Improvement of oncolytic adenovirus vectors through genetic capsid modifications

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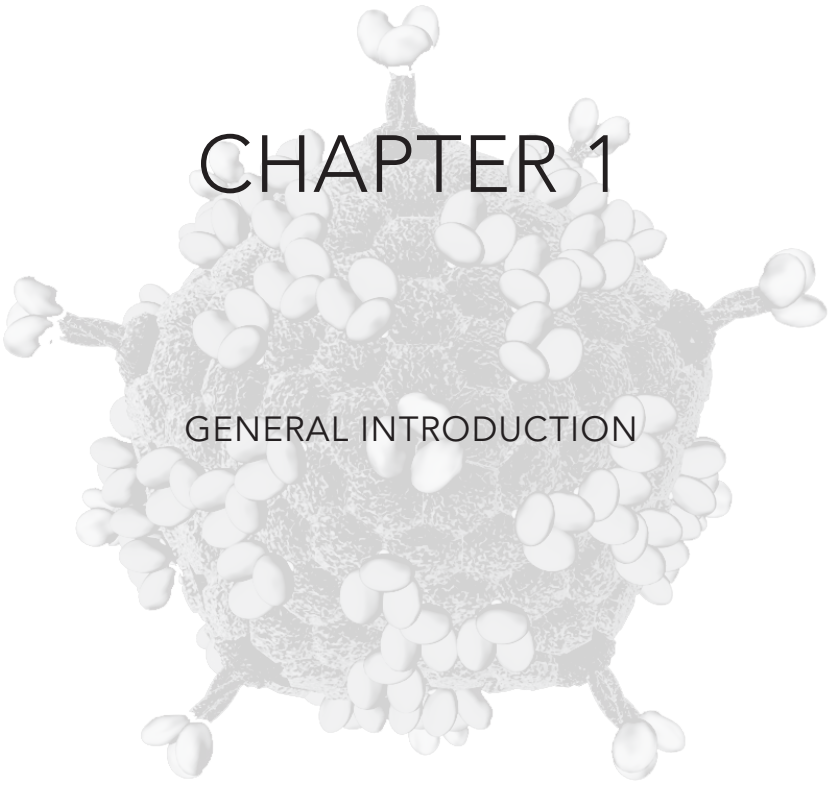

CHAPTER 1

GENERAL INTRODUCTION

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1.1 AIMS AND OUTLINE OF THIS THESIS

Aims

1. To establish the production of HAdV-5 particles decorated with protein IX-fused polypeptide ligands that have proven potential for tumor targeting, such as single-chain antibody fragments, single-chain T-cell receptors, or Affibody molecules.

2. To investigate the targeting efficacy and specificity of protein IX-ligand decorated HAdV-5 vectors to tumor cell lines.

3. To investigate the targeting of HAdV-5 to cancer-testis antigens through fusing a single-chain T-cell receptor with protein IX or fiber molecules.

4. To analyze the effect on transduction of incorporating cathepsin-cleavage sites in between HAdV-5 protein IX and its fused targeting ligand.

5. To obtain insight into the biological consequences of protein IX modification.

Outline

Chapter 1 provides a general introduction on HAdV-5, which is the best-studied adenovirus serotype and the serotype most-often used for the construction of oncolytic vectors for cancer gene therapies. Important aspects on the biology of HAdV-5 are summarized, including the virion architecture, the infection route, and the replication mechanism. A separate paragraph is devoted to the minor capsid protein IX of HAdV-5, taking into account the important role of this protein in this thesis. Chapter 1 also provides a general overview on oncolytic adenovirus vectors. Vector modification strategies aiming at improved efficacy are described, as well as strategies for reducing transduction of non-target tissues. The ins and outs are provided for replication-deficient HAdV-5 vectors, as well as for the more recently developed Conditionally Replicating Adenoviruses (CRAds).

In Chapter 2 the most recent advances in oncolytic adenovirus technology are described, focusing on vectors for prostate-cancer treatment. The most prominent bottlenecks for successful cancer gene therapy with oncolytic viruses are reviewed, and potential solutions to overcome these hurdles are outlined.

Chapters 3, 4, and 5 describe the usability of the adenovirus minor capsid protein IX as an anchor for genetically fusing tumor targeting ligands.

The feasibility of fusing large and complex polypeptides to protein IX is described in Chapter 3. As a model ligand the hyper-stable single-chain antibody fragment 13R4 was chosen, which binds with high affinity to β-galactosidase. Incorporation of protein IX-13R4 polypeptides in the virus capsid was achieved with our previously developed "protein-IX screening" system, encompassing the transduction of a protein IX-13R4 producing helper cell line with a protein IX gene-deleted HAdV-5 vector, followed by harvesting and purification of the progeny viruses. Incorporation efficiency and functionality of 13R4 in the capsid of the HAdV-5 vector is discussed.

As a next step, using the same ligand incorporation strategy, a tumor-targeting ligand was fused with protein IX (Chapter 4). Since cancer-testis (CT) antigens have been described to be truly tumor-specific (except for their expression in the testis) it was decided to fuse protein IX with a single-chain T-cell receptor (scTCR) directed

against the CT antigen MAGE-A1, in complex with HLA-A1. Efficacy of targeting to HLA-A1/MAGE-A1 positive melanoma cell lines is described, as well as various assays to analyze the specificity of targeting.

Chapter 5 describes the results on HAdV-5 viruses targeted to tumor cells through fusion of a high-affinity binding Affibody molecule to protein IX, and the effects of incorporating a cathepsin-cleavage site (ccs) in between protein IX and the Affibody molecule. Previous findings by us and by others suggested that protein IX-mediated targeting using 'high-affinity binders' (like Affibody molecules) as ligand is limited by inefficient release of protein IX-fused ligands from their cognate receptors in the endosome. This would result in inefficient endosomal escape of the virus particles. Chapter 5 comprises an extensive comparison between HAdV-5 viruses containing either wild type protein IX, protein IX-Affibody, or protein IX-ccs-Affibody in the capsid. The transduction efficiency is compared in monolayer cultures, 3-dimensional spheroid cultures, and in SKOV-3 tumors grown on the chorioallantoic membrane of embryonated chicken eggs.

In addition to the analyses of the protein IX-scTCR loaded HAdV-5 vectors, as described in Chapter 4, the usability of the HLA-A1/MAGE-A1 specific scTCR for HAdV-5 targeting was also tested in the context of fusion with the fiber protein (Chapter 6). The adenoviral fiber knob, which is responsible for attachment to the Coxsackie virus and Adenovirus Receptor (CAR) on target cells, was replaced by the scTCR molecule and an extrinsic trimerization motif in a replication-competent HAdV-5 vector. The efficacy and specificity of targeting is presented through comparison of cell killing in a panel of melanoma cell lines.

Functional consequences of deleting the protein IX gene from HAdV-5 vectors are described in Chapter 7. The findings provide novel insights into the biological role of protein IX, and may be of relevance for future development and clinical implementation of protein IX-modified HAdV-5 vectors.

Chapter 8 provides a general discussion on the potency of protein IX-mediated tumor targeting for the development of improved oncolytic HAdV-5 vectors. Recommendations for further preclinical studies are included. Also, an overview is given on the newest insights and developments in preclinical testing of oncolytic AdV vectors in general. The anticipated essence of various model systems for future vector analyses is described.

1.2 BIOLOGY OF HUMAN ADENOVIRUS TYPE 5

Adenovirus was first isolated in the 1950s from adenoid tissue-derived cell cultures. These primary cell cultures were often noted to spontaneously degenerate over time, and human adenoviruses (HAdV), (belonging to the family Adenoviridae, genus Mastadenovirus) are now known to be a common cause of asymptomatic respiratory tract infection that produces *in vitro* cytolysis in these tissues. Based on serological parameters, hemagglutination parameters, restriction enzyme digestion patterns, and nucleotide sequence analyses, 55 types of HAdV have been described (51 'serotypes' identified by traditional immunochemical methods, and 4 'types' defined by genomics). These have been classified in seven species, A to G. Most HAdV serotypes cause mild diseases in immunologically healthy people, but some serotypes

can cause considerable morbidity, especially in individuals who are compromised immunologically (e.g. transplant patients) or nutritionally (e.g. gastrointestinal infections in children in the developing world).

Adenoviruses are icosahedral, non-enveloped viruses of approximately 90 nm in size, belonging to the largest non-enveloped viruses. Recently, the structure of a HAdV has been solved at the atomic level, providing the largest high-resolution model ever.[1,2] Research on adenoviruses has yielded ample knowledge on various cell biology mechanisms, such as RNA splicing.[3] Also, laboratory experiments on adenoviruses and their derived vectors has come along with the development of important molecular-biological techniques like the calcium-phosphate DNA transfection method.[4]

Various aspects, including the relative safety, the well-known biology, and the suitability for genetic modification, have made vectors derived from HAdV type 5 (belonging to the species C HAdVs) the currently most-often used vehicles for viral gene-delivery. HAdV-5 vectors have been used extensively as vaccine, and have shown great potential for *ex vivo* and *in vivo* gene therapies for treatment of hereditary diseases or cancer.

1.2.1 Virion architecture

A fully mature HAdV-5 particle, with an approximate mass of 150 MDa, consists of an icosahedral capsid of 20 facets and a core, as schematically depicted in **Fig. 1**. The pentons, hexons and fibers form the so-called major proteins of the adenovirus capsid. The minor proteins of the capsid are proteins IIIa, VI, VIII and IX. The core consists of a double-stranded linear DNA genome (36 kb in size) and of several proteins: proteins IVa2, V, VII, terminal protein (TP), mu, and the adenovirus protease.[5,6] The HAdV-5 genome contains transcriptional units referred to as early (regions E1 to E4), intermediate (regions pIX and IVa2) and late (regions L1 to L5) depending on their temporal expression, relative to the onset of viral DNA replication.

The fiber protein in the capsid has been identified as the main cell binding protein, with the cell surface protein bound being the coxsackievirus and adenovirus receptor (CAR).[7] CAR binds to the fiber knob domain, which is located at the carboxyl-terminus of the fiber shaft domain. The fiber tail is mounted on the vertex protein penton base. Penton base is located on each vertex and has protruding arginine-glycine-aspartic acid (RGD)-domains that are involved in secondary cell binding and the initiation of virus endocytosis through integrin binding.[8,9] Penton base has also been suggested to play a role in endosomal escape of virus particles, evidenced by the blocking of adenovirus induced endosomal lysis by α-penton antibodies and addition of soluble penton.[10,11] The bulk of the capsid consists of 240 copies of trimeric hexon, contributing to approximately 50% of the mass of the capsid.[12] The hexon content of a capsid can be divided in two subgroups. The groups of nine (GON) are a group of hexons that is frequently observed after dissociation of the capsid.[13] The non-GON belonging hexons are the peripentonal hexons. These are, as the name suggests, the hexons in direct contact with the penton bases. Each trimeric hexon has three towering structures on top, with a cavity in between that can be bound by coagulation factor X in HAdV-5.[14,15]

Most minor capsid proteins have an assigned capsid localization, but their mechanism of function has often not yet been fully resolved. Protein IIIa was found to be important

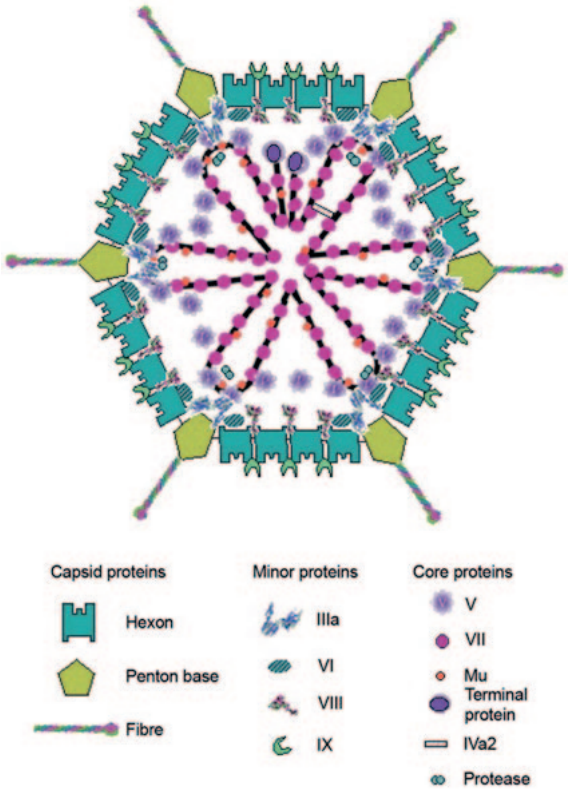

Figure 1. Schematic representation of the adenovirus structure. Taken from Russell.[6]

for packaging the viral DNA as well as capsid maturation and localizes on the inner side of the pentons.[16] Protein VI has been localized to the inner cavity of hexons and has a function in hexon transport into the nucleus during virus assembly, as well as being involved in the lysis of endosome membranes.[17-19] Protein VIII (assigned to the inner capsid) is thought to provide the capsid stability but the exact functions remains to be solved.[20] A description on the smallest but most abundant minor capsid protein, protein IX, is provided in more detail below, since this protein plays a major role in this thesis.

1.2.2 Cellular infection route

Different receptors are involved in adenovirus cell binding.[21] The main receptor for adenoviruses is CAR, which binds to the fiber knob.[7] Alternatively, species B adenoviruses utilize different receptors, that is, CD46 for species B1 (serotypes 16, 21, 35, 50), desmoglein 2 for species B2 (serotypes 3, 7, 14), and CD46 or desmoglein 2 for species B3 (serotype 11).[22] Furthermore, cell surface sialic acid molecules can be utilized as a receptor by various serotypes, such as HAdV-37 from species D.[23,24] Recently, a new mechanism of cellular uptake was discovered for HAdV-5, via the interaction of hexon with coagulation factor X (FX). This mechanism appears to be a major cause for the uptake of systemically introduced HAdV-5 vectors in the liver, through the binding of virus-bound FX to heparan sulphate molecules on hepatocytes.[14,15,25] However, it

remains to be established how important the latter mechanism is for transduction of liver cells in humans. After the initial docking of a HAdV-5 particle to its primary receptor, various secondary interactions can occur, the most prominent one being the interaction of RGD domains of penton base with integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$.[8,9] Also heparan sulphate proteoglycans have been identified as cell surface molecules for secondary interactions, through binding to the fiber shaft.[26] Shortly after cell binding the fiber is shed.[27,28] The interaction of penton base RGD domains with cellular integrins induces a conformational change of penton base monomers, suggested to be necessary for fiber release or for breaking contacts with the surrounding hexons, allowing the fiber or the penton base to be released.[29] Integrin aggregation through penton-base binding localizes the virus to clathrin coated endosomes, the main entry route for adenovirus.[30] Integrin binding also activates various intracellular signaling routes, including the MAPK/p38 and the p85/p110 PI(3) kinase routes, which is thought to influence susceptibility of adenovirus uptake by the canonical routes and by other endocytosis pathways such as macropinocytosis.[31,32] Once the virus is in the clathrin-coated endosome and the environment acidifies, the release of pVI triggers endosomal escape.[18,19] Once in the cytosol, HAdV-5 binds molecular motors that move over microtubules; a minus end directed motor (kinesin) and a plus end directed motor (dynein). These motors are engaged in a tug of war principle, resulting in speeds of movement of micrometers per minute.[33,34] Hexon seems to be the protein responsible for binding kinesins and dyneins.[35] Whether the movements of the viral particle occur according to a stochastic tug of war principle or whether all movements are coordinated is still subject of debate.[36] Alternatively, microtubule independent transport has been reported, suggesting other cytosolic transport mechanisms.[37,38] When the partially dismantled HAdV-5 particle reaches the nucleus, it binds the nuclear pore complex and is further dismantled.[39] Subsequently, the viral DNA is imported and transcription of genes can be initiated.

1.2.3 Replication

The adenoviral genes can be classified in three groups, based on their time of transcription after infection: the early genes (E1A, E1B, E2, E3, and E4), the intermediate genes (pIX and IVa2), and the late genes (L1 to L5).[40] Genes are transcribed from both DNA strands and the majority of RNA transcripts are subject to complex splicing patterns. The first messengers to be transcribed after infection are the E1A transcripts. The E1A proteins induce the cell to enter the cell cycle, necessary for replication of the adenoviral DNA, through binding cellular proteins including the retinoblastoma gene product Rb. The E1B-55kD protein binds p53, thereby preventing apoptosis or blockage of the cell cycle. The E2 proteins (terminal protein, DNA-polymerase, and single-stranded DNA binding protein) are involved in viral DNA replication, the E3 proteins play essential roles in down-regulating the host immune response against HAdVs, and the E4 proteins have multiple functions in the regulation of viral transcription, replication and mRNA transport. The late genes encode the capsid proteins, with the exception of capsid protein IX, which is a product from an intermediate gene.

1.2.4 Capsid protein IX

The 14.3 kDa protein IX is the smallest of the HAdV capsid proteins. Protein IX is unique to the Mastadenoviruses and is, in contrast to the other capsid proteins, absent in the other adenovirus genera. During the viral replication cycle, the transcription of protein IX messenger RNAs is, for unknown reasons, initiated relatively early after infection, as compared to the mRNAs of the other capsid proteins.[40] Each virus facet contains 12 molecules of protein IX, resulting in a total of 240 molecules per virion. The protein has three conserved regions, as shown by amino acid alignment, located at the amino-terminus, the middle part, and the carboxyl-terminus of protein IX. The amino-terminal regions of protein IX are positioned in the cavities between the hexon tops of hexons that belong to a GON. Each cavity contains the amino-termini of three molecules of protein IX.[41] The recent determination of the HAdV-5 structure, by means of cryoelectron microscopy and x-ray analyses, has revealed the carboxyl-terminus of protein IX to be present in the capsid as quadrimeric coiled-coils, at a location previously assigned to the minor capsid protein IIIa.[1,2,42,43] The orientation of the four coiled-coils is parallel for three of the coils, originating from one facet of the capsid, and anti-parallel for one coil, originating from a neighbouring facet.[1,2,43] Taken together, these assignments result in a large network of protein IX, spanning the entire capsid (**Fig. 2**).

Despite many years of research the definite function of protein IX is still to be assigned. The protein highly likely acts as capsid cement, since deletion of the protein causes thermal instability.[44] The amino-terminus appears to be exclusively responsible for incorporation of protein IX in the virus capsid and for providing the capsid its thermal stability, probably through stabilizing the GONs.[45] Besides its postulated role in providing the virion stability, protein IX seems to play a role in viral DNA packaging, as suggested by the finding that viruses lacking protein IX have a strong reduction in infectivity if the genome is larger than 95% of wild-type size (>35 kb).[46,47] The carboxyl-terminus of protein IX, which can be deleted from HAdV-5 without affecting thermostability, has been suggested to be essential for post-infection interactions of protein IX with cellular factors, resulting in a stimulatory effect on promoters of early viral genes.[48] However, this effect, which was found in a non-viral context, was subsequently toned down by another study, showing no significant effect of protein IX on viral transcription in the context of viral infection.[49]

Interestingly, protein IX was reported to form clear amorphic bodies in the nucleus, strongly resembling the so-called PML (promyelocytic leukemia protein) bodies in terms of size and protein contents (including PML and sp100).[50] Deleting the carboxyl-terminus of protein IX abrogated the capacity of protein IX to form PML bodies. Appreciating the importance in cellular biology of PML bodies, for example functioning in regulation of cell cycle and cell growth, it was tempting to speculate on a role of (the carboxyl terminus of) protein IX in host cell modulation.[50] However, such a role has not been confirmed in subsequent experiments. The relevance of the PML-protein IX bodies in HAdV-5 biology is questionable, based on results obtained in our laboratory (De Vrij, unpublished data, **Fig. 3**). To our surprise, expression of protein IX in normal diploid cells (VH10 cells (primary foreskin fibroblasts) and mesenchymal stem cells (MSCs)) results in protein IX localization in both cytoplasm and nucleus, with significant accumulation of the protein at certain cytoplasmic locales. Protein

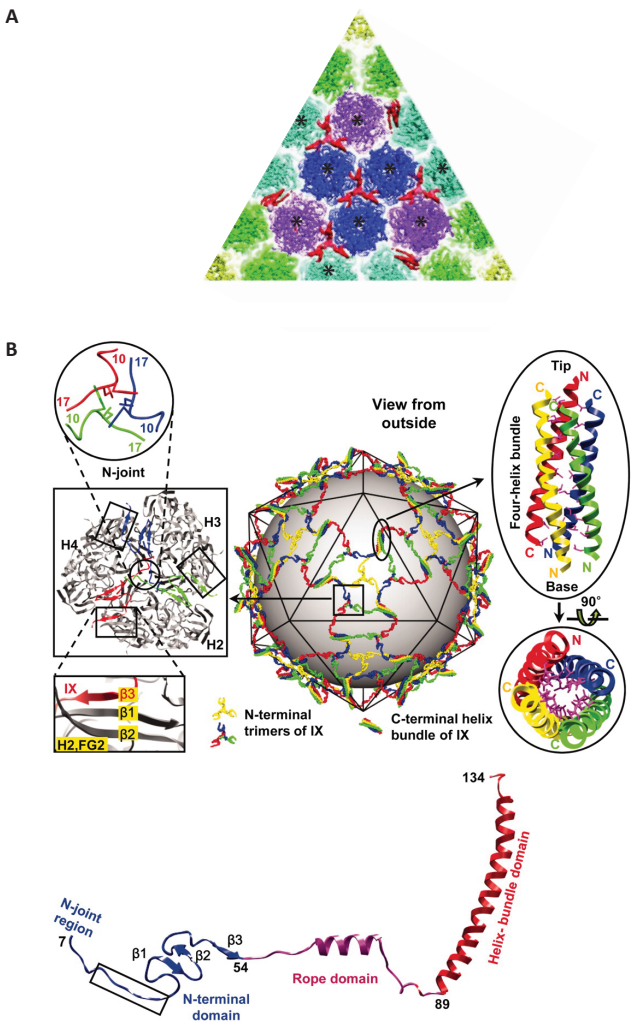

Figure 2. Capsid structure of HAdV-5. (**a**) Protein density on an exterior region of the capsid, roughly corresponding to one icosahedral facet. The model was created through overlaying a cryo-electron microscopy model of the entire virus particle (at 6-Å resolution) with X-ray crystallography structures of individual proteins. Penton base monomers are indicated in yellow, and hexons are indicated in green (position 1), cyan (position 2), blue (position 3), and magenta (position 4). Protein IX densities, as four trimeric regions and three helical bundles, are indicated in red. Hexons belonging to a group of nine (GON) are marked with an asterisk (*). Figure adapted from Saban *et al.*[42] (**b**) CryoEM (at 3.6-Å resolution) reveals a physical network of protein IX in the capsid, lashing together hexons into GON tiles. Left insets: Ribbon models of the N-terminal domains of three protein IX monomers (blue, green, and red), overlaying the models of three adjacent hexon monomers (H2, H3, and H4) (gray). The N-terminus of protein IX is in close proximity to the FG2 region of a hexon monomer (lower left inset). Right insets: Ribbon models of the C-terminal domains of protein IX. Four C-terminal domains form a bundle consisting of three parallel α-helices and one antiparallel α-helix. The helices are linked by a ladder of hydrophobic residues (leucines and valines) (magenta). Bottom inset: Ribbon model of protein IX showing three distinguished domains as well as the N-joint region. Figure adapted from Liu *et al.*[1]

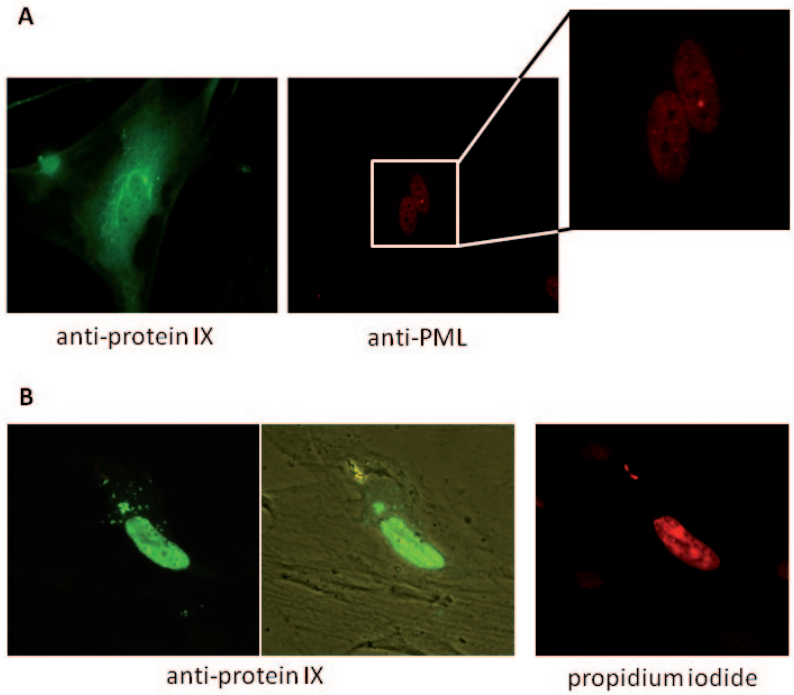

Figure 3. Subcellular localization of protein IX in mesenchymal stem cells, as visualized by immunohistochemistry. (**a**) Detection of protein IX and promyeolocytic leukemia (PML) protein after establishing lentiviral vector-mediated heterologous expression of protein IX. The cells were fixed with acetone-methanol (1:1). Staining was performed by means of primary incubation with the antibodies α-protein IX (rabbit, polyclonal)[51] and α-PML (5E10; mouse, monoclonal),[52] followed by secondary incubation with α-rabbit-FITC and α-mouse-Alexa594, respectively. (**b**) Detection of protein IX after infection with HAdV-5. Infection was performed with 10 virus particles per cell. The cells were fixed with acetone-methanol (1:1), at 72 hours post infection. Staining was performed through subsequent incubations with antibodies α-protein IX (rabbit, polyclonal) and α-rabbit-FITC. The nuclei were stained with propidium iodide.

IX appeared not to co-localize with PML. In the context of HAdV-5 infection in these primary cell cultures, protein IX localizes to the nucleus, regularly forming ring-like structures. In contrast, and in line with the results published by Rosa-Calatrava *et al.*,[50] protein IX formed nuclear bodies in transformed cell lines like A549 alveolar epithelium cells. These observations argue against a role of protein IX in nuclear sequestration of PML in non-transformed cells.

1.3 ADENOVIRUS VECTORS FOR CANCER THERAPY

Genetically modified adenoviruses have been explored extensively as gene-transfer vehicle for the purpose of gene therapy or vaccination.[53] Several characteristics make adenoviruses highly suitable as gene-transfer vehicle: a relatively low level of pathogenicity; a high stability of the viral DNA genome, thereby preventing the

development of heterogeneous populations of 'quasispecies'; the ability to transduce dividing as well as quiescent cells; the well known biology and uncomplicated genetic modification; the availability of technologies for production of clinical-grade virus batches with high titers and high purity. Adenoviruses can be used either as replication-deficient or replication-competent vectors. The replication-deficient vectors can be used as gene delivery vehicle for gene augmentation therapy (e.g. through delivery of genes that are mutated in the vector-receiving patient) or cancer therapy (e.g. through delivery of prodrug-activating genes). Also, replication-deficient vectors are being used (with proven efficacy and safety) as vaccine vector to induce immune responses against antigenic polypeptides that are displayed on the viral capsid or encoded for by the viral vector.[54] More recently, replication-competent adenovirus vectors, or Conditionally Replicating Adenoviruses (CRAds), have been developed, exploiting the lytic infection cycle of the virus to kill tumor cells. Various modifications can be introduced to CRAds to provide tumor cell selective replication.

Currently, the large majority of adenovirus vectors for cancer therapies are derived from HAdV of serotype 5, mainly as a result of its well known biology and its proven safety. In the next paragraphs a general overview is given on the development of HAdV-5-derived vectors for cancer therapy, describing replication-deficient as well as replication-competent vectors. An extensive outline is provided on rational design approaches towards improved oncolytic HAdVs, such as capsid modifications for targeting and detargeting purpose. Preclinical developments on random approaches, such as bioselection with mutagen-induced viral libraries, are summarized as well.

1.3.1 Replication-deficient vectors

Different types, or 'generations', of replication-deficient HAdV-5 vectors have been developed. The first-generation vectors have the E1 region deleted.[55] This deletion renders the recombinant virus replication-defective, providing an important safety feature. The production of E1-deleted vectors is dependent on specialized helper cells that provide the E1 functions *in trans*. The most frequently used helper cell lines are the 293 cell line (human embryonic kidney cells transformed with sheared HAdV5 DNA)[56] and the 911 and PER.C6 cell lines (human embryonic retinoblasts transformed with a plasmid carrying a defined portion of the adenovirus genome).[57,58] By combining removal of the E1 region with removal of the E3 region, which encodes for proteins involved in evasion of the immune system and is dispensable for vector growth *in vitro*, approximately 7500 base pairs of heterologous DNA can be accommodated in HAdV-5 vectors.

The first-generation HAdV-5 vectors appeared to be suboptimal for certain gene-delivery applications, mainly as a result of the induction of a strong cell-mediated immune response.[59] These responses appeared to be a result of viral protein expression. As a consequence second-generation HAdV-5 vectors were made in which deletions or mutations were introduced in the E2 and E4 region. These modifications resulted in a substantial reduction of the cellular immune response and, as a consequence, prolonged transgene expression.[55] Also third-generation or 'high-capacity' HAdV-5 vectors have been developed, which are devoid of all viral genes and can accommodate up to 35 kilo bases of heterologous DNA.[55,60] The only remaining viral sequences are the inverted terminal repeats (ITRs) and the packaging signal. Production of high-

capacity vectors requires the usage of a helper virus to provide all viral functions and structural proteins *in trans*. Elegant systems have been developed to prevent the presence of helper virus contaminants in the final high-capacity vector preparation. As intended, high-capacity HAdV vectors have a strongly improved duration of transgene expression as compared to first- or second-generation HAdV vectors, as a result of a reduced cellular immune response.[55]

As evidenced by various studies, the first-generation HAdV-5 vectors are inferior to the higher-generation vectors in these gene-delivery applications that require the *in vivo* expression of large heterologous genes for prolonged times. However, the usage of replication-deficient vectors for the (short-term) expression of oncolytic genes in cancer cells not necessarily requires the usage of higher-generation vectors. First-generation vectors can perfectly accommodate the majority of anti-tumor transgenes and, importantly, induce immune responses that might be of benefit for the efficacy of the therapy (e.g. through induction of a cellular immune response against tumor antigens). Examples of anti-tumor transgenes are genes encoding for prodrug converting enzymes (e.g. the Herpes Simplex Virus thymidine-kinase (HSV-TK) for activating gancyclovir, the bacterial nitroreductase for activating CB1954), immune stimulatory cytokines (IL-2, IL-12, GM-CSF, IL-24), or apoptotic proteins (e.g. p53).[61] Specificity of transgene expression can be provided through the inclusion of tissue-specific promoters.

To improve the specificity and efficacy of AdV vectors, a large variety of capsid modifications is being pursued. Such modifications aim, on one hand, on the enhancement of tumor cell transduction (e.g. through fusing tumor-targeting polypeptides to the viral capsid) and, on the other hand, on reduced transduction of non-target cells or tissues. Transductional targeting and detargeting approaches will be discussed in Paragraph 1.2.3.

1.3.2 Conditionally-Replicating Adenoviruses (CRAds)

Past clinical trials have defined major limitations of replication-deficient vectors for cancer gene therapy, as a result of their inability to infect the majority of cells within a clinically presented three-dimensional solid tumor mass.[62] Conditionally Replicating Adenoviruses (CRAds) are designed to overcome this limitation by making use of the natural ability of HAdV-5 to kill their host cells upon its spread throughout a tissue. To provide specificity of cell killing, CRAds are designed in such a way to restrict their replicative ability to tumor cells. Besides the direct lytic effect of CRAds, their induction of cell death might cause anti-tumor immune responses as a positive bystander effect.[63,64] Future research is necessary to fully delineate the effects of CRAd therapies on the patient's immune system.

CRAds are rendered tumor-specific by taking advantage of cancer-specific cellular changes. It was found by Berk *et al*. that disruption of the HAdV-5 E1B-55K gene in the mutant *dl1520* yielded specific replication in cancer cells.[65] Based on previous experiments showing an ability of E1B-55K to interact with the tumor-suppressor protein p53, it was initially thought that the tumor selectivity of *dl1520* was due to p53 being mutated in cancer cells. However, *dl1520* was found to replicate in p53-expressing cancer cells as well.[66,67] Despite the controversy on the mechanism behind the tumor cell preference, E1B-55K deleted CRAds are paradigm for designing of today's oncolytic

vectors. The *dl1520* virus has been tested in a variety of cancer clinical trials (under its commercial name ONYX-015) including head and neck cancer,[68] oral carcinoma,[69] colorectal carcinoma metastases to the liver,[70] hepatocellular carcinoma[71] and glioma.[72] These studies have demonstrated safety, with well tolerated doses of up to 2 x 10^{12} particles (by various routes of injection) and tumor-selective replication. The efficacy as a single agent has been relatively limited to date (0-14% local tumor regression rates), but encouraging anti-tumor activity has been demonstrated in combination with chemotherapy.[73] A very similar virus, H101, has been registered for clinical use in China.

Cancer cell-specific CRAds can also be made by mutating the E1A gene.[74] A 24-bp deletion was found to prevent E1A from binding to the cellular protein Rb for induction of the cellular S-phase. As a consequence, replication depends on the inactivation of Rb through other means (e.g. hyperphosphorylation) which is the case in most types of cancer. Clinical safety and efficacy of E1AΔ24 CRAds is subject of current research. Recently, the maximum tolerated dose, toxicity spectrum, clinical activity, and biological effects were evaluated for a E1AΔ24 CRAd (named Ad5-Δ24-RGD) in patients with ovarian cancer.[75] Besides having the 24-bp deletion, to establish tumor-selectivity, this CRAd also contained an RGD-domain fused to the fiber, to enhance efficacy of the treatment. The approach appeared to be safe, and a minor antitumor response was found.

As an alternative to the E1 deletions, CRAds can be created through the incorporation of tissue-specific promoters to control the expression of essential viral genes. As an example, expression of the viral E1A gene can be controlled by the recombinant prostate-specific PPT sequence, which is composed of a prostate-specific antigen (PSA) enhancer, a prostate-specific membrane antigen (PSMA) enhancer and a T cell receptor gamma-chain alternate reading frame protein (TARP) promoter.[76] As a result, the AdV vector replicates exclusively in normal and neoplastic prostate epithelial cells.

A large variety of modifications is being explored, to further improve the efficacy and specificity of CRAds. Similar to the replication-deficient vectors, the CRAd genome can be armed with 'cell killing transgenes', which may improve the efficacy of tumor eradication. As an example, arming the *dl1520* virus with the HSV-TK/Ganciclovir system results in increased survival rates in mice with subcutaneous colon cancer xenografts.[77] Alternatively, CRAds with improved clinical performance may be obtained by the insertion of genes coding for proteins with antitumor effect on the tumor micro-environment, such as angiogenesis inhibition or immune activation.[78] To improve the specificity and safety of CRAds, their replication can be blocked in non-target cells by incorporating microRNA (miRNA)-binding sequences in viral genes. In this way, multiple binding sites for a hepatocyte-specific miRNA, mir-122, have been placed in the 3' untranslated region of the E1A gene of a CRAd, leading to the absence of E1A gene expression (and viral replication) in murine hepatocytes and a significant reduction in hepatotoxicity.[79,80]

Furthermore, capsid modifications for targeting and detargeting approaches are pursued, as will be discussed in the next paragraph.

1.3.3 Capsid modifications for targeting and detargeting

Studies on oncolytic HAdV-5-based vectors, in preclinical- as well as early phase clinical settings, have demonstrated the necessity of introducing modifications in the viral capsid to improve the efficacy and safety in the complex environment of a patient's tumor.

One important efficacy-limiting aspect is the low-level expression of the CAR receptor on many tumor cells, necessitating capsid modifications to alter the wild-type tropism of HAdV-5.[81,82] Development of modified vectors that can infect CAR-negative cells has mainly focused on the genetic incorporation of heterologous ligands in the fiber protein, or on 'fiber-swap' strategies in which the HAdV-5 fiber is replaced by a fiber from another HAdV serotype.[83] Although effective, the applicability of incorporating large and complex ligands (e.g. single-chain antibody fragments) into fiber locales might be limited, since such modifications in many cases result in virus replication with low titers.[21] This drawback has prompted the identification of other capsid proteins (hexon, penton base, protein IIIa, and minor capsid protein IX) as usable locales for incorporating heterologous peptides (reviewed by Vellinga *et al*.[5]).

An interesting locale for the fusion of polypeptides is the minor capsid protein IX. Fusing polypeptides to the carboxyl-terminus of protein IX does not reduce the viral titers upon *in vitro* production, and has no effect on the stability of the virus particles.[45] It was found that the presentation of protein IX-fused peptides can be improved through incorporating a 75-Ångstrom alpha-helical spacer in between protein IX and the peptide.[84] Using the protein IX-spacer sequence as anchor, highly efficient coverage of the virions with heterologous peptides can be obtained with incorporation efficiencies close to the theoretical maximum of 240 molecules per virion, depending on the size and complexity of the polypeptide. The feasibility of targeting HAdV-5 to tumor cells through fusing tumor-targeting ligands to protein IX has subsequently been investigated, as described in detail in this thesis.

Nowadays, elegant systems are available for creating genetically modified HAdV-5 vectors, which enable cloning and recombination steps in a bacterial context instead of in human cell lines. Still, genetic modification of the HAdV-5 genome is a time- and effort consuming process, limiting the rapid screening of polypeptide moieties for their capsid incorporation ability. For this reason, screening-facilitating systems have been developed based on the propagation of HAdV-5 vectors on cell lines expressing heterologous peptides fused to a capsid protein. Such systems have been used successfully for expedited functional assessment of modified variants of protein IX and fiber.[85,86]

An alternative to genetically modifying the viral vector for transductional purposes has been provided by 'adapter strategies', applying bispecific targeting moieties that on one hand bind to the virus (in general to the fiber knob domain), and on the other hand to a molecule on the target cell. As an example, HAdV-5 vectors have been efficiently retargeted to HER2/neu expressing tumor cells through using designed ankyrin repeat proteins (DARPins) as bivalent adapter molecules.[87] The adapter technology has several potential advantages with respect to the genetic modification technology, in that it enables the relatively rapid screening of targeting ligands, and provides a production platform for the preparation of high-titer batches of HAdV-5 vectors that are efficiently decorated with large and complex targeting

ligands. However, special care will be required to ensure the preparation of clinical batches with defined characteristics, for example assuring low variability of ligand incorporation efficiencies between different vector preparations.

The above described modifications aim at enhanced transduction of tumor cells through coupling tumor-targeting ligands to the HAdV-5 capsid. Another strategy to improve the potency of oncolytic HAdV-5 vectors is by detargeting the vector from non-target tissues. As discussed in more detail in the next chapter, last years have witnessed an enhanced understanding on the *in vivo* mechanisms behind the disappointing anti-tumor efficacies of oncolytic HAdV-5 vectors in early-phase clinical trials. One aspect thwarting effective therapy is the high prevalence of pre-existing humoral immunity against HAdV-5 in the human population, resulting in rapid clearance of the vectors from the blood. Additionally, strong innate immune responses, e.g. by natural killer cells, are observed after intratumoral or systemic injection of oncolytic HAdV-5 vectors. Another bottleneck, especially hampering the efficacy of systemically delivered oncolytic HAdV-5 vectors, is the rapid clearance from the blood stream as a result of sequestration in the liver. It has recently been found that binding of HAdV-5 to blood coagulation factor (F) X, results in uptake of the vectors by hepatocytes in the liver.[14,15] FX appears to bind to viral capsid epitopes of the hexon protein, and bridges the virus to heparan sulphate proteoglycans on hepatocytes. Besides the FX-hepatocyte mediated removal of oncolytic HAdV-5 vectors, the vectors are also cleared form the blood by liver-residing macrophages (Kupffer cells).[88] Binding of the vectors to complement proteins, natural antibodies and platelets seems to play an important role in the uptake by these scavenging macrophages.[89,90] Another problem to tackle is the binding of HAdV-5 vectors to erythrocytes.[91,92] This binding appears to be specific for human erythrocytes, and has therefore not been noticed previously during vector analyses in rodent models. Last years have seen enormous pre-clinical improvements in the efficacy of HAdV-5 based oncolytic vectors, through applying novel types of modifications leading to improved transductional targeting and detargeting. One highly promising example is the ability to genetically modify viral hexon sequences to abolish uptake of virus particles by hepatocytes, thereby enhancing gene transfer to target cells.[93,94]

Additionally, strategies have been developed to reduce off-target binding by shielding the adenovirus vector particles with chemical polymers.[95] In animal models, this technology significantly increases the circulation time of HAdV-5 vectors in the blood stream, and simultaneously reduces liver toxicity.[96,97] Similar to targeting of the 'naked' vector particles, the polymer coatings can also be modified to achieve targeting to tumor cells.[98] Research is ongoing to further improve the polymer coating technology, for example aiming at 'low pH triggered de-shielding' to facilitate proper intracellular routing of polymer coated virus particles after their uptake in the endosome. As an alternative to the polymer coatings, 'carrier cells' might be utilized to shield HAdV-5 vectors from efficacy-limiting moieties. Various cell types with intrinsic tumor-homing properties, such as mesenchymal stem cells, T cells, and monocytes, are currently under investigation.[99]

Taken together, a large array of new targeting and detargeting approaches has been developed to facilitate improved performance of oncolytic HAdV-5 vectors. The most up-to-date developments in the field of oncolytic HAdV-5 vector engineering,

as well as prominent aspects that require further optimization, are outlined in more detail in Chapter 2.

1.3.4 Random approaches to vector development

As described in the previous sections, a plethora of rational design approaches is being pursued to develop AdV vectors with improved performance. However, last years have witnessed a renewed interest in the more traditional 'directed evolution' method of oncolytic vector development; the random creation of virus mutants followed by bioselection of the best-performing viruses. This approach has yielded improved oncolytic HAdVs with genomic mutations that would have never been picked up using rational design approaches. These findings not only benefit to the development of improved oncolytic HAdVs, but also enhance our knowledge on HAdV biology.

Following a mutagenesis and bioselection approach, Yan *et al.* plaque purified two mutants, ONYX-201 and ONYX-203, from a pool of randomly mutated HAdV-5 that was repeatedly passaged in the human colorectal cancer cell line HT29.[100] The mutants replicated more rapidly in HT29 cells than wild-type HAdV-5, and lysed HT29 cells up to 1,000-fold more efficiently. The enhanced cytotoxicity was also observed in other human cancer cell lines, but not in a number of normal primary human cells, indicating a strong enhancement of the therapeutic index of ONYX-201 and ONYX-203. Although the virus mutants contained multiple single-base-pair mutations, they shared a mutation at nucleotide 8350, which was shown to be essential for the observed phenotype. This mutation was mapped to the i-leader sequence of the HAdV-5 genome, which is (for unknown reasons) present as a 440-nucleotide leader sequence in the majority of HAdV-5 major-late transcripts. The i-leader contains an open reading frame encoding for a 16 kDa-sized protein.[101] The mutation at nucleotide 8350 introduces a stopcodon, resulting in a truncation of 21 amino acids from the C terminus of the i-leader protein. In parallel to these results, another i-leader mutant HAdV-5 was isolated by Subramanian and coworkers in a screen for large plaques on A549 alveolar epithelium cells.[102] Although the exact mechanism behind the improved oncolytic performance of i-leader mutated HAdV-5 remains to be investigated, the potential of this type of mutation was recently underscored by Van den Hengel *et al.*, who demonstrated enhanced cytopathic activity of i-leader mutated HAdV-5 in glioma cell lines and primary glioma cultures.[103]

Using similar mutagenesis and bioselection approaches, another type of HAdV-5 mutant with enhanced antitumor efficacy was found by Gros *et al.*[104] The propagation of a mutagenized HAdV-5 stock in human tumor xenografts led to the isolation of a mutant virus displaying a large-plaque phenotype *in vitro* and an enhanced antitumor activity *in vivo*. A truncating mutation in the viral E3-19K gene, resulting in relocalization of the E3-19K protein from the endoplasmatic reticulum to the plasma membrane, appeared to be responsible for the mutant's enhanced antitumor efficacy. The aberrant protein localization appeared to enhance the cellular influx of calcium ions, thereby deregulating calcium homeostasis and inducing membrane permeabilization.

Recently, Uil *et al.* presented another type of directed evolution, through serial passaging of HAdV-5 in cancer cells in the context of a 'sloppy' viral polymerase protein.[105] To this aim, the authors first identified mutations in the viral polymerase protein that lead to viral replication with increased mutation rate. The strongest identified mutators, all

having a mutation in the single-strand DNA binding region of the exonuclease domain, were exploited to generate HAdV-5 mutants with improved cytolytic activity in tumor cells. A common mutation was identified, located in a splice acceptor site preceding the gene for the adenovirus death protein (ADP). Accordingly, high and untimely expression of ADP was observed, presumably causing the enhanced cytotoxicity.

Kuhn and coworkers have used a directed evolution approach to obtain chimeric oncolytic adenoviruses, that consist of components from different HAdV serotypes.[106] An array of serotypes, representing HAdV species B to F, was pooled and passaged on tumor cell lines under conditions that invite recombination. By using this methodology, a highly potent oncolytic HAdV-3/HAdV-11p chimeric virus (named ColoAd1) was obtained. ColoAd1 demonstrated greatly enhanced potency and selectivity, as compared to its parent serotypes and ONYX/015, in colon cancer cell cultures and in a mouse tumor model.

The proven potential of random selection approaches has triggered researchers to combine this type of approach with rational design. As such, improved HAdV vectors targeted to prostate cancer cells have been isolated after genetically incorporating random peptides at viral capsid locales flanking the tumor-targeting polypeptide sequence.[107]

Directed evolution approaches are expected to lead to the isolation of novel and improved oncolytic HAdVs. Performing such strategies in clinically relevant model systems will be of great interest, acknowledging the large repertoire of efficacy-limiting *in vivo* aspects of oncolytic viral therapy, with many aspects having non-resolved mechanisms of action.

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