miR-124 inhibits the growth of glioblastoma through the downregulation of SOS1

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Abstract. Glioblastoma multiforme (GBM) is a lethal brain tumor in adults. Despite advances in treatments, such as surgery, radiotherapy and chemotherapy, high-grade glioma remains fatal. The molecular and cellular mechanisms for GBM are not entirely clear and further studies are required to elucidate these. MicroRNAs (miRNAs) are small, non-coding, endogenous RNAs that are involved in cell differentiation and proliferation, and have been suggested to play a role in a variety of types of cancer. In this study, we investigated the role of miR-124 in the inhibition of proliferation of GBM cells. The downregulation of miR-124 in human GBM tumor cell lines was detected using quantitative RT-PCR. To assess the function of miR-124, we constructed stable cell lines, U87-124 and U373-124, which overexpressed miR-124 using lentiviral vectors. Overexpression of miR-124 inhibited the proliferation of GBM cancer cells *in vitro*. Using integrated bioinformatics analysis, SOS1 was found to be a direct target for miR-124, which is frequently upregulated in gliomas. Dual-luciferase reporter assays confirmed that the SOS1 mRNA 3'-untranslated regions (UTR) was directly targeted by miR-124 and that the mutated 3'UTR was not affected. This was revealed to be mechanistically associated with the induction of SOS/Ras/Raf/ERK and the suppression of ERK activity, which was achieved by silencing SOS1. This study therefore indicates an important role for miR-124 in the regulation of growth in the molecular etiology of GBM, and offers a potential strategy for the use of miR-124 in cancer treatment.

Introduction

Glioblastoma multiforme (GBM) is the most aggressive form of astrocytoma, with a low mean survival time after diagnosis (1). Despite aggressive treatment and recently developed clinical and targeted therapies, the overall survival time for glioblastoma patients has not improved significantly over the last twenty years, despite objective initial responses (2). Thus, the definition of novel biological characteristics is required for informed diagnosis and treatment.

MicroRNAs (miRNAs) are small, non-coding single-stranded RNAs ~19-25 nt long, which regulate genes at the translational level by binding loosely to complimentary sequences in the 3'-untranslated regions (UTRs) of target mRNAs, and are involved in cell growth, differentiation, cytokine activities and angiogenesis (3,4). Mounting evidence has demonstrated that miRNAs are essential in regulating various pathways involved in tumor pathogenesis, functioning as either oncogenes or tumor suppressors (5-7).

Previous studies have shown that there is a difference in the expression of miRNAs in glioblastoma tissues compared to that in normal brain tissues, for example, miR-21 is overexpressed in glioblastoma tissues, which inhibits cell proliferation by the mitogen-activated protein kinase (MAPK) and AKT pathways (5,6). miR-381 levels were increased in GBM. By directly targeting leucine-rich repeat-containing protein 4 (LRRC4), miR-381 was regulated by LRRC4 via a feedback loop involving the MAPK pathway (8). The miR-17-92 cluster has been found to be upregulated in GBM and directly targets the connective tissue growth factor (CTGF) (9). miR-26a and miR-214 have been shown to target PTEN and appeared to be upregulated in gliomas (10-12). Conversely, levels of miR-7 were found to be lower in GBM, the targets of which include the epidermal growth factor receptor (EGFR), and the overexpression of miR-7 reduces proliferation, survival and invasiveness in cultured glioma cells. miR-124 and miR-137, which are both downregulated in GBM, induce neuronal differentiation and inhibit glioma cell growth *in vitro*.

Son of sevenless 1 (SOS1) is a dual guanine nucleotide exchange factor (GEF) for Ras and Rac1 that converts inactive Ras-GDP into active Ras-GTP in many EGF-stimulated cells (13,14). SOS1 has two binding sites for Ras, one of which is an allosteric site distal to the active site (15). RTK activation results in the translocation of SOS1, which mediates Ras activation. The Ras-specific GEF activity of SOS1 is conferred by the Cdc25 domain in the central region of the protein, which also contains a Ras-binding region designated as the Ras exchanger motif (16). Ras is a critical signaling molecule that is important in regulating cell growth (17). MAPK pathways are involved in

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a variety of cellular functions including growth, proliferation, differentiation, migration and apoptosis (18). The activation of ERK by growth factors and mitogens leads to a series of phosphorylation reactions involving Ras, Raf and ERK, and is particularly important in understanding the pathogenesis of cancer. Active ERK signaling results in the upregulation of transcriptional products, some of which allow entry into the cell cycle, and some of which repress the expression of genes that inhibited cell proliferation and the cell cycle (19).

In this study we focused on miRNA-124, a brain-enriched miRNA that has been broadly investigated in order to understand physiological neural development (20,21). We detected that miR-124 is significantly downregulated in glioma cell lines, and that the overexpression of miR-124 induced cell proliferation inhibition, which is associated with SOS1 signaling in the MAPK pathway.

Materials and methods

Cell lines and culture. Human glioma cell lines (U87, U373, SW1088 and SW1783) and HEK293T cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). U87 and U373 cells were cultured in minimal essential medium (MEM), and SW1088 and SW1783 cells were cultured in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA, USA). All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Grand Island, NY, USA). Human astrocytes (HA) and all growth media were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were cultured in a humidified 5% CO_2 atmosphere.

Quantification of mRNA using real-time qRT-PCR. Total RNA, including small RNA, was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed using the SuperScript™ III first-strand synthesis system and oligo(dT) primers (Invitrogen) were synthesized according to the manufacturer's instructions. Real-time PCR (qRT-PCR) was performed with the ABI 7500 (Applied Biosystems, Carlsbad, CA, USA). The cycling parameters were 95˚C for 10 min followed by 40 cycles of 95˚C (15 sec) and 60˚C (60 sec), followed by melting curve analysis. The primers used were: SOS1, F: 5'-CAAGAACACCGTTAACACCTC-3' and R: 5'-GGACAGGCACTTCATCAGTG-3'; GAPDH, F: 5'-GGA GTCCACTGGCGTCTT-3', and R: 5'-GAGTCCTTCCA CGATACCAA-3'. For qRT-PCR of miR-124, 50 ng total RNA was reverse-transcribed with a miRNA-specific stem-loop primer (5'-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGAGGCATT-3'), and the specific primers for U6 were sequenced as described previously (5'-CGCTTCACGAATTTGCGTGTC-3') (22). All reactions were performed in triplicate with GAPDH as a reference (internal control) and the median Ct (cycle threshold) value was used for analysis.

SOS1-3'UTR and miR-124 reporter assays. There are three predicted target sites for miR-124 in the entire 3'UTR of SOS1 (www.targetscan.org). SOS1-3'UTR reporter assays were performed in 293T cells. pCS2-Luc vector harboring SOS1-3'UTR sequences with wild-type (WT) miR-124 binding sites or mutated (MUT) miR-124 binding sites were generated by cloning the subsequent 3'UTR of SOS1 into the *Eco*RI and *Xho*I sites. The 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were transfected with miR-124 or control mimics (50 ng), pMIR-REPORT vectors containing WT or MUT miR-124 binding sites (100 ng) and pRL-SV40 (Promega, Madison, WI, USA) expressing Renilla luciferase (30 ng) for normalization. Luciferase measurements were performed 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega).

Vector constructs. The pri-miR-124 sequence was amplified and cloned into the pcDN3.1 vector and then subcloned (*Bam*HI + *Eco*RI) into the pCDH-CMV-MCS-EF1-copGFP vector (SBI) to generate pCDH-miR-124. A 2500 bp fragment of the 3'UTR of SOS1 was obtained by PCR amplification of human genomic DNA subcloned into pCS2-Luc vector using the primers: F: 5'-CGTGAATTCGCTGCAACATGGTGGG AAC-3' and R: 5'-TCACTCGAGGTGGGCTATGTAAGGCA TTTTTC-3' (reverse). The underlined sequences are the introduced *Eco*RI and *Xho*I sites, respectively.

The mutated versions, mut-1, mut-2, mut-3 and mut, were generated utilizing the SOS1-UTR plasmid as a template and modifying the miR-124 seed binding site using the QuikChange II XL site-directed mutagenesis kit. The mutagenic primers used were: Mut1: 5'-GUUUAGUAAAUUCCA CCGGCCA-3', Mut2: 5'-CAGUAGCUGCCAAAUGCCGGC CU-3' and Mut3: 5'-AAUAAUAAAGAAAAACCGGCCAC-3'. The underlined sequences indicate the mutated bases. All constructs were sequenced for verification.

Lentivirus production and transduction. Virus particles were harvested 48-60 h after pCDH-miR-124 transfection with the packaging plasmid pRSV/pREV, pCMV/pVSVG and pMDLG/pRRE transfected into HEK293T cells using Fugene® HD Transfection Reagent (Roche, Mannheim, Germany). U87 and U373 cells were infected with recombinant lentivirus-transducing units plus 8 μg/ml polybrene (Sigma, St Louis, MO, USA).

Cell proliferation assay. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Corp., Kunamoto, Japan). Cells were seeded into a 96-well plate at a density of $3x10^3$ cells in each well with 100 μl culture medium, then 10 μl CCK-8 was added. The cells were subsequently incubated for 1 h at 37˚C and the absorbance was measured at 450 nm. Three independent experiments were performed.

SDS-PAGE and western blotting. Cells were lysed in RIPA buffer, the lysate was sonicated and centrifuged for 10 min at 13,000 x g to remove cell debris. Protein concentrations were determined using a bicinchoninic acid assay (BCA) (Thermo Scientific, Waltham, MA, USA). Equal amounts of proteins (30-40 μg/lane) were separated using SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with an appropriate primary antibody and a secondary antibody conjugated to horseradish peroxidase. The following antibodies were utilized:

GAPDH (1:1,000 dilution, Cell Signalling Technology Inc., Danvers, MA, USA), SOS1 (1:1,000 dilution, Cell Signalling Technology Inc.), Ras (1:1,000 dilution, Millipore, Billerica, MA, USA), p-Raf (1:1,000 dilution, Epitomics, Burlingame, CA, USA), p-ERK (1:1,000 dilution, Epitomics) and ERK (1:5,000 dilution, Epitomics). Proteins were visualized with enhanced chemiluminescence (Millipore). Three independent experiments were performed and one representative result is shown.

Statistical analysis. Data were presented as the means ± SE. Quantified data represent an average of at least triplicate samples or as otherwise indicated. Error bars represent SE. Statistically significant differences were determined by the Student's t-test. $P<0.05$ was taken to indicate a statistically significant difference.

Results

miR-124 is downregulated in GBM cell lines and the stable overexpression of miR-124 inhibits cell growth. To explore the functional role of miR-124 in glioma carcinogenesis, we first detected the expression levels of miR-124 using real-time PCR in five GBM cell lines. The results revealed that all four GBM cell lines (U87, U373, SW1088 and SW1783) had significantly lower levels of miR-124 expression than those of the HA cell line (Fig. 1A). To further explore the theory that miR-124 is important for cell proliferation, we constructed a miR-124 overexpression model in U87 and U373 cells infected with miR-124 by the lentivirus pCDH-CMV system, designated as U87-miR-124 or U373-miR-124, respectively, and cells infected with an empty virus vector were used as a control. The overexpression of miR-124 in U87-miR-124 and U373-miR-124 cells was confirmed using qRT-PCR (Fig. 1B). The cell proliferation assays revealed that the overexpression of miR-124 suppresses the proliferation of GBM cells (Fig. 1C and D). The data indicate that a decrease in miR-124 expression exerts a growth-inhibiting function in human GBM.

miR-124 directly targets SOS1 in human GBM cell lines and miR-124 negatively regulates endogenous SOS1 expression in GBM. It is generally accepted that miRNAs regulate expression of their downstream gene targets in order to exert their function. To clarify the molecular mechanisms by which miR-124 inhibits glioblastoma cell growth, we predicted its downstream targets using the algorithms: TargetScan (23) and PicTar (24). Among the candidate target genes commonly predicted by the algorithms was SOS1. To validate that SOS1 is targeted by miR-124, we subcloned segments of the 3'UTRs of SOS1 into a pCS2-Luc reporter vector. There are three binding sites which miR-124 was predicted to target (Fig. 2A), and we constructed mutant reporters, respectively (Fig. 2B). We used 293T cells, which have very low or undetectable levels of miR-124, for the reporter assays, and tested the effects of miRNA mimics on the relative Firefly luciferase ratio. miR-124 overexpression reduced the expression of a luciferase reporter containing wild-type SOS1 3'UTR, i.e., a mutant of all binding sites did not affect luciferase activity, and every binding site wound affected luciferase activity (Fig. 2C). We then co-transfected anti-miR-124 and SOS1-3'UTR-WT or SOS1-3'UTR-MUT into miR-124-overexpressing U87 (U87-miR-124) cells and observed that the anti-miR-124 inhibitor rescued the luciferase activities of the reporter containing the wild-type SOS1 3'UTR (Fig. 2D), but the mutant did not. We detected the endogenous protein expression of SOS1 and discovered that the protein expression of SOS1 was significantly decreased in U87-miR-124 and U373-miR-124 cells which had over-

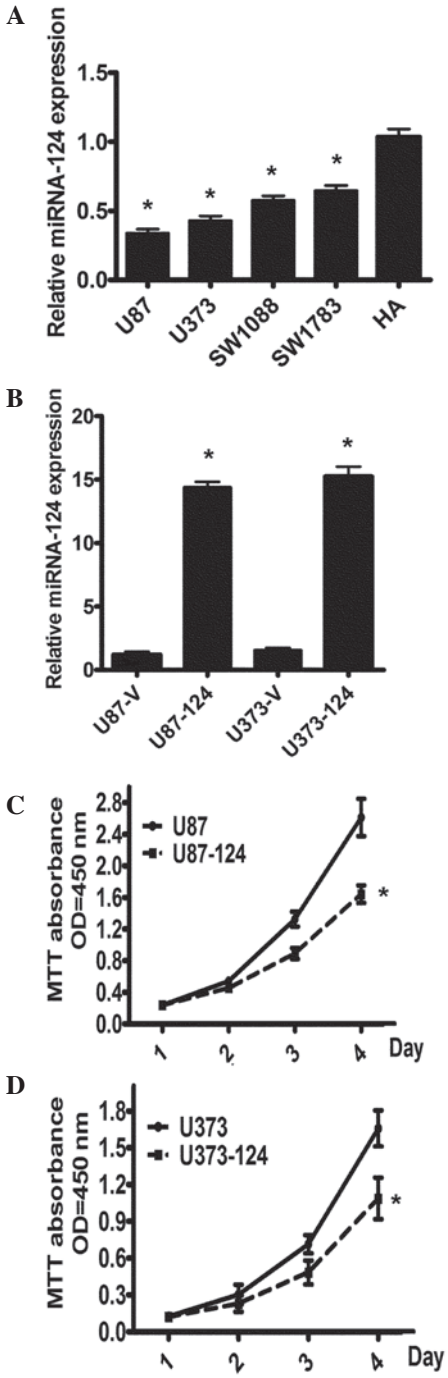

Figure 1. Expression of miR-124 in human glioma cell lines and its function in suppressing GBM cell growth *in vitro*. (A) Relative expression levels of miR-124 in glioma cell lines were assayed. (B) Relative miR-124 expression levels in U87 and U373 cells after stable transfection with miR-124 as determined by qRT-PCR. (C and D) The effect of miR-124 on cell proliferation of miR-124-overexpressing cells (U87 and U373). Expression levels were normalized for U6. All experiments were independently undertaken three times. *$P<0.05$, $P<0.01$ compared to miR-control cells. GBM, glioblastoma multiforme.

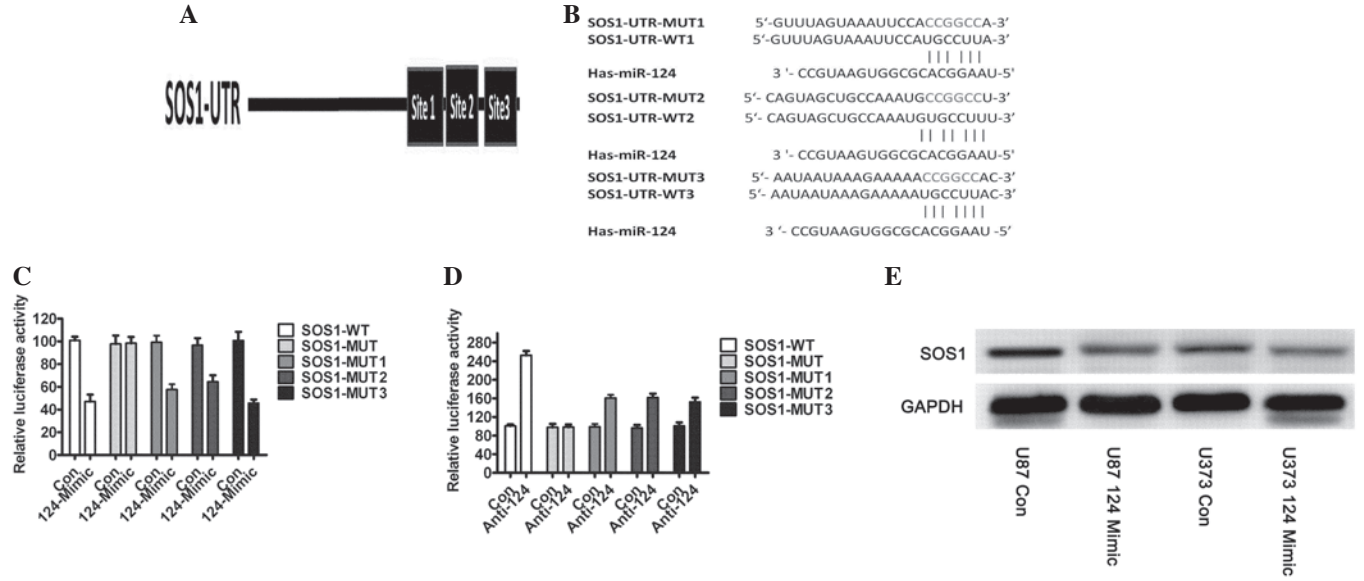

Figure 2. miR-124 downregulates SOS1 expression through a specific binding site located in the 3'UTR. (A) Schematic representation of the three predicted miR-124 binding sites; the numbers indicate the position in the human SOS1-3' UTR. (B) Three predicted target sequences of miR-124 within the 3'UTR of SOS1 mRNA. Several nucleotides within the seed region were mutated in the 3'UTR of SOS1. (C and D) Luciferase assay demonstrates a decrease in reporter activity after co-transfection of SOS1-3'UTR with miR-124 in 293T cells or anti-miR-124 in U87-miR-124, the mutant UTRs had no effect on reporter activity. (E) Western blot analysis of SOS1 expression in U87 and U373 cells infected with control or miR-124 at 96 h post-infection. GAPDH was used as an internal control.

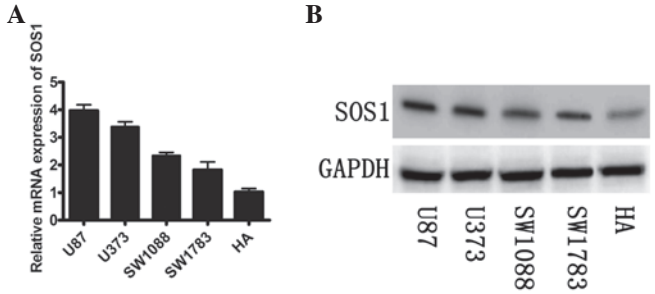

Figure 3. Expression of SOS1 in glioma cell lines. (A) Relative mRNA expression level of SOS1 was measured in glioma cell lines by qRT-PCR. (B) Western blot analysis of SOS1 expression in five glioma cell lines.

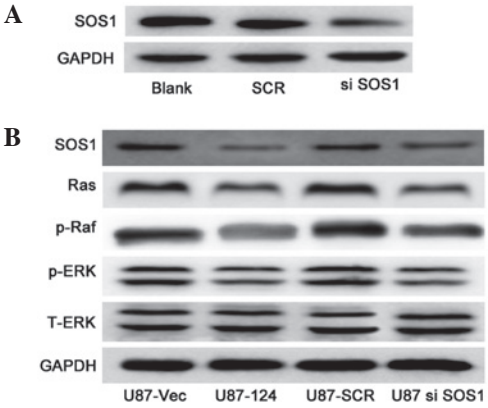

Figure 4. miR-124 suppressed the SOS1-mediated SOS1/Ras/ERK signal pathway. (A) Endogenous SOS1 expression assayed using western blotting in cells expressing siRNA directed against SOS1. (B) Expression profile changes of the Ras, p-Raf and p-ERK genes at the protein level, miR-124-overexpression significantly inhibited the expression of p-Raf and p-ERK protein. At the same time, the inhibition of SOS1 yielded similar results.

expressed miR-124 (Fig. 2E). Taken together, these results demonstrated that SOS1 was a direct target of miR-124 action in GBM.

miR-124 negatively regulates endogenous SOS1 expression. We examined the mRNA and protein expression of SOS1 in the GBM cell lines. A significant inverse correlation between the mRNA and protein expression of SOS1 and levels of miR-124 was observed (Fig. 3A and B). It was demonstrated that low levels of miR-124 were more likely to be observed in the GBM cell lines with a high expression of SOS1 mRNA and protein.

miR-124 suppresses SOS1 and negatively regulates the MAPK pathway in GBM cell lines. To investigate the molecular mechanism of miR-124-mediated cell growth, we examined the expression of vital components of the MAPK pathway, which are critical in the regulation of cell growth in U87 cells with or without miR-124 overexpression. We performed loss-of-function experiments to further verify that SOS1 targeting is involved in miR-124-mediated growth inhibition in U87 cells. The SOS1 protein can be effectively knocked down (Fig. 4A). Western blot analysis revealed that the protein levels of Ras, p-Raf and p-ERK were significantly decreased in the SOS1 knockdown cells (Fig. 4B), compared to those in the control cells. As expected, upregulation of miR-124 resulted in downregulation of the protein levels of Ras, p-Raf and p-ERK (Fig. 4B). The data indicate that miR-124 is capable of suppressing the growth of U87 cells by targeting SOS1 via the MAPK pathway.

Discussion

Over the last twenty years, miRNA has been proven to play in the regulation of a wide variety of biological processes and various studies have shown that miRNAs regulate gene

expression (25-28). Studies have has been undertaken in order to understand miRNA expression, the impact it has on malignant tumors and patient prognosis, as well as potential strategies for individualized therapy. In this study, we selected miR-124 for a detailed investigation into downregulation in glioblastoma patient samples (29,30). The detailed mechanism(s) surrounding the role of miR-124 in GBM development requires further clarification.

We identified the differential expression of miR-124 in GBM cell lines and explored the molecular mechanism by which miR-124 suppressed glioma cell growth. To identify genes that may be regulated by miR-124, we used algorithms designed to search for matching base pairs in miRNAs and mRNA targets. SOS1 was identified as a direct and functional target of miR-124, a conclusion supported by the following reasons: three complementary sequences of miR-124 were identified in the 3'UTR of SOS1 mRNA; miR-124 overexpression suppresses SOS1 3'UTR luciferase report activity and this effect was eliminated by mutation of the miR-124 seed binding site; overexpression of miR-124 led to a significant reduction in SOS1 at the protein level. SOS1 knockdown induced cell growth inhibition similar to the phenotypes induced by miR-124 upregulation. These findings indicate that miR-124 inhibits glioma cell growth by repressing SOS1 post-transcriptionally.

SOS1 is known to be overexpressed in various types of cancer and plays an important role in signaling to the Ras/ERK cascade (31-33). Grb2/Sos is central to signal transduction following growth factor engagement of receptor tyrosine kinases (RTKs). Upon further examination of the molecular mechanisms of growth inhibition induced by miR-124, we observed the expression of key components of the MAPK pathway. The results are consistent with SOS1 downregulation by siRNA, indicating that the protein levels of Ras, p-Raf and p-ERK are significantly suppressed in cells with an overexpression of miR-124. The major implication of these findings is that miR-124 is downregulated in GBM cells and directly targets SOS1 to inhibit cell growth by the MAPK pathway.

In conclusion, our study demonstrates that downregulated miR-124 is responsible for the upregulation of SOS1 in GBM, and miR-124 has an important role in inhibiting cell growth by regulating the SOS1/Raf/ERK signaling pathway. Our findings bring new insights on the targeted delivery of miR-124 to GBM cells as a potential therapeutic treatment for GBM.

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