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Original Article

ROCK2 mediates osteosarcoma progression and TRAIL resistance by modulating O-GlcNAc transferase degradation

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Abstract: Osteosarcoma is a common bone tumor, with a poor prognosis. New combinatorial therapies that sensitize anticancer drug-resistant osteosarcoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are, therefore, required. The GTPase RhoA effector, Rho-associated coiled-coil forming protein kinase 2 (ROCK2), is well known for its roles in various types of cancer; however, its involvement osteosarcoma has not yet been scrutinized. In this study, we analyzed ROCK2 expression, clinicopathological features, and prognosis in osteosarcoma patients. Apoptosis, colony formation, and cell proliferation were analyzed using flow cytometry, colony formation assays, and CCK8 assays, respectively. Proteomics analysis was used to evaluate osteosarcoma progression. We found that adjacent tissues had lower ROCK2 expression levels than osteosarcoma tissues and the level of expression was related to osteosarcoma tumor size and prognosis. Osteosarcoma prognosis was associated with ROCK2 expression level, which served as an independent marker in multivariate analysis. ROCK2 silencing inhibited proliferation *in vivo* and *in vitro* and triggered apoptotic osteosarcoma cell death. ROCK2 inhibited the TRAIL-mediated apoptotic pathway in osteosarcoma cells and promoted activation. Mechanistically, ROCK2 affected osteosarcoma progression and TRAIL resistance by modifying O-GlcNAcylation through O-GlcNAc transferase degradation. Taken together, our results demonstrated a unique mechanism whereby ROCK2 influences osteosarcoma progression and TRAIL resistance, hence improving osteosarcoma management.

Keywords: Osteosarcoma, ROCK2, TRAIL, ubiquitination degradation, O-GlcNAc transferase

Introduction

Osteosarcoma (OS) is one of the main types of bone tumor. It typically begins in adolescence and has a high degree of malignancy [1]. Despite recent advances in invasive procedures and chemotherapy, the general survival rate within five years of onset is approximately 60% [2]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine belonging to the same superfamily as tumor necrosis factor, which selectively induces apoptosis in many cancerous cells while maintaining the integrity of non-cancerous cells; therefore, it can potentially be used for anti-cancer therapy [3, 4]. As most OS cells resist TRAIL treatment, there is a demand for new therapeutic targets to sensitize tumor cells to TRAIL [5]. Studies have shown that OS progression and cellular apoptosis are linked to the dysregulated expression of tumor-suppressor genes and oncogenes. Understanding the molecular mechanisms underlying OS progression may, therefore, provide new insights and therapeutic targets.

Rho-associated coiled-coil forming protein kinase 2 (ROCK2) is the principal signaling molecule in the Rho/ROCK signal transduction pathway. It regulates target protein phosphorylation/activity levels, which modulates downstream gene expression [6]. Accumulating evidence in literature demonstrates that ROCK2 has an essential role in many biological processes, including cell adhesion, migration, and morphology [7, 8]. ROCK2 is involved in cancer

biology and has been shown to significantly promote the proliferation of many cancers. It is frequently overexpressed in hepatocellular carcinoma [9], renal cell carcinoma [10], and colorectal cancer [11]; moreover, ROCK2 knockdown inhibits cancer cell proliferation [12]. Furthermore, cancer progression and poor prognosis is associated with ROCK2 overexpression. These studies suggest that ROCK2 has an essential role in cancer progression and tumorigenesis [13, 14]. Nevertheless, the potential effects of ROCK2 expression on OS proliferation remain unclear.

The structure, polymerization, transport, and transformation of cell surface proteins are controlled by O-linked polysaccharides. The relaxation of O-linked polysaccharides affects hematological diseases and cancers [15, 16]. Recent studies have reported that O-GlcNAcylation can control the sensitivity of cancerous cells to the proapoptotic ligand, Apo2l/TRAIL [17-19]. High levels of O-glycosyltransferase expression may assist receptor reorganization in some cancerous cells, which is closely related to the sensitization of Apo2l/TRAIL. Previous studies have detected O-GlcNAcylation upregulation in liver, breast, and prostate cancers [20-22]. In contrast, O-GlcNAcylation downregulation can inhibit tumor progression. However, the mechanisms underlying the regulation of O-GlcNAcylation expression in OS remain unclear.

The results of this study demonstrate the upregulation of ROCK2 expression in OS tissues compared to non-cancerous tissues and a negative correlation between ROCK2 expression levels and patient survival rates. Multivariate analysis demonstrated that high ROCK2 expression level was a negative independent prognostic factor in OS. We also found that ROCK2 knockdown caused significant inhibition of OS cell proliferation *in vivo* and *in vitro*, in addition to initiating OS cell apoptosis. Meanwhile, ectopic ROCK2 expression showed the opposite effect. ROCK2 significantly inhibited or knocked down the TRAIL-mediated apoptotic pathway in OS cells and promoted activation. Moreover, ROCK2 influenced O-GlcNAcylation levels by regulating O-GlcNAc transferase (OGT) degradation. In conclusion, the data from this study provide new evidence regarding the clinical and biological implications of ROCK2 as a potential biomarker. This suggests that ROCK2-OGT-O-GlcNAcylation may present a new method for modulating OS progression and TRAIL resistance.

Materials and methods

Clinical specimens

Human OS specimens were collected from 106 patients who underwent surgery at the Second Affiliated Hospital of Nanchang University and the First Affiliated Hospital of Nanchang University. All patients provided informed consent and the study was approved by the Institutional Ethical Review Board of Nanchang University.

Cell culture, constructs, and plasmids

The human OS cell lines, Saos-2, U2-OS, MG-63, 143B, and the normal cell line, hfoBI-19, were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). All cell lines were cultured in ATCC adaptive medium in 5% CO_2 at 37°C. The identity of the cell lines was confirmed by short tandem repeat analysis. The study specifically used cells that were received less than 6 months prior to study commencement.

GenePharma (Shanghai, China) synthesized RNA duplexes for shRNA-mediated ROCK2 and OGT silencing. ROCK2 and OGT plasmids were purchased from GenePharma. The plasmids and shRNAs were transfected into OS cells using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA).

Immunohistochemistry

OS tissues and adjacent normal tissues were fixed, embedded in paraffin, sectioned, and then deparaffinized. ROCK2 and O-GlcNAcylation were detected using anti-ROCK2 (1:250; Abcam, Cambridge, UK) and anti-O-GlcNAcylation (1;200, Invitrogen) monoclonal antibodies. A peroxidase/3,3'-diaminobenzidine-conjugated secondary antibody was used for visualization. Relative nuclear staining intensity was used to grade ROCK2 and O-GlcNAcylation levels.

Western blotting

Human OS cell lines were collected and cell extracts were prepared with lysis buffer. The

cell extracts were boiled in the lysis buffer for 10 minutes and equal amounts (50 μg) were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated protein bands were transferred to nitrocellulose membranes by electroblotting (Millipore, Bedford, MA, USA). Membranes were incubated with anti-ROCK2 (AB-71598), anti-OGT (AB-96718), anti-O-GlcNAcylation (RL2), anti-ubiquitin (AB-7780), and anti-tubulin (AB-6046) primary antibodies (Abcam), followed by incubation with the appropriate secondary antibodies. After washing the membranes with 1× TBST, bound antibodies were observed using intensified chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA), as described previously. Quantity One software (Bio-Rad, Hercules, CA, USA) was used measured the intensity of each band.

Co-immunoprecipitation

Cell harvesting was performed on ice and cells were lysed for 15 minutes in 500 μL of immunoprecipitation lysis buffer. Cell fragments were centrifuged for 10 minutes at 1,000 rpm at 4°C and the supernatant was transferred to a new conical centrifugal tube and frozen. The relevant antibody (1:250) was added and samples were incubated at 4°C for 4 hours. After the addition of agarose and protein A/G, the mixture was shaken at rpm on a rocking platform at 4°C for 24 hours. Magnetic beads were washed three times with 800 μL of immunoprecipitation lysis buffer and centrifuged at 3,000 rpm for 5 minutes. After a final rinse, the magnetic beads were resuspended in 40 μL of 3% sodium alkyl sulphate. The samples were then boiled for 10 minutes and 20 μL of each sample was analyzed using SDS-PAGE and autoradiography.

EdU assay

OS cells were cultured with 5-ethynyl-20-deoxyuridine (EdU) for 5 hours. Cells were then treated according to the manufacturer's guidelines. In brief, OS cells were cultured in 96-well plates at a density of 1×10^4 cells/well for 24 hours. Cells were then exposed to 50 μM EdU at 37°C for 2 hours, followed by immersion in 4% formaldehyde at room temperature for 30 minutes. Cells were then treated with 0.5% Triton X-100 at room temperature for 20 minutes. Cells were washed thrice with PBS, followed by staining with 100 μL of Hoechst 33342 (5 mg/mL) for 30 minutes. Following this, cells were treated with 30 μL of 1-Bohr reaction cocktail for 30 minutes. The DNA content of each cell was determined using fluorescence microscopy. These experiments were performed in triplicate.

CCK8 assays, qRT-PCR, in vivo ubiquitination assay, and tumorigenicity assay

CCK8 assays, qRT-PCR, *in vivo* ubiquitination assays, and tumorigenicity assays were performed as previously described [11].

Flow cytometry

OS cells were harvested by 0.25% trypsinization and washed twice with PBS. The degree of apoptosis was assessed using an Annexin V-PE/7AAD apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Statistical Package for the Social Sciences 19.0 (IBM, Armonk, NY, USA) was used for data analysis. The logarithmic rank test of classified variables was used to analyze the association between variables and survival time. Patient survival curves were constructed using the Kaplan-Meier method. Differences between two groups were analyzed using Student's t-tests and one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

Results

ROCK2 expression was upregulated in human osteosarcoma

To determine the expression level of ROCK2 in tissue samples, qRT-PCR was performed in 106 OS and adjacent normal tissue samples. *ROCK2* gene expression levels were found to be markedly higher in OS tissues than in normal tissues, as shown in **Figure 1A**. In addition, OS tumor samples and matched non-tumorous tissue samples were analyzed by western blotting. ROCK2 protein levels were significantly upregulated in OS tissues compared with surrounding normal tissues (**Figure 1B** and **1C**).

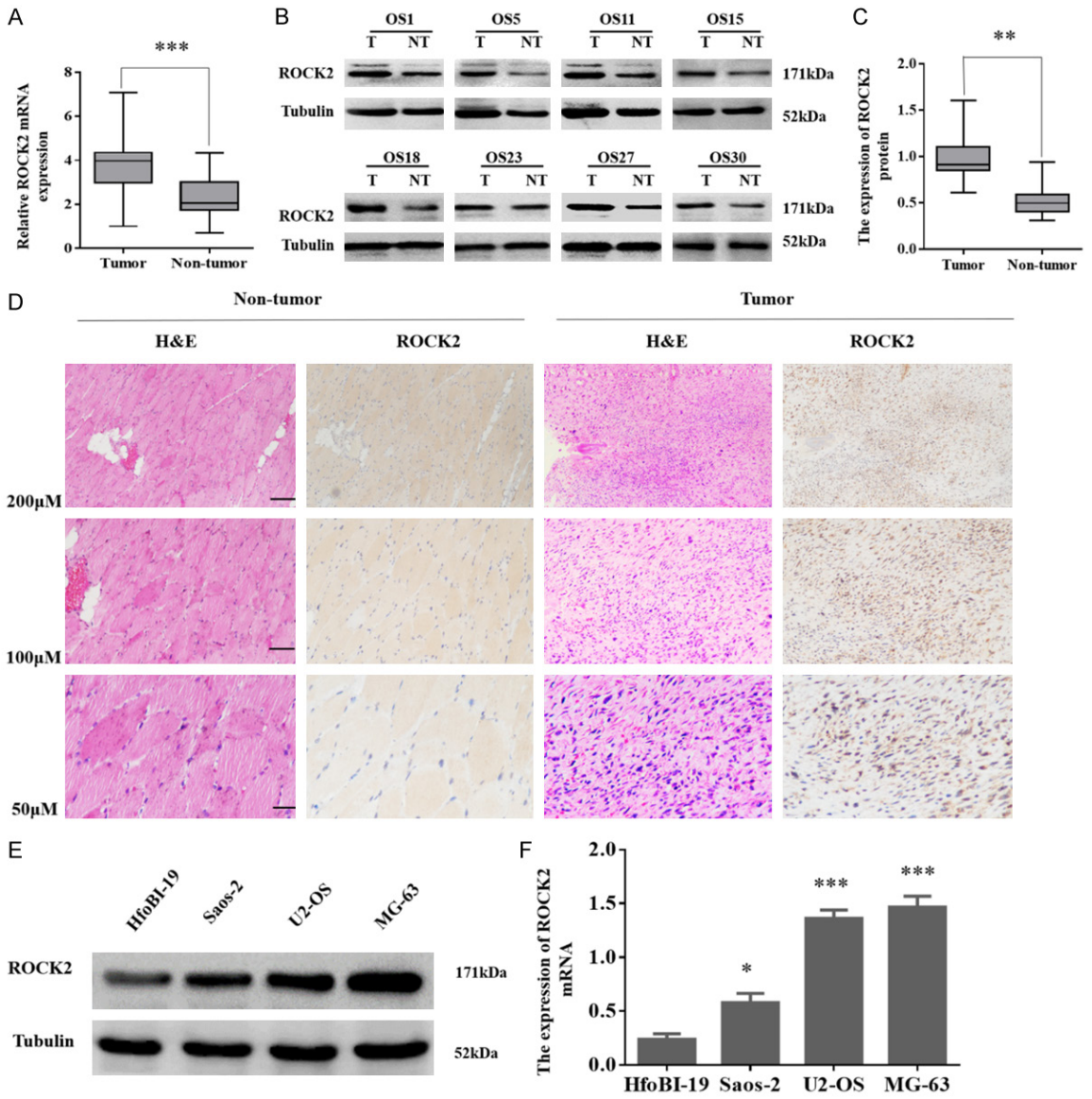


Figure 1. Relative *ROCK2* mRNA levels in osteosarcoma cells and tissues. A. Expression of *ROCK2* mRNA in paired osteosarcoma (OS) and non-tumor tissues. *ROCK2* expression was measured by qRT-PCR, using *GAPDH* as a control. B, C. Western blotting assessment of ROCK2 protein quantity in OS tissues (n=30) and paired non-tumor tissues (n=30; T, tumor, NT, tumor). D. Representative quantification (right) and images (left) of ROCK2 staining in 106 paired OS tissues. Scale bar: 50 μm. E. Determination of ROCK2 protein levels by western blotting in OS cell lines and normal hfoBI-19 cells. F. qRT-PCR assay of *ROCK2* in normal cells (hfoBI-19) and OS cell lines (Saos-2, MG-63, U2-OS) $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Moreover, ROCK2 protein levels in tumor and adjacent tissues were measured by immunohistochemistry. ROCK2 protein was found to be expressed at high levels in 67.9% (72/106) of OS tissue samples, which was consistent with the western blotting results (**Figure 1D**). These results indicated that ROCK2 expression is significantly upregulated in OS tissues.

Next, we analyzed ROCK2 expression by western blotting in normal human cells and several OS cells lines, including hfoBI-19, Saos-2, U2-OS, and MG-63, to determine the potential role of ROCK2 in OS development. ROCK2 expression was found to be higher in OS cells compared with normal cells (**Figure 1E**). qRT-PCR results indicated that *ROCK2* was highly

Table 1. Correlation between ROCK2 expression and the clinicopathological characteristics of the osteosarcoma patients

Parameters	n	ROCK2 expression		*P* value
		Low (n=34)	High (n=72)	
Age (years)				*P=0.862*
≤18	76	24	52	
>18	30	10	20	
Sex				*P=0.372*
Female	44	12	32	
Male	62	22	40	
Tumor size (cm)				*P<0.0001*
<5	46	30	16	
≥5	60	4	56	
Location				P=0.629
Upper limb bone	75	23	52	
Lower limb bone	31	11	20	
Clinical stage				*P<0.0001*
I/II	37	29	8	
III/IV	69	5	64	
Pathological differentiation				*P=0.812*
Well/Moderately	45	15	30	
Poor	61	19	42	
Lymph node metastasis				*P=0.751*
Absence	60	20	40	
Presence	46	14	32	
Distant metastasis				*P=0.454*
Absence	76	26	50	
Presence	30	8	22	
Recurrence				*P=0.665*
Absence	50	15	35	
Presence	56	19	37	

expressed in OS cells compared to hfoBl-19 cells, which was consistent with the western blotting results (**Figure 1F**). These data indicated that ROCK2 expression was significantly upregulated in OS tissues and cell lines.

ROCK2 upregulation in OS patients was positively associated with tumor size and poor prognosis

To investigate the clinical significance of ROCK2 upregulation in OS, we further analyzed the relationship between clinicopathological characteristics and ROCK2 expression in OS patients. ROCK2 overexpression was associated with tumor size and clinical stage, but not with age, sex, pathological differentiation, or tumor location (**Tables 1** and **2**). These results suggested that ROCK2 overexpression was associated with OS progression. In addition, we found that ROCK2 protein levels were associated with tumor prognosis. Survival analysis using the Kaplan-Meier method indicated that the general survival rate of patients with high ROCK2 expression was worse than that of those with lower ROCK2 expression (**Figure 2A**). Furthermore, the disease-free survival rate (DFS) in the high-ROCK2 expression group was higher than in the low-ROCK2 expression group (**Figure 2B**, *P*<0.001). Univariate analysis indicated that tumor size and high ROCK2 protein expression levels were significantly correlated with low OS incidence (*P*<0.001). Moreover, multivariate analysis indicated that ROCK2 overexpression was an independent predictor of poor prognosis in OS patients (**Table 3**).

ROCK2 knockdown inhibited OS tumor growth in vivo and in vitro

The stable transfection of OS cells with a *ROCK2*-specific short hairpin RNA (shROCK2) enabled the investigation of the possible role of ROCK2 in OS growth (Figure S1A-C). Western blotting results indicated that transfection with shROCK2 significantly reduced ROCK2 protein expression levels in MG-63 and U2-OS cells (**Figures 3A**, **3B**, S2A). As shown in **Figure 3C**, CCK8 assays confirmed that ROCK2 knockdown inhibited the proliferation of MG-63 and U2-OS cells. In addition, EdU assays showed that ROCK2 downregulation inhibited OS cell growth (**Figures 3D**, S2B). Flow cytometry results demonstrated that, in MG-63 and U2-OS cells, ROCK2 downregulation significantly inhibited cell cycle progression in G1 phase (**Figures 3E**, **3F**, S2C, S2D). Taken together, these data indicate that ROCK2 knockdown can inhibit human OS cell proliferation *in vitro*.

Table 2. Univariate and multivariate analyses of overall survival in OS patients (Cox proportional hazards regression model)

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (≥18 vs <18)	0.765	0.414-1.557	0.456	0.897	0.409-1.679	0.712
Sex (Female vs Male)	1.325	0.667-2.226	0.551	0.976	0.876-1.976	0.464
Tumor size (<5 vs ≥5)	1.994	1.225-3.342	0.005*	1.931	1.145-3.134	0.012*
Location (Upper limb vs Lower limb)	0.876	0.441-2.632	0.424	1.031	0.541-2.471	0.623
Clinical stage (I/II vs III/IV)	2.512	1.491-4.372	0.003*	2.735	1.214-4.121	0.027*
Pathological differentiation (Well/Moderately vs Poor)	0.623	0.566-1.894	0.855	0.885	0.425-1.788	0.545
Lymph node metastasis (Absence vs Presence)	1.567	0.728-2.779	0.141	1.321	0.595-2.456	0.625
Distant metastasis (Absence vs Presence)	1.751	0.895-3.135	0.690	1.526	0.623-2.563	0.461
Recurrence (Absence vs Presence)	2.153	1.3411-3.564	0.030*	1.562	0.796-2.817	0.751
ROCK2 expression (High vs Low)	2.765	1.678-5.532	<0.001*	3.252	1.874-6.431	0.006*

HR, hazard ratio; CI, confidence interval; *P<0.05.

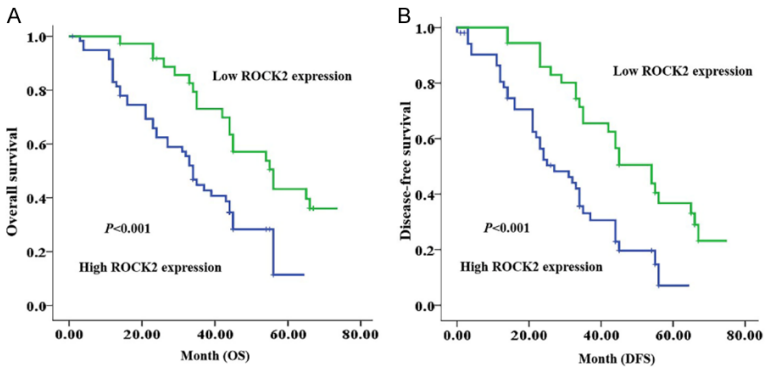


Figure 2. ROCK2 expression profile and its clinical significance. A, B. Disease-free survival (DFS) and Kaplan-Meier curves for overall survival (OS) of the two groups, based on high (n=72) or low (n=34) ROCK2 expression in OS patients. ***P<0.001.

Additionally, to investigate the effects of ROCK2 on OS tumor growth *in vivo*, we performed a tumorigenicity test in nude mice. The subcutaneous tumors in the shROCK2 group had a smaller volume and weighed less than those in the shNC group. This demonstrated that decreased ROCK2 downregulation significantly inhibited tumor growth (P<0.05, **Figure 3G** and **3H**). In conclusion, these data suggested that ROCK2 knockdown inhibits the growth of OS tumors *in vivo* and *in vitro*.

ROCK2 knockdown caused OS cell sensitivity to TRAIL-induced apoptotic processes

As a new targeted therapy, TRAIL plays an important role in tumor treatment. However, TRAIL resistance is the main cause of OS treatment failure. Therefore, the role of ROCK2 in TRAIL-induced apoptosis in human OS cell lines was investigated. OS cells were found to have increased ROCK2 mRNA and protein expression levels after TRAIL treatment (**Figures 4A**, **4B**, S4A). Furthermore, TRAIL treatment increased ROCK2 expression in a time-dependent manner in OS cells. ROCK2 mRNA and protein levels began to increase in U2-OS and MG-63 cells at 12 hours and in both cell lines, ROCK2 levels continued to increase until 48 hours. These cells showed significant TRAIL resistance (**Figures 4C**, **4D**, S4B). These results indicated that ROCK2 is upregulated in OS cells during TRAIL treatment.

We sought to further explore the potential role of ROCK2 in OS cell sensitivity to TRAIL treatment. Upon transfecting shROCK2 into U2-OS and MG-63 cells, we found that ROCK2 protein expression levels significantly decreased (**Figure 5A**). ROCK2 knockdown significantly enhanced the cellular response to TRAIL (**Figure 5B**). We then analyzed OS cell apoptosis via flow cytometry, which demonstrated that ROCK2 knockdown significantly enhanced the efficacy of TRAIL (**Figure 5C** and **5D**). In addition, caspase-3 activation was observed in shROCK2-transfected cells treated with TRAIL and this is considered a point of no return for apoptosis (**Figure 5E**). BCL-2 and BAX levels

Table 3. Spearman analysis of the correlation between ROCK2 expression and clinicopathological characteristics of the osteosarcoma patients

Characteristics	Spearman correlation	*P*-value
Age	0.142	0.765
Sex	0.232	0.832
Tumor size	0.398	<0.001
Location	0.621	0.832
Clinical stage	0.386	<0.001
Pathological differentiation	0.135	0.673
Lymph node metastasis	0.643	0.543
Distant metastasis	0.246	0.061
Recurrence	0.661	0.453

were also analyzed in shROCK2-transfected cells (**Figure 5E**).

To further elucidate the role of ROCK2 in OS during TRAIL treatment, we transfected pcDNA3.1(+)-ROCK2 into Saos-2 and 143B cells (**Figure 5F**). ROCK2 overexpression resulted in resistance to TRAIL-induced apoptosis and an increased cell proliferation rate (**Figure 5G-I**). These results indicated that ROCK2 knockdown increased the sensitivity of OS cells to TRAIL-induced apoptosis.

ROCK2 and O-GlcNAcylation levels were positively associated in OS cells and tissues

The mechanisms by which ROCK2 modulates OS progression and TRAIL resistance were investigated. Recent studies have reported that lowering O-GlcNAcylation levels in cancer cells can inhibit cancer progression and that O-GlcNAcylation modulation affects TRAIL sensitivity. Therefore, we speculated that ROCK2 may influence OS progression and TRAIL resistance by affecting O-GlcNAcylation levels. We tested this hypothesis by detecting ROCK2 and O-GlcNAcylation levels in several OS cells using western blotting. The results indicated that both ROCK2 and O-GlcNAcylation levels were higher in OS cells than in normal cells and that ROCK2 and O-GlcNAcylation levels were positively correlated (**Figure 6A** and **6B**). We stably transfected cells with shROCK2 or treated them with the ROCK2 inhibitor, Y-27632, to control ROCK2 expression levels. Western blotting analysis indicated a significant reduction in O-GlcNAcylation in MG-63 and U2-OS cells after ROCK2 downregulation (**Figure 6C** and **6D**). O-GlcNAcylation and ROCK2 levels were also measured in OS cells and normal tissues. A significant accumulation of O-GlcNAcylation was observed in OS tissues (**Figure 6E-G**). Moreover, ROCK2 and O-GlcNAcylation levels were positively correlated in OS tissues, as shown in the scatter plot in **Figure 6H**. In summary, these results highlighted a mechanism whereby ROCK2 influenced OS progression by adjusting the level of O-GlcNAcylation.

ROCK2 modified O-GlcNAcylation levels and affected OS progression and TRAIL resistance

To further confirm that ROCK2 mediates OS progression and TRAIL resistance by regulating O-GlcNAcylation levels, O-GlcNAcylation levels were increased using the glycosyl agonist, Thiamet G, in ROCK2-knockdown OS cells. We then observed the levels of ROCK2 expression, O-GlcNAcylation, and cell proliferation via western blotting and cell proliferation assays. Western blotting results indicated that ROCK2 downregulation reduced O-GlcNAcylation levels, while the upregulation of O-GlcNAcylation decreased the loss of ROCK2 expression in ROCK2-knockdown U2-OS cells (**Figure 7A-C**). Furthermore, we found that ROCK2 knockdown significantly decreased U2-OS cell proliferation, whereas increasing the levels of O-GlcNAcylation inhibited the decrease in cell proliferation caused by ROCK2 knockdown (**Figure 7D** and **7E**). We also found that increasing the levels of O-GlcNAcylation increased apoptosis in shROCK2-transfected cells after treatment with TRAIL (**Figure 7F** and **7G**).

We then decreased O-GlcNAcylation levels using the glycosyl inhibitor, OSMI-1, in ROCK2-overexpressing Saos-2 cells and measured ROCK2 and O-GlcNAcylation levels and cell proliferation. Western blotting results indicated that ROCK2 overexpression increased O-GlcNAcylation levels. Meanwhile, the inhibition of O-GlcNAcylation in Saos-2 cells significantly prevented the increase in O-GlcNAcylation levels by ROCK2 (**Figure 7H-J**). CCK8 and EdU assays showed that decreased O-GlcNAcylation levels resulted in a decrease in ROCK2-induced cell proliferation (**Figure 7K** and **7L**). Flow cytometry assay results showed that decreased O-GlcNAcylation levels increased the rate of apoptosis of p-ROCK2-transfected cells after

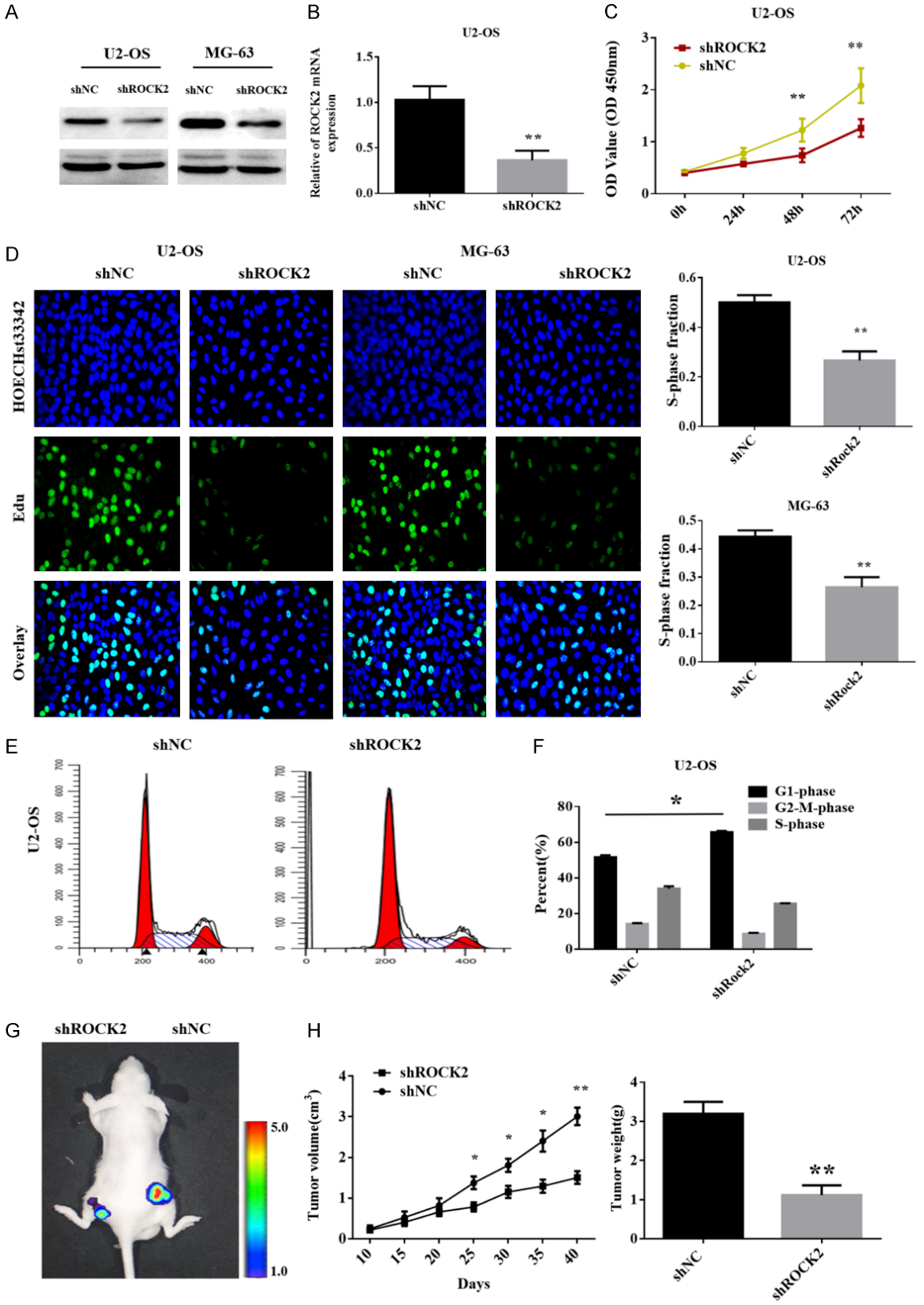


Figure 3. ROCK2 promoted osteosarcoma tumor growth *in vivo* and *in vitro*. A, B. Western blotting and qRT-PCR were used to test the efficiency of ROCK2 silencing in osteosarcoma (OS) cell lines. C. A CCK8 assay was used to examine the proliferation of U2-OS cells transfected with shNC or shROCK2 plasmids (n=3), $^{**}P<0.01$. D. The impact of ROCK2 knockdown on U2-OS and MG-63 cell proliferation, as detected by EdU assay, $^{**}P<0.01$. Scale bar: 100

μm. E, F. Impact of ROCK2 silencing on cell cycle progression in U2-OS and OS cells, *P<0.05. G and H. shNC- or shROCK2-transfected cells were injected subcutaneously into the flank of nude mice for *in vivo* tumor-formation assays (n=10), *P<0.05, **P<0.01.

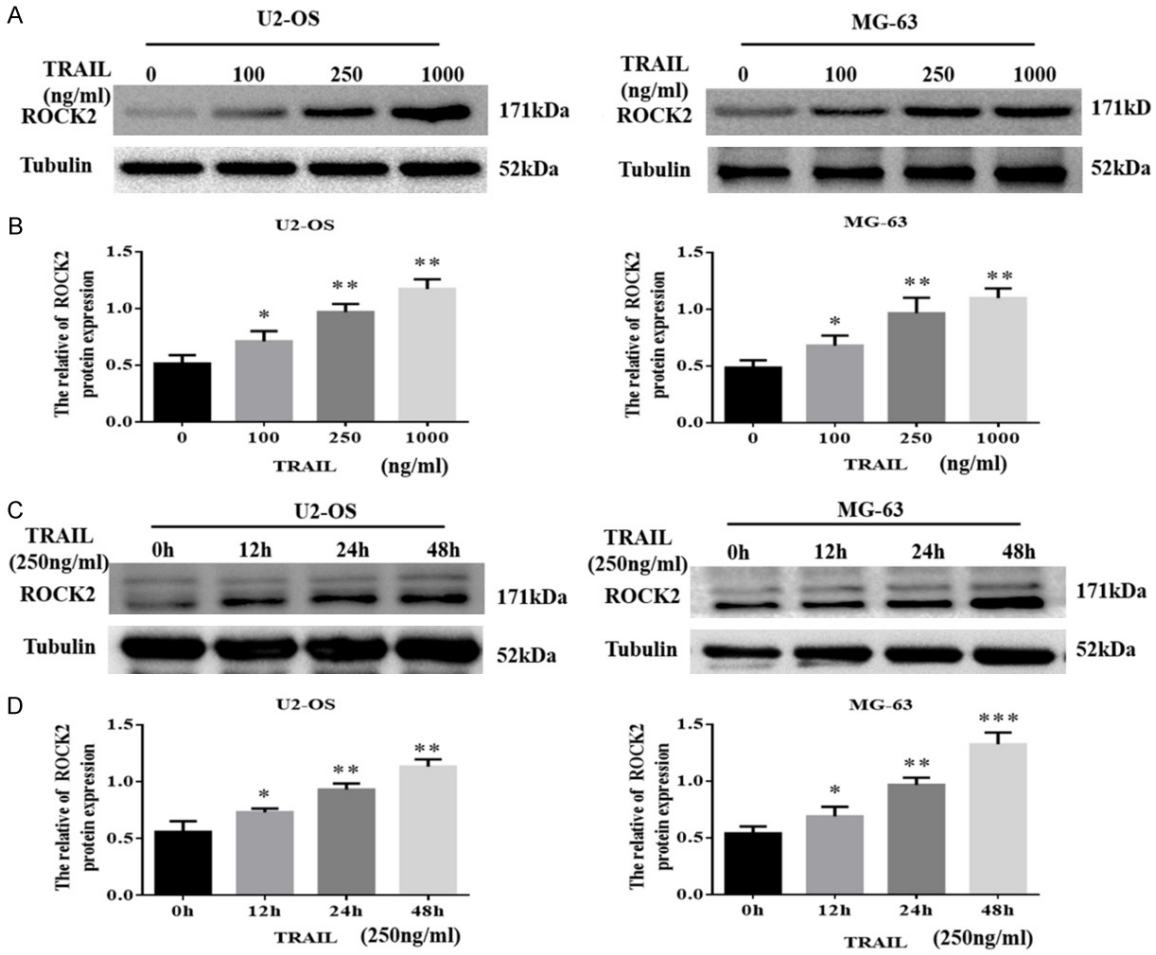


Figure 4. TRAIL promoted ROCK2 expression in osteosarcoma cells. U2-OS and MG-63 cells were incubated with different doses of TRAIL for 24 hours (A, B) or with a constant dose of TRAIL (250 ng/mL) for different periods of time (C, D). Western blotting was then used to measure ROCK2 expression, *P<0.05, **P<0.01.

TRAIL treatment (**Figure 7M** and **7N**). These results confirmed that O-GlcNAcylation levels are the key to ROCK2-mediated OS progression and TRAIL resistance.

ROCK2 regulated O-GlcNAcylation levels by modifying OGT degradation

O-GlcNAcylation is a transient post-translational modification catalyzed by OGT. O-GlcNAcylation is involved in epigenetic regulation, intracellular signal transduction, and transcription [23]. OGT expression levels are linked to metabolic diseases and cancer [24, 25]. Therefore, we hypothesized that OGT participates in the modulation of O-GlcNAcylation by ROCK2 in OS. To evaluate this possibility, OGT expression levels were measured in ROCK2-knockdown U2-OS cells. OGT expression levels were found to be significantly reduced under these conditions (**Figure 8A**), while ROCK2 upregulation had the opposite effect in Saos-2 cells (**Figure 8B**). We observed no changes in OGA expression levels when ROCK2 expression was altered (Figure S3).

In our previous study, we showed that ubiquitin-mediated CDC25A and MMP2 degradation is modulated by ROCK2 [26, 27] and therefore, we speculated that ROCK2 may also modulate

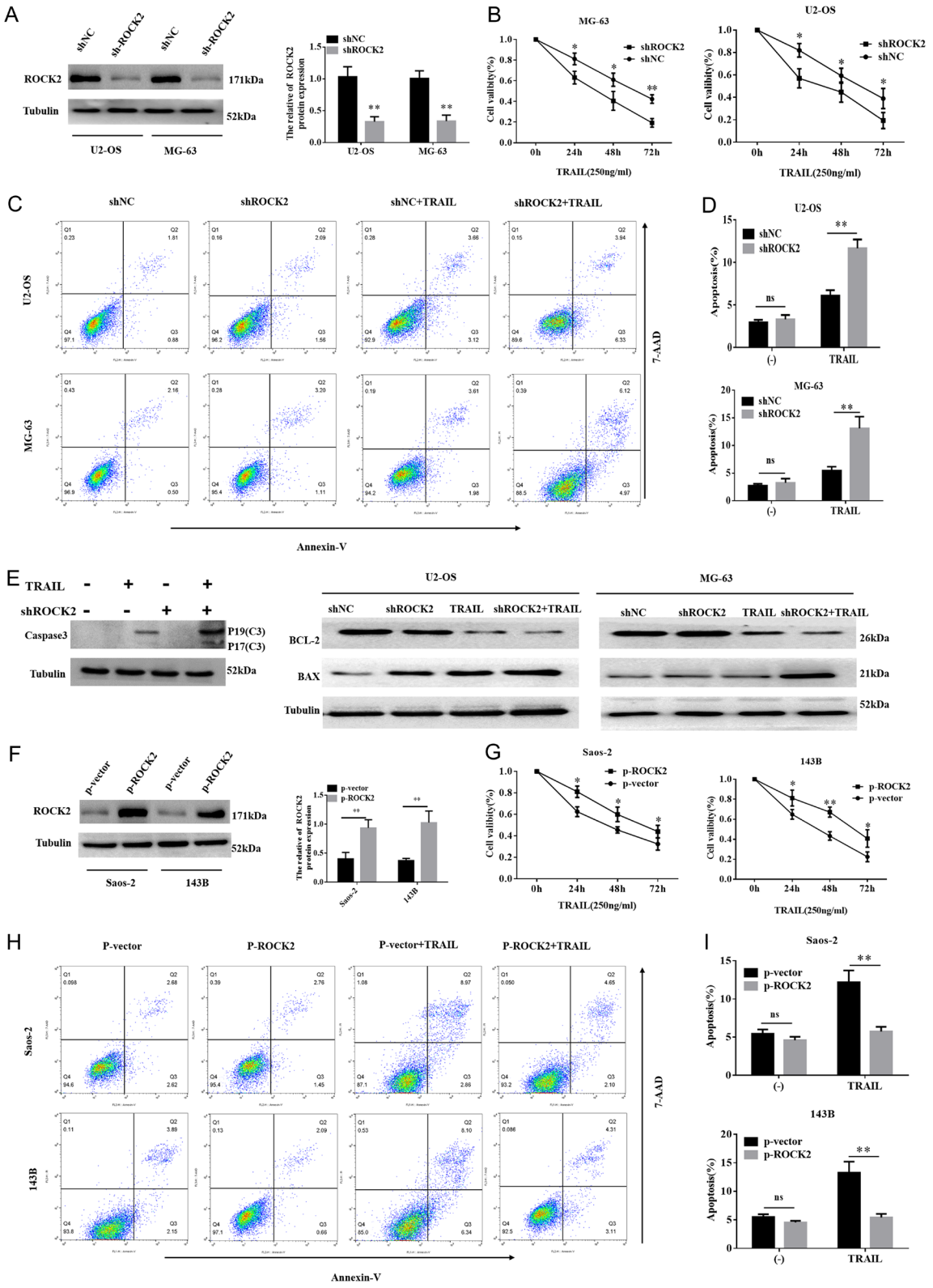


Figure 5. Downregulating ROCK2 sensitized osteosarcoma cells to TRAIL-induced apoptosis. A. Western blotting was used to test the efficiency of ROCK2 shRNA transfection in osteosarcoma (OS) cell lines. B. After transfection with a control shRNA or ROCK2 shRNA, MG-63 and U2-OS cells were treated with TRAIL for 24 hours. Cell viability was determined by CCK8 assay, **P*<0.05, ***P*<0.01. C, D. Apoptosis was analyzed by flow cytometry (n=3, ***P*<0.01). E.

Western blotting was used to measure caspase 3, BCL-2, and BAX expression. F. Western blotting was used to test the efficiency of pcDNA3.1(+)-ROCK2 transfection. G. After transfection with vector or pcDNA3.1(+)-ROCK2, the viability of MG-63 and U2-OS cells was determined by a CCK8 assay, $^{*}P<0.05$, $^{**}P<0.01$. H, I. Apoptosis was analyzed by flow cytometry (n=3), $^{**}P<0.01$.

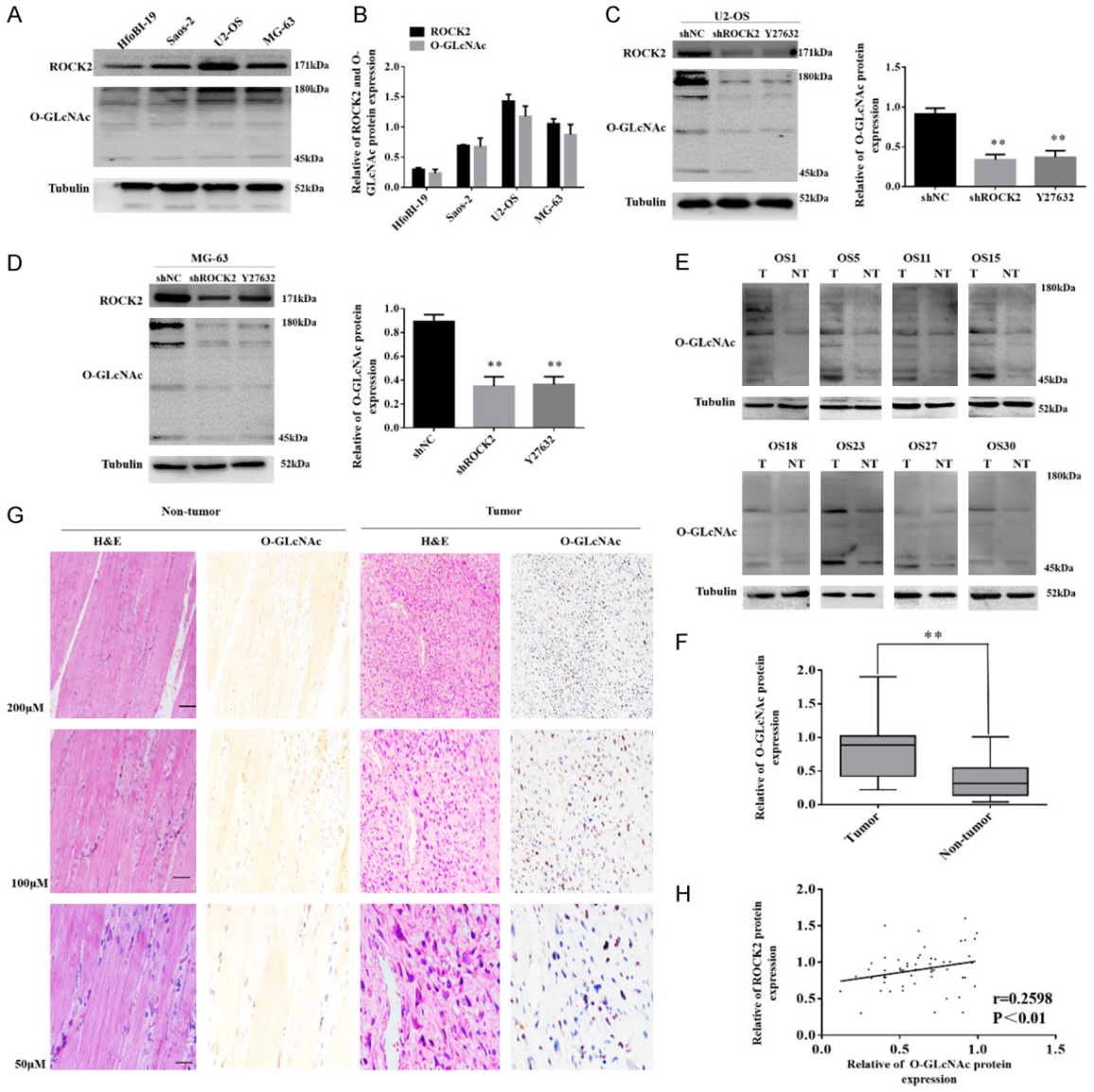
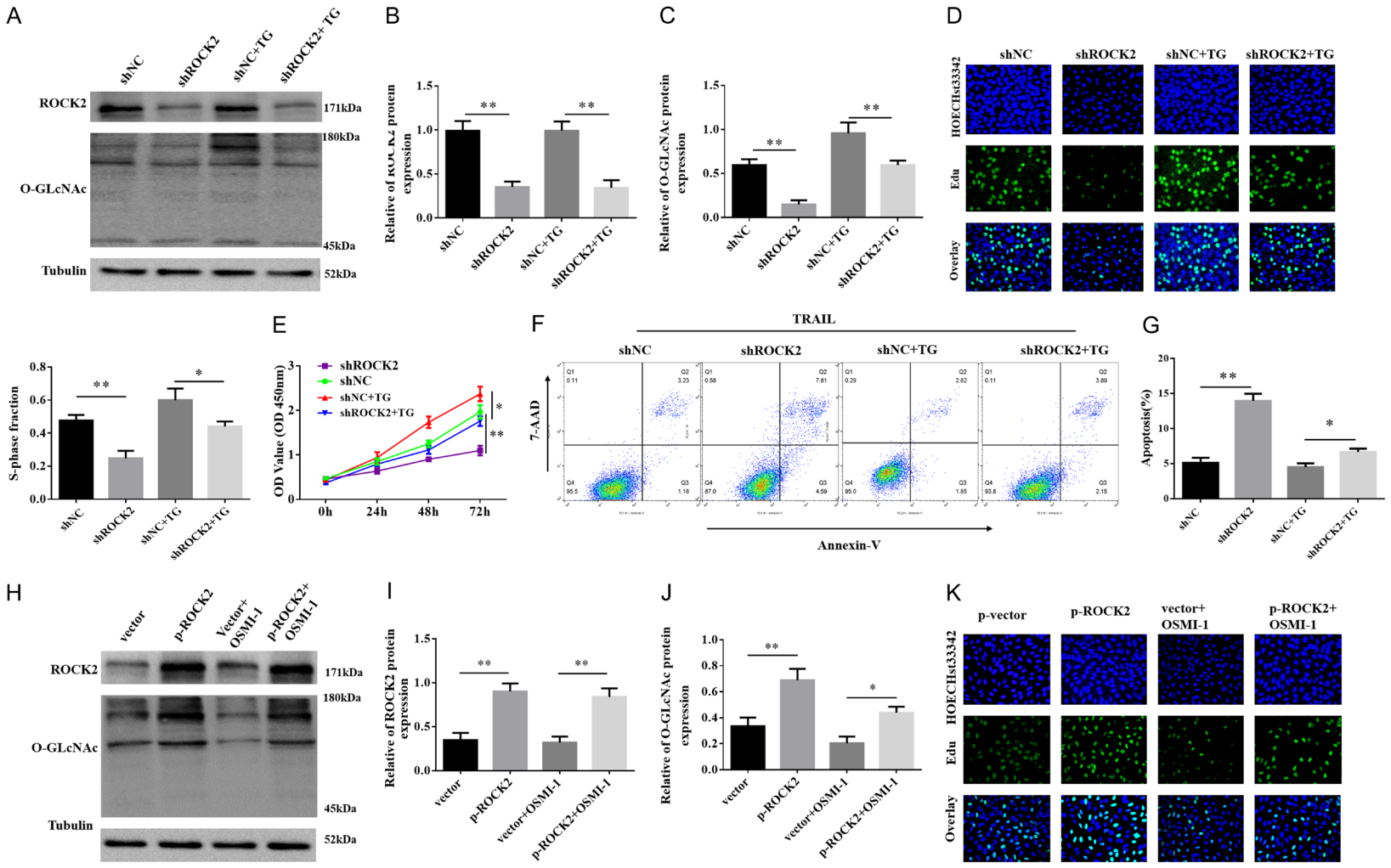


Figure 6. Stable ROCK2 knockdown reduced O-GlcNAcylation levels. A, B. O-GlcNAcylation and ROCK2 protein expression levels were determined in various osteosarcoma (OS) cells. C, D. Western blotting was performed to quantify O-GlcNAcylation in OS cells. Cells were stably transfected with shROCK2 or treated with the ROCK2 inhibitor, Y-27632, $^{*}P<0.05$, $^{**}P<0.01$. E, F. Western blotting quantification of O-GlcNAcylation and ROCK2 protein levels in OS tissues (n=30) and paired non-tumor tissues (n=30). Tubulin was used as a loading control. G. Representative quantification (right) and images (left) of O-GLcNAc staining in 106 paired OS tissues. Scale bar: 50 μm. H. Scatter plots show a positive correlation between O-GlcNAcylation and ROCK2 protein expression levels in OS tissues, $^{*}P<0.05$.

OGT levels by affecting its degradation via ubiquitin. Immunoprecipitation results demonstrated the direct binding of U2-OS and MG-63 cells and endogenous OGT and ubiquitin (**Figure 8C**). Confocal microscopic analysis confirmed the co-localization of ubiquitin and OGT in U2OS and MG-63 cells (**Figure 8D**). In addition, endogenous OGT protein levels significantly


A
shNC
shROCK2
shNC+TG
shROCK2+TG
ROCK2
171kDa
180kDa
O-GLcNAc
45kDa
Tubulin
52kDa
B
Relative of ROCK2 protein expression
C
Relative of O-GLcNAc protein expression
D
HOECHst33342
Edu
Overlay
E
S-phase fraction
OD Value (OD 450nm)
0h
24h
48h
72h
F
TRAIL
7-AAD
Annexin-V
G
Apoptosis(%)
H
vector
p-ROCK2
Vector+ OSMI-1
p-ROCK2+ OSMI-1
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Relative of ROCK2 protein expression
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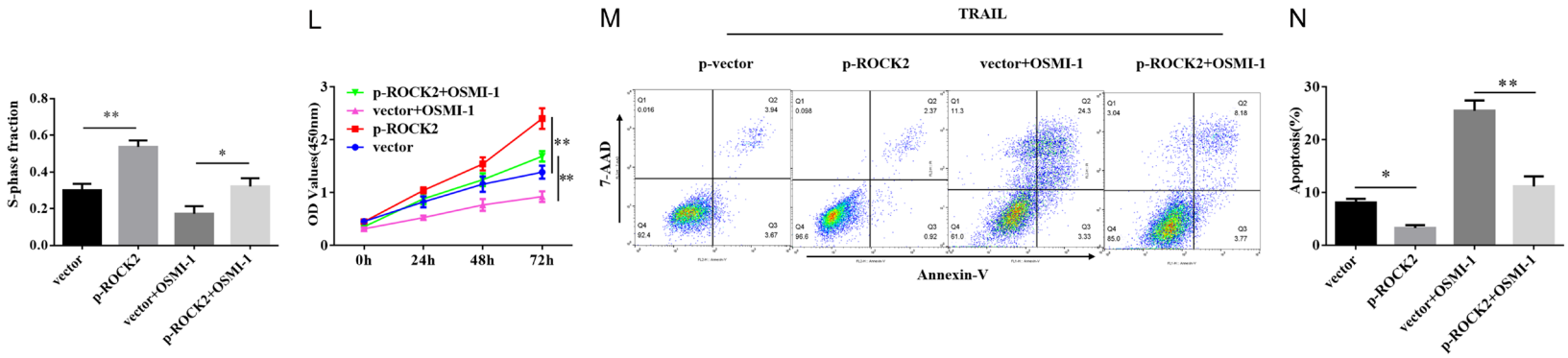

Figure 7. O-GlcNAcylation level was a key factor in ROCK2-mediated osteosarcoma cell proliferation. A-C. U2-OS cells were transfected with the indicated plasmid or a glycosyl agonist (Thiamet G, TG), *P<0.05. O-GlcNAcylation and ROCK2 protein were detected. D, E. EdU and CCK8 assays revealed that increased O-GlcNAcylation inhibited the decline in proliferation caused by ROCK2 knockdown, *P<0.05, **P<0.01. Scale bar: 100 μm. F-J. Apoptosis was analyzed by flow cytometry. H-J. Saos-2 cells were transfected with the indicated plasmid or treated with the glycosylation inhibitor, OSMI-1. O-GlcNAcylation and ROCK2 protein expression levels were detected, **P<0.01. K, L. CCK8 and EdU assays revealed that a decrease in O-GlcNAcylation levels decreased ROCK2-induced cell proliferation, *P<0.05, **P<0.01. Scale bar: 100 μm. M, N. Apoptosis was analyzed by flow cytometry (n=3), *P<0.05, **P<0.01.

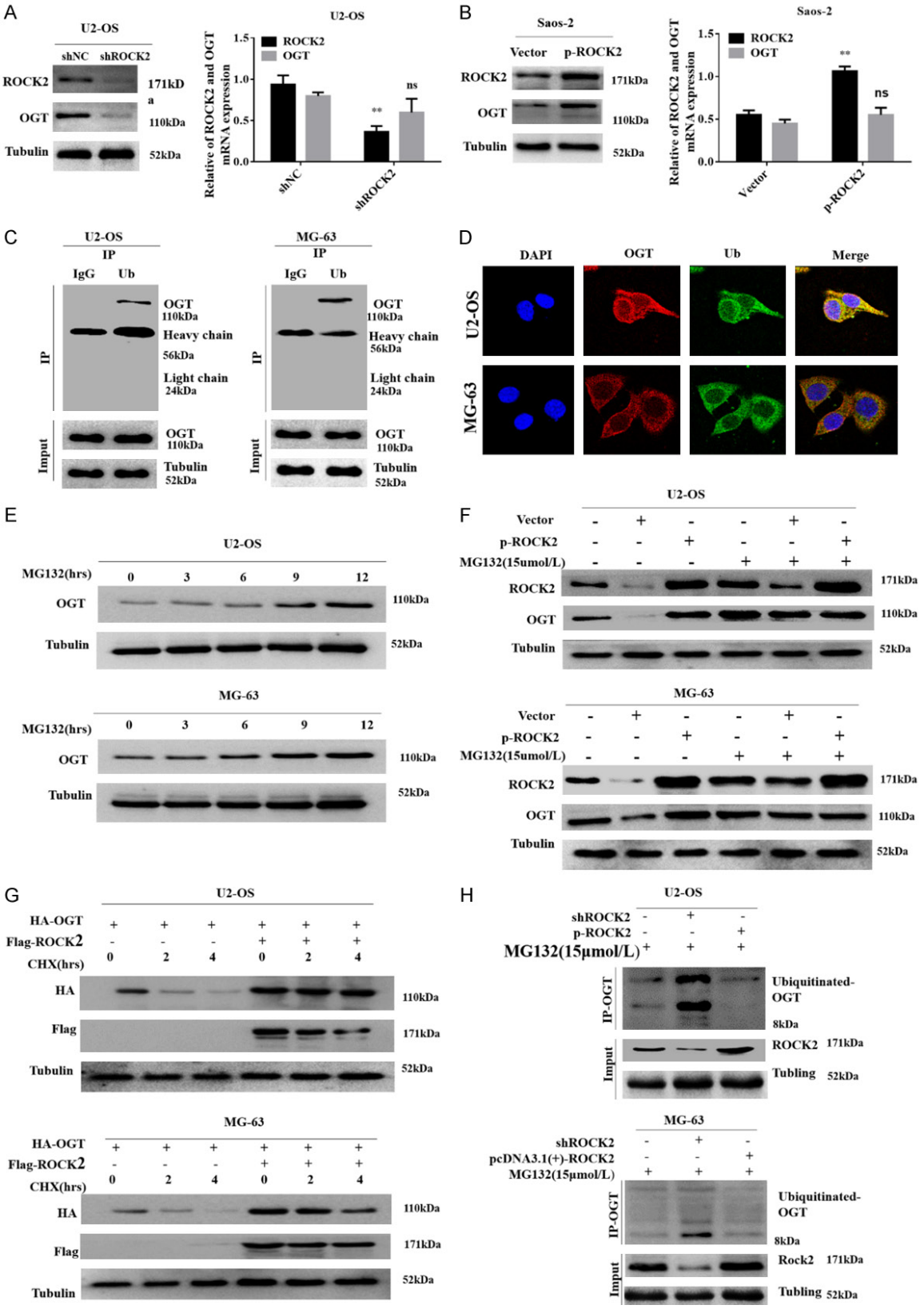

Figure 8. ROCK2 stabilized O-GlcNAc transferase expression by modifying its ubiquitination and degradation. A. O-GlcNAc transferase (OGT) expression in U2-OS cells stably transfected with shNC or shROCK2 plasmids was quantified by western blotting, *P<0.05, **P<0.01. B. OGT protein levels in Saos-2 cells stably transfected with the ROCK2

plasmid. C, D. Co-immunoprecipitation of endogenous ubiquitin and OGT. Ubiquitin and OGT colocalization. Scale bar: 9 mm. E. U2-OS cells were incubated with MG132 (15 mmol/L) as shown, followed by OGT quantification. F. U2-OS cells transfected with shROCK2 or p-ROCK2 plasmids were treated with 15 μM MG132. Cells were collected at 6 hours and immunoblotted with the indicated antibodies. G. U2-OS and MG-63 cells were transfected with an expression plasmid encoding HA-OGT, with or without the Flag-ROCK2 plasmid. The cells were then exposed to CHX (20 μM) for the indicated times, and OGT degradation was detected using an anti-HA antibody. H. Lysates from U2-OS and MG-63 cells transfected with shROCK2 or p-ROCK2 plasmids were immunoprecipitated with anti-ubiquitin and immunoblotted with anti-OGT. Cells were treated with MG132.

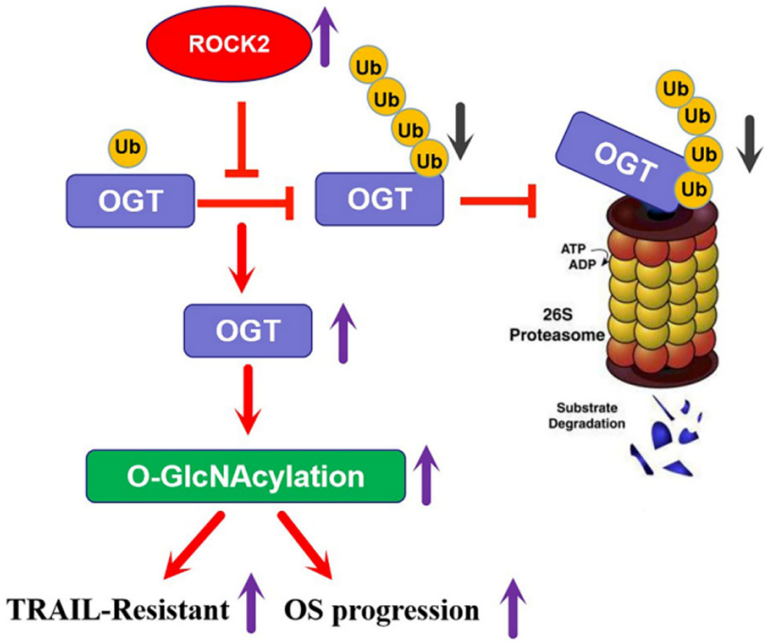

Figure 9. Proposed model by which ROCK2 mediates osteosarcoma (OS) cell progression and TRAIL resistance by modulating O-GlcNAc transferase (OGT) degradation.

increased in U2OS and MG-63 cells after treatment with the proteasome inhibitor, MG132, within a specified time frame (**Figure 8E**). These results suggested that the ubiquitin-proteasome system (UPS) degrades OGT in OS cells.

Moreover, pcDNA3.1(+)- ROCK2, and shROCK2 vectors were transfected into U2-OS and MG-63 cells to determine whether ROCK2 regulates protein degradation. The effects of ROCK2 on OGT expression levels were evaluated in the presence of MG132. ROCK2 down/upregulation had no notable effect on OGT expression in MG132-treated U2-OS cells (**Figure 8F**). The half-life of ectopic OGT was significantly higher in ROCK2-knockdown cells than in normal U2-OS cells, as shown by the degradation kinetics analysis (**Figure 8G**). ROCK2 was, therefore, shown to participate in OGT degradation. Finally, to examine the process by which ROCK2 stabilizes OGT expression, shROCK2- or pcDNA3.1(+)-ROCK2-transfected U2-OS and MG-63 cells were treated with MG132. Immunoprecipitation results demonstrated an increase in OGT ubiquitination levels due to ROCK2 inhibition. Conversely, ROCK2 overexpression decreased OGT ubiquitination levels (**Figure 8H**). From these results, we can conclude that ROCK2 modified OGT degradation and ubiquitination, subsequently stabilizing OGT expression (**Figure 9**).

Discussion

OS is a major type of primary malignant bone tumor. It usually follows an active clinical process and represents a major treatment challenge. Studies have shown that OS progress and apoptosis are linked to dysregulated expression of oncogenes and tumor suppressor genes. Understanding the molecular mechanisms associated with OS progression could, therefore, provide new information to facilitate the identification of relevant therapeutic targets.

ROCK2 mediates the function of the small GTPase, RhoA. It is overexpressed in various tumors (melanoma, colorectal cancer, hepatocellular carcinoma) and influences tumor migration and invasion by modulating signaling pathways [28, 29]. Our previous studies have shown that ROCK2 overexpression also influences the metastasis and invasion of hepatocellular carcinoma [27]. Additionally, recent evidence has suggested that ROCK2 has a role in modulating the growth and migration of tumor cells [30]. ROCK2 regulates cell division and affects breast cancer growth via BRCA2 binding [31]. Our previous studies have shown that reducing

ROCK2 levels can inhibit the proliferation of hepatocellular carcinoma cells by regulating CCAAT enhancer-binding protein delta [12]. The present study is the first to show that ROCK2 expression is linked to the proliferation of OS cells and that ROCK2 downregulation can inhibit OS cell proliferation. In particularly, high levels of ROCK2 expression were significantly correlated with advanced tumor stage and poor survival in OS patients. Moreover, functional analysis showed that ROCK2 downregulation markedly inhibited OS cell proliferation *in vitro* and tumor growth *in vivo*. Furthermore, the downregulation of ROCK2 rendered OS cells sensitive to TRAIL-induced apoptosis. The data from this study suggest that ROCK2 may have a vital role as an oncogene and may important in the development and progression of OS.

We also studied the mechanism by which ROCK2 affects OS progression. Recent studies have shown that O-GlcNAcylation has a vital role in cancer progression. Here, our results indicated that O-GlcNAcylation has a critical role in ROCK2-mediated OS progression [32], a conclusion based on several observations. Firstly, ROCK2 downregulation caused a significant reduction in the level of O-GlcNAcylation in OS cells. Secondly, O-GlcNAcylation and ROCK2 levels were significantly increased in OS tissues compared with adjacent normal tissues and they were positively correlated. Thirdly, ROCK2 knockdown significantly decreased U2-OS cell proliferation by increasing O-GlcNAcylation levels. Finally, decreased O-GlcNAcylation levels led to a decrease in ROCK2-induced cell proliferation. In summary, these data showed that the O-GlcNAcylation modification via ROCK2 affected OS progression.

Previous studies have shown that OGT-catalyzed O-GlcNAcylation is a reversible post-translational modification. O-GlcNAcylation is involved in intracellular signal transduction, epigenetic regulation, and transcription, and OGT expression levels are related to metabolic diseases and cancer. In turn, OGT expression, is modulated by post-translational modification, together with ubiquitination [33]. Importantly, this study investigated the mechanism whereby ROCK2 regulates OGT expression. As OGT is degraded by the ubiquitin/proteasome system, our previous studies indicated that ROCK2 influences CDC25A and MMP2 degradation in hepatocellular carcinoma cells via the UPS [26, 27]. Therefore, we investigated whether ROCK2 could modulate OGT expression and ubiquitination in OS cells. Our findings confirmed that ROCK2 modulated OGT expression through the UPS, thus controlling OGT protein stabilization. We initially confirmed that the UPS degraded OGT in OS cells. We then found that ROCK2 participated in the degradation of OGT and could affect the stabilization of OGT. Finally, we found that ROCK2 overexpression inhibited OGT degradation and ubiquitination. These findings demonstrated a new process by which ROCK2 may regulate OGT protein stability via the UPS.

In conclusion, this study presents the first evidence of the vital role of ROCK2 in OS progression. ROCK2 knockdown facilitated TRAIL activation and mediated OS cell apoptosis. The underlying mechanism involved the regulation of O-GlcNAcylation by ROCK2 through modulation of OGT degradation. Based on these data, there is the potential for OS diagnostic and therapeutic tools to utilize ROCK2 as a biomarker.

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Disclosure of conflict of interest

None.

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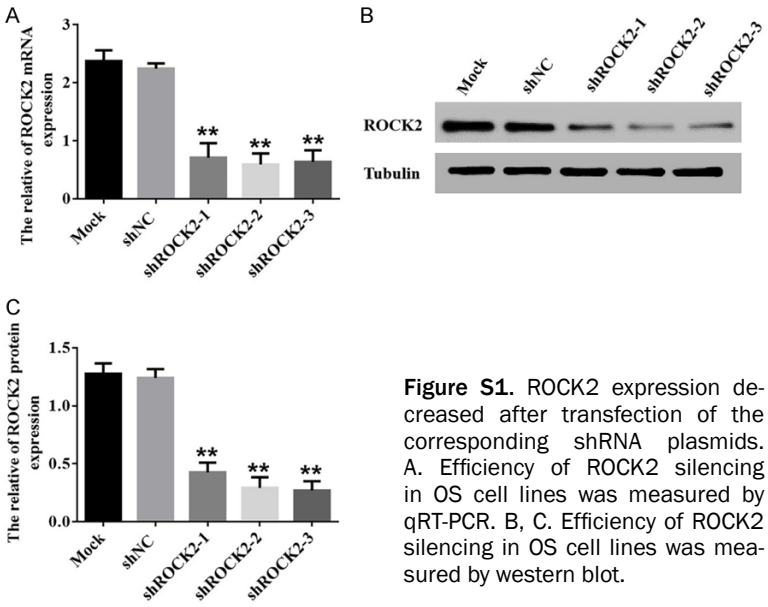

Figure S1. ROCK2 expression decreased after transfection of the corresponding shRNA plasmids. A. Efficiency of ROCK2 silencing in OS cell lines was measured by qRT-PCR. B, C. Efficiency of ROCK2 silencing in OS cell lines was measured by western blot.

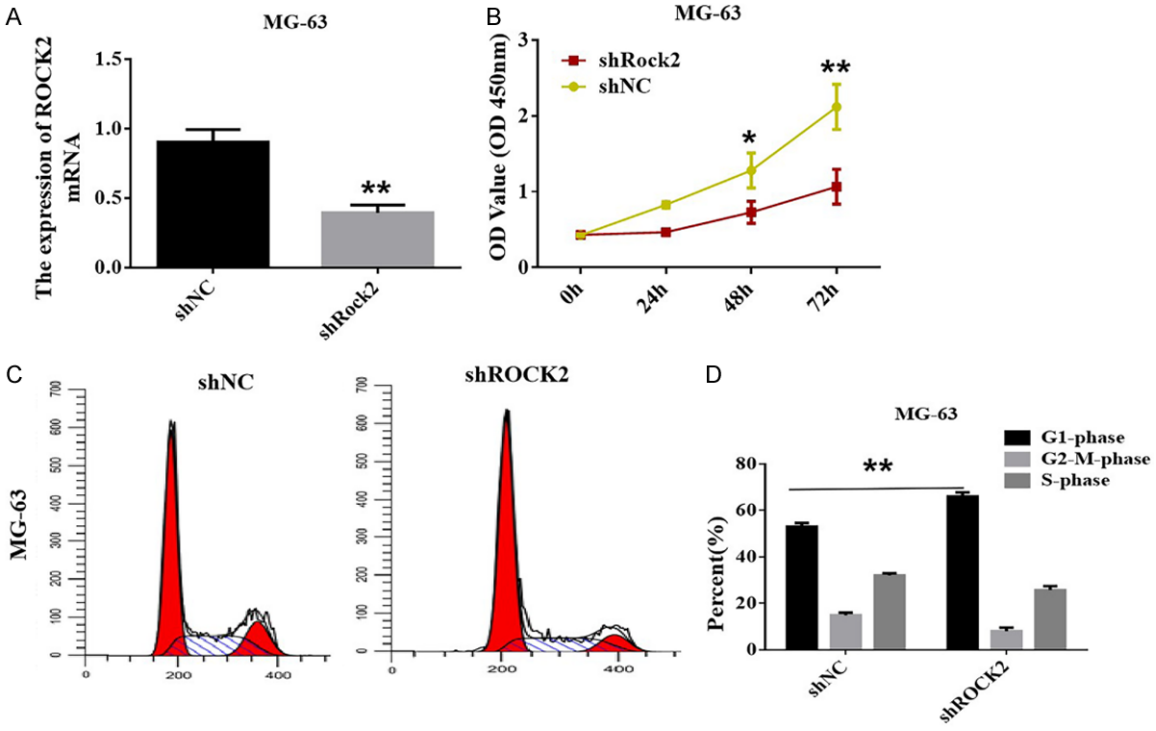

Figure S2. ROCK2 promotes osteosarcoma (OS) tumour growth inMG-63 cells. A. Efficiency of ROCK2 silencing in OS cell lines was measured by qRT-PCR in MG-63 cells. B. Proliferation capacities were detected by CCK8 assays in MG-63 cells transfected with shNC or shROCK2 plasmids $^{**}P<0.01$. C, D. Detection for cell cycle of OS cells after silencing ROCK2 expression in MG-63 cells. $^{*}P<0.05$.

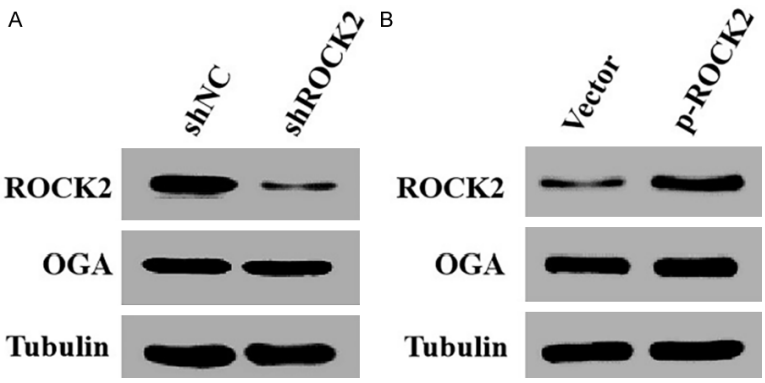

Figure S3. ROCK2 does not affect the expression of OGA. A. Western blot analyses were used to detect OGA expression in U2-OS cells stably transfected with the shNC or the shROCK2 plasmid. B. Western blot analyses were used to detect OGA expression in Saos-2 cells stably transfected with the ROCK2 plasmid.

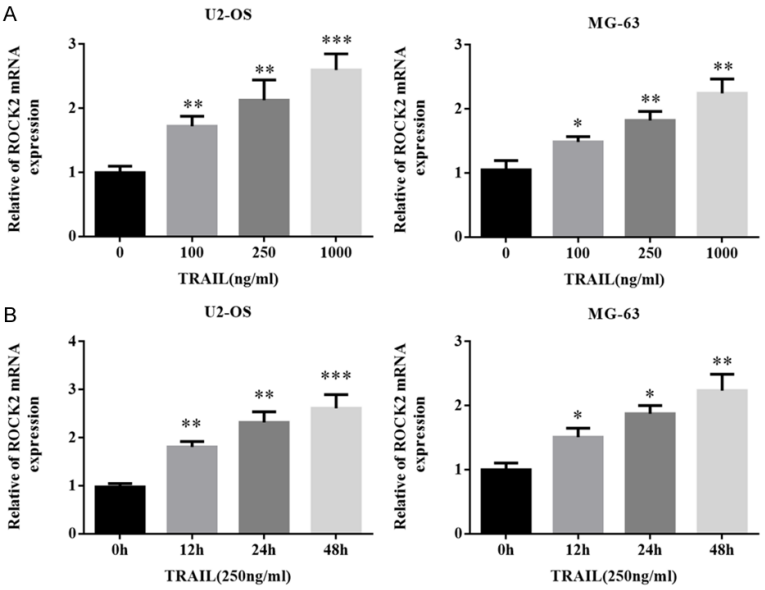

Figure S4. A. U2-OS and MG-63 cells were incubated with different doses of TRAIL for 24 hours. qRT-PCR was utilized to test ROCK2 expression, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. B. U2-OS and MG-63 cells were incubated with TRAIL (250 ng/mL) for different periods of time. qRT-PCR was utilized to test ROCK2 expression, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.