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Original Article

Down-regulation of microRNA-202-5p promotes the viability, migration and invasion of glioblastoma cells possibly via targeting EIF4E or GSKIP

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Abstract: We aimed to investigate the effects of microRNA-202-5p (miR-202-5p) on the proliferation, migration and invasion of glioblastoma cells, as well as its regulatory mechanism. The expression of miR-202-5p inglioblastoma U251 cells was detected. The mimic control, miR-202-5p mimic, si-EIF4E scramble control, si-EIF4E, si-GSKIP scramble control, si-GSKIP, pcDNA3.1 vector and pcDNA3.1-EIF4E were respectively transfected into cells. Cell proliferation, migration and invasion in different treated groups were respectively investigated. In addition, the potential targets of miR-202-5p were predicted by Target Scan Human, which was further verified by luciferase reporter analysis. Besides, the expression levels of EIF4E, GSKIP, GSK3β and β-catenin in different treated group were determined. The results showed that miR-202-5p expression in U251 cells was significantly lower than that in HEB cells. After U251 cells were transfected with miR-202-5p mimic, cell viability, migration and invasion decreased significantly compared with mimic control group. Furthermore, EIF4E and GSKIP were confirmed as the direct targets of miR-202-5p. Knockdown of EIF4E or GSKIP could significantly decreased cell viability, migration and invasion. Besides, the expression levels of EIF4E, GSKIP and β-catenin significantly decreased after miR-202-5p overexpression, while GSK3β expression markedly increased. However, overexpression of EIF4E and miR-202-5p simultaneously resulted in opposite expression changes of these proteins. Our results indicate that down-regulation of miR-202-5p may promote the glioblastoma cell proliferation, migration and invasion possibly via targeting EIF4E-β-catenin axis or GSKIP-GSK3β/β-catenin pathway. miR-202-5p may serve as a potential therapeutical target for glioblastoma.

Keywords: Glioblastoma, microRNA-202-5p, EIF4E, GSKIP, β-catenin

Introduction

Gliomas are a common malignant tumor that begins within the brain, in which glioblastoma is the most aggressive manifestation [1, 2]. Due to invasive nature of glioblastoma, current therapeutic strategies, including neurosurgery, chemotherapy and radiation and chemotherapy remain relatively ineffectiveness and the outcome is poor [3]. The survival time of most patients with glioblastoma was only 12 to 15 months following diagnosis, and only 3 to 5% of patients with glioblastoma can survive greater than five years [4]. Moreover, the prognosis and clinical outcome for glioblastoma patients have not improved although great efforts have been made [5, 6]. Therefore, it will be of great significance to identify key biomarkers for early diagnosis or effective targets for the treatment of this disease.

MicroRNAs (miRNAs), an abundant class of short non-coding RNA molecules, have been identified to exhibitcrucial functionsin many cancer types [7-9], including glioblastoma and glioblastoma cancer stem cells [10, 11]. As reported, miR-21 plays an oncogenic role in promoting tumorigenesis in glioblastoma via down-regulation of insulin-like growth factor-binding protein-3 [12]. miR-873 cansuppresstumorigenesis and metastasis in glioblastoma [13]. miR-663 is also shown to suppress glioblastoma cell proliferation, migration and invasion through targeting transforming growth factor-β

[14]. Recently, miR-202 has been identified to involved in the progression of several cancers, including lung cancer [15], cervical cancer [16] and pancreatic cancer [17]. Interestingly, miR-202 has been found downregulated inglioblastoma samples [18]. However, the association of miR-202 dys regualtion and glioblastoma tumorigenesis remains unclear.

In this study, we detected the expression of miR-202-5p in human glioblastoma cell line U251 and then investigated the effects of miR-202-5p overexpression on glioblastoma cell viability, migration and invasion. Moreover, the targets of miR-202-5p wereexplored to elucidate the underlying mechanism of miR-202-5p in glioblastoma. Our results are expected to provide theoretical basis for discovering the effective molecular therapy for glioblastoma.

Materials and methods

Cell culture

Human normal glial HEB cells and glioblastoma U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Grand Island, NY, USA), containing 10% fetal bovine serum (Gibco, USA), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (Sigma-Aldrich), and incubated at 37°C with 5% CO_2 in a fully humidified atmosphere. When the cells grew to 80% of the flask, the medium was replaced with DMEM supplemented with 1% FBS. Cells were then starved for 24 h and maintained in this low serum condition during the subsequent treatments.

Cell transfection

The miR-202-5p mimic and mimic control were designed and synthesized by Thermo Scientific (Waltham, USA). U251 cells were digested and plated in 6-well plates (the destiny was 2.5×10^5 cells/ml), with complete medium free from antibiotics, and incubated at 37°C in 5% CO_2 for 24 h. After 80-90% confluence, cells were transfected with miR-202-5p mimic, mimic control, si-EIF4E scramble control, si-EIF4E, si-GSKIP scramble control, si-GSKIP, pcDNA3.1 vector, and pcDNA3.1-EIF4E using DharmaFECT 1 (Dharmacon) depending on the manufacturer's protocol (Thermo Scientific, Waltham, USA).

MTT assay

The cell viability was determined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. U251 cells in logarithmic growth phase were adjusted to 5×10^4/mL, seeded in a 96-well plate with 200 μL per well and allowed to attach overnight. After transfection for 24 h, 48, 72 h, cells in each well were mixed with 20 μL of fresh medium with 0.5 mg/mL MTT at 37°C for 4 h. After termination, 200 μL Dimethyl Sulfoxide (DMSO) was added to each well for 15 min. The 570 nm optical density (OD) of each well was then measured with a microplate reader (BioTek, USA) The experiment was repeated for three times.

Transwell assay

Cell migration and invasion were evaluated using Transwell assay. The upper layer of Transwell chamber (8 μm pore size; Corning, USA) was enveloped with Matrigel (50 mg/L) for invasion assay, while the upper layer of Transwell chamber without Matrigel for migration assay. After transfection for 48 h, 200 μL cells (2×10^5 cells/mL) was seeded into the upper chambers of a 24-well Transwell plate (8 μm pores; Costar, USA) and 500 μL DMEM supplemented with 10% FBS was added into the lower chamber as a chemoattractant. After 48 h of incubation at 37°C in 5% CO_2, the medium was removed and cells remaining on the top of the upper chamber were scraped off with a cotton swab. Cells that invaded into the bottom surface of the membrane were fixed with methanol and stained with hematoxylin. Cells were mounted and dried at 80°C for 30 min, then 3 randomly fields were selected to count cells under a light microscope. All assays were performed in triplicate.

Dual luciferase reporter assay

The coding regions of EIF4E-3'UTR or GSKIP-3'UTR were obtained from human primary glioblastoma cell lines, amplified by PCR method and then inserted into the pMiRGLODual Luciferase miRNA reporter vector (Promega, Madison, USA) which were named EIF4E 3'UTR-wt and GSKIP 3'UTR-wt, respectively. The construction of the mutant was created by mutating the seed regions of the miR-202-5p binding sites (named EIF4E 3'UTR-mut and GSKIP 3'UTR-mut) using the Quick-Change Multisite

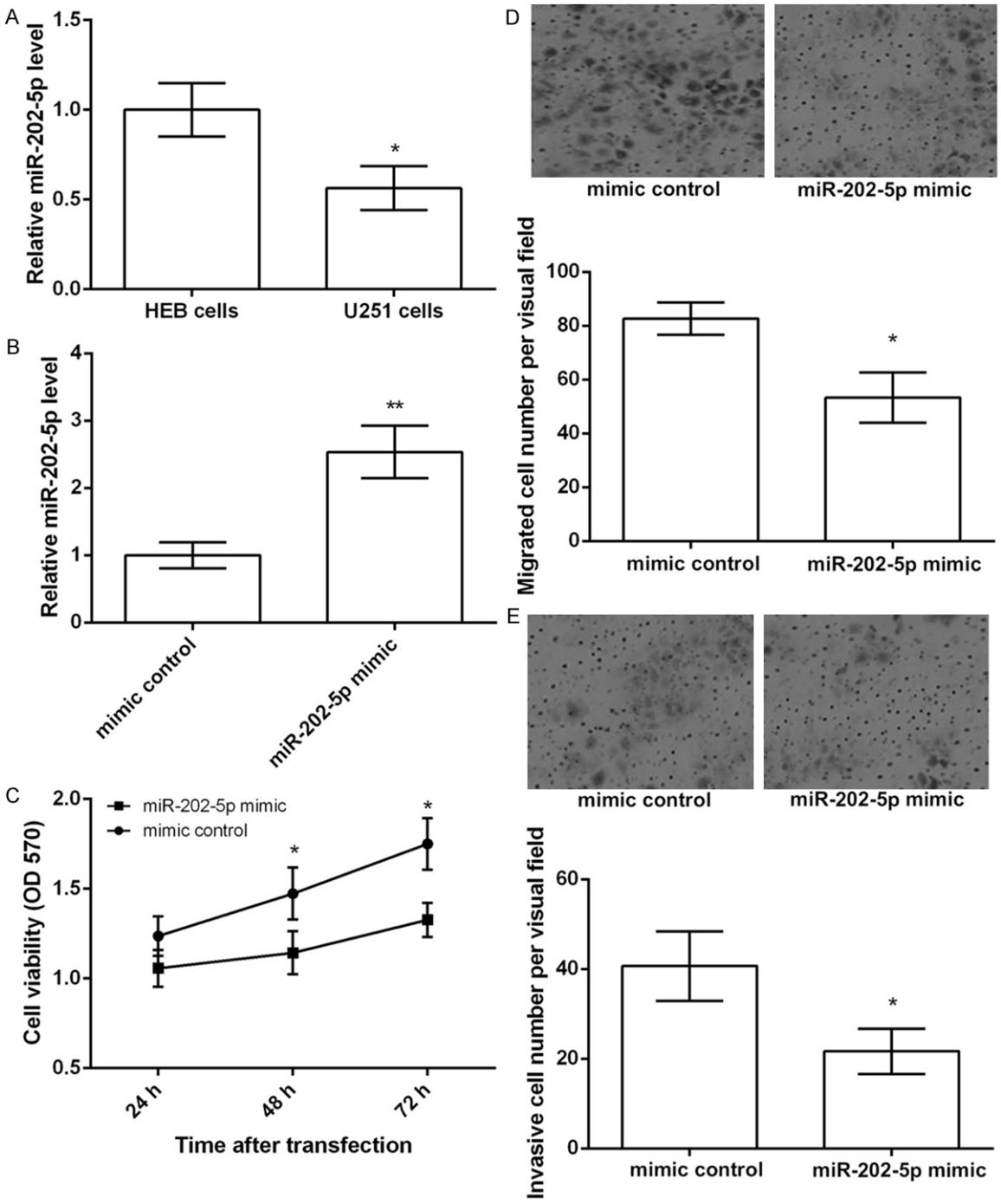


Figure 1. MiR-202-5p was down-regulated in U251 cells and overexpression of miR-202-5p inhibited U251 cells viability, migration and invasion. A: The expression of miR-202-5p in HEB cells and U251 cells. B: The expression of miR-202-5p in U251 cells after transfection with miR-202-5p mimic and mimic control. C: MTT assay showed cell viability after transfection with miR-202-5p mimic and mimic control. D: Transwell assay showed the migrated cell number after transfection with miR-202-5p mimic and mimic control. E: Transwell assay showed the invasive cell number after transfection with miR-202-5p mimic and mimic control. *, P<0.05, **, P<0.01.

mutagenesis kit (Stratagene, Santa Clara, California, USA). The sequences and mutations were verified by sequence analysis. The reporter constructs containing either EIF4E 3'UTR-wt or its mutant EIF4E 3'UTR-mut, GSKIP 3'UTR-wt or its mutant GSKIP 3'UTR-mut and miR-202-

5p mimic or mimic control were cotransfected into the U251 cells. The reporter vector without the targeting sequence containing Renilla luciferase genes was considered as a scramble-negative control. The U251 cells at 48 h of post-transfection were collected. The dual Luciferase Reporter Assay System (Promega) was used to evaluate the activity of luciferase. All transfection experiments were developed in triplicate.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from isolated from 72 h post-transfection cells using Trizol reagent (Invitrogen) following the instruction of manufacturer. The miRNAs were also isolated from these cells by the Ambionmir Vana™ miRNA isolation kit (Ambion, USA). The concentration of RNA was detected by ananodrop spectrophotometer (Gene, USA). Reverse transcription (RT) into cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). qRT-PCR was conducted using the mirVana™ qRT-PCR miRNA detection kit (Ambion, USA) according to the manufacturer's protocol. The expressions of miRNAs and mRNAs were respectively normalized to U6 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression levels of these targets were then calculated using the $2^{-\Delta\Delta Ct}$ method. Data were analyzed by Opticon Monitor Analysis Software V2.02 software (MJ Research, USA).

Western blot

After the treatments, cells were harvested, washed and lysed in RIPA buffer (Keygen Biotech), containing protease inhibitors and phosphatase inhibitors for extraction of cellular proteins. Protein concentrations were determined using the BCA assay kit (Thermo Scientific, MA, USA). Protein samples (40 μg/lane) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto Immunobilon-P nylon membranes. After blocked in 5% non-fat milk for 2 h, the members were probed with the appropriate primary antibodies against EIF4E (1:1000; Abgent, San Diego, CA, USA), GSKIP (1:1000, Cell Signaling Technology, Danvers, MA), GSK3β (1:1000, Cell Signaling) and β-catenin (1:1000, Cell Signaling) overnight at 4°C. The membranes were then washed and incubated with the appropriate alkaline horseradish peroxidase-conjugated secondary antibody for 1 h. The immunoreactive protein bands were developed by an enhanced chemiluminescence kit (ECL) reagents (Amersham Pharmacia, Buckinghamshire, UK) and visualized using the C300 imaging system (Azure Biosystem, USA). GADPH was as an internal standard to normalize loading protein. The relative concentration of the protein bands was analyzed by Quantity One software (Bio-Rad).

Statistical analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) in SPSS 19.0 statistical software. The results of multiple experiments were presented as mean ± standard deviation (SD). A value of $P<0.05$ indicated a statistically significant result.

Results

Overexpression of miR-202-5p inhibited U251 cells viability, migration and invasion

The expression of miR-202-5p in HEB cells and U251 cells were respectively determined by qRT-PCR. As shown in **Figure 1A**, compared to HEB cells, the expression of miR-202-5p were significantly lower in U251 cells ($P<0.05$). To further investigate the effects of miR-202-5p in U251 cells, we overexpressed miR-202-5p by transfection with miR-202-5p mimic. As displayed in **Figure 1B**, miR-202-5p expression in miR-202-5p mimic transfected group was significantly increased in comparison with mimic control group ($P<0.05$), indicating that miR-202-5p was successfully overexpressed. In addition, the results of MTT assay showed that cell viability of miR-202-5p mimic transfected cells significantly decreased compared with that of mimic control group (**Figure 1C**, $P<0.05$), indicating that overexpression of miR-202-5p significantly inhibited cell viability. Besides, the results of Transwell analysis showed that overexpression of miR-202-5p in U251 cells significantly inhibited cell migration and cell invasion than that of mimic control group (**Figure 1D** and **1E**, $P<0.05$).

EIF4E and GSKIP were the direct targets of miR-202-5p

According to the information of Target San Human, we found that EIF4E and GSKIP were

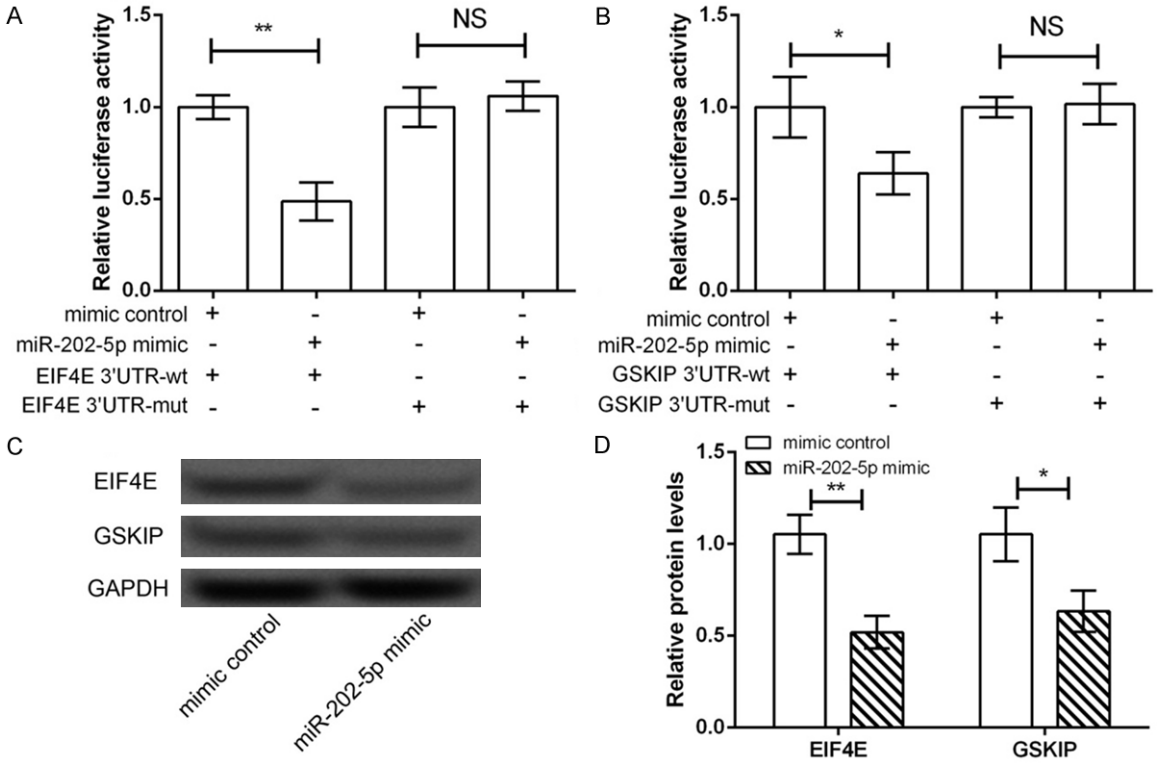


Figure 2. EIF4E and GSKIP were the direct targets of miR-202-5p. A: Dual luciferase assay confirmed that EIF4E was the direct targets of miR-202-5p. B: Dual luciferase assay confirmed that GSKIP was the direct targets of miR-202-5p. C and D: Western blot showed the protein expression levels of EIF4E and GSKIP in U251 cells after transfection with miR-202-5p mimic and mimic control. *, P<0.05, **, P<0.01.

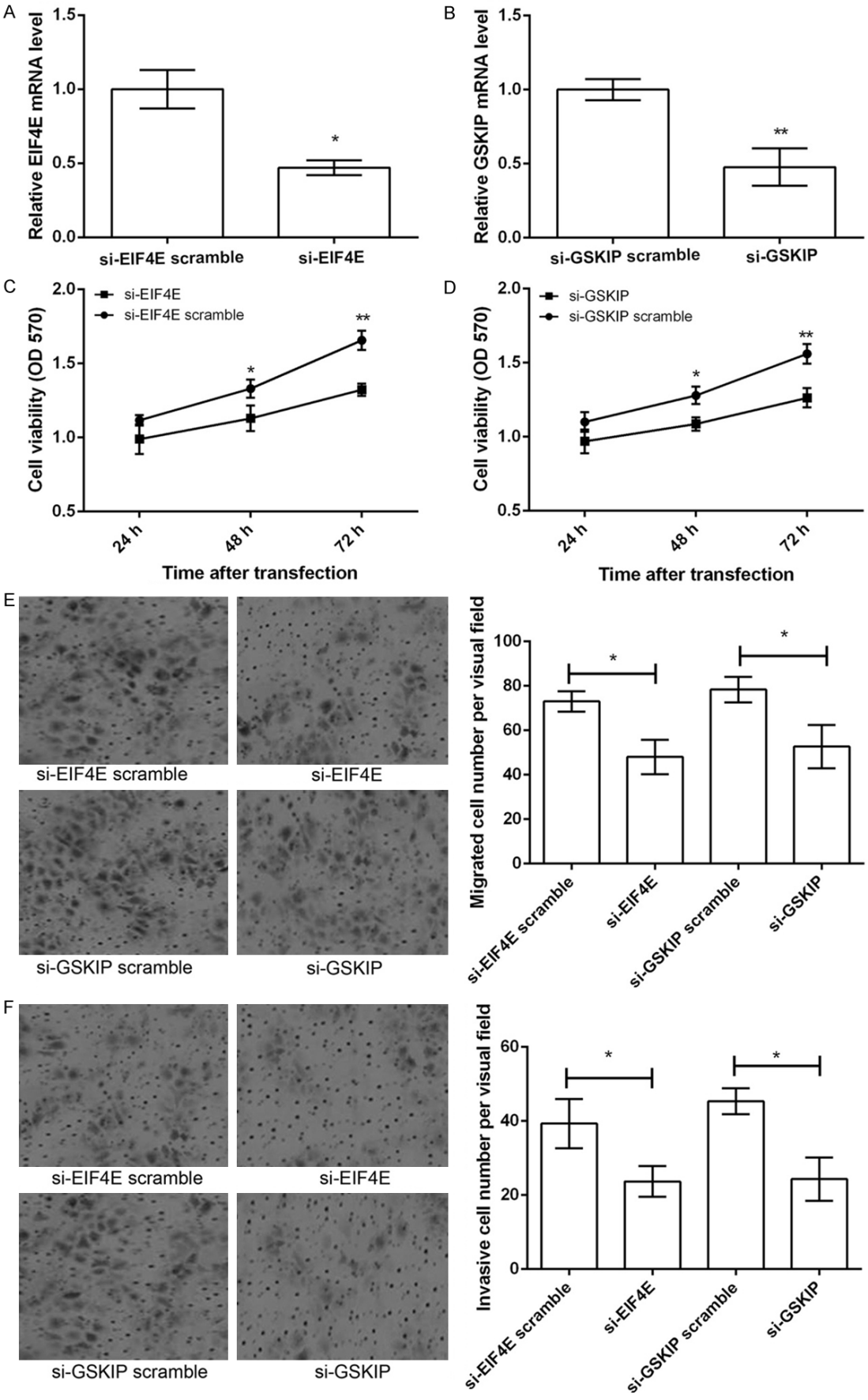
predicted as the potential targets of miR-202-5p. To further verify whether EIF4E and GSKIP were the direct targets of miR-202-5p, dual luciferase assays were conducted (**Figure 2A** and **2B**). The results showed that miR-202-5p could target wild-type EIF4E and GSKIP mRNA, but could not target mutant EIF4E and GSKIP mRNA. Furthermore, the protein expression levels of EIF4E and GSKIP in U251 cells were significantly lower after miR-202-5p mimic than mimic control transfection (**Figure 2C** and **2D**, P<0.05). These data indicated that EIF4E and GSKIP were the direct targets of miR-202-5p.

MiR-202-5p may impact inhibitory effect on U251 cells by targeting EIF4E or GSKIP

To further investigate whether miR-202-5p impacted inhibitory effects on U251 cells by targeting EIF4E or GSKIP, we knocked down EIF4E and GSKIP, respectively. In comparison with siRNA scramble control, the expression of EIF4E and GSKIP were significantly decreased after transfection with si-EIF4E and si-GSKIP (P<0.05), indicating EIF4E and GSKIP were successfully knocked down in U251 cells. Our experiment further confirmed that the cell viability, migration and invasion in U251 cells were significantly inhibited after knockdown of EIF4E or GSKIP (**Figure 3C-F**, P<0.05), indicatingthat miR-202-5p might play inhibitory effectson the viability, migration and invasion of U251 cells via targeting EIF4E and GSKIP.

β-catenin may be a critical downstream regulator of miR-202-5p to mediate glioblastoma development

To further explore the possible regulatory mechanism of miR-202-5p inglioblastoma development, the regulatory relationship between miR-202-5p and β-catenin was explored. As shown in **Figure 4**, the protein expression levels of EIF4E, GSKIP and β-catenin significantly decreased after miR-202-5p overexpression, while GSK3β expression markedly increased (P<0.05). However, overexpression of EIF4E and miR-202-5p simultaneously resulted in


A
Relative EIF4E mRNA level
1.5
1.0
0.5
0.0
*
si-EIF4E scramble
si-EIF4E
B
Relative GSKIP mRNA level
1.5
1.0
0.5
0.0
**
si-GSKIP scramble
si-GSKIP
C
si-EIF4E
si-EIF4E scramble
Cell viability (OD 570)
2.0
1.5
1.0
0.5
*
**
24 h
48 h
72 h
Time after transfection
D
si-GSKIP
si-GSKIP scramble
Cell viability (OD 570)
2.0
1.5
1.0
0.5
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24 h
48 h
72 h
Time after transfection
E
si-EIF4E scramble
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si-GSKIP
Migrated cell number per visual field
100
80
60
40
20
0
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si-EIF4E scramble
si-EIF4E
si-GSKIP scramble
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F
si-EIF4E scramble
si-EIF4E
si-GSKIP scramble
si-GSKIP
Invasive cell number per visual field
60
40
20
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*
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si-EIF4E scramble
si-EIF4E
si-GSKIP scramble
si-GSKIP

Figure 3. MiR-202-5p may impact inhibitory effect on U251 cells by targeting EIF4E or GSKIP. A: The expression levels of EIF4Eafter transfection with si-EIF4E and siRNA scramble. B: The expression levels of GSKIP after transfection with si-GSKIP and siRNA scramble. C: MTT assay showed cell viability after transfection with si-EIF4E and siRNA scramble. D: MTT assay showed cell viability after transfection with si-GSKIP and siRNA scramble. E: Transwell assay showed the migrated cell number after transfection with si-EIF4E, siRNA scramble, si-GSKIP and siRNA scramble. F: Transwell assay showed the invasive cell number after transfection with si-EIF4E, siRNA scramble, si-GSKIP and siRNA scramble. *, P<0.05, **, P<0.01.

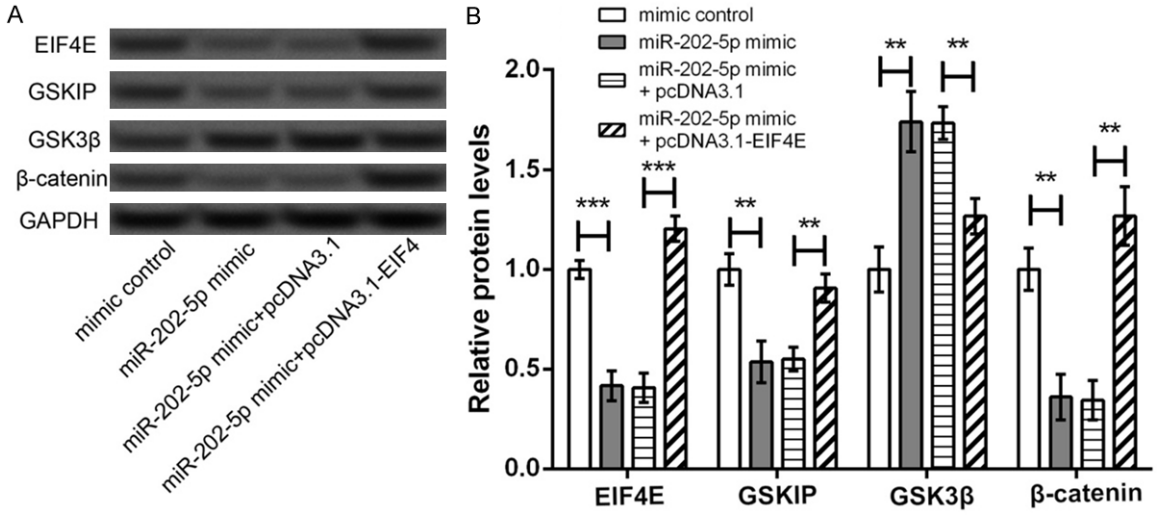

Figure 4. The protein expression levels of EIF4E, GSKIP, GSK3β and β-catenin in different groups. *, P<0.05, **, P<0.01, ***, P<0.001.

opposite expression changes of these proteins (P<0.05).

Discussion

In the present study, we found that miR-202-5p expression in U251 cells was significantly lower than that in HEB cells. After U251 cells were transfected with miR-202-5p mimic, cell viability, migration and invasion decreased significantly. Furthermore, EIF4E and GSKIP were confirmed as the direct targets of miR-202-5p. The expression levels of EIF4E and GSKIP significantly decreased after miR-202-5p overexpression. Also, knockdown of EIF4E or GSKIP could significantly decreased cell viability, migration and invasion. Besides, the expression levels of EIF4E, GSKIP and β-catenin significantly decreased after miR-202-5p overexpression, while GSK3β expression markedly increased. However, overexpression of EIF4E and miR-202-5p simultaneously resulted in opposite expression changes of these proteins.

Accumulating evidences have confirmed the suppressive roles of several miRNAs in glioblastoma, such as miR-34a [19], miR-137 [20] and let-7 [21]. Also, miR-202 has been confirmed to play crucial roles in a variety of cancers [15-17]. In our study, miR-202-5p was significantly downregulated in U251 cells, which was in line with the previous finding of Chan et al. [18]. Moreover, overexpression of miR-202-5p in U251 cells led to the significantly decreases of cell viability, migration and invasion. Although the role of miR-202-5p has not been fully investigated, our results prompt us to speculate that down-regulation of miR-202-5p contribute to the development of glioblastoma.

Furthermore, one of important aspects of our study was that EIF4E and GSKIP were confirmed as the direct targets of miR-202-5p. EIF4E, an eukaryotic translation initiation factor, is found to be overexpressed in many cancers and plays critical roles in proliferation and survival [22]. Upregulation of the EIF4E signaling pathway has been shown to result in the progression of gastric cancer [23]. Wendel et al. also demonstrated that survival signalling by EIF4E plays critical rolesin oncogenesis and cancer therapy [24]. In addition, GSKIP, the

A-kinase anchoring protein, is reported to function as a negative regulator of GSK3β [25]. GSK3β is found highly inactivated in epithelial cancers and controls the tumor metastasis [26]. Inhibition of GSK3β can suppress tumorigenesis through reducing cell proliferation, restraining cell motility and inducing apoptosis in non-small cell lung cancer [27]. In our study, the expression levels of EIF4E and GSKIP significantly decreased after miR-202-5p overexpression. Moreover, knockdown of EIF4E or GSKIP could significantly decreased cell viability, migration and invasion. Taken together, we speculate that miR-202-5p may contribute to glioblastoma development via targeting EIF4E or GSKIP.

Besides, we overexpressed EIF4E and miR-202-5p simultaneously to further explore the possible regulatory mechanism of miR-202-5p. In a recent study, EIF4E-β-catenin axis is shown to be a critical regulator of cell growth and survival in lung cancer [28]. In addition, GSKIP can regulate β-catenin through interactions with PKA and GSK3β [29]. Protein kinase D1 is found to stimulate cancer stemness and drug resistance in human breast cancer via GSK3/β-catenin signaling pathway [30]. WM130 is also reported to inhibit hepatic cancer stem-like cells through inhibiting AKT/GSK3β/β-catenin signaling pathway [31]. The results showed that the expression levels of EIF4E, GSKIP and β-catenin significantly decreased after miR-202-5p overexpression, while GSK3β expression markedly increased. However, overexpression of EIF4E and miR-202-5p simultaneously resulted in opposite expression changes of these proteins. It can be hypothesized that miR-202-5p may inhibit the protein expression levels of EIF4E and GSKIP. Overexpression of EIF4E can promote the translation of β-catenin through EIF4E-β-catenin axis. Meanwhile, GSKIP may inhibit the degradation of β-catenin by suppressing GSK3β. Thus, we speculated that overexpression of miR-202-5p may inhibit the expression of β-catenin through regulating EIF4E or GSKIP or both signal paths, thus to affect the migration and invasion of glioblastoma cells. β-catenin may be a critical downstream regulator of miR-202-5p to regulate the glioblastoma development.

In conclusion, down-regulation of miR-202-5p maypromote the glioblastoma cell proliferation, migration and invasion possibly via targeting EIF4E-β-catenin axis or GSKIP-GSK3β/β-catenin pathway. miR-202-5p may serve as a potential therapeutical target for glioblastoma.

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Disclosure of conflict of interest

None.

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