

# Forkhead box P3 regulates ARHGAP15 expression and affects migration of glioma cells through the Rac1 signaling pathway

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## Key words

ARHGAP15, epithelial–mesenchymal transition, FOXP3, glioma, Rac1 activation

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Forkhead box P3 (FOXP3), a transcriptional activator and repressor, is a member of the forkhead winged helix family of transcription factors.<sup>(1)</sup> It also is related to several tumor types, such as gastrointestinal cancers, melanomas, non-small-cell lung cancer cells, breast cancer cells, prostate cancer, and leukemias.<sup>(2–8)</sup> Forkhead box P3 suppresses cell proliferation, migration, and invasion in epithelial breast cancer,<sup>(9–11)</sup> ovarian cancer,<sup>(12)</sup> and melanoma<sup>(13)</sup> and was shown to have antimetastatic function in a colon cancer mouse model.<sup>(14)</sup> Forkhead box P3 is significantly reduced or absent in glioblastomas (GB), and has been shown to affect the migration of corresponding GB stem-like cells growing in culture as neurospheres.<sup>(15)</sup> However, the mechanism involved in FOXP3 suppression of glioma cell migration is not fully understood.

Malignant gliomas, the most common primary intracranial tumor, is very aggressive and has a poor prognosis.<sup>(16)</sup> The

Forkhead box P3 (FOXP3) plays a crucial role in the development and function of regulatory T cells and was recently identified as a tumor suppressor in different cancer types. Forkhead box P3 is expressed in normal brain tissues, but is strongly downregulated or absent in glioblastomas. In order to understand the FOXP3 adjustment mechanisms in glioma cells, we performed a DNA microarray in U87 cells overexpressing FOXP3 and validated the differences using quantitative real-time PCR, Western blot analysis, and immunohistochemistry *in vitro* and *in vivo*. We found that FOXP3 can regulate the expression of ARHGAP15. Expression of FOXP3 was also correlated with ARHGAP15 in glioma samples. Overexpression of FOXP3 inhibited glioma cell migration through ARHGAP15 upregulation and Rac1 inactivation. Silencing of FOXP3 promoted migration through ARHGAP15 downregulation and Rac1 activation. ARHGAP15, a GTPase-activating protein for Rac1, inhibits small GTPase signaling in a dual negative manner. We found that there is a correlation between expression of ARHGAP15 and glioma level. The small GTPase Rac1 plays an important role in cell migration. In addition, we found that FOXP3 regulates expression of epithelial–mesenchymal transition markers E-cadherin and N-cadherin, which is important given that epithelial–mesenchymal transition is critically involved in tumor spreading and dissemination. Thus, FOXP3 or ARHGAP15 may serve as a new molecular target for antimetastatic therapies in treating glioma.

invasive phenotype is a critical factor in a variety of ways, for instance, in the clinical progression of malignant glioma, complicating complete surgical resection, and permitting tumor regrowth and further invasion of surviving tumor cells.<sup>(17)</sup>

Cell migration is necessary for tumor metastasis. Activation of the Rho family of small GTPases included RhoA and Rac1 holds an important position in the initial steps of the metastatic cascade as well as plays a significant role in actin cytoskeletal rearrangement and cell migration.<sup>(18)</sup> The Rho family of GTPases can transform between an active GTP-bound state and an inactive GDP-bound state through inherent GTPase activity.

A series of proteins tightly regulate GTPase activation. These proteins include guanine nucleotide exchange factors, GTPase activating proteins (GAPs), and guanine nucleotide

disassociation inhibitors.<sup>(19–22)</sup> GTPase activating proteins can catalyze hydrolysis of GTP to the GDP-bound form.

The GAP ARHGAP15 has activity specific to Rac1. It includes an N-terminal pleckstrin homology domain and a C-terminal GAP domain. It is analogous to ARHGAP9 and ARHGAP12 in terms of structure and phylogenetics.<sup>(23)</sup> ARHGAP15 cannot only inhibit small GTPase signaling by targeting the GTPase itself, but also interacts with its effector, Pak kinase.<sup>(24)</sup>

Epithelial–mesenchymal transition (EMT) is an essential mechanism regulating the initial steps of metastatic progression.<sup>(25)</sup> In the process of EMT, epithelial E-cadherin expression is decreased, while expression of mesenchymal cadherins, including N-cadherin and cadherin-11, is enhanced.<sup>(26)</sup> Epithelial–mesenchymal transition can cause drug resistance by acquiring stemness in the cancer microenvironment.<sup>(27)</sup> It has been reported that E-cadherin expression is low or absent in gliomas and normal brain tissue, although N-cadherin expression is generally increased. Cadherin subtypes have been reported in various glioma grades.<sup>(28–31)</sup>

In this study, we found that FOXP3 affected glioma cell migration by regulating ARHGAP15 and the Rac1 signaling pathway, which is associated with EMT.

## Materials and Methods

**Plasmid vectors.** All plasmids were obtained from OriGene Technologies (Rockville, MD, USA). Plasmid vectors pRFP-C-RS shFOXP3 or pRS shARHGAP15 contained a specific shRNA sequence according to the manufacturer's recommendations. Four different FOXP3- or ARHGAP15-specific sequences were screened, and the most efficient sequences were selected. The pRFP-C-RS or pRS encoding non-specific shRNA were used as negative controls.

Expression vectors pCMV6-FOXP3-GFP, which contains a FOXP3 variant 1 sequence, and pCMV6-ARHGAP15 were used for overexpression; pCMV6-GFP or pCMV6-Entry were used as negative controls.

**Tumor specimens and cell culture.** Glioma specimens, including low-grade and high-grade gliomas, were obtained from the Department of Neurosurgery, Tianjin Huanhu Hospital (Tianjin, China) after the informed consent of the patients was obtained. Glioma specimens were frozen and/or placed in a saline solution after surgery. Glioma cell lines U251 and U87 were from Peking Union Medical College Cell Resource Center (Beijing, China).<sup>(32)</sup> Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. All reagents were from Invitrogen (Carlsbad, CA, USA). Cells were transfected with plasmid vectors mediated by Lipofectamine 2000 (Invitrogen).

**Human genome-wide expression profiling.** U87 glioma cells overexpressing FOXP3 and its control were extracted after 48 h of transfection. The Agilent Whole Human Genome Oligo Microarray (4X44K Shanghai, China) was used for the whole genome array assay. Microarray hybridization was carried out at CapitalBio (Beijing, China). Data analysis was undertaken using Significance Analysis of Microarray software (SAM 3.0; Stanford University, Stanford, CA, USA; <http://statistics.stanford.edu>). The original data were uploaded to NCBI's Gene Expression Omnibus (GSE89456; GSM2372590; GSM2372591; <https://www.ncbi.nlm.nih.gov/geo/>).

**Transwell migration and invasion assays.** A 24-well Boyden chamber with an 8-µm pore size polycarbonate membrane (BD Biosciences, BD Biocoat, USA) was used to test glioma cell

invasion and migration. Matrigel (BD Biosciences, USA) covered the membrane to form a matrix barrier in the invasion assay. At 48 h after transfection,  $5 \times 10^4$  cells were suspended in serum-free medium and seeded in the upper chamber. Fetal bovine serum (10%) was used as a chemoattractant. After 24 h, cells on the upper side were removed with a cotton swab, and cells that migrated through the membrane to the underside were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells were counted in four different fields with a microscope. All experiments were carried out in triplicate.

**Detection of active Rac1.** Glioma tissues were obtained by dissolution using a protein extract reagent according to the manufacturer's directions (Biotek Corporation, Beijing, China). Transfected cells were washed with ice-cold PBS buffer and lysed with lysis buffer (50 mM Tris [pH 7.6], 500 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 1% Triton, 0.5% deoxycholate [DOC], 0.1% SDS, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM PMSF, and 0.5 mM sodium vanadate). Lysates were centrifuged at 16 000 g at 4°C for 15 min. GST-PAK1-PBD fusion protein used to bind the activated form of GTP-bound Rac1 was added to cell lysates and incubated at 4°C for 1 h. Bound proteins were obtained by centrifugation and washed three times with lysis buffer. Glutathione resin-bound GTPase was eluted with SDS buffer and analyzed by Western blot. Total Rac1 was detected in corresponding cell lysates. All reagents were obtained from Cell Signaling Technology (Massachusetts, USA).

**Quantitative real-time PCR.** TRIzol (Invitrogen) was used to extract total RNA from transfected cells according to the manufacturer's instructions. Complementary DNA was obtained using the cDNA Reverse Transcription Kit (Invitrogen). Real-time PCR was carried out using Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, USA) on an ABI 7500 series PCR machine (Applied Biosystems); GAPDH was used as an endogenous control. The primers designed for quantitative real-time RT-PCR analysis were as follows: FOXP3, 5'-CA CAACATGCGACCCCTTTCACC-3' (forward) and 5'-AGGT TGTGGCGGATGGCGTTCTTC-3' (reverse); and ARHGAP15, 5'-CGGGATCCATGCAGAAATCTACAAAATC-3' (forward) and 5'-TCCCCCGGGCATCAAGACAGATGTG-3' (reverse).

**Antibodies and Western blot analysis.** Cells were lysed in RIPA lysis buffer on ice. Total proteins were separated using SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% skim milk in TBST buffer for 2 h. Membranes were then incubated with primary antibodies as follows: anti-FOXP3 (mouse mAb, 1:250; eBioscience, San Diego, CA, USA), anti-ARHGAP15 (1:1000; Proteintech, Chicago, USA), anti-GAPDH (1:10 000; Proteintech, Chicago, USA), anti-Rac1 (mouse mAb, 1:1000; Cell Signaling Technology), anti-N-cadherin, and anti-E-cadherin (mouse mAb, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight on a rocking platform. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:1000; Santa Cruz Biotechnology) for 1 h at room temperature. Relative intensity of protein bands was determined by densitometric analysis using Quantity One software (Bio-Rad, California, USA).

**Immunohistochemistry.** Glioma tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 15 min. After antigen retrieval, sections were incubated with 5% serum to avoid non-specific binding. The ARHGAP15 (1:200) and FOXP3

(1:100) antibodies were added to the sections and incubated at 4°C overnight. The sections were treated with secondary antibodies, followed by incubation with streptavidin–HRP complex (Santa Cruz Biotechnology). Immunoreactivity was visualized with diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). The sections were counterstained with hematoxylin. The stained slides were scored independently by two pathologists blinded to clinical data. The proportion of positive tumor cells was scored as follows: 0, ≤10% positive tumor cells; 1, 11–24% positive tumor cells; 2, 25–50% positive tumor cells; 3, 51–75% positive tumor cells; and 4, >75% positive tumor cells. Staining intensity was graded according to the following criteria: 1, absent or weak staining; 2, moderate staining; and 3, strong staining. Staining index was calculated as the product of the proportion of positive tumor cells and staining intensity score. The cut-off value for distinguishing positive and negative FOXP3 and ARHGAP15 expression was set as a staining index of 3.

**Tumor xenograft model in nude mice.** The U87 cells were transfected with treated vector. Transfected cells ( $3 \times 10^6$ ) were suspended by 100  $\mu$ L serum-free RPMI-1640 culture medium and were s.c. injected into 6-week-old nude mice in the flank. The *in vivo* experiment was divided into five groups with 10 nude mice in each group. All mice were killed 3 weeks after implantation. The tumors were isolated from the mice and stored at  $-80^\circ\text{C}$ .

All animal experiments were approved by the Committee on the Use of Live Animals for Teaching and Research and conducted in accordance with the Animal Care and Use Committee guidelines of Kagoshima University (Kagoshima, Japan).

**Statistical analysis.** All data are presented as the mean  $\pm$  SD and were analyzed using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA). Statistical analyses were undertaken using one-way ANOVA and Student's *t*-test. Experiments were carried out independently three times.  $P < 0.05$  was considered statistically significant.

## Results

**ARHGAP15 expression significantly regulated by FOXP3 in glioma cells *in vitro* and *in vivo*.** We downregulated or upregulated FOXP3 expression in both U87 and U251 cells using pRFP-C-RS shFOXP3 or pCMV6-FOXP3-GFP, respectively (Fig. 1a,b). In order to find the mechanism involved in FOXP3 suppression of glioma cell migration, we analyzed a DNA microarray. A heatmap of the DNA microarray showed the molecular profiles of FOXP3-overexpressing U87 cells and its control (Fig. 1c). We found that AGAP7P, ARHGAP15, and CYSLTR1 were connected to cell migration,<sup>(24,33)</sup> but AGAP7P and CYSLTR1 had no significant change (Fig. 1d). Therefore, we focused on ARHGAP15. As one replicate DNA microarray was carried out, we used quantitative real-time PCR, Western blot analysis, and *in vivo* experiments to test whether ARHGAP15 is induced by FOXP3 in glioma cells. Expression of ARHGAP15 increased dramatically at 48 h after FOXP3 transfection in U87 and U251 cells. Knockdown of FOXP3 also inhibited ARHGAP15 expression in U87 and U251 cells (Fig. 1e). To test the relevance of FOXP3 regulating ARHGAP15, a nude mouse tumor model was generated by endermic injection of treated glioma cells. Representative immunohistochemistry (IHC) images showing the change of FOXP3 and ARHGAP15 expression in tissues isolated from nude mice. The results showed that FOXP3 significantly regulates ARHGAP15 expression *in vivo* (Fig. 1f).

**ARHGAP15 expression is reduced in high-grade glioma tumors and correlated with FOXP3.** In order to understand the clinic relevance of FOXP3 and ARHGAP15, we carried out IHC assessments of ARHGAP15 and FOXP3 expression in glioma tissue samples from 178 patients. It showed that FOXP3 protein was highly expressed in 40 of 94 low-grade gliomas (42.6%) and in 21 of 84 high grade tumors (25.0%) (Fig. 2a, Table 1). The difference between two groups was statistically significant ( $P = 0.01$ , Table 1). ARHGAP15 protein was highly expressed in 61 of 94 low-grade gliomas (64.9%) and in 40 of 84 high grade tumors (47.6%) (Fig. 2b, Table 1). The difference between two groups was statistically significant ( $P = 0.02$ ; Table 1). Low FOXP3 and ARHGAP15 expression correlated with increase in tumor grade. Neither FOXP3 nor ARHGAP15 expression was significantly correlated with patient age, sex, or tumor size ( $P > 0.05$  each; Table 1).

We explored the relationship between FOXP3 and ARHGAP15 in 178 glioma patients. It showed that FOXP3 expression was positively correlated with ARHGAP15 in human glioma tissue samples ( $P < 0.05$ ; Table 2).

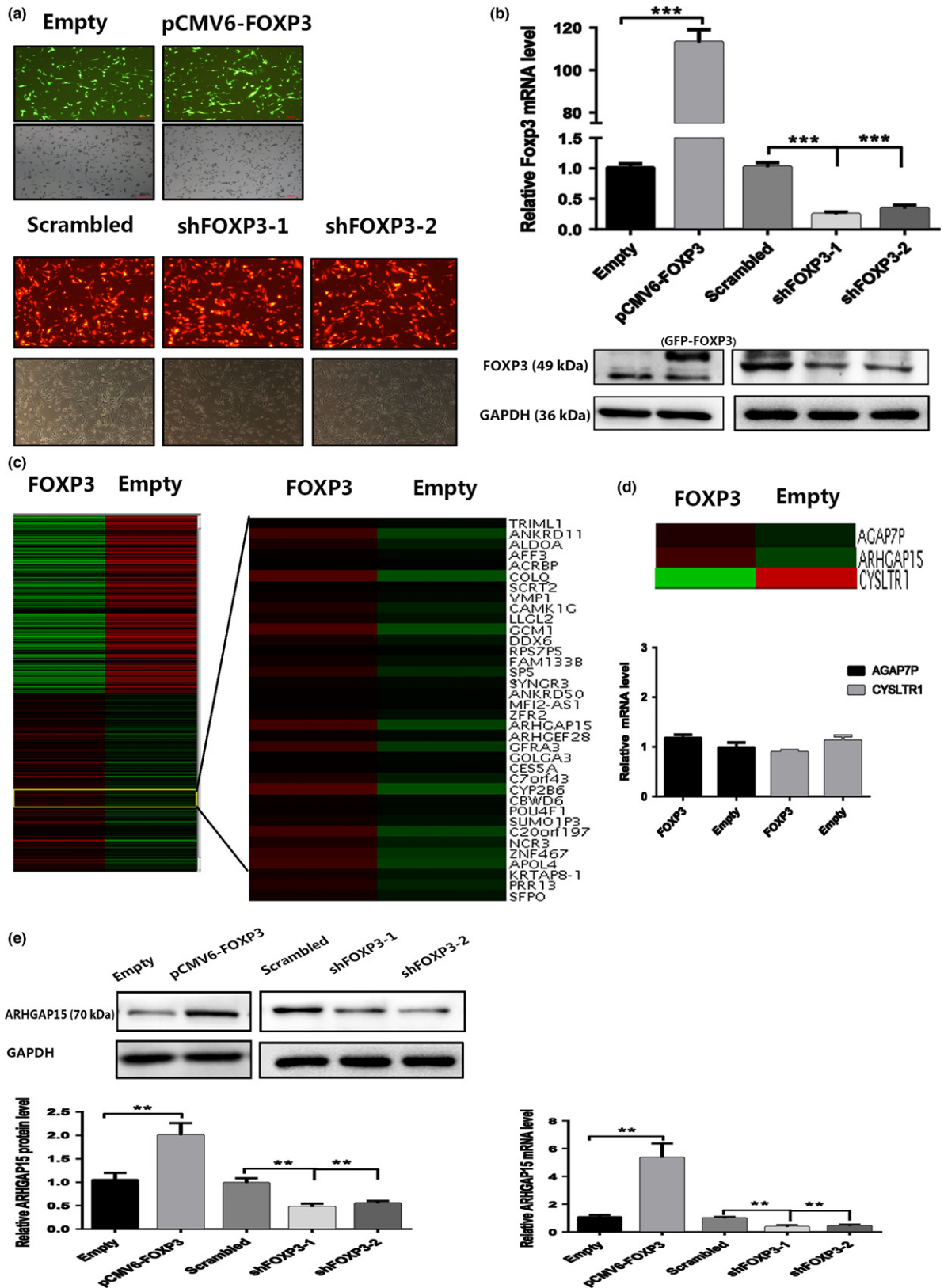
**Forkhead box P3 can regulate EMT in glioma cells and inhibit glioma cell migration and invasion.** E-cadherin and N-cadherin are key molecular markers in EMT and are both associated with an aggressive brain tumor phenotype.<sup>[30,31]</sup> Inhibition of FOXP3 enhanced migration and invasion, whereas FOXP3 overexpression attenuated invasion and migration (Fig. 3a,b). Stable knockdown of FOXP3 in U87 and U251 cells decreased E-cadherin levels, but increased N-cadherin levels. It was clear that FOXP3 increased E-cadherin levels, but did not change N-cadherin levels (Fig. 3c,d).

**ARHGAP15 results in Rac1 inactivation and regulates migration and invasion of glioma cells.** To further study the biological roles of ARHGAP15 in glioma, the effects of ARHGAP15 overexpression and knockdown were assessed. Plasmid efficiency was examined using Western blot and data quantification (Fig. 4a). Overexpression of ARHGAP15 inhibited both migration and invasion in the two cell lines evaluated. In contrast, decreasing ARHGAP15 levels promoted migration and invasion (Fig. 4b). We investigated the influence of ARHGAP15 on Rac1 and found that ARHGAP15 acts as a GAP for Rac1, resulting in Rac1 inactivation in U87 cells (Fig. 4c, d).

**ARHGAP15 knockdown counteracts inhibition of aggressive phenotype induced by FOXP3.** Our observations indicate that FOXP3 and ARHGAP15 have similar effects on aggressive phenotypes in glioma cells and that FOXP3 regulates ARHGAP15 expression. Based on these results, we undertook a rescue assay to assess whether the effects of FOXP3 on glioma cells are mediated by ARHGAP15 expression. FOXP3 was cotransfected with the shARHGAP15 plasmid, and knockdown of ARHGAP15 was confirmed to rescue the increase in ARHGAP15 protein levels caused by FOXP3 (Fig. 5a). shFOXP3 was cotransfected with the ARHGAP15 plasmid, and ARHGAP15 overexpression was confirmed to rescue the decrease in ARHGAP15 protein levels caused by shFOXP3 (Fig. 5b). As expected, restoration of ARHGAP15 expression mostly blocked the inhibitory influence of FOXP3 on migration and invasion (Fig. 5c).

**Forkhead box P3 affects the Rac1 signaling pathway mediated by ARHGAP15.** We found that ARHGAP15 contributes to malignancy in gliomas. ARHGAP15 acts as a GAP for Rac1, and is involved in the Rac1 signaling pathway. ARHGAP15 overexpression inhibited the Rac1 signaling pathway, and ARHGAP15 knockdown promoted the Rac1 signaling





**Fig. 1.** Forkhead box P3 (FOXP3) significantly regulates the expression of ARHGAP15 in glioma cells. Fluorescence expression (a) and quantitative RT-PCR and Western blot analysis (b) of FOXP3 levels in U87 cells 48 h after transfection with pCMV6-FOXP3-GFP or pRFP-C-RS shFOXP3. GAPDH was used as the internal control to normalize the levels of FOXP3. (c) DNA microarray showing the molecular profiles of U87 cells overexpressing FOXP3. (d) Relative of expression of AGAP7P and CYSLTR1. (e) Western blot assays and quantitative RT-PCR analysis were used to detect the ARHGAP15 protein level in U87 cells transfected with pCMV6-FOXP3-GFP or pRFP-C-RS shFOXP3, and the quantification of the bars are shown. (f) Representative immunohistochemical images showing the change of FOXP3 and ARHGAP15 expression in tissues isolated from nude mice.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ . All error bars indicate mean  $\pm$  SD. All experiments were repeated at least three times.

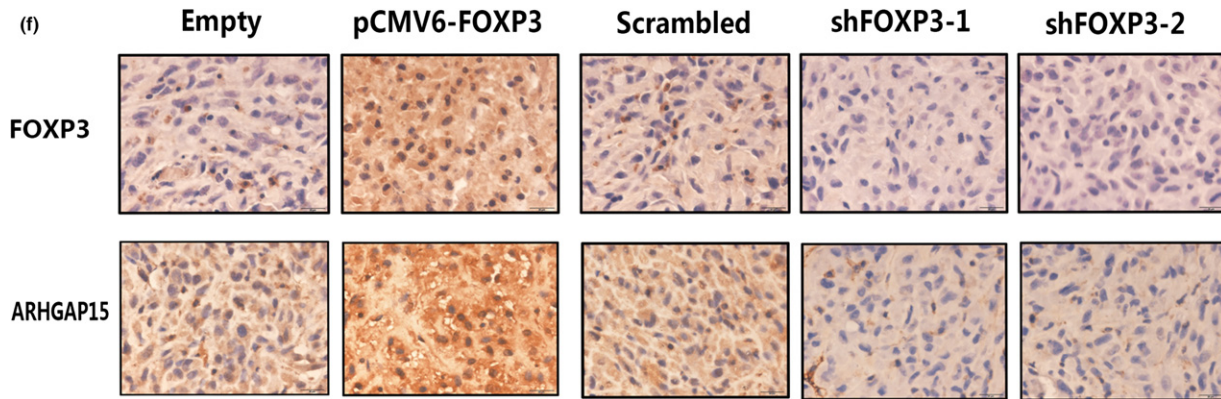
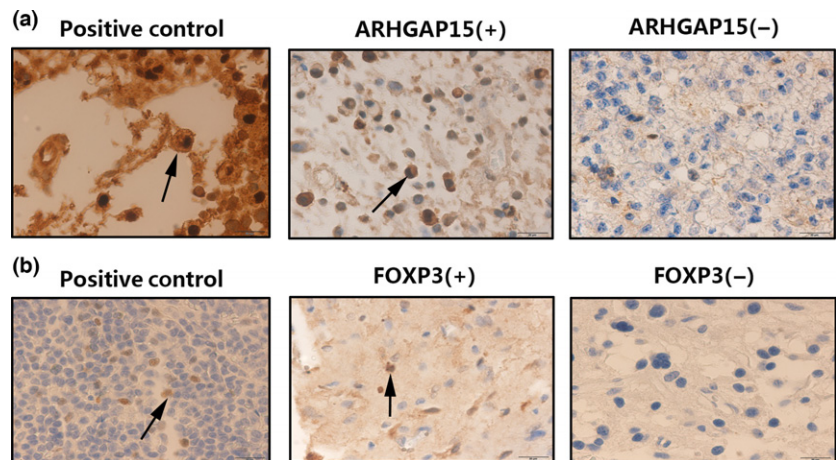


Fig. 1. continued.

**Fig. 2.** Forkhead box P3 (FOXP3) and ARHGAP15 expression in human glioma samples. (a) Left, ARHGAP15 staining as positive control is detected in lymphoma morphology (arrow, brown). Center, one glioblastoma (GB) with ARHGAP15+ cells (arrow, brown). Right, GB completely negative for ARHGAP15+ reactivity. (b) Left, FOXP3 staining as positive control is detected in lymphocyte morphology (small and round nucleus; arrow, brown). Center, one GB with FOXP3+ cells (round nucleus; arrow, brown). Right, one GB completely negative for FOXP3 reactivity. Magnification,  $\times 40$ .



**Table 1.** Correlations between forkhead box P3 (FOXP3) and ARHGAP15 expression and clinicopathological features in glioma patients

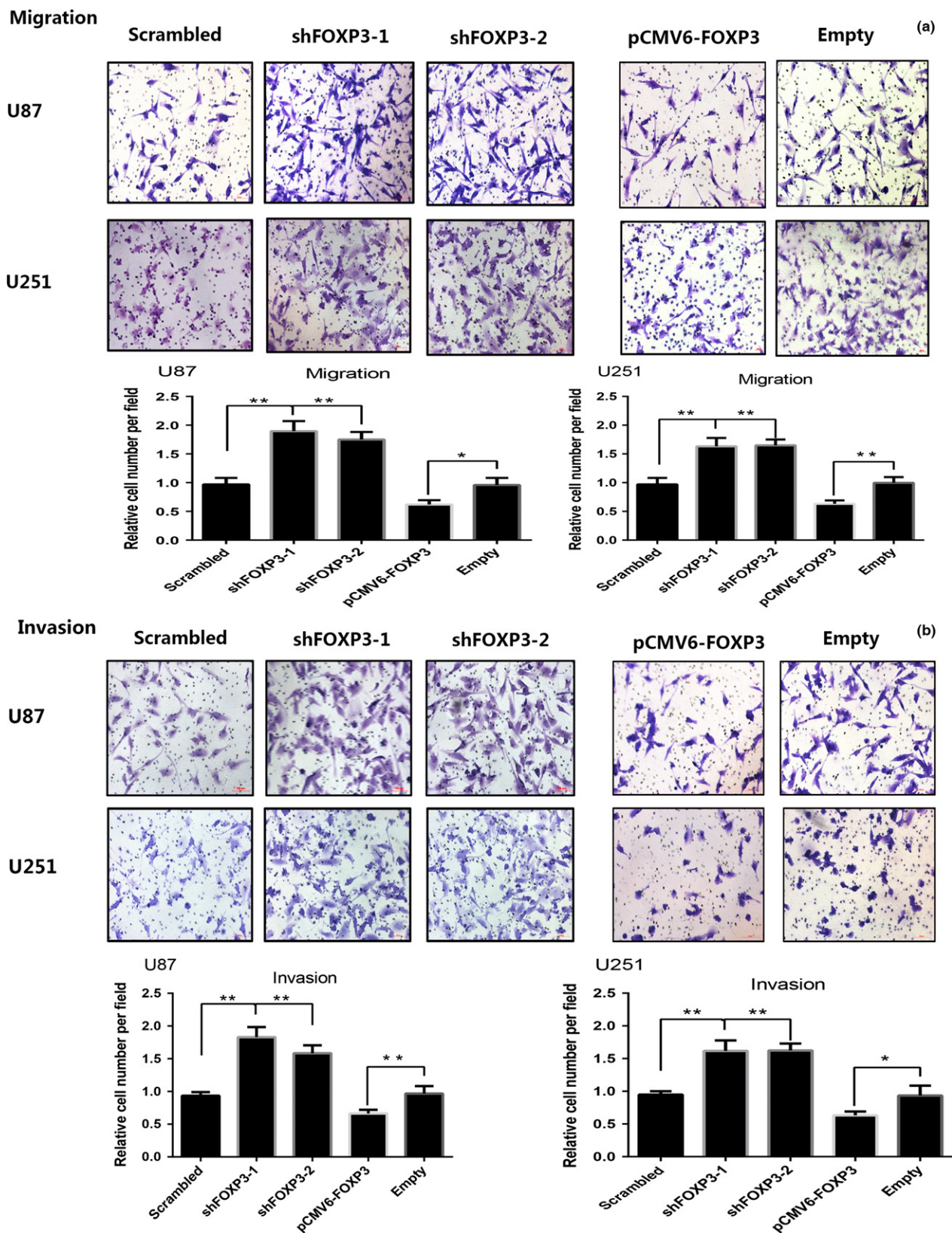
Variable	No. of cases	ARHGAP15 expression		Positive rate, %	P-value	FOXP3 expression		Positive rate, %	P-value
		Low	High			Low	High		
Age, years									
<60	151	64	87		0.58	101	50		0.44
$\geq 60$	27	13	14			16	11		
Sex									
Male	97	40	57		0.55	62	35		0.58
Female	81	37	44			55	26		
Tumor size, cm									
<4.5	91	42	49		0.43	65	26		0.10
$\geq 4.5$	87	35	52			52	35		
WHO grade									
1	46	33	61	64.9	0.02	54	40	42.6	0.01
2	48								
3	44	44	40	47.6		63	21	25.0	
4	40								

**Table 2.** Correlation analysis of forkhead box P3 (FOXP3) and ARHGAP15 in glioma patients

	ARHGAP15 (+)	ARHGAP15 (-)	Total	P-value
FOXP3 (+)	41	20	61	0.04
FOXP3 (-)	60	57	117	
Total	101	77	178	

pathway. Our results revealed that ARHGAP15 is a significant target of FOXP3. We speculated that FOXP3 may inhibit the Rac1 signaling pathway by upregulating ARHGAP15 expression. We then verified the influence of FOXP3 on Rac1 and GTP-Rac1. Results showed that FOXP3 regulates the Rac1 signaling pathway *in vivo* (Fig. 6a) and high levels of FOXP3 increased ARHGAP15 expression and decreased GTP-Rac1.





**Fig. 3.** Forkhead box P3 (FOXP3) inhibits migration invasion and regulates epithelial–mesenchymal transition-associated molecules in glioma cells. (a) Transwell migration assays were used to detect the migration ability of U87 and U251 cells transfected with pCMV6-FOXP3-GFP or pRFP-C-RS shFOXP3 or corresponding controls. (b) Transwell invasion assays were used to determine the invasion ability of U87 and U251 cells transfected with pCMV6-FOXP3-GFP or pRFP-C-RS shFOXP3 or corresponding controls. (c) Influence of FOXP3 on protein levels of epithelial–mesenchymal transition-associated molecules E-cadherin and N-cadherin were determined by Western blot analysis. (d) Quantification of data in (c). \* $P < 0.05$ , \*\* $P < 0.01$ . Error bars indicate mean  $\pm$  SD. All experiments were repeated at least three times.

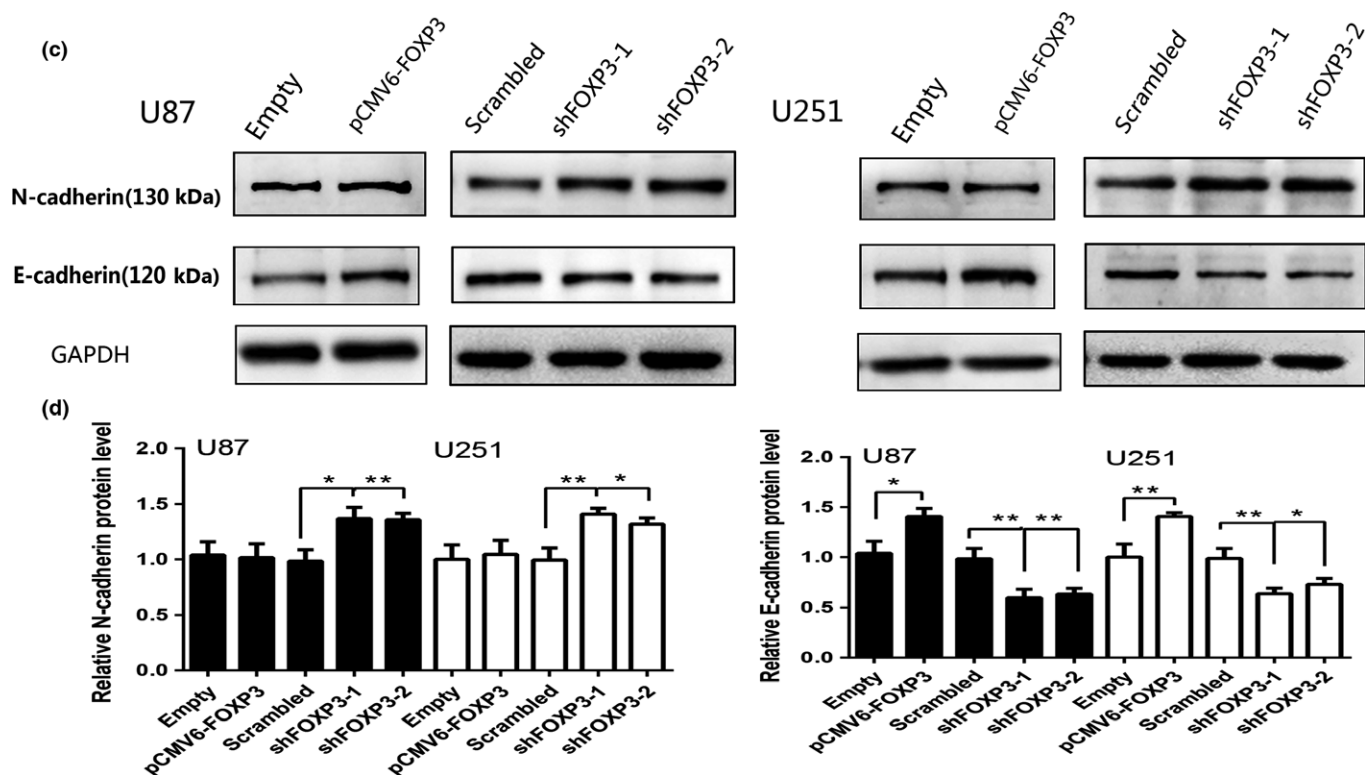


Fig. 3. continued.

Knockdown of FOXP3 decreased ARHGAP15 expression and increased GTP-Rac1 *in vitro* (Fig. 6b), which indicates that FOXP3 regulates the Rac1 signaling pathway. To confirm that the effect of FOXP3 on the Rac1 signaling pathway is mediated by ARHGAP15, we cotransfected FOXP3 overexpression and shARHGAP15 plasmids and found that ARHGAP15 can rescue suppression of the Rac1 signaling pathway caused by FOXP3 (Fig. 6c). In addition, we cotransfected shFOXP3 and ARHGAP15 overexpression plasmids and found that ARHGAP15 can rescue promotion of the Rac1 signaling pathway caused by shFOXP3. A diagram showing the regulatory relationships among FOXP3, ARHGAP15, and Rac1 in glioma cells is provided as Figure 6(D). All results indicated that FOXP3 upregulates ARHGAP15 expression, leading to inhibition of the Rac1 signaling pathway.

## Discussion

Malignant gliomas, which are extremely infiltrative tumors, migrate from the primary lesion into surrounding normal brain tissue. The invasive phenotype of malignant gliomas is bound up with the events, such as clinical progression, that complicate complete surgical resection and permit tumor regrowth and invasion of surviving tumor cells.<sup>(17)</sup> It is reported that the median survival of patients with glioblastoma is approximately 15 months,<sup>(34)</sup> even with standard treatment. Thus, understanding the molecular events involving malignant gliomas is necessary to improve therapy and prognosis.

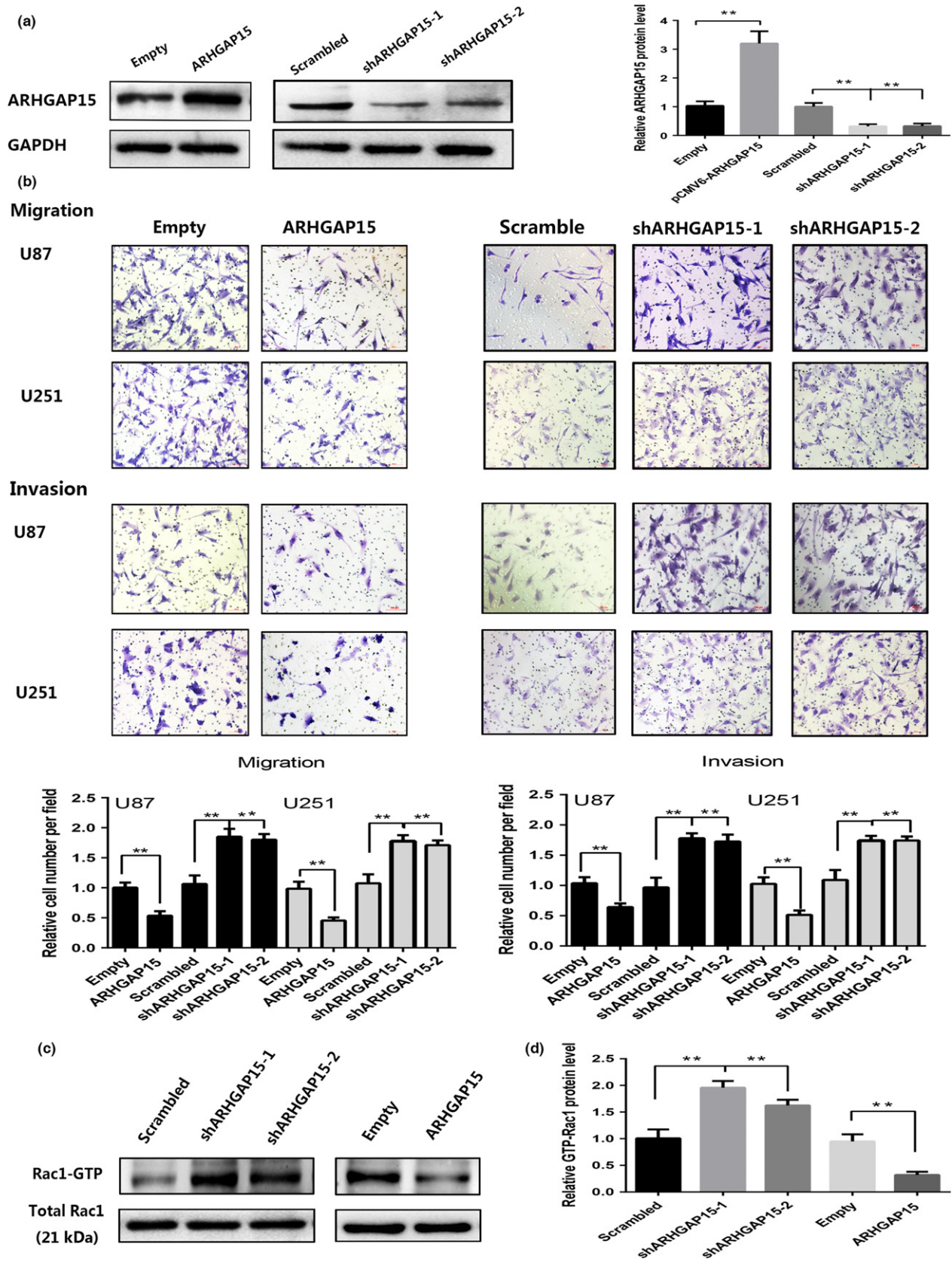
The transcription factor FOXP3 served as a master regulator of regulatory T cells, or a tumor suppressor. Cell proliferation, migration, and invasion is inhibited by FOXP3 in some cancers, including gliomas.<sup>(15)</sup> It is undeniable that the role of FOXP3,

including its effects on migration and invasion of glioma cells, remains debatable based on clinical observations of tumor progression.<sup>(35,36)</sup> It is reported that FOXP3 has been associated with some tumor suppressors, for instance p21, p18, LAT2, and ARHGAPs, in breast cancer.<sup>(9)</sup> FOXP3 acts as a transcriptional activator as well as a repressor. As an activator, FOXP3 induces tumor suppressor p21. As an inhibitor, FOXP3 can suppress cyclin-dependent kinases or Lats2.<sup>(9)</sup>

In order to understand the mechanism involved in FOXP3 suppression of glioma cell migration, we analyzed a set of DNA microarray of U87 cells overexpressing FOXP3. The result showed that ARHGAP15 had significant change, so it became our focus. In this study, we confirmed by RT-PCR and Western blot analysis that FOXP3 significantly regulates ARHGAP15 expression in U87 and U251 glioma cells. This was verified *in vivo*. Experiments indicated that FOXP3 affects migration of glioma cells by regulating ARHGAP15 expression.

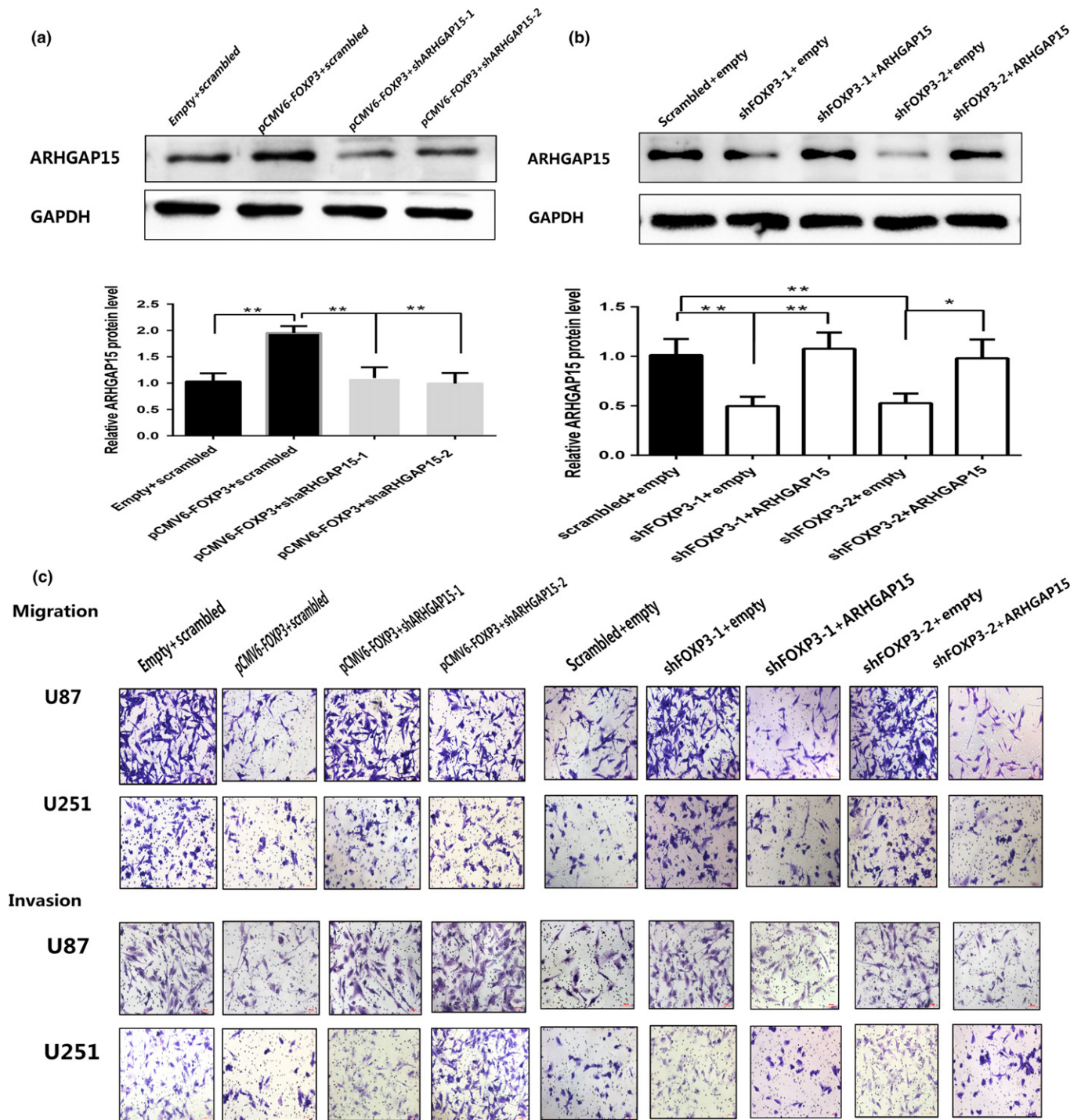
ARHGAP15 has a dual negative effect on suppressing small GTPase signaling. As a Rac-specific GAP, ARHGAP15 acts at the level of the GTPase itself and then directly inhibits an effector of GTPase, p21-activated kinase (Pak).<sup>(24)</sup> The Paks are the downstream targets of Rac. Expression of activated Pak enhances cell motility, proliferation, and resistance to apoptosis.<sup>(37–39)</sup> ARHGAP15 is a novel human RacGAP protein with GTPase binding properties,<sup>(23)</sup> but the biological roles and exact mechanism of ARHGAP15 in gliomas have not been described. In this study, we found that ARHGAP15 overexpression inhibited migration and invasion and promoted Rac1 inactivation, whereas ARHGAP15 knockdown enhanced the invasion and migration of glioma cells and activated Rac1 in U87 and U251 glioma cells. We know that FOXP3 is





**Fig. 4.** ARHGAP15 inhibits migration and invasion and contributes to Rac1 inactivation in glioma cells. (a) Western blot analysis of ARHGAP15 levels in U87 cells 48 h after transfection with pCMV6-ARHGAP15 or pRS-shARHGAP15 and quantification of data. (b) Transwell migration and invasion assays were used to detect the effect of ARHGAP15 on the migration and invasion of U87 and U251 cells. (c) Rac1-GTP was analyzed in pCMV6-ARHGAP15 or pRS-shARHGAP15 or the corresponding control-transfected U87 cells by pull-down assay. (d) Quantification of data in (c).  $**P < 0.01$ . Error bars indicate mean  $\pm$  SD. All experiments were repeated at least three times.





**Fig. 5.** Forkhead box P3 (FoxP3) affects migration and invasion of glioma cells through regulating expression of ARHGAP15. (a) U87 and U251 cells were cotransfected with pCMV6-FOXP3-GFP and pRS-shARHGAP15 or the control vector, then Western blot assay was used to test the restoration of ARHGAP15 protein by pRS-shARHGAP15 in the presence of pCMV6-FOXP3-GFP. (b) U87 and U251 cells were cotransfected with pCMV6-ARHGAP15 and pRFP-C-RS shFOXP3 or control vector, then Western blot assay was used to test the restoration of ARHGAP15 protein by pCMV6-ARHGAP15 in the presence of pRFP-C-RS shFOXP3. (c) Transwell migration/invasion assays and 3D Matrigel culture to test the cells' abilities to migrate and invade. (d) Quantification of data in (c). \* $P < 0.05$ , \*\* $P < 0.01$ . All error bars indicate mean  $\pm$  SD. All experiments were repeated at least three times.

significantly reduced or absent in GBs.<sup>(15)</sup> To clear the clinical relevance of ARHGAP15, we undertook IHC analysis. The results indicated that ARHGAP15 expression is reduced in high glioma tumors and positively correlated with ARHGAP15 in human glioma tissue samples.

We propose that FOXP3 may inhibit aggressive phenotypes by increasing ARHGAP15 expression. Overexpression of FOXP3 and shARHGAP15 plasmids were cotransfected into glioma cells. We found that ARHGAP15 can rescue the inhibitory influence on migration and invasion caused by FOXP3. In

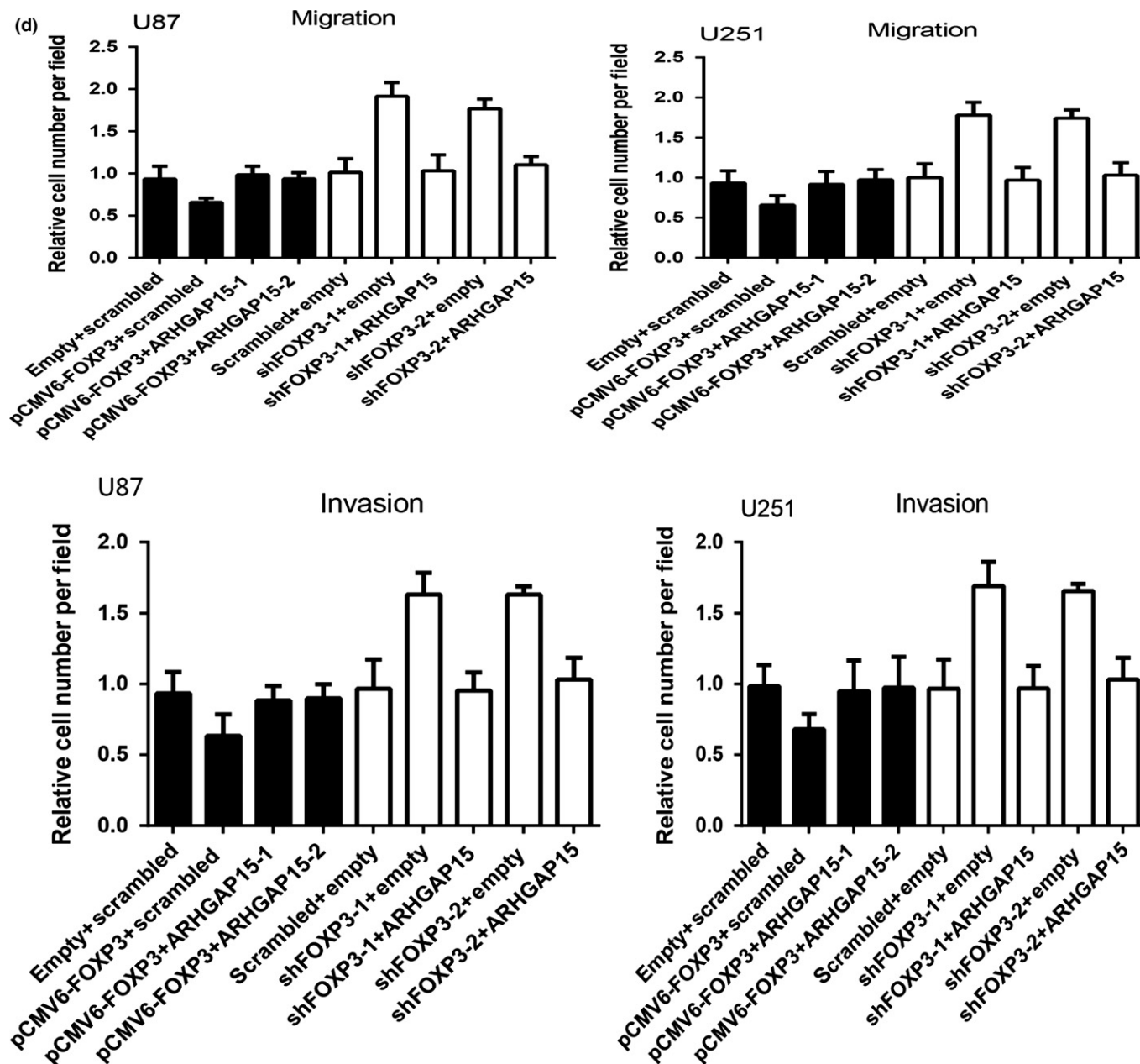


Fig. 5. continued.

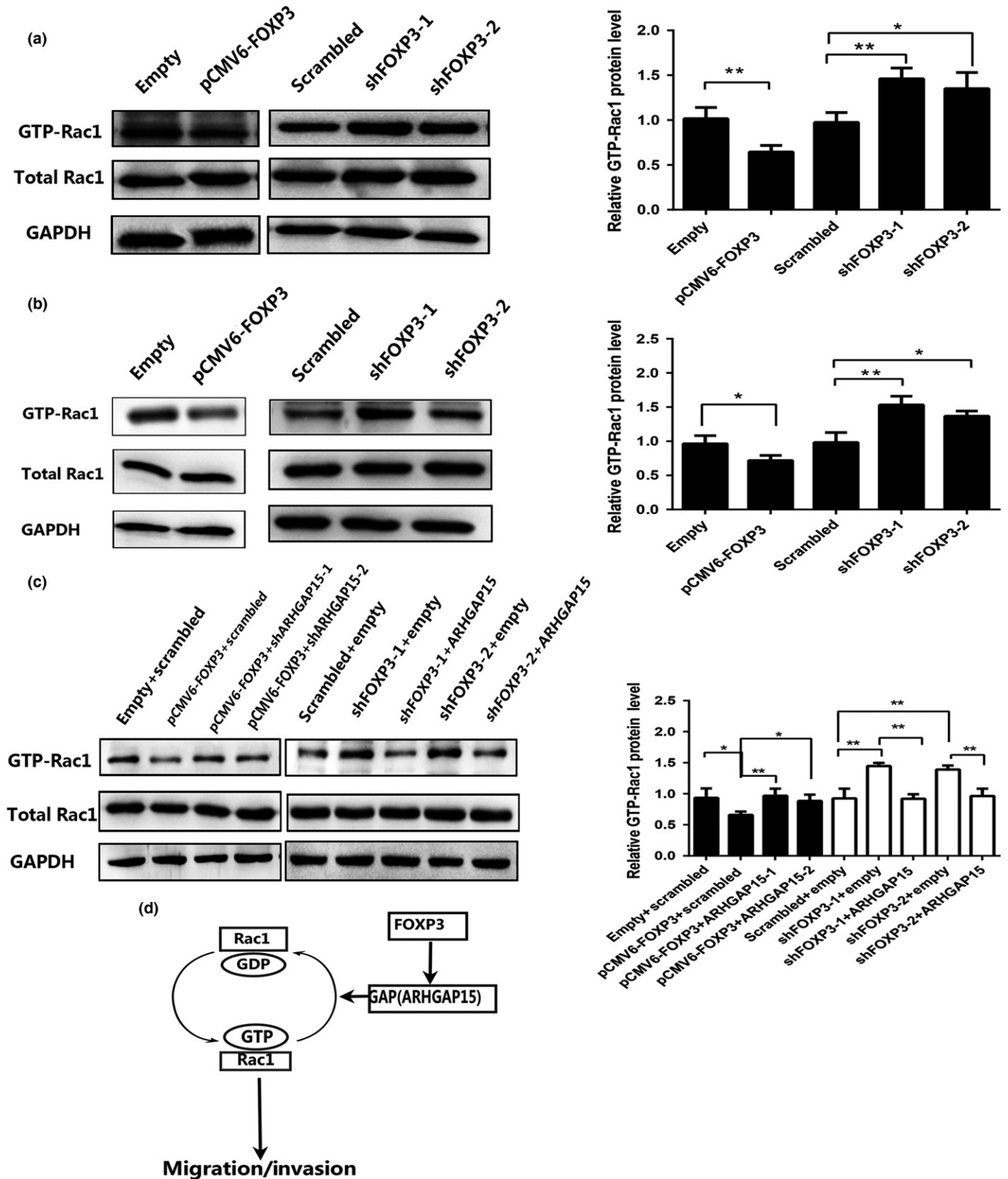
addition, we cotransfected shFOXP3 and ARHGAP15 overexpression plasmids into glioma cells and found that ARHGAP15 can rescue the promotional influence on migration and invasion caused by shFOXP3. These results prove that FOXP3 affects the migration and invasion of glioma cells partially by regulating ARHGAP15 expression.

Given that the biological roles of ARHGAP15 and FOXP3 in glioma have been confirmed, we need a better understanding of how FOXP3 affects the Rac1 signaling pathway in glioma cells. In the present study, FOXP3 overexpression suppressed Rac1 activation, whereas FOXP3 knockdown promoted Rac1 activation in U87 glioma cells. Additionally, downregulation of ARHGAP15 reduced the effects of FOXP3 on GTP-Rac1. Cotransfected shFOXP3 and ARHGAP15 overexpression plasmids showed that ARHGAP15 can rescue the

effect of FOXP3 knockdown on GTP-Rac1 in U87 glioma cells. These results reveal that the Rac1 pathway may be a mechanism through which FOXP3 regulates invasion and migration in glioma.

Further studies are required to investigate how FOXP3 mediates ARHGAP15 expression. As a member of the forkhead winged helix family of transcription factors, FOXP3 may increase the promoter activity for ARHGAP15. However, IHC results show that part of the ARHGAP15 protein is located in the nucleus of glioma cells in human glioma tissue samples. We suspect that the relation between FOXP3 and ARHGAP15 do not only reflect on the transcription level, but also the protein level. This needs further systematical analysis.

E-cadherin and N-cadherin are closely related to an aggressive brain tumor phenotype.<sup>(30,31)</sup> In the present study, stable



**Fig. 6.** Forkhead box P3 (FOXP3) regulates the Rac1 signaling pathway mediated by ARHGAP15. (a) Western blot analysis was carried out to determine the effect of FOXP3 on the expression of GTP-Rac1 and total Rac1 in U87 glioma tissue transfected with pCMV6-FOXP3-GFP or pRFP-C-R5 shFOXP3 or the corresponding controls. (b) Western blot analysis was used to determine the effect of FOXP3 on the expression of GTP-Rac1 and total Rac1 in U87 glioma cells transfected with pCMV6-FOXP3-GFP or pRFP-C-R5 shFOXP3 or the corresponding controls. (c) Western blot assays were carried out to detect the restoration of ARHGAP15 following treatment with pCMV6-FOXP3-GFP or pRFP-C-R5 shFOXP3 effected on the expression of GTP-Rac1 relationship of FOXP3, ARHGAP15 and Rac1 and total Rac1 in U87 cells. (d) Schematic plot for the regulatory relationship among FOXP3, ARHGAP15, and Rac1 in glioma cells. FOXP3 regulates ARHGAP15, which inhibits the Rac1 signaling pathway and glioma cell migration and invasion.  $**P < 0.01$ ,  $n = 3$ . All error bars indicate mean  $\pm$  SD.



knockdown of FOXP3 in U87 and U251 cells decreased E-cadherin levels, whereas N-cadherin levels were increased. Overexpression of FOXP3 increased E-cadherin levels, but there was no change in N-cadherin levels. One of two transcription variants may influence N-cadherin, which will require further investigation. Correlation between FOXP3 and EMT factors may explain the increased invasiveness and migration of glioma cells. In conclusion, these results show that FOXP3 affects migration and invasion by regulating ARHGAP15 expression and acts on the Rac1 signaling pathway through ARHGAP15 (Fig. 6d). In addition, FOXP3 is associated with EMT. These findings may provide new insights into the mech-

anisms of glioma development and present therapeutic strategies for treating gliomas.

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## Disclosure Statement

The authors have no conflict of interest.

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