Tumor Biol. (2015) 36:5965–5970
DOI 10.1007/s13277-015-3271-z

RESEARCH ARTICLE

Depletion of *ALX1* causes inhibition of migration and induction of apoptosis in human osteosarcoma

Mingfu Yang · Yong Pan · Yue Zhou

Received: 5 December 2014 / Accepted: 17 February 2015 / Published online: 4 March 2015

Abstract Osteosarcoma is the most common primary malignant tumor in children and young adults, and the molecular regulation of the invasion of osteosarcoma (OS) remains unknown. In this study, we found that increased expression of *ALX1* was associated with the progression of osteosarcoma and that *ALX1* protein levels were significantly elevated in matched distant metastases. High *ALX1* levels also predict shorter overall survival of osteosarcoma patients. We investigated the therapeutic potential of targeting *ALX1* expression using the technique of RNA silencing via short hairpin RNA (shRNA). Synthetic shRNA duplexes against *ALX1* were introduced to downregulate the expression of *ALX1* in a highly malignant osteosarcoma cell line, U2OS. The results obtained indicated that shRNA targeting of *ALX1* could lead to an efficient and specific inhibition of endogenous *ALX1* activity. Furthermore, we found that depletion of *ALX1* caused a dramatic cell cycle arrest, followed by massive apoptotic cell death, and eventually resulted in a significant decrease in migration and invasion of the osteosarcoma cell line studied.

Keywords Osteosarcoma · *ALX1* depletion · Migration · Apoptosis

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents [1–3]. Many patients experience tumor recurrence following treatment with current osteosarcoma therapy, which consists of chemotherapy and surgery. The distal femoral and proximal tibial metaphyses are the most common sites for development of osteosarcoma. Although combined chemotherapy and surgery have been applied, the 5-year survival of patients with no metastatic disease at diagnosis reaches about 70 % [4]. Nevertheless, the 5-year survival of patients with metastatic cancer becomes as low as about 20 %. However, the molecular regulation of the invasion of osteosarcoma remains elusive. To that point, there is an urgent need for studying the tumor biology of OS in order to increase our understanding so as to treat it more efficiently.

In this study, we found that Aristaless-like homeobox1 (*ALX1*), also known as Cart1, is important for the induction of morphologic changes in OS cells. A study on *ALX1* expression levels in several OS tissue was carried out. In order to investigate the possibility of turning *ALX1* into a novel therapeutic target for the treatment of osteosarcoma, U2OS was chosen to silence the expression of *ALX1* with the highly specific post-transcriptional suppression of RNAi. Thereafter, proliferation, cell cycle status, and apoptosis were also studied. The results obtained suggest that targeting of *ALX1* may be used as a potential and specific therapeutic tool for the treatment of human osteosarcoma.

Materials and methods

Immunohistochemistry

Primary osteosarcoma biopsies of 60 patients and normal bone tissue specimens were collected between June 2000 and December 2005 according to the regulations of the local ethical committee. Immunohistochemistry using anti-*ALX1* antibodies was performed as previously described [5]. Briefly sections were deparaffinized in xylene, rehydrated in alcohol and water, antigen repaired, and blocked. Anti-*ALX1* antibodies (1:150) were incubated overnight, followed by incubation with horseradish peroxidase-labeled polymer for

M. Yang · Y. Pan · Y. Zhou (✉)
Department of Orthopaedics, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China
e-mail: yzhoucq@163.com

20 min. Sections were then stained with DAB for 5 min. All sections were counterstained with hematoxylin, dehydrated, and mounted. Scoring was performed blindly by a pathologist according to the semiquantitative seventier system developed by Allred et al. [6]. This system assesses the percentage of positive cells (none=0, <10 %=1, 10–50 %=2, and >50 %=3) and the intensity of staining (none=0, weak=1, intermediate=2, and strong=3). The intensity and percentage scores are then added to give a final immunoreactivity score ranging from 0 to 6.

Cell culture

U2OS cell line was purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cells in which *ALX1* had been knocked down (U2OS ALX1-shRNA) and the control cells (U2OS control-shRNA) have been described and used in previous study [7]. They were made by the stable transfection with a shRNA-expressing vector against *ALX1* and a scrambled shRNA, respectively. All the cells were maintained in DMEM supplemented with 10 % FBS at 37 °C in a 5 % CO2 and humidified atmosphere.

Stable knockdown cell line generation

A vector-based RNAi approach was used to generate the stable *ALX1* knockdown cell line. Briefly, according to the corresponding RNAi sequences used in transient experiment, the double-stranded short hairpin RNA (shRNA) template for both the negative control and *ALX1* were designed and cloned into the Hind III/Bgl II sites of the pSUPER.retro.neo+GFP vector (Oligoengine Corporation), respectively. The recombinant RNAi constructs (pSUPER-Control-shRNA and pSUPER-*ALX1*-shRNA) were confirmed by direct sequencing and transfected into U2OS cells by Lipofectamine2000 (Invitrogen), respectively. The transfected cells were then subcultured and selected in the presence of G418 for generating the negative control and *ALX1* stable knockdown cells, designated as pControl-sh and p*ALX1*-sh, respectively.

Migration assay

Cell motility was tested in 8-μm-pore polycarbonate membrane Transwell chambers (Corning) essentially as described previously [8]. Cells were resuspended in DMEM/F12 without fetal bovine serum, and 75,000 cells were added to the top chamber of the Transwell chambers. DMEM/F12 containing 10 % FBS was added to the bottom chamber, and cells were allowed to migrate for 24 h. Nonmigrated cells were scraped off the top of the membrane. Migrated cells were fixed in 4 % formaldehyde and stained in Giemsa. Cells were counted under a microscope in five different fields in duplicate wells, in at least three independent experiments.

Apoptosis assay

Apoptosis was assayed using the Annexin V-FITC Apoptosis Kit (keygen, China) according to the manufacturer's instructions. Briefly, the cells were harvested and washed twice with PBS, followed by resuspension in Annexin-V binding buffer, and then FITC-conjugated Annexin V and PI were added. After incubation for 10 min at room temperature in the dark, another binding buffer was added, and the samples were immediately analyzed using FCM.

Quantitative real-time PCR

Total RNA was extracted from cultured cells using EZNA Total Rna Kit (OMEGA Bio-tek, USA), and complementary DNA (cDNA) was generated using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). Quantitative real-time PCR was performed using the SYBR Premix ExTaq II

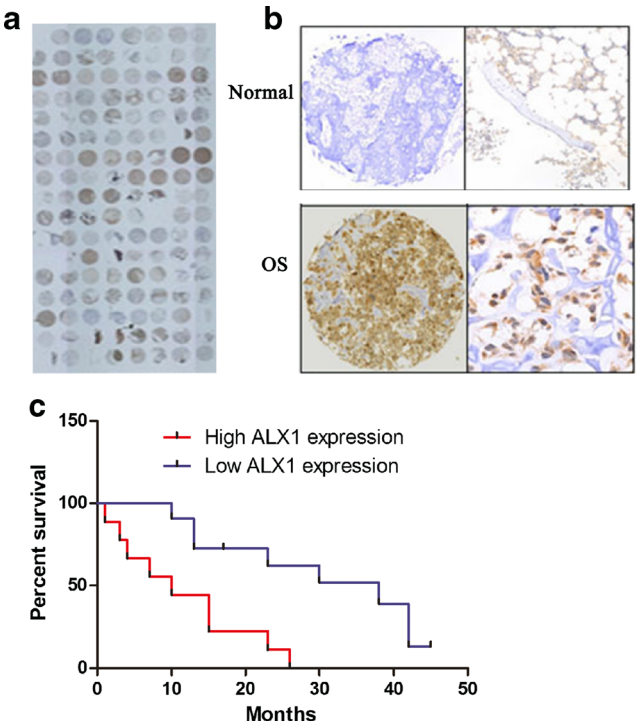

Fig. 1 Expression of *ALX1* in human osteosarcoma correlates with poor patient survival. **a** *ALX1* immunostaining of a tissue microarray consisting of control normal bone tissue and specimens from osteosarcoma biopsies. **b** *ALX1* immunostaining of a randomly selected representative tumor specimen (*below*) and nonspecific control staining of a corresponding tissue specimen in the absence of primary *ALX1* antibodies (*up*). **c** Kaplan–Meier plots of *ALX1* expression in 20 cases of osteosarcoma patients. Overall survival rate was performed by log-rank test

(TLiRNaseH Plus) (TaKaRa, Otsu, Japan) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Cells were lysed with RIPA buffer (Beyotime, China) and boiled for 5 min. The protein concentration of each lysate was measured using the BCA method (Beyotime, China). Equal quantities of protein from each cell lysate were separated on SDS-polyacrylamide electrophoresis gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5 % skimmed milk, incubated with each primary antibody overnight at 4 °C, washed with TBS-T buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05 % Tween20) and incubated with secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare Biosciences)

Statistical analysis

All variables between groups were compared using the Pearson X^2 test or Student's t test. $P<0.05$ was considered statistically significant. Numerical data were calculated using Microsoft Excel and analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

ALX1 is over-expressed in human osteosarcoma

ALX1 immunohistochemistry was performed as outlined in "Materials and methods" on an osteosarcoma tissue, consisting of tissue cores of primary tumors collected from 60 patients with a mean age of 22 years (range 5 to 66 years) (Fig. 1a, b). Twenty-two patients had metastatic disease, and five of them presented with metastases at diagnosis. A Kaplan–Meier survival analysis showed that, irrespective of metastatic or local disease, 37 patients with immunohistochemically detectable expression of *ALX1* in tumor tissue had a significantly (p=0.012) shorter overall survival of 35±9 (mean±SE) months than 23 patients with nondetectable ALX1 expression and a mean overall survival of 69±13 months (Fig. 1c).

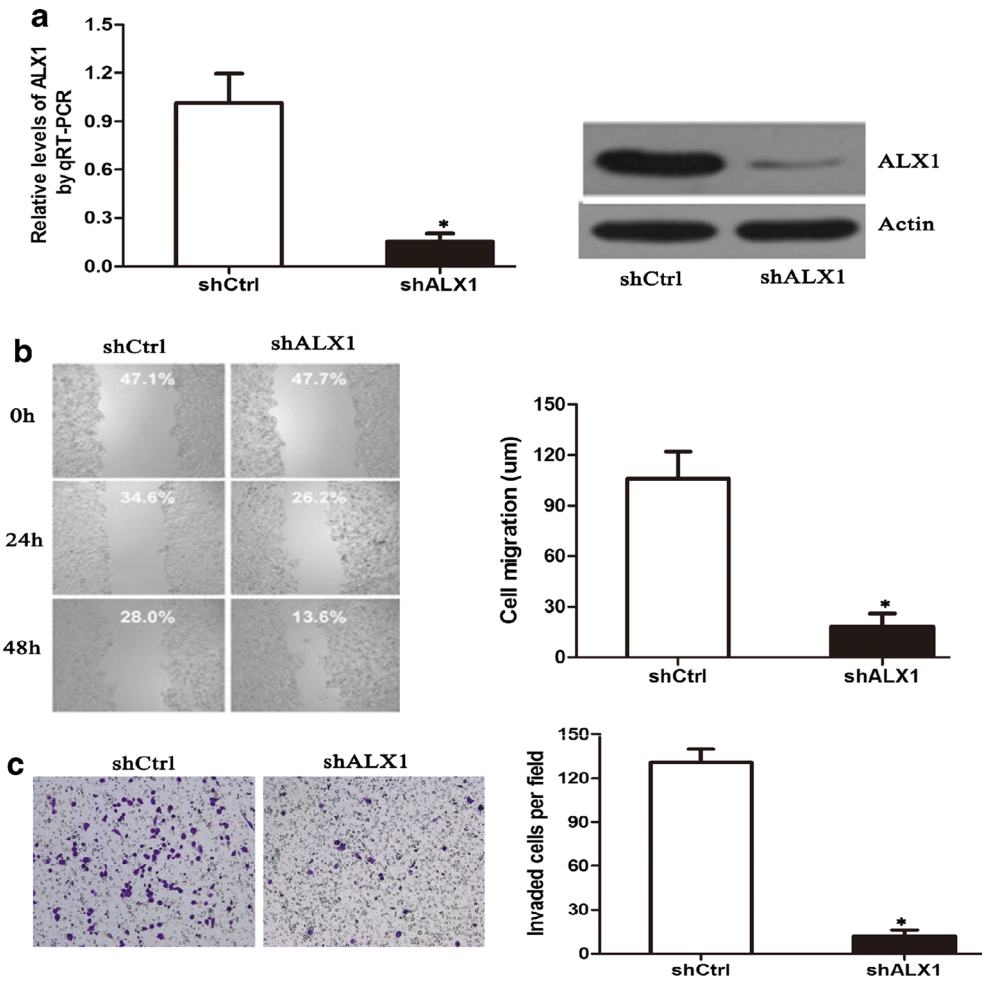

Fig. 2 *ALX1* depletion inhibits migration and invasion in osteosarcoma. U2OS cells were transiently transfected with the control shRNA and *ALX1* shRNA, respectively. Following shRNA transfection, cells were subjected to would healing assay (**a**), transwell migration (**b**), and Matrigel invasion assay (**c**), respectively. *$P<0.05$

ALX1 depletion inhibits the migration and invasiveness of U2OS cells

To explore the role of *ALX1* in maintaining the migratory and invasive traits in OS cells, we then examined the effects of *ALX1* depletion in the cell lines with high *ALX1* expression using a monolayer wound-healing assay and a Matrigel-based Boyden chamber invasion assay. Firstly, quantitative RT-PCR and Western blot analysis demonstrated that *ALX1* expression was significantly inhibited at both mRNA and protein levels 48 h after transfection, whereas the expression of actin was unchanged (Fig. 2a). Figure 2b shows a representative photograph of cell migration, and Fig. 2c shows a statistical analysis of the effects of depletion of *ALX1* on the migration. The percentage of relative migration distance in the *ALX1*-shRNA cell clone whose *ALX1* had been silenced, when compared with the percentage of relative migration distance in parent (control-shRNA, 54±6.6), is reduced. The data indicate that silencing *ALX1* decreased the cell migration, suggesting increased expression of *ALX1* in U2OS contributes to the elevated migration of these cells. Figure 2a shows a representative photograph of the cell invasion, and Fig. 2b shows the statistical analysis of the effects of silencing *ALX1* on cell invasion. Numbers of invasive cells in the *ALX1*-shRNA cell clone, when compared with numbers of invasive cells in parent (U2OS: 410±21.1) and transfection control (control-shRNA: 422±21.1). The data indicate that silencing *ALX1* decreased the cell invasion, suggesting that high expression of *ALX1* in U2OS contributes to the elevated cell invasion.

ALX1 depletion inhibits osteosarcoma cells tumorigenesis in vivo

To examine whether depletion of *ALX1* expression could inhibit the tumorigenicity in vivo, the stable *ALX1* knockdown and the control cells were injected subcutaneously into nude mice. As shown in Fig. 3, silencing of *ALX1* expression remarkably inhibited the tumor growth in both weight and size in nude mice. Mice were sacrificed 36 days after tumor cell injection and the tumor weight was determined, tumor weight and tumor size of *ALX1* knockdown group (0.12±0.04 g, 86±39 mm^3) was only 9 and 12 % of the control group (1.3±0.4 g, 720±212 mm^3) (Fig. 3c).

ALX1 depletion enhanced the apoptosis of U2OS cells

Furthermore, we determined whether or not *ALX1* depletion resulted in apoptosis in osteosarcoma cells because *ALX1* depletion has been shown to induce apoptosis in breast cancer cells. Flow cytometry analysis indicated that the cells with DNA content increased dramatically at later stages after transfection, suggesting that *ALX1*-depleted cells undergo apoptosis (Fig. 4a). About 14–76 % of *ALX1*-depleted cells displayed

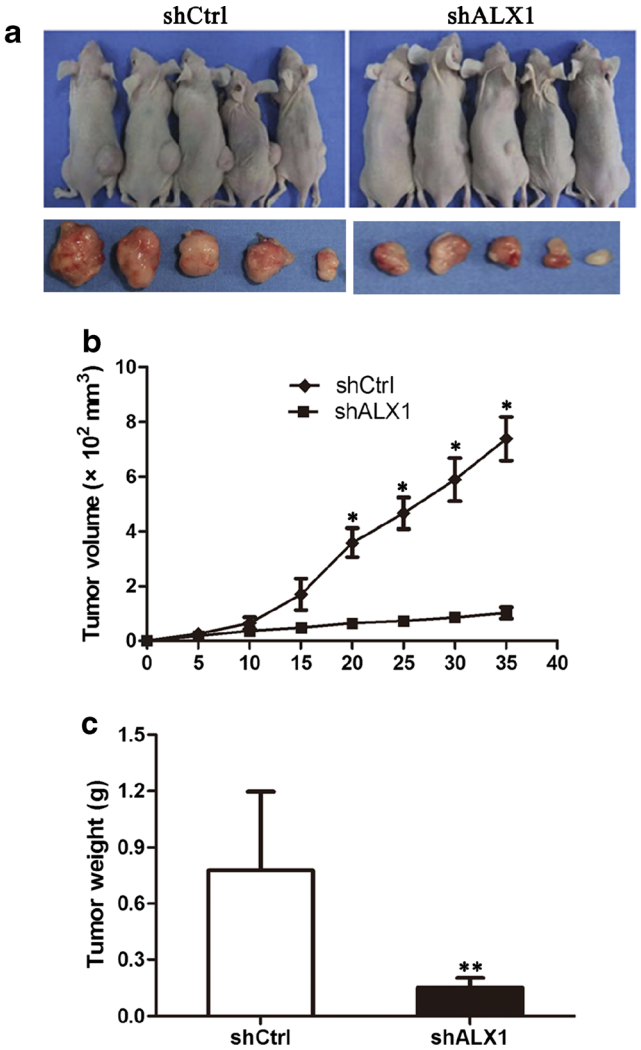

Fig. 3 *ALX1* knockdown inhibits osteosarcoma cells tumorigenicity in vivo. **a** *ALX1* knockdown cells (shCtrl) and control cells (sh*ALX1*) were injected subcutaneously into the dorsal flanks of nude mice, respectively. **b** The tumor size was measured about 5 days for tumor growth curve construction. **c** The tumor weight was measured at the end of experimental.*$P<0.05$

G1 DNA 72 h after transfection, whereas only 3–5 % of control cells had this phenotype.

ALX1 depletion induces mitotic cell cycle arrest

Next, we analyzed the effect of *ALX1* depletion on cell cycle progression using flow cytometry. *ALX1* depletion induced an obvious increase in the number of cells at G0/G1 phase and reduction in S phase, as 77.10 % of the U2OS cell in sh*ALX1* were noticed at G0/G1 phase, compared to 63.75 and 64.84 % cells in the control and shCtrl, respectively (Fig. 4b). Western blot results clearly showed a reduction in the expression of cyclin D1 and an increase of p21 in sh*ALX1* compared to the blank control and shCtrl (Fig. 4c).

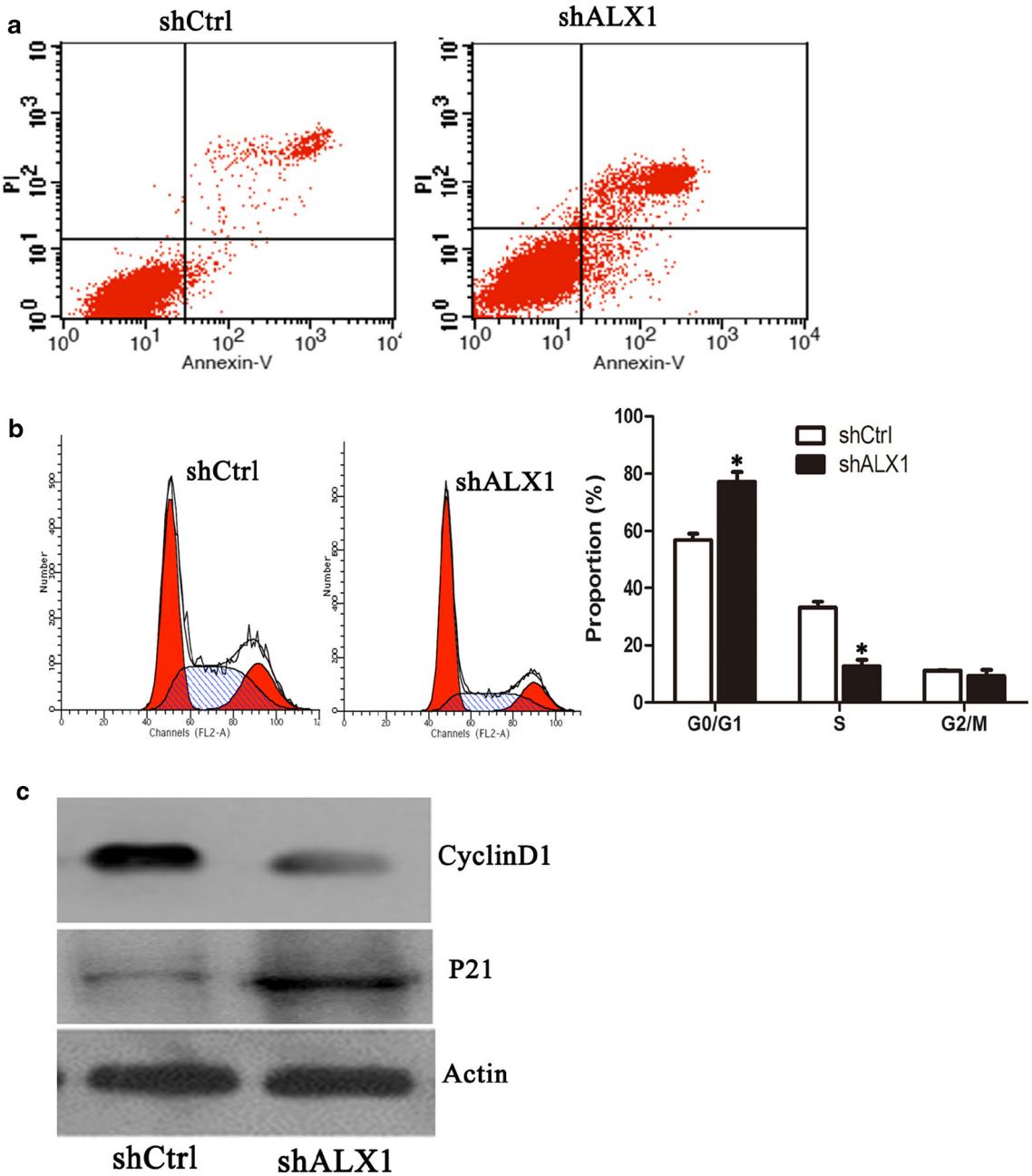

Fig. 4 *ALX1* depletion induces apoptosis and affects cell cycle progression in osteosarcoma. **a** Cells were harvested and then stained with Annexin/PI for apoptosis detection. The basal level of apoptosis in was 4.94 %, in the shCtrl and sh*ALX1* were 3.08 and 11.34 %. Significant differences in cell apoptosis were noticed among between groups (**P*<0.05). **b** The effect of *ALX1* depletion on cell cycle distribution as shown by FCM. Cells were collected and then stained with propidium iodide (PI). *ALX1* silencing induced an obvious increase in the number of cells at G0/G1 phase and reduction in S phase; there were significant differences between shCtrl and shALX1 (**P*<0.05). **c** Western blot analysis of cyclin D1 and p21 in shCtrl and sh*ALX1* U2OS

Discussion

The analysis of novel potential cancer-associated genes is of importance for developing diagnostic, preventive, and therapeutic strategies for cancer treatment and management. Osteosarcoma is the most common primary malignant bone tumor in children and young adults with a high propensity for metastasis, predominantly to the lung, and consequently is associated with poor prognosis [9–12]. Osteosarcoma treatment has undergone dramatic changes over the past 20 years, whereas the survival rate shows limited improvement. Thus so far, the 5-year survival rate is approximately 20 % with surgical treatment alone. Moreover, half of the patients exhibit pulmonary metastasis, which results in high patient mortality

[2, 13]. Thus, chemotherapy is typically employed in an adjuvant basis for improving the prognosis and long-term survival. However, recurrence frequently manifests as pulmonary metastasis or, less frequently, metastasis to distant bones or as a local recurrence [14–17]. Thus, a novel strategy that would effectively inhibit metastasis, particularly to the lungs, from the primary osteosarcoma site is highly desirable [18–21].

These in vitro and vivo study were an effort to investigate if *ALX1* could be exploited as a novel therapeutic target for the treatment of human osteosarcoma cancer. We have found that the specific shRNA-mediated depletion of *ALX1* leads to a significant decrease in cell migration, with mitotic arrest followed by massive apoptosis in the human osteosarcoma cancer cell line studied. Moreover, we also found that depletion of *ALX1* reduced tumorigenicity in nude mice in vivo. These findings indicated that *ALX1* played an important role in tumorigenesis. Our data strongly demonstrated that, apart from being of diagnostic value in osteosarcoma, inhibition of *ALX1* in osteosarcoma may additionally serve to be of therapeutic value.

In summary, our present study strongly demonstrated that the specific shRNA-mediated silencing of *ALX1* resulted in the elimination of osteosarcoma cells via the inactivation of p21/cyclin B1-mediated mitotic cell cycle arrest followed by massive apoptotic cell death. Therefore, *ALX1* may serve as a potential target in the treatment of human osteosarcoma. Consequently and conceivably, gene therapeutic approaches and/or pharmacological small molecule inhibitors aimed at *ALX1* may be developed for the management of osteosarcoma.

Conflicts of interest No potential conflicts of interest were disclosed.

References

1. Siclari VA, Qin L. Targeting the osteosarcoma cancer stem cell. J Orthop Surg Res. 2010;5:78.
2. Gorlick R, Meyers PA. Osteosarcoma necrosis following chemotherapy: innate biology versus treatment-specific. J Pediatr Hematol Oncol. 2003;25:840–1.
3. Pakos EE, Nearchou AD, Grimer RJ, Koumoullis HD, Abudu A, Bramer JA, et al. Prognostic factors and outcomes for osteosarcoma: an international collaboration. Eur J Cancer. 2009;45:2367–75.
4. Klein MJ, Siegal GP. Osteosarcoma: anatomic and histologic variants. Am J Clin Pathol. 2006;125:555–81.
5. Simmons LW, Lovegrove M and Almbro M. Female effects, but no intrinsic male effects on paternity outcome in crickets. J Evol Biol 2014.
6. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst. 1993;85:200–6.
7. Yuan H, Kajiyama H, Ito S, Yoshikawa N, Hyodo T, Asano E, et al. ALX1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of ovarian cancer cells. Cancer Res. 2013;73:1581–90.
8. Li H, Chen X, Gao Y, Wu J, Zeng F, Song F. XBP1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of breast cancer cells. Cell Signal. 2015;27:82–9.
9. Wang Q, Cai J, Wang J, Xiong C and Zhao J. MiR-143 inhibits EGFR-signaling-dependent osteosarcoma invasion. Tumour Biol 2014.
10. Savage SA, Mirabello L. Using epidemiology and genomics to understand osteosarcoma etiology. Sarcoma. 2011;2011:548151.
11. Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, et al. Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. Mol Biol Rep. 2009;36:153–8.
12. Li H, Song F, Chen X, Li Y, Fan J, Wu X. Bmi-1 regulates epithelial-to-mesenchymal transition to promote migration and invasion of breast cancer cells. Int J Clin Exp Pathol. 2014;7:3057–64.
13. Li H, Chen X, Gao Y, Wu J, Zeng F, Song F. XBP1 induces snail expression to promote epithelial- to-mesenchymal transition and invasion of breast cancer cells. Cell Signal. 2015;27:82–9.
14. Endo-Munoz L, Evdokiou A, Saunders NA. The role of osteoclasts and tumour-associated macrophages in osteosarcoma metastasis. Biochim Biophys Acta. 1826;2012:434–42.
15. Perbal B, Zuntini M, Zambelli D, Serra M, Sciandra M, Cantiani L, et al. Prognostic value of CCN3 in osteosarcoma. Clin Cancer Res. 2008;14:701–9.
16. Chen CC, Lau LF. Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol. 2009;41:771–83.
17. Mann KK, Wallner B, Lossos IS, Miller Jr WH. Darinaparsin: a novel organic arsenical with promising anticancer activity. Expert Opin Investig Drugs. 2009;18:1727–34.
18. Mei Q, Li F, Quan H, Liu Y, Xu H. Busulfan inhibits growth of human osteosarcoma through miR-200 family microRNAs in vitro and in vivo. Cancer Sci. 2014;105:755–62.
19. Liu Y, Wang W, Xu J, Li L, Dong Q, Shi Q, et al. Dihydroartemisinin inhibits tumor growth of human osteosarcoma cells by suppressing Wnt/beta-catenin signaling. Oncol Rep. 2013;30:1723–30.
20. Zhao Q, Wang C, Zhu J, Wang L, Dong S, Zhang G, et al. RNAi-mediated knockdown of cyclooxygenase2 inhibits the growth, invasion and migration of SaOS2 human osteosarcoma cells: a case control study. J Exp Clin Cancer Res. 2011;30:26.
21. Wu YF, Liang XJ, Liu YY, Gong W, Liu JX, Wang XP, et al. + Antisense oligonucleotide targeting survivin inhibits growth by inducing apoptosis in human osteosarcoma cells MG-63. Neoplasma. 2010;57:501–6.