Table 1 Up-regulated genes stimulated with the conventional Ad vector in mouse peritoneal macrophages.

Affymetrix ID	Gene symbol	GenbankID	Gene name
1416953_at	Ctgf	NM_010217	connective tissue growth factor
1417061_at	Slc40a1	AF226613	solute carrier family 40 (iron-regulated transporter), member 1
1417262_at	Ptgs2	M94967	prostaglandin-endoperoxide synthase 2
1418714_at	Dusp8	NM_008748	dual specificity phosphatase 8
1418930_at	Cxcl10	NM_021274	chemokine (C-X-C motif) ligand 10
1421008_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421009_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421578_at	Ccl4	AF128218	chemokine (C-C motif) ligand 4
1422053_at	Inhba	NM_008380	inhibin beta-A
1422311_a_at	Polr2a	NM_009089	polymerase (RNA) II (DNA directed) polypeptide A
1423252_at	Hdgfrp3	BB291880	hepatoma-derived growth factor, related protein 3
1423620_at	2610528M18Rik	AI891882	RIKEN cDNA 2610528M18 gene
1424339_at	Oasl1	AB067533	2'-5' oligoadenylate synthetase-like 1
1426065_a_at	Trib3	BC012955	tribbles homolog 3 (Drosophila)
1426208_x_at	Plagl1	AF147785	pleiomorphic adenoma gene-like 1
1427381_at	Irg1	L38281	immunoresponsive gene 1
1431153_at	Nrxn3	BB646885	Neurexin III, mRNA (cDNA clone MGC:67582 IMAGE:6406001)
1431591_s_at	G1p2	AK019325	interferon, alpha-inducible protein
1433334_at	1700024P12Rik	AK006313	RIKEN cDNA 1700024P12 gene
1433836_a_at	8430408G22Rik	AV365503	RIKEN cDNA 8430408G22 gene
1434287_at	Agpat5	BG065500	1-acylglycerol-3-phosphate O-acyltransferase 5
1434771_at	0610011F06Rik	BG070867	RIKEN cDNA 0610011F06 gene
1436058_at	Rsad2	BB132493	radical S-adenosyl methionine domain containing 2
1438511_a_at	1190002H23Rik	BB408123	RIKEN cDNA 1190002H23 gene
1440814_x_at	Hs3st2	BB267880	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
1441786_at	---	AW048005	Transcribed locus
1443670_at	2010001J22Rik	AI451392	RIKEN cDNA 2010001J22 gene
1447668_x_at	Efemp2	BB472459	epidermal growth factor-containing fibulin-like extracellular matrix protein 2
1448566_at	Slc40a1	AF226613	solute carrier family 40 (iron-regulated transporter), member 1
1449025_at	Ifit3	NM_010501	interferon-induced protein with tetratricopeptide repeats 3
1449317_at	Cflar	NM_009805	CASP8 and FADD-like apoptosis regulator
1449984_at	Cxcl2	NM_009140	chemokine (C-X-C motif) ligand 2
1450484_a_at	Tyki	AK004595	thymidylate kinase family LPS-inducible member
1450498_at	Mthfr	BB586520	5,10-methylenetetrahydrofolate reductase
1450783_at	Ifit1	NM_008331	interferon-induced protein with tetratricopeptide repeats 1
1452278_a_at	Hace1	BG922448	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1
1453409_at	Cgrrf1	AK004156	cell growth regulator with ring finger domain 1
1455324_at	LOC433022	BQ176176	hypothetical LOC433022
1456186_at	---	AV261345	Transcribed locus
1459818_x_at	Zfp261	BB527320	zinc finger protein 261
1460067_at	Ccr2	BB324415	chemokine (C-C motif) receptor 2

Table 2 Up-regulated genes stimulated with RGD-type Ad vector in mouse peritoneal macrophages.

Affymetrix ID	Gene symbol	GenbankID	Gene name
1415922_s_at	Marcksl1	NM_010807	MARCKS-like 1
1416067_at	Ifrd1	NM_013562	interferon-related developmental regulator 1
1416268_at	Ets2	BC005486	E26 avian leukemia oncogene 2, 3' domain
1416700_at	Rnd3	BC009002	Rho family GTPase 3
1417371_at	Peli1	BC016515	pellino 1
1417372_a_at	Peli1	BC016515	pellino 1
1418126_at	Ccl5	NM_013653	chemokine (C-C motif) ligand 5
1418293_at	Ifit2	NM_008332	interferon-induced protein with tetratricopeptide repeats 2
1418930_at	Cxcl10	NM_021274	chemokine (C-X-C motif) ligand 10
1418936_at	Maff	BC022952	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F
1419132_at	Tlr2	NM_011905	toll-like receptor 2
1419561_at	Ccl3	NM_011337	chemokine (C-C motif) ligand 3
1419607_at	Tnf	NM_013693	tumor necrosis factor
1419676_at	Mx2	BC007127	myxovirus (influenza virus) resistance 2
1419879_s_at	Trim25	AA960166	tripartite motif protein 25
1420330_at	Clec4e	NM_019948	C-type lectin domain family 4, member e
1420376_a_at	H3f3b	NM_008211	H3 histone, family 3B
1421008_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421009_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421269_at	Ugcg	AA591863	UDP-glucose ceramide glucosyltransferase
1421578_at	Ccl4	AF128218	chemokine (C-C motif) ligand 4
1421640_a_at	Tank	NM_011529	TRAF family member-associated Nf-kappa B activator
1422095_a_at	Tyki	AK004595	thymidylate kinase family LPS-inducible member
1422305_at	Ifnb1	NM_010510	interferon beta 1, fibroblast
1423605_a_at	Mdm2	AK004719	transformed mouse 3T3 cell double minute 2
1424067_at	Icam1	BC008626	intercellular adhesion molecule
1424339_at	Oasl1	AB067533	2'-5' oligoadenylate synthetase-like 1
1425974_a_at	Trim25	D63902	tripartite motif protein 25
1426063_a_at	Gem	U10551	GTP binding protein (gene overexpressed in skeletal muscle)
1426208_x_at	Plagl1	AF147785	pleiomorphic adenoma gene-like 1
1426276_at	Ifih1	AY075132	interferon induced with helicase C domain 1
1426721_s_at	Tiparp	BB707122	TCDD-inducible poly(ADP-ribose) polymerase
1427005_at	Plk2	BM234765	polo-like kinase 2 (Drosophila)
1427381_at	Irg1	L38281	immunoresponsive gene 1
1427718_a_at	Mdm2	X58876	transformed mouse 3T3 cell double minute 2
1427736_a_at	Ccrl2	AJ318863	chemokine (C-C motif) receptor-like 2
1428750_at	Cdc42ep2	BF453885	CDC42 effector protein (Rho GTPase binding) 2
1429060_at	Malat1	AK020483	metastasis associated lung adenocarcinoma transcript 1
1431591_s_at	G1p2	AK019325	interferon, alpha-inducible protein
1433699_at	Tnfaip3	BM241351	tumor necrosis factor, alpha-induced protein 3
1434070_at	Jag1	AV359819	jagged 1
1434401_at	Zcchc2	BM224914	zinc finger, CCHC domain containing 2
1435133_at	Ugcg	BF682223	UDP-glucose ceramide glucosyltransferase
1435458_at	Pim1	AI323550	proviral integration site 1
1436058_at	Rsad2	BB132493	radical S-adenosyl methionine domain containing 2
1436080_at	AW011738	BB528213	expressed sequence AW011738
1436202_at	Malat1	AI853644	metastasis associated lung adenocarcinoma transcript 1
1438157_s_at	Nfkbia	BB096843	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
1447685_x_at	Ets2	BB416434	E26 avian leukemia oncogene 2, 3' domain
1448830_at	Dusp1	NM_013642	dual specificity phosphatase 1
1449025_at	Ifit3	NM_010501	interferon-induced protein with tetratricopeptide repeats 3
1449134_s_at	Spic	NM_011461	Spi-C transcription factor (Spi-1/PU.1 related)
1449317_at	Cflar	NM_009805	CASP8 and FADD-like apoptosis regulator
1449984_at	Cxcl2	NM_009140	chemokine (C-X-C motif) ligand 2
1450165_at	Slfn2	NM_011408	schlafen 2
1450484_a_at	Tyki	AK004595	thymidylate kinase family LPS-inducible member
1450783_at	Ifit1	NM_008331	interferon-induced protein with tetratricopeptide repeats 1
1451567_a_at	Ifi203	BC008167	interferon activated gene 203
1452534_a_at	Hmgb2	X67668	high mobility group box 2
1453119_at	Otud1	BB530087	OTU domain containing 1
1454617_at	Arrdc3	BG072824	arrestin domain containing 3
1454742_at	Rasgef1b	BB003229	RasGEF domain family, member 1B

Table 3 Up-regulated genes by both conventional and RGD-type Ad vectors in mouse peritoneal macrophages.

Affymetrix ID	Gene symbol	GenbankID	Gene name
1418930_at	Cxcl10	NM_021274	chemokine (C-X-C motif) ligand 10
1421008_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421009_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421578_at	Ccl4	AF128218	chemokine (C-C motif) ligand 4
1424339_at	Oasl1	AB067533	2'-5' oligoadenylate synthetase-like 1
1426208_x_at	Plagl1	AF147785	pleiomorphic adenoma gene-like 1
1427381_at	Irg1	L38281	immunoresponsive gene 1
1431591_s_at	G1p2	AK019325	interferon, alpha-inducible protein
1436058_at	Rsad2	BB132493	radical S-adenosyl methionine domain containing 2
1438511_a_at	1190002H23Rik	BB408123	RIKEN cDNA 1190002H23 gene
1449025_at	Ifit3	NM_010501	interferon-induced protein with tetratricopeptide repeats 3
1449317_at	Cflar	NM_009805	CASP8 and FADD-like apoptosis regulator
1449984_at	Cxcl2	NM_009140	chemokine (C-X-C motif) ligand 2
1450484_a_at	Tyki	AK004595	thymidylate kinase family LPS-inducible member

研究成果の刊行に関する一覧表

雑誌

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Adenovirus Vector-Mediated Gene Transfer into Stem Cells

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Abstract: Stem cells, including embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), are defined by their capacity for self-renewal and multilineage differentiation. Efficient gene transfer into stem cells is essential for the basic research in developmental biology and for therapeutic applications in gene-modified regenerative medicine. Adenovirus (Ad) vectors, based on Ad type 5, can efficiently and transiently introduce the exogenous gene into many cell types via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSCs and HSCs, cannot be efficiently transduced with conventional Ad vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified Ad vectors and an Ad vector based on another serotype of Ad have been developed. Here, we review the advances in the development of Ad vectors suitable for stem cells and discuss their application in basic biology and clinical medicine.

Keywords: Adenovirus; stem cell; gene therapy; regenerative medicine; review

Introduction

Adenovirus (Ad) is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm. At least 51 serotypes of human Ad have been identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) and Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, the penton base, and the fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Figure 1A). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: the tail, the shaft, and the knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber to the Ad capsid through association with the penton base.[1] The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation.[2] By extending the knob away from the virion, the shaft facilitates its interaction with the host

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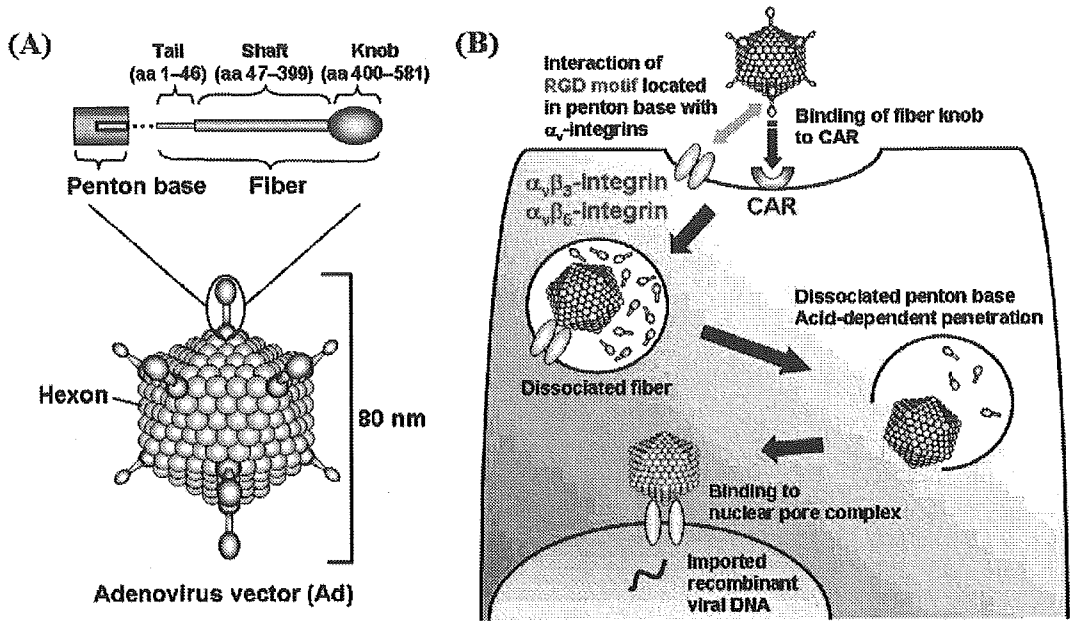

Figure 1. Structure and gene transduction pathway of the Ad vector. (A) The double-stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices. The fiber is composed of the tail, shaft, and knob domain. (B) The Ad vector binds to CAR following internalization in the cells and releases the viral DNA into the nuclei.

receptor.[1] The trimeric subunits of the carboxyl C-terminal knob domain are responsible for binding to the host's primary cellular receptor.[3,4]

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2), and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITRs) at the end of the viral genome function as replication origins. The E1A gene is the first transcription unit to be activated shortly after infection and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus propagated in E1-transcomplementing cell lines, such as 293,[5] 911,[6] or PER.C6 cells.[7] The E3 region-encoded proteins modulate the host defense but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted[8] and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer,[9] E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes.[8]

The coxsackievirus and adenovirus receptor (CAR), which is a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A and C–F.[10–12] The entry of Ad5 into cells is initiated by the

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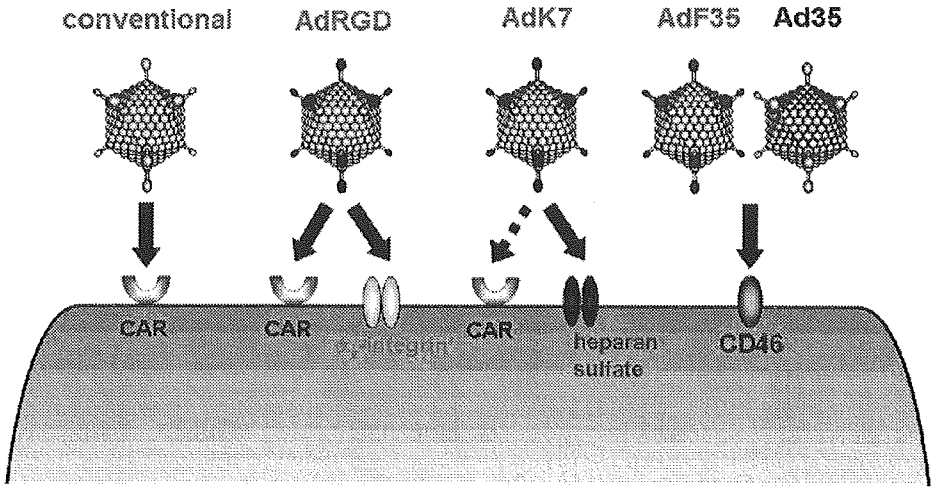

Figure 2. Characteristics of gene delivery by various types of Ad vectors. The conventional Ad vector infects via CAR. The AdRGD vector contains a RGD peptide motif in the HI loop of the fiber knob and infects via αv integrin as well as CAR. The AdK7 vector contains a polylysine peptide in the C-terminus of the fiber knob and infects via heparan sulfate as well as CAR. It is uncertain whether the AdK7 vector infects via CAR. The Ad35 and AdF35 vectors, which contain a fiber protein derived from the Ad5 fiber tail and the Ad35 fiber knob and shaft, infect via CD46.

attachment of fiber on the surface of the capsid to the CAR on the cell surface (Figure 2). The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid for the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$, aids in the internalization of Ad5 into the cell.[13−15] Furthermore, heparan sulfate glycosaminoglycans have also been reported to serve as primary attachment sites for Ad2 and Ad5.[16] The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation (Figure 1B). Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base.[17] The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus (Figure 1B). During this process, the terminal protein plays a crucial role in translocating the Ad genome into the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with the translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell.[18]

Although Ad vectors mediate extremely high transduction efficiency, gene transfer with Ad vectors is less efficient in some kinds of cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), dendritic cells, T cells, smooth muscle cells, skeletal muscle cells, and others because of the scarcity of CAR on their cell surfaces. Modification of the Ad fiber proteins has been used to successfully overcome this obstacle.[19,20] One is constructed by the addition of foreign peptides to the HI loop or C-terminus of the fiber knob of an Ad vector.[21−25] Enhanced gene transfer has been

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reported, on the basis of the use of mutant fiber proteins containing either an RGD peptide (AdRGD vector)[21-26] or a stretch of lysine residues [K7 (KKKKKKK) peptide] (AdK7 vector),[21,25,26] which target αv integrins or heparin sulfates on the cell surface, respectively (Figure 2). Altered vector tropism was reported with the substitution of the Ad5 fiber protein with that of Ad belonging to subgroup B, such as Ad types 3, 11, and 35.[27-31] These fiber-modified Ad vectors infect cells via CD46, CD80, and CD86, which have recently been identified as the cellular receptors of Ad belonging to subgroup B (Figure 2).[32-36] Mercier et al. described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing junctional adhesion molecule 1.[37]

Several groups have developed an Ad vector from the entire Ad type 35 (Ad35) or Ad type 11 (Ad11) and have demonstrated that the Ad35 and Ad11 vectors exhibit higher transduction efficiencies into hematopoietic progenitor and dendritic cells compared with the conventional Ad5 vector (Figure 2).[38-43] As other approaches to changing the vector tropism, modification of the Ad vector with the antibodies, the fusion protein composed of CAR and the cell binding domain, cationic lipid, or macromolecules has been reported.[19,20] Here, we highlight the genetic manipulations of stem cells by the Ad vector and fiber-modified Ad vector for basic research and therapeutic usage. Recent advances in Ad vector-mediated gene transfer into stem cells, such as embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), will be discussed.

Gene Transfer into Stem Cells

Stem cells are defined as cells which possess the abilities of self-renewal and multilineage differentiation. Stem cells have been isolated from a wide variety of tissues, and in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals can differentiate into specialized cells with a phenotype distinct from that of their precursor. Gene therapy applications that target stem

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cells offer great potential for the treatment of many kinds of diseases. Despite this promise, clinical success has been limited by poor rates of gene transfer and poor levels of gene expression. Therefore, an efficient gene delivery system needs to be developed for stem cell gene therapy.

Gene Transfer into Embryonic Stem Cells. ES cells are pluripotent cell lines derived from the inner cell mass of the developing blastocyst.[44−46] With the establishment of human ES (hES) cells, they have been used as a renewable source of transplantable tissue-specific stem cells.[47−49] ES cells differentiate spontaneously in vitro in a random manner into a mixture of differentiated cells. The protocols for the differentiation of ES cells enriched for a specific lineage have been developed in both the mouse ES (mES)[50,51] cell and hES cell systems,[52,53] although the differentiated cells are still relatively heterogeneous. Therefore, further research is needed to allow controlled directed differentiation of ES cells into pure cultures of committed cells. One of the most powerful techniques for controlled differentiation is genetic manipulation. Electroporation methods,[54] retroviral vectors,[55,56] lentiviral vectors,[57−59] and a supertransfection method based on a replication system using the polyoma replication origin and large T antigen[60] have been used for exogenous gene expression in ES cells, although lentiviral vectors have been shown to be ineffective at expressing exogenous genes in mES cells, but not in hES cells.[57,59] In plasmid-based systems such as eletroporation and supertransfection methods, stable cell lines are generated by selection using a drug resistance gene. All these methods mediate long-term constitutive gene expression, although a long-term gene expression system such as that as described above may be problematic for use in therapeutic applications, because the gene is continuously expressed even after cell differentiation. There is thus a need for efficient vector systems for transient expression.

The Ad vector has been thought to be inappropriate for gene transfer into ES cells.[61] It has been reported that the retrovirus vector preferentially transduced ES cells, while the Ad vector containing the cytomegalovirus (CMV) promoter preferentially transduced embryonic fibroblasts as feeders in the ES culture.[61] However, it was found that the

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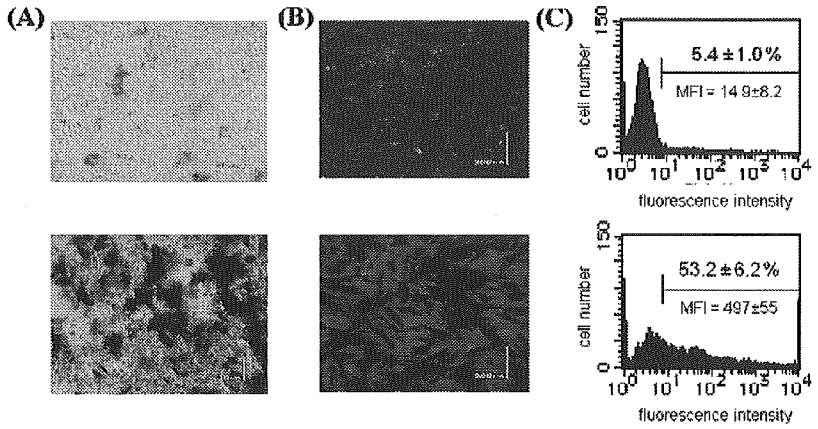

Figure 3. Improved transduction efficiency in the stem cells by the optimized Ad vectors. (A) mES cells were transduced with the LacZ-expressing conventional Ad5 vector containing the CMV promoter (top) or EF-1α promoter (bottom). (B) hMSCs were transduced with the LacZ-expressing Ad5 vector (top) or AdK7 vector (bottom). Both vectors have the CA promoter. (C) Human CD34+ cells were transduced with the GFP-expressing Ad5 vector (top) or Ad35 vector (bottom). Both vectors have the CMV promoter. MFI is the mean fluorescence intensity.

choice of a promoter is important for the efficient expression of exogenous genes in mES cells (Figure 3A). In the transient expression system using a cationic liposome−plasmid complex, the EF-1α (elongation factor 1α) and CA promoter (β-actin promoter/CMV enhancer) were shown to be highly active in mES cells while the CMV promoter was inactive.[62] More recently, we reported that the Ad vector containing the EF-1α or CA promoter has mediated the efficient expression of the reporter gene in mES cells, whereas the Ad vector containing the Rous sarcoma virus (RSV) or the CMV promoter has exhibited little expression.[63] Because CAR was highly expressed in mES cells but not in feeder cells,[63] the Ad vector could be a powerful tool for the genetic manipulation of mES cells when an appropriate promoter is used. To date, although we have no idea about the expression of CAR in hES cells, the Ad vector was reported to mediate the reporter gene expression in both mES cells and hES cells,[64] suggesting that hES cells may also express CAR on their cell surfaces.

As a result of the comparative analysis of mES cells transduced with various types of fiber-modified Ad vectors, the conventional Ad vector exhibited highly efficient and specific transduction, whereas the AdRGD and AdK7 vectors transduced mES cells and feeder cells (embryonic fibroblasts) to the same degree.[63] Therefore, the conventional Ad vector containing the EF-1α or CA promoter should be appropriate when only ES cells are transduced. In turn, the AdRGD or AdK7 vector is adequate when both ES cells and feeder cells are transduced.

The conventional Ad vector containing the EF-1α promoter was applied for the transduction of functional genes. It is well-known that the activation of signal transducer and activator of transcription 3 (STAT3) is essential for leukemia inhibitory factor (LIF)-mediated mES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation.[65] It is also known that transcription factor Nanog maintains the pluripotency of mES cells in a manner that is independent of LIF/STAT3 signaling.[66,67] Ad vector-mediated STAT3F (STAT3 dominant-negative mutant) transduction strongly promoted mES cells to cell differentiation into three germ layers without any nonspecific toxicity.[63] The co-infection of the STAT3F-expressing Ad vector and the Nanog-expressing Ad vector showed that the differentiation suppressing ability of Nanog negated the differentiation promoting function of STAT3F and that mES cells maintained their undifferentiated state.[63] Thus, the differentiation of ES cells could be controlled by the transduction of differentiation-key regulator genes with the Ad vector. ES cells might differentiate into hematopoietic progenitor, pancreatic β cells, or neurons by the Ad vector-mediated introduction of HoxB4,[68,69] Pax4,[70] or nuclear receptor-related I,[71] respectively.

Gene Transfer into Mesenchymal Stem Cells. MSCs, which reside within the stromal compartment of bone

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marrow, were first identified as bone-forming progenitor cells from rat marrow.[72] MSCs represent a very small fraction, 0.001−0.01% of the total population of nucleated cells in marrow.[73] They have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage, and muscle. Recently, it has been reported that MSCs can differentiate into other lineages, such as neurons,[74] hepatocytes,[75] and insulin-producing cells.[76] Therefore, MSCs have attracted a great deal of interest because of their potential use in regenerative medicine and tissue engineering. To date, MSCs could be differentiated in vitro into proper lineages via a change in the culture conditions.[77] Another method for the in vitro differentiation is to genetically modify MSCs.[78,79] Although exogenous gene transfer into human MSCs (hMSCs) has been reported by using a conventional Ad vector, its transduction efficiency is quite low due to the scarcity of CAR.[80,81] Therefore, hMSCs have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors.[80,81] Fiber-modified Ad vectors have been applied for hMSCs to improve the transduction efficiency.[79,82,83] hMSCs infected with the AdRGD vector containing the BMP2 gene produced larger amounts of BMP2 than cells infected with the conventional Ad vector and efficiently differentiated into the osteogenic lineage.[82,83] Highly efficient transduction of hMSCs was achieved with tropism-modified Ad5 vectors carrying fiber shaft domains and knobs of different serotypes of Ad, such as Ad16, Ad35, or Ad50.[84] In a systematic comparison with various types of fiber-modified Ad vectors, the AdK7 vector is the most efficient for hMSCs and exhibited a 460-fold higher transduction efficiency than the conventional Ad vector.[79] The AdRGD vector or the Ad vector containing the Ad35 fiber (AdF35) exhibits a 16 or 130 times higher transduction efficiency, respectively, than the conventional Ad vector.[79] hMSCs are found to express CD46, which is the primary receptor for Ad35, but not CAR.[79] In conclusion, the AdK7 or AdF35 vector is the most appropriate for the transduction of hMSCs (Figure 3B).

Gene Transfer into Hematopoietic Stem Cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and multilineage differentiation into all mature blood cells.[85] HSCs comprise only 0.01% of the whole bone marrow, the tissue in which they primarily reside.[86] Efficient transduction into HSCs would afford the opportunity to treat a number of hematopoietic disorders and would be a powerful tool for

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the study of the proliferation, differentiation, and trafficking of HSCs. Although the retroviral and lentiviral transduction of HSCs to achieve stable gene expression has been established,[87,88] stable expression is not always desirable. For example, stable expression of MDR1 gene results in HSC expansion but can cause leukemia upon transplantation to recipient mice.[89] As the Ad vector mediates the exogenous gene expression transiently, this vehicle can be safe for gene therapy. However, the application of conventional Ad vectors for the transduction into human CD34+ cells, which contain a population of HSCs, has been limited because CAR is not expressed at sufficient levels in human CD34+ cells.[90,91] It has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[90,92] We showed that the Ad35 vector, which is composed from the whole Ad35, achieved higher levels of transduction efficiency in human bone marrow CD34+ cells than both conventional Ad5 vectors and AdF35 vectors.[39,93] The expression level of reporter genes in the CD34+ cells transduced with the Ad35 vector was 12−76 and 1.4−3 times higher than that in the cells transduced with the Ad5 and AdF35 vectors, respectively.[39] The transduction efficiency of the Ad35 vector was slightly higher than that of the AdF35 vector, although the reason remains unknown. CD46 is ubiquitously expressed in almost all human cells, including human cord blood CD34+ cells.[94] Therefore, human CD34+ cells would be considered to be a suitable target for the Ad35 vector (Figure 3C). As a result of the systematic comparison of promoters with Ad35 vectors, significantly higher transduction efficiencies were achieved with the EF-1α, CA, and CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi) promoters. In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature subsets.[93] In mice, a population of mouse bone marrow highly enriched for HSC, called side population (SP) cells, has been reported to be transduced with the conventional Ad5 vector.[95] This suggests that pure mouse HSCs might express CAR on the cell surface. Further studies are needed to clarify this. The Ad vector-mediated transduction of hematopoietic regulator genes, such as HoxB4,[68,69] Bmi-1,[96] or SCL/Tal-1,[97] into HSCs may be effective for therapeutic use such as HSC expansion, although the Ad vector expressing HoxB4 was unsuccessful because of unexpected HSC differentiation due to its high transduction efficiency.[98]

Conclusions

We have reviewed recent advances in the development of improved Ad vectors for stem cells. Ad vectors have advantages over other viral vectors: the high transduction efficiency, the ease of vector preparation, and the transient expression ability. By the Ad vector-mediated introduction of a differentiation master regulator gene, we could control the differentiation of stem cells. These technical advances should greatly facilitate the analysis of gene function in the stem cells as well as the therapeutic applications of gene-modified stem cells.

Abbreviations Used

ES, embryonic stem; mES, mouse ES; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; Ad, adenovirus; CAR, coxsackievirus and adenovirus receptor; Ad5, Ad serotype 5; ITR, inverted terminal repeats; Ad35, Ad serotype 35; AdRGD vector, Ad vector containing the RGD

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peptide; Ad K7 vector, Ad vector containing a polylysine stretch; hES, human ES; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; STAT3F, dominant-negative mutant of STAT3; hMSCs, human MSCs; BMP2, bone morphogenetic protein 2; AdF35, Ad vector containing the Ad35 fiber.

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Modified Adenoviral Vectors Ablated for Coxsackievirus–Adenovirus Receptor, α_V Integrin, and Heparan Sulfate Binding Reduce *In Vivo* Tissue Transduction and Toxicity

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ABSTRACT

Coxsackievirus and adenovirus receptor (CAR), α_V integrins, and heparan sulfate glycosaminoglycans (HSGs) are the tropism determinants of adenoviral (Ad) vectors *in vivo*. For the development of a targeted Ad vector, its broad tropism needs to be blocked (or reduced). We have previously developed Ad vectors with ablation of CAR, α_V integrin, and HSG binding by mutation of the FG loop in the fiber knob (deletion of T489, A490, Y491, and T492 of the fiber protein), deletion of the RGD motif of the penton base, and substitution of the fiber shaft domain for that derived from Ad type 35, respectively, and have shown that this triple-mutant Ad vector [Ad/ΔF(FG)ΔP-S35-L2] exhibits significantly lower transduction in mouse liver compared with the conventional Ad vector [Koizumi, N., Mizuguchi, H., Sakurai, F., Yamaguchi, T., Watanabe, Y., and Hayakawa, T. (2003). J. Virol. 77, 13062–13072]. In the present study, we optimized the fiber knob mutation for further reduced *in vivo* transduction and examined toxicity of the modified Ad vectors. Ad/ΔF(AB)ΔP-S35-L2, a triple-mutant Ad vector containing a mutation of the AB loop in the fiber knob (R412S, A415G, E416G, and K417G), mediated approximately 15,000- and 500-fold lower mouse liver transduction by intravenous and intraperitoneal administration, respectively, than the conventional Ad vector, and mediated 10-fold lower mouse liver transduction than did Ad/ΔF(FG)ΔP-S35-L2. Ad/ΔF(AB)ΔP-S35-L2 also exhibited lower transduction of other organs compared with Ad/ΔF(FG)ΔP-S35-L2 and the conventional Ad vector. Levels of both liver serum enzymes (aspartate transferase [AST] and alanine transferase (ALT)] and interleukin (IL)-6 in mouse serum after intravenous administration of Ad/ΔF(AB)ΔP-S35-L2 were similar to those in the nontreatment mouse serum, whereas the conventional Ad vector led to high levels of AST, ALT, and IL-6. We therefore succeeded in further improving the mutant Ad vector, abolishing both viral natural tropism and toxicity. This new Ad vector appears to be a fundamental vector for targeted gene delivery.

OVERVIEW SUMMARY

Nonspecific distribution of adenoviral (Ad) vectors in tissue after *in vivo* gene transfer is due to the relatively broad expression of coxsackievirus and adenovirus receptor (CAR) (the primary receptor), α_V integrin (the secondary receptor), and heparan sulfate (the tertiary receptor). Ad injection *in vivo* is associated with the initiation of the inflammatory response and tissue damage. In the present study, we have generated a modified Ad vector with ablation of CAR, α_V integrin, and heparan sulfate binding and examined toxicity (liver serum enzymes and interleukin-6) of the vector as well as its transduction properties. For CAR binding ablation, the AB or FG loop mutation of the fiber knob was employed, and gene transfer activity of the triple-mutant Ad vector containing the AB loop mutation was compared with that of the triple-mutant Ad vector containing the FG loop mutation and with that of the conventional Ad vector. The triple-mu-

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tant Ad vector containing the AB loop mutation was found to mediate significantly lower tissue transduction *in vivo*. Furthermore, this mutant Ad vector reduced (or blunted) liver toxicity and innate immunity responses (interleukin-6 production). Thus, the triple-mutant Ad vector will likely be a fundamental vector for targeted gene delivery.

INTRODUCTION

RECOMBINANT ADENOVIRAL (Ad) VECTORS are attractive vehicles for *in vitro* and *in vivo* gene transfer to a wide variety of cell types. This distribution is largely due to the relatively broad expression of the primary receptor, the coxsackievirus and adenovirus receptor (CAR), and the secondary receptor, α_v integrin, and the tertiary receptor, heparan sulfate glycosaminoglycans (HSGs). The lack of specificity limits the utility of Ad vectors in gene therapy. Targeted Ad vectors would improve not only the efficacy but also the safety profiles of the vectors by permitting the use of lower doses, which would be less toxic and potentially less immunogenic (Krasnykh *et al.*, 2000; Wickham, 2000; Mizuguchi and Hayakawa, 2004).

The initial phase of Ad infection involves at least two sequential steps. The first is attachment of the virus to the cell surface through binding of the knob domain of the fiber to CAR (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). After attachment, interaction between the RGD motif of the penton bases with secondary host cell receptors, α_v integrins, facilitates internalization via receptor-mediated endocytosis (Wickham *et al.*, 1993, 1994). Furthermore, interaction between the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of Ad type 5 with HSGs and the length of the fiber shaft are involved in accumulation, in mouse and cynomolgus monkey liver, of systemically administered Ad vectors (Nakamura *et al.*, 2003; Smith *et al.*, 2003a,b; Vigne *et al.*, 2003).

Strategies to eliminate natural Ad tropism, based on modification of particular viral capsid proteins such as fiber and penton base (Wickham, 2000; Mizuguchi and Hayakawa, 2004), have been reported. To ablate CAR binding, Ad vectors containing an AB, DE, or FG loop mutation of the fiber knob (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Alemany and Curiel, 2001; Einfeld *et al.*, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002; Smith *et al.*, 2002), Ad vectors containing the Ad type 40 short fiber (which has been hypothesized not to bind to any receptors; Nakamura *et al.*, 2003), and Ad vectors containing an external trimerization motif instead of the fiber knob (Hong *et al.*, 2003) have been developed. To ablate α_v integrin binding, Ad vectors with a deletion of the RGD (Arg-Gly-Asp) motif of the penton base (Einfeld *et al.*, 2001; Mizuguchi *et al.*, 2002; Vigne *et al.*, 2003) have been developed. To ablate HSG binding, Ad vectors mutated in the KKTK motif of the fiber shaft (Smith *et al.*, 2003b) have been developed.

Several groups have reported that Ad vectors from which CAR binding has been ablated do not change the biodistribution of Ad vectors (Alemany and Curiel, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002; Smith *et al.*, 2002), although Einfeld *et al.* have reported that CAR binding-ablated Ad vectors exhibit a 10-fold decrease in liver transduction (Einfeld *et al.*, 2001). Einfeld *et al.* have also reported that Ad vectors ablated for both CAR binding ablation and α_v integrin binding exhibit a more than 700-fold decrease in liver transduction (Einfeld *et al.*, 2001). Ad vectors ablated for α_v integrin binding, however, do not change their biodistribution (Mizuguchi *et al.*, 2002; Smith *et al.*, 2003b). Smith *et al.* have shown that HSG binding-ablated Ad vectors, in which the KKTK motif on the fiber shaft of Ad type 5 is changed to GAGA (Gly-Ala-Gly-Ala), exhibit a 15-fold decrease in liver transduction (Smith *et al.*, 2003b).

We have previously developed an Ad vector ablated for CAR, α_v integrin, and HSG binding by deleting four amino acids (T489, A490, Y491, and T492) from the FG loop of the fiber knob, deleting the RGD motif of the penton base, and substituting the fiber shaft domain for that derived from Ad type 35, which does not contain the HSG-binding motif (Koizumi *et al.*, 2003a). This triple-mutant Ad vector, on intravenous administration, showed more than 1000-fold lower gene transfer activity in mouse liver than the conventional Ad vector whereas double-mutant Ad vectors (ablated for CAR and α_v integrin binding, or for CAR and HSG binding), on intravenous administration, showed 100-fold lower gene transfer activity in mouse liver than the conventional Ad vector (Koizumi *et al.*, 2003a).

In the present study, we further improve the triple-mutant Ad vector by adding a mutation of the AB loop in the fiber knob (R412S, A415G, E416G, and K417G) instead of a deletion of the FG loop in the fiber knob. The AB loop in the fiber knob interacts directly with CAR and therefore must be the key anchor for the knob–CAR complex (Bewley *et al.*, 1999). In contrast, deletion of the FG loop in the fiber knob eliminates interactions between the fiber knob and CAR by changing the structure of the fiber knob (Kirby *et al.*, 1999). We examined in detail the gene transfer activity of the triple-mutant Ad vector containing a mutation of the AB loop in the fiber knob both *in vitro* and *in vivo* (intravenous and intraperitoneal administration) in comparison with the triple-mutant Ad vector containing a deletion of the FG loop in the fiber knob and the conventional Ad vector.

Another drawback of the Ad vector is the production (release) of cytokines and chemokines, as well as hepatotoxicity, after systemic injection of the vector (Lieber *et al.*, 1997; Liu *et al.*, 2003). The cytokines play a major causative role in liver damage associated with systemic Ad infusion as well as a role in the induction of an antiviral immune response. Cytokine production and release are thought to be the direct or indirect results of Ad uptake by Kupffer cells and their subsequent activation (Lieber *et al.*, 1997; Liu *et al.*, 2003) or lysis (Schiedner *et al.*, 2003). It is important to develop an Ad vector that reduces or blunts innate immune response. In the present study, we also show that systemic injection of the triple-mutant Ad vector does not increase serum interleukin (IL)-6 levels or liver serum enzymes (aspartate aminotransferase [AST] and alanine aminotransferase [ALT], which are hepatotoxicity marker enzymes).

MATERIALS AND METHODS

Cells

SK HEP-1 (endothelial cell line derived from human liver; Heffelfinger *et al.*, 1992) and 293 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Fiber-293 cells, which are stable transformants expressing Ad type 5 fiber protein (Koizumi *et al.*,

2003a), were cultured with DMEM supplemented with 10% FCS and hygromycin (GIBCO, 200 μg/ml; Invitrogen, Carlsbad, CA).

Plasmids and Ad vectors

The vector plasmid pAdHM59, which we used to generate Ad vectors containing a mutation of the AB loop of the fiber knob (R412S, A415G, E416G, and K417G), a deletion of the RGD motif in the penton base, and a substitution of the fiber shaft domain for that derived from Ad type 35, was constructed as follows. First, a polymerase chain reaction (PCR) fragment containing a sequence surrounding the AB loop of the Ad type 5 fiber knob gene (bp 32238–32495) was generated with primers (forward, 5′-ATTAATACTTTGTGGACCACACC-AGCTCCATCTCCTAACTGTAGcCTAAATGgAGgGggt-GATGCTAAACTCACTTTGGTCTTAACAAAA-3′ [the *Ase*I site is underlined and the mutation sequence is indicated by lower-case letters]; reverse, 5′-AGATCTCCATTTCTAAAG-TT-3′ [the *Bgl*II site is underlined]) and pGEM-Teasy-knob-CAR(+) as a template (Koizumi *et al.*, 2003a). The PCR fragment was then ligated with *Eco*RV-digested pcDNA3.1-Hygro (Invitrogen), resulting in pcDNA3.1-Hyg-AB4m. pcDNA3.1-Hyg-AB4mknob was constructed by three-piece ligation of (1) the *Afl*II/*Ase*I fragment of pF35-2.3(AseI) (Mizuguchi and Hayakawa, 2002), which contains the fiber shaft of Ad type 35, (2) the *Ase*I/*Bgl*II fragment of pcDNA3.1-Hyg-AB4m, and (3) the *Afl*II/*Bgl*II fragment of pcDNA3.1-Hygro. Next, pHM-S35-K5-AB4m was constructed by three-piece ligation of (1) the *Afl*II/*Bgl*II fragment of pcDNA3.1-Hyg-AB4mknob, (2) the *Mun*I/*Bgl*II fragment of pHM-S35-K5-CAR(+) (Koizumi *et al.*, 2003a), and (3) the *Afl*II/*Mun*I fragment of pHMCMV6 (Mizuguchi and Kay, 1999). pHM-S35-K5-AB4m contains the sequence encoding the CAR-binding ablated Ad type 5 fiber knob (mutation of the sequence encoding four amino acids in the AB loop), a *Csp*45I site in the HI loop, a *Cla*I site in the C-terminal end of the fiber knob-coding sequence, and the fiber shaft sequence of Ad type 35. pS35-K5-2.2-AB4m was then constructed by ligation of *Srf*I/*Mun*I-digested pHM14-Eco2 (Koizumi *et al.*, 2003b) and *Srf*I/*Mun*I-digested pHM-S35-K5-AB4m. Next, the *Srf*I/*Mun*I fragment of pS35-K5-2.2-AB4m was ligated with the *Srf*I/*Mun*I fragment of pHM14-Eco12, resulting in the creation of pHM14-Eco2-S35-AB4m, which contains the Ad genome from bp 27331 to the end of the Ad genome and contains the sequence encoding the substitution of the fiber shaft domain for that derived from Ad type 35 and the mutation of the AB loop of the fiber knob. Finally, pAdHM59 was constructed by ligation of *Eco*RI/*Cla*I-digested pHM14-Eco2-S35-AB4m (the location of the *Eco*RI site is bp 27332 in the Ad genome, whereas the *Cla*I site is in the C-terminal end of the fiber-coding sequence) and *Eco*RI/*Cla*I-digested pAdHM43 (Koizumi *et al.*, 2003a), which contains the complete Ad genome, deletion of the RGD motif in the penton base, and a *Cla*I site in the C-terminal end of the fiber-coding sequence. pAdHM59 has a complete E1/E3-deleted Ad genome with I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region, *Pac*I sites at both ends of the Ad genome, and deletion of the RGD peptide-coding sequence of the penton base (MNDHAIRGDTFATRAE was changed to MNDTSRAE), and contains the chimeric fiber-coding sequence of the CAR binding-ablated Ad type 5 fiber knob (mutation of the AB loop-coding region of the fiber knob [R412S, A415G, E416G, and K417G]), the Ad type 35 fiber shaft sequences, and the Ad type 5 fiber tail sequence. pAdHM59 also contains a unique *Csp*45I site in the HI loop of the fiber knob-coding sequence and a *Cla*I site in the C-terminal end of the fiber knob-coding sequence. Therefore, the targeting ligands can be easily displayed in the fiber knob of the vectors by cloning the respective genes into these regions by simple *in vitro* ligation.

Ad vectors were constructed by an improved *in vitro* ligation method described previously (Mizuguchi and Kay, 1998, 1999). pAdHM59-CMVL2 and pAdHM54-CMVL2 were constructed by ligation of I-*Ceu*I/PI-*Sce*I-digested pAdHM59 and pAdHM54, respectively, and I-*Ceu*I/PI-*Sce*I-digested pCMVL1 (Mizuguchi and Kay, 1999). To construct pAdHM59-RGD-CMVL2, pAdHM59 was first digested with *Csp*45I and ligated with oligonucleotide 1 (5′-cggcctgtgactgccgcggagactgtttctgc-gatg-3′) and oligonucleotide 2 (5′-cgcatcgcagaaacagtctccgcg-gcagtcacaggc-3′), which corresponds to the RGD (RGD-4C) peptide, CDCRGDCFC, with high affinities for integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) (Koivunen *et al.*, 1995; Pasqualini *et al.*, 1997), resulting in the creation of pAdHM59-RGD. I-*Ceu*I/PI-*Sce*I-digested pAdHM59-RGD was then ligated with I-*Ceu*I/PI-*Sce*I-digested pCMVL1, resulting in the creation of pAdHM59-RGD-CMVL2.

To generate the virus, pAdHM59-CMVL2, pAdHM54-CMVL2, and pAdHM59-RGD-CMVL2 were digested with *Pac*I and purified by phenol–chloroform extraction and ethanol precipitation. Linearized DNAs were transfected into Fiber-293 cells (in the case of pAdHM54-CMVL2 and pAdHM59-CMVL2) or 293 cells (in the case of pAdHM59-RGD-CMVL2) with Super-Fect (Qiagen, Valencia, CA) according to the manufacturer's instructions. When pAdHM59-CMVL2 and pAdHM54-CMVL2 were transfected into normal 293 cells, the virus was not generated because the virus does not interact with 293 cellular receptors. Viruses [Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad/ΔF(AB)ΔP-S35-RGD-L2] were prepared by standard methods with the exception that Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 were amplified with Fiber-293 cells, and that only the last step of viral amplification was performed by infection into normal 293 cells, as described previously (Koizumi *et al.*, 2003a). Ad/ΔF(FG)ΔP-S35-L2 is identical to Ad/ΔFΔP-S35-L2 in our previous report (Koizumi *et al.*, 2003a). A conventional luciferase-expressing Ad vector, Ad-L2, had been constructed previously (Mizuguchi and Kay, 1999). Viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically by the methods of Maizel *et al.* (1968). Virus particle titers of the vector stocks, prepared from five 150-mm dishes (approximately 8×10^7 cells), were as follows: Ad-L2, 1.8×10^{12} vector particles (VP)/ml; Ad/ΔF(AB)ΔP-S35-L2, 2.8×10^{12} VP/ml; Ad/ΔF(FG)ΔP-S35-L2, 3.3×10^{12} VP/ml; Ad/ΔF(AB)ΔP-S35-RGD-L2, 2.6×10^{12} VP/ml.

Western blotting

Five hundred nanograms of virus in 1× sample buffer containing 4% 2-mercaptoethanol was loaded onto a sodium dodecyl sulfate (SDS)–polyacrylamide gel after boiling for 5 min, followed by electrotransfer to a nitrocellulose membrane. After blocking in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the filters were incubated with a rabbit fiber knob polyclonal antibody (diluted 1:3000) (kindly provided by R.D. Gerard, University of

Texas Southwestern Medical Center, Dallas, TX), followed by incubation in the presence of peroxidase-labeled anti-rabbit antibody (diluted 1:10,000) (Cell Signaling Technology, Beverly, MA). The filters were developed by chemiluminescence (ECL Western blotting detection system; GE Healthcare, Little Chalfont, UK), and signals were read with an LAS-3000 imaging system (Fujifilm Medical Systems USA, Stamford, CT).

Adenovirus-mediated gene transduction into cultured cells

SK HEP-1 cells (1×10^4 cells) were seeded in a 96-well dish. On the next day, they were transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 (3000 VP/cell) for 1.5 hr. After a 48-hr culture period, luciferase production in the cells was measured with a luciferase assay system (PicaGene LT2.0 luminescence kit; Toyo Ink, Tokyo, Japan).

In the experiment to quantify Ad uptake into SK HEP-1 cells, the cells were incubated at 37°C for 1.5 hr with the corresponding virus, washed with phosphate-buffered saline (PBS), resuspended in 0.05% trypsin–0.5 m*M* EDTA-PBS solution, and incubated at 37°C for 10 min. After this incubation, the cells were incubated at 37°C for 10 min with 0.05% DNase I–0.5 *M* $MgCl_2$–PBS, washed with PBS, and resuspended in 0.1 *M* EDTA–PBS solution. Finally, the Ad genome DNA in the cells was quantified with a TaqMan fluorogenic detection system (ABI PRISM 7700 sequence detector; Applied Biosystems, Foster City, CA). Sample DNA was isolated with an automatic nucleic acid isolation system (NA-2000; Kurabo Industries, Osaka, Japan). The Ad vector DNA standard was pAdHM4 plasmid DNA (Mizuguchi and Kay, 1999). Primers for amplification were located in the E4 region, with the sequences CACCACCTCCCGGTACCATA (sense) and CCGCACCTGGTTTTGCTT (antisense). The fluorogenic detection probe had the sequence AACCTGCCCGCCGGCTATACACTG. Samples were amplified in 50 μl for 40 cycles in the ABI PRISM 7700 sequence detector with continuous fluorescence monitoring. Data were processed with ABI PRISM 7000 SD software (Applied Biosystems).

When SK HEP-1 cells were transfected with a complex of Ad/ΔF(AB)ΔP-S35-L2 and SuperFect (Qiagen), the cells (2×10^4 cells) were seeded in a 48-well dish. The next day, the cells were transduced with Ad/ΔF(AB)ΔP-S35-L2 (10,000 VP/cell) in the presence of SuperFect (0, 0.15, or 1.5 μg; Qiagen) for 1.5 hr. After a 48-hr culture period, luciferase production in the cells was measured with a luciferase assay system (PicaGene PGL5500; Toyo Ink).

In the competition experiments, SK HEP-1 cells (2×10^4 cells) were seeded in a 48-well dish. The next day, the cells were preincubated with RGD peptide (GRGDSP; TaKaRa, Osaka, Japan) (0, 40, or 200 μg/ml) for 10 min at room temperature. The cells were then transduced with Ad/ΔF(AB)ΔP-S35-RGD-L2 (300 VP/cell) for 0.5 hr. After 48 hr in culture, luciferase production in the cells was measured by luminescence assay (PicaGene LT2.0; Toyo Ink).

Adenovirus-mediated gene transduction in vivo

Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 was intravenously (3.0×10^{10} VP) or intraperitoneally (1.0×10^{11} VP) administered to C57BL/6 mice (male, 5 weeks old; obtained from Nippon SLC, Shizuoka, Japan). Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated and homogenized as previously described (Xu *et al.*, 2001). Luciferase production was determined with a luciferase assay system (PicaGene 5500; Toyo Ink). Protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

The amounts of Ad genomic DNA in each organ were quantified with the TaqMan fluorogenic detection system (ABI PRISM 7700 sequence detector; Applied Biosystems). Samples were prepared with isolated DNA templates from each organ (25 ng) by the automatic nucleic acid isolation system (NA-2000; Kurabo Industries). The amounts of Ad DNA were quantified with the TaqMan fluorogenic detection system (Applied Biosystems), as described above.

Amounts of Ad vector DNA in liver parenchymal and nonparenchymal cells

Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was intravenously (3.0×10^{10} VP) or intraperitoneally (1.0×10^{11} VP) administered to C57BL/6 mice (male, 5 weeks old; Nippon SLC). Mice were anesthetized by intraperitoneal administration of pentobarbital sodium (Dainippon Pharmaceutical) 3 hr after Ad vector injection. Liver cells were separated into parenchymal cells (PCs; hepatocytes) and nonparenchymal cells (NPCs) (Kupffer cells and endothelial cells), as described previously (Nishikawa *et al.*, 1998). Briefly, the liver was perfused with HEPES buffer (pH 7.5) containing collagenase. The dispersed cells were separated into PC and NPC fractions by differential centrifugation. Quantitative PCR was performed to examine the amounts of Ad vector DNA in the PCs and NPCs. Total DNA, including the Ad vector DNA, was isolated from the PCs and NPCs by means of the automatic nucleic acid isolation system (NA-2000; Kurabo Industries). The amounts of Ad DNA were quantified with the TaqMan fluorogenic detection system (Applied Biosystems), as described above.

Blood clearance of Ad vectors

Blood samples were collected by retroorbital bleeding at the indicated times (2, 10, and 30 min; or 2, 60, 120, and 180 min) after intravenous (3.0×10^{10} VP) or intraperitoneal (1.0×10^{11} VP) administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. Total DNA, including the Ad vector DNA, was isolated from whole blood with a QIAamp DNA blood mini kit (Qiagen). The amounts of Ad DNA were quantified with the TaqMan fluorogenic detection system (Applied Biosystems), as described above.

Liver serum enzymes and interleukin-6 levels after systemic administration

Blood samples were collected from the inferior vena cava at the indicated times (3 or 48 hr) after intravenous (3.0×10^{11} VP) or intraperitoneal (1.0×10^{11} VP) administration of Ad-L2, Ad/ΔF(AB)ΔP-S35-L2 or Ad/ΔF(FG)ΔP-S35-L2. Serum samples were collected into separate tubes containing no anticoagulant for coagulation. The levels of AST and ALT in serum samples collected at 48 hr were measured with a Transaminase-CII kit (Wako Pure Chemical Industries, Osaka, Japan). IL-6 levels in serum samples collected 3 hr after Ad injection were

measured with an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA).

RESULTS

Construction of vectors that abolish natural viral tropism is the first step in the development of targeted Ad vectors. Identification and incorporation of a foreign ligand with high affinity for a specific cellular receptor into the capsid of Ad vectors that no longer infect cells would be the next step (Fig. 1A). This study was undertaken to improve a previously developed triple-mutant Ad vector that no longer infects cells by deletion of the FG loop of the fiber knob, deletion of the RGD motif of the penton base, and substitution of the fiber shaft domain with that derived from Ad type 35. Hepatocyte toxicity and innate immune response (IL-6 production) by systemic injection of the vectors were also examined.

Generation of several types of mutant Ad vector

To examine the effects of the various fiber knob mutations (mutation of AB loop or deletion of FG loop) in the triple-mutant Ad vector on gene transfer activity *in vitro* and *in vivo*, we constructed several types of mutant Ad vector expressing luciferase. Ad/ΔF(AB)ΔP-S35-L2 contains the Ad type 5 fiber knob

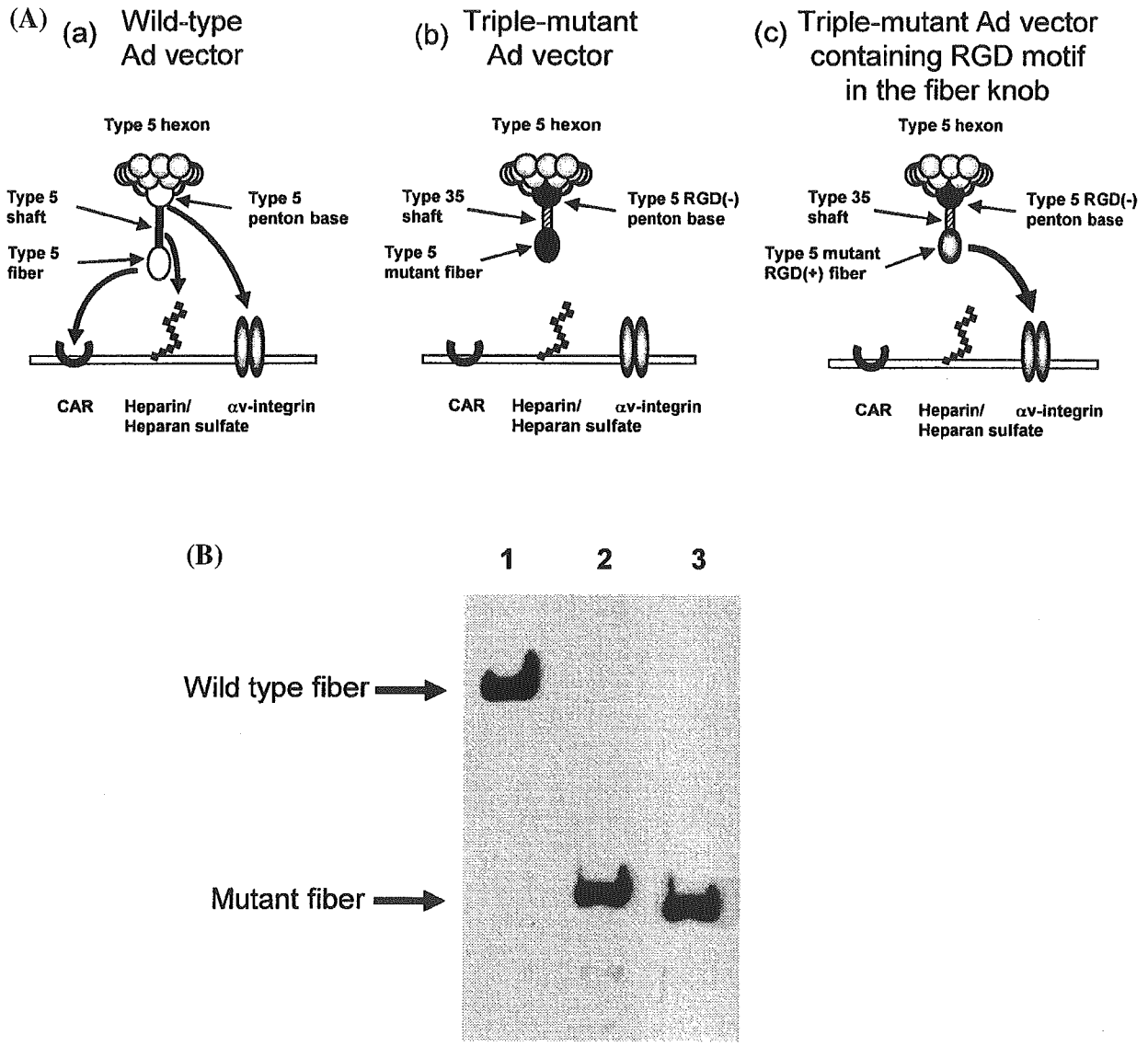

FIG. 1. Mutant Ad vectors. (**A**) Schematic diagram of the interaction of mutant Ad vectors with cells. The wild-type Ad vector infects cells by interactions of the fiber knob with CAR, the fiber shaft with HSGs, and the penton base with α_v integrin. The triple-mutant Ad vector does not have these interactions with cells. The triple-mutant Ad vector containing the RGD motif in the HI loop of the fiber knob infects cells via interaction of the RGD motif with α_v integrin. (**B**) Western blot analysis of the fiber protein in Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad/ΔF(AB)ΔP-S35-L2. Five hundred nanograms of virus was separated on a 12% SDS–polyacrylamide gel, and the fiber protein was analyzed by Western blotting using a rabbit fiber knob polyclonal antibody as described in Materials and Methods. Lane 1, Ad-L2; lane 2, Ad/ΔF(FG)ΔP-S35-L2; lane 3, Ad/ΔF(AB)ΔP-S35-L2.

Table 1. Adenoviral Vector Mutations and Potential Cell Interactions

Ad vector	*Amino acid sequence of knob domain*				*Type of Ad shaft*	*Type of Ad tail*	*Penton base*	*Type of Ad vector*[a]
	AB loop	*FG loop*	*HI loop*	*C terminus*				
Conventional Ad								
Ad-L2	–NCRLNAEKDA–	–TEGTAYTNAV–	–DTTPSA–	–QE stop	5 (22 β repeats)	5	MND–HAIRGDTFAT–RAE	a
Triple-mutant Ads								
Ad/ΔF(FG) ΔP-S35-L2	–NCRLNAEKDA–	–TEG----NAV– (Δa.a. 489–492)	–DTTSNPSA–	–QEID stop	35 (6 β repeats)	5	MND–TS---------RAE ΔRGD motif	b
Ad/ΔF(AB) ΔP-S35-L2	–NCSLNGGGDA– (4-a.a. mutation)	–TEGTAYTNAV–	–DTTSNPSA–	–QEID stop	35 (6 β repeats)	5	MND–TS---------RAE ΔRGD motif	b
Triple-mutant Ad containing RGD motif in fiber								
Ad/ΔF(AB) ΔP-S35-RGD-L2	–NCSLNGGGDA– (4-a.a. mutation)	–TEGTAYTNAV–	–DTTSACDCRG DCFCANPSA–	–QEID stop	35 (6 β repeats)	5	MND–TS---------RAE ΔRGD motif	c

[a]See Fig. 1A.

with a four-amino acid mutation of the AB loop (R412S, A415G, E416G, and K417G), the Ad type 35 fiber shaft, and a deletion of the RGD motif of the penton base. Ad/ΔF(FG)ΔP-S35-L2, which is identical to Ad/ΔFΔP-S35-L2 in our previous report (Koizumi *et al.*, 2003a), contains the Ad type 5 fiber knob with a four-amino acid deletion of the FG loop (T489, A490, Y491, and T492), the Ad type 35 fiber shaft, and deletion of the RGD motif of the penton base. Ad/ΔF(AB)ΔP-S35-RGD-L2 contains an RGD motif in the HI loop of the fiber knob in Ad/ΔF(AB)ΔP-S35-L2. Ad-L2 is a conventional Ad vector. All mutations of the mutant Ad vectors and possible interaction of each virus with the cells are summarized in Table 1 and Fig. 1A. All of the mutant Ad vectors used in this study were readily propagated with particle titers similar to that of the conventional Ad vector, Ad-L2 (see Materials and Methods).

To confirm the modification of the fiber protein in each Ad vector, Western blot analysis against fiber protein was performed with rabbit fiber knob polyclonal antibody (Fig. 1B). The mutant fiber and wild-type fiber are easily distinguished because the mutant fiber is smaller than the wild-type fiber because of the small size of the Ad type 35 fiber shaft and because Ad/ΔF(AB)ΔP-S35-L2 has a fiber protein four amino acids longer than that of Ad/ΔF(FG)ΔP-S35-L2. Western blot analysis shows the expected size of the fiber proteins, suggesting that each Ad vector should indeed contain the expected fiber protein.

Gene transfer in vitro

We examined the gene transfer activity in SK HEP-1 cells transduced with Ad/ΔF(AB)ΔP-S35-L2 in comparison with

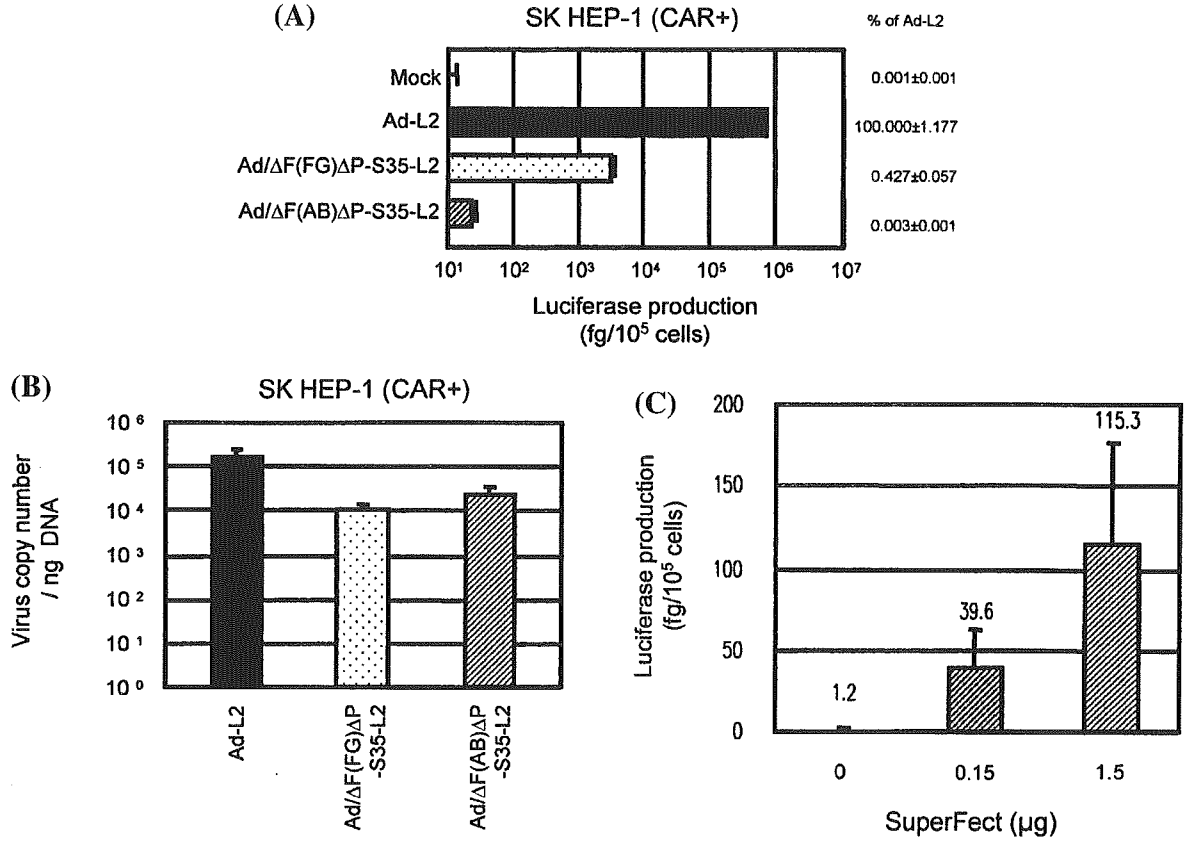

FIG. 2. Luciferase production and viral uptake in SK HEP-1 cells transduced with several Ad vectors. (**A**) Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. SK HEP-1 cells were transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 (3000 VP/cell) for 1.5 hr. After culture for 48 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means ± SD (n = 4). Relative levels of luciferase expression are described by designating the value of Ad-L2 as 100. (**B**) Viral uptake in SK HEP-1 cells. SK HEP-1 cells were transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 at 3000 VP/cell. After culture for 1.5 hr, the cells were washed with PBS, resuspended in 0.05% trypsin–0.5 m*M* EDTA–PBS solution, and incubated at 37°C for 10 min. After this incubation, the cells were incubated at 37°C for 10 min with 0.05% DNase I–0.5 *M* $MgCl_2$–PBS, washed with PBS, and 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. Data are expressed as means ± SD (n = 4). (**C**) Comparison of luciferase production in SK HEP-1 cells transduced with a complex of Ad/ΔF(AB)ΔP-S35-L2 and SuperFect. SK HEP-1 cells (2×10^4 cells) were seeded into a 24-well dish. The next day, the cells were either not transduced or were transduced with a complex of Ad/ΔF(AB)ΔP-S35-L2 and SuperFect (0.15 or 1.5 μg) (Qiagen) for 1.5 hr. After culture for 48 hr, luciferase production in the cells was measured with a luciferase assay system. Data are expressed as means ± SD (n = 4).