Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures

N. Sanjib Banerjee,[1] Angel A. Rivera,[2] Minghui Wang,[2] Louise T. Chow,[1] Thomas R. Broker,[1] David T. Curiel,[2] and Dirk M. Nettelbeck[2,3]

[1]Department of Biochemistry and Molecular Genetics and [2]Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL and [3]Department of Dermatology, University Medical Center Erlangen, Erlangen, Germany

Abstract

We have generated novel conditionally replicative adenoviruses (CRAds) targeted to melanoma cells. In these adenoviruses, the E4 region (AdΔ24TyrE4) or both E1 and E4 regions (Ad2xTyr) were controlled by a synthetic tyrosinase enhancer/promoter (Tyr2E/P) specific for melanocytes. The properties of these CRAds were compared with wild-type adenovirus (Adwt) and our previous CRAd with a targeted E1A CRII mutation (AdTyrΔ24) in submerged cultures of melanoma cells and nonmelanoma control cells. We showed that AdΔ24TyrE4 had a cell type selectivity similar to AdTyrΔ24 but had a distinct block in viral reproduction in nonmelanoma cells and that Ad2xTyr had an augmented selectivity for melanoma cells. These viruses were additionally tested in organotypic cultures of melanoma cell lines, primary human keratinocytes (PHKs), or mixed cell populations. Unexpectedly, the CRAds exhibited somewhat different cell type selectivity profiles in these cultures relative to those observed in submerged cultures, demonstrating the importance of multiple assay systems. Specifically, AdTyrΔ24 and Ad2xTyr were selective for melanoma cells, whereas AdΔ24TyrE4 exhibited no selectivity, similar to Adwt. AdTyrΔ24 and Ad2xTyr were strongly attenuated in their ability to lyse PHKs in organotypic cultures. Furthermore, Ad2xTyr had a superior melanoma selectivity in organotypic cultures of cocultivated melanoma cells and PHKs. The enhanced selectivity for melanoma cells exhibited by Ad2xTyr provides a window of opportunity for therapeutic application. These studies also demonstrate that organotypic cultures derived from mixtures of tumor and normal cells represent a promising new model for analysis of CRAd specificity and toxicity. [Mol Cancer Ther. 2004;3(4):437–449]

Introduction

Novel therapeutic approaches for cancer treatment are urgently needed because conventional treatment regimens are frequently acutely toxic, nonselective, or otherwise ineffective. Viral oncolysis or virotherapy is a promising new therapeutic strategy that has been rapidly translated into clinical trials (1, 2). Viruses are attractive anticancer agents because they allow for incorporation of biological targeting mechanisms at multiple stages, including cell surface receptor binding, transcription, and replication. Thus, tumor-specific viruses can be superior to conventional drugs from the perspective of sparing nontarget tissues and reducing nonspecific side effects while being able to amplify transiently in the targeted tumors. Adenoviruses possess the critical basic properties required for viral oncolysis (3). First, wild-type adenoviruses (Adwt) are nononcogenic in humans. Second, high titers of adenovirus particles are easy to produce and are stable. Third, the adenoviral gene functions are particularly well characterized, enabling various modifications for tumor-specific targeting. Fourth and importantly, in productive infections, host cells are lysed and release their progeny, but the viruses are usually eliminated in a few days by antibodies to the capsid proteins. This limited window of activity is an inherent safety feature. Indeed, adenovirotherapy has been well tolerated in the vast majority of clinical trials (4). These considerations have led to the development of conditionally replicative adenovirus (CRAds) for cancer therapy (5, 6).

Tumor specificity of adenoviral replication has been described for several tumor types. Several distinct strategies have been pursued toward this end. One class of CRAds is derived by mutations in adenoviral genes that are required for efficient viral replication in normal cells but are less important in tumor cells. For example, cancer cells in which the tumor suppressor proteins p53 or pRb are inactivated were reported to be permissive for efficient replication of adenoviruses that are mutated in the *E1B55K* or *E1A* genes that encode p53- or pRb-inactivating proteins, respectively (7–9). A second distinct strategy for oncolytic

Received 10/22/03; revised 1/8/04; accepted 2/6/04.

Grant support: Deutsche Forschungsgemeinschaft grant NE832/1 (D. Nettelbeck), U.S. Public Health Service grants, Komen Foundation, and NIH grants R01 CA94084, P50 CA83591, R01 CA83821, R01 CA93796, and CA36200.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: N.S. Banerjee and A. Rivera contributed equally to this work.

Requests for Reprints: Dirk M. Nettelbeck, Department of Dermatology, University Medical Center Erlangen, Hartmannstr. 14, 91052 Erlangen, Germany. Phone: 49-9131-853-4746; Fax: 49-9131-853-6417. E-mail: dirk.nettelbeck@derma.imed.uni-erlangen.de; or N. Sanjib Banerjee, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 585 MCLM Bldg., 1918 University Blvd., Birmingham, AL 35294-0005. Phone: (205) 975-8304; Fax: (205) 975-6075. E-mail: banerjee@uab.edu

CRAd development is transcriptional targeting (10) of adenoviral replication to tumors. To this end, essential adenoviral genes, primarily the key regulatory *E1A* region, have been expressed from cell type-selective promoters such as prostate-specific antigen, α-feto protein, or tyrosinase; from ''pan-tumor'' promoters, externally inducible promoters; and from tumor- or tumor endothelium-selective transcriptional control elements (for an overview, see Ref. 11). Additional strategies for achieving tumor-restricted adenoviral replication have been reported recently and include targeted homologous recombination and selective mRNA stabilization (12, 13).

Despite the advancements in CRAd development, a major deficiency has been the lack of *bona fide* model systems with which to validate their replication specificity. In this regard, animal models are of limited value because adenoviral replication is species specific and human adenovirus infection is abortive in rodents. Hence, replication selectivity of CRAds has been demonstrated mainly in normal or tumor cells or cell lines in monolayer cultures. However, these cultures recapitulate neither the three-dimensional tissue architecture nor the variety of stages of dynamic cellular differentiation present in native tissues. Thus, the development of organotypic models for analysis of CRAds is highly desirable and indeed feasible for certain applications. Notably, with respect to critical determinants of CRAd activity, such as coxsackie and adenovirus receptor (CAR) expression and cellular differentiation, these models resemble the *in vivo* situation more closely than submerged cultures. Moreover, they allow for the evaluation of viral spread in a three-dimensional tissue. In particular, primary human keratinocytes (PHKs) form a stratified squamous epithelium when grown on a dermal equivalent consisting of fibroblasts and collagen at the air-medium interface as with real skin. The epithelia developed resemble normal squamous epithelium (reviewed in Ref. 14). Such organotypic cultures of PHKs and primary oropharyngeal cells have been used to investigate the expression of the CAR, the adenoviral promoter activities during cellular differentiation, and the infectivity and progression of adenovirus infection in a three-dimensional tissue (15, 16). Balagué *et al.* (17) have taken advantage of this system in designing and testing CRAds that are specific for cells expressing human papillomavirus (HPV) oncogenes. Organotypic cultures of cocultivated melanoma cell lines and PHKs have been reported (18), but these cultures have not been used to characterize melanoma-targeted CRAds.

In recent decades, incidence and mortality rates have risen faster for malignant melanoma than for any other tumor type in developed countries (19). As a result, malignant melanoma is now one of the most common causes of cancer and cancer deaths in young adults. Melanomas typically metastasize early during tumor development. Once spread, they are highly refractory to conventional treatment modalities mainly due to the drug resistance of melanoma cells (20). These problems underline the critical need for novel therapeutic strategies such as virotherapy to treat disseminated malignant melanoma. We previously described a melanoma-targeted oncolytic adenovirus, AdTyrΔ24, in which an artificial tyrosinase enhancer/promoter drives the expression of an E1A mutant with a deletion in the pRb binding domain (21). This virus showed melanoma-selective replication and cytotoxicity as demonstrated in monolayer cultures of various melanoma and nonmelanoma cell lines, normal cells, and submerged cocultures of normal human keratinocytes and melanoma cells. However, residual viral replication and cytotoxicity were observed in nonmelanoma cells. Here, we report the engineering of adenoviruses that express *E4* genes from the tyrosinase enhancer/promoter and the combination of this strategy with the E1A mutant AdTyrΔ24. We then evaluated these CRAds in submerged cell cultures and organotypic raft cultures derived from melanoma cells, PHKs, or mixed cell types.

Materials and Methods

Cell Cultures

Human tumor cell lines SK-MEL-28 (melanoma; American Type Culture Collection, Manassas, VA) and A549 (lung adenocarcinoma; American Type Culture Collection) were cultivated in DMEM (Mediatech, Herndon, VA). The human melanoma cell line Mel624 (kindly provided by Dr. Jeffrey Schlom, Bethesda, MD) was cultivated in RPMI 1640 (Mediatech). The human ovarian adenocarcinoma cell line SKOV3.ip1 (kindly provided by Dr. Janet Price, Houston, TX) and 293 cells (purchased from Microbix, Toronto, ON, Canada) were grown in DMEM/F12 (50:50; Mediatech). Foreskin-derived primary normal human fibroblasts (kindly provided by Lisa Rivera, Birmingham, AL) and 293E4 cells (Microbix) were cultivated in Eagle's MEM (Mediatech). All media were supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all Mediatech). Growth media for 293E4 cells also contained 500 μg/ml G418. Expression of E4 was induced with 1 μM dexamethasone. PHKs were recovered from neonatal foreskins from elective circumcisions and grown in serum-free keratinocyte medium (Invitrogen, Carlsbad, CA) as described (22). All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids and Recombinant Adenoviruses

For cloning of adenoviral genomes see Supplemental Material.[4] AdΔ24TyrE4 and Ad2xTyr viruses were generated by transfection of *Pac*I-digested pAd plasmids into SK-MEL-28 cells using LipofectAMINE (Life Technologies, Inc., Rockville, MD) following the manufacturer's protocol. Viruses were amplified in SK-MEL-28 cells and purified by two rounds of CsCl equilibrium density gradient ultracentrifugation. Verification of viral genomes and exclusion of wild-type contamination was performed by PCR and restriction enzyme digestions. Replication deficient, E1-deleted AdCMVLuc (23), Adwt, and adenovirus

[4] Supplemental material is available for this article at Molecular Cancer Therapeutics online (http://mct.aacrjournals.org).

containing the Δ24 mutation (AdΔ24; Ref. 8) were amplified in 293 (AdCMVLuc and Adwt) or A549 (AdΔ24) cells and purified as described. AdCMVΔ24 and AdTyrΔ24 were described earlier (21). Physical particle concentration [viral particles (vp)/ml] was determined by OD_{260} reading and biological particle concentration (plaque-forming units/ml) was determined by standard plaque assay on 293E4 cells.

Western Blot

Adenoviral E4 expression was determined by Western blot as described previously (21). Cells were infected with the indicated viruses at 5000 vp/cell for melanoma cells or 10,000 vp/cell for SKOV3.ip1 cells or were mock infected. A high multiplicity of infection (MOI) was required for the detection of E4orf3 protein. The membranes were probed with monoclonal antibody specific for E4orf3 protein (kindly provided by Dr. Gary Ketner, Baltimore, MD).

Cytotoxicity Assay

For determination of virus-mediated cytotoxicity, 1.5×10^4 tumor cells were seeded in 24-well plates and infected with adenoviruses in 200 μl growth medium containing 2% FBS at indicated MOI or were mock infected. The infection medium was replaced with growth medium the next day. To visualize cell killing, cells were fixed and stained with 1% crystal violet in 70% ethanol for 20 min followed by washing with tap water to remove excess dye. The plates were dried and images were captured with a Kodak DC260 digital camera (Eastman Kodak, Rochester, NY).

Virus Production Assay

To determine virus production, 1.5×10^4 cells were seeded in 24-well plates and infected in 200 μl growth medium containing 2% FBS at 10 vp/cell. The next day, cells were washed thrice with HBSS (Mediatech) and further incubated with growth medium containing 10% FBS. Three days after infection, cells and medium were harvested and freeze/thawed thrice, and virus titers were determined by TCID50 assay on 293E4 cells and are presented as relative virus titers after standardization with titers of Adwt. Experiments were performed in triplicates.

Measurement of Adenovirus Genome Copy Numbers by Quantitative PCR

To quantify intracellular adenoviral genomes, 1.5×10^4 cells were seeded in 24-well plates and infected at 1 vp/cell. Cells were harvested on the indicated days after infection. DNA was purified from the samples with the Qiagen DNA Blood Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Adenoviral genome copy numbers were quantified by real-time PCR (LightCycler System; Roche Molecular Biochemicals, Indianapolis, IN) essentially as described previously (24). Primers (Applied Biosystems, Foster City, CA) were E1A forward (5′-AAC CAG TTG CCG TGA GAG TTG), E1A reverse (5′-CTC GTT AAG CAA GTC CTC GAT ACA T), and probe (6FAM-CAC AGC CTG GCG ACG CCC A-TAMRA). As an internal control, cellular genomic DNA was quantified using the PCR primers β-actin forward (5′-TAA GTA GGC GCA CAG TAG GTC TGA), β-actin reverse (5′-AAA GTG CAA AGA ACA CGG CTA AG), and probe (6FAM-CAG ACT CCC CAT CCC AAG ACC CCA-TAMRA). Data were analyzed with LightCycler software and adenoviral genome titers in copy numbers/ng cellular genomic DNA were determined. For some experiments, data are presented as relative virus genome copy numbers after standardization with genome copy numbers of Adwt. All experiments were performed in triplicates.

Organotypic Raft Cultures

Organotypic raft cultures of PHKs were prepared essentially as described previously (25, 26). For adenovirus infection, PHKs were plated in 60 mm Petri dishes, and on reaching 70% confluence, the cells were infected with Adwt or CRAds at 1 or 10 vp/cell in serum-free keratinocyte medium (Invitrogen) for 16 h. Cells were washed in PBS, trypsinized, and counted. Cells (2×10^5) were then seeded on each dermal equivalent, which consists of type 1 rat tail collagen and J2 mouse fibroblasts, followed by culturing at the air-medium interface for 10 days. For organotypic raft culture of melanoma cells, cells were cultured submerged in RPMI 1640 and 10% FBS or infected for 16 h in 2% serum containing RPMI 1640 with the specified viruses before harvesting and culturing as described for the PHK cells. For the mixed cell raft cultures, submerged cultures of melanoma cells were infected at a specified vp/cell for 16 h. Cells were then trypsinized, counted, and mixed with uninfected PHKs in proportions as described in the text to a total of 2×10^5 cells/raft culture. The cultures were grown at the air-medium interface for 10 days, harvested by a 1–2 h fixation in 10% buffered formalin, and embedded in paraffin.

Histology

Sections (4 μm) of formalin-fixed, paraffin-embedded raft cultures were stained with H&E following the standard procedure and analyzed at 20× magnification with an Olympus BH2 microscope (Olympus, Tokyo, Japan). Photomicrographs were captured using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and assembled with Adobe Photoshop 6.0 application software.

Immunofluorescence

Thin sections were deparaffinized, rehydrated, and treated with 10 mM citrate buffer (pH 6.0) at 95°C for 10 min for antigen retrieval followed by 15 min in 3% hydrogen peroxide. The slides were rinsed in PBS and blocked with 25% goat or rabbit serum for 1 h at room temperature before applying antibody. The double immunofluorescent identification of keratinocytes and melanoma cells was conducted as follows. The sections were probed with a 1:50 dilution of a monoclonal mouse anti-MART-1 antibody (Melan-A, Clone A103; Zymed, South San Francisco, CA) specific for melanoma cells. The signals were revealed with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) and streptavidin-Texas Red (Vector Laboratories). The sections were then treated with MOM mouse immunoglobulin blocking reagent (MOM, Basic Kit, BMK-2202; Vector Laboratories) before probing with a mixture of 1:1000 dilution of AE1 and AE3 monoclonal antibodies to acidic and basic subfamilies of keratins, respectively (Zymed), specific for keratinocytes. The cytokeratin signals were

then detected with biotinylated horse anti-mouse IgG followed by streptavidin-Alexa Oregon Green (Molecular Probes, Eugene, OR). Double detection of E1A and hexon were conducted as described (16). Dual detection of hexon and keratin was carried out as above but replacing anti-E1A mouse antibody with anti-keratin antibodies. All sections were mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (H-1200; Vector Laboratories). The images were captured with either a Texas Red or a FITC filter in an Olympus AX70 fluorescence microscope equipped with a Zeiss Axiocam camera (Carl Zeiss, Oberkochem, Germany). Individual images were processed and merged using Adobe Photoshop 5.5 application software.

Results

Construction of AdTyrE4 and Ad2xTyr CRAds with the Tyrosinase Enhancer/Promoter Driving E4 or Both E1A and E4

We previously reported melanoma cell-specific E1A expression by replacing the *E1A* promoter of wild-type and *E1A* mutant adenoviruses with an optimized tyrosinase promoter, hTyr2E/P, which consists of a tandem enhancer and core promoter of the human tyrosinase gene and an upstream polyadenylation signal (21). This promoter exhibits a strictly melanoma-specific activity (27, 28). The polyadenylation signal was incorporated into the engineered viruses to mitigate nonspecific gene expression from the viral inverted terminal repeat, which is located at both ends of the chromosome, directly upstream of *E1A* and *E4*. Replication and cytotoxicity of these viruses were determined to be 2 orders of magnitude stronger in melanoma cells than in various control cells. However, residual viral replication and cell killing was observed in nonmelanoma tumor cell lines and in normal cells such as PHKs and fibroblasts.

To improve tumor specificity and safety of melanoma-targeted CRAds, we evaluated the utility of expressing the adenoviral E4 genes from the same tyrosinase enhancer/promoter. In AdΔ24TyrE4 (Fig. 1), the E4 promoter (nucleotides 35576–35817) was replaced with hTyr2E/P and an upstream polyadenylation signal, whereas all other promoters were unmodified. In addition, we also constructed a CRAd that expresses E1AΔ24 from hTyr2E/P and E4 from a similar murine tyrosinase enhancer/promoter, mTyr2E/P (Fig. 1). The mTyr2E/P had an activity similar to hTyr2E/P as determined in transient transfection experiments (data not shown), but the sequences are nonhomologous to those of the hTyr2E/P. This strategy avoids adverse recombination events that might occur had we used hTry2E/P to express both E1A and E4. Viruses were generated in melanoma cells and verified as described in Materials and Methods.

The Tyrosinase Enhancer/Promoter Mediates Melanoma-Specific E4 Expression in the Context of CRAds

Next, we determined the activity and specificity of AdΔ24TyrE4. To analyze the virus properties on targeted E4 expression, other viral promoters were unmodified in

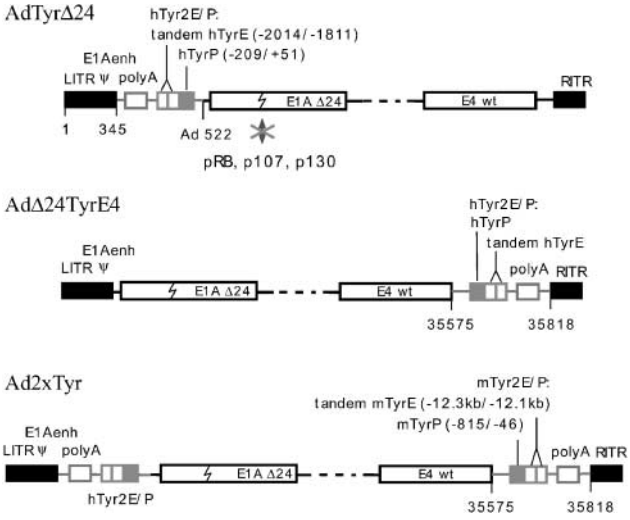

Figure 1. Schematic structures of the genomes of the CRAds generated in this study. *Numbers,* nucleotide positions of the adenoviral genome or tyrosinase genes. *LITR* or *RITR,* left or right inverted terminal repeat, respectively; *E1Aenh,* E1A enhancer; ψ, packaging signal; *polyA,* synthetic polyadenylation sequence; *hTyrE* or *mTyrE,* human or murine tyrosinase enhancer, respectively; *hTyrP* or *mTyrP,* human or murine tyrosinase promoter, respectively; *E1AΔ24,* E1A mutant with 24 nucleotide deletion in the conserved region 2.

this virus. To demonstrate melanoma-specific activity of hTyr2E/P in AdΔ24TyrE4, melanoma cell lines SK-MEL-28, and Mel624, both of which express tyrosinase (data not shown), and SKOV3.ip1, an ovarian adenocarcinoma cell line that does not express tyrosinase, were infected with AdΔ24TyrE4, AdTyrΔ24, Adwt, or AdΔ24 or were mock infected. E4orf3 expression was then assessed by Western blot analysis (Fig. 2A). AdTyrΔ24 expressed E4orf3 in melanoma and nonmelanoma cells at levels similar to Adwt and AdΔ24. In contrast, no E4orf3 expression was detected for AdΔ24TyrE4 in SKOV3.ip1 cells, whereas E4orf3 expression for this virus was clearly shown for both melanoma cell lines at expression levels similar (Mel624) or somewhat lower (SK-MEL-28) than for the other viruses. These findings imply the following: (*1*) The activity of the hTyr2E/P promoter from the adenovirus E4 region and in the context of replicating adenoviruses is restricted to melanoma cells. (2) In nonmelanoma cells, the E4 promoter is active in the absence of E1A, or the small amount of E1A expressed from hTyr2E/P is sufficient to activate the E4 promoter, accounting for the high E4 protein level from AdTyrΔ24. (*3*) The hTyr2E/P is not responsive to E1A, resulting in the loss of E4 expression from AdΔ24TyrE4 in nonmelanoma cells.

Progeny Virus Production and Cytotoxicity but not DNA Replication of AdΔ24TyrE4 Is Melanoma Selective in Submerged Cultures

We next sought to evaluate whether the expression of *E4* genes from hTyr2E/P is translated into selective cytotoxicity and replication of AdΔ24TyrE4 in melanoma cells relative to control cells. For this purpose, two melanoma cell lines (SK-MEL-28 and Mel624) and two nonmelanoma cell lines (SKOV3.ip1 and A549) were mock infected or infected

with AdΔ24TyrE4, AdTyrΔ24, Adwt, AdΔ24, or nonreplicating AdCMVLuc at 100, 10, 1, 0.1, or 0.01 vp/cell. Cytotoxicity was detected by staining of surviving cells with crystal violet (Fig. 2B). Adwt and AdΔ24 killed both melanoma and nonmelanoma cell lines, whereas AdΔ24TyrE4 exhibited a predilection for melanoma cells. Cell killing by this virus was more efficient than by Adwt and AdΔ24 in Mel624 cells. In SK-MEL-28 cells, AdΔ24TyrE4 at a 10 times higher MOI induced cell killing similar to that elicited by Adwt or AdΔ24. In contrast, substantially attenuated cytotoxicity was observed for AdΔ24TyrE4 in nonmelanoma cell lines, where 3–4 orders of magnitude higher MOIs were required to induce cell killing comparable with Adwt or AdΔ24. The specificity of cell killing by AdΔ24TyrE4 was identical to AdTyrΔ24, with the exception that the former was more efficient in Mel624 cells. In the negative control, AdCMVLuc induced no cytotoxicity in any of the cell lines.

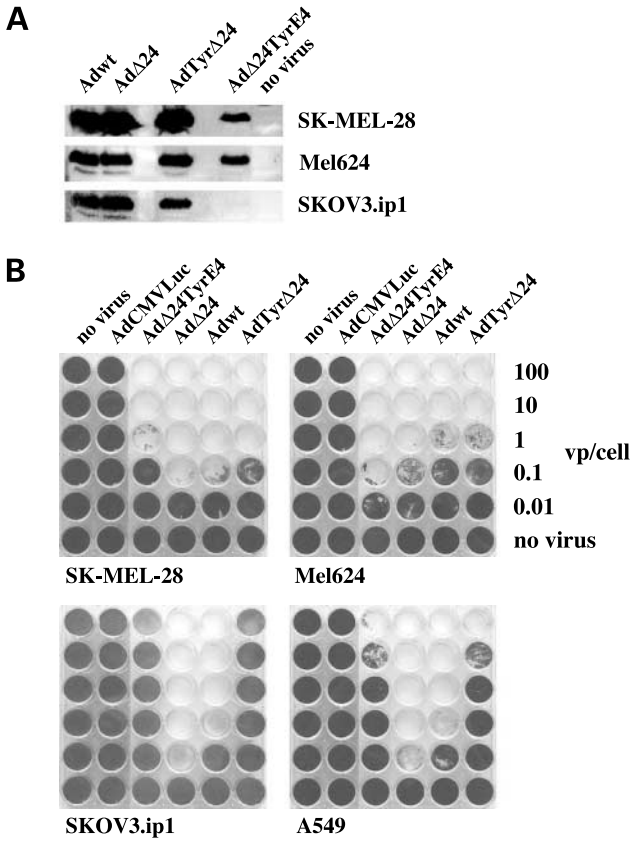

Figure 2. Melanoma-specific E4 expression and cytotoxicity of AdΔ24TyrE4. **A,** Western blot analysis of E4 expression after infection of melanoma (SK-MEL-28 and Mel624) and nonmelanoma (SKOV3.ip1) cells with indicated adenoviruses or in mocked infections. **B,** cytotoxicity of CRAds AdΔ24TyrE4, AdTyrΔ24, and AdΔ24, Adwt, and E1-deleted AdCMVLuc for melanoma cells (*upper panels*) and nonmelanoma cells (*lower panels*). Cells were infected at the indicated vp/cell or were mock infected. To show the best differential between various virus and cell type combinations, attached cells were stained with crystal violet when cell lysis was observed for Adwt or AdΔ24 at 0.01 vp/cell.

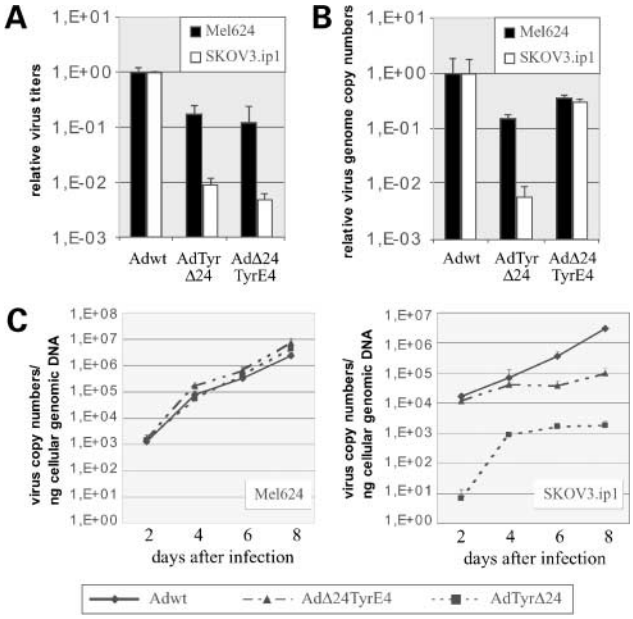

Figure 3. Analysis of melanoma-targeted CRAds for specificity of virus particle production and DNA replication. **A,** cells were infected with indicated viruses at 10 vp/cell; viruses were purified from supernatant and cells 3 days after infection and titered on 293E4 cells. *Columns,* mean relative virus titers after standardization with the titer of Adwt (triplicate experiments); *bars,* SD. **B** and **C,** cells were infected with indicated viruses at 1 vp/cell; DNA was purified from supernatant and cells 3 days (**B**) or at 2, 4, 6, and 8 days (**C**) after infection and adenoviral genomes were quantified by real-time PCR. **B,** relative virus genome copy numbers are plotted after standardization with genome copy numbers of Adwt. *Columns,* mean (triplicate experiments); *bars,* SD.

We then examined whether this preference for melanoma cell killing by AdΔ24TyrE4 correlated with progeny virus production. Using 293E4 cells, we titered progeny viruses generated 3 days after infection of Mel624 and SKOV3.ip1 cells with AdΔ24TyrE4, Adwt, or AdTyrΔ24 (Fig. 3A). After standardization with the results for Adwt, we detected AdΔ24TyrE4 at more than 1 order of magnitude higher virus titers in melanoma *versus* nonmelanoma cells. A nearly identical result was observed for AdTyrΔ24. No virus replication was observed for the negative control, AdCMVLuc, in any cells (data not shown).

We also determined adenoviral genome copy numbers generated in these cell lines 3 days after infection with AdΔ24TyrE4, Adwt, or AdTyrΔ24 (Fig. 3B). Surprisingly, AdΔ24TyrE4 generated similar genome copy numbers in melanoma and nonmelanoma cells after standardization with copy numbers of Adwt. In contrast, AdTyrΔ24 showed melanoma-specific genome replication with lower copy numbers in control cells. These results suggest a block late in the viral replication cycle in nonmelanoma cells when E4 was expressed from the cell type-restricted tyrosinase promoter.

To investigate further the above observation, we determined viral genome copy numbers on days 2, 4, 6, and 8 after infection of Mel624 and SKOV3.ip1 cells (Fig. 3C). We observed similar genome copy numbers for AdTyrΔ24,

AdΔ24TyrE4, and Adwt in melanoma cells. In SKOV3.ip1 cells, the genome copy numbers were 2 or more orders of magnitude lower for AdTyrΔ24 relative to Adwt from day 2 onward. In contrast, genome copy numbers for AdΔ24TyrE4 were similar to Adwt on days 2 and 4 but did not increase further until day 6 and only marginally until day 8, resulting in 1.5 orders of magnitude lower copy numbers relative to Adwt on day 8. Taken together, the significantly reduced expression of *E4* genes from the tyrosinase promoter in nonmelanoma cells (Fig. 2A) reduces virus production but not viral DNA replication, consistent with certain E4 protein functions late in the infection cycle (Fig. 3, A and B). However, in multiple-cycle infections over 8 days, an initial reduced virus yield then results in a lower viral DNA copy number at later time points (Fig. 3C, right panel).

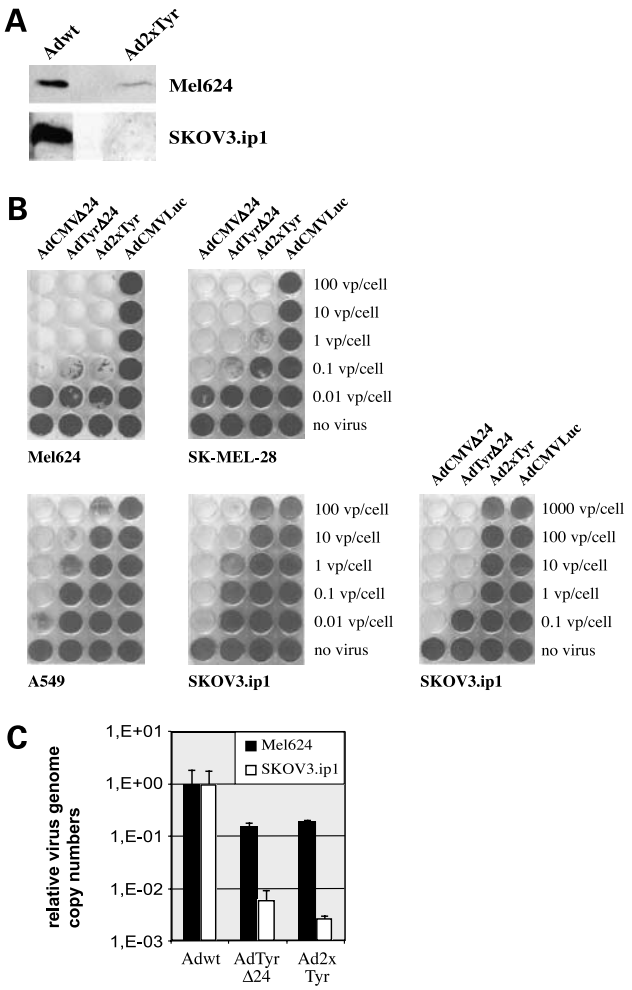

Figure 4. Melanoma-specific E4 expression and cytotoxicity of Ad2xTyr. **A,** Western blot analysis of E4 expression after infection of melanoma (Mel624) and nonmelanoma (SKOV3.ip1) cells with indicated adenoviruses or mock infection. For SKOV3.ip1, a longer exposure film is shown. **B,** cytotoxicity of CRAds AdTyrΔ24 and Ad2xTyr, nonspecific AdCMVΔ24, and E1-deleted AdCMVLuc for melanoma cells (*upper panels*) and nonmelanoma cells (*lower panels*). Cells were infected at the indicated vp/cell or were mock infected. Attached cells were stained with crystal violet when cell lysis was observed for AdCMVΔ24 at 0.01 vp/cell. For SKOV3.ip1, infections with higher MOI are also presented (*lower panel*, *right*); this plate was stained with crystal violet when cell lysis was observed for AdTyrΔ24 at 0.01 vp/cell (AdCMVΔ24 had lysed all cells at this time point). **C,** cells were infected with indicated viruses at 1 vp/cell; DNA was purified from supernatant and cells 3 days after infection and adenoviral genomes were quantified by real-time PCR. Relative virus genome copy numbers are plotted after standardization with genome copy numbers of Adwt. *Columns,* mean (triplicate experiments); *bars,* SD.

Expression of Both E1AΔ24 and E4 from Tyrosinase Enhancer/Promoter in Ad2xTyr Results in Increased Melanoma Specificity of Adenoviral Oncolysis

The properties of AdΔ24TyrE4 indicate that preferential expression of *E4* genes within replication-competent adenoviruses in melanoma cells can indeed be achieved with the optimized tyrosinase promoter. However, as the viral life cycle of this virus in nonmelanoma cells is not blocked until late stages, this strategy might not be sufficient to derive melanoma-specific CRAds for therapeutic applications. Based on these findings, we hypothesized that specific expression of both E1A and E4 within one virus may result in a CRAd with a significantly improved specificity profile because of distinct blocks to viral reproduction. Thus, we designed Ad2xTyr, a CRAd that expresses E1AΔ24 from hTyr2E/P and E4 from a similar murine construct, mTyr2E/P (Fig. 1).

Specific expression of E4orf3 by mTyr2E/P in Ad2xTyr was shown by Western blot (Fig. 4A). The signals were weaker for Ad2xTyr than for Adwt in melanoma cells and were below detection in nonmelanoma cells even after long exposure. In accord with our hypothesis, cytotoxicity of Ad2xTyr was highly specific for melanoma cells, superior to AdTyrΔ24 (Fig. 4B). We performed a crystal violet assay after infection of melanoma and nonmelanoma cells with these viruses. A nonspecific positive control was infection with AdCMVΔ24, which has a cytotoxicity similar to Adwt (21), whereas mock infection and infection with a nonreplicative AdCMVLuc were negative controls. Cell killing by Ad2xTyr was similar to AdTyrΔ24 and AdCMVΔ24 in Mel624 cells and 1 order of magnitude attenuated in SK-MEL-28 cells. In A549 cells, Ad2xTyr was 2 or 4 orders of magnitude attenuated relative to AdTyrΔ24 or AdCMVΔ24, respectively. No cell killing was observed for Ad2xTyr in SKOV3.ip1 cells even at 1000 vp/cell, thus establishing more than 3 orders of magnitude attenuation relative to AdTyrΔ24 and 6 orders of magnitude relative to AdCMVΔ24, respectively. Cytotoxicity of Ad2xTyr was also strongly attenuated in normal human fibroblasts and keratinocytes (data not shown). Furthermore, Ad2xTyr showed reduced DNA replication compared with Adwt in SKOV3.ip1 cells from day 2 after infection onward (Fig. 4C; data not shown) and thus differed from the DNA replication pattern observed for AdΔ24TyrE4. DNA replication of Ad2xTyr was reduced relative to AdTyrΔ24 in SKOV3.ip1 cells but was similar for both viruses in melanoma cells (Fig. 4C; data not shown).

Infection by Melanoma-Specific CRAds in Raft Cultures of Mel624

Next, we sought to investigate the efficiency of infection of melanoma-selective CRAds in three-dimensional melanoma tissue structures developed by culturing the

cells on a dermal equivalent at the air-medium interface (abbreviated as raft cultures). The suitability of Mel624 and SK-MEL-28 for this culture system was tested first. The three-dimensional cultures of both were fragile and became extensively fragmented during subsequent processing (Fig. 5A; data not shown). Interestingly, both raft cultures were rather refractory to adenovirus infection, which might be due at least partially to a low level of CAR expression (data not shown). We chose to use Mel624 cells in subsequent experiments because the raft cultures of this cell line were more uniform and developed more cell layers than those from SK-MEL-28 cells. Additionally, unlike Mel624, SK-MEL-28 did not form mixed raft cultures with PHKs (to be described). Rather, the cells, when observable, were distributed on top of the squames, not in direct contact with live PHKs (data not shown). This growth property would not be amenable for investigating the spreading of CRAds from melanoma cells to PHKs.

Preformed raft cultures of PHKs were refractory to infection, but infections in organotypic cultures were achieved by prior exposure of submerged cells to viruses (16, 17). This was also true with the melanoma cell lines (data not shown). To test the cell killing by different CRAds in raft cultures, Mel624 cells were infected at 1 or 10 vp/cell for 16 h in submerged cultures. The infected cells were raised onto collagen beds and cultured at the air-medium interface for 10 days. The tissues were then fixed in formalin and embedded in paraffin. Sections (4 μm) were assessed for histology after staining with H&E. Significant cytopathic effect was only observed with cultures infected with AdΔ24TyrE4 and only at 10 vp/cell (Fig. 5A, top row, right panel; data not shown). We then compared AdTyrΔ24 or Ad2xTyr at higher MOIs at 30, 100, or 300 vp/cell. The infected cells were raised as raft cultures as described and harvested on day 10. As expected, histological analysis of the tissue sections revealed extensive cytopathic effect (CPE) by both viruses. On day 10, only remnants of dead cells were observed (Fig. 5A).

Because of the limitation of histological evaluation, we conducted double immunofluorescence detection for E1A and hexon to assess the extent of adenovirus infection (Fig. 5B). We did not detect any signal for E1A or hexon in raft cultures of uninfected Mel624 cells, attesting to the specificity of the antibodies. On infection, hexon and E1A signals were often found in different cells, indicating infections proceeded to different stages, as in organotypic

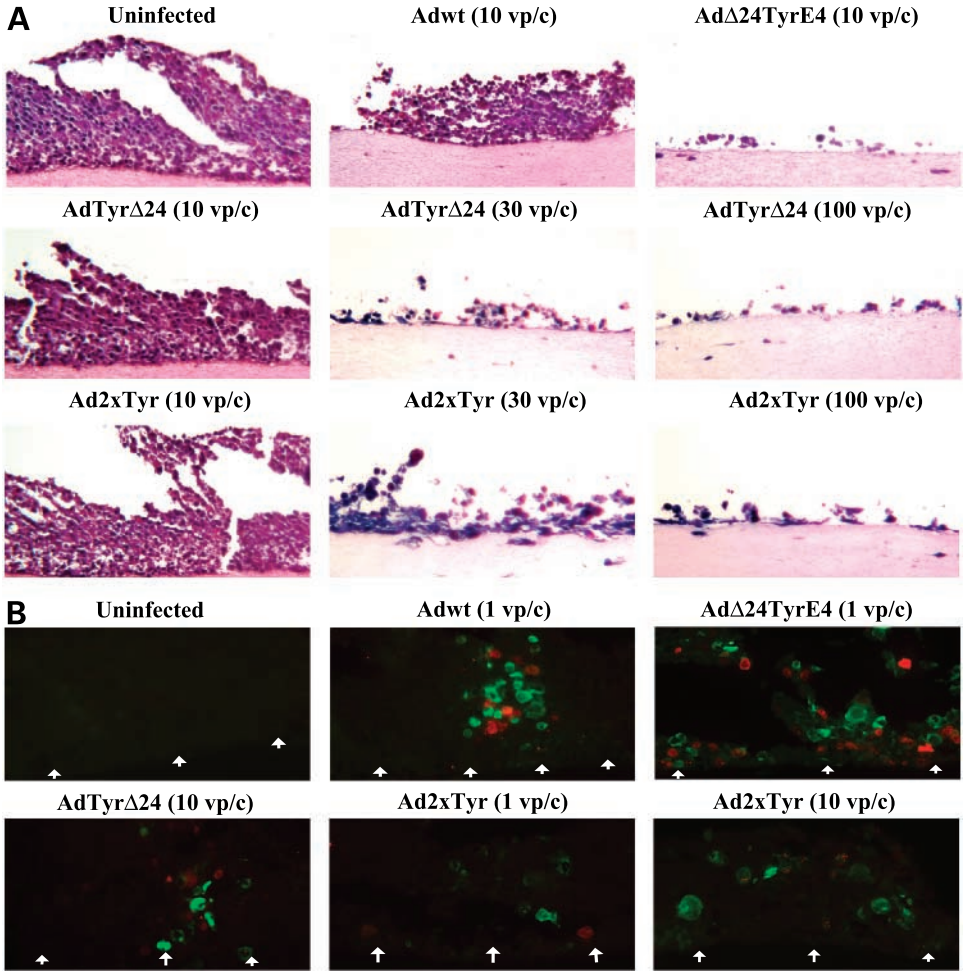

Figure 5. Infection of Adwt and tyrosinase promoter-driven CRAds in organotypic raft cultures of Mel624 cells. **A,** tissue morphology as revealed by H&E staining of thin sections of infected and uninfected cultures. The submerged Mel624 cells were infected for 16 h prior to being raised to the air-medium interface for 10 days. The virus type and the MOI (*vp/c*) are indicated. **B,** double immunofluorescence for hexon (*green*) and E1A (*red*) in serial sections. *Arrows,* cell-dermal equivalent interface. Images were captured with a 20× objective lens.

cultures of PHKs (16). In infections at 1 vp/cell by Adwt, isolated cells positive for E1A and hexon were observed. In infection by AdΔ24TyrE4 at 1 vp/cell, E1A- and hexon-positive cells were scattered throughout the entire raft culture. At 10 vp/cell, Adwt generated widespread E1A and hexon signals (data not shown). In contrast, only cell debris remained with AdΔ24TyrE4 at this virus titer (Fig. 5A). Thus, AdΔ24TyrE4 was more virulent than Adwt.

At 1 vp/cell, viral antigen signals in cultures infected by AdTyrΔ24 or Ad2xTyr were barely detectable but became evident in infections at 10 vp/cell, comparable with infections by Adwt at 1 vp/cell, but positive cells were significantly fewer than following infections by AdΔ24TyrE4 at 1 vp/cell (Fig. 5B; data not shown). There was no appreciable difference between AdTyrΔ24 and Ad2xTyr in the fraction of infected cells. These results suggest that, relative to Adwt or AdΔ24TyrE4, infections by AdTyrΔ24 or Ad2xTyr were attenuated ~10-fold in this setting, whereas Ad2xTyr was not attenuated relative to AdTyrΔ24. These observations might imply that the native E1A promoter is more active than the tyrosinase promoter in melanoma cells in raft cultures.

Attenuation of Tyrosinase Enhancer/Promoter Controlled CRAds in Raft Cultures of PHKs

Because melanomas are physically associated with cutaneous epithelium, we also evaluated the specificity of these CRAds in raft cultures of PHKs. We have previously reported adenovirus infection and gene expression in such cultures (16, 17) and attenuation of AdTyrΔ24 in submerged cultures of PHKs (21). PHKs were infected with Adwt, AdTyrΔ24, AdΔ24TyrE4, and Ad2xTyr at 1 or 10 vp/cell for 16 h and the cells were raised onto the dermal equivalent for 10 days or longer, harvested, and processed as described. The histology of the tissue sections (Fig. 6A) revealed varied CPEs induced by the different viruses. PHKs infected with Adwt and AdΔ24TyrE4, even at 1 vp/cell, did not form any epithelium and only remnants of dead cells were observed on the dermal equivalent (data not shown). Thus, in contrast to the results obtained in submerged cultures, AdΔ24TyrE4 was not selective for melanoma and was as cytolytic as Adwt in raft cultures of PHKs. In our previous studies, where infections of PHKs by Adwt or AdΔ24 at 1 plaque-forming unit/cell in submerged cultures were allowed to

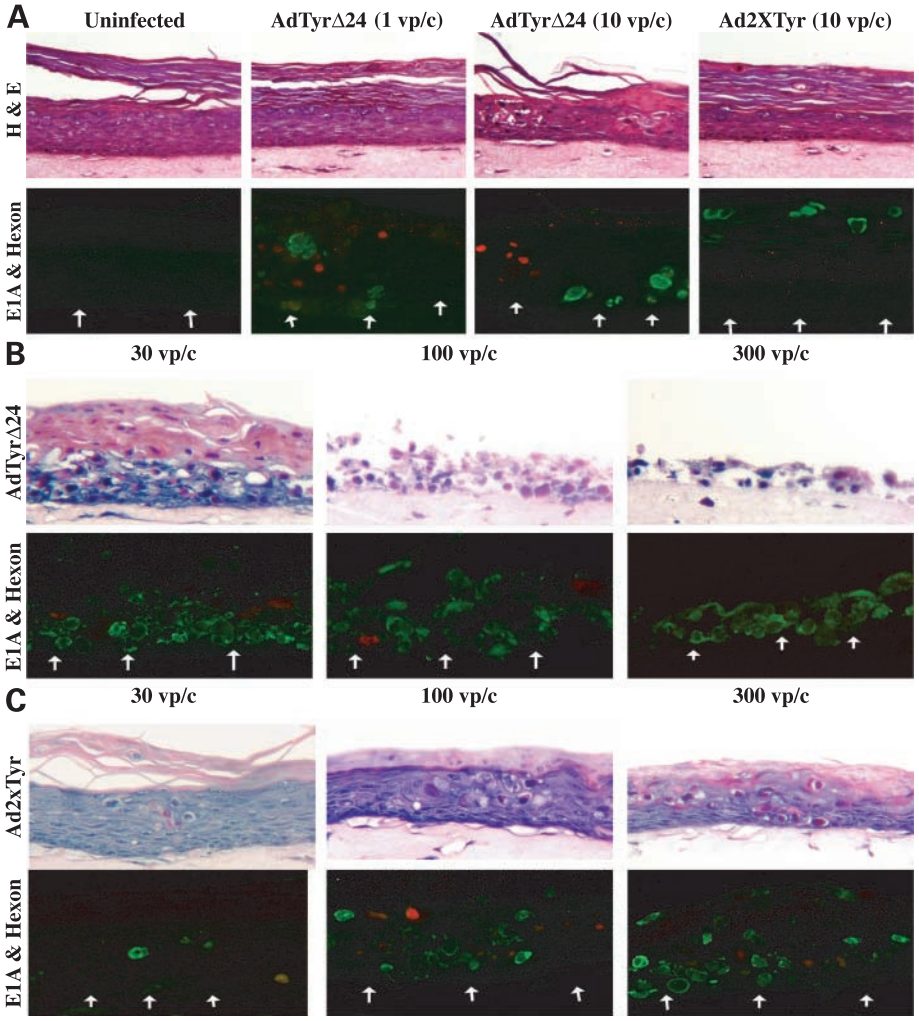

Figure 6. Infection of tyrosinase promoter-driven CRAds in organotypic raft cultures of PHKs. **A,** *top row,* tissue morphology as revealed by H&E staining of thin sections of 10-day-old raft culture of uninfected and infected PHKs; *bottom row,* double immunofluorescence to detect hexon (*green*) and E1A (*red*) on serial sections. Clear CPE in the life epithelium was detected only in infections with AdTyrΔ24 at 10 vp/cell. A few hexon-positive cells were observed in the cornified strata in infection with Ad2xTyr at 10 vp/cell. PHKs infected with Adwt and AdΔ24TyrE4 even at 1 vp/cell did not form any epithelium and only remnants of dead cells were observed (not shown). **B,** *top row,* H&E of 10-day-old PHK raft culture sections after infections with AdTyrΔ24 at 30, 100, and 300 vp/cell; *bottom row,* images of double immunofluorescence for E1A (*red*) and hexon (*green*) on serial sections. **C,** same as in **B**, except that PHKs were infected with Ad2xTyr. *Arrows,* basal stratum. Images were captured with a 20× objective lens.

proceed for only 4 h before the cells were lifted to the air-medium interface, multiple layers of dead cells were observed, suggesting that the adenovirus did not kill the cells right away until some stratification and differentiation had occurred (17). This was indeed the case when the virus was introduced by scarifying a preformed raft culture (16). In our present experiments, during the 16 h in the submerged culture, the infection had proceeded well into the late phase so that the cells died soon after being lifted onto the collagen. Thus, the outcome of the infection depends on how the experiments are conducted.

AdTyrΔ24 produced localized CPE in raft cultures only at 10 vp/cell but not at 1 vp/cell (Fig. 6A), indicating attenuated toxicity in PHK raft cultures. No CPE was detected in the raft culture of PHKs infected by Ad2xTyr at either MOI and the histologies were very similar to uninfected tissues. Thus, Ad2xTyr could not complete its infectious cycle in PHKs at these MOIs and was attenuated relative to AdTyrΔ24.

The raft cultures were further investigated by screening for E1A and hexon proteins (Fig. 6A, lower row). We did not detect any signal for E1A or hexon in uninfected raft cultures. E1A and hexon were found in a few or in a moderate number of cells, respectively, in the differentiated layers of PHK infected by AdTyrΔ24 at 1 and 10 vp/cell, indicating a reduced but dose-dependent CPE during squamous differentiation. In contrast, in Ad2xTyr-infected raft cultures, we detected neither antigen at either MOI in the basal, parabasal, spinous, or granular strata. These results agree with the lack of CPE and demonstrate that Ad2xTyr did not productively infect the PHKs at these MOIs. However, we did observe a few isolated hexon-positive cells in the cornified layers primarily at a MOI of 10 vp/cell (Fig. 6A, right panel, bottom row). These cells might have been infected at the higher MOI and infection apparently proceeded into the late phase as the keratinocytes differentiated. These infected cells were pushed up and eventually became cornified. By 16 days, no hexon signals were detected (data not shown). We suspect that the squames were shed and lost during tissue processing. At this time point, the cultures had few cell layers, but no CPE was observed and no viral antigen was detected, similar to the control cultures (data not shown). These results confirmed that the progeny CRAd did not spread in the raft cultures to elicit further rounds of infections. Collectively, these observations showed that the tyrosinase promoter-driven expression of E1A, but not of E4, strongly attenuated adenovirus replication in PHK raft cultures. Moreover, tyrosinase promoter-driven expression of both E1A and E4 confers a further attenuation of adenovirus replication in PHKs, reducing its toxicity.

We then compared Ad2xTyr and AdTyrΔ24 at higher MOIs at 30, 100, and 300 vp/cell similarly in PHK raft cultures. AdTyrΔ24 generated increasingly severe CPE with higher MOIs (Fig. 6B, top row, right panel), which became more severe on day 16 (data not shown). At 300 vp/cell, most PHKs were lysed. Only a small amount of tissue remnants was left on day 10. These observations were substantiated by immunofluorescent detection of E1A and hexon proteins (Fig. 6B, bottom row). In contrast, on day 10 of PHK raft cultures infected by Ad2xTyr at 30 vp/cell, only a few PHKs were positive for E1A or hexon (Fig. 6C). On day 16, several infected cells were found in the stratum corneum, whereas the infected cell number did not increase significantly in the live tissue (data not shown). Thus, at 30 vp/cell, this CRAd was not able to elicit an efficient productive infection in the squamous epithelium. The small number of hexon-positive cells ended up in the stratum corneum, but the progeny virus was not being able to initiate additional rounds of infection. At 100 and 300 vp/cell of Ad2xTyr, an increased number of the infected cells were observed as demonstrated by CPE and E1A and hexon staining. However, even at the MOI of 300 vp/cell, a substantial number of cells remained free of CPE and were negative for E1A and hexon (Fig. 6C). A stratified tissue, although thin, remained even on day 16 (data not shown). Consequently, both AdTyrΔ24 and Ad2xTyr showed a remarkable melanoma selectivity in organotypic cultures of melanoma cells and normal keratinocytes. Furthermore, on the basis of CPE and proportion of viral antigen-positive cells at the various MOIs, Ad2xTyr was more than 10-fold attenuated relative to AdTyrΔ24 in raft cultures of PHKs but not in raft cultures of Mel624.

Melanoma Specificity of Replication of Tyrosinase Enhancer/Promoter Controlled CRAds in Organotypic Cultures of Mel624 Cells Plus PHKs

To extend these studies, we investigated the possibility of establishing organotypic cultures containing both Mel624 and PHKs. Mel624 were mixed with PHKs at 1:3 or 1:9 ratio (Mel624 = 1) before seeding onto the dermal equivalent. The assembly was then raised to the air-medium interface, cultured for 10 days, and processed as described above. We did not detect melanoma cells in the 1:9 mixed cell raft cultures. Histological analysis of the 1:3 mixed cell raft culture revealed pockets of melanoma cells or layers of melanoma cells mostly localized to the bottom half of the raft cultures, whereas PHKs primarily occupied the upper strata (Fig. 7A, right panel). The identity of the two cells types was confirmed by double immunofluorescence analysis using melanoma-specific monoclonal antibody to Melan-A (MART-1) and a mixture of keratinocyte-specific monoclonal antibodies to keratins (AE1 and AE3; Fig. 7B). Mel624 cells were occasionally observed to grow on top of the keratinocytes as well. We did not detect any invasion by melanoma cells into the dermal equivalent as reported previously for other melanoma cell lines (18).

We then infected Mel624 cells with Adwt or with the tyrosinase promoter-containing CRAds at a range of MOIs as described previously. To detect possible virus spread to adjacent PHKs, the infected cells were then mixed at 1:3 ratio with uninfected PHKs and cultured on the dermal equivalent for 10 days or longer. The histology of the tissue sections revealed a dose- and adenovirus-dependent CPE (data not shown, but see below). To distinguish the cell types in the mixed culture, we examined MART-1 signals in raft cultures of infected Mel624 cells and cytokeratins in infected PHKs. Productive adenovirus infection virtually abolished MART-1 signals, making it impossible to identify

directly the infected Mel624 cells (data not shown). In contrast, cytokeratins that denote PHKs were very stable. Thus, we probed the sections by double immunofluorescence analysis for hexon and cytokeratins. Productive infection of Mel624 cells was inferred from being hexon positive and cytokeratin negative, whereas productive infection of PHKs would be positive for both antigens.

Infections by Adwt and AdΔ24TyrE4 at 1 or 10 vp/cell lysed virtually all of the Mel624 cells, leaving only PHKs that were positive for hexon and keratins (Fig. 8, top row; data not shown). It appeared that on lysis of the Mel624 cells, there was left with a cavity, some of which was filled in by the neighboring PHKs. The high concentrations of virions released from the lysed Mel624 cells then caused the widespread infection of PHKs. In contrast, infections by AdTyrΔ24 and Ad2xTyr at 1 vp/cell were restricted to about 20% of the Mel624 cells at the time of fixation and did not spread to the neighboring PHKs, indicative of their distinct preference for melanoma cells (data not shown). In AdTyrΔ24 infections at 10 vp/cell, ~60% of the Mel624 cells were positive for hexon and some adjacent PHKs were also positive for viral proteins (Fig. 8, middle row). At 10 vp/cell, Ad2xTyr productively infected 40–50% of melanoma cells, but only a few cocultivated PHKs were positive for E1A or hexon (Fig. 8, bottom row). Infected PHKs were fewer by at least 1 order of magnitude compared with those infected by AdTyrΔ24. These observations suggest that, although none of the Tyr-CRAds is exclusively melanoma specific, the Ad2xTyr virus is the most attenuated virus in PHKs and shows the highest degree of specificity in organotypic cultures of mixed Mel624 cells and PHKs among the viruses tested.

Discussion

Tumor specificity is the key to the realization of exploiting replicating adenoviruses as cancer therapeutics. Our study addresses this critical requirement in two ways: by developing an advanced generation tumor-targeted CRAd and by establishing a three-dimensional organotypic model derived from human tumor cells and normal cells as a novel and additional means for evaluation of the specificity and efficacy of CRAds.

Previously, several other groups have reported the development of CRAds based on targeted expression of more than one viral gene. The approaches include employing internal ribosome entry sites to express multiple genes (29, 30); the use of a tumor-specific transcription factor, tcf4, to drive expression of different adenoviral genes (31, 32); the incorporation of distinct promoters for E1A and E1B (33, 34); and the application of pS2, the E2F-1 promoter, or distinct promoters to express both E1A and E4, each from their original genomic locations (35–37). These studies have demonstrated various degrees of success as well as certain limitations. For instance, not all promoters are feasible for specific expression of adenoviral E4 genes and promoter deregulation might occur if different promoters are applied to drive distinct viral genes (35). Moreover, the restriction of additional genes did not always result in improved specificity (31, 32).

In an effort to develop melanoma-targeted CRAds, we exploited the optimized human and murine tyrosinase enhancer/promoter, hTyr2E/P and mTyr2E/P, for specific expression of adenoviral genes and examined the consequences on adenoviral DNA replication, progeny virus production, and cytotoxicity. These promoters possess key

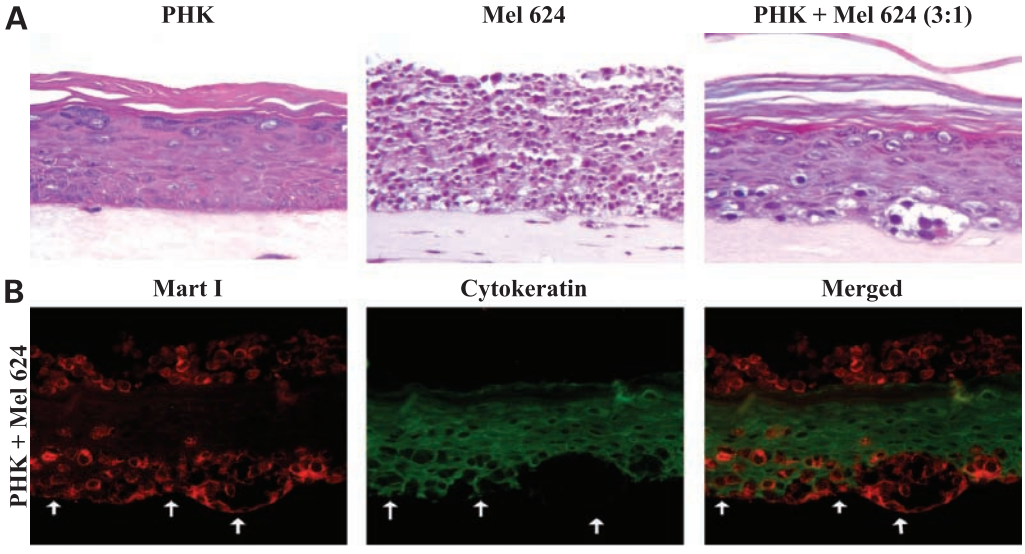

Figure 7. Organotypic cultures of PHKs, Mel624, and 3:1 mixture of PHKs and Mel624 cells. The cells were seeded on collagen bed, raised to the air-medium interface, and cultured for 10 days. The cultures were harvested, fixed in 10% buffered formalin, paraffin embedded, and thin sectioned. **A,** tissue morphology was revealed on H&E staining of thin sections of the raft cultures as specified. Mixed cell raft culture revealed pockets of melanoma cells primarily in the *lower half* of the culture. Melanoma cells were also occasionally seen on *top* of the cornified squames, apparently pushed upward during squamous differentiation of the keratinocytes, as shown in **B**. **B,** relative distribution of Mel624 and PHKs in a thin section of the mixed culture was detected by double immunofluorescence with monoclonal antibodies for melanoma cell-specific Melan-A (*red*; *left panel*) or keratinocyte-specific cytokeratins (*green*; *middle panel*) and a merged image (*right panel*). *Arrows,* cell-dermal equivalent interface. Images were captured with a 20× objective lens.

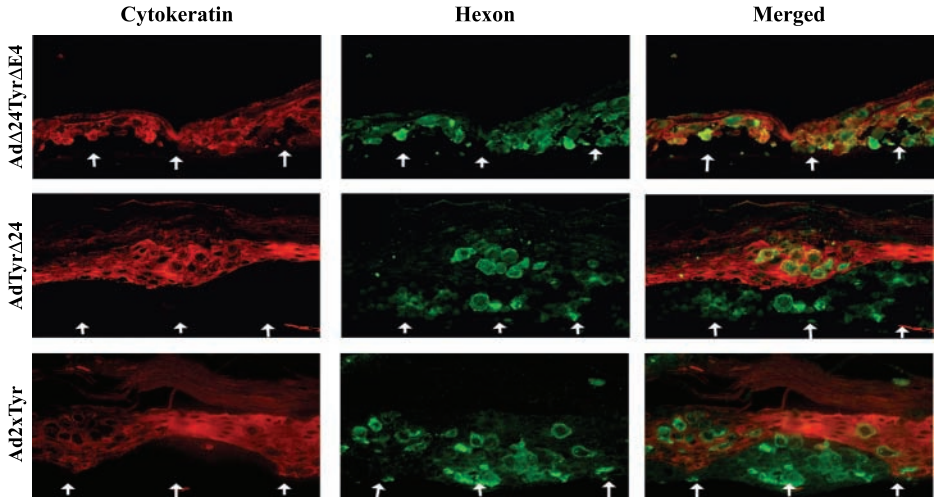

Figure 8. CRAd infection in organotypic cultures established with uninfected PHKs and infected Mel624 cells at a 3:1 ratio. Mel624 cells were infected with AdΔ24TyrE4 (*top row*), AdTyrΔ24 (*middle row*), and Ad2xTyr (*bottom row*) at 10 vp/cell for 16 h before mixing with uninfected PHK. Raft cultures (10-day-old) of infected melanoma cells and uninfected PHKs were sectioned and analyzed by double immunofluorescence for the detection of cytokeratin (*red*; *left panels*), hexon (*green*; *middle panels*), or both (*merged images*; *right panels*). *Arrows,* cell-dermal equivalent interface. Images were captured with a 20× objective lens.

features that recommend their application for transcriptional targeting of adenovirus replication to melanoma, including strong activity in melanoma cells, high specificity, and a short sequence length (27, 28). We have reported previously that the hTyr2E/P, in combination with an upstream polyA signal, retains its promoter specificity from the *E1A* locus in melanoma cells (21). Our present results show that hTyr2E/P or the similar mouse Tyr2E/P also exhibits a specific activity from the *E4* region. Targeted expression of E4 from AdΔ24TyrE4 resulted in more efficient virus production and cell killing in submerged melanoma cultures than in nonmelanoma cultures as observed with AdTyrΔ24 (Figs. 2B and 3A).

Of particular note, DNA replication was restricted to melanoma cells for AdTyrΔ24 but not for AdΔ24TyrE4 in the initial round of infection (Fig. 3, B and C). A potential explanation for this observation is that the E4 function, such as activation of E2 expression by E4orf6/7, might not be important in some tumor cells that contain high levels of free E2F because of pRb mutations. However, E4 proteins also orchestrate the early to late switch during adenoviral infection, including host cell shutoff and nuclear export of viral mRNA after replication of the viral genome. Our results are in accord with a deficiency in these latter functions for AdΔ24TyrE4 in nonmelanoma cells leading to reduced progeny virus production (Fig. 3A). The lower titer of progeny viruses then led to severely reduced viral DNA replication in subsequent round of infections (Fig. 3C).

We observed that transcriptional targeting of E4 expression restricts adenovirus propagation in control cells at least in part at a different stage of the virus life cycle than does targeted expression of E1A. In addition, because E4 proteins are cytotoxic (38), CRAds that feature targeted expression of E4 should provide a level of safeguard. We thus hypothesize that E1A and E4 targeting may synergize with each other resulting in a highly selective CRAd, the rationale for development of Ad2xTyr. A role for E1A in E4 promoter activation has been reported (39), although our results show that, at a high MOI, AdTyrΔ24 expresses E4orf3 at wild-type levels in SKOV3.ip1 cells, suggesting that targeted expression of E1A is not sufficient to restrict E4 expression. Similar results were reported by others but varied among cell lines (31, 32). Our results show improved cell type specificity of DNA replication and cell killing for Ad2xTyr in submerged cultures (Figs. 3 and 4) and organotypic cultures (Figs. 5–7).

''Δ24'' was designed to target tumor cells deficient in pRb. This mutation contains a deletion of 24 bp within conserved region 2 (CR2) of the E1A gene, preventing the mutant protein from binding to and inactivating pRb (8). Replication selectivity for tumor or proliferating cells has been reported for this mutant in certain cell lines (8, 9). The frequent inactivation of pRb in melanomas suggests that it may be beneficial to incorporate the Δ24 mutation into CRAds targeted to melanoma. However, we observed that the efficacy of DNA replication, virus particle production, and cell killing in melanoma cell lines for AdΔ24TyrE4 is identical to a matching virus with a wild-type E1A gene (D. M. Nettelbeck and A. A. Rivera, unpublished observation). Similarly, we reported identical cytotoxicity for AdTyrΔ24 and AdTyrwt, the latter containing wild-type E1A in melanoma cells (21). Thus, we can rule out the possibility that the Δ24 mutation leads to a reduced CRAd efficacy in melanoma cells relative to matching viruses that contain wild-type E1A. Interestingly, AdΔ24 also replicates as well as if not more efficiently than the Adwt in PHK raft cultures (17). However, we were able to limit this enhanced cytotoxicity attributed to Δ24 to melanoma cells by using targeted expression of E1A or E1A and E4 within CRAds (Figs. 6–8; to be discussed later).

A valuable lesson from this study is the importance of developing appropriate CRAd testing systems that simulate the native tissues *in vivo*. In our previous studies of Adwt or a CRAd targeted to genital lesions harboring HPV, we employed organotypic cultures of PHKs or keratinocytes transduced with HPV oncogenes. These cultures closely resemble native skin or HPV-induced lesions and reveal intriguing growth properties of wild-type and mutant adenoviruses in these tissues (16, 17). In the present

study, we extended this culture technique to melanoma cells and to mixed cultures of melanoma cell lines and PHKs (Figs. 5–8). Our observations demonstrate that properties of CRAds observed in submerged cell lines do not always reflect those in the organotypic culture setting of the same cells. In particular, cell killing by AdΔ24TyrE4 was at least as efficient as Adwt of both melanoma cells and PHKs (Figs. 5 and 6), a result not expected from studies in submerged cultures of nonmelanoma cells in which this CRAd was attenuated (Figs. 2 and 3). We attribute these findings to a difference in promoter activities in the two types of cell culture methods. One key example is that we have shown previously that CAR expression and viral promoter activities differ between raft cultures and submerged cultures of PHKs (16, 17, 26, 40; data not shown). Together with our present observation, these results indicate that organotypic raft cultures are a biologically valid format for CRAd analysis. Of note, the application of raft cultures might also be extended to analysis of CRAds targeted to other carcinomas, especially epithelium-associated diseases such as breast cancer and head and neck cancer.

AdTyrΔ24 and Ad2xTyr were dramatically attenuated relative to Adwt and AdΔ24TyrE4 in the skin model (Fig. 6) while exhibiting melanoma selectivity in the organotypic model of mixed cell types (Fig. 8). In these models, Ad2xTyr was superior to AdTyrΔ24 in cell type selectivity. Infection with 30 vp/cell of Ad2xTyr efficiently killed Mel624 in raft cultures, whereas a stratified tissue remained in organotypic cultures of PHKs even after infection with this virus at a 10-fold higher MOI. AdTyrΔ24 and Ad2xTyr were also attenuated relative to Adwt in melanoma cells in submerged cultures (Figs. 2B and 3; data not shown) and raft cultures (Fig. 5). However, attenuation was substantially more pronounced in submerged cultures of nonmelanoma cells (Figs. 2B and 3; data not shown) and PHK raft cultures (Fig. 6) proving cell type selectivity. These observations might reflect that the wild-type level of E1A is critical for efficient adenoviral replication and high virus titers because it activates early viral promoters necessary for viral DNA replication. We have shown previously that hTyr2E/P resulted in somewhat lower expression of E1A compared with Adwt (21). Alternatively, the distinct kinetics of E1A expression mediated by a constitutive promoter such as hTyr2E/P or the insertion of heterologous sequences in the E1 promoter region *per se* might have influenced the infectious program and thereby affect the efficiency of viral replication.

The remarkable specificity profile of Ad2xTyr establishes a platform for further CRAd development aimed at increased efficacy [*e.g.*, by increased viral spread (41, 42), incorporation of therapeutic genes (11), or a modification in viral tropism (43, 44)].

In summary, Ad2xTyr is superior to AdTyrΔ24 and AdΔ24TyrE4. (*1*) The double mutant possesses an enhanced selectivity of viral replication for melanoma cells due to a synergy between the single mutants. (*2*) This cell type selectivity is translated into a significantly reduced CPE in nonmelanoma cells, such as normal PHKs, attributed to a restricted expression of E1A and E4 and hence an impaired viral propagation. (*3*) The double mutation should feature a dramatically reduced risk of reversion to Adwt relative to single mutant. However, this enhanced cell type specificity comes at the expense of a less virulent virus. Despite this compromise, our investigation has demonstrated that the difference between the susceptibility by melanoma cells and the relative resistance of PHKs to this CRAd may comprise a window of opportunity for future clinical application. Finally, raft cultures derived from tumor cells and normal cells have great potential as three-dimensional organotypic models for validating CRAd efficacy and selectivity.

Acknowledgments

We thank Drs. Francisco Noya, Janet Price, and Jeffrey Schlom for cell lines and primary cells, Drs. Joel Glasgow, Lualhati Harkins, Ruben Hernandez-Alcoceba, Gary Ketner, Lisa Rivera, Theresa Strong, Kaori Suzuki, Andrea Volk, and Masato Yamamoto for their valuable contributions, and Ge Jin for embedding and sectioning the organotypic raft cultures.

References

1. Kirn DH, McCormick F. Replicating viruses as selective cancer therapeutics. Mol Med Today, 1996;2:519–27.

2. Nemunaitis J, Edelman J. Selectively replicating viral vectors. Cancer Gene Ther, 2002;9:987–1000.

3. Zhang WW. Development and application of adenoviral vectors for gene therapy of cancer. Cancer Gene Ther, 1999;6:113–38.

4. Reid T, Warren R, Kirn D. Intravascular adenoviral agents in cancer patients: lessons from clinical trials. Cancer Gene Ther, 2002;9:979–86.

5. Alemany R, Balagué C, Curiel DT. Replicative adenoviruses for cancer therapy. Nat Biotechnol, 2000;18:723–7.

6. Curiel DT. The development of conditionally replicative adenoviruses for cancer therapy. Clin Cancer Res, 2000;6:3395–9.

7. Bischoff JR, Kirn DH, Williams A, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science, 1996; 274:373–6.

8. Fueyo J, Gomez MC, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo.* Oncogene, 2000;19:2–12.

9. Heise C, Hermiston T, Johnson L, et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med, 2000;6:1134–9.

10. Nettelbeck DM, Jerome V, Muller R. Gene therapy: designer promoters for tumor targeting. Trends Genet, 2000;16:174–81.

11. Hermiston TW, Kuhn I. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. Cancer Gene Ther, 2002;9:1022–35.

12. Ahmed A, Thompson J, Emiliusen L, et al. A conditionally replicating adenovirus targeted to tumor cells through activated RAS/P-MAPK-selective mRNA stabilization. Nat Biotechnol, 2003;21:771–7.

13. Bernt K, Liang M, Ye X, et al. A new type of adenovirus vector that utilizes homologous recombination to achieve tumor-specific replication. J Virol, 2002;76:10994–1002.

14. Chow LT, Broker TR. *In vitro* experimental systems for HPV: epithelial raft cultures for investigations of viral reproduction and pathogenesis and for genetic analyses of viral proteins and regulatory sequences. Clin Dermatol, 1997;15:217–27.

15. Hutchin ME, Pickles RJ, Yarbrough WG. Efficiency of adenovirus-mediated gene transfer to oropharyngeal epithelial cells correlates with cellular differentiation and human coxsackie and adenovirus receptor expression. Hum Gene Ther, 2000;11:2365–75.

16. Noya F, Balagué C, Banerjee NS, Curiel DT, Broker TR, Chow LT. Activation of adenovirus early promoters and lytic phase in differentiated strata of organotypic cultures of human keratinocytes. J Virol, 2003;77: 6533–40.

17. Balagué C, Noya F, Alemany R, Chow LT, Curiel DT. Human

papillomavirus e6e7-mediated adenovirus cell killing: selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes. J Virol, 2001;75:7602 – 11.

18. Meier F, Nesbit M, Hsu MY, et al. Human melanoma progression in skin reconstructs: biological significance of bFGF. Am J Pathol, 2000; 156:193 – 200.

19. Johnson TM, Smith JW 2nd, Nelson BR, Chang A. Current therapy for cutaneous melanoma. J Am Acad Dermatol, 1995;32:689 – 707; quiz 708 – 9.

20. Helmbach H, Rossmann E, Kern MA, Schadendorf D. Drug-resistance in human melanoma. Int J Cancer, 2001;93:617 – 22.

21. Nettelbeck DM, Rivera AA, Balagué C, Alemany R, Curiel DT. 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 – 70.

22. Wilson JL, Dollard SC, Chow LT, Broker TR. Epithelial-specific gene expression during differentiation of stratified primary human keratinocyte cultures. Cell Growth & Differ, 1992;3:471 – 83.

23. Reynolds PN, Nicklin SA, Kaliberova L, et al. Combined transductional and transcriptional targeting improves the specificity of transgene expression *in vivo*. Nat Biotechnol, 2001;19:838 – 42.

24. Kanerva A, Wang M, Bauerschmitz GJ, et al. Gene transfer to ovarian cancer *versus* normal tissues with fiber-modified adenoviruses. Mol Ther, 2002;5:695 – 704.

25. Cheng S, Schmidt-Grimminger DC, Murant T, Broker TR, Chow LT. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev, 1995;9:2335 – 49.

26. Parker JN, Zhao W, Askins KJ, Broker TR, Chow LT. Mutational analyses of differentiation-dependent human papillomavirus type 18 enhancer elements in epithelial raft cultures of neonatal foreskin keratinocytes. Cell Growth & Differ, 1997;8:751 – 62.

27. Nettelbeck DM, Jerome V, Muller R. A dual specificity promoter system combining cell cycle-regulated tissue-specific transcriptional control. Gene Ther, 1999;6:1276 – 81.

28. Siders WM, Halloran PJ, Fenton RG. Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. Cancer Res, 1996;56:5638 – 46.

29. Li Y, Yu DC, Chen Y, et al. A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin. Cancer Res, 2001;61:6428 – 36.

30. Zhang J, Ramesh N, Chen Y, et al. Identification of human uroplakin II promoter and its use in the construction of CG8840, a urothelium-specific adenovirus variant that eliminates established bladder tumors in combination with docetaxel. Cancer Res, 2002;62: 3743 – 50.

31. Brunori M, Malerba M, Kashiwazaki H, Iggo R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. J Virol, 2001;75:2857 – 65.

32. Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumor cells with constitutive activation of the wnt signaling pathway. Gene Ther, 2002;9: 270 – 81.

33. Savontaus MJ, Sauter BV, Huang TG, Woo SL. Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells. Gene Ther, 2002;9:972 – 9.

34. 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 – 504.

35. Hernandez-Alcoceba R, Pihalja M, Wicha MS, Clarke MF. A novel, conditionally replicative adenovirus for the treatment of breast cancer that allows controlled replication of E1a-deleted adenoviral vectors. Hum Gene Ther, 2000;11:2009 – 24.

36. Hernandez-Alcoceba R, Pihalja M, Qian D, Clarke MF. New oncolytic adenoviruses with hypoxia- and estrogen receptor-regulated replication. Hum Gene Ther, 2002;13:1737 – 50.

37. Johnson L, Shen A, Boyle L, et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. Cancer Cell, 2002;1:325 – 37.

38. Ji L, Bouvet M, Price RE, Roth JA, Fang B. Reduced toxicity, attenuated immunogenicity and efficient mediation of human p53 gene expression *in vivo* by an adenovirus vector with deleted E1-E3 and inactivated E4 by GAL4-TATA promoter replacement. Gene Ther, 1999; 6:393 – 402.

39. Berk AJ. Adenovirus promoters and E1A transactivation. Annu Rev Genet, 1986;20:45 – 79.

40. Zhao W, Chow LT, Broker TR. Transcription activities of human papillomavirus type 11 E6 promoter-proximal elements in raft and submerged cultures of foreskin keratinocytes. J Virol, 1997;71:8832 – 40.

41. Sauthoff H, Heitner S, Rom WN, Hay JG. Deletion of the adenoviral E1b 19kD gene enhances tumor cell killing of a replicating adenoviral vector. Human Gene Ther, 2000;11:379 – 88.

42. Sauthoff H, Pipiya T, Heitner S, Chen S, Norman RG, Rom WN, Hay JG. Late expression of p53 from a replicating adenovirus improves tumor cell killing and is more tumor cell specific than expression of the adenoviral death protein. Human Gene Ther, 2002;13:1859 – 71.

43. Barnett BG, Crews CJ, Douglas JT. Targeted adenoviral vectors. Biochim Biophys Acta, 2002;1575:1 – 14.

44. Krasnykh VN, Douglas JT, van Beusechem VW. Genetic targeting of adenoviral vectors. Mol Ther, 2000;1:391 – 405.

Molecular Cancer Therapeutics

Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures

N. Sanjib Banerjee, Angel A. Rivera, Minghui Wang, et al.

Mol Cancer Ther 2004;3:437-449.