HUMAN GENE THERAPY 9:2577–2583 (November 20, 1998)
Mary Ann Liebert, Inc.

Efficient Construction of a Recombinant Adenovirus Vector by an Improved *In Vitro* Ligation Method

HIROYUKI MIZUGUCHI[1,2] and MARK A. KAY[1]

ABSTRACT

An efficient method for constructing a recombinant adenovirus (Ad) vector, based on an *in vitro* ligation, has been developed. To insert the foreign gene into an adenoviral DNA, we introduced three unique restriction sites, I-*Ceu*I, *Swa*I, and PI-*Sce*I, into the E1 deletion site of the vector plasmid, which contains a complete E1, E3-deleted adenovirus type 5 genome. I-*Ceu*I and PI-*Sce*I are intron-encoded endonucleases with a sequence specificity of at least 9–10 and 11 bp, respectively. A shuttle plasmid, pHM3, containing multiple cloning sites between the I-*Ceu*I and PI-*Sce*I sites, was constructed. After the gene of interest was inserted into this shuttle plasmid, the plasmid for E1-deleted adenovirus vector could be easily prepared by *in vitro* ligation using the I-*Ceu*I and PI-*Sce*I sites. *Swa*I digestion of the ligation products prevented the production of a plasmid containing a parental adenovirus genome (null vector). After transformation into *E. coli*, more than 90% of the transformants had the correct insert. To make the vector, a *Pac*I-digested, linearized plasmid was transfected into 293 cells, resulting in a homogeneous population of recombinant virus. The large number and strategic location of the unique restriction sites will not only increase the rapidity of production of new first-generation vectors for gene transfer but will allow for rapid further improvements in the vector DNA backbone.

OVERVIEW SUMMARY

One of the limitations of recombinant adenovirus vectors is their construction, which is a time-consuming procedure. This study demonstrates that the plasmid containing recombinant adenovirus DNA can be prepared by a simple *in vitro* ligation using three unique restriction sites, I-*Ceu*I, *Swa*I, and PI-*Sce*I, in the E1 deletion region. *Pac*I digestion of the recombinant plasmid generates the DNA for adenovirus vector, which has an inverted terminal repeat at both ends of the genome. Homogeneous recombinant virus could be obtained by the transfection of the linearized plasmid into 293 cells. This improved *in vitro* ligation system is a simple and efficient method by which to construct a recombinant adenovirus vector for gene therapy.

INTRODUCTION

RECOMBINANT ADENOVIRUS VECTORS have been shown to have great promise for gene transfer in basic research as well as for clinical treatment of many diseases (Kozarsky and Wilson, 1993; Kay and Woo, 1994). They can transduce foreign genes efficiently into both cultured cells and many target organs *in vivo*. There are more than 40 serotypes of adenovirus (Ad) identified. The Ad type 5 genome has been used most commonly to make recombinant Ad vector. The genome of human Ad is a linear 36-kb, double-stranded DNA genome that encodes more than 50 gene products. In the first-generation Ad vector, the early region 1 (E1) is replaced by the foreign gene and the virus propagated in an E1-*trans*-complementing cell line such as 293. By deleting E1 and early region 3 (E3) sequences up to about 8 kb of foreign gene can be inserted (Bett *et al.*, 1994). However, *in vitro* manipulation of Ad DNA is difficult. Unique and useful restriction sites are limited because of the large size of the genome, making the construction of Ad vectors relatively labor intensive. Two standard methods to make E1-deleted Ad vectors have been developed: an *in vitro* ligation method (Berkner and Sharp, 1983; Gilardi *et al.*, 1990; Rosenfeld *et al.*, 1991) and a homologous recombination method in 293 cells (Bett *et al* 1994; Miyake *et al.*, 1996). The *in vitro* ligation method uses whole viral DNA genomes and a plasmid containing the left end of the Ad genome with the left inverted terminal repeat (ITR), the packaging signal, and the

[1]Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA 94305.
[2]Present address: Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158, Japan.

E1A enhancer sequence (map units 0 to 1.3). After the gene of interest is inserted downstream of the viral sequence of the plasmid, the fragment containing viral sequence and the gene of interest is excised and ligated into the unique *Cla*I site (map unit 2.6), replacing a portion of the viral E1 region. Then, the ligated DNA is directly transfected into 293 cells to make recombinant virus. However, this method is rarely used now because the efficiency is low and the recombinant virus requires purification of contaminating wild-type and transgene null viruses related to incomplete restriction digestion and self-religation. One system based on homologous recombination (Bett *et al.*, 1994) uses two plasmids with overlapping fragments that recombine *in vivo*. The first plasmid contains the entire Ad genome with a deletion of the DNA packaging signal and E1 region. The second plasmid contains the left ITR, packaging signal, and overlapping sequence with the first plasmid. After the gene of interest is introduced into the second plasmid, the two plasmids are cotransfected into 293 cells. The virus, which is produced by the recombination in 293 cells, is isolated through plaque purification. The major limitation to this approach is that the recombination event occurs at a low frequency.

Newer methods for adenoviral preparation are based on homologous recombination of two plasmids, using yeast artificial chromosomes (YACs; Ketner *et al.*, 1994) or bacteria (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998). These methods, while more efficient, are more complex. The YAC system requires yeast culture and manipulation (Ketner *et al.*, 1994), while the *Escherichia coli* system requires three-step transformations using an additional, nonconventional host bacterial strain (BJ5183recBCsbcBC) (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998).

In this article we report the efficient construction of an E1-deleted Ad vector by an improved *in vitro* ligation method. This system requires a simple *in vitro* ligation using routine molecular biology reagents and transformation in commonly used bacterial strains.

MATERIALS AND METHODS

Plasmids

Vector plasmids pAdHM1, -2, -3, and -4 were constructed as follows. pHVad2 (provided by HepaVec, Berlin, Germany), which has the left end (bp 1–341 and bp 3524–5790) of the Ad type 5 genome with an E1 deletion, was cut by *Cla*I and *Eco*RI, and ligated with oligonucleotides 1 (5′ CGTAACTATAACGGTCCTAAGGTAGCGAG 3′) and 2 (5′ AATTCTCGCTACCTTAGGACCGTTATAGTTA 3′) (I-*Ceu*I recognition sequences are underlined), resulting in pAd4. pAd4 was then cut with *Eco*RI and *Sal*I, and then ligated with oligonucleotides 3 (5′ AATT*ATTTAAAT*ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA 3′) and 4 (5′ TCGATGCCATTTCATTACCTCTTTCTCCGCACCCGACATAGAT*ATTTAAAT* 3′) (PI-*Sce*I and *Swa*I recognition sequences are underlined and italicized, respectively), resulting in pAd18, which contains I-*Ceu*I, *Swa*I, and PI-*Sce*I sites. pAd19, which contains the Ad type 5 genome (bp 1 to 21562), was prepared by the insertion of the *Pac*I/*Bam*HI fragment of pTG3602 (Chartier *et al.*, 1996), which has a full-length Ad type 5 genome flanked with a *Pac*I site, in the plasmid derived from pGEM7Zf(–) (Promega, Madison, WI). The *Pac*I/*Xca*I fragment of pAd18 and pAd19 were then ligated, resulting in pAd16. The *Bam*HI/*Pac*I fragment (bp 21562 to the right end of the genome) of pTG3602 or pHVad1 (provided by HepaVec), both of which have the Ad type 5 genome with a deletion in the E3 region (bp 28133–30818), were introduced into the *Cla*I and *Bam*HI sites of pGEM7Zf(–), after the *Pac*I site of pTG3602 and pHVad1 was changed into a *Cla*I site by using a *Cla*I linker (New England BioLabs, Beverly, MA), resulting in pAd1 and pAd2, respectively. The fragment of pAd16 digested with *Pac*I and *Bam*HI was then cloned into the *Nsi*I and *Bam*HI sites of pAd1 and pAd2, respectively, after the *Nsi*I site was changed into a *Pac*I site by using a *Pac*I linker (New England BioLabs). The resulting plasmids were named pAdHM1 and pAdHM2, respectively. The *Cla*I site of pAdHM1 and pAdHM2 was changed into a *Pac*I site by using oligonucleotides 5 (5′ CGTTAATTAA 3′) and 6 (5′ CGTTAATTAA 3′) (*Pac*I recognition sequences are underlined), resulting in pAdHM3 and pAdHM4, respectively. pAdHM1, -2, -3, and -4 have I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region. pAdHM1 and -3 have the Ad genome with a deletion in the E1 region (ΔE1; bp 342–3523), while pAdHM2 and -4 have the Ad genome with a deletion in the E1 and E3 regions (ΔE1, bp 342–3523; ΔE3, bp 28133–30818). pAdHM1 and pAdHM2 have a *Pac*I site at the left end of the Ad genome and a *Cla*I site at the right end of the genome. pAdHM3 and pAdHM4 have *Pac*I sites at both ends of the Ad genome (Fig. 1A).

Shuttle plasmid pHM3 was constructed as follows. pUC18 was cut by *Aat*II and *Hin*dIII, and ligated with oligonucleotides 7 (5′ TAACTATAACGGTCCTAAGGTAGCGA A 3′) and 8 (5′ AGCTTTCGCTACCTTAGGACCGTTATAGTTA ACGT 3′) (I-*Ceu*I recognition sequences are underlined), resulting in pHM1. pHM1 was cut with *Eco*RI and *Pvu*II, and then ligated with oligonucleotides 9 (5′ AATTCTGGCAAACAGCTATTATGGGTATTATGGGT 3′) and 10 (5′ ACCCATAATACCCATAATAGCTGTTTGCCAG 3′) (PI-*Psp*I recognition sequences are underlined), resulting in pHM2. This plasmid has another intron-coded enzyme (PI-*Psp*I) recognition site. The PI-*Psp*I fragment of pHM2 was then ligated with oligonucleotides 11 (5′ ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCATTAT 3′) and 12 (5′ TGCCATTTCATTACCTCTTTCTCCGCACCCGACATA GATATAA 3′) (PI-*Sce*I recognition sequences are underlined). The resulting plasmid was named pHM3 (Fig. 1B). pHM3 contains the pUC18-derived multicloning site between the I-*Ceu*I and PI-*Sce*I sites. All mutations were sequenced with a Sequenase version 2.0 DNA sequencing kit (New England Nuclear, Boston, MA).

Construction of recombinant Ad vector DNA containing human α_1-antitrypsin expression cassette

The *Xho*I fragment of pBSRSVhAAT (Kay *et al.*, 1992), containing the Rous sarcoma virus long terminal repeat (RSV LTR) promoter, human α_1-antitrypsin (hAAT) cDNA, and bovine growth hormone polyadenylation signal, was cloned into the *Sal*I site of pHM3. Depending on the orientation of the hAAT expression cassette, the resulting plasmids were named pHM3-hAAT1 and pHM3-hAAT2 (Fig. 1C).

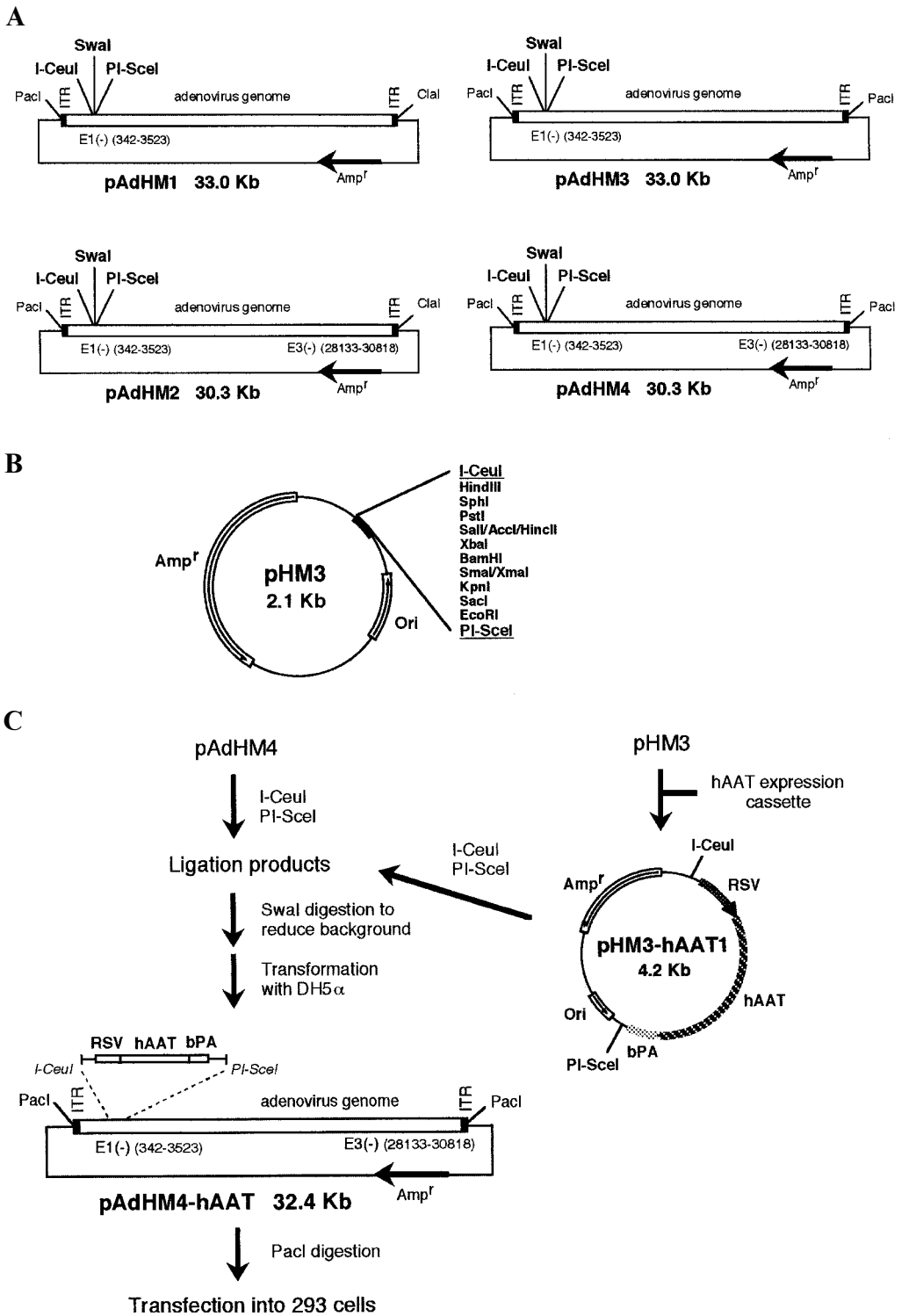

FIG. 1. Construction of recombinant adenovirus vectors by a simple *in vitro* ligation method. **(A)** Vector plasmids pAdHM1, -2, -3, and -4. **(B)** Shuttle plasmid pHM3. **(C)** The strategy for construction of E1- and E3-deleted adenovirus vector. The expression cassette of interest (RSVhAATbPA) was inserted into the *Sal*I site of the multicloning site of pHM3, and the resulting plasmid, pHM3-hAAT1, was digested with I-*Ceu*I and PI-*Sce*I. The fragment containing the hAAT expression cassette was ligated with pAdHM4 digested with I-*Ceu*I and PI-*Sce*I. Transformation into DH5α was performed after the ligation samples were digested with *Swa*I to reduce the formation of colonies containing parental vector plasmid (pAdHM4).

To construct the plasmid for a recombinant Ad vector expressing hAAT, pHM3-hAAT1 was digested with I-*Ceu*I and PI-*Sce*I, and the fragment containing the hAAT expression cassette was isolated by gel extraction after agarose gel electrophoresis. pAdHM4 was also digested with I-*Ceu*I and PI-*Sce*I, but the digested DNA was purified by phenol–chloroform extraction and ethanol precipitation. Digested pAdHM4 (0.1 μg) was then ligated to the pHM3 fragment containing the hAAT expression cassette at 16°C for more than 2 hr. To reduce the background with the parental plasmid, pAdHM4, the ligation products were treated at 65°C for 20 min to inactivate T4 DNA ligase, and then digested with *Swa*I. *Swa*I cuts the parental plasmid (pAdHM4), but not the recombinant plasmid. Finally, the DNAs were transformed with electrocompetent DH5α (chemical-competent DH5α can be also used), and the individual clones were screened by restriction analysis (Fig. 1C). Large-scale preparation of plasmid pAdHM4-hAAT was performed by using a Qiagen Plasmid Maxi-kit (Qiagen, Chatsworth, CA). No rearrangement of the plasmid during amplification was observed.

Generation of adenovirus vector

pAdHM4-hAAT was linearized by the digestion with *Pac*I and purified by phenol–chloroform extraction and ethanol precipitation. The DNA was transfected into subconfluent 293 cells plated in a 60-mm dish with SuperFect (Qiagen) according to the manufacturer's instructions. The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS) or with 0.5% overlaid agarose–DMEM containing 10% FCS. Ten days later, the cells were harvested and five independent plaques were isolated. The virus was released by four cycles of freezing and thawing, and amplified in 293 cells. Recombinant virus expressing hAAT was referred to as AdhAAT. The titer of the virus was measured by standard plaque assay in 293 cells (Kay *et al.*, 1995).

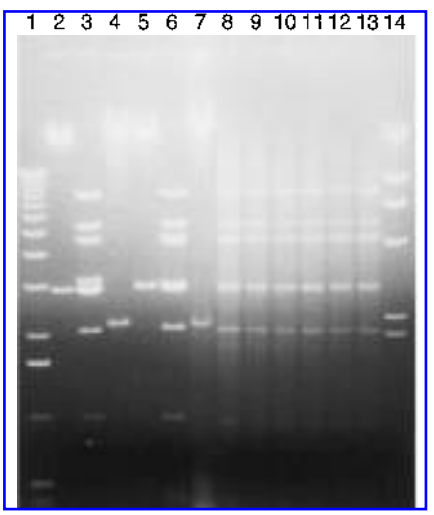

FIG. 2. Restriction endonuclease analysis. pAdHM4, pAdHM4-hAAT plasmid, or recombinant adenovirus AdhAAT DNA was digested by *Pac*I, *Pac*I/*Hin*dIII, *Hin*dIII, or I-*Ceu*I/PI-*Sce*I, separated in a 0.8% agarose gel, and ethidium bromide stained. Lane 1, 1 kb DNA ladder marker; Lane 2, *Pac*I-digested pAdHM4 plasmid DNA; lane 3, *Pac*I/*Hin*dIII-digested pAdHM4 plasmid DNA; lane 4, I-*Ceu*I/PI-*Sce*I-digested pAdHM4-hAAT plasmid DNA; lane 5, *Pac*I-digested pAdHM4-hAAT plasmid DNA; lane 6, *Pac*I/*Hin*dIII-digested pAdHM4-hAAT plasmid DNA; lane 7, I-*Ceu*I/PI-*Sce*I-digested AdhAAT (pool) viral DNA; lane 8, *Hin*dIII-digested AdhAAT (pool) viral DNA; lane 9, *Hin*dIII-digested AdhAAT (clone 1) viral DNA; lane 10, *Hin*dIII-digested AdhAAT (clone 2) viral DNA; lane 11, *Hin*dIII-digested AdhAAT (clone 3) Viral DNA; lane 12, *Hin*dIII-digested AdhAAT (clone 4) viral DNA; lane 13, *Hin*dIII-digested AdhAAT (clone 5) viral DNA; lane 14, *Hin*dIII-digested λ marker.

hAAT expression in HeLa cells infected with AdhAAT

HeLa cells (8×10^5 cells) were seeded into a 60-mm dish, and the next day they were treated with AdhAAT (at a multiplicity of infection [MOI] of 20 or 100). The cells were cultured with DMEM containing 10% FCS and 2 days later hAAT concentrations in the medium were determined by enzyme-linked immunosorbent assay as previously described (Kay *et al.*, 1995).

RESULTS

Construction and characterization of E1-deleted adenovirus vector DNA

To construct the plasmid with recombinant Ad vector DNA containing a foreign gene at the E1 deletion site by a single *in vitro* ligation, three unique restriction sites (I-*Ceu*I, *Swa*I, and PI-*Sce*I) were introduced into the E1 deletion site of the vector plasmid containing a complete vector genome. I-*Ceu*I (Marshall and Lemieux, 1991) and PI-*Sce*I (Gimble and Thorner, 1992) are intron-encoded endonucleases that recognize at least 9–10 and 11 bp, respectively. *Swa*I is a rare-cutting restriction enzyme with a sequence specificity of 8 bp. The I-*Ceu*I and PI-*Sce*I sites were used for the insertion of foreign gene, while the *Swa*I site was used to reduce the generation of parental, nonrecombined plasmid. The resulting vector plasmids, pAdHM1, -2, -3, and -4, contain the complete Ad genome minus the E1 (pAdHM1, -3) or E1/E3 region (pAdHM2, -4), have *Pac*I (pAdHM3, -4) or *Pac*I/*Cla*I (pAdHM1, -2) sites at both ends of the Ad genome, and have I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion site (Fig. 1A). *Hin*dIII and *Pac*I treatment of pAdHM4 produced the expected fragments shown in Fig. 2 (lanes 2 and 3).

A shuttle plasmid, pHM3, containing a pUC18-derived multicloning site between the I-*Ceu*I and PI-*Sce*I sites was constructed (Fig. 1B) and used for cloning an expression cassette containing human α_1-antitrypsin cDNA under the transcriptional control of the RSV LTR promoter (RSVhAATbPA); the

TABLE 1. HUMAN α1-ANTITRYPSIN EXPRESSION IN HeLa CELLS INFECTED WITH AdhAAT[a]

MOI	*hAAT concentration (μg/dish/24 hr)*
20	2.44 ± 0.65
100	12.6 ± 0.8

[a]HeLa cells were infected with AdhAAT (MOIs of 20 and 100), and hAAT concentration in the medium was determined 48 hr postinfection. Data represent means ± SD for four experiments.

product was called pHM3-hAAT1. The corresponding Ad vector DNA, pAdHM4-hAAT (Fig. 1C), was produced by *in vitro* ligation of I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and pHM3-hAAT1 (Fig. 1C). I-*Ceu*I and PI-*Sce*I digestion of pAdHM4-hAAT produced the expected 2.1-kb DNA fragment corresponding to an hAAT expression cassette in addition to the expected adenoviral fragments (Fig. 2, lane 4). The expected DNA fragments were also detected with either *Pac*I or *Pac*I/*Hin*dIII digestion (Fig. 2, lanes 5 and 6). More than 90% (15 of 16 clones) of the transformants had the correct restriction pattern.

Generation of adenovirus vector expressing hAAT

To demonstrate that pAdHM4-hAAT was able to produce Ad vector in 293 cells, *Pac*I-linearized pAdHM4-hAAT DNA was transfected into 293 cells and the cells were cultured for 10 days. The cell lysates were used to infect fresh 293 cells, followed by routine adenovirus preparation. The correctness of the viral DNA was verified by double digestion with I-*Ceu*I and PI-*Sce*I (Fig. 2, lane 7) or *Hin*dIII digestion (Fig. 2, lane 8) and found to have the same pattern as *Pac*I/*Hin*dIII-digested pAdHM4-hAAT plasmid DNA. For further confirmation, 17 independent plaques were found to have identical DNA restriction patterns (results for 5 clones are shown in Fig. 2, lanes 9–13). Virus was not produced by the transfection of circular plasmid (pAdHM4-hAAT) into 293 cells, consistent with previous reports (Chartier *et al.*, 1996; He *et al.*, 1998). Finally, to confirm the functionality of the vector, robust hAAT expression was detected in AdhAAT-infected HeLa cells (Table 1).

DISCUSSION

We have developed a simple and efficient method for constructing recombinant E1-deleted adenoviral vectors by *in vitro* ligation. Previously, it has been difficult to manipulate Ad DNA *in vitro* because of the paucity of unique useful restriction sites. Thus, by insertion of unique I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1-deleted region of the plasmid with an E1-deleted Ad genome, we could easily produce a vector by a simple *in vitro* ligation. The shuttle plasmid pHM3, which has multicloning sites between the I-*Ceu*I and PI-*Sce*I sites, allowed for the gene of interest to be easily introduced into the E1 deletion region

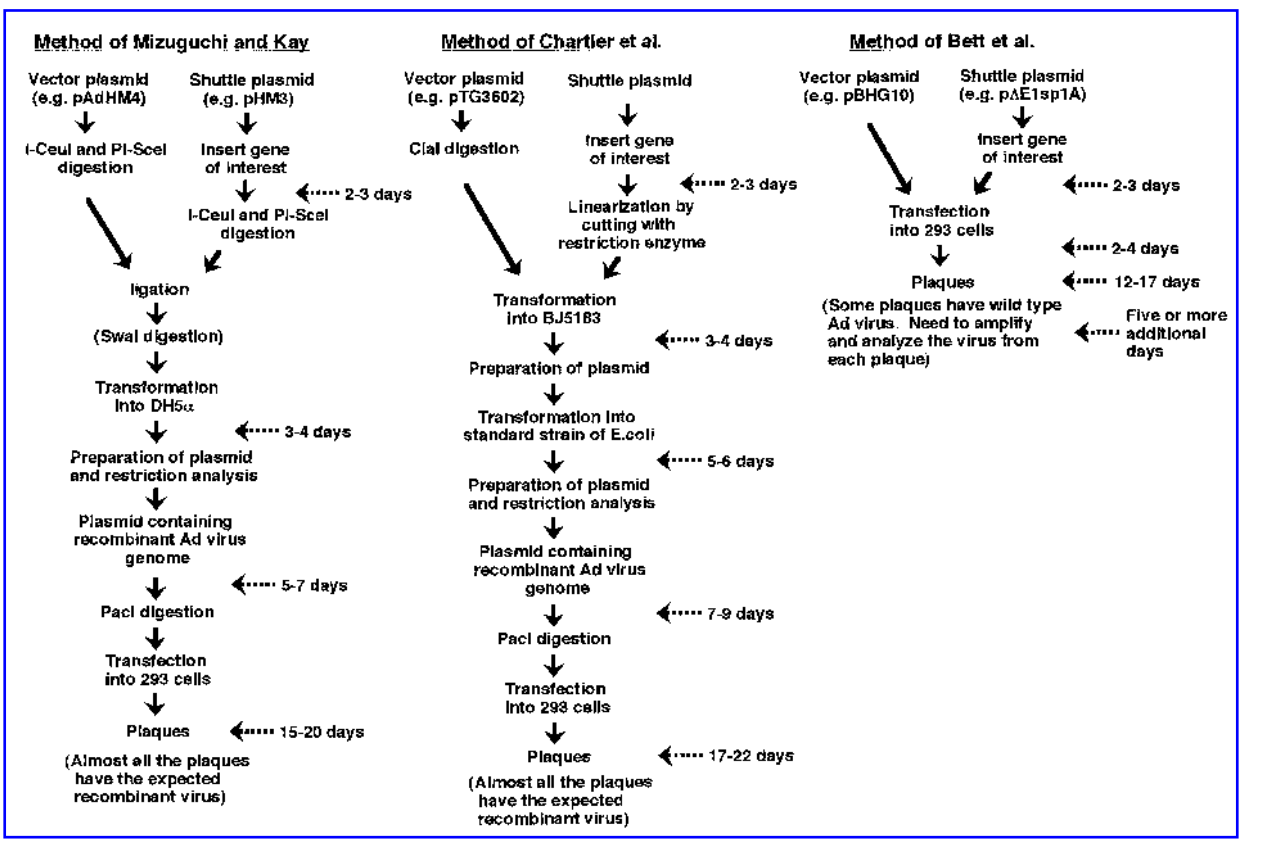

FIG. 3. A comparison flow chart of the *in vitro* ligation and homologous recombination methods in *E. coli* or 293 cells.

of the vector. The *Swa*I site of the vector plasmid nearly eliminated the possibility of generating a null cassette (nontransgene)-containing vector owing to the presence of the parental plasmid DNA.

The conventional *in vitro* ligation method (Berkner and Sharp, 1983; Gilardi *et al.*, 1990; Rosenfeld *et al.*, 1991) has a number of disadvantages. Recombinant DNA cannot be recovered as a plasmid, because the viral DNA is used as a vector and the ligation products are directly transfected into 293 cells. Therefore, in addition to the low efficiency, there is the generation of null vector and wild-type virus because of incomplete digestion and/or religation of the DNAs.

Although several other improved methods to make recombinant Ad vectors have been reported (Bett *et al.*, 1994; Chartier *et al.*, 1996; Miyake *et al.*, 1996; Crouzet *et al.*, 1997; Fu and Deisseroth, 1997; He *et al.*, 1998), they are based on inefficient homologous recombination in 293 cells (Bett *et al.*, 1994; Miyake *et al.*, 1996) or require additional steps. Miyake *et al.* (1996) reported an efficient method of vector production based on homologous recombination in 293 cells, but this required an extra step of λ packaging and wild-type adenoviral genome generation was observed. The homologous recombination method in yeast (Ketner *et al.*, 1994) and *E. coli* (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998) is probably as efficient in terms of recombinant viral production as the method described here; however, the *E. coli* system requires a three-step transformation, including the use of two different *E. coli* strains. This system does allow easy insertion of foreign genes into the E3 region as well as the E1 region. A cosmid Ad vector plasmid cloning strategy requires an additional λ packaging step (Fu and Deisseroth, 1997). A flow chart comparing the properties of the different homologous recombination systems is shown in Fig. 3.

The major advantage of our system is its simplicity; it requires only a routine two-step transformation protocol that is familiar to any molecular biologist. Because of the paucity of generation of wild-type or null vectors, the time-consuming plaque purification procedure is not absolutely required to produce virus. Furthermore, the system will allow for the easy modification of vector DNA backbone, or for addition of various expression cassettes, by routine cloning because of the many unique restriction sites in pAdHM1 and pAdHM2. These include *Pac*I (left end of the genome); I-*Ceu*I, *Swa*I, and PI-*Sce*I; *Xca*I (bp 5764, in the case of pAdHM2); *Pme*I (bp 13255); *Bam*HI (bp 21562); *Spe*I (bp27082); *Srf*I (bp 27527); and *Cla*I (right end of the genome). In addition, this system may allow for the production of vectors that have proven difficult to make because they produce a protein that interferes with DNA recombination, or because they reduce cell viability because of their prolonged expression in mammalian cells. In general, the use of this system should facilitate the construction of additional Ad vectors for gene transfer in basic research as well as gene therapy.

ACKNOWLEDGMENTS

We thank Leonard Meuse for technical assistance and Andre Lieber for helpful discussion. H.M. is the recipient of a fellowship from the Japan Society for the Promotion of Science. This work was supported by NIH DK49022.

REFERENCES

BERKNER, K.L., and SHARP, P.A. (1983). Generation of adenovirus by transfection of plasmids. Nucleic Acids Res. **11,** 6003–6020.

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.

CHARTIER, C., DEGRYSE, E., GANTZER, M., DIETERLE, A., PAVIRANI, A., and MEHTALI, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J. Virol. **70,** 4805–4810.

CROUZET, J., NAUDIN, L., ORSINI, C., VIGNE, E., FERRERO, L., LE ROUX, A., BENOIT, P., LATTA, M., TORRENT, C., BRANELLEC, D., DENEFLE, P., MAYAUX, J.F., PERRICAUDET, M., and YEH, P. (1997). Recombinational construction in *Escherichia coli* of infectious adenoviral genomes. Proc. Natl. Acad. Sci. U.S.A. **94,** 1414–1419.

FU, S., and DEISSEROTH, A.B. (1997). Use of the cosmid adenoviral vector cloning system for the in vitro construction of recombinant adenoviral vectors. Hum. Gene Ther. **20,** 1321–1330.

GILARDI, P., COURTNEY, M., PAVIRANI, A., and PERRICAUDET, M. (1990). Expression of human alpha 1-antitrypsin using a recombinant adenovirus vector. FEBS Lett. **267,** 60–62.

GIMBLE, F.S., and THORNER, J. (1992). Homing of a DNA endonuclease gene by meiotic gene conversion in *Saccharomyces cerevisiae*. Nature (London) **357,** 301–306.

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.

KAY, M.A., and WOO, S.L. (1994). Gene therapy for metabolic disorders. Trends Genet. **19,** 253–257.

KAY, M.A., LI, Q., LIU, T.J., LELAND, F., TOMAN, C., FINEGOLD, M., and WOO, S.L.C. (1992). Hepatic gene therapy: Persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery *in vivo*. Hum. Gene Ther. **3,** 641–647.

KAY, M.A., GRAHAM, F., LELAND, F., and WOO, S.L.C. (1995). Therapeutic serum concentrations of human alpha1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. Hepatology **21,** 815–819.

KETNER, G., SPENCER, F., TUGENDREICH, S., CONNELLY, C., and HIETER, P. (1994). Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. Proc. Natl. Acad. Sci. U.S.A. **91,** 6186–6190.

KOZARSKY, K.F., and WILSON, J.M. (1993). Gene therapy: Adenovirus vectors. Curr. Opin. Genet. Dev. **3,** 499–503.

LIEBER, A., HE, C.-Y., KIRILLOVA, I., and KAY, M.A. (1996). Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. J. Virol. **70,** 8944–8960.

MARSHALL, P., and LEMIEUX, C. (1991). Cleavage pattern of the homing endonuclease encoded by the fifth intron in the chloroplast large subunit rRNA-encoding gene of *Chlamydomonas eugametos*. Gene **104,** 241–245.

MIYAKE, S., MAKIMURA, M., KANEGAE, Y., HARADA, S., SATO, Y., TAKAMORI, K., TOKUDA, C., and SAITO, I. (1996). Efficient generation of recombinant adenoviruses using adenovirus DNA–terminal protein complex and a cosmid bearing

the full-length virus genome. Proc. Natl. Acad. Sci. U.S.A. **93,** 1320–1324.

ROSENFELD, M.A., SIEGFRIED, W., YOSHIMURA, K., YONEYAMA, K., FUKAYAMA, M., STIER, L.E., PAAKKO, P.K., GILARDI, P., STRATFORD PERRICAUDET, L.D., PERRICAUDET, M., *et al.* (1991). Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. Science **252,** 431–434.

Address reprint requests to:
Dr. Mark A. Kay
Department of Pediatrics
Stanford University School of Medicine
300 Pasteur Drive, Room G305A
Stanford, CA 94305

Received for publication June 3, 1998; accepted after revision September 9, 1998.

This article has been cited by:

1. Hiroyuki Mizuguchi , Naoko Funakoshi , Tetsuji Hosono , Fuminori Sakurai , Kenji Kawabata , Teruhide Yamaguchi , Takao Hayakawa . 2007. Rapid Construction of Small Interfering RNA-Expressing Adenoviral Vectors on the Basis of Direct Cloning of Short Hairpin RNA-Coding DNAs. *Human Gene Therapy* **18**:1, 74-80. [Abstract] [PDF] [PDF Plus]
2. Naoko Kanagawa, Masakazu Niwa, Yutaka Hatanaka, Yoichi Tani, Shinsaku Nakagawa, Takuya Fujita, Akira Yamamoto, Naoki Okada. 2007. CC-chemokine ligand 17 gene therapy induces tumor regression through augmentation of tumor-infiltrating immune cells in a murine model of preexisting CT26 colon carcinoma. *International Journal of Cancer* **121**:9, 2013. [CrossRef]
3. S Kurachi, K Tashiro, F Sakurai, H Sakurai, K Kawabata, K Yayama, H Okamoto, S Nakagawa, H Mizuguchi. 2007. Fiber-modified adenovirus vectors containing the TAT peptide derived from HIV-1 in the fiber knob have efficient gene transfer activity. *Gene Therapy* **14**:15, 1160. [CrossRef]
4. S Murakami, F Sakurai, K Kawabata, N Okada, T Fujita, A Yamamoto, T Hayakawa, H Mizuguchi. 2007. Interaction of penton base Arg-Gly-Asp motifs with integrins is crucial for adenovirus serotype 35 vector transduction in human hematopoietic cells. *Gene Therapy* . [CrossRef]
5. J-Q Gao, N Kanagawa, Y Motomura, T Yanagawa, T Sugita, Y Hatanaka, Y Tani, H Mizuguchi, Y Tsutsumi, T Mayumi. 2007. Cotransduction of CCL27 gene can improve the efficacy and safety of IL-12 gene therapy for cancer. *Gene Therapy* **14**:6, 491. [CrossRef]
6. Kenji Kawabata, Fuminori Sakurai, Hiroyuki Mizuguchi. 2007. Gene delivery into stem cells by modified adenovirus vectors. *Drug Delivery System* **22**:2, 148. [CrossRef]
7. S Kurachi, N Koizumi, F Sakurai, K Kawabata, H Sakurai, S Nakagawa, T Hayakawa, H Mizuguchi. 2007. Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon. *Gene Therapy* **14**:3, 266. [CrossRef]
8. Christine B. Kern, Russell A. Norris, Robert P. Thompson, W. Scott Argraves, Sarah E. Fairey, Leticia Reyes, Stanley Hoffman, Roger R. Markwald, Corey H. Mjaatvedt. 2007. Versican proteolysis mediates myocardial regression during outflow tract development. *Developmental Dynamics* **236**:3, 671. [CrossRef]
9. Kinshi Kosaka, Masakazu Yashiro, Yoji Sakate, Kosei Hirakawa. 2007. A Synergistic Antitumor Effect of Interleukin-2 Addition with CD80 Immunogene Therapy for Peritoneal Metastasis of Gastric Carcinoma. *Digestive Diseases and Sciences* **52**:8, 1946. [CrossRef]
10. Manabu Yamashita, Asami Ino, Kenji Kawabata, Fuminori Sakurai, Hiroyuki Mizuguchi. 2007. Expression of coxsackie and adenovirus receptor reduces the lung metastatic potential of murine tumor cells. *International Journal of Cancer* **121**:8, 1690. [CrossRef]
11. Ran-yi Liu, Yan Li, Ying Cheng, Zhi-hui Liang, Jiang-xue Wu, Mu-sheng Zeng, Gang Xue, Fu-zhou Tian, Wenlin Huang. 2007. Dendritic cells modified with 6Ckine/IFNγ fusion gene induce specific cytotoxic T lymphocytes in vitro. *Cancer Immunology Immunotherapy* **56**:11, 1831. [CrossRef]
12. Y Li, H Li, G Yao, W Li, F Wang, Z Jiang, M Li. 2007. Inhibition of telomerase RNA (hTR) in cervical cancer by adenovirus-delivered siRNA. *Cancer Gene Therapy* **14**:8, 748. [CrossRef]
13. Naoya Koizumi , Kenji Kawabata , Fuminori Sakurai , Yoshiteru Watanabe , Takao Hayakawa , Dr. Hiroyuki Mizuguchi . 2006. Modified Adenoviral Vectors Ablated for Coxsackievirus–Adenovirus Receptor, αv Integrin, and Heparan Sulfate Binding Reduce In Vivo Tissue Transduction and Toxicity. *Human Gene Therapy* **17**:3, 264-279. [Abstract] [PDF] [PDF Plus]
14. YinDuo Tan, Gang Xue, XiangLi Jiang, XiaoQing Li, Yue Lu, WenLin Huang, HaiPeng Xu, XiaoQin Chen, QiRong Geng, HuiJie Fan. 2006. In vitro effect of adenovirus-mediated human Gamma Interferon gene transfer into human mesenchymal stem cells for chronic myelogenous leukemia. *Hematological Oncology* **24**:3, 151. [CrossRef]

15. F Sakurai, K Kawabata, N Koizumi, N Inoue, M Okabe, T Yamaguchi, T Hayakawa, H Mizuguchi. 2006. Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice. *Gene Therapy* . [CrossRef]
16. N Okada, A Sasaki, M Niwa, Y Okada, Y Hatanaka, Y Tani, H Mizuguchi, S Nakagawa, T Fujita, A Yamamoto. 2006. Tumor suppressive efficacy through augmentation of tumor-infiltrating immune cells by intratumoral injection of chemokine-expressing adenoviral vector. *Cancer Gene Therapy* **13**:4, 393. [CrossRef]
17. F Sakurai, K Kawabata, T Yamaguchi, T Hayakawa, H Mizuguchi. 2005. Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities. *Gene Therapy* **12**:19, 1424. [CrossRef]
18. S Nagano, H Oshika, H Fujiwara, S Komiya, K Kosai. 2005. An efficient construction of conditionally replicating adenoviruses that target tumor cells with multiple factors. *Gene Therapy* **12**:18, 1385. [CrossRef]
19. Naoki OKADA. 2005. Design and Creation of Cytomedicine for Application to Cell Therapy. *YAKUGAKU ZASSHI* **125**:8, 601. [CrossRef]
20. Michael Rist, Leanne Cooper, Rebecca Elkington, Susan Walker, Chrysa Fazou, Judy Tellam, Tania Crough, Rajiv Khanna. 2005. Ex vivoexpansion of human cytomegalovirus-specific cytotoxic T cells by recombinant polyepitope: implications for HCMV immunotherapy. *European Journal of Immunology* **35**:3, 996. [CrossRef]
21. Hiroyuki Mizuguchi, Kenji Kawabata, Fuminori Sakurai, Takao Hayakawa. 2005. Efficient gene transfer into hematopoietic stem cell, mesenchymal stem cell, and ES cell by modified adenovirus vectors. *Ensho Saisei* **25**:5, 447. [CrossRef]
22. Shosuke IMAI, Masayuki KURODA, Ryusuke YAMASHITA, Yoshihito ISHIURA. 2005. Therapeutic inhibition of Epistein-Barr virus-associated tumor cell growth by dominant-negative EBNA1. *Uirusu* **55**:2, 239. [CrossRef]
23. Yuka Okada, Naoki Okada, Hiroyuki Mizuguchi, Takao Hayakawa, Shinsaku Nakagawa, Tadanori Mayumi. 2005. Transcriptional targeting of RGD fiber-mutant adenovirus vectors can improve the safety of suicide gene therapy for murine melanoma. *Cancer Gene Therapy* **12**:7, 608. [CrossRef]
24. Yusuke Eto, Jian-Qing Gao, Fumiko Sekiguchi, Shinnosuke Kurachi, Kazufumi Katayama, Mitsuko Maeda, Koichi Kawasaki, Hiroyuki Mizuguchi, Takao Hayakawa, Yasuo Tsutsumi. 2005. PEGylated adenovirus vectors containing RGD peptides on the tip of PEG show high transduction efficiency and antibody evasion ability. *The Journal of Gene Medicine* **7**:5, 604. [CrossRef]
25. Masakazu Hirouchi, Hiroshi Suzuki, Yuichi Sugiyama. 2005. Treatment of Hyperbilirubinemia in Eisai Hyperbilirubinemic Rat by Transfecting Human MRP2/ABCC2 Gene. *Pharmaceutical Research* **22**:4, 661. [CrossRef]
26. Jian-Qing Gao, Toshiki Sugita, Naoko Kanagawa, Keisuke Iida, Naoki Okada, Hiroyuki Mizuguchi, Takashi Nakayama, Takao Hayakawa, Osamu Yoshie, Yasuo Tsutsumi. 2005. Anti-tumor Responses Induced by Chemokine CCL19 Transfected into an Ovarian Carcinoma Model via Fiber-Mutant Adenovirus Vector. *Biological & Pharmaceutical Bulletin* **28**:6, 1066. [CrossRef]
27. Tetsuji Hosono , Hiroyuki Mizuguchi , Kazufumi Katayama , Zhi-Li Xu , Fuminori Sakurai , Akiko Ishii-Watabe , Kenji Kawabata , Teruhide Yamaguchi , Shinsaku Nakagawa , Tadanori Mayumi , Takao Hayakawa . 2004. Adenovirus Vector-Mediated Doxycycline-Inducible RNA Interference. *Human Gene Therapy* **15**:8, 813-819. [Abstract] [PDF] [PDF Plus]
28. Naoya Koizumi, Hiroyuki Mizuguchi, Masuo Kondoh, Makiko Fujii, Takao Hayakawa, Yoshiteru Watanabe. 2004. Efficient Gene Transfer into Human Trophoblast Cells with Adenovirus Vector Containing Chimeric Type 5 and 35 Fiber Protein. *Biological & Pharmaceutical Bulletin* **27**:12, 2046. [CrossRef]
29. Yusuke Eto, Jian-Qing Gao, Fumiko Sekiguchi, Shinnosuke Kurachi, Kazufumi Katayama, Hiroyuki Mizuguchi, Takao Hayakawa, Yasuo Tsutsumi, Tadanori Mayumi, Shinsaku Nakagawa. 2004. Neutralizing Antibody Evasion Ability of Adenovirus Vector Induced by the Bioconjugation of Methoxypolyethylene Glycol Succinimidyl Propionate (MPEG-SPA). *Biological & Pharmaceutical Bulletin* **27**:6, 936. [CrossRef]

30. Hiroyuki Mizuguchi , Zhi-Li Xu , Fuminori Sakurai , Tadanori Mayumi , Takao Hayakawa . 2003. Tight Positive Regulation of Transgene Expression by a Single Adenovirus Vector Containing the rtTA and tTS Expression Cassettes in Separate Genome Regions. *Human Gene Therapy* **14**:13, 1265-1277. [Abstract] [PDF] [PDF Plus]
31. Anja Ehrhardt , Peter D. Peng , Hui Xu , Leonard Meuse , Mark A. Kay . 2003. Optimization of Cis-Acting Elements for Gene Expression from Nonviral Vectors In Vivo. *Human Gene Therapy* **14**:3, 215-225. [Abstract] [PDF] [PDF Plus]
32. J. GUO, P. PICHURIN, Y. NAGAYAMA, B. RAPOPORT, S. M. MCLACHLAN. 2003. Insight into antibody responses induced by plasmid or adenoviral vectors encoding thyroid peroxidase, a major thyroid autoantigen. *Clinical and Experimental Immunology* **132**:3, 408. [CrossRef]
33. Kunihiko Tanaka, Shinichiro Towata, Kazuhiko Nakao, Hiroyuki Mizuguchi, Takao Hayakawa, Masami Niwa, Nobuko Ishii, Yuji Nagayama. 2003. Thyroid cancer immuno-therapy with retroviral and adenoviral vectors expressing granulocyte macrophage colony stimulating factor and interleukin-12 in a rat model. *Clinical Endocrinology* **59**:6, 734. [CrossRef]
34. Naoya Koizumi, Hiroyuki Mizuguchi, Naoki Utoguchi, Yoshiteru Watanabe, Takao Hayakawa. 2003. Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *The Journal of Gene Medicine* **5**:4, 267. [CrossRef]
35. Alysson Renato Muotri , Maria Carolina Nasser Marchetto , Luiz Fernando Celidonio Zerbini , Towia A. Libermann , Armando Morais Ventura , Alain Sarasin , Carlos Frederico Martins Menck . 2002. Complementation of the DNA Repair Deficiency in Human Xeroderma Pigmentosum Group A and C Cells by Recombinant Adenovirus-Mediated Gene Transfer. *Human Gene Therapy* **13**:15, 1833-1844. [Abstract] [PDF] [PDF Plus]
36. Ruben Hernandez-Alcoceba , Michael Pihalja , Dalong Qian , Michael F. Clarke . 2002. New Oncolytic Adenoviruses with Hypoxia- and Estrogen Receptor-Regulated Replication. *Human Gene Therapy* **13**:14, 1737-1750. [Abstract] [PDF] [PDF Plus]
37. Hiroyuki Mizuguchi, Takao Hayakawa. 2002. The tet-off system is more effective than the tet-on system for regulating transgene expression in a single adenovirus vector. *The Journal of Gene Medicine* **4**:3, 240. [CrossRef]
38. Yuka Okada, Naoki Okada, Shinsaku Nakagawa, Hiroyuki Mizuguchi, Koichi Takahashi, Nobuyasu Mizuno, Takuya Fujita, Akira Yamamoto, Takao Hayakawa, Tadanori Mayumi. 2002. Tumor Necrosis Factor alpha-Gene Therapy for an Established Murine Melanoma Using RGB (Arg-Gly-Asp) Fiber-mutant Adenovirus Vectors. *Cancer Science* **93**:4, 436. [CrossRef]
39. Moritz Hillgenberg , Frank Schnieders , Peter Löser , Michael Strauss . 2001. System for Efficient Helper-Dependent Minimal Adenovirus Construction and Rescue. *Human Gene Therapy* **12**:6, 643-657. [Abstract] [PDF] [PDF Plus]
40. Naoki OKADA. 2001. Efficient Gene Delivery into Dendritic Cells by Fiber-Mutant Adenovirus Vectors. *YAKUGAKU ZASSHI* **121**:8, 593. [CrossRef]
41. Kouji Maruyama, Yasuto Akiyama, Noriko Nara-Ashizawa, Takashi Hojo, Jin-Yan Cheng, Hiroyuki Mizuguchi, Takao Hayakawa, Ken Yamaguchi. 2001. *Journal of Immunotherapy* **24**:4, 345. [CrossRef]
42. Gudrun Schiedner , Sabine Hertel , Stefan Kochanek . 2000. Efficient Transformation of Primary Human Amniocytes by E1 Functions of Ad5: Generation of New Cell Lines for Adenoviral Vector Production. *Human Gene Therapy* **11**:15, 2105-2116. [Abstract] [PDF] [PDF Plus]
43. R.J. Parks , D.T. Cummings , C.M. Evelegh , F.L. Graham . 2000. An Enhanced System for Construction of Adenoviral Vectors by the Two-Plasmid Rescue Method. *Human Gene Therapy* **11**:5, 693-699. [Abstract] [PDF] [PDF Plus]
44. Michelle M Cummins, Philip Poronnik, Lauren M O'Mullane, David I Cook. 2000. Studying heterotrimeric G-protein-linked signal transduction using replication-deficient adenoviruses. *Immunology and Cell Biology* **78**:4, 375. [CrossRef]

45. R.J. Parks , D.T. Cummings , C.M. Evelegh , U. Sankar , F.L. Graham . 1999. A High-Efficiency Cre/loxP-Based System for Construction of Adenoviral Vectors. *Human Gene Therapy* **10**:16, 2667-2672. [Abstract] [PDF] [PDF Plus]

46. Hiroyuki Mizuguchi , Mark A. Kay . 1999. A Simple Method for Constructing E1- and E1/E4-Deleted Recombinant Adenoviral Vectors. *Human Gene Therapy* **10**:12, 2013-2017. [Abstract] [PDF] [PDF Plus]