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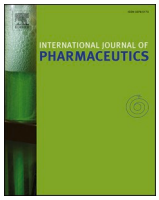

Development of a novel adenovirus serotype 35 vector vaccine possessing an RGD peptide in the fiber knob and the E4 orf 4, 6, and 6/7 regions of adenovirus serotype 5

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ABSTRACT

Adenovirus (Ad) vectors based on human adenovirus serotype 5 (Ad5) have attracted significant attention as vaccine vectors for infectious diseases. However, the effectiveness of Ad5 vectors as vaccines is often inhibited by the anti-Ad5 neutralizing antibodies retained by many adults. To overcome this drawback, we focused on human adenovirus serotype 35 (Ad35) vectors with low seroprevalence in adults. Although Ad35 vectors can circumvent anti-Ad5 neutralizing antibodies, vector yields of Ad35 vectors are often inferior to those of Ad5 vectors. In this study, we developed novel Ad35 vectors containing the Ad5 E4 orf 4, 6, and 6/7 or the Ad5 E4 orf 6 and 6/7 for efficient vector production, and compared their properties. These E4-modified Ad35 vectors efficiently propagated to a similar extent at virus titers comparable to those of Ad5 vectors. An Ad35 vector containing the Ad5 E4 orf 4, 6, and 6/7 mediated more efficient transduction than that containing the Ad5 E4 orf 6 and 6/7 in human cultured cells. Furthermore, insertion of an arginine-glycine-aspartate (RGD) peptide in the fiber region of an Ad35 vector containing the Ad5 E4 orf 4, 6, and 6/7 significantly improved the transgene product-specific antibody production following intramuscular administration in mice. The Ad35 vector containing the RGD peptide mediated efficient vaccine effects even in the mice pre-immunized with an Ad5.

1. Introduction

The prolonged pandemic of novel coronavirus infection 2019 (COVID-19) has reaffirmed not only that the emerging and re-emerging infectious diseases are a global threat, but also that vaccine development is crucial for global health and national security. Among various vaccines being developed, adenovirus (Ad) vector vaccines have attracted significant attention due to their advantages, including high transduction efficiencies, high stability during long-term storage, relatively large capacity for insertion of transgene expression cassettes, and efficient induction of both innate and adaptive immune responses (Sakurai et al., 2022; Zhang et al., 2023).

More than 80 serotypes of human Ad have been identified so far, and they are classified into species A to G (Jones, 2007; Walsh, 2011). Ad vectors that are widely used as a gene therapy vehicle are based on human adenovirus serotype 5 (Ad5), which belongs to species C. While Ad5 has the above promising characteristics, its high seroprevalence of Ad5 in adults has been a major hurdle for clinical application. Most people have been naturally exposed to Ad5 by adulthood. As a result, more than 80 % of adults have anti-Ad5 antibodies (Abbink, 2007; Parker, 2009; Barouch, 2011; Pilankatta et al., 2010). Therefore, Ad vector vaccines that are composed of Ad5 have a concern of inhibition by neutralizing antibodies in people who have pre-existing anti-Ad5 antibodies. Clinical trials of the Ad5 vector vaccine against human immunodeficiency virus (HIV) have not shown significant vaccine efficacy in the participants with pre-existing anti-Ad5 antibodies (Buchbinder,

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2008). In addition, Ad5 vectors show low transduction efficiency in cells lacking the primary receptor, coxsackievirus-adenovirus receptor (CAR). CAR expression in muscle, which is a main injection site for Ad vector vaccine, was demonstrated to be negligible (Nalbantoglu et al., 1999).

To circumvent pre-existing immunity to Ad5 vectors, several groups have developed Ad vectors composed of rare human Ad serotypes, such as Ad serotypes 26 and 11 (Abbink, 2007). Ad vectors composed of adenoviruses isolated from other animals, including chimpanzees, gorillas, and cattle, have also been developed (Sadoff, 2021; Holterman, 2004; van Doremalen, 2020; Lanini, 2022; Sayedahmed, 2018). We have previously developed an Ad vector that is composed of subgroup B Ad serotype 35 (Ad35) (Sakurai et al., 2003; Sakurai et al., 2003, 2003). Ad35 has a very low neutralizing antibody retention rate of less than 20 % (Abbink, 2007; Vogels, 2003). Ad35 vector-mediated transduction is unlikely to be inhibited by pre-existing antibodies. Ad35 recognizes human CD46, a complement regulatory protein, as an infection receptor (Gaggar et al., 2003; Marttila, 2005). CD46 is ubiquitously expressed on all human cells except for erythrocytes, indicating that an Ad35 vector can transduce a wide range of cells, including dendritic cells and skeletal fiber cells (Liszewski et al., 1991; McNearney et al., 1989; Seya et al., 1990).

Although these advantages of Ad35 vectors suggest their suitability as vaccine vectors, previous studies have reported that yields of Ad35 vectors are often inferior to those of conventional Ad5 vectors (Sakurai et al., 2003, 2003,; Sakurai, 2010; Sakurai, 2006). The low vector production efficiencies of Ad35 vectors are a drawback for clinical application. It has been reported that the Ad E1B55K protein and the Ad E4 proteins, especially the E4 open reading frame (orf) 6 protein, form a protein complex that is essential for the efficient replication of the viral genome (Babiss et al., 1985). The E1B55K and E4 protein complex induces high levels of virus late gene expression by promoting preferential transport of viral mRNA from the nucleus to the cytosol over host cell mRNA (Rubenwolf et al., 1997; Weigel and Dobbelstein, 2000). These two proteins, the E1B55K protein and the orf 6 protein, should be derived from the same Ad serotype to efficiently form the protein complex. The orf 6 and 6/7 genes in the Ad vectors composed of Ads other than human Ad5 are replaced with those of human Ad5 for efficient vector production in the packaging cells expressing the Ad5 E1 proteins, including HEK293 cells. However, not only the orf 6 gene but also the orf 4 gene is crucial for progeny virus production (Dicks, 2012). These findings led us to consider that modification of the E4 region in the Ad35 vector genome should be optimized for efficient vector production.

In this study, we have developed E4-modified Ad35 vectors which can be produced to high virus titers. The orfs 6 and 6/7 or orfs 4, 6, and 6/7 of the Ad35 E4 region were replaced with the corresponding genes of Ad5. Furthermore, the arginine-glycine-aspartate (RGD) motif, which has a high affinity for $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins, was genetically incorporated into the HI loop of the Ad35 fiber knob to enhance the transduction efficiencies and vaccine effects. This study provides important information about the potential of an Ad35 vector as a vaccine vector.

2. Materials and methods

2.1. Cell lines

HEK293 cells (a human embryonic kidney cell line) and HepG2 cells (a human hepatocellular carcinoma cell line, RCB1648; JCRB Cell Bank, Tokyo, Japan) were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, and antibiotics. H1299 cells (a human non-small cell lung carcinoma cell line) and MCF-7 cells (a human breast cancer cell line) were cultured with RPMI-1640 supplemented with 10 % FBS and antibiotics. T24 cells (a human urinary bladder carcinoma cell line) and NIH3T3 cells (a mouse embryonic fibroblast cell line) were cultured in DMEM supplemented with 10 % FBS and antibiotics. L929 cell (a mouse fibroblast cell line) were cultured with Joklik-modified Minimum Essential Medium (MEM) supplemented with 5 % FBS and antibiotics. All cells were cultured at 37℃ in 5 % CO_2 atmosphere.

2.2. Development of Ad vector plasmids

The Ad35 vector plasmids containing the Ad5 E4 region, pAdMS67R and pAdMS467R, were developed based on the pAdMS4-1 (Sakurai et al., 2005) as follows. Briefly, pAdMS4-1 with the deletion of the E1 (bp 368–3374) and E3 (bp 27761–29731) regions was modified to replace the deleted E1 region with bp 368–2912. Then the *Sfi*I and *Sbf*I sites, which are located at the E3 deletion site and the left end of the Ad35 genome, respectively, were changed to *Pme*I and *AsiS*I sites, respectively, using the oligonucleotides described in Supplementary Table 1. Next, the fragment encoding the Ad5 orfs 6 and 6/7 (bp 32914–34077) or Ad5 orfs 4, 6, and 6/7 (bp 32914–34342) was replaced with the E4 region (bp 31826–32975 or bp 31826–33246) of the Ad35 genome, respectively. The *Not*I site in these plasmids, which are located at the right end of the Ad35 genome, was changed to an *AsiS*I site using the oligonucleotides (Supplementary Table 1), generating pAdMS67R and pAdMS467R. I-*Ceu*I/PI-*Sce*I-digested pAdMS67R and pAdMS467R were ligated with I-*Ceu*I/PI-*Sce*I-digested pHM5-RBG-CMVL1, which contained the cytomegalovirus (CMV) promoter-driven firefly luciferase expression cassette, obtaining pAdMS67R-RBG-CMVL2 and pAdMS467R-RBG-CMVL2, respectively. β-galactosidase-expressing Ad35 vector plasmids, pAdMS67R-RBG-CMVLacZ2 and pAdMS467R-RBG-CMVLacZ2, were similarly constructed using pHM5-RBG-CMVLacZ1, which contained the CMV promoter-driven β-galactosidase expression cassette.

pAdMS467-HI(R), in which the RGD-4C peptide (CDCGRGDCFC) was inserted into the HI loop of the fiber knob region, was developed by referring to the strategy reported previously by Matsui *et al* (Matsui, 2009; Matsui, 2011). Ad vector plasmids pAdMS467-HI(R)-CMVL2 and pAdMS467-HI(R)-CMVLacZ2, which contained the CMV promoter-driven firefly luciferase expression cassette and β-galactosidase expression cassette, respectively, were similarly generated as described above. Details of the plasmid construction methods are available upon request.

2.3. Preparation of Ad vectors

All Ad vectors were constructed by means of an improved *in vitro* ligation method described previously (Mizuguchi and Kay, 1999; Mizuguchi and Kay, 1998). Firefly luciferase- and β-galactosidase-expressing Ad vectors, Ad5-Luc and Ad5-LacZ, respectively, were previously produced (Mizuguchi, 2001; Sakurai, 2008). Note that Ad5-Luc and Ad5-LacZ used in this study are same as Ad-L2 and Ad5LacZ, respectively, addressed in the previous study (Mizuguchi, 2001; Sakurai, 2008). pAdMS67R-RBG-CMVL2, pAdMS67R-RBG-CMVLacZ2, pAdMS467R-RBG-CMVL2, pAdMS467R-RBG-CMVLacZ2, pAdMS467-HI(R)-CMVL2, pAdMS467-HI(R)-CMVLacZ2 were digested with *AsiS*I and transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, San Jose, CA), producing Ad35-E4-Luc, Ad35-E4-LacZ, Ad35-E4.4-Luc, Ad35-E4.4-LacZ, Ad35-E4.4-HI(R)-Luc, and Ad35-E4.4-HI(R)-LacZ, respectively. All vectors were propagated in HEK293 cells, purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80 °C. The virus particles (VP) titers were determined by the method of Maizel *et al* (Maizel et al., 1968). Biological titers of Ad vectors were determined using HEK293 cells by the Adeno-X rapid titer kit (Clontech, Mountain View, CA) for Ad5-Luc and Ad5-LacZ or by the method of Kanegae *et al* (Kanegae et al., 1994) for Ad35-E4.4-Luc and Ad35-E4.4-LacZ. We have collected approximately 1 mL of virus solutions for all Ad vectors we designed above after ultracentrifugation.

2.4. Determination of Ad genome copy numbers

HEK293 cells were seeded on a 12-well plate at a density of 2.5×10^5 cells/well. On the following day, cells were treated with Ad5-Luc, Ad35-E4-Luc, and Ad35-E4.4-Luc at 25 VP/cell. Total DNA, including Ad genomic DNA, was extracted by using DNAzol (Molecular Research Center, Cincinnati, OH) at 1, 24, and 48 hrs after treatment. The Ad genome copy numbers were quantified by real-time PCR analysis (StepOnePlus System, Thermo Fisher Scientific, San Jose, CA) using the primers for the Ad5 E4 genes, and THUNDERBIRD SYBR qPCR Mix reagents (TOYOBO, Osaka, Japan). The sequences of the primers were described previously (Ono et al., 2021).

2.5. Adenovirus-mediated transduction into cultured cells

Cells were seeded at 1×10^4 cells/well onto a 96-well plate. On the following day, the cells were transduced with Ad5-Luc, Ad35-E4-Luc, Ad35-E4.4-Luc, and Ad35-E4.4-HI(R)-Luc at 100 VP/cell for H1299, HepG2, T24, and MCF-7 cells, or with Ad5-Luc, Ad35-E4.4-Luc, and Ad35-E4.4-HI(R)-Luc at 3000 VP/cell for NIH3T3 and L929 cells. After a 48 hrs incubation, luciferase production in the cells was determined using a Bright-Glo Luciferase Assay System (Promega, Madison, WI).

For the evaluation of Ad vector-mediated transduction efficiencies in the presence of human serum (KAC Co. Ltd, Kyoto, Japan), H1299 cells were seeded at 8.0×10^3 cells/well onto a 96-well plate. On the following day, Ad5-Luc and Ad35-E4.4-Luc were pre-incubated with human serum for 30 min at room temperature, and then were added to H1299 cells at 200 VP/cell. After a 72 hrs incubation, luciferase expression in the cells was determined using the Bright-Glo Luciferase Assay System. Human serums (No.1–4) used in this study correspond to the human serums (#1, 4, 2, and 6) used in the previous study (Ono et al., 2022).

2.6. Ad vector-mediated transduction in mice following intramuscular administration

Ad5-Luc, Ad35-E4.4-Luc, and Ad35-E4.4-HI(R)-Luc (1×10^{10} VP/50 μL /mouse) were intramuscularly administered to C57BL/6J (wild-type: WT; female, 6–8 weeks old) (Nippon SLC Co. Ltd., Shizuoka, Japan) or CD46 transgenic (CD46TG) mice (C57BL/6J background; female, 6–8 weeks old) (Sakurai, 2006). 48 hrs after administration, the muscles were isolated from the mice and homogenized, followed by three rounds of freezing and thawing. The homogenates were then centrifuged at 21,900 × g for 15 min at 4 °C. The protein concentration in the supernatants was measured with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Luciferase activity in the supernatants was measured with a Luciferase assay system (PicaGene 5500; Toyo Ink, Tokyo, Japan). All of the animal experiments performed in this study were approved by the Animal Experiment Committee of Osaka University.

2.7. Ad vector-mediated vaccination of mice

Ad5-LacZ, Ad35-E4.4-LacZ, and Ad35-E4.4-HI(R)-LacZ (1×10^{10} VP/50 μL /mouse) were immunized into WT and CD46TG mice (female, 6–8 weeks old) intramuscularly on days 0 and 28. On day 42, anti-β-galactosidase (β-gal) antibody titers in the serum were determined by ELISA as follows. The ELISA plates were coated with recombinant β-gal (1 μg/mL) (Sigma-Aldrich, St. Louis, MO) in carbonate buffer overnight at 4 °C. The coated plates were then incubated with 5-fold diluted ImmunoBlock (DS Pharma Biomedical, Osaka, Japan) for 1 hr at room temperature. Serum samples that were diluted using 20-fold diluted ImmunoBlock were added to the antigen-coated plates. After incubation for 2 hrs at 37 °C, the coated plates were incubated with a goat anti-mouse IgG (H+L)-BIOT (dilution 1/5000; Southern Biotech, Birmingham, AL), for 2 hrs at 37 °C. After incubation with the secondary antibody, the plates were incubated with a horseradish peroxidase (HRP)-labeled streptavidin (dilution 1/5000; Southern Biotech, Birmingham, AL), for 1 hr at room temperature. After incubation, the color reaction was developed using a Substrate Reagent Pack (catalog numbers: DY999, R&D System, Minneapolis, MN), stopped with 0.5 N HCl, and the absorbance was measured at OD450 on a plate reader.

To induce anti-Ad5 immunity, WT mice were pre-immunized intramuscularly twice, separated by a 2-week interval, with a replication-incompetent Ad5 vector encoding no transgene, Ad-null (Sakurai, 2008), at a dose of 1×10^9 VP/50 μL /mouse. Two weeks after second pre-immunization, naïve and the pre-immunized WT mice were intramuscularly administered with Ad5-LacZ and Ad35-E4.4-HI(R)-LacZ at a dose of 1×10^{10} VP/50 μL /mouse as described above.

2.8. Statistical analyses

One way-ANOVA or two way-ANOVA followed by Tukey's post hoc test or Bonferroni's post hoc test was used for statistical analyses. These analyses were conducted using Graph Pad Prism software (GraphPad Software, San Diego, CA). Data are presented as means ± SD or SEM.

3. Results

3.1. Generation of recombinant E4-modified Ad35 vector

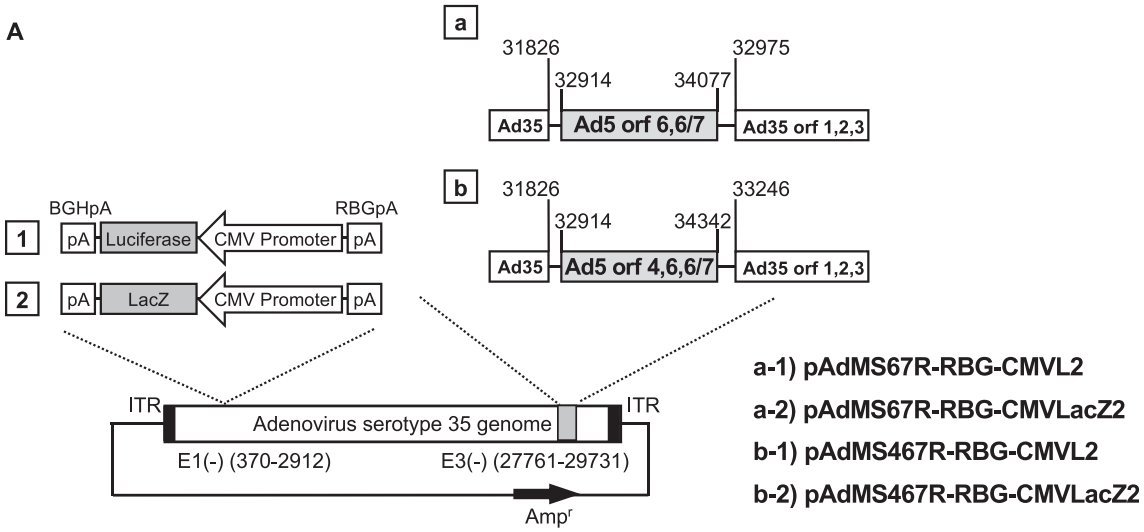
To improve the vector production of an Ad35 vector, the Ad35 E4 orf 6, 6/7 (E4orf 6, 6/7) or orf 4, 6, 6/7 (E4orf 4, 6, 6/7) gene was replaced with that of Ad5 (Fig. 1). All the E4-modified Ad35 vectors showed higher VP titers compared to the conventional Ad35 vector, Ad35-Luc (Sakurai, 2010), suggesting that the modification of the E4 region improved the production efficiencies of Ad35 vectors (Table 1). In particular, the Ad35 vectors with orf 4 modification, i.e., Ad35-E4.4-Luc and Ad35-E4.4-LacZ, exhibited more than 6.5-fold higher VP titer than Ad35-Luc. In addition, the E4-modified Ad35 vectors generated in this study exhibited titer productions at levels comparable to that of a conventional Ad5 vector, Ad5-Luc. The ratios of the particle-to-biological titers were approximately 4–12 for all the E4-modified Ad35 vectors, which were much lower than the ratio of Ad35-Luc, and similar to that of Ad5-Luc. These results indicated that modification of the E4 region significantly improved the VP and infectious titers of Ad35 vectors to the similar extent of an Ad5 vector.

3.2. Ad vector genome amplification efficiency of E4-modified Ad35 vectors in HEK293 cells

Next, to evaluate the virus genome amplification efficiencies of E4-modified Ad35 vectors in HEK293 cells, the amounts of Ad vector genome were measured after addition of the Ad vectors to HEK293 cells. Ad5-Luc amplified the Ad vector genome by 19.5-fold at 24 hrs and 97.4-fold at 48 hrs after transduction (Fig. 2). The Ad vector genomes of Ad35-E4-Luc were amplified by 77.3-fold at 24 hrs and 500.2-fold at 48 hrs following transduction, and those of Ad35-E4.4-Luc were amplified by 45.8-fold at 24 hrs and 145.8-fold at 48 hrs after transduction. These data indicated that both Ad35-E4-Luc and Ad35-E4.4-Luc propagated as efficiently as an Ad5 vector in HEK293 cells.

3.3. Transduction efficiencies of E4-modified Ad35 vectors in human cultured cells

Next, to evaluate the transduction efficiencies of the E4-modified Ad35 vectors, several types of human cultured cell lines (H1299, HepG2, MCF7, T24 cells) were transduced with the Ad35 vectors. In H1299 and HepG2 cells, which are CAR-positive cells, the transduction efficiencies of Ad35-E4-Luc and Ad35-E4.4-Luc were slightly lower than those of conventional Ad5-Luc (Fig. 3). In CAR-negative T24 cells and MCF-7 cells, which express low levels of CAR (Ono et al., 2021), the



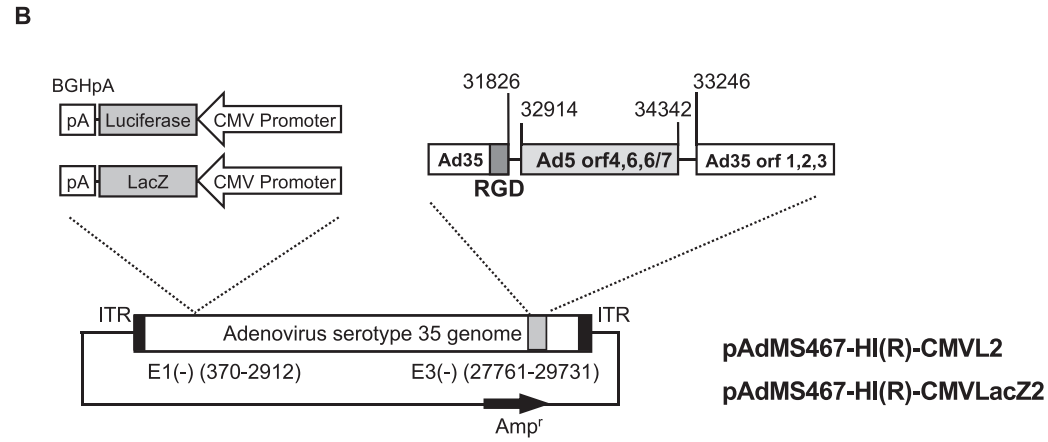


Fig. 1. Schematic diagram of the Ad vector plasmids used in this study. The luciferase expression cassette and LacZ expression cassette were inserted in the E1 region of the Ad vector genome. CMV, cytomegalovirus immediate-early promoter; Luc, firefly luciferase gene; LacZ, β-galactosidase gene; BGHpA, bovine growth hormone poly A signal; RBGpA, rabbit β-globlin poly A signal.

Table 1
Physical and Biological titers of Ad vectors.

Virus	Transgene	VP/mL	IFU or PFU/mL	Ratio
Ad5-Luc	Luciferase	2.5×10^{12}	4.9×10^{11}	5.1
Ad35-Luc (E4 non-modified)	Luciferase	2.4×10^{11}	8.4×10^{8}	286
Ad35-E4-Luc	Luciferase	7.0×10^{11}	1.3×10^{11}	4.4
Ad35-E4-LacZ	LacZ	1.5×10^{12}	1.2×10^{11}	11.9
Ad35-E4.4-Luc	Luciferase	1.6×10^{12}	3.3×10^{11}	4.8
Ad35-E4.4-LacZ	LacZ	1.8×10^{12}	2.2×10^{11}	8.3

transduction efficiencies of Ad35-E4-Luc and Ad35-E4.4-Luc were more than 2.5-fold and 5.7-fold higher than those of Ad5-Luc, respectively. These results indicated that the E4-modified Ad35 vectors efficiently transduced a broad range of human cell types, regardless of CAR expression. In addition, Ad35-E4.4-Luc mediated significantly higher transgene expression than Ad35-E4-Luc in almost all of the cultured cells examined. Thus, we focused on the Ad35-E4.4-Luc in the following experiments.

3.4. Transduction efficiencies of the E4-modified Ad35 vector in the presence of human serum

Next, in order to examine whether the E4-modified Ad35 vector efficiently transduced in the presence of human serum, Ad5-Luc and Ad35-E4.4-Luc were added to H1299 cells after pre-incubation with human serum (Fig. 4). The anti-Ad5 neutralizing antibody titers in these human serums No. 1, 2, 3, and 4 are 61, 283, 64, and 905, respectively, according to our previous study (Ono et al., 2022). The luciferase expression levels were markedly decreased for Ad5-Luc in correlation with the concentrations of human serum. The degrees of reduction in the luciferase expression in the presence of human serums were dependent on the anti-Ad5 neutralizing antibody titers, suggesting that there were sufficient levels of anti-Ad5 neutralizing antibodies to inhibit Ad5

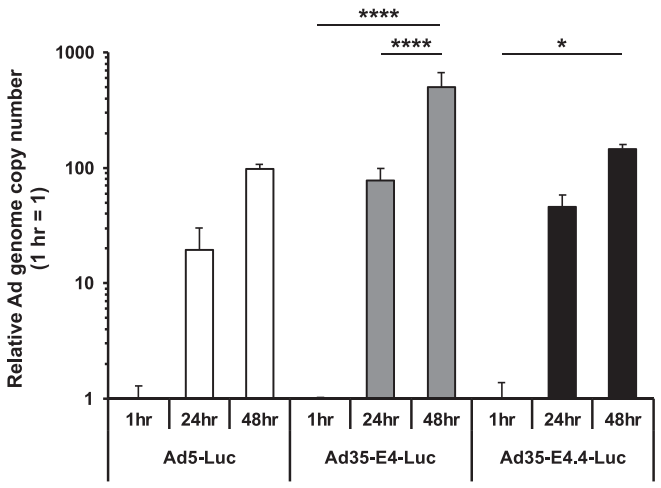


Fig. 2. Ad vector genome replication efficiencies in HEK293 cells. HEK293 cells were transduced with Ad5-Luc, Ad35-E4-Luc, and Ad35-E4.4-Luc at 25 VP/cell. After 1, 24, and 48 hrs of incubation, the Ad genome copy numbers were evaluated by real-time PCR analysis and expressed as relative values (1 hr = 1). Data are expressed as means ± S.D. (n = 3). *; $p < 0.05$, ****; $p < 0.0001$ compared with the group incubated with Ad vector for 1 hr.

vector-mediated transduction in the human serum samples used in this study. The transduction efficiencies of Ad5-Luc, when pre-incubated with 1/20 diluted human serum, were reduced to less than half in all the human serum samples used. In particular, Ad5-Luc-mediated luciferase expression levels were extensively inhibited by the human serum No. 4, which has the highest titer of anti-Ad5 neutralizing antibody in these four serums, even at the 1/500 dilution. These results indicated that the Ad5 vector-mediated transduction efficiencies were inversely correlated with the titers of anti-Ad5 neutralizing antibodies. On the other hand, Ad35-E4.4-Luc-mediated expression levels were hardly inhibited in the presence of human serum with the exception of the 1/20 dilution of sera 1, 3, and 4. Overall, the Ad35-E4.4-Luc-mediated luciferase expression was not largely decreased in the presence of human serum. These results indicated that Ad35-E4.4-Luc was able to mediate efficient transduction in the presence of anti-Ad5 neutralizing antibodies.

3.5. Transduction efficiencies of the E4-modified Ad35 vector in mice following intramuscular administration

Next, in order to examine the transduction efficiencies of the E4-modified Ad35 vector in mice, Ad35-E4.4-Luc was intramuscularly administered to WT mice and human CD46TG mice ubiquitously expressing human CD46 as in humans. Ad35-E4.4-Luc mediated low levels of luciferase expression in the muscle of WT mice, which lack CD46. On the other hand, human CD46TG mice showed approximately 8.2-fold higher luciferase expression than WT mice after administration of Ad35-E4.4-Luc. Ad35-E4.4-Luc-mediated luciferase expression levels in the muscle were comparable to that of Ad5-Luc in human CD46TG mice (Fig. 5). These results indicated that Ad35-E4.4-Luc mediated efficient transgene expression in human CD46TG mice.

3.6. Induction of anti-β-gal antibody production by intramuscular administration of E4-modified Ad35 vector

To assess the induction levels of transgene product-specific antibody following Ad vector administration, we intramuscularly administered Ad5-LacZ and Ad35-E4.4-LacZ to WT and human CD46TG mice, and evaluated anti-β-gal IgG antibody titers in the serum. Statistically significant levels of anti-β-gal antibody production were not found in the Ad35-E4.4-LacZ-treated WT or human CD46TG mice (Fig. 6), while the Ad5-LacZ-treated group showed high levels of anti-β-gal antibody production in both types of mice. Also, no significant differences in the anti-β-gal antibody titers were observed between Ad35-E4.4-LacZ-treated WT or CD46TG mice. These data indicated that Ad35-E4.4-LacZ does not induce efficient levels of transgene product-specific antibody, even in the CD46TG mice.

3.7. Transduction efficiency of the E4-modified Ad35 vector containing an RGD peptide in the fiber knob

Although human CD46 is expressed in the muscle of CD46TG mice, human CD46 expression levels in the muscle were somewhat lower than those in other tissues (Sakurai, 2006). In order to enhance the transduction efficiencies of an Ad35 vector in the muscle and induction levels of transgene product-specific antibody titers, we created an E4-modified Ad35 vector containing an RGD peptide in the HI loop of the fiber knob. An RGD peptide is known for binding with high affinity to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface, increasing the viral internalization into cells (Kolvunen et al., 1995). These integrins are expressed on broad range of cells, including muscle cells and dendritic cells (Okada, 2001; Koizumi et al., 2003). Dendritic cells play a crucial role on induction of adaptive immune responses. We first evaluated the transduction efficiencies of Ad35-E4.4-HI(R)-Luc in several types of CD46-positive cultured cell lines (H1299, HepG2, MCF7, and T24 cells). In H1299, HepG2, MCF7, and T24 cells, the transduction efficiencies of Ad35-E4.4-

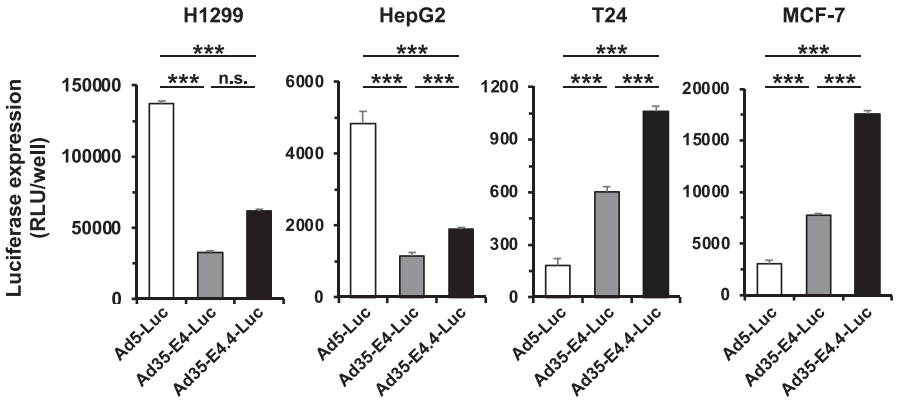


Fig. 3. In vitro transduction efficiencies of Ad vectors in human tumor cell lines. H1299, HepG2, T24, and MCF-7 cells were transduced with the Ad vectors at 100 VP/cell. Luciferase activities were measured at 48 hrs post-transduction. These data are expressed as means ± S.D. (n = 4). RLU: relative luciferase units. ***; $p < 0.001$ compared with all of the Ad vector-treated groups.

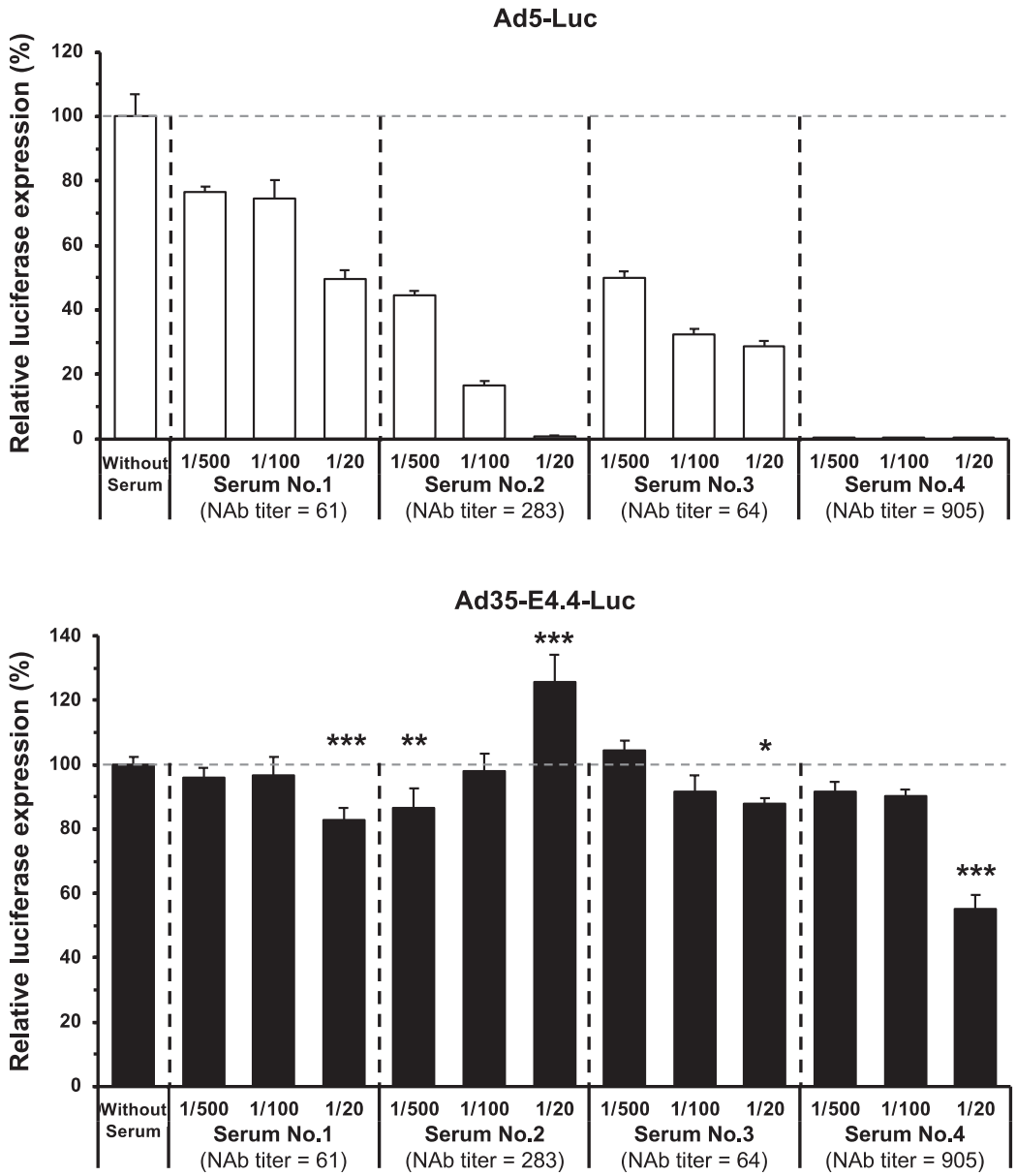


Fig. 4. In vitro luciferase expression of Ad5-Luc and Ad35-E4.4-Luc in the presence of human serum. Ad5-Luc and Ad35-E4.4-Luc were pre-incubated with diluted human serum for 30 min, followed by addition to H1299 cells at 200 VP/cell. Luciferase activities were measured 72 hrs after transduction. Luciferase activities in the cells without pre-incubation with human serum were normalized to 100 %. Neutralizing anti-Ad5 antibody titers (NAb titer) in human serums No.1–4, which are shown in the graph, were referred from our previous study (Ono et al., 2022). These data are expressed as means ± S.D. (n = 4). *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ compared with group without human serum. For Ad5-Luc treated group, all of the transduction efficiency were ***; $p < 0.001$ compared with the group without human serum.

HI(R)-Luc were more than 5-fold lower than those of Ad35-E4.4-Luc (Fig. 7A), mainly because Ad35-E4.4-HI(R)-Luc was not able to bind to CD46 (Matsui, 2009). On the other hand, in CAR-negative T24 cells, the transduction efficiency of Ad35-E4.4-HI(R)-Luc was more than 4-fold higher than those of conventional Ad5-Luc. We also examined the transduction efficiencies of Ad35-E4.4-HI(R)-Luc in NIH3T3 and L929 cells, which are CAR- and CD46-negative but $\alpha_v\beta_3$ and $\alpha_v\beta_5$-integrin-positive (Koizumi, 2001). Luciferase production by Ad35-E4.4-HI(R)-Luc was approximately 7.5-fold higher in CAR- and CD46-negative NIH3T3 cells and L929 cells, compared to luciferase production of Ad35-E4.4-Luc (Fig. 7B). Furthermore, the luciferase expression levels of Ad35-E4.4-HI(R)-Luc were comparable to those of Ad5-Luc in these cells.

In order to examine the *in vivo* transduction efficiencies of the E4-modified Ad35 vector possessing the RGD peptide, Ad35-E4.4-HI(R)-Luc was intramuscularly administered to WT mice. Ad35-E4.4-HI(R)-Luc-mediated luciferase expression levels in the muscle were approximately 4-fold lower than those of conventional Ad5-Luc, but showed approximately 2-fold higher luciferase expression than those of Ad35-E4.4-Luc, which did not include the RGD peptide (Fig. 7C). Taken together, these results indicated that inclusion of the RGD peptide in the fiber knob significantly increased the transduction efficiencies of the Ad35 vector *in vitro* and *in vivo*.

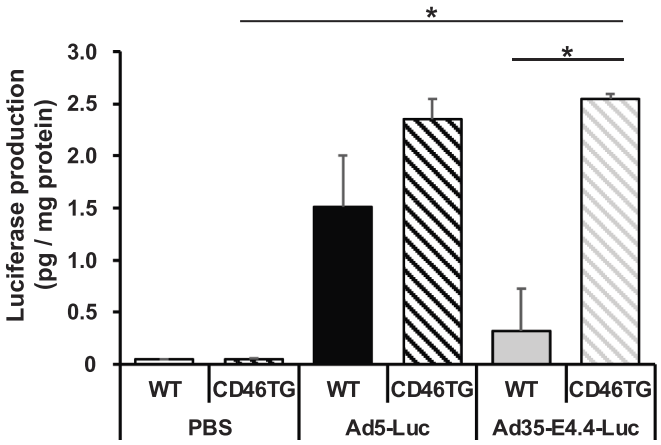


Fig. 5. *In vivo* transduction efficiencies of Ad5-Luc and Ad35-E4.4-Luc in the muscle following intramuscular administration to WT and CD46TG mice. Ad5-Luc and Ad35-E4.4-Luc were intramuscularly administered to mice at a dose of 1.0×10^{10} VP/mouse. Luciferase activity levels in the muscle were determined 48 hrs after administration. These data are expressed as means ± SEM (n = 6). *; $p < 0.05$ compared with the Ad35-E4.4-Luc- treated CD46TG mice.

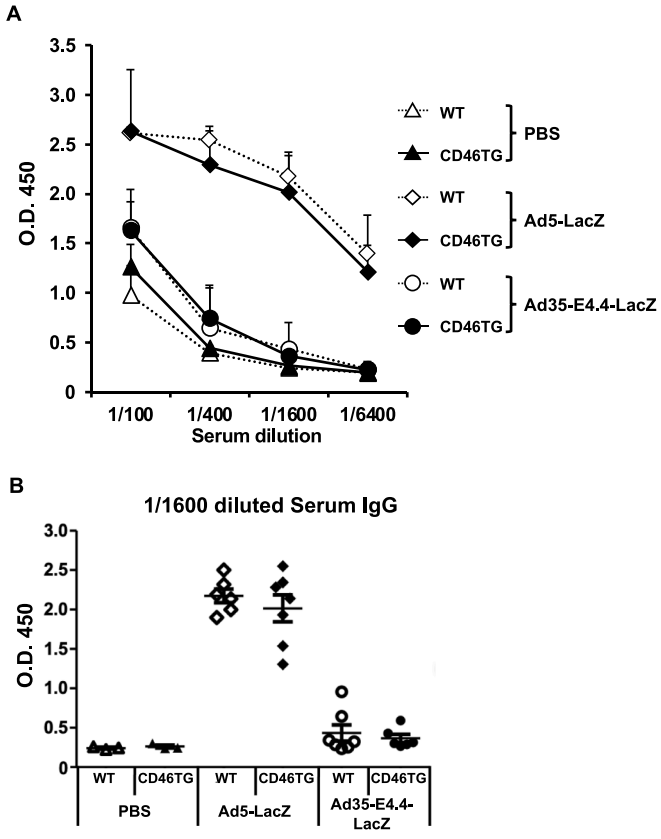


Fig. 6. Serum anti-β-gal antibody titers following intramuscular immunization with Ad35-E4.4-LacZ. WT and CD46TG mice were intramuscularly administered Ad35-E4.4-LacZ at a dose of 1.0×10^{10} VP/mouse on day 0 and day 28. Titers of anti-β-gal IgG in serum were evaluated 14 days after the final administration. The data are represented as means ± SEM (n = 7).

3.8. Vaccine effects of the E4-modified Ad35 vector containing an RGD peptide in the fiber knob

Next, to examine the induction levels of anti-β-gal antibody of Ad35-E4.4-HI(R)-LacZ, WT mice were intramuscularly administered with Ad5-LacZ, Ad35-E4.4-LacZ, and Ad35-E4.4-HI(R)-LacZ. The anti-β-gal IgG antibody titers in the serum of Ad35-E4.4-HI(R)-LacZ-treated mice were significantly higher than those of the Ad35-E4.4-LacZ-treated group (at 1/400 dilution) (Fig. 8A and B). However, the anti-β-gal IgG antibody titers in Ad35-E4.4-HI(R)-LacZ-treated mice were significantly lower than those in Ad5-LacZ-treated mice (at 1/400 dilution). These results suggested that the inclusion of an RGD peptide into the HI loop of the Ad35 fiber knob significantly improved the humoral immune responses against transgene products, but not enough to reach the levels of a conventional Ad5 vector vaccine.

In order to examine whether an Ad35 vector evaded anti-Ad5 neutralizing antibodies after *in vivo* application, we evaluated the vaccine effects of Ad35-E4.4-HI(R)-LacZ in the presence or absence of anti-Ad5 neutralizing antibodies. WT mice were pre-immunized twice with 10^9 VP of Ad-null to generate anti-Ad5 neutralizing antibodies. These pre-immunized mice had anti-Ad5 neutralizing antibody titers of 1942–16683, which titers were higher than the average titers found in humans in sub-Saharan Africa (Kostense, 2004). Although high titers of anti-β-gal IgG antibodies were found after intramuscular administration of Ad5-LacZ in naïve mice, Ad5-LacZ mediated almost background levels of anti-β-gal IgG antibody titers in the pre-immunized mice, indicating that anti-Ad5 neutralizing antibodies severely inhibited the vaccine effects of an Ad 5 vector vaccine. On the other hand, administration of Ad35-E4.4-HI(R)-LacZ to the naïve and pre-immunized mice resulted in efficient and similar levels of anti-β-gal IgG antibody titers. There was no statistical difference between the naïve and pre-immunized mice after treatment with Ad35-E4.4-HI(R)-LacZ. These results indicated that the E4-modified Ad35 vector possessing the RGD peptide effectively evaded anti-Ad5 immunity in mice and induced sufficient levels of antigen-specific antibodies.

4. Discussion

The high seroprevalence of Ad5 in adults has been a major hurdle for clinical application of Ad5 vector vaccines. Various types of Ad vectors have been developed to overcome this problem. We here focused on Ad35, which has a low seroprevalence of less than 20 % (Abbink, 2007; Vogels, 2003). However, an obstacle to the clinical use of Ad35 vectors was their low vector production efficiency. In this study, the E4 gene region of an Ad35 vector was modified to create an Ad35 vector vaccine that can be efficiently produced at levels similar to, or higher than those of an Ad5 vector vaccine. E4orf6 and E1B55K proteins have been reported to form a complex in order to increase selective export of late viral mRNA from the nucleus to cytosol (Rubenwolf et al., 1997; Weigel and Dobbelstein, 2000; Herrmann, 2020). These two proteins must be derived from the same Ad species to efficiently form a complex (Capone, 2006). HEK293 cells, which are the most common packaging cell line for Ad5 vectors, express the Ad5 E1B55K protein. In order to match the Ad serotype with the E1B55K protein expressed by HEK293 cells, the E4 region in the Ad35 vector genome was replaced with the corresponding sequences of the Ad5 genome, creating an E4-modified Ad35 vector. The ratios of particle-to-biological titers of the novel E4-modified Ad35 vectors were about 60-fold higher than those of Ad35 vectors without modification in the E4 region, suggesting that optimizing the E4 region is essential for efficient virus production. In order to circumvent the pre-existing neutralizing antibody, the Ad vector-based COVID-19 vaccines, ChAdOx1 nCov-19 (AstraZeneca) and Ad26.COV2-S (Janssen), are also based on chimpanzee Ad and human Ad serotype 26, respectively. A part of the E4 region in these Ad vector vaccines was replaced with that of Ad5 to improve the efficiency of vector production (Folegatti, 2022; Custers, 2021). Modification of the E4 region in the Ad vector genome is an effective way to improve the production efficiencies of Ad vectors other than a conventional Ad5 vectors.

The seroprevalence of Ads is different among people and Ad serotypes. Therefore, to achieve an efficient vaccine effect, it is ideal to optimize the serotype of the Ad vector to be used for the vaccine in advance (Abbink, 2007). Transduction of Ad5-Luc in the cultured cells

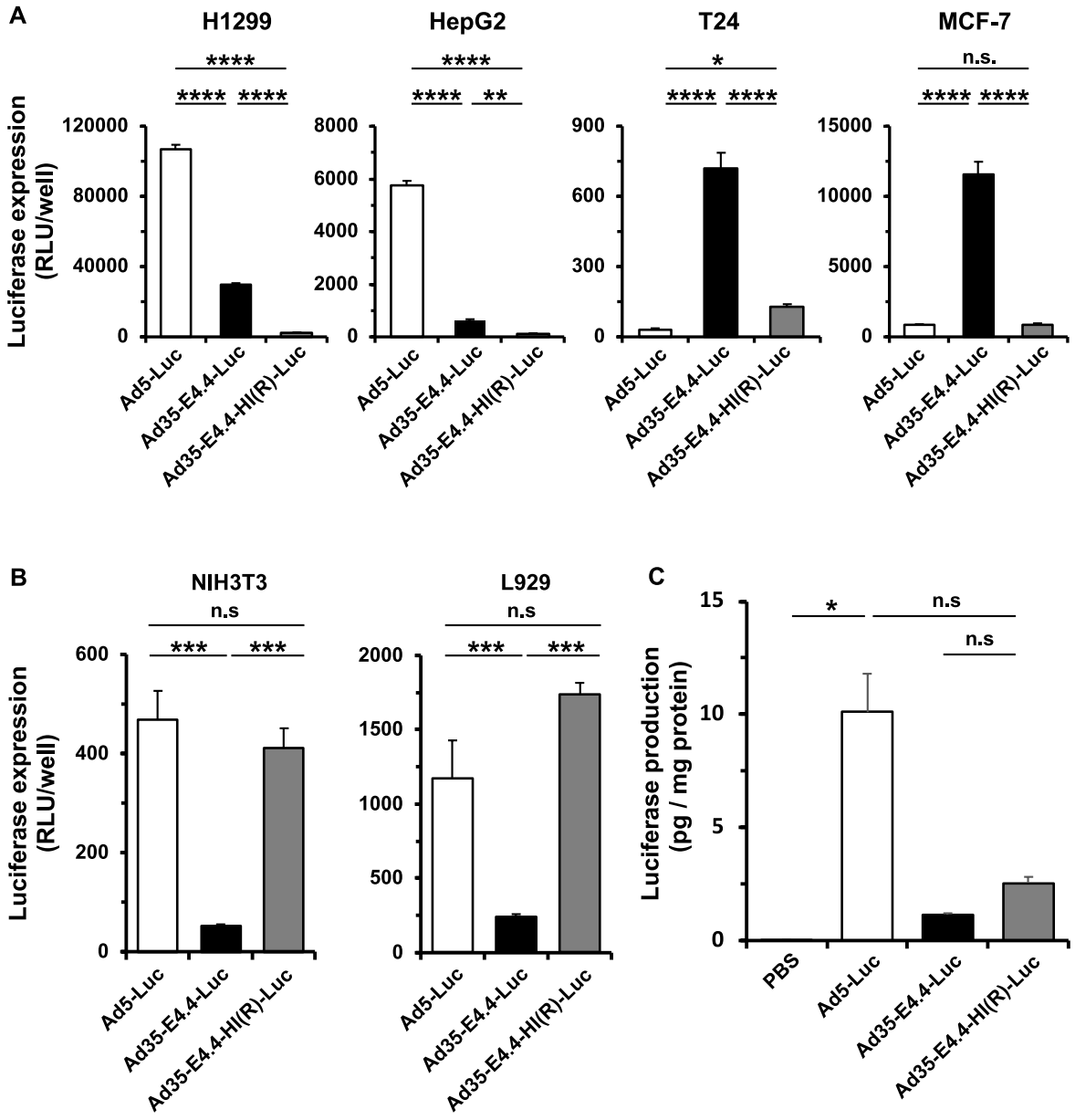


Fig. 7. Transduction efficiencies of Ad5-Luc, Ad35-E4.4-Luc, and Ad35-E4.4-HI(R)-Luc *in vitro* and *in vivo*. (A) H1299, HepG2, T24, and MCF-7 cells were transduced with the Ad vectors at 100 VP/cell. (B) NIH3T3 and L929 cells were transduced with the Ad vectors at 3000 VP/cell. Luciferase activities were measured at 48 hrs post-transduction. These data are expressed as means ± S.D. (n = 4). RLU: relative luciferase units. ***; $p < 0.001$ compared with Ad35-E4.4-Luc-treated group. (C) Ad5-Luc, Ad35-E4.4-Luc, and Ad35-E4.4-HI(R)-Luc were intramuscularly administered to mice at a dose of 1.0×10^{10} VP/mouse. Luciferase activity levels in the muscle were determined 48 hrs after administration. These data are expressed as means ± SEM (n = 6). *; $p < 0.05$ compared with the Ad5-Luc-treated mice.

was dramatically inhibited in the presence of human serum, while Ad35-E4.4-Luc was not crucially inhibited. Since the seroprevalence of Ad35 is known to be low in many humans, this Ad35 vector could be a candidate vaccine vector for numerous people. Although in most cases Ad35-E4.4-Luc was able to efficiently transduce even with a mixture of human sera, Ad35-E4.4-Luc showed a slight inhibition in the presence of low-dilution sera, indicating that the anti-Ad35 antibody titers in the serum differ among people. For these reasons, the serotype of the Ad vector used as a vaccine vector should be optimized by measuring each individual's anti-Ad antibody titer in advance in order to achieve a maximum vaccine effect in each individual.

In mice, CD46 expression is restricted to the testis in mice (Tsujimura, et al., 1998). There is only 46 % homology between mouse and human CD46. For the *in vivo* studies, we used human CD46 transgenic (CD46TG) mice, which ubiquitously express human CD46 as in humans (Sakurai, 2006). Significantly higher gene expression was detected in CD46TG mice compared to WT mice when the E4-modified Ad35 vector was administered intramuscularly. In the previous studies, the gene transduction efficiency in human CD46TG mice after intravenous and intraperitoneal administration of Ad35 vector was higher than that in wild-type mice (Sakurai, 2006). This indicates that the use of human CD46TG mice is important in order to precisely evaluate the gene transduction efficiency of Ad35 vectors.

Gene expression levels in muscle and antigen-specific antibody titers after intramuscular Ad35 vector administration did not correlate in this study, and no significant induction of antibody production was observed in either CD46TG or wild-type mice after Ad35 vector administration. The reason that gene expression levels did not correlate with antibody

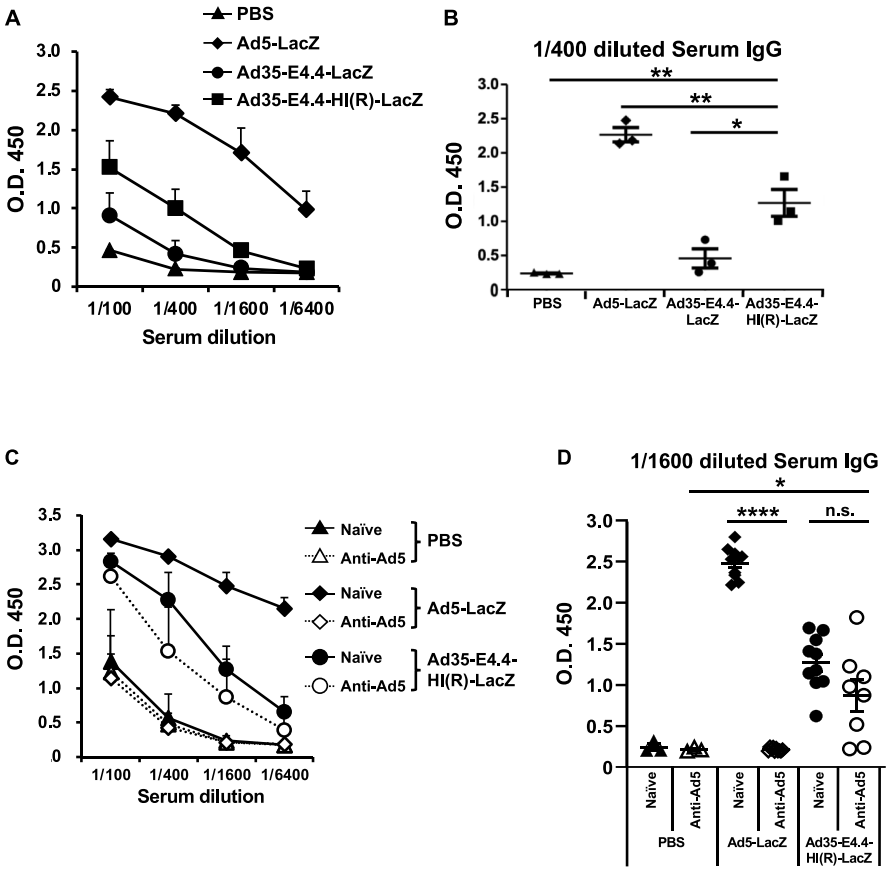


Fig. 8. Serum anti-β-gal antibody titers following intramuscular immunization with Ad35-E4.4- HI(R)-LacZ. (A, B) Mice were intramuscularly administered Ad5-LacZ, Ad35-E4.4-LacZ, and Ad35-E4.4-HI(R)-LacZ at a dose of 1×10^{10} VP/mouse on day 0 and day 28. Titers of anti-β-gal IgG in serum were evaluated 14 days after the final administration. The data are represented as means ± SEM (n = 3). *; $p < 0.05$, **; $p < 0.01$, compared with the Ad35-E4.4-HI(R)-LacZ-treated group. (C, D) Mice were intramuscularly pre-immunized twice with Ad-null at a dose of 1×10^{9} VP/mouse 14 and 28 days prior to injection of vaccine vector. As a vaccine vector, Ad5-LacZ and Ad35-E4.4-HI(R)-LacZ were administered at a dose of 1×10^{10} VP/mouse on day 0 and day 28. Titers of anti-β-gal IgG in serum were evaluated 14 days after the final administration. The data are represented as means ± SEM (n = 8–10). *; $p < 0.05$, ****; $p < 0.0001$.

titers in this study is unclear, but since the Ad35 vector was reported to strongly activate NK cells (Johnson, 2014), it is possible that antigen-expressing cells are eliminated by NK cells prior to the acquisition of humoral immunity. Further analysis of the immune response to Ad35 vector is required.

Transgene product-specific antibody titers after intramuscular administration of Ad35-E4.4-HI(R)-LacZ were significantly higher than those of Ad35-E4.4-LacZ, suggesting that the RGD peptide in the Ad35 fiber region contributed to the increase in the antibody titers. Insertion of an RGD peptide in the Ad35 fiber region may have improved the transduction to not only muscle cells but also dendritic cells *via* the interaction between an RGD peptide and αv integrins, leading to the improvement of the anti-β-gal antibody induction. Previous studies demonstrated that an Ad5 vector containing an RGD peptide in the fiber knob exhibited significantly higher levels of transduction efficiencies in the mouse bone marrow-derived dendritic cells and vaccine effects in mice, compared with a conventional Ad5 vector (Okada, 2003). Moreover, Ad35-E4.4-HI(R)-LacZ-mediated induction of anti-β-gal antibodies in the pre-immunized mice was not significantly inhibited by anti-Ad5 neutralizing antibodies, while the vaccine effects of Ad5-LacZ were markedly diminished in the pre-immunized mice. However, it is a bit of a concern that the average of anti-β-gal antibody titers in the pre-immunized mice was slightly lower than those in the naïve mouse after treatment with Ad35-E4.4-HI(R)-LacZ, although statistically significant difference was not found. Since the Ad vectors were repeatedly administered to the same muscle as pre-immunization with Ad-null, there is a possibility that pre-immunization with Ad-null induced inflammation and/or tissue damages in the muscle and that the condition in the muscle was not completely recovered when the Ad35 vector vaccines were administered, leading to the reduction in the transgene expression levels in the muscle.

5. Conclusion

In summary, in order to overcome the problem of low titer production of an Ad35 vector, we succeeded in developing E4-modified Ad35 vectors that can be produced to high titers. The inclusion of not only the orf 6 gene but also the orf 4 gene into the Ad genome improved the VP titer of Ad. In addition, the E4-modified Ad35 vector showed transduction efficiency at levels similar to the conventional Ad5 vector in various types of cultured cells. In particular, the E4-modified Ad35 vector including the orf 4 showed higher transduction efficiency than an Ad35 with only the orf 6 modification. However, significant levels of transgene product-specific antibody production were not observed in either CD46TG mice or wild-type mice following intramuscular immunization of the E4-modifided Ad35 vector. Furthermore, the insertion of an RGD peptide into the fiber knob region of the E4-modified Ad35 vector significantly elevated the transgene product-specific antibody titers. Moreover, the E4-modified Ad35 vector possessing the RGD peptide mediated efficient vaccine effects in the presence of anti-Ad5 immunity.

CRediT authorship contribution statement

Rika Onishi: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Sena Ikemoto:** Investigation, Methodology. **Aoi Shiota:** Investigation, Methodology. **Tomohito Tsukamoto:** Investigation, Methodology. **Akira Asayama:** Investigation, Methodology. **Masashi Tachibana:** Supervision. **Fuminori Sakurai:** Supervision, Writing – review & editing. **Hiroyuki Mizuguchi:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hiroyuki Mizuguchi reports financial support was provided by Ministry of Education, Culture, Sports, Sciences, and Technology of Japan. Hiroyuki Mizuguchi reports financial support was provided by Japanese Agency for Medical Research and Development. Hiroyuki Mizuguchi reports financial support was provided by BIKEN Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124480.

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