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Original Articles

MicroRNA-1 suppresses glioblastoma in preclinical models by targeting fibronectin

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ABSTRACT

Glioblastoma (GBM) is a deadly and incurable brain tumor. Although microRNAs (miRNAs) play critical roles in regulating the cancer cell phenotype, the underlying mechanisms of how they regulate tumorigenesis are incompletely understood. We found that miR-1 is expressed at relatively low levels in brain cancer patients, especially GBM. Ectopic miR-1 expression in GBM cells inhibited proliferation and migration, increased sensitivity to apoptosis induced by the DNA alkylating agent temozolomide *in vitro*, and inhibited GBM tumorigenesis *in vivo*. Expression of miR-1 in GBM cell lines directly targets fibronectin. High fibronectin expression in GBM correlates with poor patient survival and fibronectin expression is inversely correlated with miR-1 expression. Knockout of fibronectin expression in GBM cell lines inhibited proliferation and migration, increased sensitivity to apoptosis induced by temozolomide *in vitro*, and markedly suppressed GBM tumor growth and promoted animal survival. In contrast, restoring fibronectin levels in GBM cells ectopically expressing miR-1 increased tumorigenicity and decreased animal survival. Therefore, these results confirm that miR-1 has tumor suppressive activity in GBM by targeting fibronectin, and that the miR-1/fibronectin pathway may be a potential drug target in this devastating cancer.

1. Introduction

Brain tumors represent an important cause of cancer-related morbidity and mortality in the United States, with malignant gliomas being among the most aggressive and difficult tumors to treat [1]. The median survival for patients with glioblastoma (GBM), the most aggressive histological subtype of glioma in adults, is only ~15 months [1]. Despite advances in our understanding of glioma development and progression, disease course and outcome for GBM patients has not significantly improved for decades [2]. Thus, treatment of GBM patients is a significant clinical challenge requiring molecular insights into GBM tumorigenesis and novel therapeutic approaches.

MicroRNAs (miRNAs) are endogenous, small (20–24 nucleotide) single-stranded RNAs that regulate many fundamental biological processes [3,4]. Although miRNAs are noncoding RNAs, they control cellular protein expression by binding to the 3′ untranslated region (UTR) of target mRNAs, promoting their cleavage or blocking their translation [3,5]. MiRNAs play key roles in cancer initiation, progression and metastasis [4,6], and regulate the sensitivity of tumors to radiation and chemotherapy [7]. While oncogenic miRNAs have been well studied, tumor suppressor miRNAs have been less characterized. We previously identified that miR-203a is expressed at extremely low levels in glioma patient samples and has tumor suppressive activity in glioma by targeting ATM, the gene mutated in ataxia telangiectasia [8]. In the present study, we show that the expression levels of miR-1-3p, hereafter referred to as miR-1, is expressed at relatively low levels in GBM. Previous studies have shown that miR-1 is downregulated in esophageal squamous cell carcinoma and glioma [9,10]. In addition, we found that the mRNA encoding fibronectin (FN1) was a direct target of miR-1. Fibronectin is a matrix glycoprotein that is generally associated with angiogenic blood vessels, but fibronectin overexpression has been described in GBM tumor cells [11]. FN has profound effects on cell adhesion and motility, and FN depletion significantly reduced GBM tumor growth and angiogenesis. In addition, FN appears to function in GBM through a PI3K/Akt signaling pathway [12].

In this study, we examined the *in vitro* and *in vivo* roles of miR-1 and FN1 in GBM. We demonstrate that FN1 is a direct miR-1 target in GBM, and miR-1 suppresses FN1 expression. Ectopic expression of miR-1

Abbreviations: GBM, Glioblastoma; miRNAs, microRNAs; UTR, untranslated region; FN1, fibronectin; PARP, poly (ADP-ribose) polymerase; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; TCGA, The Cancer Genome Atlas; gRNA, guide RNA; TMZ, temozolomide; PI, propidium iodide; LGG, low-grade glioma; EV, empty vector

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sensitized GBM cells to the anticancer effects of the clinically used DNA alkylating agent temozolomide, and inhibited cell migration and invasion. While expression of miR-1 reduced FN protein expression, restoration of FN levels reversed the reduction of GBM cell migration and invasion by miR-1. Furthermore, miR-1 expression inhibited a PI3K/Akt signaling pathway, which was similarly reversed by fibronectin restoration. Genetic ablation of FN in GBM cells inhibited the proliferation and migration of GBM cells. Intracranial injection of GBM cells showed that miR-1 expression markedly inhibited tumorigenicity and prolonged animal survival, while fibronectin restoration in miR-1 expressing cells restored tumorigenicity. Taken together our results indicate that miR-1 is a tumor suppressor miRNA in GBM, acting through a fibronectin dependent pathway.

2. Materials and methods

2.1. Biological reagents and cell cultures

Antibodies against the following proteins were used: poly (ADP-ribose) polymerase (PARP) (Cell Signaling, Danvers, MA); fibronectin, p85, phospho-Akt, PTEN, and actin (Santa Cruz Biotechnology, Santa Cruz, CA). MT330 [13] and LN229 (American Type Culture Collection, Manassas, VA) GBM cell lines were grown in DMEM containing 10% fetal bovine serum (Hyclone) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C with 5% CO_2.

2.2. Gene expression analysis

For miRNA expression, total RNA (5 µg) was reverse-transcribed into first-strand cDNA and 30 ng of cDNA was used as a template for the PCR reaction with a forward primer specific to the mature miR-1 sequence (5′- TGGAATGTAAAGAAGTATGTAT-3′). SYBR Green-based real-time PCR was performed and miRNA expression normalized relative to U6 expression.

2.3. Lentiviral-mediated miR-1 and FN1 expression

Stable pools of miR-1 lentiviral transduced overexpressing GBM cells were isolated as previously described [14]. Restoration of FN1 levels was performed by lentiviral transduction with lentivirus expression vector encoding the FN1 open reading frame (EX-E1480-Lv102 from GeneCopeia, Rockville, MD).

2.4. Immunoblot analysis

Total cell lysates (25 µg) were separated by SDS-PAGE, immunoblotted with the indicated antibodies and visualized as previously described [15].

2.5. Construction of luciferase reporter gene plasmids and reporter assays

The 3′ UTR of FN1 containing the predicted miR-1 binding site was identified by the TargetScan algorithm and amplified by PCR from genomic DNA of human 293 T cells. After digestion with XhoI and BamHI, the PCR product was purified and cloned into pcDNA3.1-luc, resulting in the wild-type FN1 reporter plasmid, pcDNA3.1-Luc-wtUTR. The mutant FN1 reporter plasmid pcDNA3.1-Luc-muUTR was constructed by mutating the miR-1 binding site in the 3′UTR of FN1 using PCR based site-directed mutagenesis (Stratagene). The primers for amplifying the wild-type 3′-UTR were 5′- GATACTCGAGACTGTAGGA ACAAGCATGAT-3′ and 5′- GCGGATCCACAATGGTTAGAAAAGA GCA-3′, and the primers for mutant construct were 5′- GAATTCGCCA ACTTGAAGTTCATCTATTTGATAT-3′ and 5′- AAATCTTTTATTAAAAC AGTTGTCTTTCCACAGTAG-3′. Reporter gene binding assays were performed as previously described [15] by co-transfecting cells using wild-type and mutant reporter plasmids pcDNA3.1-Luc-wtUTR and pcDNA3.1-Luc-muUTR with miR-1 overexpressing plasmid, respectively.

2.6. Tumor xenograft

Animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Xenografts were established in five-week-old male NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory). Luciferase-expressing MT330 cells (10^6) were injected stereotactically into the superficial brain parenchyma of NSG mice through a burr hole in the skull as previously described [16]. NSG mice were injected with D-luciferin and subjected to live animal imaging weekly to quantify bioluminescence [16,17].

2.7. The cancer genome atlas (TCGA) data query

We queried the TCGA portal for all GBM samples with Level 3 miRNA or mRNA expression data. The dataset was filtered for samples having expression data for miR-1, FN1 and accompanying clinical data. Kaplan-Meier analysis was performed to determine the survival of the top 15% miR-1 expressing glioma and GBM patients in the TCGA database (miR-1 high) and the bottom 15% expressing patients (miR-1 low). Statistical analyses were performed using Graphpad Prism.

2.8. Cell proliferation, migration, invasion, wound healing and cell cycle phase distribution

For cell proliferation analysis, cells were plated into 96 well plates (1×10^4 cells/well) and after 24 h placed into the Incucyte live cell analysis system in a 37 °C incubator. Matrigel-coated filter invasion assays using transwell inserts (BD Biosciences) were performed as previously described [18]. Wounds were created in confluent cell monolayers with a sterile 1000 µL pipette tip, and phase-contrast images were recorded to assess wound healing. For cell cycle analysis, cells seeded into 6-well plates (2×10^5 cells/well) were treated in the presence or absence of TMZ (100 µM) for 24 h. After washing with ice-cold PBS, cells were fixed with 70% ethanol at 4 °C overnight, washed with PBS and incubated with propidium iodide (PI)-RNase solution for 30 min at 37 °C. The cell samples were analyzed on flow cytometer (Accuri model 6), and the data analyzed with FlowJo software.

2.9. Generation of FN1–KO cells

The lentiviral CRISPR/Cas9 mediated FN1 knockout vectors were constructed by cloning three FN1 guide RNAs (gRNA1: 5′- GCTCTTCG AGGCTCCCGTGG -3’; gRNA2: 5′- GCGTTGGTTTGTACTTGTTA -3’; and gRNA3: 5′- CACTGGGMCACTTACCGAG -3′) into the Bsm I site of lentiviral vector pLenti CRISPR V2. A control vector was constructed by inserting the EGFP gRNA sequence into the lentiviral vector. Lentivirus were produced by packaging in 293FT cells as we published previously [19]. Stable pools of FN1–KO cells were generated by transducing GBM cells with the lentiviral CRIPSR/Cas9 vectors and selected with 5 µg/ml puromycin.

2.10. Statistical analyses

At least two independent experiments were performed in duplicate, and data are presented as means ± sd. ANOVA and post-hoc least significant difference analysis or Student's t-tests were performed. p values < 0.05 (*), 0.01 (**) and 0.001 (***) were considered statistically significant.

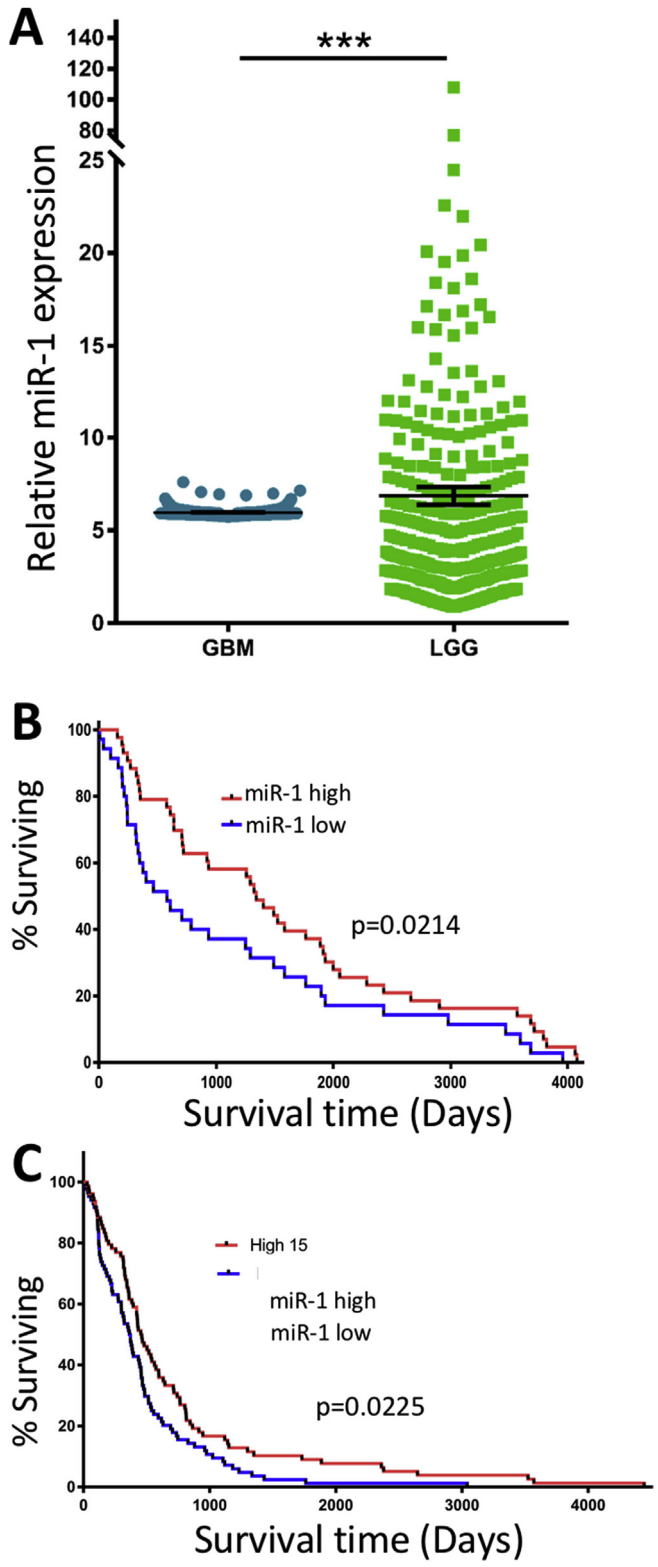

Fig. 1. Expression of miR-1 expression in brain cancer patient samples, and the relationship to patient survival. (A) Low-grade glioma (443 samples) and GBM patient samples (499) in the TCGA database were compared for miR-1 expression. (B) Kaplan-Meier analysis of survival of the top 15% miR-1 expressing glioma patients in the TCGA database (miR-1 high) and the bottom 15% expressing patients (miR-1 low). (C) Kaplan-Meier analysis of survival of the top 15% miR-1 expressing GBM patients in the TCGA database (miR-1 high) and the bottom 15% expressing patients (miR-1 low).

3. Results

3.1. Low expression of miR-1 in GBM tumor tissue is associated with poor patient survival

In previous studies, we found that miR-203a is one of several potential tumor suppressor miRNAs that were expressed at low levels in GBM patients, and ectopic miR-203a expression in GBM cell lines inhibited GBM tumorigenesis *in vitro* and *in vivo* [8,20]. While enforced expression of miR203 in GBM cells resulted in spontaneous induction of IFN-stimulated genes, enforced expression of miR-1 had no such effect [20]. To characterize the potential role of miR-1 in gliomagenesis, we analyzed miR-1 gene expression in low-grade glioma (LGG) and GBM tumor tissue in patient samples in TCGA, and we found that miR-1 was expressed at significantly lower levels in GBM patient as compared to LGG patients (Fig. 1A). LGG patients were stratified into high and low miR-1 expressers, and high miR-1 expressing patients were found to have significantly longer survival than low expressing patients (Fig. 1B). Furthermore, we analyzed patient survival in GBM, where the median survival is rather poor (~15 months), and we found that high miR-1 expressing GBM patients were found to have significantly longer survival than low miR-1 expressing GBM patients (Fig. 1C). Taken together, low miR-1 expression in GBM and the correlation with patient survival, suggested that miR-1 had tumor suppressive function in glioma.

3.2. Enforced miR-1 expression suppresses GBM cell proliferation, migration and invasion, and sensitizes GBM cell to temozolomide

We ectopically expressed miR-1 in MT330 and LN229 GBM cells, and then assessed the functional consequences of miR-1 expression (Fig. 2A). Ectopic expression of miR-1 more dramatically inhibited the proliferation of MT330 cells than LN229 cells by Incucyte live cell analysis (Fig. 2B). Furthermore, treatment with temozolomide, a DNA-alkylating agent used to treat GBM patients, also caused a decrease in GBM cell proliferation and when combined with enforced miR-1 caused a significant inhibition of proliferation in both GBM cell lines with both MT330 and LN229 nearly completely ceasing to divide by 3–5 days after treatment (Fig. 2B). Analysis of the cell cycle phase distribution by flow cytometry of propidium iodide-stained cells showed that miR-1 expression and TMZ treatment (100 μM, 24 h) caused arrest of both MT330 and LN229 GBM cells in the G1 phase of the cell cycle, and in combination resulted in marked G1 arrest (> 70%) of both GBM cells (Fig. 2C). Furthermore, enforced miR-1 expression also sensitized both LN229 and MT330 cells to TMZ induced apoptosis, as evidenced by PARP cleavage in immunoblots at lower concentrations of TMZ in miR-1 enforced GBM cells (Fig. 2D). In addition, miR-1 expression reduced the migration of MT330 and LN229 cells as assessed by wound healing (Fig. 2E), and transwell migration (Fig. 2F) assays. Expression of miR-1 also reduced the invasion of MT330 and LN229 cells through Matrigel-coated transwell plates (Fig. 2G).

3.3. FN1 is a miR-1 target gene

By binding to target mRNAs and silencing their expression, miRNAs control cellular gene expression. By bioinformatics analysis, we identified FN1 (fibronectin) as a potential miR-1 target, which has previously been shown to be overexpressed in GBM cell lines and patient tissue [11,12]. Consistent with fibronectin being a miR-1 target gene, immunoblotting of whole cell lysates prepared from empty vector (EV) and miR-1 enforced MT330 and LN229 cells showed that protein levels of fibronectin were markedly lower in GBM cells with enforced miR-1 expression, but the levels of actin were unaffected (Fig. 3A). Using the miR-1 core seed sequence (ACAUUC), we identified a complementary binding site in the 3′ UTR of FN1 (Fig. 3B). To determine whether FN1 was a direct miR-1 target, the 3′UTR of the FN1 mRNA containing the

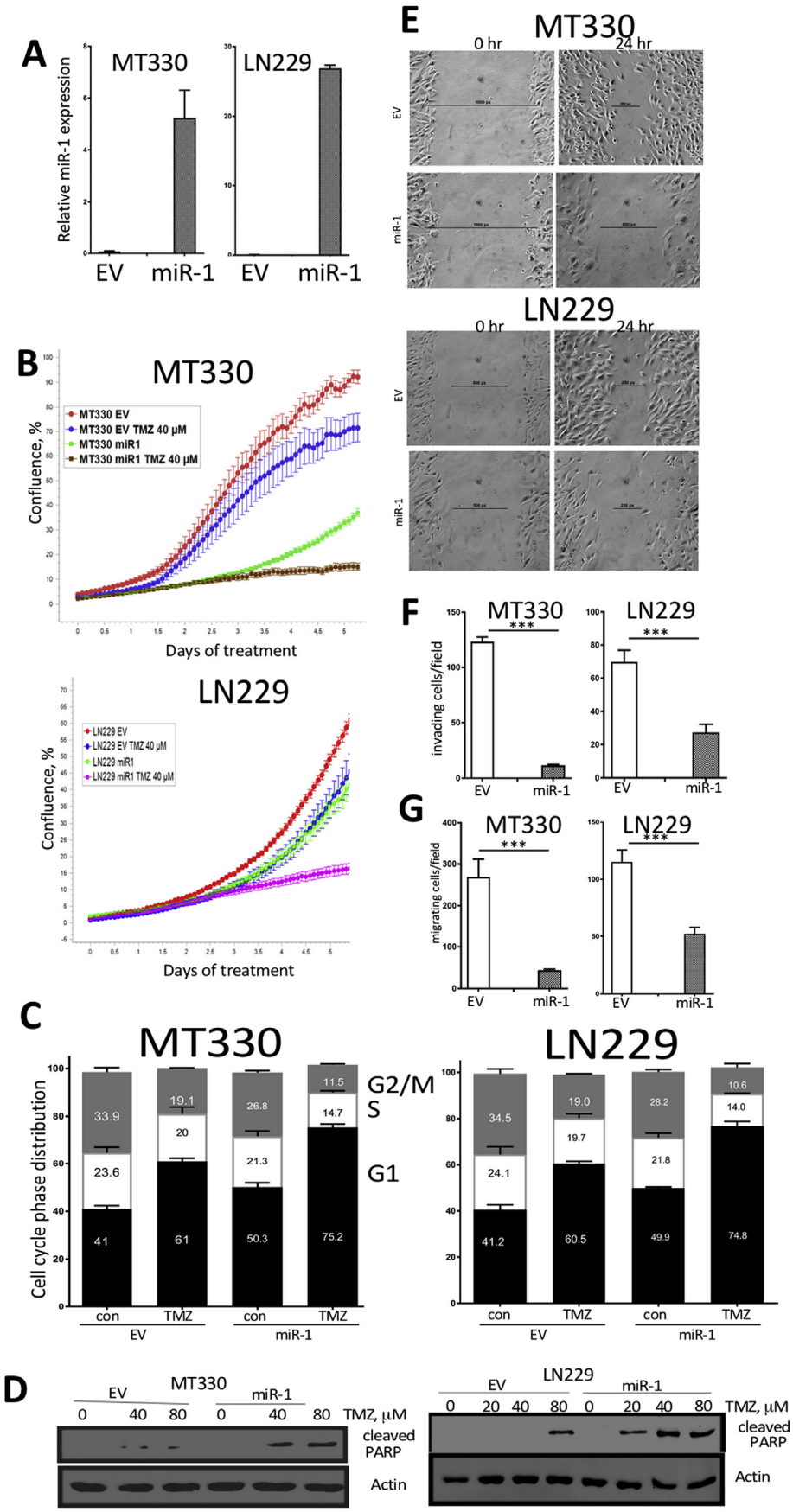

Fig. 2. Enforced miR-1 expression suppresses GBM cell proliferation, migration and invasion, and sensitizes GBM cell to temozolomide. MT330 and LN229 GBM cells were transduced with miR-1-encoding, or empty-vector (EV) lentivirus. (A) RNA extracts were assayed for miR-1 expression relative to U6A expression by qPCR. (B) MT330 and LN229 cells were plated into 96 well plates, treated with or without TMZ (40 μM), and cell proliferation determined by live cell analysis. (C) Cell cycle phase distribution was determined by flow cytometry on propidium-iodide cells treated with TMZ (100 μM, 24 h). (D) Protein lysates of cells treated in the presence of TMZ (0, 40 or 80 μM, 24 h) were immunoblotted for cleaved PARP or actin. (E) Wound healing assays on MT330 and LN229 cells. (F) Transwell plate assays on MT330 and LN229 cells, and migrating cells were stained with crystal violet and quantified. (G) Matrigel-coated transwell assays were performed to determine invasion by MT330 and LN229 cells, and invaded cells were stained with crystal violet and quantified. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

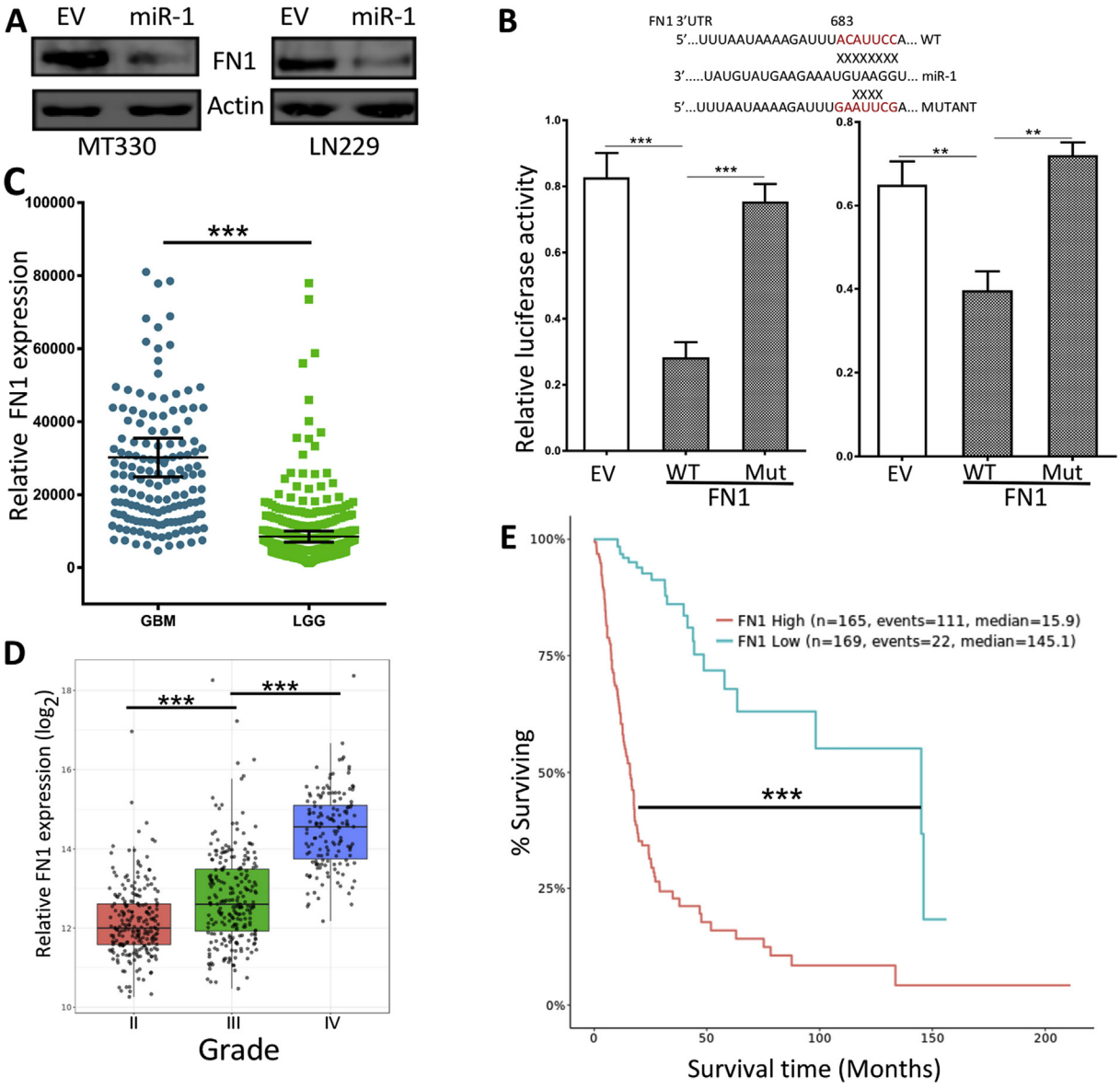

Fig. 3. FN1 is a miR-1 target gene. (A) Protein lysates of MT330 and LN229 GBM cells that were transduced with miR-1 encoding or empty-vector (EV) lentivirus were immunoblotted as indicated. (B) Sequence alignment of the miR-1 binding sequence with the 3′UTR of the wild-type (WT) and mutant (mut) FN1 constructs used in reporter assays. 293 T and MT330 cells were transiently cotransfected with miR-1 plasmid, pSV40-Renilla, and with plasmid empty-vector (pcDNA3.1-Luc), WT (pcDNA3.1-Luc-wtUTR) or mutant (pcDNA3.1-Luc-muUTR) FN1 reporter plasmids. The ratio of luciferase and Renilla activities was determined at 24 h post-transfection. (C) Low-grade glioma (443 samples) and GBM patient samples (499) in the TCGA database were compared for FN1 expression. (D) Grades II, III and IV gliomas in the TCGA database were compared for FN1 expression. (E) Kaplan-Meier analysis of the survival of high and low FN1 expressing glioma patients in the TCGA database.

predicted miR-1 target sequence as well as a corresponding mutated sequence were linked to luciferase, and a dual-luciferase (pcDNA3.1-Luc) reporter system was employed to determine miRNA:mRNA interactions [8,15,21]. Overexpression of miR-1 in HEK293T (easy to transfect cell line that drives high construct-driven expression) and MT330 GBM cells downregulated luciferase activity of the wild-type FN1-3′ UTR-fused reporter construct, while a construct fused to the mutated miR-1 binding sequence in FN1 was unaffected by miR-1 overexpression (Fig. 3B). Taken together, these results show that FN1 is a *bona fide* miR-1 target gene.

3.4. High FN1 expression correlates with poor patient survival and increasing glioma grade

To characterize the role of FN1 in GBM, we analyzed FN1 expression in the TCGA dataset and found that FN1 was expressed at significantly higher levels in GBM patient samples than in LGG ($p < 0.0001$) (Fig. 3C). We then examined FN1 expression in the TCGA database in the different glioma grades (Grades II, III and IV), and found that FN1 expression increased with increasing glioma grade with highest expression in GBM ($p = < 0.0001$) (Fig. 3D). We then examined the relationship between FN1 expression and individual glioma patient survival in the TCGA dataset. FN1-high glioma patients had significantly shorter survival than FN1-low glioma patients ($p < 0.0001$) (Fig. 3E). Taken together, these results show that high FN1 expression in glioma is associated with poor patient survival.

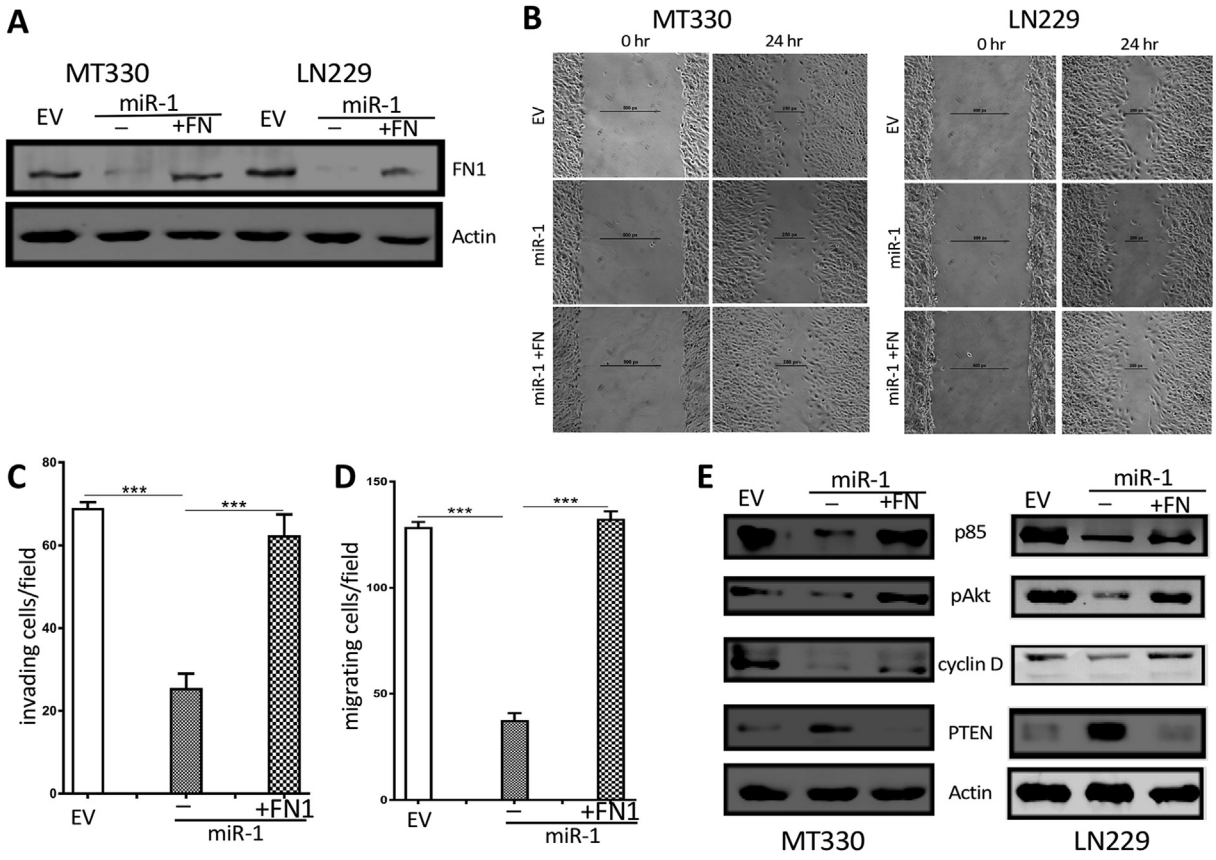

Fig. 4. Restoration of FN1 in miR-1 expressing GBM reverses the suppressive effects of miR-1 on cell migration and invasion, and on the PI3K/Akt pathway. (A) Protein lysates of MT330 and LN229 GBM cells that were transduced with miR-1 encoding or empty-vector (EV) lentivirus and with FN1 expression restored were immunoblotted as indicated. (B) wound healing, (C) transwell migration, and (D) Matrigel-coated transwell assays were performed as described in Fig. 2 (E) Protein lysates were immunoblotted as indicated for components of the PI3K/Akt pathway.

3.5. The role of fibronectin in the miR-1 biological action on GBM cells in vitro

To investigate the role of fibronectin in miR-1 action on GBM cells *in vitro*, fibronectin expression was restored in GBM cells that ectopically expressed miR-1 (Fig. 4A). Consistent with our previous finding (Fig. 2), while miR-1 expression in LN229 and MT330 cells inhibited cell migration in wound healing and transwell assays, restoration of fibronectin expression in these GBM cells resulted in cell migration equivalent to control (EV) GBM cells (Fig. 4B and D). Similarly, while miR-1 inhibited GBM cell invasion in Matrigel-coated filter invasion assays (Fig. 4C), restoration of fibronectin expression resulted in cell invasion equivalent to that in control GBM cells. Moreover, in agreement with previous findings that fibronectin acts through a PI3K/AKT signaling pathway in glioma cells [12], miR-1 expression in both LN229 and MT330 GBM cells resulted in decreased levels of p85, phospho-Akt, and cyclin D, and increased PTEN levels as determined by immunoblotting (Fig. 4E). However, restoration of fibronectin expression in miR-1 expressing GBM resulted in p85, phospho-Akt, cyclin D, and PTEN levels similar to that in control (EV) GBM cells.

Since FN1 was expressed at relatively high levels in GBM patient samples and cell lines, we examined the biological consequences of ablating FN1 expression in GBM cells by CRISPR/Cas9 mediated knockout (KO) using three individual guide RNA sequences. As determined by immunoblotting of cell lysates, fibronectin expression was nearly completely ablated in independent pools of FN1–KO MT330 cell lines generated with each guide RNA (KO1, KO2, KO3) (Fig. 5A). We next examined the biological consequences of FN1–KO in GBM cells *in vitro*. The effect of FN1–KO on cell proliferation was monitored daily with the Incucyte live cell analysis, and FN1–KO was found to inhibit the proliferation of each of these MT330 glioma cell lines (Fig. 5B). In addition, FN1–KO inhibited cell migration as evidenced by impaired wound healing, decreased cell invasion in transwell assays, and decreased numbers of migrating cells in transwell assays (Fig. 5C and D). In addition, as observed in miR-1 enforced cells, there was accumulation of cells in the G1 phase of the cell cycle as determined by flow cytometry, and a sensitization to the effect of TMZ on G1 arrest of cells (Fig. 5E). Taken together these studies indicate that FN1–KO closely resembles the effects of miR-1 expression on GBM cell behavior *in vitro*.

3.6. Enforced miR-1 expression inhibits GBM tumorigenesis

We then determined the effect of enforced miR-1 expression on the tumorigenicity of GBM cells *in vivo*. The role of miR-1 was determined in the orthotopic microenvironment for GBM by intracranial injections of luciferase-expressing control and miR-1 expressing MT330 cells, and tumorigenesis followed by live animal imaging after D-luciferin injection. Significant bioluminescent signal was evident throughout the brains of mice injected with control MT330 cells demonstrating marked tumor induction and invasion. In contrast, brain tumor formation was significantly reduced in mice injected with miR-1 expressing MT330 cells (Fig. 6A). Moreover, as shown in Fig. 6B, survival was significantly prolonged in mice injected intracranially with miR-1 cell lines. In cells ectopically-expressing miR-1, we restored FN1 expression by an ORF construct that lacks the miR-1 binding site (Fig. 4A) and examined the effect of FN1 re-expression on cell tumorigenicity upon intracranial injection. Although enforced miR-1 expression in MT330 cells markedly reduced tumorigenicity, restoration of FN1 expression increased tumorigenicity, similar to that observed with control MT330 cells (Fig. 6A). Furthermore, animal survival was markedly

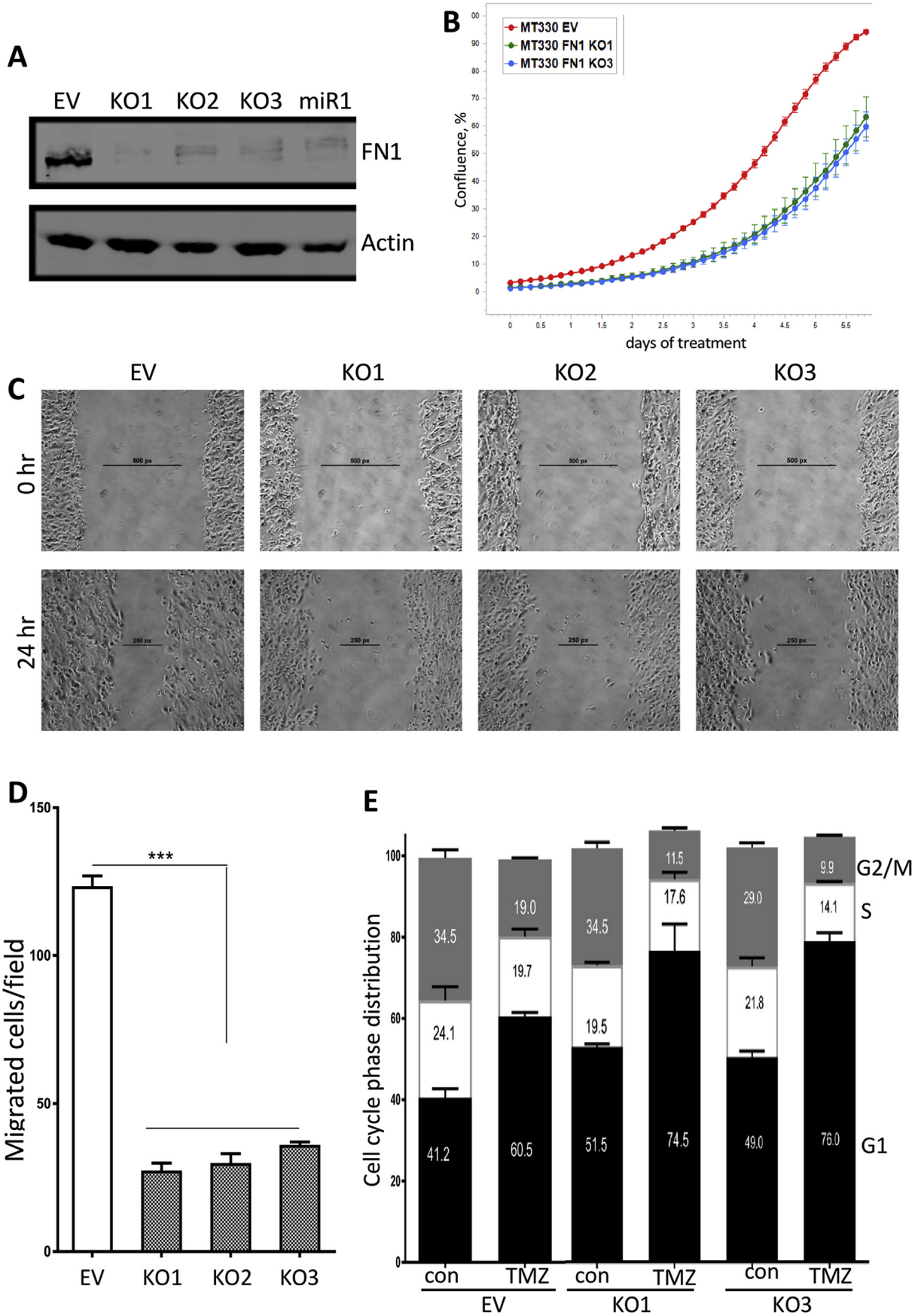

Fig. 5. Characterization of FN1–KO MT330 cells. (A) Cell lysates were prepared from pools of FN1–KO MT330 cells using 3 different gRNAs and immunoblotted as indicated. (B) Proliferation of FN1–KO1 and FN1–KO3 cells was determined by live cell analysis. (C, D) Wound healing (C), and transwell migration (D) were performed as described in Fig. 2 (E) Cell cycle phase distribution was determined by flow cytometry on propidium-iodide cells treated with TMZ (100 μM, 24 h).

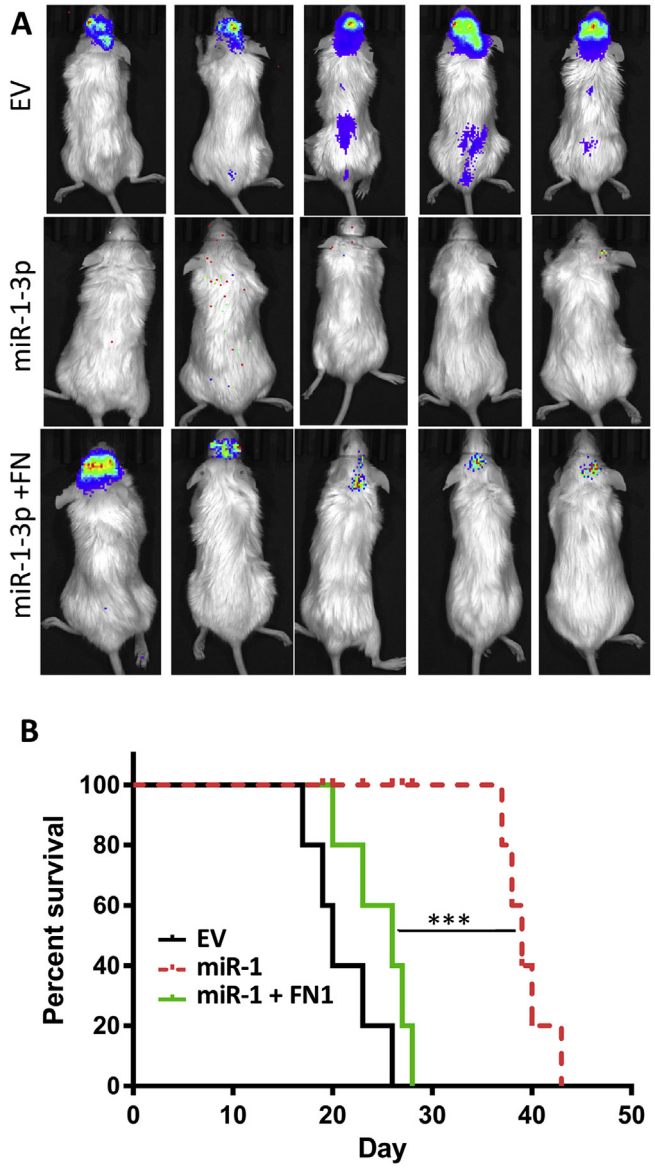

Fig. 6. The effects of miR-1 expression and restoration of FN1 on tumor formation by GBM cell lines. (A) Live animal imaging at 6 weeks after intracranial injection with 10^6 EV, miR-1 expressing or FN1 restored MT330 cells. (B) Kaplan-Meier analysis of survival data of the mice (5 mice/group) injected in Panel A was performed.

reduced after restoring FN1 expression to nearly that of mice injected with control (EV) MT330 cells (Fig. 6B). Taken together, these results show that, while miR-1 has tumor suppressive activity on GBM cells *in vivo*, FN1 has pro-tumorigenic action.

4. Discussion

GBM continues to be a malignancy that has an extremely poor prognosis. Therefore, it is critical to identify new molecular targets for potential therapeutic treatment. Many miRNAs have been implicated in GBM pathophysiology and therapy [15,20,22–25]. In the present study, we show that miR-1 is tumor suppressive miRNA in GBM. First, miR-1 is expressed at relatively low levels in GBM, and high miR-1 expression is associated with better patient survival in glioma, and even in GBM the most malignant form of glioma miR-1 is associated with better patient outcome. Expression of miR-1 has been shown to be downregulated in many cancers, including lung, gastric, breast, prostate, esophageal and brain cancer [9,10,26–30]. Then we show that through ectopic expression of miR-1 in GBM cells that miR-1 has tumor suppressive activity on GBM *in vitro* because miR-1 inhibits GBM cell proliferation, invasion, migration and sensitizes cells to the DNA alkylating agent temozolomide (TMZ) that is used in the clinical management of GBM patients. Our studies are in concordance with previous studies in GBM demonstrating that enforced miR-1 expression has marked effects on the phenotype of GBM cells *in vitro* [10].

Since miRNAs suppress the expression of their target genes, we determined genes potentially downregulated by miR-1 by bioinformatic analysis. Here we provide novel evidence the tumor suppressive activity of miR-1 is mediated through its targeting of the FN1 gene that encodes fibronectin. We demonstrate that enforced miR-1 expression inhibited the protein levels of fibronectin and that FN1 gene expression was directly regulated by miR-1 by using luciferase reporter assays driven by wild-type and mutant 3′UTR sequences of FN1. These results identified for the first time that FN1 as a *bona fide* miR-1 target in GBM cells. Consistent with our findings, a recent report shows that FN1 is a direct target gene of miR-1 in renal carcinoma cells [31]. We then examined the relationship between FN1 expression in glioma patient tumor samples and survival or severity of disease. We found that not only glioma patients but also GBM patients with the lowest overall survival had significantly higher levels of FN1 expression, and that high-FN1 expressing glioma patients had significantly shorter overall survival as compared to low-FN1 expressing glioma patients. Furthermore, we show that the FN1 gene is expressed at significantly higher levels in GBM than in low-grade glioma, and that FN1 expression increases with increasing tumor grade in glioma. Consistent with our findings, FN1 was previously shown to be gene differentially overexpressed in invasive GBM as compared to non-invasive astrocytoma [32].

To characterize the function of fibronectin in the tumor suppressive action of miR-1 in GBM cells *in vitro*, we restored FN1 in miR-1 overexpressing GBM cells, as well as ablated FN1 expression by CRISPR/Cas9-mediated knockout. We found that fibronectin expression in GBM cells restored cell migration and invasion that was inhibited by miR-1 overexpression in GBM cells. In addition, FN1 knockout in GBM cells inhibited cell migration and invasion, and FN1 knockout sensitized to cell cycle arrest induced by TMZ treatment of GBM cells. Previous studies in GBM cell lines showed that fibronectin expression promotes cell migration and invasion [11]. Moreover, consistent with the previous studies on fibronectin promoting a PI3K/Akt pathway [12], while miR-1 reduced the levels p85, cyclin D and phospho-Akt, it increased PTEN levels. In contrast, restoration of FN1 expression in miR-1 overexpressing GBM cells increased p85, cyclin D and phospho-Akt levels and reduced PTEN levels. Therefore, FN1 knockout in GBM cells mirrored the effects of miR-1 overexpression on GBM cell phenotype.

Finally, we show that miR-1 suppresses GBM tumorigenicity in GBM animal intracranial xenografts, and that this is mediated through a fibronectin-dependent pathway. While miR-1 dramatically suppressed the tumorigenicity of GBM cells when injected into the brains of immunocompromised mice, restoration of fibronectin increased the tumorigenicity of GBM cells. Furthermore, while ectopic expression of miR-1 in GBM cells markedly increased the survival of mice with intracranial GBM xenografts, restoration of fibronectin expression in miR-1 expressing GBM cells reduced animal survival similar to that of control GBM cells. Our findings are consistent with the previous studies that show that miR-1 has tumor suppressive activity in animal models of GBM [10], while fibronectin promoted orthotopic GBM tumor growth [11]. The mRNA encoding Annexin A2 was previously found to be a miR-1 target in GBM and plays an important role in miR-1 directed tumor suppressive signaling network [10]. However, our studies are the first to establish that the fibronectin pathway is an important pathway the miR-1 targets in GBM for its tumor suppressive activity. In summary, our findings suggest that miR-1 is tumor suppressive miRNA in GBM by inhibiting the pro-tumorigenic function of fibronectin.

Conflict of interest

All authors declare no potential conflicts of interest.

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