Loss of miR-204 Expression Enhances Glioma Migration and Stem Cell-Like Phenotype

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Abstract

Phenotypic similarities have long been recognized between subpopulations of glioma cells and neural stem cells. Many of these similar properties, including the robust abilities to self-renew, migrate and invade, are hallmarks of glioma cells that render them extremely aggressive. However, the molecular mechanisms underlying this character, particularly in glioma stem-like cells that drive this disease, remain poorly understood. Here we report the results of a differential miRNA expression screen that compared glioma cells and neural stem cells, where we found that miR-204 was markedly down-regulated in both types of cells. Mechanistic investigations revealed that miR-204 simultaneously suppressed self-renewal, stem cell associated phenotype and migration of glioma cells via targeting the stemness-governing transcriptional factor SOX4 and the migration-promoting receptor EphB2. Restoring miR-204 expression in glioma cells suppressed tumorigenesis and invasiveness in vivo and increased overall host survival. Further evaluation revealed that the miR-204 promoter was hypermethylated and that attenuating promoter methylation was sufficient to upregulate miR-204 in glioma cells. Together, our findings reveal miR-204 as a pivotal regulator of the development of stem cell-like phenotypes and cell motility in malignant glioma cells.
Introduction

Gliomas are the most common malignant primary brain tumors in adults and exhibit a spectrum of aberrantly aggressive phenotype. Despite advances in treatments during past decades, prognosis of the disease remains poor, with the median survival time approximately 12-14 months. The extremely poor prognosis of patients with gliomas is largely due to the high tendency of tumor invasiveness, which leads to severe structural and functional damage to the surrounding brain tissue, incomplete surgical resection and high frequency of tumor recurrence (1). The molecular mechanisms underlying the aggressive malignant phenotype of glioma cells, however, remain largely unknown.

It has been established that glioma cells, or their subpopulations, share similar biological phenotype and gene expression profiles to those of neural stem cells (2, 3). Two common phenotypic features shared by glioma cells and neural stem cells are their robust abilities to migrate through brain parenchyma (4, 5) and to self-renewal (6, 7). For neural stem cells, these characters typical of cellular “stemness” are essential for tissue homeostasis, regeneration and repairing processes in the central nervous system (CNS). In malignant gliomas, however, these properties contribute to tumor invasiveness. Understanding whether there are common factors that simultaneously modulate the malignant phenotypes of glioma cells and the stemness of neural stem cells is expected to provide new insights in developing novel and effective therapies for glioma. In this context, identification of genetic and/or epigenetic factors that modulate the “stemness” phenotype of glioma cells is of great importance.

Deregulation of miRNAs has been implicated in the development and progression of nearly all tumor types (8). miRNAs play pivotal roles in development, particularly in modulating stem cell-specific pathways (9). This prompted us to identify and study miRNAs with
common or similar expression patterns in neural stem cells and glioma cells. In this report, we describe miR-204 as a miRNA that is significantly down-regulated in both gliomas and neuron stem cells, through methylation of its promoter. Reintroduction of miR-204 into glioma cells markedly suppresses the motility and invasion of glioma cells as well as their stem cell-like phenotype through targeting multiple regulators. Furthermore, our data show that miR-204 inhibits glioma tumorigenesis and invasion in the brain of mice. These findings thus reveal a novel pathway by which epigenetic modulation of miR-204 renders glioma cells abilities of robust self-renewal and aggressive invasion.
Materials and Methods

Cell lines and primary cell culture. Primary normal human astrocytes (NHA) were purchased from Sciencell Research Laboratories (San Diego, CA) and cultured under the condition suggested by the manufacturer. Glioma cell lines, including LN443, LN444, LN464, U118MG, T98G, U251MG, U87MG, D247MG, A172, LN319, LN382T, LN229, LNZ-308 and SNB19, were shared with Dr. Shi-Yuan Cheng’s laboratory at Northwestern University, Chicago, IL and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) as previously described (10, 11). All cell lines were authenticated by short tandem repeat fingerprinting at IDEXX RADIL (Columbia, MO, USA) and Services at SYSU Forensic Medicine Lab (Guang Zhou China).

Tissue specimens and patient information. Twenty freshly dissected glioma tumor specimens were clinically and histopathologically diagnosed at the First Affiliated Hospital of Sun Yat-sen University. Six normal brain tissues were obtained by donation from individuals who died in traffic accidents and confirmed to be free of any pathologically detectable lesions. Prior donors’ consents and approvals from the Institutional Research Ethics Committee were obtained.

Plasmid, retroviral infection and transfection. A DNA fragment containing the hsa-miR-204 precursor flanked by 500bp genomic sequence at either end was inserted into retroviral vector pMSCV-puro (Clontech, Palo Alto, CA). The open reading frames (ORFs) of SOX4 and EphB2 genes generated by PCR were cloned into retroviral vector pMSCV-neo (Clontech, Palo Alto, CA). Retroviral production and infection were performed as previously described (10). 3’-UTRs of SOX4 and EphB2 were amplified and cloned into the downstream region of a luciferase gene in a modified pGL3 control vector (Promega, Madison, WI). The miR-204 mimics and miR-204 inhibitor oligonucleotides and their corresponding control
oligonucleotides were synthesized at Ribo Biotech (Guangzhou, China). Transfection of plasmids or oligonucleotides was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction.

**Single cell tracks assay.** Cells (5×10^3/well) were seeded in 6-well plates. Single cell tracks were obtained by Axio Observer-Z1 time-lapse microscopy system (Carl Zeiss, Oberkochen, Germany). Experiments were performed according to the manufacturer’s instruction and repeated for at least three independent times.

**Flow Cytometry analysis.** Cells were dissociated into single-cell populations and labeled with a phycoerythrin-conjugated CD133/2 (293C3) antibody (Miltenyi Biotec, Germany). The expression level was analyzed using EPICS XL flow cytometer with EXPO32 ADC software (Beckman Coulter, Indianapolis, IN).

**Tumor xenograft live imaging.** Glioma xenografts stably expressing firefly luciferase together with miR-204 or the corresponding control vector were orthotopically implanted in the brains of nude mice as previously described (11) and monitored using the IVIS Spectrum Live Imaging System (Caliper Life Sciences, Mountain View, CA). Image calibration and visualization were performed using the Life Imagine 4.2 software (Caliper Life Sciences).

**Bisulfite genomic sequencing.** Genomic DNA from primary NHA, glioma cell lines and clinical specimens was bisulfite-modified with the Epitect Bisulfite Kit (Qiagen, Valencia, CA). Bisulfite-treated DNA was amplified with bisulfite-sequencing PCR (BSP) primers located in the TRPM3 promoter. PCR products were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). Plasmids from single colonies were purified and sequenced.

**Microarray data visualization.** TCGA dataset were obtained from (12).GEO expression profile was obtained from (13). All microarray data were processed on the MeV 4.6 platform (14).
Statistical analysis: Statistical analyses, except the microarray data were carried out using the
SPSS 11.0 statistical software package. $P < 0.05$ in all cases was considered statistically
significant.
Results

miR-204 was down-regulated in gliomas

To identify miRNAs with similar expression patterns in glioma and neural stem cells, we retrieved miRNA expression profiles from two datasets, The Cancer Genome Atlas (TCGA) and the GEO dataset GSE29759. As shown in Fig. 1A, miR-204 emerged as one of the most prominent down-regulated miRNAs in the TCGA glioblastoma (GBM) dataset, accompanied by several other down-regulated miRNAs reported previously, namely, miR-124, 106a, 17-5p and members of the miR-181 family (15, 16). As documented previously, miRNAs up-regulated in gliomas, including miR-21 (17-19), miR-9 (20, 21) and miR-10b, were also screened out (22, 23). Comparing the miRNA profiles in the TCGA dataset with those in neural stem cells identified that miR-204 was down-regulated in both glioma and neural stem cells (Fig. 1B). To validate the expression pattern of miR-204 in gliomas, qRT-PCR was conducted with 6 normal brain tissue samples and 20 freshly dissected glioma samples. In consistence with the data presented in the TCGA profile, expression of miR-204 was markedly down-regulated in high-grade glioma samples (WHO tumor grade III and IV) and to a lesser degree, decreased in WHO tumor grade I and II glioma samples, as compared to normal brain tissues (Fig. 1C). Of note, 10 out of 14 glioma cell lines displayed significantly down-regulated expression of miR-204 relative to that in two primary cultured normal human astrocytes (NHA) (Fig. 1D).

Restoration of miR-204 in glioma cells suppressed cellular migration and invasion

To investigate whether miR-204 could modulate the migration and invasiveness of glioma cells, SNB19 and LN382T glioma cell lines, which possessed the lowest levels of miR-204 expression among all tested glioma cell lines, were chosen for further studies. By retrovirally transducing glioma cells with miR-204 at expression levels comparable with that of NHAs (Fig. S1), we first examined the effect of miR-204 on cell migration using wound
healing assay. As shown in Fig. 2A, compared with the vector-control cells, which spread to
the center line within 16 hours, miR-204-transduced cells exhibited considerably slower
migration and reduced cell spreading. Furthermore, we tracked the movement of glioma
cells, using a time-lapse imaging system, and examined whether miR-204 altered the
migrating behavior of individual glioma cells. As shown in Fig. 2B, miR-204
transduced-SNB19 and -LN382T cells generally retained their original positions, whereas
vector-control cells moved away at higher extents. Specifically, we measured the migration
tracks of 10 individual cells within 5 hours and observed that miR-204 transduced cells
moved within a very limited area. In contrast, the control cells spread out in a markedly
larger area, with approximately 1.5–3 folds of increase in migration distance (Fig. 2C). To
further determine the effect of miR-204 expression on motility of glioma cells, transwell
penetration assay were performed. As shown in Fig. 2D, less number of miR-204 cells
invaded across the membrane pre-coated with matrigels when compared with control cells.
Importantly, the three-dimensional spheroid invasion assay showed that vector-control cells
displayed a highly aggressively penetrating growth after 5 days, whereas the miR-204
transduced-cells grew in spherical colonies (Fig. 2E). Taken together, these observations
suggested that miR-204 strongly inhibited the migration and invasion of glioma cells.

**miR-204 attenuated stem cell like phenotype of glioma cells**

Malignant gliomas tend to relapse after surgical resection, and that such a character is
believed to be largely attributable to the stem cell-like properties of a fraction of cells within a
glioma tumor (2). Since miR-204 is expressed at very low levels in both glioma and neural
stem cells but at high levels in differentiated normal brain tissues, we determined the potential
role of mir-R-204 in the development and maintenance of the stem-like property of glioma
cells. Sphere forming assay showed (Fig. 3A), miR-204 overexpressing-cells formed much
smaller neural spheres after 7 days of culture as compared with control cells (~2 folds smaller
in diameter), indicating markedly decreased self-renewal ability by miR-204.

To further investigate the potential role of miR-204 in suppressing the stemness property of glioma cells, we examined the expression of pluripotency factors in neuronal spheres formed by miR-204-transduced cells and control cells. As shown in Fig. 3B, the expression levels and patterns of pluripotency factors, including SOX2, NANOG, KLF4 and OCT4, were similar between spheres formed by vector control- and miR-204 transduced-cells. Whereas, at 48 hours after stimulation with 1% serum, miR-204-expressing cells rapidly lost their expression of pluripotency factors, retaining only 2% of expression in the neuronal spheres after induction for differentiation, while the vector control-cells retained expression of pluripotency factors at approximately 30% of the initial levels (Fig. 3B). Consistent with these results, at both time points, CD133-positive proportions were significantly lower in miR-204 transduced-cells than those in control cells, and a faster declining rate of CD133 positivity was also displayed in miR-204 transduced-cells (Fig. 3C). In further experiments we co-stained for a glioma stem cell marker, Nestin, and an astrocyte differentiation marker, GFAP, during the process of differentiation. We observed a significantly faster decrease of Nestin and simultaneous increase of GFAP expression in cells transduced with miR-204 as compared with those in control cells (Fig. 3D). Taken together, these data showed that re-introduction of miR-204 in glioma cells reduced the stem cell-like population in glioma and greatly attenuated the ability of stem cell-like glioma cells to retain stemness.

**Reintroduction of miR-204 induced loss of invasion and tumorigenecity in vivo**

Next, we test whether miR-204 is involved in tumorigenesis and invasiveness of glioma cells *in vivo*. An orthotopic glioma xenograft model was employed by xenotransplanting luciferase-expressing glioma cells transduced with miR-204 or corresponding control vectors in the brains of nude mice. Tumor growth was monitored using a live animal bioluminescence imaging system during the experiment. As shown in Fig. 4A, both
miR-204-transduced cell lines exhibited significantly slower growth after implantation. For the highly proliferative SNB19 cells, results from luminescence images revealed a high degree of suppression on the growth of miR-204-transduced cells in contrast to the larger intracranial tumor formed by the control cells at day 10 after implantation. All mice bearing vector control-cells died 18 days after implantation whereas only one mouse implanted with miR-204 transduced SNB19 cells died at day 21 (Fig. 4B). Similar inhibition of tumorigenicity of miR-204 transduction was also observed in LN382T cells, which are known to grow more slowly. Specifically, one mouse with miR-204 cells died after 35 days of tumor cells implantation, and all vector cells-implanted mice were dead at day 29 (Fig. 4B). These data suggested that miR-204 was a strong inhibitor of glioma tumorigenicity in vivo.

Furthermore, IHC analysis showed that tumors formed by vector control cells exhibited extensive branch-like growing pattern that spread into the surrounding tissue. By contrast, miR-204-overexpressing cells formed oval-shaped tumor with smooth margins and non-invasive front (Fig. 4C), further validating that the invasive behavior of glioma could be suppressed by miR-204 in vivo.

Core regulators of migration and stemness were direct targets of miR-204

As miRNAs exert their functions by targeting multiple transcripts that are coordinately orchestrated in a biological process, we screened for such targets of miR-204 using the TargetScan Program and identified two conserved binding sites for miR-204 in the 3’UTR region of SOX4 gene, a known core regulator governing the stemness of both glioma and neural stem cells (24, 25). Meanwhile, EphB2, which is known to be required for the migration of both glioma and neural stem cells (26-29), was also found as a potential target gene of miR-204 (Fig. 5A). To validate whether SOX4 and EphB2 were targets of miR-204, we transiently expressed miR-204 in SNB19 and LN382T cells, both of which possess low endogenous miR-204 expression. Significantly decreased protein expression of both SOX4
and EphB2 was observed after transfection of a miR-204 mimic oligonucleotide. In contrast, inhibition of miR-204 by transfecting a miR-204 inhibitor oligonucleotide in LN443 and U251MG cells, which express high levels of endogenous miR-204, led to elevated expression of both proteins (Fig. 5B). Furthermore, in the above described in vivo glioma model, lower levels of SOX4 and EphB2 staining were detected in tumors formed by miR-204 transduced cells, as compared those in the control tumors (Fig. S2).

To determine whether inhibition of miR-204 on SOX4 and EphB2 expression is mediated by posttranscriptional effects on the 3’UTRs of both genes, we constructed luciferase reporter plasmid containing 3’UTRs for SOX4 and EphB2, respectively. As shown in Fig. 5C, ectopic expression of miR-204 markedly decreased, while inhibition of miR-204 significantly elevated, the reporter luciferase activities (Fig. 5C), suggesting that miR-204 post-transcriptionally inhibited SOX4 and EphB2 expression. Furthermore, mutagenesis in miR-204 target sites of the SOX4 or EphB2 3’UTR linked to the luciferase reporter confirmed the site-specific effect of miR-204 (Fig. 5C). To examine the interaction between miR-204-containing miRNP and target mRNAs, we pulled down Ago1 and its associated RNA in cells transfected by control miRNA or the miR-204 mimic. qPCR assessment of the SOX4 and EphB2 3’UTR regions revealed high levels of SOX4 and EphB2 transcripts enrichment in the miRNP of miR-204 mimic-transfected cells, strongly suggesting that miR-204 could guide miRNP to target SOX4 and EphB2 (Fig. 5D). Finally, we assessed the expression of SOX4 and EphB2 in 20 glioma specimens shown in Figure 1C, and found significant inverse correlation between miR-204 and its two targets (Fig. S3). This observation indicates miR-204 may act as regulator for SOX4 and EphB2 expression in clinical glioma specimens.

To determine the functional significance of SOX4 and EphB2 genes in miR-204-induced phenotype, we further stably expressed SOX4 ORF, or EphB2 ORF, that did not contain the
3'UTR in miR-204-transduced cells. As shown in Fig. 5E, restored SOX4 expression could significantly abrogate the inhibitory effect of miR-204 induced-sphere formation. Similarly, re-expression of EphB2 ORF in miR-204 transduced cells regained the invasiveness of glioma cells induced by miR-204 expression (Fig. 5F). Taken together, these data revealed that by coordinately suppressing core regulators of glioma stemness and migration, miR-204 conferred the abilities of self-renewal and invasion in glioma cells.

**Hypermethylation in the promoter region of miR-204 gene**

We next sought to investigate the molecular mechanism that mediates the down-regulation of miR-204 in glioma cells. Interestingly, miR-204 was an intronic miRNA located between exons 7 and 8 of the TRPM3 gene (30). Consistent with a previous report (30), expression of mature miR-204 and pri-miR-204 linearly correlated with that of TRPM3 in NHA and several glioma cell lines (Fig. 6A), indicating that miR-204 and TRPM3 might share common regulatory mechanism(s). To examine the epigenetic modification of this shared promoter region, we retrieved the CpG-rich loci of TRPM3 promoter from the UCSC genome browser (Fig. 6B) and assessed for their methylation status via bisulfite genomic-sequencing PCR (BSP). The BSP result showed that in NHA, as well as in glioma cells with relatively higher levels of miR-204 expression, the CpG islands were mostly not methylated (Fig. 6C). By contrast, in genomic DNA obtained from glioma cells with down-regulated miR-204 and from clinical glioma tumor samples, the promoter CpG islands of miR-204 were hypermethylated in most glioma specimens, strongly suggesting an essential role of promoter methylation in miR-204 down-regulation (Fig. 6C and Fig. S4). To further validate this finding, we treated the cells with DNA methyltransferase inhibitor 5-aza-dC for 72 hr and found that in cells with low levels of miR-204 expression, inhibition of DNA methylation significantly promoted the expression of miR-204, whereas in NHA and glioma cells without miR-204 promoter hypermethylation, miR-204 expression was not affect (Fig. 6D). Correspondingly 5-aza-dC
treatment could induce down-regulation of SOX4 and EphB2 expression in glioma cell lines examined with low basal levels of miR-204 expression but could not suppress the expression of SOX4 and EphB2 in cells with high levels of endogenous miR-204 (Fig. S5). Taken together, our data suggested that in glioma cells miR-204 down-regulation was attributable to promoter methylation.
Discussion

Development of stem cell-like properties in glioma cells has been recognized as a hallmark contributing to disease progression and generation of cellular population(s) robustly empowered to migrate and invade the surrounding brain tissue, to resistant to chemo- or radio-therapies, resulting in frequent tumor recurrence, and to proliferate and colonize from a small number of initiating cells, in addition to other aspects of tumor malignancies (31). Accumulated evidence supports the notion that such a process is regulated by signaling pathways similar to those governing the stemness of neural stem cells. Of note, activation of these pathways requires coordinated expression and cooperative action of several core regulators. For example, SOX4 has recently been identified as a determinant factor of glioma stemness, which forms a complex with OCT4 and subsequently activates the transcription of SOX2, one of the core pluripotency reprogramming factors (24, 32). This transcriptional axis is essential for the maintenance of glioma stem cell population, and more importantly, blocking this pathway greatly improves animal survival in a glioma xenograft model (24). As a short half-life (<1h) protein (33), the expression level of SOX4 is tightly controlled transcriptionally and post-transcriptionally. In endometrial cancer, down-regulation of miR-129-2 mediates the overexpression of SOX4, suggesting a significance of miRNA-mediated regulation in sustaining the expression of this stemness-associated oncoprotein (34). In glioma cells, however, the level of miR-129 has not been reported or screened out in high throughput assays to have significant change. In this study, we identified miR-204 as a miRNA commonly down-regulated in both types of neural cells. We then further determined the targets of miR-204 and the consequent modulation of stem cell-like properties of glioma cells. Our data showed that miR-204 acts to inhibit the expression of SOX4 and subsequently suppress the stem cell-like phenotype of glioma cells, and the miR-204-downregulated status in glioma cells leads to generation of a
glioma cell population in which marked stemness is developed. In miR-204 transduced cells, ectopic expression of SOX4 is able to rescue neural sphere formation ability attenuated by miR-204, confirming that SOX4 is a major target of miR-204 and responsible for maintaining the stem cell population in human gliomas. Of note, miR-204 not only suppresses self-renewal but also migration of glioma cells, which is another hallmark of neural stem cells (5). This study has also identified EphB2 as a major migration regulator directly targeted by miR-204. Previously, EphB2 has been reported to promote stem cell migration in both physiological and injury repairing conditions (5, 26). Elevated expression and enhanced phosphorylation of EphB2 has also been found in glioma cells (29, 35). More importantly, EphB2 has identified to be a critical promoter of tumor migration in glioma through interacting with the FAK and activating FAK-mediated signal (28). The mechanism regulating EphB2 expression in glioma cells, however, is still unclear. Our data thus demonstrates a novel post-transcriptional regulator of EphB2, namely, miR-204, epigenetic silencing of which enables elevation of EphB2 in glioma cells and enhances EphB2 mediated migration and invasion.

Sustained activation of regulatory programs shared by glioma and neural stem cells requires orchestrated transcription and post-transcriptional regulation of gene expression. Owing to the multi-target property and the network effect, miRNAs is of great importance in the suppression of pluripotency of embryonic stem cells by targeting reprogramming factors and initiating differentiation (9). During the process of differentiation, an optimal miRNA expression pattern enables proper development of the organism (36). In tumor cells, however, many of the stem cell-specific miRNA expression patterns are re-archived, such as the loss of neural specific miR-124 expression that promotes the stemness of glioma (15, 37). Consistently, we find that miR-204, which is down regulated and thereby expected to play significant roles in both glioma cells and neural stem cells. miR-204 down-regulation not
only promotes cellular stemness but also the biological properties essential to the lethality of
the disease, namely, migration and invasion abilities of glioma cells, \textit{in vitro} and \textit{in vivo}.
Most importantly, restoration of miR-204 in glioma cells greatly abrogates the aggressiveness
of glioma cells.

Lastly, we showed that reduction of miR-204 is attributed to down-regulated transcription
due to methylation of the promoter of its host gene TRPM3. TRPM3 is a member of the
transient receptor potential melastatin (TRPM) family that has been reported to be associated
with cancer progression. Down-regulation of TRPM1/melastatin mRNA in the primary
cutaneous tumor is a prognostic marker for metastasis in patients with localized malignant
melanoma (38). Moreover, TRPM8 is lost in prostate cancer tissues from patients treated
pre-operatively with anti-androgen therapy, suggesting that loss of TRPM8 is associated with
a more advanced form of the disease (39). Despite these findings, however, the
pathobiological role of TRPM3 in cancer remains largely unknown. In this context, our
current finding that the promoter of TRPM3 is hypermethylated and TRPM3 mRNA is
significantly down-regulated in gliomas warrants further studies on the potential
tumor-suppressive function of TRPM3 gene in glioma. Moreover, it also remains highly
interesting whether TRPM3 and miR-204 might cooperate with each other in the pathogenesis
of glioma. Taken together, our present study provides new insights in understanding the
stemness phenotype developed by glioma cells and therefore might contribute to future
development of new anti-gliomas strategies.
Acknowledgments

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Reference:

**Figure Legends**

**Figure 1. miR-204 expression is down-regulated in glioma and neuronal stem cells.**  
* A, Expression profiles of miRNA were obtained from TCGA database and differential expressed miRNAs were retrieved.  
* B, Most differentially expressed miRNA between neural stem cells (NSCs) and differentiated counterparts (Dif-NSCs) were obtained from published expression dataset GSE29759 of the NCBI GEO database.  
* C, qPCR analysis of miR-204 expression in 6 normal brain specimens without identifiable pathological lesions, 6 WHO tumor grade I and II, and 14 WHO tumor grade III and IV glioma specimens.  
* D, Relative miR-204 expression in indicated primary cultured astrocytes (NHA A and B) and glioma cell lines was obtained by qPCR. Error bars represent mean ± SD from three independent experiments. ***: P<0.001.

**Figure 2. miR-204 suppresses glioma migration and invasion in vitro.**  
* A. Wound healing assay was performed with indicated cells and image were taken at 0, 8 and 16 hr.  
* B. Growth pattern of indicated cells were monitored using time-lapse microscopy. Images comparatively demonstrate the pattern of cell growth at the 0 and 24 hr.  
* C. Ten representative cell migration tracks were obtained from 5 hr of time-lapse image of indicated cells, their initial position were aligned the origin of coordinates (left). Cumulated migration distance of indicated cells in 5 hr was measured.  
* D. Representative images (left) and quantification (right) of penetrated cells were analyzed using the Transwell matrix penetration assay.  
* E. Representative micrographs of indicated cultured cells at day 7 of culture in three-dimensional spheroid invasion assay. Error bars represent mean ± SD from three independent experiments, *: P<0.05 ,**: P<0.01.

**Figure 3. miR-204 promotes differentiation of stem cell-like population of glioma cells.**  
* A. Upper panel: Representative images of neural spheres formed by indicated cells after 7 days of culture. Lower panel: Statistic analysis of the average diameter of spheres.  
* B. qPCR
analysis of expression of pluripotency factors, including SOX4, NANOG, KLF4 and OCT4, at indicated time points after induction of differentiation in indicated sphere diverted cells. 

Expression of CD-133 was measured by FACS in indicated spheres-derived cells 24 and 48 hr after induction of differentiation. 

Co-staining of GFAP and Nestin in indicated spheres-derived cells 24 and 48 hr after induction of differentiation. Error bars represent mean ± SD from three independent experiments, *: P<0.05 , **: P<0.01.

Figure 4. Reintroduction of miR-204 suppresses glioma tumorigenesis and invasiveness in vivo. 

A. Mice with intracranial glioma xenografts were monitored by luciferase live imaging system at indicated time points. Heat map scale bar represents photon emission. 

B. Survival curves of mice with brain glioma xenograft formed by indicated cells. 

C. H&E staining of tumor boundary of glioma xenograft formed by indicated cells.

Figure 5. miR-204 targets and suppresses SOX4 and EphB2 expression. 

A. Conserved miR-204 binding sites in SOX4 and EphB2 generated by Targetscan database. 

B. Western blotting analysis of expression of SOX4 and EphB2 in indicated cells. 

C. Relative activity of reporter luciferase linked to SOX4 or EphB2 3’UTR measured following miR-204 mimic or miR-204 inhibitor transfection in indicated cells. 

D. Enrichment of SOX4 and EphB2 3’UTR in miRNP detected by qPCR following immunoprecipitation against Ago1. 

E. Representative micrographs of neural spheres formed by indicated cells after 7 days of culture (left panel). Statistic analysis of the average diameter of spheres (right panel). 

F. Representative pictures of penetrated cells analyzed using the Transwell matrix penetration assay. Error bars represent mean ± SD obtained from three independent experiments, *: P<0.05 , **: P<0.01.

Figure 6. Promoter methylation is responsible for the loss of miR-204 expression in glioma cells. 

A. Relative expression of miR-204 (left panel) or pri-miR-204 (right panel)
was plotted against expression of TRPM3 accessed by qPCR.  

**B.** Bisulfite genomic sequencing resulting from 3 individual clones reveals methylation status of promoter CpG of TRPM3 in indicated specimens.  

**C.** Schematic illustration of the spatial arrangement of the promoter region and miR-204 stem loop in TRPM3 genomic sequence.  

**D.** qPCR analysis of miR-204 expression in indicated cells treated with 5µM 5-aza-dC for 72 hr.  

Error bars represent mean ± SD obtained from three independent experiments, *: P<0.05 ,**: P<0.01.
Figure 1

A

TCGA GBM miRNA dataset (n=505)

Up Regulated in GBM
- hsa-miR-9
- hsa-let-7f
- hsa-miR-26a
- hsa-miR-29a
- hsa-let-7c
- hsa-miR-125b
- hsa-miR-9*
- hsa-let-7a
- hsa-miR-10b
- hsa-miR-21

Down Regulated in GBM
- hsa-miR-204
- hsa-miR-424
- hsa-miR-130b
- hsa-miR-26b
- hsa-miR-132
- hsa-miR-181d
- hsa-miR-17-5p
- hsa-miR-19a
- hsa-miR-210
- hsa-miR-342
- hsa-miR-27b
- hsa-miR-494
- hsa-miR-142-3p
- hsa-miR-15a
- hsa-miR-124a
- hsa-miR-106a
- hsa-miR-181a
- hsa-miR-331

B

C

D

Relative miR-204 Expression

NSCs

Dif-NSCs

miR-124

miR-204

miR-210

miR-23b

miR-27b

miR-574-3p

miR-21

Normal

n=6

T1/2

n=6

T3/4

n=14

***

Relative miR-204 Expression

NHA

NHAB

LN3827

SNB19

LN2298

LNC44

A172

U118MG

LN22

LN319

LN443

D471MG

LUM43

U215MG

n=6

n=6

n=14
Figure 4

A

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<td>Day24</td>
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B

C

SNB19       LN382T

Vector       miR-204

H&E

200μm
Loss of miR-204 expression enhances glioma migration and stem cell like phenotype

Zhe Ying, Yun Li, Jueheng Wu, et al.

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