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Regulatory Aspects of Oncolytic Virus Products

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Abstract: Many types of oncolytic viruses, wild-type virus, attenuated viruses and genetically-modified viruses, have been developed as an innovative cancer therapy. The strategies, nature, and technologies of oncolytic virus products are different from the conventional gene therapy products or cancer therapy products. From the regulatory aspects to ensure the safety, efficacy and quality of oncolytic viruses, there are several major points during the development, manufacturing, characterization, non-clinical study and clinical study of oncolytic viruses. The major issues include 1) virus design (wild-type, attenuated, and genetically engineered strains), 2) poof of concept in development of oncolytic virus products, 3) selectivity of oncolytic virus replication and targeting to cancer cells, 4) relevant animal models in non-clinical studies; 5) clinical safety, 6) evaluation of virus shedding. Until now, the accumulation of the information about oncolytic viruses is not enough, it may require the unique approach to ensure the safety and the development of new technology to characterize oncolytic viruses.

Keywords: Gene therapy, cancer therapy, replicating virus.

INTRODUCTION

Oncolytic virus therapy has been developed as a new wave of cancer therapies. These therapies are dependent on the replication-selective nature of these viruses in tumor cells, which provides the marked breaths of cancer therapy. More than one century ago, evidence of oncolytic activity caused by replicating viruses was reported, and it was known the viruses could induce tumor lysis. Using these studies as a point of departure, rare but dramatic responses in cancer patients recovering from viral infections were reported. In the early development of oncolytic virus therapy, wild-type viruses with low pathogenicity to normal tissues, or attenuated viruses were selected for the treatment of cancer patients. However, some adverse events, such as the development of encephalitis in immune compromised patients, were reported [1-3]. Other works reported the oncolytic activity of wild-type or attenuated oncolytic viruses to be transient or limited to the site of injection [4-8]. Recently, attention has focused on overcoming the disadvantages of wild-type or attenuated oncolytic virus therapy, and many genetically modified viruses have been developed for cancer treatment. Progress in the development of genetically engineered oncolytic viruses has been based on recent advances in our understanding of the molecular biology of cancer and viruses, and on advances in genetic engineering technologies of virus genomes. Although many gene therapy clinical studies for the treatment of cancer have been conducted during the past decade using replication-incompetent virus vectors, these studies have not achieved satisfying results. Tumor-selective replicating viruses have been suggested to have an advantage over conventional gene therapy vectors for cancer therapy, and oncolytic viruses, especially genetically modified viruses, must be considered to be a special type of gene therapy product since their principle is directly associated with the transfer of the viral genome as the therapeutic gene [9]. In the present report, we review the development of oncolytic viruses as gene therapy products or attenuated virus products with specific reference to the associated regulatory issues.

Oncolytic virus therapy is based on several strategies, including tumor-selective replication, tumor-selective targeting, and/or the minimization of toxicity to normal cells. Many types of viruses have been utilized in oncolytic virus therapy; including adenovirus, herpes simplex virus (HSV), reovirus, Newcastle disease virus (NDV), vaccinia, measles virus, vesicular stomatitis virus (VSV) and Sendai virus [10-13]. During the development of oncolytic virus products, a number of major issues have arisen with respect to ensuring the quality, safety and efficacy of the products: 1) virus design (wild-type, attenuated and genetically engineered strains); 2) proof of concept in the development of oncolytic virus products; 3) the selectivity of oncolytic virus replication and targeting to cancer cells; 4) relevant animal models in non-clinical studies; 5) clinical safety; and 6) the evaluation of virus shedding. Since the strategies, nature and technologies of oncolytic virus products are different from those of conventional gene therapy products or cancer therapy products, we discuss the regulatory aspects of the development of oncolytic viruses in the present paper.

VIRUS DESIGN AND PRODUCT DEVELOPMENT

While many types of viruses are utilized for oncolytic virus therapies [10, 11, 14], selective replication in tumor cells is essential for the efficacy and safety of oncolytic viruses. Wild-type viruses and naturally occurring attenuated viruses are known to possess the ability to infect and kill

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transformed cells such as tumor cells. For example, VSV, NDV and reovirus have been used as oncolytic viruses with inherent tumor-selectivity [12, 15-18]. In the case of wild-type viruses or attenuated viruses, the mechanism underlying the virus-selectivity to tumor cells has been analyzed from various points of view. For instance, reovirus has an inherent preference for replicating cells with dysregulated growth factor-signaling cascades by Ras activation [17, 19]. Attenuated strains from HSV-1 have been reported to be potential anti-cancer therapeutics and have necessitated a thorough investigation into the molecular basis of host-cell permissiveness to HSV [20-22]. Since in the development of wild-type or naturally attenuated oncolytic viruses, intentional genetic modification is not introduced into the virus genome, the tumor selective targeting, tumor-selective replication and pathogenicity of these oncolytic viruses are dependent on the method of selecting the strains. During the characterization of wild-type and attenuated oncolytic viruses, it is extremely important to analyze the molecular mechanisms of the tumor-selectivity and tumor-specific replication, as well as the genetic stability, etc.

There are several strategies used to design and construct the tumor selectivity of genetically engineered oncolytic viruses [10, 11, 23, 24]. One strategy is to engineer viruses through the deletion of virus genes critical for viral replication in normal cells but non-essential in tumor cells. For example, adenovirus E1B55K is responsible for binding and inactivating p53. E1B55K-deleted adenovirus has acquired the ability to propagate and induce cell death selectively in tumor cells, and then to spread to surrounding cells and tissues [25, 26]. Another strategy is transcriptional targeting, in which tumor- or tissue-specific promoters that are active in tumor cells are inserted into the viral genome to regulate the expression of essential viral genes and to restrict viral replication in tumor cells. The promoters used are categorized into different types; telomerase reverse transcriptase (TERT), S-phase of cell cycle promoter (E2F) and hypoxia promoter (HIF-1) are utilized as targeting promoters to all tumor cells; and prostate-specific antigen (PSA) promoter, α-fetoprotein (hepatoma) promoter and albumin promoter for hepatoma are used as tissue-specific promoters. The third strategy is the receptor-mediated targeting of replication-competent viruses to tumor cells [23, 27]. In this approach, the natural virus tropism of a replication-competent virus is adapted to the tumor cells through genetic modification of the virus coat or envelopes. This approach requires the ablation of the natural virus infection pathway and the introduction of new ligands into the virus surface without disrupting viral integrity. To improve the selectivity of oncolytic viruses to tumor cells and to improve safety, constructs with multiple modifications to tumor selectivity are developed. In addition, to improve efficacy, oncolytic viruses carrying a transgene (armed oncolytic viruses) have recently been developed [14, 28, 29].

In the endorsement of clinical trials or the approval of oncolytic virus products, the scientific rational of the design of the oncolytic virus construct must be thoroughly justified. Furthermore, non-clinical studies should be designed in each case to verify predictions of efficacy and safety. In this context, it is recommended that animal models be developed to provide valuable evidence concerning the non-clinical safety of these products and to assess their proof of concept.

MANUFACTURING AND CHARACTERIZATION OF ONCOLYTIC VIRUSES

There are to date no specific regulatory guidelines related to the manufacture and characterization of oncolytic viruses for clinical use. However, guidelines concerning the manufacturing and characterization of gene therapy products have been issued by the Food and Drug Administration (FDA), the European Agency for the Evaluation of Medicinal Products (EMEA) and the government of Japan [30-33]. While there are some differences in the format of these guidelines, the underlying scientific principles are not fundamentally different, and the scientific principles covered by the above guidelines for gene therapy products may be applicable to the evaluation of the manufacturing and characterization of oncolytic viruses.

The guidelines should require that the rational behind the selection of the virus, helper virus and cells used in the production of the virus be described, including the genetic construct of the oncolytic virus, and of the helper virus if applicable. In cases in which the manufacturing method of the oncolytic virus in question has a specific feature, a justification of the feature must be included. The DNA or RNA sequence of the oncolytic virus must be clarified as much as possible, with particular attention to any regions of the virus genome that have been modified. Sequence analysis should be performed by a validated method which must also be described. In the case of genetically modified oncolytic viruses, a full explanation must be provided of the origin and detailed derivation of all constitutive components, such as promoters, enhancers, duplication units, selection markers and other base sequence parts from other constructs of oncolytic virus DNA or RNA. When a transgene is inserted into an oncolytic virus sequence, the construing procedure, amplification method, purification method and any flanking area that may have an important effect on the transcription, translation or stability of the translation sequence must be described in detail.

Cell and Virus Bank System

It is important to establish a cell and virus banking system in order to maintain consistency in the production of oncolytic viruses. A cell banking system for manufacturing oncolytic viruses should be designed and fully characterized; in general, a cell banking system includes a Master Cell Bank (MCB) and Working Cell Bank (WCB) for producing and packaging cell lines ("International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH)" guideline Q5D [34]). The concept of a two-tiered cell bank, in which the MCB is used to generate WCBs, is generally considered to be the most practical approach to providing a supply of cell substrates for the continued manufacture of oncolytic virus products. The strategy for providing a continued supply of cells from their cell bank(s) must be described, including the anticipated utilization rate of the cell bank(s) for production, the expected intervals between the generations of new cell bank(s), and the criteria for qualification of cell bank(s). Generally, the MCB is created first, usually directly from an

initial clone or from a preliminary cell bank derived from an initial clone. A WCB is derived from one or more containers of the MCB. It is the WCB which is typically used to directly provide cells for the manufacturing process.

The characterization and testing of banked cells is critical for the control of oncolytic viruses. The MCB and WCB must be subject to extensive quality control, and the established guidelines will be applicable to evaluate these banks (ICH Q5D or regional guidelines). Characterization of the MCB allows the sponsor to assess the source with regard to the presence of cells from other lines, adventitious agents, endogenous agents and molecular contaminants (e.g., toxins or antibiotics from the host organism). A characterization of the criteria for cell banks may include appearance, identity, cell count and viability for cell banks, as well as the sterility, mycoplasma, purity, absence of adventitious viruses and absence of specific human viruses. The objective of this testing is to confirm the identity, purity and suitability of the cell substrates for manufacturing oncolytic viruses.

Another dimension of cell characterization is the appropriateness for their intended use in oncolytic virus production. There are two concerns for cell substrate stability: the consistent production of the oncolytic virus and the retention of production capacity during storage under defined conditions.

A two-tiered virus banking system, a Master Virus Bank (MVB) and a Working Virus Bank (WVB), is generally constructed for the production of oncolytic virus products. The MVB and WVB should also be characterized and should be subject to extensive quality control; the established guidelines may be applicable to evaluate these banks (ICH Q5D or regional guidelines). A characterization of the criteria for virus banks may include particle number and infectious titer, sterility, mycoplasma, purity, absence of adventitious viruses, replication-competent viruses and molecular variants, and absence of specific human viruses. A MVB is produced from an initial seed virus, and a WVB is derived from one or more containers of the MVB. The MVB and WVB should be produced under optimized culture conditions for viral growth and harvest, and be thoroughly defined, giving an efficient and reproducible downstream purification process. The quality, safety and efficacy of the final formulation of the oncolytic virus in which the virus will be stable for long periods in storage is guaranteed by the establishment of a well-defined virus banking system.

Sponsors are also encouraged to employ state-of-the-art methods and technological improvements in oncolytic virus characterization and testing as they become available, as long as the specificity, sensitivity and precision of the newer methods are at least equivalent to those of existing methods. Since oncolytic virus therapy has been developed only very recently, technologies for the characterization of oncolytic viruses remain to be fully elucidated. There remain a number of technical challenges concerning oncolytic virus testing and product characterization.

Manufacturing of Oncolytic Viruses

The manufacturing method for oncolytic viruses (vectors) must be fully described, including a description of the cells used for the production of the oncolytic viruses, and all relevant data on the name, manufacturing method, pathogenicity, propagation, growth factor dependence, phenotype, tumorigenecity, stability, etc. Changes in the character of the original cells must be clarified and the cultivation method of the cells described, including the medium, serum, antibiotics or other growth factors used. When a packaging cell is used, the manufacturing procedure, selection, identification method and isolation purification method to produce a seed cell strain must be established and characterized and the genetic stability of any sequence inserted into a packaging cell should also be described. The purification method of oncolytic viruses should be described in detail. When scaling up for manufacturing, suitable validation data to describe the contents should be made available. Additionally, descriptions must be included of the preparation and storage method of the MCB and WCB, as well as of the controlling and renewal methods. Finally, tests should be performed to confirm that the cell phenotype between the lots has not changed during the cultivation period. The test period, method and results of any safety tests necessary for quality control should be justified.

Genetic Stability, Replication-Competent Viruses (RCVs) and Molecular Variants

The biological and manufacturing consistency of oncolytic viruses depends primarily on the genetic stability of virus genomes as well as on the nature of the producer cells. A well-defined cell banking system partially ensures the genetic stability of oncolytic viruses during the manufacturing process. Relevant concerns include the generation of replication-competent viruses (RCVs) and molecular variants during manufacturing. RCVs in products can be evaluated by bioamplification assay [35]. Semiquantitative bioamplification systems are used to detect recombination that may occur during manufacturing. These assays are able both to detect contaminating wild-type viruses and to evaluate the genomic stability of an engineered virus; the oncolytic virus product tested in such assays requires multiple passages. Wild-type viruses that contaminate a preparation of engineered oncolytic virus are also typically detected using quantitative polymerase chain reaction (PCR) [36]. When the molecular variants are predicted by recombination, a preparation of engineered oncolytic virus should be tested for molecular variants using quantitative PCR [35].

The selection of the cell substrate is another strategy to minimize the appearance of recombinant RCVs. In the case of adenovirus production, the amount of replication-competent adenovirus (RCA) detected is higher in batches produced in conventional cell lines (e.g., 293 cells [37]) compared to that found in batches produced in recently engineered cell lines (e.g., PER.C6 cells [38]) because of the sequence homology between the engineered adenoviruses and the integrated sequences in the 293 cells. PER.C6 cells are reported to have produced no RCAs in large-scale adenovirus product [39]. A novel cell line, C139 derived from A549 human lung cancer cells, it has been reported that the E1a and E1b coding regions were reduced to their minimal sequences and that native promoters were deleted [40]. Additionally, it has been reported that neither RCAs nor

cytopathogenic effect (CPE)-inducing replication-deficient recombinants are generated during the production of adenoviral vector using C139.

Adventitious Agent Testing

For more information on adventitious agent testing, ICH guidance Q5A: "Guidance on Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" [41] should be referred if applicable. *In vitro* viral testing should be performed on the MCB, WCB, MVB, WVB, CAL (cells at the limit of *in vitro* cell age used for production) and oncolytic virus products. In such testing, the test sample (for example, MCB or MVB) is inoculated onto various susceptible indicator cell lines such as the human or primate cell line. The choice of cells used would depend on the species of origin of the oncolytic virus and the cell substrate used. In addition, the test would include a measure of both cytopathic and hemadsorbing viruses.

In vivo viral assays should be carried out by inoculating the test sample (MCB, MVB, etc.) into animals such as adult and suckling mice, and embryonated hen's eggs. Additional testing of guinea pigs, rabbits or monkeys should also be considered. An assay for species-specific viruses should be performed and rodent cell lines used during production should be tested for rodent-specific viruses. If human cell lines are used in the therapeutic product, testing for human pathogens, including cytomegalovirus (CMV), human immunodeficiency virus (HIV) -1 and 2, human T-cell lymphotropic virus (HTLV) 1 and 2, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus(HCV), B19, and other human viral agents should be performed if appropriate. Human viral agents may be tested using a PCR-based test system. Retroviral contamination in MCB and MVB must be analyzed using reverse transcriptase (RT) assays and electron microscopic analysis.

A Adventitious agent testing may be particularly challenging for oncolytic virus products. One strategy is based on the neutralization of the oncolytic virus with a specific antibody prior to testing for adventitious agents both for *in vitro* and *in vivo* assays. This is done to prevent the product from generating false positive results.

Batch Release

Typical release criteria for oncolytic viruses may be based on appearance, identity, virus titer, virus particles, potency, purity (including product-related and process-related impurity), safety (sterility, mycoplasma, endotoxins, adventitious viruses), and characterization.

In general, a standard potency assessment for oncolytic virus products is carried out based on the ratio of virus particle numbers to infectious titers in the final products. For replication-defective adenoviral vectors, the ratio of adenovirus vector particles to infectious titers [must] be less than 30:1 to satisfy FDA guidelines [31]. At present, however, no specific guidelines exist concerning the acceptable ratio of physical and infectious titers for oncolytic viruses. In addition to measuring tumor cell line killing in an *in vitro* assay, biological characteristics such as viral infectious titer and particles to infectious unit ratio are very useful to ensure batch-to-batch consistency.

Furthermore, it will be necessary to develop standardized testing procedures that will allow the evaluation and comparison of the selectivity, potency and toxicity of oncolytic viruses. If applicable, a wild-type strain may be useful as a positive control in order to allow normalization of infectivity and viral replication capability between different cell types. In the case of adenovirus products, the Adenovirus Type 5 Reference Material established by FDA and Adenovirus Reference Material Working Group can be used to standardize and normalize quantification methods, particle numbers and infectious titers [42].

The following numerical estimation of expressing the selectivity of an oncolytic virus effect in tumor cells compared to a normal cell line has been proposed [43]:

$$\text{Selectivity} = \frac{[\text{effect of oncolytic virus in tumor cell/effect of wt in tumor cell}]}{[\text{effect of oncolytic virus in normal cell/ effect of wt in normal cell}]}$$

"Effect" can be measured in terms of viral yield (or burst size, cytopathic effect (IC50 values), viral late protein expression or viral DNA replication level. Progeny production is considered the most relevant for the desired analytical effect [9].

NON-CLINICAL STUDIES

Non-clinical studies of oncolytic virus products are crucial to establish the safety and proof of concept in advance of clinical trials. Since oncolytic viruses have very unique safety issues, such as the emergence of genetic variants and the risk of germline transmission, international harmonized guidelines such as ICH S6 or other documents do not seem to be applicable. The design of non-clinical studies for oncolytic virus products will depend on the type and nature of the specific oncolytic virus product.

In each case, the objective and design of the animal studies, including the type of animal and the reason for selecting it, must be explained. Non-clinical studies should be designed to obtain data that demonstrate the proof-of-principle of oncolytic virus products and that provide biosafety features: target organs for toxicity, risk of shedding, etc. In general, animal models are valuable for testing non-clinical safety and assessing proof of concept, however, they have certain limitations. Some viruses have species-specific susceptibility to viral infection and replication, there may be differential tropism in tumor-xenograft models, and it is impossible to model all aspects of the immune response. Differences in the tissue architecture between animal models and humans are also an important factor, especially with respect to the role of the connective tissue and intermixed normal cells. Nevertheless, animal models are useful to address specific questions such as the choice of a route of administration, biodistribution, safety/toxicity, dose selection and dose regimen. When possible, the selectivity of virus replication has also been studied using *in vivo* models.

Non-clinical safety studies should initially include single-dose toxicity studies, repeated-dose toxicity studies if

appropriate, and biodistribution studies, which can incorporate pharmacodynamic-like endpoints. The type and duration of repeated-dose non-clinical safety studies should be considered dependent on the type of oncolytic virus and potential concerns about insertional mutagenesis, for instance.

A quantitative nucleic acid amplification test (NAT) may be used to investigate tissue distribution and the persistence of the oncolytic virus sequence in biodistribution studies. If the administered oncolytic virus sequence is detected in unintended tissues or organs by a NAT assay, this may assist in determining mRNA for the gene product by RT-PCR. Additionally, RT-NAT immunological-based assays may be used to in verify the duration and level of expression of the gene product to detect functional protein.

According to the potential risk of inadvertent germline integration of oncolytic viruses based on the vector type, route of administration and patient population, it may also be necessary to determine whether or not the nucleic acid of the oncolytic virus is incorporated into gonads. The key element in the assessment of inadvertent germline integration is a well-conducted biodistribution study in animal models.

CLINICAL STUDIES AND SAFETY EVALUATION

Due to the complexity of oncolytic virus products and the limited usefulness of animal models, many concerns, including safety issues, remain to be addressed in early-phase clinical studies. These studies must focus on safety and definitions of dose and clinical strategy.

Clinical Pharmacokinetics

With respect to the pharmacokinetics of oncolytic viruses, both quantitative PCR and infectivity assays may be used to monitor patients. In some cases, quantitative monitoring of administered oncolytic virus genomes may provide data supporting viral replication in permissive tissues.

DOSE SELECTION AND DOSE REGIMEN

The dose selection and dose regimen of oncolytic viruses in clinical use should be carefully assessed to ensure their safety and evaluate their toxicity for humans. In one case, a patient who was enrolled in a phase-one clinical trial using replication-deficient adenovirus vector died due to the injection of high-dose vector [44]. The use of replication-competent viruses poses special concerns since the replication of the virus in the patient may lead to an enhanced level of and prolonged exposure to the virus, and thus might increase the risk of virus-induced toxicity. Replication competence of the oncolytic virus does not eliminate the need to perform dose ranging studies to determine an effective dose level.

Viral Shedding and Risks of Contact Person

Since data on viral shedding are limited with respect to oncolytic viruses [29, 45-49], precautions to reduce the risk of exposure of healthcare providers, family members and other patient contacts should be taken. The possibility of virus shedding and the site of shedding may depend on the site and route of administration, dose and replication efficacy of the virus in question. During clinical trials, risk must be monitored not only in the patient but also in the general population. The monitoring of viral shedding and mobilization/recombination with wild-type strains is recommended, and the need to establish long-term follow-up programs must be evaluated. However, all of these measures should take into account the special aspects of oncolytic viruses, such as the disease spectrum and pathogenicity of wild-type strains versus modified oncolytic viruses, the level of pre-existing immunity in the general population, and the ability of the virus to evade the immune system. The onset of tropism-modified versions of some oncolytic viruses requires additional control since the tropism can be narrowed or expanded, and previous clinical experience with non-modified strains is not necessarily relevant.

Schedule for Patient Follow-Up

It is important to establish observation and follow-up schedules for patients, including investigation of the *in vivo* distribution of the administered oncolytic virus, survival and functional expression terms of the gene of interest, symptoms caused by replication-competent viruses or molecular variants, etc. If the oncolytic virus is found to be transiently distributed to the gonads in animal studies, assaying patient semen for the presence of vector may be considered. However, if the patient population is sterile, or if the patient has a severe disease condition with short life expectancy, monitoring of semen samples may not be necessary.

ABBREVIATIONS

HSV	=	Herpes simplex virus
VSV	=	Vesicular stomatitis virus
NDV	=	Newcastle disease virus
TERT	=	Telomerase reverse transcriptase
E2F	=	S-phase of cell cycle promoter
HIF-1	=	Hypoxia-inducible factor-1
PSA	=	Prostate-specific antigen
FDA	=	Food and Drug Administration
EMEA	=	European agency for the evaluation of medicinal products
MCB	=	Master cell bank
WCB	=	Working cell bank
MVB	=	Master virus bank
WVB	=	Working virus bank
RCV	=	Replication-competent virus
PCR	=	Polymerase chain reaction
RCA	=	Replication-competent adenovirus
CPE	=	Cytopathogenic effect

EOP cells = End of production cells

CMV = Cytomegalovirus

HIV = Human immunodeficiency virus

HTLV = Human T-cell lymphotropic virus

EBV = Epstein-Barr virus

HBV = Hepatitis B virus

HCV = Hepatitis C virus

RT = Reverse transcriptase

NAT = Nucleic acid amplification test

ICH = International conference on harmonization of technical requirements for registration of pharmaceuticals for human use

REFERENCES

[1] Southam, C. M.; Moore, A. E. Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus. *Cancer* **1952,** *5*, 1025-1034.

[2] Huebner, R. J.; Rowe, W. P.; Schatten, W. E.; Smith, R. R.; Thomas, L. B. Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* **1956,** *9*, 1211-1218.

[3] Russell, S. J. Replicating vectors for gene therapy of cancer: risks, limitations and prospects. *Eur. J. Cancer* **1994,** *30A*, 1165-1171.

[4] Yamanishi, K.; Takahashi, M.; Kurimura, T.; Ueda, S.; Minekawa, Y. Studies on live mumps virus vaccine. 3. Evaluation of newly developed live mumps virus vaccine. *Biken J.* **1970,** *13*, 157-161.

[5] Csatary, L. K.; Eckhardt, S.; Bukosza, I.; Czegledi, F.; Fenyvesi, C.; Gergely, P.; Bodey, B.; Csatary, C. M. Attenuated veterinary virus vaccine for the treatment of cancer. *Cancer Detect. Prev.* **1993,** *17*, 619-627.

[6] Shimizu, Y.; Hasumi, K.; Okudaira, Y.; Yamanishi, K.; Takahashi, M. Immunotherapy of advanced gynecologic cancer patients utilizing mumps virus. *Cancer Detect. Prev.* **1988,** *12*, 487-495.

[7] Okuno, Y.; Asada, T.; Yamanishi, K.; Otsuka, T.; Takahashi, M.; Tanioka, T.; Aoyama, H.; Fukui, O.; Matsumoto, K.; Uemura, F.; Wada, A. Studies on the use of mumps virus for treatment of human cancer. *Biken J.* **1978,** *21*, 37-49.

[8] Heicappell, R.; Schirrmacher, V.; von Hoegen, P.; Ahlert, T.; Appelhans, B. Prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells. I. Parameters for optimal therapeutic effects. *Int. J. Cancer* **1986,** *37*, 569-577.

[9] European Medicines Agency (EMEA). Report from the CPMP Gene Therapy Expert Group Meeting 26th-27th February 2004. EMEA/CPMP/1879/ 04/Final, **2004**.

[10] Ries, S. J.; Brandts, C. H. Oncolytic viruses for the treatment of cancer: current strategies and clinical trials. *Drug Discov. Today* **2004,** *9*, 759-768.

[11] Lin, E.; Nemunaitis, J. Oncolytic viral therapies. *Cancer Gene Ther.* **2004,** *11*, 643-664.

[12] Russell, S. J. RNA viruses as virotherapy agents. *Cancer Gene Ther.* **2002,** *9*, 961-966.

[13] Kinoh, H.; Inoue, M.; Washizawa, K.; Yamamoto, T.; Fujikawa, S.; Tokusumi, Y.; Iida, A.; Nagai, Y.; Hasegawa, M. Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases. *Gene Ther.* **2004,** *11*, 1137-1145.

[14] Aghi, M.; Martuza, R. L. Oncolytic viral therapies - the clinical experience. *Oncogene* **2005,** *24*, 7802-7816.

[15] Barber, G. N. VSV-tumor selective replication and protein translation. *Oncogene* **2005,** *24*, 7710-7719.

[16] Stojdl, D. F.; Lichty, B. D.; tenOever, B. R.; Paterson, J. M.; Power, A. T.; Knowles, S.; Marius, R.; Reynard, J.; Poliquin, L.; Atkins, H.; Brown, E. G.; Durbin, R. K.; Durbin, J. E.; Hiscott, J.; Bell, J. C. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **2003,** *4*, 263-275.

[17] Norman, K. L.; Lee, P. W. Reovirus as a novel oncolytic agent. *J. Clin. Invest.* **2000,** *105*, 1035-1038.

[18] Stoeckel, J.; Hay, J. G. Drug evaluation: Reolysin--wild-type reovirus as a cancer therapeutic. *Curr. Opin Mol. Ther.* **2006,** *8*, 249-260.

[19] Shmulevitz, M.; Marcato, P.; Lee, P. W. Unshackling the links between reovirus oncolysis, Ras signaling, translational control and cancer. *Oncogene* **2005,** *24*, 7720-7728.

[20] Kimata, H.; Takakuwa, H.; Goshima, F.; Teshigahara, O.; Nakao, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Effective treatment of disseminated peritoneal colon cancer with new replication-competent herpes simplex viruses. *Hepatogastroenterology* **2003,** *50*, 961-966.

[21] Takakuwa, H.; Goshima, F.; Nozawa, N.; Yoshikawa, T.; Kimata, H.; Nakao, A.; Nawa, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Oncolytic viral therapy using a spontaneously generated herpes simplex virus type 1 variant for disseminated peritoneal tumor in immunocompetent mice. *Arch. Virol.* **2003,** *148*, 813-825.

[22] Teshigahara, O.; Goshima, F.; Takao, K.; Kohno, S.; Kimata, H.; Nakao, A.; Nishiyama, Y. Oncolytic viral therapy for breast cancer with herpes simplex virus type 1 mutant HF 10. *J. Surg. Oncol.* **2004,** *85*, 42-47.

[23] Mathis, J. M.; Stoff-Khalili, M. A.; Curiel, D. T. Oncolytic adenoviruses - selective retargeting to tumor cells. *Oncogene* **2005,** *24*, 7775-7791.

[24] Post, D. E.; Khuri, F. R.; Simons, J. W.; Van Meir, E. G. Replicative oncolytic adenoviruses in multimodal cancer regimens. *Hum. Gene Ther.* **2003,** *14*, 933-946.

[25] Bischoff, J. R.; Kirn, D. H.; Williams, A.; Heise, C.; Horn, S.; Muna, M.; Ng, L.; Nye, J. A.; Sampson-Johannes, A.; Fattaey, A.; McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **1996,** *274*, 373-376.

[26] Ries, S.; Korn, W. M. ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus. *Br. J. Cancer* **2002,** *86*, 5-11.

[27] Nakamura, T.; Peng, K. W.; Harvey, M.; Greiner, S.; Lorimer, I. A.; James, C. D.; Russell, S. J. Rescue and propagation of fully retargeted oncolytic measles viruses. *Nat. Biotechnol.* **2005,** *23*, 209-214.

[28] Hermiston, T. W.; Kuhn, I. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther.* **2002,** *9*, 1022-1035.

[29] Freytag, S. O.; Khil, M.; Stricker, H.; Peabody, J.; Menon, M.; DePeralta-Venturina, M.; Nafziger, D.; Pegg, J.; Paielli, D.; Brown, S.; Barton, K.; Lu, M.; Aguilar-Cordova, E.; Kim, J. H. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.* **2002,** *62*, 4968-4976.

[30] US Food and Drug Administration (FDA). Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy. **1998**.

[31] US Food and Drug Administration (FDA). Guidance for FDA Review Staff and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs). DRAFT GUIDANCE. **2004**.

[32] European Medicines Agency (EMEA). Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products. CPMP/BWP/3088/99. **2001**.

[33] Ministry of Health, Labour and Welfare (MHLW). Notification of Pharmaceutical Affairs Bureau, Yaku-hatsu No.1062: Guidance for Assuring the Quality and Safety of the Gene Therapy Products. (in Japanese) **1995**.

[34] ICH Harmonized Tripartite Guideline. ICH-Q5D: Derivation and Characterization of Cell Substrates Used for Production of BioTechnological/Biological Products. **1997**.

[35] Working, P. K.; Lin, A.; Borellini, F. Meeting product development challenges in manufacturing clinical grade oncolytic adenoviruses. *Oncogene* **2005,** *24*, 7792-7801.

[36] Bernt, K.; Liang, M.; Ye, X.; Ni, S.; Li, Z. Y.; Ye, S. L.; Hu, F.; Lieber, A. A new type of adenovirus vector that utilizes homologous recombination to achieve tumor-specific replication. *J. Virol.* **2002,** *76*, 10994-11002.

[37] Graham, F. L.; Smiley, J.; Russell, W. C.; Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **1977,** *36*, 59-74.

[38] Fallaux, F. J.; Bout, A.; van der Velde, I.; van den Wollenberg, D. J.; Hehir, K. M.; Keegan, J.; Auger, C.; Cramer, S. J.; van Ormondt, H.; van der Eb, A. J.; Valerio, D.; Hoeben, R. C. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum. Gene Ther.* **1998,** *9*, 1909-1917.

[39] Lusky, M. Good manufacturing practice production of adenoviral vectors for clinical trials. *Hum. Gene Ther.* **2005,** *16*, 281-291.

[40] Farson, D.; Tao, L.; Ko, D.; Li, Q.; Brignetti, D.; Segawa, K.; Mittelstaedt, D.; Harding, T.; Yu, D. C.; Li, Y. Development of Novel E1-Complementary Cells for Adenoviral Production Free of Replication-Competent Adenovirus. *Mol. Ther.* **2006,** *14*, 305-311.

[41] ICH Harmonised Tripartite Guideline. ICH-Q5A: Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin. **1997.**

[42] Hutchins, B.; Sajjadi, N.; Seaver, S.; Shepherd, A.; Bauer, S. R.; Simek, S.; Carson, K.; Aguilar-Cordova, E. Working toward an adenoviral vector testing standard. *Mol. Ther.* **2000,** *2*, 532-534.

[43] Alemany, R.; Balague, C.; Curiel, D. T. Replicative adenoviruses for cancer therapy. *Nat. Biotechnol.* **2000,** *18*, 723-727.

[44] Raper, S. E.; Chirmule, N.; Lee, F. S.; Wivel, N. A.; Bagg, A.; Gao, G. P.; Wilson, J. M.; Batshaw, M. L. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab.* **2003,** *80*, 148-158.

[45] Lichtenstein, D. L.; Wold, W. S. Experimental infections of humans with wild-type adenoviruses and with replication-competent adenovirus vectors: replication, safety, and transmission. *Cancer Gene Ther.* **2004,** *11*, 819-829.

[46] Makower, D.; Rozenblit, A.; Kaufman, H.; Edelman, M.; Lane, M. E.; Zwiebel, J.; Haynes, H.; Wadler, S. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin. Cancer Res.* **2003,** *9*, 693-702.

[47] Small, E. J.; Carducci, M. A.; Burke, J. M.; Rodriguez, R.; Fong, L.; van Ummersen, L.; Yu, D. C.; Aimi, J.; Ando, D.; Working, P.; Kirn, D.; Wilding, G. A Phase I Trial of Intravenous CG7870, a Replication-Selective, Prostate-Specific Antigen-Targeted Oncolytic Adenovirus, for the Treatment of Hormone-Refractory, Metastatic Prostate Cancer. *Mol. Ther.* **2006,** *14*, 107-117.

[48] DeWeese, T. L.; van der Poel, H.; Li, S.; Mikhak, B.; Drew, R.; Goemann, M.; Hamper, U.; DeJong, R.; Detorie, N.; Rodriguez, R.; Haulk, T.; DeMarzo, A. M.; Piantadosi, S.; Yu, D. C.; Chen, Y.; Henderson, D. R.; Carducci, M. A.; Nelson, W. G.; Simons, J. W. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* **2001,** *61*, 7464-7472.

[49] Todo, T.; Feigenbaum, F.; Rabkin, S. D.; Lakeman, F.; Newsome, J. T.; Johnson, P. A.; Mitchell, E.; Belliveau, D.; Ostrove, J. M.; Martuza, R. L. Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus. *Mol. Ther.* **2000,** *2*, 588-595.

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Technical Report

Rapid Construction of Small Interfering RNA-Expressing Adenoviral Vectors on the Basis of Direct Cloning of Short Hairpin RNA-Coding DNAs

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ABSTRACT

In the conventional method for constructing an adenoviral (Ad) vector expressing small interfering RNA (siRNA), short hairpin RNA (shRNA)-coding oligonucleotides are introduced downstream of a polymerase III (or polymerase II)-based promoter cloned into a shuttle plasmid. An siRNA expression cassette, which is cloned into the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid by *in vitro* ligation or homologous recombination in *Escherichia coli*, and the linearized plasmid is transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step plasmid manipulation is required. In this study, we developed a method by which shRNA-coding oligonucleotides can be introduced directly into the Ad vector plasmid. To do this, we constructed a new vector plasmid into which the human U6 promoter sequence was cloned in advance. Unique restriction enzyme sites were introduced at the transcription start site of the U6 promoter sequence in the vector plasmid. Luciferase and p53 genes were efficiently knocked down by Ad vectors generated by the new method and expressing siRNA against the target gene. This method should be useful for RNA interference-based experiments, and should make it easy to construct an siRNA-expressing Ad vector library for functional screening.

INTRODUCTION

RNA INTERFERENCE (RNAi), which mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (Scherer and Rossi, 2003), is a powerful tool for the knockdown of gene expression. Transduction of synthetic small interfering RNA (siRNA; 19 to 29 nucleotides of RNA) or the promoter-based expression of siRNA in the cells results in sequence-dependent degradation of target mRNA and subsequent reduction of target gene expression. Most promoter-based RNAi systems express short hairpin RNA (shRNA), which is then trimmed by Dicer, generating functional siRNA. Polymerase III-based promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, are widely used for the expression of shRNA (siRNA) (Scherer and Rossi, 2003), although polymerase II-based promoters are also used (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). The promoter-based method has an advantage in that viral vectors as well as nonviral vectors can be used for delivery of the siRNA expression unit, whereas only nonviral vectors are used for delivery of synthetic siRNA.

Recombinant adenoviral (Ad) vectors have been used extensively to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo* (McConnell and Imperiale, 2004; Volpers and Kochanek, 2004). They can be easily grown to high titers and can efficiently transfer genes into both dividing and nondividing cells. Therefore, Ad vector-mediated

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delivery of an siRNA expression unit, in which a promoter-based shRNA expression cassette is delivered into the cell by the Ad vector, provides a valuable tool for both gene function studies and therapeutic applications.

Construction of Ad vectors used to be a time-consuming and labor-intensive procedure, but several improved methods to facilitate the construction of Ad vectors have been developed (reviewed in Mizuguchi *et al.*, 2001). The homologous recombination method in E1-complementing cell lines (i.e., 293 cells) has been the most widely used method for generating recombinant Ad vectors, and it has greatly contributed to the widespread use of Ad vectors (Bett *et al.*, 1994). The major limitations of this approach are the low frequency of the recombination event and the tedious and time-consuming plaque purification procedure required to select the recombinant virus of interest, because a relatively high percentage of the virus produced is wild type (in most cases, 20–70%), due to recombination with the Ad sequence integrated into the chromosomes of 293 cells. The improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999) and the homologous recombination method in *Escherichia coli* (He *et al.*, 1998), which are commercially available from Clontech (Palo Alto, CA) and Invitrogen (Carlsbad, CA), respectively, have now become widely used, because these systems overcome the limitations of the homologous recombination method in 293 cells. To construct an Ad vector expressing siRNA by these two methods, shRNA-coding oligonucleotides are introduced downstream of the polymerase III (or polymerase II)-based promoter cloned in a shuttle plasmid. An shRNA (siRNA) expression cassette, which is cloned in the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid, which clones a full Ad genome, by simple *in vitro* ligation or homologous recombination in *E. coli*. The resulting plasmid is then linearized and transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step *E. coli* transformation and plasmid manipulation is required for the improved *in vitro* ligation method, whereas three-step *E. coli* transformation and plasmid manipulation is required in the homologous recombination method in *E. coli* (because a special *E. coli* strain is used in the latter method, retransformation into a normal stain of *E. coli* is required) (reviewed in Mizuguchi *et al.*, 2001).

In the present study, we developed a simple method for generating an Ad vector expressing siRNA, in which shRNA-coding oligonucleotides could be directly introduced into an Ad vector plasmid containing the human U6 (hU6) promoter sequence. Unique restriction enzyme sites were introduced at the transcription start site of the hU6 promoter sequence cloned into the Ad vector plasmid. Two types of modified hU6 promoter sequence were constructed to develop this method. Using this method, only one-step *E. coli* transformation is required to generate an Ad vector plasmid containing an siRNA expression cassette.

MATERIALS AND METHODS

Cells

A549 and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). A549-Luc cells, which are stable transformants with luciferase expression, were cultured in DMEM supplemented with 10% FCS. For construction of A549-Luc cells, A549 cells were transfected with luciferase-expressing plasmid pGL3-Control-RSVneo, which contains the simian virus 40 (SV40) promoter/enhancer-luciferase cDNA-SV40 p(A) sequence and the neomycin expression cassette, using SuperFect transfection reagent (Qiagen, Valencia, CA). pGL3-Control-RSVneo was constructed by insertion of the Rous sarcoma virus (RSV) promoter-driven neomycin expression cassette into pGL3-Control (Promega, Madison, WI). Monoclonal A549 cells stably expressing luciferase (A549-Luc) were obtained by geneticin (G418) selection.

Plasmid and virus

The hU6 promoter sequence was amplified from human genomic DNA (Clontech), using the following primers: hU6-S1, hU6-AS1, and hU6-AS2 (Table 1). The hU6a and hU6b promoter sequences were amplified with hU6-S1/hU6-AS1 and hU6-S1/hU6-AS2 primer sets, respectively (see Fig. 2). These promoter sequences were introduced into pHM5 (Mizuguchi and Kay, 1999), and were then transferred into the E1 deletion region of the vector plasmid pAdHM4.1, a derivative of pAdHM4 (Mizuguchi and Kay, 1998) (the *Xba*I site outside the Ad genome of pAdHM4 was deleted), by an *in vitro* ligation method using the I-*Ceu*I and PI-*Sce*I sites (Mizuguchi and Kay, 1998, 1999), resulting in pAdHM4-hU6a and pAdHM4-hU6b, respectively (Fig. 1A). To construct a vector plasmid containing an shRNA-coding sequence against luciferase, oligonucleotides 1/2 and 3/4 were synthesized (Table 1), annealed, and cloned into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-Lu and pAdHM4-hU6b-Lu, respectively. The target sequence for siRNA is bp 158 to 176 of luciferase cDNA. For the construction of vector plasmid containing shRNA-coding sequence against p53 (Brummelkamp *et al.*, 2002), oligonucleotides 5/6 and 7/8 were used for cloning into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-p53 and pAdHM4-hU6b-p53, respectively. The target sequence for siRNA is bp 775 to 793 of human p53 cDNA.

The original intact hU6 promoter sequence, derived from an *Eco*RI/*Sal*I fragment of piGene hU6 (iGENE Therapeutics, Tsukuba, Japan), was also introduced into the *Sph*I and *Sal*I sites of pHM5 (Mizuguchi and Kay, 1999), resulting in pHM5-ihU6. pHM5-ihU6 was then digested with *Sal*I and *Xba*I, and ligated with oligonucleotides 9 and 10, resulting in pHM5-hU6. In this case, oligonucleotides 11/12 and 13/14 (for the shRNA-coding sequence against luciferase and p53, respectively) were introduced into the *Bsp*MI site of pHM5-hU6 according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); and then an siRNA expression cassette was inserted into the E1-deletion region of pAdHM4 (Mizuguchi and Kay, 1998), using the I-*Ceu*I and PI-*Sce*I sites, resulting in pAdHM4-hU6-Lu and pAdHM4-hU6-p53, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Viruses (Ad-hU6-Lu, Ad-hU6a-Lu, Ad-hU6b-Lu, Ad-hU6-p53, Ad-hU6a-p53, and Ad-hU6b-p53) were prepared by the

TABLE 1. OLIGONUCLEOTIDES USED IN THE PRESENT STUDY

Oligonucleotide	*Sequence of oligonucleotide (5′–3′)*
hU6-S1 primer	aaggtcgggcaggaagagggccta
hU6-AS1 primer	ggtctagaagt*atcgat*ttttcgtctttccacaagatatat (*Xba*I and *Cla*I recognition sequences are underlined and italicized, respectively)
hU6-AS2 primer	ggtctagaagt*atttaaat*tcgtcctttccacaagatatataa (*Xba*I and *Swa*I recognition sequences are underlined and italicized, respectively)
Oligonucleotide 1	cg*acgctgagtacttcgaaat*ttcaagaga*atttcgaagtactcagcgt*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 2	ctagatttccaaaaa*acgctgagtacttcgaaat*tctcttgaa*atttcgaagtactcagcgt* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 3	cc*acgctgagtacttcgaaat*ttcaagaga*atttcgaagtactcagcgt*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 4	ctagatttccaaaaa*acgctgagtacttcgaaat*tctcttgaa*atttcgaagtactcagcgtgg* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 5	cg*gactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 6	ctagatttccaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 7	cc*gactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 8	ctagatttccaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc*gg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 9	tcgacctgcaggcatgcaagcttc (BspMI recognition sequences are underlined)
Oligonucleotide 10	ctaggaagcttgcatgcctgcagg (BspMI recognition sequences are underlined)
Oligonucleotide 11	cacc*acgctgagtacttcgaaat*ttcaagaga*atttcgaagtactcagcgt*ttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 12	gcataaaaaa*acgctgagtacttcgaaat*tctcttgaa*atttcgaagtactcagcgt* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 13	cacc*gactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*tttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 14	gcataaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)

transfection of a *Pac*I-linearized vector plasmid (pAdHM4-hU6-Lu, pAdHM4-hU6a-Lu, pAdHM4-hU6b-Lu, pAdHM4-hU6-p53, pAdHM4-hU6a-p53, and pAdHM4-hU6b-p53, respectively) into 293 cells as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the original intact hU6 promoter sequence (without a target sequence; Ad-hU6) were similarly constructed with pHM5-hU6 and pAdHM4. The virus was purified by $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol; and stored in aliquots at −70°C. Determination of virus particle (VP) titers and infectious titers was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and with an Adeno-X rapid titer kit (Clontech), respectively. The infectious titer-to-particle ratio was 1:36 for Ad-hU6, 1:31 for Ad-hU6-Lu, 1:28 for Ad-hU6a-Lu, 1:24 for Ad-hU6b-Lu, 1:22 for Ad-hU6-p53, 1:12 for Ad-hU6a-p53, and 1:15 for Ad-hU6b-p53.

Adenovirus-mediated gene transduction and luciferase assay

A549 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. Determination of luciferase production in the cells and extraction of cellular protein for Western blotting were performed after a 72-hr culture period. Luciferase production in the cells was measured with a luciferase assay system (PicaGene LT 2.0; produced by Toyo Ink [Tokyo, Japan] for Wako [Kyoto, Japan])

Western blotting for p53

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured

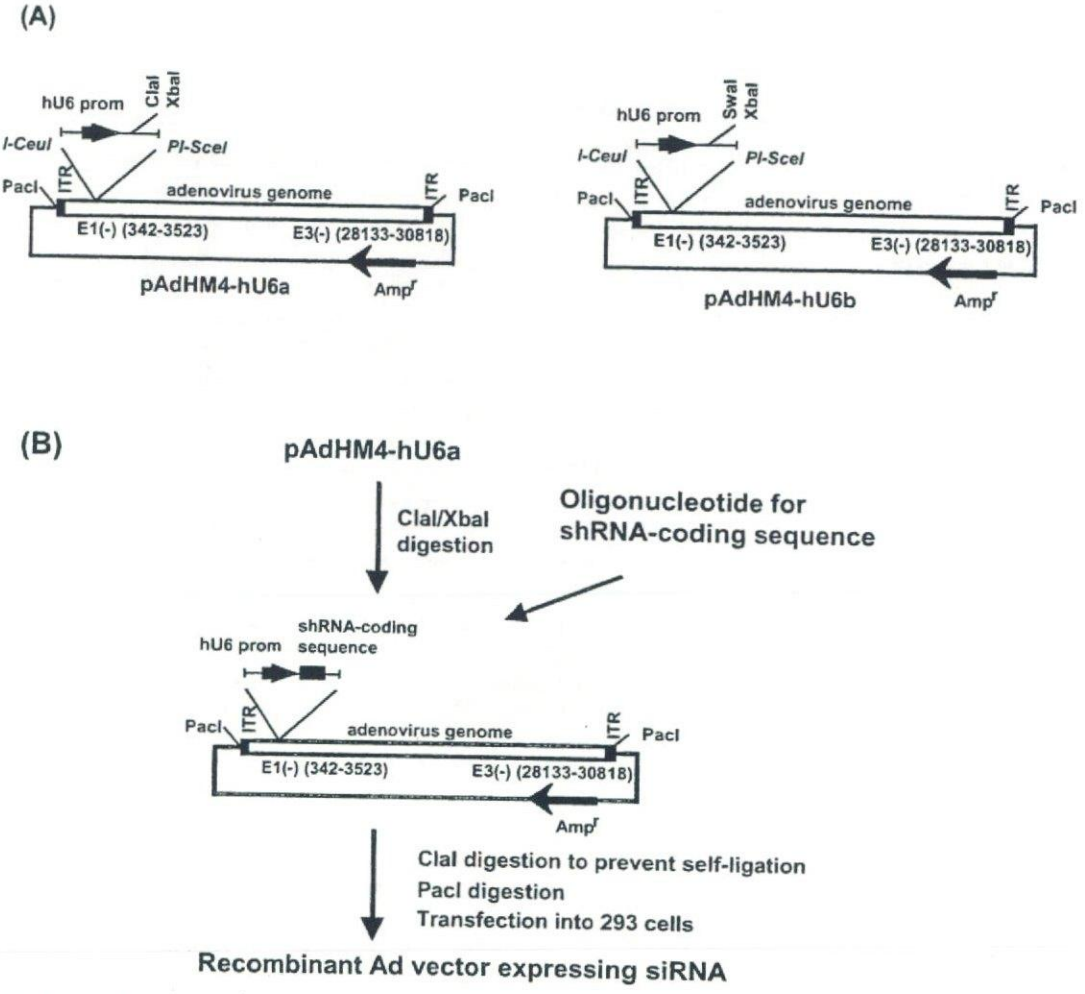

FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. (**A**) Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *ClaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. pAdHM4-hU6b contains a unique *SwaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. (**B**) Construction strategy for the Ad vector expressing siRNA. pAdHM4-hU6a was digested with *ClaI*/*XbaI* and ligated with oligonucleotides for the shRNA-coding sequence. Ligation products were then digested with *ClaI* to prevent the generation of nonrecombinant parental plasmid. The resulting plasmid was linearized by digestion with *PacI* and transfected into 293 cells, generating recombinant Ad vectors expressing siRNA. pAdHM4-hU6b was similarly used.

with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on sodium dodecyl sulfate (SDS)– 12.5% polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in nonfat dry milk, the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Oncogene Research Products/EMD Biosciences, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products/EMD Biosciences). The filters were developed by chemiluminescence (ECL Western blotting detection system; GE Healthcare, Piscataway, NJ). The signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified with Image Gauge software (Fujifilm).

RESULTS AND DISCUSSION

Rapid and efficient construction of Ad vectors expressing siRNA offers the promise of using RNAi in the context of both gene function analysis and therapeutic applications. In the present study, we developed a simple method for constructing Ad vectors expressing siRNA, based on only one-step *in vitro* ligation. To do this, we first constructed an Ad vector plasmid containing the E1- and E3-deleted Ad genome and the hU6 pro-

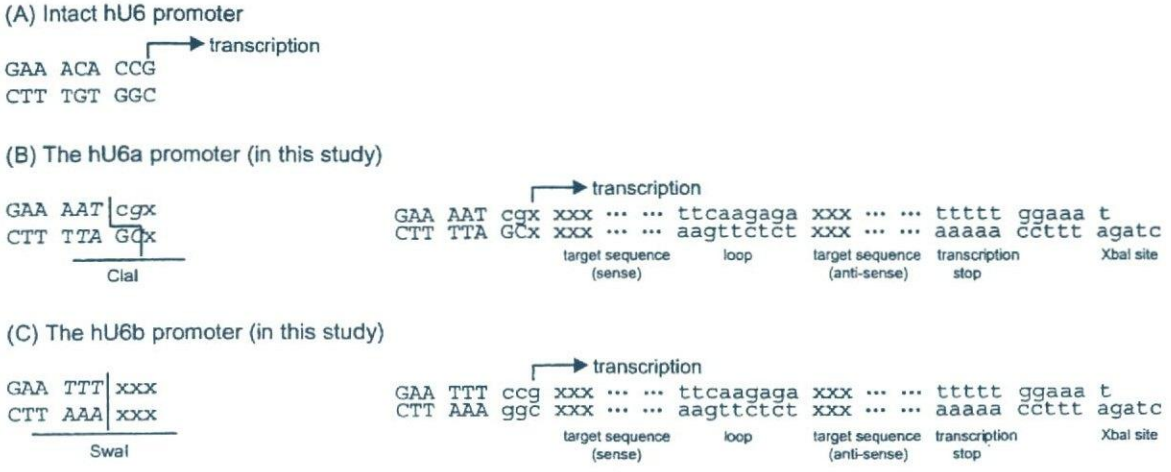

FIG. 2. Sequences at the transcription start site of the new hU6 promoter. (**A**) Intact hU6 promoter sequence. (**B**) hU6a promoter sequence. In this promoter, the *Cla*I site is placed at the transcription start site. (**C**) hU6b promoter sequence. In this promoter, a *Swa*I site is placed at the transcription start site. shRNA-coding oligonucleotides to be synthesized for each promoter are shown as lower-case letters on the right-hand side.

moter sequence in the E1 deletion region, pAdHM4-hU6a and pAdHM4-hU6b (Fig. 1A). By introducing the hU6 promoter sequence into the vector plasmid in advance, the cloning step of the gene of interest from the shuttle plasmid to the vector plasmid, which is an essential step in the conventional method for constructing Ad vectors (namely, the improved *in vitro* ligation method [Mizuguchi and Kay, 1998, 1999] and homologous recombination method in *E. coli* [He *et al.*, 1998]), can be skipped. To make it possible to directly clone the shRNA-coding oligonucleotides downstream of the hU6 promoter sequence, hU6 promoters containing unique restriction enzyme sites at the transcription start site have been developed. The new hU6 promoter sequences contain a *Cla*I or *Swa*I site around the transcription start site and an *Xba*I site downstream from the promoter (Figs. 1 and 2). These enzyme sites were selected because they do not cut the E1- and E3-deleted Ad genome. Because the transcription of shRNA might be influenced by the mutated sequences around the transcription start site, two types of hU6 promoters, differing by only a few nucleotides, were constructed. The hU6a promoter sequence contains a *Cla*I site, whereas the hU6b promoter sequence contains a *Swa*I site. *Cla*I, *Swa*I, and *Xba*I sites are unique in the vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. To generate a recombinant vector plasmid for Ad vectors expressing siRNA, oligonucleotides for shRNA against the target gene were synthesized, annealed, and ligated with *Cla*I/*Xba*I-digested pAdHM4-hU6a or *Swa*I/*Xba*I-digested pAdHM4-hU6b. Oligonucleotides were designed so that recombinant vector plasmid containing the shRNA-coding sequence is redigested with *Xba*I, but not with *Cla*I or *Swa*I. By designing oligonucleotides like the one described above, the generation of self-ligated plasmid can be avoided by digestion of the ligation products with *Cla*I or *Swa*I. On the right side of Fig. 2, DNA sequences, including the shRNA-coding sequence around the transcription start site of the hU6 promoter, are shown. Oligonucleotides that must be synthesized for the shRNA-coding sequence are shown as lower-case letters. By using the method developed in the present study, we could easily generate Ad vectors expressing siRNAs against luciferase and human p53. More than 90% of the recombinant Ad vector plasmids contained the correct insert. Because the *Cla*I- (or *Swa*I-) and *Xba*I-digested pAdHM4-hU6a and pAdHM4-hU6b can be stored at −20°C, only the ligation-based introduction of oligonucleotides into these sites of the vector plasmid would be required for the construction of an appropriate vector.

To examine the function of Ad vectors expressing siRNA against luciferase (Ad-hU6a-Lu and Ad-hU6b-Lu), the efficiency of knockdown of luciferase expression in A549-Luc cells, which stably express luciferase, was examined by treatment with Ad-hU6a-Lu or Ad-hU6b-Lu (Fig. 3). Ad-hU6-Lu, in which the hU6 promoter contains the original intact sequence even after introduction of an shRNA-coding sequence, was used as a positive control. To generate Ad-hU6-Lu, the shRNA-coding sequence was first introduced downstream from the hU6 promoter sequence cloned into the shuttle plasmid, according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); the shRNA expression cassette was then introduced into the E1 deletion region of the Ad vector plasmid pAdHM4 (Mizuguchi and Kay, 1998). Transfection of a *Pac*I-digested vector plasmid into 293 cells generated Ad-hU6-Lu. Ad-hU6, which contains the intact hU6 promoter without the shRNA-coding sequence, and Ad-hU6-p53, which contains the intact hU6 promoter with the shRNA-coding sequence against human p53, were similarly constructed and used as negative controls. Data showed that Ad-hU6a-Lu and Ad-hU6b-Lu suppressed luciferase expression in A549-Luc cells as efficiently as Ad-hU6-Lu, in a dose-dependent manner (Fig. 3). Ad-hU6 and Ad-hU6-p53 showed no effects on luciferase expression. Ad-hU6a-p53 and Ad-hU6b-p53 (these Ad vectors are used in Fig. 4) also had no influence on luciferase expression (data not shown). The RNAi effect of luciferase expression was relatively weak compared with that of p53 (de-

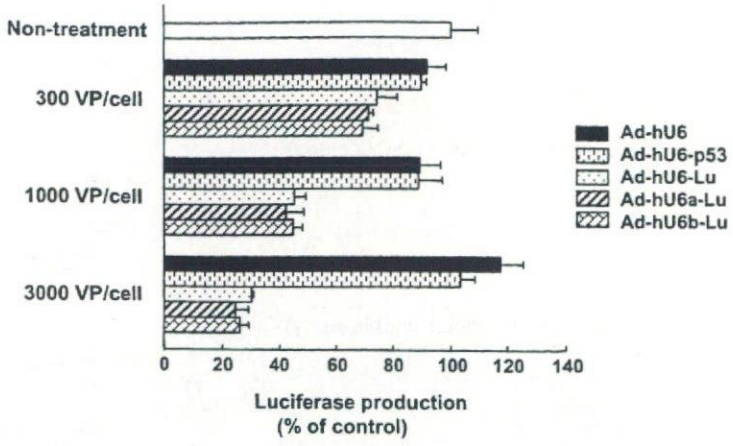

FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means and SD ($n = 4$).

scribed below). This difference probably occurred because the A549-Luc cells were expressing luciferase from a strong viral promoter (SV40 promoter and enhancer) and because the levels of luciferase expression were higher than those of endogenous p53 expression.

We next examined the RNAi effect of the siRNA-expressing Ad vector generated in the present study on the endogenous gene. As a model, we silenced p53 expression in A549 cells (Fig. 4). Ad-hU6a-p53 and Ad-hU6b-p53 were generated, and Ad-hU6, Ad-hU6-Lu, and Ad-hU6-p53 were also used. Ad-hU6-p53 contains the intact hU6 promoter sequence, including the transcription start site, even after introduction of the shRNA-coding sequence. A549 cells were transduced with a 300- or 1000-VP/cell of each Ad vector, and cultured for 3 days. Levels of p53 expression were examined by Western blotting. Expression of actin was also measured as an internal control. Expression of p53 in A549 cells was efficiently decreased by treatment with Ad-hU6a-p53 and Ad-hU6b-p53 as well as with Ad-hU6-p53. Levels of p53 expression in cells treated with Ad-hU6-p53, Ad-hU6a-p53, or Ad-hU6b-p53 at 1000 VP/cell were decreased to 7, 2, and 5%, respectively, relative to cells treated with Ad-hU6, according to Image Gauge software (Fujifilm) (in the case of 300 VP/cell, they were decreased to 53, 24, and 30%, respectively). The efficiency of p53 silencing by treatment with Ad-hU6-p53 was slightly lower than that with Ad-hU6a-p53 or Ad-hU6b-p53. This reduced efficiency is likely due to the approximately 1.5 to 2 times lower infectious titer-to-particle titer ratio of Ad-hU6-p53 in comparison with those of Ad-hU6a-p53 and Ad-hU6b-p53. Ad-hU6 and Ad-hU6-Lu did not decrease the level of p53 expression (Fig. 4). These results indicate that new hU6 promoters containing *Cla*I or *Swa*I sites at the transcription start site should transcribe as efficiently as the original hU6 promoter, and that Ad vectors containing the new hU6 promoters efficiently silence target gene expression. Different vector systems (pAdHM4-hU6a and pAdHM4-hU6b) should be used according to the specific purpose.

To facilitate the construction of an siRNA expression plasmid, the U6 and H1 promoters, which contain *Apa*I, *Bbs*I, *Bgl*II, *Eco*RV, *Sal*I, and *Xba*I sites, etc., at the transcription start site, have been developed (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Boden *et al.*, 2003). All types of promoters worked efficiently, and could be widely used for efficient RNAi, although the efficiency (activity) of the mutated promoters described above has not been compared with that of the intact promoter. The present study clearly showed that the mutated hU6 promoter, at least one having a *Cla*I or *Swa*I site at the transcription start site and an *Xba*I site downstream of the promoter sequence, is similar in activity to the intact hU6 promoter and would not influence the function of the promoter.

The method using polymerase chain reaction (PCR)-based amplification of shRNA together with the U6 promoter followed by subsequent cloning of the complete expression cassette directly into the Ad vector genome is another strategy for one-step construction of recombinant Ad plasmids containing an siRNA expression cassette. In this method, however, the procedures described below are required for preparation of insert DNA: (1) ordering of the PCR primer, (2) PCR, (3) purification of the PCR product, (4) restriction enzyme digestion and purification of the PCR product, and (5) ligation. In our present system, only the following procedures are required: (1) ordering of the oligonucleotides, (2) hybridization of the oligonucleotides, and (3) ligation. Thus, the present method would be much easier and would allow any laboratory to easily construct

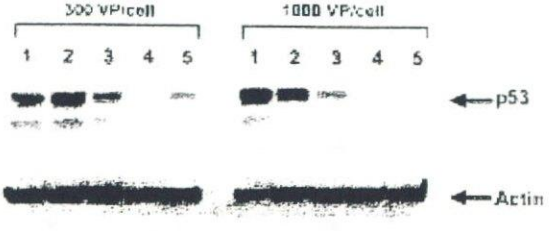

FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted from the cells, and the levels of p53 expression were examined by Western blotting. The actin bands served as an internal control for equal total protein loading. The extra (lower) bands of p53 are nonspecific.

Ad vectors expressing siRNA for gene transfer studies and therapeutic applications.

Various types of promoters that are based on polymerase II as well as polymerase III have been developed to transcribe shRNA (siRNA) (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). Although the present study applied the most commonly used U6 promoter for simple and efficient construction of siRNA-expressing Ad vectors, this method could easily be applied to vectors using other promoters including polymerase II-based promoters. This method can also easily be combined with various types of improved Ad vectors, such as Ad vectors containing capsid modification (Koizumi *et al.*, 2003, 2006; Mizuguchi and Hayakawa, 2004; Kurachi *et al.*, 2006) or Ad vectors belonging to different subgroups to modify tropism (Sakurai *et al.*, 2003), and Ad vectors containing a tetracycline-inducible RNAi system (Hosono *et al.*, 2004). The method developed in the present study should be a powerful tool for the application of RNAi, and might facilitate the development of an siRNA-expressing Ad vector library for functional screening.

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REFERENCES

BETT, A.J., HADDARA, W., PREVEC, L., and GRAHAM, F.L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. U.S.A. **91,** 8802–8806.

BODEN, D., PUSCH, O., LEE, F., TUCKER, L., SHANK, P.R., and RAMRATNAM, B. (2003). Promoter choice affects the potency of HIV-1 specific RNA interference. Nucleic Acids Res. **31,** 5033–5038.

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science **296,** 550–553.

HE, T.C., ZHOU, S., DA COSTA, L.T., YU, J., KINZLER, K.W., and VOGELSTEIN, B. (1998). A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. U.S.A. **95,** 2509–2514.

HOSONO, T., MIZUGUCHI, H., KATAYAMA, K., XU, Z.L., SAKURAI, F., ISHII-WATABE, A., KAWABATA, K., YAMAGUCHI, T., NAKAGAWA, S., MAYUMI, T., and HAYAKAWA, T. (2004). Adenovirus vector-mediated doxycycline-inducible RNA interference. Hum. Gene Ther. **15,** 813–819.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2003). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. **5,** 267–276.

KOIZUMI, N., KAWABATA, K., SAKURAI, F., WATANABE, Y., HAYAKAWA, T., and MIZUGUCHI, H. (2006). Modified adenoviral vectors ablated for coxsackievirus–adenovirus receptor, α_v integrin, and heparan sulfate binding reduce *in vivo* tissue transduction and toxicity. Hum. Gene Ther. **17,** 264–279.

KURACHI, S., KOIZUMI, N., SAKURAI, F., KAWABATA, K., SAKURAI, H., NAKAGAWA, S., HAYAKAWA, T. and MIZUGUCHI, H. (2006). Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon. Gene Ther. (in press).

LEE, N.S., DOHJIMA, T., BAUER, G., LI, H., LI, M.J., EHSANI, A., SALVATERRA, P., and ROSSI, J. (2002). Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. Nat. Biotechnol. **20,** 500–505.

MAIZEL, J.V., Jr., WHITE, D.O., and SCHARFF, M.D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology **36,** 115–125.

McCONNELL, M.J., and IMPERIALE, M.J. (2004). Biology of adenovirus and its use as a vector for gene therapy. Hum. Gene Ther. **15,** 1022–1033.

MIYAGISHI, M., SUMIMOTO, H., MIYOSHI, H., KAWAKAMI, Y., and TAIRA, K. (2004). Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J. Gene Med. **6,** 715–723.

MIZUGUCHI, H., and HAYAKAWA, T. (2004). Targeted adenovirus vectors. Hum. Gene Ther. **15,** 1034–1044.

MIZUGUCHI, H., and KAY, M.A. (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum. Gene Ther. **9,** 2577–2583.

MIZUGUCHI, H., and KAY, M.A. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum. Gene Ther. **10,** 2013–2017.

MIZUGUCHI, H., KAY, M.A., and HAYAKAWA, T. (2001). Approaches for generating recombinant adenovirus vectors. Adv. Drug Deliv. Rev. **52,** 165–176.

PADDISON, P.J., CAUDY, A.A., BERNSTEIN, E., HANNON, G.J., and CONKLIN, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16,** 948–958.

PAUL, C.P., GOOD, P.D., WINER, I., and ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat. Biotechnol. **20,** 505–508.

SAKURAI, F., MIZUGUCHI, H., and HAYAKAWA, T. (2003). Efficient gene transfer into human $CD34^+$ cells by an adenovirus type 35 vector. Gene Ther. **10,** 1041–1048.

SCHERER, L.J., and ROSSI, J.J. (2003). Approaches for the sequence-specific knockdown of mRNA. Nat. Biotechnol. **21,** 1457–1465.

SHINAGAWA, T., and ISHII, S. (2003). Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. Genes Dev. **17,** 1340–1345.

SUI, G., SOOHOO, C., AFFAR EL, B., GAY, F., SHI, Y., and FORRESTER, W.C. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 5515–5520.

VOLPERS, C., and KOCHANEK, S. (2004). Adenoviral vectors for gene transfer and therapy. J. Gene Med. **6,** S164–S171.

XIA, H., MAO, Q., PAULSON, H.L., and DAVIDSON, B.L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo*. Nat. Biotechnol. **20,** 1006–1010.

YU, J.Y., DERUITER, S.L., and TURNER, D.L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 6047–6052.

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SHORT COMMUNICATION

Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice

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We previously demonstrated that systemic administration of adenovirus serotype 35 (Ad35) vectors to mice does not mediate efficient transduction in organs, probably because expression of the mouse analog of the subgroup B Ad receptor, human CD46 (membrane cofactor protein), is limited to the testis. Here, we describe the in vitro *and* in vivo *transduction characteristics of Ad35 vectors by using homozygous and hemizygous human CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46. An Ad35 vector more efficiently transduced the primary dendritic cells and macrophages prepared from CD46TG mice than those from wild-type mice.* In vivo *transduction experiments demonstrated that CD46TG mice are more susceptible to Ad35 vector-mediated* in vivo *transduction than are wild-type mice. In particular, homozygous CD46TG mice, which express higher levels of CD46 in the organs than hemizygous CD46TG mice, tend to exhibit higher transduction efficiencies after intraperitoneal administration than hemizygous CD46TG mice. Intraperitoneal administration of Ad35 vectors resulted in efficient transduction into the mesothelial cells of the peritoneal organs in homozygous CD46TG mice. These results indicate that an Ad35 vector recognizes human CD46 as a cellular receptor in CD46TG mice. However, the* in vivo *transduction efficiencies of Ad35 vectors in CD46TG mice are much lower than those of conventional Ad5 vectors in wild-type mice.*
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Keywords: *adenovirus serotype 35 vector; human CD46; human CD46-transgenic mice; receptor; biodistribution*

The human adenoviruses (Ads) comprise a group of 51 serologically distinct viruses.[1,2] Among them, Ad vectors widely used for gene therapy are based on Ad serotype 5 (Ad5), which belongs to subgroup C. Adenovirus serotype 5 vectors have several attractive features as gene delivery vehicles; for example, they have noteworthy *in vivo* transduction efficiency and transduction ability into both proliferating and non-proliferating cells. In addition, Ad5 vectors can be grown to high titer, and large stretches of foreign DNA can be inserted into the Ad5 vector genome.

However, recent studies have revealed several disadvantages associated with the clinical use of Ad5 vectors. One disadvantage is the high prevalence of adult humans (>50%) who produce neutralizing antibodies to Ad5.[3,4] Pre-existing neutralizing antibodies prevent Ad vectors from transducing cells *in vivo*.[5] Furthermore, Ad vector preimmunization in mice has been demonstrated to significantly increase vector-mediated liver toxicity on re-exposure.[6] Therefore, pre-existing immunity to Ad5 vectors greatly hampers the *in vivo* application of Ad5 vectors. Inefficient transduction with Ad5 vectors of cells lacking expression of a primary receptor for Ad5, coxsackievirus and adenovirus receptor (CAR), is also highly problematic. Several important target cells for gene therapy, including hematopoietic stem cells,[7] dendritic cells (DCs)[8] and malignant tumor cells,[9] express low levels of CAR.

To overcome these drawbacks of Ad5 vectors, several groups (including ours) have developed Ad vectors composed of other human Ad serotypes, such as Ad serotype 7a,[10] 11[4,11] and 35,[12–15] and Ads of animal origin, such as chimpanzee,[16] bovine,[17] mouse[18] and ovine.[19] Among these non-Ad5 vectors, those composed of human Ad11 and Ad35, which belong to subgroup B, are highly promising as gene transfer vectors for the following reasons. First, Ad11 and Ad35 are serotypes least neutralized by serum from healthy human blood donors: less than 20% of serum samples are positive for anti-Ad11 and -Ad35 neutralizing antibodies.[14] Second, human subgroup B Ads, including Ad11 and Ad35, recognize human CD46 (membrane cofactor protein) as a cellular receptor,[20,21] although Ads belonging to subgroups A, C, D, E and F use CAR as a primary receptor. CD46 is a single-chain type I transmembrane glycoprotein that is ubiquitously expressed in all cells (except erythrocytes) in humans, suggesting that human sub-

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group B Ads can infect almost all human cell types, regardless of CAR expression. We previously demonstrated that Ad35 vectors show broad tropism toward human cells,[12,13,22] including CAR-negative cells, because of the ubiquitous expression of CD46. Furthermore, CD46 expression is highly upregulated in human malignant tumor cells,[23,24] suggesting that these cells would be suitable targets for Ad35 vector-mediated transduction.

Adenovirus serotype 35 vectors exhibit efficient transduction in a variety of human cells *in vitro*. In contrast, systemic administration of Ad35 vectors into mice mediates low levels of transduction efficiencies in organs.[13,14] The refractoriness of mice to Ad35 vectors would be owing to the expression pattern of CD46 in this host. Whereas CD46 is ubiquitously expressed in humans, expression of mouse CD46 is limited to the testes. In addition, mouse and human CD46 s are only 46% similar.[25] These differences indicate that the conventional mouse is not a suitable small animal model for characterization of Ad35 vector-mediated *in vivo* transduction. A small animal model in which the transduction properties of Ad35 vectors can be characterized appropriately is essential to estimate the efficiency of Ad35 vector-mediated transduction in humans.

In the present study, to evaluate the *in vivo* transduction properties of Ad35 vectors in an animal model that ubiquitously expresses CD46 (as do humans), we administered Ad35 vectors intravenously and intraperitoneally into homozygous and hemizygous human CD46-transgenic (CD46TG) mice, which have a human CD46 gene inserted into the mouse genome. Our results indicate that CD46 acts as an attachment receptor for Ad35 after *in vivo* administration, but the transduction efficiencies in organs were lower than we had expected.

First, Western blot analysis was performed to examine human CD46 expression levels in the organs of homozygous and hemizygous CD46TG mice. As shown in Figure 1, human CD46 was ubiquitously expressed in all organs examined of homozygous and hemizygous CD46TG mice. In particular, amounts of CD46 were higher in the liver, spleen, lung and kidney than in other organs. CD46 expression patterns in CD46TG mice mimicked those observed in humans, which were reported previously,[26] and homozygous CD46TG mice expressed CD46 more abundantly than hemizygous mice. CD46 expression in the liver, spleen and diaphragm of homozygous mice was 3.2, 3.7 and 3.2 times that, respectively, in these organs of the hemizygous mice. We also confirmed that human CD46 expression levels in the primary hepatocytes, splenocytes and thymocytes of the homozygous mice were similar to, or lower than, those in human cultured cell lines (human hepatoma and leukemia lines; data not shown). Flow cytometric and Western blotting analysis failed to detect CD46 expression in the erythrocytes of our CD46TG mice (data not shown), although detectable levels of CD46 were expressed in the erythrocytes of some CD46TG mice lines used in other studies.[27,28]

Next, we performed *in vitro* transduction of an Ad35 vector into bone marrow-derived dendritic cells (mBM-DC) and peritoneal macrophages prepared from CD46TG and wild-type mice. Recently, the potential utility of Ad35 vectors as vaccine vectors has been proposed,[29,30] and DC and macrophages are considered

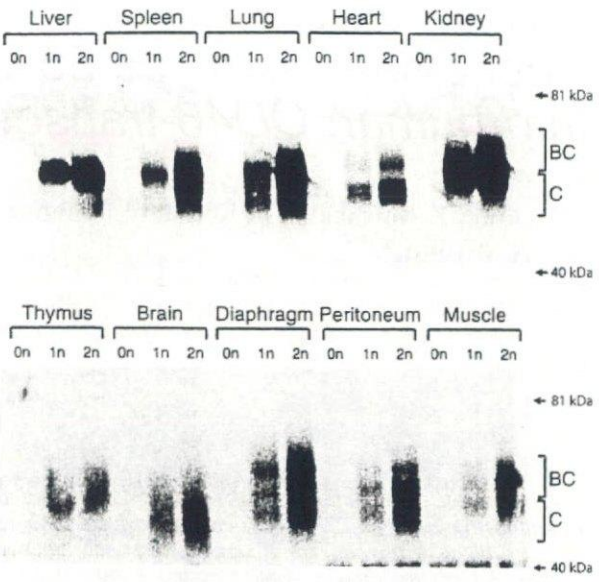


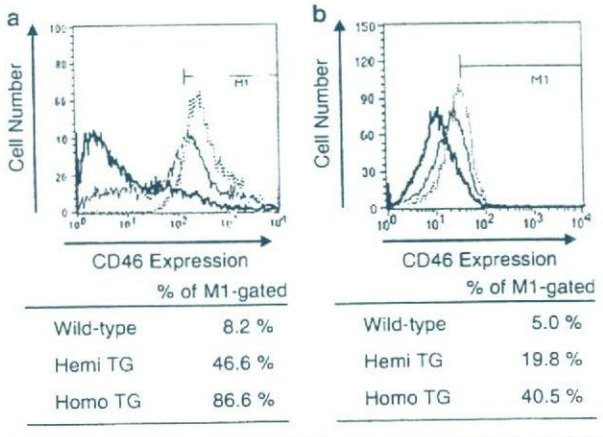
Figure 1 Human CD46 expression in organs harvested from CD46TG mice. The protein samples were prepared from wild-type mice (0n), hemizygous CD46TG mice (1n) and homozygous CD46TG mice (2n). The molecular masses of marker proteins (kDa) and approximate positions of the two major isoforms of the CD46 proteins (BC and C isoforms) are indicated on the right. CD46TG mice were produced as follows. Spermatozoa were dispersed from the epididymis of mature (>12 weeks old) B6D2F1 male mice into 400 μl of TYH medium[50] and were frozen in liquid nitrogen immediately after dilution with TYH medium to 1×10^7/ml. The bacterial artificial chromosome DNA carrying human CD46 (5 μg/ml in TE) (GenomeSystems Inc., St Louis, MO, USA) was added to thawed sperm after purification by using the Large-Construct Kit (Qiagen, Valencia, CA, USA). The mixture was incubated for 5 min at room temperature and then diluted with 9 volumes of 12% PVP-HCZB. Metaphase II oocytes for microinjection were prepared from B6D2F1 female mice, as described previously.[51] These oocytes were maintained in potassium simplex optimized medium (kSOM) under mineral oil equilibrated in 5% (v/v) CO_2 in air at 37°C until use. For microinjection, sperm heads were aspirated into a pipette attached to a piezoelectric pipette-driving unit, and a sperm head was injected into each oocyte, as described previously.[51] After injection, the eggs were incubated in kSOM until two-cell stage, and were transferred to ICR pseudo-pregnant foster mothers. CD46TG mice were detected among the pups born by using genomic PCR as described previously.[40] After backcrossing to the C57Bl6 background for more than five generations, homozygous CD46TG mice were obtained by mating hemizygous mice. Homozygous CD46TG mice were identified by mating CD46TG mice with wild-type mice. For Western blotting analysis, organs collected from wild-type mice (C57Bl6, female, 5 weeks old, obtained from Nippon SLC Co. Ltd, Shizuoka, Japan) and CD46TG mice (female, 5 weeks old) were homogenized in phosphate-buffered saline containing 1% Triton-X, 2 mM EGTA and proteinase inhibitor cocktail (1 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin). After centrifugation of the homogenates, the supernatants (7.5 μg protein per sample) underwent nonreducing sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific binding, CD46 was detected with anti-CD46 rabbit serum (1:5000; kindly provided by Dr T Seya, Hokkaido University, Japan), followed by incubation in the presence of peroxidase-labeled anti-rabbit antibody (1:6000). Signals on the membrane were visualized and analyzed as described previously.[52]

to be ideal targets for immunotherapy using Ad35 vector-based vaccines. Bone marrow-derived dendritic cells[31] and peritoneal macrophages[32] were prepared as

described previously. An Ad35 vector that expresses green fluorescence protein (GFP), Ad35GFP, was prepared by means of an improved *in vitro* ligation method[22,33,34] using 293-E1B cells as a packaging cell line. 293-E1B cells are stable transformants expressing Ad35E1B proteins, which were generated by transfection of pEF-Ad35E1B into 293 cells and after selection with G418 (Invitrogen, Carlsbad, CA, USA). pEF-Ad35E1B was constructed by insertion of the fragment of the Ad35 genome (bp 1911–3413), which contains the Ad35 E1B-55 K gene, into pEF/myc/nuc (Invitrogen). The plaque-forming unit (PFU)-to-particle ratios of Ad35GFP in 293-E1B cells was 1:66.

Flow cytometric analysis showed that mBM-DC from hemizygous and homozygous CD46TG mice express considerable amounts of human CD46 (Figure 2a): 47% of the mBM-DC from hemizygous mice and 87% of those from homozygous mice were CD46-positive (% of M1-gated). Transduction experiments demonstrated that Ad35GFP mediated more efficient transduction in mBM-DC from CD46TG mice than from wild-type mice (Figure 3a). Ad35GFP at a dose of 3000 vector particles (VP)/cell successfully transduced about 42% of the mBM-DC from the hemizygous CD46TG mice and 83% of those from homozygous mice. In contrast, only 3.8% of the mBM-DC from wild-type mice were positive for GFP expression, a rate that is only slightly above background level. In addition, mean fluorescence intensity data revealed that the mBM-DC from CD46TG mice were more susceptible to Ad35 vector than those from wild-type mice. Similar results were obtained for peritoneal macrophages. Human CD46 was expressed in 20% of the peritoneal macrophages from hemizygous CD46TG mice and in 41% of those from homozygous animals (Figure 2b). Infection by Ad35GFP resulted in 10% GFP-positive macrophages from hemizygous CD46TG mice and in 20% GFP-positive macrophages among those from homozygous transgenic mice. In contrast, the macrophages from wild-type mice were refractory to Ad35 vector-mediated transduction (Figure 3b). These results indicate that human CD46 expression greatly increases the transduction efficiency of Ad35 vector in mouse primary cells and that the *in vitro* transduction efficiency of Ad35 vector depends on CD46 expression density.

The refractoriness of mBM-DC and macrophages from wild-type mice also suggests inefficient interaction between the RGD motif in the penton base of Ad35 vector and αv-integrins on the cells. The hypervariable RGD loop in the penton base of the Ad35 vector is supposed to be shorter than that of conventional Ad5 viruses because the RGD loop of Ad11 (55 amino acids), the sequence of which is identical to that of Ad35, is shorter than that of Ad2 (74 amino acids).[35] It suggests that αv-integrins on the cell surface might be less easily accessible to the RGD motif in the penton bases of Ad35 viruses, compared with Ad5 vectors. Conventional Ad5 vectors transduce to varying degrees via interaction



a	% of M1-gated
Wild-type	8.2 %
Hemi TG	46.6 %
Homo TG	86.6 %

b	% of M1-gated
Wild-type	5.0 %
Hemi TG	19.8 %
Homo TG	40.5 %

Figure 2 CD46 expression in (**a**) bone marrow-derived dendritic cells (mBM-DC) and (**b**) peritoneal macrophages from wild-type mice and CD46TG mice. Thick lines, thin lines and dotted lines represent cells from wild-type mice (C57Bl6, 5 weeks old), and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice, respectively. Bone marrow-derived dendritic cells and peritoneal macrophages were incubated with fluorescein isothiocyanate-conjugated anti-human CD46 antibody (E4.3; Pharmingen, San Diego, CA, USA) after incubation with anti-FcγRII/III monoclonal antibody (2.4G2; Pharmingen) to block nonspecific binding of the anti-human CD46 antibody. After being washed thoroughly, 10^4 stained cells were analyzed using a FACSCalibur (Becton Dickinson, Tokyo, Japan) and CellQuest software (Becton Dickinson).

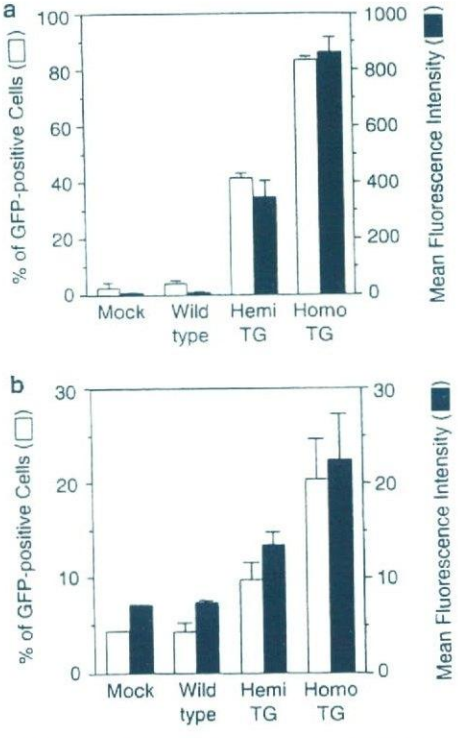


Figure 3 Ad35 vector-mediated green fluorescence protein (GFP) expression in (**a**) bone marrow-derived dendritic cells (mBM-DC) and (**b**) peritoneal macrophages prepared from wild-type mice and CD46TG mice. Open bars represent percentages of cells positive for GFP, and closed bars indicate mean fluorescence intensity. Bone marrow-derived dendritic cells (5×10^5 cells/well) and peritoneal macrophages (2×10^5 cells/well) prepared from wild-type mice and hemizygous (Hemi TG) and homozygous (Homo TG) CD46TG mice were seeded into 12-well plates the day before transduction. The cells were then transduced with Ad35GFP at 3000 VP/cell for 1.5 h. After a total of 48 h of incubation, GFP expression in cells was evaluated by flow cytometric analysis. The results are represented as mean ± s.d. ($n = 3$).

between the RGD motif in the penton base and αv-integrins, even in the absence of the primary receptor for Ad5, CAR.[36]

Next, to assess the *in vivo* transduction properties of Ad35 vectors in CD46TG mice, we intravenously and intraperitoneally administered an Ad35 vector that expressed luciferase (Ad35L). Ad35L was prepared in the same way as Ad35GFP. The PFU-to-particle ratio of Ad35L in 293-E1B cells was 1:625. Intravenous administration of Ad35L to hemizygous and homozygous CD46TG mice increased the transduction efficiencies for the liver, lung and kidney over those in wild-type mice. In contrast, no apparent increase in luciferase production occurred in the spleen or thymus of CD46TG mice (Figure 4a). There were no clear differences in the transduction efficiencies of Ad35 vectors between the homozygous and hemizygous mice, except in the case of liver.

In contrast, transduction efficiencies of Ad35L in the liver, spleen and kidney of CD46TG mice after intraperitoneal administration were much higher than those after intravenous administration (Figure 4b). Intraperitoneal injection into homozygous mice led to transduction efficiencies in the liver and kidney that were 83 and 271 times that after intravenous administration, respectively. Furthermore, even larger differences in transduction efficiency occurred between CD46TG mice and wild-type mice after intraperitoneal injection. Luciferase production from the liver, kidney and diaphragm of homozygous mice was 536, 492 and 83 times, respectively, that of wild-type mice. Comparison of the homozygous and hemizygous mice demonstrated that after intraperitoneal administration of vector, the homozygous mice appeared to be more susceptible to Ad35 vectors. Transgene expression levels in the liver and diaphragm of homozygous mice were 6.8 and 5.5 times, respectively, those of the hemizygous mice. The increased transduction efficiencies in the homozygous mice are probably owing to their increased levels of CD46 expression (Figure 1). These results indicate that CD46TG mice are more susceptible to Ad35 vectors than wild-type mice. However, the transduction efficiencies of Ad35 vectors in CD46TG mice after both intraperitoneal and intravenous injection were much lower than those of conventional Ad5 vectors in wild-type mice.[13] Luciferase production in the liver and spleen by Ad35 vectors intravenously administered to homozygous CD46TG mice was approximately 20 000 and 57 times lower, respectively, than that from Ad5 vectors administered intravenously to wild-type mice at the same dose as for the Ad35 vector in the present study (Ad5 vector-mediated luciferase expression levels in wild-type mice at a dose of 1.5×10^{10} VP/mouse after intravenous administration: liver, 2266 pg/mg protein; spleen, 0.893 pg/mg protein; kidney, 0.768 pg/mg protein; heart, 2.13 pg/mg protein; lung, 0.252 pg/mg protein).[13]

Next, to compare the fate of Ad35 vectors after *in vivo* administration to wild-type versus CD46TG mice, we used real-time PCR at 48 h post-administration to measure the amounts of Ad35L genomic sequences that had accumulated in various organs. After intravenous administration to CD46TG mice, the vector DNA in the sampled organs of CD46TG mice (except for liver) exceeded that in those of wild-type mice (Figure 5a). In addition, homozygous mice appeared to take up higher amounts of Ad35L than hemizygous mice. For example,

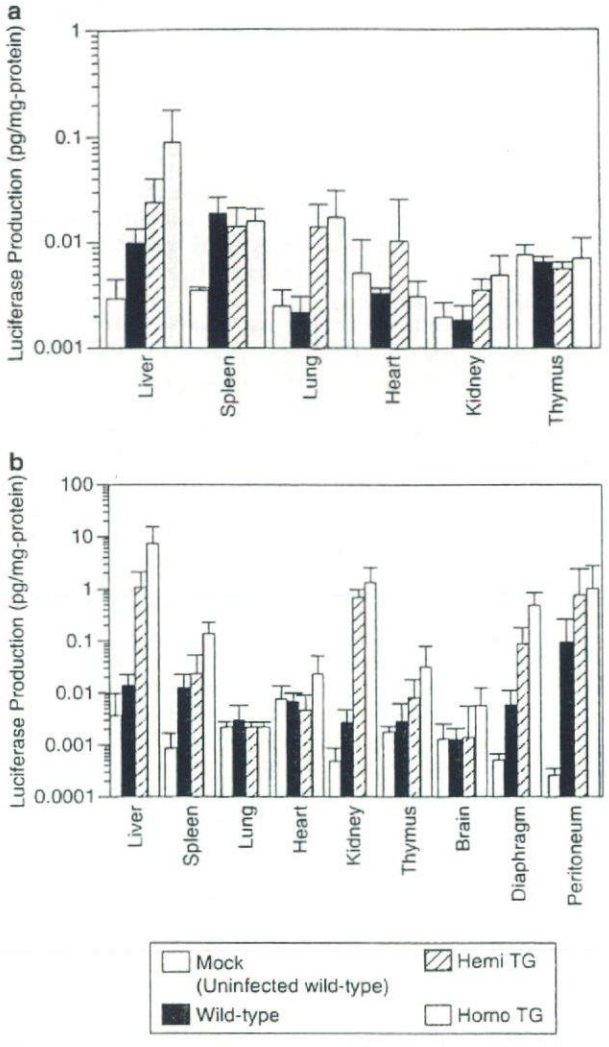


Figure 4 Luciferase production in CD46TG and wild-type mice after intravenous and intraperitoneal administration of Ad35L. (a) Luciferase production after intravenous administration of the vector. (b) Luciferase production after intraperitoneal administration. Ad35L (1.5×10^{10} VP) was administered to wild-type mice (C57Bl6, 5 weeks old) and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice. After 48 h, the organs were harvested and homogenized as described previously,[53] and luciferase production was measured by a luminescence assay system (PicaGene 5500; Toyo Inki, Japan). The data are represented as mean ± s.d. ($n = 4$, intravenous administration; $n = 6$, intraperitoneal administration).

Ad35 vector quantities in the spleen, which contained the most Ad35 vector DNA among the organs tested, of hemizygous and homozygous mice were 8 and 69 times, respectively, that in wild-type mice. In contrast, Ad35 vector DNA did not accumulate to high levels in the liver of CD46TG mice. Moreover, these quantities were similar, or slightly lower than, those in other organs of CD46TG mice, even though liver is well known to be a predominant organ for sequestration of Ad5 vectors administered intravenously to mice.[37,38]

After intraperitoneal injection, Ad35L was accumulated more efficiently in the liver, kidney, peritoneum

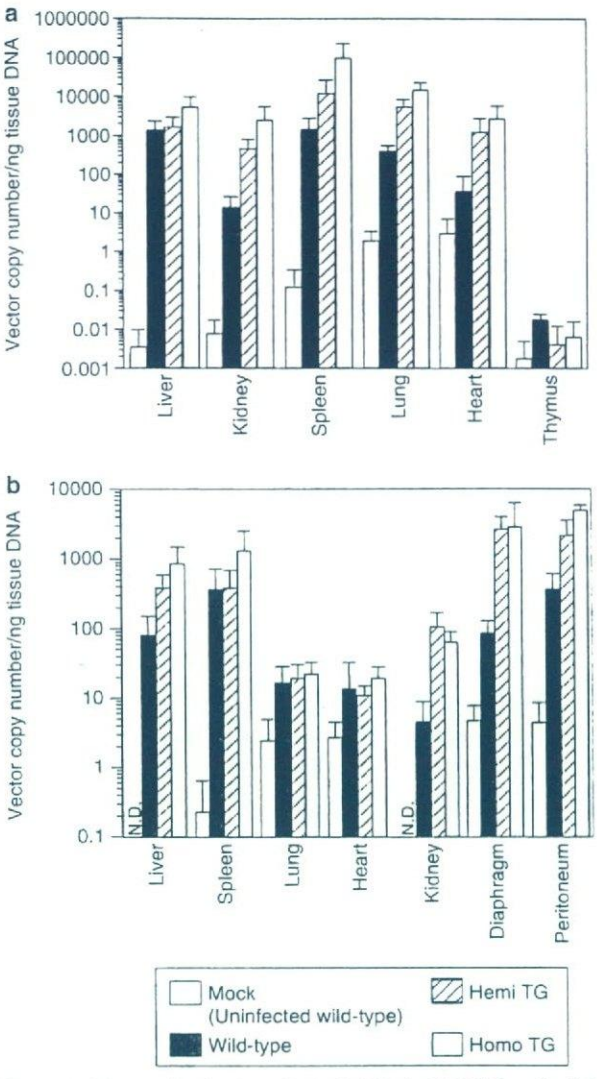


Figure 5 Tissue distribution of viral DNA in CD46TG and wild-type mice after intravenous and intraperitoneal administration of Ad35L. (a) Ad35L vector DNA detected in organs after intravenous administration. (b) Ad35L vector DNA detected in organs after intraperitoneal administration. Ad35L (1.5×10^{10} VP) was administered to wild-type mice (C57Bl6, 5 weeks old) and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice. After 48 h, the organs were harvested, and total DNA including viral DNA was extracted from the tissues after proteinase K digestion, and 25 ng samples of total DNA were subjected to quantitative real-time PCR, as described previously.[22] The data are represented as mean ± s.d. ($n = 4$).

and diaphragm of CD46TG mice than in those of wild-type mice (Figure 5b). The quantities of vector DNA in the liver and kidney of homozygous CD46TG mice were 11 and 14 times, respectively, those of wild-type mice. Furthermore, Ad35 vector DNA tended to be accumulated more efficiently in the liver, spleen, peritoneum and diaphragm of the homozygous mice than in those of the hemizygous mice. In addition, low levels of viral DNA were detected in the lung and heart, which are not directly accessible to intraperitoneally injected Ad35L from the injection point, with no significant difference between CD46TG mice and wild-type mice in the amounts that were detected. The data on the *in vivo* transduction efficiencies and viral DNA accumulation indicate that Ad35 vectors administered *in vivo* use human CD46 in CD46TG mice. In both intravenous and intraperitoneal dosing experiments, the total amounts of Ad35 vector DNA recovered from wild-type mice were lower than those from CD46TG mice. The decreased recovery of Ad35 vector DNA from wild-type mice could be due to its degradation in phagocytic cells, such as liver Kupffer cells. We speculate that the absence of human CD46 expression in organs results in decreased infection of the organs and increased uptake of Ad35 vector by phagocytic cells, leading to degradation of Ad35 vector DNA. In the previous study, we demonstrated that Ad35 vectors predominantly were taken up by liver nonparenchymal cells (endothelial and Kupffer cells) after intravenous administration in wild-type mice, and that the internalized Ad35 vector DNA was degraded rapidly.[13]

Finally, to examine the types of cells that Ad35 vectors transduce in CD46TG mice, we performed X-gal staining of the peritoneal organs after intraperitoneal administration of Ad35LacZ, an Ad35 vector expressing β-galactosidase (dose, 7.5×10^{10} VP/mouse). The vector was prepared by means of an improved *in vitro* ligation method[22,33,34] using pAdMS18 and pHMCMV6-LacZ. pAdMS18 was constructed by ligating oligonucleotides encoding I-*Ceu*I/*Swa*I/PI-*Sce*I into the *Pac*I site of pFS2-Ad35–7.[12] pHMCMV6-LacZ was generated by cloning the *Escherichia coli* β-galactosidase gene derived from pCMVβ (Clontech, Palo Alto, CA, USA) into the multicloning site of pHMCMV6.[34] The PFU-to-particle ratio of Ad35LacZ in 293-E1B cells was 1:315.

The peritoneal organs (liver, kidney and peritoneum) were efficiently transduced with Ad35LacZ (Figure 6a–f). However, X-gal staining of liver and kidney sections revealed that mainly the mesothelial cells on the surface of the liver and kidney were transduced; few deeper cells were transduced (Figure 6g–j). These results indicate that after injection, Ad35 vectors directly access the mesothelial cells of these organs, leading to efficient transduction.

In the present study, we assessed the *in vitro* and *in vivo* transduction properties of Ad35 vectors by using homozygous and hemizygous human CD46TG mice, a small animal model in which human CD46 is expressed with human-like tissue specificity. Human CD46 serves as a cellular receptor for not only subgroup B Ads but also several human pathogens, including measles virus, human herpes virus 6 and two types of bacteria.[39] Therefore, CD46TG mice already have been used in several studies, which have reported that replication of the pathogens and inflammatory responses occur in CD46TG mice after exposure to the pathogens, demonstrating the utility of CD46TG mice as animal models (note that for study of measles virus, the alpha/beta-interferon receptor gene is usually knocked out with insertion of the human CD46 gene).[28,40,41] In addition, in most of the CD46TG mice lines used in these studies, including in our present study, expression of human CD46 is driven by the human CD46 promoter, not the promoter of a ubiquitously expressed gene, leading to a pattern of CD46 expression similar to that in humans.[27,28,40] This conservation of the expression pattern is another advantage of using CD46TG mice as an animal model.

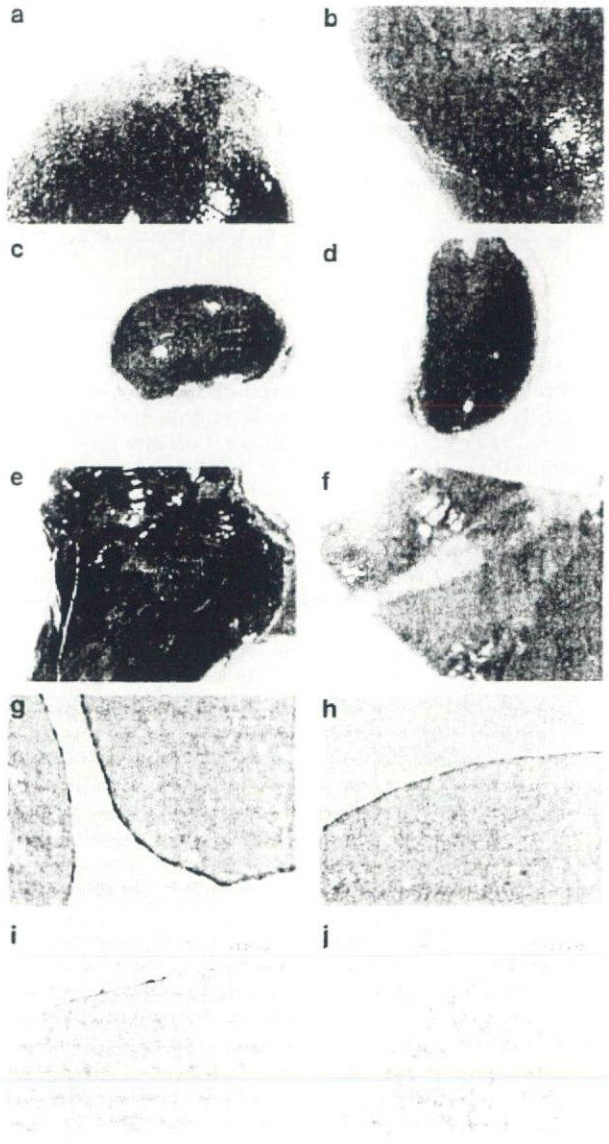


Figure 6 X-gal staining of the peritoneal organs of homozygous CD46TG mice receiving β-galactosidase-expressing Ad35 vectors. (**a**) Liver, (**c**) kidney, (**e**) peritoneum, (**g**) liver and (**i**) kidney sections from homo TG mice injected with Ad35LacZ. (**b**) Liver, (**d**) kidney, (**f**) peritoneum, (**h**) liver and (**j**) kidney sections from mock-infected homo TG mice. Ad35LacZ was injected intraperitoneally into homozygous CD46TG mice at a dose of 7.5×10^{10} VP/mouse. At 2 days postadministration, the organs were recovered after perfusion with 0.5% glutaraldehyde solution and then fixed and stained as described previously[54] by using 0.5% glutaraldehyde instead of 4% paraformaldehyde. For X-gal staining of liver and kidney, 10 μm sections were cut, fixed with 0.5% glutaraldehyde, and stained as described previously.[55]

In vivo transduction experiments using CD46TG mice showed that Ad35 vectors mediated higher transduction efficiencies in CD46TG mice than wild-type mice, indicating that Ad35 vectors recognize human CD46 as an attachment receptor on *in vivo* application. However, the transduction efficiencies of Ad35 vectors in CD46TG mice were much lower than expected. Addition of human CD46 expression to mouse primary cells greatly enhanced Ad35 vector-mediated transduction (Figure 3). Our previous study demonstrated that the transduction activities of Ad5 and Ad35 vectors were nearly equivalent in human cultured cell lines,[13] which express high levels of CD46. These results suggest that, in cells expressing sufficient CD46, the transduction efficiencies of Ad35 vectors could be similar to those of Ad5 vectors. Therefore, we had expected that the transduction efficiencies of Ad35 vectors in CD46TG mice would greatly increase to levels comparable to those of Ad5 vectors. However, the transduction efficiencies of Ad35 vectors in the organs of homozygous CD46TG mice after intravenous administration were approximately 20- to 20 000-fold lower than those of conventional Ad5 vectors at the same dose (1.5×10^{10} VP/mouse) in wild-type mice.[13] Why the *in vivo* transduction efficiencies of Ad35 vectors in CD46TG mice were lower than expected remains to be clarified. One possibility is that Ad35 vectors administered to CD46TG mice cannot access the human CD46 that is expressed on the cells. Human CD46 primarily is expressed on the basolateral surfaces of polarized epithelial cells,[42,43] and measles virus preferentially infects cells from their basolateral, rather than apical, sides.[44] Anatomical barriers such as the tightness between the basal membrane and extracellular matrix might impair the access of Ad35 vectors to the human CD46 on the basolateral cell surface. Another possibility is that an unidentified receptor or co-receptor for Ad35 is expressed in humans but not in CD46TG mice. Segerman *et al*.[45] suggest that there are two different receptors in human cells for subgroup B Ads. Interaction between Ad35 vectors and blood components (blood cells or serum proteins) might also inhibit Ad35 vector-mediated transduction after intravenous administration. In particular, the soluble form of human CD46, which is found in normal human serum,[46] might block infection by Ad35 vectors. However, preincubation of Ad35 vectors with serum or blood cells recovered from CD46TG mice did not reduce the transduction efficiencies of Ad35 vectors *in vitro* (data not shown). Further evaluation is necessary to clarify whether studies using CD46TG mice appropriately evaluate the transduction properties of Ad35 vectors. Currently, we are examining the transduction properties of Ad35 vectors in non-human primates, which express CD46 in all organs. The findings should greatly help us to understand characteristics of Ad35 vector-mediated transduction, including the validity and utility of CD46TG mice as model animals for Ad35 vectors.

The fiber shaft of Ad35 lacks the KKTK (Lys-Lys-Thr-Lys) motif, which is located in the fiber shaft of Ad5 and is considered to bind to heparan sulfate,[47] and its absence may partly explain the lower transduction efficiencies of Ad35 vectors than Ad5 vectors. Smith *et al*.[47] demonstrated that amino-acid substitution of the KKTK motif dramatically decreased the transduction efficiencies of Ad5 vectors in the mouse liver, whereas ablation of CAR- and integrin-binding sites did not significantly reduce the transduction efficiency of Ad5 vectors in the liver; these findings indicate a potential role for heparan sulfate binding in Ad5 vector-mediated liver transduction. In addition, we reported that replacement of the Ad5 fiber shaft with the Ad35 shaft, in addition to the ablation of CAR and integrin binding, decreases Ad5 transduction efficiencies in the liver.[48] Heparan sulfate