while the single Ad vector containing the original tet-responsive promoter showed higher induced (maximum) transgene expression. The modified tet-responsive promoter was used in the following experiments.

3.3. Fiber-modified Ad vectors containing the tet-off system

Fiber-modified Ad vectors containing the tet-off system were developed to transduce various types of cells, including CAR-deficient cells. By using pAdHM51-RGD and pAdHM49, Ad vectors containing both the tet-off system and the fiber modification, such as the addition of the RGD peptide in the HI loop of the fiber knob or substitution of Ad type 5 fiber for Ad type 35 fiber, were easily generated. The resulting Ad vectors, AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, were functionally compared with AdOff-SEAP6. CHO and CHO–CD46 cells were used (Fig. 3). Neither of these cells expresses CAR, but both express αv integrin. CHO cells are CD46 negative, while CHO–CD46 cells are CD46 positive (Fig. 3A,B). AdRGD-Off-SEAP6 achieved approximately 46-times higher SEAP production in CHO cells than AdOff-SEAP6 in the absence of doxycycline (Fig. 4). In contrast, in the case of CHO–CD46 cells, AdF35-Off-SEAP6 achieved approximately 40-times higher SEAP production than AdOff-SEAP6 in the absence of doxycycline (Fig. 4B). In both cases, fiber-modified Ad vectors showed higher regulation of SEAP production by doxycycline. AdOff-SEAP6 showed very low regulation of SEAP production by doxycycline, probably due to the lower transduction efficiency.

In order to further examine the functionality of AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, SEAP expression profiles were examined in SK HEP-1, LNZ308, and NIH3T3 cells (Fig. 5). SK HEP-1 cells express sufficient levels of CAR, αv integrin [25], and CD46 (Fig. 3C). LNZ308 cells express αv integrin and CD46 (Fig. 3D), but not CAR [25]. NIH3T3 cells

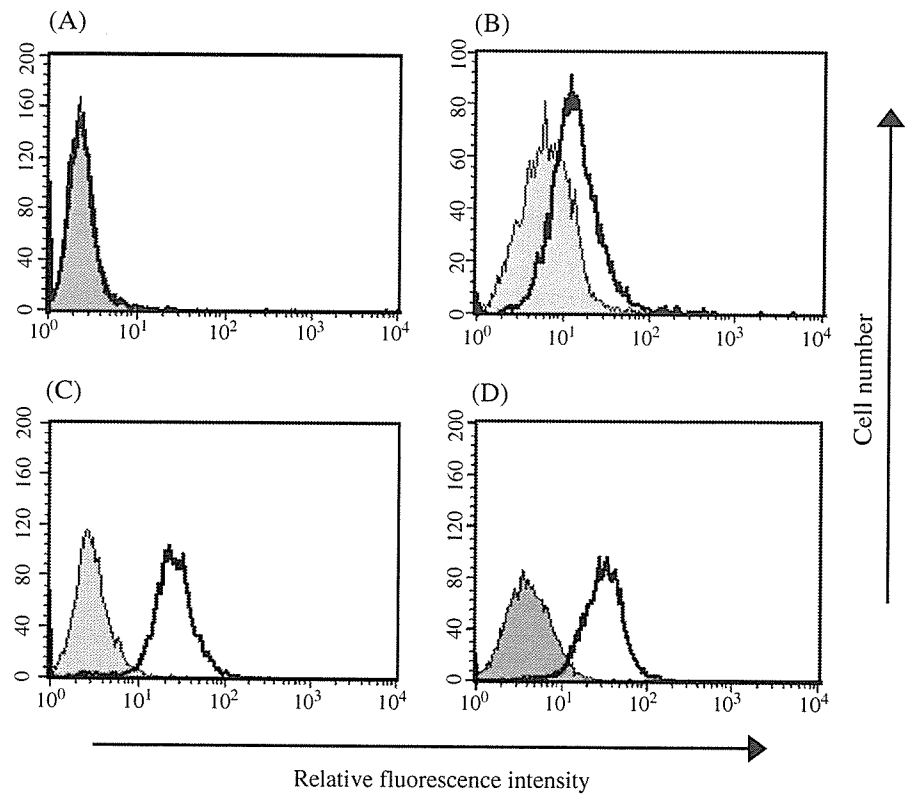

Fig. 3. CD46 expression in CHO, CHO–CD46, SK HEP-1, and LNZ308 cells. CHO (A), CHO–CD46 (B), SK HEP-1 (C), and LNZ308 (D) cells were stained with FITC-conjugated anti-CD46 antibodies, and subsequently analyzed by a flowcytometer.

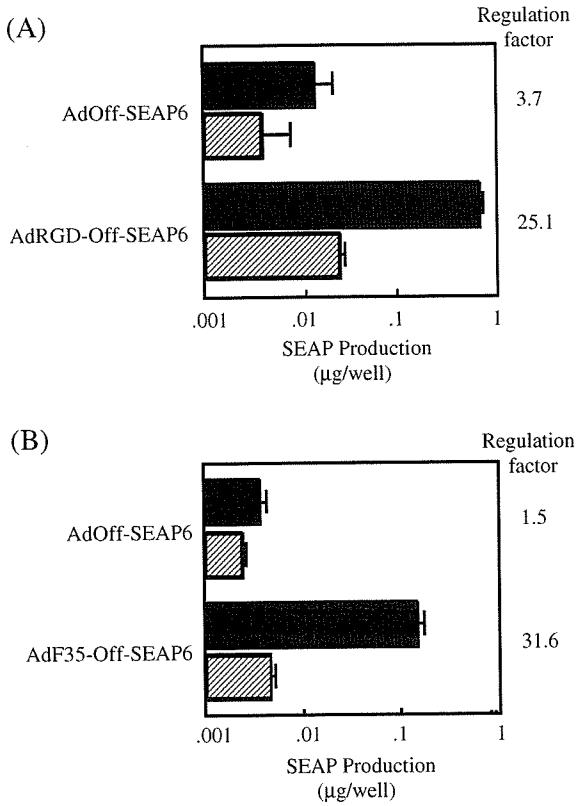

Fig. 4. Fiber-modified Ad vector-mediated tet-off systems in CHO and CHO–CD46 cells. CHO (A) and CHO–CD46 (B) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGD-Off-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the two cell types were as follows: CHO, 0.0008; CHO–CD46, 0.0002 (μg/well).

express αv integrin, but not CAR [25] or CD46. In SK HEP-1 cells, all the vectors, AdOff-SEAP6, AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, showed high SEAP production in the absence of doxycycline as well as high regulation factors by doxycycline (Fig. 5A). In contrast, only AdRGD-Off-SEAP6 and AdF35-Off-SEAP6 achieved high regulation factor by doxycycline and high SEAP production in the absence of doxycycline in LNZ308 cells (Fig. 5B), while only AdRGD-Off-SEAP6 achieved high regulation factor and high SEAP production in NIH3T3 cells (Fig. 5C). AdOff-SEAP6 showed lower SEAP production in LNZ308 and NIH3T3 cells (Fig. 5B, C). Taken together, these results indicate that fiber-modified Ad vectors containing the tet-off system overcame the

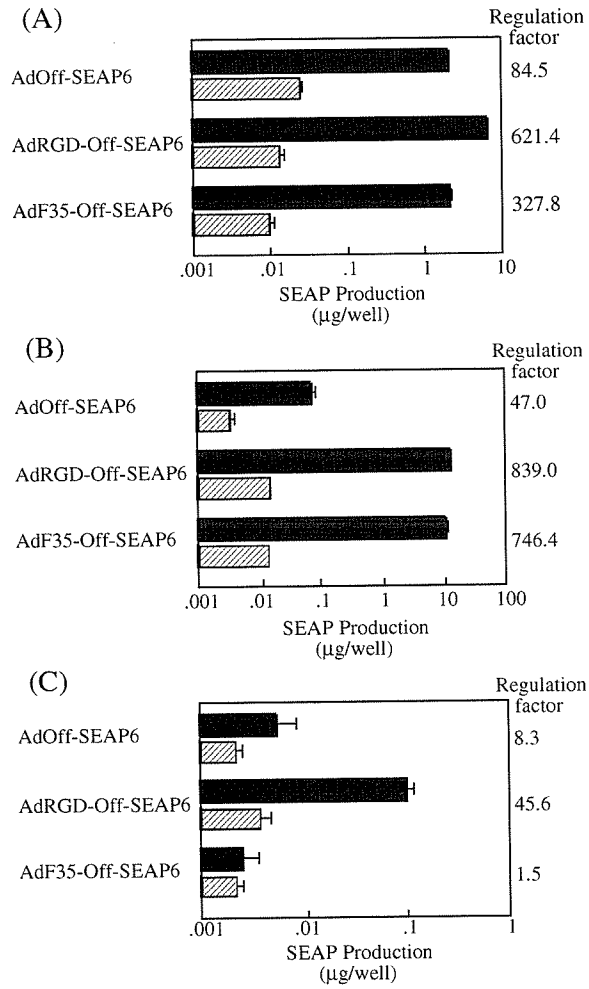

Fig. 5. Fiber-modified Ad vector-mediated tet-off systems in various types of cells. SK HEP-1 (A), LNZ308 (B), and NIH3T3 (C) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGDOff-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the three cell types were as follows: SK HEP-1, 0.0023; LNZ308, 0.0008; NIH3T3, 0.0004 (μg/well).

lower transduction efficiency and regulation of transgene expression in the cells lacking CAR expression.

Finally, SEAP production from Ad-SEAP2 which contains the putative strong CMV promoter/enhancer and was also transduced at 500 VP/cell, was used as a reference to evaluate the maximal SEAP production levels of each regulated Ad vectors. SK HEP-1 cells were used in this experiment. As shown in Table 2, AdOff-SEAP6, AdRGD-Off-SEAP6 and AdF35-Off-SEAP6 showed comparable SEAP production to Ad-SEAP2 in the absence of doxycycline. AdOff-SEAP6 achieved similar levels of SEAP production in LNZ308 and NIH3T3 cells compared with Ad-SEAP2 in the absence of doxycycline (AdRGD-Off-SEAP6 and AdF35-Off-SEAP6 showed 1–2 log orders higher SEAP production than Ad-SEAP2) (data not shown). These results suggested that fiber-modified Ad vectors containing the tet-off system are practical as a regulated gene expression system in terms of high maximal expression as well as high regulation activity.

We previously reported that an Ad vector containing the tet-on system, in which the gene of interest lies in the E1 deletion region and the reverse *tTA* (*rtTA*) (transactivator for the tet-on system) gene lies in the E3 deletion region, exhibited much lower regulation of transgene expression by doxycycline than Ad vectors containing the tet-off system [3]. This lower regulation can be overcome by a triple-Ad vector carrying the gene of interest, the *rtTA* gene, and the tetracycline transcriptional silencer (*tTS*) gene in the E1 deletion region, the E3 deletion region, and the region between the E4 and 3′ITR, respectively [4]. The vector plasmids pAdHM49 and −51 developed in the present study contain a unique restriction enzyme site in the region between the E4 and 3′ITR as well as in the E1 and E3 deletion region. Thus, the triple-Ad vectors which combine the fiber-modification and improved tet-on system can be easily generated by an in vitro ligation-based method [4].

Table 2
Comparison of the maximal induced SEAP production in SK HEP-1 cells transduced with Ad vectors containing the tet-off system to that transduced with Ad vectors containing the CMV promoter-driven SEAP expression cassette

Name	SEAP production (μg/well)
AdOff-SEAP6	2.14 ± 0.14
AdRGD-Off-SEAP6	6.91 ± 0.33
AdF35-Off-SEAP6	2.26 ± 0.18
Ad-SEAP2	5.55 ± 0.32

SK HEP-1 cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGDOff-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured with medium containing doxycycline (10 ng/ml). The cells were also transduced with Ad-SEAP2 at 500 VP/cell, and cultured with normal medium without doxycycline. Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The data are expressed as mean ± S.D. (n = 3). Mean background value of SEAP production in the cells was 0.0023 (μg/well).

Here, we showed that Ad vectors containing RGD peptides in the HI loop of the fiber knob and Ad type 35 fiber mediated a wide range of efficient gene transfer and strict regulation of transgene expression by doxycycline. Several types of re-targeted Ad vectors other than RGD-modified Ad vectors and Ad35-fiber substituted Ad vectors have been developed; Ad vectors containing a stretch of lysine residues (K7 (KKKKKKK) peptide) in the C-terminal of the fiber knob [26,27], Ad vectors containing the substitution of the Ad type 5 (Ad5) fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad37, the reovirus attachment protein σ1 and others [10,11,28–35]. These vectors target heparan sulfates (Ad vectors containing K7), CD46 (and/or CD80 and CD86) (Ad vectors containing Ad3, Ad11, Ad16, Ad37 fiber) [16–18,36], sialic acids (Ad vectors containing Ad37 fiber) (Note that Ad37 fiber has been reported to bind CD46 and sialic acid [16,37,38], junctional adhesion molecule 1 (Ad vectors containing the reovirus attachment protein σ1) [34]. These types of fiber modification could be also applied for the fiber-modified Ad vectors containing the tetracycline-controllable expression system.

Since αv integrins are expressed on most types of cells, the exception being certain blood cells, and CD46 is expressed on most human cells, including blood cells, Ad vectors containing either RGD peptides on the fiber knob or Ad type 35 fiber are potentially powerful tools for efficient transduction to the cells lacking sufficient CAR expression. Furthermore, the peptide of interest displayed in the HI loop of the fiber knob can be freely changed by an in vitro ligation-based method [12,14]. The present Ad vector system should have great potential for a variety of applications.

4. Conclusions

In conclusions, we developed highly efficient self-contained Ad vectors having the fiber modification and the tetracycline-controllable expression system. The present Ad vectors system will greatly facilitate the in vitro and in vivo analyses of gene function and may be useful for gene therapy.

Acknowledgments

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A trial of somatic gene targeting *in vivo* with an adenovirus vector

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Abstract

Background: Gene targeting *in vivo* provides a potentially powerful method for gene analysis and gene therapy. In order to sensitively detect and accurately measure designed sequence changes, we have used a transgenic mouse system, MutaMouse, which has been developed for detection of mutation *in vivo*. It carries bacteriophage lambda genome with *lacZ*$^+$ gene, whose change to *lacZ*-negative allele is detected after *in vitro* packaging into bacteriophage particles. We have also demonstrated that gene transfer with a replication-defective adenovirus vector can achieve efficient and accurate gene targeting *in vitro*.

Methods: An 8 kb long DNA corresponding to the bacteriophage lambda transgene with one of two *lacZ*-negative single-base-pair-substitution mutant allele was inserted into a replication-defective adenovirus vector. This recombinant adenovirus was injected to the transgenic mice via tail-vein. Twenty-four hours later, genomic DNA was extracted from the liver tissue and the lambda::*lacZ* were recovered by *in vitro* packaging. The *lacZ*-negative phage was detected as a plaque former on agar with phenyl-beta-D-galactoside.

Results: The mutant frequency of the *lacZ*-negative recombinant adenovirus injected mice was at the same level with the control mouse (~1/10000). Our further restriction analysis did not detect any designed recombinant.

Conclusion: The frequency of gene targeting in the mouse liver by these recombinant adenoviruses was shown to be less than 1/20000 in our assay. However, these results will aid the development of a sensitive, reliable and PCR-independent assay for gene targeting *in vivo* mediated by virus vectors and other means.

Background

Gene targeting, which is the precise alteration of genomic information by homologous recombination, has provided a powerful means of genetic analysis in

microorganisms and mammalian systems [1]. In mouse systems, embryonic stem-cell lines modified *in vitro* can be used to generate mice that are altered at the germ-line level. If the gene targeting of somatic cells is made possible by gene transfer *in vivo*, it will facilitate the analysis of gene function, and provide a means of gene therapy for genetic and other diseases [2].

There are two major inherent problems with the use of gene targeting *in vivo*. First, its low efficiency makes it difficult to detect and analyze. A sensitive and accurate measurement system is therefore needed to detect such low-frequency events. Although there have been several reports of gene targeting in the rat liver with specifically designed oligonucleotides [3,4], their reproducibility remains controversial [5]. PCR-based detection methods might thus be inaccurate and prone to various artifacts. In order to detect and measure gene targeting in mice with sufficient sensitivity, we used a bacteriophage transgenic-mouse system, MutaMouse, which has been developed for the detection of mutagenesis *in vivo* (Figure 1) [6]. The MutaMouse carries tandem repeats of the bacteriophage lambda genome with the *lacZ*$^+$ gene, in which the change to a *lacZ*-negative allele is detected after its *in vitro* packaging into viable bacteriophage particles.

The second major problem with gene targeting *in vivo* is that non-homologous recombination is much more frequent than homologous recombination in mammalian cells. Rare accurately modified cells are selected and purified in the case of embryonic stem cells that are treated *in vitro*. For gene targeting *in vivo*, imprecise modification would be detrimental for analytical uses and therapeutic purposes. Accurate gene modification has been achieved efficiently using replication-defective adenovirus vectors for gene delivery *in vitro* [7,8]. Fujita and colleagues used a mammalian plasmid as a model target [7]. The gene targeting was frequent (~10^{-4} per cell) and analysis of the products revealed that homologous recombination was more frequent than non-homologous recombination. One possible reason for this high accuracy was protection of the viral DNA by the terminal protein, which is covalently attached to the ends of the viral DNA and to other viral proteins during its transfer to the nucleus and target DNA. Breaks in unprotected DNA would lead to non-homologous recombination.

The adenovirus is useful for gene delivery *in vivo* because it has a broad host-range, is easy to prepare to a high titer and only rarely integrates into the host genome by non-homologous recombination [9,10]. To date, more than 170 clinical studies have used recombinant adenovirus vectors to express cDNA in humans [11]. Numerous adenovirus-infection experiments have been carried out with mice, and have established that the injection of adenovirus recombinants into the mouse tail-vein leads to the expression of their genes in approximately one-half of the liver cells [12,13].

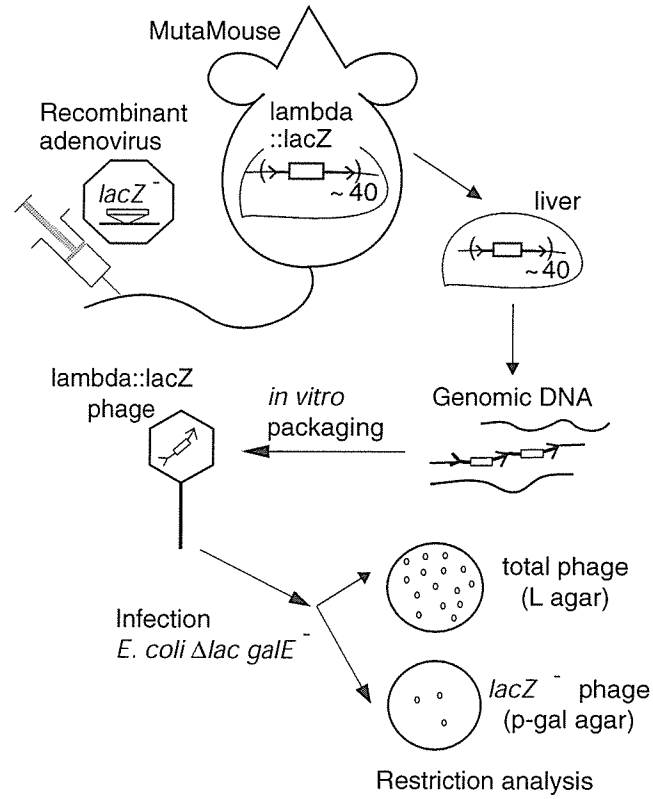

Figure 1
Experimental steps to detect gene targeting *in vivo*. Gene targeting *in vivo* in liver cells was attempted after the delivery of donor DNA with an adenovirus vector. The gene with the required sequence change (*lacZ*$^-$) on the lambda transgene in the mouse will be detected after its recovery in bacteriophage particles. Only *lacZ*-negative mutants can form plaques under the selective conditions.

In the present study, we investigated gene targeting in the mouse liver using a replication-defective adenovirus vector and a transgenic mouse system (Figure 1). Although our initial attempts did not detect the predicted gene targeting (the frequency of the expected recombinants was less than 1/20,000 per lambda genome), the strategy and methods detailed here will aid the development of virus-mediated gene targeting *in vivo*.

Materials and methods

Bacteria, bacteriophages and plasmids

The bacteria, bacteriophages and plasmids used in this study are listed together with details of their construction in Additional file 1.

BIK12001 was used for the titration of bacteriophage lambda and the measurement of *lacZ*-negative bacteriophage lambda by phenyl beta-D-galactoside (p-gal) selection (see below). BIK1564 was used for the growth of all bacteriophage lambda strains in this study. BIK2206 was used for confirmation of the LacZ-negative phenotype of the bacteriophage selected with p-gal using 5-bromo-4-chloro-3-indlyl-beta-D-galactose (X-gal).

The construction of the plasmids used in this study is detailed in additional file 1. The construction of pAdNY58 is also illustrated in Figure 2. The construction of pAdNY57 was as follows. The SmaI(1)-SacI fragment of LIA7 within the *lacZ* gene (Figure 2) was used to replace the shorter SmaI-SacI fragment of pUC18. The Glu461Gly mutation (Figure 3) was introduced into the resulting plasmid (pNY15) by site-directed mutagenesis using PCR [14] as follows. The PCR products generated with the primer pair LZG-U (5'-ACCGGCGATGAGCGAA-3') and LZG-MA (5'-GCCTGATCCATTCCCCAGCGACCA-3'), and the primer pair LZG-MS (5'-GGGAATGGATCAG-GCCACGGCCGC-3') and LZG-D (5'-GGGCTGGTCT-TCATCC-3'), were mixed and used as templates for the second round of PCR with the primer pair LZG-U and LZG-D. The MluI-BssHII fragment of the wild-type *lacZ* gene of pNY15 was replaced by the MluI-BssHII fragment of the PCR product. The targeted change in the resulting plasmid (pNY15G3.11) was confirmed by sequencing. pNY20 was produced by replacing the smaller SmaI-SacI fragment of pNY19 with the homologous SmaI-SacI fragment of pNY15G3.11, which carries the mutant sequence.

These two *lacZ* mutations were transferred back to lambda by homologous recombination *in vivo* [15] in order to generate LIA15 and LIA11, respectively. The recombinational transfer was carried out as follows. Cells of BIK12015 or BIK12018 were grown to OD_{600} = ~0.3 in LB (10 g bactotrypton, 5 g yeast extract and 10 g NaCl per liter) containing 20 μg/ml chloramphenicol, 0.2% maltose and 10 mM $MgSO_4$. LIA7 was adsorbed onto the cells at a multiplicity of 1.0 at 37°C for 15 minutes. The mixture was shaken at 37°C until the OD_{600} dropped below 0.3. One drop of $CHCl_3$ was added to the mixture, which was then shaken for 30 seconds. The mixture was centrifuged and the supernatant was recovered. The supernatant was assayed for BIK12001 on agar plates containing p-gal as detailed below. The plaques on the p-gal plates were isolated and analyzed for the designed sequence change by restriction of the PCR products (see *Analysis of the mutant bacteriophage DNA*).

Selection of lacZ-negative bacteriophage with p-gal

The lacZ-negative bacteriophage particles were detected using positive selection [15,16]. BIK12001 cells were grown with shaking at 37°C to OD_{600} = 1.0 in LB containing ampicillin (50 μg/ml), kanamycin (20 μg/ml) and 0.2% maltose. The culture was centrifuged at 3,500 rpm for 15 minutes at 4°C. The pellets were dissolved into one-half the volume of LB containing 10 mM $MgSO_4$. The bacteriophage was adsorbed onto these cells at room temperature for 20 minutes. To estimate the total number of bacteriophages, 2.5 ml molten 1/4 LB top agar (5 g LB broth base (Gibco BRL, Rockville, MD, USA), 6.4 g NaCl and 7.5 g Bactoagar per liter) was added to 0.25 ml of the mixture of cells and bacteriophages, and the entire content was poured onto a 1/4 LB plate (5 g LB broth base, 6.4 g NaCl and 15 g Bactoagar per liter). To estimate the number of lacZ-negative bacteriophages, 2 ml of the mixture of cells and bacteriophages, and 22 ml of molten 1/4 LB top agar containing 0.3% p-gal (Sigma Chemical Co., MO, USA), were mixed and poured onto four 1/4 LB plates. The plates were incubated at 37°C for 12 hours.

Construction of recombinant adenoviruses

pNY56 was constructed by replacing the shorter XbaI-BamHI fragment of pHM5 by the XbaI-BglII fragment of pNY19 (Figure 2). pAdHM4 includes the entire genome of the recombinant adenovirus vector. The plasmid pAdNY56 was constructed by replacing the shorter I-CeuI-PI-SceI fragment of pAdHM4 by an I-CeuI-PI-SceI fragment of pNY56. The PacI fragment of pAdNY56 was transfected into cells of cell-line 293, which allows replication of the replication-defective adenoviruses. The recombinant adenovirus AdNY56 was prepared and purified as described previously [18]. Similarly, AdNY57 was constructed from pNY20 via pNY57 (Additional file 1), and AdNY58 was constructed from pNY21 via pNY58 (Figure 2, Additional file 1).

Adenovirus infection

Female MutaMice (7 weeks old) were obtained from Covance Research Products Inc. (Denver, PA, USA). The MutaMice were maintained under specific pathogen-free conditions in the animal faculty of the Institute of Medical Science at the University of Tokyo, Japan. After the animals were anesthetized with Nembutal (Dainippon Pharmaceutical Co., Osaka, Japan), 3×10^9 plaque-forming units (PFU) of the recombinant adenovirus in 200 μl of PBS (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.33 mM $MgCl_2$) was injected into the tail-vein of each mouse using a 30-gauge needle. AdNY56 was injected into one mouse, AdNY57 was injected into two mice and AdNY58 was injected into two mice.

Isolation of genomic DNA, recovery of lambda bacteriophage and measurement of mutant frequency

Twenty-four hours after injection, the mice were sacrificed. A lobe of the liver of each animal was excised, frozen by submersion in liquid nitrogen and stored in a 1.5-ml

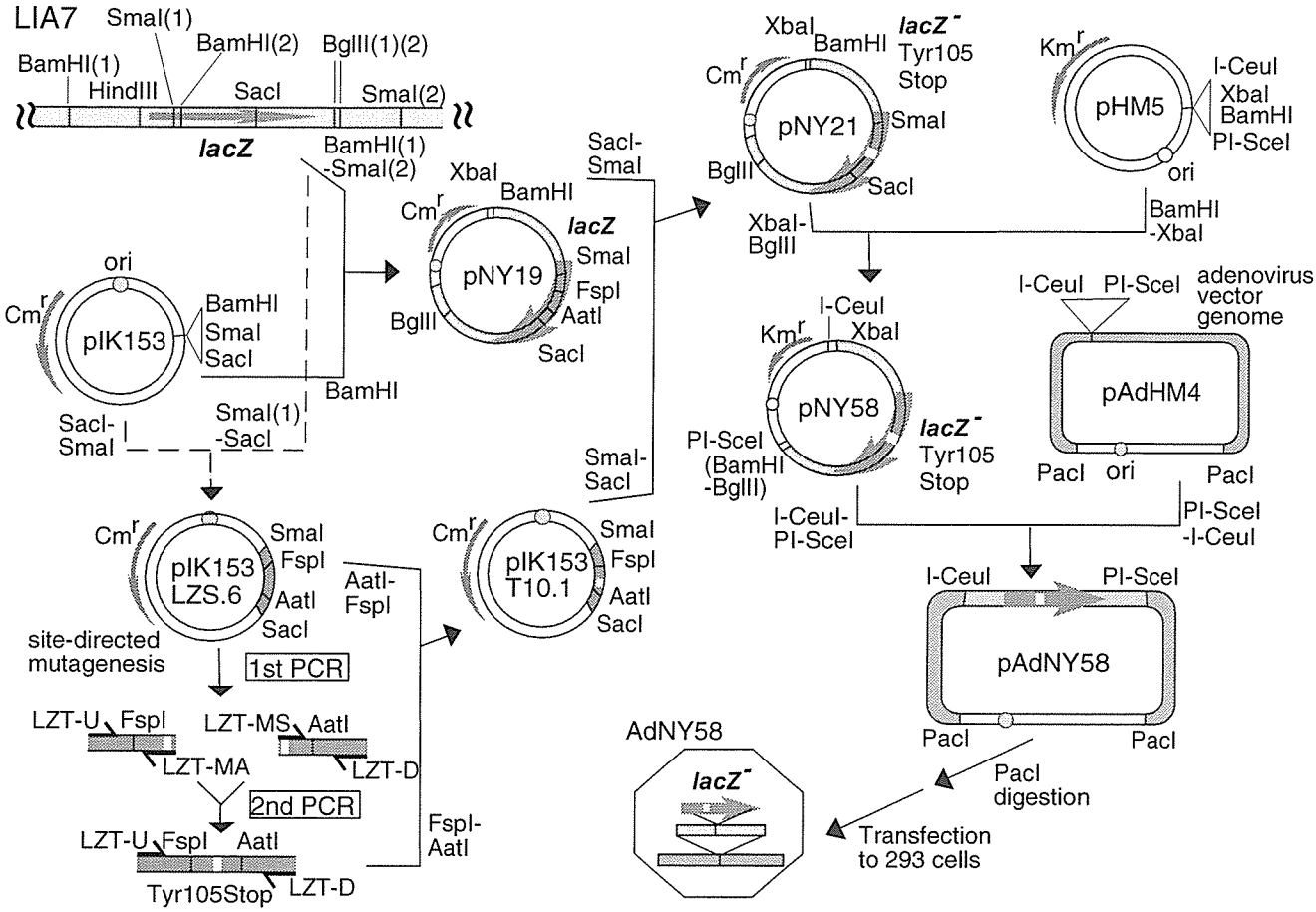

Figure 2

Construction of the recombinant adenovirus AdNY58. The bacteriophage lambda LIA7 was recovered from the Muta-Mouse by *in vitro* packaging. An SmaI-SacI fragment of LIA7 within its *lacZ* gene was inserted into pIK153. The Tyr105Stop mutation (Figure 3) was introduced into the resulting plasmid (pIK153LZS.6) using site-directed mutagenesis by PCR as follows. The PCR products generated with the primer pair LZT-U (5'-CGAAGAGGCCCGCAC-3') and LZT-MA (5'-TAAT-GGGCTAGGTTACGTTGGTGTAG-3'), and the primer pair LZT-MS (5'-TAACCTAGCCCATTACGGTCAATCC-3') and LZT-D (5'-GGCAACATGGAAATCGC-3') were mixed and used as templates for the second PCR with the primer pair LTZ-U and LZT-D. Replacement of an FspI-AatII fragment of pIK153LZS.6 by the FspI-AatII fragment of the resulting PCR product resulted in pIK153 T10.1. A BamHI-SmaI fragment covering the *lacZ* gene of LIA7 was inserted into the BamHI site of pIK153 (resulting in pNY19). pNY21 was made by replacing the smaller SmaI-SacI fragment of pNY19 with the homologous SmaI-SacI fragment of pIK153T10.1, which carries the mutant sequence. An XbaI-BglII fragment of pNY21 was used to replace the smaller XbaI-BamHI fragment of pHM5 (resulting in pNY58). pAdNY58 was made by replacement of the smaller I-CeuI-PI-SceI fragment of pAdHM4 with an I-CeuI-PI-SceI fragment of pNY58. The longer PacI fragment of pAdNY58 was transfected into 293 cells. The recombinant adenovirus AdNY58 was prepared and purified from the cell culture.

plastic tube at -80°C. Genomic DNA was isolated from the liver tissue with phenol-chloroform and precipitated by ethanol/sodium as described in the manual for Muta-Mouse. Lambda bacteriophage particles were recovered from the isolated DNA by incubation with packaging extracts (Mutaplax, Epicentre, WI, USA). The lacZ-negative mutants were detected by p-gal selection as described above. Each plaque on the selective agar was recovered in 100 µl of SM buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 100 mM NaCl and 0.01% gelatin). In order to verify the lacZ-negative phenotype, each isolate was assayed on agar with X-gal using a spot assay as follows. BIK2206 was grown in LB containing ampicillin (50 µg/ml) and tetracycline (10 µg/ml). Twice-concentrated cul-

ture (1.25 ml) was mixed with 6 ml molten LB/MM agar (100 ml LB medium, 0.75 g Bactoagar, 10 mM $MgSO_4$, 0.2% maltose and 0.35 mg/ml X-gal) and spread on agar. A 10-μl aliquot of each bacteriophage sample was spotted onto these cells. The plates were incubated overnight at 37°C. The mutant frequency was estimated by dividing the number of PFU on the selective plate (as verified with X-gal) by the number of total PFU on 1/4 LB agar.

Analysis of the mutant bacteriophage DNA

The lacZ-negative lambda bacteriophage DNA from the mice was analyzed using restriction enzymes following PCR. For the lacZ-negative lambda DNA from the AdNY57-treated mouse, PCR was carried out with the primer pair LG-1 (5'-TACCGGCGATGAGCGAAC-3') and LG-2 (5'-CTCCAGGTAGCGAAAGCC-3'). The 288-bp product was purified by ethanol/sodium precipitation, digested with TfiI (New England Biolabs, Beverly, MA, USA) (recognition site, 5'-G|AWTC-3' (W = A or T)) at 65°C and analyzed using agarose electrophoresis. The mutant sequence was resistant to TfiI, while the wild-type sequence was sensitive, yielding 204 and 84 bp fragments. The primer pair Lam-1 (5'-TACTGTCGTCGTCCCCTC-3') and Lam-2 (5'-CGCAGATGAAACGCCGAGT-3') was used for the lacZ-negative lambda DNA from the AdNY58-treated mouse. The 213-bp PCR product was digested with XspI (Takara Bio Inc., Shiga, Japan) (recognition site, 5'-C|TAG-3') at 37°C and analyzed using agarose electrophoresis. The wild-type sequence was resistant to XspI, while the mutant sequence was sensitive, yielding 146 and 67 bp fragments.

Results

Experimental design for the detection of gene targeting in vivo

Figure 1 illustrates our experimental design for the sensitive detection of gene targeting in vivo. The MutaMouse carries approximately 40 copies of bacteriophage lambda gt10lacZ on a chromosome [6,19]. The single integration site is located in band C on chromosome 3 [20]. Our target sequence was the wild-type lacZ gene. The donor DNA was delivered to the liver cell nuclei by tail-vein injection of the recombinant adenovirus. Genomic DNA was isolated from the liver and its in vitro packaging allowed the recovery of the lambda genome in viable bacteriophage particles. A lacZ-negative mutant bacteriophage was selected as a plaque-former in an Escherichia coli mutant defective in the galE gene on an agar plate containing p-gal. This chemical is converted by the lacZ gene product (beta-galactosidase) into UDP-galactose, which accumulates in the absence of the GalE protein to induce cell death. The ratio of the mutant plaque-formers to the total plaque-formers was used to estimate the fraction of the mutated gene. The mutant gene was further analyzed using restriction enzymes.

Replication-defective recombinant adenoviruses constructed by an *in vitro*-ligation method were used to deliver the donor DNA [18,21]. Figure 3 shows the structure of the recombinant adenoviruses used in the present study (see Figure 2, Additional file 1, and Materials and methods for further details). An 8077-bp fragment of lambda gt10*lacZ* was inserted into the E1 deletion site of the mutant adenovirus [18,21]. AdNY56 had wild-type *lacZ*, while AdNY57 and AdNY58 had a point mutation in *lacZ* (Figure 3B).

AdNY57 was constructed so as to introduce a point mutation at the active site of LacZ. The target sequence was the 5' GAA that codes for Glu461, which is essential for the activity of LacZ [22,23]. AdNY57 was expected to change its second base (that is, the 1437 th base) from A to G, thereby generating the Glu461Gly mutant, which shows a 76-fold decrease in activity [23]. The mutant and wild-type sequences can be distinguished using the restriction enzyme TfiI (Figure 3B).

AdNY58 was constructed so as to introduce a point mutation at the 5' TAT that codes for Tyr105. AdNY58 was expected to change its third base (that is, the 369th base) from T to G, thereby generating the Tyr105Stop mutant. The mutant and wild-type sequences can be distinguished using the restriction enzyme XspI (Figure 3B).

Control experiments

We demonstrated that lacZ mutants that were predicted to be generated by the recombinant adenovirus could be selected with p-gal as follows. Bacteriophage lambda strains carrying the mutations were produced by transferring each mutation on a plasmid back to lambda through homologous recombination in E. coli (as detailed in Materials and methods). The two bacteriophage strains, lambda gt10lacZ$^-$ Tyr105Stop (LIA11) and lambda gt10lacZ$^-$ Glu461Gly (LIA15), were then used in the p-gal selection. As shown in Table 1, lambda with wild-type lacZ showed a plaque-formation efficiency of less than 1/10,000 on the selective agar relative to that on the non-selective agar. By contrast, each of the mutant lambda strains showed similar or slightly decreased plaque-formation efficiency on the selective agar. We concluded that the expected targeted product with AdNY57 and AdNY58, if it was produced, should be selected and measured using the p-gal-selection procedure.

Delivery of donor DNA and measurement of mutant frequency

The recombinant adenovirus particles (3×10^9 PFU in 200 μl of PBS) were injected into the tail-vein of a MutaMouse. It is well established that the adenovirus genome accumulates in the liver cell nuclei after tail-vein injection [12,13]. Most of the hepatocyte nuclei are expected to receive sev-

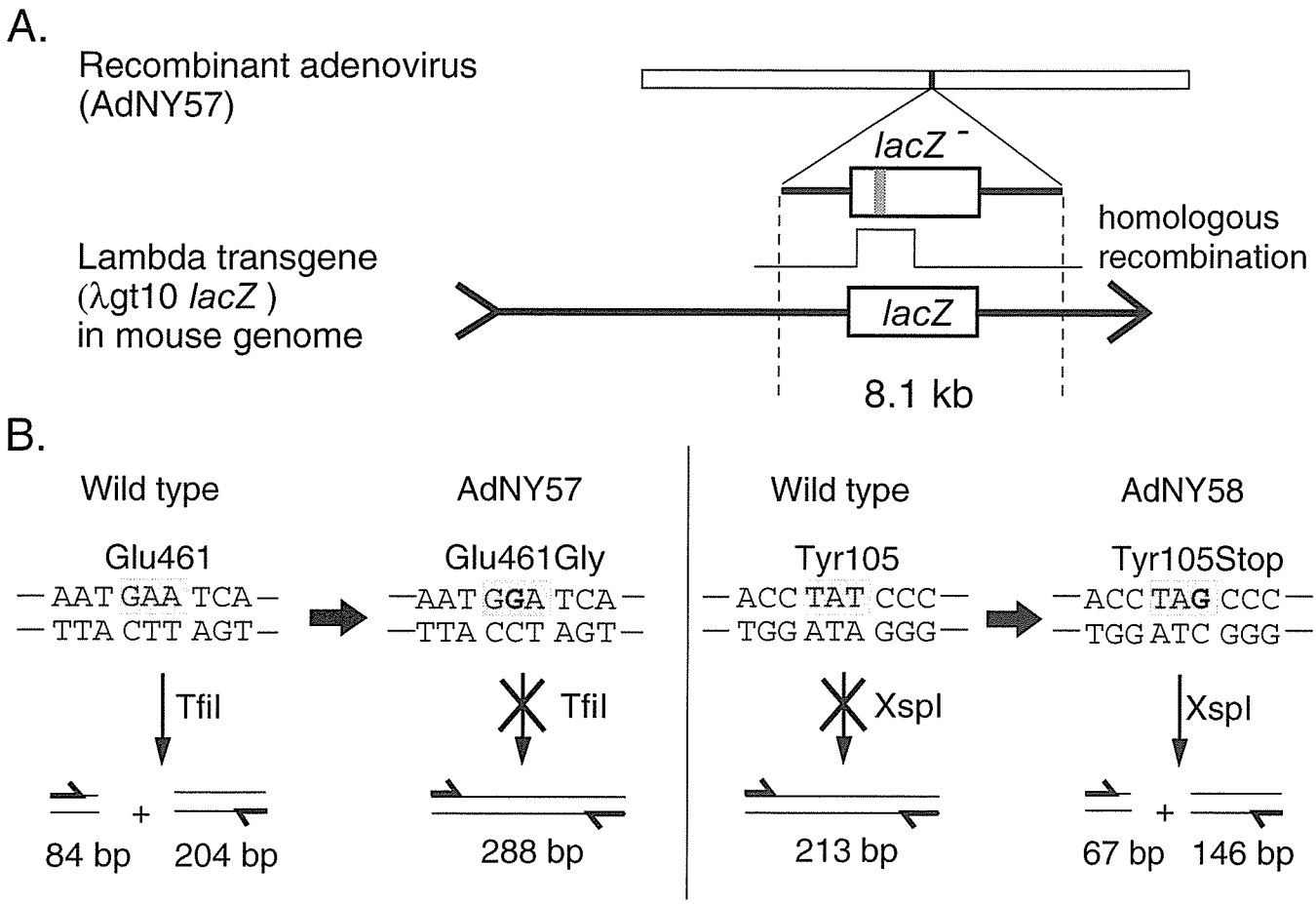

Figure 3
Design for gene targeting and its detection. (**A**) The donor carrying the mutant *lacZ* gene is inserted into an adenovirus vector. The *lacZ* mutation will be transferred to the *lacZ* gene of the lambda transgene in the mouse genome. (**B**) Expected sequence changes and their detection using restriction analysis.

Table 1: Selection efficiency of lambda *lacZ*-negative mutants

Lambda	Genotype	Titer	Titer on p-gal selective plate	Relative plaque formation
LIA7	*lacZ*$^+$	2.2×10^{10}	1.9×10^{6}	8.6×10^{-5}
LIA11	*lacZ*$^-$ (Tyr105Stop)	9.6×10^{10}	8.8×10^{10}	9.2×10^{-1}
LIA15	*lacZ*$^-$ (Glu461Gly)	1.4×10^{10}	1.3×10^{10}	9.1×10^{-1}

eral copies of the adenovirus genome under these conditions (see Discussion). After 24 hours, the liver was excised from the MutaMouse, genomic DNA was isolated from the liver tissue and the lambda genome was recovered as a bacteriophage particle by in vitro packaging. The lacZ-negative phage was detected selectively on agar with p-gal. The plaques on these selective plates were isolated and the LacZ-negative phenotype was confirmed on agar plates containing X-gal. The mutant frequency was estimated as the fraction of the lacZ-negative phage (Table 2).

The control mouse (animal number 0) received no injections.

The mutant frequencies of the AdNY56-injected and control mice were similar (Table 2, Experiment 1), and did not differ significantly from those reported previously using this method (see [15] and the references cited therein). No significant increase in the mutant form of the gene was induced by injection of the recombinant adenovirus: the mutant frequency of the AdNY57- and AdNY58-injected mice was similar to that of the control mouse, which was approximately 1/10,000 (Table 2).

All of the *lacZ*-negative bacteriophages were purified and their *lacZ* genes were analyzed using restriction-enzyme treatment of the PCR products (Figure 4). As shown in Figures 3B and 4A, the PCR product of the Glu461Gly mutant, as predicted from the AdNY57 injection, could not be cut with TfiI. By contrast, the wild-type and most of the other possible mutants could be cut with TfiI. In fact, all of the *lacZ*-negative bacteriophages from the AdNY57-injected mouse were cleavable with this restriction enzyme. As shown in Figure 3B and 4B, the PCR product of the Tyr105Stop mutant, as predicted from the AdNY58 injection, could be cut with XspI. By contrast, the wild-type and most of the other mutants could not be cut with XspI. None of the *lacZ*-negative bacteriophages from the AdNY58-injected mice were cleavable with this restriction enzyme.

We did not detect the expected gene replacement in any of the isolates. Moreover, the gene-correction frequency by these adenovirus constructs was shown to be less than 1/20,000 in the present system.

Discussion

Here we attempted to perform gene targeting in a transgenic mouse system that allowed the sensitive detection of mutagenesis by various agents, such as those directly interacting with DNA in the liver and other organs [24,25]. The limit of sensitivity in this system was 1/20,000 (see also [15]). This procedure might provide an alternative to the PCR-based assay for gene targeting *in vivo*, although our initial trials did not detect any of the expected recombinants.

In the present system, the sensitivity appeared to be limited by the high level of spontaneous mutagenesis in the target gene. The MutaMouse system was produced to detect mutagenesis at numerous sites within a gene, rather than to study gene targeting. Experimental designs involving the specific selection of homologous recombination events, such as those used in the previous work *in vitro* [7], would therefore be preferred.

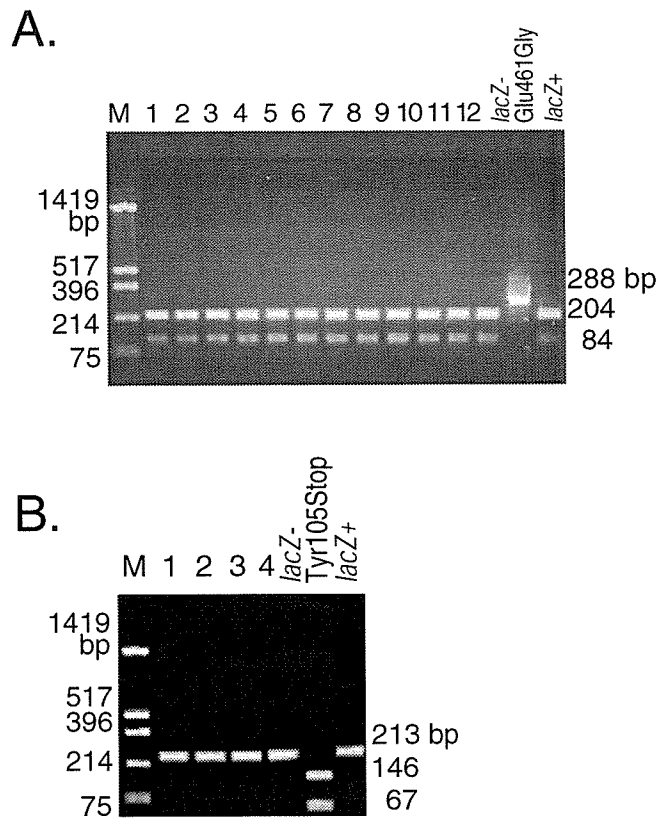

Figure 4
Restriction analysis of the *lacZ*-negative gene from mice treated with a recombinant adenovirus. (A) AdNY57-injected mouse. The PCR product of the lambda bacteriophage DNA with primers that flank the target site is 288 bp long. The wild-type PCR product is cut with TfiI into 84 and 204 bp fragments, whereas the Glu461Ala mutant PCR product is not cut. Lane M: Marker DNA prepared by HinfI digestion of the plasmid pUC19; 1–12, *lacZ*-negative bacteriophages from animal number 2; *lacZ*$^+$: Lambda bacteriophage recovered from control mouse; *lacZ*-Glu461Gly: lambda bacteriophage LIA15. **(B)** AdNY58-injected mouse. The PCR product of the lambda bacteriophage DNA with primers that flank the target site is 213 bp long. The Tyr105Stop mutant PCR product is cut with XspI into 146 and 67 bp fragments, whereas the wild-type product is not. Lane M: Marker DNA prepared by HinfI digestion of plasmid pUC19; 1–4, *lacZ*-negative bacteriophages from animal number 3; *lacZ*$^+$: Lambda bacteriophage recovered from control mouse; *lacZ*-Tyr105Stop: lambda bacteriophage LIA11.

Also, in the present system, a successful gene-targeting event would not be distinguishable in the phenotype of the mouse cell. In transgenic mice with a single copy of the mutant *lacZ* gene [26], correction to the wild-type

Table 2: Detection of *lacZ*⁻ phage

Packaging exp.	RAd	Genotype	Animal number	Packaging	Total number of plaque formers	*lacZ*⁻ plaques	Mutant Frequency	Expected genotype
1	None	Not relevant	#0	Tube 1	4.5×10^4	4	8.9×10^{-5}	n.t.
				Tube 2	3.2×10^4	3	9.4×10^{-5}	n.t.
				Tube 3	8.5×10^4	8	9.4×10^{-5}	n.t.
							average 9.2×10^{-5}	
	AdNY56	*lacZ*⁺	#1	Tube 4	8.5×10^4	8	9.4×10^{-5}	n.t.
				Tube 5	6.4×10^4	3	4.7×10^{-5}	n.t.
				Tube 6	8.8×10^4	1	1.1×10^{-5}	n.t.
							average 5.1×10^{-5}	
2	None	Not relevant	#0	Tube 7	4.9×10^4	5	10×10^{-5}	n.t.
				Tube 8	6.6×10^4	4	6.1×10^{-5}	n.t.
				Tube 9	5.1×10^4	10	20×10^{-5}	n.t.
							average 12×10^{-5}	
	AdNY57	*lacZ*⁻ (Glu461Gly)	#2	Tube 10	3.8×10^4	6	16×10^{-5}	0/6
				Tube 11	3.0×10^4	7	23×10^{-5}	0/7
				Tube 12	4.5×10^4	9	20×10^{-5}	0/9
							average 20×10^{-5}	total 0/22
3	None	Not relevant	#0	Tube 13	3.7×10^4	6	16×10^{-5}	n.t.
				Tube 14	6.0×10^4	5	8.3×10^{-5}	n.t.
				Tube 15	4.4×10^4	4	9.1×10^{-5}	n.t.
							average 11×10^{-5}	
	AdNY57	*lacZ*⁻ (Glu461Gly)	#2	Tube 16	1.3×10^4	9	69×10^{-5}	0/9
				Tube 17	3.9×10^4	19	49×10^{-5}	0/19
				Tube 18	6.5×10^4	26	40×10^{-5}	0/26
							average 53×10^{-5}	total 0/54
4	None	Not relevant	#0	Tube 19	2.6×10^5	8	8.5×10^{-5}	n.t.
	AdNY57	*lacZ*⁻ (Glu461Gly)	#3	Tube 20	1.6×10^5	5	6.3×10^{-5}	0/5
				Tube 21	4.1×10^5	9	8.6×10^{-5}	0/9
							average 1.5×10^{-5}	total 0/14
5	None	Not relevant	#0	Tube 22	3.3×10^4	3	9.1×10^{-5}	n.t.
	AdNY58	*lacZ*⁻ (Tyr105 Stop)	#4	Tube 23	8.6×10^4	4	4.7×10^{-5}	0/4
				Tube 24	3.1×10^4	3	9.7×10^{-5}	0/3
							average 7.2×10^{-5}	total 0/7

RAd: Recombinant adenovirus
n.t.: Not tested.

gene would result in a direct positive readout in the mouse body (for example, through staining with dye). However, as the authors admit, it would be difficult to detect the targeting events with a high sensitivity. The presence of multiple copies of the target gene would improve the sensitivity because the *lacZ*$^+$ allele is dominant over, and epistatic to, the *lacZ*$^-$ alleles with respect to the above phenotype. The MutaMouse carries multiple (approximately 40) copies of the target gene, which amount to 0.4% of the genome. This should be able to improve the sensitivity of detection of gene targeting, although the sensitivity is limited by spontaneous mutagenesis. In addition, the presence of tandem repeats might have other types of negative effect on gene targeting, as detailed below.

How efficient is adenovirus infection and delivery to the hepatocyte nucleus? Tail-vein injection is an established method for the delivery of adenovirus to liver cells. The average copy number of a replication-defective recombinant adenovirus genome per liver cell has been estimated as 14–28 copies using Southern hybridization after tail-vein injection of 5×10^9 PFU of the virus [12]. This corresponds to 40% of the injected adenovirus. Fluorescence *in situ* hybridization revealed that, after tail-vein injection of 2×10^9 PFU, all of the hepatocyte nuclei had 1–100 copies of a recombinant adenovirus genome, with an average of 20 copies [27]. After tail-vein injection of 2×10^8 PFU of a recombinant adenovirus with the *lacZ* expression cassette, 40% of the hepatocytes expressed beta-galactosidase [13]. We assumed that the majority of the liver cells received several copies of the adenovirus genome, at least sufficient for gene expression, after injecting 3×10^9 PFU in our experiment. (We cannot raise the titer any more because of the toxicity of the virus.) This type of information can be confirmed by Southern hybridization and fluorescence *in situ* hybridization.

The gene-targeting frequency with recombinant adenoviruses *in vitro* varies from $\sim 10^{-7}$–10^{-4} per cell [7,8,28]. We did not detect any signal using recombinant adenovirus for gene delivery in the mouse liver. In order to achieve gene targeting *in vivo* using an adenovirus vector or any other means, it will be necessary to increase the frequency of gene targeting. So how can we achieve this goal?

The efficiency of gene targeting *in vitro* varies from one locus to another [29,30]. Such locus-dependence might reflect drastic effects of the chromatin structure on the frequency of homologous recombination [30,31]. Thus, the target transgene could be placed at a different locus that is known to be a hot spot in gene targeting in embryonic stem (ES) cells.

Repetitive sequences are methylated in the mouse genome [32]. Ikehata and colleagues suggested that the whole coding region of the MutaMouse *lacZ* transgene is methylated to a high degree at every CpG site [33]. One possible reason for this phenomenon is that the CpG content of the *lacZ* gene (9%) [34] is much higher than the average CpG content of the mouse genome (~1%) [35]. Methyl-CpG binding protein 2 (MeCP2) might bind to methylated CpG and somehow compact chromatin [36]. Furthermore, Manuelidis analyzed the structure of a mouse chromosome bearing a huge (~11 Mb) insert of a tandem-repeated transgene (~1,000 copies) [37]. This transgene was localized on an arm of chromosome 3 at a distance from the centromere. According to Manuelidis, the transgene is heterochromatic and highly condensed. Therefore, the MutaMouse transgene might be heterochromatic. The accessibility of nucleases to the heterochromatic structure is lower than that of euchromatin [38,39]. Reducing the copy number of the transgene and/or using another transgene that is lower in CpG content might increase gene targeting, although the decrease in copy number might affect the sensitivity of detection. An important experiment that can be done is to test whether the coding region of the MutaMouse lacZ transgene is really heterochromatic, using, for example, CHIP assay with the antibody against the methylated histones and PCR primers on the lacZ genes.

Chromosome replication is known to stimulate homologous recombination. Partial hepatectomies in mice might stimulate liver cell proliferation and DNA replication, which in turn might stimulate recombination. Hara et al. (1999) reported that partial hepatectomies increased mutagenesis with *N*-ethyl-*N*-nitrosourea, which is a direct-acting DNA-ethylation agent, in the MutaMouse [40].

It might be easier to modify the donor DNA than the recipient DNA. One can generate recombinogenic damage on the donor DNA. Irradiating adenovirus particles with ultraviolet light of 1500 J/m^2 resulted in an approximately three-fold increase in their mutual homologous recombination [41]. Recombinogenic cross-links are induced by some mutagens, such as psoralens, cisplatin (*cis*-diamminedichloroplatinum) and mitomycin C [42]. Such agents, both mutagenic and recombinogenic, might be suitable for gene targeting *in vivo* if they are shown to be active in mutagenesis in a transgenic-reporter mouse system. The effect of such recombinogenic damage might be much larger with replication-defective adenovirus recombinants than with replication-competent adenoviruses, because their replication-intermediates are responsible for their high recombination frequency [41,43-45].

The gene-targeting frequency is strongly dependent on the length of homology; the frequency increases as the homology length increases up to 10 kb [46-48]. If the deviation from this rule above 10 kb is due to the shearing and/or degradation of longer DNA after electroporation in embryonic stem cells, donor DNAs that are protected by the DNA binding proteins in the adenovirus particle might show greater length dependence over a wider range of values. Adenoviral vectors with a larger capacity for inserts, which are known as high-capacity 'gutless' vectors [49-51] might therefore be suitable for use in this approach.

Conclusion

Here we attempted to perform gene targeting in a transgenic mouse system that allowed the sensitive detection of mutagenesis. The frequency of gene targeting in the mouse liver by these recombinant adenoviruses was shown to be less than 1/20000 with the sensitive and PCR-independent detection system.

List of abbreviations

PCR, polymerase chain reaction; PFU, plaque-forming unit; RFLP, restriction fragment length polymorphism; p-gal, phenyl-beta-D-galactoside; X-gal, 5-bromo-4-chloro-3-indlyl-beta-D-galactose

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AI carried out the injection of the recombinant adenovirus and the analysis of the mouse DNA. YN and HM constructed the recombinant adenovirus. NH injected the recombinant adenovirus to the mouse. YN constructed the experimental design as well as cloning of the part of lambda DNA from the MutaMouse genomic DNA. IK provided the original experimental idea and coordinated the experimental design. All authors read and approved the final manuscript.

Additional material

Additional file 1

Bacterial strains, plasmids, bacteriophage strains and recombinant adenovirus constructs.

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RESEARCH ARTICLE

Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV

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Immunization involving a DNA vaccine prime followed by an adenovirus type 5 (Ad5) boost elicited a protective immune response against SHIV challenge in monkeys. However, the hepatocellular tropism of Ad5 limits the safety of this viral vector. This study examines the safety and immunogenicity of a replication-defective chimeric Ad5 vector with the Ad35 fiber (Ad5/35) in BALB/c mice and rhesus monkeys. This novel Ad5/35 vector showed minimal hepatotoxicity after intramuscular administration with the novel Ad5/35 vector. In addition, an Ad5/35 vector expressing HIV Env gp160 protein (Ad5/35-HIV) generated strong HIV-specific immune responses in both animal models. Priming with a DNA vaccine followed by Ad5/35-HIV boosting yielded protection against a gp160-expressing vaccinia virus challenge in BALB/c mice. The Ad5/35-HIV vector was significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector. These findings indicate that an Ad5/35 vector-based HIV vaccine may be of considerable value for clinical use.
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Keywords: *Ad5/35 vector; HIV; animal model; vaccine; immune response*

Introduction

A vaccine capable of preventing HIV infection is needed to control the global AIDS pandemic. In the past decade, multiple strategies to produce an immunogenic HIV vaccine have been explored. This included production of HIV subunit peptide vaccines,[1] DNA vaccines,[2] recombinant virus-vector vaccines (including modified vaccinia virus,[3] adenovirus (Ad),[4,5] rabies virus,[6] flavivirus,[7] sendai virus,[8] Venezuelan equine encephalitis virus,[9] and adeno-associated virus[10,11]), and bacterial vector-vaccines (bacille Calmette–Guerin,[12,13] and *Lactococcus lactis*[14]). Each of these strategies showed some promising results in animal models, either alone or in combination.

Among these vectors, the replication-defective human Ad type 5 (Ad5) recombinants (with the deletion of a replication-essential gene, E1) and the replication-defective modified vaccinia Ankara (MVA) elicited the most potent $CD8^+$ T-cell responses and provided the highest degree of protection in non-human primates.[3,4,15,16] A major limitation for the clinical application of the Ad5 and MVA vectors is the pre-existing immunity against these viruses in humans, since most of the human population has been infected with Ad5[17] and vaccinia virus on being administered the smallpox vaccine. The pre-existing antiviral immunity may strongly influence the efficacy of the HIV vaccine using Ad5 and MVA vectors.

Human Ads are classified into six subgroups from A–F.[18] Most of Ad serotypes belonging to subgroups A, C, D, E, and F use the coxsackievirus and adenovirus receptor (CAR) as a cellular receptor.[19] The Ad5 (subgroup C) has well-defined biological properties and has been widely used as a vector for gene therapy and vaccine. The replication-defective Ad5 vector can easily be produced in high titers and is highly effective in boosting HIV-specific immunity.[4,15] However, this virus uses CAR as its primary attachment receptor, which confers tropism for liver parenchymal cells.[19–22] This raises important safety concerns,[22] particularly because the administration of an Ad5-based vector for gene therapy resulted in the death of a patient.[23] In response to these shortcomings, our laboratory has examined the immunogenicity and safety of a replication-defective chimeric Ad5 vector with Ad type 35 fiber (Ad5/35) (Ad35 virus was classified as subgroup B). The Ad35 fiber showed 25% amino-acid homology with the Ad5 fiber.[24] Cell entry of Ad35 is CAR independent and may involve CD46 receptor, which expresses on most human cells.[25] Ad35 can be transducted to liver nonparenchymal cells on a level 4–5 log orders lower than Ad5, but not to

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liver parenchymal cells.[20] In the present study, we found that the Ad5/35 recombinants not only induced strong antigen-specific humoral and cellular immune responses and exhibited minimal hepatotoxicity in both mice and non-human primates, but were also significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector.

Results

Biodistribution of Ad in mice

In the initial experiments, mice were injected intramuscularly (i.m.) with 10^{11} viral particles (vp) of a luciferase-expressing Ad5 (Ad5-Luc) or Ad5/35 vector (Ad5/35-Luc). Luciferase expression was monitored using an *in vivo* imaging system (IVIS) on days 3 and 10 after administration. As shown in Figure 1a, all of the Ad5/35-Luc vector remained at the injection site. In contrast, substantial amounts of the Ad5 vector migrated to the liver. This difference in vector distribution was confirmed by studies involving LacZ-expressing Ad5 and Ad5/35 vectors (data not shown). Studies on serum glutamic-oxaloacetic transaminase (GOT) and serum glutamic-pyruvic transaminase (GPT) levels revealed that mice injected with the Ad5-Luc vector had changes indicative of liver damage (Figure 1b). We also analyzed serum levels of key proinflammatory cytokines (IFN-γ and IL-6) on days 0, 3, and 10 after administration of virus vectors. The levels of IFN-γ and IL-6 were significantly elevated following administration of Ad5-Luc vector, but not of Ad5/35-Luc vector (Figure 1c). Thus, the hepatotoxicity caused by the Ad5 vector was circumvented by the use of an Ad5/35 vector.

Time-course study of HIV-specific immune responses in mice.

Ad5/35 vector can efficiently transfect antigen-presenting cells[18,21,26,27] and muscular cells (Figure 1a). In order to explore whether the virus vector can be used as a vaccine vector, we constructed an HIV Env gp160-expressing Ad5/35 vector (Ad5/35-HIV). The expression of HIV gp160 was confirmed by Western blotting (Figure 2a). The HIV Env gp160-expressing DNA vaccine (DNA-HIV) used in this study was reported previously.[28] The mice were immunized with 10^{10} vp of Ad5/35-HIV vector, and the HIV-specific cellular immune response was periodically monitored by the intracellular cytokine staining (ICS) assay. The assay has been widely utilized to distinguish the relative contributions of $CD8^+$ cells to the overall T-cell responses.[29] On day 3, HIV-specific IFN-γ-secreting $CD8^+$ T cells can be detected (Figure 2b) and peaked 2 weeks after immunization. On day 50 and month 7 after final immunization, 2.5 and 1.2% of HIV-specific IFN-γ-secreting $CD8^+$ T cells still persisted, respectively.

Mice were vaccinated with Ad5/35-HIV vector to explore the humoral immune response 7 weeks after the final immunization. The animals immunized with 10^{10} vp of Ad5/35-HIV vector developed a high-tittered anti-gp160 antibody (Ab) response (Figure 2c). The specificity of the Ab response was confirmed by Western blotting (Figure 2c, upper panel). The magnitude of this response was not significantly altered by preimmunization with the DNA-HIV vaccine (Figure 2c). DNA-HIV vaccination alone generated a low level of HIV-specific serum Ab (Figure 2c, bottom panel). HIV-specific neutralizing Ab was only detectable in the Ad5/35-HIV

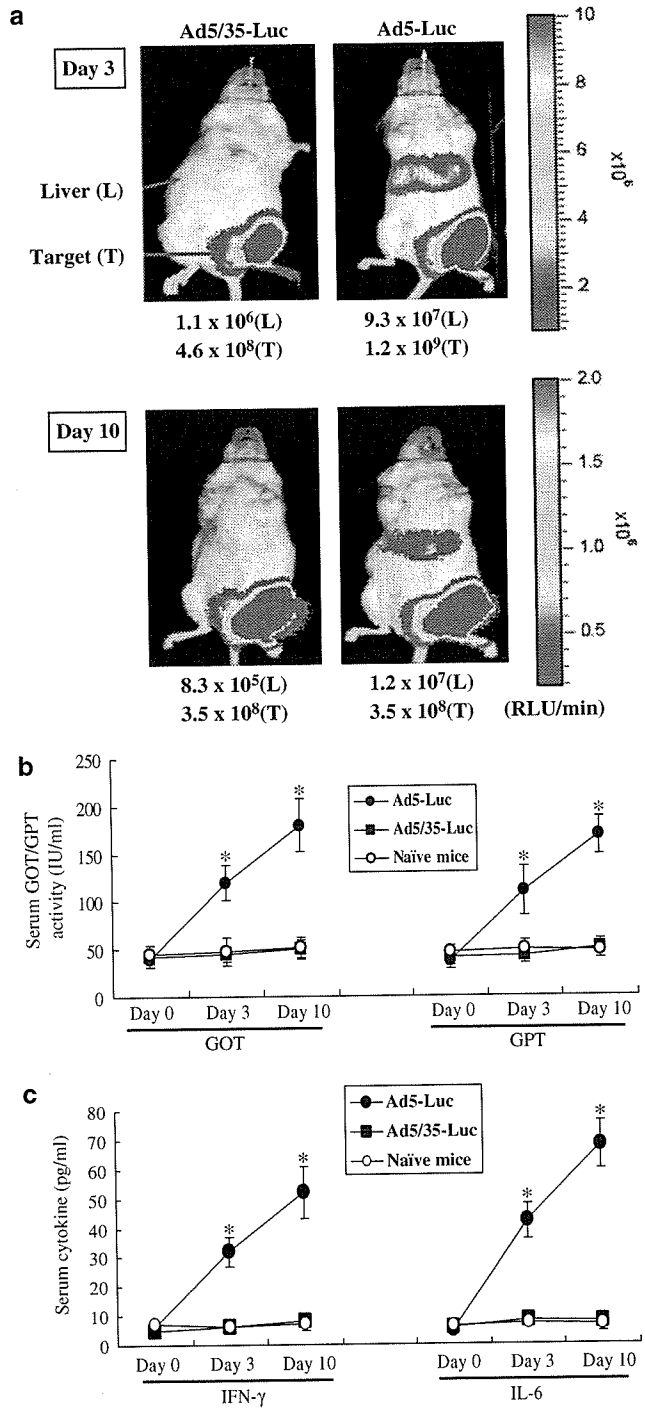

Figure 1 *Biodistribution and safety of Ad vectors. BALB/c mice were injected i.m. with 10^{11} vp of the Ad5-Luc or Ad5/35-Luc vector. (a) Using an IVIS CCD camera, vector distribution was detected after the addition of luciferin (3 mice/group) (expressed in relative light units (RLU)). One of the mice is represented and other mice used show the same pattern. (b) Serum GOP and GPT levels were measured on days 0, 3, and 10 after injection (5 mice/group). IU: international unit. (c) Serum IFN-γ and IL-6 levels were measured on days 0, 3, and 10 after injection (5 mice/group). *Mean values are significantly different between Ad5-Luc-administered mice and Ad5/35-Luc-administered mice or naïve mice at the same time point.*

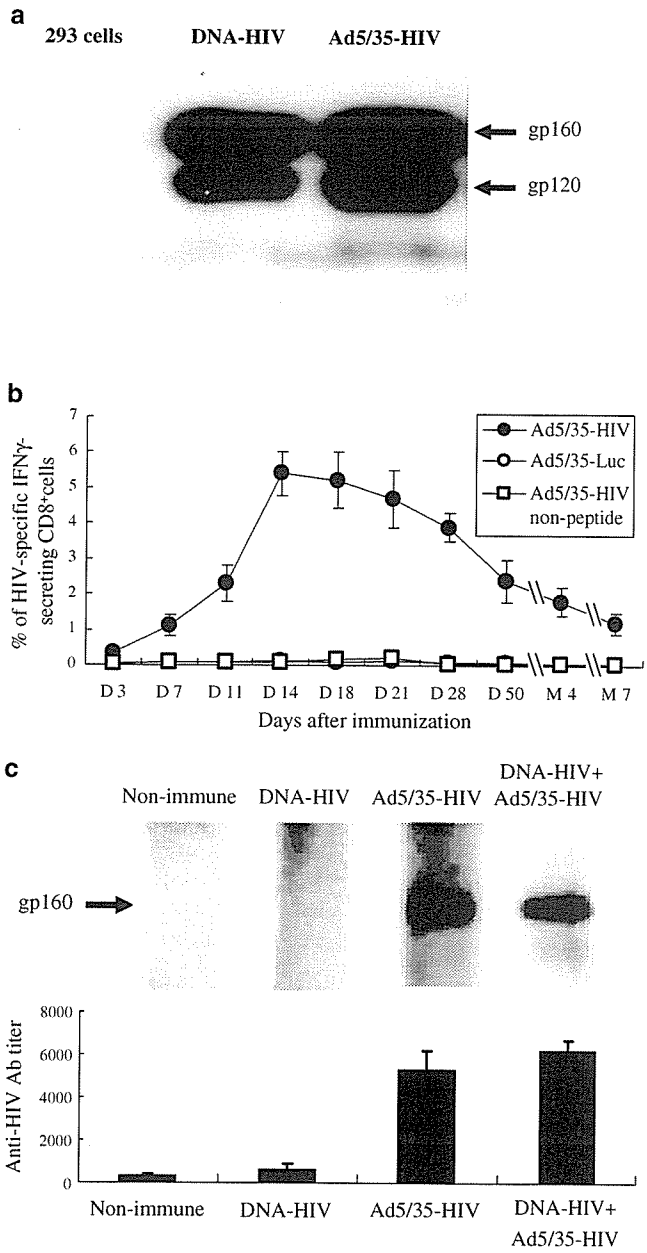

Figure 2 *Time course of HIV-specific immune responses in mice. (a) HIV Env protein expression of DNA-HIV vaccine and Ad5/35-HIV on HEK293 cells was confirmed by Western blotting using an HIV Env-specific mAb. (b) Time-course study of cellular immune responses after a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector (3 mice/time point). D: day; M: month. (c) HIV-specific Ab was detected by Western blotting using 100-fold diluted antisera (serum pool of 10 mice/group) (upper panel) and ELISA (10 mice/group) (bottom left panel).*

vaccinated mice (1:186) and DNA prime/Ad5/35-HIV boosted mice (1:206).

Immune responses and challenge in mice 2 weeks after vaccination. There is growing evidence that cellular immunity contributes to protecting the host against HIV infection.[3,4,30,31] The ability of the Ad5/35 vector to trigger the activation and proliferation of antigen-specific T cells was monitored. Vaccination with the DNA-HIV vaccine induced the number of HIV-specific IFN-γ-secreting $CD8^+$ T cells to increase from background levels (<0.2–0.7%) ($P<0.05$) (Table 1). This was significantly less than the effect of vaccination with the Ad5/35-HIV vector (10^{10} vp/mouse) that increased the IFN-γ-secreting $CD8^+$ T cells to 5.5% ($P<0.05$). Priming with the DNA-HIV vaccine followed by an Ad5/35-HIV vector boost led to a further three-fold increase in the number of IFN-γ-secreting $CD8^+$ T cells ($P<0.05$).

A tetramer-binding assay was used to verify that the IFN-γ-secreting cells were MHC class I-restricted HIV-specific $CD8^+$ T cells.[32] A single immunization with Ad5/35-HIV vector elicited a significant increase in the number of tetramer-binding $CD8^+$ T cells (Table 1). When compared with DNA-HIV vaccination alone, immunization with the Ad5/35-HIV vector yielded five-fold more HIV-specific $CD8^+$ T cells ($P<0.05$). Priming with the DNA-HIV vaccine, followed by Ad5/35-HIV boosting, further increased the tetramer binding ($P<0.05$).

To examine the protective activity of the Ad5/35-HIV vector, immunized mice were challenged with 10^8 plaque forming units (PFU) of vPE16 2 weeks after final immunization. The animals that were vaccinated with the Ad5/35 vector alone or in combination with the DNA-HIV vaccine were completely protected from infection (Table 1); however, the DNA-HIV vaccination alone had little impact on the susceptibility to infection by vPE16.

Long-term cell-medicated immune responses and challenge in mice. The durability of these vaccine regimens was explored. HIV-specific cellular immune responses persisted through 7 months after final immunization (Table 1 and Figure 2b). To determine whether this immune response was protective, vaccinated mice were challenged with vPE16 (10^8 PFU/mouse) 7 weeks after final immunization. The viral load of Ad5/35-HIV-immunized mice was reduced by 10^5 as compared with that of the control mice ($P<0.05$). DNA-HIV vaccination by itself was not protective, but the combination of DNA-HIV priming and Ad5/35-HIV boosting yielded a prolonged and complete protection (Table 1).

Biodistribution of Ad in rhesus macaques

To study the biodistribution of Ad in monkeys, 10^{11} vp of Ad5-Luc and Ad5/35-Luc vectors was injected i.m. into two rhesus monkeys for each vector. The luciferase activity in the tissues was detected 3 days after administration, because high luciferase activity in the mouse liver was observed at that time point. Liver infection with Ad5 vector was 20- to 40-fold stronger than that with Ad5/35 vector (Figure 3a). It is important to note that the luciferase activity of the cerebellum and the posterior cerebrum in the monkeys that received the Ad5-Luc vector was two- and four-fold higher, respectively, than that of the monkeys that received the Ad5/35-Luc vector.

Immune response in rhesus monkeys after vaccination

To explore the immunogenicity of the Ad5/35-HIV vector in monkeys, two rhesus macaques were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector. A detectable HIV-specific serum Ab response developed

Table 1 HIV-specific cell-mediated immune responses and virus challenge after vaccination

	Week 2			Week 7			Month 4	Month 7
	ICS (%)	*Tetramer (%)*	*Ovary viral titer*	*ICS (%)*	*Tetramer (%)*	*Ovary viral titer*	*Tetramer (%)*	*Tetramer (%)*
Nonimmune	0.1±0.1	0.1±0.1	$8\times10^{8}\pm35$	0.1±0.1	0.1±0.1	$1\times10^{9}\pm65$	0.0±0.0	0.0±0.0
DNA-Empty	0.1±0.1	0.1±0.1	$2\times10^{9}\pm45$	0.0±0.0	0.0±0.0	$8\times10^{8}\pm32$	0.0±0.0	0.0±0.0
Ad5/35-Luc	0.2±0.1	0.2±0.2	$2\times10^{9}\pm25$	0.0±0.0	0.0±0.0	$4\times10^{8}\pm46$	0.0±0.0	0.0±0.0
DNA-HIV	0.7±0.1	1.0±0.3	$6\times10^{6}\pm42$	0.4±0.2	0.6±0.1	$5\times10^{7}\pm51$	0.3±0.1	0.1±0.1
Ad5/35-HIV	5.5±0.3	5.2±0.3	ND	2.5±0.8	3.1±0.2	$2\times10^{3}\pm34$	2.5±0.5	1.2±0.4
DNA-HIV+Ad5/35-HIV	17.2±0.8	19.4±2.1	ND	8.2±1.2	8.9±0.8	ND	7.1±0.6	4.1±0.3

Mice were immunized with DNA plasmid or Ad5/35 vector, either alone or in combination. At 2 weeks, 7 weeks, 4 months, and 7 months after final immunization, HIV-specific cellular immune responses were detected by ICS assay and tetramer assay. The data represent the percentage of IFN-γ- or tetramer-positive $CD8^+$ T cells (5–10 mice/group). The backgrounds were less than 0.1% IFN-γ-secreting $CD8^+$ T cells when cells were stimulated with control peptide (influenza NP peptide, TYQRTRALV). The vaccinated mice (10 mice/group) were challenged with vaccinia virus vPE16 2 or 7 weeks after final immunization. At 6 days after the challenge, the vPE16 titer in mouse ovaries was measured. ND, not detectable.

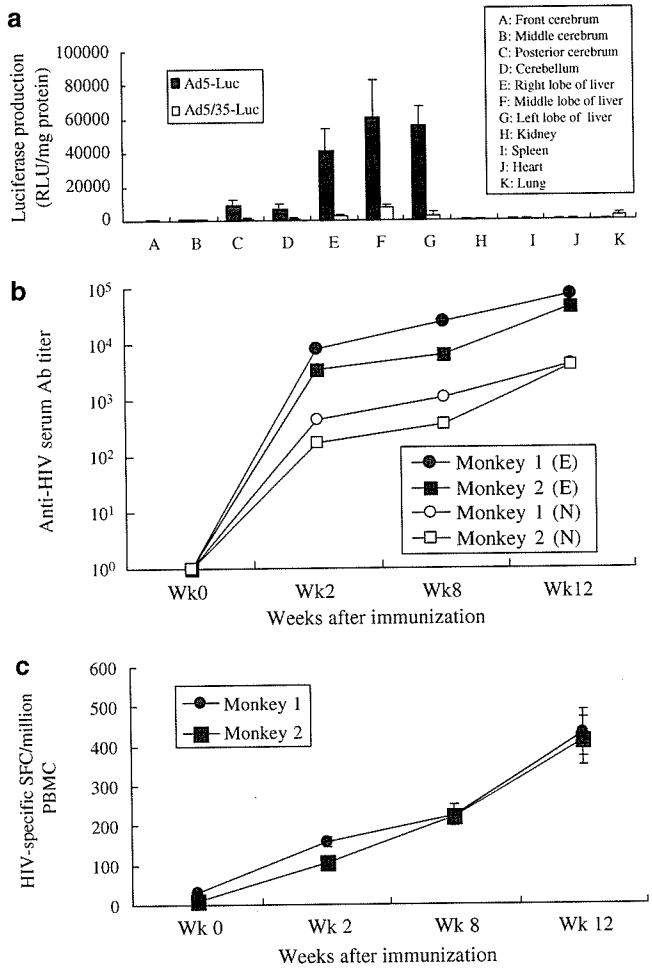

Figure 3 Biodistribution and HIV-specific immune responses in rhesus monkeys. Rhesus monkeys (2 monkeys/group) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc vector. The luciferase activity in the organs of the monkey (expressed in RLU) was examined 3 days after administration (a). Rhesus monkeys were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector at 0 and 8 weeks. PBMCs were isolated at weeks 0, 2, 8, and 12. HIV-specific Ab titers were measured in triplicate by ELISA (E) (●, ■) and neutralizing assay (N) (○, □) (b). The detecting limitation of the neutralizing assay was 100 ND_{50}/ml. PBMCs were stimulated with HIV Env gp120 protein, and the number of cells activated to secrete IFN-γ was determined in triple wells by ELISPOT (c). SFC: spot-forming cells.

within 2 weeks of immunization (Figure 3b). The animals were boosted at 8 weeks. After 4 weeks, titers in excess of 1:50 000 were achieved. Similar results were observed in neutralizing Ab. A increase in the number of HIV-specific IFN-γ-secreting T cells was also detected in the peripheral blood mononuclear cells (PBMCs) (Figure 3c). Boosting with Ad5/35-HIV vector further increased this T-cell response.

Effect of pre-existing immunity on vaccination

To evaluate the effect of the anti-Ad5 neutralization Ab (found in 60% of the adult human population)[17] on the Ad5/35 vector, the infectivity of the vector was examined after incubation with serially diluted serum from subjects with high titers of anti-Ad5 Abs (anti-Ad5 neutralizing titer = 1:64). As shown in Figure 4, the human antisera had 1:8 anti-Ad5/35 neutralizing titer and normal human sera against either Ad5 or Ad5/35 vector was less than 1:4. The sera derived from Ad5/35-HIV-immunized monkeys showed two-fold higher neutralizing Ab titer against Ad5/35 vector than Ad5 vector.

To examine the effect of pre-existing anti-Ad5 immunity on the activity of the Ad5/35 vector *in vivo*, mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc. After 8 weeks, these animals were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV. The HIV-specific responses were detected by the tetramer assay 2 weeks after immunization. Although pre-existing immunity to Ad5 reduced the immune response elicited by both vectors, Ad5/35-HIV was significantly more immunogenic than Ad5-HIV ($P<0.05$; Figure 4).

Discussion

This study demonstrates that an Ad5/35-HIV vector vaccine induces strong cellular and humoral immune responses with minimal toxicity in mice and rhesus macaques. A prime–boost strategy involving the DNA-HIV vaccine and the Ad5/35-HIV vector generated protective immunity against viral infection in mice.

A widely used HIV vaccine should have high immunogenicity, low cost of production, and low or no pathogenicity. Replication-defective Ad5 is one of the best vectors for HIV vaccine development. Vaccination