airway hyper-responsiveness, inflammation, remodeling, and eosinophil function in asthma (51). Okunishi *et al* (52) reported that HGF suppresses antigen-induced T-cell priming by regulating the functions of dendritic cells through IL-10 downregulation in the antigen-sensitization phase. By contrast, they found that repeated treatment with HGF induced Th2 immune responses with the upregulation of IL-10 by DCs in the chronic inflammation phase of a mouse model of collagen-induced arthritis. Thus, it is clear that HGF induces various immune responses in different disease models. However, further analysis is required to clarify the effects of HGF on the immune system.

In conclusion, we have shown that a single round of intramuscular injections of adenoviral hHGF is sufficient to inhibit apoptosis and reconstitute the epithelium in a mouse model of DSS-induced colitis. Based on these results, this approach shows promise for clinical application in IBD.

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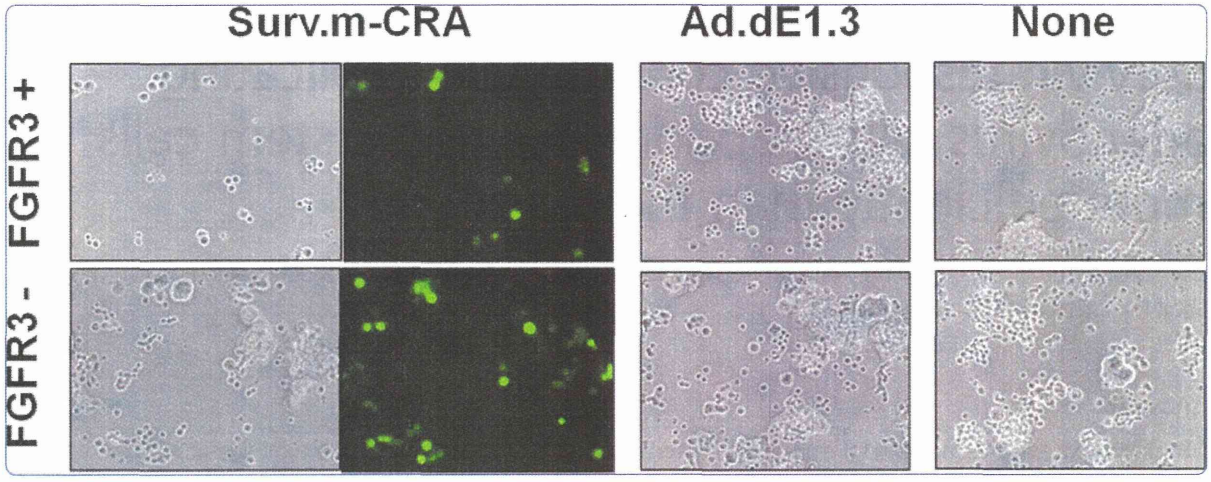

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Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny

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Abstract

Background: Effective methods for eradicating cancer stem cells (CSCs), which are highly tumorigenic and resistant to conventional therapies, are urgently needed. Our previous studies demonstrated that survivin-responsive conditionally replicating adenoviruses regulated with multiple factors (Surv.m-CRAs), which selectively replicate in and kill a broad range of cancer-cell types, are promising anticancer agents. Here we examined the therapeutic potentials of a Surv.m-CRA against rhabdomyosarcoma stem cells (RSCs), in order to assess its clinical effectiveness and usefulness.

Methods: Our previous study demonstrated that fibroblast growth factor receptor 3 (FGFR3) is a marker of RSCs. We examined survivin mRNA levels, survivin promoter activities, relative cytotoxicities of Surv.m-CRA in RSC-enriched (serum-minus) vs. RSC-exiguous (serum-plus) and FGFR3-positive vs. FGFR3-negative sorted rhabdomyosarcoma cells, and the *in vivo* therapeutic effects of Surv.m-CRAs on subcutaneous tumors in mice.

Results: Both survivin mRNA levels and survivin promoter activities were significantly elevated under RSC-enriched relative to RSC-exiguous culture conditions, and the elevation was more prominent in FGFR3-positive vs. FGFR3-negative sorted cells than in RSC-enriched vs. RSC-exiguous conditions. Although Surv.m-CRA efficiently replicated and potently induced cell death in all populations of rhabdomyosarcoma cells, the cytotoxic effects were more pronounced in RSC-enriched or RSC-purified cells than in RSC-exiguous or progeny-purified cells. Injections of Surv.m-CRAs into tumor nodules generated by transplanting RSC-enriched cells induced significant death of rhabdomyosarcoma cells and regression of tumor nodules.

Conclusions: The unique therapeutic features of Surv.m-CRA, *i.e.*, not only its therapeutic effectiveness against all cell populations but also its increased effectiveness against CSCs, suggest that Surv.m-CRA is promising anticancer agent.

Keywords: Cancer stem cells, Conditionally replicating adenovirus, Fibroblast growth factor receptor 3, Gene therapy, Oncolytic adenovirus, Promoter, Rhabdomyosarcoma, Survivin, Tumor-initiating cell, Virotherapy

Background

Accumulating data have suggested that cancer stem cells (CSCs), also called tumor-initiating cells, are a small but specialized population of tumor cells that possess high capacity for tumor initiation, invasion, and metastasis, as well as for self-renewal [1]. After most cells in the tumor are killed by conventional chemotherapy or radiotherapy, residual CSCs are believed to give rise to the bulk populations of tumor-cell progeny and recapitulate the original tumor nodule [2]. From the standpoints of clinical oncology and therapeutics, the most critical feature of CSCs is that they are highly resistant to conventional chemoradiotherapies [3,4], because they are predominantly in a dormant or slow-growing phase of the cell cycle [3] and they express high levels of multiple drug-resistance transporters [4]. Because the poor prognosis of patients with malignant tumors is caused, at least in part, by CSCs, the development of effective therapies against CSCs is urgently needed.

Rhabdomyosarcoma is the most common soft-tissue malignancy in children and adolescents [5]. Metastatic rhabdomyosarcoma is often incurable, and is associated with poor prognosis; approximately 20% of rhabdomyosarcoma

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patients have disseminated disease at the time of diagnosis [6]. Whereas current treatment for rhabdomyosarcoma relies on chemotherapy, the cytotoxic actions of chemotherapeutic agents are not only ineffective but also non–tumor-specific in treatment of advanced and metastatic tumors. Therefore, these agents can impair normal development and cause secondary cancers in some growing children [5]. To develop a novel and innovative therapy against malignant rhabdomyosarcoma, we previously identified rhabdomyosarcoma stem cells (RSCs) and showed that fibroblast growth factor receptor 3 (FGFR3) is a marker of RSCs [7]. For instance, implantation of a single FGFR3-positive KYM-1 rhabdomyosarcoma cell can form a tumor nodule *in vivo* consisting of histologically defined rhabdomyosarcoma cells, whereas a single FGFR3-negative cell cannot form such nodules [7]. Likewise, the careful analyses in our previous study characterized FGFR3-positive rhabdomyosarcoma cells as RSCs.

Conditionally replicating adenoviruses (CRAs), also called oncolytic adenoviruses, replicate predominantly in tumor cells, which they kill via apoptosis mediated by adenoviral proteins; therefore, CRAs are promising anticancer agents [8,9]. We previously developed a method to efficiently construct diverse CRAs that can specifically target and/or efficiently treat malignant tumors using multiple factors (m-CRAs) [10]. Our m-CRA construction system expedited the process of generating, modifying, and testing diverse m-CRAs with the goal of developing an ideal m-CRA for tumor therapy; indeed, our m-CRA strategy increased the potential cancer specificity of virotherapy [10-12]. Survivin, a new member of the inhibitor of apoptosis (IAP) gene family, is expressed at high levels in cancerous but not normal tissues, and high survivin expression levels are positively correlated with poor prognosis, an accelerated rate of recurrence, and increased resistance to therapy in cancer patients [13,14]. We developed several types of survivin-responsive m-CRAs (Surv.m-CRAs) in which adenoviral E1A was regulated by the promoter of survivin; in some versions of these viruses, the p53-binding domain in E1B was deleted (*i.e.*, E1B55KD), the Rb-binding domain in E1A was deleted, or the native E1B promoter was replaced with another cancer-specific promoter [11,12]. All Surv.m-CRAs induced potent *in vitro* and *in vivo* cytotoxic effects against a variety of malignant tumors, and exhibited stronger and more cancer-selective phenotypes than telomerase reverse transcriptase (Tert)-responsive m-CRAs (Tert.m-CRAs), which are currently among the best CRAs [11,12]. Furthermore, certain types of Surv.m-CRAs significantly increased cancer specificity (*i.e.*, safety) without reduced anticancer effects [11].

CSCs are resistant to conventional chemoradiotherapies, and the therapeutic potentials of Surv.m-CRAs against CSCs have not been well examined. In order to evaluate the clinical usefulness of Surv.m-CRAs against malignant and incurable tumors, it will be necessary to perform careful comparative studies of endogenous survivin expression levels, activity of transduced survivin promoters, and relative antitumor effects on CSCs and their progeny. More generally and importantly, it has not yet been clearly elucidated whether transcriptional targeting using CRAs is a useful strategy for treating CSCs. Because FGFR3-positive RSCs are a useful model for CSCs, we examined the biological features of survivin and compared the therapeutic potentials of Surv.m-CRA against RSCs and progeny tumor cells.

Methods

Cells and cell culture

KYM-1 cell lines were purchased from Health Sciences Research Resources Bank (Tokyo, Japan). KYM-1 cells were cultured in DMEM, supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). In some experiments, KYM-1 cells were cultured in serum-free S-Clone (Eidia Co., Ltd, Tokyo, Japan) containing 10 ng/ml basic fibroblast growth factor (bFGF).

Flow-cytometric analysis and cell sorting

Cells were conjugated with anti-FGFR3 antibody (R & D, Minneapolis, MN, USA) for 30 min on ice. Cells were resuspended in the same buffer at 1.0×10^7 per ml, and then kept on ice until analysis. Flow-cytometric analysis was performed using CyAn™ ADP (Beckman Coulter, Fullerton, CA, USA). For further analyses, cells were sorted using a FACSAriaII (BD Biosciences, San Jose, CA) to isolate pure populations of FGFR3-positive and FGFR3-negative cells.

Generation of adenoviruses

The following viruses were propagated and purified as described previously [15-19]: E1-deleted replication-defective adenoviruses; two types of Ads-LacZ that expressed the LacZ gene under the transcriptional control of the Rous sarcoma virus long terminal repeat (RSV promoter) (Ad.RSV-LacZ) or the survivin promoter (Ad. Surv-LacZ); two types of Ads-EGFP that expressed the enhanced green fluorescent protein (EGFP) gene under the cytomegalovirus immediate early gene enhancer/promoter (CMV promoter) (Ad.CMV-EGFP) or the cytomegalovirus enhancer and β-actin promoter (CA promoter) (Ad.CA-EGFP); Ad.dE1.3 that expressed no gene, and Ad.CA-EGFP/RGD, in which an Arg-Gly-Asp (RGD)-containing peptide was added to the HI loop of the fiber-knob domain of Ad.CA-EGFP. Surv.m-CRA with wild-type E1A downstream of the survivin promoter, E1B55KD downstream of the CMV promoter, and the EGFP gene downstream of the CMV promoter was generated as described previously and used for this study [10-12].

Adenoviral gene transduction efficiencies

The adenoviral gene transduction efficiency (AGTE) for each cell type *in vitro* was assessed by infecting cells with Ad.CMV-EGFP at several different multiplicities of infection (MOIs), detaching the cells 48 h after infection, and analyzing the percentage of EGFP-positive cells by flow cytometry [20].

Promoter activities

Promoter activities were examined as described previously with some modification [15,21]. Briefly, cells (8×10^5 cells per plate) were infected with Ad.Surv-LacZ or Ad.RSV-LacZ at an MOI of 30 for 1 h, and then incubated with fresh media. The cells were collected 48 h post-infection, and β-gal activity was measured using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA) as described previously [15,21]. In addition, expression levels of β-galactosidase in individual KYM-1 cells were examined by flow cytometry using the FluoReporter lacZ Flow Cytometry Kit (Molecular Probes, Leiden, The Netherlands).

Real time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis

RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) or the CellAmp Direct RNA Prep Kit (Takara Bio Inc., Ootsu, Japan), and was subsequently reverse-transcribed using the PrimeScript II First Strand cDNA Synthesis Kit (Takara Bio Inc.) [22,23]. RT-PCR using QuantiFast SYBR Green PCR (Qiagen, Venlo, The Netherlands) was performed on a Rotor Gene RG-3000 (Qiagen). The relative mRNA expression levels were determined by the comparative C_t method; expression levels of individual genes were normalized against the levels of the reference gene *HPRT*, which encodes hypoxanthine guanine phosphoribosyl transferase. The following primer sets and annealing temperatures were used: survivin, 5- CCAGTGTTTCTTCTGCTTCAA-3 and 5-GAATG CTTTTTATGTTCCTCTATG-3 at 60°C; *HPRT*, 5- TGA CCTTGATTTATTTTGCATACC-3 and 5-CTCGAGCA AGACGTTCAGTC-3 at 60°C [11,12].

Cytotoxic effects *in vitro*

Cells in 96-well plates were infected with each adenovirus at an MOI of 1, and cell viability was determined after 3 and 5 days using the WST-8 assay (Dojindo Laboratories, Mashiki, Japan) as described previously [11,12,24].

Therapeutic effects *in vivo* in animal experiments

KYM-1 cells (1×10^6 cells), which had been cultured in serum-minus media containing S-Clone and 10 ng/ml bFGF, were mixed with Matrigel (BD Biosciences) and subcutaneously inoculated into 5-week-old BALB/c nude mice. After a tumor nodule reached 6–10 mm in diameter, the mice were randomly divided into three groups. On day 0, a mouse in each group was given a single intratumoral injection of 150 μL of buffer (10 mmol/L Tris–HCl pH 7.4, 1 mmol/L $MgCl_2$, 10% glycerol, and 20 μg/mL hexadimethrine bromide) containing 1×10^9

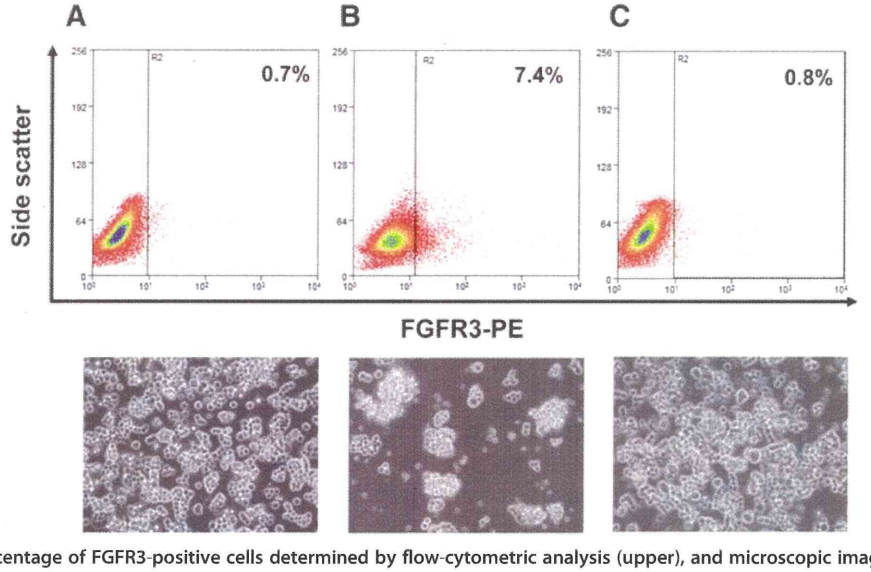

Figure 1 The percentage of FGFR3-positive cells determined by flow-cytometric analysis (upper), and microscopic image of KYM-1 cells (200× magnification; lower) in each culture condition. KYM-1 cells were cultured in serum-plus media **(A)**, then cultured for 3 weeks in serum-minus media containing 10 ng/ml bFGF **(B)**, and subsequently cultured in the serum-plus media for 1 week **(C)**. The percentages of FGFR3-positive cells and spheres were significantly increased by the serum-minus culture and decreased by serum-plus culture.

plaque-forming units (pfu) of Surv.m-CRA (n = 6), Ad. dE1.3 (n = 7), or phosphate-buffered saline (PBS) (n = 8). Subsequently, tumor size was measured twice a week, and tumor volume was calculated according to the following formula: volume = long axis × (short axis)^2 × 0.5.

For histopathologic analysis, tumors were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin.

All animal studies were performed in accordance with National Institutes of Health guidelines and with the approval of the Division of Laboratory Animal Science, Natural Science Center for Research and Education, Kagoshima University. All reasonable efforts were made to minimize suffering.

Statistical analysis

Data were represented as the means ± standard errors (s.e.). Statistical significance was determined using Student's *t* test. $P < 0.05$ was defined as statistically significant.

Results

RSCs can be maintained, expanded, and differentiated *in vitro*

We characterized FGFR3-positive KYM-1 cells as RSCs in a previous study [7]. Therefore, in this study, we first reproduced two distinct culture conditions that successfully induced FGFR3-positive RSC-enriched and FGFR3-negative RSC-exiguous cell fractions *in vitro*. Flow-cytometric analysis revealed that FGFR3-positive cells accounted for only 0.7% of the population when cells were cultured in regular serum-plus media (Figure 1A). However, the percentage of FGFR3-positive cells significantly increased, to 7.4% of the population, after 3-week culture in serum-minus media (Figure 1B). The population of FGFR3-positive cells was drastically decreased to 0.8%, *i.e.*, almost to the original level, only 1 week after the RSC-enriched cell fractions were returned to culture in regular serum-plus media (Figure 1C). Sphere formation, which represented clonogenic growth of the floating cells and was therefore a useful indication of enrichment of CSCs, was observed

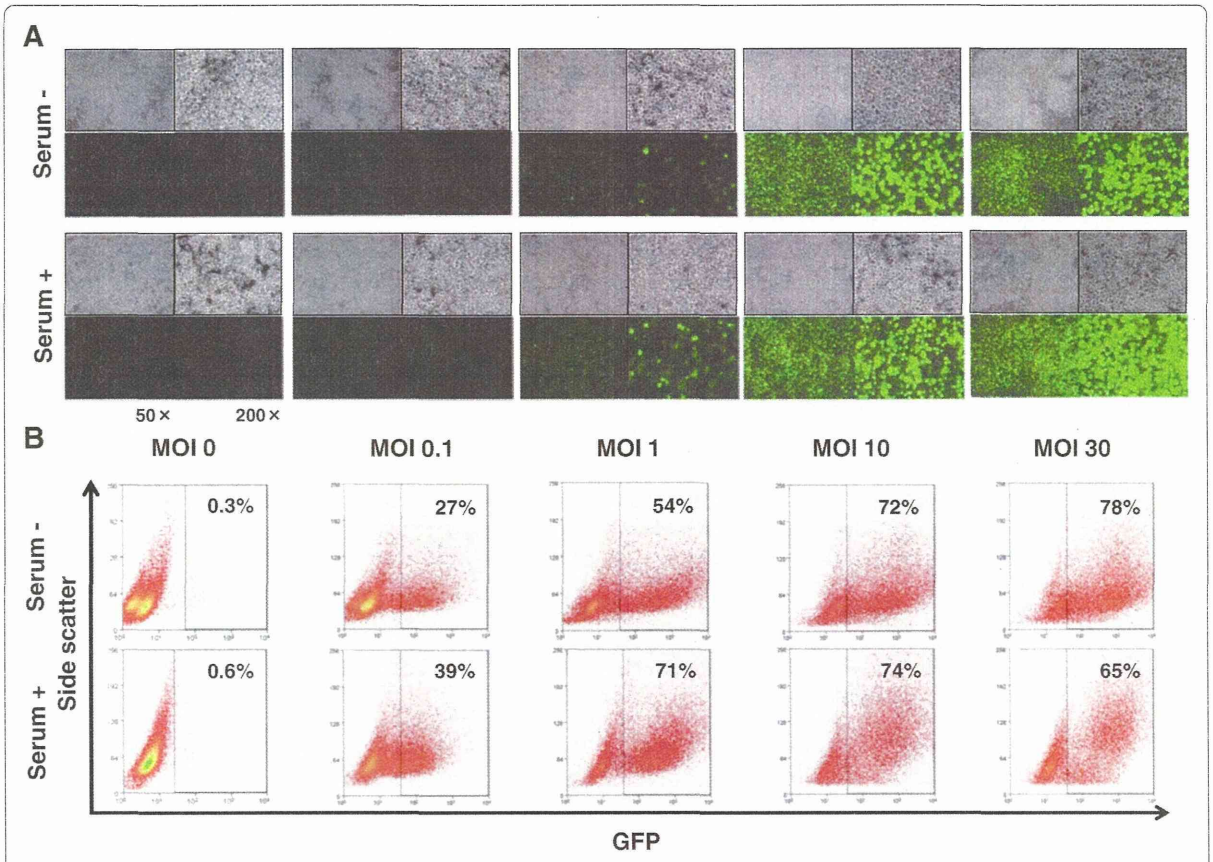

Figure 2 Adenoviral gene transduction efficiencies (AGTEs) in each culture condition. Cells that had been cultured under either serum-minus or serum-plus conditions were infected for 48 h with Ad.CMV-EGFP at MOI of 0 (none), 0.1, 1, 10, or 30. Subsequently, fluorescence-microscopic analysis **(A)** and flow-cytometric analysis **(B)** were performed to assess EGFP-expressing cells. **(A)** Representative images of phase-contrast (upper) and the fluorescence (lower) microscopy are shown at 50× (left) and 200× (right) magnification. No difference in AGTEs between the two groups was apparent.

solely in the serum-minus condition. Thus, we established two distinct *in vitro* conditions that allow accurate assessments of survivin and Surv.m-CRAs in RSCs and their progeny.

AGTEs in KYM-1 cells

KYM-1 cells, cultured in either serum-minus or serum-plus media, were infected with Ad.CMV-EGFP at various MOIs and analyzed by fluorescence microscopy or flow cytometry to assess AGTEs (Figure 2). AGTEs in KYM-1 cells were higher than those in other previously examined cell types [11,12]; however, the difference in AGTEs between RSC-enriched and RSC-exiguous cells was not significant, or at least was not drastic. We further compared AGTEs between infections with Ad.CA-EGFP and Ad.CA-EGFP/RGD, because a number of previous studies demonstrated that modification of the fiber knob with the RGD peptide increased AGTEs in some cell types [19]. Fiber modification did not drastically increase or change AGTEs in KYM-1 cells, either in RSC-enriched or RSC-exiguous fractions (Figure 3). Based on these results, we decided to use replication-defective adenoviral vectors and m-CRAs with wild-type fibers for subsequent experiments.

Higher expression levels of *survivin* mRNA in the FGFR3-positive KYM-1 cell

To assess whether the expression levels of survivin mRNA were changed in RSCs, we performed qRT-PCR analyses to make comparisons between RSC-enriched (serum-minus) and RSC-exiguous (serum-plus) cell fractions and between sorted FGFR3-positive and FGFR3-negative cells. The RSC-enriched cell fraction exhibited a slight increase in survivin mRNA level; however, the difference between the RSC-enriched and RSC-exiguous cell fractions was not statistically significant (Figure 4A). We next examined survivin mRNA levels in sorted FGFR3-postive and FGFR3-negative cells, because such an examination in purified FGFR3-positive cells should make their characteristic phenotypes more apparent. In accordance with that speculation, survivin mRNA levels in sorted FGFR3-positive KYM-1 cells were significantly higher than in FGFR3-negative cells (Figure 4B). These results suggest that survivin is more highly expressed in FGFR3-positive RSCs than in FGFR3-negative progeny.

Higher activities of survivin promoter in FGFR3-positive RSCs

We examined the activities of the survivin and control RSV promoters using replication-defective adenoviral vectors with the LacZ gene downstream of each of these promoters. Flow-cytometric analysis revealed that almost 100% of cells expressed LacZ after Ad.RSV-LacZ infection, and that less than 0.6% of cells were nonspecifically positive after no infection or infection with Ad.dE1.3 (lacking the transgene); thus, the experimental conditions were appropriate (Figure 4C). The percentages of cells expressing LacZ under control of the survivin promoter were 8.3 and 5.6% under the RSC-enriched (serum-minus) and RSC-exiguous (serum-plus) conditions, respectively. Next, we examined the activity of the survivin promoter relative to that of the RSV promoter, a representative control promoter with constitutively strong activity, by measuring β-galactosidase activities after infection with Ad.Surv-LacZ (Figure 4D and E). The survivin promoter activity was significantly higher in RSC-enriched (serum-minus) than in RSC-exiguous (serum-plus) conditions, and also significantly higher in sorted FGFR3-positive than in FGFR3-negative cells. This result, together with the same tendency in endogenous survivin gene expression, suggests that the survivin promoter, which is highly active in tumor cells but minimally active in normal cells [11,12], is more

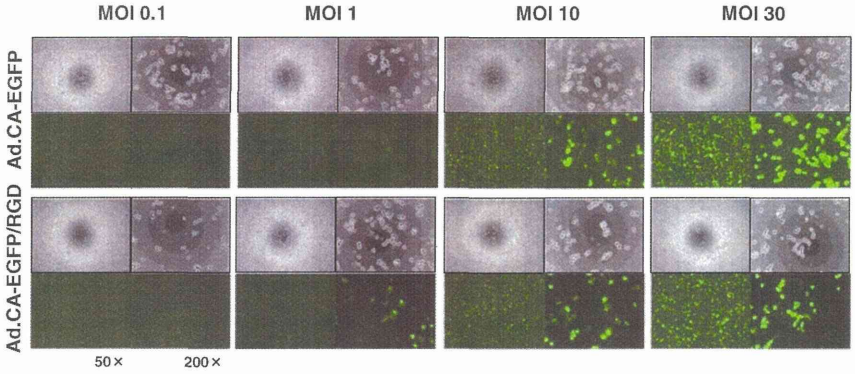

Figure 3 AGTEs of adenoviral vectors with wild or modified fiber. KYM-1 cells were infected with either Ad.CA-EGFP or Ad.CA-EGFP/RGD, which have wild-type or modified fiber (RGD-peptide added to the fiber knob), respectively, at an MOI of 0.1, 1, 10, or 30, and then observed under phase-contrast and fluorescence microscopy 48 h later. Representative phase-contrast (upper) and fluorescence (lower) images are shown at 50× (left) and 200× (right) magnification. There was no apparent difference in AGTEs between the two groups.

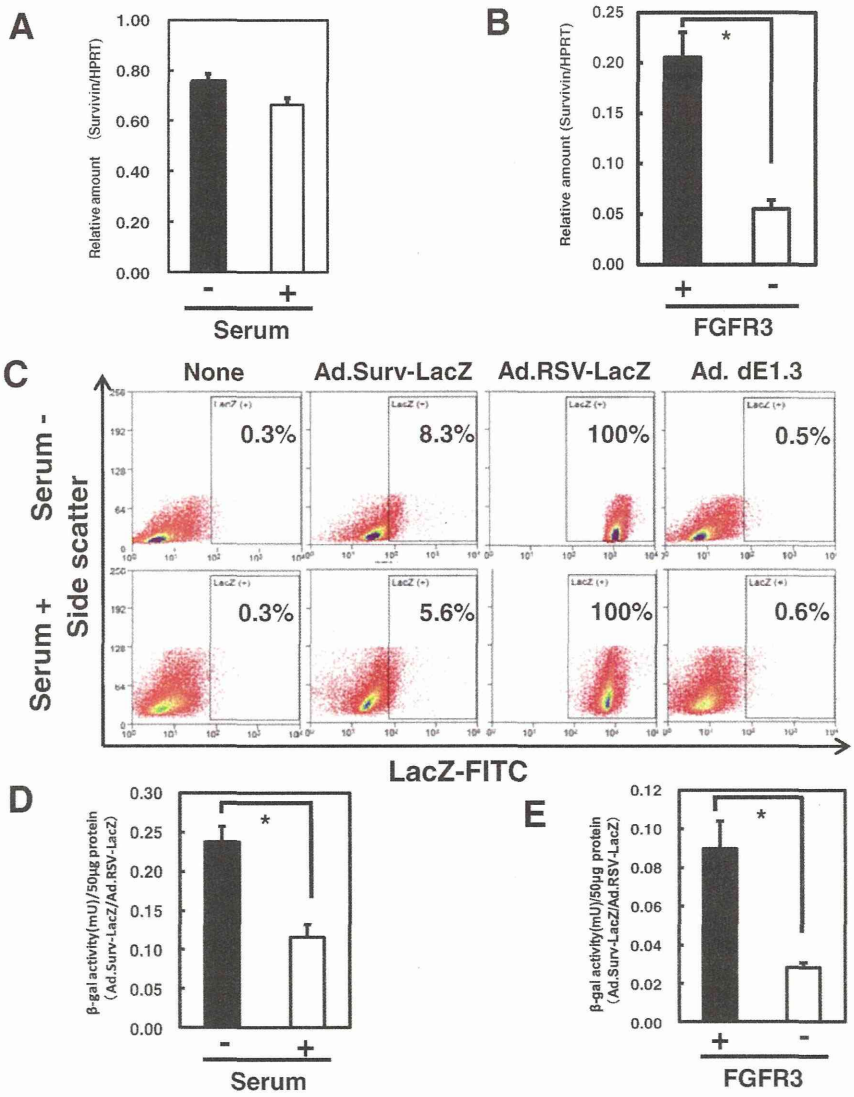

Figure 4 Survivin mRNA expressions and survivin promoter activity. (A, B) Expression levels of survivin mRNA under serum-minus and serum-plus conditions **(A)** and in sorted FGFR3-positive and FGFR3-negative cells **(B)** were measured by real-time qRT-PCR. The *HPRT* gene was amplified as an internal 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

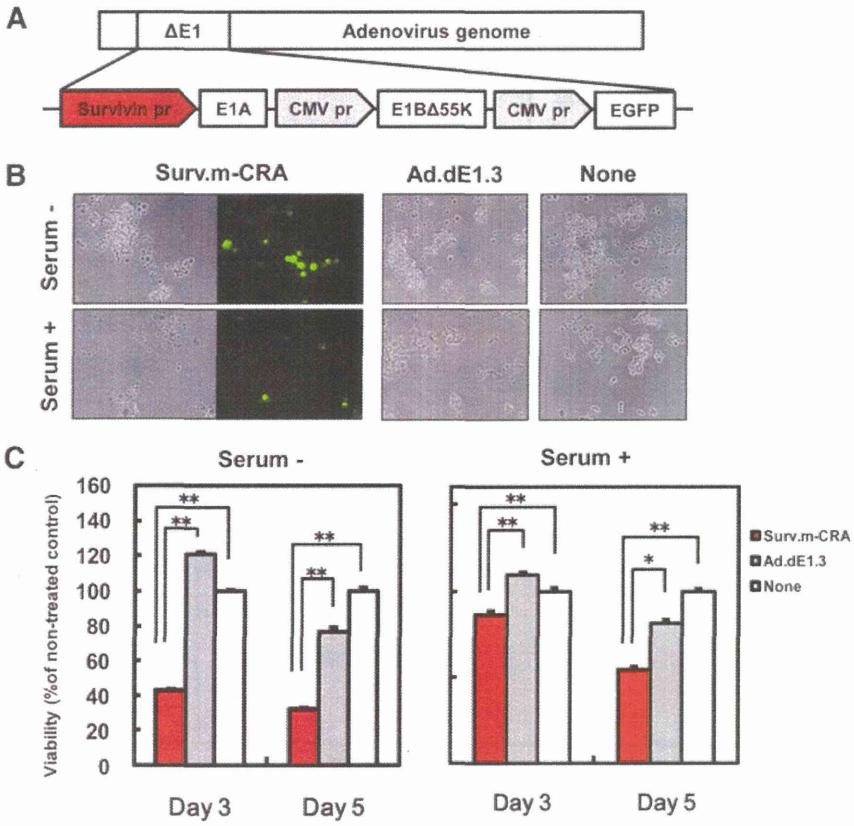

Figure 5 Viral replication of Surv.m-CRA and cytotoxicity against KYM-1 cells cultured in serum-minus and serum-plus media. **(A)** Schematic representation of the construction of Surv.m-CRA. **(B)** Representative images of phase-contrast and fluorescence-microscopic pictures 5 days after infection 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