



Rhoj Is a Novel Target for Progression and Invasion of Glioblastoma by Impairing Cytoskeleton Dynamics

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Published online: 21 August 2020

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Abstract

Rho GTPase family members were identified as critical regulators of cell morphology, actin cytoskeleton organization, cell movement, and cell cycle and also contributed to tumor progression, which have been implicated in various types of cancer metastasis and growth. Here, we firstly reported the dysregulation of Rhoj in glioblastoma multiforme (GBM) and aimed to investigate the role and mechanism of Rhoj in GBM. We analyzed the expression of 21 Rho GTPases family members and validated the expression of Rhoj in GBM by immunohistochemistry. We further investigated the role and mechanism of Rhoj in GBM both *in vitro* and *in vivo*. We observed that Rhoj is significantly overexpressed in GBM and associated with patients' survival. However, the role and underlying molecular mechanism of Rhoj in GBM are still unclear. We demonstrated that transcription factor c-Jun regulated the expression of Rhoj, and Rhoj interacted with moesin to promote GBM cell proliferation and migration by potentiating the activation of Rac1/PAK pathway and cytoskeletal dynamics. Rhoj may promote migration and invasion of GBM cells by regulating epithelial-mesenchymal transition (EMT)-like process. In conclusion, the Rhoj/Rac1/PAK signaling mediates invasion and progression of GBM and is a potential therapeutic target for GBM treatment. Rhoj may also be a promising biomarker for GBM diagnosis and prognosis.

Key Words Rhoj · GBM · actin cytoskeleton · invasion · moesin

Introduction

Glioblastoma multiforme (GBM) is a common malignant adult primary brain tumor in the central nervous system

(CNS) and is a lethal malignant tumor characterized by rapid cell division, proliferation, and unparalleled invasion with concomitant neovascularization [1]. Over the past decade, several large, publicly molecular studies demonstrated that

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13311-020-00910-w>) contains supplementary material, which is available to authorized users.

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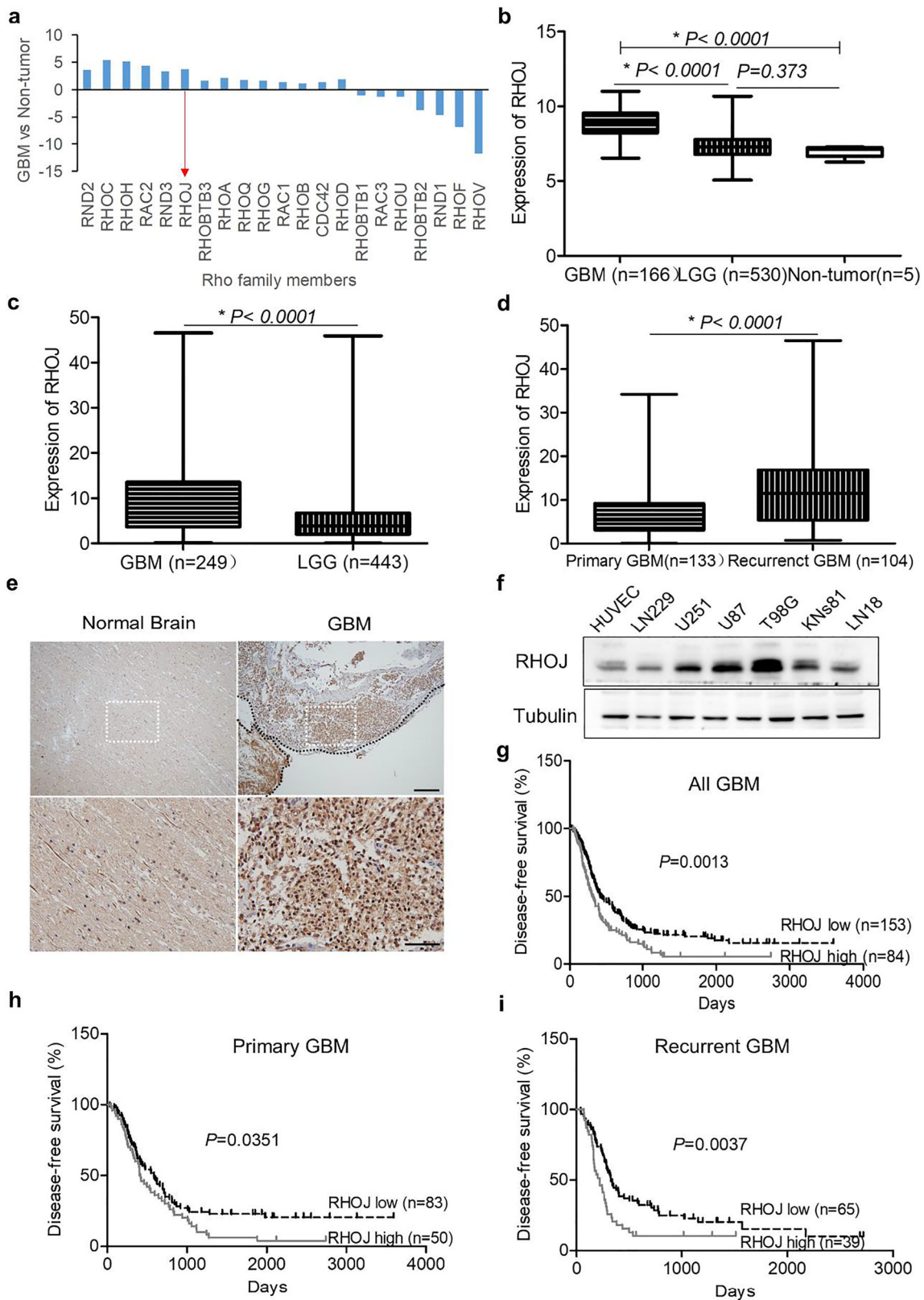
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genetic events drive human GBMs progression and poor clinical response [2, 3]. However, patients treated by combined surgery, radiotherapy, and chemotherapy still survive no more

than 15 months [4]. To better understand the mechanisms and develop more effective targeted molecular therapies are necessary for this incurable disease.

Fig. 1 Rhoj is highly overexpressed in GBM and its overexpression is strongly associated with poor patients' survival. (a) The normalized relative expression of Rho family members in GBM *versus* nontumor tissues based on the TCGA dataset (based on the log₂ transformed and normalized data download from <http://firebrowse.org/>). (b) The relative expression of Rhoj in GBM, LGG, and nontumor tissues based on the TCGA dataset. (c, d) The normalized relative expression difference of Rhoj in GBM *versus* LGG, or recurrent GBM *versus* primary GBM in the CGGA dataset. (e) IHC staining of Rhoj in the normal brain and GBM tissues. Representative images were obtained at $\times 100$ magnification (upper) and $\times 400$ magnification (lower). (f) Western blotting analysis of Rhoj expression in HUVEC and human GBM cell lines. (g–i) Kaplan–Meier analysis of survival for all GBMs (g), primary (h), and recurrent (i) Chinese glioma patients showed that high expression of Rhoj was significantly associated with poor survival of patients based on CGGA dataset

Rho GTPase family, as a part of Ras superfamily, is highly conserved and occurs in almost all eukaryotes. In mammals, the Rho family contains ~ 21 members. Rho GTPases are involved in several cellular processes, including actin and microtubule cytoskeleton dynamics, gene expression regulation, vesicle transport, cell cycle progression, and cell morphogenesis and migration, in turn promoting cancer progression, inflammation, wound repair, and other pathological processes [5]. More recent functional studies revealed that small GTPases family Rac1 activation leads to glioma growth and invasion [6]. Epithelial-mesenchymal transition (EMT) process has been thought to be essential for tumor metastasis. The EMT-like process has been proposed to participate in GBM metastasis invasion, and Rho GTPases Rac1 and RhoA pathways have been reported to crucially regulate EMT process [7, 8].

In this study, we analyzed the expression of 21 Rho family members in both TCGA and HPA datasets. We observed that Rhoj is significantly overexpressed in GBM. However, the role and underlying molecular mechanism of Rhoj in GBM are still unclear. Small Rho GTPase Rhoj belonging to the Cdc42 subfamily has been reported to regulate tubule formation, actomyosin contractility, and endothelial motility [9]. Recent studies have demonstrated that Rhoj acts as a positive angiogenic role in many cancers. Rhoj was found highly expressed in human melanomas and to regulate tumor metastasis and progression through its downstream PAK-BAD signaling [10]. As an endothelial cell-enriched small protein, Rhoj was demonstrated critical in tumor angiogenesis and the metastasis of Lewis lung carcinoma (LLC) tumor and spontaneous breast cancer model [11]. Intriguingly, few studies showed its expression and role in GBM. We aimed to demonstrate Rhoj expression and the correlation with GBM' progression as well as to reveal its putative function and molecular mechanisms in GBM.

Materials and Methods

Cell Lines and Cell Culture

The human glioma cell lines LN229, U251, U87, T98G, KNS81, and LN18 and human renal epithelial cell line 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA) and penicillin/streptomycin (Sangon Biotech, Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 200 containing large vessel endothelial supplement (LVES). All cells were cultured at 37 °C and 5% CO₂.

Flow Cytometry–Cell Cycle Analysis

5×10^5 transfected cells were collected in 1.5 mL 75% ethyl alcohol overnight at -20°C . For flow cytometry assay, the cells were centrifuged and washed with PBS, then resuspended in RNase A and treated with propidium iodide (PI) for 30 min, followed by detection with flow cytometry (BD Bioscience, CA, USA) and data were analyzed using the Modfit software.

Xenograft Mouse Model

For *in vivo* animal study, the established U87 cells expressing shNC or shRhoj were injected into the oxters of 5- to 6-week-old BALB/c nude mice (the Animal Research Center, Nanjing Medical University, China). The tumor size was measured every 5 days after injection and calculated according to the formula: $(\text{length} \times \text{width})^2/2$. Tumors were collected for immunoblotting and immunohistochemistry at 26 days. All animal experiments were performed under the approval of the National Institutes of Health of China.

Dual-Luciferase Assay

pGL3-Basic reporter plasmid, pGL3-Rhoj WT, or mutant plasmids and Renilla were transfected into 293T cells or U251 cells. c-Jun expression plasmid or vector were cotransfected for 24 h, and the luciferase activity was analyzed by the Dual-Luciferase Assay System (Cat.No:E1910, Promega, Madison, WA, USA) followed the instructions and normalized to the activity of Rluc.

Co-immunoprecipitation (co-IP)

We added a flag tag to Rhoj expressing vector and transfected U87 cells. Stable U87 cells expressing Flag-Rhoj were lysed, and protein was extracted for follow co-IP assay. Flag-beads were washed by PBS for two times, and then protein was divided into two equal parts, incubated with flag-beads or

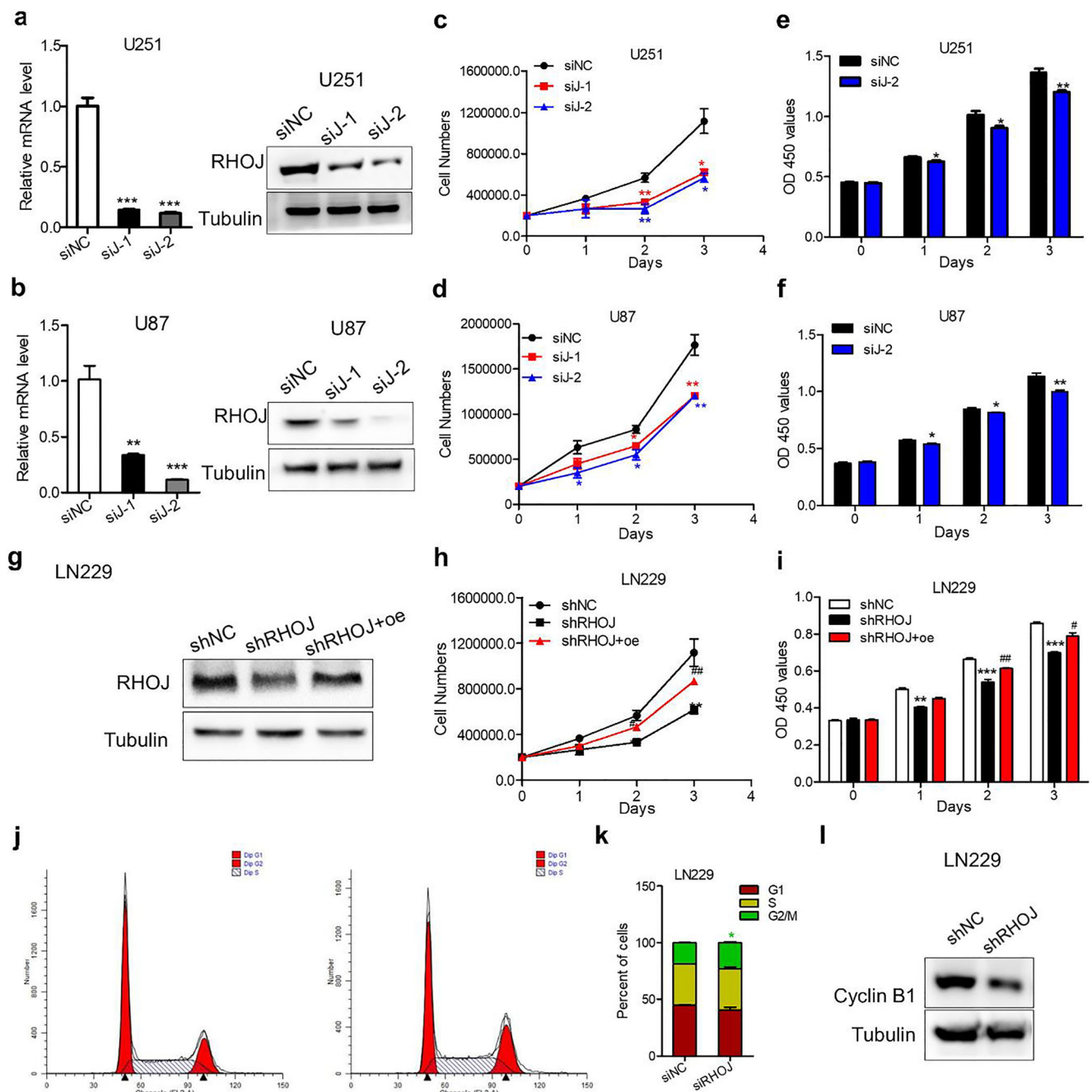


Fig. 2 The depletion of Rhoj suppresses the proliferation of GBM cells and induces cell cycle arrest in the G2 phase. (a, b) The knockdown effect of Rhoj by siRNA. U251 and U87 cells were harvested for RT-qPCR or western blotting. (c, d) The growth curve and (e, f) the Cell Counting Kit-8 (CCK-8) assays of two GBM cell lines after applying siRNA targeting Rhoj or negative control. (g) The stable knockdown and rescue effect by overexpression of Rhoj in LN229 cells by western blotting analysis. (h)

Cell growth curve and (i) CCK-8 assays. (j, k) Knockdown of Rhoj by RNAi in LN229 cells induced cell cycle arrest in the G2 phase after transduction for 24 h. (l) Western blot revealed that the expression of cyclin B1 was significantly decreased, which mediated cell cycle arrest partially. The p value was analyzed by a two-tailed Student t test as means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

IgG overnight, and, on the second day, centrifuged at 3000 rpm for 3 min, with supernatant being discard, and washed five times with wash buffer. Then SDS loading buffer was added, with boiling at 95 °C for 10 min, and followed by western blotting.

Statistical Analysis

All statistical analyses were performed using analysis of a two-tailed Student test with GraphPad Prism 5 (GraphPad Software). $p < 0.05$ was considered statistically significant

and $p < 0.001$ highly statistically significant. All data shown in figures are represented as mean \pm standard deviation (error bars).

Results

Rhoj Is Significantly Overexpressed in Glioblastoma and Correlates with Poor Patients' Survival

We analyzed the expression of 21 Rho family members in both the TCGA and HPA datasets. We observed that the expression of Rhoj is significantly higher in GBM than in LGG or nontumor tissues (Fig. 1a, b; Fig. S1d). The different expression of the 21 Rho family members in all glioma (GBM + LGG) versus nontumors, GBM versus LGG, or LGG versus nontumors is shown in the Fig. S1a–c. Meanwhile, the expression of Rhoj is also significantly higher in GBM versus LGG or recurrent GBM versus primary GBM in the Chinese Glioma Genome Atlas (CGGA) dataset (Fig. 1c, d). Overexpression of Rhoj in GBM was also verified by IHC staining in the GBM tissues of our samples. Rhoj was highly expressed in GBM tissues compared with normal brain tissues (Fig. 1e). Furthermore, consistent with the human tissues, we also found that Rhoj protein levels were increased in the human GBM cell lines that we studied when compared with HUVEC cells which were reported to have high expression of Rhoj (Fig. 1f). Next, the Kaplan–Meier analysis of survival for primary and recurrent Chinese GBM patients showed that high expression of Rhoj was significantly associated with poor survival of GBM patients based on the CGGA dataset (Fig. 1g–i). According to the TCGA dataset, Rhoj was relatively low expressed in many types of cancers compared with their matched normal, but its expression in GBM or GBM/LGG tissues is significantly higher than normal tissues (Fig. S2a). The overexpression of Rhoj was also significantly associated with both worse overall survival and disease-free survival (Fig. S2b, c). Consistently, high expression of Rhoj was also significantly associated with poor survival of both primary glioma or recurrent glioma in the CGGA dataset (Fig. S2d, e). These data suggest that Rhoj is overexpressed in GBM tissues and associated with poor patient survival.

Silencing Rhoj Inhibits Proliferation and Tumorigenesis of GBM Cells and Induces Cell Cycle Arrest

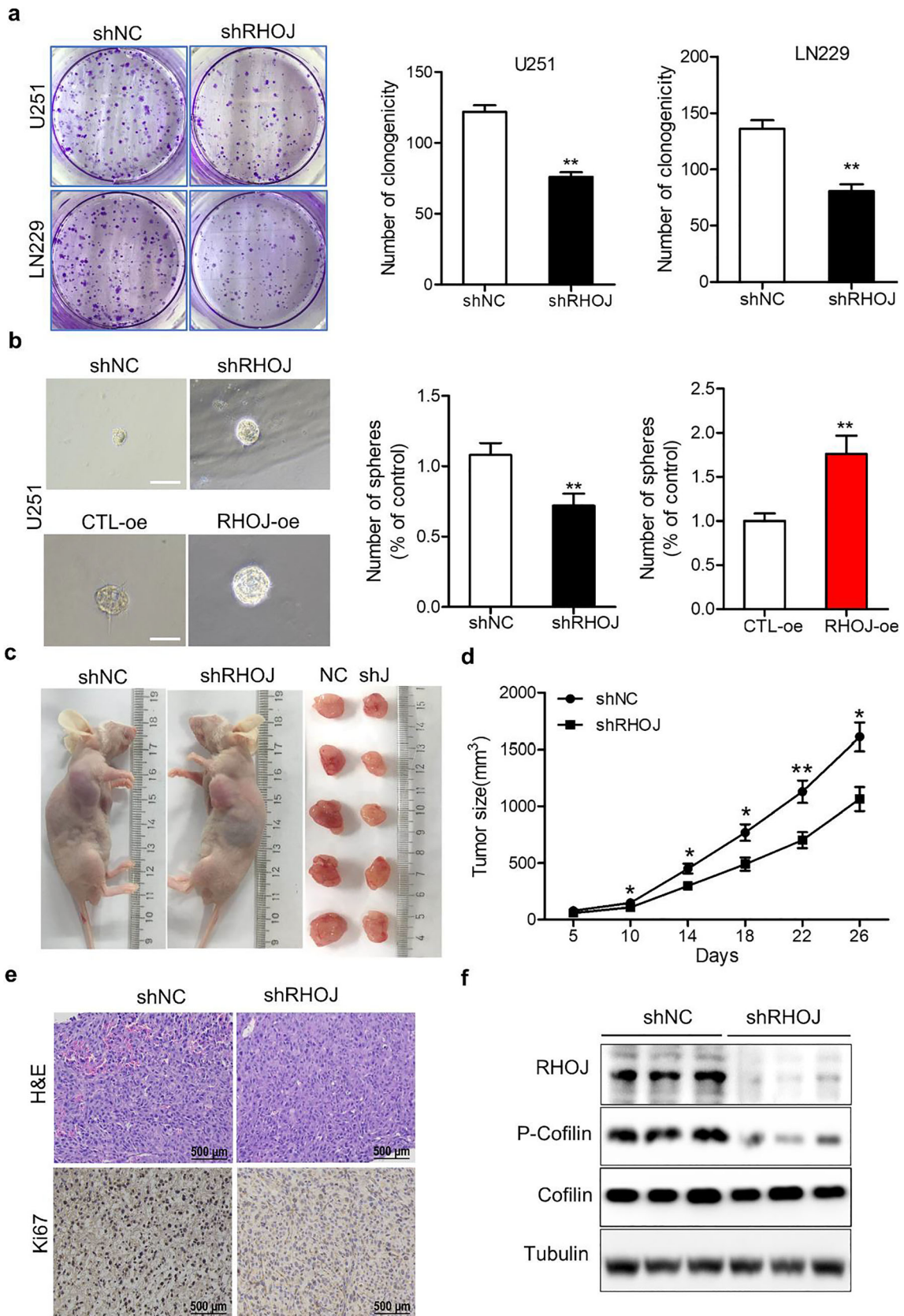
To determine whether Rhoj has functions in GBM, we first applied siRNAs to knockdown the expression of Rhoj in GBM cells, and the transient knockdown effects were examined by RT-qPCR and immunoblotting analysis (Fig. 2a, b). The knockdown of Rhoj significantly reduced U251 and U87 cell growth (Fig. 2c, d) and viability (Fig. 2e, f). Then, we

constructed shRNA targeting negative control or Rhoj and restoring Rhoj expression in shRhoj cells (Fig. 2g). The stable knockdown of Rhoj can also attenuate the growth and viability of LN229 GBM cells, and overexpressed Rhoj based on shRhoj can rescue the cell growth and viability inhibition phenotypes (Fig. 2h, i). These data indicated that cell proliferation was inhibited by Rhoj downregulation. Moreover, we examined the cell cycle distribution by flow cytometry analysis. We found that G2 phase cells showed a significant enrichment after transfected with siRhoj in the LN229 cell line (Fig. 2j, k). Cyclin B1, as a cell cycle parameter, was downregulated detected by western blot (Fig. 2l).

To evaluate Rhoj oncogenic and tumorigenic properties in GBM, monolayer colony formation assay and tumorsphere formation assay were performed to investigate the effects of Rhoj knockdown or overexpression on glioblastoma cell malignant proliferative ability. The colony formation and tumorsphere formation abilities were significantly suppressed by Rhoj stable knockdown compared with negative control cells (Fig. 3a, b; Fig. S3). Besides, Rhoj overexpression robustly promoted tumorsphere formation (Fig. 3b; Fig. S3). Next, we used a subcutaneous and intracranial xenograft U87 tumor model to investigate whether the above *in vitro* findings are applicable in animals. We found that Rhoj stable knockdown showed significantly smaller tumors and slowed tumor growth compared with negative control knockdown ones (Fig. 3c, d; Fig. S4). Then we collected the tissue sections for H&E staining and immunoblotting analysis. The results showed few blood vessels, and the proliferation marker Ki67 was significantly decreased in tumor tissues derived from shRhoj U87 cells (Fig. 3e). Immunoblotting studies demonstrated that the expression of Rhoj was effectively inhibited in xenografted tumors (Fig. 3f).

The Deficit of Rhoj Restrained Cell Migration and Invasion of GBM Cells *In Vitro* and Was Related to EMT-Like Phenotype

Rhoj has previously been demonstrated to modulate migration and invasion in melanoma cells [12]. To further identify whether Rhoj plays a role in GBM progression, based on the wound healing assay and transwell assay, Rhoj knockdown cells showed significantly poor migration and invasion abilities in U251 and LN229 cell lines (Fig. 4), and restoration of Rhoj expression in Rhoj knockdown cells rescued the inhibition of migration. EMT has been reported to be able to drive morphogenesis and initiate tumor progression [13]. We observed increased cell sizes and lower phalloidin-conjugated F-actin fluorescence intensity in Rhoj depletion cells (Fig. 5a–d; Fig. S5). Next, we detected the expression of EMT-related proteins E-cadherin and vimentin by immunofluorescence and immunoblotting and found upregulated E-cadherin and lower level of vimentin which indicated silencing of Rhoj



◀ **Fig. 3** Stable knockdown of Rhoj by shRNA inhibits tumorigenicity and angiogenesis of U87 cells in mice. (a) Rhoj stable knockdown (shRhoj) decreases colony numbers by U251 and LN229 cells. (b) Representative images of tumorsphere formation assay for shRhoj and Rhoj overexpression (Rhoj-oe) of U251 cells (500 cells/well) and the quantification of tumorsphere number after 9 days culture; scale bar, 50 μm . (c, d) 1.5 million shRhoj or control-shRNA (shNC) expressing U87 cells were inoculated subcutaneously to BALB/c nude mice under their armpits. Tumor sizes were measured every 4–5 days ($n=6$). At 26 days after injection, the mice and tumors were photographed and preserved for IHC (immunohistochemistry) and western blot. (e) Representative images of control- or Rhoj shRNA-expressing xenografts for H&E staining and IHC analysis of the proliferation marker Ki-67. Scale bar, 500 μm . (f) Western blotting analysis of Rhoj, p-cofilin (Ser3), total cofilin, expression in xenografts extracts. Data represent means \pm SEM analyzed by a two-tailed Student *t* test of three independent experiments. * $p < 0.05$, ** $p < 0.01$

suppressed EMT and lead to the changed cell morphology and increased cytoskeletal dynamics.

The Transcription Factor c-Jun Directly Regulated Rhoj Expression

To further investigate the upstream molecular mechanism that caused the upregulation of Rhoj in GBM and lead to its tumorigenesis and cell migration, we analyzed the promoter of Rhoj through JASPAR and ALGGEN-PROMO databases. We found that there are three conserved binding sites of transcription factor c-Jun in the upstream of the Rhoj transcription initiation site (Fig. 6a). To examine whether Rhoj transcription was regulated by the transcription factor c-Jun in GBM, we performed luciferase reporter assay using luciferase reporter vectors containing WT or mutant Rhoj promoters. We first confirmed the Rhoj promoter activity by transfecting the pGL3 basic and pGL3 vector containing the Rhoj promoter in 293T cells (Fig. 6b). In c-Jun overexpressing cells, Rhoj promoter-luciferase activity augmented compared with vector control. Accordingly, mutated c-Jun binding sites in Rhoj promoters abolished the luciferase activity (Fig. 6c). In line with these findings, we treated glioma cells with a c-Jun small molecular inhibitor, SP600125, the endogenous Rhoj expression at both the mRNA and protein levels was drastically suppressed (Fig. 6d, e), and the downregulated Rhoj expression was also confirmed by shRNA targeted JNK which belongs to the MAPK pathway and is the upstream regulator of c-Jun (Fig. 6f). All these findings indicated that c-Jun is a direct target transcription factor of Rhoj.

Rhoj Interacts with Moesin To Regulate Cell Proliferation and Migration by Rac1-PAK Pathway

To further explore Rhoj-mediated molecular mechanisms in the regulation of cell proliferation and migration in GBM

cells. We firstly constructed the flag-tagged Rhoj vectors for co-IP assay, after Coomassie blue staining, specific protein bands were collected for mass spectrometry (MS), and a total of 147 candidate proteins were identified after removing the common false-positive hits. The results showed that actin-based cytoskeleton protein membrane-organizing extension spike protein (moesin, MSN) was the most likely cargo protein for Rhoj in GBM cells. MSN was also highly expressed in malignant GBM and is a direct target of miR-200c to regulate several types of glioma tumor cell proliferation and migration [14]. We confirmed that ERM family protein MSN could directly interact with Rhoj by an endogenous reciprocal co-IP assay (Fig. 7a). We also found that subcellular colocalization of Rhoj and moesin in U87 cells (Fig. 7b). Our study showed Rhoj could interact with moesin to activate its downstream signaling pathway to regulate glioma progression.

Given that Rhoj can modulate the Rac1-PAK pathway in regulating actin dynamic and cell migration. Immunoblotting results showed silencing of Rhoj caused a striking decrease in p-PAK4, p-PAK2, p-cofilin, and Rac1. However, the overexpression of Rhoj caused the activation of these proteins (Fig. 7c–e). To further characterize whether Rhoj promotes Rac1 activation, we applied siRNA targeted Rhoj or Rac1 and found that interruption the expression of Rhoj can inhibit Rac1 protein level, but only depletion of Rac1 cannot change the Rhoj expression, indicating that Rac1 was a targeted downstream molecule of Rhoj (Fig. 7f, g). Taken together, our data indicate that transcription factor c-Jun regulates the expression of Rhoj to promote GBM cell proliferation and migration of PAK-Rac1 pathway and cytoskeletal dynamics (Fig. 7h).

Discussion

Rho GTPases as key regulators of cytoskeletal rearrangement are important in tumor progression and metastasis [15], and in recent years, a growing number of studies focused on the GTP-binding proteins. Rho signaling members can have crosstalk with other oncogenic pathways such as Ras-MAPK [16] and Yes-associated protein (YAP) [8], to participate in the EMT process and cancer progression. In this study, we firstly demonstrated that Rhoj directly regulated by transcription factor c-Jun could interact with moesin to modulate the Rac1-PAK-cofilin pathway to suppress actin dynamics and promote an EMT-like process, therefore expediting glioma cell progression.

Compared with the endothelial HUVECs, Rhoj expressed more prevalingly in glioma cells. With the correlation analysis of Rhoj expression and overall survival of GBM and LGG patients, we can declare that Rhoj is a negative prognostic factor, and it makes far-reaching sense

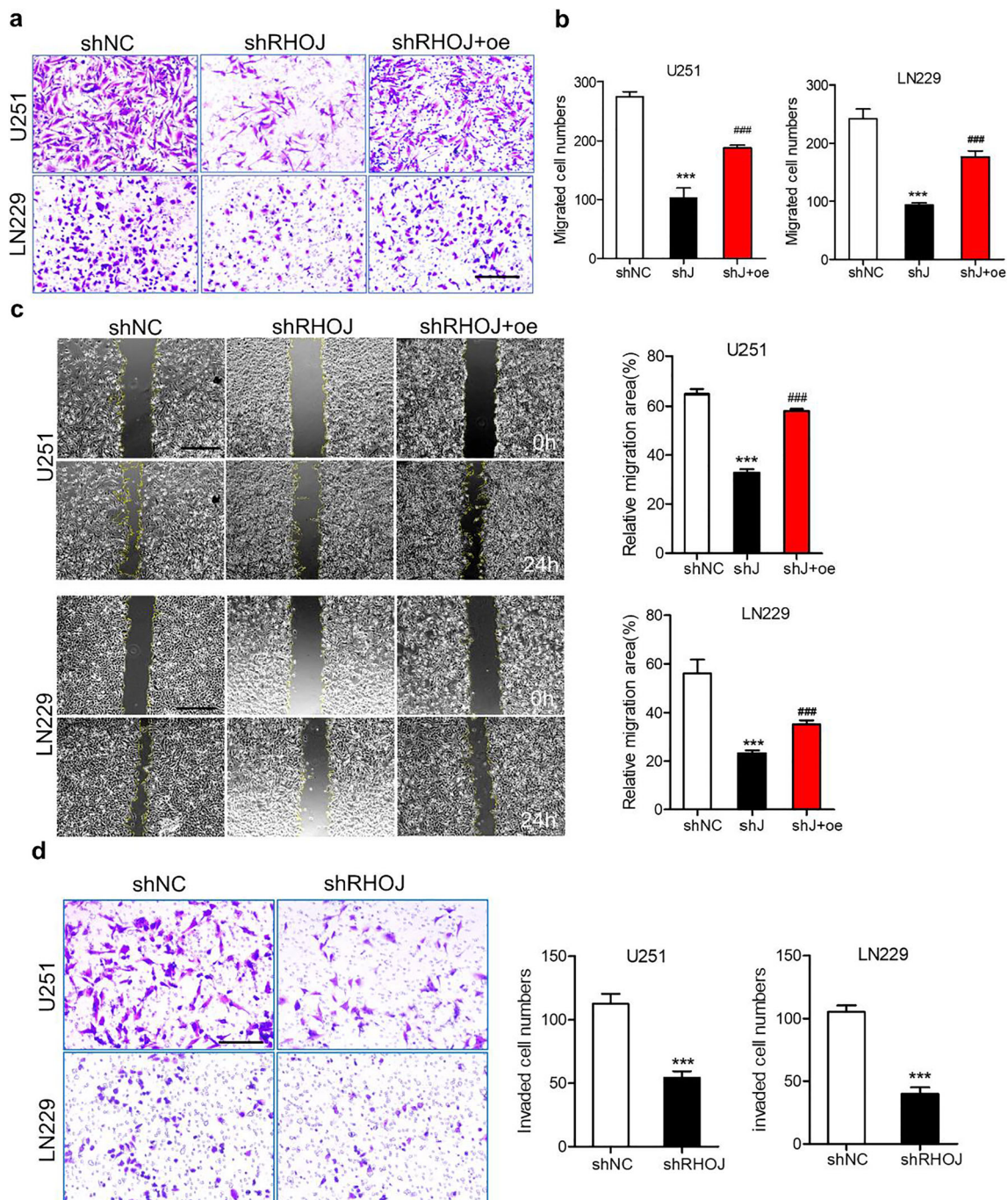


Fig. 4 Silencing Rhoj inhibits cell migration and invasion of GBM cells. (a) Representative images and (b) graphical representation of transwell migration assay of GBM cells expressing control or Rhoj shRNAs or shRhoj + Rhoj overexpression plasmid. Scale bar, 50 μ m. (c) The representative images of wound healing assay with GBM cells and relative migration area were counting statistics. (d) Representative

images of transwell invasion assay and the number of invaded cells counting statistics. Three independent experiments were performed for the *in vitro* assay, including cell migration and invasion assay and the wound healing assay, respectively. Data were expressed as the mean \pm SEM of three experiments. *** $p < 0.001$

to research the molecular mechanism of Rhoj for the targeted therapy for glioma patients. Through a series of

cell experiments, we drew the link between Rhoj expression and glioma cell proliferation, migration, and invasion

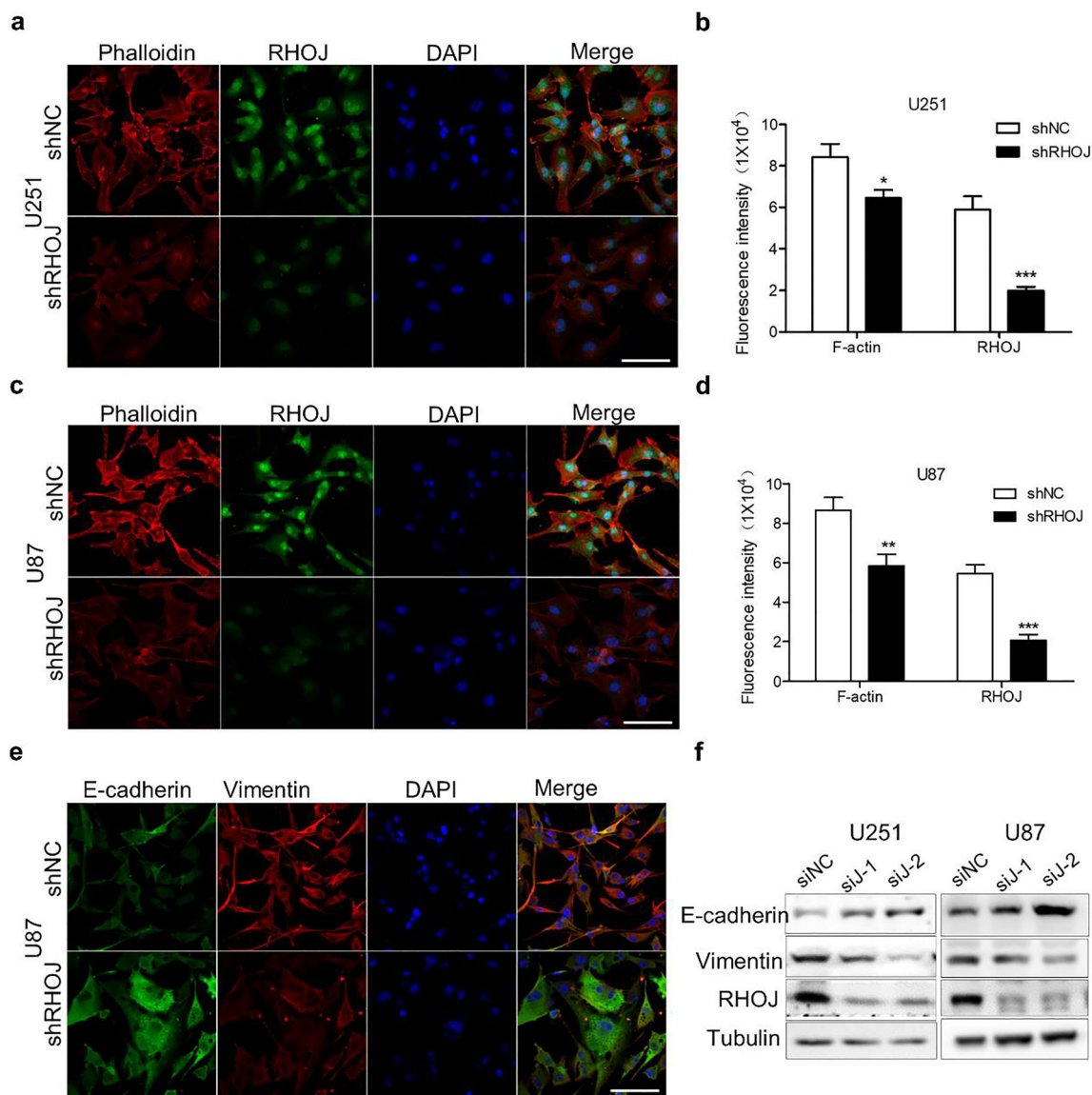


Fig. 5 Rhoj expression was associated with cellular morphology and the EMT phenotype of GBM cells. Representative confocal micrograph of (a) U251 and (c) and U87 GBM cells showing Rhoj (green), rhodamine-conjugated phalloidin-labeled F-actin (red), DAPI (blue), and merge staining. (b, d) Quantitative analysis of fluorescence intensity of Rhoj and F-actin in the panel. Scale bar, 200 μm . (e) Representative

immunofluorescence staining of E-cadherin (green), vimentin (red), and DAPI (blue) in U87 GBM cells. Scale bar, 50 μm . (f) Representative immunoblots to detect the expression of Rhoj and the EMT markers E-cadherin and vimentin. Data are expressed as means \pm SEM, and a two-tailed Student *t* test analyzed the *p* value. **p* < 0.05, ***p* < 0.01

and found that Rhoj expression blocks cytoskeletal dynamics and promotes an EMT-like process. However, it is unclear what caused the overexpression of Rhoj in glioma and which key pathways are influenced by Rhoj to promote glioma progression. Previous studies showed that Rhoj is a direct downstream of ETS transcription factor family ERG and associated with endothelial cells (ECs) lumen formation [17]. In our research, we initially found that oncogene c-Jun contributes to the upregulation of

Rhoj. c-Jun as a member of transcription factor AP-1 family (c-Jun, Fra-1, Jun-D, c-Fos, and Jun-B) was activated in GBM cells mediated by IL-13/IL-13R α 2 axis and served as a putative immunotherapy target of GBM [18]. According to the previous study, Rhoj targets Rac1 and activates Pak2 and Pak4 to influence lumen formation and sprouting of the cell. Our data confirmed that Rhoj played a critical role in the regulation of the PAK-Rac1-cofilin pathway. Cofilin was also known as an actin-

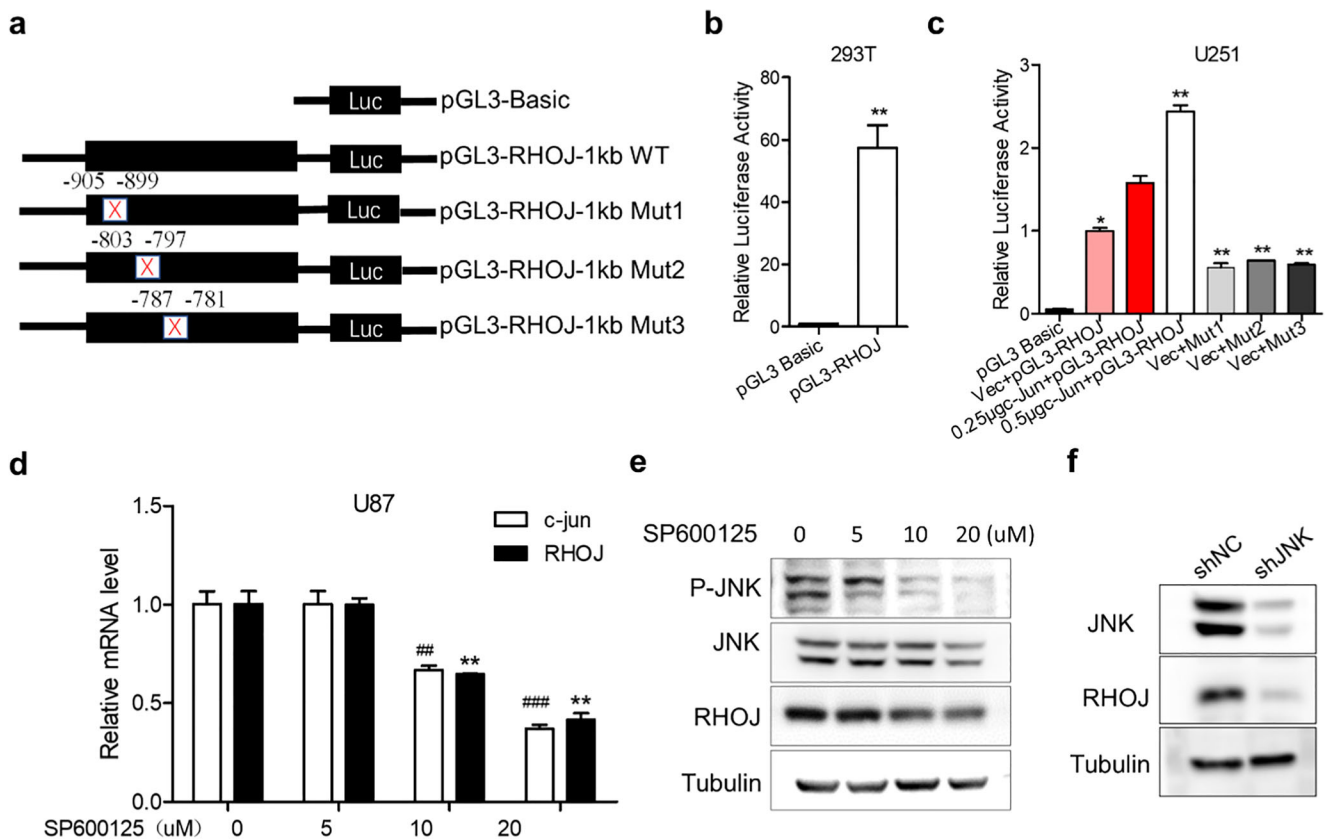


Fig. 6 c-Jun-regulated Rhoj expression. (a) Predicted transcription factor c-Jun binding site in the upstream region of Rhoj between -1 kb and 0 kb. WT contained c-Jun binding sites. Mutations in putative binding sequences are listed as Mut1, Mut2, and Mut3. (b) Luciferase assay established using HEK-293T cells transfected with PGL3 basic or pGL3-Rhoj (pGL3-plasmid containing the Rhoj DNA promoter region [$-1000/0$]), Luciferase activities were normalized to Renilla luciferase activities. (c) The dual-luciferase assay. pGL3 Basic, pGL3B-Rhoj, or the

mutant plasmids Mut1, 2, or 3 were used to transfect c-Jun-overexpressing or empty vector control U251 cells. Luciferase activities were normalized to Renilla luciferase activities. (d, e) The Jun inhibitor SP600125 suppressed Rhoj mRNA (d) and protein (e) expression in U87 cells and showed a significant dose dependence. (f) The downregulated Rhoj expression was confirmed by shRNA targeted JNK. Results represent means \pm SEM analyzed by a two-tailed Student *t* test of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

depolymerizing factor, and its dephosphorylated form (active cofilin) is essential for the actin cytoskeleton dynamic [19] and EMT-like process.

Because preceding studies have shown the role of Rhoj in modulating cytoskeleton proteins and how it is attributed to tumor progression, we hypothesized that the protein interactions might be the cause of the oncogenic role. We found the Rhoj interacting protein moesin by mass spectrometry and verified by co-IP assay combined with immunofluorescence staining. Rhoj was recruited to the membrane and transformed into an activation form [20]. In recent studies, Rhoj was reported to interact with glutamine synthetase palmitoylates and sustain Rhoj palmitoylation and activation form located at the membrane [21]. Moreover,

the N terminus (N) was important for Rhoj plasma membrane localization and nucleotide exchange [22]. The MSN protein interacts with the membrane receptor by the N-terminal FERM domain and binds with F-actin at the C-terminal cytoskeleton interacting domain [23, 24]. However, with regard to how they bind with each other and the covalent binding domain, further research is needed.

Angiogenesis is a critical process in the progression of glioma. Rhoj, which is highly expressed in endothelial cells, has been reported to rank seventh in the up-regulated angiogenesis-related common genes among several profiles [25], and it has been found to be expressed in vessels during tumor growth; mutual inhibition of Rhoj and VEGF has a better anti-tumor effect

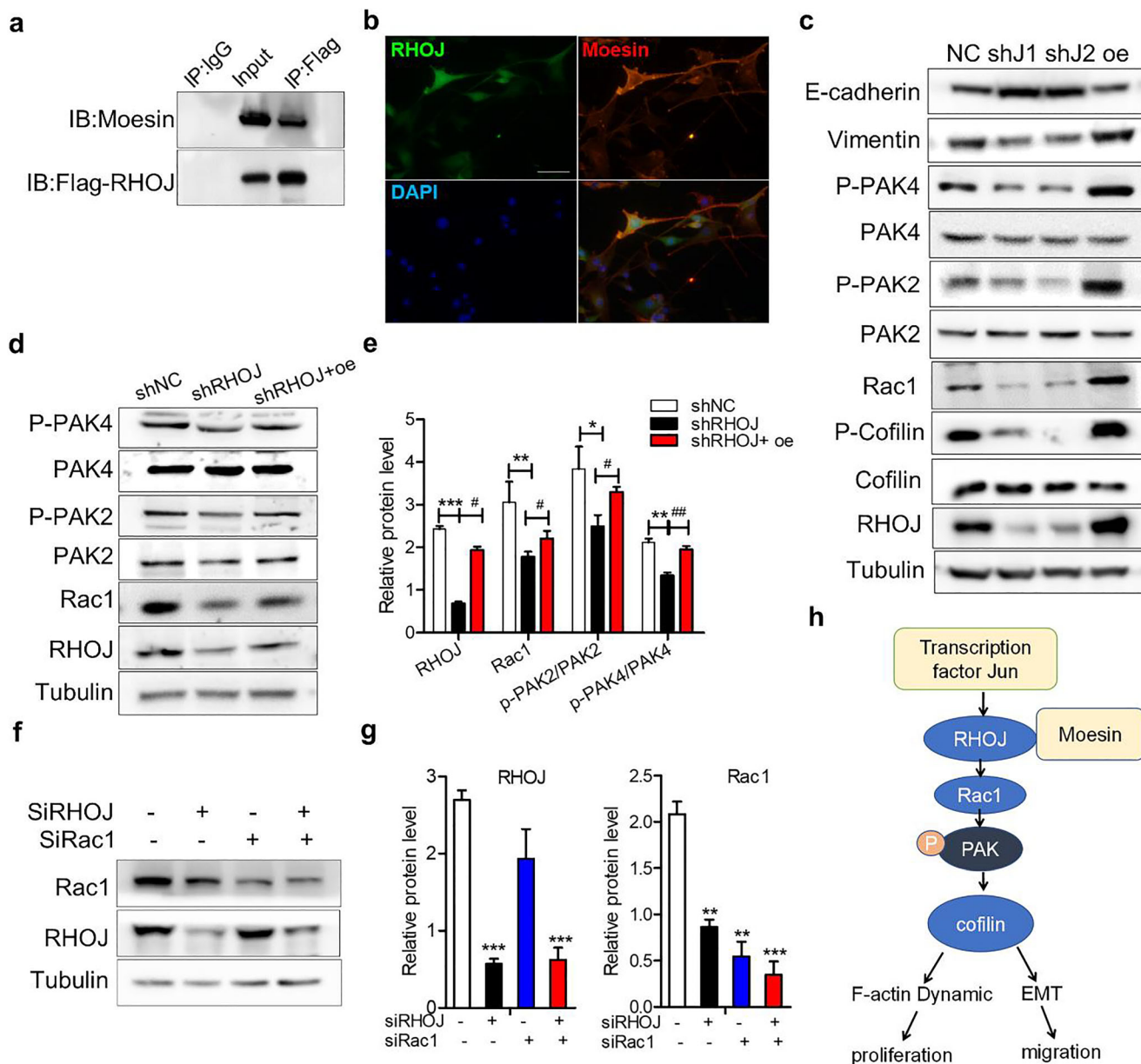


Fig. 7 Rhoj interacts with ERM family protein moesin to drive its downstream Rac1-PAK-cofilin exchange, cell migration, and proliferation. (a) Western blotting assay was used to further validate the interaction between Rhoj and moesin followed by co-IP. (b) Representative fluorescence images showed the colocalization of Rhoj and moesin in U87 cells. Scale bar, 50 μ m. (c) Negative control (NC), Rhoj shRNA, and stable overexpression cells were subjected to immunoblotting and with antibodies that recognize downstream proteins. (d) Immunoblotting assays for PAK2/4 activity, Rac1, and Rhoj expression were prepared in U251 cells expressing negative

control or Rhoj shRNA, or shRhoj and Rhoj overexpression plasmids. Tubulin was used as a loading control. (e) Quantification of the panels. (f) U251 cells were transfected with Rhoj or Rac1 siRNA for 72 h. Cell lysates were analyzed for Rhoj and Rac1 by immunoblotting. (g) Relative quantification of Rhoj and Rac1 of each group is shown, this experiment had been independently repeated three times. (h) Diagram depicting upstream and downstream signals of Rhoj that might be contributing to GBM cell migration and proliferation. Data represent means \pm SEM analyzed by a two-tailed Student *t* test of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

[11]. In the xenograft mouse model, we also found that Rhoj knockdown may inhibit angiogenesis. This

indicates that Rhoj may be a therapeutic target in the anti-angiogenesis therapy of glioma.

Taken together, our findings demonstrate Rhoj as a new player in GBM, and we understand how Rhoj is involved in tumor progression. Further development of Rhoj-specific inhibitors is needed to validate their therapeutic efficacy and safety.

Acknowledgments This study was supported by grants from the National Natural Science Foundation of China, National High-Level Talent Youth Program of China, Jiangsu Province Education Department Grant, Jiangsu Province “Innovative and Entrepreneurial Team” and “Innovative and Entrepreneurial Talent” Grant, Southeast University-Nanjing Medical University Cooperative Research Project, and the China Scholarship Council (201906090247).

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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