Rational and random approaches to adenoviral vector engineering

Uil, T.G.

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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

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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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