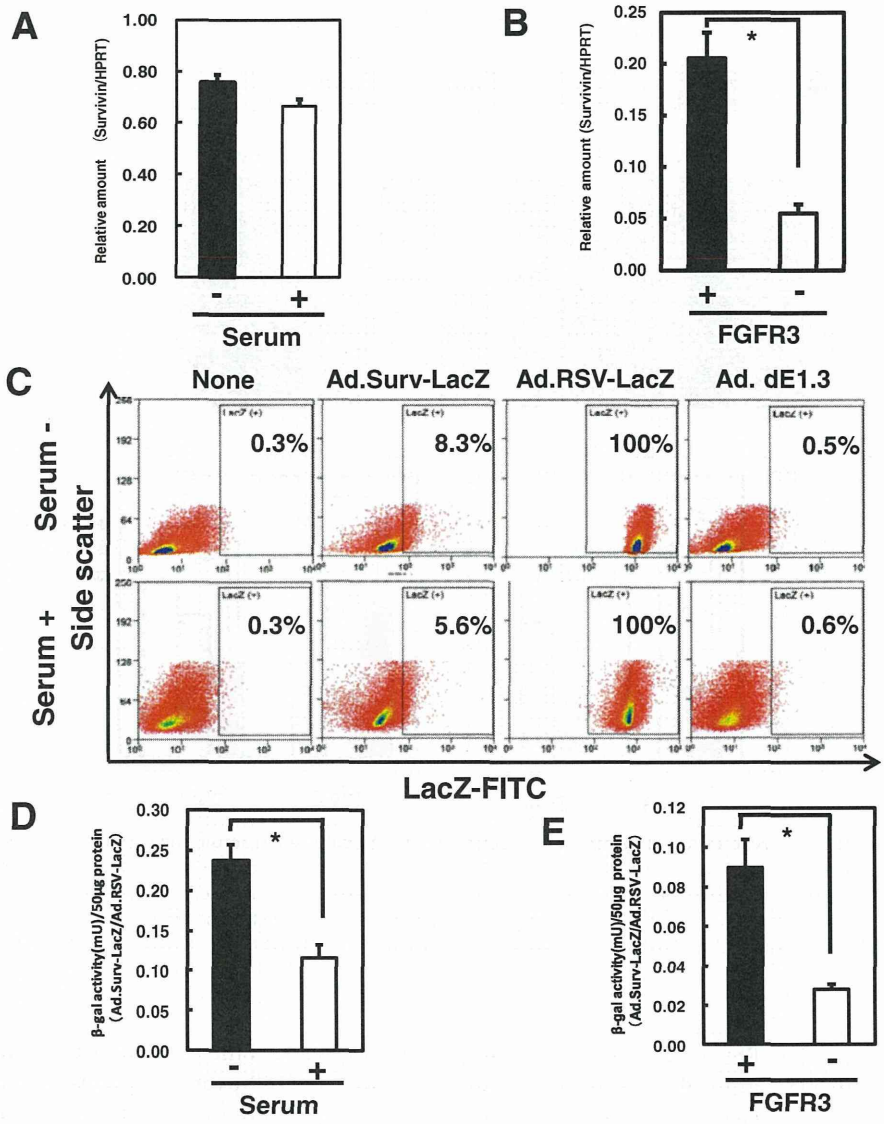


**Figure 4 Survivin mRNA expressions and survivin promoter activity. (A, B)** Expression levels of survivin mRNA under serum-minus and serum-plus conditions **(A)** and in sorted FGFR3-positive and FGFR3-negative cells **(B)** were measured by real-time qRT-PCR. The *HPRT* gene was amplified as an internal control. **(C)** Flow-cytometric analysis to detect LacZ-expressing cells 48 h after Ad.Surv-LacZ or Ad.RSV-LacZ infection at MOI of 30 demonstrated the activities of the survivin and control RSV promoters in individual cells. **(D, E)** β-galactosidase activities in cells *cultured in serum-minus or serum-plus media* **(D)** *and in sorted FGFR3-positive and FGFR3-negative cells* **(E)** *were measured after the same* infection described in **(C)**. *, $P < 0.05$.

active in RSCs than in progeny. Furthermore, these results confirm that the adenovirally transduced promoter region functions well in RSCs.

### Surv.m-CRA exhibited more efficient replication and cytotoxicity against RSC-enriched cell fractions *in vitro*

The construction of the Surv.m-CRA used in this study is shown in Figure 5A. We first explored apparent viral replication and the cytotoxic effects of Surv.m-CRAs against RSC-enriched and RSC-exiguous cell fractions by microscopically observing the spread of cells expressing EGFP and the swollen cells that are a characteristic feature of the adenoviral cytopathic effect (Figure 5B). In both fractions, Surv.m-CRA induced prominent viral replication and cytotoxic effects as early as 3 days after infection at an MOI of 1. To accurately and quantitatively analyze the cytotoxic effect, we performed the WST viability assay (Figure 5C). Surv.m-CRA potently induced cell death in both groups. The cytotoxicity was somewhat more prominent under the RSC-enriched

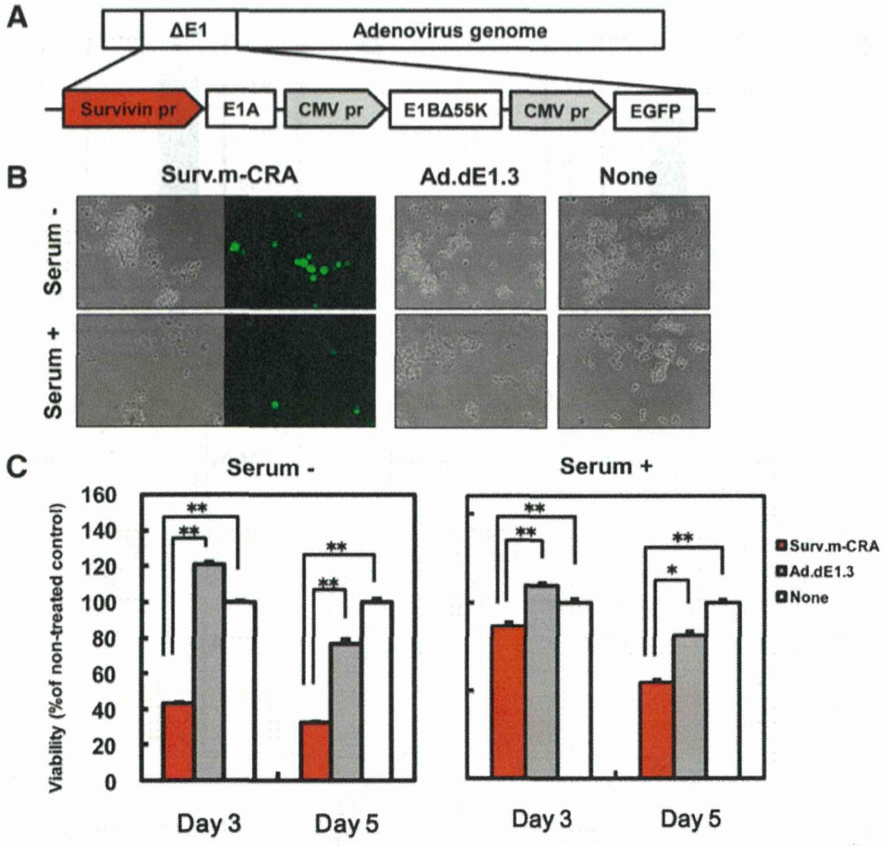


**Figure 5 Viral replication of Surv.m-CRA and cytotoxicity against KYM-1 cells cultured in serum-minus and serum-plus media.** **(A)** Schematic representation of the construction of Surv.m-CRA. **(B)** Representative images of phase-contrast and fluorescence-microscopic pictures 5 days after infection with Surv.m-CRA, Ad.dE1.3, or no virus (none) of KYM-1 cells cultured in serum-minus or serum-plus media. Viral replication and cytopathic effects were prominent in both groups after Surv.m-CRA infection. **(C)** Cell viability was determined by WST-8 assay 3 or 5 days after infection.*, $P < 0.05$. **, $P < 0.005$. Surv.m-CRA efficiently replicated and potently induced cell death in both cell fractions cultured in serum-minus and serum-plus media.

(serum-minus) than the RSC-exiguous (serum-plus) conditions (e.g., $P < 0.005$ and $P < 0.05$ between Surv.m-CRA and Ad.dE1.3 in RSC-enriched and RSC-exiguous, respectively, on day 5).

### Surv.m-CRA exhibited more efficient replication and cytotoxicity against sorted FGFR3-positive cells *in vitro*

To directly examine the therapeutic potentials of Surv.m-CRA against RSCs and progeny, FGFR3-positive and -negative cells were sorted and subsequently cultured in serum-minus and serum-plus media, respectively. Whereas FGFR3-positive cells proliferated somewhat more rapidly than FGFR3-negative cells after sorting and regrowth, the difference was not drastic (Figure 6A and Figure 7). Both cell types were infected with Surv.m-CRA, the control Ad.dE1.3, or no virus (none) 3 days after the sorting (*i.e.*, when the cells had recovered from possible damage related to the sorting procedure), and viral replication and cytotoxic effects were assessed 1, 3, or 5 days later. Efficient viral replication, assessed by the spread of EGFP-expressing cells, was observed in both cell types (Figure 6B). In the case of cells infected with Surv.m-CRA, the number of FGFR3-positive cells was noticeably smaller than the number of FGFR3-negative cells after 5 days of growth (Figure 6B and Figure 7). This result suggests that viral replication and the resulting cytotoxic effects of Surv.m-CRA were more prominent in RSCs than in the progeny. According to the microscopic analysis, the viability assay accurately and quantitatively demonstrated that the cytotoxicity of Surv.m-CRA was higher in FGFR3-positive RSCs than in FGFR3-negative progeny (Figure 6C and Figure 7). A statistically significant difference in the percentage of viable cells between Surv.m-CRA–treated and the control replication-defective Ad.dE1.3–treated groups was observed in sorted FGFR3-positive cells, but not in sorted FGFR3-negative cells. Together, the results from all the *in vitro* experiments revealed that Surv.m-CRA

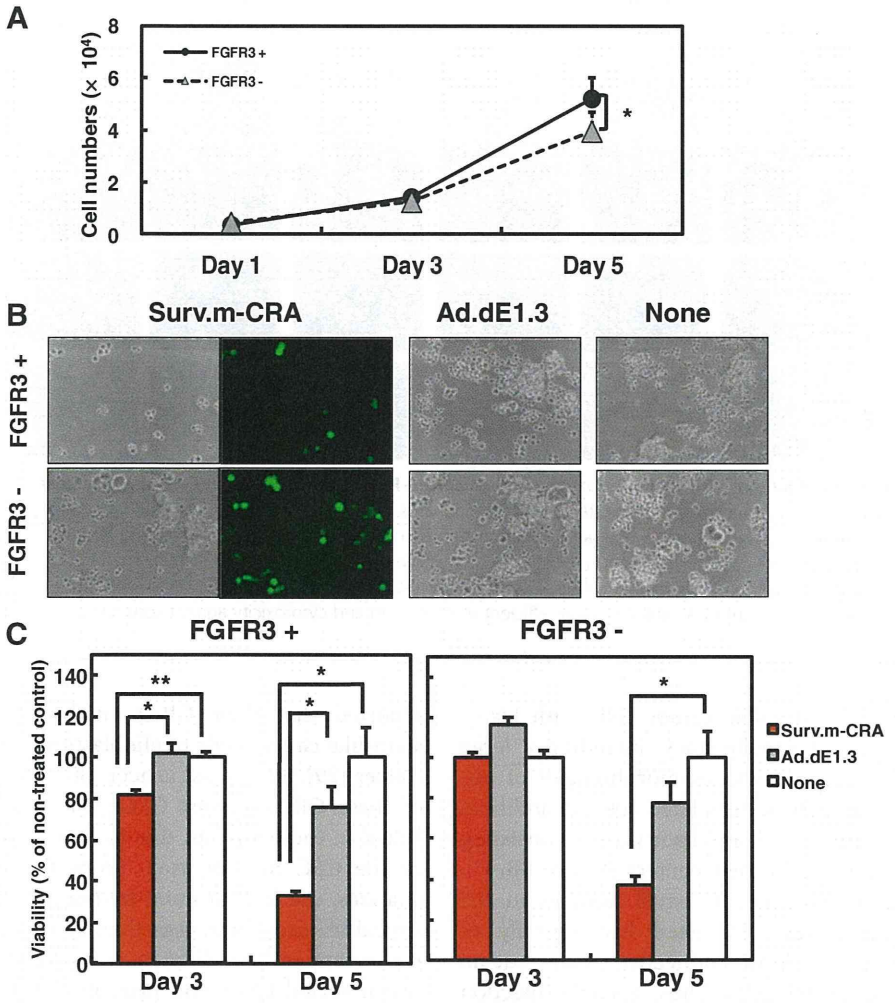


**Figure 6 Viral replication of Surv.m-CRA and cytotoxicity against sorted FGFR3-positive and FGFR3-negative cells. (A)** Growth curves of sorted cells. Whereas FGFR3-positive cells proliferated somewhat more rapidly than FGFR3-negative cells, the difference was not drastic. *, $P < 0.05$. **(B)** Representative images of phase-contrast and the fluorescence-microscopic pictures 5 days after infection with Surv.m-CRA, Ad.dE1.3, or no virus. Whereas viral replication and cytopathic effects of Surv.m-CRA were prominent in both groups, the proportion of remaining cells 5 days after Surv.m-CRA infection at an MOI of 1 was lower in sorted FGFR3-positive cells than in sorted FGFR3-negative cells. **(C)** Cell viability was determined by WST-8 assay 3 or 5 days after infection. Cytotoxic effects of Surv.m-CRA were statistically significantly higher than those of Ad.dE1.3 solely in the sorted FGFR3-positive cells. *, $P < 0.05$; **, $P < 0.005$ between Surv.m-CRA and either Ad.dE1.3 or no virus.

induced prominent viral replication and cell death in all rhabdomyosarcoma cell fractions, including RSCs, and that such effects were more potent in RSCs than in progeny. This is consistent with the higher survivin expression levels and promoter activities in RSCs relative to progeny, shown above (Figure 3).

### Surv.m-CRA had potent *in vivo* therapeutic effects on tumors generated from RSC-enriched rhabdomyosarcoma cells in mice

We subcutaneously inoculated RSC-enriched rhabdomyosarcoma cells into mice to generate a tumor nodule, into which Surv.m-CRA ($1 \times 10^9$ pfu), the control Ad.dE1.3, or no virus (none) was subsequently directly injected. Periodic measurement of tumor size revealed that a single intratumoral injection of Surv.m-CRA significantly inhibited tumor growth in comparison to Ad.dE1.3 42 days after adenoviral injection (Figure 8A, B). By contrast, there was no significant difference in tumor size on day 42 between the controls, *i.e.*, Ad.dE1.3–treated and PBS–treated mice. Furthermore, histopathologic analysis of viable and dead tumor cells clearly demonstrated the potent therapeutic effects of Surv.m-CRAs beyond what could be shown by the macroscopic analysis (Figure 8B, C): the macroscopically large tumor nodules in control mice treated with either Ad.dE1.3 or

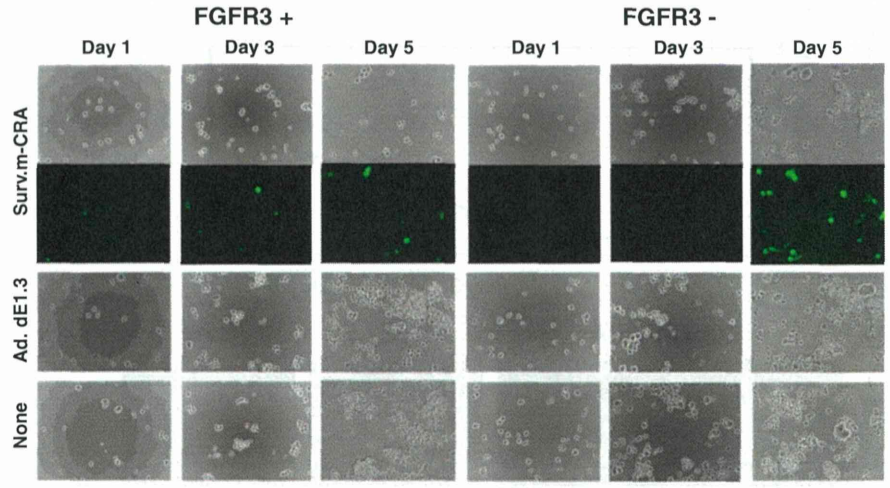


**Figure 7 Viral replication of Surv.m-CRA and cytotoxicity against sorted FGFR3-positive and FGFR3-negative cells.** Sorted FGFR3-positive and FGFR3-negative cells were infected with Surv.m-CRA, Ad.dE1.3, or no virus (none) at an MOI of 1. The cells were observed under phase-contrast and fluorescence microscopy 1, 3, and 5 days after infection. Representative phase-contrast (upper) and fluorescence (lower) images are shown at 50× magnification. In the case of no infection (none), FGFR3-positive cells proliferated more rapidly than FGFR3-negative cells after 3 and 5 days. By contrast, the proportion of remaining cells 5 days after Surv.m-CRA infection was lower in FGFR3-positive than in FGFR3-negative cells. *These results suggest that Surv.m-CRA exhibited more efficient replication in and cytotoxicity against sorted FGFR3-positive than* FGFR3-negative cells.

PBS consisted mainly of viable tumor cells with histological features of active malignancy, including a large number of mitoses, heterogeneous morphologies of nuclei and cells, and densely accumulated cells; in addition, these tumors contained small and spotty areas consisting of spontaneous necroses in their centers (Figure 8B, C). By contrast, the macroscopically small nodules in the Surv.m-CRA–treated mice consisted histologically of large necrotic areas with loose connective tissues but no apparent viable malignant cells. Thus, a single injection of Surv.m-CRA into tumor nodules generated by implantation of RSC-enriched rhabdomyosarcoma cells in mice exhibited a potent therapeutic effect *in vivo*.

## Discussion

We previously showed that Surv.m-CRAs could treat a broad range of cancer types more efficiently and safely (*i.e.*, cancer-specifically) than Tert.m-CRAs, which are among the best CRAs [11,12,25]. The results of this study demonstrate not only that Surv.m-CRAs can efficiently kill all populations of rhabdomyosarcoma cells, including both RSCs and their progeny (*i.e.*, the bulk of malignant cells), but also that the antitumor effects of Surv.m-CRAs are higher against RSCs. This feature is clinically meaningful and promising because the therapeutic mode of Surv.m-CRAs is opposite to that of conventional chemoradiotherapies, and because Surv.m-CRA may overcome, at least in part, the serious drawbacks of current cancer treatments.

Some previous studies assessed the cytotoxic effects of oncolytic viruses on CSCs [26], and several groups reported that their CRAs might be effective against stem-like cancer cells of glioblastoma [27,28], esophageal cancer [29], and breast cancer [30]. However, the efficacy of these CRAs against CSCs was not accurately established in these studies, due to limitations of the analyses or the CSC models used. From the standpoint of the analyses, the lack of point-by-point comparisons of biological features, both regarding genes that regulated viral replication and the differences in the effects of CRAs between sorted CSCs and progeny, led to unclear conclusions regarding the most important question: how efficiently do these viruses kill CSCs in comparison to their progeny [27,28]? From the standpoint of the CSC models, some previous studies used radioresistant cancer cells as cancer stem-like cells, but did not examine sorted CSCs [28]. Although radiation treatment may enrich CSCs, the radioresistant cancer cells are not equivalent to CSCs. Thus, although the previous studies did provide some important information, their results may not allow a generalized assessment regarding the potentials of CRAs against CSCs. Therefore, the efficacy of each oncolytic virus and CRA against CSCs should be individually and carefully assessed in the proper experimental models.

To accurately assess the biological features and therapeutic potential of Surv.m-CRAs against CSCs, in a previous study we identified FGFR3 as a useful marker that allows accurate monitoring and purification of RSCs; a single implanted FGFR3-positive rhabdomyosarcoma cell could form a tumor *in vivo*, whereas FGFR3-negative

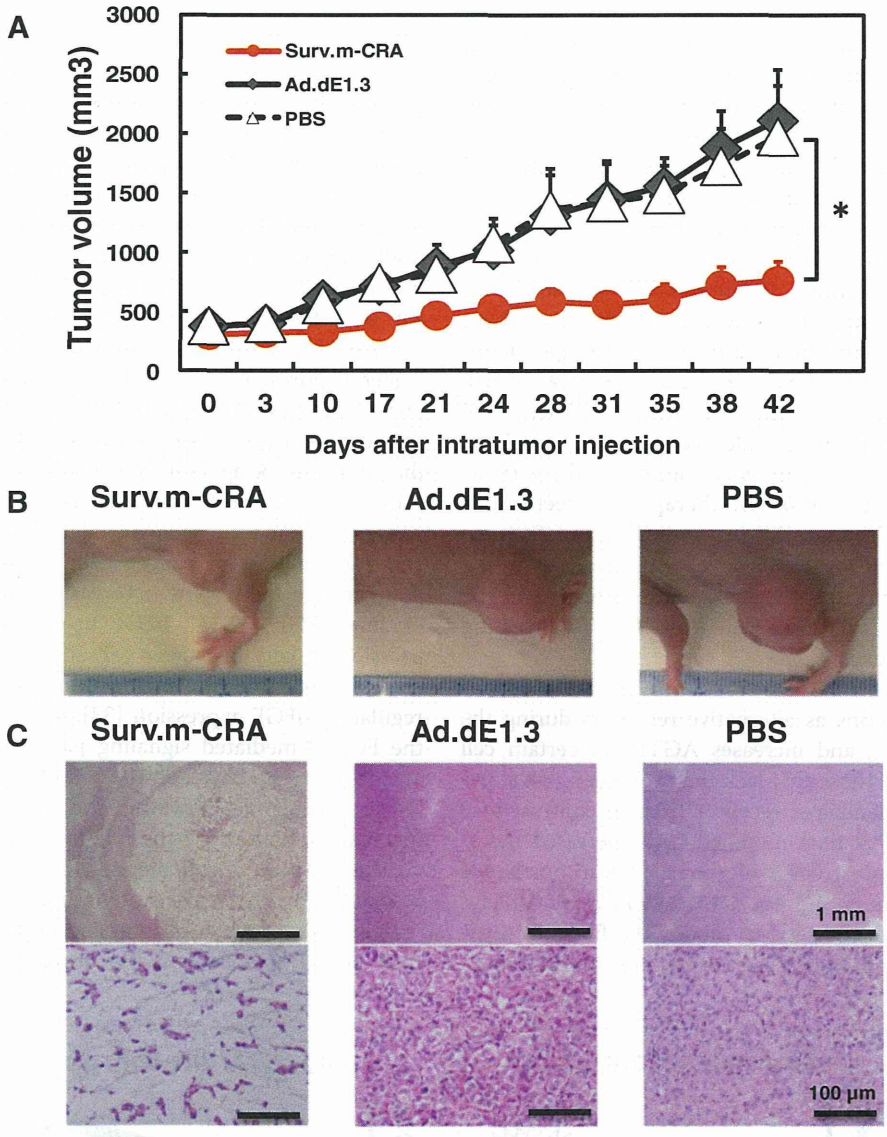


**Figure 8 *In vivo* therapeutic effects of Surv.m-CRA against tumors in mice.** Tumor nodules were generated in mice by implantation of RSC-enriched rhabdomyosarcoma cells, and a single intratumoral injection of Surv.m-CRA, Ad.dE1.3, or PBS was subsequently administered to each nodule. **(A)** The macroscopic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

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

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

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

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

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

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

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

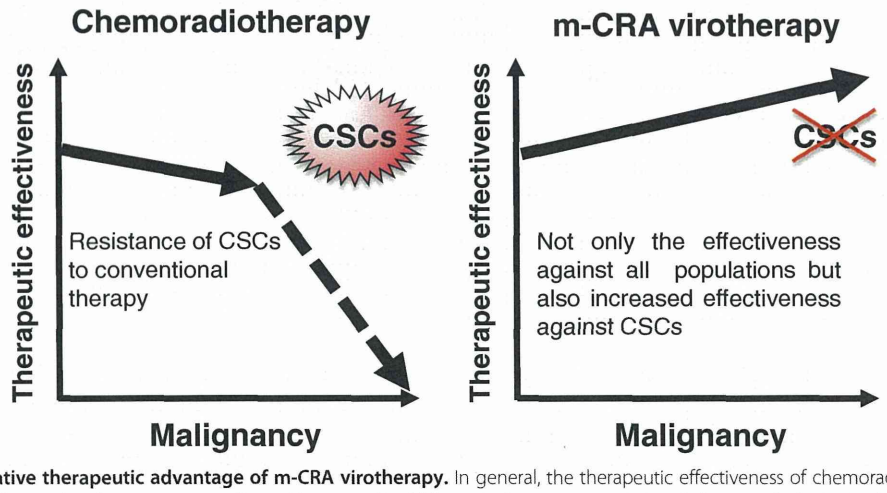


**Figure 9 The putative therapeutic advantage of m-CRA virotherapy.** In general, the therapeutic effectiveness of chemoradiotherapy is decreased in the advanced malignant tumor cells, and in particular, CSCs are resistant to conventional chemoradiotherapies. In contrast, not only m-CRA virotherapy can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

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

## Conclusion

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

### Abbreviations

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

### Competing interests

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

### Authors' contributions

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

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Original Articles

# Ribosomal protein S3 regulates GLI2-mediated osteosarcoma invasion

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ABSTRACT

It has been reported that *GLI2* promotes proliferation, migration, and invasion of mesenchymal stem cell and osteosarcoma cells. To examine the molecular mechanisms of GLI2-mediated osteosarcoma metastasis, we performed a microarray analysis. The gene encoding ribosomal protein S3 (*RPS3*) was identified as a target of GLI2. Real-time PCR revealed that *RPS3* was upregulated in osteosarcoma cell lines compared with normal osteoblast cells. Knockdown of *GLI2* decreased *RPS3* expression, whereas forced expression of a constitutively active form of GLI2 upregulated the expression of *RPS3*. *RPS3* knockdown by siRNA decreased the migration and invasion of osteosarcoma cells. Although forced expression of constitutively active GLI2 increased the migration of human mesenchymal stem cells, knockdown of *RPS3* reduced the up-regulated migration. In contrast, forced expression of *RPS3* increased migration and invasion of osteosarcoma cells. Moreover, reduction of migration by *GLI2* knockdown was rescued by forced expression of *RPS3*. Immunohistochemical analysis showed that RPS3 expression was increased in primary osteosarcoma lesions with lung metastases compared with those without. These findings indicate that GLI2–RPS3 signaling may be a marker of invasive osteosarcoma and a therapeutic target for patients with osteosarcoma.



## Introduction

Although osteosarcoma is a rare malignant tumor, it is the most common malignancy in pediatrics [1]. Because osteosarcomas have a high tendency to metastasize, they are ranked among the most frequent causes of cancer-related death in childhood tumors [2]. After the introduction of adjuvant chemotherapy in the 1970s, survival has increased from 10–20% to 50–80% [1,3]. However, during the last two decades no further improvements have been made in terms of survival [4]. An estimated 15%–20% of patients with osteosarcoma have detectable pulmonary metastases at initial presentation [5,6], and these patients have worse prognosis compared with patients without metastasis [7–9]. There are substantial differences in prognosis for patients who have localized disease compared with metastases at the time of diagnosis. The molecular mechanisms underlying the metastasis of osteosarcoma remain poorly understood.

The Hedgehog pathway plays an important role during embryonic development by regulating cell proliferation and differentiation [10]. Hedgehog ligands (Sonic, Indian, and Desert hedgehog) bind to Patched1 (PTCH1) receptor, which results in release of Smoothened (SMO) to promote dissociation of the suppressor of fused (SUFU) glioma-associated oncogene homolog (GLI) complex. This dissociation promotes nuclear translocation and activation of the GLI transcription factors (GLI1/-2/-3) to induce transcription of several target genes [11,12].

Recently, abnormal activation of Hedgehog signaling has been reported in many malignancies [13–15]. Aberrant expression of GLI1 promotes tumorigenesis and invasiveness of glioblastoma as

*Abbreviations:* RPS3, ribosomal protein S3; PTCH1, Patched1; SMO, Smoothened; SUFU, suppressor of fused; GLI, glioma-associated oncogene homolog; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NHOst, normal human osteoblast cells; RNAi, RNA interference; ACTB, β-actin; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline Tween-20; rSHH, recombinant sonic hedgehog; ATO, arsenic trioxide.

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well as prostate and breast cancers [13,16]. Additionally, GLI2 promotes proliferation of hepatocellular carcinoma and prostate cancer [17,18]. We and other groups have reported that the expression levels of GLI2 were associated with poor prognosis in osteosarcoma patients [19], and it is known that GLI2 plays roles in proliferation, cell apoptosis, and sensitivity to chemotherapeutic drugs in osteosarcoma [19–21].

In addition, we showed that inhibition of GLI2 suppresses metastasis of osteosarcoma cells [22].

The human ribosomal protein S3 (RPS3), which is a component of the small 40S ribosomal subunit, is mainly involved in ribosomal maturation and initiation of translation through association with initiation factors [23]. RPS3 plays various extra-ribosomal functions including DNA repair [24–29], apoptosis [30], cell signaling [25,31–33], transcriptional regulation [34–36], and transformation [25,28].

In this study, we identified RPS3 as a novel downstream factor of GLI2 that mediates migration and invasion of osteosarcoma.

## Materials and methods

### *Cell lines and reagents*

The osteosarcoma cell lines 143B, Saos-2, and USOS, HOS, and MG63 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HS-Os-1 cell line and HuO9 were obtained from Riken Bioresource Center (Tsukuba, Japan). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The human hTERT-immortalized mesenchymal stem cell line (YKNK-12) was kindly provided by Dr. Kobayashi (Okayama University) [37]. YKNK-12 cells were grown in the culture medium described above. Cell lines were maintained for up to 20 passages at 37 °C in 5% $CO_2$. Recombinant Sonic Hedgehog (rSHH) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). ATO (Arsenic Trioxide) was purchased from Nippon Shinyaku Co., Ltd. (Tokyo, Japan).

### *Patient specimens*

Human osteosarcoma tissue and normal bone tissue were obtained from osteosarcoma patients at the time of surgery. Normal bone tissues from the same resected specimens were obtained from areas adjacent to the tumor in each patient. Clinicopathological information is described in Table 1. The study protocol was approved by the Institutional Review Board of Kagoshima University. Informed consent was obtained from all patients.

### *Immunohistochemistry*

Immunohistochemical analyses for osteosarcoma were performed as previously described [19,22]. The duration of decalcification was between 10 and 15 min. For tissue staining, antigen retrieval was performed using ER1 antigen retrieval buffer (Leica Microsystems, Inc, Wetzlar, Germany) for all sections. Following incubation with RPS3 antibody (1:4000; 66046-1-Ig; Proteintech Inc., Chicago, IL, USA), sections were stained on a Leica Bond-III (Leica Microsystems) using the Bond Refine Polymer detection kit (Leica Microsystems). We counted the number of cells stained above a moderate degree in the cytoplasm. The results of immunohistochemical staining were evaluated by calculating the percentage of positively-stained osteosarcoma cells. Immunostaining was considered positive if at least 5% of osteosarcoma cells were stained. The positive rates of GLI2 and RPS3 in osteosarcoma cells were calculated from three visual fields in each tissue. Pancreatic tissue was used as a positive control for the RPS3 antibody [38] (Appendix: Supplementary Fig. S1A).

### *Reverse transcriptase quantitative-PCR*

Real-time PCR analyses were performed as previously described [39]. Total RNA was extracted from the cell lines and tissue specimens using miR-Vana RNA isolation kits (Life Technologies, Grand Island, NY, USA) and reverse transcribed using ReverTra Ace-T (Toyobo Co, Ltd, Osaka, Japan). The cDNA was amplified by real-time PCR using SYBR Green (Life Technologies). Real-time PCR was performed on MiniOpticon™ (Bio-Rad Laboratories K.K., Tokyo, Japan). Comparative Ct (ΔΔCt) analysis was performed to evaluate the fold-change of mRNA expression using the expression of β-actin (*ACTB*) as a reference. PCR was performed in triplicate for all samples. All primers were designed using Primer 3 software. For rSHH treatments, $1 \times 10^5$ cells were seeded in 6-well plates overnight. The medium was changed to 0.5% FBS/DMEM and 500 ng/mL rSHH was added. After 48 h, real-time PCR was performed. For ATO treatments, $1 \times 10^5$ cells were seeded in 6-well plates overnight. The medium was changed to 0.5 μM ATO/DMEM. After 24 h, real-time PCR was performed. The following primers were used: *ACTB*, 5-AGAAAATCTGGCACCACACC-3 and 5-AGAGGCGTACAGGGATAGCA-3; *RPS3*, 5-AACTGGTAAGATTGGCCCTAAGAAG-3 and 5-TGTTATGCTGTGGGGACTGG-3.

### *Plasmid constructs and gene transfer*

Control siRNA (S20C-0600) was purchased from B-Bridge International, Inc. (Cupertino, CA, USA). *RPS3* siRNA (sc-96950) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). All siRNA transfection experiments were performed using Lipofectamine RNAiMAX (Life Technologies) transfection reagent according to the manufacturer's instructions and GLI2ΔN expression vector was constructed as previously described [20]. The pCMV6-Entry Vector (PS100001) and RPS3 expression vector (RC205936) were purchased from OriGene Technologies (Rockville, MD, USA). All plasmid transfection experiments were performed using FuGENE6 transfection reagents (Roche, Basal, Switzerland) according to the manufacturer's protocol.

### *Western blotting*

Western blotting analyses were performed as previously described [40]. Cells were lysed using NP40 buffer, which contained 0.5% NP40, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 3 mM PMSF (Wako Chemicals, Kanagawa, Japan), 5 mg/mL aprotinin (Sigma-Aldrich Co. LLC, St. Louis, MO, USA), 2 mM sodium orthovanadate (Wako Chemicals), and 5 mM EDTA. Lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS–polyacrylamide gel electrophoresis (Bio-Rad Laboratories KK), and transferred to polyvinylidene fluoride membranes (Caliper Life Sciences, Mountain View, CA, USA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) buffer and incubated in primary antibodies diluted in TBST for 1 h at room temperature or overnight at 4 °C. Blots were washed using TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) in TBST buffer for 45 min at room temperature. Immunocomplexes were visualized using an enhanced chemiluminescence kit (GE Healthcare, Tokyo, Japan). Primary antibodies used were RPS3 (1:1000; #9538, Cell Signaling Technology, Boston, USA) and d in primary anti; DM1A, Sigma-Aldrich Co. LLC). Densitometric analysis of samples was performed using NIH Image J software.

### *Migration assay*

Cells seeded ($1 \times 10^5$) in 12-well plates were carefully scratched with the tip of a 200-luted in to create a gap. Cells were washed with phosphate-buffered saline to remove detached cells and then supplied with new growth medium. Ten hours after incubation, images of the gap width were acquired using an Olympus DP12-2

**Table 1**
Clinicopathological data and RPS3 and GLI1 expression in osteosarcoma patients.

| Case no. | Age | Gender | Site | Type | Lung metastasis | Chemotherapy | TNM classification | Survival time (month) | RPS3 positive tumor cells (%) | GLI2 positive tumor cells (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| #1 | 9 | Male | Right tibia | Conventional | – | + | IIB | 160, alive | 55 | 46.3 |
| #2 | 18 | Male | Right tibia | Conventional | + | + | IVA | 42, death | 65.9 | 73.9 |
| #3 | 10 | Male | Right tibia | Fibroblastic | – | + | IIB | 144, alive | 60 | 60.4 |
| #4 | 48 | Female | Right femur | Conventional | + | + | IVA | 70, death | 70.5 | 78.1 |
| #5 | 15 | Male | Left femur | Conventional | + | + | IVA | 71, death | 78.3 | 77.5 |
| #6 | 21 | Male | Right femur | Telangiectatic | – | + | IB | 79, alive | 68.4 | 27.3 |
| #7 | 18 | Male | Left femur | Conventional | – | + | IIB | 81, alive | 61.2 | 47.9 |
| #8 | 19 | Female | Left femur | Conventional | – | + | IIB | 48, death | 48.8 | 69 |
| #9 | 18 | Male | Right femur | Chondroblastic | – | + | IIB | 171, alive | 37 | 54 |

Survival time in months following first diagnosis. Immunohistochemical staining results were evaluated by calculating the percentage of positively-stained osteosarcoma cells. Tumor cells were considered RPS3- or GLI2-positive if at least 5% of osteosarcoma cells were stained.