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Rational and random approaches to adenoviral vector engineering

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Citation
Uil, T. G. (2011, January 28). *Rational and random approaches to adenoviral vector engineering*. Retrieved from https://hdl.handle.net/1887/17743

Version:	Corrected Publisher's Version
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Note: To cite this publication please use the final published version (if applicable).

RATIONAL AND RANDOM APPROACHES TO ADENOVIRAL VECTOR ENGINEERING

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ISBN: 978-94-90371-95-1

Layout and printing: Off Page, www.offpage.nl

Cover: A rendered photograph of a sculpture by Nicolas Dings, located in front of the Amsterdam City Hall. The artwork consists of a bronze statue of Baruch de Spinoza and an icosahedron of polished granite, which refers to the sharpening of the mind. The birds on Spinoza's cloak – 'exotic' ring-necked parakeets intermingled with native sparrows – symbolize Amsterdam's multicultural society.

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RATIONAL AND RANDOM APPROACHES TO ADENOVIRAL VECTOR ENGINEERING

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 28 juni 2011
klokke 16.15 uur

door

Taco Gilles Uil

geboren te Delft
in 1977

PROMOTIECOMMISSIE

Promotor:	Prof. dr. R.C. Hoeben
Overige leden:	Prof. dr. A.J. van Zonneveld Prof. dr. E.J. Snijder Prof. dr. E.J.H.J. Wiertz (Universiteit Utrecht)

The research described in this thesis was performed at the department of Molecular Cell Biology of the Leiden University Medical Center, Leiden, the Netherlands.

The work described in this thesis was supported by the European Union through the 6th Framework Program GIANT (contract no. 512087).

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1



INTRODUCTION

PART I

GENERAL INTRODUCTION & AIMS AND OUTLINE OF THIS THESIS

GENERAL INTRODUCTION

An old concept of exploiting viruses for human clinical use is that of viral therapy of cancer (1). The initial incentive for using viruses for this purpose came from reports that cancer patients occasionally experienced partial clinical remission upon contraction of an infectious disease. These early observations (from up to more than a hundred years ago), as well as more recent ones, mostly concerned leukemia or lymphoma patients who had contracted either influenza (2), chickenpox (3), measles (4-8), hepatitis (9), or glandular fever (i.e. Epstein-Barr virus) (10). Thus, stimulated by such cases, researchers ventured to deliberately use natural viruses to combat various types of cancer. In this regard, several early clinical virotherapy trials that showed significant results made use of for example hepatitis B virus (11), West Nile virus (12), adenovirus (13), and mumps virus (14). However, despite the occasional evidence of an anti-tumor effect, oncolytic virotherapy using unmodified viruses generally lacked in efficacy and/or safety (1).

With the advent of recombinant DNA technologies (15), it became possible to specifically modify viral genomes and to develop viruses that incorporate foreign genes (16-18). This development marked the birth of the concept 'viral gene therapy' (19), which entails virus-mediated transfer of therapeutic genes into target cells. Compared to previous, non-viral 'gene therapy' methods, virus-mediated gene delivery represented a dramatic advancement regarding the efficiency of gene transfer. As a result, the original idea of gene therapy – i.e. *in vivo* or *ex vivo* transfer of therapeutic genes into cells in order to complement for a genetic defect or to counteract a disease phenotype – became a much more realistic prospect.

Ever since the first promising results of efficient gene transfer using retroviruses in 1981 (17,18), many different viruses – enveloped or non-enveloped, DNA- or RNA-based, with or without host genome integration ability – have been explored as platforms for gene delivery (20,21). In this regard, several types of replication-incompetent gene transfer vectors are currently being developed. For example, vectors are being developed not only for the purpose to 'heal' cells by gene correction or augmentation, but also to kill cancer cells by the introduction of lethal genes (e.g. for prodrug-converting enzymes, cytokines, or fusogenic or apoptotic proteins). Further, replication-defective vectors are also being engineered to serve as viral vaccine vectors that encode and/or display pathogenic antigens (22,23). Finally, revisiting the old concept of viral therapy of cancer, the modern molecular design approaches are also being used in the context of replication-competent vectors, i.e. to generate more selective and effective oncolytic viruses (24,25).

Human adenoviruses (Ads) are the most widely used viruses as vectors for gene delivery or oncolytic virotherapy (26,27). Reasons for the popularity of Ad-based vectors include Ad's relative non-pathogenicity in immunocompetent

adults, its genetic stability, and its ability to infect a wide range of cells, both quiescent and replicating. Furthermore, Ad's biology has been extensively studied, both regarding natural infection in humans and in the context of experimental infections with wild-type or recombinant Ad in humans and animal models. Also, Ad has been used as a model for multiple cellular or viral processes. For example, early work on Ad has led to the discovery of RNA splicing (28), while work on Ad replication provided the first example of a mammalian cell-free DNA replication system (29). Thus, fundamental knowledge about Ad, combined with the availability of methods for genetic modification of Ad, has allowed for the development or improvement of Ad-based gene delivery vehicles and oncolytic agents.

All-important for any Ad vector – replication-competent or not – is that transduction of the target cells is sufficiently efficient and specific (21,30,31). However, especially for systemically administered Ad vectors this has been difficult to achieve. Blood-borne Ad is known to quickly become localized mainly to the liver, putatively as a consequence of the larger size of the endothelial fenestrations of the liver compared to those of other organs. Consequential to this biodistribution, Ad is readily taken up by scavenging liver macrophages (Kupffer cells) or, alternatively, efficiently transduces hepatocytes (mainly via interaction with blood coagulation factor X). These mechanism, as well as those involving other interactions with blood and/or cellular components, make that intravenously administered Ad is very poorly available for transduction of target cells.

Thus, major topics in Ad vectorology are de- and retargeting, both at the level of biodistribution and cell transduction (30,32). An ideal Ad vector would be modified to avoid sequestration by macrophages and blood components, be ablated for direct and indirect interactions with its native receptors, and, importantly, would efficiently and specifically enter cells via a new target receptor. Ways to achieve this are genetic capsid modifications and/or chemical modification of Ad.

AIMS AND OUTLINE OF THIS THESIS

The overall aim of this thesis is to contribute to the engineering of more selective and effective oncolytic Ad vectors. Two general approaches are taken for this purpose: (i) genetic capsid modification to achieve Ad retargeting (**Chapters 2** to **4**), and (ii) directed evolution to improve the cytolytic potency of Ad (**Chapter 5**). In order to provide some context for these approaches, **Chapter 1**, **part II** gives a brief background on Ad biology and vectorology. Further, in **Chapter 1**, **part III**, a broad overview is provided of the ways that evolution-based engineering has previously been used to generate or improve viral vectors.

Chapters 2 and **3** focus on the modification of the minor Ad capsid protein IX (pIX). pIX is present on the faces of the Ad capsid icosahedron, functioning as 'cement' between the much larger hexon proteins (33). Previously, the C-terminus of pIX proved serviceable as an anchor for the genetic capsid incorporation of targeting ligands and other heterologous moieties (34-36). In **Chapter 2**, a new system is described that allows for the rapid functional testing of new pIX-ligand fusion proteins. In this system, lentiviral vectors are used to generate cells stably expressing the pIX variant of interest. Large-scale infection on such cells with a pIX-deleted Ad vector subsequently yields an Ad vector preparation phenotypically pseudotyped with the new pIX variant. This system thus allows rapid analysis of new pIX-ligand fusions in the context of the Ad capsid without having to genetically modify the Ad genome. In **Chapter 3**, the lentiviral vector-based pIX-pseudotyping system is put to use for the analysis of a new pIX fusion protein harboring a single-chain T-cell receptor (scTCR) as a targeting ligand. The concerning scTCR was directed against the intracellular cancer-testis antigen melanoma-associated antigen-A1 (37-39). Importantly, this chimeric pIX molecule proved to be efficiently incorporated into the Ad capsid. Moreover, Ad transduction studies showed evidence of the capsid-displayed scTCR to mediate a degree of specific target cell transduction via the cognate peptide-MHC complex.

Analogously as done for pIX, **Chapter 4** describes a phenotypical pseudotyping approach for fiber. The Ad-encoded fiber protein is present as a trimeric rod-like structure that extends from the vertices of the Ad capsid icosahedron (40). Its outward-facing, C-terminal 'knob' domain is responsible for binding the Coxsackie and adenovirus receptor (CAR), Ad's *in vitro* primary cell surface attachment protein (41,42). With its prominent role in native receptor binding, the Ad fiber is logically subject to many capsid modification strategies that aim at altering Ad tropism (30). Thus to facilitate expedited testing of new fiber variants, a lentiviral vector-based, fiber-pseudotyping system was set up. This involved optimization of the fiber (variant) expression cassettes by inclusion of the tripartite leader sequence of Ad's major late transcription unit (28). A second objective of this study was to functionally assess a new chimeric fiber harboring a tumor antigen-directed single-chain variable fragment (scFv) antibody (43). Although this fiber variant showed some degree of target binding and formed stable trimers, it displayed problems regarding capsid incorporation ability, functionality within the capsid, and folding of its scFv constituent. Thus, this particular fiber proved not suitable for Ad retargeting.

Finally, **Chapter 5** describes the development and validation of a novel evolution-based engineering approach for Ad. To date, most Ad-based vectors have been generated through molecular design. Although this rational tailoring of Ad has led to significant vector improvements, it is often still hampered by our limited understanding of the intricate viral function-

structure relationships. Therefore, 'random' virus engineering strategies (see **Chapter 1**, **part III**) may be a useful alternative or complementary approach for the generation of new or improved viral vectors. In this regard, the high mutation rates of RNA viruses have proven readily exploitable in adaptation studies to achieve vectorological goals (44-54). Thus, it was hypothesized that a mutator Ad polymerase-based, 'accelerated evolution' procedure would likewise be of use for Ad vector engineering. To develop such a system, the intrinsic mutation rate of Ad replication was sought to be increased by modification of the Ad-encoded DNA polymerase (Ad pol) (55). This was done by mutation of residues within regions putatively important for nucleotide selection or proofreading. A mutation-accumulation and deep sequencing strategy was subsequently used to identify any mutators among the Ad pol mutants. Finally, the mutator polymerase-based directed evolution approach was validated by conducting an evolution procedure aimed at increasing Ad's oncolytic potency, and by subsequent characterization of resultant bioselected virus populations and isolated clones.

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PART II

ADENOVIRUS BIOLOGY & ADENOVIRAL VECTORS

ADENOVIRUS BIOLOGY

Human adenoviruses, of family *Adenoviridae*, genus *Mastadenovirus*, are a frequent cause of respiratory, gastrointestinal, urogenital, and ocular infections (1). Adenovirus infections in healthy individuals are mostly associated with only mild symptoms, but occasionally they can take a more severe course, leading to, for example, gastroenteritis or pneumonia (especially in children) (2-6). The diversity among adenoviruses is great: for human adenovirus there are 51 'serotypes' identified by traditional immunochemical methods, and 4 more 'types' defined recently by genomics (1,3,7-9). These different viruses are chronologically numbered 1 to 55 and further subgrouped in 7 distinct species, A to G. Human adenoviruses of serotypes 2 and 5 (Ad2 and Ad5), of species C, were thus among the earliest to be discovered and have been best characterized. Here we focus mainly on Ad5, the virus used most for gene therapy purposes.

Virus structure and cell entry mechanisms

Adenovirus is a non-enveloped, icosahedral virus with a linear double-stranded DNA genome (10). The capsid, of about 90 nm, is formed by three major proteins, 'hexon' (II), 'penton base' (III), and 'fiber' (IV), in addition to four minor polypeptides (IIIa, VI, VIII, and IX). Hexons make up the greater part of the capsid, with each of the 20 icosahedron faces being formed by 12 hexon homotrimers. The penton base proteins in turn are located at the 12 vertices of the virus particle, with per vertex one penton base homopentamer. Further, from each of these vertices protrudes a trimeric fiber, each N-terminally anchored to the penton base structure. For prototypic Ad5, the genome contained within the capsid is about 36 kb long, has 103-nucleotide long inverted terminal repeats (ITRs) (11,12), and is covalently linked – at either of its 5' ends – to a terminal protein (TP) (13). Besides with TP, the genome is associated with several other core proteins (V, VII, and μ) (14). Further contained within the viral capsid is the Ad protease, which plays an important role in viral maturation (15) as well as in Ad cell entry (16-18).

Adenoviruses enter cells through specific interactions with cell surface receptors. Such interactions may occur directly – between capsid protein and receptor – or indirectly via a 'bridging' function provided by a soluble host factor (19). *In vitro*, Ad5 infects cells via a well-defined 2-step process. First, the C-terminal 'knob' domain of Ad fiber binds to the Coxsackie and adenovirus receptor (CAR), which is a primary cell surface attachment molecule for members of species A, C, D, E, and F (19-21). Second, an RGD motif within the penton base protein binds to cellular integrins, thereby providing a trigger for internalization via receptor mediated endocytosis (22). This CAR-mediated infection pathway is a highly efficient process, making that CAR-

expressing cells are highly permissive to Ad5 infection, while non-CAR cells are comparatively virtually refractory.

In vivo, however, the canonical CAR-mediated cell entry mechanism is not the (only) major pathway of Ad5 infection. For example, CAR does not mediate the predominant transduction of the liver seen upon intravascular delivery of Ad5 (23,24). Instead, the hepatic tropism of blood-borne Ad5 is largely due to interaction of the virus with the blood coagulation factor X (FX) (25-27). In this regard, FX was shown to specifically bind Ad5 hexon and thereby to provide a bridging function to interact with heparan sulfate proteoglycans on the cell surface (26). Like CAR-mediated entry, this FX-mediated infection pathway is dependent – for efficient internalization via endocytosis – on integrin binding via penton base (28). Interestingly, besides being responsible for hepatocyte transduction by Ad5 present in blood (an artifactual situation), this FX-based cell entry pathway may also be relevant in the context of the natural, primary infections of respiratory or ocular epithelial cells (19,29).

Early viral transcription

Following their internalization via receptor-mediated endocytosis (22), partially dismantled adenoviruses disrupt the endosome (17,30), transport to nuclear pore complexes (31), and release their core protein-coated genomes into the nucleoplasm to allow viral transcription and replication to take place. The transcription program that subsequently begins can be divided into an early and a late phase, with the latter starting upon the onset of viral DNA replication. The early transcription phase has several general aims: to coax the infected cell (e.g. a terminally differentiated epithelial cell) to enter the S phase of the cell cycle, which is necessary to allow viral DNA synthesis; to suppress intra- and extracellular antiviral responses; and, finally, to produce the proteins necessary to carry out viral replication.

Transcription units expressed in the early transcription phase can be divided in the 'immediate early' (E1A) and 'early' (E1B, E2A, E2B, E3, and E4) units. As a consequence of differential splicing and alternative use of start codons and/or polyadenylation signals, these units generate multiple distinct mRNAs encoding more than 25 proteins in total (32). The first viral transcription unit to be expressed after Ad infection is E1A (33). The protein products of E1A, which are essential for efficient transcription of the other early units, function by directly and indirectly influencing viral and cellular gene expression as well as cellular regulatory pathways. For example, E1A proteins 12S and 13S, which for the largest part overlap in amino acid sequence, act by sequestration of retinoblastoma tumor suppressor pRb (34), thereby freeing the transcription factor complex E2F (35). Free E2F in turn promotes transcription of cellular genes important for the regulation of cell cycle progression, thereby forcing cell cycle progression to the S phase.

Additionally, the freed cellular E2F promotes transcription of the viral E2A unit (36).

Subordinately to E1A activation, the other early transcription units are activated (32,33,36). Early transcription unit E1B encodes two proteins, E1B 55K and E1B 19K. E1B 55K acts to prevent p53-dependent apoptosis by inhibiting the transactivating function of p53 (37). Additionally, in a complex with E4 ORF6, E1B 55K functions to promote p53 degradation by proteasomes (38). The other E1B protein, E1B 19K, is a functional homologue of cellular apoptosis suppressor Bcl-2 and acts to prevent p53-independent apoptotic pathways (32). Transcription units E2A and E2B, the former of which is activated earlier than the latter, harbor genes required for viral DNA replication (39-41). E2A encodes the single-stranded DNA-binding protein (DBP), while E2B encodes the precursor of the terminal protein (pTP) and the Ad DNA polymerase (Ad pol). Further, transcription unit E3 encodes several proteins that counteract or prevent host innate and cellular immune responses to the infected cell (42,43). For example, E3 gp19K, a membrane glycoprotein that localizes to the endoplasmic reticulum, functions to avoid recognition of the infected cells by cytotoxic T lymphocytes (CTLs). It does so by blocking the transport of major histocompatibility complex class I antigens (MHC-I) to the cell surface, thereby preventing the display of MHC-I-complexed viral peptides to CTLs. Finally, transcription unit E4 encodes proteins with various different functions: influencing viral mRNA transport and splicing, promoting virus DNA replication, and forcing shutoff of host protein synthesis (32,44). In this regard, a complex containing E4 ORF6 and E1B 55K would function to bring about selective nuclear export of late viral mRNAs and to inhibit transport of host mRNAs (45).

Viral genome replication

As a result of the coordinated action of the immediate early and early transcription events, replication of the viral genome begins about 5 to 6 hours after infection (46). Adenovirus genome replication takes place via a protein-primed strand-displacement mechanism that requires at least three adenovirus-encoded proteins: pTP, Ad pol, and DBP (40,41). Additionally, several cellular factors are necessary for efficient replication, including the transcription factors NFI/CTF and NFIII/Oct-1, and the type I DNA topoisomerase NFII. Ad genome replication can start at both the left and right ITR. The 103-bp long ITR consists of a terminal sequence of two 3-bp direct repeats (positions 1 to 6), a pTP/pol binding site (position 9 to 18), and an auxiliary region (positions 19 to 49) (40). The latter contains binding sites for transcription factors NFI/CTF and NFIII/Oct-1. Prior to DNA replication, Ad pol and pTP already associate to form a heterodimer complex (47,48). When bound to the ITR, the pTP/pol complex together

with host transcription factors NFI/CTF and NFIII/Oct-1 represents the pre-initiation complex (41).

Upon formation of the pre-initation complex, replication is initiated using a 'jumping back' mechanism (41,49). First, using pTP as a protein primer, Ad pol carries out template-directed DNA polymerization starting at position 4 of the ITR. The first nucleotide to be incorporated, dCTP, is covalently attached to a Serine at position 580 of pTP, thereby creating 'pTP-C'. Then, after the addition of two more nucleotides (i.e. dATP and dTTP), the resultant 'pTP-CAT' molecule jumps back three positions, allowing the pTP-linked nascent primer strand (i.e. CAT) to hybridize with the complementary first three positions of the template strand. After this operation, DNA synthesis by Ad pol commences again, quickly causing the pTP/pol complex to dissociate (after incorporation of the 7th nucleotide). Ad pol subsequently replicates the complete duplex genome, meanwhile displacing the nontemplate strand. DBP, which coats the displaced strand during replication, assists in the elongation process by helping to unwind the parental duplex DNA (50).

Late viral transcription and virus assembly

With the onset of Ad DNA replication, the late transcription phase starts. General aims of this phase of the infection cycle are to produce the structural and regulatory proteins required for efficient virus assembly and release. During this phase, the so-called 'intermediate' (IVa2 and IX) and 'late' (major late transcription unit, MLTU) transcription units are activated.

The intermediate units, IVa2 and IX, are activated at the beginning of viral DNA synthesis (51,52). For IVa2, the mechanism of this replication-dependent activation is based on the relief from a titratable cellular transcriptional repressor (53). In case of IX, the basis for replication dependence is thought to lie in IX being entirely contained within the E1B transcription unit (54). Due to this nested arrangement, active E1B transcription would occlude the IX promoter, and IX expression would thereby only be possible using newly replicated templates not committed to E1B transcription (54). The single gene product encoded by IX, protein IX (pIX), has a structural role as a minor capsid protein and further has several regulatory functions (55). The other intermediate gene product, pIVa2, is an essential, multifunctional protein that supports encapsidation of the viral genome (56,57), assists in capsid assembly (58), and acts as a transcriptional enhancer of the viral major late promoter (MLP) (59-61). In this latter role, pIVa2 is instrumental for the replication-dependent activation of the MLTU.

The primary major late transcript extends from the MLP all the way to the right end of the viral genome. By alternative use of five polyadenylation sites and through the excision of multiple introns, this 30-kb primary transcript gives rise to five different families of late mRNAs (L1 to L5) (62). Attached

to the 5' end of all major late mRNAs is a ~200-nucleotide leader sequence consisting of three noncoding exons. This 'tripartite' leader (TPL) sequence ensures that Ad's late mRNAs are efficiently translated in the face of a general shutoff of protein synthesis induced by viral protein 100K (63-67). 100K, a protein encoded by L4 transcripts, blocks the function of host cell translation initiation factor eIF-4F (a cap-binding protein complex), thus causing a general inhibition of mRNA translation (65). Ad's late mRNAs are not affected by this shutoff because their TPL sequences facilitate an alternative, eIF-4F-independent form of translation initiation (called 'ribosome shunting') (68). Apart from the TPL, additional leader sequences can be found in late transcripts. For example, in case of the fiber-coding L5 transcripts, so-called x-, y-, and z-leaders are sometimes found spliced – alone or combined – between the TPL and the fiber sequence (69). The presence of such ancillary leaders within the L5 transcripts has been found to directly correlate with the efficiency of fiber synthesis (70).

The late mRNA transcripts encode proteins that make part of the Ad capsid, that assist in virus assembly (e.g. L1 52/55K and IVa2), or that have other regulatory functions (32,62). The assembly of progeny Ad particles begins about 8 hours after infection and takes place in the nucleus (71,72). It proceeds in an ordered series of steps involving the successive generation of several defined assembly intermediates. Light particles consist of all major capsid proteins, are devoid of DNA, and contain several non-capsid proteins, putatively for scaffolding purposes. Heavy intermediate particles contain all outer capsid proteins as well as the viral DNA genome complexed with the core proteins. Mature particles finally have undergone numerous proteolytic cleavages by the co-packaged Ad-encoded protease (18). These proteolytic cleavages are essential for full infectivity of the virus. At 30 to 40 hours after infection, the nucleus of the cell is packed with 10^4 to 10^5 progeny viruses (46). Subsequent viral release from the cell involves the action of the Adenovirus Death Protein (ADP), an Ad species C-specific glycoprotein that promotes cell lysis through an as yet unknown mechanism (73,74). The gene for ADP is embedded in Ad's E3 region but it makes part of both the E3 and the ML transcription units (75). While the level of E3-derived ADP mRNAs remains low throughout infection, ML-derived ADP mRNAs become highly abundant at late stages of infection.

ADENOVIRAL VECTORS

Adenoviruses display many qualities that favor them as vectors for gene therapy. Such qualifying traits include the ability to infect many different cell types, the capacity of generating high amounts of progeny, and genetic and physical stability (76). Furthermore, the sustained build-up – since the

early 1950's – of fundamental insights into adenovirus (Ad) biology and genomic organization has made genetic manipulation of adenoviruses a straightforward practice and, concomitantly, has provided the vectorologist with leads for developing safe and effective gene therapy vectors (77,78). As a consequence, the field has seen many incremental improvements in vector technology, successful cases of preclinical assessments, and progression to clinical trials (77-79).

There are various gene therapy and vaccination purposes for which adenoviruses have been studied and altered. Major aims in this regard are to use adenoviruses as replication-incompetent gene delivery vehicles for gene augmentation therapy, suicide gene therapy, and immonotherapy (76). Additionally, replication-incompetent recombinant adenoviruses are currently extensively being developed as vaccine vectors that can induce immune responses against antigenic polypeptides displayed on the viral capsid and/or against antigens encoded for by the viral vector (80). Finally, another major aim is to use replication-competent adenoviruses as oncolytic vectors, i.e. to harness their replicative ability to fight cancers (81).

Gene delivery vectors

First-generation Ad gene delivery vectors are deleted for the E1 region, and sometimes additionally for the E3 region (76). The deletion of the E1 region serves to rid the virus of the essential transactivation and regulatory functions of E1. The absence of these functions prevents setting off the adenoviral gene expression program and thus essentially avoids viral replication. For their propagation, E1-deleted vectors are dependent on E1 proteins being provided *in trans*. E1-complementing cell lines used for this purpose are 293 (82), 911 (83), PER.C6 (84), and N52.E6 (85), which are all cell lines transformed through the uptake in their genomes of E1 coding sequences. Unlike the E1 region, the E3 region, which mostly encodes immunoregulatory functions (42,43), is dispensable for viral growth *in vitro*. Consequently, its deletion in E1- and E3-deleted vectors needs not to be complemented for. The lack of E1 and E3 sequences together provides space for up to 8 kb of transgenic insertions.

Although these first-generation vectors have proven effective gene delivery *vehicles in vitro* and *in vivo* (86), there have been some limitations. Most prominently, the vectors elicited strong innate and adaptive (cell-mediated and humoral) immune reactions, thus curtailing prolonged transgene expression and making repeated administrations ineffective (87,88). Cell-mediated immune responses causing eradication of transduced cells were found to be directed against the transgene product, but also against viral gene products, thus implicating low-level viral gene expression despite the E1 deletion. Another issue with the first-generation vectors was the

emergence during virus propagation of E1-positive, replication-competent viruses due to recombination with the E1 sequences genomically present in the complementing cell line (89). However, the risk for the occurrence of such replication competent Ads (RCAs) has been virtually eliminated with the newer E1-complementing cell lines whose integrated Ad sequences lack homology with E1-deleted viral vector genomes (84,85).

Second-generation Ad vectors have been made in which more of the early genes were deleted (76). This was done mainly with the aim to further restrict viral protein expression in order to avoid eliciting the cell-mediated immune response. Additional aims were to increase the genetic space for transgenes and to practically exclude the risk for RCAs. The observed low-level expression of viral genes observed in the first generation vectors might partially be a consequence of some residual replication of the viral genome. Therefore, new vectors contained deletions or mutations affecting the E2 genes for proteins necessary for replication (90-94). Additionally, vectors were engineered to lack E4 genes (95,96). To propagate these viruses, cell lines were developed expressing E2 or E4 genes either in a constitutive or inducible manner. The viruses carrying these additional deletions were found to elicit reduced innate and cell-mediated responses and, importantly, showed prolonged transgene expression.

Finally, third generation or 'high-capacity' vectors are devoid of all viral genes (76,97,98). The only viral sequences their genomes contain are the ITRs and the packaging signal. These vectors have to be grown using a helper virus that provides all the viral functions and structural proteins *in trans*. Important for this approach is to avoid that the final high-capacity vector preparations are contaminated with helper viruses (be they replication-competent or not). One strategy that limits contamination entails the inclusion within the helper virus genome of recombinase recognition sites flanking the viral packaging signal (98,99). In this way, helper virus genomes are excluded from encapsidation when co-propagated – with the high-capacity vector – on cells expressing the recombinase. With viral genes completely lacking, these vectors have the unique capacity to incorporate up to 35 kb of heterologous sequence and, furthermore, would in principle be unable to elicit a cell-mediated immune response. Indeed, high-capacity vectors have been reported to maintain transgene expression in immune competent animals for durations much longer than previous generations (100-102).

The optimal vector design depends of course on the specific gene therapy goal. For gene augmentation applications (e.g. to counteract a monogenic effect by the introduction of a functional gene), where long-term expression is desired, the choice of vector might be for one deleted for multiple or all viral genes, as these are associated with reduced eradication of transduced cells by the immune system. However, the retention or reintroduction of

some of the viral genes might be beneficial. For example, E3 genes with anti-immune function may help prolong the survival of the transduced cells (103). Further, the inclusion of E4 ORF3 might be necessary for maintaining sufficient levels of transgene expression (104,105). For other applications, vector-induced immunity may be a primary goal [e.g. for immunotherapy of cancer (106,107) or viral vector-based vaccination against pathogen antigens (80)] or a potentially beneficial circumstance [e.g. for approaches that combat cancer by p53 gene replacement therapy (108,109) or by suicide gene therapy using prodrug-activating enzymes (110,111)]. Thus, for these applications an E1-deleted vector (with or without E3) may be a more likely choice.

For safety and efficacy reasons, a common goal for many gene therapy applications is to direct gene delivery and expression to specific target cells or tissues. Therefore, much research has focused on endowing vectors with target cell specificity (112-114). Distinct approaches taken to achieve target specificity are transcriptional and transductional retargeting. Transcriptional retargeting entails restricting the expression of a transgene to desired targets by making use of tumor- or tissue-specific promoters (TSPs). For example, genes for prodrug-converting enzymes have been put under the control of TSPs to mitigate the toxicity of suicide gene therapy (115). Further, retargeting at the transductional level involves modification of the adenoviral capsid such that Ad more efficiently and/or selectively transduces certain target cells. Transductional retargeting of Ad can be induced by genetic means, e.g. through the incorporation of polypeptide targeting ligands into major or minor capsid components of Ad, or by non-genetic means like chemical modification or usage of bi-specific adaptor molecules (113,114). Ad targeting through genetic capsid modification is discussed in more detail below.

Oncolytic vectors

A unique gene therapy approach to the treatment of cancer is oncolytic virotherapy (116,117). This approach makes use of the natural ability of lytic viruses to kill their host cells and to amplify their cell-lytic effect by replication and viral spread. Thus, unlike replication-incompetent gene delivery vectors, oncolytic viruses must express all viral genes necessary to efficiently perform their lytic life cycles. Further, for extra efficacy, they may be armed with therapeutic genes or modified with respect to their immunomodulatory functions. Most importantly, however, in order to kill tumors while leaving non-malignant tissues intact, they must be cancer selective.

Adenovirus is not particularly cancer selective by nature and must therefore be rendered such. There are two general approaches by which this achieved. First, Ad can be rendered defective for functions that are essential for growth in normal cells but are redundant in tumor cells. For example, Ad5-Δ24 carries a 24-bp deletion in E1A, and the modified E1A gene products fail to bind the

cellular protein Rb for induction of S-phase (118). Due to this defect Ad5-Δ24 exhibits selective replication in cells in which induction of S-phase is not necessary, e.g. most tumor cells. The second general approach to render Ad cancer-selective involves replacement of endogenous viral promoters with TSPs. TSPs have mostly been placed to control E1A, sometimes in combination with distinct TSP driving another early gene like E1B or E4 (112). The above approaches for selectivity have also been combined with a TSP driving the 24-bp deletion mutant of E1A (119,120).

Genetic capsid modification for Ad retargeting

Despite the many advances made for both replication-defective and replication-competent Ad vectors, major barriers persist, especially relating to the specificity and efficiency of target cell transduction. In this regard, one of the most testing issues has been that intravenously injected Ad5-based vectors preferentially transduce hepatocytes (121), which severely limits the availability of these vectors for transduction of non-liver target cells. This phenomenon, which was found to be largely independent of binding to the known Ad5 receptors, (23,24,121,122) has recently been demonstrated to be primarily attributable to interaction of Ad with coagulation factor (F)X, which was found to provide a bridging function between Ad hexon and cell surface heparan sulfate proteoglycan (26,123). Another long-standing issue with intravenously injected Ad is the high uptake by the scavenging action of hepatic macrophages (i.e. Kupffer cells) (124-126). Although the exact mechanism is unknown, Ad clearance by these cells has been found to be predominantly mediated by scavenger receptors, with a contributory role for opsonization (by natural antibodies and complement) and interaction with platelets (127-130). Furthermore, alongside sequestration by the liver, an additional challenge is potential vector uptake by (other) non-target tissues owing to the widespread distribution of the primary receptor for Ad5, the coxsackievirus and adenovirus receptor (CAR). All these matters of off-target transduction limit bioavailability of the vector and may cause side-effects. This, together with the fact that many target cell types are relatively refractory to CAR-dependent transduction, has thwarted the systemic use of Ad5-based vectors.

A rational way to overcome the obstacles associated with native Ad tropism is represented by transductional retargeting by genetic capsid modification (113,114). The main goals for genetic Ad retargeting is to restrict Ad's broad infectivity profile and, simultaneously, to redirect Ad infection to specific target cells. To achieve the former of these goals, researchers have previously sought to ablate the direct interactions between capsid components and cellular receptors, including those between fiber and CAR (23), and penton base and integrins (131-134). While these interventions were successful in avoiding infection through the concerned receptors, they did not greatly influence Ad's

hepatotropism or general biodistribution after systemic delivery. However, the recent elucidation of the FX-mediated infection pathway provided a new rationale for avoiding liver transduction (26). Mutation of hexon to disrupt binding of FX binding indeed resulted in viruses with markedly lower liver cell transduction (135). Moreover, FX-binding ablation of Ad5 combined with incorporation of a CD46-targeted fiber (specifically, a high affinity Ad35 fiber) yielded viruses showing much better lung targeting than control viruses (136). Thus these data indicate that genetic strategies to ablate liver tropism of Ad are feasible and, moreover, can be successfully combined with modifications that introduce new receptor specificities.

In this regard, genetic capsid modification strategies aimed at introducing new receptor specificities have been numerous and involved alteration of the major capsid proteins fiber, penton base (137-139), and hexon (140-142), as well as minor capsid protein pIX (143-148). With its role as Ad's primary receptor-binding protein (*in vitro* at least), fiber was the first Ad capsid protein to be modified for retargeting purposes. Ad5 fiber consists of three domains: (1) an N-terminal 'tail' sequence that provides anchorage to the capsid via penton base, (2) a rod-like 'shaft' domain consisting of 22 β-spiral repeats, and (3) a C-terminal globular 'knob' domain consisting of a β-barrel structure and harboring the CAR-binding motifs. Fiber modifications to affect Ad tropism included swapping of the fiber knob domain (149,150), the fiber knob and shaft domains (151), or the complete fiber (152), with those of non-CAR binding Ad serotypes. These approaches led to Ad5 vectors displaying new receptor specificities, e.g. for CD46. Another approach is the incorporation of targeting ligands. Locations in the knob domain that have been found to tolerate ligand insertions are the C-terminus and the so-called HI loop (i.e. a certain exposed loop located between β-strands 'H' and 'I' of the fiber knob) (153-156). Especially the HI loop has been shown to tolerate ligands of considerable size (157-159). Finally, the complete fiber knob domain (possibly combined with all or part of the shaft) can be deleted and replaced with a targeting ligand. Strategies that took this approach have compensated for the loss of the trimerization functionality contained intrinsically within the knob. This has been accomplished by the inclusion of either the trimerization domain of a retroviral envelope glycoprotein (160), the neck region peptide (NRP) of human surfactant protein D (161), the 'foldon' domain of the bacteriophage T4 fibritin protein (162), or the oligomerization domain of the reoviral σ1 protein (163). However, another study found that inclusion of such extrinsic trimerization domains was not necessary owing to a putative trimerization initiation ability contained within the shaft (164). Although general issues regarding the encapsidation of such rigorously modified fibers seem to exist, this strategy was successful in displaying different types of large and/or complex ligands on Ad (164-167).

Protein IX (pIX) has also been extensively exploited for displaying targeting ligands on Ad. pIX is the smallest of the minor capsid proteins and is located in the crevices between the hexons that constitute the faces of the capsid icosahedron. It is involved in stabilizing the interactions between neighboring hexons (168), and is known to be necessary for packaging of full-length viral genomes (169). Present with 240 monomer copies per particle, pIX is about 7 times more abundant than fiber, and therefore represents an interesting locale for incorporation of targeting ligands, vaccine antigens, or other functional groups. The C-terminus of pIX was found to be accessible in the context of the intact particle and (170), moreover, proved to tolerate genetic anchorage of targeting peptides (143). Further it was found that inclusion of α-helical spacer sequences increased the accessibility and targeting ability of peptide ligands (148). Finally, many later studies showed that different classes of large and complex moieties (e.g. single chain antibody fragments, green fluorescent protein, pathogen antigens) were readily displayed on Ad by genetic fusion to pIX (144-147,171,172).

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PART III

RANDOM APPROACHES TO VIRAL VECTOR ENGINEERING

INTRODUCTION

Numerous viruses have been explored for their applicability as gene delivery vehicles or as oncolytic agents (1,2). By nature, viruses are well-suited for employment for these purposes. They are inherently able to introduce their genomes into cells, to reproduce themselves, and, in case of lytic viruses, to kill their host cells. However, despite these abilities, viruses – or the vectors derived from them – often still fall short regarding both the efficiency and the selectivity with which they deliver therapeutic genes or eradicate cancer cells. Therefore, viruses are being genetically altered to better fit our therapeutic needs.

Before the introduction of recombinant DNA technologies, genetically altered viruses with desired traits could only be obtained by way of phenotypic screens on genetically diverse virus populations. Such classical or 'forward' genetics procedures typically made use of chemical or physical mutagens to randomly mutate viral genomes. Alternatively, these procedures relied on spontaneous mutation during repeated viral passaging. In either case, following the selection for a particular phenotype, genetic analysis of isolated viral variants allowed for the identification of the underlying genotypes.

Later, with the advent of molecular cloning techniques (3), it became possible to genetically modify viruses through 'reverse' genetics: viruses could now be specifically genetically modified at will, after which recombinant viruses could be phenotypically tested. This development allowed for the reengineering of viruses based on rational design, and, with the novel possibility to introduce heterologous genes into viral genomes, made possible the concept 'viral gene therapy' (4). Thus, fundamental knowledge of viral life cycles, cellular targets, and therapeutic genes could now be applied to viral vector design. This led to many improvements to viral gene delivery vehicles and oncolytic vectors (1,2).

However, we still lack a full understanding of the many aspects relevant to successfully reengineer or create a particular viral trait. Viruses have evolved compact genomes in which many coding and non-coding functions overlap. For example, many viral genes are expressed through intricate alternative splicing dynamics. This serves to save space and also to allow for the execution of balanced and time-regulated gene expression programs. However, due to these and other functional and structural constraints, not much of the viral genome can be changed without affecting functions critical for virus viability. As a consequence, the outcome of rational engineering of viruses is inherently unpredictable and often unsuccessful.

Therefore, there has been a renewed interest in random virus engineering approaches. These approaches include conventional forward genetics (involving the use of mutagens for whole-genome random mutagenesis), but also for instance strategies that employ *in vitro* genetic diversification. Regarding the latter, owing to the availability of modern molecular cloning

techniques and efficient library generation methods, virus engineering approaches can now benefit from powerful randomization procedures such as PCR-based mutagenesis and *in vitro* DNA shuffling (5-11). Thus, presently, many random engineering approaches – both classical and modern – are undertaken. These approaches are aimed to avoid the need for any detailed mechanistic knowledge and, furthermore, to allow for the unprejudiced selection of (possibly new types of) virus engineering solutions.

Here, we explore the various random engineering approaches that have been used to obtain genetically modified viruses with desired traits. Besides giving an overview of the different methods, we provide examples of how and to what ends they were employed to various different viruses. First, this review covers the classical genetics procedures that depend on either spontaneous viral mutation or on mutagen-induced mutagenesis. Subsequently, we describe several ways by which phage display strategies have been used for virus targeting purposes. Further, we review the various viral library-based engineering strategies. These approaches – employing *in vitro* randomization techniques and viral library-based selection – have been especially fruitful for some viruses with relatively small genomes and for which sufficiently complex viral libraries are readily made.

SPONTANEOUS MUTATION

One way to obtain genetically altered viruses with desired new phenotypes is to exploit the natural ability of viruses to evolve. Spontaneous mutations (including recombination events) occurring during viral replication are the major source of a virus' potential to adapt to new circumstances. Whether this adaptive potential can be effectively used to purposely generate viruses with desired phenotypes depends both on the mutation rate of the concerning virus and on the nature of the target phenotype. Generally, the higher the mutation rate, the higher the probability is that beneficial mutations can be isolated within reasonable timeframes and in experiments of manageable size.

In this regard, riboviruses (i.e. all RNA viruses excluding the retroviruses) are well-known to exhibit the highest of viral mutation rates. The genomic mutation rates of riboviruses during their replication – by RNA-dependent RNA polymerases – have been estimated to be centered at around 0.76 mutations per replication round (12). Considering the replication mechanism of these viruses (involving two replications per progeny genome) (12,13), this rate would translate to 1.5 mutations per genome per viral generation. Interestingly, with such high mutation rates, riboviruses are thought to exist close to an error-threshold, beyond which viral fitness would become ever lower due to degeneration (13,14). The answer to 'why' riboviruses evolved such high – and burdensome – mutation rates would lie in part in the

associated benefit of being able to rapidly evolve in the face of changing host conditions (13). Thus, the spontaneous mutation rates of riboviruses, but possibly also those of retro- and DNA viruses [which can be several orders of magnitudes lower (15,16)], may be exploitable to evolve viruses towards desired properties.

The concept of viral genetic adaptation under laboratory-controlled conditions has long been illustrated by the common observation that repeated passage of a pathogenic virus in tissue culture can lead to an attenuated phenotype *in vivo*. This makes that tissue culture-adapted strains are natural candidates for live-attenuated vaccines. For example, the repeated passage – at low temperature – of the respiratory syncytial virus (RSV), a ssRNA virus of the paramyxo family, yielded strains that, while remaining immunogenic, exhibited attenuated phenotypes in infants (17). This phenomenon of attenuation by repeated tissue culturing illustrates a very common process in evolution: prolonged subjection to a new set of conditions might result in a loss of ability to live under the old conditions.

However, the opposite of attenuation is also bound to occur: extensive viral growth on a particular cell type will enhance viral fitness on those cells. In this regard, because laboratory virus strains have mostly been propagated on transformed cells, their adaptation to these cells often led to a general enhanced ability to replicate in tumor cells. Below, we discuss several such cases of enhanced, and/or more selective, tumor cell killing by tissue culture-adapted strains. Furthermore, examples are given of how viral adaptability has been exploited – purposely or coincidentally – to achieve other vectorological goals, such as altered protease specificity or receptor usage.

Riboviruses

Many studies illustrate the high adaptive potential of riboviruses. For example, pathogenic riboviruses have been reported to evolve escape mutants during anti-viral therapy (18-21). Furthermore, in laboratory experiments, different riboviruses have been found to readily adapt to new cell types (22,23), new host organisms (23-25), or altered growth conditions (26). In this regard, even under the relatively 'constrained' conditions imposed by alternated passaging on two highly divergent host cell types (i.e. from vertebrate and invertebrate origin), were several different riboviruses – i.e. sindbis virus, vesicular stomatitis virus, and Eastern equine encephalitis virus – able to evolve increased levels of fitness for either of the selective environments (27-29).

Regarding the use of riboviruses as oncolytic agents, there are many reported cases of tissue culture-adapted strains having an enhanced and preferential ability to kill cancer cells. In fact, several of the most promising oncolytic riboviruses are attenuated lab strains or recombinant vectors derived from them (30). Examples of such attenuated oncolytic strains are

73-T (31) and HUJ (32) of Newcastle disease virus, Edmonston-B of measles virus (33,34), and A7(74) of semliki forest virus (35). All these strains were obtained after extensive viral passaging *in vitro* and/or *in vivo*, e.g. in tissue culture cell lines, tumor cells, chicken eggs, or mice.

The Edmonston-B strain of measles virus (MV-Edm), for example, was generated by subjection of a primary measles isolate to multiple passages (82 in total) in human embryonic kidney cells, human amnion cells, chick embryos, and chick embryo fibroblasts. This measles strain was observed to be low pathogenic while remaining immunogenic (36). Its derivatives have therefore successfully been used worldwide as live attenuated vaccines. Additionally, however, MV-Ed was coincidentally found to potently and selectively kill various tumor cell types (30) and, subsequently, to have efficacy as an oncolytic virus in animal models and in a phase I clinical trial (33,34,37). The enhanced oncolytic phenotype displayed by MV-Ed is thought to be due, at least in part, by altered receptor specificity (30). Owing to mutations in the viral attachment glycoprotein, the virus has an enhanced ability to interact with CD46 (38,39), a cancer-associated antigen expressed widely and highly on the cell surface of many tumor cells.

The enhanced oncolytic phenotypes of the above strains were mostly obtained unintentionally, as the result of attenuation processes. More recently however, the high spontaneous mutation rates of riboviruses have been exploited more purposely to realize specific vectorological goals. Examples in this regard are directed evolution experiments – performed with rhinovirus, coxsackievirus, vesicular stomatitis virus, sindbis virus (VSV), tick-borne encephalitis virus, and avian influenza virus – to achieve enhanced growth on certain target cells and/or to achieve altered receptor or protease specificity (22,40-46). All these studies show that with proper selective conditions imposed, riboviruses can be readily forced to evolve towards desired new phenotypes.

An example of how the high adaptability of riboviruses can be exploited for vector engineering is given by a study that performed directed evolution to optimize a sub-optimally functional, transductionally retargeted VSV (40). A recombinant replicating VSV, engineered to express a chimaeric glycoprotein containing a single-chain antibody directed to human Her2/*neu*, exhibited a preferential ability to infect Her2/*neu*-expressing target cells over non-target cells. However, despite this, the virus grew very poorly on its target cells over multiple infection rounds. Therefore, to optimize this retargeted virus, it was serially passaged on the Her2/*neu*-expressing target cells, eventually yielding viruses – after fifteen passages – showing much better growth on these cells. Isolated infectivity-enhanced viruses were found to carry an improved version of the chimaeric glycoprotein (with two mutations in the single-chain antibody segment) that, putatively as a consequence of being more abundantly expressed on the viral surface, was better capable of promoting infection. Therefore, showing that a 'suboptimally retargeted' riboviruses can quickly be

adapted for improved functionality, this study provides an illustration of the power of combining rational and random engineering approaches.

Retroviruses

Retroviruses are RNA viruses that replicate through a host genome-integrated DNA intermediate, i.e. a 'provirus' (47). Per retrovirus infection cycle (from provirus to provirus), the viral genome undergoes three error-prone replication events (15). First, transcription from the provirus is carried out by a host RNA polymerase, generating an RNA viral genome that is packaged into the virus particle (along with a second genome). Then, in a newly infected cell, two replications by the virus-encoded reverse transcriptase convert the incoming RNA genome into double stranded DNA (dsDNA), which becomes integrated into the host genome.

The fidelity of the above replication process has been found to be intermediate to that of riboviruses and DNA viruses (13,15,48). Mutation rates for retroviruses have been estimated at around 0.1 per genome per replication, thus corresponding to 0.3 per genome per round of cell infection (15). This spontaneous mutation level, although being lower than that found for riboviruses, provides the basis for the notoriously high adaptive potential of retroviruses in the face of the immune system or anti-viral drugs (49,50). Additionally, this inherent mutation level has been found to readily facilitate the directed evolution of retroviruses for vector engineering purposes. Indeed, it has become somewhat of a general approach to exploit retroviral adaptability for the improvement of sub-optimal recombinant retroviral vectors (51-55).

Especially regarding *env* gene modifications, has forced evolution proven a useful tool for vector optimization. The *env* gene encodes for the precursor of the polypeptides that constitute Env, the envelope glycoprotein complex that mediates cellular receptor binding and membrane fusion. In general, modification of Env and Env-pseudotyping are powerful ways to endow retroviruses with new functionalities like altered cell tropisms (56,57). However, these targeted genetic interventions of Env are often associated with difficult-to-foresee functional defects that affect for instance viral infectivity or stability. Therefore, evolution-based strategies – in the context of replicating retroviruses – have been used to optimize the functionality of modified Env molecules.

Studies where *env*-modified viruses were subjected to forced evolution include those aiming to reengineer HIV-I Env as an effective vaccine antigen (51,52). In these studies, new Env variants – with impaired ability to mediate infection – were genetically incorporated in the context of a replication competent virus in order to allow for their functional optimization by evolution. This was done for example for variants lacking one or more of the variable loops of Env (51). These deletion mutants have improved immunogenic profiles

but are often, when non-optimized, problematic for production processes because of their relative structural instability. Importantly, forced evolution in the context of HIV-I to restore their functionality successfully yielded variable loop-deletion mutants exhibiting improved characteristics regarding the expression and secretion as stable multi-subunit Env complexes.

Other examples of using forced evolution to optimize *env*-modifed viruses are provided by studies on an avian retrovirus genetically pseudotyped with the amphotropic murine leukemia virus (MLV) Env protein (53,54). This chimaeric virus was optimized as a mammalian cell-gene delivery vehicle by two successive evolution steps. First, it was adapted to restore its ability to replicate on avian cells (this ability had been impaired by the *env* gene swap) (53). Then, to aid the establishment of stable producer cell lines, the virus was further adapted to be less toxic for avian cells (54). Interestingly, these two subsequent adaptations were respectively caused by two subsequent substitutions at a single amino acid position in Env.

DNA viruses

With the exception of several ssDNA viruses, DNA-based viruses generally replicate with much lower spontaneous mutation rates than RNA viruses (13,15,16). For example, herpes simplex virus I (HSV-I), which carries a dsDNA genome of 152 kb, is estimated to have a genomic mutation rate of around 0.0027 per replication or 0.006 per infection cycle (this latter number corresponds to Drake and Hwang's estimation of 1.36×10^{-4} mutations per 1128-bp *tk* sequence per three infection cycles) (16,58). These rates are up to more than two orders of magnitudes lower those of riboviruses and retroviruses (see above). Still, despite their relative genetic stability, viruses from different dsDNA virus families – including the adeno-, herpes-, and poxviridae – have proven amenable, to some extent at least, to evolution-based engineering.

Adenoviruses. For adenovirus (Ad), for instance, it is long known that the genetic diversity obtained during normal virus propagation can be sufficient to isolate heat-stable or host range mutants from virus stocks (59,60). Recently, this was illustrated again in the context of a heat-liable Ad mutant deleted for the Ad core protein V (61). Subjection of a preparation of this virus to a simple incubation procedure (3 days at 37°C) demonstrated the *a priori* presence – within the virus stock – of thermostable viral mutants carrying compensatory mutations (within the precursor of the Ad core protein X/μ). Thus, these second-site mutations had occurred, and were presumably strongly selected for, during normal virus propagation rounds.

Another example demonstrative of Ad's natural adaptability comes from early work from Harald zur Hausen's lab (62,63). Human adenovirus type 12 was serially passaged in two different tumor cells, yielding virus mutants with an enhanced growth potential. Interestingly, the isolated tumor-adapted

viruses all carried rearrangements at their genome termini, with a common feature of the right terminal alterations being a duplication of the inverted terminal repeat.

Finally, a recent special case of exploiting adenoviral mutability is one that employed Ad's spontaneous recombination ability (64). Specifically, an array of Ad serotypes was used in a high MOI-infection to invite recombination among the different, co-infected viruses. The genetically diverse viral pools resulting from this procedure were subsequently adapted to growth on different tumor cell lines. Ultimately this led to the isolation of an oncolytic viral mutant – a complex Ad3/Ad11p chimaera – that showed efficacy in models for colon cancer treatment.

Herpesviruses. Also herpesviruses have shown sufficiently high levels of genetic diversification to purposely isolate interesting new mutants in selection experiments. In this regard, several herpesviruses have been evolved towards envelope glycoprotein D-independent infection (65-67). In case of pseudorabies virus (PrV), glycoprotein D (gD) mediates a secondary cell attachment step that is essential for PrV cell entry. However, PrV-encoded gD is not required for the direct viral cell-to-cell spread of PrV. Therefore, a gD-deleted PrV, which is unable to infect cells by itself, can be propagated through the co-cultivation of infected and non-infected cells. In this regard, Schmidt *et al*. found that the repeated passage of a gD-deleted PrV, by the co-cultivation procedure, eventually led to the evolution of variants that had regained the ability to infect cells (65). These infective gD-deleted mutants carried putative compensatory mutations within the fusion effectors glycoproteins B (gB) and H (gH) (68).

In similar types of adaptation experiments, gD-independent infection has also been achieved for bovine herpesvirus (BHV) (66) and herpes simplex virus (HSV) (67), viruses for which gD normally is essential for viral cell entry as well as direct viral cell-to-cell spread. In case of HSV, the adapted, gD-independent viruses contained a compensatory double mutation in the fusion effector gB (67). Interestingly, the gB mutant was found to be hyperactive, allowing for membrane fusion in the absence of the gD-receptor binding trigger. Interestingly, this hyperactive phenotype was found to be very beneficial in the context of a retargeted HSV; the combination of the gB mutant and a ligand-bearing gD was found to dramatically increase target-specific infection.

Spontaneous mutation by herpesviruses has also been exploited for genetic selection in cancer cells to increase viral antitumor activity. In this regard, several passages of an attenuated, γ34.5 gene-deleted HSV-I variant in neoplastic cells resulted in the isolation of second-site 'suppressor' mutants exhibiting a regained ability to replicate in cancer cells (69). Surprisingly, while these mutants showed a much improved *in vitro* and *in vivo* oncolytic efficacy, they still exhibited the severely attenuated parental phenotype (69,70). The

common suppressor mutation seen in the isolated mutants – a discrete 583 bp deletion – was found to cause a much earlier onset of expression of a viral inhibitor (Us11) of cellular PKR. Thus, suppressor viruses are thought to have overcome the PKR-induced shutoff of protein synthesis that occurs in the absence of the γ34.5 gene product. Similar results have been obtained in another adaptation study using γ34.5 mutant viruses (71). This study extended the above approach by performing the serial passage procedure *in vivo* (in tumor xenografts), and by additionally making use of a cytokine-armed virus (expressing murine interleukin-12 from γ34.5 gene loci) (71).

Poxviruses. Finally, also spontaneous mutation by poxviruses has proven to facilitate adaptation procedures. For example, a recent study showed that several tissue culture adapted strains of vaccinia virus have evolved, relatively to the parental strain, enhanced abilities to replicate in tumor cells (72). This ability is likely due to that these strains were adapted to growth, by continual passage, in transformed cells. One of the strains that proved exceptionally potent and tumor-selective (the WR strain) served in this study as the backbone for the development of a new recombinant oncolytic vaccinia virus. The resulting virus, JX-963, proved to have systemic efficacy against primary carcinomas and metastases in immunocompetent mouse and rabbit models.

MUTAGEN-INDUCED MUTAGENESIS

Physical and chemical mutagens can be used to generate genetically diverse virus populations from which new phenotypes can be selected. Such mutagen-based random mutagenesis and selection procedures have logically mostly been applied to genetically stable DNA viruses – e.g. adenovirus, HSV, vaccinia virus, and polyomavirus – and much less to the faster mutating RNA viruses. Early forward genetics studies used mutagens to facilitate genetic screens for identifying and investigating viral functions. Recently, however, mutagen-induced mutagenesis has also been employed with the purpose of optimizing or generating new oncolytic viral vectors. Among the mutagens that have been used for viral mutagenesis are nitrous acid, hydroxylamine, nitrosoguanidine, ultraviolet radiation, and the nucleotide analog BrdU. Mutagenesis by mutagens is brought about through various different mechanisms, but is always ultimately dependent on cellular or viral functions like replication and DNA repair.

Mutagenesis by nitrous acid, the most-used mutagen to mutate viruses, involves deamination of adenines and cytosines and therewith the formation of respectively hypoxanthine and uracil. This reaction affects the base pairing potential of the concerning nucleosides: hypoxanthine prefers pairing with cytosine and uracil with adenine. Therefore, during viral replication, altered templating properties – of the affected nucleosides – will cause certain base

pair substitutions to occur, namely the transitions A:T to G:C, and C:G to T:A. However, besides deamimation of bases, nitrous acid also causes interstrand cross-links in duplex DNA (73). If left unrepaired, this type of lesion will block viral replication, thus causing virus inactivation. Additionally, if cross-links do get repaired – by the cellular repair mechanism – they are likely to leave behind deletions (73), which are generally much more deleterious than substitutions. Thus, nitrous acid has mutagenic properties – causative of substitutions and deletions – as well as direct inactivating effects (74). In general, it is thought that the greater part of the drop in virus viability seen after treatment with a mutagen is the consequence of such direct inactivating effects rather than of the biological effects of genetic alterations (75).

Theoretical considerations have been made as to how strong a mutagenic pulse should be for a maximized chance to select a desired phenotype (75). Exposure of a virus population to a mutagen will cause a level of virus inactivation (due to both the direct inactivating effects as well as lethal genetic mutations). This level of inactivation – or its reciprocal, the surviving fraction – is a parameter that can be set by the investigator to influence the chance of success in a mutagenesis and selection experiment. In this regard, Bull showed that in the context of a single-pulse mutagenic treatment, the theoretical optimum for the surviving fraction can be calculated by e^{-D}, where D is the phenotype dimensionality, i.e. the number of mutations needed to create the phenotype (75). Thus, if a certain phenotype can be reached by 1 mutation, then a surviving fraction of 37% would give the highest chance of success. Likewise, for selection of a phenotype dependent on 2 mutations, then 14% survival would be best. Interestingly, most studies that performed viral mutagenesis using mutagens mutated to survival levels that are considerably lower than the percentages above.

As said, in classical experimental virology, mutagen-induced mutagenesis was used to facilitate the screening for new phenotypes, especially in case of the relatively genetically stable DNA viruses. Phenotypes that were commonly isolated in genetic screens are those for which appropriate selective conditions could be readily imposed. Such phenotypes included for example temperature sensitivity (76-81) and altered host range (60,82,83). Through recombination mapping and functional analysis (e.g. complementation assays), the selected phenotypic mutants subsequently aided the identification of new genes and functions. For example, temperature-sensitive (84) and host range mutants (83) of polyoma virus, a tumorigenic double stranded DNA virus, have proven useful in the elucidation of mechanism of neoplastic transformation (85,86).

Recently, this forward genetics approach has been revisited to generate viruses with enhanced oncolytic properties (87-91). For example, Fu and Zhang sought to increase the tumor cell killing capacity of HSV G207, a well-studied, but sub-optimally potent oncolytic HSV exhibiting a tumor cell-selective phenotype (as a consequence of an inactivated *ICP6* gene) and a

non-virulent profile (by virtue of the deletion of its γ34.5 loci) (89). HSV G207 was mutagenized by replication of the virus – for one infection round – in the presence of BrdU, a nucleoside analog of thymine that can cause mutations through mistemplating. Subsequently, from the mutagenized virus preparation, mutants were isolated that showed a syncytial phenotype in plaque assays. Characterization of one of the mutants – exhibiting a strong syncytial activity – demonstrated a significantly increased antitumor potency *in vitro* and *in vivo*. Thus this study showed that random mutagenesis can readily facilitate a non-fusogenic HSV to acquire a strong membrane fusion capability.

Investigators have also sought to improve adenovirus as an oncolytic virus (87,88,91). Yan *et al*. mutated HAdV-5, using nitrous acid, and subsequently performed repeated passages on a tumor cell line to enrich for viral mutants with an acquired growth advantage (87). This 'bioselection' procedure yielded mutants displaying an enhanced cytotoxicity in different cancer cell lines but not on a number of primary human cell lines. The observed phenotype was associated with a mutation causing a C-terminal truncation of the i-leader protein, a protein with as yet no known function. Interestingly, another genetic screen (involving UV-induced viral mutagenesis), by Subramanian *et al*., yielded a similar i-leader-truncating mutation (91). This mutation was selected as a second-site suppressor mutation in the context of a small-plaque mutant of Ad (dl327, which lacks most of Ad's E3 region). Thus, both studies implicate a stimulating role of the i-leader protein in viral spread.

Gros *et al*. extended the above oncolytic adenovirus bioselection strategy by performing several *in vivo* selection rounds in human tumor xenografts-bearing mice (88). This approach led to the isolation of an oncolytic mutant showing a large-plaque, enhanced-spread phenotype *in vitro* and enhanced systemic efficacy *in vivo*. Intriguingly, the causative mutation was found to rid the E3/19K protein of its endoplasmic reticulum retention domain, thus relocating it to the plasma membrane. There, at the cell membrane, the mutant protein might exert a viroporin-like function: it might render the cell permeable to ions and destabilize the membrane, thereby promoting the observed enhanced viral release. With the identification of this unanticipated, putative gain-of-function mutation, this study illustrates the relevance and the feasibility of using the *in vivo* tumor environment as a selective pressure for the isolation of enhanced oncolytic viruses.

PHAGE DISPLAY-BASED SELECTION OF VIRUS TARGETING MOIETIES

Virus targeting – to specific receptors, cells, or tissues – is an all-important goal in viral gene therapy (2). Therefore, viral capsid or surface proteins have been

used as sites for the genetic incorporation of various targeting ligands (92,93). Additionally, viruses have been retargeted using bispecific adaptor molecules consisting of targeting ligands conjugated to virus-binding moieties (94-97). However, the natural repertoire of high-affinity, virus incorporable, target-specific ligands is limited. Therefore, random peptide display on phage has been explored as a means to select peptides capable of binding specific cellular targets (98).

Phage peptide display is the expression of a peptide library on the outside of bacteriophage particles (99). The peptides are expressed as fusions of a phage coat protein, e.g. minor coat protein pIII or the major coat protein (pVIII) of M13 filamentous phage. Importantly, each phage virion represents a physical linkage between a peptide variant and its genetic code. This phenotype-genotype connection is what makes phage library-based selection possible: selection for a certain peptide or protein variant will co-select for its encoding sequence (which is then simply recovered and identified by PCR and sequencing). The power of the phage display technology is that very large and complex libraries are readily made, allowing for instance virtually all possible variants of a 7-mer peptide to be represented in a single library (100).

Phage display-based selection to obtain new virus targeting motifs can be performed both *in vitro* and *in vivo*. *In vitro*, selection has been performed to isolate peptides binding specific cell types as for example ciliated airway epithelial cells, vascular endothelial cells, and vascular smooth muscle cells (96,101-103). *In vivo* biopanning procedures selected for peptides that home to certain tissues, like organ-specific vasculatures (104-107), renal tubular epithelium and glomeruli (108), and tumor xenografts (109,110). After such selection procedures, the individual targeting peptides are genetically incorporated into predefined sites – known to be able to accommodate heterologous peptides – of for example the adenovirus fiber protein or the AAV capsid protein. These sites are respectively the HI loop and C-terminus of the Ad fiber (111-113), and amino acid 587 of AAV capsid (92). Additionally, isolated targeting ligands have been chemically coupled to Ad (101), or genetically fused to an adenovirus specific binding moiety such as antibody fragments (94,96) or the extracellular domain of the coxsackie and adenovirus receptor (95).

Several studies successfully retargeted AAV and Ad by genetic incorporation of *in vivo* selected targeting peptides (106-108,114). Grifman *et al*. showed that AAV capsid-incorporation of a previously isolated tumor-targeting peptide, NGR (109), altered the tropism of the recombinant AAV vector, leading to preferential transduction of cells expressing the target of NGR, CD13 (114). Further, the group of Baker performed *in vivo* phage selection in rats to obtain peptides that home to the lung or brain (107). Incorporation of some of these peptides into the the AAV-2 capsid resulted in both independence from the natural receptor, heparan, and relative redirection of *in vivo* infection to the

vascular beds of the concerning targeted organs. Finally, two studies of the same lab incorporated *in vivo* selected targeting peptides into Ad (106,108). Cardiac and kidney vasculature-homing peptides were inserted into the HI loop of Ad19p serotype fiber in the context of an Ad5-based vector. The resulting vectors showed selective transduction of certain vascular cells of the respective targeted organs.

An important issue of these strategies is whether the isolated targeting peptide will retain its cell-binding or tissue-homing functionality in the context of the virus particle. A targeting peptide selected in the context of a phage virion – and displayed as either a linear or cyclic epitopes – might lose its functional configuration when inserted in the virus capsid protein. Moreover, the functionality of the peptide might differ per location in the capsid protein or per exact insertional configuration. Also, certain peptide insertions may compromise virion integrity. Therefore, to avoid selecting peptides that could be non-functional out of the bacteriophage context, attempts have been made to select peptides immediately in the right context. The most rigorous approach is to perform selection in the complete viral context, thus to perform virus library-based selection. This approach has been well established for AAV and to a much lesser extent for Ad, as discussed in the next sections. However, a less rigorous but potentially useful approach is to perform context-specific phage display: the peptide library is displayed on phage in the context of the viral capsid protein or a portion of it.

For Ad retargeting, a number of context-specific phage display systems have been developed. The 'contexts' that were displayed on phage in these systems were either the complete fiber knob domain (115,116) or only the two β-sheets (H and I) that flank the HI loop (117). Biopanning experiments using these context-specific systems identified peptides that, upon their incorporation in the complete virus, enhanced Ad's ability to transduce the target cells (mouse fibroblasts and skeletal muscle cells) (116,117). Of note, an advantage of the approach displaying only the H and I sheets of fiber knob, as opposed to approach using the intact fiber knob, is that larger libraries can be generated.

Besides for peptide affinity biopanning, phage display has also been used for the engineering of protein domain-based affinity ligands (118-121). In these cases, instead of a peptide library, a small protein domain with randomized protein binding site is displayed on phage. The advantage of using small protein domain scaffolds instead of peptides for obtaining affinity ligands is that target binding can occur in the context of a stable secondary structure, which potentially allows for better and more selective binding of targets. One type of affinity ligand that has been engineered in this way is the 'affibody' (121). Affibody libraries are made by randomization of a limited number of solvent-accessible surface residues of a staphylococcal protein A-derived alpha-helical bacterial receptor domain Z. Because of their relatively simple and stable structure that does not rely on disulfide bridges,

affibodies have potential as virus targeting ligands. This is especially the case for non-enveloped viruses, like adenovirus, for which genetic capsid incorporation of more complex, disulfide bridge-containing secretory ligands (e.g. antibody-derived ligands) has proven problematic (122-124). In this regard, affibodies directed against the tumor-associated antigen HER2/*neu* have been successfully incorporated into the adenovirus capsid with retention of target binding functionality (125-127).

VIRAL LIBRARY-BASED SELECTION

Owing to the availability of modern molecular cloning and diversification techniques and efficient viral library generation methods, various powerful viral library-based engineering strategies have been developed (128,129). These strategies have proven extremely useful to obtain viruses with desired traits, especially in the fields of retrovirus and AAV vectorology. Although diversification techniques and combinatorial selection strategies are evolving, these new random engineering approaches generally entail one or more rounds of the following steps: (a) *in vitro* diversification of a bacterially cloned viral gene or genome, (b) conversion of the resultant plasmid-based library into a viral library, and (c) viral library-based phenotypic selection. *In vitro* methods that have been used in the diversification step are transposon-mediated mutagenesis, cloning of degenerated oligonucleotides, error-prone PCR, and *in vitro* recombination. After the diversification step, the diversified cloned fragment or genome must be efficiently packaged (or 'rescued') into virus particles to create a viral library. Importantly, for each viral particle within this library the genotype and the phenotype must correspond. Then, after phenotypical selection from the viral library (e.g. for functionality, target binding, target cell entry, temperature stability, neutralizaing antibody avoidance), the selected genotype can be recovered by PCR and be analyzed. Viral library-based selection strategies have been best established and proven most fruitful for AAV and retroviruses, viruses for which viral genome handling and/or viral library generation is relatively efficient compared to viruses like adenovirus and herpesvirus.

Random insertional mutagenesis

The concept of random insertional mutagenesis is the insertion of a known sequence at random positions into the viral genome or genome fragment. This can be achieved by *in vitro* transposition (in bacteria) of a transposable element into plasmids (or bacterial artificial chromosomes) carrying the viral sequences. This technique, originally used to identify essential and non-essential genes in herpesvirus (130), has emerged as a general means to study viral functions. More recently, however, it has additionally become a

useful tool for vector engineering purposes, e.g. to identify new sites in viral genomes that can tolerate transgene or targeting peptide insertions.

Different studies used transposon-based scanning to find permissive insertion sites within the envelope proteins of MLV and VSV. For example, the bacteriophage Mu transposon system was employed by Rothenberg *et al*. to construct a library of 15-nucleotide insertion mutations within the *env* gene of MLV (131). Selection for virus viability subsequently resulted in identification of multiple sites within the Env glycoprotein that are permissive for five-amino-acid insertions. Likewise, Yu and Schaffer used a similar retrovirus-based strategy to scan the vesicular stomatitis virus glycoprotein (VSV-G) for sites that tolerate a six-histidine (His_6) tag-containing 13-amino acid sequence (172). Repeated selection for retroviral replication ability and affinity for Ni-nitrilotriacetic (Ni-NTA) led to the identification of His_6-tag insertion mutants of VSV-G that facilitated virus purification using Ni-NTA columns. Other vectorological goals achieved for retroviruses by random insertional mutagenesis include the isolation of MLV variants capable of infecting non-dividing cells (132) and the selection of MLV mutants with altered host genome integration properties (133). These results were accomplished through the generation of respectively a nuclear localization signal (NLS) insertion library and a DNA-binding domain insertion library.

Random insertional mutagenesis has also proven useful for the generation of new adenoviral vectors. Two recent studies from Hermiston's lab showed that a modified transposon Tn7-based system could be efficiently used to scan the Ad genome for identification of insertion sites compatible with viral replication. The first study identified several unique sites for the insertion of (promoter-based) transgene expression cassettes (134). The second study performed Ad genome scanning using a splice acceptor-containing reporter gene expression cassette (135). This study identified sites allowing Ad major late promoter-driven expression of inserted transgenes. Together these studies provided new rationales for the development of new oncolytic Ad vectors armed with therapeutic genes.

Random peptide display / targeted randomization

Targeted randomization in conjunction with viral library-based selection has emerged as an important and fruitful way of viral vector engineering. A major application of this approach is 'viral peptide display', which entails the display and selection of randomized polypeptides in the context of viral capsid or envelope proteins (136). This application, which is conceptually analogous to phage display, allows for example for the selection of peptides capable of mediating cell receptor-specific or cell protease-dependent viral cell entry. Importantly, the viral display procedure ensures that polypeptides are

selected directly for functionality in the relevant viral context, which is a major advantage over peptide selection in the heterologous context of phage.

Protease-dependent retroviral cell entry via protease activatable Env. The first study demonstrating the feasibility of random peptide display on a virus was one that sought to achieve protease-dependent replication of a retroviral vector (136). A randomized seven-residue linker was inserted between a heterologous receptor-binding ligand and MLV Env. The concerning ligand, EGF, is known to have an inhibitory effect on cell entry because it directs EGF receptor (EGFR)-bound MLV to lysosome-mediated degradation (137). Screening of the viral library for the ability to transduce EGFR-overexpressing cells selected peptide linkers that served as substrates for furin-like proteases. Thus, this study provided proof-of-principle that viral peptide display strategies can be employed to render the MLV cell entry mechanism dependent on proteases.

However, despite the fact that the above selection strategy involved serial passage on a tumor cell line, the selected peptide linker sequences were substrates for ubiquitously expressed proteases, not cancer-associated proteases (136). Therefore, attempting to achieve cancer selectivity, a subsequent study adapted the strategy by inserting instead of a completely random sequence, a matrix metalloprotease-2 (MMP-2) substrate motif semi-randomized such to avoid the possibility for furin-sensitive substrate (138). Two cycles of diversification and screening on human fibrosarcoma cells led to substrate sequences that were specifically cleaved by a tumor-associated MMP-2. Thus, MLVs carrying the concerning Env-EGF fusions transduced EGFR-overexpressing cells in an MMP-2-dependent manner. A subsequent strategy sought to achieve a mechanism of MMP-2-dependency of MLV transduction that did not require high levels of EGFR on the cell surface (139). This was done by using instead of EGF, another cell entry-blocking moiety, CD40L, which acts in a general way, not requiring cognate receptor binding on the cell surface. Viral peptide library screening using the new chimaeric Env configuration successfully yielded virus whose replication is restricted to MMP-positive cells.

Selection of targeting peptides in context of retroviral Env. Random peptide display on retroviruses has also been used to select targeting peptides in the context of the Env glycoprotein. Bupp and Roth explored the receptor specificity-determining variable region A (VRA) of feline leukemia virus (FeLV) as a locale for targeted randomization (140). In FeLV, the VRA of Env is the sole major determinant of cell surface receptor specificity; exchange of a defined VRA segment between different FeLV subgroups altered the viral host range (141). Viral libraries were made by randomizing a 10- or 11-amino acid stretch within the VRA of FeLV Env (140,142,143). Screening of these libraries for the ability to infect certain target cell lines yielded host-range mutants with altered receptor usage abilities. Importantly, one isolated Env protein proved

to mediate cell entry via a receptor protein not known to be used by any other retrovirus (143,144). Further, another isolated Env protein uses a receptor known to be utilized by an endogenous retrovirus from a distant species (145). Of note, this latter targeted receptor, a G-protein-coupled receptor that functions as the human gamma-hydroxybutyrate (GHB) receptor, is up-regulated in different human malignancies and therefore may be a useful anti-cancer target. Although the above studies clearly demonstrate the power of this method to target retroviruses to alternative receptors, they also implicate a limitation regarding the repertoire of targetable cell surface receptors. In this regard, an efficient retroviral receptor appears to be required to be a multipass transmembrane receptor capable of providing a specific facilitatory role (e.g. perhaps regarding membrane fusion or intracellular trafficking) (145).

Selection of targeting peptides in context of the AAV capsid. After random peptide display on viruses was first demonstrated for retroviruses, the principle was soon also applied with much success to AAV vectors (146-148). Amino acid position 587 of the AAV-2 capsid is located within a motif required for viral binding to heparan sulfate proteoglycan (149-151), the primary receptor of AAV-2 (152). This particular position in the AAV capsid, which had already been found to tolerate the insertion of targeting peptides (92), was successfully used by different groups as a locale for the display of random peptide libraries (146,147). Perabo *et al*. and Muller *et al*. independently generated 7-amino-acid peptide display libraries and performed multiple-round selections for the ability to infect non-permissive target cells. Importantly, selected mutants showed enhanced infection of the respective target cell lines (i.e. a human megakaryocytic and a B-cell chronic lymphocytic leukemia cell line, and human coronary artery endothelial cells).

Several studies expanded the utility of the AAV capsid display approach by performing *in vivo* biopanning. Grimm *et al*. displayed a peptide library in the context of a chimaeric AAV (which itself was obtained by DNA family shuffling and phenotypical selection as discussed below), and subsequently subjected the resultant viral library to a selection regime involving serial passaging in mouse lungs (153). The procedure led to retargeting of the chimaeric AAV to distinct alveolar cells. Another *in vivo* biopanning study (using an AAV-displayed peptide library) conducted multiple rounds of intravenous injection and subsequent viral recovery from mammary tumor tissue (154). Recombinant vectors displaying the peptides selected by this procedure were found to mediate significantly enhanced reporter gene expression in the target tissue. However, increased reporter expression was also observed in other tissues, especially that of the heart. Thus, the procedure led to a general expansion of AAV tropism despite the putative ablation of heparin binding ability. Finally, Ying *et al*. performed *in vivo* screening of an AAV-2 display peptide library to select vectors specifically homing to heart tissue after systemic delivery (155).

Indeed, selected viral clones showed better specificity of gene transfer to the heart than control vectors.

Selection of targeting peptides in context of the adenovirus fiber. Viable strategies for high-throughput random peptide display in the context of Ad particles have only recently begun to emerge (156-159). A general bottleneck for the development of such strategies for Ad has been that plasmid-based genomes are very inefficiently converted – upon transfection in cells – into packaged Ad virions. Furthermore, Ad's relatively large genome size, in comparison to AAV and retrovirus, poses an additional efficiency bottleneck for Ad library generation. Two recent studies took very similar approaches to bypass these issues (156,157). Both studies exploited the 'cre-lox' site-specific recombination system to achieve efficient transfer of a plasmid-based Ad fiber-peptide library directly into 'live', replicating Ad vectors. Benefits of this strategy are that initial fiber-peptide library generation and handling takes place on a small shuttle plasmid and, more importantly, that subsequent transition to a viral library does not entail virus 'rescue' from a plasmid-derived genome. Both methodologies used the HI loop of the Ad fiber knob domain as the site for peptide library insertion. Lupold *et al*. subsequently screened their CAR-binding ablated Ad peptide display library for replication ability on the standard Ad propagation cell line 293 (which expresses CAR) (156). This led to the isolation of viruses exhibiting enhanced, CAR-independent infectivity on different cell lines. Similarly, Miura *et al*. screened their library on a glioma cell line, leading to the selection of variants showing enhanced gene transduction on the target cell line but not on many other cell lines (157). Thus, in parallel, these studies established a general methodology for relatively high-throughput selection of new Ad targeting peptides.

The potential of random peptide display on Ad in the context of the fiber knob was soon further exemplified by the same two groups (158,159). Nishimoto *et al*. performed screening on a human pancreatic cancer cell line, and subsequently inserted the selected targeting peptide in a replication-competent, CAR-binding ablated (ΔCAR) Ad (158). Compared to the parental ΔCAR vector, the resulting retargeted vector showed enhanced cytolytic potency on the original target cell line as well as several other pancreatic cancer cell lines. Moreover, the new oncolytic vector showed enhanced efficacy in subcutaneous tumors after intratumoral injection without displaying increased ectopic liver gene transduction. Further, in a more recent study, Wu *et al*. significantly expanded the utility of their random peptide Ad display methodology by employing it to optimize the positioning of a fiber knob-incorporated Ad targeting ligand (159). The concerning ligand, a phage display-derived peptide with affinity for the prostate specific membrane antigen (PSMA), was incorporated in the HI loop of the Ad fiber protein and was flanked by random peptide sequences. The resulting

viral library was then screened against PSMA-expressing cell and tumors, leading to the selection of viruses displaying preferential infection of PSMA-expressing cells via the incorporated targeting peptide. Additionally, these viruses infected human prostate adenocarcinoma cell-derived tumors after intravenous administration. The unique engineering approach exemplified in this study may be highly valuable for Ad retargeting purposes: it represents a general way to improve the suboptimal functionality seen often for Ad capsid-incorporated heterologous ligands. Thus, by using this approach, Ad targeting might better benefit from the repertoire of ligands generated by phage-display-based engineering [e.g. simple targeting peptides but also more complex polypeptides like affibodies (121) or designed ankyrin repeat proteins (118)].

Viral genome segment diversification

Another concept of viral library-based engineering is that entailing *in vitro* diversification of a viral genome segment, for instance a viral gene or possibly the whole viral genome. Particularly powerful diversification methods that are used in this regard are error-prone PCR (5,6) and *in vitro* recombination (7-11). These techniques, which are widely used for protein engineering purposes, have recently been introduced into the realm of viral library-based vector engineering. While error-prone PCR introduces random (point) mutations within an amplified (viral) segment, *in vitro* recombination generates genetic diversity through homology-based recombination between homologous but distinct (viral) segments. These diversification methods, which can be used together or in combination, are especially useful in the context of multiple-round genetic diversification and selection schemes.

Retroviruses. *In vitro* recombination for viral library generation was first performed in the context of retroviruses (160,161). Soong *et al*. shuffled the Env sequences from six different MLV strains, which led to a diverse library of Env recombinants (161). This library was subsequently subjected to a selection procedure that selects for Chinese Hamster Ovary (CHOK1) cell infectivity. The procedure involved passaging the library on CHOK1 cells, which are non-permissive for the parental MLV strains, in the presence of a minority population of a cell line that is permissive for some of the parental strains. This setup allowed for the enrichment of a chimaeric viral clone with a tropism for CHOK1 cells. A subsequent study by the same group also reported screening using an *env* region-shuffled library of six MLV strains (160). This time, the library was subjected to a selection scheme involving several repetitions of the 'harsh' concentration step of the normal virus manufacturing procedure. This led to the isolation of complex chimaeras that were physically more stable than the parental viruses.

Another example of *in vitro* recombination being applied to diversify retroviral sequences comes from a study that shuffled the *gag-pol* regions from several different HIV-I isolates (162). The diversified region was cloned in an HIV-I backbone (containing SIV-encoded *nef*) and the resulting library was subsequently successfully used for evolution of an HIV-I variant with enhanced ability to replicate in certain cells of the pig-tailed macaque. Unlike any of the parental HIV-I strains, the recombinant viruses that emerged were able to continuously replicate on the target cells. Interestingly, the genomes of the enhanced viruses, besides being modified for the gag-pol region, also carried putatively adaptive mutations outside the shuffled regions.

AAV. Within the last few years, PCR-based mutagenesis and *in vitro* recombination techniques have been extensively explored for AAV vector engineering. In 2006, Maheshri *et al*. developed a platform for directed evolution of AAV vectors based on these two methods (163). Mutant AAV-2 *cap* libraries with random point mutations were generated by performing error-prone PCR followed by *in vitro* recombination. The recombination step in this procedure is important for effective sequence space sampling: it lessens the chance that any beneficial mutations remain linked to any deleterious ones. Different selection processes were subsequently applied to mutant capsid libraries, leading to the isolation of mutants with altered affinities for heparin or with an ability to evade antibody neutralization. In the latter case, after the first selection phase (involving three infection rounds in the presence of escalating concentrations of neutralizing serum), the enriched virus population was used for a second round of mutagenesis and selection. These operations yielded numerous successful viral mutants, one of which showed a 96-fold improvement in gene delivery in the presence of the antiserum. Contemporary to this study, Perabo *et al*. also performed AAV library-based selection for evasion of neutralizing antibodies (164). In this case, *env* was diversified by one round of error-prone PCR, and selection against serum samples led to the isolation of mutants with up to 5.5-fold increased resistance to neutralization.

Instead of using error-prone PCR as a source of genetic diversity, several subsequent studies employed 'family DNA shuffling' to tap into the natural repository of AAV diversity (153,165,166). Such studies generated AAV *cap* gene libraries based on up to 9 different AAV serotypes. More recently, family DNA shuffling of AAV *cap* genes has been combined for the first time with PCR-based mutagenesis (167). In this case, a multiple-round mutagenesis and selection procedure on an organotypic human airway model yielded AAV vectors exhibiting highly efficient gene delivery to pulmonary cells. Other recent studies successfully used shuffled AAV cap libraries in similar directed evolution protocols to increase AAV transduction into human ciliated airway epithelium or glial cells (168,169). Finally, chimeric AAV cap libraries have also

been subjected to *in vivo* biopanning procedures. In the first study to report this, a library was successfully screened for muscle-homing AAV after tail vain injection of mice (170). Another study employing *in vivo* biopanning reported the selection of chimeric AAVs capable of crossing the seizure-compromised blood-brain barrier (171).

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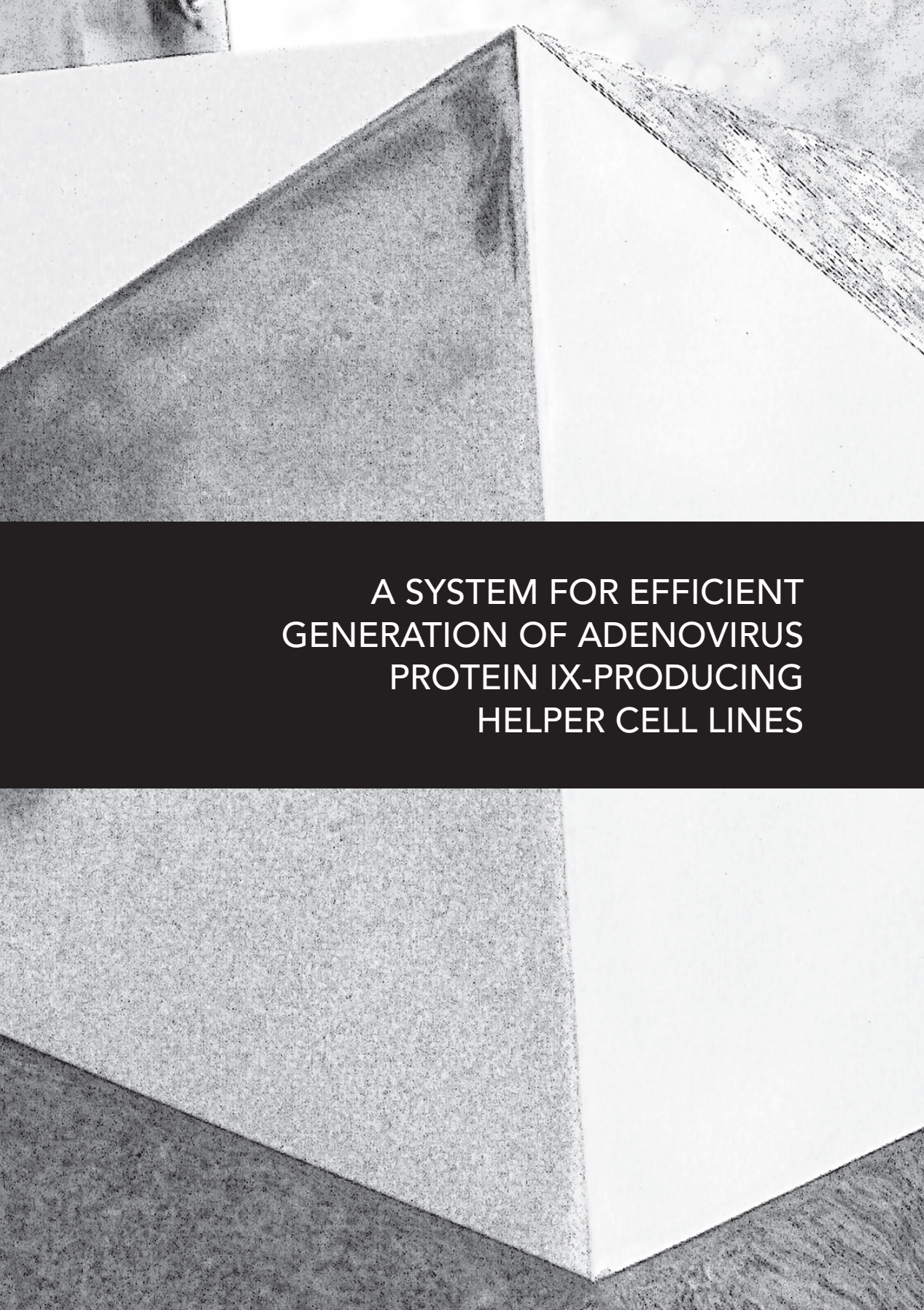
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2



A SYSTEM FOR EFFICIENT GENERATION OF ADENOVIRUS PROTEIN IX-PRODUCING HELPER CELL LINES

ABSTRACT

Background: The adenovirus 14.3 kDa hexon-associated protein IX (pIX) functions in the viral capsid as 'cement' and assembles the hexons in stable groups-of-nine (GONs). Although viruses lacking pIX do not form GONs, and are less heat-stable than wild-type (wt) viruses, they can be propagated with the same kinetics and yields as the wt viruses. To facilitate 'pseudotyping' of adenoviral vectors we have set up an efficient system for the generation of pIX-producing helper cell lines.

Methods: With a lentiviral pIX-expression cassette, monoclonal and polyclonal helper cell lines were generated, which express wt or modified pIX genes at levels equivalent to wt HAdV-5 infected cells. The incorporation efficiency into pIX gene deleted viruses was examined by Western analysis, immuno-affinity electron microscopy, and heat-stability assays.

Results: Immuno-affinity electron microscopy on viruses lacking the pIX gene demonstrated that more than 96% of the particles contain pIX protein in their capsids after propagation on the pIX-expressing helper cell lines. In addition, the pIX level in the helper cells was sufficient to generate heat-stable particles. Finally, the ratio between pIX and fiber was equivalent to that found in wt particles. The pIX-producing cell lines are very stable, demonstrating that pIX is not toxic to cells.

Conclusion: These data demonstrate that lentivirus vectors can be used for the establishment of pIX-complementing helper cell lines.

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Journal of Gene Medicine 2006;8(2):147-54

INTRODUCTION

The human adenoviruses (HAdVs) have been studied extensively as gene transfer vehicles for experimental gene therapy. The modification of the vector tropism to enhance the efficiency and cell-type specificity of gene transfer is an important topic in the adenovirus (Ad)-derived vector design. Successful transgene delivery with HAdV vectors is dependent on efficient transduction of target cells, with minimal transduction of non-target cells (1). One approach to enhance the efficiency and specificity relies on the coupling of targeting devices to the viruses after their production and purification. Such targeting devices could be single-chain antibodies, synthetic ligands, synthetic soluble receptors, etc. (2). Alternatively, the tropism of HAdV receptors can be changed by genetic modification of capsid components. Modification of the major capsid proteins II (hexon) (3–5), III (penton base) (6), and IV (fiber) (7–9) has been exploited for insertion of targeting ligands. More recently, the use of the minor capsid protein IX (pIX) of human and bovine Ad as an anchor for linking targeting ligands has been evaluated (10–13). Protein IX is a 14.3 kDa protein, of which 240 copies are present in the capsid. It arranges the hexons in stable assemblies consisting of groups-of-nine (GONs) (14,15). HAdVs deficient for pIX can be propagated with yields similar to wild-type (wt) Ads; however, such particles are heat-labile and rapidly inactivated at 48 °C (16). Image analyses have positioned pIX at a buried position, approximately 65 Ångstroms lower than the tops of the hexons (17). *In vitro* transduction data and immuno-affinity electron microscopy techniques have demonstrated that the ligand accessibility is augmented by the insertion of an alpha-helical spacer between the carboxyl terminus of pIX and the targeting ligand. The lengths of the inserted spacers positively correlate with the accessibility of the ligands at the outer surface of the viral capsid (18). These data make pIX a promising candidate for the addition of foreign ligands (2).

The modified pIX gene can be inserted into the vector backbone. Alternatively, helper cell lines can be used to provide the pIX *in trans*. The latter approach would lead to particles that do not carry the genetic information for the tropism-modifying capsid modification. Previous data showed that expression of the pIX gene had an adverse effect on cell viability (19). Nevertheless, monoclonal pIX-producing cell lines could be isolated that could restore the pIX deficiency (19–21).

Here we describe a new efficient and robust technique for the generation of stable pIX-producing helper cell lines without the need for clonal selection. The resulting cell populations could effectively complement the pIX deficiency and, as a result, the heat stability of the pIX-gene deleted viruses was fully restored. The availability of a technique for the efficient generation of pIX trans-complementing helper cells will facilitate the use of 'pseudotyping', as opposed to genetic retargeting, as a strategy for modifying the tropism of replication-deficient HAdV vectors.

MATERIALS AND METHODS

Cells

The HAdV-5 E1-transformed cell lines 293 (22), PER.C6 (23), and 911 (23,24) were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS; Gibco-BRL) and 0.3% glucose (J.T. Baker, Deventer, The Netherlands). For PER.C6 cells this medium was supplemented with 10 mM $MgCl_2$. The 911 cells were used to propagate and titer adenovirus vectors. Infections of the cells with HAdVs were carried out in infection medium containing 2% FBS. 293T cells were transfected with the calcium phosphate co-precipitation technique (25). 911 cells were transfected with the calcium phosphate co-precipitation technique using pEGFPc1 (Clontech, BD Biosciences, The Netherlands).

Production of recombinant lentiviruses

The lentiviral vectors used in this study are so-called SIN vectors (26), and contain the Rev-responsive element sequence (27), the central polypurine tract (cPPT) (28–30), and the human hepatitis B virus-derived post-transcriptional regulatory element. All lentivirus vectors were derived from the plasmid pRRL-cPPT-CMVeGFP- PREsense-SIN (31), here named pLV-CMV-eGFP. Plasmids pLV-CMV-IRES-eGFP, pLV-CMV-pIX-IRES-eGFP, pLV-CMV-pIX-IRES-NPTII, pLV-CMV-pIX.MYC-IRESNPTII, and pLV-CMV-pIX.flag.75.MYC-IRES-NPTII were constructed by standard cloning procedures. The encephalomyocarditis virus internal ribosomal entry site (IRES) was obtained from pTM3 (32) and the NPTII-coding region was isolated from pEGFP-N2 (Clontech, BD Biosciences, The Netherlands). The genes for pIX, pIX.MYC, and pIX.flag.75.MYC were obtained from the pCDNA3.1-based constructs pAd5pIX, pAd5pIX.MYC, and pAd5pIX.MYC.flag.75.MYC, respectively (18). A schematic outline of these vectors is provided in Figure 1.

The lentiviral vectors were produced as described previously (33). Briefly, the three 'helper' plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope were co-transfected together with the pLV construct into 293T cells. After overnight incubation, the cells were washed and cultured in fresh medium. The medium was harvested 48 and 72 h post-transfection, passed through a 0.45-μm filter, and stored at −80 °C until use. The viruses were quantified by antigen-capture enzyme-linked immunosorbent assay (ELISA) measuring HIV p24 amounts (ZeptoMetrix Corp., New York, NY, USA) as described previously (34). Approximately 1 ng p24 is equal to 2×10^3 transducing units (30).

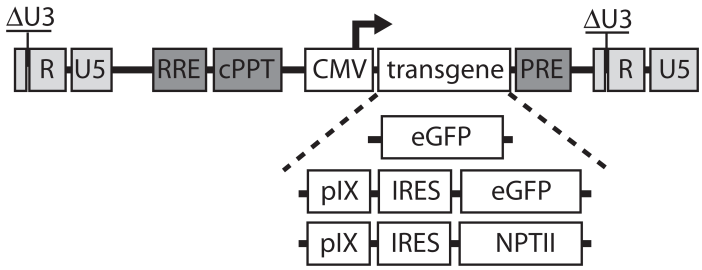


Figure 1. Schematic representation of the lentiviral vectors. The vectors are derived from a self-inactivating, third-generation HIV-1-derived vector. Upon integration, the vector loses its capacity to produce RNA other than the mRNA from the transgene-expression cassette. The positions of the Rev-responsive element (RRE), the central polypurine tract (cPPT), and the posttranscriptional regulatory element (PRE) are indicated. The pIX transgenes were inserted upstream of the internal ribosome entry site (IRES) and the neomycin phosphotransferase (NPTII) selection marker or the enhanced green fluorescent protein (eGFP).

Lentiviral transduction

For transduction the lentiviral supernatant was added to fresh medium supplemented with 8 µg/ml Polybrene (Sigma Aldrich, Zwijndrecht, The Netherlands). After overnight incubation the medium was replaced with fresh medium. Cells transduced with lentiviral vectors containing the neomycin selection gene were cultured in medium supplemented with 200 µg/ml G418 (Invitrogen, Breda, The Netherlands).

Adenovirus vectors

The pTrackCMV-GFP/LUC is an E1-deleted HAdV-5- based shuttle plasmid that carries a green fluorescent protein (GFP) and a firefly luciferase (LUC) transgene, each under the control of the human cytomegalovirus (CMV) immediate-early promoter (35). The ΔpIX pTrack CMV.GFP/LUC plasmid was constructed as described previously for ΔpIX pTrackCMV-GFP (18). Replication-incompetent HAdV-5 vectors HAdV-5CMV.GFP/LUC and HAdV-5ΔpIX.CMV.GFP/LUC were generated with the pAdEasy-1 system as described elsewhere (35). Wild-type (wt) HAdV-5 and HAdV-5-*dl*313 were obtained from the virus collection of the Department of Molecular Cell Biology of the Leiden University Medical Center. The HAdV-5-*dl313* virus lacks 2307 bp of the E1 region including the 5′ portion of the pIX gene. This mutant does not express detectable amounts of pIX (16). The infectious titer of the adenovirus vectors was determined by plaque assay (24). For heat-stability assays, 150-µl aliquots were incubated at 45 °C for 0, 4, 6, 8, and 10 min and then quickly cooled on ice for 5 min. U2OS cells were infected with 100 µl virus suspension from each aliquot. After 24 h viral titers were determined by measuring the luciferase activity, as described previously (36).

Western analysis

Cell lysates were made in RIPA lysis buffer (50 mMTris.Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC and 1% NP40). Protein concentrations were measured with the BCA protein assay (Pierce, Perbio Science B.V., Etten-Leur, The Netherlands). Virus lysates were prepared by adding 5×10^9 virus particles [measured by a standard OD260 protocol (37)] directly to Western sample buffer. The Western blotting and detection procedures have been previously described (18). For detection of fiber the 4D2 antibody was used (1 : 2000; Abcam, Cambridge, UK).

FACS analysis

For fluorescence-activated cell sorting (FACS) analyses the cells were trypsinized and suspended in phosphate-buffered saline supplemented with 1% FBS, and subsequently kept on ice. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson), as described (18). Data analysis was performed using CellQuest 3.1 software (Becton Dickinson). For each sample, 10 000 events were collected.

Immuno-affinity electron microscopy

The presence of the modified pIX molecules in the viral capsids was visualized with antisera and gold-labeled protein-A, as described (18). Subsequently, these samples were fixed in 1.5% glutaraldehyde in cacodylate buffer and negatively stained with 1% uranyl acetate for 15 min. The viruses were examined with a Philips EM 410-LS transmission electron microscope at 80 kV.

RESULTS

It has been shown previously that pIX is an attractive candidate for use as anchor for polypeptide ligands (10–12,18,38–40). Since pIX is dispensable in the capsid, generation of helper cell lines that synthesize pIX may allow 'pseudotyping' of HAdV vectors. Therefore, we designed and evaluated a lentivirus-based system for the generation of new pIX-complementing cell lines. To test the efficiency of a lentivirus-based system for generating E1- and pIX-complementing helper cell lines, 911 cells were transduced with LV-CMV-eGFP at 40 ng p24 per 10^5 cells. After 48 h the transduced cells were harvested and the number of eGFP-positive cells was determined by FACS analysis (Figure 2). The 911 cell line was efficiently transduced. Similar results were obtained when using the E1-complementing helper cell lines 293 and PER.C6 (data not shown). Note that the expression of the transgene is generally higher and more homogenous than in cells transfected with the calcium phosphate co-precipitation technique (Figure 2).

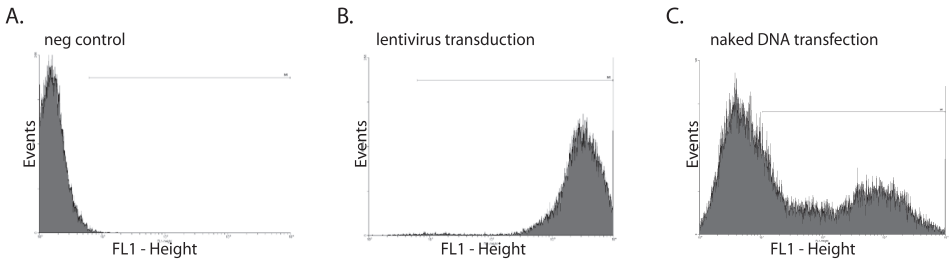


Figure 2. Transduction-efficiency assay. GFP-positive cells were visualized by FACS analysis. (A) Negative control. (B) The helper cell line 911 transduced with LV-CMV-eGFP at 40 ng p24 per 10^5 cells. (C) 911 cells transfected with the calcium phosphate co-precipitation technique using 10 µg pEGFP-C1 per semi-confluent 9-cm dish.

To examine the pIX production after lentivirus-mediated pIX-gene transfer, 911 cells were exposed to LV-CMV-pIX-IRES-NPTII at 40 ng p24 per 10^5 cells, and nine monoclonal G418-resistant cell lines were isolated after limiting dilution. Similar pIX amounts are detected in all 911-pIX cell lines (Figure 3A), which is consistent with the homogenous pIX signals observed by immunohistochemistry (data not shown). Therefore, in all subsequent experiments, polyclonal cultures of G418-resistant lentivirus-transduced cells were used.

Next, pIX amounts were assessed during wt HAdV-5 replication in 911 cells. For this, 911 cells were infected with wt HAdV-5 at multiplicity of infection (MOI) of 5. HAdV-5-*dl313*, which lacks a functional pIX gene, was used as a control. At various time points the cells were collected in lysis buffer and the pIX amounts were determined by Western analysis (Figure 3B). The pIX amount peeked at 32 h post-infection. The peak amounts of pIX after HAdV-5 infection were compared with the amounts of pIX in LV-CMV-pIX-IRES-NPTII-transduced polyclonal cell populations established after transduction of 911 cells with LV-CMV-pIX-IRES-NPTII at 40 ng (N1) and 200 ng (N5) p24 per 10^5 cells. After 7 days in medium containing 200 µg/ml G418, cells were harvested and protein samples were analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS-PAGE). Equivalent amounts of pIX were detected in the LV-CMVpIX- IRES-NPTII-transduced cells and in HAdV-5-infected cells at 32 h post-infection. In the 911-pIX.N5 cells more pIX was present than in 911-pIX.N1 cells (Figure 3C). This probably reflects a higher copy number of integrated pIX vector in the 911-pIX.N5 cells. We noted such a strict copy-number dependency of reporter gene expression previously in differentiated adipocytes and 293T cells (33).

To test the stability of these cell lines, the cells were passaged for 5 months on G418 selection. Only a moderate reduction in pIX amounts occurred over the course of this period, demonstrating that these cell lines are reasonably

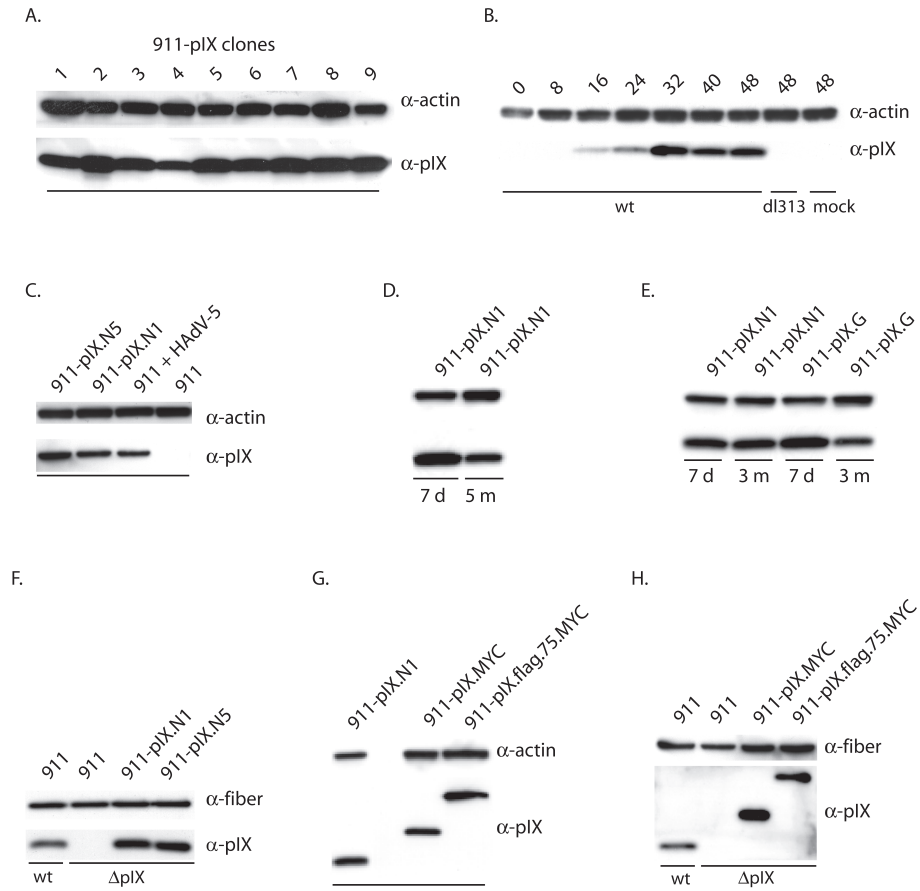


Figure 3. Western blot analyses. (**A**) Comparison of pIX amounts of the monoclonal 911 cell lines after lentivirus-mediated transfer of the pIX gene. The cell lines were generated after transduction of 911 cells with LV-CMV-pIX-IRES-NPTII. (**B**) Protein IX amounts during wt HAdV-5 and HAdV-5-*dl313* infection. 911 cells were infected with HAdV-5 (MOI = 5). Cell lysates were prepared at 0, 8, 16, 24, 32, 40 and 48 h post-infection and a Western analysis was performed using anti-pIX serum. (**C**) pIX amounts of the 911-pIX.N1 and 911-pIX.N5 cell lines. Equal amounts of lysates of the 911-pIX.N1, 911-pIX.N5, and as control 911 cells infected with HAdV-5 at 32 h post-infection, were assayed by Western analysis. (**D**) pIX amounts of the 911-pIX.N1 cell line 7 days (7 d) and 5 months (5 m) post-transduction. (**E**) pIX amounts of the 911-pIX.G cells and 911-pIX.N1 cells 7 days (7 d) and 3 months (3 m) post-transduction. (**F**) Complementation efficiency of the 911-pIX.N1 and 911-pIX.N5 helper cell lines. To test the incorporation efficiency of pIX produced by the 911-pIX.N1 and 911-pIX.N5 cells, HAdV-5ΔpIX.CMV.GFP/LUC was propagated on the cell lines and purified by CsCl centrifugation, after which protein lysates of the purified viruses samples were made for Western analysis. The amounts of pIX in HAdV-5ΔpIX.CMV.GFP/LUC propagated on 911-pIX.N1 and 911-pIX.N5 cell lines were compared with wt HAdV-5, with anti-pIX serum and, as a virus-particle loading control, the 4D2 antibody directed against the fiber protein. (**G**) pIX.MYC and pIX.flag.75.MYC amounts in the complementing cell lines 911-pIX.MYC and 911-pIX.flag.75.MYC were compared with the pIX amounts in the 911-pIX.N1 cell line. Equal amounts of cell lysate were assayed as described above. (**H**) The incorporation efficiency of the pIX.MYC and pIX.flag.75.MYC variants produced by the helper. The HAdV-5ΔpIX.CMV.GFP/LUC vector was propagated and purified as described above and pIX amounts were compared with the amount of pIX in wt HAdV-5 particles.

stable (Figure 3D). To test the stability of pIX-producing cell lines without selection, 911 cells were transduced with LV-CMV-pIX-IRES-eGFP at 40 ng p24 per 10^5 cells (911-pIX.G). The pIX amounts in the unselected 911-pIX.G cells were compared with that in 911-pIX.N1 cells grown in parallel in medium containing G418 (Figure 3E). Only a slight reduction in pIX amounts was detected after 3 months without selection in the 911- pIX.G cells, while there was no decrease in pIX amounts in the 911-pIX.N1 cells. This demonstrates that pIX is not toxic for 911 cells. For further experiments we used cells transduced with the lentiviral vectors harboring the NPTII selection gene.

Next, we examined the incorporation of pIX into the capsid of the HAdV-5 vector HAdV-5ΔpIX.CMV.GFP/LUC. This vector lacks a functional pIX gene and carries the eGFP and the firefly luciferase reporter genes under control of two separate CMV promoters. HAdV- 5ΔpIX.CMV.GFP/LUC viruses was propagated on the 911-pIX.N1 and 911-pIX.N5 cell lines, and harvested and purified via the conventional CsCl purification method. During purification, the particle-associated pIX is separated from the non-associated pIX since variants of pIX that cannot be incorporated into the capsid do not co-purify with the particles of CsCl gradients (18). To evaluate the amount of pIX in the particles, 5×10^9 CsCl-gradient purified particles from each virus preparation were analyzed by Western blot analysis (Figure 3F). The amounts of pIX in the pIX-loaded HAdV- 5ΔpIX.CMV.GFP/LUC particles is similar to, or maybe even slightly higher than, the amounts in wt HAdV-5 particles, while the fiber contents are identical. The yield of the HAdV-5ΔpIX.CMV.GFP/LUC vector in the pIX-complementing cells was similar to the yield routinely obtained on 911 cells (data not shown).

To further characterize the pIX loading in HAdV- 5ΔpIX.CMV.GFP/LUC particles that were propagated on the pIX-complementing cells 911-pIX.N1 and 911-pIX.N5, we tested these viruses for their heat-stability. The HAdV-5ΔpIX.CMV.GFP/LUC rapidly decreased in titer in response to incubation at 45 °C, whereas the vector containing a wt pIX gene (HAdV-5.CMV.GFP/LUC) was thermostable (Figure 4A) (16). The HAdV-5ΔpIX.CMV.GFP/ LUC particles that were loaded with pIX via propagation on complementing cell lines 911-pIX.N1 and 911-pIX.N5 were as thermostable as HAdV-5.CMV.GFP/LUC.

To verify that the majority of particles carried pIX upon propagation on the pIX-complementing cell lines, we used the two MYC-tagged pIX variants pIX.MYC and pIX.flag.75.MYC (18). New 911 cell lines were generated via transduction with LV-CMV-pIX.MYC-IRES-NPTII and LV-CMV-pIX.flag.75.MYC-IRES-NPTII, resulting in the 911-pIX.MYC and 911-pIX.flag.75.MYC helper cell lines, respectively. To test the expression level in the newly produced cell lines, samples were taken and used for Western analysis (Figure 3G). The amounts of pIX.MYC and pIX.flag.75.MYC are similar to the pIX amount in the 911-pIX.N1 cell line. After propagation of HAdV- 5ΔpIX.CMV.GFP/LUC

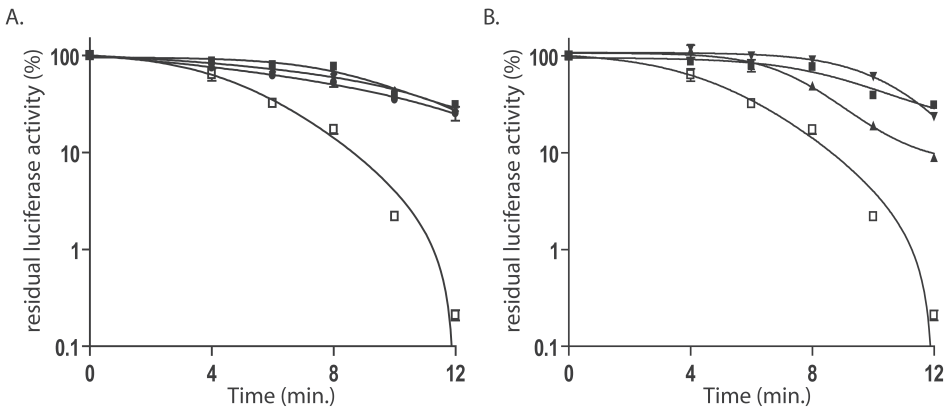


Figure 4. Virus-stability assay. Thermostability of pIX gene-deleted HAdV vectors carrying pIX molecules produced by the trans-complementing helper cells and, as control, pIX gene-containing HAdV vectors. Infectivity of HAdV-5ΔpIX.CMV.GFP/LUC particles produced on pIX trans-complementing helper cells, and on 911 cells, as determined by their capacity to transfer the luciferase reporter gene to indicator cells. The results are presented as the percentage residual luciferase activity. Each bar represents the mean ± standard deviation (SD) of triplicate analyses. (**A**) Comparison of HAdV-5ΔpIX.CMV.GFP/LUC viruses propagated on the 911-pIX.N1 (●) and 911-pIX.N5 (♦) with HAdV-5.CMV.GFP/LUC (■) and HAdV-5ΔpIX.CMV.GFP/LUC (□) produced on 91 cells. (**B**) HAdV-5_pIX.CMV.GFP/LUC viruses produced on 911-pIX.MYC (▼) and 911-pIX.flag.75.MYC (▲) cells, compared with HAdV-5.CMV.GFP/LUC (■) and HAdV-5ΔpIX.CMV.GFP/LUC (□) produced on 911 cells. The data from (**A**) and (**B**) are derived from the same experiment.

on the 911-pIX.MYC and 911- pIX.flag.75.MYC cell lines, the viruses were harvested and purified by CsCl banding. The MYC-tagged proteins efficiently incorporated (Figure 3H). The presence of the MYC-tagged pIX in the HAdV-5ΔpIX.CMV.GFP/LUC particles was visualized by immuno-affinity electron microscopy, with gold-labeled anti-MYC antibodies, as described previously (18). Of over 1000 particles counted, more than 96% of the particles bound the gold-labeled antibodies (Figure 5). There was no gold label present on HAdV-5ΔpIX.CMV.GFP/LUC lacking the MYC tag.

Subsequently, we examined the heat stability of the adenoviruses containing the MYC-tagged pIX molecules (Figure 4B). The HAdV-5ΔpIX.CMV.GFP/LUC loaded with pIX.MYC was as stable as HAdV-5.CMV.GFP/LUC. Whereas the virus loaded with pIX.flag.75.MYC was significantly stabilized compared to the pIX-deficient HAdV-5ΔpIX.CMV.GFP/LUC particles, it was less stable than the particles loaded with either pIX or pIX-MYC.

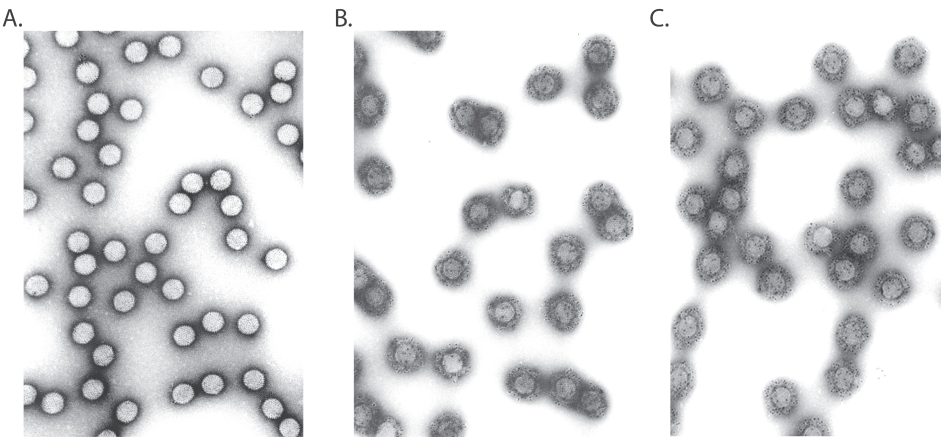


Figure 5. Immuno-affinity electron microscopic analysis of HAdV-5ΔpIX.CMV.GFP/LUC particles loaded with pIX.MYC and pIX.flag.75.MYC. The fraction of particles loaded with the MYC-tagged pIX was assessed by detection of the MYC tag on the virions visualized by the presence of gold particles. There is no MYC tag detected on the HAdV-5ΔpIX.CMV.GFP/LUC particles (**A**), whereas at least 96% of the virions are loaded with pIX.MYC (**B**) and pIX.flag.75.MYC (**C**).

DISCUSSION

Here we describe the use of lentiviral vectors for the generation of pIX-complementing helper cell lines. Lentiviruses are a versatile and efficient tool to transfer transgenes (33,41–44). We have used a third-generation nonreplicating HIV-1-derived vector. The VSV-G-pseudotyped lentiviral vectors can transduce a wide variety of cell types of different species (45). Also the conventional helper cells for adenovirus production can be efficiently transduced. The transduction of cells with lentiviral vectors results in modest changes in gene expression and cellular transcription (46). The propagation of HAdV-5 vectors on the transduced helper cells 911-pIX.N1 and 911-pIX.N5 was not impaired.

Previously, we evaluated the use of modification of pIX for retargeting of HAdV vectors (18). This system was based on transient expression of modified pIX genes in helper cells. The use of lentiviral vectors circumvents the need to sub-clone the stably modified clones after transduction. After lentiviral-vector-mediated pIX gene transfer, the cells in a polyclonal cell population have homogenous pIX amounts as evident from immunohistochemistry and Western analyses.

HAdV-5 lacking the pIX gene can be efficiently propagated on the pIX-producing helper cell lines 911- pIX.N1 and 911-pIX.N5. Intriguingly, the amounts of pIX in the particles seem even slightly higher than in wt HAdV-5 particles. Contamination of the purified virus with non-virus-associated

pIX protein can be excluded: a variant-pIX protein with a deletion in the N-terminus is not present in the purified virus preparation (18). There are two possible alternative explanations. Firstly, if the pIX-gene-deleted virions have lost their pentons (47), we could have loaded more particles, without increasing the fiber signal on the blots. Alternatively, the pIX-gene expression may be deregulated if a wt HAdV-5 virus is grown on helper cells. This could be the result of deregulation of the transcription of the E1B gene of the virus in the helper cells, and E1B transcription affects pIX-gene expression (48). However, the heat-inactivation data, the electron microscopy data, and our particle quantization data do not support these explanations.

By propagation on the transduced cell line populations the heat-stable phenotype could be fully restored. Previous studies have demonstrated that pIX can be incorporated into the HAdV capsid upon heterologous expression in helper cell lines, after transient expression (18), and after generating stable cell lines using EBV replicons and plasmid vectors (20). In the latter studies selected stable cell lines could restore the heat-stable phenotype. Moreover, pIX-deficient HAdV vectors with a genome size larger than wt HAdV-5 could be efficiently packaged on the pIX-producing cell lines, but not on the parental, non-modified helper cells (20). However, the selection and characterization of monoclonal cell lines is a laborious and time-consuming process, making the efficient lentivirus-based system described here a valuable extension of this technology.

To further exploit the possibility to examine pIX-fusion proteins via this system we tested two MYC-tagged pIX variants, i.e. pIX.MYC and pIX.flag.75.MYC, which can be efficiently incorporated into the virus capsid (18). Here we showed that the two MYC-tagged pIX variants incorporate with efficiencies equivalent to that of wt HAdV-5. However, the variant with the flag.75.MYC extension exhibited moderately reduced heat stability. This suggests that the flag.75.MYC extension has an effect on the capsid-stabilizing function of pIX. This is unlikely to be due to inhibition of the pIX-trimer formation, since recent data demonstrated that trimer formation is required neither for pIX capsid incorporation, nor for the heat-stable phenotype (49). The addition of relatively large ligands has previously been shown to influence the capsid stability (38), suggesting that steric hindrance may hinder bona fide capsid stabilization.

In conclusion, the use of lentiviral vectors for the generation of pIX-producing helper cell lines may allow development of systems for 'pseudotyping', rather than genetic retargeting, of HAdV-vectors. By pseudotyping, HAdV vectors are retargeted to a specific cell type or tissue by propagating them on the appropriate pIX-complementing helper cell line. So far few studies have been focused on HAdV peudotyping. Stable cell lines expressing the fiber gene have been described but it has proven difficult to achieve expression levels sufficiently high to fully load all particles (50,51). The availability of a robust

system for generating pIX-producing cell lines, together with the observation that trimer formation of pIX is not required for its incorporation into the capsid, makes modification of pIX a feasible approach for developing a system for development and generation of batches of 'pseudotyped' HAdV vectors.

ACKNOWLEDGEMENTS

We thank Keith N. Leppard (University of Warwick, UK) for supplying the anti-pIX serum, and Hans van der Meulen (Leiden UniversityMedical Center) for help with immuno-affinity electron microscopy. This work was supported in part by the Technology Foundation STW (program LGN66.3977), and the European Union through the Sixth Framework Program GIANT (Contract no.: 512087).

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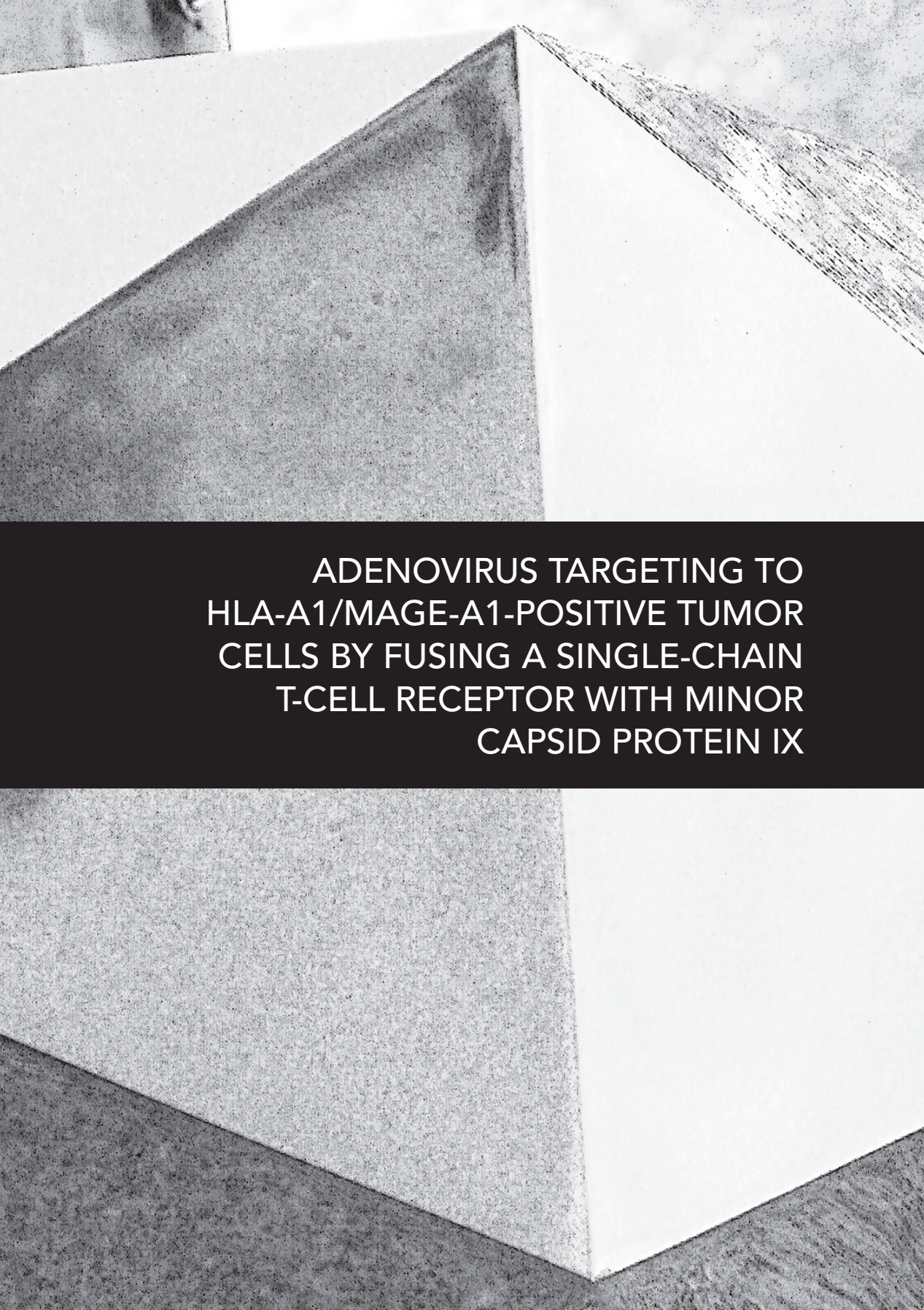
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3



ADENOVIRUS TARGETING TO HLA-A1/MAGE-A1-POSITIVE TUMOR CELLS BY FUSING A SINGLE-CHAIN T-CELL RECEPTOR WITH MINOR CAPSID PROTEIN IX

ABSTRACT

Adenovirus vectors have great potential in cancer gene therapy. Targeting of cancer-testis (CT) antigens, which are specifically presented at the surface of tumor cells by human leukocyte antigen (HLA) class I molecules, is an attractive option. In this study, a single-chain T-cell receptor (scTCR) directed against the CT antigen melanoma-associated antigen (MAGE)-A1 in complex with the HLA class I molecule of haplotype HLA-A1 is fused with the C terminus of the adenovirus minor capsid protein IX. Propagation of a protein-IX (pIX)-gene-deleted human adenovirus 5 (HAdV-5) vector on cells that constitutively express the pIXscTCR fusion protein yielded viral particles with the pIXscTCR fusion protein incorporated in their capsid. Generated particles specifically transduced melanoma cell lines expressing the HLA-A1/MAGE-A1 target complex with at least 10-fold higher efficiency than control viruses. Whereas loading of HLA-A1- positive cells with MAGE-A1 peptides leads to enhanced transduction of the cells, the efficiency of virus transduction is strongly reduced if the HLA-A1 molecules are not accessible at the target cell. Taken together, these data provide proof of principle that pIXscTCR fusions can be used to target HAdV-5 vectors to tumor cells expressing intracellular CT antigens.

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Gene Therapy 2008;15(13):978-89

INTRODUCTION

Recombinant viral vectors hold great promise in the field of cancer gene therapy. Much effort is devoted to generating vectors that have the ability to specifically transduce tumor cells. In this respect it will be of major interest to develop vectors that are targeted to antigens that are specifically expressed at the surface of tumor cells to prevent transduction of noncancerous cells.

Intriguingly, in many cases cancer cells have been found to induce a tumor cell-specific response of the immune system. Cytotoxic T lymphocytes (CTLs) have been discovered that eradicate tumor cells via the recognition of tumor-specific antigens, while leaving healthy tissue intact. Such tumor-specific antigens might belong to the group of so-called 'cancer-testis' (CT) antigens, which are presented at the tumor cell surface by major histocompatibility complex (MHC) class I molecules and are recognized by CTLs (1). CT antigens are expressed in a variety of cancerous tissues and are generally silent in normal tissues, except for the testis (2). To date, 89 CT genes or isoforms, which are organized into 44 families, have been described. From these, 19 families are testis restricted, 11 show additional expression in one or two somatic tissues, 9 are expressed in three to six tissue types besides testis and 5 are ubiquitously somatically expressed. With the exception of the testis-restricted CT antigens, the others also show expression in the pancreas but at levels as much as 10 times lower than in the testis (2). Expression of CT antigens was first shown in melanoma and all the classic CT antigens are expressed in this type of tumor, but since the 1980s, expression in various other tumors has been recognized (2). The highly specific expression profiles of the CT antigens make them interesting target molecules for cancer therapies. Importantly, CT antigens belonging to the melanoma-associated antigen (MAGE) A, B and C family seem to be involved in oncogenesis, providing protection against apoptosis in tumor cells (3). The expression of other tumor antigens, such as 'overexpression' antigens p53 and HER-2/*neu*, or 'differentiation antigens' gp100 and Mart-1, is less restricted to tumor cells and their use for cancer-drug targeting may be associated with negative side effects.

The principle of targeting human MHC class I molecules (human leukocyte antigen, HLA class I), in complex with tumor-specific antigens, has been the subject of many studies on T-cell targeting to tumor cells (4). So far, HLA class I/peptide targeting of viral vectors has not been extensively explored. Promising results have been obtained by targeting measles virus particles to a specific HLA class I/peptide complex via fusion of a single-chain T-cell receptor (scTCR) to the attachment protein H of the virus (5). Recently, adenovirus has been also retargeted to CT antigens by replacing the knob domain of the fiber protein with a CT antigen-specific scTCR (6).

Adenoviral vectors are among the most promising viral vectors for cancer gene therapy for several reasons. They can be produced in large quantities,

they do not lead to stable genetic modification of the transduced cells and they have a good safety profile (7). Genetic modification of adenoviral capsid proteins might lead to the development of vectors that are specifically targeted to tumor cells, thereby improving safety and efficacy. Cell binding of subgroup C-derived human adenovirus (HAdV) vectors (including HAdV-5 and HAdV-2), which are widely used in gene therapy, involves two distinct steps. First, they bind with high affinity to the coxsackie virus and adenovirus receptor (CAR) at the cell surface. Second, interaction of penton base Arg-Gly-Asp (RGD) motifs with cellular integrins, including αvβ3 and αvβ5 leads to endocytosis (8). Many tumor cells are relatively refractory to infection by subgroup C-derived HAdV vectors due to the paucity of CAR receptors (9). Development of genetically modified vectors that can infect CAR-negative cells has mainly focused on incorporation of heterologous ligands in the fiber knob, or on replacement of the entire knob domain by a heterologous ligand (7). The complexity of incorporating ligands into the adenovirus fiber locale has prompted the identification of other capsid proteins amendable for ligand incorporation. These approaches have the potential to incorporate an increased number of complex ligands per virion. To date, the capsid proteins hexon (10,11), penton base (12), minor capsid protein IX (pIX) (13-18), and protein pIIIa (19) have been explored as platforms for the incorporation of heterologous peptides [reviewed by Vellinga *et al*. (20)].

We have been exploiting the adenovirus minor capsid pIX as an anchor to genetically incorporate large proteins (17,18). Protein IX is a small (14.3 kDa) protein of unknown structure that acts as capsid cement, stabilizing the interactions between hexon trimers on each facet of the virion (21). Twelve molecules of pIX are associated with each facet of the icosahedron, with an overall stoichiometry of 240 pIX monomers per virion (22). Although the main mass of pIX is thought to be located in the cavities between the so-called group-of-nine hexon capsomers, the postulated position of pIX in the capsid is being challenged (23-26).

Nevertheless, we have recently demonstrated efficient and functional incorporation of the hyperstable single-chain antibody fragment 13R4 that was fused with pIX via a 75-Ångstrom spacer (18). The 13R4 was functional in the capsid as was evidenced by its capacity to bind Escherichia coli β-galactosidase.

Here we report the production of an adenoviral vector which is targeted to tumor cells presenting peptides of the CT antigen MAGE-A1 on HLA class I molecules of haplotype HLA-A1, via a scTCR (TCR^{A1M1}) fused with adenovirus minor capsid pIX. To ensure enhanced protrusion of the scTCR at the virus surface, a 75-Ångstrom α-helical spacer was included between pIX and the TCR (17). We produced virus particles that were efficiently loaded with the pIX_TCR^{A1M1} fusion protein. The transduction efficiency of the HLA-A1/MAGE-A1-positive melanoma cell lines MZ2-MEL3.0 and MZ2-MEL43 was

strongly increased upon incorporation of the fusion protein. These findings represent (a) the first demonstration of a pIXscTCR-mediated adenovirus targeting of a cell type that is normally refractory to HAdV-5, and (b) further proof-of-principle of targeting the highly tumor-specific CT antigen/HLA class I complexes at the surface of human tumor cell lines.

RESULTS

Establishment and characterization of the pIX_TCR^{A1M1}-producing helper cell line

To establish a helper cell line stably expressing the pIX_TCR^{A1M1} fusion protein, 911 cells were transduced with the recombinant lentivirus LV.pIX_TCR^{A1M1}. A schematic overview of the pro-lentiviral DNA construct and the sequence eventually incorporated in the 911 genome is provided in Figure 1.

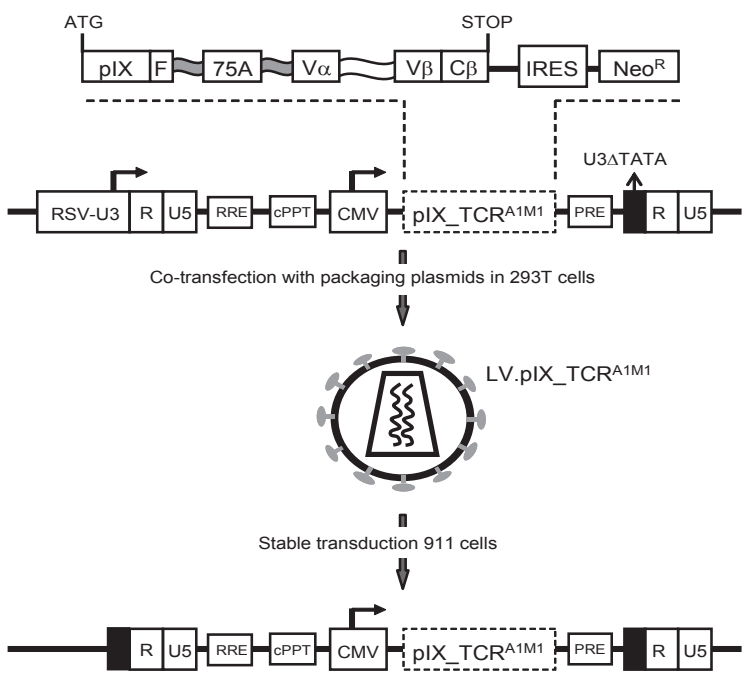


Figure 1. Schematic representation of the lentivirus system used to establish the 911 helper cell line stably expressing pIX_TCR^{A1M1}. The vector used in this study is a third-generation, self-inactivating (SIN) vector, with a major part of the 3' U3 region deleted, including the TATA box (27). The Rev-responsive element (RRE) (28), the central polypurine tract (cPPT) (29–31), and the human hepatitis B virus-derived posttranscriptional regulatory element (PRE) are indicated. The encephalomyocardin virus internal ribosomal entry site (IRES) was obtained from pTM3 (32). The NPTII coding region (Neo^R), which mediates resistance to G418, was isolated from pEGFP-N2 (Clontech, Leusden, the Netherlands).

After growing the cells for several weeks in selection medium (containing 200 µg/ml G418), western analysis on cell lysate was performed. This revealed pIX_TCR^{A1M1} protein amounts that were similar to the pIX level in 911 cells infected with HAdV-5.LUC virus (Figure 2A). The protein size of pIX_TCR^{A1M1} was as expected (65.8 kDa). The percentage of pIX_TCR^{A1M1}-positive cells was determined by immunohistochemistry analysis (Figure 2B). This showed more than 90% of the cells to be positive for pIX_TCR^{A1M1}. The pIX fusion protein appeared to be located mainly in the cytoplasm. Surprisingly, visualizing pIX_TCR^{A1M1} protein in the cells by using a conformation-dependent antibody recognizing the variable domain of the scTCR (Vα12.1) was dependent on adenovirus infection of the cells (Figure 2C). At 24 h postinfection, a Vα12.1-mediated signal could be observed (cells were infected with the

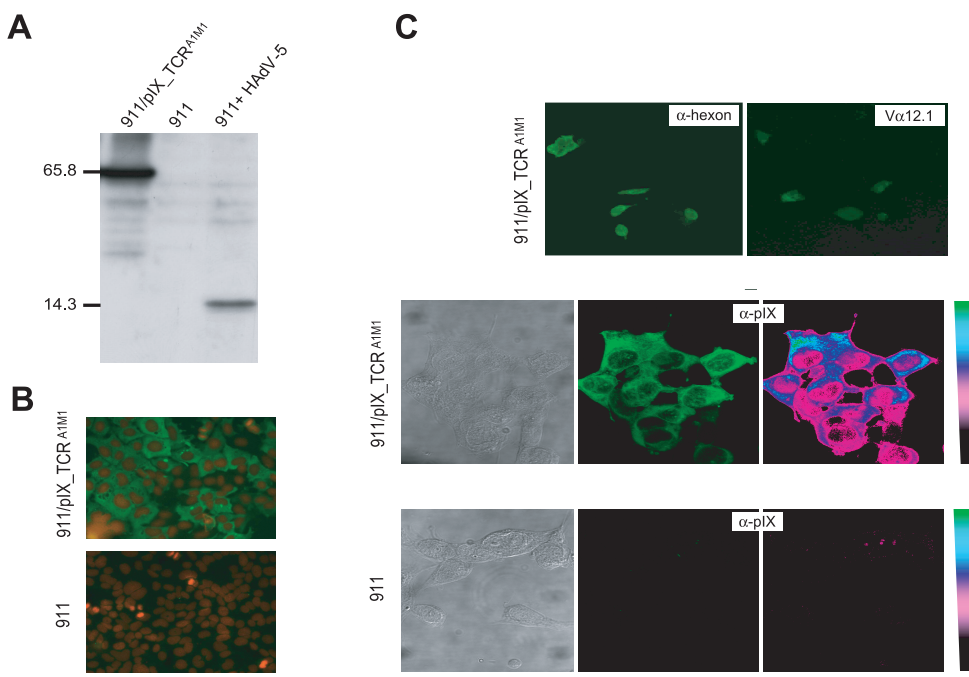


Figure 2. Characterization of the 911/pIX_TCR^{A1M1} helper cell line. (**A**) Western analysis on lysates of the 911/pIX_TCR^{A1M1} cells. Production of pIX_TCR^{A1M1} was compared to the production of wild-type (wt) pIX in 911 cells infected with HAdV-5. Used antibodies were α-pIX and horseradish peroxidase (HRP)-conjugated secondary antibody. The predicted size of 14.3 kDa for wt pIX and 65.8 kDa for pIX_scTCR^{A1M1} was confirmed by the SDS–polyacrylamide gel electrophoresis (PAGE), as indicated in the figure. (**B**) Immunohistochemistry assay on 911/pIX_TCR^{A1M1} cells. The pIX_TCR^{A1M1} was visualized by using α-pIX antibody. The nuclei were stained with propidium iodide. (**C**) Immunohistochemistry assays on 911/pIX_ TCR^{A1M1} cells, infected with HAdV-5ΔpIX. Fixation was performed at 24 h postinfection. The upper panel shows wide-field microscopy images of α-hexon and Vα12.1-stained cells. The lower panel shows confocal microscopy images of α-pIX-stained 911/pIX_TCR^{A1M1}. Infected 911 cells were included as negative control. To illustrate the presence of fluorescence signal in the nuclei more clearly, pseudocolor images of the cells are shown. Fluorescent intensities range from purple (low) to green (high).

viral vector HAdV-5.CMVLUCΔE1ΔE3ΔpIX, see next paragraph). Detection of pIX_scTCR^{A1M1} by Vα12.1 antibody was absent at earlier time points of infection and in pIX_TCR^{A1M1}-negative 911 cells (result not shown). Confocal laser scanning microscopy was used to analyze the subcellular localization of the pIX_scTCR^{A1M1} protein after virus infection (Figure 2C). Although the majority of the pIX_TCR^{A1M1} protein was observed in the cytoplasm, significant amounts were present in the nuclei of infected cells. In >90% of the infected cells the pIX_TCR^{A1M1} fusion protein was readily detectable in the nucleus.

Efficient incorporation of pIX_TCR^{A1M1} in the virus Capsid

Next, we tested the incorporation of pIX_TCR^{A1M1} into the capsid of the vector HAdV-5.CMVLUCΔE1ΔE3ΔpIX. This vector lacks a functional pIX gene and carries the firefly luciferase reporter gene under control of the cytomegalovirus (CMV) promoter. After transduction of the 911/pIX_TCR^{A1M1} cells with the ΔpIX virus, the offspring virus particles were harvested and purified via the conventional cesium chloride (CsCl) purification method. This resulted in the virus HAdV-5/ pIX_TCR^{A1M1}. During purification, particle-associated pIX molecules were separated from the nonassociated pIX molecules, as nonassociated variants do not co-purify with the virus particles in the CsCl gradient (17,33). A schematic representation of the pIX_TCR^{A1M1} fusion protein, with its exposed single-chain TCR^{A1M1} positioned above the hexon capsomers, is depicted in Figure 3A.

The presence of pIX_TCR^{A1M1} in the virus particles was detected by western assay (Figure 3B). The amount of pIX_TCR^{A1M1} in the HAdV-5/pIX_TCR^{A1M1} particles is slightly lower than the amounts of pIX in wild-type (wt) HAdV-5 particles. Loading was similar to the loading of pIX_13R4 in the previously produced virus HAdV-5/pIX_13R4 (18). The 13R4 is a single-chain antibody fragment directed against β-galactosidase, which is approximately 14 kDa smaller than the scTCR^{A1M1}.

Incorporation of pIX_TCR^{A1M1} in the virus capsid was also shown by spot-blot analyses (Figure 3C). Virus particles were spotted on a nitrocellulose membrane, followed by incubation with various antibodies. Upon spotting, the virus particles remain intact, indicated by the inability to detect the adenovirus core protein VII (pVII). Only after denaturation of the virus particles the pVII could be detected. From the appearance of the a-flag signal it can be concluded that flag epitopes of the pIX_TCR^{A1M1} fusion protein were accessible to immunoglobulins in the context of intact virus particles. For the α-flag and the α-pIX detection longer exposure times were used, resulting in increased background signals.

To further investigate the accessibility of the single-chain TCR^{A1M1} at the surface of the virus particles we performed an immunoprecipitation assay, in which the ability of the pIX_TCR^{A1M1} virus to bind to anti-TCR antibody-coated

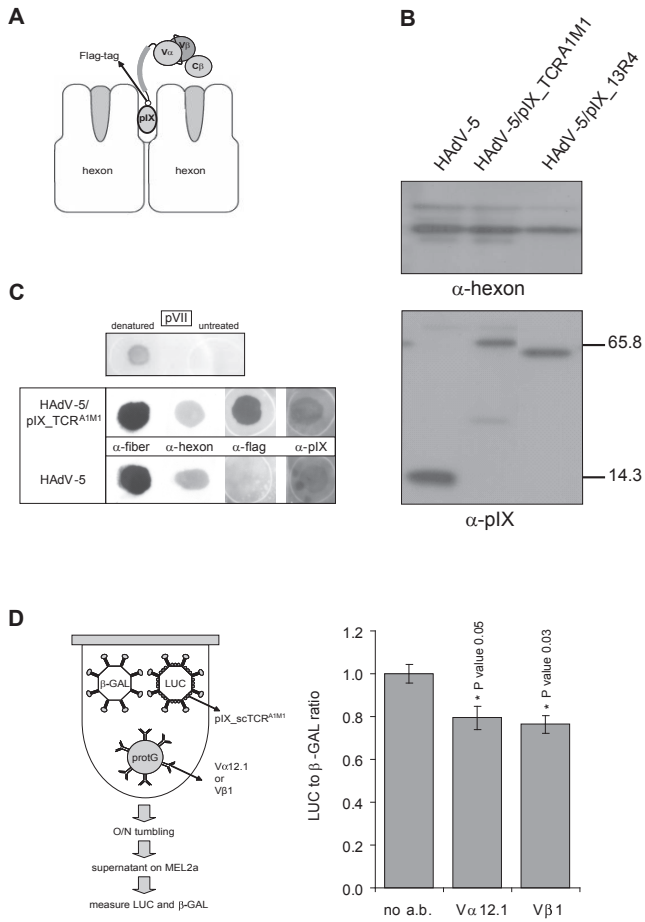


Figure 3. Analysis of pIX_TCR^{A1M1} incorporation in the virus capsid. (**A**) Schematic representation of the pIX_TCR^{A1M1} fusion protein exposing the single-chain TCR^{A1M1} above the hexon capsomers. A spacer of 75 Ångstrom is included to improve presentation of the single-chain T-cell receptor (scTCR). A flag tag is present in between the C terminus of pIX and the 75-Ångstrom spacer. Additional linkers flank the 75-Ångstrom spacer (with amino-acid sequence 'ser-gly-gly-gly') to enhance the flexibility of the scTCR. (**B**) Western analysis on virus lysates of cesium chloride (CsCl)-purified viruses to analyze incorporation of pIX_TCR^{A1M1} in the particles. To compare incorporation efficiencies, virus lysates of HAdV-5 and HAdV-5/pIX_13R4 were included. The α-hexon antibody was included as a virus particle loading control. The predicted size of 14.3 kDa for wild-type pIX and 65.8 kDa for pIX_scTCR^{A1M1} was confirmed by the SDS–polyacrylamide gel electrophoresis (PAGE), as indicated in the figure. (**C**) Spot-blot analysis to detect the presence of pIX_TCR^{A1M1} in intact virus particles. Virus was spotted onto a membrane followed by incubation with multiple antibodies. As a control to show integrity of the particles upon the spot-blot treatments, incubation with an antibody directed against the core protein VII (pVII) was included. Only after denaturation could pVII be detected. (**D**) Binding of pIX_TCR^{A1M1}-loaded virus particles on beads containing α-TCR antibodies (Vα12.1 or Vβ1). After incubation of beads with the pIX_TCR^{A1M1}-loaded virus (containing the luciferase reporter as a transgene) or a control virus (containing the *Escherichia coli* β-galactosidase gene as a transgene), the supernatant was applied to MEL2A cells. The ratio of luciferase to β-galactosidase expression in the cells was subsequently measured, and was normalized to the ratio obtained on mock beads (beads without antibody).

beads was analyzed (Figure 3D). The pIX_TCR^{A1M1} virus was mixed with a control virus and subsequently incubated with Vα12.1 or Vβ1 antibody precoated beads. Both antibodies specifically recognize the variable (V) domain of the TCR^{A1M1}. After incubation, supernatant fraction, containing virus particles that were not bound to the beads, was applied to MEL2a cells, and the infection ratio of pIX_TCR^{A1M1} virus (containing the luciferase transgene) to control virus (containing the β-galactosidase transgene) was determined. As a result, significantly lower ratio for the Vα12.1- or Vβ1-treated samples was observed when compared to the ratio obtained from the samples without antibody treatment. This shows the binding of intact pIX_TCR^{A1M1} virus particles to the Vα12.1 and Vβ1 antibodies. Thus, it can be concluded that the V domains of the TCR^{A1M1} at the surface of the virus particles are accessible, suggesting that the scTCR may be free to interact with the HLA-A1/MAGE-A1 complex at the cell surface.

Targeting of the pIXscTCR-containing virus to HLA-A1/ MAGE-A1-positive MZ2-MEL3.0 tumor cells

To test the targeting potential of the HAdV-5/ pIX_TCR^{A1M1} virus to HLA-A1/ MAGE-A1-expressing cells, transduction of MZ2-MEL3.0 melanoma cells (CAR^{neg}, HLA-A1^{pos}, MAGE-A1^{pos}) was analyzed and compared to control virus transduction. The absence of CAR expression is an important aspect to test the targeting potential of the pIX_TCR^{A1M1}-containing virus, since the virus is not ablated for its natural CAR-binding ability, which occurs via the fiber attachment protein. In parallel to the infection of MZ2-MEL3.0, the CAR-positive melanoma cell line MEL2a (CAR^{pos}, HLA-A1^{pos}, MAGE-A1^{neg}) was infected with the viruses to analyze CAR-mediated transduction. Transduction efficiencies of the viruses were determined by measuring luciferase production 24 h after transduction. As represented in Figure 4, presence of the pIX_TCR^{A1M1} fusion protein in the virus capsid results in a highly increased transduction of the target cell line MZ2-MEL3.0. Whereas CAR-mediated transduction on the MEL2a cell line was highest for the pIXscTCR-lacking virus (indicated by a twofold higher luciferase activity), the opposite pattern was obtained for the target cell line MZ2-MEL3.0, which was approximately fourfold better transduced by the pIXscTCR-loaded virions. When setting the targeting ratio (MZ2-MEL3.0/MEL2a) for HAdV-5 at one, the normalized targeting effect for HAdV-5/ pIX_TCR^{A1M1} on the MZ2-MEL3.0 cell line is 9.5. The approximate 10-fold increase in transduction specificity on the target cell line did not significantly change by increasing (multiplicity of infection, MOI = 10.000) or decreasing (MOI = 100) the MOI (results not shown).

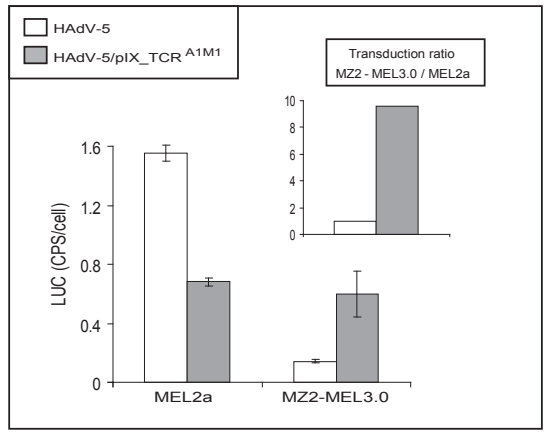


Figure 4. Targeting of HAdV-5/pIX_TCR^{A1M1} to HLA-A1/MAGE-A1-presenting MZ2-MEL3.0 cells. The target cell line MZ2-MEL3.0 (HLA-A1^{pos}/MAGE-A1^{pos}, CAR^{neg}) was transduced with HAdV-5 and HAdV-5/pIX_TCR^{A1M1}. In parallel, transduction of the MEL2a cell line was performed to determine fiber-coxsackie adenovirus receptor (CAR)-mediated transduction of both vectors. As transduction readout, luciferase production was determined 24 h after transduction. Multiplicity of infection was 1000 virus particles per cell. The insert graph shows the ratio of MZ2-MEL3.0 to MEL2a transduction for the targeted virus HAdV-5/pIX_TCR^{A1M1}, which is normalized to the ratio for the control virus. The presence of pIX_TCR^{A1M1} results in a 10-fold improved transduction of the MZ2-MEL3.0 cell line.

Downmodulation of HLA-A1/MAGE-A1 availability results in decreased targeting by HAdV-5/ pIX_TCR^{A1M1}

To demonstrate that HAdV-5/ pIX_TCR^{A1M1} mediates transduction through binding to HLA molecules at the cell surface, MZ2-MEL3.0 cells were incubated with α-HLA-ABC antibodies, prior to adding the virus. This resulted in a significant decrease in transduction with the pIXscTCR virus, whereas no decrease was observed for the control virus (Figure 5A). This observation indicates that transduction of the MZ2-MEL3.0 cells is inhibited by immunoglobulins binding to HLA molecules.

As an alternative blocking strategy, HLA class I presentation at the surface of MZ2-MEL3.0 was downmodulated by the expression of the human cytomegalovirus (HCMV) US11 protein. The US11 protein causes rapid degradation of newly synthesized HLA class I heavy chains by mediating their retrograde transport or 'dislocation' from the endoplasmic reticulum (ER) into the cytosol, where they are degraded by proteasomes (34). MZ2-MEL3.0 cells were transduced using retroviruses encoding US11 or a control vector and surface HLA class I levels were analyzed by flow cytometry (Figure 5B). The MZ2-MEL3.0 cells were efficiently transduced with the retroviruses, since the majority of the cells was positive for the vector-mediated green fluorescent protein (GFP) expression. The downshift of the US11-expressing cells in the dot plots indicates the downregulation of HLA class I presentation. Next,

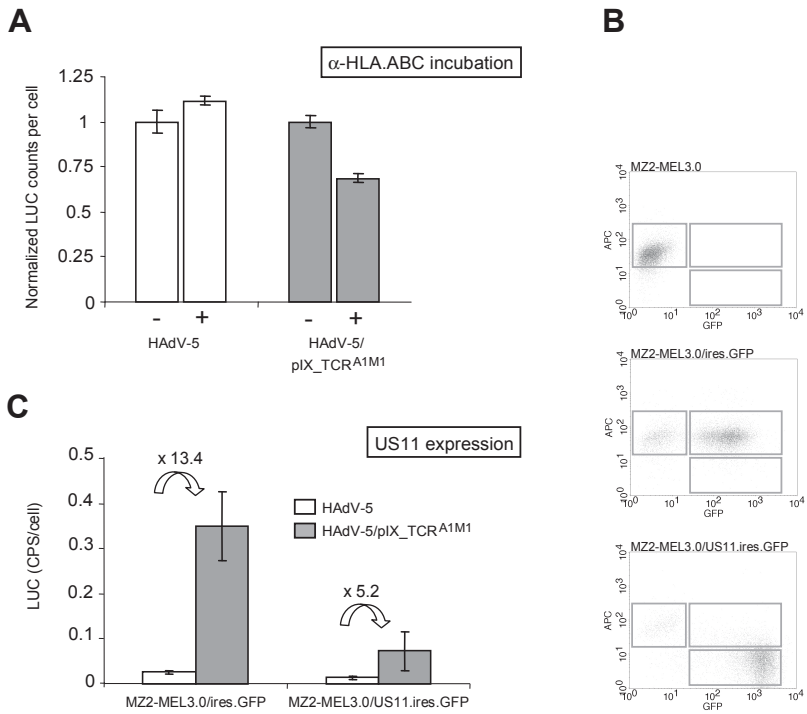


Figure 5. Downmodulation of human leukocyte antigen (HLA) availability results in decreased targeting. (**A**) Incubation of MZ2-MEL3.0 with α-HLA-ABC antibody results in significant decrease in HAdV-5/pIX_TCR^{A1M1} transduction. Transduction of the control virus is not downregulated after α-HLA-ABC loading. (**B**) Fluorescence-activated cell sorting (FACS) analysis shows downregulation of HLA class I presentation at the cell surface of MZ2-MEL3.0 cells after infection with the retroviral vector pLZRS.US11.ires.GFP or the control vector pLZRS.ires.GFP. The dot plots show for both transductions the presence of green fluorescent protein (GFP)-positive cells (shift to the right). The downshift in case of the pLZRS.US11.ires.GFP transduction indicates the downregulation of HLA class I presentation (detection via B9.12.1 antibody plus allophycocyanin (APC)-conjugated secondary antibody). (**C**) Decrease in HAdV-5/pIX_TCR^{A1M1} targeting after US11-mediated downregulation of HLA class I molecules. Compared to the control cell line, the ratio HAdV-5/pIX_TCR^{A1M1} to HAdV-5 transduction is lower for the US11-expressing cell line (ratios of respectively 13.2 and 5.2). Multiplicity of infection was 1000 virus particles per cell. Luciferase production was measured 24 h after transduction.

luciferase production after HAdV-5/ pIX_TCR^{A1M1} and HAdV-5 transduction was measured in the cell lines MZ2-MEL3.0/US11.ires.GFP and MZ2- MEL3.0/ ires.GFP (Figure 5C). This revealed a decrease in pIX_TCR^{A1M1}-mediated targeting for the US11-expressing cells. Complete blocking of targeting did not occur, probably as a result of incomplete downregulation of HLA class I expression.

Further analysis on different cell lines to confirm specificity of targeting to HLA-A1/MAGE-A1

During the time course of our study, the cell line MZ2-MEL43 became available. This line is CAR^{neg}, $HLA\text{-}A1^{pos}$ and $MAGE\text{-}A1^{pos}$. Since the level of HLA-A1 expression appeared to be at least equal to the HLA-A1 level of the target cell line MZ2-MEL3.0 (Figure 6A), the targeting efficiency of HAdV-5/pIX_TCR^{A1M1} to MZ2-MEL43 and MZ2-MEL3.0 was analyzed in parallel (Figure 6B). A third cell line, MZ2-MEL2.2, which is a derivative from MZ2-MEL3.0 but does not express MAGE-A1, was included as well. As expected, transduction with the pIXscTCR virus was enhanced in cell line MZ2-MEL43. Interestingly, the targeting efficiency was 45% higher to MZ2-MEL43 than to MZ2-MEL3.0. As expected, no targeting was obtained on the MAGE-A1-negative cell line MZ2-MEL2.2, demonstrating the absolute requirement for MAGE-A1 presentation. The enhanced transduction in the two HLA-A1/MAGE-A1-positive cell lines was blocked by incubation of the pIXscTCR virus with the antibody Vα12.1 (Figure 6B), confirming that the targeting is dependent on the scTCR in the capsid.

To further analyze the specificity of targeting, MZ2- MEL2.2 cells (CAR^{neg}, $HLA\text{-}A1^{pos}$, $MAGE\text{-}A1^{neg}$) were loaded either with a control peptide (an irrelevant peptide derived from influenza virus A nucleoprotein), a MAGE-A1 peptide or were mock treated, and were subsequently exposed to the pIXscTCR virus (Figure 6C). Incubation of the cells with MAGE-A1 peptide, but not with the control peptide, resulted in a significant increase in transduction by HAdV-5/pIX_TCR^{A1M1}. This effect did not occur in HAdV-5 transduction, confirming the dependency of HAdV-5/pIX_TCR^{A1M1} transduction on the presentation of the MAGE-A1 on HLA-A1. Compared to the MZ2-MEL3.0 cell line, the targeting efficiency to MAGE-A1-loaded MZ2-MEL2.2 cells was rather low. This can be explained by the significantly lower number of HLA-A1 molecules at the cell surface (Figure 6D).

DISCUSSION

We demonstrate successful targeting of HAdV-5 vectors to HLA-A1/MAGE-A1-presenting tumor cells using an scTCR incorporated in the capsid as a genetic fusion with pIX. The pIX_TCR^{A1M1}-loaded virions transduced HLAA1/ MAGE-A1-expressing cells and HLA-A1-expressing cells loaded with MAGE-A1 peptides. These findings warrant further exploration of minor capsid pIX as an anchor for the insertion of targeting moieties.

The fusion of targeting proteins to pIX has some potential advantages. The use of pIX allows the incorporation of larger numbers of targeting molecules since it is present in 240 copies whereas 36 fiber molecules are present per virion. Furthermore, incorporation of large targeting ligands in the fiber, such

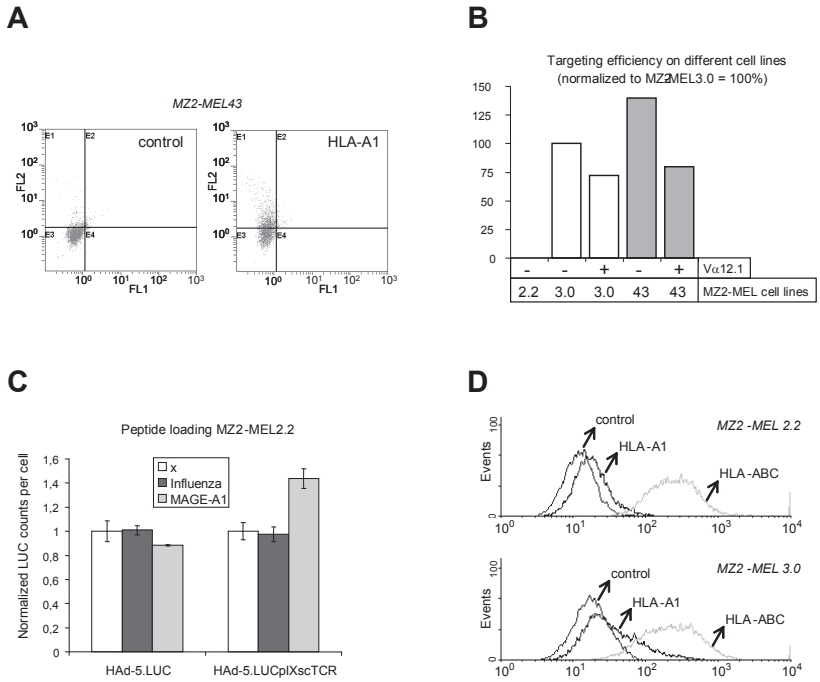


Figure 6. Comparison of the targeting on multiple cell lines to further confirm HLA-A1/MAGE-A1 specificity. (**A**) Flow cytometry analysis of human leukocyte antigen (HLA)-A1 expression at the surface of MZ2-MEL43 cells. The control plot represents incubation with secondary antibody only. (**B**) The targeting efficiency of the HAdV-5/pIX_TCR^{A1M1} virus on three different cell lines was determined, and normalized to the targeting on MZ2-MEL3.0 cells equal to 100%. Enhanced transduction of the pIXscTCR virus compared to the control virus was observed for the cell line MZ2-MEL43. This cell line was more efficiently targeted than the MZ2-MEL3.0 cells. No specific targeting was obtained on the melanoma-associated antigen (MAGE)-A1-negative cell line MZ2-MEL2.2. A decrease in targeting was obtained after incubation of the pIXscTCR virus with Vα12.1 antibody. (**C**) Improved transduction of MZ2-MEL2.2 cells after loading with MAGE-A1 peptide. MAGE-A1 peptide or an irrelevant peptide derived from influenza virus A nucleoprotein antigen was added to the wells 2 h before transduction with HAdV-5/pIX_TCR^{A1M1}. Luciferase production was measured 24 h after transduction and was normalized to transduction on non-peptide-loaded cells. (**D**) Flow cytometry analysis to compare the presentation of HLA-A1 and HLA-ABC at the cell surface of MZ2-MEL2.2 and MZ2-MEL3.0 cells. The control graphs represent incubation with secondary antibody only. The MZ2-MEL3.0 cell line had significantly more α-HLA-A1 events in the higher phycoerythrin (PE) range, indicating a higher number of HLA-A1 molecules per cell.

as scTCRs or scFv's, may lead to a reduced number of fiber molecules per virion (35). Intriguingly, pIX fusion proteins with sizes of up to 120 kDa can be accommodated in the capsid, although incorporation efficiency of the modified pIX (linked to an HSV1-TK/luciferase fusion protein) was slightly decreased.36 However, this may be improved by the use of α-helical spacers (17).

To produce our targeting virus, with pIX_TCR^{A1M1} incorporated in the capsid, we used pIX-producing helper cell lines generated via lentiviral transduction

(37). By using this strategy of incorporation of pIX in a ΔpIX virus, the time-consuming process of making viruses with pIX modifications in the genome can be avoided. Efficient incorporation of modified pIX, for example, linked to single-chain antibody fragments, can be obtained (18). After transduction of 911 helper cells that produce the pIX_TCR^{A1M1} protein, production of pIX_TCR^{A1M1} in the cells was verified by immunohistochemistry. Infection of the transduced helper cells with ΔpIX virus resulted in the production of virus particles with close to wt level of the pIX_TCR^{A1M1} protein incorporated in their capsid, demonstrating the usefulness of this approach.

Interestingly, pIX_TCR^{A1M1} was detected predominantly in the cytoplasm of the LV.pIX_TCR^{A1M1}- transduced 911 helper cells. This is in contrast to the location of the wt pIX, which is mainly nuclear (38). As can be concluded from our results, the aberrant subcellular localization of pIX_TCR^{A1M1} does not hamper incorporation in the virus capsid. Since adenovirus particles are assembled in the nucleus, these results imply that a sufficient amount of pIX_TCR^{A1M1} was present in the nuclei during formation of the HAdV-5/pIX_TCR^{A1M1} virus. Indeed, we could confirm by confocal microscopy that during the adenovirus infection significant amounts of pIX_TCR^{A1M1} are localized in the nucleus.

The targeting specificity to tumor cells expressing the MAGE-A1-derived epitope in the context of HLA-A1 was demonstrated via different approaches. First, transduction of the HLA-A1/MAGE-A1-positive MZ2- MEL3.0 cells was increased up to 10-fold by the incorporation of pIX_TCR^{A1M1} in the virus capsid. The targeting efficiency in another HLA-A1/MAGE-A1-positive cell line, MZ2-MEL43, appeared to be higher. Specificity was also evident from experiments in which HLA-A1-positive/MAGE-A1-negative MZ2- MEL2.2 cells were loaded with MAGE-A1 peptides. This resulted in a significant increase in transduction efficiency. The targeting specificity was lower than achieved on MZ2-MEL3.0 cells. This may be due to distinct presentation of synthetic peptides when compared to endogenously processed and presented peptides at the cell surface. Also, MZ2-MEL2.2 cells express fewer HLAA1 molecules at their cell surface than MZ2-MEL3.0 cells (Figure 6D). Furthermore, the targeting efficiency to MZ2-MEL3.0 could be reduced by blocking HLA, either via incubation of the cells with α-HLA-ABC antibody, or alternatively, via the expression of the HCMV US11 gene. The US11 causes degradation of newly synthesized MHC class I heavy chains by mediating their dislocation from the ER into the cytosol (34). For the antibody incubation, as well as the US11-mediated downregulation, the blocking of targeting was not 100%. Apparently, the incubation of cells with a-HLA-ABC antibody was not sufficient to block all HLA-A1 molecules. In case of the US11- mediated downmodulation, flow cytometry analysis showed that a small but detectable fraction of the cell population had not been transduced (GFP-negative), and thus did not downregulate HLA class I presentation, which probably explains the incomplete block of targeting.

We report successful targeting of HAdV-5 via fusion of a specific targeting moiety to capsid pIX. It has been speculated that targeting via pIX results in entrapment of the virions in the endosomes, caused by high-affinity interaction between the pIX fusion protein and the cellular receptor (16). However, binding of T-cell receptors to their target MHCI/peptide complex is known to have low affinity (39). This may allow the scTCR-containing virions to escape from the endosome. Alternatively, binding of the virus particles to the HLA-A1/MAGEA1 complex may have resulted in a distinct internalization, that is, differing from the normal HAdV-5 internalization via clathrin-coated vesicles. Cellular internalization mediated by binding to MHCI molecules is exploited by Simian virus 40, which enters the cell via a unique pathway that involves caveolae, rather than clathrin-coated pits (40). Interestingly, HLA class I has been suggested as an alternative receptor for HAdV-5 (41).

The fact that the scTCR used in this study is biologically active at the surface of the adenovirus supports the feasibility of targeting adenovirus vectors by fusing complex polypeptide molecules, such as scTCRs or scFv fragments, to capsid pIX. Normally, such complex polypeptides are routed via the protein secretory pathway. This aspect might hamper functional incorporation in the capsid of adenovirus particles, which are assembled in the nucleus. The reducing environment in the cell prevents the formation of disulfide bridges, which may result in improper folding of these proteins (42). Another obstacle for the implementation of scTCRs in adenoviral vectors might be that the relatively large scTCRs hamper correct virion formation or might interfere with crucial processes necessary for virus propagation. Initial attempts to produce adenovirus vectors with an scTCR fragment genetically fused to a knobless fiber protein were unsuccessful (43). Incorrect folding and/or trimer formation of the fiber protein due to the presence of the scTCR fragment was reported to be the most likely explanation. The incorrect fiber formation might have caused the inability to rescue the virus, even though the mutated viral DNA was introduced into the target cell line. However, more recently the successful development of a scTCR-containing adenovirus has been reported (6). Our results, with a scTCR bound to the minor capsid pIX, emphasize the potential of scTCRs for obtaining transductional specificity in adenoviral vectors. The pIX_TCR^{A1M1} fusion protein was incorporated in the capsid with high efficiency. Although it remains to be established whether all scTCRs are functional if fused with pIX, our results clearly demonstrate the functionality of at least part of the scTCRs. The variable domains of the scTCR, which mediate binding to the HLA-A1/MAGE-A1 target complex, were accessible to antibodies in the context of intact virions, which was shown via immunoprecipitation of the virus on Vα12.1- or Vβ1-coated beads, and alternatively, via downregulation of transduction as a result of Vα12.1 incubation. The presence of the 75-Ångstrom α-helical spacer (17) in between the scTCR and pIX domains might have been crucial to generate sufficient

flexibility for proper orientation of the scTCR at the capsid surface. Detailed studies on elucidating the process of scTCR folding during virus infection would be of great interest. Our immunohistochemistry analysis on the 911/pIX_TCR^{A1M1} cell line yielded staining with the conformation-dependent antibody Vα12.1, only after adenovirus infection. It is tempting to speculate that the scTCR only adopts its proper conformation upon change of the intracellular milieu upon induction of adenovirus-induced cell death.

The pIXscTCR-containing virus used in this study was not de-targeted, as the CAR-binding elements, the heparan sulfate proteoglycans-binding elements and the plasma protein-binding elements are still present in the capsid. For the final aim of *in vivo* tumor therapy via delivery of an HAdV-5 vector, these elements should be abolished. Also, it would be interesting to test whether shortening of the fiber shaft or complete removal of the fiber improves targeting efficiency, for instance by reducing steric hindrance of the pIXscTCR molecules by the protruding fiber proteins. Currently, pIXscTCR targeting in the context of fiber mutations is under investigation.

Our approach as described here utilizes the targeting of CT antigen epitopes that are presented at the cell surface of tumor cells by HLA class I molecules. This principle differs from previously reported retargeting approaches of adenovirus-based vectors, which encompass the targeting of 'overexpression' receptors like Her2/*neu* (44) or the epidermal growth factor receptor (45). The expression profile of the CT antigens is generally much more specific compared to the expression profile of the 'more conventional' target molecules such as the overexpressed receptors. The fact that HLA class I/CT antigen complexes can elicit a highly specific response of the immune system has prompted many studies on immune cell modifications with the aim to direct the modified cells toward tumor cells (4). However, tumor therapies via such approaches may not be feasible since the process of isolating, modifying and expansion of patient immune cells is likely to be difficult and time consuming. Viral vector-based cancer gene therapy might be a more suitable strategy. Genetic modification of adenoviral vectors, which are the most widely used virus vectors in clinical tumor gene therapy studies, is relatively easy. Besides, adenoviruses have a good safety profile and can be produced with high titers.

Our results show that HAdV-5 vectors can be genetically modified to mediate greatly enhanced gene delivery into tumor cells by targeting HLA class I/CT antigen complexes. The successful combination of two aspects with great potential in cancer gene therapy, adenovirus as a vector and CT antigens as a target, clearly warrants further exploration. Follow-up studies are under way, and especially the fusion of other scTCRs to pIX (directed against other HLA class I/CT antigen complexes), and the evaluation of *in vivo* performance of the modified HAdV-5 vectors will be of great interest.

MATERIALS AND METHODS

DNA constructs

The lentiviral vectors used in this study were described in earlier studies (37). Plasmid pLV.CMV.pIXflag75AscTCR^{A1M1}.bc.neo, abbreviated as pLV.pIX_TCR^{A1M1}, was constructed by standard cloning procedures. The pLV plasmid contained a G418 resistance gene (neo) downstream of the pIX_TCR^{A1M1} sequence, separated by an internal ribosome entry site (bc). In between the pIX and scTCR sequence, sequences are present that encode a flag tag and a 75-Ångstrom spacer. Insertion of this spacer has been shown to greatly enhance presentation of pIX-fused proteins at the virus capsid (17). The gene for pIXflag75 was obtained from the pcDNA3.1-based construct pAd5pIXflag75MYC (17). The gene encoding scTCR^{A1M1} was subcloned from the retroviral vector pBullet. VαVβCβ (46). The scTCR^{A1M1} sequence of pBullet. VαVβCβ was originally constructed from the Vα, Vβ and Cβ sequences from an HLA-A1/MAGE-A1-specific CTL clone MZ2-82/30 of patient MZ2 (47).

Cells

All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, the Netherlands) supplemented with 8% fetal bovine serum (FBS, Gibco-BRL) and penicillin-streptomycin mixture. Cell lines used for the adenovirus targeting experiments were the melanoma cell lines MEL2a, MZ2-MEL2.2, MZ2-MEL3.0 and MZ2-MEL43. Expression status of the CAR and HLA-A1/MAGE-A1 has been described before for the cell lines MEL2a (CAR^{pos}/HLA-A1^{pos}/MAGE-A1^{neg}), MZ2-MEL2.2 (CAR^{neg}/HLA-A1^{pos}/MAGE-A1^{neg}) and MZ2-MEL3.0 (CAR^{neg}/HLA-A1^{pos}/MAGE-A1^{pos}) (6). The status of the previously uncharacterized cell line MZ2- MEL43 is CAR^{neg}/HLA-A1^{pos}/MAGE-A1^{pos}, as determined by flow cytometry analysis and shown in Figure 6A in this paper. The HAdV-5 E1-transformed cell line 911 was used to propagate and titer adenovirus vectors (48).

Lentiviral and retroviral transductions

The recombinant lentivirus LV.pIX_TCR^{A1M1} was produced as described elsewhere (37). The lentivirus titer was determined by p24 enzyme-linked immunosorbent assay, assuming that 1 ng p24 equals 2.5 $\times 10^3$ transducing units (49). To establish a helper cell line stably expressing pIX_TCR^{A1M1}, 911 cells were transduced with the recombinant lentivirus, resulting in the cell line 911/pIX_TCR^{A1M1}. Infection was done with five transducing units per cell. To select for stably transduced cells, the cell line 911/pIX_TCR^{A1M1} was cultured in medium supplemented with 200 mg l^{-1} G418 (Invitrogen, Breda, the Netherlands).

The retroviral vector pLZRS-US11-IRES-EGFP was made by subcloning the HCMV US11 cDNA fragment into the pLZRS-IRES-EGFP vector (50). This construct was used, together with the wt enhanced green fluorescent protein (EGFP)-expressing retroviral vector, to produce amphotropic retrovirus by transfection of the Phoenix cell line with the calcium phosphate method (www.stanford.edu/group/nolan/retroviral_systems/retsys.html). Transfected cells were grown under puromycin selection (2 μg ml^{-1}), which was removed 24 h before collecting the virus. MZ2-MEL3.0 cells, grown on retronectin-coated dishes (Takara, Japan), were transduced with the recombinant viruses to create cells stably expressing US11 and the control construct.

Adenovirus vectors

The 911/pIX_TCR^{A1M1} helper cell line was transduced with the replication-deficient adenoviral vector HAdV- 5.CMVLUCΔE1ΔE3ΔpIX. Production and characteristics of this vector are described elsewhere (17). The vector carries a firefly luciferase transgene under control of the human CMV immediate-early promoter. In this vector the pIX gene had been deleted from the genome. Three days after transduction, the offspring virus was harvested and purified by CsCl banding, resulting in the virus HAdV-5.CMVLUCΔE1ΔE3ΔpIX+pIX_TCR^{A1M1} (indicated in this paper as HAdV-5/pIX_TCR^{A1M1}). Adenovirus particle titers were determined by measuring optical density at 260 nm, using a standard protocol (51). The control virus used in all retargeting experiments was HAdV-5.CMVLUCΔE1ΔE3 (indicated as HAdV-5).

Immunohistochemistry

Immunohistochemistry was performed on the helper cell line 911/pIX_TCR^{A1M1}, with or without HAdV- 5.CMVLUCΔE1ΔE3ΔpIX infection. Infection was performed with 1000 virus particles per cell, 24 h before fixation. After washing with phosphate-buffered saline (PBS), the cells were fixed in acetone/methanol (1:1) for 10 min at room temperature. Staining was performed with the antibodies α-pIX (rabbit polyclonal (1:500), kindly provided by Dr Keith Leppard, University of Warwick, UK) (52), Vα12.1 (Pierce-Endogen Biotechnology, Rockford, IL, USA) and α-hexon (clone BOD604, fluorescein isothiocyanate (FITC)-conjugated, Biodesign International, Saco, ME, USA). FITC-labeled antibodies (Jackson ImmunoResearch, France) were used as secondary antibody. Upon analysis of the uninfected cells, the nuclei were stained using propidium iodide. Exact detection procedure has been described previously (18).

Wide-field microscopy was performed with a Leica DM-IRBE microscope. Confocal laser scanning microscopy was performed on a confocal microscope system (model TCS/SP2; Leica). Z-series images (slice spacing 0.45 μm) were acquired with a 63_ NA 1.4 plan Apo objective and were analyzed with Leica confocal software.

Immunoblotting procedures

Western analysis was performed to analyze production of pIX_TCR^{A1M1} in the helper cells and to analyze incorporation efficiency of pIX_TCR^{A1M1} in the virus capsid. Cell lysates were made in radioimmunoprecipitation assay lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40). Protein concentrations were measured with the bicinchoninic acid protein assay (Pierce Biotechnology, Perbio Science BV, Etten-Leur, the Netherlands). Virus lysates were prepared by adding 5×10^9 virus particles directly to western sample buffer. The pIX variants were visualized with rabbit polyclonal α-pIX serum (1:2000) (52). Goat polyclonal α-hexon (1:1000) (Abcam, Cambridge, UK) was used as a virus particle loading control. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat-α-rabbit and rabbit-α-mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The western blotting and detection procedures have been previously described (17,37).

A spot-blot type analysis was used to detect viral capsid proteins in intact virus particles. Immobilon membrane (Millipore, Etten-Leur, The Netherlands) was activated with methanol, washed for 10 min with PBS and virus (3×10^9 particles) was spotted onto the membrane. Virus was spotted as intact particles (untreated) or as virus lysate (denatured through adding western sample buffer plus 5 min incubation at 98 °C). This was followed up by blocking for 1 h in Soja milk (Alpro soja, Breda, The Netherlands), and washing twice for 10 min with 0.2% Tween20 in PBS. Thereafter, probing of capsid proteins was performed according to the same protocol as applied for the western analysis. The primary antibodies used were mouse monoclonal α-fiber knob (1D6.14, 1:1000) (53), goat polyclonal α-hexon (1:2000; Abcam), α-flag (1:500) (α-flag M2 Affinity Gel Freezer-Safe; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands), rabbit polyclonal α-pIX (1:2000) (52) and α-pVII antibody (1:500) (kindly provided by Saw See Hong, Laboratoire de Virologie et Pathoge´ne`se Virale, Lyon, France). Secondary antibodies used were HRP-conjugated goat-α-rabbit and rabbit-α-mouse (Santa Cruz Biotechnology).

Binding of virus particles to anti-TCR-coated beads

The ability of the pIX_TCR^{A1M1} virus to bind to anti-TCR-coated beads was investigated through an immunoprecipitation assay. Protein G Sepharose beads (30 µl; Pierce Biotechnology) were incubated with 1 µg of antibody (Vα12.1 or Vβ1), for 2 h at 4 °C in 600 µl cold PBS. Antibody-coupled beads were added to 1 ml DMEM without serum, containing two types of virus; HAdV-5.CMVLUCΔE1ΔE3ΔpIX (5×10^{10} particles), and HAdV-5.CMVLacZΔE1 (5×10^{10} particles). Control samples were incubated with beads only. After incubation for 12 h at 4 °C, 50 µl of the supernatant was added to 450 µl DMEM (containing 8% FBS), which was subsequently applied on MEL2a cells in a 24-well plate. After 24 h, the expression levels of intracellular luciferase

and β-galactosidase were determined. To this end, the cells were washed once with PBS, and lysed in 100 µl LUC-lysis mix (25 mM Tris-phosphate (pH 7.8), 2 mM CDTA, 2 mM DTT, 10% glycerol and 1% Triton-X in PBS). After shaking for 15 min at room temperature, lysates were centrifuged (10000 g, 10 min). Luciferase production was determined with Promega Luciferase Assay, and β-galactosidase production was determined with the Tropix Galacto-Light reporter gene assay (Applied Biosystems, Bedford, MA, USA). Light intensity measurement was performed in a Victor Wallac 2 microplate reader (PerkinElmer, Inc., Waltham, MA, USA).

Virus transduction assays

Transduction efficiencies with HAdV-5/pIX_TCR^{A1M1} and HAdV-5 were analyzed by measuring luciferase production. For all virus transduction assays, transduction was performed in 24-well plate, with 1000 viral particles per cell, in 500 µl DMEM/8% FBS. After 24 h, luciferase production was measured as described above (25 µl luciferase assay reagent was added to 10 µl lysate).

Incubation of cells with α-HLA-ABC antibody (ascites) was performed to test downmodulation of targeting. The cells were incubated for 12 h with 20 µg ml^{-1} α-HLA-ABC (in medium), which was removed from the cells before adding the virus. TCR blocking analysis was performed by incubating the virus (10^8 particles) for 12 h (4 °C) with 150 ng Vα12.1 antibody in 100 µl PBS supplemented with 2% horse serum. After adding 400 µl medium, the virus was added to 10^5 cells (resulting in a titer of 1000 particles per cell).

For peptide loading of the MZ2-MEL2.2 cells, 500 ng MAGE-A1 peptide, or 500 ng of an irrelevant peptide derived from influenza virus A nucleoprotein antigen (54) was added per well 2 h prior to adding the virus.

Flow cytometry

To perform flow cytometry analysis on the different cell lines, the cells were trypsinized, resuspended in PBS (containing 0.5% bovine serum albumin and 0.02% sodium azide), and were incubated with antibodies. The cells were incubated with saturating conditions of α-CAR antibody (55), α-HLA-ABC antibody (W6/32, Cedarlane, ON, Canada) or α-HLA-A1 antibody (One Lambda, CA, USA) for 30 min on ice, followed by incubation with phycoerythrin (PE)-labeled secondary antibody (Caltac Laboratories, Burlingame, CA, USA) for 30 min on ice. The status of HLA-ABCGE expression at the cell surface of the US11-expressing MZ2-MEL3.0 cell line and the control lines was performed by using α-HLA-ABCGE (B9.12.1, Immunotech, Marseille, France) as primary antibody and allophycocyanin (APC)-conjugated goat-α-mouse immunoglobulin G (Jackson ImmunoResearch) as secondary antibody. Flow cytometry data were analyzed with CellQuest software (Becton Dickinson).

ACKNOWLEDGEMENTS

We thank Karien Wiesmeijer (Molecular Cell Biology, LUMC, Leiden) for performing the confocal microscopy, and Saw See Hong (Laboratoire de Virologie et Pathoge ´ne`se Virale, Lyon, France) for providing us with the α-pVII antibody. This work was supported by the European Union through the 6th Framework Program GIANT (contract no. 512087).

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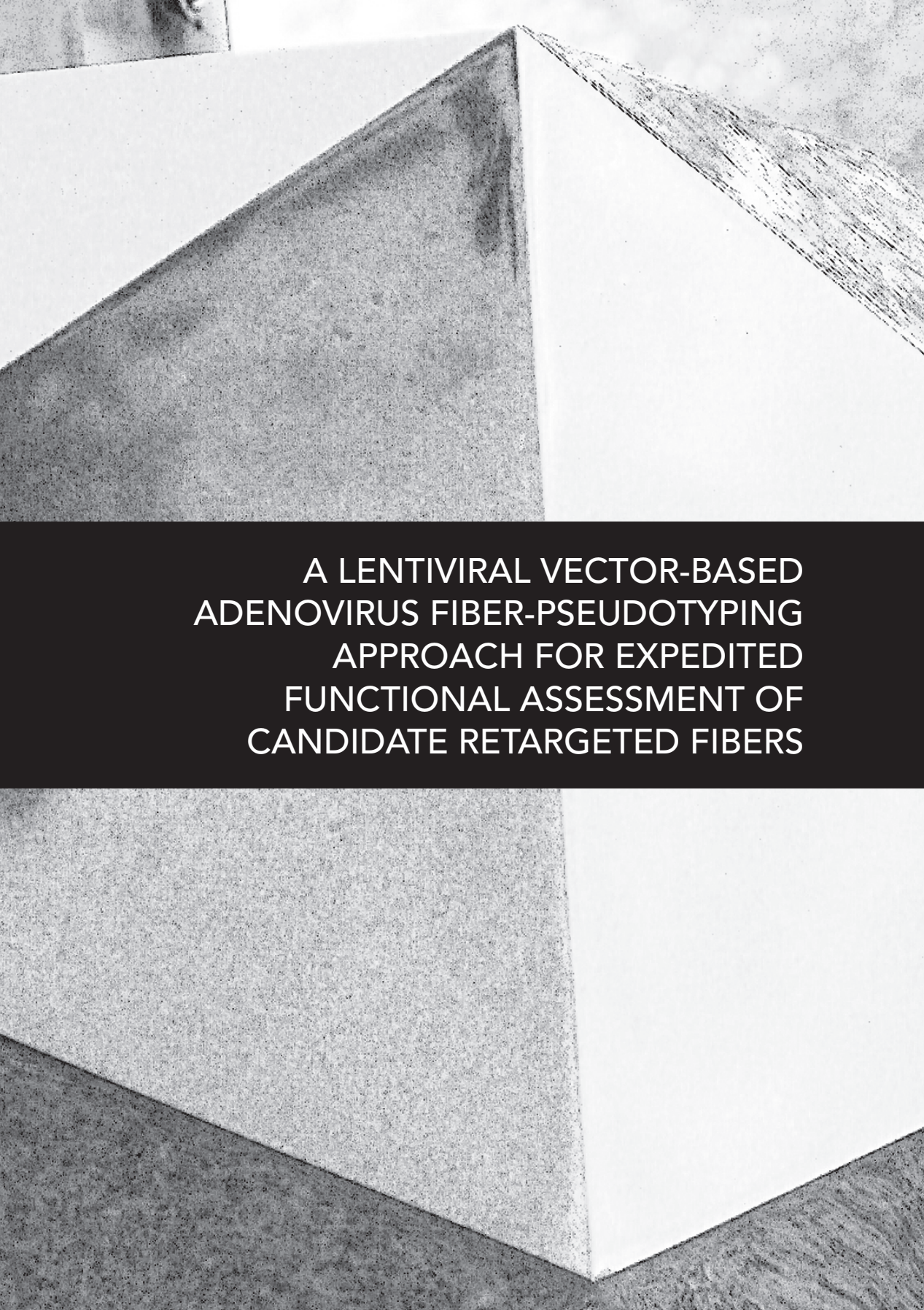
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A LENTIVIRAL VECTOR-BASED ADENOVIRUS FIBER-PSEUDOTYPING APPROACH FOR EXPEDITED FUNCTIONAL ASSESSMENT OF CANDIDATE RETARGETED FIBERS

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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Journal of Gene Medicine 2009;11(11):990-1004

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 fibermodels 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% CO_2.

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 FibΔCAR-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 *Pac*I-digested recombinant genome into 293HER2/*neu*. AdGLΔF/F and its fiber-pseudotyped versions AdGLΔF/R7Knob and AdGLΔF/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 pAdGLΔF, 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 *Eco*RI 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, pΔFS, a pFS derivative containing the above mentioned 1658-bp fiber gene deletion, was made by inversed polymerase chain reaction (PCR) on pFS using oligonucleotides FIB-Ct-SwaI-s and FIB-Nt-as (Table 1) and subsequent self-ligation of the resulting 8.0-kbp PCR product. Subsequently, within pΔFS, 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-SwaI-s	ATTTAAATTTTCATGGGACTGGTCTGG
FIB-Nt-as	CTGCAACAACATGAAGATAGTGG
Fib-FWD-PstI	CAGCACTGCAGACCATGAAGCGCGCAAGACCGTC
Fib-REV-XhoI	CGACACTCGAGCTATCATTCTTGGGCAATGTATGAAAAAGTG
TPL-PstI-s	CAGCACTGCAGACTCTCTTCCGCATCGCTG
TPL-FIB-as	GGTCTTGCGCGCTTCATCTTGCGACTGTGACTGGTTAGACG
TPL-FIB-s	CGTCTAACCAGTCACAGTCGCAAGATGAAGCGCGCAAGACC
F-BsrGI-as	GACGTACTGTACACTATCATTCTTGGGCAATGTATG
800E6-NcoI-MluI-F	GCATCGAAGCCATGGGACGCGTCGACTCGAAGC
800E6-BsrGI-R	CTTACCGGATGTACATTAGTTACCGGATCCC

[a]Restriction sites referred to in the Ma
terials and Methods are underlined.

*Spe*I site-containing, 1.3-kbp *Mfe*I-*Eco*RI pAdEasy-I fragment into the unique *Eco*RI site of pΔFS, yielding pΔFS2. Finally, pAdGLΔF was generated by homologous recombination (in *E. coli* BJ5183) (33,34) between the 5.7-kbp *Bgl*I-fragment of pΔFS2 and *Spe*I-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 *Pac*I-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-XPRE- SIN (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 *Pst*I and *Xho*I, and ligation of the resulting fragments in between the *Pst*I and *Xho*I 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 *Pst*I and *Bsr*GI 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 *Pst*I-*Pml*I 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 *Nco*I 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 *Bsh*TI 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 *Pst*I-*Bsr*GI 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 treatedwith 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, Philedelphia, PA, USA) (43) or, as a control, BSA was diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6) to a concentration of 5 μg/ml. Subsequently, 100-μl 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

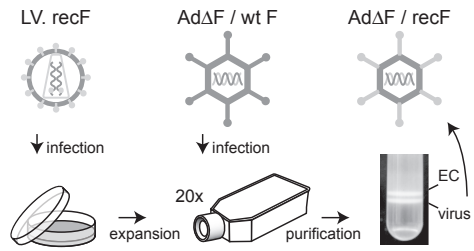


Figure 1. A lentiviral vector-based adenovirus fiber-pseudotyping system for rapid testing of new fiber variants in the virus particle context. Cells stably expressing a recombinant fiber of interest are generated by a small scale transduction with a lentiviral vector (LV.recF). Expansion of these cells 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, TPLcontaining 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,

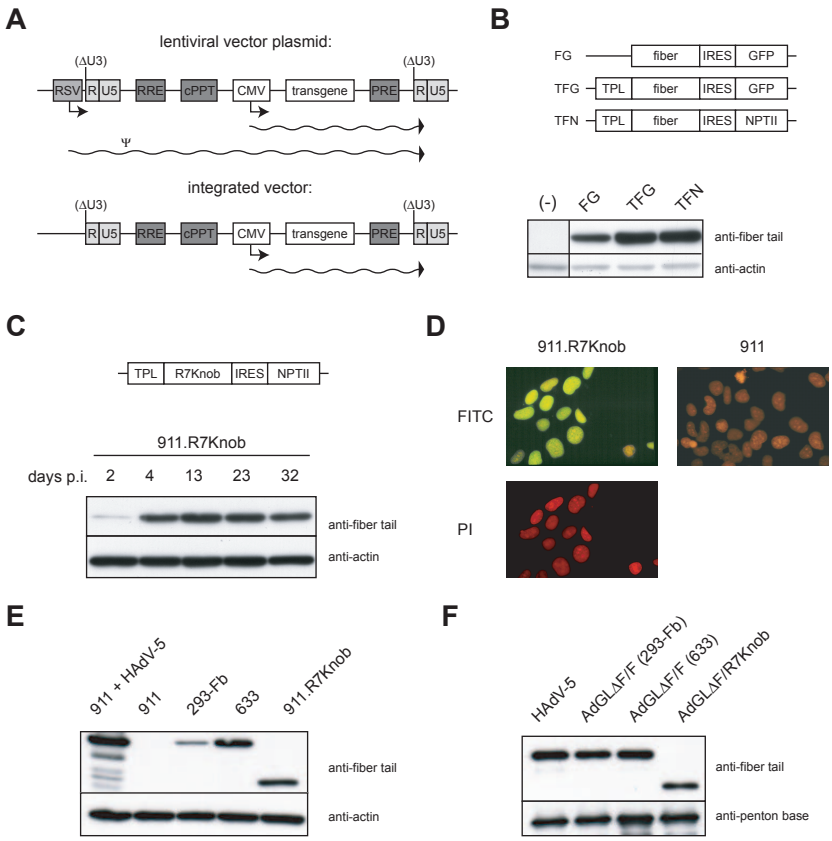


Figure 2. Usage of lentiviral vectors for the generation of packaging cells employable for fiber-pseudotyping of Ad. (**A**) Schematic outline of the lentiviral vector system. The lentiviral vectors employed are based on self-inactivating, third-generation HIV-I-derived vectors (46,47) and are further optimized for enhanced integration capacity and transgene expression (35). RSV, Rous sarcoma virus promoter; ΔU3, deletion of (most of) the U3 region; RRE, HIV-I Rev responsive element; cPPT, HIV-I 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 HAdV-5. (**F**) Western analysis on purified virus particles demonstrating capsid incorporation of R7Knob. Per lane, 4×10^9 particles were loaded of HAdV-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 levelwas 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, AdGLΔF/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 AdGLΔF/F. After 72 h, virus progeny was harvested and purified by a standard CsCl gradient-based protocol, yielding a purified AdGLΔF/R7Knob preparation. Subsequently, to evaluate capsid incorporation capacity of the R7Knob fiber supplied by the complementing cells, AdGLΔF/R7Knob was subjected to western blot analysis alongside with HAdV-5 and two AdGLΔF/F preparations (Figure 2F). The results clearly show that the R7Knob fiber was incorporated into the AdGLΔF/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-tocell variability (Figure 3B). With respect to localization, however, expression appeared to be somewhat aberrant because the 800E6 fiberwas 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

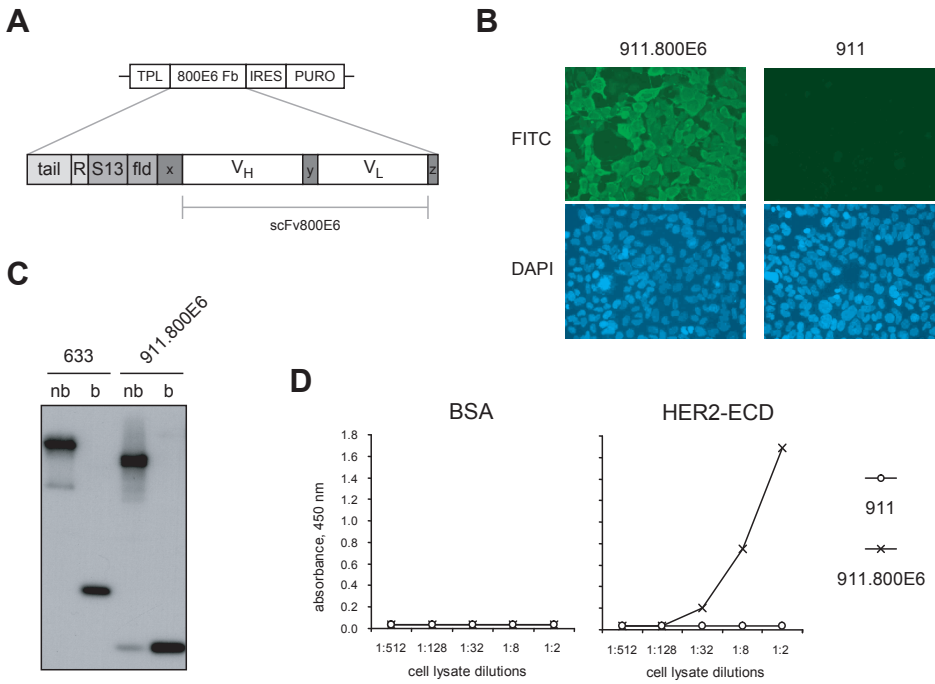


Figure 3. Design, LV-mediated expression, and functional characterization of 800E6 fiber. (**A**) Transgene construct configuration for lentiviral vector-mediated expression of 800E6 fiber. The components of the 800E6 fiber gene are depicted to scale. PURO, puromycin resistance gene; tail, fiber tail domain (44 amino acids); R, fiber shaft first repeat (15 amino acids) followed by residues Gly and Met; S13, fibritin coiled coil segment 13 (39 amino acids); fld, fibritin foldon domain (30 amino acids); x, spacer (25 amino acids), VH and VL, variable fragments of monoclonal antibody 800E6 (53) heavy and light chain, respectively; y, $(G_4S)_3$ 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-liable 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 AdGLΔF/F, a CsCl purified stock of 'AdGLΔF/800E6 Fb', a fiber gene-deleted Ad vector transiently loaded with the 800E6 fiber.

The chimeric fiber loadwas investigated by western blot analysis of purified AdGLΔF/800E6 Fb and, as a control, wild-type HAdV-5 virions (Figure 4A). Qualitatively, this experiment demonstrated that AdGLΔF/800E6 Fb incorporates the full-size 800E6 fiber. However, quantification of fiber content by normalization to hexon shows that the chimeric fiber load in AdGLΔF/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-stoichiomeric 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

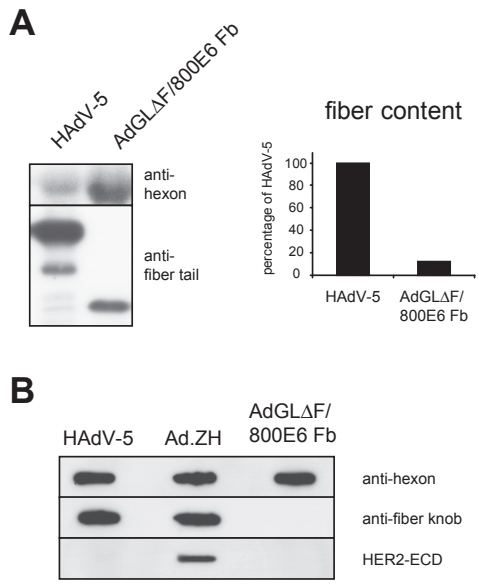


Figure 4. Functional assessment of the 800E6 fiber in the context of fiber-pseudotyped Ad particles. (**A**) Western analysis on purified virus particles to assess the capsid incorporation capacity of 800E6 fiber. Of HAdV-5 and AdGLΔF/800E6 Fb, 2×10^{10} and 4.6×10^{10} CsCl-purfied particles were loaded, 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 AdGLΔF/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-

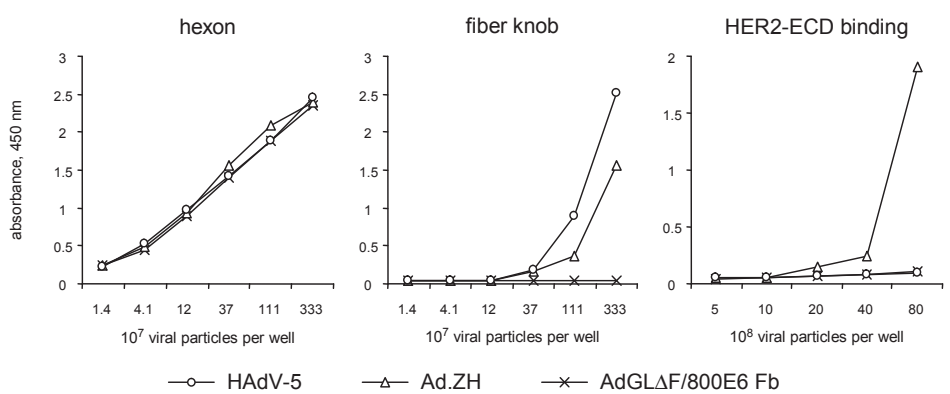


Figure 5. ELISA-based binding experiment to assess antigen binding capacity of capsid-incorporated 800E6 fiber. CsCl-purified HAdV-5 (negative control), Ad.ZH (positive control), and AdGLΔF/800E6 Fb were immobilized on an ELISA plate and probed with either anti-hexon antibody (left graph), anti-fiber 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.

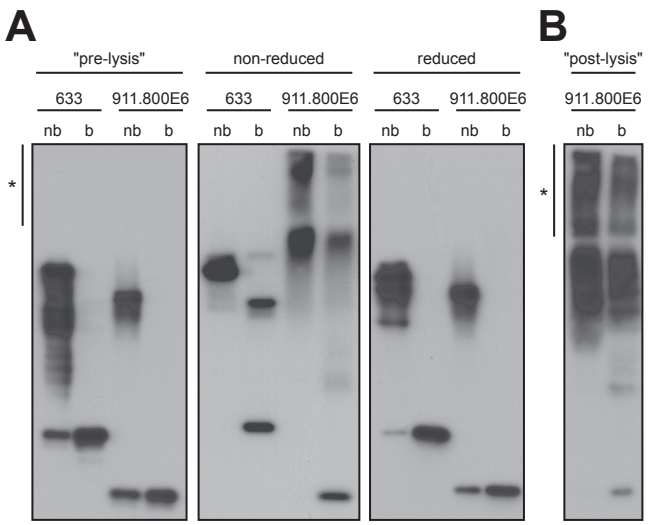


Figure 6. Western blot analysis to assess the redox state of 800E6 fiber before and after cell lysis. (**A**) Analysis of the pre-lysis redox states of 800E6 fiber and wild-type fiber. Nonreduced samples representing the pre-lysis redox states (left panel) of 800E6 fiber-expressing (911.800E6) and wild-type 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, fibritin 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).

ACKNOWLEDGEMENTS

We thank Jurgen Seppen for providing plasmids of the lentiviral vector system, Ralph Willemsen for a fiber-fibritin fusion construct, Dan von Seggern formaking the 633 cell line available, Monika Lusky for the 293-Fb cell line, Victor van Beusechem for the 1D6.14 antibody, Patrizia Galeffi for helpful suggestions during the preparation of the manuscript, and Kaori Suzuki for critically reading the manuscript. This work was supported in part by the European Union through the 6th Framework Program GIANT (contract no. 512087).

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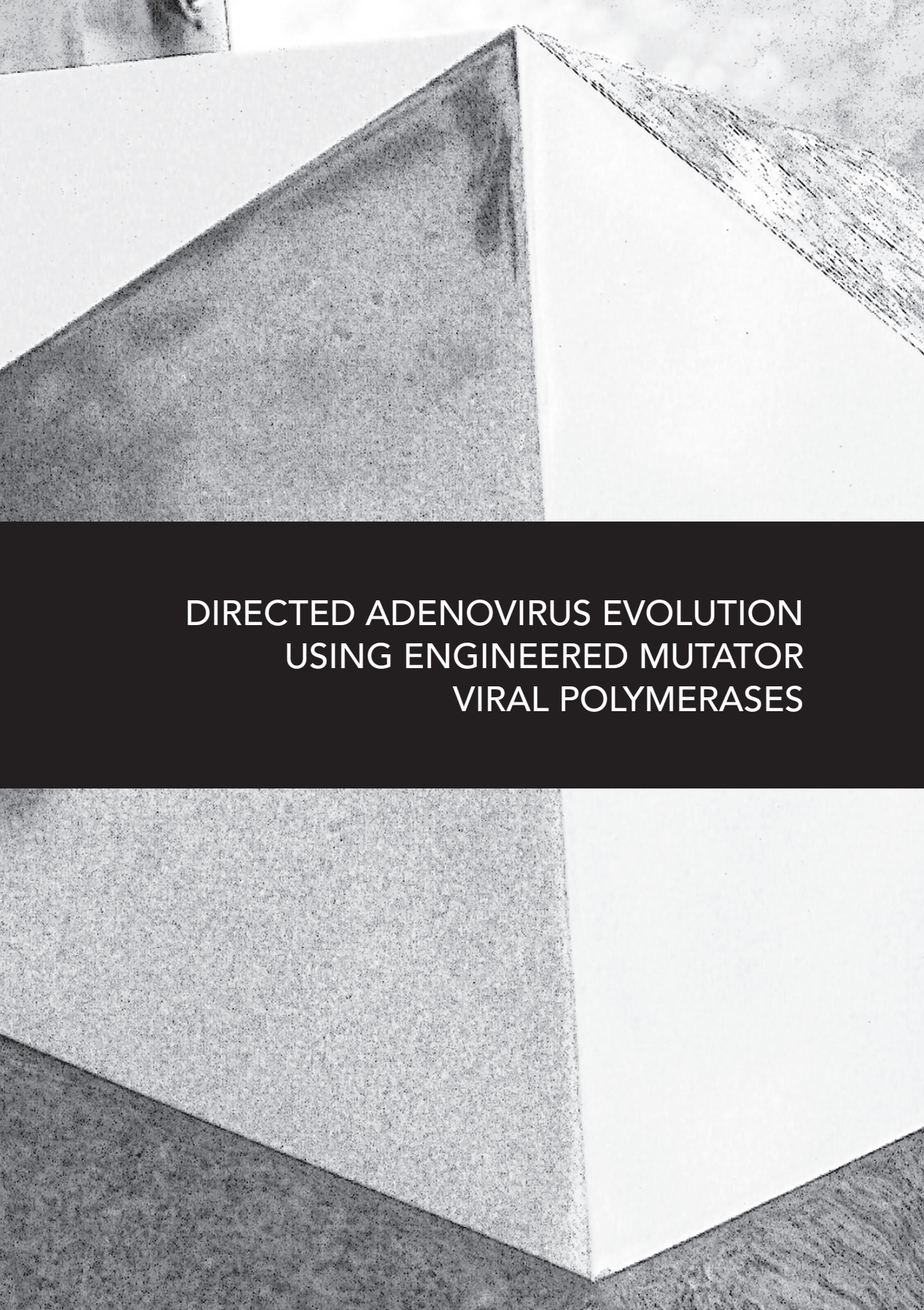
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5a



DIRECTED ADENOVIRUS EVOLUTION USING ENGINEERED MUTATOR VIRAL POLYMERASES

ABSTRACT

Adenoviruses (Ads) are the most frequently used viruses for oncolytic and gene therapy purposes. Most Ad-based vectors have been generated through rational design. Although this led to significant vector improvements, it is often hampered by an insufficient understanding of Ad's intricate functions and interactions. Here, to evade this issue, we adopted a novel, mutator Ad polymerase-based, 'accelerated-evolution' approach that can serve as general method to generate or optimize adenoviral vectors. First, we site-specifically substituted Ad polymerase residues located in either the nucleotide binding pocket or the exonuclease domain. This yielded several polymerase mutants that, while fully supportive of viral replication, increased Ad's intrinsic mutation rate. Mutator activities of these mutants were revealed by performing deep sequencing on pools of replicated viruses. The strongest identified mutators carried replacements of residues implicated in ssDNA binding at the exonuclease active site. Next, we exploited these mutators to generate the genetic diversity required for directed Ad evolution. Using this new forward genetics approach, we isolated viral mutants with improved cytolytic activity. These mutants revealed a common mutation in a splice acceptor site preceding the gene for the adenovirus death protein (ADP). Accordingly, the isolated viruses showed high and untimely expression of ADP, correlating with a severe deregulation of E3 transcript splicing.

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Nucleic Acids Research 2011; 39(5):e30

INTRODUCTION

Viruses with life cycles involving lytic disruption of host cells are being explored for their use as oncolytic agents (1). Oncolytic viruses are unique anticancer agents owing to their ability to amplify their cell-lytic effect through replication and viral spread. This ability, combined with the promise of tumor selectiveness (2), fosters the hope that 'oncolytic virotherapy' could ultimately be more efficient and cause less side effects than existing therapies.

Adenovirus (Ad) is one of the most-studied viruses for oncolytic virotherapy and its potential has been demonstrated by promising preclinical studies and clinical trials (2,3). So far, however, the clinical efficacy of Ad-based virotherapy has not been spectacular; only when viral treatment was combined with more conventional therapies were the results unequivocally positive (4,5). Therefore, many seek to develop improved oncolytic Ads endowed with enhanced tumor cell killing abilities (2,3,6–11). In this regard, in addition to strategies based on rational design, 'bioselection'- or 'directed evolution'-type processes—i.e. methods based on genetic diversification and phenotypic selection—have proven useful to generate new oncolytic Ads (12–15). In studies that took such approaches, whole-genome genetic diversification was achieved either by chemical mutagens, by ultraviolet radiation or by recombination among co-infected Ad serotypes.

Here, we describe the development and validation of a new directed Ad evolution approach that is based on the high mutation rates achieved by engineered mutator Ad polymerases. This 'accelerated evolution' approach is distinct, conceptually and practically, from classical procedures employing chemical or physical mutagens. First, the use of mutator viral polymerases avoids the direct virus inactivating effects normally associated with mutagens (i.e. damage to the virus particle and irresolvable DNA lesions) (16,17). Second, and relatedly, our approach is inherently capable of bringing about genetic diversity over repeated viral infection rounds. Importantly, this property allows for multistep viral adaptation processes to occur, i.e. a virus may successively acquire multiple beneficial mutations. Thus, owing to the above aspects, this Ad engineering approach resembles not so much the classical genetic screens, but rather the adaptation processes by which the rapidly mutating RNA viruses—and their recombinant derivatives—can be readily altered or optimized (18–28). In this regard, many such RNA virus adaptation procedures have already led to potency-enhanced oncolytic viruses and/or optimized recombinant vectors.

First, to set up this system, we modified the Ad-encoded polymerase (Ad pol), a protein-primed family B DNA polymerase with proofreading function (29,30). Any mutator activities of the new Ad pol mutants were revealed by a deep-sequencing strategy allowing direct assessment of mutational buildups

in replicated viruses. Then, to validate our approach, several of the identified mutator polymerases were used in a directed evolution scheme aimed at increasing Ad's oncolytic potency. Interestingly, this procedure isolated viruses with a common mutation causing untimely expression—due to altered splicing—of the adenovirus death protein (ADP) (31,32).

Thus, our data demonstrate that mutator mutants of a viral DNA polymerase can serve to provide the genetic diversity needed for efficient directed evolution of a normally genetically very stable DNA virus. The methodology outlined in our study may represent a general strategy to generate or optimize Ad-based gene delivery vehicles and oncolytic vectors.

MATERIALS AND METHODS

Cell culture

HAdV-5 E1-transformed human embryonic retinoblast cell line 911 and human untransformed diploid foreskin fibroblast cell line VH10 were described previously (33,34). Human ovarian (SKOV-3), breast (SKBR-3) and prostate (PC-3) adenocarcinoma cell lines were obtained from American Type Culture Collection (ATCC). All cell lines were grown at 37°C in DMEM (Gibco BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (Gibco BRL) in a humidified atmosphere with 5% CO_2. Cells transduced with lentivirus LV.PP were grown in the presence of 0.7 µg/ml puromycin (MP Biomedicals, Amsterdam, The Netherlands). Cells stably transduced with lentiviruses were named after their parental constituents. For example, an SKOV-3 cell population transduced with lentivirus LV.PP is designated as SKOV-3.PP.

Lentiviral vectors

All lentivirus (LV) plasmids were based on pRRL-cPPT-CMV-X-PRE-SIN (35) or its derivative pLV.CMV.IRES.PURO (36), the latter of which carries an internal ribosomal entry site (IRES) followed by a puromycin resistance gene. LV vectors generated for this study were LV.AdPol, LV.PP and LV.pol-HA, which respectively encode for Ad5 pol, Ad5 pol bicistronically with puromycin and 'pol-HA', a C-terminally HA-tagged version of Ad5 pol. Furthermore, we developed a panel of 23 LV vectors, each encoding a single amino acid substitution mutant of pol-HA. Lentivirus stocks were titrated using a HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., New York, NY, USA). For the rationale for selecting the Ad pol residues to be mutated, and for further details on vector generation, see Supplementary Data.

Lentivirus-mediated expression of Ad pol variants

Cells seeded in 3-cm wells and grown, in one day, to a confluency of 50–70% were lentivirally transduced overnight in a 2-ml volume in the presence of 8 µg/ml polybrene. Transductions with LV.AdPol, LV.pol-HA and LVs encoding the Ad pol mutants were performed at 50 ng/ml p24 for 911 cells and 150 ng/ml p24 for SKOV-3 cells. Transductions with LV.PP were at 50 ng/ml p24 for SKOV-3 and VH10 cells and at 200 ng/ml p24 for SKBR-3 and PC-3 cells. For immunofluorescence detection of Ad pol variants' expression in transduced cell populations, cells were grown on glass coverslips, fixed and permeabilized with methanol, probed successively with an anti-HA tag and a fluorescien isothiocyanate (FITC)-conjugated antibody, and mounted in Dabco-glycerol for viewing under a fluorescence microscope.

Ad vectors

Two polymerase-defective Ad vectors, AdGLΔPOL (E1- and E3-deleted; reporter genes in place of E1) and HAdV-5ΔPOL (wildtype in sequence apart from the polymerase gene-affecting deletion), were generated as described in Supplementary Data. Viruses were purified by centrifugation on CsCl gradients by standard protocols. The physical viral particle titers (VP/ml) were determined using the fluorescent dye PicoGreen as described by Murakami and McCaman (37).

Ad pol *trans*-complementation assays

For all assays, semiconfluent parallel cultures of 911 cells stably expressing Ad pol variants were incubated with AdGLΔPOL at a multiplicity of infection (MOI) of 0.15 pfu/cell for 1.5–3.5 h. For assays with luciferase-based readout, the infected cells were lysed in lysis mix [25 mM Tris-phosphate (pH 7.8), 2 mM CDTA, 2 mM DTT, 10% glycerol and 1% Triton-X in PBS] and stored at −80°C until luciferase activities were measured (Luciferase Assay System, Promega). See Supplementary Data for the initial setup of the complementation system.

Mutation-accumulation procedure

AdGLΔPOL was subjected to 10 serial viral passages on semiconfluent cultures of 911 cells expressing Ad pol variants. For the first passage, the infections were carried out in 24-well plate wells at 0.15 pfu/cell. All subsequent passages were performed in six-well plate wells using 300, 30 and 3 µl of the harvested previous infection for inoculation. At 3 or 4 days after each inoculation, those wells were harvested where virtually all cells had been infected—as judged from green fluorescent protein (GFP) expression on day 2—and for which the cytopathic effect (CPE) had become abundant. Harvested infections, consisting of dislodged cells and medium, were freeze-

thawed and cleared by centrifugation before being used as inocula for the next passage. To test for any polymerase-proficient recombinants within the virus populations, we performed PCRs on the polymerase region and screened for replication ability on non-complementing cells (i.e. 911 cells). Although one of the initial AdGLΔPOL stocks tested positive for a minute subpopulation of recombinants, all the passaged virus populations—intermediate and final—were negative.

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Preparation of viral pools containing approximately 50 clones each

Two different approaches were taken to prepare 50-clone virus pools from the passaged virus populations of the mutation-accumulation experiment. The first approach entailed infection of 911.pol-HA cells in duplicate at 100, 50 and 25 pfu/well in a six-well plate. Of the duplicate sets of infections, one served as an intra-experiment plaque assay titration and was therefore overlaid with 0.5% agarose (Invitrogen) medium. From the other set, the infection containing nearest to 50 clones (as inferred from the intra-experiment titration) was harvested—by collecting cells and medium—at full CPE (day 14). The second, presumably more accurate approach to obtain near 50-clone pools involved the pooling of 50 mostly single-clone infections. For this approach, multiple 96-well plates—seeded with 911.pol-HA cells—were infected with such an MOI that the majority (i.e. about 80%) of the wells were non-infected. The contents of 50 randomly selected infection-positive wells were harvested—after the occurrence of full CPE—and pooled together. In this procedure, the observed fraction of wells found to be infection-negative allowed for the calculation—using the Poisson distribution—of the average number of clones in an infection-positive well. Multiplication of this average clone number with 50 (i.e. the number of pooled infections) gave an approximation of the total number of clones in the pooled population.

Massively parallel sequencing and data analysis

(Procedures for the pilot massively parallel sequencing (MPS) run performed on the spiked test sample can be found in Supplementary Data.) From each 50-clone virus pool obtained in the mutation-accumulation experiment, a 6.5-kb fragment was PCR amplified by Phusion DNA polymerase (Finnzymes/NEB), purified and size selected using the SureClean PCR cleanup kit (Bioline) and, after electrophoresis, the JetSorb gel extraction kit (Genomed) (see Supplementary Data for primer sequences). For each purified product, 100 ng was fragmented by nebulization according to Illumina's 'Preparing Samples for Sequencing Genomic DNA' document. Fragment libraries—size selected for fragments of 200–300 bp—were subsequently prepared using Illumina's 'Preparing Samples for ChiP Sequencing of DNA' document. The libraries were subjected to 32 cycles of single-end sequencing by an Illumina Genome

Analyzer II (GAII) instrument using one flowcell lane per sample. Image analysis and base calling were performed by Illumina's Genome Analyzer pipeline (GAPipeline) using default parameters. Sequence reads passing the default Illumina chastity filter (threshold = 0.6) were outputted—by the GERALD module of the GAPipeline—in read files of the SCARF format. These files served—after their conversion to a suitable format—as input for the short read analysis pipeline SHORE (http://1001genomes.org/downloads/shore.html) (38). SHORE carried out alignments [through invocation of GenomeMapper (39)], performed quality filtering (i.e. masking of bases with assigned quality values below a set threshold) and generated forward and reverse consensus summaries (i.e. base counts per position in the reference sequence). Details on the workflow and parameter settings used can be found in Supplementary Data. MPS was also employed to obtain the complete sequences of several individual Ad genomes. Briefly, an Illumina multiplexed sequencing run was performed in one GAII flow cell, after which the resulting data sets were analyzed using the ELAND aligner of the GAPipeline and the Velv*et al*gorithm for de novoassembly (http://www.ebi.ac.uk/~zerbino/velvet/) (40).

Directed evolution procedure

HAdV-5ΔPOL was subjected to 10 serial passages on SKOV-3 cells expressing the Ad pol variants and then successively to 10 such rounds on SKOV-3. pol-HA cells. The passaging procedure was as described for the mutation-accumulation procedure except for the following distinctions. First, each infection was harvested by collection of only culture medium (i.e. without cells). Second, harvesting took place at the first sign of CPE, which was mostly at day 3. Third, the MOIs of the successive infections were strived to be kept relatively low. The latter was sought to be achieved by choosing, for each passage, a suitable narrow range of inoculation doses (e.g. 36, 12 and 4 µl of the harvested media of the previous passage) and by subsequently harvesting from among the resultant infections one exhibiting only a modest level of CPE. Virus stocks and passaged populations of HAdV-5ΔPOL all tested negative for any polymerase-proficient recombinants (see mutation-accumulation procedure above).

Cell killing assays

For cytotoxicity assays, semiconfluent cell cultures grown in 96-well plates were infected at the indicated MOIs. The infections were performed in duplicate (pools) or in triplicate (clones). Cell viabilities were measured on day 6 (911.pol-HA) or between days 10 and 15 (SKOV-3.pol-HA, SKOV-3. PP, SKBR-3.PP, PC-3.PP and VH10.PP) using the WST-1 reagent (Roche). For plaque size assays, semiconfluent SKOV-3.pol-HA cell cultures in six-well plates were infected for 2 h at low MOI, washed and then overlaid with 0.5%

agarose (Invitrogen) medium. On day 11, cells were fixed by adding either 1% formaldehyde on top of the overlays or pure methanol directly to the cells. Cells were then, respectively, stained by applying a 0.5% crystal violet solution in 25% methanol or by performing 3,3'-diaminobenzidine (DAB) immunohistochemistry, probing for Ad fiber expression (4D2, Abcam). DAB reactions were enhanced by complementing the DAB solution (product D5905, Sigma-Aldrich) with $NiCl_2$ and $CuSO_4$ (both to a final concentration of 0.03% w/v).

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Protein immunoblot analysis

Semiconfluent cultures of SKOV-3.pol-HA cells in 24-well plate wells (~1.5×10^5 cells/well) were incubated with 1000 VP/cell for 2 h. At 24 and 36 h after infection, cell lysates were made using 100 µl/well of RIPA lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% NP40) supplemented with protease inhibitors. Lysate aliquots (10 µl) were boiled in reducing sample buffer and subjugated to sodium dodecyl sulfate–polyacrylamide gel electrophoreses (SDS–PAGE) using a two-layer resolving gel composed of 12% (top) and 15% (bottom) polyacrylamide. After electroblotting to polyvinylidene difluoride membranes, blots were probed by anti-fiber tail (4D2, Abcam) and anti-E1A [M73 (41)] monoclonals, a rabbit antiserum raised against an ADP peptide [P63-77 (42); a kind gift from Dr W. S. M. Wold] and matching horseradish peroxidase-coupled secondary antibodies. Immunologically detected proteins were visualized by enhanced chemiluminescence.

Reverse transcriptase-polymerase chain reaction

SKOV-3.PP cells in six-well plate wells (~5×10^5 cells/well) were infected at 1000 VP/cell for 1.5 h. At 8 and 24 h after infection, total RNA was isolated according to the Total RNA Isolation/NucleoSpin RNA II kit (Macherey-Nagel). First-strand cDNA synthesis, using oligo(dT)20 primers, was performed on 250 ng of total RNA by the SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. One microliter portions of the reverse transcriptase reactions were subsequently used in PCRs using specific primer pairs listed in Supplementary Data.

Statistical analysis

The *P*-values assigned to the observed forward and reverse frequencies of a given minor variant indicate the probabilities of obtaining such frequencies or higher if they were to be due to sequencing errors. For the calculation of *P*-values, we assumed a Poisson distribution of errors, analogously to Wang *et al*. (43), and took as error rate estimates the 97.5^{th} percentiles of the observed minor variant frequency distributions (see Supplementary Data for

summary statistics). Usage of such high estimates of the error rate—instead of taking for instance the average of the minor variant frequency distributions—is thought to accommodate, at least partially, the presumed heterogeneity of the actual error rate (44).

RESULTS

Candidate mutator Ad polymerases and their ability to support Ad replication

To render Ad pol more error prone, we mutated residues putatively involved in maintaining polymerization fidelity. By extrapolation from data on other polymerase species, 19 Ad pol residues were selected for mutation (Figure 1). The selected residues roughly fall into two categories, those putatively involved with proofreading and those implicated in (geometric) selection of the incoming nucleotide. In total, 23 Ad pol mutants were generated (Supplementary Table S1), each engineered with a single amino acid substitution. Three of the targeted residues—N417, F421 and M689 (i.e. no. 4, 5 and 13 in Figure 1)—were represented by more than one mutant.

The newly engineered Ad pol mutants were assessed for their ability to support productive Ad replication. To this end, a *trans*-complementation assay was set up that involves lentiviral vector-mediated expression of Ad pol variants in cells, and the subsequent infection of these cells by a polymerase-defective reporter Ad vector (Supplementary Figure S1).

A first complementation assay revealed that nine of the Ad pol mutants supported productive replication of the polymerase-defective vector (Figure 2A shows green fluorescent protein (GFP) expression on day two for this experiment). The complementing mutants were T286I, N417A, F421Y, S506T, V585A, M689N, Y690F, D827A and S834E. Besides that they were associated with infected cells becoming brightly green fluorescent (indicating viral genome replication), these mutants also gave rise, eventually, to 'green comets' and/or plaques, demonstrating the production of viable progeny virus. Regarding the size and onset of such foci, marked variation was observed among the mutants, with M689N and Y690F lagging furthest behind. In summary of this assay and several subsequent, independent assays, all Ad pol mutants were assigned arbitrary complementation classes (Figure 2B).

Identification of mutator Ad polymerases

Ad pol mutants capable of supporting viral replication were subsequently analyzed for their proneness to cause mutations. For this purpose, a mutation-accumulation and sequencing scheme was devised for the direct assessment of mutational buildups in viruses replicated by virtue of the various Ad pol

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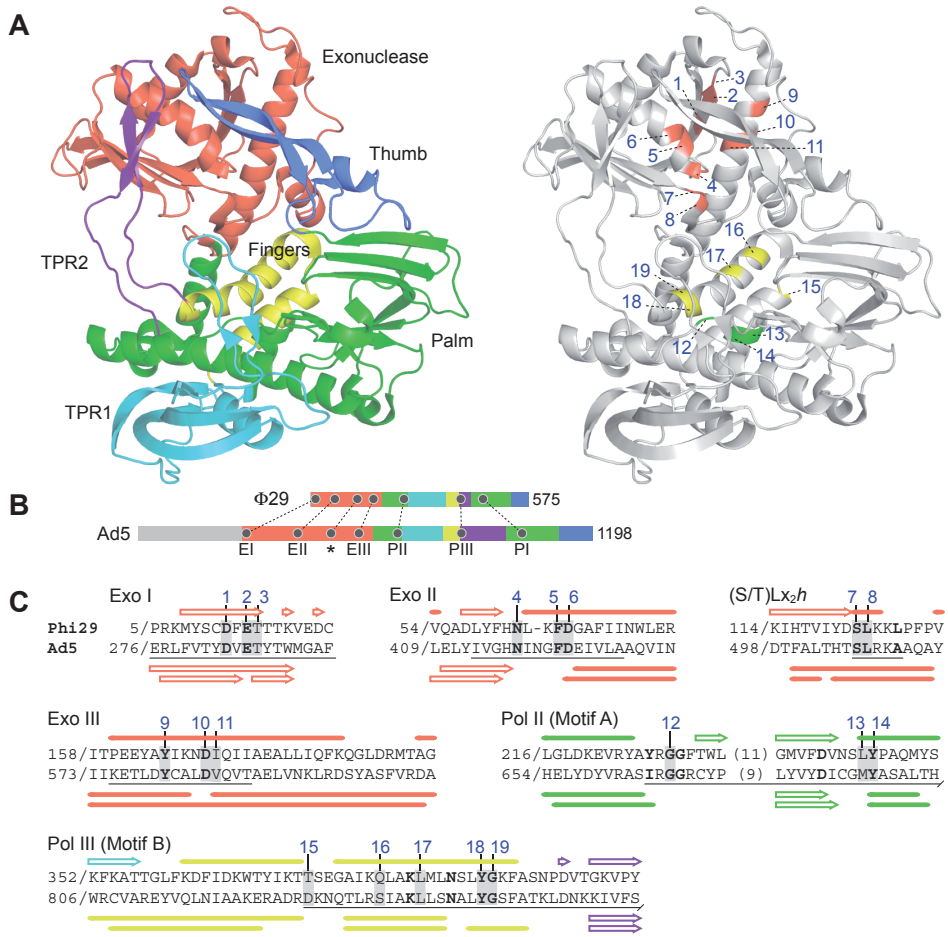
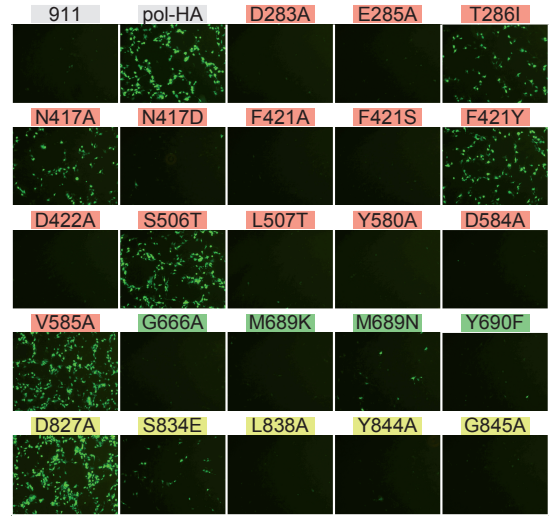


Figure 1. Ad pol residues targeted for mutation. Twenty-three single-amino acid substitution mutants of Ad pol were generated by mutation of nineteen selected residues implicated in governing replication fidelity (see also Supplementary Table S1). (**A**) The respective putative ϕ29 pol homologues of the targeted Ad pol residues are depicted in the context of a ϕ29 pol structure (Protein Data Bank code 1XI1) determined by Kamtekar *et al*. (47). The cartoon representation on the left shows the domain organization of ϕ29 pol while the right diagram points out the targeted residues. Protein structure visualizations were by Polyview-3D (http://polyview.cchmc.org/) using image rendering by PyMol. (**B**) The mutated residues reside in several of the most highly conserved polymerase sequence motifs, the locations of which are shown in linear representations, to scale, of the ϕ29 and Ad polymerases. Depicted are the 'Exo' motifs I, II and III (59), the (S/T)Lx2h motif (46) (indicated by the asterisk) and the 'Pol' regions I, II and III (72), which are also, respectively, known as polymerase motifs C, A and B (73). (**C**) Sequence alignments of conserved motifs of Ad and ϕ29 polymerases. The respective conserved motifs are underlined. Invariable residues are shown in bold face. Blue numbers point to the targeted Ad pol residues and their respective putative ϕ29 pol homologues. Secondary structures known for ϕ29 pol (47) and those predicted for Ad pol are, respectively, shown above and below the alignments. Closed bars indicate α-helices while open arrows represent β-strands. The upper and lower predictions for Ad pol were, respectively, by PredictProtein (http://www.predictprotein.org) and SABLE (http://sable.cchmc.org).

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B

#	mutant	complementation	#	mutant	complementation
	pol-HA	+++	9	Y580A	-
1	D283A	-	10	D584A	-
2	E285A	-	11	V585A	+++
3	T286I	++	12	G666A	-
4	N417A	++	13	M689K	-
4	N417D	-	13	M689N	+
5	F421A	-	14	Y690F	+
5	F421S	-	15	D827A	+++
5	F421Y	++	16	S834E	+
6	D422A	-	17	L838A	-
7	S506T	+++	18	Y844A	-
8	L507T	-	19	G845A	-

Figure 2. Ability of candidate mutator Ad polymerases to support full-fledged Ad replication. (**A**) A *trans*-complementation system (see Supplementary Figure S1 for its components) was employed to assess the ability of the various Ad pol mutants to support Ad replication. Control 911 cells or 911 cell populations stably expressing the respective Ad pol variants were infected with a polymerase-defective Ad vector (AdGLΔPOL). The mutants' abilities to *trans*-complement AdGLΔPOL were assessed by monitoring vector-encoded reporter gene expression. The images show GFP at 2 days post-infection. 'pol-HA' stands for HA-tagged Ad pol, which is parental to the other Ad pol variants. The exposure time was the same for all pictures. Not discernible under this setting was the very weak GFP signal in infected cells that do not *trans*-complement the polymerase defect (e.g. 911 cells). Mutants of the different polymerase domains are color-coded as in Figure 1 (exonuclease, red; palm, green; fingers, yellow). (**B**) Summary of the abilities of the Ad pol mutants to complement the polymerase-defective vector. The numbers in the columns denoted by a pound symbol (#) correspond to the residue numbers assigned in Figure 1. The depicted complementation classes (-, +, ++, and +++) were arbitrarily assigned to reflect the differential patterns of complementation abilities observed for the mutants in several independent experiments (e.g. Figure 2A).

variants (Figure 3A). Central to this scheme is the employment of a MPS technology to perform 'deep sequencing' of virus DNA obtained from multi-clone virus pools.

To implement this strategy, we first established a safe maximum to the number of viral clones that could be pooled while permitting detection of single-clone mutations. For this, we performed a pilot sequencing run on a 3.1-kb DNA fragment spiked with several minority mutants (Table 1). Analysis of this run demonstrated false positive-free mutation scoring using a minimal minor variant prevalence cutoff value of as low as 0.17% (Supplementary Figure S2), a cutoff that would theoretically allow a single-clone mutation to be detected in a pool of 588 even-sized clones. Based on this outcome,

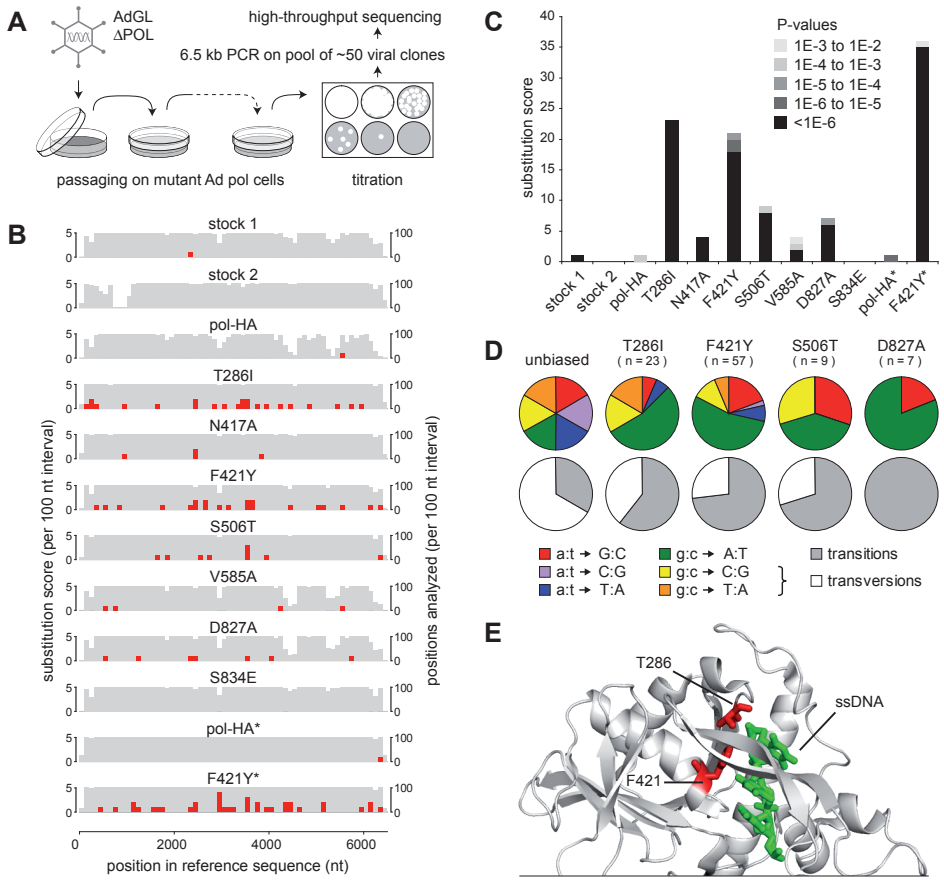


Figure 3. Identification of mutator Ad polymerases. (**A**) Mutation-accumulation and deep sequencing scheme. A polymerase-defective vector, AdGLΔPOL, was subjected to 10 serial infection rounds on candidate mutator Ad pol-expressing cell populations. For each passaged virus population, the potential mutation load buildup was assessed by deep sequencing (using a MPS technology) of a 6.5-kb virus DNA fragment obtained from a '50-clone' virus pool. After mapping of the sequence reads to the reference sequence, single-base substitutions were scored using a minor variant frequency cutoff value of 0.25% and a minimal sequencing depth requirement of 1200, both of which conditions were to be met for both the forwardly and reversely mapped read distributions. See Supplementary Figure S3 for details on mappings and minor variant detection. (**B**) Substitution score distributions over the length of the sequenced fragment. The grey background columns indicate, per 100-nt interval, the number of positions included in the analysis (i.e. the number of positions for which the minimal sequencing depth requirement was met). The red columns display the single-base substitutions scores. 'Stock 1' and 'stock 2' represent two independently prepared 50-clone pools of non-passaged viruses. Sample names marked with an asterisk (*) indicate the use of an alternative pooling method to obtain the '50-clone' pools. See text for details. (**C**) Total number of substitutions found per analyzed pool. For a given substitution, the assigned *P*-value range (shade of gray) relates to the higher of the two *P*-values estimated for the forward and reverse occurrence of that substitution. See the statistics section for *P*-value estimations. Further, see Supplementary Table S2 for the substitution scores expressed relative to the amount of DNA analyzed. (**D**) Base substitution spectra of the identified mutator Ad polymerases. The relative frequencies of the different types of substitutions were corrected for the GC content (of ~60%) of the ▶

Table 1. Detection of spiked variants by deep sequencing[a]

Spike no.	Position	Base substitution	Spiked frequency (%)	Observed frequency (%)		*P*-values[b]	
				F	R	F	R
1	1393	A → C	25.0	16.9	14.8	$<1\times10^{-14}$	$<1\times10^{-14}$
	1395	A → C	25.0	22.0	14.0	$<1\times10^{-14}$	$<1\times10^{-14}$
2	1398	C → T	6.25	6.75	6.07	$<1\times10^{-14}$	$<1\times10^{-14}$
3	1790	A → G	1.56	1.78	1.84	$<1\times10^{-14}$	$<1\times10^{-14}$
	1792	C → T	1.56	1.71	3.22	$<1\times10^{-14}$	$<1\times10^{-14}$
4	1806	A → C	0.39	0.61	0.56	6.5×10^{-13}	$<1\times10^{-14}$
5	2293	C → T	0.10	0.09	0.03	0.56	1.0
	2295	T → C	0.10	0.08	0.13	0.63	0.46

[a]This pilot deep sequencing run was performed on a spiked test sample consisting of a 3.1-kb DNA fragment spiked with several minority fragments, each containing a single or double nucleotide polymorphism. Depicted in this table are the frequencies with which the spiked variants occurred in the sequence read alignments. See Supplementary Data and Supplementary Figure S2 for more information on the sequencing run, the read alignment, and for an exemplification of minor variant detection.
[b]The *P*-values associated with the observed forward (F) and reverse (R) frequencies indicate the probabilities of obtaining such frequency levels or higher if they were due to sequencing errors. See the statistics section for the estimation of *P*-values.

we opted to sequence a 6.5-kb fragment from virus pools containing ~50 clones each, thus allowing for some experimental variations in clone size and number.

All Ad pol mutants capable of Ad replication, including the parental HA-tagged Ad pol (i.e. pol-HA), were included in a mutation-accumulation experiment involving 10 serial passages of the polymerase-defective Ad vector (Figure 3A). However, passaging using the two most weakly complementing mutants, M689N and Y690F, was discontinued prematurely as these mutants proved unable to sustain multiple-round viral propagation. From the 10 times passaged virus populations and, additionally, from 2 non-passaged virus stocks, virus pools were prepared containing near 50 clones each. Subsequently, from each prepared viral pool, a 6.5-kb Ad genome fragment, spanning from the gene for pIX to that for pTP, was amplified and sequenced by MPS. After mapping of the short sequence reads, base substitutions were scored by imposing—on both the forwardly and reversely mapped read

▶ analyzed sequence. (**E**) The ϕ29 pol homologues of Ad Pol residues T286 and F421 have previously been shown to entertain direct contacts with single-stranded DNA (ssDNA) (47). The concerned residues are depicted, with side chains, in a cartoon representation of ϕ29 pol, additionally displaying the cocrystallized ssDNA bound in the exonuclease active site. See Figure 1 for protein structure and visualization.

distributions—a local sequencing coverage depth requirement of 1200 and a minor variant frequency cutoff value of 0.25% (Supplementary Figure S3).

The base substitution scores found for the analyzed viral pools revealed that among the Ad pol mutants tested, all but one (i.e. S834E) displayed a degree of mutator activity (Figures 3B and C and Supplementary Table S2). Mutants T286I and F421Y were identified as the most distinctive mutators. With respect to the types of substitutions found, these two, but also the intermediate-level mutators S506T and D827A, exhibited a strong bias toward transitions, especially the transition involving the exchange of a G:C base pair for an A:T base pair (Figure 3D). Interestingly, the strongest of the identified mutators (T286I and F421Y) are both mutants of residues likely involved in stabilization of the displaced primer terminus at the exonuclease active site during proofreading (Supplementary Table S1). The putative homologues of these residues in Φ29 polymerase entertain direct contacts with single-stranded DNA (Figure 3E; 45–47).

Directed evolution using mutator polymerases yields virus pools with enhanced tumor cell killing potency

Next we investigated whether the identified mutator Ad polymerases could serve to facilitate the directed evolution of Ad. To this end, Ad pol variants N417A, F421Y, S506T, D827A, S834E and pol-HA, which together represent a wide range of mutator activities, were put into service in a directed evolution scheme involving multiple viral infection rounds on the human ovarian carcinoma cell line SKOV-3 (Figure 4A). Subjected to this selection scheme was an Ad5-based virus that, apart from the polymerase-disrupting deletion, is completely wild type. Further, the passaging conditions were chosen to strongly select for any faster growing mutants (see 'Materials and Methods' section for details).

Of the virus populations that had underwent the evolution procedure, several were found to have gained an enhanced cytolytic activity on the target SKOV-3 cells (Figure 4B). Interestingly, the observed cytotoxic potencies (Figure 4C) of the populations correlate with the mutator abilities (Figure 3C) of the Ad pol mutants used during bioselection. For example, directed evolution employing the highest level mutator, F421Y, proved to have yielded the most cytolytically potent virus population. Subsequent plaque size assessment on SKOV-3 cell monolayers further revealed the heterogeneity of the F421Y-bioselected population, with a considerable fraction of the viruses displaying a large plaque phenotype (Figure 4D).

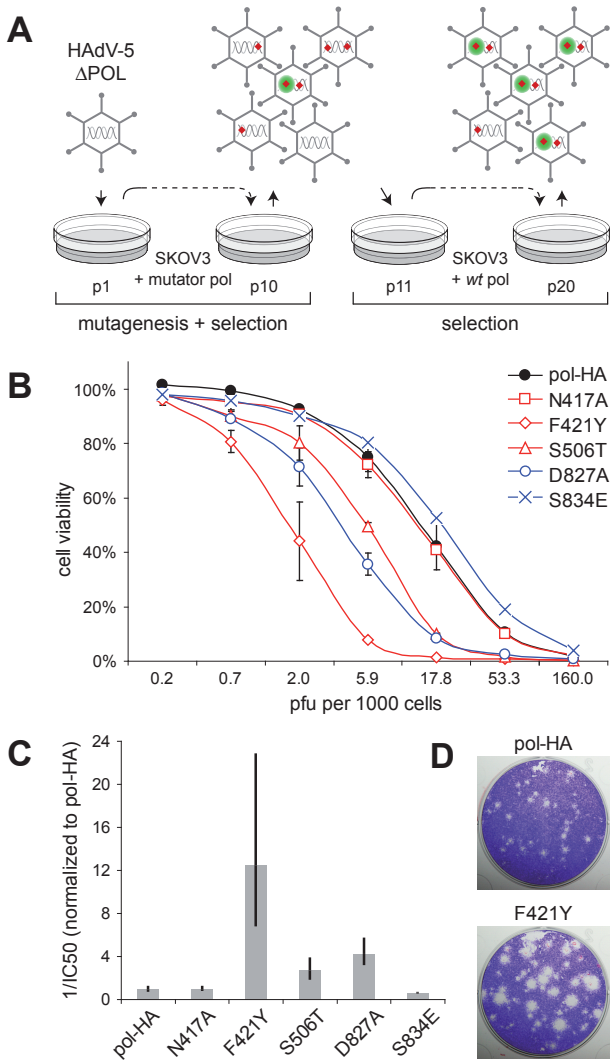


Figure 4. Directed evolution of Ad using mutator Ad polymerases. (**A**) Conceptual directed evolution scheme to achieve enhanced tumor cell killing. A HAdV-5-based virus, which otherwise from its polymerase-disrupting deletion is fully wild-type in sequence, is subjected to iterative rounds of infection on tumor cell populations. Initial virus passaging on mutator Ad pol expressing cells results in genetically diverse pools from which viruses with an acquired growth advantage are enriched for in later passages. Red diamonds represent mutations. Green circles indicate an acquired selective advantage. (**B**) Virus populations bioselected using the indicated Ad pol variants were analyzed for cytotoxicity on their target cells. SKOV-3 cells were infected at the indicated MOI's and cell viabilities were assessed by WST-1 assay. Exonuclease domain mutants are shown in red, fingers domain mutants in blue. (**C**) A 12-fold decrease in IC50 was observed for the virus population bioselected using Ad pol mutator F421Y. The inversed IC50 values were calculated from the cytotoxicity assay above and normalized to that of the pol-HA-bioselected pool. Error bars indicate 95% confidence intervals. (**D**) A subpopulation of the F421Y-bioselected viruses forms large plaques on SKOV-3 cell monolayers. Cells were grown under an agarose layer and stained with crystal violet.

Characterization of bioselected virus clones reveals overexpression of the adenovirus death protein

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From the F421Y-bioselected pool, two individual viral clones (F421Y-c1 and F421Y-c2) were isolated and further characterized. In a cell killing assay, both clones displayed an increased ability to kill SKOV-3 cells (Figure 5A), with each registering a difference with the non-bioselected 'stock' virus of at least a 100-fold (Figure 5B). Furthermore, on SKOV-3 cell monolayers, both clones were found to give rise, exclusively, to 'large' plaques (shown in Figure 5C for clone F421Y-c1). Apart from on SKOV-3, increased cell killing abilities were also observed on human breast (SKBR-3) and prostate (PC-3) carcinoma cell lines, and, to a lesser extent, on untransformed fibroblasts (VH10) (Supplementary Figure S4). In contrast, killing of a standard Ad producer cell line (911) was found to be either unaffected (F421Y-c1) or slightly attenuated (F421Y-c2).

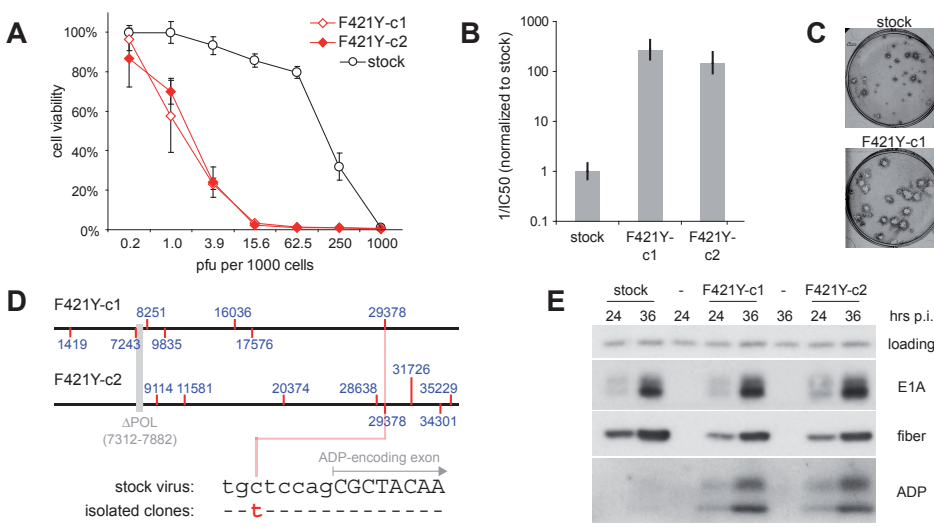


Figure 5. Characterization of isolated bioselected viral clones F421Y-c1 and F421Y-c2. Two clones with a large plaque phenotype were isolated from the F421Y-bioselected virus population and further characterized. Cell killing ability on SKOV-3 cells was assessed by a cytotoxicity (**A** and **B**) and a plaque size (**C**) assay. Error bars for the inversed IC50 columns indicate 95% confidence intervals. For the plaque size assay, cells were grown under an agarose layer and were visualized by immunohistochemistry for Ad fiber protein expression. See Supplementary Figure S4 for cell killing abilities on other (tumor) cell lines. (**D**) Sequencing of the genomes of the two clones revealed a shared mutation located within the splice acceptor site of the ADP-encoding exon. Mutations are indicated by red ticks. Blue numbers correspond to the respective positions in the wildtype HAdV-5 genome (accession no. AC_000008). See Supplementary Tables S3 and S4 for all detected mutations and their predicted consequences for primary protein structures. (**E**) The bioselected clones show highly increased levels of ADP expression. SKOV-3 cells were harvested for protein immunoblot analysis at 24 and 36 h after their infection. Blots were immunologically probed for Ad proteins E1A, fiber and ADP.

Viral genome sequencing revealed seven and eight mutations for the respective clones, including one shared by the two (Figure 5D and Supplementary Table S3). All mutations were scrutinized for having any conceivable relevant functional consequences. Of those that were found to change primary protein structure (Supplementary Table S4), none were obvious candidates to underlie the observed phenotype. Further, with respect to non-protein-coding functions, only the single common mutation, at position 29378 of the HAdV-5 genome, was identified by us as being potentially functionally relevant. The concerned mutation would affect a splice acceptor (SA) site preceding the exon encoding the adenovirus death protein (ADP) (31,42,48), a protein known to be required for efficient virus-mediated cell lysis and concomitant virus release (32).

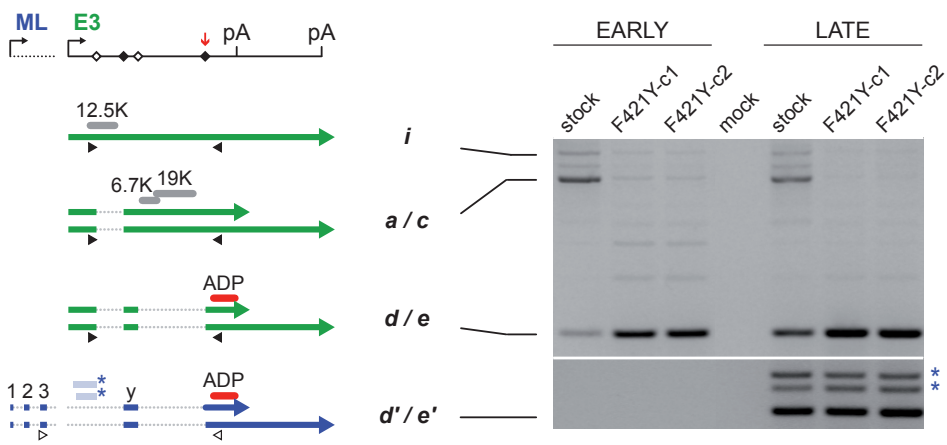


Figure 6. Viral clones F421Y-c1 and F421Y-c2 show a deregulated E3 splicing pattern strongly favoring the production of ADP-encoding mRNAs. RT–PCRs were performed with primer sets discriminating between E3 and ML promoter-derived ADP-encoding mRNAs. These primer sets, respectively indicated by closed and open triangles, have a common reverse primer (targeting the ADP-coding sequence) but consist of different forward primers (targeting the first E3 exon and the 3rd leader of the ML tripartite leader sequence). Depicted are all mRNA species recognized by these primer sets [according to Ad2 and Ad5 transcritption maps (42,48,49)]. E3 mRNAs (i.e. *a*, *c*, *d*, *e* and *i*) are shown in green while ML mRNAs (i.e. *d'* and *e'*) are depicted in blue. Within the E3 transcription unit, the splice donor and acceptor sites involved with splicing of the shown mRNAs are, respectively, depicted by open and closed diamonds. The red arrow points to the splice site found to be mutated in the bioselected clones. Further depicted are the relevant polyadenylation signals (pA), E3 proteins (12.5K, 6.7K, 19K and ADP) and leader sequences of the ML transcription unit (1, 2, 3 and y). The RT–PCR results show highly increased levels of E3- but not ML-derived ADP mRNAs. Furthermore, the altered alternative splicing of E3 transcripts was found to go at the expense of mRNA species *a*/*c* and *i*. The two asterisks next to the gel-image denote newly identified minority ML-derived ADP mRNAs. These variants presumably carry instead of the y-leader sequence one of two longer 'x-leader' exons (also denoted by asterisks) that would overlap with the first E3 exon. For control RT–PCRs on cellular β-actin and viral E1A mRNAs see Supplementary Figure S5.

Therefore, the ADP levels were investigated by immunoblot analysis at different time points after virus infection (Figure 5E). Importantly, both bioselected clones were found to exhibit highly elevated levels of ADP expression. Moreover, considerable levels of ADP were already detectable 24 h after infection, at which time the control virus showed no noticeable levels of ADP.

The overexpression of ADP correlates with altered splicing of E3 transcripts, not major late transcripts

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The ADP gene, although embedded in the Ad E3 region, is considered an element of both the E3 and the major late (ML) transcription units (Figure 6, on the left). Early in infection, ADP is translated from two scarce E3 promoter-derived mRNAs, termed *d* and *e* (48,49). Later in infection, however, ADP is mostly translated from two highly abundant ML promoter-derived mRNAs, termed *d*′ and *e*′ (42). Importantly, splicing of both ADP mRNA types—E3 and ML-derived—critically involves utilization of the splice acceptor site found to be mutated in the bioselected viruses.

To provide evidence for any altered splicing dynamics involving ADP-encoding transcripts, we performed a reverse transcriptase–PCR analysis using primer sets allowing discrimination between the two ADP mRNA types (Figure 6). Control reactions were performed on cellular β-actin and viral E1A mRNAs (Supplementary Figure S5). Significantly, both bioselected clones exhibited markedly increased levels of the E3-derived ADP mRNAs (i.e. *d* and/or *e*), especially early and also late in infection. By marked contrast, no such elevations were observed for the ML-derived ADP mRNAs (*d*′ and/or *e*′). Interestingly, the elevated levels of *d* and/or *e* mRNAs were found to be accompanied by an all-but-disappearance of the two normally abundant E3-derived mRNA species, *a* and *c* (48,49), which code for E3 proteins 6.7K and 19K. Furthermore, the normally lesser abundant mRNA *i*, encoding E3-12.5K, was also virtually absent. Thus, for both bioselected viruses, the splicing dynamics of E3 transcripts were found to be severely altered, displaying a marked shift in favor of the production of ADP-expressing mRNAs.

DISCUSSION

In the present study, we describe the development and validation of a conceptually new ‘accelerated evolution’ approach to Ad engineering that depends—for obtaining genetic diversity—on the error-prone viral genome replication achieved by engineered versions of the Ad polymerase. First, we report on the generation of several mutator Ad polymerases that sustain Ad replication. Next, we demonstrate that viral replication using such ‘sloppy’ Ad polymerases accelerates the adaptation to the selective pressures exerted

by a directed evolution scheme aimed at increasing the oncolytic potency of Ad. Together, these findings demonstrate that mutator Ad polymerases can facilitate bioselection protocols for the isolation of Ads with new phenotypes. Interestingly, the specific application of our approach to enhance oncolytic Ad was found to yield viral mutants exhibiting high and early overexpression of the ADP, an outcome that is directly relevant for designing and engineering new, more potent oncolytic Ads (2,3,8–11). Importantly, neither the identified mutation nor any other mutations with similar implicated effects (i.e. early cell lysis due to untimely ADP overexpression) have previously been isolated in bioselection studies involving chemical or physical mutagens (12,14).

To render Ad pol more error prone, we mutated Ad pol residues implicated in maintaining polymerization accuracy. In general, replication fidelity of proofreading-proficient DNA polymerases is governed by two main mechanisms: incoming nucleotide selection—based mainly on nascent base pair geometry—and exonucleolytic excision of misinserted nucleotides, i.e. proofreading (50,51). Accordingly, we aimed to compromise either of these functions of Ad pol by introducing substitutions within conserved motifs of either the nucleotide-binding pocket or the exonuclease active site. Since for Ad pol there is only limited structure–function information, we drew heavily from data available on other polymerase species (Supplementary Table S1).

Most of the Ad pol mutants constructed in this study were unable to support full-fledged Ad replication. Very generally, mutants non-supportive of virus replication might be either directly or indirectly—i.e. through global structural changes—impaired with respect to one or more essential aspects of viral genome replication (e.g. DNA binding, protein-primed initiation, transition between initiation and elongation, polymerase catalytic activity, strand-displacement and processivity). Strikingly, among the defective mutants were all those with substitutions of the universally conserved exonuclease residues contributing to the metal ion binding framework of the exonuclease active site (i.e. mutants D283A, E285A, D422A, Y580A and D584A). Previously, analogous substitutions in other viral polymerases—those of RB69, HSV and CMV—did prove compatible with virus replication (52–54). In contrast, for Φ29 polymerase, the concerned residues are known not only to be vital for exonucleolytic activity but also to play an essential role in strand-displacement functionality during polymerization (55,56). Speculatively, our data on Ad pol reflects a similar such dual role for these invariantly conserved residues.

To reveal any mutator activities of Ad pol mutants that were supportive of Ad replication, we performed 'deep sequencing'—by MPS—on pools of viruses replicated by the respective polymerases. Previously, deep sequencing has proven a powerful means to detect low-prevalence mutations in heterogeneous samples, such as virus populations, tumors and/or pooled samples (43,44,57,58). Here, we exploited Illumina's MPS technology to detect point mutations in a 6.5-kb viral DNA fragment obtained from near

50-clone pools of viruses that had undergone 10 viral replication rounds. Importantly, this approach enabled us to obtain direct molecular evidence for any mutational buildups in a procedural context that, with its multiple-round viral passaging on cells, resembles a directed evolution regime.

Of the Ad pol mutants that sustained multiple-round viral replication, several displayed a degree of mutator activity. The strongest of these were T286I and F421Y, respectively, mutants of exonuclease motifs I and II (59). These two mutators, as well as the weaker mutator N417A, carry replacements of residues whose counterparts in Φ29 are important for primer terminus stabilization—putatively through direct binding (47)—at the exonuclease active site during proofreading (45,46). Another of the identified mutators, S506T, carries a substitution within the 'S/TLx$_2$*h*' motif of the exonuclease domain (46). Akin to the above mutants, its mutator activity could result from a lesser ability to stabilize primer termini during proofreading. However, considering the buried state of the homologous residue in Φ29 polymerase (47), any such impact might be through indirect effects. Further, also D827A was identified as an intermediate-strength mutator. Residue D827, which is non-highly conserved, is putatively located on a loop structure connecting two α-helices of the fingers domain (see Figure 1, residue no. 15). Thus, the D827A substitution might have indirect effects on incoming nucleotide selection, perhaps through an altered or more flexible relative positioning of the two helices, as has been suggested for mutator activity-yielding substitutions at similar positions in *Pyrococcus furiosus* polymerase (60).

The mutation rates—at the viral level—achieved by Ad pol mutants T286I and F421Y are within the range of those reported for highly evolvable RNA viruses, indicating perhaps a particular suitability of these mutants for directed Ad evolution. For the two pools of F421Y mutant-replicated viruses (i.e. pools 'F421Y' and 'F421Y*'), the observed respective substitution loads were 73 and 100 per million base pairs (Supplementary Table S2). These numbers would correspond to a substitution rate, per viral passage, of 0.26 and 0.36 per Ad5 genome length equivalent (i.e. 36 kb). Considering that the latter of these measures is probably the more accurate (given the pooling methods used) and, furthermore, that these measures only represent the non-lethal mutations, we expect that viruses replicated by the F421Y mutant—and, by analogy, the T286I mutant—underwent genomic mutation rates of at least, but probably greater than, 0.4 per viral generation. By comparison, for several 'rapidly evolving' riboviruses (i.e. measles virus, poliovirus, rhinovirus and vesicular stomatitis virus), estimates for the per-genome per-generation mutation rate ranged from 0.26 to 2.3 (61). Likewise, the genomic mutation rates for the fast adapting retroviruses have been estimated at a mean of 0.3 per viral generation (62).

Mutator Ad polymerases successfully served in a directed evolution regime aimed at increasing the oncolytic potency of Ad. Ad pol mutants

were employed to replicate Ad—through trans-complementation—in a viral passaging scheme performed on the human ovarian carcinoma cell line SKOV-3. Resultant 'bioselected' virus populations that had undergone replication by either intermediate level (S506T and D827A) or strong (F421Y) mutators were found to have gained an increased cytolytic activity on the target cell line. In contrast, populations subjected to replication by weak and non-mutators (i.e. N417A and S834E, respectively) showed no evidence of any fitness gain relative to the control passaged virus population. These outcomes, combined with the mutation loads found in individual bioselected clones, demonstrate that engineered mutator Ad polymerases can be used to generate the genetic diversity needed to effectively facilitate directed Ad evolution.

A common mutation seen in the two isolated bioselected clones (at position 29 378 of the HAdV-5 genome) affects a splice acceptor site known to be critically involved in the generation of ADP-encoding mRNAs (42,48). Given that ADP is crucial for efficient cell death and virus release (32), its implication with the observed enhanced cytotoxic phenotype is highly conceivable. The ADP gene resides in the E3 region and is accordingly expressed, albeit relatively scarcely, from E3 promoter-derived mRNAs early in infection (42,48,49). However, ADP is additionally and much more abundantly expressed from major late promoter-derived transcripts arising later in infection (>24 h p.i.) (42). Both isolated virus clones exhibited dramatically elevated levels of ADP, the overproduction of which already became noticeable early in infection. Furthermore, the splicing pattern of E3 transcripts was found to be severely affected, with the generation of ADP-encoding mRNAs being strongly favored at the expense of other E3 mRNA species. In contrast, the levels of ADP-encoding mRNAs originating from the major late promoter were relatively unaffected. Thus, these data indicate an untimely expression of ADP that is mechanistically attributable to an increased utilization—within E3 transcripts—of the mutated splice acceptor site preceding the ADP-encoding sequence.

Several previous studies that aimed to enhance Ad's oncolytic potency have rationally sought to overexpress ADP or other cell death-inducing proteins (2,3,6–11). The ADP-overexpressing viruses generated in these studies were found to exhibit accelerated cell-to-cell viral spread *in vitro*, and improved antitumor efficacy *in vivo*. These data thus illustrate the direct relevance of our bioselected, ADP-overexpressing viral mutants for oncolytic virotherapy. Notably, the 'ADP splice-site mutation' identified here may be directly implementable in the context of existing designs of oncolytic Ad vectors. For example, vectors already endowed with tumor specificity might greatly benefit from the anticipated boost in oncolytic potency conferred by this mutation.

Taken together, our data validate a new, evolution-based bioengineering approach for Ad. In general, evolution-based engineering methods—i.e. methods based on genetic diversification and phenotypic selection—are powerful tools to isolate viruses with new or improved properties (63). Important advantages of these 'random' engineering methods over rational design are that they do not require an in-depth mechanistic insight and, furthermore, that they have the potential to yield unanticipated solutions to virus engineering problems. Existing viral random engineering methods can be divided into two general types: conventional forward genetics, which employs mutagens to bring about genome-wide mutagenesis, and the more modern approaches that combine *in vitro* genetic diversification techniques (64,65) with viral library-based selection (63). Both of these methods have been applied with some success to Ads in the past (12–14,66,67). However, the usefulness of the latter of these methods for Ad engineering has—in comparison to that for AAV (68) or retrovirus (69) engineering—been limited due to the difficulty of generating sufficiently complex adenoviral libraries. Recently, there has been significant progress in this respect. Different studies explored a site-specific recombination system to transfer plasmid-based Ad fiber-peptide libraries directly into replicating Ads, thus bypassing major efficiency bottlenecks in Ad library generation (i.e. large genome handling and transfection, and virus rescue from naked viral DNA) (66,67).

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With the ability to achieve genome-wide genetic diversification, our viral polymerase-based approach bears some similarity to traditional strategies relying on mutagens. However, major conceptual and practical distinctions exist. First, while mutator viral polymerases cause mutations directly, mutagens do so through the infliction of DNA damage. This is of relevance because not all mutagen-induced DNA lesions—like cross-links produced by nitrous acid (70)—can be bypassed by polymerases or repaired (16,70). In this regard, the greater part of the lethality seen after mutagen exposure may be due to collateral viral inactivation—consequential to irresolvable DNA lesions and damaged protein components—rather than bona fide genetic mutations (17). Importantly, this mutagen-associated excess lethality compromises the effective complexities of the diversified populations (17). While these issues are not so much a problem in single mutational-pulse experiments (because the amount of input virus can be chosen sufficiently high), they thwart schemes involving repeated mutagenic treatments, e.g. following each replication round. This points to a second, very important distinctive aspect of the polymerase-based approach, namely that it naturally allows repeated rounds of mutagenesis and selection. Thus, our approach is particularly suitable for directed evolution procedures involving multiple mutagenic viral passages, allowing for the possibility of generating compound mutants.

Thus, the Ad engineering approach described here is essentially an accelerated form of evolution that, regarding viral mutation rate levels,

matches RNA virus evolution (see above). This is of interest because it is well established that the rapid evolution of RNA viruses can be effectively exploited for directed evolution purposes. For example, many of the most promising oncolytic riboviruses were the (coincidental) result of multiple-passage adaptation to growth in tissue culture cells (19). Furthermore, there are many recent examples where the adaptabilities of riboviruses (such as rhinovirus, coxsackievirus, vesicular stomatitis virus, sindbis virus, tick-borne encephalitis virus and avian influenza) and retroviruses (HIV-I and MLV) have been deliberately exploited to achieve specific vectorological goals. For example, riboviruses were evolved to exhibit enhanced target cell killing (18), altered or expanded receptor specificity (18,20,27,28), altered protease specificity (21) and ability to grow in cells of other host species (22,23). Likewise, retrovirus adaptability has been exploited to optimize viral envelope glycoprotein-pseudotyped recombinant vectors (24–26). Thus, with appropriate selective conditions imposed, similar aims for Ad may be achievable by our rapid evolution approach. In this regard, a general aim would be to restore or optimize recombinant Ad vectors (e.g. retargeted vectors) with impaired or limited function. Further, a captivating specific goal would be to generate human Ad derivatives capable of productive infection of mouse cells. Such mouse-adapted viruses would help realization of a permissive mouse model for replication-competent human Ad infection, potentially an invaluable tool for preclinical safety and efficacy studies (71).

The mutator polymerase-based Ad engineering approach presented here should be easily implementable by others, offering great practical flexibility and choice regarding the design of evolution regimes. There are two general ways recommended to adopt the system. Mutator viral polymerases might be stably expressed in target cells—e.g. using lentiviral vectors, as in our study—or, alternatively, be encoded for by the virus itself. In the first approach, one might consider using instead of a polymerase-defective virus, a polymerase-proficient one. This would save the reintroduction of the polymerase gene into any promising viral mutants (or, conversely, the introduction of promising acquired mutations into a wild-type virus). However, in such a setting, due to the co-presence of wild-type and mutator polymerases within the infected cells, the level of mutation is likely to be compromised somewhat. Therefore, it may be advantageous for this purpose to use Ad pol mutants exhibiting hyper-mutator phenotypes. In this regard, our unpublished data shows that some of the viable polymerase mutations identified in this study can be combined, thereby generating yet higher level mutators. Finally, the second approach to adopt the system—introduction of the mutator polymerase gene into the virus genome—might be especially attractive in the context of *in vivo* directed evolution schemes.

FUNDING

European Union through the 6th Framework Program GIANT (contract no. 512087). Funding for open access charge: Leiden University Medical Center and European Union through the 6th Framework Program GIANT (contract no. 512087).

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ACKNOWLEDGEMENTS

We thank William Wold and Ann Tollefson (St Louis University, St Louis, MO, USA) for providing the ADP antibody. Jeroen Bakker (Amsterdam Medical Center, Amsterdam, The Netherlands) is acknowledged for providing technical assistance. Peter C. van der Vliet (University Medical Center Utrecht, Utrecht, The Netherlands) is thanked for critical reading of the manuscript and for giving valuable suggestions.

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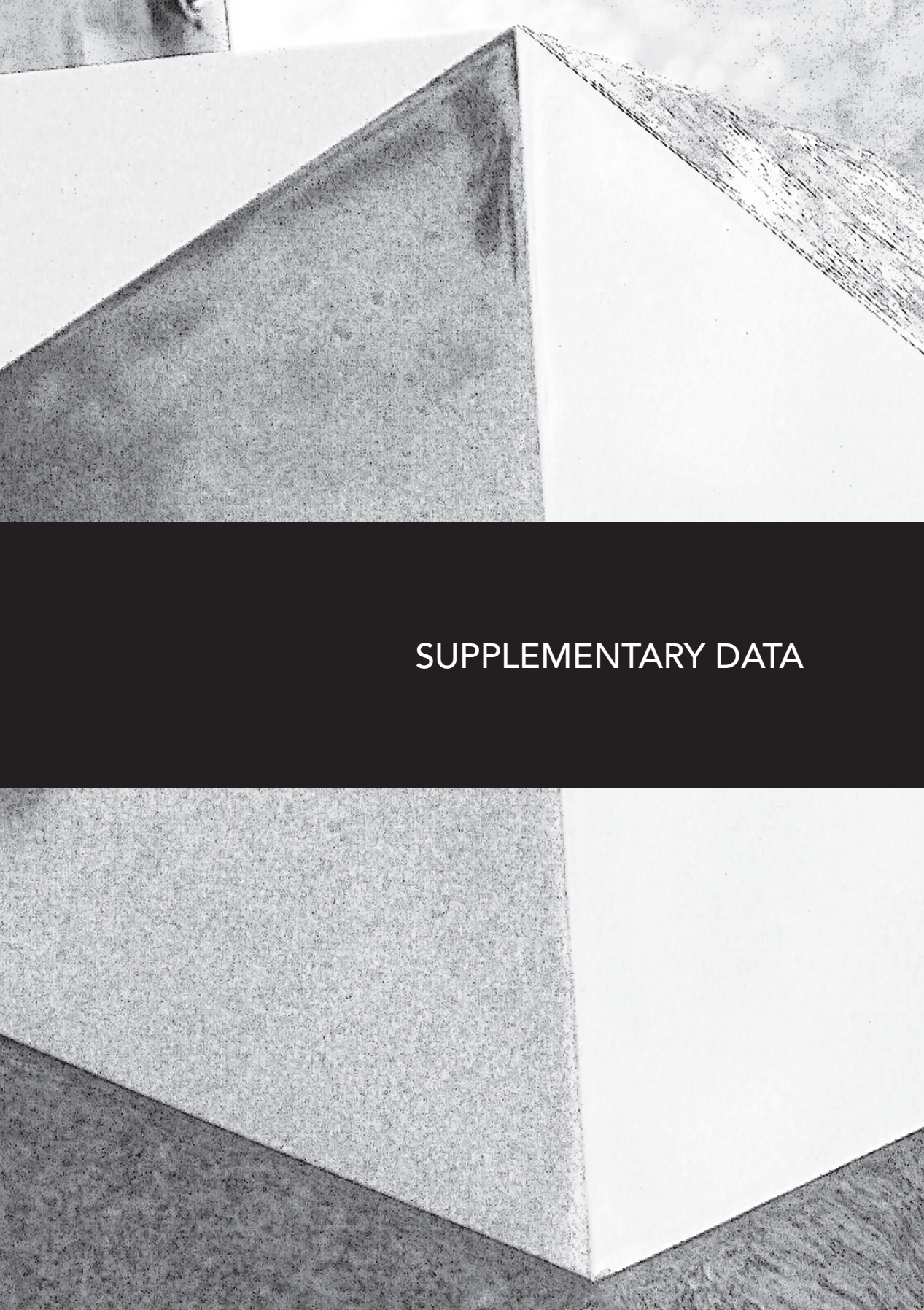
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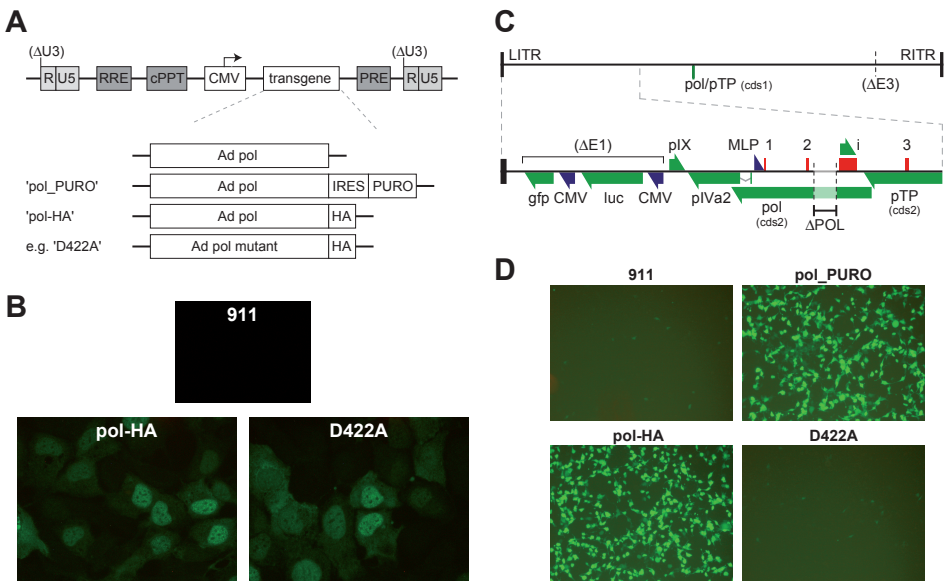
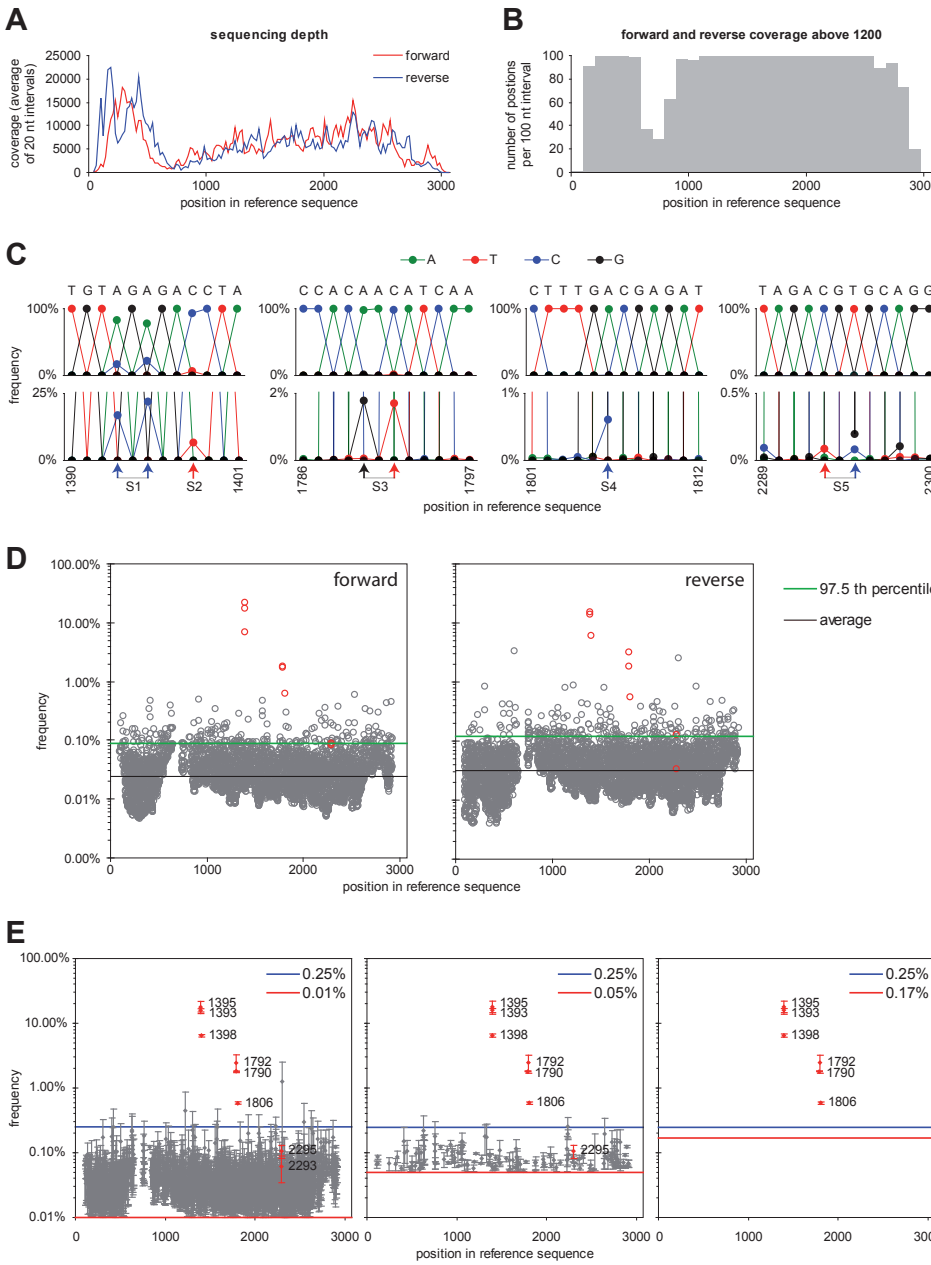


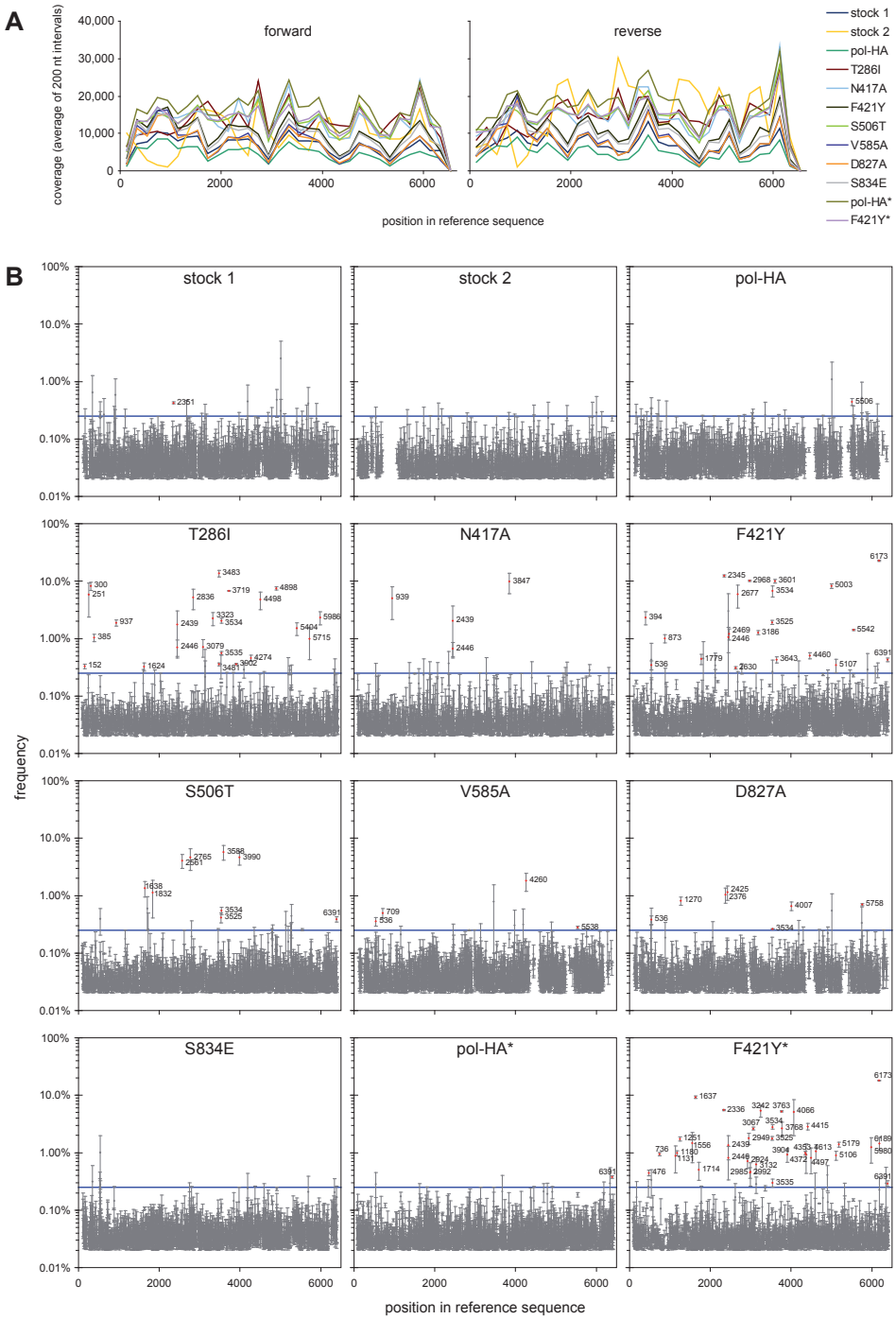
Figure S1. Components of the Ad pol *trans*-complementation system. (**A**) Lentiviral vectors for the stable expression of Ad pol variants in cells. Shown are representations of a lentiviral vector (in the proviral state) and the different transgenic configurations used. IRES, internal ribosomal entry site; PURO, puromycin resistance gene; HA, hemagglutinin (HA) tag; ΔU3, deletion of the U3 region; RRE, HIV-I Rev responsive element; cPPT, HIV-I central polypurine tract; CMV, human cytomegalovirus promoter; PRE, human hepatitis B virus post-transcriptional regulatory element. (**B**) Lentiviral expression – in the E1-complementing cell line 911 – of HA-tagged versions of wild-type Ad pol (pol-HA) and the D422A mutant Ad pol (D422A). Immunofluorescence analysis was performed using an HA-tag-specific primary antibody. (**C**) Representation of the genome of AdGLΔPOL, a polymerase-defective HAdV-5 based reporter vector. E1 and E3 regions are deleted, with the former replaced by GFP and luciferase (luc) gene expression cassettes. The polymerase-disrupting deletion 'ΔPOL' corresponds to positions 7312 to 7882 of the wild-type HAdV-5 genome (accession no. AC_000008). Green and blue arrows respectively represent coding and promoter sequences. Adenovirus genes depicted are polymerase (pol), pTP, pIX, pIVa2, and the i-leader protein (i). Red bars represent leader exons (1, 2, i, and 3) of the major late transcription unit. All components are depicted to scale. MLP, major late promoter. (**D**) Trans-complementation of the polymerase-defective reporter virus by both wild-type Ad pol ('pol_PURO') and pol-HA but not by D422A. 911 cells and 911 cells stably expressing the respective polymerases were infected with AdGLΔPOL. Images showing GFP fluorescence were made 2 days after infection.

5b


A
sequencing depth
forward
reverse
coverage (average of 20 nt intervals)
position in reference sequence
B
forward and reverse coverage above 1200
number of postions per 100 nt interval
position in reference sequence
C
A
T
C
G
frequency
position in reference sequence
S1
S2
S3
S4
S5
D
forward
reverse
97.5 th percentile
average
frequency
position in reference sequence
E
0.25%
0.01%
0.05%
0.17%
frequency
position in reference sequence

◀ **Figure S2. Deep sequencing to detect low-prevalence mutations: analysis of a spiked test sample.** Deep sequencing was performed on a 3.1-kb DNA fragment that was spiked with five minority sequences, each of which contained a single or double nucleotide polymorphism. The sequence reads were mapped against the reference sequence allowing up to two mismatches, after which low-quality bases were masked. (**A**) Forward and reverse sequencing depth distributions over the length of the sequenced fragment. (**B**) Positions in the reference sequence for which both the forward and reverse mappings passed a minimal sequencing depth requirement of 1200. (**C**) Local frequencies of base-calls around the spiked positions. Results represent those of the forwardly mapped reads. S1 to S5 specify the positions of the spiked polymorphisms. The color coding of the arrows correspond to that of the spiked minority bases. (**D**) Minor variant frequencies for the forward and reverse mappings. Depicted per position are the frequencies of all three possible minor variants (i.e. of the non-consensus base occurrences). The spiked minor variants are shown in red. The indicated 97.5th percentiles of the observed minor variant frequencies were used as error rate estimates for the calculation of *P*-values (see Statistics section in the main article). The data shown are for positions for which both the forward or reverse coverage level were above 1200. (**E**) Exemplification of mutation scoring using a minimal coverage requirement of 1200 and minor variant frequency requirements of 0.01%, 0.05%, or 0.17%. Shown – for positions with forward and reverse sequencing depths of minimally 1200 – are the minor variant occurrences for which both the forward and reverse observed frequencies were above a set cutoff value, indicated by the red line. For a given minor variant, the diamond and its associated 'error bars' respectively represent the average and the respective absolute values of the observed forward and reverse frequencies. The spiked minor variants are shown in red; their associated numbers indicate their respective positions in the reference sequence. The blue line (at 0.25%) represents a prevalence cutoff value considered safe for mutation scoring.

5b


A
forward
reverse
coverage (average of 200 nt intervals)
position in reference sequence
stock 1
stock 2
pol-HA
T286I
N417A
F421Y
S506T
V585A
D827A
S834E
pol-HA*
F421Y*
B
frequency
position in reference sequence

◀ **Figure S3. Deep sequencing to detect minor variants in pools of passaged viruses.** Viral DNA fragments obtained from pools of viruses replicated by different Ad pol variants were subjected to deep sequencing. Resultant sequence reads were mapped to the reference sequence allowing up to two edit operations (i.e. mismatches or gaps), after which low-quality base calls were masked. Using the forwardly and reversely mapped base-call distributions, substitutions were scored by imposing a local coverage depth requirement of 1200 and a minor variant frequency cutoff value of 0.25% (both of which requirements were to be met for both the forward and reverse mappings). (**A**) Forward and reverse sequencing depth distributions over the length of the sequenced fragment. (**B**) Minor variant frequency plots showing the scored substitutions. Depicted are all minor variant occurrences for which both the forward and reverse frequencies were above 0.02% (including only those positions for which the minimal sequencing depth requirement was met). Diamonds and their associated 'error bars' respectively represent the average and the respective absolute values of the observed forward and reverse frequencies. Minor variants were scored as bona fide substitutions (indicated in red) when their forward and reverse frequencies were both above the prevalence cutoff value of 0.25% (indicated by the blue line).

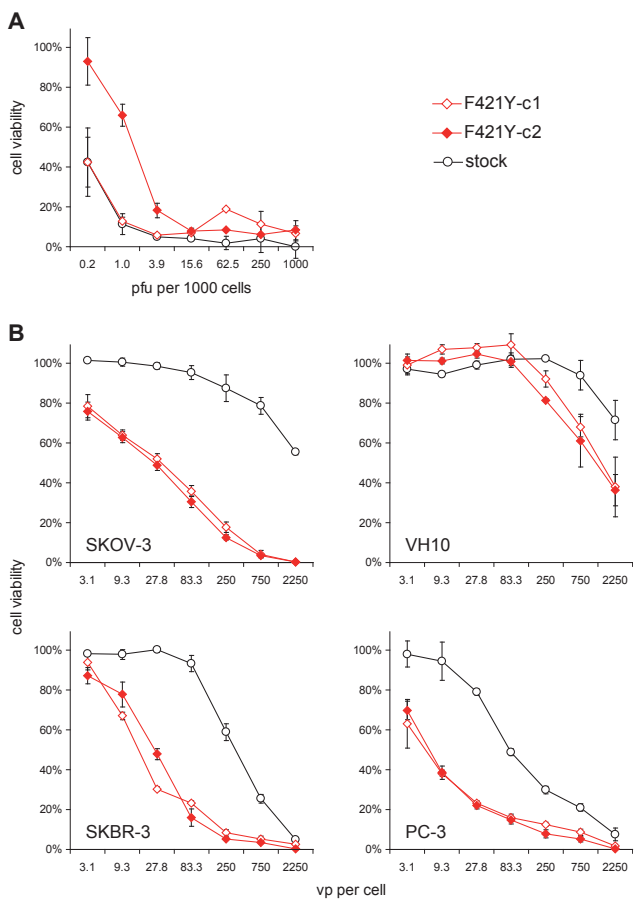


Figure S4. Cell killing abilities of the bioselected viral clones F421Y-c1 and F421Y-c2. Shown are cytotoxicity assays on 911 cells (**A**) and SKOV-3, SKBR-3, PC-3, and VH10 cells (**B**). Cells were infected at the indicated MOI's and cell viabilities were assessed by WST-1 assay.

5b

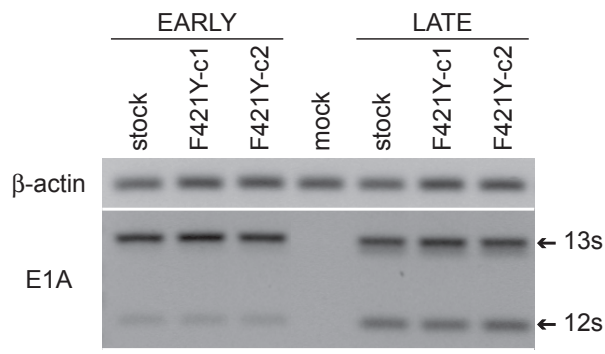


Figure S5. Reverse transcriptase-PCR analysis controls: cellular β-actin and viral E1A. These are controls reactions for the RT-PCRs performed to detect ADP-encoding mRNAs (main article, Figure 6). 12s and 13s are splice variants of E1A.

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SUPPLEMENTARY TABLES

Table S1. Ad pol mutants examined in this study

No.[a]	Ad pol mutant	Putative functions of the substituted residue[b]
1	D283A	Exo catalytic activity; metal ion binding; strand displacement (Φ29) (1)
2	E285A	Exo catalytic activity; metal ion binding; strand displacement (Φ29) (1)
3	T286I	Stabilization of the primer terminus at exo active site
4	N417A/D	Stabilization of the primer terminus at exo active site
5	F421A/S/Y	Stabilization of the primer terminus at exo active site
6	D422A	Exo catalytic activity; metal ion binding; strand displacement (Φ29) (1)
7	S506T	Involvement in exo activity; requirement for interaction with the terminal protein (11); primer binding (?)
8	L507T	Involvement in exo activity; primer binding (?)
9	Y580A	Involvement in exo activity; strand displacement (Φ29) (1)
10	D584A	Exo catalytic activity; metal ion binding; strand displacement (Φ29) (1)
11	V585A	Formation of part of the exo active site
12	G666A	dsDNA binding; tuning between pol and exo activities
13	M689K/N	Formation of part of the hydrophobic pocket that binds the base and ribose portions of the incoming nucleotide; role in base discrimination and/or partitioning to exo site
14	Y690F	Interaction with the ribose of the incoming dNTP; role in preventing incorporation of NTPs
15	D827A	Indirect involvement in incoming nucleotide binding (?)
16	S834E	Indirect involvement in incoming nucleotide binding (?)
17	L838A	Formation of part of the nascent base-pair binding pocket; involvement in positioning the templating nucleotide; controlling base-pairing correctness
18	Y844A	Hydrophobic interaction with the base of the incoming – or the templating (*Eco* I) – nucleotide; critical role in checking correctness of base pairing
19	G845A	dsDNA binding (Φ29) (39); role in the insertion of correct nucleotides (*Sce* α)

[a]The residue numbers 1 to 19 correspond to those assigned to the selected Ad pol residues in Figure 1.
[b]Listed are functions ascribed to homologous residues in polymerases of other organisms. Uncertain or debated functions are followed by a parenthesized question mark. (See Supplementary References for literature cited here).
[c]Depicted are those substitutions that were found – in the referenced studies – to either diminish exonuclease activity, affect the pol/exo balance, lower replication fidelity, or give a mutator phenotype *in vivo*. Substitutions of residues not evidently homologous to that of Ad pol are parenthesized. (See Supplementary References for literature cited here).
Abbreviations: exo, exonuclease; pol, polymerase; *Eco*, *Escherichia coli*; *Sce*, *Saccharomyces cerevisiae*; HSV, herpes simplex virus; CMV, cytomegalovirus ; *Taq*, *Thermus aquaticus* ; *Pfu*, *Pyrococcus furiosus*.

Mutator characteristics-yielding substitutions of homologous residues in other polymerases[c]

Φ29: D12A (2,3); T4: D112A/N (4); *Sce* δ: D321G/N (5); HSV: D368A (6)

Φ29: E14A (2,3); T4: E114A (4); *Sce* δ:E323Q (5); HSV: E370A (6)

Φ29: T15I (7); *Sce* δ: C324R (5)

Φ29: N62D (7); PRD-1: N71D (8)

Φ29: F65Y/S (9)

Φ29: D66A (2,3); T4: D219A/N (4); *Sce* δ: D407N (5); HSV: D471A (6); CMV: D413A (10)

Φ29: S122T/N (9)

Φ29: L123T/A (9); T4: L287A (4); *Sce* δ: L479S (5)

Φ29: Y165F/C (3,12); T4: Y320A/Q/F (4); RB69: Y323F (13); HSV: Y577F/H (6,14)

Φ29: D169A (3,12); T4: D324A/N (4); HSV: D581A (6,14)

T4: V325A (15)

Φ29: G228A (16)

T4: L412M (17); *Sce* δ: L612F/M/K/G/N (18); RB69: L415F/G (19); *Eco* I: I709A/F/M/N (20); *Sce* α: L868F/M/W/V (21); *Taq* I: I614K/N/Q (22); *Sce* ε: M644F (23)

Φ29: Y254F/V (24,25); *Eco* I: E710A (26)

(*Pfu*: T471G/A, Q472G/A/P, D473G/A (27))

(*Taq* I: A661E (28))

Φ29: L384R/Q (29); RB69: L561A (30,31); *Taq* I: T664R (28)

Φ29: Y390F/S (24); RB69 Y567A (31-34); *Eco* I: Y766S/A (35-37); *Sce* α: Y951E/P (38)

Sce α: G952Y/A (40)

Table S2. Substitution loads relative to the analyzed amount of viral DNA.

Sample	Pool size[a]	Positions analyzed[b]	Total amount analyzed (bp)[c]	Absolute substitution score[d]	Substitution load per million bp[e]
stock 1	38	5869	2.2×10^5	1	4.5
stock 2	54	5809	3.1×10^5	0	0.0
pol-HA	26	5189	1.3×10^5	0	0.0
T286I	48	6320	3.0×10^5	23	76.3
N417A	42	6296	2.6×10^5	4	15.3
F421Y	47	6162	2.9×10^5	21	72.7
S506T	36	6291	2.3×10^5	8	35.3
V585A	41	5619	2.3×10^5	2	8.7
D827A	33	5670	1.9×10^5	7	37.4
S834E	44	6109	2.7×10^5	0	0.0
pol-HA*	58	6341	3.6×10^5	1	2.7
F421Y*	55	6314	3.5×10^5	35	100.1

[a]Virus pool sizes were estimated based on intra-experiment titrations during pool preparations.
[b]Positions in the reference sequence for which both the forwardly and reversely mapped base distributions had a minimal coverage level of 1200.
[c]The estimated total amount of analyzed DNA sequence is the arithmetic product of 'pool size' and 'positions analyzed'.
[d]The substitution scores as found in Figure 3C. Shown are only the substitution scores for which both the forward and reverse observed minor variant frequencies had associated *P*-values smaller than 1×10^{-4}.
[e]The substitution scores (column 'absolute substitution score') are expressed here relative to the respective DNA amounts analyzed (column 'total amount analyzed').

Table S3. Mutations found in two bioselected clones.

Clone	Position[a]	Base change[b]
F421Y-c1	1,419	G ⇨ A
	7,243	G ⇨ A
	8,251	C ⇨ A
	9,835	C ⇨ T
	16,036	G ⇨ A
	17,576	G ⇨ A
	29,378	C ⇨ T
F421Y-c2	9,114	C ⇨ G
	11,581	G ⇨ A
	20,374	C ⇨ T
	28,638	T ⇨ A
	29,378	C ⇨ T
	31,726	A ⇨ G
	34,301	C ⇨ T
	35,229	A ⇨ Δ

[a]Position in HAdV-5 genome (accession no. AC_000008)
[b]The indicated base change is for the direct strand. 'Δ' stands for a deleted base.

Table S4. Protein coding changes in two bioselected viral clones.

Clone	Position[a]	Base Change[b]	Strand	Protein	Coding Change	Conservation[c]
F421Y-c1	8,251	C ⇨ A	direct	i-leader protein	L92M	C,D
	9,835	C ⇨ T	opposite	pTP	R255H	A,B,C,D,E,F
	17,576	G ⇨ A	direct	pV	V346M	A,B,C,D,E,F
F421Y-c2	9,114	C ⇨ G	opposite	pTP	Q495H	A,B,C,D,E,F
	11,581	G ⇨ A	direct	L1 52/55K	V178M	A,B,C,D,E,F
	28,638	T ⇨ A	direct	E3 6.7K	I31N	C[e]
	31,726	A ⇨ G	direct	fiber	T229A	C
	35,229	A ⇨ Δ	opposite	E4orf1	Frameshift[d]	A,B,C,D,E[f]

[a]Position in HAdV-5 genome (accession no. AC_000008)
[b]The indicated base change is for the direct strand. 'Δ' stands for a deleted base.
[c]HAdV subgroups across which the affected amino acids are conserved.
[d]The base deletion results in a frameshifted, shorter protein: the last 27 amino acids of E4orf1 are replaced by 7 other residues.
[e]E3 6.7K does not exist outside HAdV-5 subgroup C.
[f]Six of the 27 deleted residues are conserved across the indicated subgroups. E4orf1 is not known by us to exist in subgroup F.

SUPPLEMENTARY PROCEDURES AND MATERIAL

Rationale for selecting Ad pol residues to be mutated

A panel of twenty-three single-amino-acid substitution mutants of Ad pol was generated by site-directed mutation of nineteen selected residues thought to be important for governing replication fidelity (Figure 1 and Supplementary Table S1). In our picking of residues to modify, we were led by the consideration that only Ad pol's accuracy of polymerization was to be targeted – any other detrimental effects on functions necessary for faithful adenovirus genome replication were ideally to be avoided. For example, the efficiency of polymerization – or its protein-primed initiation – was preferably not to be compromised. To achieve this goal, in addition to drawing from what is known about Ad pol itself, we tapped into the wealth of data available on DNA-polymerases of other organisms, such as the Φ29, T4, RB69, and PRD1 bacteriophages, herpes simplex virus (HSV), cytomegalovirus (CMV), *Escherichia coli*, *Thermus aquaticus* (*Taq*), *Pyrococcus furiosus* (*Pfu*), and *Saccharomyces cerevisiae*. Eleven of the selected Ad pol residues, all located within the exonuclease domain, have been implicated – mostly through their homologues in other polymerase species – with proofreading function. The eight other targeted residues make part of either the fingers domain or the palm domain and are thought to play important roles in incoming base selection and/or tuning between polymerase and exonuclease activities.

Among the mutations of residues putatively involved in proofreading were those aimed at specifically abolishing the catalytic activity of the exonuclease domain. These substitutions, which were meant to rigorously knock-out exonucleolytic activity, concern the highly conserved residues D283, E285, D422, Y580, and D584 (Figure 1 and Supplementary Table S1, assigned amino acid no. 1, 2, 6, 9 and 10). These are the key residues thought to be involved in metal ion binding and catalysis. Importantly, substitutions of the homologues of these residues in polymerases of Φ29, T4, RB69, and HSV have previously been shown to render these polymerases gravely impaired (or effectively inactive) with respect to the ability to catalyze exonucleolytic hydrolysis (2-4,6,12-14). Furthermore, a D422A mutant of Ad pol has already been shown by Brenkman *et al*. to be exonuclease deficient (41).

Ad pol residues thought to be otherwise (than catalytically) involved in proofreading were also subjected to mutation. Three of these, namely T286, N417, and F421 (no. 3-5), were selected for their suspected direct involvement – based on studies on their homologues in Φ29 – in stabilization of the primer terminus at the exonuclease active site (7,9). Two other residues targeted for mutation were S506 and L507 (no. 7 and 8), both of which make part of to the (S/T)Lx2*h* motif (9) (Figure 1). These residues putatively play important roles in proofreading, possibly through (indirect) interaction with the ssDNA

primer, as has been suggested for their homologous counterparts in Φ29 polymerase (9). Finally, the last residue within the exonuclease domain that was mutated in this study was V585 (no. 11). Substitution of its homologue in T4 polymerase had led to an *in vivo* mutator phenotype, possibly due to functional disturbance of a neighboring catalytic residue, which, in Ad pol, is D584 (no. 10) (15).

Further singled out for mutation were three amino acid residues residing in the conserved Pol II (or Motif A) region of the palm domain (Figure 1). One of these residues, G666 (no. 12), is located in the Y/IxGG/A motif, a region believed to be involved in tuning between polymerase and exonuclease activities (16,42). The other two residues, M689 and Y690 (no. 13 and 14), might make part of the incoming nucleotide binding pocket and, as such, could play important roles in the geometric selection of nucleotides, which has been suggested for their putative homologues in other polymerases (17-26).

Within the fingers domain, with its prominent role in the binding of incoming nucleotides, a total of five residues were selected for mutation. Two non-highly conserved residues were chosen based on the speculation that their respective substitutions could have indirect effects on base selecting properties. One of these residues, D827 (no. 15), is seemingly located in the connecting loop between two helices of the fingers domain (Figure 1). Positioned as such, it might functionally resemble similarly located *Pfu* DNA polymerase residues, mutation of which previously led to strong mutator phenotypes (27). The other lesser-conserved residue that was targeted, S834 (no. 16), which seems to be situated in a second helix of the fingers domain of Ad pol (Figure 1), is positioned just before highly conserved residues thought to be directly involved in catalytic and base-selection functions. Mutation of residues at this approximate location in *Taq* DNA polymerase I has previously given rise to increased *in vitro* and *in vivo* mutation rates (28). Further targeted for mutation in the fingers domain were two highly conserved residues, L838 and Y844 (no. 17 and 18), both of which were selected based on their presumed critical roles in controlling base-pairing correctness (24,28-38). Finally, another target was G845 (no. 19), a highly conserved residue to which different functions have been ascribed for different polymerases. In Φ29 it would be involved in dsDNA binding (39), while in *S. cerevisiae* polymerase alpha, a familiy A polymerase, it is suspected to be critical for the insertion of correct nucleotides (40).

Construction and production of lentiviral vectors

Lentiviruses used in this study were self-inactivating, third-generation HIV-I-derived vectors (43,44). LV plasmids expressing wild-type Ad5 pol and the mutant Ad5 pol D422A were made by insertion of previously described cDNAs

(41) into the basal LV plasmids pRRL-cPPT-CMV-X-PRE-SIN (45) or its derivative pLV.CMV.IRES.PURO (46). C-terminal HA-tagging of these two polymerases involved PCR-amplification using a reverse primer harboring an HA-tag coding sequence (see below for the primer sequence). For the generation of polymerase mutants other than D422A, point mutations were introduced – in a LV plasmid expressing HA-tagged Ad5 pol – by a site-directed mutagenesis technique involving 'inversed' PCR of the parental plasmid and subsequent self-ligation of the PCR product (see below for the primer sequence pairs). Lentivirus production, which was performed as described previously (47), involved the co-transfection of LV plasmids with helper plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope. Lentivirus stocks were titrated using a HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., New York, NY, USA).

Generation of polymerase-defective adenovirus vectors

Generation of the polymerase-defective Ad vectors involved two basal Ad genome-containing plasmids. The first of these, pAdGL, was described previously and contains an E1- and E3-deleted HAdV-5 genome, with, at the place of E1, two tandem expression cassettes encoding GFP and firefly luciferase (46). The second basal plasmid, pAd, carries within its plasmid backbone of pShuttle (48) the complete HAdV-5 genome of pTG3602 (49). These plasmids were modified, in several cloning steps, to contain a 571-bp, polymerase-affecting deletion (ΔPOL; corresponding to nt 7312 to 7882 of the HAdV-5 genome; accession no. AC_000008). First, through splicing by overlap extension-PCR (see below for primer sequences), a chimeric DNA fragment was generated that consisted of two 'deletion-flanking' fragments (of approximately 1 kb each) with, spliced in between them, a chloramphenicol resistance gene (Cam^r), obtained from pGP618 (50). Subsequently, this 'ΔPOL.CAM' fragment served as a selectable donor in homologous recombination [in *E. coli* BJ5183 (51)] with acceptor plasmids pAdGL and pAd, allowing the generation of pAdGLΔPOL.CAM and pAdΔPOL.CAM. Finally, the excision of Cam^r (by virtue of two PCR-introduced *Swa*I sites) yielded pAdGLΔPOL and pAdΔPOL. The viral genomes of these two plasmids were released by *Pac*I digestion and subsequently transfected into 911.AdPol cells for viral rescue of, respectively, AdGLΔPOL and HAdV-5ΔPOL.

Initial setup of the Ad pol *trans*-complementation system

A polymerase complementation system was set up to test Ad pol mutants for their ability to support the functions necessary for productive Ad replication (Supplementary Figure S1). This system employs a polymerase-defective Ad reporter vector that is completely dependent – for its replication – on a functional Ad pol (mutant) being provided in *trans*. Such a polymerase

complementation strategy is analogous to previously taken approaches in studies that investigated variants of HSV (14,52) and RB69 (32) polymerases.

The E1- and E3-deleted polymerase-defective Ad vector generated for this study, AdGLΔPOL, accommodates a polymerase gene-disrupting deletion at a location not known to harbor any other essential elements than the polymerase sequence (Supplementary Figure S1C). Importantly, at the site of this partial polymerase gene-deletion, an extra stop codon was introduced such that only a severely truncated Ad pol (encoded by the first forth of the Ad pol open reading frame) was producible. Of note, Amalfitano *et al*. previously showed that an Ad vector with an essentially similar polymerase deletion was effectively rendered unable to replicate, except when provided with Ad pol *in trans* (53). To facilitate monitoring of viral replication, the AdGLΔPOL genome was additionally equipped with CMV promoter-driven reporter genes for GFP and firefly luciferase. Transfection of the AdGLΔPOL genome into (E1-complementing) cells engineered to express wild-type Ad pol resulted in the rescue of viable AdGLΔPOL virions.

The Ad polymerase complementation system further involved the use of lentivirus (LV) vectors for achieving stable expression of Ad pol variants in cells (Supplementary Figure S1A). In this regard, the above mentioned viral rescuing of AdGLΔPOL was conducted using cells transduced with a LV-vector encoding wild-type Ad pol. Initially, in order to detect the heterologous expression of Ad pol in these cells, immunofluorescence (IF) was performed for which we made use of a lab-generated anti-serum raised against an Ad pol/pTP complex. Although detection by this means proved to be achievable, the procedure suffered from a low signal-to-background ratio (data not shown). Therefore, since more unambiguous detectability was preferred, we opted to equip the Ad pols of this study with HA-tags at their C-termini. Supplementary Figure S1B shows that lentiviraly expressed HA-tagged versions of wild-type Ad pol and a mutant Ad pol, namely D422A, were readily detectable by IF using an HA-tag specific antibody. Both these polymerases were found to localize to both the nucleus and the cytoplasm, with a higher intensity of detection observed in the nucleus.

To ascertain that Ad pol's ability to replicate the Ad genome was not affected by its fusion to an HA-tag, a pilot complementation experiment was carried out (Supplementary Figure S1D). Cells expressing the HA-tagged versions of either wild-type Ad Pol or the D422A mutant were infected with the polymerase-defective vector AdGLΔPOL. Alongside these infections were those of positive control cells expressing (non-tagged) wild-type Ad Pol and negative control cells not expressing any Ad pol. The results of GFP expression analysis at 52 hours post infection show that HA-tagged Ad pol is as efficient as non-tagged Ad pol to complement AdGLΔPOL. Thus, a C-terminal HA-tag proves to be compatible with Ad pol's functions necessary for Ad genome

replication. Interestingly, the results further reveal that the one mutant taken along, D422A, is unable to complement the polymerase-defective vector. Thus, although the D422A mutant had previously been shown to achieve polymerization in an *in vitro* setting (41), it seems not to sustain complete Ad replication.

Pilot deep sequencing run on a spiked test sample

To probe the utility of massively parallel sequencing (MPS) for the detection of minority mutations, we performed a sequencing run on a spiked test sample. A 3.1-kilobase-pair (kb) DNA fragment was spiked with several minority fragments, each of which differing from the original fragment by only a 1- or 2-nucleotide variation. The resulting sample, which contained spikes with theoretical prevalences of 25, 6.25, 1.56, 0.39, and 0.1%, was run on a Solexa/Illumina Genome Analyzer (I) instrument to obtain approximately 20 million 20-mer raw reads. These reads were mapped, allowing up to two mismatches, against the 3.1-kb reference sequence using the ELAND aligner of Illumina's Genome Analyzer data analysis pipeline. Resultant forward and reverse mappings – representing only reads passing the default Illumina chastity filter (threshold = 0.6) – were subjected to single-base masking to exclude bases with phred quality values lower than 29. These operations finally led to forwardly and reversely mapped base-call distributions exhibiting average coverage depths of respectively 7096 and 6134 high-quality bases. Notably, considerable variation in the coverage depth was observed along the length of the reference sequence (Supplementary Figure S2A). Nonetheless, as much as 84% of the reference sequence was found to be covered – in both the forward and the reverse orientation – by at least 1200 high-quality base-calls per position (Supplementary Figure S2B).

Of the spiked mutations, those with prevalences of 25, 6.25, 1.56, and 0.39% were found to be distinctively present in both the forward and the reverse distributions (Supplementary Figures S2C and S2D). Furthermore, each of these spikes was found with approximately its expected prevalence (Table 1). By contrast, the spike with expected prevalence of 0.1% (spike no. 5) did not as prominently stand out from local levels of 'background' base-calls.

Directed by the above-obtained base-call distribution data we defined generalizable filtering rules that allowed for the confident scoring of minority mutations. The first of these rules disallows from the analysis any positions for which either forward or reverse coverage levels are below a certain set minimum. This rule was found necessary to protect against the relatively high background base-call levels seen at positions where the coverage levels dropped. The second filtering rule conditions that a given minor sequence variant may only be scored as a bona fide mutation when present – with

prevalences above a set cutoff value – in both the forward and reverse distributions. Applied to the spiked test sample data, the combination of these rules allowed the scoring of spiked mutations 1 to 4 without picking up any false-positives. Specifically, with the minimal coverage level set arbitrarily at 1200, a mutation prevalence-cutoff value of as low as 0.17% permits scoring of only those four spikes (Supplementary Figure S2E). Of note, for all the thusly 'scored' spikes, both the forward and reverse observed frequencies would have very low estimated probabilities of occurring solely as a consequence of random sequencing errors, i.e. all *P*-values were below 1×10^{-12} (Table 1). By contrast, the 'non-scored' spike could not be significantly distinguished from background sequencing errors (all *P*-values above 0.4).

Oligonucleotides used for cloning, sequencing, and reverse transcriptase-PCRs

Name	Sequence
HA-tag (YPYDVPDYA) addition to the Ad pol C-terminus[a]	
Forward	GACGTATGTTCCCATAGTAACGC
Reverse	GTCACGTGCT**CTA**AGCGTAATCCGGAACATCGTATGGGTACGGCATCTCGATC
Construction of a polymerase-defective adenovirus vector[b]	
Lpol 1	TCGATACGCGTTGGACAGCAACTTGG
Lpol 2	CGGATGAATGGCAGAAATTTAAATGGTCAGGGACACCTTTGC
Cam 1	GGTGTCCCTGACCATTTAAATTTCTGCCATTCATCCGC
Cam 2	CCTTCATGCTGGTCATTTAAATCAGTAAGTTGGCAGCATTC
Rpol 1	GCTGCCAACTTACTGATTTAAATGACCAGCATGAAGGGCA
Rpol 2	CGTATGTATACGCCTTCTCGCAGCTC
Amplification of a 6.5-kb DNA fragment from virus pools for deep sequencing	
Forward	GTAGTTTTGTATCTGTTTTGCAGCAG
Reverse	CGAATTTATCCACCAGACCAC
Reverse transcriptase-PCRs[c]	
β-actin 1	GGCATCCTCACCCTGAAGTA
β-actin 2	GGGGTGTTGAAGGTCTCAAA
E1A 1	GTCCGGTTTCTATGCCAAAC
E1A 2	GATAGCAGGCGCCATTTTAG
E3 ADP 1	CCTGAAACACCTGGTCCACT
E3 ADP 2	GCGTTGGTTGTGTTGGTCAT
ML ADP 1	CGAGAAAGGCGTCTAACCAG
ML ADP 2	GCGTTGGTTGTGTTGGTCAT

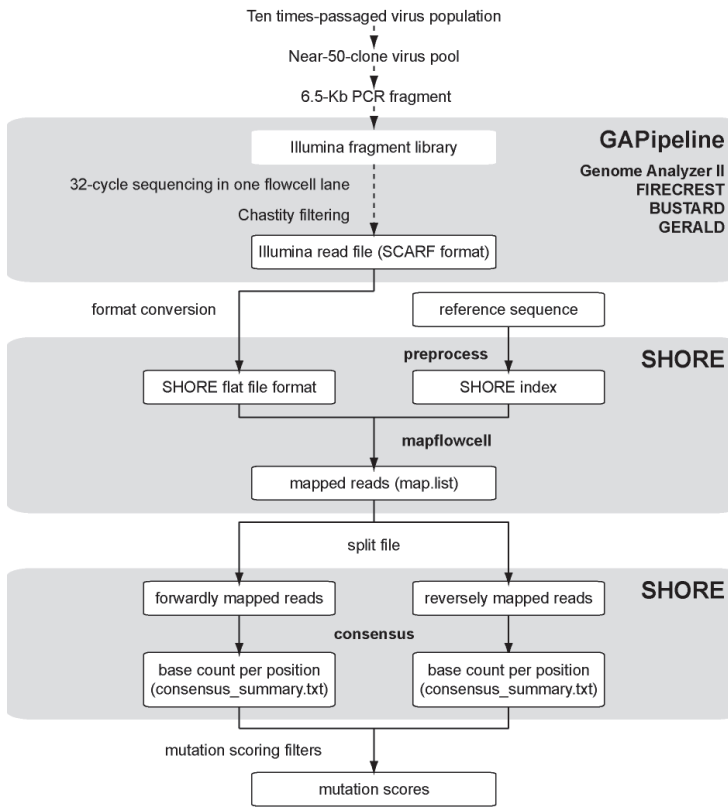
[a]The forward primer is CMV promoter-specific. The reverse primer anneals to the C-terminus of Ad pol. The HA-tag encoding sequence is shown in red font. The new stop codon is in boldface.
[b]The indicated primers were used to construct a recombination donor fragment ('ΔPOL.CAM') carying a chloramphenicol resistance gene (Cam^r) at the site of a partial Ad polymerase gene deletion. First, two 'deletion flanking' fragments were generated using the Lpol and Rpol primer sets, and a Cam^r-containing fragment using the Cam primer set. Then, to generate ΔPOL.CAM, these three fragments were fused by performing two sequential splicing by overlap extension-PCRs using appropriate combinations of primers. The respective sequences providing the overlaps between the three fragments are color-coded blue and green. Underlined sequences indicate introduced restriction enzyme recognition sites.
[c]Primer sets E3 ADP and ML ADP have a common reverse primer.

Oligonucleotides used for the construction of Ad pol mutants

Mutant	Primer 1 (forward)[a]	Primer 2 (reverse)[a]	RE site
D283A	GTACGCTGTAGAGAC-CTATACTTGGATGG	GTAACAAAGAGACGCTCG-GTGC	SnaBI
E285A	CGACCTATACTTGGAT-GGGGGC	CGACATCGTAGGTGACAAA-GAGACG	NruI
T286I	TCTATACTTGGATGGGGGCCT	TCTCTACATCGTAGGT-GACAAAGAG	BglII
N417A	TGCCATCAACGGCTTTGAC-GAGA	TGCCCCACAATGTAAAGT-TCCAAG	SphI
N417D	ATCAACGGCTTTGACGAGATC	ATCGTGGCCCACAATG-TAAAGTTC	EcoRV
F421A	GCCGACGAGATCGTGCTCGCC	GCCGTTGATGTTGTGGCC	KasI
F421S	TCCGACGAGATCGTGCTCGC	TCCGTTGATGTTGTGGCC	BamHI
F421Y	AATGGGTACGACGAGATCGT-GCTCGC	AATGTTGTGGCCCACAAT-GTA	AseI
S506T	GTTGCGGAAGGCCGCGCAG	GTTGTGTGGGTGAGCG-CAAAG	AclI
L507T	ACTCGGAAGGCCGCGCA	ACTGGTGTGGGTGAGCG-CA	ScaI
Y580A	TGCGCCCTAGACGTGCAG	TGCGTCCAGGGTTTCCTT-GATGATG	SphI
D584A	ACAGGTCACCGCCGAGCTG	ACAGCTAGGGCGCAG-TAGTCCAG	BsrGI
V585A	GCAGGTCACCGCCGAGC	GCATCTAGGGCGCAG-TAGTCCAG	FspI
G666A	GGCAGATGCTACCCTACAT-ATCTTGG	GGCGCGGATGCTGGCG	NaeI
M689K	CGTCCGCGCTCACCCACC	CGTATTTGCCGCAAAT-GTCGTAAAC	MluI
M689N	CGTCCGCGCTCACCCACC	CGTAGTTGCCGCAAAT-GTCGTAAAC	MluI
Y690F	CGAGCGCGCTCACCCACCC	CGAACATGCCGCAAAT-GTCG	NruI
D827A	GAACCAAACCCTGCGCTC	TTAGCGCGATCGGCGCG	DdeI
S834E	AGGGAGATCGCCAAGTTGCT-GTCC	CAGGGTTTGGTTTTTGTCGC	Bsu36I
L838A	CTCTCCAACGCCCTCTACGG	GGCCTTGGCGAT-GGAGCGC	EcoO109I
Y844A	AGCAGGGTCGTTTGCCACCA	AGCGCGTTGGACAG-CAACTTG	NheI
G845A	CGTCGTTTGCCACCAAGC	CGTAGAGGGCGTTGGACA	MluI

[a]The indicated primer pairs were used for an 'inversed' PCR on a lentiviral vector plasmid encoding HA-tagged HAdV-5 DNA polymerase. Resultant PCR products were subsequently self-ligated to obtain plasmids encoding the respective mutants. Red fonts indicate base substitutions. Boldfaced nucleotides show the codons mutated to encode the amino acid changes indicated in the first column. Underlined sequences represent the newly generated restriction enzyme recognition sites (depicted in the last column) used for identification of positive clones.

Workflow for the analysis of deep sequencing data obtained from passaged virus pools



Sequencing data obtained from the passaged virus pools were outputted by the Genome Analyzer Pipeline (GAPipeline) in read files of Illumina's SCARF file format. These files included only reads passing Illumina's default chastity filter (i.e. reads that among their first 25 bases have maximally one base with a chastity value less than 0.6). Subprograms 'preprocess' and 'mapflowcell' of the short read analysis pipeline SHORE (http://1001genomes.org/downloads/shore.html) were used for mapping of the reads against the reference sequence. The alignments were performed allowing up to 2 mismatches and/or gaps. The SHORE subprogram 'consensus' was used – separately for the forward and reverse mappings – to generate base counts per position in the reference sequence. Individual base masking was performed (by SHORE consensus) using a quality value cutoff of 30. Resultant forward and reverse 'consensus_summary.txt' files served for mutation scoring using a sequencing depth requirement of 1200 and a prevalence cutoff value of 0.25%. See the table on the next page for further details on the data analysis workflow.

Command line programs and parameters used for the analysis of deep sequencing data obtained from passaged virus pools

Program[a]	Relevant parameters	Notes
Custom Perl script[b]	s_N_sequence.txt	Input read file (Illumina SCARF format)
	reads_0.fl	Output read file (SHORE format)
SHORE preprocess	-f Ref.fa	Reference sequence file (fasta format)
	-i IndexFolder	
SHORE mapflowcell	-o FlowcellFolder	See note below[c]
	-f IndexFolder/Ref.fa.shore	
	-n 2	Maximum no. of mismatches plus gaps
	-g 2	Maximum no. of gaps
Custom Perl script[d]	map.list	Input file (= output of mapflowcell)
	CustomOutputFolder	Output files: for.list and rev.list
SHORE consensus	-n 0001	Arbitrary ID
	-f IndexFolder/Ref.fa.shore	
	-o AnalysisFolder	
	-i for.list *(or rev.list)*	Input: for.list or rev.list (not map.list)
	-q 30	Quality value cutoff for base masking
	-v	Required for 'consensus_summary.txt'
	-r	
Custom Perl script[e]	consensus_summary.txt	Forward input file
	consensus_summary.txt	Reverse input file
	MutationScoreFolder	Output: mutation score files
	1200	Coverage depth requirement
	0.25	Minor variant frequency cutoff (%)

[a]See workflow diagram above for an overview of the data analysis workflow. SHORE is a short read analysis pipeline (http://1001genomes.org/downloads/shore.html) (54) . Its subprogram 'mapflowcell' invokes the aligner GenomeMapper (55). (See Supplemental References for literature cited here)

[b]A Perl script was used to convert between SCARF and SHORE read file formats. Of note, this conversion entailed inferring 'Sanger' base quality scores (needed for SHORE) from the 'Illumina' base quality scores (present in the SCARF format). Furthermore, since SCARF files lack chastity value information (which SHORE files do accommodate), each base in the generated 'reads_0.fl' files was assigned the maximum chastity value.

[c]The read files-containing folder structure normally built by the SHORE subprogram 'illumina2flat' – i.e. ProjectFolder/FlowcellFolder/LaneFolder/ReadFolder/LengthFolder – was created manually and appropriately populated – in its 'LengthFolders'– by the above-generated 'reads_0.fl' files.

[d]This script divides the forwardly and reversely mapped reads of the 'map.list' file (generated by the SHORE subprogram 'mapflowcell') over two new alignment files: 'for.list' and 'rev. list'.

[e]This mutation scoring Perl script uses as its input the forward and reverse mapping-derived 'consensus_summary.txt' files. These files, which were outputted by the SHORE subprogram 'consensus' in the folder 'AnalysisFolder/ConsensusAnalysis/supplementary_data', contain A, G, C, and T base counts – both quality filtered and unfiltered – per position in the reference sequence.

Summary statistics for minor variant frequencies found in deep sequencing data

	Mean[a]		97.5th percentile[a,b]	
	forward	reverse	forward	reverse
Spiked test sample sequencing run				
with spikes	2.4×10^{-4}	3.1×10^{-4}	8.9×10^{-4}	1.2×10^{-3}
without spikes	1.8×10^{-4}	2.7×10^{-4}	8.9×10^{-4}	1.2×10^{-3}
Virus pool sample sequencing runs				
stock 1	1.9×10^{-4}	1.7×10^{-4}	1.0×10^{-3}	9.0×10^{-4}
stock 2	1.6×10^{-4}	1.5×10^{-4}	8.1×10^{-4}	6.8×10^{-4}
pol-HA	1.9×10^{-4}	1.8×10^{-4}	1.1×10^{-3}	1.0×10^{-3}
T286I	1.9×10^{-4}	1.8×10^{-4}	7.5×10^{-4}	6.7×10^{-4}
N417A	1.7×10^{-4}	1.6×10^{-4}	7.9×10^{-4}	6.6×10^{-4}
F421Y	2.2×10^{-4}	1.9×10^{-4}	7.8×10^{-4}	6.8×10^{-4}
S506T	1.9×10^{-4}	1.8×10^{-4}	8.1×10^{-4}	7.2×10^{-4}
V585A	1.8×10^{-4}	1.7×10^{-4}	9.3×10^{-4}	8.3×10^{-4}
D827A	1.7×10^{-4}	1.6×10^{-4}	8.2×10^{-4}	7.5×10^{-4}
S834E	1.8×10^{-4}	1.6×10^{-4}	7.7×10^{-4}	6.8×10^{-4}
pol-HA*	1.8×10^{-4}	1.8×10^{-4}	8.6×10^{-4}	7.6×10^{-4}
F421Y*	2.1×10^{-4}	1.9×10^{-4}	7.4×10^{-4}	6.4×10^{-4}

[a]The statistics are for positions for which both forward and reverse coverage levels were above 1200.
[b]The 97.5th percentiles were taken as error rate estimates used for the calculation of *P*-values for minor variant occurrences (See the statistics section in the main article).

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6



SUMMARIZING DISCUSSION

Human adenovirus (Ad)-derived vectors are widely being developed and used for gene therapy (1), oncolytic virotherapy (2) and vaccine delivery (3). Despite the many advances made in these fields, existing vectors are often not effective, specific, and/or safe enough. Therefore, major goals in Ad vectorology currently are to improve the selectivity and efficiency of target cell transduction (4), and, concerning oncolytic vectors, to enhance cell killing ability and viral spread (5). This thesis describes studies aimed at improving Ad vectors with respect to both these aspects.

The first part of this thesis is about genetic capsid modification for Ad retargeting. It focuses on exploiting Ad capsid proteins pIX and fiber as sites for the incorporation of cell-targeting ligands. **Chapter 2** describes a new, transient pIX-pseudotyping approach that facilitates rapid analysis of pIX-ligand fusions in the context of the Ad capsid. This new system is used in **Chapter 3** to analyze the utility of a single-chain T-cell receptor (scTCR) as targeting ligand anchored to pIX's C-terminus. Finally, in **Chapter 4**, analogously to the pIX-pseudotyping approach, a fiber-pseudotyping approach is taken to allow convenient functional testing of new recombinant Ad fibers. Also in this chapter, a new fiber variant displaying a single-chain antibody fragment (scFv) was assessed for its suitability for Ad retargeting. Following these chapters on genetic capsid modifications, **Chapter 5** describes the development and validation of a new directed evolution approach for Ad that is based on mutator versions of the Ad-encoded polymerase. This evolution approach is employed to increase Ad's ability to lyse cancer cells.

Protein IX is a minor capsid protein located buried between the hexons that constitute the faces of the capsid icosahedron. Its capsid surface-exposed C-terminus (6) has previously shown to be a very useful locale for the introduction heterologous polypeptides, including small peptide ligands but also larger protein domains (7-11). In this regard, the inclusion of an alpha-helical spacer sequence into liganded pIX constructs was shown to improve accessibility of the ligand (9). Thus, pIX has general utility as a genetic anchor for viral display of foreign protein sequences. However, the conventional way to test new pIX variants for functionality in the virus particle – i.e. genetic incorporation into the virus – is cumbersome as it involves time-consuming steps as *in vitro* modification of the viral genome and subsequent virus rescue from naked viral DNA. Therefore, a more convenient system for routine functional testing of new pIX fusion proteins in the context of the Ad particle was developed (**Chapter 2**). The new approach entailed the use of LV vectors to generate helper cell lines stably expressing the pIX variant of interest. These cells then served to trans-complement a pIX-deleted Ad vector, thus yielding Ad vectors that were phenotypically pseudotyped with the concerning pIX variant. The use of LV vectors for providing pIX *in trans* proved very robust and practical: the pIX levels were at least as high as seen late during Ad5

infection, showed low variability among cells, and were sustained over time. Moreover, the pIX-expressing cells were fully able to *trans*-complement the pIX-deleted vector, both regarding capsid loading of pIX and restoration of heat stability of the virus. Thus, LV vector-based pIX-pseudotyping of Ad is feasible and provides a system for easy testing of new pIX variants in the context of Ad particles.

Next, the above pIX-complementation system was put into service to test a single-chain T-cell receptor (scTCR) for applicability as an Ad targeting ligand genetically displayed on pIX (**Chapter 3**). T-cell receptors (TCRs) determine a T-cell's specificity by binding to intracellular protein-derived peptide fragments in complex with major histocompatibility complex (MHC) class I molecules. TCRs specific for tumor-associated peptide-MHC complexes exist (12) and have been extensively explored to target genetically engineered T-cells to tumors (13). Further, Peng *et al*. recently demonstrated – for the first time – that TCRs, or derivatives thereof, also have utility as virus targeting devices (14). They showed that genetic fusion of a scTCR to the attachment protein of measles virus led to peptide-MHC complex-specific virus-cell entry and cell-to-cell fusion. The thusly demonstrated concept of TCR-based viral targeting is very attractive as it grants access to a new class of targets: intracellular antigens. In this regard, targets of particular interest may be cancer-testis (CT) antigens, a class of antigens that is highly restricted to tumors (15). Previously, another immunoglobulin superfamily member derivative, a single-chain antibody fragment, already proved functionally displayable on Ad via genetic fusion to pIX (16). Therefore, it was hypothesized that a pIX-scTCR fusion may likewise be functionally incorporated into Ad capsids. A scTCR directed against the CT antigen melanoma-associated antigen (MAGE)-A1 was fused – via an alpha-helical spacer sequence – to the C-terminus of pIX. The pIX-scTCR proved capsid incorporable and, moreover, showed evidence of mediating viral transduction by specific interaction with the cognate peptide-MHC complex. These results represent a first indication that scTCRs can be used as genetic Ad targeting ligands by fusion to pIX.

Given the usefulness of LV vector-based pIX-pseudotyping for the functional assessment of novel liganded pIX molecules (described in **Chapter 2**), an analogous approach was taken to test recombinant Ad fibers. Previously, it has been shown that wild-type fiber-expressing cell lines – generated by stable transfection through clonal selection – were able to *trans*-complement a fiber gene-deleted Ad vector (17,18). Additionally, a 'transient transfection/infection' procedure has proven to allow phenotypical fiber-pseudotyping of Ad (19). This latter procedure entails a large-scale transfection with fiber variant-expressing plasmids followed by the coordinated infection with a fiber-gene deleted virus. Aiming to extend this pseudotyping concept, **Chapter 4** describes that LV vectors can be used as a convenient alternative

tool to generate fiber variant-expressing cell populations employable for fiber-pseudotyping of Ad. Importantly, the fiber (variant)-expression cassettes used for this purpose had to be engineered to include the tripartite leader (TPL) sequence. TPL – a short 5' untranslated region found in major late-promoter-derived mRNAs – is known to function *in cis* to allow translation of late mRNAs in the context of host protein synthesis shut-off (20-24). Additionally, the TPL sequence has been shown to have a cis-stimulatory effect on transgene-expression in the absence of Ad infection (25,26). LV vectors incorporating the TPL sequence – proximally to the fiber start codon – were shown to generate fiber variant-expressing cell populations with characteristics favorable for pseudotyping experiments (i.e. expression levels were considerable, sustainable in time, and homogeneous among individual cells). Moreover, cells lentivirally transduced to express a modified fiber allowed for phenotypic fiber-pseudotyping of a fiber gene-deleted Ad vector.

Additionally in **Chapter 4**, the newly established transient Ad fiber-pseudotyping system was employed to assess the suitability – for Ad retargeting purposes – of a new, scFv-bearing chimeric fiber. Generally, antibodies, and their derivatives like scFvs, are not likely candidates for genetic Ad capsid incorporation because of biosynthetic incompatibilities: antibody polypeptides are routed to the endoplasmic reticulum (ER), while Ad capsid proteins are synthesized in the cytoplasm and migrate to the nucleus. Thus, when fused to a capsid component (and rid of their signal sequence), scFvs are forced to fold in the reducing environment of the cytosol, which is potentially problematic due to the lack of chaperone assisted folding and disulfide bridge formation (27). Indeed, a previous study showed that a scFv was not functional when displayed on a de-knobbed fiber (28). However, scFvs may be engineered for optimized stability in a reducing milieu, resulting in intrabodies or hyperstable antibodies (29-33). Previously, such stabilized antibodies have indeed been shown to be functionally displayable on the Ad capsid by genetic fusion to either pIX or fiber (16,34). In view of these favorable results, scFv800E6, a tumor antigen-directed scFv that was previously reported be structurally robust and thus to functionally fold in a reducing environment (35-37), was evaluated here for Ad capsid incorporation as part of a genetic fusion to a de-knobbed chimeric fiber. It was found that this '800E6 fiber' was able to trimerize (by virtue of a heterologous trimerization domain) and to achieve a degree of specific binding to its cognate target Her2/*neu*. However, this fiber showed only a low degree of capsid incorporation and, moreover, was unable to detectably mediate antigenic target binding by Ad. Further investigation into the intra- and extracellular redox states of the 800E6 fibers subsequently revealed that immediately upon cell lysis these fibers form large covalent aggregates through intermolecule disulfide bonds. Thus, it appears that a large fraction of the cysteines within the scFv moieties tend to engage

in non-canonical disulfide bridges (rather than the canonical intradomain bridges). Importantly, these findings strongly suggest improper cytosolic folding of the scFv. Thus, the particular chimeric fiber tested here was found not suited for Ad retargeting. Moreover, its scFv constituent, despite being reported to have robust structural framework, appeared to suffer from typical problems associated with the biosynthetic incompatibility between antibodies and Ad capsid proteins.

The folding difficulties encountered for the scFv in **Chapter 4** are thought to be generic to complex, disulfide bridge-containing secretory proteins being artifactually expressed in the cytosol (28). Therefore, other immunoglobulin superfamily member-based molecules, like the scTCR used in **Chapter 3**, could be expected to be similarly affected. While for the concerning pIX-scTCR fusion this was not specifically tested, preliminary unpublished data on scTCRs fused to fiber indeed show evidence of exactly the same phenomenon as seen for 800E6 fiber (i.e. covalent aggregation due to disulfide linkages upon cell lysis). Therefore, barring fusion partner-specific effects, many of the scTCR moieties present in pIX-scTCR fusion molecules may be improperly folded and thereby putatively be non-functional. The seeming discordance between the pIX and fiber data – regarding the successful use of an immunoglobulin-like retargeting ligand – may then be explained by pIX-scTCR being present with many more copies per capsid than 800E6 fiber (with a ~67 times difference if assuming 100% pIX and 10% fiber loading).

While **Chapters 2** to **4** describe ways to improve Ad vectors by rational design, **Chapter 5** deals with a new random engineering procedure for Ad. In general, virus engineering approaches based on genetic diversification followed by phenotypic selection have great potential to generate new or improved gene therapy vectors and oncolytic agents (38-43). The major benefit of such procedures is that they do not require *a priori* mechanistic knowledge to obtain new desired viral traits. Various distinct types of methodologies exist that could be considered a form of random virus engineering (see **Chapter 1**, **part III**). First there are the conventional forward genetics screens that make use of mutagens to cause genome-wide mutagenesis. Second, there are the more recent approaches that employ *in vitro* genetic diversification – mostly on viral genomic subfragments – followed by viral library-based selection. Finally, there are the viral adaptation schemes – mostly conducted with RNA viruses – that rely on spontaneous viral mutation.

In **Chapter 5**, a new concept for Ad engineering is described that relies on error-prone viral genome replication by modified versions of the adenovirus-encoded DNA polymerase (Ad pol) (44). To set up this 'accelerated evolution' approach, individual Ad pol residues implicated in maintaining polymerization fidelity, as extrapolated from other polymerase species, were targeted for mutation. This generated several single-amino-acid substitution

mutants of Ad pol that, while supportive of viral replication, exhibited an increased proneness to cause mutations. The respective mutator abilities of these mutants were revealed by a deep sequencing strategy allowing direct assessment of mutational buildups in viral pools. The two strongest of the identified mutator polymerases, T286I and F421Y, were both mutants of residues implicated in stabilizing the primer terminus at the exonuclease active site during proofreading. To validate the utility of the approach, the newly engineered mutator mutants of Ad pol were employed in an evolution regime that was aimed at increasing Ad's cytolytic activity in ovarian carcinoma cells. Importantly, this multi-passage evolution procedure led to the accelerated evolution towards the desired, selected-for phenotype. Characterization of individual viral clones generated in this manner demonstrated a strong and early overexpression of the Adenovirus Death Protein (ADP), a protein necessary for efficient cell lysis and viral release (45-47). Further analysis of these bioselected clones provided evidence for an increased utilization – within Ad's early E3 transcripts – of a mutated splice acceptor site preceding the ADP-encoding exon. Altogether, this work demonstrated that engineered 'sloppy' Ad polymerases can serve to provide the genetic diversity needed for efficient directed evolution of adenovirus. A distinctive feature of this mutator viral polymerase-based Ad engineering approach is that it intrinsically permits mutagenesis and selection over successive viral infections rounds. This property makes this approach particularly suitable for multiple-passage viral adaptation schemes, allowing for the possibility of individual viruses to acquire multiple beneficial mutations.

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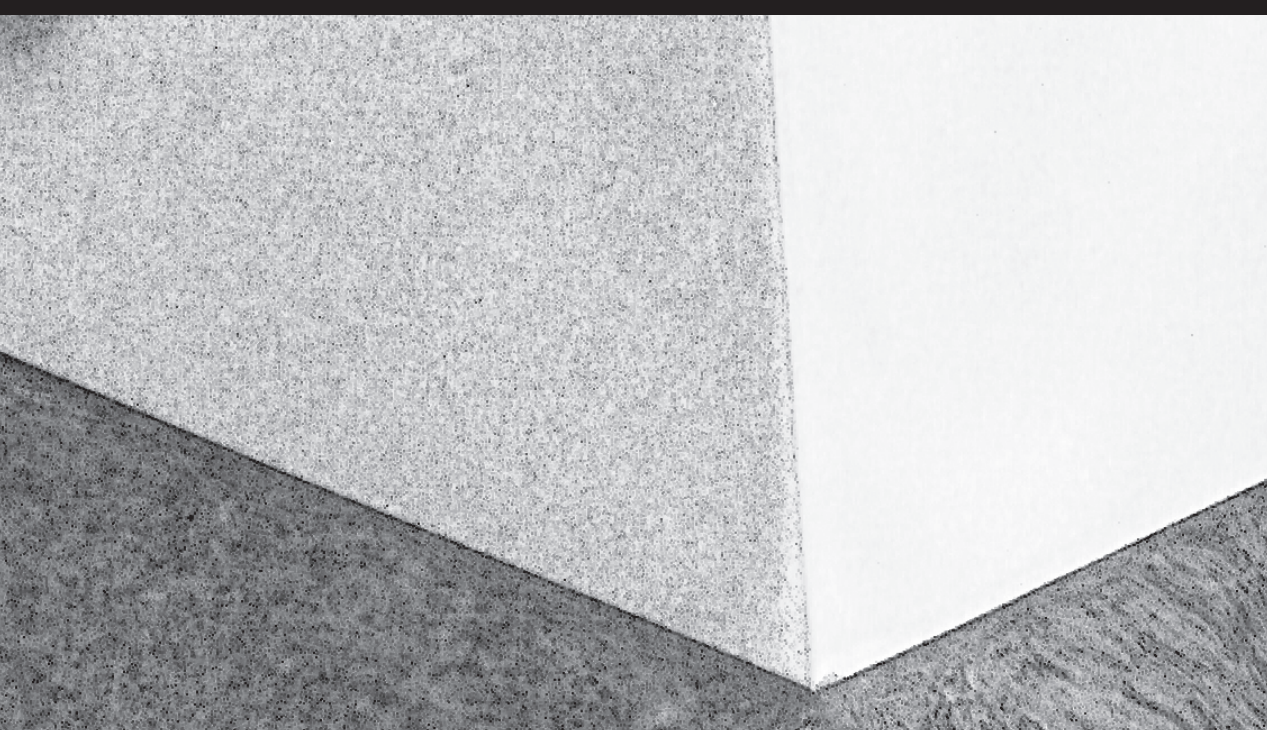
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NEDERLANDSE SAMENVATTING
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NEDERLANDSE SAMENVATTING

Het doel van het werk dat beschreven is in dit proefschrift is het verbeteren van de selectiviteit en effectiviteit van oncolytische adenovirusvectoren. Twee benaderingen zijn genomen om dit doel te bereiken: (i) genetische modificatie van virale capside-eiwitten om het infectieprofiel (op celtransductieniveau) van het virus te verbeteren (hoofdstukken 2 t/m 4), en (ii) artificiële evolutie om de cytolytische potentie van adenovirus te verhogen (hoofdstuk 5).

In hoofdstuk 1, deel II wordt kort de biologie van de adenovirussen geïntroduceerd en wordt beschreven hoe van deze virussen vectoren kunnen worden gemaakt. Vervolgens wordt in hoofdstuk 1, deel III een overzicht gegeven van de verschillende artificiële evolutiemethoden die gebruikt zijn om virale vectoren te genereren en te verbeteren.

De hoofdstukken 2 en 3 richten zich op modificatie van het adenovirus capside-eiwit 'IX' (pIX). Doel hiervan is door de incorporatie van 'targeting' liganden een veranderde receptorspecificiteit te bewerkstelligen. Het pIX is aanwezig op het oppervlak van het icosahedrische adenoviruscapside, waar het functioneert als 'cement' tussen de veel grotere hexoneiwitten. Eerder is gebleken dat de C-terminus van pIX gebruikt kan worden als anker om peptideliganden (en andersoortige polypeptiden) genetisch in het adenovirale capside te bouwen. In hoofdstuk 2 wordt een nieuw 'pseudotyping' systeem beschreven waarmee het mogelijk wordt om op snelle wijze nieuwe pIX-ligand combinaties te testen op functionaliteit. Hierbij worden lentivirale vectoren gebruikt om celpopulaties te maken die stabiel de nieuwe pIX-varianten tot expressie brengen. Door zulke celpopulaties met een pIX-gedeleteerd adenovirus te infecteren kunnen virussen gemaakt worden die fenotypisch gepseudotypeerd zijn met de betreffende pIX fusies. Dit systeem maakt het mogelijk om nieuwe pIX varianten te testen zonder dat daarvoor het adenovirale genoom hoeft te worden gemodificeerd.

In hoofdstuk 3 wordt het bovengenoemde pIX-pseudotyperingssysteem toegepast voor de analyse van een nieuw pIX fusie-eiwit. Dit eiwit bevat een enkele-keten T-cel receptor (scTCR) als 'targeting' ligand. De gebruikte scTCR is gericht tegen het intracellulaire 'cancer-testis' (CT) antigeen 'melanoma-associated antigen-A1'. Het chimère pIX molecuul bleek efficiënt te worden geïncorporeerd in het adenovirale capside. Bovendien gaven virustransductiestudies aan dat het capside-gekoppelde scTCR een zekere mate van doelceltransductie kon bewerkstelligen via specifieke interactie met het corresponderende peptide-MHC complex.

Volgens dezelfde methode is een fenotypisch pseudotyperingssysteem opgezet voor modificatie van de 'fiber' van het adenovirus (hoofdstuk 4). De adenovirale fiber bestaat als een homotrimere staafvormige structuur die verankerd is aan de 'penton-base'. Deze bevindt zich op elk van de twaalf hoekpunten van het icosahedrische adenovirale capside. Het naar

buiten gerichte C-terminale 'knob' domein van de fiber is verantwoordelijk voor binding met de primaire receptor van het adenovirus, de 'Coxsackie and adenovirus receptor' (CAR). Met zijn natuurlijke rol in de binding van celoppervlakkenreceptoren in het achterhoofd, ligt het voor de hand de adenovirus fiber te modificeren in strategieën die beogen het infectieprofiel van adenovirus te veranderen. Om het versneld testen van nieuwe fibervarianten mogelijk te maken werd een op lentivirale vector-gebaseerde, adenovirus fiber-pseudotyperingssysteem opgezet en gevalideerd. Dit systeem werd gebruikt om een nieuwe chimère fiber te testen. De betreffende fiber bevatte als 'targeting' ligand een enkele-keten antilichaam (scFv) gericht tegen het tumor-geassocieerde antigeen Her2/*neu*. De fiber-scFv fusie was in staat stabiele trimeren te vormen en bleek bovendien een zekere mate van specifieke binding met de corresponderende receptor te bewerkstelligen. Echter, de nieuwe fiber vertoonde problemen met betrekking tot capside incorporatie-efficiëntie, Her2/*neu*-bindingsfunctionaliteit in de context van het capside, en de vouwing van het scFv-gedeelte. De betreffende nieuwe fibervariant bleek daardoor niet geschikt voor adenovirus 'targeting'. Bovendien wezen de resultaten op een algemene biosynthetische incompatibiliteit tussen adenovirale capside-eiwitten en immunoglobuline-achtige 'targeting' liganden.

Hoofdstuk 5 beschrijft de ontwikkeling en validatie van een nieuwe op evolutie gebaseerde methode ter verbetering van adenovirale vectoren. Tot op heden zijn de meeste adenovirale vectoren gegenereerd door gerichte genetische modificatie. Hoewel deze rationele manier van virale vector ontwikkeling goede resultaten heeft geboekt, wordt ze vaak gehinderd door onze beperkte kennis van alle complexe virale structuur-functie relaties. Daarom kunnen strategieën gebaseerd op mutatie en selectie (zie hoofdstuk 1, deel III) een nuttig alternatief en/of aanvullende aanpak vormen voor het verbeteren van virale vectoren. Het is bijvoorbeeld al veelvuldig aangetoond dat de hoge mutatiesnelheden van RNA-virussen eenvoudig gebruikt kunnen worden om in evolutie-experimenten specifieke vectorologische doelen te bereiken. Daarom werd in Hoofdstuk 5 onderzocht of een artificiële evolutieprocedure gebaseerd op 'mutator' versies van het adenovirus polymerase gebruikt kon worden voor het genereren van verbeterde adenovirale vectoren. Om een dergelijk systeem te ontwikkelen, werd getracht de intrinsieke mutatie snelheid van adenovirus replicatie te verhogen door gerichte modificatie van het door adenovirus-gecodeerde DNA-polymerase. Dit werd gedaan door mutatie van residuen in de gebieden die geïmpliceerd zijn in nucleotideselectie en 'proofreading'. Sneller muterende polymerase mutanten konden vervolgens geïdentificeerd worden dankzij een mutatie-accumulatie en 'deep-sequencing' strategie. Tenslotte werd de mutator polymerase-gebaseerde artificiële evolutiemethode gevalideerd door een evolutie-experiment gericht op het verhogen van de oncolytische potentie van

adenovirus. Dit leverde virale mutanten op met een verhoogde en vervroegde expressie van het Adenovirus Death Protein (ADP), een adenovirus eiwit dat betrokken is bij gastheercellysis en virale spreiding

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 23 juni 1977 geboren in Delft. Na het behalen van het VWO diploma aan de scholengemeenschap Vincent van Gogh te Assen, werd in 1996 begonnen met de studie Medische Biologie aan de Vrije Universitieit (VU) in Amsterdam. Tijdens de doctoraal fase werd een stage gedaan op de afdeling Pathologie van het VU Medisch Centrum te Amsterdam onder supervisie van prof. dr. J.M. Middeldorp en ing. M.B.H.J. Vervoort. Hierna werd stage gelopen bij het Gene Therapy Center aan de Univeristy of Alabama at Birmingham (UAB) in de Verenigde Staten onder supervisie van prof. dr. D.T. Curiel en dr. T. Seki. Vervolgens werd een scriptie geschreven bij de afdeling Therapeutische Genmodulatie aan de Universiteit van Groningen onder leiding van prof. dr. H.J. Haisma en prof. dr. M.G. Rots. Na het behalen van het doctoraal examen in 2002 werd in datzelfde jaar begonnen met het promotie onderzoek op de afdeling Moleculaire Celbiologie van de Universiteit Leiden en het Leids Universitair Medisch Centrum onder leiding van prof. dr. R.C. Hoeben. In 2010 begon de auteur als onderzoeker in de afdeling Vaccine Research van Crucell Holland B.V. te Leiden.