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The knock-down of overexpressed EZH2 and BMI-1 does not prevent osteosarcoma growth

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Abstract. Polycomb group proteins control the transcriptional memory of cells by maintaining the stable silencing of specific sets of genes through chromatin modifications. Polycomb group protein complexes control gene repression through recruitment of histone deacetylase. This recruitment leads to trimethylation of Lys_{27} of histone H3 (H3K27). Histone H3K27 trimethylation is a property of stably silenced heterochromatin. EZH2 and BMI-1 are pivotal components of polycomb group protein complexes. Increased *EZH2* levels have been found in several malignancies and reported as a molecular biomarker of poor prognosis. Similarly, *BMI-1* has also been found to be associated with malignant transformation. In addition, inhibition of *EZH2* or *BMI-1* inhibits the growth of various types of malignancies. The expression of *BMI-1* and *EZH2* in human osteosarcoma has not been clearly determined. We examined the potential involvement of aberrant polycomb group protein expression in the pathogenesis of osteosarcoma. Real-time PCR revealed that expression of *EZH2* in 143B, HOS, NOS-1 and Saos2 was increased compared to normal osteoblasts. *BMI-1* was also up-regulated in 143B, HOS and NOS-1. Expression of *EZH2* and *BMI-1* were up-regulated in osteosarcoma patient biopsy specimens compared to normal bone. Immunohistochemical examinations showed that EZH2 and BMI-1 were up-regulated in osteosarcoma cells and that trimethylation of histone H3K27 was increased. We examined the effects of knock down of *EZH2* and *BMI-1* by shRNA. Unexpectedly, the knock-down of *EZH2* and *BMI-1* did not prevent osteosarcoma growth either *in vitro* or *in vivo*. Our findings suggest that EZH2 and BMI-1 may be tumor-associated antigens of osteosarcoma, but are not useful molecular targets of osteosarcoma treatment.

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Introduction

Osteosarcoma is the most common primary bone cancer occurring mainly in children (1). Standard treatment involves the use of 'up-front' multi-agent chemotherapy, definitive surgery of the primary tumor and postoperative chemotherapy. In recent years, great effort has been made aiming at elucidating the molecular events underpinning the biology of osteosarcoma including dysregulation of cell division and apoptotic processes. Although such dysregulation may constitute a potent source of new therapeutic targets, the molecular mechanisms of regulation of osteosarcoma cell proliferation are largely unknown.

Polycomb group (PcG) proteins control the transcriptional memory of cells by maintaining the stable silencing of specific sets of genes through chromatin modifications (2). Two distinct and evolutionarily conserved PcG complexes have been identified, consisting of various PcG proteins and non-PcG proteins. The polycomb repressive complex 1 (PRC1) contains the BMI-1, MEL-18, RING1, HPH and HPC PcG proteins, while the polycomb repressive complex 2 (PRC2) contains the EZH2, EED, YY1 and SUZ PcG proteins (3-15). EZH2 is a histone methyltransferase associated with transcriptional repression. EZH2 catalyzes trimethylation of histone H3 at lysine 27 (H3K27) (16-19).

Recent findings have linked deregulated expression of human PcG genes to malignant transformation, loss of differentiation in tumor cells, and metastatic behavior (20). Increased *EZH2* levels have been found in several epithelial tumors (21-26) and in various hematological malignancies (27-29). Similarly, *BMI-1* has also been associated with malignant transformation (23,27,30-38). The expression of *BMI-1* and *EZH2* in human osteosarcoma cell lines and osteosarcoma patient specimens have not been well defined. To explore the potential involvement of aberrant PcG expression in the pathogenesis of osteosarcoma, we investigated the expression of *EZH2* and *BMI-1* in osteosarcoma cell lines and patient samples. We next examined the status of trimethylation of H3K27. In addition, we examined the effect of the knock-down of *EZH2* and *BMI-1* by shRNA *in vitro* and *in vivo*.

Materials and methods

Cell culture. HOS, 143B and Saos2 cells were purchased from the American Type Culture Collection (ATCC). NOS-1 was purchased from RIKEN cell bank (39). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Human osteoblast cells (NHOst) were purchased from Sanko Junyaku (Tokyo, Japan). Cells were cultured with OBM™ (Cambrex, NJ, USA) or DMEM supplemented with 10% FBS. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37˚C.

Patient osteosarcoma biopsy specimens. All human osteosarcoma biopsy specimens were obtained from primary lesions. Biopsy was performed before chemotherapy or radio therapy to make the diagnosis.

RT-PCR. Each sample was run minimally at three concentrations in triplicate. All primer sets amplified 100- to 200-bp fragments. Total RNA was extracted using the miR-Vana RNA isolation system (Ambion, TX, USA) or TRIzol (Invitrogen, CA, USA). Reactions were run using SYBR-Green (Bio-Rad, CA, USA) on a MiniOpticon™ machine (Bio-Rad). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using ßII-microglobulin. Each sample was run minimally at three concentrations in triplicate. The following primers were used. EZH2: 5-TTCA TGCAACACCCAACACT-3, 5-GAGAGCAGCAGCAAAC TCCT-3; BMI-1: 5-TTCATTGATGCCACAACCAT-3, 5-GTA CTGGGGCTAGGCAAACA; ßII-microglobulin: 5-TCAATG TCGGATGGATGAAA-3, 5-GTGCTCGCGCTACTCTC TCT-3.

Cell proliferation assay. MTT assay: Cells were incubated with substrate with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 4 h and washed with PBS and lysed to release formazan from cells. Then cells were analyzed in a Safire microplate reader (Bio-Rad) at 562 nm. shRNAs were purchased from (SABiosciences, MD, USA). Lipofection of siRNA was performed every other day as recommended in the supplier's protocol using FuGENE 6 (Roche, Basel, Switzerland).

Immunohistochemistry. The following primary antibodies were used: anti-EZH2 (diluted 1:200 Zymed Laboratories, CA, USA), anti-BMI-1 (diluted 1:200 R&D Systems, MN, USA), and anti-trimethylated H3K27 (diluted 1:200 Abcam, Cambridge, UK). The following secondary antibodies were used: fluorescein-conjugated goat anti-mouse IgG antibody (diluted 1:200; Jackson ImmunoResearch, PA, USA) and rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, CA, USA). The cells were counterstained with Hoechst 33258 to identify nuclei. Immunohistochemistry with each second antibody alone without primary antibody was performed as a control.

Animal experiments. shRNA-transfected 143B cells ($1x10^5$) were mixed with collagen gel in a 1:1 volume and inoculated subcutaneously in 5-week-old nude mice. Tumor size was measured, and tumor volume was calculated using the formula $LW^2/2$ (with L and W representing the length and width of tumors). All experimental procedures were performed in compliance with the guiding principles for the Care and Use of Animals described in the American Journal of Physiology and with the Guidelines established by the Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize possible alternatives to *in vivo* techniques.

Data analysis. Each sample was analyzed in triplicate and experiments were repeated three times. In figures the error bar means standard error. Data were analyzed by the STASTISCA (StatSoft, OK, USA). Differences between mean values were evaluated by the unpaired t-test and differences in frequencies were evaluated by Fisher's exact test. Results were considered statistically significant at $P<0.05$.

Results

Overexpression of EZH2 and BMI-1 in osteosarcoma. RT-PCR was performed to examine the expression of *EZH2* and *BMI-1* in osteosarcoma cell lines. RT-PCR revealed that NOS-1, HOS and 143B osteosarcoma cell lines expressed EZH2 more strongly than normal human osteoblasts (NHOst) (Fig. 1A). More sensitive real-time PCR analyses revealed that expression of *EZH2* in 143B, HOS, NOS-1 and Saos2 was increased 13-, 11-, 4.9- and 4.4-fold, respectively (Fig. 1B). RT-PCR revealed that NOS-1, HOS and 143B osteosarcoma cell lines expressed *BMI-1* more strongly than NHOst (Fig. 1C). Real-time PCR revealed that expression of *BMI-1* in 143B, HOS and NOS-1 was increased 6.7-, 3.7- and 3.7-fold, respectively, while that in Saos2 did not change appreciably (Fig. 1D). We next examined the expression of *EZH2* and *BMI-1* in osteosarcoma patient biopsy samples. RT-PCR revealed that 3 osteosarcoma patient samples expressed EZH2 more strongly than normal bone tissue (Fig. 1E). Real-time PCR revealed that expression of *EZH2* in patient samples was increased 1.4- to 4.2-fold (Fig. 1F). RT-PCR revealed that 3 osteosarcoma patient samples expressed *BMI-1* more strongly than normal bone (Fig. 1G). Real-time PCR revealed that expression of *BMI-1* in patient samples increased 4.5- to 9.4-fold (Fig. 1H). To extend these findings, we performed immunohistochemistry for EZH2 and BMI-1 examination revealed that osteosarcoma cell lines and osteosarcoma patient samples expressed EZH2 and BMI-1 more strongly than normal bone tissue (Fig. 2A and B). EZH2 and BMI-1 were localized in the nucleus of osteosarcoma cells (Fig. 2A and B). These findings showed that EZH2 and BMI-1 are overexpressed in osteosarcomas.

Histone H3-K27 is trimethylated in osteosarcoma. To determine if overexpression of polycomb proteins promoted histone H3K27 trimethylation, we performed immunohistochemical examination using trimethylated histone H3K27-specific antibody. Histone H3K27 was found to be trimethylated more strongly in osteosarcoma cells lines and osteosarcoma patient samples than in normal osteoblasts and bone tissue (Fig. 2C).

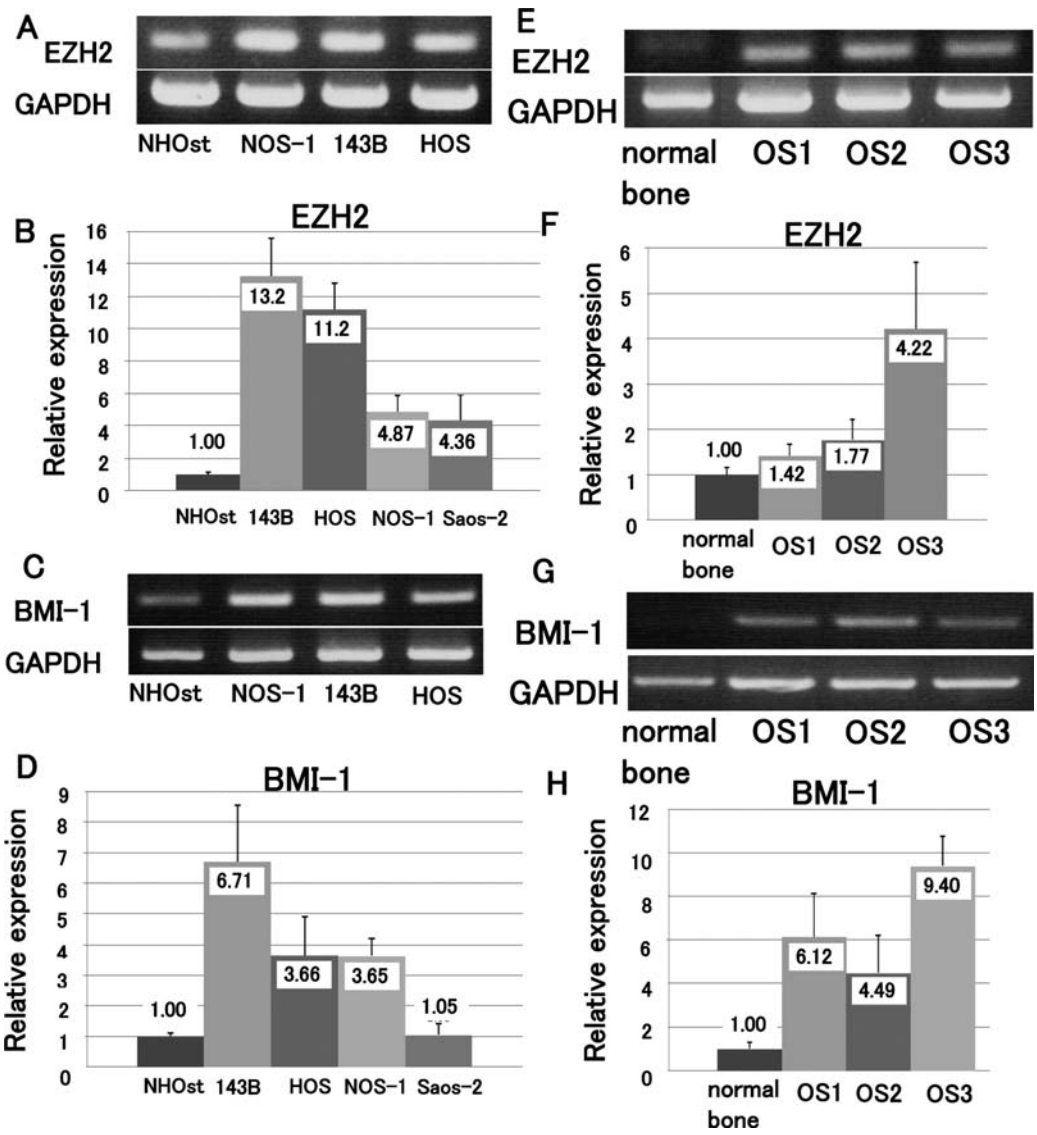

Figure 1. Overexpression of *EZH2* and *BMI-1* in osteosarcoma. (A) RT-PCR revealed that 3 osteosarcoma cell lines including NOS-1, 143B and HOS expressed *EZH2* more strongly than NHOst (normal osteoblasts). (B) Real-time PCR revealed that expression of *EZH2* in 143B, HOS, NOS-1 and Saos2 was increased 13-, 11-, 4.9- and 4.4-fold, respectively. (C) RT-PCR revealed that 3 osteosarcoma cell lines including NOS-1, 143B and HOS expressed *BMI-1* more strongly than NHOst. (D) Real-time PCR revealed that expression of *BMI-1* in 143B, HOS and NOS-1 was increased 6.7-, 3.7- and 3.7-fold, respectively, while that in Saos2 did not change appreciably. (E) Total RNA extracted from osteosarcoma biopsy samples were used for RT-PCR. RT-PCR revealed that osteosarcoma biopsy sample 1 (OS1), OS2 and OS3 expressed *EZH2* more strongly than normal bone. (F) Real-time PCR revealed that expression of *EZH2* in patient samples was increased 1.2- to 4.2-fold. (G) RT-PCR revealed that 3 osteosarcoma samples expressed *BMI-1* more strongly than normal bone. (H) Real-time PCR revealed that expression of *BMI-1* in patient samples increased 4.5- to 9.4-fold.

Knock-down of overexpressed EZH2 and BMI-1 does not prevent osteosarcoma growth in vitro or in vivo. It has been reported that overexpression of EZH2 or BMI-1 promotes malignant transformation (21,36,38,40-47). In addition, inhibition of EZH2 or BMI-1 inhibits growth of various types of malignancies (38,41,43,45,46). To determine whether knock-down of *EZH2* and *BMI-1* prevents osteosarcoma growth, we examined the effects of *EZH2* and *BMI-1* shRNA. We used 143B and HOS, which strongly express EZH2 and BMI-1. Real-time PCR revealed that shRNA effectively knocked-down *EZH2* and *BMI-1* (Fig. 3A). 143B and HOS were transfected with *EZH2* shRNA, *BMI-1* shRNA and *EZH2* shRNA plus *BMI-1* shRNA. Unexpectedly, MTT assay revealed that the knock-down of *EZH2*, *BMI-1* and *EZH2* plus *BMI-1* did not prevent osteosarcoma growth *in vitro* (Fig. 3B-D). To confirm the effects of *EZH2* and *BMI-1* knock-down, we examined xenograft models. Nude mice were inoculated with control shRNA-transfected 143B cells, *EZH2* shRNA-transfected 143B cells and *BMI-1*-shRNA-transfected cells intradermally and tumor sizes were measured. Tumor sizes did not significantly differ among these three groups (Fig. 4).

Discussion

The PcG genes encode a family of evolutionarily conserved regulators that were discovered in *Drosophila* as repressors of homoeotic genes, which are involved in establishing body segmentation patterns during development. In mammalian systems, PcG proteins regulate genes involved in development

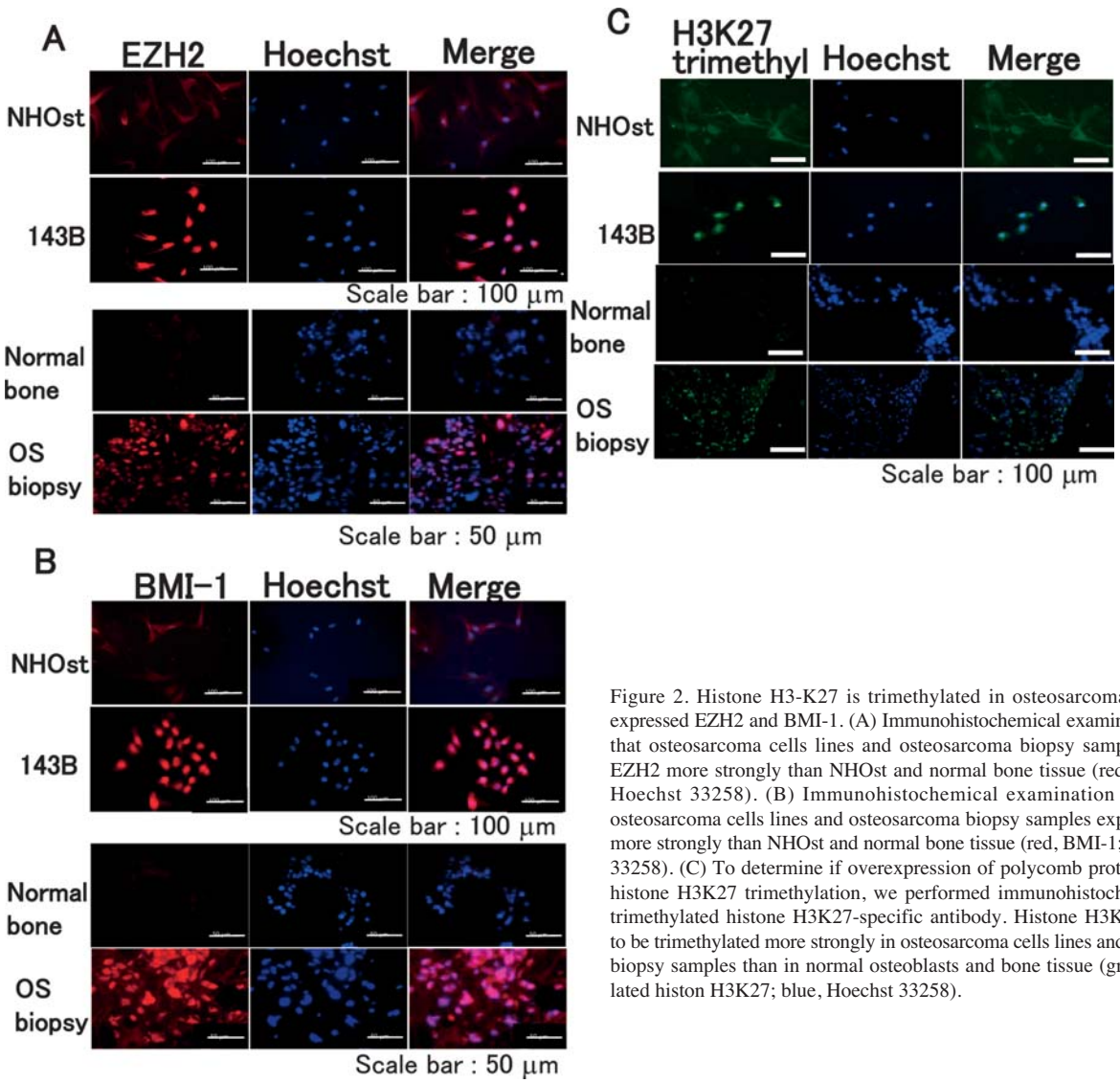

Figure 2. Histone H3-K27 is trimethylated in osteosarcoma which overexpressed EZH2 and BMI-1. (A) Immunohistochemical examination revealed that osteosarcoma cells lines and osteosarcoma biopsy samples expressed EZH2 more strongly than NHOst and normal bone tissue (red, EZH2; blue, Hoechst 33258). (B) Immunohistochemical examination revealed that osteosarcoma cells lines and osteosarcoma biopsy samples expressed BMI-1 more strongly than NHOst and normal bone tissue (red, BMI-1; blue, Hoechst 33258). (C) To determine if overexpression of polycomb proteins promoted histone H3K27 trimethylation, we performed immunohistochemistry using trimethylated histone H3K27-specific antibody. Histone H3K27 was found to be trimethylated more strongly in osteosarcoma cells lines and osteosarcoma biopsy samples than in normal osteoblasts and bone tissue (green, trimethylated histon H3K27; blue, Hoechst 33258).

and differentiation via epigenetic mechanisms. Transcriptional profiling of human tumor samples holds significant promise for the advancement of cancer therapy, both in terms of improving diagnosis as well as predicting patient responses to treatment. Recently, an RNA expression signature associated with 'stem-cell-ness', based partly on PcGs-driven transcriptional changes, was postulated to predict poor therapeutic outcome in patients with various types of cancers (48). Although these claims await further validation, they suggest that levels of PcGs expression might prove valuable as prognostic markers, particularly because *EZH2* and *BMI-1* overexpression appears to be tightly correlated with poor prognosis in various types of cancers (49,50). *BMI-1* was originally identified as an oncogene (8). *BMI-1* up-regulation induces development of B- and T-cell lymphomas (7,41,42). In this study, we found that *EZH2* and *BMI-1* RNAs are up-regulated in osteosarcoma cell lines and patient samples, following the study of overexpression of *EZH2* in the U2OS human osteosarcoma cell line (51). Steele *et al* reported that $CD8^+$ T-cell epitopes derived from EZH2 and BMI-1 elicited T-cell responses as assessed by IFN-γ release confirming the presence of CD8 responses against these proteins in patients with cancer (52). These findings suggest that EZH2 and BMI-1 may be useful targets for cancer immunotherapy of osteosarcoma.

The PRC2 containing EZH2 controls gene repression through recruitment of histone deacetylase. This recruitment leads to local chromatin deacetylation and subsequent trimethylation of Lys_{27} of histone H3 (H3K27). Histone H3K27 trimethylation is a property of stably silenced heterochromatin. The PRC1 complex containing BMI-1 subsequently binds to histone H3K27, suppresses gene expression and contributes to the maintenance of epigenetic memory (53). In this study, we found that histone H3K27 was trimethylated both in osteosarcoma cell lines and patient samples. These findings suggest the possibility that overexpressed EZH2 and BMI-1 are functionally active and promote histone H3K27 trimethylation in osteosarcoma as in stem cells and other types of cancer cells (45,54,55). In addition, trimethylated histone H3K27 suppresses target gene expression via epigenetic regulation (45,55,56). The gene suppression may contribute to the pathogenesis of osteosarcoma. BMI-1 represses the transcription of cell cycle repressors encoded by the ink4a locus (41,57-59). Although PcG proteins are generally

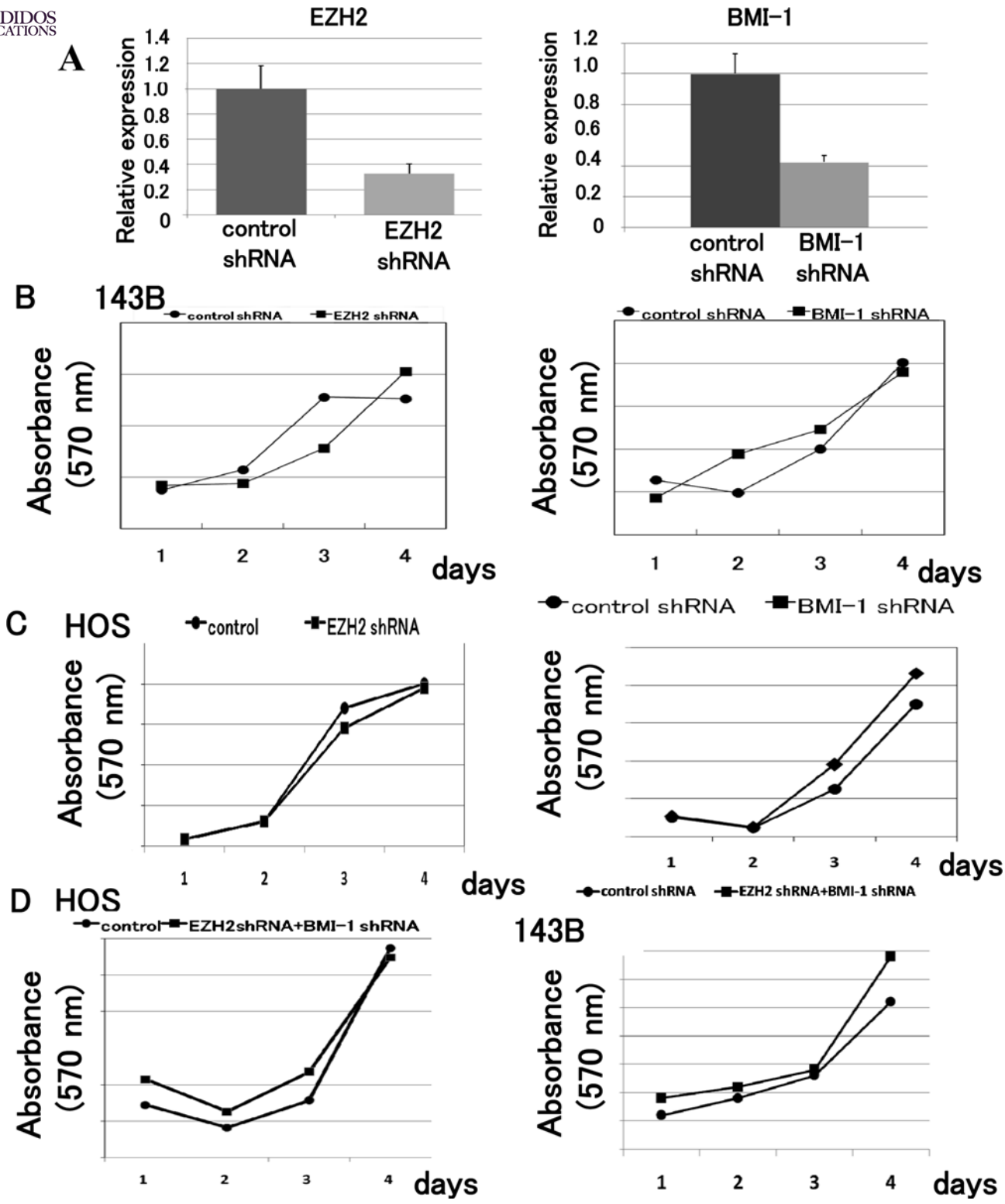

Figure 3. the knock-down of *EZH2* and *BMI-1* does not inhibit osteosarcoma growth *in vitro*. (A) 143B cells were transfected with *EZH2* shRNA and *BMI-1* shRNA. Real-time PCR revealed the knock-down effect by *EZH2* shRNA or *BMI-1* shRNA. (B) MTT assay showed that knock down of *EZH2* and *BMI-1* did not prevent 143B growth *in vitro*. (C) MTT assay showed that knock down of *EZH2* and *BMI-1* did not prevent HOS growth *in vitro*. (D) Double knock-down of *EZH2* plus *BMI-1* did not prevent HOS and 143B growth *in vitro*.

recognized as suppressors of target gene transcription, Shi *et al* reported that EZH2 enhances the transcription of c-myc and cyclin D1 (60). We previously found that transcription of c-myc is activated and expression of the ink4a locus are suppressed in osteosarcoma (61). These findings suggest that these genes may be targets of *EZH2* and *BMI-1* in osteosarcoma.

It has been reported that overexpression of *EZH2* or *BMI-1* promotes malignant transformation (21,36,38,40-47,49). In addition, inhibition of *EZH2* or *BMI-1* inhibits growth of various types of malignancies (38,41-43,45,46,49). These findings suggest that *EZH2* and *BMI-1* play roles in regulating cell proliferation and survival and that *EZH2* or *BMI-1* may be useful as molecular targets in various types of malignancies.

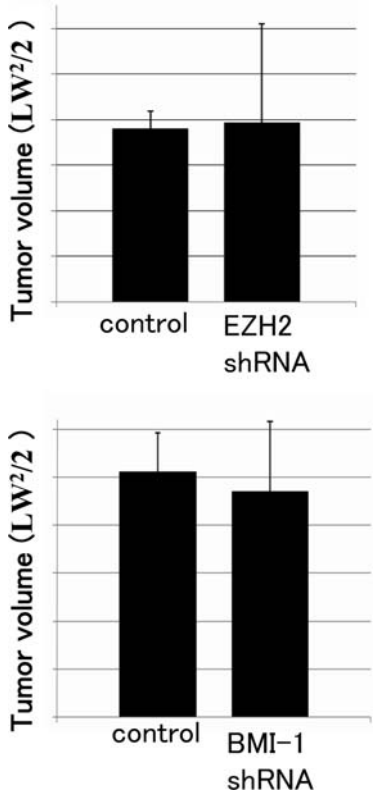

Figure 4. The knock-down of *EZH2* and *BMI-1* does not inhibit osteosarcoma growth *in vivo*. (A) Control shRNA-transfected 143B cells, *EZH2*-shRNA-transfected 143B cells and *BMI-1*-shRNA-transfected cells ($1x10^5$) were inoculated subcutaneously. Established 143B tumors were measured. The tumor volume was evaluated 5 weeks after transplantation (n=3, each group. Error bar, mean standard division).

In fact, pharmacologic interference of EZH2 function induces selective apoptosis of cancer cells but not normal cells (62). In the present study, we examined the effect of *EZH2* and *BMI-1* knock-down in osteosarcoma and found unexpectedly that *EZH2* or *BMI-1* knock-down by shRNA did not prevent osteosarcoma growth *in vitro* or *in vivo*. These findings are contrary to those reported in previous studies. Two groups reported that although PcG protein overexpression appeared to be correlated with poor prognosis for some types of malignancies, low BMI-1 expression was correlated with poor prognosis of endometrial carcinomas and malignant melanocytic lesion (63,64). These studies suggest that osteosarcoma may be included among these types of malignancies. In addition, McGarvey *et al* reported that *EZH2* knock-down results in increased expression of unmethylated and basally expressing genes but not of completely silenced and hypermethylated tumor suppressor genes (65). These findings suggest that important regulator genes for osteosarcoma growth may be hypermethylated. *BMI-1* co-overexpression with other inducers, such as *H-RAS*, *hTERT* and $p16^{INK4a}$ shRNA, resulted in efficient malignant transformation (36,40,41,44). These findings in turn suggest that other factors might be regulated in addition to *BMI-1* to suppress osteosarcoma growth. Taken together, these findings suggest that inhibition of PcG proteins may not be useful for treatment of some other malignancies in addition to osteosarcoma.

In conclusion, we found that *EZH2* and *BMI-1* are up-regulated in osteosarcoma. *EZH2* and *BMI-1* may be useful targets for cancer immunotherapy of osteosarcoma, although knock-down of *EZH2* and *BMI-1* could not prevent osteosarcoma growth. Further investigation of the functions of EZH2 and BMI-1 in osteosarcoma is needed.

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