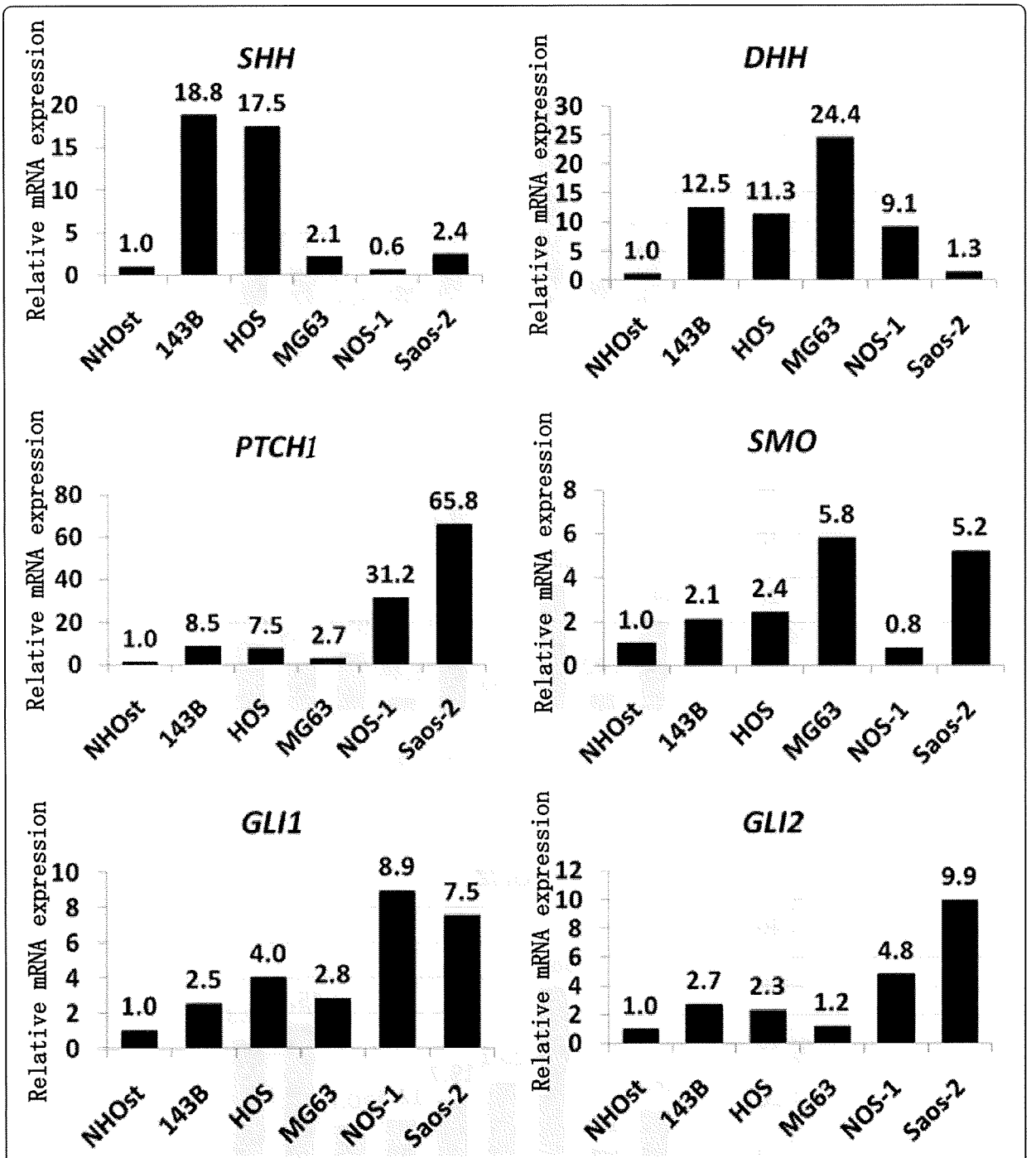

Figure 1 Expression of activated Hh-GLI pathway molecules. Total RNA extracted from osteosarcoma cell lines were used for real-time PCR. Real-time PCR revealed that 4 of 5 human osteosarcoma cell lines increased *Sonic Hedgehog* (*SHH*) 2.1- to 18.8-fold (Fig. 1). In addition, 5 of 5 osteosarcoma cell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

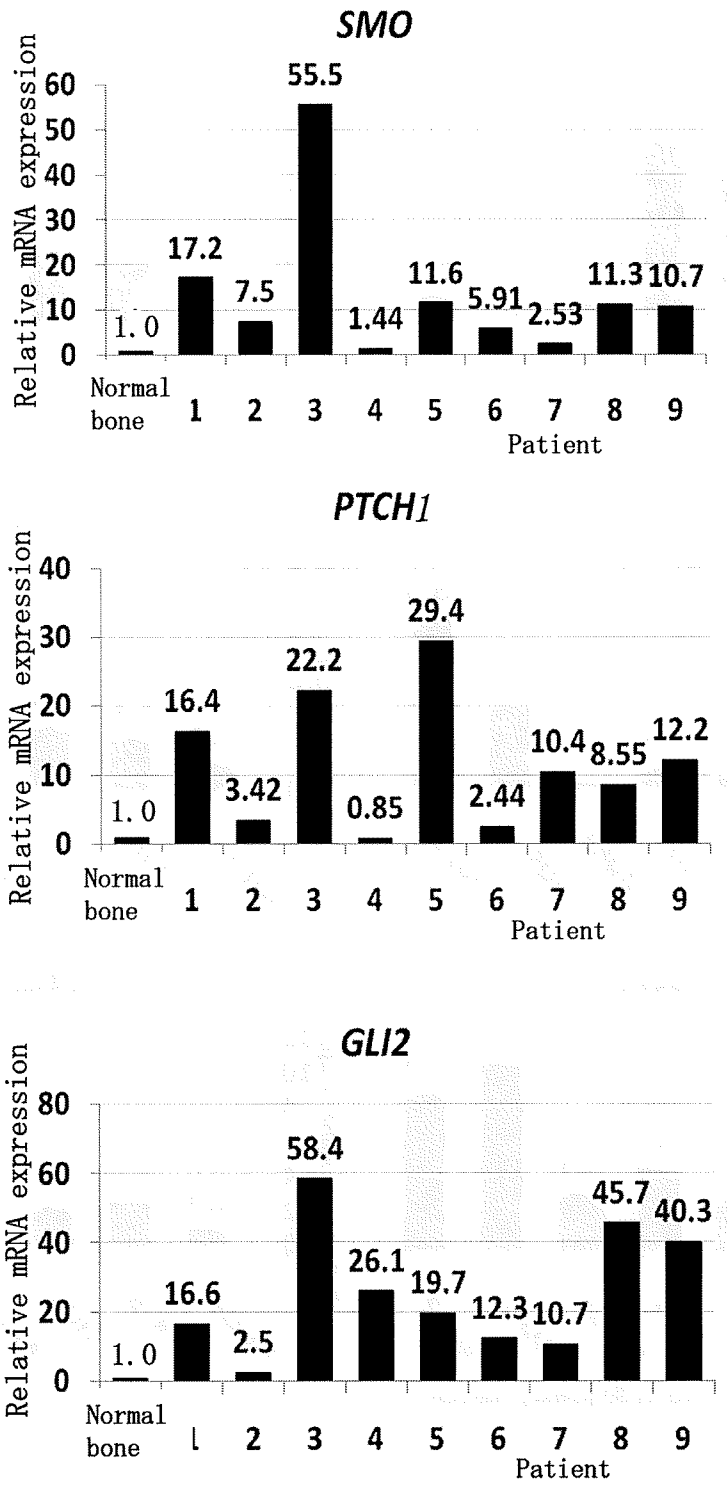

Figure 2 Activation of Hh pathway in patient' biopsy samples. Real-time PCR revealed that 9 of 9 human biopsy specimens of osteosarcoma increased *SMO* 1.44- to 55.5-fold. Real-time PCR revealed that expression of *PTCH1* was increased in 8 of 9 patients' biopsy samples 2.44- to 29.4-fold. *GLI2* was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

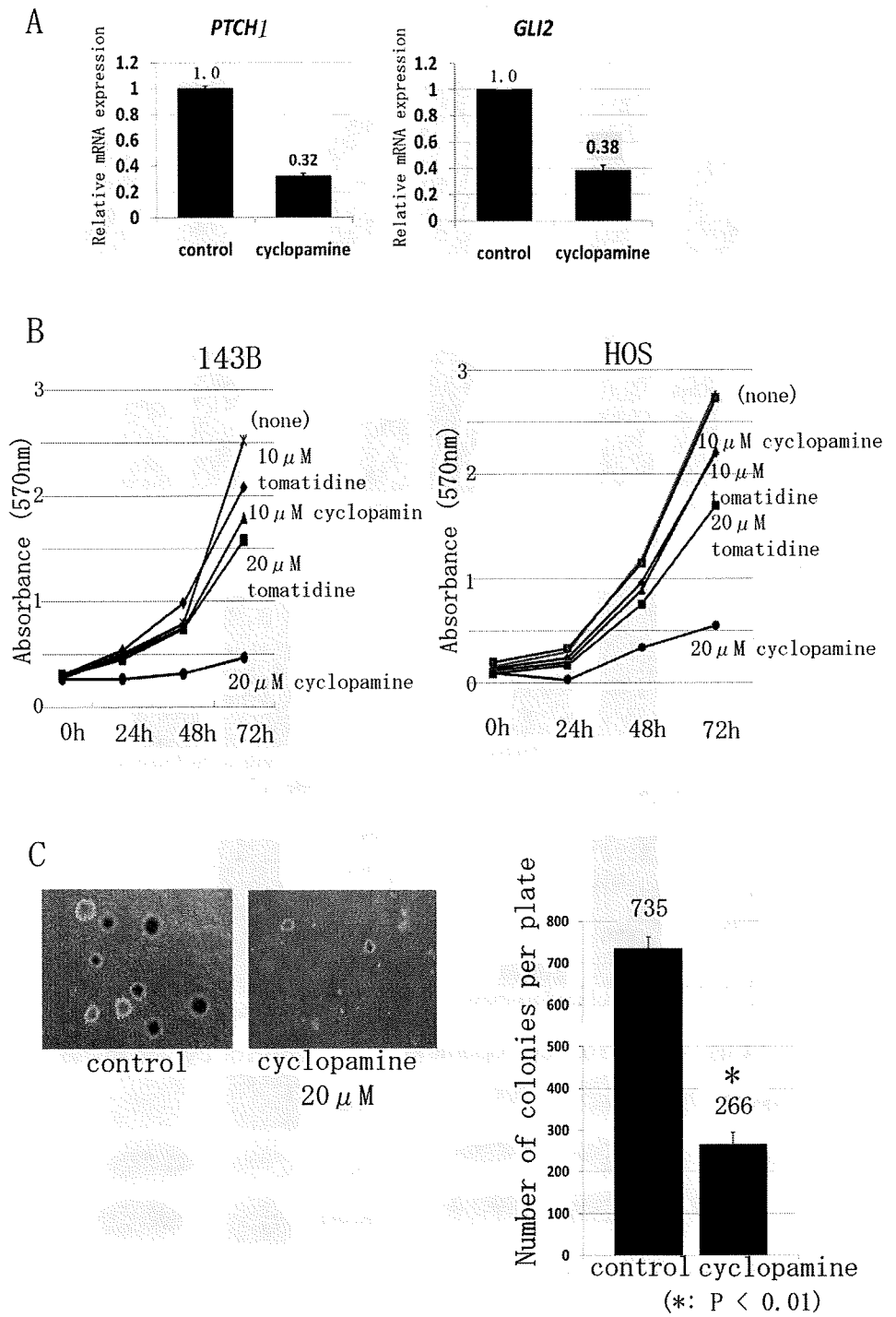

Figure 3 Inhibition of Hh pathway prevents osteosarcoma growth in vitro. A, We performed real-time PCR to determine which concentration of cyclopamine effectively inhibited Hh-GLI activity in osteosarcoma cells, and then measured the expression of the Hh-GLI pathway target *PTCH1* and *GLI2*. Cyclopamine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

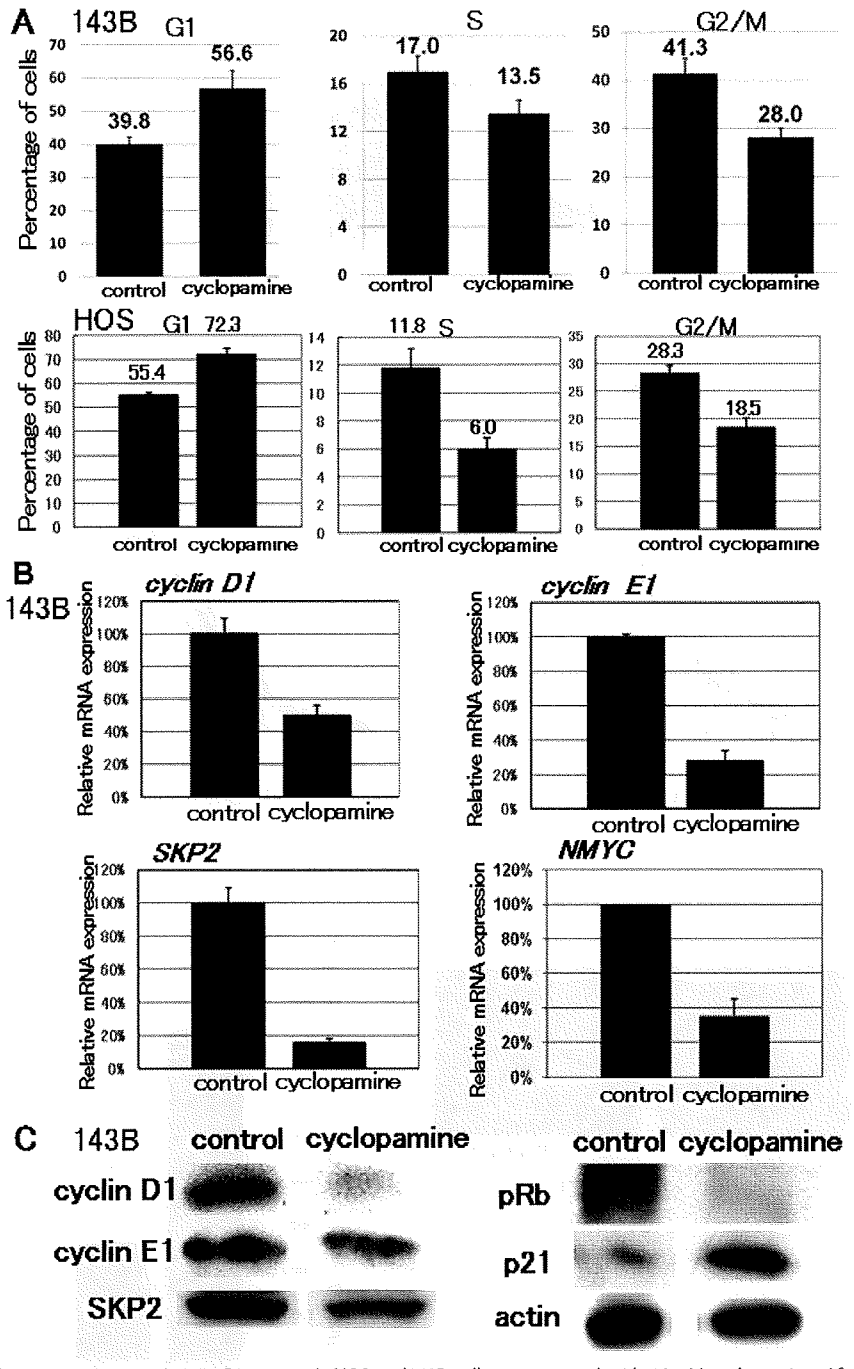

Figure 4 Cyclopamine treatment promotes G1 arrest. A, HOS and143B cells were treated with 10 μM cyclopamine. After 48-hour treatment cells were collected and subjected to cell cycle analysis. When 143B cells were cultured without cyclopamine, 39.8% of cells were in G1 phase. On the other hand, when cultured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

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

Discussion

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

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

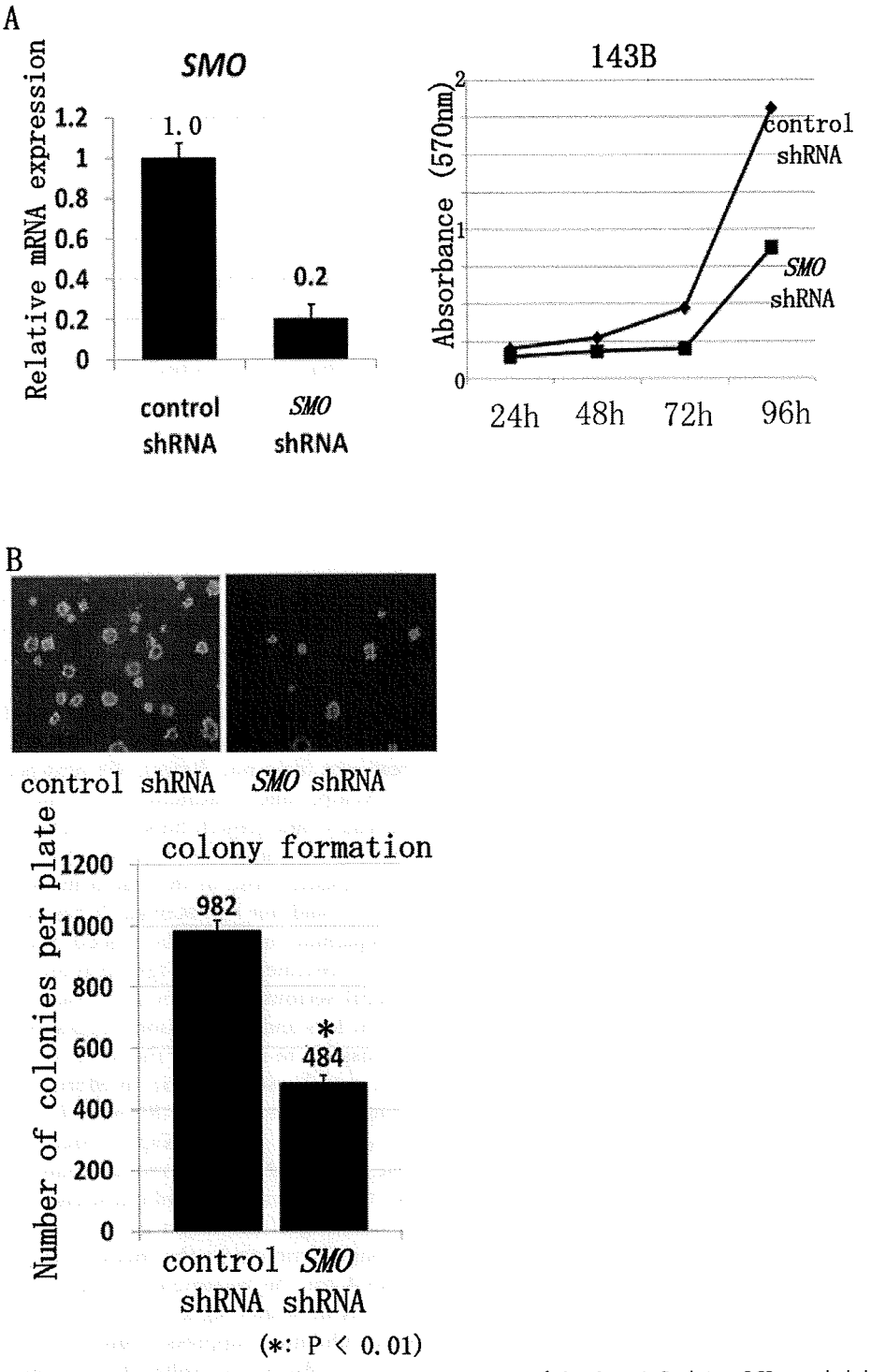

Figure 5 Knock down of *SMO* by *SMO* shRNA prevents osteosarcoma growth in vitro. A, Real-time PCR revealed that *SMO* shRNA effectively knock down *SMO* mRNA. (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using ACTB. Each sample was run minimally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

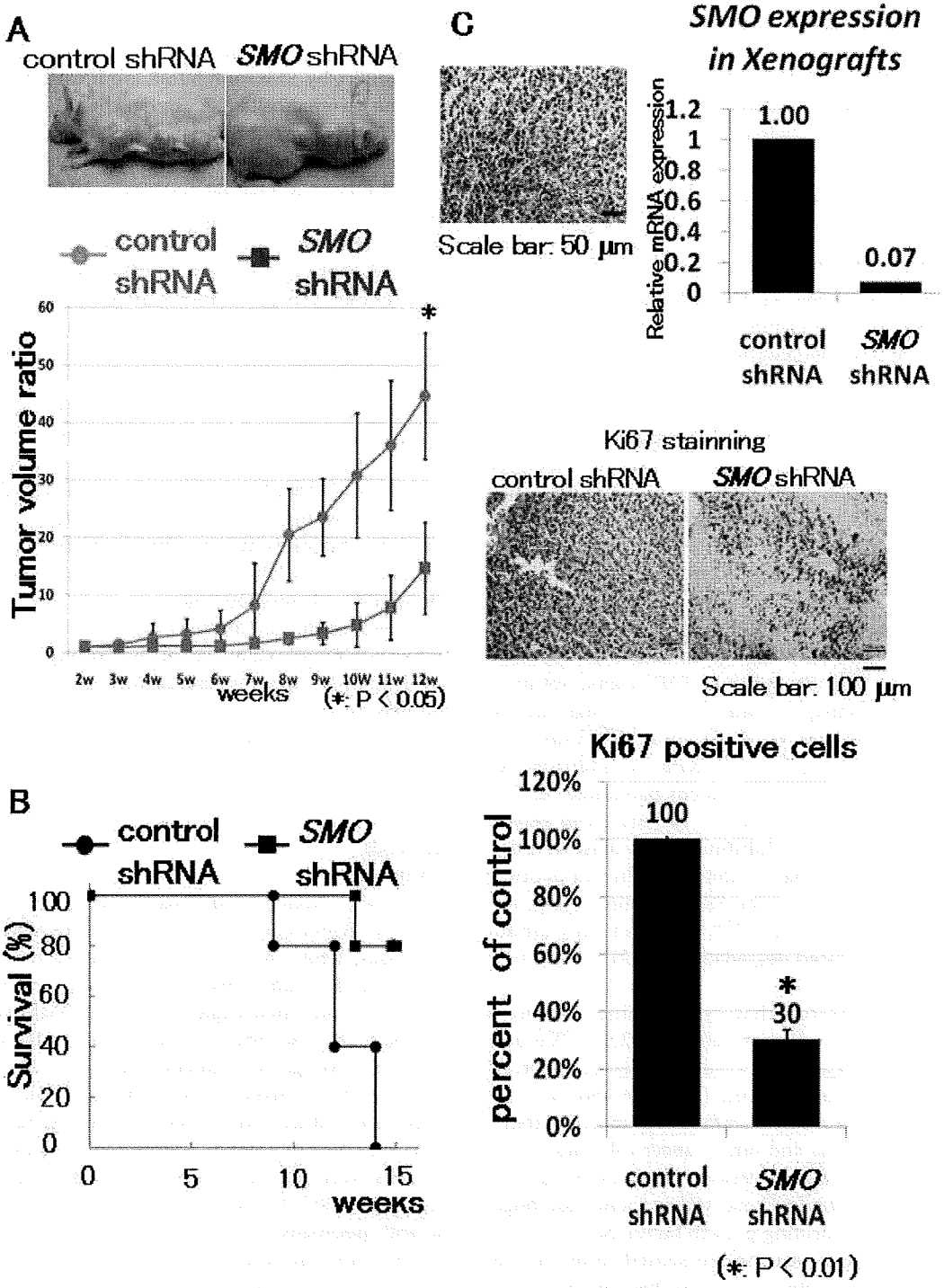

Figure 6 *SMO* shRNA prevents osteosarcoma xenograft growth in vivo and prolongs survival. A, *SMO* shRNA or control shRNA transfected 143B cells (1×10^6) were inoculated subcutaneously. Established 143B tumors were measured. The tumor volume at day 7 was set at 1, and tumor volumes at subsequent time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

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

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

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

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

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

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

Methods

Cell culture

HOS, 143B, MG63, and Saos-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). NOS-1 was purchased from RIKEN cell bank (Tsukuba, Japan) [67]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human osteoblast cells (NHOst) were purchased from Sanko Junyaku (Tokyo, Japan). Cells were cultured with OBM™ (Cambrex, East Rutherford, NJ, USA) or DMEM supplemented with 10% FBS. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Patient' specimens

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

MTT assay

Cells were incubated with substrate for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h, and washed with PBS and lysed to release formazan from cells. Then cells were analyzed in a Safire microplate reader (BIO-RAD, Hercules, CA, USA) at 562 nm. Cyclopamine and tomatidine was purchased from Funakoshi (Tokyo, Japan). 143B cell were serum starved for 12 h, and then cultured with recombinant human sonic hedgehog (R&D Systems, Minneapolis, Japan).

SMO shRNA was purchased from SABiosciences (Maryland, USA). *SMO* and control shRNAs were cloned into pGeneClip™ Neomycin Vector, which express shRNA under the control of the U1 promoter. Lipofection of shRNA was performed every other day as recommended in the supplier's protocol using FuGENE 6 (Roche, Basel, Switzerland).

Colony formation assay

Colony formation assay was performed as previously described [68]. 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.

Real-time PCR

All primer sets amplified 100- to 200-bp fragments. Total RNA was extracted using the miR-Vana RNA isolation system or TRIzol (Invitrogen, Carlsbad, CA, USA). Reactions were run using SYBR Green (BIO-RAD, Hercules, CA, USA) on a MiniOpticon™ machine (BIO-RAD, Hercules, CA, USA). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *βII-microglobulin*, or *GAPDH*, or *ACTB*. Each sample was run at three concentrations in triplicate. The following primers were used. *Desert hedgehog*: 5-TGATGACCGAGCGTTGTAAG-3, 5-GCCAGCAACCCATACTTGTT-3; *Indian Hedgehog*: 5-ACTTCTGCCTGGTCCTGTTG-3, 5-AGCGATCTTGCCTTCATAGC-3; *Sonic hedgehog*: 5-ACCGAGGGCTGGGACGAAGA-3, 5-ATTTGGCCGCCACCGAGTT-3; *PATCHED*: 5-TAACGCTGCAACAACTCAGG-3, 5-GAAGGCTGTGACATTGCTGA-3; *SMOOTHENED*: 5-GGGAGGCTACTTCCTCATCC-3, 5-GGCAGCTGAAGGTAATGAGC-3; *GLI1*: 5-GTGCAAGTCAAGCCAGAACA-3, 5-ATAGGGGCCTGACTGGAGAT-3, *GLI2*: 5-CGACACCAGGAAGGAAGGTA-3, 5-AGAACGGAGGTAGTGCTCCA-3; *cyclin D1*: 5-ACAAACAGATCATCCGCAAACAC-3, 5-TGTTGGGGCTCCTCAGGTTC-3; *cyclin E1*: 5-CCACACCTGACAAAGAAGATGATGAC-3, 5-GAGCCTCTGGATGGTGCAATAAT-3; *SKP2*: 5-TGGGAATCTTTTCCTGTCTG-3, 5-GAACACTGAGACAGTATGCC-3; *NMYC*: 5-CTTCGGTCCAGCTTTCTCAC-3, 5-GTCCGAGCGTGTTCAATTTT-3; *βII-microgloblin*: 5-TCAATGTCGGATGGATGAAA-3, 5-GTGCTCGCGCTACTC TCTCT-3; *GAPDH*: 5-GAAGGTGAAGGTCGGAGTC-3, 5-GAAGATGGTGATGGGATTTC-3; *ACTB*: 5-AGAAAATCTGGCACCACACC-3, 5-AGAGGCGTACAGGGATAGCA-3.

Immunohistochemistry

The following primary antibodies were used; anti-SMO (diluted 1:200, Santa Cruz, CA. U.S.A), anti-GLI2 (diluted 1:200, Abcam, Cambridge, UK), and anti-ki67 (Zymed laboratories, San Francisco, USA). The following secondary antibodies were used:; fluorescein-conjugated goat anti-mouse IgG antibody (diluted 1:200; Jackson ImmunoResearch, PA, USA) and rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Temecula, CA). The cells were counterstained with Hoechst 33258 (Molecular Probes, Carlsbad, CA, USA) to identify nuclei. Immunohistochemistry with each second antibody alone without primary antibody was performed as a control.

Western blot

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 with antibodies to cyclin D1, E1, p21, SKP2, and pRb (Santa Cruz, CA. U.S.A). Detection was performed using the ECL detection system (Amersham, Giles, UK).

Animal experiments

143B cells (1×10^6) were mixed with a collagen gel in a 1:1 volume, and were inoculated subcutaneously in 5-week-old nude mice. The mice were randomly assigned to receive either cyclopamine (25 mg/kg-10 mg/kg) or an equal volume of DMSO as control. Cyclopamine and saline solution were administered by intraperitoneal injection. The treatment with cyclopamine was initiated 1 week after tumor inoculation when the tumors had grown to visible size. The injections were repeated every other day. Tumor size was measured with calipers weekly, and tumor volume was calculated using a formula of $LW^2/2$ (L and W representing the length and width of tumors). SMO shRNA (SABiosciences, Maryland, USA) transfected 143B cells (1×10^6) or control shRNA (1×10^6) cells were mixed with a collagen gel in a 1:1 volume, and were inoculated subcutaneously in 5-week-old nude mice. Tumor size was measured with calipers weekly, and tumor volume was calculated using a formula of $LW^2/2$ (L and W representing the length and width of tumors). All experimental procedures were performed in compliance with the guiding principles for the Care and Use of Animals described in the American Journal of Physiology and with the Guidelines established by the Institute of Laboratory Animal Sciences,

Faculty of Medicine, Kagoshima University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize possible alternatives to in vivo techniques.

Cell cycle analysis

Cell cycle analysis was performed by Reprocell (Tokyo, Japan). At 48 h after cyclopamine treatment, cells were collected by trypsinization and washed with DPBS. Cells were fixed in 70% (v/v) ethanol at 4°C, washed with PBS, and resuspended with 500 μl of staining solution [PBS pH 7.4, 100 μg/ml DNase-free RNase, 1 mg/ml propidium iodide]. Cells were then analyzed by flow cytometry using a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ). Data were gated using pulse width and pulse area to exclude doublets, and the percentage of cells present in each phase of the cell cycle was calculated using FlowJo software (Tree Star, Ashland, OR, USA).

Statistics and supplemental data

Each sample was analyzed in triplicate, and experiments were repeated three times. In all figures, error bars are standard divisions. 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 $P < 0.05$.

List of abbreviations

(Hh): Hedgehog; (SMO): SMOOTHENED; (PTCH1): PATCHED; (SHH): Sonic hedgehog; (DHH): Desert hedgehog.

Additional file 1: A, Immunohistochemical examination revealed that SMO was expressed on cytoplasm of 143B and GLI2 was localized in the nucleus of 143B. B, MTT assay showed that Sonic hedgehog promote proliferation of osteosarcoma cells. The experiment was triplicate with similar results.
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Additional file 2: Real-time PCR was performed to quantify mRNAs of cell cycle related genes. *SMO* shRNA reduced levels of *cyclin D1*, *cyclin E1*, *SKP2*, and *E2F1* transcription (error bar means S.D.). The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *ACTB*. The experiment was triplicate with similar results.
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Additional file 3: We performed real-time PCR using formed tumors. Real-time PCR revealed that transcription of *GLI1*, *GLI2*, and *PTCH1* was decreased in tumors formed by *SMO* shRNA-transfected 143B. In addition, *SMO* shRNA reduced levels of *Cyclin E1*, *SKP2*, and *E2F1* transcription. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *ACTB*. The experiment was triplicate with similar results.
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Additional file 4: Cyclopamine prevents proliferation of osteosarcoma *in vivo*. Immunohistochemical examination of ki67 was performed in xenograft tumors. Ki67 staining revealed that proliferation of osteosarcoma cells was decreased by cyclopamine treatment. The numbers of Ki67-positive cells was decreased to 50% of control revel by cyclopamine administration at day 14 (error bar means S.D.).
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Acknowledgements

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Authors' contributions

TS was involved in the design and execution of the experiments, drafted the manuscript and contributed to the overall experimental design. MH conducted most of the experiments. HS was conducted a most of experiments. HG was conducted a part of experiments. YM was conducted a part of experiments. HN was conducted a part of experiments. OK was conducted a part of experiments. SK contributed to the overall experimental design. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tumour formation by single fibroblast growth factor receptor 3-positive rhabdomyosarcoma-initiating cells

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BACKGROUND: The hypothesis that malignant tumours are generated by rare populations of cancer stem cells that are more tumourigenic than other cancer cells has gained increasing credence. The objective of this study was to identify and characterise a subpopulation of human sarcoma-initiating cells.

METHODS: We examined established rhabdomyosarcoma cell lines by flow cytometry. Tumourigenesis was examined by xenograft models. Real-time PCR and immunohistochemistry were performed to examine the gene expression using cell lines and biopsy specimens.

RESULTS: Rhabdomyosarcoma cell lines included small populations of fibroblast growth factor receptor 3 (FGFR3)-positive cells. FGFR3-positive KYM-1 and RD cells were more strongly tumourigenic than FGFR3-negative cells. In addition, xenoengraftment of 33% of single FGFR3-positive KYM-1 cells yielded tumour formation. Stem cell properties of FGFR3-positive cells were further established by real-time PCR, which demonstrated upregulation of undifferentiated cell markers and downregulation of differentiation markers. We showed that in the absence of serum, addition of basic fibroblast growth factor maintained and enriched FGFR3-positive cells. On the other hand, ciliary neurotrophic factor reduced the proportion of FGFR3-positive cells. Real-time PCR and immunohistochemical examination revealed that embryonal rhabdomyosarcoma patient biopsy specimens were found to over-express FGFR3.

CONCLUSIONS: Our findings suggest that rhabdomyosarcoma cell lines include a minor subpopulation of FGFR3-positive sarcoma-initiating cells, which can be maintained indefinitely in culture and which is crucial for their malignancy.

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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 (Clarke and Fuller, 2006). TICs were initially identified in acute myeloid leukaemia (AML), and were found capable of inducing AML in immunodeficient mice (Lapidot *et al*, 1994; Bonnet and Dick, 1997). TICs have since been identified in numerous other tumours, including melanoma, lung, head, neck, pancreatic, prostate, colon, squamous cell cancers, and benign tumours (Collins *et al*, 2005; Fang *et al*, 2005; Kim *et al*, 2005; Dalerba *et al*, 2007; Prince *et al*, 2007; Loebinger *et al*, 2008; Xu *et al*, 2009). Although the AML TICs resemble and probably originate from the transformation of a stem cell, it is possible that other TICs originate from transformation of early or late progenitor cells. Thus, the definition of a TIC is not related to the cell of origin for a tumour but rather to its ability to self-renew, initiate cancer, and give rise to more differentiated cells that have lost the capacity for self-renewal and tumourigenic potential. The notion that cancer is driven by TICs has obvious therapeutic implications (Al-Hajj *et al*, 2004; Raguz and Yague, 2008). The efficacy of tumour response to systemic therapy has traditionally been assessed based on the bulk of tumour cells by monitoring of changes in tumour size (Therasse *et al*, 2000). However, if only a small fraction of TICs are capable of initiating cancer, then curative therapy should be designed to target these rare TICs rather than the bulk of nontumourigenic cells. Analysis of TICs might thus yield novel therapeutic targets.

In this study, we attempted to identify rhabdomyosarcoma-initiating cells (RICs) using cell surface markers. We examined many markers of undifferentiated cells. We found that human rhabdomyosarcoma cell lines include a small proportion of fibroblast growth factor receptor 3 (FGFR3)-positive cells. Single FGFR3-positive cells have the potential for tumour formation *in vivo*. In addition, tumours formed by FGFR3-positive cells could be used for serial propagation of tumours in animals. Moreover, basic fibroblast growth factor (bFGF) could maintain and enrich FGFR3-positive RICs in the KYM-1 cell line in the absence of serum. The easy method of preparation we describe will be useful for the development of anti-RICs target therapy.

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MATERIALS AND METHODS

Cell culture

KYM-1 and RD rhabdomyosarcoma cell lines were purchased from Health Sciences Research Resources Bank (Tokyo, Japan). The A204 cell line was purchased from ATCC (Manassas, VA, USA). These cells were cultured in DMEM, supplemented with 10% FCS, 100 units per ml penicillin G, and 100 $\mu g\,ml^{-1}$ streptomycin (Invitrogen, Carlsbad, CA, USA). In some experiments, KYM-1 cells were cultured in serum-free S-Clone (Sanko Junyaku, Japan) containing 10 $ng\,ml^{-1}$ bFGF, 10 $ng\,ml^{-1}$ epidermal growth factor (EGF), 2.5 $ng\,ml^{-1}$ transforming growth factor beta (TGF-β), 10 $ng\,ml^{-1}$ ciliary neurotrophic factor (CNTF), 10 $ng\,ml^{-1}$ platelet-derived growth factor (PDGF)-AA, 10 $ng\,ml^{-1}$ PDGF-BB, and 5 $\mu g\,ml^{-1}$ heparin. Normal human skeletal muscle cells (HSkMC) were purchased from TOYOBO (Osaka, Japan). HSkMC were cultured in skeletal muscle cell growth medium (Cell Applications Inc., San Diego, CA, USA) or DMEM, supplemented with 10% FCS, 100 units per ml penicillin G, and 100 $\mu g\,ml^{-1}$ streptomycin (Invitrogen). In all experiments, cells were maintained in 100 mm culture dishes (Nunc, New York, NY, USA) at 37°C in a humidified 5% CO_2 per 95% air atmosphere.

Flow cytometry analysis and sorting

Fluorescence-activated cell sorting analysis was performed using an Epics Altrta (Beckman Coulter, Fullerton, CA, USA). Cells were conjugated with anti-FGFR3 antibody (R&D, Minneapolis, MN, USA) for 30 min on ice. Cells were washed three times in PBS, re-suspended in the same buffer at 5×10^6 per ml, and kept on ice until analysis. Live single cells (fixed FSC-A/FSC-W ratio; PI negative) were gated for analysis.

Rhabdomyosarcoma patients' biopsy specimens

We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Biopsy was performed before chemotherapy or radiotherapy to make the diagnosis. Control muscle was obtained from surgery for scoliosis. 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

Each sample was run minimally at three concentrations in triplicate. All primer sets amplified 100–200 bp fragments. Primers were designed by Primer3. Total RNA was extracted using the miR-Vana RNA isolation system (Ambion, Austin, TX, USA) or TRIzol (Invitrogen). Reactions were run using SYBR Green (Bio-Rad, Hercules, CA USA) on a MiniOpticon machine (Bio-Rad). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using *βII-macroglobulin*, *ACTB*, or *GAPDH*. The following primers were used: *CD34*: 5- CACCCTGTGTCTCA ACATGG-3, 5-GGCTTCAAGGTTGTCTCTGG-3; *PAX3*: 5- GCCT GACGTGGAGAAGAAAA-3, 5-GCCTCCTCCTCTTCACCTTT-3; *PAX7*: 5-GAACCTGACCTCCCACTGAA-3, 5-CCTCTGTCAGCTTGGTCCTC-3; *MYF5*: 5-AATTTGGGGACGAGTTTGTG-3, 5- CATGGTGGTGGACTT CCTCT-3; *NANOG*: 5-AATACCTCAGCCTCCAGCAGATG-3, 5-TGCGT CACACCATTGCTATTCTTC-3; *OCT-4*: 5-GAGAACCGAGTGAGAGG CAACC-3, 5-CATAGTCGCTGCTTGATCGCTTG-3, *SOX2*: 5-AGAAC CCCAAGATGCACAAC-3, 5-CGGGGCCGGTATTTATAATC-3; *MYH1*: 5-GCTCATCGAGAAGCCTATGG-3, 5-CAAAGAGAAGTGGGCCTCAG -3; *desmin*: 5-CATCGCGGCTAAGAACATTT-3, 5-GCCTCATCAGGG AATCGTTA-3; *myogenin*: 5-TGGGCGTGTAAGGTGTGTAA-3, 5-CGA TGTACTGGATGGCACTG-3; *dystrophin*: 5-ACCACCTCTGACCCTAC ACG-3, 5-GCAATGTGTCCTCAGCAGAA-3; *β2-microglobulin*: 5-TCAA TGTCGGATGGATGAAA-3, 5-GTGCTCGCGCTACTCTCTCT-3; *ACTB*: 5-AGAAAATCTGGCACCACACC-3, 5-AGAGGCGTACAGGGATAGCA-3; *GAPDH*: 5-GAAGGTGAAGGTCGGAGTC-3, 5-GAAGATGGTGAT GGGATTTC-3.

Immunohistochemical examination

We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Anti-FGFR3 (diluted 1:200, R&D) was used as a primary antibody. Rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Billerica, MA, USA) was used as a secondary antibody. The cells were counterstained with Hoechst 33258 to identify nuclei. Immunohistochemistry with second antibody alone without primary antibody was performed as a control.

Animal experiments

KSN/SLC nude mice were purchased from SLC. FGFR3-positive cells were collected by magnetic sorting by MACS according to the manufacturer's recommendations (Miltenyi Biotec, Gladbach, Germany). The following antibodies were used: PE-conjugated anti-FGFR3 antibody (R&D) and anti-PE Microbeads (Miltenyi Biotec). Cell inoculation was performed as reported earlier (Tanaka *et al*, 2009). Cells were mixed with a collagen gel, and were inoculated subcutaneously in 5-week-old nude mice. Grafts were excised and small portions of tumour (20 mg) were serially inoculated into other nude mice. In addition, graft was excised and trypsinised. Each number of cells was serially inoculated into other mice. Grafts were fixed with 10% buffered formaldehyde and stained with hematoxylin and eosin. All experimental procedures were performed in compliance with the guiding principles for the Care and Use of Animals described in the American Journal of Physiology and with the Guidelines established by the Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University. All efforts were made to minimise animal suffering, to reduce the number of animals used, and to use possible alternatives to *in vivo* techniques.

RESULTS

Rhabdomyosarcoma cell lines include a small portion of FGFR3-positive cells

To determine whether any of the established osteosarcoma and rhabdomyosarcoma cell lines included small portions of undifferentiated cell marker-positive cells, we performed flow cytometry. We examined many markers of undifferentiated cell, such as side population (SP), CD9, CD10, CD13, CD29, CD31, CD34, CD44, CD117, CD133, FLT3, LNGFR, and FGFR3 (Caligaris-Cappio *et al*, 1985; Robinson *et al*, 1999; Erices *et al*, 2000; Singh *et al*, 2003; Kondo *et al*, 2004; Bobis *et al*, 2006; Jones *et al*, 2006; Small, 2008). We found that three rhabdomyosarcoma cell lines, KYM-1, RD, and A204, each included a small proportion of FGFR3-positive cells (1.6–2.6%) (Figure 1).

The malignancy of KYM-1 and RD cells *in vivo* depends to a large extent on FGFR3-positive RICs

To determine whether the subset defined by FGFR3 was enriched for RICs, we compared the abilities of FGFR3+ and FGFR3− rhabdomyosarcoma cells to initiate tumour formation *in vivo*. After 8W, all mice inoculated with 100 KYM-1 cells had formed tumours. After 5W, in 5 out of 6 of 10 FGFR+ KYM-1 cells

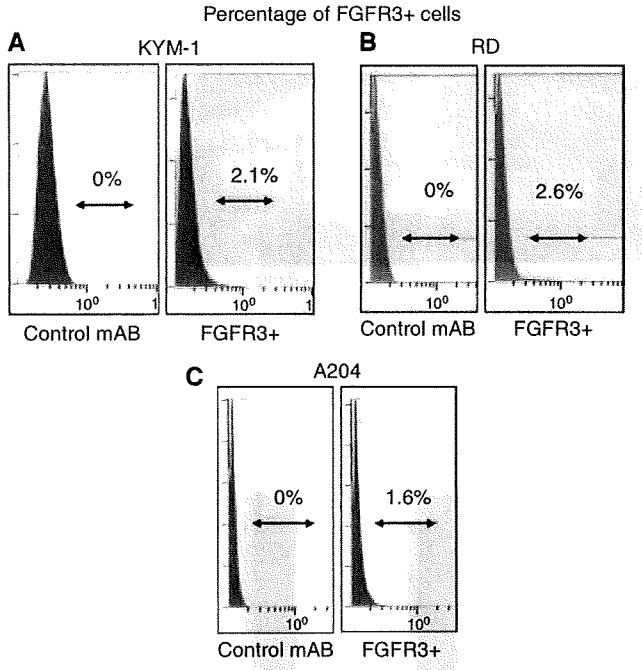

Figure 1 Rhabdomyosarcoma cell lines include a small portion of FGFR3-positive cells. Cells of human KYM-1 rhabdomyosarcoma (**A**), human RD rhabdomyosarcoma (**B**), and human A204 rhabdomyosarcoma (**C**) cell lines were labelled with anti-FGFR3 antibody and then analysed by flow cytometry. These three human rhabdomyosarcoma cell lines included small subpopulations of FGFR3-positive cells. These experiments were repeated at least three times with similar results.

inoculated mice, there was tumour formation. In contrast, in 1 out of 6 of 10 FGFR− KYM-1 cells inoculated mice, there was tumour formation. Surprisingly, 2 out of 6 of only single FGFR3+ KYM-1 cell inoculated mice also exhibited tumour formation. (Figure 2A). Next, we examined RD cells. After 6W, in 2 out of 3 of 100 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 100 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. In addition, in 1 out of 3 of 10 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 10 FGFR− RD cells inoculated mice, there was tumour formation (Figure 2C). We next performed serial transplantation. Small portions of formed tumour (20 mg) were excised and then inoculated into other nude mice. Six of six tumours formed by FGFR3+ cells inoculated into mice formed tumour. In contrast, none of six tumours were formed by FGFR− KYM-1 cells. In addition, 3 out of 3 of 1000 cells prepared from FGFR3+ tumour inoculated mice formed tumour (Figure 2D). In contrast, 0 out of 3 of 1000 cells prepared from FGFR− tumour formed tumour (Figure 2D). Immunohistochemical examination revealed that tumours formed by FGFR3+ KYM-1 cell contained both FGFR3+ cells and FGFR3− cells *in vivo* (Figure 2E).

RICs express undifferentiated cell markers

We next examined the expression of genes specific to skeletal muscle development or embryonic stem cells. RNA from FGFR3+ KYM-1 or FGFR3− KYM-1 cells was analysed by real-time PCR for *CD34*, *PAX3*, *PAX7*, *MYF5*, *NANOG*, *OCT4*, *SOX2*, *myosin heavy chain 1* (*MYH1*), *desmin*, *myogenin*, and *dystrophin*. Real-time PCR revealed that expression of *CD34* and *PAX3* in FGFR3+ cells was markedly increased by 7.23- and 2.47-fold, respectively. In addition, the expression of *PAX7*, *MYF5*, *NANOG*, *OCT3*, and *SOX2* was slightly increased by 1.15-, 1.13-, 1.35-, 1.56-, and 1.5-fold, respectively. On the other hand, the expression of the differentiated muscle markers *MYH1*, *desmin*, *myogenin*, and *dystrophin* was decreased to 0.85-, 0.91-, 0.81-, and 0.25-fold baseline levels, respectively (Figure 3).

RICs can be maintained and enriched by bFGF

We then examined which factor(s) can maintain KYM-1 FGFR3+ cells in serum-free culture media. We tested bFGF, EGF, TGF-β1, CNTF, PDGF-AA, and PDGF-BB as candidates. These mitogens are important factors in maintaining many types of progenitor cells (Marmur *et al*, 1998; Kondo *et al*, 2004; Vallier and Pedersen, 2005). We first cultured unfractionated KYM-1 cells in serum-free culture medium alone or with a mixture of bFGF, EGF, TGF-β, CNTF, PDGF-AA, and PDGF-BB. KYM-1 cells could not grow without growth factors. On the other hand, this growth factor cocktail promoted KYM-1 cell growth. We next examined which mitogen is essential for KYM-1 cell growth, by withdrawing each mitogen individually. All culture conditions promoted KYM-1 growth but bFGF withdrawal (Figure 4A). These findings suggested that bFGF is essential for KYM-1 survival and proliferation. We cultured 1000 KYM-1 cells in each condition and counted 20 days after culture. Addition of EGF to serum-free culture medium with bFGF appreciably stimulated KYM-1 cell growth approximately three-fold, the same as neural progenitor cells (Figure 4B) (Kitchens *et al*, 1994). On the other hand, when cultured in serum-free culture medium with EGF alone, KYM-1 cells could not survive. We then stained cells with anti-FGFR3 antibody and analysed them by flow cytometry. When cultured in serum-free medium with bFGF, FGFR3+ cells were maintained, and their proportion increased to 7.6–9.2%. In addition, when cultured in both bFGF and EGF, FGFR3+ cells increased to 4.2–6.0 % and total cell number increased three-fold (Figure 4C). We next examined which factor prevents expansion of FGFR3+ cells. When KYM-1 cells were cultured with bFGF plus CNTF, CNTF reduced the proportion of FGFR3+ cells by approximately 15% (Figure 4D), although CNTF did not affect the total number of KYM-1 cells. These findings suggest that RICs can be maintained and increased in bFGF alone and that a combination of bFGF and EGF can increase cell numbers. On the other hand, CNTF decreased the proportion of RICs.

FGFR3 was upregulated in rhabdomyosarcomapatient biopsy specimens

We next examined the expression of FGFR3 in patient biopsy specimens. Real-time PCR revealed that FGFR3 was upregulated in embyonal rhabdomyosarcoma patient biopsy specimens (Figure 5A). Immunohistochemical examination revealed that a portion of rhabdomyosarcoma cells expressed FGFR3. The intensity of FGFR3 expression differed among rhabdomyosarcoma cells (Figure 5B).

DISCUSSION

Although there is an expanding literature supporting the existence of cancer stem cells, important caveats of these studies continue to provoke debate. The current definitive test for a cancer stem cell is the capacity to propagate tumours as xenografts in immunocompromised mice (Clarke *et al*, 2006). We have described here the isolation of a highly tumourigenic subpopulation of cells from human rhabdomyosarcoma cell lines in accord with terminology. To our knowledge, this is the first isolation of

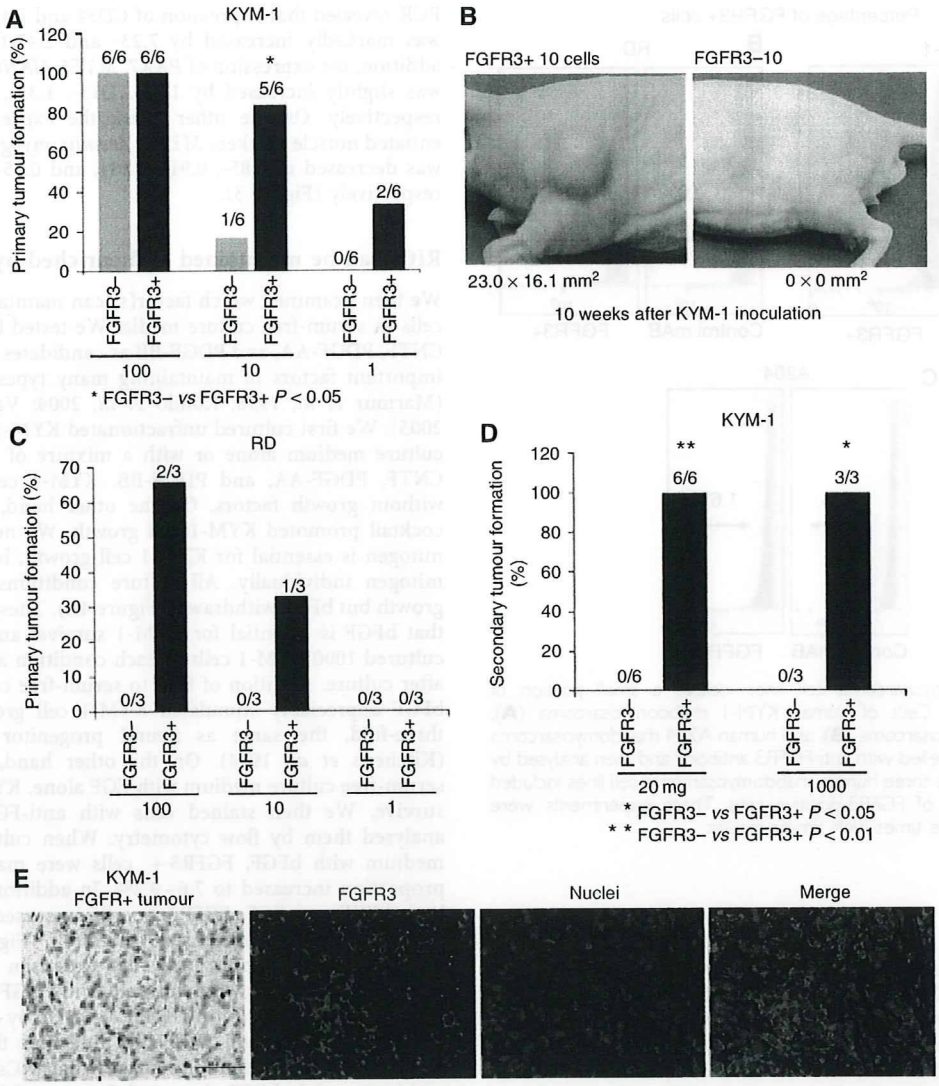

Figure 2 The malignancy of rhabdomyosarcoma cells *in vivo* depends to a large extent on the FGFR3-positive cells. FGFR3-dependent cell sorting was performed using immunomagnetic selection, with culture for one night to exclude dead cells by mechanical stress followed by inoculation of cells from either population interdermally into nude mice. (**A**) Primary tumour formation by KYM-1, FGFR3−, or FGFR3+ cells. After 8W, all mice inoculated with 100 KYM-1 cells had formed tumours. After 5W, in 5 out of 6 of 10 FGFR+ KYM-1 cells inoculated mice, there was tumour formation. In contrast, in 1 out of 6 of 10 FGFR− KYM-1 cells inoculated mice, there was tumour formation after 12W inoculation. Surprisingly, 2 out of 6 of only single FGFR3+ KYM-1 cell inoculated mice also exhibited tumour formation. In contrast, 0 out of 6 of single KYM-1 FGFR cell inoculated mice formed tumour after 12W inoculation. (**B**). Ten FGFR3-positive cells form tumour 8 weeks after inoculation. (**C**). Primary tumour formation by RD, FGFR3−, or FGFR3+ cells. After 6W, in 2 out of 3 of 100 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 100 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. In addition, in 1out of 3 of 10 FGFR+ RD cells inoculated mice, there was tumour formation. In contrast, in 0 out of 3 of 10 FGFR− RD cells inoculated mice, there was tumour formation after 12W inoculation. (**D**) Secondary tumour formation by FGFR3− or FGFR3+ KYM-1 cells. We next performed serial transplantation. Small portions of formed tumour (20 mg) were excised and then inoculated into other nude mice. Six of six tumours formed by FGFR3+ KYM-1 cells inoculated into mice formed tumour. In contrast, none of six tumours formed by FGFR− KYM-1 cells inoculated into mice formed tumour after 12W inoculation. In addition, 3 out of 3 of 1000 cells prepared from FGFR3+ tumour inoculated mice formed tumour. In contrast, 0 out of 3 of 1000 cells prepared from FGFR− tumour inoculated mice formed tumour after 12W inoculation. (**E**) HE staining of tumour formed by FGFR3+ KYM-1cells. Immunohistochemical examination revealed that tumour formed by FGFR3+ KYM-1cells contains both FGFR3+ and FGFR3− cells (red: FGFR3, blue: Hoechst). * FGFR3− *vs* FGFR3+ $P<0.05$, * * FGFR3− *vs* FGFR3+ $P<0.01$.

malignant progenitors from human rhabdomyosarcoma to be described.

Initially, to identify candidate RICs, we used a side population method as reported earlier (Kondo *et al*, 2004; Setoguchi *et al*, 2004). We detected approximately 1–3% SP cells among KYM-1 cells. We sorted SP and non-SP cells and then inoculated them into nude mice subcutaneously, but could not detect differences between them in tumourigenisity (data not shown). We next examined CD133, which has been reported to be a cancer stem cell marker (Singh *et al*, 2003; Hermann *et al*, 2007; Ricci-Vitiani *et al*, 2007; Chearwae and Bright, 2008; Mizrak *et al*, 2008). KYM-1 cells also included a small proportion of CD133-positive cells. We sorted CD133+ and CD133− cells and then inoculated them into nude mice subcutaneously, but found no

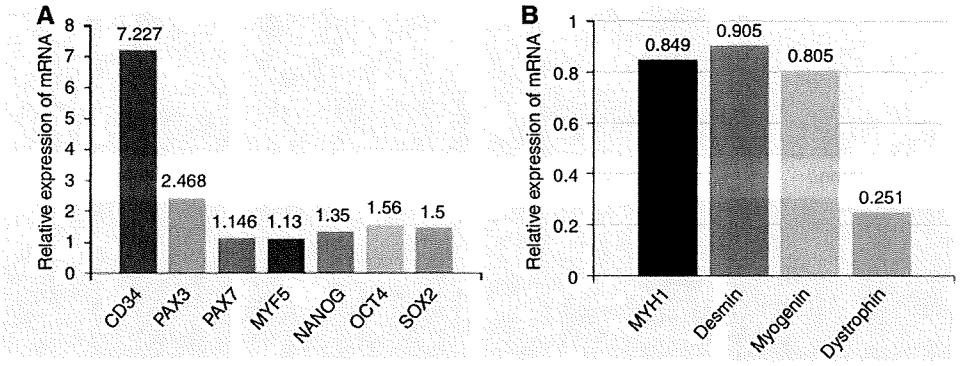

Figure 3 FGFR3-positive cells over-expressed undifferentiated cell genes. (**A**) As demonstrated by real-time PCR, FGFR3 + cells (RICs) over-express several undifferentiated cell marker genes compared with FGFR3− cells (**B**). Real-time PCR revealed that FGFR3 + cells (RICs) exhibited downregulation of several differentiated cell marker genes compared with FGFR3− cells. The comparative Ct (ΔΔCt) method was used to determine fold change in expression using *GAPDH* or *βII-microglobulin*. Each sample was run minimally at three concentrations in triplicate. The experiment was triplicate with similar results.

differences between them in tumourigenisity (data not shown). In addition, we were unable to identify subpopulations by other undifferentiated cell markers. In our study, RICs were enriched in rhabdomyosarcoma subpopulations defined by FGFR3 alone. These findings suggest that sarcomas may differ from other epithelial malignancies, including cancers of the breast, head and neck, lung, pancreas, colon, and prostate (Al-Hajj *et al*, 2003; Collins *et al*, 2005; Fang *et al*, 2005; Kim *et al*, 2005; Dalerba *et al*, 2007; Li *et al*, 2007; Prince *et al*, 2007). It has been reported that FGFR3 is expressed in human muscle from 11 weeks of gestation and is decreased in adult muscle (Sogos *et al*, 1998). Muscle stem cells (muscle satellite cells) express FGFR3 whereas muscle-derived fibroblasts do not (Sheehan and Allen, 1999). These findings suggest that FGFR3 is expressed not only in RICs but also in muscle stem cells. In addition to exhibiting aggressive tumourigenisity, RICs expressed stem cell markers intensely with fewer markers of differentiation. These findings suggest that RICs have the characteristics of undifferentiated cells. In particular, RICs upregulated *CD34* and that downregulated *dystrophin*. These genes are muscle cell linage specific. These findings suggest that RICs are already to some extent committed to the muscle cell linage from more undifferentiated stages such as mesenchymal stem cells.

Rhabdomyosarcoma is composed of embryonal and alveolar subtypes. KYM-1 and RD is established from embryonal rhabdomyosarcoma (McAllister *et al*, 1969; Sekiguchi *et al*, 1985). The subtype of A204 was not described in article of cell line establishment (Giard *et al*, 1973). Embryonal rhabdomyosarcoma contain primitive undifferentiated round cells (Gallego Melcon and Sanchez de Toledo Codina, 2007). Consistent with these findings, we showed that embryonal rhabdomyosarcoma cell lines contain undifferentiated RICs. In addition, real-time PCR revealed that the amount of FGFR mRNA in the embryonal rhabdomyosarcoma biopsy sample was more than that in the normal skeletal muscle or alveolar rhabdomyosarcoma sample.

Mammals have four FGFR tyrosine kinase genes (FGFR1–4) (Eswarakumar *et al*, 2005). FGFRs are composed of an extracellular ligand-binding domain, a transmembrane domain, and a split cytoplasmic tyrosine kinase domain. In this study, we examined only FGFR3. Whether related members of the FGFR family are markers of RICs requires further study. We found that bFGF could maintain and expand RICs. It has been reported that bFGF promotes proliferation and inhibits differentiation of muscle satellite cells (Guthridge *et al*, 1992; Lefaucheur and Sebille, 1995). The bFGF binds to FGFR1, FGFR2, FGFR3, and FGFR4. The binding of bFGF to FGFR3 activate FGF signalling pathway (Ornitz and Leder, 1992; Maric *et al*, 2007). These data suggest that FGFR3 is not only a cell surface marker for RICs but also mediates signals important for RICs maintenance and proliferation. In addition, we found that CNTF reduced the proportion of RICs. There is increasing evidence that chemotherapy and radiation can each efficiently eradicate the majority of malignant cells within neoplastic lesions. However, these regimens frequently fail to eliminate a minor subpopulation of resistant cancer stem cells (Trumpp and Wiestler, 2008). Inhibition of FGFR3 signalling or activation of CNTF signalling might thus be a good candidate for anti-cancer stem cell therapy for rhabdomyosarcoma.

Recent studies have suggested that FGFR3 has a significant function in the pathogenesis and progression of some malignancies including thyroid carcinoma, bladder carcinoma, multiple myeloma, and peripheral T-cell lymphoma (Cappellen *et al*, 1999; Onose *et al*, 1999; Kastrinakis *et al*, 2000; Yagasaki *et al*, 2001; Wolff *et al*, 2005). Whether FGFR3 is a marker of TICs in these malignancies requires further study.

In our study, 33% of single KYM-1 RICs formed tumours. This TIC frequency is somewhat higher than previously reported for other TICs (Singh *et al*, 2003; Hermann *et al*, 2007; Ricci-Vitiani *et al*, 2007; Mizrak *et al*, 2008). These more strong tumourigenic RICs may be more useful than other TICs for examining the molecular mechanisms of tumour initiation, proliferation, anti-apoptotic capacity, and metastasis. Although RICs are enriched in the rhabdomyosarcoma subpopulations defined by FGFR3, not every FGFR3 + cell is an RIC, as 67% of purified single RICs did not form tumours. Quintana *et al* reported that frequency of tumourigenisity in mice depends to a large extent on the status of immunodeficiency (Quintana *et al*, 2008). When melanoma cells were transplanted into NOD/SCID mice, 1 in 111 000 cells formed tumour. When transplanted into highly immunocompromised NOD/SCID interleukin-2 receptor γ chain null mice, 27% of single cells formed tumours. The tumourigenisity of RICs might vary depending on the experimental conditions used, such as the tissue site of xenotransplantation, or differences among recipient immunodeficient mice.

In summary, we identified FGFR3-positive RICs in human rhabdomyosarcoma cell lines. RICs were more strongly tumourigenic than other previously reported TICs. Our easy method of preparing RICs may prove useful for further exploration of

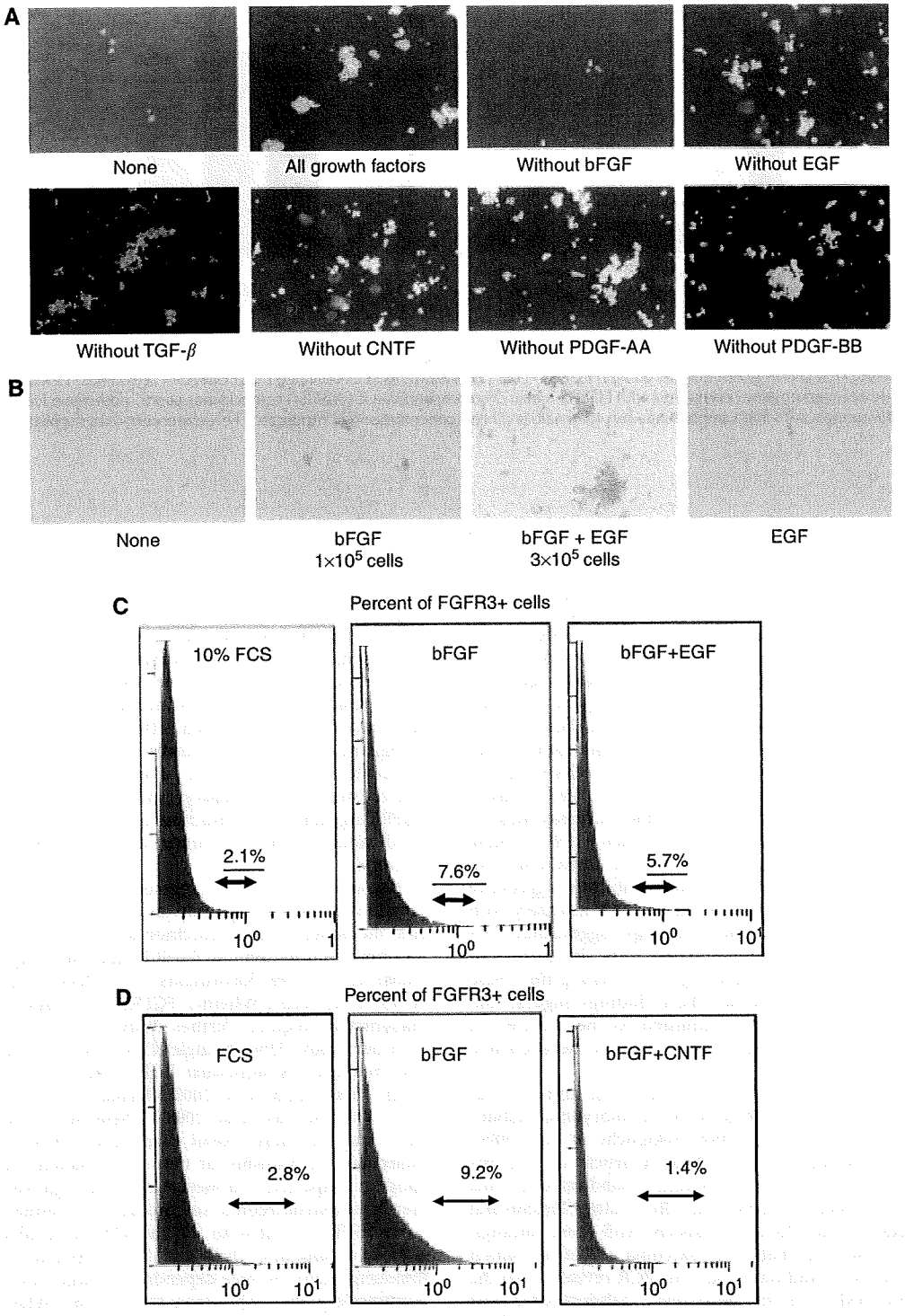

Figure 4 Increase in proportion of KYM-1 FGFR3-positive cells with bFGF. (**A**) KYM-1 cells were cultured in serum-free medium with or without growth factor cocktails. Cells were cultured for 3 weeks and then photographed. When cultured without bFGF, KYM-1 cells did not increase in proportion. (**B**) 1000 KYM-1 cells were cultured for 3 weeks in serum-free medium with bFGF, EGF, or both or neither and were then photographed. When cultured with bFGF, KYM-1 cells were maintained. Cell number was increased with bFGF plus EGF. (**C**) Proportion of FGFR3 + cells was analysed by flow cytometry. KYM-1 cells were cultured in FCS, bFGF, or bFGF plus EGF for 3 weeks. The proportion of FGFR3-positive RICs was increased by bFGF. When cultured with bFGF plus EGF, total number of cells was increased three-fold compared with bFGF alone. All experiments were repeated at least three times with similar results. (**D**) Proportion of FGFR3 + cells was analysed by flow cytometry. KYM-1 cells were cultured in FCS, bFGF, or bFGF plus CNTF for 3 weeks. When cultured with bFGF plus CNTF, the proportion of FGFR3 + cells was markedly decreased compared with bFGF alone or FCS. All experiments were repeated at least three times with similar results.

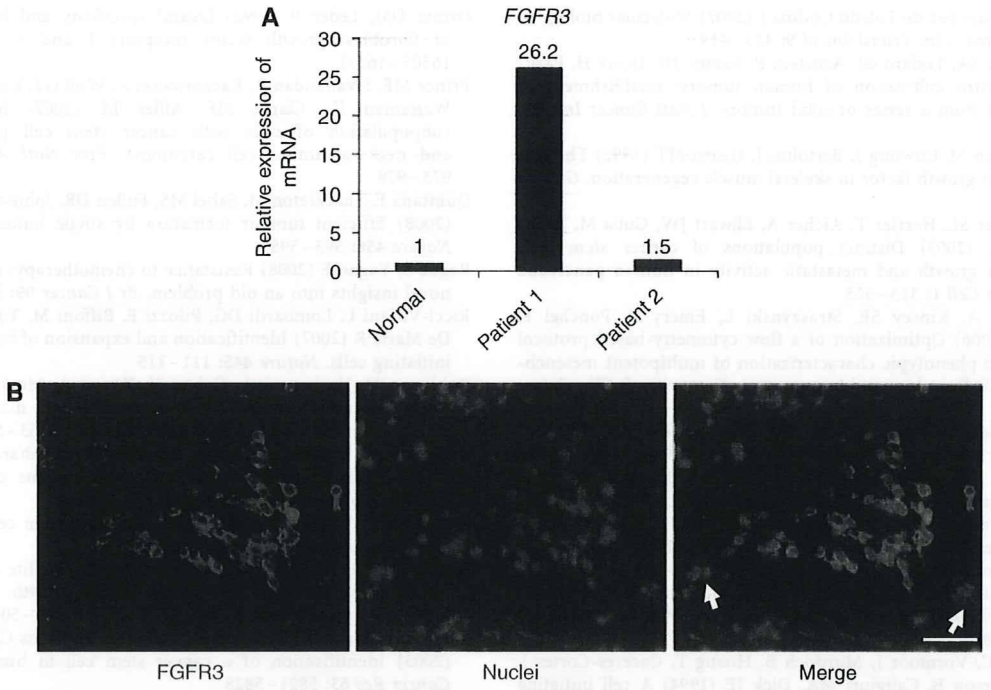

Figure 5 FGFR3 is over-expressed in rhabdomyosarcoma patient specimens. (**A**) We obtained two biopsy specimens of human rhabdomyosarcoma from primary lesions. Pathological examination revealed that patient 1 had embryonal rhabdomyosarcoma and patient 2 had alveolar rhabdomyosarcoma. Real-time PCR revealed that the amount of FGFR mRNA in the embryonal rhabdomyosarcoma biopsy sample was more than that in the normal skeletal muscle or alveolar rhabdomyosarcoma sample. (**B**) Immunohistochemistry revealed that FGFR3 was expressed in a portion of rhabdomyosarcoma patient 1 biopsy specimens. Arrows indicate FGFR3-negative cells. Immunohistochemical examination showed that 11.2 ± 2.8% cells were positive for FGFR3.

pathogenesis of rhabdomyosarcoma and molecular characterisation of cancer stem cells.

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Conflict of interest

The authors declare no conflict of interest.

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