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RASSF4 Overexpression Inhibits the Proliferation, Invasion, EMT, and Wnt Signaling Pathway in Osteosarcoma Cells

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RASSF4, a member of the RASSF family, is broadly expressed in normal tissues but often inactivated in human cancers. Despite various studies on RASSF4, its role in osteosarcoma remains unclear. Therefore, in this study, we investigated the effects of RASSF4 expression on osteosarcoma cells and explored the underlying mechanism. The results of our study showed that RASSF4 was lowly expressed in osteosarcoma tissues and cells. RASSF4 overexpression significantly inhibited proliferation, migration, and invasion as well as the EMT process in osteosarcoma cells. Meanwhile, we found that RASSF4 overexpression markedly decreased the protein expression of β-catenin, cyclin D1, and c-Myc in osteosarcoma cells. In conclusion, our findings showed that RASSF4 overexpression inhibits proliferation, invasion, EMT, and Wnt signaling pathway in osteosarcoma cells. Thus, RASSF4 may be considered a novel target for osteosarcoma treatment.

Key words: RASSF4; Osteosarcoma; Proliferation; Invasion; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Osteosarcoma, the most prevalent bone tumor, is characterized by a highly malignant and metastatic potential and often occurs in children and adolescents (1). In the past, surgery alone was employed for osteosarcoma treatment, resulting in a 20% survival rate (2). Currently, osteosarcoma treatment has significantly advanced with options including surgery, radiotherapy, and chemotherapy. However, patient outcomes remain poor as a result of relapse and metastasis (3–5). Therefore, identifying the molecular mechanisms involved in osteosarcoma development is desperately needed for improvement of osteosarcoma treatment.

The Ras-association domain family (RASSF) proteins have 10 members, known as RASSF1 to RASSF10 (6). The RASSF proteins play a significant role in tumor suppression and are involved in many important biological functions such as proliferation, cell cycle, apoptosis, autophagy, and DNA repair (7,8). In addition, these proteins carry out their functions by interacting with diverse proteins and are modulated via complex mechanisms including protein posttranslational modifications, histone modifications, promoter hypermethylation, and polymorphic changes (8). Increasing evidence has shown that many RASSF members are downregulated in different types of cancers including prostate, lung, brain, and breast cancers, and their overexpression can inhibit cell proliferation and promote cell death (8,9).

RASSF4, a member of the RASSF family, is broadly expressed in normal tissues but is often inactivated in human cancers (6,10,11). Moreover, it is found to have an effect on growth suppression and cell death (6). Eckfeld et al. reported that overexpression of RASSF4 could promote apoptosis in breast tumor cells and inhibit growth of lung tumor cells (12). Despite various studies on RASSF4, its role in osteosarcoma remains unclear.

In the present study, we investigated the effects of RASSF4 expression on osteosarcoma cells and explored the underlying mechanism. Our results indicated that RASSF4 overexpression inhibits proliferation, invasion, epithelial–mesenchymal transition (EMT), and Wnt signaling pathway in osteosarcoma cells.

MATERIALS AND METHODS

Tissue Specimens

Twenty paired osteosarcoma and matched normal noncancerous tissues were obtained from patients at the

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Department of Oncology, China–Japan Union Hospital, Jilin University (Changchun, P.R. China). All the tissues were immediately stored in liquid nitrogen before use. All the patients agreed to take part in the study and provided written informed consent. The study was approved by the Ethics Committee of Jilin University.

Cell Culture

Human osteosarcoma cell lines (MG63, 143B, and U2OS) and osteoblast cell line (hFOB 1.19) were purchased from the American Type Culture Collection (Manassas, VA, USA). The osteosarcoma cells were cultured in RPMI-1640 medium (Hyclone, Tauranga, New Zealand) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 IU/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 mg/ml streptomycin (Sigma-Aldrich). The osteoblast cells were maintained in DMEM/F12 medium (Gibco) supplemented with 10% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin. All the cells were kept at 37°C in an incubator containing 5% CO_2.

Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the human tissues or the cultured cells using the TRIzol reagent (TaKaRa, Dalian, P.R. China). The synthesis of cDNA was performed through the PrimeScript RT Reagent kit (TaKaRa) in accordance with the manufacturer's protocol. RT-PCR was carried out with the following primers: RASSF4, 5′-AGGATAYGATATATGTAGTGGTTTTTGGATT-3′ (forward) and 5′-ATTATAACCCCTAAATTACTTAACAAAAATACCAAA-3′ (reverse); β-actin, 5′-AGAAAATCTGGCACCACACC-3′ (forward) and 5′-TAGCACAGCCTGGATAGCAA-3′ (reverse). β-Actin was used as an internal control. The comparative CT method ($2^{-\Delta\Delta Ct}$) was applied to measure the expression level of genes (13).

Western Blot

The cells or tissues were lysed in ice-cold lysis buffer. The proteins were separated using 10% SDS-PAGE (Beyotime, Shanghai, P.R. China) and then transferred onto a nitrocellulose membrane (Life Technologies, Gaithersburg, MD, USA). Subsequently, the membrane was blocked for 1 h in phosphate-buffered saline and 5% nonfat milk, followed by overnight incubation at 4°C with the primary antibodies against RASSF4, E-cadherin, N-cadherin, vimentin, β-catenin, cyclin D1, c-Myc, and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membrane was further incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.). Protein bands were visualized by enhanced chemiluminescence (Millipore, Boston, MA, USA).

RASSF4 Expression Vector and Transfection

To ensure stable transfection, pcDNA3.1 expression vectors (Genesil, Wuhan, P.R. China) were applied for insertion of RASSF4 cDNA. MG63 and U2OS cells were transfected with the pcDNA3.1-RASSF4 expression vector or the empty pcDNA3.1 vector using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfected cells were incubated at 37°C for 24 h. Positive clones were obtained from the pcDNA3.1-RASSF4 vector transfection group (MG63-RASSF4 and U2OS-RASSF4) and the empty pcDNA3.1 vector transfection group (MG63-PC and U2OS-PC). The clones were identified and frozen for future experiments. Western blot analysis was carried out to detect RASSF4 expression in MG63-RASSF4, MG63-PC, U2OS-RASSF4, and U2OS-PC cells.

Cell Proliferation Assay

The MTT assay was performed to examine the proliferative capacity of the osteosarcoma cells. In brief, the transfected cells were added onto 96-well plates at a density of 1×10^4 cells/well and then cultured for 24, 48, 72, or 96 h. Next, each well was filled with 20 μl of MTT (Sigma-Aldrich). After 4 h of incubation with 5% CO_2 at 37°C, the medium was discarded and then 150 μl of DMSO (Sigma-Aldrich) was administrated into each well. Optical densities were measured at a spectral wavelength of 490 nm using a microplate reader (BD Bioscience, Bedford, MA, USA).

Cell Migration and Invasion Assays

Transwell chambers (Costar, Corning, NY, USA) were used to measure the migration and invasion abilities of osteosarcoma cells. For the migration assay, 1×10^4 cells were plated into the upper chamber. For the invasion assay, 1×10^4 cells were plated into the upper chamber coated with Matrigel (Trevigen, Gaithersburg, MD, USA). In both assays, serum-free medium was added to the upper chamber to maintain cells, while medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 48 h of incubation, cells remaining on the upper surface of the membrane were removed. Then the membrane was fixed and stained with 0.1% crystal violet. Four random optical fields (200×) were examined to determine the average number of cells that migrated to the lower surface of the membrane.

Statistical Analysis

Data from at least three independent experiments were expressed as mean ± SD and analyzed with SPSS 16.0. Student's *t*-tests were conducted to determine the statistical significance. A value of $p < 0.05$ was considered statistically significant.

RESULTS

RASSF4 Is Lowly Expressed in Osteosarcoma Tissues and Cell Lines

Both RT-PCR and Western blot analysis were performed to measure RASSF4 expression in human osteosarcoma and matched normal tissues. As shown in Figure 1A and B, RASSF4 was remarkably decreased in osteosarcoma tissues in comparison with the matched normal tissues. Moreover, as shown in Figure 1C and D, a lower expression level of RASSF4 was shown in three osteosarcoma cell lines (MG63, 143B, and U2OS) in comparison with the osteoblast cell line hFOB 1.19.

RASSF4 Overexpression Inhibits Osteosarcoma Cell Proliferation

The pcDNA3.1 expression vector was used to upregulate RASSF4. Transfection efficiency was confirmed by Western blot. As shown in Figure 2A, RASSF4 increased

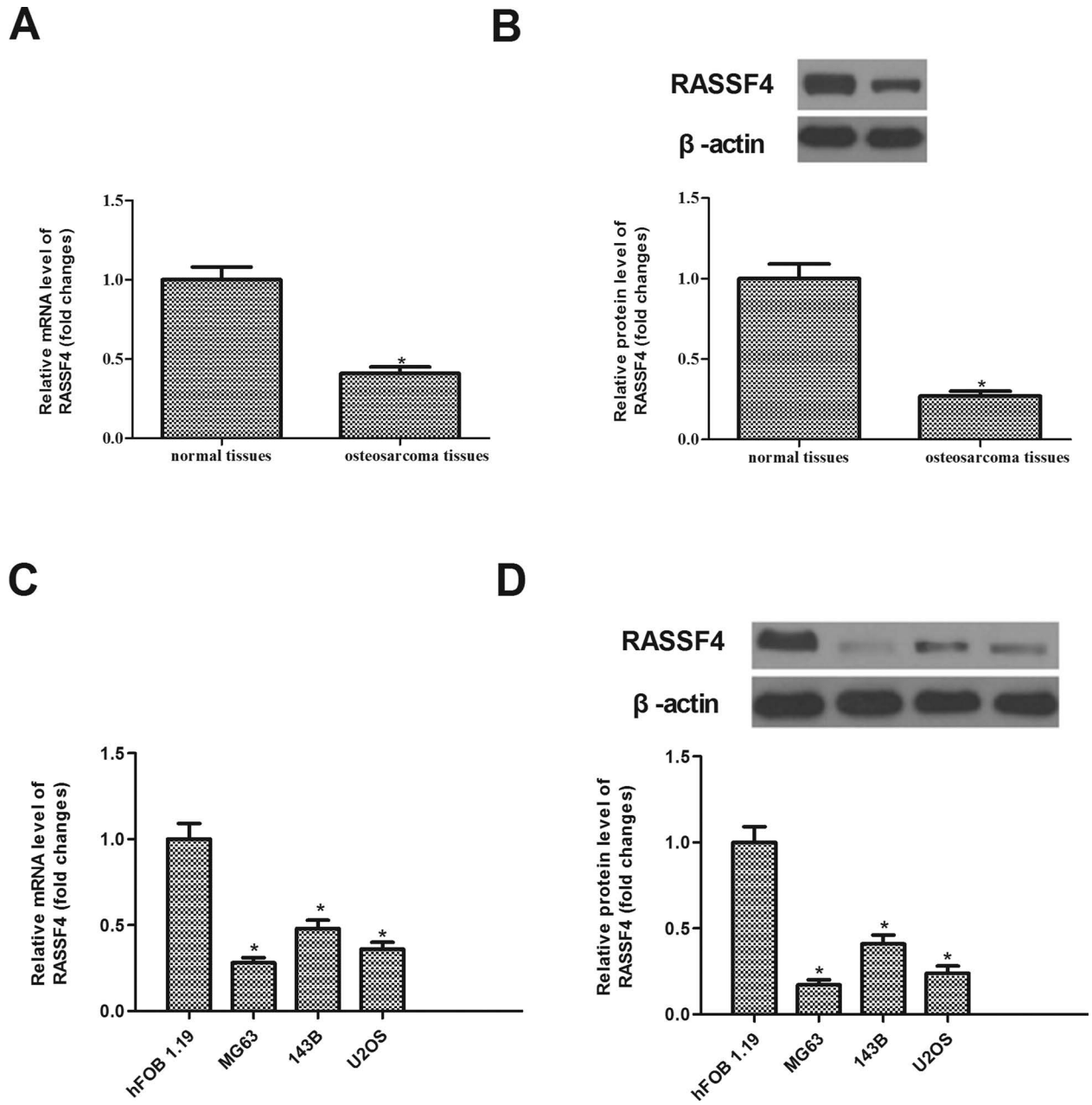

Figure 1. RASSF4 is lowly expressed in osteosarcoma tissues and cell lines. (A, B) The expression of RASSF4 in osteosarcoma tissues and corresponding normal tissues was measured by RT-PCR and Western blot. (C, D) The mRNA and protein expression of RASSF4 in three osteosarcoma cell lines (MG63, 143B, and U2OS) and the osteoblast cell line hFOB 1.19. *$p<0.05$.

markedly in MG63-RASSF4 cells in comparison with MG63-PC. Similar results were discovered in U2OS-RASSF4 cells (Fig. 2B).

As shown in Figure 2C, RASSF4 overexpression inhibited the cell growth rate of MG63-RASSF4 cells in comparison with that of MG63-PC. RASSF4 overexpression exerted similar effects on U2OS-RASSF4 cells (Fig. 2D).

RASSF4 Overexpression Inhibits Osteosarcoma Cell Migration and Invasion

We conducted Transwell assays to detect the effects of RASSF4 overexpression on the migration and invasion of MG63 and U2OS cells. The experiment results indicated that the migratory and invasive capabilities of MG63-RASSF4 (Fig. 3A and B) and U2OS-RASSF4

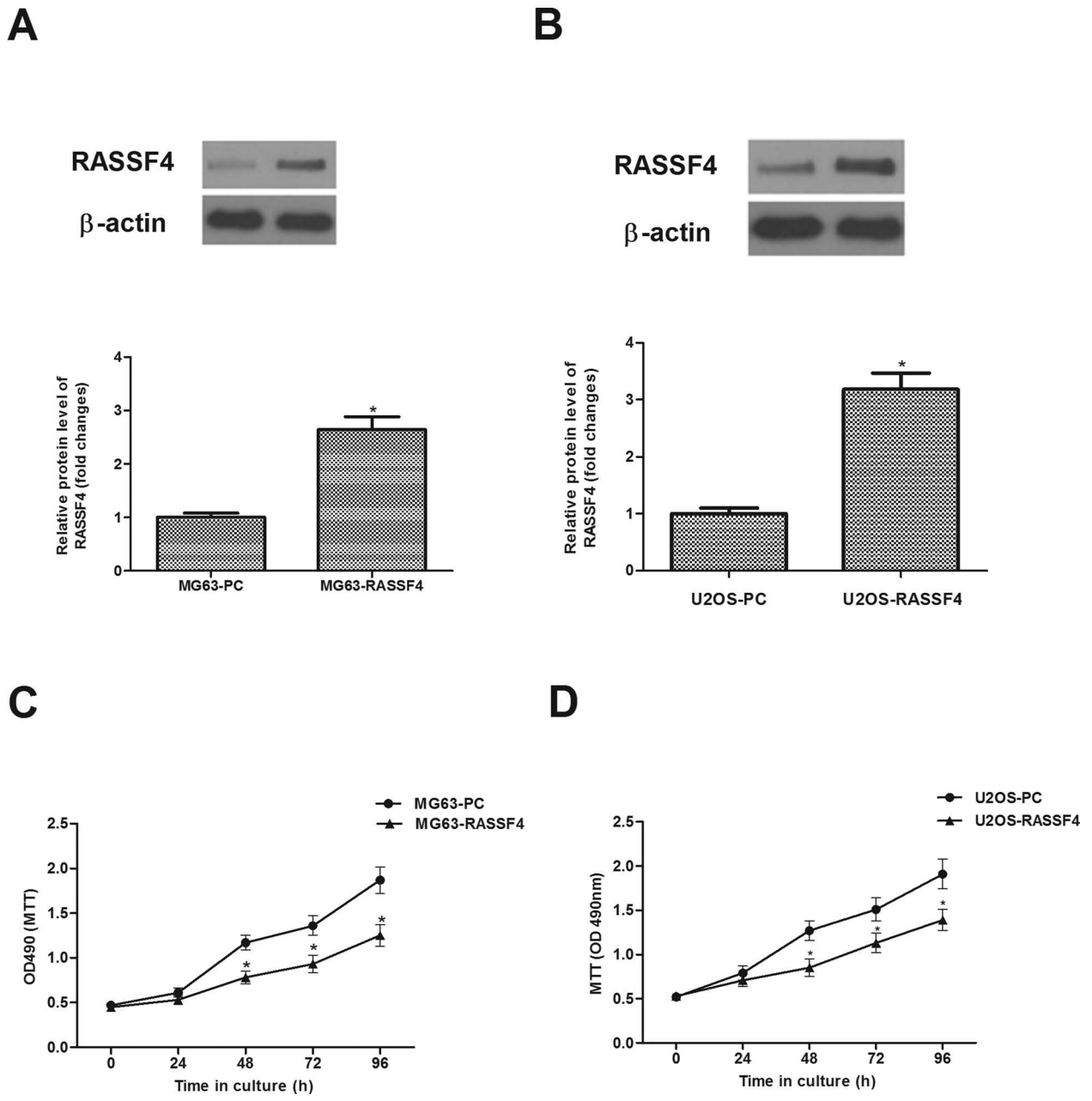

Figure 2. RASSF4 overexpression inhibits osteosarcoma cell proliferation. (A, B) The corresponding transfection efficiency was detected by Western blot in MG63 cells and U2OS cells, respectively. (C, D) Growth curves of MG63 and U2OS cells, showing suppression of cell proliferation after RASSF4 transfection. *$p<0.05$. MG63-RASSF4, MG63 cells transfected with pcDNA3.1-RASSF4 vector; MG63-PC, MG63 cells transfected with the empty pcDNA3.1 vector; U2OS-RASSF4, U2OS cells transfected with the pcDNA3.1-RASSF4 vector; U2OS-PC, U2OS cells transfected with the empty pcDNA3.1 vector.

cells (Fig. 3C and D) were significantly reduced in comparison with their corresponding controls.

RASSF4 Overexpression Inhibits the EMT Process in Osteosarcoma Cells

To detect the effects of RASSF4 on EMT of osteosarcoma cells, Western blot was performed to measure the expression of EMT-related factors in MG63 and U2OS cells after transfection with RASSF4. As shown in Figure 4, RASSF4 overexpression significantly elevated the protein expression of E-cadherin and meanwhile reduced the protein expression of N-cadherin and vimentin in MG63-RASSF4 (Fig. 4A) and U2OS-RASSF4 cells (Fig. 4B), compared to the control groups. The results showed that RASSF4 overexpression remarkably obstructed the EMT process.

RASSF4 Overexpression Inhibits the Activity of Wnt/β-Catenin Signaling Pathway

To identify whether RASSF4 affected Wnt/β-catenin signaling pathway, we conducted Western blot assays to measure the protein expression of β-catenin and its

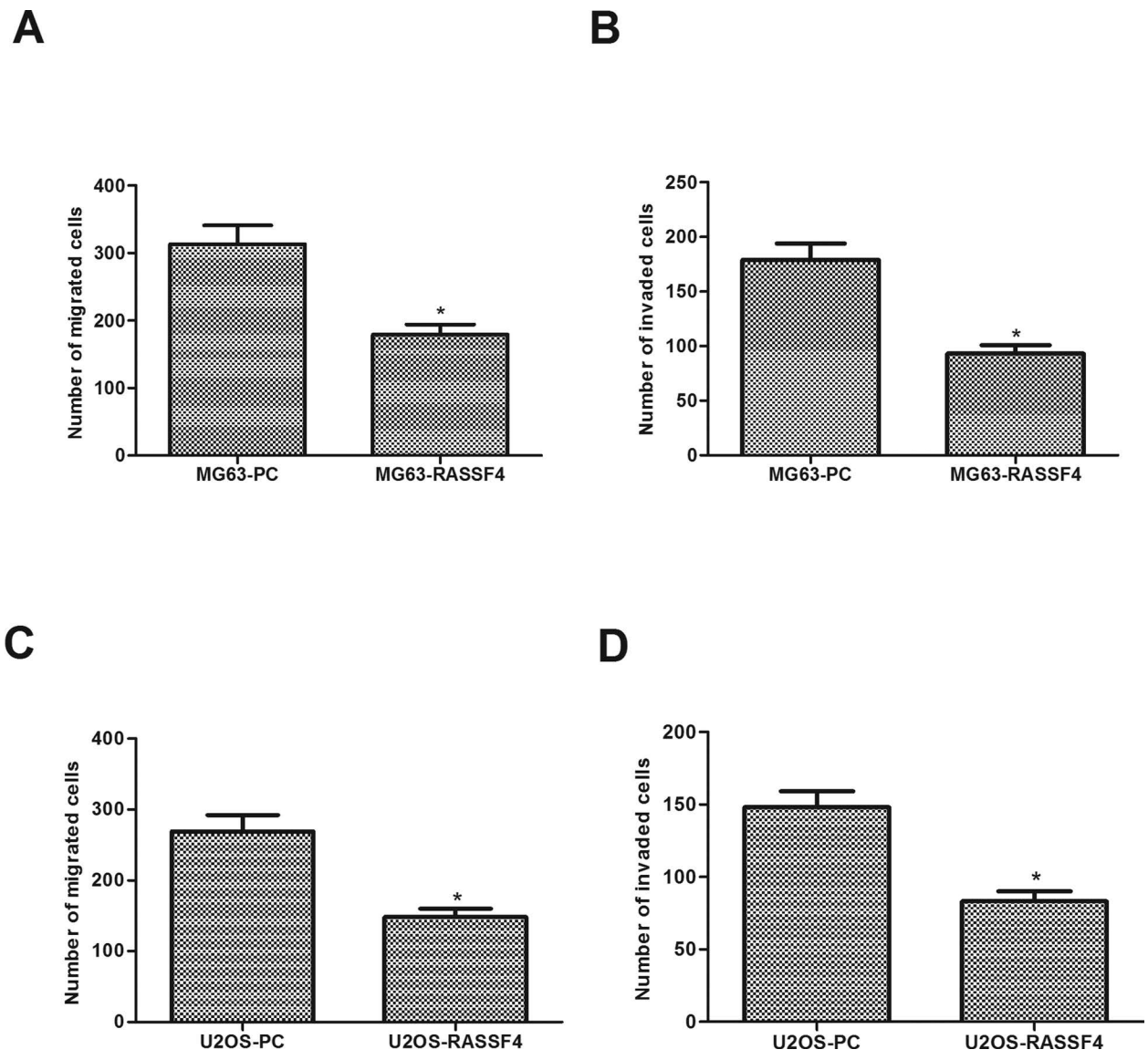

Figure 3. RASSF4 overexpression inhibits osteosarcoma cell migration and invasion. (A, B) As determined by the Transwell assay, RASSF4 overexpression significantly reduced the migratory and invasive capabilities of MG63-RASSF4 in comparison with MG63-PC cells. (C, D) Similar results were found in U2OS-RASSF4 cells. *$p<0.05$. MG63-RASSF4, MG63 cells transfected with the pcDNA3.1-RASSF4 vector; MG63-PC, MG63 cells transfected with the empty pcDNA3.1 vector; U2OS-RASSF4, U2OS cells transfected with pcDNA3.1-RASSF4 vector; U2OS-PC, U2OS cells transfected with the empty pcDNA3.1 vector.

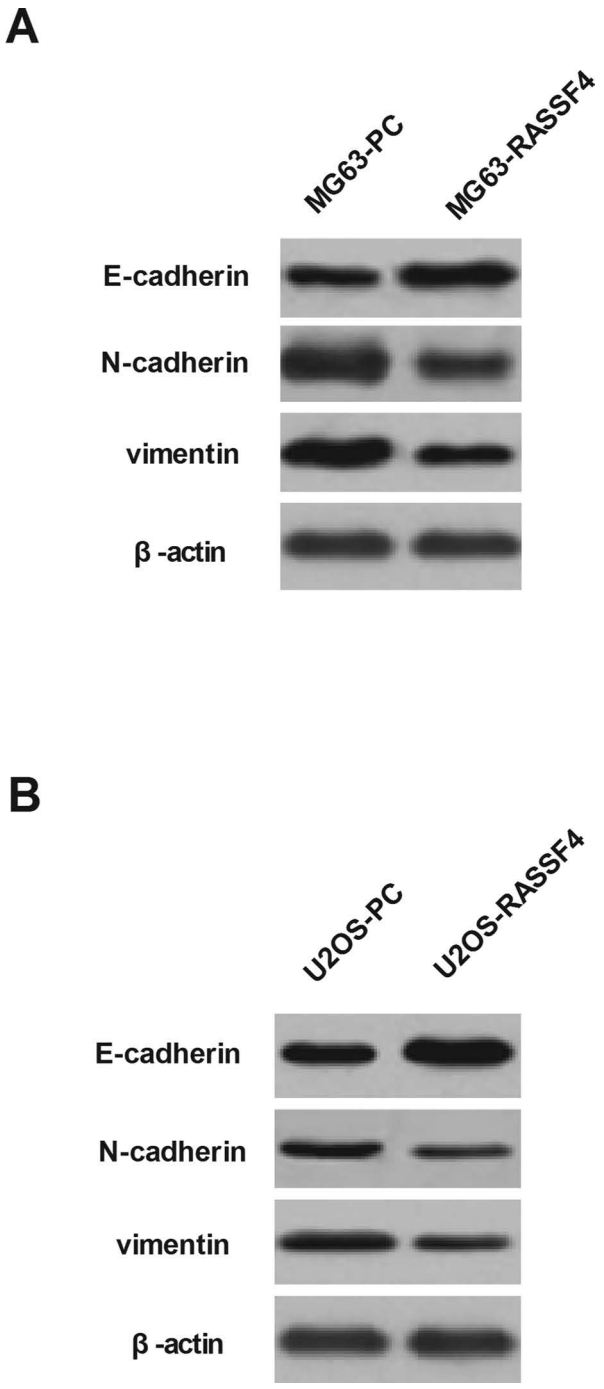

Figure 4. RASSF4 overexpression inhibits the EMT process in osteosarcoma cells. (A) The Western blot assay indicated that RASSF4 overexpression markedly increased the protein expression of E-cadherin and decreased the protein expression of N-cadherin and vimentin in MG63-RASSF4 cells in comparison with the control groups. (B) Similar results were found in U2OS-RASSF4 cells. *$p<0.05$. MG63-RASSF4, MG63 cells transfected with the pcDNA3.1-RASSF4 vector; MG63-PC, MG63 cells transfected with the empty pcDNA3.1 vector; U2OS-RASSF4, U2OS cells transfected with the pcDNA3.1-RASSF4 vector; U2OS-PC, U2OS cells transfected with the empty pcDNA3.1 vector.

downstream targets cyclin D1 and c-Myc in MG63 cells. As shown in Figure 5A, a significant decrease in the protein expression of β-catenin, cyclin D1, and c-Myc was found in MG63-RASSF4 cells in comparison with the control cells. Quantification analysis of β-catenin, cyclin D1, and c-Myc is shown in Figure 5B.

DISCUSSION

Despite an advancement in treatments, osteosarcoma is still the main reason for cancer-related mortality in the pediatric age group (14,15). Therefore, it is essential to develop a more effective therapeutic method for osteosarcoma treatment.

RASSF4 has been given much attention for its involvement in the development of several human cancers and for its role as a potential tumor suppressor of the RASSF family (16). Han et al. demonstrated that RASSF4 could inhibit cell proliferation and invasion in non-small cell lung cancer (17). Similarly, Eckfeld et al. suggested that RASSF4 overexpression could suppress growth of lung tumor cells (12). In addition, RASSF4 was reported to have an inductive effect on cell death in breast cancer (18). In spite of extensive studies and reports on RASSF4, there has been no research on its role in osteosarcoma. Therefore, we investigated in this study the effects of RASSF4 on osteosarcoma and explored the underlying mechanism.

First, we conducted RT-PCR and Western blot to measure the expression of RASSF4 in osteosarcoma tissues and cell lines. The results showed that RASSF4 had a lower mRNA and protein expression in osteosarcoma tissues and cell lines in comparison with the control groups. These results were consistent with the previous reports that RASSF4 was broadly found in normal human tissues but was often downregulated in human tumor cell lines (12). Then we used pcDNA3.1 expression vectors to upregulate the expression of RASSF4 in osteosarcoma cells (MG63 and U2OS) by transfection and performed related assays to explore the specific role of RASSF4 overexpression on osteosarcoma cell proliferation, migration, and invasion. The assay results indicated that RASSF4 overexpression had a suppressive effect on those biological processes. EMT played a key role in cancer progression for its association with invasive and metastatic behaviors (19). Therefore, in this study, we also investigated the effect of RASSF4 overexpression on EMT and found a similar result that RASSF4 overexpression could inhibit the EMT process with increased expression of E-cadherin and decreased expression of N-cadherin and vimentin. All the results mentioned above provided additional evidence for the role of RASSF4 as a potential tumor suppressor.

To clarify the mechanism underlying the inhibitory effect of RASSF4 on osteosarcoma cells, we explored the canonical Wnt signaling pathway, which is referred

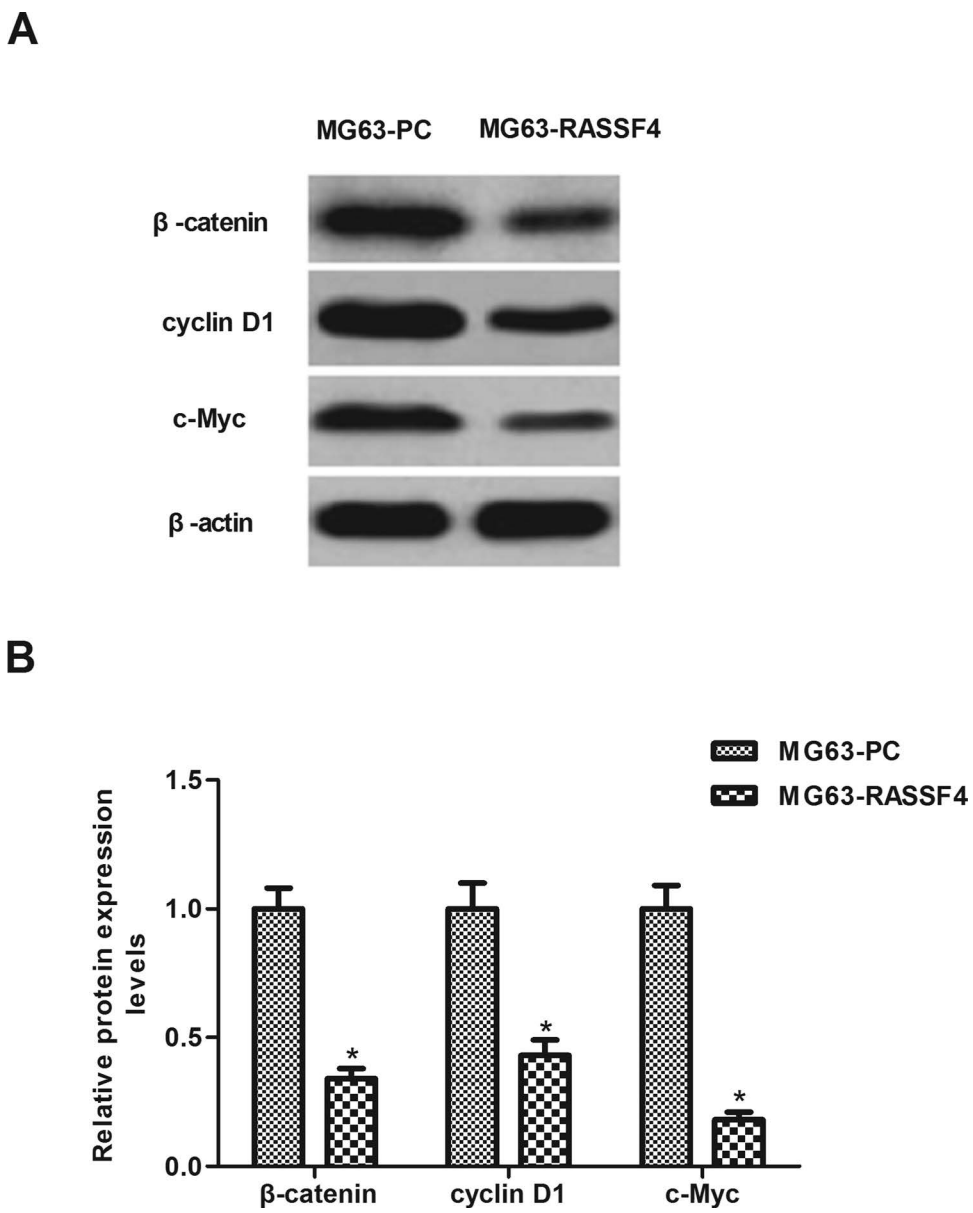

Figure 5. RASSF4 overexpression inhibits the activity of the Wnt/β-catenin signaling pathway. (A) Western blot was performed to detect the protein expression of β-catenin, cyclin D1, and c-Myc in MG63-RASSF4 cells. β-Actin was used as an internal control. (B) Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. *$p < 0.05$. MG63-RASSF4, MG63 cells transfected with the pcDNA3.1-RASSF4 vector; MG63-PC, MG63 cells transfected with the empty pcDNA3.1 vector.

to as the Wnt/β-catenin signaling pathway. As shown by previous studies, the Wnt/β-catenin signaling pathway played an essential role in modulating cell proliferation and migration as well as cell death (20,21). Moreover, the signaling pathway was associated with the pathogenesis of a number of diseases (22–26). Dysregulation of this signaling pathway could result in numerous types of human cancers (27–29). Among the signaling pathways, β-catenin is a vital member, and its excessive expression has been reported to be a cause of tumorigenesis in the central nervous system, bone, colorectum, and other tissues (30,31). A growing number of studies have identified the mutation in β-catenin in various cancers, thereby confirming the role of β-catenin as a potential target for cancer treatment (32–34). Zou et al. applied β-catenin as a drug target for osteosarcoma treatment and suggested that downregulated β-catenin could inhibit proliferation of osteosarcoma cells (35). In this study, we also chose β-catenin as a main object to measure the effect of RASSF4 on the Wnt/β-catenin signaling pathway in

osteosarcoma. The study results revealed that RASSF4 overexpression remarkably decreased the protein expression of β-catenin. Furthermore, we observed a similar decrease in the protein expression of cyclin D1 and c-Myc, which are the downstream targets of β-catenin. On the basis of the above results, we demonstrated that RASSF4 overexpression could inhibit the activity of the Wnt/β-catenin signaling pathway.

In conclusion, we suggest that RASSF4 overexpression inhibits proliferation, invasion, EMT, and Wnt signaling pathway in osteosarcoma cells, on basis of which RASSF4 may be considered a novel target for osteosarcoma treatment.

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