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A new targeting approach for breast cancer gene therapy using the Heparanase promoter

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Abstract

Gene therapy with adenoviral (Ad) vectors is a promising new approach in the treatment of cancer. Strategies to restrict adenoviral-mediated transgene expression are important to avoid gene transfer into normal cells. Heparanase (HPR) is overexpressed in breast cancer but downregulated in differentiated normal tissue. Expression of the HPR gene was evaluated in breast cancer cells. Biodistribution and liver tropism was evaluated in a mouse model. HPR is highly expressed in breast cancer tissue. The HPR promoter retained its fidelity in an adenovirus context and was activated in breast cancer cells but showed low activity in normal breast cells and the murine liver. We conclude that the HPR pathway is a promising target for the development of breast cancer directed gene therapy strategies

Keywords: Heparanase; Tissue-specific promoters; Transcriptional targeting; Biological therapy

1. Introduction

Breast cancer is a leading cause of death in women in the United States. This year, an estimated 192,200 new cases will be diagnosed, and 40,800 women will die of the disease, making breast cancer second only

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to lung cancer as the cause of cancer death in women in the United States [15]. Despite surgical removal of the primary tumor in patients with apparently local disease, relapse at local or distant sites may occur because of the presence of micrometastases at the time of the diagnosis. Treatment of metastatic breast cancer with chemotherapy is often empirical and based on histological tumor parameters, in the absence of individual molecular characteristics. Once breast cancer becomes resistant to hormone- or chemotherapy, there is no effective therapy at present. Consequently, survival of patients with metastatic breast cancer remains poor.

One recent novel approach for breast cancer is gene therapy, representing a targeted therapeutic intervention. Ad-mediated gene therapy is a new therapeutic approach and has been shown to achieve efficient in situ gene delivery to tumors [8,9,17]. In the context of adenovirus-based gene therapy, a variety of therapeutic strategies have been explored for advanced breast cancer including antiangiogenesis gene therapy [12] and suicide gene therapy [4]. Unfortunately, Ad vectors can have unacceptable side effects due to inefficient restriction of the toxic gene to target cells and subsequent delivery of genes at non-tumor sites such as the liver [2,3,10]. The risk of incidental toxicity is especially relevant for systemic application of Ad vectors due to widespread expression of the primary adenovirus receptor CAR [25]. Therefore, strategies to restrict adenoviral-mediated transgene expression to target cells are needed. The strategy of transcriptional targeting is based upon the use of promoters that display preferential display in tumor cells [19]. An ideal tumor specific promoter (TSP) for transcriptional targeting exhibits a selective 'tumor on' phenotype. To mitigate hepatotoxicity upon systemic delivery, candidate promoters additionally exhibit a 'liver off' phenotype. However, TSPs for breast cancer gene therapy have not been systematically explored here-to-fore. Heparanase (HPR) is a heparan sulfate-specific *endo-β*-D-glucuronidase which plays an important role in tumor cell metastasis due to its capability of cleaving heparan sulfate and degrading extracellular matrix heparan sulfate proteoglycans [11]. HPR has been implicated in many important physiological and pathological processes including tumor cell metastasis, angiogenesis and leukocyte migration [18,20,28]. Furthermore, HPR is expressed in many tumor types including hepatocellular carcinoma [29], gastric cancer [24], malignant melanoma [23], head and neck cancer [21] and metastatic breast cancer [16]. Based upon these considerations, we performed this study to evaluate the usefulness of the HPR promoter in the context of Ad-based gene therapy applications for breast cancer. A 1.8-kb upstream sequence of human HPR gene, containing the promoter region, was inserted into an Ad vector and used to drive expression of the luciferase reporter gene. This vector was then assessed in vitro and in vivo for activity and tumor specificity of transgene expression.

2. Materials and methods

2.1. Cell lines

The human breast cancer cell lines MDA-MB-435 (ductal carcinoma) was a kind gift of Dr J. Price, M.D. Anderson Cancer Center (Houston, TX). The normal human breast epithelial cell line MCF-12A and the breast cancer cell lines MCF-7 and MDA-MB 231, derived from adenocarcinomas of mammary gland, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). GI-101 is an estrogen-independent metastatic human breast cancer cell line and has been obtained from the Rumbaugh–Goodwin Institute for Cancer Research (Plantation, Florida). The transformed human embryonic kidney cell line, 293, was obtained from Microbix (Toronto, Canada). All cell lines were cultured in recommended media, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 25 μg/ml streptomycin, and 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were maintained in a humidified 37 °C incubator containing 5% carbon dioxide.

2.2. Primary breast cancer samples

Human primary breast cancer samples were obtained from patients with breast cancer and grossly visible lesions who underwent surgery for primary treatment. Tissue was obtained from these lesions prior to initiation of surgery and following removal of the surgical specimen and confirmed to be breast

cancer by a clinical pathologist. Ethical approval has been obtained and all patients gave informed consent prior to initiation of surgical procedure. To obtain purified primary breast cancer cells, tissues were mechanically disrupted and digested with 0.1% Collagenase type III (Life Technologies, Grand Island, NY). Epithelial cells were isolated from the digest by differential centrifugation followed by culture in selective media as previously described [22]. These cells thus prepared were primarily tumor cells (≧95%) as judged by morphologic examination following H&E staining. To generate breast cancer tissue slices, tissue was cut in consecutive 0.5 mm-thick slices using the Krumdieck tissue slicer (Alabama Research and Development Corporation, Birmingham, AL). Sequential slices were then cultured in 24 well-plates in RPMI medium supplemented with 10% bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml insulin. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Three tissue slices were included per group.

2.3. Adenoviral vector constructs

E1-deleted, replication-defective adenovirus vectors based on human adenovirus serotype (Ad5) were used for this study (Fig. 1). The human Heparanase promoter was previously described [5,26]. To create the recombinant Ad vector AdHPRLuc encoding firefly luciferase under control of the HPR promoter, we used the 'AdEasy' method as reported previously [7]. Briefly, the luciferase reporter gene from pGL3 basic (Promega, Madison, WI), was excised as a *KpnI-SalI* fragment and ligated into *pShuttle* (Quantum Biotechnologies, Montreal, Canada). The Heparanase promoter was excised from pXP1 (based upon pBR322 plasmid) with *Bgl2* and *SacI* and then inserted into the multiple cloning site of *pShuttle* to create *pShuttle.HPR.Luc*. The resulting plasmid was linearized using *PmeI* and subsequently co-transfected into *Escherichia coli* BJ5183 with the pAdEasy-1 backbone plasmid. Recombinant Ad was generated by homologous recombination with pAdEasy1 in *E. coli*. The control vectors AdCMVluc, AdSLPILuc and AdVEGFLuc are replication-defective adenoviruses with a luciferase reporter gene, driven by the CMV promoter and vascular endothelial growth factor promoter (VEGF), respectively and have been reported previously [1,13,30]. The viruses are isogenic and were propagated in 293 cells and purified by double CsCl density centrifugation. Physical particle concentration (viral particles (vp)/ml) was determined by OD_{260} reading, and functional virus titers (plaque-forming units (pfu)/ml) were determined by plaque assay in 293 cells. The vp/pfu ratio was 9.7, 23.4, 28.3 and 25.1 for AdCMVluc, AdHPRLuc, AdSLPILuc and AdVEGFLuc, respectively.

2.4. In vitro gene transfer

Cell lines were plated on day 1 at 30,000 cells/well on 24-well plates in 1 ml of 10% growth medium (GM). On day 2, cells were infected with recombinant adenoviruses at 10 pfu/cell for 2 h in 200 μl of 2% GM on a rocker. Afterwards, cells were washed once with 1 ml of PBS, and 1 ml of 10% GM was added per well. Purified breast cancer primary cells were plated on day 1 at 10,000 cells/well on 96 well plates in 100 μl GM on a rocker. On day 2, cells were infected with 5 and 50 pfu/cell for 2 h in 20 μl of 2% GM on a rocker. Breast cancer tissue slices were infected in

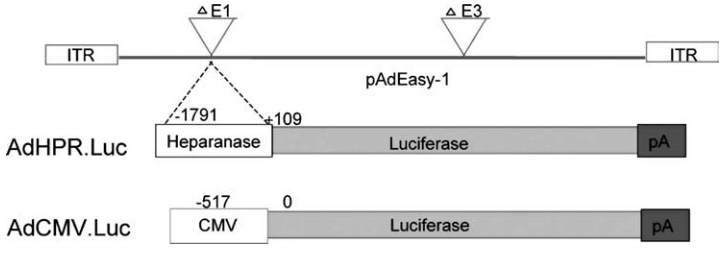

Fig. 1. Structures of Recombinant Adenoviruses. AdHPRLuc was constructed by inserting a DNA fragement in E1 deleted region using KpnI-Sal I restriction sites. This DNA fragment contains the 1860 bp HPR promoter, as well as full length of luciferase cDNA, and simian virus 40 polyadenylation signal. The control vector AdCMVLuc contains the CMV promoter and is otherwise isogenic to AdHPRLuc.

24 well plates with 5 and 50 pfu/cell for 2 h in 100 μl of 2% GM on a rocker. Cell number for tissue slices was estimated at 1×10^6 cells per slice based on a 10-cell thick slice (250 μm) and 8-mm slice diameter. Afterwards, cells/tissue slices were washed once with PBS, and 60 μl 10% GM was added per well. After 24 h, the GM was removed; cells were washed once with PBS and lysed with 200 μl (cell lines and tissue slices) or 20 μl (primary cells) of lysis buffer (Reporter Lysis Buffer; Promega, Madison, WI) and freeze-thawed once. Twenty microlitres of these samples were mixed with 100 μl of luciferase assay reagent (Promega) and measured with Berthold Lumat LB 9501. Standardization was accomplished by setting values obtained with AdCMVluc as 100% for each cell line.

2.5. RNA Preparation and quantitative RT-PCR

Total cellular RNA of tumor cells was extracted from 2×10^5 cells using the RNeasy mini prep kit (Qiagen, Santa Clarita, CA) and treated with DNase I (Life Technologies, Rockville, MD) for 30 min. PCR products from Heparanase were used for creation of the standard curve. GeneAmp RNA PCR core kit (Applied Biosystems) was used for cDNA synthesis and PCR amplification of cDNA products. TaqMan primers and probes were designed by the Primer Express 1.0 software and synthesized by Applied Biosystems (Foster City, CA). The sequences to amplify the Heparanase gene were forward primer, 5′-TTT TCC AGG TGG TTG AGA GC-3′, reverse primer 5′-TCC GCT CCA TAT GCA GAG-3′ and probe CCT AAC CAG ACC TTC TTG CCA GGC CTG 6F AM-TAMRA.

Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as house keeping gene for internal control. The sequences to amplify GAPDH gene were forward primer 5′-GGT TTA CAT GTT CCA ATA TGA TTC CA-3′, reverse primer 5′-ATG GGA TTT CCA TTG ATG ACA AG-3′ and probe 6FAM-CGT TCT CGC CTT GAC GGT GCC AT-TAMRA. With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a final volume of 9 μl per reaction containing 1X TaqMan® EZ RT-PCT Kit (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 100 nM probe and 0.025%BSA. For the assay, pre-determined amount of GAPDH template DNA (10^8, 10^6, 10^4 and 10^2 copies/μl) was amplified to generate a standard curve for quantification of the GAPDH copy numbers of study samples. Pre-determined amount of human total RNA (200, 20, 2 and 0.2 ng/μl) was amplified to generate a standard curve for determination of the concentration of study samples. One microlitre of total RNA sample was added to 9 μl of PCR mixture in each reaction capillary. A no template control received 1 μl of water. All capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, Indiana) to facilitate mixing. All PCR reactions was carried out using a LightCycler™ System (Roche Molecular Biochemicals, Indianapolis, Indiana). Thermal cycling conditions were subjected to 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C and 40 cycles of 20 s at 94 °C and 1 min at 60 °C. Data were analyzed with LightCycler software.

2.6. Animal experiments

Mice were obtained at 4–6 weeks of age and quarantined at least 1 week before the study. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of UAB.

2.7. In vivo gene transfer

For determination of luciferase gene expression in mouse organs, C57/BL6 mice (Charles River Laboratories; $n=8$/group) received 1×10^9 pfu of AdHPRLuc, or AdCMVLuc i.v. through the lateral tail vein in a volume of 300 μl of Opti-MEM, or Opti-MEM only. After 48 h mice were killed and livers, kidneys, lungs, spleens and hearts were harvested and representative sections were snap frozen. The frozen organ samples were ground to a fine powder using a mortar and pestle cooled in a dry ice-ethanol bath. Organ powders were lysed using Cell Culture Lysis Buffer (Promega) at room temperature for 20 min. Lysates were frozen once, then centrifuged at 10, $000\times g$ for 15 min. Luciferase activity was measured

as before. Mean background luciferase activity was subtracted from the data. The luciferase activity was normalized by protein concentration in the tissue lysate.

2.8. Statistics

Data are presented as a mean value ± standard deviation. For the in vivo gene expression patterns and the mRNA copy number analysis, results with the HPR promoter constructs were compared with other groups using a two tailed student's *t*-test where a P (*) value <0.05.

3. Results

3.1. Evaluation of HPR mRNA Expression in breast cancer cell lines and human primary breast cancer samples

Quantitative RT-PCR was performed to examine the level of HPR mRNA relative to a housekeeping gene, GAPDH in the breast cancer cell lines GI 101, MDA MB-435, MDA MB-231 and MCF7. The normal human breast epithelial cell line MCF-12A was used as negative control. Whereas similar levels of expression were seen for the housekeeping gene GAPDH among all cell lines used (not shown), the mRNA levels of the HPR gene varied between the different cell lines examined. Of note the mRNA signals for HPR in GI 101, MDA MB-435, MDA MB-231 are 4.5–44.6 fold ($P<0.05$) increased compared to the negative control cell line MCF-12A (Fig. 2A). Interestingly, the low metastatic breast cancer cell line MCF 7 only shows slightly increased mRNA level for HPR compared to MCF-12A cells, whereas GI 101, MDA MB-435, MDA MB-231, known for their high metastatic potential in nude mice [27], showed significantly higher HPR mRNA expression. To more closely model, the clinical situation with the most stringent substrate, we compared HPR expression in six primary breast cancer patient samples and four healthy breast tissue samples from patients who underwent breast reconstructive surgery. In primary breast cancer samples, the average HPR mRNA copy number was significantly (90 fold, $P<0.05$) increased compared to the normal breast tissue samples (Fig. 2B).

3.2. Evaluation of the HPR promoter activity in breast cancer cell lines and breast cancer tissue samples

After having detected high expression levels of the HPR gene, we tested the activity of the HPR promoter in breast cancer cell lines and primary breast cancer tissue samples. To this end, we constructed a nonreplicative Ad, AdHPRLuc, where the HPR promoter was placed upstream of the luciferase reporter gene. Promoter activity was compared to that of the constitutive cytomegalovirus (CMV) promoter and promoters for vascular endothelial growth factor (VEGF) and secretory leucoprotease inhibitor (SLPI) (Fig. 3A). The VEGF and SLPI genes have recently been shown to

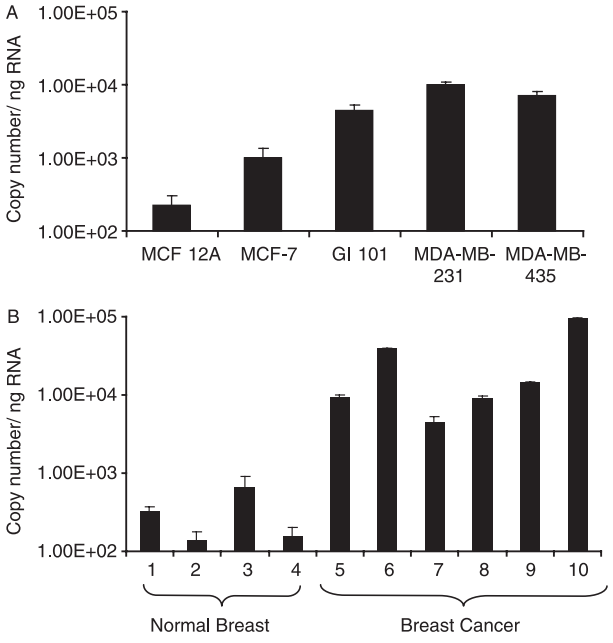

Fig. 2. The human HPR gene is upregulated in established and primary breast cancer cells. (A) RNA was extracted from four breast cancer cell lines (MCF-7, GI 101, MDA-MB-231, MDA-MB-438) and was reverse-transcribed into cDNA. The normal breast cell line MCF 12A was used as negative control. (B) RNA was extracted from six human primary breast cancer patient samples and four normal breast tissue samples and reverse-transcribed into cDNA. Real-time PCR analysis was performed to quantify the expression of the HPR gene. The gene copy numbers are normalized by the GAPDH copy number. Each bar represents triplicate experiments. Error bars indicate SD.

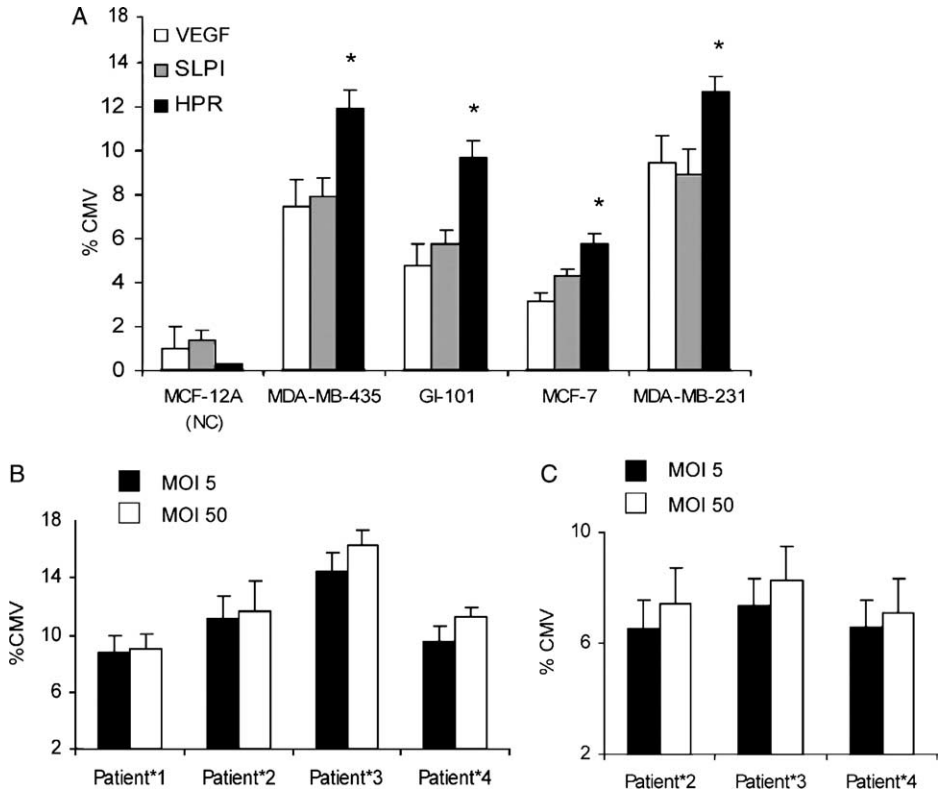

Fig. 3. The human HPR promoter is specifically activated in breast cancer. Breast cancer cell lines (A), purified primary breast cancer cells (B) and tissue slices of primary breast cancer tissue (C) were infected with adenoviruses containing *luciferase* regulated by the CMV promoter, HPR promoter, VEGF promoter and SLPI promoter, respectively at an MOI of 10. Primary patient samples were infected at two different viral doses: MOI 5 and 50. Luciferase activity of candidate promoters is expressed as relative light units (RLU) as a percentage of the CMV promoter-driven activity. Each point represents the mean of three experiments. Error bars indicate standard deviation. $*P<0.05$ versus VEGF and SLPI promoter-driven activity.

be frequently overexpressed in breast cancer tissues. At a viral dose of 50 pfu/cell, gene expression controlled by the HPR promoter resulted in 11.9, 9.7, 12.7 and 5.5% of the expression achieved with the CMV promoter in MDA MB-435, GI 101, MDA MB-231 and MCF7 cells, respectively. AdHPRLuc displayed a significantly ($P<0.05$) increased expression rate compared to both, Ad5SLPILuc (5.8–8.9% of the CMV promoter activity) and Ad5VEGFLuc (4.8–9.5% of the CMV promoter activity) in the highly metastatic, estrogen receptor negative cell lines MDA MB-435, GI 101, MDA MB-231. The negative control cell line MCF-12A displayed a significantly lower promoter activity with 0.3% of the CMV promoter activity. The promoter activity of AdHPRLuc reflects the HPR gene expression levels in the breast cancer cell lines (Fig. 2).

To more closely model the clinical situation with the most stringent substrate, gene transfer experiments were performed on human primary breast cancer cells. Purified, unpassaged breast cancer cells (Fig. 3B) were examined as well as precision cut breast cancer tissue slices (Fig. 3C). At a viral dose of 50 pfu/cell, gene expression controlled by the HPR promoter resulted in 8.8–14.4% of the expression achieved with the CMV promoter in purified breast cancer cells and 6.6–7.3% of CMV promoter activity in breast cancer tissue slices.

3.3. The human HPR Promoter is repressed in the murine liver

A key limitation to the use of a systemic gene therapy approach is the potential toxicity to non-target

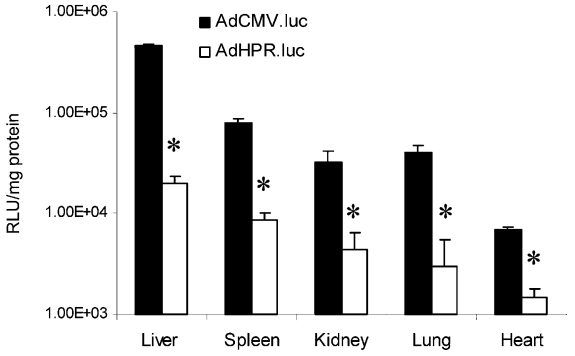

Fig. 4. The human HPR promoter is repressed in the murine liver. HPR promoter activity in major organs. Fourty-eight hours after tail vein administration of HPR or CMV promoter-driven expression vectors (AdHPRLuc and AdCMVLuc, 10^9 pfu), Organs were harvested and luciferase activities were analyzed in five major organs. The luciferase activities are shown as RLU/mg protein. Error bars indicate standard deviation. *$P<0.05$ versus AdCMVLuc.

organs. Due to the high tropism of Ad-based gene therapy vectors to the liver, we were especially interested to determine whether the HPR promoter would have a low liver activity in vivo. To this end, AdHPRLuc and AdCMVLuc (as a positive control) were injected intravenously (i.v.) into mice. At 48 h, liver, spleen, kidney, lung and heart were harvested and luciferase activity and protein concentration of tissue lysate were measured. In this assay, transgene expression induced by the HPR promoter was a mean 11.5-fold less expressed than that with the CMV promoter. The AdHPRLuc-induced expression in the liver was 23.3 fold ($P<0.05$) lower compared to that of AdCMV.Luc (Fig. 4). These results demonstrate that the HPR promoter in the context of an Ad vector possesses the key element, of repression in the liver, for consideration for use for cancer gene therapy.

4. Discussion

Transcriptional targeting is a useful approach for Ad targeting as it seeks to genetically limiting gene expression to specific cells through the use of tissue specific promoters (TSP). Although a wide range of promoters have been evaluated in different tumor types, TSPs for breast cancer should be systematically explored. In the present study, we demonstrate that the HPR promoter has tissue specific fidelity in the Ad backbone. These findings indicate that this promoter may be a good candidate for breast cancer specific gene therapy. When we evaluated HPR promoter-driven reporter gene expression in the context of Ad vector gene transfer, on average the HPR promoter activity reached 10% of the level observed for the highly active CMV promoter in breast cancer cell lines. More importantly in purified primary breast cancer patient samples the values were even slightly higher. This level of promoter activity can be regarded sufficient for driving therapeutic genes or oncolytic conditionally replicative adenovirus (CRAD) vectors in vivo. Recent studies have shown that estrogen receptor-negative breast tumors, which may account for the more aggressive metastatic phenotype, poor prognosis and failure of antiestrogen therapy, have shown increased HPR expression [16,26]. Another study [5] has demonstrated the estrogen responsiveness of the Heparanase gene promoter in estrogen receptor-positive breast cancer cell lines. These results are consistent with our findings of a significantly higher HPR gene expression rates and HPR promoter activitiy in three highly metastatic, receptor-negative cell lines compared to a weakly metastatic, estrogen receptor-positive cell line. The recently developed cDNA microarray technique has led to the identification of genes for SLPI and VEGF, which are overexpressed in breast cancer but not in the liver [6,31]. We have used isogenic Ads to compare HPR, SLPI and VEGF promoter driven gene expression in established breast cancer cell lines. Among these promoters, highest activity was seen for the HPR promoter. It is important to note, that the normal human breast epithelial cell line MCF-12A, showed a significantly lower activity relative to the CMV promoter. The high CMV-to-HPR promoter ratio in non-breast cancer cell types indicates the relative specificity of the HPR promoter, which could be used to mitigate toxicity to normal cells after local or systemic administration in vivo. The data generated with primary breast cancer tissue from patients with advanced or metastatic breast cancer confirm the results obtained from established breast cancer cell lines. To validate the use of HPR as tissue specific promoter for breast cancer gene therapy, we performed experiments with purified primary breast cancer cells as well as organ cultures of primary breast cancer tissue slices. The latter displayed a lower HPR

promoter activity compared to the purified cancer cells. This can be explained with methodological differences, specifically the fact that tissue slices, obtained with precise and reliable automated slicers, represent a tissue portion with well-saved tissue architecture [14] including non-tumor cell types such as fibroblasts. In primary breast cancer tissue samples and all breast cancer cell lines tested, levels of HPR promoter activity correlated with levels of HPR gene expression. Another key feature of the HPR promoter is repression in the liver.

In conclusion, our studies delineate the HPR promoter as a novel candidate for transcriptional targeting of breast cancer, by virtue of its very low hepatic activity, high tumor activity and fidelity in the Ad vector. This new approach may have utility not only for breast cancers, but other HPR-positive neoplasms such as cervical cancer, prostate cancer, gliomass and multiple myelomas.

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