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MiRNA-125a-5p inhibits glioblastoma cell proliferation and promotes cell differentiation by targeting TAZ

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ABSTRACT

Glioblastoma (GBM) is the most lethal brain tumor due to the resistance to conventional therapies, such as radiotherapy and chemotherapy. TAZ, an important mediator of the Hippo pathway, was found to be up-regulated in diverse cancers, including in GBM, and plays important roles in tumor initiation and progression. However, little is known about the regulation of TAZ expression in tumors. In this study, we found that miR-125a-5p is an important regulator of TAZ in glioma cells by directly targeting the *TAZ* 3′ UTR. MiR-125a-5p levels are inversely correlated with that of TAZ in normal astrocytes and a panel of glioma cell lines. MiR-125a-5p represses the expression of TAZ target genes, including CTGF and survivin, and inhibits cell proliferation and induces the differentiation of GBM cells; whereas over-expression of TAZ rescues the effects of miR-125a-5p. This study revealed a mechanism for TAZ deregulation in glioma cells, and also demonstrated a tumor suppressor role of miR-125a-5p in glioblastoma cells.

1. Introduction

Glioblastoma (GBM) is the most lethal brain tumor with median survival of about 15 months using current standard of care for patients [1]. A subgroup of stem cell-like cells in GBMs (known as glioma stem cells) was identified and proved to be responsible for the initiation of glioma and the resistance of GBMs to conventional therapies [2,3]. Therefore, it is important to reveal the mechanisms regulating glioma stem cell, and the initiation and progression of GBMs.

TAZ is an important transducer of the Hippo tumor-suppressor pathway. Upon activation of the Hippo pathway, TAZ was phosphorylated by LATS1/2, which promotes its cytoplasmic sequestration and then functional inhibition [4]. Moreover, phosphorylation by the Hippo pathway also promotes TAZ degradation [5]. Deregulation of TAZ has been reported at a high frequency in many kinds of cancers and often correlates with poor prognosis [6–10]. In addition, TAZ was required to sustain the self-renewal of cancer stem cells (CSCs) and induce the tumorigenic potential of CSCs [11,12]. In glioma, TAZ expression was lower in low grade gliomas and proneural GBMs compared with GBMs that had a mesenchymal phenotype [13]. Knockdown of TAZ in glioma stem cell decreases the expression of mesenchymal markers, as well as invasion, self-renewal and tumor formation [13]. However, the regulation of TAZ expression in high grade tumors is largely unknown.

MicroRNAs (miRNAs) are a large family of endogenous small RNAs (19–24 nt) and act as important post-transcriptional regulators of gene expression [14]. It has been firmly established that miRNAs were involved in the regulation of diverse physiological and pathological processes [15], especially in tumorigenesis [16,17]. MiR-125a-5p is derived from the 5′ end of pre-miR-125a. It was reported that miR-125-5p was deregulated in some kinds of cancers, and acts as a tumor suppressor by targeting genes associated with cell proliferation and tumor progression [18,19]. In glioblastoma, miR-125a-3p, another mature miRNA derived from pre-miR-125a, was proved to be down-regulated and inhibited the invasion and proliferation of GBM cells [20]. Moreover, miR-125a-3p was down-regulated in $CD133^+$ GBM cells compared with the $CD133^-$ GBM cells, suggesting its involvement in the regulation of glioma stem cells [20]. However, little is known about the expression and roles of miR-125a-5p in glioblastoma.

In this study, we revealed the regulation of TAZ by miR-125a-5p in glioma cells. We found that expression of TAZ and miR-125a-5p are inversely correlated in normal astrocytes and different glioma cell lines. Mechanically, miR-125a-5p directly targets the 3′ UTR of *TAZ* mRNA and represses TAZ expression. MiR-125a-5p inhibits

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GBM cell proliferation and promotes cell differentiation by repressing the TAZ target genes including CTGF and survivin. Our study revealed a mechanism for TAZ deregulation in glioma cells, and also demonstrated a tumor suppressor role of miR-125a-5p in glioblastoma cells.

2. Materials and methods

2.1. Cell culture and transfection

Normal Human Astrocytes (NHA), glioma cell lines (HS683, SW1783, LN229, U87MG, T98G, HFU-251MG) and HEK293 cells were all cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO_2 humidified incubator at 37 °C. Transient cell transfections of plasmids or small RNAs were performed using Lipofectamine 2000 (Life Technologies). siRNA and miRNA mimics were transfected at a final concentration of 50 nm, and miRNA antagomirs were transfected at a final concentration of 100 nm.

2.2. RNA oligoribonucleotides and antibodies

The control RNA mimics, miR-125a-5p mimics, miR-125a-5p antagomirs and TAZ siRNA were all obtained from GenePharma (Shanghai, China). The target sequence of TAZ siRNA is: 5′-AGGTACTTCCTCAATCACA-3′. Antibodies against TAZ (#2149) and CD133 (#3663) were from Cell Signaling Technology. Antibodies against CTCG (sc-14939), survivin (sc-17779), GFAP (sc-6170) and β-actin (sc-47778) were from Santa Cruz.

2.3. Plasmid construction and luciferase assay

The full length cDNA of human *TAZ* gene (*NM_000116*) was amplified by PCR and cloned into pcDNA3 vector. Primers used for cDNA amplification are: TAZ-F, 5′-CCCAAGCTTATGCCTCTGC ACGTGAAGTG-3′; TAZ-R: 5′-AAATATGCGG CCGCCTATCTCCCAG GCTGGAGGT-3′. The sequence of the cDNA was verified by DNA sequencing.

To construct the TAZ 3′ UTR reporter plasmids, 50 nt forward and reverse DNA oligos of the TAZ 3′ UTR harboring the potential targeting sites of miR-125a-5p were synthesized, annealed and cloned into psiCHECK2 vector (Promega) in the XhoI/NotI sites. For mutation analysis of the miR-125a-5p target site in TAZ 3′ UTR, DNA oligos with mutations were also synthesized and cloned as above. Oligos used in this study are: Wt-F, 5′-TCGAGACCAGGAGCTGCTCA CTACCTCCTCAGGG ATGGCCGTTGGCCACGTCTTCGC-3′; Wt-R, 5′-GG CCGCGAAGACGTGGCCA ACGGCCATCCCTGAGGAGGTAGTGAGCAG CTCCTGGTC-3′; Mt-F, 5′-TCGA GACCAGGAGCTGCTCACTACCTCC GCCGATATGGCCGTTGGCCACGTCTTCGC-3′; Mt-R, 5′-GGCCGCGAA GACGTGGCCAACGGCCATATCGGCGGAGG TAGTGAGCAGCTCCT GGTC-3′.

For luciferase assay, HEK293 or HS683 cells in 24-well plates were transfected with 100 ng psiCHECK2-UTR vectors and the miR-125a-5p mimics or antagomirs at a final concentration of 50 or 100 nM. 48 h after transfection, cells were lysed and reporter gene expression was determined using the Dual-luciferase reporter assay system (Promega). Experiments were performed three times in triplicates.

2.4. Western blotting

Cells in 6-well plates were lysed in RIPA Buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). Protein samples were separated by 10% SDS PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were then incubated with primary antibodies, followed by HRP-linked secondary antibodies (Santa Cruz). Blots were developed using enhanced chemiluminescence reagent (Pierce) and exposed to films.

2.5. RNA extraction, reverse transcription, and real time PCR analysis

Total RNAs were extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. RNAs were reverse transcribed using the PrimeScript™ RT-PCR Kit (Takara). Primer used for miR-125a-5p reverse transcription was: 5′-GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACTCACAG-3′. SYBR-Green-based real-time quantitative PCR was performed using Takara SYBR Green Master Mix (Takara). U6 snRNA was used as an internal control. The final results were expressed as fold changes between the samples and the controls after being corrected with the internal control. Primers used for miR-125a-5p real-time PCR are: forward, 5′-GGTCATTCCCTGAGACCCTTTAAC-3′; reverse, 5′-GT GCAGGGTCCGAGGT-3′. Primers used for TAZ real-time PCR are: forward, 5′-ACCCACCCACGATGACCCCA-3′; revere, 5′-GCACCCTA ACCCCAGG CCAC-3′.

2.6. Cell growth assay

HFU-251 or HS683 cells were seeded in 6-well plates and then transfected with miR-125a-5p mimics, TAZ siRNA, or miR-125a-5p antagomirs. Cells were maintained in DMEM containing 10% FBS for 10 days. Colonies were fixed with methanol, stained with 0.1% crystal violet for 10 min and photographed using a digital camera. Experiments were performed three times.

2.7. Immunofluorescence assay

HFU-251 MG cells were fixed and incubated with CD133 or GFAP antibodies, and then incubated with Alexa Fluor dye-conjugated secondary antibodies and Hoechst 33342 according to standard protocols. Cells were examined using a deconvolution microscope (Zeiss). Axio Vision software from Zeiss was used to deconvolute Z-series images.

2.8. Statistical analysis

All data are expressed as the mean ± standard deviation from at least three independent experiments. Differences between groups were analyzed using a Student's t test. $p < 0.05$ was considered statistically significant.

3. Result

3.1. TAZ expression is repressed by miR-125a-5p in human glioma cells

To reveal the functional relationship between miR-125a-5p and TAZ, we detected the levels of miR-125a-5p and TAZ protein in different glioma cell lines. We used seven cell lines including one cell line derived from normal human astrocytes (NHA), two cell lines from low-grade glioma (HS683 and SW1783), and four from high-grade glioma (LN229, U87 MG, T98G and HFU-251 MG). MiR-125a-5p was highest in NHA cells, relative lower in HS683, SW1783 and LN229 cells (Fig. 1A). However, miR-125a-5p had the lowest expression in other three high-grade cell lines including U87 MG, T98G and HFU-251 MG (Fig. 1A). In contrast to miR-125a-5p expression, TAZ protein was highest in U87 MG, T98G and HFU251 MG cell lines, moderate in HS683 and SW1783, and low in NHA cells (Fig. 1B). Note that LN229 showed relative high miR-125a-5p and low TAZ protein (Fig. 2A and B). These results

indicated that miR-125a-5p expression is inversely correlated with TAZ in glioma cell lines.

We next determined to reveal whether miR-125a-5p regulates TAZ expression. MiR-125a-5p mimics were transfected into U87 MG and HFU-251 MG cells, both of which have low endogenous miR-125a-5p expression. Results showed that transfection of miR-125a-5p mimics significantly decreased TAZ expression in both U87 MG and HFU-251 MG cell lines (Fig. 1C). To exam the effect of in vivo miR-125a-5p on TAZ expression, we inhibited miR-125a-5p expression by transfection of miR-125a-5p specific antagomirs. TAZ expression was increased after miR-125a-5p inhibition in both HS683 and SW1783 cells (Fig. 1D). These results suggested that miR-125a-5p represses TAZ expression in glioma cells.

3.2. MiR-125a-5p directly targets the 3′ UTR of TAZ mRNA

To reveal the mechanism of miR-125a-5p in repressing TAZ expression, we searched for the potential target sites of miR-125a-5p in the 3′ UTR of TAZ mRNA using the prediction algorithm TargetScan (Release 6.2, http://www.targetscan.org/). One conserved target site was predicted (Fig. 2A). We synthesized and cloned the 3′ UTR segments harboring the wild type or mutant type candidate motif targeted by miR-125a-5p into psiCHECK2 vector. Transfection of miR-125a-5p mimics significantly repressed the luciferase activity in the wild type TAZ 3′ UTR construct, but not in the mutant 3′ UTR (Fig. 2B). In line with this result, inhibition of miR-125a-5p in HS683 cells increased the luciferase activity in the wild type TAZ 3′ UTR construct, but not in the mutant 3′ UTR (Fig. 2C). Moreover, in both U87 MG and HFU-251 MG cells, transfection of miR-125a-5p mimics decreased the TAZ mRNA levels as determined by real-time PCR (Fig. 3D). These results indicated that miR-125a-5p directly targets the 3′ UTR of TAZ mRNA and promotes TAZ mRNA degradation.

3.3. MiR-125a-5p represses TAZ target genes and inhibits glioma cell growth

We next examined the effect of miR-125a-5p on TAZ target gene expression. CTGF and survivin were reported to be targeted by TAZ [11], and therefore were selected for the validation. Transfection of miR-125a-5p mimics significantly reduced both CTGF and survivin expression in HFU-251 MG cells as done by TAZ siRNA (Fig. 3A). In addition, cell growth was attenuated by miR-125a-5p and TAZ siRNA using monolayer colony formation assays (Fig. 3B). Furthermore, over-expression of TAZ rescued the effects of miR-125a-5p on the expression of CTGF and survivin and HFU-251 MG cell growth (Fig. 3C and D). To identify the in vivo function of miR-125a-5p, we next inhibited miR-125a-5p expression in HS683 cells and found that the expression of TAZ, CTGF, and survivin were all increased (Fig. 3E). Moreover, inhibition of miR-125a-5p promoted HS683 cell growth compared with the control (Fig. 3F). Thus, these results demonstrated that miR-125a-5p represses the expression of TAZ target genes and inhibits glioblastoma cell growth.

3.4. MiR-125a-5p induce the differentiation of GBM cells

The TAZ target genes CTGF and survivin were reported to be involved in the regulation of cancer stem cells [11,21]. Therefore, we examined the effect of miR-125a-5p on the differentiation of HFU-251 MG cells, which was derived from high grade glioblastoma and possesses features of glioma stem cells [22]. Transfection of miR-125a-5p decreased the expression of CD133, a self-renewal marker of glioma stem cells; whereas, the differentiation marker GFAP was induced by transfection of miR-125a-5p as done by TAZ siRNA (Fig. 4A). Moreover, over-expression of TAZ rescued the effect of miR-125a-5p on the expression of CD133 and GFAP (Fig. 4B). The effect of miR-125a-5p and TAZ knockdown on the differentiation of HFU-251 MG cells was further confirmed by immunofluorescence assays (Fig. 4C). Thus, these results indicated that miR-125a-5p represses the glioma stem cell features and promotes the differentiation of glioblastoma cells.

4. Discussion

TAZ is an important transducer of the Hippo pathway and play important roles in tumor initiation and progression. However, little is known about the regulation of TAZ expression in tumors. In this

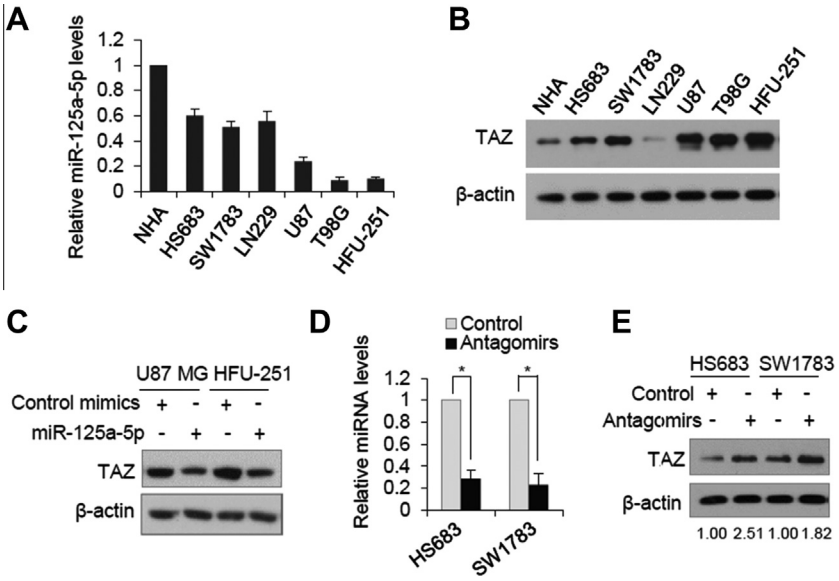

Fig. 1. TAZ expression is repressed by miR-125a-5p in human glioma cells. (A) The expression of miR-125a-5p was analyzed by real-time PCR in the Normal Human Astrocytes (NHA) and six glioma cell lines. U6 snRNA was used as an internal control. Data was from three independent assays. (B) TAZ expression in NHA and six glioma cell lines was detected by immunoblotting. β-Actin was used as a loading control. (C) The control mimics or miR-125a-5p mimics were transfected into U87 MG or HFU-251 MG cells, and TAZ expression was examined by immunoblotting. (D) MiR-125a-5p expression was analyzed by real-time PCR after the transfection of miR-125a-5p antagomirs in HS683 and SW1783 cells. $^{*}p < 0.05$. (E) TAZ expression was examined by immunoblotting after the transfection of miR-125a-5p antagomirs in HS683 and SW1783 cells.

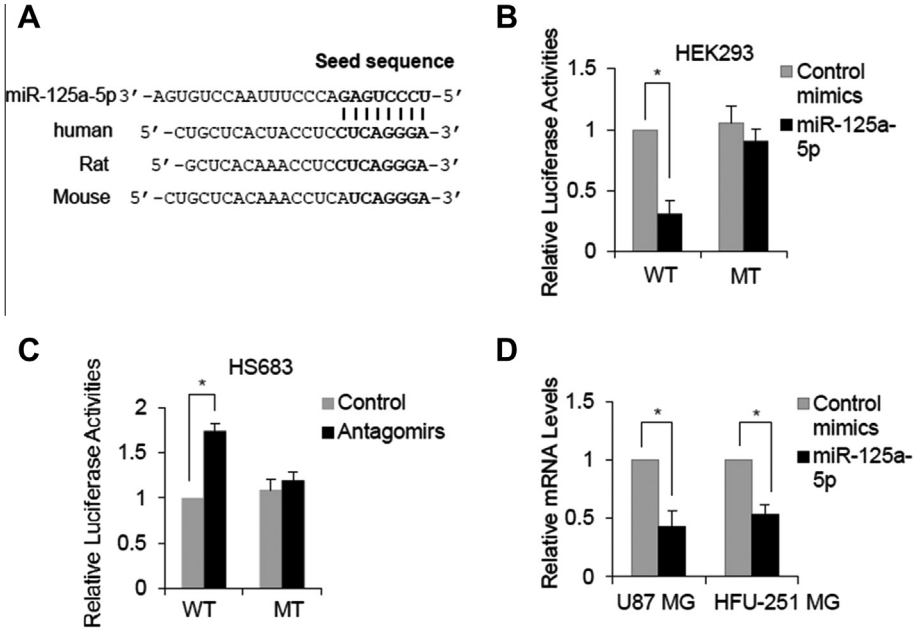

Fig. 2. MiR-125a-5p directly targets the 3′ UTR of TAZ mRNA. (A) Predicted target sequence of miR-125a-5p in the wild type TAZ 3′ UTR. (B) Reporter gene expression was analyzed by luciferase assays after transfection of miR-125a-5p mimics and psiCHECK2-TAZ-3′ UTR-WT or psiCHECK2-TAZ-3′ UTR-MT in HEK293 cells. The relative luciferase activities are the ratios of *Renilla* luciferase normalized to the control mimics. (C) Luciferase assays shows the reporter gene expression after transection of miR-125a-5p antagomirs and psiCHECK2-TAZ-3′ UTR-WT or psiCHECK2-TAZ-3′ UTR-MT in HS683 cells. (D) MiR-125a-5p mRNA expression was detected after transfection of miR-125a-5p mimics compared with the control mimics. All data were from three independent assays. $^{*}p < 0.05$.

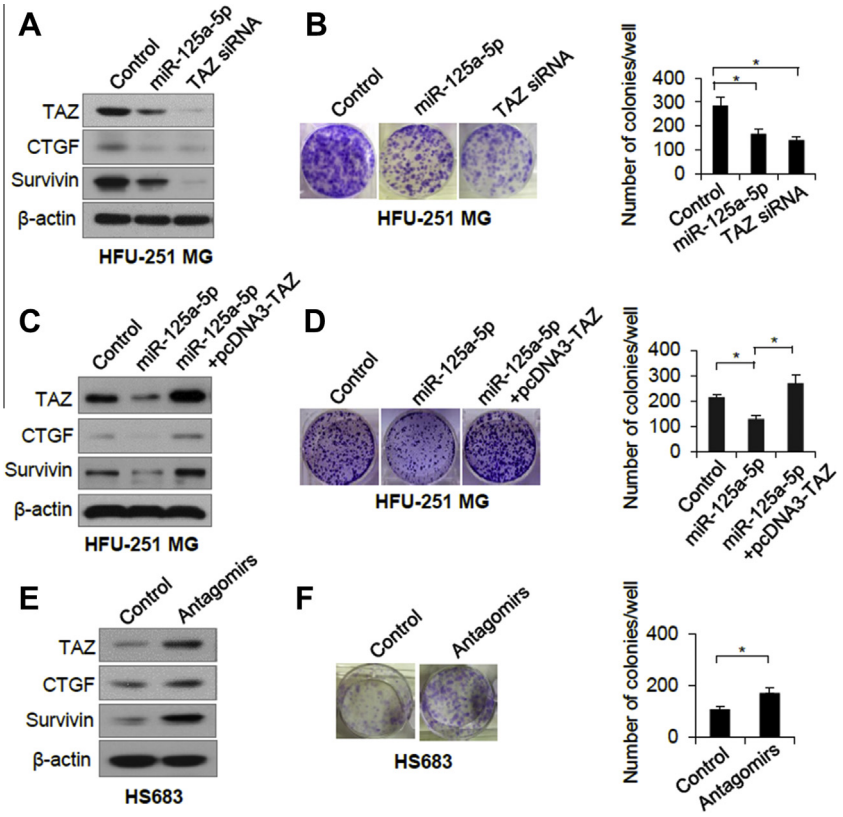

Fig. 3. MiR-125a-5p represses TAZ target genes and inhibits glioma cell growth. (A) TAZ, CTGF, and survivin were examined by immunoblotting after transfection of miR-125a-5p or TAZ siRNA. (B) Representative images show the cell growth after transfection of miR-125a-5p or TAZ siRNA compared with the control in HFU-251 MG cells (left panel). Average colonies in each well for each group were counted from three independent experiments (right panel). (C) The miR-125a-5p transfected HFU-251 cells were rescued by transfection of pcDNA3-TAZ and the expression of TAZ, CTGF, and survivin were examined by immunoblotting. (D) HFU-251 MG cell growth was analyzed by colony formation assay after transfection of miR-125a-5p or miR-125a-5p+pcDNA3-TAZ. (E) TAZ, CTGF, and survivin were detected by immunoblotting after transfection of miR-125a-5p antagomirs or control in HS683 cells. (F) Cell growth of HS683 cells after transfection of miR-125a-5p antagomirs or control was analyzed by colony formation assay. Colonies with more than 50 cells were counted in each well. Data was from three independent experiments. $^{*}p < 0.05$.

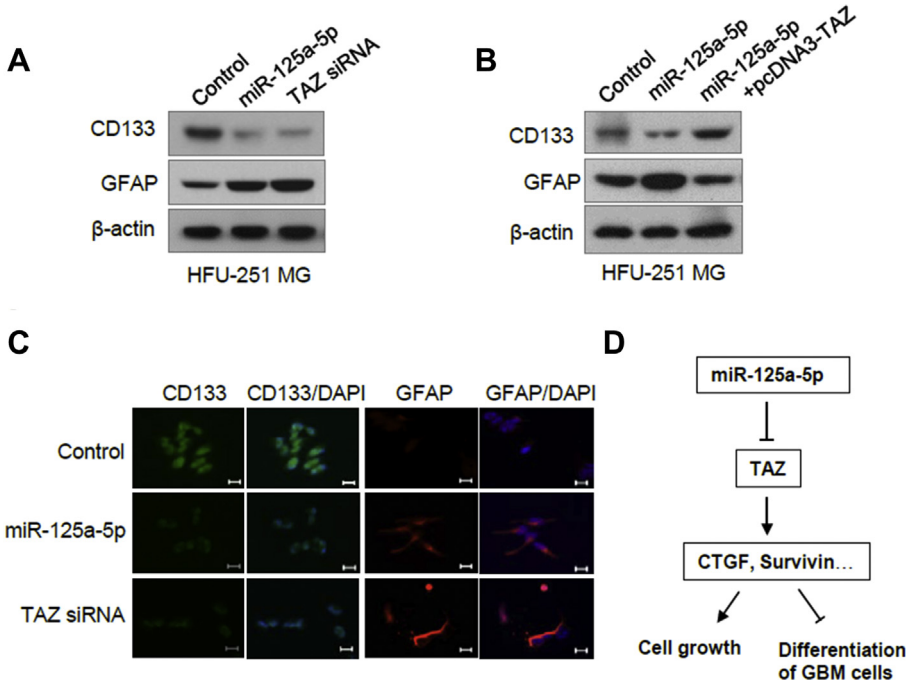

Fig. 4. MiR-125a-5p induces the differentiation of GBM cells. (A) CD133 and GFAP expression were analyzed by immunoblotting after transfection of miR-125a-5p or TAZ siRNA in HFU-251 cells. (B) The miR-125a-5p transfected HFU-251 cells were rescued by transfection of pcDNA3-TAZ and the expression of CD133 and GFAP were analyzed by immunoblotting. (C) Immunofluorescence assays show the expression of CD133 and GFAP after transfection of miR-125a-5p or TAZ siRNA in HFU-251 cells. (D) A scheme shows the miR-125a-5p-TAZ-CTGF/survivin pathway in cell proliferation and differentiation of glioblastoma cells.

study, we revealed that miR-125a-5p represses TAZ expression by directly targeting the 3′ UTR of TAZ mRNA. MiR-125a-5p also represses the expression of CTGF and survivin, two important target genes of TAZ, and inhibits glioma cell proliferation and promotes GBM cell differentiation (Fig. 4D). This study revealed a mechanism for the regulation of TAZ and also demonstrated the anti-proliferative and differentiation-promoting effects of miR-125a-5p in glioma cells.

Our results showed that TAZ and miR-125a-5p were inversely correlated in NHA and different glioma cell lines, which further confirmed that TAZ was targeted by miR-125a-5p for repression in glioma cells. Deregulation of TAZ was found to be a common event in cancers [6,9,13]. Wnt/β-catenin pathway was shown to stabilize TAZ by inhibiting its binding with the ubiquitin ligase β-TrCP [23], which revealed a mechanism for the deregulation of TAZ in cancers. Our result demonstrated, for the first time, that TAZ expression was regulated post-transcriptionally by miR-125a-5p, which provides a new mechanism for the deregulation of TAZ in cancers.

In this study, we also showed that miR-125a-5p expression was related with the grades of glioma. MiR-125a-5p had high expression in normal astrocytes (NHA), median expression in low grade glioma (HS683 and SW1783), and low expression in high grade glioma (U87 MG, T98G and HFU-251 MG). LN229 is an exception, which was shown to have higher miR-125a-5p expression than U87 MG, T98G and HFU-251 MG.

CTGF and *survivin* is two important target genes of TAZ [11], and was found to be repressed by miR-125a-5p in our study. CTGF was proved to be a prognostic for tumor progression and survival of gliomas [24], and targeting CTGF was correlated with improved survival of glioblastoma [25]. Survivin expression levels also are of prognostic values in human gliomas [26], and high expression of survivin enhances radiation resistance in glioblastoma [27]. Moreover, both CTGF and survivin are involved in the regulation of cancer stem cell self-renewal [11,27]. In our study, we also proved that miR-125a-5p down-regulated, at least partly by repressing CTGF and survivin, the expression of glioma stem cell marker CD133 and induced the differentiation marker GFAP. These results suggest an important role of miR-125a-5p in the differentiation GBM cells.

In summary, we found that miR-125a-5p represses the expression of TAZ in glioma cells. MiR-125a-5p expression is reversely related with TAZ in normal astrocytes and glioma cell lines. MiR-125a-5p represses the expression of TAZ target genes including CTGF and survivin, and inhibits GBM cell proliferation and induces differentiation. Further studies are required to determine whether miR-125a-5p can be used as a marker of diagnosis for glioma and a strategy for the treatment of GBM.

Conflict of interest

There are no conflict of interest for all authors.

Acknowledgments

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