

Armadillo Repeat-Containing Protein 8 (ARMC8) Silencing Inhibits Proliferation and Invasion in Osteosarcoma Cells

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Armadillo repeat-containing protein 8 (ARMC8) plays an important role in regulating cell migration, proliferation, tissue maintenance, signal transduction, and tumorigenesis. However, the expression pattern and role of ARMC8 in osteosarcoma are still unclear. In this study, our aims were to examine the effects of ARMC8 on osteosarcoma and to explore its underlying mechanism. Our results demonstrated that ARMC8 was overexpressed in osteosarcoma cell lines. Knockdown of ARMC8 significantly inhibited osteosarcoma cell proliferation in vitro and markedly inhibited xenograft tumor growth in vivo. ARMC8 silencing also suppressed the epithelial–mesenchymal transition (EMT) phenotype, as well as inhibited the migration and invasion of osteosarcoma cells. Furthermore, knockdown of ARMC8 obviously inhibited the expression of β -catenin, c-Myc, and cyclin D1 in MG-63 cells. In conclusion, this report demonstrates that ARMC8 silencing inhibits proliferation and invasion of osteosarcoma cells. Therefore, ARMC8 may play an important role in the development and progression of human osteosarcoma and may represent a novel therapeutic target in the treatment of osteosarcoma.

Key words: Armadillo repeat-containing protein 8 (ARMC8); Osteosarcoma; Proliferation; Invasion

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor and a leading cause of cancer death in adolescents (1). During the last decade, several strategies have been suggested for the treatment of osteosarcoma patients, but unfortunately some patients died of pulmonary metastases (2–5). Therefore, it is urgent to explore the molecular mechanisms underlying osteosarcoma carcinogenesis and progression in order to improve the prevention and treatment of this disease.

Armadillo repeat-containing protein 8 (ARMC8) proteins are novel proteins that are conserved in eukaryotes. Increasing evidence has reported that ARMC8 plays an important role in regulating cell migration, proliferation, tissue maintenance, signal transduction, and tumorigenesis (6–8). Jiang et al. reported that the expression level of ARMC8 was obviously higher in colon cancer in comparison to that in the adjacent normal colon tissues, and ARMC8 expression was associated with TNM stage, lymph node metastasis, and poor prognosis of colon cancer (9). Another study demonstrated that ARMC8 was also highly expressed in human non-small cell lung cancer tissues, and overexpression of ARMC8 α

significantly promoted growth, colony formation, and invasion in H1299 lung cancer cells (10). However, the expression pattern and role of ARMC8 in osteosarcoma are still unclear. In this study, our aims were to examine the effects of ARMC8 on osteosarcoma and to explore its underlying mechanism.

MATERIALS AND METHODS

Cell Culture

The human osteosarcoma cell lines (U2OS and SaOS2) and the normal osteoblast cell line (hFOB1.19) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37°C with 5% CO₂ in an incubator (Life Technologies, Baltimore, MD, USA).

RNA Interference and Transfection

The short hairpin RNA targeting ARMC8 (ARMC8-shRNA) and its negative control were synthesized by RiBo Biotech (GuangZhou RiBo Biotech). The ARMC8-shRNA

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or scr-shRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the cell density reached 70%–80%. The cells were harvested for assays 48 h after transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was isolated from human osteosarcoma cells using TRIzol reagent (Invitrogen). About 5 μ g of total RNA for each sample was reverse transcribed into first-strand cDNA for qRT-PCR analysis. The primer sequences used for PCR amplification are ARMC8, 5'-GTGTAGAGCTGGAGCAATTCGG-3' (forward) and 5'-AGGCAAGTGTCTCAGCTCCTTCGTCCCCTC GAGGAGTTGTGT-3' (reverse); β -actin, 5'-ATCACCA TTGGCAATGAGCG-3' (forward) and 5'-TTGAAGGT AGTTTCGTGGAT-3' (reverse). The amplification protocol used was as follows: an initial 3-min denaturation at 94°C, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 45 s. β -Actin was used as internal control. The relative amount of each cDNA was analyzed by means of $2^{-\Delta\Delta Ct}$.

Western Blot

Cells were lysed using RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate) on ice for 30 min, and then the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). A total of 20 μ g of protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was blocked with 2% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature (RT) followed by incubation in primary antibodies (antibodies against ARMC8, E-cadherin, N-cadherin, β -catenin, c-Myc, cyclin D1, and β -actin; diluted 1:1,000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the blocking buffer overnight at 4°C. Subsequently, the blots were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. Protein bands were visualized by enhanced chemiluminescence (Millipore, Boston, MA, USA) according to the manufacturer's protocol.

Cell Proliferation Assay

Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, osteosarcoma cells (1×10^5 cells per well) transfected with ARMC8-shRNA or scr-shRNA were seeded in 96-well flat-bottom microtiter plates (Pierce) and then cultured at 24-h intervals for 4 days. Cell proliferation was then evaluated using the CCK-8 (Sigma-Aldrich, St. Louis, MO, USA) assay according to the manufacturer's instructions.

The absorbance value was read spectrophotometrically at 450 nm on a microtiter plate reader (Merek, Whitehouse Station, NJ, USA).

Cell Migration and Invasion Assay

The cell migration capability was measured using the Transwell assay. In brief, osteosarcoma cells transfected with ARMC8-shRNA or scr-shRNA suspended in RPMI medium were added to the upper chamber, and the lower

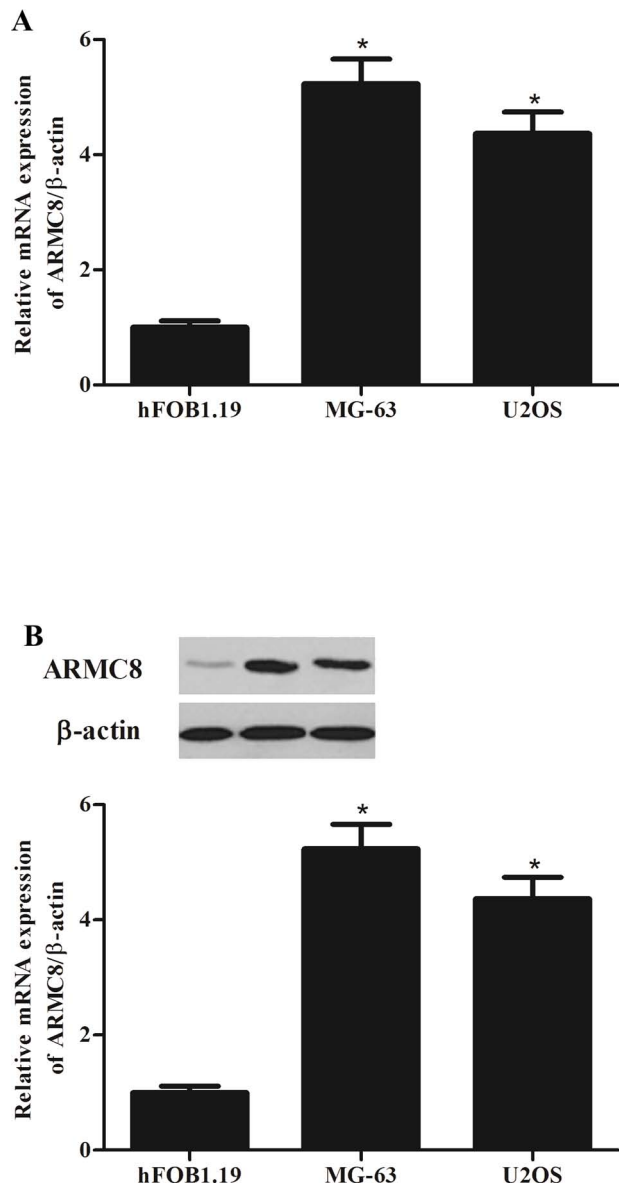


Figure 1. Armadillo repeat-containing protein 8 (ARMC8) is overexpressed in human osteosarcoma cell lines. (A) Representative mRNA expression of ARMC8 in human osteosarcoma cell lines. (B) Representative Western blot image of ARMC8 protein in human osteosarcoma cells lines. Data represent mean \pm SD from three independent experiments. * $p < 0.05$.

chamber of the Transwell plates was filled with 500 μ l of RPMI medium containing 10% FBS (Invitrogen). After 24 h of incubation, the cells that migrated through the membrane were fixed in 4% paraformaldehyde for 20 min, stained with 0.1% crystal violet for 30 min, and counted under the microscope (Olympus, Tokyo, Japan) by counting five independent visual fields. The invasion assay was

done by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier.

In Vivo Xenograft Tumor Assay

All mice were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of Huaihe Hospital of Henan

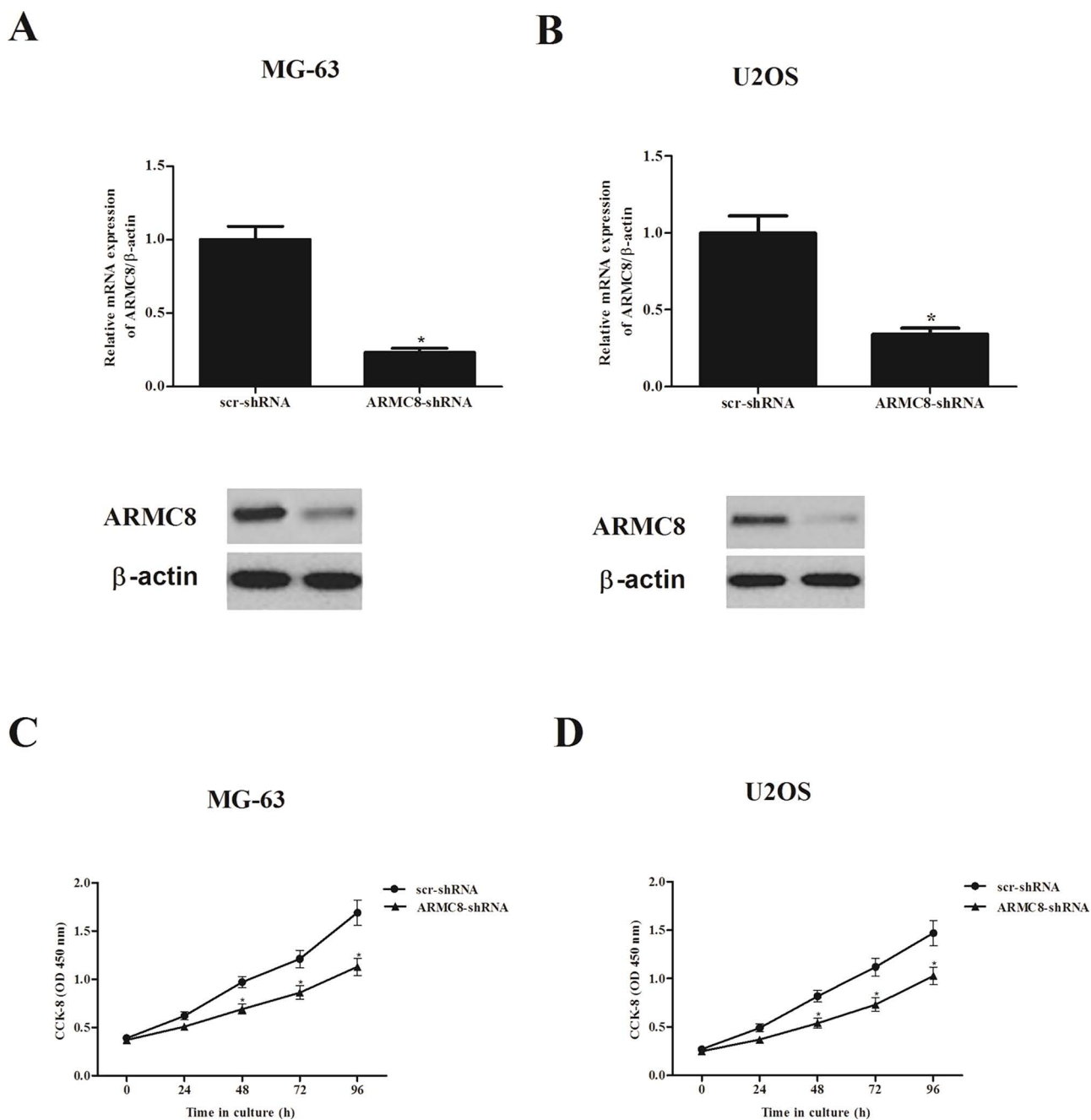


Figure 2. ARMC8 silencing inhibits osteosarcoma cell proliferation in vitro. (A) mRNA and protein levels of ARMC8 in ARMC8-shRNA-transfected MG-63 cells. (B) mRNA and protein levels of ARMC8 in ARMC8-shRNA-transfected U2OS cells. (C) Knockdown of ARMC8 inhibited the proliferation of MG-63 cells in a time-dependent manner. (D) Knockdown of ARMC8 inhibited the proliferation of U2OS cells in a time-dependent manner. Data represent mean \pm SD from three independent experiments. * p < 0.05.

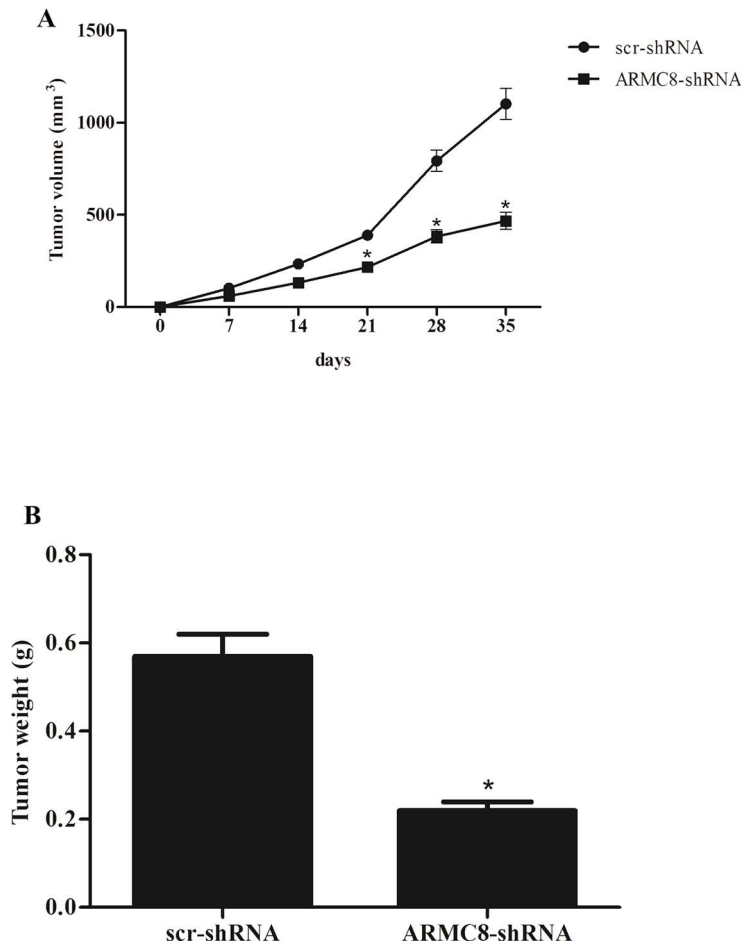


Figure 3. ARMC8 silencing inhibits the growth of osteosarcoma in vivo. MG-63 cells (1×10^6 cells/0.1 ml) transfected with ARMC8-shRNA or scr-shRNA were injected subcutaneously into the flank of nude mice. (A) The tumor volumes were calculated in each group every 7 days from day 0 to day 35. (B) The tumor weights were measured at day 35. Data represent mean \pm SD. * $p < 0.05$.

University. MG-63 cells (1×10^6 cells/0.1 ml) transfected with ARMC8-shRNA or scr-shRNA were injected subcutaneously into the flank of nude mice. About 5 weeks after inoculation, mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg), and the tumors were weighed. Tumor size was measured every 7 days using a caliper and calculated using the formula: volume = length \times width² \times $\pi/6$.

Statistical Analysis

All statistical analyses were performed using the SPSS software (ver. 13.0; SPSS, Chicago, IL, USA), and all results are expressed as mean \pm SD. Statistical

significance was analyzed with one-way analysis of variance followed by the Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

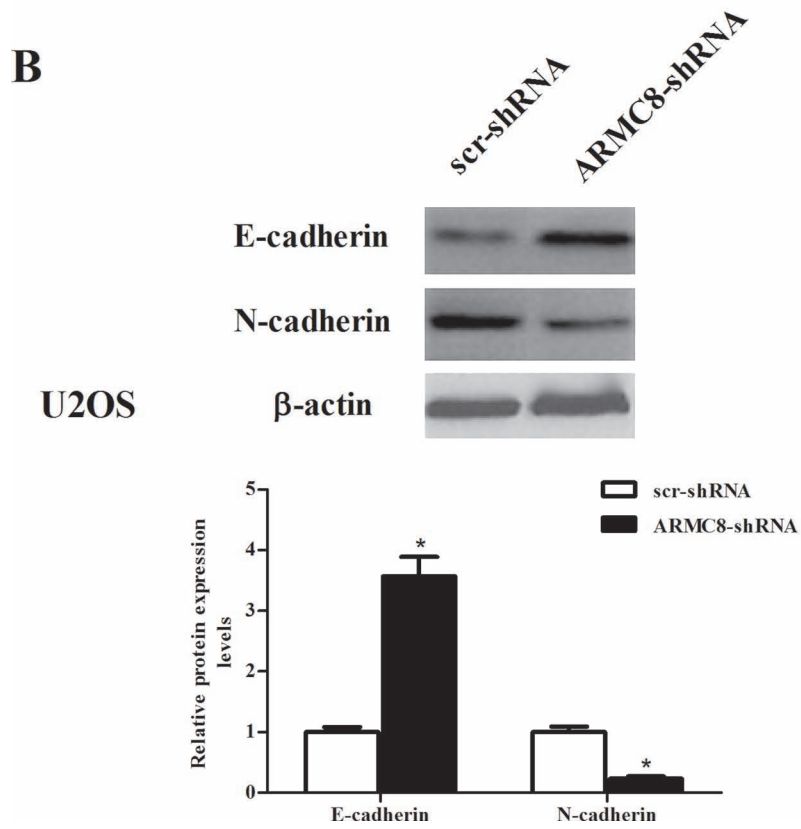
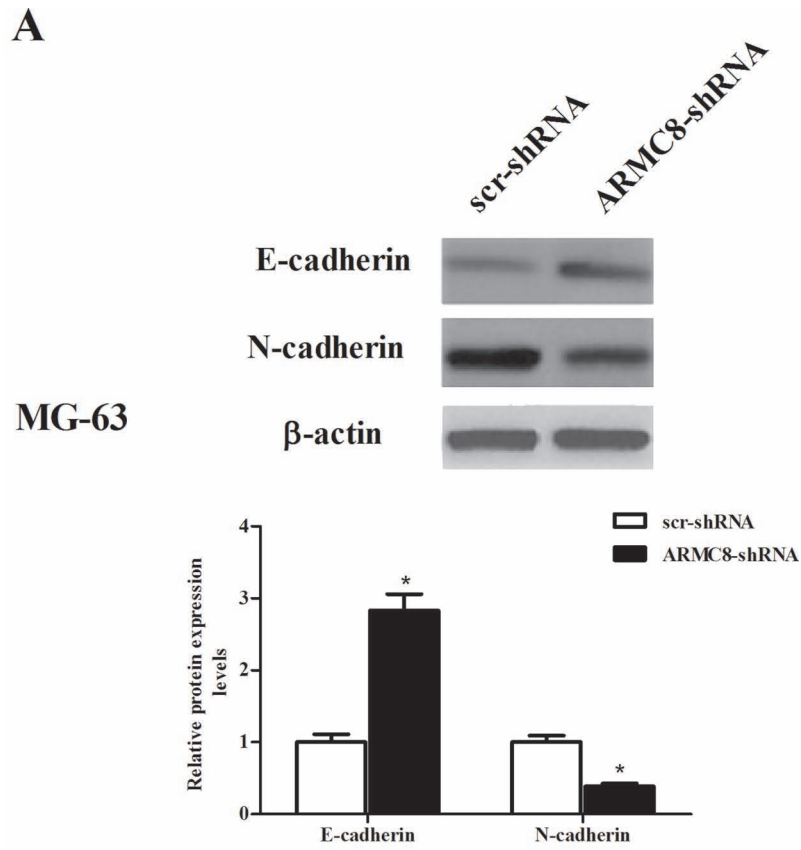
RESULTS

ARMC8 Is Overexpressed in Human Osteosarcoma Cell Lines

To verify the expression of ARMC8 in osteosarcoma, we detected the mRNA level and protein level of ARMC8 in different osteosarcoma cell lines using qRT-PCR and Western blot assays. The results of the qRT-PCR analysis demonstrated that compared with

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Figure 4. ARMC8 silencing inhibits the EMT process in osteosarcoma cells. MG-63 or U2OS cells were transfected with ARMC8-shRNA or scr-shRNA for 24 h. (A) The protein expression levels of E-cadherin and N-cadherin were detected by Western blot in MG-63 cells and quantification of protein levels from three independent experiments. (B) The protein expression levels of E-cadherin and N-cadherin were measured by Western blot in U2OS cells and quantification of protein levels from three independent experiments. Data represent mean \pm SD. * $p < 0.05$.



the normal cells, the mRNA expression of ARMC8 was overexpressed in osteosarcoma cell lines (Fig. 1A). Consistent with the results of the qRT-PCR, Western blot analysis also showed that the protein expression of ARMC8 was higher in osteosarcoma cell lines than that in normal cells (Fig. 1B).

ARMC8 Silencing Inhibits Osteosarcoma Cell Proliferation In Vitro

To investigate the biological role of ARMC8 in osteosarcoma tumorigenesis, we generated ARMC8 silencing

osteosarcoma cell lines of MG-63 and U2OS. The transfection efficiency was confirmed by qRT-PCR and Western blot. The results indicated that the mRNA and protein expression levels of ARMC8 were significantly reduced in MG-63 (Fig. 2A) and U2OS (Fig. 2B) cells, respectively. We then observed the effect of ARMC8 silencing on osteosarcoma cell proliferation. The results of the CCK-8 assay showed that knockdown of ARMC8 significantly inhibited the growth of MG-63 cells (Fig. 2C). Similarly, knockdown of ARMC8 also suppressed the proliferation in U2OS cells (Fig. 2D).

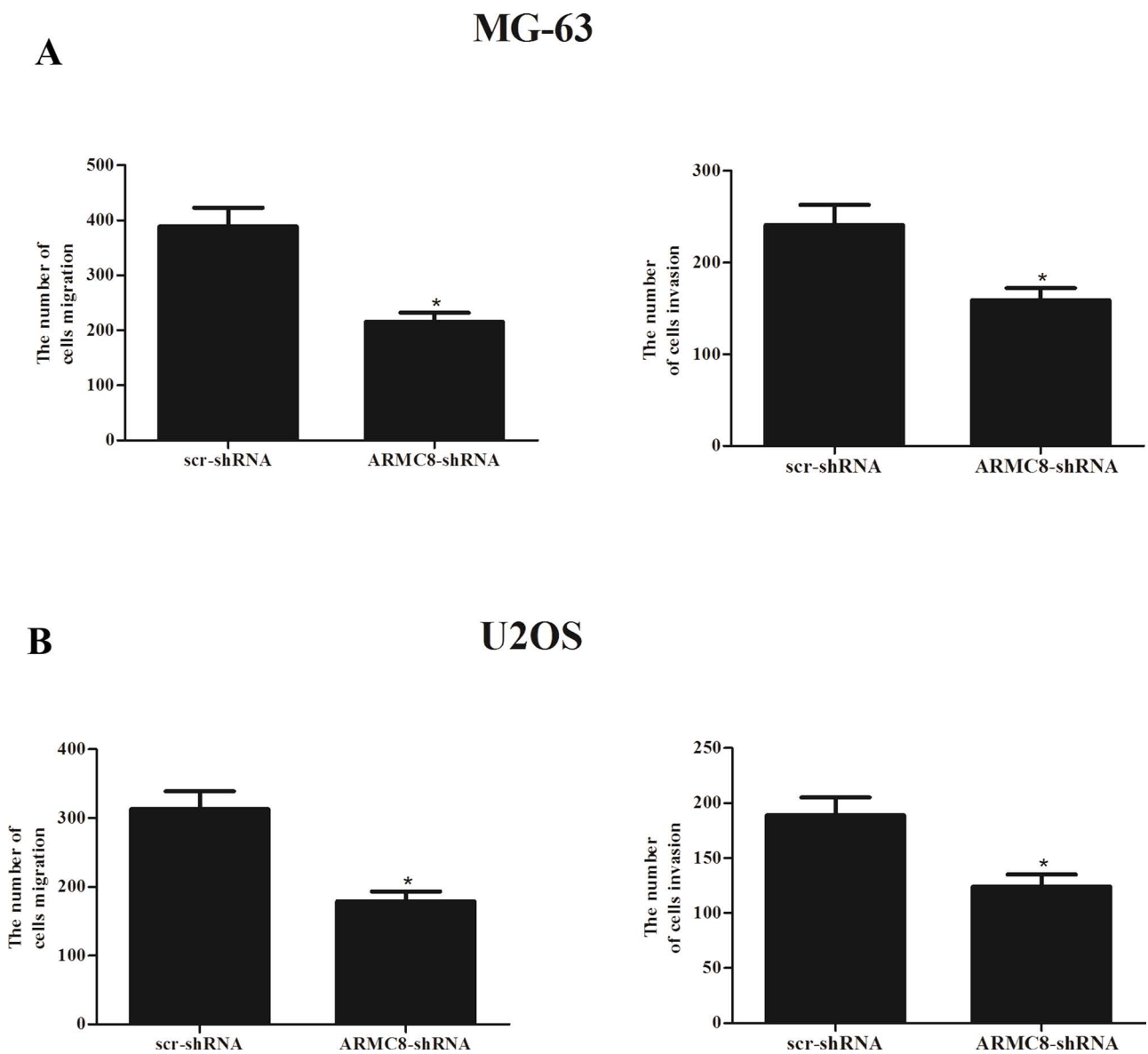


Figure 5. ARMC8 silencing inhibits OS cell migration and invasion in vitro. MG-63 or U2OS cells were transfected with ARMC8-shRNA or scr-shRNA for 24 h. (A) The number of migrated/invaded cells in the ARMC8-shRNA-transfected MG-63 cells was obviously decreased, compared with the scr-shRNA group. (B) Knockdown of ARMC8 significantly inhibited the migration and invasion of U2OS cells. Data represent mean \pm SD from three independent experiments. * $p < 0.05$.

ARMC8 Silencing Inhibits the Growth of Osteosarcoma In Vivo

Furthermore, we examined the effect of ARMC8 silencing on osteosarcoma growth in vivo. We found that knockdown of ARMC8 significantly reduced tumor volume compared to control mice (Fig. 3A). In addition, knockdown of ARMC8 obviously reduced the weight of the tumor, compared with the control tumor (Fig. 3B).

ARMC8 Silencing Inhibits the Epithelial–Mesenchymal Transition (EMT) Process in Osteosarcoma Cells

We investigated the effect of ARMC8 silencing on EMT phenotype in osteosarcoma cells. The results

uncovered that knockdown of ARMC8 in MG-63 cells induced an increase in the protein expression of E-cadherin, paralleling with a decrease in the protein expression level of N-cadherin (Fig. 4A). Similar results were observed in U2OS cells (Fig. 4B).

ARMC8 Silencing Inhibits OS Cell Migration and Invasion In Vitro

We next investigated the effect of ARMC8 silencing on the migration and invasion of OS cells. As shown in Figure 5A, for MG-63 cells, there was a significantly lower number of migrated cells in ARMC8-shRNA-transfected cells than in scr-shRNA-transfected cells.

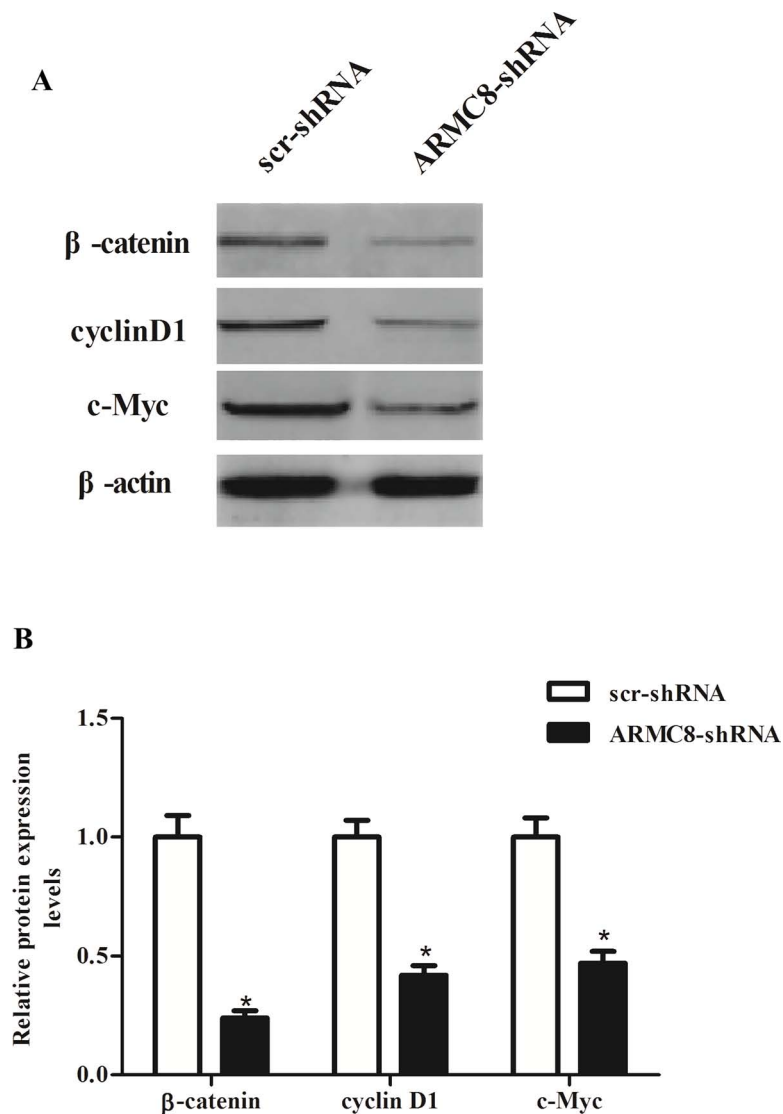


Figure 6. ARMC8 silencing inhibits the activation of Wnt/ β -catenin signaling pathway in OS cells. (A) MG-63 cells were transfected with ARMC8-shRNA or scr-shRNA for 24 h. The protein expression of β -catenin, cyclin D1, and c-Myc was detected by Western blot. (B) Relative expression of β -catenin, cyclin D1, and c-Myc was quantified using Image-Pro Plus 6.0 software after normalization with β -actin. Data represent mean \pm SD from three independent experiments. * $p < 0.05$.

Furthermore, the Matrigel assays showed that the number of cells that passed through the Matrigel-coated membrane into the lower chamber was significantly lower in the ARMC8-shRNA-transfected cells than in empty scr-shRNA-transfected cells. At the same time, we observed that knockdown of ARMC8 greatly suppressed migration and invasion of U2OS cells (Fig. 5B).

ARMC8 Silencing Inhibits the Activation of Wnt/ β -Catenin Signaling Pathway in Osteosarcoma Cells

To gain insights into the downstream signaling pathways modulated by ARMC8 silencing in tumor inhibition, we investigated the effect of ARMC8 silencing on the activation of Wnt/ β -catenin signaling pathway in osteosarcoma cells. The results of the Western blot analysis indicated that knockdown of ARMC8 obviously inhibited the expression of β -catenin, c-Myc, and cyclin D1 in MG-63 cells, compared with scr-shRNA-transfected cells (Fig. 6A). Quantification analysis of β -catenin, c-Myc, and cyclin D1 is shown in Figure 6B.

DISCUSSION

To the best of our knowledge, the present study represents the first demonstration that ARMC8 was overexpressed in osteosarcoma cell lines. Knockdown of ARMC8 significantly inhibited osteosarcoma cell proliferation *in vitro* and markedly inhibited xenograft tumor growth *in vivo*. ARMC8 silencing also suppressed the EMT phenotype, as well as inhibited the migration and invasion in osteosarcoma cells. Furthermore, knockdown of ARMC8 obviously inhibited the expression of β -catenin, c-Myc, and cyclin D1 in MG-63 cells.

Recently, research reported that the expression of ARMC8 was upregulated in ovarian cancer and the borderline ovarian tumor tissues, in comparison with the benign ovarian tumors and the normal ovarian tissues (11). Fan et al. confirmed that ARMC8 expression in breast carcinoma including breast carcinoma *in situ*, infiltrating ductal carcinoma, and infiltrating lobular carcinoma was higher than that in normal breast epithelia, usual ductal hyperplasia, benign breast tumors, and breast epithelia with dysplasia (12). Consistent with the above data, in this study we found that ARMC8 was overexpressed in osteosarcoma cell lines. All these data suggest that ARMC8 may act as an oncogenic factor in osteosarcoma.

Previous studies demonstrated that ARMC8 was implicated in the growth of malignant tumors (9,10,13). For example, Xie et al. confirmed that ARMC8 α downregulation by siRNA knockdown inhibited growth and colony formation in A549 lung cancer cells, while ARMC8 α overexpression promoted growth and colony formation in H1299 lung cancer cells (10). In agreement with the prior

study, herein we observed that knockdown of ARMC8 significantly inhibited osteosarcoma cell proliferation *in vitro* and markedly inhibited xenograft tumor growth *in vivo*. Collectively, these data obtained from both *in vitro* and *in vivo* experiments strongly suggest that ARMC8 may play an important role in the development and progression of human osteosarcoma.

EMT is known to be a central mechanism responsible for invasiveness and metastasis of various cancers (14–16). A particular characteristic of EMT is downregulation of the epithelial marker E-cadherin and increase in mesenchymal markers such as N-cadherin, vimentin, and fibronectin (17). Furthermore, previous studies indicated that ARMC8 plays an important role in tumor cell migration and invasion (9–11). We observed that ARMC8 silencing also suppressed the EMT phenotype, as well as inhibiting the migration and invasion in osteosarcoma cells. These data suggest that ARMC8 positively regulates EMT, consequently markedly inducing osteosarcoma cell migration and invasion *in vitro*.

The Wnt/ β -catenin signaling pathway plays a pivotal role in regulating osteosarcoma cell growth, EMT, invasion, and metastasis (18–21). Its aberrant activation is a key event in the pathogenesis and progression of human osteosarcoma. β -Catenin is a main downstream effector of the canonical Wnt signaling pathway and induces the transcription of several target genes, such as cyclin D1 and c-Myc, which are involved in cell survival, proliferation, and metastasis (22). Most interestingly, in a recent study ARMC8 α knockdown downregulated canonical Wnt signaling pathway activity and the expression of cyclin D1 and matrix metalloproteinase-7 (MMP-7) in non-small cell lung cancer cells (10). Similarly, in this study we found that ARMC8 obviously inhibited the expression of β -catenin, c-Myc, and cyclin D1 in MG-63 cells. These results suggest that ARMC8 silencing inhibits proliferation and invasion of osteosarcoma cells through suppression of the Wnt/ β -catenin signaling pathway.

In conclusion, this report demonstrates that ARMC8 silencing inhibits proliferation and invasion in osteosarcoma cells. Therefore, ARMC8 may play an important role in the development and progression of human osteosarcoma and may represent a novel therapeutic target in the treatment of osteosarcoma.

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