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Inhibition of JMJD6 expression reduces the proliferation, migration and invasion of neuroglioma stem cells

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Neuroglioma is the most common form of human primary malignant brain tumor, more and more studies recently showed only a small subpopulation of glioma cells which called glioma stem cells have true tumorigenic potential. Meanwhile, it was reported the overexpression of JMJD6 protein is closely involvement with the occurrence and development of multiple tumors, and JMJD6 is required for the differentiation of multiple organ, tissues and cells during embryogenesis. However, the influence of JMJD6 overexpression on neuroglioma development is unclear now. Hence, to explore the effects of JMJD6 expression on neuroglioma, we firstly isolated glioma stem cells by using CD133 MicroBead Kit, and identified via neurosphere-forming assay and Immunofluorescence staining. At the same time, we investigated the effects and mechanism of JMJD6 on the proliferation, migration and invasion of glioma stem cells through MTT, transwell assays and the Cignal finder cancer 10-pathway reporter array. The results demonstrated that the glioma neurosphere cells positively expressed stem cell marker SOX2, neuroectodermal stem cell marker Nestin, and also expressed astrocytes marker GFAP and neurons marker β-tubulin III fter FBS-induced differentiation for a week, which proved the glioma neurosphere cells have the self-renewal and multipotential differentiation capacity. Moreover, shRNA lentiviral vector mediated knockdown of JMJD6 in glioma stem cells led to decreased proliferation, migration and invasion, the underlying molecular mechanism is related to the weaken of Wnt signaling pathway and strengthen of p53 signaling pathway.

Key words: JMJD6, neuroglioma, neuroglioma stem cell

Neuroglioma, also called gliocytoma, is the most common form of human primary malignant brain tumor at present. It occupies more than 50% of brain cancers, with about three new cases per 100,000 populations per year [1, 2], and is characterized with high invasion, morbidity and mortality, and endangers human life and health seriously [3, 4]. In the neuroglioma patients, fewer than 10% of them could survive beyond 5 years [5]. The neuroglioma pathogenesis is related to multiple processes as affected by dozens of regulatory factors [6]. Up to now, our knowledge about the potential underlying factors regulating neuroglioma progression is still not entirely clear.

Jumonji domain-containing 6 (JMJD6) is a member of the Jumonji C domain-containing family of proteins. It was first considered as a phosphatidylserine receptor on cell membrane mediating phagocytosis in apoptotic cells [7]. Subsequently, various studies showed that JMJD6 has a catalytic activity as dioxygenase in the nucleus and is not involved in apoptotic cell clearance [8, 9]. Now, JMJD6 is mainly described as a protein with bifunctional histone arginine demethylation and lysyl-5-hydroxylase activity [10], although the potential functional importance of these activities remains unclear. Recently, more and more studies showed that over-expression of JMJD6 protein is strongly involved with poor prognosis in multiple cancers, such as breast cancer, lung cancer, Colon cancer, et al [8, 10, 11]. It promotes the cellular proliferation and invasion, and plays an oncogene-like role in the development of these cancers. Moreover, It was reported that the function of hydroxylating RNA splicing factors mediated by JMJD6 is essential for the differentiation of multiple tissues and cells during embryogenesis [12]. And silencing of JMJD6 weakens angiogenic functions of endothelial cells via regulating the gene expression and the splicing of the VEGF receptor 1 (Flt1) [13].

All the evidence above showed JMJD6 participates the differentiation of stem cells and exerts an important role in

tumor biological functions. However, the role of JMJD6 in neuroglioma is still unclear. Hence, in this study, we sought to clarify the molecular mechanism in the occurrence, development of neuroglioma by investigating the functions of JMJD6 in neuroglioma stem cells, and provide the new theoretical basis for the treatment of neuroglioma in clinical.

Materials and methods

Material and reagent. Glioma cells U87 and U251 were purchased from ATCC. FBS was purchased from Gibco. Dulbecco's modified eagle medium was purchased from Hyclone. CD133 MicroBead Kit was purchased from Miltenyi Biotec. EGF, bFGF and B27 were purchased from Gibco. Anti-SOX2 were purchased from Cell Signaling Technology. Anti-Nestin, anti-JMJD6, anti-GAPDH, anti-p53, anti-TCF7L2, anti-GFAP and anti-β-tubulin III, Anti-Mouse IgG H&L (FITC) , Anti-Rabbit IgG H&L (FITC), Anti- Mouse IgG H&L (Alexa Fluor® 647) and Anti-Rabbit IgG H&L (Alexa Fluor® 647) were purchased from Abcam. Goat anti-rabbit or goat anti-mouse IgG-HRP was purchased from Beyotime. Lipofectamine 2000 was purchased from Invitrogen. Cignal Finder Cancer 10-Pathway Reporter Array CCA-101L was purchased from QIAGEN.

Culture and isolation of glioma stem cells. U87 and U251 cells were cultured with complete DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37 °C. When glioma cell confluence reached 80%, cells were harvested by trypsinization collected by centrifugation and washed with PBS for 3 times. Then the cells were incubated with mAbs against CD133-PE and CD133 MicroBeads according to the instruction manual. Finally, Glioma stem cells (CD133+ cells) were isolated from the tubular fraction by magnetic sorting, using the MACS system.

Sphere-forming assay. Glioma stem cells (1000 cells per well) were plated onto a 24-well ultra-low attachment plate and cultured in serum-free DMEM/F12 medium supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, 2% B27 in 5% CO_2 at 37 °C. After 7 days of culture, the spheres were observed under an inverted microscope and identified by immunofluorescence staining.

Immunofluorescence staining. The U87 and U251 stem cells (U87-SC or U251-SC) were seeded onto a 24-well plate and fixed in 4% paraformaldehyde for 20 min, permabilized with 0.1% Triton X-100 and subsequently blocked with 10% donkey serum for 1 h. Then the cells were incubated at 4 °C overnight with one of the following antibodies: mouse anti-Nestin (1:1000), rabbit anti-SOX2 (1:400), rabbit anti-GFAP (1:1000) and mouse anti-β-tubulin III (1:1000). Next day, cells were washed with PBS for 3 times and incubated with corresponding secondary antibodies including Alexa Fluor 647-conjugated goat anti-mouse or goat anti-rabbit (1:500) and FITC-conjugated goat anti-rabbit or goat anti-mouse (1:1000). Nuclei were counterstained with DAPI and imaged under a fluorescence microscope.

Transfection and western blot. Glioma stem cells were divided into shRNA-NC group and shRNA-JMJD6 group. JMJD6-shRNA lentiviral vector and vector control were constructed in FulenGen (Guangzhou, China). Cells were harvested using trypsin and lysed with RIPA buffer, the supernatants were collected by centrifugation. Then the protein was denatured by boiling and detected the concentration by BCA Protein Assay reagent kit. The protein was fractionated using SDS-PAGE and transferred onto PVDF membrane, and membranes were blocked for 1 h in 5% skim milk before being incubated with antibodies. For detection, the membrane were incubated at 4 °C overnight with one of the following antibodies: rabbit anti-JMJD6 (1:500), mouse anti-GAPDH (1:5000), rabbit anti-p53 (1:1000), rabbit anti-TCF7L2 (1:25000), and washed 3 times in TBST, each time for 10 min. Then the membrane was incubated with secondary antibodies (goat anti-mouse or goat anti-Rabbit IgG H&L) (1:1000) for 1 h at room temperature. Finally, the membrane was washed with TBST repeatedly again and the protein was visualized using the ECL kit and observed by GeneGnome mechine (Syngene).

MTT assay. Cell proliferation was evaluated after transfected with JMJD6-shRNA lentiviral vector. Firstly, cells in the logarithmic growth phase were harvested by trypsin and centrifugation, and resuspended. Then 100 μL suspension containing 1000 cells were plated into 96-well plates per well and cells were grown for 24~72 h. Four parallel wells were designed for each experimental group. Then 20 μL MTT (5 mg/ml) was added to each well and incubated for 4 h. Finally, the reaction was terminated by 150 μL DMSO. And the optical density at 490 nm was detected using Microplate Reader.

Cell migration and invasion assay. 200 μL Serum-free DMEM containing 1.5×10^5 U87-SC or U251-SC were placed into the upper room of a Transwell insert, while the lower room was filled with completed DMEM containing 10% FBS as a chemoattractant. The filter of Transwell insert without Matrigel were used for transwell migration assay and coated with Matrigel to act as extracellular matrix were used for invasion assay. Then the Transwell system was incubated at 37 °C for 24 h. And migrated and invaded cells were fixed with methanol for 20 min, rinsed with PBS and stained with crystal violet for 15 min. Cells were counted in four randomly selected fields under microscope.

Cignal finder cancer 10-pathway reporter array. The Cignal finder cancer 10-pathway reporter array was performed as described to pinpoint pathways which regulated the effect of JMJD6 possibly in glioma stem cells. After preparation of complex formation according to the instruction manual, the logarithmic growth phase cells were harvested by trypsin and centrifugation, and resuspended. Then 50 μL suspension (1×10^6/ml) were plated into 96-well plates per well and cultured at 37 °C with 5% CO_2 for 6 h, and previous medium was replaced with medium which containing 10% FBS without antibiotics subsequently. Next day, complete medium were added and cells were cultured continuously for 48 h. Finally,

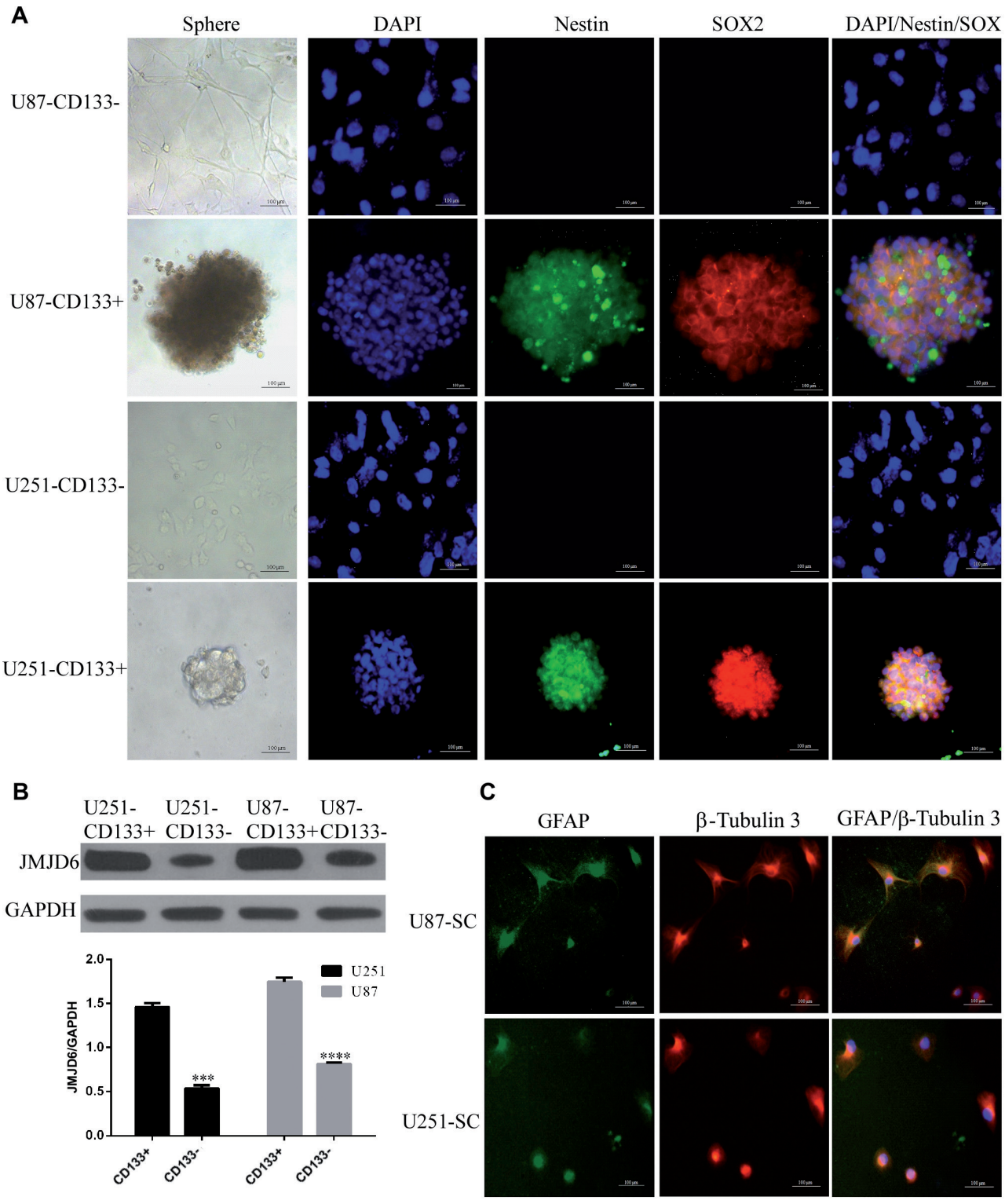

Figure 1. Isolation and identification of glioma stem cells. (A) Line1: The sphere morphology of glioma stem cells (CD133+ cells) was observed and imaged under invert microscope; Line2~ Line5: Before differentiation, neurosphere was identified by immunefluorescence staining, and the stem cell marker SOX2 and neuroectodermal stem cell marker Nestin were both expressed positively in neurosphere. (B) The expression of JMJD6 were detected via Western blot. Compared with CD133- cells, the expression of JMJD6 was significantly increased in glioma stem cells (CD133+ group). CD133- group as control, compared to control group, ***P<0.001; ****P<0.0001. (C) After FBS-induced for a week, the markers of differential neural cell lineages like GFAP and β-tubulin III were expressed in CD133+ cells.

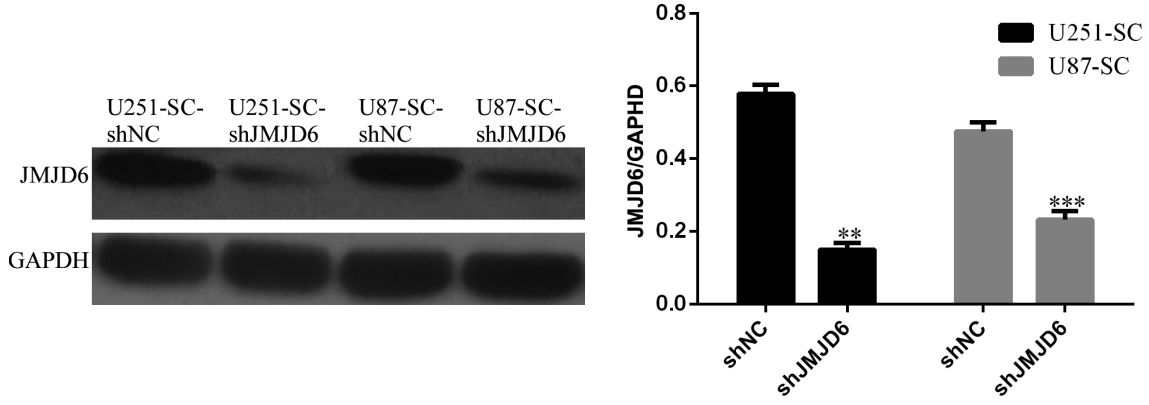

Figure 2. JMJD6 protein expression levels of Glioma stem cells (U87-SC/U251-SC). Cells transfected with shRNA-JMJD6 lentiviral vector and NC vector were considered as experimental groups and control groups, respectively. Western blot was performed to verify transfection efficacy. Left: The expression of JMJD6 was down-regulated notably in glioma stem cells after transfected with shRNA-JMJD6 lentiviral vector. Right: Relative expression of JMJD6. Compared to shNC group, **P<0.01; ***P<0.001.

the luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega, WI, USA).

Statistical analysis. All experiment data were expressed as mean ± SD, and Statistical analyses were performed using SPSS 19.0. Significance between two groups was examined with unpaired Student's *t* test for two groups; one-way ANOVA for multiple groups. Differences were considered statistically significant at P<0.05.

Results

Isolation and identification of neuroglioma stem cells. In this study, we firstly isolated glioma stem cells (U87-SC and U251-SC) using CD133 MicroBead Kit. For the CD133+ cells, Sphere-forming assay was performed in a 24-well ultra-low attachment. The result showed CD133+ cells have the neurosphere-forming ability. The sphere morphology was formed after cultured for a week, and imaged under an invert microscope (Figure 1A, Line1).

In order to identify whether the CD133+ cells were neuroglioma stem cells, immunofluorescence staining was used to detect the expression of stem cell specific markers. As shown in Figure 1A (Line2~Line5), stem cell marker SOX2 and neuroectodermal stem cell marker Nestin were both expressed positively in U87-SC and U251-SC. Moreover, after FBS-induced differentiation for a week, we detected the markers of different neural cell lineages including astrocytes (GFAP) and neurons (β-tubulin III) were also expressed positively (Figure 1C), all evidences above suggested the glioma neurosphere cells we isolated had the capacities of self-renew and multipotential differentiation.

The expression of JMJD6 in neuroglioma stem cells. The expression of JMJD6 protein was analyzed by Western blot in CD133- and CD133+ glioma cells (U87 and U251), respectively. Compared with CD133-glioma cells, JMJD6 expression increased notably in CD133+ glioma cells (P<0.001, Figure 1B).

Lentiviral vector infection and JMJD6 silencing. After understanding the difference of JMJD6 expression between CD133- and CD133+ glioma cells, the silencing efficacy of JMJD6 in CD133+ glioma cells (neuroglioma stem cells) was determined by Western blot, and the results showed the expression level of JMJD6 decreased dramatically compared with that in negative control groups (P<0.01, Figure 2).

JMJD6 promotes neuroglioma stem cell proliferation. The effect of JMJD6 on neuroglioma stem cell proliferation was assessed via MTT assay. And results showed cell viability decreased significantly in U87-SC and U251-SC after transfected with JMJD6-shRNA lentiviral vector (P<0.05 or P<0.01; Figure 3). That is to say JMJD6 could promote the proliferation of neuroglioma stem cell.

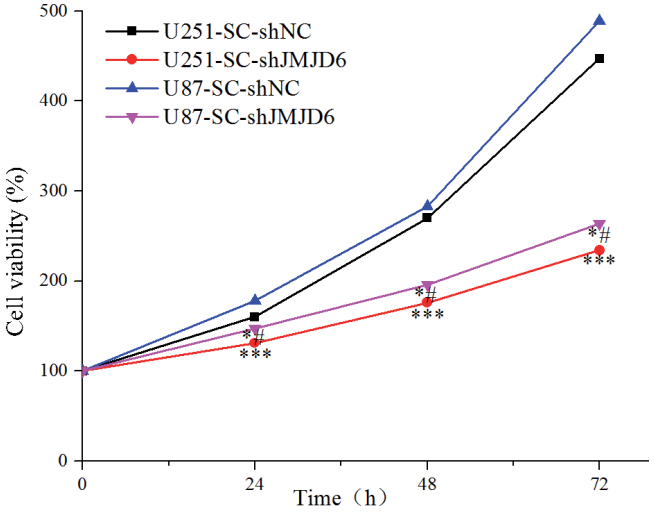

Figure 3. MTT assay was used to detected cell viability. The proliferation ability of glioma stem cells reduced after JMJD6-shRNA lentiviral vector transfecion. Compared with shNC group, ***P<0.001; *#P<0.01.

JMJD6 accelerates neuroglioma stem cell migration and invasion. Transwell migration and matrix penetration assays were used to examine the effect of JMJD6 on migratory and invasive abilities. Compared to control group, the migration speed was reduced in neuroglioma stem cells (U87-SC and U251-SC) after transfected with JMJD6-shRNA lentiviral vector ($P<0.01$, Figure 4A). Moreover, cell invasion ability was reduced as well after JMJD6-shRNA lentiviral vector transfection ($P<0.01$, Figure 4B). In brief, JMJD6 could promote migration and invasion of neuroglioma stem cells.

JMJD6 exerts its function by activating Wnt signaling and suppressing P53 signaling. To investigate the mechanisms by which JMJD6 promotes glioma stem cell proliferation, migration and invasion, we firstly adopted the Cignal finder cancer 10-pathway reporter array to explore signaling pathways involved in these processes. The results showed that Wnt signaling was suppressed and p53/DNA damage signaling was activated in neuroglioma stem cells after transfected with JMJD6-shRNA lentiviral vector (Figure 5A, 5B). To verify the result above, we measured the expression levels of TCF7L2 and p53, which is the

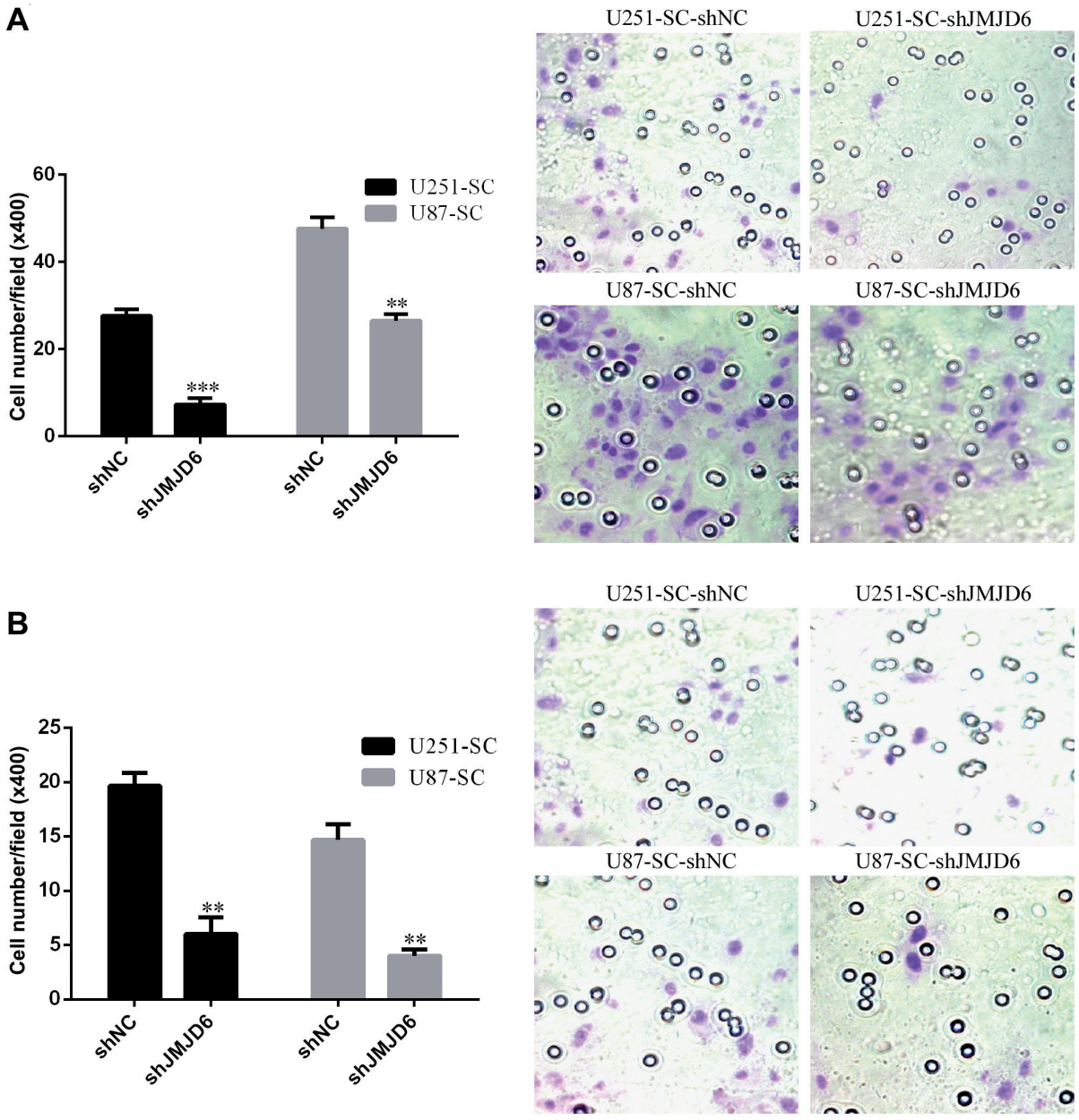

Figure 4. Inhibition of JMJD6 could reduce migration and invasion ability of glioma stem cells (U87-SC and U251-SC). (A) Cell migration; (B) Cell invasion. U87-SC-shNC/ U251-SC-shNC as control groups. Compared with control group, **$P<0.01$; ***$P<0.001$.

important molecule in Wnt and p53/DNA damage signaling pathway, respectively. And results indicated that silencing of JMJD6 decreased the expression of TCF7L2 and increased the expression of p53 notably in JMJD6-shRNA lentiviral transfection groups (Figure 6A, 6B).

Discussion

Neuroglioma is the most frequent malignant primitive tumors of central nervous system, and statistical data show that the annual morbidity is about 10-20/100,000 people. This disease has a wide range of onset age, it occurs to people in age of 20-50 years old with 5-year survival rate lower than 5% [14]. Currently, despite great advances in therapy, none of the treatments is curative. Previous studies show that the malignant degree has a positive correlation with the ability cytotoxin resistance, invasion transfer and proliferation which induced by tumor vessel, the main reason is neuroglioma always presents invasive growth and has no obvious demarcation in normal brain tissues [15]. The pathogenesis of neuroglioma is complex and involves with dysregulation of many biological pathways at multiple levels [16], and despite the main view which caused neuroglioma is due to the interaction between genetic factors and environmental factors, the specific mechanism is not yet clear [17-19]. Hence, it is important to deeply understand and research the occurrence and development mechanism of neuroglioma.

Previous studies show heterogeneous cell populations exist in glioma, these different subpopulation cells are result due to the loss of genomic stability, or due to the dysregulated proliferation and differentiation processed in glioma cells, and the subpopulations exhibit distinct abilities regarding their contribution to glioma growth and development [20]. A small part of glioma subpopulation cells which characterized with tumor-initiating/stem cells function are defined as glioma initiating/stem cells (GICs/GSCs) [4]. Interesting, it was reported that glioma stem cells are shown to have significant similarities to normal neural stem cells (NSCs), including the self-renew ability, similar transcriptome profiles, and ability differentiation into different neuronal lines [21]. The self-renew and differentiation capacity of normal neural stem cells can be evaluated via surrogate neurosphere-forming assay [22-26]. Therefore, in this study, we isolated glioma stem cells from glioma cells by using CD133 MicroBead Kit, then identified via neurosphere-forming assay, immunofluorescence staining and FBS-induced differentiation experiments. The result showed the glioma stem cells (U251/U87-CD133+ cells) had neurosphere-forming ability (Figure 1A, Line1) and positively expressed stem cell marker SOX2 and neuroectodermal stem cell marker Nestin (Figure 1A, Line2~Line5). Moreover, after FBS-induced differentiation for a week, the neurosphere cells expressed astrocytes marker GFAP and neurons marker β-tubulin III (Figure 1C), which suggested the glioma neurosphere cells have the self-renew and multipotential differentiation capacity.

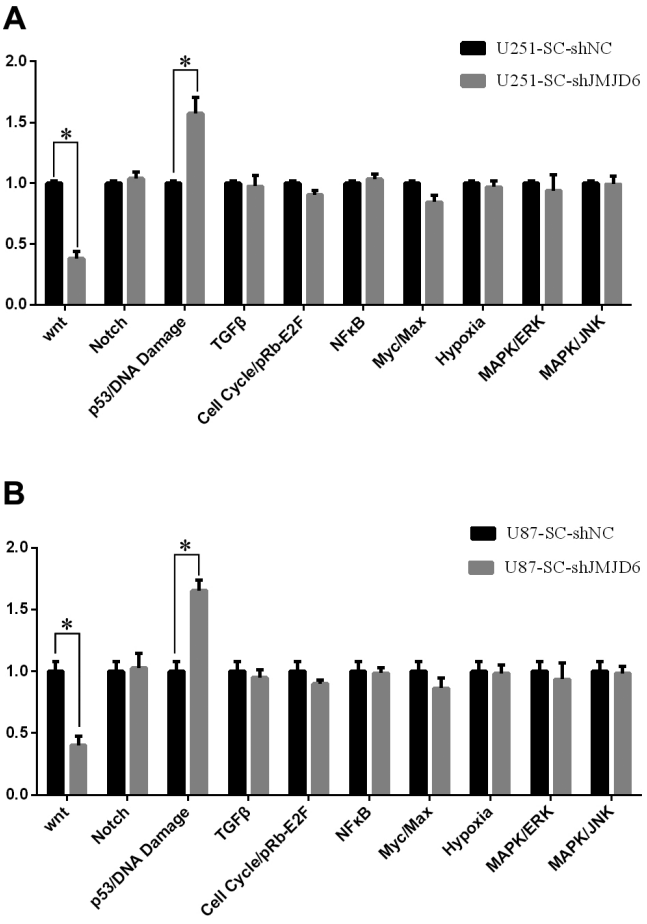

Figure 5. JMJD6 stimulates Wnt and p53/DNA damage signaling in glioma stem cells. Glioma stem cells transfected with JMJD6-shRNA or NC vectors were used in these studies. (A, B) Wnt and p53/DNA damage signaling were both stimulated in U251-SC and U87-SC, respectively, while the other signaling pathways showed no significant change.

JMJD6 protein has been identified for many years, the mainly functions are arginine demethylase and lysyl-5-hydroxylase activities. A structural study had suggested that the substrates of JMJD6 might be methyl groups on single-stranded RNAs (ssRNAs) [27]. Recent years, multiple studies demonstrate that overexpression of JMJD6 is closely involvement with the occurrence and development of breast, lung cancer, colon cancer, et al [8, 11, 28]. Meanwhile, it was reported that JMJD6 is required for the differentiation of multiple organ, tissues and cells during embryogenesis [9]. However, the role of JMJD6 in neuroglioma is still unclear. Based on the functions of tumor stem cells and JMJD6, we detected the expression of JMJD6 in glioma stem cells via Western blot and found a significant difference in the expression between glioma stem cells (CD133+) and giloma cells (CD133-) (Figure 1B). Furtherly, we investigated the effects of JMJD6 on the proliferation, migration and invasion of glioma stem cells through MTT and transwell assays. The

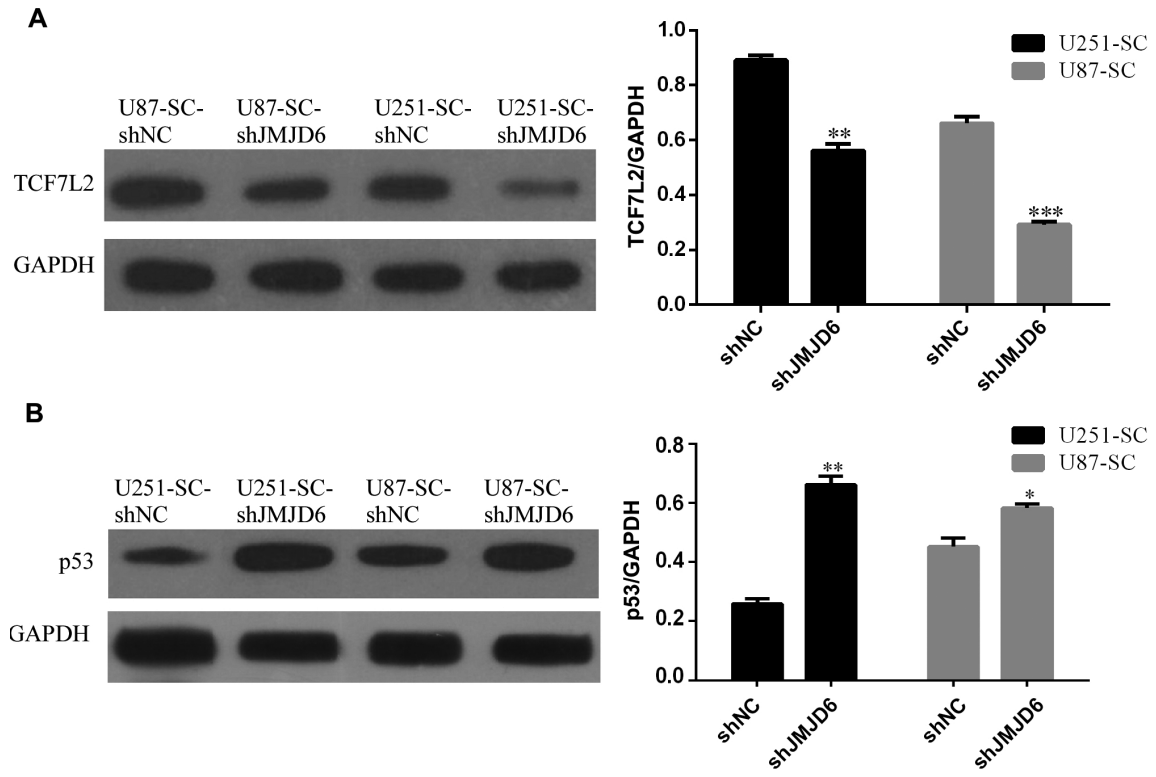

Figure 6. The expression of TCF7L2 and p53 protein levels. (A) The expression of TCF7L2, which is an important downstream molecule in Wnt signal pathway, was detected via Western blot. And TCF7L2 expression of glioma stem cells was reduced significantly after JMJD6-shRNA lentiviral vector transfection. (B) The expression of critical molecule in p53 signal pathway was increased compared to control groups. Compared with control group, *P<0.05, **P<0.01, ***P<0.001.

results demonstrated that shRNA lentiviral vector mediated knockdown of JMJD6 in glioma stem cells led to decreased proliferation, migration and invasion (Figure 3, 4). To explore the underlying mechanism, the Cignal finder cancer 10-pathway reporter array was performed to pinpoint pathways which regulated the effect of JMJD6 possibly in glioma stem cells. The results showed Wnt signaling pathway was weakened and p53 signaling pathway was enhanced in U251-SC/U87-SC-shJMJD6 groups (Figure 5). As we know, p53 is a tumor suppressor, it plays a critical role in maintaining genomic stability and preventing tumor formation [29]. In previous study, Wang et al [30] has reported that JMJD6 could negatively regulate p53 signaling through hydroxylation which occurs on lysine 382 site of p53 protein and promote colon carcinogenesis. Meanwhile, JMJD6 antagonizes p53 acetylation and promotes the association with its negative regulator MDMX, and represses transcriptional activity of p53. And Wnt signaling also has a close relationship with tumor, its abnormal activation acts an important role in the occurrence and development of human tumors [31]. In this process, Wnt/β-catenin signaling is mediated by protein complexes of individual members of Tcf/Lef family of DNA-binding factors [32]. Zhang et al [33] had reported that JMJD6 could enhance β-catenin-induced gene activation through antagonizes the repression effect of Tcf7l1 on downstream gene transcription. In addition, in Wetering's study [34], it was reported that disruption of β-catenin/TCF-4 activity in colorectal cancer cells induces a rapid G1 arrest and controls proliferation and differentiation, inhibition the activity of β-catenin/TCF-4 complex could block the proliferation of colorectal cancer cells effectively. All the evidence above show Wnt and p53 signaling pathways possibly make an important role in caner. Hence, investigating the correlation between JMJD6 and Wnt (or p53) signaling furtherly may provide a novel view and therapeutic target as well as a prognostic marker in neuroglioma.

In conclusion, our study above demonstrated that JMJD6 acts an important role in the processes of migration and invasion in neuroglioma, and the underlying molecular mechanism may involve Wnt and p53 signaling. However, the detailed interaction mode and processes between JMJD6 and Wnt (or p53) signaling pathway in glioma stem cells has not studied in this article and need to be investigated further.

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