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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.

Keywords: osteosarcoma; Hedgehog; GLI2; cell cycle

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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′-GGGAGGCTACTTCCTCATCC-3′, 5′-GGCAGCTGAAGGTAATGAGC-3′; *GLI2*: 5′-CGACACCAGGAAGGAAGGTA-3′, 5′-AGAACGGAGGTAGTGCTCCA-3′; *cyclin D1*: 5′-ACAAACAGATCATCCGCAAACAC-3′, 5′-TGTTGGGGCTCCTCAGGTTC-3′; *SKP2*: 5′-TGGGAATCTTTTCCTGTCTG-3′, 5′-GAACACTGAGACAGTATGCC-3′; *GAPDH*: 5′-GAAGGTGAAGGTCGGAGTC-3′, 5′-GAAGATGGTGATGGGATTTC-3′; *ACTB*: 5′-AGAAAATCTGGCACCACACC-3′, 5′-AGAGGCGTACAGGGATAGCA-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.

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 $8 \times 3'$ 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

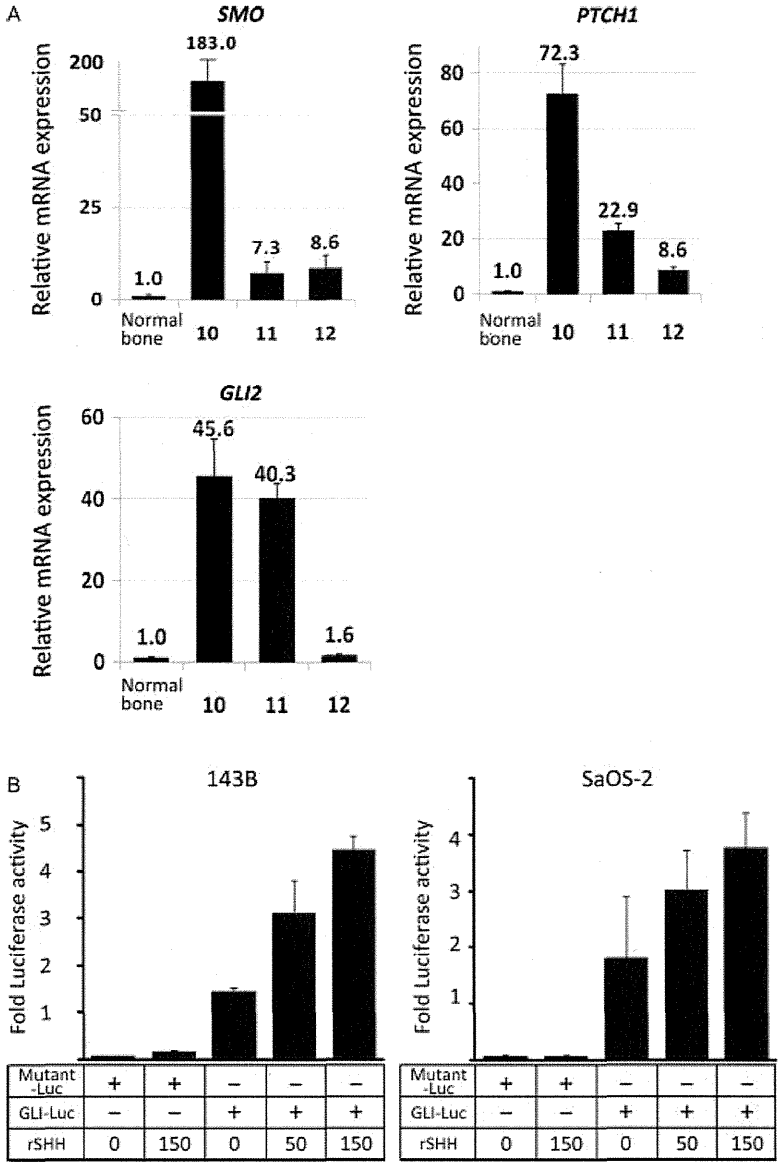


Figure 1. Activation of the Hedgehog pathway in human osteosarcoma. (A) Total RNA obtained from osteosarcoma biopsy tissues was examined by real-time quantitative PCR. Comparative Ct ($^{\Delta\Delta}$ Ct) analysis was performed to evaluate fold changes of mRNA expression using *GAPDH* or *ACTB*. All 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 × 3′ Gli-BS–δ51LucII (GLI–Luc), 8 × 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

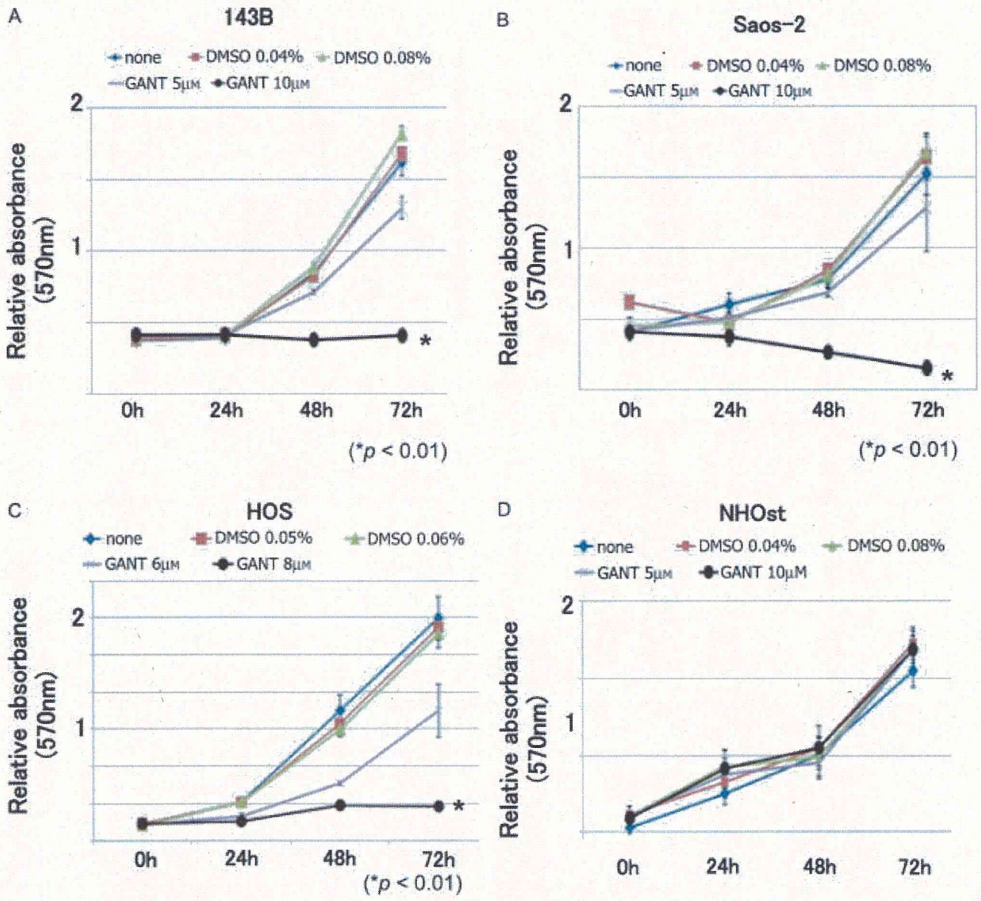


Figure 2. Inhibition of GLI prevents osteosarcoma cells proliferation. (A–C) GANT61 dose-dependently inhibited the growth of 143B, Saos-2 and HOS cells at 72 h (*$p < 0.01$). (D) At the same time point, GANT61 did not affect the growth of normal osteoblast cells (NHOst) ($n > 3$; error bars indicate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

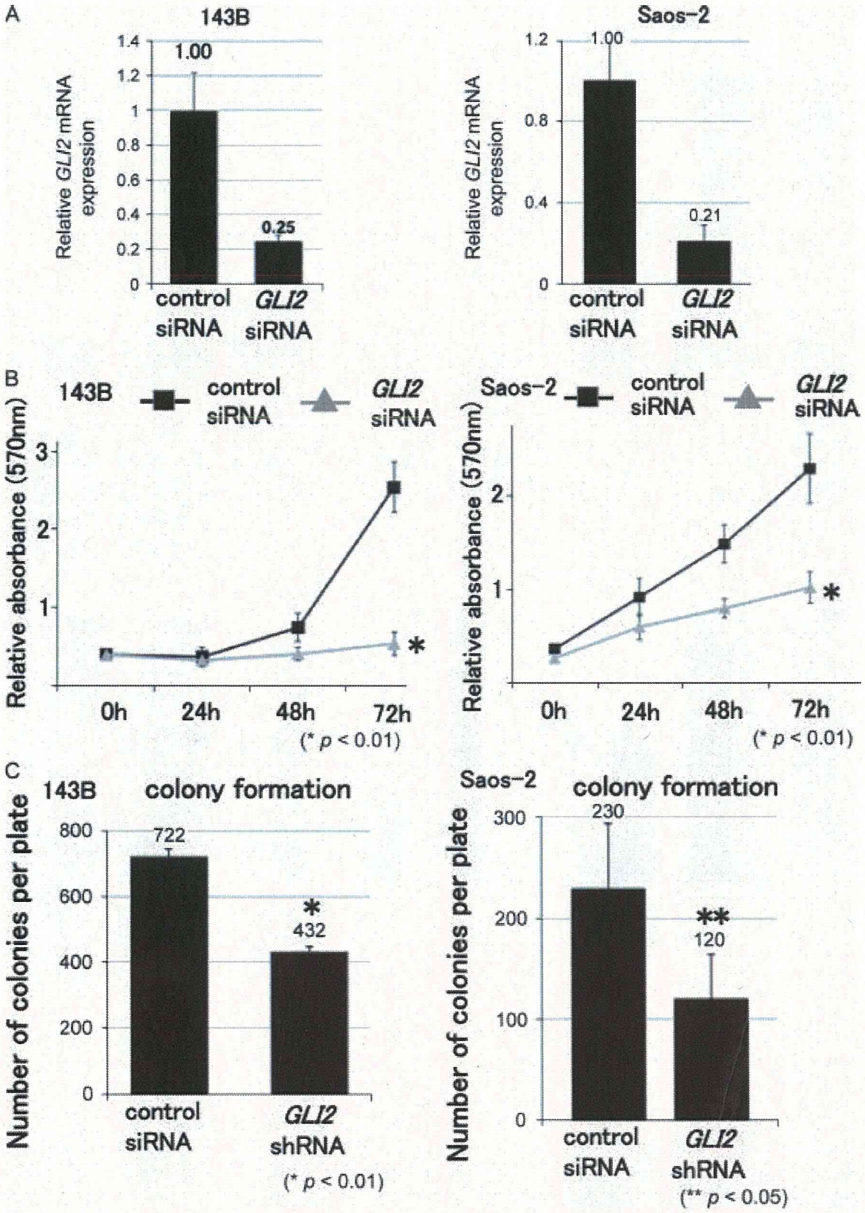
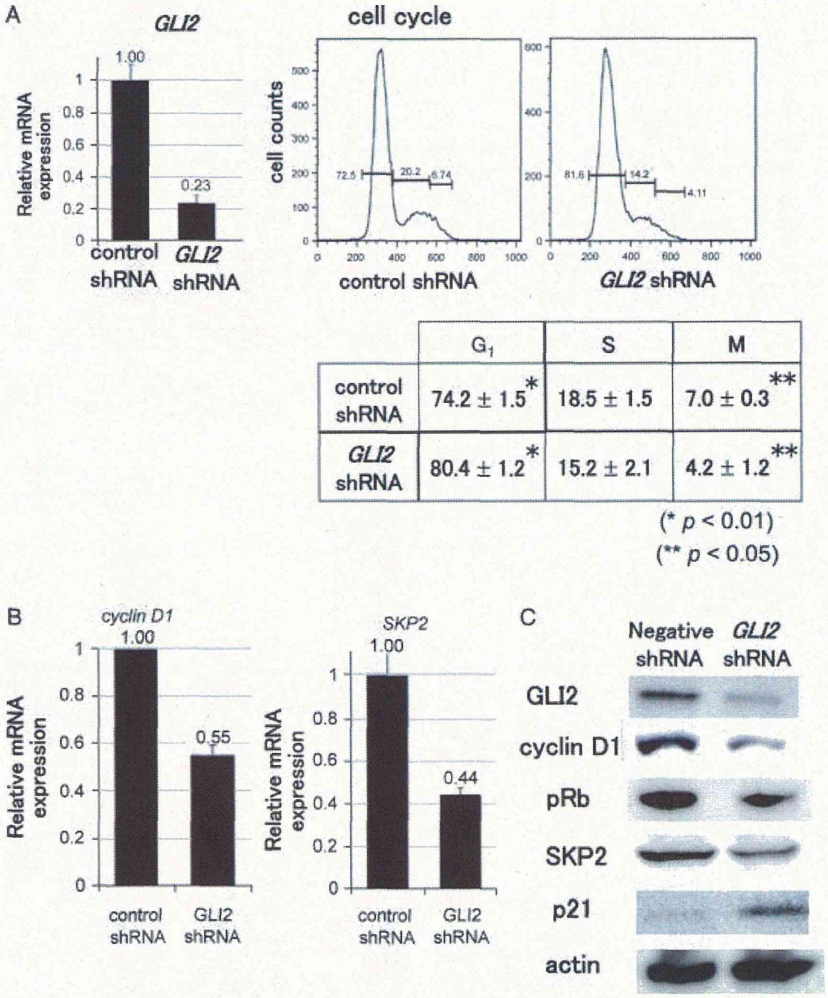


Figure 3. *GLI2* knockdown inhibits proliferation of osteosarcoma cells. (A) Transfection of *GLI2* siRNA resulted in a >70% knockdown efficiency of GLI2 [error bars represent mean (SD)]. $^{\Delta\Delta}$ Ct analysis was performed to evaluate the fold change in *GLI2* mRNA expression, using *GAPDH* or *ACTB*. (B) Growth at 72 h of 143B and Saos-2 cells was inhibited by *GLI2* siRNA. The experiment was performed in triplicate with similar results ($^{*}p < 0.01$) [error bars represent mean (SD)]. (C) A reduced number of colonies was observed in soft agar following *GLI2* knockdown. These experiments were performed in triplicate with similar results ($^{*}p < 0.01$; $^{**}p < 0.05$) [error bars represent mean (SD)].

between the osteosarcoma and prostate cancer cells *in vivo*. Nonetheless, these two studies independently suggest that low-molecular-weight compounds can inhibit malignant tumours *in vitro*. Moreover, these findings suggest that other GLI-specific inhibitors may have a powerful therapeutic potential for the management of osteosarcoma and other malignancies characterized by constitutive activation of the Hedgehog signalling pathway.

For *in vivo GLI2* RNA interference studies, we inoculated 143B osteosarcoma cells that had been previously transfected with *GLI2* shRNA. Although knockdown of *GLI2* by shRNA significantly inhibited osteosarcoma growth in nude mice, this method is not clinically applicable. Recently, the potential clinical usefulness of RNA interference in mammalian cells has been demonstrated, with no reported interferon activation [25]. In addition, Davis *et al* [26] reported a human phase I clinical trial involving the systemic administration of siRNA to patients with solid cancers; they demonstrated that siRNA administered systemically to a human can inhibit a specific gene. These findings strongly suggest that administration of *GLI2* siRNA might be a promising new treatment for osteosarcoma.



	G_1	S	M
control shRNA	74.2 ± 1.5*	18.5 ± 1.5	7.0 ± 0.3**
GLI2 shRNA	80.4 ± 1.2*	15.2 ± 2.1	4.2 ± 1.2**

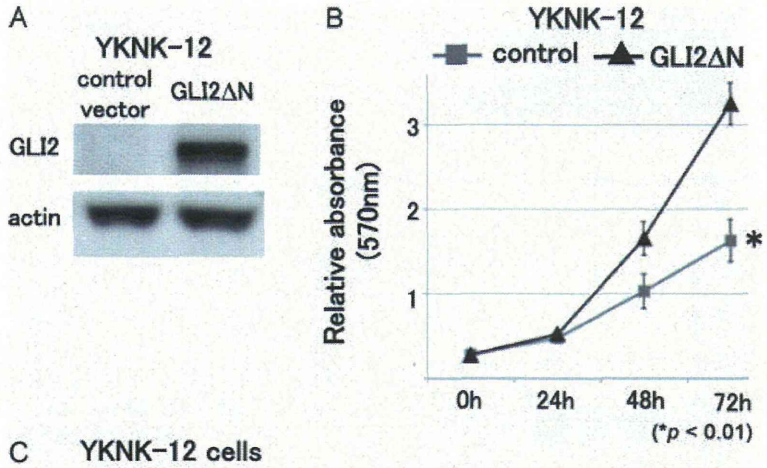
(* $p < 0.01$)
(** $p < 0.05$)

Figure 4. Knockdown of *GLI2* promotes cell cycle arrest in the G_1 phase. (A) Following transfection of *GLI2* shRNA, the efficacy of *GLI2* knockdown was >75%. $^{\Delta\Delta}$ Ct analysis was performed to evaluate the fold change in mRNA expression, using *GAPDH* or *ACTB*. (B) When 143B cells were transfected with control shRNA, 74.2% of them were in G_1 phase, while when they were transfected with *GLI2* shRNA, 80.4% of the cells were in G_1 phase (*$p < 0.01$; **$p < 0.05$). (B) Real-time PCR was employed to examine the expression of cell cycle-related genes. $^{\Delta\Delta}$ Ct analysis was performed to evaluate fold changes of mRNA expression, using *GAPDH* or *ACTB*. Knockdown of *GLI2* decreased the expression of the cell cycle accelerators, *cyclin D1* and *SKP2* [error bars represent mean (SD)]. (C) Western blot analysis revealed that knockdown of *GLI2* decreased the protein levels of cyclin D1, pRb and SKP2. Western blot analysis revealed that knockdown of *GLI2* increased the expression of $p21^{cip1}$, a negative regulator of cell cycle progression.

We previously reported that inhibition of SMO by cyclopamine or by *SMO* RNA interference reduced the growth of osteosarcoma via cell cycle regulation [21]. Compared to several potential mutational targets within the Hedgehog pathway downstream of SMO already discovered, the group of tumours that would benefit from direct GLI inhibition is substantial and likely to increase. For instance, it has been reported that inhibition of GLI, but not SMO, induced apoptosis in chronic lymphocytic leukaemia cells [27].

In order to examine the molecular mechanisms of GLI2 up-regulation, we examine genomic amplification of the GLI2 locus. We performed cytogenetic studies in three osteosarcoma specimens. FISH analysis using specific probes for the GLI2 locus revealed no chromosomal abnormalities in our osteosarcoma biopsy tissues (data not shown); this region of the genome is known to be amplified in some tumour specimens [28–32]. Further examinations should be done to elucidate the molecular mechanisms of GLI2 up-regulation.

We showed that knockdown of *GLI2* decreased the expression of SKP2 [33,34]. In addition, we found that knockdown of *GLI2* increased the expression of $p21^{Cip1}$. SKP2 is a subunit of the SCF^{SKP2} complex, a ubiquitin-dependent ligase. Down-regulation of the SCF^{SKP2} complex may promote a cell cycle arrest in G_1 phase by inhibition of $p21^{Cip1}$ degradation. Several key signalling pathways, including Hedgehog, TGFβ, BMP, Notch and Wnt, are engaged in essential processes of embryonic development. Recently, it has been clarified that these pathways also play important roles in the pathogenesis of malignant tumours (reviewed in [35]). In addition, it has been shown that there is a direct interaction or crosstalk among these key pathways (reviewed in [36]). We previously reported that



	G_1	S	M
control vector	72.0 ± 0.5*	9.8 ± 1.1**	17.0 ± 1.3*
GLI2ΔN	62.9 ± 0.9*	12.5 ± 1.0**	22.9 ± 1.5*

(*$p < 0.01$)
(**$p < 0.05$)

Figure 5. Over-expression of GLI2 accelerates mesenchymal stem cell proliferation. (A) Western blot analysis revealed that cells transfected with the GLI2ΔN expression vector reacted positively with the anti-GLI2 antibody. (B) We assessed the proliferation of YKNK-12 cells following transfection with the GLI2ΔN expression vector, which exhibits a potent transcriptional activity. The MTT assay showed that forced expression of GLI2ΔN promoted YKNK-12 cell proliferation to a greater extent than transfection with control vector (*$p < 0.01$) [error bars represent mean (SD)]. (C) Cell cycle analysis of YKNK-12 cell revealed that 62.9% and 72.0% of the cells were in G_1 phase following forced expression of GLI2ΔN and transfection with control vector, respectively. Furthermore following forced expression of GLI2ΔN, 12.5% and 22.9% of the cells were in the S and G_2–M phase, respectively, whereas 9.8% and 17.0% of the control vector-transfected cells were in the S and G_2–M phase, respectively (*$p < 0.01$; **$p < 0.05$).

the Notch pathway is activated in human osteosarcoma and that its activation promotes osteosarcoma cell growth [37]. In turn, activation of the Notch pathway promotes transcription of SKP2. SKP2 might thus mediate the crosstalk between the Notch and Hedgehog pathways. Further studies are needed to elucidate the role of interaction between these pathways in the pathogenesis of osteosarcoma.

Several recent studies have demonstrated that the anti-tumour effects of Hedgehog pathway inhibitors are mediated by their effects on tumour stromal cells [38,39]. Other studies have demonstrated that Hedgehog pathway inhibitors directly affect cancer cells [21,22,40–44]. Our findings showed that both GLI inhibition and *GLI2* knockdown directly inhibit osteosarcoma cell growth. Further studies are needed to establish the role of GLI2 activation in response to paracrine and autocrine Hedgehog signalling in osteosarcoma cells.

The hypothesis that malignant tumours are generated by rare populations of tumour-initiating cells (TICs), also called cancer stem cells, that are more tumourigenic than other cancer cells, has gained increasing credence [22,45]. We and others have reported that some bone and soft tissue sarcomas are generated by TICs [16,46]. The Hedgehog pathway has been implicated in the maintenance of normal stem cell or progenitor cells in many tissues, including the epithelia of many internal organs and brain [47]. Magali *et al* [48,49] reported that inhibition of Hedgehog

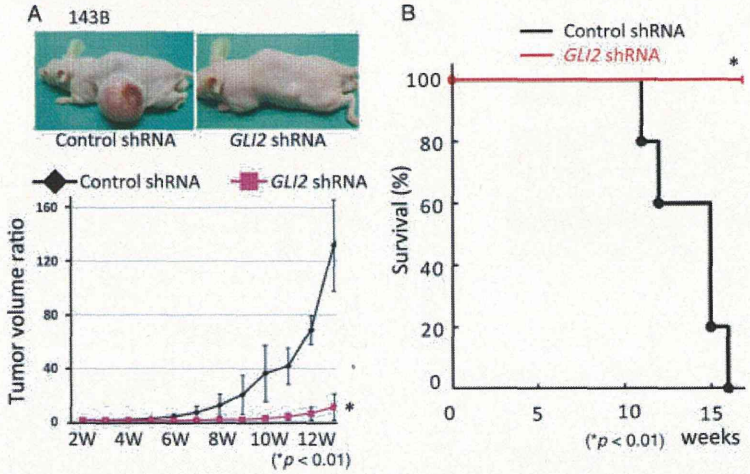


Figure 6. *GLI2* knockdown inhibits osteosarcoma growth in nude mice. (A) Following transfection of control shRNA or *GLI2* shRNA, 1×10^6 143 B cells were subcutaneously inoculated in nude mice. Tumour size was calculated weekly by using the formula $LW^2/2$ (where L and W represent the length and width of tumours). Seven days after inoculation, tumour volume was set as 1 and the increase in tumour volume was calculated at different time points, using the above formula. *GLI2* shRNA-transfected cells demonstrated a significant inhibition of tumour growth as compared with control shRNA-transfected cells ($n = 6$; $^*p < 0.01$) [error bars represent mean (SD)]. Kaplan–Meier analysis revealed that knockdown of *GLI2* provided a significant survival benefit ($n = 6$; $^*p < 0.01$).

signalling depletes TICs, whereas constitutive activation of Hedgehog signalling increases the number of TICs and accelerates tumour progression. These findings suggest that inhibition of the Hedgehog pathway might decrease the proportion of osteosarcoma TICs. The presence of a high aldehyde dehydrogenase (ALDH) activity has been used to identify TICs in malignant tumours [50–52]. Recently, Wang *et al* [53] reported that TICs obtained from osteosarcoma can be identified by a high ALDH activity. In this regard, we determined the proportion of cells with a high ALDH activity following *GLI2* siRNA transfection. At baseline, 30.6% of 143B cells showed a high ALDH activity. Seven days after *GLI2* siRNA transfection, there was no change in the proportion of cells with a high ALDH activity (data not shown). Further studies are needed to determine the impact of Hedgehog pathway inhibition on the proportion of TICs in other osteosarcoma cell lines or using other methodologies to identify TICs. In conclusion, our findings demonstrate that inhibition of GLI2 prevents osteosarcoma growth. These finding improves our understanding of osteosarcoma pathogenesis and suggest that inhibitions of GLI2 may be regarded as an effective treatment for patients with osteosarcoma.

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Author contributions

NH, IY, NS, KS and ST conceived and designed the experiments. NH, HM, YT and TT performed the experiments. IK, NK, KS and ST analysed the data. NH and HM contributed reagents/materials/analysis tools. ST drafted the manuscript.

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