

TRIM2 regulates the development and metastasis of tumorous cells of osteosarcoma

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Abstract. The present study aimed to investigate candidate genes involved in the development and metastasis of osteosarcoma. Candidate genes were screened preliminarily from the Gene Expression Omnibus database and then validated using actual tumor tissues collected from patients with osteosarcoma. The cells were prepared and transfected with specific gene-targeted small interfering RNA followed by an MTS assay for cell viability detection and Transwell assays for cell migration and invasion capacity detection. The cell apoptosis was determined by flow cytometry and the protein level of the genes was detected by western blot analysis. An *in vivo* nude model was used and injected with cells to detect the functions of the genes. Transcriptome sequencing was performed to verify the regulation network, followed by reverse transcription-quantitative polymerase chain reaction and western blot analyses for validation. Increased tripartite motif-containing protein 2 (TRIM2) was detected in the osteosarcoma tumor tissues compared with normal tissues. The inhibition of TRIM2 induced lower cell viability and cell invasion capacity, and increased the rate of cell apoptosis. Decreased TRIM2 also inhibited the development and metastasis of osteosarcoma in the nude mouse models. The transcriptome sequencing revealed that the regulation of TRIM2 may be correlated with genes, Sirtuin 4, DNA damage inducible transcript 3, cAMP responsive element binding protein 5, G protein-coupled receptor 65 (GPR65) and ADP-ribosyltransferase 5. Western blot analysis indicated that TRIM2 regulated the development and metastasis of osteosarcoma via the phos-

phoinositide 3-kinase/protein kinase B signaling pathway. Therefore, TRIM2 performs important functions in regulating the development and metastasis of osteosarcoma.

Introduction

Osteosarcoma is a primary mesenchymal tumor, which is considered an aggressive type of malignancy. Metastasis is an important process in malignant tumor formation (1,2). Conventional chemotherapy is not efficient in patients with osteosarcoma, and ~80% of patients with distant metastasis develop lung metastasis (3,4). Evidence has shown that 10-20% of patients with osteosarcoma are at risk of developing pulmonary metastasis, and ~40-50% of the patients develop pulmonary heterochrony although without metastasis (5). By contrast, patients with osteosarcoma with metastasis usually present with a lower 5-year survival rate, compared with those patients without metastasis (6). Conventional chemotherapy is not efficient for treatment of the disease, and 30-50% of patients eventually succumb to mortality as a result of the pulmonary metastasis (7). As the occurrence and development of the disease is closely associated with molecular biology, investigating the molecular mechanism of osteosarcoma has significant value for developments in early diagnosis and treatment.

The underlying biological mechanism of osteosarcoma tumors and metastasis has been widely investigated, and genes and pathways involved in osteosarcoma have been identified. For example, Notch proteins are important for normal bone development and homeostasis, and also for angiogenesis (8,9). Dysregulation of the Notch pathway is present in human diseases associated with congenital skeletal abnormalities. The Notch pathway genes have been found to be overexposed in osteosarcoma cells, compared with normal bone cells, which include Hes related family bHLH transcription factor with YRPW motif 1 (HEY1), Hes family bHLH transcription factor 1 (HES1) and NOTCH2 (10-12). Runt related transcription factor 2 (RUNX2) is an important transcription factor involved in osteoblast differentiation (13). Previous studies have suggested that the Notch pathway transcription of Runx2 is decreased via HES and HEY proteins. As a result, it is hypothesized that the increase of immature osteoblasts may be one of the potential factors in malignant transformation (14,15).

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The Wnt pathway manages several processes, including cell growth, normal bone development and carcinogenesis (16,17). According to previous studies, the Wnt pathway was found to be highly activated in human osteosarcoma, therefore, it may be also potential pathway involved in osteosarcoma (18-20). Mammalian target of rapamycin (mTOR), one of the phosphoinositide 3-kinase (PI3K) family members, is important in the regulation of cell cycle, growth and development (21,22). It was shown that, by suppressing the expression of mTOR or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α , growth arrest was induced in a murine osteosarcoma model (23). Based on the evidence so far, molecular regulation appears to be important in the occurrence and development of osteosarcoma. The investigation of genes which are correlated with osteosarcoma are likely to be beneficial for the diagnosis and treatment of the disease.

RNA sequencing (RNA-seq) is an advanced technique based on the deep-sequencing approach. It provides a relatively unbiased and more precise measurement for the quantitation of the transcripts and their isoforms (24). As RNA-seq technology presents apparent advantages against conventional hybridization-based microarrays, it has been widely applied and utilized in the screening the differentially expressed genes in multiple experimental conditions. In the present study, candidate genes involved in osteosarcoma were screened by analyzing the Gene Expression Omnibus (GEO) dataset. A large number of differentially expressed genes were screened based on the results of the microarray (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21257>). The present study also verified the differentially expressed genes, which have not been reported in osteosarcoma, in six cell lines (hFOB, SW1353, SAOS-2, MG-63, 143B and U2-OS) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The results indicated that the expression levels of Kelch domain containing 1 (KLHDC1), TRIM2, Laccase domain-containing protein 1 (LACC1), Mitochondrial transcription factor A (TFAM), Laminin subunit β 1 (LAMB1), Stanniocalcin 2 (STC2) and Vimentin (VIM) in the osteosarcoma cell lines were significantly higher than those in normal osteoblasts (data not shown). As the TRIM2, STC2 and VIM genes were significantly expressed in osteosarcoma cells, they were selected for metastatic analysis and analysis of prognosis. The prognosis and metastasis analyses indicated that TRIM2 was important in the pathogenic process. Relevant cell assays and RNA-seq were performed in order to verify the mechanism underlying the effect of TRIM2.

Materials and methods

Prognosis and metastasis analysis. The expression profiles and clinical data of the patients with osteosarcoma were downloaded from the NCBI GEO database (accession no. GSE21257). The methods of pretreatment of specimens, RNA isolation, and microarray assay setup were described on the website. The expression values were used for statistical analysis. The expression differences are presented via box-plots. Differences in overall survival rates between 'high' and 'low' expression groups were compared using Kaplan-Meier curves, with P-values calculated via the log-rank test, using the Survival package in R version 3.2.3 (<https://www.r-project.org/>).

Table I. Clinicopathological information of the 53 patients with osteosarcoma.

Characteristic	n
Age (years)	
<20	42
>20	11
Sex	
Male	34
Female	19
Distant metastasis	
Absent	19
Present	34
Huvos grade	
Unknown	6
1	13
2	16
3	13
4	5
Status	
Alive	30
Deceased	23

Immunohistochemical staining. Fresh primary osteosarcoma samples and paired adjacent non-tumor soft tissues from 53 patients undergoing surgical resection at Zhuhai Hospital, Jinan University (Zhuhai, China) between April 2010 and June 2011 were collected, formalin-fixed and paraffin-embedded. The present study was approved by the Institutional Research Ethics Committee of Jinan University. Informed consent was provided by all participants prior to the study. The clinicopathological information of the 53 patients were recorded and are presented in Table I.

The formalin-fixed and paraffin-embedded (FFPE) tissue sections (5 μ m) were subjected to antibody thermal remediation, the activity of endogenous peroxidase was inhibited by hydrogen peroxide and then closed with goat or mouse serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following dewaxing and hydration. The primary antibodies TRIM2 (cat. no. ab3942; 1:1,000), E-cadherin (cat. no. ab76055; 1:1,000), N-cadherin (cat. no. ab18203; 1:500) or Vimentin (cat. no. ab92547, 1:500) (all from Abcam, Cambridge, UK) diluted in 1X PBS solution were used to cover the tissue sections and incubated overnight at 4°C. Anti-mouse immunoglobulin G (IgG) (cat. no. sc-516102; 1:2,000), anti-rabbit IgG (cat. no. sc-3753; 1:500) or anti-goat IgG (cat. no. sc-2489; 1:500) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) secondary antibodies were used to bind the specific primary antibodies at 37°C for 30 min, and DAB was applied for coloration. The stained tissues were observed using IX71 inverted microscopy (Olympus Corporation, Tokyo, Japan). Six samples for each group were used for the immunohistochemical staining.

Cell culture. The hFOB, U2-OS, SW1353, MG63, 143B and SAOS-2 human osteosarcoma cell lines provided by American

Type Culture Collection (Manassas, VA, USA) were used in the present study. The cells were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.). The medium was supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and the cells were all maintained at 37°C in 5% CO₂. On reaching 70-80% confluence, the cells were washed with PBS and detached with 0.25% trypsin/0.2% EDTA. Cell morphology was viewed under a light microscope, and suspended to the concentration of 1x10⁶/ml.

Lentivirus production and transfection. The night prior to transfection, 3x10⁶ 293T (CRL-3216™; American Type Culture Collection, Manassas, VA, USA) cells were seeded into 10 cm dishes. Small interfering RNA (siRNA) sequences targeted to TRIM2 were cloned into the PLKO.1 lentiviral vector (Dharmacon, Inc., Lafayette, CO, USA). The cells were transfected with the TRIM2 siRNA-expressing PLKO.1 lentiviral vectors and control empty vector along with packaging vectors (psPAX2 and pMD2.G) using FuGENE-6 transfection reagent (Promega Corporation, Madison, WI, USA). The lentivirus-containing supernatants were harvested 72 h following transfection and were filtered through 0.45-μm PVDF filters. The supernatant was then concentrated by ultracentrifugation (at 100,000 x g for 2 h at room temperature) in a Beckman Optima L-90K ultracentrifuge (Beckman Coulter, Inc., Brea, CA, USA). The virus-containing pellet was dissolved in DMEM, aliquoted and stored at -80°C. The sequences of the siRNAs were as follows: siTRIM2-1 (human), forward, 5'-CCTGGAACG GTACAAGAAT-3' and reverse, 5'-ATTCTTGTACCGTTCC AGG-3'; siTRIM2-2 (human), forward, 5'-CCAGTGAAGCA CCAACAT-3' and reverse, 5'-ATGTTGGTGCCTTCACTGG-3'.

The U2OS and MG63 cells were infected by adding 1 ml of concentrated virus supplemented with 2 μg/ml polybrene to 4x10⁵ cells in 12-well plates. After 36 h at room temperature, the viral supernatant was replaced with standard growth medium, and transduction efficiency was monitored by GFP expression 48 h following replacement of the virus-containing medium with normal medium.

Cell viability assay. Cell viability was examined using the MTS included in the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation) according to the manufacturer's protocol. The U2OS and MG63 cells were seeded in a 96-well plate at a cell density of 200 cells per well. The cells were incubated with 15 μl of MTS reagent solution for 4 h and the produced formazan was measured at 490 nm in a Sunrise Basic Tecan cell plate reader with Magellan 6 software (both from Tecan Group, Ltd., Grödig, Austria).

Transwell migration and invasion assays. For the Transwell migration assays, 2.5-5x10⁴ cells were plated in the top chamber with a non-coated membrane (8-μm pore size; Corning Incorporated, Corning, NY, USA). For the cell invasion assay, the Transwell chambers were pretreated with Matrigel (EMD Millipore, Billerica, MA, USA) and the cells were resuspended at a density of 2x10⁵ cells/ml in serum-free medium at 48 h post-transfection. In the two assays, the upper chamber was filled with 300 μl cell suspensions and the lower well was filled with complete medium. After 24 h, the cells in

the upper chambers were removed with a cotton swab, and the migrated or invasive cells in the lower chambers were fixed with 4% formaldehyde and stained with crystal violet. The cells were observed and counted by IX71 inverted microscopy (Olympus Corporation).

Cell apoptosis assay. The cells were seeded in 6-well plates and incubated for 48 h. The cells were detached with trypsin and the apoptosis assay was performed using an Annexin V assay kit according to the manufacturer's protocol. The assay was performed with a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The apoptosis data were analyzed with FACSCanto II flow cytometer software (BD Biosciences).

RNA-seq. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. mRNA was isolated from the total RNA using oligo(dT) magnetic beads, and fragmentation buffer was subsequently added for the fragmentation of mRNA. The mRNA fragments were subsequently used as templates for first-strand cDNA synthesis using random primers. The second-strand cDNA was synthesized using DNA polymerase I. Adaptors were ligated to the fragments and 200-bp fragments were subsequently enriched by PCR amplification. The quality of the library was confirmed using the Agilent 2100 Bioanalyzer. The sequencing was performed on the Hiseq2000 platform.

The primary raw reads were trimmed of adaptors and filtered to remove low quality reads using SOAP nuke software version 1.5.3 (<http://soap.genomics.org.cn>). The length of raw reads used were 150-bp in pair end. The cleaned reads were aligned to the hg19 reference genome using hierarchical indexing for spliced alignment of transcripts. DEGseq2 were used to detect the differentially expressed genes. The P-value threshold was determined by the false discovery rate (FDR) using an FDR threshold ≤0.001. Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using the Blast2GO program version 5.1 (<https://www.blast2go.com/>).

RT-qPCR assay. Total RNA was prepared using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The genomic DNA-free RNA was then converted into cDNA using M-MLV Reverse Transcriptase (Promega Corporation), according to the manufacturer's protocol. PCR was carried out on the CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a 20 μl reaction mixture containing 10 μl GoTaq® Green Master Mix (Promega Corporation), 1.5 μl cDNA, 0.5 μl each primer and 7.5 μl nuclease-free water under the following conditions: Initial denaturation at 95°C for 120 sec, followed by 40 cycles of denaturation at 95 °C for 15 sec and extension at 60°C for 30 sec. The primer sequences were as follows: Sirtuin 4 (SIRT4), forward, 5'-AAG CCTCCATTGGGTTATTTGT-3' and reverse, 5'-TGTAGTCTG GTATCCCCGATTC-3'; DNA damage-inducible transcript 3 (DDIT3), forward, 5'-GAACGGCTCAAGCAGGAA ATC-3' and reverse, 5'-ATTCACCATTCGGTCAATCAGAG-3'; cAMP responsive element binding protein 5 (CREB5), forward,

5'-AAAGACTGCCCAATAACAGCC-3' and reverse, 5'-AAGCTGGGACAGGACTAGCA-3'; G protein-coupled receptor 65 (GPR65), forward, 5'-GAAATGGCAAATCAACCTCAAC-3' and reverse, 5'-TTCCTTGTTTTCCGTGGCTT-3'; frizzled class receptor 8 (FZD8), forward, 5'-GGAGTGGGGTTACCTGTTGG-3' and reverse 5'-CTTGCGTGTCTGTTGAA-3'; SHC adaptor protein 2 (SHC2), forward, 5'-TACGTCGTGCGGTACATGG-3' and reverse, 5'-AAGCGAAGGTTGCTCTTGC-3'; ADP-ribosyltransferase 5 (ART5), forward, 5'-CAGATTGTAATGCCACCCTC-3' and reverse, 5'-CAAAGCCAGAATGGAATAGC-3'; TRIM2, forward, 5'-AGGACAAGACGGTGAGCTG-3' and reverse, 5'-CTCTTCACGCCTTCTGTGGT-3'; and GAPDH, forward, 5'-GAGTCAACGGATTGCTCGT-3' and reverse, 5'-CCCAGTAGCAGTTCAGGTGG-3'. GAPDH was used as the internal control. The relative expression levels of genes were quantified using the $2^{-\Delta\Delta C_q}$ method, as described previously (25). At least three biological repeats and three technical replicates were performed.

Western blot analysis. Total protein from the osteosarcoma cells was lysed and extracted using RIPA lysis buffer and quantified using a BCA Protein Assay kit (both from Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The protein samples (40 μ g) were separated by 10% SDS-PAGE and transferred onto NC membranes (EMD Millipore). The membranes were blocked in 5% non-fat milk and incubated with the following primary antibodies: TRIM2 (cat. no. ab3942; 1:1,000), matrix metalloproteinase (MMP)9 (cat. no. ab38898; 1:1,000), MMP2 (cat. no. ab37150; 1:500), N-cadherin (cat. no. ab18203; 1:500), E-cadherin (cat. no. ab76055; 1:500), Snail (cat. no. ab180714; 1:1,000), Vimentin (cat. no. ab92547; 1:1,000), protein kinase B (AKT; cat. no. ab8805; 1:500), phosphorylated-AKT (cat. no. ab38449; 1:1,000), protein kinase A (PKA; cat. no. ab75991; 1:2,000), CREB (cat. no. ab31387; 1:1,000), phosphorylated-CREB (cat. no. ab32096; 1:3,000) and anti-GAPDH (cat. no. ab9485; 1:2,000) (all from Abcam) at 4°C overnight. The membranes were then incubated with secondary antibodies, anti-rabbit IgG (cat. no. ab205718; 1:2,000), anti-goat IgG (cat. no. ab6566; 1:2,000) or anti-mouse IgG (cat. no. ab6728; 1:2,000) (all from Abcam) for 2 h at room temperature. The complexes were detected by ECL (Forevergen Biosciences, Guangzhou, China). GAPDH was applied as the internal standard. Six samples for each group were used for western blot analysis.

In vivo nude mouse models. All animal experiments were approved by the Institute Research Medical Ethics Committee of Sun Yat-Sen University (Guangzhou, China). Male BALB/C nude mice (age, 5-6 weeks; weight, 18-22 g) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). Animals were maintained in a specific pathogen-free environment throughout the experiments under the following conditions: Controlled humidity, 50 \pm 10%; temperature, 23 \pm 2°C; 12-h light/dark cycle and *ad libitum* access to food and water. U2OS cells (2 \times 10⁶) in a volume of 100 μ l were subcutaneously injected into the caudal veins of the mice. Post-implantation, five of the mice were sacrificed and the tumors were surgically dissected at 5 days. The weight and size of the cancerous tissues were then examined.

Statistical analysis. Data are presented as the mean \pm standard error of the mean unless otherwise indicated. Significance was established using SPSS PASW Statistics 18.0 (SPSS, Inc., Chicago, IL, USA) software and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) software. For data with a normal distribution, comparisons were performed using independent t-tests, one-way analysis of variance (ANOVA) and two-way ANOVA, with the LSD-t-test used for the post hoc test. The non-parametric Mann-Whitney U test, K-S test, Kruskal-Wallis test and Wilcoxon test were performed if the data did not have a normal distribution, and the Nemenyi test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. The significant level was corrected if necessary.

Results

High expression of TRIM2 in osteosarcoma tissue is correlated with lower survival rates and induction of metastasis. The expression profiles were collected from the NCBI GEO (GSE21257). By combining the expression profiles and the clinical properties, the association between the expression levels of TRIM2, STC2 and VIM and survival rate were analyzed. The results showed that the survival difference was statistically significant between the high and low expression level of TRIM2 and VIM. Regardless of whether overall survival or distal metastasis-free survival rate, a high expression level of TRIM2 and VIM led to a reduced rates of survival and distal metastasis-free survival (Fig. 1A-C). Higher expression levels of TRIM2 and VIM reduced the survival percentage by 50% at 40 days. However, for STC2, although the survival percentage was reduced when its expression level was high, the difference between a low expression level of STC2 was not significant ($P > 0.05$). Therefore, the evidence confirmed that a high expression of TRIM2 was correlated with lower survival rate and distal metastasis-free survival rate. By contrast, when the expression level of TRIM2 was compared in primary tumor and metastatic tumor tissues, only the metastatic tissues exhibited a significantly higher level of TRIM2, compared with the primary tumor tissues or in tumor tissues with metastasis within 5 years (Fig. 1D and E). Therefore, actual osteosarcoma tumor tissues and normal tissues were collected for the immunohistochemical staining to determine the expression level of TRIM2. The TRIM2 molecules were stained a brown color specifically. The results showed that the level and density of the TRIM2 molecules were significantly higher in the osteosarcoma tissues compared with the normal tissues (Fig. 1F). According to the preliminary analysis, it was concluded that TRIM2 in osteosarcoma tissues performs specific functions that lead to lower survival rate and induces metastasis. Therefore, the high expression level of TRIM2 may also be an indicator of osteosarcoma.

TRIM2 performs functions in regulating cell proliferation, migration, invasion and apoptosis. In order to verify the functions of TRIM2, *in vitro* cell assays were performed. The cell lines were examined for the expression level of TRIM2 in order to identify the appropriate line for the following assays. From the results, the hFOB cell line exhibited the lowest expression signal of TRIM2 among the examined cell lines. In the remaining

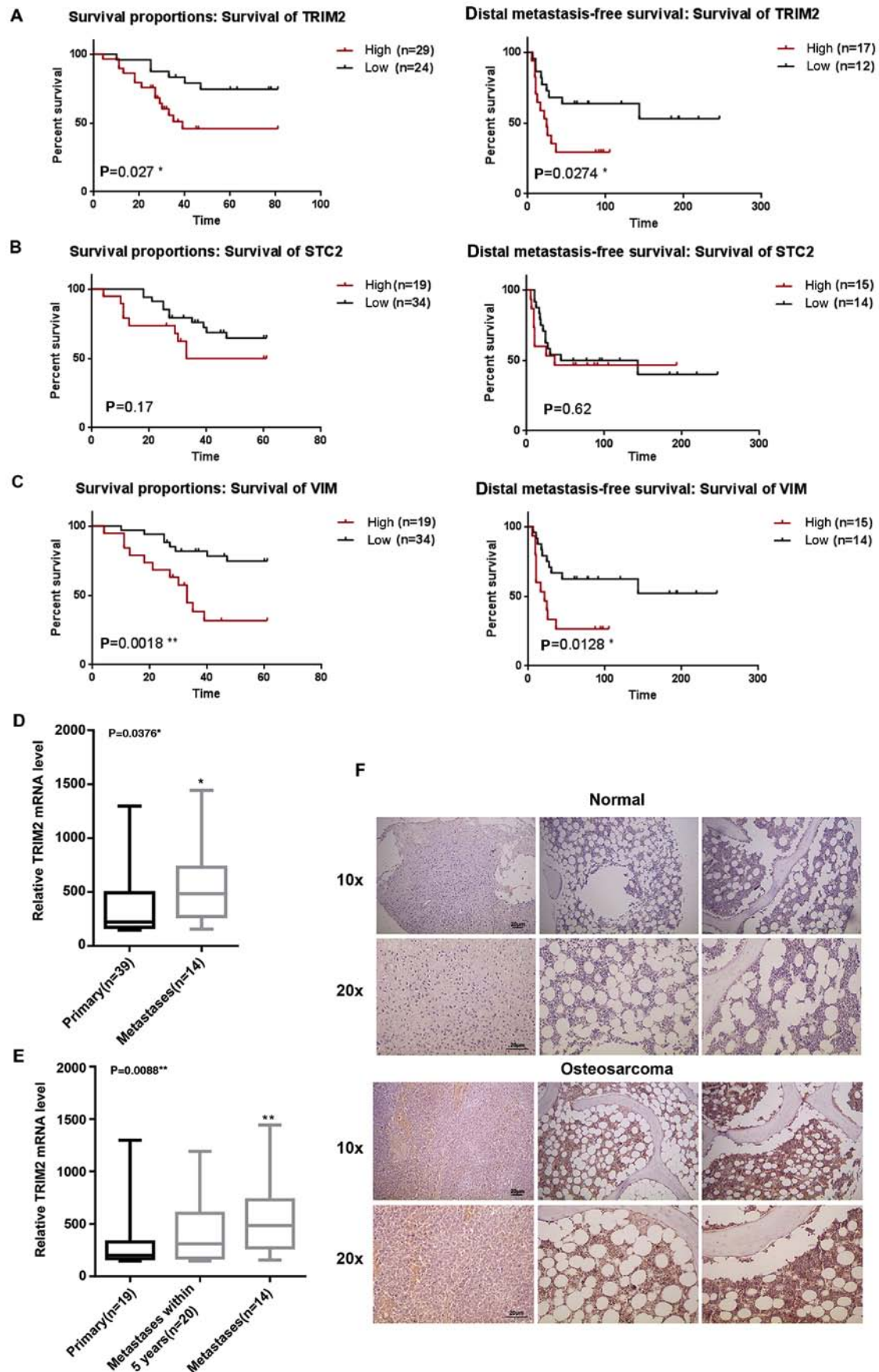


Figure 1. Prediction of the prognosis and metastasis of osteosarcoma by candidate genes. Survival percentage of cells with expression of (A) TRIM2, (B) STC2, and (C) VIM (survival and distal metastasis-free survival). Comparison of relative mRNA level of TRIM2 between primary tumor tissues and (D) metastatic tumor tissues and (E) metastasis within 5 years by combining the expression profiles and the clinical properties. (F) Immunohistochemical staining showing the amounts and density of the TRIM2 molecules in the osteosarcoma and normal tissues. *P<0.05 and **P<0.01. TRIM2, tripartite motif-containing protein 2; STC2 stanniocalcin 2; VIM, vimentin.

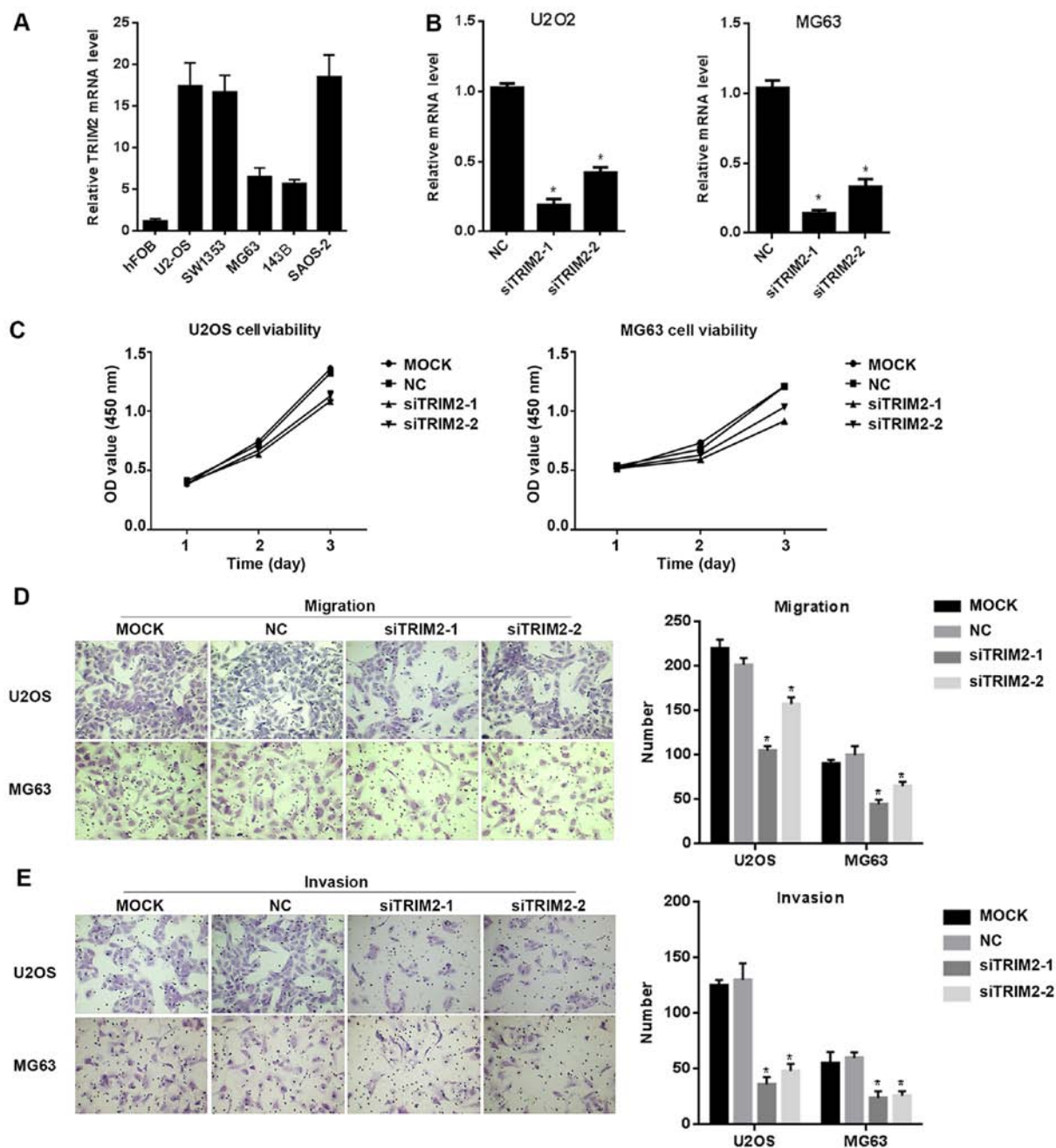


Figure 2. Levels of TRIM2 in cell lines and design of siRNAs targeting TRIM2. (A) *In vitro* cell assays were performed to examine the expression level of TRIM2 in cell lines. (B) Expression levels of TRIM2 in U2OS and MG63 cells following transfection with TRIM2-targeted siRNAs. (C) Cell viability of U2OS and MG63 cells transfected with siTRIM2-1 or siTRIM2-2, determined via MTS assay. Levels of (D) migrated and (E) invasive cells among groups, determined by immunohistochemistry (original magnification, x200). * $P < 0.05$. siRNA, small interfering RNA. TRIM2, tripartite motif-containing protein 2; NC, negative control; OD, optical density.

cell lines, U2OS, SW1353 and SAOS2 cells exhibited relatively high expression levels of TRIM2 among the six cell lines, whereas MG63 and 143B expressed comparatively lower levels of TRIM2 (Fig. 2A). Therefore, U2OS and MG63 were selected for the following assays, which exhibit high and low expression level of TRIM2, respectively. siRNAs targeting TRIM2 were designed. The sequences of the siRNAs were synthesized and integrated into the vector, and the vector was packed into the lentivirus and transfected into U2OS and MG63 cells in order to suppress the expression of TRIM2. The results revealed that siTRIM2-1 and siTRIM2-2 successfully decreased the expression level of TRIM2 (Fig. 2B). Among the two siRNAs,

siTRIM2-1 decreased the level to ~25% whereas siTRIM2-2 decreased the level to ~50%, compared with the control group, in the U2OS and MG63 cell lines. Cell viability was then determined via an MTS assay. The assay was performed every day for a total of 3 days. The results showed that, for the two cell lines, the viability of the cells transfected with empty vector or without treatment exhibited similar optical density (OD) values at 450 nm. However, for those cells transfected with siTRIM2-1 and siTRIM2-2, the cell viability decreased by ~25%, which was detected on day 3 (Fig. 2C). When the cell migration and invasion capacity of those cells were determined, the migrated cells and invasive cells in the control group and the mock group

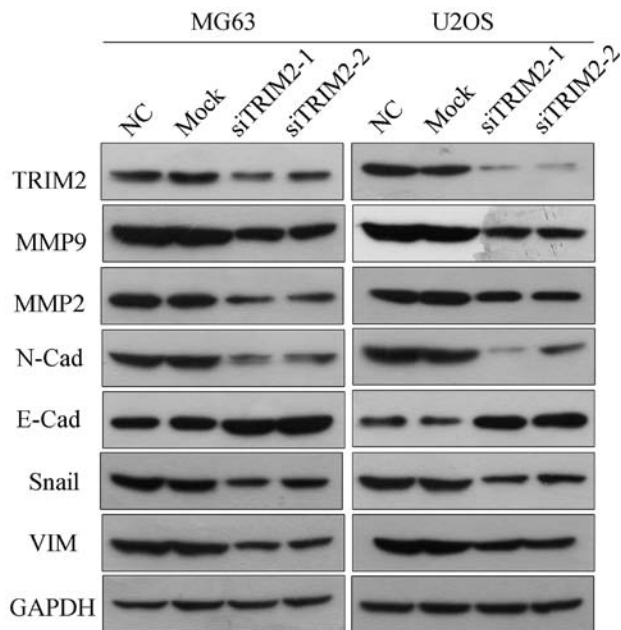


Figure 3. Protein levels of TRIM2 and migration-related genes as detected via western blot analysis. Protein levels of cell migration- and invasion-related molecules, including MMP2, MMP9, N-Cadherin, E-Cadherin, Snail and VIM, were determined by western blot analysis. GAPDH was applied as the internal standard. Six samples were used for each group. TRIM2, tripartite motif-containing protein 2; si, small interfering RNA; NC, negative control; MMP, matrix metalloproteinase; VIM, vimentin.

were increased, with cells exhibiting in higher density in the area; in the siTRIM2-1 and siTRIM2-2 treatment groups, fewer migrated and invasive cells were observed than in the control group and mock group (Fig. 2D and E). This indicated that the migration and invasion capacities of the cells were inhibited when the level of TRIM2 was decreased. Subsequently, the protein from these cells was extracted in order to determine the protein levels of TRIM2 via western blot analysis. According to the results, the protein level of TRIM2 was decreased in the MG63 and U2OS cell lines when transfected the TRIM2-targeted siRNAs (Fig. 3). Therefore, the protein levels of cell migration- and invasion-related molecules, including N-Cadherin, E-Cadherin, Snail and Vimentin, were examined. The results corresponded to those of the cell migration and invasion assays: When the expression of TRIM2 was inhibited, the levels of MMP9, MMP2, N-Cadherin, Snail and Vimentin were accordingly decreased, whereas the protein level of E-Cadherin was increased, indicating weak cell migration and invasion capacities.

Flow cytometry was also performed to determine cell apoptosis. This involved comparing the cells with and without treatment of TRIM2-targeted siRNA transfection. As shown when siTRIM2-1 and siTRIM2-2 inhibited the level of TRIM2 and thereby led to lower cell viability, the cell apoptosis was increased when TRIM2 was decreased in the results (Fig. 4A). The apoptotic percentage increased ~5-fold in the siRNA-transfected cells compared with the cells in the control group and mock group. In addition, the cell cycle assay demonstrated that the proportion of MG63 and U2OS cells in S phase was decreased, whereas the proportion in G1 phase was increased post-transfection with siTRIM2-1 and siTRIM2-2 (Fig. 4B). These findings indicated that most of the

cells exhibited reduced viability due to the occurrence of cell apoptosis. Based on the evidence above, nude mice were used to perform the tumor formation assay. When the cells were injected into nude mice, the tumors formed gradually. The tumors were collected and compared on day 20 (Fig. 5). The tumor was shown to be marginally smaller in the siTRIM2 group than that in negative control (NC) group. The volume of the tumors were determined every 5 days for a total of 20 days. The difference between the NC group and siTRIM2 group was significant on day 10, and the size of the tumor in the NC group was ~2-fold larger than that in the siTRIM2 on day 20. In terms of the weights of the tumor tissues, the tumor weight in the NC group was 2-fold higher than that in the siTRIM2 group, and the difference was statistically significant. According to the immunohistochemical staining of the metastasis-related proteins, the expression of E-Cadherin was upregulated, whereas the expression of Vimentin and N-Cadherin were downregulated expression when TRIM2 was inhibited (Fig. 5D). These results suggested that the tumor metastasis was suppressed when TRIM2 was at a lower level compared with that in the control group. Therefore, according to the results above, TRIM2 appeared to be an activator of cell proliferation, cell migration and cell invasion, thus inducing tumor metastasis.

Verifying the regulation network of TRIM2. In order to further verify the regulation performed by TRIM2 at the molecular level, transcriptome sequencing was performed for the cells transfected with siTRIM2 and empty vector. The criteria for the significant expression was considered as $FDR \leq 0.01$ and $\log_2 \text{Ratio} \geq 1$. Under these criteria, the significantly differential expressed genes were tagged in red and green (Fig. 6A). The hierarchical clustering for the overview of the genes in the transcriptome sequencing is shown in Fig. 6B. In the graph, the genes with upregulated expression are tagged in red, and those with downregulated expression are tagged in green. According to the results, the effect of the inhibition of TRIM2 was different between the MG63 and U2OS cells. For the MG63 cells, the differentially expressed genes were predominantly upregulated, whereas, the majority of the genes in the U2OS cells were downregulated (Fig. 6B). The genes were then subjected to GO and KEGG pathway analysis. As shown in the plot graphs (Fig. 6C), the genes involved in transcriptome analysis were involved in several cellular components, molecular functions, and biological processes. From the cellular component perspective, 'cell', 'cell part', and 'organelle' were the top three significantly enriched terms. From the molecular function perspective, 'binding' was the top significantly overrepresented term. From the perspective of biological process, 'biological regulation', 'cellular process' and 'metabolic process' were the top three significantly enriched terms. As shown in Fig. 6D, the 'Wnt signaling pathway' and 'MicroRNAs in cancer' were the top enriched term in the MG63 cells, whereas 'Viral carcinogenesis', 'PI3K-Akt signaling pathway', 'Pathways in cancer', and 'cAMP signaling pathway' were the most significantly enriched terms in the U2OS cells.

According to the data from the cell assays, the expression level of TRIM2 differed in the MG63 and U2OS cells, with MG63 cells showing lower expression of TRIM2 and U2OS

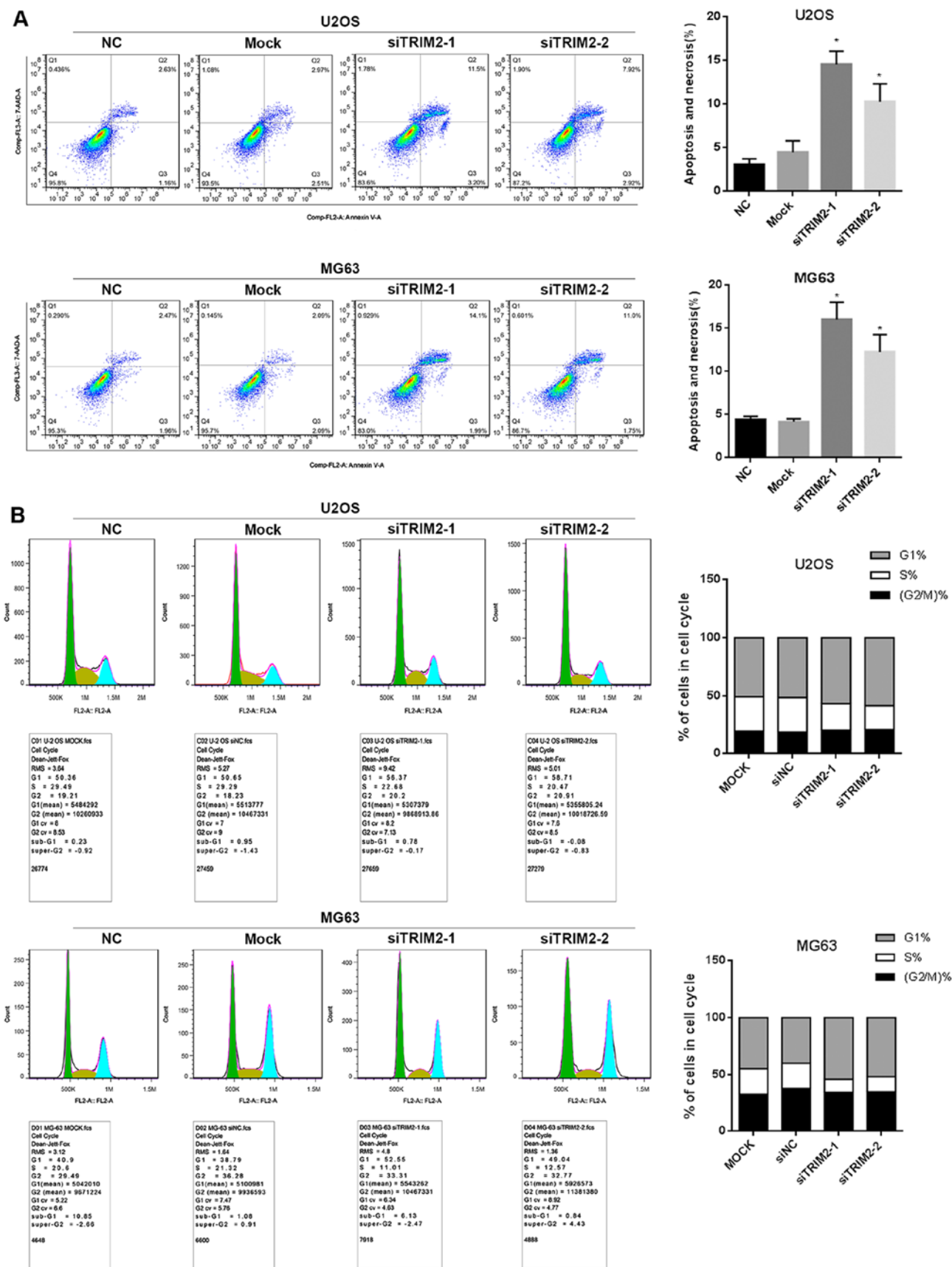


Figure 4. Cell apoptosis was detected under the regulation of TRIM2 by cell flow cytometry. Flow cytometry was performed to determine (A) cell apoptosis and (B) cell cycle in U2OS and MG63 cells. * $P < 0.05$. TRIM2, tripartite motif-containing protein 2; NC, negative control; si, small interfering RNA.

cells showing higher expression of TRIM2. However, as the two cell lines showed similar results in terms of lower cell viability and capacity to migrate and invade when TRIM2 was inhibited, regulation was expected to be common in these cell lines.

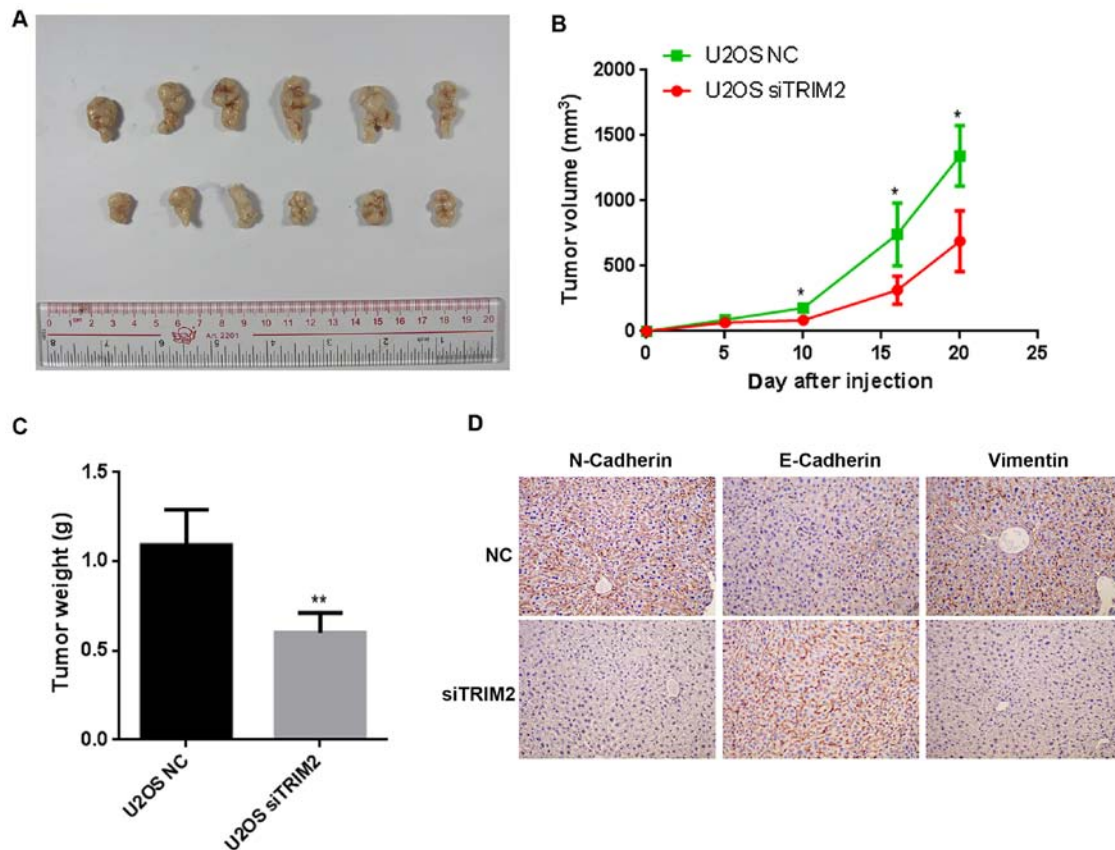


Figure 5. *In vivo* nude mouse models were used and injected to investigate the regulation of TRIM2 on tumor formation and metastasis. (A) Tumor external dimension, (B) tumor volume and (C) tumor weight were compared between the control and mock groups. * $P<0.05$ and ** $P<0.01$. (D) Metastasis-related proteins were determined by immunohistochemical staining in the NC group and siTRIM2 group (original magnification, x200). Six samples were used for each group for immunohistochemical staining. TRIM2, tripartite motif-containing protein 2; NC, negative control; si, small interfering RNA.

Therefore, seven significantly differentially expressed genes related to TRIM2 were extracted from the expression profiles and further validated via RT-qPCR analysis. In the U2OS cells, the TRIM2 gene was significantly downregulated, whereas the remaining genes, with the exception of FZD8 and SHC2, were significantly upregulated in the two cell lines (Fig. 7). For FZD8 and SHC2, they were significantly increased in the MG63 cells but did not in the U2OS cells. These two genes may not be closely associated with TRIM2. As the consistently significantly expressed genes are involved in the regulation of cell cycle, it was hypothesized that TRIM2 performs its functions in osteosarcoma by regulating these genes.

Western blot analysis was used to verify those genes that may involved in the regulatory pathways indicated by the KEGG analysis. The results, as shown in Fig. 8, indicated that, when TRIM2 was inhibited in the U2OS and MG63 cell lines, the protein level of p-AKT was decreased, whereas AKT was not changed. The protein levels of PKA, CREB and phosphorylated CREB were not affected by the inhibition of TRIM2. The results suggested that TRIM2 regulates the development and metastasis of tumorous cells of osteosarcoma via the PI3K/AKT pathway.

Discussion

TRIM2 was selected and its effects were verified using TRIM2 siRNA *in vitro* and *in vivo* in the present study; TRIM2 was

shown to be correlated with tumorous cell proliferation, invasion, migration and apoptosis. It may be a novel therapeutic target for the diagnosis and treatment of osteosarcoma. TRIM2 is a member of the TRIM-NHL protein family, which functions as an E3 ubiquitin ligase. It can interact with B-cell lymphoma 2-interacting mediator (Bim) of cell death (26). TRIM2 usually binds to Bim when it is phosphorylated by the p42/p44 mitogen-activated protein kinase (MAPK) pathway. As TRIM2 was expressed at a higher level in patients with osteosarcoma, the level of Bim is expected to be accordingly decreased, thus leading to the enhanced cell proliferation of the tumorous cells (26,27). In previous studies, the association between TRIM2 and osteosarcoma have not been reported. In studies focused on different types of carcinoma, TRIM2 has been detected to be significantly differentially expressed in ovarian cancer, breast cancer, cervical carcinoma, buccal mucosa carcinoma and follicular carcinoma (27-32). In a study of ovarian cancer, the expression of TRIM2 was detected as upregulated, and negatively regulated Bim (7). This finding is consistent with the results of the present study. However, certain studies have shown the opposite results, with the expression of TRIM2 found to be downregulated (28,31,32). The bias between different types of cancer suggests that TRIM2 is an important gene involved in the pathogenic process of cancer, although the underlying mechanism may be different and complex. However, the dysfunction of TRIM2 may be one of the factors involved in the occurrence and development of osteosarcoma to a certain extent.

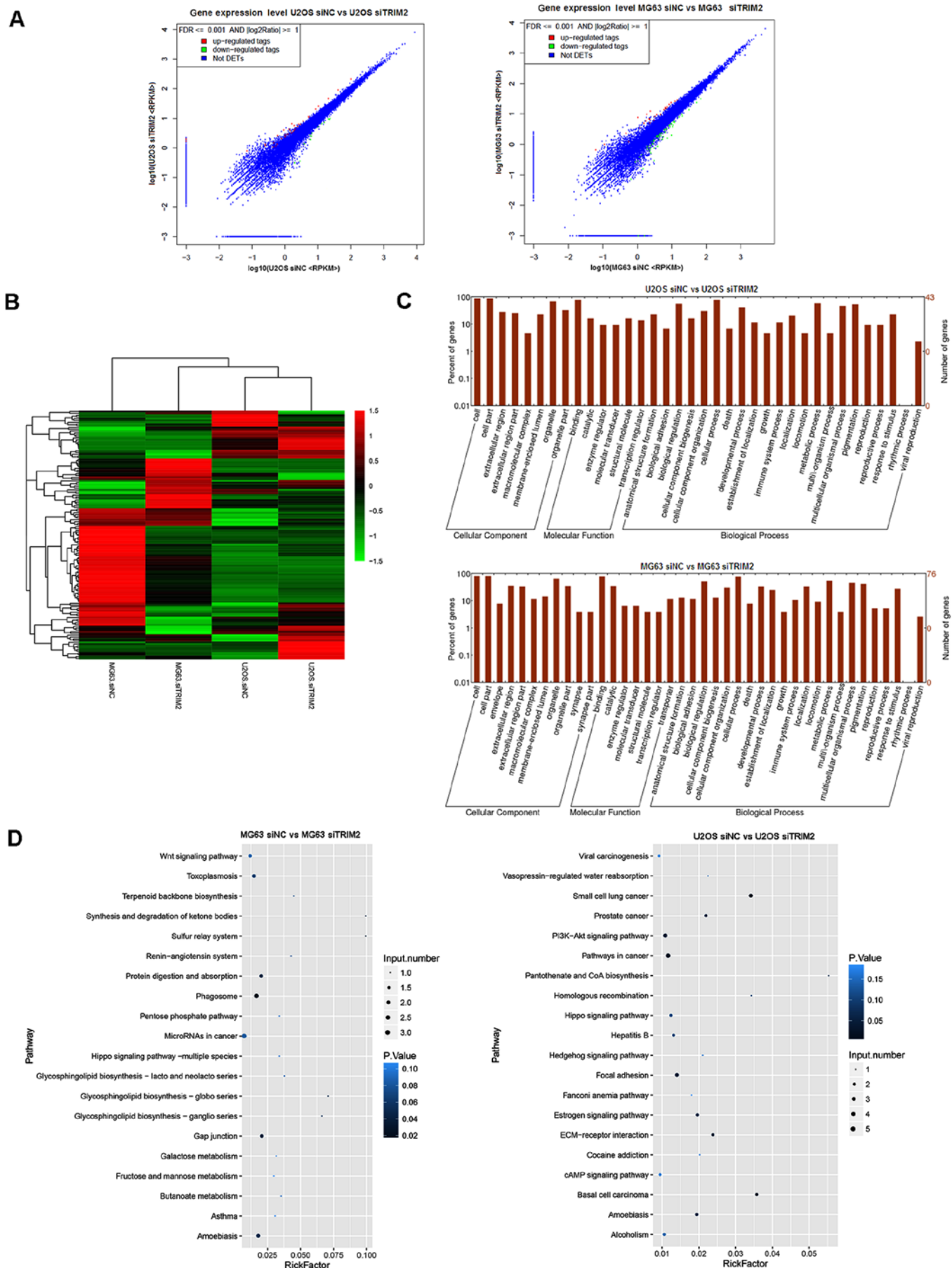


Figure 6. Expression profile and GO analysis of the MG63 and U2OS cells. (A) Hierarchical clustering for the overview of the genes in transcriptome sequencing. (B) Visualization of significantly differentially expressed genes of the MG63 and U2OS cells using a scatter plot. (C) GO analysis and (D) pathways of the MG63 and U2OS cells. GO, Gene Ontology; si, small interfering RNA; NC, negative control; TRIM2, tripartite motif-containing protein 2.

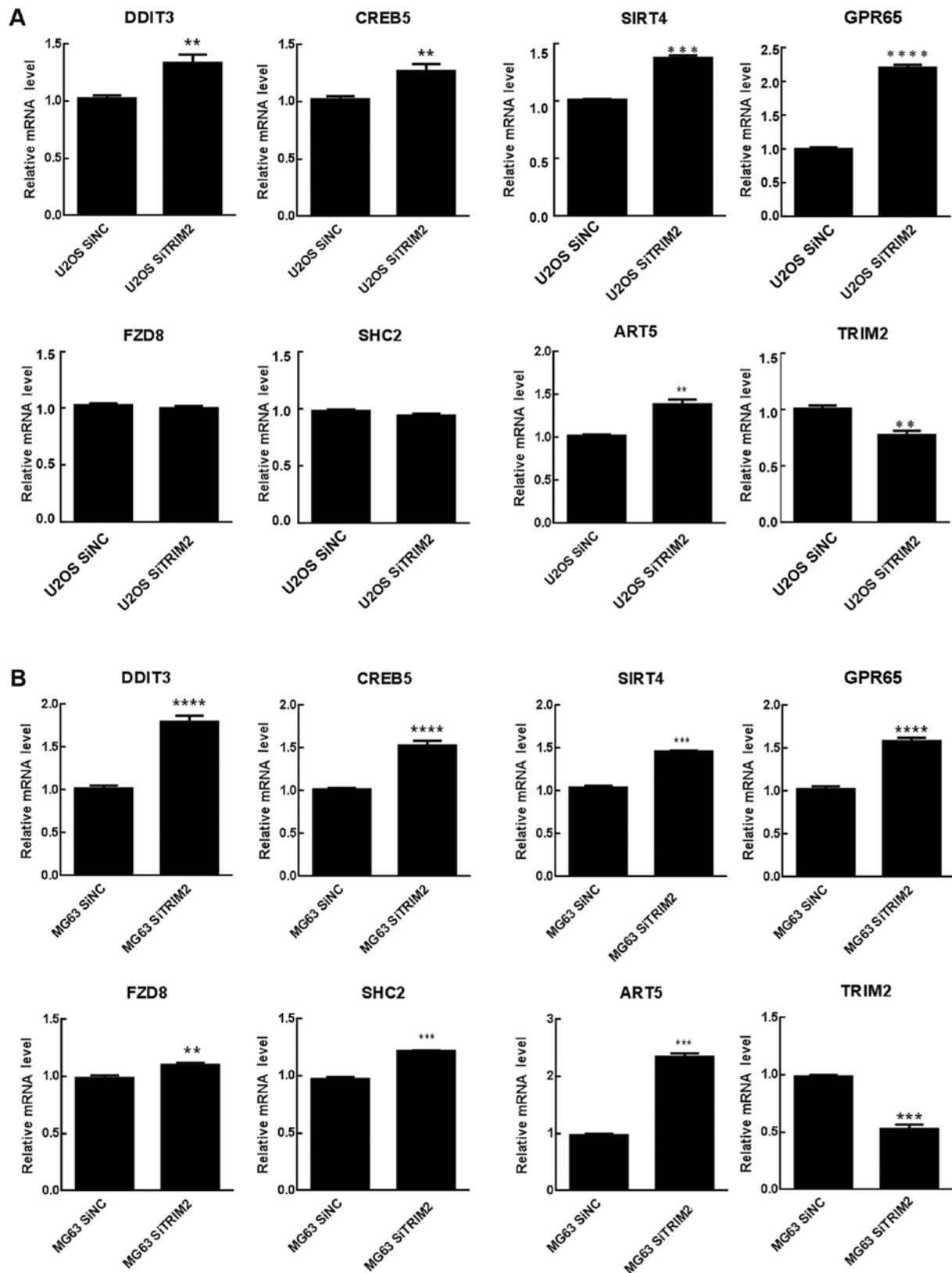


Figure 7. Genes involved in the regulation network of TRIM2. Relative mRNA level of genes associated with TRIM2 were detected in (A) U2OS and (B) MG63 cells by reverse transcription-quantitative polymerase chain reaction analysis. **P<0.01, ***P<0.001 and ****P<0.0001. ART5, ADP-ribosyltransferase 5; CREB5, cAMP responsive element binding protein 5; DDIT3, DNA damage-inducible transcript 3; FZD8, frizzled class receptor 8; GPR65, G protein-coupled receptor 65; NC, negative control; SHC2, SHC adaptor protein 2; si, small interfering RNA; SIRT4, Sirtuin 4; TRIM2, tripartite motif-containing protein 2.

The epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells transition into cells of the mesenchymal phenotype, and it has long been known to be

involved in cellular differentiation during development and tumor invasion. EMT results in cells with migratory and invasive properties, the mesenchymal state is associated

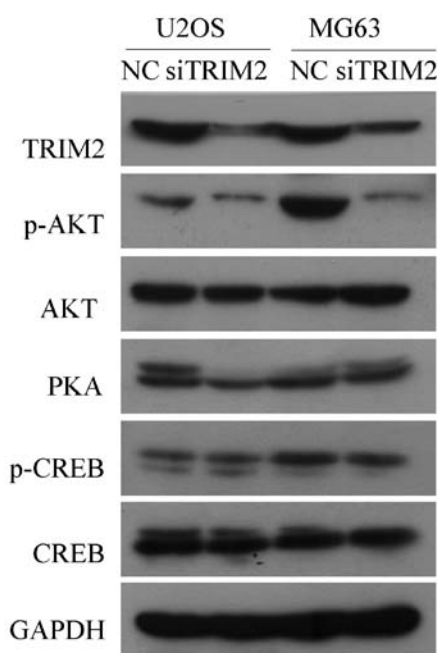


Figure 8. Western blot analysis to verify genes that may be involved in the regulating pathways of TRIM2. Protein levels of the genes that may be involved in the regulating pathways of TRIM2, including p-AKT, AKT, PKA, p-CREB and CREB, were detected by western blot analysis. GAPDH was applied as the internal standard. Six samples were used for each group. AKT, protein kinase B; CREB, cAMP response element binding protein; NC, negative control; p-, phosphorylated; PKA, protein kinase A; si, small interfering RNA; TRIM2, tripartite motif-containing protein 2.

with the capacity of cells to migrate to distant organs and maintain stemness, enabling their subsequent differentiation into multiple cell types during development and the initiation of metastasis (33). EMT can be induced by various factors. Transforming growth factor- β is a major inducer of EMT, it downregulates genes expressed in epithelial cells, including E-cadherin, and upregulates genes normally expressed in mesenchymal cells, including N-cadherin and vimentin (34,35). The degradation of extracellular matrix (ECM) through activating MMPs is an essential step in tumor cell migration. The expression of MMP-2 and MMP-9 is reported to be closely linked with tumor progression and metastasis (36). In the present study, the protein levels of the above-mentioned genes, which are associated with cancer cell development and metastasis, were examined *in vitro* and *in vivo*. The results indicated that the inhibition of TRIM2 led to decreased cell migration and invasion capacities, which confirmed that TRIM2 is important in regulating tumorous cell proliferation, migration and invasion.

To investigate the underlying regulatory mechanism of TRIM2, the gene expression was inhibited in expression and screened by RNA-seq for candidate genes. There are few reports on the association between TRIM2 and the candidate genes screened in the present study. Among these genes, SIRT4 and CREB5 are reported to be functionally correlated with tumor development. SIRT4, it is a member of the sirtuin family of NAD⁺-dependent enzymes, which have various roles in metabolism, stress response and longevity (37,38). It has been reported that SIRT4 negatively regulates mitochondrial glutamine metabolism via the inhibition of glutamate

dehydrogenase and has tumor-suppressive functions (39). The decreased level of SIRT4 can lead to poor prognosis in breast cancer (40), and the increased level of SIRT4 contributed to tumorous cell proliferation when TRIM2 was inhibited in the present study. It has been reported that CREB5 may be key in the metastatic signal network of colorectal cancer. A previous report confirmed that metastasis was promoted when CREB5 was expressed at a high level (41). In this study, CREB5 was increased when TRIM2 was downregulated. Therefore, the increase of CREB5 may enhance the metastatic process that leads to inactive cell migration and invasion. However, migrated and invasive cells in the present study were decreased in number when TRIM2 was suppressed. This suggests other genes are likely to regulate the events, rather than CREB5 alone.

For the remaining three genes, ART5, DDIT3 and GPR65, their expression was upregulated when TRIM2 was inhibited. Reports on the functions of these genes in tumor occurrence and development are limited. In analyzing the gene functions independently, ART5 can interact with TIM23. It has been reported that the overexpression of ART5 suppresses TIM23, and defects in cell growth were observed (42). This also explains the decreased cell viability in the TRIM2 inhibition group in the present study. DDIT3 is able to function in inducing DNA damage. The overexpression of DDIT3 can damage cell migration and invasion according to a previous study (43,44). Therefore, it may be an important gene contributing to the decreased cell migration and invasion observed when TRIM2 was suppressed. GPR65, is a gene involved in the p38 MAPK signaling pathway, which is correlated with the cell cycle (45). However, its exact function, particularly involving tumor development, remains to be fully elucidated.

The results from KEGG analysis indicated that 'Wnt signaling pathway' and 'MicroRNAs in cancer' were the top enriched terms in MG63 cells, and 'Pathways in viral carcinogenesis', 'PI3K-Akt signaling pathway', 'Pathways in cancer', and 'cAMP signaling pathway' were the most significantly enriched terms in U2OS cells. The present study mainly focused on PI3K/AKT and cAMP signaling pathways and examined whether TRIM2 was implicated in these regulating pathways. A number of studies have shown that the PI3K/AKT pathway is involved in osteosarcoma (46-48). mTOR, one of the PI3K family members, is important in the regulation of cell cycle, growth and development; the PI3K/AKT/mTOR signaling pathway is reported to be involved in the proliferation, migration and survival of osteosarcoma (49). The results of the present study showed that, in the U2OS and MG63 cell lines, the expression of TRIM2 was inhibited, the protein level of phosphorylated-AKT was decreased, and the level of AKT was unchanged, which indicated that the AKT signaling was implicated in the regulatory pathway of TRIM2. A previous study demonstrated that the cAMP/PKA signaling pathway was involved in osteosarcoma chemoresistance (50). Parathyroid hormone (PTH) stimulates bone formation in animals and humans. Studies have indicated that the cAMP/PKA pathway is involved in PTH signaling transduction, which subsequently affects the development of osteosarcoma cells (51,52). The present study failed to show evidence that the cAMP/PKA pathway was implicated in the development of osteosarcoma, which may due to the small sample size, and further

investigation is required to confirm the results. Taken together, the results suggested that TRIM2 regulated the development and metastasis of osteosarcoma tumor cells via the PI3K/AKT pathway.

In conclusion, the present study determined the functions of TRIM2 in osteosarcoma cell lines. TRIM2 is important in regulating the tumorous cell proliferation, migration and invasion. The SIRT4, ART5 and DDIT3 genes appear to be functionally correlated with TRIM2, which regulates the pathogenic processes of osteosarcoma, and the PI3K/AKT signaling pathway may be involved in the regulatory role of TRIM2 in the development and metastasis of osteosarcoma, however, comprehensive assays are required to validate this. The importance of TRIM2 requires further validation in clinical samples, and the comprehensive analysis of clinical samples is likely to benefit the development of diagnosis and treatment for osteosarcoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YQ and JY conceived and designed the study, and critically revised the manuscript. JY performed the experiments, analyzed the data and drafted the manuscript. FZ, SH and SW was involved in study design, study implementation and manuscript revision. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Research Ethics Committee of Jinan University. All animal experiments were approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University. Informed consent was provided by all participants prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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