Transcriptional targeting modalities in breast cancer gene therapy using adenovirus vectors controlled by α-lactalbumin promoter

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Abstract
The breast-specific antigen α-lactalbumin is expressed in >80% of breast cancer tissues. To evaluate the effect of gene therapy for breast cancer by controlling adenovirus replication with human α-lactalbumin promoter, we investigated the activity of a 762-bp human α-lactalbumin promoter. α-Lactalbumin promoter showed significantly higher activity in MDA-MB-435S and T47D breast cancer cells than in normal breast cell lines or other tumor cell lines. We then developed two novel breast cancer–restricted replicative adenoviruses, AdALAE1a and AdE1aALAE1b. In AdALAE1a, expression of adenoviral E1a gene is under the control of α-lactalbumin promoter, and in AdE1aALAE1b, expression of both E1a and E1b genes is under the control of a single α-lactalbumin promoter. Both breast cancer–restricted replicative adenoviruses showed viral replication efficiency and tumor cell-killing capability similar to wild-type adenovirus in MDA-MB-435S and T47D cells. The replication efficiency and tumor cell-killing capability of both viruses were attenuated significantly in cells that did not support α-lactalbumin promoter. AdE1aALAE1b showed better breast cancer–restricted replication than AdALAE1a, suggesting that a transcriptional targeting modality with α-lactalbumin promoter controlling both E1a and E1b gene expression is superior to α-lactalbumin promoter controlling only E1a gene expression. Importantly, we found that AdE1aALAE1b could be used to target hormone-independent breast tumors in vivo by inhibiting the growth of MDA-MB-435S s.c. tumors. These data showed that α-lactalbumin promoter could regulate the replication of adenovirus to target hormone-independent breast cancers, suggesting that α-lactalbumin promoter can be used to develop a novel therapeutic modality for hormone-independent breast cancer.

Introduction
Breast cancer is the most commonly diagnosed malignancy in women. It is estimated that >1 million new cases appear annually, representing 18% of all female cancers (1). In the United States, an estimated 211,300 new cases of invasive breast cancer were diagnosed and 39,800 women died from this disease in 2003 (2). It is the second leading cause of cancer death in the United States. The conventional therapeutic approaches for hormone-refractory breast cancer include chemotherapy, radiation therapy, and surgery. Chemotherapy and radiation kill many normal cells along with tumor cells, damage the human immune system, hamper the functions of bone marrow hematopoietic stem cells, and easily incur drug resistance (3). Despite surgical methods that can remove the primary and local sites of breast cancer, relapse at local or distant sites is still possible because of the presence of micrometastases undetectable at the time of diagnosis (3). Advances in understanding genetic alterations associated with breast cancer progression (4) have made gene therapy an exciting alternative for treating hormone-refractory breast cancers. The present gene therapy strategies for carcinoma of the breast include genetic correction (5), genetic ablation (6), genetic toxins (7), genetic immunotherapy (8), and proapoptotic gene therapy strategies (9). However, these strategies cannot be maximized until better gene transfer technologies are developed to target breast cancer cells more specifically.

Two principal adenovirus-mediated gene therapy strategies have been attempted. The first approach is a transcriptional targeting modality, which selectively targets tumors with a tissue/tumor-specific promoter driving the expression of therapeutic genes or viral replication in a tissue- or tumor-specific manner (10). The second approach is a transductional targeting modality, which redirects...
adenoviral tropism from its native receptor to a new one preferentially expressed on target cells (11) through either direct modification of viral fiber protein (12, 13) or conjunction with bispecific single-chain antibodies (14) that interact with specific cell surface receptors.

One of the biggest challenges facing transpositional targeting gene therapy is the development of tissue- or tumor-restricted replicative adenovirus (TRRA) to deliver therapeutic genes (15), especially in breast cancer. Although mammary tissues produce a great number of different antigens and proteins, few of them are specific to breast tissue or breast tumors (16). Even whether the breast cancer cell lines in in vitro experiments are truly representative of the tumors from which they originated remains a matter for debate, reflecting the difficulty of investigating specific target markers in vitro (17). For breast cancer gene therapy, a few promoters have already been used to drive the expression of genetic toxins [e.g., DF3/MUC1 promoter (18, 19), ErbB-2 promoter (20), ErbB-2 promoter/ MUC1 enhancer (21), human α-lactalbumin and ovine β-lactaglobulin promoter (16)].

α-Lactalbumin, a major whey protein synthesized in differentiated breast epithelial cells, was shown to be expressed in >60% of breast cancer tissue in the clinical cases tested (16, 22–26). It is a key protein for lactogenesis and acts as a modifier protein in the lactose synthetase reaction by interacting with UDP-galactosyltransferase to form lactose synthetase (27). It is also a calcium metalloprotein and has been found in all milks studied thus far. α-Lactalbumin RNA and protein levels are low in virgin glands and, during most of pregnancy, increase sharply near parturition and decrease rapidly following involution (28). This suggests that due to the hormonal perturbation that occurs in many breast carcinomas the usually tightly regulated human α-lactalbumin gene is transcriptionally active (16). Other functional properties have been attributed to this protein, such as cell lytic activity, induction of cell growth inhibition, or apoptosis (29). Human α-lactalbumin regulatory and coding sequences were reportedly able to functionally rescue lactose production in α-lactalbumin-null transgenic mice (30, 31), suggesting that the promoter sequences responsible for breast tissue specificity existed within the 770 bp upstream sequences used in the transgenic mouse study (32, 33). This human α-lactalbumin promoter has been used to direct therapeutic thymidine kinase and cytosine deaminase gene expression targeting breast cancers in animal models (16, 34).

Although the replication-deficient first-generation adenovirus provided high transduction rates, clinical trials suggested that the single-agent antitumor effect may not be sufficient for all treatment approaches, requiring multiple rounds of readministration (11). Replication-competent adenoviruses that spread specifically inside the targeted tumor cells have been suggested as a way to improve the targeting efficiency for gene therapy (11). In replication-competent adenoviral vectors, tissue/tumor-specific promoters are used to replace the Ela promoter (35–37), with the rationale that expression of Ela, which is the crucial regulator of adenoviral replication and therefore of the whole adenoviral transcription program, will be controlled by these tissue- or tumor-specific promoters. However, leakage of foreign promoters in controlling the Ela expression, yielding low levels of Ela, may result in loss of specificity (38). Separate promoter control of the Ela and E1b genes is shown to control viral replication more stringently and to further improve the specificity significantly (39, 40).

In this report, we investigated the tissue/tumor specificity of α-lactalbumin promoter and developed two novel breast cancer–restricted replicative adenoviruses (BRRA), AdALAE1a and AdE1aALAE1b, in which α-lactalbumin promoter either controls adenovirus Ela gene expression alone or controls both Ela and E1b expression simultaneously. We investigated their tissue-specific replication efficiency and cell-killing ability in vitro and tumor suppression effect in vivo.

**Materials and Methods**

**Cells and Cell Culture**

MCF10A, MCF12A, PC-3, DU145, and HEK293 (transformed human embryonic kidney cell line that expresses complementing adenoviral E1 protein supporting the replication of E1-deleted recombinant adenoviruses) were cultured as described previously (41). MDA-MB-435S, T47D, and PER.C6 [a packaging cell line containing the adenovirus 5 (Ad5) Ela- and E1b-encoding sequences (nucleotides 459–3,510) under the control of the human phosphoglycerate kinase promoter] were cultured in DMEM containing 10% fetal bovine serum.

**Plasmid Construction**

The α-lactalbumin promoter was cloned from pAd.A-LA(tk) (ref. 4; a gift from DirectGene, Inc., Annapolis, MD) into pGL3-basic (Promega, Madison, WI) to make pGL3-ALA construct. The first 353 bp of Ad5 left end (Genbank accession no. 000008), which contains the adenoviral inverted terminal repeats (ITR), packaging sequences, and Ela enhancer, was cloned in the upstream of α-lactalbumin promoter in pGL3-ALA plasmid to make pGL3-AdALA construct.

**Transient Reporter Assay**

Cells (2.5 × 10^5 per well) were seeded in 12-well plates and transfected with 1.6 μg pGL3-ALA, pGL3-TATA, or pGL3-AdALA together with 10 ng Renilla luciferase-expressing vector pRL-SV40 (as an internal control) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA; ref. 42). Cells were collected 48 hours after transfection for dual luciferase assay (Promega) according to the manufacturer’s protocol (42). Data are expressed as fold pGL3-ALA or pGL3-AdALA activity over pGL3-TATA and expressed as the mean ± SE of triplicate wells.

**Construction and Amplification of Recombinant Adenovirus**

The adenovirus construction system was developed by Dr. Xavier Danthinne (O.D.260, Inc., Boise, ID). The system contains a cloning vector pAd1020SfidA containing adenovirus left ITR and packaging signal (1–358 bp) and an
adeno-viral genome vector pAd288 containing the right arm of adeno-virus from 3,504 bp to the end with E3. An α-lactalbumin promoter, E1a (without TATA box), and part of the E1b gene (from TATA box to 3,924) were cloned into pAd1020SfidA. Then, the left arm of the adeno-virus was cloned into pAd288 vector via a three-way ligation, SfiI-AflII (from pAd1020SfidA cloning vectors), AflII-PmeI, and PmeI-SfiI (from pAd288). λ Phages were applied to pack the ligation mix and to transduce bacteria that were then plated onto an agar plate containing kanamycin. Plasmids were digested with PacI to release adeno-viral genomes and transfected into PER.C6 cells using LipofectAMINE 2000 to generate recombinant adeno-viruses. An agarose mixture (0.6% agarose with 10% fetal bovine serum in MEM) was added onto the cells 2 days after transfection and incubated for 7 to 10 days to allow plaque formation. Plaques were isolated for amplification in PER.C6 cells. The viruses were screened by PCR (α-lactalbumin forward CTGGAGAGGAAAAAGTAG, E1a reverse CGGGAAAAATCTGCGAAACC, α-lactalbumin reverse AATTGGAGAGGGGTGTATGTC, and E1b reverse AAAAAGCAACACAGCCCAACG) to choose the correct plaques after two or three additional runs of plaque isolations. AdALAE1a and AdE1aALAE1b were then amplified in PER.C6 cells and purified by CsCl gradient centrifugation according to procedures described previously (43). Viral titer was determined by plaque assay using HEK293 cells.

Adeno-viral Infectivity Assay

Cells were seeded (2.5 × 10^5 per well) in 12-well plates 1 day before viral infection and infected with serial dilutions of AdCMV-Luc (an E1/E3-deleted recombinant adeno-virus carrying the luciferase reporter gene controlled by cytomegalovirus promoter) from 0.05 to 31.25 multiplicities of infection (1 multiplicity of infection = 1 plaque-forming unit/cell). The media were changed 24 hours after addition of the virus. The cells were harvested 48 hours after infection for luciferase assay. The luciferase activities obtained were used as a reference to adjust the virus titer for each cell line to obtain a similar infectivity for the proceeding experiments. The accuracy of the above titer assay was checked by infecting cells with the amount of virus derived from the above assay.

Western Blot Analysis

Cells were seeded in six-well plates and infected with AdALAE1a, AdE1aALAE1b, and a wild-type Ad5 (Ad-WT) the next day. Each cell line was infected with standardized doses of virus according to the luciferase activity obtained above to achieve similar infectivity. Cell lysates were collected for Western blot analysis as described previously (41). The membrane was probed with an anti-Ad5 E1a antibody (BD Biosciences PharMingen, San Diego, CA) followed by a horseradish peroxidase–conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Reverse Transcription-PCR Analysis

Cells were seeded in P100 dishes (5 × 10^6 per dish) and infected with AdALAE1a or AdE1aALAE1b the next day with standardized doses of virus as described above. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Genomic DNA was checked by PCR without reverse transcriptase as control. The RNeasy Mini kit (Qiagen, Valencia, CA) was used to purify RNA if DNA contamination was identified. Reverse transcription-PCR was done using a kit from Invitrogen. β-Actin was used as an internal control. The PCR primers were chosen in adeno-virus E1b region (E1b forward CTGGGAGAGGAAAAGTAG and E1b reverse CCGGAAAAATCTGCGAAACC).

Viral Replication Assay

Cells were seeded in six-well plates (1 × 10^6 per well) and infected with standardized doses of AdALAE1a, AdE1aALAE1b, or Ad-WT the next day as described above. The media were changed 24 hours later, and the viral supernatants were harvested 3 days after the infection. Then, the titers of the harvested virus supernatants were checked by titer assay using HEK293 cells (41). HEK293 cells were seeded in 96-well plates (5 × 10^4 per well) 1 day before infection. The cells were infected with serial dilutions of the harvested supernatants ranging from 1 to 10^{-11} μL/well. A row of eight wells was used for each dose. The media were changed on day 4, and the cells were examined under the microscope on day 7. The titers of the produced viruses were shown as the LD_{50} value (the dilution factor that causes a cytopathic effect in at least four wells of cells in a row on a 96-well plate on day 7). A tissue specificity index was obtained by dividing the LD_{50} value of therapeutic virus to that of Ad-WT.

Cell-Killing Assay

Cells were seeded in 96-well plates (1 × 10^4 per well) 1 day before viral infection. The cells were infected with serial doses ranging from 50 to 5 × 10^{-9} multiplicities of infection of AdALAE1a, AdE1aALAE1b, or Ad-WT. A row of eight wells was used for each dose. The media were changed on day 4, and the cells were examined under a light microscope on day 7. The viral-killing activity was represented as the LD_{50} value. A tissue specificity index was obtained by dividing the LD_{50} value of therapeutic virus to that of Ad-WT expressed as a log_{10} scale. The evaluation method is the same as the replication assay described above.

Animal Studies

All animal procedures were approved by the Indiana University Institutional Animal Care and Use Committee. MDA-MB-435S mouse tumor models were established by injecting s.c. 0.5 × 10^5 MDA-MB-435S cells into the flanks of 6-week-old female athymic nude mice (Harlan, Indianapolis, IN). A total of 21 mice were used for cancer cell inoculation and two inoculations were done per mouse on each side of the flank. Mice were randomized 10 weeks after cancer cell inoculation when the tumors reached an average volume of ~80 mm^3 and divided into three groups. Mice were then treated with 1 × 10^7 plaque-forming units of AdE1aALAE1b, AdPSES-Luc (a replication-deficient adeno-virus used as a negative control), or PBS via intratumoral injection. A second treatment was...
done 3 weeks later. Tumor size was measured once weekly with a caliper, and the following formula was applied to calculate tumor volume: length × width² × 0.5236. Statistical analysis using ANOVA and multiple range tests was done to analyze the difference between groups.

**Histology and Immunohistochemistry**

Breast tumors were removed, fixed in formalin, and embedded in paraffin. Sections (4 μm thick) were cut and stained with H&E for histologic evaluation. Immunohistochemistry was carried out as described previously (41). Rabbit polyclonal antibody to Ad5 (Abcam, Cambridge, MA) was used as the primary antibody at a dilution of 1:200, and a normal rabbit IgG was used as a control.

**In situ Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay**

The in situ apoptosis detection kit was purchased from Roche Diagnostics (Indianapolis, IN). Briefly, tumor sections were treated with 10 mmol/L Tris solution containing proteinase K and incubated with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction mixture. The slides were then incubated with TUNEL POD and 3,3'-diaminobenzidine solution and counterstained with hematoxylin.

**Results**

α-Lactalbumin Promoter Showed Higher Activity in MDA-MB-435S and T47D Breast Cancer Cells Than in Normal Breast Epithelial Cells or Prostate Tumor Cells

To evaluate the tissue/tumor-specific activity of α-lactalbumin promoter, we constructed the pGL3-ALA plasmid by cloning the 762-bp α-lactalbumin promoter into the pGL3-basic vector, which carries a luciferase reporter gene. pGL3-ALA was transfected into nontumorigenic human normal mammary epithelial cell lines MCF10A and MCF12A, human breast cancer cell lines MDA-MB-435S and T47D, and human prostate cancer cell lines PC-3 and DU145. Consistent with other reports (16, 34), α-lactalbumin promoter showed higher activity in breast cancer cell lines MDA-MB-435S and T47D than in normal breast cell lines or prostate cancer cell lines (Fig. 1A). These data indicated that α-lactalbumin promoter could be used as a breast cancer–specific promoter to direct adenovirus replication for targeting breast cancers.

To control adenovirus replication in a tissue/tumor-specific manner, we developed two adenoviruses, AdALAE1a and AdE1aALAE1b. AdALAE1a was constructed by replacing adenovirus E1a gene promoter with α-lactalbumin promoter. The expression of E1a gene is thereby controlled by α-lactalbumin promoter, and the expression of E1b is controlled by its own promoter. AdE1aALAE1b was generated by placing the α-lactalbumin promoter between E1a and E1b genes, and E1a and E1b genes were oriented to face in opposite directions so that α-lactalbumin promoter can drive both E1a and E1b gene expression simultaneously (Fig. 1B). The gene structures of AdALAE1a and AdE1aALAE1b produced in PER.C6 was confirmed by PCR. For AdALAE1a (Fig. 1C), the use of α-lactalbumin forward (primer 1) and E1a reverse (primer 3) produced an expected DNA fragment of 423 bp, whereas α-lactalbumin reverse (primer 2) and E1a reverse (primer 3) did not produce a PCR product because they face the same direction. For AdE1aALAE1b (Fig. 1C), use of α-lactalbumin reverse (primer 2) and E1a reverse (primer 4) produced an expected DNA fragment size of 769 bp, and use of α-lactalbumin forward (primer 1) and E1b reverse (primer 3) produced an expected DNA fragment size of 961 bp. The above results showed that no gross rearrangement of the inserted gene occurred during virus production.

Because various cell lines express different amounts of Coxsackie adenovirus receptor for adenovirus, their adenoviral infectivities were expected to vary accordingly. Therefore, it is necessary to establish individual infection conditions for each cell line to achieve similar infectivity

![Figure 1](image_url)

**Figure 1.** Construction of α-lactalbumin promoter-based adenoviral vectors. A, α-lactalbumin promoter activity in various cell lines. A 762-bp human α-lactalbumin promoter was cloned into the pGL3-basic vector to construct pGL3-α-lactalbumin. MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145 cells were seeded in 12-well plates and transfected with pGL3-TATA or pGL3-ALA. Luciferase activities were determined 48 h after transfection. Data are expressed as fold pGL3-ALA activity over pGL3-TATA activity. Columns, mean of triplicate wells; bars, SD. B, structure of AdALAE1a and AdE1aALAE1b. C, the gene structure of AdALAE1a and AdE1aALAE1b produced in PER.C6 was confirmed by PCR using genomic DNA extracted from AdALAE1a-infected or AdE1aALAE1b-infected cells. The primers selected are labeled with arrows: α-lactalbumin forward (1), α-lactalbumin reverse (2), E1a reverse (3), and E1b reverse (4).
among cell lines. We conducted an experiment to normalize the susceptibility of tumor cell lines to adenoviral infection using AdCMV-Luc, an E1/E3-deleted replication-deficient adenovirus that carries a luciferase reporter gene under the control of cytomegalovirus promoter. Figure 2A illustrated various viral doses required for similar infectivity in MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145. To test if the different viral doses determined in Fig. 2A for each cell line result in similar luciferase activity, cells were infected with AdCMV-Luc. As shown in Fig. 2B, similar luciferase activity (~10^5 luciferase units) could be obtained among cell lines when adenoviral infectivities were standardized. This result was applied to all subsequent in vitro experiments.

AdALAE1a and AdE1aALAE1b Directed E1a Protein Expression Specifically in Breast Cancer Cells

To test whether α-lactalbumin promoter can control E1a protein expression in a breast cancer-specific manner, we infected MCF10A, MDA-MB-435S, and T47D with AdALAE1a, AdE1aALAE1b, or Ad-WT. We found that Ad-WT was able to direct E1a protein expression in all three cell lines tested (Fig. 3A). Importantly, AdALAE1a and AdE1aALAE1b directed the expression of E1a proteins only in breast cancer MDA-MB-435S and T47D cells but not in normal breast MCF10A cells (Fig. 3A). Furthermore, our data indicated that AdALAE1a and AdE1aALAE1b were able to directly induce the expression of E1a proteins specifically in breast cancer cells.
MCF10A and MCF12A cells or prostate cancer PC-3 and DU145 cells (Fig. 3B). The results showed that the presence of adenoviral packaging signal greatly enhanced α-lactalbumin promoter activity.

**AdE1aALAE1b Directed E1b mRNA Expression in Breast Cancer Cells**

To test whether α-lactalbumin promoter is able to control E1b in AdE1aALAE1b, we checked E1b mRNA expression in T47D, MDA-MB-435S, MCF10A, and PC-3 cells. E1b mRNA expression was examined, instead of the E1b protein, due to the lack of E1b-specific antibodies for Western blot analysis. AdE1aALAE1b expressed high amounts of E1b mRNA only in α-lactalbumin high-activity breast cancer MDA-MB-435S and T47D cells but not in α-lactalbumin low-activity MCF10A and PC-3 cells, because the E1b gene was under the control of α-lactalbumin promoter in AdE1aALAE1b. On the other hand, AdALAE1a directed E1b mRNA expression in all cell lines tested because E1b was under its own promoter control in AdALAE1a (Fig. 3C). The results showed that a single α-lactalbumin promoter could control both E1a and E1b expression in AdE1aALAE1b, thus enhancing the tumor specificity of the conditionally replicative adenovirus.

**AdALAE1a and AdE1aALAE1b Propagated Efficiently in Breast Cancer Cell Lines but not in Normal Breast Cell or Prostate Cancer Cells**

We did an *in vitro* viral replication assay to compare the viral replication efficiency of AdALAE1a and AdE1aALAE1b (Table 1). Viral titers for AdALAE1a and AdE1aALAE1b were normalized for Ad-WT, and values were represented as log_{10} phase to address the tissue-specific replication of α-lactalbumin viral vectors. As shown in Fig. 4A, AdALAE1a and AdE1aALAE1b propagated in MDA-MB-435S and T47D cells as efficiently as Ad-WT. On the other hand, AdALAE1a propagated poorly in normal breast cell lines as well as in prostate cancer cell lines. Compared with Ad-WT, AdALAE1a produced 500-fold fewer viruses in MCF10A, 250-fold fewer in MCF12A, 1,000-fold fewer in PC-3, and 100-fold fewer in DU145. AdE1aALAE1b showed a more restricted replication in nontarget cells. Compared with Ad-WT, AdE1aALAE1b produced 1,000-fold fewer viruses in MCF10A, 1,000-fold fewer in MCF12A, 10,000-fold fewer in PC-3, and 1,000-fold fewer in DU145. The contrast in the tumor-specific replication activity of the two adenoviruses suggested that the breast cancer specificity of AdE1aALAE1b is much better than that of AdALAE1a.

**AdALAE1a and AdE1aALAE1b Showed Specific Cell Killing Ability in Breast Cancer Cells**

To test the breast cancer cell-specific killing activity of AdALAE1a and AdE1aALAE1b, MCF10A, AdE1aALAE1b showed breast cancer-specific replication activity. MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145 cells were infected with standardized doses of AdALAE1a or AdE1aALAE1b and viral replication assay was done. Ad-WT was used as a control. The value is expressed as a log_{10} scale, such that a value of 0 indicates that the therapeutic virus has the same replication activity as a wild-type virus in a particular cell line and that a value of −1 indicates that the therapeutic virus has 10 times less replication activity compared with a wild-type virus in a particular cell line. AdALAE1a and AdE1aALAE1b showed breast cancer-specific killing activity. Serial dilutions of AdALAE1a or AdE1aALAE1b were applied to MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145 cells and cell-killing assay was done as described in Materials and Methods.

**Table 1. Replication efficiency of AdALAE1a and AdE1aALAE1b**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>AdALAE1a</th>
<th>AdE1aALAE1b</th>
<th>Ad-WT</th>
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<td>MCF10A</td>
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<td>5 × 10^2</td>
<td>5 × 10^5</td>
</tr>
<tr>
<td>MCF12A</td>
<td>2.5 × 10^4</td>
<td>5 × 10^3</td>
<td>5 × 10^6</td>
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<tr>
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<td>2 × 10^4</td>
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<td>10^6</td>
</tr>
<tr>
<td>DU145</td>
<td>10^4</td>
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</table>

NOTE: Cells were seeded and infected with AdALAE1a, AdE1aALAE1b, or Ad-WT. The supernatants were harvested for titer assay as described in Materials and Methods.

Viral yield is the LD_{50}, value of the titer of viral particles produced in each cell line in replication assay. LD_{50} value was the dilution factor that caused a cytopathic effect in at least four of eight wells of cells in a row on a 96-well plate on day 7 after addition to HEK293 cells.

Figure 4. Tissue-specific viral replication and cell killing by AdALAE1a and AdE1aALAE1b. A, AdALAE1a and AdE1aALAE1b showed breast cancer-specific replication activity. MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145 cells were infected with standardized doses of AdALAE1a or AdE1aALAE1b and viral replication assay was done. Ad-WT was used as a control. The value is expressed as a log_{10} scale, such that a value of 0 indicates that the therapeutic virus has the same replication activity as a wild-type virus in a particular cell line and that a value of −1 indicates that the therapeutic virus has 10 times less replication activity compared with a wild-type virus in a particular cell line. B, AdALAE1a and AdE1aALAE1b showed breast cancer-specific killing activity. Serial dilutions of AdALAE1a or AdE1aALAE1b were applied to MCF10A, MCF12A, MDA-MB-435S, T47D, PC-3, and DU145 cells and cell-killing assay was done as described in Materials and Methods.
killing was analyzed. As shown in Fig. 4B, AdALAE1a and AdE1aALAE1b were able to kill MDA-MB-435S and T47D cells at the same doses as Ad-WT. On the other hand, AdALAE1a and AdE1aALAE1b required much higher doses than Ad-WT to kill MCF10A, MCF12A, PC-3, and DU145 cells. AdALAE1a required 100-fold more virus than Ad-WT to kill MCF10A, 500-fold more to kill MCF12A, 100-fold more to kill PC-3, and 1,000-fold more to kill DU145. AdE1aALAE1b required 1,000-fold more virus than Ad-WT to kill MCF10A, 1,000-fold more to kill MCF12A, 10,000-fold more to kill PC-3, and 5,000-fold more to kill DU145. These data indicated that the killing activity of AdALAE1a and AdE1aALAE1b was the same as Ad-WT toward breast cancer cells and that both AdALAE1a and AdE1aALAE1b exhibited significantly better cell killing in breast cancer cells compared with normal breast cells or prostate cancer cells.

AdE1aALAE1b Was Highly Effective in Inhibiting the Growth of Hormone-Refractory MDA-MB-435S Breast Tumor In vivo

Our in vitro data showed that AdE1aALAE1b achieved better breast cancer–specific replication than AdALAE1a. We then examined the efficacy of AdE1aALAE1b in inhibiting the tumor growth of hormone-refractory MDA-MB-435S breast cancer cells in athymic nude mice. We found that the tumor growth rate in the AdE1aALAE1b-treated group was much lower than the PBS-treated group (P < 0.001) or the AdPSES-Luc-treated group (P < 0.001; Fig. 5). This result suggested that AdE1aALAE1b greatly inhibited breast tumor growth and was a potent therapeutic agent for breast cancer gene therapy. Although one tumor in each of the PBS or AdPSES-Luc groups showed regression, the tumor size for these two tumors were the smallest in each group when injected with PBS or AdPSES-Luc. It is very likely that these tumors were not the established tumors at the time of injection, but the residual nodules.

Tumor histology was evaluated conventionally. In AdE1aALAE1b-treated tumors, we found bigger patchy and uneven necrotic areas inside the tumor (Fig. 6A), separated from the tumor tissues by strands of the connective tissue component of the tumor, with the cell nuclear compartments squeezed to the side (Fig. 6B). This phenomenon is rare in the control virus and PBS-treated groups (Fig. 6C and D). We estimated that AdE1aALAE1b-treated tumors contain ~70% necrotic tissue area and that AdPSES-Luc-treated tumors contain ~38% necrotic tissue area. Student’s t test indicated that AdE1aALAE1b-treated tumors contain significantly larger necrotic tissue area (P < 0.01). Anti-adenovirus immunohistochemical staining using antibody against Ad5 revealed that viral-infected cells could be detected in the AdE1aALAE1b-treated group, in the bordering areas of tumor necrosis and around the fibrosis (Fig. 6E), which illustrated persistent viral protein expression in AdE1aALAE1b-treated tumors. Anti-adenovirus staining was absent in AdPSES-Luc- and PBS-treated tumors (Fig. 6F). In situ TUNEL assays were done to detect apoptotic bodies in the AdE1aALAE1b-treated tumors. Dark brown nuclear-stained cells were found around the bordering areas of the tumor necrosis and the fibrosis (Fig. 6G), but no dark brown cells could be found in either the tumor or necrosis areas (Fig. 6H), which

Figure 5. AdE1aALAE1b inhibited MDA-MB-435S breast tumor formation in vivo. MDA-MB-435S breast tumors were established s.c. in athymic nude mice and treated with AdE1aALAE1b, AdPSES-Luc (a replication-deficient adenovirus used as a negative control), or PBS via intratumoral injection. A, average of tumor growth in each group. B, individual tumor growth in each group.
reflected programmed cell death in the tumor-killing process. Apoptotic cells with DNA fragmentation could also be identified in H&E-stained section in AdE1aALAE1b-treated tumor (Fig. 6I).

Discussion

Currently, effective therapy for breast cancer treatment is still limited to estrogen receptor (ER)–positive tumors that respond well to hormonal therapy. Few choices are available for hormone-independent breast cancer. Several clinical trials have been launched to evaluate the safety and efficacy of adenoviral-based gene therapy in cancer. The most exciting results come from the study of TRRA that allows virus to propagate from a limited number of infected cells to the whole tumor mass, overcoming the problem of inadequate in vivo infectivity or biodistribution of adenovirus. TRRA has been explored to treat breast cancers in animal models (44, 45) by using estrogen and hypoxia-responsive elements to limit the adenoviral replication in ER-positive cancer cells and in a hypoxic condition. Because the majority of ER-positive breast cancers can be effectively treated by hormonal therapy, the application of this strategy is limited for breast cancer treatment.

In this report, we explored the use of α-lactalbumin promoter to control adenoviral replication in a breast cancer–specific manner. α-Lactalbumin is expressed in >60% of breast cancer tissues, and its promoter is active in both ER-positive T47D and MCF7 breast cancer cells and ER-negative MD-MBA231 breast cancer cells (34). We also detected its high activity in hormone-refractory MDA-MB-435S cells. Our demonstration that α-lactalbumin promoter can specifically control adenoviral replication in breast cancer cells exhibiting high α-lactalbumin transcriptional activation supports the idea that retargeting breast cancer cells is feasible using a promoter-based strategy. Other reports (18, 19) have used MUC1 antigen promoter to target breast cancer cells expressing MUC1 (such as MCF7 and T47D cells). MUC1 promoter-based TRRA has no efficacy in MUC1-negative cells (such as MDA-MB-231 cells). Interestingly, MCF7 and T47D cells are ER positive and MDA-MB-231 cells are ER negative. Therefore, these studies showed that MUC1-based adenoviral vector has no therapeutic efficacy in ER-negative MDA-MB-231 cells. This is different from our α-lactalbumin-based TRRA; α-lactalbumin promoter-based TRRA does have therapeutic efficacy in ER-negative breast cancer cells. For treating ER-negative breast cancer, our α-lactalbumin-based TRRA seems to be a better choice than MUC1 promoter-based TRRA.

Some studies have explored the advantages and disadvantages of using promoters to control the expression of Ad5 E1a and/or E1b. Hsieh et al. observed that two copies of the same promoter that drive E1a and E1b genes resulted in the deletion of the E1a sequence during viral replication (15). Juxtaposing promoters with a homologous region could result in homologous recombination and deletion of transgenes important for viral expression. Conversely, juxtaposing promoters with heterogenous sequences could prevent homologous recombination and result in promoter competition and the squelching of transcription factors.
Using a single promoter to drive both E1a and E1b genes could avoid the homologous recombination, promoter competition, and squelching of transcription factors during gene transcription (15). In this report, the breast cancer–specific replication of AdE1aALAE1b was better than AdALAE1a, indicating that the TRRA with both E1a and E1b genes controlled by tissue- or tumor-specific promoter exhibited higher tumor specificity. Yu et al. also reported that controlling both Ad5 E1a and E1b genes achieved better tissue/tumor-restricted viral replication than controlling the E1a genes alone (39, 40). The growth inhibition of the MDA-MB-435S tumors showed the therapeutic potential of AdE1aALAE1b for hormone-refractory breast cancers.

It is interesting to note that the expression of E1a protein was higher in AdALAE1a-infected than in AdE1aALAE1b-infected breast cancer cells. The possible reason is that the α-lactalbumin promoter is next to the ITR of the adenoviral genome in AdALAE1a. Somehow, the enhancer elements located in the ITR enhance the α-lactalbumin promoter activity in a breast cancer–specific manner. However, we could not rule out the possibility that this enhancing effect might result in higher, undesirable basal level activity in certain organs. With this consideration, AdE1aALAE1b presents another way to avoid complications with adenoviral ITR.

Histologic evaluation of AdE1aALAE1b-treated MDA-MB-435S tumors revealed that the tumors were surrounded by strands of connective tissue, possibly protecting them from adenoviral infection. This may explain why AdE1aALAE1b could not completely eliminate the tumor in our study. This represents a critical problem to overcome for the future success of TRRA-based gene therapy. Incorporation of proteases in TRRA may be a practical way to break down connective tissues within the tumor mass and further enhance the therapeutic efficacy of TRRA. The observation that cells stained positive for adenoviral proteins and apoptosis were located in the same areas bordering the tumor necrotic center indicated persistent viral replication accompanied by cell killing in the tumors. Because adenovirus expresses apoptosis-inducing proteins (46, 47) at a late stage of infection to facilitate cell lysis and release of adenovirus from infected cells, it is not surprising to see apoptotic cells in the area of viral replication. In TUNEL assays to identify apoptotic cells, we found that not all positively stained cells showed characteristic apoptotic features. However, apoptotic cells with DNA fragmentation could be also identified in regions bordering the tumor necrotic center in H&E-stained slides. In a recent report, we also observed that not all positively stained cells showed characteristic apoptotic features in TUNEL assay (41).

Anderson et al. (16) have shown that α-lactalbumin promoter was strongly active in murine lactating normal breast tissue. However, the model used in this report was a lactating BALB/c mouse model. It is true that α-lactalbumin promoter activity will be high in normal lactating mammary gland, because α-lactalbumin is a milk protein present in lactating mammary gland. However, α-lactalbumin activity is extremely low in normal mammary gland in nonlactating animals and in normal human breast epithelial cells, suggesting that α-lactalbumin-based TRRA will not replicate in normal breast tissue. Furthermore, most breast cancer patients will not undergo lactation once cancer is diagnosed. Therefore, AdALAE1a or AdE1aALAE1b will not be activated in nonlactating normal breast tissue and should not pose any risk in nonlactating nonmalignant tissue. In a worst case scenario, breast tissue is not a vital organ and a small degree of damage to normal breast epithelial cells should be tolerable in light of the potential benefits of treating advanced breast cancer. Our experimental approach presented in this article is a proof-of-concept strategy to show the usefulness of α-lactalbumin promoter-based TRRA in treating breast tumors. To further reduce tumor recurrence, systemic delivery of α-lactalbumin promoter-based TRRA could be used to treat breast tumor metastasis, a major cause of breast cancer mortality.

In conclusion, we have developed and compared two BRRAs controlled by α-lactalbumin promoter and showed that a single α-lactalbumin promoter can control both E1a and E1b gene expression to achieve breast cancer–restricted control of adenoviral replication. α-Lactalbumin promoter-based BRRAs replicate as efficiently as wild-type adenovirus in both hormone-dependent and hormone-independent breast cancer cells but not in normal breast cells or prostate cancer cells. These promising results warrant further development of an α-lactalbumin promoter-based gene therapy modality.

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Transcriptional targeting modalities in breast cancer gene therapy using adenovirus vectors controlled by $\alpha$-lactalbumin promoter

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