Triple-Targeted Oncolytic Adenoviruses Featuring the Cox2 Promoter, E1A Transcomplementation, and Serotype Chimerism for Enhanced Selectivity for Ovarian Cancer Cells

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Conditionally replicating adenoviruses (CRAd’s) feature selective replication in and killing of tumor cells. Initial clinical studies with relatively attenuated early generation agents have resulted in promising safety and efficacy data. Nevertheless, increased specificity may be advantageous for an emerging generation of infectivity-enhanced CRAd’s. Further, increased specificity could translate into a larger tolerated dose. An approach for increasing specificity is dual control of E1A expression. We constructed six CRAd’s featuring two variants of the cyclo-oxygenase 2 (cox2) promoter, combined with three versions of E1A. Transcriptional targeting was supplemented with transducational targeting utilizing the serotype 3 knob. In vivo and in vitro results suggest that cox2 can be utilized for enhancing the specificity of E1A deletion mutants and that combination with the Delta24 mutation increases specificity without reducing potency. Combination with Delta2-Delta24 was specific but somewhat attenuated. The promoter variants behaved similarly, although the longer 1554-bp version displayed a trend for improved specificity. Transcriptional modifications were compatible with transducational targeting and resulted in up to 100,000-fold increase in the therapeutic window for Ad5/3cox2Ld24 vs wild-type adenovirus. Thus, the proposed triple-targeting strategy may be useful for increasing the safety and efficacy of adenoviral gene therapy for ovarian cancer.

Key Words: adenoviridae, ovarian neoplasms, gene therapy, virus replication, cyclooxygenase 2, capsid

INTRODUCTION

Despite numerous innovations in therapy concepts, ovarian cancer remains one of the leading gynecological mortality factors for women in Western countries, with an estimated 25,400 new cases and 14,300 deaths in 2003 in the United States [1]. New therapy regimes have improved outcomes in terms of disease-free and overall survival for early stages, but women with metastatic disease remain incurable.

Conditionally replicating adenoviruses (CRAd’s) take advantage of cancer-specific changes, allowing preferential replication in tumor cells. Replication causes oncolytic death of the cell, release of virions, and subsequent infection of surrounding cells, resulting in efficient tumor penetration and amplification of effect. Therefore, the anti-tumor effect is caused by replication per se. Consequently, limiting replication to tumor cells is important to minimize side effects. The anti-tumor effect...
of CRAd’s is determined by their capability of infection of tumor cells. Unfortunately, recent evidence suggests that the expression level of the coxsackie–adenovirus receptor (CAR) is highly variable and often low in many human tumors [2]. As most normal epidermal tissues express CAR, use of untargeted Ad may result in transduction of mainly nontarget cells. Nevertheless, even first-generation CRAd’s have shown some clinical utility [2]. This suggests that if the capacity of the agents to transduce cancer cells could be improved, significant progress in clinical efficacy might be gained. Therefore, infectivity enhancement strategies have been studied. With regard to ovarian cancer, modification of the Ad5 capsid with the serotype 3 knob (Ad5/3) has been demonstrated as a useful approach [3–5].

Heretofore, clinical results with CRAd’s have demonstrated good safety [6]. However, most agents studied have featured low replicativity and CAR-dependent entry. Therefore, it is possible that when more effective agents are studied, side effects may become dose limiting. Further, it is possible that side effects may be related to the specificity of replication and therefore improvements in selectivity may reduce side effects and allow higher doses.

Two main approaches have been used in the design of CRAd’s [2]. “Type I CRAd’s” such as dl1520 and Ad5Δ24RGD harbor deletions in early genes that are transcomplemented in tumor but not normal cells [7,8]. “Type II CRAd’s” feature tumor-specific promoters for control of early genes, most often E1A [9]. Nevertheless, neither approach renders virus replication completely specific to target cells and some replication in normal cells is usually seen [2]. Theoretically, the combination of types I and II is feasible and could be useful for specificity [10].

We hypothesized that we could increase the selectivity of CRad replication by combining the cyclo-oxygenase 2 (cox2) promoter [11] with tumor-complemented E1A mutants. The Delta24 variant of E1A (aka dl922-947) cannot bind to Rb and therefore replicates selectively in cells deficient in the Rb/p16 pathway [12–14], including most if not all human tumor cells [15]. Also, reintroduction of Rb has been shown to abrogate virus replication [12]. The Delta2–Delta24 variant has an additional 3-bp mutation in the p300 binding site of E1A [10,16]. It has been suggested that the inability of Delta24 to bind pRb might be compensated for by other proteins such as p300 [16], and thus a mutation of this site might increase specificity [10].

The cox2 promoter is induced during carcinogenesis and might be a useful tumor-specific promoter for many types of cancer, including ovarian cancer [17,18]. We have previously identified two variants of the cox2 promoter (Medium (M), 988 bp, and Long (L), 1554 bp), which might be feasible for ovarian cancer gene therapy [11]. Here, we constructed six different CRAd’s with either the cox2L or the cox2M version of the cox2 promoter for controlling three different variants of E1A (wild type, Delta24, and Delta2–Delta24), while each capsid was modified with the serotype 3 knob for infectivity enhancement. These six viruses were compared with regard to efficacy and specificity in ovarian cancer and nonmalignant cells. In particular, human hepatocytes were studied, as the liver is the main organ responsible for clearance of adenovirus, and—consequently—a crucial organ with regard to toxicity [3,19]. Further, we studied human fibroblasts. Adenovirus type 5 does not enter fibroblasts due to lack of CAR, but 5/3 chimerism renders these cells susceptible, and therefore they might be a stringent model system for detecting differences in replication specificity and potentially important with regard to toxicity mediated by tropism expansion.

**RESULTS**

**Cloning of Ad5/3cox2 CRAd’s**

We performed cloning of six different CRAd’s featuring cox2L and cox2M, for driving wild-type E1A, E1A with a 24-bp deletion (Delta24), and E1A with a deletion of the second amino acid as well as a 24-bp deletion (Delta2–Delta24) (Fig. 1). The viruses had the following physical and functional titers: Ad5/3cox2LE1 4.0 × 10¹² viral particles (vp)/ml, 2.6 × 10¹¹ plaque-forming units (pfu)/ml, 15 vp/pfu; Ad5/3cox2Ld24 4.0 × 10¹² vp/ml, 1.2 × 10¹¹ pfu/ml, 33 vp/pfu; Ad5/3cox2Ld2d24 2.0 × 10¹² vp/ml, 1.2 × 10¹¹ pfu/ml, 17 vp/pfu; Ad5/3cox2ME1 3.5 × 10¹² vp/ml, 1.15 × 10¹¹ pfu/ml, 30 vp/pfu; Ad5/3cox2Md24 3.0 × 10¹² vp/ml, 1.0 × 10¹¹ pfu/ml, 30 vp/pfu; Ad5/3cox2Md2d24 1.1 × 10¹² vp/ml, 1.0 × 10¹¹ pfu/ml, 11 vp/pfu. Similar vp/pfu ratios suggest that each of these viruses is packaged effectively in cells allowing replication.

**Oncolytic Potential of the E1A Variants in Combination with the Cox2L Promoter**

In all four ovarian cancer cell lines, the MTS cell killing assay showed oncolysis with all three cox2L promoter-driven CRAd’s and the wild-type control Ad5wt (Fig. 2). In OV-4 and SKOV3.ip1, significantly more cells were lysed by the CRAd’s in comparison to Ad5wt (P < 0.01). Therefore, on ovarian cancer cells in vitro, the cox2L promoter-driven and Ad5/3 infectivity-enhanced CRAd’s show higher efficacy than wild-type adenovirus. In BT474 cells, which are low in cox2 expression [17], the CRAd’s were attenuated in comparison to Ad5wt. Interestingly, there was little or no additional advantage in selectivity mediated by the Delta2 deletion, when combined with Delta24. The CRAd’s showed little oncolysis of fibroblasts when incubation times similar to those of the ovarian cancer cells were used (not shown). However, to detect differences in specificity sensitively, a longer incubation...
time was used, resulting in oncolysis with all viruses featuring the serotype 3 knob. The latter phenomenon is caused by the serotype 3 modification of the knob allowing entry into fibroblasts (data not shown).

**Ad5/3cox2 CRAd’s Have No Oncolytic Activity in Human Hepatocytes in Vitro**

Whereas Ad5wt shows complete oncolysis with 10 and 100 vp/cell on hepatocytes, there was no measurable oncolysis with the cox2 promoter-driven CRAd’s (Figs. 2 and 3). This demonstrates cox2 as a liver-off promoter and that virus replication is tightly regulated by both promoter variants.

**Oncolytic Potential of the Different E1A Variants in Combination With the Cox2M Promoter**

In all four ovarian cancer cell lines, the MTS cell killing assay showed oncolysis with all three different cox2M promoter-driven CRAd’s and Ad5wt (Supplementary Fig. 1). The CRAd’s lysed significantly more OV-4 and SKOV.3ip1 cells than Ad5wt ($P < 0.01$). Therefore, on ovarian cancer cells in vitro, the cox2M promoter-driven and Ad5/3 infectivity-enhanced CRAd’s show higher efficacy than wild-type adenovirus. Again, while the addition of the Delta24 deletion seemed to increase the specificity of the CRAd’s, there seemed to be little if any advantage due to an additional Delta2 deletion.

**The Relative Specificity of Cox2L vs Cox2M in the Context of Wild-Type E1A**

In all ovarian cancer cell lines tested, the efficacy was similar for the CRAd’s with the cox2L and the cox2M promoters controlling wild-type E1A (Supplementary Fig. 2). For the positive control cell line A549 and the control cells low in cox2 (BT474) the differences were small. There seemed to be a tendency for the cox2L promoter to be more specific than cox2M, as the CRAd with the former caused less oncolysis in BTB474 cells ($P = 0.01$). In human hepatocytes neither CRAd demonstrated oncolysis in comparison to the E1-deleted control virus Ad5/3Luc1.

**The Relative Specificity of Cox2L vs Cox2M in Combination with the Delta24 Deletion**

On A549 cells (positive control for cox2 and CAR) Ad5wt and cox2L-driven Delta24 showed similar oncolytic potency, whereas cox2M-driven Delta24 seemed to be slightly weaker. On BT474 cells (low for cox2), both CRAd’s were similar and ca. 100-fold weaker than Ad5wt. In the ovarian cancer cell lines there were only slight differences between the cox2L and the cox2M promoter-driven Delta24 (Fig. 3). In human hepatocytes both CRAd’s showed nearly no oncolysis comparable to the nonreplicating control virus Ad5/3Luc1, and the selectivity in fibroblasts was similar.

**The Relative Specificity of Cox2L vs Cox2M in Combination with the Delta2–Delta24 Deletions**

There was not much difference between the two promoter variants in the context of the Delta2–Delta24 deletion (Supplementary Fig. 3). In human hepatocytes, the CRAd’s demonstrated little if any oncolysis comparable to the nonreplicating control virus Ad5/3Luc1.

**The Relative Specificity of Cox2-Driven Delta24 Compared to Delta24 without a Promoter (Ad5/3d24)**

On most ovarian cancer cell lines, cell killing by viruses featuring the cox2 promoter was similar to that of the isogenic virus without the promoter (Ad5/3d24). With ES-2 ovarian cancer cells (Fig. 4), the cox2-driven CRAd’s were weaker than Ad5/3d24 but all three were more oncolytic than wild type ($P < 0.01$ for Ad5/3d24 compared to cox2 CRAd’s at 0.1 vp/cell; $P < 0.01$ for each CRAd compared to wild type at 1 vp/cell). Notably, in comparison to Ad5/3d24, the cox2-driven CRAd’s had reduced oncolytic activity in human fibroblasts grown at 2% FBS (Fig. 5). In human peripheral blood mononuclear cells (PBMCs), none of the viruses caused oncolysis (Fig. 5).

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**FIG. 1.** Cloning strategy. The adenoviral E1 region was deleted and replaced by three variants of E1A driven by either a long (cox2L) or a medium (cox2M) version of the cox2 promoter. In each case, the fiber was modified with the adenovirus type 3 fiber knob replacing the original type 5 knob.
Viral Replication Depends on Cox2 Status of the Cells

Whereas Ad5/3cox2Ld24 replicates rapidly in the cox2-positive cell line SKOV3.ip1 (Fig. 6), replicativity is reduced in low-cox2 BT474 cells. We used an isogenic virus (Ad5/3d24) lacking the promoter as a positive control. In SKOV3.ip1 the replication of wt adenovirus is poor compared to both CRAd’s, as the latter are infectivity enhanced with 5/3 chimerism. In BT474 replication of wt adenovirus seems to be superior to that of both CRAd’s, probably due to high CAR and low Ad3 receptor. Nevertheless, Ad5/3cox2Ld24 replicates ca. 100-fold less in BT474 than in the ovarian cancer cell line SKOV3.ip1, suggesting preferential replication in cox2-positive tumor cells.

Anti-tumor Efficacy in Vivo

To compare the CRAd’s in vivo, we established ovarian cancers in nude mice and injected them with viruses (Fig. 7A). Ad5/3Cox2Ld24 had more anti-tumor effect than the positive control Ad300wt (P = 0.0001), was more effective than negative controls Ad5LacZ (P < 0.01) and mock (P < 0.001), and had efficacy similar to that of Ad5/3d24 (P = 0.4). In this aggressive model, Ad5/3cox2Ld24 and Ad5/3cox2LE1 were not more effective than negative controls (P > 0.01).

Replication of CRAd’s in Vivo in a Subcutaneous Mouse Model

To evaluate replication in vivo, we co-infected CRAd’s with a luciferase-expressing virus (Ad5/3Iic1), which replicates only in the presence of E1 produced by replication of the CRAd’s (Fig. 7B). In comparison to tumors co-infected with replication-deficient virus (Ad5LacZ), tumors co-infected with CRAd’s demonstrated more luciferase expression (all P > 0.01).

Anti-tumor Effects in an Orthotopic Model of Intraperitoneally Disseminated Ovarian Cancer

To compare the CRAd’s in vivo, we established SKOV3.ip1 carcinomatosis and then injected mice intraperitoneally with virus on 3 consecutive days (Fig. 7C). After 90 days, Ad300wt (P = 0.002), Ad5/3cox2Ld24 (P ≤ 0.001), and Ad5/3d24 (P ≤ 0.001) resulted in significantly better survival than mock. Ad5/3cox2Ld24 and Ad5/3d24 seemed to improve survival over the positive control Ad300wt, although P values were not significant (P = 0.120 and P = 0.148, respectively). Ad5/3cox2Ld24 had efficacy similar to that of Ad5/3d24 (P = 0.971).

**DISCUSSION**

In the context of anti-tumor efficacy, most approaches utilizing replication-deficient viruses for gene delivery have been disappointing [20,21]. Although randomized clinical evidence is still pending, it is possible that the capacity of conditionally replicating adenoviruses for local amplification and subsequent penetration of tumor masses may be useful for anti-tumor effect [2,6]. Furthermore, transcriptional and transductional targeting strategies could improve the therapeutic window of clinical approaches [22].

First-generation CRAd’s current in clinical testing typically feature a single modification for restricting replication. Type I CRAd’s feature deficiencies in early genes, which are transcomplemented in tumor cells. One example is dl1520 (aka ONYX-015), which has been tested in a number of trials [23]. Type II CRAd’s feature a promoter for regulation of E1A expression. CN706 (aka CV706 and CG0706) is an example of such a virus and has been tested in trials [24]. Safety data have been good heretofore, but it should be noted that all trials published thus far have featured relatively attenuated viruses. For example, both dl1520 and CN706 harbor partial or complete deletions in the E3 region, which could result in reduced replication. Also, while infectivity enhancement and transductional targeting strategies have been tested preclinically with impressive results, no trials have been completed yet. Given the potential for severe and even fatal toxicity with large doses of adenovirus [19], toxicity could become an important issue when more effective CRAd’s enter trials.

Recent publications have suggested the feasibility of constructing CRAd’s with multiple levels of replication specificity, but little is known about the relative importance of promoter vs transcomplementation control [10,14,25]. In this paper we constructed six novel viruses, each controlled by a long or medium variant of the cox2 promoter [17,26]. These promoter variants have been evaluated previously for potential utility for ovarian cancer gene therapy [11,27]. The cox2 promoter has been suggested as a potentially useful tumor-specific promoter given low expression in livers of mice and relatively high expression in many tumor types including ovarian cancer [11,17,18,27]. Three variants of the central adenoviral replication regulator gene (E1A) were used: wild-type E1, a Delta24-modified version (24-bp deletion in Rb binding site of E1A), or a Delta2–Delta24-modified version (additional incorporation of a 3-bp deletion encoding the second amino acid of E1A, i.e., the p300 binding site [10]). The specificity and efficacy of Delta24-modified viruses...
have been demonstrated previously [5,8,10,28,29]. Theoretically, additional control over E1A might result in higher specificity allowing an improved therapeutic window (difference in replication between normal and cancer cells). An earlier publication suggested tumor specificity for the Delta2 mutation in the context of cervical cancer cells[16]. All viruses constructed here were infectivity enhanced with the adenovirus type 3 fiber knob in the adenovirus type 5 fiber shaft. Our previous work suggests that this may be the “best currently available” transductional targeting moiety for ovarian cancer [3–5,29].

We compared two variants of the cox2 promoter; a longer version (cox2L, 1554 bp), which has been reported to be more specific, and a medium version (cox2M, 988 bp), which might feature a higher expression level[17,26]. Here, we saw similar results in that the cox2L promoter was slightly more specific, especially in the context of wild-type E1A (Supplementary Figs. 2a and 2b). In tumor cells we did not see significant differences between the promoters, but there seemed to be a tendency for the cox2M promoter to allow slightly higher rates of cell killing when driving wild-type E1 or driving Delta2–Delta24 (Fig. 3 and Supplementary Figs. 2 and 3).

When the different versions of E1 were compared, Delta24 resulted in the most promising data. The rates of oncolysis were higher (cox2L) or similar (cox2M) to those of wild-type E1A. Other studies have corroborated the high replicativity of Delta24-type viruses [8,13]. Oncolysis caused by Delta2–Delta24 was approximately 10-fold less than with the other versions of E1. This is in concordance with other publications showing that multiple modified viruses have longer and slower rates of replication [16,30]. Importantly, we saw little or no additional specificity when the Delta2 mutation was added to Delta24. Thus, the double-modified E1A does

FIG. 3. Evaluation of cox2 promoter variants in the context of the Delta24 E1A mutation. Four ovarian cancer cell lines (ES-2, Hey, OV-4, SKOV3.ip1), human primary hepatocytes (nHEPs), human fibroblasts (FHS173WE), the cox2/CAR-positive A549 cells, and a control cell line low in cox2 (BT474) were infected with cox2 promoter-driven Delta24 CRAd’s and the appropriate control viruses at four different doses and the oncolytic capacity was measured with the MTS assay. Error bars indicate standard error deviation.

FIG. 4. Comparison of the cox2 promoter-controlled Delta24 CRAd’s with the isogenic Delta24-based CRAd (Ad5/3d24). Ovarian cancer cell lines ES-2, Hey, OV-4, and SKOV3.ip1 were infected with cox2 promoter-driven Delta24 CRAd’s and Ad5/3d24 at four different vp/cell concentrations and the oncolytic capacity was measured with the MTS assay. Error bars indicate standard error deviation.
not seem to provide benefits over the single-modified E1A (Delta24).

The specificity of heterologous promoters is affected by viral elements such as the inverted terminal repeats. Therefore, it is important to note that the specificity of the cox2 promoter was retained: in the low-cox2-expressing cell line BT474 the rates of replication were 100-fold lower than in the wild type. In contrast, in the cox2-positive cell line A549, replication of the CRAd’s was similar to that of Ad5 wild type, except for the double-modified Delta2–Delta24, which was slower. A549 cells express CAR to a high degree [31] and therefore this cell line allows comparison of the CRAd’s to Ad5wt in a situation in which all viruses infect cells effectively.

Ovarian cancer cells have been reported to be refractory to infection with adenovirus. Therefore, we sought to target the novel CRAd’s transductionally by utilization of the serotype 3 knob. In OV-4 and SKOV3.ip1 cells Ad5wt resulted in little oncolysis, while the Ad5/3 CRAd’s were up to 1000-fold more effective in infecting and killing cells, due to higher expression of the serotype 3 receptor [4]. In Hey and ES-2 there was a ca. 10-fold difference in cell killing. The same was true for the fibroblast cell line FHS173WE, which has no CAR but seems to express the adenovirus 3 receptor, because it allows viruses with the serotype 3 knob to enter cells. To investigate this further, we are in the process of determining the cox2 expression of this fibroblast line and if adenovirus infection can induce cox2 in these cells. Although it is difficult to interpret this with regard to potential toxicity, as most fibroblasts in vivo would not be dividing (as opposed to fibroblasts in vitro), we felt that this might be a stringent model for detecting potential differences with regard to specificity between the CRAd’s.

Interestingly, all 5/3 CRAd’s seemed to replicate in cycling FHS173WE cells, and the promoter increased the selectivity of the virus significantly compared to Ad5/3d24 (P < 0.01 at 1 vp/cell, Fig. 5) with a 10-fold difference in cell killing. Furthermore, these findings raise an important safety question: if this happens also in vivo, is there potential for toxicity not seen with serotype 5-based viruses? Unfortunately, this is difficult to study preclinically, as no animal models are known to support productive replication of human serotype adenoviruses to the same degree as human cells [32]. Therefore, it is important to initiate Phase I trials rapidly, to assess the
feasibility of 5/3 serotype chimerism as a cancer gene therapy strategy.

In addition, we demonstrated that the cox2 promoter-driven CRAd's killed tumor cells with comparative or even superior efficacy compared to Ad5/3d24, which is the isogenic Delta24 virus with the serotype 3 knob (Figs. 4, 6, and 7). This is most likely due to a higher amount of E1A proteins produced, when expressed from the cox2 promoter. This could improve replication; or increased cell killing might be due to the anti-tumor effect of E1A proteins per se [33]. In nonreplicating fibroblasts replication of the cox2-driven CRAd's was reduced and none of the tested viruses lysed human PBMCs, which might be useful from a safety standpoint [34].

As the liver is an important organ with regard to toxicity from adenoviral gene therapy [19], we evaluated primary human hepatocytes (nHEPs) for replication permissivity. None of our CRAd's replicated in these cells, resulting in at least 100-fold difference in replication compared to Ad5 wild type. These results suggest the potential for low liver toxicity. Nevertheless, in addition to hepatocytes, human liver contains other cell types such as endothelial cells and specialized macrophages known as Kupffer cells. Also, as the tertiary structure of the liver may also be important with regard to adenoviral gene transfer, clinical trials are needed to evaluate toxicity reliably.

In conclusion, our results suggest increased specificity and efficacy for viruses featuring the cox2 promoter with the Delta24 deletion, in comparison to viruses without the deletion. Further, CRAd’s with Delta24 seemed advantageous over viruses with Delta2–Delta24 due to stronger replication, while the specificity was similar. Further, cox2-Delta24 was more oncolytic than cox2E1 or
Ad5/3d24. When data from ovarian cancer cells (cox2-Delta24 100-fold more effective) are combined with data from hepatocytes (Ad5wt 100- to 1000-fold more toxic), the cox2-Delta24 configuration increased the therapeutic window by 10,000- to 100,000-fold in comparison to Ad5wt. With regard to promoter configuration, cox2L was more specific than and as effective as cox2M. Therefore, cox2L-Delta24 emerges as the agent of choice for further studies, which may eventually facilitate clinical testing of the agent.

**MATERIALS AND METHODS**

**Cell culture.** ES-2 and OV-4 ovarian cancer cell lines, FHS173WE human fibroblast cell line, BT474 mammary adenocarcinoma cell line (negative control for cox2 [17,26]), and human lung adenocarcinoma cell line A549 (positive control for cox2 [17,26]) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SKOV3.ip1 and Hey were obtained from Dr. Janet Price and Dr. Judy Wolf (both M. D. Anderson Cancer Center, Houston, TX, USA). All cell lines were maintained in a humidified 37°C atmosphere containing 5% CO2 and cultured with the recommended media. Infections were performed in medium with 2% FBS (HyClone, Logan, UT, USA).

**Viruses.** The different CRAd’s were constructed by digesting pShuttleGL3box-2pl [17,26] and pShuttleGL3box-2M [17,26] with KpnI and HindIII, respectively, to achieve the different cox2 promoter sequences. pSE1, pSE1d24, and pSE1d2d24 [10] were digested with XhoI and NotI to remove the natural E1 promoter. After blunt-ending with T4 polymerase, the two different cox2 promoter sequences were included into different pSE shuttles. Direction of the inserted promoter was checked by PCR. Viral backbone was rescued with PacI-digested pTU-5/3 [35], which includes GFP and luciferase instead of E1. Specifically, the six different PacI-digested pSE’s were cotransfected with the digested pTU-5/3 into 911 cells. Plaques were picked 7 to 20 days after infection and checked for presence of the expected promoter–E1 combination and Ad5/3 fiber modification as well as absence of wt E1 and GFP by PCR.

Propagation was performed on A549 cells to avoid wild-type contamination. Ad5wt (ATCC) and Ad5/3luc1 [4] were propagated on 293 cells (Microbix, Toronto, ON, Canada). All viruses were purified with double CsCl gradients using standard methods. The presence of the E3 region and 5’/3’ fiber modification was confirmed with PCR. E3 promoter–E1 combination and Ad5/3 fiber modification was checked for presence of the expected promoter–E1 combination and Ad5/3 fiber modification as well as absence of wt E1 and GFP by PCR. The different CRAd’s were constructed by digesting pShuttleGL3box-2pl, pShuttleGL3box-2M [17,26] with KpnI and HindIII, respectively, to achieve the different cox2 promoter sequences. pSE1, pSE1d24, and pSE1d2d24 [10] were digested with XhoI and NotI to remove the natural E1 promoter. After blunt-ending with T4 polymerase, the two different cox2 promoter sequences were included into different pSE shuttles. Direction of the inserted promoter was checked by PCR. Viral backbone was rescued with PacI-digested pTU-5/3 [35], which includes GFP and luciferase instead of E1. Specifically, the six different PacI-digested pSE’s were cotransfected with the digested pTU-5/3 into 911 cells. Plaques were picked 7 to 20 days after infection and checked for presence of the expected promoter–E1 combination and Ad5/3 fiber modification as well as absence of wt E1 and GFP by PCR.

**Oncolytic assay.** MTS assay was performed as described [8]. Briefly, all cell lines were infected in 96-well plates with 0, 0.1, 1, or 10 vp/cell for 1 h in 50 µl medium (2% FBS) on a rocker. Cells were incubated in medium with 5% FBS, half of which was changed every other day. On day 7 (nHEPs), day 9 (A549, BT474), day 10 (FHS173WE), day 14 (SKOV3.ip1), day 17 (OV-4), or day 18 (Hey and ES-2), an MTS assay (CellTiter96Aqueous One Solution Reagent; Promega, Madison, WI, USA) was performed. Results are displayed in proportion to uninfected cells and error bars indicate standard deviation.

**Detection of viral replication with RT-PCR.** Cells were infected on 96-well plates and after 1 h incubation the virus was collected after 2, 4, 6, 8, and 10 days and snap-frozen. DNA was extracted with the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) and quantitative PCR for the E4 gene was performed as described [36]. E4 copy numbers were normalized to human β-actin.

**Detection of viral replication with TCID50.** SKOV3.ip1 or BT474 cells (1.5 × 105/well) were cultured on 96-well plates and after 24 h infected with 10 vp/cell. Quadruplicate wells (cell lines and supernatant) were collected after 2, 4, 6, and 8 days and carried over to 48-well plates. Cells were plated and infected with 0, 1, or 10 vp/cell. Quadruplicate wells (cell lines and supernatant) were collected after 2, 4, 6, and 8 days and carried over to 48-well plates. Cells were incubated in medium with 5% FBS, half of which was changed every other day. On day 7 (nHEPs), day 9 (A549, BT474), day 10 (FHS173WE), day 14 (SKOV3.ip1), day 17 (OV-4), or day 18 (Hey and ES-2), an MTS assay (CellTiter96Aqueous One Solution Reagent; Promega, Madison, WI, USA) was performed. Results are displayed in proportion to uninfected cells and error bars indicate standard deviation.
were lysed with three freeze-thaw cycles. TCID₅₀ was performed as standard procedure with 10²⁹ cells/well with dilutions up to 10⁻⁵ and detected after 10 days.

**In vivo analyses.** Hey ovarian cancer cells were grown sc in nude mice and when tumors were ca. 10 mm³, 3 x 10⁶ virus of each CRAd was injected on 3 consecutive days. Tumor size was followed and plotted relative to initial size. Luciferase-expressing, nonreplicating Ads/3Luc1 (3 x 10⁶ vp) was co-infected with each virus and in vivo luciferase imaging was performed 3 days later. For imaging, 4.5 mg of d-luciferin (Promega) was injected ip in 100 μl of 0% growth medium and images were captured with the IVIS 100 system using Living Image version 2.50 (Xenogen, Inc., Alameda, CA, USA).

SKOV3.ip1 ovarian cancer cells were injected at 10⁷ cells per mouse and tumors were allowed to grow intraperitoneally for 7 days. Then, 10⁸ vp of Ads300wt, Ads5/3d24, or Ads3/3cox2Ld24 was injected intraperitoneally on 3 consecutive days and survival was monitored.

**Statistical analysis.** The F test was performed to see if there were differences between the oncolytic potency of the viruses within each dose and cell line. If there was unequal distribution of the results, a two-sided t test was utilized to assess statistical significance in comparison to the nonreplicating virus, the Delta24 5/3-chimeric virus without TSP, and the wild-type controls. For all analyses a two-sided P value of < 0.05 was deemed statistically significant. In vivo, mean tumor size and standard deviations were calculated for each group of animals for each time point. A nonparametric change-point test was used to show a systematic change in the pattern of observations as opposed to fluctuation due to chance. The Proc Mixed procedure in SAS version 6.12 (SAS Institute, Cary, NC, USA) was used to examine the effects of group and time on tumor growth. Pair-wise comparisons were performed to compare groups. In the survival experiment, data were plotted into a Kaplan–Meier curve and groups were compared pair-wise with a log-rank test using SPSS 11.5.