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ORIGINAL ARTICLE

Assessment of an altered *E1B* promoter on the specificity and potency of triple-regulated conditionally replicating adenoviruses: implications for the generation of ideal m-CRAs

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Although previous studies modified two components of conditionally replicating adenoviruses (CRAs), which selectively replicate in and kill cancer cells, the most accurate ways to achieve increased cancer specificity (that is, safety) without reducing the anticancer (that is, therapeutic) effects are unknown. Here, we generated two types of *survivin*-responsive m-CRAs (Surv.m-CRAs), Surv.m-CRA-CMVp and Surv.m-CRA-OCp, which use two and three different mechanisms to target cancer, that is, early region 1A (*E1A*) regulated by the *survivin* promoter and mutated *E1BΔ55K* regulated by the ubiquitously active *cytomegalovirus* promoter and cancer/tissue-specific *osteocalcin* promoter, respectively, and carefully examined their safety and anticancer effects. Endogenous *osteocalcin* mRNA was expressed and further enhanced by vitamin D_3 in all osteosarcoma and prostate cancer cell lines and human osteoblasts, but not in human fibroblasts. The *osteocalcin* promoter activity was weak even with vitamin D_3 treatment in these osteocalcin-expressing cancers, leading to low *E1BΔ55K* expression after Surv.m-CRA-OCp infection. Nevertheless, Surv.m-CRA-OCp had significantly increased cancer specificity without reduced anticancer effects in both *in vitro* and *in vivo* experiments. The unexpected but favorable fact that strong activity of an altered *E1B* promoter is unnecessary indicates that the majority of cancer/tissue-specific promoters may be used to generate ideal m-CRAs and will advance the development of m-CRA-based cancer therapies.

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Introduction

Conditionally replicating adenoviruses (CRAs) selectively replicate in and kill cancer cells and are therefore attractive anticancer agents.[1,2] CRAs are predominantly classified into two categories.[3] The first CRA category has attenuated viral replication only in normal cells because of specific mutations in the adenoviral *early gene 1* (*E1*), such as *E1AΔ24* and *E1BΔ55K* that lack the *Rb*- and *p53*-binding sites in the *early region 1A* (*E1A*) and *1B* (*E1B*), respectively.[4–6] In the second category, *E1* expression is altered in a cancer-specific manner because the promoter driving *E1*, particularly *E1A*, is replaced.[7] Although previous studies demonstrated that both strategies are efficacious, it is possible that a single cancer-specific factor will be insufficient to strictly target cancer, potentially leading to adverse side effects in future clinical trials.[8] A combination of multiple cancer-specific factors may be a promising approach to increase the safety of CRAs;[1,9,10] however, it is largely unclear whether and to what degree the anticancer effects are decreased by introducing multiple cancer-specific factors. These insufficient analyses have partially occurred because constructing diversely modified CRAs is time consuming and labor intensive.

To solve this problem, we previously developed a method to efficiently construct diverse CRAs that can specifically target cancers using multiple factors (m-CRAs).[8] Using this method, we previously generated two types of *survivin*-responsive m-CRAs (Surv.m-CRAs), in which *E1B* was mutated to *E1BΔ55K* and either the wild-type *E1A* or mutant *E1AΔ24* gene was regulated by the promoter of *survivin*, a new member of the inhibitor of apoptosis gene family.[7,11,12] These modified CRAs were promising because the *survivin* promoter and the resulting Surv.m-CRAs exhibited both a stronger and more cancer-selective phenotype (that is, transgene expression and anticancer effects, respectively)

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than the telomerase reverse transcriptase promoter and telomerase reverse transcriptase-responsive m-CRA (Tert.m-CRA), which is currently one of the best CRAs.[7,13] A careful comparative study of both Surv.m-CRAs revealed that additional modifications of *E1A* to *E1AΔ24* downstream of the *survivin* promoter provided neither increased cancer specificity nor reduced the anticancer effects, suggesting that this combination is not essential to improve m-CRAs, at least Surv.m-CRAs.[7]

On the other hand, a few previous studies obtained increased cancer specificity by replacing the *E1B* promoters with cancer-specific promoters in addition to regulating *E1A* with a different cancer-specific promoter.[9,10] However, the actual utility of this combination remains unclear because the original and modified CRAs were not sufficiently compared. In particular, several crucial and unanswered questions remain, including how much *E1B* or *E1BΔ55K* (*E1B*/*E1BΔ55K*) expression is necessary and how strong of a different cancer-/tissue-specific promoter is necessary to drive *E1B*/*E1BΔ55K* expression to increase cancer specificity without reducing the anticancer effects. Because many cancer/tissue-specific promoters have weak activity,[13–15] it is unclear whether this strategy will consistently yield clinically useful m-CRAs.

To clarify this issue, we carefully analyzed the characteristics of two types of Surv.m-CRAs, the original Surv.m-CRA-CMVp and a newly constructed Surv.m-CRA-OCp that express *E1BΔ55K* under the ubiquitously strong, human *cytomegalovirus immediate-early gene* enhancer/promoter (*CMV* promoter) and cancer/tissue-specific *osteocalcin* promoter, respectively. We chose the *osteocalcin* promoter because osteocalcin, a major non-collagenous *Gla* protein that is natively produced by osteoblasts, has a vitamin D_3 (VD_3)-responsive element that is activated by VD_3.[16–18] These analyses showed that the strong activity of an altered cancer/tissue-specific *E1B* promoter is not essential. This finding is unexpected but meaningful because many popular cancer/tissue-specific promoters can now be used to generate ideal m-CRAs, which will open up an innovative therapeutic strategy.

Materials and methods

Cells and cell culture

Four human osteosarcoma cell lines (HOS-MNNG, MG-63, KHOS-NP and SaOS-2), two human prostate cancer cell lines (DU145 and PC-3), human primary lung fibroblasts (WI-38) and human osteoblasts (NHOst) were obtained and cultured as described previously.[7,13,19,20]

Reverse transcription-polymerase chain reaction analysis

At 2 days after culturing cells with media in the presence or absence of 50 nM VD_3 (Sigma-Aldrich, Tokyo, Japan), total RNA was extracted from cells and reverse transcription-polymerase chain reaction analyses were performed using the primer sets and annealing temperatures that were described previously.[7,14,21]

The *osteocalcin*/*hypoxantine-phosphoribosyl-transferase* (a representative housekeeping gene) mRNA ratio was calculated to compare the relative effects of VD_3 in individual cells and standardize the unavoidable variability in the polymerase chain reaction data as described.[21]

Generation of adenoviruses

The human *osteocalcin* promoter (−886 to +3) was cloned by polymerase chain reaction using genomic DNA from SaOS-2 cells and the following primers: sense-hOC.pr, 5′-CTGCAGGGTCAGGAGGAGAA-3′ and antisense-hOC.pr, 5′-CATGGTGTCTCGGTGGCTGC-3′. Surv.m-CRA-CMVp and Surv.m-CRA-OCp, which contained wild-type *E1A* downstream of the *survivin* promoter, *E1BΔ55K* downstream of the *CMV* promoter or the human *osteocalcin* promoter, and the *enhanced green fluorescent protein* (*EGFP*) gene downstream of the *CMV* promoter, were generated using the novel m-CRA construction method that we developed previously.[7,8] An *E1*-deleted replication-defective adenovirus that expressed no gene (Ad.dE1.3), the *LacZ* gene under the transcriptional control of the *Rous sarcoma virus* long-terminal repeat (*RSV* promoter; Ad.RSV-LacZ) or the *osteocalcin* promoter (Ad.OC-LacZ), or *EGFP* under the cytomegalovirus enhancer and *β-actin* promoter (Ad.CA-EGFP) were generated and prepared as described previously.[15,19,20,22–24]

Adenoviral gene transduction efficiencies and promoter activities

The adenoviral gene transduction efficiency (AGTE) for each cell *in vitro* was assessed by infecting the cells with Ad.CA-EGFP at several multiplicity of infections (MOIs), detaching the cells after 48 h, and then counting the EGFP-positive cells among the total cells under a fluorescent microscope. The promoter activities were examined as previously described with some modifications.[7,13,15] Briefly, the cells (5×10^5 cells per plate) were infected with Ad.OC-LacZ or Ad.RSV-LacZ at an MOI of 30 for 1 h and then incubated with fresh media in the presence or absence of 50 nM VD_3. The cells were collected at 48 h post-infection, and the *β-gal* activity was measured using a *β-Galactosidase* Enzyme Assay System (Promega, Madison, WI) as described previously.[13,15]

Western blot analysis

Cells were either mock infected or infected with Surv.m-CRA-CMVp, Surv.m-CRA-OCp or Ad.dE1.3 at an MOI of 5 for 1 h and then incubated with fresh media in the presence or absence of 50 nM VD_3 for 3 days. The cells were lysed in buffer containing 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.5% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride (Nacalai Tesque, Kyoto, Japan) and a Protease inhibitor cocktail (Sigma-Aldrich). After electrophoresing 50 μg of protein, western blot analysis was performed using an anti-adenovirus 2 *E1B* 19-kDa primary antibody (Calbiochem, Darmstadt, Germany), horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary

antibody (Invitrogen, Carlsbad, CA) and Chemi-Lumi One (Nacalai Tesque) according to the manufacturers' protocols and as described previously.[14,19] β-Actin was used as an internal control and detected in the same manner.

Cytotoxic effects in vitro

Cells in 96-well plates were infected with each adenovirus at various MOIs, and cell viability was determined after 3 and 5 days using the WST-8 assay (Dojindo Laboratories, Mashiki, Japan) as described previously.[7,15]

Therapeutic effects in vivo *animal experiments*

Subcutaneous tumor mouse models were generated using HOS-MNNG cells and examined for therapeutic effects as previously described with the minor modification that the number of viral injections was increased from one to two.[7] Briefly, HOS-MNNG cells (5×10^6 cells) were injected subcutaneously into athymic nude mice. A measure of 50 μl of vehicle containing 1×10^8 plaque-forming units (PFU) of Surv.m-CRA-CMVp ($n=8$), Surv.m-CRA-OCp ($n=8$) or Ad.dE1.3 ($n=7$) or 50 μl of phosphate-buffered saline alone ($n=9$) was injected two times on days 0 and 3 into the subcutaneous tumor with a size ranging from 6 to 10 mm in diameter. Tumor size was monitored twice weekly and the tumor volume was calculated as described previously.[7]

For histopathological analyses, the tumors were collected 35 days after the initial viral injections, fixed and stained with hematoxylin and eosin.

The animal protocols were approved by the Animal Research Committee of Kagoshima University. All animal experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Statistical analysis

Data are represented as the means ± standard errors (s.e.). Statistical significance was determined using the *Tukey*'s test. $P<0.05$ was considered statistically significant.

Results

Expression of endogenous survivin *and* osteocalcin *mRNAs in osteosarcoma and prostate cancer cell lines*

In accordance with our study and others,[7,25] *survivin* mRNA was expressed at moderate to high levels in all of the examined osteosarcoma and the prostate cancer cell lines, but the expression level was very low or undetectable in normal fibroblasts and osteoblasts (Figure 1). On the other hand, *osteocalcin* mRNA was expressed at various levels and further enhanced by VD_3 not only in osteosarcoma and prostate cancer cell lines, but also in normal osteoblasts (Figure 1). *Osteocalcin* mRNA expression was very low or undetectable and was not prominently enhanced by VD_3 in normal fibroblasts.

Various AGTEs in cells

Ad.CA-EGFP infection at serially diluted MOIs yielded various AGTEs among individual cell types. The AGTEs

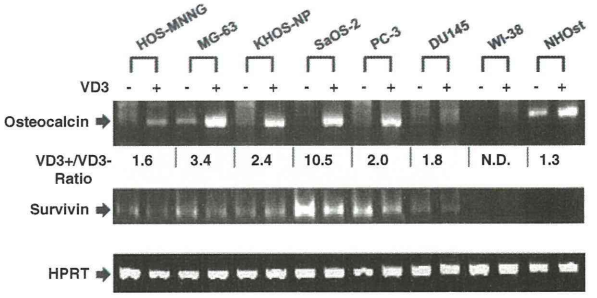

Figure 1 *Osteocalcin* and *survivin* mRNA expression in osteosarcoma cells (HOS-MNNG, MG-63, KHOS-NP, and SaOS-2), prostate cancer cells (PC-3 and DU145) and normal cells (WI-38 and NHOst). Cells were cultured in the presence or absence of 50 nM vitamin D_3 (VD_3) for 48 h and then harvested. Both *osteocalcin* and *survivin* as well as the *hypoxanthine guanine phosphoribosyl transferase* (*HPRT*; an internal control) gene were amplified by reverse transcription-polymerase chain reaction (RT-PCR). The *osteocalcin*/*HPRT* mRNA ratio in the presence of VD_3 divided by that in the absence of VD_3 is shown below the *osteocalcin* band in each cell line. ND: not determined because of very low expression levels.

were high in two osteosarcoma cell lines (HOS-MNNG and KHOS-NP) and normal fibroblasts (WI-38), moderate in two prostate cancer cell lines (PC-3 and DU145), and low in two osteosarcoma cell lines (MG-63, SaOS-2) and normal osteoblasts (NHOst) (Figure 2).

VD_3-dependent enhancement of the osteocalcin *promoter activity*

The activities of the *osteocalcin* and control *RSV* promoters and their VD_3-dependent enhancement in individual cells were examined after infecting with Ad.OC-LacZ or Ad.RSV-LacZ and then measuring the *β-gal* activity. Whereas the level of *β-gal* activity after infecting with the control Ad.RSV-LacZ, which includes the actual *RSV* promoter activity and yielded different AGTEs, varied among cell types, it was not prominently enhanced by VD_3 in any of the cell types (Figure 3a). On the other hand, the *osteocalcin* promoter activity was much lower than the *RSV* promoter activity in all of the osteosarcoma cells and fibroblasts without the addition of VD_3. Notably, the *osteocalcin* promoter activity was drastically enhanced by VD_3 in *osteocalcin*-expressing cells (HOS-MMMG, MG-63 and KHOS-NP), except for SaOS-2 cells that had a low AGTE. In contrast, the *osteocalcin* promoter activity was not enhanced by VD_3 in WI-38 fibroblasts that demonstrated a high AGTE. Interestingly, the *osteocalcin* promoter activities in both of the prostate cancer cell lines (DU145 and PC-3) were much higher than those in all of the osteosarcoma cell lines and were enhanced three- to fourfold by VD_3 (Figures 3b and c). Thus, the activity of the *osteocalcin* promoter transduced by the adenoviral vector was successfully enhanced by VD_3 in the *osteocalcin*-expressing cells with a sufficient AGTE, and interestingly, the activity levels were much lower in the osteosarcoma cell lines than the prostate cancer cell lines. More importantly

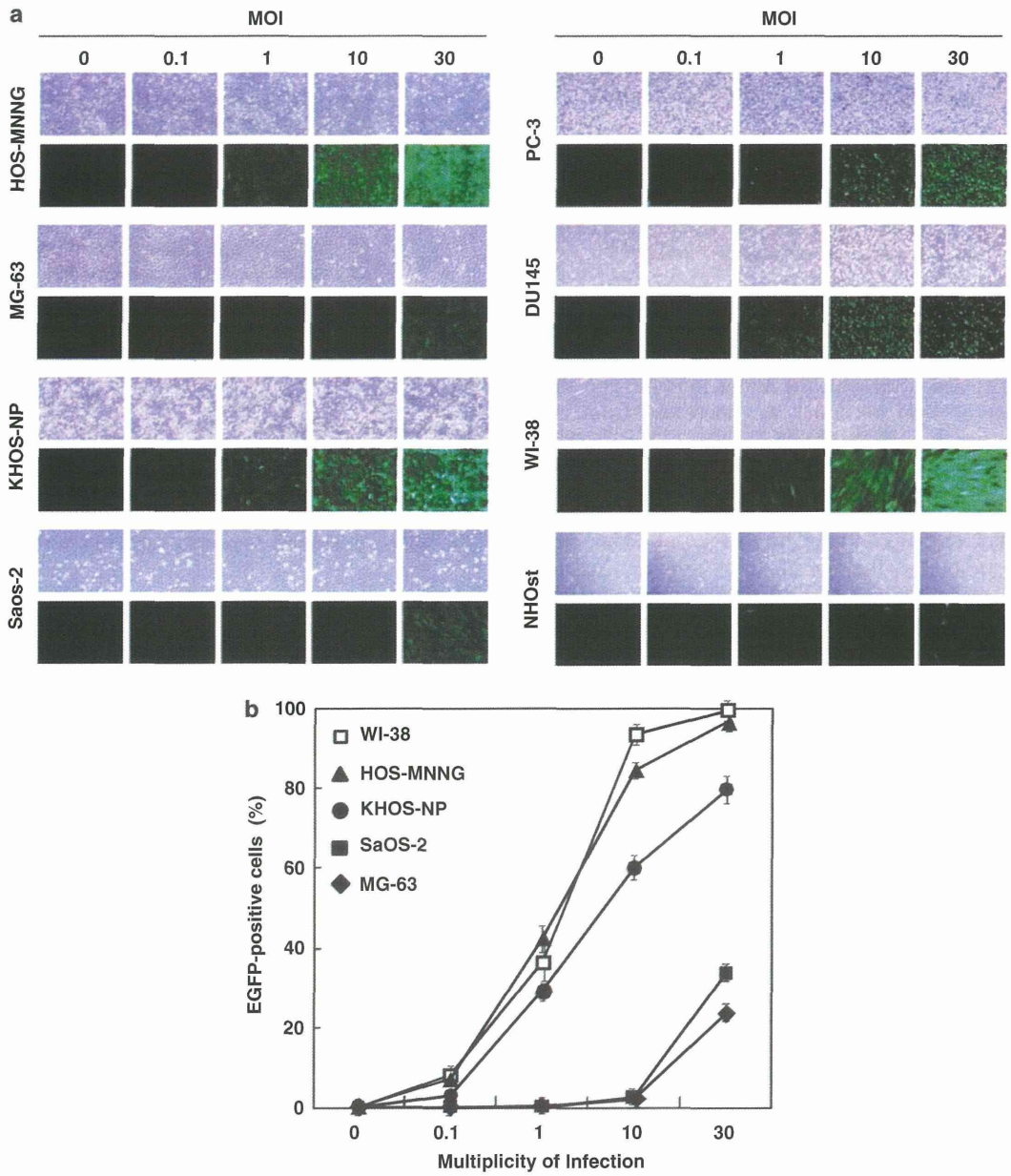

Figure 2 Adenoviral gene transduction efficiency (AGTE). (**a**) Representative phase-contrast (top) and fluorescent microscopic images (bottom) 2 days after the cells were infected with Ad.CA-enhanced green fluorescent protein (EGFP) at various multiplicity of infections (MOIs). Original magnification, ×40. (**b**) Quantitative AGTE by counting the EGFP-positive cells after Ad.CA-EGFP infection. HOS-MNNG and WI-38 cells showed similar AGTEs.

and notably, the activity of the *osteocalcin* promoter in the most of these cells, including the VD_3-enhanced activity in *osteocalcin*-expressing cells, was much lower than that of the *RSV* promoter.

Low E1BΔ55K *expression levels after Surv.m-CRA-OCp infection*

Because the *osteocalcin* promoter had weak activity that was enhanced by VD_3, the *osteocalcin* promoter is an ideal model to answer our question, that is, how much activity of the altered *E1B* promoter is necessary for exhibiting sufficient therapeutic effects. To this end, we generated Surv.m-CRA-CMVp and Surv.m-CRA-OCp, in which *E1BΔ55K* is regulated by the ubiquitously active *CMV* promoter and *osteocalcin* promoter, respectively, and *E1A* is regulated by the *survivin* promoter (Figure 4a). We used the *CMV* promoter because we previously determined that the *CMV* promoter had stronger (almost

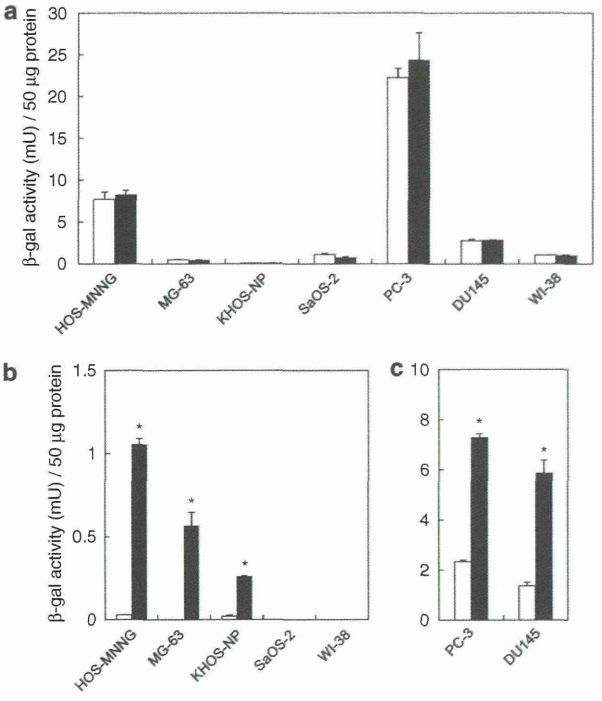

Figure 3 Activities of the *Rous sarcoma virus* (*RSV*) and *osteocalcin* promoters in the presence or absence of vitamin D_3 (VD_3). Cells were infected with Ad.RSV-LacZ (**a**) or Ad.OC-LacZ (**b**, **c**) at a multiplicity of infection (MOI) of 30 for 1 h, and subsequently cultured for 48 h in the presence (black bars, $n=3$) or absence (white bars, $n=3$) of VD_3. The cells were harvested and the *β-galactosidase* activities were measured. There were significant differences between the presence and the absence of VD_3 in all osteosarcoma and prostate cancer cells, except in SaOS-2 cells that were treated with Ad.OC-LacZ infection ($^*P<0.05$).

10-fold) activity than the *RSV* promoter in most cell types.[15] *E1BΔ55K* protein expression was quantified by western blot analyses of cell extracts that were collected 3 days after the HOS-MNNG cells were infected with the noted adenoviruses in the presence or absence of VD_3 (Figure 4b). Surv.m-CRA-CMVp infection resulted in high *E1BΔ55K* expression regardless of VD_3 stimulation, whereas uninfected cells and cells infected with the control *E1*-deleted adenovirus had no *E1BΔ55K* expression. Surv.m-CRA-OCp infection induced much lower *E1BΔ55K* expression than Surv.m-CRA-CMVp infection, even when *E1BΔ55K* expression was successfully enhanced by VD_3. Thus, the weak activity of the *osteocalcin* promoter consistently resulted in low *E1BΔ55K* expression in *osteocalcin*-expressing cells that had been infected with Surv.m-CRA-OCp.

Weak E1BΔ55K *expression levels are sufficient to induce significant m-CRA-associated cytotoxicity*

To examine whether the *E1BΔ55K* expression levels correlate with the efficiency of viral replication and the resulting anticancer effects of m-CRA, the viability of HOS-MNNG osteosarcoma and PC-3 prostate cancer cells were analyzed 7 days after they had been infected with Surv.m-CRA-OCp, and then cultured with or without VD_3. Both Surv.m-CRA-CMVp and Surv.m-CRA-OCp demonstrated significant cytotoxic effects regardless of VD_3 compared to the control replication-deficient adenovirus (Ad.dE1.3). Notably, culturing HOS-MNNG osteosarcoma cells with VD_3, which clearly enhanced *osteocalcin* promoter activity and *E1BΔ55K* expression in Surv.m-CRA-OCp (Figures 3b and c and 4b), did not increase the cytotoxic effects of Surv.m-CRA-OCp (Figure 4c). The same result was found with PC-3 prostate cancer cells. Thus, even drastic differences in the *E1BΔ55K* expression levels did not lead to significant differences in cytotoxicity, and it was particularly interesting that very weak *E1BΔ55K* expression was sufficient to achieve significant cytotoxic effects with m-CRAs.

Surv.m-CRA-OCp has similar anticancer effects against osteocalcin*-expressing cancers as Surv.m-CRA-CMVp, but significantly less cytotoxicity in normal cells*

To carefully assess whether Surv.m-CRA-OCp induces anticancer effects against *osteocalcin*-expressing cancers as potently as Surv.m-CRA-CMVp and whether Surv.m-CRA-OCp significantly reduces the nonspecific cytotoxicity of Surv.m-CRA-CMVp in normal cells, cell viabilities were analyzed 3 or 5 days after the cells were infected with Surv.m-CRA-OCp or Surv.m-CRA-CMVp, and then cultured without VD_3 (Figure 5). At 2 days after infection, Surv.m-CRA-OCp-infected HOS-MNNG cells that had not been treated with VD_3 and expressed low levels of *E1BΔ55K* (Figure 4b) started exhibiting cytopathic effects that were microscopically characteristic of oncolytic adenovirus, that is, cell swelling and detaching from the dish, and were similar to those observed in Surv.m-CRA-CMVp-infected cells (data not shown). On day 3, both groups showed a prominent and significant reduction in cell viability, as accurately determined by the WST-8 assay (Figure 5). Although Surv.m-CRA-CMVp demonstrated slightly more efficient cytotoxic effects than Surv.m-CRA-OCp in HOS-MNNG cells on day 3, there was no difference between the two groups on day 5. On the other hand, the viability of PC-3 prostate cancer cells, which have stronger *osteocalcin* promoter activity than osteosarcoma cells, but weaker *osteocalcin* promoter activity than *RSV* promoter activity (Figure 3), was not significantly different between Surv.m-CRA-CMVp and Surv.m-CRA-OCp on day 3 or 5 (Figure 5). Thus, Surv.m-CRA-OCp may generally confer sufficient anticancer effects to *osteocalcin*-expressing cancer cells, including not only prostate cancer cells, but also osteosarcoma cells in which the *E1BΔ55K* expression levels are very low in the absence of VD_3.

On the other hand, our previous study demonstrated that low doses of Surv.m-CRA-CMVp, which may be one of the best CRAs both in terms of cancer specificity and anticancer efficiency,[7] did not induce significant cell death in normal cells. Nevertheless, there is a possibility that higher doses of any CRA may induce nonspecific cytotoxic effects in normal cells. To examine whether Surv.m-CRA-OCp increases the target specificity to

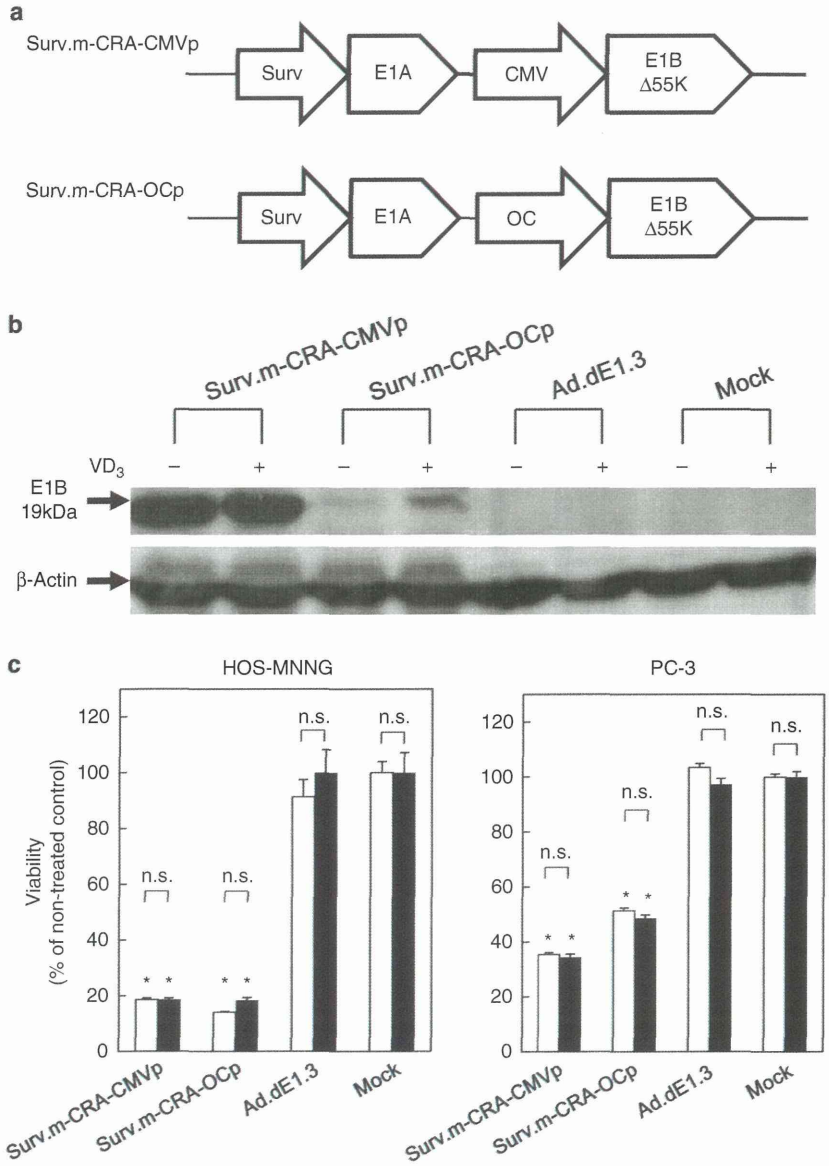

Figure 4 E1BΔ55K expression and cytotoxicity after *survivin*-responsive m-CRA (Surv.m-CRA) infection in the presence or absence of vitamin D_3 (VD_3). (**a**) Schematic representation of the construction of Surv.m-CRA-CMVp and Surv.m-CRA-OCp. (**b**) HOS-MNNG cells were either mock infected or infected with Surv.m-CRA-CMVp, Surv.m-CRA-OCp or Ad.dE1.3 at a multiplicity of infection (MOI) of 5, and then cultured in the presence or absence of 50 nM VD_3 for 3 days. The protein expression levels for E1B19 kDa (E1BΔ55K) and β-actin (internal control) were detected by western blot analysis. (**c**) HOS-MNNG osteosarcoma cells and PC-3 prostate cancer cells were infected with each adenovirus at an MOI of 5 and then cultured in the presence (black bars) or absence (white bars) of 50 nM VD_3 for 7 days. The cells were collected and the viability was determined by the WST-8 assay ($n=8$, each group). There were significant differences between Surv.m-CRAs and the control Ad.dE1.3 ($^*P<0.05$) in both HOS-MNNG and PC-3 cells, but no difference between Surv.m-CRA-CMVp and Surv.m-CRA-OCp in HOS-MNNG or PC-3 cells (NS).

osteocalcin-expressing cancers, leading to a reduction in the nonspecific death of normal cells, the same comparative study was performed using high doses of both Surv.m-CRAs in WI-38 fibroblasts and NHOst osteoblasts (Figure 5). WI-38 cells that were infected with Surv.m-CRA-CMVp at MOIs of 5 and 10 demonstrated some cytotoxic effects after 3 and 5 days, and the degree of cytotoxicity was more prominently manifested at later time points at a higher MOIs. WI-38 cells that were infected with Surv.m-CRA-OCp at both MOIs demonstrated significantly less death on both days 3 and 5 than those infected with Surv.m-CRA-CMVp.

Interestingly, Surv.m-CRA-OCp also induced significantly less cytotoxicity than Surv.m-CRA-CMVp in

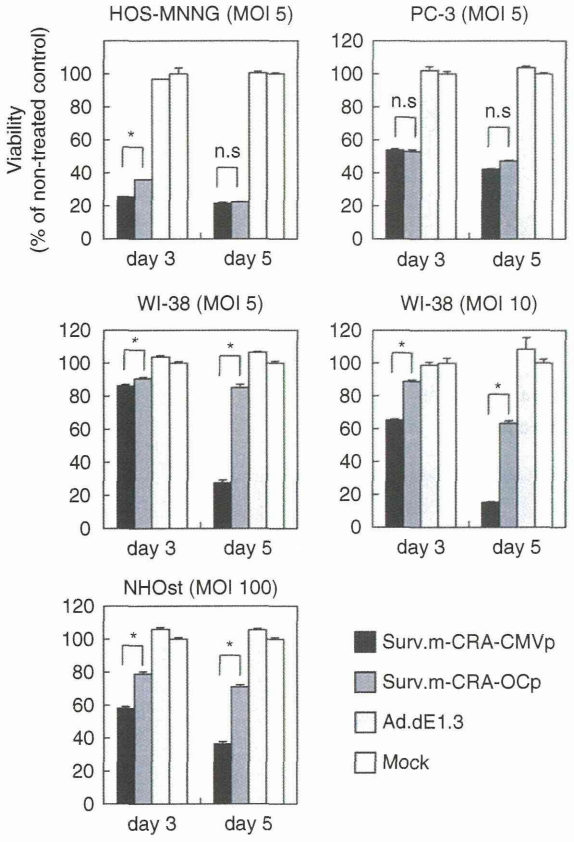

Figure 5 Comparison of the cytotoxic effects *in vitro* between Surv.m-CRA-CMVp and Surv.m-CRA-OCp in cancerous and normal cells. HOS-MNNG osteosarcoma cells, PC-3 prostate cancer cells, WI-38 normal fibroblasts and NHOst normal osteoblast were either mock infected or infected with Surv.m-CRA-CMVp, Surv.m-CRA-OCp or Ad.dE1.3 at the indicated multiplicity of infections (MOIs) and then cultured for 3 and 5 days in the absence of vitamin D_3 (VD_3). Cell viability was determined by the WST-8 assay. There were significant differences between Surv.m-CRA-CMVp and Surv.m-CRA-OCp in WI-38 and NHOst cells on the days 3 and 5 and in HOS-MNNG cells on day 3 ($^*P<0.05$), but no difference between Surv.m-CRA-CMVp and Surv.m-CRA-OCp in HOS-MNNG cells on day 5 or in PC-3 cells on days 3 and 5 (NS).

NHOst osteoblasts when these cells were infected with Surv.m-CRAs at an MOI of 100 (Figure 5). NHOst cells that were infected with any of the Surv.m-CRAs at an MOI of 10 did not undergo cell death after 3 or 5 days (data not shown).

These *in vitro* data suggest that the regulation of *E1BΔ55K* expression by a cancer/tissue-specific promoter, regardless of its activity, results in more effective m-CRAs that efficiently kill the targeted cancer, but have significantly reduced nonspecific toxicity in normal cells.

Surv.m-CRA-OCp and Surv.m-CRA-CMVp exhibit the same anticancer effects in vivo

Using an animal model of pre-established subcutaneous tumors, we examined whether Surv.m-CRA-OCp had the same *in vivo* therapeutic potential as Surv.m-CRA-CMVp in the absence of VD_3. Two intratumoral injections of Surv.m-CRA-OCp (1×10^8 PFU per time) on days 0 and 3 significantly inhibited tumor growth compared with the same dose of Ad.dE1.3. There were statistically significant differences in the tumor size between the Surv.m-CRA- and Ad.dE1.3-treated mice, but there were no differences between Surv.m-CRA-OCp- and Surv.m-CRA-CMVp-treated animals (Figures 6a and b). It should be noted that the actual therapeutic effects of both Surv.m-CRAs were more significant based on microscopic analyses (Figure 6c). The tumor nodules in Surv.m-CRA-treated mice contained large necrotic areas, but Ad.dE1.3-treated mice consisted primarily of viable tumor cells that histologically exhibited active malignant features. It should also be noted that the viral dose (1×10^8 PFU per injection) in this study was much smaller than that used in many previous studies (approximately 1×10^9 PFU), suggesting that more viral doses in future preclinical and clinical studies may result in enhanced efficacy.[26,27] Thus, both the *in vitro* and *in vivo* therapeutic potential of Surv.m-CRA-OCp and Surv.m-CRA-CMVp for osteosarcoma in the absence of VD_3 was comparable, suggesting that low *E1BΔ55K* expression, that is, extremely weak activity of the altered *E1B* promoter, is sufficient to exert actual anticancer therapeutic effects.

Discussion

It has been uncertain whether replacing the *E1B* promoter with a cancer/tissue-specific promoter in addition to modified *E1A* regulation is more beneficial because previous studies did not examine the reduced anticancer effects.[9,10] Furthermore, it is important to note that none of the previous studies examined the necessary activity of an altered *E1B* promoter to increase the cancer specificity without reducing the anticancer effects. In this regard, the following two points should be noted. First, the activities of cancer/tissue-specific promoters vary to a large degree, and many of them are typically lower than the characteristic constitutively and ubiquitously active promoters, including the *RSV* and *CMV* promoters.[13–15] Second, it is necessary to achieve optimal expression levels of certain genes and optimal activities of promoters driving certain genes to obtain correct and preferential functions. For instance, we previously revealed that an overly robust promoter clearly induced nonspecific cytotoxicity without increasing the anticancer effects and that a relatively weak promoter was suitable to drive a suicide gene from a clinical perspective.[15] Conversely, we also demonstrated that the activities of some tissue-specific promoters are too weak to directly visualize the reporter *EGFP* gene in target cells, and that it was necessary to enhance the activity of some tissue-specific promoters for this purpose.[14] These lessons illustrate the importance of determining the necessary expression levels of E1B/E1BΔ55K and the necessary activity of the cancer/tissue-specific promoter driving *E1B*/*E1BΔ55K* to

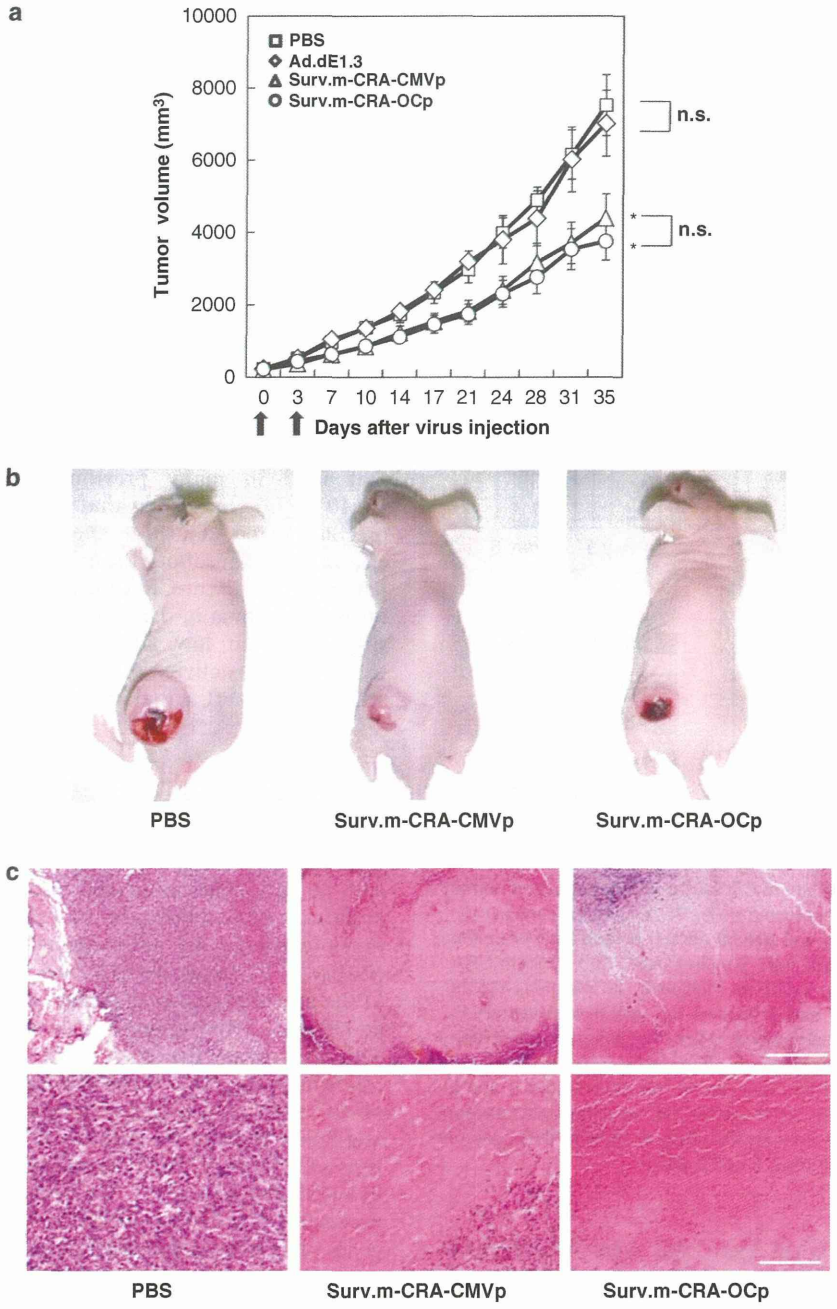

Figure 6 Therapeutic effects of Surv.m-CRAs *in vivo*. (**a**) Tumor volume was measured after phosphate-buffered saline (PBS) ($n=9$) or 1×10^8 PFU Surv.m-CRA-CMVp ($n=8$), Surv.m-CRA-OCp ($n=8$) or Ad.dE1.3 ($n=7$) was injected twice on days 0 and 3 (arrows) into athymic nude mice with subcutaneous tumors that were 6–10 mm in diameter. There was a significant difference between Surv.m-CRAs and the control Ad.dE1.3 ($^*P<0.05$), but no difference between uninfected tumors and Ad.dE1.3 or between Surv.m-CRA-CMVp and Surv.m-CRA-OCp (NS). (**b**) Representative macroscopic pictures 21 days after the initial viral injection. (**c**) Representative histological images at the time of being killed. Hematoxylin- and eosin-stained sections from mice treated with either of the Surv.m-CRAs had large necrotic areas in the tumor nodules. In contrast, the control tumor nodules contained primarily viable tumor cells without large necrotic areas. Original magnification: $\times$40 (top; scale bar, 1 mm) and $\times$200 (bottom; scale bar, 200 μm).

develop clinically useful m-CRAs. This study has clarified this issue and shown that the strong activity of an altered cancer/tissue-specific *E1B* promoter is not essential to generate ideal m-CRAs with increased cancer specificity (that is, safety) without reducing its anticancer (that is, therapeutic) effects. These findings are somewhat

unexpected, but beneficial because these results indicate that most cancer/tissue-specific promoters can be used and illustrate that this principle may be, at least in part, generalized for the future development of this type of m-CRA (that is, two different promoters driving *E1A* and *E1BΔ55K*). On the other hand, previous studies showed that the *E1A* and *E1B* proteins must function in concert to activate effectively the transcription of other adenoviral genes, leading to efficient viral replication.[28] One widely accepted speculation on the role of *E1B*/*E1BΔ55K* in adenoviral replication is that the antiapoptotic effects of *E1BΔ55K* prevent the early onset of cell death, providing extended periods for efficient viral replication in living cells.[29] A detailed examination of this molecular mechanism is beyond the scope of this study because part of the independent and diverse biological functions of the *E1B* protein may not simply reflect the overall virological functions of *E1B* in concert with other viral proteins. Nevertheless, these results further highlight the importance of the *E1BΔ55K* protein in adenoviral replication, particularly in the development of ideal m-CRAs.

This study also provides important information for future clinical trials that examine the efficacy of Surv.m-CRAs to treat a variety of cancers, including the ability of Surv.m-CRA-OCp to treat osteosarcoma or prostate cancer. Clinical trials that have examined intratumoral injections of simple CRAs, in which *E1A* is regulated by a single cancer-specific promoter, demonstrated sufficient safety with no reports of lethal adverse effects.[30,31] However, to date, there are no reports of clinical trials that have examined or sufficiently assessed the safety of systemic injections of simple CRAs, even though systemic injections may be more effective to treat disseminated cancer cell metastases, including metastatic osteosarcoma in lung, for which current therapies are ineffective.[1,20,32] The improved m-CRAs that were generated based on these results, including Surv.m-CRA-OCp, may facilitate clinical trials on systemic m-CRA therapy. However, careful and extensive preclinical studies should be performed.

In conclusion, replacing the *E1B* promoter with a different cancer/tissue-specific promoter, in addition to cancer-specific regulation of *E1A*, results in more effective m-CRAs that have high anticancer efficacy and increased safety. Surprisingly, but favorably, it is not essential for m-CRAs to use an altered *E1B* promoter with strong activity, indicating that the majority of cancer/tissue-specific promoters can be used to generate ideal m-CRAs. All of these results will greatly contribute to the development of highly effective m-CRA-based cancer therapy.

Conflict of interest

K Kosai is the founder of WyK BiotechPharma Inc., but does not earn a salary from the company. No other potential conflict of interest was disclosed.

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