



Upregulation of exosomal integrin $\beta 4$ causes osteosarcoma cell proliferation via the PI3K-Akt-mTOR signaling pathway

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Background: Osteosarcoma is the most common primary bone malignant tumor in adolescents and young adults. Although the molecular pathogenesis of osteosarcoma is well studied, the role of integrin $\beta 4$ in the progression of osteosarcoma is still largely unknown.

Methods: Using blood samples obtained from 46 patients and 38 healthy donors as well as human osteosarcoma cell lines MG-63 and U2-OS, we investigated the roles of integrin $\beta 4$ in the proliferation of osteosarcoma cells.

Results: Integrin $\beta 4$ was commonly overexpressed in the serum and exosomes of patients with osteosarcoma, and this phenomenon significantly correlated with advanced tumor stage. Furthermore, *in vitro* experiments indicated that a knockdown of integrin $\beta 4$ could notably suppress proliferation of both MG-63 and U2-OS cells, and upregulate the p16, p21, and p27 proteins in osteosarcoma cells. By means of PI3K inhibitor LY294002, we found that the PI3K-Akt-mTOR signaling pathway may be involved in the effect of integrin $\beta 4$ on the proliferation of osteosarcoma cells.

Conclusions: This study for the first time demonstrates that $\beta 4$ integrin is highly expressed in osteosarcoma cell lines and in high-grade osteosarcoma tumors, and the larger amount of $\beta 4$ integrin promotes proliferation of osteosarcoma cells (i.e., cancer progression) possibly via the PI3K-Akt-mTOR signaling pathway.

Keywords: Osteosarcoma; integrin $\beta 4$; proliferation; PI3K-Akt-mTOR

Submitted Mar 01, 2018. Accepted for publication Sep 06, 2018.

doi: 10.21037/tcr.2018.09.02

View this article at: <http://dx.doi.org/10.21037/tcr.2018.09.02>

Introduction

Osteosarcoma is the most common bone cancer and is the second-leading cause of cancer deaths among adolescents and young adults (1,2). Although there have been many advances in the diagnosis and treatment strategies for osteosarcoma (patients now have a higher survival rate, at 60–80%), the prognosis remains unsatisfactory, especially

for patients at advanced stages (3). Thus, further elucidation of the pathogenesis of osteosarcoma is urgently needed to improve the rates of the diagnosis, therapy, and prognosis of osteosarcoma patients.

Integrins belong to the family of heterodimeric transmembrane cell surface receptors, which contain α and β subunits that conjointly connect the cytoskeleton to elements in the extracellular matrix or to adjacent cells (4).

Table 1 The clinical and pathological characteristics of included osteosarcoma patients and healthy donors

Variables	Osteosarcoma (n=46)	Healthy (n=38)
Gender, n (%)		
Male	25 (54.3)	21 (55.3)
Female	21 (45.7)	17 (44.7)
Age (years)		
Median	16	14
Range	4–25	4–23
Stage, n (%)		–
I	5 (10.9)	
II	16 (34.8)	
III	7 (15.2)	
IV	18 (39.1)	
Tumor site, n (%)		–
Distal femur	22 (47.8)	
Proximal tibia	10 (21.7)	
Distal humerus	4 (8.7)	
Distal fibular	5 (10.9)	
Proximal humerus	4 (8.7)	
Pelvis	1 (2.2)	
Histological classification, n (%)		–
Conventional	41 (89.1)	
Telangiectatic	2 (4.3)	
Periosteal	3 (6.5)	

The $\alpha 6\beta 4$ integrin (referred to as integrin $\beta 4$) is an adhesion receptor for the laminins that performs a crucial function both in normal tissue development and cell homeostasis as well as in carcinoma progression (5). Integrin $\beta 4$ has been reported to play a pivotal role in progression of many types of tumors, including osteosarcoma, prostate cancer, breast cancer, and ovarian cancer in a ligand-independent manner to promote proliferation, migration, and invasion (6–10). A recent report showed that exosomal integrins may predict organ-specific metastasis, and exosomal integrin $\beta 4$ is associated with lung metastasis (7). Thus, it can be said that exosomal integrin $\beta 4$ is involved in carcinoma progression; however, its role in osteosarcoma remains elusive. Taken together, these reasons inspired us to further explore the usefulness of exosomal integrin $\beta 4$ for predicting the risk of

osteosarcoma progression.

Accumulating evidence has suggested that PI3K-Akt-mTOR signaling is involved in the progression of many types of tumors including osteosarcoma (11,12). PI3K-Akt-mTOR signaling practically takes part in the whole progression of a tumor: from tumorigenesis, cancer cell proliferation, metastasis, and angiogenesis to chemoresistance (13). A previous work indicated that integrin $\beta 4$ cooperates with the PI3K-Akt-mTOR signaling pathway to promote invasive growth and metastasis of breast cancer cells (14). These clues prompted us to investigate the interaction between integrin $\beta 4$ and PI3K-Akt-mTOR signaling in osteosarcoma.

The present study was aimed at investigating the role of integrin $\beta 4$ in the pathogenesis of osteosarcoma as well as the underlying molecular mechanism in enhancing osteosarcoma cell proliferation with regard to regulation of the PI3K-Akt-mTOR signaling pathway.

Methods

Patients and blood sample collection

Blood samples were obtained from 46 patients with a diagnosis of osteosarcoma and 38 healthy donors in the Xijing Hospital, The Fourth Military Medical University, from June 2014 to October 2016. All the samples from patients were collected immediately after diagnosis, without any treatment. Written consent for participation in the study was obtained from all the participants or, where participants were children, from a parent or guardian. This study protocol was approved by the ethics committee of the Xijing Hospital, The Fourth Military Medical University (approval No. ChiCTR-OPC-17010758). Patients' clinical information is showed in *Table 1*.

Cell culture and treatment methods

MG-63 and U2-OS cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in the DMEM medium with 10% of fetal bovine serum (FBS, Gibco, USA) in a humidified atmosphere containing 5% of CO₂ and 95% of air at 37 °C. For PI3K inhibitor LY294002 treatment experiments, the cells in culture medium were incubated with or without LY294002. Cells where the final concentration of LY294002 reached 20 mmol/L were regarded as a treatment group, and the cells incubated without LY294002 served as the

control group. All unmentioned chemical reagents used in this study were purchased from Sigma-Aldrich (USA).

Enzyme-linked immunosorbent assay (ELISA)

Integrin $\beta 4$ expression in peripheral blood or in the cell culture medium was measured via an ELISA kit (Sigma, RAB0212). The absorbance was detected by Thermo Multiskan Ascent at 450 nm (Thermo, USA). Each experiment was performed three times.

Isolation of exosomes by differential centrifugation

Exosomes were isolated from a cell culture medium or serum by differential centrifugation on Eppendorf Centrifuge 5804 R. Serum and cells were centrifuged at 10,000 $\times g$ for 30 min at 4 °C in a A-4-44 swinging-bucket rotor, and the supernatant was centrifuged at 10,000 $\times g$ for 30 min at 4 °C in the identical rotor to remove shed vesicles and other vesicles of bigger sizes, and then centrifuged at 100,000 $\times g$ for 60 min at 4 °C in a Type 60 Ti rotor to obtain purified exosomes. Finally, the pellets were resuspended in phosphate-buffered saline (PBS) and stored at -80 °C for subsequent analysis.

Isolation of exosomes by means of markers CD63 and CD81

Exosomal markers CD63 and CD81 were detected by flow-cytometric analysis. Exosomes from serum or a cell culture medium were incubated with 5 μL of an antibody to CD81 (PE-Cy7 conjugate; eBioscience, San Diego, CA, 1:100) or to CD63 (FITC conjugate; eBioscience, San Diego, CA, 1:100) for 30 min at 4 °C away from light. Then, these exosomes were resuspended in the 400 μL of a buffer for flow staining. The percentage of exosomes was determined by flow cytometry, and the results were analyzed in the CXP software.

Transmission electron microscopic analysis

For transmission electron microscopy, the isolated exosomes were dissolved in a droplet of 4% paraformaldehyde in PBS, and a drop of the suspension was placed on a carbon-coated copper grid for 5 min, then removed from the grid, and we drained the excess liquid with filter paper. The grid

was stained with saturated uranyl acetate for 5 min, allowed to dry for 10 min, and then examined under a transmission electron microscope (Hitachi H-7600; Hitachi Ltd., Tokyo, Japan) at 100 kV.

Particle size analysis

Exosome size distribution was tracked using the qNano system (Izon Science, Christchurch, New Zealand). Exosomes were diluted in PBS, and the size was detected by means of the NP100 nanopores and CPC100 calibration particles. Finally, the data were analyzed by means of the Izon Control Software Suite (version 2.2).

Quantitative reverse-transcription RT-PCR

Total RNA (2.5 μg) in the exosomes isolated from cell culture medium or serum was reverse-transcribed into cDNAs (Sangon Biotech, Shanghai) and subjected to quantitative PCR. The primer sequences for RT-PCR amplification were as follows: integrin $\beta 4$, 5'-CTGGTCTTCTCCACCGAGTCA-3' (F), 5'-ATCGTTGCGGCTCATGATG-3' (R); GAPDH, 5'-TGGGTGTGAACCATGAGAAGT-3' (F), 5'-TGAGTCCTTCCACGATACCAA-3' (R). The expression level of GAPDH served as a control. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of target genes. All the reactions were conducted in triplicate.

Western blotting

Exosomes isolated from a cell culture medium were homogenized and lysed in RIPA lysis buffer, and protein concentration was quantified by means of the BCA kit (Thermo Scientific). Next, the samples were resolved by SDS-PAGE on a 5% gel, and then transferred to a porous polyvinylidene fluoride membrane (Millipore, USA). The primary antibodies used in the present study were specific to the following proteins: integrin $\beta 4$, phospho (p)-PI3K, PI3K, p-Akt, Akt, p-mTOR, mTOR, p16, p21, p27, and β -actin (Abcam, USA). The membranes were incubated with anti-mouse IgG antibodies (1:10,000) (secondary antibodies) with gentle shaking. Finally, each specific protein was visualized with a chemiluminescence detection system. Quantitative data were analyzed in the Quantity One software.

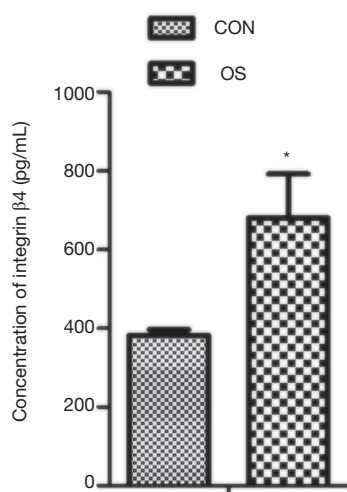


Figure 1 The concentration of integrin $\beta 4$ in the serum of patients with osteosarcoma and the healthy donors as detected by an ELISA. Data are expressed as the mean \pm SD, *, $P < 0.01$ compared with a control.

Cell proliferation assays

Cell proliferation was measured by the CCK-8 assay (Dojindo, Japan). After cell incubation, the CCK-8 reagent was added into each well for 1.5 h incubation. A microplate reader was used to measure the absorbance (OD value) at a wavelength of 450 nm. A colorimetric assay was performed, and all the experiments were conducted three times.

A knockdown of integrin $\beta 4$ and siRNA transfection

Integrin $\beta 4$ silencing was implemented by means of the following siRNA sequence: 5'-GTGGATGAGTTCCGGAATAAA (Hilden, Germany). Cell transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). An siRNA scrambled sequence was included as a control (Santa Cruz Biotechnology). Western blotting and qRT-PCR were performed to confirm knockdown efficiency.

Statistical analysis

All the data are presented as the means \pm standard deviation. Student's t test or One-way ANOVA was employed to test the significance of differences between groups. Spearman's rank correlation was calculated to assess the relation between a tumor stage and integrin $\beta 4$ expression in the serum. Data with $P < 0.05$ were considered statistically significant.

Results

Integrin $\beta 4$ concentration in the serum from patients with osteosarcoma

To explore the changes in integrin $\beta 4$ concentration, we measured the level of integrin $\beta 4$ in the serum from patients with osteosarcoma and healthy donors by the ELISA. Figure 1 shows that the concentration of integrin $\beta 4$ in the serum of patients with osteosarcoma was higher than that in the healthy donors (Student's t test; $t = -17.95$, $P < 0.01$). We next analyzed the correlation between the grade of osteosarcomas and the concentration of integrin $\beta 4$ and found that a high serum integrin $\beta 4$ significantly correlated with an advanced tumor stage (Spearman's rank correlation; $r = 0.673$, $P < 0.01$). Thus, it can be said that integrin $\beta 4$ change may be involved in osteosarcoma progression.

Integrin $\beta 4$ expression in the exosomes isolated from serum (Figure 2)

To further examine the effect integrin $\beta 4$ on osteosarcoma progression, we isolated exosomes from the serum of patients with osteosarcoma and healthy donors. Transmission electron microscopy was employed to verify the isolation of the exosomes from the serum. The results revealed that images of round microvesicles with diameters of 50–100 nm were captured successfully (Figure 2A). We examined exosomal markers CD63 and CD81 by flow cytometry and confirmed that the exosomes expressed CD63 and CD81 at 76.58% and 75.04% prevalence, respectively (Figure 2B,D,F). ELISA and RT-PCR were carried out to evaluate the level of integrin $\beta 4$ in the exosomes. The results showed that integrin $\beta 4$ in the exosomes from patients with osteosarcoma was significantly upregulated as compared with that in the healthy donors (ELISA, Student's t -test; $t = -10.06$, $P < 0.01$; RT-PCR, Student's t -test; $t = -21.45$, $P < 0.01$; Figure 2C,E). These results suggested that integrin $\beta 4$ in the exosomes participates in the osteosarcoma progression.

siRNA-silenced integrin $\beta 4$ expression in the exosomes isolated from the cell culture medium

We silenced integrin $\beta 4$ in U2OS and MG63 cells by means of a specific siRNA sequence and observed reduced integrin amounts in their exosomes and then measured the expression of integrin $\beta 4$ by Western blotting and RT-PCR. As is shown in Figure 3A, the sizes of these exosomes

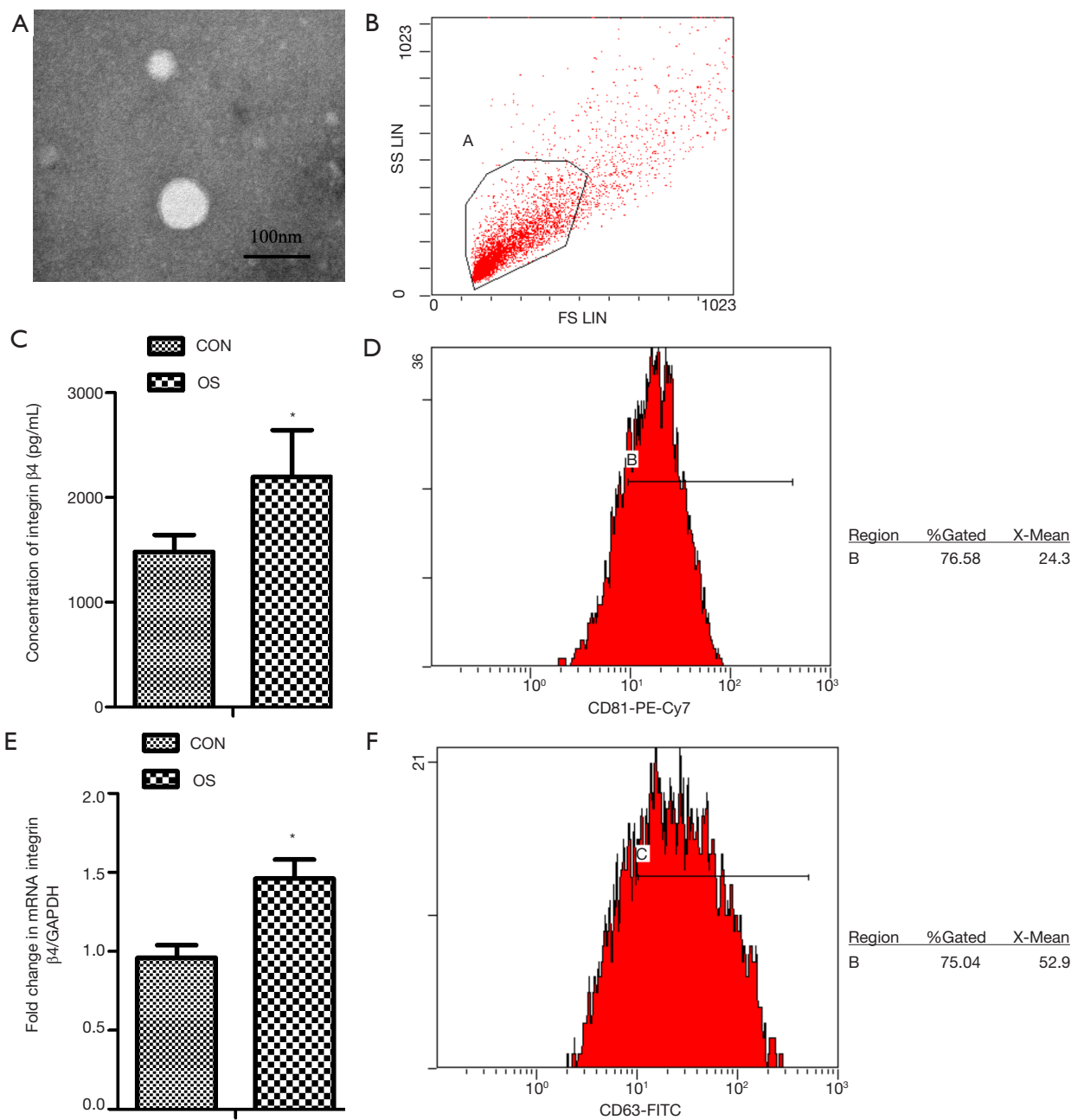


Figure 2 The analysis of integrin $\beta 4$ in the exosomes isolated from the serum of patients with osteosarcoma and healthy donors. (A) Exosomes isolated from serum were scanned by a transmission electron microscope. (B,D,F) Exosomal markers CD63 and CD81 were expressed according to flow-cytometric analysis. (C) The concentration of integrin $\beta 4$ in the exosomes isolated from the patients with osteosarcoma and the healthy donors as detected by an ELISA. (E) The level of integrin $\beta 4$ mRNA in the exosomes isolated from the patients with osteosarcoma and the healthy donors, as detected by RT-PCR. Data are expressed as the mean \pm SD, *, $P < 0.01$ compared with a control.

were mostly ~70–100 nm. The positivity rates of exosomal markers CD63 and CD81 were 80.43% and 72.64%, respectively (Figure 3B,C,D). These results indicated that exosomes were successfully isolated by differential centrifugation from the two cell lines. The protein and mRNA levels of integrin $\beta 4$ in the exosomes isolated from MG-63 cells [one-way ANOVA, Western blotting; $F_{(2,21)}=15.50$, $P<0.001$; RT-PCR; $F_{(2,21)}=58.14$, $P<0.001$] and U2-OS cells [one-way ANOVA, Western blotting; $F_{(2,21)}=12.15$, $P<0.001$; RT-PCR; $F_{(2,21)}=38.13$, $P<0.001$] were downregulated by siRNA as compared with the siRNA scrambled group (Figure 3E,F). These results suggested that the specific siRNA sequence effectively reduced the expression of integrin $\beta 4$ in the exosomes isolated from the MG-63 and U2-OS cells.

The knockdown of integrin $\beta 4$ inhibits osteosarcoma cell proliferation in MG-63 and U2-OS cells

One study revealed that integrin $\beta 4$ is involved in cancer cell proliferation (9), and then we selected these cell lines transfected with integrin $\beta 4$ siRNA that causes a transient knockdown to perform CCK-8 cell assays. As illustrated in Figure 4A,B, the knockdown integrin $\beta 4$ significantly inhibited proliferation of both MG-63 and U2-OS cell lines. Proteins p16, p21, and p27 play an important part in the progression of malignant tumors and can inhibit cell proliferation by binding to a variety of cyclins and cyclin-related protein kinases to hinder the procession of the cell cycle (15). Thus, we measured the protein expression of p16, p21, and p27 and found that siRNA significantly increased the p16, p21, and p27 protein levels in both MG-63 and U2-OS cell lines as compared with that in the siRNA scrambled group ($P<0.001$, $P=0.006$, $P<0.001$ for p16, p21, and p27 in MG-63 cells; $P=0.001$, $P<0.001$, $P<0.001$ for p16, p21, and p27 in U2OS cells, respectively; Figure 4C). Taken together, these results indicated that the knockdown of integrin $\beta 4$ inhibited osteosarcoma cell proliferation both in MG-63 and U2-OS cells.

The effect of PI3K-Akt-mTOR signaling pathway on the osteosarcoma proliferation

One study gave a clue that the PI3K-Akt-mTOR signaling pathway crucially participates in the osteosarcoma proliferation (16). In the current study, we tested whether integrin $\beta 4$ regulated osteosarcoma proliferation via PI3K-Akt-mTOR pathway signaling. Surprisingly, we found

that after the knockdown of integrin $\beta 4$, the protein levels of p-PI3K, PI3K, p-Akt, Akt, p-mTOR, and mTOR all remarkably decreased in both MG-63 and U2-OS cells as compared with the controls (all $P<0.05$; Figure 5). To confirm these results, we also used the PI3K inhibitor LY294002-treated MG-63 and U2-OS cells to examine the change in osteosarcoma cell proliferation. As presented in Figure 6A,B, the cell proliferation inhibition was totally reversed by the PI3K inhibitor LY294002 through decreasing the level of $\beta 4$ integrin in MG-63 and U2-OS cell lines. These markers of proliferation—p16, p21, and p27—were also upregulated by LY294002 (all $P<0.05$), while there was no significant effect of LY294002 on the p16, p21 and p27 levels in the siRNA integrin $\beta 4$ group (all $P>0.05$) (Figure 6C). In conclusion, these results suggested that LY294002 mimics the effect of integrin knockdown.

Discussion

Exosomal integrins have been reported to be deeply involved in the tumorigenesis and malignant progression of osteosarcoma (17,18). However, the underlying mechanism is poorly understood. In the present study, we sought to understand the involvement and molecular mechanisms of action of integrin $\beta 4$ in the regulation of osteosarcoma cell proliferation. Our data suggested that the concentration of integrin $\beta 4$ in the serum of patients with osteosarcoma was higher than that in the healthy donors, and the high integrin $\beta 4$ level in the serum significantly correlated with advanced tumor stage. We next isolated exosomes from the serum of patients with osteosarcoma and healthy donors and found that integrin $\beta 4$ in the exosomes from patients with osteosarcoma was also significantly more abundant as compared with that in the healthy donors. Exosomes are cell-derived small membrane microvesicles with a diameter of 40–150 nm (12)—present in the body fluids such as blood and urine—and contain a variety of biomolecules such as proteins, lipids, RNA, and DNA (13). Exosomes have been known to mediate tumor progression, such as activation of fibroblasts, promotion of angiogenesis, and enhancement of tumor cell proliferation and invasiveness as well as chemoresistance (10,17). Integrin $\beta 4$ has been reported to be highly expressed in osteosarcoma cell lines and high-grade tumors from patients, at metastatic sites and in primary tumors (19). Furthermore, integrin $\beta 4$ binds to multiple oncogenic receptor tyrosine kinases, such as EGFR, ErbB2/Neu, and Met, and promotes cell survival, proliferation, invasion, and migration (20–22).

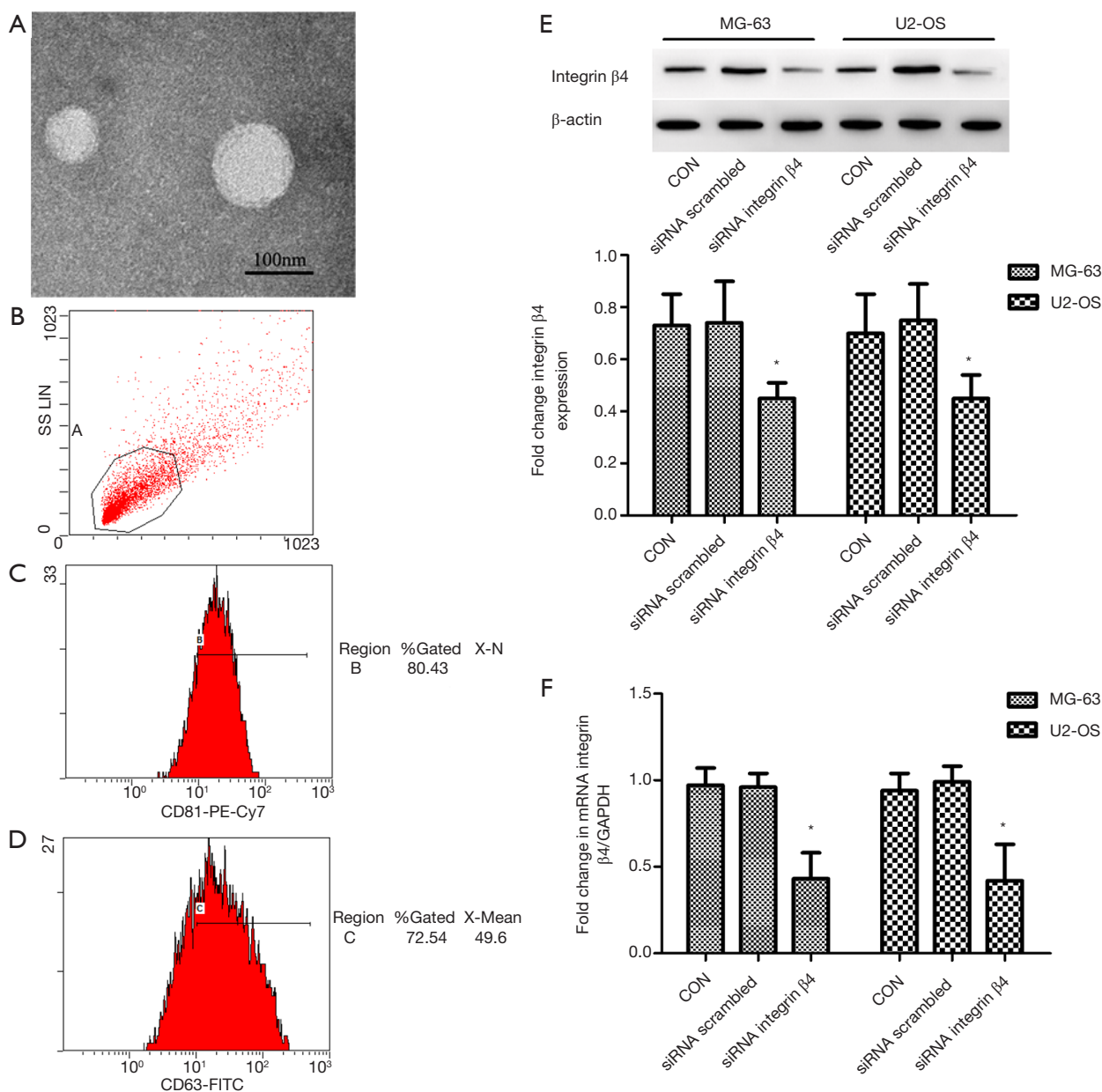


Figure 3 The analysis of integrin β4 in the exosomes isolated from the culture supernatants of MG-63 and U2-OS cells. (A) Exosomes isolated from cell culture medium scanned by a transmission electron microscope. (B,C,D) Exosomal markers CD63 and CD81 were expressed according to flow-cytometric analysis. (E) The protein expression of integrin β4 in the exosomes isolated from the cell culture medium, as detected by western blotting. (F) The level of integrin β4 mRNA in the exosomes isolated from the cell culture medium, as determined by RT-PCR. Data are expressed as the mean ± SD. *, P<0.01 as compared with a control.

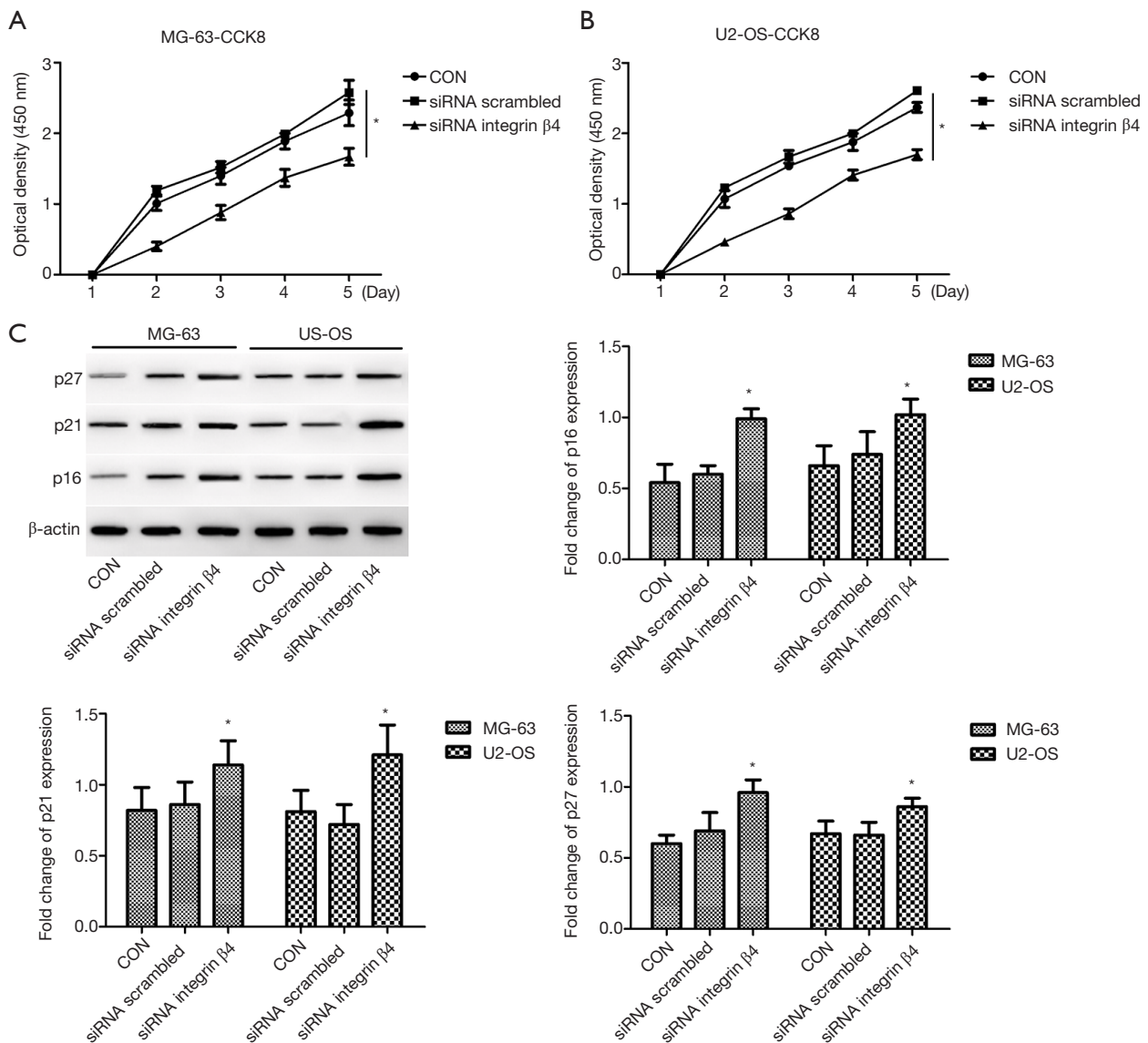


Figure 4 The knockdown of integrin $\beta 4$ reduces osteosarcoma cell proliferation *in vitro*. (A) The knockdown of integrin $\beta 4$ reduces osteosarcoma cell proliferation (MG-63 cells) as evaluated by the CCK8 assay. (B) The knockdown of integrin $\beta 4$ reduces osteosarcoma cell proliferation (U2-OS cells) evaluated using the CCK8 assay. (C) The protein expression of p16, p21, and p27 in both MG-63 and U2-OS cell lines was detected by western blotting. Data are expressed as the mean \pm SD. *, $P < 0.01$ as compared with a matched control.

These data suggest that exosomal integrin $\beta 4$ is involved in osteosarcoma progression.

To verify these data, MG-63 and U2-OS cells were applied to investigate the mechanism of action of integrin $\beta 4$ in osteosarcoma. The results of Western blotting and RT-PCR demonstrated that $\beta 4$ integrin was highly upregulated in both MG-63 and U2-OS cell lines. When we silenced integrin $\beta 4$ by a specific siRNA sequence, in

the exosomes isolated from the culture supernatants of both knockdown cell lines, the integrin $\beta 4$ expression was lower as compared with the supernatant of the control cells. Furthermore, we measured the effect of the integrin $\beta 4$ knockdown on osteosarcoma cell proliferation and found that this knockdown significantly inhibited the proliferation of both MG-63 and U2-OS cell lines. Accordingly, the protein expression of p16, p21, and p27, which are markers

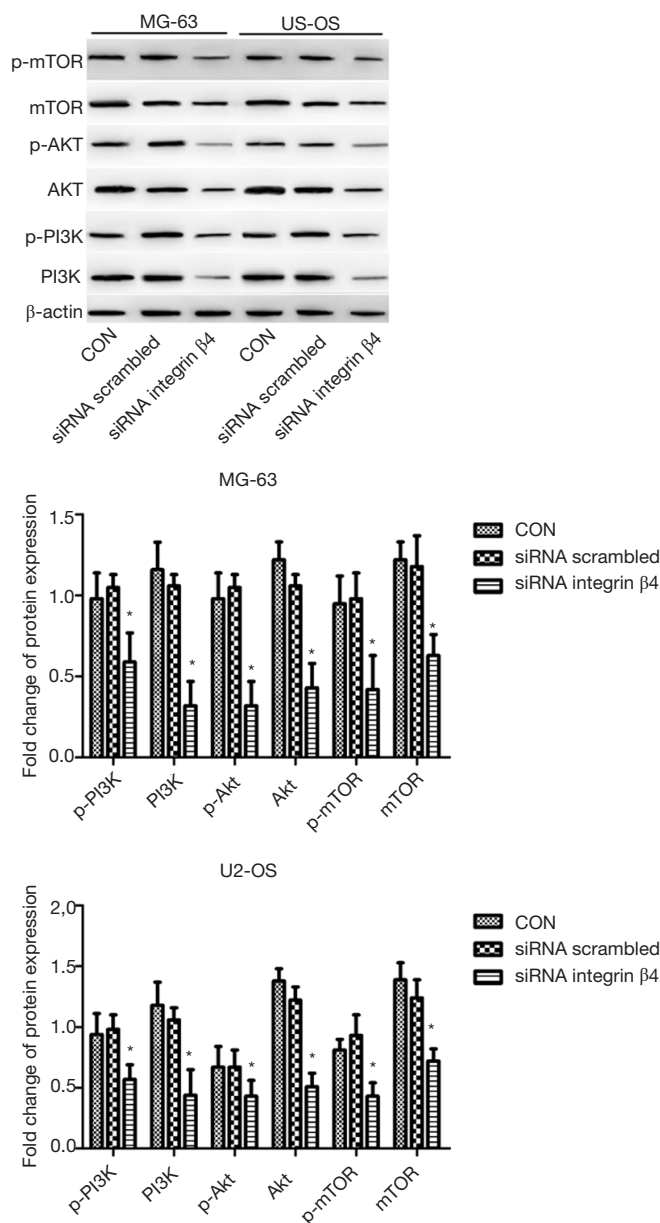


Figure 5 The protein levels of phospho (p)-PI3K, PI3K, p-Akt, Akt, p-mTOR, and mTOR were measured by western blotting. Data are expressed as the mean ± SD. *, P<0.05 as compared with a matched control.

of cell proliferation and can inhibit cell proliferation, was also upregulated by siRNA targeting integrin β4 in both MG-63 and U2-OS cell lines. Previous works have reported that integrin β4 can activate PI3K-Akt (23), PKC (24), and Ras-ERK (25) signaling pathways, which are associated with cell migration and proliferation. Our present results indicate that siRNA to integrin β4 significantly downregulated the protein expression of p-PI3K, PI3K, p-Akt, Akt, p-mTOR,

and mTOR in both MG-63 and U2-OS cell lines. To confirm these results, we also employed the PI3K inhibitor LY294002 to treat MG-63 and U2-OS cells and to examine the possible change of osteosarcoma proliferation. The data showed that the cell proliferation inhibition was totally reversed by LY294002 in both MG-63 and U2-OS cell lines. The markers of proliferation—p16, p21, and p27—were also upregulated by LY294002. In conclusion, these

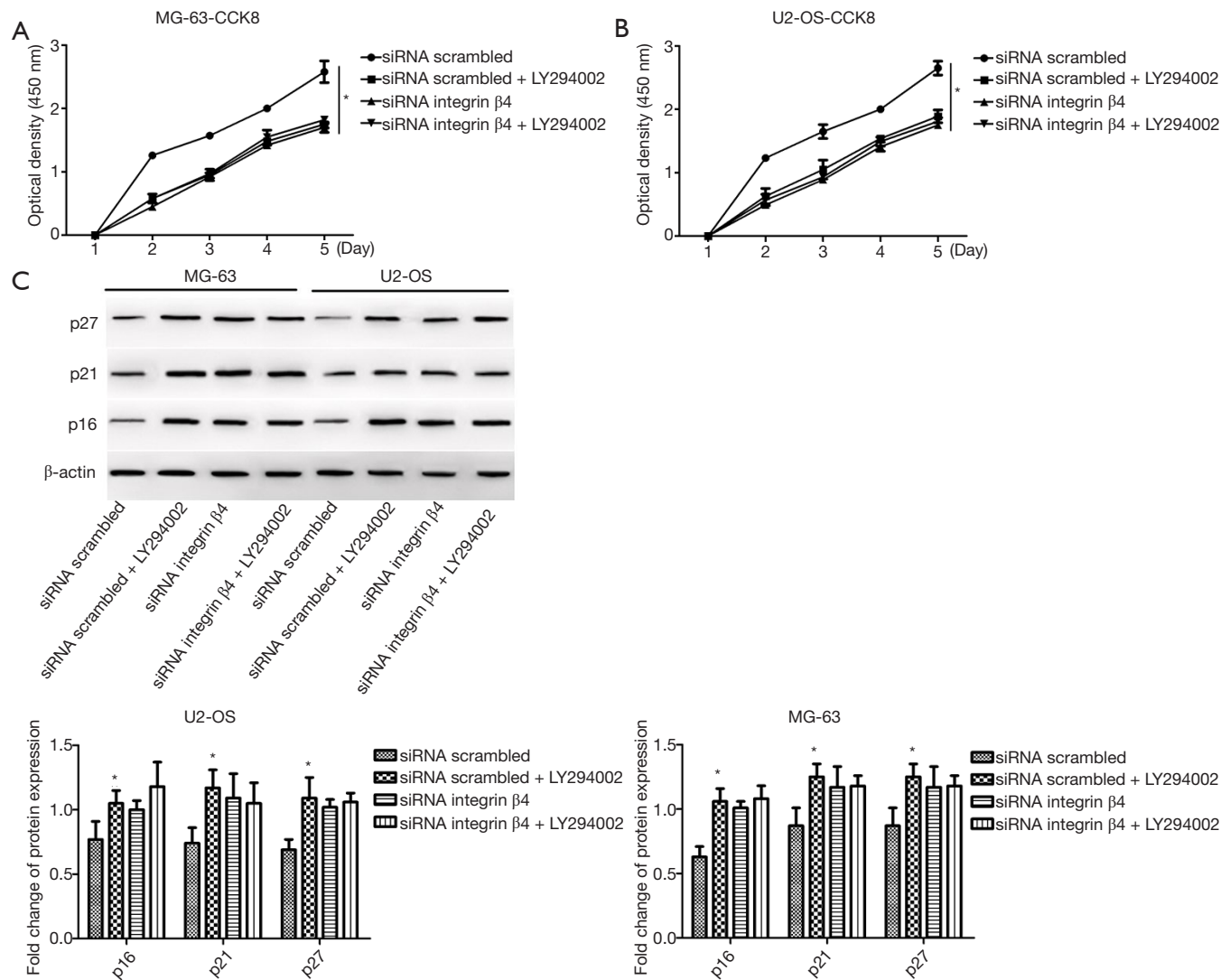


Figure 6 The effect of PI3K inhibitor LY294002 on osteosarcoma proliferation and p16, p21, and p27 protein expression. (A) LY294002 mimics the effect of integrin knockdown on osteosarcoma cell proliferation in MG-63 cells evaluated by the CCK-8 assay. (B) LY294002 mimics the effect of integrin knockdown on osteosarcoma cell proliferation in U2-OS cells evaluated using the CCK8 assay. (C) LY294002 increased the protein expression of p16, p21, and p27 in both MG-63 and U2-OS cell lines examined by western blotting. Data are expressed as the mean \pm SD. *, $P < 0.01$ as compared with a matched control.

findings suggest that $\beta 4$ integrin promotes cell proliferation probably via the PI3K-Akt-mTOR signaling pathway.

Conclusions

The present study for the first time demonstrated that $\beta 4$ integrin is highly upregulated in osteosarcoma cell lines and high-grade osteosarcoma tumors from patients, and

the larger amount of $\beta 4$ integrin promotes cell proliferation (osteosarcoma progression) probably via the PI3K-Akt-mTOR signaling pathway.

Acknowledgments

Funding: This study was supported by the National Natural Science Foundation of China (81572699).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2018.09.02>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study protocol was approved by the ethics committee of the Xijing Hospital, The Fourth Military Medical University (approval No. ChiCTR-OPC-17010758). Written consent for participation in the study was obtained from all the participants or, where participants were children, from a parent or guardian.

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Cite this article as: Wang Q, Shi Z, Zhang H, Liu W, Yu Z, Zhang Q. Upregulation of exosomal integrin $\beta 4$ causes osteosarcoma cell proliferation via the PI3K-Akt-mTOR signaling pathway. *Transl Cancer Res* 2018;7(5):1209-1220. doi: 10.21037/tcr.2018.09.02