Original Article
MicroRNA-141 inhibits glioma cells growth and metastasis by targeting TGF-β2

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Abstract: MicroRNA-141 (miR-141) has been reported to function as tumor suppressor in many types of cancer. However, the molecular function and underlying mechanisms of miR-141 in glioma is still unknown. The aims of this study were to investigate miR-141 expression and determine its biological function and underlying mechanism in glioma. In this study, we found that miR-141 expression levels, both in glioma cell lines and in tissues, were significantly lower than that in a normal human astrocyte cell line or adjacent non-cancerous tissues. Overexpression of miR-141 significantly inhibited glioma cell proliferation, colony formation, migration and invasion *in vitro*, as well as suppressed glioma tumor growth *in vivo*. In addition, transforming growth factor beta 2 (TGF-β2) was identified as a target of miR-141 in glioma cells. TGF-β2 expression was also found to be upregulated, and negatively associated with miR-141 in glioma tissues. TGF-β2 over-expression partly reversed the effect caused by transfection of miR-141 mimic. These findings together suggested that miR-141 functioned as tumor suppressor by targeting TGF-β2, and that miR-141 might be a promising therapeutic strategy for future treatment of glioma.

Keywords: Glioma, miR-141, TGF-β2, proliferation, migration, invasion

Introduction

Human brain glioma is the most common endocranial tumor with high mortality and morbidity rates worldwide [1, 2]. Although the 5-year survival rate of patients with glioma has improved in recent years due to the combination of surgery, radiotherapy and chemotherapy, the prognosis of high-grade glioma remains gloomy mainly due to the low sensitivity to radio-/ chemo- therapeutic agents, and tumor metastasis [3, 4]. Therefore, improved understanding of the mechanisms that involved in progression and metastasis of glioma is urgent essential to develop more effective therapies.

MicroRNAs (miRNAs) are a group of small non-coding RNAs that regulate gene expression by translation repression or messenger RNA (mRNA) degradation by binding to the 3'-un-translated region (3'-UTR) of target mRNA [5]. miRNAs have been shown to involve in diverse biological processes, such as cell differentiation, tumorigenesis, cell death, proliferation, metastasis, and drug resistance [6-8]. Accumulating evidence has indicated that microRNAs, with anomalous expression and functions, may act as both classical oncogenes and tumor suppressor in various malignant cancers [9]. In glioma, a larger number of miRNAs, such as miR-92b, miR-21, miR-221, miR-34a and miR-218 have been found to be associate with the initiation and progression of glioma [10, 11].

miR-141 is a member of the miR-200 family that was associated with the growth, apoptotic response and regulation of metastasis. miR-141 has been reported to inhibit migration, invasion, proliferation and drug resistance in nasopharyngeal carcinoma [12], hepatocellular carcinoma [13], gastric cancer [14], and breast cancer [15, 16]. However, the detail biological function and underlying molecular mechanism of miR-141 in glioma is still unclear. Therefore, the aims of this study were to investigate the miR-141 expression and analyze its biological

role and underlying molecular mechanism in glioma.

Materials and methods

Clinical samples

Glioma tissues and matched adjacent normal tissue samples were obtained from 36 patients with primary gliomas who underwent surgical treatment at the Department of Neurosurgery, the first of Hospital of Jilin University during July 2012 to December 2014 in accordance with the national regulation of clinical sampling in China. None of patients had received chemotherapy, immunotherapy, radiotherapy or other therapy prior to surgery. All tissues specimens were immediately sectioned from the resected glioma tissues, frozen in liquid nitrogen and stored at -80°C until use. This study was approved by the Medical Ethics Committee of Jilin University (Changchun, China) and written informed consent was obtained from all patients.

Cell lines and transfection

The glioma cell lines U251, U87, U118 and LN18 and primary normal human astrocytes (NHA) were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely maintained in complete Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma, St- Louis, MO, USA) and 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere containing 5% CO_2.

miR-141 mimic (miR-141) and corresponding miRNA negative control (miR-NC) were brought from Ribobio Co (Guangzhou, China). TGF-β2 overexpressed plasmid was designed and synthesized by GenePharma Co., Ltd (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Transfection efficiencies were determined in every experiment at 48 h after transfection.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from cultured cells or tissues using TRIzol Reagent (Invitrogen) acco-

rding to the manufacturer's protocols. The purity and concentration of total RNA were determined using a ultraviolet spectrophotometer (Eppendorf, Germany). To quantify miR-141, total RNA was reverse transcribed using the TaqMan® MicroRNA reverse transcription kit (Invitrogen), and quantitative PCR was performed using TaqMan microRNA assays with specific primers for miR-141 (Applied Biosystems, Foster City, CA, USA) under an ABI PRISM7900 Sequence Detection System (Applied Biosystems). To quantify TGF-β2 mRNA, total RNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit (Takara, Dalian, China), and quantitative PCR was performed using Real-time PCR Mixture Reagent (Takara) under an ABI PRISM7900 Sequence Detection System. The primers for TGF-β2 and GAPDH were used in this study as described previously [17]. The miRNA and mRNA expression levels were normalized to those of U6 and, respectively, using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and colony formation assay

Cell proliferation was measured by MTT assay. In briefly, transfected cells (100 μl/ well at a density of 1×10^4 cells/well) were seeded in 96-well plates. In indicated time (24 h, 48 h and 72 h), 10 μl MTT solution (5 mg/ml, Sigma, St. Louis, MO, USA) was added to each well, and the plate was incubated at 37°C for 4 h. Afterward, 150 μl dimethyl sulfoxide (DMSO, Sigma) were added to each well, and the plate was vortexed for 10 min at room temperature. Finally, the optical density value (OD) of each well was measured at 490 nm with a Synergy 2 microplate reader (BioTek, USA).

For colony formation assay, the 1,000 transfected cells were seeded in 6-well plates and cultured in DMEM medium containing 10% FBS at 37°C under 5% CO_2 for 14 days. After washed three times with PBS, the colonies were fixed with 75% ethanol for 10 min, dried and stained with 0.1% crystal violet solution (Sigma, USA) for 10 min. Finally, the colonies were counted and taken pictures using light microscope (Olympus, Tokyo, Japan).

Cell migration and invasion assays

Wound healing assay was performed to determine cell migration of glioma cells. In briefly,
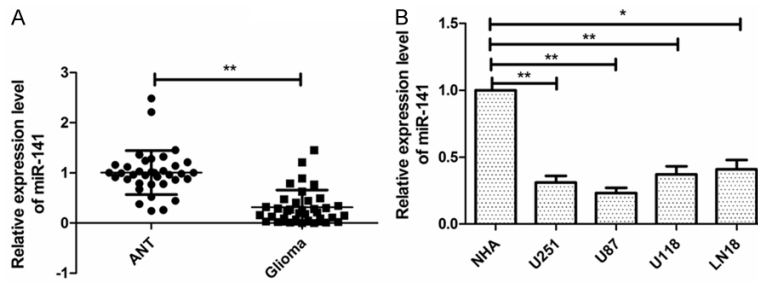
Figure 1. miR-141 expression was downregulated in glioma tissues and cell lines. A. The relative expression of miR-141 was examined by RT-qPCR in glioma tissues (glioma) and adjacent noncancerous tissues (ANT). U6 was used as loading control. B. The relative expression of miR-141 was examined in four human glioma cell lines (U251, U87, U118 and LN18) and in the normal human astrocyte (NHA) cell line. U6 was used as loading control. *P < 0.05, **P < 0.01.

10^5 cells were plated in 24-well plates and cultured for 24 h, then cells were co-transfected with 50 ng of TGF-β2 -Wt or TGF-β2 -Mut reporter plasmid, and 50 nM of miR-141 mimic or miR-NC using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, both firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization.

transfected cells were plated in 6-well plates at density of 1×10^6 cells per well. When cell was reached 90% confluency, cells were scratched with a 200-uL filter tip to create an artificial wound. After wounding, the medium was changed to fresh serum-free DMEM medium to remove cellular debris. The wounds in each well were photographed at 0 and 24 h using light microscope (Olympus). To assess the migration rate, we measured the fraction of cell coverage across the line.

Cell invasion was determined using the Matrigel Invasion Chamber (BD Biosciences). Transfected cells (1×10^5) were seeded on transwell chambers with Matrigel in serum-free medium. DMEM medium containing 20% FBS were added to the lower chamber as the chemo-attractant. After 48 h of incubation at 37°C with 5% CO_2, the invasive cells that attached to the lower surface of the membrane insert were fixed with 90% alcohol and stained with 0.1% crystal violet for 5 min, then photographed. The cells that had migrated to the bottom of the insert were counted in five randomly selected fields under a light microscope (Olympus).

Luciferase assay

Wild-type (Wt) human TGF-β2 3'-UTR and mutated (Mut) TGF-β2 3'-UTR (with mutant sequence on the miR-141 binding site) were amplified by PCR from a human brain cDNA library and inserted into pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and XhoI restriction sites. For luciferase assays, the 1 ×

Western blot assay

The method used for Western blot analysis has been described in our previous study [18]. The primary antibodies were anti-TGF-β2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-GAPDH (Santa Cruz Biotechnology).

Xenograft experiments

Ten male BALB/c mice (4-5 weeks old) were obtained from Experiments Animal Center of Changchun Biological Institute (Changchun, China), and kept under specific pathogen-free (SPF) conditions. All animal studies were approved by the Institutional Animal Care and Use Committee of Jilin University (Changchun, China).

2×10^6 U87 cells stably expressing miR-141 or miR-NC diluted in 200 µl of PBS were inoculated subcutaneously into the right flank of nude mice (n = 5), respectively. The tumor volume was measured every five days using a vernier caliper and was calculated according to the formula: Volume (mm³) = 1/2 × width² × length. About 30 days after inoculation, the mice were euthanized using a subcutaneous injection with sodium pentobarbital (50 mg/kg), and the tumor tissues were striped and weighted.

Statistical analysis

Data from at least three independent experiments are expressed as mean ± standard deviation (SD). One-way ANOVA or Student's t test were utilized to determine the significant differ-
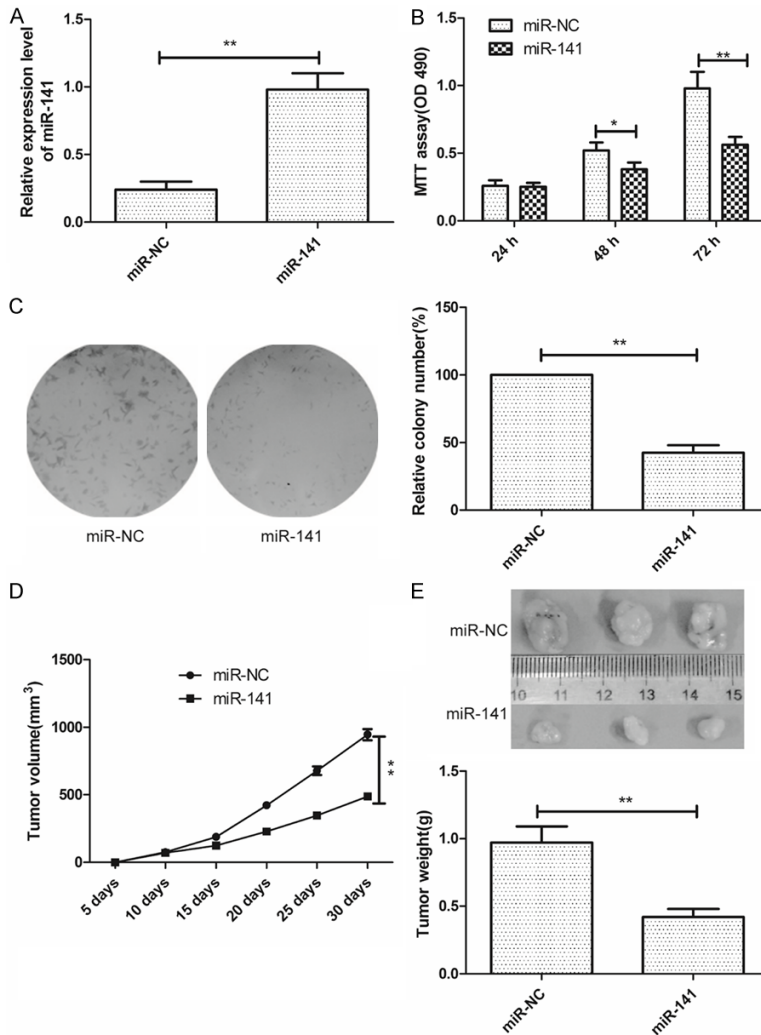
Figure 2. miR-141 inhibits glioma growth *in vitro* and *in vivo*. A. qRT-PCR was used to detect the expression of miR-141 in U87 cells after transfection with miR-141 mimic or miR-NC. U6 was used as the loading control. B. Effect of miR-141 on the proliferation of U87 cells was detected by the MTT assay. C. Effect of miR-141 on the colony formation of U87 cells was detected by colony forming assay. D. Tumor growth curves were established by measurement tumor volume every five days during treatment. E. Tumor tissues image and weight. *P < 0.05, **P < 0.01.

detected by qRT-PCR. Compared with the adjacent non-cancerous tissues, glioma tissues had significantly lower miR-141 expression levels (*P* < 0.001; **Figure 1A**). Moreover, the expression of miR-141 was detected in four human glioma cell lines (U251, U87, U118 and LN18) and normal human astrocytes (NHA) by qRT-PCR. The miR-141 expression in four glioma cell lines was significantly decreased compared to normal human astrocytes (NHA) (**Figure 1B**). The U87 cell line, possessed the lowest expression of miR-141 among four cell lines, was therefore selected for below studies.
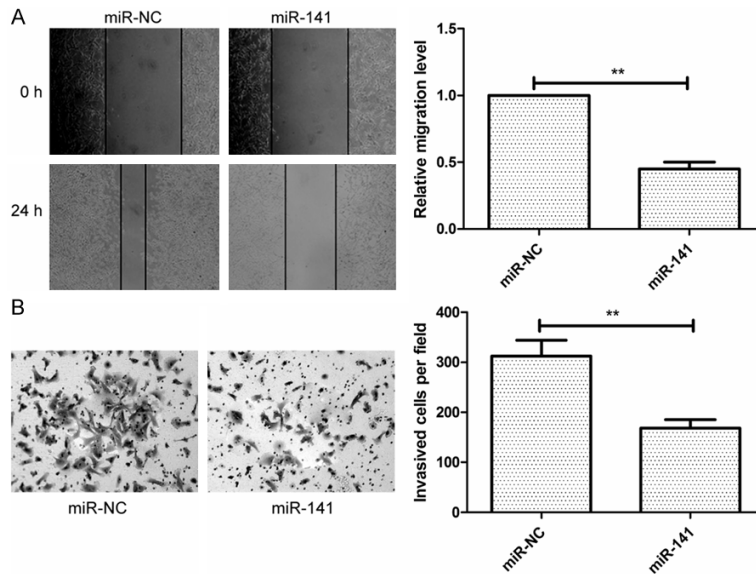
miR-141 inhibits glioma growth in vitro and in vivo

To investigate the effect of miR-141 on glioma growth, miR-141 mimic were transfected into the U87 cell line, and the effect of miR-141 on the proliferation and colony formation of U87 cells was assessed by MTT and colony forming assays, respectively. miR-141 expression was significantly increased in the U87 cells transfected with miR-141 mimics compared to those transfected with negative control mimic (miR-NC), as shown by qRT-PCR (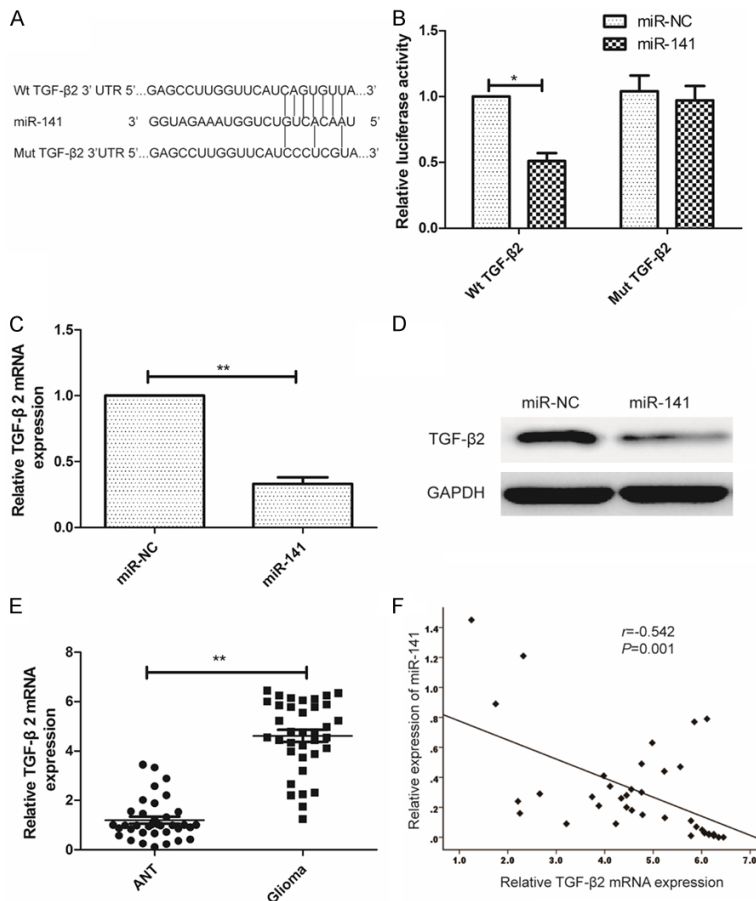**Figure 2A**). MTT assay showed that miR-141 overexpression significantly inhibited cell proliferation in U87 cells (**Figure 2B**). Consistent with this result, we found that miR-141 overexpression significantly inhibited cell colony formation in U87 cells (**Figure 2C**).

To further confirm the effect of miR-141 on glioma tumor growth *in vivo*, U87 cells stable expression of miR-141 or miR-NC were subcutaneously inoculated into nude mice. Tumor volumes were measured every five days during treatment. The growth curve of tumor xenografts showed that miR-141 overexpression significantly slowed down tumor growth compared

ence. Associations of miR-141 expression and TGF-β2 expression were estimated using Spearman's correlation analysis. Statistical analyses were undertaken using the SPSS® statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA) for Windows®. *P* value of less than 0.05 was considered as significant.

Results

miR-141 is downregulated in glioma tissues and cell lines

The levels of miR-141 in 36 glioma tissues and adjacent non-cancerous tissues (ANT) were

Figure 3. miR-141 inhibits cell migration, invasion in human glioma cells. A. Effect of miR-141 on the cell migration of U87 cells was detected by wound healing assay. B. Effect of miR-141 on cell invasion of U87 cells was detected by transwell invasion assay. *P < 0.05, **P < 0.01.

sequences of mutant-type were shown. (B) The relative luciferase activities were determined in the U87 cells cotransfected with wild-type/mutant-type TGF-β2 reporter vector and miR-141/miR-NC. Firefly luciferase activity was normalized to Renilla luciferase. *P < 0.05 versus miR-NC. (C, D) TGF-β2 mRNA level (C) and protein level were determined in U87 cells after transfection of miR-141 or miR-NC. GAPDH was used as loading control. **P < 0.01 versus miR-NC. (E) TGF-β2 mRNA level was determined in glioma tissues and adjacent non-cancerous tissues (ANT). GAPDH was used as loading control. (F) The reverse relationship between TGF-β2 and miR-141 expression was explored by Spearman's correlation in glioma tissues (n = 36). *P < 0.05, **P < 0.01.

with the miR-NC (**Figure 2D**). At the end of the experiments, the tumor tissues were isolated and the weight of the tumors was measured. It was found that the size and weight of the miR-141-expressing U87 tumors was lower than that of miR-NC-expressing U87 tumors (**Figure 2E**). These findings indicated that miR-141 was able to inhibit glioma growth in vitro and in vivo.
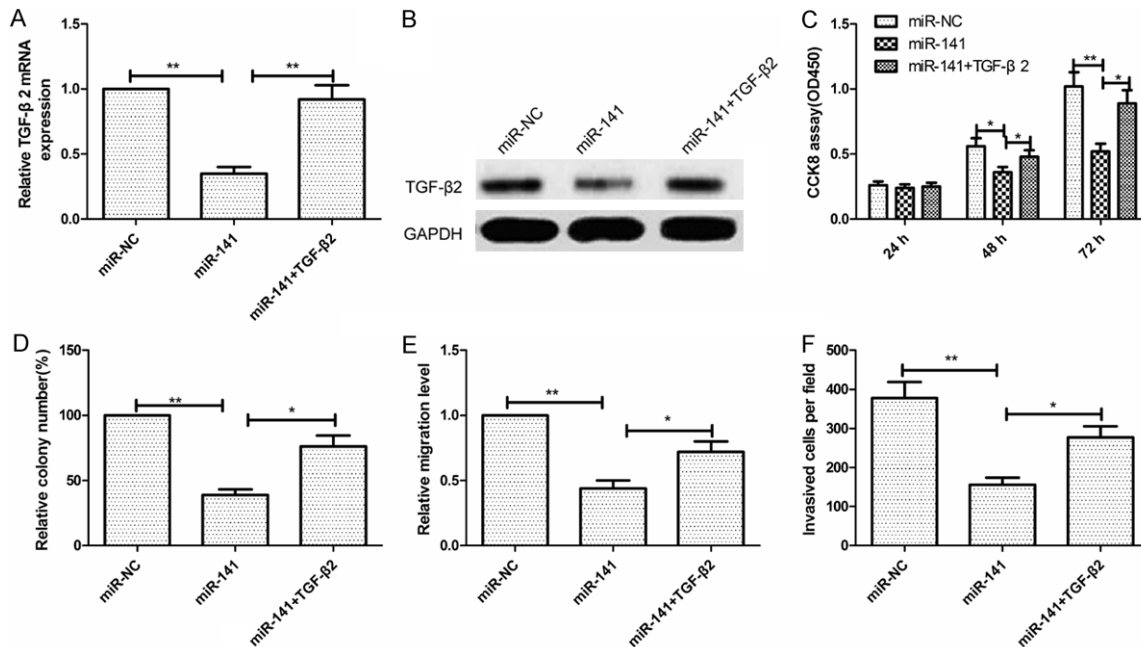
miR-141 inhibits cell migration, invasion in human glioma cells

To investigate the effect of miR-141 on glioma metastasis, would healing and transwell invasion assays were performed in U87 cells transfected with miR-141 mimic or miR-NC. It was found that miR-141 overexpression significantly inhibited migration (**Figure 3A**) and invasion (**Figure 3B**) of glioma cells.

TGF-β2 is a specific target gene of miR-141 in glioma

As for the important biological effects of miR-141 in glioma,

Figure 4. TGF-β2 is a specific target gene of miR-141 in glioma. (A) Putative binding sites of miR-141 within the 3'-UTR region of TGF-β2 mRNA, and the

Figure 5. TGF-β2 overexpression reverses the inhibitory effects of miR-141 in glioma cells. (A, B) TGF-β2 mRNA expression (A) and protein expression (B) was measured by in U87 cells cotransfected with TGF-β2 overexpression plasmid and miR-141/miR-NC. GAPDH was used as an internal control. (C-F) Cell proliferation, colony formation, migration and invasion were determined in U87 cells transfected with miR-141 with/without TGF-β2 overexpression plasmid. *$P < 0.05$, **$P < 0.01$.

further mechanism analysis is becoming necessary. Using the publicly available databases, we found a conserved binding site in 3'-UTRs of TGF-β2 (109-116 bp) for miR-141 (**Figure 4A**). The luciferase reporter assay was performed to identify the direct interaction between miR-141 and TGF-β2. As shown in **Figure 4B**, the relative luciferase activity was decreased in glioma cells cotransfected with Wt-TGF-β2-3'UTR vector and miR-141 mimic, while Mut-TGF-β2-3'UTR vector combine with miR-141 mimic had no effect on luciferase activity (**Figure 4B**), suggesting that miR-141 specifically binds to the seed zone of TGF-β2 3'UTR to inhibit luciferase expression. qRT-PCR and western blot assays showed that miR-141 overexpression obviously decreased TGF-β2 expression both on mRNA level (**Figure 4C**) and protein level (**Figure 4D**). In addition, we found that the mRNA expression of TGF-β2 in glioma tissues was upregulated compared with adjacent noncancerous tissues (**Figure 4E**), and was negatively correlated with miR-141 in glioma tissues, as shown by Spearman's correlation analysis ($r = -0.542$, $P = 0.001$) (**Figure 4F**). These results suggested that TGF-β2 might be a direct functional target of miR-141 in glioma.

TGF-β2 overexpression reverses the inhibitory effects of miR-141 in glioma

To further confirm that miR-141 mediates its inhibition effects through *TGF-β2*, we cotransfected with miR-141 mimic or miR-NC and TGF-β2 overexpression plasmid without specific miR-141 binding sequences in the 3'-UTR in U87 cells. As shown in **Figure 5A** and **5B**, the expression of TGF-β2 was decreased in cells transfected with miR-448 mimic, while the expression of TGF-β2 could been restore in cells cotransfected with miR-141 mimic and TGF-β2. In addition, we showed that ectopic expression of TGF-β2 effectively reversed the inhibition effect on cell proliferation, colony formation, migration and invasion of U87 cells induced by miR-141 overexpression (**Figure 5C-F**). These findings demonstrate that miR-141 exerted suppressive role in glioma cells, at least in part, by suppression of TGF-β2.

Discussion

Accumulating evidence has shown that miRNAs are involved in the pathogenesis of glioma as oncogenes or tumor suppressor genes by regu-

lating their target gene [10]. Recently, numerous of miRNAs has been identified to have potential tumor diagnostic and prognostic values, as well as therapy values in human glioma [11]. For example, miR-506 inhibited the proliferation, colony formation, migration and invasion of glioma cells in vitro, and suppress glioma tumor growth in vivo by targeting STAT3 [18]. miR-508-5p was frequently decreased and might act as a tumor suppressor gene in glioma by targeting Glycoprotein Non-metastatic Melanoma B (GPNMB) [19]. miR-338-3p can inhibit malignant biological behaviors of glioma cells partly through directly silencing MACC1 expression [20]. In the current study, we provide the first demonstration that miR-141 expression was down-regulated in a human glioma tissues and cell lines. We also demonstrated that miR-141 overexpression inhibited glioma growth in vitro and in vivo, as well as suppressed glioma cell migration and invasion. Thus, our in vitro and in vivo findings together suggest that miR-141 might play crucial roles in growth and metastasis in glioma.

As one important member of the miR-200 family, microRNA (miR)-141 has been reported to be upregulated in ovarian cancer [21], prostate cancer [22], bladder cancer [23], colorectal cancer [24] and non-small lung cancer cells [25], while be down-regulated in nasopharyngeal carcinoma [12], hepatocellular carcinoma [13], gastric cancer [14], and breast cancer [15, 16]. These studies suggested that miR-141 functions as oncogene or tumor suppressor, which depended on the specfic cancer type. However, the molecular function and mechanisms of miR-141 in glioma are still unclear. Here, we demonstrated that miR-141 expression was downregulated in a human glioma tissues and cell lines compared to adjacent non-cancerous tissues and normal cell line. Our results also showed that overexpression of miR-141 significantly inhibited glioma cell proliferation, colony formation, migration and invasion in vitro, as well as suppressed glioma tumor growth in vivo. These findings suggested that miR-141 might function as tumor suppressor in glioma.

TGF-β2 belongs to transforming growth factor β (TGFB) family that regulate various cell procession including proliferation, differentiation, adh-

esion and migration by signal transduction through combinations of transmembrane type I and type II receptors and their downstream effectors, the SMAD proteins [26, 27]. The expression level of TGF-β2 is upregulated in many types of cancer including glioma [28]. TGF-β2 had been showed to play important roles in initiation and development of glioma [29]. It was found that downregulation of TGF-β2 through the antisense oligonucleotide trabedersen (AP 12009) could effective inhibit glioma growth [30]. These studies suggest that TGF-β2 might function as oncogene in glioma. In the present study, TGF-β2 was identified as a direct target gene of miR-141 in glioma by dual-luciferase reporter assay, qRT-PCR and Western blot. TGF-β2 expression was upregulated, and was negatively correlated with the expression level of miR-141 in glioma tissues. Of note, TGF-β2 overexpression reversed the inhibition effect on cell proliferation, colony formation, migration and invasion of glioma cells mediated by miR-141 overexpression. These findings suggested that miR-141 exerted a tumor suppressor in glioma, at least in part, by repressing TGF-β2.

In summary, to our knowledge, our study first provides evidence that miR-141 is frequently down-regulated in glioma tissues and cell lines, and that miR-141 overexpression inhibits proliferation, colony formation, migration and invasion of glioma cells *in vitro*, and suppress glioma tumor growth in vivo, at least in part, by targeting TGF-β2. These findings suggested that miR-141 might function as a novel tumor suppressor in glioma, and that miR-141 might be a novel potential therapeutic target for glioma.

Disclosure of conflict of interest

None.

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