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Suppression of Osteosarcoma Cell Invasion by Chemotherapy Is Mediated by Urokinase Plasminogen Activator Activity via Up-Regulation of EGR1

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Abstract

Background: The cellular and molecular mechanisms of tumour response following chemotherapy are largely unknown. We found that low dose anti-tumour agents up-regulate *early growth response 1* (*EGR1*) expression. EGR1 is a member of the immediate-early gene group of transcription factors which modulate transcription of multiple genes involved in cell proliferation, differentiation, and development. It has been reported that EGR1 act as either tumour promoting factor or suppressor. We therefore examined the expression and function of *EGR1* in osteosarcoma.

Methods: We investigated the expression of EGR1 in human osteosarcoma cell lines and biopsy specimens. We next examined the expression of EGR1 following anti-tumour agents treatment. To examine the function of EGR1 in osteosarcoma, we assessed the tumour growth and invasion in vitro and in vivo.

Results: Real-time PCR revealed that *EGR1* was down-regulated both in osteosarcoma cell lines and osteosarcoma patients' biopsy specimens. In addition, EGR1 was up-regulated both in osteosarcoma patient' specimens and osteosarcoma cell lines following anti-tumour agent treatment. Although forced expression of EGR1 did not prevent osteosarcoma growth, forced expression of EGR1 prevented osteosarcoma cell invasion in vitro. In addition, forced expression of EGR1 promoted down-regulation of urokinase plasminogen activator, urokinase receptor, and urokinase plasminogen activity. Xenograft mice models showed that forced expression of EGR1 prevents osteosarcoma cell migration into blood vessels.

Conclusions: These findings suggest that although chemotherapy could not prevent osteosarcoma growth in chemotherapy-resistant patients, it did prevent osteosarcoma cell invasion by down-regulation of urokinase plasminogen activity via up-regulation of EGR1 during chemotherapy periods.

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Introduction

Osteosarcoma is the most frequent primary malignant bone tumor. After initial diagnosis is made by biopsy, treatment consists of preoperative chemotherapy, followed by definitive surgery and postoperative chemotherapy. The Survival rates for patients treated with intensive multidrug chemotherapy and aggressive local control have been reported at 60–80% [1–5]. Indeed, patients with non-metastatic disease have a 70% chance of long-term survival. Eighty percent of patients die of metastatic disease, most commonly in the lungs [3]. Unfortunately, patients with metastatic disease at diagnosis or 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 [6,7].

The early growth response gene 1 (*EGR1*) is a member of the immediate-early gene group of transcription factors which modulate transcription of multiple genes involved in cell proliferation, differentiation, and development [8]. Expression of *EGR1* is significantly reduced in a number of tumor cells [9,10], and loss of expression of it is closely associated with tumor formation in mammalian cells and tissues [10]. On the other hand, stable expression of *EGR1* inhibited cell proliferation and soft agar growth in NIH3T3 cells transformed with v-*sis*, indicating that *EGR1* functions as a tumor suppressor [11]. We therefore examined the expression and function of *EGR1* in osteosarcoma. Here, we report that expression of *EGR1* is down-regulated in human osteosarcoma cell lines and patient' biopsy specimens. In addition, treatment with anti-tumour agents promoted up-regulation of *EGR1*. Although forced expression of EGR1 did not affect osteosarcoma growth,

forced expression of EGR1 inhibited osteosarcoma cell invasion by down-regulation of urokinase plasminogen activator (uPA) and urokinase receptor (uPAR).

Materials and Methods

Cell culture

143B, Saos-2, HOS, and MG63 cells were purchased from the American Type Culture Collection (ATCC, USA). NOS-1 was provided by the RIKEN BRC through the National Bio-Resource Project of The MEXT, Japan (Tsukuba, Japan) [12]. 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). NHOst was 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.

Anti-tumor agents

Doxorubicin, methotrexate, and etoposide were purchased from Sigma-Aldrich (MO, USA). Cisplatin was purchased from LKT laboratories (MN, USA).

Patient' specimens

All human osteosarcoma biopsy specimens were obtained from primary lesions. Biopsy was performed before chemotherapy or radio therapy to make the diagnosis. Normal bone tissue was obtained from femur during total hip arthroplasty. Specimens of OS6, OS8, and OS9 tumors were obtained during tumor resection in osteosarcoma patients who received chemotherapy. Doxrubicin, methotrexate, and cisplatin were given to these three patients according to COSS-86 protocol. We compared the EGR1 expressions in the biopsy specimens and the resected tumor specimens obtained from these patients. The study protocol was approved by the institutional review board of the Kagoshima University. All patients and controls gave written informed consent.

Real-time PCR

For real-time PCR, total RNA was obtained 24 h, 48 h, and 5 days following drug treatment. DNase-treated and reverse-transcribed using oligo(dT) primers as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Reactions were run using SYBR Green (BIO-RAD, Hercules, CA, USA) on a MiniOpticon™ machine (BIO-RAD). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH* or *ACTB*. Each sample was run minimally at three concentrations in triplicate. All primer sets amplified 150- to 200-bp fragments. The primers sequences used were follows: for *EGR1*: 5-CAG-CACCTTCAACCCTCAG-3, 5- CACAAGGTGTTGCCACT-GTT-3; *uPA*: 5- TGTGAGATCACTGGCTTTGG-3, 5- GTCA-GCAGCACACAGCATTT-3; *uPAR*: 5- TGAAGAACAGTGC-CTGGATG-3, 5- TGTTGCAGCATTTCAGGAAG-3; *GAPDH*: 5- GAAGGTGAAGGTCGGAGTC-3, 5- GAAGATGGTGAT-GGGATTTC-3; *ACTB*: 5-AGAAAATCTGGCACCACACC-3, 5-AGAGGCGTACAGGGATAGCA-3.

MTT assay

Following 100 ng–1 μg cisplatin, 1 ng–10 ng methotrexate, 50 ng–1000 ng etoposide, or 10 ng–100 ng doxorubicin treatment, we performed MTT assay to evaluate the osteosarcoma growth as previously reported [13]. In addition, we transfected control vector or EGR1 expression vector, and examined osteosarcoma cell growth by MTT assay. Cells were incubated with substrate for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 hours, 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.

Vector transfection

EGR1 expression vector was purchased from Origene (Maryland, USA). EGR1 was cloned into pCMV6-Entry Neomycin Vector. Lipofection of expression vector was performed as recommended in the supplier's protocol using FuGENE 6 (Roche, Basel, Switzerland). All transfected cells were treated with neomycin constitutively to obtain stable transfectants. EGR stable transfectants were used for invasion assay, examinations of uPA and uPAR expressions, and in vivo experiments.

Colony formation assay

Colony formation assay was performed as previously described [14]. Briefly, cells were suspended in DMEM containing 0.33% agar and 10% fetal bovine serum and plated onto the bottom layer containing 0.5% agar. The cells were plated at a density of 5×10^3 per well in a 24-well plate, and colonies were counted 14 days later. Each condition was analyzed in triplicate, and all experiments were repeated three times.

Invasion assay

Invasion of osteosarcoma cells was measured using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Bioscience, NJ, U.S.A.) according to the manufacturer's protocol. Briefly, the cells were transfected with plasmids and selected by neomycin. Osteosarcoma cells were seeded onto the membrane of the upper chamber of the transwell at a concentration of $3–5\times10^5$/ml in 2 ml of DMEM medium. The medium in the upper chamber was serum-free. The medium in the lower chamber contained 5% fetal calf serum as a source of chemoattractants. Cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan) and photographed.

Western blot

Western blot analysis was performed as previously reported [15]. Briefly, cells were lysed using NP40 lysis buffer (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, StLouis, USA), 2 mM sodium orthovanadate (Wako Chemicals, Kanagawa, Japan), and 5 mM EDTA). Lysates were subjected to SDS-PAGE and subsequent immunoblotting was performed. Following antibodies were used: ant- EGR1 and anti-beta actin (Santa Cruz, CA. U.S.A). Detection was performed using the ECL detection system (Amersham, Giles, UK).

uPA activity assay

uPA activity assay was performed with cell extracts according to the manufacturer's instructions, with absorption measured at 340 nm (Innovative Research, MI, U.S.A.). The assay measures only the active species of uPA, and a standard curve was generated using recombinant active uPA. The assay conditions were optimized so that the amount of tissue extract or cell extract added gave rise to uPA activity within the linear range of detection. Each reaction was performed in triplicate, and all experiments were repeated for three times.

Xenograft model of osteosarcoma

For subcutaneous xenograft models, 143B cells were suspended in 100 μL Matrigel (BD, NJ USA.). Cell suspensions were

subcutaneously inoculated in nude mice. Three weeks after inoculation, 4 μg/kg doxorubicin was administered by intraperitoneal injection. One day after treatment, mice were scarified and tumors were examined. For metastasis experiments, 143B cells (5×10^5) were transfected with GFP lentiviral particles (Santa Cruz, CA. U.S.A). Stably-GFP-expressing 143B cells (1×10^6) were mixed with a collagen gel in a 1:1 volume, and inoculated into the left knee joint of 6-weeks-old nude mice. Five weeks after inoculation, the mice were sacrificed. GFP-positive-143B cells were counted in 50 μl blood aspirates from hearts using the M165 FC microscope (Leica Microsystems, Wild Heerbrugg, Switzerland). Metastatic nodules in the lungs were evaluated by direct microscopic visualization using an M165 FC microscope. Lung metastasis area was calculated by Lumina Vision (Mitani Corporation, Tokyo, Japan). 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 (approval number: 20064). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize possible alternatives to in vivo techniques.

ELISA

Expression levels of uPA and uPAR proteins were assayed using specific enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Abnova, Taipei, Taiwan). Cell lysates were collected by EGR1 stable transfected osteosarcoma cells.

Statistics

Each sample was analyzed in triplicate, and experiments were repeated three times. In all figures, error bars are standard deviations. All statistical analyses were performed using Microsoft Office Excel (Microsoft, Albuquerque, New Mexico, USA) and STASTISCA (StatSoft, Tulsa, OK, USA). Differences between mean values were evaluated by the unpaired *t*-test, and differences in frequencies by Fisher's exact test. Differences were considered significant at $P<0.05$.

Results

EGR1 is down-regulated in osteosarcoma cell lines and patient' specimens

Real-time PCR was performed to examine the gene expression of *EGR1* in osteoblast and osteosarcoma cell lines including NHOst, 143B, Saos-2, HOS, MG63, and NOS-1. Real-time PCR revealed that the 5 of 5 osteosarcoma cell lines exhibited 0.002- to 0.369-fold decreased in expression of *EGR1* (**Figure 1A**). In addition, we performed real-time PCR using patient' biopsy specimens. Real-time PCR revealed that *EGR1* was decreased 0.01-to 0.2-fold in 8 of 10 human biopsy specimens (**Figure 1B**). These findings suggest that the *EGR1* is down-regulated in human osteosarcomas.

Anti-tumour agent treatment promoted up-regulation of *EGR1*

To examine the effects of anti-tumour agents on *EGR1* expression, we performed real-time PCR after anti-tumour agent treatment. We attempt to clarify the changes in EGR1 expression following low-dose anti-tumor agent treatment, and determined anti-tumor drug concentrations required to prevent osteosarcoma cell proliferation. MTT assay revealed that 250 ng/ml cisplatin, 1 ng/ml methotrexate, 50 ng/ml etoposide, or 10 ng/ml doxorubicin treatment did not prevent 143B cell growth. Growth of Saos-2 cells was not inhibited by 1 ng/ml methotrexate, 5 ng/ml methotrexate, 10 ng/ml methotrexate, 50 ng/ml etoposide, or 10 ng/ml doxorubicin. On the other hand, Growth of 143B cell and Saos-2 cell was inhibited by higher dose of each drug (**Figure 2 A, B**). Following 24 h treatment with these concentrations of anti-tumor drugs, *EGR1* was up-regulated (**Figure 3 A–D**). Following 48 h or 5 days treatment, cisplatin, methotrexate, etoposide or doxorubicin increased *EGR1* expression in 143B cell and Saos-2 cells (**Figure S1**). We next examined the expression of *EGR1* following chemotherapy in biopsy specimens. Specimens of OS6, OS8, and OS9 tumors were obtained during tumor resection in osteosarcoma patients who received chemotherapy. We compared the *EGR1* expressions in the biopsy specimens and the resected tumor specimens obtained from these patients. In 3 of 3 patient' specimens examined, *EGR1* expression was increased 7.87- to 1.71 following chemotherapy (**Figure S2A**). To examine the expression of *EGR1* following low-dose chemotherapy in vivo, we used a novel osteosarcoma murine xenograft model with 143B cells. We injected 4 μg/kg doxorubicin which is less than one-hundred dose of COSS-86 protocol for osteosarcoma patients. Real-time PCR showed that low dose doxorubicin treatment promoted up-regulation of *EGR1* in vivo (**Figure S2B**).

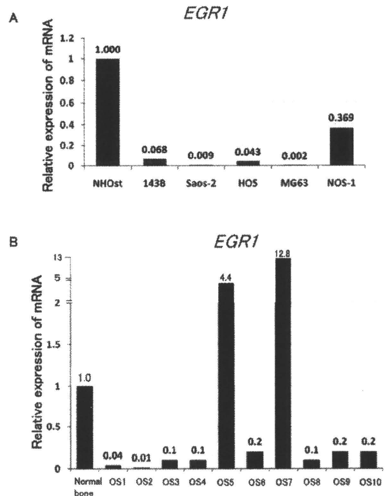

Figure 1. Down-regulation of *EGR1* in human osteosarcoma. Total RNA extracted from osteosarcoma cell lines (A) and osteosarcoma patients' biopsy specimens (B) were analyzed by real-time PCR. Results revealed that 5 of 5 human osteosarcoma cell lines and 8 of 10 human biopsy specimens of osteosarcoma had decreased *EGR1* expression. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH*. These experiments were performed in triplicate with similar results.
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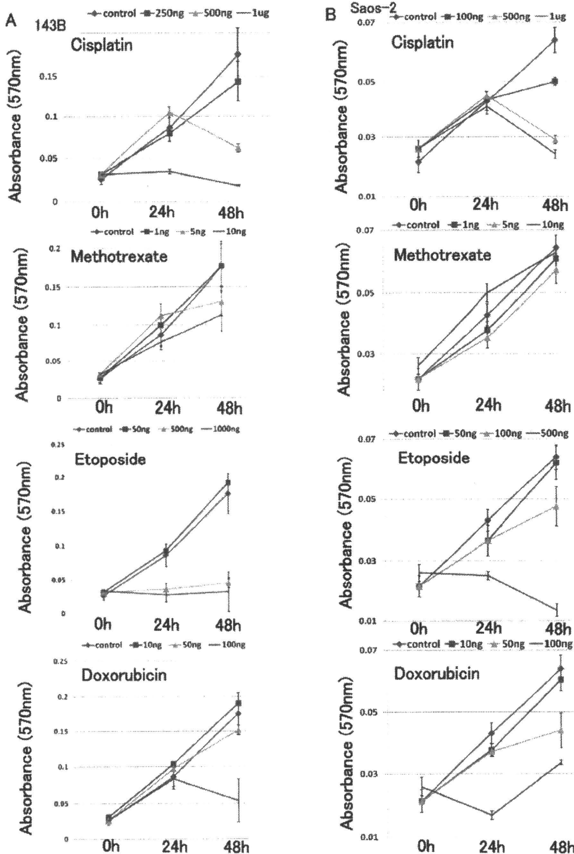

Figure 2. Osteosarcoma cell growth following anti-tumor drug treatment. MTT assay showed that growth at 48 h of 143B cells was not inhibited by 250 ng cisplatin, 1 ng/ml methotrexate, 50 ng/ml etoposide, or 10 ng/ml doxorubicin. Growth of 143B cell was inhibited by higher dose of each drug (*A*) ($P<0.05$). Growth at 48 h of Saos-2 cells was not inhibited by 1 ng/ml methotrexate, 5 ng/ml methotrexate, 10 ng/ml methotrexate, 50 ng/ml etoposide, or 10 ng/ml doxorubicin. Growth of Saos-2 cell was inhibited by higher dose of each drug (*B*) ($P<0.05$). The experiment was performed in triplicate with similar results [error bars represent mean (SD)].
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Over-expression of EGR1 does not prevent osteosarcoma growth

It has been reported that EGR1 over-expression suppresses the growth of cell in soft agar and tumor growth in nude mice [10,16]. We therefore, transfected the EGR1 expression vector and examined osteosarcoma cell growth. Western blot analysis showed up-regulation of EGR1 in 143B, Saos-2, and HOS cells (**Figure 4A**). MTT assay revealed that forced expression of EGR1 did not prevent osteosarcoma growth in vitro (**Figure S3A**). We next examined the effects of EGR1 on anchorage-independent osteosarcoma growth. Colony formation assay revealed that forced expression of EGR1 did not affect the

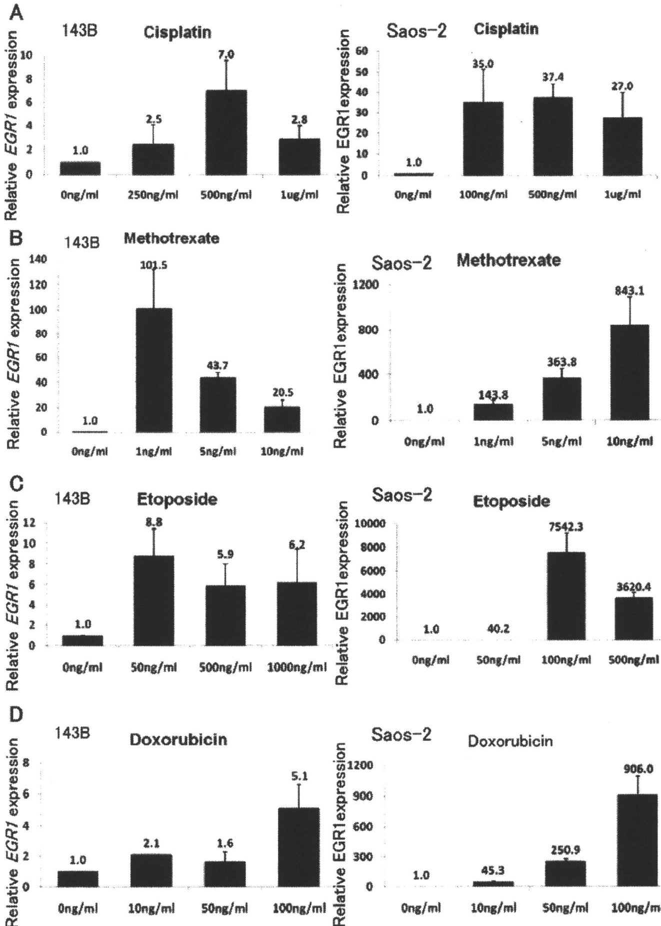

Figure 3. Anti-tumor agent treatment increased the expression of *EGR1*. Following 24 h drug treatments, total RNA extracted from osteosarcoma cell lines were analyzed by real-time PCR. Treatment with cisplatin, methotrexate, etoposide or doxorubicin increased *EGR1* expression in 143B and Saos-2 cells. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH* or *ACTB*. Experiments were performed in triplicate with similar results [error bars represent mean (SD)].
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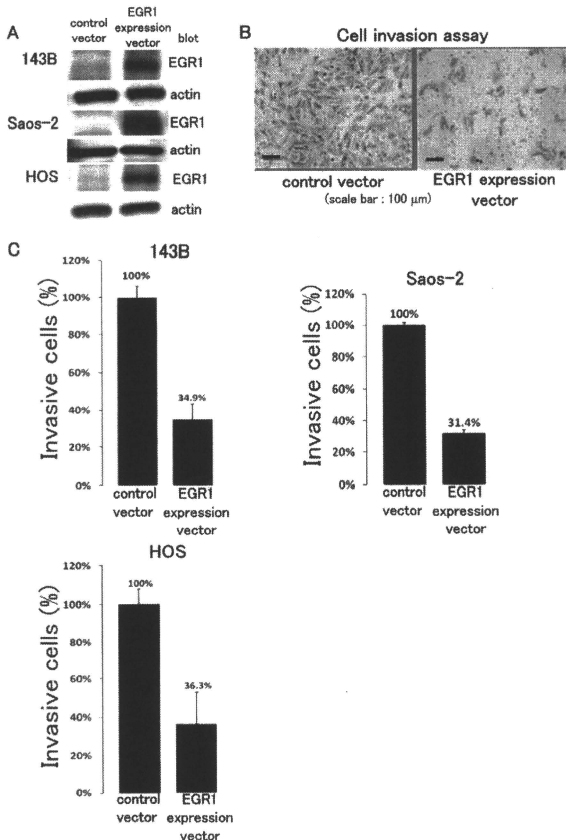

Figure 4. EGR1 prevents osteosarcoma cell invasion in vitro. Western blot analysis revealed that lysates of EGR1 expression vector-transfected cells were positive for anti-EGR1 antibody (*A*). Cell invasion assay showed that forced expression of EGR1 decreased 143B, Saos-2, and HOS cell invasion (*B*) ($P<0.05$). These experiments were performed in triplicate with similar results [error bars represent mean (SD)].
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number of colony formation (**Figure S3B**). These findings suggest that up-regulation of EGR1 following anti-tumor agent treatment had no effect on osteosarcoma cell growth.

Over-expression of EGR1 prevents osteosarcoma cell invasion in vitro

To examine the effects of EGR1 up-regulation after anti-tumour agent treatment in modulating the invasive activity of osteosarcoma cells, in vitro invasive activity assays were performed to assess the proportion of osteosarcoma cells transfected with EGR1 expression vector or control vector that invaded through matrigel-coated membranes. Significantly lower proportions of 143B, Saos-2, and HOS cells transiently transfected with EGR1 expression vector migrated through matrigel-coated chambers than osteosarcoma cells transfected with control vector (**Figure 4B, C**).

Down-regulation of uPA and uPAR by EGR1

We then examined the cellular mechanisms by which EGR1 exerts its effects on osteosarcoma cell invasion. Several investigations have shown that EGR1 plays an important role in the control of tumor metastasis through regulation of cancer invasion-related genes, including TGF-β1, thrombospondin-1, and plasminogen activator inhibitor-1 [17,18]. We examined whether EGR1 affects the expression of cancer invasion-related genes. Real-time PCR revealed that forced expression of EGR1 in 143B, Saos-2, and HOS osteosarcoma cell lines decreased the expression of *uPA* and *uPAR* (**Figure 5**). ELISA revealed that forced expression of EGR1 decreased the expression of uPA and uPAR proteins (**Figure S4**). Further, we examine the effects of anti-tumour agents on *uPA and uPAR* expression in vitro, we performed real-time PCR after anti-tumor agent treatment. Treatment of low dose anti-tumor drugs decreased the expression of *uPA* and *uPAR* (**Figure S5**). To examine the effects of low dose chemotherapy on *uPA and uPAR* expression in vivo, we used osteosarcoma murine xenograft model with 143B cells. Nude mice were treated with 4 μg/kg doxorubicin. Real-time PCR showed that low dose doxrubicin

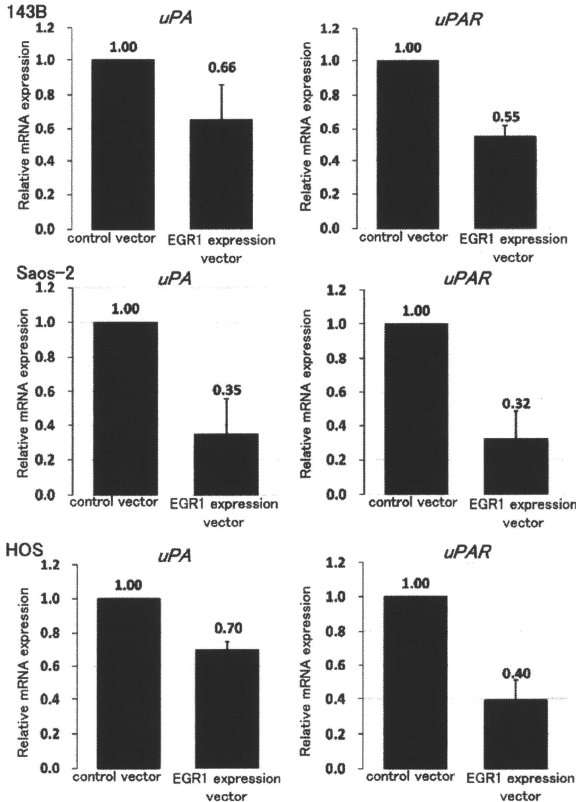

Figure 5. EGR1 decreased expression of *uPA* and *uPAR*. We examined whether EGR1 affects the expression of uPA and uPAR. RNA was prepared from control vector or EGR1 expression vector stably transfected cells. Real-time PCR revealed that forced expression of EGR1 decreased *uPA* and *uPAR* expression in 143B, Saos-2, and HOS cells ($P<0.05$). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH*. These experiments were performed in triplicate with similar results [error bars represent mean (SD)].
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treatment decreased expression of *uPA* and *uPAR* in vivo (**Figure S6A**).

EGR1 down-regulates uPA activity

uPA is produced and secreted as an inactive single-chain polypeptide, termed pro-uPA, which lacks plasminogen-activating activity. The binding of pro-uPA to uPAR induces its activation which in turn converts plasminogen to the active serine protease plasmin [19]. In this regard, we examined whether EGR1 exerts effects on uPA activity by performing uPA activity ELISA. ELISA showed that forced expression of EGR1 in osteosarcoma cell lines down-regulated the activity of uPA (**Figure 6**).

EGR1 suppresses osteosarcoma migration into blood vessels in vivo

To investigate the effects of EGR1 on osteosarcoma tumor migration and invasion in vivo, we used a novel osteosarcoma murine xenograft model with 143B cells. Intrajoint inoculation of GFP-positive 143B cells in nude mice induced primary osteosarcoma tumor formation by 2 weeks after inoculation. These primary tumors gave rise to microscopically detectible micro metastases in the lungs within 5 weeks after inoculation. Although we attempted to determine the volume of the primary tumors, we were unable to do so because tumor had extended into muscle and bone. RNA was prepared from tumor formed by control vector or EGR1 expression vector transfected cells. Real-time PCR revealed that forced expression of EGR1 decreased *uPA* and *uPAR* expression in vivo (**Figure S6B**). After 5 weeks, we counted GFP-positive- 143B cells within 50 µl blood aspirates from hearts. The vector control group had an average of 51.2 cells, whereas the EGR1 group averaged only 18.7 cells (**Figure 7A, B**). Lung metastases were found in 6 of 6 control cell-inoculated mice. In contrast, there were lung metastases in 4 of 6 EGR1-expressing 143B-inoculated mice. The percent of lung metastasis area was calculated. The vector control group had an average of 0.6% metastasis area, whereas the EGR1 group averaged 0.31% metastasis area (**Figure 7C**). These findings show that EGR1 prevented osteosarcoma migration into blood vessel in vivo.

Discussion

Current standard regimens for osteosarcoma treatment include preoperative and postoperative chemotherapy. The benefits of chemotherapy have been demonstrated in many studies. Preoperative chemotherapy induces tumor necrosis in the primary tumor facilitating surgical resection and enabling early treatment of micrometastatic disease [20,21]. Among those patients who received neoadjuvant treatment, chemotherapy-related tumor necrosis was good in 62% and poor in 38% of patients [22].

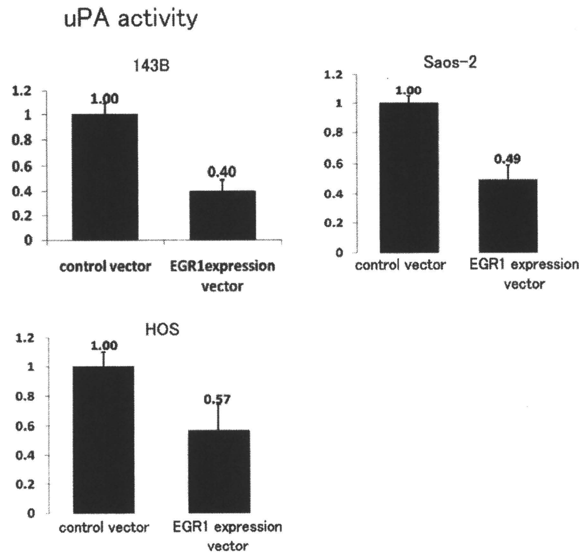

Figure 6. EGR1 decreased uPA activity. uPA activity was examined using cell lysates. Cell lysate were prepared from control vector or EGR1 expression vector transfected cells. ELISA assay showed that EGR1 decreased uPA activity 0.40-fold in 143B (A). uPA activity was decreased 0.49-fold by EGR1 in Saos-2 (B). Luciferase assay showed that EGR1 decreased uPA activity 0.57-fold in HOS (C). These experiments were in triplicate with similar results [error bars represent mean (SD)] ($P<0.05$).
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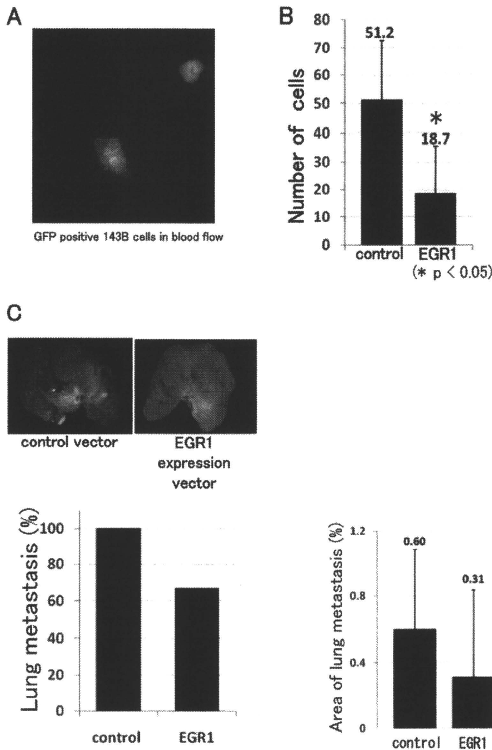

Figure 7. EGR1 prevents osteosarcoma cell migration into blood vessel in vivo. GFP expression virus-transfected 143B cells were inoculated into the knee joint. To examine tumour cell invasion of blood vessels, we counted GFP- positive- 143B cells within 50 µl blood aspirates from hearts at 5 weeks after inoculation (A). The number of GFP- positive cells in blood was decreased in EGR1 -expressing 143B- inoculated mice (B) [error bars represent mean (SD)] ($P<0.05$). Metastatic nodules in lungs were evaluated under fluorescence microscopy. Six of 6 control cell-inoculated mice exhibited lung metastases. Four of six (66.7%) EGR1-expressing cell-inoculated mice exhibited lung metastases. The percent of lung metastasis area was calculated by Lumina Vision (C).
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These findings showed that one-third of osteosarcoma patients are even poor responders to chemotherapy. Even though chemotherapy is quite benefit for osteosarcoma patients, administration of preoperative chemotherapy results in delay of surgical resection of primary tumor. It is possible that new lung metastases will develop during preoperative chemotherapy in poor or non-responders. We showed that low-dose anti-tumor agent treatment up-regulated EGR1 expression and that EGR1 prevented osteosarcoma invasion via uPA/uPAR down-regulation. These findings suggest that preoperative chemotherapy prevents the development of new lung metastases in poor or non-responders. In addition, osteosarcoma incidence rates in the United States peak in adolescence and in the elderly [23]. Many elderly patients cannot tolerate aggressive chemotherapy. Our findings suggest that low-dose chemotherapy might be useful for elderly osteosarcoma patients by preventing new metastasis when used in combination with radiation therapy or as maintenance therapy.

EGR1 has received much attention recently because of its wide range of activities as a transcription factor. Remarkably, EGR1 can exert effects as either a growth promoter or a tumor suppressor. EGR1 may induce or suppress cell proliferation or induce apoptosis of cancer cells [10,16,24–27]. Our MTT assay

and colony formation assay showed that EGR1 over-expression had no effect on osteosarcoma cell growth. Our findings suggest that the expression of EGR does not have general effects on growth and instead exerts regulatory effects that appear to be cell-type-specific.

We showed up-regulation of EGR1 following cisplatin, methotrexate, etoposide, or doxorubicin treatment, each of which exerts cytotoxic effects by different pharmacological mechanisms. EGR1 can be rapidly induced by many stimuli, including growth factors, cytokines, ultraviolet light, anti-tumour agents, and various stresses [8,10,24, Cao, 1992 #88,25,28–35]. The distinct types of stress caused by anti-tumor drugs might promote up-regulation of EGR1, although anti-tumor drugs exert different pathways. Further, we examined which signaling pathway promotes EGR1 expression following anti-tumor agent treatment. We treated osteosarcoma cell lines with anti-tumor agent and some specific inhibitors including ERK inhibitor, HIF1-α inhibitor, JAK2 inhibitor, LY294002, and others but we were unable to inhibit EGR1 expression effectively. Further examination for regulation mechanisms of EGR1 expression is needed.

The principle mode of action of doxorubicin, an anthracycline antibiotic, appears to be its ability to cross-link DNA and RNA, thereby affecting DNA and RNA synthesis [36,37]. However, recent studies have demonstrated that genotoxic (*i.e.*, DNA damaging) agents, including many important cancer chemotherapy drugs, can have significant and selective effects on the expression of certain inducible genes [38]. It has also been demonstrated that noncytotoxic doses of the DNA cross-linking cancer chemotherapy drugs MMC, cisplatin, and carboplatin were effective at significantly altering the expression of the *MDR1* gene coding for the multidrug resistance protein P-glycoprotein [37]. We were therefore interested in whether chemotherapy agents might similarly alter the expression of inducible invasion-related genes, and thereby potentially alter tumor invasiveness, and found that anti-tumour agents increased the expression of EGR1, and EGR1 decreased that of uPA and uPAR.

The uPA system is thought to play roles in several different processes important to tumor progression including angiogenesis, tumor growth, and metastasis [39]. Expression of uPA and uPAR frequently indicates a poor prognosis, and is in some cases predictive of invasion and metastasis. uPAR is also thought to play roles in the growth and metastasis of human osteosarcoma [40–44]. We showed that forced expression of EGR1 inhibited expression of uPA and uPAR. In addition, EGR1 decreased the activity of uPA. These findings suggest that up-regulation of EGR1 following chemotherapy inhibits osteosarcoma migration via uPA system. Many signaling pathways activate transcription factors that act on the uPAR promoter, driving uPAR expression in cancer [45]. uPAR transcription is controlled by ERK through activator protein 1 transcription factors [46]. Hypoxia-inducible factor 1α drive uPAR expression through a hypoxia responsive element in the uPAR promoter [47]. Nuclear factor-κB also activates uPAR expression [48]. Thus, multiple signaling inputs can up-regulate uPAR transcription in tumors. We could not detect the pathways that promote down-regulation of uPA/uPAR. Further examination for regulation mechanisms of uPA/uPAR system is needed.

Recently, many molecular target drugs have been developed [49–52]. In addition, several Notch signal inhibitors have been tested as molecular target drugs [53–55]. We previously reported that activation of Notch signaling promotes the progression of human osteosarcoma [56]. We examined the EGR1 expression by γ-secretase inhibitor, a pharmacological agent known to effectively block Notch activation. EGR1 was up-regulated by γ-secretase inhibitor in human osteosarcoma cell lines (data not shown). These findings suggest that EGR1 expression will also be up-regulated by molecular target drugs.

In summary, anti-tumor agents increased the expression of EGR1, and EGR1 decreased osteosarcoma invasion. Our findings suggest that even though chemotherapy could not prevent osteosarcoma growth in chemotherapy poor responders, chemotherapy prevents osteosarcoma cell migration into blood vessel by down-regulation of urokinase plasminogen activation via up-regulation of EGR1 during chemotherapy periods.

Supporting Information

Figure S1 Anti-tumor agent treatment increased the expression of *EGR1*. Following 48 h or 5 days drug treatments, total RNA extracted from osteosarcoma cell lines were analyzed by real-time PCR. Following 48 h treatment, cisplatin, methotrexate, etoposide or doxorubicin increased *EGR1* expression in 143B cell and Saos-2 cells. Following 5 days treatment, cisplatin increased *EGR1* expression in 143B cell. Following 5 days treatment, etoposide or doxorubicin increased *EGR1* expression in Saos-2 cells. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH* or *ACTB*. Experiments were performed in triplicate with similar results [error bars represent mean (SD)].
(TIF)

Figure S2 Chemotherapy increased *EGR1* expression. Total RNA extracted from osteosarcoma patients' biopsy specimens and excised tumors following chemotherapy were used for real-time PCR. Real-time PCR revealed that 3 of 3 excised specimens of osteosarcoma increased *EGR1* expression 7.87- to 1.73-fold (A). One day after 4 μg doxorubicin treatment, RNA was extracted from tumor in nude mice xenograft models. Real-time PCR revealed that low dose chemotherapy increased *EGR1* expression in vivo (B) ($P<0.05$). The comparative Ct (ΔΔCt) method was used to determine fold change in expression. These experiments were performed in triplicate with similar results [error bars represent mean (SD)].
(TIF)

Figure S3 Forced expression of EGR1 does not affect osteosarcoma cell growth in vitro. We transfected control vector or EGR1 expression vector, and examined osteosarcoma cell growth. MTT assay revealed that growth of viable 143B, Saos-2, and HOS cells over 8 days was not affected by forced expression of EGR1 (A). These experiments were performed in triplicate with similar results [error bars represent mean (SD)]. Colony formation assay revealed that forced expression of EGR1 did not affect the number of colonies in soft agar (B). These experiments were performed in triplicate with similar results [error bars represent mean (SD)].
(TIF)

Figure S4 Forced expression of EGR1 decreased the expression of uPA and uPAR. Cell lysate were prepared from control vector or EGR1 expression vector stably transfected cells. ELISA assay showed that forced expression of EGR1 decreased the expression of uPA and uPAR proteins in 143B ($P<0.05$) (A). The expression of uPA and uPAR decreased in Saos-2 and HOS ($P<0.05$) (B, C). These experiments were in triplicate with similar results [error bars represent mean (SD)].
(TIF)

Figure S5 Low dose anti-tumor agent treatment decreased the expression of *uPA* and *uPAR*. Following 24 h drug treatments, total RNA extracted from osteosarcoma cell lines were analyzed by real-time PCR. Treatment with cisplatin, methotrexate, etoposide or doxorubicin decreased *uPA and uPAR* expression in 143B and Saos-2 cells ($P<0.05$). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using *GAPDH* or *ACTB*. Experiments were performed in triplicate with similar results [error bars represent mean (SD)].
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Figure S6 Chemotherapy prevents expression of *uPA* and *uPAR* by down-regulation of EGR1. Twenty four hours after 4 µg doxorubicin treatment, RNA was extracted from tumour in nude mice xenograft model. Real-time PCR revealed that chemotherapy decreased *uPA* and *uPAR* expression in vivo (A) ($P<0.05$). To examined whether EGR1 affects the expression of uPA and uPAR in vivo. RNA was prepared from tumor formed by control vector or EGR1 expression vector transfected cells. Real-time PCR revealed that forced expression of EGR1 decreased *uPA* and uPAR expression in vivo (B) ($P<0.05$). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using GAPDH. These experiments were performed in triplicate with similar results [error bars represent mean (SD)].
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Acknowledgments

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Author Contributions

Conceived and designed the experiments: KI SN SK TS. Performed the experiments: YM YI TY HN. Analyzed the data: YM SK TS. Contributed reagents/materials/analysis tools: YM HN. Wrote the paper: TS.

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ORIGINAL PAPER

Role of GLI2 in the growth of human osteosarcoma

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Abstract

The Hedgehog pathway functions as an organizer in embryonic development. Aberrant activation of the Hedgehog pathway has been reported in various types of malignant tumours. The GLI2 transcription factor is a key mediator of Hedgehog pathway but its contribution to neoplasia is poorly understood. To establish the role of GLI2 in osteosarcoma, we examined its expression by real-time PCR using biopsy tissues. To examine the function of GLI2, we evaluated the growth of osteosarcoma cells and their cell cycle after *GLI2* knockdown. To study the effect of GLI2 activation, we examined mesenchymal stem cell growth and the cell cycle after forced expression of GLI2. We found that *GLI2* was aberrantly over-expressed in human osteosarcoma biopsy specimens. *GLI2* knockdown by RNA interferences prevented osteosarcoma growth and anchorage-independent growth. Knockdown of *GLI2* promoted the arrest of osteosarcoma cells in G_1 phase and was accompanied by reduced protein expression of the cell cycle accelerators cyclin D1, SKP2 and phosphorylated Rb. On the other hand, knockdown of *GLI2* increased the expression of $p21^{cip1}$. In addition, over-expression of GLI2 promoted mesenchymal stem cell proliferation and accelerated their cell cycle progression. Finally, evaluation of mouse xenograft models showed that *GLI2* knockdown inhibited the growth of osteosarcoma in nude mice. Our findings suggest that inhibition of GLI2 may represent an effective therapeutic approach for patients with osteosarcoma.
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No conflicts of interest were declared.

Introduction

Osteosarcoma is a highly malignant bone tumour and is the most commonly encountered malignant bone tumour in children and adolescents [1,2]. Furthermore, a large number of patients with osteosarcoma eventually develop pulmonary metastases and die, despite conventional multi-agent chemotherapy and surgical excision of the tumour mass [3]. The survival rate of patients treated with intensive multidrug chemotherapy and aggressive local control interventions has been reported to be 60–80% [4,5]. In patients with a high-grade osteosarcoma, the clinical detection of a metastatic disease at first diagnosis is predictive of a poor outcome, with long-term survival rates in the range 10–40% [6]. It has been reported that aberrant activation of cell cycle progression affects the pathogenesis of osteosarcoma [7]. Although inactivation of the deregulated cell cycle seems promising, the molecular mechanisms of osteosarcoma cell growth remain unclear.

Hedgehog–GLI signalling is involved in various steps of development and is induced via the Patched (PTCH1) and Smoothened (SMO) Hedgehog receptors. Activated SMOs promote the translocation of GLI zinc-finger transcription factors into the nucleus [8,9]. In mammals, three transcription factors, viz GLI1, GLI2 and GLI3, activate the transcription of Hedgehog target genes [10,11]. The transcription induced by Gli2 is crucial for development, because *Gli2* knockout mice die prenatally and show defects of the central nervous system [12]. Aberrant activation of Hedgehog pathway is associated with malignant tumours (reviewed in [13]). Our findings indicate that GLI2 is actively involved in the patho-aetiology of osteosarcoma, because suppression of GLI2 inhibits osteosarcoma growth via cell cycle regulation.

Materials and methods

Cell culture

The osteosarcoma cell lines 143B, Saos-2 and HOS were purchased from the American Type Culture Collection (ATCC; Manassas, USA). Osteosarcoma cells

were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). The human hTERT-immortalized mesenchymal stem cell line (YKNK-12) was kindly provided by Dr Kobayashi (Okayama University) [14]. YKNK-12 cells were grown in the culture medium described above. Normal human osteoblast cells (NHOsts; Sanko Junyaku, Tokyo, Japan) were grown in OBM™ medium (Cambrex, East Rutherford, NJ, USA). All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

Biopsy samples

Human osteosarcoma biopsy tissues were collected from primary lesions before any diagnostic or therapeutic treatment. Control specimens were collected from the femoral bone of patients undergoing total hip arthroplasty. The study protocol was approved by the Review Board of Graduate School of Kagoshima University. Written informed consent was obtained from all patients.

Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell proliferation, as previously described [15]. Briefly, cells cultured on microplates were incubated with the MTT substrate for 4 h, and subsequently lysed. The developed optical density of the compound was then analysed using a microplate reader (Bio-Rad, Hercules, CA, USA). GANT61 was obtained from Alexis Biochemicals (CA, USA). The pCS2–MT GLI2ΔN plasmid was provided by Addgene (MA, USA). *GLI2* siRNA was purchased from Santa Cruz Biotechnology (CA, USA). An shRNA plasmid for human *GLI2* was purchased from SA Biosciences (MD, USA). *GLI2* and control shRNAs were cloned into the pGeneClip™ neomycin-resistant vector, which is under the control of the U1 promoter. Transfection of the plasmid was performed according to the supplier's recommendations, using FuGENE6 (Roche, Basel, Switzerland).

Soft agar assay

Cells were suspended in DMEM containing 0.33% soft agar and 5% FBS, and then plated on a 0.5% soft agar layer. The cells were cultured at a density of 5×10^3 cells/well in six-well plates. Fourteen days later, the number of colonies was counted. Every experiment was performed in triplicate, and all experiments were performed three times.

Real-time quantitative PCR assay

Real-time quantitative PCR assay was performed as previously described [16]. Each primer set used amplified a 150–200 bp amplicon. The miR-Vana RNA isolation kit or TRIzol (Invitrogen, Carlsbad, CA, USA) were used for total RNA purification. PCR was performed using SYBR Green as the dye for quantification (Bio-Rad) and analysed using MiniOpticon™ (Bio-Rad). The comparative Ct ($^{\Delta\Delta}$ Ct) analysis method was used to evaluate the fold change of mRNA expression, using the expression of *GAPDH* or *ACTB* as a reference. All PCR reactions were performed in triplicate. All primers were designed using Primer3 software. The following primers were used: *PTCH1*: 5′-TAACGCTGCAACAACTCAGG-3′, 5′-GAAGGCTGTGACATTGCTGA-3′; *SMO*: 5′-GGG AGGCTACTTCCTCATCC-3′, 5′-GGCAGCTGAAGG TAATGAGC-3′; *GLI2*: 5′-CGACACCAGGAAGGAA GGTA-3′, 5′-AGAACGGAGGTAGTGCTCCA-3′; *cyclin D1*: 5′-ACAAACAGATCATCCGCAAACAC-3′, 5′-TGTTGGGGCTCCTCAGGTTC-3′; *SKP2*: 5′-TGG GAATCTTTTCCTGTCTG-3′, 5′-GAACACTGAGAC AGTATGCC-3′; *GAPDH*: 5′-GAAGGTGAAGGTCG GAGTC-3′, 5′-GAAGATGGTGATGGGATTTC-3′; *ACTB*: 5′-AGAAAATCTGGCACCACACC-3′, 5′-AG AGGCGTACAGGGATAGCA-3′.

Western blotting

Cells were lysed using NP40 buffer including 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, Kanagawa, Japan) and 5 mM EDTA. SDS–PAGE and immunoblotting were subsequently performed and the following antibodies used: GLI2, cyclin D1, p21, SKP2, pRb and actin (Santa Cruz). The ECL reagent was used and chemiluminescence detected (Amersham, Giles, UK).

Plasmid construction

A fragment containing the GLI2ΔN region was obtained from the pCS2-MT GLI2ΔN plasmid (Addgene) and subcloned into the pcDNA3 plasmid.

Luciferase assay

8 × 3′Gli-BS–δ51LucII (GLI–Luc) and 8 × m3′Gli-BS–δ51LucII (mutant-Luc) reporter genes were kindly provided by Dr Sasaki H. [17,18]. Luciferase assays were carried out as described previously [19]. In brief, cells (1.5×10^4 cells/well) were transfected with 400 ng/well of firefly luciferase expression vectors and 1 ng/well internal control vector, pGL4.74 (Promega, Madison, WI, USA) using the FuGENE6 followed by the incubation for 24 h. Recombinant Sonic Hedgehog (R&D Systems, Minneapolis, MO, USA) was added to the well and after 24 h the activities of luciferase were measured, using the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's instructions.

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Animal model

Xenograft experiments were performed as previously described [20]. Briefly, control or *GLI2* shRNA-transfected 143B cells (1×10^6) were suspended in 100 µl Matrigel (BD, NJ, USA). Cell suspensions were subcutaneously inoculated in 5 week-old nude mice. Tumour size was calculated weekly, using the formula $LW^2/2$ (where L and W represent the length and width of the tumour). Mice were randomly treated with GANT61 (50 mg/kg) or an equal volume of DMSO as control. GANT61 or DMSO was also injected subcutaneously. Injection of GANT61 started 1 week after inoculation of 143B cells. Treatments with GANT61 or DMSO were performed every other day. All animal experiments were performed in compliance with the guidelines of the Institute of Laboratory Animal Sciences, Graduate School of Medical and Dental Sciences, Kagoshima University. Every effort was employed to minimize the number of animals used and animal pain.

Cell cycle analysis

Cells were harvested with trypsin–EDTA. The cells were rinsed with PBS, fixed with 70% ethanol for 2 hr at 4 °C, washed three times with cold PBS and resuspended with 500 µL staining buffer containing PBS, pH 7.4, RNase A and 50 µg/ml propidium iodide (Wako). DNA content was examined by flow cytometry, using FACS Vantage SE (Becton-Dickinson, Franklin Lakes, NJ, USA) or CyAn™ ADP (Beckman Coulter, CA, USA) with FlowJo software (Tree Star) and Summit software (Beckman Coulter), respectively.

Statistical analysis

All experiments were performed three times unkess otherwise stated, and samples were analysed in triplicate. Results are presented as mean (SD). The statistical difference between groups was assessed by applying Student's t-test for unpaired data, using Microsoft Office Excel (Microsoft, Albuquerque, NM, USA) and Statistica (StatSoft, Tulsa, OK, USA).

Results

Activation of Hedgehog pathway in human osteosarcoma

We previously reported that several genes of the Hedgehog pathway were increased in five osteosarcoma cell lines and nine osteosarcoma biopsy specimens [21]. In the present study, we examined the expression of *SMO, PTCH1* and *GLI2* in three additional osteosarcoma biopsy tissues. We found that *SMO* expression was up-regulated in all osteosarcoma patient tissues, from 7.3- to 183-fold (Figure 1A). Similarly, the expression of *PTCH1* and *GLI2* was up-regulated in all three biopsy samples, from 8.6- to 72.3-fold and from 1.6- to 45.6-fold, respectively (Figure 1A). In agreement with these results, recombinant Sonic Hedgehog activates a reporter gene carrying 8 × 3′ Gli-BS-δ51LucII (GLI–Luc) in 143B and Saos-2 human osteosarcoma cells (Figure 1B) [17,18]. These findings corroborate our previous findings and indicate that the Hedgehog pathway is active in human osteosarcoma [21].

Inhibition of GLI prevents osteosarcoma cell proliferation

To examine the effects of GLI inhibition, we used GANT61, a pharmacological agent known to effectively block GLI transcription [22]. The MTT assay revealed that GANT61 dose-dependently inhibited the proliferation of 143B, Saos-2 and HOS cells (Figure 2A–C). In contrast, the same concentration of GANT61 did not affect the proliferation of normal human osteoblasts (NHOst) (Figure 2D). These findings suggest that inhibition of GLI prevents osteosarcoma proliferation *in vitro*.

Knockdown of *GLI2* prevents osteosarcoma proliferation *in vitro*

In order to evaluate the function of GLI in osteosarcoma, we knocked down GLI expression by using siRNA; scrambled siRNA was used as a negative control. MTT assay revealed that knockdown of *GLI1* did not affect the osteosarcoma cell proliferation (data not shown). On the other hand, MTT assay showed that knockdown of *GLI2* inhibited the proliferation of 143B and Saos-2 cells (Figure 3B). To rule out the possibility of an artifact due to off-target effects, we transfected the cells with two other shRNA sequences and obtained results comparable to those observed with *GLI2* siRNA (data not shown). We next examined the effects of *GLI2* knockdown on anchorage-independent osteosarcoma growth. The colony formation assay revealed that knockdown of *GLI2* reduced the number of colonies formed in soft agar (Figure 3C). These findings revealed that *GLI2* knockdown inhibits osteosarcoma growth.

GLI2 knockdown prevents cell cycle progression of human osteosarcoma cells

We next examined the role of GLI2 in the regulation of cell cycle. In 143B cells, following *GLI2* knockdown by *GLI2* shRNA, the proportion of cells in G_1 phase increased from 74.2% to 80.4% (Figure 4A). In Saos-2 cells, following *GLI2* knockdown by *GLI2* shRNA, the proportion of cells in G_1 phase increased from 60.5% to 68.7% (see Supporting information, Figure S1A), indicating that knockdown of *GLI2* promoted cell cycle arrest. We further examined the expression of cell cycle-related genes. Real-time PCR revealed that knockdown of *GLI2* decreased the expression of cell cycle accelerators, such as *cyclin D1* and

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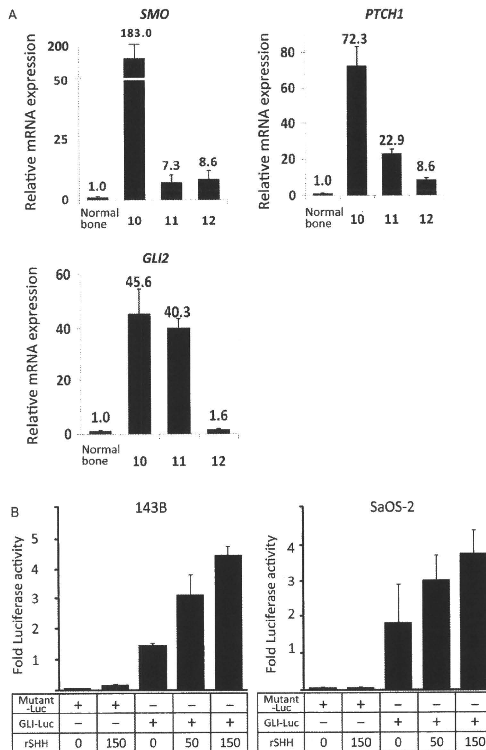

Mutant -Luc	+	+	−	−	−
GLI-Luc	−	−	+	+	+
rSHH	0	150	0	50	150

Mutant -Luc	+	+	−	−	−
GLI-Luc	−	−	+	+	+
rSHH	0	150	0	50	150

Figure 1. Activation of the Hedgehog pathway in human osteosarcoma. (A) Total RNA obtained from osteosarcoma biopsy tissues was examined by real-time quantitative PCR. Comparative Ct ($^{\Delta\Delta}$ Ct) analysis was performed to evaluate fold changes of mRNA expression using *GAPDH* or *ACTB*. All three human osteosarcoma biopsy specimens showed increased expression of *SMO* (7.3–183.0-fold), *PTCH1* (8.6–72.3-fold) and *GLI2* (1.6–45.6-fold). (B) 143B and Saos-2 cells were co-transfected with $8 \times 3'$ Gli-BS-δ51LucII (GLI-Luc), $8 \times$ m3′ GLI-BS-δ51LucII (mutant-Luc) and internal control luciferase vector. The cells were treated with recombinant sonic hedgehog (rSHH). The luciferase activity was analysed after 24 h transfection and normalized to internal control luciferase activity. Values represent mean ± SD ($n = 3$).

SKP2 (Figure 4B). In mammals, cell cycle regulators are short-lived proteins that are regulated by protein degradation. Western blot analysis further confirmed that knockdown of *GLI2* decreased the protein levels of cyclin D1, pRb and SKP2 (Figure 4C). We next examined the expression of p21^{cip1}, a negative regulator of cell cycle progression. Western blot analysis revealed that p21^{cip1} was up-regulated following knockdown of *GLI2* (Figure 4C). Taken together, these findings indicate that knockdown of *GLI2* promoted cell cycle arrest in G_1 phase by inhibiting the progression of the cycle from G_1 to S phase.

Over-expression of GLI2 accelerates mesenchymal stem cell proliferation

To examine the role of GLI2 in the pathogenesis of osteosarcoma, we over-expressed GLI2. Although the origin of osteosarcoma is still controversial, it is believed that it originates from osteoblasts or mesenchymal stem cells [23]. In this regard, we studied the effects of GLI2 over-expression in the immortalized human mesenchymal stem cell line (YKNK-12) [14]. We assessed the proliferation of YKNK-12 cells following transfection with the GLI2ΔN expression vector, which exhibits potent transcriptional activity *in vivo* [24]. The MTT assay showed that forced

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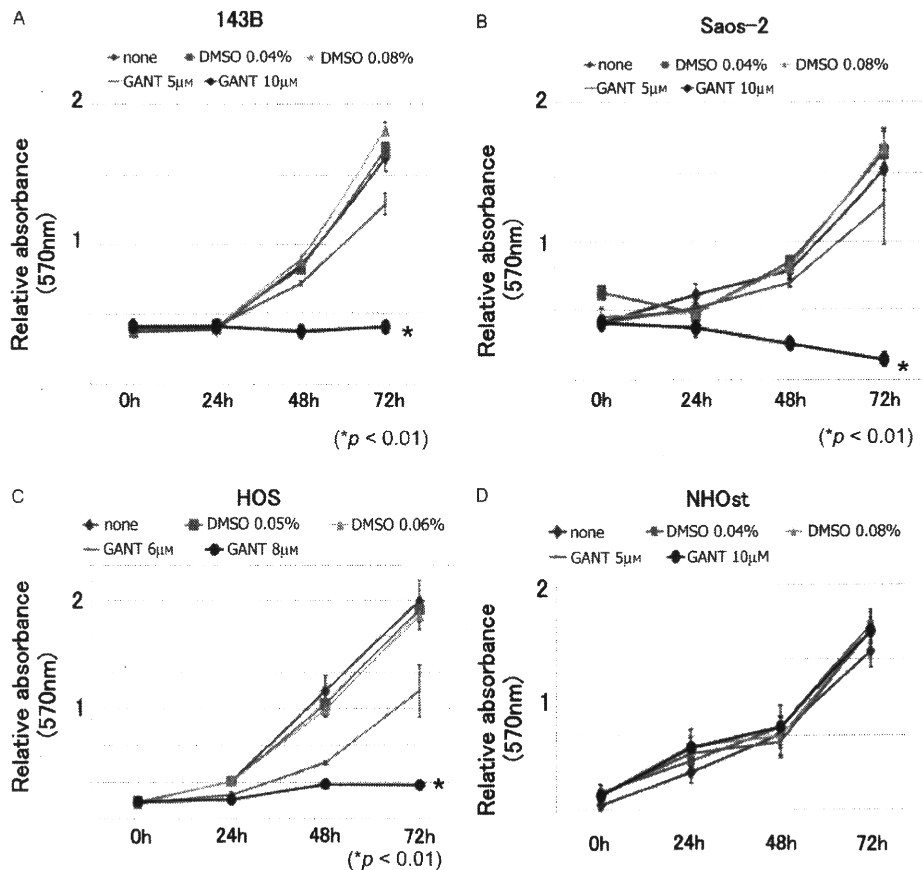

Figure 2. Inhibition of GLI prevents osteosarcoma cells proliferation. (A–C) GANT61 dose-dependently inhibited the growth of 143B, Saos-2 and HOS cells at 72 h (* $p < 0.01$). (D) At the same time point, GANT61 did not affect the growth of normal osteoblast cells (NHOst) ($n > 3$; error bars indicate SD).

expression of GLI2ΔN promoted YKNK-12 proliferation to a greater extent than transfection with control vector (Figure 5B). These findings suggest that GLI2 promotes mesenchymal stem cell proliferation. We also examined the role of GLI2 in regulating cell cycle in mesenchymal stem cells. Following forced expression of GLI2ΔN, 62.9% of the cells were in G_1 phase, 12.5% were in S phase and 22.9% were in the G_2–M phase, whereas 72.0%, 9.8% and 17.0% of cells were in G_1, S and G_2–M phases, respectively, following transfection with the control vector (Figure 5C). These findings suggest that GLI2 accelerates cell cycle progression of mesenchymal stem cells.

GLI2 knockdown inhibits osteosarcoma growth in nude mice

To confirm the role of *GLI2* knockdown in osteosarcoma growth, we tested the effects of *GLI2* knockdown in nude mice. Inoculation of 143B cells, previously transfected with *GLI2* shRNA, resulted in a statistically significant reduction of tumour growth as compared with inoculation of 143B cells transfected with control shRNA (Figure 6A). Kaplan–Meier analysis revealed that knockdown of *GLI2* in 143B cells provided a statistically significant survival benefit in mice (Figure 6B). These findings show that *GLI2* knockdown inhibits osteosarcoma cell growth in nude mice.

Discussion

Our findings demonstrate that GLI2 transcription factor significantly contributes to the growth of osteosarcoma cells. Our findings thus suggest that GLI2 might be an attractive target for therapeutic intervention, particularly in patients with high-grade and/or metastatic osteosarcoma. Small-molecule inhibitors of GLI transcription factors, such as GANT61, that efficiently inhibit the proliferation of prostate cancer cells have recently been identified [22]. MTT assay showed that GANT61 effectively inhibited osteosarcoma cell proliferation *in vitro*. We used 50 mg/kg GANT61 to inhibit GLI in a mouse xenograft model as previously described [22]. All injections were performed at a distance of 2–3 cm from the tumour site. We found no differences in osteosarcoma growth between the GANT61- and the control DMSO-treated groups (see Supporting information, Figure S1B). One possible explanation for this discrepancy is given by the difference in cell viability or permeation of GANT61

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