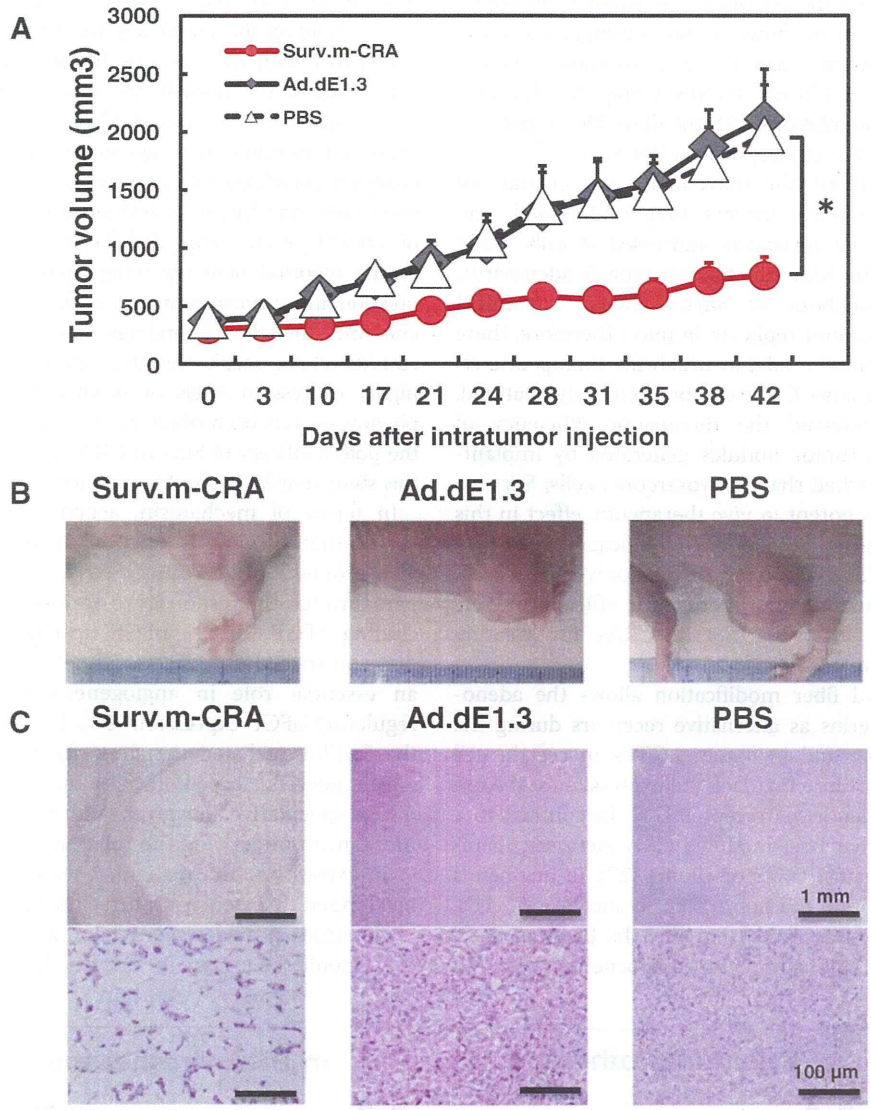


**Figure 8** ***In vivo*** **therapeutic effects of Surv.m-CRA against tumors in mice.** Tumor nodules were generated in mice by implantation of RSC-enriched rhabdomyosarcoma cells, and a single intratumoral injection of Surv.m-CRA, Ad.dE1.3, or PBS was subsequently administered to each nodule. **(A)** The macroscopic tumor size after each treatment. A significant difference was found between mice treated with Surv.m-CRA and those treated with either control (Ad.dE1.3 or PBS) ($P < 0.05$). **(B)** Representative macroscopic pictures of a tumor nodule 42 days after each treatment. **(C)** Representative histopathologic pictures of hematoxylin/eosin-stained sections in the tumor nodule 42 days after each treatment. In Ad.dE1.3-treated and the PBS-treated mice, tumor nodules mainly consisted of viable tumor cells exhibiting malignant features without large necrotic areas. By contrast, in Surv.m-CRA–treated mice, tumor nodules mainly consisted of large necrotic areas with loose connective tissues and without viable tumor cells. Original magnification: 40× (top; scale bar, 1 mm) and 400× (bottom; scale bar, 100 mm).

cells did not form tumors [7]. Because FGFR3-positive rhabdomyosarcoma cells, including KYM-1 cells, were characterized as RSCs in our previous study, it was not necessary to repeat this characterization in this study. Based on those results, in this study we carefully compared the biological features of survivin (both endogenous gene expression and the transduced promoter activity) and Surv.m-CRAs, both between RSC-enriched and RSC-exiguous conditions and between purified FGFR3-positive RSCs and purified FGFR3-negative progeny cells. We used both of these experimental systems because the former (enriched CSCs together with some progeny cells) may, at least in part, reflect the *in vivo* microenvironment, whereas the latter (purified CSCs) may facilitate clarification of the biological differences between CSCs and progenitor cells. Analyses in both experimental systems

clearly revealed that the activity of the survivin promoter, which has already been shown to have stronger and more cancer-specific activity than the Tert promoter [11,12], was further increased in RSCs; indeed, Surv.m-CRAs efficiently killed all populations with the desirable property of increased therapeutic efficacy against RSCs.

On the other hand, the movements and changes of CSCs within the body are not fully understood, and these points can be accurately addressed in only a few animal models. In addition, human type 5 adenovirus, which is the backbone of Surv.m-CRAs, can infect mouse cells but cannot replicate in mice; therefore, there is no available animal model in which the therapeutic efficacy of CRAs against CSCs can be accurately analyzed. Therefore, we assessed the therapeutic efficiency of Surv.m-CRAs in tumor nodules generated by implantation of RSC-enriched rhabdomyosarcoma cells; Surv.m-CRAs exhibited a potent *in vivo* therapeutic effect in this animal model. Although the *in vivo* efficacy of Surv.m-CRAs against CSCs cannot be quantitatively assessed, this result demonstrates the therapeutic efficacy and the possible clinical utility of Surv.m-CRAs for treating rhabdomyosarcoma.

The RGD-based fiber modification allows the adenovirus to use integrins as alternative receptors during the cell entry process, and increases AGTEs in certain cell types, particularly those that lack the expression of the native Coxsackie-adenovirus receptor [31]. In contrast to a previous report that fiber-modified CRA increased therapeutic efficacy against CSCs of glioma [27], in our hands the fiber modification did not drastically increase AGTEs. Therefore, we did not need to modify the fibers of Surv.m-CRAs in order to obtain therapeutic benefits, at least in this model. The clinical utility of the fiber modification may depend on the adenoviral infectivity in each cell type.

Together with the previous findings, the results of this study suggest the possible therapeutic efficacy of Surv.m-CRAs against other types of CSCs. Clinical studies previously demonstrated that high survivin expression levels are positively correlated with poor prognosis, accelerated rate of recurrence, and increased resistance to therapy in a variety of cancer types, including rhabdomyosarcoma [5,13,14]. Our results reported here regarding up-regulated survivin expression and survivin promoter activity in RSCs are consistent with the clinical findings, and should therefore be considered reasonable. Because a close relationship between higher expression levels of survivin and more malignant phenotypes has been observed in a variety of cancer types, the potent efficacy of Surv.m-CRAs to the RSCs revealed in this study may be applicable to other types of CSCs.

In terms of mechanism, accumulated data have revealed that survivin is involved in apoptosis resistance and proliferation of cancer cells, mediated at least in part through the responses to various growth factors, including bFGF [32,33]. bFGF up-regulates survivin expression in certain cancer cells [32], and survivin plays an essential role in angiogenesis in tumors by up-regulating bFGF expression [33], leading to activation of the FGFR3-mediated signaling pathway [7]. Any mechanistic inference based on these findings would necessarily be speculative, however, and the overall molecular mechanism underlying the relationship between the survivin expression and malignant features of CSCs should be clarified by extensive future studies.

This study provides general and important information that should be useful in the development of oncolytic

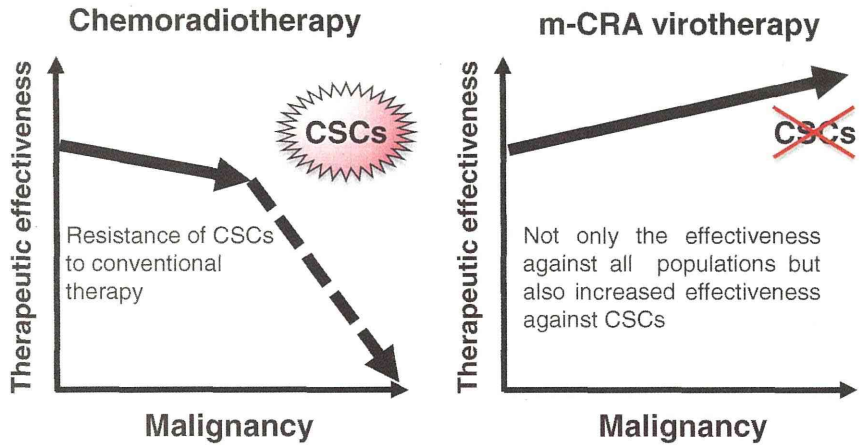


**Figure 9 The putative therapeutic advantage of m-CRA virotherapy.** In general, the therapeutic effectiveness of chemoradiotherapy is decreased in the advanced malignant tumor cells, and in particular, CSCs are resistant to conventional chemoradiotherapies. In contrast, not only m-CRA virotherapy can efficiently kill all populations of malignant tumor cells, including CSCs and their progeny, but also the antitumor effects of m-CRA virotherapy are higher against CSCs.

virotherapies against CSCs. To date, there have been very few successful reports of transcriptional targeting of oncolytic viruses against CSCs. In particular, none of the previous reports clearly showed that oncolytic viruses successfully acquired increased therapeutic efficacy against CSCs in parallel to increases in promoter activity and expression of a target gene. In this study, expression and promoter activity of survivin were further increased in CSCs; as a result of these transcriptional changes, Surv.m-CRAs exerted increased therapeutic efficacy against CSCs. Although the replicative mechanisms of adenoviruses have not been fully elucidated, the results described here suggest that the promising features of Surv.m-CRA may be due partly to specific features of adenoviruses and partly to the function of the survivin gene. Taken together, these findings demonstrate that Surv.m-CRA is an effective anticancer agent, but more generally, the results indicate that the use of m-CRAs represents a promising strategy for the development of effective anticancer agents against CSCs (Figure 9). In other words, the results described here pave the way to future development of several effective m-CRA–based therapies against CSCs; future progress will proceed via identification of new genes that target CSCs and generation of new m-CRAs using the promoters of these genes.

## Conclusion

Surv.m-CRAs demonstrated not only therapeutic efficacy against all the populations of rhabdomyosarcoma, but also increased efficacy against RSCs. These results will facilitate the clinical application of Surv.m-CRAs, and should be useful for future development of oncolytic virotherapies that target CSCs.

### Abbreviations

CSCs: Cancer stem cells; Surv.m-CRAs: Survivin-responsive conditionally replicating adenoviruses regulated with multiple factors; RSCs: Rhabdomyosarcoma stem cells; FGFR3: Fibroblast growth factor receptor 3; CRAs: Conditionally replicating adenoviruses; AGTE: The adenoviral gene transduction efficiency; IAP: Inhibitor of apoptosis; Tert.m-CRAs: Telomerase reverse transcriptase-responsive m-CRAs; bFGF: basic fibroblast growth factor; RSV promoter: Rous sarcoma virus long terminal repeat; EGFP: Enhanced green fluorescent protein; CMV: Cytomegalovirus; MOI: Multiplicities of infection; PBS: Phosphate-buffered saline.

### Competing interests

K. Kosai is the founder of WyK BiotechPharma Inc., but does not earn a salary from the company. All other authors declare no competing interest.

### Authors' contributions

KT and YW designed the experimental protocol, performed the most of experiments, analyzed the data, and wrote the manuscript. MI, KM, and RI performed some experiments. TS provided the materials and information regarding rhabdomyosarcoma stem cells. SK and SN partially supervised the preclinical study. KK conceived and designed the study, supervised all the experiments, assessed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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# Arsenic Trioxide Prevents Osteosarcoma Growth by Inhibition of GLI Transcription via DNA Damage Accumulation

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## Abstract

The Hedgehog pathway is activated in various types of malignancies. We previously reported that inhibition of SMO or GLI prevents osteosarcoma growth in vitro and in vivo. Recently, it has been reported that arsenic trioxide (ATO) inhibits cancer growth by blocking GLI transcription. In this study, we analyzed the function of ATO in the pathogenesis of osteosarcoma. Real-time PCR showed that ATO decreased the expression of Hedgehog target genes, including *PTCH1*, *GLI1*, and *GLI2*, in human osteosarcoma cell lines. WST-1 assay and colony formation assay revealed that ATO prevented osteosarcoma growth. These findings show that ATO prevents GLI transcription and osteosarcoma growth in vitro. Flow cytometric analysis showed that ATO promoted apoptotic cell death. Comet assay showed that ATO treatment increased accumulation of DNA damage. Western blot analysis showed that ATO treatment increased the expression of γH2AX, cleaved PARP, and cleaved caspase-3. In addition, ATO treatment decreased the expression of Bcl-2 and Bcl-xL. These findings suggest that ATO treatment promoted apoptotic cell death caused by accumulation of DNA damage. In contrast, Sonic Hedgehog treatment decreased the expression of γH2AX induced by cisplatin treatment. ATO re-induced the accumulation of DNA damage attenuated by Sonic Hedgehog treatment. These findings suggest that ATO inhibits the activation of Hedgehog signaling and promotes apoptotic cell death in osteosarcoma cells by accumulation of DNA damage. Finally, examination of mouse xenograft models showed that ATO administration prevented the growth of osteosarcoma in nude mice. Because ATO is an FDA-approved drug for treatment of leukemia, our findings suggest that ATO is a new therapeutic option for treatment of patients with osteosarcoma.

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## Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents [1,2]. Osteosarcoma is a highly aggressive neoplasm that is resistant to current therapeutic approaches, including radiation, chemotherapy, and surgical treatment. The survival rate of patients treated with neoadjuvant chemotherapy and local control therapy is 60–80% [3]. The predicted outcome is poor in patients with lung metastasis at first diagnosis, with long-term survival rates ranging between 10% and 40% [4]. Therefore, more effective treatments and more personalized therapies (i.e., treatments targeting a specific signaling pathway or gene) are essential for patients with osteosarcoma.

The Hedgehog pathway is involved in various aspects of development. The Hedgehog pathway is activated via the PATCHED (PTCH1) and SMOOTHENED (SMO) Hedgehog receptors. Activation of SMO promotes the activation of GLI family transcription factors (GLI1, GLI2, and GLI3) to regulate the transcription of target genes [5–7]. Aberrant activation of the Hedgehog pathway is associated with malignant tumors (reviewed in ref [8].). We have previously reported that aberrant

activation of the Hedgehog pathway is involved in the pathoetiology of osteosarcoma. Inhibition of the Hedgehog pathway by knockdown of SMO or GLI2 prevents osteosarcoma growth in vitro and in vivo [9,10]. Although several SMO inhibitors have been developed, they have several limitations, including constitutive activation of SMO, spontaneous mutation of SMO that impairs its binding to the drug, and constitutive activation downstream of SMO [11–21]. Arsenic trioxide (ATO) is an FDA-approved drug used for the treatment of patients with acute promyelocytic leukemia (APL) who show relapse after first-line chemotherapy (reviewed in 22. ATO promotes complete remission without myelosuppression and causes few adverse reactions. Recently, it has been reported that ATO prevents human cancer cell growth by inhibiting activation of the Hedgehog pathway [23–25]. In the present study, we examined the effect of ATO treatment on GLI transcription and osteosarcoma growth in vitro and in vivo. Our findings show that ATO inhibits Hedgehog pathway signaling and prevents human osteosarcoma cell growth via accumulation of DNA damage.

## Materials and Methods

### Cell culture

The osteosarcoma cell line 143B, Saos-2, and U2OS were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HsOs1 cell line was purchased from the Riken cell bank (Tsukuba, Japan). Osteosarcoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). For analyzing DNA damage, recombinant Sonic Hedgehog protein (R&D Systems, Minneapolis, MN, USA), ATO (Nihon Shinyaku, Kyoto, Japan), and cisplatin (CDDP) (LKT Laboratories, Minneapolis, USA) were used. Cell lines were cultured in a humidified incubator with 5% $CO_2$ at 37°C.

### Real-time polymerase chain reaction

Human osteosarcoma cells were cultured with or without 1 μM ATO. A vehicle (aqueous sodium hydroxide and hydrochloric acid to adjust to pH 7.5) was used as the control. Primer sets amplified amplicons of 150 to 200 bp in size. Polymerase chain reactions (PCRs) were performed using SYBR Green (BIO-RAD) on a MiniOpticon™ machine (BIO-RAD). The comparative Ct (ΔΔCt) method was used to evaluate the fold change in mRNA expression using *β-actin* as the reference gene. All PCR reactions were performed in triplicate, with 3 different concentrations of cDNA. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The following primers were used: *PTCH1*: 5′-TAACGCTGCAACAACTCAGG-3′, 5′-GAAGGCTGTGACATTGCTGA-3′; *GLI1*: 5′-GTGCAAGTCAAGCCAGAACA-3′, 5′-ATAGGGGCCTGACTGGAGAT-3′, *GLI2*: 5′-CGACACCAGGAAGGAAGGTA-3′, 5′-AGAACGGAGGTAGTGCTCCA-3′; *β-actin*: 5′-AGAAAATCTGGCACCACACC-3′, 5′-AGAGGCGTACAGGGATAGCA-3′.

Each experiment was performed in triplicate, and all experiments were performed 3 times.

### WST-1 assay

Human osteosarcoma cells were cultured with or without 1 μM or 3 μM ATO. An equivalent volume of vehicle (aqueous sodium hydroxide and hydrochloric acid to adjust to pH 7.5) was used as the control. The cells were treated with WST-1 substrate (Roche, Basel, Switzerland) for 4 h, washed with phosphate-buffered saline, and lysed to release formazan. Then, the cells were analyzed on a microplate reader (BIO-RAD, Hercules, CA, USA). Each experiment was performed in triplicate, and all experiments were performed 3 times.

### Colony formation assay

Cells were cultured in DMEM containing 0.33% soft agar and 5% fetal bovine serum, and plated on 0.5% soft agar layer. Cells were cultured in 6-well plates at a density of $5 \times 10^3$ cells per well. Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. Fourteen days later, the number of colonies was evaluated. Each experiment was performed in triplicate, and all experiments were performed 3 times.

### Cell cycle analysis

Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Cell cycle analysis was performed as previously reported [9]. Cells were collected, fixed with 70% ethanol for 2 h at 4°C, washed with phosphate-buffered saline, and treated with 500 μL staining buffer containing RNase A and 50 μg/mL propidium iodide (Wako Chemicals, Kanagawa, Japan). The DNA content was examined by flow cytometry using CyAn™ ADP (Beckman Coulter, CA, USA) and Summit software (Beckman Coulter). Each experiment was performed in triplicate, and all experiments were performed 3 times.

### Comet assay

Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. Cells were trypsinized and electrophoresed on agarose gels as previously reported [26]. Tail moment (TM) and tail length (TL) were used to evaluate DNA damage in individual cells. Image analysis and quantification were performed using NIH ImageJ software. TM = % DNA in the tail × TL, where % of DNA in the tail = tail area (TA) × tail area intensity (TAI) × 100/(TA × TAI) + [head area (HA) × head area intensity (HAI)].

### Western blotting

Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. The cells were dissolved in NP40 buffer containing 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3 mM pAPMSF (Wako Chemicals, Kanagawa, Japan), 5 mg/mL aprotinin (Sigma, St. Louis, MO, USA), 2 mM sodium orthovanadate (Wako Chemicals), and 5 mM EDTA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and

immunoblotting were performed subsequently. The following antibodies were used: phospho-histone H2AX (Ser139) (γH2AX) (Cell Signaling Technology, MA, USA), cleaved caspase-3 (Asp175) (Cell Signaling Technology), poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology), Bcl-xL (Cell Signaling Technology), SAPK/JNK (Cell Signaling Technology), Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology), NF-κB p65 (Cell Signaling Technology), phospho-NF-κB p65 (Ser468) (Cell Signaling Technology), and tubulin (Santa Cruz, California, USA). Bands were visualized using the ECL chemiluminescence system (Amersham, Giles, UK).

### Xenograft model

143B cells ($1 \times 10^6$) and 100 μL Matrigel (BD, NJ, USA) suspension were subcutaneously inoculated into 5-week-old nude mice. The mice were randomly allocated to treatment with either ATO (10 μg/g) or an equivalent volume of vehicle (30 mM NaOH, pH 7.0). ATO and vehicle were administered intraperitoneally every day. ATO and vehicle treatment was started at 1 week after inoculation, at which time, the tumors had grown to a visible size. The tumor size was measured using the formula $LW^2/2$ (L and W represent the length and width of tumors, respectively). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Kagoshima University. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee, Graduate School of Medical and Dental Sciences, Kagoshima University (Permit Number: MD11017). All surgeries were performed under general anesthesia, and every effort was made to minimize the number of animals used and animal pain.

### Immunohistochemistry

ApopTag® Peroxidase In Situ Apoptosis Detection Kit was used for TUNEL staining according to the supplier's protocol (MerckMillipore, Billerica, MA, USA). The sections were stained with methyl green (Merck-Chemicals, Darmstadt, Germany) to identify nuclei.

### Statistical analysis

All examinations were performed 3 times, except where otherwise stated, and all samples were analyzed in triplicate. All results are presented as mean (SD). Statistical differences between groups were assessed by Student's *t*-test for unpaired data using Microsoft Office Excel (Microsoft, Albuquerque, NM, USA) and Kaplan 97.

## Results

### ATO prevents GLI transcription and proliferation of osteosarcoma cells

To determine whether ATO prevents GLI transcription in osteosarcoma cells, real-time PCR was performed for ATO-treated cells. Four human osteosarcoma cell lines showing upregulation of GLI transcription were examined [9,10]. The human osteosarcoma cell lines were treated with ATO at previously reported concentrations, which inhibit human cancer cell proliferation by inhibiting activation of the Hedgehog pathway [25]. Real-time PCR revealed that ATO prevented the transcription of GLI target genes, including *PTCH1*, *GLI1*, and *GLI2*, in human osteosarcoma cell lines (Figure 1). The WST-1 assay showed that proliferation of the 143B, Saos2, HsOs1, and U2OS cell lines was inhibited by ATO (Figure 2). We next evaluated the effects of ATO on anchorage-independent growth of osteosarcoma cells. The colony formation assay showed that ATO treatment decreased the number of colonies in soft agar (Figure 3). These findings showed that ATO treatment prevents GLI transcription and growth of osteosarcoma cells in vitro.

### ATO promotes DNA damage and apoptotic cell death

To examine whether ATO treatment promoted cell death or cell cycle arrest, we performed flow cytometric analysis. The results showed that ATO treatment increased the population of sub-G1 cells (Figure 4). These findings show that ATO treatment promotes apoptotic cell death in osteosarcoma cells. To examine whether ATO promotes DNA damage, we performed a comet assay, which can be used to detect single cell DNA damage by the cellular elution pattern through agarose gels. The comet assay showed that ATO treatment altered the elution profiles (Figure 5). These findings show that ATO treatment promotes the accumulation of DNA damage in osteosarcoma cells. In addition, we used western blotting to examine the expression of DNA damage markers and apoptosis-related proteins after ATO treatment. Western blot analysis showed that ATO treatment increased the expression of γH2AX, a marker of double-strand breaks, cleaved poly (ADP-ribose) polymerase (PARP), and cleaved-caspase 3. In contrast, ATO treatment decreased the expression of Bcl-2 and Bcl-xL (Figure 6A). These findings suggest that ATO treatment promotes apoptotic cell death caused by accumulation of DNA damage.

It has been reported that ATO promotes apoptotic cell death and phosphorylation of JNK [27]. Although western blot analysis showed that ATO treatment increased the amount of phosphorylated JNK, inhibition of JNK activity had no effect on osteosarcoma cell proliferation with or without ATO, as seen with Ewing sarcoma cells (Figure S1) [23]. It has been reported that ATO treatment decreases the phosphorylation of NF-κB and promotes cell death [28]. Our findings showed that ATO treatment did not affect the status of NF-κB phosphorylation (Figure S1).

### Hedgehog signaling prevents DNA damage caused by CDDP treatment

To examine whether activation of Hedgehog signaling affects accumulation of DNA damage, we performed western blot analysis after cisplatin (CDDP) treatment. Western blotting showed that CDDP treatment upregulated the expression of γH2AX. Treatment with Sonic Hedgehog attenuated the upregulation of γH2AX (Figure 6B). In addition, we examined the effect of ATO treatment on the attenuation of DNA damage by Hedgehog activation. The attenuation of DNA damage caused by Hedgehog activation was reversed by ATO

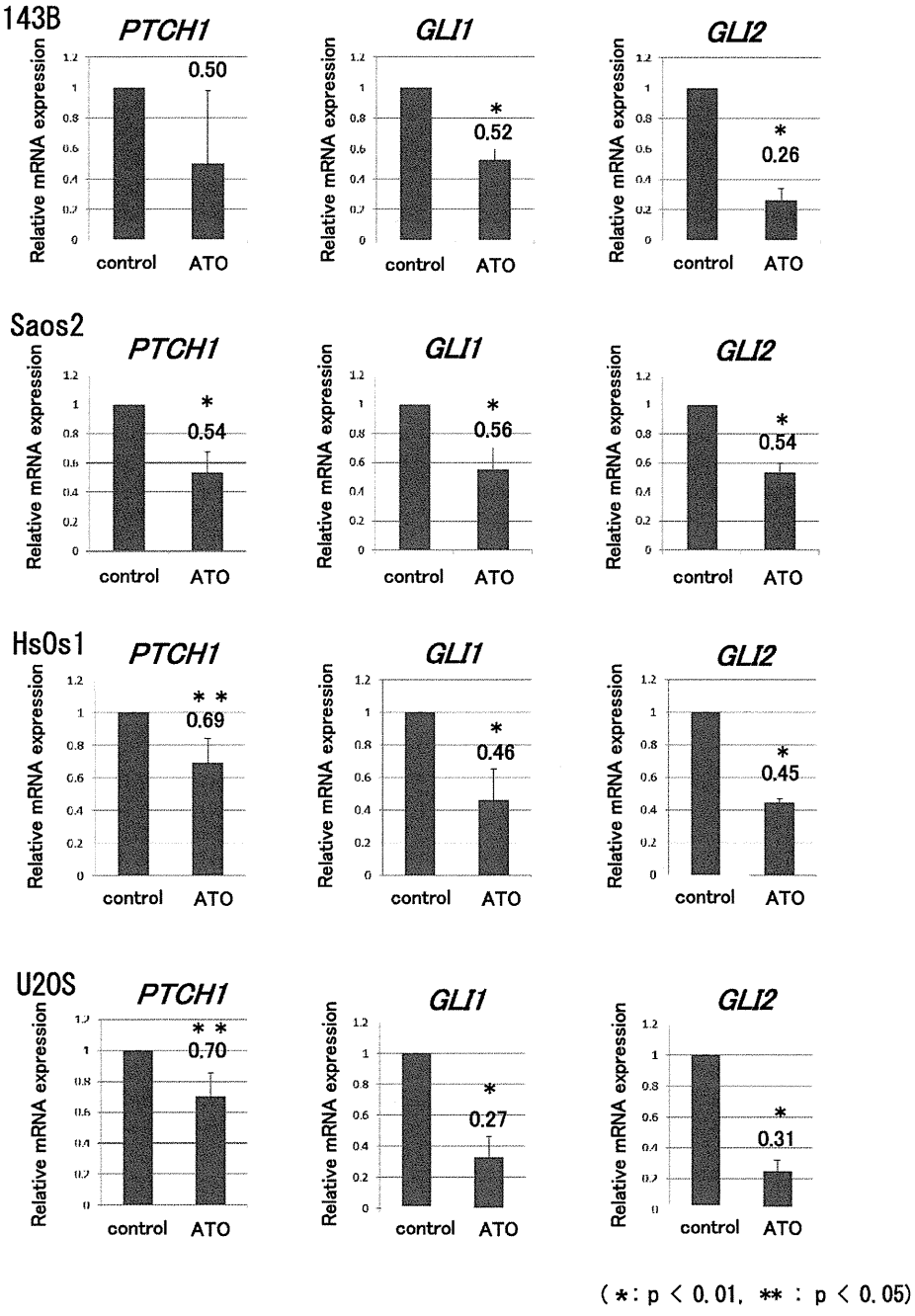


**Figure 1. ATO prevents the transcription of GLI target genes.** Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Total RNA collected from osteosarcoma cell lines was examined by real-time polymerase chain reaction (PCR). A comparative Ct (ΔΔCt) analysis was performed to examine fold changes in mRNA expression compared with *β-actin*. Real-time PCR showed that ATO decreased the transcription of GLI target genes, including *PTCH1*, *GLI1*, and *GLI2*, in 143B, Saos2, HsOs1, and U2OS cells. The experiment was performed in triplicate with similar results (error bars represent mean [SD]) (*P < 0.01, **P < 0.05).

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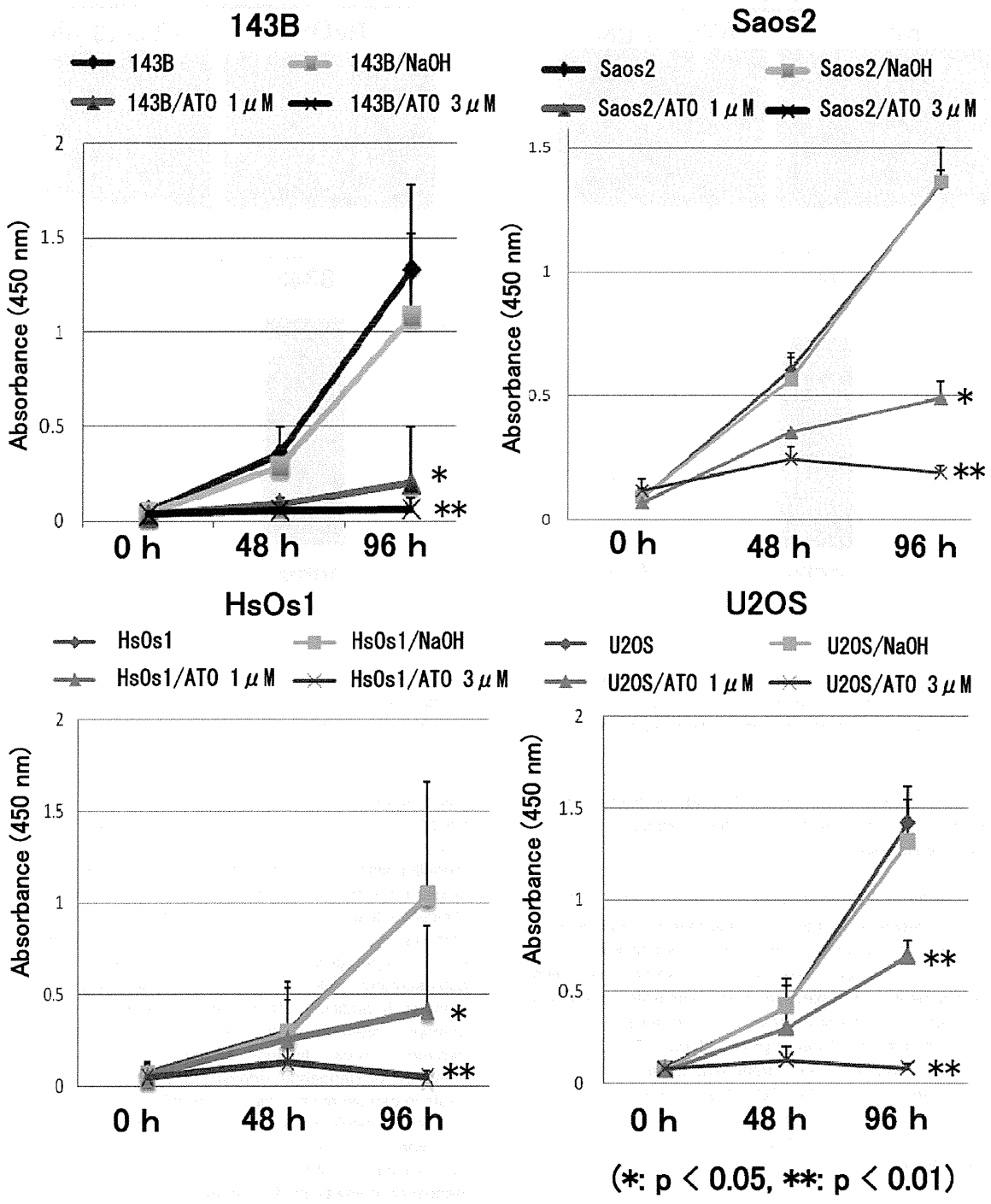


**Figure 2. ATO prevents human osteosarcoma cell proliferation.** WST assay showed that the growth of 143B, Saos-2, HsOs1, and U2OS cells was prevented by 1 μM or 3 μM ATO treatment for 96 h. An equivalent volume of vehicle was used as the control. The experiment was performed in triplicate with similar results (*P < 0.05, **P < 0.01) (error bars represent mean [SD]).

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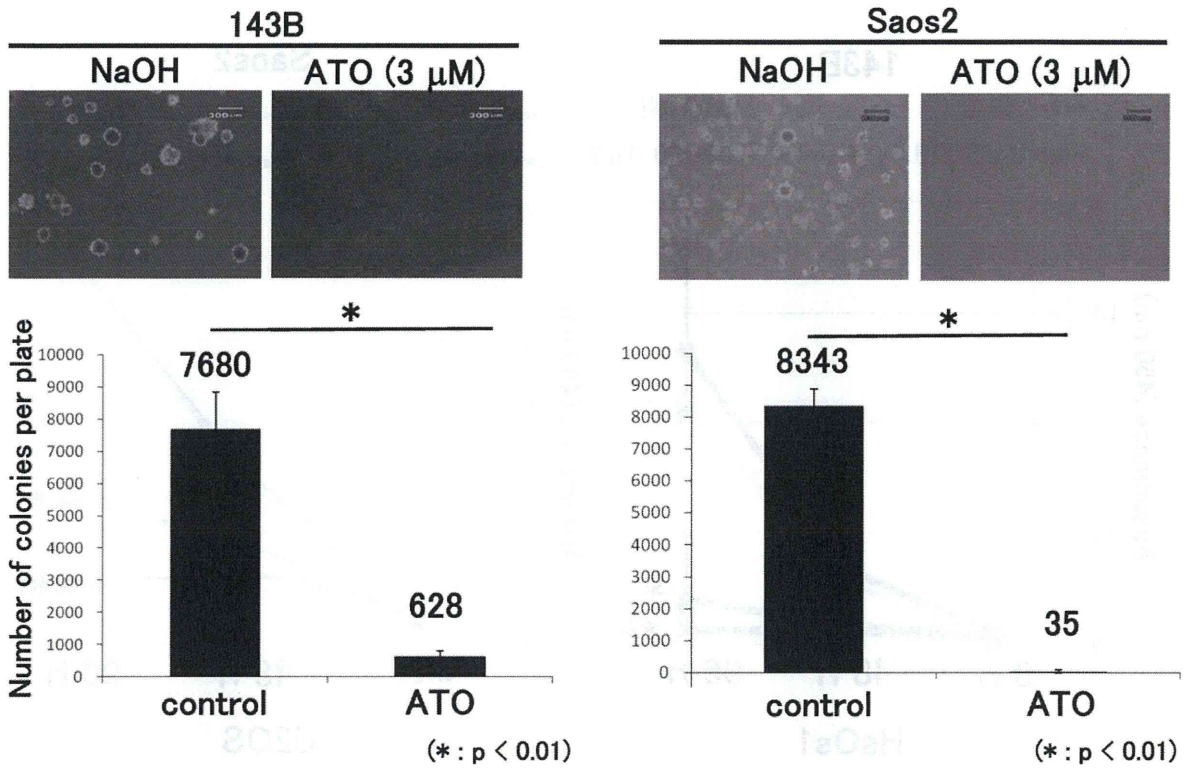


**Figure 3. ATO inhibits anchorage-independent osteosarcoma growth.** Treatment of 143B and Saos2 cells with 3 μM ATO reduced the number of colonies in soft agar at 14 days. An equivalent volume of vehicle was used as the control. These experiments were performed in triplicate with similar results (*P < 0.01) (error bars represent mean [SD]).

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treatment (Figure 6C). These findings suggest that ATO promotes the accumulation of DNA damage by inhibiting Hedgehog signaling.

### ATO prevents osteosarcoma growth in vivo

143B osteosarcoma cells were intradermally inoculated into nude mice, and palpable tumors were formed within 7 days. Then, ATO or an equivalent volume of vehicle was injected intraperitoneally. The injections were administered every day. Compared with vehicle treatment, treatment with ATO significantly prevented tumor growth (Figure. 7). Kaplan-Meier analysis showed that ATO treatment provided a significant survival benefit (Figure 7A). TUNEL staining showed that ATO treatment induced apoptotic cell death. The number of apoptotic cells was significantly increased in ATO-treated tumors (Figure 7B).

## Discussion

We and other researchers have previously reported that inhibition of the Hedgehog pathway prevented the growth of osteosarcoma cells [9,10,29]. In particular, we showed that knockdown of GLI2 prevented osteosarcoma cell growth in vitro and in vivo [9]. ATO prevents Ewing sarcoma, medulloblastoma, and basal cell carcinoma growth by inhibition of GLI transcription [23–25]. To apply our previous findings in clinical settings, we examined the effects of ATO in human osteosarcoma. We showed that ATO prevents the transcription of GLI target genes and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, ATO re-induces the accumulation of DNA damage attenuated by recombinant Sonic Hedgehog treatment. These findings suggest that ATO inhibits the activation of Hedgehog signaling and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, our findings showed that ATO decreased the expression of Bcl-2 and Bcl-xL. GLI1 and GLI2 upregulate the transcription of Bcl-2 and Bcl-xL [30–33]. Inhibition of the Hedgehog pathway by ATO treatment may downregulate Bcl-2 and Bcl-xL to promote apoptotic cell death in osteosarcoma cells. Singh et al. reported that ABCG2, a drug transporter protein, is a direct transcriptional target of Hedgehog signaling [33]. These findings suggest that activation of Hedgehog