lial [70–72], lymphocytes [69,73,74], fibroblasts [68,69,75], hematopoietic cells [76] and monocyte-derived dendritic cells [77,78] and require an escalating dose of vector in order to achieve efficient gene transfer. This in turn increases vector-associated immunogenic toxicities. Hence, lack of Ad vector specificity is directly linked to the induction of massive systemic immune responses. Furthermore, localizing gene transfer by Ad vectors to specific cell types is likely to reduce immunogenic toxicities by allowing lower does to be administered. Therefore, there is a strong rationale for the development of tropism-modulated Ad vectors of enhanced specificity and gene transfer efficiency. In recent years, there have been significant efforts to improve Ad transduction efficiency to targets that are resistant to Ad infection due to CAR deficiency [79,80]. Ad vectors with the native tropism completely ablated have also been successfully developed [42,81].

*4.2.1. Increasing transduction of target cells by bispecific conjugates*

Douglas et al. [82] first reported the bispecific conjugate-based approach. They conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and was shown to redirect the Ad infection of target cells via the folate receptor at a high efficiency. Furthermore, when complexed with an Ad vector carrying the gene for herpes simplex virus thymidine kinase, the Fab–folate conjugate mediated the specific killing of cells that overexpress the folate receptor [82]. After that, the Fab fragment of the anti-fiber monoclonal antibody has been utilized to conjugate with several other ligands. For example, the Fab has been conjugated with (1) basic fibroblast growth factor (FGF2) to target various cells [83], including Kaposi's sarcoma cell lines [84], and ovarian cancer cells [85–87]; (2) mAb against the epidermal growth factor receptor (EGFR) to target glioma cells [64] and squamous cell carcinoma [88]; (3) an anti-CD40 mAb fragment to target dendritic cells [77]; (4) anti-angiotensin converting enzyme (ACE) mAb to target pulmonary endothelial cells [89]; (5) and an Hc fragment of tetanus toxin to target neuronal cells [90]. Theoretically, in this approach, any conjugates with one component directed against the Ad capsid and the second component directed against the cell surface protein can be applied to increase transduction of the target cells. The component directed against the Ad capsid can be the neutralizing Fab fragment of an anti-fiber monoclonal antibody as described above, a neutralizing anti-Ad knob single-chain antibody (scFV) [91–95] or the extracellular domain of CAR [96,97]. The targeting cell-binding moiety can either be natural molecules or man-made peptides identified by phage display technique [92,98,99].

*4.2.2. Increasing transduction of target cells by genetic modification of the fiber*

Since the fiber stretches out from the capsid and plays a central role for Ad binding to the native receptors, many attempts have focused on genetic modifications of the fiber. This approach can be divided into two main sub-approaches.

One is to incorporate ligands into the fiber knob. In order not to destroy the fiber trimerization, and to facilitate the ligands to access their cognate receptor, the HI loop and C-terminal of the Ad fiber knob have been found to be most appropriate to accommodate the foreign ligands [100,101]. We and other groups showed that Ad vectors containing the RGD motif in the HI loop greatly increased by as much as 3 orders the efficiency of gene delivery to a variety of CAR-deficient cells including primary and established ovarian cancer cells [102,103], squamous cell carcinoma [104], leukemia [105,106], rhabdomyosarcoma [107], dendritic cells [108,109], glioma [94,105], pancreatic cancer cells and primary human endothelia cells [103]. These results indicate that the integrin family could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by RGD-modified Ad vectors. Currently, the RGD-modified Ad vector is being tested in a phase I clinical trial of ovarian cancer and recurrent cancer of the oral cavity and oropharynx [80]. Besides the RGD motif, there are reports of inserting the peptide SIGYPLP (Ser-Ile-Gly-Tyr-Pro-Leu-Pro), which was discovered by phage display to show high affinity to vascular endothelial cells [92], in the HI loop to increase transduction of vascular endothelial cells [110] and cancer cells [111]. In terms of the incorporation of foreign peptide into the C-terminal of fiber knob, we and another group found that a peptide containing seven lysine residues could be

inserted to increase transduction efficiency to a variety of CAR-deficient cells [69,112].

Another sub-approach is fiber-pseudotyping. Since Ads that belong to subgroup B, such as Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50, recognize CD46 as the primary cellular receptors [113,114], fiber (knob, or knob and shaft) substitution could alter the tropism of Ad5 vectors. This strategy was first reported by Gall et al. [115]. They constructed a chimeric Ad5 vector by replacing the Ad5 fiber gene with the fiber gene from Ad7 (although Ad7 belongs to subgroup B, its receptor has not been identified), and found altered tropism to Ad5 vectors. Shayakhmetov et al. [76] constructed an Ad5 vector with a chimeric fiber (Ad5 tail/Ad35 shaft/Ad35 knob) and showed increased transduction of CD34+ cells relative to the unmodified Ad5 vector. Also, the same group incorporated the Ad11 fiber to Ad5 and found enhanced infection of human hematopoietic progenitor cells [116]. An Ad5-based vector containing the Ad16 fiber shaft and knob domains yielded an 8- and 64-fold increase in gene transfer to endothelial and smooth muscle cells as compared to Ad5 [117] and an 150-fold increase in gene transfer to cultured synoviocytes as compared to Ad5 [118]. Replacement of only the knob domain of the fiber can also alter viral tropism. Stevenson et al. [119] demonstrated that replacement of the Ad5 fiber knob with the Ad3 fiber knob (which, though it also belongs to subgroup B, has been shown not to use CD46 as a high-affinity attachment receptor [113,114,120]) improved gene delivery to human fibroblasts and head and neck cancer cells when compared to unmodified Ad5. Takayama et al. [121] generated a dual-knob mosaic Ad virus by incorporating both Ad5 and Ad3 knobs in the same particle, which displayed infectivity enhancement and tropism expansion by utilizing either receptor, CAR or the Ad3 receptor, for virus attachment to cells.

In addition to the approaches at the level of transduction, increasing transduction of the target cells can also be achieved to some extent at the level of transcription by using cell-specific promoters. Furthermore, combining transductional and transcriptional targeting seems to be an attractive strategy to enhance the targeting effect of Ad vectors. Reynolds et al. [122] reported that in an Ad vector, the combination of transductional targeting by linking the Fab fragment of an anti-Ad5 knob antibody to the anti-ACE (pulmonary endothelial marker) monoclonal antibody mAb, and an endothelial-specific promoter (flt-1) resulted in a synergistic, 300,000-fold improvement in the selectivity of transgene expression for the lung versus the usual site of vector sequestration, the liver. Barnett et al. [123] obtained great synergistic targeting effect in cancer cells using a similar dual targeting strategy with the target molecule being epidermal growth factor receptor (EGFR), which is overexpressed on many tumor cells; the specific promoter was the osteocalcin gene 2 promoter, which has specificity for osteoblasts and osteoblastic metastatic lesions. Nicklin et al. [111] also observed the synergistic targeting effect in certain cancer cells by combining transductional targeting (incorporating the SIGYPLP peptide into the fiber knob) with transcriptional targeting (via the FLT-1 promoter).

#### *4.2.3. Ablation of the native tropism*

Though the above tropism-modified Ad vectors could greatly improve transduction efficiency to many CAR-deficiency cells, when systemically administered, sometimes vector dissemination, resulting in liver accumulation, is still unavoidable. To create a strictly targeted Ad vector, two basic requirements are thought to be necessary: interaction of Ad with its native receptors must be completely removed and novel tissue-specific ligands must be added to the virus capsid (Fig. 5).

The capsid proteins determine the tropism of Ad. The fact that Ad5 uses multiple receptors such as CAR, αv integrin and heparan sulfate to transduce various cells implies that the Ad5 capsid must be multi-engineered to abolish its native tropism. Several groups including us have shown that vectors with the ablation of only CAR-binding, i.e., vectors in which the AB, DE, or FG loop of the fiber knob was mutated, do not change the systemic gene-transfer properties [124–127]. Vectors with the ablation of only αv-integrin-binding also show similar or slightly decreased liver transduction compared with wild-type Ad vectors [126]. Furthermore, the length [128–130] and the KKTK motif of the fiber shaft [81] have been reported to influence Ad5-mediated in vivo gene transfer. We supposed that Ad5 tropism would be determined by at least three factors: the fiber knob, the fiber shaft and the RGD motif at the penton base. Thereby, we developed a triple-mutant Ad5 vector by

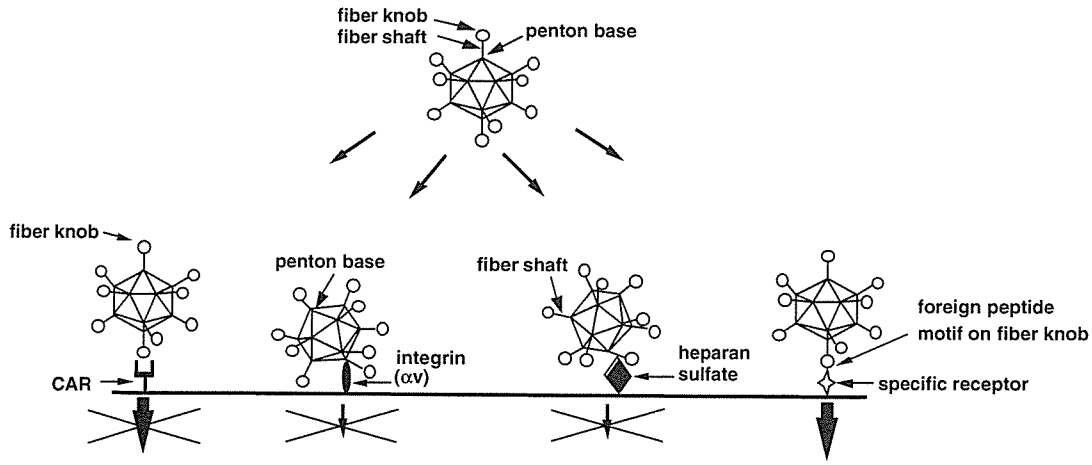


Fig. 5. Schematic diagram of targeted Ad vectors with the original tropism ablated. With the triple mutations in the fiber knob, fiber shaft and penton base, the CAR-, αv intergrin- and heparan sulfate-binding activities of the Ad viral capsid are completely ablated. Targeting effects can be achieved by either the bispecific conjugate or the foreign ligands incorporated into the fiber knob.

(1) mutating the fiber knob to ablate CAR-binding interaction, (2) replacing the Ad5 shaft with a shorter shaft from Ad35, which contains no KKTK motif to ablate binding with heparan sulfate, and (3) depleting the RGD motif at the penton base to ablate αv-integrin-binding interaction. As expected, this triple-capsid-mutant Ad5 vector exhibited little tropism to any organs (Fig. 6). Compared with the wild-type Ad5 vector, it showed 30,000-fold lower mouse liver transduction [42]. This indicates that to ablate the original tropism of Ad vectors, all three parts of the capsid (fiber knob, shaft and penton base) associated with the original tropism should be simultaneously blocked. Smith et al. [81] utilized a similar strategy and reached the same conclusion as ours.

Due to the multiple mutation of the capsid, the triple-mutant Ad vectors could not be produced by the original protocol using 293 cells. To support the propagation of our triple-mutant Ad5 vector, we generated a mutant 293 cell line stably expressing wild-type Ad5 fiber protein (Fiber-293 cells). It is possible to produce this mutant Ad vector to high titer using Fiber-293 cells. Furthermore, for the convenient display of foreign ligands, both the HI loop and C-terminal region of the fiber knob and the region of the RGD motif of the penton base were designed to have unique restriction sites. Therefore, by using our simple in vitro ligation method, the targeting ligands can easily be displayed in the capsid of our triple-mutation Ad vector [42]. We suggest that our triple-mutation Ad vector provide for a platform for future targeted Ad vector development. Future efforts should be directed into exploring novel ligands for specific tissue targeting.

Our triple-mutant Ad vector described here should be easily combined with other approaches such as transductional targeting, transcriptional targeting and even deletion of viral genomes to create less immunogenic vectors. Such combination will no

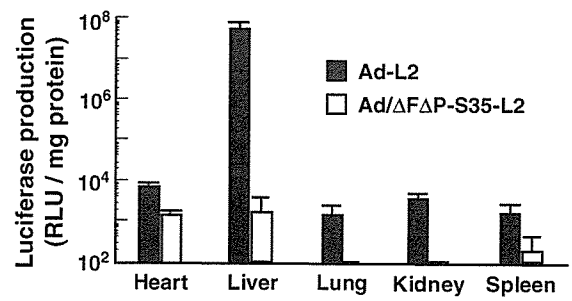


Fig. 6. Biodistribution of a transgene product (luciferase) in mice after the systemic administration of Ad-L2 (conventional Ad vectors) or Ad/ΔFΔP-S35-L2 (with triple mutations in the regions of fiber knob, fiber shaft and penton base). Ad-L2 or Ad/ΔFΔP-S35-L2 ($3.0\times10^{10}$ VP) were intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean±S.E. of five mice. The data in this figure were published in our previous paper [42].

doubt improve the kinetics of Ad vectors to better meet the requirements of clinical trial.

### 4.3. Cationic liposome complexation or PEGylation

The most straightforward approach for circumventing Ad-induced immunogenic toxicities has been the use of cationic liposome and polyethylene glycol (PEG) to shield the vectors from the host immune system.

Cationic liposomes have shown promise as a gene delivery tool for plasmid DNA due to their simplicity, safety and efficiency in some tissues [131]. Several groups have observed that cationic liposome-conjugated Ad vectors greatly increase gene transduction efficiency to a variety of cell and tissue types that are especially resistant to Ad infection, including vascular tissue [132], human smooth muscle cells [133], airway epithelia [134] and human glioma cells [135]. Fasbender et al. [134] suggested that viral binding was dependent on an electrostatic interaction with the cell surface, that viral entry did not require an interaction of the Ad fiber protein with the cell surface, and that cationic liposome-conjugated Ad vectors entered cells via a pathway different from that utilized by Ad alone. Use of a more efficient delivery system could allow a smaller dose of Ad vector to be administered for therapeutic effects, thereby decreasing the total immune response. Yoshida's group noted that mice injected with cationic liposome-conjugated Ad vectors produced fewer anti-Ad antibodies compared with an equivalent dose of unconjugated Ad vectors, and cationic liposome-conjugated Ad vectors were less susceptible to inactivation by neutralizing antibodies than Ad vectors alone [136,137]. The increased transduction efficiency, reduced antigenicity, and attenuated susceptibility to neutralizing antibodies might be beneficially multiplied for redosing.

Complexation with PEG (PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase blood persistence of therapeutic proteins such as erythropoietin (EPO), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) etc. [138–140]. Covalent attachment of PEG to the surface of the Ad vector might prolong persistence in the blood and circumvent neutralization of Ad vectors by antibodies [141]. There is a report that PEGylated Ad vectors exhibited a 4-fold slower clearance rate than the non-PEGylated Ad vectors [38]. Furthermore, coupling of PEG to the viral capsids attenuates the ability of the vector to infect antigen presenting cells, thereby reducing inflammatory responses. Animals administered with the PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression upon readministration of unmodified Ad vectors in the lung [141,142]. However, PEGylation might lead to loss of infectivity [38]. The activated PEG reacts preferentially with the ε-amino terminal of lysine residues on the capsid, including the hexon, fiber and penton base, which might lead to decreased infectivity. Experimental optimization of the PEGylation reaction so that PEG occupies only 70 to 80% of the available sites on virus capsid proteins shielded vectors from antibody neutralization and retained viral infectivity [143,144]. Nonetheless, compared with local readministration, the results of systemic readministration of the PEGylated Ad vectors were less encouraging. Levels of transgene expression achieved by systemic readministration of the PEGylated Ad vectors were on the order of 1000- to 10,000-fold lower than that seen in animals carrying no neutralizing antibodies against Ad; these levels were also significantly lower than those observed with readministration of these modified vectors in the lung [142]. Thus, the exact nature of the immune response against the PEG modified Ad must be characterized further.

### 4.4. Immune intervention

The success of long-term gene therapy by Ad vectors depends on finding ways to avoid/attenuate the induction of immune responses to both the vector and the transgene product. Some advances have been made in the development of novel strategies to disrupt or modulate immune responses in various animal models; these approaches include inhibiting cell-mediated immune responses to prolong transgene expression, and inhibiting humoral immune responses to permit readministration of the vector.

One straightforward approach is to disrupt the inflammatory immune response by inhibition of NF κB activation, thereby blocking release of inflammatory cytokines like TNF-α and IL-6 [145,146]; other relatively simple approaches include pretreatment by

or coadministration of anti-inflammatory drugs such as steroids [147–150]. Lieber et al. [145] obtained persistent Ad-delivered hAAT [human alpha (1)-antitrypsin] expression in bcl-2 transgenic mice for longer than 3 months by coadministrating an Ad vector expressing an IκBα supersuppressor to unravel the role of virus-induced NF κB activation [151,152]. They found that to confer vector persistence, simultaneous expression of bcl-2, an antiapoptotic protein [153], was required to block virus-induced apoptosis, while NF κB protection was inactivated by the IκBα supersuppressor. Kolb et al. [147] investigated the use of topical corticosteroids in improving gene expression after repeated injection of Ad vectors into mouse lungs. They showed that budesonide given around exposure to Ad to the lung significantly helped maintain high levels of the expressed transgene protein in bronchoalveolar lavage fluid after as many as four consecutive injections of virus at 2-week intervals. Furthermore, they observed that the improved transgene expression in budesonide-treated animals was associated with a reduction, but not prevention of neutralizing antiviral antibodies.

Another approach is the use of immunosuppressive drugs to inhibit cell-mediated immune functions, just as they are used in clinical trials for organ transplantation. The central role of CD4+ T cells in the activation of cellular and humoral immune response has focused immunosuppressive strategies towards blockade of costimulatory molecules. Recombinant murine CTLA4Ig (an inhibitor of the CD28/B7 pathway) and the anti-CD40 ligand antibody block costimulatory interactions between T cells and antigen presenting cells. Blocking of costimulatory signals has been shown to inhibit T-cell activation in several animal models of Ad vector gene therapy. Kay et al. [154] observed that when the anti-CD40 ligand and recombinant murine CTLA4Ig were coadministered around the time of primary Ad vector administration, Ad-mediated gene expression was maintained up to 1 year in mouse livers, and persistent secondary Ad-mediated gene expression lasted for at least 200-300 days even after the immunosuppressive effects of these agents were no longer present. However, neither agent alone allowed transduction after secondary vector administration. Jooss et al. [155] described a strategy that aimed to inhibit CD4+ T cell activation by transiently administering CTLA4Ig at the time an E1-deleted Ad vector is administered to the liver or lung. In the lung, CTLA4Ig treatment significantly blocked the formation of neutralizing antibodies, allowing efficient readministration of the vector, whereas transgene expression was only moderately prolonged. In contrast, CTLA4Ig did not suppress neutralizing antibody formation in the context of liver gene therapy, but resulted in more stable gene expression. These observations suggest that it may be possible to obtain persistence as well as secondary Ad-mediated gene transfer with transient inhibition of the CD28/B7 pathway at the time of virus instillation, especially in a local route. However, immune intervention in systemic Ad administration seems to be much complex. Moreover, this immunomodulation might compromise the host immune response. Thus, the clinical utility is uncertain.

### *4.5. Optimization of in-cis acting elements*

As discussed in the previous section, the immunogenic toxicities of Ad vectors can lead to clearance of the transduced cells and only transient transgene expression. The severity of the immunogenic toxicities is intimately connected with vector dose. However, to achieve anatomically detectable and physiologically relevant levels of transgene expression, the number of Ad particles to be injected is always too large to avoid severe immunological side effects [156,157]. One simple but practical way to alleviate this kind of immunogenic toxicities is to improve the expression efficiency of each viral particle unit so that the number of Ad particles to be injected can be decreased.

Transgene expression can be modulated at both the transcriptional and post-transcriptional levels. We systematically investigated the ability of transcriptional regulatory elements [promoter, enhancer, intron and poly(A) sequences] and post-transcriptional regulatory elements to maximize the transgene expression efficiency from each Ad vector unit [158]. Our optimized Ad vector, Ad-WCMVL2, with an expression cassette containing the human CMV promoter/enhancer, intron A, the luciferase gene, the Woodchuck hepatitis virus post-transcriptional regulation element (WPRE), and bovine growth hormone (BGH) poly (A), showed more than 700-fold luciferase expression in mouse liver than the Ad vector, Ad-

CMVL1, with an expression cassette containing only the human CMV promoter/enhancer, the luciferase gene and BGH poly (A), when they were intravenously administered into the mouse [158]. It may be deduced that to express transgenes at a therapeutic level, the in-cis acting elements of our optimized Ad vector, Ad-WCMVL2, may decrease, by several orders of magnitude, the number of viral particles that must be injected as compared with Ad-CMVL1 with the expression cassette containing only the CMV promoter/enhancer and the BGH poly(A) as in-cis acting elements which are considered to be one of the most strongest in-cis acting element combination and are widely used in clinical trials and gene function studies [159]. It also means that immunogenic toxicities induced by Ad particles could be greatly alleviated by using the in-cis acting elements of Ad-WCMVL2 to construct therapeutic Ad vectors. Gerdes et al. [160] demonstrated that the murine CMV promoter was much stronger than the human CMV promoter in the context of Ad vectors in all the cell lines tested, including nonhuman primate and human cell lines. By using this murine CMV promoter, the investigators observed that the Ad vector dose required to achieve sufficient transduction could be reduced 100-fold and the cellular inflammation and viral cytotoxicity associated with the delivery of Ad vectors into the rat brain could be completely eliminated.

In the context of HD Ad vectors, the inclusion of a human centromeric region and a matrix attachment region as in-cis acting elements improved the maintenance of the Ad vector genome and the transgene expression level [57]. It can be concluded that even for the Ad vectors that have an extremely high transduction efficiency, the in-cis acting elements can be optimized to obtain maximized transgene expression.

### *4.6. Integrating Ad vectors*

For replication-deficient Ad vectors, the existence of episomal Ad DNA presents the disadvantage of not integrating for long-term expression of the delivered foreign gene. In the case that stable genetic alternation needs to be maintained in dividing cells, integrating vectors are at present the tools of choice. Though integration is not a guarantee of stable transcription, since transgene expression from integrated vector genomes can be gradually silenced over time in some cases [161], at present the best way to get long-term transgene expression is to integrate the foreign gene into the host genome.

To incorporate integration machinery to Ad, a variety of hybrid vectors combining the highly efficient DNA delivery of Ad with the integrating machinery of adeno-associated viruses (AAV), retroviruses, and transposons have been emerging [162]. The unique biology of AAV has stimulated considerable efforts toward the development of AAV-based integrating Ad vectors. Such integrating Ad-AAV hybrid vectors devoid of all Ad viral genes were successfully developed by Lieber et al. [163] and Recchia et al. [164]. Lieber's hybrid Ad-AAV vectors displayed in vitro an integration frequency comparable to that reported for AAV vectors, and high transgene expression at a level comparable to the first-generation Ad vectors [163].

Baun's group did extensive studies exploiting the retrovirus integrating machinery to realize permanent Ad-delivered transgene expression. By flanking the transgene cassette in the Ad vector with the cis-acting elements from Moloney murine leukemia virus in a unique arrangement, they obtained high integration frequencies (between 5% and 11%) in rat spleen cells [165]. In consideration of the high transduction efficiency, this integration rate should be high enough to stably keep transgene expression at therapeutic levels for many gene therapy applications.

Kay and colleagues addressed the challenge of genome persistence by exploiting the promiscuous integration capabilities of the Sleeping Beauty (SB) transposon in Ad-mediated gene delivery [166]. SB transposon is a member of the Tc1/mariner superfamily of transposons, and undergoes cut-and-paste transposition through a DNA intermediate, mediated by the SB transposase [167]. By incorporating the SB integration machinery into an HD Ad vector containing human coagulation factor IX, therapeutic levels of human coagulation factor IX were sustained for more than 6 months in mice undergoing extensive liver proliferation [166].

However, at the current time there are great concerns about the risk of insertional mutagenesis due to random integration of vectors into the host genome. The recent clinical trial in France using

retroviral vectors in a gene therapy approach for X-linked severe combined immunodeficiency disease (X-SCID) had been taken as the milestone success in human gene therapy [168]. Unfortunately, two of the 11 patients treated during this X-SCID trial developed a leukemia-like disorder [169,170]. Now it is known that the cause of induced leukemia in X-SCID trial is attributed to the retrovirus vector integration in proximity to the LMO2 proto-oncogene promoter, which leads to aberrant transcription and expression of LMO2 [171]. Recent study suggests that the AAV vector preferentially integrates into active genes, though at present it is not clear if this will contribute to the risk of developing a malignancy [172]. Recchia's Ad/AAV hybrid vector system showed 35% of DNA integration was site-specific at the AAVS1 site in hepatoma cells, with one HD Ad vector expressing Rep78 and the second HD vector carrying a transgene flanked by AAV-ITRs [164]. However, excess Rep expression is toxic to the cells and may result in rearrangement of AAVS1 without transgene integration [173]. To avoid potential mutation risks of random integration, episomally replicating Ad vectors might be a potential alternative choice for long-term expression. Leblois et al. [174] inserted a loxP flanked Epstein-Barr virus (EBV) replicon into an Ad vector. Expression of Cre recombinase from a second Ad vector has been shown to release the transcription unit that could replicate coordinately during the cell cycle. Kreppel et al. [175] incorporated the episomally replicating machinery to an HD vector by utilizing the DNA recombinase FLPe to circularize the genome containing the EBV replicon, and obtained long-term transgene expression in proliferating cells. However, data of episomally replicating Ad vectors from in vivo studies are lacking.

### *4.7. Serotype switch or animal Ad vectors for readministration*

In many gene therapy applications, lifelong expression might be needed, which would require readministration of vectors following the eventual loss of therapeutic transgene expression if the vectors have no integration or episomal replication machinery. However, readministration of an Ad vector will require the circumvention of the humoral immune response directed against the original vector capsid. Studies indicated that fiber-substituted Ad5 vectors containing fiber proteins of another serotype could not evade the humoral immune response against Ad5 [115], because hexon proteins of the capsid are the major targets of host-neutralizing antibodies in Ad5 infection [115,176,177]. This suggests that capsid partially modified Ad5 vectors do not meet the long-term need. Hence, the most practical approach to overcoming the obstacle of pre-existing antibodies is the use of alternative serotypes.

Morral et al. [178] observed in baboons that the hurdle of readministration due to the humoral response to an Ad5 vector was overcome by use of an Ad2 vector expressing hAAT. Their data further suggest that long-term expression of transgenes should be synergized by combining the reduced immunogenicity and toxicity of HD vectors with sequential delivery of vectors of different serotypes. However, it might be difficult to completely avoid cross-humoral response between Ad2 and Ad5 in all species including humans, as both Ads belong to the same subgroup and have high homogenicity. To address this issue, we and other groups have developed Ad35 vectors (subgroup B) [179–182] and have found that antibodies to Ad5 vector do not affect the transduction efficiency by Ad35 vector (Sakurai et al. unpublished observation, 181). Furthermore, while there is more than a 50% prevalence in adult humans of neutralizing antibodies to Ad5, less than 10% of individuals have anti-Ad35 neutralizing antibodies [181].

Since many humans are pre-exposed to human Ad, Ads from non-humans have been developed as vector systems for gene delivery. Mouse [183], avian [184], bovine [185], canine [186], porcine [187] and chimpanzee [188] Ad have been tested as vectors for various applications to overcome the barriers of pre-existing neutralizing antibodies. These animal-derived Ad vectors might be useful in evading humoral immune responses to human Ad5 for readministration. However, prior to their clinical application, safety issues such as oncogenicity should be addressed.

Readministration might be a solution to the transient transgene expression of Ad vectors when it is necessary. To balance the gain and loss in safety, efficacy and economy are the key points. The potential serotypes and animal origin Ads are limited.

Furthermore, this approach needs a complex set of clinical products.

## 5. Summary

Among all the vectors for gene delivery, no single one can meet all the requirements for all gene therapy applications. Similarly, for Ad vectors, no single approach can overcome the hurdle of immunogenic toxicities. It seems that HD vectors might serve as basis for combination with other approaches. It is clear that the ultimately ideal Ad vectors will have low or no immunogenic toxicities, and specifically transduce interested tissues/cells, with high, persistent and regulatable transgene expression. It is also clear that there is still a long way before research reaches this ideal.

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# Efficient regulation of gene expression using self-contained fiber-modified adenovirus vectors containing the tet-off system

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**Abstract**

Previously, we developed single adenovirus (Ad) vectors that contained the gene of interest in the E1 deletion region and the transactivator gene for the tetracycline-controllable expression system in the E3 deletion region. In the present study, we improved the Ad vector-mediated tetracycline-controllable expression system by the fiber modification of Ad. We developed fiber-modified Ad vectors containing the tet-off system, which are effective in overcoming the limitations of conventional Ad vectors, specifically their inefficient gene transfer into cells lacking the primary receptor, the coxsackievirus and adenovirus receptor (CAR). Ad vectors containing the tet-off system with an Arg–Gly–Asp (RGD) peptide in the HI loop of the fiber knob or the Ad type 35 fiber greatly improved transduction efficiency (more than 1–2-log orders) into the cells lacking CAR expression but expressing αv integrin or CD46, respectively. They exhibited vastly higher regulation of gene expression by doxycycline. The combination of fiber-modified Ad vectors and the tetracycline-controllable expression system should offer a powerful tool for gene therapy and gene transfer experiment.


*Keywords:* Gene therapy; Adenovirus vector; Fiber; Tetracycline; Gene regulation

## 1. Introduction

Regulated transgene expression systems provide a valuable tool for both studies of gene function and safe, effective gene therapy. The tetracycline-controllable expression system has been most widely used, because it has several advantages over other regulated

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gene expression systems; e.g., tight on/off regulation, high inducibility, fast response times, lack of a pleiotropic effect due to the use of tetracycline operons derived from bacteria, and a well-characterized inducer (tetracycline), etc. [1,2]. The tet-off system [1], which uses the tetracycline-responsive transcriptional activator (tTA), and the tet-on system [2], which uses the reverse tetracycline-responsive transcriptional activator (rtTA), provide negative and positive control of transgene expression, respectively.

Previously, we developed single adenovirus (Ad) vectors containing the gene of interest in the E1 deletion region and *tTA* or *rtTA* gene in the E3 deletion region, and showed that Ad vectors containing the tet-off system mediated high regulation of gene expression by doxycycline [3]. However, Ad vectors containing the tet-on system exhibit a much lower level of regulation (less than 10–30-fold of regulation factor (ratio of maximum transgene expression to minimum transgene expression)) of transgene expression than Ad vectors containing the tet-off system [3]. We therefore developed Ad vectors that overcame this lower level of regulation in the tet-on system by introducing the latest generation rtTA and tetracycline-controlled transcriptional silencer [4].

In the present study, in order to further improve the self-contained Ad vectors having a tetracycline-controllable expression system, fiber-modified Ad vectors were employed to deliver the transgene cassette. Ad type 5 along with Ad type 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. However, Ad vector-mediated transduction is quite low when the cells do not express sufficient levels of the primary receptor, called the coxsackievirus and adenovirus receptor (CAR) [5,6]. Modification of fiber protein is an attractive strategy for overcoming the limitations imposed by the CAR-dependence of Ad infection [7]. We and other groups have shown that Ad vectors containing the RGD motif in the HI loop of the fiber knob and Ad vectors containing the Ad type 35 fiber greatly increase the efficiency of transduction to a variety of CAR-deficient cells [7–16]. αv integrin [8] and CD46 (membrane cofactor protein) [17,18] could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by fiber-modified Ad vectors containing the RGD peptides or Ad type 35 fiber, respectively [7]. We here show the successful generation of self-contained Ad vectors having both fiber-modification and the tet-off system. We report the application and characteristics of gene transfer and regulation of fiber-modified Ad vectors containing the tet-off system.

## 2. Materials and methods

### 2.1. Cells

SK HEP-1 (endothelial cell line from human liver) [19], LNZ308 (human glioblastoma multiforme; kindly provided by Dr. M. Tada, Hokkaido University, Hokkaido, Japan) [20], and 293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). NIH3T3 cells (mouse embryo fibroblast; Human Science Research Resources Bank, Japan, JCRB0615) were cultured with minimum essential medium (MEM) supplemented with 10% FCS. CHO (Chinese hamster ovary) cells were cultured with MEM alpha medium supplemented with 10% FCS. CHO–CD46 cells were stable transformants generated by the transfection of pcDNA3.1-CD46 (described below) into CHO cells and selection with hygromycin (GIBCO-BRE, Rockville, MD). CHO–CD46 cells stably expressed the BC2 isoform of human CD46 (Fig. 3B).

### 2.2. Plasmid and virus

Vector plasmid pAdHM49 was constructed as described below. First, pHM15-ITR10 was constructed by the ligation of NdeI/MunI-digested pHM15-ITR16 [4] and NdeI/MunI-digested pEco-ITR10, a derivative of pEco-ITR9 [13]. pHM15-ITR10 contains the right end of the Ad genome (bp 27332-right end with deletion of bp 27865 to bp 30995) with a Csp45I site in the E3 deletion region, an XbaI site between the E4 region and 3′ inverted terminal repeat (ITR), and an Ad type 35 fiber (Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail)-coding sequence. Then, Csp45I/XbaI-digested pAdHM19 was ligated with Csp45I/NdeI-digested pHM15-ITR10, resulting in pAdHM49-1. Finally, pAdHM49 was constructed by changing the ClaI site at the right end of the Ad genome of pAdHM49-1 into a PacI site using

oligonucleotides (5′-CGTTAATTAA-3′) (PacI recognition sequences are underlined). pAdHM49 has a complete E1/E3-deleted Ad genome with an I-CeuI, a SwaI, and a PI-SceI site in the E1 deletion region, a Csp45I site in the E3 deletion region, an XbaI site between the E4 region and 3′ITR, PacI sites at both ends of the Ad genome, and the chimeric fiber-coding sequence of the Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail. pAdHM51, which contains an I-CeuI, a SwaI, and a PI-SceI site in the E1 deletion region, a ClaI site in the E3 deletion region, an XbaI site between the E4 region and 3′ITR, PacI sites at both ends of the Ad genome, and a Csp45I site between positions 32679 and 32680 of the Ad genome (residues threonine-546 and proline-547 of the fiber protein), was similarly constructed (Fig. 1). pAdHM51-RGD was constructed by insertion of oligonucleotide 1 (5′-CGGCCTGTGACTGCCGCGGAGACTGTTTCTGCGATG-3′) and oligonucleotide 2 (5′-CGCATCGCAGAAACAGTCTCCGCGGCAGTCACAGGC-3′), which correspond to the RGD (RGD-4C) peptide (CDCRGDCFC) with high affinities to integrins (αvβ3 and αvβ5) [21], into a Csp45I site of pAdHM51.

pHM13-CMVi-tTA was constructed by cloning the cytomegalovirus (CMV) promoter with intron A (derived from pGeneGrip (Gene Therapy Systems, San Diego, CA)), *tTA* gene (derived from pTet-Off (Clontech, Palo Alto, CA)), and SV40 late P(A) signal sequences into pHM13 [22]. pAdHM20-tTA1, pAdHM49-tTA1, and pAdHM51-RGD-tTA1 were constructed by the ligation of ClaI-digested pHM13-CMVi-tTA into ClaI-digested pAdHM20 [22], Csp45I-digested pAdHM49, and ClaI-digested pAdHM51-RGD, respectively. pHM5-TRE-SEAP and pHM5-TRE2-SEAP are shuttle plasmids for insertion of the gene of interest into the E1 deletion region of the vector plasmid. pHM5-TRE-SEAP contains the sequence of the tet-responsive promoter (derived from pTRE (Clontech)), the secreted alkaline phosphatase (SEAP) gene (derived from pSEAP2-Control (Clontech)), and the bovine growth hormone (BGH) poly (A) signal. pHM5-TRE2-SEAP contains the tight tet-responsive promoter (derived from pTRE-Tight (Clontech)) instead of the tet-responsive promoter in pHM5-TRE-SEAP. pAdHM20-tTA1-SEAP4 was constructed by the ligation of I-CeuI/PI-SceI-digested pHM5-TRE-SEAP with I-CeuI/PI-SceI-digested pAdHM20-tTA1. pAdHM20-tTA1-SEAP6, pAdHM49-tTA1-SEAP6, and pAdHM51-RGD-tTA1-SEAP6 were constructed by the ligation of I-CeuI/PI-SceI-digested pHM5-TRE2-SEAP with I-CeuI/PI-SceI-digested pAdHM20-tTA1, pAdHM49-tTA1, and pAdHM51-RGD-tTA1, respectively.

To generate the virus, pAdHM20-tTA1-SEAP4, pAdHM20-tTA1-SEAP6, pAdHM49-tTA1-SEAP6,

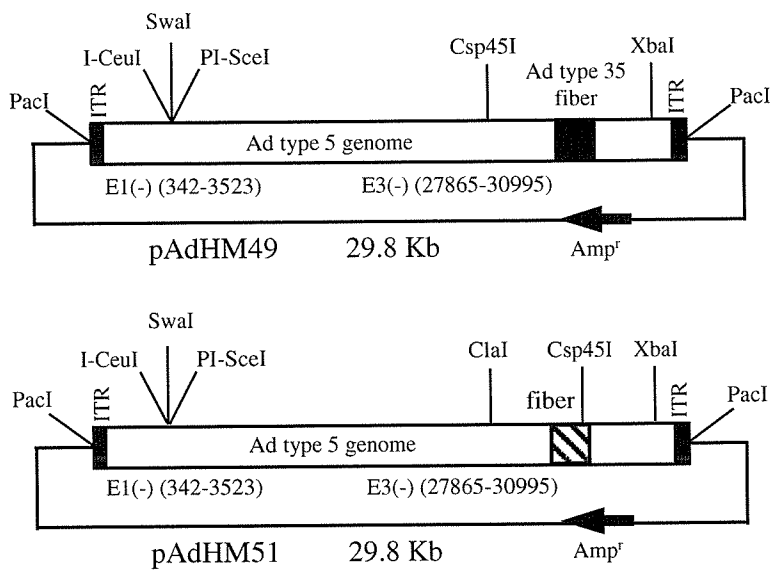


Fig. 1. The structure of the vector plasmids pAdHM49 and -51.

and pAdHM51-RGD-tTA1-SEAP6 were digested with PacI and purified by phenol–chloroform extraction and ethanol precipitation. Linearized DNAs were transfected into 293 cells plated in a 60 mm dish with SuperFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Viruses (AdOff-SEAP4, AdOff-SEAP6, AdF35-Off-SEAP6, AdRGD-Off-SEAP6) were prepared as described previously [23]. Viruses were purified with CsCl gradient centrifugation, dialyzed with the solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −70 °C. Ad-SEAP2, which contains the CMV promoter/enhancer-driven SEAP expression cassette in the E1 deletion region, was previously prepared [4]. Determination of virus particle titers was accomplished spectrophotometrically by the methods of Maizel et al. [24]. The Ad vectors used in the present study are summarized in Table 1.

pcDNA3.1-CD46 was constructed by insertion of the gene coding the human CD46 BC2 isoform, which was generated by PCR using primers (forward, 5′-atggagcctcccggccgccgcgagtgtccc-3′; reverse, 5′-cgcggccgcctattcagcctctctgctctgctg-3′), into pcDNA3.1-Hyg (Invitrogene). Sequencing of the gene coding the human CD46 BC2 isoform in pcDNA3.1-CD46 verified that the clone contained the appropriate sequences.

### 2.3. Adenovirus-mediated gene transduction in vitro

The cells ($1 \times 10^4$ cells) were seeded into a 96-well dish. On the following day, they were transduced with the Ad vector (500 vector particles (VP)/cell) for 1.5 h. The cells transduced with the Ad vector containing the tet-off system were cultured with medium containing 10 ng/ml of doxycycline (Clontech). Tet-system-proved FCS (Clontech), a tetracycline-free serum determined to be optimal for the tetracycline-controllable expression system, was used as FCS. Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, the SEAP level in the medium was measured using a Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech).

### 2.4. Flow cytometric analysis

CHO, CHO–CD46, SK HEP-1, and LNZ308 cells were suspended in staining buffer (phosphate buffered saline containing 1% bovine serum albumin) containing fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD46 antibody (Pharmingen, San Diego, CA, USA). After washing with the sorting solution, the stained cells ($10^4$ cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson).

## 3. Results and discussion

### 3.1. Newer vector plasmids

In order to combine fiber-modified Ad vectors and the tetracycline-controllable expression system, newer Ad vector systems were first developed (Fig. 1). The vector plasmid pAdHM49 contains the chimeric fiber proteins (Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail), and an I-CeuI/SwaI/PI-SceI site, a Csp45I site, and an XbaI site in the E1 deletion region, the E3 deletion region and the region between the E4 region and 3′ITR, respectively. The vector plasmid pAdHM51 contains a unique Csp45I site in the HI loop-coding sequence of the fiber knob as well as an I-CeuI/SwaI/PI-SceI site in the E1 deletion region, a ClaI site in the E3 deletion region, and an XbaI site between the E4 region and 3′ITR. Any foreign ligands can be easily displayed in the HI loop of the fiber knob of Ad vectors by cloning its gene into their regions using simple in vitro ligation as

Table 1
Adenovirus vectors used in the present study

| Name | Fiber type | E1 deletion region | E3 deletion region |
|---|---|---|---|
| Ad-SEAP2 | Type 5 fiber | CMV promoter+SEAP | |
| AdOff-SEAP4 | Type 5 fiber | TRE promoter+SEAP | CMV promoter+tTA |
| AdOff-SEAP6 | Type 5 fiber | TRE-Tight promoter+SEAP | CMV promoter+tTA |
| AdRGD-Off-SEAP6 | RGD peptide in the HI-loop of the fiber knob | TRE-Tight promoter+SEAP | CMV promoter+tTA |
| AdF35-Off-SEAP6 | Chimeric type 5 fiber tail and type 35 fiber knob and shaft | TRE-Tight promoter+SEAP | CMV promoter+tTA |

previously described [12,14]. In both vector plasmids, the gene of interest can also be introduced into the E1 deletion region as well as the E3 deletion region and the region between the E4 region and 3′ITR by simple in vitro ligation using a series of shuttle plasmids developed previously [4,22]. In the present study, the coding sequence of the RGD peptide, which binds with high affinities to both αvβ3 and αvβ5 [21], was introduced into the HI loop-coding region of the fiber knob (a Csp45I site) of pAdHM51.

### 3.2. Effect of the tet-responsive element on the regulated gene expression

Previously, we developed self-contained Ad vectors having a tetracycline-controllable expression system [3,22]. These vectors contain the original tet-responsive promoter, which is a hybrid of the tet-responsive element (TRE) containing a seven tet-operator (tetO) sequence and the minimal CMV promoters. Recently, a modified tet-responsive promoter derived from pTRE-Tight (Clontech) was developed. Both promoters contain a seven tetO sequence. The sequences among the tetO sequence and the minimal CMV promoter differ between the original and the modified tet-responsive promoters. The modified tet-responsive promoter contains a deleted minimal CMV promoter.

First, we performed a functional comparison of these two promoters cloned in the Ad vectors (Fig. 2). AdOff-SEAP4 contains the original tet-responsive promoter-driven SEAP expression cassette in the E1 deletion region as well as the tTA expression cassette in the E3 deletion region. AdOff-SEAP6 contains a modified tet-responsive promoter instead of the original tet-responsive promoter in AdOff-SEAP4. SK HEP-1 and HeLa cells, both of which express sufficient levels of CAR, were transduced with AdOff-SEAP4 or AdOff-SEAP6 at 500 VP/cell, and the basal and induced SEAP production in the medium, in which the cells were cultured without doxycycline or with 10 ng/ml of doxycycline, respectively, were measured. Both cell types transduced with AdOff-SEAP6 showed lower basal SEAP production than those transduced with AdOff-SEAP4, although the induced SEAP production in the cells transduced with AdOff-SEAP6 was also lower than that with AdOff-SEAP4. Importantly, the regulation factor[s]

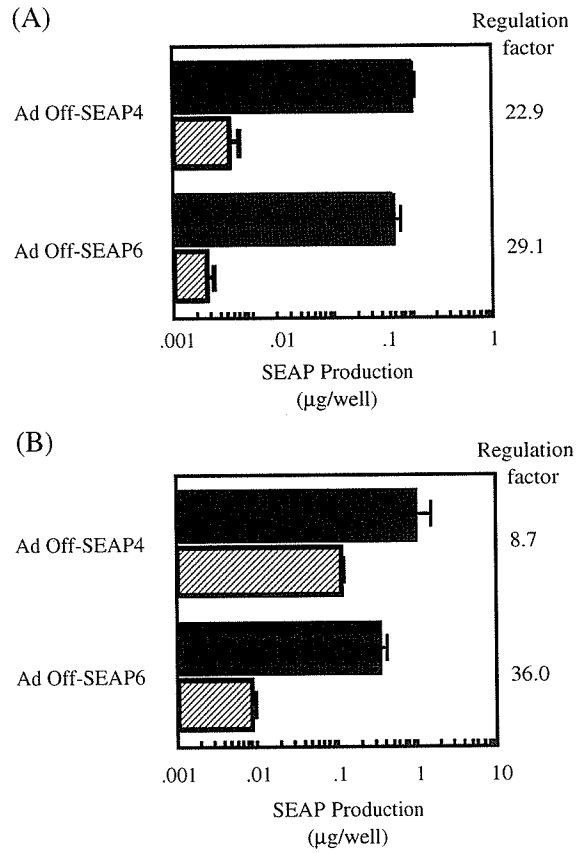


Fig. 2. Effect of the tet-responsive element on the regulated SEAP production. SK HEP-1 (A) and HeLa (B) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP4 or AdOff-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the two cell types were as follows: SK HEP-1, 0.0006; HeLa, 0.0009 (μg/well).

(ratio of maximum SEAP production to basal SEAP production) by AdOff-SEAP6 were higher than those by AdOff-SEAP4; the regulation factor by AdOff-SEAP6 were 29.1 (SK HEP-1 cells) and 36.0 (HeLa cells), while those by AdOff-SEAP4 were 22.9 (SK HEP-1 cells) and 8.7 (HeLa cells). These results suggest that the single Ad vector containing the modified-tet responsive promoter and the tet-off system showed lower basal transgene expression and higher regulation of transgene expression by doxycycline,