doi: 10.4149/gpb_2019018

Knockdown long non-coding RNA PEG10 inhibits proliferation, migration and invasion of glioma cell line U251 by regulating miR-506

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Abstract. Glioma is a serious malignant tumor without effective therapies till now. lncRNA PEG10 was reported to have some biological activities in cancers. Hence, we explored the effects of PEG10 on the human glioma cell line U251 cells. U251 cells were transfected with sh-PEG10 and/or miR-506 inhibitor. The expression of PEG10 and miR-506 was measured by qRT-PCR. Cell viability, cell apoptosis, cell migration and invasion were detected by CCK-8 assay, flow cytometry and Transwell chamber assay, respectively. The cell proliferation and apoptosis related p16, p53, Bcl-2, Bax, and pro-/Cleaved-Caspase-3/9, migration and invasion related-protein: matrix metalloproteinases MMP-2, MMP-9 and vimentin, and Raf/MEK/ERK and JAK1/STAT3 pathways-related proteins were accessed by Western blot. Transfection with sh-PEG10 inhibited cell viability, migration and invasion, and increased cell apoptosis. Meanwhile, PEG10 silence upregulated the expression of p16 and p53, Bax, cleaved-Caspase-3/9 expression, and downregulated Bcl-2 expression. PEG10 silence upregulated miR-506 expression. Co-transfection with sh-PEG10 and miR-506 inhibitor impaired the tumor suppressive effects. PEG10 knockdown decreased the phosphorylation of Raf/MEK/ERK and JAK1/STAT3-related proteins Raf, MEK, ERK, JAK1 and STAT3. PEG10 knockdown inhibited cell viability, migration and invasion, induced cell apoptosis through miR-506 upregulation, as well as inactivation of Raf/MEK/ERK and JAK1/STAT3 signal pathways.

Key words: Glioma — PEG10 — miR-506 — Raf/MEK/ERK — JAK1/STAT3

Introduction

Glioma is a serious malignant tumor which belongs to one of the most common and fatal intracranial tumors (Li et al. 2018b). This kind of cancer possesses features in widespread and fast invasion throughout the brain and what makes it worse due to its strongly resists to the traditional and newer targeted therapies (Winter et al. 2017). Even though more understanding about the pathogenesis is obtained, the outcome about survival percentage following surgery and prognostic factors of the patients is still not optimistic (Miller et al. 2017; Winter et al. 2017). New therapy or novel medicine was urgently needed for the diagnosis and treatment of gliomas.

Long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides, do not have the functions of protein synthesis as the name described (Xing et al. 2014). Increasing evidence revealed that lncRNAs might be involved in various biological activities in several diseases, including glioma (Zhang et al. 2012; Necsulea et al. 2014). For example, lncRNA ADAM metallopeptidase with thrombospondin type 1 motif, 9 (ADAMTS9) antisense RNA 2 (ADAMTS9-AS2) demonstrated anti-tumor functions in glioma *via* inhibiting cell migration (Yao et al. 2014); lncRNA taurine upregulated gene 1 (TUG1) could promote cell apoptosis to suppress glioma tumor (Li et al. 2016b). Among all these experimental validated lncRNAs, paternally expressed 10 (PEG10, Gene ID: 23089) was first reported by Ono et al. and was a maternally imprinted and novel paternally expressed gene (Ono et al. 2001). Previous studies have suggested that PEG10 participated in modulating several biological processes in cancers, such as esophageal cancer (Tang et al. 2015), gastric cancer (Ishii et al. 2017) and hepatocellular carcinomas (Kobayashi 2014). However, from our limited knowledge, no studies were performed to explore the func-

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tions of PEG10 in glioma cells. Hence, we aimed to investigate the the roles of PEG10 in glioma.

miRNAs, which referred to a class of noncoding RNA species (~22 nts) that are involved in gene regulation at the post-transcriptional levels (Chou et al. 2016). lncRNAs were also found to be interaction with miRNA, act as miRNA sponges (Paraskevopoulou et al. 2016), including PEG10. PEG10 was reported to be down-modulated by miR-122 to trigger its tumor suppressive effects in human hepatocellular carcinoma (Shyu et al. 2016). In addition, miR-491 and PEG10 could cooperate as an axis to influence cell proliferation and apoptosis in HCT-116 cells (Xu et al. 2013). miR-506 is a widely reported miRNA which functions as a tumor suppressor in glioma (Luo et al. 2015; Peng et al. 2016). Based on that, we further explored whether miR-506 and PEG10 could cooperate to function in glioma.

In this study, we used human glioma cell line U251 to establish cell model *in vitro* and investigated the effects of PEG10 on U251 cells as well as the underlying mechanisms. Our study might provide a new insight for the treatment of glioma.

Material and Methods

Cell culture

The human glioma cell line U251 was purchased from Procell life science and technology Co, Ltd (Cat. No: CL-0237, Wuhan, China). The specific growth medium for U251 cells were Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco). Cells were cultured under the condition of 95% air with 5% CO_2 at the temperature of 37°C. The culture medium was renewed every two to three days.

Transfection

In order to alter the expression of PEG10 and miR-506, sh-PEG10 (lncRNA PEG10 silence) and miR-506 inhibitor (miR-506 silence) and their corresponding negative control (NC) (GenePharma Co, Shanghai, China) were transfected into U251 cells. Pre-treated cells at the density of 2×10^5 cells/well were seeded and incubated until the cells arrived at 70–80% confluence, they were transfected with sh-PEG10, miR-506 inhibitor and NC using Lipofectamine 2000 reagent (Invitrogen).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from U251 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The Taqman MicroRNA Reverse Transcription Kit (Cat. No: 4366596, Thermo Fisher Scientific, Rockford, IL, USA) was used for converting miRNA to cDNA. The Taqman Universal Master Mix II (Cat. No: 4440040, Thermo Fisher Scientific) was used for cDNA be generated in a reverse transcription reaction. These two cooperate with TaqMan MicroRNA Assay of PEG10 and miR-506 (Thermo Fisher Scientific) were used for determining the PEG10 and miR-506 expression in U251 cells. U6 and GAPDH were used for the internal controls for miR-506 and PEG10, respectively.

Cell viability assay

Cell viability was enacted by cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China). Firstly, U251 cells were seeded in in 96-well plate. Cells were maintained at 37°C in humidified air with 5% CO_2. Secondly, added 10 μl CCK-8 solution and cells were incubated for 1 h. After incubation, absorption values for detecting cell viability were read at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining (Yeasen, Shanghai, China) was used for determining cell apoptosis. In brief, the cells at the density of 100,000 cells/well were seeded in 6 well-plate. Treated cells were washed twice with precooling phosphate buffer saline (PBS) and centrifuged to resuspend in binding buffer. Then added 5 μl Annexin V-FITC, mixed gently and put in the dark and incubated for 15 min. In addition, added 5 μl PI to the plates. The apoptotic cells rate was measured with flow cytometer (Beckman Coulter, USA) according to the manufacture's instruction.

Migration and invasion assay

Cell migration was evaluated by using a modified two-chamber migration assay (pore size: 8 μm). Cell suspension 100 μl (around 2×10^5 cells/ml) without serum was added to upper Transwell. Then 600 μl culture medium with 10% FBS was added in the lower Transwell. U251 cells were maintained for 24 h at 37°C with 95% air and 5% CO_2. After incubation, cells at the upper surface of the filter were removed by a cotton swab, and the filter was fixed with methanol for 5 min. U251 cells at the lower surface of the filter were stained by crystal violet for 15 min. Cells were counted under microscope (100× magnification). Cell invasion was conducted in the similar process as cell migration, but the inserts were coated with 50 μg of Matrigel (BD Biosciences, Bedford, MA, USA).

Western blot

Western blot was used in our study to detect all the proteins expression. Proteins were obtained from U251 cells using

RIPA lysis buffer (Cat. No: R0010, Solarbio, Beijing, China) supplemented with protease inhibitors (Thermo Fisher Scientific). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used for determining proteins concentration. The Western blot system was established using a Bio-Rad Bis-Tris Gel system following the manufacturer's instructions. Primary antibodies included: anti-p16 antibody (ab51243), anti-p53 antibody (ab75754), anti-B-cell lymphoma 2 (Bcl-2) antibody (ab32124), anti-Bcl-2-associated X protein (Bax) antibody (ab32503), anti-pro Caspase-3 antibody (ab32499), anti-pro Caspase-9 antibody (ab135544), anti-cleaved Caspase-3 antibody (ab32042), anti-cleaved Caspase-9 antibody (ab2324), anti-matrix metalloproteinases MMP-2 antibody (ab92536), anti-MMP-9 antibody (ab76003), anti-vimentin antibody (ab137321), , anti-beta actin antibody (ab8227), all from Abcam (Cambridge, UK); anti-Raf antibody (53745), anti-extracellular signal-regulated kinase (ERK) antibody (4695), anti-phospho-ERK, antibody (4370), anti-mitogen-activated protein kinase ERK kinase (MEK) antibody (9126), anti-phospho-MEK, antibody (9154), anti-janus kinase (JAK) 1 antibody (3344), anti-phospho-JAK1 antibody (74129), anti-signal transducers and activator of transcription (STAT) 3 antibody (12640), anti-phospho-STAT3 antibody (9145), all from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies were prepared in 5% blocking buffer and diluted according to the product instruction. These primary antibodies were incubated in membrane and maintained at 4°C overnight at recommended concentration. Then for second antibody incubation, incubate with horseradish peroxidase (HRP) conjugated second antibody. Detection was performed by capturing the signals and analyzing the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

Results are shown as the mean ± standard deviation (SD) of three independent experiments. Statistical analyses were performed using GraphPad 6.0 statistical software (GraphPad, San Diego, CA, USA). The *p*-values were calculated using a one-way analysis of variance (ANOVA). $p < 0.05$, $p < 0.01$, and $p < 0.001$ reveal statistically significant differences.

Results

PEG10 knockdown inhibited cell proliferation and induced cell apoptosis

To clarify the functions of PEG10, U251 cells were transfected with sh-PEG10 to silence PEG10. The expression of PEG10 was significantly downregulated, indicating high transfection efficiency ($p < 0.01$, Figure 1A). Cell viability, cell apoptosis and their corresponding factors were detected. Results showed that transfection with sh-PEG10 significantly decreased cell viability within each time interval especially in 96 h ($p < 0.01$, Figure 1B). Based on this, we verified the expression of proliferation-related factors p16 and p53. Obviously, we found that the expression of p16 and p53 was upregulated by transfection with sh-PEG10 (both $p < 0.05$, Figures 1C and D). In addition, we found that cell apoptosis was significantly increased by transfection with PEG10 in U251 cells ($p < 0.01$, Figure 1E). Moreover, the expression of anti-apoptotic protein Bcl-2 was downregulated while pro-apoptotic proteins Bax, cleaved-Caspase-3 and cleaved-Caspase-9 were upregulated by transfection with sh-PEG10 (Figure 1F). Taken together, transfection with sh-PEG10 in U251 cell inhibited cell growth by decreasing cell viability and increased cell apoptosis.

PEG10 knockdown inhibited cell migration and invasion

Cell migration and invasion are vital for cancer cells moving forward to the target tissue (Hood and Cheresh 2002). Hence, we measured the effects of sh-PEG10 transfection in U251 cells. Results showed that transfection with sh-PEG10 significantly decreased cell migration and cell invasion (both $p < 0.05$, Figures 2A and D). Consistent with this, we found that the expression of MMP-2, MMP-9 (Figures 2B and C) and vimentin (Figures 2E and F) were all downregulated by transfection with PEG10 (all $p < 0.05$). In a word, transfection with sh-PEG10 inhibited cell migration and invasion in U251 cells.

PEG10 knockdown upregulated the expression of miR-506

MiR-506 has been revealed to have tumor suppressive functions in glioma (Peng et al. 2016). Herewith, we proposed that miR-506 might also associate with the anti-tumor effects of sh-PEG10 in glioma cell line U251 cells. Results in Figure 3 demonstrated that transfection with sh-PEG10 significantly upregulated the expression of miR-506 compared with NC ($p < 0.05$), which indicated that miR-506 might be involved in the anti-tumor effects of PEG10 downregulation.

PEG10 knockdown decreased cell viability and induced cell apoptosis via miR-506 upregulation

To further explore the functions of miR-506, miR-506 inhibitor and NC were transfected into U251 cells. Statistically downregulation of miR-506 by transfection with miR-506 inhibitor indicated high transfection efficiency ($p < 0.01$, Figure 4A). We experimentally determined cell viability, cell apoptosis and the related factors. Surprisingly, co-transfection with sh-PEG10 and miR-506 inhibitor impaired the anti-tumor effects of transfection with sh-PEG10 by increasing cell viability ($p < 0.05$, Figure 4B) and increasing cell

apoptosis ($p < 0.05$, Figure 4E). In addition, the expression of corresponding factors by co-transfection with sh-PEG10 and miR-506 inhibitor presented in Figures 4C, D and F led to the opposite results with transfection with sh-PEG10 in U251 cells. Taken together, the anti-tumor effects of PEG10 knockdown were disappeared after transfected miR-506 inhibitor into cells, which indicated that the PEG10 knockdown decreased cell viability and induced cell apoptosis *via* miR-506 upregulation.

PEG10 knockdown decreased cell migration and invasion via miR-506 upregulation

How does transfection with miR-506 influence the effects of PEG10 knockdown? Similarly we evaluated the effects of co-transfection with sh-PEG10 and miR-506 inhibitor on U251 cells. We found that both migration and invasion were induced after transfection with miR-506 (both $p < 0.05$, Figures 5A and D). Furthermore, the expression of MMP-2,

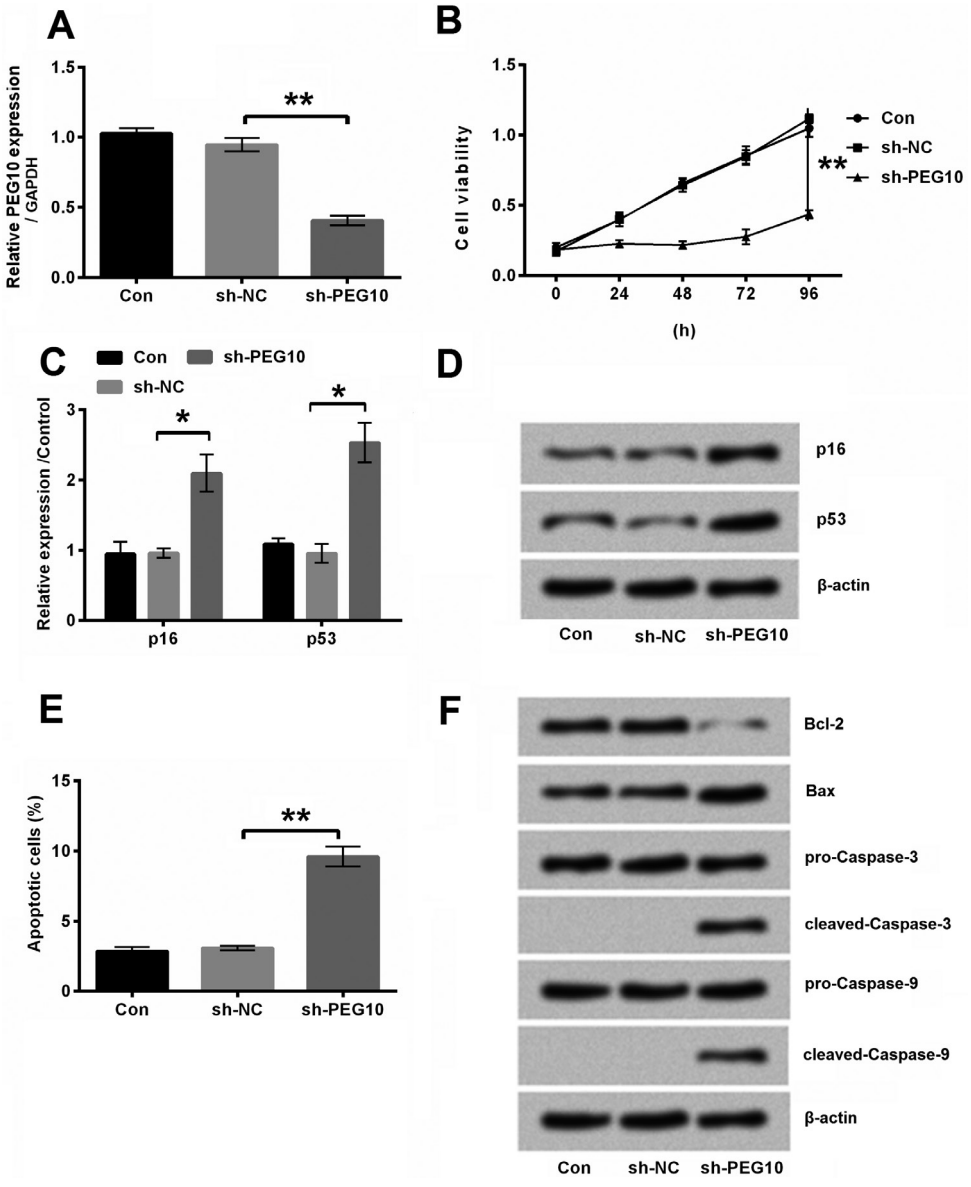

Figure 1. PEG10 downregulation inhibited cell viability and induced cell apoptosis in U251 cells. sh-PEG10 was transfected into U251 cells (**A**). Transfection with sh-PEG10 decreased cell viability in a time-dependent manner (0, 24, 48, 72 and 96 h; **B**), upregulated the expression of p16 and p53 (**C, D**), increased cell apoptosis (**E**), and upregulated the expression of Bax, cleaved Caspase-3 and cleaved Caspase-9, and downregulated the expression of Bcl-2 (**F**) compared with transfection with sh-NC group. The data are shown as the mean ± SD. * $p < 0.05$ and ** $p < 0.01$) are both considered as statistically significant.

MMP-9 and vimentin were all upregulated (all $p < 0.05$, Figures 5B, C and F). Overall, these results suggested that PEG10 knockdown decreased cell migration and invasion *via* modulating miR-506 expression.

PEG10 knockdown inactivated Raf/MEK/ERK and JAK1/STAT3 signal pathways by upregulation of miR-506

The expression of Raf, the phosphorylation of MEK and ERK were downregulated by transfection with sh-PEG10 (all $p < 0.05$) while were reversed by co-transfection with sh-PEG10 and miR-506 inhibitor (all $p < 0.01$, Figures 6A and B). Similarly, the phosphorylation of JAK1 and STAT3 were downregulated by PEG10 knockdown while upregulated by transfection with sh-PEG10 and miR-506 inhibitor ($p < 0.01$, Figures 6C and D). Taken together, treatment with PEG10 knockdown inactivated Raf/MEK/ERK and JAK1/STAT3 signal pathways by upregulation of miR-506.

Discussion

In the present study, we investigated the effects of lncRNA PEG10 on human glioma cell line U251 cells *in vitro*. The main results showed that PEG10 downregulation presented tumor suppressive effects by decreasing cell viability, inducing cell apoptosis, alleviating cell migration and invasion. Further experiment showed that PEG10 knockdown upregulated the expression of miR-506. In addition, the tumor suppressive effects of PEG10 knockdown were through upregulation of miR-506. This process might be modulated by inactivating of Raf/MEK/ERK and JAK1/STAT3 signal pathways.

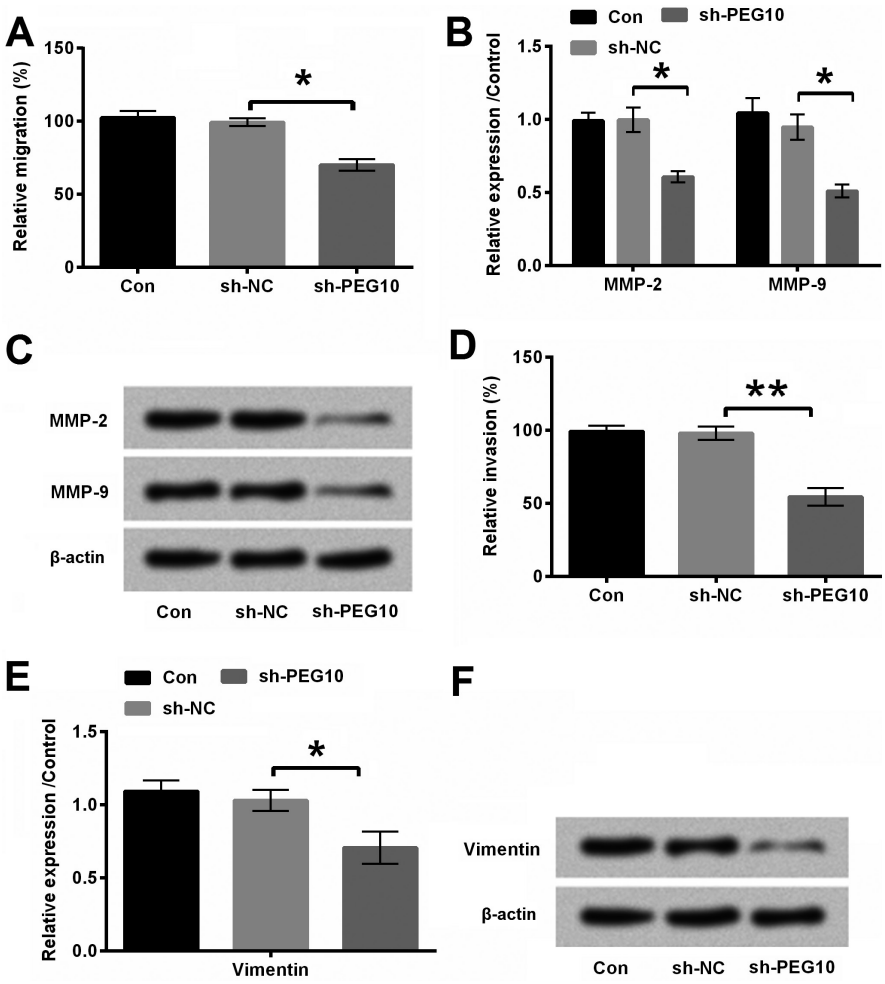

Figure 2. PEG10 downregulation inhibited cell migration and invasion. Transfection with sh-PEG10 decreased cell migration (**A**), downregulated the expression of matrix metalloproteinases MMP-2 and MMP-9 (**B, C**), inhibited cell invasion (**D**), downregulated the expression of vimentin (**E, F**) compared with transfection with sh-NC. The data are shown as the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ are both considered as statistically significant.

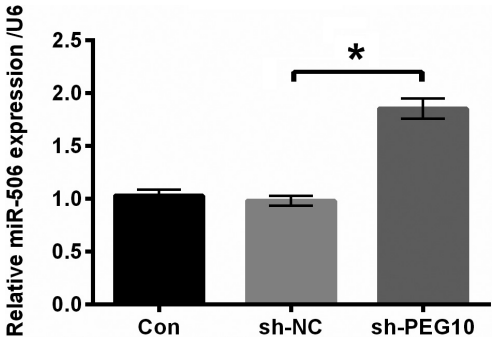

Figure 3. PEG10 downregulation upregulated the expression of miR-506. The data are shown as the mean ± SD. * $p < 0.05$ is considered as statistically significant.

Malignant glioma is the most catastrophic disease and often led to widespread and progressive disability and death. What make it worse is its difficult to diagnose and no standard effective treatments till now (Stewart 2002). lncRNAs demonstrated potential effects on glioma cell biogenesis, development and differentiation of gliomas (Zhang et al. 2012). In our study, we investigated the effects of lncRNA PEG10 expression on glioma cell growth. Results revealed that PEG10 knockdown inhibited cell growth by increasing cell viability and induced cell apoptosis. Previous studies from Hiroshi Okabe et al. found that PEG10 upregulation decreased cell apoptosis in human hepatocellular carcinogenesis, which supported our results on the other side (Okabe et al. 2003).

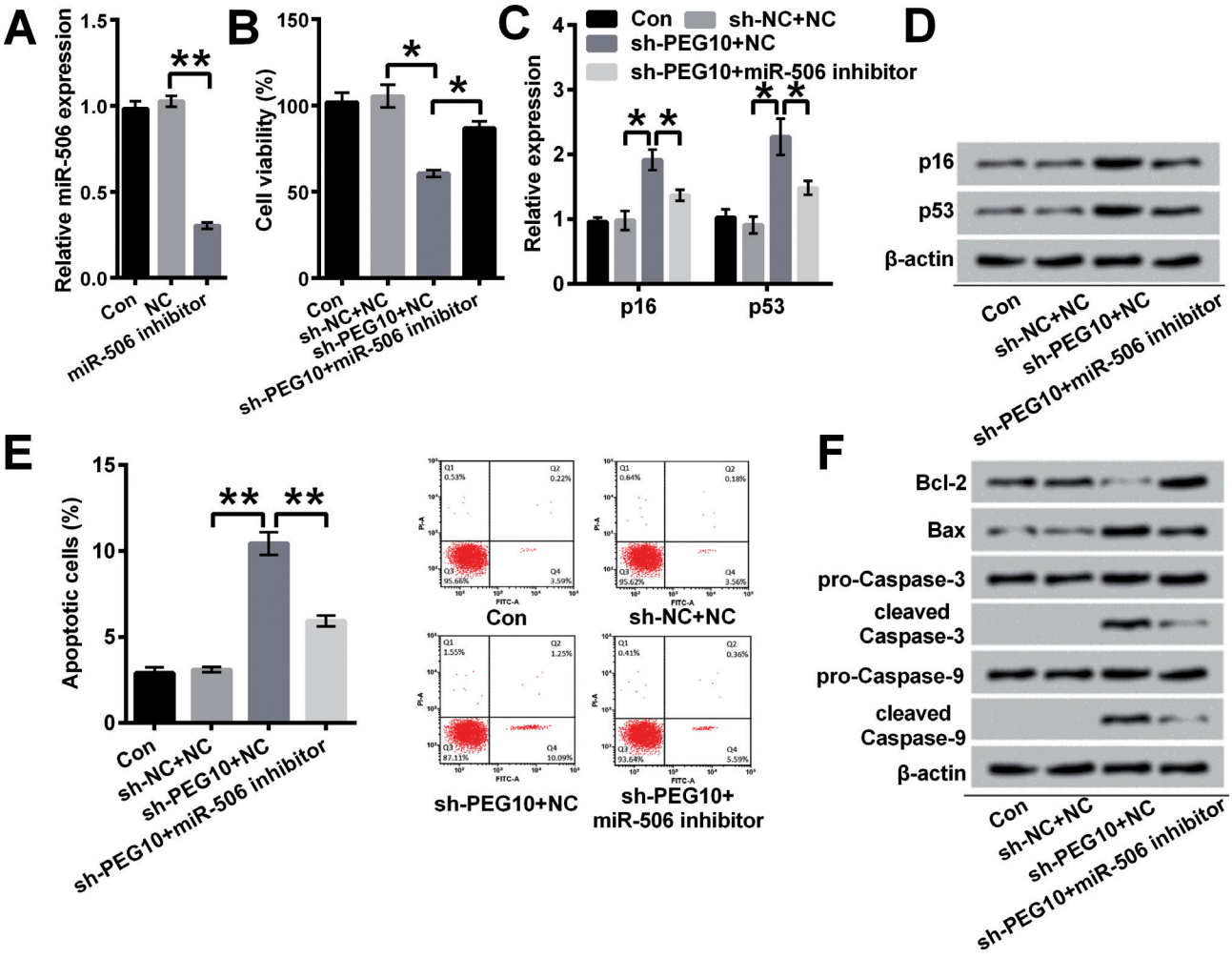

Figure 4. PEG10 downregulation decreased cell viability and induced cell apoptosis by miR-506 upregulation miR-506 inhibitor transfected into U251 cells (**A**). Co-transfection with sh-PEG10 and miR-506 inhibitor impaired the tumor suppressive effects by increasing cell viability (**B**), downregulating expression of p16 and p53 (**C, D**), decreasing cell apoptosis (**E**), upregulating Bcl-2 and downregulating Bax, cleaved-Caspase-3 and cleaved-Caspase-9 (**F**) compared with transfection with sh-PEG10 and NC. The data are shown as the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ are both considered as statistically significant.

In addition, migration and invasion are features of malignant cancer and cancer therapeutics are aimed to slow down tumor progress in migration and invasion (Friedl and Wolf 2003). Importantly, glioma was characterized by its widespread and rapid migration and invasion which made it difficult to treat (Miller et al. 2017). In our study, gladly, we found that PEG10 knockdown dramatically reduced cell migration and invasion, indicating the potential tumor suppressive effects of PEG10 knockdown. Our results supported these previous studies that PEG10 overexpression enhanced cell migration and invasion in human breast cancer (Li et al. 2016c), pancreatic cancer (Peng et al. 2017) and esophageal cancer cells (Zang et al. 2015).

We further explored the underlying mechanisms about how PEG10 knockdown could trigger tumor suppressive effects in U251 cells. Previous study pointed out that the functions of lncRNAs are often found to be connected with miRNAs which are involved in most of biological activities (Jalali et al. 2013). Therefore, we hypothesized that lncRNA PEG10 achieved its functions through regulating miRNAs. As mentioned in the forgone literature, lncRNA PEG10 affected gastric cancer cell growth and development through

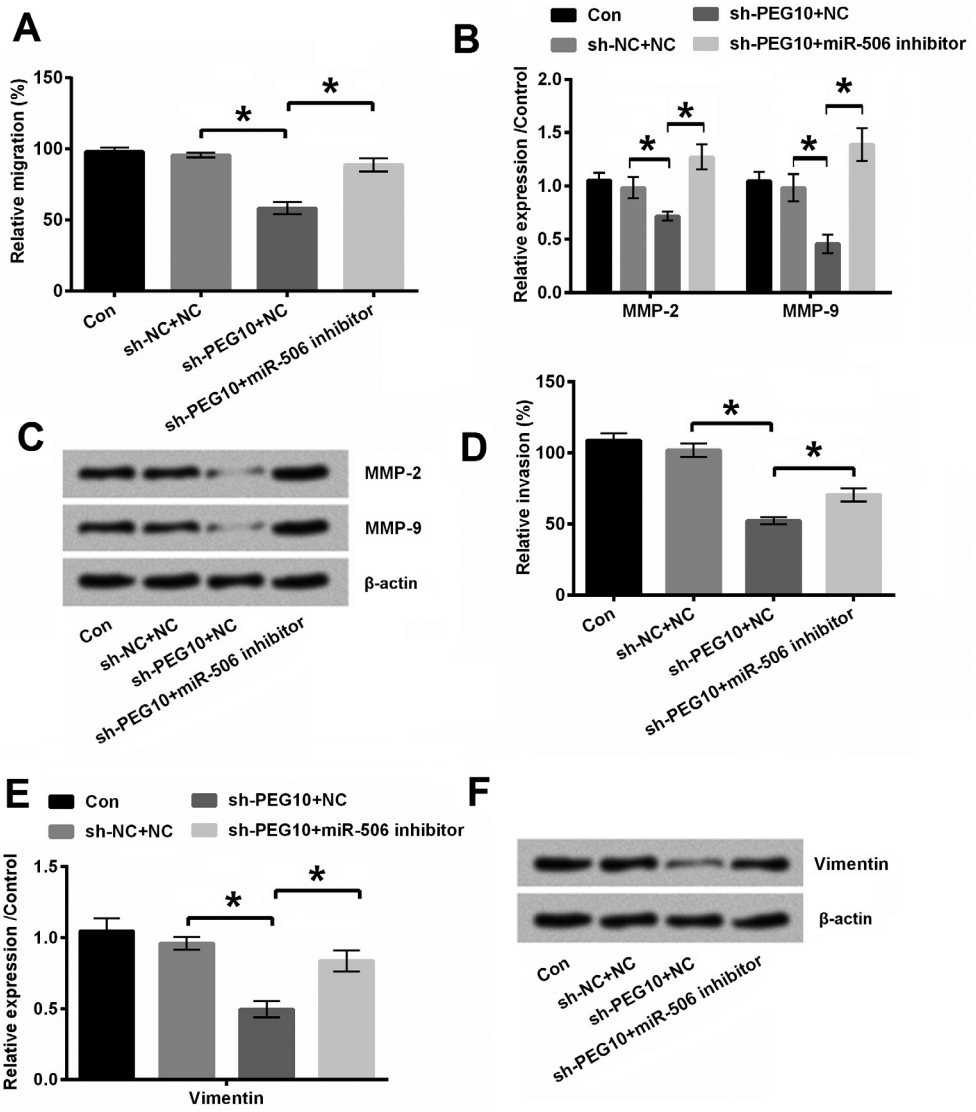

Figure 5. PEG10 downregulation decreased cell migration and invasion by miR-506 upregulation. Co-transfection with sh-PEG10 and miR-506 inhibitor increased cell migration (**A**) upregulated the expression of matrix metalloproteinases MMP-2 and MMP-9 (**B, C**), increased cell invasion (**D**), upregulated the expression of vimentin (**E, F**) compared with transfection with sh-PEG10 and NC. The data are shown as the mean ± SD. * $p < 0.05$ is considered as statistically significant.

regulation of miR-3200 (Wang et al. 2018), and miR-491/PEG10 axis functions in modulating HCT-116 cells growth (Li et al. 2018a). Even though several recent studies started to pay attention to explore miRNAs and lncRNA PEG10, there are still more possibilities in this field. In our study, we induced miR-506 to investigate the possibility between PEG10 and miR-506. miR-506 was a well-studied tumor suppressor in various cancers, such as nasopharyngeal carcinoma (Zhang et al. 2015), colorectal cancer (Chen et al. 2015), pancreatic cancer (Li et al. 2016a) and in glioma (Luo et al. 2015; Peng et al. 2016). In our study, it is the first time that the correlation between lncRNA PEG10 and miR-506 was studied. Surprisingly, after transfection with miR-506 inhibitor into U251 cells, the anti-tumor effects of PEG10 knockdown was impaired with increased cell viability, migration and invasion, and inhibited cell apoptosis. In agreement with our work, we found that the roles of miR-506 in glioma U251 cells were consistent with what roles miR-506 possess in the other cancers (Chen et al. 2015; Luo et al. 2015; Zhang et al. 2015; Li et al. 2016a; Peng et al. 2016) that miR-506 have anticarcinogenic functions. On the other hand, our study also revealed that the mechanism about how PEG10 knockdown could inhibit glioma cell growth and metastasis, that is by miR-506 upregulation.

Raf/MEK/ERK and JAK1/STAT3 pathways play vital roles in tumorigenesis on cell growth, apoptosis, cell cycle arrest and inducible resistance to drugs (McCubrey et al. 2007; Wen et al. 2014). In addition, Raf/MEK/ERK and JAK1/STAT3 pathway were also reported to be correlated in glioma (Liu et al. 2014; Malla et al. 2011). Inactivation of both of these signal pathways can be treated as resultant anti-tumor effect (De Luca et al. 2012; van der Zee et al. 2015). In our study, transfection with PEG10 inactivated both of Raf/MEK/ERK and JAK1/STAT3 pathways while transfection with miR-

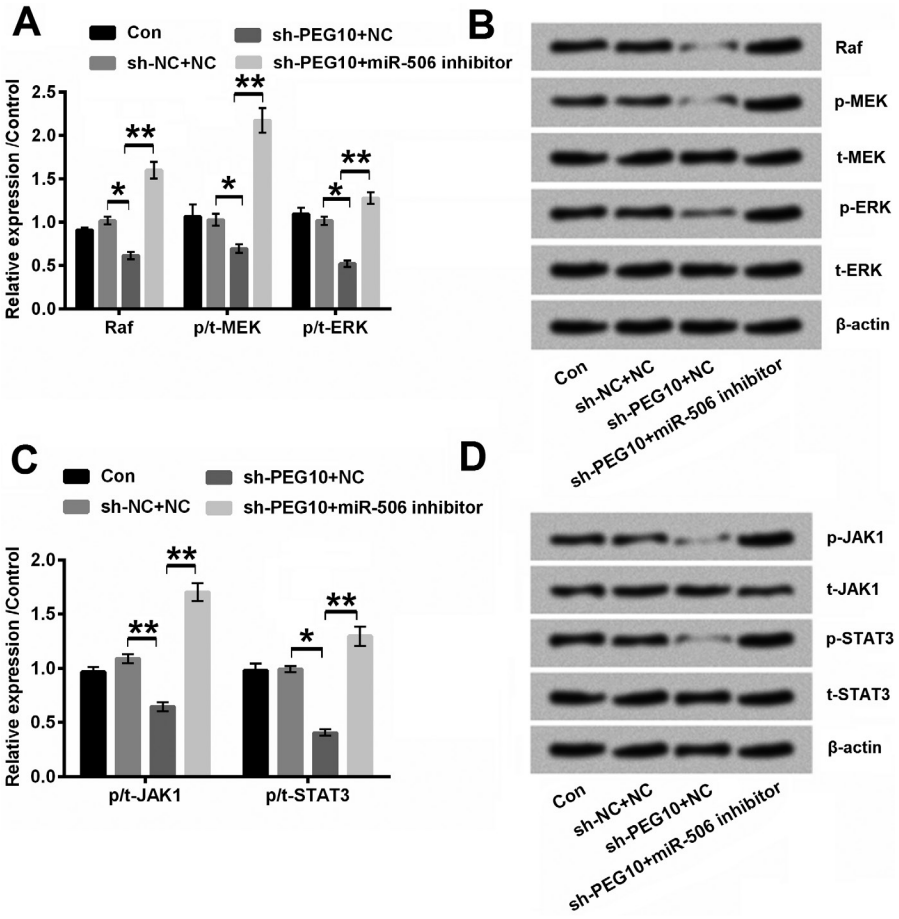

Figure 6. PEG10 downregulation inactivated Raf/MEK/ERK and JAK1/STAT3 signal pathways. Transfection with sh-PEG10 downregulated the expression of Raf, decreased the ratio of p/t-MEK and p/t-ERK (**A, B**), decreased the ratio of p/t-JAK1 and p/t-STAT3 (**C, D**) compared with control. Of contrast, co-transfection with sh-PEG10 and miR-506 inhibitor reversed this trend. The data are shown as the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ are both considered as statistically significant. ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase ERK kinase; JAK, janus kinase; STAT, signal transducers and activator of transcription.

506 inhibitor reversed these results which indicated that the effects of PEG10 knockdown in U251 cells could be *via* modulating of signal pathways by upregulation of miR-506.

In conclusion, our study revealed that PEG10 knockdown inhibited glioma cell growth and migration and invasion through regulation of miR-506. This process might be through inactivation of Raf/MEK/ERK and JAK1/STAT3 pathways. Further studies need to be done whether PEG10 could be treated as a biomarker for the treatment of glioma in the further.

Funding. This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Conflict of interest. The authors declare no conflict of interest.

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Received: December 19, 2018
Final version accepted: May 10, 2019
First published online: June 26, 2019