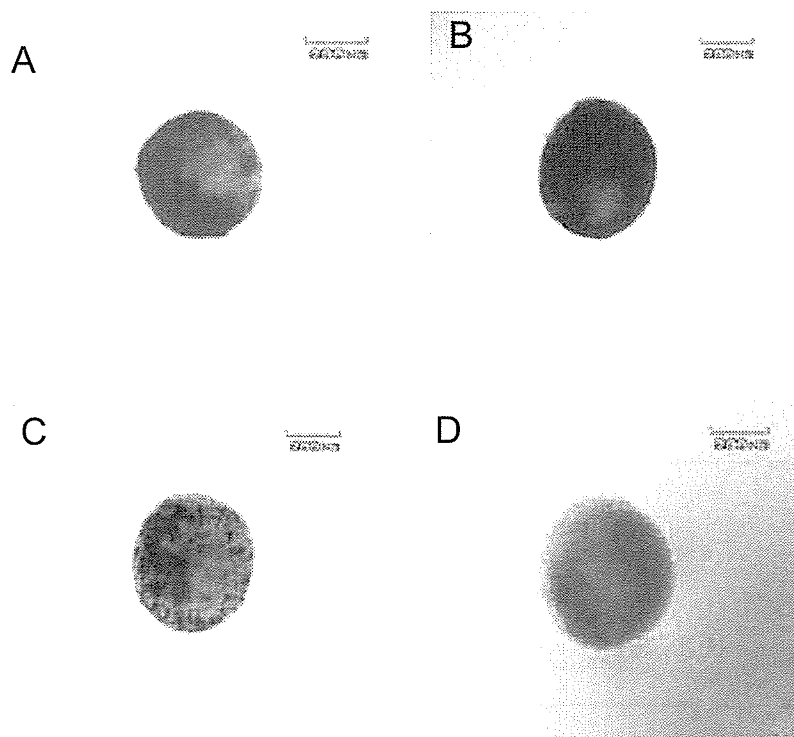


**Fig. 24 X-gal staining of Ad vector-transduced EBs.**
EBs were transduced with 3000 VP/cell Ad-EF-LacZ (A), Ad-CA-LacZ (B), Ad-RSV-LacZ (C), or Ad-CMV-LacZ (D). Two days after infection, X-gal staining was performed.

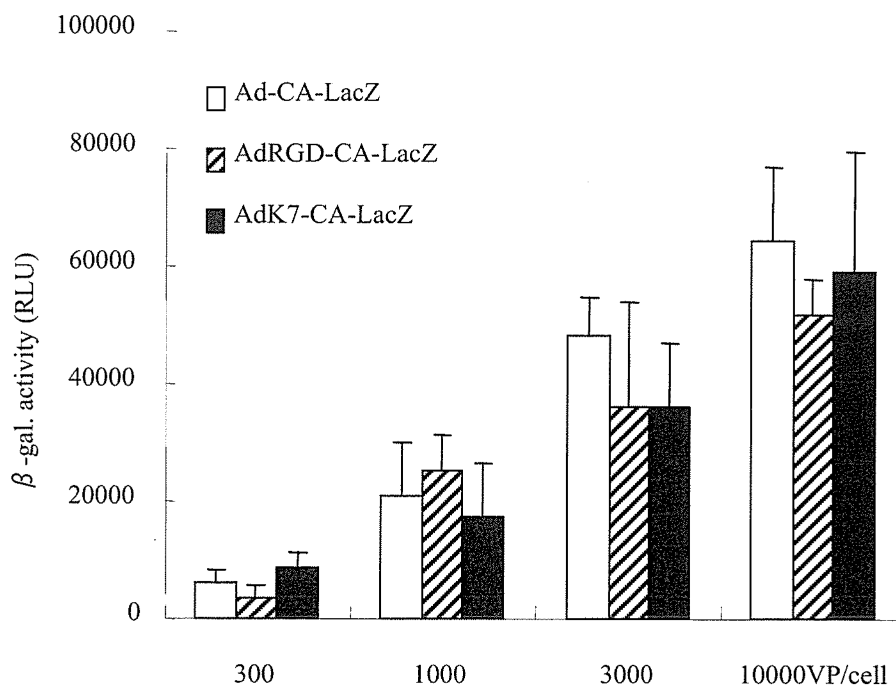


**Fig. 25 Dose dependent LacZ expression by fiber-modified Ad vectors in EBs.**

**Table 4 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 5 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(HI)-L2 | MND-TS----------------RAE ΔRGD motif | 5 | 35 (6 β-repeats) | - NCSLNGGGDA- - 4 a.a. mutation | TEGTAYTNAV- | -DTTSACDCR GDCFCANPSA- | -QEID stop |

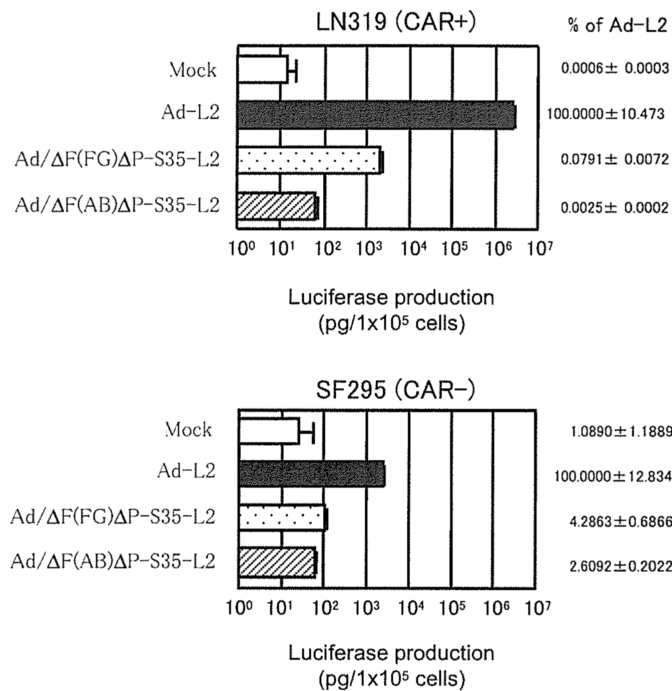


**Fig. 26 Comparison of luciferase production in cultured cells transduced with modified Ad vectors.**
Cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2 or, Ad/ΔF(AB)ΔP-S35-L2, for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean ± S.D. (n = 4).

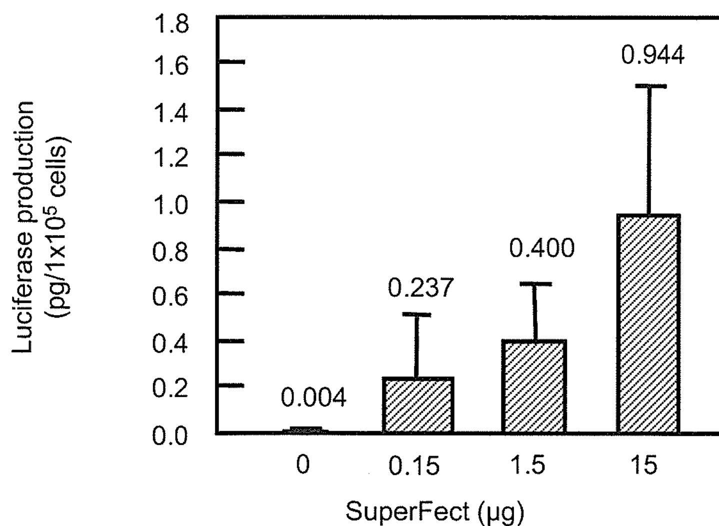


**Fig. 27 Luciferase production in SF295 cells trsnsduced by Ad/ΔF(AB )ΔP-S35-L2 in the presence of Superfect Transduction Reagent.**
Cells were transduced with 30000 VP/cell of Ad/ΔF(AB)ΔP-S35-L2 in the presence of polyamidoamine dendrimer reagent SuperFect for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean ± S.D. (n = 6).

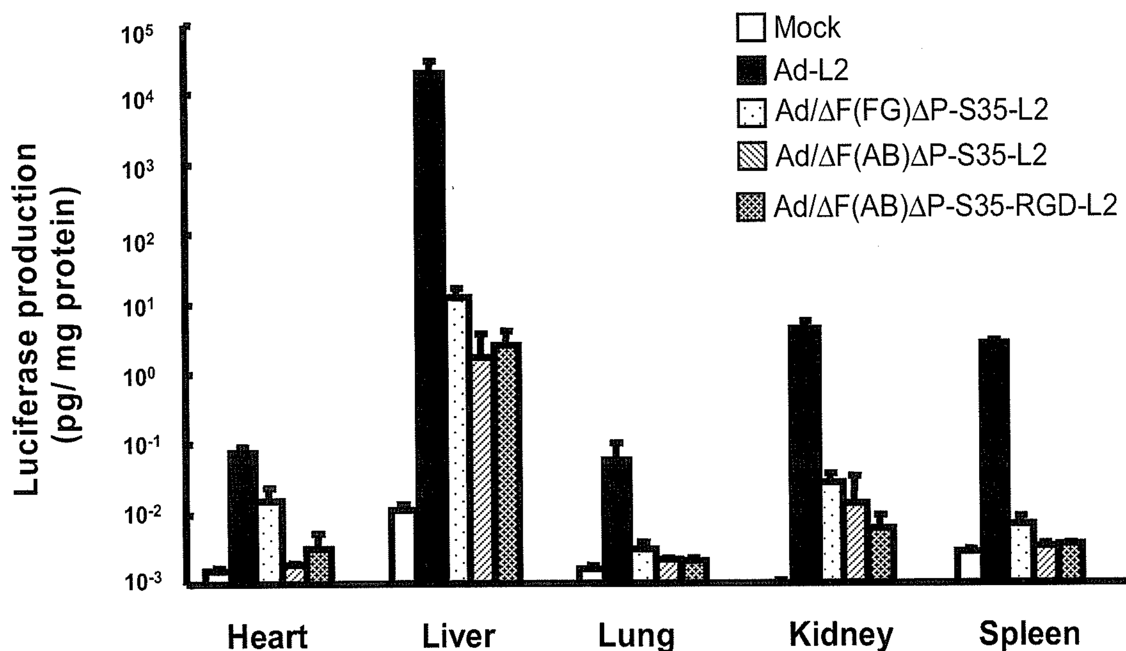


**Fig.28 Luciferase production in mice after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 were intravenously ($3.0 \times 10^{10}$ VP) injected into the mice. Forty-eight 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.

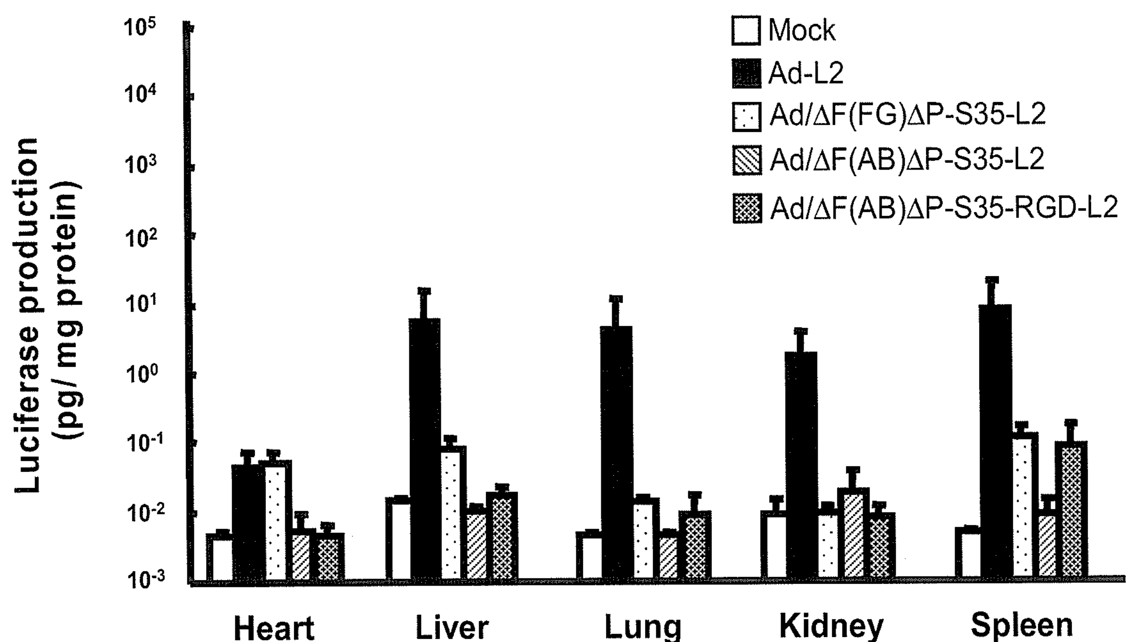


**Fig. 29 Luciferase production in mice after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 were intraperitoneally ($1.0 \times 10^{11}$ VP) injected into the mice. Forty-eight 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.

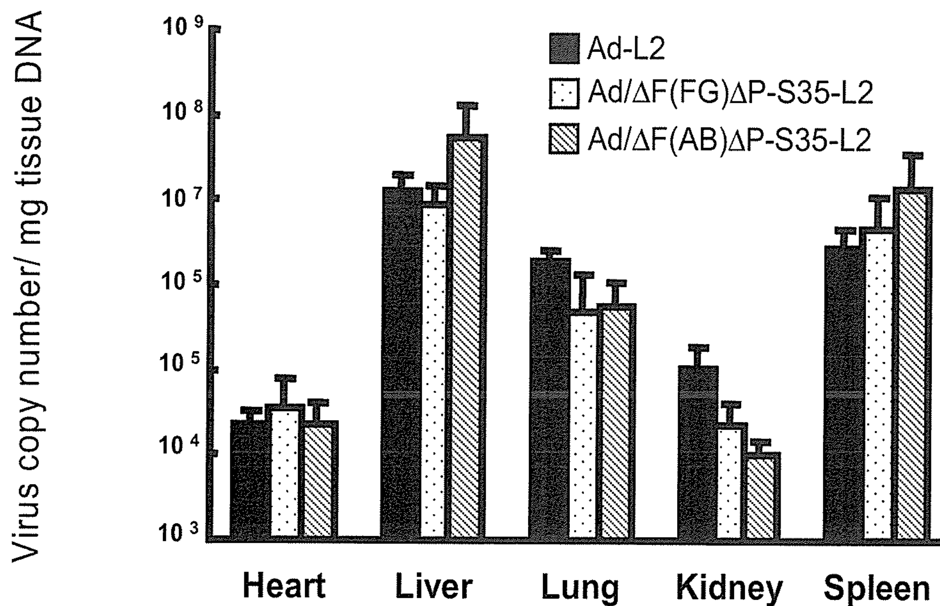


**Fig. 30 Biodistribution of viral DNA after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously ($3.0 \times 10^{10}$ VP) injected into the mice. Three hours later, the heart, lung, liver, kidney, and 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.

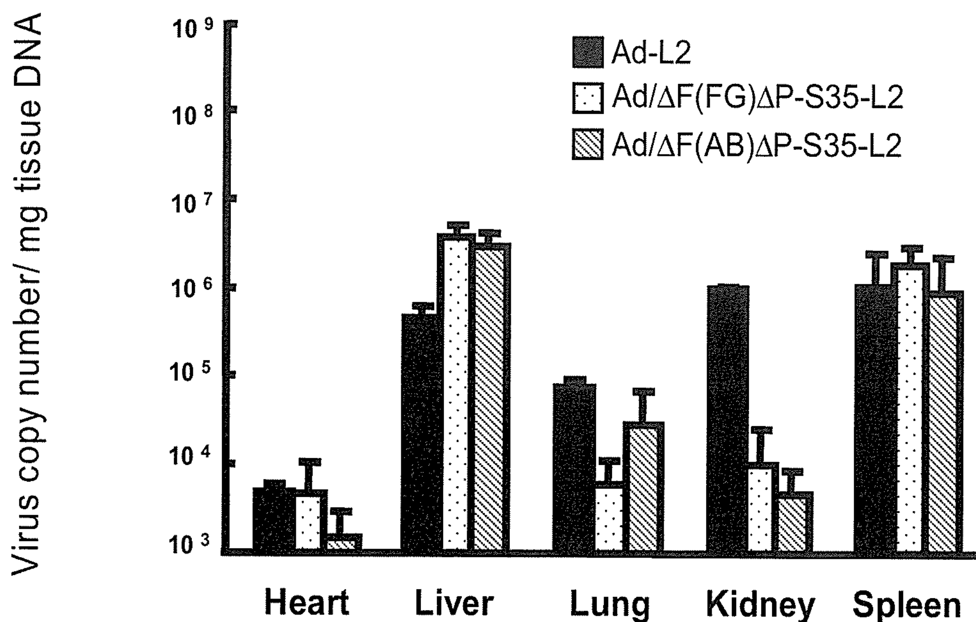


**Fig. 31 Biodistribution of viral DNA after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally ($1.0 \times 10^{11}$ VP) injected into the mice. Three hours later, the heart, lung, liver, kidney, 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.

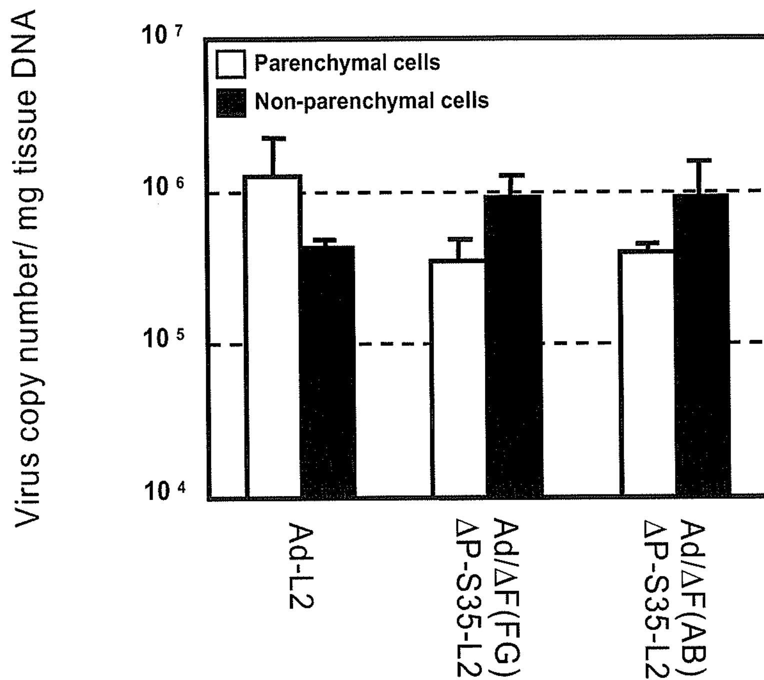


**Fig. 32 Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously ($3.0x10^{10}$ VP) injected in the mice. Collagenase perfusion was performed 3 hours after injection of the Ad vector to separate liver PC and NPC. 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.

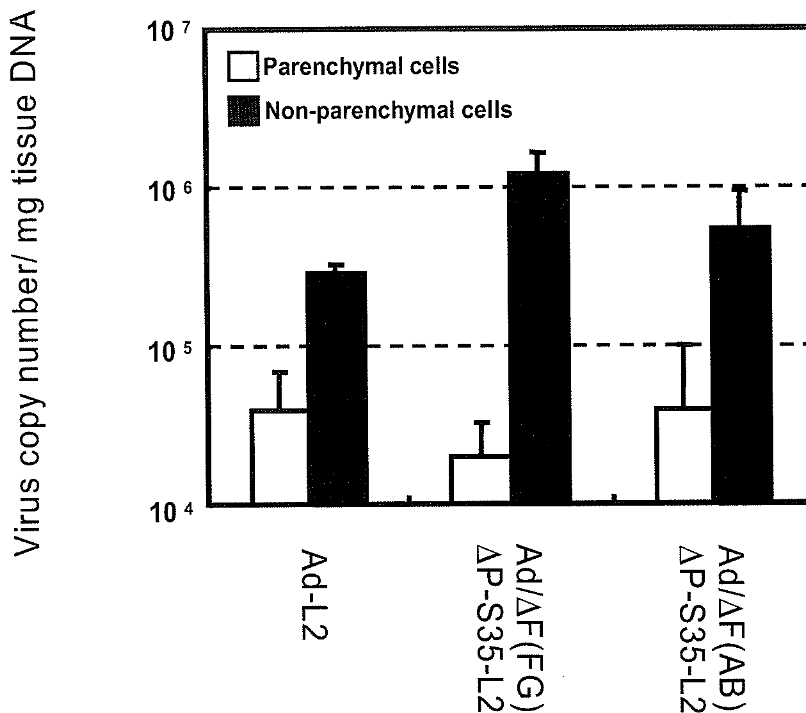


**Fig. 33 Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally ($1.0x10^{11}$ VP) injected in the mice. Collagenase perfusion was performed 3 hours after injection of the Ad vector to separate liver PC and 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.

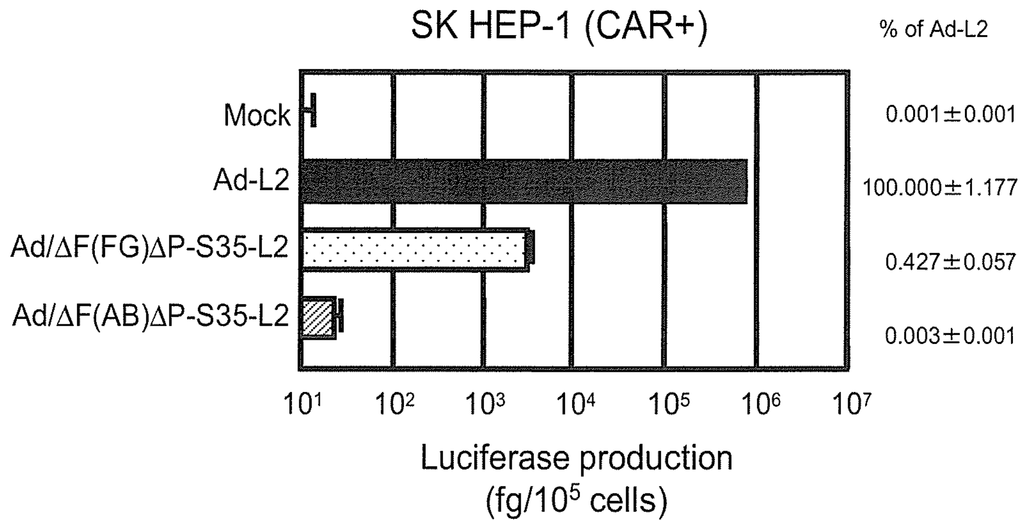


**Fig. 34 Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2.**
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 for 1.5 hours. After culture for 48 hours, luciferase production in the 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.

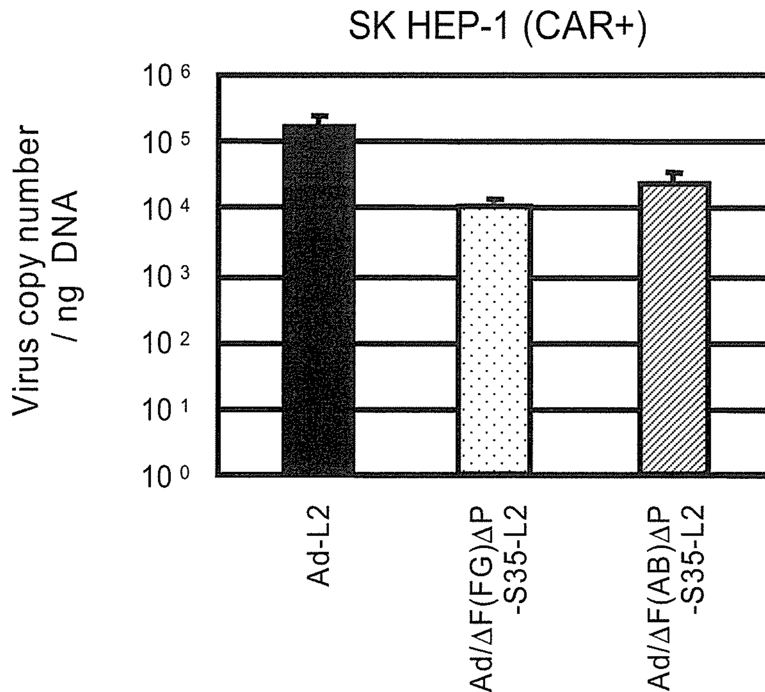


**Fig. 35 The amounts of viral uptake in SK HEP-1 cells.**
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. After culture for 1.5 hours, the cells were washed with PBS, resuspended in 0.05% trypsin–0.5 mM EDTA-PBS solution, and incubated at 37° C for 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).

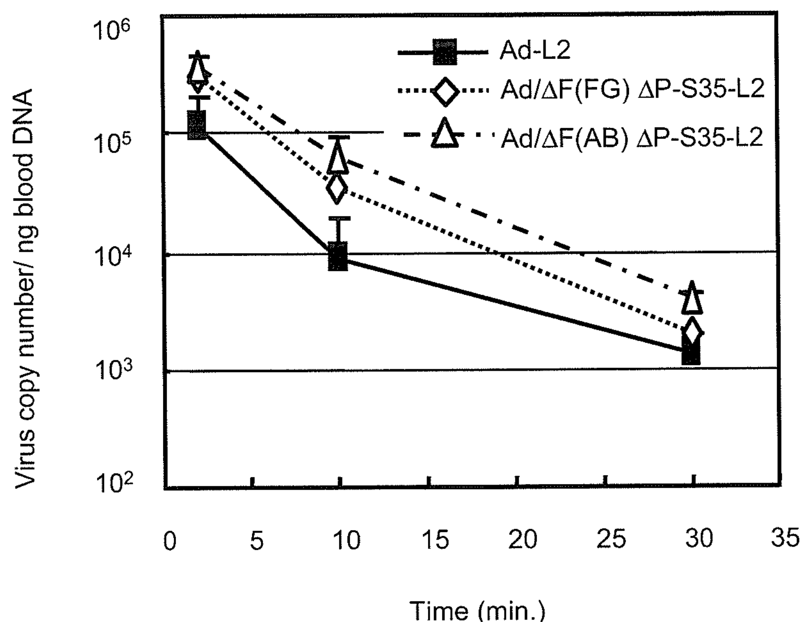


**Fig. 36 Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intravenously ($3.0x10^{10}$ VP) injected, and blood was drawn from the retro-orbital at the indicated times post injection. Total 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.

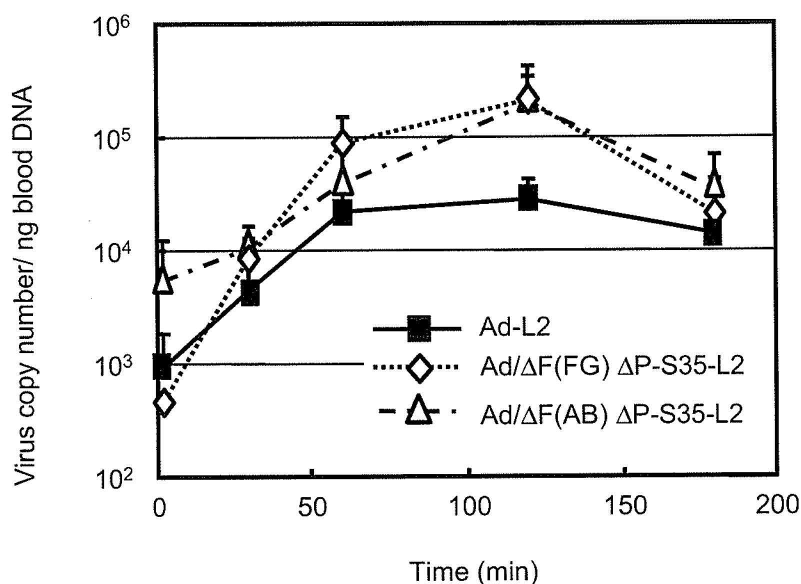


**Fig. 37 Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice.**
Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 were intraperitoneally ($1.0x10^{11}$ VP) injected, and blood was drawn from the retro-orbital at the indicated times post injection. 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.

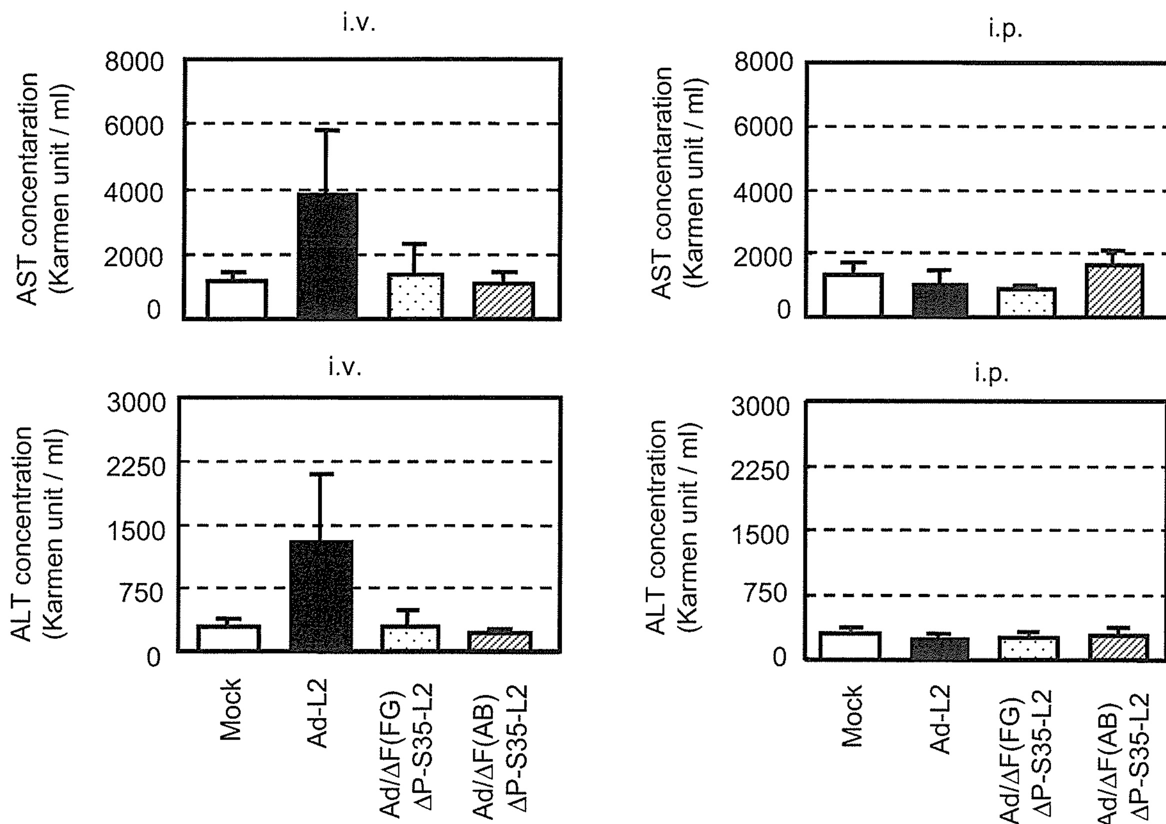


**Fig. 38 Serum enzymes levels after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice.**
Blood samples were collected by inferior vena cave at 48 hours after intravenous ($3.0 \times 10^{11}$ VP) or intraperitoneal ($1.0 \times 10^{11}$ VP) injection of Ad-L2, 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.

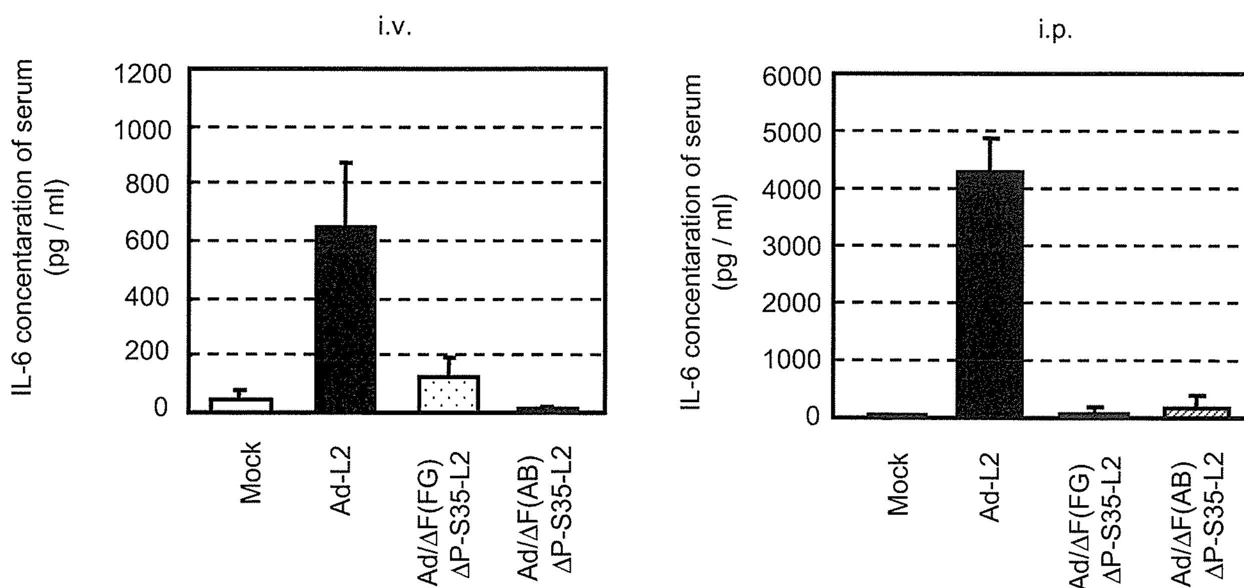


**Fig. 39 Interleukin (IL)-6 levels in serum after the systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice.**
Blood samples were collected by the inferior vena cave at 3 hours after intravenous ($3.0 \times 10^{11}$VP) or intraperitoneal ($1.0 \times 10^{11}$ VP) injection 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.

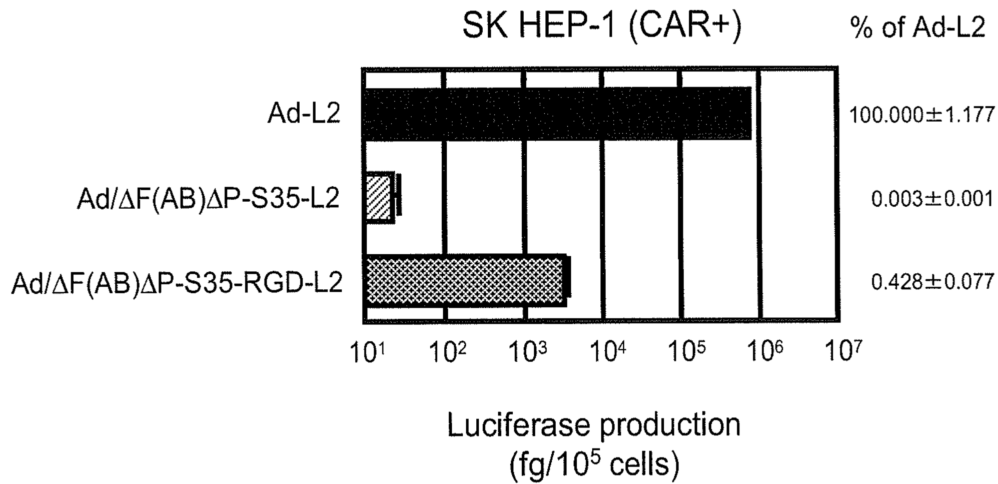


**Fig. 40 Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2.**
SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 for 1.5 hours. After culture for 48 hours, luciferase production 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.

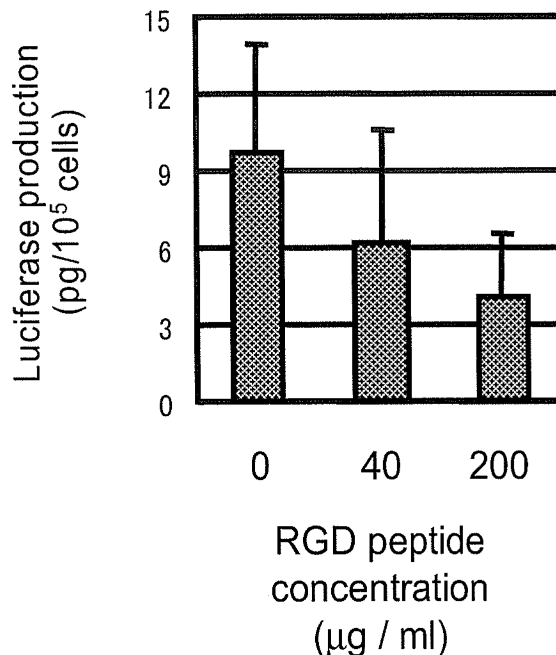


**Fig. 41 Effects of RGD peptide on the transduction efficiency of Ad/ΔF(AB)ΔP-S35-RGD-L2 into SK HEP-1 cells.**
SK HEP-1 cells were preincubated with RGD peptide (0,40, 200 mg/ml) for 10 min. The cells were then transduced with 300 VP/cell of Ad/ΔF(AB)ΔP-S35-RGD-L2 for 0.5 hours in the presence of RGD 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).

**Table 6 Ad vectors used in the present study**

| Ad vectors | vector plasmids | fiber HI loop | fiber C-terminus | pIX | hexon (HVR5) |
|---|---|---|---|---|---|
| Ad-L2 | pAdHM4-L2 | ― | ― | ― | ― |
| Ad-FLAG(HI)-L2 | pAdHM41-FLAG(HI)-L2 | DYKDDDDK | ― | ― | ― |
| Ad-FLAG(C)-L2 | pAdHM41-FLAG(C)-L2 | ― | $(GS)_4$DYKDDDDK | ― | ― |
| Ad-FLAG(pIX)-L2 | pAdHM56-FLAG-L2 | ― | ― | GSDYKDDDDKGS | ― |
| Ad-FLAG(pIX/75)-L2 | pAdHM56-FLAG75-L2 | ― | ― | alpha-helical linker plus $(GS)_4$DYKDDDDK | ― |
| Ad-FLAG(hexon)-L2 | pAdHM62-FLAG-L2 | ― | ― | ― | GSDYKDDDDKGS |
| Ad-His(pIX)-L2 | pAdHM56-His-L2 | ― | ― | GSHHHHHHGS | ― |
| Ad-His(pIX/75)-L2 | pAdHM56-His75-L2 | ― | ― | alpha-helical linker plus $(GS)_4$HHHHHH | ― |
| Ad-RGD(HI)-L2 | pAdHM15-RGD-L2 | ACDCRGDCFCD | ― | ― | ― |
| Ad-RGD(C)-L2 | pAdHM41-RGD-L2 | ― | $(GS)_4$ACDCRGDCFCG | ― | ― |
| Ad-RGD(pIX)-L2 | pAdHM56-RGD-L2 | ― | ― | GSCDCRGDCFCGS | ― |
| Ad-RGD(pIX/75)-L2 | pAdHM56-RGD75-L2 | ― | ― | alpha-helical linker plus $(GS)_4$CDCRGDCFC | ― |
| Ad-RGD(hexon)-L2 | pAdHM62-RGD-L2 | ― | ― | ― | GSCDCRGDCFCGS |
| Ad-EGFP(pIX)-L2 | pAdHM56-pIX/GFP-L2 | ― | ― | EGFP (enhanced green fluorescence protein) | ― |

Each modified Ad vector has additional amino acids derived from unique restriction enzyme sites (Csp45I, ClaI, or XbaI) in each region, but not be described here.

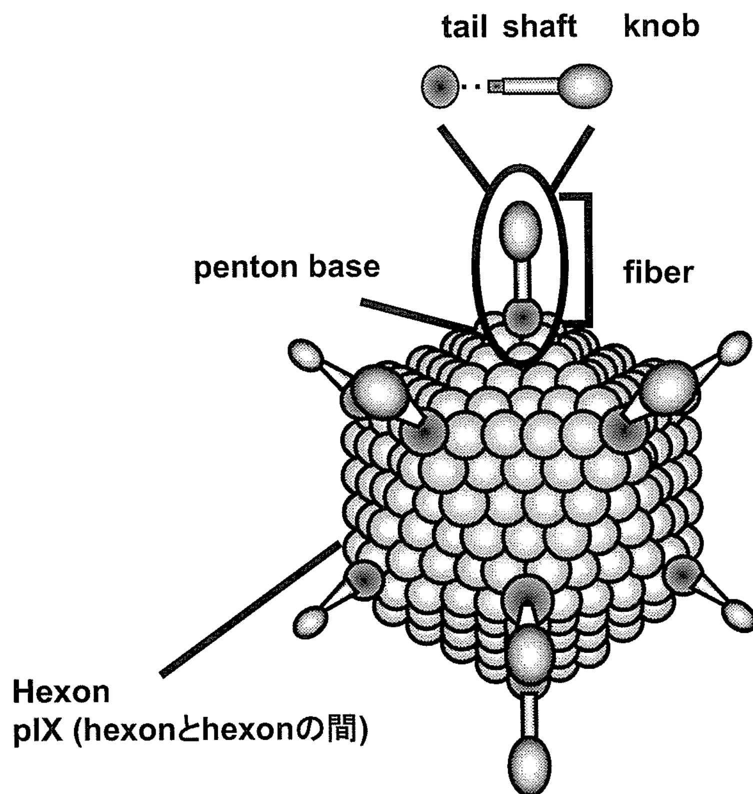


Fig. 42 Structure of adenovirus vector

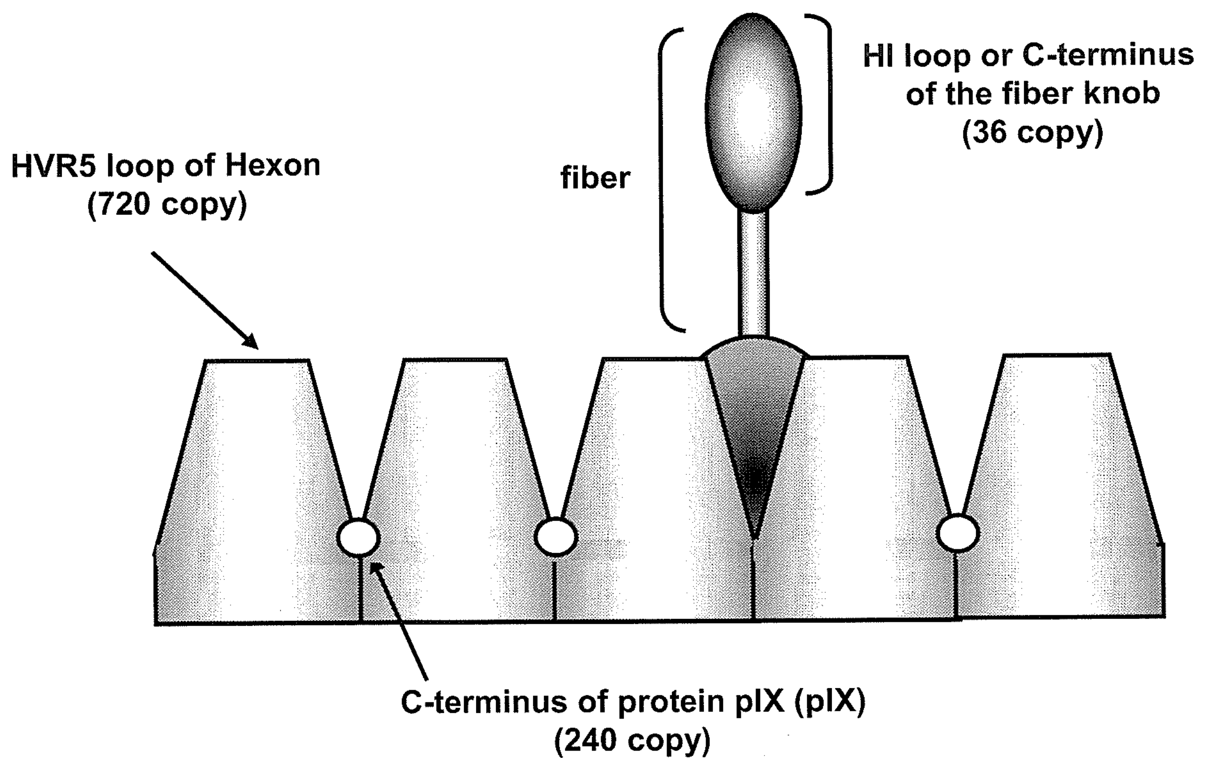


Fig. 43 Candidate location on Ad capsid for the presentation of foreign peptide

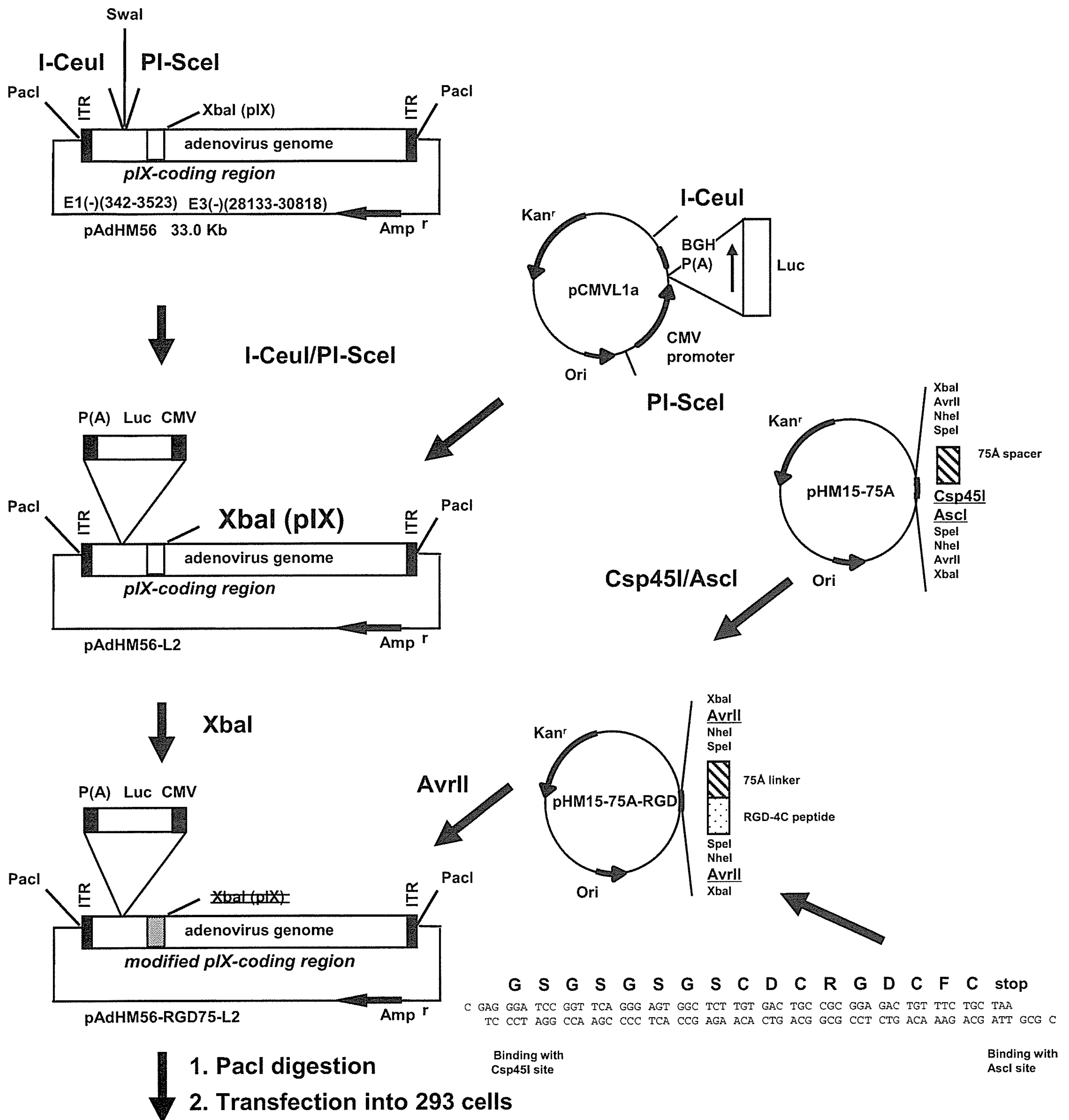


**Fig. 44 The construction strategy for pIX-modified Ad vectors containing foreign peptides.**
pAdHM56 was digested by I-CeuI/PI-SceI and ligated with I-CeuI/PI-SceI-digested pCMVL1a, which contains CMV promoter-driven luciferase expression cassette, resulting in pAdHM56-L2. The shuttle plasmid pHM15-75A-RGD, which cloned oligonucleotide corresponding to GS linker plus RGD peptide into pHM15-75A, was digested with AvrII and ligated with XbaI-digested pAdHM56-L2, resulting in pAdHM56-RGD75-L2. 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 by 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-L2 into 293 cells.

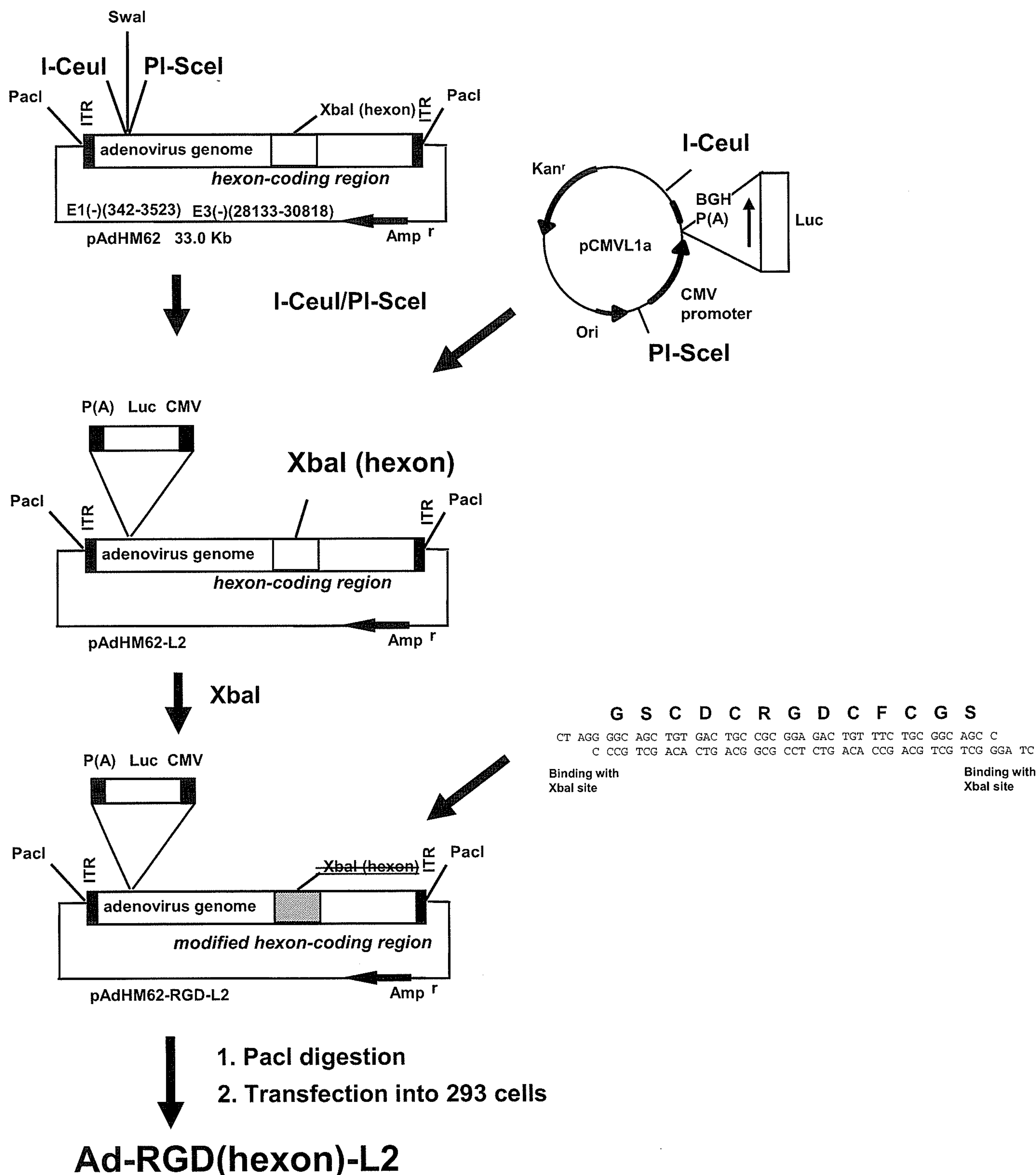


**Fig. 45 The construction strategy for pIX-modified Ad vectors containing foreign peptides.** pAdHM62-L2 was constructed by the ligation of I-CeuI/PI-SceI-digested pAdHM62 and I-CeuI/PI-SceI-digested pCMVL1a. Then, pAdHM62-L2 was digested with XbaI and ligated with an oligonucleotide corresponding to the linker (GS) and RGD peptide that contains a binding site with XbaI-digested fragment, resulted in pAdHM62-RGD-L2. 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-L2 into 293 cells.

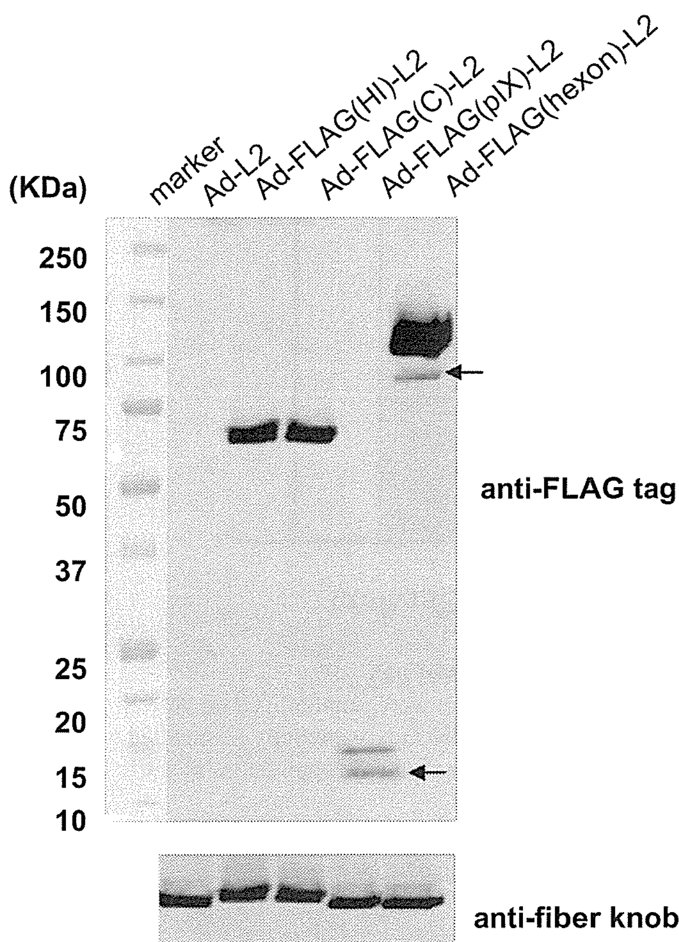


**Fig. 46 Western blotting of FLAG tag-modified Ad vectors.**
The total protein (1μg) of each vector in 1 × sample buffer containing 4% β-mercaptoethanol was separated on a 4-20% SDS-PAGE gel, and the expression of the FLAG tag peptide was analyzed by Western blotting using mouse anti-FLAG tag monoclonal antibody. As a control, the membrane was also incubated with anti-fiber knob antibody. The band of the fiber of Ad-FLAG(HI)-L2 and Ad-FLAG(C)-L2 was higher than that of the other vectors, reflecting the insertion of the FLAG tag into the HI loop or C-terminus of the fiber knob. The extra bands marked with an arrow are proteolytic degradation products.

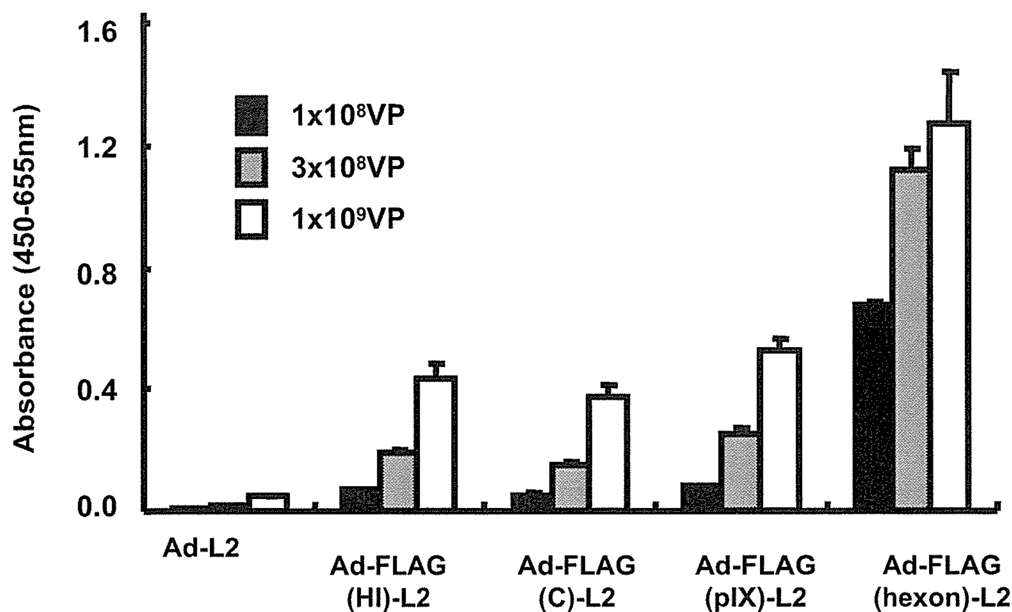


**Fig. 47 ELISA of FLAG tag-modified Ad vectors.**
Ad-L2, Ad-FLAG(HI)-L2, Ad-FLAG(C)-L2, Ad-FLAG(pIX)-L2, or Ad-FLAG(hexon)-L2 ($10^9$ VP/well, $3 \times 10^8$ VP/well, or $1 \times 10^8$ VP/well) were immobilized on a 96-well immunoplate. Mouse FLAG tag antibody was applied and then detected by anti-mouse IgG HRP-linked antibody. Absorbance at 450-655nm was measured by microplate reader. The data are expressed as means ±S.D. (n=3)

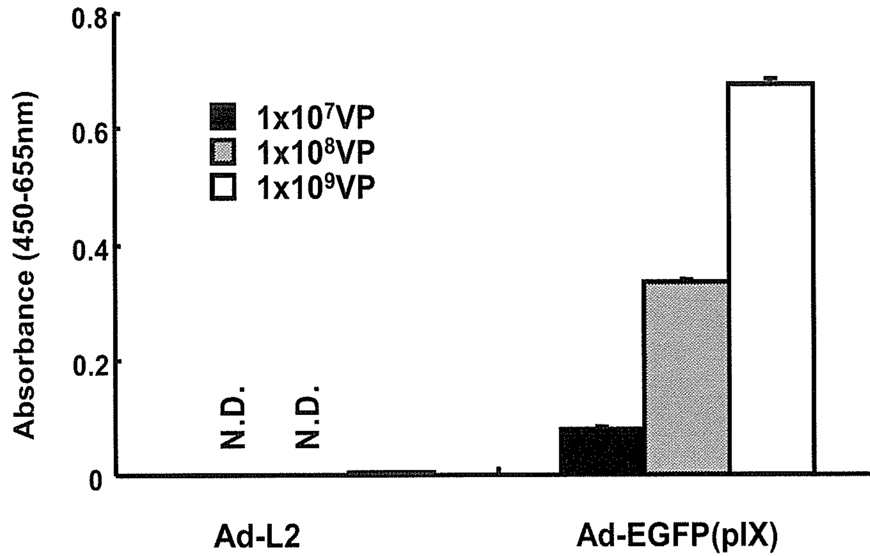


**Fig. 48 ELISA of Ad vector containing EGFP at the C-terminus of pIX**
Ad-L2 and Ad-EGFP(pIX) ($10^{10}$VP/well, $10^9$ VP/well, $10^8$ VP/well) were immobilized on 96 well immunoplate. Primary antibody was applied and then detected by secondary antibody conjugated with HRP. Absorbance at 450-655nm was measured by microplate reader. The data are expressed as mean ± S.D. (n=3). N.D., not detectable

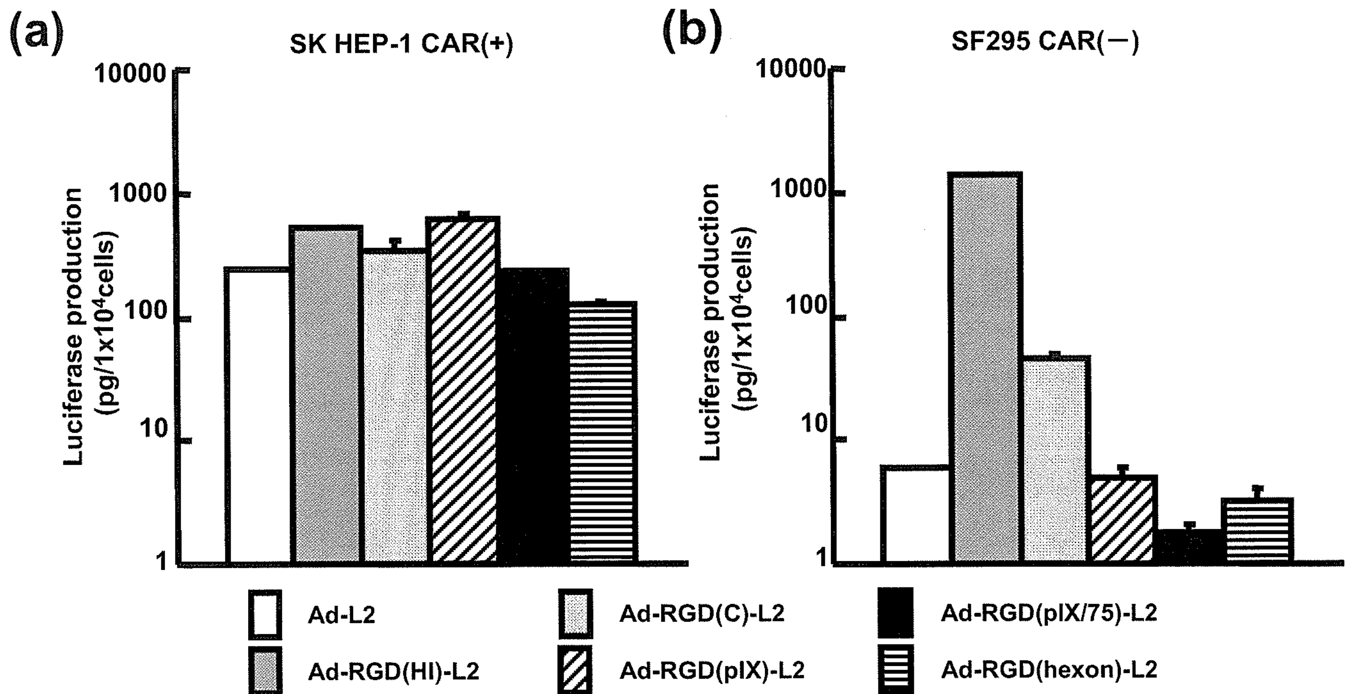


**Fig. 49 Transduction efficiency of RGD-modified Ad vectors.**
SK HEP-1 (a) and SF295 (b) cells were transduced with 3000 VP/cell of Ad-L2, Ad-RGD(HI)-L2, Ad-RGD(C)-L2, Ad-RGD(pIX)-L2, Ad-RGD(pIX/75A)-L2 or Ad-RGD(hexon)-L2 for 1.5 hr, respectively. After culturing for 48 hr, luciferase production was determined. The data are expressed as means ±S.D. (n=3).

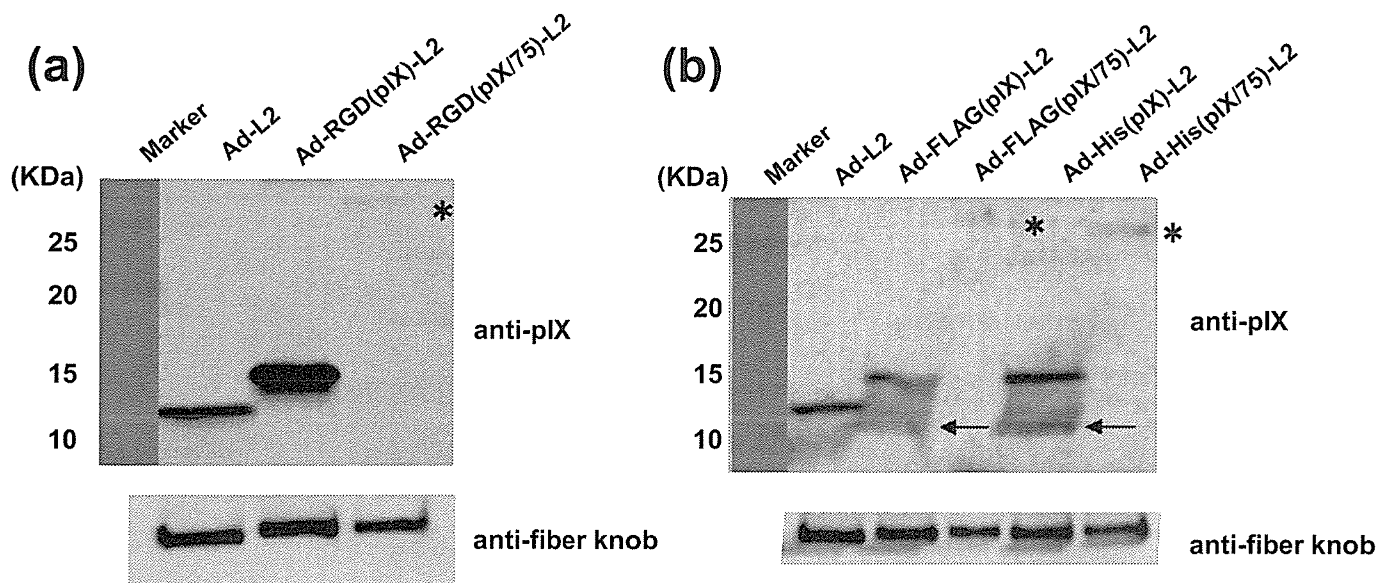




**Fig. 50 Analysis of the incorporation efficiency of modified-pIX.**
Incorporation efficiency of modified-pIX into the virus particles was determined by Western blotting. The total protein (a and b, 1 μg ; c, 10 μg) of each vector in 1 × sample buffer containing 4% β-mercaptoethanol was separated on a 4-20% SDS-PAGE gel, and pIX was detected by the anti-pIX antibody. As a control, the anti-fiber knob antibody was used. (a) pIX-modified Ad vector containing the RGD peptide with or without a 75Å alpha-helical spacer. (b) pIX-modified Ad vector containing the FLAG tag or His tag peptide with or without a 75Å alpha-helical spacer. (c) The pIX-modifed Ad vector containing the RGD, FLAG tag, or His tag peptide with a 75Å alpha-helical spacer. The extra bands marked with an arrow are proteolytic degradation products. The asterisks indicate the band of the modified-pIX with a 75Å alpha-helical spacer.

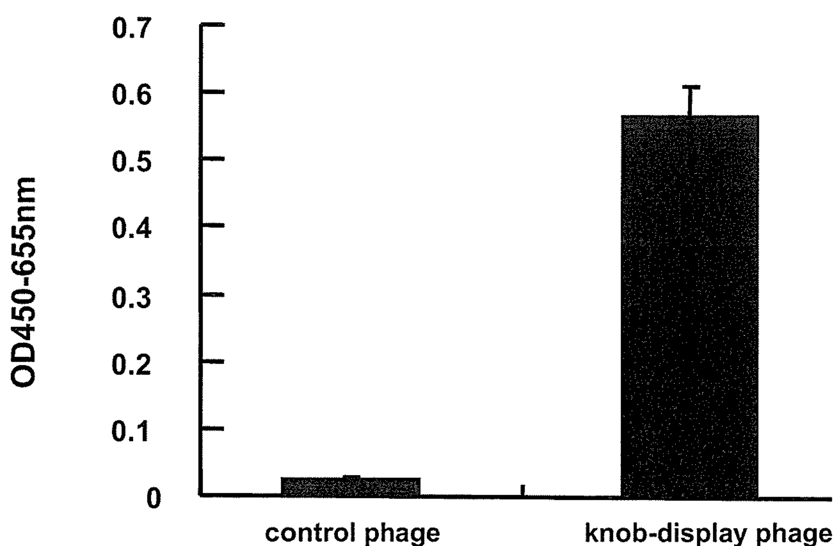


**Fig. 51 Binding assay of fiber knob displayed on g3p of filamentaous phage to the anti-fiber knob antibody**
Anti-fiber knob antibody (diluted 1:1000) were immobilized on a 96-well immunoplate. Each phage ($1x10^{10}$ CFU) was applied and then detected by anti-M13 filamentous phage HRP-linked antibody. Absorbance at 450-655nm was measured by microplate reader. The data are expressed as means ± S.D. (n=3)

**Table 7. Vector plasmid and Ad vector used in the present study**

| Ad vectors | vector plasmids | Fiber: HI loop | Fiber: C-terimnus | pIX |
|---|---|---|---|---|
| Ad-L2 | pAdHM4-L2 | — | — | — |
| Ad-TAT(HI)-L2 | pAdHM41-HITAT-L2 | GRKKRRQRRRPQ | — | — |
| Ad-TAT(C)-L2 | pAdHM41-CTAT-L2 | — | $(GS)_4$GRKKRRQRRRPQ | — |
| Ad-TAT(pIX75)-L2 | pAdHM56-TAT75-L2 | — | — | α-helical spacer plus $(GS)_4$GRKKRRQRRRPQ |
| Ad-RGD(HI)L2 | pAdHM15-RGD-L2 | ACDCRGDCFCD | — | — |
| Ad-K7(C)-L2 | pAdHM41-K7(c)-L2 | — | $(GS)_4$KKKKKKK | — |

Each modified Ad vector has additional amino acids derived from unique restriction enzyme sites (Csp45I, ClaI, or XbaI) in each region, but not be described here. The underlines indicate TAT peptide.

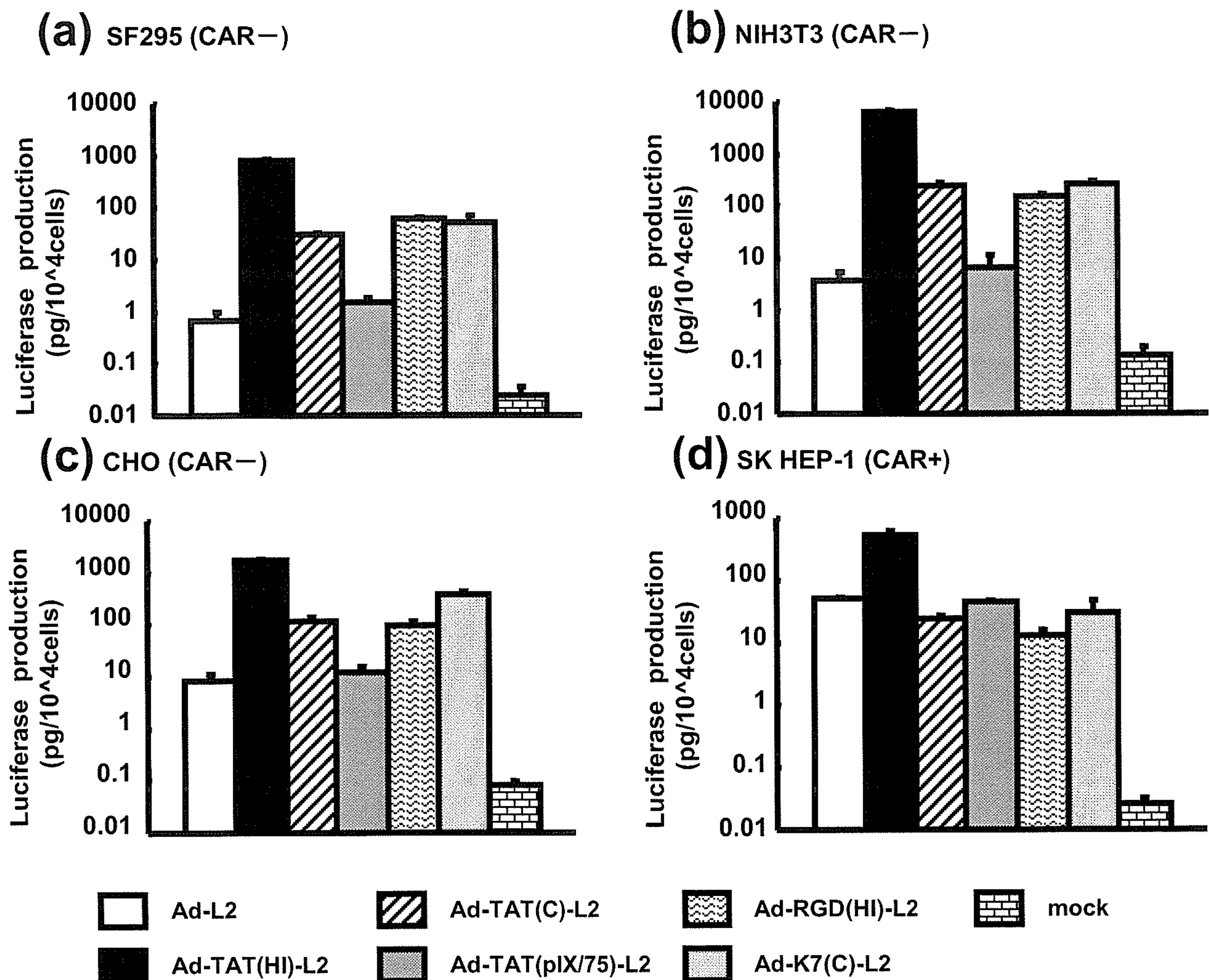


**Fig. 52 Transduction efficiency of TAT-modified Ad vectors.**
SF295 (a), NIH3T3 (b), CHO (c), and SK HEP-1 (d) cells were transduced with Ad-L2, Ad-TAT(HI)-L2, Ad-TAT(C)-L2, Ad-TAT(pIX/75)-L2, Ad-RGD(HI)-L2, or Ad-K7(C)-L2 ((a, b, and c);3000 VP/cell, (a); 300 VP/cell) for 1.5 hr, respectively. After culturing for 48 hr, luciferase production was determined. The data are expressed as means ±S.D. (n=4).

Table 8 Ad vectors and vector plasmids used in the present study

| Ad vectors | Vector plasmid | Fiber | pIX※1 | Hexon※2 |
|---|---|---|---|---|
| Ad-L2 | pAdHM4-CMVL2 | intact | ——— | ——— |
| Ad/ Δ fiber-L2* | pAdHM63-CMVL2 | partial deletion | ——— | ——— |
| Ad/ Δ fiber-L2 | pAdHM63-CMVL2 | complete deletion | ——— | ——— |
| Ad-His(pIX)-L2 | pAdHM56-His-CMVL2 | intact | GSHHHHHHGS | ——— |
| Ad-His(hexon)-L2 | pAdHM62-His-CMVL2 | intact | ——— | GSHHHHHHGS |
| Ad/ Δ fiber-His(pIX)-L2 | pAdHM65-His-CMVL2 | complete deletion | GSHHHHHHGS | ——— |
| Ad/ Δ fiber-His(hexon)-L2 | pAdHM64-His-CMVL2 | complete deletion | ——— | GSHHHHHHGS |

※1 His tag sequence (HHHHHH) was added at the C-terminal of pIX

※2 His tag sequence (HHHHHH) was added at the HVR5 of hexon

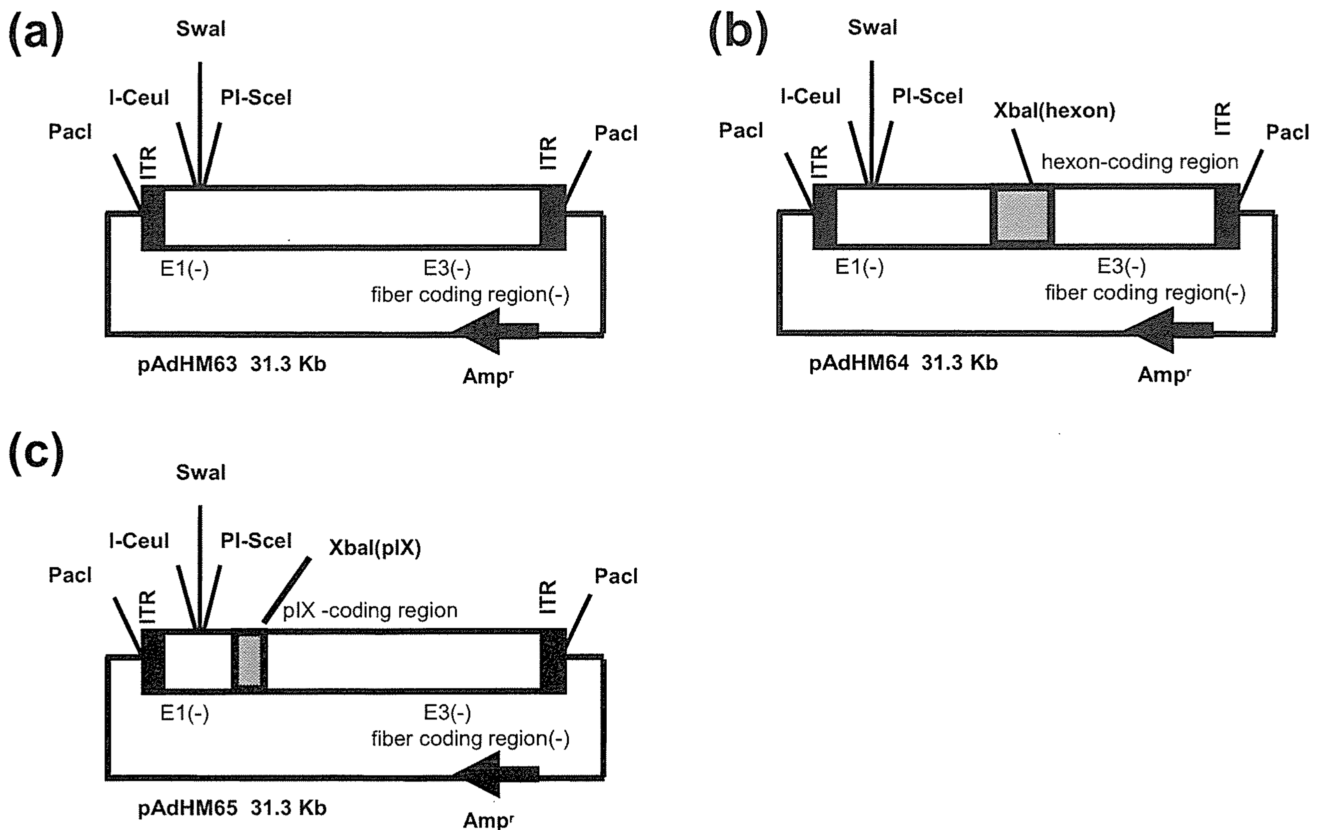


**Fig. 53 The vector plasmids used in this study.**
The vector plasmids for generation of several types of fiber-less Ad vectors, (a) unmodified fiber-less Ad vector, (b) hexon-modified fiber-less Ad vector, and (c) pIX-modified Ad vector.

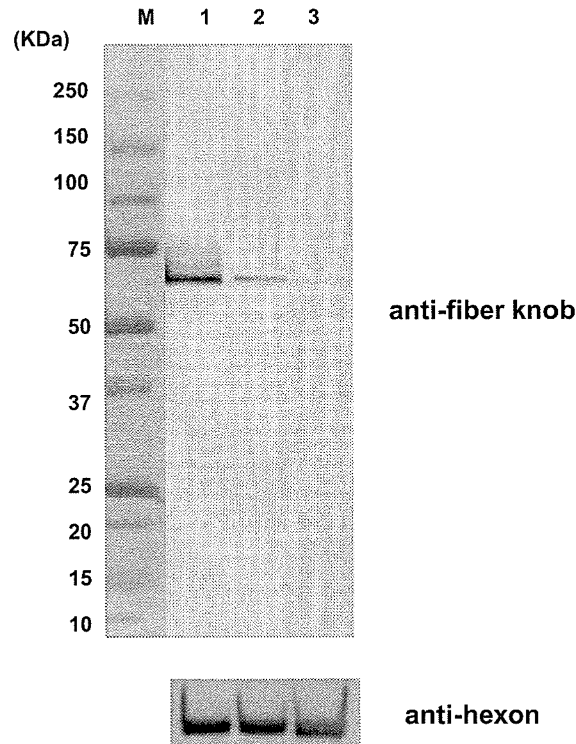


**Fig. 54 Western blotting of fiber-less Ad vectors.**
The total protein (500 ng) of each Ad vector in 1 × sample buffer containing 4% β-mercaptoethanol was separated on a 4-20% SDS-PAGE gel, and the fiber protein or hexon (as a control) was analyzed by Western blotting using a rabbit fiber knob polyclonal antibody or a goat hexon antibody as described in Material and Methods. Lane 1, Ad-L2; Lane 2, Ad/Δfiber-L2*; Lane 3, Ad/Δfiber-L2.

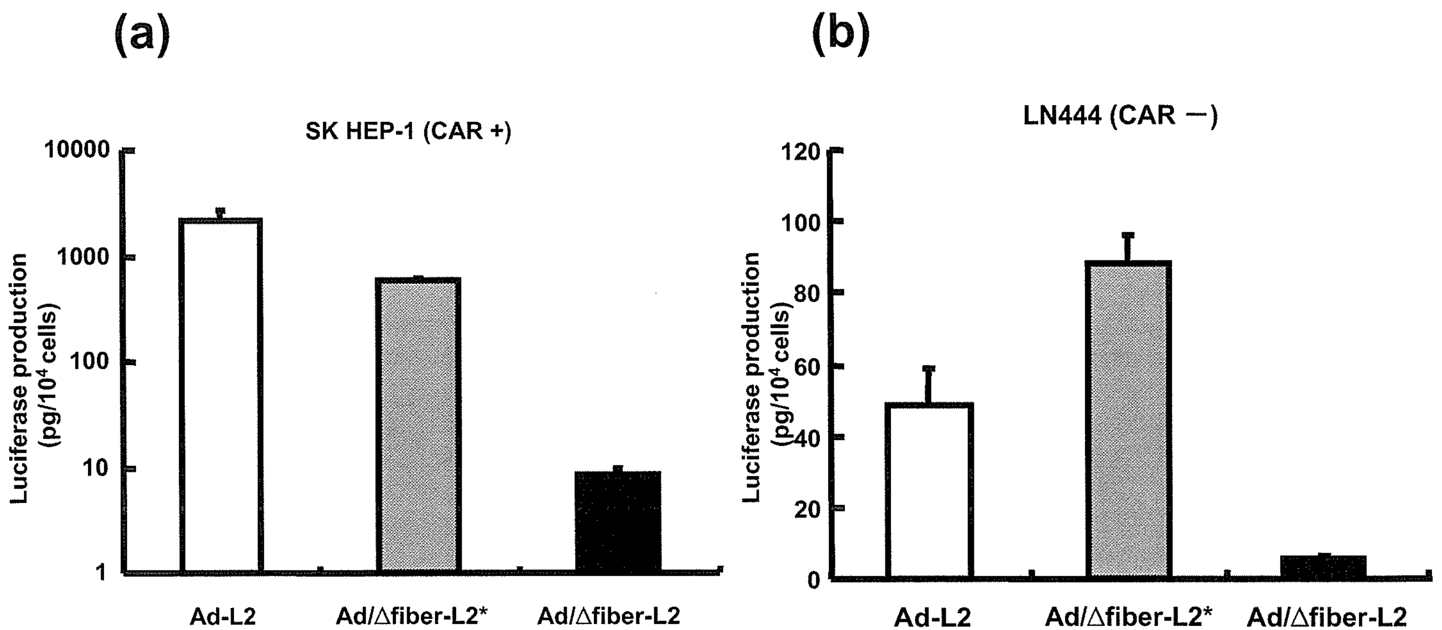


**Fig. 55 Transduction efficiency of fiber-less Ad vectors *in vitro***
SK HEP-1 (a) and LN444 (b) cells were transduced with 3000 VP/cell of Ad-L2, Ad/Δfiber-L2*, or Ad/Δfiber-L2 for 2 hr, respectively. After culturing for 2 days, luciferase production in the cells was determined. The data are expressed as means ±S.D. (n=4).