

Chapter 2

Recent Progress in Strategies for Adenovirus Mediated Therapeutic Cell Targeting

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Abstract Increasing numbers of therapeutic genes and cellular targets are available for gene therapy. Many clinical trials using virus-derived delivery systems are devoted to combat cancer, to correct single-gene malfunctions or to regenerate tissues. To develop gene delivery vectors with high efficiency through target cell selectivity, in particular under in situ conditions, remains a major challenge. The most widely used vector systems to transduce cells are based on adenoviruses. Recent approaches to develop selective adenoviral vectors (Ad) that exclusively target cells or tissues of interest without interfering with all others have focused on the modification of the broad natural tropism of adenoviruses. A popular way of Ad targeting is attained by directing vector particles towards distinct cellular receptors. Retargeting can be accomplished by linking custom-made peptides with unique specificity and reasonable affinity to cellular surface protein moieties via genetic alteration, chemical coupling or bridging with dual-specific adaptor molecules. Ideally, target-specific vectors are incapable of entering cells via their native receptors. Such altered vectors offer new opportunities to delineate functional genomics in the native environment and should enable efficient systemic therapeutic approaches. This review provides a summary of current state-of-the-art techniques to target specifically adenovirus-derived gene delivery vector systems.

Keywords Gene therapy • Vector targeting • Vector tropism • Adaptor molecules

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2.1 Introduction

2.1.1 *The Concept of Gene Therapy*

Somatic gene therapy is defined as the transfer of nucleic acids into a patient's cells to achieve a lasting therapeutic effect without influencing the germline. A myriad of conditions has been tackled where a cure is envisaged by intervening at the genetic level rather than via conventional application of drugs. Thereby, influence on or correction of inherited or acquired malfunctioning genes should be lasting. The best known tools to affect a cell's genetic makeup are virus-based vectors as the vehicles of choice to intervene on the gene expression profile of cells and ideally, to correct a malfunction, to suppress the expression of an undesired cellular gene or to aim at quantitative elimination of a population of cells out of control. Still, therapeutic approaches to alter inherited or acquired genetic disorders as well as those to eliminate neoplastic cells once disseminated in the patient are still far from regular clinical application.

Effective in vivo transfer of a gene using an adenoviral vector was shown in 1991 by Ronald Crystal's group (Rosenfeld et al. 1991), and the first clinical application of a vector delivering an intact cystic fibrosis transmembrane conductance regulator gene was done in 1993 as reviewed by Crystal (2014). In his review, the author reiterates in retrospect the success and feasible future applications for adenovirus-based vectors in human gene therapy. Due to the strong immune response against vector proteins, Crystal concludes that Ads may serve best in temporary applications to build new structures, to selectively destroy cells or as vehicles to induce immunity against a transgene. The authors of this chapter put the focus on the concepts of targeted adenoviral gene transfer by ablating the native tropism and reroute Ad vectors by altering their physical composition or by covering the native particle with moieties directed against selected cell plasma surface structures. At the time of assembling this article, cumulatively over 2200 gene therapy trials in different clinical phases were completed or are ongoing with more than half of these clinical trials being dedicated to combat cancer. Amongst those, Ads are the most widely used gene transfer vehicles (Wirth et al. 2013; Das et al. 2015).

2.1.2 *The Tools for Gene Therapy*

The isolation of adenoviruses dates back to 1953 (Rowe et al. 1953). The family of Adenoviridae consists of four accepted genera (Davison et al. 2003; Cupelli and Stehle 2011) with some species awaiting assignment. Adenoviruses infect numerous vertebrates including man. Nowadays, about 57 human adenoviruses have been identified and were subclassified on the basis of parameters regarding classical standard methods, and more recently, by comparison of the adenoviral genomes (Seto et al. 2011). In the genus Mastadenovirus, the human pathogenic species are

further divided into the subgroups A to G (Russel 2000; Berk 2013). The virions are built up of an icosahedral capsid that carries a single molecule of double-stranded DNA as the viral genome. The particles are studded with 12 fibers consisting of trimeric rigid structural elements linked to the icosahedron's vertices. The capsid components forming the vertices are called penton bases and the fibers are non-covalently linked to these structures. Most virus serotypes bind to their target cells via the interaction of the fiber knob, the most distal part of the fiber with their counterpart, a protein structure on the cell plasma membrane. This moiety, the coxsackie adenovirus receptor (CAR) represents the main high-affinity receptor for many adenoviruses. CAR is a 46-kDa transmembrane protein in the superfamily of immunoglobulins (Bergelson et al. 1997; Roelvink et al. 1998; Bewley et al. 1999). Internalization of the virus depends on the subsequent secondary interaction of an Arginine-Glycine-Aspartate (RGD) protein structure on the penton base with $\alpha V\beta 3$ or $\alpha V\beta 5$ integrins also present on the cell plasma surface (Wickham et al. 1993). CAR is highly abundant in many tissues and therefore, adenoviruses display a wide tropism and infect a broad spectrum of cells (Wickham 2000; Arnberg 2009; Chailertvanitkul and Pouton 2010).

All of these characteristics drew an early focus on this virus family as masterpieces for gene therapy approaches. Unlike other viral vector systems, Ad virions have a high packaging capacity and easily replicate to high titers. Moreover, Ad-derived vectors maintain high stability *in vivo* and transduce both, dividing and non-dividing cells (Douglas 2004). Once internalized into the cell, the genome predominantly persists as episomal DNA with an extremely low frequency of integration into the host genome (Rauschhuber et al. 2012), and thereby, insertional mutagenesis does not occur. Considering this safety aspect, adenovirus based vectors are particularly attractive for gene therapy applications, where temporary gene expression is desired or preferred over permanent genetic modifications. Due to their apathogenicity and non-oncogenic properties, the most commonly utilized Ad vectors for gene therapy are derivatives from adenovirus Serotype 5 (Ad5) in the subgroup C of human adenoviruses (Arnberg 2009; Armendariz-Borunda et al. 2011). What ultimately converts the replicating agent into a one way delivery system is the purposeful removal of a part of the viral genome (Haj-Ahmad and Graham 1986).

Tissue tropism of adenoviral vectors is greatly influenced by the viral serotype and receptor presence and density, depending on a cell type's provenance (Wickham et al. 1993; Hashimoto et al. 1997; Takayama et al. 1998; Havenga et al. 2002; Arnberg 2009). Ad5 are best explored and most widely used in preclinical studies as well as clinical trials. Ad5 particles bind exclusively to CAR. However, in the case of Ads made from Serotype 41, a subgroup F member, only one of its two distinct types of fibers can recognize CAR (Russel 2000; Zhang and Bergelson 2005). Some serotypes enter host cells via other receptors such as CD46, desmoglein 2, CD80 or CD86 or the sialic acid moiety (reviewed by Sharma et al. 2009). Altogether, Ad5 vectors are efficient vehicles for delivering foreign genes into target cells *in vitro* and *in vivo* (Haj-Ahmad and Graham 1986; Pützer et al. 1997). Based on the favourable attributes, Ad5 remains the vector of choice in human gene therapy trials due to their proven safety profile (Hedley et al. 2006; Das et al. 2015).

Whenever the utilization of adenoviral vectors as targeted gene delivery systems is desired, the Ad5 vector's broad tropism for a wide range of cells and tissues is a challenging obstacle (Coughlan et al. 2010). If a gene transfer system is required that exclusively alters a single cellular compartment or a particular kind of tissue that spares all other cells and tissues from transduction, alteration of vector particles becomes essential. Another major obstacle after systemic adenoviral vector administration is that 80 % of circulating particles are sequestered in the liver after an interaction with coagulation factors (Shayakhmetov et al. 2005; Stone et al. 2007), and thereby, most particles may not reach the tissue to be addressed. A number of researchers demonstrated that after the virus is recognized by the coagulation system, the immune system is activated, and in turn, an acute inflammatory response is initiated (Reynolds et al. 2001; Shayakhmetov et al. 2004; Khare et al. 2011; Doronin et al. 2012; Xu et al. 2013). In addition, regarding Ad transport in the bloodstream, Duffy and colleagues (2013) identified a number of small molecules capable of efficiently blocking the intracellular virus transport independently of factor X-associated inactivation. Considering these hurdles, a selective gene transfer by wild-type adenoviral vectors imposes an increased risk of toxicity due to vector dissemination to non-targeted cells, even if the particles are administered close to or directly into the tissue of interest. Other undesired side effects of systemic virus administration are virus-associated immunogenic toxicity, thrombocytopenia, intense periportal polymorphonuclear lymphocyte infiltration and elevated liver enzyme secretion (Morrall et al. 2002; Thacker et al. 2009a; Coughlan et al. 2012).

The reverse obstacle is the question of how to reach cell types refractory to adenoviral infection, due to their lack of or insufficient CAR expression. Such cells include, for instance, many cancer cells, as well as hematopoietic and neural stem cells (McConnell and Imperiale 2004; Schmidt et al. 2005). To achieve gene transfer into those cell types and to ensure efficient integrin receptor-mediated virus uptake, extremely high vector doses are required. High vector doses in turn increase inadvertent side effects, like viral sequestering in Kupffer cells in the liver (Haisma et al. 2009), and once vectors surpass the latter's binding capacity, hepatocytes will absorb the remaining vector particles.

The restrictions outlined above can be overcome by strategies to modify the vector's cellular tropism, as reviewed by Beatty and Curiel (2012). Redirecting vectors towards cells of interest can also enhance the therapeutic potential with increased safety by reduction of immune responses, since simultaneous re- and de-targeting allows lower vector doses to be administered systemically (Schmidt et al. 2007; Dorer und Nettelbeck 2009; Haisma et al. 2010; Schmidt et al. 2011). In this review, we present and discuss three different methods to alter the natural Ad vector tropism. We describe genetic integration of peptide sequences into the fiber, peptide conjugation via chemical modification using polyethylene glycol (PEG) and bridging the vector and cell of choice with bispecific adaptor molecules. The advantages and benefits, as well as restrictions and limitations of these technological approaches are reported and debated. The initial considerations towards targeting, however,

relate to the identification of suitable moieties on the plasma membrane of the cells or the tissue to be addressed that fulfil the following characteristics: singularity, abundance, and affinity.

2.2 Screening Methods for Cell-Specific Ligands

The ultimate gene “taxi” for systemic gene therapy purposes should exclusively recognize the cells to be treated and leave all others unaffected. Directed gene delivery can be achieved by addressing selective moieties on the cells of interest. Specifically selected peptides possess appropriate properties to serve as targeting agents and are valid alternatives to antibody-based targeting approaches, since unique cellular receptors are often unknown.

The simplest way to design a specific binding peptide for a receptor is to analyse structural data and the expression profile of cells to identify candidate peptides. Often however, structural data are not available or incomplete. To solve this, the phage display method is a frequently used technique to determine specific binding peptides. We and others have used the phage display technology to screen for and to identify tissue- or cell-specific ligands in cell culture systems and animal models (Böckmann et al. 2005a, b; Vives et al. 2008, Reetz et al. 2013). Already in 1990, researchers constructed an epitope library that yielded a mixture of filamentous phage clones with each one displaying a single peptide sequence on the virion’s surface (Scott and Smith 1990). After the interaction of the phage with the specific binding partner, the expansion of the phage comprehends several rounds of infection followed by selection. The display of polypeptide repertoires on the surface of phages, together with the efficient enrichment and amplification of the desired binding specificities was shown to be a valuable route towards isolation of unique peptides suitable to serve as vehicles for targeting applications (Arap et al. 1998; Nicklin et al. 2000; Essler and Ruoslahti 2002; Dias-Neto et al. 2009; Chen et al. 2010; Kügler et al. 2013). Taken together, the phage display technique identifies peptides in a range from 7 to 12 amino acids (Vives et al. 2008).

Phage display was successfully employed to acquire peptides that specifically recognize human embryonic progenitor cells (Bignone et al. 2013) and bind normal or aberrant tissues, like vascular endothelium (Pasqualini and Ruoslahti 1996, 2000; White et al. 2001), lymphatic vessels (Laakkonen et al. 2002), kidney tubules (Odermatt et al. 2001), hepatocytes (Piccolo et al. 2014), and several others (Barry et al. 1996; Ravera et al. 1998; Ivanenkov et al. 1999; Mazzucchelli et al. 1999; Cheung et al. 2013; Sclavons et al. 2013). Furthermore, the lack of gene transfer systems that are potent in selectively targeting tumor tissue prompted the search for cancer-specific peptide molecules for yet unknown tumor-associated receptors (Araki et al. 2010; Deutscher et al. 2010; Ahmadvand et al. 2011; Nishimoto et al. 2012). Many novel peptides homing to angiogenic vessels showed cross-affinity with several tumor types (Liu and Wu 2008). In this regard, we conducted biopanning on

human medullary thyroid carcinoma (MTC) cells *in vitro* and transplanted tumor xenografts *in vivo* (Böckmann et al. 2005a). MTC, which is caused by dominant activating mutations in the RET proto-oncogene encoding a transmembrane tyrosine kinase receptor, is characterized by aggressive growth and early metastasis and therefore, provides a perfect model for targeting disseminated cancer cells (Drosten and Pützer 2006). The selected phages bound with highest specificity to and were internalized by these tumor cells in culture as well as after systemic injection into nude mice (Böckmann et al. 2005a). The same 7-mer cyclic phage peptide library was injected into the tail vein of RET oncogene transgenic mice carrying bilateral orthotopic tumors in their thyroid glands (Böckmann et al. 2005b). This ligand that also binds efficiently to human MTC cells was covalently linked to adenoviral capsids carrying a RET oncogene inhibitor as therapeutic gene. Systemic delivery of this peptide-tagged adenovirus led to a substantial growth reduction of orthotopic and disseminated xenograft tumors, while the interaction with other organs, such as the liver, was abolished (Schmidt et al. 2011). This precedent opens a promising new road towards using peptide-mediated adenoviral gene transfer to achieve an efficient and selective therapeutic response also against circulating and metastasis initiating tumor cells detected in an advanced stage of metastatic disease that possess features of cancer stem cells. Beyond that, other researchers took the first step in developing a molecular map of the human vasculature by screening a peptide library in patients (Arap et al. 2002; Chang et al. 2009; Seung-Min et al. 2009). Rangel and co-workers developed a novel technique that enables receptor-independent phage particle entry into mammalian cells. Phage particles provide a unique discovery platform for combinatorial intracellular targeting of organelle ligands along with their corresponding receptors and for fingerprinting functional protein domains in living cells (Rangel et al. 2012, 2013).

An alternative approach that aims at detecting molecules with high affinity, adequate specificity and suitable pharmacokinetic properties for *in vivo* applications is represented by single-stranded nucleic acid ligands, termed aptamers. Aptamers are isolated by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology. Applying this technology against whole-living cells in culture or *in vivo* allows direct selection of aptamers even against rare antigens without prior purification of membrane-bound targets, access to membrane proteins in their native conformation and identification of targets related to a specific phenotype. Their thermal stability, low cost, unlimited applications and high binding affinity to disease-associated proteins or non-protein targets (Song et al. 2012; Wang et al. 2014; Mahlknecht et al. 2015) make them attractive, even in clinical trials for the treatment of distinct medical conditions, as reviewed extensively elsewhere (Sundaram et al. 2013; Tan et al. 2013; Zimbres et al. 2013). The potential of aptamers as tools for tumor targeting gene delivery systems with high transduction efficiency was summarized by others (Zhu et al. 2012; Hu and Zhang 2013). In this perspective, an innovative step towards targeted therapies would certainly be a combination of both technologies, cell-specific aptamers and adenoviral vectors.

2.3 Methods to Specifically Target Adenovirus-Derived Vectors

2.3.1 Genetic Fiber Engineering

To increase the selectivity of adenovirus for target tissues, novel approaches in Ad vector design exploit the concept of tissue-specific expression of therapeutic transgenes or virus replication. With the fiber being the major determinant of adenovirus tropism, the development of genetically targeted vectors has, consequently, focused on this distant protruding entity of the particle. The fiber is a rigid homotrimeric protein structure characterized by a domain organization with an N-terminal tail domain anchoring it in the Ad capsid. A C-terminal globular domain, termed the knob, mediates binding to CAR and a central shaft domain extends the knob away from the particle. Any manipulations of the fiber knob per se significantly reduce the transduction efficiency of CAR-positive cells by Ad vectors (Wickham et al. 1997; Krasnykh et al. 1998; Einfeld et al. 2001). Different strategies of adenovirus fiber modification have been employed, like genetic replacement of the fiber or ligand incorporation into the fiber knob. In this regard, the development of a fiber phage display system (Nishimoto et al. 2012) or a fiber-shuttle library for the adenoviral knobs (Wu et al. 2010) provide tools to alter Ad vector binding specificity.

Several studies narrowed down the insertion positions for targeting peptides within the fiber knob to two locations where the vector system as such tolerates the genetic alterations without structural impairment. The sites of choice for targeting ligand incorporation are the fiber knob's HI loop, which connects the β -strands H and I, and the C-terminus of the protein (Dmitriev et al. 1998; Krasnykh et al. 1998; Mizuguchi et al. 2001; Koizumi et al. 2003; Glasgow et al. 2004; Nettelbeck et al. 2004; Coughlan 2009; Wang et al. 2011). These findings indicated that ligands whose sizes exceed 25 to 30 amino acid residues cannot be configured into the carboxy-terminus of the fiber, as they destabilize the fiber structure (Wickham et al. 1997) and thus, limit the range of potential ligand candidates to short peptides. The structural properties of the HI loop of the Ad fiber, however, favor the insertion of larger ligands and expand the size of potential targeting moieties. Meanwhile Behr and colleagues (2014) elaborated on insertion of a peptide ligand to target the receptor tyrosine kinase EphA2 by introducing a previously selected peptide into different positions of a chimeric fiber derived from Ad5 and Ad 41 with striking permissiveness concerning the insertion site into several loops of the fibers.

When testing the resilience of fiber modification, Belousova and co-workers (2002) incrementally increased the size of the peptides integrated in the HI loop and generated Ad vectors with fiber inserts ranging from 13 to 83 amino acid residues. The authors concluded that the incorporation of heterologous sequences in the examined size ranges was essentially tolerated without a negative impact or compromising the production yield or transductional properties of the vectors. HI-loop incorporation of rather short 7- and 9-mer peptides was performed to transduce CAR-deficient primary tumor cells, such as ovarian cancer cells, vascular endothelia,

vascular smooth muscle cells, and brain microcapillary endothelia in culture (Dmitriev et al. 1998; Nicklin et al. 2000, 2003; Xia et al. 2000; Work et al. 2004). The last step of Ad entry into target cells depends on the interaction between RGD motif at the penton base protein and the host cell integrins (Cao et al. 2012). Ad vectors containing this RGD peptide in the HI loop of the fiber showed higher yields of gene transfer than vectors containing the identical peptide attached at the fiber's C-terminus, due to the easy access to the receptor (Kurachi et al. 2007; Tanaka et al. 2007; Schmidt et al. 2011). Several researchers efficiently transduced different types of tumor cells by inserting this RGD motif into the HI loop of the Ad fiber in vitro (Terao et al. 2009) and in vivo (Bayo-Puxan et al. 2009; Katayama et al. 2011). Rojas et al. (2012) improved systemic antitumor therapy with oncolytic adenoviruses by replacing the fiber shaft heparin sulfate glycosaminoglycan-binding domain with RGD in order to achieve simultaneously liver de- and tumor re-targeting.

Each fiber knob monomer forms an eight-stranded antiparallel β -sandwich structure. The β -strands are connected with turns and loops (Bewley et al. 1999). To further reduce the transduction efficiency of Ad vectors to CAR-positive cells, elaborate mutation studies on the AB, DE or FG loop of the fiber knob have been reported (Roelvink et al. 1999; Jakubczak et al. 2001; Leissner et al. 2001). In order to completely ablating a vector particle's tropism from CAR binding and eventually entering the cell, genetic fiber modification is not sufficient. Rather, the secondary minor interaction of the RGD motif at the penton base with the αv -integrin receptor should be depleted as well (Mizuguchi et al. 2002), to fully ablate Ad vectors from the native binding moieties. Whereas the dual mutation markedly reduces the retention of the vector in the liver (Einfeld et al. 2001; Koizumi et al. 2003), single mutations in the fiber knob or penton base did not alter the biodistribution of adenoviral vectors injected into mice (Alemany and Curiel 2001; Leissner et al. 2001; Nakamura 2003; Smith et al. 2003a, b). In order to use Ads in cancer gene therapy, gene transduction to tumor cells is limited by the weak expression of CAR on these cells, as reviewed by (Tanaka et al. 2007). Magnusson and co-workers (2007) efficiently transduced human ovarian and breast cancer cell lines with a vector that carried a tandem repeat of the human epidermal growth factor receptor 2 (HER2/neu) reactive affibody molecule in the HI loop of a CAR ablated fiber knob. Later, the group generated a vector with dual specificity by incorporating the HER2/neu-binding (ZH) and Taq polymerase-binding (ZT) sequences at different positions within the HI-loop. Receptor-binding studies revealed that ZT in the first position and ZH in the second position bound to both receptors, whereas the reverse order of both motifs was devoid of binding to HER2/neu (Myhre et al. 2009). Subsequently, these researchers designed a vector to transduce efficiently HER2/neu-presenting cell lines, by altering the RGD motif to EGD (Glu-Gly-Asp) and substituting the KKTK motif in the third shaft repeat to RKSK (Arg-Lys-Ser-Lys). This new vector at last gained the ability to efficiently infect prostate cancer cells in vitro (Magnusson et al. 2012).

Genetic modification also covers the replacement of the entire fiber or just the knob domain with that derived from other adenovirus serotypes (Wu et al. 2002).

Belousova et al. (2003) targeted an Ad vector with bacteriophage T4 fibrin to the CD40 receptor. The tropism was modified by incorporating into the virion capsid a recombinant protein comprising structural domains of the Ad Serotype 5 fiber, phage T4 fibrin, and the human CD40 ligand. The authors achieved specific gene delivery in monocyte-derived dendritic cells *in vivo*. In a pilot vaccination study, Thacker and colleagues (2009b) successfully targeted these cells in a canine model by integrating the CD40 ligand into the fiber knob. The same group reported later that Ad vectors bound to the CD40 ligand failed to infect integrin-deficient canine lymphoma cells. This study demonstrates that the lack of virus internalization signals can impair targeting approaches (O'Neill et al. 2011).

Yu and co-workers (2011) modified the Ad5 hexon protein by inserting the protein transduction domain from the HIV-1 Tat protein. The resulting viral vector showed significantly higher transduction efficiency on many tumor cells compared to the parental vector. In the next step, this group increased the infection efficiency of human primary cell types even further after swapping wild-type Ad5 fiber against a Serotype 35 fiber specific for the CD46 receptor, a surface marker often upregulated in malignant tumors. This surface modified Ad vector was developed to transduce cells that are otherwise difficult to transduce in basic, pre-clinical, and clinical research (Yu et al. 2013). Another strategy to reroute adenoviral vectors from healthy towards cancer tissue is the utilization of recombinant adenovirus. The Lieber group constructed a capsid-modified adenovirus that expresses the TNF-related apoptosis-inducing ligand (TRAIL) and specifically replicates in tumor cells (Sova et al. 2004). Their Ad capsid contains the Serotype 35-derived short-shafted fibers, which recognize the CD46 receptor. In combination with TRAIL, expression of this oncolytic vector induces apoptosis in tumor cell lines derived from human colorectal, prostate, lung, and liver cancer. Both, the cell culture and xenograft tumor models tested in these experiments showed efficient intratumoral spread of the virus.

Yet others designed Ad vectors presenting the short fibers of Ad41 as a ligand insertion tool, achieving higher infection efficiency when compared to viruses presenting the same ligand cloned into another part of the fiber (Hesse et al. 2007). Even an enhanced transduction efficiency of recombinant adenovirus Serotype 5 vectors with Serotype 35 fibers was observed by Matsui and co-workers (2011). Using a feasible *in vitro* ligation, the group incorporated two copies of the RGD peptide in two different loops of the fiber 35 knob and observed high infection efficiencies in CD46-positive cells.

Overall and despite these positive results, genetic modifications on the native Ad5 fiber knob remain a laborious technical challenge and its benefit is hard to calculate. The repertoire of incorporable ligands to yield functional retargeted vectors for gene therapy is restricted to a small number of peptides that do not impair correct folding and assembly of the fiber trimer (Magnusson et al. 2002). A general limitation of this approach is the necessity to tediously re-engineer a given Ad vector for every further target cell. On the other hand, if common motifs were found for instance on the surface of cancer cells, corresponding metastases, and/or circulating cancer stem cells, the workload and more fine-tuning on fiber manipulations may pay off. With oncolytic viruses, the agents engineered from wild-type replicating

pathogens and designed to specifically deplete neoplastic cells, both, in solid tumors and straying through the body, chimeric fibers gain further attention. Oncolytic viruses are by definition restricted to replicate only in neoplastic cells and supposed to deliver a payload that attracts the immune system or restores apoptotic pathways (Dias et al. 2012; Schipper et al. 2014) and many ongoing clinical trials rely on the power of this concept (Pol et al. 2014; Jiang et al. 2015). To target pancreatic cancer, Nishimoto and co-workers (2009) screened a peptide-display adenovirus library. They came up with a selected peptide incorporated in the fiber of a virus ablated for CAR interaction. The technique based on a previous paper of the same group showing the validity of incorporating random sections of genomic DNA and screening of the resulting viruses for specificity for a desired cell type (Miura et al. 2007). The virus yielded potent cancer cell killing than the parental virus only deficient for CAR binding.

More recent setups towards fiber modification came for instance from the Curiel group. In a think out of the box approach, the researchers utilized antibodies from a member of the camelid family, the alpaca. Immunoglobulin of these animals consists of heavy chains only. The group immunized the animals with the human carcinoembryonic antigen and, in brief, replaced the adenovirus knob and part of the shaft with protein fragments directed against the antigen which transduced target cells that carried the original epitope (Kaliberov et al. 2014b). Jose and colleagues (2014) made a tumor selective adenovirus altering the fiber in a way that it gets activated by cancer-specific proteases. This approach can be fruitful for both, vector design and the development of virotherapeutic agents.

2.3.2 Alteration of Vector Tropism by PEGylation

Targeting an adenoviral vector can be achieved without altering the fiber by genetic modification. One option to ablate and redirect an adenoviral vector's tropism is to coat the otherwise native particle via covalently attached small molecules. This can be achieved by means of bispecific non-toxic spacer molecules. Polyethylene glycol (PEG) is a linear hydrophilic polymer and often used in pharmaceutical formulations involving proteins and peptides to prolong their blood persistence (Mizuguchi and Hayakawa 2004). In addition, PEG is, therefore, considered as a bridging entity between the capsid and peptide of choice for a given cell plasma membrane target. Optimized transduction by targeted Ad vectors can be accomplished by linking cell-specific peptides, antibodies or antigens to the particle's surface by a chemical process called PEGylation (Kreppel and Kochanek 2008). PEG forms a covalent bridge between the proteins of the virion's surface and the targeting molecule of choice, resulting in a vector coated all over with the desired ligand. This approach of redirecting viral vectors does not require genetic modification, including the efforts to ablate the native tropism. Additional benefits of PEGylated vectors are reduced immunogenicity (Elkon et al. 1997; Zaiss et al. 2002; Croyle et al. 2004; Mok et al. 2005), fewer hepatotoxic side effects (Gao et al. 2007), less cytokine secretion and prolongation of the vector plasma half-life (Alemany et al. 2000).

Such PEG-driven Ad vector modifications have been adapted in a number of targeting approaches (O’Riordan et al. 1999; Alemany et al. 2000; Croyle et al. 2002; Lee et al. 2005; Hofherr et al. 2008; Eto et al. 2010; Wonagan and Croyle 2010). The success of such approaches might depend on the ligand size. Romanczuk and co-workers (1999) were the first to link biologically selected peptides to Ads surface via PEGylation. For instance, coupling of a short RGD motif to the tip of PEG has shown both, high in vitro transduction efficiency (Lanciotti et al. 2003; Eto et al. 2004, 2005, Ogawara et al. 2004) and overall improvement of systemic gene delivery (Xiong et al. 2006; Gao et al. 2007). To target ovary cancer cells, the full-length fibroblast growth factor 2 (FGF2) was linked to an Ad vector by PEGylation. This vector mediated increased transgene expression in tumor tissue and reduced localization of adenovirus to non-target cells when compared to the native vector (Lanciotti et al. 2003). To silence the proinflammatory activation status of endothelial cells, Kuldo and colleagues (2013) demonstrated the potential of an E-selectin targeted adenoviral vector to deliver a therapeutic transgene into microvascular endothelial cells in inflammation and downregulate the endothelial adhesion molecule. Besides ablating the native tropism and to redirect a vector to another cellular receptor, the PEGylation treatment prolongs the circulation time and decreases the formation of neutralizing antibodies as shown by Kim and colleagues (2011). A PEGylated Ad that recognizes Her2/neu receptor-positive cancer cells showed longer circulation times than the unmodified control and decreased the level of neutralizing antibodies. These observations raise positive expectations for future therapeutic applications of PEGylated vectors against late-stage cancer diseases. Exploring the suitability of PEGylated Ad vectors to address metastatic tumors, a dual cancer-specific strategy was described using this technology for transductional targeting with transgene expression under control of the telomere reverse transcriptase promoter for transcriptional targeting (Yao et al. 2009).

With regard to the conclusion that the molecular weight of PEG and the PEG modification ratio significantly affects the characteristics of conjugates (Kaneda et al. 2004), Eto and colleagues (2010) optimized adenovirus PEGylation in a way that after systemic administration of PEGylated adenoviral vector expressing tumor necrosis factor-alpha, an antibody reduction against Ad and an increased therapeutic response was observed against metastatic cancer. In a rather elegant experimental setup, Yao and co-workers demonstrated that the CGKRR (Cys-Gly-Lys-Arg-Lys) peptide conjugated to Ad with PEG was highly selective and yielded good gene expression in tumors and tumor vasculatures after systemic administration (Yao et al. 2011, 2012). Their results indicate an important aspect to consider if working with Ads coated with PEG. The appropriate ratio between PEG and targeting ligand concentration is crucial to achieve specific tissue transduction. As described above, the latest success in the treatment of disseminated tumors in mice was made by injecting a low dose with 10^8 plaque forming units per animal of Ad vector encoding RET oncogene inhibitor coated with MTC-specific 7-mer peptide via PEG into the tail vein, which led to the regression of multiple orthotopic and xenograft tumors in mouse models (Schmidt et al. 2011). The same Ad-PEGylation approach using a short artificial peptide selected by phage display, which in this case, specifically

binds neural stem cells isolated from the hippocampus of adult mice, was highly effective after injecting the vector into the brain (Schmidt et al. 2007). Such tools could eventually serve to exclusively manipulate neural stem cells either by direct injection in the brain or systemic vector application with the potential as a delivery system for therapeutic genes to treat various disorders of the central nervous system.

Another chemical Ad modification using diblock copolypeptides as an alternative for PEG was first described by Jiang and co-workers (2013). Copolypeptides are well-defined polypeptide sequences (Deming 1997) which provide non-covalent Ad vector modification resulting in efficiently altering Ad tropism with an obvious potential to target cancer metastases. PEGylation has also been used to link cell-penetrating peptides to adenoviral particles to overcome obstacles with the CAR ablation (Nigatu et al. 2015). Whether this treatment will have advantages when targeting the particles to specific cells remains to be seen.

2.3.3 Bifunctional Non-Covalently Linked Adaptor Molecules

Another way for re-directing and widening Ad vector tropism is the application of bifunctional adaptor molecules or other bispecific protein fragments often derived from antibodies. Such adaptors recognize with one binding site the vector and the other the desired structure on the cell membrane. Bispecific antibodies are usually composed of an anti-fiber binding portion and a component specific for a cell-specific receptor or secondary antibody conjugated with a peptide moiety against a selected cell surface antigen. Since CAR does not play any role in virus internalization, the Ad fiber knob's CAR binding domain accessibility is dispensable and therefore, the candidate of choice to link heterologous binding sites, for instance a bispecific adaptor molecule. A fully studded Ad vector particle with a bridging molecule prevents any interaction with CAR and thus, ablates Ad's native tropism (Curiel 1999; Everts and Curiel 2004; Glasgow et al. 2006).

In an initial demonstration of CAR-independent targeting, a conjugate consisting of folate and a fragment derived from an antibody directed against the fiber was used as a recombinant protein to bind the Ad fiber as well as the target, the folate receptor, which is widely expressed on the surface of numerous malignant cells (Douglas et al. 1999). In a similar strategy, a conjugated FGF was used to target ovarian carcinoma cells (Rancourt et al. 1998). The approach reached a clinical trial, where FGF2-conjugated Ad vector expressing human herpes simplex virus thymidine kinase was applied in patients (Bauerschmitz et al. 2002). Reynolds and colleagues (2000) succeeded in targeting pulmonary endothelial cells in vivo by i.v. injection of Ad vectors bearing fibers studded with a bispecific antibody directed against the Ad fiber knob and the angiotensin-converting enzyme.

In light of the development of new therapeutic strategies for diseases in which angiogenesis plays an important role and considering that physiological barriers for high molecular weight components prevent the transduction of the majority of

tumor cells, vascular targeting became a worthwhile approach in cancer gene therapy (Griffioen and Molema 2000). Targeting of adenovirus to endothelial cells by a bispecific fusion protein directed against the human endoglin CD105 receptor for antivascular cancer gene therapy was published by Nettelbeck and coworkers (2001). In 2004, the same group designed a single-chain adaptor molecule that binds the fiber protein and the high molecular weight melanoma-associated antigen. This antigen is widely and specifically expressed on the surface of melanoma cells and its expression is associated with tumor development and progression (Nettelbeck et al. 2004). Other bispecific constructs directing Ad fibers to cells were developed for endothelial receptors (Haisma et al. 2010), the epidermal growth factor receptor (Haisma et al. 2000; van Beusechem et al. 2000), and the lymphocyte antigen 6 complex (van Zeeburg et al. 2010). An elegant approach uses a truncated soluble form of CAR as the virus attachment site fused to human epidermal growth factor (EGF) to direct a vector against cancer cells that express the EGF receptor (Dmitiriev et al. 2000; Kashentseva et al. 2002; Hemminki et al. 2002). In addition, a number of authors described the adaptor-based strategy to target CAR-deficient dendritic cells as a therapeutic vaccination against cancer or infectious diseases (Tillmann et al. 2000; Pereboev et al. 2004; Kim et al. 2010; Echeverria et al. 2011; Hangalapura et al. 2012; Williams et al. 2012). Figure 2.1 provides a schematic representation of all three strategies used to alter virus tropism described above.

Watkins and colleagues (1997) utilized a construct that encodes a fusion protein derived from a single-chain neutralizing anti-adenovirus fiber antibody, designated S11, fused to a specific peptide ligand directed against cellular receptors, termed the

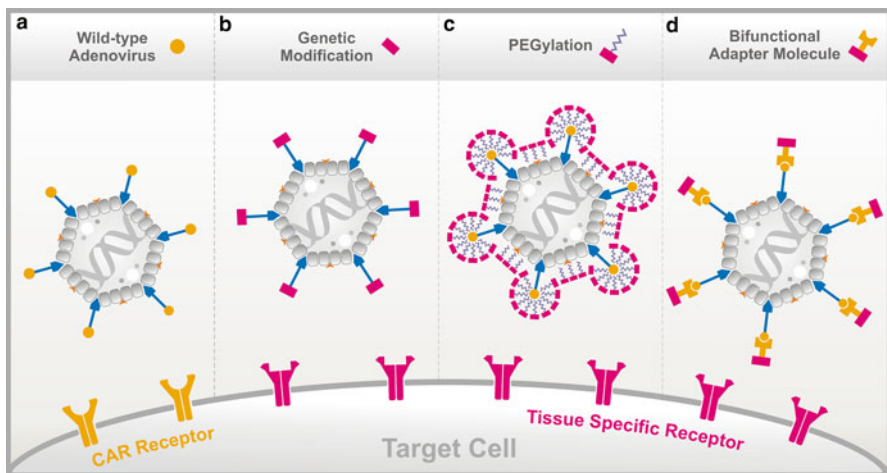


Fig. 2.1 Methods to alter adenovirus tropism. Wild-type adenovirus enters target cells after binding the coxsackie-adenovirus receptor (CAR), an entity present on a wide number of cell types (a). Ablation of CAR binding and re-directing adenovirus-derived vectors towards the cells of choice by means of specific peptides can be achieved by genetically integrating the peptide into the fiber knob (b), chemically coating the vector particle with bi-specific polyethylene glycol (c) or through bridging by means of a bifunctional adapter molecule (d)

bispecific adaptor molecule. Coating virus with this adaptor molecule ablates CAR binding and directs the viral particle to the desired cellular receptor. S11 can be produced in eukaryotic, as well as prokaryotic cells. By means of its 6-His-tag, purification and concentration of the fusion protein can be easily performed by nickel-affinity chromatography. This procedure ensures the high yield of pure protein without the loss of activity.

Based on the S11 strategy, we intended to specifically transduce *in vitro* and *in vivo* activated hepatic stellate cells (HSCs), whose number is increased in fibrotic livers (Reetz et al. 2013). Therefore, we picked a peptide derived from nerve growth factor (NGFp) with specific affinity for the p75 neurotrophin receptor (p75NTR) present on activated HSCs. Coating the GFP-expressing Ad vector with NGFp was done either via chemical conjugation using bifunctional PEG or, alternatively, by molecular bridging with an S11-based fusion protein specific for viral fiber knob and p75NTR (S11-NGFp; Fig. 2.2). After systemic administration of the targeted viral particles, we observed that Ad.GFP-S11-NGFp transduced activated HSCs better than Ad.GFP-PEG-NGFp. The latter's low transduction potential could be explained either by an improper ratio between PEG and targeting ligand concentration that prevented successful and specific tissue transduction or due to the ablation of the viral internalization signals such as the RGD motif by the chemical procedure. These experiments contributed to the development of a targeted gene transfer system to specifically deliver antifibrotic compounds into activated HSCs by systemically applied adenoviral vectors modified by the NGFp ligand. In our study, we demonstrate that adenoviral-mediated targeting of HSCs via p75NTR, concurrently avoiding its binding to hepatocytes, provides a potentially feasible and effective strategy for therapeutic gene delivery to activated HSCs in the liver *in vivo* and the technique may be useful to support approaches to regenerate liver tissue (Best et al. 2015; Salazar-Montez et al. 2015). Beyond, Haisma and co-workers (2010) observed a selective targeting of Ad5 to the endothelial receptors *in vitro* and obtained viral transgene expression only in tumors infected with adenobody retargeted adenovirus from mice bearing subcutaneously colon carcinoma cell derived tumors.

Most recently, we utilized the S11-based Ad targeting method to transduce exclusively neural stem cells in the subventricular zone of adult mice (Reetz et al. 2015). Like in our previous *in vivo* study on HSC transduction, a relevant peptide to be fused to S11 was beforehand detected with the phage-display and biopanning technique (Schmidt et al. 2007). For easy production of the fusion protein, we established by means of lentiviral transduction, a eukaryotic cell line that permanently secretes the bivalent adaptor. Thinking further, bivalent vector targeting approaches may also allow the creation of so-called mosaic modified vectors for instance for patients suffering from metastatic cancer in analogy to work by Pereboeva and colleagues (2004, 2007). The researchers targeted EGFR with a metabolically biotinylated fiber-mosaic adenovirus and demonstrated enhanced binding of vectors with heterologous fiber trimming. Utilizing heterologous fibers, a recent pancreatic cancer therapy approach improved the effect of conditionally replicating adenovirus-

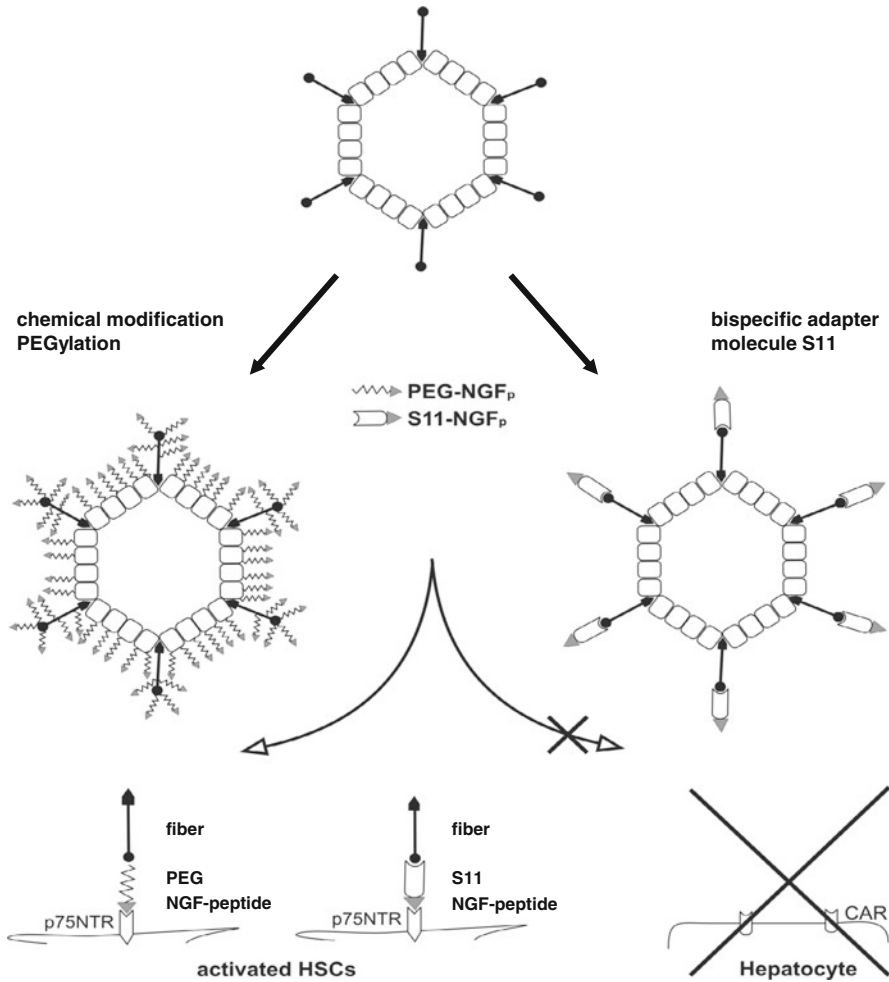


Fig. 2.2 Specific targeting of hepatic stellate cells (HSC). Shown are two different HSC-specific retargeting strategies for adenoviral vectors by linking NGFp. This peptide has specificity for p75NTR, an entity expressed by HSC but not hepatocytes. NGFp was attached to the vector’s surface via chemical coupling by PEGylation or as part of the bispecific single-chain immunoglobulin-derived adapter molecule S11. *Top*: Wild-type vectors enters target cells after binding the coxsackie-adenovirus receptor (CAR), the receptor present on a wide range of cell types including hepatocytes and HSC. *Center, left*: the edging formed by PEGylation shields the entire vector surface including RGD-binding motifs and exposes NGFp. *Center, right*: the S11 adapter molecule is stoichiometrically attached to the fiber knobs. *Bottom, left*: p75NTR-binding via PEG-NGFp. *Bottom, center*: S11-NGFp-mediated binding of the HSC-specific moiety. *Bottom, right*: both coupling methods ablate the interaction of the vector particle with CAR (Reetz et al. 2013)

based oncolytic virus (Kaliberov et al. 2014a). This approach opens applications adenoviral vectors to target cells where single targeting molecules are sparse. Different bivalent adaptors on the same particle may enhance transduction of such cells.

A potential alternative to targeting approaches by single-chain antibodies are Ad vectors coated with adaptor molecules based on designed ankyrin repeat proteins (DARPin). DARPins differ from antibodies in size, structure, binding pattern, and stability. These properties paired with high-yield, easy production in *E. coli* makes them promising candidates for targeting purposes. Dreier and colleagues (2011, 2013) designed an adaptor molecule consisting of two DARPin modules fused to each other. One binding site anchors the molecule to the Ad fiber knob and the other enables the particle to attach to tumor cell markers, like the human epidermal growth factor receptor or the epithelial cell adhesion molecule. In their work, the authors convincingly demonstrate that DARPins are high-affinity adaptor molecules that allow efficient gene transfer and are a promising tool to rapidly target Ad vectors to any desired receptor.

To alter the tropism of adenoviral vectors, the recombinant fusion protein technology offers a number of technical advantages compared to the methods of chemical conjugation. The conveniences of rerouting adenoviral tropism using recombinant proteins include simplified production in prokaryotic or, preferably, in eukaryotic expression systems, as well as vector purification. In addition, this approach may allow the application of different fusion proteins suitable for retargeting Ad to other receptors, simply by the substitution of the peptide ligand. This procedure offers, according to our experience, the method of choice to retarget Ad vectors.

2.4 Conclusions

In conclusion, adenoviral vectors have been proven to serve as efficient tools for gene delivery when temporary gene expression is beneficial. The major challenge towards applying the technology remains the development of a target system for specific gene delivery that reaches a high level of efficiency. Genetic approaches to modify the fiber require tediously re-engineering of a given Ad vector, and PEGylation causes decreased transduction efficiencies due to improper PEG to ligand ratios as well as RGD ablation, bifunctional adaptor molecules seem to be the most favorable targeting approach. An expeditious and simple production followed by a broad portfolio of different fusion proteins suitable to retarget Ad by substitution peptide ligands offers a standardized method to retarget vectors for both *in vitro* and/or *in vivo* applications. Moreover, engineering of bifunctional adaptors may be customized more easily than fiber modifications and chemical treatment of vector preparations. Increased knowledge of adenovirus biology and powerful techniques such as the phage display method to identify new cell or tissue specific targets as well as unique receptors on neoplastic cells provide the opportunity to develop innovative strategies for gene therapy. Custom-made gene shuttles that

exclusively deliver into cells to be addressed a therapeutic nucleic acid or a potent inhibitor of pathogenic genes may, in future, allow success in the treatment of patients with systemic disease first and foremost with metastasized cancer.

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