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Biochemical and Biophysical Research Communications 335 (2005) 205–214
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Reovirus σ1 fiber incorporated into adenovirus serotype 5 enhances infectivity via a CAR-independent pathway

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Received 23 June 2005
Available online 25 July 2005

Abstract

Adenovirus serotype 5 (Ad5) has been used for gene therapy with limited success because of insufficient infectivity in cells with low expression of the primary receptor, the coxsackie and adenovirus receptor (CAR). To enhance infectivity in tissues with low CAR expression, tropism expansion is required via non-CAR pathways. Serotype 3 Dearing reovirus utilizes a fiber-like σ1 protein to infect cells expressing sialic acid and junction adhesion molecule 1 (JAM1). We hypothesized that replacement of the Ad5 fiber with σ1 would result in an Ad5 vector with CAR-independent tropism. We therefore constructed a fiber mosaic Ad5 vector, designated as Ad5-σ1, encoding two fibers: the σ1 and the wild-type Ad5 fiber. Functionally, Ad5-σ1 utilized CAR, sialic acid, and JAM1 for cell transduction and achieved maximum infectivity enhancement in cells with or without CAR. Thus, we have developed a new type of Ad5 vector with expanded tropism, possessing fibers from Ad5 and reovirus, that exhibits enhanced infectivity via CAR-independent pathway(s).

Keywords: Gene therapy; Adenovirus; Reovirus; σ1 spike protein; Tropism expansion; Coxsackie and adenovirus receptor

Adenoviral vectors, in particular human serotype 5 (Ad5), have been widely employed for cancer gene therapy applications, owing to their unparalleled ability to accomplish in vivo gene transfer [1]. Despite this capacity, the limited efficacies noted in human gene therapy trials have suggested deficiencies of this vector vis-à-vis the achievement of efficient gene delivery. In this regard, it has been observed that human tumor cells frequently manifest a relative deficiency of the primary Ad receptor, coxsackie and adenovirus receptor (CAR) [2]. This CAR deficiency renders many tumor cells resistant to Ad infection, undermining cancer gene therapy strategies that require efficient tumor cell transduction. Thus, this unanticipated aspect of tumor biology potentially confounds direct exploitation of current generation of human Ad-based vectors.

To address this issue, strategies have been proposed to alter the tropism of Ad to accomplish CAR-independent infection of tumor cells [2]. Initial efforts to this end focused on the use of so-called retargeting adaptors that cross-link Ad to non-CAR receptors that are overexpressed on tumor cells [2,3].

Genetic capsid modification has also been endeavored to achieve these same functional ends. This approach has rationally focused on the fiber knob domain, the primary determinant of Ad tropism, to achieve CAR-independent cell entry. Ad fiber pseudotyping, the genetic substitution of either the entire fiber or the fiber knob domain with its structural counterpart from another human Ad serotype, has been realized. Fiber-pseudotyped vectors display CAR-independent

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doi:10.1016/j.bbrc.2005.07.054

tropism by virtue of the natural diversity in receptor recognition found in species B and D Ad fibers. This approach has identified vectors with superior infectivity to Ad5 in several clinically relevant cell types [4–6]. These studies clearly established that genetic capsid modification can achieve the goal of enhanced transduction of tumor cells via CAR-independent cell entry.

On this basis, advanced strategies to achieve further infectivity enhancement have been proposed. We previously derived a fiber mosaic Ad5 vector that incorporates two distinct fibers: the Ad5 fiber and a chimeric fiber that incorporates the Ad3 fiber knob domain. This strategy provided viral entry via two different pathways with additive gains in infectivity [7]. We next explored a strategy to expand Ad tropism by means of exploiting the tropism of non-Ad viruses. Specifically, we endeavored the construction and characterization of fiber mosaic Ad5 vectors that contained fibers of Ad5 and reovirus. In particular, the receptor-binding molecule of serotype 3 Dearing (T3D) reovirus, called the σ1 protein, was incorporated into fiber mosaic Ad5 vectors together with the wild-type Ad5 fiber. This fiber-like σ1 attachment protein is known to bind sialic acid [8] and junction adhesion molecule 1 (JAM1) [9], which together determine T3D reovirus tropism. Since T3D reovirus tropism is clearly distinct from that of Ad, the σ1 protein is a promising candidate for incorporation into a fiber mosaic Ad vector, which could bind to neoplastic cells using the widely expressed cell receptors JAM1, sialic acid, and CAR. Our study establishes a novel strategy to achieve infectivity enhancement based on a fiber mosaic Ad5 vector, which contains fibers from different virus families.

Materials and methods

Cell lines. The 293 cells were purchased from Microbix (Toronto, Ontario, Canada). Human embryonic rhabdomyosarcoma RD cells, human glioma cell line U118MG, human head and neck tumor cell line FaDu, human ovarian cancer cell lines ES-2, OV-3, SK-OV-3, and OVCAR-3, Chinese hamster ovary (CHO) cells, and Lec2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Human ovarian adenocarcinoma cell lines OV-4 and Hey were a kind gift from Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA) and Dr. Judy Wolf (M.D. Anderson Cancer Center, Houston, TX), respectively. L929 cells, U118MG-hCAR-tailless cells, and 211B cells were maintained as described previously [8,10,11]. Cell lines were cultured in media recommended by suppliers (Mediatech, Herndon, VA, and Irvine Scientific, Santa Ana, CA). FBS was purchased from Hyclone (Logan, UT). All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Primary human ovarian carcinoma cells were established in culture from fresh malignant ascites fluid obtained from patients with pathologically confirmed ovarian adenocarcinoma during surgery at the University of Alabama at Birmingham (UAB) Hospital. Approval was obtained from the UAB Institutional Review Board before acquisition of samples. Cancer cells were purified from ascites fluid by a previously described immunomagnetic-based method [12].

Generation of the σ1 chimeric fiber construct. A schematic of the σ1 chimeric fiber structure is shown in Fig. 1A. To design the σ1 chimeric fiber, the fiber tail domain of Ad5 was amplified by PCR from plasmid pNEB.PK.3.6 [5]. The PCR product was cloned into pGEM4ZT3DS1 that encodes T3D reovirus σ1, resulting in pGEM4ZT3DS1delBamAd5tail. The Ad5tail and entire σ1 sequence was PCR-amplified from pGEM4ZT3DS1delBamAd5tail, using the primers 5′-GCCATG AAGCGCGCAAGACCGTCTGAA (sense), 5′-TTTACTAGATGA AATGCCCCAGTGCCGC (the first antisense for addition of stop codon and polyadenylation signal), and 5′-GAAATCAATTGTTTAC TAGATGAAATGCCC (the second antisense for addition of *Mun*I restriction site). The resultant PCR product of the σ1 chimeric fiber was cloned into pNEB.PK.3.6, resulting in pNEB.PK.3.6Ad5tail/σ1. The sequence of pNEB.PK.3.6Ad5tail/σ1 was confirmed by DNA sequencing. This σ1 chimeric fiber was designated as F5S1.

To design the expression vector for the σ1 chimeric fiber, we cloned the F5S1 sequence into plasmid pShuttle-CMV (Qbiogene, Carlsbad, CA). The resultant expression plasmid was designated as pShuttle-CMV-AdSig.

Generation of shuttle plasmids for fiber mosaic Ad5 genome. The construct was based on a fiber mosaic Ad genome that encodes two fiber genes (the wild-type Ad5 fiber and Fiber-Fibritin, designated as FF) in the L5 region, which has been described previously [13]. Our strategy was to replace the fibritin part in the chimeric fiber FF with the σ1 sequence and create the σ1 chimeric fiber (F5S1H). Coding sequence of σ1 was amplified from plasmid pNEB.PK.3.6Ad5tail/σ1 with the primers 5′-CAGAACGTTGGGGATCCTCGCCTACG TGAAGAAGTAGTAC and 5′-TCCTCTAGATCCGCCCGTGAAA CTACGCGGGTACGAAAC. The PCR product was cloned into *Acl*I/*Xba*I sites of FF in plasmid pZpTG 5FF3 [13]. We replaced the fibritin sequence with σ1 coding sequence in-frame with a carboxy-terminally encoded 6-histidine (6-His) stretch, resulting in F5S1H. The resulting plasmid was designated as pZpTG5F/S1.6H. An *Age*I/*Age*I fragment of this plasmid was cloned into the *Age*I site of pNEB.PK.FSP [5] to obtain a shuttle plasmid, designated as pNEB.PK.FSPF5S1/F5, which contains tandem fiber genes: the σ1 chimeric fiber, F5S1H, and the wild-type Ad5 fiber.

Generation of recombinant Ad. A schematic of the viruses used in this study is shown in Fig. 1B. Recombinant Ad5 genomes containing the tandem fiber genes were derived by homologous recombination in *Escherichia coli* BJ5183 with *Swa*I-linearized rescue plasmid pVK700 and the tandem fiber-containing *Pac*I and *Kpn*I-fragment of pNEB.PK.FSPF5S1/F5 essentially as described previously [14]. The recombinant region of the genomic clones was sequenced prior to transfection into 293 cells. All vectors were propagated in 293 cells and purified using a standard protocol [15]. The resultant fiber mosaic virus was Ad5-σ1. Viral particle (v.p.) concentration was determined by the method of Maizel et al. [16]. An infectious titer was determined according to the AdEasy Vector System (Qbiogene).

PCR amplification of viral genome fragments. Viral DNA was amplified using the Taq PCR Core Kit (Qiagen, Valencia, CA). The sequences of the primers were as follows: Ad5tail-sense 5′-ATGAAGC GCGCAAGACCGTCTGAAGAT; Ad5knob-antisense 5′-TTATTCT TGGGCAATGTATGAAAAAGT; and σ1head-antisense 5′-ATTCT TGCGTGAAACTACGCGG.

Protein electrophoresis and Western blotting. To detect the incorporation of fibers in virus particles, Ad vectors equal to 5.0×10^9 v.p. were resolved by SDS–PAGE and Western blotting as described previously [13].

To detect trimerization of the σ1 chimeric fiber, F5S1, the expression plasmid pShuttle-CMV-AdSig was transiently transfected into 293 cells using SuperFect Transfection Reagent (Qiagen). Cell lysates were used for SDS–PAGE and Western blotting. For JAM1 detection, a panel of cell lines was harvested for SDS–PAGE and analyzed by Western blotting using anti-JAM1 monoclonal antibody (BD Biosciences Clontech, Palo Alto, CA).

Recombinant proteins. Recombinant T3D σ1 was produced as described in Chandran et al. [17]. Ad5 fiber knob domain recombinant

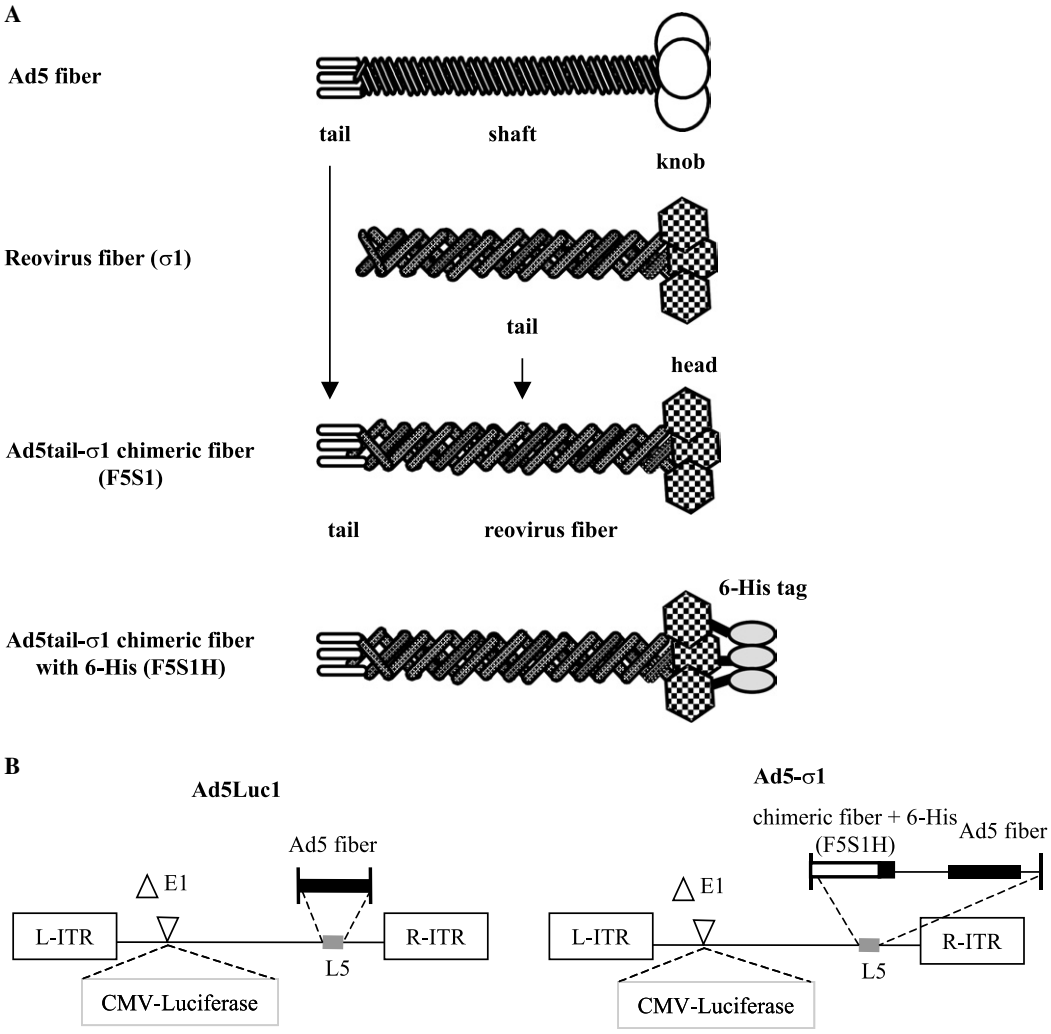

Fig. 1. Schema of fiber mosaic Ad5 genomes. (A) Key components of the σ1 chimeric fiber. In the σ1 chimeric fiber, the tail of Ad5 fiber is fused to the reovirus fiber protein σ1 (designated as F5S1). A six-histidine (6-His) tag is fused to the carboxy-terminus of the σ1 chimeric fiber through a linker (designated as F5S1H). (B) Map of Ad5 genomes with fiber modification. In both vectors, the E1 region is replaced by CMV promoter/luciferase transgene cassette. Ad5Luc1 is a control virus that carries the wild-type Ad5 fiber. Ad5-σ1 is a fiber mosaic vector that carries the σ1 chimeric fiber with a carboxy-terminal 6-His tag (F5S1H) as well as the wild-type Ad5 fiber.

protein was produced and purified as described previously [5]. Concentration of protein in all experiments was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

In vitro gene transfer assays. Cells were infected with virus at 37 °C for 1 h and unbound virus was washed away. After 24 h of incubation at 37 °C, a luciferase assay was performed (Promega, Madison, WI) according to the manufacturer's instructions. Data are presented as mean values ± SD.

Competitive inhibition assay. Recombinant Ad5 fiber knob proteins or anti-JAM1 polyclonal antibody (c-15, Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the cells at 37 °C for 15 min prior to infection. Alternatively, cells were treated with 333 milliunits/ml of *Clostridium perfringens* neuraminidase type X (Sigma–Aldrich, St. Louis, MO) at 37 °C for 30 min to remove cell-surface sialic acid, followed by two washes with PBS. Cells were then adsorbed with viruses at 37 °C for 1 h. Unbound virus and blocking agents were washed away. After 24 h of incubation at 37 °C, the cells were processed for luciferase assay, as described above. For σ1 blocking experiments, anti-T3D σ1 antibody (9BG5, a gift from Dr. Patrick W.K. Lee, University of Calgary, Calgary, Canada) [18] was incubated with virus at room temperature for 1 h prior to cell infection. Subsequent procedures were same as described above. Data are presented as mean values ± SD.

Flow cytometry. For CAR detection, the cells were incubated with 2 μg/ml of the anti-human CAR monoclonal antibody RmcB (hybridoma was purchased from ATCC) or normal mouse IgG1κ (Sigma–Aldrich) for 1 h on ice. Subsequently, the cells were washed and incubated with FITC-conjugated anti-mouse IgG (Sigma–Aldrich) for an additional 1 h. For sialic acid measurement, cells were incubated with 1 μg/ml FITC-labeled wheat germ agglutinin (WGA; Sigma–Aldrich) on ice for 1 h. After washing with 1% BSA/PBS, the cells were analyzed by flow cytometry at the UAB FACS Core Facility.

Results

Generation of a chimeric fiber (F5S1) containing reovirus σ1

To create a functional chimeric fiber structurally compatible with Ad5 capsid incorporation, we designed the

σ1 chimeric fiber to comprise the amino-terminal tail segment of the Ad5 fiber sequence genetically fused to the entire T3D σ1 protein, with (F5S1H) or without (F5S1) a carboxy-terminal 6-His tag as a detection marker (Fig. 1A).

Prior to Ad5 vector design, we evaluated the trimerization capacity of the σ1 chimeric fiber (F5S1), using pShuttle-CMV-AdSig, a fiber expression plasmid. Following transfection of 293 cells, cell lysates were subjected to SDS–PAGE and Western blot analysis using two primary antibodies, the 4D2 monoclonal antibody (Neomarkers, Fremont, CA) that recognizes the Ad5 fiber tail domain common to both the wild-type Ad5 and the σ1 chimeric fiber, and an anti-T3D σ1 polyclonal antibody (a gift from Dr. Max L. Nibert, Harvard Medical School, Boston, MA) that recognizes σ1. We detected a band for Ad5 fiber from 211B cell lysates [10] at approximately 180 kDa with the 4D2 antibody (Fig. 2, lane 1). This band corresponds to the trimeric fiber molecule, while an approximately 60 kDa band in boiled lysates represents fiber monomers (lane 2). The chimeric fiber was detected with both the 4D2 antibody (lane 3) and an anti-T3D σ1 antibody (data not shown) at an apparent molecular mass of 160–170 kDa. When heat denatured, the monomeric chimeric fiber was detected at an apparent molecular mass of 50 kDa (lane 4). This analysis demonstrates that the σ1 chimeric fiber F5S1 is capable of trimerization, which is required for Ad capsid incorporation.

Construction of fiber mosaic viruses

We sought to create a fiber mosaic Ad5 encoding both the Ad5 fiber and chimeric fibers in the L5 region of the Ad5 genome. We employed a tandem-fiber cassette wherein the F5S1H σ1 chimeric fiber was positioned upstream of the wild-type fiber gene. In this configuration, each fiber was positioned before the untranslated sequences of the wild-type fiber to provide equal transcription, splicing, polyadenylation, and regulation by the major late promoter. We constructed E1-deleted recombinant Ad genomes (Ad5-σ1) containing the wild-type Ad5 fiber, the σ1 chimeric fiber (F5S1H), and a firefly luciferase reporter gene controlled by the CMV immediate early promoter/enhancer. Following virus rescue and large-scale propagation in 293 cells, we obtained Ad5-σ1 vector at concentrations ranging from 1.1×10^{11} v.p./ml to 5.31×10^{12} v.p./ml, depending on the individual preparation. These concentrations compared favorably with that of Ad5Luc1 at 3.74×10^{12} v.p./ml. In addition, the v.p./plaque-forming unit (PFU) ratios determined for Ad5-σ1 and Ad5Luc1 were 22 and 13.3, respectively, indicating excellent virion integrity for both species. Of note, the control vector used throughout this study, Ad5Luc1, is isogenic to Ad5-σ1 in all respects except for the fiber locus.

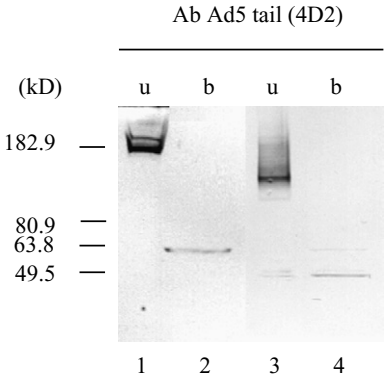

Fig. 2. Western blot analysis of F5S1 chimeric fiber protein in lysates of transiently transfected cells. Fiber proteins were detected by anti-Ad5 fiber tail antibody (4D2). Lanes 1 and 2, 211B cell lysate as a positive control for the wild-type Ad5 fiber; lanes 3 and 4, pShuttle-CMV-AdSig transfected 293 cell lysate for F5S1 chimeric fiber detection (without 6-His). We used cell lysates from 211B cells expressing the wild-type Ad5 fiber as a positive control for detection of Ad5 fiber. The samples in lanes 2 and 4 were heat denatured (b), which resulted in dissociation of trimeric proteins to monomers, while lanes 1 and 3 contain proteins in their native trimeric configuration (unboiled (u)).

Definition of fiber gene configurations for fiber mosaic Ad

We confirmed the fiber genotype of Ad5Luc1 and Ad5-σ1 via diagnostic PCR, using Ad5 fiber or the σ1 chimeric fiber primer pairs and genomes from purified virions as PCR templates (Fig. 3A). To confirm that Ad5-σ1 virions contained both trimerized fibers, we performed SDS–PAGE followed by Western blot analysis on viral particles. Using the 4D2 antibody we observed fiber bands at approximately 180 kDa for unboiled samples of Ad5Luc1 and Ad5-σ1 virions, corresponding to fiber trimers (Fig. 3B, lanes 1 and 3). In boiled samples, the 4D2 antibody detected bands of apparent molecular mass of approximately 60 kDa, indicative of fiber monomers (lanes 2 and 4). Due to the near-identical sizes of the σ1 chimeric and the wild-type Ad5 fiber proteins, it was difficult to visualize both fibers simultaneously via Western blot with 4D2.

To confirm the presence of the σ1 chimeric fiber protein in virions, we used the anti-Penta His monoclonal antibody (Qiagen) that recognizes 6-His tags (Fig. 3C) and the anti-T3D σ1 antibody (Fig. 3D). Using the anti-Penta His antibody, we observed the fiber bands corresponding to both trimeric and monomeric σ1 chimeric fiber proteins (Fig. 3C, lanes 2 and 3). The trimeric band of the σ1 chimeric fiber was also detected with the anti-T3D σ1 antibody (Fig. 3D, lane 3), however, the monomeric band of the σ1 chimeric fiber protein was faint because of relatively weak binding affinity of the anti-T3D σ1 antibody (Fig. 3D, lanes 2 and 4). These results confirm that the trimeric F5S1H σ1 chimeric fiber was incorporated into Ad5-σ1 virions.

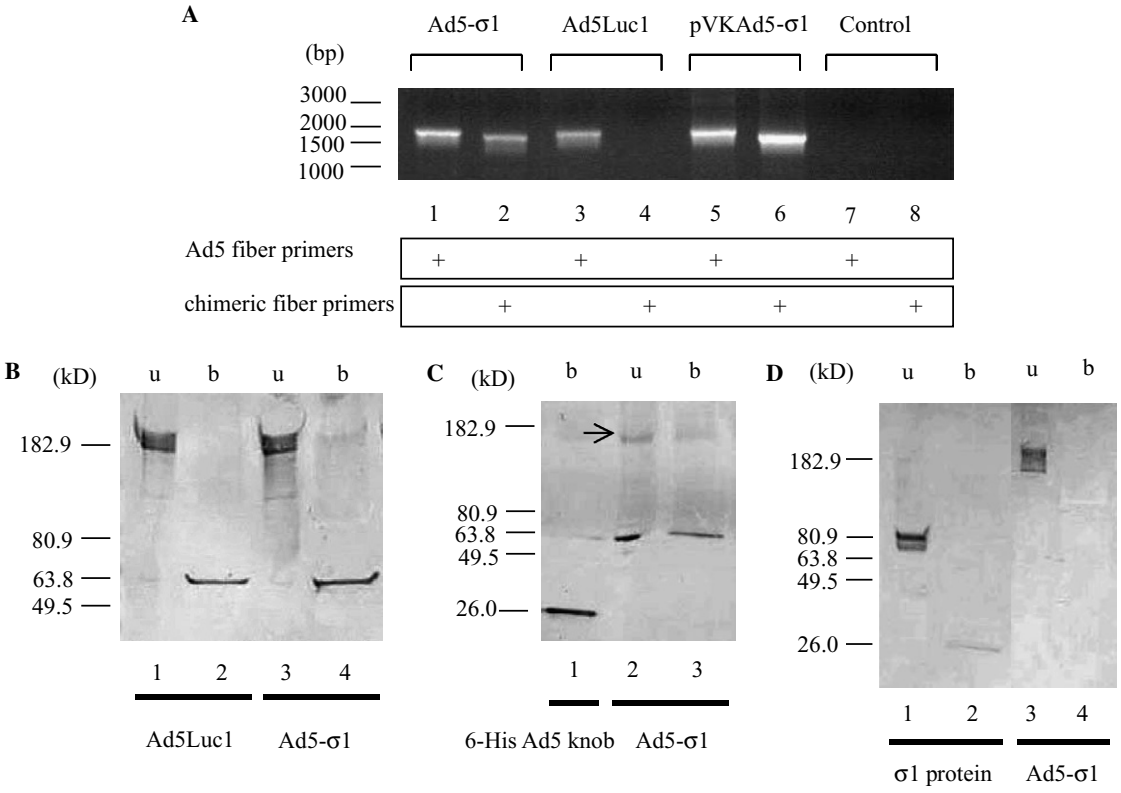

Fig. 3. Analysis of fibers in rescued viral particles. (A) Detection of fiber genes in Ad genome. Rescued viral particles were analyzed with PCR, using pairs of Ad5 fiber primers or σ1 chimeric fiber primers. pVKAd5-σ1 was used as a positive control for both fibers. No PCR template is designated as Control. (B–D) Western blot analysis of fiber proteins in purified virions. (B) A total of 5.0×10^9 v.p. per lane of Ad5Luc1 with the wild-type Ad5 fiber (lanes 1 and 2) or Ad5-σ1 with dual fibers (lanes 3 and 4) were resuspended in Laemmli buffer prior to SDS–PAGE and electrotransfer and detected with the 4D2 anti-Ad5 fiber tail antibody. The samples in lanes 2 and 4 were boiled (b), while lanes 1 and 3 (unboiled (u)) contain proteins in their native trimeric configuration. (C) A total of 5.0×10^9 v.p. per lane of Ad5-σ1 with dual fibers (lanes 2 and 3) was probed with an anti-6-His antibody. Lane 1, recombinant Ad5 knob with a 6-His tag as a positive antibody control; lane 2, unboiled Ad5-σ1 virions; and lane 3, boiled Ad5-σ1 virions. The arrow indicates the position of the trimeric σ1 chimeric fiber protein. (D) A total of 5.0×10^9 v.p. per lane of Ad5-σ1 with dual fibers (lanes 3 and 4) were probed with an anti-T3D σ1 antibody. Lanes 1 and 2, recombinant σ1 protein. The samples in lanes 2 and 4 were boiled, while lanes 1 and 3 contain proteins in their native trimeric configuration (unboiled).

Ad5-σ1 vector exhibits native Ad5 tropism

Our hypothesis was that the inclusion of the σ1 chimeric fiber, F5S1H, into an Ad5 vector would provide infectivity enhancement to Ad-refractory cell types via expanded vector tropism. To test whether this vector retained CAR-dependent tropism, we evaluated Ad5-σ1 infection in a pair of tumor cell lines that vary only in their CAR expression. The human U118MG glioma cell line is CAR-deficient [11]. The U118MG-hCAR-tailless cell is a CAR-positive variant line that artificially expresses the extracellular domain of human CAR [11]. Ad5Luc1 was used as a positive control for CAR binding. As shown in Fig. 4A, Ad5Luc1 exhibited CAR-dependent tropism, as shown by a 40-fold increase in luciferase transgene expression in U118MG-hCAR-tailless cells relative to the parental CAR-deficient U118MG cells. Similarly, Ad5-σ1 exhibited CAR-dependent tropism, as demonstrated by a 53-fold increase in transgene expression in U118MG-hCAR-tailless cells relative to the CAR-deficient U118MG cells (Fig. 4A). Incubation of U118MG-hCAR-tailless cells with recombinant Ad5 knob protein at 50 μg/ml prior to infection efficiently inhibited over 70% of Ad5Luc1 and Ad5-σ1 luciferase activity (Fig. 4B). These data indicate that Ad5-σ1 retains CAR-based tropism, confirming the functionality of the wild-type fiber in our fiber mosaic Ad5.

Ad5-σ1 vector exhibits sialic acid- and JAM1-dependent tropism

To confirm that Ad5-σ1 exploits the non-CAR receptor sialic acid and JAM1 by virtue of the chimeric fiber, we further characterized Ad5-σ1 tropism by performing competitive blocking experiments using 9BG5, a σ1-specific monoclonal antibody that recognizes the T3D σ1 head domain and blocks σ1/JAM1 interaction [18]. Pre-incubation of Ad5-σ1 with 9BG5 prior to infection blocks over 50% of Ad5-σ1 transgene expression in L929 cells, a sialic acid and JAM1-positive cell line commonly used for propagating reovirus (Fig. 4C).

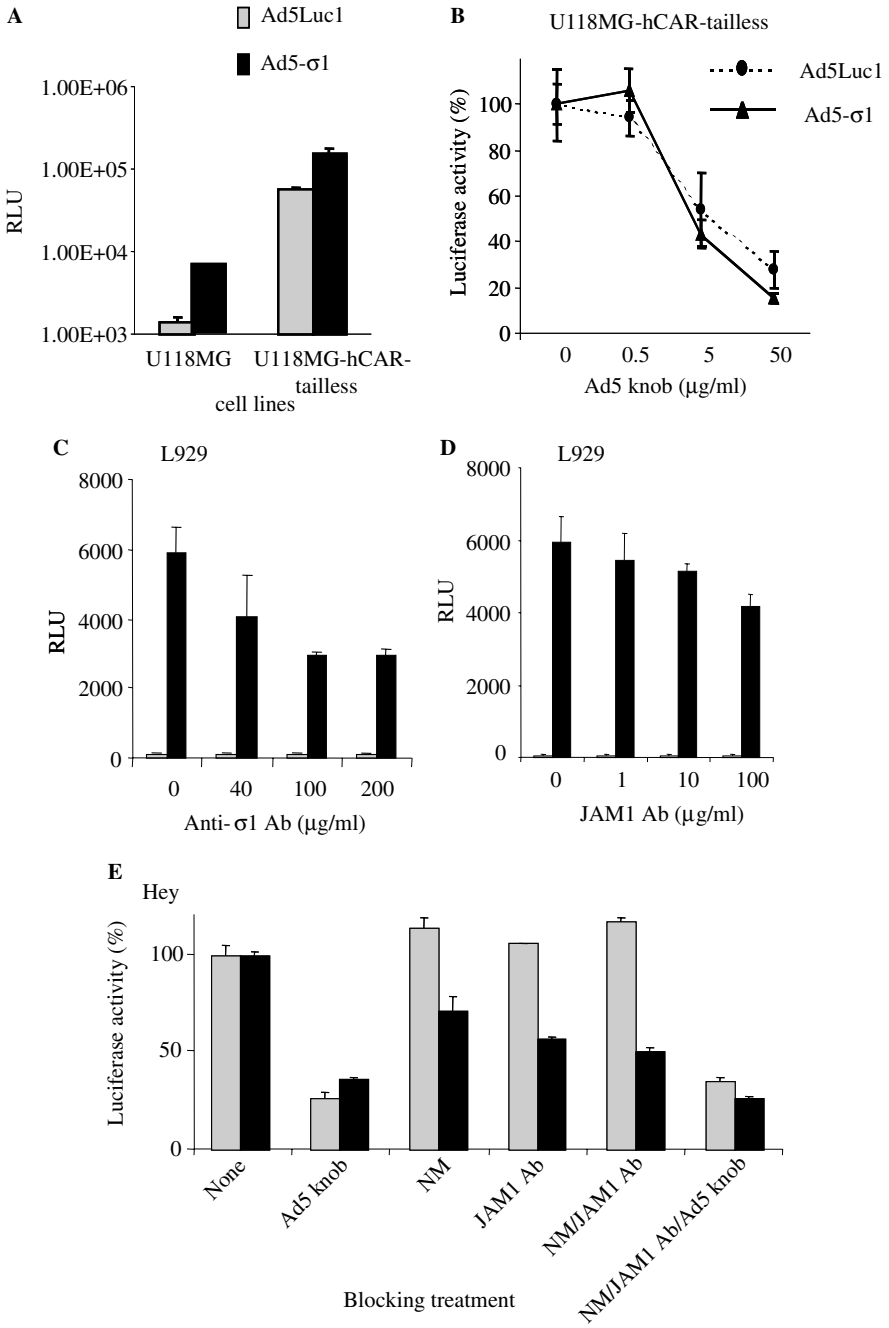

Fig. 4. Evaluation of the efficacy and receptor specificity of Ad5-σ1 mediated gene transfer. (A) Ad5-σ1 infection of a pair of tumor cell lines that vary only in their CAR expression. U118MG is a parental CAR-deficient human glioma cell line, whereas U118MG-hCAR-tailless stably expresses the extracellular domain of human CAR. Cells were infected with Ad5Luc1 (gray bar) and Ad5-σ1 (black bar) at 10 v.p./cell. Luciferase activity was measured 24 h post-infection and is expressed as relative light units (RLU). (B) Recombinant Ad5 fiber knob protein blocks Ad5-σ1 and Ad5Luc1 gene transfer. U118MG-hCAR-tailless cells were incubated with 100 v.p./cell of Ad5-σ1 or Ad5Luc1 with or without recombinant Ad5 fiber knob protein at the indicated concentrations. Luciferase activity was determined 24 h post-infection. All luciferase values were normalized against the activity of controls receiving no knob valued at 100%. (C) σ1 antibody blocks Ad5-σ1 gene transfer. σ1 antibody was incubated with Ad5-σ1 or Ad5Luc1 at 100 v.p./cell for 1 h prior to the infection to L929 cells. Luciferase activity was determined 24 h post-infection. (D) An anti-JAM1 antibody blocks Ad5-σ1 infection. L929 cells were incubated with 100 v.p./cell of Ad5-σ1 or Ad5Luc1 with or without anti-JAM1 antibody (JAM1 Ab) at the indicated concentrations. Luciferase activity was determined 24 h post-infection. (E) Analysis of Ad5-σ1 receptor usage in Hey cells. *C. perfringens* neuraminidase (NM), an anti-JAM1 antibody (JAM1 Ab), and recombinant Ad5 fiber knob protein (Ad5 knob) were employed to block Ad5-σ1 infection. Hey cells were either untreated or treated with 333 milliunits/ml neuraminidase, 100 μg/ml anti-JAM1 antibody, 50 μg/ml recombinant Ad5 fiber knob protein, both neuraminidase and an anti-JAM1 antibody or combined reagents with neuraminidase, anti-JAM1 antibody, and recombinant Ad5 fiber knob protein. Cells were incubated with Ad5-σ1 or Ad5Luc1 at 100 v.p./cell and harvested 24 h later for luciferase activity. All luciferase values were normalized against the activity of controls receiving no blocking treatment valued at 100%. Each data point is an average of four replicates. The error bars indicate standard deviation.

Table 1
Ad5-σ1 luciferase gene expression and co-receptor expression in various cell lines

Cell line	JAM1[a]	Sialic acid[b]	CAR[c]	CAR reference	Activity vs. Ad5Luc1[d]
Fold increase in luciferase					
L929	P	P	L/N	Flow cytometry data	45.3
OV-4	P	P	L/N	Flow cytometry data	5.8
Hey	P	P	L/N	Flow cytometry data	7.0
OV-3	P	P	L/N	Flow cytometry data	4.7
ES-2	P	P	L/N	Flow cytometry data	10.7
SK-OV-3	P	P	L/N	Kashentseva et al. [22]	4.4
OVCAR-3	P	P	M	Kelly et al. [21]	8.5
U118MG	L/N	P	L/N	Kim et al. [11]	6.1
U118MG-hCAR-tailless	L/N	P	P	Kim et al. [11]	5.5
RD	L/N	P	L/N	Cripe et al. [20]	6.4
FaDu	P	P	L/N	Kasono et al. [19]	3.9
CHO	L/N	P	L/N	Flow cytometry data	6.8
Lec2	L/N	N	L/N	Flow cytometry data	2.3

[a] P, highly JAM1-positive; L/N, little or none. As determined by Western blot analysis.
[b] P, highly sialic acid-positive; N, none. As determined by flow cytometric analysis.
[c] P, highly CAR-positive; M, moderate; L/N, little or none.
[d] Multiplicity of infection ranged from 10 to 1000 v.p./cell. Luciferase activity was measured at 24 h post-infection.

The σ1 protein has been reported to utilize the co-receptors JAM1 and sialic acid [8,9]. It has been shown that the σ1 knob-like head domain binds to JAM1 localized on the cell surface and that an anti-JAM1 antibody reduced reovirus replication 10- to 100-fold [9]. Similarly, in the presence of anti-σ1 or anti-JAM1 monoclonal antibodies, a 4-fold decrease in sialic acid-independent σ1 binding has been reported [9]. To further explore the role of JAM1 in Ad5-σ1 infection, we performed competitive blocking experiments using an anti-JAM1 antibody. We used the JAM1-positive L929 cell line for these studies. Exposure of L929 cells to 100 μg/ml anti-JAM1 antibody resulted in approximately 30% inhibition of Ad5-σ1 transgene expression (Fig. 4D). To further clarify Ad5-σ1 tropism, we performed neuraminidase treatment to remove cell-surface sialic acid and competitive blocking experiments using an anti-JAM1 antibody, and Ad5 knob protein. For this analysis, we used the low-CAR human ovarian cancer Hey cell line due to its high sialic acid and JAM1 expression. Transduction by Ad5-σ1 was inhibited 29% by neuraminidase, 42% by an anti-JAM1 antibody, and 50% by combined treatment with neuraminidase and an anti-JAM1 antibody (Fig. 4E). Combined treatment with neuraminidase, an anti-JAM1 antibody, and Ad5 knob protein reduced transduction 74% compared to controls receiving no blocking agent (Fig. 4E), with similar results in U118MG and OV-3 cells (data not shown). Together, these findings confirm that the Ad5-σ1 vector utilizes sialic acid and the JAM1-binding domain of the σ1 chimeric fiber (F5S1H) for cell transduction.

Ad5-σ1 vector exhibits increased transduction of CAR-deficient cells

To demonstrate the contribution of the σ1 chimeric fiber to Ad tropism expansion, we evaluated Ad5-σ1 infectivity in several cell lines. Table 1 shows the co-receptor expression profiles and infectivity of cell lines tested [19–22]. As expected, Ad5-σ1 provided the maximum increase in gene transfer (45-fold) relative to Ad5-Luc1 in L929 cells, which express σ1 receptors sialic acid and JAM1, but no detectable CAR (Table 1, Fig. 5A). In other sialic acid/JAM1-positive and low-CAR cancer cell lines, Ad5-σ1 also provided increased luciferase activity from 3.9-fold (FaDu) to 10.7-fold (ES-2) (Table 1, Fig. 5A). Furthermore, in cancer cells expressing only sialic acid that are JAM1/CAR-negative, Ad5-σ1 provided more than 6-fold higher luciferase activity relative to Ad5Luc1 in RD and U118MG cells (Table 1, Fig. 5A). Thus, we found that the presence of sialic acid and/or JAM1 co-receptors in cell lines contributed to the Ad5-σ1 infection via the usage of the σ1 chimeric fiber. To further demonstrate the σ1 chimeric fiber contribution to Ad5-σ1 infection, we selected a pair of cell lines, JAM1/CAR-negative CHO cells and its sialic acid-negative derivative Lec2 cells (Table 1, Fig. 5B). In CHO cells, Ad5-σ1 provided a 6.8-fold augmentation in luciferase activity versus Ad5Luc1, while infectivity enhancement of Ad5-σ1 on sialic acid-negative Lec2 cells was negligible, suggesting that Ad5-σ1 can exploit sialic acid as a co-receptor.

Many clinically relevant tissues are refractory to Ad infection, including ovarian cancer cells, due to negligible CAR levels [21]. To evaluate the Ad5-σ1 infectivity of patient tissue, we analyzed Ad5-σ1 transduction of primary human ovarian carcinoma cells. Importantly, Ad5-σ1 increased gene transfer to three unpassaged primary ovarian cancer patient samples from 3.9- to 13.5-fold versus Ad5Luc1 (Fig. 5C).

Herein, we have outlined the construction, rescue, purification, and initial tropism characterization of a novel vector containing a non-Ad fiber molecule. Our

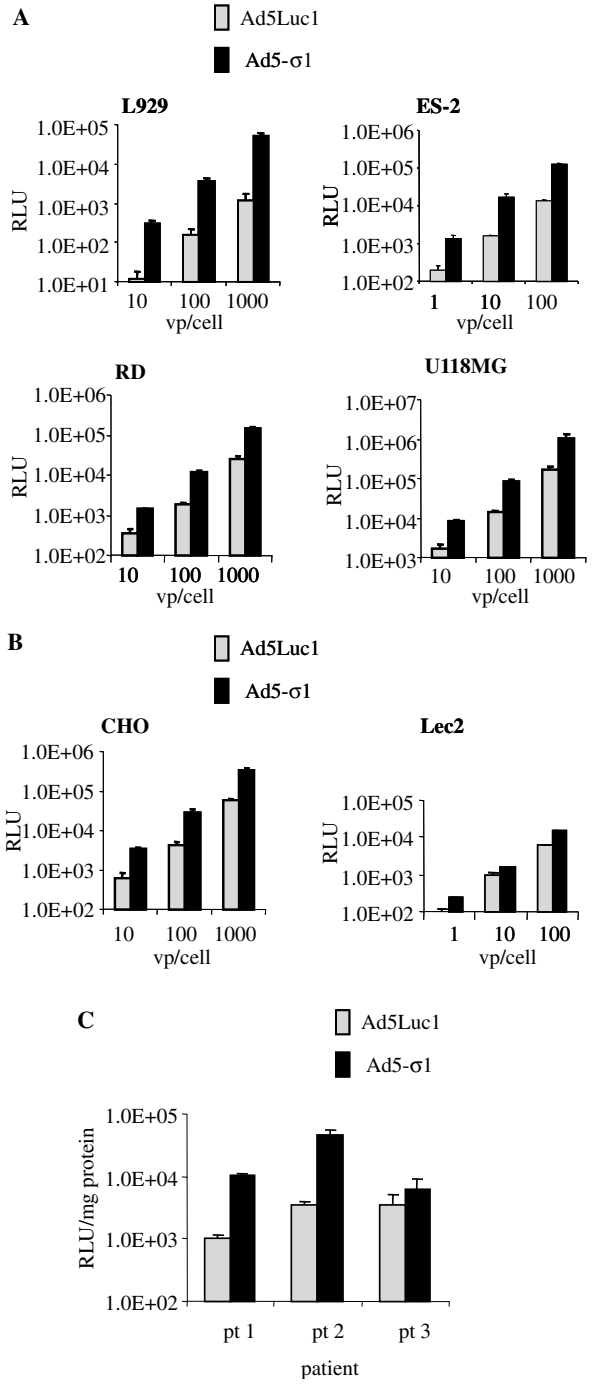

Fig. 5. Infectivity profiles of Ad5-σ1. (A,B) Representative cell lines. (A) Mouse fibroblast cells (L929), ovarian cancer cells (ES-2), human glioma cells (U118MG), and human embryonic rhabdomyosarcoma cells (RD), and (B) sialic acid-positive CHO and sialic acid-negative CHO derivative Lec2 cells were infected with Ad5Luc1 (gray bar) and Ad5-σ1 (black bar) at 1, 10, 100, and 1000 v.p./cell. Luciferase activity was measured 24 h post-infection and is expressed as relative light units (RLU). Each bar represents the mean of three experiments. The error bars indicate standard deviation. (C) Unpassaged primary ovarian cancer cells purified from patient ascites were infected with Ad5Luc1 (gray bar) and Ad5-σ1 (black bar) at 10 v.p./cell. Luciferase activity was measured 24 h post-infection and is expressed as RLU/mg protein. Each bar represents the mean of four experiments. The error bars indicate standard deviation.

results show that in low-CAR cells, Ad5-σ1 provides expanded tropism and increased gene transfer compared to wild-type Ad5 via an alternate infection pathway utilizing the reovirus co-receptors JAM1 and sialic acid. The expanded tropism of this vector represents a crucial attribute for Ad-based gene therapy vectors.

Discussion

A major obstacle to be overcome in Ad5-based cancer gene therapy is the paucity of the primary receptor, CAR, on human primary tumor cells. Variable, but usually low, expression of CAR has been documented in many cancer cell types including glioma, rhabdomyosarcoma, and ovarian cancer [4,11,20]. Thus, Ad gene therapy vectors with CAR-independent and/or expanded tropism may prove valuable for maximal transduction of low-CAR tumors at minimal vector doses.

To achieve expanded tropism by utilizing distinct receptors, we have established a new type of fiber mosaic Ad5 vectors, wherein two fibers derived from different virus families are incorporated in a single virion. This is a novel approach to genetically modify Ad5 vector tropism by means of adding the reovirus receptor-binding spike (σ1) protein to the Ad5 capsid. We selected the spike from reovirus T3D due to its ability to infect numerous tumor cell types that express either JAM1 or sialic acid [23,24].

The main technical feasibility for this new vector is the structural similarity between Ad fiber and the reovirus σ1 protein, which is a trimeric fiber-like molecule protruding from the 12 vertices of the icosahedral reovirus virion [25]. The crystal structure of the reovirus σ1 attachment protein reveals an elongated trimer with two domains: a compact head with a β-barrel fold and a fibrous tail containing a triple β-spiral [25]. The σ1 protein contains two receptor-binding domains: one within the fibrous tail that binds sialic acid [8] and the other in the globular head that binds to JAM1 [9].

In designing a σ1 chimeric fiber, we considered the Ad5 tail portion to be indispensable for incorporation of a σ1 chimeric fiber into the Ad5 penton base. We therefore designed our σ1 chimeric fiber to contain the Ad5 tail and reovirus σ1. The entire σ1 molecule was included since it contains receptor-binding domains in both the tail and head regions [26]. In addition, we engineered a 6-His tag into the C-terminus of σ1 for protein detection. We were initially concerned that the incorporation of the 6-His motif could alter JAM1 recognition, since the C-terminus is proximal to the predicted JAM1-binding motif in the D-E loop of the β-barrel structure [25]. To our knowledge, however, there are no reports suggesting that C-terminal additions are deleterious to normal σ1/JAM1 interaction(s). Indeed, it is unlikely that the 6-His tag interferes significantly with the σ1/JAM1 interaction

since the contribution of JAM1 to the tropism of our mosaic virus was sufficient to result in a 42% decrease in luciferase activity in the presence of an anti-JAM1 antibody (Fig. 5E). Further, we expect that the sialic acid-binding domain localized to the σ1 tail domain would remain unaffected by the C-terminal tag [8,27].

We confirmed the trimerization of the F5S1 σ1 chimeric fiber and constructed an Ad5 genome encoding a tandem fiber cassette, resulting in an Ad5 vector expressing both the Ad5 fiber and a σ1 chimeric fiber. We confirmed that the fiber mosaic Ad5 virions incorporated both fibers by Western blot analysis and by characterizing the functional ability of both fibers to utilize the appropriate receptor(s) for viral transduction.

Consistent with our hypothesis of enhanced infectivity, we observed augmented gene transfer with Ad5-σ1 in all cell lines tested, ranging from 2.3- to 45-fold. This augmentation was variable and often occurred in cell lines with supposedly similar receptor expression profiles. We believe this variation is due to variable receptor expression between cell lines. In this regard, we wish to highlight that the semi-quantitative methodology (Western blot analysis and FACS) used to determine receptor expression in these lines likely masks minor receptor variations between cell lines that accounts for the observed results. Importantly, the observed expanded tropism of Ad5-σ1 extended to a stringent clinical substrate, human primary ovarian tumor tissue, although the augmentation of gene transfer was also variable. While primary ovarian cancer cells are often low in CAR, the specific receptor profiles are unknown. On this basis, the variability in gene transfer very likely reflects natural variability of CAR, JAM1, and sialic acid expression between individual patient samples. These results serve to highlight the utility of Ad vectors that exhibit expanded tropism via utilization of multiple receptors. This vector capacity would be of importance in any future clinical application wherein tissue receptor expression is poorly defined.

During the course of this work, Mercier et al. [28] reported the construction and characterization of an Ad vector containing only the reovirus σ1 fiber. This vector demonstrated JAM1- and SA-dependent tropism that was CAR-independent, resulting in an Ad vector with reovirus tropism only. Mercier demonstrated a 3-fold infectivity enhancement in human dendritic cells relative to Ad5, but did not report any infectivity enhancement in human cancer cells or other substrates. In contrast, we have used the concept of "fiber mosaicism," the use of two separate fibers with distinct receptor recognition, to provide maximum enhanced infectivity via use of multiple receptors. On this basis, we assert that our fiber mosaic Ad5-σ1 vector could offer distinct advantages over Ad vectors with single receptor recognition in the context of an infectivity-enhanced vector for cancer applications.

Our goal was to create a vector with expanded tropism to achieve maximum infectivity enhancement utilizing multiple receptors in CAR and non-CAR cell entry pathways. In this study, the fiber mosaic Ad5-σ1 vector provided enhanced infectivity in low-CAR cancer cell lines resulting from multi-receptor binding properties derived from different virus families. It is our ultimate intent to exploit mosaic adenovirus paradigms in various combinations in order to accomplish additivity or synergism of infectivity enhancements. On this basis, the infectivity gains we demonstrate in this study may contribute to a combinational approach of clinical relevance.

Acknowledgments

This work was supported by the following grants from the National Institute of Health: R01 CA83821, R01 CA94084, and R01 CA93796; Department of Defense W81XWH-05-1-0035; Ovarian Cancer SPORE P50 CA83591 and Breast Cancer SPORE P50 CA89019. We thank Dr. Victor Krasnykh for providing plasmids pVK700 and pNEB.PK.3.6. We also thank Dr. Joanne T. Douglas for providing cell line U118MG-hCAR-tailless, Dr. Dan J. Von Seggern for the cell line 211B, and Dr. Jerry L. Blackwell for the RmcB antibody. We also thank Dr. Maaike Everts for critical reading of the manuscript.

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