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expression is best associated with luminal subtype A and FOXA1 immunoreactivity is shown as a significant predictor of cancer-specific survival for patients with ERα-positive tumors (Badve et al., 2007; Mehta et al., 2012). The prognostic relevance of FOXA1 in the breast cancers with relatively low risk will be useful for the determination of therapeutic methods (Badve et al., 2007; Thorat et al., 2008).

Altered expression of FOXP1 is associated with various types of tumors, including breast cancer (Banham et al., 2001, 2007; Barrans, Fenton, Banham, Owen, & Jack, 2004; Bates et al., 2008; Craig et al., 2011; Fox et al., 2004; Goatly et al., 2008; Hoeller, Schneider, Haralambieva, Dirnhofer, & Tzankov, 2010; Prown et al., 2008; Sagaert et al., 2006; Takayama et al., 2008; Wang et al., 2004; Zhang et al., 2010). FOXP1 immunoreactivity may be associated with the immunoreactivity of ERα and PgR in breast cancer, which may predict favorable prognosis in patients (Banham et al., 2005; Rayoo et al., 2009). A recent study showed that FOXP1 immunoreactivity was significantly enhanced in breast cancer samples for tamoxifen-treated patients without relapse, compared with samples for those with relapse within 5 years after surgery (Shigekawa et al., 2011). It was also demonstrated that a positive immunoreactivity for either FOXP1 or FOXA1 significantly correlated with better relapse-free and overall survivals for breast cancer patients with adjuvant tamoxifen therapy, compared with either FOXP1- or FOXA1-negative immunoreactivity (Ijichi et al., 2012). Univariate and multivariate proportional analyses showed that the relapse-free and overall survival rates were associated with FOXA1 and FOXP1 immunoreactivities. For the relapse-free survival, either FOXP1 or FOXA1 immunoreactivity was found to be a significant prognostic predictor through univariate analysis ($P=0.001$ and 0.002, respectively), whereas only FOXP1 immunoreactivity was a better prognostic predictor based on multivariate analysis ($P=0.026$). On the other hand, neither FOXP1 nor FOXA1 was significantly associated with overall survival by multivariate analysis. Notably, double-positive FOXP1 and FOXA1 immunoreactivities were significantly associated with more favorable prognosis for the relapse-free and overall survivals compared with either FOXP1- or FOXA1-negative immunoreactivity based on multivariate analyses ($P=0.002$ and 0.002, respectively). These findings suggest that the combined analyses of the FOXA1 and FOXP1 immunoreactivities provide powerful prognostic indicators for the patients with ERα-positive breast cancers treated with adjuvant tamoxifen therapy.

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Carroll and his colleagues showed that FOXA1 also plays a role in the differential ER-binding events in the tumors with a poor outcome (Ross-Innes et al., 2012). Notably, an siRNA-mediated knockdown study showed that ERα signals, including ERα occupancy and estrogen-dependent cell growth, are FOXA1 dependent in both tamoxifen-sensitive and tamoxifen-refractory MCF-7 cells (Hurtado et al., 2011). Further studies are required to answer the question whether the ER/FOXA1-driven growth is associated with tumor recurrence in various stages of the disease.

4. CONCLUSIONS AND FUTURE DIRECTIONS

A recent genome-wide study using ChIP analysis with high-throughput sequencing revealed that FOXA1 is a critical transcription factor that contributes to most of the ERα-chromatin interactions and estrogen-dependent changes of gene expression (Hurtado et al., 2011). FOXA1 influences genome-wide chromatin accessibility of ERα in response to different ligands, including both estrogen and tamoxifen (Hurtado et al., 2011). Thus, FOXA1 is considered as a major determinant for estrogen–ERα activity and endocrine response in breast cancer cells. Since FOXP1 exhibits functions analogous to those of FOXA1 in the ER-mediated transcription and its immunoreactivity has a clinicopathological significance along with FOXA1 immunoreactivity in breast cancer, it is assumed that FOXP1 also plays an important role in the regulation of ERα activity and tamoxifen responsiveness in breast cancer, functioning cooperatively with FOXA1. Future genome-wide studies of FOXP1 binding as well as ERα and FOXA1 occupancy will elucidate the precise interactions of these transcription factors in the ERα-mediated signaling pathways.

In summary, *FOXP1* and *FOXA1* are primary ERα target genes and critical transcription factors that regulate the ERα activity. Both FOX proteins will be potential biomarkers for the prediction of breast cancer prognosis. Pharmacological modulation of FOXP1 and FOXA1 activities may be clinically useful in the prevention and/or treatment of breast cancer.

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

Shunsuke Nakamura[1], Satoshi Nagano[1], Hiroko Nagao[1], Yasuhiro Ishidou[2], Masahiro Yokouchi[1], Masahiko Abematsu[1,3], Takuya Yamamoto[1], Setsuro Komiya[1], Takao Setoguchi[3*]

1 Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan, **2** Department of Medical Joint Materials, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan, **3** The Near-Future Locomotor Organ Medicine Creation Course (Kusunoki Kai),Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

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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* E-mail: setoro@m2.kufm.kagoshima-u.ac.jp

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 5×10^3 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 (1×10^6) 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

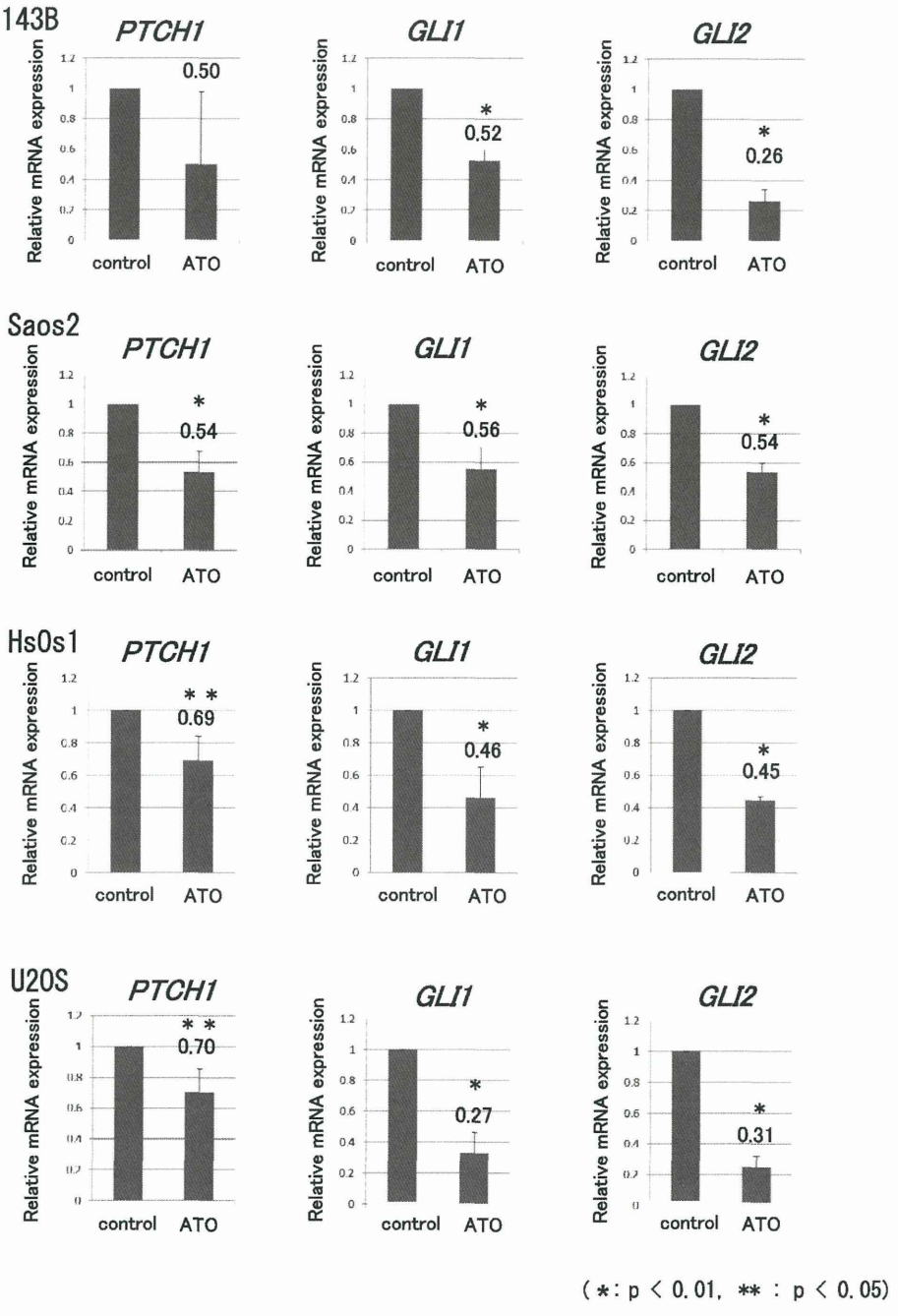

Figure 1. ATO prevents the transcription of GLI target genes. Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Total RNA collected from osteosarcoma cell lines was examined by real-time polymerase chain reaction (PCR). A comparative 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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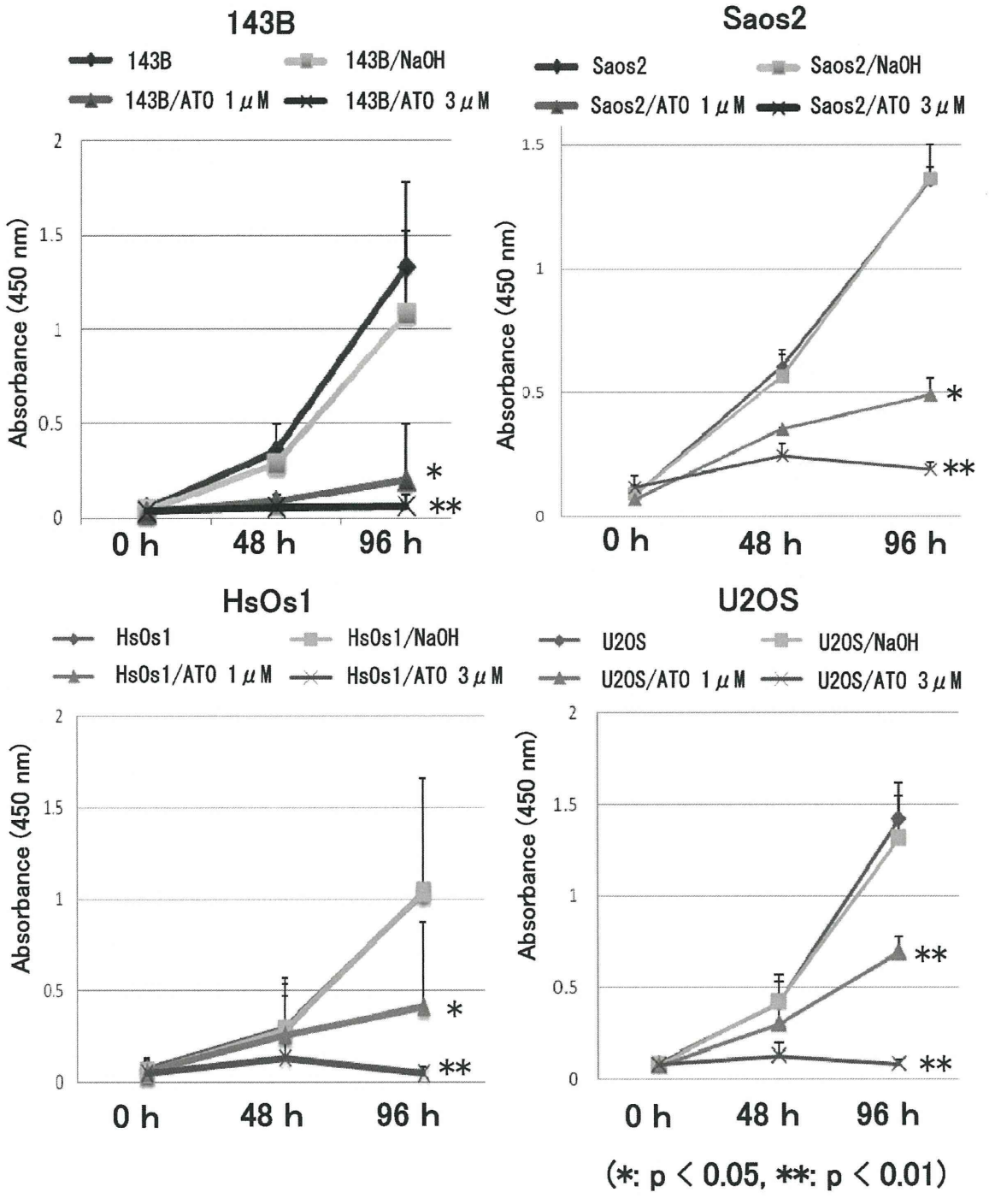

Figure 2. ATO prevents human osteosarcoma cell proliferation. WST assay showed that the growth of 143B, Saos-2, HsOs1, and U2OS cells was prevented by 1 μM or 3 μM ATO treatment for 96 h. An equivalent volume of vehicle was used as the control. The experiment was performed in triplicate with similar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

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