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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.03.022>Involvement of $\alpha 5$ integrin in survivin-mediated osteosarcoma metastasisXiao-Zhou Liu^{1,2}, Cheng-Jun Li², Su-Jia Wu², Xin Shi², Jian-Ning Zhao^{1,3*}¹Southern Medical University, Guangzhou 510515, China²Department of Orthopedics, Jinling Hospital, Medical School of Nanjing University, Nanjing 210002, China³Southern Medical University, Guangzhou 510515, Guangdong Province, China

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ABSTRACT

Objective: To investigate the role of survivin in osteosarcoma metastasis.**Methods:** Small interfering RNA (siRNA) was used to knockdown the expression of survivin and $\alpha 5$ integrin in the human osteosarcoma cell line MG63. Western blotting and immunostaining methods were used to assess the effect of survivin knockdown on the expression of $\alpha 5$ integrin through flow cytometry and fluorescence microscopy detection. Meanwhile, the invasion and migration of transfected cells in Transwell and wound healing assays were probed, and the growth situation of these cells transplanted into nude mice was monitored.**Results:** Knockdown of survivin expression could inhibit the invasion and migration of osteosarcoma MG64 cells *in vitro* and the expression of $\alpha 5$ integrin on osteosarcoma MG64 cell surface, suggesting that survivin can inhibit the invasion and migration of osteosarcoma cells through downregulation of $\alpha 5$ integrin. Anti- $\alpha 5$ integrin antibody could also markedly decrease the capability of invasion and migration of osteosarcoma MG64 cells. Additionally, knockdown of survivin expression could slow the growth of osteosarcoma MG63 cells transplanted into nude mice.**Conclusions:** Survivin-directed anti-tumor strategies might be an effective method in the treatment of osteosarcoma.

1. Introduction

Osteosarcoma, the most frequently-encountered primary malignant tumor of bones, represents over 56% of all bone tumors, and it is the third most frequent cause of cancer in adolescents [1–3]. Conventional treatments for osteosarcoma include surgical resection, chemotherapy and radiotherapy, but the prognosis remains poor due to aggressive infiltration into local

tissue and extremely-high metastatic rate [4]. Hence, novel therapeutic approaches, such as biological therapies against specific targets, are urgently sought [5].

Survivin is an inhibitor of apoptosis and has been reported to confer tumor resistance to apoptotic stimuli [6]. It is abundantly expressed during fetal development, but its expression is low in most adult human tissues [7]. In some tumor cells, survivin expression increases aberrantly, which makes it be an attractive anti-tumor target. Elevated survivin expression is associated with enhanced proliferation, reduced apoptosis, resistance to chemotherapy and increased tumor recurrence [8,9]. In an animal model, survivin has been reported to contribute to tumor metastasis [10]. The level of survivin expression was correlated with malignant degree of osteosarcoma [11], even could be a prognostic indicator for osteosarcoma [12–14]. However, little is known regarding the specific role of survivin in osteosarcoma. Inhibition of survivin was previously reported to reduce proliferation and increase apoptosis of the

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human osteosarcoma cell lines [15,16], and meanwhile, it was also associated with spontaneous pulmonary metastasis of osteosarcoma in a mouse model. Nevertheless, the precise mechanisms that survivin regulates tumor cell metastasis are poorly understood.

McKenzie *et al.* [17] found that in melanoma, survivin could promote the proliferation of tumor cells via enhancement of integrin expression. The integrin family can not only act as receptors for cell adhesion molecules, but also can mediate cell proliferation, differentiation, apoptosis and motility [18–24]. These cell–cell interactions are crucial for tumor cell motility and growth [20]. Integrin expression is associated with metastatic phenotype of various tumors including osteosarcoma [18–21]. Blocking the expression of integrins with specific antibodies can decrease the invasiveness of tumor cells *in vitro* and *in vivo* [25].

In this study, the expression of survivin in the human osteosarcoma cell line MG63 was knocked down, and its effect on the properties of these cells important for metastasis was investigated. It was found that knockdown of survivin expression could inhibit MG63 invasion and migration, and downregulate the expression of $\alpha 5$ integrin. Subsequently, it was observed that decreased expression of $\alpha 5$ integrin mimicked the effect of survivin knockdown on MG63 invasion and migration. The study further confirmed that the growth of osteosarcoma MG63 cells transplanted into nude mice was suppressed obviously. All these findings suggest that survivin may promote osteosarcoma metastasis by means of expression of $\alpha 5$ integrin.

2. Materials and methods

2.1. Cell culture

Human osteosarcoma cell line MG63 cells (ATCC, Manassas, VA, USA) were cultured and incubated in DMEM containing 10% FBS at 37 °C.

2.2. Transwell migration and invasion assays

The migratory response of MG63 cells was measured by Transwell migration assay. Specific operations [26] were as follows: The experiment was performed using a Transwell cell culture plate (Corning Costar, Rochester, NY, USA). The Transwell inserts (8 μ m pore size) were pre-coated with Matrigel (Becton Dickinson, Bedford, MA). The upper chamber contained the cells in culture medium (5×10^5 /mL) with 1% FBS, and the lower chamber contained culture medium with 20% FBS. The cells transfected with survivin-siRNA (si-survivin) or scramble RNA were cultured in transwell inserts for 48 h. Non-migrated cells were scraped from the upper surface of the membrane with a cotton swab, and migrated cells on the underside of the filters were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. The number of cells was counted under a microscope.

2.3. Wound healing assay

MG63 cells were cultured as confluent monolayers and wounded by scratching with a sterile pipette tip. Wounded

monolayers were washed twice with PBS to remove detached cells and incubated in fresh medium for an additional 48 h. Images of the wounds were taken immediately at 0 h and 48 h. Migration of cells into the wound was recorded using a phase contrast microscope. The mean percentage of remaining cell-free area was quantified using ImageJ software, and compared with the area of initial wound to indicate wound healing.

2.4. Flow cytometry

MG63 cells were seeded in 6-well plates at 5×10^4 cells per well and transfected with siRNAs (sense: 5'-CAGACUUGGCCCA-GUGUUU-3', antisense: 5'-AAACACUGGGCCAAGUCUG-3') obtained from Shanghai GeneChem Co. Ltd (China). After 24 h, the cells were harvested using 0.05% trypsin solution (Sigma, St. Louis, MO, USA), fixed in 70% ethanol, washed and stained with anti-human $\alpha 5$ integrin mAb (1: 2 000, ebioscience, San Diego, CA, USA), prior to analysis using a FACSort flow cytometer (BD, NJ, USA).

2.5. Western blotting analysis

The cells transfected with si-survivin were harvested after two days and cell lysates were loaded onto a 4%–12% Bis-Tris gel, electrophoresed and transferred to a polyvinylidenedifluoride membrane. The membrane was subsequently immunoblotted with rabbit polyclonal anti-survivin (Novus) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce). The signals were visualized using an enhanced ECL kit from Amersham Pharmacia Biotech.

2.6. Immunofluorescence

Transfected cells were washed, fixed with methanol, and air-dried, then incubated with rabbit polyclonal anti-survivin antibody (Novus Biologicals) and secondary fluorescent antibody. The slides were washed and mounted with 4',6-diamidino-2-phenylindole. Images were captured by Zeiss Axioplan 2 imaging microscope.

2.7. Xenograft tumor formation

Six-week-old nude mice (Shanghai SLAC Laboratory Animal Co. Ltd, China) were bred and maintained under specific pathogen-free conditions in accordance with the Shanghai Medical Experimental Animal Care Commission. MG63 cells (5×10^6) transfected with either the scramble RNA or si-survivin were inoculated into the left and right flanks of nude mice, respectively. Three weeks after inoculation, the mice were sacrificed and the tumors were weighed.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean \pm SD of at least three independent experiments. Statistical significance was analyzed using a Student's *t*-test (two-tailed). *P* < 0.05 was considered to be statistically significant.

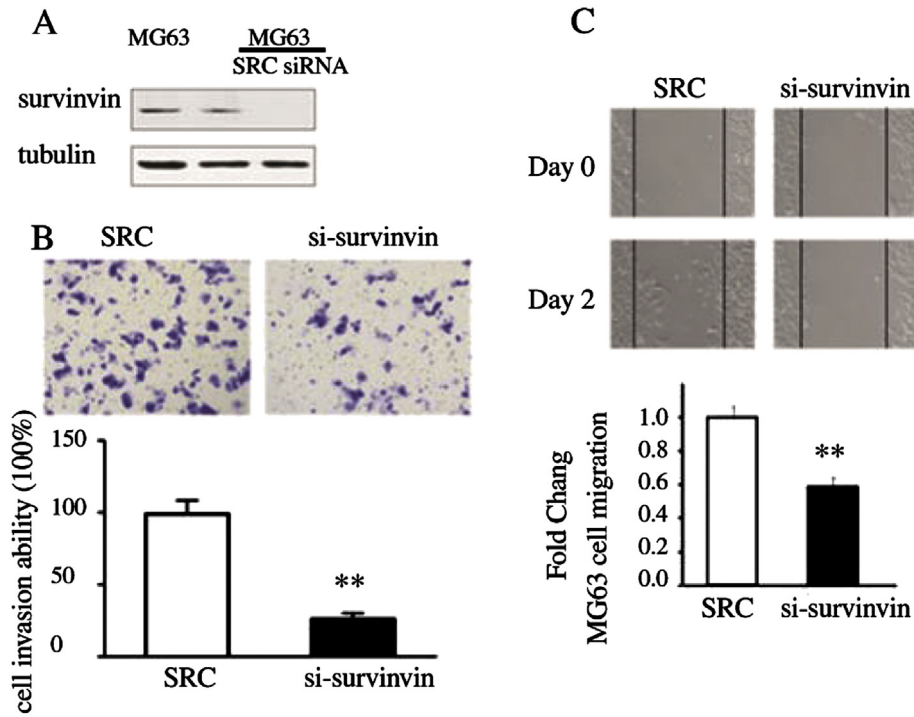


Figure 1. Knockdown of survivin expression in MG63 cells by siRNA and its role in cell invasion.

(A) Western blotting assay. Tubulin expression was used as a loading control. Src: Scramble RNA. (B) Transwell assay was employed to evaluate the invasion of these cells. The quantitative results for each group are shown as the number of invaded cells 24 h after incubation. (C) Wound healing assay. The quantitative results for each group are shown as the fold change of migrated cells vs. scramble control 48 h after incubation. ** $P < 0.01$.

3. Results

3.1. Survivin was required for human osteosarcoma MG63 cell migration

After transfection with siRNA, the level of survivin in MG63 cells was detected first. As shown in Figure 1A, the content of survivin in the cells transfected with si-survivin was significantly lower than that in the cells transfected with scramble RNA, indicating successful knockdown of survivin expression.

The effect of survivin on invasiveness of MG63 cells was evaluated by Transwell assay. The percentage of survivin knockdown cells that migrated was significantly lower than that of cells transfected with scramble RNA (Figure 1B). The effect of survivin on MG63 cell migration was further assessed by wound healing assay. It was found that survivin knockdown profoundly inhibited the MG63 cell migration (Figure 1C). Collectively, these results demonstrate that survivin is required for human osteosarcoma MG63 cell motility and its knockdown greatly attenuate MG63 cell migration, suggesting that survivin might be involved in the process of osteosarcoma metastasis.

3.2. Knockdown of survivin expression led to down-regulation of $\alpha 5$ integrin in MG63 cells

Considering the possibility that knockdown of survivin expression in MG63 cells might reflect decreased cell adhesion to extracellular matrix, it was concluded that $\alpha 5$ integrin might be

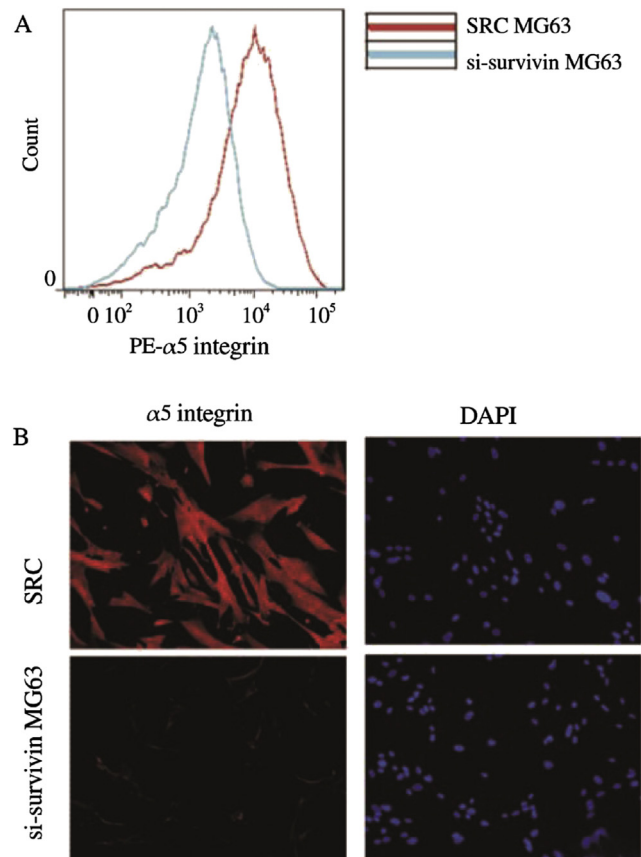


Figure 2. Knockdown of survivin expression led to decreased expression of $\alpha 5$ integrin.

(A) Flow cytometry. (B) Fluorescent microscopy.

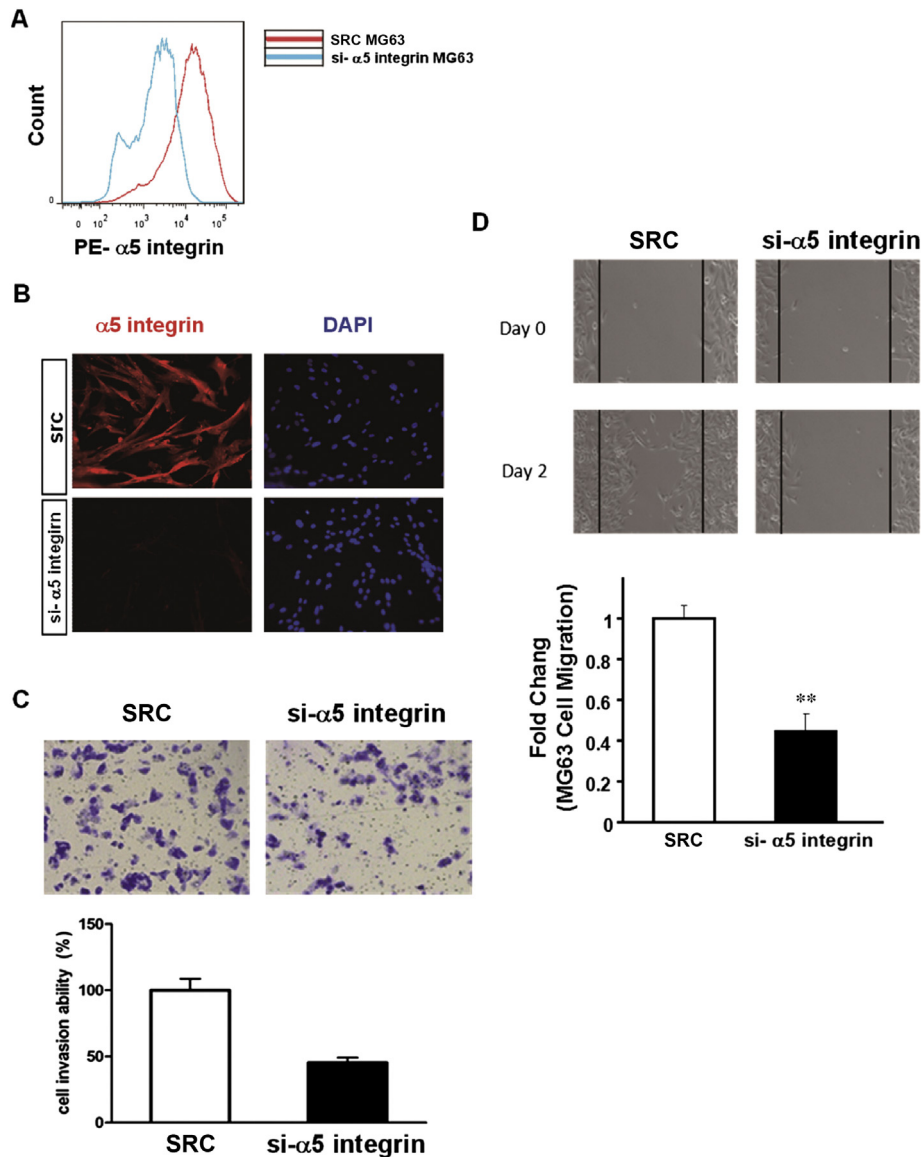


Figure 3. Downregulation of $\alpha 5$ integrin contributed to MG63 cell metastasis. (A) Flow cytometry. (B) Fluorescent microscopy. (C) Transwell assay. (D) Wound healing assay. ** $P < 0.01$.

involved in survivin-induced tumor cell metastasis. It was found that the expression of $\alpha 5$ integrin was markedly reduced in si-survivin transfected cells using flow cytometry (Figure 2A) and fluorescent microscopy (Figure 2B), suggesting that survivin is likely an upstream regulator of $\alpha 5$ integrin.

3.3. Knockdown of $\alpha 5$ integrin resulted in decreased metastatic ability of MG63 cells

MG63 cells with $\alpha 5$ integrin targeting siRNA were transduced. The knockdown of $\alpha 5$ integrin expression on the cell surface was confirmed by flow cytometry (Figure 3A), fluorescent microscopy (Figure 3B) and immunostaining assay.

Transwell and wound healing assays were conducted repeatedly in MG63 cells with knockdown of $\alpha 5$ integrin. As shown in Figure 3C, the cell invasiveness was significantly

weakened after knockdown of $\alpha 5$ integrin. Additionally, wound healing assay revealed that the migration of cells was over two fold in $\alpha 5$ integrin knockdown cells than scramble RNA transfected cells (Figure 3D). These results indicate that $\alpha 5$ integrin is related to the migration of human osteosarcoma cells.

3.4. Anti- $\alpha 5$ integrin antibody could effectively reduce MG63 motility

The invasiveness and migration of MG63 cells in Transwell and wound healing assays were measured under the action of anti- $\alpha 5$ integrin antibody (M200). It was found that the invasiveness and migration of tumor cells could be inhibited in the treatment of MG63 tumor cells using $\alpha 5$ integrin (Figure 4), suggesting that anti- $\alpha 5$ integrin can effectively block the metastasis of MG63 cells.

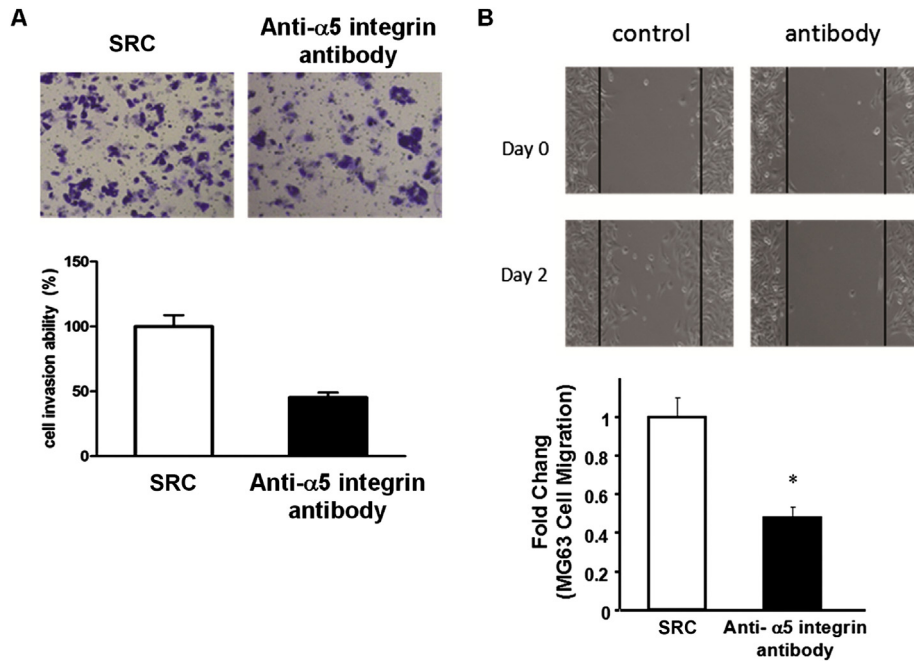


Figure 4. Anti-α5 integrin antibody decreased migration and invasiveness of MG63 cells. (A) Transwell assay. (B) Wound healing assay. **P* < 0.05.

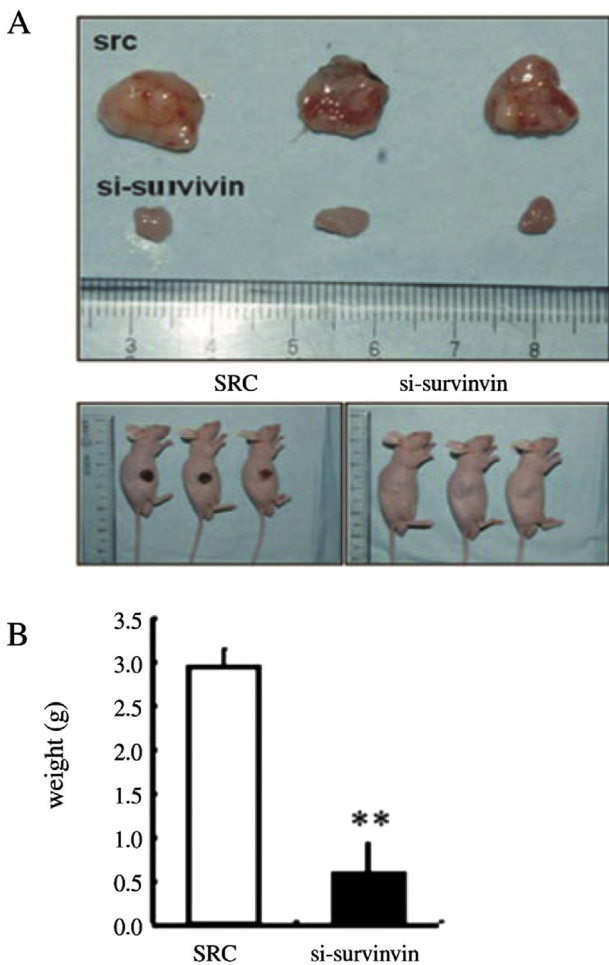


Figure 5. Tumorigenicity of scramble RNA and si-survivin cells (1×10^6) after subcutaneous injection in the flanks of nude mice (*n* = 10). (A) One representative mouse and formed tumors are shown. (B) The quantification of tumor weight is shown on the bottom (*n* = 10) (***P* < 0.01).

3.5. Knockdown of survivin expression could reduce the tumor growth in vivo

As shown in Figure 5, the tumor volume of survivin knockdown was smaller than that of cells transduced with scramble RNA. These results suggest that inhibition of survivin expression may suppress xenograft tumor formation in osteosarcoma cells *in vivo*.

4. Discussion

Survivin can protect osteosarcoma cells from apoptosis and enhance proliferation *in vitro* [15,16], but its role in osteosarcoma is not well understood. In this study, the role of survivin in the human osteosarcoma metastasis was explored, and siRNA knockdown of survivin expression was established in the human osteosarcoma cell line. It was found that knockdown of survivin expression could reduce the invasion and migration of this human osteosarcoma cell line *in vitro*, suggesting that survivin may be required for the metastasis of human osteosarcoma.

α5 integrin is a sort of protein that can make cells adhere to the extracellular matrix. The results in this study displayed that knockdown of survivin expression could decrease the expression of α5 integrin on the cell surface. On the contrary, knockdown of α5 integrin could also reduce the invasion and migration of this human osteosarcoma cell line, suggesting that knockdown of survivin expression may decrease the metastasis via inhibition of α5 integrin expression, thus reducing the capability of tumor cells adhering to the extracellular matrix.

In this study, a mouse model of osteosarcoma was established by transplantation of the human osteosarcoma cell line into nude mice so as to probe the role of survivin in osteosarcoma metastasis *in vivo*. It was found that knockdown of survivin expression could slow the tumor growth of the

transplanted human osteosarcoma cell line, indicating that survivin may involve in the process of osteosarcoma proliferation.

The results in this study also revealed that inhibition of survivin was detrimental to human osteosarcoma cell lines, which were consistent with the previous studies [15,16]. Upregulation of survivin-mediated $\alpha 5$ integrin could promote the metastasis of melanoma [17]. It is reported that survivin is associated with spontaneous pulmonary metastasis of osteosarcoma in another mouse model [10]. All of these findings point to a potential target for osteosarcoma. As survivin and $\alpha 5$ integrin are required for osteosarcoma metastasis, suppressing the expression of survivin or $\alpha 5$ integrin in tumor cells or blocking the activity of these proteins may reduce the tumor metastasis. For instance, survivin siRNA, antagonizing survivin function by dominant-negative surviving and anti- $\alpha 5$ integrin antibody all are conducive to the development of osteosarcoma treatment. Additionally, it was also found in this study that blocking $\alpha 5$ integrin antibody *in vitro* could inhibit the invasion and migration of the human osteosarcoma cell line.

It still needs further studies about the role of survivin in tumor cells and downstream signaling processes involved in survivin-induced metastasis. Survivin is expressed in some adult cells, but its inhibition is not associated with restrictive side effects in animal models [7]. In recent years, a small molecule inhibitor has been reported to inhibit the proliferation of human osteosarcoma cells and induce its apoptosis in a subcutaneous xenograft tumor model via downregulation of survivin pathway [27]. Hence, studying the drugs targeting to survivin may provide a new idea for osteosarcoma.

Conflict of interest statement

We declare that we have no conflict of interest.

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