

## ORIGINAL ARTICLE

## Lentiviruses with trastuzumab bound to their envelopes can target and kill prostate cancer cells

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In this study, we took advantage of the overexpression of human epidermal growth factor receptor 2 (HER-2) in prostate cancers to design lentiviruses with modified envelope proteins that bind antibodies to specific cell-surface antigens. When bound to trastuzumab (Herceptin, Genentech, CA), lentiviruses were able to selectively infect androgen-sensitive LNCaP and castration-resistant C4-2 human prostate cancer cell lines, both of which express high levels of HER-2. To test for a therapeutic effect, we engineered our antibody-binding lentiviruses to express thymidine kinase, which can convert the non-toxic pro-drug ganciclovir (GCV) into a cytotoxic form. LNCaP and C4-2 cells infected by these viruses were sensitive to GCV killing. *In vivo*, C4-2 xenograft tumors treated either intratumorally or i.v. with trastuzumab-bound lentivirus expressed luciferase, although the latter route was less tumor specific. When a prostate-specific promoter for governing luciferase expression was combined with trastuzumab-mediated delivery, there was a further enrichment in targeting viral gene expression in prostate tumors. In conclusion, we found that although prostate cancers that express high levels of HER-2 are resistant to the killing effects of trastuzumab, they can be targeted for selective gene expression and destruction by viruses with envelope proteins engineered to bind this antibody.

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**Keywords:** prostate cancer; lentivirus; HER-2; trastuzumab; viral gene therapy

## Introduction

Prostate cancer is the most frequently diagnosed non-skin cancer in adult males in North America and is the second leading cause of cancer-related mortality. This year in the United States, an estimated 186 000 new cases will be diagnosed and ~29 000 men will die of the disease.<sup>1</sup> Although standard treatments such as radical prostatectomy, radiation therapy and androgen ablation improve the overall survival rates in prostate cancer patients, the emergence of recurrent, metastatic forms of castration-resistant/androgen-independent prostate cancer continues to be a major challenge, with no cure presently available.<sup>2–4</sup> Clearly, new therapeutic modalities are needed to deal effectively with advanced, end-stage prostate cancer.

The *c-erbB-2* gene (human epidermal growth factor receptor 2; *HER-2*), a member of the family of oncogenes associated with tyrosine protein kinase activity, has been

reported to be overexpressed in metastatic prostate cancer.<sup>5–11</sup> Overexpression of *HER-2*, which causes activation of the PI3k/AKT pathways and promotion of cell proliferation, has been proposed to be a survival factor for prostate cancer cells in the absence of androgens.<sup>10,12</sup> Moreover, this can lead to the activation of the androgen receptor and enhancement of binding of this nuclear receptor to the promoters of androgen-regulated genes.<sup>13</sup> Overall, overexpression of *HER-2* in prostate cancer samples has been associated with lower survival.<sup>7</sup> Although trastuzumab (Herceptin), a humanized monoclonal antibody, which neutralizes the *HER-2* receptor, has been shown to be very effective in treating breast cancers, treatment of patients with prostate cancer showed poor efficacy.<sup>14</sup> Furthermore, systematic administration of trastuzumab, especially in combination with other chemotherapeutic drugs, caused serious side effects, such as cardiotoxicity.<sup>14,15</sup>

A possible alternative therapeutic application of *HER-2* overexpression in advanced prostate cancers would be for targeted, viral gene therapy. In this scenario, pseudotyped viruses with an antibody to *HER-2* on their envelope could bind specifically to the surface of prostate tumor cells, infect the cells and then deliver genes whose expression would mediate cell killing. Lentiviral vectors, derived from human immunodeficiency virus, have been

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proven to be highly efficient, versatile and safe vehicles for gene delivery into mammalian cells.<sup>16</sup> As a retrovirus, lentivirus vector integrates into the cell genome and mediates efficient and long-term expression of the genes transferred.<sup>16</sup> The most commonly used lentivirus vectors are pseudotyped with vesicular stomatitis virus G (VSV-G) protein and possess a wide tropism for different cell types. Pseudotyping of lentivirus has recently been accomplished using Sindbis virus envelope glycoproteins.<sup>17,18</sup> Sindbis virus belongs to the alphavirus family and is classified within the *Togaviridae* virus family (genus Alphavirus).<sup>19</sup> The alphaviruses have highly organized icosahedral protein shells with an associated membrane.<sup>20–22</sup> The outer protein shell contains 240 copies of glycoproteins, E1 and E2.<sup>20,23,24</sup> E1 causes cell fusion, whereas E2 is predominantly the protein responsible for cell membrane attachment.<sup>19,25–29</sup> Importantly, the Sindbis E1 protein can fuse to cells independently of the receptor binding E2 protein.<sup>26</sup> Dubuisson and Rice<sup>30</sup> showed that the receptor-binding properties of Sindbis virus could be disrupted by insertion of short peptides (11 aa) into defined regions of the E2 glycoprotein but with retention of all other replication functions. An IgG-binding domain (ZZ domain) of *Staphylococcus aureus* protein A has been inserted within the E2 glycoprotein<sup>31</sup> and lentivirus or Sindbis vectors have been constructed with this modified envelope. By incubating with any IgG antibody, the ZZ domain and the Fc region of IgG form a tight complex. The Fab regions of the antibody determines the target specificity of the vector for any cell surface antigen.<sup>17,32</sup> Through selection of appropriate antibodies, these customized lentiviruses have been shown to be capable of specifically targeting many different cell types.<sup>17,18,31,33</sup>

In this study, we bound the therapeutic antibody trastuzumab to lentiviruses pseudotyped with an engineered Sindbis virus envelope protein and then used these vectors to mediate very efficient *in vitro* and *in vivo* delivery into androgen-sensitive LNCaP and castration-resistant/metastatic C4-2 human prostate cancer cells for cell-selective expression of either a reporter gene or the therapeutic gene, thymidine kinase, which is able to activate the pro-drug ganciclovir (GCV) into a cytotoxic form.

## Materials and methods

### Cell culture

Prostate cancer cell lines LNCaP, C4-2, PC3 and DU145 were obtained from American Type Culture Collection. The breast cancer cell line, SK-BR3 and its trastuzumab-resistant subline, SK-BR3 P1, were kindly provided by R Nahta (University of Texas, MD Anderson Cancer Center).<sup>34</sup> LNCaP and C4-2 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The other cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were grown at 37°C under a 5% CO<sub>2</sub> atmosphere.

### Immunohistochemistry, immunocytochemistry and western blotting

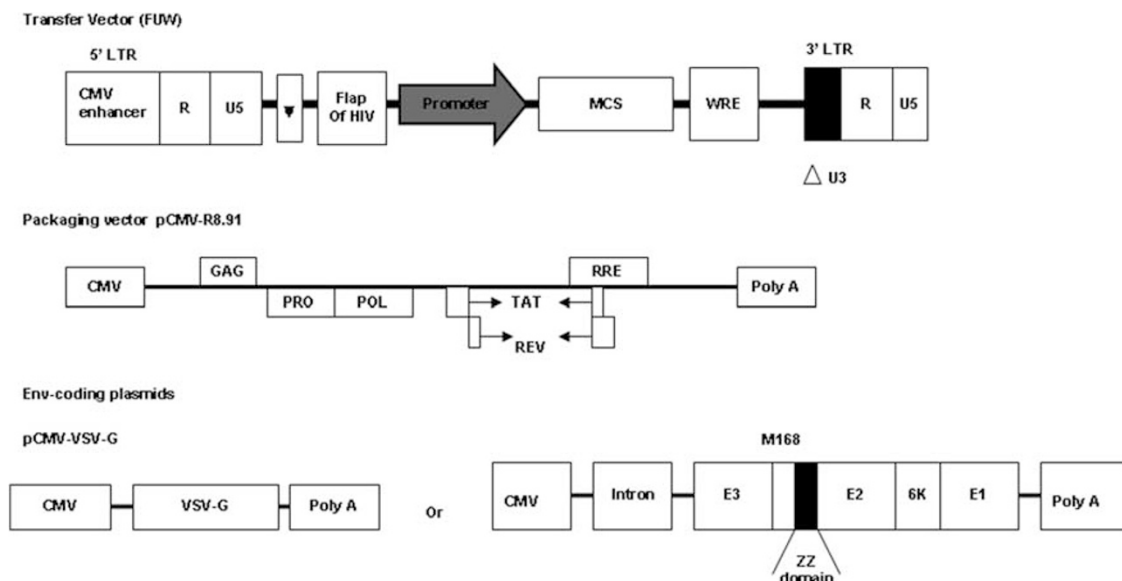
Tissuemicroarrays (TMAs) of human prostate cancer was constructed at the Prostate Centre at Vancouver General Hospital. TMAs were composed of low stage (21 cases) as well as androgen-independent and metastases samples (14 cases). Each tumor was sampled three times for a total number of 105 cores per TMA. The TMAs were stained with monoclonal anti-HER-2 antibody (R&D Systems Inc., Minneapolis, MN) and expression was showed by goat-anti-mouse secondary antibody conjugated to horse radish peroxidase reacting with Nova Red (Vector laboratories, Burlingame, CA). Sections were counter-stained with hematoxylin and then the slide was scanned on a BLISS workstation at ×20 magnification (Bacus Laboratories, Lombard, IL) and reviewed by a pathologist (LF).

To study the expression of HER-2 in prostate cancer cell lines, LNCaP, C4-2, PC3 and DU145 cells were plated on coverslips for 3 days. Cells were washed with phosphate buffered saline and blocked with 5% BSA in phosphate buffered saline for 30 min. After removing the BSA, cells were exposed to anti-HER-2 antibody for 60 min. After washing three times, cells were fixed with methanol for 45 min and then exposed to Alexa 594 conjugated goat anti-mouse antibody. Cells were further counter-stained with 5 μM 4',6-Diamidino-2-phenylindole (DAPI). The coverslips were mounted on the slides with anti-fade mounting medium (Promega Corporation, Madison, WI).

To confirm the expression of HER-2 by western blotting, 20 μg of whole cell lysates from prostate cancer cells, prepared in a lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% NP40 and 5 mM EDTA) in the presence of protease and phosphatase inhibitor cocktail (Sigma, St Louis, MO), were subjected to an 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were first blocked with Odyssey blocking buffer for 30 min and then stained with anti-HER-2 antibody at the dilution of 1:1000. Expression was shown by IRDye 800CW goat anti-mouse IgG and scanned on an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). Equivalent loading controls were performed by blotting β-actin.

### Construction and preparation of lentiviral vectors

The lentiviral vector, FUGW, which expresses enhanced green fluorescent protein (EGFP) under control of an ubiquitin promoter, was a gift from David Baltimore (California Institute for Technology) and was used as the backbone for lentiviral vector construction in this study (Figure 1). Herpes thymidine kinase (hTK) or firefly luciferase coding sequences were amplified by PCR and both *Bam*H1 and *Eco*R1 flanking site sequences were introduced. After digestion of PCR products with *Bam*H1 and *Eco*R1, the coding sequence for hTK or luciferase was purified with a gel purification kit (Invitrogen, Carlsbad, CA) following the manufacture's instruction. The DNA coding for these proteins were subsequently cloned in FUGW vector earlier digested with *Bam*H1 and



**Figure 1** Diagram of plasmid constructs used to prepare the lentiviruses used in this study.

*Eco*R1 and gel purified. The vectors so constructed are referred as FUTK or FULW, respectively. A modified probasin promoter ( $ARR_2PB$ )<sup>35–38</sup> was cloned at *Pac*I and *Bam*H1 site into vector FUGW earlier cut with *Pac*I and *Bam*H1 to remove the ubiquitin promoter. The DNA fragment coding for luciferase was cloned into the vector under the control of the  $ARR_2PB$  promoter to create the lentiviral vector FALW. Authenticity of these constructs was confirmed through sequence analysis.

Preparation of lentiviruses was accomplished using the ProFection Mammalian Transfection System (Promega) according to manufacturer's instructions with some modifications. Briefly, 10  $\mu$ g of each transducing vector, FUGW, FULW, FUTKW or FALW, were mixed with 10  $\mu$ g of packaging vector pR8.91 and 5  $\mu$ g of plasmid encoding either vesicular stomatitis virus glycoprotein (VSV-G) or M168, a plasmid that encodes the Sindbis virus envelope protein modified to bind the Fc fragment of immunoglobulins. In these mixtures, 37  $\mu$ l of  $CaCl_2$  (2 M, pH 7.2) were added and the final volume brought to 250  $\mu$ l with sterile water. The DNA–calcium complexes were further mixed with 250  $\mu$ l of 2  $\times$  HEPES buffered solution for 30 min at room temperature. The mixtures were subsequently added onto 6 ml culture medium in a 10 cm Petridish containing 293T cells at a density of ~75–80%. After 24 h, the medium was replaced with 15 ml of phenol red-free and fetal bovine serum-free Dulbecco's Modified Eagle Medium. The supernatant was collected 48 h later and the cell debris was cleared by centrifugation at 3750 g for 5 min at 4  $^{\circ}C$  in a bench top centrifuge (Beckman, Fullton, CA). The clear supernatants were filtered through a 0.45  $\mu$ m filter and then used to infect cells directly or after further concentration by ultra-centrifugation (126 000 g for 90 min at Beckman ultracentrifuge with the rotor S28). For viruses pseudotyped with modified Sindbis virus envelope protein, the supernatants were filtered through a Centricon Plus-70

filter (Millipore, Billerica, MA) at 2500 g for 20 min and collected at 3500 g for 5 min. Lentiviruses pseudotyped with VSV-G were referred to as FUGW/VSV-G, FULW/VSV-G, FUTKW/VSV-G; those pseudotyped with modified Sindbis virus envelope protein were referred to as FUGW/SB, FULW/SB, FUTKW/SB and FALW/SB, respectively. For this latter group of lentiviruses, the antibodies targeting cell membrane proteins were incubated with the supernatant at room temperature for 30 min and then added to the cells to be infected. Expression of the reporter protein, EGFP, in infected cells was monitored under an inverted microscope (Axiovert 200M, Zeiss, Goettingen, Germany).

#### Quantification of lentivirus

Concentrated or non-concentrated supernatant containing VSV-G pseudotyped lentivirus FUGW/VSV-G virus was serially diluted and used to infect a specific number of LNCaP cells. At 5 days after infection, cells were trypsinized and analyzed by flow cytometry. A transducing unit per ml of virus was defined as percentage EGFP-positive cells after infection with 1 ml of virus multiplied by the number of cells plated for infection. Alternatively, serial dilutions of virus lysed in buffer (0.75 M KCl, 0.45% Tritonx-100, 12.5 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 0.125 mM EDTA and 25% glycerol) were quantified by reverse transcription and amplification using primers for Woodchuck hepatitis B virus response element (forward: 5'-CGGCTGTTGGGCAC TGA-3'; reverse: 5'-CCAAGGAAAGGACGATGATT TC-3') on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) after the SYBR Green PCR Master Mix protocol. Correlation of viral particles with the transducing unit was calculated as amount of viral particles capable of infection of a given amount of cells analyzed through flow cytometry.

### *In vitro cell proliferation assay*

To test the effect of trastuzumab on viability, prostate cancer cells were plated at a density of 100 000 per well in 12-well plates or 10 000 per well in 96-well plates. At 24 h after plating, trastuzumab was added at different concentrations. After 5 days, cells in 12-well plates were collected and their viability was assessed using the trypan blue exclusion method and by direct counting with a haemocytometer (Neubauer, Hausser Scientific, Horsham, PA). The viability of cells grown in 96-well plates was assessed using the CellTiter96 AQueous Non-Radioactive Cell Proliferation Assay MTS assay (Promega).

To test the effects of the cytotoxic pro-drug, GCV (La Roche, Mississauga, ON, Canada), LNCaP cells or C4-2 cells were infected with  $10 \times$  concentrated supernatant containing lentivirus FUGW/SB or FUTK/SB bound to anti-EGFP or trastuzumab, respectively. Expression of EGFP or hTK was first confirmed by immunoblotting cell lysates with anti-epithelial growth factor receptor (EGFR) or anti-hTK. Cells expressing EGFP or hTK after lentiviral infection were grown in 96-well plates in 100  $\mu$ l of media at a density of 10 000 cells per well. A range of concentrations of GCV was added to cells in 100  $\mu$ l medium. After 5 days, cell viability was determined by MTS assay. Data was analyzed using Prism 4.03 (GraphPad, San Diego, CA) and normalized to controls of non-drug treated cells. Each experiment was performed with three replicates and repeated three times.

### *Bioluminescent imaging of prostate cancer tumor xenografts*

We used the castration-resistant and metastatic human prostate cancer cell line, C4-2, to conduct our *in vivo* animal studies. C4-2 xenograft tumors were established by subcutaneous injection of  $2 \times 10^6$  cells in 50  $\mu$ l phosphate buffered saline mixed with 50  $\mu$ l of Matrigel. Briefly, 8-week old male athymic nude mice (Harlan, Indianapolis, IN) were anesthetized with isoflurane and inoculated subcutaneously into the upper left and lower right back of each mouse with 100  $\mu$ l of Matrigel per cell mixture. After 2 weeks, when the tumors were palpable, 100  $\mu$ l of  $100 \times$  concentrated lentivirus FULW/VSV-G or FULW/SB (corresponding to  $\sim 10^9$  viral particles per ml) were incubated with 20  $\mu$ g antibody for 30 min and then injected intra-tumorally or i.v.. To visualize the expression of luciferase in C4-2 tumors, the mice were imaged on days 1, 3, 5 and 10 with an IVIS 200 Imaging System (Xenogen, Alameda, CA) 10 min after intraperitoneal injection of 150 mg per kg luciferin. At 24 h before injection of lentivirus, mice were treated with 80 mg per kg cyclophosphamide by intra-peritoneal injection. The results were analyzed using Living Image software version 2.50 (Xenogen) with 3–4 tumor-bearing animals per group. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care.

### *Analysis of background infection in vivo*

Mice were killed by CO<sub>2</sub> narcosis after imaging, organs from each mouse were excised and genomic DNA was

isolated using a DNeasy kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's protocol. Quantification of the vector copy number in the DNA isolates was performed using real-time PCR as described above.

### *Statistical analysis*

Results of cell viability were expressed as means  $\pm$  s.e.. Statistical tests were performed using Prism 4.03 software and InStat 3.06. *P*-values  $< 0.05$  were considered statistically significant.

## **Results**

### *Expression of HER-2 in human prostate cancer tissue and prostate cancer cell lines*

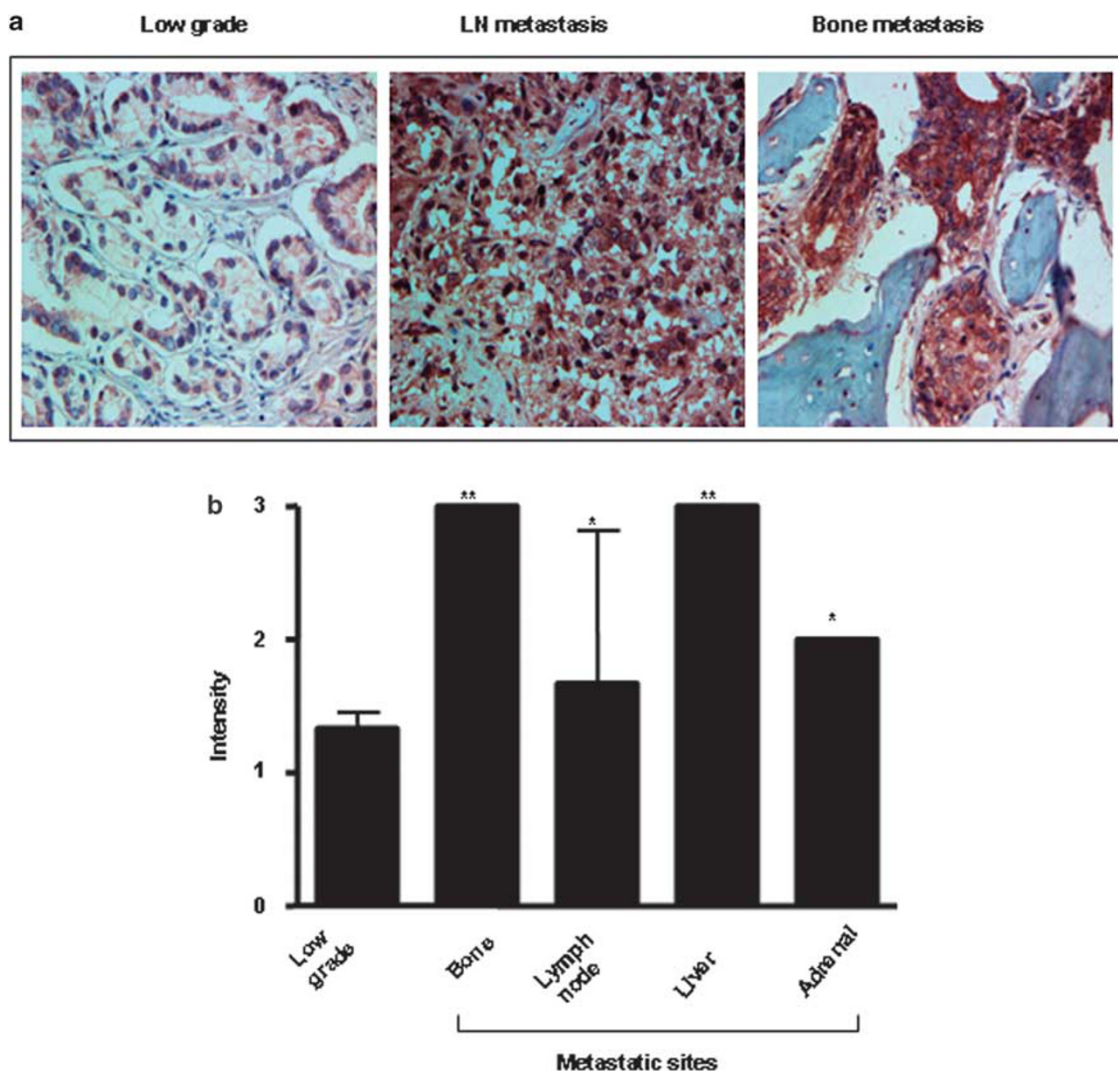
Human epidermal growth factor receptor-2 is a tyrosine kinase and member of the EGFR family, which has been shown to be overexpressed in advanced breast, ovarian and prostate cancers. In prostate cancers, overexpression of HER-2 has been reported to be associated with progression to androgen independence,<sup>5–8,10,11</sup> although some contradictory results have also been reported.<sup>39,40</sup>

To normalize variables such as fixation and staining procedures, which could account for the reported differences, we used a TMA approach where 105 unique prostate tissue cores from 35 patients were immunostained for HER-2 simultaneously. As shown in Figure 2, expression of HER-2 was found in all the prostate cancer cores with the highest intensity associated with castration-resistant metastases from prostate cancer bone, liver and adrenal sites ( $P < 0.001$ ).

With respect to prostate cancer cell lines, immunofluorescent measurement and western blotting for HER-2 expression in androgen-sensitive LNCaP and castration-resistant C4-2 prostate cancer cells showed comparable levels of this protein in each, which were approximately similar to the amount of EGFR detected (Figures 3a and b). Immunoblots for HER-2 with two androgen-receptor negative human prostate cancer cell lines, PC3 and DU145 cells, also showed relatively high levels of this receptor, whereas immunocytochemistry with an antibody recognizing the extracellular domain of HER-2 failed to stain for this receptor (data not shown).

### *Effects of trastuzumab treatment on prostate cancer cell survival*

Although advanced, castration-resistant prostate cancers are associated with high levels of HER-2 expression, the use of trastuzumab as a single agent in clinical trials to treat hormone refractory prostate cancer has shown no therapeutic efficacy.<sup>14</sup> To test whether androgen-sensitive or resistant prostate cancer cell lines are responsive to trastuzumab treatment, we determined, by direct counting of viable cells with a haemocytometer and by MTS assay, the extent of cell survival of LNCaP, C4-2, PC3 and DU145 prostate cancer cells after 5 days of treatment with this monoclonal antibody (Figure 4). Although  $\sim 50\%$  of SK-BR-3 cells, a trastuzumab-sensitive human breast cancer cell line, were effectively killed by exposure to



**Figure 2** TMA analyses of HER-2 expression in low grade and metastatic prostate cancers. (a) Immunohistochemical staining for HER-2 in low-grade prostate cancer as well as castration-resistant lymph node and bone metastasis. (b) Relative mean expression levels ( $\pm$  s.e.) of HER-2 as scored by a pathologist (LF). \*\* $P < 0.001$ ; \* $P < 0.05$ , when compared with low-grade tumor.

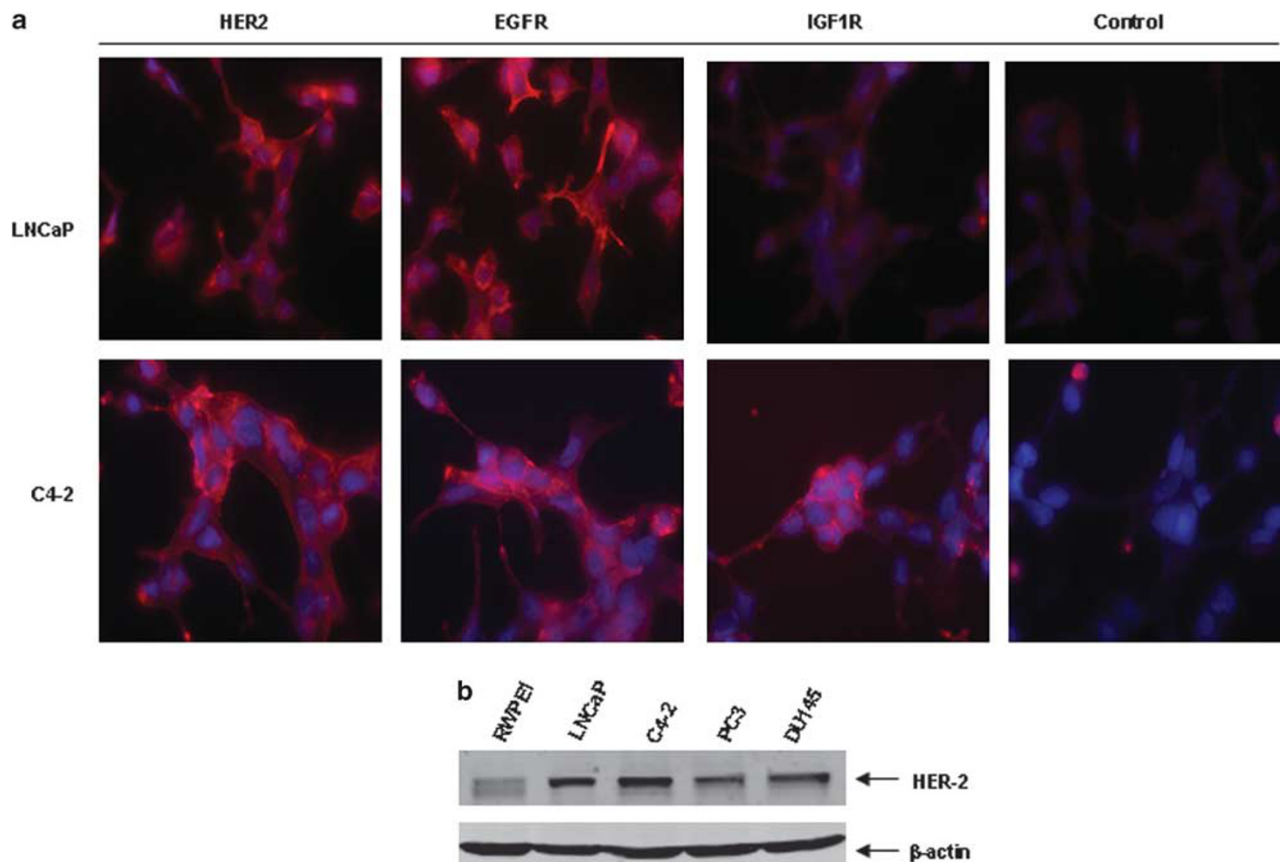
concentrations  $>4 \mu\text{g ml}^{-1}$  of this agent ( $P < 0.01$ ), the trastuzumab-resistant derivative line, SK-BR-3 p1 cells, as well as all of the prostate cancer cell lines tested were refractory to this treatment ( $P > 0.05$ ).

#### Targeting lentivirus to prostate cancer cells using trastuzumab

Although prostate cancer cells-expressing HER-2 are resistant to the killing effects of trastuzumab, the presence of this receptor on their cell membrane provided a potential opportunity for targeting viruses to these cells. To test the feasibility of this, lentiviruses (FUGW), which express EGFP, were engineered such that the normal VSV-G envelope protein used to attach to cells was switched with the Sindbis virus envelope protein, and modified to bind the Fc fragment of immunoglobulins (referred to as FUGW/SB; see Figure 1), such as trastuzumab. FUGW/SB lentiviruses were incubated for

30 min with trastuzumab, anti-EGFR antibodies or an antibody control (anti-IGFR1, which recognizes the intracellular domain of IGFR1). C4-2 cells were incubated with lentiviruses and then 3 days later, collected and measured for expression of EGFP by flow cytometry (Figure 5). On the basis of EGFP expression, lentivirus FUGW/SB with anti-EGFR bound was the most efficient infectious agent, followed by virions with bound trastuzumab. No infectibility was apparent after the cells were exposed to FUGW/SB virus alone (data not shown) or bound with the antibody control. Although lentivirus bound to anti-EGFR had a higher infectivity compared with lentivirus bound to trastuzumab, the latter may be more selective for targeting cancer cells as overexpression of HER-2 is more restricted to malignant cells than is expression of EGFR.<sup>6</sup> In addition to that, the difference can be abrogated if concentrated virus bound to anti-EGFR or to trastuzumab were incubated with cells.





**Figure 3** Expression of HER-2 and EGFR in human prostate cancer cells. (a) LNCaP and C4-2 Prostate cancer cell lines grown on coverslips were stained with antibodies recognizing extra-cellular domains of HER-2, EGFR, IGF1R or the intracellular domain of IGF1R (control) visualized fluorescently with Alexa 594 conjugated goat anti-mouse antibody. Cells were further counter-stained with DAPI. Microphotographs were taken at a magnification of  $\times 400$ . (b) To further confirm the expression of HER-2, whole cell lysates of normal prostate (RWPE1) and prostate cancer cell lines (LNCaP, C4-2, PC3 and DU145) were analyzed by western blotting with anti-HER-2 antibody and shown with IRDye 800CW goat anti-mouse IgG. The membrane was scanned with a LI-COR odyssey infrared imaging system. The equivalence of loading was confirmed by blotting the  $\beta$ -actin on the same membrane.

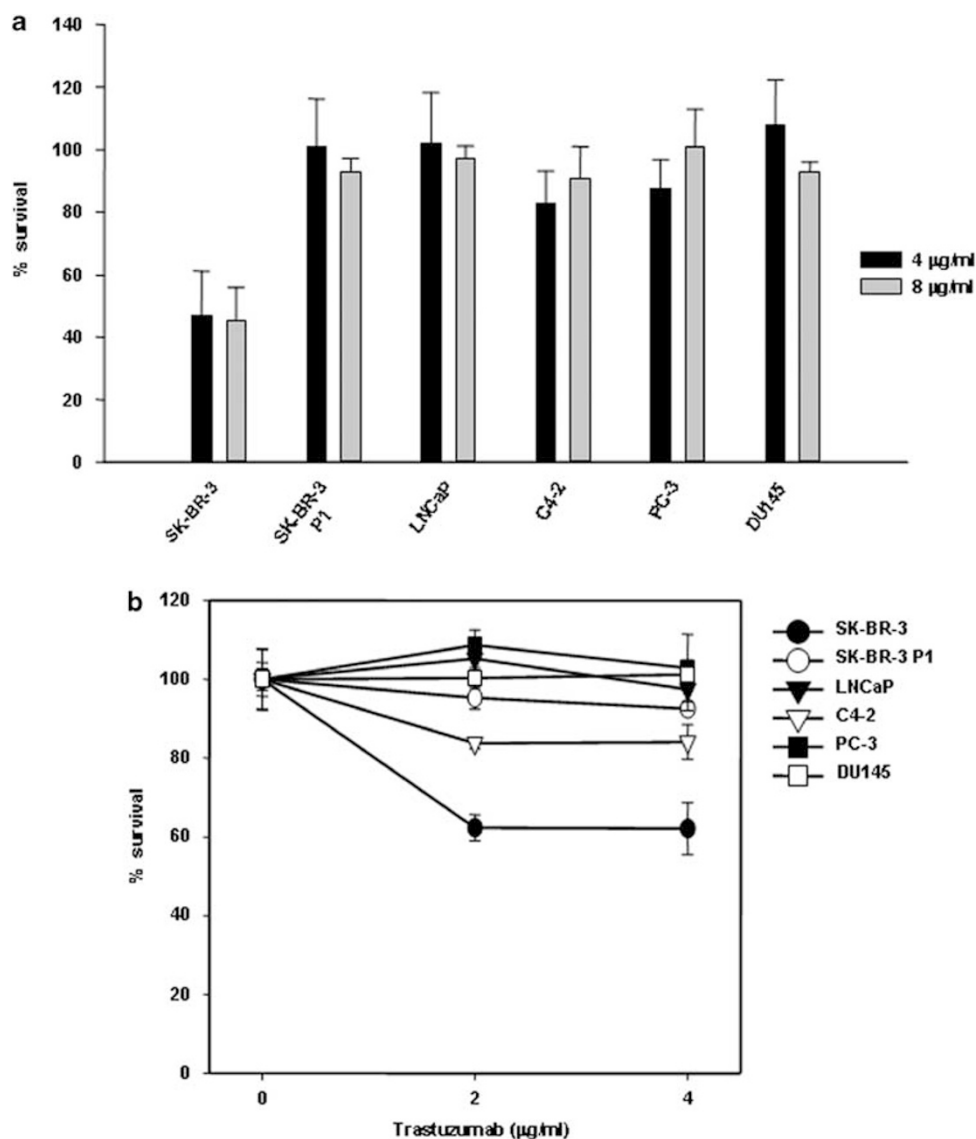
#### *Killing of prostate cancer cells using trastuzumab-bound lentivirus-expressing Herpes thymidine kinase*

Having found that lentivirus bound with trastuzumab could effectively mediate the expression of the reporter gene, *EGFP*, in prostate cancer cells, we next tested whether this viral vector could deliver and mediate expression of the therapeutic gene, *hTK*, in these cells. At 5 days after infection of C4-2 cells and LNCaP cells with lentivirus FUGW/SB alone or with FUTK/SB having trastuzumab or anti-EGFR antibody attached, cells were lysed and analyzed by western blotting for expression of EGFP and hTK. As shown in Figure 6, lentivirus with trastuzumab bound can infect C4-2 and LNCaP cells and mediate the expression of either EGFP or hTK. When hTK-expressing cells are exposed to the pro-drug, GCV, cell killing occurred in a dose-dependent manner. Although some non-specific toxicity to GCV was observed in C4-2 cells not expressing hTK, the killing effects of this drug were far more selective and potent in C4-2 cells targeted with anti-EGFR or trastuzumab-expressing hTK ( $P < 0.001$ ). By comparison, LNCaP cells were relatively insensitive to the non-specific toxicity of

GCV. However, western blot showed that both lentivirus FUTK/SB + anti-EGFR and FUTK/SB + trastuzumab-mediated expression of hTK the similar level in either LNCaP or C4-2 cells.

#### *In vivo targeting of prostate tumors with lentivirus bound to trastuzumab*

As lentivirus bound to trastuzumab could selectively infect prostate cancer cells *in vitro*, we next sought to determine whether these viruses could efficiently target prostate tumors *in vivo*. Groups of nude mice were inoculated with C4-2 cells, and then after  $\sim 2$  weeks, at which time the tumors became palpable, the mice were injected intra-tumorally or i.v. (through the tail vein) with concentrated lentivirus ( $\sim 10^9$  viral particles per ml). Before 30 min injection, FULW/SB vectors, which express luciferase, were incubated with trastuzumab or a control antibody. To visualize the expression of luciferase in C4-2 tumors, the mice were injected with luciferin and imaged on days 1, 3, 5 and 10 using an IVIS 200 Imaging System. After 5 days intra-tumor virus injection, expression of luciferase because of infection by FULW/SB with bound



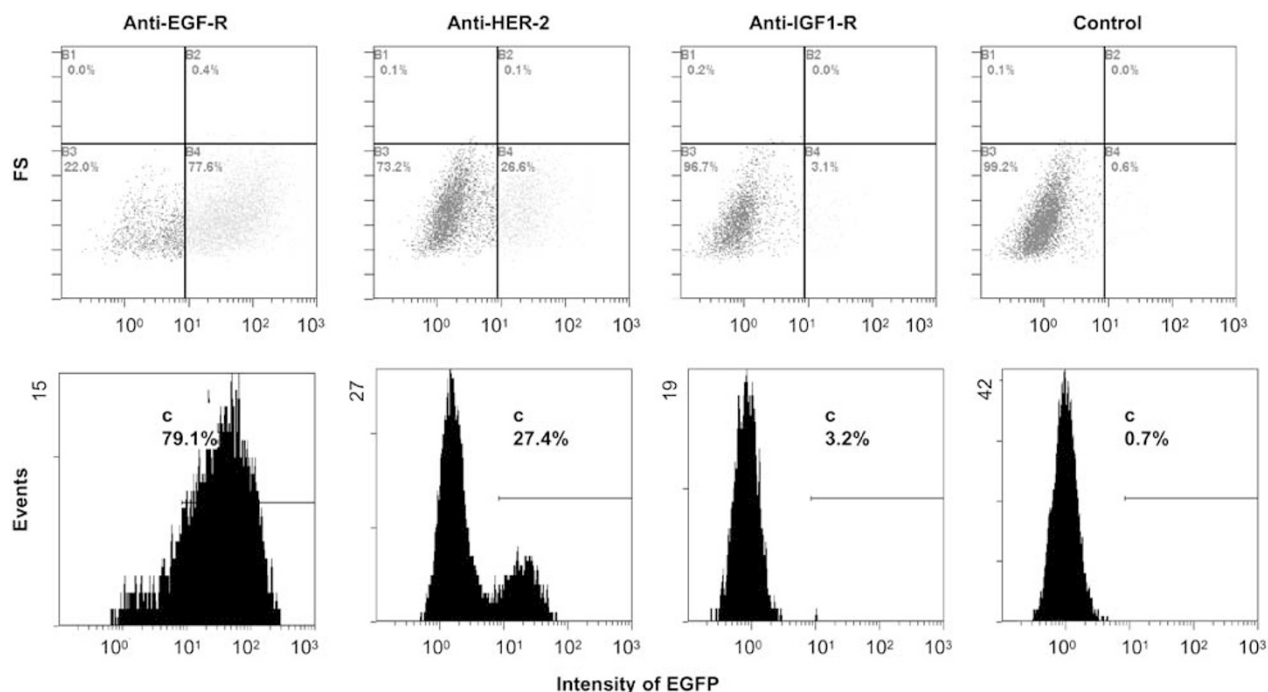
**Figure 4** Effects of trastuzumab on the survival of breast and prostate cancer cell lines. **(a)** Prostate cancer (LNCaP, C4-2, PC3 and DU145) and breast cancer (SK-BR-3 and SK-BR-3 P1 (trastuzumab resistant)) cells were plated in triplicate in 12-well plates at the cell density of  $10^5$  cells per well. One day after plating, trastuzumab was added and 5 days later, the cells were collected and viable cells counted directly using the trypan blue exclusion method with haemocytometer. **(b)** Cells were plated in triplicate in 96-well plates at  $10^4$  cells per well. One day after plating, trastuzumab was added and 5 days later, cell survivability was measured by the MTS assay. Results are expressed as percentage of survival of control cells without trastuzumab treatment. Tukey's multiple comparison test following one-way ANOVA was performed to test effect of trastuzumab on survivability of different cell lines. Comparison SK-BR-3 vs SK-BR-3 P1,  $P < 0.01$ ; comparison between SK-BR-3 P1 vs different prostate cancer cell lines,  $P > 0.05$ .

trastuzumab was clearly observed (Figure 7a.2), although the levels of expression corresponding to intensity of the luminescent signal were lower than after non-selective FULW/VSV-G infection (Figure 7a.1). In either case, little or no luminescent signals were observed outside the tumor region.

Before i.v. administration of lentivirus, mice bearing C4-2 tumors were treated with low doses of cyclophosphamide ( $80 \text{ mg kg}^{-1}$ , intra-peritoneal injection), as it has been reported that cyclophosphamide enhances virotherapy by inhibiting innate immune responses.<sup>41,42</sup> At 24 h later, concentrated lentivirus FULW/VSV-G or FULW/SB

bound to trastuzumab were injected i.v.. At 10 days after the first injection of FULW/SB with bound trastuzumab, luminescent signal on the C4-2 tumors were clearly observed although some signals from the spleen area could still be seen (Figure 7a.4). When there was no pretreatment with cyclophosphamide, luminescent signals were increased in the spleen, which is enriched in macrophages (data not shown). Compared with FULW/SB-trastuzumab, i.v. injection of FULW/VSV-G resulted in a more diffuse distribution of luminescence (Figure 7a.3).

In an attempt to further enhance viral targeting after systemic injections, the luciferase reporter gene was placed



**Figure 5** Relative infectivity of C4-2 prostate cancer cells by antibody-bound lentiviruses. Supernatants (not concentrated) containing lentivirus FUGW/SB produced in 293T cells were incubated with antibodies recognizing extra-cellular domains of EGFR, HER-2 (trastuzumab), IGFR or the intracellular domain of IGFR (control), respectively, for 30 min and then used to infect C4-2 cells. At 3 days after infection, cells were collected and the expression of EGFP was analyzed by flow cytometry (Beckman Coulter) in the FL1 channel.

under the control of a modified probasin promoter (ARR<sub>2</sub>PB),<sup>35–38</sup> whose expression is prostate specific.<sup>38</sup> This viral vector, referred to as FALW/SB, was incubated with trastuzumab and then injected i.v. into the tail veins of C4-2 tumor-bearing mice. After 10 days, luminescent signals were only seen concentrated in the tumors and not elsewhere in the mice (Figures 7b.1 and 7b.2). Overall, these results indicate that although lentivirus with bound trastuzumab allows for enriched infection and luciferase expression in HER-2-expressing prostate tumors, additional selection pressures, such as a prostate-specific promoter, may further enhance the prostate tumor targeting after systemic administration of the viruses.

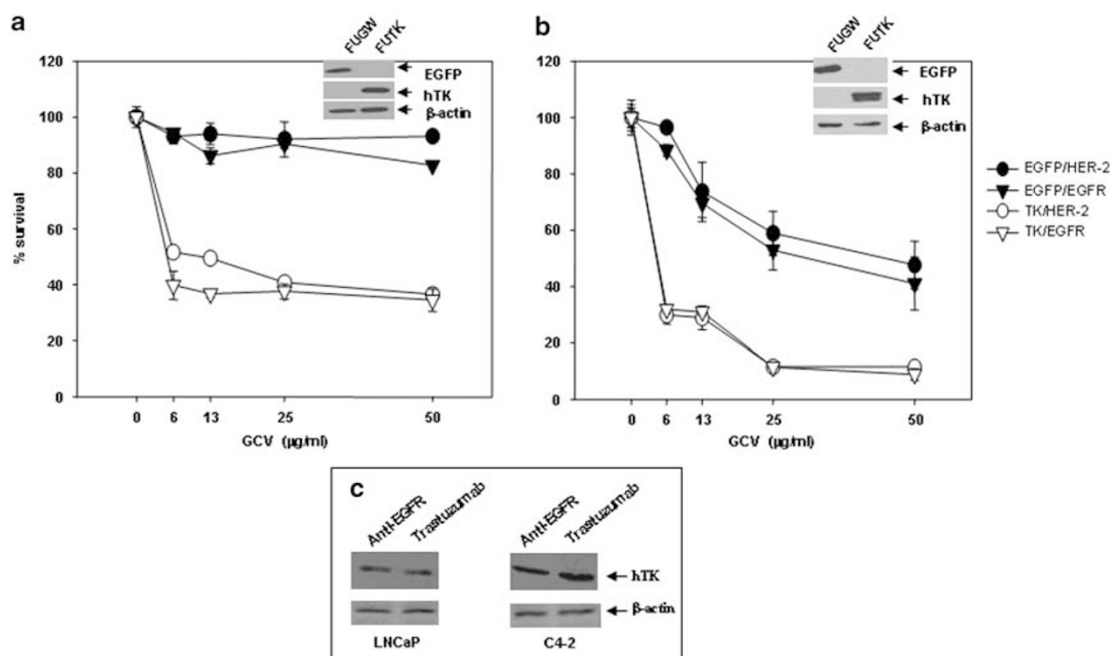
## Discussion

Although considerable progress has been made in reducing mortality rates and stabilizing incidence rates, prostate cancer is still the most commonly diagnosed cancer in men in North America and the second leading cause for cancer-related death.<sup>1,43–45</sup> The high mortality from this cancer is largely because of the lack of an effective treatment for when the disease becomes metastatic and refractory to androgen deprivation.<sup>3</sup> Docetaxel is the most effective cytotoxic agent for the treatment men with hormone-resistant metastatic prostate cancer, but unfortunately survival is only extended by a few months and many patients may suffer from adverse side

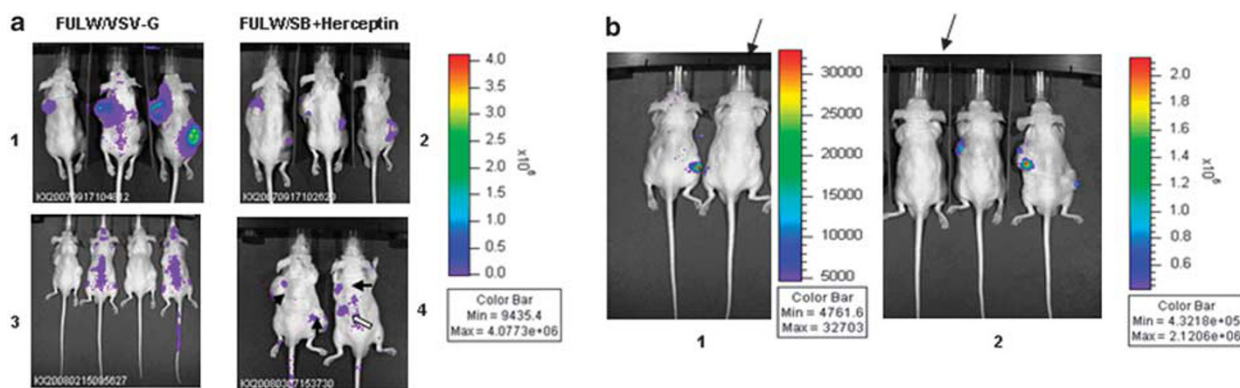
effects.<sup>46</sup> A possible alternative is viral gene therapy, which holds the promise of targeted therapeutic delivery of a cytotoxic gene with little morbidity to normal cells. In this regard, we focused on establishing whether HER-2 is a viable target for *in vitro* and *in vivo* antibody-mediated targeting of lentiviral vectors to prostate cancers.

There is some controversy in the literature concerning the association of HER-2 overexpression with the malignant phenotype of prostate cancers, with most laboratories reporting an association with progression to androgen independence,<sup>5–8,10,11</sup> whereas others have not observed this.<sup>39,40</sup> These contradictory results may be a consequence of the variable procedures involved in sample preparation and staining in different research groups. In the current investigation, we used a TMA approach so that variables, such as fixation and staining procedures are normalized and minimized. Our results on prostate cancer TMAs, composed of low grade and metastatic samples,<sup>47</sup> supported the observation that HER-2 is overexpressed in primary human prostate tumors and that this expression is substantially elevated in cancers that have progressed to the castration-resistant and metastatic stage (Figure 2). Furthermore, in prostate cancer cell lines, we found that androgen-sensitive LNCaP cells and castration-resistant C4-2 cells also overexpressed this protein (Figure 3). Our findings are consistent with the majority of patient-related reports in literature,<sup>5–8,10,11</sup> as well as some *in vitro* and animal model studies.<sup>13,48,49</sup> Furthermore, it has been recently reported that in men with long-term follow-up after





**Figure 6** Effects of GCV treatment on lentiviral infected prostate cancer cells. LNCaP (a) and C4-2 (b) were infected with  $10 \times$  concentrated FUGW/SB or FUTK/SB lentiviruses with bound trastuzumab or antibodies recognizing EGFR. At 5 days after infection, cells were lysed and subjected to immunoblotting with anti-EGFP or anti-hTK with equivalence of loading confirmed by blotting the  $\beta$ -actin on the same membrane (inserts). Cells expressing EGFP or hTK were then plated in 96-well plates at a density of  $10^4$  cells per well. One day after plating, GCV was added and cell survival was measured by MTS assay 5 days later. Results were expressed as percentage survival relative to control cells without GCV treatment. The experiments were repeated in triplicate. Bonferonni's multiple comparison tests following two-way ANOVA was used to compare response of hTK expressing cells with EGFP expressing cells to GCV at different concentrations. In all cases,  $P < 0.001$  when hTK expressing cells were compared with EGFP expressing cells. (c). Expression of hTK mediated by lentivirus bound to anti-EGFR or trastuzumab in LNCaP and C4-2 cells.



**Figure 7** *In vivo* targeting of prostate tumors with lentivirus bound to trastuzumab. (a) Targeting of trastuzumab-bound lentivirus expressing luciferase under control of ubiquitin promoter to C4-2 xenograft tumors established in nude mice. When the tumors were palpable,  $100 \mu\text{l}$  of  $100 \times$  concentrated lentivirus FUGW/VSV-G or FUGW/SB bound to trastuzumab were injected intra-tumorally (a.1 and a.2) or intravenously (a.3 and a.4), respectively. Expression of luciferase was monitored with an IVIS200 Imaging System. Black arrow indicates tumor area, whereas white arrow shows spleen area. (b) Intravenous targeting of trastuzumab-bound lentivirus expressing luciferase under control of a prostate-specific promoter (ARR2PB) to C4-2 tumors. Arrows indicate tumor-free control mice.

radical prostatectomy, HER-2 expression when combined with percentage DNA index can be used clinically for prediction of progression, metastasis and prostate cancer-specific death.<sup>50</sup>

However, as anticipated from the results of clinical trials,<sup>14,51</sup> prostate cancer-cell lines overexpressing

HER-2 were resistant to the killing effects of trastuzumab (Figure 4), the humanized antibody to HER-2, which has proven to be very effective in treating breast cancers where HER-2 is overexpressed. In breast cancer cells, such as the SK-BR-3 cells used in this study, HER-2 is overexpressed and activates multiple cellular signaling

pathways, including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase cascades. Trastuzumab binds to HER-2 and interferes with signaling in these pathways, which increases p27<sup>Kip1</sup> levels, promoting cell cycle arrest and apoptosis.<sup>52</sup> However, many prostate cancers,<sup>53–55</sup> as well as LNCaP, PC-3 and C4-2 cell lines, have a PTEN deficiency<sup>56</sup> which removes negative regulation of the PI3K/Akt pathway<sup>53,57</sup> and is presumed to make a major contribution to prostate cancer development and progression. Thus, increased constitutive signaling through the PI3K/Akt pathway could contribute to trastuzumab resistance because of activation of multiple receptor pathways that include HER-2-related receptors, as well as non-HER-2 receptors such as EGFR or insulin-like growth factor 1 receptor, which seem to be involved in a cross talk with HER-2.<sup>52</sup> In an unpublished study, we indeed observed that after blocking HER-2 activation with trastuzumab, PI3K/mTOR/eIF-4E are still partially activated and EGF expression is slightly elevated, which functions to activate EGFR. Nevertheless, when trastuzumab was affixed to a modified lentivirus, it proved to be a very effective antibody for targeting prostate cancers.

Lentiviruses pseudotyped with the modified Sindbis virus envelope protein have been shown to bind and enter cells when an appropriate monoclonal antibody is attached.<sup>17,18,31,33</sup> We used this approach to show that lentiviruses engineered to express this Fc-binding domain of protein A and then incubated with antibodies to EGFR or HER-2 could readily infect C4-2 (Figure 5) and LNCaP cells (data not shown). As EGFR is more ubiquitous and often associated with normal epithelia cells, HER-2 is the preferred target antigen for malignant cells.

Herpes thymidine kinase in combination with the pro-drug GCV is perhaps the most commonly used anticancer gene therapy system both in experimental models and clinical trials.<sup>58–61</sup> HTK, whose substrate specificity is distinct from cellular TKs, can convert GCV to a toxic phosphorylated form allowing killing of cells that express it as well as regional cell killing through a bystander effect.<sup>62</sup> When trastuzumab was bound to lentiviruses carrying an hTK expression cassette, C4-2 and LNCaP cells could be readily infected and shown to express hTK (Figure 6). Addition of GCV resulted in substantial cell killing compared with cells infected with trastuzumab-bound lentivirus carrying a GFP expression cassette. Although some non-specific killing was observed with GCV-treated C4-2 control cells, cells-expressing hTK were much more sensitive to GCV cytotoxicity. Interestingly, LNCaP cells not expressing hTK were very resistant to non-specific killing by this drug.

Luminescence from luciferase-expressing cells infected after intra-tumor injections of these viruses remained largely confined to the tumors. Interestingly, intensity of the luminescent signal was much higher in viruses with the wild-type VSV-G envelope protein compared with those with the HER-2 antibody attached (Figures 7a.1 and 7a.2). This may be attributed to the

less efficient viral infection through the engineered SB viral envelope.

Tumor-specific targeting by the trastuzumab-coated lentivirus vector was best shown when these viruses were injected i.v.. In contrast to a non-specific diffused luminescent signal seen with the VSV-G packaged lentivirus, the trastuzumab-bound lentivirus gave rise to a clearly tumor-enriched luminescence (Figures 7a.2 and 7a.4). Considering the significant dilution effect through i.v. injection, it is encouraging that a small fraction of injected virus vector was able to infect prostate cancer tumors through the antibody-mediated targeting. Furthermore, the immune system also played a role in the efficiency of virus targeting. It has been reported that cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses.<sup>41,63</sup> We observed that treatment of mice with cyclophosphamide before injection of trastuzumab-bound virus enhanced the luminescent signal in the tumors (Figure 7a.4), whereas in the absence of cyclophosphamide, luminescence was only seen in the spleen area (data not shown).

Tissue-specific expression of genes can be achieved by controlling gene expression at the transcription level with a tissue-specific promoter. As the probasin-derived ARR<sub>2</sub>PB promoter has been shown to be capable of directing tightly controlled prostate-specific gene expression,<sup>35–38</sup> we placed it in front of the firefly luciferase gene in an attempt to increase tumor-specific expression. Indeed, the luminescent signal from cells infected with the trastuzumab-bound lentivirus containing the ARR<sub>2</sub>PB promoter was entirely restricted to the tumors (Figure 7b). Given that safety is a primary concern of any viral-based gene therapy, the ability to more stringently limit viral-directed gene expression to tumor target cells is a very important consideration. On the downside, with each modification of the viral envelope or gene promoters to further increase tumor specificity, there is an apparent attenuation of the viral infectivity and level of reporter or therapeutic gene expression. Accordingly, it is likely that in order to achieve adequate efficiency of metastatic cancer cell killing together with specific tumor targeting and safety, consideration must be given to amplifying the potency of the viral therapeutic. With our current technology this could most likely be attained using some sort of strategy employing a targeted, replication-competent oncolytic virus.<sup>64,65</sup> Alternatively, lentivirus coated with a targeting antibody could be used to express secretable proteins with anti-cancer properties and hence render a high-local concentration within the tumor.

In conclusion, although HER-2 is overexpressed in advanced prostate cancers yet resistant to trastuzumab treatment, this protein can serve as an ideal tumor antigen for specifically targeting and restricting viral-based gene therapies to prostate cancers, particularly when used in combination with a prostate-specific gene promoter. Furthermore, this strategy may also be applied to other trastuzumab-resistant, HER-2 overexpressing human malignancies such as those found in breast cancers.

## Conflict of interest

The authors declare no conflict of interest.

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