50 nm stem–loop RT primer, 1× RT buffer, 0.25 mm each of dNTPs, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl RNase inhibitor. The 7.5 μl reactions were incubated in an Applied Biosystems 9700 Thermocycler in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then maintained at 4°C.

Western Blotting. Western blotting was performed according to standard protocol. The primary antibodies were: anti-FOXA2 (rabbit, 1: 500, Cell Signaling Technology, Inc., MA), and anti-GAPDH (1:1000, Sigma, MO). After treatment with appropriate condition, GSCs were lysed and proteins were collected. Proteins were separated by SDS-PAGE (Novex® Tris-Glycine 4-20% gradient gel, Invitrogen, Waltham, MA), transferred to Hypond-P PVDF membranes (Amersham Bioscience, Piscataway, NJ), and incubated with appropriate primary antibodies overnight at 4°C. Proteins were then incubated with secondary antibody (1:10000 dilution) (GE Healthcare UK Ltd., England) at room temperature for 1 hour. Bound antibody complexes were detected by SuperSignal® West Femto Maximum Sensitivity Substance (Thermo Scientific, Rockford, IL).

shRNA-induced FOXA2 Knockdown. $2.5x10^5$ GSCs were plated into each well of a six-well plate. Anti-FOXA2 shRNA constructs (See *Construction of Lentivirus*) were added (MOI of 3). After three days, cells were examined microscopically for GFP expression and also analyzed for FOXA2 expression via western blotting. Cell viability was analyzed by WST1 assay.

FOXA2 Rescue. $2.5x10^5$ GSCs were plated into each well of a six-well plate. Lentiviruses overexpressing FOXA2 were constructed using FOXA2 Precision LentiORF vectors (See *Construction of Lentivirus*) (Dharmacon, Clone ID: PLOHS_100010592) and added (MOI of 3) to the cells. This FOXA2 construct was insensitive to miR-124a by manipulating the

FOXA2 protein coding sequence of the vector. On day three, cells were examined for GFP expression, and on day 4, Blasticidin S was added (5 μg/mL). Cells were maintained under selection for six days, replacing medium or passaging every 2-3 days as needed. Cell viability was analyzed and western blotting for FOXA2 was performed.

Lipid Staining. $2.5x10^5$ GSCs were transduced with appropriate lentivirus. After three days, GSCs were fixed in 10% formalin for 5 min at room temperature. Cells were washed once with 60% isopropanol. *Oil Red O* solution was added and incubated for 10 min. After three washes with dH2O, cells were examined with phase contrast microscope.

Animal studies. Male athymic mice (nu/nu) (Department of Experimental Radiation Oncology, MDACC, TX) were anesthetized by intra-peritoneal injections of ketamine (100 mg/kg)/xylazine (10 mg/kg). To test the ability of exosomes carrying miRs-of-choice to inhibit tumorigenesis *in vivo*, GSCs treated with miR-124-carrying exosomes (10^4 exosomes/cell), scrambled miR-carrying exosomes (10^4 exosomes/cell) or PBS for three days and injected into the caudate nucleus at 10^5 cells/mice in 5 μl volume using a guide screw and a multiport microinfusion syringe pump (Harvard Apparatus, MA) as previously described (36). All manipulations were performed under an approved protocol.

To test the efficacy of systemic administration of miR-containing exosomes, GSCs were implanted into mice in the same manner as described above (10^5 cells/animal, N=8 animals/group). After 7 days, mice were treated with miR-124-carrying exosomes, scrambled miR-carrying exosomes, or PBS three times per week (Monday, Wednesday, Friday), delivered intraperitoneally at a dose of 10^8 exosomes/100 μl. On day 7 and day 21, animals also received an intracarotid injection of exo-miR124, exo-miRControl, or exo-empty (dose: 10^8

exosomes/100 μl) or PBS (100 μl). All animals were followed until moribund at which time they were sacrificed and the brains removed.

Statistical Analysis. Statistical differences were assessed by the two-tailed, paired Student's t-test with significance if <0.05. The data are represented as the mean ± standard deviation.

RESULTS

miR-124a is a miR-of-choice for inhibiting GSC proliferation. In order to determine the extent to which specific miRs are capable of directly inhibiting the growth of GSCs, we generated lentivirus vectors to overexpress miRs-of-choice in transduced cells. These lentiviruses contained the precursor cDNA of each of eight miRs (miR-27a, miR-100, miR-124a, miR-122, miR-133, miR-138, miR-145, and Let-7b) along with the cDNA for green fluorescent protein (GFP) (called lenti-miR-of-choice-GFP, respectively). Prior to this screen, miR-491 was also tested independently and was not identified as an effective miR against GSCs. These miRs were chosen based on a literature review of anti-glioma, tumor suppressor miRs. Lentivirus containing a scrambled miR precursor (lenti-miRControl-GFP) was generated as a control.

To determine the extent to which each of these miRs was capable of altering the proliferation of gliomas, we used a panel of five GSCs, including GSC267, GSC6-27, GSC8-11, GSC2-14, and GSC20, each of which was extensively characterized as described in Methods and as shown in Table 1 and Supplementary Fig. 1. Treating each of the GSCs with the lentiviral constructs and analyzing the percentage of GFP-positive cells demonstrated that for each lentiviral construct 90-95% of cells in each GSC were effectively transduced at an MOI of 3

(Fig. 1A). Because the miR precursor gene was regulated by a stronger promoter (CMV) than the GFP promoter (EF1), GFP production indicated concurrent expression of the miRs.

To screen the effects of the eight miRs on the growth of gliomas, each of the five GSCs was transduced with each of the eight miRs (MOI 3). Lenti-miRControl-GFP (MOI 3) and medium alone (no virus) were used as negative controls. One week after plating, cell proliferation was determined using the WST1 assay. In all experiments, there was no significant difference between the viability of GSCs treated with lenti-miRControl-GFP or with medium alone. Of the eight miRs in these screening experiments, miR-124a resulted in the most significant decrease in GSC viability across all five GSCs ($P<0.010$ *versus* miRControl in all GSCs, Student's t-test) (Fig. 1B and Supplementary Fig. 2), identifying miR-124a as an effective anti-glioma miR.

To verify the effects of miR-124a, GSC viability after treatment was analyzed through direct cell count. GSC267, GSC6-27, GSC8-11 and GSC20 were treated with lenti-miR124-GFP, lenti-miRControl-GFP, or medium alone. Treatment with lenti-miR124-GFP significantly inhibited the viability of each of the GSCs. Specifically, treatment with lenti-miR124-GFP resulted in a 58.9% reduction in viability of GSC267 ($P=0.019$ *versus* miRControl), a 43.8% reduction in GSC20 ($P=0.023$), a 58.5% reduction in GSC8-11 ($P=0.010$), and a 39% reduction in GSC6-27 ($P=0.009$) (Fig. 1C).

To determine whether long term exposure would enhance the effects of miR-124a, GSC6-27, a GSC line that grew more slowly in culture than the other GSCs, was treated with lenti-miR124-GFP and allowed to grow in culture for two weeks. A more robust response was seen after this long term exposure, as there was an 83.2% viability reduction when compared with lenti-miRControl-GFP (data not shown).

To directly prove that miR-124a was overexpressed in GSCs transduced with lenti-miR124-GFP, GSC267 was treated with lenti-miR124-GFP, lenti-miRControl-GFP, or medium alone, and 72 hours later miR-124a levels were analyzed by qRT-PCR. GSCs transduced with lenti-miR124-GFP contained 81 times more miR-124a compared with GSCs treated with medium alone or transduced with lenti-miRControl-GFP (P=0.0001) (Fig. 1D).

Taken together, these results indicated that miR-124a is an effective anti-glioma miR against a variety of GSCs. Therefore, we used miR-124a in subsequent proof-of-principle analyses of MSC and exosome delivery.

MSCs loaded with lenti-miR-124a-GFP inhibits GSC viability. Previous work has shown that MSCs can be used to deliver a variety of biological molecules to gliomas (14, 18, 19, 34). Therefore, we sought to determine whether we could use MSCs engineered to secrete miR-124a in the treatment of gliomas. Specifically, we treated MSCs with lenti-miR124-GFP (MOI 3), generating MSCs that overexpressed miR-124a (abbreviated MSC-miR124), or with lenti-miRControl-GFP, generating MSCs that overexpressed the scrambled miRControl (MSC-miRControl), or with medium alone (no virus) (MSC-empty). After 48 hours, fluorescent microscopy revealed that 90-95% of MSCs-miR124 and MSCs-miRControl expressed GFP, indicating a high rate of MSC transduction (Fig. 2A). MSCs remained viable despite miR-124a overexpression, indicating that miR-124a was not lethal to MSCs.

To determine the extent to which the MSCs carrying miR-124a could inhibit the proliferation of GSCs, and to mimic the clinical scenario where miR-containing MSCs are delivered to gliomas, we performed *in vitro* Transwell culture experiments using MSCs-miR124, MSCs-miRControl, and MSCs-empty. MSCs were plated in the upper wells of Transwell plates (10^5 cells/well). In the lower wells, GSC6-27 or GSC20 ($2.5x10^4$ cells/well) was plated. The

upper and lower wells were separated by a filter with 0.4 micron pores, which only allowed passage of soluble factors. After 7 days of co-culture, the proliferation of the GSCs was analyzed by WST1 assay. Co-culture with MSCs-miRControl had little effect on GSC20 compared with GSC20 co-cultured with MSCs-empty. However, co-culture with MSCs-miR124 resulted in a 45.6% reduction in viability of GSC20 (P=0.004 *versus* MSC-miRControl) (Fig. 2B). Likewise, GSC6-27 co-cultured with MSCs-miR124 resulted in a 52.9% reduction in viability when compared with MSC-miRControl (P=0.004) (Fig. 2B). Therefore, treatment with MSCs that overexpress miR-124a is capable of inhibiting the growth of GSCs.

To verify the result, in an independent Transwell experiment, GSC267 (5.0×10^5 cells per well, lower wells) was co-cultured with MSCs-miR124, MSCs-miRControl, or MSCs-empty (10^6 cells per well, upper wells). GSC proliferation was analyzed by direct cell count. GSCs co-cultured with MSCs-miR124 grew 58.3% less compared with GSCs co-cultured with MSCs-miRControl, MSCs-empty, and no MSCs (P=0.015 *versus* MSC-miRControl) (Fig. 2C). These results confirmed that MSCs engineered to overexpress miR-124a are capable of inhibiting the growth of GSCs.

MSCs can be engineered to package miR-124a into exosomes. Previous studies have shown that miRs can be packaged into exosomes (24, 26), and the Transwell experiments indicated that MSCs are able to transfer miRs to GSCs through a secretory process, given that the MSCs could not pass through the filter. Thus, we hypothesized that MSCs package miR-124a into exosomes, that they secrete these exosomes, and that these exosomes are taken up by the GSCs. To test this hypothesis, we sought to analyze the extent to which overexpression of miR-124a in MSCs resulted in overexpression of miR-124a in exosomes. Specifically, MSCs were transduced with lenti-miR124-GFP, lenti-miRControl-GFP, or medium alone, as described

above, generating MSCs-miR124, MSCs-miRControl, or MSCs-empty. These transduced MSCs were then plated, the supernatant was collected after 48 hrs, and exosomes were isolated from the supernatant by differential centrifugation (called exo-miR124, exo-miRControl, and exo-empty, respectively).

To verify that the isolated particles were indeed exosomes, electron microscopy of the particles revealed spherical structures of 50-100 nm diameter with a bilayer membrane, consistent with the definition of exosomes (Fig. 3A). The vesicles were also lysed, protein collected and analyzed by western blotting for known exosomes markers. Comparisons were made to whole cell protein lysates derived from MSCs. Consistent with the published criteria of exosomes, the isolated particles expressed high levels of CD9, CD63 and CD81, which were not evident in the whole cell lysates (Fig. 3B). In addition, the mitochondrial protein cytochrome c was not identified in the isolated exosomes, whereas it was identified in the whole cell lysates, consistent with the definition of exosomes (Fig. 3B). Lastly, the isolated particles were examined using Nanosight™ technology, which demonstrated a sharp peak of particles at 105nm, indicating the high purity of the isolated particles and a size consistent with previous reports of exosomes using this method (Fig. 3C). These results demonstrate that the particles isolated from the supernatant of the MSCs were in fact exosomes.

To prove that these isolated exosomes contained high levels of miR-124a, the exosomes were lysed, and RNA was collected and analyzed by qRT-PCR using primers for miR-124a and for miR-16, a commonly used internal control for quantifying miRs in exosomes. The level of miR-124a in the collected exosomes was 59-fold greater than the level of miR-124a in exosomes derived from MSCs-miR-Control or MSCs-empty ($P<0.0001$), both of which contained essentially no miR-124a (Fig. 4A). These results indicate that MSCs treated with lenti-miR-124-

GFP package the overexpressed miR-124a into exosomes, which are then secreted into the surrounding medium. Therefore, lentiviral-engineered *ex vivo*-cultured MSCs can be used to produce exosomes containing an anti-glioma miR-of-choice.

MSC-derived exosomes containing miR-124a are efficacious against GSCs in vitro. In order to demonstrate that the miR-124a-engineered, MSC-derived exosomes are therapeutically effective, and to mimic the clinical scenario in which exosomes carrying miR-124a are delivered to gliomas, we first cultured MSCs-miR124, MSCs-miRControl, or MSCs-empty, collected the supernatant, and isolated the exosomes from the culture medium by differential centrifugation (generating exo-miR124, exo-miRControl, and exo-empty). GSC267 or GSC6-27 were cultured ($2.5x10^4$ cells/ well), and after 24 hrs, GSCs were treated with exo-miR124, exo-miRControl, or exo-empty ($1x10^4$ particles/cell) daily for three days. As a further negative control, GSCs were treated with medium alone (no exosomes). One week after plating, the viability of the GSCs was analyzed by WST1 assay. Treatment with exo-miRControl had little effect compared with treatment with exo-empty or with medium alone. However, treatment of GSC267 with exo-miR124 resulted in a 52% reduction in proliferation compared with treatment with exo-miRControl (P=0.007) (Fig. 4B). Similarly, treatment of GSC6-27 with exo-miR124 resulted in a 78% reduction in proliferation compared with treatment with exo-miRControl (P=0.001) (Fig. 4B).

To verify this result and to further prove that exo-miR124 was able to inhibit the clonogenicity of GSCs, a clonogenic stem cell assay was performed. Specifically, all five GSCs (GSC267, GSC2-14, GSC6-27, GSC8-11, and GSC20) were cultured and 24hrs later treated with exo-miRControl, exo-miR124, or medium alone (no exosomes) for three days. Spheroids were then dissociated and single cells were placed into each well of a 96-well plate. After one week,

GSC viability was analyzed by counting the number of wells containing a viable spheroid. For each of the five GSCs, treatment with exo-miR124 resulted in a statistically significant >50% reduction in spheroid formation compared with treatment with exo-miRControl or exo-empty ($P<0.05$ *versus* exo-miRControl in all GSCs) (Fig. 4C).

MSC-derived exosomes containing miR-124a are efficacious against GSCs in vivo. We next sought to determine the ability of exo-miR124 to inhibit tumorigenicity *in vivo*. To this end, GSC267 was treated with exo-miR124, exo-miRControl, or exo-empty, and implanted (10^5 cells/animal, N=10 animals/group) into the brains of nude mice. All animals treated with exo-miRControl or exo-empty died within 45 days with median survival of 41±2 days. However, treatment with exo-miR124 significantly increased animal survival with a median of 104±3 days ($p<0.0001$; log-rank test) (Fig. 4D).

To determine the extent to which systemic administration of exo-miR124 could recapitulate the *ex vivo* treatment and thereby assess the efficiency of using exosomes to deliver therapeutic miRs to gliomas, GSC267 was implanted (10^5 cells/animal, N=8 animals/group) into the brains of nude mice. After 7 days, mice were treated with PBS (no exo), exo-miRControl, or exo-miR124, delivered intraperitoneally 3 times per week. Exosomes were also injected intra-arterially on day 7 and 21. Treatment with exo-miR124 resulted in a significant improvement in survival compared with controls. Whereas all controls were dead by day 62 after tumor implantation (exo-miRControl median survival = 54 days; PBS median survival = 55 days), about 60% of treated mice are currently alive after 110 days ($P<0.0001$, log-rank test) (Fig. 4E).

Taken together, these experiments indicate that MSC-derived exosomes engineered to contain miR-124a have the ability to inhibit the growth of GSCs *in vitro* and *in vivo*, including, most importantly, after systemic injection.

Exo-miR124 effectively inhibits FOXA2 and alter GSC lipid metabolism. In order to further show that the miR-124a delivered into GSCs by MSC-derived exosomes is functional, we analyzed a variety of known targets of miR-124a (Fig. 5A and Supplementary Fig. 3). Specifically, GSC267 or GSC6-27 were plated and after 24 hrs, treated with exo-miR124, exo-miRControl, or medium alone. GSCs were also treated with lenti-miR124-GFP to test the effect of direct overexpression of miR-124a on gene expression. Three days after treatment, protein lysates were collected and analyzed by western blotting for the expression of SOX2, SOX9, STAT3, PTBP1, REST and FOXA2. Although miR-124a did not alter SOX2, SOX9, STAT3, PRBP1, or REST in our GSCs, treatment with lenti-miR124-GFP or exo-miR124 significantly inhibited the expression of FOXA2 (Fig. 5A), which is a known oncogene (38) and a target of miR-124a (39).

We next sought to prove that FOXA2 knockdown results in reduced GSC viability. We constructed three shRNA against FOXA2 (shRNA1/2/3-FOXA2). GSC267 were cultured and after 24hrs, treated with the anti-FOXA2 shRNA constructs. Three days after treatment, FOXA2 expression was determined using western blotting and, one week after treatment, GSC proliferation was analyzed using a WST1 assay. Based on western blotting, treatment with shRNA1-FOXA2 and shRNA3-FOXA2 resulted in a significant decrease in FOXA2 expression, whereas shRNA2-FOXA2 had minimal effect (Fig. 5B). Consistent with this finding, shRNA1-FOXA2 and shRNA3-FOXA2 significantly inhibited the viability of GSC267 ($P<0.01$ *versus* shRNA-Control), whereas shRNA2-FOXA2 had minimal effect compared with shRNA-Control (Fig. 5C). These results indicate a causal and dose dependent effect of inhibiting FOXA2 on GSC viability.

To demonstrate a causal relationship between miR-124a expression, FOXA2 inhibition, and GSC proliferation, we performed a rescue experiment using a lentivirus vector for a miR-124a-insensitive FOXA2 (lenti-FOXA2). This lenti-FOXA2 construct only included the protein-coding sequence of FOXA2. Therefore, by transducing cells with these exogenously expressed constructs, it is possible to express a FOXA2 that is resistant to cleavage by miR-124a, which targets the 3'-UTRs of endogenous FOXA2, and thereby permit phenotypic rescue. After lenti-FOXA2 construction, GSC267 was cultured and after 24 hrs, treated with medium alone (no lentivirus), lenti-FOXA2 alone, lenti-miR124-GFP alone, or both lenti-FOXA2 and lenti-miR124-GFP. As expected, treatment with lenti-miR124-GFP alone resulted in a significant reduction in the expression of FOXA2 (by western blotting) and a concomitant inhibition of GSC viability (by WST1 assay) compared with control (Fig. 5D and 5E). Also as expected, treatment with lenti-FOXA2 alone resulted in a slight increase in FOXA2 expression and a slight, but not statistically significant, increase in GSC viability compared with control (Fig. 5D and 5E). However, co-treatment with miR-124a and lenti-FOXA2 showed that lenti-FOXA2 was capable of restoring the expression of FOXA2 after treatment with miR-124a and also of rescuing the survival of GSCs (Fig. 5D and 5E). This result proves that the mechanism by which miR-124a inhibits GSC viability is, at least partly, through knockdown of FOXA2.

Although it is known that FOXA2 is important for GBM pathogenesis (40), its mechanism of action in GBM is obscure. FOXA2 plays an important role during animal development and in the regulation of cellular metabolism, especially in lipid metabolism (41-44). The recognition that metabolic reprogramming is a key feature of transformed cells has led to significant interest in targeting metabolism as a cancer therapy (45). This rationale lead us to assess how the miR-124a/FOXA2 axis might regulate lipid metabolism in GSCs. We

hypothesized that the miR-124 knockdown of FOXA2 causes cell death by perturbing lipid metabolism in GSCs. To begin to demonstrate a link between treatment with miR-124a and abnormal lipid metabolism, we treated GSC267 and GSC8-11 with lenti-miRControl-GFP, lenti-miR124-GFP, or medium alone (no lentivirus), and analyzed lipid production by Oil Red O staining. GSCs treated with lenti-miRControl-GFP showed no significant difference in accumulation of lipids compared with GSCs treated with medium alone (Fig. 5F). In contrast, GSCs treated with lenti-miR124-GFP demonstrated dramatic increases in lipid accumulation in the GSCs, indicating an association between treatment with miR-124a and perturbation of lipid metabolism, through downregulation of FOXA2 (Fig. 5F).

DISCUSSION

In this study, we showed that overexpression of miR-124a using lentivirus vectors is capable of inhibiting the growth of a panel of diverse GSCs. Thus, we identified miR-124a as an effective anti-glioma agent, which is consistent with previously reported results (34, 46). We then showed that MSCs engineered to overexpress miR-124a using lentivirus technology (MSCs-miR124) can effectively inhibit the growth of GSCs in Transwell experiments, thereby providing proof-of-principle that MSCs can be used to deliver anti-glioma miRs-of-choice to gliomas. Next, we showed, for the first time to our knowledge, that exosomes isolated from the supernatant of *ex vivo*-cultured MSCs-miR124 contain supraphysiological levels of miR-124a and that these exo-miR124 can inhibit the growth of GSCs both *in vitro* and, more importantly, *in vivo*. Lastly, mechanistic studies show that the effects of exo-miR124 are mediated through downregulation of FOXA2 and through the alteration of lipid metabolism in GSCs. These results

suggest that MSCs can be engineered to function as *in vivo* "mini-pumps" or as *ex vivo* "bio-factories" for the production of therapeutic exosomes carrying a miR-of-choice, like miR-124a.

Although overexpression of miR-124a was more effective against GSCs than was overexpression of the other eight miRs (miR-491, miR-145, miR-100, miR-27a, miR-122, miR-133, miR-138, and Let-7), it is noteworthy that there was significant variability in the ability of miR-124a to inhibit the growth of the individual GSC lines, with growth reduction rates varying from 39% to 59% among the GSCs. In addition, other miRs were effective against several of the GSCs, e.g. miR-122 was particularly effective against GSC11, and miR-138 and Let-7 had inhibitory effects on several lines. These observations indicate that further studies identifying miRs that are most effective against particular GSCs would be of value. For example, screening a large library of hundreds of miRs for the ability to kill GSCs would be useful as it would identify those miRs that are particularly effective against specific GSCs. Likewise, the heterogeneous effects on different GSCs emphasizes the need to test a large number of GSCs in order to identify the most effective miRs against particular molecular subtypes of GSCs. Nevertheless, our studies support the notion that specific miRs, particularly miR-124a, are potentially powerful therapies against a variety of GBMs.

Past results have shown that MSCs home to gliomas *in vivo* after systemic injection and can be used to deliver therapeutic agents (14, 15, 18, 19). Based on this concept, *in vitro* Transwell experiments using MSCs and GSCs were designed to mimic the conditions surrounding MSCs after they home to and settle in a GBM. We showed that, within these conditions, MSCs driven to overexpress miR-124a inhibited GSC proliferation. These results suggest that miR-124a, or any anti-glioma miR-of-choice, can be overexpressed in MSCs and that these MSCs can be used as delivery vehicles that act as "intratumoral biofactories,"

producing and transferring miR-124a to the tumor. Therefore, miRs should be added to the list of biological agents that MSCs can deliver to tumors, as previously suggested by Lee et al. (34).

Our results also indicate that MSCs transfer miR-124a to GSCs at least in part by packaging functional miR-124a into exosomes. We showed that lentivirus-mediated overexpression of miR-124a in MSCs results in miR-124a overexpression in exosomes secreted into the supernatant by the MSCs. Importantly, we proved that the particles isolated from the supernatant of MSCs were, in fact, exosomes based on criteria using electron microscopy, western blotting of exosomal (CD9, CD63, CD81) and nonexosomal (cytochrome c) proteins, and Nanosight™ technology. Our results are consistent with Lee et al., who also found that MSCs package miRs in exosomes (34). Interestingly, whereas overexpression of miR-124a inhibited GSC growth, miR-124a overexpression in MSCs had no effect on MSC proliferation, demonstrating that MSCs are able to proliferate despite overexpression of anti-glioma miRs.

Next, in experiments designed to mimic the use of exosomes as the delivery vehicle, we showed for the first time that directly treating GSCs with MSC-derived exosomes overexpressing miR-124a inhibits GSC survival based on a colorimetric proliferation assay, on a stringent limiting-dilution clonogenic stem-cell assay, and on an *in vivo* tumorigenesis study. Most importantly, *in vivo* systemic treatment with exo-miR124 of mice harboring orthotopic patient-derived glioma xenografts significantly extended the survival of the mice compared with controls. Interestingly, the increase in survival after systemic treatment with exo-miR124 was greater than the increase in survival after *ex vivo* treatment of the same GSC cell line and subsequent implantation in the brain. This result suggests that exo-miR124 very efficiently deliver the miR into the tumor. Together these results support the notion that MSCs can be used as *ex vivo* “biofactories” for the packaging and production of exosomes carrying anti-glioma

miRs-of-choice and that these exosomes can then be isolated, purified, and used therapeutically, particularly in systemic delivery strategies. In other words, rather than delivering MSCs, which require intratumoral injection or intracarotid injection, exosomes derived from the MSCs can themselves be used as a intravenously-administered delivery system. Intravenous administration is advantageous because it is easily conducted and can be performed daily, thereby allowing for a much wider range of dose manipulation and dose scheduling.

Lastly, our results suggest a possible new mechanism for the effects of miR-124a on GSCs. Specifically, we show that miR-124a downregulates FOXA2, an oncogenic transcription factor (38), and that FOXA2 downregulation results in reduced GSC viability in our study. It has recently been shown that FOXA2 regulates lipid metabolism (42-44). Recent reports also suggest that a variety of cancers, including GBM, are dependent on capturing and metabolizing exogenous lipids for their growth, demonstrating a metabolic adaptation to facilitate tumor growth and survival (47, 48). Indeed, we show that GSCs overexpressing miR-124a and GSCs treated with exosome containing miR-124a result in intracellular lipid accumulate. This result indicates that miR-124-treated GSCs are unable to metabolize lipids leading to toxic levels compared with untreated GSCs. Thus, our findings support the notion that downregulation of FOXA2 by miR-124a is a mechanism of reduced viability of GSCs, perhaps due to an induced inability to utilize lipids. Further investigation is required to completely elucidate the role of FOXA2 in glioma lipid metabolism and its link to tumor growth.

CONCLUSION

Here we show that miRs-of-choice, particularly miR-124a, can effectively be delivered to GSCs using MSCs engineered to overexpress the miR-of-choice or using exosomes derived from

these MSCs. Given the success of preliminary *in vivo* experiments demonstrating the feasibility of systemic exosomal administration, continued *in vivo* experiments, including dose response, dose scheduling, and repeated experimentation in other GSC lines, are the next logical step to test the effectiveness of this therapeutic approach. The ultimate translation of this approach to patients in clinical trials in patients would be the final goal. Although this investigation analyzed the effects of delivering a single miR-of-choice, future work should test the effect of delivering multiple anti-glioma miRs, perhaps in a "multi-miR cocktail." It is anticipated that such a cocktail would result in greater inhibition in proliferation of GSCs through the simultaneous downregulation of multiple genes that drive GSC growth. Given the ability of MSCs to package miRs in exosomes to inhibit GSC growth, there is great potential for this novel approach to change the outcome of the 13,000 people who currently die of GBM each year.

FIGURES

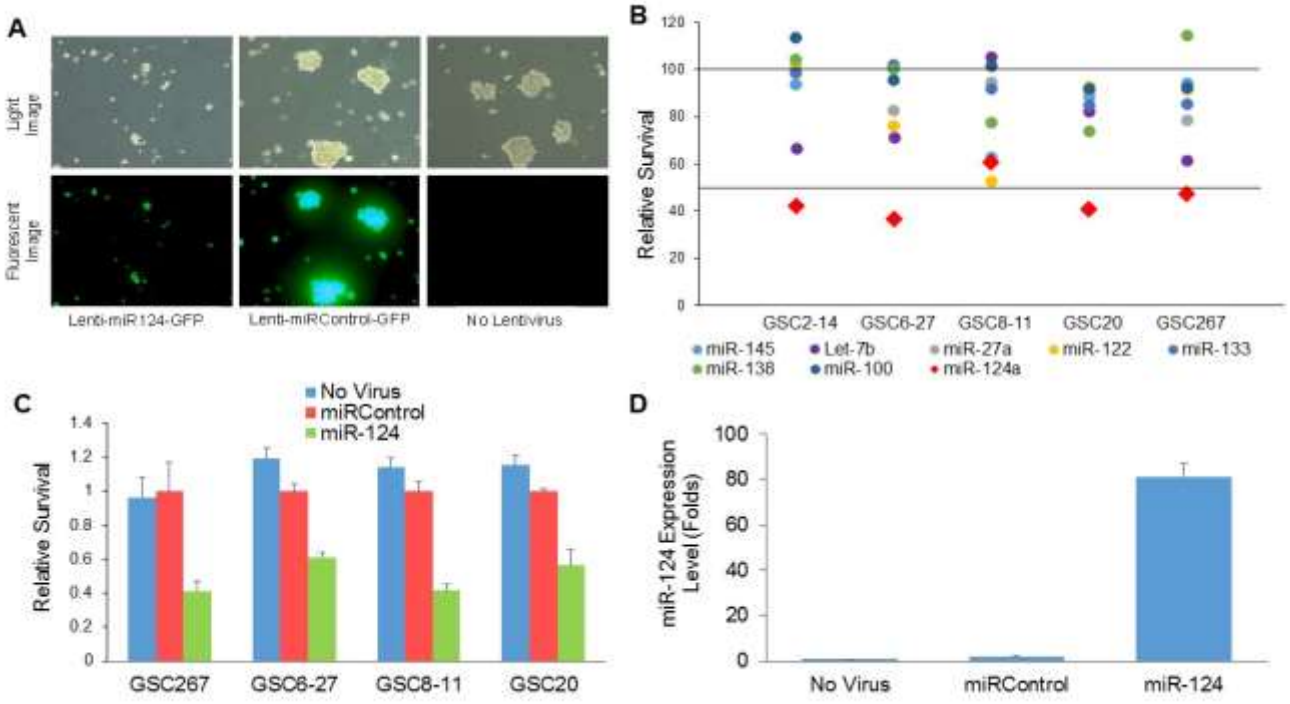


Figure 1. A) Representative photomicrographs of GSC267 spheroids 24 hours after treatment with indicated lentivirus vector. Upper panel shows light microscopy images and lower panel shows view under fluorescent microscope. 90-95% transduction is evident by GFP-labeled cells. Spheres of GSCs transduced with lenti-miR124-GFP are smaller because of anti-proliferative effects of miR-124. Similar results were obtained for all other GSC lentivirus transductions. **B)** Viability of GSCs after treatment with the indicated miRs and assayed for proliferation using WST1. Only miR-124a resulted in a significant decrease in survival in all cell lines. Values are relative to miRControl. **C)** Direct cell count assay of GSCs after treatment with indicated condition. miR-124a treatment significantly reduced GSC viability. **D)** qRT-PCR analysis of miR-124a expression level in GSC267 after treatment with lentivirus vectors. Treatment with miR-124a vector resulted in significant increase in miR-124a expression.

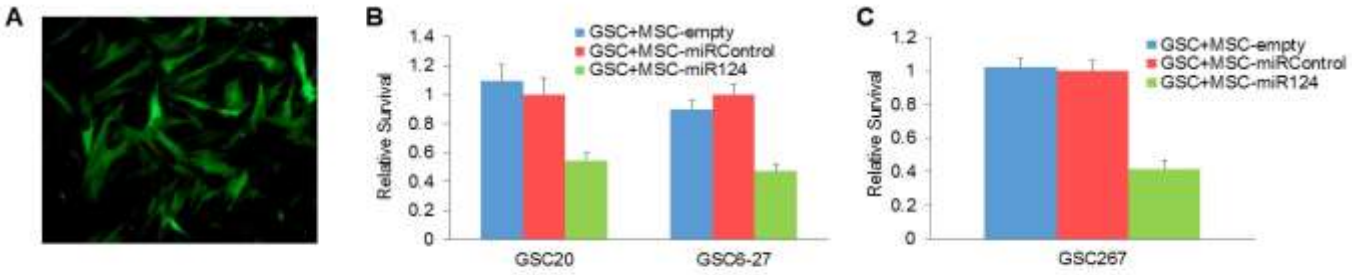


Figure 2. A) Representative photomicrograph of MSCs 24 hours after treatment with lenti-miR124-GFP. 90-95% transduction is evident by GFP-labeled cells, and MSCs retain their spindle shape. **B)** WST1 assay of GSCs after Transwell plate co-culture with indicated condition. MSCs overexpressing miR-124a significantly reduced GSC20 and GSC6-27 viability. Values are relative to GSC+MSC-miRControl. **C)** Direct cell count assay of GSCs after Transwell plate co-culture with indicated condition. MSCs overexpressing miR-124a significantly reduced GSC267 viability.

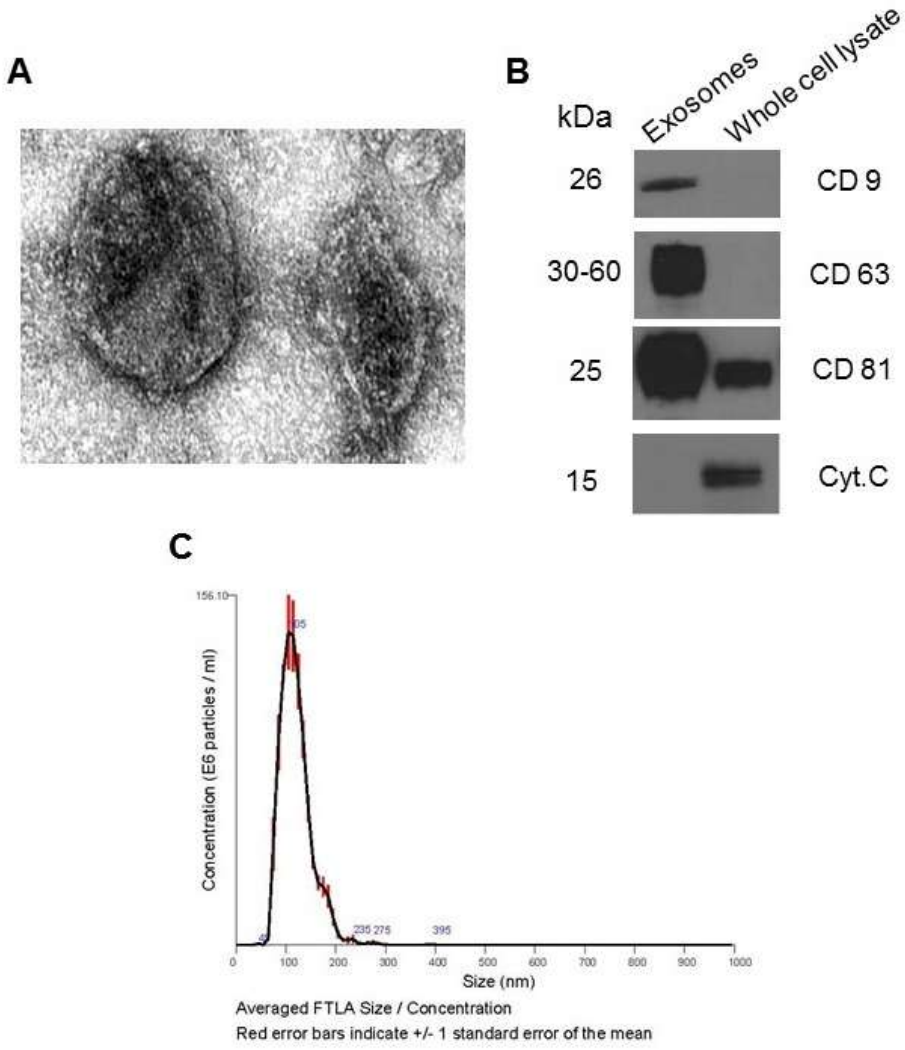


Figure 3. A) Representative photomicrographs of isolated exosomes using electron microscopy. **B)** Western blot analyzing exosomal (CD9, CD63, CD81) and nonexosomal (Cytochrome C) markers in the isolated particles versus a whole MSC lysate. **C)** Graph obtained using Nanosight^{TM} technology showing size (100 nm) and concentration of isolated particles.

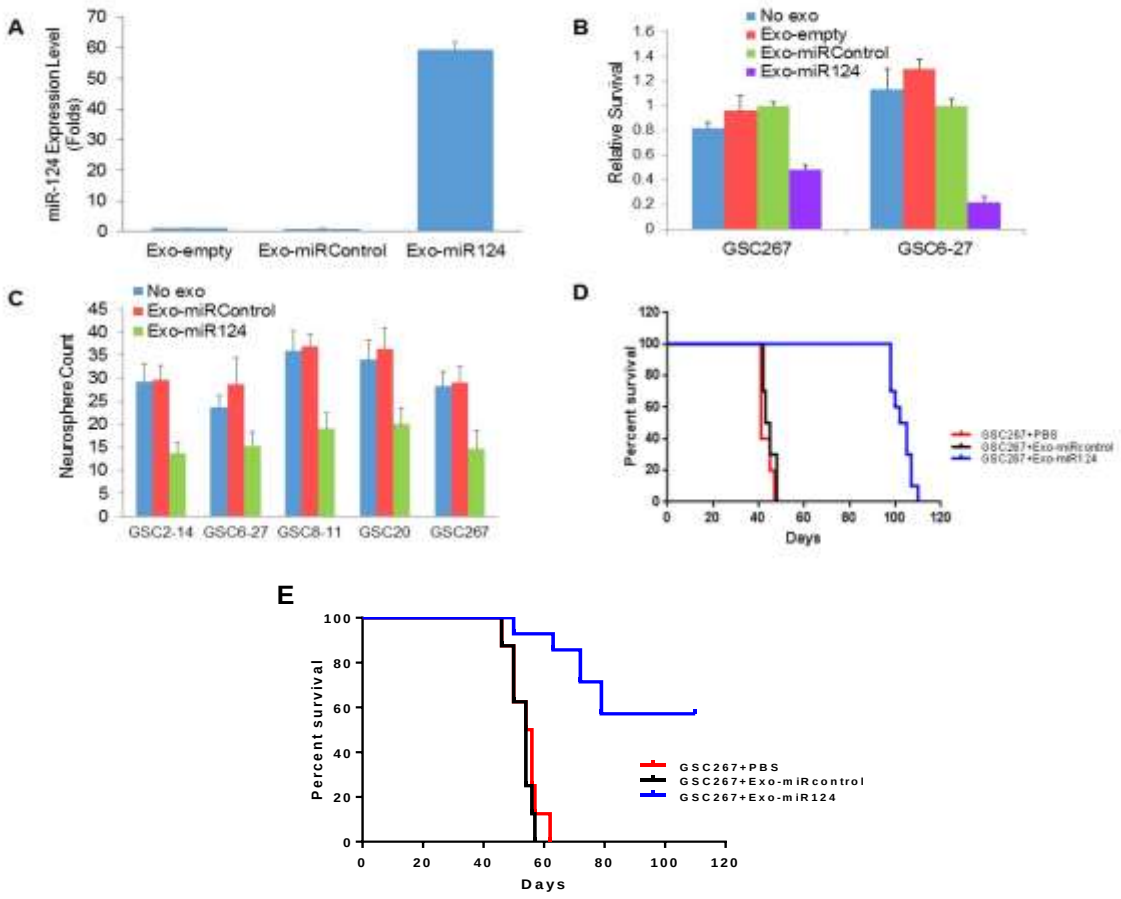


Figure 4. A) qRT-PCR analyzing expression level of miR-124 in MSC-derived exosomes. Exosomes from MSCs transduced with lenti-miR124-GFP exhibited high miR-124a expression levels. **B)** WST-1 assay of GSCs after treatment with exosomes. Exosomes engineered to carry miR-124a significantly inhibited GSCs. Values are relative to exo-miRControl. **C)** Clonogenic assay of GSCs demonstrates the ability of exo-miR124 to inhibit GSC tumorigenicity and colony formation. **D)** Survival curve of *in vivo* tumorogenicity experiment, where mice were implanted with *ex-vivo*-treated GSC267. Mice treated with exo-miR124 lived significantly longer than controls. **E)** Survival curve of *in vivo* systemic administration experiment, where mice were implanted with GSC267, then treated with exosomes three days per week. >50% of mice treated with exo-miR-124 lived past 110 days, while all control mice died by day 62.

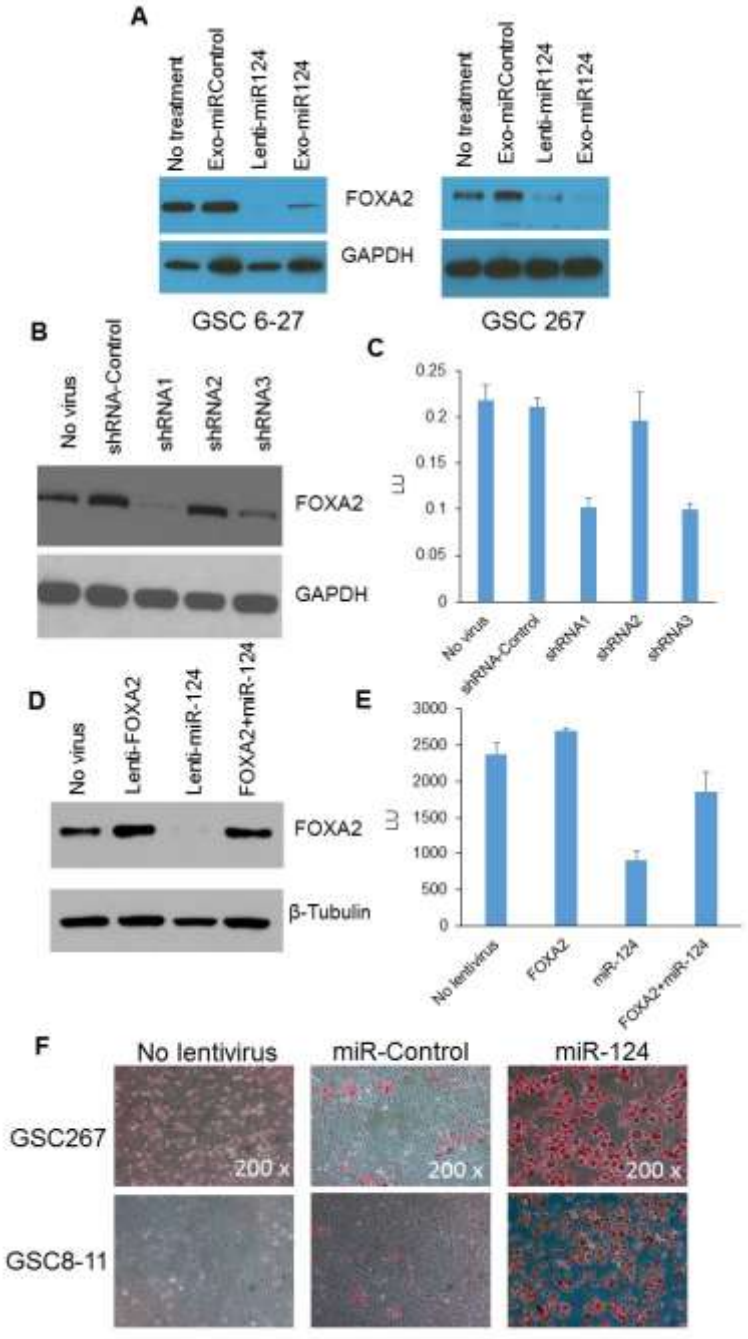


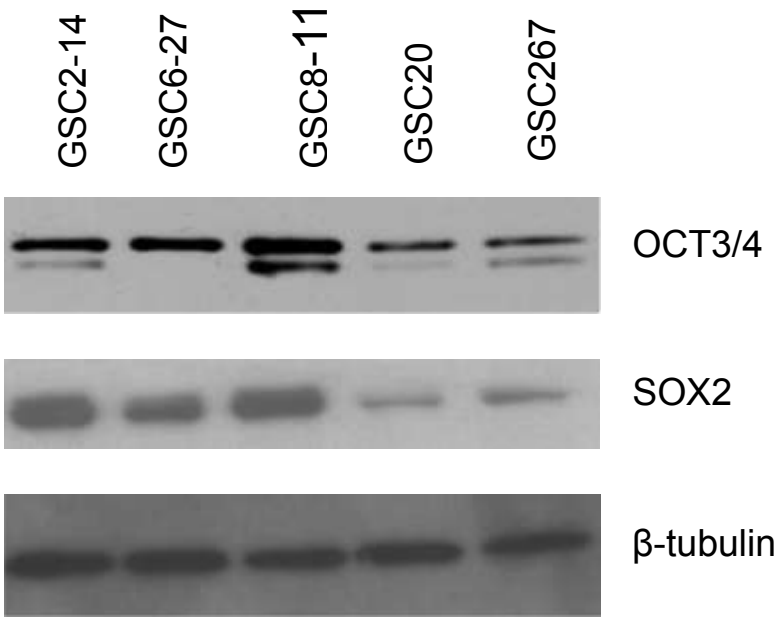
Figure 5. A) Western blot showing FOXA2 expression, a known miR-124 target, after treatment of GSCs with indicated condition. GAPDH was used as a loading control. Treatment with lenti-miR124-GFP or exo-miR124 inhibited FOXA2 expression in GSCs. **B/C)** Western blot showing FOXA2 expression and graph showing GSC267 viability after treatment with anti-FOXA2 shRNAs. FOXA2 knockdown correlated with GSC viability reduction. **D/E)** Western blot showing FOXA2 expression and graph showing GSC267 viability after treatment with indicated condition. FOXA2 expression and GSC proliferation were rescued when treating with both lentiviruses. β-Tubulin was used as a loading control. **F)** Representative photomicrographs of GSCs treated with lenti-miR124 and stained with Oil Red O solution for lipids. Lipid accumulation can be seen in GSCs treated with lenti-miR124-GFP.

SUPPLEMENTARY FIGURES AND TABLES

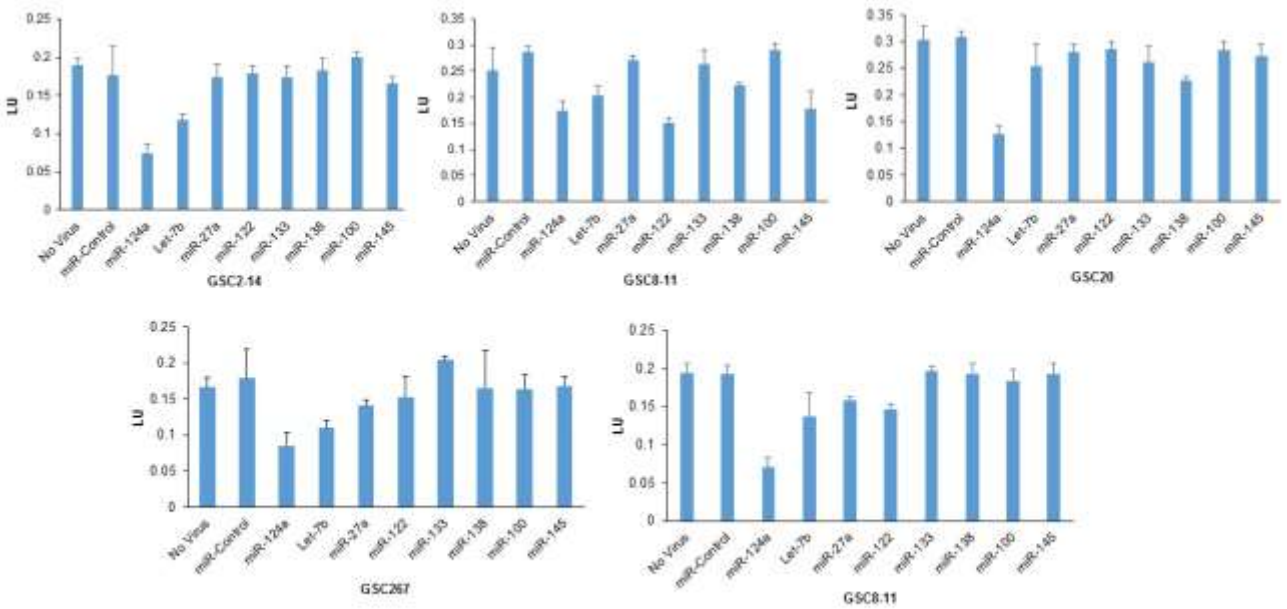
GSC	TCGA subtype	MGMT-Methylation	Radiation Sensitivity	Mouse Survival (days)	7p11.2-EGFR Amp.	10q23.31-PTEN Deletion	17p13.1-TP53 Deletion	PTEN Mut.	EGFR Mut.	TP53 Mut.	IDH1 Mut.	CD133 Express (%)	CD15 Express (%)	CD44 Express (%)
GSC6-27	Classical	Un-methyl.	Resistant	>90	0	Deleted	0	Wild	Wild	Mutated	Wild	0.93	86.05	62.26
GSC2-14	Classical	Methylated	Sensitive	>90	Amplified	0	0	Mutated	Wild	Mutated	Wild	0.06	52.43	0.52
GSC20	Mesenchymal	Methylated	Sensitive	>90	0	Deleted	0	Wild	ND	ND	ND	0.05	91.44	0.82
GSC267	Mesenchymal	Un-methyl.	Sensitive	<90	0	Deleted	Deleted	Wild	ND	ND	ND	2.6	0.72	99.25
GSC8-11	Proneural	Un-methyl.	ND	<90	Amplified	0	0	Mutated	Wild	Mutated	ND	0.29	47.17	99.95

ND = Not Done

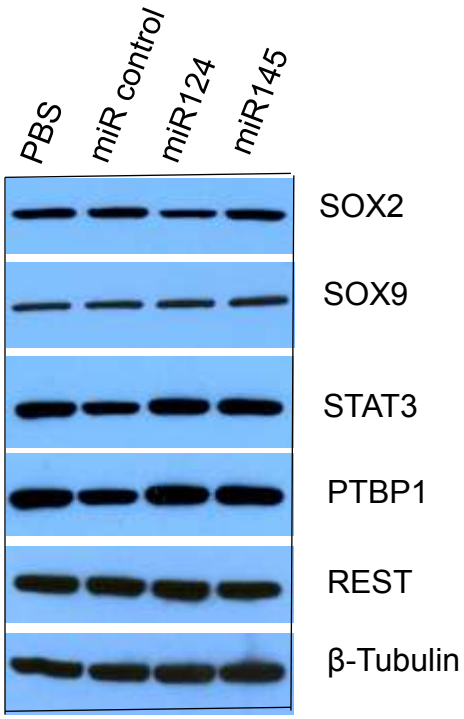
Table 1. Defining characteristics of the 5 GSC lines used in this study.



Supplementary Figure 1. Western blotting analyzing expression level of stem cell markers, OCT3/4 and SOX2, in GSCs. These markers denote the stem cell, tumor-driving property of GSCs. β-Tubulin was used as a loading control.



Supplementary Figure 2. Graphs showing viability of GSCs (N=5) after treatment with the indicated miRs and assayed for viability using the WST1 assay. Only miR-124a resulted in a significant reduction in viability across all GSC lines.



Supplementary Figure 3. Western blotting analyzing expression level of putative miR-124 targets in GSC26. miRControl and miR-124 were used as negative controls. Lentivirus-induced miR-124a overexpression did not result in a reduction in expression level of any of these 5 proteins in our GSCs. β-Tubulin was used as a loading control.

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ADDENDUM

I spent my first summer in the laboratory conducting preliminary experiments to identify microRNAs that were capable of effectively inhibiting GSCs. I studied miR-100, a purported oncomiR, and miR-128, a purported tumor suppressor miR. My initial findings were inconclusive because I was learning the techniques of gene transfer and cell culture. Nevertheless, surprisingly, my miR-100 emerged as a potentially promising inhibitor of glioma cells, despite lack of statistical significance. During my second summer, armed with knowledge of experimental techniques, I broadened my experimental methods and tested the ability of miR-100, miR-124, and miR-491 to inhibit the growth of several GSC lines. In the experiments, I transferred these miRs into the tumor cells using lentivirus technology and found that miR-124 was a highly effective inhibitor of GSCs (Fig. 1A and 1C). These results were independently verified by my mentor Dr. Anwar Hossain, who added several other microRNAs to the analysis (based on recommendations from the reviewers of our submitted paper). He confirmed my initial result showing that miR-124 was the most effective miR capable of killing GSCs (Fig. 1B and Supp. Fig. 2). I also used PCR technology to show that the lentivirus produced high levels of miR-124 in the cells (Fig 1C).

After identifying miR-124 as an anti-glioma miR, during my second summer in the laboratory, I then conducted Transwell experiments showing that MSCs loaded with miR-124 were capable of killing GSCs (Fig. 2A, 2B, and 2C). After, I carried out PCR and exosome isolation techniques proving that MSCs transduced with lenti-miR-124 secreted exosomes that harbored miR-124 (Fig. 4A). I then isolated these miR-124-containing exosomes and performed exosome treatment/feeding experiments on several GSC lines (Fig. 4B).

My third summer in the laboratory was crucial for finding FOXA2 as the critical target of miR-124 (Fig. 5A). I determined multiple possible targets of miR-124 through a literature search, and I tested multiple purported targets, including STAT-3, SOX2, SOX-9, PTBP-1, and REST, using western blotting of proteins isolated from lenti-miR-124-treated GSCs, which were found to be unaffected by miR-124 upregulation (Supplemental Figure 3). Ultimately, after additional literature searches, I tested FOXA2 which proved to be down-regulated by miR-124 in our system (Fig. 5A). Because I had established the basic proof-of-concept that miRs could be used to treat gliomas and that MSCs or exosomes could be used to deliver these miRs, we submitted the paper for publication and received feedback from reviewers requesting clarification of several issues.

Dr. Hossain was responsible for conducting these additional experiments. The reviewers asked that we show that the GSCs had stem-like features, and so Supplementary Figure 1 was added showing that the GSCs expressed proteins found in stem cells. The reviewers also requested that we provide further evidence that our isolated nanoparticles were indeed exosomes. Therefore, Dr. Hossain, with the help of Joy Gumin, analyzed the isolated particles by electron microscopy, nanoparticle tracking technology (NanoSight™), and Western Blotting, proving that the particles I was delivering were in fact exosomes (Fig. 3A, 3B, and 3C). Finally, per the reviewers' request, Dr. Hossain conducted a neurosphere assay to accompany the proliferation assays (Fig. 4C), further explored FOXA2 as a miR-124 target (Fig. 5B-F), and began in vivo studies (Fig. 4D). After the completion of these experiments, we submitted the paper for publication an additional time and received feedback to gather more *in vivo* data. Using these peer reviews, I helped Dr. Hossain design animal trials testing our novel glioma treatment, which have been ongoing for the past few months.

I thank Dr. Anwar Hossain for his great mentorship and his incredible contribution throughout the project. Throughout this process, Joy Gumin was also extremely helpful in maintaining cell lines for experiment and helping with the additional, reviewer-recommended experiments. I thank everyone in the Lang laboratory, including Dr. Tal Shahar and Dr. Shinji Yamashita, for teaching me several of the experimental techniques, and I thank Dr. Lang for opening his laboratory to me. Finally, I thank Dr. John Sampson for his mentorship throughout the thesis process.