effects [8]. In fact, delivery of growth factor genes that promote regeneration of neurons also has shown to promote glial scar formation [9–11].

Several attempts have been made for selective gene delivery into neurons. Steffen *et al.* [12] reported neuroblastoma-specific gene expression using tyrosine hydroxylase promoter. In addition, Huang *et al.* [13] developed vectors using a combination of neuron restrictive silencer elements, hypoxia responsive elements, and CMV minimal promoter. These vectors allowed gene expression selectively in neurons in a hypoxia-regulated manner. By contrast to these promoter driven approaches, there is another approach via specific target receptors. Thus far, the receptor for glial cell line-derived neurotrophic factor and high-affinity laminin receptors have been proposed as neuron-specific targets [14,15]. However, optimal target receptors for gene therapy for neurological disorders remain to be defined.

Recently, fiber mutant forms of adenovirus designated as FZ33 [16] and FdZ [17–19] have been introduced as targeting tools. Both FZ33 and FdZ adenovirus vectors contain the immunoglobulin (Ig) G binding motif, thereby incorporating into target cells together with specific IgG monoclonal antibody (mAb). In the present study, we screened hybridomas to define those that produce IgG mAb specifically reacting with the rat neuronal cell line PC12. Subsequently, we evaluated the efficacy and specificity of gene transfer into the neuron cells by FZ33 and FdZ adenovirus vectors along with the established mAb.

Materials and methods

Cell culture

The cell lines used were rat pheochromocytoma cell lines, PC12; rat kidney cell line, NRK; rat glioma cell line, C6; rat thymoma cell line, MTHC-1; human embryonic kidney cell line, 293T; and mouse myeloma cell line, P3U1. All cell lines except P3U1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). P3U1 was cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS. All the cells were cultured at 37 °C in a 5% CO_2 incubator.

Construction of plasmids

FZ33 type adenovirus vector containing the IgG binding Z33 motif at the HI loop of the Ad5 fiber knob was generated as Adv-FZ33, as described previously [20]. Adv-FZ33 has been proven to have the ability to incorporate into target cells via both coxsackie and adenovirus receptor (CAR) and the IgG binding motif [20].

FdZ type adenovirus vector is a modified form of Adv-FZ33 wherein four amino acids, TAYT (d489–492) in the FG loop, were deleted to ablate the CAR-binding motif [17–19]. Detailed information regarding these vectors is available on request. For plasmid construction: the FdZ fragment containing d489–492 and Z33 motif was generated by polymerase chain reaction (PCR) using the primers #2201 (71-mer; TGCCAGAATATTGGAATTTTCGAAACGGAGATCT-CACAGAGGGTAACGCTGTTGGATTTATGCCTAACCTA) and #2202 (29-mer; GTGGATCCTTATTCTTGGGCAAT-GTATGA), with the pSKII + Z33 plasmid as template. The PCR product was digested with *Bst*XI and *Bam*HI, and the 438 bp fragment was inserted into the *Bst*XI/*Bam*HI sites of pWE6.7R-F/wt2 [21], resulting in pWE6.7R-FdZ. pAx3isi was derived from pAx3 by inserting an I-*Sce*I linker/*Cla*I adapter sequence (ATCGATATTACCCT-GTTATCCCTAATCGAT) into the *Cla*I site of pAx3. The *Rsr*II/I-*Sce*I fragment from pAx3isi containing the left end of the Ad5 genome (4 kbp), I-*Sce*I/*Eco*RI fragment from pAx3isi containing the central 24 kbp of the Ad5 genome, and *Eco*RI/*Rsr*II fragment from pWE6.7R-FdZ were ligated together, resulting in pAx3i-FdZ. From the plasmid pCAZ3 [21], β-Gal expression cassette (CAZ3, 5153 bp) was cut by *Bam*HI/*Bgl*II digestion, blunted, and ligated into the SwaI site of pAx3i-FdZ, resulting in pAx3iCAZ3-FdZ.

Construction of recombinant adenoviruses

Each cosmid was linearized by *Pac*I digestion and transfected into 293 cells using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA). Plaques arising from the transfected 293 cells were isolated and evaluated by restriction enzyme digestion of the viral genome. The resultant adenoviral vectors were expanded in the 293 cells and purified by cesium chloride ultracentrifugation. Purified viruses were dialysed in phosphate-buffered saline (PBS) with 10% glycerol and stored at −80 °C until further use. To determine the viral concentration (vp/ml), the viral solution was incubated in 0.1% sodium dodecyl sulfate (SDS), and the absorbance at 260 nm (A_{260}) was measured [22]. The concentration was defined as vp/ml $= A_{260} \times (1.1 \times 10^{12})$. Before using adenovirus, contamination with replication-competent viruses in the viral stock was ruled out by PCR analysis using primers described previously [23]. Adenoviral infection was performed essentially as described previously [24].

Production and screening of hybridomas

A female Balb/c mouse, aged 6 weeks, was injected intraperitoneally with PBS containing 1×10^6 PC12 cells every 10 days. The final boost was then performed with the same number of PC12 cells intravenously 10 days after the fifth injection. Three days after the final boost, the mouse was sacrificed and 1×10^8 splenocytes from the mouse were fused with 2×10^7 P3U1 cells

using polyethylene glycol. When the hybridomas had grown to approximately 50% confluence, the culture supernatant fluid was tested for antibody activity. The PC12 cells were prepared in 96-well microplates at a concentration of 1×10^4 cells per well. After the removal of the culture medium, supernatant fluid was added to each well and the microplates were incubated for 1 h at 4 °C. The supernatant fluid was then removed and the microplates were washed with PBS. Fifty microliters of AdvZ3-FZ33 prepared in FBS-free DMEM at a concentration of 6×10^7 vp/ml [multiplicity of infection (MOI) = 300 vp/cell] was added to each well, and the microplates were incubated for 1 h at 4 °C. Then, the microplates were washed twice with PBS and incubated at 37 °C in a 5% CO_2 incubator. Twenty-four hours after infection, chemiluminescent β-Gal reporter gene assays (Roche Diagnostics, Mannheim, Germany) were performed according to the company's recommendations. The hybridomas that showed high β-Gal activity were selected. The same assays were performed several times on the selected wells. Finally, the hybridomas were cloned by limiting dilution. Using a commercial kit (Roche Diagnostics), one, 6E3, was shown to be IgG_1 kappa isotype. The mouse monoclonal antibody was purified using protein G-sepharose beads (Amersham Bioscience, Uppsala, Sweden).

Flow cytometric analysis

In the experiments examining the efficacy of gene transfer by Adv-FZ33 in combination with mAb6E3, rat cell lines were prepared in 12-well plates at a concentration of 3×10^5 cells per well, respectively. After culturing overnight, the culture medium was replaced with 500 µl of FBS-free DMEM in the presence or absence of the mAbs (2 µg/ml of mAb6E3 or $mIgG_1$) and incubated for 1 h at 4 °C. $mIgG_1$ (MOPC-21; BD PharMingen, San Diego, CA, USA) was used as a control. Then, the microplates were washed with PBS. Next, 500 µl of AdvEGFP-FZ33 in FBS-free DMEM at a concentration of 1.8×10^8 vp/ml (MOI = 300 vp/cell) was added to each well, and the plates were incubated for 1 h at 4 °C. The wells were then washed twice with PBS and the culture medium was added to each well. They were incubated at 37 °C in a 5% CO_2 incubator for 24 h. The efficiency of EGFP gene transfer was determined by measuring green fluorescent protein activity using a FACS-Calibur system (Becton Dickinson, San Jose, CA, USA).

The reactivity of cell lines with mAb6E3 was also examined by flow cytometry using FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) as the secondary antibody.

Immunofluorescent staining

Immunofluorescent staining was conducted with the PC12 cell cultures, neuron–glia cocultures, and rat spinal cord sections.

The PC12 cells were prepared on collagen-coated slides (BD Bioscience, Bedford, MA, USA) at a concentration of 2×10^5 cells per well and incubated overnight. After washing with PBS, the culture medium was replaced with FBS-free DMEM in the presence of the mAbs (1 µg/ml of mAb6E3 or $mIgG_1$) and incubated for 1 h at 4 °C. The medium was again replaced with FBS-free DMEM containing AdvZ3-FZ33 or AdvZ3-FdZ (1.8×10^8 vp/ml: MOI = 300 vp/cell) and incubated for 1 h at 4 °C. Next, the wells were washed twice with PBS and incubated at 37 °C for 24 h. Samples were soaked in paraformaldehyde (4% in 0.1 M phosphate buffer, pH7.4) for 30 min at room temperature. Then, the samples were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked with 0.2% Triton X-100, 0.2% bovine serum albumin, and 5% normal goat serum (Dako, Glostrup, Denmark) in PBS for 30 min at room temperature. Mouse anti-β-Gal monoclonal antibody (DC1-4C7: Promega, Madison, WI, USA: 2 µg/ml) was added to the blocking buffer and incubated for 1 h. The samples were then incubated with Alexa Fluor 594 goat anti-mouse IgG (1 : 500 Molecular Probes, Eugene, OR, USA) as the secondary antibody for 1 h. After washing, the samples were counterstained with 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI; Vector Laboratories, Inc. Burlingame, CA, USA) and examined under a fluorescence microscope.

For immunostaining the neuron–glia cocultures, the Nerve-Cell Culture System (Sumitomo Bakelite Co., Tokyo, Japan) was used. Cells were prepared on poly L-lysine-coated slides (BD Bioscience, Bedford, MA, USA). After the cells had adhered and sprouted fibers, they were treated with the mAbs (1 µg/ml of mAb6E3 or $mIgG_1$), followed by infection with AdvZ3-FdZ as described above at the MOI of 100,300 and 1000. They were double-stained with DC1-4C7 and each of the rabbit anti-MAP-2 polyclonal antibody (1 : 500; Chemicon, Temecula, CA, USA), rabbit anti-GalC polyclonal antibody (1 : 500; Chemicon) or rabbit anti-glial fibrilliary acidic protein (GFAP) polyclonal antibody (1 : 500; Dako, Glostrup, Denmark). Alexa Fluoro 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) was used as the secondary antibody. The number of β-Gal-positive neurons was counted at three randomly selected fields and expressed as a percentage of all neurons at the fields.

For immunostaining of the spinal cord, 12-week-old, female Sprague-Dawley rats were perfused transcardially with PBS followed by 4% paraformaldehyde under deep anesthesia. The spinal cord was excised at the Th10 level and fixed with 4% paraformaldehyde overnight. The fixed spinal cord was then equilibrated in PBS containing 30% sucrose for 24 h and then embedded in OCT compound. Horizontal sections of the spinal cord were made. Following permeabilization and blocking, the sections were double-stained with mAb6E3 and each of rabbit anti-MAP-2 polyclonal antibody, rabbit anti-GalC polyclonal antibody or rabbit anti-GFAP polyclonal

DOI: 10.1002/jgm

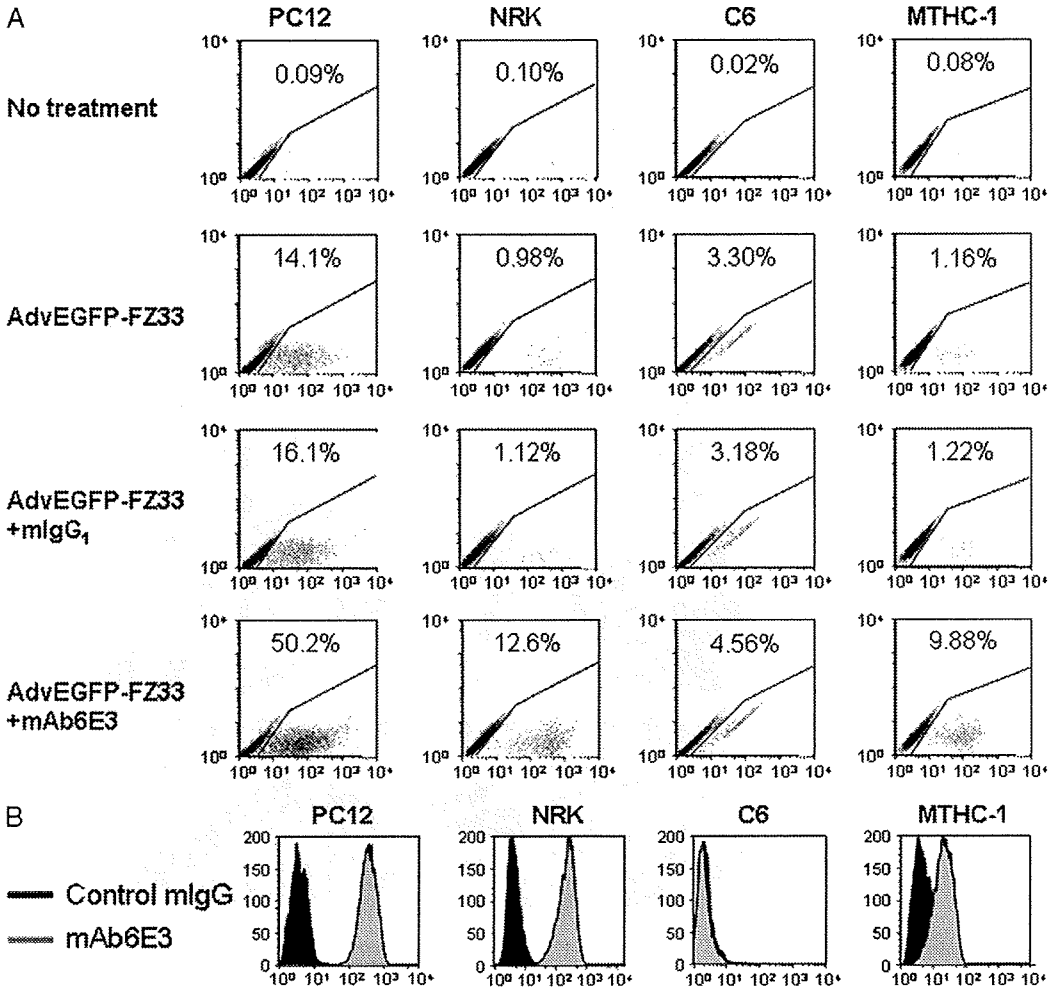

Figure 1. Efficacy of mAb6E3-targeted gene transfer and expression of the 6E3 antigens. (A) Efficiency of gene transfer into cell lines using AdvEGFP-FZ33 and mAb6E3. Numbers in each panel indicate the percentage of cells expressing EGFP. Cell lines used were PC12 (rat pheochromocytoma cell line), NRK (rat kidney cell line), C6 (rat glioma cell line), and MTHC-1 (rat thymoma cell line). Treatment with AdvEGFP-FZ33 and mAb6E3 resulted in enhanced gene transfer efficacy in PC12, NRK and MTHC-1. (B) Cell surface expression of the 6E3 antigen. Reactivity of cell lines with mAb6E3 was determined by flow cytometry. The 6E3 antigen was expressed by PC12, NRK and MTHC-1, but not C6, which failed to show enhanced gene transfer efficacy by mAb6E3-targeted gene transfer

antibody, followed by an incubation with the secondary antibody.

Chemiluminescent β-Gal reporter gene assay

PC12 and C6 cell lines were prepared in 96-well plates at a concentration of 1×10^4 cells per well, respectively. They were divided into six groups: AdvZ3-FZ33 alone, AdvZ3-FdZ alone, AdvZ3-FZ33 with control $mIgG_1$, AdvZ3-FdZ with control $mIgG_1$, AdvZ3-FZ33 with mAb6E3, and AdvZ3-FdZ with mAb6E3. Each group was assayed with the MOI of 300, 1000, 3000 and 10000 vp/cell. After removing the culture medium, 50 µl of FBS-free DMEM containing 100 ng of mAb was added to each well and incubated for 1 h at 4 °C. The medium was removed and the microplates were washed with PBS. Fifty microliters of adenovirus prepared in FBS-free DMEM at the indicated concentrations was added to each well and incubated for 1 h at 4 °C. The microplates were then washed twice with PBS and incubated at 37 °C in a 5% CO_2 incubator. Twenty-four hours after infection, chemiluminescent β-Gal reporter gene assays were performed according to the manufacturer's instructions. The experiments were repeated four times.

Immunoprecipitation and mass spectrometry

PC12 cells (4×10^7) were prepared and the cell surfaces were biotinylated (Pierce, Rockford, IL, USA). Membranes were solubilized on ice for 30 min in

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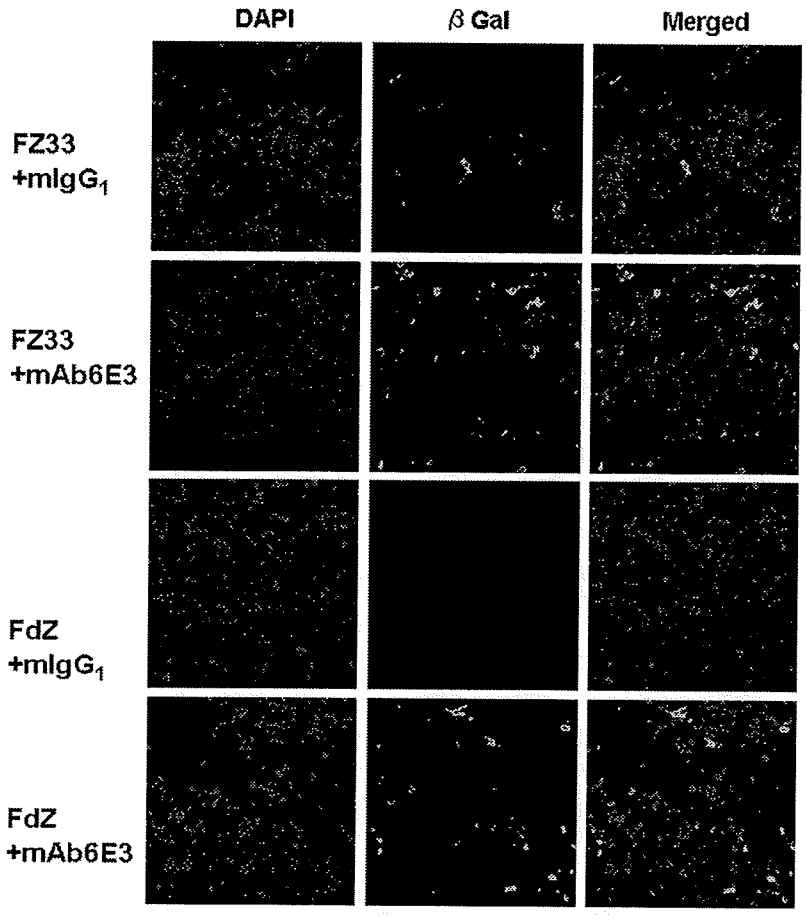

Figure 2. Expression of β-Gal in PC12 cells treated with fiber-modified adenovirus vectors and mAb6E3. PC12 cells were treated with adenovirus vectors (AdvZ3-FZ33 or AdvZ3-FdZ) and mAbs (control $mIgG_1$ or mAb6E3). DAPI indicates counterstained nuclei. PC12 cells treated with AdvZ3-FZ33 showed positive reactivity with anti-β-Gal mAb regardless of whether mAb6E3 was applied in combination. By contrast, AdvZ3-FdZ required mAb6E3 for successful gene transfer because none of the PC12 cells treated with AdvZ3-FdZ and $mIgG_1$ were positive for anti-β-Gal mAb

1 ml buffer containing 1% NP40, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and protease inhibitor cocktail (Roche Diagnostics). Samples were cleared of nuclear fragments by centrifugation for 20 min at 15 000 *g* at 4 °C. The samples were mixed with protein G-Sepharose beads and incubated for 2 h at 4 °C, and the beads were then centrifuged to remove nonspecifically bound proteins. Five micrograms of mAb6E3 and control $mIgG_1$ (eBioscience, San Diego, CA, USA) were added to the supernatant of each sample and allowed to incubate for 1 h at 4 °C. The immunocomplexes were precipitated by the addition of protein G-Sepharose beads to each sample and incubated for 2 h at 4 °C. The supernatant was discarded and the beads were washed six times with solubilization buffer. The immunocomplexes binding with beads were boiled for 5 min in 20 µl of SDS sample buffer containing 5% 2-mercaptoethanol. The samples were separated using 5–20% gradient polyacrylamide gels (BioRad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (Millipore, MA, USA). After blocking with 5% milk in TBS [10 mM Tris-HCl, (pH 7.5), 150 mM sodium chloride, 0.05% Tween-20], the membranes were incubated for 1 h at room temperature with 20 µl (per 40 ml) of avidin-horseradish peroxidase (Amersham Bioscience, Bucks, England). Detection was conducted by enhanced chemiluminescence (Amersham Bioscience) according to the manufacturer's instructions.

For mass spectrometry, The PC12 cells (1×10^9) were solubilized in 20 ml buffer as described above. After the removal of nuclear fragments by centrifugation, the samples were mixed with protein G-Sepharose beads and incubated over night at 4 °C to remove nonspecifically bound proteins. Five micrograms of mAb6E3 and control $mIgG_1$ were added to the supernatant of each sample and allowed to incubate for 2 h at 4 °C The immunocomplex was precipitated by addition of protein G-Sepharose beads to each sample and incubated for 2 h at 4 °C. The samples were then separated by SDS/PAGE as described above. The polyacrylamide gel was silver stained using a Silver Stain kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according

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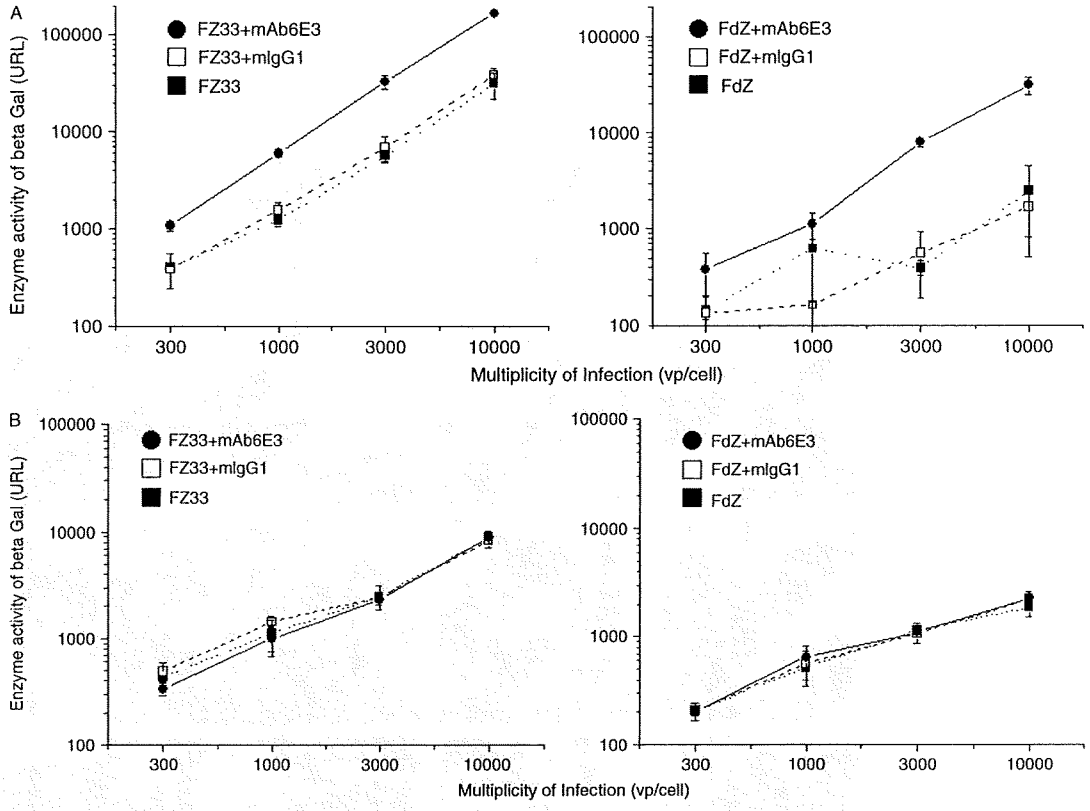


Figure 3. Chemiluminescent β-Gal reporter assays with fiber-modified adenovirus vectors and mAb6E3. (A) Transfer efficiency into PC12 cells. The cells were lysed and assayed for β-Gal activity using a commercial kit. The left panel indicates the efficiency of gene transfer into PC12 cells with AdvZ3-FZ33 alone (FZ33), AdvZ3-FZ33 and control $mIgG_1$ (FZ33 + $mIgG_1$), and AdvZ3-FZ33 and mAb6E3 (FZ33 + mAb6E3). The right panel indicates the efficiency of gene transfer with AdvZ3-FdZ alone (FdZ), AdvZ3-FdZ and control $mIgG_1$ (FdZ + $mIgG_1$), and AdvZ3-FdZ and mAb6E3 (FdZ + mAb6E3). Each dot represents the mean ± SD of the four experiments. (B) Transfer efficiency into C6 cells. In the same experiments with C6 cells, there were no increases in mAb6E3-targeted gene transfer efficacy mediated by AdvZ3-FZ33 or AdvZ3-FdZ

to the manufacturer's instructions. Specifically stained protein bands were extracted from the gel, digested by trypsin, and analysed by oMALDI-Qq-TOF MS/MS QSTAR Pulsar i (Applied Biosystems Japan Ltd, Tokyo, Japan).

Cloning and transfection of Rat Na,K-ATPase cDNAs

The cDNAs of rat Na,K-ATPase α1–4 (pRc alpha 1–4) were provided by Dr Lingrel (University of Cincinnati, USA). Total RNA of rat Na,K-ATPase β1–3 and γ a, b and c was isolated from the PC12 cells using RNeasy mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcriptase-PCR. The oligonucleotide primers used to examine gene expression were: rat Na,K-ATPase β1 forward primer 5′-AGCAGACACCATGGCCCGCGGAA-3′, reverse primer 5′-GTGCTTGTGATCAGCTCTTAACTTCAA-3′; rat Na,K-ATPase β2 forward primer 5′-CGTGCCTCCAAGATGGTC-ATCCAAA-3′, reverse primer 5′-GGAGCCTCAGGCTTTGT-TGATTCGA-3′; rat Na,K-ATPase β3 forward primer 5′-TTCCACTCGCCCATCATGACGAAGA-3′, reverse primer 5′-GCCTACTCCTAGGCATGTGCTATGA-3′; rat Na,K-ATPase γa forward primer 5′-GAAATGACAGAGCTGTCA-GCTAACCA-3′; rat Na,K-ATPase γ b forward primer 5′-TCAGCTAACCATGGTGGCAGTGCAG-3′; rat Na,K-ATPase γ c forward primer 5′-CCACCATGGACAGGTGGTACTTG-GGTGGC-3′; rat Na,K-ATPase γ a, b, and c reverse primer 5′-TCTTCCGTCACAGCTCATCTTCATTG-3′. The resulting cDNA was ligated into the pTarget vector (Promega). Each cDNA was transfected into 293T cells using LipofectAMINE Plus reagent (Invitrogen) by incubating in Opti-MEM medium (Gibco BRL) at 37 °C for 48 h. The 293T cells transfected with each cDNA of Na,K-ATPase and the rat cell lines were suspended in staining medium [PBS, 2% FBS, 0.05% NaN_3, and 1 mg/ml propidium iodide (PI)] containing saturating amounts of mAb6E3 or $mIgG_1$ (MOPC-21; BD PharMingen) as a control. The reactivity of cells with mAb6E3 and $mIgG_1$ was determined by flow cytometry using a FACS Calibur system. Dead cells defined by positive staining with PI were gated out from the analysis.

DOI: 10.1002/jgm

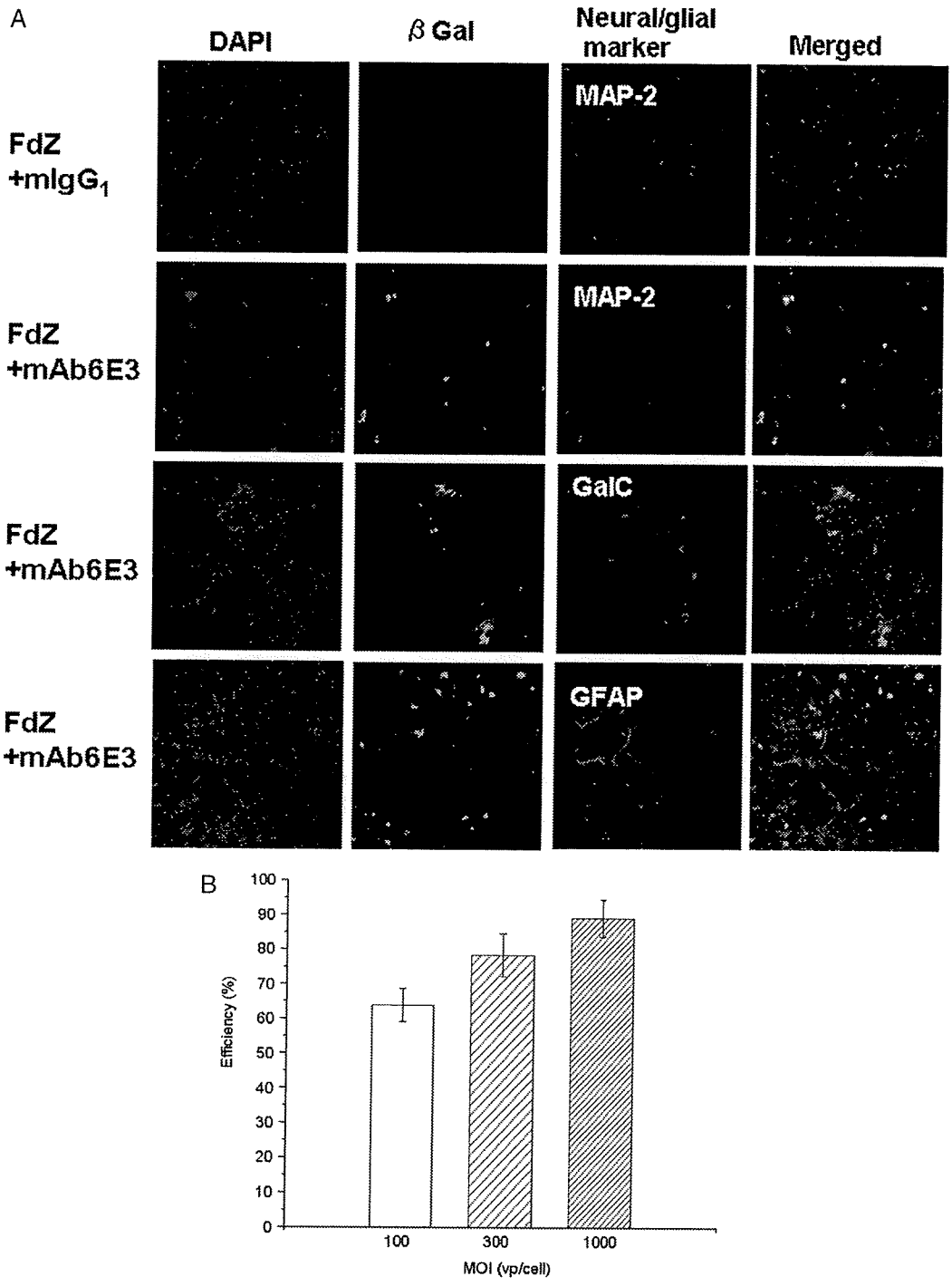


Figure 4. *In vitro* gene transfer into neuron–glia cocultures. (A) Immunofluorescent image at the MOI of 300. Cocultured neural cells were treated with adenovirus vectors (AdvZ3-FZ33 or AdvZ3-FdZ) and mAbs (control $mIgG_1$ or mAb6E3). Then, they were immunolabeled with anti-β-Gal mAb (green) and antibodies for neural markers (red; MAP2, GalC and GFAP). DAPI (blue) was used for counterstaining of the nuclei. (B) Quantification of gene transfer efficiency into the primary neuron. The number of β-Gal-positive neurons was counted at random in three selected fields and expressed as a percentage of all neurons at the fields

RNA interference transfection

PC12 cells were transfected with rat Na,K-ATPases β1-specific siRNAs (Qiagen Atp1b1 siRNA, catalog number SI01490307 and SI01490314) or a negative control siRNA (Qiagen AllStars negative control siRNA, catalog number 1 027 280) using HiPerfect reagent (Qiagen). The transfection mixtures were prepared in total 100 μl

containing 3 μl of HiPerfect and siRNA (final 5 nM) in serum-free Opti-MEM medium. The transfection mixtures were then transferred to 24-well plate and overlaid with 0.5 ml of PC 12 cell suspension (10^5/ml) in serum-containing medium. The cells were then placed at 37 °C for the indicated times. The surface antigen recognized by mAb6E3 was measured by flow cytometry. Mean fluorescence intensity (MFI) ratios were calculated as MFI of the mAb6E3 divided by MFI of corresponding mIgG1 k (MOPC-21) isotype control. Experiments were carried out in triplicate. MFI ratio of transfectants were compared and statistically analysed by Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Development of monoclonal antibody, 6E3, and targeted gene transfer

To develop mAbs suitable for neuron-cell specific gene transfer using FZ33 fiber-modified adenovirus vectors, we immunized PC12 cells in mice and screened hybridomas by β-Gal reporter gene assay. β-Gal activity reflects the transfer efficiency of β-Gal gene into the PC12 cells via the antigen recognized by the antibodies secreted from the hybridoma. We found that β-Gal activity of the 6E3 well was constantly higher than that of other wells in the repeated assays. Thus, we cloned the hybridomas from the 6E3 well by limiting dilution and established the hybridoma, 6E3, that secrets mAb6E3 of the IgG_1 kappa isotype.

Subsequently, we examined the effects of mAb6E3 in Adv-FZ33-mediated gene transfer into several cell lines. As shown in Figure 1A, the efficacy of EGFP gene transfer by Adv-FZ33, as defined by the EGFP activity of cells, increased dramatically in the PC12 cells when the cells were treated with both AdvEGFP-FZ33 and mAb6E3. Such increase in the gene transfer efficacy was also observed in the NRK kidney and MTHC-1 thymoma cell lines, but not in the C6 glioma cell line. Notably, there were approximately 15% of the EGFP activities in the PC12 cells even when the cells were treated with AdvEGFP-FZ33 alone or AdvEGFP-FZ33 with the control mouse IgG_1 ($mIgG_1$). These activities are likely mediated via endogenous CAR-binding domain in Adv-FZ33.

Figure 1B shows cell surface expression of the 6E3 antigen in these cell lines. In line with mAb6E3-targeted gene transfer efficacy, PC12, NRK and MTHC-1 cells, but not C6 cells, expressed the mAb6E3 antigen.

mAb6E3-targeted gene transfer via Adv-FdZ

To specifically target mAb6E3 in gene transfer, we generated FdZ type adenovirus (Adv-FdZ) that lacks a CAR-binding motif. Thus, Adv-FdZ predominantly uses the IgG-binding motif to incorporate into cells. Figure 2 shows the efficacy of mAb6E3-targetted gene transfer into the PC12 cells via AdvZ3-FZ33 and AdvZ3-FdZ. As shown, AdvZ3-FZ33 transferred β-Gal gene into 5.2% of PC12 cells in the absence of mAb6E3, as defined by positive staining with anti-β-Gal mAb. This is probably due to the CAR-binding domain of AdvZ3-FZ33. By contrast, none of the PC12 cells treated with AdvZ3-FdZ and control $mIgG_1$ were positive for anti-β-Gal mAb. Notably, 40% of PC12 cells were stained positively with anti-β-Gal mAb when they were treated with both AdvZ3-FdZ and mAb6E3. These findings indicate the dependency of AdvZ3-FdZ-mediated gene transfer on mAb6E3.

In addition, we compared the capacity of AdvZ3-FZ33 and AdvZ3-FdZ in mediating mAb6E3-targetted gene transfer into the PC12 cells and C6 cells by chemiluminescent β-Gal reporter assays (Figure 3). In PC12 cells (Figure 3A), there was a linear correlation between β-Gal activity and viral concentration in all the experimental conditions, especially in those involving AdvZ3-FZ33. AdvZ3-FZ33 exhibited gene transfer efficacy, which was approximately five-fold higher in the conditions with mAb6E3 than in those without mAb6E3. By contrast, AdvZ3-FdZ showed approximately a 20-fold difference in gene transfer efficacy between the conditions in the presence and absence of mAb6E3. By contrast to PC12 cells, addition of mAb6E3 did not increase the gene transfer efficacy mediated by AdvZ3-FZ33 or AdvZ3-FdZ (Figure 3B) in C6 cells, indicating the dependency of gene transfer on the expression of the 6E3 antigen. The difference in gene transfer efficacy in the absence of mAb6E3 between PC12 and C6 cells likely reflects their distinct expression levels of integrins and CAR.

These findings are consistent with the structural characteristics of AdvZ3-FdZ that lacks the CAR-binding domain, thereby depending on IgG-binding domain for its gene transfer. Based upon these findings, we then focused on Adv-FdZ in the subsequent experiments.

Neuron-selective gene transfer in neuron–glia cocultures

Using Adv-FdZ, we examined whether mAb6E3-targeted gene transfer led to neuron-specific expression of the gene product. To address this *in vitro*, we used a kit composed of primary cultures from fetal rat cerebral cortex. The culture consists of three types of cells; neuron cells, oligodendrocytes, and astrocytes, as defined by the expression of specific markers MAP-2, GalC, and GFAP, respectively (Figure 4A). These mixed cell cultures were treated with AdvZ3-FdZ or a combination of AdvZ3-FdZ and mAb6E3. Figure 4A shows the immunofluorescent image at the MOI of 300. β-Gal gene was introduced into the cell culture by AdvZ3-FdZ depending on mAb6E3. Notably, the cells positive for anti-β-Gal mAb simultaneously were positive for anti-MAP-2 antibody. No such correspondence was observed in the expression of β-Gal and GalC or GFAP, indicating the specificity of mAb6E3-targeted gene transfer via Adv-FdZ into neuron

DOI: 10.1002/jgm

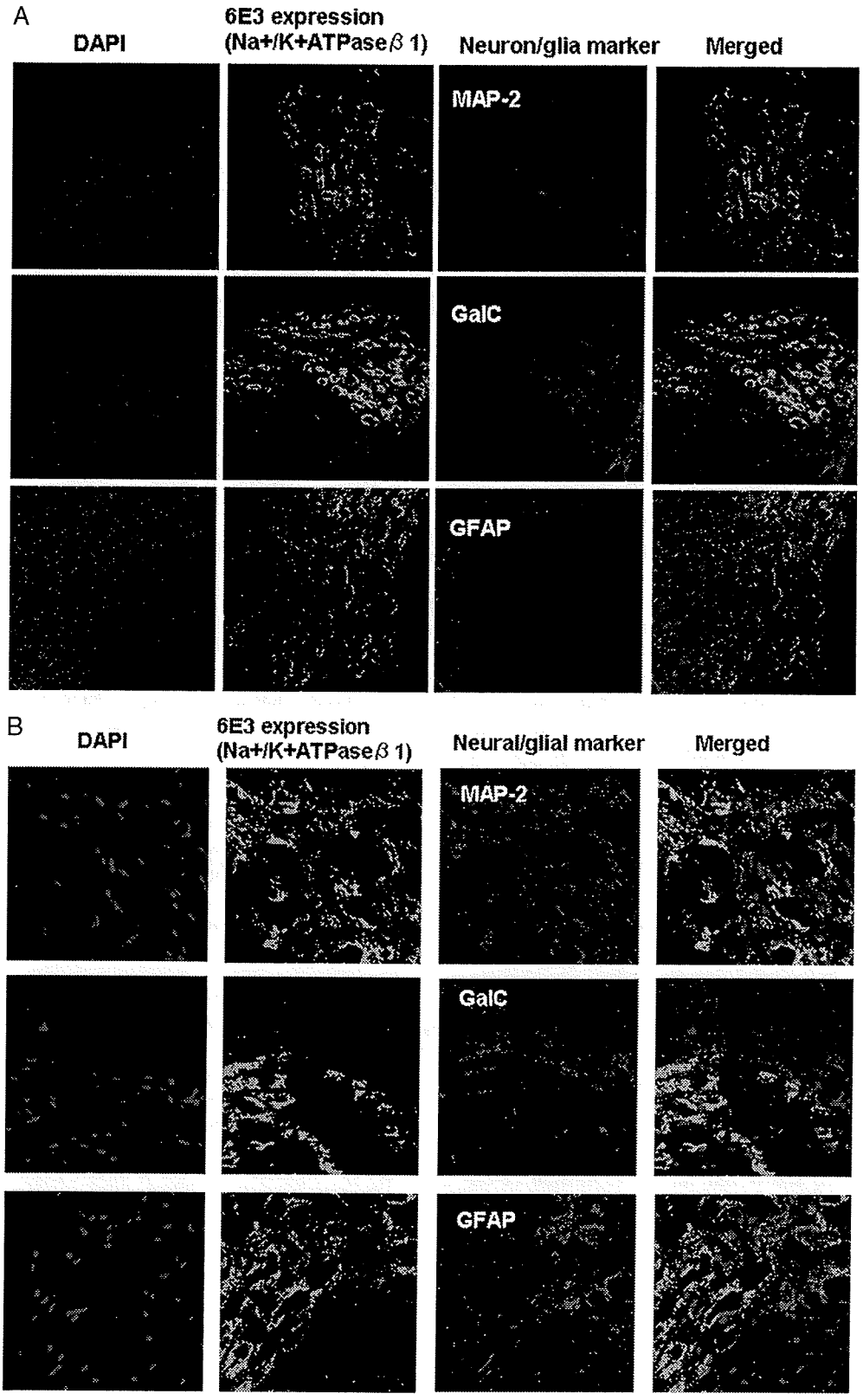
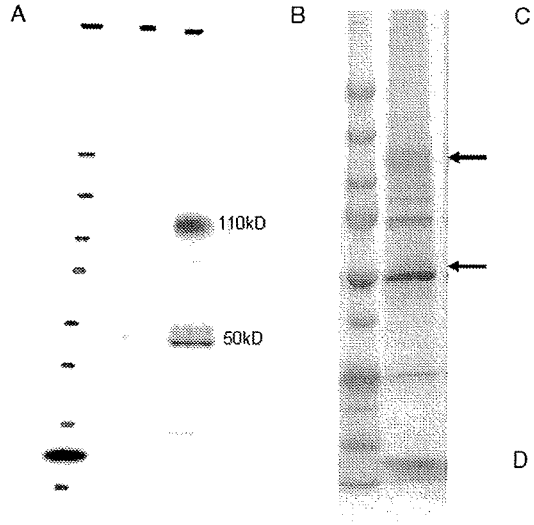


Figure 5. Immunostaining of rat spinal cord tissue with mAb6E3. (A) Representative images at low magnification (×20). (B) Representative images at high magnification (×63). Sections of rat spinal cord tissue were immunolabeled with mAb6E3 (green) and with antibodies for neural markers (red; MAP2, GalC and GFAP). DAPI (blue) was used for counterstaining. The expression of MAP-2 and Na,K-ATPase $\beta 1$ was observed mainly in the gray matter, whereas GalC and GFAP were observed in the white matter



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801  PLGTVTILCI DLGTDMVPAI SLAYEQAESD IMKRQPRNPK TDKLVNERLI
851  SMAYGQIGMI QALGGFTYFV ILAENGFLPF HLLGIRETWD DRWINDVEDS
901  YGQQWTYENR KIVEFTCHTA FFVSIVVVQW ADLVICKTRR NSVFQQGMKN
951  KILIFGLFEE TALAAFLSYC PGMGAALRMY PLKPTWWFCA FPYSLLIFVY
1001 DEVRKLIIRR RPGGWVEKET YY
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D

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1    MARGKAKEEG SWKKFIWNSE KKEFLGRTGG SWFKILLFYV IFYGCLAGIF
51   IGTIQVMLLT ISELKPTYQD RVAPPGLTQI PQIQKTEISF RPNDPKSYEA
101  YVLNIIRFLE KYKDSAQKDD MIFEDCGSMP SEPKERGEFN MERGERKVCR
151  FKLDWLGNCS GLNDESYGYK EGKPCIIIKL NRMLGFKPKP PKNESLETYP
201  LTHKYNPNVL PVQCTGKRDE DKDKVGNIEY FGMGGFVGFP LQYYPYYGKL
251  LQPKYLQPLL AVQFTNLTLD TEIRIECKAY GENIGYSEKD RFQGRFDVKI
301  EVKS
```

Figure 6. Identification of mAb6E3 antigen. (A) Immunoprecipitation of PC12 cell lysate with mAb6E3. Lysates of PC12 cells were immunoprecipitated with control mIgG$_1$ (left lane) and mAb6E3 (right lane). In the right lane, biotinylated proteins were immunoprecipitated at the molecular weight of 110- and 50-kDa. (B) Silver staining of PC12 cell lysate. PC12 cell lysate was immunoprecipitated with mAb6E3 on SDS/PAGE and then silver stained. From the sliver-stained gel, two bands at 110- and 50-kDa (indicated by arrows) were excised from the gel and analysed by mass spectrometry, respectively. (C) Mass spectrometry of the 110-kDa protein. A band at 110 kDa was extracted from the gel, digested by trypsin, and analysed by oMALDI-Qq-TOF MS/MS QSTAR Pulsar. Figure 6C indicates the amino acid sequence of Na,K-ATPase α1. The sequence of the peptide detected from the 110-kDa protein band is indicated in bold. (D) Mass spectrometry of the 50-kDa protein. Similar to the 110-kDa protein band, the 50-kDa protein band was analysed. Figure 6D indicates the amino acid sequence of Na,K-ATPase β1. The sequence of the peptide detected from the 50-kDa protein band is indicated in bold

cells. The efficacy of neuron-selective gene transfer by a combination of AdvZ3-FdZ and mAb6E3 was in the range 65–90% in a dose-dependent manner (Figure 4B).

Distribution of mAb6E3 antigen in rat spinal cord

To determine whether mAb6E3 is useful for neuron-selective gene transfer *in vivo*, we stained rat spinal cord tissue with mAb6E3 and a specific marker of neural cells. Low magnification images (Figure 5A) showed the reactivity of mAb6E3 and anti-MAP-2 antibody (a specific marker of neurons) in the gray matter. By sharp contrast, GalC (a specific marker of oligodendrocytes) and GFAP (a specific marker of astrocytes) are expressed mainly in the white matter. The high magnification images (Figure 5B) show that the distribution of the cells reacting with mAb6E3 corresponds with the distribution of MAP-2-expressing cells, but not with GalC- or GFAP-expressing cells. These findings indicate that mAb6E3 react specifically with neuron cells in the neural tissue.

Identification of mAb6E3 antigen

Subsequently, we identified the antigen recognized by mAb6E3. As shown in Figure 6A, mAb6E3 immunoprecipitated 110- and 50-kDa proteins from the PC12 cell lysates. In western blot analysis with the PC12 lysate, mAb6E3 failed to detect any proteins (data not shown). Silver stain of SDS/PAGE where PC12 lysate had been immunoprecipitated with mAb6E3 revealed several protein bands, including two bands defined by immunoprecipitation using mAb6E3 (Figure 6B). The 110- and 50-kDa protein were cut and analysed by mass spectrometry. This analysis identified the extracted protein bands as rat Na,K-ATPase α1 and β1, respectively (Figures 6C and 6D).

Na,K-ATPase has three subunits (α, β and γ) that usually form complexes. The α subunit has 1–4 isoforms, the β subunit has 1–3 isoforms [25,26], and the γ subunit has three splice variants including variant a [27], b [28] and another [29] that we named c in the present study. To determine the antigen recognized by mAb6E3, we transfected each cDNA of these subunits and isoforms into 293T cells and assessed the reactivity of these transfectants with mAb6E3. As shown in Figure 7A, mAb6E3 did not cross-react with human cell-derived 293T cells (NT and pTarget). Among ten transfectants, mAb6E3 reacted only with the 293T transfectant expressing the Na,K-ATPase β1 subunit.

To further confirm the specificity of mAb6E3 to Na,K-ATPase β1 subunit, we carried out RNA interference transfection experiments using siRNAs specific to Na,K-ATPase β1 subunit. As shown in Figure 7B, transfection of PC12 with Atp1b1 (1) siRNA and Atp1b1 (2) siRNA resulted in significant decreases in the reactivity of PC12 with mAb6E3 as compared to PC12 cells transfected with a control siRNA.

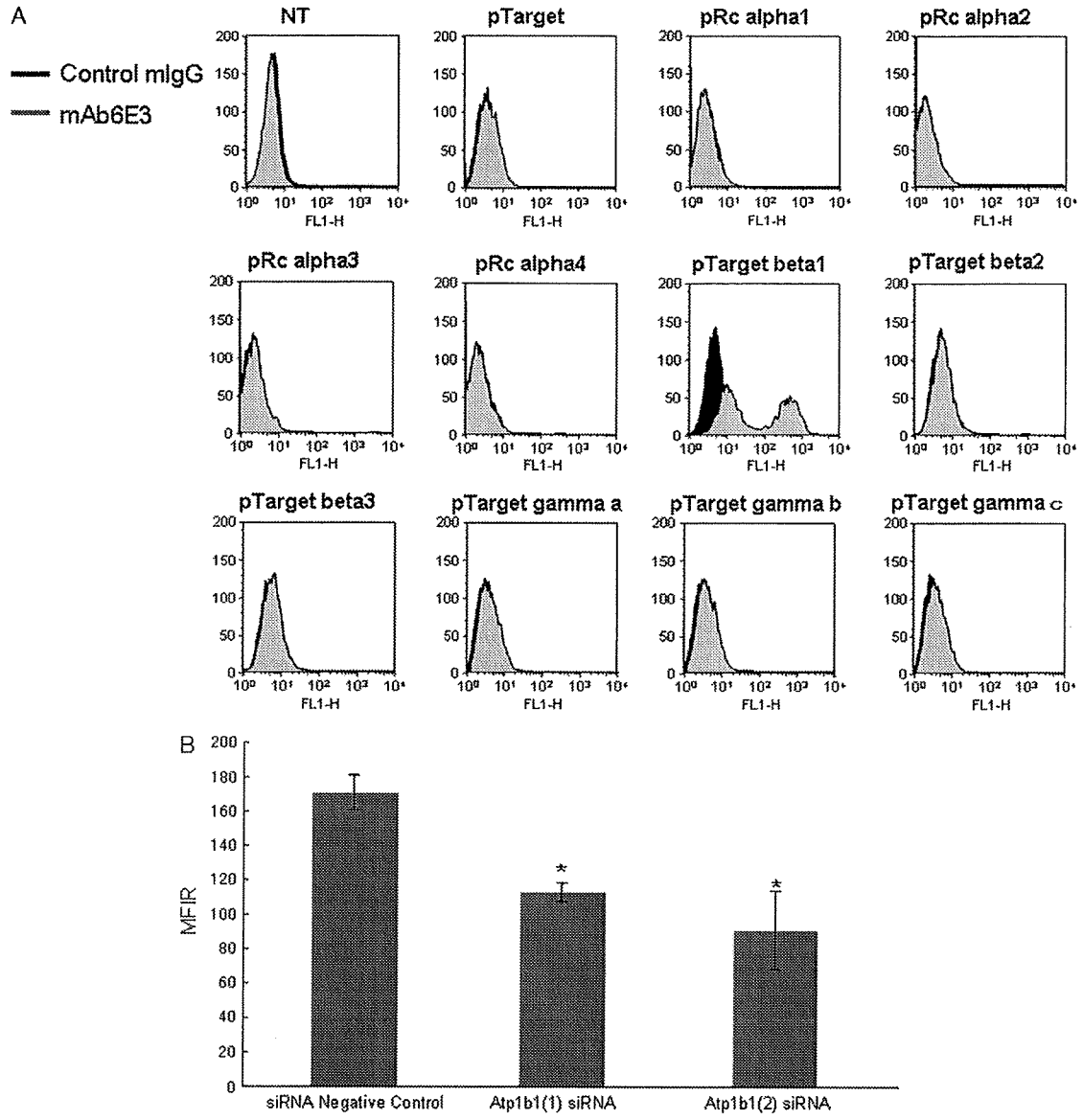

Figure 7. Transfection analysis of 6E3 antigen. (A) Reactivity of mAb6E3 with 293T cells transfected with cDNA of each subunit and isozyme of rat Na,K-ATPase. The *x*-axis indicates the intensity of fluorescein isothiocyanate (FITC)-labeled cell surface molecule. The *y*-axis indicates cell counts. The 293T human cell line was transfected with each cDNA of ten isoforms and variants of Na,K-ATPase. mAb6E3 only reacted with 293T cells transfected with cDNA of rat Na,K-ATPase $\beta 1$. NT, no treatment; pTarget, transfectants of pTarget vector; pRC alpha 1–4, transfectants of 1–4 isoforms of the α subunit; pTarget beta1–3, transfectants of 1–3 isoforms of the β subunit; pTarget gamma a–c, transfectants of a–c variants of the γ subunit. (B) Reactivity of mAb6E3 with PC12 cells transfected with siRNAs of rat Na,K-ATPase. PC12 were transfected with rat Na,K-ATPases $\beta 1$-specific siRNAs or a negative control siRNA using HiPerfect. The surface antigen recognized by mAb6E3 level were measured by flow cytometry. Expression levels were calculated as mean fluorescence intensity ratio and statistically analysed by Student's *t*-test. $^*p < 0.05$ compared to control siRNA

Discussion

The primal requirements for successful gene therapy include: (i) abundant expression of target molecules on the cell surface of target cells; (ii) incorporation of the gene into the target cell; and (iii) expression of the gene products. Our hybridoma screening procedure is designed to quantitatively evaluate these requirements. Through the first screening by β-Gal reporter gene assay, we were able to efficiently assess the efficacy of mAb-targeted gene transfer via fiber-modified adenovirus vectors. The subsequent screening focused on the specificity of mAb-targeted gene transfer and the identification of antigens recognized by the mAb. As a result of these screening procedures, we developed the hybridoma 6E3 and identified Na,K-ATPase $\beta 1$ as the antigen.

The immunostaining and chemiluminescent β-Gal reporter assays revealed that the mAb-targeted pathway

DOI: 10.1002/jgm

showed dose-dependent increases that were the same as the CAR-dependent pathway in gene transfer. These findings indicate that Na,K-ATPase $\beta 1$ functions the same as CAR as a target of gene transfer.

The Na,K-ATPase $\beta 1$ subunit has been shown to play a fundamental role in K^+ transport [30] and is distributed in almost all organs, especially in the brain and kidney [31]. Thus, Na,K-ATPase $\beta 1$ is not a neuron-specific molecule. However, previous reports [32–36] showed that Na,K-ATPase $\beta 1$ is expressed abundantly on the surface of neurons but poorly expressed on other types of neural cells, such as oligodendrocytes and astrocytes. Consistent with this, we found that the expression of Na,K-ATPase $\beta 1$ corresponded with that of MAP-2 but not with that of GalC or GFAP in the rat spinal cord tissue. In addition, mAb6E3-targeted gene transfer was specific to neuron cells in primary cultures of fetal rat cerebral cortex. The efficacy of mAb6E3-targeted gene transfer was in the range 65–90% depending on the virus doses. Since Cameron *et al.* [34] reported that the expression of Na,K-ATPase $\beta 1$ in immature neurons is lower than that of mature neurons, more efficient and selective gene transfer would perhaps be possible in mature neural tissue. Therefore, it is conceivable that Na,K-ATPase $\beta 1$ is a neuron-specific molecule in the neural tissues where it may serve as an target for neuron-specific gene transfer. Identification of an optimal ratio of antibodies to viruses as well as the development of new vectors superior to adenovirus are prerequisites for *in vivo* gene transfer.

In terms of clinical relevance, targeting of the Na,K-ATPase $\beta 1$ subunit may have adverse effects on cells since it plays a fundamental role in K^+ transport. In this regard, mAb6E3 did not affect the viability or form of the PC12 cells (data not shown). Neuron-specific expression of Na,K-ATPase $\beta 1$ in the neural tissues prefers the use of a mAb6E3-targeted gene transfer approach in local neurological disorders. In this regard, Agrawal and Fehlings [37] reported that Na,K-ATPase was involved in the mechanism of secondary spinal cord injury. Thus, gene transfer via Na,K-ATPase $\beta 1$ may target neurons that are secondarily injured.

Clearly, further studies including animal treatment models are necessary to demonstrate the feasibility of therapeutic targeting of transgene expression to neurons using mAb6E3 and AdvZ3-FdZ. For clinical application, it is also important to determine whether mAb6E3-targeted gene transfer can circumvent immunological reactions to adenovirus vectors. However, there are some problems with respect to performing an *in vivo* study. For example, animal models for neural disorders and methods of administration are not yet established. These are the critical themes for us to overcome in our next study.

In conclusion, the present study has demonstrated that hybridoma screening using FZ33 fiber-modified adenovirus vectors serves as an efficient approach for detecting antigens in mAb-targeted gene transfer. This approach may be more broadly applicable to other research fields. Na,K-ATPase $\beta 1$ defined by mAb6E3 represents a candidate molecule for neuron-selective gene transfer within the neural tissues.

Acknowledgements

We would like to thank Hiroshi Isogai and Noriko Kawano for their help with the animal experiments, Misumi Matsuo for her technical assistance and Dr Lingrel for providing the cDNAs. This work was supported in part by grants from the Japan Ministry of Education and Science, and by a grant from The General Insurance Association of Japan (03-25).

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The Prostate 67:1163 – 1173 (2007)

Exploration of Target Molecules for Prostate Cancer GeneTherapy

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BACKGROUND. Focusing on Adv-FZ33, a modified adenovirus in which a synthetic 33-amino-acid immunoglobulin G-binding domain was inserted into the adenoviral fiber protein, we tried to identify suitable target molecules for prostate cancer-specific gene therapy.
METHODS. Hybridomas were established from mice immunized with prostate cancer cell lines. The hybridomas were screened using Adv-FZ33 to create monoclonal antibodies (mAbs) that induced high gene transfer efficiency for PC-3 cells. Furthermore, we identified target antigens of the mAbs by immunoprecipitation and mass spectrometry, and investigated the expression of target molecules by flow cytometry and immunocytochemistry.
RESULTS. Using Adv-FZ33, we established four different mouse mAbs that increased transduction efficiency for PC-3. The target antigens identified were Ep-CAM, CD155, HAI-1, and Na,K-ATPase β1. These antigens were expressed in several cancer cell lines, including prostate cancer. Human prostatic myofibroblast cells lacked expression of Ep-CAM and HAI-1.
CONCLUSIONS. We established anti-Ep-CAM mAb and anti- HAI-1 mAbs. Gene transduction via Ep-CAM and HAI-1 may be a novel strategy for treatment of prostate cancer. *Prostate 67: 1163–1173, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: **gene therapy; modified adenovirus vector; prostate cancer; monoclonal antibody**

INTRODUCTION

Prostate cancer is the most common cancer in the West and the second leading cause of male cancer-related death [1]. Advanced androgen-insensitive prostate cancer exhibits little or no response to conventional therapies [2]. Thus, gene therapy is anticipated as an alternative treatment for this type of disease [3].

Adenoviral vectors are commonly used in gene therapy. This is due to their ability to produce high titers and infect various cell types [4]. Genes are transferred to target cells via the Coxsackie adenovirus receptor (CAR), which is a cell-surface receptor required for adenovirus attachment [5]. However, because CAR is widely expressed on normal cells, its lack of specificity is still an obstacle for its clinical application in cancer-gene therapy. In this context, we focused on the function of fiber Z33 type adenovirus (Adv-FZ33). This adenovirus has a synthetic 33-amino-acid immunoglobulin G (IgG)-binding domain (Z33) derived from staphylococcal protein A inserted into the virus having fiber protein [6]. This modified fiber binds IgG with high affinity and allows an antibody to redirect the vector to a new target molecule on the cell surface.

In this study, we established hybridomas from mice splenocytes immunized with prostate cancer cell lines. Then we selected from them monoclonal antibodies

Grant sponsor: Stiftelsen Japanese-Swedish Research Foundation; Grant sponsor: Cancer Research from the Ministry of Health and Welfare of Japan; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology.

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Received 12 January 2007; Accepted 4 April 2007
DOI 10.1002/pros.20613
Published online 21 May 2007 in Wiley InterScience (www.interscience.wiley.com).

(mAbs) that increased transduction efficiency by bridging Adv-FZ33 with prostate cancer.

MATERIALS AND METHODS

Cell Lines

We used human prostate cancer cell lines (PC-3, LNCaP, and DU145), a human renal cell carcinoma cell line (Caki-1), a bladder cancer cell line (T24), an ovarian cancer cell line (SKOV-3), normal dermal fibroblasts (PDF), normal prostate myofibroblasts (PrMFB), human embryonic kidney cells (293 T), and mouse myeloma (P3U1). These cell lines were purchased from American Type Culture collection (Manassas, VA). PrMFB was established in our department [7]. Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1 mm sodium pyruvate, and 1% streptomycin/penicillin solution.

Adenoviral Vectors

We generated adenoviral vectors containing the IgG-binding Z33 motif from Staphylococcal protein A at the HI-loop of the adenovirus type 5 (Ad5) fiber knob, and designated it Adv-FZ33 (represented in Fig. 1A). Details of Adv-FZ33 construction were described in a previous study [8].

Production and Screening of Hybridomas

Screening protocol for targeting mAbs is represented in Figure 1C. A Balb/c mouse was injected intraperitoneally with a mixture of PC-3, LNCaP, and DU145 cells (total: 2×10^6) every 14 days. Three days after the 5th injection, the mouse was sacrificed and 1×10^8 mouse splenocytes were fused with 2×10^7 P3U1 cells using polyethylene glycol (PEG). When hybridomas grew to about 50% confluence, culture supernatants were tested for antibody activity. PC-3

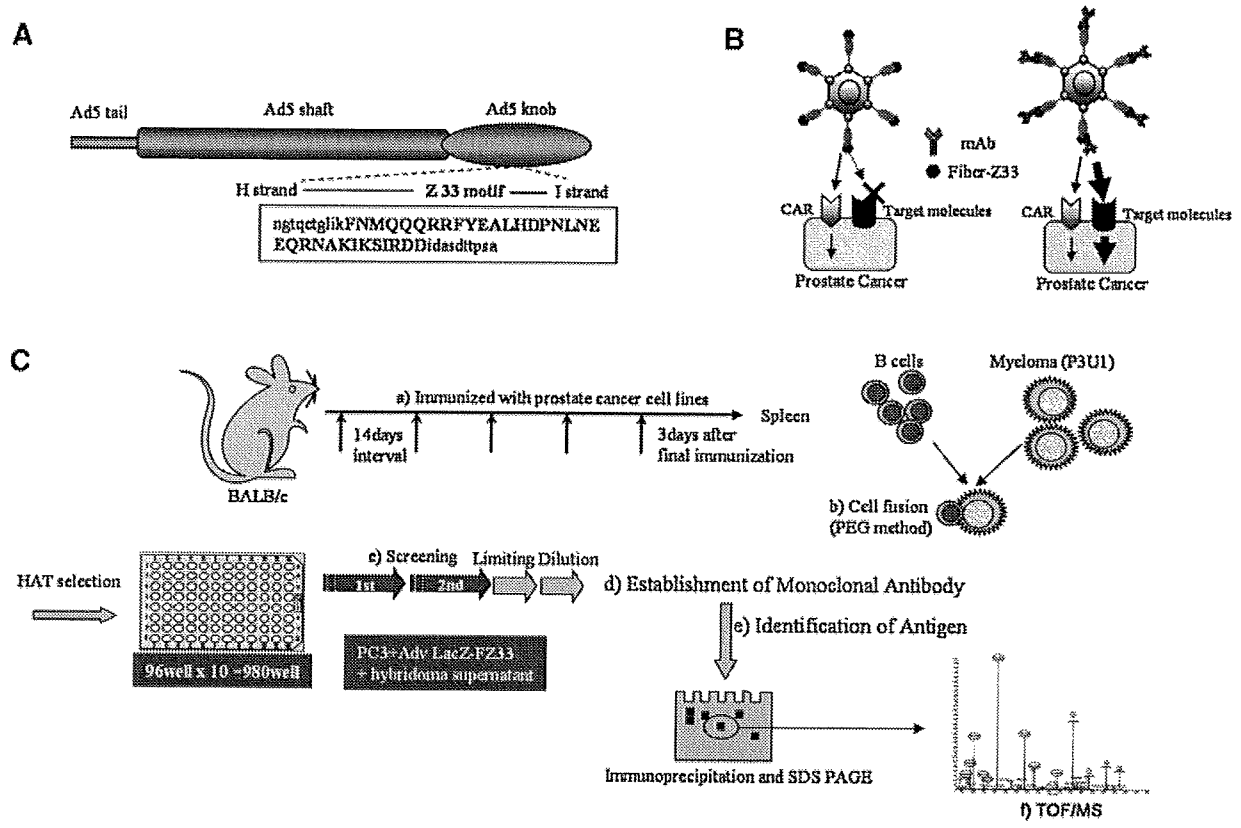

Fig. 1. Schematic representation of Adv-FZ33 and procedure for the establishment of cancer-targeting antibodies. **A**: Z33-modified Ad5 fiber A synthetic 33-amino acid IgG-binding domain (Z33), derived from staphylococcal protein A, was inserted into the HI loop of knob protein. **B**: Targeting with Adv-FZ33 Adv-FZ33 binds immunoglobulins and allows an antibody to redirect the vector to a new target molecule on the cell surface. Our Adv-FZ33 had intact CAR-binding structure and retained CAR-binding ability. **C**: Methods for establishment of novel cancer-targeting antibodies using Adv LacZ-FZ33. (a) Immunization with prostate cancer cell lines. (b) Cell fusion with PEG method. (c) Screening and limiting dilution. (d) Establishment of monoclonal antibody. (e) Identification of antigen by immunoprecipitation and SDS–PAGE. (f) Detection of molecule by mass spectorometry.

cells were prepared in 96-well microplates. After the removal of the culture medium, supernatants were added to each well and incubated for 1 hr at 4°C, after which supernatants were removed and microplates were washed with PBS. Adv-FZ33 inserted LacZ reporter gene (Adv LacZ -FZ33) prepared in FBS-free RPMI-1640 at a multiplicity of infection (MOI) of 1,000 vp/cell was added to each well and incubated for 1 hr at 4°C. Then microplates were washed twice with PBS and incubated at 37°C in a 5% CO_2 incubator. Twenty-four hours after infection, chemiluminescent β-Gal reporter gene assays (Roche Diagnostics, Mannheim, Germany) were performed according to the company's recommendations. Hybridomas that showed high β-Gal activity were picked through first and second screenings and cloned by twice limiting dilution. These hybridomas were injected into nude mice intraperitoneally. The mouse monoclonal antibody was purified from ascites of nude mice using protein G sepharose beads (Amersham Bioscience, Uppsala, Sweden). A commercial kit (Roche Diagnostics) was used to detect the isotypes of antibodies.

Identification of Target Molecules

Immunoprecipitation of biotinylated protein and detection of molecular weight. First, 2×10^6 PC-3 cells were prepared and the cell surface was biotinylated (PIERCE, Rockford, IL). Membranes were solubilized on ice for 30 min in 1 ml of buffer containing 1% NP40, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and protease inhibitor cocktail (Roche Diagnostics). Samples were cleared of nuclear fragments by centrifugation for 20 min at 15,000*g* at 4°C, then mixed with protein G sepharose beads and incubated for 2 hr at 4°C, after which the beads were centrifuged to remove non-specifically bound proteins. Five μg of the mAbs established in this study and control mouse IgG (eBioscience, San Diego, CA) were added to the supernatant of each sample and allowed to incubate for 2 hr at 4°C. The immunocomplexes were precipitated by addition of protein G sepharose beads to each sample and incubated for 2 hr at 4°C. The supernatant was discarded and the beads were washed six times with solubilization buffer. Immunocomplexes binding with beads were boiled for 5 min in 20μl of SDS sample buffer containing 5% 2-mercaptethanol. Samples were separated using 5–20% gradient polyacrylamide gels (BioRad, Hercules, CA) and transferred onto nitrocellulose membranes (Millipore, MA). After blocking with 5% milk in TBS consisting of 10 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, and 0.05% Tween-20, the membranes were incubated for 1 hr at room temperature with avidin-horseradish peroxidase (dilution 1:2,000; Amersham Bioscience, Buckinghamshire, England). Detection was carried out by enhanced chemiluminescence (Amersham Bioscience) according to the manufacturer's instructions.

Silver stain and mass spectrometry. For this procedure, 1×10^9 PC-3 cells were solubilized in 40 ml of buffer as described above. After the removal of nuclear fragments by centrifugation, samples were mixed with protein G sepharose beads and incubated overnight at 4°C to remove non-specifically bound proteins. Five micrograms of the mAbs established in this study and control mIgG1 were added to the supernatant of each sample and allowed to incubate for 2 hr at 4°C. The immunocomplex was precipitated by addition of protein G sepharose beads to each sample and incubated for 2 hr at 4°C. Samples were separated by SDS–PAGE as described above. The polyacrylamide gel was stained using a Silver Stain kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according to the company's recommendations. Specifically stained protein bands were extracted from the gel, digested by trypsin, and analyzed by oMALDI-Qq-TOF MS/MS QSTAR Pulsari (Applied Biosystems Japan Ltd, Tokyo, Japan).

Confirmation of results of mass spectrometry. The cDNAs of target molecules identified by mass spectrometry were synthesized by reverse transcription or obtained from Open Biosystems, Inc. (Huntsville, AL). Some cDNAs were ligated into the expression vector with pTarget vector (Promega, Madison, WI) or pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). cDNA was transfected into 293 T cells or CHO cells using Lipofect AMINE Plus reagent (Invitrogen). Forty-eight hours after transfection, transfected cells were washed and then suspended in staining medium (2% FBS/PBS) containing saturating amounts of mAbs established in this study and negative control IgG1 (MOPC-21, BD PharMingen, San Diego, CA) as controls. The reactivity of each mAb was analyzed by flow cytometry using a FACS-Calibur® (Becton Dickinson, San Jose, CA).

Transduction Efficiency in PC-3

Flow cytometric analysis. To examine transduction efficiency using Adv-FZ33 with established mAbs, PC-3 cells were prepared in six-well plates at the concentration of 1×10^5 cells/well. After removal of the culture medium, FBS-free RPMI-1640 containing the mAbs created in this study at a concentration of 2 μg/ml was added to each well and incubated for 1 hr at 4°C. Adv-FZ33 inserted the DNA fragment encoding

the enhanced green fluorescence protein (Adv EGFP - FZ33) prepared in FBS-free RPMI-1640 at the MOI of 1,000 vp/cell was added to each well and incubated for 1 hr at 4°C. Then the wells were washed twice with PBS and incubated at 37°C in a 5% CO_2 incubator. Twenty-four hours after infection, cells were collected and their transduction efficiencies were analyzed by flow cytometry using a FACS-Calibur®.

Chemiluminescent β-Gal reporter gene assay. PC-3 cells were prepared in 96-well plates at the concentration of 5×10^3 cells/well and divided into five groups by the concentration of the mAbs and control IgG1, that is 0.001, 0.01, 0.1, 1.0, and 10 μg/ml. After removal of the culture medium, 50 μl of FBS-free RPMI-1640 at the concentrations of the mAb described above was added to each well and incubated 1 hr at 4°C. Medium was removed and microplates were washed with PBS. Fifty microliters of Adv LacZ-FZ33 at MOI of 1,000 vp/cell prepared in FBS-free RPMI-1640 was added to each well and incubated for 1 hr at 4°C. The microplates were then washed twice with PBS and incubated at 37°C in a 5% CO_2 incubator. Twenty-four hours after infection, chemiluminescent β-Gal reporter gene assays were performed. Furthermore, we compared transduction efficiency of Adv-FZ33 with wild type adenovirus (Ad5). The concentration of virus was divided into 30, 100, 300, 1,000, 3,000, and 10,000 vp/cell. The concentration of mAb and control IgG1 was 1 μg/ml.

Distribution of Target Antigens

Flow cytometric analysis. The reactivity of the mAbs with human cell lines (PC-3, LNCaP, DU145, Caki-1, T24, SKOV-3, PDF, and PrMFB) was analyzed by flow cytometry. Cells in the logarithmic growth phase were tripsinized and washed. A cell pellet containing 1×10^5 cells was suspended in staining medium (2% FBS/PBS) containing 2 μg of mAb or isotype control IgG as controls for 60 min at 4°C in the dark. After three rinses with PBS, cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig antibody (diluted 1:100) (TAGO, Inc., Burlingame, CA) for 45 min at 4°C. The cell suspension obtained was washed three times with PBS and then analyzed by flow cytometry.

Immunohistochemistry. Study specimens of 30 patients were selected from the clinical pathology archives of the Sapporo Medical University Hospital. They included 30 specimens consisting of 13 needle-core biopsies, 14 prostatectomies, and 3 cystoprostatectomies obtained between 2001 and 2002. All H&E-stained slides were reviewed and the respective diagnoses were confirmed. All of these specimens included prostatic adenocarcinoma (22 patients with Gleason scores of 5–8, and 8 with Gleason scores of 9).

Immunohistochemistry with mAbs created in this study was performed on 5-μm thick, formalin-fixed paraffin-embedded tissue sections mounted on poly L-lysine-coated slides. The concentration of the mAb as the primary antibody was 5 μg/ml. Details of immunohistochemistry methods were described in a previous study [9].

RESULTS

Establishment of Hybridomas and Mouse Monoclonal Antibodies

Cell fusions done three times produced hybridoma colonies in 2,500 wells. We cloned the hybridomas from wells with high β-Gal activity by limiting dilution, because the β-Gal activity of each well reflected the transfection efficiency into PC-3 cells via the antigen recognized by the antibodies secreted from the hybridoma. We thereby established hybridomas secreting mAb 1B7, 2H7, 6F8, and 9B10. Isotypes of mAb 1B7, 2H7, and 6F8 were determined to be IgG1 kappa and, for mAb 9B10, IgG2a kappa.

Identification of mAbs 1B7, 2H7, 6F8, and 9B10 Antigens

Biotinylated proteins were detected at 40 kDa by immunoprecipitation using mAb 1B7 (Fig. 2A). Silver stain detected the same proteins. The epithelial cell adhesion molecule (Ep-CAM) was detected by mass spectrometry (Fig. 2B,C). cDNA of Ep-CAM was transfected into 293 T cells. Flow cytometry revealed that mAb 1B7 reacted with transfectants expressing Ep-CAM (Fig. 2D). We therefore concluded that the antigen recognized by mAb 1B7 was Ep-CAM.

In immunoprecipitation using mAb 2H7, an 80 kDa protein was detected (Fig. 3A). The protein was identified as poliovirus receptor (CD155) by mass spectrometry (Fig. 3B,C). cDNA of human CD155 was transfected into CHO cells and mAb 2H7 reacted with cells expressing CD155 (Fig. 3D).

In immunoprecipitation using mAb 6F8, 110, and 50 kDa biotinylated proteins were detected (Fig. 4A). The 110 kDa silver-stained protein, which was sharper than the 50 kDa band, was extracted and analyzed by mass spectrometry (Fig. 4B,C). The protein was identified as Na,K-ATPase β1. We obtained the cDNAs of human Na,K-ATPase α1, Na,K-ATPase α2, Na, K-ATPase α3, Na,K-ATPase α4 transcript variant 2, Na, K-ATPase β1, Na,K-ATPase β2, and Na,K-ATPase β3 to determine the antigens. Each cDNA was transfected into CHO cells and it was found that mAb6F8 reacted

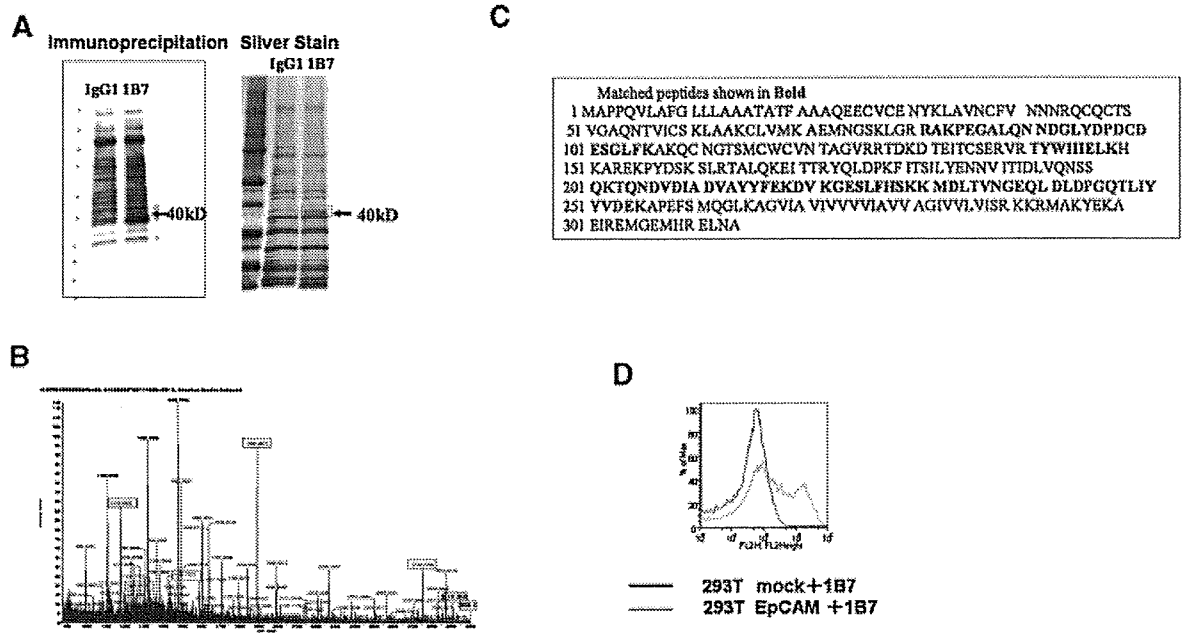

Fig. 2. Identification of mAb 1B7 antigen. **A**: Lysates of PC-3 cells were immunoprecipitated with mAb 1B7; proteins (40 kDa) were detected. The band that appeared at 40 kDa (indicated by an arrow) was excised from the gel and analyzed by mass spectrometry. **B**: High-intensity spectra indicated by a rectangles indicate the peptide, the sequence of which corresponded to the amino acid sequence of human Ep-CAM. **C**: Boldface indicates the sequence of the detected peptide. **D**: Flow cytometry of the reactivity of mAb 1B7 with 293T cells transfected with cDNA of Ep-CAM. mAb 1B7 reacted only with 293T cells transfected with cDNA of Ep-CAM.

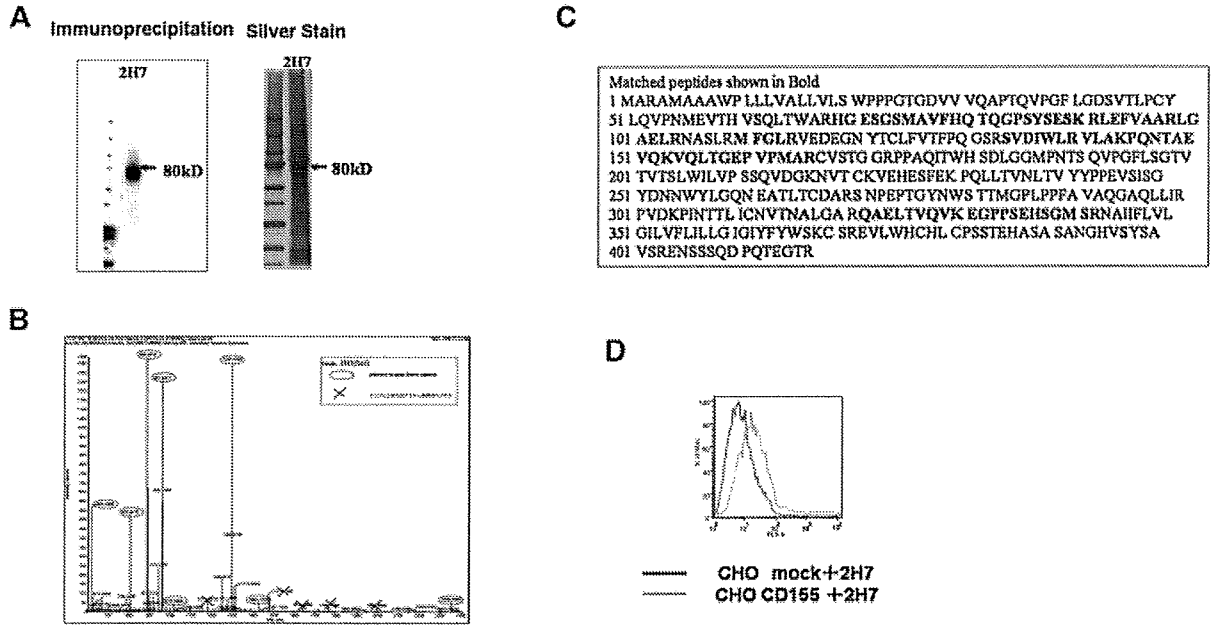

Fig. 3. Identification of mAb 2H7 antigen. **A**: Immunoprecipitation with mAb 2H7. The band that appeared at 80 kDa (indicated by an arrow) was excised from the gel and analyzed by mass spectrometry. **B**: Encircled high-intensity spectra indicate the peptide, the sequence of which corresponded to the amino acid sequence of human CD155. **C**: Boldface indicates the sequence of the detected peptide. **D**: Flow cytometry of the reactivity of mAb 2H7 with CHO cells transfected with cDNA of CD155. mAb 2H7 reacted only with CHO cells transfected with cDNA of CD155.

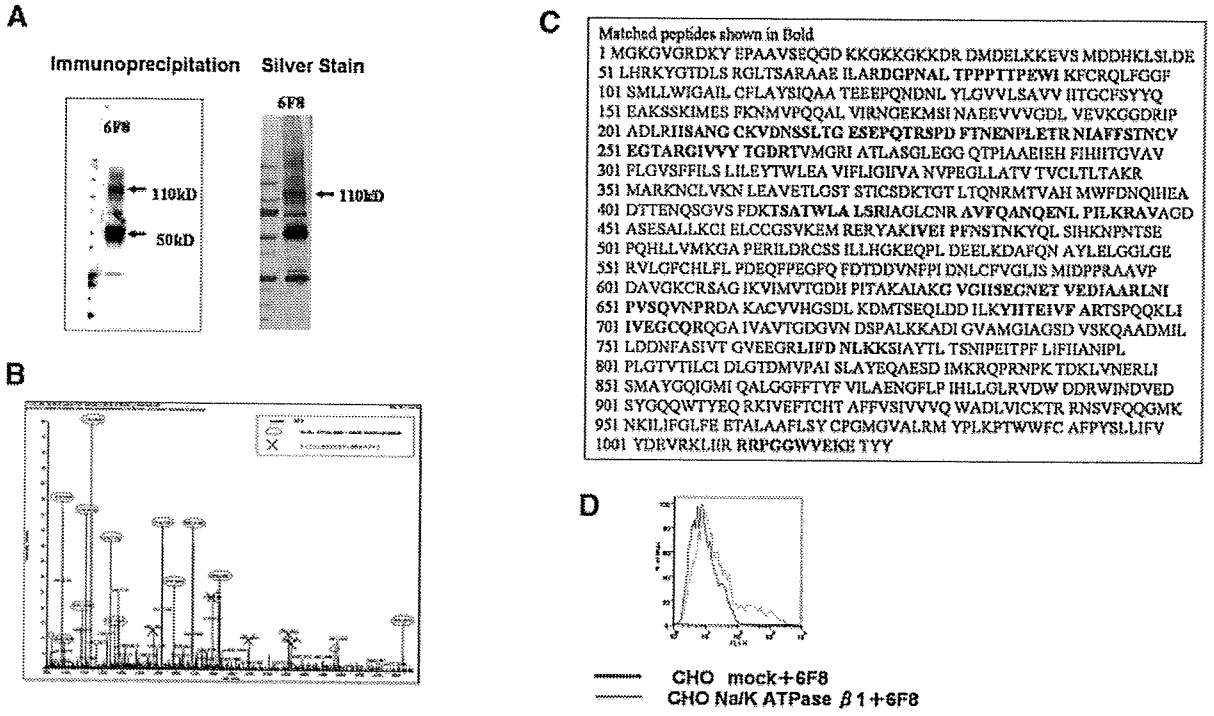

Fig. 4. Identification of mAb 6F8 antigen. **A**: immunoprecipitation with mAb 6F8; proteins (110 and 50 kDa) were detected. The band that appeared at 110 kDa was excised from the gel and analyzed by mass spectrometry. **B**: Encircled high-intensity spectra indicate the peptide, the sequence of which corresponded to the amino acid sequence of human Na,K-ATPase αI. **C**: Boldface indicates the sequence of the detected peptide. **D**: Flow cytometry of the reactivity of mAb 6F8 with CHO cells transfected with the cDNA of each subunit and isozyme of human Na,K-ATPase. mAb 6F8 reacted only with CHO cells transfected with cDNA of human Na,K-ATPase βI.

only with the transfectant expressing the Na,K-ATPase β1 subunit (Fig. 4D). We therefore concluded that the antigen recognized by mAb 6E3 was Na,K-ATPase β1.

In immunoprecipitation using mAb 9B10, a 55 kDa protein was detected (Fig. 5A). The protein was identified as HAI-1 by mass spectrometry (Fig. 5B,C). cDNA of human hepatocyte growth factor activator inhibitor type 1 (HAI-1) transcript variant 2 and HAI-1 transcript variant 3 were obtained. Each cDNA was transfected into CHO cells. mAb 9B10 reacted with each transfectant (Fig. 5D). Therefore, we concluded that mAb 9B10 recognized HAI-1.

Transfection Efficiency Into PC-3 With mAbs and Adv-FZ33

Flow cytometric analysis. Transfection efficiency was evaluated with various mAbs. Cells transfected using AdvEGFP-FZ33 together with mAb 1B7, 2H7, 6F8, and 9B10 showed enhanced expression EGFP compared with those together with mouse IgG1 (Fig. 6A).

Chemiluminescent β-Gal reporter gene assay. β-Gal activity in mAb 1B7, 2H7, and 6F8 at the concentration 1.0 μg/ml showed about 70-fold enhancement compared with control mouse IgG1. In mAb 9B10, transfection efficiency showed about 10-fold enhancement (Fig. 6B). Adv LacZ-FZ33 with mAb 6F8 showed significantly high expression of β-Gal compared with wild type—fiber adenovirus with or without mAb (Fig. 6C).

Distribution of Target Antigens

Expression in several cell lines. We examined the reactivities of mAb 1B7, 2H7, 6F8, and 9B10 with cancer and non-cancer cell lines by flow cytometry. mAb 2H7 and 6F8 reacted strongly with all cell lines (Fig. 7B,C). mAb 1B7 reacted with all cancer cell lines but not with PrMFB and PDF (Fig. 7A). mAb 9B10 did not react with SKOV-3, PrMFB, or PDF (Fig. 7D).

Histologic findings on the specimens. All of the prostate cancer cells (Fig. 8A,C) and most of the normal epithelial cells (Fig. 8B) showed strong immunoreactivity for mAb 1B7 in all the samples. Some of the normal epithelial cells (Fig. 8C,D) and all of the stromal cells (Fig. 8A–D) showed negative staining. No samples were stained with the other three mAbs.

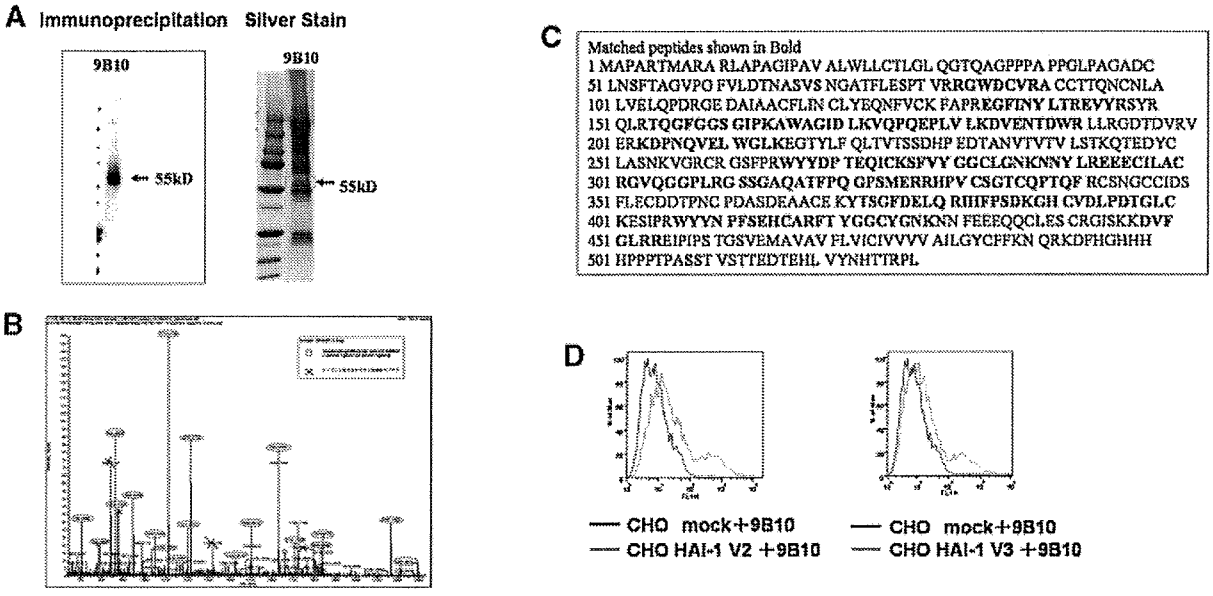

Fig. 5. Identification of mAb 9B10 antigen. **A**: immunoprecipitation with mAb 9B10. The band that appeared at 55 kDa (indicated by an arrow) was excised from the gel and analyzed by mass spectrometry. **B**: Encircled high-intensity spectra circle indicate the peptide, the sequence of which corresponded to the amino acid sequence of human HAI-1. **C**: Boldface indicates the sequence of the detected peptide. **D**: Flow cytometry of the reactivity of mAb 9B10 with CHO cells transfected with cDNA of each subunit of HAI-1. mAb9B10 reacted with CHO cells transfected with cDNA of each subunit of HAI-1.

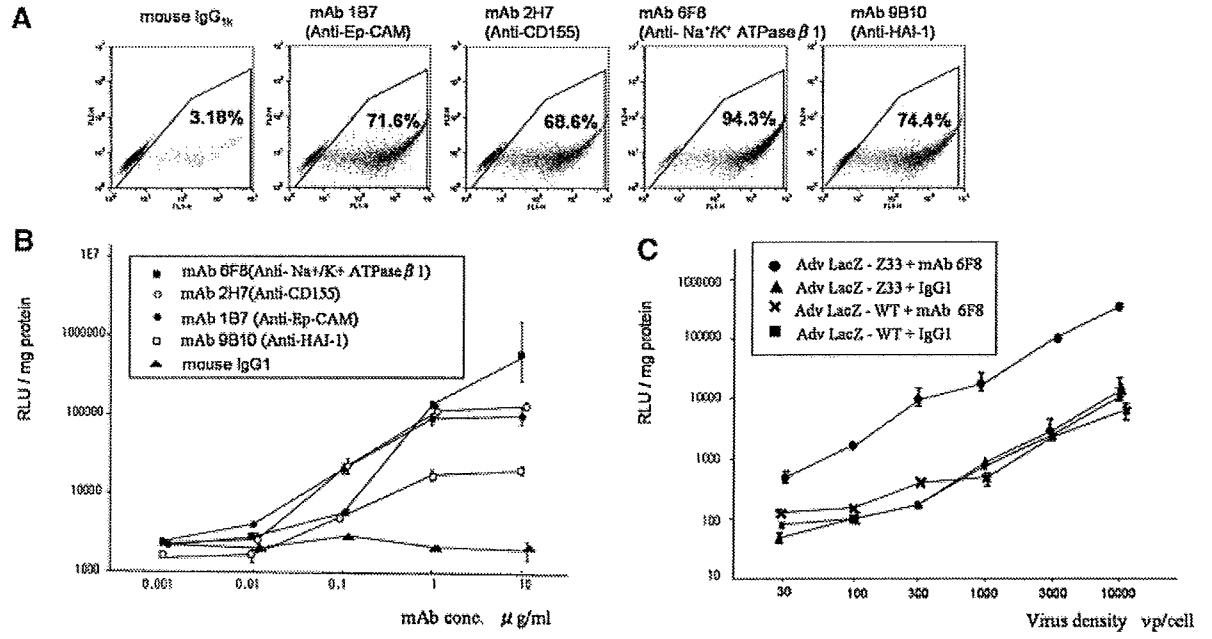

Fig. 6. Transfection efficiency into PC-3 cells with each fiber mutant adenovirus-mediated mAb. **A**: Numbers presented in each panel indicate the percentage of cells expressing EGFP. Cells transfected using AdvEGFP-FZ33 together with mIgG1 showed low expression of EGFP. Cells transfected using AdvEGFP-FZ33 together with mAbs 1B7(anti-Ep-CAM mAb), 2H7(anti-CD155 mAb), 6F8(Na,K-ATPase β1 mAb), and 9B10(anti-HAI-1 mAb) showed enhanced expression of EGFP. **B**: The cells were lysed, and assayed for β-Gal activity using a commercial kit ($n = 4$). AdvEGFP-FZ33 together with mAbs 1B7, 2H7, 6F8, and 9B10 showed high transduction efficiency compare with control IgG. **C**: Adv LacZ - FZ33 together with mAb 6F8 showed high transduction efficiency compared with wild type adenovirus (Adv Lacz–WT).

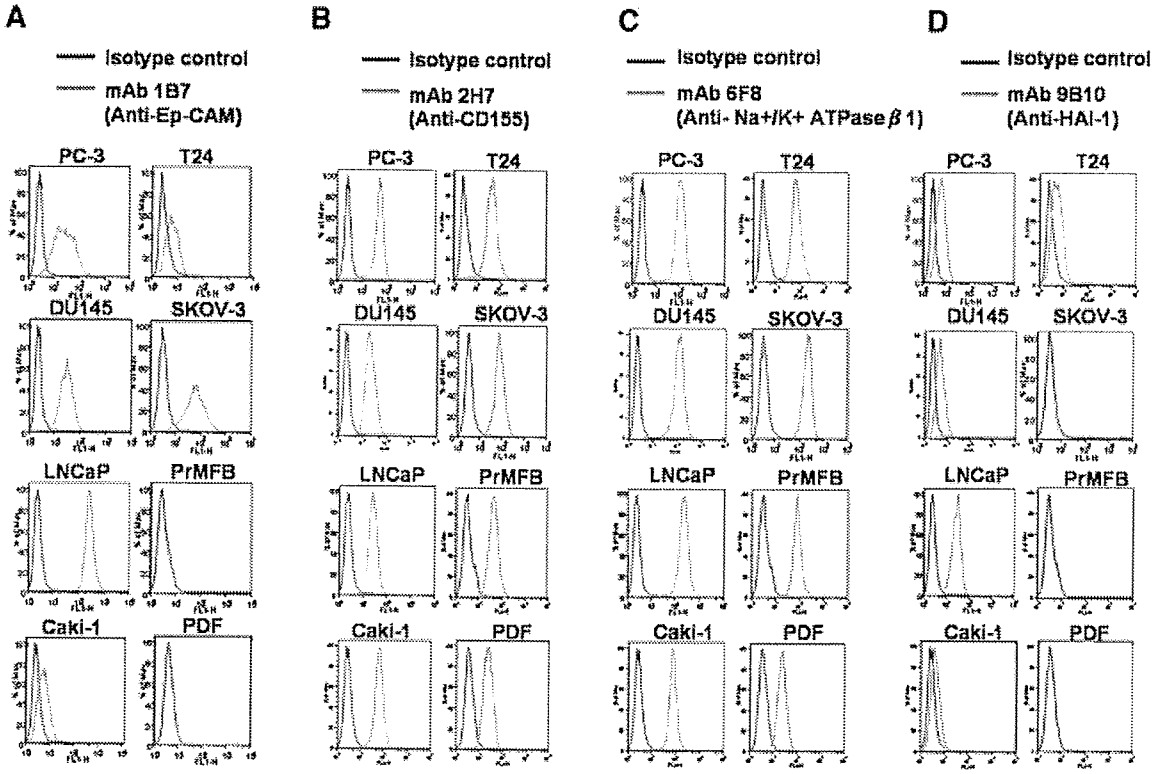

Fig. 7. Reactivities of established mAbs with various human cell lines. **A**: Antigen of mAb 1B7 was expressed on the surface of PC-3, DU145, LNCaP, Caki-1, T24, and SKOV-3 but not PrMFB and PDF. **B,C**: Antigens of mAbs 1B7 and 6F8 were expressed on the surface of each cell line. **D**: Antigen of mAb 9B10 was expressed on the surface of PC-3, DU145, LNCaP, Caki-1, and T24 but not SKOV-3, PrMFB, or PDF.

DISCUSSION

Volspers et al. [6] constructed a Z33-modified adenovirus vector and reported that the transduction efficiency of this modified vector in epidermal growth factor receptor (EGFR)-expressing cells was strongly and dose-dependently enhanced by combination with an EGFR-specific monoclonal antibody. They suggested that antibody-mediated targeting of the Z33-modified adenovirus vector could be applied for directed gene transfer to a wide variety of cell types by simply changing the target-specific antibody. Tanaka et al. [8] evaluated, both in vitro and in vivo, the extent of retargeting toward and therapeutic effectiveness against carcinoembryonic antigen (CEA)-positive gastric cancers when using the fully human CEA antibody complex with Adv-FZ33. They generated Ax3CAUP-FZ33 (UP-FZ33), an Adv-FZ33 derivative vector expressing a therapeutic gene (*Escherichia coli* uracil phosphoribosyltransferase) that converted 5-fluorouracil (5-FU) directly to 5-fluoro-UMP. UP-FZ33 with the anti-CEA mAb enhanced the cytotoxicity of 5-FU by 10.5-fold in terms of the IC_{50} against a CEA-positive gastric cancer cell line compared with control IgG4. In a nude mouse peritoneal dissemination model, tumor growth in mice treated by UP-FZ33 premixed with the anti-CEA mAb was significantly suppressed, and the median survival time was significantly longer than in the control group.

Surface antigens may be a viable target for antibody-mediated gene therapy. Although, PSA is a clinically important biomarker in prostate cancer [10,11], PSA is not a surface antigen [12]. Although, prostate-specific membrane antigen (PSMA) [13] is a type II membrane antigen, the PSMA expression pattern is not fully restricted to the prostate [14,15]. Therefore, we investigated target molecules amenable to gene therapy with Adv-FZ33. In this study, we immunized mice with three human cell lines that were androgen-independent (PC-3 and DU145) or androgen-dependent (LNCaP) [16], for generating various types of mouse mAbs. We have performed to screening the target antibodies using human prostate cancer cell line PC-3. Although, our Adv-FZ33 has intact CAR-binding structure and retains CAR-binding ability, CAR protein is downregulated in the highly tumorigenic PC-3 cell line [17] and the transduction efficiency of adenovirus is quit low (Fig. 6). Furthermore, PC-3 does