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 Medicines Agency (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 cytopathic 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.

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, CPE (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

CMV = Cytomegalovirus

CPE = Cytopathic effect

E2F = S-phase of cell cycle promoter

EBV = Epstein-Barr virus

EMEA = European Medicines Agency

EOP cells = End of production cells

FDA = Food and Drug Administration

HBV = Hepatitis B virus

HCV = Hepatitis C virus

HIF-1 = Hypoxia-inducible factor-1

HIV = Human immunodeficiency virus

HSV = Herpes simplex virus

HTLV = Human T-cell lymphotropic virus

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

MCB = Master cell bank

MVB = Master virus bank

NAT = Nucleic acid amplification test

NDV = Newcastle disease virus

PCR = Polymerase chain reaction

PSA = Prostate-specific antigen

RCA = Replication-competent adenovirus

RCV = Replication-competent virus

RT = Reverse transcriptase

TERT = Telomerase reverse transcriptase

VSV = Vesicular stomatitis virus

WCB = Working cell bank

WVB = Working virus bank

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Adenovirus Vectors Composed of Subgroup B Adenoviruses

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Abstract: Recombinant adenovirus (Ad) vectors have gained attention as gene delivery vehicles because they efficiently introduce foreign DNA into host cells, can be produced in high titers, and are able to transduce terminally differentiated cells. Conventional Ad vectors commonly used in the world, including clinical trials, are derived from subgroup C Ad serotype 5 (Ad5). Although Ad5 vector-mediated transduction provides encouraging results, preclinical and clinical applications have revealed several disadvantages of Ad5 vectors, such as high seroprevalence of anti-Ad5 antibodies in adults and low transduction efficiencies of Ad5 vectors in cells lacking the primary receptor for Ad5, the coxsackievirus and adenovirus receptor (CAR). To overcome these problems, novel recombinant Ad vectors, which are derived entirely from subgroup B Ads, including Ad serotypes 3, 7, 11, and 35, have been developed. These subgroup B Ad vectors can infect cells via human CD46 (membrane complement protein), which is ubiquitously expressed in almost all human cells, and/or via unidentified receptors other than CAR, leading to efficient transduction of subgroup B Ad vectors in most human cells, including CAR-negative cells. In addition, transduction efficiencies of subgroup B Ad vectors do not decrease in the presence of anti-Ad5 antibodies, and seroprevalences of most subgroup B Ads are lower than that of Ad5, indicating that transduction with subgroup B Ad vectors is unlikely to be hampered by preexisting anti-Ad antibodies. In this paper, we review the advances in subgroup B Ad vector research.

Keywords; Adenovirus vector, gene therapy, transduction, neutralizing antibodies, CD46, fiber, seroprevalence, coxsackievirus and adenovirus receptor (CAR), receptors.

1. INTRODUCTION

Adenoviruses (Ads) are non-enveloped, double-stranded DNA virus with icosahedral symmetry, and have been isolated from numerous mammalian species. To date, 51 human adenovirus (Ad) serotypes have been identified and categorized into six subgroups (A to F) on the basis of genomic and biological properties (Table **1**) [De Jong *et al.*, 1999]. Among the 51 serotypes, most studies of Ad vector-mediated transduction are performed using Ad vectors based on subgroup C Ad serotype 5 (Ad5), along with Ad serotype 2 (Ad2), which also belongs to subgroup C, since Ad2 and Ad5 are the most thoroughly studied and characterized serotypes. Ad5 vectors show promise as a gene transfer vector in studies on not only gene therapy but also gene function analysis; they can be grown to high titers and have extremely high transduction activity *in vivo* and *in vitro*. Additionally, a relatively large size of foreign DNA can be inserted into Ad5 vector genomic DNA.

Unfortunately, however, preclinical and clinical studies using Ad5 vectors have revealed their limited efficacy and propensity for side effects [Knowles *et al.*, 1995, Sterman *et al.*, 1998, Raper *et al.*, 2003], suggesting at least two major limitations associated with the use of Ad5 vectors in human gene therapy. The first hurdle is the inhibition in adults of Ad5 vector-mediated transduction by preexisting anti-Ad5 neutralizing antibodies, which are induced as a result of natural Ad infection. Ad5 are efficiently neutralized by the majority of human sera examined [Chirmule *et al.*, 1999, Seshidhar Reddy *et al.*, 2003, Vogels *et al.*, 2003, Kostense *et al.*, 2004, Ophorst *et al.*, 2006]. Neutralizing anti-Ad5 antibodies efficiently block Ad5 vector-mediated transduction *in vivo*, especially via systemic injection [Chen *et al.*, 2000]. In addition, preexisting anti-Ad antibodies in human blood may increase Ad5 vector-mediated liver toxicity upon re-administration [Vlachaki *et al.*, 2002] and activate the complement system to induce inflammatory reactions in the presence of Ad [Cichon *et al.*, 2001]. Another limitation is the poor transduction efficiencies of Ad5 vectors in cells showing insufficient expression levels of the primary receptor for Ad5, the coxsackievirus and adenovirus receptor (CAR). Infection efficiencies of Ad5 vectors largely depend on the levels of CAR expression. CAR is sufficiently expressed in some types of cells, including hepatocytes and epithelial cells; however, several target cells important for gene therapy, such as dendritic cells (DCs), hematopoietic stem cells, skeletal muscle cells, and malignant tumor cells, express low or negligible levels of CAR. High vector doses are required for efficient transduction in these cells, but infection with high multiplicity of infection (MOI) is associated with cytotoxicity and immunogenicity *in vivo*.

Several approaches have been employed to address these problems, including development of novel Ad vectors derived from other human Ad serotypes and Ads of animal origin [Hofmann *et al.*, 1999, Nguyen *et al.*, 1999, Rasmussen *et al.*, 1999, Reddy *et al.*, 1999, Farina *et al.*, 2001], and chemical modification by polymers conjugated with targeting ligands [Lanciotti *et al.*, 2003, Ogawara *et al.*,

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2004, Eto *et al.*, 2005]. Among these strategies, Ad vectors composed entirely of subgroup B Ads show attractive properties as gene delivery vehicles. Subgroup B Ad vectors can infect via receptors other than CAR, leading to efficient transduction in both CAR-positive and –negative cells. Neutralizing anti-Ad5 antibodies do not prevent subgroup B Ad vectors from efficient transduction. In addition, the seroprevalences of most subgroup B Ads have been found to be lower than that of Ad5 vectors. In this paper, we review the development and properties of subgroup B Ad vectors.

2. PROPERTIES OF SUBGROUP B AD VECTORS

Ad subgroup B is comprised of Ad serotypes 3, 7, 11, 14, 16, 21, 34, 35, and 50 (Table **1**). These subgroup B Ads can be subdivided into subgroups B1 (Ad serotypes 3, 7, 16, 21, and 50) and B2 (Ad serotypes 11, 14, 34, and 35) based on DNA homology. Most subgroup B1 Ads cause respiratory infection, while subgroup B2 Ads are mainly associated with infections of kidneys and the urinary tract. The first replication-incompetent subgroup B Ad vectors to be developed were Ad serotype 7a vectors, developed in 1997 by Abrahamsen *et al.* [Abrahamsen *et al.*, 1997], followed by replication-incompetent Ad serotype 3 (Ad3) [Sirena *et al.*, 2005], 11 (Ad11) [Holterman *et al.*, 2004, Stone *et al.*, 2005], and 35 (Ad35) vectors [Gao *et al.*, 2003, Sakurai *et al.*, 2003a, Vogels *et al.*, 2003] (Table **2**). In addition to subgroup B Ad vectors, fiber-substituted Ad5 vectors bearing fiber proteins of other subgroup Ads have been exploited [Gall *et al.*, 1996, Stevenson *et al.*, 1997, Shayakhmetov *et al.*, 2000, Rea *et al.*, 2001, Stecher *et al.*, 2001, Kanerva *et al.*, 2002, Mizuguchi *et al.*, 2002a, Lu *et al.*, 2006]. Fiber substitution can change the tropism of Ad5 into the tropism of Ads offering fiber proteins because fiber proteins play a crucial role in the virus tropism. However, other capsid proteins, including hexon and penton base proteins, also contribute to the infectivity of viruses [Wickham *et al.*, 1993, Wickham *et al.*, 1994, Medina-Kauwe, 2003]. Transduction with fiber-substituted Ad5 vectors is hampered by neutralizing anti-Ad5 antibodies, as described below. In this review, we mainly introduce Ad vectors fully derived from subgroup B Ads.

2.1. Receptors For Subgroup B Ad Vectors

Infection of Ads, including subgroup B Ads, is initiated by binding of the fiber knob, which is located on the C-terminal of the trimeric fiber protein, to their primary receptors on cell surface. Previous studies have demonstrated that the fiber knob of most Ads belonging to subgroup A, C, D, E and F binds to CAR as a primary receptor; by contrast, subgroup B Ads use receptors other than CAR [Roelvink *et al.*, 1998]. Cross-competition binding studies using Ad3 and Ad35 suggest that at least two receptors exist for subgroup B Ads [Segerman *et al.*, 2003a]; however, these receptors remained unknown for a long time. In 2003, human CD46 (membrane cofactor protein) was demonstrated to be a cellular receptor for most subgroup B Ads (Fig. **1**) [Gaggar *et al.*, 2003, Segerman *et al.*, 2003b].

Human CD46 is a single-chain type I transmembrane glycoprotein with a molecular mass of 45-70 kDa, and plays an important role in protecting autologous cells from complement attack by serving as a cofactor for factor I-mediated inactivation of C3b and C4b, thus blocking the complement cascade at the C3 activation stage [Adams *et al.*, 1991, Seya *et al.*, 1999]. Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine-threonine-proline-rich region, a short region of unknown function, a hydrophobic transmembrane domain, and a carboxy-terminal cytoplasmic domain. Subgroup B Ads bind to the short consensus repeat 2 (SCR2) and/or SCR1, which are in the upper regions of human CD46 [Fleischli *et al.*, 2005, Gaggar *et al.*, 2005, Sakurai *et al.*, 2006b]. The HI loop in the fiber knob of subgroup B Ads is critical for interaction with human CD46 [Gustafsson *et al.*, 2006]. Almost all human cells except for erythrocytes ubiquitously express CD46, leading to broad tropism of subgroup B Ads. Ad11 and Ad35 vectors efficiently transduce various types of human cells, including human bone-marrow $CD34^+$ cells, DCs, synoviocytes, and smooth muscle cells, which are refractory to Ad5 vectors due to their lack of CAR expression [Gao *et al.*, 2003, Sakurai *et al.*, 2003a, Sakurai *et al.*, 2003b, Vogels *et al.*, 2003, Holterman *et al.*, 2004, Stone *et al.*, 2005]. Ad3 vectors also exhibit higher transduction efficiencies in human cultured

Table 1. Human Adenovirus Serotypes

Subgroup	Serotypes	Receptor*)
A	12, 18, 31	CAR
B1	3, 7, 16, 21, 50	CD46
B2	11, 14, 34, 35	CD46
C	1, 2, 5, 6	CAR
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	CAR
E	4	CAR
F	40, 41	CAR

CAR: coxsackievirus and adenovirus receptor

*) Some Ad serotypes recognize other receptors different from CAR and CD46.

Table 2. Replication-Incompetent Subgroup B Ad Vectors

Serotype	E1 Region	E1-deleted Region	Complement Cell Lines (Introduced genes)	Groups
Ad3	E1A bp 576-1351 E1B bp 1603-3386	bp 561-3029	911-Ad3E1B cells (Ad3 E1B-55K gene)	Sirena *et al.* (2005)
Ad7	E1A bp 577-1455 *) E1B bp 1603-3386 *)	bp 476-2799	293-ORF6 cells (Ad5 E4-ORF6 gene)	Abrahamsen *et al.* (1997)**)
Ad11	E1A bp 476-1440 E1B bp 1556-3486	bp 465-3340	PER.C6/55K cells (Ad35 E1B-55K gene)	Holterman *et al.* (2004)
		bp 389-3348	293-Ad11-E1B55K cells (Ad11 E1B-55K gene)	Stone *et al.* (2005)
Ad35	E1A bp 569-1441 E1B bp 1611-3400	bp 365-2912	VK10-9 cells (Ad5 E4 gene)	Sakurai *et al.* (2003)
		bp 465-3400	PER.C6/55K cells (Ad35 E1B-55K gene)	Vogels *et al.* (2003)
		bp 338-1454	PER.C6 cells	Reddy *et al.* (2003)
		bp 561-3074	CRE8 cells expressing E1B proteins of Ad35 (Ad35 E1B gene)	Gao *et al.* (2003)

*) This shows E1 region of Ad7 Gomen strain.
**) Abrahamsen *et al.* constructed recombinant Ad7 vectors based on Ad7a.

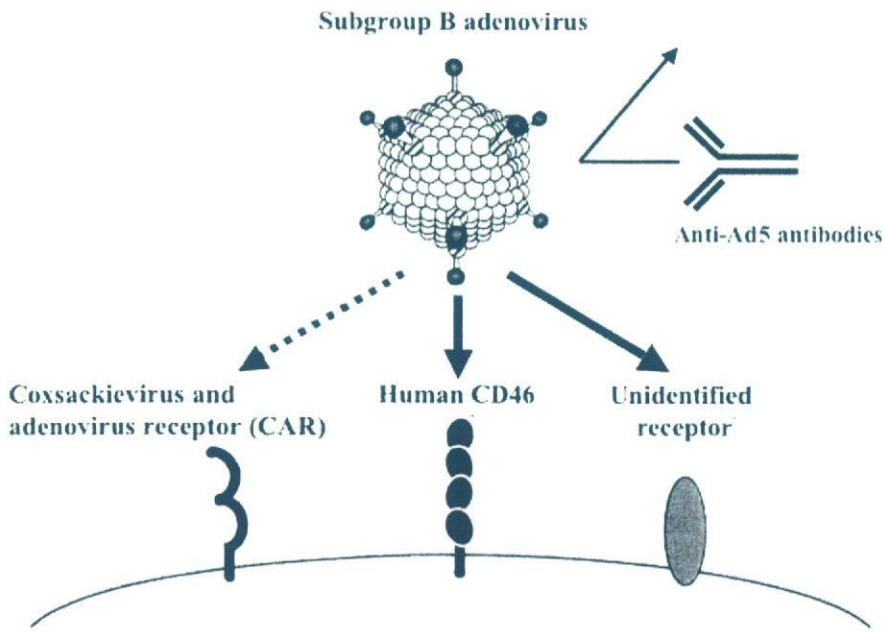

Fig. (1). Characteristics of subgroup B Ad vectors. Subgroup B Ad vectors infect cells via interaction with human CD46 and/or an unidentified receptor other than CAR. Subgroup B Ad vectors can escape neutralizing anti-Ad5 antibodies.

cell lines than Ad5 vectors [Sirena *et al.*, 2005]. In addition, human CD46 is known to be frequently overexpressed in cancer cells compared with normal cells [Fishelson *et al.*, 2003], suggesting that cancer cells overexpressing human CD46 would be a suitable target for subgroup B Ad vectors. Ad35 vectors show significantly increased ability to transduce primary glioma cells compared with Ad5 vectors [Brouwer *et al.*, 2006]. CD46 expression levels are an important determinant of the transduction efficiencies of CD46-utilizing Ad vectors [Anderson *et al.*, 2004]. Although most subgroup B Ads utilize CD46 on infection, usage of CD46 by Ad serotypes 3 and 7 (Ad7) is controversial. Ad3 and Ad7 have been demonstrated not to bind to human CD46 [Marttila *et al.*, 2005]; however, Sirena *et al.* have demon-

strated human CD46-dependent infection with Ad3 [Sirena *et al.*, 2004]. The reason for this discrepancy is unclear. On the other hand, rodent cells are refractory to subgroup B Ad vectors because rodent CD46 is expressed only in the testis, and the homology between human and rodent CD46 is low [Tsujimura *et al.*, 1998, Mead *et al.*, 1999]. Rodent cell lines, including CHO cells (Chinese hamster ovary cell line), BHK-21 cells (baby hamster kidney cell line), and B16 (mouse melanoma cell line), are refractory to subgroup B Ads [Gaggar *et al.*, 2003, Seshidhar Reddy *et al.*, 2003, Marttila *et al.*, 2005, Sirena *et al.*, 2005, Verhaagh *et al.*, 2006].

Another unidentified receptor for subgroup B Ads, other than human CD46, has been proposed. In an earlier study before identification of human CD46 as a receptor for most subgroup B Ads, Ad3, as well as the Ad3 fiber knob, have been demonstrated to bind to a membrane protein of approximately 130 kDa in a divalent cation-dependent manner [Di Guilmi *et al.*, 1995]. Two groups have also indicated the existence of a receptor for subgroup B Ads other than human CD46, which is trypsin-sensitive and requires divalent cations for virus binding to the receptor [Segerman *et al.*, 2003a, Tuve *et al.*, 2006]. Segerman *et al.* suggest that this unidentified receptor is a common subgroup B Ad receptor [Segerman *et al.*, 2003a], but Tuve *et al.* have reported that Ad serotypes 3, 7, 11, and 14 utilize the unidentified receptor while Ad serotypes 16, 21, 35, and 50 do not use it [Tuve *et al.*, 2006]. It has been found that human CD80 and CD86 are also attachment receptors for all members of subgroup B Ads [Short *et al.*, 2004, Short *et al.*, 2006]. CD80 and CD86 are co-stimulatory molecules that are primarily expressed on mature DCs and B lymphocytes. DCs are permissive to infection with Ad11 and Ad35 vectors, suggesting that subgroup B Ads might utilize CD80 and CD86 for the infection of DCs.

Efficient infection of hematopoietic cells, including hematopoietic stem cells, with subgroup B Ad vectors is another attractive characteristic of subgroup B Ad vectors. Gene transfer into hematopoietic cells provides opportunities not only for treatment of genetic immune and blood disorders, but also for characterization of gene functions in cellular growth, differentiation and survival. However, conventional Ad5 vectors poorly transduce hematopoietic cells, including hematopoietic stem cells, due to the lack of CAR. Subgroup B Ads, in particular, Ad serotypes 11, 35, and 50, have shown a much higher ability to bind to human hematopoietic cells [Segerman *et al.*, 2000, Shayakhmetov *et al.*, 2000, Knaan-Shanzer *et al.*, 2001]. Ad3, Ad11, and Ad35 vectors have been shown to efficiently transduce human primary hematopoietic cells and cultured hematopoietic cell lines [Sakurai *et al.*, 2003a, Sakurai *et al.*, 2005, Sirena *et al.*, 2005, Stone *et al.*, 2005]. In particular, human bone-marrow and cord-blood $CD34^+$ cells, and the more primitive subpopulations ($CD34^+CD38^-$ cells and $CD34^+AC133^+$ cells), which contain human hematopoietic stem cells, are much more susceptible to Ad11 and Ad35 vectors than to Ad5 vectors [Sakurai *et al.*, 2003a, Sakurai *et al.*, 2005, Stone *et al.*, 2005]. Human bone-marrow and cord-blood $CD34^+$ cells sufficiently express human CD46 [Manchester *et al.*, 2002, Sakurai *et al.*, 2005]. Furthermore, bone-marrow $CD34^+$ cells transduced with Ad35 vectors have a capacity for proliferation and differentiation [Sakurai *et al.*, 2005]. Ad vector-mediated transgene expression does not persist due to the lack of genomic integration of Ad vector DNA; however, this property would be suitable when stable transgene expression is not desirable, such as in *ex vivo* manipulation of hematopoietic cells and function analysis of genes that should be downregulated for differentiation. By contrast, when prolonged transgene expression after cell proliferation and differentiation is desired, the vector DNA should be integrated into the host genome. Several groups have developed hybrid Ad vectors such as adenovirus/adeno-associated virus vectors, which can integrate viral genomes into the host genome, to obtain persistent gene expression [Ueno *et al.*, 2000, Murphy *et al.*, 2002, Shayakhmetov *et al.*, 2002, Kubo *et al.*, 2003]. Furthermore, gutted Ad5 vectors carrying integration machinery into the host genome, such as sleeping beauty, bacteriophage-derived integrase phiC31, and retrotransposition, have been emerging [Yant *et al.*, 2002, Calos, 2006, Ivics *et al.*, 2006, Kubo *et al.*, 2006, Ehrhardt *et al.*, 2007]. Use of these Ad5 vectors also permits long-term gene expression. Subgroup B Ad vectors would be a promising framework for the development of these improved vectors.

Incorporation of foreign peptides into the fiber knob is used to modify the tropism of Ad5 vectors, enabling Ad5 vectors to transduce in a CAR-independent manner [Wickham *et al.*, 1997, Dmitriev *et al.*, 1998, Hidaka *et al.*, 1999, Mizuguchi *et al.*, 2001, Koizumi *et al.*, 2003]. Suitable sites for incorporation of foreign peptides, the HI loop and the C-terminal, have been identified in the Ad5 fiber knob. Foreign peptides include an Arg-Gly-Asp (RGD) peptide incorporated into the HI loop, which binds to αv-integrins, and a stretch of lysine residues (KKKKKKK [K7] peptide) inserted into the C-terminal, which binds to heparan sulfate. This approach has been applied to fiber-chimeric Ad5 vectors displaying the fiber knob of subgroup B Ads to expand vector tropism and to increase transduction activities of the Ad vectors. Incorporation of single RGD peptides and six-histidine tags into either the HI loop or the C-terminal of the Ad3 fiber knob has been tried [Uil *et al.*, 2003, Borovjagin *et al.*, 2005, Tyler *et al.*, 2006]. Insertion of the RGD peptide into the C-terminal domain of the Ad3 fiber knob significantly increases the transduction activity of the Ad vectors in malignant glioma cell lines and primary brain tumor cells. Fiber-mutant subgroup B Ad vectors containing foreign peptides would largely enhance the utility of subgroup B Ad vectors.

2.2. *In Vivo* Tropism

In vivo transduction properties of subgroup B Ad vectors remain to be elucidated because conventional rodents cannot be used as an animal model for subgroup B Ads. Conventional mice and rat express CD46 only in the testis. In addition, mouse and rat CD46 shows low homology with human CD46 [Tsujimura *et al.*, 1998, Mead *et al.*, 1999]. Several attempts at subgroup B Ad vector-mediated *in vivo* transduction in conventional mice were reported before human CD46 was identified as the main attachment receptor for subgroup B Ads. Intravenously administered Ad35 vectors produced lower levels of transgene expression in organs than Ad5 vectors and were mainly taken up by reticuloendothelial systems, including liver Kupffer cells, leading to degradation of

viral DNA [Sakurai *et al.*, 2003b]. Intravenous administration of Ad11 vectors also resulted in less efficient transduction in organs than that of Ad5 vectors [Stone *et al.*, 2005], while Ad7 vectors transduced liver and spleen after systemic administration [Abrahamsen *et al.*, 1997]. After identification of human CD46 as an attachment receptor for subgroup B Ads, evaluation of *in vivo* transduction properties of Ad35 vectors has been performed using human CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46 in a similar pattern to that of human. Ad35 vectors mediate higher levels of transduction efficiencies in the organs of CD46TG mice than those of conventional mice by intravenous, intraperitoneal, and intramuscular administration [Sakurai *et al.*, 2006a, Verhaagh *et al.*, 2006]; however, despite the sufficient expression of human CD46, the transduction efficiencies of Ad35 vectors in human CD46TG mice are still much lower than transduction efficiencies of Ad5 vectors in conventional mice. The reasons for the much lower transduction efficiencies of Ad35 vectors in CD46TG mice remain unclear; however, Ad35 vectors might not have access to human CD46 because human CD46 is mainly expressed on the basolateral side of cells [Ichida *et al.*, 1994, Maisner *et al.*, 1997]. Second, efficient infection with Ad35 vectors might require other cellular receptors that have not yet been identified. Identification of another receptor for subgroup B Ads would greatly contribute to advances in the evaluation of transduction properties of subgroup B Ad vectors. The absence of a heparan sulfate binding domain in the fiber shaft of Ad11 and Ad35 might also explain the low levels of *in vivo* transduction activities in mice. *In vivo* studies using Ad5 vectors have revealed that the Lys-Lys-Thr-Lys (KKTK) motif in the fiber shaft, which is believed to interact with heparan sulfate on the cell surface, plays an important role in *in vivo* transduction, especially in the liver [Smith *et al.*, 2003].

On the other hand, subgroup B Ad vectors are promising, as they appear to be safer than Ad5 vectors in the two ways. First, the poor transduction efficiencies of subgroup B Ad vectors after systemic administration suggest that locally administered subgroup B Ad vectors would not cause unwanted side effects in other organs except for injected tissues when draining from injected sites into the bloodstream. A large portion of Ad5 vectors injected into local tissues, such as tumors, drain into the bloodstream and target into other organs, mainly the liver, causing unwanted side effects [Mizuguchi *et al.*, 2002b, Okada *et al.*, 2003]. Second, subgroup B Ad vectors would be less likely potential to activate innate immunity after *in vivo* application than Ad5 vectors. Activation of innate immunity by Ad vectors consists primarily of rapid induction of inflammatory cytokines, such as interleukin (IL)-6 and -12 and subsequent tissue damages after administration [Muruve, 2004, Nazir *et al.*, 2005, Koizumi *et al.*, 2006]. Innate immunity induced by subgroup B Ad vectors has not yet been reported; however, fiber-substituted Ad5 vectors bearing the fiber protein of Ad35 elicit less innate immunity, such as inflammatory cytokine productions, after intravenous administration in mice than Ad5 vectors [DiPaolo *et al.*, 2006, Ni *et al.*, 2006]. These results suggest that Ad35 vectors have less innate toxicity than Ad5 vectors, which would be another advantage of subgroup B Ad vectors.

In *in vivo* studies, subgroup B Ad vectors are best studied as vaccine vectors by local administration of subgroup B Ad vectors encoding antigen proteins. Intradermal delivery of Ad35 vectors in a human skin model leads to efficient transduction in DCs, migration and maturation of DCs, and DC-mediated activation of specific $CD8^+$ effector T cells [de Gruijl *et al.*, 2006]. Ad11 and Ad35 vectors intramuscularly administered into conventional mice effectively elicit antigen-specific immunity even in the presence of anti-Ad5 immunity [Barouch *et al.*, 2004, Lemckert *et al.*, 2005, Nanda *et al.*, 2005, Ophorst *et al.*, 2006]. These studies indicate that Ad11 and Ad35 vectors are highly promising vaccine vectors, even though Ad11 and Ad35 vectors have lower potential to induce transgene-specific immune responses than Ad5 vectors in naïve mice [Barouch *et al.*, 2004, Lemckert *et al.*, 2005]. The lower ability of the Ad35 vector to activate immune responses is able to be partly overcome by replacing its fiber shaft with that of Ad5 [Nanda *et al.*, 2005]. It remains unclear why conventional mice are successfully vaccinated by intramuscular administration of subgroup B Ad vectors despite the absence of CD46 expression. Ad35 vectors fail to transduce mouse DCs derived from conventional mice due to the lack of CD46 expression [Sakurai *et al.*, 2006a, Verhaagh *et al.*, 2006], although intramuscular injection of Ad35 vectors in conventional mice results in detectable levels of transgene expression in the muscle, approximately two- to four-fold lower than that of Ad5 vectors [Sakurai *et al.*, 2003b, Vogels *et al.*, 2003]. Mercier *et al.* suggest that cross-priming from cells expressing Ad vector-mediated transgene products is not a key event in eliciting a $CD8^+$ T cell response [Mercier *et al.*, 2004].

2.3. Low Seroprevalence

The high seroprevalence in various human populations of Ad5 vector-neutralizing antibodies, which hamper *in vivo* transduction, has been problematic for a long time. Due to common exposure to Ad5, more than 50 % of people are seropositive for Ad5 [Seshidhar Reddy *et al.*, 2003, Vogels *et al.*, 2003, Kostense *et al.*, 2004, Ophorst *et al.*, 2006]. Neutralizing anti-Ad5 antibodies dramatically reduce the *in vivo* transduction activity of Ad5 vectors. Even in intramuscular administration, the utility of Ad5 vectors as a vaccine vector is significantly suppressed in mice preimmunized with Ad5 vectors [Ophorst *et al.*, 2004, Lemckert *et al.*, 2005, Nanda *et al.*, 2005]. In contrast, subgroup B Ad vectors successfully circumvent anti-Ad5 antibodies. Ad11 and Ad35 vectors efficiently induce transgene-specific immunity after intramuscular injection in the presence of anti-Ad5 immunity [Barouch *et al.*, 2004, Lemckert *et al.*, 2005, Nanda *et al.*, 2005, Ophorst *et al.*, 2006]. In addition, the seroprevalence of most subgroup B Ads, especially Ad11 and Ad35, is significantly lower than that of Ad5 in adults [Seshidhar Reddy *et al.*, 2003, Vogels *et al.*, 2003, Holterman *et al.*, 2004, Stone *et al.*, 2005], indicating that most subgroup B Ad vector-mediated transduction is less likely to be blocked by pre-existing immunity. Titers of neutralizing anti-Ad35 antibodies are also low in cancer and AIDS patients [Kostense *et al.*, 2004, Brouwer *et al.*, 2006], although subgroup B Ads are often isolated from immune-suppressed patients [De Jong *et al.*, 1999], suggesting that Ad35 vectors would not be hampered in cancer and AIDS patients. Titers of neutralizing

anti-Ad3 and anti-Ad7 antibodies have been found to be higher compared with other subgroup B Ads because Ad3 and Ad7 cause disease in humans more frequently than other subgroup B Ads [Vogels *et al.*, 2003].

Fiber-substituted Ad5 vectors containing fiber proteins of subgroup B Ads instead of Ad5 fibers show a tropism reflected from Ads offering fiber proteins, allowing efficient transduction in a CAR-independent manner [Gall *et al.*, 1996, Stevenson *et al.*, 1997, Shayakhmetov *et al.*, 2000, Rea *et al.*, 2001, Stecher *et al.*, 2001, Kanerva *et al.*, 2002, Mizuguchi *et al.*, 2002a, Lu *et al.*, 2006]. However, fiber-substituted Ad5 vectors are not able to circumvent inhibition by neutralizing anti-Ad5 antibodies [Havenga *et al.*, 2006b] since the majority of neutralizing anti-Ad5 antibodies are directed against the hexon protein [Sumida *et al.*, 2005], which is still present in fiber-substituted Ad5 vectors.

3. CONSTRUCTION OF SUBGROUP B AD VECTORS

Recombinant, replication-incompetent subgroup B Ad vectors are constructed by deletion of the E1 region and insertion of a transgene expression cassette into the E1-deleted region in a similar way as with Ad5 vectors. However, attention should be paid to two aspects, the first of which is the deletion size of the E1 region. Although the location and size of the E1 region of subgroup B Ads are almost the same as those of Ad5 (Table **2**), complete deletion of the E1 region of Ad35 impairs pIX expression, leading to a reduction in capsid stability, because pIX-promoter elements of Ad35 are located within the E1B region [Havenga *et al.*, 2006a]. pIX is a minor protein that increases the structural integrity of particles by stabilizing hexon-hexon interactions. In order to maintain the expression levels of pIX, the promoter region of pIX should be retained [Havenga *et al.*, 2006a], or a foreign promoter should be inserted in the front of the pIX gene [Stone *et al.*, 2005].

The second aspect to consider is E1-complementing packaging cell lines for subgroup B Ad vectors. Conventional 293 cells, which stably express E1 proteins of Ad5, do not support production of replication-incompetent subgroup B Ad vectors with both E1A and E1B deleted. Instead of 293 cells, 293 transformants stably expressing the E1B-55K protein of subgroup B Ads, or E4 open-reading frame (ORF)6 proteins of Ad5, can support the production of subgroup B Ad vectors as complement cell lines (Table **2**). This suggests that E1B-55K and E4 ORF6 proteins should be derived from the same subgroup for viral growth. The Ad5 E1B-55K protein, which is encoded from bp 2019 to 3509 in the Ad5 genome, has been reported to form a complex with the Ad5 E4 ORF6 protein and to in turn increase selective export of late viral mRNA [Rubenwolf *et al.*, 1997, Weigel *et al.*, 2000]. E1B-55K proteins from one subgroup Ad might not be able to be complexed with E4 ORF6 proteins derived from another subgroup Ad. Although the appearance of replication-competent Ad is of great concern in an Ad5 vector production system using 293 cells, these complement cell lines for subgroup B Ad vectors offer one important advantage: replication-competent subgroup B Ads do not emerge in culture by homologous recombination because there is no homologous region between the genomic DNA of subgroup B Ad vectors and the complement cell lines.

In almost all studies which have developed subgroup B Ad vectors, subgroup B Ad vector DNA containing a transgene expression cassette has been constructed by homologous recombination in packaging cell lines or *E. coli*; however, these methods are inefficient and difficult to operate. In order to more rapidly and easily obtain subgroup B Ad vectors, we have applied an improved *in vitro* ligation method, which was developed by Mizuguchi and Kay [Mizuguchi *et al.*, 1998, 1999], to the construction of Ad35 vectors by introducing *I-CeuI* and *PI-SceI* sites in the E1-deleted region [Sakurai *et al.*, 2005]. Using this method, recombinant Ad35 vectors can be produced as easily and efficiently as Ad5 vectors.

4. DRAWBACKS OF SUBGROUP B AD VECTORS

As mentioned above, subgroup B Ad vectors possess various advantages as gene delivery vehicles; on the other hand, there are some hurdles to be overcome for successful gene therapy using subgroup B Ad vectors. First, the plaque-forming unit (PFU)-to-particle ratios of subgroup B Ad vectors are often lower than those of Ad5 vectors. We have confirmed that the PFU-to-particle ratios of Ad35 vectors are approximately in the range of 1:50 to 1:250 [Sakurai *et al.*, 2003a, Sakurai *et al.*, 2006a], while those of Ad5 vectors are usually in the range of 1:10 to 1:50. Stone *et al.* reported that Ad11 vectors are found to have PFU-particle ratios in the range of 1:150 to 1:250 [Stone *et al.*, 2005]. It is now unclear why PFU-to-particle ratios of subgroup B Ad vectors are lower than those of Ad5 vectors; wild-type Ad3 and Ad7 also have PFU-to-particle ratios 10-fold lower than wild-type Ad2 and Ad5 [Defer *et al.*, 1990]. Immune responses induced by Ad vectors, including innate and acquired immunity, are activated by both infectious and noninfectious virus particles, indicating that preparation of Ad vectors at high PFU-to-particle ratios is preferable to circumvent immune responses by Ad vectors.

Second, fundamental properties of subgroup B Ads, such as cellular receptors and intracellular trafficking, have been poorly characterized, compared with Ad5, which is the most extensively characterized Ad. Subgroup B Ads utilize receptors different from Ads of other groups, suggesting that distribution after *in vivo* application, intracellular trafficking, and immune responses induced by subgroup B Ad vectors would be largely different from those of Ad5 vectors. Notably, *in vivo* application of subgroup B Ad vectors might show markedly unique immune responses because interaction between CD46 and CD46 ligands is associated with immune events [Kemper *et al.*, 2003, Sanchez *et al.*, 2004, Oliaro *et al.*, 2006]. Other CD46-utilizing viruses, such as measles virus and herpesvirus type 6, cause specific immune responses, such as immune suppression and downregulation of CD46 [Schneider-Schaulies *et al.*, 1995, Santoro *et al.*, 1999, Dockrell, 2003]. We have confirmed that *in vitro* transduction with Ad35 vectors also induces downregulation of surface CD46 expression in human peripheral blood mononuclear cells (PBMC) [Sakurai *et al.*]. Cytokine productions induced by interferon-gamma (IFN-γ) and lipopolysaccharide in PBMC are significantly suppressed by preinfection with CD46-utilizing Ad vectors [Iacobelli-Martinez *et al.*, 2005]. Further analysis is necessary to clarify the transduction properties of subgroup B Ad vectors.

CONCLUSION

In this review, we have described the features of subgroup B Ad vectors. Subgroup B Ad vectors have some advantages over conventional Ad5 vectors; however, there are hurdles to be overcome before their successful application to gene therapy. Further improvement of subgroup B Ad vectors, including characterization of the basic properties of subgroup B Ad viruses, should greatly facilitate their usefulness in clinical applications.

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ABBREVIATIONS

Ad	=	Adenovirus
Ads	=	Adenoviruses
HSCs	=	Hematopoietic stem cells
IL	=	Interleukin
IFN	=	Interferon
CAR	=	Coxsackievirus and adenovirus receptor
CD46-transgenic	=	CD46TG
DC	=	Dendritic cell
DCs	=	Dendritic cells
RGD motif	=	Arginine-glycine- asparagine motif
PFU	=	Plaque forming unit
PBMC	=	Peripheral blood mononuclear cells
MOI	=	Multiplicity of infection
ORF	=	Open reading frame
SCR	=	Cysteine-rich short consensus repeat

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改良型アデノウイルスベクターを用いた各種幹細胞への遺伝子デリバリー

特集　遺伝子・核酸医薬品のデリバリー

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Gene delivery into stem cells by modified adenovirus vectors

The application of adenovirus (Ad) vectors, which are widely used in gene therapy, depends on CAR (coxsackievirus and adenovirus receptor) expression on the cells. To overcome this problem, the capsid proteins of Ad vectors have been genetically modified. Here, we introduce several types of capsid-modified Ad vectors. Furthermore, we describe the application of capsid-modified Ad vectors into some kinds of stem cells for regenerative medicine.

アデノウイルスベクターは，遺伝子治療や基礎研究に幅広く用いられている．しかしながら，アデノウイルスベクターの受容体であるCARの発現が乏しい細胞では，アデノウイルスベクターによる遺伝子導入効率は低い．そこで筆者らは，CAR非依存的に遺伝子導入可能な種々のカプシド改変型アデノウイルスベクターを開発してきた．本稿では，これらのカプシド改変型アデノウイルスベクターの特徴と，その応用例として，近年，再生医療分野で注目を浴びている各種幹細胞への高効率遺伝子導入法について解説する．

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key words : adenovirus vector, gene delivery, stem cells, regenerative medicine, gene therapy

ヒトアデノウイルスは，赤血球凝集活性の違いからAからFまでのサブグループにわけられ，計51種の血清型が存在する．遺伝子治療用ベクターとして繁用されているアデノウイルスベクターは，サブグループCに属するヒト5型アデノウイルスを基盤としている．5型アデノウイルスの感染は，カプシド蛋白質のファイバーが細胞表面に存在するCAR(coxsackievirus and adenovirus receptor) と結合することにより起こる．そのため，従来の5型アデノウイルスベクターは，CAR陽性細胞へは効率よく遺伝子導入可能であるが，CARの発現が乏しい細胞への遺伝子導入効率はきわめて低いことが課題であった．

CARの発現が乏しい細胞としては，造血幹細胞や間葉系幹細胞などの幹細胞，血液細胞，悪性度の高いがん細胞，血管内皮細胞などがあげられ，このような細胞へはアデノウイルスベクターの適用は不向きであった．

本稿では，CAR陰性細胞に対しても高効率遺伝子導入が可能な種々の改良型アデノウイルスベクターについて概説し，つぎにその応用例として，近年，再生医療への応用が期待されている幹細胞への高効率遺伝子導入法について紹介する．

改良型アデノウイルスベクター

1. ファイバー改変型アデノウイルスベクター

アデノウイルスのファイバーはノブ，シャフト，テール領域にわけられ，ノブ領域がCARと結合する(**図1a**)．アデノウイルスベクターの感染域を拡大するためのアプローチの一つとして，ファイバーノブのHIループやC末端領域に細胞接着活性などを有する外来ペプチドを遺伝子工学的に挿入することがあげられる．

筆者らは，これらの部位に外来ペプチドをコードした遺伝子をきわめて簡便に挿入できるファイバー

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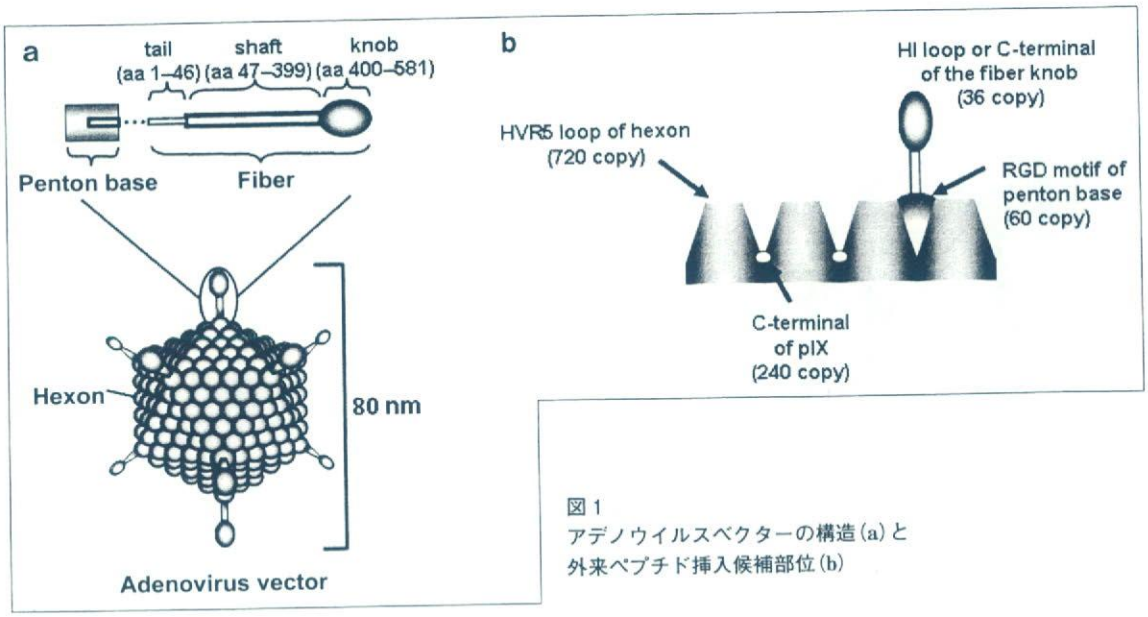

図1
アデノウイルスベクターの構造(a)と
外来ペプチド挿入候補部位(b)

改変型アデノウイルスベクター作製法を開発済みであり[1,2]，この技術と *in vitro* ライゲーションに基づいたE1欠損領域への外来遺伝子挿入法[3,4]を組み合わせることにより，CAR陰性細胞に対しても高効率に遺伝子導入が可能なアデノウイルスベクターを簡便に作製する方法を開発した(**図2，表1**)．ファイバーノブのHIループにRGD(リジン-グリシン-アスパラギン酸)からなるペプチドを挿入しインテグリンと親和性を保持させることにより(RGD型ベクター)，種々のがん細胞[5,6]や樹状細胞[7]，血管内皮細胞[8]に高効率な遺伝子導入が可能となった．また，ファイバーノブのC末端領域に七つのリジン残基からなるポリペプチドを挿入したファイバー改変型(K7型)アデノウイルスベクターでは，ヘパラン硫酸と親和性を有するようになり，種々のCAR陰性細胞に効率よく遺伝子導入が可能である[2,9]．

さらに筆者らは，最近，HIV(human immunodeficiency virus)由来のprotein transduction domain(PTD：蛋白質導入ドメイン)として知られているTatペプチド[10]をファイバーノブに付与することで，RGD配列やポリリジン配列を付与したベクターよりも，より広範に効率よく外来遺伝子を発現可能であることを見いだした．したがって，Tatペプチドを付与したアデノウイルスベクターは，遺伝子治療用ベクターや基礎研究におけるツールとしてきわめて有用であると考えられる．

2. ファイバー置換型アデノウイルスベクター

サブグループBに属する11型あるいは35型などのアデノウイルスはCARではなく，補体制御因子として知られているCD46を受容体として感染することが知られている[11,12]．

そこで筆者らは，5型アデノウイルスベクターのファイバー領域のみを35型アデノウイルスのものに置換したベクター(F35型ベクター)やすべての構造蛋白質を35型アデノウイルスからなるベクターを開発した(**図2**)[13~15]．ヒトにおいては，CD46は赤血球を除くほぼすべての細胞に発現していることが知られており，これらのベクターは，多くのヒト由来細胞だけではなく，たとえば5型アデノウイルスベクターでの遺伝子導入が困難で，遺伝子治療の重要な標的細胞であるCD34陽性ヒト造血幹細胞にも効率よく遺伝子導入可能であることが明らかとなった[14,16]．

3. ヘキソン，pIX改変型アデノウイルスベクター

ファイバーは，ウイルス1粒子当たり12分子存在するが(ファイバーは3量体を形成するため，ノブは36コピー存在する)，主要なカプシド蛋白質のヘキソンは240分子(同じく3量体をとっている

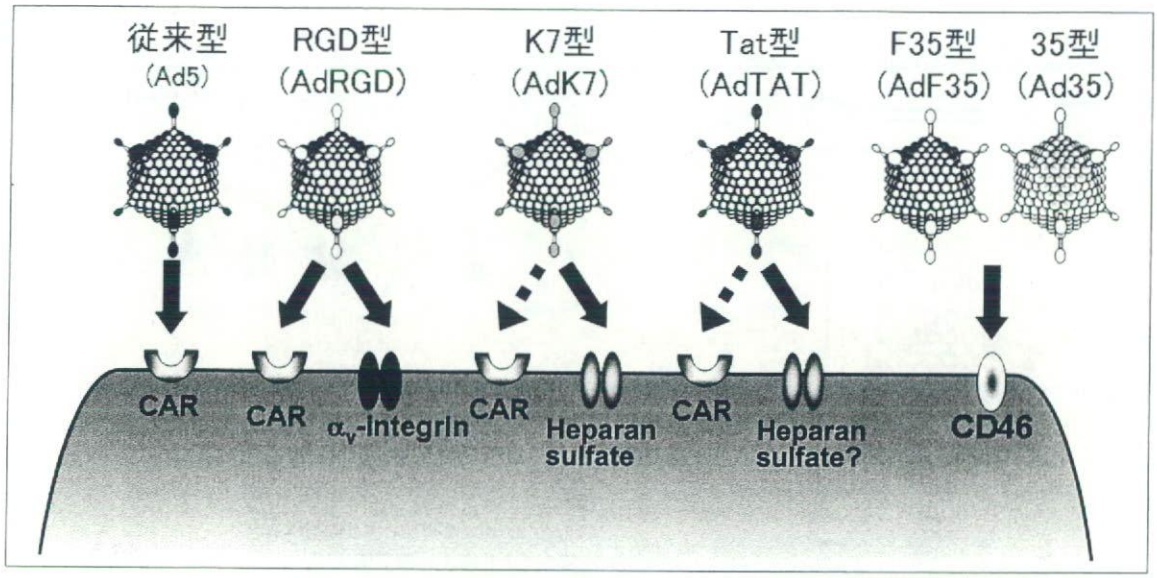

図2　筆者らが独自に開発した改良型アデノウイルスベクター

野生型のファイバーを持った従来の5型アデノウイルスベクターは細胞表面上の受容体であるCARを認識して感染するが，RGD配列やポリリジン配列をファイバーに有したファイバー改変型ベクターはCARだけでなく，αvインテグリンやヘパラン硫酸を認識しても感染できる．また，35型のアデノウイルスのファイバーを有したベクターや，すべての構造蛋白質が35型アデノウイルスからなるベクターは，CD46を認識して感染する．Tat型ベクターは未知の機構により(ヘパラン硫酸を介するという報告もある)細胞内に取り込まれる．

表1　改良型アデノウイルスベクターを用いた各種細胞への遺伝子導入効率

	従来型 Ad	改良型 Ad
ヒト造血幹細胞 (CD34陽性細胞)	5%以下	50%以上(Ad35)
ヒト間葉系幹細胞	10%以下	100%(AdK7)
マウスES細胞	10%以下	90%以上 (Ad5-EF1α-プロモーター)
樹状細胞 (ヒト・マウス)	10%以下	90%以上(AdRGD)
CAR陰性がん細胞 (ヒト・マウス)	10%以下	100%(AdRGD)
マウス脂肪細胞	10%以下	50%以上(AdK7)

ため，720コピー存在する)，pIX(プロテインIX)は240分子(240コピー)存在するため，これらの領域を改変できれば，より効率のよい遺伝子導入が可能になることが期待される(図1b)．ヘキソンは，ウイルス粒子の中で最も豊富に存在する蛋白質であり，カプシドの構造を維持する役割を有する．またpIXは，ヘキソンカプソマーの間に挟まれた形で存在し，ヘキソン同士の結合を補助する．

そこで，ヘキソンのhypervariable region 5 (HPV5)およびpIXのC末端に外来ペプチドを挿入できるベクター系を構築し，ファイバー改変型，ヘキソン改変型，pIX改変型各アデノウイルスベクターの遺伝子発現効率について比較検討した[17]．

各挿入部位にRGDペプチドを挿入した結果，ファイバーノブのHIループにRGDペプチドを挿入したファイバー改変型アデノウイルスベクターが最も高い遺伝子発現効率を示した．これは，ヘキソンやpIXと比較し，ファイバーノブは最も外側に位置するので，宿主細胞と結合しやすくなっていること，およびヘキソンやpIXに発現させたペプチドはファイバーによる立体障害のため，細胞表面に作用しにくくなっている可能性が原因として考えられる．

したがって，ヘキソンやpIXを改変する場合，ファイバーを遺伝子工学的に欠損させた(ファイバーレス)アデノウイルスベクターを基盤ベクターとすることにより遺伝子発現効率が改善する可能性が考えられ(この場合，同時にCAR経由による遺伝子導入も起こらないため，ターゲティングアデノウイルスベクターの開発にもつながる)，現在検討中である．

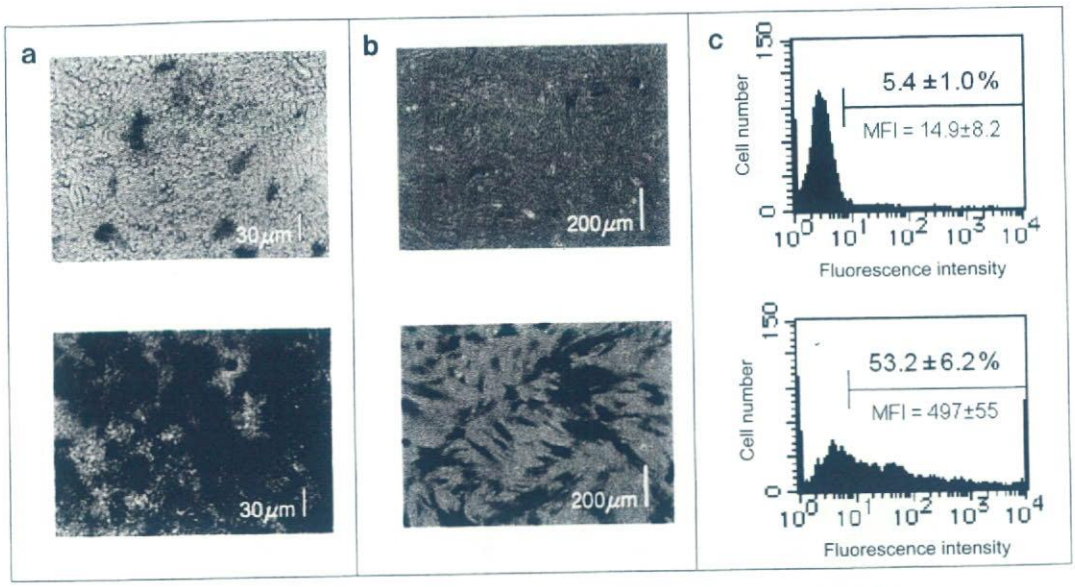

図3　改良型アデノウイルスベクターによる各種幹細胞への高効率遺伝子導入

a：マウスES細胞に対し，CMVプロモーター(上段)あるいはEF-1αプロモーター(下段)有するβ-ガラクトシダーゼ遺伝子を発現する従来型アデノウイルスベクターを感染させた．

b：ヒト間葉系幹細胞に対し，従来型(上段)あるいはK7型(下段)アデノウイルスベクターを用いてβ-ガラクトシダーゼ遺伝子を発現させた．

c：ヒト造血幹細胞に対し，従来型(上段)あるいは35型(下段)アデノウイルスベクターを用いてGFP遺伝子を発現させた．

ES細胞への高効率遺伝子導入法

ES細胞は胚盤胞内部細胞塊由来の細胞であり，無限に増殖するとともにすべての機能細胞に分化する性質を有する．近年，ヒトES細胞が樹立されたことにより，これを再生医療へ応用するための基礎研究が活発に行われている[18]．しかしながら，ES細胞の分化を自由に制御する技術はいまだ確立されておらず，その原因の一つとしてES細胞への効率よい遺伝子導入法が確立されていないことがあげられる．

これまで，ES細胞に対しては，プラスミドDNAを用いたエレクトロポレーション法(プラスミドDNAを電気的刺激により細胞内に導入し，染色体にわずかに目的遺伝子と薬剤耐性遺伝子が組み込まれた細胞を薬剤で選択する方法)[19]，レトロウイルスベクター[20]，レンチウイルスベクター[21]，ポリオーマウイルスの複製機構を利用したスーパートランスフェクション法(ポリオーマウイルスの複製起点を含んだプラスミドDNAがマウスES細胞ではエピゾーマルに増幅できる性質を利用した方法)[22]などが外来遺伝子導入法として用いられてきた．

しかしながら，これらは半永久的に導入遺伝子を発現しつづける方法であり，ES細胞の分化制御，特に医療目的などの細胞分化後には発現を停止させたい場合には好ましくない．アデノウイルスベクターは，導入遺伝子が宿主染色体へ組み込まれることなく，染色体外にエピゾームとして存在することから(増幅しない)，遺伝子発現が一過性であり，ES細胞を目的の機能細胞に分化させたあとは導入遺伝子の発現が消失するものと期待される．

そこで，筆者らは，マウスES細胞に最も適したアデノウイルスベクターによる遺伝子導入法の確立を試みた．その結果，マウスES細胞はアデノウイルス受容体CARを高発現しており，従来型アデノウイルスベクターが最適であることが明らかとなった[23]．また，RSV，CMV，CA(β-actin promoter/CMV enhancer)，EF-1αの4種のプロモーターを用いて検討した結果，ES細胞にはCAおよびEF-1αプロモーターを用いた場合にのみ遺

伝子発現がみられ，RSV やCMV プロモーターはほとんど機能しなかった(**図 3a**)．

これまでアデノウイルスベクターは，ES 細胞への遺伝子導入には不適と考えられてきたが，これは多くの場合，最も一般的に用いられているCMV プロモーターを用いて検討されてきたためであり，ウイルスの細胞へのエントリー自体には問題がないことが示された．ただし，CA プロモーターを用いた場合には，ES 細胞のみならずその支持細胞(フィーダー細胞)である胚線維芽細胞にも遺伝子発現がみられたのに対し，EF-1α プロモーターを用いた場合には，ほぼES 細胞特異的に遺伝子発現可能であった．これは，EF-1α プロモーターの活性が胚線維芽細胞にくらべES 細胞において相対的に高いことが原因と考えられる．したがって，目的により両プロモーターを使いわけることによって，再生医療への幅広い応用が期待できる．

つぎに，最適化されたアデノウイルスベクターを用いてES 細胞に機能遺伝子を導入し，実際にES 細胞の分化を制御できるかどうかについて検討した．マウスES 細胞は，フィーダー細胞由来のサイトカインLIF(leukemia inhibitory factor)がその未分化維持に必須であることが知られている．LIF は受容体に結合後，下流のSTAT3(signal transducer and activator of transcription 3)を介してシグナルを伝達する．

そこで，EF-1α プロモーターを有した従来型アデノウイルスベクターを用いて，STAT3 のdominant-negative 変異体(STAT3F) のcDNA をマウスES 細胞に導入することにより，LIF の下流シグナルを阻害させたところ，LIF 存在下でもES 細胞は三胚葉すべての細胞に分化することが明らかとなった．ES 細胞の未分化維持には，LIF 以外にもNanog などの転写因子が必須であることが明らかとなっている．

そこで，先述のベクターを用いてSTAT3F とNanog を同時に発現させたところ，STAT3F による細胞分化シグナルがNanog 発現により阻害され，ES 細胞は未分化状態を維持しつづけた．したがって，アデノウイルスベクターを用いることでES 細胞の分化を自由に制御できる可能性が示された[23]．現在，筆者らはES 細胞に対し分化に関与するマスター遺伝子などを導入することにより，特定の細胞への分化制御が可能かどうか検討中である．

間葉系幹細胞への高効率遺伝子導入法

間葉系幹細胞は骨髄由来のストローマ細胞であり，骨，軟骨，脂肪，心筋系列などの中胚葉系細胞に分化することができ，未分化状態で細胞を容易に増殖させることが出来る[24]．また，最近では，間葉系幹細胞は神経細胞，肝細胞，インスリン産生細胞などの外胚葉や内胚葉系の細胞へも分化するという報告もあり，再生医療や組織工学への応用が強く期待されている．

間葉系幹細胞の分化を制御する手段の一つとして，細胞分化に関与する遺伝子を導入することがあげられる．アデノウイルスベクターを用いた間葉系幹細胞への遺伝子導入も試みられてきたが，ヒト間葉系幹細胞はCAR を発現していないためにその導入効率はきわめて低く，遺伝子導入には高タイターのベクターを必要としていた[25,26]．

筆者らは，種々のファイバー改変型アデノウイルスベクターを用いて間葉系幹細胞にレポーター遺伝子を導入し，その発現効率を比較検討した．その結果，ヒト間葉系幹細胞にはK7 型ベクターが最も適しており，従来型ベクターの460 倍の遺伝子導入効率を示すことが明らかとなった(**図 3b**)[27]．RGD 型ベクターやF35 型ベクターは，従来型ベクターに比較しそれぞれ16 倍，130 倍の導入効率を示した．また，種々のプロモーターを用いて比較検討したところ，CA プロモーターが最適であった．

したがって，間葉系幹細胞にはCA プロモーターを有するK7 型アデノウイルスベクターを用いることにより，最も高効率に遺伝子導入できることが明らかとなった．間葉系幹細胞は，さまざまな系列の細胞に分化するというだけではなく，担がんマウスに投与された場合には腫瘍に集積する性質を有している[28]．したがって，間葉系幹細胞は分化させた細胞自身を治療に利用するだけでなく，抗腫瘍性サイトカインなどを発現する間葉系幹細胞を，がんに対する細胞治療薬として利用できる可能性があり，現