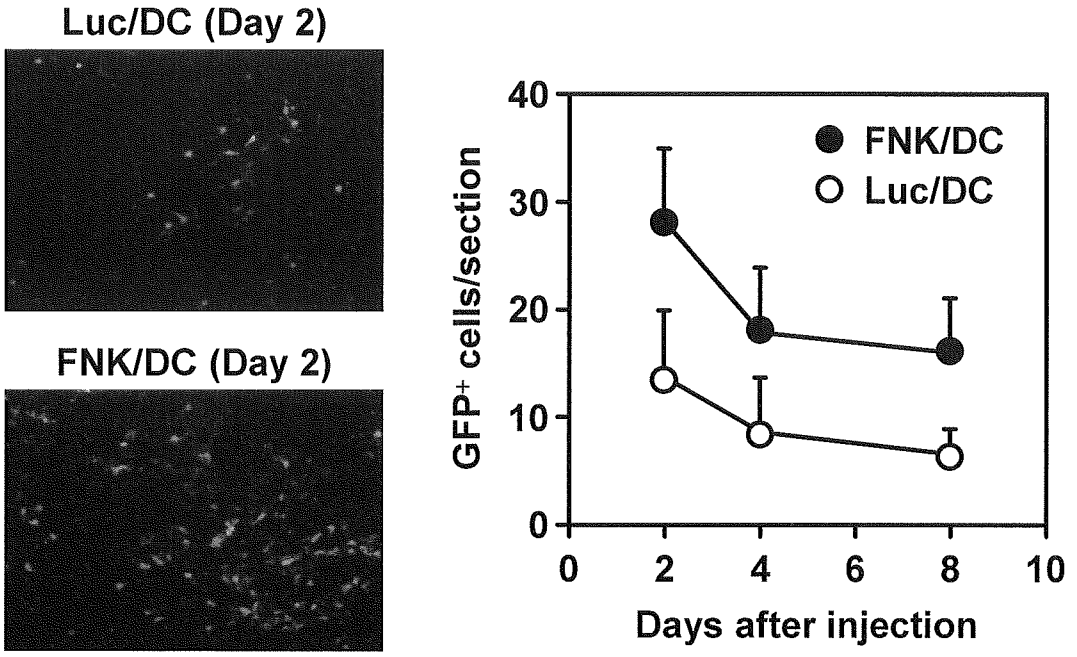

Fig.32 Enhanced accumulation of FNK/DCs from administration site to regional lymph node.
DCs derived from GFP transgenic mice were transfected with AdRGD-FNK or AdRGD-Luc at 25 MOI, and then were cultured for 24 h. These transduced GFP^+ DCs were injected intradermally into right flank of wild type C57BL/6 mice at 2×10^6 cells. The draining inguinal lymph nodes were harvested on days 2, 4, and 6 after injection. Frozen sections (6-μm thickness) of lymph node were prepared, and then the number of GFP^+ DCs was counted under fluorescence microscopy. Each point represents the mean ± SE of results from three mice.

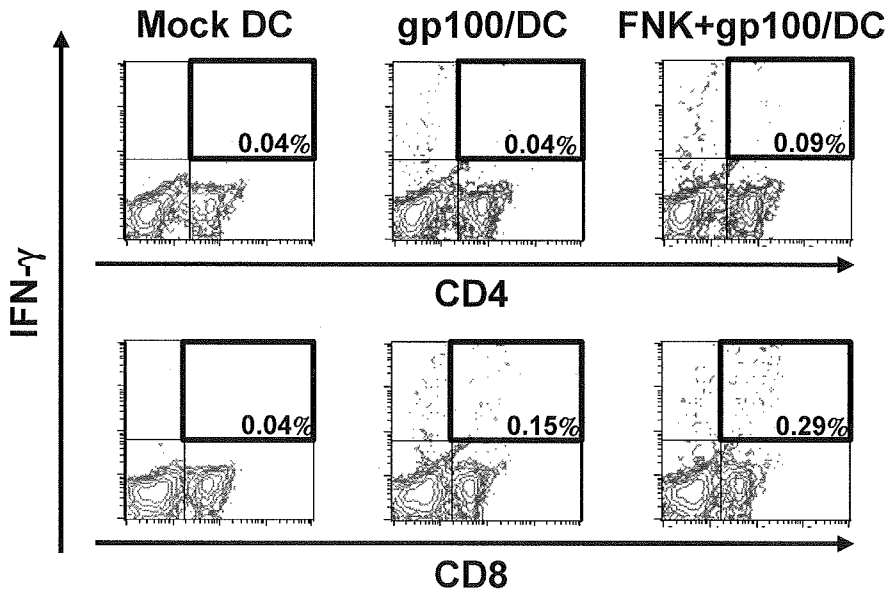

Fig.33 gp100-specific $CD4^+$ or $CD8^+$ T cell immune response in mice immunized with DCs cotransduced with gp100 and Bcl-xFNK genes.
gp100/DCs and FNK+gp100/DCs were prepared using corresponding vectors at 25 MOI, and then culture for 24 h. These transduced cells and mock DCs were vaccinated once intradermally into C57BL/6 mice at 1.5×10^6 cells. At 1 week after immunization, regional lymph node cells were prepared from these mice, and were re-stimulated *in vitro* with mitomycine C- inactivated B16BL6 cells for 24 h. The number of IFN-γ-producing $CD4^+$ or $CD8^+$ T cells was analyzed by intracellular IFN-γ staining followed by flow cytometry.

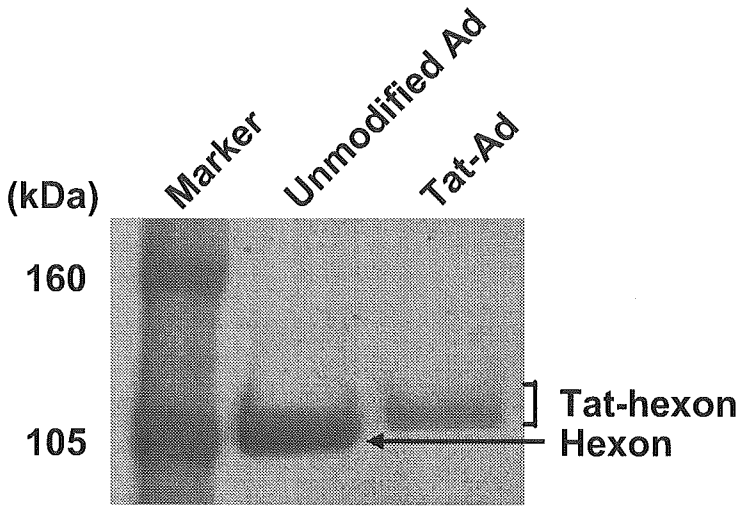

Fig.34 SDS-PAGE analysis of Tat-Ad.

Table 4. The surface charge of Tat-Ad.

Vector	Surface charge (mV)
Unmodified Ad	-18.7
Tat-Ad	+2.3

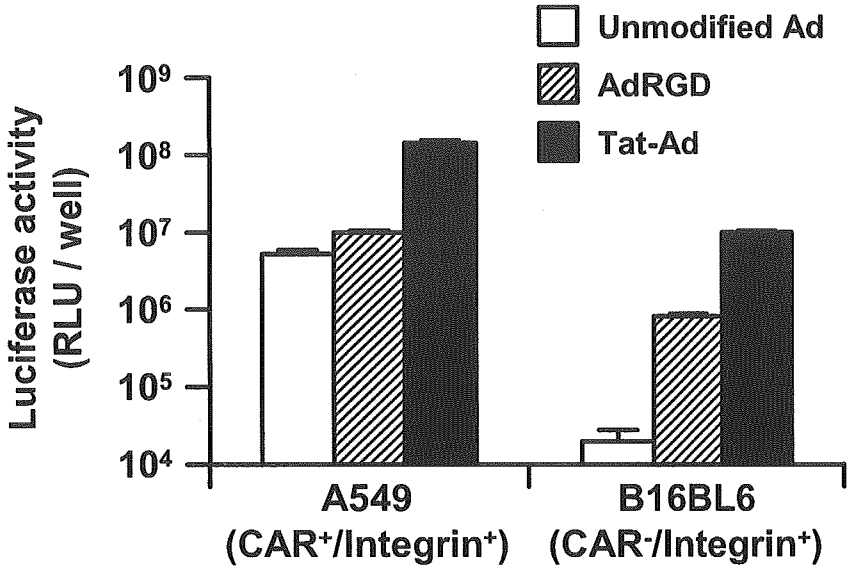

Fig.35 Transduction efficiency of Tat-Ad in A549 and B16BL6 cells.
A549 and B16BL6 cells were transduced with unmodified Ad-Luc, AdRGD-Luc, or Tat-Ad-Luc at 10000 VP/cell. After 24 h-cultivation, luciferase activity was measured using the kit according to the manufacture's instructions. Data represent the mean ± SD of results from triplicate culture.

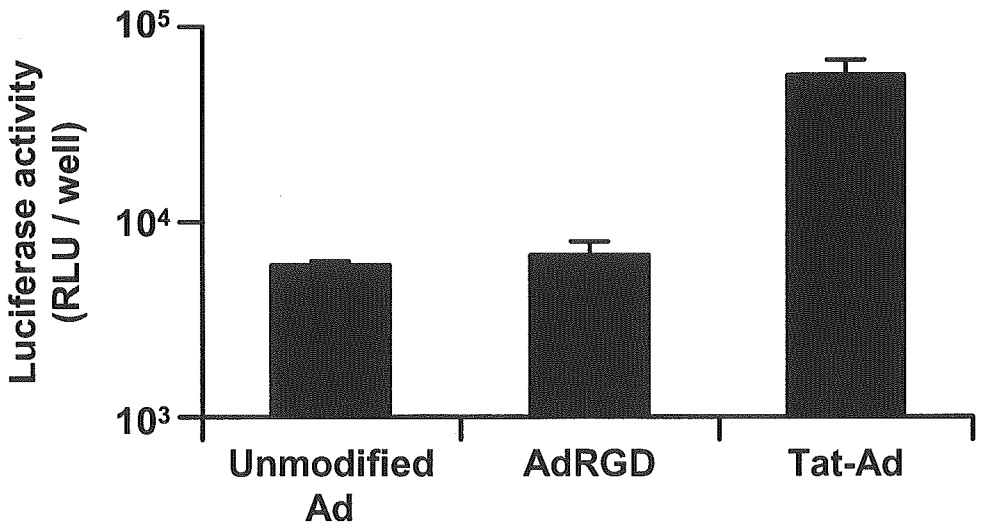

Fig.36 Transduction efficiency of Tat-Ad in KG-1a cells.
KG-1a cells were transduced with unmodified Ad-Luc, AdRGD-Luc, or Tat-Ad-Luc at 10000 VP/cell. After 24 h-cultivation, luciferase activity was measured using the kit according to the manufacture's instructions. Data represent the mean ± SD of results from triplicate culture.

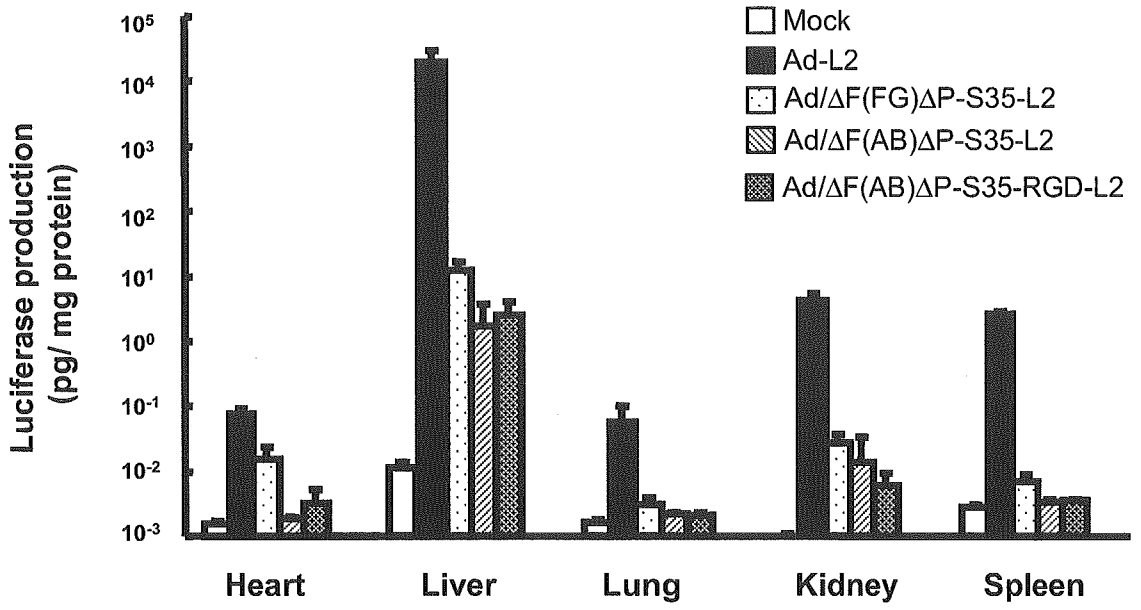

Fig.37 Luciferase production in mice after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 were intravenously (3.0×10^{10} VP) injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production was measured by a luciferase assay system. All data represent the means ± S.D. of 4-6 mice.

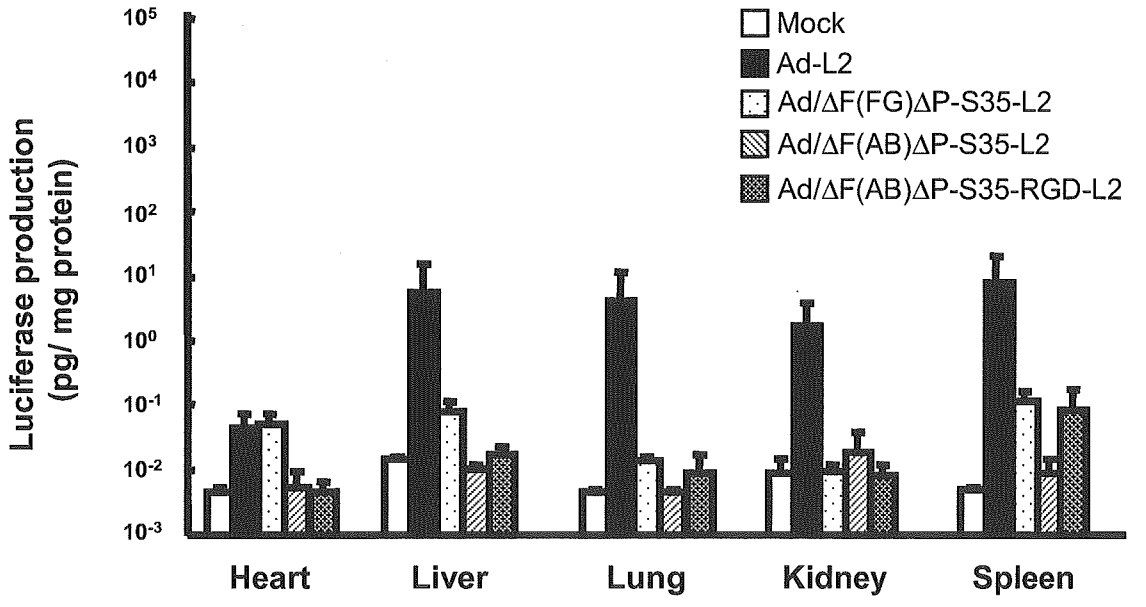

Fig. 38 Luciferase production in mice after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 were intraperitoneally (1.0×10^{11} VP) injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production was measured by a luciferase assay system. All data represent the means ± S.D. of 4-6 mice.

Table 5. Adenovirus vectors used in the present study

Ad vectors	The RGD motif of the penton base	Type of the fiber shaft	The fiber knob			
			AB loop	FG loop	HI loop	C-terminus
Ad-L2	intact	5	intact	intact	——	——
Ad/ΔF(FG)ΔP-S35-L2	mutation	35	intact	mutation	——	——
Ad/ΔF(AB)ΔP-S35-L2	mutation	35	mutation	intact	——	——
Ad/ΔF(AB)ΔP-S35-RGD(HI)-L2	mutation	35	mutation	intact	RGD motif	

Table 6. Adenovirus vectors used in the present study

Ad vector	Penton base	Ad type of tail	Ad type of shaft	Amino acid sequence of knob domain			
				AB loop	FG loop	HI loop	C-terminus
Conventional Ad							
Ad-L2	MND-HAIRGDTFAT-RAE	5	5 (22 β-repeats)	- NCRLNAEKDA--	TEGTAYTNAV-	-DTTPSA-	-QE stop
Mutant Ad							
Ad/ΔF(FG) ΔP-S35-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCRLNAEKDA-	- TEG - - - - NAV- Δ a.a. 489-492	-DTTSNPSA-	-QEID stop
Ad/ΔF(AB) ΔP-S35-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCSLNGGGDA-- 4 a.a. mutation	TEGTAYTNAV-	-DTTSNPSA-	-QEID stop
Ad/ΔF(AB)ΔP -S35-RGD-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCSLNGGGDA-- 4 a.a. mutation	TEGTAYTNAV-	-DTTSACDCR GDCFCANPSA	-QEID stop

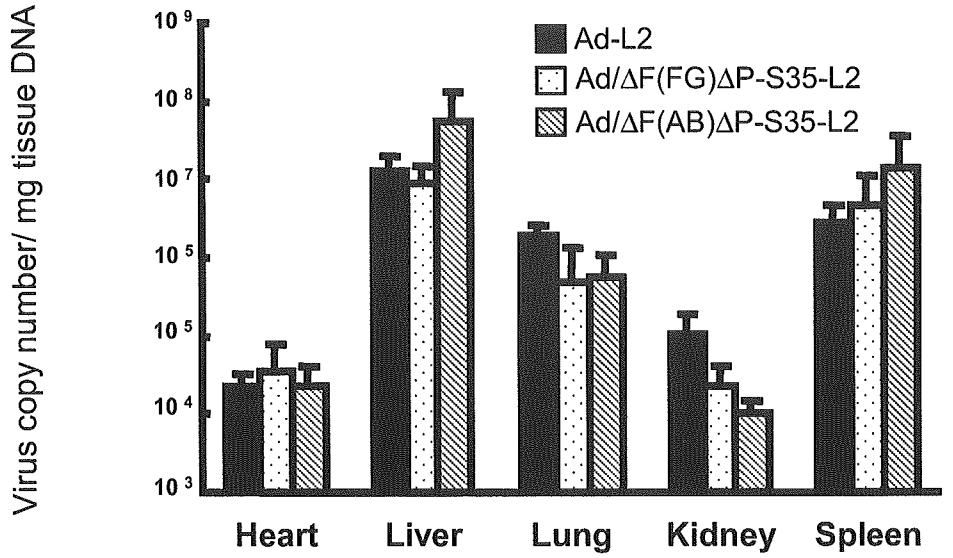

Fig. 39 Biodistribution of viral DNA after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously (3.0×10^{10} VP) injected into the mice. Three hours later, the heart, lung, liver, kidney, and spleen were harvested, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4 to 6.

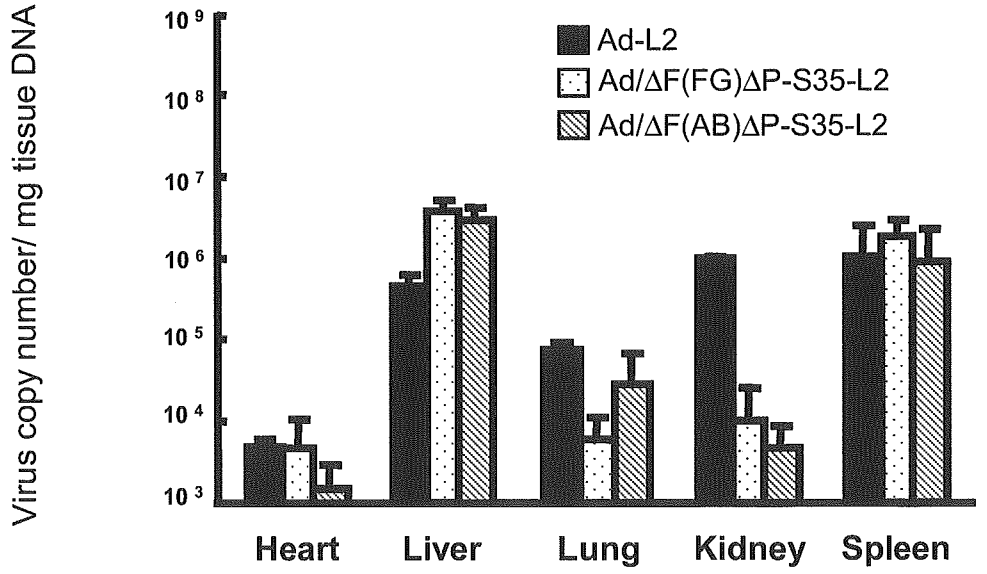

Fig. 40 Biodistribution of viral DNA after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally (1.0×10^{11} VP) injected into the mice. Three hours later, the heart, lung, liver, kidney, and spleen were harvested, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4 to 6.

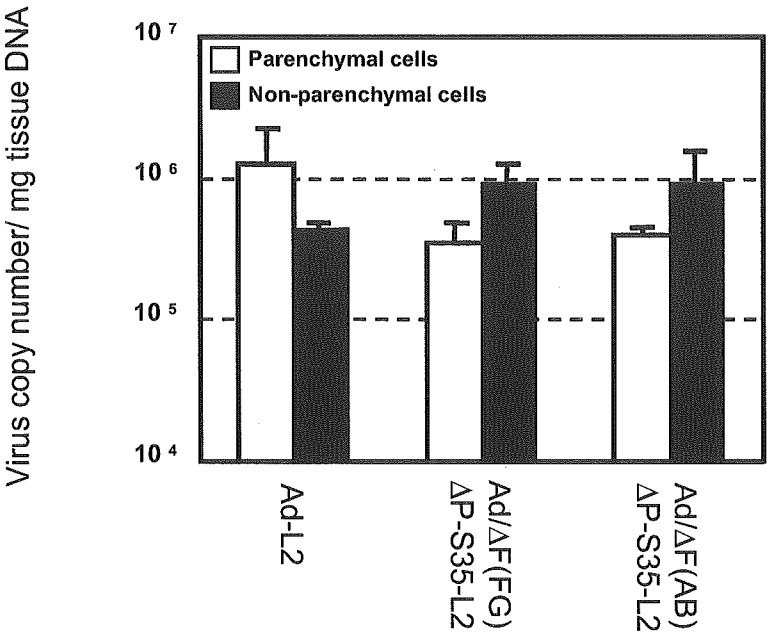

Fig. 41 Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously ($3.0x10^{10}$ VP) injected in the mice. Collagenase perfusion was performed 3 hours after injection of the Ad vector to separate liver PC and NPC. Total DNA, including the Ad vector DNA, was isolated from the cells, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4-6 mice.

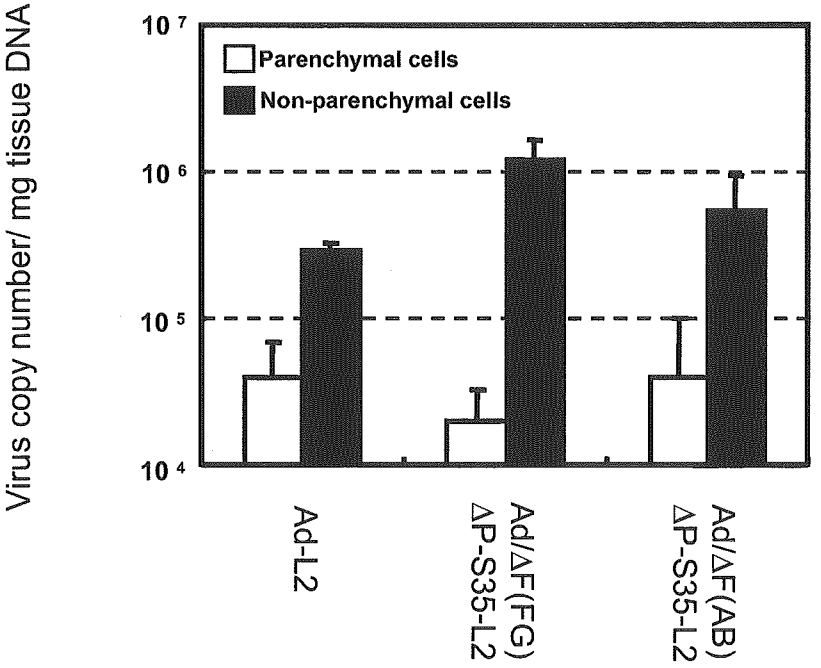

Fig. 42 Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally ($1.0x10^{11}$ VP) injected in the mice. Collagenase perfusion was performed 3 hours after injection of the Ad vector to separate liver PC and NPC. Total DNA, including the Ad vector DNA, was isolated from the cells, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4-6 mice.

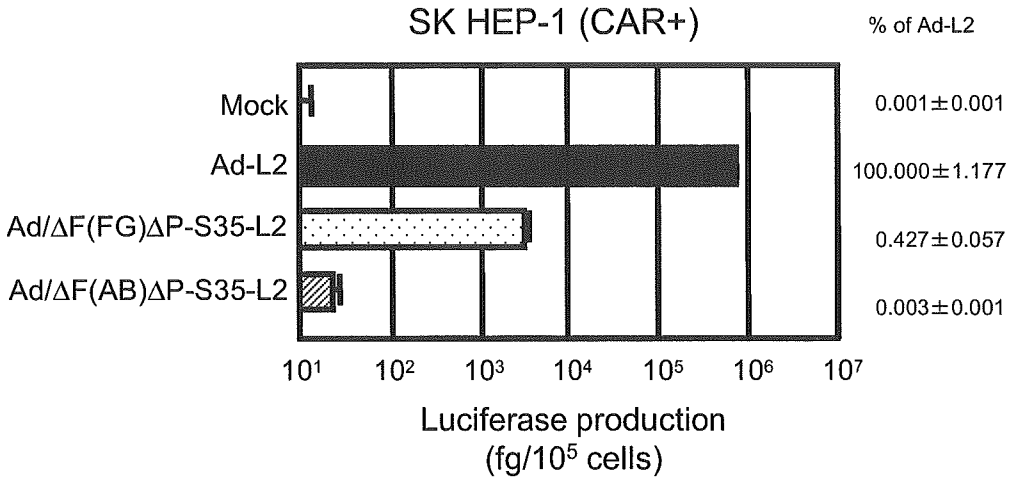

Fig. 43 Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 for 1.5 hours. After culture for 48 hours, luciferase production in the cell was measured by luminescent assay. The data are expressed as means ± S.D. (n=4). The relative levels of luciferase expression are described by designating the value of Ad-L2 as 100.

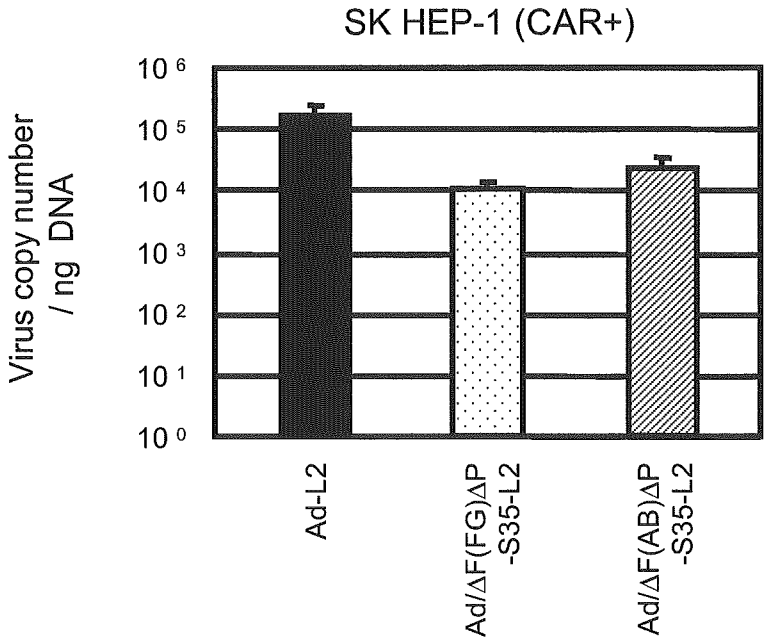

Fig. 44 The amounts of viral uptake in SK HEP-1 cells.
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. After culture for 1.5 hours, the cells were washed with PBS, resuspended in 0.05% trypsin–0.5 mM EDTA-PBS solution, and incubated at 37° C for 10 min. Following this incubation, the cells were incubated at 37° C for 10 min with 0.05% DNase I-0.5 M MgCl2-PBS, washed with PBS and were resuspended in 0.1 M EDTA-PBS solution. The amounts of Ad genome DNA isolated from the cells were quantified with the TaqMan fluorogenic detection system. The data are expressed as means ± S.D. (n=4).

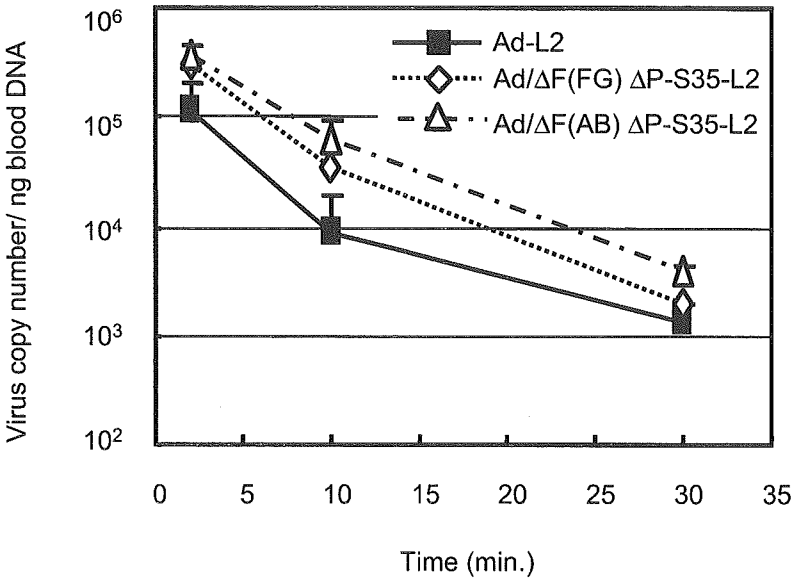

Fig. 45 Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously (3.0×10^{10} VP) injected, and blood was drawn from the retro-orbital at the indicated times post injection. Total DNA, including the Ad vector DNA, was isolated from the blood, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4-6 mice.

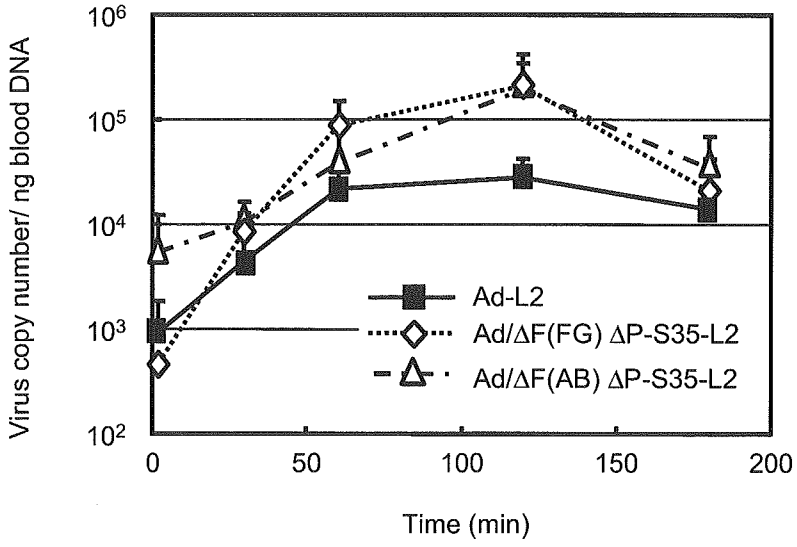

Fig. 46 Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice.
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally (1.0×10^{11} VP) injected, and blood was drawn from the retro-orbital at the indicated times post injection. Total DNA, including the Ad vector DNA, was isolated from the blood, and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± S.D. of 4-6 mice.

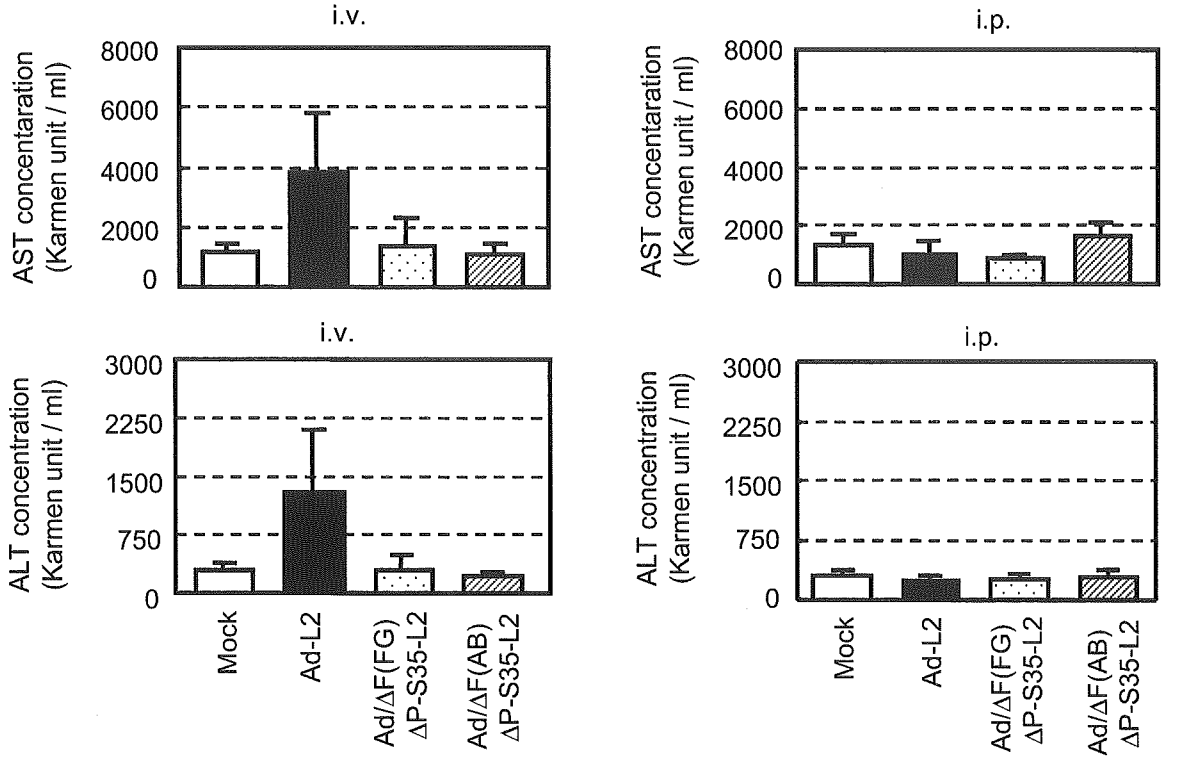

Fig. 47 Serum enzymes levels after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice.

Blood samples were collected by inferior vena cave at 48 hours after intravenous ($3.0x10^{11}$ VP) or intraperitoneal ($1.0x10^{11}$ VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. Serum samples were collected into separate tubes containing no anticoagulant for coagulation, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum were measured using a Transaminase-CII kit. All data represent the means ± S.D. of four mice.

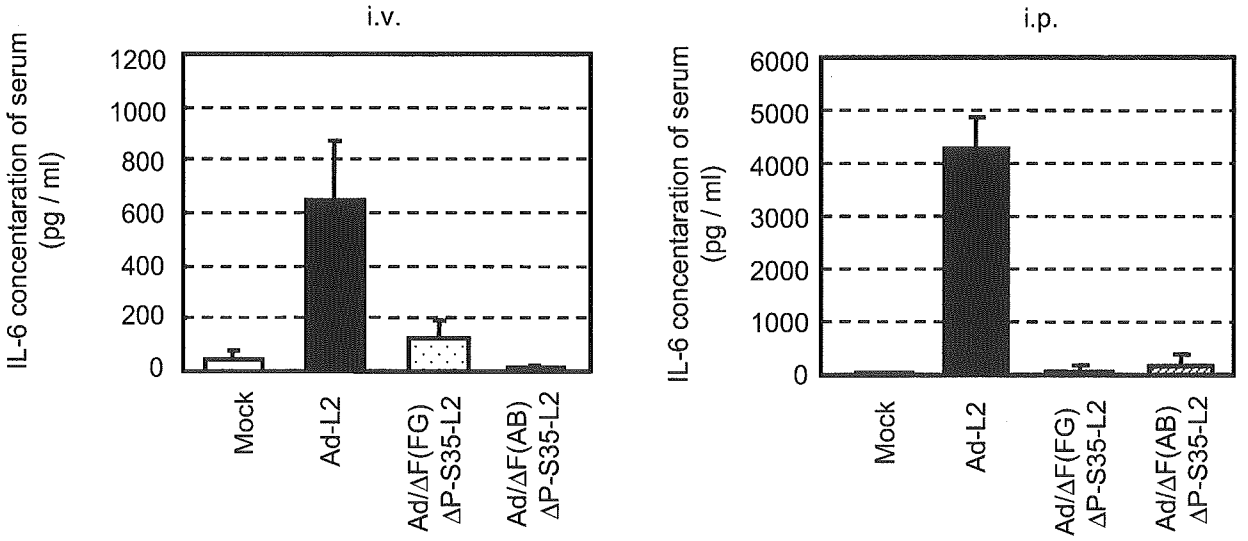

Fig. 48 Interleukin (IL)-6 levels in serum after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice.

Blood samples were collected by the inferior vena cave at 3 hours after intravenous ($3.0x10^{11}$VP) or intraperitoneal ($1.0x10^{11}$ VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. Serum samples were collected into separate tubes containing no anticoagulant for coagulation, and IL-6 levels in the serum were measured by ELISA. All data represent the means ± S.D. of six mice.

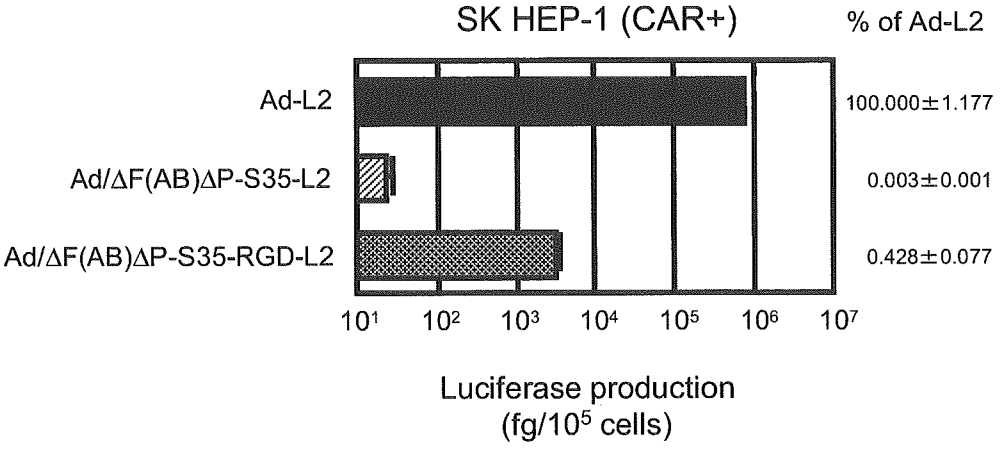

Fig. 49 Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2.
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 for 1.5 hours. After culture for 48 hours, luciferase production in the cells was measured by a luciferase assay system. The data are expressed as means ± S.D. (n=4). The relative expression levels are described by designating the value of Ad-L2 as 100.

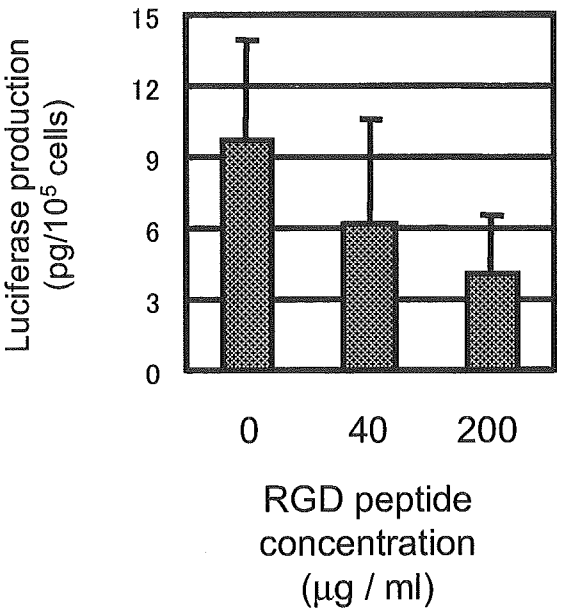

Fig. 50 Effects of RGD peptide on the transduction efficiency of Ad/ΔF(AB)ΔP-S35-RGD-L2 into SK HEP-1 cells.
SK HEP-1 cells were preincubated with RGD peptide (0,40, 200 mg/ml) for 10 min. The cells were then transduced with 300 VP/cell of Ad/ΔF(AB)ΔP-S35-RGD-L2 for 0.5 hours in the presence of RGD peptide. After culture for 48 hours, luciferase production was measured by a luciferase assay system. The data are expressed as means ± S.D. (n= 6).

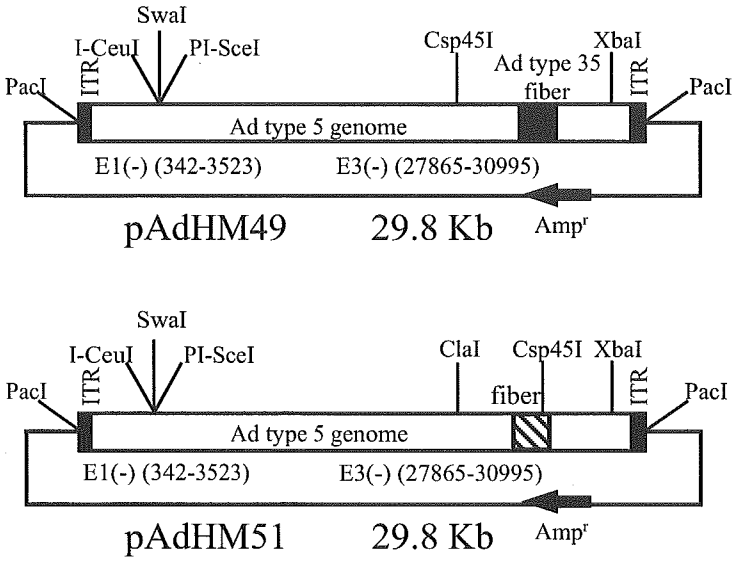

Fig. 51 The structure of the vector plasmids pAdHM49 and -51.

Table 7. Adenovirus vectors used in the present study

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

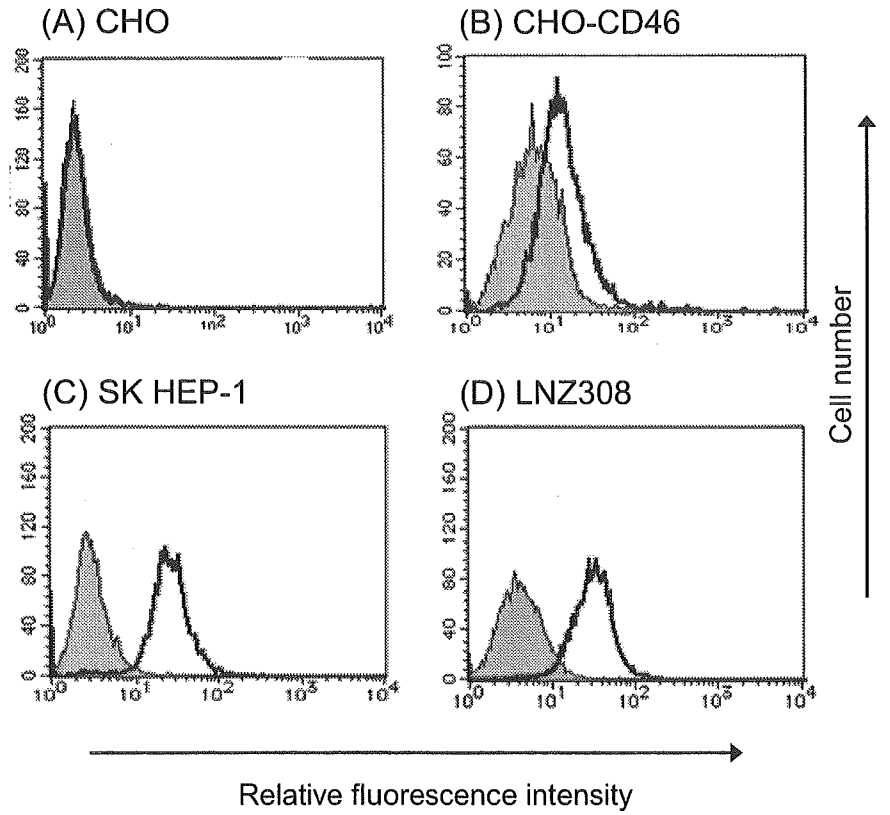

Fig. 52 CD46 expression in CHO, CHO-CD46, SK HEP-1, and LNZ308 cells.
CHO (A), CHO-CD46 (B), SK HEP-1 (C), and LNZ308 (D) cells were stained with FITC-conjugated anti-CD46 antibodies, and subsequently analyzed by a flowcytometer.

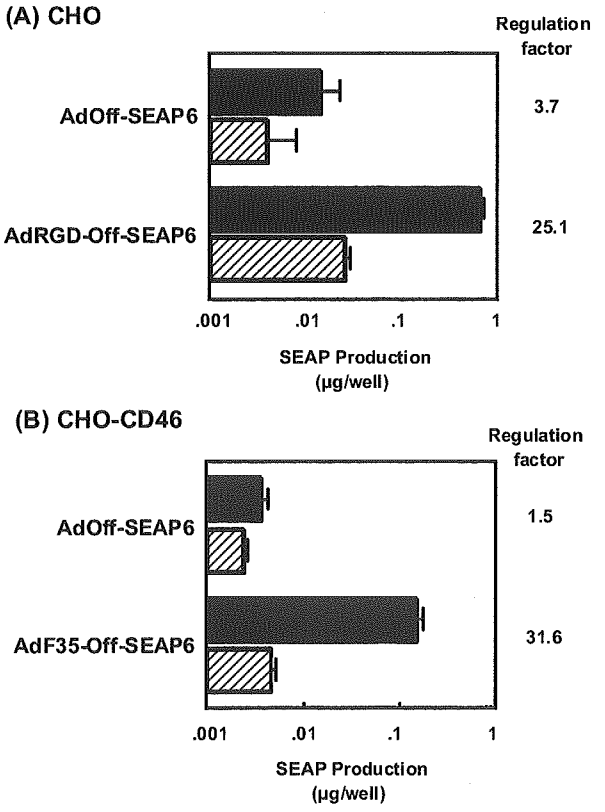

Fig. 53 Fiber-modified Ad vector-mediated tet-off systems in CHO and CHO-CD46 cells.
CHO (A) and CHO-CD46 (B) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGDOff-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 hours. 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).

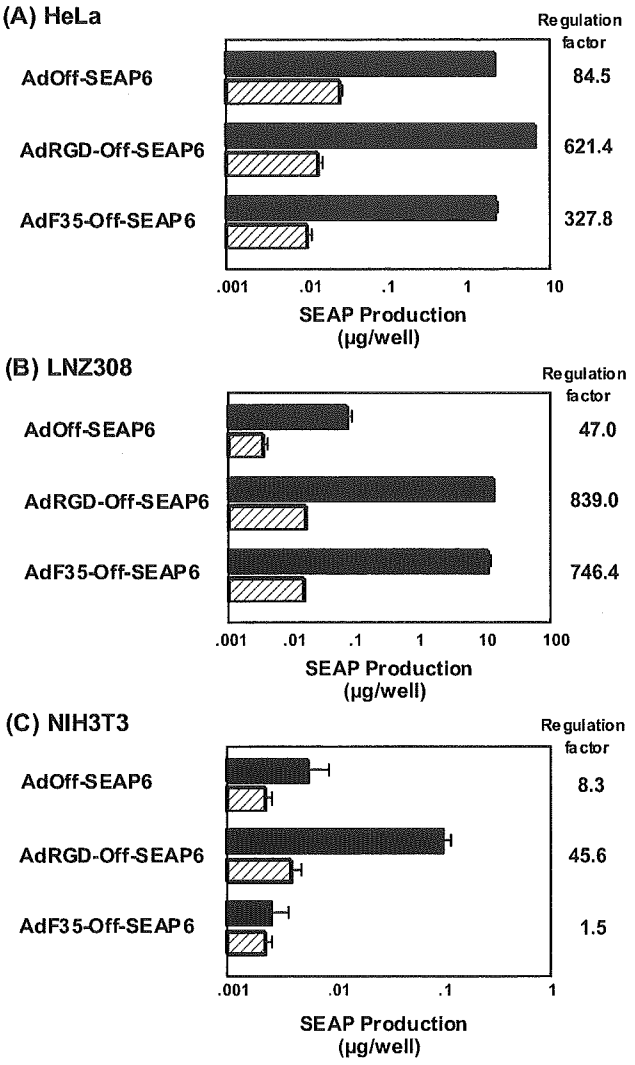

Fig. 54 Fiber-modified Ad vector-mediated tet-off systems in various types of cells. SK HEP-1 (A), LNZ308 (B), and NIH3T3 (C) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGDOff-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 hours. 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).

Table 8 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 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 hours. 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).

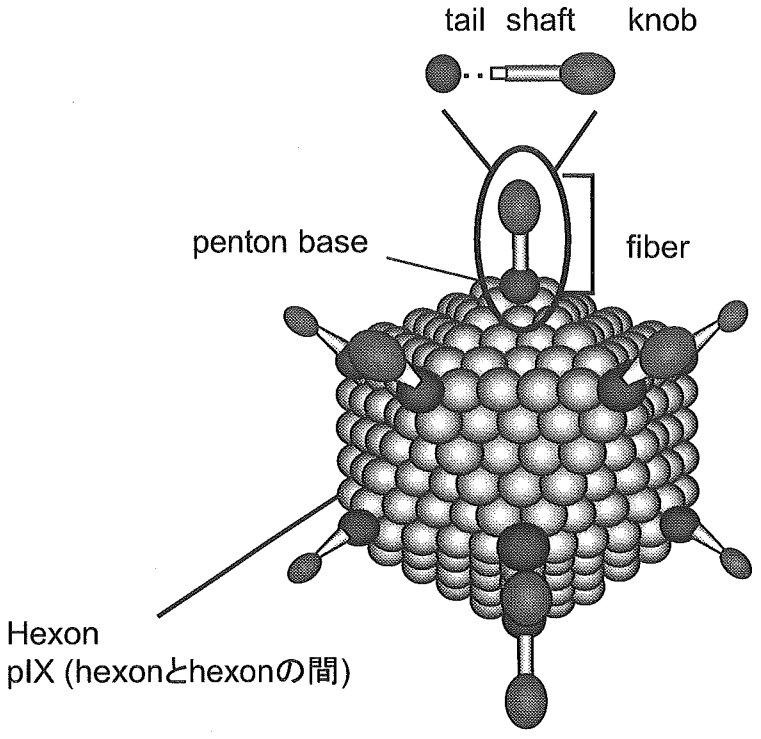

Fig.55 Structure of adenovirus vector

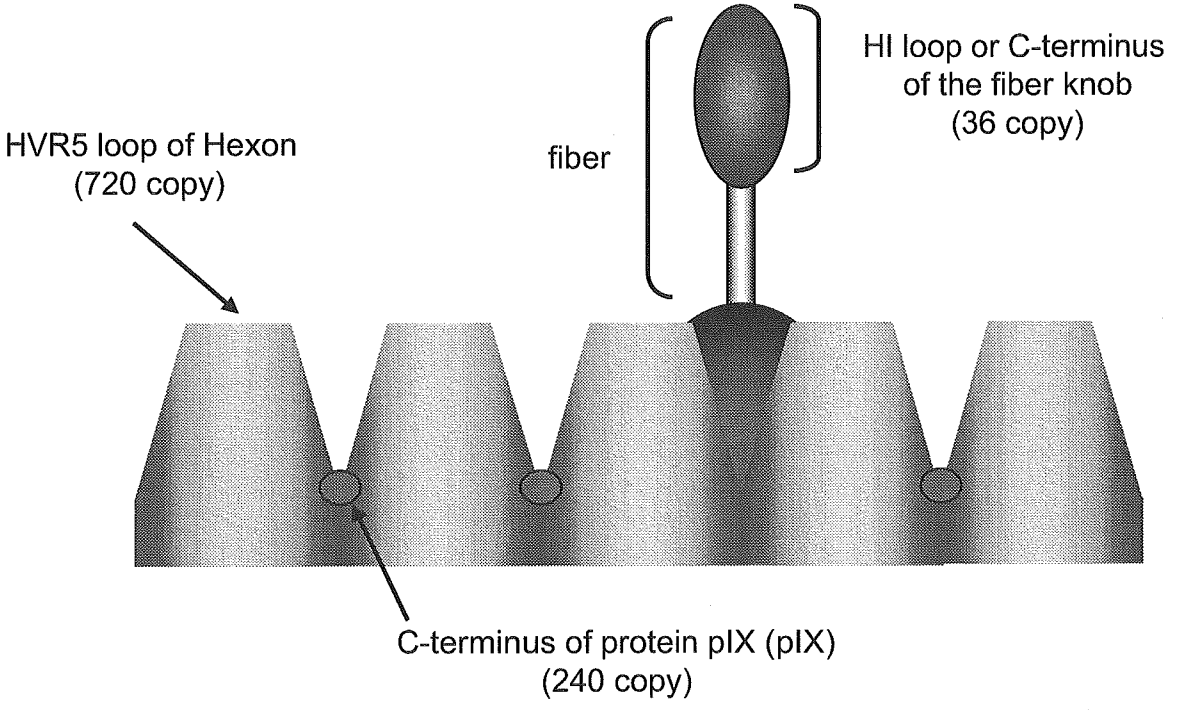

Fig.56 Candidate location on Ad capsid for the presentation of foreign peptide

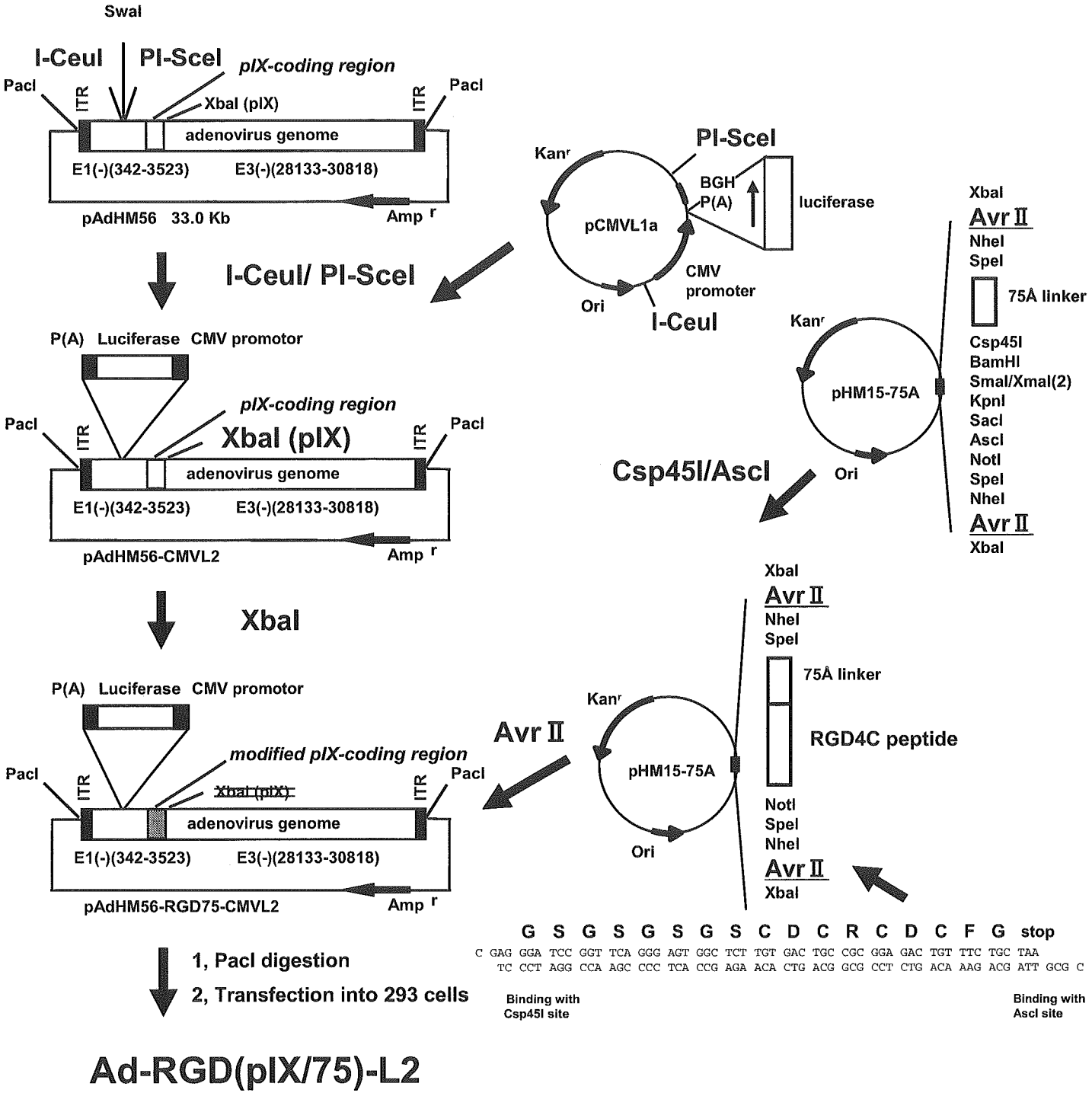

Fig.57 The construction strategy for pIX-modified Ad vectors containing foreign peptides.
pAdHM56 was digested by I-CeuI/PI-SceI and ligated with I-CeuI/PI-SceI-digested pCMVL1a, which contains CMV promoter-driven luciferase expression cassette, resulting in pAdHM56-CMVL2. The shuttle plasmid pHM15-75A-RGD, which cloned oligonucleotide corresponding to GS linker plus RGD peptide into pHM15-75A, was digested with AvrII and ligated with XbaI-digested pAdHM56-CMVL2, resulting in pAdHM56-RGD75-CMVL2. Because XbaI, AvrII, NheI or SpeI produce compatible ends, AvrII, NheI, and SpeI sites as well as the XbaI site can be used for cloning into the XbaI site of pAdHM56. When AvrII, NheI, or SpeI sites of shuttle plasmid are used for cloning into the XbaI site of the vector plasmid, the positive recombinant plasmid lacks a XbaI site. Therefore, generation of the self-ligated plasmid is reduced by the digestion of the ligation sample with XbaI. Luciferase-expressing Ad vectors containing RGD peptide in the C-terminal of pIX with alpha-helical spacer, Ad-RGD(pIX/75)-L2, was produced by transfection of the PacI-digested pAdHM56-RGD75-CMVL2 into 293 cells.

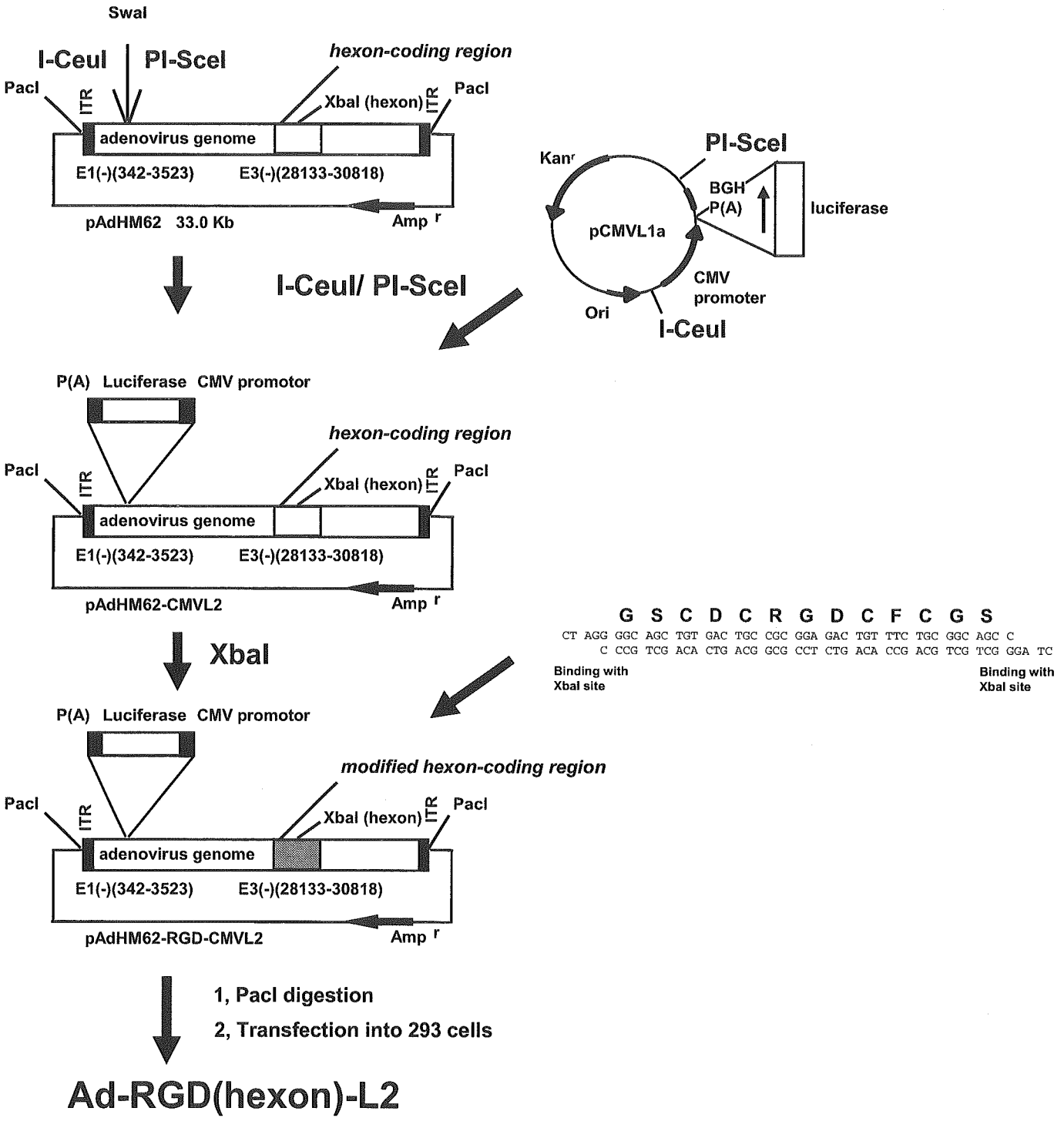

Fig.58 The construction strategy for pIX-modified Ad vectors containing foreign peptides.
pAdHM62-CMVL2 was constructed by the ligation of I-CeuI/PI-SceI-digested pAdHM62 and I-CeuI/PI-SceI-digested pCMVL1a. Then, pAdHM62-CMVL2 was digested with XbaI and ligated with an oligonucleotide corresponding to the linker (GS) and RGD peptide that contains a binding site with XbaI-digested fragment, resulting in pAdHM62-RGD-CMVL2. The oligonucleotide was designed so that the positive recombinant plasmid lacks a XbaI site. Generation of the self-ligated plasmid was reduced by the digestion of the ligation sample by XbaI. Luciferase-expressing Ad vectors containing RGD peptide in the HVR5 of hexon, Ad-RGD(hexon)-L2, was produced by transfection of the PacI-digested pAdHM62-RGD-CMVL2 into 293 cells.

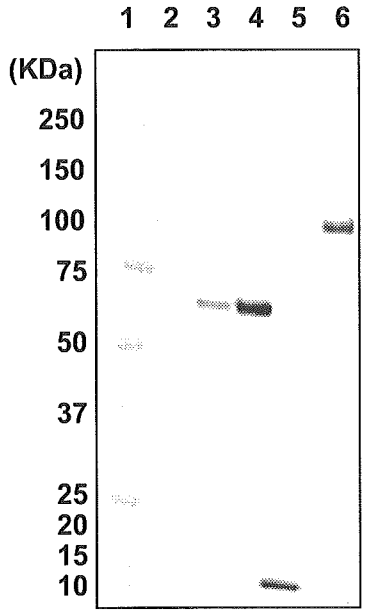

Fig. 59 Western blotting of His tag-modified Ad vectors. Total protein (1 μg) of each vector in 1×sample buffer containing 4% β-mercaptoethanol was loaded on the SDS-PAGE gel after boiling 5 min, followed by electrotransfer to a nitrocellulose membrane. After blocking in Block Ace the filters were incubated with His Tag Monoclonal Antibody (1:1000), followed by incubation in the presence of peroxidase-labeled anti-mouse IgG. The filters were developed by Chemi-Lumi One. The signals were read by using an LAS-3000 machine.
Lane 1: molecular weight marker, Lane 2: Ad-L2, Lane 3: Ad-His(HI)-L2, Lane 4: Ad-His(C)-L2, Lane 5: Ad-His(pIX)-L2, Lane 6: Ad-His(hexon)-L2

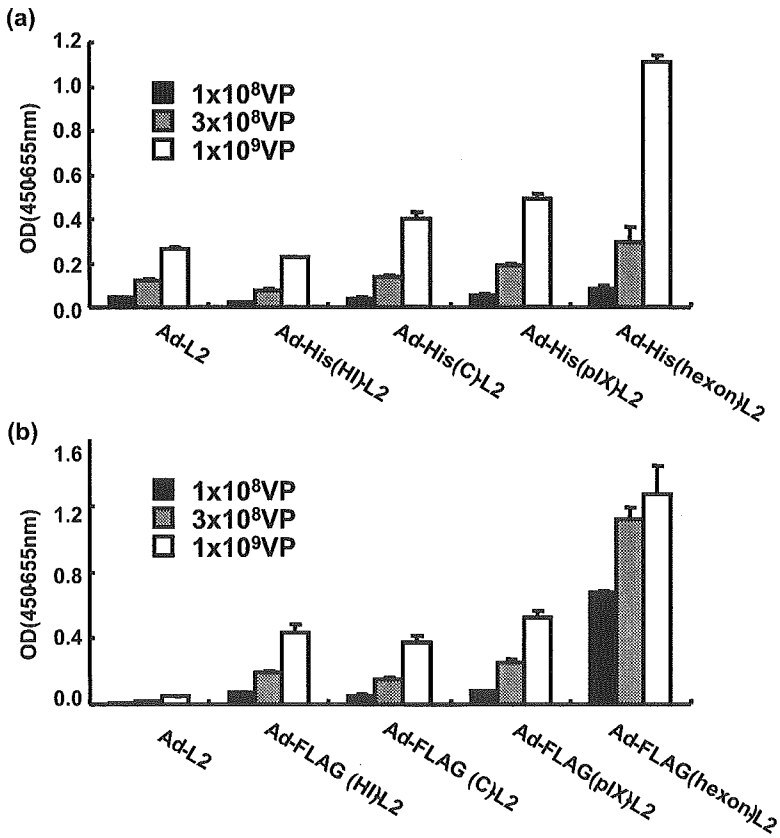

Fig. 60 ELISA of capsid-modified Ad vectors containing (a) His tag or (b) FLAG tag sequence. (a) Ad-L2, Ad-His(HI)-L2, Ad-His(C)-L2, Ad-His(pIX)-L2, or Ad-His(hexon)-L2 (10^{10}VP/well, 10^9VP/well, 10^8 VP/well) were immobilized on 96 well immunoplate. Mouse anti-His tag antibody (primary antibody) was applied and then detected by anti-mouse IgG antibody conjugated with HRP (secondary antibody). Absorbance at 450-655nm was measured by microplate reader. (b) Ad-L2, Ad-FLAG(HI)-L2, Ad-FLAG(C)-L2, Ad-FLAG(pIX)-L2, and Ad-FLAG(hexon)-L2 (10^{10}VP/well, 10^9 VP/well, 10^8 VP/well) were immobilized on 96 well immunoplate. Mouse anti-FLAG tag antibody (primary antibody) was applied and then detected by anti-mouse IgG antibody conjugated with HRP (secondary antibody). Absorbance at 450-655nm was measured by microplate reader. The data are expressed as mean ±S.D. (n=3).

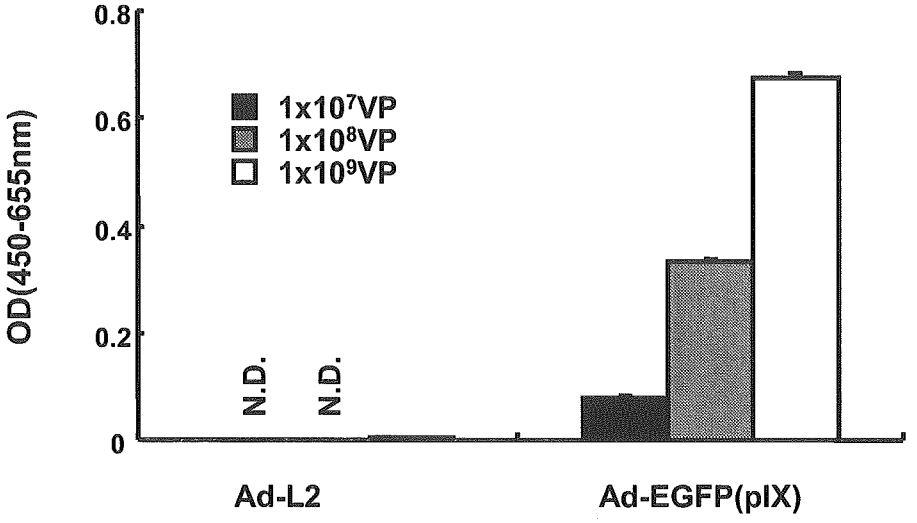

Fig. 61 ELISA of Ad vector containing EGFP at the C-terminus of pIX
Ad-L2 and Ad-EGFP(pIX) (10^{10}VP/well, 10^9 VP/well, 10^8 VP/well) were immobilized on 96 well immunoplate. Primary antibody was applied and then detected by secondary antibody conjugated with HRP. Absorbance at 450-655nm was measured by microplate reader. The data are expressed as mean ± S.D. (n=3). N.D., not detectable

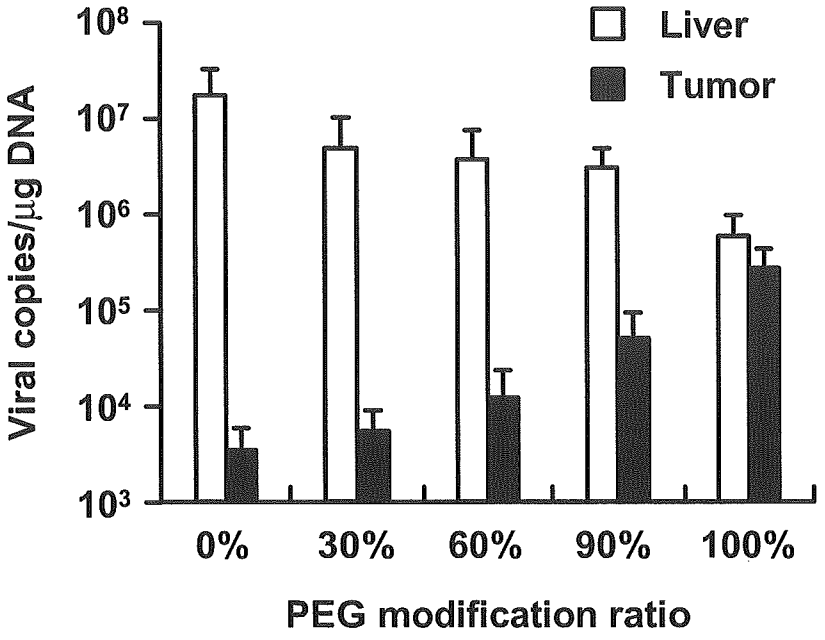

Fig.62 Biodistribution of PEG-Ad vector in Meth-A tumor-bearing mice.
BALB/c mice were intradermally inoculated with Meth-A cells into the flank at 2×10^6 cells/mouse. One week later, these mice were intravenously injected with unmodified Ad-Luc or PEG-Ad-Luc with various modification ratios at 10^{11} VP/mouse. At 6 h after vector injection, tumor and liver were harvested, and then real-time PCR was carried out for detecting viral geneme in DNA isolated from them. Data represent the mean ± SD of results from four mice.

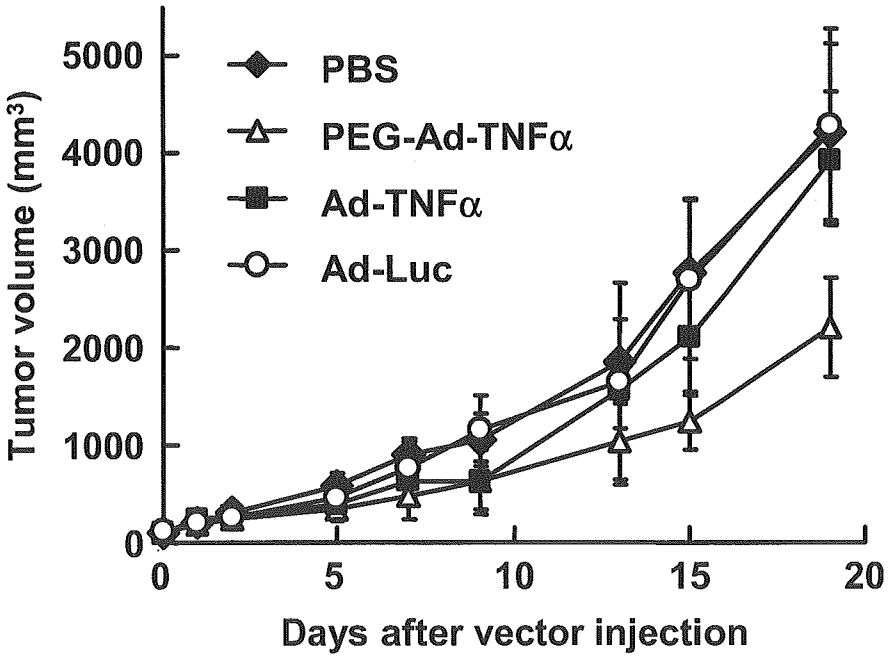

Fig.63 Anti-tumor efficacy of systemic injection of PEG-Ad-TNFα.
Meth-A tumor-bearing BALB/c mice were intravenously injected with Ad-Luc, Ad-TNFα, or PEG-Ad-TNFα (89% modification ratio) at 10^{10} VP/mouse. Tumor volume was measured two or three times per week. Each point represents the mean ± SE from 6-8 mice.