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## A heparan sulfate-targeted conditionally replicative adenovirus, Ad5.pk7- $\Delta$ 24, for the treatment of advanced breast cancer

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### Abstract

Conditionally replicating adenoviruses (CRADs) that replicate in tumor but less in normal cells are promising anticancer agents. A major determinant of their potency is their capacity for infecting target cells. The primary receptor for serotype 5 adenovirus (Ad5), the most widely used serotype in gene therapy, is the coxsackie-adenovirus receptor (CAR). CAR is expressed variably and often at low levels in various tumor types including advanced breast cancer. We generated a novel p16/retinoblastoma pathway-dependent CRAd, Ad5.pK7- $\Delta$ 24, with a polylysine motif in the fiber C-terminus, enabling CAR-independent binding to heparan sulfate proteoglycans (HSPG). Ad5.pK7- $\Delta$ 24 mediated effective oncolysis of all breast cancer cell lines tested. Further, we utilized noninvasive, fluorescent imaging for analysis of antitumor efficacy in an orthotopic model of advanced hormone refractory breast cancer. A therapeutic benefit was seen following both intratumoral and intravenous delivery. Murine biodistribution similar to Ad5, proven safe in trials, suggests feasibility of clinical safety testing. Interestingly, upregulation of CAR was seen in low-CAR M4A4-LM3 breast cancer cells *in vivo*, which resulted in better than expected efficacy also with an isogenic CRAd with an unmodified capsid. These results suggest utility of Ad5.pK7- $\Delta$ 24 and the orthotopic model for further translational studies.

### Keywords

adenovirus; breast cancer; oncolytic virus; heparan sulfate proteoglycans

### Introduction

A total of 20–85% of patients diagnosed with breast cancer, depending on the initial stage, tumor biology and adjuvant treatment, will develop metastatic disease, which features median survival of approximately 2 years.<sup>1</sup> Despite extensive research and advances in treatments, metastatic breast cancer remains essentially incurable and is the most common cause of cancer-related mortality in women. The disease is particularly difficult to treat when resistant to antiestrogen therapies, that is, becomes hormone refractory. Adenoviral

cancer gene therapy has been proposed to possess potential as an effective treatment alternative for disseminated disease refractory to available modalities. In clinical cancer trials, nonreplicating and replicating adenoviruses have been remarkably safe, with hundreds of patients treated without treatment-related mortality.<sup>2</sup> Further, recently completed randomized trials have validated the dramatic inherent potential of even early generation viruses.<sup>3-5</sup> However, in most trials, when faced with bulky disease, antitumor efficacy has been low owing to the limited dispersion of the therapeutic agent in advanced tumor masses.

In an attempt to improve transduction of solid tumors, conditionally replicating adenoviruses (CRAds) have been constructed. Their therapeutic effect is potentiated owing to replication and subsequent oncolysis of tumor cells followed by further infection by the progeny virions.<sup>6</sup> Type I CRAds utilize the similarity between the requirements of DNA viruses and carcinogenesis for inactivation of the major growth control pathways.<sup>7</sup> Inactivation of such functions from the virus genome retains the ability for replication in tumor but not normal tissue. Specifically, a useful prototype approach has been deletion of 24 bp in the constant region 2 (CR2) of the E1A gene, which results in a mutated E1A protein unable to bind the retinoblastoma (Rb) protein.<sup>8,9</sup> In quiescent normal cells, this binding is required for locking the cell cycle into S-phase for subsequent effective viral replication.<sup>10</sup> The selectivity of this mutation has been demonstrated previously.<sup>8,9</sup> The Rb/p16 pathway is mutated in a majority of human tumors including advanced breast cancers.<sup>11,12</sup> In contrast to E1B region mutations,<sup>13</sup> the CR2 deletion does not hinder replication in tumor cells.<sup>8,9</sup>

An issue concerning the use of adenoviruses for cancer therapy is the variable and often low expression of the primary receptor, coxsackie-adenovirus receptor (CAR), which has been reported for various tumor types,<sup>14</sup> including breast cancer.<sup>15,16</sup> Previously, it has been demonstrated that the oncolytic potency of CRAds is mostly related to their capability for entering target cells.<sup>17,18</sup> Strategies have been proposed to circumvent dependence on CAR by using CAR-independent entry pathways, including retargeting complexes and genetic capsid modifications.<sup>19</sup> With regard to genetic capsid modifications, an Arg-Gly-Asp (RGD) moiety has been introduced into the C-terminus<sup>20</sup> or the HI-loop of the fiber<sup>21</sup> to allow internalization via  $\alpha v\beta$ -integrins. Another useful approach has been utilization of non-CAR binding fibers for CAR-independent entry.<sup>22-24</sup>

Heparan sulfate proteoglycans (HSPGs) are common constituents of the extracellular matrix and have angiogenic and growth-promoting characteristics.<sup>25</sup> Further, HSPGs have been found highly expressed in advanced breast cancers.<sup>26-29</sup> Here, we incorporated an HSPG binding polylysine moiety into the C-terminus of a CR2 mutated (i.e. Rb/p16 pathway selective) CRAAd. We hypothesized that this might lead to effective transduction and subsequent oncolysis of advanced breast cancer cells.

## Results

### Ad5.pK7- $\Delta$ 24 displays efficient oncolysis of breast cancer cells in vitro

Seven lysines and a glycine-serine (GS)-linker sequence were incorporated in the C-terminus of the fiber of serotype 5 adenovirus (Ad5).pK7- $\Delta$ 24 enabling HSPG binding. As a control virus, we utilized an isogenic virus, Ad5 $\Delta$ 24E3+, which has a wild-type fiber (Figure 1). Oncolytic potency of Ad5.pK7- $\Delta$ 24 was analyzed by the mitochondrial activity-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Monolayers of MDA-MB-435, M4A4-LM3, MDA-MB-436, ZR-75-1, CAMA-1 and MCF-7 breast cancer cell lines were infected with Ad5.pK7- $\Delta$ 24, Ad5 $\Delta$ 24E3+ and a nonreplicating control virus, Ad5luc1 (Figure 2). In all but one cell line (MDA-MB-436), significantly higher cell killing efficacy was observed for Ad5.pK7- $\Delta$ 24 when compared to Ad5 $\Delta$ 24E3+. In MCF-7, Ad5.pK7- $\Delta$ 24 was significantly more effective

in all viral doses ( $P<0.05$ ,  $P<0.05$ ,  $P<0.01$  for doses 0.1, 1 and 10 virus particles (VP)/cell, respectively) than Ad5 $\Delta$ 24E3+. In MDA-MB-435 and M4A4-LM3, significantly more effective cell killing was observed with the highest dose ( $P<0.0000001$  and  $P<0.01$ , respectively). The difference was statistically significant on CAMA-1 cells with 1 VP/cell ( $P<0.01$ ) and in ZR-75-1 with doses 0.1 and 1 VP/cell ( $P<0.05$  and  $P<0.01$ , respectively). The E1-deleted control virus Ad5luc1 did not cause oncolysis in any of the cells tested (a significant difference versus Ad5.pK7- $\Delta$ 24 in all cell lines).

### **Efficacy of an intratumoral administration of Ad5.pK7- $\Delta$ 24 in an orthotopic murine model of advanced hormone refractory breast cancer**

M4A4-LM3 cells expressing green fluorescent protein (GFP) were injected to both left and right uppermost mammary fat pads of nude mice. These cells metastasize rapidly to local and distant lymph nodes whereas lung metastases occur later.<sup>30</sup> To establish advanced disease, the primary tumor was allowed to grow for 17 days before intratumoral injections of Ad5.pK7- $\Delta$ 24, Ad5 $\Delta$ 24E3+ or growth medium on 3 successive days. Antitumor response was monitored by noninvasive imaging of fluorescence emission and by measuring the tumor with calipers in three dimensions (Figures 3a-c, and 4). All Ad5.pK7- $\Delta$ 24-treated mice had significantly smaller tumors starting from day 9 after treatment when compared to the mock group (all  $P$ -values less than 0.01). One of the Ad5.pK7- $\Delta$ 24-treated mice showed a complete tumor regression of one tumor on day 20 after the treatment but relapsed and had to be killed on day 46. Another mouse from the Ad5.pK7- $\Delta$ 24-treated group also showed a complete tumor regression of one tumor and relapse did not occur.

Ad5 $\Delta$ 24E3+-treated mice showed significant tumor regression compared to the mock group starting on day 4 until the last day of the experiment (all  $P$ -values less than 0.05). All of these mice showed a complete tumor regression of both tumors and relapse occurred in only one tumor. Ad5 $\Delta$ 24E3+ was significantly more effective than Ad5.pK7- $\Delta$ 24 from day 4 to day 11 (all  $P$ -values  $<0.05$ ).

The survival of the mice was also monitored (data not shown). Mice ( $n = 4$ ) were killed when either tumor reached the size of 1 cm in any diameter. For Ad5.pK7- $\Delta$ 24-treated mice and mock mice, median survival was 55.0 and 27.0, respectively (difference not significant). All of the mock mice had large primary tumors in both mammary fat pads as opposed to the Ad5.pK7- $\Delta$ 24-treated mice, which frequently had a good response in one tumor whereas the other was distinctly smaller.

The difference between measurement of tumor size and photon emission is that only live tumor cells are expected to produce GFP, whereas stromal cells, cellular debris and extracellular matrix contribute to physical tumor size. Therefore, it was of interest to compare size and photon emission (Figure 3c). Good correlation was seen ( $P<0.01$ ), although there seemed to be a trend for more rapid response in the photon count, as indicated by a more rapidly declining curve (Figure 3a).

### **Comparison of transductional efficacy of the viruses and CAR/HSPG levels *in vitro* and *in vivo***

The discrepancy between *in vitro* and *in vivo* data on the efficacy of Ad5.pK7- $\Delta$ 24 versus Ad5 $\Delta$ 24E3+ urged us to study whether there is a difference in CAR and HSPG levels in M4A4-LM3 cells *in vitro* and in M4A4-LM3 xenografts *in vivo*. To study transduction, we performed gene transfer assays with viruses containing the luciferase gene. *In vitro*, M4A4-LM3 cells were infected with Ad5.pK7 (GL), Ad5 (GL) or no virus and gene transfer efficacy was assessed. Ad5.pK7 (GL) was over 300 times more effective in gene transfer

than Ad5 (GL) ( $P < 0.00001$ , Figure 5a). *In vivo*, however, there was no significant difference between Ad5.pK7 (GL) and Ad5 (GL) (Figure 5b).

To study CAR and HSPG expression in M4A4-LM3 cells and xenograft tumors, we performed flow cytometric analysis. CAR expression was significantly higher in tumor cells *in vivo* versus *in vitro*: over 98.3% ( $\pm 0.8\%$ ) were CAR positive *in vivo* compared to just 3.2% ( $\pm 2.5\%$ ) when the same cells were grown *in vitro* ( $P < 0.01$ ) (Figure 5c–f). No significant differences were seen in HSPG expression levels *in vitro* versus *in vivo* ( $83.2 \pm 15.2$  and  $78.7 \pm 7.1\%$  positive cells, respectively). As an additional control, we compared gene delivery with Ad5 to delivery with Ad5-RGD *in vitro* versus *in vivo*, and the same upregulation of the Ad5 receptor CAR was seen, whereas the receptors involved with Ad5-RGD seemed unaffected (not shown).

### Immunohistochemical staining and histology

Immunohistochemical staining of M4A4-LM3 xenograft tumors showed that HSPG staining was pronounced in blood vessel endothelial cells (see arrowheads in Figure 5g) but less pronounced in viable cancer tissue. In contrast, necrotic areas seemed to stain strongly (not shown). CAR was highly expressed in the cytoplasm of the healthy tumor tissue but not in the necrotic areas (Figure 5h), blood vessels nor the basement membrane. Tumors had large necrotic areas (shown as lighter color in Figure 5i). Blood vessels (see arrowhead in Figure 5j) rarely penetrated deep into the tumor and tumors were lined by a continuous basement membrane. Tumors were composed mostly of cancer cells and normal tissue was sparse (Figure 5i and j).

### Ad5.pK7- $\Delta$ 24 in a systemic treatment model of orthotopic hormone refractory advanced breast cancer

We allowed tumors to grow for 10 days for establishing advanced disease before intravenous injection of Ad5.pK7- $\Delta$ 24, Ad5 $\Delta$ 24E3+ or growth medium. Twenty-three days after the first injection, mice received another injection (Figure 6a). In general, antitumor efficacy was less dramatic than with intratumoral injection. However, 32 days after the first virus injection, Ad5.pK7- $\Delta$ 24-treated mice showed less GFP expression in the tumors when compared to both the mock-or Ad5 $\Delta$ 24E3+-treated mice ( $P < 0.05$ ). Despite a promising trend, there was no significant difference in survival between the groups. The median survivals were 27, 32 and 32 days in the mock, Ad5.pK7- $\Delta$ 24 and Ad5 $\Delta$ 24E3+ groups, respectively. The capsid modification did not seem to have a big impact on the biodistribution of the agent (Figure 6b).

### Discussion

Encouraging antitumoral efficacy and safety have been demonstrated in animal models with various CRAds. Nevertheless, this has not fully translated into similar clinical efficacy, which may be partly owing to poor transduction of a sufficient proportion of cells in solid tumor masses.<sup>31</sup> A major determinant of oncolytic efficacy is infectivity, and therefore infectivity enhancement has emerged as an important goal.

In this study, we added seven lysine residues (pK7) into the fiber C-terminus of a CRAd to create Ad5.pK7- $\Delta$ 24. HSPGs are expressed ubiquitously on cell surfaces and they may have a minor role in the binding of Ad2 and Ad5.<sup>32</sup> However, advanced breast cancers have been reported to express significantly higher levels of HSPGs than normal breast cells.<sup>26–29</sup> pK7 modification resulted in enhanced cell killing *in vitro* (Figure 2). Only in one cell line, MDA-MB-436, was the isogenic control CRAd Ad5 $\Delta$ 24E3+ superior to the pK7-modified counterpart, which may be a direct consequence of high CAR expression previously

reported for this cell line.<sup>15</sup> These data were well in accord with a previous paper where a polylysine modified CRAd was studied for therapy of glioma.<sup>33</sup>

To study the oncolytic efficacy of Ad5.pK7- $\Delta$ 24 in a situation that models the clinical situation as closely as possible, we utilized a murine model of advanced breast cancer. M4A4-LM3 cells were labeled with GFP to allow noninvasive imaging. These cells are not hormone sensitive, which is another characteristic of the putative clinical target population, as anti-estrogen therapies would have priority over experimental approaches. Implantation of M4A4-LM3 xenografts into mammary fat pads results in early metastasis to regional lymph nodes, with later spread to distant lymph nodes and lungs.

Locally administered Ad5.pK7- $\Delta$ 24 displayed significantly higher efficacy in comparison to the mock-treated group, but the isogenic control virus Ad5 $\Delta$ 24E3+ was even more effective. Oncolytic killing of tumor cells correlated closely with the reduction of fluorescent signal emitted from the tumors (Figure 3c). Importantly, photon emission was more sensitive and rapid in detecting antitumor response (Figure 3a and b). The tumors could be easily followed before and after treatment by noninvasive imaging. However, a slight drawback of GFP in noninvasive imaging is that organs, especially the liver, feature autofluorescence and that GFP is not surface-weighted. In other words, anything closer to the surface will appear brighter than structures deeper in the tissue.<sup>34</sup>

Interestingly, there seemed to be a discrepancy between the *in vitro* and *in vivo* efficacy of Ad5.pK7- $\Delta$ 24 versus Ad5 $\Delta$ 24E3+. *In vitro*, the former was more oncolytic, whereas *in vivo* the latter seemed almost as effective after intravenous (Figure 6) or even superior following local delivery (Figures 3a, b and 4). Generally, it has been assumed that adenovirus receptors are expressed to a similar degree *in vitro* and *in vivo*. Further, this has been corroborated in many reports demonstrating similar infectivity data *in vitro* and in orthotopic models.<sup>21,22,35-37</sup> Also, relatively good correlation has been seen between tumor cell lines grown two-dimensionally on plates or as three-dimensional spheroids.<sup>38-40</sup> With regard to breast cancer, this has not been studied previously. However, a recent report suggested that the level and location of CAR were different when the same breast cancer cells were grown on plates versus in a three-dimensional model system.<sup>41</sup> Furthermore, it was demonstrated with brain tumors that xenografts had higher CAR mRNA expression than the parental tumor cells.<sup>42</sup> Moreover, with lung cancer cells, it was suggested that CAR may be required for effective xenograft formation.<sup>43</sup>

Our results suggest that orthotopic M4A4-LM3 tumors feature a higher CAR/HSPG ratio than the same cells grown *in vitro* (Figure 5c-f). Therefore, the infectivity of cells *in vitro* is not always the whole truth with regard to gene transfer and oncolysis *in vivo*. Also, given the nature of HSPGs as important components of the extracellular matrix of tumors, it is possible that binding to HSPGs may in fact hinder access of the virus to cancer cells. Nevertheless, binding to basement membrane HSPG might allow the virus to retain localization in the vicinity of the tumor for a longer time, resulting in a biological extended release system. It is also noteworthy that with intratumoral inoculation, high viral concentrations ensue, and part of the dose may be delivered to peripheral or even necrotic areas of the tumor. Both phenomena might negate differences in the transduction efficacy. Nevertheless, differences in CAR/HSPG-mediated transduction may help to shed some light on the biology of these moieties and their feasibility as utilization for viral antitumor approaches.

Given the systemic nature of the disease, it would be useful if efficacy could be achieved via intravenous delivery. Therefore, we utilized this route in mice with established advanced M4A4-LM3 tumors but saw only limited antitumor activity with Ad5.pK7- $\Delta$ 24.

Optimization of dose and schedule may yield improved results. Interestingly, Ad5.pK7- $\Delta$ 24 seemed at least as effective as Ad5 $\Delta$ 24E3+ in this experiment. It is tantalizing to speculate that HSPG binding and subsequent extended localization near tumor cells might have played a role. High expression of HSPG in vasculature may contribute to the accumulation of systemically delivered Ad5.pK7- $\Delta$ 24 into tumors (Figure 5i). Further, HSPGs have angiogenic characteristics,<sup>25</sup> which might be a mechanism for HSPG expression in neo-vasculature and subsequent accumulation of pK7 modified virions to the tumor. Ad5.pK7- $\Delta$ 24 may even have ‘antiangiogenic’ activity, if it results in the destruction of perivascular tissues. Further, as Ad5.pK7- $\Delta$ 24 is not CAR-binding ablated, the virus may be able to utilize both CAR and HSPGs for spreading within the tumor.

In summary, Ad5.pK7- $\Delta$ 24 is a novel CRAd that allows CAR-independent killing of tumor cells. Further studies are needed to investigate the full potential of this agent in the treatment of advanced cancers. One use of this virus might be re-treatment of patients previously treated with a CRAd with the Ad5 capsid, as utilization of a virus with a distinct capsid allows partial avoidance of pre-existing neutralizing antibodies, which are conformation specific.<sup>21,35,44</sup> ‘Sero-switching’ the capsid might be a useful approach for retaining antitumor activity, as any CRAd will likely result in a neutralizing antibody response. Also, as many common and deadly tumor types such as colorectal, lung, pancreatic and ovarian cancers have been reported to frequently feature dysregulation of HSPG expression,<sup>45,46</sup> Ad5.pK7- $\Delta$ 24 might be useful for the treatment of a number of advanced cancer types. The capsid modification does not seem to affect the biodistribution much (Figure 6b), which may be useful with regard to safety, as Ad5-based CRAds have been safe in dozens of trials.

## Materials and methods

### Cell lines

GFP-expressing human breast cancer cell line M4A4-LM3<sup>30</sup> is an aggressive metastatic, hormone refractory subline derived from MDA-MB-435. Human transformed embryonic kidney cell line 293 and hormone refractory breast cancer cell lines MDA-MB-435, ZR-75-1, CAMA-1, MDA-MB-436 and MCF-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in the recommended conditions.

### Construction of Ad5.pK7- $\Delta$ 24

Ad5.pK7- $\Delta$ 24 was constructed by co-transfecting (Effectene Transfection Reagent, Qiagen Corporation, Hilden, Germany) ethanol-precipitated Ad5.pK7 (GL) DNA<sup>47</sup> with *PacI*- and *PmeI*-digested pShuttle $\Delta$ 24<sup>8</sup> plasmid DNA in a 1:1 weight to weight ratio into 293 cells. Cells were incubated in a humidified incubator at +37°C with 5% CO<sub>2</sub> for 8 h. Individual plaques were collected and purified for two rounds on A549 cells. Presence of the 24-bp deletion in *E1A*, absence of the wild-type *E1A* and the presence of an intact *E3* region were confirmed with PCR as described.<sup>23</sup> A PCR product from the fiber C-terminus (primers: F 5'-TCAGTCAAGTTTACTTAAACGGAG-3' and R 5'-CTGTTACCCATGATATGATGCT-3') was sequenced to verify the presence of the pK7 modification (sequencing primers: F 5'-GACACAACCTCCAAGTGCATA-3' and R 5'-GTTGAATACTAGGGTTCTGTGAG-3'). Ad5.pK7- $\Delta$ 24 was amplified on A549 cells and purified using a standard double cesium chloride gradient method. Standard plaque assay and optical density (OD)<sub>260</sub> were used for titering the virus. Production on A549 cells yielded titers of  $1.6 \times 10^{11}$  plaque-forming units (PFU)/ml and  $2.2 \times 10^{12}$  VP/ml.

### Other recombinant adenoviruses

Conditionally replicating Ad5 $\Delta$ 24E3<sup>+48</sup> and nonreplicating viruses Ad5lucI<sup>49</sup> Ad5.pK7 (GL) and Ad5 (GL)<sup>47</sup> have been described previously. Nonreplicating viruses were propagated on 293 cells, Ad5 $\Delta$ 24E3<sup>+</sup> was propagated on A549 cells and all viruses were purified and titered as described previously. Production of Ad5 $\Delta$ 24E3<sup>+</sup>, Ad5 (GL), Ad5lucI and Ad5.pK7 (GL) yielded titers of  $3.1 \times 10^{12}$  VP/ml and  $4 \times 10^{11}$  PFU/ml,  $2.9 \times 10^{12}$  VP/ml and  $2.7 \times 10^{11}$  PFU/ml,  $1.6 \times 10^{12}$  VP/ml and  $1.6 \times 10^{11}$  PFU/ml and  $3.2 \times 10^{11}$  VP/ml and  $2.7 \times 10^{10}$  PFU/ml, respectively.

### In vitro cytotoxicity assay

Cells in quadruplicate were infected with 0.1, 1 or 10 VP/cell for 1 h at 37°C in 50  $\mu$ l of growth medium with 2% fetal calf serum (FCS), and incubated thereafter with growth medium with 5% FCS. Cell viability was measured using the CellTiter 96 AQUAEUOUS One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, USA) on day 7 (MCF-7), day 11 (CAMA-1, M4A4-LM3) or 12 (MDA-MB-435).

### Orthotopic breast cancer model in nude mice

Pathogen free 3-to 4-week-old female Naval Medical Research Institute (NMRI) nude mice were purchased from Taconic (Ejby, Denmark) and quarantined for 2 weeks. Mice were injected orthotopically into the left and right uppermost mammary fat pad with  $2 \times 10^6$  M4A4-LM3 cells and a tumor was allowed to develop. In the local treatment model, when tumors reached the diameter of approximately 0.5 cm, mice were randomized into groups and injected with growth medium or  $3 \times 10^8$  VP of Ad5.pK7- $\Delta$ 24 or Ad5 $\Delta$ 24E3<sup>+</sup> (all groups:  $n = 8$  tumors in four mice). Viruses were diluted with minimum essential growth medium without supplements and injected intratumorally in a total volume of 50  $\mu$ l on 3 subsequent days (days 17, 18 and 19). Starting on day 20, mice were anesthetized using medetomidine (Domitor) and ketamine (Ketalar, Orion Pharma, Espoo, Finland) and imaged three times a week with the IVIS imaging system as recommended (Xenogen Corp., Alameda, CA, USA). Fixed size regions of interest were drawn around each tumor and chosen threshold values were set for minimum and maximum emission measured. Values were normalized to values obtained from a region of interest with no tumor. Tumors were measured in three dimensions using a caliber. The size was calculated by using formula  $\frac{3}{4} \pi((0.5 \times L)(0.5W)(0.5H))$ .

In the systemic treatment model, either growth medium or  $3 \times 10^{10}$  VP of Ad5.pK7- $\Delta$ 24 ( $n = 14$  tumors, seven mice) or Ad5 $\Delta$ 24E3<sup>+</sup> (all groups:  $n = 14$  tumors in seven mice) were injected in a total volume of 100  $\mu$ l through the tail vein 10 days after the injection of cells, and again on day 33 (23 days after the first viral injection). On day 10, mice were imaged and tumor sizes were measured as above. When tumors reached 1 cm in any diameter, the mice were killed as required by animal regulations. Animal experiments were approved by the Provincial Government of Southern Finland.

### Gene transfer assays

M4A4-LM3 cells in quadruplicates were infected for 30 min in room temperature with 40 VP/cell by adding Ad5.pK7 (GL) or Ad5 (GL) or no virus diluted in 200  $\mu$ l of growth medium with 2% FCS. Cells were washed once and complete growth medium was added. Cells were incubated for 24 h at 37°C after which luciferase assay was performed (Luciferase Assay System, Promega). Background luciferase activities were subtracted from the data.

For *in vivo* studies,  $2 \times 10^6$  cells of M4A4-LM3 were injected into the left uppermost mammary fat pad of NMRI nude mice and primary tumors were allowed to grow for 20

days. On day 20,  $5 \times 10^{10}$  VP of Ad5.pK7 (GL), Ad5 (GL) or no virus were injected through the tail vein. After 48 h, mice were killed and tumors were harvested. Tumors were homogenized in Cell Culture Lysis Buffer (Luciferase Assay System, Promega) and luciferase activity was analyzed as before. Mean background luciferase activity was subtracted from the data and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) to allow normalization of the gene expression data for the number of cells.

### Determination of receptor expression by flow cytometric analysis

M4A4-LM3 cells grown in cell culture flasks were washed with phosphate-buffered saline (PBS), harvested by incubating in 0.1% trypsin-ethylenediaminetetraacetic acid and suspended in PBS containing 2% FCS. Cells ( $2 \times 10^5$ ) were incubated with either anti-CAR primary antibody RmcB (1:100), anti-HSPG primary antibody 10E4 (SEIKAGAKU Co., Falmouth, MA, USA) (1:100) or buffer only for 30 min at 4°C. Cells were then rinsed with 2% FCS and incubated with 1:100 dilution of phycoerythrin (PE)-labeled goat anti-mouse immunoglobulin polyclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 4°C. After cells were rinsed with 2% FCS, cells were immediately analyzed with flow cytometry.

To analyze receptor expression in M4A4-LM3 tumors grown in mammary fat pads, tumors were harvested on day 30, roughly homogenized with scalpels and further homogenized by exposing tissue to enzymatic dissociation for 2 h in 37°C using 0.2 Wünsch Units/ml of Liberase Blendzyme 1 (Roche Diagnostics, Indianapolis, IN, USA). Cells ( $2 \times 10^5$ ) were treated as described previously and analyzed by flow cytometry.

### Immunohistochemical stainings

M4A4-LM3 xenograft tumors were harvested on day 30 and fixed in buffered formalin (10%), mounted on paraffin and deparaffinized by treating with xylene and decreasing ethanol gradient. Deparaffinized sections were treated with citrate buffer and methanol before blocking with 1.5% horse serum (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA). Slides were incubated overnight with 100  $\mu$ l of antibody dilutions (10E4 for HSPG and RmcB for CAR, 1:200) and washed with PBS three times. Biotinylated secondary antibody (mouse IgG, 6  $\mu$ l in 100  $\mu$ l) was applied on the slides, and visualized by the Vectastain ABC system.

### Histopathology

M4A4-LM3 xenograft tumors were harvested on day 30 and fixed in buffered formalin (10%) and cut in 5  $\mu$ m sections. Deparaffinized sections were stained with hematoxylin and eosin.

### Biodistribution

Tumors were allowed to grow for 30 days.  $3 \times 10^{10}$  VP of Ad5 (GL) or Ad5.pK7 (GL) were injected through the tail vein. After 48 h, the mice ( $n = 7$ ) were killed and organs were collected and analyzed for luciferase expression as described previously.<sup>35</sup> Results were normalized to protein content of the organs by DC Protein Assay (Bio-Rad, Hercules, Manassas, CA, USA).

### Statistical analysis

Cell viability and tumor size data were analyzed by two-tailed Student's *t*-test. *P*-value of <0.05 was considered statistically significant. Survival data were plotted into a Kaplan–Meir curve and groups were compared pairwise with log-rank test using SPSS 11.5.



## Acknowledgments

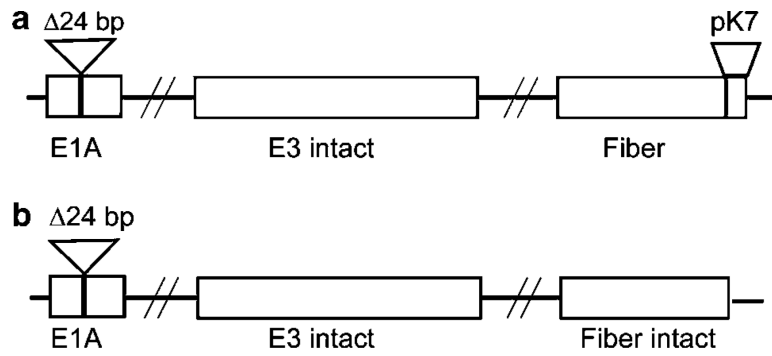
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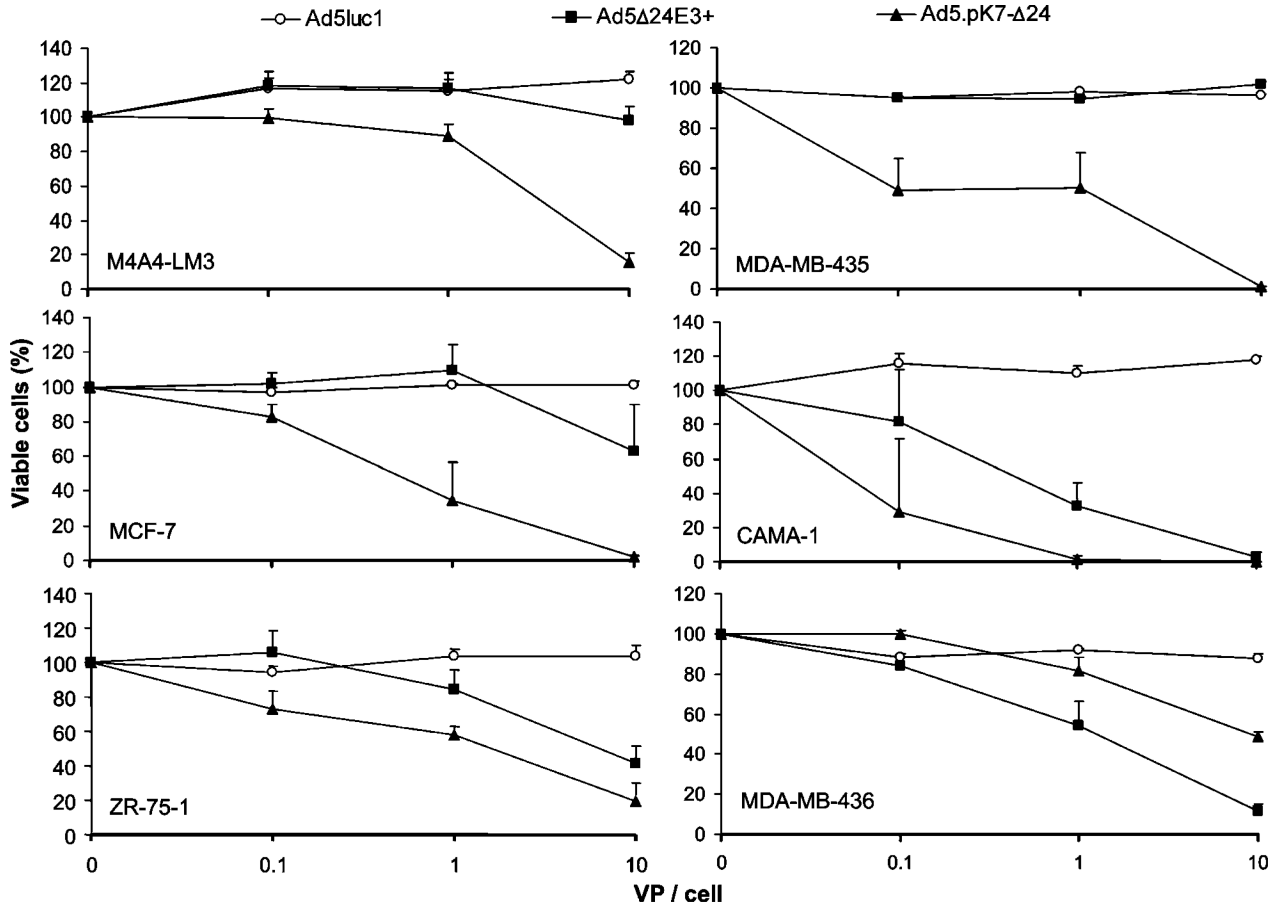
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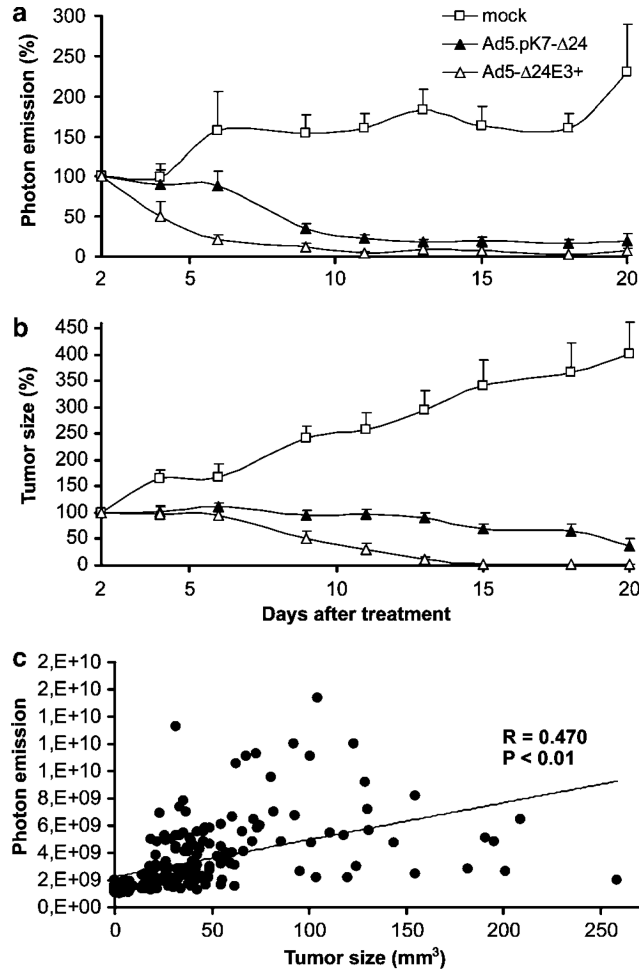


**Figure 1.** Structure of the oncolytic viruses used in the study. Ad5.pK7-Δ24 (**a**) and Ad5Δ24E3+ (**b**) have a 24-bp deletion in the Rb-binding site of the adenoviral E1A, resulting in selective replication in cells deficient in the Rb/p16 pathway, such as breast cancer cells. Seven lysines and a GS linker sequence are incorporated into the C-terminus of the fiber of Ad5.pK7-Δ24, enabling binding to HSPGs.

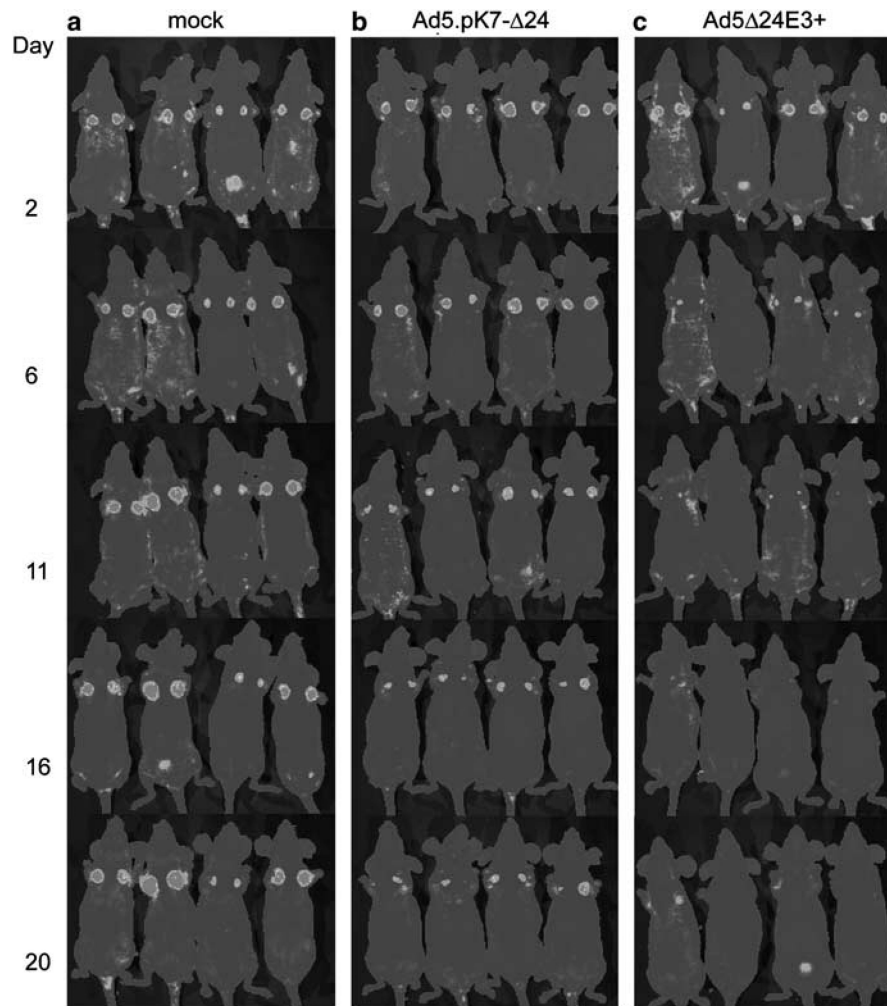


**Figure 2.**

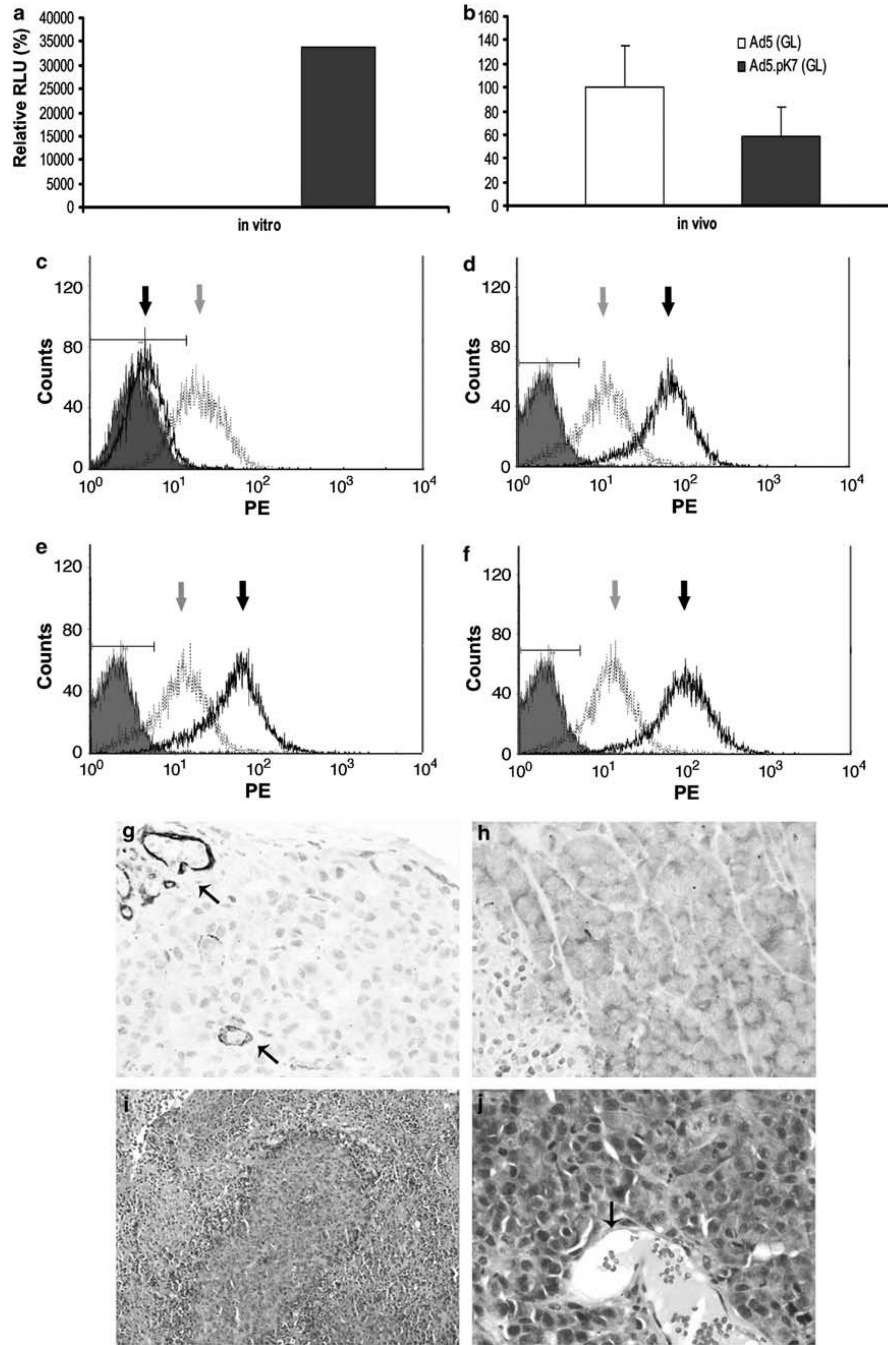
Ad5.pK7-Δ24 displays effective killing of breast cancer cell lines. Cells were infected with 0, 0.1, 1 or 10 VP/cell of Ad5.pK7-Δ24, Ad5Δ24E3+ (an isogenic control virus with a wild-type fiber) or Ad5luc1, a nonreplicating control virus, which was included to control for the effect of virus alone. Cell viability was measured with the MTS assay. The OD<sub>490</sub> values of uninfected cells were set as 100%. Data are expressed as means±s.e. of quadruplicate experiments. In all but one cell line (MDA-MB-436), oncolysis was significantly enhanced with Ad5.pK7-Δ24 compared to Ad5Δ24E3+ or Ad5luc1 (all  $P < 0.05$ ).

**Figure 3.**

Therapeutic effect of intratumorally injected Ad5.pK7-Δ24 in an orthotopic animal model of advanced hormone refractory breast cancer. GFP-expressing M4A4-LM3 cells were injected into the mammary fat pads of NMRI nude mice and primary tumors were allowed to grow for 17 days. Mice received intratumoral injections of  $3 \times 10^8$  VP of Ad5.pK7-Δ24, Ad5Δ24E3+ or no virus on 3 successive days (days 17, 18 and 19 after injections of tumor cells). Tumor size was followed by assessment of the number of viable tumor cells by noninvasive imaging of fluorescence (a) or by physical measuring of the tumors with a caliper in three dimensions (b). Photon count and tumor size are depicted as a percentage of initial values. Ad5.pK7-Δ24-treated mice had significantly less viable tumor cells when compared to the mock group ( $P < 0.01$ ). (c) A significant correlation was seen between the GFP expression and tumor size ( $P < 0.01$ ).



**Figure 4.** Noninvasive imaging of viable tumor cells following treatment with Ad5.pK7- $\Delta$ 24. Hormone refractory GFP-positive M4A4-LM3 breast cancer cells were injected into the mammary fat pads of NMRI nude mice and primary tumors were allowed to grow for 17 days. On days 17–19, mice were treated with intratumoral injections of either virus or growth medium. Fluorescence was imaged three times a week using a cooled CCD camera. A pseudocolor image was obtained (blue represents the lowest intensity and red the highest) and overlaid on a gray scale photographic image. (a) Mock-treated mice (b) Ad5.pK7- $\Delta$ 24-treated mice and (c) Ad5 $\Delta$ 24E3+-treated mice. The left column indicates the imaging time point (days after last virus injection). (For color figure see online version.)

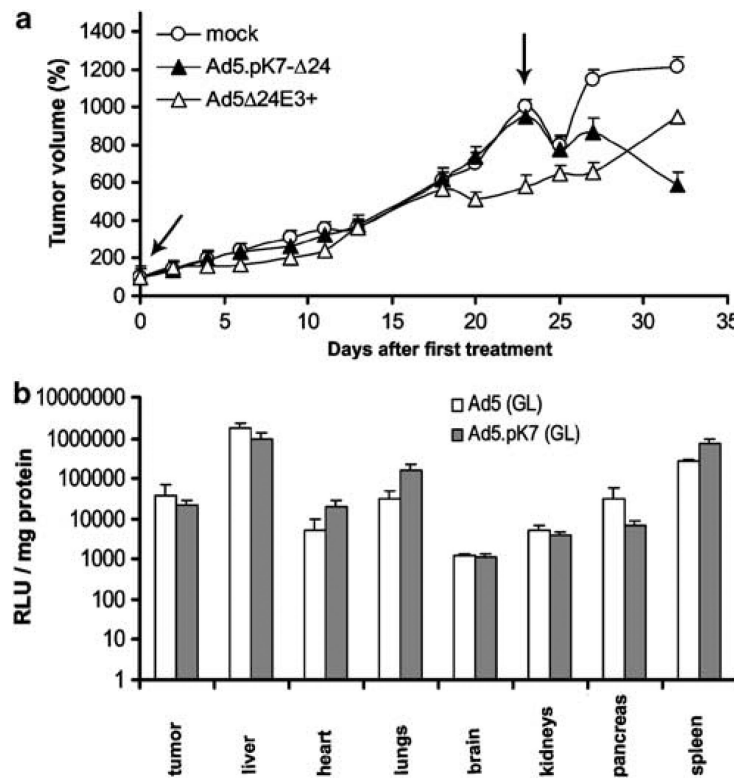


**Figure 5.**

Difference between relative gene transfer efficacy with a pK7-modified Ad versus an unmodified Ad5 capsid virus and between CAR and HSPG levels *in vitro* and *in vivo*. (a) M4A4-LM3 cells were infected *in vitro* with 40 VP/cell of either Ad5.pK7 (GL) or Ad5 (GL) and luciferase expression was assessed as a measure of gene transfer. The relative luciferase units (RLU) values of unmodified Ad5 (GL) were set as 100%. (b) *In vivo* M4A4-LM3 xenografts were allowed to grow for 28 days and  $5 \times 10^{10}$  VP of virus were injected intravenously and luciferase expression was assessed 48 h later from homogenized tumors. *In vitro*, Ad5.pK7 (GL) displays 300 times more efficient gene transfer when compared to Ad5 (GL) (a), but *in vivo* (b) there is no significant difference between the two



viruses. **(c–f)** CAR expression was significantly upregulated in the M4A4-LM3 xenograft tumors grown in mice when compared to the cell line grown *in vitro*. M4A4-LM3 cells *in vitro* **(c)** or homogenized M4A4-LM3 tumors **(d–f)** were analyzed for their CAR (black line and arrow) and HSPG (gray line and arrow) expression by flow cytometric analysis. Grey shading is negative control. **(g, h)** Immunohistochemical staining was used to analyze whole-tumor sections for CAR and HSPG expression. Blood vessel endothelium (arrows) stained positive for HSPG, whereas the bulk of the tumor stained less intensively **(g)**. In contrast, viable cancer cells stained positive for CAR and necrotic areas were negative **(h)**. **(i, j)** Tumors were stained with hematoxylin and eosin to study their histopathology. Necrotic areas stained lighter and viable tumor tissue darker **(i)**. Blood vessels (arrow) were surrounded by viable cancer cells with atypic morphology with enlarged/variable size nucleus **(j)**. (For color figure see online version.)

**Figure 6.**

(a) Therapeutic effect of intravenously injected Ad5.pK7-Δ24 in an orthotopic animal model of advanced hormone refractory breast cancer. GFP-expressing M4A4-LM3 cells were injected into the mammary fat pads of NMRI nude mice and primary tumors were allowed to grow for 10 days. Mice received two intravenous injections ( $3 \times 10^{10}$  VP) of Ad5.pK7-Δ24, Ad5Δ24E3+ or no virus on days 10 and 33 (marked with an arrow) after the injection of cells (i.e. days 0 and 23 after treatment, respectively). Ad5.pK7-Δ24-treated mice had significantly fewer viable tumor cells on day 33 when compared to Ad5Δ24E3+ or mock-treated mice ( $P < 0.05$ ). Signal (photons) is depicted as a percentage of the initial photon count. (b) Biodistribution of Ad5.pK7 (GL) is similar to the isogenic Ad5 (GL) control. Bars denote s.e.