Rational and random approaches to adenoviral vector engineering

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SUMMARIZING DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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