Ushikosi H, Nagano S, Fujiwara H, Kosai K : Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2. Circ J, 2004 (in press)

15) Minatoguchi S, Takemura G, Chen XH, Wang N, Uno Y, Koda M, Arai M, Misao Y, Lu C, Suzuki K, Goto K, Komada A, Takahashi T, Kosai K, Fujiwara T, Fujiwara H : Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation, 109 : 2572 - 2580, 2004

RESEARCH ARTICLE

An efficient construction of conditionally replicating adenoviruses that target tumor cells with multiple factors

S Nagano[1,2,3], H Oshika[4], H Fujiwara[5], S Komiya[2] and K Kosai[1,3,4]

[1]Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, Kurume, Japan; [2]Department of Neuro-musculoskeletal Disorder, Orthopaedic Surgery, Graduate School of Medicine and Dentistry, Kagoshima University, Kagoshima, Japan; [3]Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan; [4]Department of Gene Therapy and Regenerative Medicine, Gifu University School of Medicine, Gifu, Japan; and [5]Department of Cardiology, Respiratory and Nephrology, Regeneration & Advanced Medical Science, Graduate School of Medicine, Gifu University, Gifu, Japan

Despite the enormous potential of conditionally replicating adenoviruses (CRAs), the time-consuming and laborious methods required to construct CRAs have hampered both the development of CRAs that can specifically target tumors with multiple factors (m-CRA) and the efficient analysis of diverse candidate CRAs. Here, we present a novel method for efficiently constructing diverse m-CRAs. Elements involving viral replication, therapeutic genes, and adenoviral backbones were separately introduced into three plasmids of P1, P2, and P3, respectively, which comprised different antibiotic resistant genes, different ori, and a single loxP (H) sequence. Independently constructed plasmids were combined at 100% accuracy by transformation with originally prepared Cre and specific antibiotics in specific Escherichia coli; transfection of the resulting P1+2+3 plasmids into 293 cells efficiently generated m-CRAs. Moreover, the simultaneous generation of diverse m-CRAs was achieved at 100% accuracy by handling diverse types of P1+2 and P3. Alternatively, co-transfection of P1+3 and P2 plasmids into Cre-expressing 293 cells directly generated m-CRA with therapeutic genes. Thus, our three-plasmid system, which allows unrestricted construction and efficient fusion of individual elements, should expedite the process of generating, modifying, and testing diverse m-CRAs for the development of the ideal m-CRA for tumor therapy.

Gene Therapy (2005) 0, 000–000. doi:10.1038/sj.gt.3302540

Keywords: conditionally replicating adenovirus; cancer; tumor-specific; Cre/lox recombination

Introduction

One of the major obstacles to cancer gene therapy is inefficient and nonspecific gene delivery to cancer cells, leading to unsatisfactory outcomes in clinical trials due to recurrence from nontransduced tumor cells, even though some of the effective strategies, such as suicide gene therapy,[1,2] immunological gene therapy,[3,4] and their combinations[5] may treat nontransduced tumor cells to some degree and partially circumvent this problem. Conditionally replicating adenoviruses (CRAs), which selectively replicate in tumor cells, but not in normal cells, have the potential to circumvent this problem and to achieve tumor-specific gene delivery.[6,7] Moreover, CRA itself may be an attractive tool for innovative cancer therapy because selectively propagated adenovirus (Ad) induces the lysis of tumor cells. While various CRAs have been reported to date, the majority of them may be classified into two groups.[8] One is CRA that expresses E1 in a tumor-specific manner by the replace-

ment of a native E1 promoter with various tumor-specific promoters.[9–12] The other is CRA with a partial deletion of the E1 gene; the representatives are the mutant (mt) type of Ad lacking a p53-binding protein that is encoded by E1B55kD (ONYX-015),[13] and the mt Ad lacking an Rb-binding site of E1A (Δ24).[14,15] In the case of infection with the wild type of Ad, E1B55kD inhibits the p53-induced apoptosis of the host cell and enables Ad to continuously replicate in cells.[13,16] In addition, the interaction of adenoviral E1A with cellular Rb leads to the release of E2F transcription factor, which induces S-phase transition of the host cell in order to facilitate viral replication.[16] Based on these theories, neither mt ONYX-015 nor Δ24 may efficiently replicate in normal cells with intact Rb and p53, whereas both CRAs may actively replicate in the majority of tumor cells, disrupting the Rb-E2F pathway and/or p53 function.

However, a perfect CRA, which replicates efficiently in cancer cells but is completely attenuated in normal cells, has not yet been established in reality. Especially, the crucial problem of current CRAs is the insufficient or incomplete cancer specificity; that is, these CRAs do replicate in and cause some cytopathic effects, while greatly attenuated, in normal cells.[17–19] Recent studies suggested that CRAs with two or three tumor specific

2

factors enhanced their tumor specificity: mt E1A and mt E1B,[20] two tumor-specific promoters,[21] or a tumor-specific promoter and mt E1A.[12,22] In this regard, a promising approach to circumvent this obstacle might be combining and introducing multiple (more than three) tumor-specific factors into a single CRA. However, extensive and comparative studies on CRAs that are regulated with multiple tumor-specific factors (m-CRAs) are currently hampered by the lack of standardized methods to efficiently construct m-CRAs in contrast to well-established methods for efficiently constructing E1-deleted replication-incompetent Ad vectors.[23–25] It remains time-consuming and laborious to construct diverse m-CRAs using current methods; the requirement of additional modification steps hampers efficient production of diverse m-CRAs in large numbers by the same protocol. In addition, although functions of individual viral proteins have been largely elucidated,[16] the controversy over p53-dependent replication of the most representative CRA, ONYX-015,[13,17,18] suggests the necessity of extensive biological and systematic virological analyses of a large number of diverse m-CRAs in practice.

Here, we develop a novel method for the efficient construction of m-CRAs; this system simplifies and expedites the generation and modification of m-CRAs.

Results

Constitution of m-CRAs

One of the characteristic features of our method is the independent and unrestricted construction of three different regulatory elements in m-CRA, involving viral replication, therapeutic genes, and Ad backbones. To this end, these elements were separately introduced into three plasmids (Figure 1a). Replication-controllable plasmid P1 consists of wt or mt E1A and E1B sequences. Therapeutic gene-cloning plasmid P2 characteristically contains the tetracycline resistance gene (tet^r) and $R6K\gamma$ ori, which render this plasmid selectively amplified in only a specific type of Escherichia coli expressing the pir gene.[26] The Ad backbone plasmid P3 was described previously.[23] Potentially, more than seven tumor-specific factors can be introduced into the m-CRA (Figure 1a).

The use of different antibiotic resistance genes in all three plasmids, characteristically specific ori in P2 and unique I-CeuI and PI-SceI restriction sites in P1 and P3, enable the independent and unrestricted construction of three plasmids, and, subsequently, the feasible and rapid fusion of these three plasmids to generate a single CRA plasmid without using a regular ligation procedure. This is accomplished in the following manner (Figure 1b). Four variants of P1 vector with different combinations of wt or mt of E1A and E1B can be chosen at present. After the therapeutic gene and the promoters of interest were inserted into the multiple-cloning sites in P1 and P2, these two vectors were mixed and incubated with Cre aliquot. DH5α E. coli was transformed using all of the mixtures, and then grown on LB plates containing 5 μg/ml tetracycline. As P1 or P2 has either the kanamycin resistance gene (kan^r) or $R6K\gamma$ ori but not both, only the DH5α clone containing successfully recombined plasmid P1+2 will grow and form a colony on the LB plates containing tetracycline. P3 is digested with I-CeuI and PI-

SceI, and ligated with I-CeuI/PI-SceI-digested P1+2, yielding a single P1+2+3 plasmid. Finally, this P1+2+3 plasmid, that is, Ad vector plasmid containing a replication-regulatory element and therapeutic gene, is linearized by PacI, and transfected into 293 cells, as described previously.[5,23] Miniprep DNA can be used in all of the procedures, including the transfection; this feature increases the rapidity of this method and allows the handling of numerous samples simultaneously.

Preparation of Cre recombinase

Commercial Cre recombinase is so expensive that it prohibits the manipulation of a large number of samples in the present system. To circumvent this obstacle, we developed a feasible and inexpensive way to obtain a solution containing highly active Cre recombinase as follows. HepG2 cells, which demonstrated the highest level of transgene expression and the highest adenoviral gene transduction efficiency (data shown elsewhere), were infected with Ad.CA-Cre (Ad expressing Cre under the strongest CA promoter, which was kindly donated by I Saito) at an MOI of 30 for 2 days, and were then harvested and lysed in 200 μl buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% glycerol by three rounds of freeze–thawing. The supernatants after centrifugation were collected and stored in aliquots at −80°C until use.

We compared the activity of our Cre aliquot with commercial products using two plasmids which had a single loxP sequence. At 1 day after the transformation of E. coli with our Cre aliquot and growth on 10 cm LB plates, several hundred colonies appeared and all colonies were correctly recombined to form a single plasmid (data not shown). Unexpectedly, two of the three commercial lots from two representative companies did not work well (no and one colony). Thus, the Cre activity in our aliquot was sufficiently high for reliable Cre/lox recombination in E. coli; we used this Cre aliquot for the following m-CRA construction (supernatant from one 10 cm dish allowed 200 samples of reaction).

Construction of CEA-responsive m-CRAs

To test the efficiency and the feasibility of this system, we generated carcinoembryonic antigen (CEA) responsive m-CRAs as an example, in which either wt or mt E1A was expressed under the transcriptional control of the CEA promoter (CEApr). Both types of m-CRA have mt E1B (E1BΔ55kD) downstream from the cytomegalovirus immediate-early gene enhancer/promoter (CMVpr). As a P2 plasmid, pUni/CMVpr-EGFP was used.

After the recombination of P1 and P2 with Cre aliquot, followed by the transformation of DH5α and growth, 10–50 colonies per 10 cm LB-tetracycline plate appeared. Notably, restriction enzyme analyses demonstrated that all of the clones contained the correctly recombined plasmid (pCEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP or pCEApr-E1AΔ24-CMVpr-E1BΔ55kD/CMVpr-EGFP; we term each P1+2 plasmid as 'pP1-component/P2-component') (Figure 2a). The somewhat lower titer here than that shown in an earlier section was due to tetracycline selection, but not dur to Cre/lox recombination efficiency, according to our preliminary studies (data not shown), whereas the result of 100% accuracy in 10–50 colonies was rather encouraging and sufficient for the present purpose.
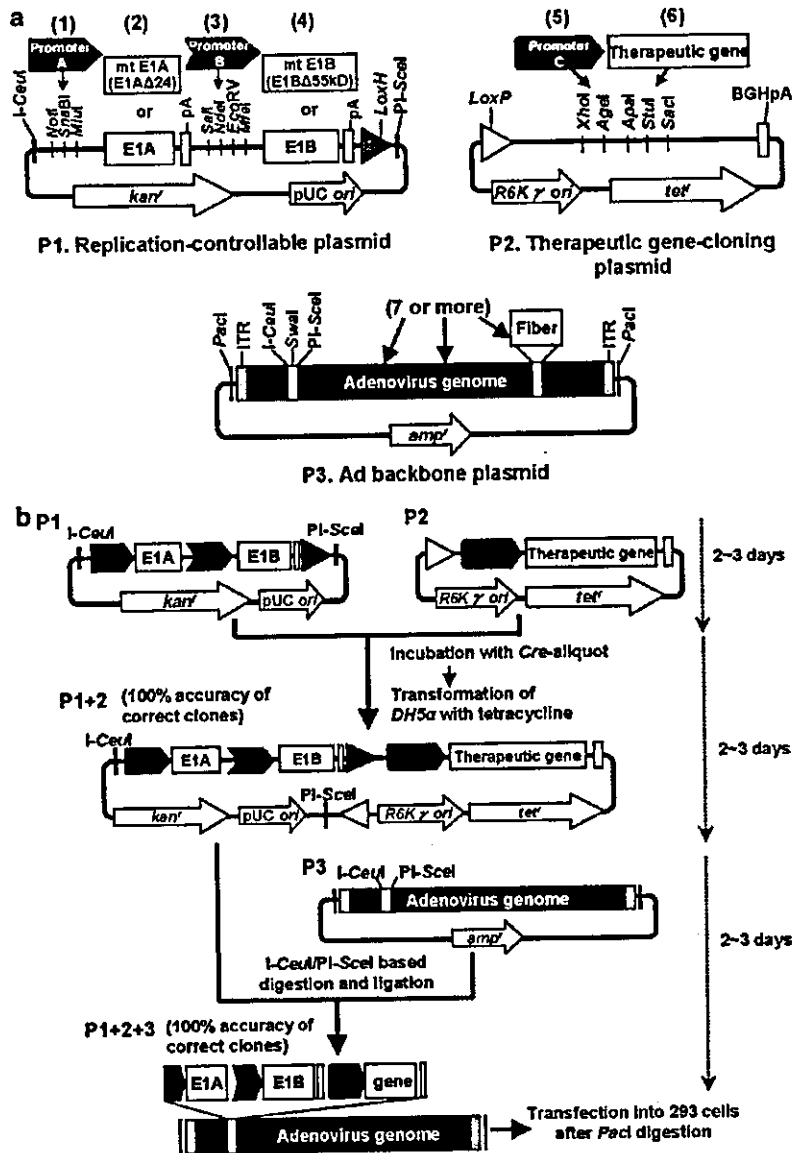
Figure 1 The constitution and construction of m-CRA. (a) The constitution of the vector plasmids. Potentially, more than seven tumor-specific factors can be introduced into the m-CRA as follows. (1) Promoter A, which drives wt or mt E1A. (2) mt E1A, which lacks an Rb-binding site (E1AΔ24). (3) Promoter B, which drives wt or mt E1B. (4) mt E1B, which lacks a p53-binding protein that is encoded by E1B55kD (E1BΔ55kD). (5) Promoter C, which drives a therapeutic gene. (6) A therapeutic gene (seven or more). Modification of Ad backbone, such as a fiber modification to modulate an infectivity. (b) The schematic representation of the m-CRA construction. All procedures, including the transfection into 293 cells, can be carried out using miniprep DNA.

Elements involving viral replication and the therapeutic gene were transferred from P1+2 to P3 (pAd.HM4) to generate P1+2+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP or pAd.HM4-CEApr-E1AΔ24-CMVpr-E1BΔ55kD/CMVpr-EGFP; we term each P1+2+3 adenoviral plasmid as 'pAd.P3-component.P1-component/P2-component'). The accuracy of such unique I-CeuI/PI-SceI-based ligation was almost 100% (Figure 2b), in accordance with the previous results.[23] Over 10 plaques appeared on 6 cm dishes 12 days after the transfection of PacI-digested P1+2+3 into 293 cells. Notably, all of the plaques were EGFP-positive under fluorescent microscopy (Figure 2c). Accordingly, the PCR analyses of the DNA extracted from these m-

CRAs verified that all of them were correct CEA-responsive m-CRA (CRA.CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP or CRA.CEApr-E1AΔ24-CMVpr-E1BΔ55kD/CMVpr-EGFP; we term each m-CRA as 'CRA.P1-component/P2-component') (Figure 2d).

Simultaneous construction of diverse types of m-CRAs
It would further facilitate extensive analyses of m-CRAs if numerous and diverse types of m-CRAs could be constructed at one time. To investigate this possibility, we first investigated whether initially constructed P1+3, that is, Ad backbone plasmid with a replication-regulatory element, might be recombined with therapeutic-gene
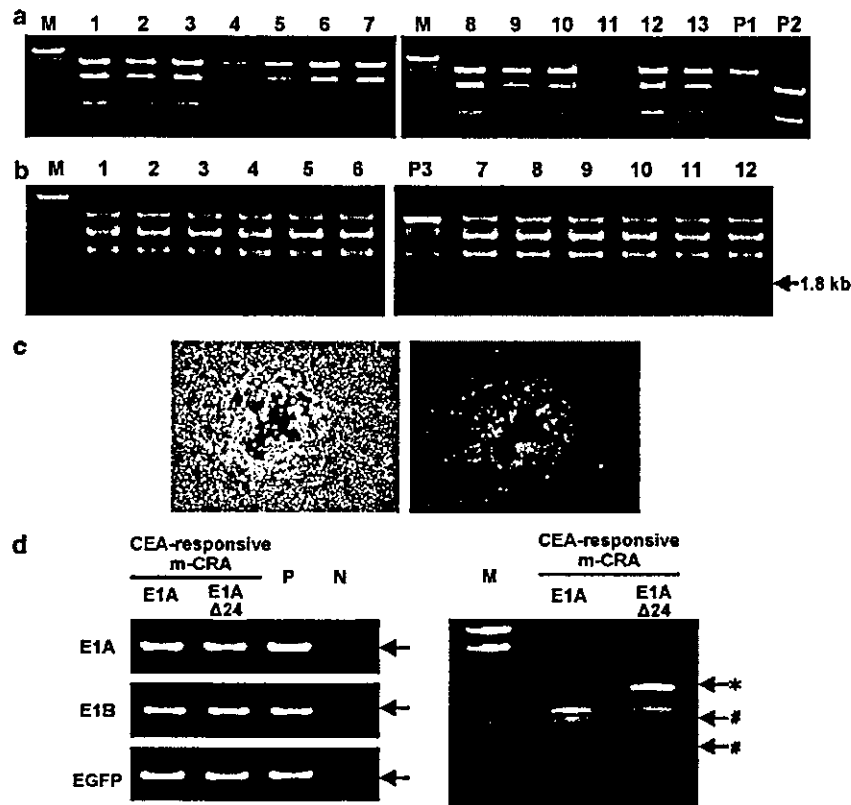
4

Figure 2 *The efficiency and accuracy in the m-CRA construction on the protocol shown in Figure 1b. (a) Restriction enzyme analysis of P1+2 plasmid. After the reaction of pCEApr-E1A-CMVpr-E1BΔ55kD (P1) and pUni-CMVpr-EGFP (P2), 13 miniprep samples from E. coli colonies were digested by SalI and electrophoresed (M, marker. Lanes 1–13, each sample; P1, P1 plasmid; P2, P2 plasmid). The correct pattern (three bands consisting of P1-derived 6.3 kb band and P2-derived 3.2 and 1.2 kb bands) was seen in all samples, demonstrating 100% accuracy of correct clones containing P1+2 plasmid (pCEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP) among all of the transformed clones. (b) Restriction enzyme analysis of P1+2+3 plasmid. After the reaction of P1+2 (pCEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP) and P3 (pAd.HM4), 12 miniprep samples from E. coli colonies were digested with HindIII and electrophoresed (M, marker. Lanes 1–12, each sample; P3, P3 plasmid). The correct pattern was seen in all samples; the 1.8 kb band was indicative of the correct clone. (c) Phase-contrast (left) and fluorescent (right) microscopic pictures of one representative of m-CRA plaques (CRA.CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP) on 293 cells 10 days after transfection of PacI-digested pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP. (d) PCR analyses of genomic DNA extracted from m-CRA plaques. PCR was performed with three different primer sets of S-E1A-1/AS-E1A-1, S-E1B-1/AS-E1B-1, and S-EGFP/AS-EGFP to detect E1A, E1A, and EGFP DNA, respectively, in CEA-responsive m-CRAs (E1A; CRA.CEApr-E1A-CMVpr-E1BΔ55KD/CMVpr-EGFP, and E1AΔ24; CRA.CEApr-E1AΔ24-CMVpr-E1BΔ55kD/CMVpr-EGFP) (the left picture). P, positive control plasmid DNA corresponding to each of primer sets. N, nontemplate DNA. To distinguish these two CEA-responsive m-CRAs, PCR products amplified with the primer sets of S-HM5 and AS-E1A-1 were digested with BstXI, of which recognition sites existed in the Rb-binding domain of E1A and Ad backbone (the right picture). The correct pattern was seen in all samples of both types of m-CRA; the 0.8 kb band (*), and the 0.5 and 0.3 kb bands (#) were indicative of m-CRA with mt E1A (E1AΔ24), and that with wtE1A, respectively.*

cloning vector P2 in the same way as the recombination of P1 and P2 (Figure 3a). P1+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD) was constructed by transferring replication-regulatory elements from P1 (pCEApr-E1A-CMVpr-E1BΔ55kD) to P3 (pAd.HM4) with I-CeuI/PI-SceI-based ligation. After incubating P1+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD) and P2 (pUni/CMVpr-EGFP) with Cre aliquot, DH5α was transformed with all of the mixtures and grew on LB plates with tetracycline. After 1 day, about 30 colonies appeared on a 10 cm dish, and all of them contained correctly recombined P1+2+3 plasmid (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP) (Figure 3b). Thus, both the efficiency and accuracy of the recombination/transformation of P1+2 and P3 were similarly high in comparison with those of P1 and P2, as shown in the earlier section, although P1+3 was a much larger plasmid than P1 alone.

Next, the feasibility of simultaneously constructing several different types of m-CRAs was tested. DH5α E. coli in each of 10 tubes containing the same P2 (pUni/CMVpr-EGFP) and Cre aliquot was transformed by each of 10 different types of P1+3 plasmid. At 1 day after the growth on LB-tetracycline plates, 3–52 colonies appeared on each of the 10 cm plates, and all colonies were correctly recombined plasmids (Figure 3c).

Furthermore, we examined whether more different tumor-specific factors including therapeutic genes can be correctly inserted into m-CRAs with the present system. Eight different m-CRAs that contain six tumor-specific factors, that is, (1) human telomerase reverse transcriptase promoter (TERTpr) driving E1A, (2) wt or mt E1A, (3) human E2F promoter (E2Fpr) driving E1B, (4) mt E1B, (5) mouse survivin promoter (Surv.pr) or CMVpr driving therapeutic gene, and (6) therapeutic gene (p53 or herpes
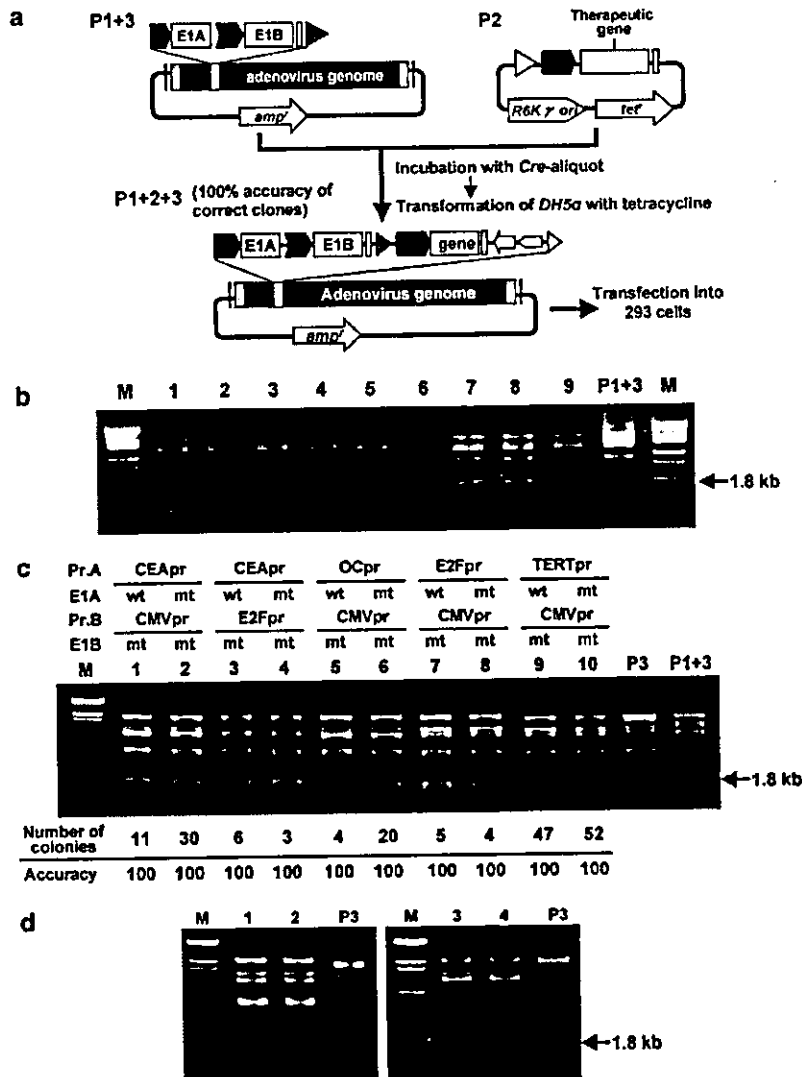
Figure 3 Simultaneous construction of diverse types of m-CRA plasmids. (a) The schematic representation. Initially constructed P1+3, that is, Ad backbone plasmid with a replication-regulatory element, can be recombined with therapeutic-gene cloning vector P2 to yield a single P1+2+3 plasmid. (b) Restriction enzyme analysis of genomic DNA extracted from clones transformed with P1+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD) and P2 (pUni/ CMVpr-EGFP). HindIII digestion of miniprep samples demonstrated the correct pattern of P1+2+3 plasmid (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD/ CMVpr-EGFP) in all of the transformed clones (M, marker. Lanes 1–9, each sample; P1+3, P1+3 plasmid); the 1.8 kb band was indicative of the correct clone. (c, d) Simultaneous construction of several different types of m-CRAs with this protocol. (c) DH5α E. coli in each of 10 tubes containing the same P2 (pUni/CMVpr-EGFP) and each of 10 different types of P1+3 plasmid, which were preincubated with the Cre aliquot, were transformed and grew with tetracycline. HindIII-digestion analysis was carried out in the same manner as above, and one representative picture per group was shown here. Numbers of colonies per 10 cm plate appeared and the accuracy (the percentage of the correct clones; clones in each group were carefully analyzed with several different types of restriction enzymes although data were not shown here) in each group is shown below the picture (M, marker. Lanes 1–10, each sample; Pr.A, promoter driving wt or mt E1A; Pr.B, promoter driving wt or mt E1B; P3, P3 plasmid; P1+3, P1+3 plasmid). The 1.8 kb band was indicative of the correct clone. (d) Four different CRAs which contain six tumor-specific factors were constructed and the P1+2+3 plasmids were analyzed by HindIII digestion (M, marker. Lane 1, pAd.HM4-TERTpr-E1A-E2Fpr-E1BΔ55kD/Surv.pr-p53; Lane 2, pAd.HM4-TERTpr-E1AΔ24-E2Fpr-E1BΔ55kD/Surv.pr-p53; Lane 3, pAd.HM12-TERTpr-E1A-E2Fpr-E1BΔ55kD/Surv.pr-tk; each sample; Lane 4, pAd.HM12-TERTpr-E1AΔ24-E2Fpr-E1BΔ55kD/Surv.pr-tk; P3, P3 plasmid).

simplex virus thymidine kinase (HSV-tk), were successfully constructed (Figure 3d and data not shown).

Thus, it was shown that simultaneous construction of diverse m-CRAs was, in fact, feasible using the present system.

Therapeutic gene insertion into m-CRA directly in Cre-expressing 293 cells (alternative protocol)

We further hypothesized that two types of plasmids, P1+3 and P2, might be recombined directly in Cre-expressing 293 cells (Figure 4a). To assess this possibility, P1+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD) and
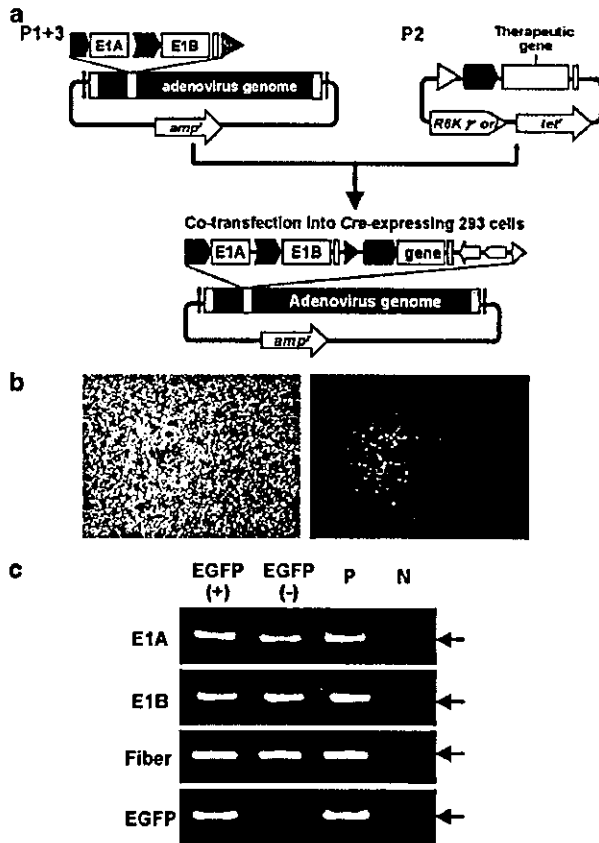
Figure 4 Therapeutic gene insertion into m-CRA directly in Cre-expressing 293 cells (alternative protocol). (a) The schematic representation. (b) Phase-contrast (left) and fluorescent (right) microscopic pictures of an m-CRA plaque (CRA.CEApr-E1A-CMVpr-E1BΔ55kD/CMVpr-EGFP) on Cre-expressing 293 cells 12 days after co-transfection of P1+3 (pAd.HM4-CEApr-E1A-CMVpr-E1BΔ55kD) and P2 (pUni/CMVpr-EGFP). (c) PCR analyses of genomic DNA extracted from m-CRA plaques that do or do not show EGFP fluorescence (two left lanes, respectively). PCR analyses were performed using four different primer sets of S-E1A-1/AS-E1A-1, S-E1B-1/AS-E1B-1, S-Fiber/AS-Fiber, and S-EGFP/AS-EGFP to detect E1A, E1B, fiber, and EGFP DNA, respectively. P, positive control plasmid DNA corresponding to each of primer sets. N, nontemplate DNA.

P2 (pUni/CMVpr-EGFP) were cotransfected into Cre-expressing 293 cells. Some plaques became visible 6 days afterwards, and 10 plaques per 10 cm dish appeared 12 days after cotransfection. One of these 10 plaques showed an EGFP-positive finding under fluorescent microscopy (Figure 4b). PCR analysis of the DNA isolated from these plaques verified that only this fluorescence-positive plaque contained a correctly recombined m-CRA with EGFP cDNA (Figure 4c). Therefore, using a marker gene such as EGFP, the correct m-CRA derived from P1+2+3 plasmid can be easily isolated from the m-CRA derived from P1+3 plasmid.

Discussion

The large size of the adenoviral DNA (36 kB) hampered the feasible modification of the Ad vector, due to limited numbers of available unique restriction enzyme recognition sites. For constructing the E1-deleted Ad, a two-plasmid system, that is, separate construction of the therapeutic gene and adenoviral backbone in two plasmids, was recently utilized.[23] However, when constructing CRAs, a modification of the E1 region is additionally necessary; currently, there is no standardized method to construct diverse types of m-CRAs in large numbers. Owing to this deficiency, many molecular biologists or gene therapy researchers, who are not CRA specialists, may not be able to efficiently construct CRAs, whereas they can feasibly construct E1-deleted Ad using commercially available kits. Detailed protocols, incorporating the careful consideration of available restriction enzyme sites, should be formulated each time a new CRA is constructed or any time a currently available CRA is modified. The procedures used currently may vary in the details of constructing different m-CRAs, and additional modifications of their own protocols in individual laboratories may be necessary depending on individual CRAs. Thus, even though it may be possible to construct an m-CRA, the present methods are not yet standardized and are deficient in their potential to be widely utilized by a large number of researchers.

In this respect, the notable feature of our system is that it allows the independent and unrestricted construction of individual elements of viral replication, therapeutic genes, and adenoviral backbones in three plasmids, and subsequently permits the accurate fusion of these plasmids within the same protocol into a single m-CRA plasmid without regular ligation procedures. This feature enables a normally trained molecular biologist to feasibly construct m-CRAs, and to construct and/or modify diverse m-CRAs in large numbers. To this end, we introduced Cre/lox recombination and I-CeuI/PI-SceI-based ligation for combining the three plasmids, and different types of antibiotic resistance genes and ori for specifically selecting only the correctly combined clones, into this system. In this context, the factors needed for this system to function at its maximum efficiency were the use of both an optimized concentration of tetracycline, and high-activity Cre. Due to the fact that there have been no reports of tetracycline selection together with the Cre/lox recombination in E. coli, and because we obtained a lower transformation efficiency with tetracycline than with kanamycin or ampicilin, we initially determined the optimal concentration of tetracycline for this system. Unexpectedly, the optimal concentration of tetracycline was 5 μg/ml in the LB plate, which is lower than that described in textbooks (12 μg/ml),[27] and the effective range was narrow (data not shown). Higher Cre activity is also essential, especially due to the use of tetracycline selection; the use of lower Cre activity did not result in the appearance of the correct clone. We established a feasible and inexpensive way to obtain aliquots containing highly active Cre. With these conditions, sufficient numbers of clones always appeared, and all of them contained the correct recombined plasmid. The distinct advantage of the present method is 100% accuracy of correct clones among all of the transformed clones in E. coli; this feature, together with the use of miniprep DNA throughout the procedure, allowed us to handle multiple diverse samples at one time, and we in fact simultaneously constructed diverse types of m-CRAs in this study. The remnant components of tet[r], R6Kγ ori, and a single lox sequence in m-CRAs, which are indispensable to achieve 100% accuracy of correct clones,

are not troublesome, at least for the experimental purpose, probably in general, described herein because neither *tet*^r lacking a proper mammalian promoter or poly A sequence, nor *R6Kγ ori* efficiently function in mammalian cells. In fact, we found that several m-CRAs, including the ones shown in Figure 3c in this study, function well with these components without any harmful effects.

An introduction of a marker gene into a CRA, such as *EGFP* gene used in the present study, which allows researchers to monitor the spread of a CRA *in vitro* and *in vivo*, is quite useful for carefully analyzing the virological features of CRA. It was recently shown that CRA expressing a therapeutic gene may enhance the cytotoxicity and the therapeutic potential in addition to the oncolytic activity of CRAs.[19] In this regard, an advantage of our system is the ability to subsequently insert a therapeutic or marker gene either into an m-CRA plasmid in *E. coli* or directly into m-CRA in *Cre*-expressing 293 cells. To maximize both protocols, diverse types of P1+3 and P2 may be initially prepared using the regular subcloning procedure, because combining different types of P1+3 and P2 at the later stage can be carried out simultaneously, as shown in the present study. The latter protocol, that is, direct transfection of P1+3 and P2 into *Cre*-expressing 293 cells, has the advantage of further eliminating one step in *E. coli*, leading to an increase in the rapidity of the procedure. *EGFP* or other fluorescent genes alongside the therapeutic gene in P2, which allows direct identification of the correct m-CRA plaque containing therapeutic genes under fluorescent microscopy, may maximize this benefit.

The unrestricted and independent construction of individual elements in the three-plasmid system should not only expedite the process of generating diverse m-CRAs but also make feasible the modification of individual elements in m-CRAs. Several combination patterns of different P1, P2, and P3 vectors allow the generation of diverse types of m-CRAs in large numbers, including those in which some elements were modified with diverse combinations. Lessons from previous gene therapy studies, that is, discrepancies between experimental data and actual clinical outcomes, suggest the necessity of the systematic and extensive examination of the biological and virological characteristics of diverse m-CRAs in practice. In this respect, the ability to efficiently generate large numbers of modified m-CRAs with diverse combinations of gene elements may be the most significant advantage of our system. Particularly, unrestricted selection of the adenoviral backbone may provide two potential advantages. First, any suitable adenoviral backbone, such as fiber-modified Ads to achieve tumor-specific infection, which may be determined by other types of extensive studies, can be directly used in the m-CRA studies; additional steps in constructing new m-CRAs that contain both characteristic elements may be omitted. Second, Ad can package 105% of its genome, and such DNA size limitation may potentially complicate or hamper the modification of pre-constructed m-CRAs. However, the present system allows the change of the P3 backbone plasmid to circumvent this problem; P3 with a longer deletion can be used if additional DNA should be inserted in P1 or P2 to modify pre-established m-CRA. Namely, the three-plasmid system in the present m-CRA construction may

maximize the benefit of the *in vitro* ligation system.[23] For instance, up to 5.4, 5.9 or 8.7 kb of DNA elements can be introduced in P1 and P2 when pAdHM4, pAdHM10, or pAdHM12 is used as P3 vector, respectively, as described previously.[23]

In conclusion, the present study reports a novel method for efficiently constructing and/or modifying diverse m-CRAs; this system may be useful for the development of the ideal m-CRA for tumor therapy.

Materials and methods

Cell lines
The human hepatoma cell line, Hep-G2, was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Hep-G2 was cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The *Cre*-expressing 293 cell line was generated as described elsewhere.[28]

Plasmids
Plasmid P1 was constructed as follows. The E1A coding sequence and pA without the native E1A promoter were obtained by PCR from pXC1 (Microbix, Toronto, Canada) with the primers S-E1A/AS-E1A (Table 1). This E1A fragment was digested with *Sph*I and *Sal*I and inserted into *Sph*I/*Sal*I-digested pHM5.[23] The resulting plasmid was designated pΔPrE1A. The mt E1B coding sequence (1684–2285), which lacked the E1B55kD coding sequence, native E1B promoter, and pA, was obtained by PCR from pXC1 with the primers S-E1BΔ55kD/AS-E1BΔ55kD (Table 1) and inserted into pΔPrE1A by *Sal*I/*Bam*HI sites, resulting in pΔPrE1A-ΔPrE1BΔ55kDΔpA plasmid. BGHpA obtained by PCR with the primers S-BGHpA/AS-BGHpA (Table 1) from pRc/CMV plasmid (Invitrogen, Carlsbad, CA, USA) was inserted into pΔPrE1A-ΔPrE1BΔ55kDΔpA by *Bam*HI/*Eco*RI sites, resulting in pΔPrE1A-ΔPrE1BΔ55kD plasmid. The full length of E1B (1684–4073) was obtained by PCR from pXC1 with the primers S-E1B/AS-E1B (Table 1) and inserted into *Kpn*I/*Eco*RI-digested pΔPrE1A-ΔPrE1BΔ55kD, resulting in ΔPrE1A-ΔPrE1B plasmid. All sequences obtained by PCR were confirmed using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

E1A sequence without a 24 bp sequence for pRb binding was obtained by site-directed mutagenesis using sequential PCR steps[27] with the primers AS-E1AΔ24/S-E1AΔ24 (Table 1). The obtained product was inserted into pΔPrE1A-ΔPrE1B or pΔPrE1A-ΔPrE1BΔ55kD plasmid by *Not*I/*Sal*I sites, resulting in pΔPrE1AΔ24-ΔPrE1B or pΔPrE1AΔ24-ΔPrE1BΔ55kD plasmid, respectively.

Plasmid P2 was constructed as follows. *kan*^r was removed from pUni/V5-HisC (Invitrogen) by *Bgl*II/*Sma*I digestion. A blunt-end fragment of *tet*^r obtained from pBR322 (Invitrogen) was inserted into the vector to yield pUni/V5-HisC-*tet*^r. The construction of plasmid P3 was performed as described previously.[23]

CEApr[29] and CMVpr were obtained by PCR from AxCEAprTK (Riken gene bank, Tsukuba, Japan) and pRc/CMV with the primer sets of S-CEApr/AS-CEApr and S-CMVpr/AS-CMVpr, respectively (Table 1). The fragment containing CEApr or CMVpr was excised by

8

Table 1 PCR primers

Primer	Sequence	Annealing temperature (°C)
S-E1A	5'-TCAGTCGCATGCGCGGCCGCTACGTAACGCGTTACCCGGTGAGTTCCTCAAGAGGC-3'	57
AS-E1A	5'-GGACGTCCTAGGGTCGACGCCCCATTTAACACGCCATGCAAG-3'	
S-E1BΔ55kD	5'-TCAGTCCCTAGGGTCGACCATATGGATATCCAATTGCGTGGGCTAATCTTGGTTACATCT-3'	57
AS-E1BΔ55kD	5'-GGACGTGGATCCGCGTCTCAGTTCTGGATACAGTTC-3'	
S-E1B	5'ATAAATGGAGCGAAGAAACC -3'	57
AS-E1B	5'-GGACGTGAATTCATAACTTCGTATAATGTATGCTATATGAGGTAATCTTGATCCAAATCCAAACAGAGTC-3'	
S-BGHpA	5'-TCAGTCGGATCCGCATGCATCTAGAGCTCGCTGATC-3'	57
AS-BGHpA	5'-GGACGTGAATTCATAACTTCGTATAATGTATGCTATATGAGGTAATTCAGAAGCCATAGAGCCCACCGCA-3'	
S-Δ24	5'-TTGTACCGGAGGTGATCGATCCACCCAGT-3'	57
AS-Δ24	5'-TCCTCGTCGTCACTGGGTGGATCGATCACC-3'	
S-CEApr	5'-TCAGTCGCGGCCGCATCATCCCACCTTCCCAGAG-3'	57
AS-CEApr	5'-GGACGTACGCGTCCATGGTCTCTGCTGTCTGC-3'	
S-CMVpr	5'-TCAGTCGTCGACCGTTGACATTGATTATTGAC-3'	57
AS-CMVpr	5'-GGACGTCAATTGGCTTGGGTCTCCCTATAGTG-3'	
S-E1A-1	5'-CCTGTGGCATGTTTGTCTAC-3'	57
AS-E1A-1	5'-CAACTGGTTTAATGGGGCAC-3'	
S-E1B-1	5'-AAGGAGGATTACAAGTGGGA-3'	57
AS-E1B-1	5'-AGTAGCAGGCGATTCTTGTG-3'	
S-EGFP	5'-CACAAGTTCAGCGTGTCC-3'	59
AS-EGFP	5'-CTTGATGCCGTTCTTCTG-3'	
S-Fiber	5'-GTTCCTGTCCATCCGCACCCACTATCTTCATGTTG-3'	59
AS-Fiber	5'-AGTGGCAGTAGTTAGAGGGGGTGAGGCAGTGATAG-3'	
S-pHM5	5'-AACGGTCCTAAGGTAGCGAA-3'	59

NotI/MluI or *SalI/MfeI*, respectively, and ligated into similarly digested pΔPrE1A-ΔPrE1BΔ55kD or pΔPrE1AΔ24-ΔPrE1BΔ55kD, resulting in pCEApr-E1A-CMVpr-E1BΔ55kD or pCEApr-E1AΔ24-CMVpr-E1BΔ55kD. Human osteocalcin promoter (OCpr, −834 to +34)[30] and Surv.pr (−173 to −19)[31] were obtained by PCR from genomic DNA (details should be described elsewhere). E2Fpr (−218 to +51)[32] and TERTpr (−181 to +78)[33] were kindly provided by H Fine (National Cancer Institute, Bethesda, MD, USA) and S Kyo (Kanazawa University, Kanazawa, Japan), respectively. P1 plasmids pCEApr-E1A-E2Fpr-E1BΔ55kD, pCEApr-E1AΔ24-E2Fpr-E1BΔ55kD, pE2Fpr-E1A-CMVpr-E1BΔ55kD, pE2Fpr-E1AΔ24-CMVpr-E1BΔ55kD, pOCpr-E1A-CMVpr-E1BΔ55kD, pOCpr-E1AΔ24-CMVpr-E1BΔ55kD, pTERTpr-E1A-CMVpr-E1BΔ55kD, pTERTpr-E1AΔ24-CMVpr-E1BΔ55kD, pTERTpr-E1A-E2Fpr-E1BΔ55kD, and pTERTpr-E1AΔ24-E2Fpr-E1BΔ55kD were constructed in the same manner as described above. pUni/V5-HisC-*tet*[r] was digested with *XhoI* and blunted, and the blunt-end fragment of CMVpr, which was excised from pCEApr-E1A-CMVpr-E1BΔ55kD, was inserted to yield pUni/CMVpr. The EGFP coding sequence from pEGFP-C1 (Clontech, Palo Alto, CA, USA) was inserted into pUni/CMVpr to obtain pUni/CMVpr-EGFP. In the same manner, Surv.pr was inserted into pUni/V5-HisC-*tet*[r], resulting in pUni/Surv.pr. pUni/CMVpr-p53, pUni/CMVpr-tk, pUni/Surv.pr-p53, and pUni/Surv.pr-tk were constructed by insertion of p53 and HSV-tk fragment excised from pCMV-p53 (Clontech) and pAd.RSV-tk (kindly provided by Z Sheng Guo, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA) into pUni/CMVpr or pUni/Surv.pr.

PCR

The primer sets used in this study are listed in Table 1. The PCR conditions that were used for the m-CRA construction are described earlier. For verification of the correct m-CRAs, PCR of genomic DNA, which was extracted from viral plaques with proteinase K digestion phenol/chloroform purification and ethanol precipitation, was performed with primer sets, as shown in Table 1. The amplified DNA was analyzed by electrophoresis on 1% agarose gel.

Preparation of Ads

All Ads were generated and amplified in 293 cells, and purified in CsCl gradients, as described previously.[5,23] The titer of the Ads (plaque-forming unit (PFU)/ml) was measured by a plaque assay on 293 cells.

Acknowledgements

References

1 Terazaki Y et al. An optimal therapeutic expression level is crucial for suicide gene therapy for hepatic metastatic cancer in mice. Hepatology 2003; 37: 155–163.

2 Fukunaga M et al. Adenoviral herpes simplex virus thymidine kinase gene therapy in an orthotopic lung cancer model. Ann Thorac Surg 2002; 73: 1740–1746.

3 Caruso M et al. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. Proc Natl Acad Sci USA 1996; 93: 11302–11306.

4 Nagano S et al. Gene therapy eradicating distant disseminated micro-metastases by optimal cytokine expression in the primary lesion only: novel concepts for successful cytokine gene therapy. Int J Oncol 2004; 24: 549–558.

5 Chen SH et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci USA 1995; 92: 2577–2581.

6 Kruyt FA, Curiel DT. Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. Hum Gene Ther 2002; 13: 485–495.

7 Hallden G, Thorne SH, Yang J, Kirn DH. Replication-selective oncolytic adenoviruses. Methods Mol Med 2004; 90: 71–90.

8 Curiel DT. The development of conditionally replicative adenoviruses for cancer therapy. Clin Cancer Res 2000; 6: 3395–3399.

9 Rodriguez R et al. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res 1997; 57: 2559–2563.

10 Hallenbeck PL et al. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum Gene Ther 1999; 10: 1721–1733.

11 Adachi Y et al. A midkine promoter-based conditionally replicative adenovirus for treatment of pediatric solid tumors and bone marrow tumor purging. Cancer Res 2001; 61: 7882–7888.

12 Nettelbeck DM et al. Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter. Cancer Res 2002; 62: 4663–4670.

13 Bischoff JR et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 1996; 274: 373–376.

14 Fueyo J et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene 2000; 19: 2–12.

15 Heise C et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med 2000; 6: 1134–1139.

16 Shenk T. Adenoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howly PM (ed). Fields Virology. Lippincott-Raven: Philadelphia, 1996, pp 2111–2147.

17 Harada JN, Berk AJ. p53-Independent and -dependent requirements for E1B-55 K in adenovirus type 5 replication. J Virol 1999; 73: 5333–5344.

18 Rothmann T et al. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. J Virol 1998; 72: 9470–9478.

19 Geoerger B et al. Oncolytic activity of p53-expressing conditionally replicative adenovirus Ad{Delta}24-p53 against human malignant glioma. Cancer Res 2004; 64: 5753–5759.

20 Gomez-Manzano C et al. A novel E1A-E1B mutant adenovirus induces glioma regression in vivo. Oncogene 2004; 23: 1821–1828.

21 Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res 1999; 59: 1498–1504.

22 Johnson L et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. Cancer Cell 2002; 1: 325–337.

23 Mizuguchi H, Kay MA. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. Hum Gene Ther 1998; 9: 2577–2583.

24 Miyake S et al. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc Natl Acad Sci USA 1996; 93: 1320–1324.

25 Chartier C et al. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J Virol 1996; 70: 4805–4810.

26 Metcalf WW, Jiang W, Wanner BL. Use of the rep technique for allele replacement to construct new Escherichia coli hosts for maintenance of R6 K gamma origin plasmids at different copy numbers. Gene 1994; 138: 1–7.

27 Ausubel FM (ed). Current Protocols in Molecular Biology. John Wiley & Sons: New York, 1999.

28 Chen L, Anton M, Graham FL. Production and characterization of human 293 cell lines expressing the site-specific recombinase Cre. Somat Cell Mol Genet 1996; 22: 477–488.

29 Osaki T et al. Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. Cancer Res 1994; 54: 5258–5261.

30 Yeung F et al. Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. J Biol Chem 2002; 277: 2468–2476.

31 Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. Cancer Res 1999; 59: 3143–3151.

32 Parr MJ et al. Tumor-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector. Nat Med 1997; 3: 1145–1149.

33 Takakura M et al. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res 1999; 59: 551–557.

Survivin-responsive conditionally replicating adenovirus exhibits cancer-specific and efficient viral replication

Junichi Kamizono[1,2]*, Satoshi Nagano[1,2,3]*, Yoshiteru Murofushi[1], Setsuro Komiya[2], Hisayoshi Fujiwara[4], Toyojiro Matsuishi[1,3] and Ken-ichiro Kosai[1,3]

(*These authors equally contributed to this work)

[1]Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, and [3]Department of Pediatrics and Child Health, Kurume University, Kurume, Japan. [2]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan. [4]Department of Cardiology, Respiratory and Nephrology, Regeneration & Advanced Medical Science, Graduate School of Medicine, Gifu University, Gifu, Japan.

Correspondence to:

Ken-ichiro Kosai, M.D., Ph.D.

Division of Gene Therapy and Regenerative Medicine,

Cognitive and Molecular Research Institute of Brain Diseases, Kurume University

67 Asahi-machi, Kurume, 830-0011, Japan.

Phone: +81-942-31-7910; Fax: +81-942-31-7911 E-mail: kosai@med.kurume-u.ac.jp

Abstract

Although a conditionally replicating adenovirus (CRA) exhibiting cancer-selective replication and induction of cell death is an innovative potential anti-cancer agent, current imperfections in cancer specificity and efficient viral replication limit the usefulness of this technique. Here, we constructed survivin-responsive CRAs (Surv.CRAs), in which expression of the wild-type or mutant adenoviral early region 1A gene is regulated by the promoter of survivin, a new member of the inhibitor of apoptosis gene family. We explored the cancer specificity and effectiveness of viral replication of Surv.CRAs, evaluating their potential as a treatment for cancer. The survivin promoter was strongly activated in all cancers examined at levels similar to or even higher than those seen for representative strong promoters; in contrast, low activity was observed in normal cells. Surv.CRAs efficiently replicated and potently induced cell death in most types of cancer. In contrast, minimal viral replication in normal cells did not induce any detectable cytotoxicity. A single injection of Surv.CRAs into a pre-established tumor expressing survivin, even at relatively low levels, induced significant tumor death and inhibition of tumor growth. Furthermore, Surv.CRAs were superior to telomerase-dependent CRAs, one of the most effective CRAs that have been examined to date, both in terms of cancer-specificity and efficiency. Thus, Surv.CRAs are an attractive potential anti-cancer agent that could effectively and specifically treat a variety of cancers.

Introduction

Conditionally replicating adenoviruses (CRAs), which selectively replicate in and kill tumor cells, may be an attractive tool for innovative cancer therapy(1). Achievement of both cancer-specificity and efficient viral replication is critical for any CRA-oriented strategy. The CRAs that have been reported to date can primarily be classified into one of two groups (1). The first category employs the strategy of attenuating viral replication in normal cells by mutating cell cycle-inducing adenoviral genes necessary for viral replication; representatives of this group are the mutant type (MT) adenoviruses lacking an RB-binding site within early region 1A (E1A) and the MT adenoviruses lacking a p53-binding protein encoded by the early region 1B (E1B)-55K gene (2-4). Although these CRAs exhibit potential in cancer cells, these viruses do replicate and cause some cytopathic effects (CPEs) in normal cells (4-6). The second group of CRAs alters the regulation of E1A expression. E1A is the first gene to be transcribed after infection with wild-type (WT) adenoviruses, transactivating the viral and cellular genes critical for producing infective adenoviruses. CRAs of this category reproduce in a tumor-specific manner by replacing the native E1A promoter with a tissue- and tumor-specific promoter, such as the prostate-specific antigen promoter (7),

the α-fetoprotein promoter (8), the midkine promoter (9), or the tyrosinase promoter (10). Although previous studies of this CRA strategy have been promising, the use of tissue-specific promoters has the disadvantage of targeting only limited types of cancer. In addition, these viruses demonstrate insufficient cancer specificity (leaky transactivation in normal cells) and weak activity even in cancer cells. As a result, viral targeting and replication for previously reported CRAs may not have achieved sufficient efficiency or cancer-specificity. The use of a novel and ideal promoter able to induce strong expression in a cancer-specific manner is crucial to circumventing these problems.

Survivin, a new member of the inhibitor of apoptosis (IAP) gene family, was reported to be expressed in high levels in cancerous, but not normal, tissues (11). Clinical studies have indicated a positive correlation between high survivin expression levels and a poor prognosis, an accelerated rate of recurrence, and an increased resistance to therapy in cancer patients (12). Survivin is predominantly expressed during the G2/M phase of the cell cycle, functioning in mitosis via interactions with microtubules (13). In addition, the survivin promoter successfully regulates transgene expression in a cancer-specific manner (14). Moreover, studies have suggested that a putative region of the survivin promoter is likely to be responsible for the induction of

cancer-specific expression in tumors at high levels (15, 16). The promoter contains

multiple cell cycle-dependent elements (CDEs) and a cell-cycle gene homology region

(CHR), which may control expression of various G2/M-regulated genes, including the

survivin gene, in a manner correlating with G2/M-cell-cycle periodicity (13, 15-17).

In this study, we generated and analyzed two survivin-responsive CRAs

(Surv.CRA). Surv.CRAwt and Surv.CRAmt expressed WT and MT E1A under the

control of the survivin promoter, respectively. We finally compared these Surv.CRAs to

a recently reported CRA, in which E1A is regulated by the telomerase reverse

transcriptase (TERT) promoter (Tert.CRA), currently one of the best CRAs available

(18-20).

Materials and Methods

Cell Lines.

The human cell lines MKN-1 and MKN-45 (gastric cancer cell lines), HCT-15,

LoVo and Colo-205 (colon cancer cell lines), HepG2 and Hep3B (hepatoma cell lines),

Hela (a cervical cancer cell line), SaOS-2, HOS-MNNG, and KHOS-NP

(osteosarcoma cell lines), and WI-38 (a primary lung fibroblast) were maintained in

DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum.

Primary human osteoblasts, obtained from BioWhittaker (Walkersville, MD), were

maintained according to the manufacturer's protocol.

Generation of Adenoviruses.

A region of the mouse survivin gene promoter (-173 to -19), which contains

two CDEs and one CHR, was obtained from mouse genomic DNA by polymerase chain

reaction (PCR) using the following primers: sense (S)-Surv.pr (5'-

AGATGGGCGTGGGCGCGGAC-3') and antisense (AS)-Surv.pr (5'-

TCCGCCAAGACGACTCAAAC-3'). Generation of Surv.CRAwt, Surv.CRAmt and

Tert.CRAwt viruses, which contained WT or MT E1A downstream of either the survivin

or TERT promoter (-181 to +79; kindly provided by Dr. S. Kyo, Kanazawa University

School of Medicine)(21), E1BΔ55K downstream of the CMV immediate-early gene

enhancer/promoter (CMV promoter) and the enhanced green fluorescent protein

(EGFP) gene downstream of the CMV promoter, was performed using a novel method

developed by our group (22).

An E1-deleted replication-defective adenovirus expressing EGFP (Ad.ΔE1)

and E1-deleted adenoviruses expressing the LacZ gene under the control of the Rous

sarcoma virus long-terminal repeat (RSV promoter), the CMV promoter, the *survivin*

promoter, or the TERT promoter (Ad.RSV-LacZ, Ad.CMV-LacZ, Ad.Surv-LacZ and

Ad.Tert-LacZ, respectively) were generated and prepared as described previously (23).

Reverse Transcription-PCR Analysis.

Extraction of total RNA from the cells and the semi-quantitative reverse

transcription-PCR (RT-PCR) analyses were performed as described previously(24), with

the following primer sets and annealing temperatures: S-Surv (5'-

CCCTTGGTGAATTTTTGAAA-3') and AS-Surv (5'-

TGGTGCCACTTTCAAGACAA-3') for human *survivin* at 56°C; S-TERT (5'-

TTCCTGCACTGGCTGATGAGTGT) and AS-TERT (5'-

CGCTCGGCCCTCTTTTTCTG for human *TERT* at 59°C (25); and S-HPRT (5'-

CCTGCTGGATTACATTAAAGCACTG-3') and AS-HPRT (5'-

AAGGGCATATCCAACAACAA-3') for *hypoxanthine guanine phosphoribosyl*

transferase (HPRT) as an internal control at 57°C (24, 26).

Promoter Activities and Adenoviral Gene Transduction Efficiency.

Cells (5x10^5 cells per plate) were infected with Ad.CMV-LacZ, Ad.RSV-LacZ,

Ad.Surv-LacZ, or Ad.Tert-LacZ at a multiplicity of infection (MOI) of 30 for 24 hours.

After harvesting, cellular β-galactosidase (β-gal) activity was measured as previously

described (27).

The adenoviral gene transduction efficiency (AGTE) for each cell *in vitro* was

determined 48 hours after infection with Ad.CMV-LacZ at an MOI of 30, as previously

described (27-29).

Flow Cytometric Analysis.

After infection with each adenovirus, cells were detached with trypsin and

fixed in 4% paraformaldehyde. The percentage of EGFP-positive cells was then

analyzed by flow cytometry on a FACSCalibur using CELLQuest software (Becton

Dickinson, San Jose, CA).

Cytotoxic Effects *In vitro.*

After plating in 96-well plates, cells were infected with each adenovirus at a

variety of MOIs. Cell viability was determined three and five days after adenoviral

infection using a WST-8 assay (Dojindo Laboratories, Mashiki, Japan) according to the

manufacturer's protocol.

Therapeutic Effects *In vivo* in Animal Experiments.

HOS-MNNG cells (5 x10^6 cells) were injected subcutaneously into the back of

5-week-old male BALB/c athymic nude mice. After the subcutaneous tumors reached 6-

10 mm in diameter, the mice were randomly divided into three groups. Each group

was given a single injection of 1 x 10^8 pfu Surv.CRAwt (n=9), Surv.CRAmt (n=8), or

Ad.ΔE1 (n=8) in 50 μl of 10 mM Tris-HCl (pH 7.4) / 1 mM MgCl$_2$ / 10% (vol / vol)

glycerol / hexadimethrine bromide (20 μg / ml) into the subcutaneous tumor. In another

comparative experiment, tumor-bearing mice were infected with Tert.CRAwt (n=9),

Surv.CRAwt (n=8), or Ad.ΔE1 (n=11) as described above. Tumor size was then

monitored twice a week using digital calipers. Tumor volume was calculated according

to the following formula: volume = long axis x (short axis)^2 x 0.5 (29, 30).

For histopathological analysis, the tumors were fixed in 10% buffered-formalin,

embedded in paraffin, cut into 4-μm serial sections, and stained with hematoxylin and

eosin (H&E).

The protocol for this animal experiment was approved by the Animal Research

Committee of Kurume University. All animal experiments were performed in

accordance with the National Institutes of Health Guidelines for the Care and Use of

Laboratory Animals.

Statistic Analysis.

Data are represented as the means +/- SEM. Statistical significance was

determined using the Student's *t*-test. Values of *P*<0.05 were considered to indicate

statistical significance.

Results

Survivin mRNA Was Expressed in Various Cancer Cell Lines.

The RT-PCR analyses demonstrated that *survivin* mRNA was expressed in multiple cancer cells derived from a variety of tissue origins; this finding was consistent with previous reports (11) (Fig. 1A). The levels of *survivin* mRNA, however, varied widely among the different cancer cell lines. *Survivin* mRNA expression was remarkably high in both hepatoma cell lines tested, HepG2 and Hep3B, and in one of the osteosarcoma cell lines, SaOS-2. The levels in the other cell lines were only moderate or relatively low. *Survivin* mRNA was also detected in normal WI-38 human fibroblasts and primary human osteoblasts; these levels, however, were relatively low in comparison to those seen in the cancer cell lines.

Strong Cancer-specific Activity of the *Survivin* Promoter.

The *survivin* promoter provided strong transcriptional activation in all of the cancer cell lines that demonstrated sufficient viral transduction (Fig. 1B). The low levels or absence of β-gal activity after infection with adenoviruses in either Colo-205 or KHOS-NP cells was apparently due to very low levels of AGTE in these cells, not to a

low activity of the *survivin* promoter; β-gal activity was not detected in this group even after infection with Ad.RSV-LacZ or Ad.CMV-LacZ at the same MOI (MOI of 30). The apparent variability in β-gal levels was also due to both the variability of AGTE levels in individual cells and the cellular activity required to express the transgenes, not the variability in *survivin* promoter activity. In seven of the remaining nine cancer cell lines, the *survivin* promoter exhibited stronger activity than either the RSV promoter or the CMV promoter, two representative ubiquitously strong promoters (27, 28). Notably, the *survivin* promoter was stronger than both the RSV and CMV promoters in HepG2 cells. In two additional cell lines, HCT-15 and LoVo, the *survivin* promoter displayed activity levels very similar to those observed for the RSV and CMV promoters.

In contrast, *survivin* promoter activity was not detected in normal WI-38 fibroblasts, despite both high levels of RSV and CMV promoter activity and moderate to high AGTE levels. Despite detectable, albeit low, levels of endogenous *survivin* expression, no detectable transactivation could be observed in normal cells with the use of this *survivin* promoter (Fig. 1B). Thus, the *survivin* promoter region and length of the transcriptional regulatory element used in these experiments are suitable to induce strong transactivation in all cancer types examined here in a tumor-specific manner.

Surv.CRAs Efficiently and Selectively Replicated in Cancer.

After infection with Surv.CRAwt or Surv.CRAmt, the number of EGFP-positive cells increased in a time-dependent manner in all of the cancer cell lines analyzed, indicating the efficient replication of both Surv.CRAs (Fig. 2). CPE was efficiently induced within a short period of time after the appearance of EGFP-positivity. The speed of the Surv.CRA spreading was consistent with the observed levels of β-gal activity (Fig. 1B), except for MKN-45 cells. In these cells, the Surv.CRAs replicated very rapidly, spreading throughout the entire culture dish at a rate similar to that seen in LoVo, HepG2, and Hep3B cells. The slow, yet still apparent, spread of the Surv.CRAs was even observed in Colo-205 cells, despite a low AGTE in the initial infection. This phenomenon likely results from high levels of endogenous survivin expressed, suggesting that efficient viral replication within cells may overcome the disadvantage of low AGTE. In contrast, the percentage of EGFP-positive cells did not clearly increase over a seven-day period in normal WI-38 cells, although the EGFP fluorescence intensity within each cell increased minimally. In addition, no CPE was observed in WI-38 cells even at seven days after infection with Surv.CRAs.

To verify tumor-specific replication of both Surv.CRAs accurately and quantitatively, we performed flow cytometric analysis using two representative cancer

cell lines, HepG2 and HOS-MNNG, as well as normal WI-38 cells. HepG2 cells exhibited the highest levels of survivin expression, the highest AGTE levels, and the strongest survivin promoter activity, resulting in rapid amplification of the Surv.CRAs (Fig. 2). HOS-MNNG demonstrated low to moderate levels of these properties, resulting in lower, but significant, viral replication. Twenty-four hours after infection with either of the adenoviruses at the MOI that initially provided approximately 20% AGTE, Surv.CRAs propagated rapidly, spreading to greater than 90% of HepG2 and HOS-MNNG cells. Under these conditions, we could not observe any significant amplification or spread of the control replication-defective Ad.ΔE1 (Fig. 3). In contrast, the propagation and resulting spread of Surv.CRAs remained minimal in WI-38 cultures. Thus, both Surv.CRAs replicated more efficiently in cancer cells, even those expressing survivin at relatively low levels with moderate AGTE levels, than in normal WI-38 cells. In addition, we did not detect any significant differences in the phenotypic characteristics of Surv.CRAwt and Surv.CRAmt in any of the cell types tested.

Surv.CRAs Specifically Kill Cancer Cells In vitro.

To assess the selective killing of cancer cells by Surv.CRAs, we conducted a cell viability assay (Fig. 4). In two representative cell lines demonstrating both high

AGTE and high levels of *survivin* expression, HepG2 and Hep3B cells, Surv.CRAs induced prominent cytotoxic effects as early as three days, even when infection was performed at a low MOI (0.1). Both hepatoma cell lines were sensitive to adenoviral cytotoxicity; cytotoxic effects were minimally, but clearly, seen five days after infection at an MOI of 0.1 with the control, E1-deleted Ad.ΔE1. In HOS-MNNG cells, which exhibited low expression of *survivin* and moderate AGTE levels, both Surv.CRAs induced more prominent cytotoxicity than Ad.ΔE1. The cytotoxic effects were amplified in a dose-dependent manner when initial infection at increasingly higher MOI (Fig. 4). In contrast to these results in cancer cell lines, neither Surv.CRA induced cytotoxic effects in normal WI-38 fibroblast cells, even five days after infection at an MOI of 1. Thus, both Surv.CRAs efficiently induced cell death in three cancer cell lines in contrast to the lack of clear toxicity observed in normal WI-38 cells. In addition, we did not observe any significant differences in the cytotoxicity of Surv.CRAwt and Surv.CRAmt between the cell types tested, including the normal WI-38 cells, RB-intact HepG2 cells, and RB-deficient Hep3B cells.

Surv.CRA Inhibited Tumor Growth *In vivo*.

Using an animal model of pre-established subcutaneous tumors, we examined

the therapeutic potential of both Surv.CRAs *in vivo*. We intentionally used an HOS-MNNG osteosarcoma cell line that expressed relatively low levels of *survivin* and demonstrated moderate levels of AGTE to assess the therapeutic potentials of these vectors in a wider range of cancers. A single intratumoral administration (1×10^8 pfu) of Surv.CRAwt or Surv.CRAmt significantly inhibited tumor growth in comparison to the same dose of Ad.ΔE1 (Fig. 5A). Statistically significant differences in the tumor size were seen between Surv.CRAs-treated and Ad.ΔE1-treated mice as early as 11 days after administration and continuing thereafter. As assessed by macroscopic and microscopic examination, the therapeutic effects of both Surv.CRAs were more significant; the tumor nodules in Surv.CRA-treated mice contained large necrotic areas, while the nodules in Ad.ΔE1-treated mice consisted primarily of viable tumor cells histologically demonstrating active malignant features (Fig. 5B & C). These results suggest the therapeutic potential and general utility of Surv.CRAs for the treatment of cancer.

The Superiority of Surv.CRAs to a Tert.CRA.

We compared the viral properties of Surv.CRAs to those of Tert.CRA. The expression levels of endogenous TERT varied among cancer cell lines; HOS-MNNG

cells, as well as HepG2 cells, expressed TERT mRNA at very high levels (Fig. 6A), in

contrast to the relatively low level of *survivin* expression in HOS-MNNG cells (Fig.

1A). Nevertheless, the activity of the *survivin* promoter in HOS-MNNG cells was

higher than that of the TERT promoter, as well as in HepG2 cells (Fig. 6B). These

results suggest that the *survivin* promoter may be more active than the TERT promoter

among multiple cancer cell types.

To precisely analyze the differences in the efficiency and attenuation of viral

replication between Surv.CRAs and Tert.CRA in cancerous and normal cells, we

performed flow cytometric analysis after infection of three types of cells at low MOI

(Fig. 6C). Surv.CRAwt exhibits more efficient replication in both HepG2 and HOS-

MNNG cells than that seen in Tert.CRAwt cells, although the former virus is more

quiescent in normal WI-38 cells than the latter.

We compared the therapeutic potentials of Surv.CRAwt and Tert.CRAwt in

tumor-bearing animals (Fig. 6D). Although we did not find a statistically significant

difference in the effects of Surv.CRAwt and Tert.CRAwt, both viruses significantly

decreased tumor size in animals from the tumor volumes observed in mice treated with

the control Ad.ΔE1 virus. Tumor volumes in Surv.CRAwt-treated mice were smaller

than those in Tert.CRAwt-treated animals; in addition, the difference between the

Surv.CRAwt and control Ad.ΔE1 groups was more significant (smaller *P* value) than the

difference between the Tert.CRAwt and Ad.ΔE1 groups.