1 INTRODUCTION

Glioma, originating from neoplastic glial cells, is the most prevalent type of intracranial tumour in adults, with an annual incidence of around 0.005% (www.cbtrus.org). According to the World Health Organization (WHO) classification, there are four grades of gliomas. Among them, anaplastic oligoastrocytoma (Grade III), anaplastic oligodendroglioma (Grade III), and glioblastoma (Grade IV) are defined as malignant gliomas.1 The etiology of most malignant gliomas remains unclear. Despite extensive efforts having been made to explain the molecular pathology of glioma and a few specific molecules being found to be differentially expressed in different glioma types, the molecular pathogenesis of glioma is largely unknown.2 The complex pathogenesis of glioma is the main reason leading to the poor prognosis in glioma patients undergoing surgical resection and chemotheraphy. Thus, identification of biological markers for early detection of glioma is critical for the development of novel therapeutic strategies in order to improve the survival of glioma patients.

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Summary
As the most prevalent and lethal type of brain tumours, gliomas, especially malignant ones, are relatively resistant to conventional therapies. Gremlin 1 (GREM1) is a secreted glycoprotein that is implicated in the maintenance of cancer stem cells in tumour hierarchy. In the current study, the role of GREM1 in the carcinogenesis of glioma was studied using a knockdown approach. We first examined the expression level of GREM1 in the clinical samples, and then evaluated the effect of GREM1 knockdown on the viability and colony formation capacity of U87-MG cells. Moreover, the migration ability, invasiveness, cell cycle, and apoptosis of GREM1-silenced cells were assessed. Furthermore, the involvement of functional GREM1 in the epithelial-mesenchymal transition (EMT) process of glioma was investigated by detecting the expression levels of glioma-associated oncogene homologue 3 (GLI3) and EMT-related molecules. Our results demonstrated that knockdown of GREM1 reduced cell viability, suppressed migration and invasion, and inhibited GLI3 expression and the EMT process in U87-MG cells. Meanwhile, GREM1 silencing promoted apoptosis in U87-MG cells through the accumulation of Bax, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) as well as the downregulation of Bcl-2. In addition, GREM1 knockdown abolished transforming growth factor (TGF)-β1-mediated activation of the Smad pathway, which may underlie the mechanism of GREM1-regulated EMT. In conclusion, GREM1 plays an important role in the development of glioma, and it may serve as a potential target in glioma therapy.

KEYWORDS
epithelial-mesenchymal transition, glioma, glioma-associated oncogene homologue 3, Gremlin 1, Smad, TGF-β1
A recent study by Yan et al. showed that Gremlin 1 (GREM1)-overexpressing glioma cells exhibited enhanced growth and tumorigenicity, whereas downregulation of GREM1 expression impaired the growth of glioma cancer stem cells. Thus, the authors conclude that GREM1 plays an important role in the maintenance of CSCs in glioma. In addition, ubiquitous expression and pro-tumour effect of GREM1 was also verified by Snedden et al. in basal cell carcinoma. GREM1 is a secreted glycoprotein. It antagonizes bone morphogenetic proteins (BMPs) from binding to their receptors, and thereby inhibits the transduction of transforming growth factor-β (TGF-β) signals. TGF-β-mediated Smad signalling plays an essential role in the epithelial-to-mesenchymal transition (EMT) process that is associated with tumour progression and metastasis. In glioma patients, the pre-treatment EMT status contributes to the risk assessment of micro-metastasis and multifocal growth. Whether GREM1 is involved in the regulation of EMT through the TGF-β-mediated signalling pathway is still to be elucidated, and it will help with the understanding of GREM1 as an anti-glioma target and underline the mechanism for GREM1-driven progression of glioma.

The aim of the current study was to assess the potential of GREM1 as a therapeutic target for glioma and to provide preliminary demonstration on the role of GREM1 in the tumorigenicity and growth of glioma cells. Furthermore, we studied the effect of GREM1 knockdown on the expression of glioma-associated oncogene homologue 3 (GLI3), a key member in the Hedgehog signalling pathway in human glioma-initiating cells, as well as on TGF-β1/Smads-mediated EMT process.

2 | RESULTS

2.1 | GREM1 expression was positively associated with the histopathological grade of human glioma specimens

Human glioma specimens of different histopathological grades were subjected to immunohistochemistry for GREM1, and eight representative images are shown in Figure 1A. GREM1-positive cells were characterized by the presence of yellowish-brown particles. Based on the Wilcoxon rank sum test results, clinicopathological features including age, gender, and tumour location had no impact on GREM1 expression, whereas the histopathological grade of gliomas was positively associated with the expression level of GREM1 (Figure 1B). Up-regulation of GREM1 was detected in the glioma specimens of a higher histopathological grade as compared with those of a lower grade (P<.05).

2.2 | Knockdown of GREM1 reduced GLI3 expression at both mRNA and protein levels

GLI3 is highly expressed in multiple types of gliomas and is one of the essential players in the Hedgehog signalling pathway. Here we found that knockdown of GREM1 in U87-MG cells reduced the expression of GLI3 at both mRNA and protein levels (Figure 2), the differences were statistically significant between the shGREM1 group and the other two groups (P<.05). The result revealed an association in the expression of these two molecules.

2.3 | Silencing of GREM1 inhibited the EMT process in U87-MG cells and suppressed TGF-β1/Smad signalling pathway

A switch from E-cadherin to N-cadherin expression indicates the commitment to the EMT process. In the current study, the expression of E-cadherin was up-regulated while the expression of N-cadherin was downregulated after knockdown of GREM1 in U87-MG cells (Figure 3A), implying a marked inhibition of the EMT process in GREM1-silenced U87-MG cells. In addition, the TGF-β1-mediated Smad signalling pathway also plays a supportive role in transcriptomic reprogramming during EMT. As shown in Figure 3A, the levels of TGF-β1, p-Smad2, and p-Smad3 were all decreased after knockdown of GREM1. The expression of BMP7, a TGF-β1 inhibitor that is negatively regulated by GREM1, was elevated when GREM1 expression was silenced. Moreover, immunofluorescent staining revealed that silencing of GREM1 blocked nuclear translocation of Smad2/3 complexes in U87-MG cells (Figure 3B).

2.4 | Knockdown of GREM1 inhibited viability and anchorage-independent growth of U87-MG cells

To evaluate the effect of GREM1 knockdown on the viability and growth of U87-MG cells, we measured viable cells using the BrdU assay. As shown in Figure 4A, knockdown of GREM1 significantly inhibited the growth of U87-MG cells. The inhibitory effect was time-dependent, and the differences in the cell number were statistically significant between the shGREM1 group and the control group or the NC groups at 48, 72, 96, and 120 hours (P<.05). Additionally, the capability of anchorage-dependent growth of U87-MG cells was assessed by soft agar colony formation assay. After GREM1-knockdown, the colony formation rate in the shGREM1 group (17.8±3.0) was significantly lower compared with the other two groups (40.8±6.0 for the control group and 37.3±5.6 for the NC group; P<.05; Figure 4B).

2.5 | GREM1 knockdown reduced mobility and invasiveness of U87-MG cells

Transwell and scratch assays were conducted to evaluate the effect of GREM1 knockdown on the invasion and migration of U87-MG cells. In the shGREM1 group, the number of cells invading through the matrigel-coated membrane was significantly lower than that in the other two groups (P<.05), indicating that the invasiveness of U87-MG cells was inhibited upon GREM1 knockdown. The scratch assay showed that GREM1 knockdown delayed the process of wound closure compared with the control cells (Figure 5B), representing markedly suppressed migration of GREM1-silenced cells. To further explore the underlying molecular mechanism, the expression levels and activities of matrix metalloproteinase-2 (MMP2) and MMP9 were quantified by western blotting and gelatin zymography. Both assays
FIGURE 1  GREM1 expression was associated with the histopathological grade in human glioma specimens. (A) Representative images of immunohistochemical staining of GREM1 in human glioma specimens. GREM1-positive cells were characterized by the presence of yellowish-brown particles. Scale bars, 50 μm. According to the WHO classification, specimens No. 1, No. 2, No. 3 and No. 4 were stage II gliomas, specimens No. 5 and No. 6 were stage III-IV gliomas, and specimens No. 7 and No. 8 were stage IV gliomas. (B) Quantitative analysis of the immunohistochemical results grouped by different clinicopathological features. Significance of group-wise differences was analyzed using Wilcoxon rank sum test. Only the histopathological grade showed a significant impact on the immunohistochemical score of GREM1. *Indicates a significant difference from the scores of the Grade I-II gliomas, P<.05
demonstrated that MMP2 and MMP9 were markedly downregulated in U87-MG cells with GREM1 knockdown (Figure 6A,B).

## 2.6 Knockdown of GREM1 induced cell cycle arrest and apoptosis in U87-MG cells

Cell cycle distribution and apoptosis in U87-MG cells with different treatment were analyzed by flow cytometry. As illustrated in Figure 7A, the cells with GREM1 knockdown showed increased percentage of cells in the G1 phase (72.6%), which was significantly different from the percentage of G1 cells in the other two groups (58.8% for control group and 62.8% for NC group). Downregulation of GREM1 in U87-MG cells also resulted in a concomitant decrease in the fraction of cells in the S phase (Figure 7A). In addition, knockdown of GREM1 led to significant increases in the early and late apoptotic rates in the shGREM1 group as compared with the other two groups (Figure 7B). Hoechst staining showed that the cell nuclei in the control and NC groups were regularly shaped and stained in dark blue by DAPI, while the cell nuclei in the shGREM1 group were fragmented and stained by Hoechst bright blue (Figure 7C).

The possible pathways involved in the GREM1 knockdown-induced cell cycle arrest and apoptosis were examined by western blotting. As shown in Figure 8, accumulations of cleaved caspase-3, cleaved PARP, and Bax were detected in the shGREM1 group while the expression of Bcl-2 was downregulated after the knockdown of GREM1 (Figure 8). The quantitative analyses of relative expression...
Knockdown of GREM1 inhibited epithelial-to-mesenchymal transition (EMT) and inactivated the TGF-β1/Smad pathway in U87-MG cells. (A) Representative images and quantitative analysis of western blotting assays. The expressions of E-cadherin and BMP7 were upregulated while the protein levels of N-cadherin, TGF-β1, p-Smad2 and p-Smad3 were reduced in GREM1-silenced cells. *Indicates a significant difference from the control group, \( P < .05 \). # indicates a significant difference from the NC group, \( P < .05 \). (◽) control; (◼) NC; (◾) shGREM1. (B) Representative images of immunofluorescent assay. The Smad2/3 complexes, stained in red, were localized in the nuclei of control U87-MG cells, whereas nuclear localization of Smad2/3 complexes were abated after GREM1 knockdown. Scale bar, 100 μm.
levels of these proteins indicated statistically significant differences between the shGREM1 group and the control or NC group \((P<.05)\). Moreover, the ratio of Bcl-2 to Bax decreased dramatically as a result of GREM1 knockdown, suggesting initiation of mitochondria-mediated apoptosis in GREM1-silenced U87-MG cells.

2.7 | Silencing of GREM1 abrogated TGF-β1-induced EMT in U87-MG cells

To further explore the mechanism underlying GREM1-regulated EMT, U87-MG cells with or without GREM1 knockdown were incubated with TGF-β1 for 48 hours. Following TGF-β1 treatment, the levels of p-Smad2, and p-Smad3 in U87-MG cells were significantly increased \((P<.05)\), while knockdown of GREM1 abolished TGF-β1-induced phosphorylation of Smads (Figure 9A). Moreover, GREM1 knockdown abrogated TGF-β1-induced EMT in U87-MG cells, as evidenced by the decreased E-cadherin to N-cadherin ratio (Figure 9A). Furthermore, knockdown of GREM1 reduced the invasiveness of U87-MG cells regardless of the presence or absence of TGF-β1 (Figure 9B).

3 | DISCUSSION

Glioma cells and normal neural stem cells (NSC) share several pathways, including those activated by Notch, Wnt, and Hedgehog. These pathways all appear to be important for gliomagenesis, but only the Hedgehog signalling pathway is indispensable for the self-renewal and tumourigenicity of glioma-initiating cells through a Hedgehog→Gli→Cdc2 signalling cascade. In the present study, we found that knockdown of GREM1 led to downregulation of GLI3, a bifunctional transcriptional regulator in the Hedgehog pathway. Reduced expression of GLI3 upon GREM1 knockdown implied that the activation of the Hedgehog pathway was toned down. Moreover, GREM1 knockdown also inhibited the EMT process in U87-MG cells. These findings suggest that targeting of GREM1 might be the cure of aggressive glioma. In the present study, a positive association was detected between the histopathological grade and the GREM1 expression level in human glioma specimens, suggesting that GREM1 may serve as a potential prognostic marker for glioma. In addition, we demonstrated that knockdown of GREM1 reduced cell viability and anchoragedependent growth of human glioma cells, indicating that GREM1 was required to maintain the oncogenic characteristics of glioma cells.

Knockdown of GREM1 decreased the cell mobility and invasiveness of U87-MG cells as demonstrated by transwell and scratch assays. To explore the underlying molecular mechanism, we measured the expression levels and activities of MMP2 and MMP9 in U87-MG cells with and without GREM1 knockdown, and found that downregulation of GREM1 led to reduced expression and activity of MMP2 and MMP9 in glioma cells. It is known that overexpression of MMPs contributes to the aggressiveness of tumour cells in multiple cancer types. Interference with MMP expression by GREM1 knockdown suggests that targeting GREM1 may suppress invasion and metastasis of high-graded gliomas by inhibiting the expression of MMPs.

Apoptosis induced by anticancer agents constitutes one major aspect for evaluating the therapeutic efficacy. In the present study, we showed that silencing of GREM1 induced cell cycle arrest and apoptosis in U87-MG cells. In the meanwhile, the levels of cleaved caspase-3, cleaved PARP, and Bax were increased while the expression of Bcl-2 was downregulated in GREM1-silenced cells. The Bcl-2 family proteins can either promote cell survival (Bcl-2) or induce apoptosis (Bax). An increased Bax-to-Bcl-2 ratio causes a disturbance of the mitochondrial membrane potential, leading to the cleavage of caspase-3 and PARP for the execution of cell apoptosis. Our results demonstrated that GREM1 knockdown-induced apoptosis in glioma cells through the intrinsic (mitochondrial) pathway.

According to Kahlert et al’s review, EMT is generally considered to be a relevant molecular event in malignant gliomas. Hence,
therapies with an anti-EMT potential is a promising direction for the development glioma therapy. The role of GREM1 in the EMT process of glioma cells was investigated in the present study. Our data demonstrated that GREM1 knockdown led to an increased ratio of E-cadherin to N-cadherin in U87-MG cells, which indicated an inhibited EMT process. To reveal the molecular pathway through which GREM1 played a regulatory role in EMT, we examined the expressions of BMP7 and other molecules involved in the TGF-β1/Smad pathway. Considering that BMP7 can counteract TGF-β1-induced EMT, increased BMP7 level by GREM1 knockdown and concurrent suppression of the TGF-β1/Smad pathway suggest blockade of the EMT process in GREM1-silenced U87-MG cells. Moreover, GREM1 knockdown inhibited TGF-β1-stimulated Smad activation and cell invasion, which further supports the conclusion that GREM1 knockdown impairs the EMT process in glioma cells through an anti-TGF-β1 manner.

In summary, our study demonstrated that GREM1 played a positive role in the oncogenesis and development of glioma. Knockdown of GREM1 not only inhibited growth, migration and invasion, but also induced apoptosis of glioma cells. In addition, knockdown of GREM1 blocked the EMT process in glioma cells through an anti-TGF-β1 manner. Our findings suggest that GREM1 may serve a novel prognostic marker of glioma. As GREM1 may regulate glioma cells at multiple levels, the potential value of GREM1 as anti-glioma target is to be further assessed in future studies.

4 METHODS

4.1 Reagents and cell culture

Antibodies against GREM1 (Cat. no. sc-18274), GLI3 (Cat. no. sc-6154), p-Smad2/3 (Ser 423/425) (Cat. no. sc-11769), TGF-β1 (Cat. no. sc-57447), and β-actin (Cat. no. sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against cleaved caspase-3 (Cat. no. ab2302), cleaved PARP (Cat. no. ab32561), E-cadherin (Cat. no. ab76051), N-cadherin (Cat. no. ab18203), and BMP7 (Cat. no. ab56023) were purchased from Abcam (Cambridge, UK). Antibodies against Bcl-2 (Cat. no. BA0412), Bax (Cat. no. BA0315), MMP-2 (Cat. no. BA0569), MMP-9, Smad2 (Cat. no. BA4557) (Cat. no. BA0573), and Smad3 (Cat. no. BA4559) were purchased from Boster (Boster, Wuhan, China). Antibodies against Smad2/3 (Cat. no. #5678) used for immunofluorescent staining were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). Human glioblastoma (GBM) cell line U87-MG was obtained from the Cell Bank of Shanghai Institutes of Biological Sciences, Chinese
Academy of Sciences. The cells were cultured in MEM with 15% FBS and 1% (v/v) antibiotics mix and maintained in an atmosphere of 95% air and 5% CO$_2$ at 37°C.

4.2 | Patients and collection of glioma specimens

Glioma specimens were collected from 30 glioma patients in The First Affiliated Hospital of China Medical University. The specimens were fixed and prepared in paraffin sections. All the patients enrolled in the present study met the following criteria: (i) Diagnosed to be primary gliomas; (ii) All the patients had complete information on clinicopathological and prognostic characteristics. The study was approved by the ethic committee of The First Affiliated Hospital of China Medical University for related screening, inspection, and data collection. All the subjects had signed a written informed consent form, and all the procedures were performed in accordance with the Declaration of Helsinki.
For the immunohistochemical assay, the glioma sections were incubated at 60°C for 2 hours before incubation with dimethylbenzene for dewaxing. The sections were then hydrated with different concentrations of alcohol (95% for 2 minutes, 85% for 2 minutes, and 75% for 5 minutes), and washed with ddH$_2$O for 2 minutes. Subsequently, the sections were fixed using 3% H$_2$O$_2$ for 15 minutes and washed with PBS three times, followed by incubation with a primary antibody (1:50) at 37°C for 30 minutes and then at 4°C overnight. After three washes with 0.01 mol/L PBS (5 minutes each), a secondary antibody (1:200) was added to the sections for 30 minutes incubation at 37°C, followed by five cycles of PBS wash. Thereafter, the sections were incubated with HRP-labeled avidin for 30 minutes at 37°C, and reacted with DAB for 3-10 minutes before the reaction was stopped by ddH$_2$O. The sections were re-stained with haematoxylin and dehydrated. The immunohistochemical scores were determined by scanning the sections using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) at 400× magnification. Aperio ImageScope software (Aperio Technologies) was then used to evaluate the scanned images based on the percentage of positively stained cells and the staining intensity. Generally, the sections with a score of one or two were defined as low expression of GREM1, the sections with a score of three or four were defined as moderate expression of GREM1, and the sections with a score of five to seven were defined as high expression of GREM1.

4.4 | Knockdown of GREM1

GREM1-specific shRNA (shGrem1_676 target sequence, 5’-GAAGCAGTGTCGTTGCATA-3’) and a nontargeting control shRNA sequence were obtained from Genechem Biotech (Shanghai, China). The shRNAs were inserted into pRNA-H1.1 plasmid to construct pRNA-H1.1-GREM1 and pRNA-H1.1-control plasmids. For transfection, U87-MG cells were transfected with pRNA-H1.1-GREM1 or pRNA-H1.1-control using Lipofectamine 2000 (Invitrogen, CA, USA).
FIGURE 9  GREM1 knockdown abrogated TGF-β1-induced EMT and invasion in U87-MG cells. (A) Representative images and analysis of western blotting assay. TGF-β1 stimulated downregulation of E-cadherin and elevated the levels of N-cadherin, p-Smad2, and p-Smad3, and these TGF-β1-induced changes were abolished by GREM1 knockdown. (●) Control; (◼) NC; (◼) shGREM1; (◼) control+TGF-β1; (◼) NC+TGF-β1; (◼) shGREM1+TGF-β1. (B) Representative images and analysis of transwell assay. Knockdown of GREM1 inhibited the invasiveness of U87-MG cells regardless of the presence or absence of TGF-β1 treatment. *Indicates a significant difference from the shGREM1 group, P<.05
The cells were divided into three groups: (i) Control group, parental U87-MG cells; (ii) NC group, U87-MG cells transfected with pRNA-H1.1-control plasmids; and (iii) shGREM1 group, U87-MG cells transfected with pRNA-H1.1-GREM1. Each group was represented by three replicates. The expression of GREM1 and GLI3 in U87-MG cells under different treatments were determined using reverse transcription quantitative PCR (RT-qPCR) and western blotting as described below.

4.5 | Reverse transcription quantitative PCR

Total RNA in the cells from different groups was extracted using RNA Purified Total RNA Extraction Kit according to the manufacturer’s instructions (Cat. no. RP1201; BioTeke, Beijing, China). β-actin was selected as the internal reference gene. cDNA templates were obtained by reverse transcription of RNA using Super M-MLV reverse transcriptase and the final RT-qPCR reaction mix in 20 μL volume contained 10 μL SYS BR Primix Ex Taq 2 (Cat. No. PR1702, Bioteke, China), 0.5 μL of each primer (Table S1), 1 μL cDNA template, and 8 μL Rnase free H$_2$O. Thermal cycling parameters for the amplification were set as follows: a denaturation step at 95°C for 10 minutes, followed by 40 cycles of amplification of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. The relative expression levels of the target genes were calculated by the Data Assist Software (Applied Biosystems/Life Technologies, version 3.0, Foster City, CA, USA) using the formula of 2$^{-\Delta\Delta C_{t}}$.

4.6 | Western blotting

Total cellular protein was extracted using the Total Protein Extraction Kit according to the manufacturer’s instructions (Cat. no. WLA019; Wanleibio, Shenyang, China). β-actin was used as the internal reference protein. The concentrations of the protein samples were determined using the BCA method, and 40 μg protein from each sample was subjected to 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, the membranes were rinsed with TTBS and blocked with skimmed milk solution for 1 hour. Thereafter, the membranes were incubated with the primary antibodies against the target proteins (GREM1 [1:5000], GLI3 [1:5000], p-Smad2/3 [Ser 423/425] [1:5000], TGF-β1 [1:5000], cleaved caspase-3 [1:5000], cleaved PARP [1:5000], E-cadherin [1:5000], N-cadherin [1:5000], BMP7 [1:5000], Bcl-2 [1:5000], Bax [1:5000], matrix metalloproteinase-2 [MMMP-2] [1:5000], MMP-9 [1:5000], Smad2 [1:5000], Smad3 [1:5000]) or β-actin (1:1000) at 4°C overnight. After four washes with TTBS, the membranes were incubated with secondary HRP-conjugated IgG antibodies (1:5000) for 45 minute at 37°C. Following six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent and the images were recorded in the Gel Imaging System. The relative expression levels of GREM1 and GLI3 were calculated by the Gel-Pro-Analyzer (Media Cybernetics, Rockville, MD, USA).

4.7 | Immunofluorescent assay

Nuclear translocation of Smad2/3 after GREM1 knockdown was detected by immunofluorescent staining. Briefly, the transfected cells were seeded in 14-well chambers, allowed adhesion, and fixed with 4% paraformaldehyde for 15 minutes. Then the cells were permeabilized with 0.5% Triton X-100 for 30 minutes. After washing with PBS, the cells were blocked with 10% goat serum for 15 minutes, and incubated with primary rabbit polyclonal antibodies against Smad2/3 (1:200; with 1% goat serum) overnight at 4°C. Subsequently, the cells were incubated with a fluorescein isothiocyanate-labeled secondary antibody (1:200) for 1 hour. After that, the cells were washed and stained with 4,6-diamino-2-phenyl indole (DAPI) for 5 minutes at room temperature. After three cycles of five-min washes with PBS, the cells were imaged with a fluorescent microscope at 400x magnification.

4.8 | Brdu (bromodeoxyuridine) assay

The viability and proliferation of U87-MG cells were assessed by the Brdu assay using a BrdU ELISA kit (Cat. no. CEL-BRDU, Maibio, Shanghai, China) according to the manufacturer’s instructions. The number of viable cells was represented by the optical density (OD) at 450 nm measured with a Microplate Reader (ELX-800, BIOTEK, Winooski, VA, USA).

4.9 | Colony formation assay

The capability of anchorage-independent growth of glioma cells was measured by the colony formation assay. The transfected cells were suspended in 10% serum media containing 0.35% agarose, and inoculated into 35 mm plates at a density of 200 cells per plate. After 1-week culture, the colonies were stained with Wright-Giemsa stain and the number of colonies in each plate was recorded. The colony formation rate was the ratio of colony number to inoculated cell number per plate.

4.10 | Scratch assay

To evaluate cell mobility after GREM1 knockdown, scratching assay were performed on the transfected cells. The cells were seeded in a 24-well plate at a density of 2×10$^4$ cells/well. Reference points were marked to guarantee the same area of image acquisition. The cells were allowed to grow into a confluent monolayer at 37°C for 2 days. Then the cell layer was scratched to generate a cell-free straight line. The cells were washed with PBS twice to remove debris at the edges of the scratch. Afterwards, cell migration towards the midline of the scratch was recorded in reference with the reference points. For each well, three images (at 0, 24, and 48 hours, respectively) were captured with a phase-contrast microscope, and the gap distances were measured. Wound healing rate was defined as the percentage of gap closure, and the data were analyzed by the ImageJ software (US National Institutes of Health, Bethesda, MA, USA).
4.11 | Transwell assay

Transwell assay was conducted to assess the invasiveness of U87-MG cells. Briefly, 200 µL medium containing 2×10⁴ cells were plated in the upper chamber of a transwell system (BSA-coated porous polycarbonate membrane with a pore size of 8 µm; Corning Star, Cambridge, MA, USA). Each polycarbonate membrane was pre-coated with 40 µL matrigel (1.5 mg/mL; BD Biosciences, San Jose, CA, USA) and incubated at 37°C for 2 hour to form a reconstituted basement membrane. The cells were allowed to migrate through the porous membrane at 37°C for 4 hour. The cells in the upper surface of the membrane were completely removed, and the cells on the lower surfaces of the membrane were stained with 1% (w/v) crystal violet for 30 seconds. The cell number was determined using the Image-Pro Plus 6.0 software (Nikon, Melville, NY, USA). The effect of GREM1 knockdown on cell invasion was also evaluated by detecting expressions and activities of MMP2 and MMP9 using western blotting and gelatin zymography, respectively.²⁵

4.12 | Flow cytometry

Cell cycle was determined by flow cytometry. Briefly, Propidiod Iodide (PI) was added to the cells to stain DNA in the dark. After 20 minute incubation at room temperature, the DNA contents were analyzed using a FACS flow cytometer (Accuri C6; BD, San Diego, CA, USA).

To assess the effect of GREM1 knockdown on the apoptotic process in U87-MG cells, a PI/Annexin V-FITC Apoptosis Detection Kit (JinMei Biotech, Beijing, China) was used according to the manufacturer’s instructions. The apoptotic rates were analyzed using a FACScan flow cytometer (Accuri C6; BD). The total apoptotic rate was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis).

4.13 | Hoechst staining

The morphological changes of apoptotic cell nuclei were detected using a Hoechst staining kit according to the manufacturer’s instructions (Beyotime Biotechnology, Shanghai, China). The stained cells were observed under a fluorescence microscope at a magnification of 400×.

4.14 | Determination of the effect of GREM1 knockdown on TGF-β1-induced EMT

The cells were treated with 10 ng/mL TGF-β1 to stimulate Smad activation in the following groups: (i) Control group, parental U87-MG cells; (ii) NC group, U87-MG cells transfected with pRNA-H1.1-control plasmids; (iii) shGREM1 group, U87-MG cells transfected with pRNA-H1.1-GREM1; (iv) Control+TGF-β1 group, parental U87-MG cells treated with TGF-β1 for 48 hour; (v) NC+TGF-β1, pRNA-H1.1-control-transfected U87-MG cells treated with TGF-β1 for 48 hour; (v) shGREM1+TGF-β1 group, GREM1-silenced U87-MG cells treated with TGF-β1 for 48 hour. Each group was represented by five replicates. Following 48 hour TGF-β1 treatment, the invasion ability of U87-MG cells in each group was assessed by the transwell assay as describe above. The levels of p-Smad2, p-Smad3, E-cadherin, and N-cadherin were quantified by western blotting assay.

4.15 | Statistical analysis

The data were expressed as means±SD, and each assay was performed with at least three replicates. The differences in the immunohisto-chemical scores between groups grouped by various clinicopathological indicators were analyzed using Wilcoxon rank sum test. Multiple comparisons were performed using ANOVA followed by post-hoc Duncan test in order to control type I error. Difference between two groups was analyzed using Student’s t test. Significance was accepted when the two-tailed P value was smaller than 0.05. All the statistical analyses and graph plotting were conducted using R language version 3.2.1.²⁶

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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