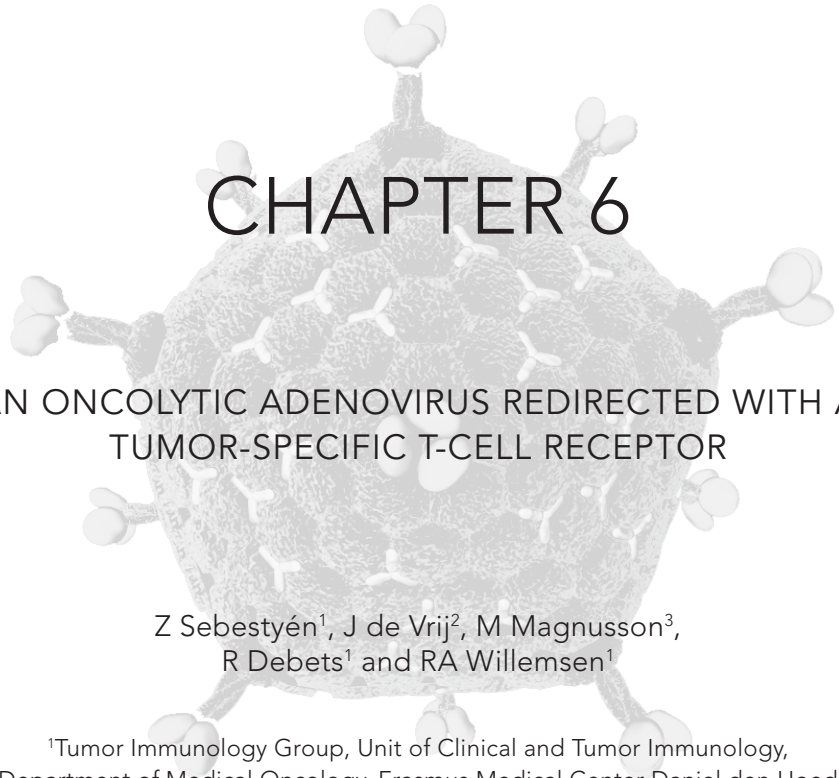


CHAPTER 6

AN ONCOLYTIC ADENOVIRUS REDIRECTED WITH A TUMOR-SPECIFIC T-CELL RECEPTOR

Z Sebestyén[1], J de Vrij[2], M Magnusson[3], R Debets[1] and RA Willemsen[1]

[1]Tumor Immunology Group, Unit of Clinical and Tumor Immunology, Department of Medical Oncology, Erasmus Medical Center-Daniel den Hoed Cancer Center, Rotterdam, the Netherlands; [2]Virus and Stem Cell Biology Laboratory, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands and [3]Got-A-Gene AB, Kullavik, Sweden

Cancer Research 2007;67:11309-11316

ABSTRACT

To improve safety and specificity of oncolytic adenoviruses, we introduced T-cell receptors (TCR) specific for a unique class of truly tumor-specific antigens into the adenoviral fiber protein. The adenoviral fiber knob responsible for attachment to the coxsackie-adenoviral receptor (CAR) on target cells was replaced by a single-chain TCR (scTCR) molecule with specificity for the melanoma-associated cancer-testis antigen MAGE-A1, presented by HLA-A1, and an extrinsic trimerization motif in a replicating Ad5 vector (Ad5.R1-scTCR). The production of the recombinant virus was initiated in a novel producer cell line that expressed an antibody-based hexon-specific receptor (293T-AdR) in the cell membrane. This new production system allowed CAR-independent and target antigen-independent propagation of Ad5.R1-scTCR. Infection with adenovirus bearing the scTCR-based fiber resulted in an efficient killing of target tumor cells. The infection was cell type specific because only HLA-A1$^+$/MAGE-A1$^+$ melanoma cells were killed, and thus, this retargeting strategy provides a versatile tool for future clinical application.

INTRODUCTION

A prerequisite for the safe and effective application of therapies that are based on antigen-driven tumor eradication is the nature of the target antigen, which has to be truly tumor specific. Studying tumor cell eradication by CTL immunologists identified such tumor-specific antigens that were targets for CTL while leaving healthy tissue intact.[1,2] Tumor cells may express a group of antigens termed "cancer-testis antigens" that are presented as antigenic peptides by MHC molecules to CTL.[3] In fact, cancer-testis antigens are immunogenic in cancer patients as they may elicit an anticancer response.[4-6] They exhibit highly tissue-restricted expression and are considered promising target molecules for immunotherapies. To date, 44 cancer-testis antigen gene families have been identified and their expression has been studied in numerous cancer types.[7] For example, bladder cancer, non-small lung cancer, and melanoma are high cancer-testis antigen gene expressers, with 55%, 51%, and 53% of the cancer-testis antigen transcripts examined by reverse transcription-PCR detected in 20% or more of the specimens examined, respectively. With the exception of testis-restricted cancer-testis antigen transcripts, all remaining cancer-testis antigen transcripts were expressed in normal pancreas. Other antigens that were shown to elicit potent antitumor responses in cancer patients include differentiation antigens, such as the melanoma antigens gp100, Mart-1, and tyrosinase, or antigens that are overexpressed on tumor cells, such as p53, Her-2/neu, and WT-1.[7,8] Both groups of antigens are also expressed in healthy tissue and may therefore elicit autoimmune disease when targeted.

Oncolytic adenoviral vectors hold great promise for cancer gene therapy because they potently eradicate tumor cells.[9] Therefore, efforts are currently invested in improving replication-competent adenoviruses with respect to safety and specificity to fulfill criteria for clinical application.[10] Among several strategies, genetic modification of the adenoviral fiber, which is responsible for cell binding, may result in a logical and preferable site to carry structures that specifically bind to target antigens of choice, thereby changing viral tropism.

Application of adenoviruses in a tumor cell-specific fashion is highly hampered because the natural cellular receptor of adenovirus is widely expressed on normal tissues and, on the other hand, often reported to be down-regulated or even absent on tumor cells.[11] To address this issue, one needs strategies to alter the tropism of adenoviral vectors and retarget them against tumor-specific antigens.

Recent developments to change the natural tropism of adenoviral vectors into tumor-specific recognition are based on the genetic engineering of capsid proteins, such as pIX,[12,13] hexon, and fiber.[14-19]

Genetic modification of the fiber protein has been achieved either through exchange of the Ad5 fiber knob with the Ad3 knob,[20,21] knob mutagenesis,[22,23] or incorporation of small ligands into the knob domain.[21,24] However, it should be noted that an effective and safe retargeting strategy should include complete ablation of the natural tropism, which is not guaranteed by the above-mentioned modifications, and preferably include deletion of the fiber knob. This can be accomplished by replacing the fiber knob by new antigen-binding structures and an extrinsic trimerization signal.[18,21] Antibody or T-cell receptor (TCR) fragments [e.g., single-chain Fv (scFv)

and single-chain TCR (scTCR)] mediate tumor cell recognition and are able to redirect T cells[25] and viruses[26] and, as such, are candidate structures to genetically redirect adenoviruses to tumor cells. Previous attempts to produce adenoviruses with fibers that include scFv have failed, most likely as a consequence of improper folding of the chimeric fiber in the cellular cytoplasm.[18]

Here, we show that an oncolytic adenovirus bearing chimeric fibers, comprising an extrinsic trimerization signal and scTCR with HLA-A1–restricted MAGE-A1 specificity, can be produced. To this end, we generated a novel producer cell line expressing an anti-hexon receptor, which was needed to initiate production of virus that specifically infects HLA-A1/MAGE-A1$^+$ melanoma cells but not MAGE-A1$^-$ or HLA-A1$^-$ target cells. The presented strategy to produce genetically retargeted oncolytic adenoviruses holds great promise to develop clinically applicable anticancer agents.

RESULTS

Construction of a replication-competent adenovirus with HLA-A1/MAGE-A1 specificity

A knobless fiber containing fiber tail plus NH_2-terminal first shaft repeat (R1, 61 amino acids), an extrinsic trimerization motif (NRP, 36 amino acids) from lung surfactant protein D, a linker derived from *Staphylococcus* protein A (13 amino acids), and scTCR VαVβCβ (377 amino acids), specific for the melanoma antigen MAGE-A1, presented by HLA-A1 (which replaced the natural fiber knob), was constructed (**Fig. 1**) and introduced into replication-competent adenovirus serotype 5 essentially as described.[17,18] To construct the scTCR, TCR α and β chains were cloned from an HLA-A1–restricted, MAGE-A1–specific CTL clone, MZ2-82/30, and reformatted into the scTCR VαVβCβ as described.[27] Specific binding of the scTCR was verified by expression on primary human T lymphocytes, which showed scTCR-directed immune functions such as specific tumor cell kill and cytokine production. The apparent molecular weight of the R1-scTCR fiber (54 kDa) is similar to that of the WT Ad5 fiber (59 kDa).

Generation of 293T-AdR cells to propagate fiber-modified adenoviruses

As a consequence of ablating the natural tropism, we expected that recombinant Ad5-scTCR virus would require the presence of its target antigen, HLA-A1/MAGE-A1, on the surface of the producer cell line for the primary attachment and entry. Therefore, we generated 293T cells expressing the HLA-A1/MAGE-A1 antigen. 293T cells were infected with retroviral vectors pBullet HLA-A1 and pBullet MAGE-A1 (full-length cDNA and minigene). Next to these antigen$^+$ 293T cells, we also used MZ2-mel 3.0 melanoma cells, which naturally present MAGE-A1 in the context of HLA-A1. Neither the antigen-transduced 293T cells nor MZ2-mel 3.0 cells were able to initiate production of the recombinant virus starting with transfection of the adenoviral DNA (data not shown). To support initiation of viral production and propagation of fiber-modified adenoviruses that depend neither on CAR nor on MAGE-A1/HLA-A1 antigen (or any ligands of interest for that matter), we generated a novel producer cell line based on the introduction of an adenovirus-binding antibody into 293T cells.

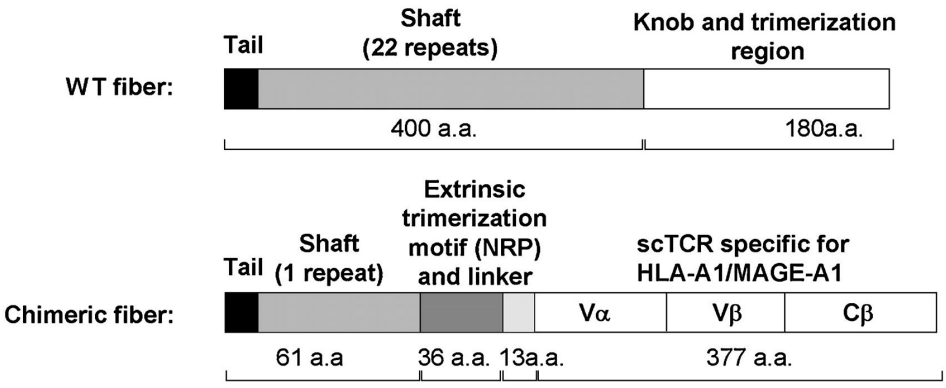


Figure 1. Diagram of WT Ad5 fiber and R1-scTCR fiber. Distinct domains of the Ad5 WT fiber and R1-scTCR fiber as well as the amino acid (a.a.) composition of the distinct domains. Vα, variable domain of TCRα chain; Vβ, variable domain of TCRβ chain; Cβ, constant domain of TCRβ chain.

To this end, we constructed two membrane-anchored anti-adenovirus receptors, AdR and AdR-cMyc/ζ, from hybridoma cells producing a hexon-specific antibody (**Fig. 2a**) that cross-reacts with many adenovirus subtypes and introduced it via retroviral transduction into 293T cells. Due to a lack of antibodies binding to the scFv directly, demonstration of cell surface expression of the anti-adenovirus receptors on 293T cells was only possible for AdR-cMyc/ζ using anti-c-Myc mAb (**Fig. 2b**).

The ability of AdR and AdR-cMyc/ζ to serve as universal receptors for Ad5 was analyzed in 293T cells. 293T cells with AdR, termed 293T-AdR, 293T cells with AdR-cMyc/ζ, termed 293T-AdR-cMyc/ζ, or parental 293T cells were transfected with a fiberless Ad5 vector encoding the *EGFP* gene. We observed a severely impaired propagation of fiberless adenovirus in 293T cells in line with previous reports and most likely due to a lack of CAR-fiber knob interactions.[21,28] We hypothesized that the presence of the AdR receptor would at least in part restore the ability of 293T cells to produce fiber-deleted viruses. As shown in **Fig. 2c**, on day 1 following transfection, the expression of EGFP was comparable in both 293T and 293T-AdR cells. The ratio of EGFP in normal 293T cells did not improve on day 2 or 3. However, in 293T-AdR cells, we observed a robust spread of the reporter gene together with comet-like formation that was most significant on day 3 after transfection. Production of virus particles in culture supernatant was confirmed by an adenovirus-specific ELISA (data not shown). When 293T cells were stably expressing the AdR-cMyc/ζ receptor, we also observed an increase in reporter gene expression and release of viral particles. However, the ability of 293T-AdR-cMyc/ζ to induce adenovirus production was significantly less than that of 293T-AdR cells (data not shown).

We then introduced the Ad5.R1-scTCR construct into 293T-AdR cells and showed that, 3 days following transfection, virus was produced at a titer of 6×10^7 particles/mL (= physical particles, determined by ELISA), starting from 3×10^6 293T-AdR cells.

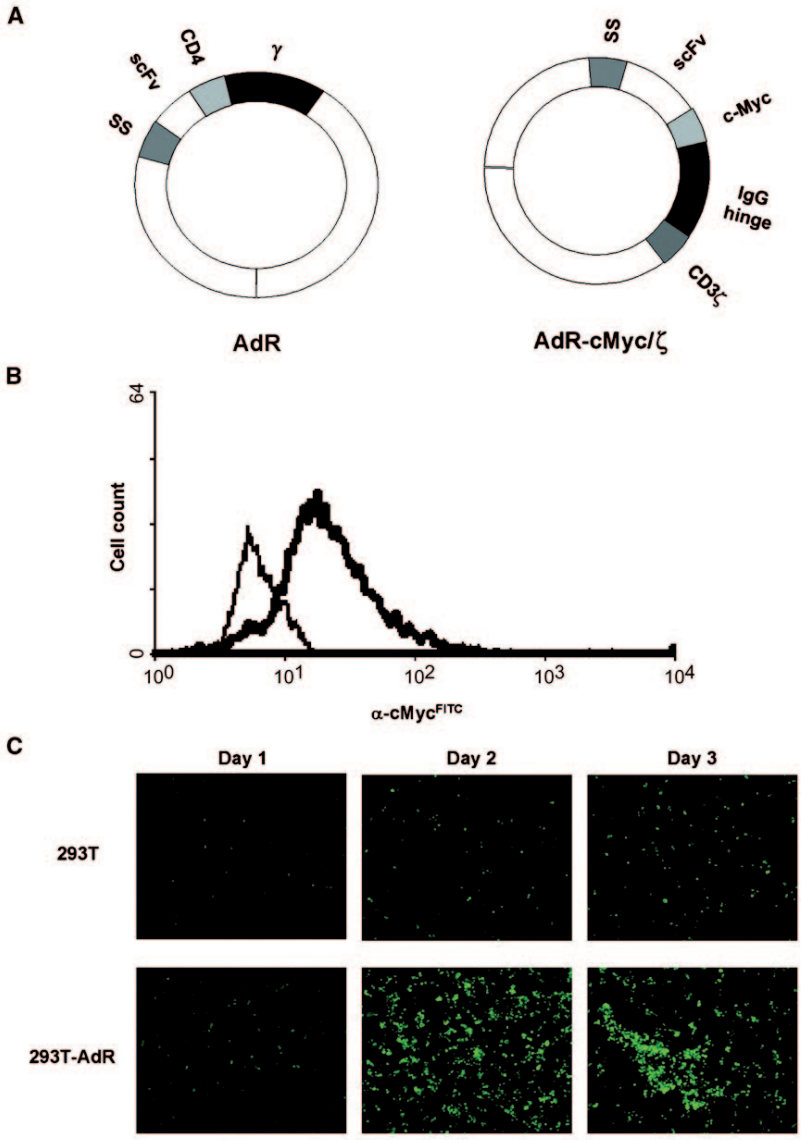


Figure 2. (**a**) Diagram of expression vectors encoding the "adenoreceptor." SS, Igκ signal sequence; γ, intracellular domain of FcεR1-γ chain. (**b**) Surface expression of AdR-cMyc/ζ on 293T cells. 293T cells were retrovirally transduced with the adenoreceptor fused to a c-Myc tag (AdR-cMyc/ζ). Cells were stained with FITC-conjugated c-Myc-specific mAb (9E10) and samples were measured by flow cytometry. Histograms represent nontransduced 293T cells (*thin line*) and receptor-positive 293T cells (*thick line*). (**c**) Infection of 293T cells by fiberless adenovirus requires the expression of AdR on the cell surface. 293T and 293T-AdR cells were seeded and transfected with fiberless pAdeasy EGFP construct. Kinetics of green fluorescent protein expression was monitored by fluorescence microscopy at days 1, 2, and 3 after transfection. Representative images (×10 magnification) from one of three experiments.

Ad5.R1-scTCR virus is produced, specifically binds to HLA-A1/MAGE-A1 complexes, and replicates in HLA-A1$^+$/MAGE-A1$^+$ tumor cells

To show production, specific binding to peptide/MHC complexes, and fiber incorporation of adenoviral particles that incorporate the chimeric R1-scTCR fiber, we did the following experiments: (*a*) electron microscopy, to show presence of viral particles in MZ2-mel 3.0 cells (**Fig. 3a**); (*b*) flow cytometry analysis of HLA-A1/MAGE-A1–specific binding of Ad5-R1-scTCR (**Fig. 3b**); (*c*) Western blot analysis, to show incorporation of the R1-scTCR fiber (**Fig. 3c**); and (*d*) ELISA, to show production of Ad5.R1-scTCR in 293T AdR and MZ2-mel 3.0 cells.

To show production of Ad5.R1-scTCR particles, MZ-2-mel 3.0 cells were infected with viral supernatant and analyzed by electron microscopy. **Fig. 3a** shows the presence of viral particles in the nucleus of MZ2-mel 3.0 cells 72 h after infection.

To show specific binding to HLA-A1/MAGE-A1, supernatant from 293T-AdR cells producing Ad5-R1-scTCR was incubated with magnetic beads that were loaded with HLA-A1/MAGE-A1 complexes or HLA-A1 complexes that present an irrelevant peptide derived from influenza virus A nucleoprotein.

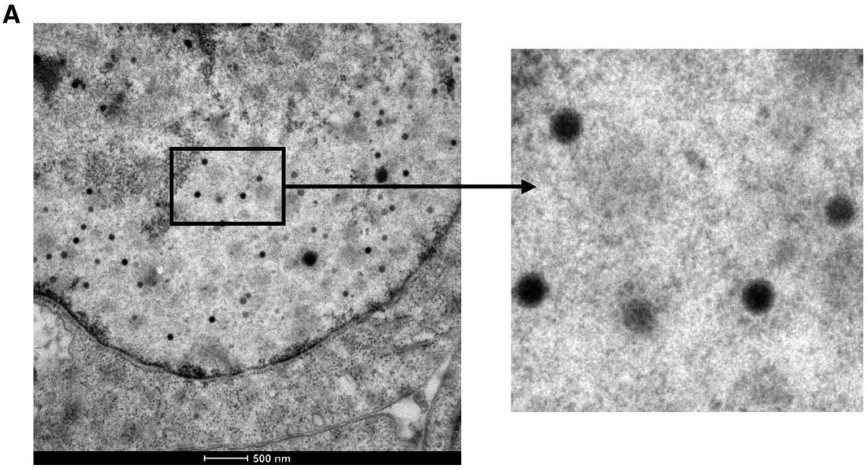
As shown, Ad5.R1-scTCR virus only bound to HLA-A1/MAGE-A1 complexes and not to HLA-A1 complexes presenting an irrelevant influenza virus peptide (**Fig. 3b**).

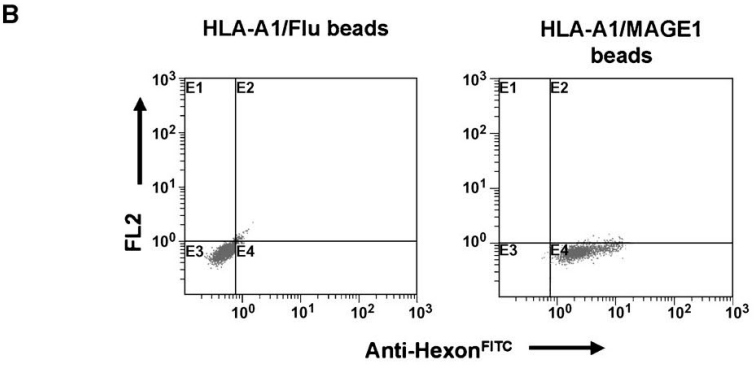
Ad5.R1-scTCR virus bound to HLA-A1/MAGE-A1–coated magnetic beads was then analyzed by Western blotting using fiber tail-specific mAb 4D2. As shown in **Fig. 3c**, chimeric scTCR fibers were incorporated into adenoviral particles.

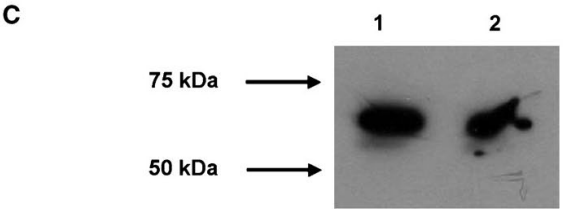
To determine whether Ad5.R1-scTCR virus is able to infect HLA-A1$^+$/MAGE-A1$^+$ tumor cells, we incubated MZ2-mel 3.0 melanoma cells with supernatant obtained from 293T AdR cells transfected with either Ad5.WT or Ad5.R1-scTCR DNA. MZ2-mel 3.0 cells lack CAR expression (**Table 1**), making them refractory to infection by WT virus. As shown in **Fig. 3d**, Ad5.R1-scTCR virus produced by 293T AdR cells infected MZ2-mel 3.0 cells and was able to replicate in these cells, shown by the presence of viral particles in the tissue culture supernatant 3 days after infection. In contrast, WT virus at comparable virus particle-to-cell ratio did not result in adenoviral infection.

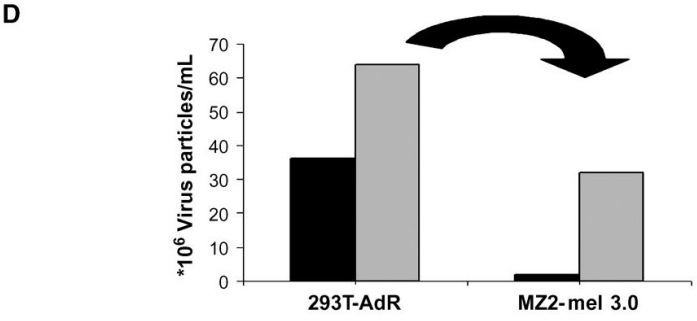
Cellular localization of Ad5.R1-scTCR epitopes during replication

It has been suggested that only those recombinant fibers that assemble correctly in the nucleus may be incorporated into an infectious adenoviral particle.[24] To analyze intracellular localization of adenoviral proteins, we infected MZ2-mel 3.0 cells with Ad5.R1-scTCR and did intracellular immunofluorescent staining with mAbs specific for hexon, fiber tail, and scTCR. Hexon and fiber tail molecules showed a comparable cellular distribution, localized almost exclusively to the nucleus 24 h after infection (**Fig. 4, *top***). Interestingly, at that time point, hexon molecules were detected in the cytoplasm to some extent, whereas fiber molecules were not. In control experiments, we observed that adenoviruses displaying WT fiber showed similar cellular localization of hexon and fiber tail (data not shown). In contrast, staining for the R1-scTCR fiber with the anti-TCR-specific antibody did not result in any detectable fluorescent signal at 24 h after infection (**Fig. 4, *top***).


A
500 nm


B
HLA-A1/Flu beads
HLA-A1/MAGE1 beads
E1
E2
E3
E4
FL2
Anti-Hexon^FITC


C
1
2
75 kDa
50 kDa


D
*10^6 Virus particles/mL
293T-AdR
MZ2-mel 3.0

◀ **Figure 3.** Characterization of Ad5.R1-scTCR particles. (**a**) Electron microscopic image of Ad5.R1-scTCR in MZ2-mel 3.0 cells. Presence of Ad5.R1-scTCR particles in the nucleus of MZ2-mel 3.0 cells was shown by electron microscopy 72 h after incubation of MZ2-mel 3.0 cells with viral supernatant. Right, a five times enlargement of a region of the left. (**b**) Ad5.R1-scTCR particles from 293T-AdR supernatant specifically bind to HLA-A1/MAGE-A1 complexes only. Tissue culture supernatants derived from 293T-AdR cells producing Ad5.R1-scTCR particles were incubated with HLA-A1/MAGE-A1 or irrelevant HLA-A1/Flu complex-coated magnetic beads. Ad5.R1-scTCR particles were detected by flow cytometric analysis using anti-hexonFITC mAb. (**c**) Ad5.R1-scTCR viral particles incorporate the R1-scTCR fiber. Ad5.R1-scTCR virus bound to HLA-A1/MAGE-A1 complex-coated beads and cesium chloride-purified WT Ad5 were loaded on 7% SDS-PAGE, transferred to nitrocellulose membrane, and detected with anti-fiber tail antibody 4D2. *Lane 1*, Ad5.WT; *lane 2*, Ad5.R1-scTCR. (**d**) Adenovirus expressing the scTCR fiber infects and replicates in HLA-A1/MAGE-A1+ melanoma cells. MZ2-mel 3.0 cells were incubated with viral supernatant obtained from 293T AdR cells transfected with either Ad5.R1-scTCR or Ad5.WT DNA. Adenoviral titer in tissue culture medium was detected by ELISA after (*a*) transfection of the producer cell 293T-AdR with Ad5.WT (*black columns*) or Ad5.R1-scTCR (*gray columns*) and (*b*) 3 d after infection of MZ2-mel 3.0 cells by crude lysates from *a*.

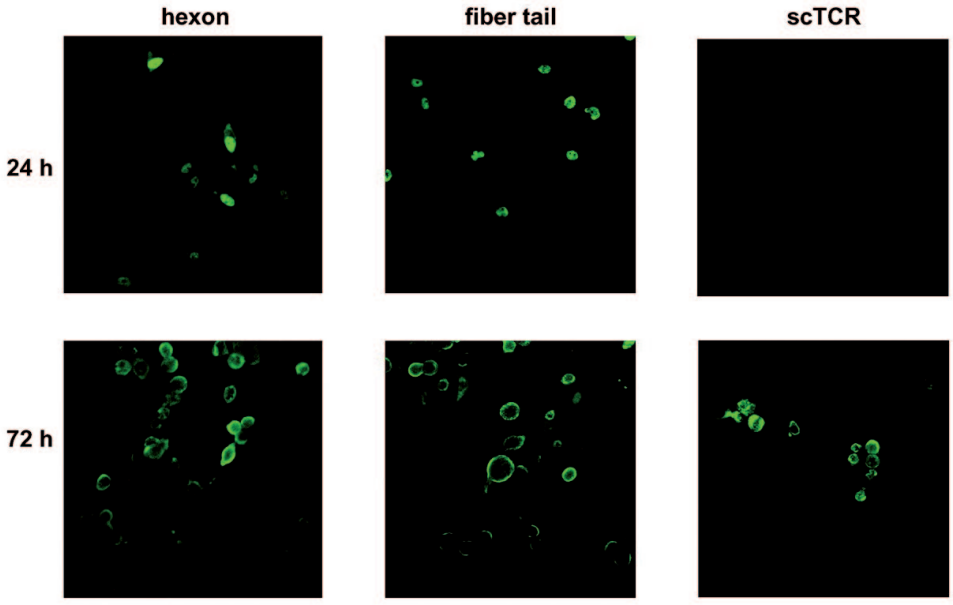


Figure 4. Localization of adenoviral proteins during replication. MZ2-mel 3.0 cells were infected with Ad5.R1-scTCR at a virus particle-to-cell ratio of 50, and cell-associated adenoviral proteins were stained with mAbs against hexon (α-hexon), fiber tail (4D2.5), and scTCR Vα domain (Vα12.1) at 24 and 72 h after infection. Images were collected with a fluorescent microscope.

Localization of both hexon and fiber proteins changed at 72 h after infection from a prominent nuclear localization to accumulation at the cell periphery, most likely at the plasma membrane. At this stage of replication, presumably on virus release, R1-scTCR could be detected with anti-TCR antibody and also located to the plasma membrane or to its proximity, indicating an identical cellular compartmentalization of hexon, fiber tail, and scTCR (**Fig. 4, *bottom***).

Infection by Ad5.R1-scTCR is epitope specific

Specificity of infection of Ad5.R1-scTCR was analyzed by infecting the melanoma cells: MZ2-mel 3.0 (HLA-A1$^+$/MAGE-A1$^+$) and MEL.2A (HLA-A1$^+$/MAGE-A1$^-$). Also included were 293T and 293T-AdR cells. Target cells were infected at different virus particle-to-cell ratios and monitoring the production of hexon protein at 2 days after infection. In this assay, cells expressing the hexon molecule represent infected cells and constitute an indirect readout for viral titers as an alternative to plaque assay. As shown in **Fig. 5**, Ad5.R1-scTCR virus reached maximum infectivity at ~20 virus particle-to-cell ratio when infecting antigen$^+$ MZ2-mel 3.0 cells and 293T-AdR, approximately corresponding to a MOI of 4. When using antigen$^-$ MZ2-mel 2.2 cells, Ad5.R1-scTCR infectivity remained low even at high virus particle-to-cell ratio and showed a similar titration curve when using 293T cells.

Specificity studies were expanded by the use of a larger panel of target cells, including the following melanoma cell lines: MZ2-mel 3.0; 9303-A; 518-A2; MZ2-mel 2.2, a MAGE-A1 antigen lost mutant obtained from MZ2-mel 3.0; MEL.2A; and FM-3. We also included Nemeth renal cell carcinoma cell lines and the 293T cells. Five days after infection, surviving tumor cells were stained with methylene blue. HLA-A1/MAGE-A1 expression as well as infectivity data of all target cells are summarized in **Table 1**. As shown in **Table 1** only HLA-A1$^+$/MAGE-A1$^+$ melanoma cells were infected.

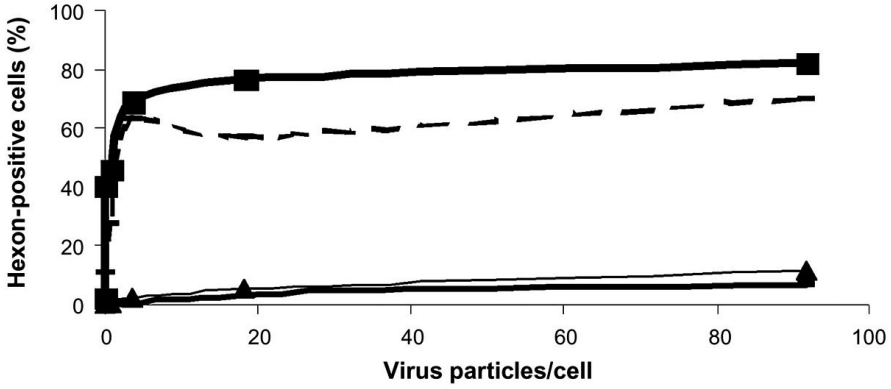


Figure 5. Ad5.R1-scTCR specifically infects melanoma cells expressing the HLA-A1/MAGE-A1 epitope at a low virus particle-to-cell ratio. Cells (5×10^5) of MZ2-mel 3.0 (*solid line with black box*), MEL.2A (*solid thin line*), 293T (*solid line with triangle*), and 293T-AdR (*dashed line*) were infected at various virus particle-to-cell ratios of Ad5.R1-scTCR. Cells were harvested 2 d after infection and stained with FITC-labeled mAb against hexon molecule. Percentage of hexon-expressing cells analyzed by flow cytometry.

Table 1. Antigen expression and infection of melanoma and renal cell carcinoma cell lines by Ad5.R1-scTCR.

Target cell	HLA-A1	MAGE-A1	CAR	Infection by Ad5.R1-scTCR*
MZ2-mel 3.0	+	+	–	+
9303-A	+	+	+	+
518-A1	+	+	+	+
MZ2-mel 2.2	+	–	–	–
MEL2A	+	–	+	–
FM-3	–	–	+	–
Nemeth	–	–	+	–
293T	–	–	+	–

* Represents infection of target cells analyzed by methylene blue staining. + or – is based on qualitative comparison with noninfected control cells.

DISCUSSION

Our aim of this study was to provide oncolytic adenoviral vectors with a truly tumor cell specificity to improve safety and efficacy, which are major criteria for the clinical use of replicating vectors. TCR recognizing MHC-restricted cancer-testis antigens, such as MAGE-A1, which are distributed in a highly tumor tissue-specific manner, may constitute promising molecules to retarget adenoviruses. This study shows for the first time that even complex molecules such as TCR can genetically replace the fiber knob and be expressed on the adenoviral fiber, thereby ablating its natural tropism. The Ad5 fiber knob was replaced by an extrinsic trimerization motif and an HLA-A1/MAGE-A1–specific scTCR. Critical to the experimental use of this recombinant adenovirus was the generation of a novel producer cell line, 293T-AdR, which was successfully used to initiate production, starting by transfection with adenoviral DNA, and supported propagation of adenovirus, which depended neither on CAR nor on HLA-A1/MAGE-A1. Our failure to use 293T expressing the HLA-A1 and MAGE-A1 cDNA for the initiation of adenovirus production was most likely attributed to unstable expression of the *HLA-A1* gene. Within 3 days after infection with retrovirus encoding the *HLA-A1* gene, HLA-A1 molecules disappeared from the cell surface, resulting in lack of MAGE-A1 antigen presentation to the chimeric fibers (data not shown). There may be more reasons why we were unable to initiate adenovirus production in MZ2-mel 3.0 cells. In general, the production of adenovirus after DNA transfection is inefficient because plasmid DNA does not contain the protein binding to the viral inverted terminal repeats and therefore requires several rounds of amplification.[29] When DNA transfection efficiencies become limiting, as might be the case when using MZ2-mel 3.0 cells, low numbers of virus-producing cells may result in undetectable levels of virus even after serial amplification. In addition, MZ2-mel 3.0 cells may produce lower numbers of viral particles than, for example, 293 cells.

Importantly, the retargeted viruses specifically bound to relevant HLA-A1/MAGE-A1 complexes only (**Fig. 3b**), specifically infected target cells expressing HLA-A1–restricted MAGE-A1 antigen (**Fig. 5; Table 1**), and killed these melanoma cells.

The initiation of production when starting from recombinant Ad5 DNA seemed to be a critical step during propagation. As a consequence of ablation of native Ad5 tropism, the recombinant Ad5.R1-scTCR is not able to use the natural CAR-mediated cellular entry pathway during propagation in conventional packaging cell lines, such as 293 or 911. Although there are reports on possible solutions to overcome the limitations of fiber-modified adenoviral vector production,[28,30] there is no precedent on virus retargeting via complex molecules, such as TCR or Igs, and an alternative strategy had to be developed. A major factor that can hamper genetic retargeting of adenoviral vectors, especially when including complex molecules into the viral genome, is the proper folding of these new molecules in the nucleus. According to Pecorari *et al.*,[31] the formation of intrachain disulfide bridges, which are crucial for the correct folding and stability of IgG or TCR, is suboptimal in the reducing environment of the cytoplasm and nucleus. One could address this issue by using small molecules, such as affibodies, which possess the binding properties of an antibody but do not require intrachain disulfide bounds.[16] Magnusson *et al.*[18] reported that, although the scTCR fiber was able to form homotrimers and bound its ligand, the recombinant fiber protein misfolded, thereby possibly explaining the unsuccessful propagation of Ad5.R1-scTCR in MZ2-mel 3.0 melanoma cells. However, our studies on scTCR fiber expression suggest that at the proximity to the cell membrane (**Fig. 4, *bottom***) proper folding of the scTCR fiber occurs, resulting in exposure of a fully functional scTCR fiber on the virus particle. We assume that at this stage of virus assembly restricting intracellular conditions no longer limits the formation of sulfide bridges. Furthermore, we now also succeeded in the initiation of production of two other recombinant viruses, one with a scTCR fiber and a virus equipped with an affibody fiber (data not shown), which shows the universal applicability of this production system.

These findings open new and safer strategies for cell-specific retargeting of oncolytic adenoviruses, providing a versatile tool for future clinical application.

MATERIALS AND METHODS

Cells and antibodies

Target cell lines used in this study are the melanoma cell line MZ2-mel 3.0, MZ2-mel 2.2, and MEL.2A (kindly provided by T. Boon and P. Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium). The melanoma cell lines 9303-A, 518-A1, and FM-3; the renal cell carcinoma cell line Nemeth (kindly provided by Dr. E. Oosterwijk, University Medical Center Nijmegen, Nijmegen, the Netherlands); and the human embryonic kidney cell line 293T and its derivative 293T-AdR (further described in the Results section) were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone). HLA-A1/MAGE-A1 and coxsackie-adenovirus receptor (CAR) expression status of the target cell lines is further described in **Table 1** (see Results).

Antibodies used in this study were against fiber tail (4D2.5; NeoMarkers), hexon (clone BOD604, FITC conjugated; Biodesign), rabbit polyclonal anti-Ad5 (Abcam), TCR Vα12.1 (FITC or nonconjugated; Endogen), CAR (USBiological), and c-Myc (9E10, FITC conjugated; Convance). FITC-conjugated rabbit anti-mouse IgG Fab fragment

(Jackson ImmunoResearch) or horseradish peroxidase–conjugated goat anti-rabbit IgG (Becton Dickinson Biosciences) was used as secondary antibodies.

Infectivity assay with fiberless virus

293T and 293T-AdR cells were transfected with a fiberless pAdeasy-EGFP construct (a kind gift of Wim Jongmans, University Medical Center, Nijmegen, the Netherlands) using the CellPhect Transfection kit (Amersham Biosciences). The expression of the reporter gene *EGFP* was monitored using a Leica DMIL inverted fluorescence microscope (Leica Microsystems). At time points indicated, culture supernatant was collected and virus release (particle count) was analyzed using the IDEIA Adenovirus ELISA kit (DakoCytomation).

DNA constructs

Ad5.R1-scTCR adenoviral DNA was generated as described.[18] Briefly, recombinant fiber genes were constructed using methods based on ligation, PCR, and splicing by overlap extension. The gene encoding the Ad5 wild-type (WT) fiber was obtained from pAB26 (Microbix, Inc.) by PCR introducing an upstream *Bam*HI and downstream *Xho*I site, respectively. The knob domain in recombinant fibers was deleted and replaced by a 36-amino acid extrinsic trimerization motif derived from the neck region peptide (NRP) of human lung surfactant protein D.[18] The NRP sequence followed by a linker sequence from *Staphylococcus* protein A was ligated to the COOH-terminal end of fiber shaft with one repeat and named R1, and the scTCR VαVβCβ was added to the COOH-terminal end of the Staph-A linker. The resulting R1-scTCR fiber was then cloned into a fiberless Ad5 genome as described.[18]

Retroviral vectors encoding the *HLA-A1* gene, MAGE-A1 complete cDNA, or MAGE-A1 minigene (encoding the 9-amino acid antigenic epitope EADPTGHSY) were generated as follows: HLA-A1 and MAGE-A1 cDNA cloned in pCDNA-3 (a kind gift from Pierre van der Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium) were reamplified to introduce *Nco*I and *Xho*I sites and cloned into the retroviral vector pBullet. The MAGE-A1 minigene was introduced into a version of pBullet that contains a signal sequence from the G250 antibody heavy chain[32] by ligation of a small linker encoding the MAGE-A1 minigene next to the signal sequence.

To construct the membrane-bound adenovirus-specific receptor (AdR), first an scFv was generated from the hexon-specific hybridoma 2Hx-2 (American Type Culture Collection). In short, RNA isolated from the 2Hx-2 hybridoma was reverse transcribed using SuperScript II (Invitrogen) and amplified using Ig variable heavy and variable light chain primers (Amersham scFv module, Amersham Biotech). The variable heavy and variable light chain DNA fragments were then reamplified to fuse them together by introducing a linker sequence between the two fragments and to introduce *Sfi*I and *Not*I restriction sites. The resulting scFv was then introduced into the retroviral expression cassette pBullet-CD4γ, and pBullet-cMyc/ζ, which allows for membrane expression of the scFv.[32,33]

Generation of the recombinant virus

293T-AdR cells were transfected with *Pac*I-digested recombinant adenovirus plasmid (Ad5.R1-scTCR), and after 3 days, culture supernatant was harvested and used

immediately for infection or further analysis. Adenovirus particle count (semiquantitative) was determined by IDEIA Adenovirus ELISA kit. Infectious adenovirus particle number [multiplicity of infection (MOI)] was determined by the Adeno-X Rapid titer kit (BD Clontech) on 293T-AdR cells.

Analysis of adenoviral particles

Electron microscopy

For electron microscopy, MZ2-mel 3.0 cells were fixed in 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 1 h at room temperature, postfixed in 1% OsO_4 in the same buffer for 1 h at 4°C, dehydrated in a graded ethanol series, and embedded in epon. Ultrathin sections were poststained with uranyl acetate and lead citrate and viewed with a Tecnai 12 electron microscope at 80 kV (FEI).

Flow cytometry

Supernatant derived from Ad5.R1-scTCR–producing 293T-AdR cells (12.5 mL containing 10^8 particles/mL) was incubated overnight with magnetic beads (Dynal Biotech ASA) that were loaded with *in vitro*-generated HLA-A1 complexes (1 µg total) presenting the MAGE-A1 nonapeptide (EADPTGHSY) or an irrelevant peptide derived from influenza virus A nucleoprotein (CTELKLSDY). After three wash steps with PBS, the beads were incubated with a saturating concentration of anti-hexon^{FITC} monoclonal antibody (mAb) and incubated for 30 min at 4°C. Specific binding of Ad5.R1-scTCR virus to the beads was then analyzed by flow cytometry on a Cytomics FC-500 flow cytometer (Beckman Coulter).

Western blotting

Ad5.R1-scTCR virus bound to the HLA-A1/MAGE-A1–coated magnetic beads was eluted from the beads by addition of high-affinity Fab fragments that specifically bind to HLA-A1/MAGE-A1 (15 min at room temperature, 39 µg total in 1 mL PBS).[34] Excess high-affinity Fab fragments were then removed by addition of Ni-NTA agarose (Qiagen) that binds to the 6× His tag present in the Fab fragment. Purified Ad5.R1-scTCR virus was then separated on SDS-PAGE, immobilized on a nitrocellulose membrane, and detected with fiber tail–specific mAb (4D2).

Detection of adenoviral infection

Flow cytometry of infected cells

One million cells were infected at indicated virus particle-to-cell ratios using virus supernatant diluted in DMEM supplemented with 10% FBS for 2 h at 37°C/5% CO_2. After infection, cells were seeded in six-well plates. Cells were harvested 2 days after infection by scraping, after which they were spinned and permeabilized in FACSPerm2 solution (Becton Dickinson). Following a PBS wash, cells were incubated in the presence of FITC-hexon mAb (1:10 dilution) for 30 min at room temperature in the dark, washed again, and analyzed on a Cytomics FC-500 flow cytometer.

Methylene blue staining of infected cells

Half a million cells were infected using virus supernatant as described above. After infection, cells were seeded in gelatin-coated (0.1% gelatin in PBS) six-well plates in the presence of 2 mL 1.25% agar in DMEM culture medium. Cells were stained

with methylene blue 5 days (after infection) and photographed (LEICA DMIL inverted microscope).

Expression and localization of adenoviral proteins

A quarter of a million cells were infected using virus supernatant (at virus particle-to-cell ratio of 50) as described above. After infection, cells were seeded in 24-well plates and cultured for the indicated times. Cells were carefully washed with PBS and fixed with a 1:1 solution of ice-cold methanol and acetone for 10 min on ice. After repeated washing steps with PBS, cells were blocked using 1% bovine serum albumin in PBS for 30 min at room temperature. Cells were then shortly air dried and stained with primary and secondary antibodies (diluted in blocking buffer). Kinetics of expression and cellular localization of fluorescently labeled adenoviral proteins were monitored (LEICA DMIL inverted fluorescence microscope).

ACKNOWLEDGEMENTS

Grant support: European Union grant QLK3-1999-01262.

We thank Prof. Rob Hoeben (Virus and Stem Cell Biology Laboratory, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands) for helpful suggestions and critical reading of the manuscript, Mirjam Heuveling for technical assistance, and Ronald Limpens and Mieke Mommaas-Kienhuis for doing the electron microscopic analysis (Section Electron Microscopy, Leiden University Medical Center).

REFERENCES

1. Zinkernagel RM, Doherty PC. The discovery of MHC restriction. *Immunol Today* 1997; **18**: 14-17.
2. van der BP, Traversari C, Chomez P, *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643-1647.
3. Boon T, Coulie PG, Van den Eynde B. Tumor antigens recognized by T cells. *Immunol Today* 1997; **18**: 267-268.
4. Schultz ES, Schuler-Thurner B, Stroobant V, *et al.* Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 2004; **172**: 1304-1310.
5. Couly G, Creuzet S, Bennaceur S, Vincent C, Le Douarin NM. Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* 2002; **129**: 1061-1073.
6. Lonchay C, van der Bruggen P, Connerotte T, *et al.* Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. *Proc Natl Acad Sci USA* 2004; **101** Suppl 2: 14631-14638.
7. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 2004; **4**: 1.
8. Renkvist N, Castelli C, Robbins PF, Parmiani G. A listing of human tumor antigens recognized by T cells. *Cancer Immunol Immunother* 2001; **50**: 3–15.
9. Nemunaitis J, Khuri F, Ganly I, *et al.* Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol* 2001; **19**: 289-298.

10. Kanerva A, Hemminki A. Modified adenoviruses for cancer gene therapy. *Int J Cancer* 2004; **110**: 475-480.
11. Kanerva A, Wang M, Bauerschmitz GJ, *et al*. Gene transfer to ovarian cancer versus normal tissues with fiber-modified adenoviruses. *Mol Ther* 2002; **5**: 695-704.
12. Vellinga J, Rabelink MJWE, Cramer SJ, *et al*. Spacers increase the accessibility of peptide ligands linked to the carboxyl terminus of adenovirus minor capsid protein IX. *J Virol* 2004; **78**: 3470-3479.
13. Dmitriev IP, Kashentseva EA, Curiel DT. Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J Virol* 2002; **76**: 6893-6899.
14. Gaden F, Franqueville L, Magnusson MK, et al. Gene transduction and cell entry pathway of fiber-modified adenovirus type 5 vectors carrying novel endocytic peptide ligands selected on human tracheal glandular cells. *J Virol* 2004; **78**: 7227-7247.
15. Henning P, Andersson KME, Frykholm K, *et al*. Tumor cell targeted gene delivery by adenovirus 5 vectors carrying knobless fibers with antibody-binding domains. *Gene Ther* 2005; **12**: 211-224.
16. Hong SS, Magnusson MK, Henning P, Lindholm L, Boulanger PA. Adenovirus stripping: a versatile method to generate adenovirus vectors with new cell target specificity. *Mol Ther* 2003; **7**: 692-699.
17. Magnusson MK, Hong SS, Boulanger P, Lindholm L. Genetic retargeting of adenovirus: novel strategy employing "deknobbing" of the fiber. *J Virol* 2001; **75**: 7280-7289.
18. Magnusson MK, Hong SS, Henning P, Boulanger P, Lindholm L. Genetic retargeting of adenovirus vectors: functionality of targeting ligands and their influence on virus viability. *J Gene Med* 2002; **4**: 356-370.
19. Nilsson M, Ljungberg J, Richter J, *et al*. Development of an adenoviral vector system with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells. *J Gene Med* 2004; **6**: 631-641.
20. Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol* 1996; **70**: 6839-6846.
21. Wickham TJ, Tzeng E, Shears LL, *et al*. Increased *in vitro* and *in vivo* gene transfer by adenovirus vectors containing chimeric fiber proteins. *J Virol* 1997; **71**: 8221-8229.
22. Jakubczak JL, Rollence ML, Stewart DA, *et al*. Adenovirus type 5 viral particles pseudotyped with mutagenized fiber proteins show diminished infectivity of coxsackie B-adenovirus receptor-bearing cells. *J Virol* 2001; **75**: 2972-2981.
23. Kirby I, Davison E, Beavil AJ, *et al*. Mutations in the DG loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. *J Virol* 1999; **73**: 9508-9514.
24. Borovjagin AV, Krendelchtchikov A, Ramesh N, Yu DC, Douglas JT, Curiel DT. Complex mosaicism is a novel approach to infectivity enhancement of adenovirus type 5-based vectors. *Cancer Gene Ther* 2005; **12**: 475-486.
25. Willemsen RA, Debets R, Chames P, Bolhuis RLH. Genetic engineering of T cell specificity for immunotherapy of cancer. *Hum Immunol* 2003; **64**: 56-68.
26. Peng KW, Holler PD, Orr BA, Kranz DM, Russell SJ. Targeting virus entry and membrane fusion through specific peptide/MHC complexes using a high-affinity T-cell receptor. *Gene Ther* 2004; **11**: 1234-1239.
27. Willemsen RA, Weijtens ME, Ronteltap C, et al. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther* 2000; **7**: 1369-1377.
28. Von Seggern DJ, Kehler J, Endo RI, Nemerow GR. Complementation of a fibre mutant adenovirus by packaging cell lines stably expressing the adenovirus type 5 fibre protein. *J Gen Virol* 1998; **79**: 1461-1468.
29. van Bergen BG, van der Ley PA, van Driel W, van Mansfeld AD, van der Vliet PC. Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. *Nucleic Acids Res* 1983; **11**: 1975-1989.
30. Douglas JT, Miller CR, Kim M, *et al*. A system for the propagation of adenoviral vectors with genetically modified

receptor specificities. *Nat Biotechnol* 1999; **17**: 470-475.

31. Pecorari F, Tissot AC, Pluckthun A. Folding, heterodimeric association and specific peptide recognition of a murine αβ T-cell receptor expressed in Escherichia coli. *J Mol Biol* 1999; **285**: 1831-1843.
32. Willemsen RA, Debets R, Hart E, Hoogenboom HR, Bolhuis RL, Chames P. A phage display selected fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes. *Gene Ther* 2001; **8**: 1601-1608.
33. Turatti F, Figini M, Alberti P, Willemsen RA, Canevari S, Mezzanzanica D. Highly efficient redirected anti-tumor activity of human lymphocytes transduced with a completely human chimeric immune receptor. *J Gene Med* 2005; **7**: 158-170.
34. Chames P, Willemsen RA, Rojas G, *et al*. TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity. *J Immunol* 2002; **169**: 1110-1118.