Tissue-Specific Promoters Active in CD44$^+$CD24$^{-/low}$ Breast Cancer Cells

Gerd J. Bauerschmitz,[1,2,8] Tuuli Ranki,[1,2] Lotta Kangasniemi,[1,2] Camilla Ribacka,[1,2] Minna Eriksson,[1,2] Marius Porten,[8] Isabell Herrmann,[8] Ari Ristimäki,[2,3,4] Pekka Virkkunen,[5] Maija Tarkkanen,[6] Tanja Hakkarainen,[1,2] Anna Kanerva,[1,2,7] Daniel Rein,[8] Sari Pesonen,[1,2] and Akseli Hemminki[1,2]

[1]Cancer Gene Therapy Group, Molecular Cancer Biology Program and Transplantation Laboratory, University of Helsinki, [2]HUSLAB, [3]Department of Pathology, Helsinki University Central Hospital, [4]Genome Scale Biology Program, Biomedicum Helsinki, [5]Medical Imaging Center, [6]Department of Oncology, and [7]Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland; and [8]Department of Obstetrics and Gynecology, Duesseldorf University Medical Center, Duesseldorf, Germany

Abstract

It has been proposed that human tumors contain stem cells that have a central role in tumor initiation and posttreatment relapse. Putative breast cancer stem cells may reside in the CD44$^+$CD24$^{-/low}$ population. Oncolytic adenoviruses are attractive for killing of these cells because they enter through infection and are therefore not susceptible to active and passive mechanisms that render stem cells resistant to many drugs. Although adenoviruses have been quite safe in cancer trials, preclinical work suggests that toxicity may eventually be possible with more active agents. Therefore, restriction of virus replication to target tissues with tissues-specific promoters is appealing for improving safety and can be achieved without loss of efficacy. We extracted CD44$^+$CD24$^{-/low}$ cells from pleural effusions of breast cancer patients and found that modification of adenovirus type 5 tropism with the serotype 3 knob increased gene delivery to CD44$^+$CD24$^{-/low}$ cells. α-Lactalbumin, cyclo-oxygenase 2, telomerase, and multidrug resistance protein promoters were studied for activity in CD44$^+$CD24$^{-/low}$ cells, and a panel of oncolytic viruses was subsequently constructed. Each virus featured 5/3 chimerism of the fiber and a promoter controlling expression of E1A, which was also deleted in the Rb binding domain for additional tumor selectivity. Cell killing assays identified Ad5/3-cox2L-d24 and Ad5/3-mdr-d24 as the most active agents, and these viruses were able to completely eradicate CD44$^+$CD24$^{-/low}$ cells *in vitro*. *In vivo*, these viruses had significant antitumor activity in CD44$^+$CD24$^{-/low}$–derived tumors. These findings may have relevance for elimination of cancer stem cells in humans. [Cancer Res 2008;68(14):5533–9]

Introduction

Decades ago, it was proposed that aberrant stem cells might underlie the heterogeneous nature of many advanced tumors. However, it is only recently that this theory has gained support from molecular evidence. Nonetheless, because cancer stem cells have not yet been fully defined, it cannot conclusively be proven that they exist, and there are also competitive hypotheses (reversible epidermal to mesenchymal transition, mutator phenotypes, etc.), which might explain some of the puzzling features of certain tumor cell populations. With regard to breast cancer, the cancer stem cell hypothesis is supported by the finding that CD44$^+$CD24$^{-/low}$ cells, frequently found in primary tumors and metastases, exhibit stem cell characteristics, including self-renewal and differentiation along various mammary epithelial lineages (1–3). A low number of CD44$^+$CD24$^{-/low}$ cells, as opposed to unsorted cells, is sufficient for initiation of tumors in mice (4).

Because of slow turnover and the ability for expelling antitumor drugs, putative cancer stem cells are resistant to many conventional cancer therapies (5). Therefore, they may have an important role in relapse after treatment and might therefore be causative of the incurable nature of many advanced solid tumors including metastatic breast cancer. Because most antitumor agents have been approved based on tumor response, agents preferentially active on cancer stem cells (which form a minority of the bulk of the tumor) may have been missed.

Viruses capable of selectively killing tumor cells, including oncolytic adenoviruses, enter cells through infection and kill both proliferating and quiescent cells. Such viruses are rendered replication deficient in normal cells by engineered genetic changes that are transcomplemented in tumor cells. One useful approach in this regard is utilization of tissue or tumor-specific promoters (TSP), which are activated in target cells, whereas nontumor cells are spared (6).

As there are no previous reports on which promoters might be useful in the context of cancer stem cells, we constructed viruses featuring the α-lactalbumin (ala; ref. 7), cyclo-oxygenase 2 (Cox-2; ref. 8), telomerase (hTERT; ref. 9), and multidrug resistance (mdr; ref. 10) promoters. Given that many stem cell types express low levels of the rate-limiting coxsackie-adenovirus receptor (11–14), we studied the utility of viral capsid modification for enhanced delivery. Then, we constructed the respective capsid modified, promoter-controlled oncolytic adenoviruses, and investigated their utility for killing CD44$^+$CD24$^{-/low}$ breast tumor cells *in vitro* and *in vivo*.

Materials and Methods

Cells. Pleural effusions were obtained with ethics committee–approved informed consent and washed with DMEM/F12 supplemented with 10ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 5 μg/mL insulin, and 0.4% bovine serum albumin (all from Sigma). All three patients (Fig. 2*A–C* corresponds to patients PL11-13) had metastatic ductal breast cancer. PL11: 58 y, grade 1, ER+, PR+, Her2− (CISH); PL12: 59 y,

Note: G.J. Bauerschmitz, T. Ranki, and L. Kangasniemi contributed equally to this work.

Requests for reprints: Akseli Hemminki, Biomedicum, Haartmaninkatu 8, 00014 University of Helsinki, Helsinki, Finland. Phone: 358-9-1912-5464; Fax: 11-358-9-1912-5155; E-mail: akseli.hemminki@helsinki.fi.

doi:10.1158/0008-5472.CAN-07-5288

grade 3, ER+, PR−, Her2−. PL13: 85 y, grade 3, ER+, PR+, Her2−. JIMT-1 cells were cultured as reported (15). Cells were sorted with FITC-labeled anti-CD44 and phycoerythrin-labeled anti-CD24 antibodies (BD PharMingen), collected with magnetic beads (Miltenyi Biotech). Cells (911, 293, and A549) are from American Type Culture Collection (ATCC).

Viruses. Ad5/3ala-d24, Ad5/3mdr-E1, and Ad5/3mdr-d24 were constructed by cutting promoter sequences from pGL3-ALA (7) and pM3mdr-p-hTNF (10) into pSE1 and pSE1d24 (16), from which the *E1* promoter was removed. The viral backbone was rescued in 911 cells with PacI-digested pTU-5/3 (17). For construction of Ad5/3-hTERT-Δgp, the hTERT was cloned into pSE1 (16), resulting in pShTERTE1. pAd5/3-hTERT-E1-Δgp was generated by homologous recombination in BJ5183 cells using PmeI-digested pShTERTE1 and SrfI-linearized pAdEasy-1.5/3-Δgp (serotype 3 knob, a 24-bp deletion in *E1A* and a 965-bp *6.7K/gp19K* deletion in *E3A*) for transfection into 911 cells. Propagation of these viruses and of Ad5/3-d24 (14) and Ad5/3-Cox2L-D24 (12) was performed on A549 cells.

The same promoter sequences were cloned to replace cytomegalovirus (CMV) in pShuttle-CMV (AdEasy; QBiogene) and *luciferase* was cut from pGL3-Basic (Promega) for homologous recombination with pAdEasy. Wild-type adenovirus (Ad5wt) is from ATCC. Ad5luc1, Ad5lucRGD, and Ad5/3luc1 have been reported (12, 13). *E1*-deleted viruses were propagated on 293 cells (Microbix). All viruses were purified on double CsCl gradients using standard methods. The *E1* and *E3* regions, fiber, and TSPs were checked with PCR followed by sequencing (11, 16). The viruses were titered for viral particles (VP) at 260 nm. Functional titer was determined with plaque assay with an initial overnight infection of 293 cells.

Luciferase assay. Cell lines were plated, infected the next day, and washed once. After 24 h, the medium was removed, cells were lysed with 200 μL of Reporter lysis buffer (Promega), and freeze thawed once followed by luciferase assay (Reporter Lysis Buffer; Promega). Standardization was accomplished by comparing to CMV (set as 100%).

Gene expression analysis. Messenger RNA from sorted $CD44^+CD24^{low/-}$ JIMT-1 cells was isolated using The RNeasy Mini RNA extraction kit (Qiagen). Expression was analyzed with the OneStep reverse transcription-PCR kit (Qiagen). Primers were as follows: ala, 5′-GGCCAAGCAATTCACAA-AAT-3′ (forward) and 5′-CCAAGGACAGCAGACACTCA-3′ (reverse); cox-2, 5′-TCTGGTGCCTGGTCTGATGA-3′ (forward) and 5′-GGTCAATGGAGGC-CTGTGAT-3′ (reverse); hTERT, 5′-AACGTTCCGCAGAGAAAAGA-3′ (forward) and 5′-GAGGAGCTCTGCTCGATGAC-3′ (reverse); mdr, 5′-GACT-GAGCCTGGAGGTGAAG-3′ (forward) and 5′-CCACCAGAGAGCT-GAGTTCC-3′ (reverse); and β-actin, 5′-AAACTGGAACGGTGAAGGTG-3′ (forward) and 5′-TCAAGTTGGGGGACAAAAAG-3′ (reverse).

Oncolysis assay. Cell lines were infected for 1 h and incubated in medium with 5% fetal bovine serum, half of which was changed every other day. Cells were checked daily and when the most oncolytic virus seemed to have killed most of the cells at 1 VP per cell, MTS assay (CellTiter96 Aqueous One Solution Reagent; Promega) was performed.

***In vivo* analyses.** Sorted JIMT-1 cells (2×10^6) were injected into the topmost mammary fat pads of nude mice (18). Mice were injected with 1 mg/kg Estradurin (Pfizer) every 3 wk as reported (1). Intratumoral injections were performed with 10^9 VP thrice weekly for 5 wk. Tumor volume = length × width^2 × 0.5. Animal experiments were approved by the Provincial Government of Southern Finland.

Flow cytometry. Expression of CD44 and CD24 in tumors (n = 7 per group) was measured by flow cytometry after 17 d of treatment. Briefly, tumors were dissociated with collagenase (Blend type F; Sigma) overnight in +4°C. Cells (2×10^6) from each tumor were labeled with antibodies as above and samples were analyzed with FACSaria (Becton Dickinson).

Statistical analysis. The F-test was performed to see if there were differences between the oncolytic potency of the viruses *in vitro*. If there was unequal distribution of the results, a two-sided Student's t test was used to assess significance, defined as a two-sided value of $P < 0.05$. *In vivo*, a nonparametric change-point test was used to determine a systematic change in the pattern of observations as opposed to chance. Proc Mixed (SAS v.6.12; SAS Institute) was used to examine the effects of group and time on tumor growth. Pairwise comparisons were performed.

Results

Breast cancer cells were sorted for CD44 and CD24 and infected by viruses featuring TSPs (Fig. 1*A*). Mdr showed the strongest activity with 17.8% or 6% of the highly active but nonselective CMV promoter, respectively, in JIMT-1 and fresh patient cells. hTERT also gave high activity at 3.1% and 12.8% of CMV, whereas Cox-2 was also active at 5.2% and 7.9%, respectively. Expression levels of ala were 0.4% and 1.8% of CMV and seem too low for successful control of oncolytic adenoviruses. RT-PCR analysis confirmed expression of genes that showed high luciferase expression (Fig. 1*A, insert*). However, correlation was not perfect between the assays suggesting that factors other than promoter activity may play a role in mRNA expression (e.g., mRNA stability factors present in untranslated regions).

For comparison, CD24+ JIMT-1 cells were analyzed for TSP expression (Fig. 1*A, center*). The main difference was that mdr was expressed to lower and ala to higher degree than in the $CD44^+CD24^{-/low}$ population.

Capsid modified luciferase expressing viruses were used to investigate if gene transfer to $CD44^+CD24^{-/low}$ or CD24+ cells could be improved (Fig. 1*B*). 5/3 modification seemed the optimal capsid configuration as it achieved 10- and 100-fold higher gene transfer to $CD44^+CD24^{-/low}$ and CD24+ cells, respectively. These results prompted us to construct the respective oncolytic adenoviruses featuring the respective TSPs and incorporating the 5/3 capsid (Fig. 1*C*). In many cases, an Rb binding site deletion was included as our previous data suggests that this can improve selectivity without loss of efficacy (12).

When $CD44^+CD24^{-/low}$ cells fresh from pleural effusions were infected, we found that 5 of 3 modified viruses were more oncolytic than the wild-type Ad5 control (Fig. 2). In 2 of 3 samples, Ad5/3-mdr-Δ24 was most oncolytic, with Ad5/3-hTERT-Δgp and Ad5/3-Cox2L-Δ24 closely following. Impressively, despite including a TSP, these viruses were sometimes even more potent than the highly active Ad5/3-Δ24.

Adding the Rb binding site deletion to Ad5/3-mdr-E1A to make Ad5/3-mdr-Δ24 did not reduce the activity of the virus but actually increased potency. As expected from the luciferase data, the ala promoter–driven oncolytic virus did not show oncolytic activity. $CD44^+CD24^{-/low}$ cells fresh from pleural effusions did not propagate actively *in vitro*, which may support their "stemness". Nevertheless, this precluded analysis of all viruses on all samples. For experiments that required large numbers of cells, the JIMT-1 explant was used (Figs. 3–4).

To look at the kinetics of cell killing between the different viruses, JIMT-1 cells were infected and daily cell viability assays were performed (Fig. 3*A*). Ad5/3-mdr-Δ24 and Ad5/3-hTERT-Δgp were the most effective TSP viruses and similar in the efficacy with the highly active Ad5/3Δ24, included as a positive control. Because probably not all $CD44^+CD24^{-/low}$ cells are "stem" cells (progenitors may be included), it is important to note that viruses were able to kill 100% of cells, therefore including also the actual "cancer stem cells." As an alternative method for analyzing cell killing, trypan blue assays were performed as a function of time (Fig. 3*B*), with results quite similar to the MTS assay.

To analyze the effect of the viruses on $CD44^+CD24^{-/low}$ cells *in vivo*, tumors were allowed to establish, followed by intratumoral virus or mock injections every other day. In histologic analysis of mock-treated tumors, central necrosis was present in 20% of the tumor section, whereas the remainder seemed healthy. In Ad5/

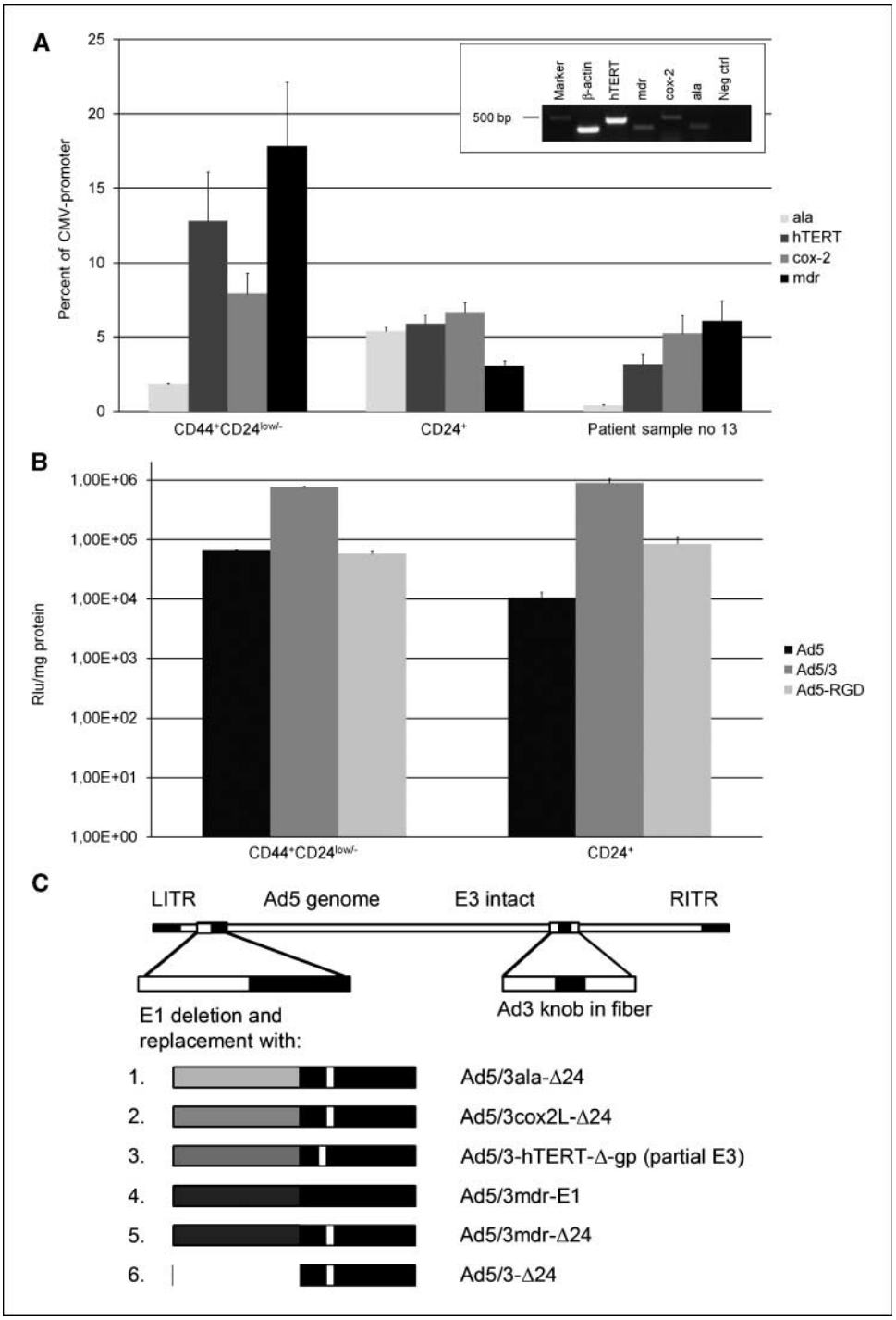

Figure 1. *A,* patient sample no 13 (*right*) and JIMT-1 pleural effusion explant (*left*) were sorted for CD24-negative and CD44-positive cells and seeded at 25,000 cells per well on 24-well plates. Twenty-four hours after infection with 5 VP per cell of TSP-driven luciferase-expressing viruses, cells were lysed and luciferase activity was determined. Results are presented as relative values of TSP-driven adenoviruses compared with CMV-driven virus. For comparison, CD24+ JIMT-1 cells were analyzed for TSP expression (*center*). *Insert,* RT-PCR analysis of $CD44^+CD24^{low/-}$ JIMT-1 cells for β-actin (350 bp), hTERT (462 bp), mdr (373 bp), cox-2 (482 bp), and ala (393 bp). All were positive except the negative control (*neg ctrl*). *B,* $CD44^+CD24^{low/-}$ and $CD24^+$ JIMT-1 cells were infected with 500 VP per cell of Ad5, a virus featuring serotype 5/3 chimeric capsid or a RGD-4C modified capsid. Twenty-four hours after infection, cells were lysed and luciferase activity was determined and normalized to the cellular protein content. *Columns,* mean; *bars,* SE. *C,* oncolytic adenoviruses featuring the TSPs and 5/3 modification of the capsid were constructed. All viruses have intact E3 except for Ad5/3-hTERT-Δgp, which is deleted for *gp19K*. For additional tumor selectivity, Δ24 viruses have a 24-bp deletion in the constant region 2 of E1A, which renders them unable to bind Rb, and therefore, these viruses replicate selectively in p16-Rb pathway–deficient cells.

3-mdr-Δ24–treated tumors, 50% of the section was necrotic and also non-necrotic regions displayed condensed nuclei, suggesting initiation of cell death (data not shown).

To assess antitumor efficacy, $CD44^+CD24^{-/low}$–derived tumors were established and injected with oncolytic viruses, all of which resulted in significantly smaller tumor size versus mock (all $P < 0.001$; Fig. 4*A*). Ad5/3cox2LΔ24 and Ad5/3-mdr-Δ24 showed the greatest antitumor efficacy and Ad5/3-mdr-Δ24 was superior even to Ad5/3-Δ24, a highly active positive control ($P < 0.001$). Interestingly, despite promising activity *in vitro*, Ad5/3-hTERT-Δgp was less effective *in vivo* than Ad5/3-Δ24 ($P < 0.001$).

Putative stem cells would be predicted to divide asymmetrically; each division producing one new stem cell and one progenitor. Progenitors would then divide to produce differentiated tumor cells. To assess this in a preliminary manner, tumors were induced by injection of 100% $CD44^+CD24^{-/low}$ cells, and the proportion $CD44^+CD24^{-/low}$ cell was analyzed again when mice died or were killed. In mock-injected tumors, the proportion of $CD44^+CD24^{-/low}$ cells had decreased from 100% to 6.2% ($n = 2$; SD, 6.8). In Ad5/3cox2L-Δ24–injected tumors, the proportion reduced to 1.2% ($n = 1$), whereas in Ad5/3mdr-Δ24, Ad5/3-Δ24, and Ad5/3-hTERT-Δgp–injected tumors, the respective numbers were 3.8%

(n = 3; SD, 3.5), 1.2% (n = 3; SD, 0.4), and 1.6% (n = 1). The absolute number of $CD44^{+}CD24^{-/low}$ cells decreased from 2 million to a median of 669,000 (n = 8; SD, 1.2 million) in virus-injected tumors.

However, because the number of tumors available for analysis was limited by practical aspects (duration of analysis and freshness of tumor), and the time points of analysis were not standardized in the efficacy experiment, we performed another set of experiments in more rigorous conditions (Fig. 4*B* and *C*). Tumors were established and treated with mock, Ad5/3mdr-Δ24 (able to kill CD44+/CD24− cells *in vitro*; Figs. 2–3), or Ad5/3-ala-Δ24 (not able to kill CD44+/CD24− cells *in vitro*) for 17 days, followed by fluorescence-activated cell sorting (FACS; Fig. 4*B* and *C*). Interestingly, the proportion of $CD44^{+}CD24^{-/low}$ cells was nearly identical [3.1% (SD, 1.35) versus 2.6% (SD, 1.17) in Ad5/3mdr-Δ24

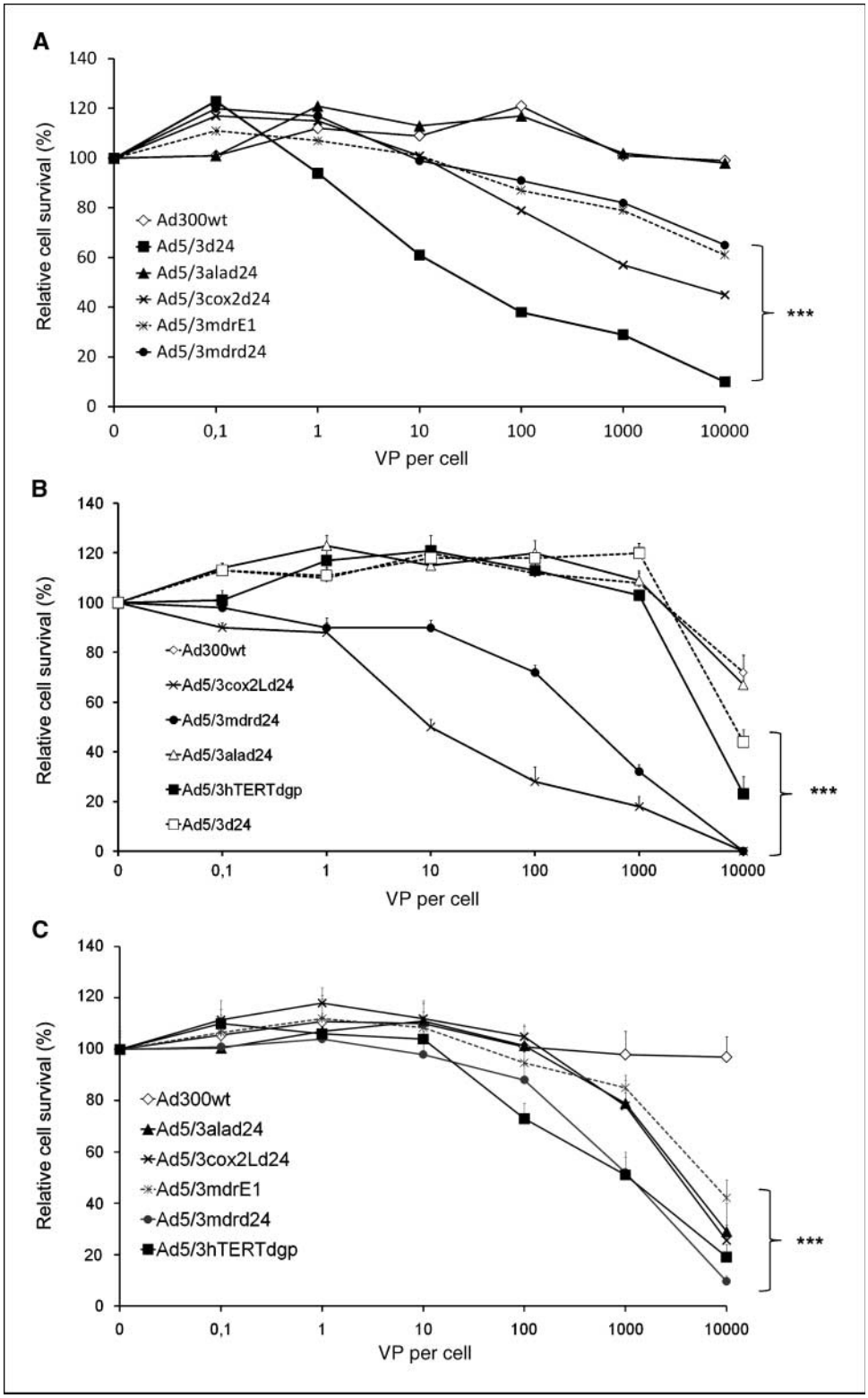

Figure 2. Primary breast cancer pleural effusion samples from 3 different patients (*A–C*) were sorted for CD24-negative and CD44-positive cells and seeded at 20,000 cells per well on 96-well plates. After infection with promoter-driven oncolytic viruses, cells were checked daily by light microscopy, and MTS cell viability assay was performed when the most potent virus had lysed virtually all cells with the second lowest viral concentration. Results are presented as relative survival compared with mock-infected cells. *Points,* mean; *bars,* SE. ***, $P < 0.001$ versus Ad5wt.

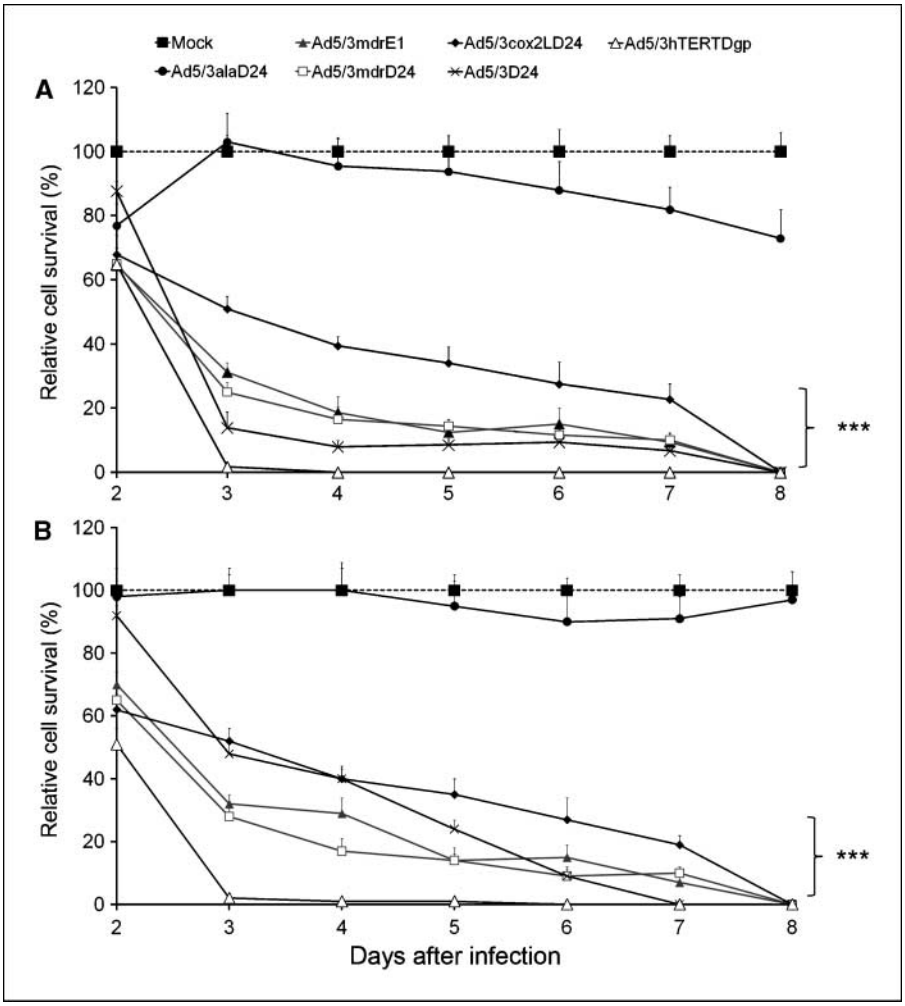

Figure 3. JIMT-1 pleural metastasis explant cells were sorted for CD24-negative and CD44-positive cells and seeded at 20,000 cells per well on 96-well plates. After infection with promoter-driven oncolytic viruses in triplicates, daily MTS (*A*) or trypan blue cell viability assays (*B*) were performed. Results are presented as relative survival compared with mock-infected cells. *Points,* mean; *bars,* SE. ***, $P < 0.001$ versus mock.

versus mock-treated groups, and 3.3% (SD, 0.27) versus 3.4% (SD, 0.53) in Ad5/3-ala-Δ24 versus mock-treated groups, respectively; Fig. 4*C*].

Importantly, because Ad5/3mdr-Δ24 reduced tumor size, it also reduced the total number of CD44+/CD24- cells (Fig. 4*A–C*). In contrast, Ad5/3-ala-Δ24 did not affect tumor size nor the proportion of CD44+/CD24− (Fig. 4*B* and *C*). In mock versus Ad5/3mdr-Δ24–treated groups, other cell populations had larger differences: 5.4% (SD, 4.72) versus 2.3% (SD, 0.97), 9.2% (SD, 6.75) versus 4.3% (SD, 1.37), 82.3% (SD, 11.3) versus 90.8% (SD, 3.35) for $CD44^{-/low}CD24^{+}$, $CD44^{+}CD24^{+}$, and $CD44^{-/low}CD24^{-/low}$, respectively. In mock versus Ad5/3-ala-Δ24–treated groups, the proportions of these cell populations were similar: 4.3% (SD, 1.93) versus 4.3% (SD, 1.07), 6.8% (SD, 1.07) versus 5.6% (SD, 1.44), 85.5% (SD, 2.88) versus 86.8% (SD, 2.54), respectively.

Discussion

Previous work suggests that cancer stem cells may underlie the relapsing nature of advanced cancer (1–4). Eradication of these cells seems daunting as they are resistant to chemotherapy and radiation (5). Nevertheless, oncolytic viruses may have potential in this regard, as they are not susceptible to ion pumps or lack of cell cycling. Although oncolytic adenoviruses have been quite safe in the dozens of trials, most of them have been performed with relatively attenuated early generation agents (19). A new generation of infectivity enhanced, armed, and highly effective viruses is rapidly emerging, and the first such viruses have already been tested in patients with good safety.[9] Nevertheless, preclinical data suggests that enhanced potency can sometimes result in toxicity, and therefore, improved control of virus replication may become clinically relevant (20).

TSPs may be a useful way to prevent expression of adenoviral *E1A* in nontarget tissues (6, 12, 16). Dual control may be particularly useful in the context of nontumor cells positive for the TSP. For example, normal tissue stem cells might express hTERT or mdr. Nevertheless, they would be expected to be intact in the Rb-p16 pathway, and therefore, Δ24-type viruses should not replicate in them.

Given the paucity of TSPs known to be active in putative cancer stem cells, we focused on a handful of promoters theoretically useful. Luciferase assays indicated that all of these promoters, except ala, were active in $CD44^{+}CD24^{-/low}$ cells. 5/3 chimerism was identified as the optimal capsid configuration, and oncolytic viruses were subsequently constructed (Fig. 1). The respective viruses were effective in killing $CD44^{+}CD24^{-/low}$ cells (Figs. 2–3). However, in the most stringent assay (Fig. 4), Ad5/3-mdr-Δ24 and Ad5/3-Cox2L-Δ24 emerged as the most promising agents, with

[9] A. Hemminki, personal communication.

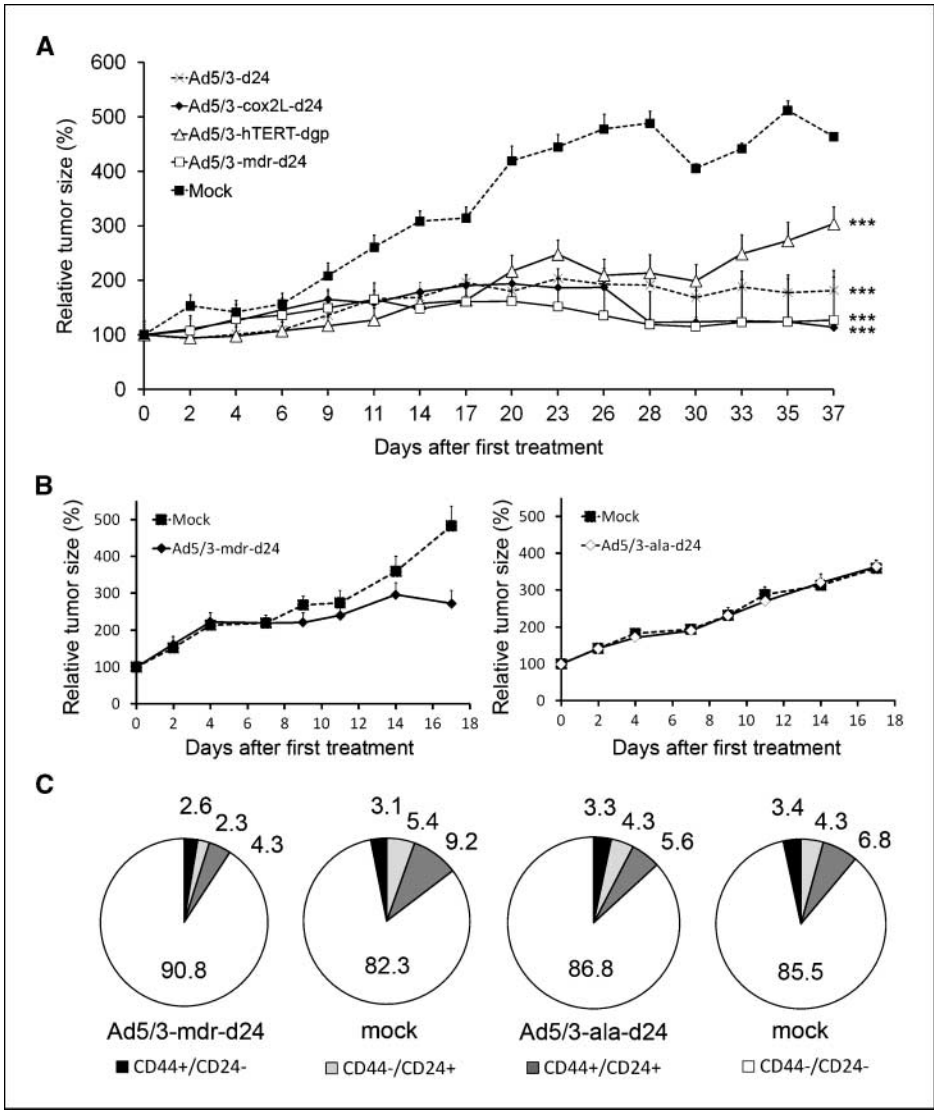

Figure 4. To assess antitumor efficacy, $CD44^+CD24^{-/low}$ cells were injected into mammary fat pads of nude mice and tumors were allowed to grow for 14 d, followed by intratumoral injection of viruses or mock every second day until all mock mice died (*A*). Data points represent relative tumor volume of eight tumors per group compared with mean tumor volume at first day of treatment. *Points,* mean; *bars,* SE. ***, $P < 0.001$ versus mock. For evaluation of the effect of the viruses on $CD44^+CD24^{-/low}$ cells, tumors were allowed to grow for 14 d, followed by intratumoral injection of virus or mock thrice weekly (*B*). Mock or Ad5/3-mdr-Δ24 (active in $CD44^+CD24^{-/low}$ cells) and mock or Ad5/3-ala-Δ24– treated tumors (not active in $CD44^+CD24^{-/low}$ cells; $n = 7$ per group) were collected for FACS analysis after 17 d of treatment (*C*). *Numbers,* percentage.

Ad5/3-mdr-Δ24 perhaps holding the advantage given slightly superior data on clinical specimens (Fig. 2). Nevertheless, Ad5/3-Cox2L-Δ24 also had activity in clinical samples, especially at lower doses, which may be promising with regard to *in vivo* application.

Importantly, *in vivo* data suggested that asymmetrical cell division does occur. Furthermore, sorting and FACS data from treated tumors supports *in vivo* killing of $CD44^+CD24^{-/low}$ cells by promoter controlled oncolytic viruses. Because virus-treated tumors were much smaller than mock-treated tumors, and had a similar or even slightly smaller proportion of $CD44^+CD24^{-/low}$ cells (Fig. 4*C*), it seems likely that viruses were able to kill $CD44^+CD24^{-/low}$ cells *in vivo*, similarly to what was seen *in vitro*. If viruses would only be able to kill differentiated tumor cells, the proportion of $CD44^+CD24^{-/low}$ cells would be higher, not lower, in virus versus mock-treated tumors. Nevertheless, neither tumors nor $CD44^+CD24^{-/low}$ cells were completely eradicated *in vivo* and, thus, work remains.

Finally, it was interesting that despite 100% $CD44^+CD24^{-/low}$ cells injected, the proportion found in tumors was between 2.6% and 3.8% with or without treatment and regardless of how long injections were performed. This suggests that the number of $CD44^+CD24^{-/low}$ cells may determine tumor size and each initiator cell is surrounded by a fixed number of differentiated cells. Taken together, these results suggest that mdr, hTERT, and Cox-2 promoters are active in $CD44^+CD24^{-/low}$ breast cancer cells. Also, oncolytic adenoviruses controlled by these promoters seem to be able to kill $CD44^+CD24^{-/low}$ cells. Viruses such as Ad5/3-mdr-Δ24 and Ad5/3-Cox2L-Δ24 may be appealing for testing in breast cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/13/2007; revised 5/6/2008; accepted 5/15/2008.

Grant support: European Union grants THERADPOX and APOTHERAPY, Helsinki University Central Hospital, Sigrid Juselius Foundation, Academy of Finland, Emil Aaltonen Foundation, Finnish Cancer Society, University of Helsinki, Helsinki Biomedical Graduate School, and the Finnish Cultural Foundation. Akseli Hemminki is K. Albin Johansson Research Professor of the Foundation for the Finnish Cancer Institute.

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.

We thank Eerika Karli and Aila Karioja-Kallio for superb assistance.

References

1. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983–8.
2. Kelly PN, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. Science 2007;317:337.
3. Hill RP, Perris R. "Destemming" cancer stem cells. J Natl Cancer Inst 2007;99:1435–40.
4. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 2003;3:895–902.
5. Jordan CT, Guzman ML, Noble M. Cancer stem cells. N Engl J Med 2006;355:1253–61.
6. Saukkonen K, Hemminki A. Tissue-specific promoters for cancer gene therapy. Expert Opin Biol Ther 2004;4:683–96.
7. Li X, Zhang J, Gao H, et al. Transcriptional targeting modalities in breast cancer gene therapy using adenovirus vectors controlled by α-lactalbumin promoter. Mol Cancer Ther 2005;4:1850–9.
8. Yamamoto M, Alemany R, Adachi Y, Grizzle WE, Curiel DT. Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. Mol Ther 2001;3:385–94.
9. Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res 1999;59:826–30.
10. Walther W, Wendt J, Stein U. Employment of the mdr1 promoter for the chemotherapy-inducible expression of therapeutic genes in cancer gene therapy. Gene Ther 1997;4:544–52.
11. Bauerschmitz GJ, Lam JT, Kanerva A, et al. Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res 2002;62:1266–70.
12. Bauerschmitz GJ, Guse K, Kanerva A, et al. Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells. Mol Ther 2006;14:164–74.
13. Kanerva A, Mikheeva GV, Krasnykh V, et al. Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. Clin Cancer Res 2002;8:275–80.
14. Kanerva A, Zinn KR, Chaudhuri TR, et al. Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. Mol Ther 2003;8:449–58.
15. Tanner M, Kapanen AI, Junttila T, et al. Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. Mol Cancer Ther 2004;3:1585–92.
16. Nettelbeck DM, Rivera AA, Balague 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.
17. Uil TG, Seki T, Dmitriev I, et al. Generation of an adenoviral vector containing an addition of a heterologous ligand to the serotype 3 fiber knob. Cancer Gene Ther 2003;10:121–4.
18. Eriksson M, Guse K, Bauerschmitz G, et al. oncolytic adenoviruses kill breast cancer initiating CD44(+) CD24(-/low) cells. Mol Ther 2007;15:2088–93.
19. Kanerva A, Raki M, Hemminki A. Gene therapy of gynecological diseases. Expert Opin Biol Ther 2007;7:1347–61.
20. Raki M, Kanerva A, Ristimaki A, et al. Combination of gemcitabine and Ad5/3-Δ24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. Gene Ther 2005;12:1198–205.