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Rational and random approaches to adenoviral vector engineering

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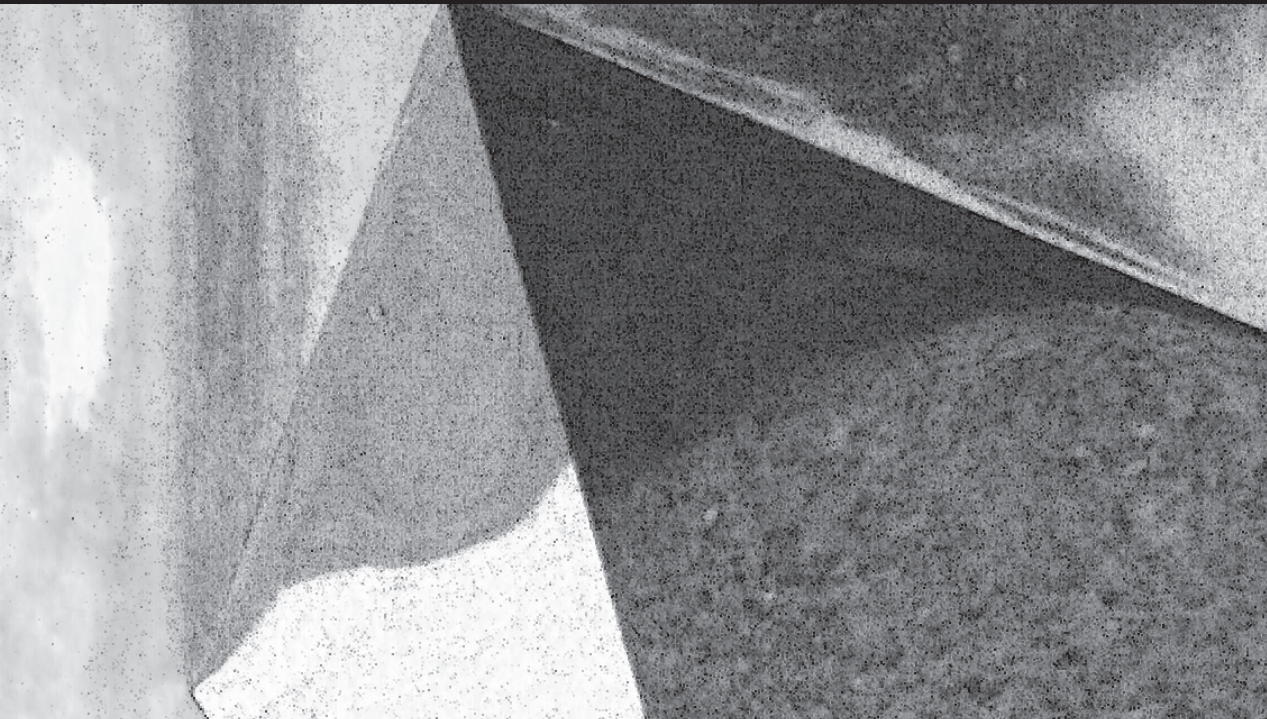
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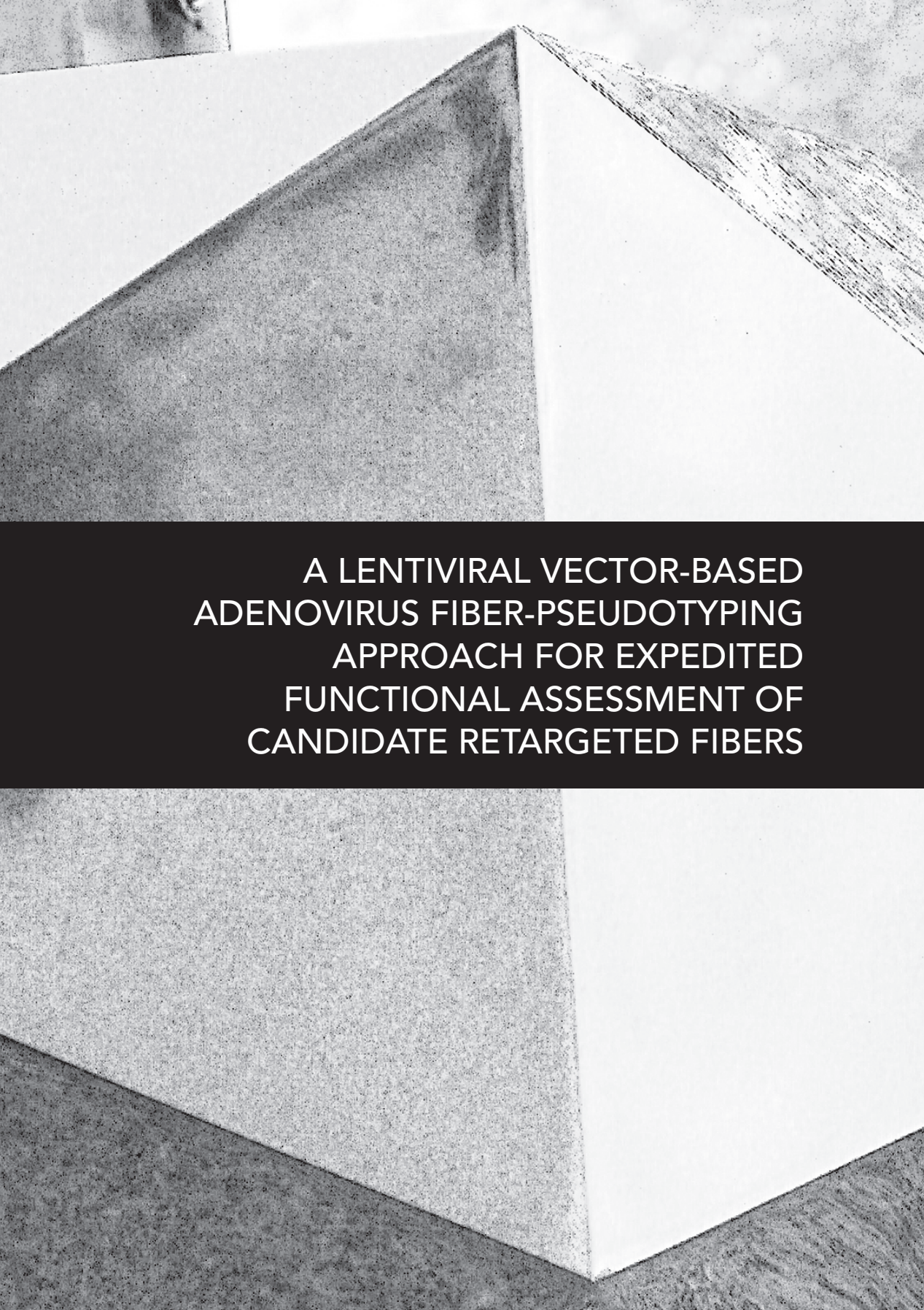
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**A LENTIVIRAL VECTOR-BASED
ADENOVIRUS FIBER-PSEUDOTYPING
APPROACH FOR EXPEDITED
FUNCTIONAL ASSESSMENT OF
CANDIDATE RETARGETED FIBERS**

ABSTRACT

Background: Many studies aimed at retargeting adenovirus (Ad) rationally focus on genetic modification of fiber, which is the primary receptor-binding protein of Ad. Retargeted fibers ultimately require functional validation in the viral context.

Methods: Lentiviral vectors (LV) were used to express fiber variants in cells. Infections with a fiber gene-deleted Ad vector yielded fiber-pseudotyped viruses. An enzyme-linked immunosorbent assay and slot blot-based assays probed target binding-ability of retargeted fibers. Differential treatments with an alkylating agent prior to western blot analysis allowed for examination of intra- and extracellular redox states of fibers.

Results: In the present study, LV-based fiber-pseudotyping of Ad is presented as an accelerated means to test new fibers. LV-mediated gene transfer yielded stable and uniform populations of fiber variant-expressing cells. These populations were found to effectively support fiber-pseudotyping of Ad. As a secondary objective of the study, we functionally assessed a chimeric fiber harboring a tumor antigen-directed single-chain antibody fragment (scFv). This fiber was shown to trimerize and achieve a degree of binding to its antigenic target. However, its capsid incorporation ability was impaired and, moreover, it was unable to confer a detectable level of target binding upon Ad. Importantly, subsequent analyses of this fiber revealed the improper folding of its scFv constituent.

Conclusions: LV-based fiber-pseudotyping was established as a convenient method for testing modified fibers for functionality within Ad particles. Furthermore, a new chimeric fiber was found to be inadequate for Ad retargeting. The folding difficulties encountered for this particular fiber might be generally inherent to the use (i.e. for genetic Ad capsid incorporation) of complex, disulfide bridge-containing natural ligands.

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INTRODUCTION

Aiming to improve safety and efficacy of systemic Ad-mediated gene therapy, diverse genetic capsid modification strategies are being employed to retarget Ad, which involves restricting the broad infectivity profile of Ad and, simultaneously, redirecting Ad infection to specific target cells (1,2). In this regard, because the canonical infection route of HAdV-5 entails a primary docking step that involves Ad fiber protein binding to cellular coxsackievirus and adenovirus receptor (CAR) (3), many genetic strategies for Ad retargeting rationally focus on modification of the fiber (4,5). These efforts typically seek to generate new fiber variants that are ablated for interaction with CAR and additionally incorporate heterologous ligands for alternative, target cell-specific receptors. Similarly, other capsid proteins are being genetically modified to ablate native viral interactions with cell or blood components and/or to incorporate targeting ligands (6–10). In this respect, with the recent recognition that hexon-to-FX binding is largely responsible for the hepatic tropism of Ad, new prospects have opened up for genetic capsid modification strategies aiming to detarget the liver.

Genetic strategies aimed at altering Ad tropism through modification of fiber have been numerous (1,2,4,5). One method to endow HAdV-5-based vectors with novel tropism involves swapping of the fiber, or the fiber knob domain, with that of other Ad serotypes with distinct cellular receptors (11–13). Other fiber modification approaches focus on the insertion of targeting ligands at specific sites found to be accommodating for this purpose, such as the C-terminus and the so-called HI-loop (14–17). Furthermore, several studies have combined the above approaches by inserting peptide ligands into the knob domains of non-HAdV-5 serotype fibers (18–20). Finally, more rigorous strategies involve the complete deletion of the fiber knob domain (by which both CAR-binding and trimerization functionalities of the fiber are removed) and its functional replacement by an extrinsic trimerization domain and a targeting ligand (21–24). This approach would allow for the insertion of more complex ligands, which otherwise, in the knob-incorporated context, would be incompatible with correct fiber folding.

New fiber designs drawn up to achieve Ad retargeting ultimately require experimental validation by functional testing of the new fiber models in the context of Ad virions. Presently, this is mostly achieved by genetic incorporation of the new fiber construct into the Ad genome by means of homologous recombination in *Escherichia coli*, subsequent virus rescuing followed by upscaling in a suitable cell line, and, finally, functional assessment of the new fiber in its capsid-incorporated context. Although extensively proven to be functional, this scheme does have a significant drawback in that it is rather time-consuming.

An alternative fiber-testing system is represented by transient fiber-pseudotyping of Ad. This approach, as first established by von Seggern *et al.* (25), involves the use of Ad packaging cells expressing the fiber variant of interest. Infection of these cells with a fiber gene-deleted (ΔF) Ad vector allows for the generation of virus particles carrying the modified fiber. Such transiently fiber-pseudotyped viruses can then be used for functional testing of the incorporated new fiber. In previous manifestations of this approach, the fiber variant-expressing cell populations used for the pseudotyping infection were either generated by means of transient transfection or by clonal selection and expansion of stably transfected cells (18,25,26). In this regard, the 'transient transfection/infection' procedure first described by Jakubczak *et al.* (26) comprises a major advancement in terms of time and labour expended on testing new fibers.

In the present study, as a first objective, we established the feasibility of a fiber-pseudotyping system that makes use lentiviral vectors (LVs) for the generation of fiber variant-expressing cells. Importantly, this lentivirus-based system brings together two distinct benefits associated with previously described fiber-pseudotyping approaches. First, analogous to the transient transfection-based protocol (26), the lentivirus-based method is a procedurally quick way to test new fibers. Second, akin to the approach relying on stably transfected cell clones (25), the lentivirus-based procedure ensures that the cell population used for fiber-pseudotyping expresses the fiber of interest in a stable and uniform fashion. Thus, with its relative procedural speediness and its practical robustness, the system described in the present study is considered to represent a useful toolbox addition for testing candidate retargeted fibers.

A secondary objective of the present study was to use this newly-established fiber-pseudotyping system for the functional assessment of a chimeric fiber harboring a scFv directed to an important tumor-associated antigen. Importantly, the scFv used in the present study was previously found to be capable of functional folding in a reducing environment, a finding suggestive of potential compatibility with the nuclear process of Ad particle assembly. The results obtained in the present study with this new scFv-fiber chimera are considered to be relevant to genetic adenovirus targeting strategies aiming to exploit complex ligands whose natural biosynthetic routes are distinct from those of Ad capsid components.

MATERIALS AND METHODS

Cells

The HAdV-5 E1-transformed cell line 911 has been described previously (27). Fiber-expressing cell lines 633 and 293-Fb were, respectively, obtained from Dan von Seggern (The Scripps Research Institute, La Jolla, CA, USA) and Monika Lusky (Transgene SA, Strasbourg, France) (25,28). 293HER2/*neu* cells were described previously (29). All cell lines were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (Gibco BRL) in a humidified atmosphere with 5% CO₂.

Adenovirus vectors

HAdV-5 was obtained from the virus collection of the Department of Molecular Cell Biology of the Leiden University Medical Center. Ad.ZH is an HAdV-5-based vector genetically modified to possess a previously described recombinant fiber termed Fib Δ CAR-HI-Link-ZHZH (29), which incorporates, in its HI-loop, a head-to-tail dimer of ZH, a Her2/*neu* targeted affibody (30). This virus was generated using previously described procedures based on homologous recombination and cosmid cloning (24), and subsequent virus rescuing by transfection of the *PacI*-digested recombinant genome into 293HER2/*neu*. AdGL Δ F/F and its fiber-pseudotyped versions AdGL Δ F/R7Knob and AdGL Δ F/800E6 Fb were generated as detailed below. All viruses were purified by centrifugation on CsCl gradients by standard protocols. The physical particle titers were determined spectrophotometrically by the method of Maizel *et al.* (31), using a conversion factor of 1.1×10^{12} viral particles per absorbance unit at 260 nm.

Construction of a fiber gene-deleted Ad vector genome plasmid

A series of genetic manipulations led to the generation of pAdGL Δ F, a plasmid containing an E1- and E3-deleted HAdV-5 genome further deleted for HAdV-5 nucleotides 31042–32699, which correspond to all but the last 85 nucleotides of the fiber gene open reading frame. First, an intermediate 'fiber shuttle' plasmid, pFS, was constructed by self-ligation of the fiber gene-containing, 9.6-kbp *EcoRI* fragment of pAdEasy-I, an adenoviral genome plasmid lacking the left-end (including the E1 genes) and most of the E3 region of the HAdV-5 genome (32). Then, p Δ FS, a pFS derivative containing the above mentioned 1658-bp fiber gene deletion, was made by inverted polymerase chain reaction (PCR) on pFS using oligonucleotides FIB-Ct-Swal-s and FIB-Nt-as (Table 1) and subsequent self-ligation of the resulting 8.0-kbp PCR product. Subsequently, within p Δ FS, the pAdEasy-I derived Ad genome sequence upstream of the fiber gene deletion was extended by ligation of the

Table 1. Oligonucleotide list.

Oligonucleotide	Sequence (5'-3') ^a
FIB-Ct-Swal-s	ATTTAAATTTTCATGGGACTGGTCTGG
FIB-Nt-as	CTGCAACAACATGAAGATAGTGG
Fib-FWD-PstI	CAGCA <u>CTGCAG</u> ACCATGAAGCGCGCAAGACCGTC
Fib-REV-XhoI	CGACA <u>CTCGAG</u> CTATCATTCTTGGGCAATGTATGAAAAAGTG
TPL-PstI-s	CAGCA <u>CTGCAG</u> ACTCTCTTCCGCATCGCTG
TPL-FIB-as	GGTCTTGCGCGCTTCATCTTGCGACTGTGACTGGTTAGACG
TPL-FIB-s	CGTCTAACCAGTCACAGTCGCAAGATGAAGCGCGCAAGACC
F-BsrGI-as	GACGTA <u>CTGTACA</u> CTATCATTCTTGGGCAATGTATG
800E6-NcoI-MluI-F	GCATCGAAG <u>CCATGGG</u> ACGCGTCTGACTCGAAGC
800E6-BsrGI-R	CTTACCGGAT <u>GTACA</u> TTAGTTACCGGATCCC

^aRestriction sites referred to in the Materials and Methods are underlined.

SpeI site-containing, 1.3-kbp *MfeI-EcoRI* pAdEasy-I fragment into the unique *EcoRI* site of pΔFS, yielding pΔFS2. Finally, pAdGLΔF was generated by homologous recombination (in *E. coli* BJ5183) (33,34) between the 5.7-kbp *BglI*-fragment of pΔFS2 and *SpeI*-linearized pAdGL, a plasmid that itself is the product of homologous recombination between pAdEasy-I and pAdTrack-CMV.Luc. The latter plasmid was constructed by insertion of a firefly luciferase gene into the multiple cloning site of pAdTrack-CMV, a shuttle vector of the AdEasy system (32).

Generation of a fiber gene-deleted Ad vector and fiber-pseudotyped vector stocks

A fiber gene-deleted Ad vector was generated by transfection of *PacI*-digested pAdGLΔF into the wildtype fiber-complementing cell line 293-Fb. The resulting rescued virus was propagated further on 293-Fb cells and purified as described above, giving AdGLΔF/F (293-Fb). This virus was subsequently used for the generation of purified preparations of AdGLΔF/F (633), AdGLΔF/R7Knob, and AdGLΔF/800E6 Fb through large-scale infection of 633, 911.R7Knob, and 911.800E6 cells, respectively. The latter two of these fiber-complementing cell types were made in the present study by means of lentiviral transduction (see below).

Construction of LV plasmids

The LV plasmids used in the present study are based on pLV.CMV.IRES.GFP and pLV.CMV.IRES.NPTII, which both originally stem from pRRL-cPPT-CMV-XPRESIN (35) through multiple genetic manipulations that effectively resulted into insertion (between the *Bst*Z17I and *Nsi*I sites of the original

plasmid) of an internal ribosomal entry site (IRES) sequence followed by a green fluorescent protein (GFP) gene or a neomycin phosphotransferase II (NPTII) gene, respectively. Furthermore, within these plasmids, the original multiple cloning site (MCS) had been extended by sequential insertions of additional polylinker sequences. A third basal LV plasmid, pLV.CMV.IRES.PURO, was generated for the present study by the replacement of the GFP gene of pLV.CMV.IRES.GFP with a puromycin resistance gene obtained by PCR from plasmid pBABEpuro (36).

Plasmids pLV.CMV.Fiber.IRES.NPTII, pLV.CMV.Fiber.IRES.GFP, and pLV.CMV.R7Knob.IRES.NPTII were constructed by PCR-amplification of the genes for wild-type HAdV-5 fiber and R7Knob fiber (37) using primers Fib-FWD-PstI and Fib-REV-XhoI (Table 1), digestion of the PCR products with *PstI* and *XhoI*, and ligation of the resulting fragments in between the *PstI* and *XhoI* sites of pLV.CMV.IRES.NPTII or pLV.CMV.IRES.GFP. Of note, the resulting cloned fiber genes lack the poly A signal that normally overlaps the HAdV-5 fiber stop codon. To construct wild-type and R7Knob fiber genes fused to an upstream tripartite leader (TPL) sequence, PCR-mediated splicing by overlap extension (SOE) was employed. First, the TPL sequence was amplified from pMad5 (38) using primers TPL-PstI-s and TPL-FIB-as, whereas the fiber genes were amplified from pLV.CMV.Fiber.IRES.NEO and pLV.CMV.R7Knob.IRES.NEO using primers TPL-FIB-s and F-BsrGI-as (Table 1). Then, mixing of TPL and fiber PCR products and subsequent amplification using only the 'outer' primers (TPL-PstI-s and F-BsrGI-as) resulted in the generation of the desired fusion products.

These TPL-fiber fusion products were subsequently cloned into pLV.CMV.IRES.NEO using *PstI* and *BsrGI* restriction sites, yielding pLV.CMV.TPL.Fiber.IRES.NEO and pLV.CMV.TPL.R7Knob.IRES.NEO. Furthermore, pLV.CMV.TPL.Fiber.IRES.GFP was made by replacing the fiber gene-containing *PstI*-*PmlI* fragment of pLV.CMV.Fiber.IRES.GFP with that of pLV.CMV.TPL.Fiber.IRES.NEO.

The generation of an 800E6 fiber-expressing LV vector plasmid involved several cloning steps, which, in effect, amount to the following. The coding sequence for scFv800E6 was PCR-amplified from pIVEX 2.4d-His(6x)-ad-N-ScFv800E6 (39) using primers 800E6-NcoI-MluI-F and 800E6-BsrGI-R (Table 1). Subsequently, employing the introduced *NcoI* site, the obtained scFv800E6 sequence was ligated at its 5' end to a chimeric gene fragment (made by PCR-mediated SOE) encoding the tail and first shaft repeat of the HAdV-5 fiber protein followed by the coiled coil segment 13 and foldon domain of the bacteriophage T4 fibritin protein. Next, the resulting 800E6 fiber gene was fused (by virtue of the *BshTI* site existing within the fiber tail sequence) to the TPL sequence present in pLV.CMV.TPL.Fiber.IRES.NEO. Finally, the TPL-800E6 fiber fusion sequence obtained was introduced as a *PstI*-*BsrGI* fragment into pLV.CMV.IRES.PURO, yielding pLV.CMV.TPL.800E6-Fb.IRES.PURO.

Production of LV vectors

LVs were produced by a previously described procedure involving transient cotransfection of a LV vector plasmid (expressing the transgene of interest) together with 'helper' plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope (40). For quantification of virus yield in the harvested medium, p24 antigen levels were determined using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corp., New York, NY, USA).

Transient fiber expression

911 cells seeded in six-well plate wells were grown to 70% confluency and subsequently transfected with plasmids pLV.CMV.Fiber.IRES.GFP, pLV.CMV.TPL.Fiber.IRES.GFP, and pLV.CMV.TPL.Fiber.IRES.NPTII by the calcium phosphate coprecipitation technique (41). An equal molar amount of each plasmid was separately transfected (approximately 1 µg per well). Twenty-four hours after transfection, the cells were washed and cultured in fresh growth medium. The cells were harvested for western blot analysis after another 24 h.

Lentiviral transductions

911 cells seeded in wells of six-well plates and grown, in 1 day, to a confluency of 50% (approximately half a million cells) were transduced with LV vectors (diluted in fresh growth medium) at 200 ng of p24 per well for 24 h in the presence of 8 µg/ml polybrene. After transduction, the virus containing medium was replaced by fresh growth medium and the transduced cells were passaged 1 day later. Selection for antibiotic resistance was initiated at the first passage. Cells transduced with LV.CMV.TPL.R7Knob.IRES.NPTII were grown in medium containing 400 µg/ml G418 (Invitrogen, Breda, The Netherlands), whereas those transduced with LV.CMV.TPL.800E6-Fb.IRES.PURO were grown in the presence of 0.6 µg/ml puromycin (MP Biomedicals, Amsterdam, The Netherlands). The antibiotic-resistant cell populations obtained were, respectively, termed 911.R7Knob and 911.800E6.

Western analysis

Except otherwise specified, cell lysates were made in RIPA lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, and 1% NP40) supplemented with protease inhibitors. Protein concentrations were determined with a BCA protein assay (Pierce, Etten-Leur, The Netherlands) with bovine serum albumin (BSA) as the standard. Typically, cell lysate samples for western analysis were prepared by boiling of 20 µg of total protein in reducing sample buffer (final composition: 33 mM Tris-Cl, pH 6.7, 9% glycerol, 2% SDS, and 2.2% β-mercaptoethanol, and 2.2% from a 1 : 20 dilution of a saturated bromophenol blue solution) for 5–10 min at 100°C. Virus samples

for western analysis were prepared by adding purified viruses directly to the reducing sample buffer, followed by boiling.

For assessment of the intracellular redox state of expressed fibers, cells were lysed in the presence of either 10 mM dithiothreitol (DTT) or 10 mM *N*-ethylmaleimide (NEM), or in the absence of either of these agents. In the case of the DTT-treated lysates, Western samples were subsequently prepared using the reducing sample buffer, whereas, for the other two treatments, a nonreducing sample buffer was used (e.i. without β -mercaptoethanol). For assessment of the post-lysis redox state of 800E6 fiber, cells were lysed by freeze-thawing and subsequently treated with NEM (final concentration of 10 mM). Western samples were then prepared using nonreducing sample buffer.

All samples were subjugated to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 8% polyacrylamide running gels, and subsequent protein transfer to poly (vinylidene fluoride) (PVDF) membranes (Immobilon-P transfer membrane; Millipore, Amsterdam, The Netherlands) by electroblotting. After immunological probing of the blots, horse radish peroxidase (HRP)-conjugated antibodies were detected by enhanced chemiluminescence (ECL). Primary antibodies used were mouse monoclonal anti-actin (1 : 5000, clone C4; Millipore), goat polyclonal anti-hexon (1 : 2000, ab19998; Abcam, Cambridge, UK), rabbit polyclonal anti-penton base (1 : 2000; laboratory made), and mouse monoclonal anti-fiber tail (1 : 5000, 4D2; Abcam) (42).

For 800E6 fiber incorporation analysis, chemiluminescence-exposed X-ray films were scanned and the specific signals were quantified by a digital image analysis program (ImageJ, version 1.40g; <http://rsb.info.nih.gov/ij/>).

Immunofluorescence analysis

For analysis of fiber variant expression in LV-transduced cells, 911.R7Knob or 911.800E6 cells were grown on glass coverslips in six-well plates, fixed and permeabilized with methanol, and probed with the mouse anti-fiber tail primary antibody 4D2 (1 : 1000; Abcam) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody. Nuclei were visualized by either propidium iodide or 4',6-diamidino-2-phenylindole (DAPI). Cells were mounted in Dabco-glycerol (glycerol, 0.02M Tris-Cl, pH 8.0, and 2.3% 1,4-diazabicyclo-[2.2.2]-octane) and viewed under a fluorescence microscope.

ELISA-based HER2-ECD binding assays

To assess the binding of lentivirally expressed 800E6 fiber to HER2-ECD, an ELISA was employed. Her2/*neu* extracellular domain (HER2-ECD; Fox Chase Cancer Centre, Philadelphia, PA, USA) (43) or, as a control, BSA was diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6) to a concentration of 5 μ g/ml. Subsequently, 100- μ l aliquots were added to wells of 96-well Nunc F96

Maxisorp plates (Nunc/Sanbio, Uden, The Netherlands). After overnight incubation at 4°C, the wells were emptied and blocked with blocking buffer [2% BSA, 0.05% Tween 20 in phosphate-buffered saline (PBS)] for 1 h at room temperature, and subsequently washed with washing buffer (0.05% Tween 20 in PBS). Per confluent 10-cm dish, 911 and 911.800E6 cells were resuspended in 1.4 ml of PBS and subsequently lysed by freeze-thawing. These freeze-thaw cell lysates were serially diluted by first making a one- to two-fold dilution in 2 × binding buffer (1% BSA, 0.1% Tween 20 in PBS) and subsequently, from this dilution, serial four-fold dilutions in 1 × binding buffer (0.5% BSA, 0.05% Tween 20 in PBS). The binding buffers were supplemented with protease inhibitors. One hundred microliter aliquots of these dilutions were then added to the coated wells and left to incubate at room temperature for 1 h, after which the wells were washed with washing buffer. The wells were subsequently probed with mouse anti-fiber tail antibody (4D2; Abcam) diluted 1 : 2000 in 1 × binding buffer. After incubation for 1 h, the wells were washed and then incubated for 1 h with HRP-conjugated goat anti-mouse IgG antibody diluted 1 : 5000 in 1 × binding buffer. After a final wash, the plates were developed with the tetramethylbenzidine (TMB) substrate solution from a HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corp) as recommended by the manufacturer.

Another ELISA-based protocol was used to assess the binding of purified Ad virions to HER2-ECD. HAdV-5, Ad.ZH, and AdGLΔF/800E6 Fb were serially diluted in PBS and 100-μl aliquots of the virus dilutions were added to wells of Nunc Maxisorp plates. After overnight coating at 4°C, the wells were washed with PBS and blocked with 2.5% BSA, 2.5% protifar (Nutricia, Zoetermeer, The Netherlands) in PBS. The wells were then washed with 0.025% Tween 20 in PBS. Subsequently, wells were incubated with 100-μl aliquots of a 1 ng/μl HER2-ECD dilution in 1.25% BSA, 1.25% protifar, 0.025% Tween 20 in PBS. Control wells were incubated with mouse monoclonal anti-fiber knob 1D6.14 antibody (44) (1 : 100; obtained from V. van Beusechem, VU University Medical Center, Amsterdam, The Netherlands) or goat polyclonal anti-hexon (1 : 1000, ab19998; Abcam). Incubation was overnight at 4°C, after which the plates were washed with 0.025% Tween 20 in PBS. The bound primary reagents were then probed with corresponding HRP-conjugated secondary reagents, which, in the case of primary reagent HER2-ECD, was a goat polyclonal anti-six histidine (His-tag) antibody (ab1269; Abcam).

Slot blot-based HER2-ECD binding Assay Binding of purified Ad virions to HER2-ECD was assessed by a slot blot-based protocol that was adapted from a soluble CAR virus-binding assay described by Roelvink *et al.* (45). Purified Ad virions were diluted in PBS and blotted onto a PVDF membrane (Millipore) using a slot blot apparatus. After blocking with 2.5% BSA, 2.5% protifar in PBS for 2 h, blots were immunologically probed using the same primary and

secondary reagents as used in the ELISA-based virus binding assay above. Briefly, blots were incubated overnight at 4°C with either HER2-ECD (300 ng/ml), 1D6.14 (1 : 250), or antihexon antibody (1 : 5000) diluted in PBS with 1.25% BSA, 1.25% protifar, and 0.025% Tween 20. Then, after washing, incubation followed with the respective HRPconjugates, which, after washing again, were detected by ECL.

RESULTS

Feasibility of a lentiviral vector-based adenovirus fiber-pseudotyping approach

Newly-designed, candidate fibers for adenovirus retargeting require assessment for their functionality in terms of capsid incorporation capacity, target molecule binding, and, ultimately, the ability to mediate target cellspecific transduction. For the rapid analysis of modified fiber proteins in the context of the viral particle, Jakubczak *et al.* (26) previously established a transient fiber-pseudotyping system based on transient transfection of a fiber-expression plasmid followed by infection with a fiber gene-deleted (ΔF) Ad vector. In the present study, we explored the practical feasibility of an adapted transient fiber-pseudotyping system that relies on lentiviral vector transduction, rather than transient transfection, for the expression of novel fiber variants in cells (Figure 1). In this approach, expansion of the lentivirally transduced cell population and its subsequent large-scale transduction with a ΔF Ad vector allows for the derivation of a purified virus preparation suitable for the functional testing (in the virus particle context) of newly-designed fibers.

To establish the feasibility of such a LV vectorbased fiber-pseudotyping system we make use of a previously described recombinant fiber termed 'R7Knob' (37). R7Knob is a variant of the HAdV-5 fiber containing only the first seven of the 22 shaft repeats. Additionally, in between its shaft and knob domains, it harbors a heterologous trimerization domain. Previously shown to be capsid incorporable, this fiber variant can serve here to provide proof of principle as to whether LV vectors can be used for the generation of stable, modified fiber-producing cells that, upon infection with a ΔF vector, will sustain production of fiber-pseudotyped progeny virus.

For the generation of cells stably expressing fiber variants, we made use of LV vectors that are based on the self-inactivating, third-generation HIV-1-derived vectors originally developed by the Trono group and Cell Genesys (46,47). These LV vectors (Figure 2A), which are devoid of any HIV-1 coding sequences and are thus produced by co-transfection with helper plasmids, are self-inactivating by virtue of a deletion of most of the 3' U3 region. This deletion is designed to preclude, upon host cell genome integration of the

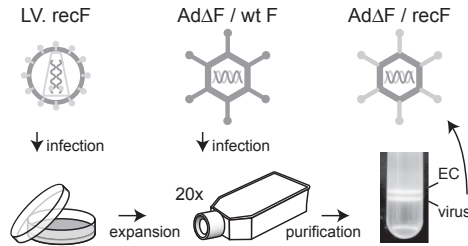


Figure 1. A lentiviral vector-based adenovirus fiber-pseudotyping system for rapid testing of new fiber variants in the virus particle context. Cells stably expressing a recombinant fiber of interest are generated by a small scale transduction with a lentiviral vector (LV.recF). Expansion of these cells followed by a large-scale infection with a fiber gene-deleted Ad vector equipped with wild-type fibers (AdΔF/wt F) allows for the derivation of a purified stock of recombinant fiber-pseudotyped virus (AdΔF/recF). EC, empty capsids.

vector, the production of any RNA other than that promoted by the transgene expression cassette (Figure 2A).

First, prior to transduction experiments with LV vectors, we performed DNA transfections with lentiviral vector plasmids for the evaluation of different fiber expression cassette configurations (Figure 2B). In normal Ad infection, three noncoding exons are spliced to the 5' end of messenger RNAs from the major late transcription unit. This so-called TPL plays a role in ensuring viral protein production when general host cell protein synthesis is shut down (48,49). Furthermore, TPL-containing transcripts have also been shown to exhibit enhanced translation in the absence of Ad infection (50). Therefore, analogous to a previous study by von Seggern *et al.* (51), we included the TPL sequence at the 5' end of the fiber gene in our expression cassettes. Specifically, the complete TPL sequence was inserted immediately upstream of the fiber start codon, exactly mirroring the configuration of viral late transcripts. Additionally, as shown in Figure 2B, the expression cassettes were furnished with an internal ribosomal entry site downstream of the fiber gene followed by either genes encoding GFP or NPTII. Evaluation of fiber production in cells transfected with these constructs indeed showed enhanced expression for the TPL-containing cassettes compared to the TPL-lacking construct (Figure 2B).

Next, LV vectors were used to generate cells stably expressing the R7Knob fiber variant. With this intent, the E1-complementing cell line 911 (27) was transduced with a LV vector containing the TPL-R7Knob-IRES-NPTII cassette (Figure 2C). The transduced cell population, termed 911.R7Knob, was first passaged at 48 h and subsequently after every four or five days. Selection for G418 resistance, conferred to the cells by the NPTII gene, was initiated after the first passage. Western blot analysis of samples from different time points after transduction demonstrated that, once NPTII-positive cells had been selected for, R7Knob levels were sustained over time. By immune fluorescence,

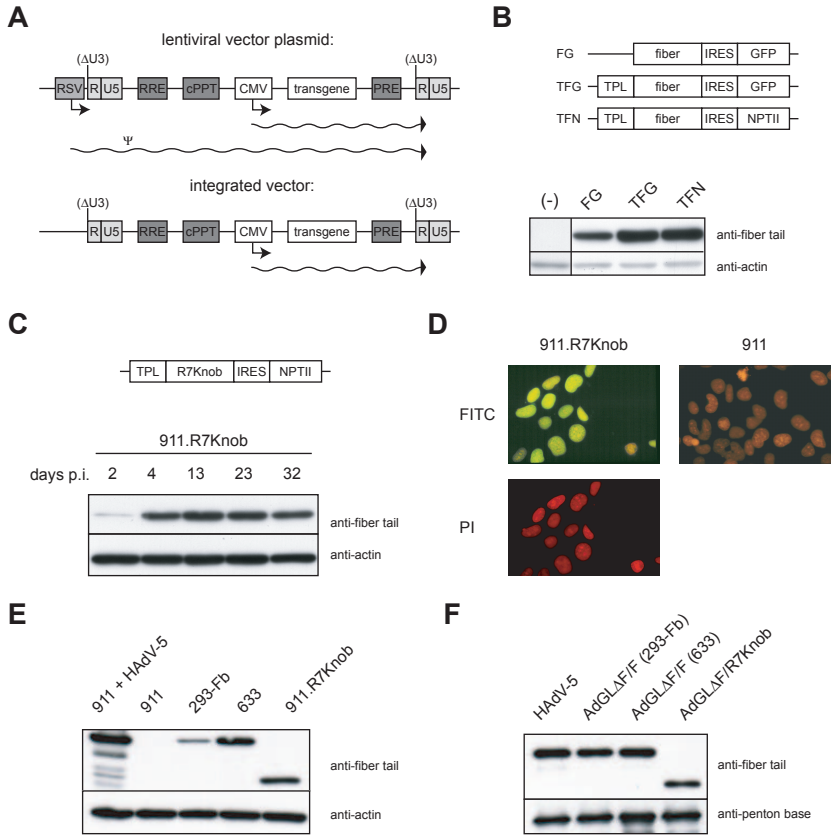


Figure 2. Usage of lentiviral vectors for the generation of packaging cells employable for fiber-pseudotyping of Ad. (A) Schematic outline of the lentiviral vector system. The lentiviral vectors employed are based on self-inactivating, third-generation HIV-1-derived vectors (46,47) and are further optimized for enhanced integration capacity and transgene expression (35). RSV, Rous sarcoma virus promoter; Δ U3, deletion of (most of) the U3 region; RRE, HIV-1 Rev responsive element; cPPT, HIV-1 central polypurine tract; CMV, human cytomegalovirus promoter; PRE, human hepatitis B virus post-transcriptional regulatory element; wavy arrows, RNA transcripts; ψ , packaging signal. (B) Effect of TPL sequence inclusion in fiber expression cassettes. Western blot analysis was conducted on cells transfected with lentiviral vector plasmids with transgenic configurations as depicted. TPL, tripartite leader; IRES, internal ribosomal entry site; GFP, green fluorescent protein; NPTII, neomycin phosphotransferase II. (C) Western blot analysis of lentiviral vector-mediated R7KnoB expression at different time points post-infection. 911.R7KnoB cells were generated by transduction with a lentiviral vector with the indicated transgenic configuration and were maintained under G418 selection pressure from day 2 post-infection onward. (D) Immunofluorescence analysis of lentiviral vector-mediated R7KnoB expression. Cells were probed with anti-fiber tail antibody followed by a FITC-conjugated secondary antibody (top). Nuclei were visualized by staining with propidium iodide (bottom). 911.R7KnoB cells had been selected for G418 resistance prior to the analysis. (E) Comparison, by western blot analysis, of the R7KnoB expression levels achieved in 911.R7KnoB with fiber expression levels found in established fiber-complementing cell lines 293-Fb (28) and 633 (25), and 911 cells at 32 h post-infection with HAAdV-5. (F) Western analysis on purified virus particles demonstrating capsid incorporation of R7KnoB. Per lane, 4×10^9 particles were loaded of HAAdV-5 or of viruses obtained by trans-complementation of a fiber-gene deleted Ad vector by final amplification in 293-Fb, 633, or 911.R7KnoB.

it was further shown that almost all G418 resistant cells were positive for the R7Knob fiber, that cell-to-cell variability in expression level was minimal, and, importantly for adenovirus packaging purposes, that sub-cellular localization of the fiber variant was nuclear (Figure 2D).

To further evaluate the feasibility of a LV vector-based fiber-pseudotyping system, we subsequently compared the level of R7Knob expression achieved by LV transduction with the levels of fiber expression obtained in the previously described fiber-expressing cell lines 293-Fb and 633, both of which have been successfully used in the past for fiber-complementation experiments (25,28). A western blot analysis featuring 911.R7Knob set side by side with these fiber-expressing cell lines and, as additional reference, 911 cells infected with HAdV-5, showed that expression of the R7Knob fiber was of an intermediate, but substantial level (Figure 2E). This result, together with the data above, demonstrates that lentiviral vectors can be used for the generation of a homogenous population of cells constitutively expressing a fiber variant, with the fiber expression level being within a range suitable for the purpose of fiber-complementation.

Final validation of this LV-based approach was sought to be provided by a fiber-pseudotyping experiment that entails infection of the 911.R7Knob cells by a fiber gene-deleted Ad vector. For this purpose, an E1-, E3-, and fiber gene-deleted HAdV-5-based vector, AdGL Δ F/F, was generated. This vector, having been produced on wild-type fiber expressing cells, was transiently loaded with normal fibers (hence the additional 'F' in its designation), enabling it to infect cells via interaction with CAR. Von Seggern *et al.* (25,52) previously showed that a vector carrying an essentially similar fiber gene-deletion served well in the context of fiber-pseudotyping experiments. For the generation of a large-scale R7Knob-pseudotyped virus preparation, 911.R7Knob cells were expanded, readily, to obtain 20 semi-confluent 175-cm² flasks that were subsequently used for infection with AdGL Δ F/F. After 72 h, virus progeny was harvested and purified by a standard CsCl gradient-based protocol, yielding a purified AdGL Δ F/R7Knob preparation. Subsequently, to evaluate capsid incorporation capacity of the R7Knob fiber supplied by the complementing cells, AdGL Δ F/R7Knob was subjected to western blot analysis alongside with HAdV-5 and two AdGL Δ F/F preparations (Figure 2F). The results clearly show that the R7Knob fiber was incorporated into the AdGL Δ F/R7Knob viral particles and, moreover, indicate that the level of R7Knob incorporation was considerable in relation to the fiber content of the wildtype fiber-bearing viruses. Thus, these findings demonstrate a case of successful fiber-pseudotyping of Ad involving lentiviral vectors for the generation of fibervariant producing cells.

Design, expression, and functional characterization of a novel liganded fiber chimera incorporating an intrinsically stable, tumor antigen-directed scFv

With the fiber-pseudotyping system described above in place, we subsequently used it for the functional assessment of a new candidate retargeted fiber. In this regard, we sought to functionally test a chimeric fiber molecule harboring a scFv directed against tumor antigen HER2/*neu* (39,53,54). Importantly, this particular single chain antibody, named scFv800E6, was specifically chosen for its intrinsic capability to fold efficiently and functionally in a reducing environment, a recognized prerequisite for bona fide assembly into the Ad capsid as part of a genetic capsid protein fusion (37,55–57).

In the design of the fiber chimera harboring scFv800E6, we paralleled a genetic fiber modification strategy implemented previously by us and others in which the fiber knob domain is removed and its intrinsic functions (i.e. trimerization and receptor binding) uncoupled and, respectively, restored by the introduction of a heterologous trimerization motif and a ligand domain (21,24,37,55). Specifically, the '800E6 fiber' constructed for the present study was a genetic fusion of the tail and first shaft repeat of the HAdV-5 fiber protein, the coiled coil segment 13 and the trimerization domain 'foldon' of the bacteriophage T4 fibritin protein, and, separated by a spacer, the scFv800E6 moiety (Figure 3A).

In our scheme to test this new fiber for Ad targeting capability, we first set out to generate and characterize cells stably expressing this new, liganded fiber chimera. With this objective, 911 cells were transduced with a LV vector containing an 800E6 fiber expression cassette functionally arranged in the same way as that successfully used before to express the R7Knob fiber (Figure 3A). Subsequent probing for the fiber tail by immune fluorescence showed that, before initiation of selection with puromycin, the LV-transduced cell population (911.800E6) was almost 100% positive for 800E6 fiber expression and exhibited minimal cell-to-cell variability (Figure 3B). With respect to localization, however, expression appeared to be somewhat aberrant because the 800E6 fiber was found both in the nucleus and the cytoplasm (whereas wild-type fiber normally localizes only to the nucleus).

Subsequently, the lentivirally expressed 800E6 fiber was characterized with respect to its ability to trimerize, which is considered a key functional requirement for insertion into the capsid. In this regard, the T4 fibritin foldon domain had previously been shown to be successful in conferring trimerization ability to knobless, chimeric fiber-fibritin proteins (23). Although outfitted with this same foldon domain, the 800E6 fiber is not entirely structurally similar to these fiber-fibritin molecules and therefore its capacity to trimerize had yet to be determined. To this end, boiled and nonboiled lysates of 911.800E6 and the wild-type fiber-expressing 633 cells were analysed, under reducing

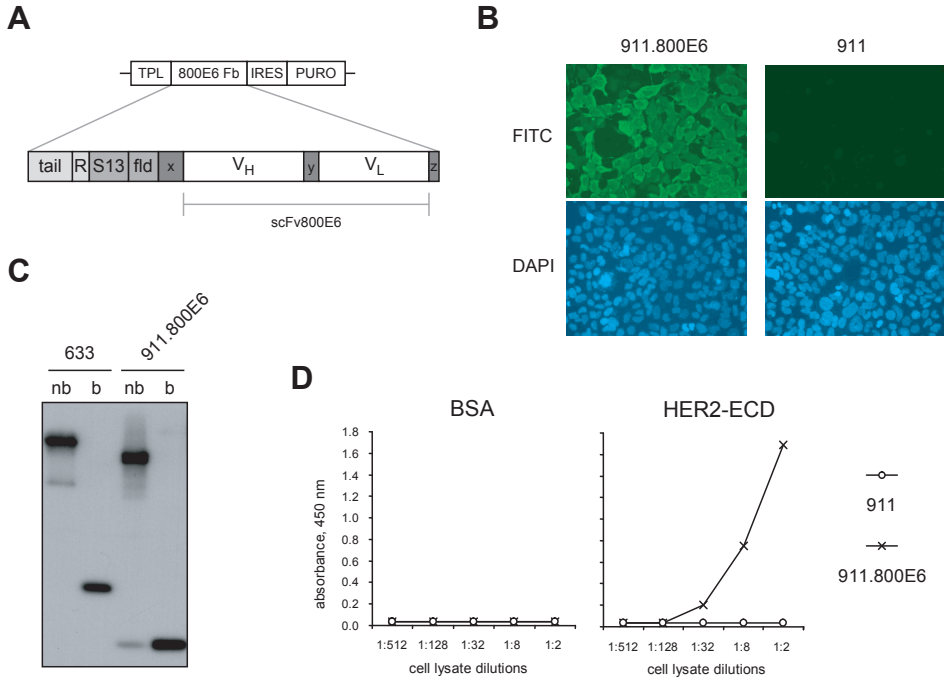


Figure 3. Design, LV-mediated expression, and functional characterization of 800E6 fiber. (A) Transgene construct configuration for lentiviral vector-mediated expression of 800E6 fiber. The components of the 800E6 fiber gene are depicted to scale. PURO, puromycin resistance gene; tail, fiber tail domain (44 amino acids); R, fiber shaft first repeat (15 amino acids) followed by residues Gly and Met; S13, fibrin coiled coil segment 13 (39 amino acids); fld, fibrin foldon domain (30 amino acids); x, spacer (25 amino acids), V_H and V_L, variable fragments of monoclonal antibody 800E6 (53 heavy and light chain, respectively); y, (G₄S)₃ flexible linker; z, additional C-terminal residues (10 amino acids). (B) Immunofluorescence analysis of lentiviral vector-mediated 800E6 fiber expression. Cells were probed with anti-fiber tail antibody followed by a FITC-conjugated secondary antibody (top). Nuclei were visualized by staining with DAPI (bottom). 911.800E6 cells had not been selected for puromycin resistance prior to the analysis. (C) Western analysis of the lentivirally expressed 800E6 fiber protein. Reduced nonboiled (nb) and boiled (b) samples of 911.800E6 and, as a wild-type fiber control, 633 cell lysates were loaded for SDS-PAGE. Western blots were probed by anti-fiber tail antibody. (D) Binding experiment to assess the antigen recognition capability of 800E6 fiber. HER2-ECD or an irrelevant protein control (BSA) were immobilized in the wells of an ELISA plate and subsequently incubated with dilution ranges of freeze-thaw cell lysates of either 911.800E6 or control cells. Bound 800E6 fiber was detected by immunological probing using anti-fiber tail antibody and a HRP-conjugated secondary antibody followed by development with TMB substrate and absorbance reading at 450 nm.

conditions, by SDS-PAGE and immunoblotting (Figure 3C). For both fiber types, the nonboiled samples exhibited fiber tail-specific detection of proteins that were three times the predicted size of the respective fiber monomers, indicating the presence of fiber trimers. Furthermore, when the samples were denatured by boiling, these trimeric proteins were shown to be degraded

to their respective fiber monomers. Thus, the results obtained show that the 800E6 fiber, similar to the control wild-type fiber, was able to form detergent and reducing agent-resistant, heat-labile trimers.

In these preliminary characterizations of the 800E6 fiber, an all-important query was whether the scFv constituent had preserved its functionality with respect to antigen binding. To answer this question, we conducted an ELISA experiment that employed the extracellular domain of Her2/*neu* (HER2-ECD) (Figure 3D). Immobilized HER2-ECD and, separately, an irrelevant protein (BSA) were incubated with freeze-thaw cell lysates of the 800E6 fiber expressing cells, 911.800E6, or 911. With subsequent probing for the fiber tail, this assay demonstrated that the 800E6 fiber was able to bind to HER2-ECD, whereas no affinity for BSA could be detected.

Taken together, these experiments demonstrate that the 800E6 fiber maintains the important properties of trimerization capability and antigen recognition, suggesting the suitability of this chimeric fiber for Ad capsid incorporation and vector targeting.

Functional assessment of the liganded fiber chimera in the context of fiber-pseudotyped Ad particles

The essential step in testing this new scFv-harboring chimeric fiber was to evaluate it for capsid incorporation efficiency and for functionality in the capsid-incorporated context. For this purpose, we put into service the fiber pseudotyping system established in the present study to generate, via large-scale infection of 911.800E6 cells with AdGL Δ F/F, a CsCl purified stock of 'AdGL Δ F/800E6 Fb', a fiber gene-deleted Ad vector transiently loaded with the 800E6 fiber.

The chimeric fiber load was investigated by western blot analysis of purified AdGL Δ F/800E6 Fb and, as a control, wild-type HAdV-5 virions (Figure 4A). Qualitatively, this experiment demonstrated that AdGL Δ F/800E6 Fb incorporates the full-size 800E6 fiber. However, quantification of fiber content by normalization to hexon shows that the chimeric fiber load in AdGL Δ F/800E6 Fb is considerably lower than that of wild-type fiber in HAdV-5. Digital image analysis of chemiluminescence-exposed film scans showed an 800E6 fiber incorporation level of approximately 12%. This analysis therefore revealed gravely impaired capsid incorporability of the 800E6 fiber.

Because the 800E6 fiber was shown to be present, albeit at a sub-stoichiometric level, in the fiber-pseudotyped vector particles, it could, in principle, serve to mediate vector binding to its target antigen, Her2/*neu*. Therefore, we investigated the functionality of the capsid-incorporated 800E6 fiber by performing a slot blot-based, HER2-ECD binding assay (Figure 4B). Specifically, membrane immobilized virus particles were incubated with HER2-ECD, after which (virus-) bound HER2-ECD was detected by virtue of a

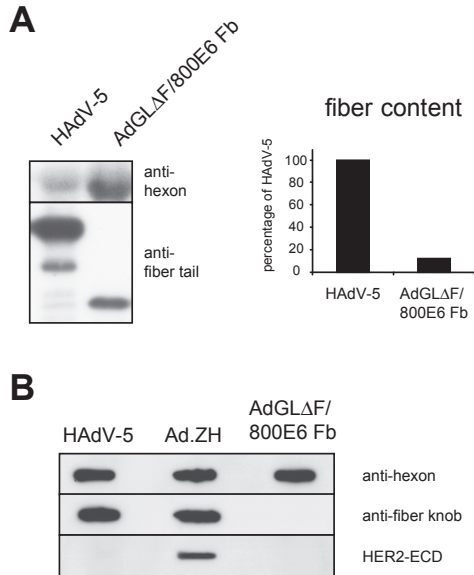


Figure 4. Functional assessment of the 800E6 fiber in the context of fiber-pseudotyped Ad particles. (A) Western analysis on purified virus particles to assess the capsid incorporation capacity of 800E6 fiber. Of HAdV-5 and AdGLΔF/800E6 Fb, 2×10^{10} and 4.6×10^{10} CsCl-purified particles were loaded, respectively. The depicted incorporation level of 800E6 fiber in AdGLΔF/800E6 Fb (in relation to wild-type fiber load in HAdV-5) was calculated using digitally quantified hexon- and fiber-specific signals. (B) Slot-blot-based binding experiment to assess the antigen binding capacity of 800E6 fiber in the capsid-incorporated context. CsCl-purified HAdV-5, Ad.ZH, and AdGLΔF/800E6 Fb were blotted to a PVDF membrane (2×10^9 viral particles per slot) and subsequently incubated with either HER2-ECD, anti-fiber knob antibody, or anti-hexon antibody. Bound primary reagents were detected using appropriate HRP-conjugated antibodies, which were subsequently detected by ECL. Serving as negative and positive controls in this experiment are, respectively, HAdV-5 and Ad.ZH, the latter of which genetically incorporates a fiber carrying HER2-ECD-specific affibody ligands in its knob domain (29).

fused histidine-tag. In this assay, negative and positive control viruses were represented, respectively, by HAdV-5 and Ad.ZH, the latter of which bears a recombinant fiber that genetically incorporates, in the HI loop of the knob domain, two copies of a Her2/*neu* specific 'affibody' termed ZH (29). This affibody-containing fiber had previously been shown to mediate Ad vector binding to HER2-ECD and therefore appropriately serves as a positive control in the present study for binding functionality in the capsid-incorporated context. The results of this slot-blot experiment indeed demonstrate Ad.ZH binding to HER2-ECD. However, no binding of AdGLΔF/800E6 Fb to this target could be detected.

We subsequently sought to confirm the negative outcome for HER2-ECD binding obtained above by employing another technique. Accordingly, we employed an ELISA-based approach that involved coating of ELISA-plate

wells with the viruses under investigation, subsequent incubation with HER2-ECD, and, finally, detection of bound HER2-ECD using an anti-His tag antibody (Figure 5). The assay showed that, although Ad.ZH is clearly positive for binding, there is no indication that AdGL Δ F/800E6 Fb can bind to HER2-ECD. These results thus corroborate those achieved with the slot-blot based method.

In conclusion, the 800E6 fiber, although proven to exhibit target binding functionality when produced in cells (Figure 3D), was found unable to achieve (detectable) target binding in the Ad capsid-incorporated context (Figures 4B and 5).

Analysis of intra- and extracellular redox states reveals aberrant folding of the liganded fiber chimera

The encountered impediments to functional capsid incorporation of 800E6 fiber could point towards folding and/or solubility issues, especially in light of the fact that cellular localization of this fiber appeared to be aberrant (Figure 3B). Therefore, to obtain more clues with respect to the ability of 800E6 fiber (and in particular its scFv constituent) to fold properly, we sought to investigate its redox state. On this topic, scFv800E6 had previously been found to be functionally folded when produced within a reducing environment (39,53,54), a property on which the candidacy for being part of a capsid incorporable fiber chimera was based. This preserved functionality suggests that the correct folding of scFv800E6 is independent of the immunoglobulin-

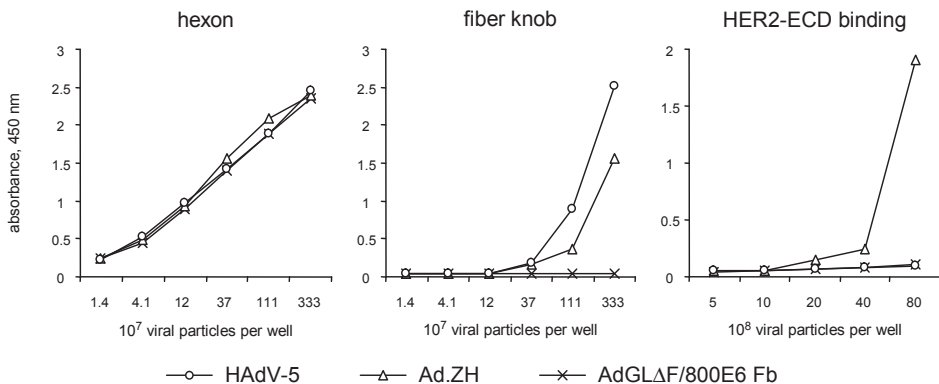


Figure 5. ELISA-based binding experiment to assess antigen binding capacity of capsid-incorporated 800E6 fiber. CsCl-purified HAdV-5 (negative control), Ad.ZH (positive control), and AdGL Δ F/800E6 Fb were immobilized on an ELISA plate and probed with either anti-hexon antibody (left graph), anti-fiber knob antibody (middle graph), or HER2-ECD (right graph). Bound primary reagents were detected using appropriate HRP-conjugated antibodies, followed by development with TMB substrate and absorbance reading at 450 nm.

superfamily canonical intradomain disulfide bridges, of which this scFv has the potential to form two per polypeptide.

We initially set out to demonstrate that, as expected, the intracellular 800E6 fibers are devoid of disulfide bridges. To address this, a western blot experiment was conducted with samples representing the intracellular (or 'pre-lysis') redox state of 633 and 911.800E6 cells (Figure 6A, left panel). These samples were obtained by lysing cells in nonreducing conditions in the presence of NEM, an alkylating agent that nonreversibly modifies non-oxidized cysteine residues, and thereby precludes the formation of new disulfide bonds, at the same time as leaving intact those already formed. As a reference, non-NEM treated cells were also analysed, both under nonreducing and reducing conditions (Figure 6A, middle and right panels, respectively). Analysis of the protein samples representing the intracellular state of 911.800E6 cells gave bands corresponding to the reduced-state monomer and trimer sizes of 800E6 fiber, indicating that, within the cell, 800E6 fiber is indeed present in a reduced state.

The analyses further revealed, however, that, in the non-NEM treated, nonreduced protein samples, the 800E6 fiber is present (even when boiled) in much-larger-than-trimer-sized multimers (Figure 6A, middle panel), indicating a high occurrence of interpolypeptide (and probably intertrimer) disulfide linkages. By contrast, such large covalent meshes were not observed for the control samples with wild-type fiber.

These observations may implicate that many of the fiber-incorporated scFv moieties are actually improperly folded; a misfolded state would allow for the formation of noncanonical, intermolecule disulfide bridges upon entrance to an oxidative milieu. To determine whether this implication holds true, we checked whether the observed intermolecule meshes arose immediately upon cell lysis and not artifactually at a later stage in the western blot procedure. To this end, a nonreducing Western blot was conducted with samples representing the post-lysis redox state of 911.800E6 cells (Figure 6B). In this case, the cells were first lysed by freeze-thawing and then immediately treated with NEM to fix their redox state (i.e. to prevent the occurrence of any disulfide bridges at a later stage). The analysis shows, for both the nonboiled and the boiled 'post-lysis' sample, that the majority of the 800E6 fibers are in the 'aggregated' state, indicating that the formation of intermolecular disulfide bridges already occurs quickly after, or during, cell lysis.

Thus, collectively, the results obtained in the present study demonstrate that the 800E6 fiber, although intracellularly present in a completely reduced state, has a strong propensity to form intermolecule disulfide bridges upon encountering a shift to an oxidative environment. This tendency to form intermolecule bonds rather than the canonical intradomain disulfide bridges indicates that at least a sub-population of the scFv moieties within 800E6 fibers is not folded properly.

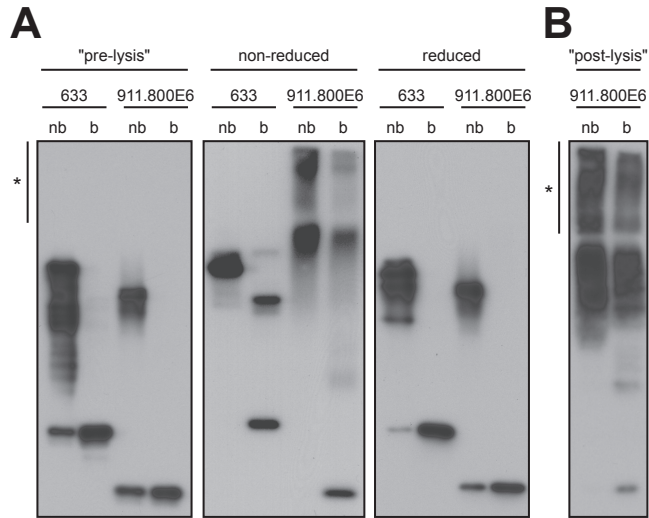


Figure 6. Western blot analysis to assess the redox state of 800E6 fiber before and after cell lysis. (A) Analysis of the pre-lysis redox states of 800E6 fiber and wild-type fiber. Nonreduced samples representing the pre-lysis redox states (left panel) of 800E6 fiber-expressing (911.800E6) and wild-type fiber-expressing (633) cells were made by cell lysis in the presence of NEM. Additionally, nonreduced (middle panel) and reduced samples (right) were included, with the latter providing a reference to the molecular weights of the reduced-state monomers and trimers of 800E6 and wild-type fiber. Of all these samples, both nonboiled (nb) and boiled (b) aliquots were loaded for SDS-PAGE. Western blots were probed with anti-fiber tail antibody. Of note, to allow for the detection of excessively high-molecular-weight proteins (aggregates), the stacking gel had not been removed prior to the electroblotting. The portion of the blots containing proteins transferred from the stacking gel is indicated by an asterisk. (B) Post-lysis redox state. Nonreduced samples representing the post-lysis redox state of 800E6 fiber-expressing cells (911.800E6) were made by treatment with NEM after cells were lysed by freeze-thawing. Western blotting procedures were performed as in the above experiment.

DISCUSSION

A long-standing goal in adenovirus-mediated gene therapy is the development of systemically deliverable genetically retargeted Ad vectors. In this regard, many previous efforts rationally focused on modification of the Ad fiber protein in order to achieve abrogation of native interactions of the fiber with cellular receptors and, furthermore, to realize the introduction of novel cell specificities. With the recent elucidation of the mechanisms responsible for liver entrapment of systemically-administered HAdV-5 vectors and the consequent opening of new genetic and pharmacological avenues to improve Ad bioavailability (58,59), genetic modification of the fiber holds all the more promise for the achievement of selective transduction of target cells in an *in vivo* context. Therefore, efficient means are required to assess newly-designed candidate retargeted fibers for their ability to confer desired

properties to Ad vectors. However, the conventional method of testing a new fiber is a rather time-consuming process, involving the generation of a fiber-modified Ad genome and the subsequent rescuing of the fiber-modified virus.

In the present study, a new, lentiviral vector-based adenovirus fiber-pseudotyping system is presented as a means for expedited functional screening of new fiber variants designed to retarget Ad. This system entails the use of lentiviral vectors to generate 'packaging cells' stably expressing fibers of interest. Infection of these cells with a fiber gene-deleted adenovirus vector subsequently allows for the incorporation of the new fibers into the capsids of progeny virus. This fiber-pseudotyping approach enables new fiber variants to be rapidly tested (without the need for genomically modifying Ad) for capsid incorporation efficiency and for functionality (with respect to target binding and, ultimately, the capability to mediate specific transduction) in the context of Ad viral particles.

Our system elaborates on a previous endeavor by Jakubczak *et al.* (26) that involves large-scale transient transfection with fiber expression constructs and subsequent coordinated infection of the transfected cells with an ΔF Ad vector (18,26). Similar to this transient transfection/infection system, the LV-based system described in the present study is a significantly quicker and less labour-consuming way to evaluate modified fibers compared to the conventional method involving the rescue of viruses genomically incorporating the new fiber. However, because this new system entails an additional step (i.e. the generation of lentiviral vectors), it is evident that it does not fully match the transfection-based method in this respect. Nonetheless, for a number of reasons, the lentivirus-based system is considered to be a valuable addition to the repertoire of tools at the disposal of investigators seeking to test new fibers. First, in contrast to the transfection-based method, the lentivirus-based system allows for cell manipulations (i.e. for achieving fiber expression) to be carried out at a small scale and without the need for close coordination with the subsequent fiber-pseudotyping infection. Second, fiber variant-expression by means of lentiviruses proves to be associated with low cell-to-cell variability, is stable over multiple passages, and can be selected for. Therefore, taken together, the lentivirus-based system provides a relatively facile and robust procedure for reliably generating a homogenous population of cells suitable for large-scale fiber-pseudotyping.

In our demonstration of the lentiviral vector-based Ad fiber-pseudotyping system, we made use of R7Knob, a seven-shaft repeat-containing Ad5-based fiber. This fiber served to establish that lentiviral vectors can be used to generate fiber-variant producing cells and, furthermore, that such cells can achieve transient fiber-pseudotyping of Ad. However, because R7Knob is a non-retargeted fiber, it could not serve to illustrate directly the concept of using transiently fiber-pseudotyped vector stocks for the functional evaluation of candidate de- and/or retargeted fibers. The feasibility of this concept,

however, has already been demonstrated previously in several studies by others. First, Seggern *et al.* (25) reported that fiber-pseudotyping with a chimeric fiber (i.e. consisting of the Ad5 fiber tail and shaft fused to the Ad3 fiber knob) resulted in infection being directed away from CAR and redirected to the Ad3 receptor. Furthermore, Jakubczak *et al.* (26) identified putative CAR-binding ablating mutations by assessment of transduction abilities of transiently fiber-pseudotyped vectors. Finally, in two recent studies, which both adopted the 'transient transfection/infection' system of Jakubczak *et al.* (26), pseudotyping with different types of liganded fibers was shown to result in accordant changes in vector tropism (18,55).

In the second part of the present study, the newly established LV-based fiber-pseudotyping system was employed to evaluate a new fiber variant harboring a scFv as targeting ligand. In this regard, for endowing viral vectors with new target cell specificity, scFvs represent an important class of ligands. This is especially the case in the field of enveloped viral vector-retargeting, in which genetic fusion of scFvs to viral envelope proteins has, in multiple instances, resulted in the successful generation of viruses with modified cell specificities (60–63). However, because of the distinct natural biosynthetic routes of Ad capsid proteins and antibody fragments, scFvs are not considered as the optimal type of targeting moiety when it comes to adenovirus retargeting through genetic capsid modifications (37,55). As the assembly of Ad particles takes place in the nucleus, scFv-based ligands genetically fused to Ad capsid proteins are forced to fold in an unnatural environment in which secondary modifications associated with the secretory route (e.g. intra-domain disulfide bridges) are excluded. This could lead to improper or unstable folding of the scFv moiety and, consequently, to further complications as loss of antigen recognition capacity, aggregation, and disturbance of the capsid assembly process. On this matter, in a previous study, attempts to retarget Ad by genetically fusing a scFv or other disulfide bridge-containing ligands to the Ad fiber protein were unsuccessful and identified problems with respect to solubility, folding, and incorporation efficiency (37).

To overcome the difficulties associated with the genetic Ad capsid incorporation of antibody-based ligands, recent focus in the field has been on the use of a class of particularly stably structured scFvs (56,57). These scFvs, termed intrabodies or hyper-stable scFvs, have been selected for their solubility and stability under the reducing conditions of the cytosol (64–68) and therefore are considered as promising candidates for functional and stable folding as part of a genetic Ad capsid protein fusion. Indeed, two such stabilized scFvs were successfully displayed on Ad particles by genetic anchorage to, respectively, fiber and pIX (56,57). Importantly, these capsid-incorporated scFvs were shown to have retained their functionality with respect to specific antigen binding.

In view of the favorable results obtained with stabilized scFvs, we hypothesized that scFv800E6 (39,53,54), an intrinsically stable scFv directed to the important tumor associated antigen Her2/*neu* (69), would also be suited for the purpose of genetic Ad capsid incorporation. This scFv was previously found to possess, by nature, a structural framework of particular robustness, and it was observed to functionally fold in a reducing environment in a variety of expression platforms (39,53,54). Thus, for reasons of both therapeutic relevance and technical feasibility, scFv800E6 represents an interesting moiety to test for functionality as a targeting ligand genetically displayed on an Ad capsid protein. Therefore, analogous to previously established genetic fiber modification strategies (21,23,24), we chose to genetically insert scFv800E6 into a de-knobbed, extrinsic trimerization domain-containing fiber scaffold.

Functional assessment of lentivirally expressed 800E6 fiber demonstrated that this new chimeric fiber was capable of exerting two important functions that are considered as a key utility in genetic Ad targeting, namely trimerization and target binding. The intactness of these functionalities suggests that the respective responsible functional domains, fibrin foldon and scFv800E6, can both fold correctly in the context of this particular chimeric fiber configuration. The indication that scFv800E6 is able to acquire a functional fold as part of an Ad capsid fusion protein would further illustrate the stability and versatility previously reported for this scFv (39,53,54). Thus, these preliminary characterizations suggested the suitability of the 800E6 fiber for Ad capsid incorporation and vector targeting.

However, functional assessment of the 800E6 fiber in Ad particles revealed that this fiber is significantly impaired in its capsid incorporation ability (Figure 4A). Moreover, in two independent binding experiments, 800E6 fiber-pseudotyped Ad particles failed to detectably bind HER2-ECD (Figures 4B and 5). These negative outcomes, which we suspected to indicate the improper folding of the scFv constituent of the 800E6 fibers, prompted analysis of the pre- and post-lysis redox states of the 800E6 fiber (Figure 6). This led to the observation that 800E6 fiber molecules have the tendency, when subjected to an oxidative environment, to form large covalent aggregates. We found that 800E6 fibers, although in a completely reduced state in the intracellular environment, immediately form intermolecular disulfide bridges upon a shift, by cell lysis, to the extracellular (oxidative) milieu. This finding suggests the poor formation of the canonical intra-domain disulfide bridges after cell lysis, which would be indicative of improper, unstable folding of the scFv moiety in the context of the 800E6 fiber. Taking all this into consideration, we are led to speculate that, in the HER2-ECD-binding experiment with loose 800E6 fibers (Figure 3D), it was only a subpopulation of functionally folded scFvs (amid perhaps a majority population of misfolded domains) that accounted for the observed target binding capacity.

It is suspected that the observed impairment in capsid incorporation ability of the 800E6 fiber is, to a large extent, directly consequential to the solubility and folding issues identified above. However, other factors most likely contribute to the observed low level of 800E6 fiber loading. For example, the structure of the fiber scaffold itself and/or its combination with the incorporated scFv could be a limitation to optimal display on the capsid. Furthermore, the observed low 800E6 fiber content in viral particles might also be partially attributable to a loss of incorporated fibers as a result of the physical strains imposed by post-lysis association (via disulfide bonds) with other viral particles, loose fibers, or other proteins with accessible cysteines.

With respect to the failure of 800E6 fiber-pseudotyped Ad particles to detectably bind HER2-ECD, we recognize two chief causal factors. The first is the much-lower-than-stoichiometric presence of 800E6 fiber in the capsid and the second is the suspected nonfunctional folding of a considerable fraction of the scFv domains.

The folding issues identified in the present study for the fiber-incorporated scFv800E6 appear to be representative of generic problems considered to be inherent to the use (as Ad targeting ligands) of complex proteins that contain disulfide bridges under natural conditions (37,55). Thus, in relation to the two artificially stabilized scFvs that have recently been reported to be functionally displayable on Ad (56,57), scFv800E6 appears a less favorable ligand for the purpose of genetic Ad targeting. Of relevance, in contrast to those successfully incorporated scFvs, scFv800E6 had not been specifically engineered for intracellular stability but was found to be particularly robust by nature (39,53,54). Therefore, considering this difference in origin, it is possible that the relatively unfavorable results obtained for 800E6 fiber are, after all, a result of a lesser stable structure of scFv800E6 compared to artificially stabilized scFv frameworks.

In conclusion, in the present study, we establish a new LV vector-based transient adenovirus fiber-pseudotyping system that provides a convenient method for testing newly-designed retargeted fibers. Using this fiber testing system, a particular combination of a fiber scaffold with an incorporated antibody-based ligand was found not to be suited for Ad retargeting. Furthermore, as a result of being confronted with problems associated with solubility, folding, and functional capsid display of the new liganded chimeric fiber, the present study provides support for the existing doubts on the usefulness (i.e. in the context of genetic Ad targeting) of naturally occurring ligands that depend on disulfide bridges for stability and functionality. In this respect, the employment of a means to artificially optimize scFvs for stability in a reducing milieu comprises a very rational and promising approach to overcome fundamental incompatibilities between the natural biosynthetic routes of antibodies and adenovirus capsid proteins (64–68). However,

possibly the most suitable candidates for genetic Ad capsid incorporation are the new classes of non-immunoglobulin, artificial ligands (e.g. affibodies and designed ankyrin repeat proteins) made from existing protein scaffolds by randomization at selected amino acid positions, followed by selection against desired targets (30,70,71). Because of their biosynthetic compatibility with Ad capsid components and their low structural complexity, these molecules represent likely candidates for serving as genetic Ad capsid incorporable ligands, as already exemplified for affibodies in a number of previous studies (29,55,72).

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