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Fiber Modification of Adenoviral Vectors for Cancer Gene Therapy

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MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Cancer still remains a major public health concern despite improvements in primary prevention, early detection and advanced treatments. Cancer gene therapy using human adenovirus serotype 5 (HAdV-5) as a vector has been explored as a new therapeutic approach. HAdV-5 infection is initiated by binding to the coxsackie virus and adenovirus receptor (CAR), its primary cellular receptor. However, the levels and patterns of expression of CAR vary greatly in clinical tumor tissue samples, and the expression levels tend to decrease as the tumors progress. The low level expression of CAR in target cancer cells diminishes the utility of HAdV-5 as a vector for cancer gene therapy. To overcome this expression problem, we have developed and investigated novel modifications of HAdV-5 vectors for: 1) efficient gene delivery via a CAR-independent mechanism, and 2) tropism expansion from CAR-positive to CAR-negative tumor cells. For the first strategy, a range of chimeric HAdV vectors displaying the fiber shaft and knob domains of species B HAdVs (HAdV-3, -11, or -35) has been developed, since species B HAdVs utilize CD46 as a receptor for infection. CD46 is highly expressed in the majority of cancer cells. The fiber chimeric HAdV vectors were compared with the original HAdV-5 vector for transductional efficiency in a variety of cancer cell lines, using luciferase as a reporter gene. The chimeric HAdV vectors had distinct infectivity in different types of cancer cells as well as a CAR-independent mechanism for infection. In particular, of the developed chimeric HAdV vectors, a vector incorporating the HAdV-3

fiber shaft and knob domains achieved the highest ratio of gene transfer ratio in advanced prostate cancer as compared to normal prostate cells, suggesting that this vector could potentially be used for prostate cancer gene therapy. For the second strategy, we have genetically developed a fiber-mosaic HAdV vector incorporating both a chimeric fiber protein displaying the HAdV-3 shaft and knob domains, as well as the native HAdV-5 fiber protein. Both types of the fiber proteins functionally utilized their two distinct receptors, CAR and CD46. Importantly, in a mixed population of two different types of cells, which simulates a tumor-like environment, the fiber-mosaic HAdV vector transferred a transgene into both cell types utilizing CAR and CD46, demonstrating tropism expansion. These results indicate that fiber modification of the HAdV vector is a suitable platform for future HAdV vectors capable of a variety of clinical applications for cancer gene therapy.

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LIST OF ABBREVIATIONS

Ad	adenovirus
CAR	coxsackie B and adenovirus receptor
CHO	Chinese hamster ovary cells
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
HAdV	human adenovirus
HSV-TK	herpes simplex virus-1 thymidine kinase
MLP	Adenovirus major late promoter
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
RGD	extracellular matrix tripeptide arginine-glycine-asparate motif
SDS	sodium dodecyl sulfate
TP	Adenovirus terminal protein
TPL	Adenovirus tripartite leader sequence
VP	viral particle

CHAPTER 1

INTRODUCTION

Cancer Status and Therapy

The incidence and cancer death rates from all cancers combined significantly decreased from 1996 to 2006 (1). These decreases were seemingly due to improved treatments and early detections. However, despite these improvements, cancer remains a major public health concern. The National Institute of Health (NIH) estimates the overall costs of cancer in 2008 at $228.1 billion. Cancer is responsible for one in four deaths in the United States (2) and is the leading of cause of death under age 85 due to the decline of heart disease mortality (3). This is because, despite the improved treatments in some cancer types, the outcome of treatment in other cancers is poor. For example, treatment for childhood leukemia has improved, resulting in 5 years survival rates approaching 90% (4). However, the treatment of mixed lineage leukemia, which is aggressive and predominantly occurs in pediatric patients, has not achieved satisfactory results (5). This discouraging outcome is one of the reasons that leukemia is still the most common fatal cancer among our youth (2). Brain cancer is the second leading cause of cancer death in childhood (2). As another example, treatment of one type of malignant brain tumor, medulloblastoma, has successfully improved patient outcomes (6), whereas survival of high grade glioma in childhood remains poor.

Even with the improved outcome of a majority of cancer types, another question that arises is the effect of treatment on quality of life. For example, one of the effective

treatments for medulloblastoma includes surgical resection, craniospinal irradiation and chemotherapy; however, this has caused a decline in the intelligence of the patients (7), resulting in diminished quality of life (8). The breast cancer death rate, the most common fatal cancer among females at ages 20 to 59 years, decreased by 37% between 1991 and 2005 (2). Chemotherapy is one of the treatment methods for breast cancer that has contributed to this positive outcome. However, it has caused a negative impact on reproductive function (9) and thus reduced the quality of life of young patients. These considerations rationalize the development of a novel therapeutic approach for cancers.

Cancer Gene Therapy

Gene therapy is a relatively new cancer therapeutic approach. The National Cancer Institute (NCI) has defined gene therapy as "a treatment that alters a gene" (http://www.cancer.gov/). Based on this concept, gene therapy has initially been used to compensate for genetic disorders, such as severe combined immunodeficiency syndromes (SCID) (10), cystic fibrosis (CF) (11), hemophilia (12) and muscular dystrophies (13). However, the meaning of gene therapy has extended along with the development of gene delivery systems (14). The term of gene therapy now encompasses a wide range of treatment types that all use genetic material to modify cells (either *in vitro* or *in vivo*) to help effect a cure (15). Thus, gene therapy includes the application of different viral and non-viral gene therapy vectors (16) for any human disease, including cancer. Cancer gene therapy using viral vectors, which is the focus of this introduction, can be divided into three sections: immunotherapy, mutation compensation and oncolytic virotherapy (17).

Genetic Immunotherapy

The goal of immunotherapy for the treatment of cancer is to boost host anti-tumor immunity, and genetic immunotherapy can be categorized into three strategies. The first strategy is to modify the cancer cells by the delivery of a cytokine gene, with the expressed cytokine subsequently stimulating the host immune-system. The cytokines used in clinical trials include granulocyte-macrophage colony stimulating factor (GM-CSF) (18, 19), interleukin (IL)-2 (20-22), IL-12 (23-26), interferon (IF)-γ (27) and IF-β (28-30). The second strategy is to unmask cancer cells from the immune-system by transferring immunostimulatory genes, including major histocompatibility complex class I (31) and T-cell co-stimulatory molecules: B7-1 (26) and B7-2 (18). The expression of theses molecules is usually down-regulated in cancer cells (32-35). Transferring and thus expressing genes encoding these molecules on cancer cells assists the immune system to discern and eliminate the cancer cells. The third strategy is to directly stimulate the patient's immune system by transferring genes encoding tumor-associated antigens (TAAs) into antigen-presenting cells, such as dendritic cells (36, 37). These cells will subsequently mediate and coordinate an immune response against cancer cells.

Virus-directed Prodrug Therapy

A second type of cancer gene therapy is to transfer genes, including suicide genes and cellular stasis genes. In the first step of suicide gene therapy, genes encoding enzymes are transferred and expressed in cancer cells. In the next step, a non-toxic prodrug is administered, which is subsequently transformed to be active or toxic by the expressed enzymes. As the enzymes are only expressed inside or on the surface of the

cancer cells, the activated drug kills only the transduced cancer cells, leaving healthy cells unharmed. The genes to be transferred include herpes simplex virus-1 thymidine kinase (HSV-TK) (38-41), *Escheria coli* cytosine deaminase (CDA) (42-45) and *Escheria coli* purine nucleoside phosphorylase (PNP) (45), for which a phase I clinical trial was initiated in 2008.

Mutation Compensation

In many cancer cells, loss of tumor suppression function is generally observed (46). Transferring tumor suppressor genes, including p53 and $p16^{INK4a}$ (47, 48) into cancer cells is another strategy in the gene transfer section for cancer therapy. For example, the p53 gene is mutated or deleted in more than 50% of human cancers (49). It has been demonstrated that induction of the p53 genes arrest growth of p53 mutated cancer cells (50-52); a phase II clinical trial for squamous cell carcinoma using an adenoviral vector encoding p53 genes has been performed (53). In 2003, China officially approved the world's first gene therapy strategy, an injection of non-replicative HAdV vectors encoding p53 (Gendicine; SiBiono, Shenzhen, China), for commercial use (54).

Oncolytic Virotherapy

The forth kind of cancer gene therapy is oncolytic virotherapy. The notion that the natural cytolysis capability of viruses can be utilized to treat cancer was appreciated long time ago. The first case of the application based on this concept was presented in 1904 in a report of tumor regression following receiving a rabies vaccination in a patient with cervical carcinoma (55). Around the late 1940s and early 1950s, investigations of

oncolytic viruses in human cancer patients were started (56). The focus of these investigations was the natural cytolysis of the viruses. However, based on these early pioneering studies, the focus of oncolytic viruses moved to the ability to genetically engineer oncolytic viruses into increasingly potent and tumor-specific vectors (56). At present, a number of different viruses have been utilized as oncolytic viruses in late phase or completed clinical trials, including adenovirus, HSV, reovirus, vaccina virus and Newcastle disease viruse (57). Subsequently to Gendicine as described above, in 2005, China approved the second gene medicine, replication competent oncolytic HAdV vector (H101; Sunway Biotech, Shanghai, P.R. China), for market (54).

Adenovirus

Adenovirus has been extensively used as a vector in gene therapy clinical trials (58) (http://www.wiley.co.uk/genetherapy/clinical/). Adenoviruses belong to the family of adenoviridae (59). Human adenovirus was first extracted from human adenoids in the 1950s (60). Currently, at least 51 serotypes of human adenoviruses (HAdVs) have been identified and grouped into six species (A-F) (61). The six species have different natural sites of infection in the body (Table I).

Table I. Classification of human adenovirus

Species	Serotypes	Sites of infection	Receptor(s)
A	12, 18, 31	Gastrointestinal tract	CAR[a]
B	3, 7, 16, 21, 50 (B1) 11, 14, 34, 35 (B2)	Lung, Urinary tract	CD46, CD80, CD86
C	1, 2, 5, 6	Upper respiratory tract	CAR
D	8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26, 27, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51	Eye, Gastrointestinal tract	CAR, Sialic Acid (8, 19, 37)[b], CD46 (37)[b]
E	4	Respiratory tract	CAR
F	40, 41	Gastrointestinal tract	CAR

[a] CAR is the coxsackie virus and adenovirus receptor

[b] The number(s) in parentheses in species D indicates HAdV serotype(s) which utilizes the receptor.

The diameter of the adenovirus icosahedral capsid is about 90 nm (62). The nonenveloped icosahedrally shaped virion is composed of multiple copies of 12 different structural proteins, including three major proteins, hexon (II), penton base (III), and fiber (IV), along with minor proteins, VI, VIII, IX, and IIIa (63). The linear, double-strand DNA of 30-40 kb with a terminal protein (TP) is connected with the protein, V, VII and mu (64) **(Fig. 1)**. The Ad genome contains two groups of genes: the early transcription units (five units: E1A, E1B, E2, E3 and E4) and the one late unit (major late) that produces five families of late mRNA (L1 to L5) **(Fig. 2)**. E1A, E1B, IX, major late, VA RNA and E3 units are transcribed using the sense reading strand, while E4, E2 and IVa2 units are transcribed using the anti-sense reading strand **(Fig. 2)**.

The adenovirus infection cycle can be divided into two phases: the early phase, the first 6 to 8 hours, and the late phase, another 4 to 6 hours **(Fig. 3)**. For the initiation of the infection of HAdV, receptor-mediated interaction with the host cell is crucial. All HAdVs, except those of species B, bind to CAR (65). In 1997, a complementary DNA clone of CAR was first isolated and transfected into hamster cells, resulting in HAdV-2 binding to the cells (66). Most of species B HAdVs have been reported to utilize CD46 (67-71), or CD80 and CD86 (72, 73) as a cellular attachment receptor. HAdV-37 from species D has also demonstrated binding to CD46 (74). Several species D HAdVs, such as HAdV-37, -8 and -19, have been reported to bind to α(2-3)-linked sialic acid as a cellular receptor (75-77). These receptors will be discussed in a later section of this introduction.

Subsequent to the cell binding of HAdVs, secondary interaction of the arg-gly-asp (RGD) motif on the viral penton base with αvβ3 and αvβ5 integrins promotes virus

internalization (78). After the initial interaction of viruses with receptors, clathrin-mediated endocytosis leads to the viruse cell entry (79). Partial disassembly of the HAdV particle occurs prior to disruption of endosome at low pH (80), facilitating entry of particles into the cytoplasm. Subsequently, the viral genome associated with core protein VII is imported through the nuclear pore into the nucleus (81), where the primary transcription occurs.

In the early phase of the infection, the early transcription units, E1A, E1B, E2, E3 and E4, undergo the first transcriptional events. The E1A protein, one of two sets of E1 proteins, regulates transcription of these early genes, including the E1A gene itself, as well as host cell gene expression and proliferation, by binding to cellular regulatory proteins. Specifically, it can bind to the retinoblastoma (Rb) protein. This interaction releases the E2F protein, and the released E2F activates viral and cellular gene expression (82). The E1A protein also modulates cell division by regulation of NF-κB and p53. E1B proteins are involved in inhibition of apoptosis by directly binding to p53 or binding to Bax and other apoptosis inducers (83).

The products of the E2 transcription unit include adenovirus DNA polymerase (Ad Pol), preterminal protein (pTP) and DNA binding protein (DBP), which are involved in viral DNA replication. The products of the E3 transcription unit function to interfere with host immune responses (84, 85). The products of the E4 transcription unit exhibit a broad range of functions, including viral DNA replication, viral mRNA transport and splicing, inhibition of host cell protein synthesis and regulation of apoptosis (64).

After the onset of viral DNA replication, the major late promoter (MLP) is activated by several transcriptional factors. When the MLP is fully functional, it

facilitates production of the late transcription unit, which is involved in expression of adenoviral structural proteins including the fiber proteins. The major late pre-mRNA contains the sequences of at least 15 different mRNAs, and the length is almost to the right end of the genome (86). The major late pre-mRNA undergoes polyadenylation and falls into five families (L1 to L5) (86). Subsequently, the splicing of three short exons results in assembly of the tripartite leader sequence (TPL) at the end of 5' ends of all late mRNAs, which is important for efficient translation late in the adenoviral infectious cycle (86). The resulting structural proteins are transported into the nucleus; and the viral capsids are assembled from the structural proteins. Eventually, the newly formed viruses are released by lysis of the host cells.

Adenovirus Fiber Protein

The adenovirus fiber protein plays a critical role during the initial cellular attachment phase of the viral infection. Its fiber amino acid sequence was first identified by Herrise *et al.* in 1981 (87). Subsequently, Green *et al.* determined the organization of the fiber protein, which is composed of three different structural components: the tail, shaft and knob domains (88). The amino-terminal tail domain interacts with the penton base protein, mediating the anchoring of the fiber protein into the HAdV capsid. The amino acid sequence of the fiber tail domain is strongly conserved among HAdVs (89). The fiber tail domain is important as it contains a nuclear localization signal, which targets the synthesized fiber proteins to the nucleus (90).

The components of the fiber shaft domain contribute to the variation in the fiber flexibility as well as in the length among different serotypes. A variable number of

repeating sequence motifs of approximately 15 amino acids in the fiber shaft domain results in a large difference in the fiber length among different serotypes (89) (Table II). Each repeat contains two β-strands connected by a short linker (88). These repeats form a shaft of three intertwined strands, thus providing high stability to the fiber protein (91).

Table II. General features of human adenovirus fiber proteins and their receptors

Species	Receptor(s)	Fiber length	Number of β-repeats	Flexibility
A	**CAR**	**~ 330 Å**	**20.5[b] or 22.5**	**Yes**
B	**CD46, CD80, CD86**	**~ 130Å**	**5.5 or 7.5[c]**	**No**
C	**CAR**	**~ 330 Å**	**18.5[d] or 21.5**	**Yes**
D	**CAR, Sialic Acid, CD46**	**~ 150 Å**	**7.5**	**No**
E	**CAR**	**Unknown**	**11.5**	**Unknown**
F	**CAR**	**~ 220 Å or ~ 330 Å[a]**	**11.5, 20.5, or 21.5[a]**	**Unknown**

CAR is the coxsackie virus and adenovirus receptor.
[a] Species F HAdVs incorporate two different types of fiber proteins; one short and one long.
[b] HAdV-31 fiber contains two fewer β-repeats than HAdV-12.
[c] HAdV-16 fiber contains two more β-repeats than any other species B HAdVs.
[d] HAdV-6 fiber contains two fewer β-repeats than any other species C HAdVs.

The flexibility of the fiber proteins, provided by the fiber shaft domain, also varies among serotypes. The location of the bend of the fiber shaft domain of many HAdVs observed by electron microscopy analysis matches a four amino acid insertion in the third repeat of the fiber shaft domain (89, 92). Certain species D HAdVs, such as HAdV-37, do not possess the four amino acid insertion in the third repeat of the fiber shaft domain, which is rigid, whereas many of species A, C and E HAdVs possess it there which thus is flexible (89). Wu *et al.* has genetically replaced the flexible third repeat of the HAdV-5 fiber with that of the HAdV-37 fiber, which is rigid (93). The modified HAdV-5 fiber was more rigid than the wild-type HAd-5 fiber; and the fiber rigidity decreased binding and infection of CAR expressing cells (93). This suggests that the fiber-shaft domain, as well as the fiber-knob domain, contributes to the receptor binding interaction that is needed for viral infection.

The carboxy terminal knob domain is absolutely crucial for trimerization of the fiber protein and receptor binding. Self-trimerizationof the fiber protein was observed (94-96) without any interaction needed with any other adenovirus-encoded proteins or cell nuclear matrix components (97). Trimerrization was prevented by deletions in the knob sequence, indicating its role in this process (98). Only fiber trimers, but not monomers, appear to bind to penton bases (97), suggesting that the fiber-knob domain indirectly plays a critical role for the incorporation of the fiber proteins into the viral capsid. The fiber trimerization process takes place in the cytoplasm as the deletion of the nuclear transport signal resulted in the accumulation of trimeric HAdV-2 fiber in the cytoplasm (90).

Another function of the fiber knob domain is to bind to a cellular receptor and mediate the initial step of infection. X-ray crystallography of the HAdV-5 knob domain performed by Xia *et al.* provided the first atomic resolution information for the fiber protein (99, 100). The monomer structure of the HAdV-5 knob domain contains a sandwich of two antiparallel β-sheets (V and R β-sheets), containing 8 strands with 6 prominent loops: AB, CD, DG, GH, HI and IJ (99, 100). The mutagenesis study performed by Roelvink *et al.* (101) and crystal structure analysis performed by Bewley *et al.* (102) have determined that the majority of amino acid residues crucially involved in CAR-binding sites of HAdVs are in the AB loop in the fiber-knob domain.

Receptors for Adenoviruses

The coxsackie virus and adenovirus receptor (CAR)

The coxsackie virus and adenovirus receptor (CAR) was first identified as a receptor for HAdV-2 and -5 by Bergelson and his colleagues in 1997 (66). Subsequently, Roelvink *et al.* demonstrated that CAR is a cellular receptor for serotypes from species A, C, D, E and F, but not B (65). The predicted molecular weight of CAR is approximately 38 kDa, but it migrates at 46 kDa on sodium dodecyl sulfate (SDS) polyacrylamide gels, probably due to glycosylation (103). CAR is a transmembrane protein, which is composed of an intracellular domain of 107 residues and a 216- residue extracellular domain, including two immunoglobulin (Ig)-like domains, D1 and D2 (102) (**Fig. 3**). HAdVs bind to the D1 domain of CAR (102); the transmembrane region and cytoplasmic domain of CAR are unnecessary for adenovirus infection (104). The amino acid residues in the AB loop in the knob domain are very important for the CAR-binding interaction (101, 102) as described above. These amino acid residues in the AB loops are conserved in the fiber proteins among HAdVs serotypes utilizing CAR as a cellular receptor (102, 105). Contrarily, these residues are substituted in the AB loops of the fiber proteins of HAdVs serotypes utilizing receptors other than CAR (102, 105). Binding of the virion to multiple CAR molecules simultaneously results in the high affinity binding constant (Kd = 1 nM), whereas the affinity of a single CAR for the fiber knob is relatively modest (78).

The normal tissue distribution of CAR in humans, assessed by Northern blots analysis, shows the highest RNA expression levels were detected in pancreas, brain, heart, small intestine, testis and prostate. The liver and lung had small amounts of CAR RNA, while no signal could be detected in kidney, placenta, peripheral blood leukocytes,

thymus, and spleen (106). CAR normally functions in the formation of the tight junctions in polarized epithelial cells (107, 108). Many tumor samples have been examined for CAR expression as the number of studies utilizing HAdV vectors for clinical applications has increased. With the exception of the breast tumor, in which CAR expression is increased with increasing grade of tumor (109), the expression levels of CAR are likely to be low in advanced tumors in the majority of tumor types (110-117). However, of these tumor types, immunohistochemistry of ovarian tumor demonstrated CAR-positive regions adjacent to completely negative areas (117). Therefore, the expression of CAR differs greatly among individual tumor cells, even within the same tumor.

CD46

In 1998, species B HAdVs were found to not bind to CAR for the initial step of viral infection (65). Subsequent to this finding, the lack of the conserved CAR binding sequence in this species was demonstrated (101, 105) as described above. In 2003, Gaggar *et al.* reported that instead of CAR, CD46 was the receptor for most of species B HAdVs (68). CD46 is a membrane co-factor protein, that functions as a regulator of complement activation on host cells (118). The molecular weight of CD46 is 45-70 kDa with cell line- or tissue specific phenotypic variations (119). CD46 contains four short consensus repeats (SCRs), a serine/threonine-rich domain, a small region of unknown function, a transmembrane domain and a short cytoplasmic tail (120). Of the SCRs, SCR-1, -2 or -4 contains N-linked oligosaccharides. Using mutagenesis analyses, SCR-1 and -2 were identified as HAdV-35 binding sites on CD46 (121). Subsequently, Persson *et al.* reported the co-crystal structure of the HAdV-11 fiber knob domain with SCR-1 and -2

of CD46 (122). The HAdV-11 fiber knob domain interacts with both SCR-1 and -2 (Kd = ~2 nM); and a glutamic acid residue in SCR-2 docking with arginine 280 of the fiber knob domain plays a crucial role in the interaction (122). A conformational change of CD46 was observed after the HAdV-11 fiber knob domain binding (122). Other groups have indentified two key residues in HAdV-11 fiber knob domain: arginine 279 and 280, also based on mutagenesis analysis (123, 124). Pache *et al.* has reported the affinities of CD46 for the HAdV-11 or -16 fiber knob domain (124). The affinity of CD46 for the HAdV-16 fiber knob domain is much lower than that for the HAdV-11 fiber knob domain. Species B is divided into two groups: B1, including HAdV-3, -7, -16, -21 and -50, and B2, including HAdV-11, -14, -34 and -35. In addition to the low HAdV-16 binding affinity for CD46, HAdV-3, also from species B1, has a low binding affinity for CD46 (68, 71, 125). HAdV-37, from species D, has also been demonstrated to utilize CD46 as a cellular receptor (74). CD46 serves as a cellular receptor for not only some of HAdVs but also a wide variety of other pathogens, including measles virus (126, 127), *Neisseria gonorrhoeae* and *meningitides* (128, 129), human herpesvirus 6 (127, 130), and *Streptococcus pyogenes* (131, 132).

CD46 is ubiquitously expressed throughout the human body with the level of expression ranging from very strong to very weak, depending on the tissue (133). Among the tissues examined by Western blotting, the relative intensities of CD46 bands were very strong in salivary and adrenal gland, kidney and pancreas, whereas they were weak in lung and brain. (133). When compared with their normal counterparts, CD46 is over-expressed in several cancers (134), including breast, colorectal (135), liver (136) and endometrial (137) cancer tissues, and some leukemia cells (138). Since the expression of

CD46 in CD55 (decay-accelerating factor; DAF) deficient cell lines provided protection of the cells from complement-mediated cell lysis (138), CD46 has been thought to be involved in mechanisms of tumor cells escape from complement-mediated cytotoxicity.

CD80 (B7-1) and CD86 (B7-2)

Short *et al.* has identified CD80 and CD86 as cellular receptors for HAdV-3 (72). CD80 was first identified as a co-stimulatory molecule providing T-cell activation by binding to CD28 (139-141). Later studies suggested the existence of additional co-stimulatory molecules, such as CD86 (142-147). These co-stimulatory signals are required to trigger an optimal immune reaction. Both CD80 and CD86 are members of the immunoglobulin gene superfamily, containing an Ig-V like and an Ig–C like domain (148).

The expression of CD80 and CD86 is restricted to lymphoid cells including T cells, B cells, dendritic cells (DCs) and macrophages (149, 150). In cancer cells, the expression of CD80 and/or CD86 is rarely observed (32-35, 151). In contrast to the Short *et al.* findings, one of the groups that examined the expression levels of CD80 and CD86 demonstrated that there was no relationship between the expression of CD80 or CD86 and HAdV-3 fiber protein binding or HAdV-3 cell entry (32).

Sialic Acid

Several species D HAdVs, such as HAdV-37, -8 and -19, have been found to utilize α(2-3)-linked sialic acid as a cellular receptor (75-77). Infection with these serotypes causes epidemic keratoconjunctivitis (75-77). Sialic acid is a family of acidic

nine-carbon sugars that are typically located at the terminal positions of a variety of glycoconjugates; and widely distributed in the human body (152).

Adenoviral Vectors as Gene Delivery Vehicles for Cancer Therapy

Human adenovirus serotype 5 (HAdV-5) has been extensively explored as vectors for cancer therapy since they possess several favorable features. The HAdV-5 vectors are able to transfer large therapeutic genes (153, 154) extending to nearly 37 kb (155). For in vivo gene delivery and clinical applications, the HAdV-5 vectors can be amplified to high titer concentrations (156). They are relatively safe, as the genome of HAdV does not integrate into the host genome (157, 158).

Despite these advantages, the HAdV- 5 vector has limitations for its use in cancer therapy. The HAdV-5 vectors cannot achieve high transduction into some cancer cells, mainly due to insufficient expression of CAR, the primary receptor for HAdV-5, as described above (159-161). Moreover, the distribution of the CAR expression is based on the origin of the tumor cells (106). With the exception of the breast tumor, in which CAR expression is increased with increasing grade of tumor (109), the expression levels of CAR tend to be low in advanced tumors of the majority of tumor types (110-117). In addition, CAR expression in ovarian tumor exhibited marked distributional heterogeneity as described above (117). Therefore, the expression of CAR varies greatly in individual cells of the same tumor; and the insufficient CAR expression hampers HAdV-5 transducion, especially for advanced tumors.

Several approaches have been developed to address this problem. These approaches are based on strategies that either ablate or expand the HAdV-5's native

tropism, and can be divided into two categories: 1) the use of adapter molecules, including antibodies and bi-specific fusion proteins, 2) genetic capsid modification, especially of the fiber protein.

For the approach using adapter molecules, Douglas *et al*. developed a monoclonal antibody (mAb) against the HAdV-5 fiber protein, and conjugated its Fab-fragment to folate, which would target the folate receptor (162). The folate receptor is up-regulated in several cancer cells (163, 164). In addition, several anti-fiber protein antibodies have been chemically conjugated to receptor-specific ligands such as basic fibroblast growth factor (FGF2) (165) and a mAb directed against the epidermal growth factor receptor (EGFR) (166). Similarly, bi-specific fusion proteins have been generated by several groups. These proteins include an anti-Ad fiber knob single-chain antibody (scFv) (167, 168) or the extracellular domain of CAR (169), genetically fused to a cell receptor-specific ligand, such as the epidermal growth factor (EGF). This strategy has the advantage of a uniform population of retargeting molecules, as opposed to the less uniform molecules resulting from chemical conjugation of the two components. However, both strategies have limitations, including the possibility of dissociation of the HAdV-5 vector and the adapter molecules before reaching the target cell, as well as being lack of applicability to replicative HAdV-5 vectors, where the progeny viral particles would not be targeted.

In order to overcome these limitations, genetic modification of the fiber protein has been explored. As described above, the fiber protein is responsible for the interaction with the cellular receptor, CAR, which is the initial step of the infection. This genetic modification of the fiber protein can be categorized into two strategies: 1) incorporation

of targeting ligands into the fiber knob domain and 2) replacement of the fiber protein region, such as the fiber knob domain, the fiber shaft and knob domains, or even the whole fiber protein. In the first strategy, the most extensively studied modification is the incorporation of an Arg-Gly-Asp (RGD)-containing peptide in the fiber knob domain (170-173). The purpose of the incorporation of RGD-containing peptide is to target αv-integrins, which are highly expressed in cancer cells (174-176). Another example of this first strategy is an adenoviral vector containing lysine residues in the fiber knob domain (170, 177). 'Polylysine' has been demonstrated to interact with heparan sulfates (178), which are widely expressed throughout the human body (179). Thus, the HAdV vectors containing polylysine have expanded the native tropism and achieved high infection or transduction in glioma cells (180), myeloma (181), lymphoma (182), and leukemia (183), which are all usually refractory to the HAdV-5 infection. Since incorporation of peptides in the C-terminus of the fiber hinders the fiber trimerization (98), the HI loop of the fiber knob domain (100), has been utilized as the location of the peptide-incorporation. However, the size of the peptide matters despite the competence of the HI loop (184), thus limiting what ligands can be incorporated with this strategy.

Alternatively, the latter strategy, replacement of a fiber protein region, has been explored by several groups. Krasnykh *et al.* has developed chimeric molecules containing the N-terminal region of the HAdV-5 tail domain, genetically fused to the bacteriophage T4 fibritin protein from which N-terminal was deleted (185). This molecule was chosen because the fibritin has been reported to form homotrimers; and, as mentioned earlier, trimarization is necessary for the alternative fiber to be incorporated into the HAdV particles (97). Subsequently, the same group has shown the successful incorporation of

this ‘fiber fibritin’, fused with a targeting moiety, being either CD40L (186) or affibodies specific for human epidermal growth factor receptor type 2 (Her2) (187), into the HAdV-5 particle (186).

The most common replacement strategy is, however, replacement of the fiber portion of the HAdV-5 vector with that of other HAdV serotypes, including human or non-human adenoviruses. Gall *et al.* and Nakamura *et al.* explored the incorporation of whole fiber proteins derived from HAdV-7 (188) or from HAdV-40 (189) into HAdV-2 or -5 viral particles respectively. Since the fiber-tail sequence is highly conserved among HAdVs (89), the replacement of whole fiber protein into the different HAdVs would be possible. However, preserving the fiber-tail sequence of the capsid (usually of HAdV-5 or -2) is more favorable in general as the fiber-tail domain is docked into the penton base on the capsid.

Several groups have reported the replacement of only the fiber knob domain of HAdV-5 with that of HAdV-3 (190, 191) or non human adenoviruses (192, 193). These genetically modified HAdV-5 vectors have achieved more efficient transduction in cancer cells compared to the unmodified HAdV-5 vectors. However, *in vivo*, Smith *et al.* has reported that the shaft of HAdV-5 mediates the HAdV-5 liver tropism (194), which is one of the limitations of using HAdV-5 as a vector for cancer therapies. The optimal strategy when replacing portions of the fiber protein therefore would include replacing both the fiber shaft and knob domains. For example, Nakayama *et al.* have developed an HAdV-5 vector that incorporates the fiber shaft and knob domains from ovine adenovirus type 7 (161). However, the fiber protein from species B HAdVs is most often used for fiber replacement, as species B HAdVs do not utilize CAR as a cellular receptor (65).

Another advantage is that species B HAdVs bind to CD46, which is up-regulated in cancer cells in comparison with their normal counter parts (134-137, 195), as described above. In fact, chimeric species B HAdV fibers have successfully been incorporated into HAdV-5 viral particles, achieving infection/transduction in insufficient CAR-expressing cells (196-198). Hence, the effect on viral particle structure by replacing the fiber shaft and knob domains of HAdV-5 with those of the species B fibers has not been thoroughly evaluated. Therefore, in the next chapter of this thesis, we investigated whether the replacement of the HAdV-5 fiber would impact the HAdV biological and physiological properties. Furthermore, we evaluated the infectivity of the chimeric HAdV-5 vectors incorporating the fiber shaft and knob domains from HAdV-3, -11, or -35 in cancer cell lines derived from different origins.

In order to target not only one kind of cellular receptor but also multiple receptors in tumor tissues, which are composed of a heterogeneous population of cells, our group has developed several fiber-mosaic HAdV vectors, which incorporate two distinct kinds of fiber proteins. These mosaic vectors have been produced using two different methods: 1) co-infection of cells with two different HAdV vectors (199) and 2) genetic modification of the fiber genes (200-203). With the first method, we have previously generated fiber-mosaic viral particles that incorporate both the native HAdV-5 fiber protein and a chimeric HAdV-5 fiber protein possessing the HAdV-3 fiber knob domain (199). The fiber-mosaic Ad was produced by co-infection of two different kinds of the HAdV-5 vectors encoding either the native HAdV-5 fiber gene or the chimeric HAdV-3 fiber knob gene. However, one possibility is that a single type of HAdV vector infects an

individual cell, with the progeny of the HAdV vectors incorporating only one kind of fiber protein.

With the latter method, we previously genetically generated fiber-mosaic HAdV vectors encoding additional fiber genes including the bacteriophage T4 fibritin (200), the bacteriophage T4 fibritin containing the 1.3S subunit of *propionibacterium shermanii* transcarboxylase (202), or reovirus attachment protein sigma 1 along with wild type HAdV-5 fiber gene (201), and reovirus attachment protein sigma 1 along with the HAdV-5 fiber gene containing HAdV-3 knob sequence (203). These studies showed that the additional protein was able to function as a ligand for its cellular receptor (201-203). Thus, mosaic system adenoviral vectors are suitable for targeting tumors that are composed of a heterogeneous cell population (204, 205). In the final chapter of this thesis, we report a different cloning strategy compared to previous studies to genetically generate fiber mosaic HAdV vectors; the biological properties and infectivity of these fiber-mosaic Ad vector was evaluated.

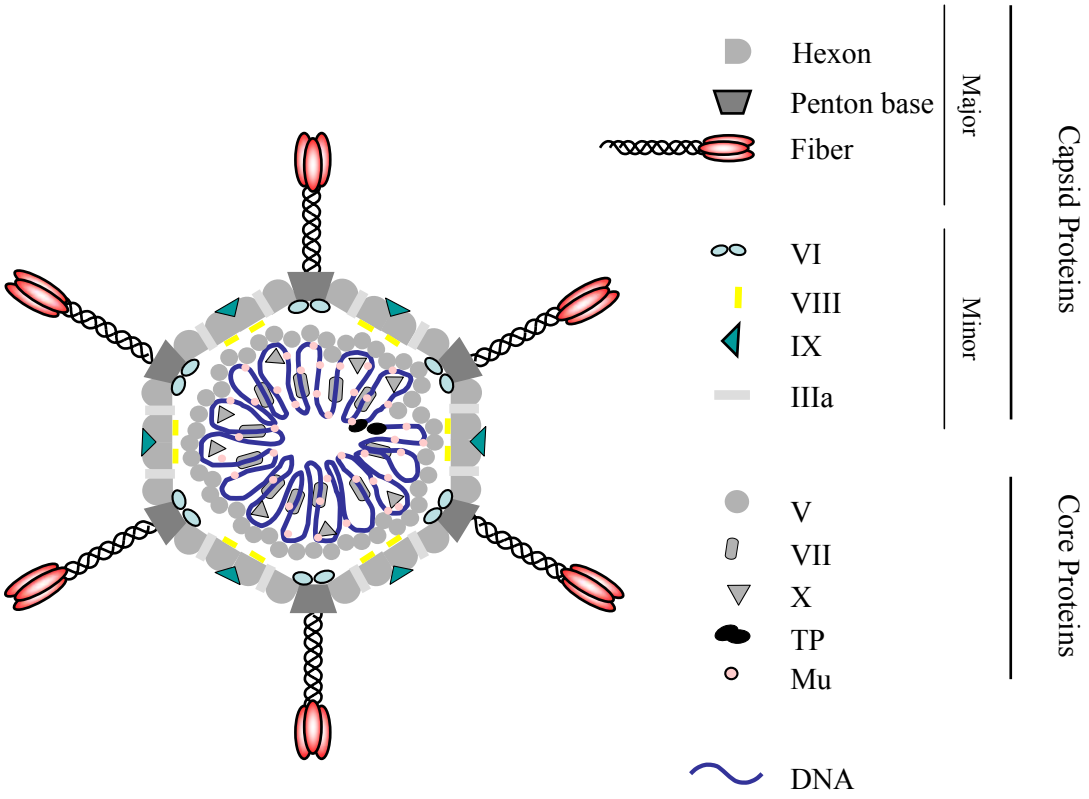

Figure 1 Structure of adenovirus and the adenovirus proteins. Adenovirus is nonenveloped icosohedral capsid containing a linear double-stranded DNA genome of approximate 36 kb for human adenovirus serotype 5 (HAdV-5). The virion is composed of multiple copies of 12 different structural proteins, including three major proteins, hexon (II), penton base (III), and fiber (IV), along with minor proteins, VI, VIII, IX, and IIIa. The linear double-stranded DNA genome is connected with the protein, a terminal protein (TP),V, VII and mu.

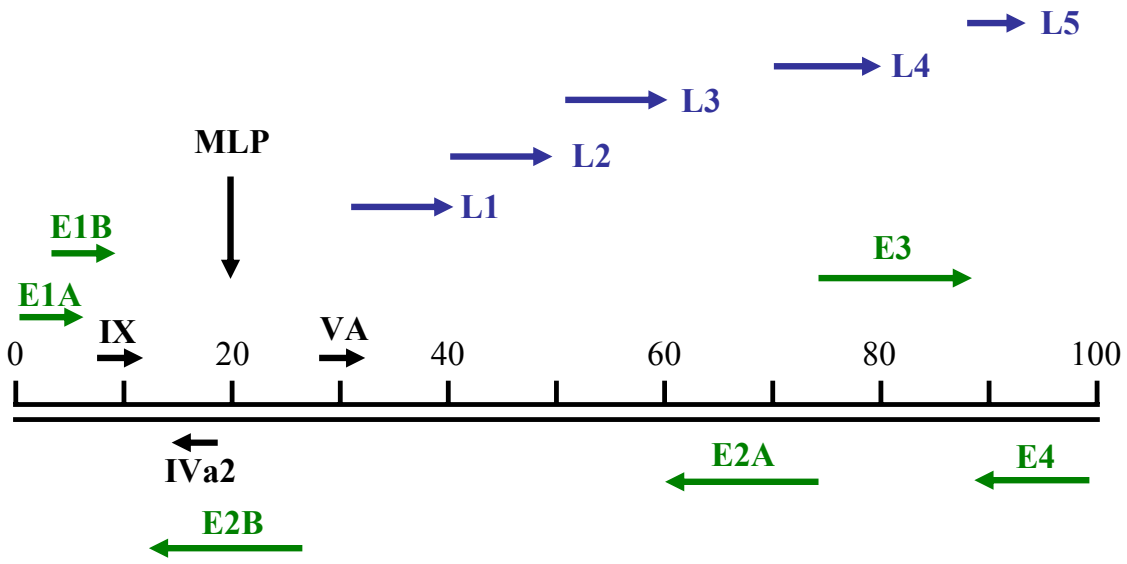

Figure 2 Transcription map of the adenovirus genome. The early transcription units are indicated in green, one late unit in blue. Arrows indicate the direction of transcription. MLP, Major late promoter. The numbers represent the map units along the adenovirus genome.

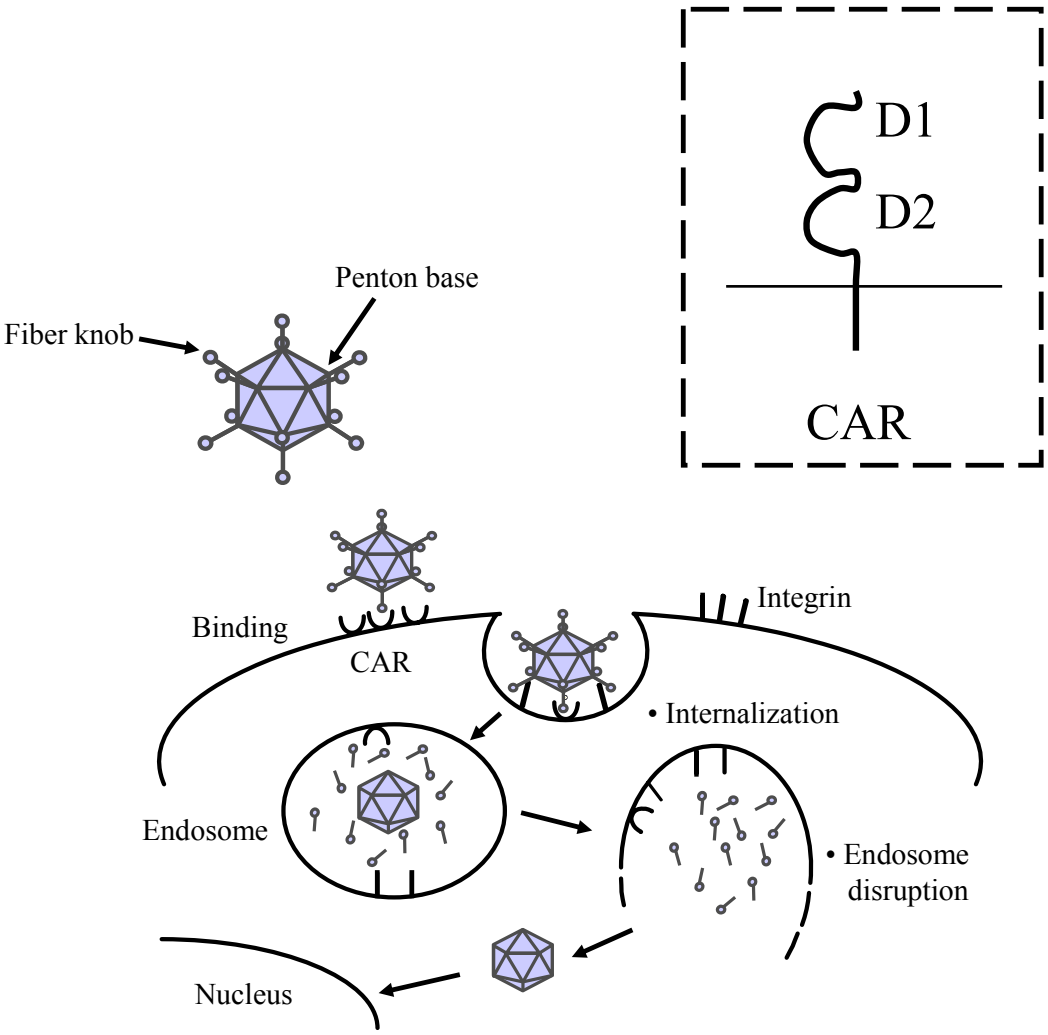

Figure 3 The pathway of Human Adenovirus serotype 5 (HAdV-5) infection. The cell entry of HAdV-5 initiate through the initial binding of the knob domain of fiber protein to the primary cellular receptor, CAR. Subsequent internalization of the HAdV-5 is triggered by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with integrins on the cell surface. After a sequential disassembly of the viral particle, the viral genome is released and imported into the nucleus.

CHAPTERII: CHIMERIC ADENOVIRAL VECTORS INCORPORATING A FIBER OF HUMAN ADENOVIRUS 3 EFFICIENTLY MEDIATE GENE TRANSFER INTO PROSTATE CANCER CELLS

by

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ABSTRACT

Background. We have developed a range of adenoviral (Ad) vectors based on human adenovirus serotype 5 (HAdV-5) displaying the fiber shaft and knob domains of species B viruses (HAdV-3, HAdV-11, or HAdV-35). These species B Ads utilize different cellular receptors than HAdV-5 for infection. We evaluated whether Ad vectors displaying species B fiber shaft and knob domains (Ad5F3Luc1, Ad5F11Luc1, and Ad5F35Luc1) would efficiently infect cancer cells of distinct origins, including prostate cancer.

Method. The fiber chimeric Ad vectors were genetically generated and compared with the original Ad vector (Ad5Luc1) for transductional efficiency in a variety of cancer cell lines, including prostate cancer cells and primary prostate epithelial cells (PrEC), using luciferase as a reporter gene.

Result. Prostate cancer cell lines infected with Ad5F3Luc1 expressed higher levels of luciferase than Ad5Luc1, as well as the other chimeric Ad vectors. We also analyzed the transductional efficiency via monitoring of luciferase activity in prostate cancer cells when expressed as a fraction of the gene transfer in PrEC cells. In the PC-3 and DU145 cell lines, the gene transfer ratio of cancer cells versus PrEC was once again highest for Ad5F3Luc1.

Conclusion. Of the investigated chimeric HAdV-5/species B vectors, Ad5F3Luc1 was judged to be the most suitable for targeting prostate cancer cells as it showed the highest transductional efficiency in these cells. It is foreseeable that an Ad vector incorporating the HAdV-3 fiber could potentially be used for prostate cancer gene therapy.

KEY WORDS

adenovirus; species B; fiber; prostate cancer; gene therapy

INTRODUCTION

Prostate cancer is one of the most common cancers in men worldwide. In the U.S., prostate cancer is not only the most frequently diagnosed cancer in men (approximately 25%), it is also one of the most common fatal cancers in men in recent years (1). During the initial stages of this disease, tumor growth depends upon androgen; and thus androgen ablation or anti-androgen treatments can be therapeutically useful (2). However, tumor growth eventually becomes androgen independent leading to decreased effectiveness of these treatments during this stage of locally advanced or metastatic disease (3). These considerations rationalize the development of new treatment strategies for prostate cancer therapy.

One of the new treatment strategies is gene therapy. The U.S. National Institutes of Health has approved a number of gene therapy clinical trials for prostate cancer (www.clinicaltrials.gov). These approaches can be divided into at least five categories, i.e. tumor-suppressor gene therapy, suicide gene therapy, immunomodulatory gene therapy, anti-oncogene therapy, and oncolytic virus therapy. Vectors based on human adenovirus serotype 5 (HAdV-5), Ad5 vectors, have been widely employed as the vehicles for these therapies as they have several advantages over other gene delivery systems. They can deliver large therapeutic genes (4, 5), up to approximately 37 kb (6). Also, these vectors can be amplified to very high titers, which is critical for *in vivo* gene delivery and clinical

applications (7). Moreover, Ad5 vectors are relatively safe as the HAdV genome does not integrate into the host genome (8, 9).

Currently, at least 51 serotypes of HAdVs have been identified, which have been grouped into 6 species, A through to F (10). Of these, HAdV-5, a serotype from species C, is mainly used as a vector for *in vitro* and *in vivo* studies of cancer gene therapy. However, clinical trials utilizing Ad5 vectors have provided disappointing results (11-13), mainly due to inefficient gene delivery to human cancer cells (14-16). One explanation for this observation is that the expression level of the coxsackie virus and adenovirus receptor (CAR), which mediates cell attachment via the fiber protein of HAdV-5 (17), is low in many cancers (14, 18). For example, CAR expression is decreased in specimens of prostate tumors at varying degrees of progression when compared with normal prostate tissue (19). Thus, new Ad vectors which can utilize receptors other than CAR should be used for prostate cancer gene therapy. Since the fiber protein mediates Ad binding to its receptor, the fiber protein of the Ad5 vector will require modification in order to achieve efficient gene delivery via a CAR-independent mechanism.

Previous studies have shown that the fibers of species A, C, D, E, and F bind to soluble recombinant CAR, but those of species B do not (20). This result shows that HAdVs from species B achieve cell entry via a CAR-independent pathway. In addition, several recent studies have identified cellular receptors for species B HAdVs, including human adenovirus 3 (HAdV-3), 11 (HAdV-11), and 35 (HAdV-35). Our group has identified CD80 and CD86, which are co-stimulatory molecules providing T cell activation (21), as receptors for HAdV-3 (22). The expression of CD80 and CD86 is, however, restricted to lymphoid cells (23, 24). The expression of CD80 and/or CD86 is

rarely observed in cancer cells (25-27), making targeting via CD80 and CD86 a particularly unsuitable strategy for cancer therapy. Recent studies have shown that HAdV-3 also binds to CD46 as a cellular receptor for cell entry (28, 29). Further, it has been demonstrated that HAdV-11 and HAdV-35 utilize CD46 as a cellular receptor (30-32). CD46 is ubiquitously expressed throughout the human body (33) but, more importantly, it is generally overexpressed on various human cancer cells when compared with their normal counterparts (34-38). In addition to CD46, a yet unidentified receptor X has been determined to act as an alternative receptor for some serotypes of species B, including HAdV-3 and HAdV-11 (39). Of note is the discovery that receptor X is highly expressed on human cancer cell lines (39). The binding to and subsequent infection in tumor cells can thus be facilitated by targeting CD46 or receptor X. During the initial cellular attachment phase of viral infection, the fiber protein plays a critical role. We therefore hypothesized that the incorporation of a species B fiber in a HAdV-5 based vector would confer CAR-independent tropism to this vector as well as increase its infectivity in CAR-deficient cancer cells.

In this study, the fiber shaft and knob domains of a HAdV-5 vector were genetically replaced with the corresponding domains from HAdV-3, HAdV-11, or HAdV-35, resulting in Ad5F3Luc1, Ad5F11Luc1, and Ad5F35Luc1 respectively. We examined the biological and physical properties of the fiber chimeric Ad vectors and evaluated the infectivity in cancer cell lines derived from different origins, with a focus on prostate cancer cell lines. The results highlight the potential utility of these chimeric Ad vectors in prostate cancer gene therapy approaches.

MATERIALS AND METHODS

Cell Lines

Chinese hamster ovary (CHO), human embryonic kidney (HEK) 293 (40), human lung epithelial A549, human breast epithelial MCF-7, human chronic myelogenous leukemia (CML) K562 cells and three human prostate epithelial cell lines, LNCaP, PC-3, and DU145 cells were all obtained from the American Type Culture Collection (ATCC; Manassas, VA) and HEK293A cells were purchased from Invitrogen (Carlsbad, CA). All cells above except K562 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, (DMEM/F12; Sigma-Aldrich; St. Louis, MO) containing 10% fetal bovine serum, (FBS; Hyclone; Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech, Inc; Herndon, VA). K562 cells were cultured in Iscove's DMEM (Mediatech, Inc; Herndon, VA) containing 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293F28 cells (41), which are derivatives of HEK293 cells that express the wild type HAdV-5 fiber, were used to upscale the fiber chimeric Ad vectors and were cultured in the same medium as HEK293 cells but supplemented with 100 μg/ml zeocin (Invitrogen; Carlsbad, CA). Human glioblastoma U118 and their derivatives U118-hCAR cells, which stably express human CAR were previously described (42). CHO-CD80 and CHO-CD86 stably expressing human CD80 and human CD86, respectively, were derivatives of CHO (22, 43). These cell lines were cultured in DMEM/F12 as above. A CHO-derived cell line, CHO-C2, which stably expresses the C2 isoform of human CD46 was previously reported (44) and was cultured in Eagle's minimum essential medium (MEM; Mediatech) containing 10% FBS, 2 mM L-glutamine, 10 mM MEM non-essential amino acids

(Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. Primary prostate epithelial cells (PrEC; Lonza, Walkersville, MD), including the ones infected with Ad vectors, were cultured using the prostate epithelial cell medium bulletkit (Lonza, Walkersville, MD). All cells were propagated at 37°C in a 5% CO_2 atmosphere. The cell lines infected with Ad vectors were maintained using the corresponding cell culture medium but containing 2% instead of 10% FBS.

Construction of Recombinant Plasmids

All recombinant plasmids were constructed by "sticky-end" polymerase chain reactions (sePCR), which generate PCR products containing cohesive ends compatible with the intended restriction sites (45). Table I summarizes the nucleotide sequences for the primers and the oligonucleotides used in this study. For the construction of the recombinant plasmid encoding the HAdV-3 fiber shaft and knob domains, the nucleotide sequence of these domains was amplified using primers SK3LF and 3KnR1 or primers SK3SF and 3KnR2 with pPIC9Ad3F (an unpublished construct) containing the HAdV-3 fiber gene as template. The resulting two PCR products were subsequently mixed, denatured, and annealed to generate a XmaI restriction enzyme overhang at the 5' end of the HAdV-3 shaft domain (shown in bold and underlined for SK3LF and underlined for SK3SF in Table I) and four overhanging nucleotides at the 3'end of the HAdV-3 knob domain (shown in bold for 3KnR2 in Table I) corresponding to the overhang of BfuAI restriction enzyme site. The HAdV-5 fiber tail DNA sequence was amplified using plasmid pKAN3.1 (46) as template with primers SacSwa5T and Xma5T.R; SacI site for SacSwa5T and XmaI site for Xma5T.R are underlined in Table I. The PCR product for

the HAdV-5 fiber tail domain was digested with SacI and XmaI. The two PCR products, i.e. the HAdV-3 fiber shaft and knob domains as well as the HAdV-5 fiber tail domain, were mixed and ligated simultaneously into the SacI and BfuA1 digested pNEB193/Swa5T/3ShaftBfu2x plasmid (an unpublished construct) containing only the nucleotide sequences for the HAdV-5 fiber tail and the HAdV-3 fiber shaft domains. This process resulted in plasmid pNEB193.5T3SK, which contained the nucleotide sequences for the HAdV-5 fiber tail, the HAdV-3 fiber shaft, as well as the HAdV-3 fiber knob domain.

For the construction of the recombinant plasmids encoding the HAdV-11 or HAdV-35 fiber shaft and knob DNA sequences, a similar cloning strategy was utilized. HAdV-11 and HAdV-35 were purchased from the ATCC to amplify DNA sequences of their respective fiber shaft and knob domains utilizing two sets of primers: SK11/35LF and 11KnR1, or SK11/35SF and 11KnR2 for the HAdV-11 shaft and knob domains as well as SK11/35LF and 35KnR1 or SK11/35SF and 35KnR2 for the HAdV-35 shaft and knob domains. The resulting two PCR products for each Ad vector were subsequently mixed, denatured, and annealed to generate a XmaI restriction enzyme overhang at the 5' end of the HAdV-11 or HAdV-35 shaft domain (shown in bold and underlined for SK11/35LF and underlined for SK11/35SF in Table I) and four overhanging nucleotides at the 3' end of the HAdV-11 or HAdV-35 knob domain required for the ligation to another fragment containing a MfeI restriction enzyme site (shown for 11KnR2 or 35KnR2 in bold in Table I). Two kinds of oligonucleotides, MfeLF and MfeSR were synthesized and annealed to generate the double strand DNA fragment (overhanging four nucleotides at 5' end to be used for the next step is shown in bold, a half site of

nucleotides to be used for the next step recognized by SwaI is underlined, and the MfeI site to be used for cloning into the plasmid pKAN3.1, is also underlined in Table I). In addition, the HAdV-5 fiber tail DNA sequence was amplified using pKAN3.1 as template with primers SacCla5T and Xma5T.R, introducing a SacI site and a XmaI site (underlined for SacCla5T and Xma5T.R in Table I). The resulting PCR product for the HAdV-5 fiber tail domain was digested with the restriction enzymes SacI and XmaI. The PCR products for the shaft and knob domains, the DNA fragment containing the MfeI site, as well as the SacI and XmaI digested HAdV-5 fiber tail fragment were mixed and ligated simultaneously into the SacI and SwaI digested pNEB193/Cla5T/3ShaftBfu2x plasmid (an unpublished construct) containing the nucleotide sequences for the HAdV-5 tail and the HAdV-3 shaft. This process resulted in two plasmids, pNEB193.5T11SK and pNEB193.5T35SK. All three resulting plasmids (pNEB193.5T3SK, pNEB193.5T11SK, and pNEB193.5T35SK) were digested with AgeI and MfeI before ligation into the pKAN3.1 plasmid which was digested with the same enzymes. This additional cloning step was required for the subsequent homologous recombination step with a plasmid DNA carrying the HAdV-5 genome. The new plasmids were named pKAN5T3SK, pKAN5T11SK, and pKAN5T35SK. The nucleotide sequences of the DNA fragments cloned into all plasmids were confirmed by the UAB DNA sequencing core facility.

TABLE I. Primers used for cloning the recombinant plasmid DNA and PCR analysis

Name of oligonucleotides	Direction	Sequence (5' to 3')	Position
SK3LF	Forward	5'-**ccGG**GGTaTTAAGTCTTAAATGT-3'	31,497 – 31,517[a,b]
3KnR1	Reverse	5'-ttcttcaGTCATCTTCTCTAATATAG-3'	32,324 – 32,306[a]
SK3SF	Forward	5'-GGTaTTAAGTCTTAAATGTGTTAATCC-3'	31,499 – 31,525[a,b]
3KnR2	Reverse	5'-**acga**ttcttcaGTCATCTTCTCTAATATAG -3'	32,324 – 32,306[a]
SacSwa5T	Forward	5'-taagagctcatttaAATGTCAGTTTCCTC-3'	30,948 – 30,998[c]
Xma5T.R	Reverse	5'-gtaCCCCgGGtGGACTCTCTT-3'	31,177 – 31,160[c, b]
SK11/35LF[f]	Forward	5'-**ccgg**ggtaCTTACTTTAAAATGT-3'	30,946 – 30,960[d] 30, 962 – 30,976[e]
11KnR1	Reverse	5'-ttctTCAGTCGTCTTCTCTGATGTAG-3'	31,788 – 31,767[d]
SK11/35SF[f]	Forward	5'-ggtaCTTACTTTAAAATGTTTAACCC-3'	30,946 – 30,967[d] 30, 962 – 30,983[e]
11KnR2	Reverse	5'-**acga**ttctTCAGTCGTCTTCTCTGATGTAG-3'	31,788 – 31,767[d]
35KnR1	Reverse	5'-ttctTTAGTTGTCGTCTTCTGTAATG-3	31,798 – 31,777[e]
35KnR2	Reverse	5'-**acga**ttctTTAGTTGTCGTCTTCTGTAATG-3'	31,798 – 31,777[e]
MfeLF	Sense	5'-**TCGT**TTGTGTTATGTTTCAACGTGTTTATTTTTCAATTG*attt*-3'	32, 792 – 32,830[c]
MfeSR	Anti-sense	5'-*aaat*CAATTGAAAAATAAACACGTTGAAACATAACACAA-3'	32,830 – 32, 796[c]
SacCla5T	Forward	5'-taagagctcatcgatTGTCAGTTTCCTC-3'	30,946 – 30,998[c]
Fiber –100	Forward	5'-CCTCCTGGCTGCAAACTTTCTC-3'	30,948 – 30,969[c]
Fiber +100	Reverse	5'-GAGGTGGCAGGTTGAATACTAG-3'	32,945 – 32,924[c]

[a] Position 1 refers to the beginning of the right arm of the HAdV-3 genome (accession No. DQ086466).
[b] Point mutation(s) are in the nucleotide sequence corresponding to the HAdV genome
[c] Position 1 refers to the beginning of the right arm of the HAdV-5 genome (accession No. AC_000008).
[d] Position 1 refers to the beginning of the right arm of the HAdV-11 genome (accession No. AC_000015).
[e] Position 1 refers to the beginning of the right arm of the HAdV-35 genome (accession No. AC_000019).
[f] These primers anneal to the fiber genes for both HAdV-11 and HAdV-35.
Overhang is shown in bold.
Restriction enzyme sites that were used for cloning are underlined.

Construction of the Adenoviral Plasmids Encoding a Fiber Chimeric Gene

The constructed plasmids (pKAN5T3SK, pKAN5T11SK, and pKAN5T35SK) were used for homologous recombination with the *Swa*I-linearized pVK700 genomic plasmid. Plasmid pVK700 includes the HAdV-5 genome containing a firefly luciferase reporter gene controlled by the cytomegalovirus (CMV) promoter in the deleted early region 1 (E1) region (47). The homologous recombination resulted in pMM703, pMM711, and pMM735 carrying chimeric fiber genes of HAdV-3, HAdV-11 and HAdV-35, respectively.

Adenovirus Generation, Propagation, Purification, and Titration

The recombinant plasmids pMM703, pMM711, and pMM735 were linearized by *Pac*I and transfected into HEK293 cells using UniFECTOR transfection reagent (B-Bridge International Inc., Sunnyvale, CA) in order to generate Ad vectors, Ad5F3Luc1, Ad5F11Luc1, and Ad5F35Luc1. The cells were harvested in two to three weeks when cytopathic effect (CPE) was observed and disrupted by three freeze/thaw cycles. The cell lysates were centrifuged at 3,500 × g for 10 min at 4°C and used for reinfection of HEK293F28 cells. Subsequent to this, all Ad vectors were propagated by the two-step procedure developed by Von Seggern *et al* (48). In brief, HEK293F28 cells were infected with the crude viral lysates to upscale mosaic Ad vectors incorporating wild type Ad5 fiber and fiber from species B HAdVs (41).

HEK293 cells were then infected with cell lysates containing mosaic Ad vectors producing the vectors incorporating only one type of fiber. The resulting Ad vectors were Ad5F3Luc1 incorporating the HAdV-5 fiber tail domain and the HAdV-3 fiber shaft and

knob domains, Ad5F11Luc1 incorporating the HAdV-5 fiber tail domain and the HAdV-11 fiber shaft and knob domains, and Ad5F35Luc1 incorporating the HAdV-5 fiber tail domain and the HAdV-35 fiber shaft and knob domains (Table II). A control Ad vector, Ad5Luc1 is a replication-defective E1-deleted HAdV-5 based vector containing a firefly luciferase reporter gene driven by a CMV promoter (49). All vectors produced in HEK293 cells were purified by two rounds of CsCl gradient ultracentrifugation (50). CsCl was removed by dialysis using 10 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 10% glycerol (dialysis buffer). The Ad vectors were stored at –80°C prior to the next experiments. The infectious titer (plaque forming units [PFU]/ml) of the purified Ad vectors were determined by triplicate 50% Tissue Culture Infective Dose ($TCID_{50}$) assays using HEK293A cells, as previously described (50). The physical titer (viral particles [VP]/ml) were determined by Maizel's method with a conversion factor of 1.1×10^{12} VP/ml per absorbance unit at 260 nm (51).

Polymerase Chain Reaction (PCR) Analysis of the Fiber Region

To confirm the presence of chimeric fiber genes in the Ad genomes, 10^7 VP of purified Ads were boiled at 95ºC for 5 min and analyzed by PCR reactions using the TaqPCR Master Kit (Qiagen Inc., Valencia, CA). The sequences for primers and oligonucleotides used in this study are summarized in Table I. Forward (Fiber –100) and reverse (Fiber +100) primers were used to amplify the region containing the full length of the nucleotide sequences for all four types of fibers. Forward (SK3LF) and reverse (3KnR1) primers were used to amplify the region containing the HAdV-3 fiber shaft and knob nucleotide sequence. Forward (SK11/35LF) and reverse (11KnR1) primers were

used to amplify the region containing the HAdV-11 fiber shaft and knob nucleotide sequence. Forward (SK11/35LF) and reverse (35KnR1) primers were used to amplify the region containing the HAdV-35 fiber shaft and knob nucleotide sequence (Table I). The following PCR conditions were applied: 1 min denaturation at 96ºC, 1 min annealing at 60ºC, and 3 min extension at 72ºC to amplify the region of the whole fiber gene, or 1 min extension at 72ºC, to amplify the nucleotide sequence of the shaft and knob domains.

Analysis of Adenoviral Proteins by GELCODE Blue Staining and Western Blotting

Aliquots of purified Ad vectors equal to 10^{10} VP were denatured by boiling in Laemmli sample buffer (Bio-Rad laboratories Inc., Hercules, CA) at 95ºC for 10 min. The proteins were separated by electrophoresis in sodium dodecyl sulfate 12% polyacrylamide gel (SDS-PAGE) and stained with GELCODE Blue Stain Reagent according to the manufacturer's instructions (Pierce, Rockford, IL).

For western blot analysis of the fiber proteins, aliquots of purified Ad vectors equal to 5×10^9 VP were denatured, and the viral proteins were separated by the same method described above. The separated viral proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and detected with a mouse monoclonal anti-HAdV-5 fiber tail domain antibody (4D2; Lab Vision Corp., Fremont, CA), followed by a horseradish peroxidase-conjugated secondary anti-mouse antibody (Dako North America, Inc. Carpinteria, CA). The blot was developed using an ECL Plus Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ). Pre-stained protein ladder of Kaleidoscope Standards (Bio-Rad Laboratories, Inc.) was used in the stained gel and the western blot.

Virus Thermostability Assay

Thermostability of Ad vectors was examined by the method described by Ugai *et al.* (52, 53). For each purified Ad, 10^{10} VP were incubated for three and seven days in the dialysis buffer at –80ºC, 4ºC, and 37ºC. The resulting infectious titers of Ad vectors were determined by $TCID_{50}$ using HEK293A cells.

One-step Growth Curve Analysis of Adenoviral Vectors

HEK293 cells, grown to 80% confluence in 6-cm dishes, were infected with Ad vectors at a multiplicity of infection (MOI) of 10 PFU/cell. The infected cells were maintained in 3 ml of medium containing 2% FBS. The culture medium and infected cells were scraped and collected in a 15-ml tube at various times (1, 12, 24, and 48 hours) post-infection. The cell suspension was centrifuged at 1,000 × g for 5 min at 4°C, and the cell pellet was resuspended in 2 ml of medium. The cells in the suspension were disrupted by three freeze/thaw cycles. The lysates were centrifuged at 3,500 × g for 10 min at 4°C and the supernatants were used for the subsequent infection.

Gene Transfer Assay

U118, U118-hCAR, HEK293, A549, MCF-7, DU145, LNCaP, PC-3, and PrEC cells grown in 24-well plates were infected with Ad vectors at an MOI of 10 PFU/cell. For blocking experiments, 0.2 ml of FBS-free medium containing the recombinant HAdV-5 knob protein (54) at a final concentration of either 0.5 or 5 µg/ml was added to the wells. No blocking agent was added to the control wells. Cells were incubated with the recombinant HAdV-5 knob protein at 4°C for one hour, subsequently Ad vectors at

an MOI of 10 PFU/cell were added to the cells in 0.2 ml of medium containing 4% FBS. After incubation at 37ºC for 24 hours, infected cells were washed with phosphate buffered saline (PBS; pH 7.4) and lysed in passive lysis buffer (Promega, Madison, WI). The cell lysates' luciferase activities were measured according to the manufacturer's protocol. Each data point was measured in triplicate and calculated as the mean of these three determinations.

To evaluate gene transfer efficiency of the Ad vectors at various incubation times (0, 12, 24, and 48 hours) at 37ºC, the cells were infected with Ad vectors at an MOI of 10 PFU/cell in 0.4 ml of medium containing 2% FBS. After various incubation times the cell lysates were prepared, and the luciferase activities were measured as described above. Each data point was measured in triplicate and calculated as the mean of these three determinations.

The protein concentration was determined using the D_C protein assay kit (Bio-Rad laboratories Inc., Hercules, CA) according to the manufacturer's instructions, and the luciferase activity was normalized for protein concentration.

Flow Cytometry

Adherent cells (CHO, CHO-C2, CHO-CD80, CHO-CD86, U118, U118-hCAR, HEK293, A549, MCF-7, LNCaP, PC-3, and DU145) were detached from a 75-cm^2 tissue culture flask by treatment with Versene solution (0.53 mM EDTA in PBS [pH 7.4], UAB Media Preparation Shared Facility, Birmingham, AL) and washed with SM buffer (PBS [pH 7.4] containing 0.1% bovine serum albumin [BSA] and 0.01% sodium azide, stored at 4ºC). K562 cells grown in suspension were harvested by centrifugation and washed

with SM buffer. Subsequent to washing, aliquots of 5×10^5 cells were incubated for 1 hour at 4ºC with either the anti-CD46 MAb J4-48 (Beckman Coulter, Fullerton, CA), the anti-CD80 MAb L307.4 (BD Pharmingen, San Jose, CA), the anti-CD86 MAb 2331 (BD Pharmingen), the anti-CAR MAb RmcB (a kind gift from Dr. Douglas (42)), or the anti-αv integrin antibody L230 (Axxora, San Diego, CA). Following this incubation, the cells were washed three times with SM buffer and incubated with Alexa fluor 488-conjugated goat anti-mouse immunoglobulin G (Invitrogen, Carlsbad, CA). After two additional washing steps, the cells were fixed with 0.5% paraformaldehyde in PBS (pH 7.4) and analyzed with a FACScan (Becton Dickinson, Mountain Vies, CA) at the UAB Cell Sorting Facility.

Determination of the Gene Transfer Ratio in Cancer versus PrEC Cells

As a means of showing transductional selectivity of the vectors for prostate tumor cells versus normal prostate, we divided the luciferase activity values in prostate cancer cells (LNCaP, PC-3, and DU145) with each vector's luciferase activity in PrEC cells at 12 hours post-infection. Each vector's gene expression ratios were normalized to the control vector by dividing them by the ratio of Ad5Luc1 in cancer cell lines compared with PrEC cells. This results in a value of 1 for the control Ad5Luc1 vector. Values greater than 1 indicate transductional selectivity for prostate cancer cells compared to Ad5Luc1.

Statistical Analysis

Statistical analysis was performed with two-tailed unpaired *t*-tests among groups. P values <0.05 were considered statistically significant.

RESULTS

Generation of Fiber Chimeric Adenoviral Vectors

Plasmids pKAN5T3SK, pKAN5T11SK, and pKAN5T35SK encoding chimeric fibers were constructed by replacing the nucleotide sequence of the HAdV-5 fiber shaft and knob domains with the corresponding sequences from HAdV-3, HAdV-11, or HAdV-35 as described in the Materials and Methods section. Plasmids containing the recombinant Ad genomes with the chimeric fiber genes were subsequently derived by homologous recombination between the above plasmids and pVK700 (47). The Ad vectors were generated in HEK293 cells, upscaled in HEK293F28 cells (41), and finally amplified in HEK293 cells to yield Ad vectors displaying only one type of fiber. Figure 1A shows a schematic representation of the fiber chimeric Ad vectors generated in this study. The composition of the fiber domain of the fiber chimeric Ad vectors is summarized in Table II.

TABLE II. Composition of the fiber domain of chimeric Ad vectors

Name of an Ad vector	Fiber		
	Tail	Shaft	Knob
Ad5Luc1	**5**	**5**	**5**
Ad5F3Luc1	**5**	**3**	**3**
Ad5F11Luc1	**5**	**11**	**11**
Ad5F35Luc1	**5**	**35**	**35**

All numbers refer to serotype of human adenovirus

To confirm the replacement of the chimeric fiber gene in each vector, PCR analyses were done with the purified Ad vectors. The PCR analyses were performed with HAdV-5-specific primer sets, which anneal at 100 base pairs (bp) upstream and downstream of the HAdV-5 fiber gene, as well as serotype-specific primers for the fiber shaft and knob domains. Using the HAdV-5 specific primers, the PCR amplification produced a 1990 bp long HAdV-5 fiber product and approximately 1200 bp short fiber products for species B fiber (**Fig.1B**, Upper left panel). The PCR analysis, using serotype-specific primers demonstrated the amplification of the corresponding fiber region in each of the fiber chimeric Ad vector (**Fig. 1B**, upper right panel for Ad5F3Luc1, **Fig. 1B**, lower left panel for Ad5F11Luc1, and **Fig. 1B**, lower right panel for Ad5F35Luc1).

To confirm that the viral structural proteins were not affected by the exchange of the fiber shaft-knob domains, the protein composition of the purified Ad5Luc1 and the chimeric Ad vectors was analyzed by protein staining analysis. The capsid proteins were separated by SDS-PAGE and stained with GELCODE blue. The fiber chimeric Ad vectors contained the Ad structural proteins in proportions similar to Ad5Luc1 (**Fig. 1C**).

It was also confirmed by western blot analysis with anti-HAdV-5 fiber tail domain 4D2 MAb that the viral particles incorporated the fiber shaft and knob domains from HAdV-3, HAdV-11, or HAdV-35 (**Fig. 1D**). The western blot analysis indicated that the molecular mass of the fiber monomer of Ad5F3Luc1, Ad5F11Luc1, and Ad5F35Luc1 is approximately 35-37 kDa, corresponding to the mass predicted by the amino acid sequences. These results clearly demonstrated the incorporation of the species B fiber proteins into the Ad5Luc1 viral particles. In addition, wild type HAdV-5 fiber,

expressed by HEK293F28 cells which were used for virus propagation, was not detected by western blot analysis of the purified viral particles (**Fig. 1D**).

Thermostability of Fiber Chimeric Adenoviral Vectors

To determine if the thermostability of the resulting fiber chimeric Ad vectors was affected, a thermostability assay was performed (52, 53). Equal numbers of Ad vectors (10^{10} VP) containing either HAdV-5 or chimeric fibers were incubated at various temperatures (–80°C, 4°C, and 37°C) for 3 or 7 days followed by a $TCID_{50}$ assay to analyze the remaining infectivity. All the Ad vectors demonstrated similar infectivity profiles subsequent to various time and temperature incubations, with all the Ad vectors displaying a decrease in infectivity after 7 days incubation at 37°C (**Fig. 2**). This data indicated that replacement of the fiber shaft-knob domain from species B HAdVs into the HAdV-5 viral particle does not influence the stability of the viral particles.

Analysis of Viral Replication

Since the fiber of Ad5Luc1 was replaced with species B fibers, we sought to determine whether this change affected the replication of the chimeric Ad vectors. A one-step growth curve analysis was performed in HEK293 cells using the lysate harvested and prepared from the infection of each Ad vector at an MOI of 10 PFU/cell in HEK293 cells. The kinetics of viral replication of each chimeric Ad vector were similar to that of Ad5Luc1 during 12 to 24 hours (**Fig. 3**). Therefore, replacement of the fiber shaft and knob domains did not affect viral replication.

The Fiber of the Chimeric Adenoviral Vectors Dictates CAR-independent Tropism

As shown previously, the luciferase activity of Ad5Luc1-infected U118-hCAR cells, a cell line that stably overexpresses CAR (42), was 100-fold higher than that of CAR-negative U118 cells (**Fig. 4A**). These data indicate that gene transfer of Ad5Luc1 is dependent on the expression level of CAR. To verify whether the fiber chimeric Ad vectors achieve CAR-independent gene transfer, a well-established gene transfer experiment with HAdV-5 knob-blocking was performed (14, 54, 55). Figure 4B demonstrates that the recombinant HAdV-5 knob protein inhibits transduction of Ad5Luc1, but not that of the chimeric Ad vectors. When blocking with the recombinant HAdV-5 knob protein at a final concentration of 0.5 μg/ml, the luciferase activity of Ad5Luc1-infected cells, but not that of cells infected by fiber chimeric Ad vectors, was lowered by more than 50% when compared to the activity in the absence of the blocking protein (**Fig. 4B**). Likewise, a final concentration of 5 μg/ml achieved an approximate 86% reduction in the luciferase activity of the cells infected with Ad5Luc1 when compared to unblocked controls. Some reduction in luciferase activity was observed for the fiber chimeric Ad vectors during the blocking experiment (**Fig. 4B**), but it was much lower compare to Ad5Luc1. These findings confirm that the fiber chimeric Ad vectors achieve cell entry via a CAR-independent route.

Expression Analysis of Adenoviral Receptors in Cancer Cell lines

Previous studies have identified CD46 as a primary receptor for human adenovirus species B (28-32). Flow cytometry was performed to determine the expression levels of CD46 on the following cell lines: HEK293, A549, K562, MCF-7,

DU145, LNCaP, and PC-3; with CHO-C2 cells utilized as a positive control (data not shown) for CD46 expression (44). All cancer cell lines highly expressed CD46 (**Fig. 5**). In addition to the CD46 expression levels, the expression levels of CAR and αv-integrins, which are required for the HAdV-5 infection were analyzed (56). For this analysis, U118-hCAR cells (42) were used as a positive control for CAR expression and HEK293 cells were used as a positive control for the expression of αv-integrins (57). The cell lines A549, HEK293, DU145, and LNCaP expressed very high levels of CAR and the cell line K562 expressed medium levels of CAR (**Fig. 5**). MCF-7 and PC-3 cells, however, expressed very low levels of CAR (**Fig. 5**). Expression of αv-integrins was high in all cancer cell lines except K562, which expressed only moderate levels (**Fig. 5**). The expression levels of CD80 and CD86 were also determined, as it has been shown that CD80 and CD80 are the receptors for HAdV-3 (22). The flow cytometry data showed that K562 cells expressed medium levels of CD80 (41% of cells were positive), and A549 cells expressed very little CD80 (3 % of cells were positive). All other cell lines were CD80 negative (less than 1 % of cells were positive). All cell lines were CD86 negative (less than 1 %of cells were positive) except for K562, which expressed very low levels (1.7% of cells were positive) (data not shown).

Gene Delivery by the Fiber Chimeric Adenoviral Vectors in Cancer Cell Lines

The level of luciferase gene expression in the chimeric Ad vectors-infected cells was compared with that of Ad5Luc1-infected cells in the same cell lines used for receptor analysis by flow cytometry (**Fig. 6**). In HEK293 cells, which are positive for both CAR and CD46, all Ad vectors, including Ad5Luc1, provided the same expression pattern of luciferase. The levels of luciferase in all CD46-positive cells that had low to medium

levels of CAR (K562, MCF-7 and PC-3), were higher for cells infected with the fiber chimeric Ad vectors compared to Ad5Luc1 (**Fig. 6**). Except for HEK293 cells, the expression levels obtained with Ad5F3Luc1 were higher than those obtained with the other vectors in CAR- and CD46-positive cells (A549, LNCaP, and DU145). The expression levels obtained with Ad5F11Luc1, AdF35Luc1, and Ad5Luc1, however, were equally low in these same cells as compared to Ad5F3Luc1. Of note, the Ad5F3Luc1 vector provided higher gene delivery in prostate cancer cell lines than did Ad5Luc1 or the other chimeric Ad vectors. Further, the luciferase activities in all cells reached a plateau by 12 hours post-infection.

Transduction Efficiency of the Fiber Chimeric Adenoviral Vectors in Normal Human Prostate Cells (PrEC)

Ad5F3Luc1 demonstrated a higher transduction efficiency in prostate cancer cell lines when compared with the other Ad vectors. We then sought to determine the transduction efficiency of these Ad vectors in primary prostate epithelial cells (PrEC). To this end, we initially determined the expression of CD46, CAR, and αv-integrins on these cells. As shown in Fig. 7A, PrEC cells express high levels of CD46, CAR and αv-integrins. At 12 hours post-infection, Ad5F3Luc1 achieved 14 to 70-fold higher transduction than the other fiber chimeric Ad vectors or Ad5Luc1 in PrEC cells. Luciferase activity reached a plateau at 12 hours post-infection, similar to what was observed in the cancer cell lines (**Figs. 6 and 7B**).

The viral infectivity was further evaluated by comparing the ratio of the luciferase activity in prostate cancer cells to that in PrEC cells. Figure 7C shows the ratios of

luciferase activity in prostate cancer cell lines versus PrEC cells at 12 hours post-infection relative to the luciferase activity of Ad5Luc1, with increased values being indicative of greater cancer cell gene transductional specificity. The chimeric vectors other than Ad5F3Luc1 had significantly lower specificity for LNCaP cells (CD46+/CAR+) compared to Ad5Luc1 ($p = 0.0045$ and $p = 0.0042$ respectively), and the ratios of Ad5F3Luc1 and Ad5Luc1 were not significantly different ($p = 0.1172$) for LNCaP cells. Ad5F3Luc1 demonstrated the highest specificity in PC-3 (CD46+/CAR–) and DU145 (CD46+/CAR+) cell lines, with respective luciferase activity ratios of approximately 6 and 13 fold higher than that of Ad5Luc1 ($p = 0.0142$ and $p = 0.0168$ respectively). Ad5F11Luc1 and Ad5F35Luc1 also showed significantly higher specificity in CD46+/CAR- PC-3 cell lines with respective luciferase activity ratios of approximately 3.5 and 2, ($p = 0.0057$ and $p = 0.0274$ respectively) but no specificity in DU145 cells (CD46+/CAR+) compared to Ad5Luc1. These results clearly indicate that Ad5F3Luc1 has the most favorable prostate tumor-selective tropism.

DISCUSSION

This study clearly demonstrates that chimeric Ad vectors incorporating a fiber from species B (HAdV-3, -11, or -35) have distinct infectivity in different types of cancer cells. In particular, Ad5F3Luc1, which incorporates the HAdV-3 fiber shaft and knob domains, seems to be a very suitable vector for prostate cancer. These conclusions are based on the observed biological and physical properties of the fiber chimeric Ad vectors, as well as their infectivity in cancer cell lines derived from different tumors. In fact, many groups have replaced the fiber shaft and knob domains or only the fiber knob

domain of HAdV-5 with that of other human Ads, including those of species B (54, 58-60) and non-human Ads (14, 55), in order to develop HAdV-5 based vectors that achieve CAR-independent infectivity. However, the effect on viral particle structure by the replacement of the fiber shaft and knob domains of HAdV-5 with the species B fiber shaft and knob domains has not been thoroughly evaluated. We first investigated whether the replacement of the HAdV-5 fiber would negatively impact the Ad biological and physical properties. It was found that all the fiber chimeric Ad vectors had the same protein composition as Ad5Luc1 (**Fig. 1C**), and appeared to be equally thermostable (**Fig. 2**). The fiber chimeric Ad vectors had kinetics of viral replication similar to that of Ad5Luc1 from 12 to 24 hours, although the replacement of the HAdV-5 fiber with the chimeric fiber of HAdV-3 or HAdV-11 appeared to reduce the total production of adenovirus at 48 hours post-infection in 293 cells (**Fig. 3**). Altogether, our results indicate that the incorporation of species B fiber shaft and knob domains into the Ad5 vector has no significant effects on the viral particles, their thermostability or infectious dynamics.

We have demonstrated that the chimeric Ad vectors incorporating species B fiber shaft and knob domains achieve cell entry without utilizing CAR (**Fig. 4B**). Moreover, we analyzed the transductional efficiency of the fiber chimeric Ad vectors in various prostate cancer cell lines with known CAR, CD46 and integrin expression levels (**Fig. 5**). In this regard, the results of our receptor expression analyses were similar to the findings previously reported by Sandberg *et al.* (27). Our group previously identified CD80 and CD86 as receptors for HAdV-3 attachment; however, neither of these two receptor types were expressed on the prostate cancer cell lines we examined (data not shown). Despite these findings, we found that Ad5F3Luc1 had significantly increased transductional

efficiency in prostate cancer cell lines compared to the other Ad vectors (**Fig. 6**). Therefore, the transduction of Ad5F3Luc1 into the prostate cancer cells was mediated via receptors other than CD80 and CD86.

It has also been shown that HAdV-3 can utilize CD46 as a cellular attachment receptor (28, 29), which is overexpressed in cancer cells (34-38). However, the binding affinity of HAdV-3 for CD46 is lower than that of HAdV-11 and HAdV-35 (28, 31, 39). In addition to the findings regarding HAdV-3 receptor binding, Tuve *et al.* concluded that HAdV-3 utilized an as yet unknown receptor called receptor X (39). These results indicate that HAdV-3 may utilize a broad range of receptors to achieve the high transduction observed.

It would be advantageous to develop a vector with a high infectivity of prostate cancer cells, but a low infectivity of normal cells. We therefore examined the transductional efficiency of species B fiber chimeric Ad vectors in primary normal prostate epithelial cells (PrEC). PrEC cells express CD46, CAR, and αv-integrins, as determined by flow cytometry (**Fig. 7A**). Once again, Ad5F3Luc1 had the highest transductional efficiency in PrEC when compared to the other vectors (**Fig. 7B**). However, as stated before, the transductional efficiency of Ad5F3Luc1 was also the highest in all prostate cancer cell lines (**Fig. 6**). To gauge the relative transductional efficiency in prostate cancer versus normal prostate cells, we calculated the ratio of the gene transfer obtained in prostate cancer cells to the gene transfer obtained in PrEC cells. In the PC-3 and DU145 cell lines, the gene transfer ratio of prostate cancer versus normal prostate cells for Ad5F3Luc1 was the greatest among the tested vectors (**Fig. 7C**). The result indicates that, of the investigated species B chimeric Ad vectors, Ad5F3Luc1 is the

best vector to target prostate cancer cells. Of note, both PC-3 and DU145 cell lines are androgen-insensitive and can be considered as examples of more progressed cancer than LNCaP, which is androgen-sensitive (61). Since androgen-insensitive prostate cancer is especially in need of alternative treatments than the currently available methods, the development of Ad5F3Luc1-based vectors is more clinically relevant.

Using prostate samples from patients, Loberg *et al.* demonstrated that the expression level of CD46 was not significantly different among normal prostate tissue, prostatic intraepithelial neoplasia, localized prostate cancer or metastatic prostate cancer specimens (62). The replacement of the HAdV-5 fiber with that of HAdV-11 and HAdV-35 may not be a good strategy to target only prostate tumor for clinical application as CD46 is a high affinity cellular receptor for HAdV-11 and HAdV-35.

In summary, replacement of the fiber shaft and knob domains of HAdV-5 did not affect viral growth properties, and the transduction efficiency of these fiber chimeric Ad vectors dramatically increased in CAR-negative cancer cell lines. Furthermore, Ad5F3Luc1 achieved the highest level of gene transfer in prostate cancer cells. Of note, Ad5F3Luc1 achieved the greatest ratio of gene transfer in progressed prostate cancer to normal prostate cells. Also, a future extension of Ad5F3Luc1 vector usage for clinical application could be to generate a conditionally replicative adenoviral vector (CRAd) containing the HAdV-3 fiber shaft-knob. Such a CRAd would more efficiently target and kill prostate cancer cells compared to normal prostate cells, thus contributing to the development of new treatments for prostate cancer.

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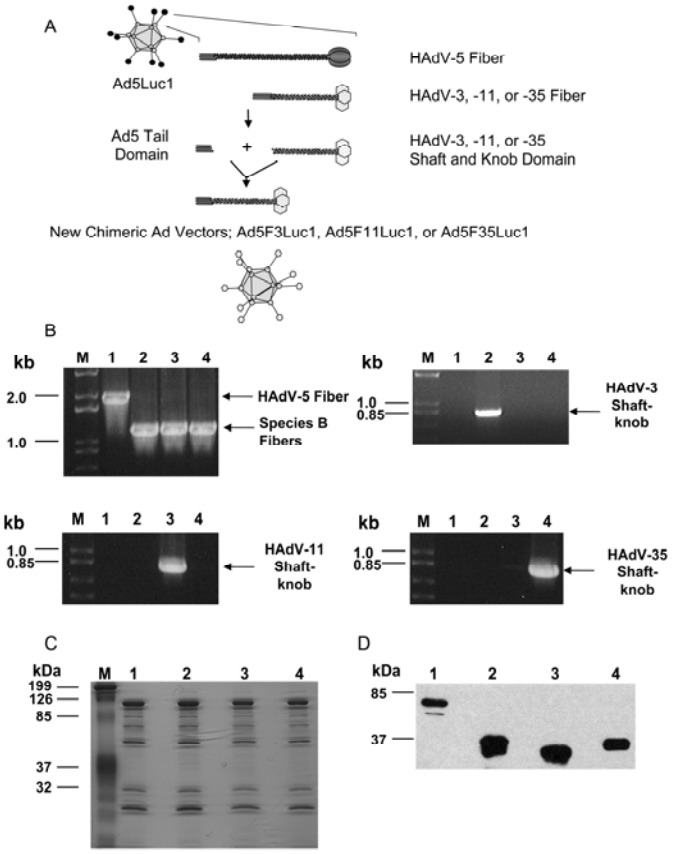

Figure 1 Characterization of chimeric Ad vectors. **A:** Schematic representation of a chimeric fiber of HAdV-3, HAdV-11, and HAdV-35 with HAdV-5 tail domain. **B:** Validation of the replacement of an HAdV-5 fiber gene with that of either HAdV-3, HAdV-11, or HAdV-35 in the HAdV-5 genome by PCR; all panels: M: DNA size markers; Lane 1: PCR using DNA from purified Ad5Luc1 as a template; Lane 2: PCR using DNA from purified Ad5F3Luc1 as a template; Lane 3: PCR using DNA from purified Ad5F11Luc1 as a template; Lane 4: PCR using DNA from purified Ad5F35Luc1 as a template. Upper left panel: PCR with pan fiber-specific primers; upper right panel: PCR with HAdV-3 fiber shaft knob-specific primers; lower left panel: PCR with HAdV-11 fiber shaft knob specific-primers; and lower right panel: PCR with HAdV-35 fiber shaft knob-specific primers. **C:** Analysis of protein composition of viral particles by GelCode blue staining of a 12% SDS-PAGE gel. A total of 10^{10} VP of purified Ad5Luc1 and chimeric Ad vectors was loaded per lane. M: protein molecular mass markers in kilodalton (kDa); Lane1: Ad5Luc1; Lane2: Ad5F3Luc1; Lane3: Ad5F11Luc1; Lane 4: Ad5F35Luc1. **D:** Detection of fiber proteins incorporated into purified viral particles by Western blot with an antibody against HAdV-5 fiber tail. A total of 5×10^9 VP of the purified Ad vectors were run on a 12% SDS-PAGE; separated viral proteins were transferred to a PVDF membrane and probed by an antibody against the HAdV-5 fiber tail (4D2). Lane1: Ad5Luc1; Lane2: Ad5F3Luc1; Lane3: Ad5F11Luc1; Lane 4: Ad5F35Luc1; protein molecular mass markers (in kDa) are indicated on the left.

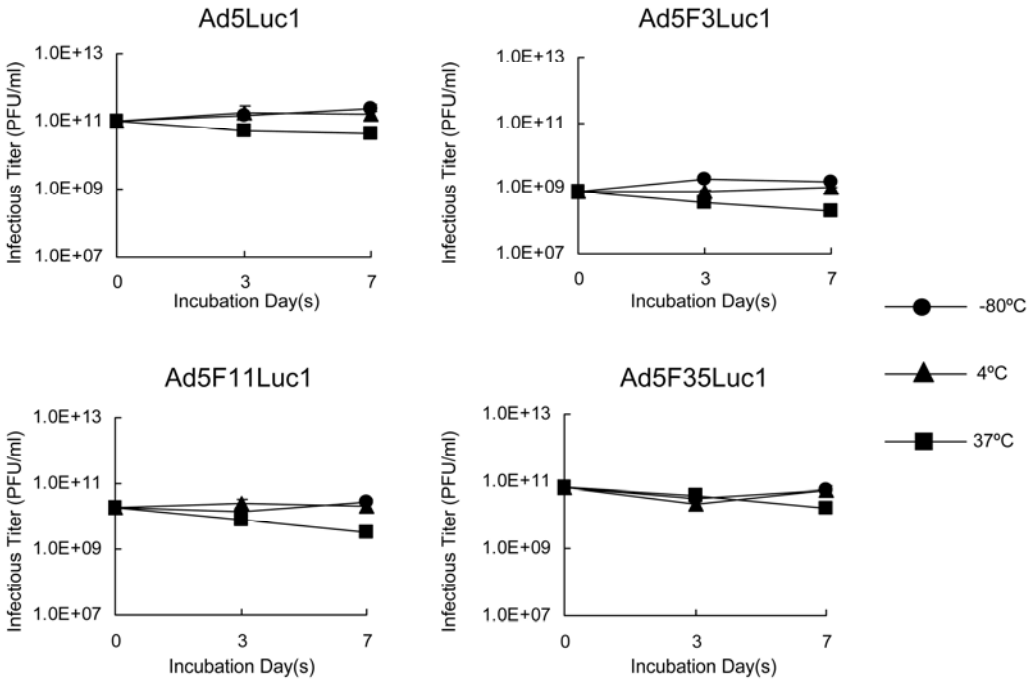

Figure 2 Thermostability of Ad5Luc1 and fiber chimeric Ad vectors. Purified viral particles (10^{10} VP) were incubated at indicated temperatures for 3 and 7 days, and the resulting infectivity was examined by titration in a triplicate $TCID_{50}$ on HEK293 cells. –80°C (●), 4°C (▲), and 37°C (■). Data points represent mean + standard deviation (n = 3).

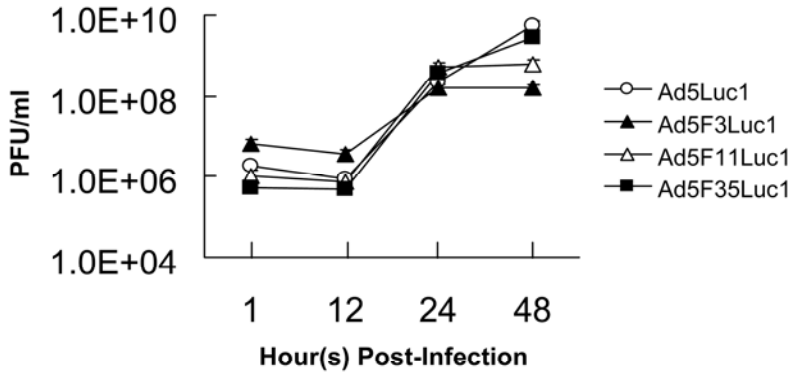

Figure 3 Comparison of a one step growth curve obtained by infection of HEK293 cells with Ad5Luc1 (○), Ad5F3Luc1 (▲), Ad5F11Luc1 (Δ), and Ad5F3Luc1 (■). HEK293 cells were infected with the vectors above at an MOI of 10 PFU/cell. Data points represent mean + standard deviation (n = 3).

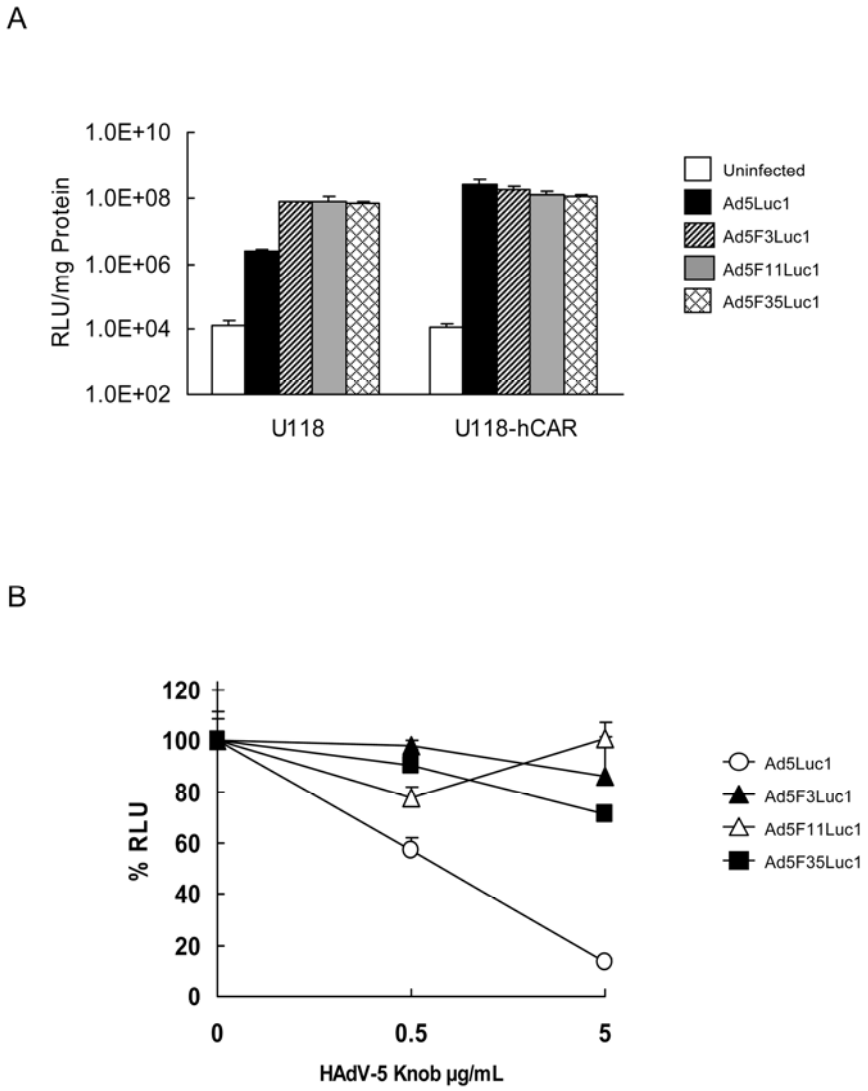

Figure 4 Gene transfer of Ad5Luc1 and chimeric Ad vectors in CAR-negative U118 and CAR expressing U118-hCAR cells, and gene transfer in A549 cells with HAdV-5 knob blocking. **A:** Luciferase activities in the lysates of CAR-negative U118 and CAR expressing U118-hCAR cells infected with no virus (white bar), Ad5Luc1 (black bar), Ad5F3Luc1 (diagonal bar), Ad5F11Luc1 (gray bar), and Ad5F35Luc1 (cross-hatch bar) at an MOI of 10 PFU/cell. The luciferase activity was measured 24 hours post-transduction. Data points represent mean ± standard deviation (n = 3). **B:** The percentage of the luciferase activities in the lysates of A549 cells infected with Ad5Luc1 (○), Ad5F3Luc1 (▲), Ad5F11Luc1 (Δ), and Ad5F35Luc1 (■) at an MOI of 10 PFU/cell after being blocked by the recombinant HAdV-5 knob protein at various concentrations. Final concentration of the recombinant HAdV-5 knob protein used to block transduction is indicated in µg/ml. The luciferase activity was measured at 24 hours post-transduction. Bars represent the mean + S.E.M. (n = 3).

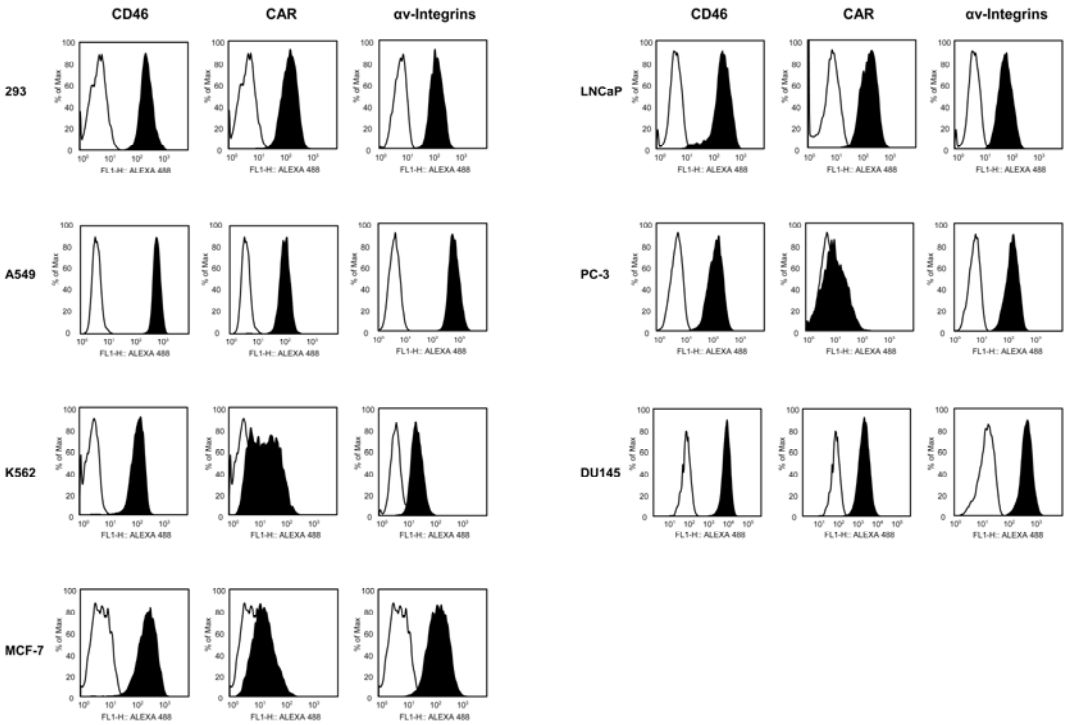

Figure 5 Analysis of expression of CD46, CAR, and αv-integrins on various cancer cell lines by flow cytometry. Filled, black histograms indicate stained cells; open, white histograms indicate unstained control cells.

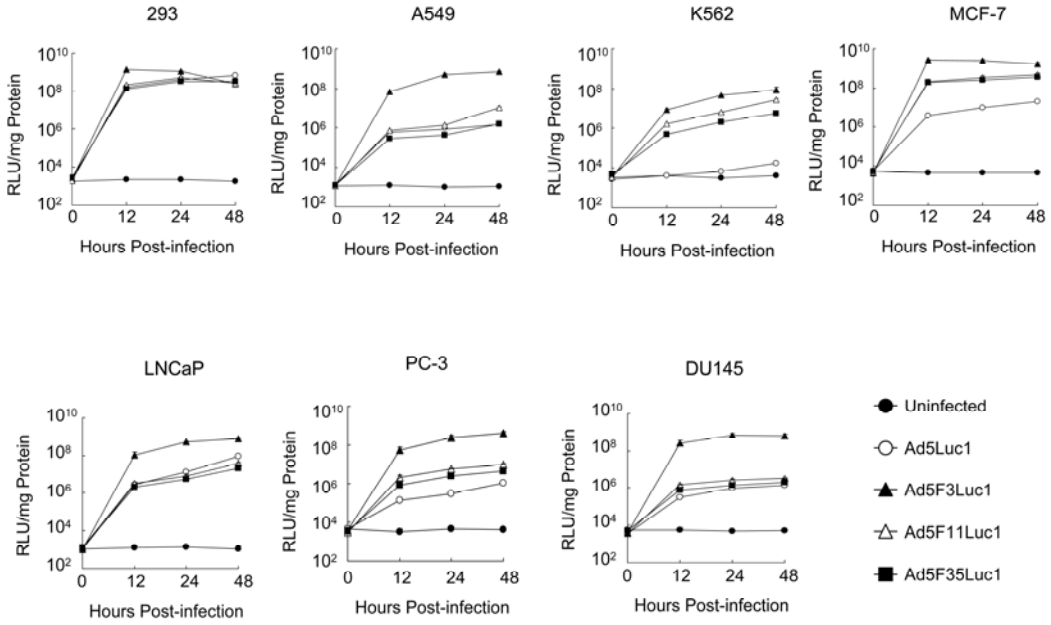

Figure 6 Comparison of transductional efficiency in various human cancer cells (HEK293, A549, K562, and MCF-7) and prostate cancer cells (LNCaP, DU145, and PC-3) with Ad5Luc1 and chimeric Ad vectors at various times. The luciferase activities in the lysates of cells infected with Ad5Luc1 (○) and chimeric Ad vectors Ad5F3Luc1 (▲), Ad5F11Luc1 (Δ), and Ad5F35Luc1 (■) at an MOI of 10 PFU/cell were measured at various time points (0, 12, 24, and 48 hours post-transduction) and normalized for protein concentration. Data points represent mean + standard deviation (n = 3). Closed circles (●) indicate background luciferase activity.

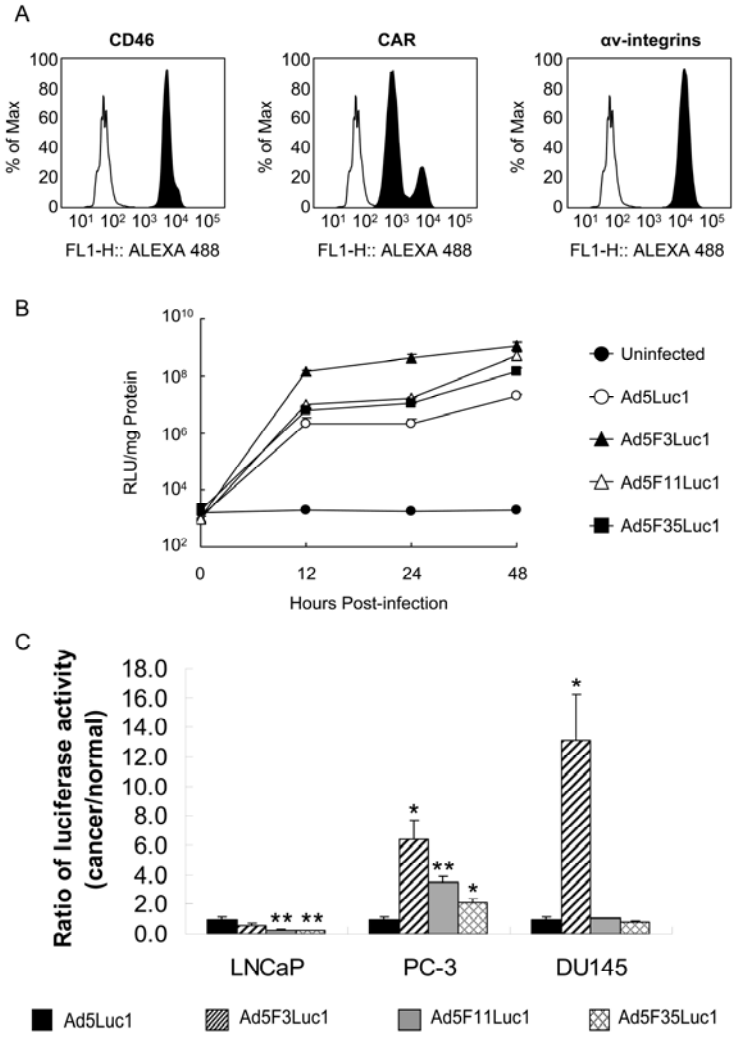

Figure 7 Analysis of gene transfer in PrEC cells. **A:** Analysis of CD46, CAR, and αv-integrins expression in prostate epithelial cells (PrEC) by flow cytometry. Filled, black histograms indicate stained cells; open, white histograms indicate unstained control cells. **B:** Comparison of transduction efficiency in primary prostate epithelial cells with Ad5Luc1 and chimeric Ad vectors. The luciferase activities in the lysates of cells infected with Ad5Luc1 (○) and the chimeric Ad vectors Ad5F3Luc1 (▲), Ad5F11Luc1 (Δ), and Ad5F35Luc1 (■) at an MOI of 10 PFU/cell were measured at various time points (0, 12, 24, and 48 hours post-transduction) and normalized for protein concentration. Data points represent mean ± standard deviation (n = 3). Closed circles (●) indicate background luciferase activity. **C:** Gene transfer in prostate cancer cells (LNCaP, PC-3, and DU145) expressed as a ratio of the gene transfer in primary prostate epithelial cells at 12 hours post transduction, normalized by that of Ad5Luc1; Ad5Luc1 (black bar), Ad5F3Luc1 (diagonal bar) Ad5F11Luc1 (gray bar), and Ad5F35Luc1 (cross-hatch bar). Bars represent the mean ± S.E.M. A *t*-test statistical analysis was performed with respect to Ad5Luc1 and significance is indicated by * $P < 0.05$; ** $P < 0.01$ (n = 3).

CHAPTER III: VALIDATION OF A GENETICALLY GENERATED ADENOVIRAL VECTOR INCORPORATING FIBERS OF HUMAN ADENOVIRUS 5 AND 3 FOR TARGETING A HETEROGENEOUS CELL POPULATION WITHIN TUMORS

by

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Abstract

The human adenovirus serotype 5 (HAdV-5) vector binds to its primary receptor, the coxsackie and adenovirus receptor (CAR), for infection. However, levels of CAR expression tend to decrease as tumors progress; thus, this low CAR expression diminishes the utility of the HAdV-5 vector for cancer therapy. Contrariwise, aggressive tumor cells highly express CD46, which is a cellular receptor for HAdV-3. Therefore, we hypothesize that a genetically generated fiber-mosaic HAdV-5 vector incorporating a chimeric HAdV-3 fiber protein as well as the native HAdV-5 fiber protein will expand tropism to multiple cancer cell stages by utilizing distinct receptors. The herein generated fiber-mosaic HAdV-5 vector transduced both CAR- and CD46-expressing Chinese Hamster Ovary cells. Importantly, the fiber-mosaic vector was demonstrated to have expanded tropism considering its infectivity in different kinds of cells in a mixed population. The herein used method to generate fiber-mosaic vectors is thus a promising method for future cancer gene therapy applications.

Introduction

Viral vector-mediated gene therapy is a new tool currently under development to treat tumors. In particular, adenoviral vectors based on human adenovirus 5 (HAdV-5) have been developed and used for cancer gene therapy (1, 2). HAdV-5 has several features that are useful for cancer gene therapy application, including a high insert capacity (up to approximately 37kb) (3) to deliver large therapeutic genes (4, 5), ease of production at high titers applicable for clinical use (6), and relative safety as the viral genome is not integrated into the host genome (7, 8). HAdV-5 efficiently attaches to cells

expressing the coxsackie virus and adenovirus receptor (CAR) (9), via its fiber protein and is able to infect various cancer cell lines in culture.

However, the distribution of CAR expression in the human body is dependent on the origin of the tumor cells (10). With the exception of breast tumors, in which CAR expression is increased with increasing tumor grade (11), the expression levels of CAR tend to be low in the majority of advanced types of tumors (12-19). In addition, CAR expression in ovarian tumors exhibited marked distributional heterogeneity (19). Thus, the varied and often insufficient expression of CAR hampers HAdV-5 transduction in individual cells, especially for advanced tumors.

In contrast to the CAR expression, aggressive tumor cells highly express CD46 (a membrane cofactor protein); for example, invasive carcinoma appears to preferentially retain CD46 expression (20-25). Additionally, CD46 is over-expressed in cancer cells as compared to normal cells (21-25). making CD46 an optimal target for cancer therapy. It has been demonstrated that the HAdV-3 fiber protein binds to CD46 (26, 27). We have previously reported that a chimeric HAdV-5 vector incorporating a HAdV-3 fiber protein efficiently transduces cancer cell lines with low CAR expression (28). Accordingly, we suggest that the utilization of the HAdV-3 fiber protein along with the native HAdV-5 fiber protein will facilitate effective cellular transduction of the HAdV-5 vector, even into cells expressing low levels of CAR, resulting in tropism expansion.

We have previously developed HAdV-5 vectors incorporating two different kinds of fiber proteins - called fiber-mosaic HAdV vectors - using two different methods: co-infection of cells with two different HAdV ‘parent’ vectors (29) and genetic insertion of an additional fiber gene in the HAdV-5 genome (30-33). However, when using the first

method (29), a considerable possibility is that the viral progeny incorporates only one kind of fiber protein, when cells are not co-infected with both kinds of the HAdV-5 'parent' vectors. Using the second method, we genetically generated fiber-mosaic HAdV vectors encoding additional fiber genes including the bacteriophage T4 fibritin domain (30), the bacteriophage T4 fibritin containing the 1.3S subunit of *propionibacterium shermanii* transcarboxylase (32), or reovirus attachment protein sigma 1 (31). These additional fibers were combined with either a wild type HAdV-5 fiber gene or a chimeric HAdV-3 fiber gene in the case of sigma 1 (33). These studies showed that the additional fiber protein was able to function as a ligand for its respective cellular receptor, and thus achieve tropism expansion (31-33). Since tumors, as mentioned above, are composed of a heterogeneous cell population (34, 35), this fiber-mosaic system would thus be very suitable for efficiently targeting different cells within tumors.

On this basis, we hypothesize that a genetically generated fiber-mosaic HAdV-5 vector incorporating two different types of HAdV-derived fiber proteins - a chimeric fiber protein displaying the HAdV-3 shaft and knob domains, as well as the native HAdV-5 fiber protein - will expand tropism to cancer cells by utilizing distinct receptors. We first determined the best cloning strategy for the genomic insertion of the second fiber gene in order to gain similar levels of incorporation of the two different kinds of fiber proteins. After the fiber-mosaic HAdV vector was genetically generated, we tested the tropism expansion hypothesis by comparing its gene transfer efficacy in various cell types with the control HAdV vectors with only a single type of fiber.

RESULTS

Generation of Fiber-Mosaic Adenoviral Vectors

We explored three different cloning strategies for insertion of the second fiber into the genome; the schematic structures are shown in Figure 1. The composition of the inserted fiber genes in the fiber-mosaic HAdV genome and the results of the three cloning strategies are summarized in Table I. Cloning strategy 1, a method developed in a previous study (30), was to insert the tandem fiber genes in the late region 5 (L5) transcription unit. The HAdV vector was generated in HEK293 cells. Western blot analysis demonstrated that the additional fiber protein was not incorporated in the HAdV particles (data not shown). We also observed that one fiber gene was deleted from the HAdV genome, probably due to homologous recombination between the identical fiber tail sequences (data not shown).

Table I
Summary of the result of the three cloning strategies of additional fiber protein

Orientation	Promoter	Generation	Expression	Incorporation
Sense	Major late	Yes	No	No
Anti-sense	E4	Yes	Little	No
Anti-sense	CMV	Yes	Yes	Yes

We therefore devised a different cloning strategy that would avoid deletion of a fiber gene from the HAdV genome encoding two fiber genes. We utilized cloning strategy 2, inserting the additional fiber gene with an internal ribosome entry site (IRES) gene, downstream of the early region 4 (E4) transcription unit (**Fig. 1**). With the second fiber gene in anti-sense orientation with respect to the native fiber gene, potential homologous recombination between the fiber tail sequences would result in *both* fiber genes being deleted from the genome, thereby prohibiting virus production all together.

The HAdV vector was successfully generated using this strategy. However, a western blot of infected cell lysate showed a low level of additional fiber expression whereas the native HAdV-5 fiber protein was highly expressed (data not shown). Therefore, mainly the native HAdV-5 fiber protein was incorporated in the purified HAdV particles (data not shown). These data indicate that the second strategy using IRES under the E4 promoter was not optimal for the additional fiber expression.

Considering the low expression level of the additional fiber protein utilizing the IRES method as described above, we utilized a cytomegalovirus (CMV) promoter and also added a poly A site at the 3' end of the additional fiber gene, thereby hoping to gain higher expression levels of the additional fiber protein (cloning strategy 3, **Fig. 1**). The plasmid encoding two different fiber genes, pKAN3.1F5CMV5T3SKpA, was constructed as described in the Materials and Methods section. The recombinant HAdV genomic plasmid containing the two fiber genes was subsequently derived by homologous recombination between pKAN3.1F5CMV5T3SKpA and the genomic plasmid pVK900. The HAdV vector was generated in HEK293 cells, resulting in Ad5F5-F3EGFP. To confirm the additional fiber gene in the HAdV genome, polymerase chain reaction (PCR) analysis was performed with the purified HAdV vectors, as well as recombinant plasmids as a positive control, and Ad5EGFP and Ad5F3EGFP as negative controls. The HAdV-5-specific primer set, which anneal at 100 base pairs (bp) upstream and downstream of the HAdV-5 fiber gene were used for the PCR analysis (**Fig. 2A**). The DNA fragment containing the additional fiber gene was amplified by PCR (**Fig. 2B**, Lane 4). Subsequently, both types of the fiber proteins were detected in the purified HAdV viral particles by Western blot analysis (**Fig. 2C**). We also confirmed that both of the fiber

proteins incorporated in the purified Ad5F5-F3EGFP viral particles were homotrimerized but not heterotrimerized (**Fig. 2D**). We observed significant expression of the additional fiber protein in infected cells by Western blot analysis (**Fig. 2E**). The expression of the HAdV-3 as well as the native HAdV-5 fiber proteins was detected from 12 hr to 48 hr post-infection in the investigated time period.

Analysis of the Viral Particle Morphology and Confirmation of Incorporation of Fiber Proteins by Transmission Electron Microscopy

To directly observe Ad5F5-F3EGFP viral particles and to confirm the incorporation of the two different types of the fiber proteins, we took electron micrographs of Ad5F5-F3EGFP viral particles as well as the control vectors using a transmission electron microscope. The viral particles of Ad5EGFP, Ad5F3EGFP or Ad5F5-F3EGFP, had the expected icosahedral morphology (**Fig. 3** Upper panel). The electron micrograph of Ad5EGFP showed the incorporation of the HAdV-5 fiber proteins (Black arrows in **Fig. 3**, Lower panel, left), whereas that of Ad5F3EGFP demonstrated the incorporation of the HAdV-3 fiber proteins (Orange arrows in **Fig. 3**, Lower panel, middle). The electron micrograph of Ad5F5-F3EGFP displayed the incorporation of the HAdV-5 fiber (Black arrows in **Fig. 3**, Lower panel) as well as the HAdV-3 fiber proteins (Orange arrows in **Fig. 3**, Lower panel, right).

Thermostability of Fiber-mosaic Adenoviral Vectors

We determined if the thermostability of Ad5F5-F3EGFP was affected by incorporation of the two different kinds of the fiber proteins (36, 37). Equal numbers of

the HAdV vectors (10^{10} viral particles [VP]), Ad5EGFP, Ad5F3EGFP, or Ad5F5-F3EGFP, were incubated at various temperatures (–80°C and 37°C) for 3 or 7 days, followed by a $TCID_{50}$ assay to analyze the remaining infectious titer. The infectivity of Ad5F5-F3EGFP, as well as that of Ad5EGFP or Ad5F3EGFP, was similarly decreased from 3 to 7 days of incubation at 37°C (**Fig. 4**). This data indicated that incorporation of another kind of fiber proteins into the HAdV-5 viral particle did not influence its thermostability, considering the similar reduction between Ad5F5-F3EGFP and the control HAdV vectors, Ad5EGFP or Af5F3EGFP.

Analysis of Viral Replication

We sought to determine how incorporation of two different kinds of fibers affected the replication of Ad5F5-F3EGFP. A one-step growth curve analysis was performed in HEK293 infected with each HAdV vector at a multiplicity of infection (MOI) of 10 PFU/cell. The total production of infectious Ad5F5-F3EGFP particles at 48hr post-infection in HEK293 cells was similar to the control vectors. The kinetic of viral replication of AdV5F5-F3EGFP was similar to that of Ad5F3EGFP, but different from that of Ad5EGFP from 12 to 24 hr (**Fig. 5**). Therefore, the replication kinetics of Ad5F5-F3EGFP tends to be similar to the kinetics of the fiber-chimeric vector incorporating the HAdV-3 fiber protein.

Gene Transfer of the Fiber-mosaic Adenoviral Vectors Utilizing Two Different Fiber Proteins

To verify whether Ad5F5-F3EGFP would transfer the EGFP reporter gene through binding to both CAR and CD46, CHO-CAR and CHO-C2 cells were infected with the HAdV vectors at an MOI of 10 PFU/cell. CHO-CAR and CHO-C2 cells are CHO-derived cell lines expressing human CAR (9) and the C2 isoform of human CD46 (9, 38), respectively. After 48 hours post-infection (hpi), fluorescent signal for EGFP was observed by a fluorescence microscope. EGFP signal was observed in both cell lines infected with Ad5F5-F3EGFP, but only in the CHO-CAR cells or in the CHO-C2 cells infected with Ad5EGFP or Ad5F3EGFP, respectively (**Fig. 6**). These data demonstrated that Ad5F5-F3EGFP utilizes both CAR and CD46.

Subsequently, we performed a blocking experiment of Ad5F5-F3EGFP in A549 cells using recombinant fiber knob proteins in order to confirm that both fiber proteins are functional for attachment to the cells. When using the HAdV-3 and -5 knob together as blocking reagents, the EGFP signal provided by Ad5F5-F3EGFP was dramatically attenuated, as well as that provided by Ad5EGFP or Ad5F3EGFP (**Fig. 7**). Addition of either the HAdV-3 or HAdV-5 knob protein did not inhibit the transduction of Ad5F5-F3EGFP (**Fig. 7**). These data confirm that both kinds of fiber proteins in Ad5F5-F3EGFP were used for the cellular attachment (**Fig .7**).

Next, we investigated gene transfer of Ad5F5-F3EGFP in a simulated tumor-like environment, where heterogeneous cell population exists, by mixing two different cell lines. To distinguish between the two cell lines, we first labeled CHO-CAR cells with a red fluorescent protein, by infection of these cells with a recombinant lentiviral vector

encoding the mCherry gene (CHO-CARmCherry). Subsequently, CHO-CARmCherry, as well as PC-3, which is a CAR-negative and CD46 positive human prostate cancer cell line (28), were seeded in a single 6-well plate. As anticipated, both CHO-CARmCherry and PC-3 cells were transduced by Ad5F5-F3EGFP, whereas CHO-CARmCherry cells were transduced by Ad5EGFP, and CHO-C2 cells were transduced by Ad5F3EGFP (**Fig 8**). These results indicated that Ad5F5-F3EGFP was efficiently able to infect both of the cell types in the mixed cell population.

DISCUSSION

In this study, we have genetically generated a fiber-mosaic HAdV vector incorporating both a chimeric fiber protein displaying the HAdV-3 shaft and knob domains, as well as the wild type HAdV-5 fiber protein. We demonstrated that Ad5F5-F3EGFP utilizes distinct receptors using the two different fiber proteins, thus achieving tropism expansion compared to the parent vectors (**Fig. 8**). It proved to be challenging to achieve equal incorporation of two different types of fiber proteins in a virus population (39). We attempted three cloning strategies to insert the second fiber gene in the viral genome. Only one of these strategies succeeded at generating a fiber-mosaic HAdV vector incorporating two different types of fiber proteins. We propose that insertion of the second fiber gene in an anti-sense orientation, as done in the successful strategy, prevents the production of vectors that encode only one fiber gene (Table I). In addition, a CMV promoter ensured sufficiently high expression of the additional fiber protein (**Fig 2E**), resulting in similar incorporation levels of the two distinct fibers in the HAdV particles (**Fig. 2C and 2D**).

To determine whether both fibers are incorporated into individual viral particles in a sample is nearly impossible using current technologies: Western Blotting would show that a composite viral population contains both kinds of fiber proteins, and enzyme-linked immunosorbent assays (ELISA) or immunoprecipitation (IP)-Western blot analysis is not suitable due to a tendency for aggregation by the HAdV viral particles (**Fig. 3**, upper panel) in such assays. We thus chose to visualize the fiber proteins incorporated in a single viral particle by electron microscopy in order to confirm whether a single viral particle contains both kinds of fiber proteins (**Fig. 3**). The micrograph of Ad5F5-F3EGFP was the first to prove incorporation of two different kinds of fiber protein in a single viral particle that was generated by genetic modification, in addition to an existing micrograph of wild type HAdV-41 done by a different research group(40).

Subsequently, we investigated whether the incorporation of two distinct fiber proteins into the HAdV-5 viral particles would harmfully affect HAdV physical properteis. Fortunately, incorporation of another type of fiber protein did not affect thermostability (**Fig. 4**). The replication kinetic of the fiber-mosaic HAdV vector was similar to that of a control vector with only HAdV-3-derived fibers; and the production of the progeny of the fiber-mosaic HAdV vector was similar to the both of the control vectors (**Fig. 5**). We therefore concluded that Ad5F5-F3EGFP retains its biological properties upon its incorporation of two different kinds of fiber proteins, thus justifying a continuation with *in vivo* validation of its tropism.

Our purpose for generating fiber-mosaic HAdV vectors is to efficiently infect the heterogeneous cancer cell population that exists within tumors, through binding of the vector to two distinct receptors. Ad5F5-F3EGFP, as developed in this study, was able to

deliver a transgene into both CHO cells expressing CD46 as well as CHO cells expressing CAR, whereas the respective 'parent' vectors, either HAdV-5 or the chimeric HAdV-5 containing an HAdV-3-derived fiber, were able to only transduce CHO cells expressing CAR or CD46, respectively (**Fig. 6**). Thus, we actually confirmed that Ad5F5-F3EGFP utilized two different receptors: CAR and CD46. Using blocking experiments with recombinant HAdV-5 or/and HAdV-3 knob proteins, we also demonstrated that both types of fiber proteins incorporated in the fiber-mosaic HAdV vector were functionally utilized for cellular transduction (**Fig. 7**). Furthermore, we demonstrated that Ad5F5-F3EGFP delivered a transgene to cells that insufficiently express either CAR or CD46 in a mixed population environment, which confirmed our hypothesis that incorporating two different types of fibers would expand the virus' tropism for different cell types (**Fig. 8**). Since tumors contain a heterogeneous population of cells (34, 35), this mosaic HAdV vector might thus be able to efficiently achieve high transduction levels in tumor tissues.

Ad5EGFP and Ad5F3EGFP efficiently achieved high transduction in the target cells, CHO-CAR and CHO-C2 cells, respectively (**Fig. 6**). Therefore, simultaneous infection of two kinds of these viruses would also achieve gene transfer in the widespread area of a heterogeneous population of tumors. However, it was shown that simultaneous infection of two kinds of the HAdV-5 vectors resulted in a reduced transduction as compared with the mosaic HAdV-5 vector (29), emphasizing the advantage of the latter.

An important utility of using a genetically generated mosaic HAdV vector is its application in conditionally replicative HAdV vectors (CRAds), where the progeny of the replicative HAdVs would also encode two different fiber genes, thus also utilizing distinct receptors to facilitate the next round of infection within the tumor tissue.

Therefore, this genetic approach of the fiber mosaic HAdV vector system that will avoid the production of viral progeny that encode only a single fiber gene, would be a promising approach for further utility of fiber-mosaic HAdV vectors for cancer therapy.

MATERIALS AND METHODS

Cell Lines

Chinese hamster ovary (CHO) cells, as well as CAR and CD46 positive cells, human embryonic kidney (HEK) 293 (41) and human lung epithelial A549 cells (28) were all obtained from the American Type Culture Collection (ATCC; Manassas, VA) and HEK293A cells were purchased from Invitrogen (Carlsbad, CA). All cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, (DMEM/F12; Sigma-Aldrich; St. Louis, MO) containing 10% fetal bovine serum, (FBS; Hyclone; Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech, Inc; Herndon, VA). CHO-CAR cells stably expressing human CAR, were derivatives of CHO (9) and were cultured in MEM Alpha medium (MEM Alpha; Invitrogen) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. A CHO-derived cell line, CHO-C2, which stably expresses the C2 isoform of human CD46 was previously reported (38) and was cultured in Eagle's minimum essential medium (MEM; Mediatech) containing 10% FBS, 2 mM L-glutamine, 10 mM MEM non-essential amino acids (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. The CHO-CAR and CHO-C2 cells expressing mCherry were generated by infection of these cells with lentiviral vector containing the mCherry gene (Dr. Justin C. Roth, Division of Human Gene Therapy, University of Alabama at Birmingham, unpublished

vector). Briefly, the corresponding cell culture medium including the lentiviral vector and hexademethrine bromide (Sigma) at a final concentration of 8 μg/ml was added to the CHO-CAR cells grown in a 6-well plate. After 24 hrs incubation, the medium was changed to the fresh cell culture medium. After 48 hrs incubation, the cells were trypsinized, reseeded, and maintained in the corresponding medium with puromycin (Mediatech) at a final concentration of 10 μg/ml until the control cells were killed.

All cells were propagated at 37°C in a 5% CO_2 atmosphere. The cell lines infected with the HAdV vectors were maintained using the corresponding cell culture medium but containing 2% instead of 10% FBS.

Construction of Recombinant Plasmids

We had three construction strategies to encode two different fiber genes in the HAdV genome. With the first construction strategy as described elsewhere (30), we generated the recombinant plasmids encoding the nucleotide sequences of the HAdV-5 fiber tail domain and species B HAdV fiber shaft and knob domains as the second fiber gene next to the wild type HAdV-5 fiber gene (**Fig. 1**). The plasmid encoding the HAdV-5 fiber tail domain and the HAdV-3 fiber shaft and knob domains, pNEB193.5T3SK (28), were digested with the restriction enzymes, BssHII and MfeI. The BssHII- and MfeI-digested DNA fragments of the HAdV-5 fiber from the plasmid pKAN3.1 (42) were ligated into the BssHII and MfeI site of pNEB193.5T3SK. The resulting plasmid was named as pNEB.F2.5TSK. The plasmid pNEB.5T11SK (28) or pNEB.5T35SK (28) was digested with SacI and SwaI restriction enzymes, and the DNA fragment of pNEB.5T11SK or pNEB.5T35SK was ligated into the SacI and SwaI sites of

pNEB.F2.5TSK. The resulting plasmid was named as pNEB5T11SK.5TSK or pNEB5T35SK.5TSK, digested with ClaI and MfeI restriction enzymes, and the DNA fragment of pNEB5T11SK.5TSK or pNEB5T35SK.5TSK was ligated into the ClaI and MfeI sites of pKAN3.1.Cla. The resulting plasmid was named as pKAN5T11SK.5TSK or pKAN5T35SK.5TSK, respectively. The plasmid pKAN3.1.Cla is a plasmid, based on pTGbxCla (30), but encoding kanamycin resistant gene instead of ampicilin resistant gene. For the construction of the plasmid pKAN3.1.Cla, pTGbxCla plasmid was digested with BsmI and NdeI before ligation into the BsmI and NdeI sites of pKAN3.1 plasmid (42).

With the second construction strategy, we generated the recombinant plasmid encoding the sequence of the HAdV-5 fiber tail domain and species B HAdV fiber shaft and knob domains, as the second fiber gene, in the E4 untranslation region using an internal ribosome entry site (IRES), in addition to the wild type HAdV-5 fiber gene (**Fig. 1**). The additional fiber insertion site (Positions between 32863 bp and 32864 bp on the HAdV-5 genome) was determined based on the study done by Kretschmer *et al.* (43). For the insertion of the additional fiber gene, we first planned to insert multiple cloning sites into the plasmid pKAN5T3SK (28). The partial nucleotide sequence of the HAdV-3 knob was amplified using primers SacIScaI1280 5'-TTG**GAGCTC**AGTACTACAGCGTATCCATT-3' (Position 32061 bp-32080 bp on the HAdV-3 genome) and i-Not1622 5'-AGA**GCGGCCGC**ACTACTGAATGAAAAATGAC-3' (Position 32863 bp-32844 bp on the HAdV-5 genome), with pKAN5T3SK plasmid (28) containing the nucleotide sequence of the HAdV-3 fiber shaft and knob domain, as a template. The bold and

underline showed the restriction enzyme sites used in this study. The resulting PCR products were digested with the SacI and NotI restriction enzymes, and ligated into the SacI and NotI sites of the plasmid pBluescript II KS (–) (Stratagene, La Jolla, CA), resulting in the plasmid, pBlueSacIScaI-NotI. The plasmid pKAN5T3SK (28) was digested with the the XhoI and KpnI restriction enzymes, and the DNA fragment of pKAN5T3SK was ligated into the XhoI and KpnI sites of pBlueSacIScaI-NotI. The resulting plasmid was named as pBlueSacIScaI-NotI-XhoI-KpnI, and subsequently digested with ScaI and KpnI. The DNA fragment of pBlueSacIScaI-NotI-XhoI-KpnI was ligated into the SacI and KpnI sites of pKAN5T3SK, resulting in the plasmid pKAN5T3SK1622MCS. Subsequently, the nucleotide sequences of the IRES and the spacer were amplified using primers XhoBglSp 5'-GAC**CTCGAG**AGGCAGTTATTGGTGCCCTT-3'and i-ClaNcoIRES 5'-CTT**ATCGAT**CCATGGTATCATCGTGTTTT-3'using pfiberIL (44) as a template. The PCR product was digested with XhoI and ClaI restriction enzymes, and ligated into the XhoI and ClaI sites of pKAN5T3SK1622MCS, resulting in the plasmid pKAN5T3SK.IRES.sp1622. To construct a plasmid but encoding the HAdV-5 fiber gene, pKAN5T3SK.IRES.sp1622 was digested with BspHI and MfeI restriction enzymes, and the DNA fragment of pKAN5T3SK.IRES.sp1622 was ligated into the BspHI and MfeI sites of pKAN3.1 (42) plasmid encoding HAdV-5 fiber gene, resulting in the plasmid pKAN3.1.IRES.sp1622. The plasmid pKAN5T11SK5TSK or pKAN5T35SK5TSK, as described in the first cloning strategy above, was digested with ClaI and SwaI, and the DNA fragment of pKAN5T11SK5TSK or pKAN5T35SK5TSK ligated into the ClaI and

SwaI sites of the plasmid pKAN3.1.IRES.sp1622. The resulting plasmid was named as pKAN3.1.5T11SK.IRES.sp1622 or pKAN3.1.5T35SK.IRES.sp1622, respectively.

For the third construction strategy, we cloned the recombinant plasmid encoding the HAdV-5 fiber tail domain and the HAdV-3 HAdV fiber shaft and knob domains as an additional fiber gene with the cytomegalovirus (CMV) promoter and bovine growth hormone polyadenylation signal (BGH polyA) in the E4 untranslation region (**Fig. 1**). The plasmid pcDNA3.1_Ad5T-3SK (Anand Anan, Division of Human Gene Therapy, University of Alabama at Birmingham, unpublished plasmid) is pcDNA3.1/Zeo (Invitrogen) encoding the HAdV-5 fiber tail, the HAdV-3 fiber shaft and knob nucleotide sequence. The plasmid pcDNA3.1_Ad5T-3SK was digested with the NruI and PvuII restriction enzymes, and the DNA fragment of pcDNA3.1_Ad5T-3SK was ligated into the EcoRV site of pKAN3.1F5-MCS (Dr. Hideyo Ugai, Division of Human Gene Therapy, University of Alabama at Birmingham, unpublished plasmid), which encodes HAdV-5 fiber gene. The resulting vector encodes the HAdV-5 fiber gene in sense orientation, and the additional fiber expression cassette containing the nucleotide sequences for CMV promoter, the HAdV-5 fiber tail and the HAdV-3 fiber shaft and knob, and BGH polyA in anti-sense or sense orientation. We selected the plasmid encoding the second fiber expression cassette in anti-sense orientation detected by the restriction enzyme analysis. The plasmid was named as pKAN3.1F5CMV5T3SKpA.

The nucleotide sequences of the DNA fragments cloned into all plasmids were confirmed by the UAB DNA sequencing core facility.

Construction of the Adenoviral Plasmids

The constructed plasmids (pKAN5T11SK.5TSK, pKAN5T35SK.5TSK, pKAN3.1.5T11SK.IRES.sp1622, pKAN3.1.5T35SK.IRES.sp1622 and pKAN3.1F5CMV5T3SKplA) were used for homologous recombination with the SwaI-linearized pVK900 genomic plasmid (pVK700 (45)-based plasmid encoding the enhanced green fluorescence protein [eGFP] controlled by the cytomegalovirus [CMV] promoter in the deleted early region 1 [E1], Dr. Victor Krasnykh, Department of Experimental Diagnostic Imaging, MD Anderson Cancer Center, The University of Texas, Houston unpublished plasmid). The homologous recombination resulted in pMM900.5T11SK.F5, pMM900.5T35SK.F5, pMM900.F5.5T11SK.IRES.sp1622, pMM900.F5.5T35SK.IRES.sp1622 and pMM900.F5CMV5T3SKpA, respectively. To construct the control HAdV vectors which encodes the HAdV-5 fiber or the HAdV-3 fiber gene, pKAN3.1 (42) and pKAN5T3SK (28) were used for homologous recombination with the SwaI-linearized pVK900 plasmid, resulting in the plasmid pMM905 and pMM903, respectively.

Adenovirus Generation, Propagation, Purification, and Titration

The recombinant adenoviral plasmids were linearized by PacI and transfected into HEK293 cells using Lipofectamine^{TM} LTX reagent (Invitrogen) in order to generate those HAdV vectors. Table I shows the recombinant HAdV vectors generated in this study. All vectors propagated in HEK293 cells were purified by two rounds of CsCl gradient ultracentrifugation (46). CsCl was removed by dialysis using PBS (pH 7.4) containing 10% glycerol. The HAdV vectors were stored at –80°C prior to the next

experiments. The infectious titer (plaque forming units [PFU]/ml) of the purified HAdV vectors were determined by triplicate 50% Tissue Culture Infective Dose ($TCID_{50}$) assays using HEK293A cells, as previously described (46). The physical titer (viral particles [VP]/ml) were determined by Maizel's method with a conversion factor of 1.1×10^{12} VP/ml per absorbance unit at 260 nm (47).

Polymerase Chain Reaction (PCR) Analysis of the Fiber Region

To confirm the presence of the two fiber genes in the HAdV genomes, 10^7 VP of the purified HAdV vectors were boiled at 95ºC for 5 min and analyzed by PCR using the TaqPCR Master Kit (Qiagen Inc., Valencia, CA). Forward (Fiber –100) and reverse (Fiber +100) primers (28) were used to amplify the region containing the full length of the nucleotide sequences for one or two kinds of fiber. The following PCR conditions were applied: 1 min denaturation at 96ºC, 1 min annealing at 60ºC, and 5 min extension at 72ºC.

Western Blot Analysis of Adenoviral Proteins

Aliquots of the purified HAdV vectors equal to 5×10^9 VP and 5 μg of total proteins extracted from virus-infected cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) at various time points (1, 3, 6, 12, 18, 24, 36 and 48 hours post-infection [h.p.i]) were denatured by boiling in Laemmli sample buffer (Bio-Rad laboratories Inc., Hercules, CA) at 95ºC for 10 min. The proteins were separated by electrophoresis in sodium dodecyl sulfate 10 or 12% polyacrylamide gel (SDS-PAGE). Subsequently, Western blot analysis was performed as

described elswere (28). The protein concentration was determined using the D_C protein assay kit (Bio-Rad laboratories Inc., Hercules, CA) according to the manufacturer's instructions.

Electron Microscopy

A total of 1.0×10^8 VP of purified HAdV vector was transferred into a 1.5-ml tube and vortexed for 20 sec (s) to be separated to each VP. Subsequently, the HAdV vector was fixed with an equal volume of fixative buffer (1.25% glutaraldehyde and 2% paraformaldehyde in 30 mM Hepes-NaOH buffer [pH 7.4]) and incubated at room temperature for 30 min. Ten microliters of the fixed adenoviral particles were placed on parafilm, and a glow discharged grid (400-mesh, pure carbon support films; Electron Microscopy Sciences, Hatfield, PA) was placed on the viral solution for 10 s. The carbon grid was washed with 100 µl of filtered 10 mM Tris-HCl buffer ([pH 7.4] containing 30 mM NaCl,) on parafilm six times at room temperature for 30 s. The adenoviral particles on the grid were negatively stained with 1% (wt/vol) uranyl acetate at room temperature for 10 s and, subsequently, the grids were air dried. Electron micrographs for the adenoviral particles were taken with FEITecnai F20 200 kV at the High-Resolution Imaging Facility of the University of Alabama at Birmingham.

Virus Thermostability Assay

Thermostability of the HAdV vectors was examined by the method described elsewhere (28, 36, 37). For each purified HAdV, 10^{10} VP were incubated for three and

seven days in the dialysis buffer at –80ºC and 37ºC. The resulting infectious titers of HAdV vectors were determined by $TCID_{50}$ using HEK293A cells.

One-step Growth Curve Analysis of Adenoviral Vectors

HEK293 cells, grown to 80% confluence in 6-cm dishes, were infected with HAdV vectors at a multiplicity of infection (MOI) of 10 PFU/cell. The infected cells were maintained in 3 ml of medium containing 2% FBS. The culture medium and infected cells were scraped and collected in a 15-ml tube at various times (1, 12, 24, and 48 hours) post-infection. The cell suspension was centrifuged at 1,000 × g for 5 min at 4°C, and the cell pellet was resuspended in 2 ml of medium. The cells in the suspension were disrupted by three freeze and thaw cycles. The lysates were centrifuged at 3,500 × g for 10 min at 4°C and the supernatants were used for titration.

Gene Transfer Assay

Cells were grown in 6-well plates, and the cell numbers were counted to determine MOI. Cells were infected with the HAdV vectors at an MOI of 10 PFU/cell in 2 ml of medium containing 2% FBS.

For blocking experiments, 1 ml of the recombinant HAdV-5 knob protein and/or HAdV-3 knob protein (48) diluted to a final concentration 50 μg/ml in FBS-free medium was added to the wells. No blocking agent was added to the control wells. Cells were incubated with the recombinant HAdV-5 knob protein and/or HAdV-3 knob protein at 4°C for one hour, subsequently HAdV vectors at an MOI of 10 PFU/cell were added to the cells in 1 ml of medium containing 4% FBS. After incubation at 37ºC for 48 hours,

we observed the fluorescent signal for EGFP by fluorescence microscopy. The signal for the fluorescent proteins was detected at a low magnification (× 200) by a fluorescent microscope.

For the gene transfer experiment using mixed population of two different cell lines, cells were grown in a 75-cm flask, and the cell numbers were counted for the subsequent seeding. PC-3 (2.25 x 10^5 cells per well) and CHO-CARmCherry (0.75 x 10^5 cells per well) cells were seeded and grown in the 6-well plates. The mixed cells were counted to determine MOI and were infected with HAdV vectors at an MOI of 10 PFU/cell in 2 ml of medium containing 2% FBS. After incubation at 37ºC for 48 hours, we observed the fluorescent signal for EGFP by fluorescence microscopy. The signal for the fluorescent proteins was detected at a low magnification (× 200) by a fluorescent microscope.

Statistical Analysis

Statistical analysis was performed with ANOVA among groups. *P* values <0.05 were considered statistically significant.

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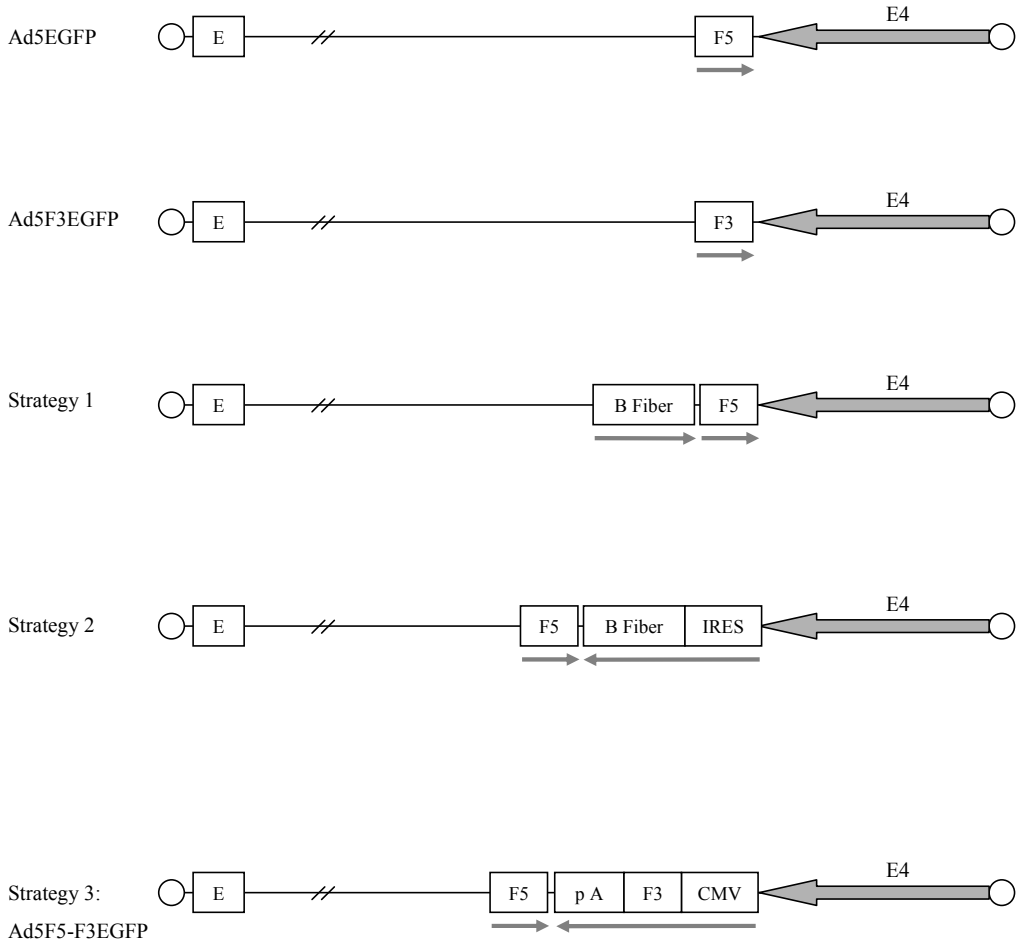

Figure 1 Schema of genome organization of the recombinant HAdV vectors used in this study. E, the expression cassette encoding cytomegalovirus (CMV) promoter, an enhanced green fluorescent protein (EGFP) gene and polyadenylation signal in the deleted E1 region; F5, the HAdV-5 fiber gene; F3, the chimeric HAdV-3 fiber gene containing the HAdV-5 tail, the HAdV-3 shaft and knob domain nucleotide sequence; B fiber, the chimeric species B fiber gene containing the HAdV-5 tail, the species B HAdV shaft and knob domain; CMV, cytomegalovirus promoter; pA, bovine growth hormone polyadenylation signal. The arrows indicate the orientation of the genes.

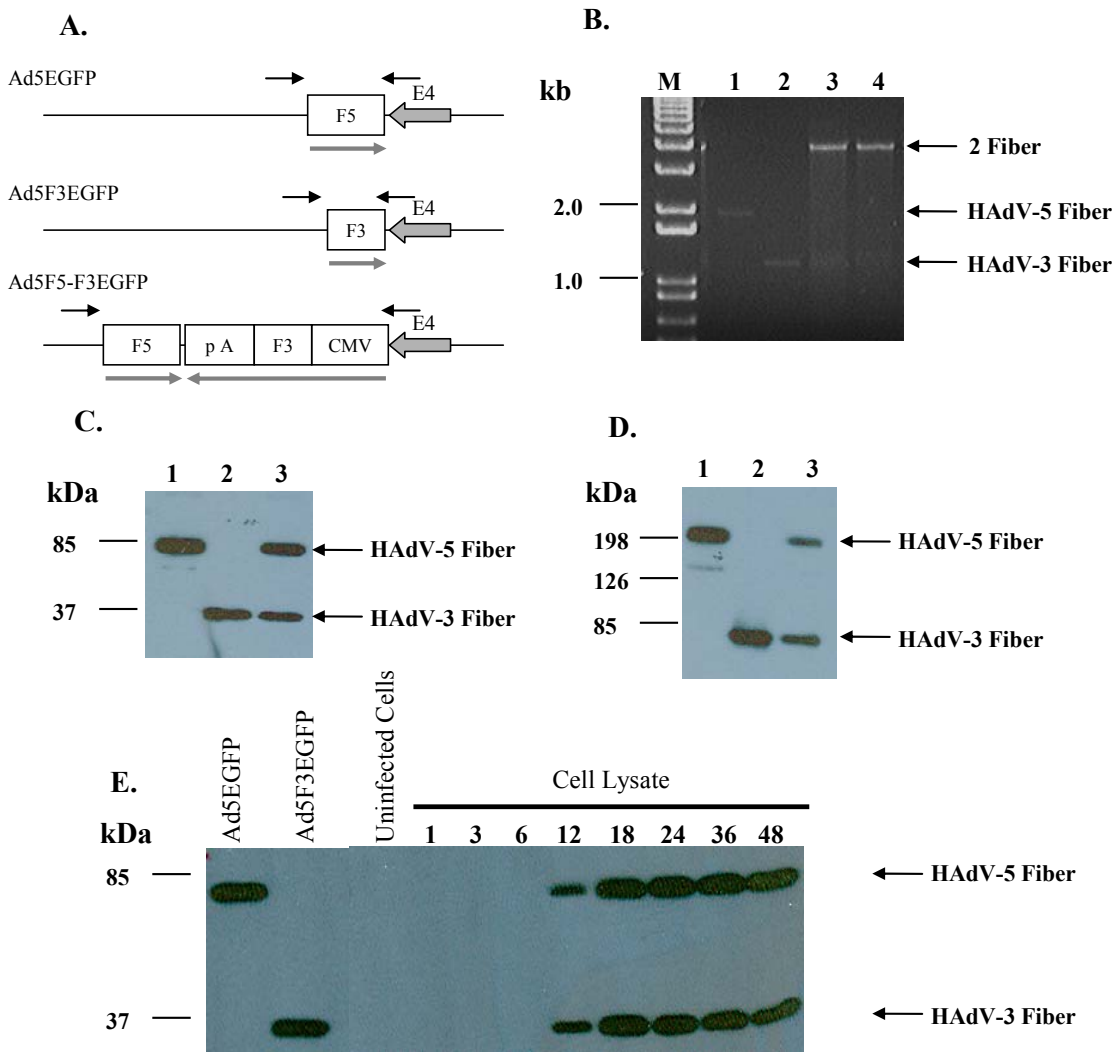

Figure 2 Characterization of a fiber-mosaic HAdV vector. **A:** Schema of the fiber gene(s) of Ad5EGFP, Ad5F3EGFP or Ad5F5-F3EGFP as well as the primer binding positions for PCR analysis. The bold arrows indicate the orientation of the genes. Localization of primers is indicated by the black thin arrows. **B:** Validation of the presence of two fiber genes in the HAdV-5 genome by PCR; M: DNA size markers; Lane 1: PCR product using DNA from purified Ad5EGFP as a template; Lane 2: PCR product using DNA from purified Ad5F3EGFP as a template; Lane 3: PCR product using the recombinant HAdV genome encoding two different fiber genes as a template; Lane 4: PCR product using DNA from purified Ad5F5-F3plA.EGFP as a template. **C, D:** Detection of fiber proteins incorporated into the purified viral particles by Western blot analysis. A total of 5×10^9 VP of the purified HAdV vectors were boiled and run on a 12% SDS-PAGE in C, or run without boiling on a 10% SDS-PAGE in D; separated viral proteins were transferred to a PVDF membrane and probed by an antibody against the HAdV-5 fiber tail (4D2). Lane 1: Ad5EGFP; Lane 2: Ad5F3EGFP; Lane 3: Ad5F5-F3EGFP; protein molecular mass markers (in kDa) are indicated on the left. **E:** Detection of the expression of the fiber proteins from aliquots of the purified HAdV vectors equal to 5×10^9 VP or 5 μg of total proteins extracted from virus-infected cells at various time points

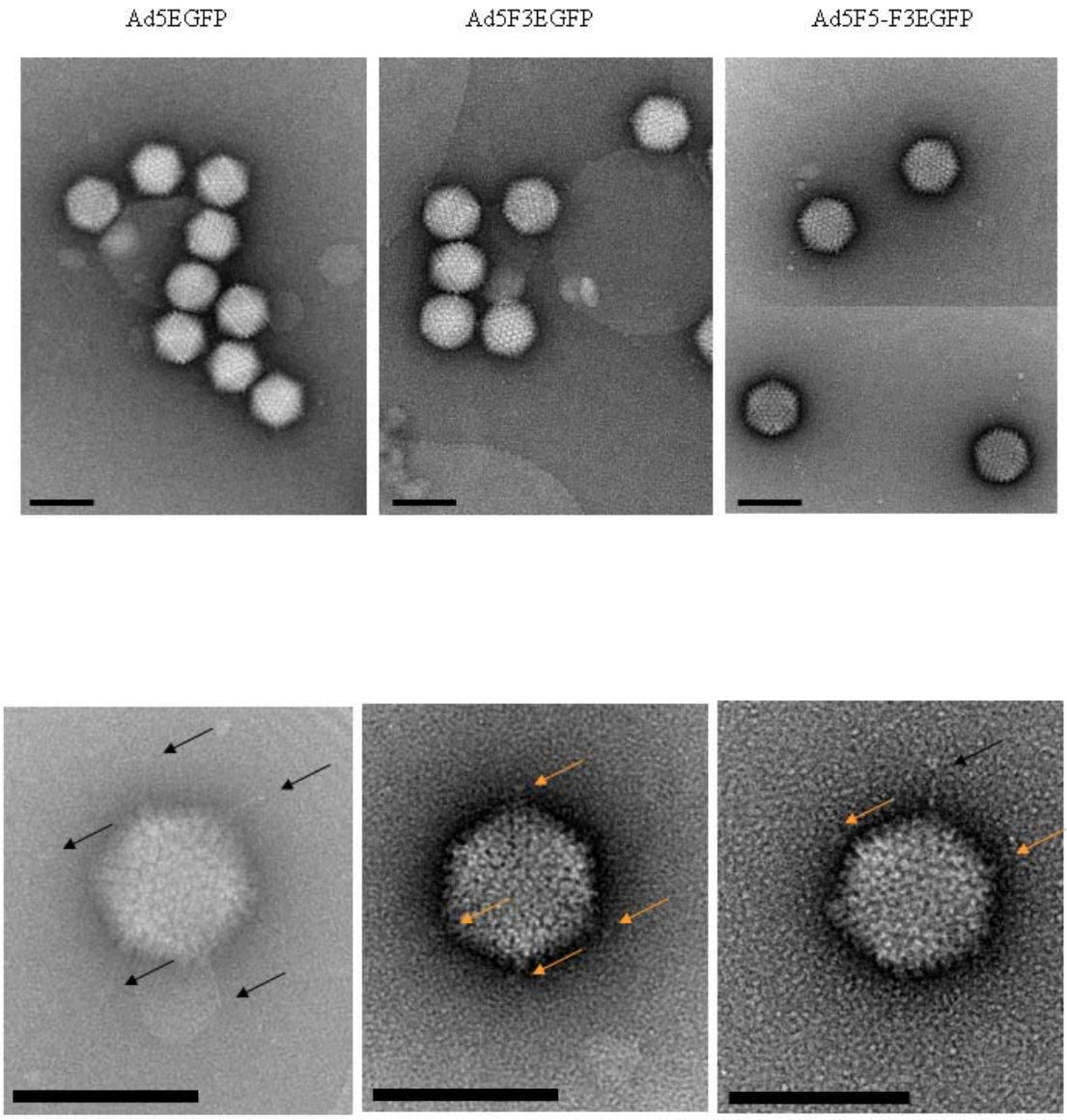

Figure 3 Electron micrograph of negatively stained viral particles of Ad5EGFP, Ad5F3EGFP and Ad5F5-F3EGFP. Black arrows indicate the incorporation of the HAdV-5 fiber. Orange arrows indicate the incorporation of the HAdV-3 fiber. Scale bar represents 100 nm.

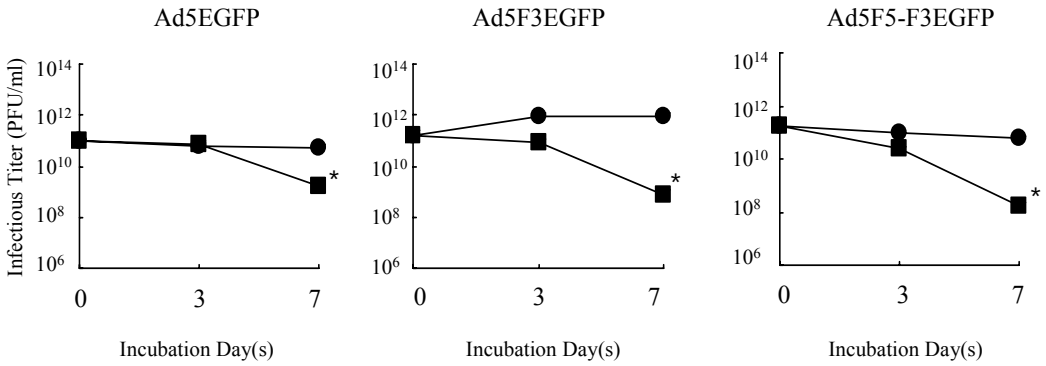

Figure 4 Thermostability of Ad5EGFP, Ad5F3EGFP and Ad5F5-F3EGFP. Purified viral particles (10^{10} VP) were incubated at indicated temperatures for 3 and 7 days, and the resulting infectivity was examined by titration in a triplicate $TCID_{50}$ on HEK293 cells. –80°C (●) and 37°C (■). Data points represent mean + standard deviation (n = 3). ANOVA statistical analysis was performed with respect to the infectious titer at day 7 compared to that at day 3, and significance is indicated by * $P < 0.05$.

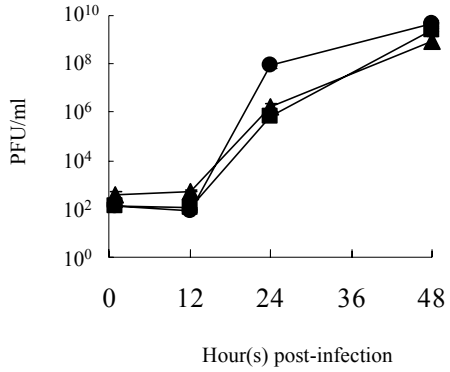

Figure 5 Comparison of a one step growth curve obtained by infection of HEK293 cells with Ad5EGFP (●), Ad5F3EGFP (▲), and Ad5F5-F3EGFP (■). HEK293 cells were infected with the vectors above at an MOI of 10 PFU/cell. Data points represent mean ± standard deviation (n = 3).

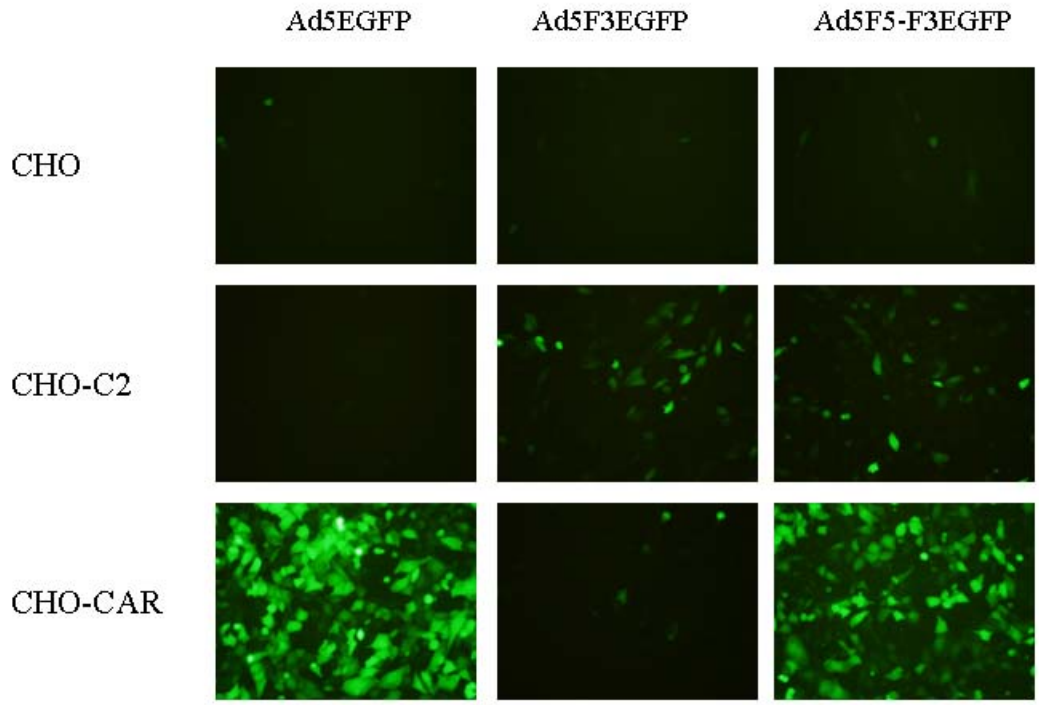

Figure 6 Gene transfer of Ad5EGFP, Ad5F3EGFP and Ad5F5-F3EGFP in CAR-negative CHO, CAR expressing CHO-CAR cells, and CD46 expressing CHO-C2 cells infected at an MOI of 10 PFU/cell.

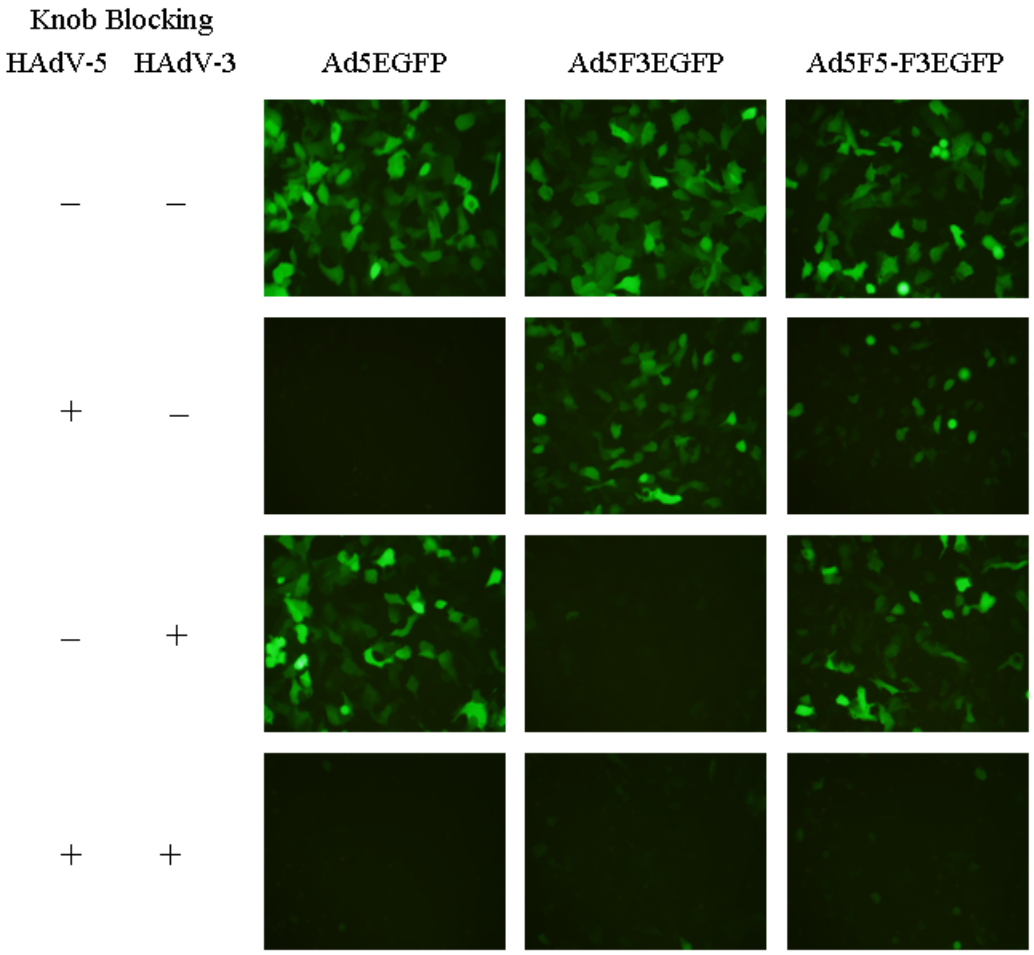

Figure 7 Gene transfer of Ad5EGFP, Ad5F3EGFP and Ad5F5-F3EGFP in A549 cells infected at an MOI of 10 PFU/cell with or without adding recombinant HAdV-5 or/and -3 knob proteins diluted to a final concentration of 50 μg/ml in FBS-free medium prior to the infection.

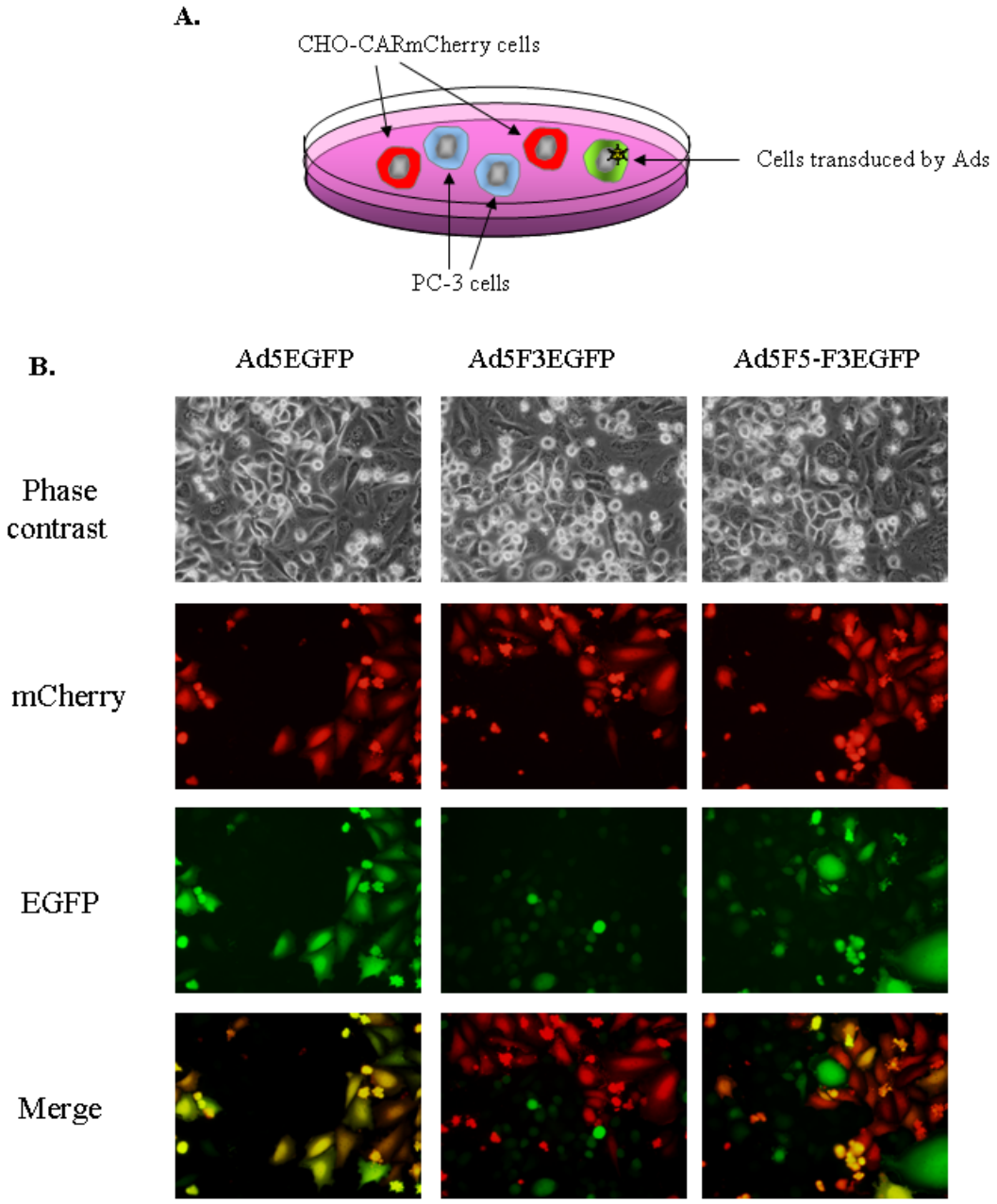

Figure 8 Gene transfer of Ad5EGFP, Ad5F3EGFP and Ad5F5-F3EGFP in the mixed population of PC-3 cells and CHO-CARmCherry cells at an MOI of 10 PFU/cell. **A:** Illustration of color indication of fluorescence signals. **B:** Detection of green and red fluorescence signals in mixed population.

SUMMARY AND GENERAL DISCUSSION

Mortality and incidence of all types of cancer combined have been decreasing in recent years. These decreases are the result of a combination of improvements in primary prevention, early detection and advanced treatments including chemotherapy, surgery and radiotherapy. However, these currently broadly used treatments are not sufficient for some types of cancer (5, 6, 206-208). In addition, the side effect of these treatments is problematic (209-212), especially for patients' quality of life (7-9, 213, 214). Therefore, the development of new novel therapies is mandatory if a cure for these cancers is ever to be found.

One of these novel therapies currently under evaluation is gene therapy. Gene therapy was first designed to repair or replace the gene for inherited single-gene disorders. It is a relatively new cancer therapeutic approach, ever since the prospect of treating cancer with gene therapy rapidly progressed in the 1980s (215). A number of clinical trials of cancer gene therapy have been performed, including trials using immunotherapy (18-31, 36), virus-directed prodrug therapy (38-45), mutation compensation (53) and oncolytic virotherapy (54, 57, 216).

Human adenoviruses (HAdVs) have been developed as a vector for cancer therapy and well-studied by a number of researchers because of its suitable features (155-158) for cancer therapy applications. However, an increased number of studies utilizing adenoviral vectors have revealed several obstacles of this vector's usage for

cancer treatments in humans. One of the obstacles is the natural tropism of HAdVs, as explained in Chapter 1. In this Chapter, a general review of the adenovirus structure and its infection mechanism, including receptor interaction, is described. The most well-studied HAdV, HAdV serotype 5 (HAdV-5), utilizes the coxsackie virus and adenovirus receptor (CAR) as a cellular receptor (217) with high binding affinity (78). Thus, it would logically follow that the effectiveness of using the HAdV-5 vector for cancer therapy depends on the CAR expression/distribution. The patterns and levels of expression of CAR differ greatly among individual tumor cells, even within the same type of the tumor; and tend to decrease as the tumor grade increase. Thus, the HAdV-5 vectors inefficiently infect tumors and can not efficiently deliver its therapeutic cargo.

To overcome this issue, numerous researchers have focused on engineering an adenoviral vector that has a new tropism. The use of adapter molecules has been investigated in this regard (162, 165-169, 218, 219). Although this approach has shown the promise of retargeting Ad to the desired surface receptors other than CAR, most of the adaptor systems have the potential risk of disassociation of the targeting molecules from the HAdV vector. In addition, this approach cannot be utilized for replicative HAdV vectors, since the progeny viral particles would not be targeted.

To circumvent the limitations of the adapter systems, a genetic approach has been attempted by numerous investigators. Genetic approaches include incorporation of targeting ligands into and replacement of the fiber protein region. One of the more fruitful targeting moieties is the Arg-Gly-Asp (RGD)-containing peptide, which upon incorporation into the fiber knob domain targets vectors to integrins (170-173). Poly-lysine (pK7) is another fruitful targeting moiety (170, 177), and targets vectors to heparan

sulfates. Both of these modifications have achieved the desired tropism expansion of the vectors. However, as the trimerization of the knob domain is often hampered by the large ligands, the size of the peptide that can be incorporated remains an issue (98, 184).

Replacement of the HAdV fiber region is an alternative way to change the HAdV native tropism. Some adenoviruses, including non-human adenoviruses and species B HAdVs, do not utilize CAR as a cellular receptor. Chimeric fiber proteins, which contain the fiber knob domain, or the fiber shaft and knob domains from non-human adenoviruses, have achieved CAR-independent transduction (161, 192, 193). However, the tropism of these vectors that contain a non-human adenovirus fiber component might be unpredictable in the human body, due to the unknown distribution of the unidentified receptors for these non-human adenoviruses.

In contrast, the cellular receptors for HAdV-3, CD80 and CD86, have been identified by Short *et al.* (72). CD80 and CD86 critically co-stimulate the T-cells through CD28 binding (148). The expression of CD80 and CD86 are usually restricted to lymophoid cells (149, 150) and are thus rarely observed in cancer cells (32-35, 151). However, CD46 is also identified as a cellular receptor for the species B HAdVs (68), including HAdV-3 (67, 71). CD46 is a membrane co-factor protein, which controls complement activation on host cells (118) and is generally up-regulated in cancer cells (134-138). In addition to CD46, a yet unidentified receptor X, which is highly expressed on human cancer cell lines, has been determined to function as an alternative receptor for HAdV-3 and -11 (125). Thus, replacement of the HAdV-5 fiber shaft and knob domains with those of species B HAdVs is suitable to target the vector to cancer cells that express CD46 and/or a receptor X.

The study reported in Chapter 2 demonstrates that chimeric HAdV-5 vectors incorporating a fiber from species B (HAdV-3, -11, or 35) have increased infectivity in low CAR expressing cancer cells. In this study, the fiber shaft and knob domains of a HAdV-5 vector were genetically replaced with the corresponding domains of HAdV-3, -11 or -35. No deleterious impact on the HAdV biological properties by the replacement of the fiber and shaft domains has been observed in the study. Cancer cell lines, including those derived from prostate cancer, expressed various levels of CAR, CD46, and α-integrins, which are critical for HAdVs infection. However, neither CD80 nor CD86 was expressed on the cancer cell lines used in this study, even though the chimeric HAdV vectors containing the HAdV-3 fiber protein provided the highest amount of gene delivery in most of the cancer cell lines,. These data suggested that the transduction of the chimeric HAdV vector containing the HAdV-3 fiber protein into prostate cancer cells was mediated via receptors other than CD80 and CD86, such as CD46, albeit with low binding affinity or receptor X with high binding affinity.

This study has also demonstrated that, of the chimeric HAdV vectors, the chimeric vector, which incorporates the HAdV-3 fiber shaft and knob domains, seems to be the most suitable vector for prostate cancer gene therapy. It would be promising if the vector had a high infectivity in cancer cells but a low infectivity in normal cells. The chimeric HAdV vector, containing the HAdV-3 fiber shaft and knob domains, achieved the greatest ratio of gene transfer in progressed prostate cancer to normal prostate cells. Replacement of the HAdV-5 fiber protein with that of HAdV-11 and -35 may not be as useful for prostate cancer therapy, since unlike for HAdV-3, CD46 is a high affinity receptor for HAdV-11 and -35 (68, 71, 125), and it is similarly expressed in normal

prostate tissue as well as various levels of prostate cancer specimens (220). Therefore, for prostate cancer therapy, it is foreseeable that modification of the HAdV fiber by replacing the shaft and knob domains with the corresponding HAdV-3 fiber domains would be a promising targeting strategy. Interestingly, even though all species B HAdVs utilize CD46, they infect cancer cells of distinct origins. The chimeric HAdV-5/species B fibers might be able to be useful for targeting many kinds of cancers. Hence, a further comparison of the CD46 expression levels in target cancer tissues and in adjacent normal tissues is required. In future studies, identification of the main HAdV-3 receptors, other than CD46, CD80, or CD86, would further extend the utility of the chimeric vector containing HAdV-3 fiber for other types of cancer therapy.

The study described in Chapter 3 demonstrates the utility of the fiber-mosaic HAdV vector incorporating two distinct fiber variants for cancer therapy. The rationale for this study was that using a mosaic system with different binding moieties in a single adenoviral vector would be suitable for targeting tumors that are composed of a heterogeneous cell population. It has proven, however, to be challenging to achieve the desired incorporation ratio of the two different fibers (221). In addition to this difficulty, the genetic modification strategy has a potential risk of homologous recombination, resulting in elimination of one fiber gene from the virus' genome. The cloning strategy identified in this Chapter to generate the fiber-mosaic HAdV vector prevents the production of a vector containing only one fiber gene. Importantly, no negative impact on the HAdV biological properties by incorporating two distinct fiber protein variants was observed in the study. The fiber-mosaic HAdV vector was able to deliver the gene to two different cell types in a mixed population through binding multiple receptors.

It is especially important for replicative HAdV vectors that the genome is genetically manipulated to achieve tropism expansion. Conditionally replicative HAdV (CRAd) vectors have become a powerful tool for cancer gene therapy. CRAds are designed to replicate within tumor under the control of tissue-specific promoters. The genetic modification will allow the progeny viral particles to be targeted to the cancer cells, resulting in having the same potency for targeting as their 'parent' virions. The combination of expanded HAdV tropism by using the mosaic system in addition to promoter-based targeting of the replicative HAdV system would be additively beneficial for cancer therapy than use of one type of modification.

The utilization of fiber-mosaic HAdV vectors for cancer therapy not only includes targeting heterogeneous population of cells such as those inside a solid tumor, but also the utilization of the two different fiber proteins for different purposes. For example, one type of the fiber protein might be capable to bind to a cell vehicle, while another would be capable to target tumor cells. In that case, temporary incorporation of one type of the fiber protein might be more advantageous than the enduring incorporation by genetic modification. For example, after being internalized in a cell vehicle using a temporary fiber protein, the progeny of the HAdV in the vehicle can contain only another single type of the fiber protein that can target the cancer cells. In that case, the method to produce mosaic fiber HAdV vectors, by infection of a cell line stably expressing the other type of fiber, is more suitable. Another advantage of this production method is to quickly analyze the effect of incorporation of two types of the fiber protein a fiber mosaic HAdV vector.

In summary, in this thesis we have developed fiber-modified HAdV vectors incorporating chimeric fiber proteins of species B HAdVs. In addition, we have shown how to incorporate two different types of fiber proteins in a single viral particle using genetic modification of the fiber genes. We have also demonstrated the potential utilization of each vector for cancer therapy in *in vitro* systems. For a further understanding of the vector tropism as well as its potential recognition and clearance by the immune system, thorough *in vivo* investigation is required. Hopefully, our findings in this study would contribute to the progress in the development of a practical vector for cancer therapy in human use.

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APPENDIX

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE
DESIGNATION OF NOT HUMAN SUBJECTS RESEARCH

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

DATE: 2/16/10

MEMORANDUM

TO: **Miho Murakami**
Principal Investigator

FROM: Sheila Moore, CIP
Sheila Moore, CIP
Director, UAB OIRB

RE: Request for Determination—Human Subjects Research
IRB Protocol #N100110002 – Fiber Modification of Adenoviral Vectors for Cancer Gene Therapy

An IRB Member has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is **not** subject to FDA regulations and is **not** Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

SM/cro

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