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ARTICLE

# Efficient Retroviral Vector Targeting of Carcinoembryonic Antigen-Positive Tumors

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**Many gene therapy approaches require specific, efficient gene delivery to cells *in vivo*. To target colorectal tumors we fused a single-chain variable fragment (scFv) directed against carcinoembryonic antigen (CEA) to the amphotropic murine leukemia virus envelope. A proline-rich hinge and matrix metalloprotease (MMP) cleavage site linked the two proteins. Following attachment to CEA, MMP cleavage of the envelope at the cell surface removed the scFv and proline-rich hinge, allowing transduction. This allowed selective targeting of CEA-positive cells *in vivo* after injection of producer cells at the site of the tumor, with up to 10% of cells within a CEA-positive tumor xenograft becoming transduced. Intraperitoneal injection of amphotropic producer cells resulted in transduction of cells in spleen, liver, and kidney, which was not detected when CEA-targeted producer cells were used. These results demonstrate the feasibility of using targeted retroviral vectors for *in vivo* gene delivery to tumors. Furthermore, the lack of transduction of host cells eliminates the risk of insertional mutagenesis leading to transformation of host hematopoietic cells.**

**Keywords: retroviral vector, tumor targeting, CEA**

## INTRODUCTION

Many gene therapy approaches have failed to achieve initial expectations because current technology cannot deliver therapeutic genes to target cells *in vivo* with sufficient efficiency. Retroviral vectors remain an attractive option for clinical gene delivery because integration of the vector genome allows stable gene expression in the infected cell and its progeny. Also, because viral coding regions are deleted from the vector, viral proteins are not expressed in the infected cells, avoiding stimulation of an inappropriate immune response. As retroviral vectors transduce only dividing cells they have been used to deliver therapeutic genes to tumors *in vivo*, with surrounding normal tissue being largely refractory to transduction [1,2].

Recently, X-linked SCID gene therapy patients who received retrovirally transduced bone marrow have developed T cell leukemia caused by retroviral vector integration leading to insertional mutagenesis [3]. This highlights the need to target retroviral gene delivery specifically to tumors, if vectors or packaging cells are to be injected *in vivo* for cancer gene therapy. Most tumors induced by the viruses from which retroviral vectors are derived, in our case murine leukemia virus (MLV), involve hematopoietic cells transformed by insertional mutagenesis [4]. Thus, particular care is needed to avoid transduction and potential transformation of these cells. The development of surface-targeted retroviral vectors should also allow more efficient transduction of tumors, as the pool of viral particles will not be depleted by other cells. To date, the only surface-targeting strategies that have allowed efficient transduction by retroviral vectors *in vivo* are those in which retargeted binding has been followed by fusion using the natural viral receptor [5–8].

We have previously described retroviral vectors targeted to high-molecular-weight melanoma-associated antigen (HMWMAA) [9]. The chimeric envelope surface domain (SU) contained a single-chain antibody (scFv) recognizing HMWMAA followed by a proline linker and a matrix metalloprotease (MMP) cleavage site. The proline linker prevented binding of the chimeric SU to Pit-2.



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However, when these vectors bound to HMWMAA they were then cleaved by cell surface MMPs, revealing the amphotropic 4070A (MLV-A) backbone that mediated transduction via its Pit-2 receptor. The targeted vector (LMH2/ProMMP, previously named ScLPMA) infected HMWMAA-positive cells when injected into human tumor xenografts [6]. Here we report a retroviral vector targeted to carcinoembryonic antigen (CEA). CEA is expressed by a number of tumors of epithelial origin, most notably colorectal carcinoma, but not in most normal adult tissue [10]. MFE23 is an scFv against CEA that was selected using a combinatorial phage library of $10^7$ scFv's generated using the cDNA from spleens of mice immunized with CEA [11]. The ability of MFE23 to target CEA *in vivo* has been shown by radioimaging studies [12]. In a study of 10 patients with CEA-expressing tumors MFE23 was able to localize all known tumor deposits and demonstrated advantages over current imaging technology.

The envelope MFE23/ProMMP was constructed by linking MFE23 to the amino terminus of MLV-A SU using a proline-rich spacer followed by a cleavage site for MMPs. Fortuitously, retroviral vectors incorporated the MFE23/ProMMP envelope as efficiently as the unmodified MLV-A envelope, in contrast to the relatively poor incorporation of many chimeric envelopes, including LMH2/ProMMP [7,9,13]. Perhaps because of this efficient envelope incorporation, retroviral vectors carrying the MFE23/ProMMP envelope could specifically transduce CEA-positive cells or tumors with high efficiency.

## RESULTS

### MFE23/Pro and MFE23/ProMMP Chimeric Envelopes

In the envelope chimera MFE23/Pro, MFE23 [11], an scFv that recognizes CEA, was linked to codon 5 of the mature MLV-A SU by a 59-amino-acid proline-rich (Pro) linker derived from 4070A SU. Into MFE23/ProMMP, we introduced the MMP cleavage site PLGLWA [14] between the Pro linker and the envelope protein (Fig. 1).

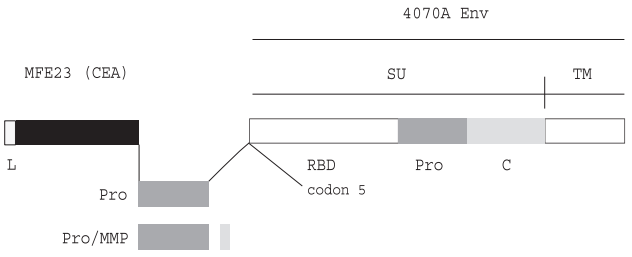



**FIG. 1.** Construction of CEA-targeted envelopes. MFE23, an scFv recognizing CEA, was fused to the N terminus of amphotropic 4070A surface protein (SU) using a proline-rich spacer (Pro) derived from 4070A SU. In MFE23/ProMMP an MMP cleavage site was introduced after the inserted scFv and Pro. L, leader peptide; RBD, receptor-binding domain; TM, transmembrane protein; C, carboxy-terminal domain.

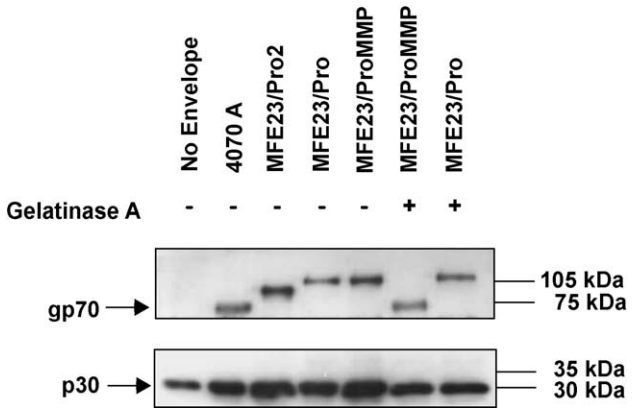



**FIG. 2.** Targeted envelope incorporation into retroviral vector particles. Concentrated supernatants from TELCeB6 cells (No envelope) and TELCeB6 cells transfected with 4070A, MFE23/Pro2 [16], MFE23/Pro, or MFE23/ProMMP envelopes were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel, electroblotted, incubated with goat anti-Rauscher leukemia virus SU (gp70) and anti-Rauscher leukemia virus CA protein (p30) antisera followed by anti-goat horseradish peroxidase, and then developed using ECL (Amersham). The last two lanes show the effects of treatment of viral supernatants MFE23/ProMMP and MFE23/Pro with gelatinase A (as described under Materials and Methods).

We transfected plasmids expressing these envelopes or the 4070A envelope into TELCeB6 cells that harbor the MFGnlslacZ vector genome and a MLV Gag–Pol expression plasmid [15]. We selected transfected cells with phleomycin and analyzed supernatants from pools of phleomycin-resistant clones. We analyzed the incorporation of the chimeric envelope glycoproteins into retroviral particles by immunoblotting of pelleted viral particles (Fig. 2). Both MFE23/Pro and MFE23/ProMMP chimeras showed high levels of envelope expression in viral pellets with Env-to-capsid ratios comparable to that of 4070A. Virion incorporation of this CEA-targeted chimera is significantly better than that seen with chimeras such as epidermal growth factor (EGF) and the C-terminal domain of the CD40 ligand fused to SU via an MMP-cleavable linker [7,13] or the LMH2/ProMMP chimera, an identical construct with a different scFv [9]. This higher level of incorporation of MFE23/Pro and MFE23/ProMMP probably reflects features of MFE23 that allow correct folding and assembly of SU trimers, as we have observed similar efficient incorporation of another chimeric envelope, MFE23/Pro 2 ([16] and Fig. 2). To assess whether the introduced MMP cleavage site was accessible, we incubated MFE23/ProMMP vectors with activated gelatinase A. This reduced the size of the chimeric envelope to that of SU (Fig. 2).

### Targeted Transduction

Fig. 3 shows transduction by vectors incubated with target cells for 4 h at 37°C in the presence of Polybrene

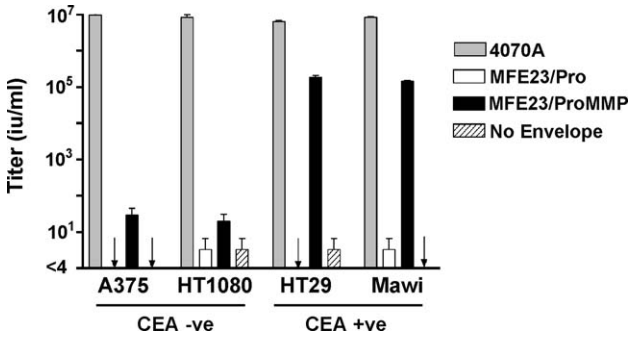


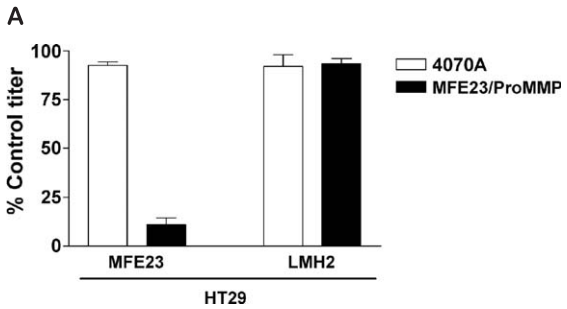

**FIG. 3.** Titers of targeted vectors. Retroviral vectors with MFE23-targeted chimeras, 4070A, or no envelope were added to CEA-negative or -positive cell lines as indicated. Target cells were transduced with serial dilutions of vector and LacZ-positive cells counted after 48 h. Titer was calculated from values in the range in which the number of infectious events was directly proportional to the volume of vector added.

and then washed and analyzed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining after 48 h [17]. Vectors with the MFE23/ProMMP envelope were able selectively to infect CEA-positive cells. All four of the target cell lines expressed similar levels of surface MMP activity (data not shown). MFE23/Pro-enveloped vectors were not infectious (Fig. 3). We propose that the incorporation of the single-chain antibody and proline linker into the retroviral envelope blocks the Pit-2 receptor-binding domain of the wild-type 4070A backbone and prevents transduction. In this model the binding of the scFv to the tumor antigen allows the chimeric envelope to undergo a conformational change that exposes the MMP cleavage site. Cleavage of the scFv and Pro reveals the 4070A Pit-2 receptor-binding domain and allows subsequent fusion and transduction. Even the MFE23/ProMMP chimera is 10- to 100-fold less infectious than the wild-type 4070A envelope, which implies that the process of binding, cleavage, and Pit-2 interaction is not 100% efficient.

### Transduction Requires CEA Binding, MMP Cleavage, and Pit-2 Function

To test this model, we first investigated whether transduction required binding to CEA. Fig. 4A shows that the addition of MFE23 could inhibit targeted transduction by approximately 90%. Presumably, complete blocking with the monovalent scFv is hard to achieve because of the higher avidity of viral binding. We also demonstrated that CEA binding was required by transducing a population of transfected 293T cells that express cell-associated MMP activity (data not shown), approximately 50% of which expressed CEA and GFP from a bicistronic transcript. Fig. 4B shows that only the CEA/GFP-positive cells were infected by the MFE23/ProMMP vector. This suggests that MFE23/ProMMP vectors become activated at the CEA-positive target cell membrane, resulting in transduction of these but not neighboring cells.

After binding to CEA, MMP cleavage of the chimera should be necessary to expose the receptor-binding domain (RBD) of the 4070A SU. Fig. 5A shows that the inhibition of MMP activity by TIMP-2 decreased the titer of the MFE23/ProMMP-enveloped vectors to approximately 15%. A role for MMP cleavage is also demonstrated by the fact that MFE23/Pro-enveloped vectors were not infectious (Fig. 3). To demonstrate that the RBD of the 4070A SU mediates transduction via interaction with Pit-2 after the blocking domain has been proteolytically cleaved, we performed receptor interference assays using cells ''chronically infected'' (2 weeks of passage) with replication-competent 4070A MLV [18]. Fig. 5B shows that transduction of 4070A-infected HT29 cells by MFE23/ProMMP was reduced by approximately 500-fold, similar to the reduction of infection by vectors carrying the unmodified 4070A envelope. The titer of amphotropic MLV pseudotyped with the GALV enve-




B

TRANSFECT : HR/IRESGFP | HR/CEA/IRESGFP

| | SORT –VE | SORT +VE | SORT –VE | SORT +VE |
|---|---|---|---|---|
| | 293T | 293T/GFP | 293T | 293T/CEA/GFP |
| 4070 A | 92% | 97% | 94% | 96% |
| MFE23/ProMMP | 0.05% | 0.02% | 0.03% | 36% |
| No envelope | 0.04% | 0.08% | 0.1% | 0.07% |

**FIG. 4.** Targeted transduction requires CEA. (A) ScFv blocking. $CEA^+$ cells (HT29) were treated with LMH2 or MFE23 scFv prior to transduction by vectors with 4070A or MFE23/ProMMP envelopes. Titer is expressed as a percentage of that on untreated cells. (B) Transfection of CEA. Cell mixtures were obtained by transfection of 293T cells with HR/IRESGFP or HR/CEA/IRESGFP plasmids (as described under Materials and Methods). Cell mixtures were transduced by vectors expressing 4070A, MFE23/ProMMP, or no envelope and then separated by cell sorting into GFP-positive and -negative populations. The sorted cells were plated and LacZ-positive cells were counted after 48 h.

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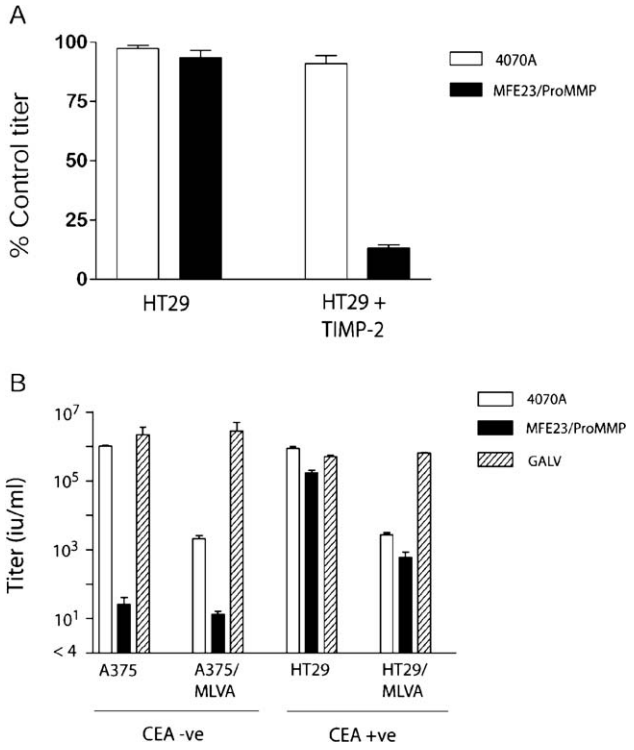


**FIG. 5.** Targeted transduction requires MMP activity and Pit-2 receptor. (A) MMP inhibition. $CEA^+$ cells (HT29) were treated with TIMP-2 at the time of transduction by vectors with 4070A or MFE23/ProMMP envelopes. Titer is expressed as a percentage of that on untreated cells. (B) Pit-2 blocking. $HMWMAA^+$ (A375) and $CEA^+$ (HT29) cells were infected with replication-competent amphotropic MLV. After 2 weeks A375, HT29, infected A375/MLVA, and infected HT29/MLVA cells were transduced by vectors with 4070A, MFE23/ProMMP, or GALV envelopes and LacZ-positive cells were counted after 48 h.

lope, which recognizes a different surface receptor, Pit-1 [19], was unaffected.

### Targeted Transduction of Tumor Xenografts

Fig. 6 shows transduction of cells within tumor xenografts after injection of irradiated viral producer cell lines at the site of the tumor. This approach has been used clinically to treat brain tumors by direct injection of murine retroviral producer cells; some response of small tumors was seen [1]. In xenograft injections the MFE23/ProMMP vectors maintained their selectivity for CEA-positive tumors (HT29 and Mawi) with no transduction (among $10^6$ disaggregated tumor cells) of the CEA-negative tumors (A375 and HT1080). Thus, MFE23/ProMMP vectors are able to maintain their specificity and efficiency *in vivo* with transduction efficiencies of approximately 20% of that seen with the amphotropic vectors. This was achieved without enhancement agents, such as Polybrene or Lipofectamine, which is advantageous as the use of cations *in vivo* would be undesirable due to complement activation [20].

### Analysis of Host Cell Transduction

To analyze vector specificity it is necessary to examine other nontarget organs using a technique that is more sensitive than staining for β-galactosidase. It was initially planned to examine organs from animals infected as above by PCR using LacZ1/LTR1 primers that amplify a vector fragment. However, a previous study had shown that transduced tumor cells are likely to be detected as micrometastases [6] and thus this method is unable to differentiate vector spread from metastasis in tumor-bearing animals. An alternative method was developed using irradiated producer cells injected intraperitoneally [6]. We injected amphotropic, MFE23/ProMMP, or unenveloped irradiated producer cells intraperitoneally into nude mice. To compensate for the higher titer seen with amphotropic vectors we injected 10 times as many MFE23/ProMMP and unenveloped producer cells. Two weeks after injection we sacrificed the mice and extracted the DNA from the spleen, liver, and kidneys for nested PCR analysis. The nested PCR used primers that amplified a 480-bp DNA fragment located between the 3′ end of the β-galactosidase gene and the 3′ LTR of the integrated vector.

Fig. 7A shows the results of the nested PCR for the three vectors in the three organs analyzed. This figure shows that in all three mice injected with amphotropic producer cells detectable levels of proviral DNA were seen in the liver and spleen. When we analyzed the kidneys from these mice they were shown to be positive in two of the mice. However, none of the mice injected with MFE23/ProMMP producer cells or unenveloped producer cells showed detectable levels of proviral DNA in the spleen, liver, or kidney. We performed semiquantitative analysis of proviral content in spleen, liver, and kidney to determine the level of amphotropic transduction in these organs. We used DNA from the mouse labeled A in the 4070A group (Fig. 7A). We

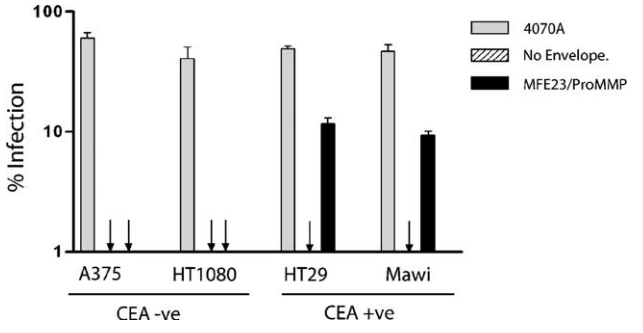


**FIG. 6.** *In vivo* targeting. CEA-negative and -positive tumors were established in nude mice. $10^6$ lethally irradiated packaging cells producing 4070A, MFE23/ProMMP, or unenveloped vectors were injected into the xenografts on 2 successive days. 7 days after the second injection tumors were excised and disaggregated and the cells were plated overnight before LacZ-positive cells were counted.

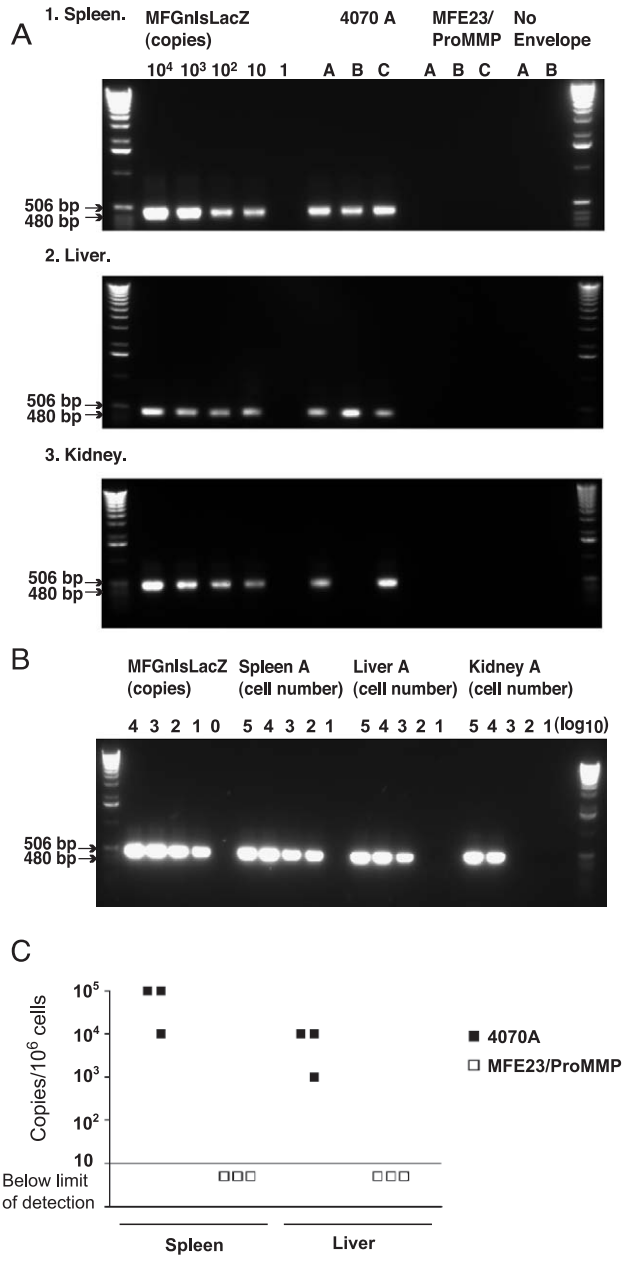


**FIG. 7.** Analysis of vector spread. (A) Lethally irradiated producer cells expressing 4070A, MFE23/ProMMP, or no envelope were injected intraperitoneally in nude mice. Proviral analysis was carried out by nested PCR using primers that amplify a 480-bp DNA fragment located between the 3′ end of the β-galactosidase gene and the 3′ LTR. DNA from the spleens, livers, and kidneys of eight mice (lanes A–C) were analyzed for proviral content. Plasmid DNA serial dilutions (from $10^4$ to 1 plasmids) of MFGnlslacZ vector were used to determine sensitivity. (B) Serial dilutions of DNA from the spleen, liver, and kidney from mouse A injected with producer cells for 4070A-enveloped vectors were used for nested PCR. (C) Quantitation from all mice. Estimated proviral number was calculated based on the number of cells used in the last dilution in which positive amplification was found and the sensitivity of the nested PCR.

carried out nested PCR using serial dilutions of DNA and the same primers (Fig. 7B). The limit of detection of this assay is 10 copies, as shown by the serial dilution of MFGnlslacZ. Thus, from Fig. 7B the likely number of copies of proviral DNA per $10^6$ cells can be calculated. For spleen, liver, and kidney this is $10^5$, $10^4$, and $10^3$ copies of proviral DNA per $10^6$ cells, respectively. No proviral DNA was detected in any organ from mice injected with MFE23/ProMMP (Fig. 7C) or unenveloped producer cells. Based on these results and taking into account that the limit of detection is 10 copies per $10^6$ cells it appears that MFE23/ProMMP vectors are approximately $10^4$ times less likely to infect host cells than amphotropic vectors. It may be argued that this effect is partly due to their lower infectivity but this was addressed by injecting a higher number of MFE23/ProMMP producer cells into each mouse to compensate for their lower titer.

## DISCUSSION

These experiments have shown that it is possible to achieve selective targeting to CEA both *in vitro* and *in vivo* by tropism restriction of amphotropic MLV. Transduction is dependent on cells expressing a specific tumor antigen and a specific protease on their cell surface. This dual requirement provides an extra degree of specificity and hence safety as it decreases the likelihood that nontarget cells will be transduced. CEA is a proven clinical marker of colorectal tumors and MMPs are upregulated in colorectal cancer [21]. The Pit-2 amphotropic receptor is essentially ubiquitously expressed, so natural tumors are likely to be good targets for the MFE23/ProMMP vector. Initial retroviral attachment to cells is not dependent on envelope/receptor interaction and is probably mediated by proteoglycans on the surface of the vector and/or target cell [22–25]. Indeed, ligand- or scFv-targeted retroviral vectors have been shown initially to interact indiscriminately with target and nontarget cells [22]. The fact that the CEA-targeted vector shows a high degree of specificity implies that binding to CEA, as opposed to cell matrix attachment, promotes MMP cleavage. This might be because CEA binding induces a conformational change that exposes the MMP cleavage site or because vector attached to CEA is retained longer on the cell surface. Lack of transduction of CEA-negative cells in a mixed population demonstrates that once this cleavage occurs transduction via SU/Pit-2 interaction at the same cell surface must be efficient. Greater selectivity was seen with the MFE23/ProMMP-enveloped vectors than when EGF-displaying or CD40L-displaying protease-activated vectors were used to infect cell mixtures [7].

The titer achieved with MFE23/ProMMP is 100-fold higher than that seen in a previous study in which MFE23

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was fused to ecotropic MMLV and coexpressed with wild-type envelope [26]. Khare and colleagues also developed a chimera in which an scFv that recognizes CEA is fused to ecotropic MMLV envelope and coexpressed with wild-type ecotropic envelope [27]. The titer they achieved with this model was 10-fold lower than that seen with MFE23/ProMMP and cocentrifugation of vector and target cells was used in addition to Polybrene to enhance transduction. We have also previously used MFE23 to target retroviral transduction to CEA using an approach based on receptor cooperation [16]; again this vector (MFE23/Pro2) showed an approximately 10-fold lower titer than did MFE23/ProMMP. It is encouraging that the targeted vector developed by Khare and colleagues was able to produce tumor suppression with a 70% reduction in tumor weight when used to express HSV-tk in tumors [28]. This would suggest that a useful therapeutic effect could be achieved by using the MFE23/ProMMP model to deliver suicide genes *in vivo*.

Targeted gene delivery to tumors may increase tumor transduction *in vivo* by preventing vector loss on other cells and also decrease host cell transduction. The latter is an important safety consideration as retroviral vectors may be able to transform host hematopoietic cells by insertional mutagenesis. Targeting using blocking domains that restrict transduction on certain cells, such as the EGF-displaying chimeras, will not necessarily prevent host cell transduction. This model has shown selective transduction of MMP-rich tumors *in vivo* but this study did not assess the extent of nontarget cell transduction [7]. Another promising *in vivo* gene delivery system is based on matrix-targeted retroviral vectors [5,29,30]. This strategy incorporated matrix-targeting motifs (i.e., collagen-binding peptides) on amphotropic MLV vector particles and demonstrated enhanced vector penetration and transduction of tumor nodules after local or systemic delivery with significant tumor regression. Again, such preferential targeting is unlikely to be highly specific and to reduce significantly infectivity of nontarget cells.

Further modifications to improve efficiency of our targeted vector could involve engineering scFv or linker to allow more efficient postbinding conformational changes or MMP cleavage. It is possible to optimize the MMP cleavage site on particular target cells using retrovirus display libraries [31]. Finally, delivery of the targeted vector to the site of the tumor remains a challenge. Systemic retroviral delivery to tumor metastases is impractical, because of limitations of viral titer and access. Injections of irradiated packaging cells are also impractical in most human malignancies. One approach may be the use of host cells, which can infiltrate tumors to produce vectors. A recent report described the use of T cells to deliver targeted retroviral vectors and demonstrated therapeutic effects following systemic delivery to lung and liver metastases [32].

## MATERIALS AND METHODS

***Chimeric envelopes.*** The scFv that recognizes CEA, MFE23, was removed from full-length MFE23 [11] by digestion with *Sfi*I and *Not*I. MFE23/Pro and MFE23/ProMMP were made by inserting the scFv coding sequence into the respective plasmids LMH2/Pro and LMH2/ProMMP (previously named ScLPA and ScLPMA, respectively) [9] in place of the coding sequence for LMH2 by digestion with *Sfi*I and *Not*I. The 4070A envelope expression plasmid (ALF) has been described previously [15]. To construct the CEA expression plasmid, the CEA coding sequence of 2108 nucleotides was amplified by PCR from an expressed sequence tag (IMAGE 587714, from the UK Human Genome Mapping Project Resource Centre) using the primers Forward, GCGCTGATCACCAGCCATGGAGTCTCCCC-TCGCCCC, and Reverse, GCGCCTCGAGCTATATCAGAGCAACCC-CAACC. This fragment was subcloned into the *Bam*HI–*Xho*I sites of vector plasmid pHR′-CMVLacZ-IRESGFP [33,34] to give the plasmid pHR′-CMVCEA-IRESGFP.

***Cell culture, vector production, and concentration.*** TELCeB6 cells are derived from the TE671 cell line (American Tissue Culture Collection (ATCC) CRL-8805) and harbor the MFGnlslacZ genome and an MLV Gag–Pol expression plasmid, CeB [15]. A375 is a human melanoma cell line (ATCC; CRL-1619), HT1080 is a human fibrosarcoma cell line (ATCC; CCL-121), HT29 is a human colonic adenocarcinoma cell line (ATCC; HTB-38), and Mawi is a human colonic adenocarcinoma cell line [35] (obtained from C. Porter, Chester Beatty Laboratories, Institute of Cancer Research, London, UK). The 293T cell line is a human embryonic cell line that expresses the SV40 large T antigen (obtained from M. Calos, Stanford University, Stanford, CA, USA). CEA expression by target cells was determined by Western blotting of cell lysates with an anti-CEA antibody (Dako Ltd., UK) or by immunostaining using anti-CEA antibody A5B7 [36]. Surface MMP activity of cell lines was determined using the fluorogenic peptide 2,4-dinitrophenol-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-$NH_2$ (Bachem, Switzerland) as previously described [9]. All cell lines used were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator at 10% $CO_2$.

Envelope expression plasmids MFE23/Pro2, MFE23/ProMMP, and ALF were transfected into TELCeB6 cells using Lipofectamine (Invitrogen). Forty-eight hours later transfected cells were selected using medium containing phleomycin (Cayla) (50 μg/ml) and pools of phleomycin-resistant clones were used for vector production. To harvest vectors, producer cells were grown at 37°C until they became confluent and then cultured at 32°C for 48 h with feeding of fresh DMEM supplemented with 10% FCS every 24 h. The medium was then replaced with serum-free OptiMEM and supernatant was collected 14 to 16 h later. The harvested vector was filtered through 0.45-μm-pore filters and, in some cases, concentrated by centrifugation at 2500 g at 4°C for 12 h. Concentrated vector was kept frozen at −70°C.

***Envelope incorporation and gelatinase A cleavage.*** Viral pellets were subjected to Western blot analysis using goat antisera against the Rauscher leukemia virus gp70 (SU) and p30 (CA) proteins as described previously [37]. Accessibility of the MMP cleavage site to protease cleavage was assessed by treatment of pelleted vectors with activated gelatinase A (Boehringer Mannheim). Vectors were centrifuged at 100,000 g for 1 h at 4°C; resuspended in 50 μl of 100 mM Tris (pH 7.5), 200 mM NaCl, 1 U activated gelatinase; incubated for 6 h at 37°C; and then subjected to Western blot analysis using anti-RLV gp70 antibody.

***Analysis of viral transduction.*** Target cells were seeded in 24-well plates at a density of $5 \times 10^4$ cells/well 18 h before transduction. Viral supernatants were serially diluted in OptiMEM. Cells were incubated in the presence of the viral dilutions and Polybrene (4 μg/ml) for 4 h at 37°C. The cells were then washed once in OptiMEM and then cultured in DMEM–10% FCS for 48 h. X-Gal staining was performed as previously described [17]. To inhibit targeted vector transduction, cells were preincubated with 50 μg/ml MFE23 for 2 h at 37°C. Viral transductions

were then performed in the presence of 50 μg/ml MFE23. To inhibit MMP activity, viral transductions were performed with TIMP-2 (at a final concentration of 5 μg/ml) [38] and Polybrene (4 μg/ml) for 4 h at 37°C. After transduction cells were washed once in OptiMEM, cultured in DMEM–10% FCS for 48 h, and then X-Gal stained. To show that Pit-2 was required for transduction receptor A375 and HT29 cells were infected with replication replication-competent 4070A and then passaged for 2 weeks [18].

***Transduction of mixed cell populations.*** Transient expression of CEA by 293T cells was achieved by Lipofectamine (Invitrogen) transfection of pHR′-CMVCEA-IRESGFP; the plasmid pHR′-CMVv-FLIP-IRESGFP, which expresses the Kaposi sarcoma-associated viral FLICE inhibitory protein, was used as a control. Forty-eight hours posttransfection the percentage of GFP-positive cells was determined and CEA expression was confirmed by immunoblotting, and then viral transductions were performed. After transduction cells were separated by fluorescence-activated cell sorting using an Epics Elite (Coulter) flow cytometer into GFP-positive and -negative populations. The cells were replated, cultured in DMEM–10% FCS for 48 h, and then X-Gal stained.

***Growth of tumor xenografts in nude mice.*** Tumor xenografts were established in athymic female Balb/c nu/nu mice by injection of $5 \times 10^6$ cells suspended in 0.2 ml HBBS subcutaneously into the left side of the abdomen. When the tumors had reached 5–7 mm in diameter they were injected with $1 \times 10^6$ lethally irradiated (40 Gy) producer cells on 2 successive days. One week after the last producer cell injection the animals were sacrificed and the tumors excised and disaggregated before incubation with 2 volumes of 5 mg/ml collagenase 1a (Sigma) for 2 h at 37°C with occasional agitation. The separated tumor cells were pelleted, washed once in HBBS, and resuspended in DMEM supplemented with 10% FCS. After 2 h of incubation, nonadherent cells were discarded and adherent tumor cells were incubated overnight before being stained for β-galactosidase expression. Up to $10^6$ disaggregated tumor cells were analyzed for tumor transduction.

***Vector transduction of host cells.*** Nude mice were injected intraperitoneally with $10^7$ irradiated (40 Gy) MFE23/ProMMP or unenveloped producer cells or $10^6$ irradiated (40 Gy) amphotropic producer cells in 0.2 ml of HBBS. Two weeks after injection the mice were sacrificed and the spleen, liver, and kidneys were removed and snap frozen at −80°C. DNA was extracted from 5–10 mg of tissue using a DNAeasy tissue kit (Qiagen) as per the manufacturer's instructions. Proviral analysis was performed by nested PCR using primers that amplify a 480-bp DNA fragment located between the 3′ end of the β-galactosidase gene and the 3′ LTR of the integrated vector.

External primers were LacZ1, 5′-GCACATGGCTGATATCGAACGG-3′, and LTR1, 5′-GCTTCAGCTGGTGATATTGTTGAG-3′. Internal primers were LacZ2, 5′-ATTGGTGGCGACGACTCCTG-3′, and LTR2, 5′-AGCCTGGACCACTGATATCCTG-3′. PCR conditions were 35 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C. DNA was quantified by the ratio of optical densities at 260 and 280 nm in a spectrophotometer (Hitachi; U-1500). Ten micrograms of DNA (approximately $10^6$ mouse cell equivalents) was used for the first PCR (LTR1/LacZ1) and 1/10 of this reaction was used as template for the second round of the nested PCR (LTR2/LacZ2). Semiquantitative PCR was carried out by serial dilutions of the DNA samples. Plasmid DNA harboring one copy of the MFGnlslacZ vector genome was used to determine the sensitivity of the system (1 plasmid = 0.1 fg).

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