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ORIGINAL PAPER

Let-7b inhibits the malignant behavior of glioma cells and glioma stem-like cells via downregulation of E2F2

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Abstract Glioblastoma multiforme (GBM), the most common and lethal primary brain tumor in adults characterized by high proliferative ability and mortality rate, contains a small subpopulation of cancer stem-like cells (CSCs), which is responsible for GBM progression and therapeutic resistance. Numerous microRNAs are strongly implicated in the malignancy of glioma. However, their specific functions and roles have yet to be fully demonstrated. In the present study, we revealed that the upregulation of Let-7b, a member of the Let-7 microRNA family, inhibited proliferation, migration, and invasion in glioma cell lines. Using bioinformatics, expression analysis, and luciferase assay, E2F2 was confirmed as a candidate target of Let-7b. Moreover, we also observed that elevated levels of Let-7b resulted in a reduction of tumor sphere growth and stemness of glioma stem-like cells. Furthermore, we found that knockdown of E2F2 expression could reduce the proliferation of glioma and GSCs, while overexpression of E2F2 partially abrogated the inhibitory effect of Let-7b on the proliferation of glioma and GSCs. In conclusion, we suggest that Let-7b could be developed into a promising anticancer target in glioma.

Keywords Glioma · Glioma stem cell · miRNA · Let-7b · E2F2

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Introduction

Glioblastoma is the most common and deadly primary brain tumor of the central nervous system in adults, with the characteristics of being highly proliferative, invasive, and vascularized. The average survival rate of patients suffering from glioblastoma, following treatment with surgery, radiotherapy, or chemotherapy, is still less than 16 months [5, 17, 19]. The poor prognosis of glioma patients is due to the oncogenic nature of glioma cell and the existence of a small subpopulation of glioma stem-like cells (GSCs), which has the capability of self-renewing and differentiation [20, 32]. Therefore, understanding the mechanism involved in the rapid growth and metastasis of glioma cells and the stemness of GSCs are urgently required to develop new approaches to overcome this disease.

MicroRNAs (miRNAs) are endogenous, single-stranded, small non-coding RNAs, approximately 18–22 nucleotides in length, which regulate the expression of other genes at the posttranscriptional level via directly targeting the 3′ untranslated region (UTR) of target mRNAs [1]. Cumulative evidence suggested that miRNAs played a critical role in the diverse physiological processes of various cancers, including tumori

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genesis, apoptosis, metastasis, and maintenance of cancer stem cells [8, 11, 34]. In particular, growing researches denote that miRNAs may act as tumor promoters or tumor suppressors in glioma cells. For example, miR-92b was found to influence cell proliferation and migration, as well as invasion and induced apoptosis of glioma cells by negatively regulating PTEN expression [27]. It was also reported that ectopic expression of miR-145 has a negative impact on the progression and development of glioma cells by directly targeting Sox9 and adducin3 [22]. However, the involvement of miRNAs in the pathogenesis of glioma remains elusive, and further studies are needed to fully elucidate their contributions to this malignancy. Current studies demonstrated that the low expression of the Let-7 family may be responsible for the poor prognosis of glioma patients [16, 21]. Furthermore, Tian et al. confirmed that Let-7b was a tumor suppressor in glioma [30]. However, the precise role of Let-7b is not fully explored and its function in GSCs has never been characterized.

E2F2, an important member of the E2F transcriptional activator family, was a cellular factor which could initiate E2 gene transcription in adenovirus [12, 13]. Previous studies indicated that E2F2 is associated with a variety of crucial cellular processes, including proliferation, cell cycle, and tumorigenesis of many cancers [23, 24]. Although latest research demonstrated that the expression level of E2F2 is correlated with the growth and metabolism of glioma cells [33], the mechanisms for E2F2 underlying the genesis of glioma and GSCs are not well addressed.

In the present study, we identified that Let-7b could suppress the proliferation, migration, and invasion of glioma cells. It also reduced the tumor sphere growth and self-renewal ability of GSCs. In addition, we demonstrated that E2F2 is a direct target of Let-7b in glioma. Furthermore, we confirmed that E2F2 expression is critical for Let-7b-induced inhibitory effect on the proliferation of glioma and GSCs, implying that the Let-7b/E2F2 signaling might be a possible therapeutic target for the treatment of glioma.

Materials and methods

Cell culture

Human glioblastoma cell lines, U251 and U87, were obtained from the Chinese Academy of Sciences Cell Bank. All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, CA, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C under 5 % CO_2.

Enrichment for glioma stem-like cells

The serum-free culturing technology was applied to select and enrich glioma stem-like cells. U251 and U87 cells maintained in conventional culture media were dispersed with trypsin (Beyotime Biotech, China) and resuspended in neurobasal medium (Invitrogen) supplemented with B27 (20 mg/ml, Invitrogen), 20 ng/ml EGF (PeproTech, NJ, USA), and 20 ng/ml bFGF (Sigma, MO, USA). EGF and bFGF were added twice a week. Tumor spheres were dissociated with TrypLE (Thermo Fisher Scientific, MA, USA) and passaged with fresh medium every 7–10 days.

Immunofluorescence staining of tumor stems cells

Immunofluorescence staining was performed as described previously [32]. Briefly, tumor spheres were collected and plated on glass coverslips coated with poly-L-lysine. Spheres were then fixed in 4 % paraformaldehyde and permeabilized with 0.3 % Triton X-100. After being blocked with 10 % bovine serum albumin (BSA) for 30 min, the spheres were incubated at 4 °C overnight with CD133 (Santa Cruz Biotechnology, Santa Cruz, USA; mouse monoclonal, 1:100 diluted) and nestin (Santa Cruz Biotechnology; rabbit polyclonal, 1:100 diluted) antibodies, rinsed with PBS three times and incubated for 1 h with Texas red-conjugated or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA; 1:500 diluted) at room temperature. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen) for 10 min. Images were captured utilizing a fluorescence microscope (Olympus, Tokyo, Japan).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent (Vazyme, China) following the manufacturer's protocol, and 1000 ng of RNA was reverse transcribed into cDNA by the HiScript First Strand cDNA Synthesis Kit (Vazyme). Relative expression levels of miRNA and

mRNA were tested by qRT-PCR using the SYBR® Green Real-Time PCR Master Mix (Vazyme) in StepOnePlus™ real-time PCR System (ABI, CA, USA). Human small nuclear U6 RNA and β-actin were amplified as internal control. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method. For miRNA analysis, the sequences of RT primers were: Let-7b 5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACAACCAC-3′ U6 5′-AAAAATATGGAACGCTCACGAATTTG-3′. The sequences of quantitative PCR primers were Let-7b 5′-CGGCTCGCATGAGGTAGTAGG-3′ (forward) and 5′-CCAGTGCAGGGTCCGAGGTA-3′ (reverse). U6 small nuclear RNA, 5′-GTGCTCGCTTCGGC AGCACATATAC-3′ (forward) and 5′-AAAA ATATGGAACGCTCACGAATTTG-3′ (reverse); E2F2, 5′-CGTCCCTGAGTTCCCAACC −3′ (forward) and 5′-GCGAAGTGTCATACCGAGTCTT-3′ (reverse); β-Actin, 5′-AAAGACCTGTACGC CAACAC-3′ (forward) and 5′-GTCATACTCCTGCT TGCTGAT-3′ (reverse).

Oligonucleotide synthesis, plasmid and cell transfection

Let-7b mimics (5′-UGAGGUAGUAGGUUG UGUGGUU-3′) and negative control miRNA mimics (5′- GCGACGAUCUGCCUAAGAU-3′, miR-NC) were chemically synthesized from GenePharma (Shanghai, China). E2F2 siRNA (On-targetplus SMARTpool, no. L-003,260-00-005, siRNA) and negative control siRNA (ON-TARGETplus non-targeting pool, no. D-001,810-10-05, siRNA-NC) were purchased from Dharmacon (Lafayette, CO). The E2F2-overexpressing plasmid pCMVHA-E2F2 was a generous gift from Kristian Helin (Addgene plasmid no. 24,226). All transfections were conducted using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions in serum-free medium. For miRNA analysis, cells were transfected with Let-7b mimics or miR-NC at a final concentration of 100 nM. All siRNA duplexes were used at a final concentration of 100 nM.

Target prediction and luciferase reporter assay

A luciferase reporter construct for the wild-type or mutant E2F2 3′UTR containing the predicted binding site for Let-7b was synthesized and cloned downstream of the luciferase gene in the pGL3-Control Vector (Promega, WI, USA). Cells were cultured in 96-well plates and cotransfected with wild or mutant luciferase reporters and Let-7b mimics using Lipofectamine 2000 (Invitrogen). Following 48 h of incubation, luciferase activity was measured using a dual-luciferase reporter system (Promega). Renilla luciferase activity was used for normalization among samples.

Cell proliferation assay

Cell proliferation assay was detected using the CCK8 Cell Counting Kit (Vazyme). All cells were seeded in 96-well microtiter plates at a density of 2000 cells per well with three replicates for each group. Ten microliters of CCK8 was added into each well and incubated for 3 h ahead each time point (24, 48, and 72 h, respectively) after transfection. The absorbance at the wavelength of 450 nm was documented using the SpectraMax M5 microplate reader (Molecular Devices, CA, USA).

Cell migration and invasion assay

Transwell filters (diameter 12 mm, pore size 8 μm, Millipore, MA, USA), coated without or with Matrigel (BD Biosciences, CA, USA), were utilized to determine the role of Let-7b on migration and invasion, respectively. Transfected glioma cells (2×10^5) were resuspended in 400-μl serum-free medium and plated on the top side of the chamber; a 600-μl medium supplemented with 20 % FBS acted as a chemoattractant in the lower side of the chamber. After being incubated at 37 °C for 48 h, cells on the upper membrane surface was removed via a cotton swab. Migrated or invaded cells on the bottom chamber were fixed with 100 % methanol for at least 15 min, dried, and stained with 1 % crystal violet for 20 min. Stained cells were counted under a microscope in three independent 200× fields for each well.

Self-renewal assessment

Tumor sphere formation assay was employed to measure the self-renewal capability of glioma stem-like cells. Primary tumor spheres were dissociated to single cells and then seeded into 96-well ultralow attachment plates in 200-μl neurobasal medium at a dilution of 200 cells per well. To replenish nutrition for the longtime culture, 25 μl of fresh media was added every 2 days. The numbers of formation tumor spheres in each well was quantified at day 7.

Western blot analysis

Western blot analysis was performed as previously described [27]. The primary antibodies and dilutions were used as follows: β-actin and E2F2 were obtained from Abcam (Cambridge, UK; 1:2000 diluted). CD133 and nestin were purchased from Santa Cruz Biotechnology (1:1000 diluted).

Statistical analysis

All the results were expressed as mean ± S.D. The GraphPad Prism 6.01 (GraphPad Software Inc.) was used for the experimental statistical analyses. The statistical analysis involving two groups was tested by Student's *t* test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was performed. $p < 0.05$ was considered statistically significant.

Results

Let-7b modulates proliferation, migration, and invasion in human glioma cells

A previous study denoted that the poor prognosis of glioma patients may contribute to the abnormal expression of the Let-7 family [16, 21]. We proposed that Let-7b may be a tumor suppressor in glioma cells. To investigate the regulatory role of Let-7b in the malignancy of human glioma cells, we first tested if the transient overexpression of Let-7b could alternate the proliferation, migration, and invasion in U251 and U87 cell lines. As illustrated in Fig. 1a, the expression of Let-7b in glioma cells was dramatically elevated more than 14-fold, 48 h posttransfection with Let-7b mimics compared with cells transfected with miR-NC. Then, cell proliferation was analyzed by using the CCK8 assay. The results revealed that increasing Let-7b led to considerable decrease of proliferation in glioma cells from 24 to 72 h (Fig. 1b). Meanwhile, transwell assays indicated that upregulation of Let-7b restrained both glioma cell migration and invasion. The migratory ability was inhibited to roughly 67.9 % in U251 cells and to nearly 63.2 % in U87 cells (Fig. 2a). Likewise, similar effects on invasiveness were also observed in parallel invasion assays. The invasion rate in the Let-7b mimics-treated group was only half of that found in the control group (Fig. 2b). Together, these data indicate that miR-92a affects the proliferation and metastasis properties of glioma cells.

Let-7b directly targets E2F2

To dissect the mechanisms by which Let-7b inhibits glioma pathogenesis, we next explored potential targets for Let-7b involved in glioma malignancy. By using the TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/) algorithms, E2F2 is suggested as a candidate target. Sequence analysis revealed that the seed region of Let-7b could directly target with the 3′UTR region of E2F2 (Fig. 3a). To confirm this possibility, the effect of Let-7b on endogenous E2F2 expression was examined in glioma cells. qRT-PCR and western blot analysis showed that there was a decrease in the E2F2 mRNA and protein levels 48 h after overexpressing Let-7b (Fig. 3b, c). To determine whether Let-7b directly targets E2F2, we constructed two types of luciferase reporter plasmids containing either the wild-type or mutant (the seed region binding Let-7b was changed from CUACCUC to CUUCGAG) 3′ UTR of E2F2. Luciferase assay indicated that the overexpression of Let-7b reduced the activity of a luciferase reporter fused to the wild-type E2F2 3′ UTR. However, the activity with the mutated 3′ UTR was not influenced by Let-7b in two glioma cells (Fig. 3d). These data suggested that E2F2 is a direct target of Let-7b in glioma cells.

Isolation and identification of glioma stem-like cells

It has been reported that glioma stem-like cells are implicated in glioma recurrence and therapeutic resistance [20, 32]. We are interested in finding whether Let-7b also has negative effects on glioma stem-like cells. To obtain the stem-like cells, we used the serum-free culturing technology to enrich tumor spheres (Fig. 4a). Subspheres generated from the primary sphere were examined for cancer stem cell markers, CD133 and nestin, by immunofluorescence staining. Stained images showed expression of CD133 (green) and nestin (red) in the tumor spheres (Fig. 4b), suggesting these tumor spheres could be identified as glioma stem-like cells.

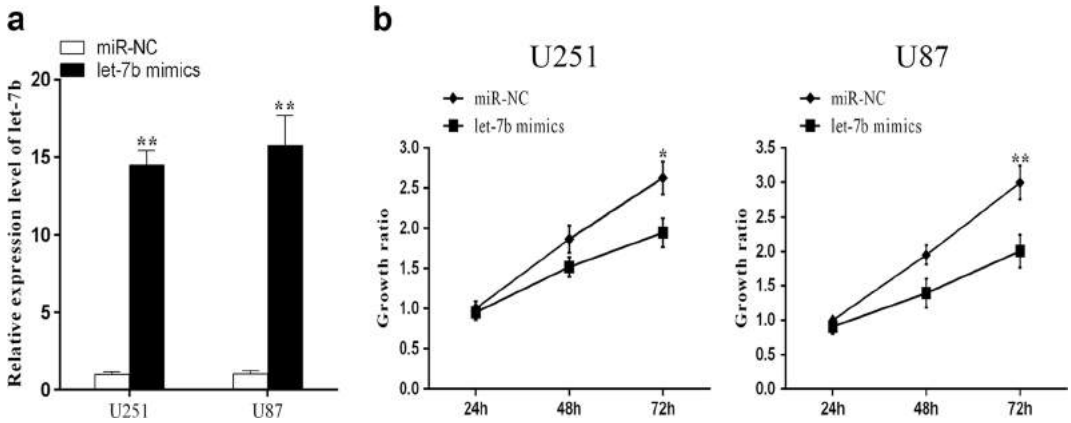

Fig. 1 Overexpression of Let-7b suppresses proliferation of glioma cells. **a** Real-time PCR analysis of Let-7b expression in glioma cells (normalized to U6 RNA). **b** The growth ratio of two glioma cell lines was measured using a CCK8 assay. All data were performed as means ± SD for three independent measurements. *$p < 0.05$ vs. miR-NC group; **$p < 0.01$ vs. miR-NC group

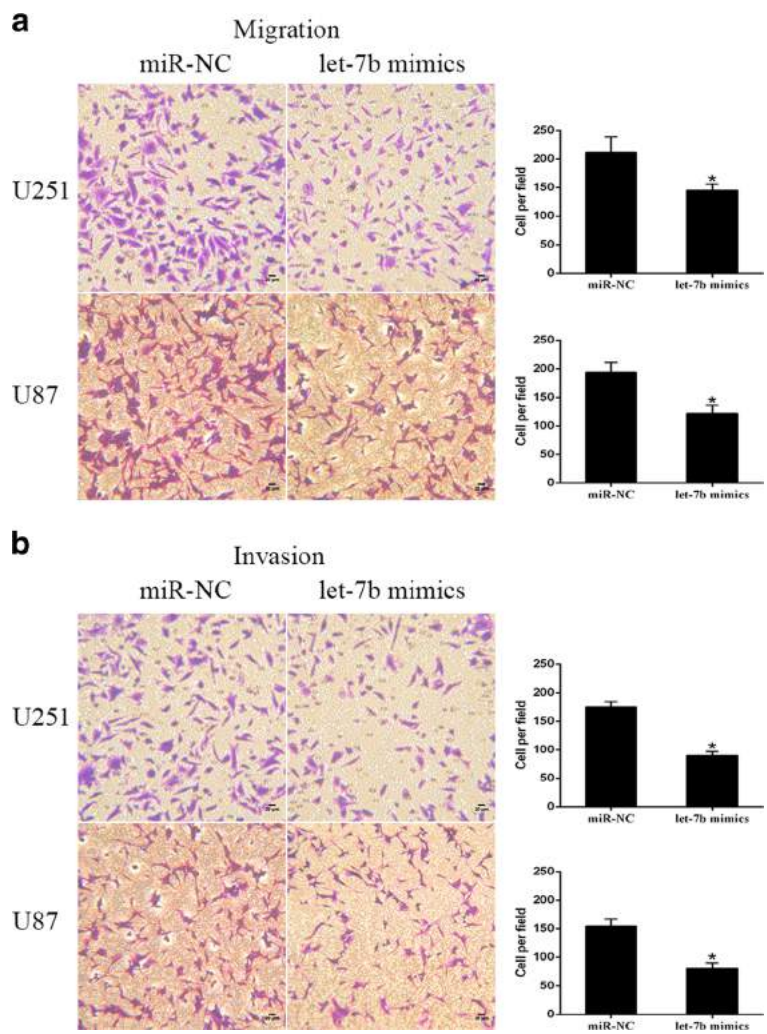

Fig. 2 Upregulation of Let-7b inhibits migration and invasion in glioma cells. Migration (**a**) and invasion (**b**) ability was detected using Transwell assay. The migratory and invasive cells were stained with crystal violet and counted. Each *bar* represents the mean of three independent experiments. *$p < 0.05$ vs. miR-NC group. *Scale bar* represents 20 μm

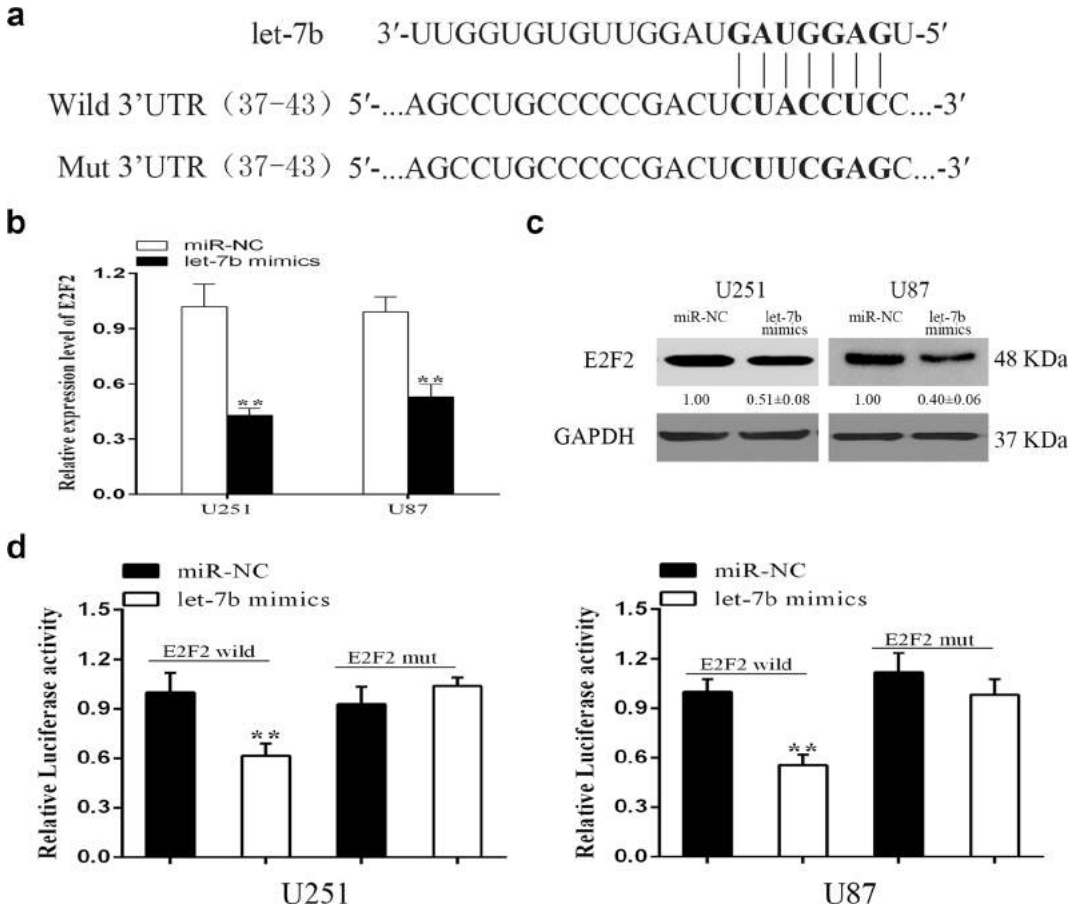

Fig. 3 E2F2 is a direct target of Let-7b. **a** Bioinformatic analysis predicts Let-7b target sequences and mutated nucleotides in 3′ UTR of E2F2. Expression of E2F2 mRNA levels (**b**) and protein levels (**c**) in glioma cells, 48 h posttransfection with Let-7b mimics. **d** Relative Renilla luciferase (Rluc) activity for E2F2 WT 3′UTR and E2F2 mutant 3′UTR, normalized to the average Rluc activity of each individual replicate experiment in glioma cells. All data were obtained from three independent experiments. $^{**}p < 0.01$ vs. miR-NC group

Let-7b suppressed the tumor sphere growth and self-renewal ability of glioma stem-like cells

To determine the role of Let-7b in glioma stem-like cells, we employed Let-7b mimics to increase the expression level of Let-7b in two glioma stem-like cell lines, U251-SC and U87-SC, which are derived from U251 and U87 cells, respectively. qRT-PCR was used to verify the results and showed a significant increasing of Let-7b in cells (Fig. 5a). As shown in Fig. 5b, the proliferation of glioma stem-like cells was decreased in Let-7b mimics-transfected group, suggesting that Let-7b was also a suppressor in regulating the proliferation of GSCs. In addition, we found a significant decrease in the volume of tumor spheres 72 h after transfection of Let-7b mimics compared to the control group (Fig. 5c), which are consistent with the results that Let-7b has a negative effect on the proliferation of GSCs. The self-renewal ability of GSCs was measured by tumor sphere formation assay. As shown in Fig. 5d, overexpression of Let-7b in GSCs led to a markedly decrease in their ability to form tumor spheres. We also examined the effect of Let-7b on the expression level of cancer stem cell markers, CD133 and nestin, by western blot. The expression of both cancer stem cell markers were decreased in U251-SC and U87-SC cells (Fig. 5e). Similarly, we also found that Let-7b could decrease the expression level of E2F2 at both mRNA and protein levels in GSCs (Fig. 5f, g). Taken together, these results demonstrated that Let-7b could reduce the tumor sphere growth and self-renewal ability of glioma stem-like cells through regulating E2F2.

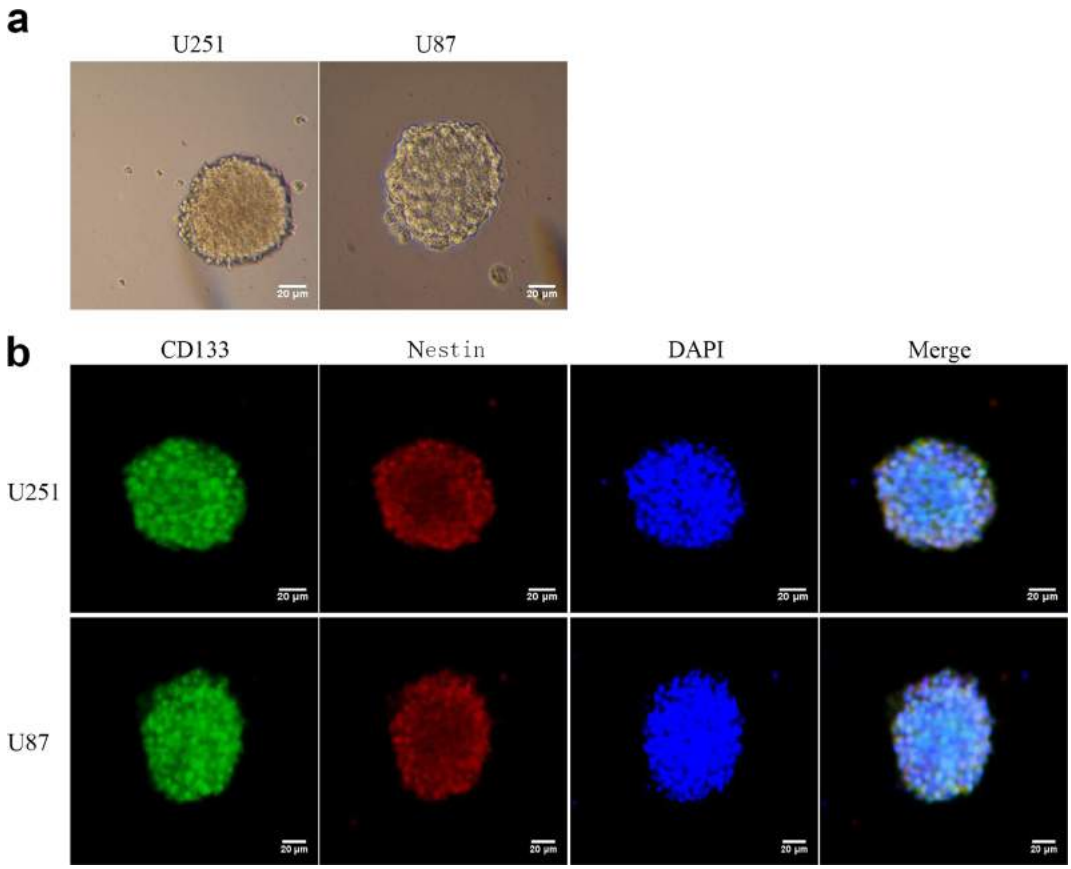

Fig. 4 Isolation and identification of glioma stem-like cells. **a** The primary tumor spheres of GSCs were observed after being cultured in a serum-free neurosphere culture medium for 7 days. **b** Individual tumor spheres of GSCs stained for CD133 (*green*) and nestin (*red*) by immunofluorescence analysis. Images are representative of three independent experiments. *Scale bar* represents 20 μm

E2F2 plays a critical role in the Let-7b-mediated inhibitory effect on glioma cells and GSCs

To examine whether Let-7b indeed exerts its function through its target E2F2, specific siRNA-targeting E2F2 was applied to suppress the protein expression of E2F2 in glioma cells and GSCs (Fig. 6a). As illustrated in Fig. 6b, c, knockdown of E2F2 expression inhibits the proliferation of glioma cells and the tumor sphere growth in GSCs markedly. In addition, all cells were cotransfected with Let-7b mimics or NC with E2F2 overexpression plasmid or control plasmid p-NC, followed by western blot analysis (Fig. 7a, b). We confirmed that the upregulation of E2F2 partially reversed the inhibitory effect of Let-7b on the proliferation in glioma cells (Fig. 8a) and GSCs (Fig. 8b, c). Interestingly, little effect was found on the migration and invasion of glioma cells, as well as the self-renewal ability of GSCs (data not shown) no matter the overexpression or knockdown of E2F2, suggesting that E2F2 may mainly function on Let-7b-regulated glioma cell progression and development.

Discussion

Previous studies have reported that a growing number of miRNAs participate in cancer carcinogenesis as activators or inhibitors [4]. It would be valuable if therapeutically promising miRNAs against cancer can be identified. The Let-7 family of miRNAs, which is highly conserved in both sequence and function across species, is one of the first discovered tumor-inhibitory miRNAs [2, 6]. Prior work has demonstrated that increased expression of Let-7a is associated with the decreased tumorigenicity in osteosarcoma cells [9]. Let-7b has been

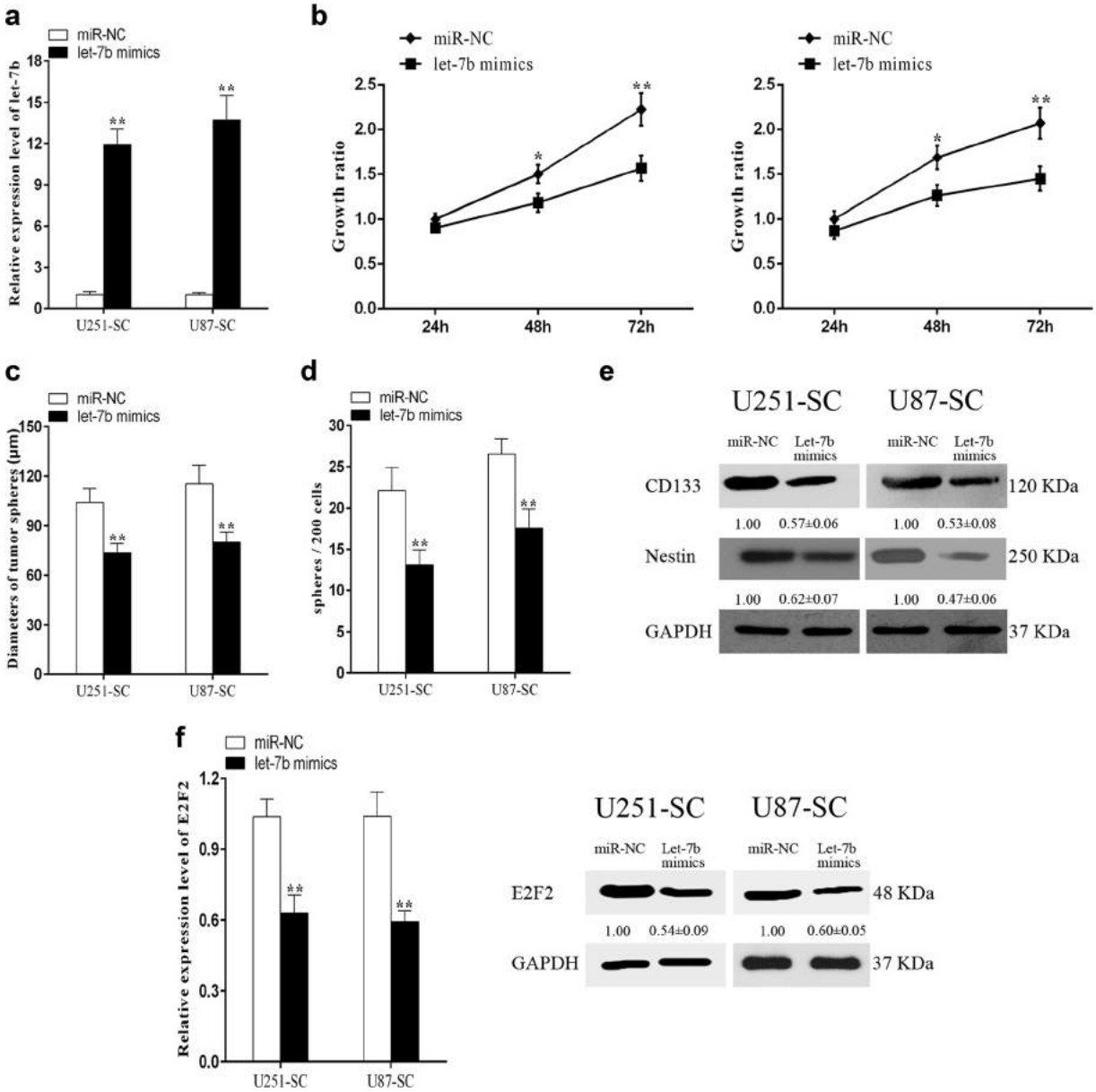

Fig. 5 Increasing Let-7b represses tumor sphere growth and self-renewal of GSCs. **a** Real-time PCR analysis of Let-7b expression in GSCs (normalized to U6 RNA). **b** CCK8 assay was applied to evaluate the role of Let-7b on the proliferation of GSCs. **c** The diameters of the tumor spheres were recorded and statistically analyzed. **d** The tumor sphere formation assay was employed to measure the self-renewal capability of glioma stem-like cells. **e** The expression level of cancer stem-like cell markers CD133 and nestin were analyzed by western blotting. Expression of E2F2 mRNA levels (**f**) and protein levels (**g**) in GSCs, 48 h posttransfection with Let-7b mimics. Data were presented as mean ± SD from three independent experiments. *$p < 0.05$ vs. miR-NC group, **$p < 0.01$ vs. miR-NC group

found to be significantly downregulated and act as a tumor suppressor in non-small cell lung cancer [10], malignant melanoma [25], and gastric cancers [7]. In addition to its involvement in cancer carcinogenesis, Let-7b was also reported to be a possible regulator of the stemness and differentiation in cancer stem cells [26, 29]. In the present study, Let-7b expression level was upregulated in glioma cells and GSCs by Let-7b mimics. Functional study found that increasing Let-7b expression reduced the proliferation, migration, and

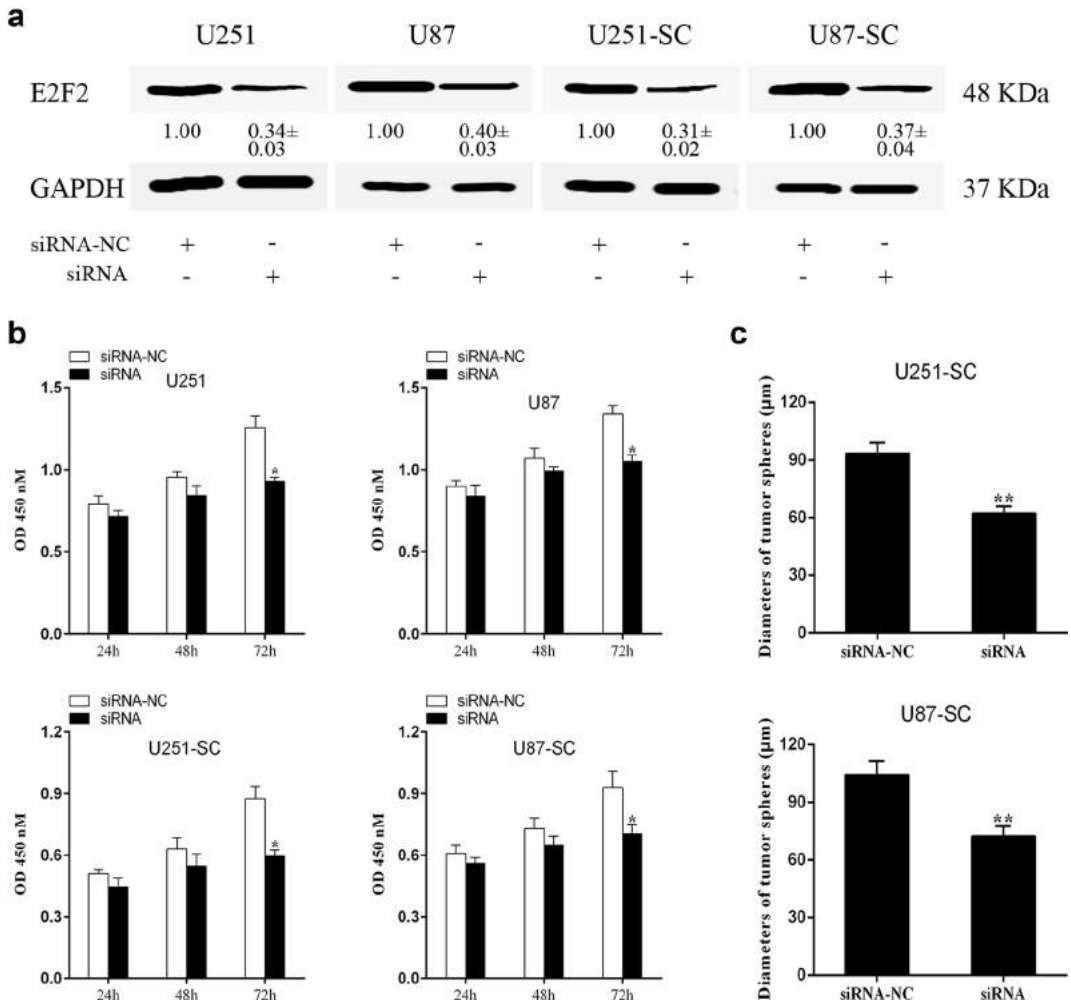

Fig. 6 Downregulation of E2F2 inhibits the proliferation of glioma cells and the tumor sphere growth of GSCs. **a** Western blotting analysis revealed the protein expression level of E2F2 48 h post-treatment with specific E2F2 siRNA. **b**, **c** CCK8 assay was used to detect the proliferation ability of glioma cells and GSCs. **d** The diameters of the tumor spheres were measured and statistically analyzed. The data were expressed as the mean ± SD and were representative of an average of three independent experiments. *$p < 0.05$ vs. siRNA-NC group; **$p < 0.01$ vs. siRNA-NC group

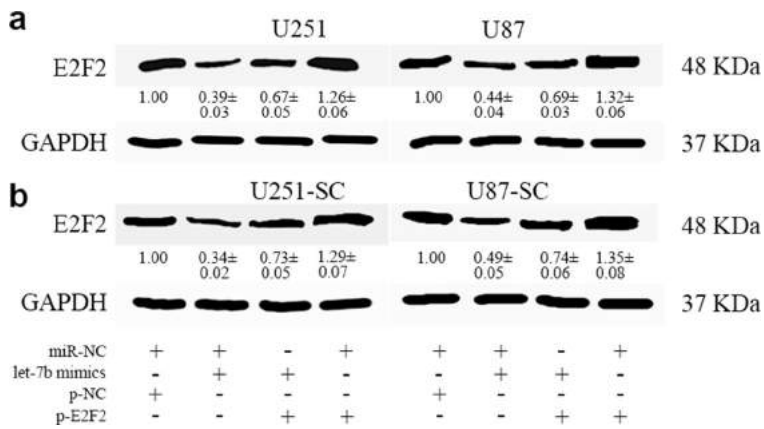

Fig. 7 Western blot analysis showed that E2F2 expression in glioma cells (**a**) and GSCs (**b**) were significantly reduced after transfection with Let-7b mimics, while it was increased after transfection with E2F2 overexpression plasmid compared with the negative control group. All of the data shown above are representative of three independent experiments

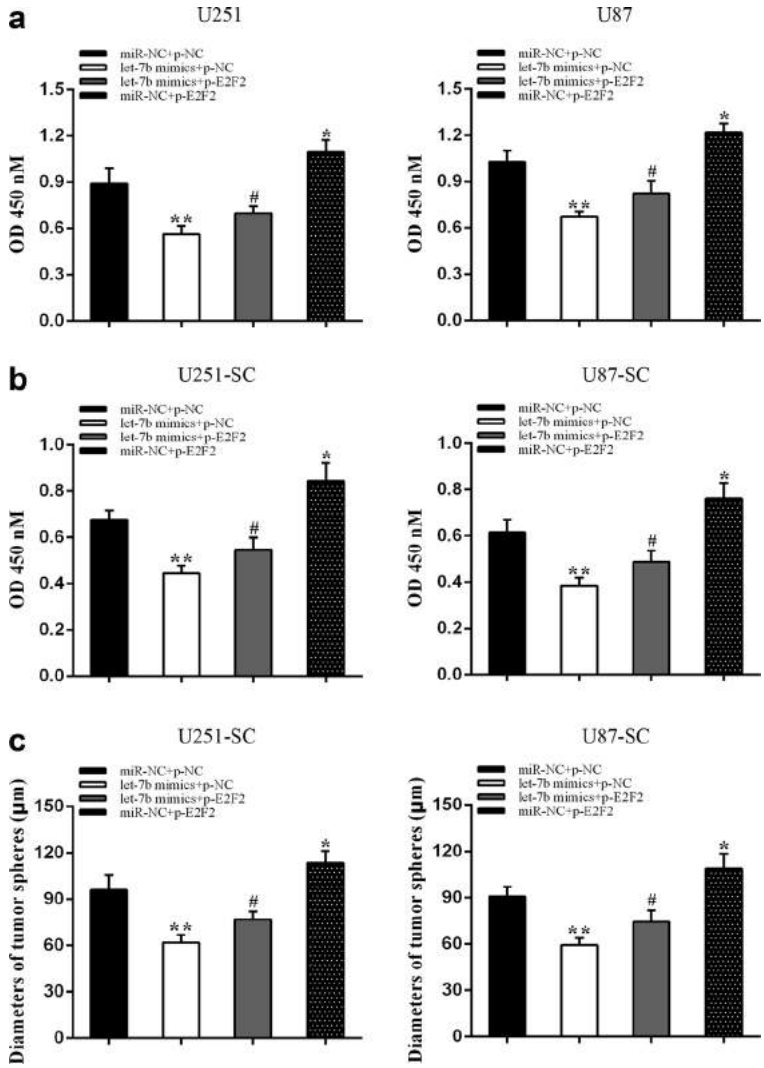

Fig. 8 Overexpression of E2F2 partly rescued Let-7b-induced inhibitory effects on glioma cells and GSCs. CCK8 assay was applied to evaluate the proliferation ability of glioma cells (**a**) and GSCs (**b**). **c** The diameters of tumor spheres were recorded and statistically analyzed. Similar results were obtained in three independent experiments. *$p < 0.05$ vs. miR-NC + p-NC group; **$p < 0.01$ vs. miR-NC + p-NC group; #$p < 0.05$ vs. Let-7b mimics + p-NC group

invasion in glioma cells, while it inhibited the tumor sphere growth and the maintenance of stemness in glioma stem cells, which further confirmed that Let-7b is a tumor-inhibiting factor.

To further characterize the mechanism of Let-7b's effects on glioma, we conducted bioinformatics analysis and found E2F2 might be the downstream target of Let-7b in glioma. We performed a dual-luciferase reporter system to prove our hypothesis. The results of the luciferase assay revealed that the relative luciferase activity was decreased when the Let-7b mimics and a reporter vector containing the wild-type 3′UTR of E2F2 cotransfected. Moreover, the relatively higher expression of Let-7b was significantly correlated with not only the decreased mRNA levels but also the protein levels of E2F2. Together, we confirmed that E2F2 is a direct target of Let-7b. Interestingly, E2F2 was also identified as a direct target of Let-7a in osteosarcoma cells [9], suggesting that different miRNAs may potentially regulate the same mRNA in human.

Members of the E2F family play a critical part in proliferation, apoptosis, and cell cycle [3], which are essential for the development of cancers. However, the role of E2F2 in cancer is still unclear. For example, silencing the expression of E2F2 improved the sensitivity of gastric cells to cisplatin-based chemotherapies, suggesting that E2F2 acts as an oncogene [31]. This result is consistent with our findings that E2F2 was involved in cancer progress. Interestingly, Li et al. reported that E2F2 acted as a tumor suppressor by repressing the expression of survivin and regulating the expression of cell cycle regulators in colon cancer

[15]. In addition, it has been reported that knockdown of E2F2 could preserve the stemness of human embryonic stem cells [28]. However, a recent study showed that the expression of E2F2 is upregulated in CD133 (+) cells isolated from human astrocytomas and associates with transformation of human astrocytes [18]. These results suggested the various roles of E2F2 in different types of cancers. Our study showed that downregulation of E2F2 by Let-7b mimics resulted in a marked decrease in the proliferation and diameters of tumor spheres in GSCs.

Subsequently, to verify the hypothesis that Let-7b mimics regulate glioma malignancy and proliferation and stemness of GSCs via downregulation of the expression of E2F2, we changed the expression of E2F2 in glioma cells and GSCs by transient transfection of E2F2 siRNA or E2F2 overexpression plasmid. As we anticipated, the results above indicated that the down regulation of E2F2 could suppress the proliferation of glioma cells and GSCs. Upregulation of E2F2 largely reversed the proliferation of glioma cells and the tumor sphere growth of GSCs inhibited by Let-7b mimics, whereas a higher E2F2 expression increased proliferation in both glioma cells and GSCs. Surprisingly, little change was observed on glioma cell migration, invasion, and the stemness of GSCs. As microRNAs target a diverse set of genes in cells [14], the function of Let-7b on migration and invasion in glioma and on maintenance of stemness in GSCs may involve in other genes. For example, Tian et al. has demonstrated that Let-7b could inhibit the metastasis of glioma cells though directly downregulating IKBKE [30]. These results revealed that the tumor-suppressor effects of Let-7b are in part mediated by the negative regulation of E2F2. Studies on Let-7b in glioma are very few until now. Further investigations on other target genes of Let-7b are needed to fully understand the regulatory mechanism of Let-7b in glioma.

In summary, the results of this study provided evidences that increasing Let-7b could reduce the malignancy of glioma cells. Importantly, the overexpression of Let-7b inhibited the tumor sphere growth and stemness of GSCs in vitro. In addition, we confirmed that Let-7b could directly target the 3′UTR region of E2F2 transcripts and consequently decrease E2F2 expression level. These data provide new insight for understanding how Let-7b is involved in regulating gliomas.

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