

## Original Article

# MicroRNA-524 inhibits the progress of glioma via the direct targeting of NCF2

Tuoye Xu<sup>1,2\*</sup>, Wan Yu<sup>3\*</sup>, Qingquan Li<sup>1</sup>, Xiaojian Li<sup>4</sup>, Yan Shi<sup>4</sup>, Boqiang Cao<sup>1</sup>, Yaxuan Zhang<sup>1</sup>, Seng Wang<sup>1</sup>, Yan Zhang<sup>1</sup>, Tianlu Wang<sup>1</sup>, Baosheng Huang<sup>1</sup>

<sup>1</sup>Department of Neurosurgery, Sir Run Run Hospital, Nanjing Medical University, Nanjing, Jiangsu Province, China; <sup>2</sup>Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China; <sup>3</sup>Department of Neurosurgery, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu Province, China; <sup>4</sup>Department of Neurosurgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu Province, China. \*Equal contributors.

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**Abstract:** In recent years, a large amount of research has reported that microRNA (miRNA) dysregulation is closely related to glioma progression. miR-524, a member of the miRNA family, has been confirmed to be involved in many human diseases, including glioma. However, the role and molecular mechanism of miR-524 in glioma have not been clarified. In our study, we showed that miR-524 expression was significantly decreased in glioma and was associated with glioma recurrence. Next, we performed a series of assays and confirmed that the upregulation of miR-524 suppressed glucose uptake, proliferation, migration and invasion in glioma cell lines. Then, through bioinformatics software and a dual luciferase assay, we demonstrated that NCF2 was a target gene of miR-524. In addition, we found that NCF2 reintroduction restored the inhibitor effect of miR-524 on glioma progression. These results elucidate the mechanism of miR-524 in glioma development and provide a potential therapeutic strategy for glioma patients.

**Keywords:** miR-524, NCF2, glucose uptake, glioma

### Introduction

Glioma is a devastating type of primary malignant tumor in the nervous system, characterized by high recurrence, rapid proliferation and strong aggression [1]. Although there has been progress in glioma diagnosis and treatment in the last decade, the prognosis of patients with glioma is still poor [2]. Therefore, it is necessary to continue investigations to discover effective and novel therapeutic strategies for glioma patients.

MicroRNAs (miRNAs) are a group of endogenous non-coding RNA molecules, approximately 18-24 nucleotides in length, which play a critical role in gene expression via binding to the complementary sequences at the 3'-untranslated regions (3'-UTRs) of their target messenger RNAs (mRNAs) and then cleaving or repressing the translation of their target mRNAs [3-6]. Mounting evidence has demonstrated that miRNAs participate in the progress of various tumors, including glioma, melanoma and

gastric cancer. For example, miR-421 is down-regulated in glioma and regulates the cell migration and angiogenesis [7]. Simultaneously, downregulation of miR-1294 is closely related to the chemosensitivity of glioma to temozolomide [8]. There is also research showing that miR-137 is downregulated in malignant melanoma and could regulate the glutamine catabolism of melanoma [3]. Moreover, there are studies reported that miRNAs are closely related to glioma glucose metabolism [9-11]. Through these studies, we insist that it is meaningful to devote ourselves to the research of the function of miRNAs, which may provide novel treatment options for glioma.

NCF2 (neutrophil cytosolic factor 2) is a necessary component for reactive oxygen species (ROS) production in phagocytes, and it plays a critical role in innate immunity and phagocytic microbicidal activity [12-14]. Recently, many studies showed that NCF2 plays a critical role in human diseases, including cancers. For example, Li et al. reported that NCF2 may act as

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an accurate predictor of colonic adenocarcinoma [15]. Tan et al. concluded that high expression of NCF2 was correlated with the risk of recurrence in renal carcinoma [16]. Although, NCF2 has a significant role in tumor progress, its function and molecular mechanism in glioma development remains unclear.

In this study, we demonstrate that miR-524 is downregulated in human glioma tissues compared to normal brain tissues (NBTs). The expression of miR-524 regulates glucose uptake, proliferation, migration and invasion in glioma cell lines. Moreover, NCF2 is the direct target of miR-524. According to these findings, we report that miR-524 is a tumor suppressor in glioma and might serve as a novel therapeutic target and prognostic evaluation indicator for glioma patients.

### Materials and methods

#### *Clinical tissue samples*

mRNA and miRNA expression microarray data from 158 gliomas were downloaded from the Chinese Glioma Genome Atlas (CGGA) data portal (<http://www.cgga.org.cn/portal.php>). A total of 5 normal human brain tissues (NBTs) and 12 glioma specimens were resected during surgery at the Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, China. Surgically excised tumor specimens were immediately frozen in liquid nitrogen and stored until total RNA and protein were extracted. This study was approved by the hospital's Ethics Committee, and written informed consent was obtained from all the patients.

#### *Cell culture*

The glioma cell lines U87 and U251 were both purchased from the Chinese Academy of Science Cell Bank (Shanghai, China). All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, ScienCell) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). These cell lines were all incubated in an atmosphere containing 5% CO<sub>2</sub> at 37°C.

#### *Oligonucleotides and cell transfection*

Oligonucleotides were all purchased from GenePharma (Shanghai, China). The sequences were as follows: NCF2-small interfering RNA:

Forward, 5'-CATCTGCCCCAGAAAGTGA-3', and reverse, 5'-CTTCATTCCAGAGGCTGATGG-3'. The hsa-miR-524 mimic and hsa-miR-524 inhibitor were also purchased from GenePharma. Cell transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions.

#### *Real-time quantitative PCR*

RNA was extracted from cells and tissues using TRIzol (Invitrogen), according to the manufacturer's protocols. Quantitative RT-PCR was used to detect the levels of NCF2 and miR-524. To detect the expression of NCF2, RT-qPCR was performed using Fermentas reverse transcription reagents and SYBR Green PCR Master Mix (Applied Biosystems, USA), according to the manufacturer's protocols. The primer sequence of NCF2 is forward 5'-ATCAGCCTCTGGAATGAAGGGG-3' and reverse 5'-GCAGCCAATGTTGAAGCAAATCC-3'. To detect the expression of miR-524, RT-qPCR was performed using TaqMan miRNA assays (Applied Biosystems, USA). The expression of U6 was measured as an endogenous control. The fold change in expression was calculated using the 2<sup>-ΔΔCt</sup> method. All reactions were performed in triplicate.

#### *Western blot analysis*

The total protein from tissues and cells was extracted by using RIPA buffer (KenGEN, China), according to the manufacturer's instructions. Protein concentrations were determined by BCA Protein Assay Kit (Beyotime, China). The protein lysates were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Next, 5% nonfat milk was used to block the membrane. The membrane was incubated overnight with diluted anti-NCF2 antibodies (1:1000, Abnova), followed by incubation with an HRP-conjugated secondary antibody (1:2000, YI FEI XUE BIOTECHNOLOGY, China). Finally, the bands were visualized under an Image Quant LAS 4000 mini (GE, USA).

#### *Glucose uptake assay*

The glucose uptake was determined using a 2-Deoxyglucose Glucose Uptake Assay Kit (Fluorometric, Abcam, USA), according to the manufacturer's instructions. The U87 and U251 cells were seeded into 96-well plates (1000 cells/well) overnight. After 24 hours, the cells were incubated in darkness with 2-deoxyglucose (10 mM) for 20 min at 37°C under a CO<sub>2</sub> humidified atmosphere and subjected to

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the measurement of 2-edoxyglucose uptake using a fluorescence microplate reader at Ex/Em = 535/587 nm [17].

### CCK-8 assay

The CCK-8 assay (Dojindo Laboratories, Japan) was used to quantify the viable cells of U87 and U251. The transfected U87 and U251 cells (3000 cells) were seeded into a 96-well microplate with 100  $\mu$ l of culture media. The medium of each well was subsequently replaced with 100  $\mu$ l of fresh culture media with 10% CCK-8 solution at different times (1, 2, 3, and 4 days), and then, the cells were incubated for an additional 3 hours. The absorbance was measured at an optical density of 450 nm using a microplate reader (Tecan Infinite 200 PRO; Salzburg Austria) [8].

### Transwell migration and invasion assay

Transwell migration and invasion assays were performed using an 8- $\mu$ m pore polycarbonate membrane Boyden chamber insert in a Transwell apparatus (Corning, NY, USA). For the invasion assay, the chamber inserts were pre-coated with 45  $\mu$ l of Matrigel (1:8 dilution; BD Biosciences, USA). For both assays, transfected cells were harvested and mechanically dissociated into a single cell suspension. Subsequently,  $5 \times 10^4$  cells in FBS-free medium were added into the upper chamber, and 500  $\mu$ l of culture medium containing 20% FBS was placed in the lower chamber. The chambers were then incubated for 48 h at 37°C in a 5% CO<sub>2</sub> incubator. Cells on the upper surface were scraped and washed away, whereas cells on the lower surface were fixed in 100% methanol and stained with 0.1% crystal violet. The values for migration and invasion abilities were quantified by photographing 3 independent visual fields under a microscope [18]. The magnifications of U87 and U251 are 100 $\times$  and 200 $\times$  respectively.

### Xenograft tumor assay

Ten immunodeficient female nude mice (Beijing Laboratory Animal Center, Beijing, China) were used to test the effects of miR-524 on glioma *in vivo*. Nude mice were divided into two groups (5 mice per group). Then,  $2 \times 10^6$  logarithmically growing U87 cells stably expressing negative control or miR-524 mimics were subcutaneously injected into nude mice. After 30 days, the nude mice were sacrificed, and the tumor

tissues were stripped and weighed [8]. Total RNA and protein were extracted from the tissues, and the expression of NCF2 was detected by qRT-PCR, western blot and immunohistochemistry. The magnification of immunohistochemistry is 200 $\times$ .

### Statistical analysis

All the experiments except the animal experiments were conducted at least three times. All values in this study are shown as the mean  $\pm$  SD. The difference between the groups was considered significant and very significant when  $P < 0.05$  (\* or #) and  $P < 0.01$  (\*\* or ##), respectively.

## Results

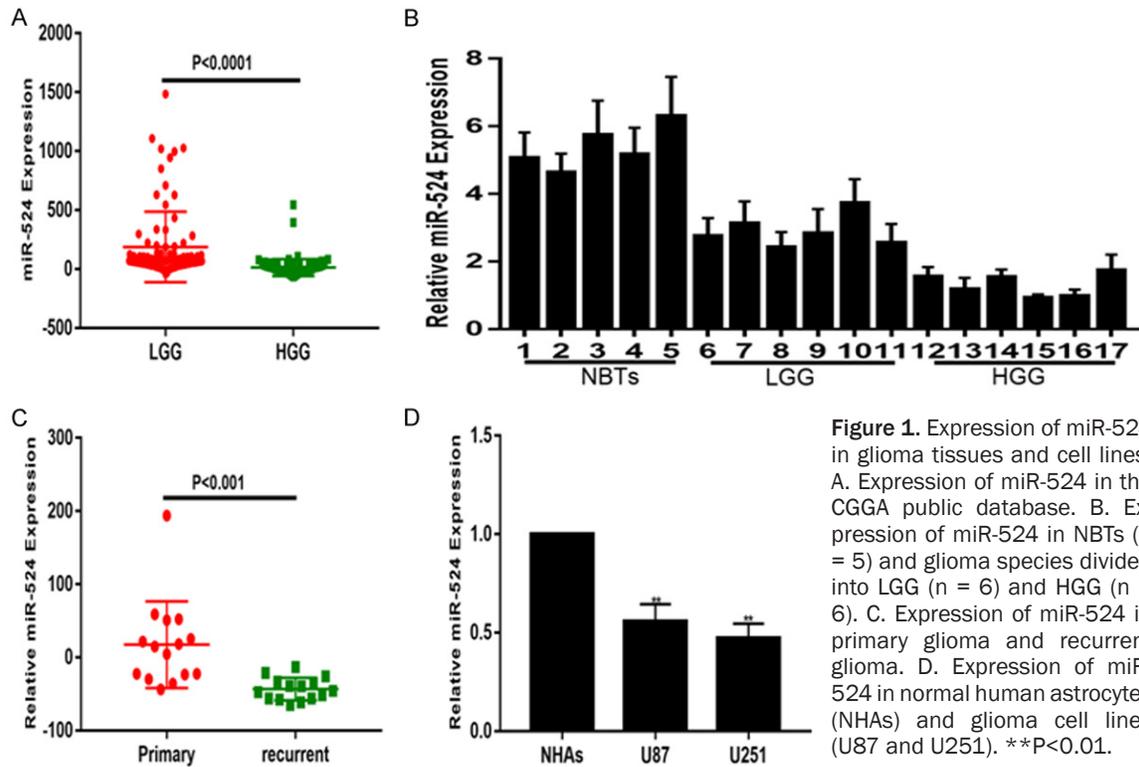
### *miR-524 is downregulated in glioma tissues and cell lines and related to glioma recurrence*

First, we investigated the expression of miR-524 in the CGGA, and the result showed that miR-524 expression was markedly decreased in high-grade glioma (HGG) compared to low-grade glioma (LGG) (**Figure 1A**). Next, we checked the expression pattern of miR-524 in 5 normal human brain tissues (NBTs) and 12 glioma specimens using RT-qPCR. As shown in **Figure 1B**, the miR-524 expression was significantly decreased in glioma compared to NBTs. In addition, we analyzed the expression pattern of miR-524 in recurrent glioma tissues and found that, compared with primary glioma tissues, miR-524 expression was markedly reduced in recurrent glioma tissues (**Figure 1C**). Last, we detected the miR-524 levels in human glioma cell lines and normal human astrocytes (NHAs). Consistent with the expression pattern in glioma specimens, miR-524 was considerably lower in glioma cell lines than in NHAs (**Figure 1D**). These findings indicated that miR-524 is closely related to the progression of glioma.

### *Increased miR-524 expression is correlated with glucose uptake, proliferation, migration and invasion in glioma cell lines*

In order to ulteriorly explore the biological function of miR-524 in glioma cell lines, we assessed the effects of miR-524 on glioma cell glucose uptake, proliferation, migration and invasion. First, we transfected both cell lines with the

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**Figure 1.** Expression of miR-524 in glioma tissues and cell lines. A. Expression of miR-524 in the CGGA public database. B. Expression of miR-524 in NBTs (n = 5) and glioma species divided into LGG (n = 6) and HGG (n = 6). C. Expression of miR-524 in primary glioma and recurrent glioma. D. Expression of miR-524 in normal human astrocytes (NHAs) and glioma cell lines (U87 and U251). \*\*P<0.01.

commercially synthesized miR-524 mimic to upregulate the expression of miR-524, and the validity of transfection was proven by qRT-PCR (Figure 2A, 2B). The glucose uptake assay showed that overexpression of miR-524 markedly repressed the glucose uptake of U87 and U251 cells (Figure 2C, 2D). In a CCK-8 assay, the proliferation ability of the miR-524 mimic-transfected cells was suppressed compared to the control group (Figure 2E, 2F). Migration assays and transwell assays revealed that the miR-524 mimic inhibited the migratory and invasive abilities of U87 and U251 cells (Figure 2G-J). Overall, these results suggest that miR-524 suppresses glucose uptake, proliferation, migration and invasion in glioma cell lines.

### *NCF2 is the direct functional target of miR-524 in glioma cell lines*

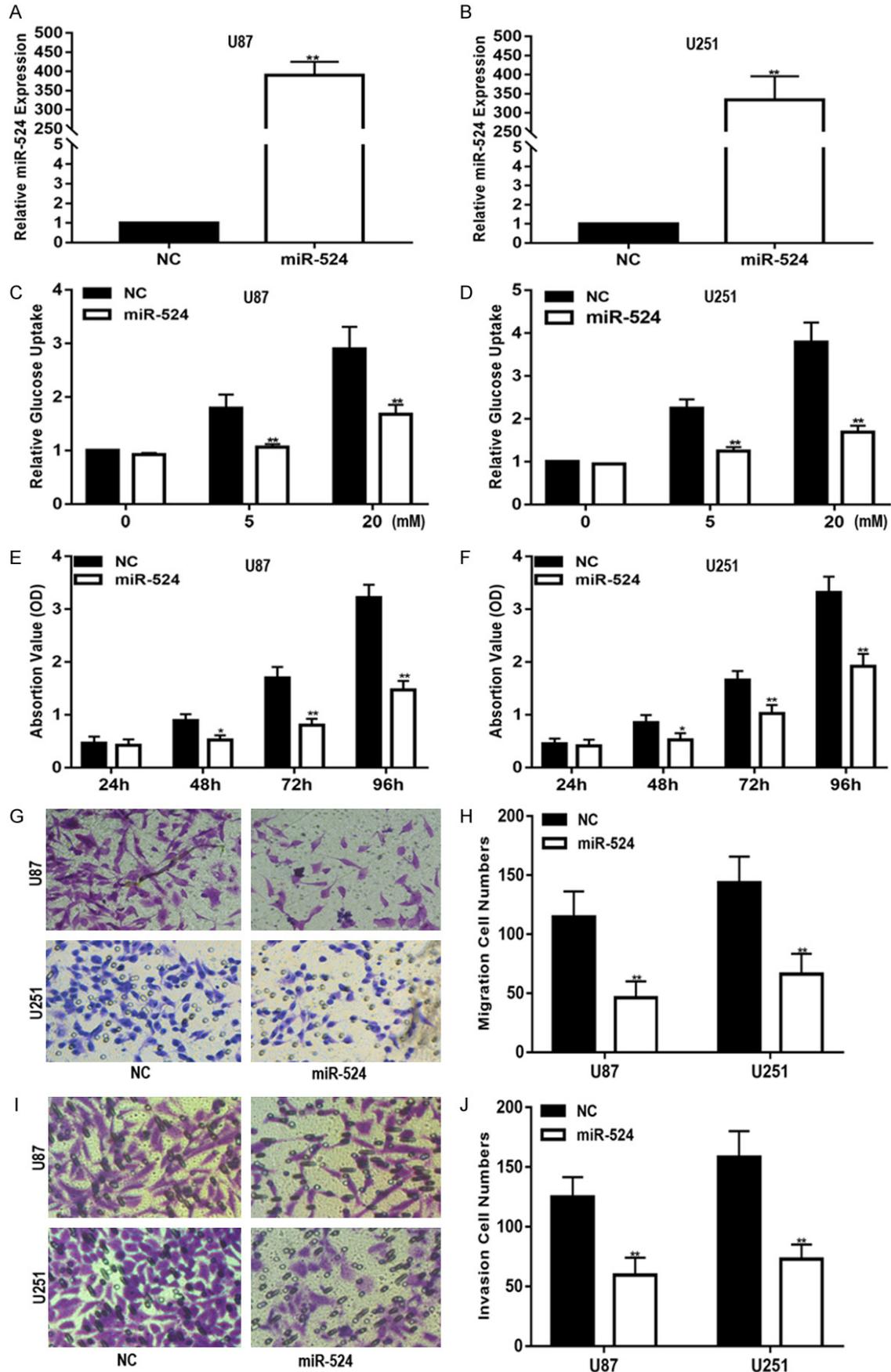
To further study the molecular mechanism of miR-524 in glioma cell lines, bioinformatics analysis was performed to find the potential target of miR-524. Though starBase v2.0 (<http://starbase.sysu.edu.cn/>), we found that NCF2 was a target of miR-524 (Figure 3A). Next, in order to examine whether miR-524 mediates the expression of NCF2, we performed the dual luciferase reporter assays, and the results showed that overexpression of miR-524 obvi-

ously suppressed luciferase activity in cells with the wild-type plasmid, but there was no significant effect detected in cells transfected with the mutant plasmid (Figure 3B). Meanwhile, we also examined the expression level of NCF2 in CGGA and clinical tissue samples. The results showed that the NCF2 levels were significantly higher in HGG compared to LGG (Figure 3C, 3D). In addition, though qRT-PCR and western blot, we found that the NCF2 protein level was decreased or increased in glioma cell lines when transfected with miR-524 mimics or inhibitors, respectively (Figure 3E, 3F). Taken together, these findings demonstrated that NCF2 is the functional target of miR-524 in glioma.

### *miR-524 regulates glucose uptake, proliferation, migration and invasion in glioma cell lines by targeting NCF2*

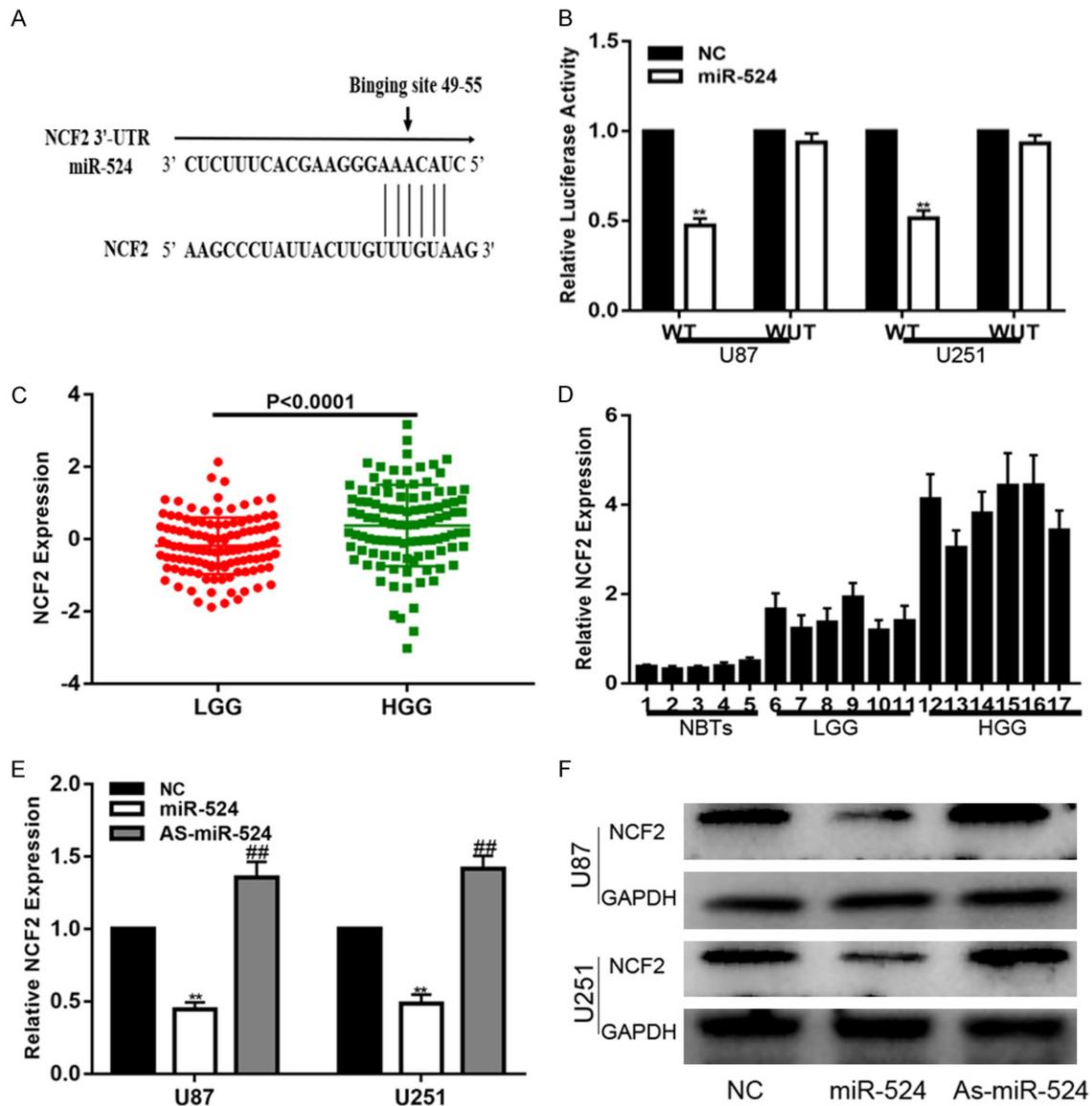
In order to test the biological functions of NCF2, we transfected both cell lines with siRNA targeting NCF2 (si-NCF2) to knock down NCF2 expression. Through qRT-PCR assays, we found that the interference was effective (Figure 4A, 4B). Through a series of experiments, we demonstrated that downregulation of NCF2 inhibited glucose uptake, proliferation, migration and

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**Figure 2.** miR-524 suppresses glioma cell glucose uptake, proliferation, migration and invasion in vitro. A, B. qRT-PCR analysis of miR-524 expression in U87 and U251 cells transfected with NC or miR-524 mimic. C, D. Relative glucose uptake in U87 and U251 cells transfected with NC or miR-524 mimic and treated with the indicated concentrations of glucose (0, 5, or 20 mM). E, F. The CCK-8 assay for U87 and U251 cells transfected with NC or miR-524 mimic. G, H. The migration assay for U87 and U251 cells transfected with NC or miR-524 mimic. I, J. The invasion assay for U87 and U251 cells transfected with NC or miR-524 mimic. \* $P < 0.05$ , \*\* $P < 0.01$ .

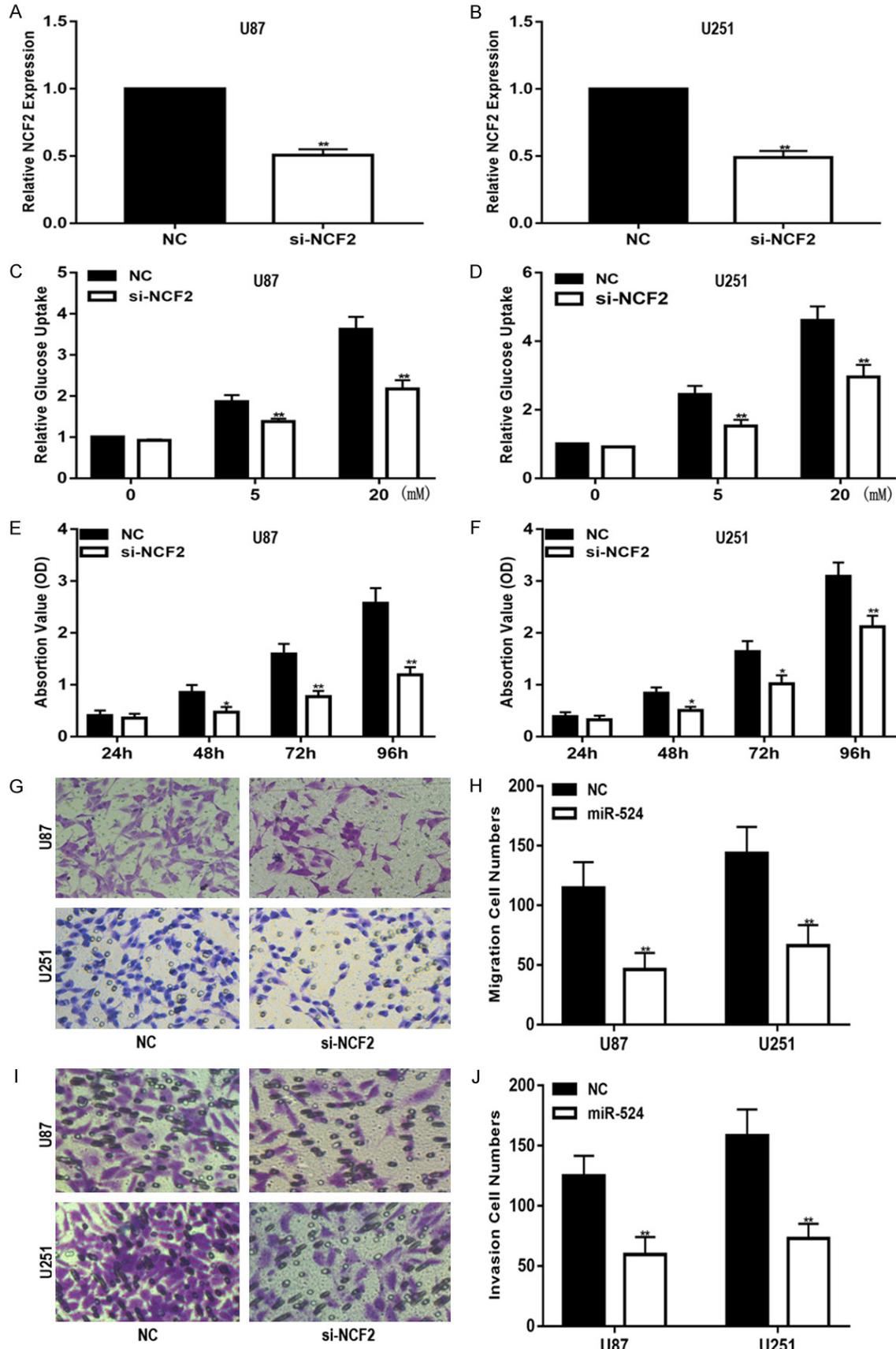


**Figure 3.** NCF2 is a direct target of miR-524 in glioma cell lines. A. Predicted miR-524 target sequence in the 3'-UTR of NCF2 mRNA. B. miR-524 downregulates the luciferase activity of the wild-type NCF2 3'-UTR expression vector but does not reduce the expression of mutant NCF2. C. Expression of NCF2 in the CGGA public database. D. The expression of NCF2 in glioma species. E, F. Expression of NCF2 in U87 and U251 cells transfected with NC, miR-524 mimic or miR-524 inhibitor. \*\* $P < 0.01$  means cells treated with NC and cells treated with miR-524 comparison, ## $P < 0.01$  means cells treated with NC and cells treated with As-miR-524 comparison. GAPDH used as control.

invasion in glioma cell lines (Figure 4C-J). Next, we transfected both cell lines with either the miR-524 mimic or miR-524 mimic together with the NCF2 plasmids and performed a series of functional assays. The results of the qRT-PCR

assays illustrated that the NCF2 plasmids could partially restore the inhibitory effect of the miR-524 mimic in both cell lines (Figure 5A, 5B). Meanwhile, though a series of functional assays, we confirmed that the restoration of

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**Figure 4.** Downregulation of NCF2 suppresses glioma cell glucose uptake, proliferation, migration and invasion *in vitro*. A, B. qRT-PCR analysis of NCF2 expression in U87 and U251 cells transfected with NC or si-NCF2. C, D. Relative glucose uptake in U87 and U251 cells transfected with NC or si-NCF2 and treated with the indicated concentrations of glucose (0, 5, or 20 mM). E, F. The CCK-8 assay for U87 and U251 cells transfected with NC or si-NCF2. G, H. The migration assay for U87 and U251 cells transfected with NC or si-NCF2. I, J. The invasion assay for U87 and U251 cells transfected with NC or si-NCF2. \* $P < 0.05$ , \*\* $P < 0.01$ .

NCF2 expression could partially rescue the effects of miR-524 on the glucose uptake, proliferation, migration and invasion (**Figure 5C-J**). These results indicated that NCF2 is a pivotal target of miR-524 in glioma cell lines.

### *miR-524 suppresses tumor growth of glioma in nude mice*

In order to investigate whether the results *in vitro* are reproducible in the mouse xenograft model, we transfected the U87 cells with negative control or the miR-524 mimic and then subcutaneously injected the cells into nude mice. Consistent with the results *in vitro*, miR-524 significantly inhibited tumor growth *in vivo*. The size of subcutaneous tumors derived from miR-524 overexpressing cells was significantly smaller than that of control cells (**Figure 6A, 6B**). Simultaneously, compared to the control, the weight of subcutaneous tumors derived from miR-524 overexpressing cells was significantly lighter (**Figure 6C**). Next, through qRT-PCR, western blot and immunohistochemistry analyses, we confirmed that the NCF2 expression in the tumor tissues from miR-524 overexpressing cells was decreased (**Figure 6D-F**). Finally, we concluded that miR-524 inhibits glioma growth *in vivo* by targeting NCF2.

### Discussion

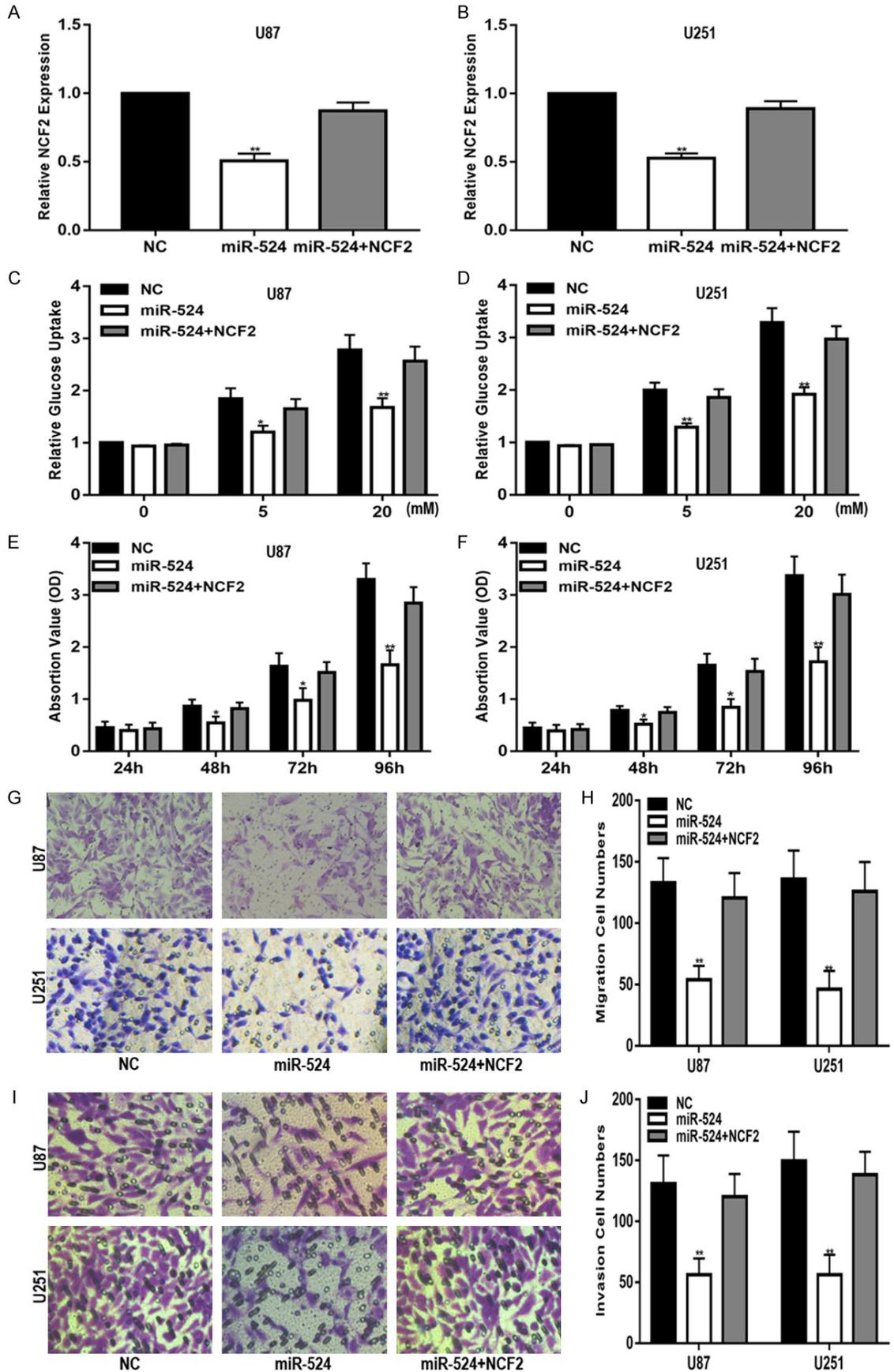
Previous studies have shown that in the spectrum of human diseases, the incidence of cancer increases year by year [19]. Gliomas are an important part of cancer and threaten human health. In recent years, despite advances in the treatment of gliomas, including electric field therapy and ketogenic diets, the prognosis of patients with gliomas has not improved significantly [20-22]. The main reason is that the mechanism of gliomagenesis has not been elucidated completely. Therefore, it is very necessary and meaningful to thoroughly study the molecular mechanism of gliomagenesis.

miRNA is a class of RNA molecules that do not have coding capabilities [23]. Mounting evidence shows that miRNA participates in numer-

ous physiological and pathological processes by binding to special sequences at the 3'-untranslated regions of target genes and then regulating gene expression. Thorough investigations show that miRNAs play a significant role in tumorigenesis, including glioma. For example, Liu et al. reported that miR-93 inhibits the proliferation, invasion and migration of glioma by targeting RBL2 [24]; Lin et al. suggested that miR-128 inhibits the proliferation and invasion of glioma through COX2 [25]. miR-524 is a relatively new member of the miRNA family which has been confirmed to have an important role in angiotensin II-induced hypertension and cellular reprogramming [26]. Although there is research showing that the EGFR/c-myc axis regulates the TGF $\beta$ /Hippo/Notch pathway via epigenetic silencing of miR-524 in glioma [27], the specific mechanism of miR-524 in glioma progression remains unclear. In this study, we illustrated that miR-524 expression is decreased in glioma tissues and glioma cell lines, which relates to glioma recurrence as well. In addition, we performed a series of assays and found that miR-524 suppressed glucose uptake, proliferation, migration and invasion of glioma cells. In order to clarify the underlying mechanisms of miR-524 in glioma, we used bioinformatics software to investigate the targets of miR-524, and NCF2 was considered a potential target. Further experimental studies indicated that there was a negative correlation between the expression of miR-524 and NCF2 in glioma tissues and cell lines. Simultaneously, NCF2 knockdown could suppress glioma glucose uptake, proliferation, migration and invasion. Next, we performed a series of functional assays and the results showed that transfection of miR-524-overexpressing cells together with the NCF2 plasmid significantly abolished the effect of miR-524 on glioma cell glucose uptake, proliferation, migration and invasion. Finally, through a tumorigenicity assay, we demonstrated that miR-524 also inhibits glioma progression by regulating NCF2 *in vivo*.

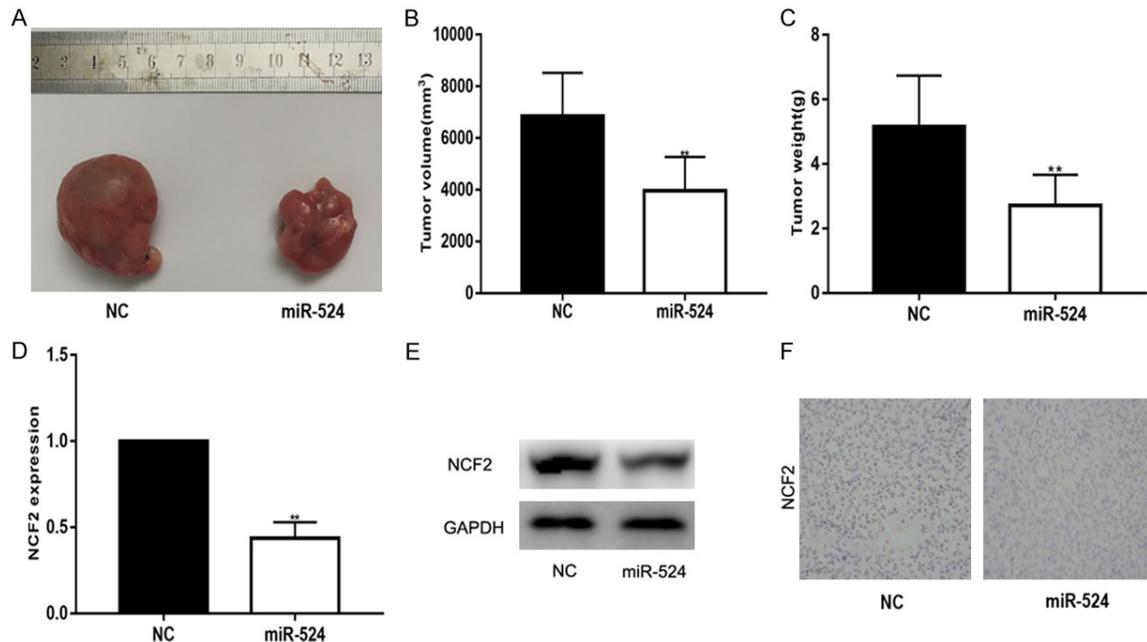
In summary, we demonstrated that miR-524 acts as a tumor suppressor in glioma by target-

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**Figure 5.** NCF2 restores the inhibitor effect of miR-524 on glioma cell glucose uptake, proliferation, migration and invasion *in vitro*. A, B. qRT-PCR analysis of NCF2 expression in U87 and U251 cells transfected with NC, miR-524 mimic or miR-524mimic together with NCF2. C, D. Relative glucose uptake in U87 and U251 cells transfected with NC, miR-524 mimic or miR-524mimic together with NCF2 and treated with the indicated concentrations of glucose (0, 5, or 20 mM). E, F. The CCK-8 assay for U87 and U251 cells transfected with NC, miR-524 mimic or miR-524mimic together with NCF2. G, H. The migration assay for U87 and U251 cells transfected with NC, miR-524 mimic or miR-524mimic together with NCF2. I, J. The invasion assay for U87 and U251 cells transfected with NC, miR-524 mimic or miR-524mimic together with NCF2. \*P<0.05, \*\*P<0.01.



**Figure 6.** miR-524 inhibits glioma growth *in vivo*. A. Tumor formation was performed in nude mice and the excised tumor of U87 xenografts. B. The volume of the excised tumor was calculated by the formula:  $V(\text{mm}^3) = 0.5 * a * b^2$  (a represents the longest axis and b the shortest axis). C. The weight of the excised tumor. D-F. Expression of NCF2 in tumor tissue. \*P<0.05, \*\*P<0.01.

ing NCF2, thus regulating tumor cell glioma glucose uptake, proliferation, migration and invasion. In addition, the expression level of miR-524 may be associated with glioma recurrence. Thus, miR-524 may be a potential therapeutic target and an indicator for assessing prognosis for glioma patients in the future.

### Acknowledgements

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Baosheng Huang, Department of Neurosurgery, Sir Run Run Hospital

of Nanjing Medical University, Nanjing, Jiangsu Province, China. Tel: +86 13851797739; E-mail: bs.huang@njmu.edu.cn

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