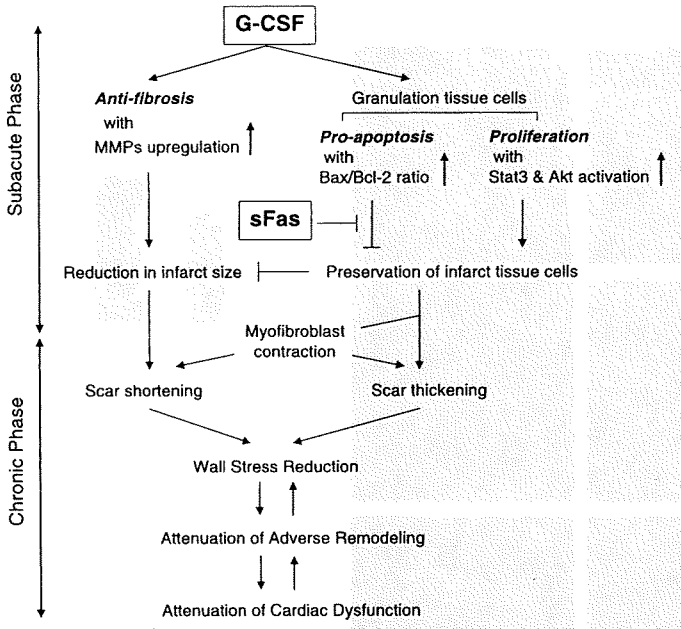

Fig. 9. Proposed mechanism underlying beneficial effect of combined use of G-CSF administration and soluble Fas (sFas) gene therapy on postinfarction cardiac remodeling and dysfunction. G-CSF exerts a beneficial effect on infarct tissue dynamics through antifibrotic and proliferative effects on granulation tissue; however, it also exerts an adverse proapoptotic effect that leads to thinning of the infarct scar. sFas appeared to offset the latter drawback.

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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 (ΔΔCt) 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).

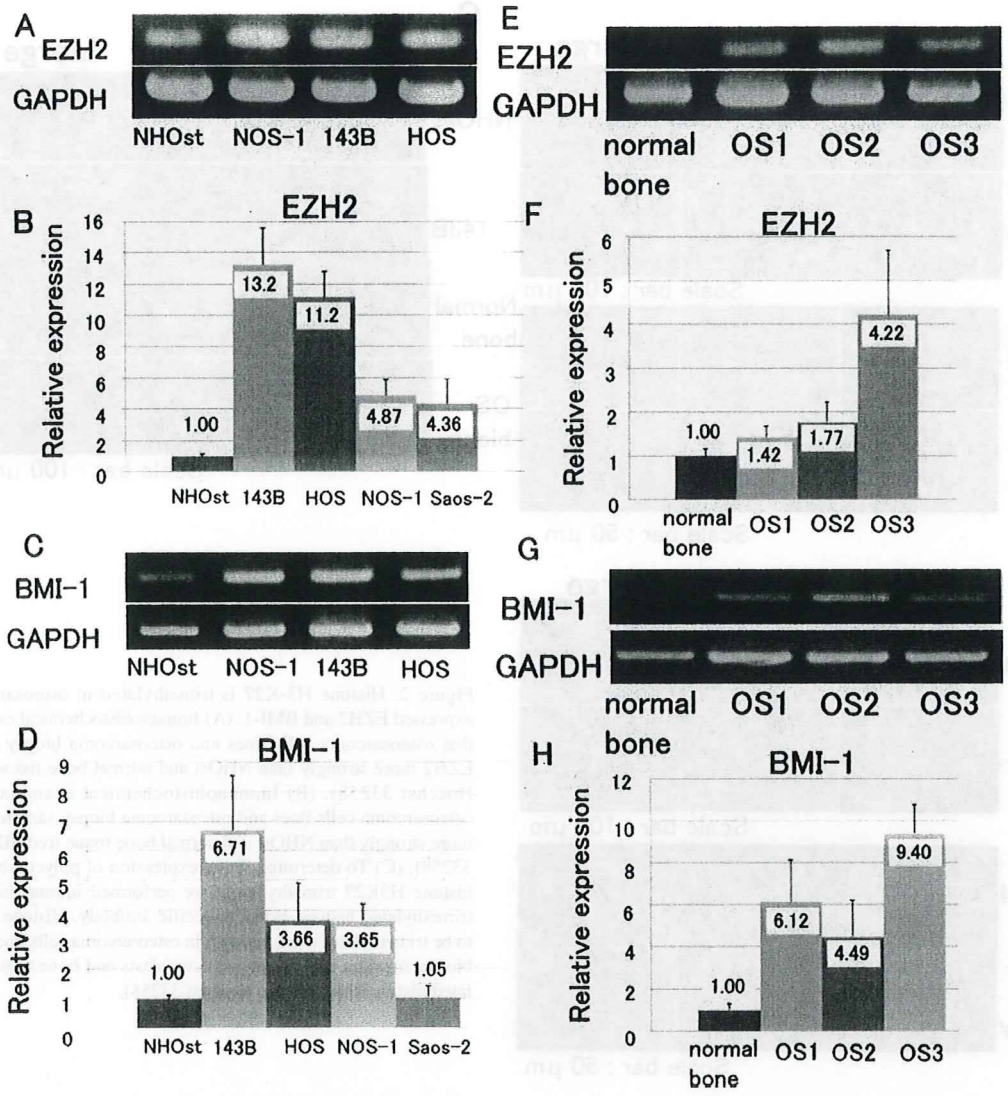

Figure 1. Overexpression of *EZH2* and *BMI-1* in osteosarcoma. (A) RT-PCR revealed that 3 osteosarcoma cell lines including NOS-1, 143B and HOS expressed *EZH2* more strongly than NHOst (normal osteoblasts). (B) Real-time PCR revealed that expression of *EZH2* in 143B, HOS, NOS-1 and Saos2 was increased 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

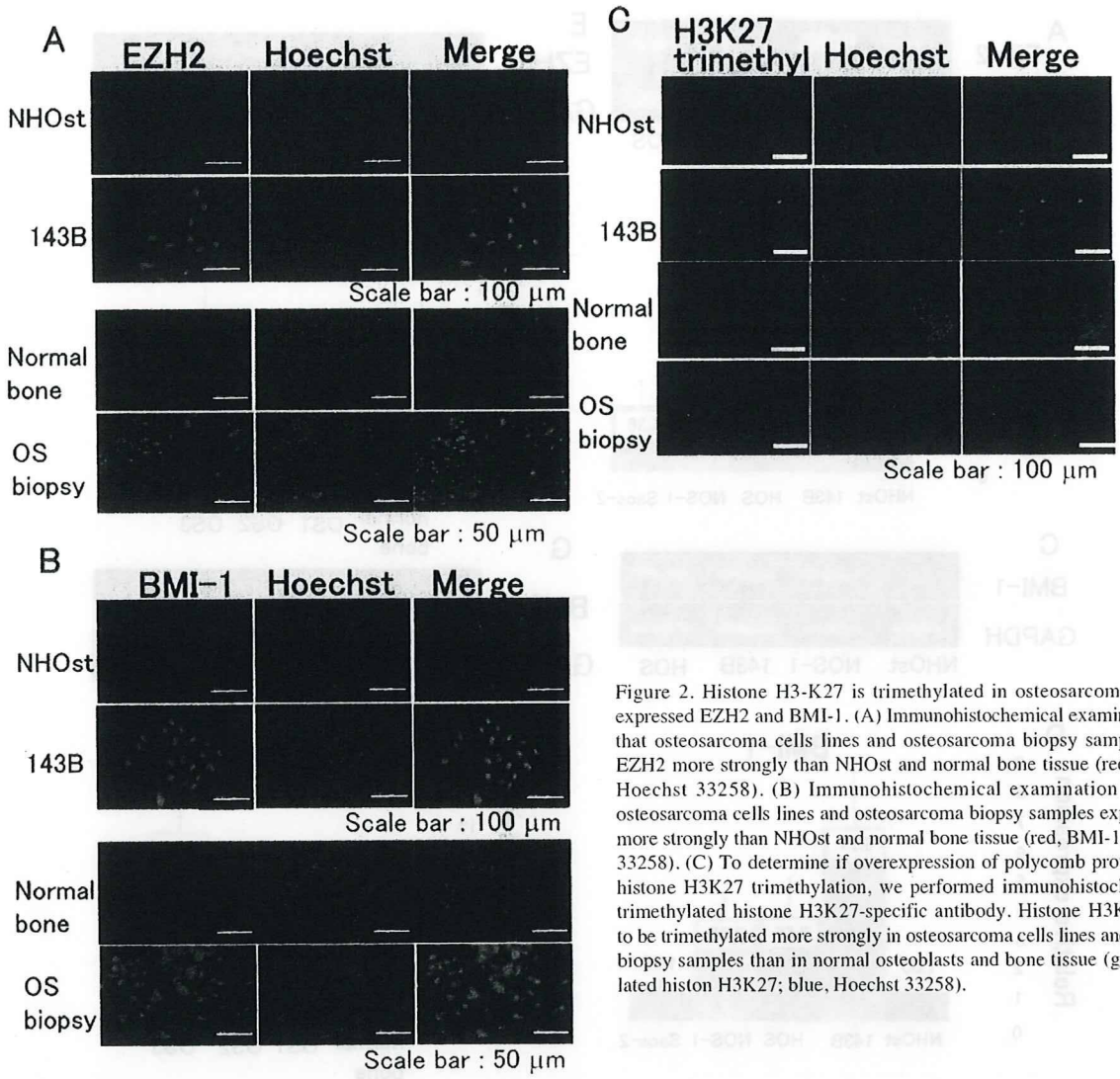

Figure 2. Histone H3-K27 is trimethylated in osteosarcoma which over-expressed EZH2 and BMI-1. (A) Immunohistochemical examination revealed that osteosarcoma cells lines and osteosarcoma biopsy samples expressed EZH2 more strongly than NHOst and normal bone tissue (red, EZH2; blue, Hoechst 33258). (B) 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

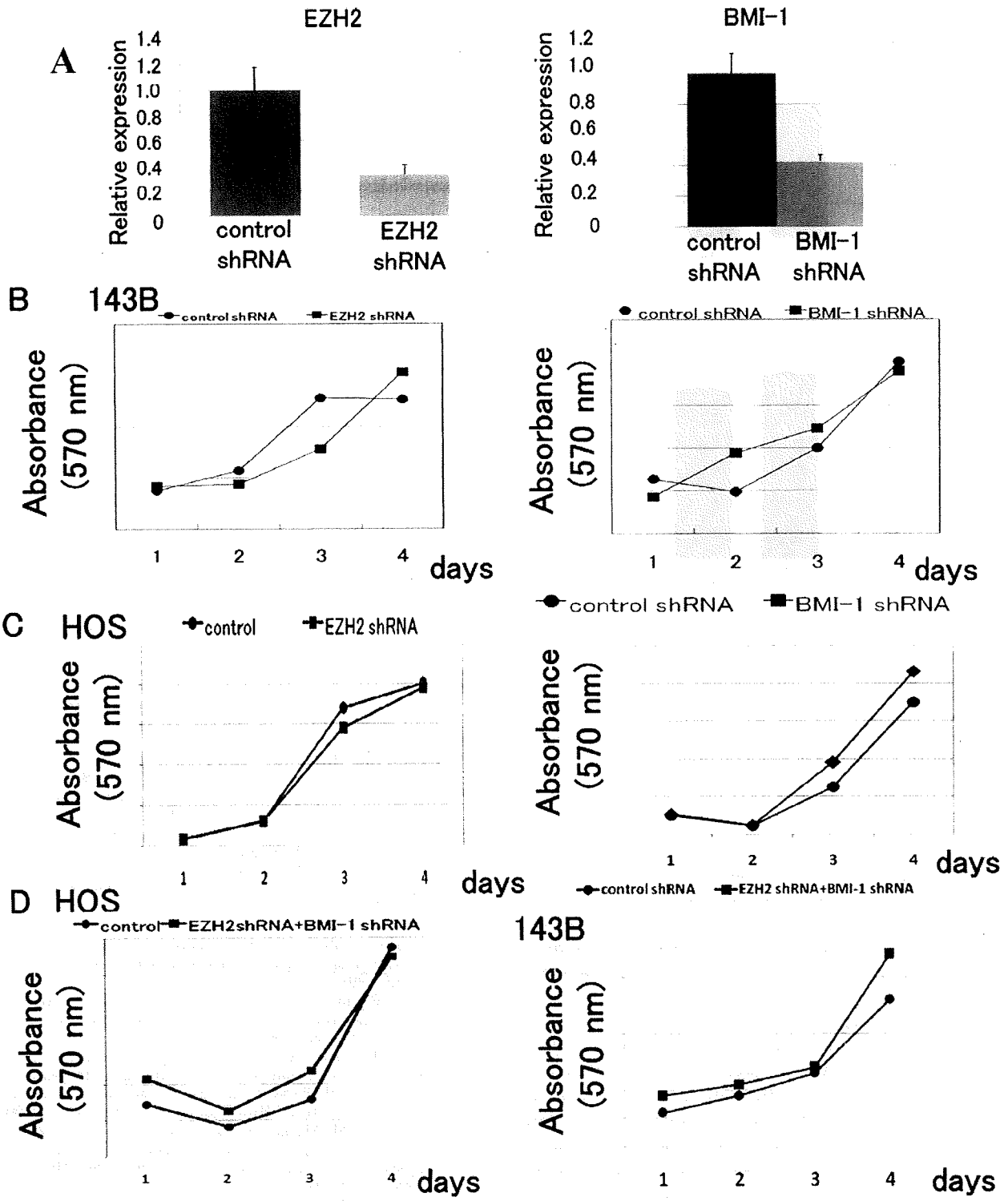

Figure 3. the knock-down of *EZH2* and *BMI-1* does not inhibit osteosarcoma growth *in vitro*. (A) 143B cells were transfected with *EZH2* shRNA and *BMI-1* shRNA. Real-time PCR revealed the knock-down effect by *EZH2* shRNA or *BMI-1* shRNA. (B) MTT assay showed that knock down of *EZH2* and *BMI-1*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

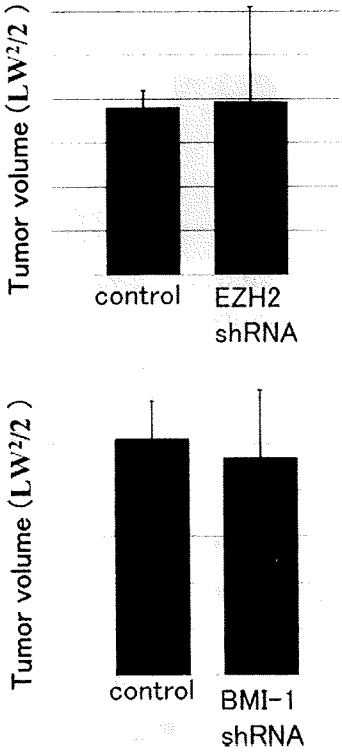

Figure 4. The knock-down of *EZH2* and *BMI-1* does not inhibit osteosarcoma growth *in vivo*. (A) Control shRNA-transfected 143B cells, *EZH2*-shRNA-transfected 143B cells and *BMI-1*-shRNA-transfected cells ($1x10^5$) were inoculated subcutaneously. Established 143B tumors were measured. The tumor 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.

Acknowledgements

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Smoothened as a new therapeutic target for human osteosarcoma

Masataka Hirotsu†, Takao Setoguchi*†, Hiromi Sasaki†, Yukihiro Matsunoshita, Hui Gao, Hiroko Nagao, Osamu Kunigou, Setsuro Komiya

Abstract

Background: The Hedgehog signaling pathway functions as an organizer in embryonic development. Recent studies have demonstrated constitutive activation of Hedgehog pathway in various types of malignancies. However, it remains unclear how Hedgehog pathway is involved in the pathogenesis of osteosarcoma. To explore the involvement of aberrant Hedgehog pathway in the pathogenesis of osteosarcoma, we investigated the expression and activation of Hedgehog pathway in osteosarcoma and examined the effect of SMOOTHENED (SMO) inhibition.

Results: To evaluate the expression of genes of Hedgehog pathway, we performed real-time PCR and immunohistochemistry using osteosarcoma cell lines and osteosarcoma biopsy specimens. To evaluate the effect of SMO inhibition, we did cell viability, colony formation, cell cycle *in vitro* and xenograft model *in vivo*. Real-time PCR revealed that osteosarcoma cell lines over-expressed *Sonic hedgehog*, *Indian hedgehog*, *PTCH1*, *SMO*, and *GLI*. Real-time PCR revealed over-expression of *SMO*, *PTCH1*, and *GLI2* in osteosarcoma biopsy specimens. These findings showed that Hedgehog pathway is activated in osteosarcomas. Inhibition of SMO by cyclopamine, a specific inhibitor of SMO, slowed the growth of osteosarcoma in vitro. Cell cycle analysis revealed that cyclopamine promoted G1 arrest. Cyclopamine reduced the expression of accelerators of the cell cycle including cyclin D1, cyclin E1, SKP2, and pRb. On the other hand, $p21^{cip1}$ wprotein was up-regulated by cyclopamine treatment. In addition, knockdown of *SMO* by *SMO* shRNA prevents osteosarcoma growth in vitro and in vivo.

Conclusions: These findings suggest that inactivation of SMO may be a useful approach to the treatment of patients with osteosarcoma.

Background

Osteosarcoma is the most common primary bone malignant tumor occurring mainly in children [1]. After initial diagnosis is made by biopsy, treatment consists of preoperative chemotherapy, followed by definitive surgery and postoperative chemotherapy. Survival has improved over the past several decades. Indeed, patients with non-metastatic disease have a 70% chance of long-term survival. Unfortunately, patients with metastatic disease at diagnosis and those who have recurrent disease have a poor prognosis, with only 20% surviving at 5 years, indicating that new therapeutic options for them need to be actively explored. In cancer cells, dysregulation of cell division and apoptotic processes contribute to both drug resistance and metastatic potential [2,3]. It has been reported that inactivation of the cell cycle regulatory pathway centered around the Rb gene is a critical step in the pathogenesis of osteosarcoma [4]. Although such dysregulation may constitute a potent source of new therapeutic targets, the molecular mechanisms of regulation of osteosarcoma cell proliferation are largely unknown.

Hedgehog (Hh) pathway has been implicated in different aspects of animal development, acting through several components, including the transmembrane proteins PATCHED (PTCH1) and SMOOTHENED (SMO), to activate the GLI zinc-finger transcription factors [5,6]. Hh pathway is critical for many processes during embryonic and postnatal development, including proliferation, differentiation, specification of cell fate,

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left-right asymmetry, and morphogenesis [7]. Sporadic and familial mutations in the Hh pathway genes, PTCH1, suppressor-of-fused, and SMO, leading to elevated expression of downstream target genes including GLI, have been reported in basal cell carcinoma and the pediatric brain tumor medulloblastoma [8,9]. In addition, the growth of many cancers has been suggested to depend on continuous Hh pathway even in the absence of activating mutations in the pathway (reviewed in ref. [10]).

To explore the involvement of Hh pathway in the pathogenesis of osteosarcoma, we investigated the expression and activation of the Hh pathway genes in osteosarcoma and examined the effect of inhibition of SMO by cyclopamine, a specific inhibitor of SMO [11] or *SMO* shRNA.

Results

Over-expression of Hh-GLI pathway molecules in osteosarcoma

To examine the role of Hhï¿½GLI pathway in osteosarcoma, we tested for the expression of Hh in osteosarcoma cell lines. Real-time PCR revealed that 4 of 5 human osteosarcoma cell lines increased *Sonic Hedgehog* (*SHH*) 2.1- to 18.8-fold (Fig. 1). In addition, 5 of 5 osteosarcoma cell lines increased *Desert Hedgehog* 1.3- to 24.4-fold (Fig. 1). To further examine Hh pathway molecules expression, we performed real-time PCR for Hh receptors and Hh target genes. *PTCH1* was up-regulated 2.7-to 65.8-fold in 5 of 5 human osteosarcoma cell lines. *SMO* was up-regulated 2.1-to 5.8-fold in 4 of 5 human osteosarcoma cell lines. *SMO* was up-regulated 2.1-to 5.8-fold in 4 of 5 human osteosarcoma cell lines. *GLI1* was up-regulated 2.5-to 8.9-fold in 5 of 5 human osteosarcoma cell lines. *GLI2* was up-regulated 1.2-to 9.9-fold in 5 of 5 human osteosarcoma cell lines. To extend these findings, we performed immunocytochemistry for SMO and GLI2, and found that only osteosarcoma cells expressed detectable levels of SMO and GLI2. GLI2 was located in the nuclei of osteosarcoma cells (see additional file 1). We next examined *SMO* expression in osteosarcoma patient' biopsy specimens. Real-time PCR revealed that 9 of 9 human biopsy specimens of osteosarcoma increased *SMO* 1.44- to 55.5-fold (Fig. 2). In addition, real-time PCR revealed that expression of *PTCH1* was increased in 8 of 9 patients' biopsy samples 2.44- to 29.4-fold (Fig. 2). *GLI2* was up-regulated 2.5-to 58.4-fold in 9 of 9 human biopsy specimens of osteosarcoma (Fig. 2). Of most importance was the finding that markers of active Hhï¿½GLI signaling, *GLI2* and *PTCH1* were consistently up-regulated in the examined osteosarcoma cells, demonstrating the aberrant Hh-GLI pathway activation [12-14]. Our findings suggest that Hh-GLI signaling is active in osteosarcomas.

Inhibition of SMO prevents osteosarcoma growth in vitro

To determine whether activation of Hh-GLI signaling is required for osteosarcoma cell growth, we used cyclopamine, a pharmacological agent known to effectively block Hh-GLI signaling by inhibiting SMO activation [11]. We performed real-time PCR to determine whether cyclopamine effectively inhibited the expression of the GLI target gene *PTCH1* and *GLI2* [14]. Cyclopamine at 20 μM reduced mRNA levels of *PTCH1* and *GLI2* in osteosarcoma cells by more than 60%, consistent with the expected down-regulation of Hh-GLI signaling (Fig. 3A). As cyclopamine was used to prevent cancer cells growth at 10 to 20 μM [15-17] we decided 20 μM was appropriate concentration for osteosarcoma. MTT assay showed that cyclopamine slowed the growth of HOS and 143B in dose-dependent fashion (Fig. 3B). On the other hand, MTT assay showed that proliferation of osteosarcoma cells was enhanced by SHH. We next used a clonogenic assay to determine whether cells capable of forming anchorage-independent colonies were depleted by cyclopamine. This assay revealed cyclopamine reduced colony formation in soft agar (Fig. 3C). These findings suggest that inhibition of SMO inhibited osteosarcoma growth in vitro.

Hh signaling regulates cell cycle of osteosarcoma

We examined cell cycle characteristics by flow cytometry. Of 143B cells cultured without cyclopamine, 39.8% of cells were in G1 phase, while 56.6% of cells were in G1 phase following treatment with cyclopamine. In the case of HOS cells were cultured without cyclopamine, 55.4% cells were in G1 phase. On the other hand, when cultured with cyclopamine, 72.3% of cells were in G1 phase (Fig. 4A). These findings suggested that cyclopamine promoted G1 arrest. We then examined the transcription of cell cycle-related genes. Real-time PCR revealed that cyclopamine prevented the transcription of accelerators of the cell cycle including *cyclin D1*, *cyclin E1*, *SKP2*, and *NMYC* (Fig. 4B). In mammalian cells, cyclin D, cyclin E, and $p21^{cip1}$ are short-lived proteins that are controlled by ubiquitin-dependent proteolysis. We performed western blot analysis to determine protein levels, and found that cyclopamine reduced the levels of expression of cyclin D1 and cyclin E1 proteins. Cyclopamine also reduced the levels of expression of cyclin D1, cyclin E1, pRb, and SKP2 proteins (Fig. 4C). We next examined the expression of $p21^{cip1}$, and found that $p21^{cip1}$ protein was up-regulated by cyclopamine treatment (Fig. 4C). These findings suggested that cyclopamine promoted G1 arrest by inhibition of G1-S phase progression. These findings suggest that inhibition of SMO inhibited osteosarcoma growth via cell cycle regulation.

Knock down of SMO prevents osteosarcoma growth in vivo

To confirm the effect of SMO suppression, we examined the effect of *SMO* shRNA. 143B was transfected with

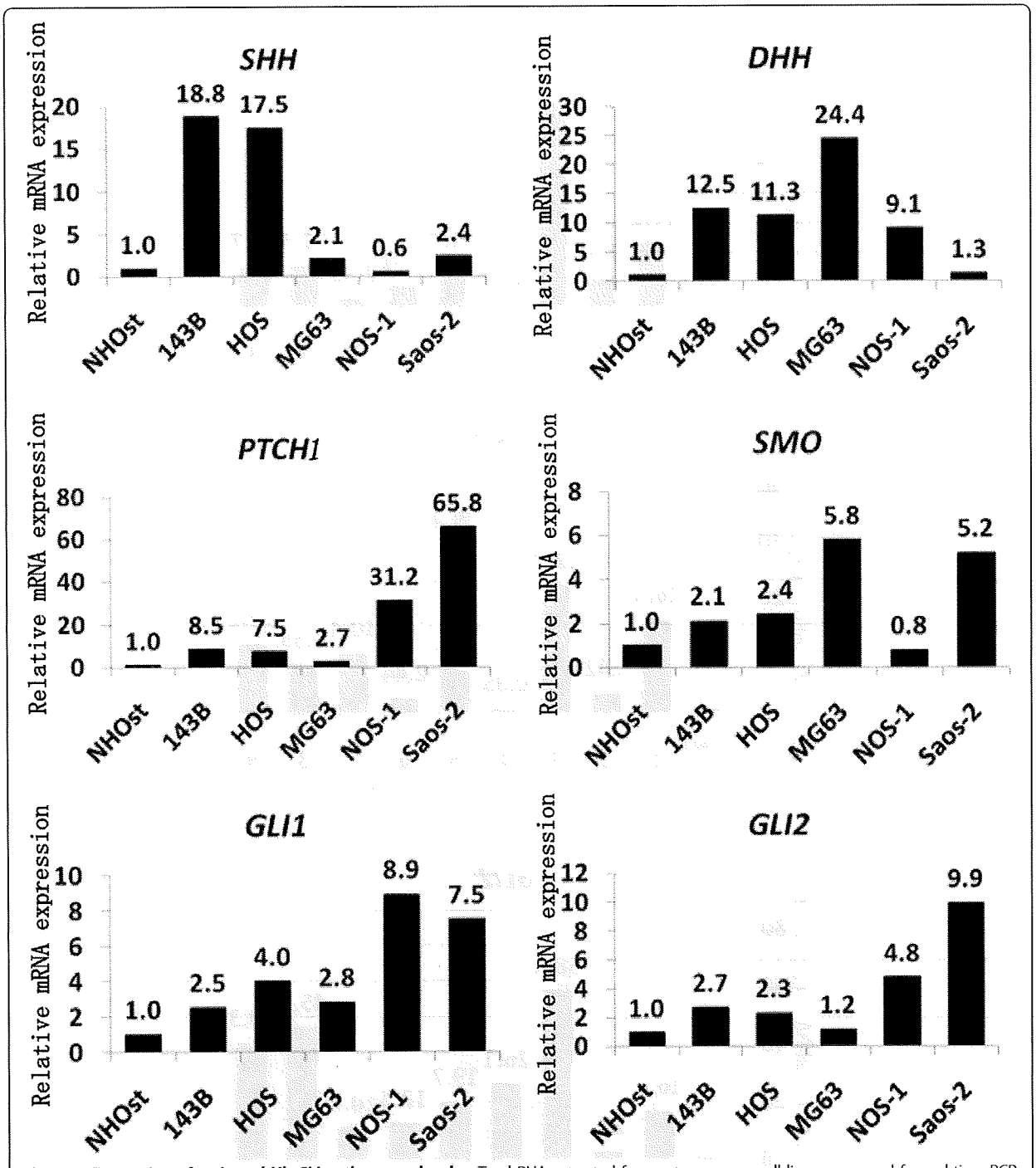

Figure 1 Expression of activated Hh-GLI pathway molecules. Total RNA extracted from osteosarcoma cell lines were used for real-time PCR. Real-time PCR revealed that 4 of 5 human osteosarcoma cell lines increased *Sonic Hedgehog* (*SHH*) 2.1- to 18.8-fold (Fig. 1). In addition, 5 of 5 osteosarcoma cell lines increased *Desert Hedgehog* 1.3- to 24.4-fold (Fig. 1). To further examine Hh pathway molecules expression, we performed real-time PCR for Hh receptors and Hh target genes. *PTCH1* was up-regulated 2.7-to 65.8-fold in 5 of 5 human osteosarcoma cell lines. *SMO* was up-regulated 2.1-to 5.8-fold in 4 of 5 human osteosarcoma cell lines. *SMO* was up-regulated 2.1-to 5.8-fold in 4 of 5 human osteosarcoma cell lines. *GLI1* was up-regulated 2.5-to 8.9-fold in 5 of 5 human osteosarcoma cell lines. *GLI2* was up-regulated 1.2-to 9.9-fold in 5 of 5 human osteosarcoma cell lines. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *βII-microglobulin*, *GAPDH* or *ACTB*. Each sample was run minimally at three concentrations in triplicate (error bar means S.D.). The experiment was triplicate with similar results.

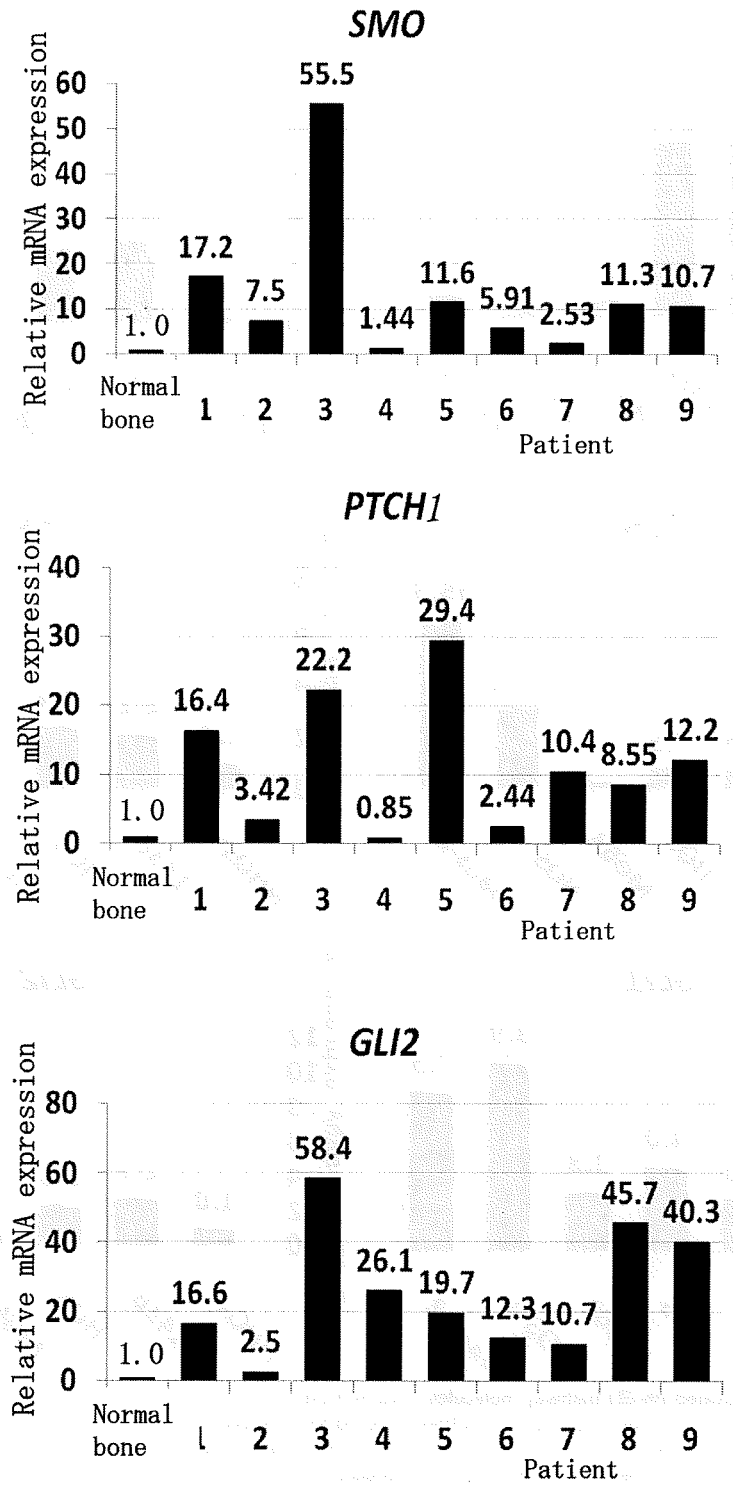

Figure 2 Activation of Hh pathway in patient' biopsy samples. Real-time PCR revealed that 9 of 9 human biopsy specimens of osteosarcoma increased *SMO* 1.44- to 55.5-fold. Real-time PCR revealed that expression of *PTCH1* was increased in 8 of 9 patients' biopsy samples 2.44- to 29.4-fold. *GLI2* was up-regulated 2.5-to 58.4-fold in 9 of 9 human biopsy specimens of osteosarcoma. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *βII-microglobulin*, *ACTB*, and *GAPDH*. Each sample was run minimally at three concentrations in triplicate (error bar means S.D.). The experiment was triplicate with similar results.

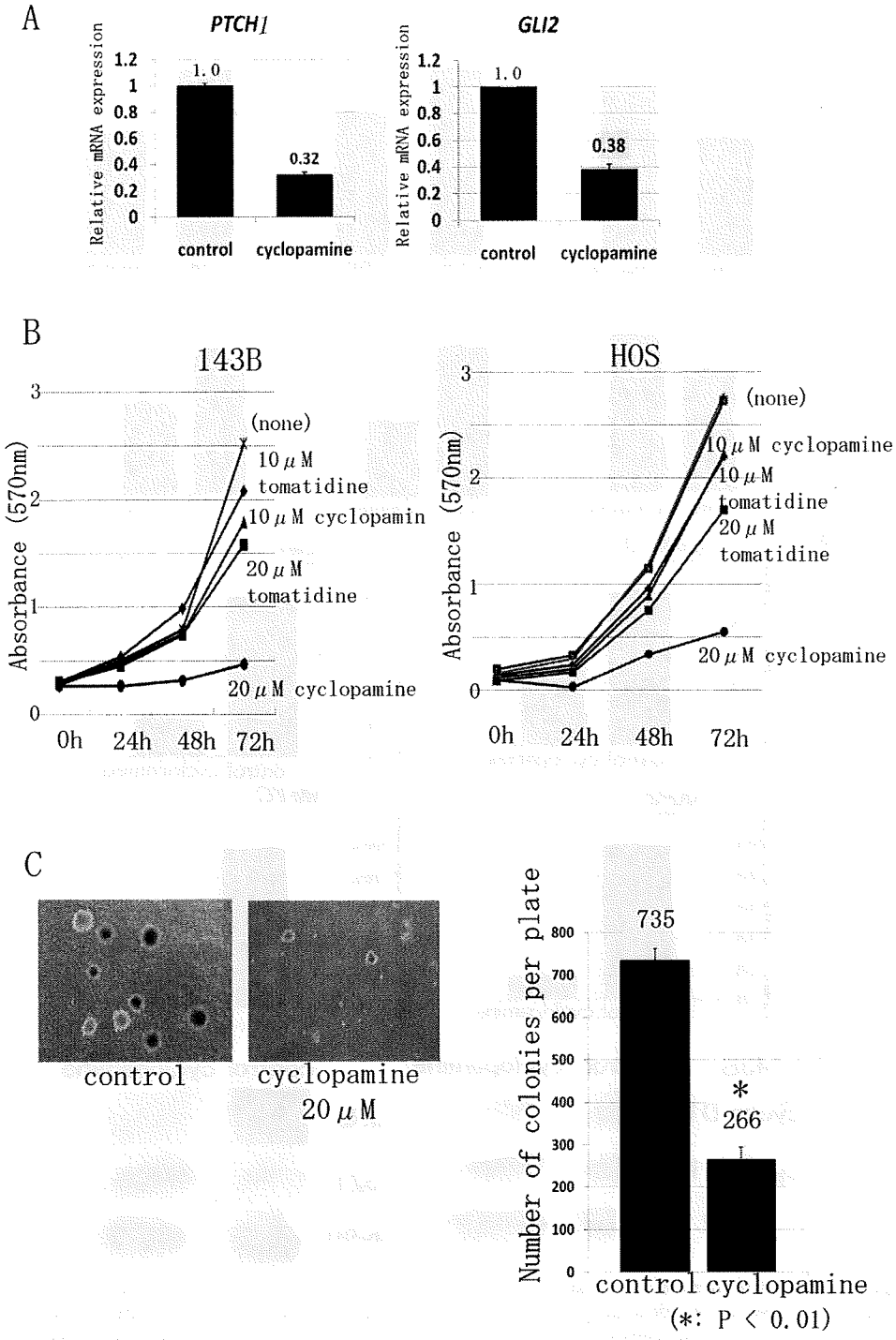

Figure 3 Inhibition of Hh pathway prevents osteosarcoma growth in vitro. A, We performed real-time PCR to determine which concentration of cyclopamine effectively inhibited Hh-GLI activity in osteosarcoma cells, and then measured the expression of the Hh-GLI pathway target *PTCH1* and *GLI2*. Cyclopamine at 20 μM reduced mRNA levels of PTCH1 in 143B cell (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *ACTB*. Each sample was run minimally at three concentrations in triplicate (error bar means S.D.). The experiment was triplicate with similar results. B, Growth of viable 143B and HOS cells over 3 days was slowed in dose-dependent fashion by cyclopamine treatment. The experiment was triplicate with similar results. C, Colony formation assay revealed cyclopamine reduced colony formation in soft agar. The experiment was triplicate with similar results. (*: $P < 0.01$) (error bar means S.D.)

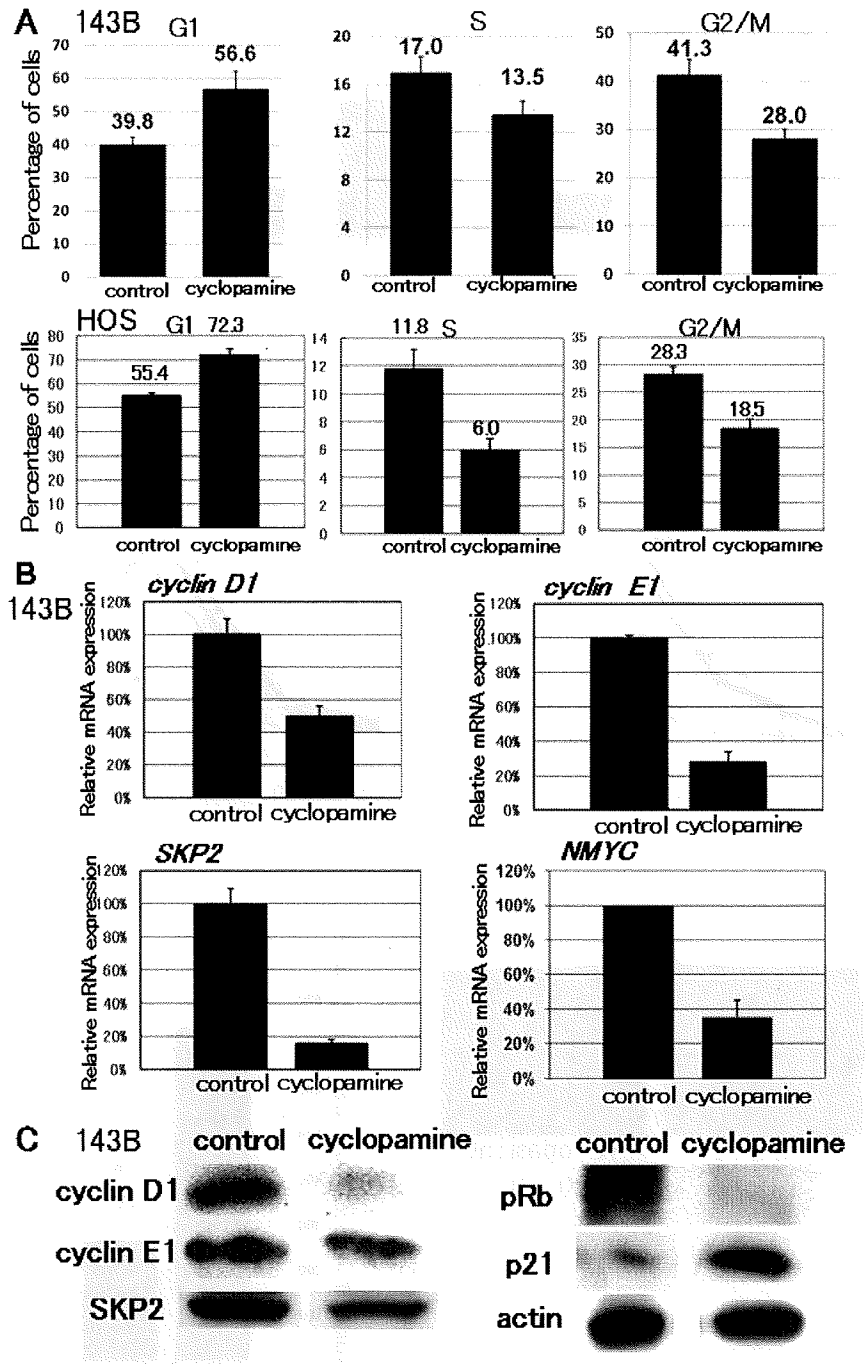

Figure 4 Cyclopamine treatment promotes G1 arrest. A, HOS and143B cells were treated with 10 μM cyclopamine. After 48-hour treatment cells were collected and subjected to cell cycle analysis. When 143B cells were cultured without cyclopamine, 39.8% of cells were in G1 phase. On the other hand, when cultured with cyclopamine, 56.6% of cells were in G1 phase. In the case of HOS cells cultured without GSI, 55.4% of cells were in G1 phase, while 72.3% of cells were in G1 phase when treated with cyclopamine (error bar means S.D.). B, Real-time PCR was performed to quantify mRNAs of cell cycle related genes. Twenty-four-hour treatment with cyclopamine reduced levels of *cyclin D1*, *Cyclin E1*, *SKP2*, and *NMYC* transcription (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *βII-microglobulin* and *GAPDH*. Each sample was run minimally at three concentrations in triplicate (error bar means S.D.). The experiment was triplicate with similar results. C, Western blot analysis of levels of cell cycle-related genes. Forty-eight-hour treatment with cyclopamine reduced levels of expression of cyclin D1, cyclin E1, SKP2, and phosphprylated RB (pRb) proteins. Expression of $P21^{cip1}$ protein was upregulated by cyclopamine treatment. The experiment was triplicate with similar results (cyclopamine: 10 μM).

control shRNA or *SMO* shRNA. *SMO* shRNA reduced the expression of *SMO* mRNA (Fig. 5A). MTT assay revealed that knock-down of *SMO* prevented osteosarcoma growth in vitro (Fig. 5A). We next used a clonogenic assay to determine whether cells capable of forming anchorage-independent colonies were depleted by *SMO* shRNA. This assay revealed *SMO* shRNA reduced colony formation in soft agar (Fig. 5B). These findings show that suppression of SMO prevents osteosarcoma growth in vitro. We then examined the transcription of cell cycle-related genes. Real-time PCR revealed that *SMO* shRNA prevented the transcription of accelerators of the cell cycle including *cyclin D1*, *cyclin E1*, *SKP2*, and *E2F1* (see additional file 2). To examine the in vivo effect of *SMO* shRNA, nude mice were inoculated with control shRNA or *SMO* shRNA transfected 143B osteosarcoma cells intradermally. Results demonstrated significant inhibition of tumor growth *SMO* shRNA versus control shRNA (Fig. 6A, B). Kaplan-Meier analysis showed that *SMO* shRNA conferred a significant survival benefit (Fig. 6B). Next, we performed real-time PCR using formed tumors. Real-time PCR revealed that transcription of *GLI1*, *GLI2*, and PTCH1 was decreased in tumors formed by *SMO* shRNA-transfected 143B. These findings showed that *SMO* shRNA prevented the transcription of Hh target genes in vivo. In addition, *SMO* shRNA prevented the transcription of accelerators of the cell cycle including *cyclin E1*, *SKP2*, and *E2F1* (see additional file 3). Histological analysis indicated that *SMO* shRNA prevented cell proliferation. The control tumors exhibited a number of cells positive for Ki67, a marker of cell proliferation. In contrast, *SMO* shRNA transfected tumors exhibited little evidence of proliferation, as evidenced by lack of Ki67 positivity. The number of Ki67-positive cells was decreased to 30% of control revel by *SMO* shRNA (Fig. 6C). These findings suggest that inhibition of SMO prevents osteosarcoma growth by cell cycle regulation in vivo.

Discussion

Although the role of Hh signaling in various cancers [18-21], it's role in the pathogenesis of osteosarcoma has not been reported. In the present study, we found that *Shh*, *Dhh*, *PTCH1*, *SMO*, *GLI1* and *GLI2* transcripts were over-expressed in osteosarcoma cell line. In addition, *SMO*, *PTCH1*, and *GLI2* were over-expressed in osteosarcoma biopsy specimens'. In general, it is accepted that enhanced Hh pathway activation leads to downstream expression of target genes including *PTCH1* and *GLI*, and hence, the levels of these transcripts are often used as surrogate markers of Hh pathway activity [22]. In addition, SHH promoted osteosarcoma cells proliferation. Our findings suggest that Hh pathway is activated in osteosarcomas. On the other hand, *GLI1* was down-regulated in human osteosarcoma biopsy specimens (data not shown). The reason for *GLI1* down-regulation could not be determined. One possibility is that the *GLI1* promoter is inactivated in human osteosarcomas by epigenetic modification. We found that GLI1 promoter contains a CG-rich region. Wong et al. reported that Hh pathway activity down-stream of SMO is mediated by GLI2 [23]. These data suggest that Hh activity down-stream of SMO is mediated by GLI2 instead of GLI1 in osteosarcoma.

SMO is a central transducer of the Hh signal and important anticancer drug target [11,14,19,22,24-33]. Warzecha et al reported that cyclopamine is able to inhibit proliferation of osteosarcoma cell lines [34]. In agreement with their findings, our results showed that inhibition of SMO by cyclopamine or *SMO* shRNA is efficient in suppressing tumourigenic properties of osteosarcoma cells both in vitro and in vivo. We used cyclopamine to inhibit SMO in xenograft model at first. We performed that treatment with 25 mg/kg cyclopamine reduced numbers of ki67-positive cells (see additional file 4). These findings suggest that inhibition of SMO prevents osteosarcoma growth by cell cycle regulation in vivo. Although it appeared that osteosarcoma growth was prevented by cyclopamine, all mice died for undetermined reasons by 1 month after cyclopamine treatment (data not shown). We next performed 10 mg/kg cyclopamine treatment, and found no difference in osteosarcoma growth between cyclopamine treatment and the control group (data not shown). Unfortunately, a therapeutic dose of this agent in the 143B xenograft model could not be obtained. It has been reported that cyclopamine might not be a good candidate for a drug in the treatment of malignant tumors because it had several serious side effects in young mice, including weight loss and dehydration, suggesting that it may not be possible to achieve a therapeutic dose in our xenograft model system [28,35]. In efforts to solve these problems, we used *SMO* shRNA. *SMO* shRNA inhibited osteosarcoma growth. Kaplan-Meier analysis showed that *SMO* shRNA conferred a significant survival benefit. It was reported that administration of RNAi resulted in silencing of the target genes in vivo [36-41]. These findings demonstrate the therapeutic potential of *SMO* shRNA for the treatment of osteosarcoma. Although SMO is the major signal transducer of the Hh pathway, SMO inhibition suppresses tumorigenesis by down-regulation of β-catenin mediated Wnt signaling [42]. It was reported that deregulation of β-catenin signaling is implicated in the pathogenesis of osteosarcoma [43,44]. Further examination might be needed the relationship between SMO inhibition and Wnt-β-catenin signaling in osteosarcoma.

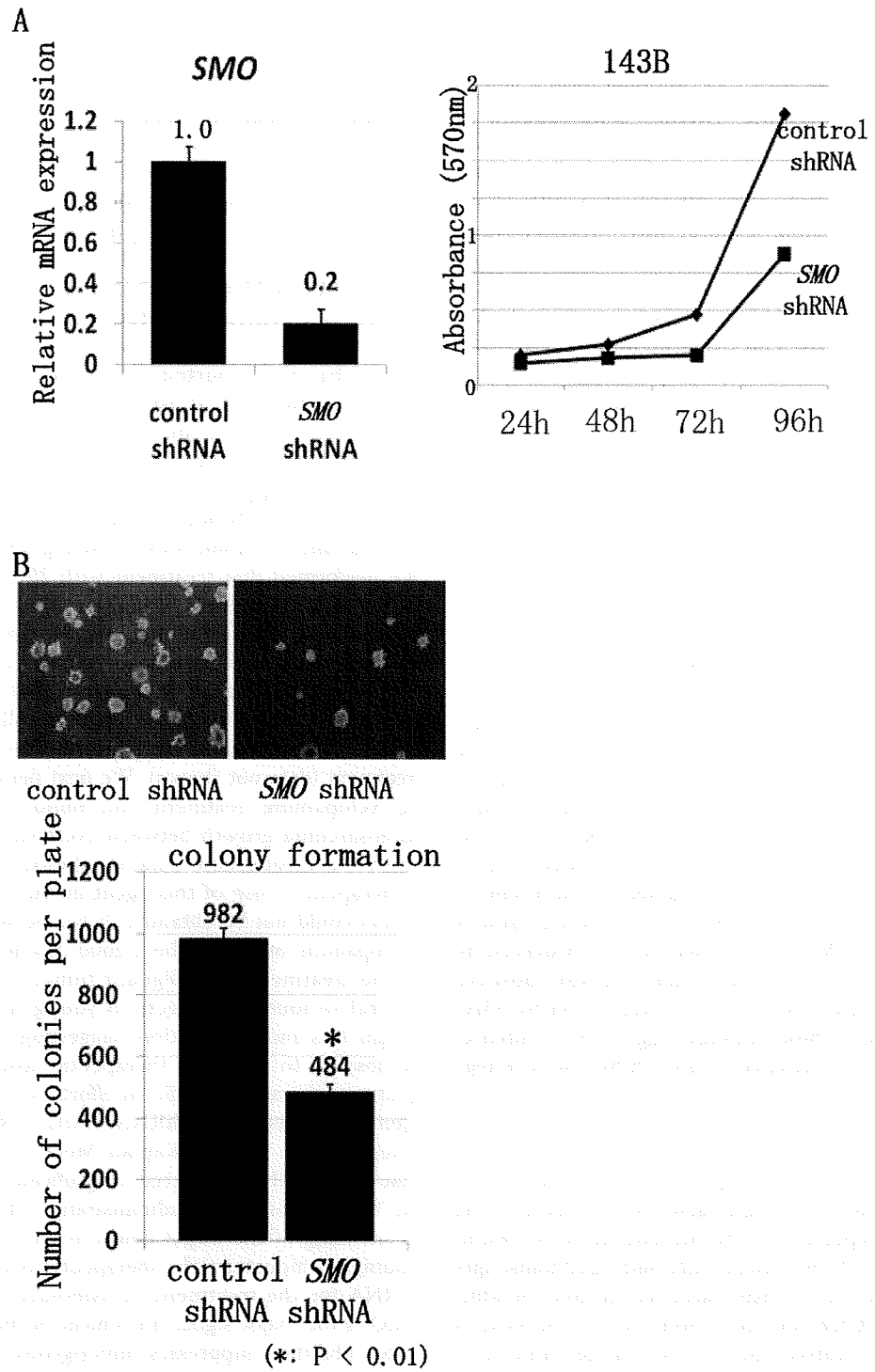

Figure 5 Knock down of *SMO* by *SMO* shRNA prevents osteosarcoma growth in vitro. A, Real-time PCR revealed that *SMO* shRNA effectively knock down *SMO* mRNA. (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using ACTB. Each sample was run minimally at three concentrations in triplicate (error bar means S.D.). The experiment was triplicate with similar results. B, Growth of viable 143B cells over 4 days was slowed by *SMO* shRNA. The experiment was triplicate with similar results. C, Colony formation assay revealed that *SMO* shRNA reduced colony formation in soft agar. The experiment was triplicate with similar results. (*: $P < 0.01$) (Error bar means S.D.)

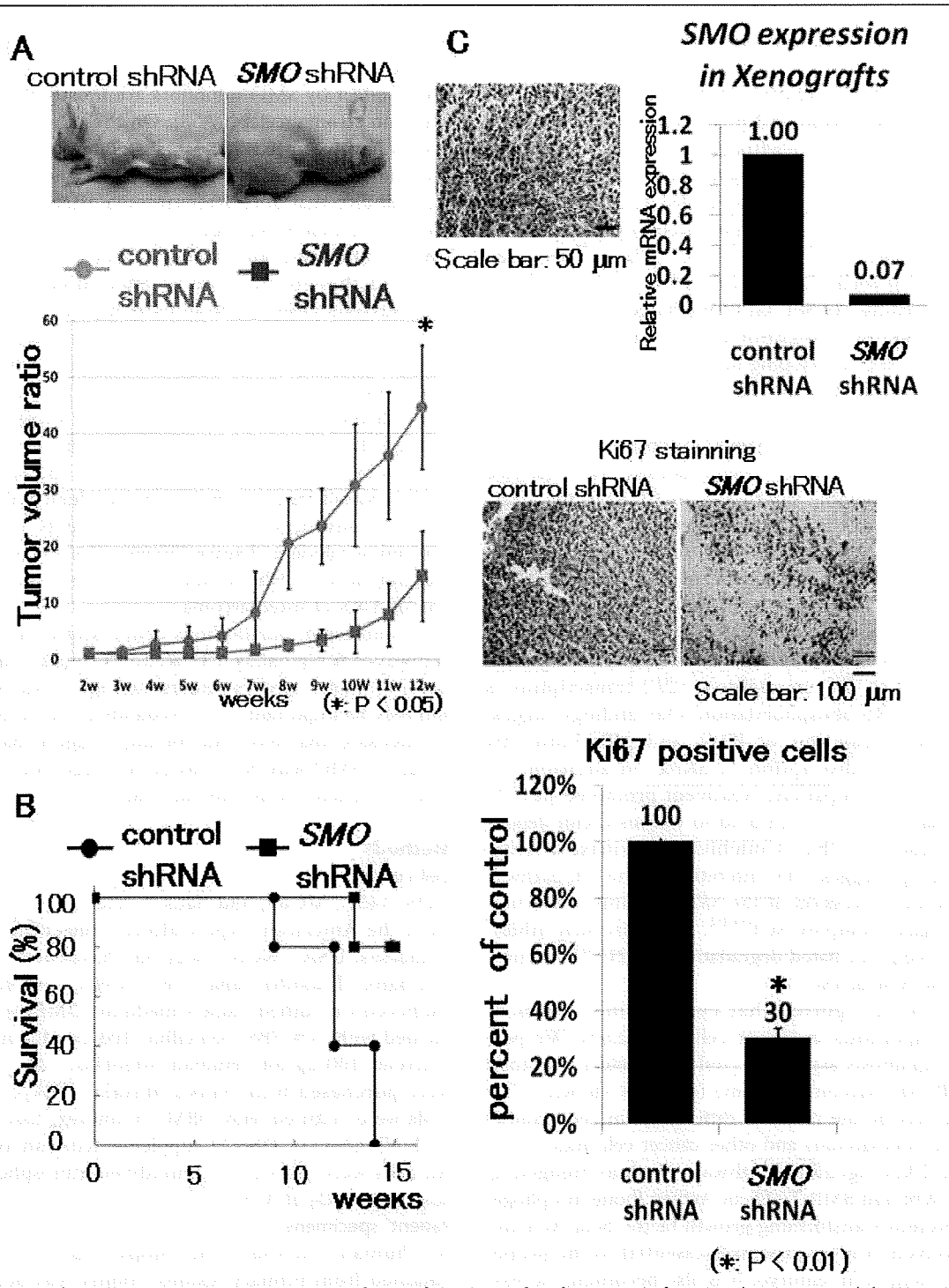

Figure 6 *SMO* shRNA prevents osteosarcoma xenograft growth in vivo and prolongs survival. A, *SMO* shRNA or control shRNA transfected 143B cells (1×10^6) were inoculated subcutaneously. Established 143B tumors were measured. The tumor volume at day 7 was set at 1, and tumor volumes at subsequent time points were calculated. *SMO* shRNA inhibited tumor growth at 8 weeks compared to control shRNA. B, Kaplan-Meier survival curves from *SMO* shRNA groups and control shRNA. Kaplan-Meier analysis showed that *SMO* shRNA conferred a significant survival benefit ($n = 6$, $p < 0.05$). C, Immunohistochemical examination of ki67 was performed in xenograft tumors. *SMO* shRNA decreased *SMO* RNA in vivo. Ki67 staining revealed that proliferation of osteosarcoma cells was decreased by GSI treatment. The number of Ki67-positive cells was decreased to 30% of control revel by *SMO* shRNA (error bar means S.D.) (*: $P < 0.01$).

Cyclopamine promoted G1 arrest in osteosarcoma in vitro. We also found that cyclopamine treatment regulated the expression of cell cycle regulators. Quantitative real-time PCR and western blot analysis revealed that cyclin D1, E1, SKP2, and pRB were down-regulated upon SMO inhibition with cyclopamine. Cyclin D1, cyclin E1, SKP2, and pRb have been reported to promote G1-S phase progression [45-48]. Our findings suggest that cyclopamine promoted cell cycle arrest via down-regulation of cyclins and pRb. It has been reported that cyclin D1 and cyclin E1 are direct targets of Hh signaling [49,50]. GLI2 mediated the mitogenic effects of Shh by transcriptional activation of cyclin D1 and cyclin D2 in developing hair follicles [51]. Our findings are consistent with the results of these previous studies. We showed that cyclopamine decreased the transcription of *SKP2*. The relationship between Hh signaling and SKP2 have not been reported. We attempted to find a GLI binding site (GACCACCCA) in the -1000 to +20 region of the 5' flanking sequence of *SKP2*, but found no GLI binding consensus sequence. These findings suggest that transcription of *SKP2* might not be regulated by GLI. It has been reported that the *SKP2* gene contains a functional E2F response element and is transcribed by E2F1 [52]. *E2F1* is an early transcriptional target of GLI2 [53]. In addition, *E2F1* transcription is activated by Rb phosphorylation. Our findings suggest that down-regulation of E2F1 and pRb indirectly reduced the transcription of *SKP2*. In addition, we showed that cyclopamine treatment promoted $p21^{Cip1}$ up-regulation. $p21^{cip1}$ can bind to various cyclin dependent kinases and that it inhibits their kinase activity. Our findings suggest that inhibition of the Hh pathway reduces the expression of the *SKP2* subunit of the ubiquitin-ligase complex SCF^{SKP2}, which in turn inhibit proteasomeï¿½mediated degradation of $p21^{Cip1}$ and promote cell cycle arrest.

It has been reported that cyclopamine treatment induced apoptosis in tumor cells [20,32,54]. We performed apoptosis assay, but could not detect apoptosis of 143B osteosarcoma cell line (data not shown). This finding may be the result of differences in cell viability between osteosarcoma and other cancer cell lines.

Several key signalling pathways, such as Hedgehog, Notch, Wnt and BMP-TGFbeta-Activin (bone morphogenetic protein-transforming growth factor-beta-Activin), are involved in most processes essential to the proper development of an embryo. It is also becoming increasingly clear that these pathways can have a crucial role in tumorigenesis (reviewed in [19]). We previously reported that activation of Notch signaling promote the progression of human osteosarcoma [55]. Additionally, some recent reports have provided evidence for direct interaction or cross-talk between these pathways (reviewed in [56]). Further examination should be performed to elucidate these pathways interaction in osteosarcoma pathogenesis.

Several recent papers have demonstrated that antitumor effect by SMO inhibitors are mostly due to their effect on stromal cells [57,58]. On the other hand some papers have reported that Hh signaling pathway is activated in cancer cells [14,17,21,23,59]. Although, there is a possibility that anti-osteosarcoma effect by cyclopamine was partially dependent to the effect on bone marrow stromal cell, anti-tumor effect of *SMO* shRNA revealed that inactivation of SMO directly inhibits osteosarcoma proliferation in vitro and in vivo.

The hypothesis that malignant tumours are generated by rare populations of Tumour-initiating cells (TIC), also called cancer stem cells, that are more tumourigenic than other cancer cells has gained increasing credence [31,60]. Some reports have shown the existence of TICs in bone and soft tissue sarcomas [61-65]. Magali et al. reported that loss of Smo causes depletion of TICs whereas constitutively active Smo augments TICs number and accelerates disease [20,66]. These data suggest that inhibition of Hh pathway might affect the proliferation of TICs of osteosarcoma.

In conclusion, our findings demonstrate that the Hh pathway is functionally activated in osteosarcoma. This novel finding improves understanding of osteosarcoma and may be important in understanding the proliferation of osteosarcoma cells. Our findings suggest that inactivation of SMO may be an attractive target for the treatment of patients with osteosarcoma.

Methods

Cell culture

HOS, 143B, MG63, and Saos-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). NOS-1 was purchased from RIKEN cell bank (Tsukuba, Japan) [67]. 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, East Rutherford, NJ, USA) or DMEM supplemented with 10% FBS. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Patient' specimens

All human osteosarcoma biopsy specimens were obtained from primary lesions. Biopsy was performed before chemotherapy or radio therapy for diagnostic purpose. Normal bone tissue was obtained from femur during total hip arthroplasty. The study protocol was approved by the institutional review board of the Kagoshima University. All patients and controls gave written informed consent.