Bangari DS, Shukla S, Mittal SK. 2005. Comparative transduction efficiencies of human and nonhuman adenoviral vectors in human, murine, bovine, and porcine cells in culture. Biochem.Biophys.Res.Commun. 327:960-966. PMID: 15649439

Comparative transduction efficiencies of human and nonhuman adenoviral vectors in human, murine, bovine and porcine cells in culture

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ABSTRACT

Clinical usefulness of human Ad serotype 5 (HAd5) based vectors is limited primarily because of preexisting Ad immunity and lack of targeting to specific cell types. Alternative vectors based on less prevalent HAd serotypes as well as nonhuman adenoviruses such as porcine Ad serotype 3 (PAd3) and bovine Ad serotype 3 (BAd3) are being developed to overcome these shortcomings. Using virus neutralization assay, we examined whether preexisting Ad immunity in humans would cross-neutralize PAd3 or BAd3. To further evaluate the potential of PAd3 and BAd3 vectors as gene delivery vehicles we compared their transduction efficiencies in a panel of human, murine, bovine and porcine cell lines to those obtained with a HAd5 vector. Transduction by the HAd5 vector in the majority of human cell lines correlated with the expression levels of coxsackievirus B-adenovirus receptor (CAR), the primary HAd5 receptor; while transduction by PAd3 and BAd3 vectors was CAR-independent. The results suggest that PAd3 and BAd3 vectors are promising gene delivery vehicles for human gene therapy as well as for recombinant vaccines for human and animals use.

Key words: Nonhuman adenoviral vectors, adenoviral vectors, bovine adenovirus, porcine adenovirus, comparative transduction, CAR

INTRODUCTION

Adenoviral (Ad) vectors are excellent delivery vehicles for gene therapy and vaccination purposes. Numerous advantages offered by Ad vectors include the ease of large-scale production, well-characterized biology, a broad host range, and their ability to transduce post-mitotic cells. However, the practical usefulness of Ad vectors is severely hampered by the wide prevalence of preexisting adenoviral immunity in the majority of human population[1-3], and the lack of specific tissue tropism of human Ad serotype 5 (HAd5), the most widely used Ad vector. Alternative vectors derived from the less prevalent HAd serotypes such as HAd35 [4] or nonhuman Ad [5] are being developed to circumvent these shortcomings of HAd5 vectors.

Nonhuman Ad are not naturally prevalent in the human population and their use is expected to elude preexisting HAd immunity in humans.[1; 6; 7] Several nonhuman Ad have been investigated as potential vectors for gene delivery including porcine Ad serotype 3 [1; 8] and bovine Ad serotype 3 [9; 10]. We have previously [1] shown that preexisting Ad immunity in humans did not cross-neutralize PAd3. Furthermore, PAd3-, HAd5- and BAd3-specific neutralizing antibodies were found to be not cross-neutralizing and sequential administration of these viruses could circumvent Ad immunity in a mouse model [11].

In order to be relevant for application in human gene therapy, nonhuman Ad vectors should be capable of efficiently transducing human cells apart from eluding preexisting Ad immunity. Since most preclinical evaluations using various Ad vectors are conducted in the mouse model, it is equally important that the prospective nonhuman Ad vectors efficiently transduce murine cells. To assess the potential of nonhuman Ad vectors for developing recombinant vaccines for humans, pigs and cattle, it is critical to determine the repertoire of cell types of various species that are efficiently transduced with these vectors. In this study we show that preexisting human Ad immunity in humans does not cross-neutralize PAd3 or BAd3. We also compare transduction efficiencies of PAd3 and BAd3 vectors relative to those of a HAd5 vector in a variety of human, murine, bovine or porcine cell lines and examine their correlation with CAR expression levels.

MATERIALS AND METHODS

Cells lines

Various cell lines of human, murine, bovine and porcine origin were propagated following the standard procedures recommended by American Type Culture Collection (ATCC). The origin and source of these cell lines are given in Table 1.

Recombinant viruses

The construction and propagation of replication-defective HAd-GFP (HAd5 vector carrying the green fluorescent protein [GFP] gene) and PAd-GFP (PAd3 vector carrying the GFP gene) has been described elsewhere [1]. Construction of the replication-defective BAd-GFP (BAd3 vector carrying the GFP gene) is described below. HAd-GFP, PAd-GFP and BAd-GFP were grown in 293, FPRT HE1-5[1] and FBRT HE1[12] cells, respectively and were purified by cesium chloride density gradient centrifugation and titrated by plaque assay as described [1].

Construction of BAd-GFP

For foreign gene insertion into the E1A region of BAd3 genome, we constructed a BAd3 E1 transfer plasmid (pBAd-E1T) containing the left (1.6 kb) and right (1.2kb) ends of BAd3

genome flanked by *Pac*I sites and having a 0.6 kb deletion in the E1 region (E1A) replaced with an unique *XbaI* site. The GFP gene under the control of the CMV promoter and the bovine growth hormone polyA was inserted into pBAd-E1T at the *Xba*I site in the E1-parallel orientation to generate pBAd-E1T-GFP. The unique *Hind*III site present between the left (1.6 kb) and right (1.2kb) ends of BAd3 genome was used to linearize pBAd-E1T-GFP to facilitate recombination with BAd-ΔE3 (BAd3 having a 1.2 kb deletion in the E3 region) genome to obtain an infectious clone (pBAd-GFP) by homologous recombination in *E.coli* BJ5183 (*recBC* and *sbcBC*) [12]. DNA from the infectious clone was digested with *Pac*I and used for transfecting FBRT HE1 cells to generate infectious virus (BAd-GFP). Initially the presence of the GFP gene in the BAd3 genome was identified by agarose gel electrophoresis of vector DNA digested with suitable restriction enzyme/s. Subsequently GFP expression by BAd-GFP in infected cells was confirmed by Western blot analysis using a GFP-specific antibody as well as by fluorescence microscopy.

Virus neutralization assay

Collection and processing of human serum samples were the same as described previously [1]. For determining BAd3 cross-neutralizing antibodies in human serum samples, wild-type BAd3 was used and the virus was titrated on MDBK cells. A hyperimmune serum raised in a rabbit against BAd3 [11] was used as a positive control. The virus neutralization titer was the reciprocal of the highest serum dilution that completely prevented the development of cytopathic effect.

Western blot analysis for CAR expression

Cell lysates were prepared and subjected to Western blotting for CAR expression as described previously [1]. Rabbit polyclonal anti-human CAR antibody at 1:200 dilution, and HRP conjugated goat anti-rabbit IgG at 1:2000 were used as primary and secondary antibodies, respectively.

Comparative transduction experiments

Each cell line was seeded in 12-well tissue culture plates at the rate of 1-2 x 10^5 cells per well. Twenty-four h post-infection, triplicate wells were infected either with HAd-GFP, BAd-GFP, or PAd-GFP at a multiplicity of infection (m.o.i.) of 25 plaque forming units (p.f.u.) per cell. After 45 min adsorption at 37^{o}C, the wells were supplemented with minimum essential medium containing 2% fetal calf serum. Infected cells were harvested by trypsinization at 36 h post-infection, and washed once with phosphate buffered saline (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) and cells expressing GFP were sorted by flow cytometry as described below. Mock-infected cells served as negative controls.

Flow cytometry

Cells were resuspended at 10^6cell/ml in PBS containing 2% formaldehyde. Green fluorescent cells were enumerated by flow cytometry at Purdue University Flow Cytometry Laboratories using Cytomics™ FC-500 Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA) with Ar 488 nm excitation filter and FL-1 525 nm BP emission filter. For each sample, a total of 5000-10000 cells were counted.

RESULTS AND DISCUSSION

Absence of PAd3 or BAd3 cross-neutralizing antibodies in humans

Preexisting HAd immunity in the majority of humans is a major obstacle to the use of adenoviral vectors in vivo [13]. Although nonhuman adenoviruses are not expected to be prevalent in human population, it was important to know whether neutralizing antibodies against some of the >50 HAd subtypes would cross-neutralize PAd3 or BAd3 or exposure to PAd or BAd subtype/s occurs naturally in humans. Using 50 randomly collected human serum samples we previously reported that preexisting HAd-specific humoral immune response in humans did not cross-neutralize PAd3 [1]. These same samples were tested for BAd3 cross-neutralizing antibodies by virus neutralization assay. HAd5-specific neutralizing titers of <4, 4-64, 64-256, and >256 were observed in approximately 6, 36, 42 and 16% of the samples, respectively (Fig. 1). Importantly, PAd3 or BAd3 cross-neutralizing titers in all the samples were <4 (the lowest serum dilution in our assay) (Fig. 1) suggesting that preexisting HAd immunity did not cross-neutralize PAd3 or BAd3. These findings strongly favor the use of PAd3 or BAd3 vectors for gene delivery in humans in situations where high titers of preexisting HAd neutralizing antibody response are observed. In addition, such vectors will also facilitate readministration of the therapeutic gene(s) after the first use of a HAd vector.

Coxsackievirus-adenovirus receptor (CAR) expression in various cell lines

Coxsackievirus-adenovirus receptor (CAR) is a immunoglobulin superfamily protein that has been implicated as a primary receptor mediating entry of HAd5 [14] and several other HAd serotypes [15]. Poor transduction of a variety of cancer cell types by HAd5 vectors has been attributed to the loss of CAR receptor in such cell types [16]. While receptors involved in PAd3 and BAd3 internalization are not currently known, we have observed that transduction by these vectors was CAR-independent [2]; unpublished observation). To ascertain if CAR expression on various cell types correlates with transduction of HAd5, PAd3 and BAd3 vectors, we evaluated the levels of CAR expression by Western blot in a number of human, murine, bovine and porcine cell lines (Fig.1). Since 293 cells are known to express high levels of CAR and NIH 3T3 cells are CAR-deficient [2; 17], these cell lines served as positive and negative controls, respectively. Expression levels of CAR in cell lines of human origin were as follow: high in HepG2, DU145 and LNCaP, moderate to low in MDA-MB-231 and MCF-10A, and below the detection level in T24, TCCSUP, PC-3, Jurkat and HRT cell lines (Fig. 2A). Expression levels of CAR in the cell lines of murine origin were high in MT1A2 and EL4, moderate in MS-K68, and below the detection level in NIH 3T3, WEHI and AML12 cell lines (Fig. 2B). In bovine cell lines, high levels of CAR expression were observed in FBRT HE1, MDBK and FBK-34 cell lines, whereas in BT-5705 and EBL cell lines, CAR expression was below the detection level (Fig. 2C). Among the porcine cell lines, FPRT HE1-5 and PK-15 showed high, and PK-M12 showed moderate levels of CAR expression, while in PT-K75, EPL and SBC-1765 cell lines CAR expression was below the detection level (Fig. 2D).

These results indicated that CAR expression in cell lines of various species was highly variable. This variability in CAR expression reflected the diverse origins of these cell lines. CAR expression in vivo is highly variable depending on the tissue type [18], and it also varies with the developmental stage [19]. Increased expression of CAR was recently reported in osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma [20]. CAR expression levels on various human cell lines, as observed in the present study were in agreement with several previous reports [2; 16; 21].

Transduction efficiencies of HAd5 vectors in human, murine, bovine and porcine cell lines

In order to determine transduction efficiencies of HAd5 vectors in human, murine, bovine and porcine cell lines, a HAd5 vector expressing GFP as a reporter (HAd-GFP) was used. Various cell lines were infected with HAd-GFP at an m.o.i. of 25 p.f.u. per cell and at 36 h post-infection, the percent of vector transduced cells was determined by flow cytometry. The HAd-GFP transduction efficiencies of ≥70, 50-69, 25-49, 15-24, and ≤14% were taken as very high, high, moderate, low, and very low, respectively. In human cell lines, transduction efficiencies were very high in 293, MDA-MB-231, HepG2 and DU145, high in MCF-10A, TCCSUP, LNCaP, and PC-3, moderate in T24, and very low in Jurkat and HRT (Fig. 3). In murine cell lines, transduction efficiencies were very high in MT1A2, moderate in MSK68 and AML12, and very low in NIH-3T3, WEHI and EL4 (Fig. 3). The transduction efficiencies of HAd-GFP in bovine cells were very high in FBRT and MDBK, moderate in BT-5705 and FBK-34 and very low in EBL (Fig. 3). Transduction efficiencies in the cell lines of porcine origin were very high in FPRT HE1-5, SBC-1765, PK-15 and PK-M12, high in EPL, and low in PT-K75 (Fig. 3).

In general, there was a correlation between levels of CAR expression and efficiency of HAd-GFP transduction, except in human (T24, TCCSUP and PC-3), murine (AML12), and porcine (SBC-1765 and EPL) cell lines, which showed significant transduction in the absence of detectable levels of CAR expression. These results suggested that either very low levels of CAR expression in these cell lines might be enough for vector transduction or HAd vector internalization in these cell lines may be independent of CAR. Whereas, mouse EL4 and bovine FBK-34, which expressed high levels of CAR were poorly transduced by HAd-GFP suggesting that CAR expression alone may not be sufficient for HAd5 internalization in these two cell lines. Inefficient transduction of human cell lines Jurkat and HRT and only moderate transduction of T24 and TCCSUP cell lines was in agreement with previous reports [16; 21] suggesting that absence or loss of CAR from cell surface renders the cells poorly transducible with HAd5 vectors.

Comparative transduction efficiencies of PAd3, BAd3 and HAd5 vectors in human, murine, bovine and porcine cell lines

To assess the usefulness of BAd3 or PAd3 vectors as a potential tool for gene delivery, we determined the transduction efficiencies of PAd-GFP and BAd-GFP in a number of human, murine, bovine or porcine cell lines and compared them with those obtained with HAd-GFP. Comparative transduction efficiencies of PAd-GFP or BAd-GFP in various cell lines represent the percent transduction relative to transduction efficiency of HAd-GFP for each cell line. PAd-GFP transduced PC-3, MCF-10A and MDA-MB-231 cell lines somewhat better than HAd-GFP and transduction of 293 cells with PAd-GFP was approximately 90% of that obtained with HAd-GFP (Fig. 4A). Jurkat cell line was poorly transduced by these three vectors (Figs. 3 and 4A). Transduction efficiencies of PAd-GFP in TCCSUP, T24, HepG2, LNCaP, DU145 and HRT cell lines were significantly ($p<0.5$) lower that those observed with HAd-GFP. These results suggested that PAd3 vectors might be less promiscuous than HAd5 vectors and could have an advantage in specific targeting of the breast tissue. BAd-GFP on the other hand seemed to be more limited in transducing human cells. Except 293 cells, BAd-GFP failed to efficiently transduce any of the human cell lines tested (Fig. 4A) indicating that BAd3 vectors may have an advantage for developing modified vectors to target a specific tissue or organ. Limited transduction of BAd3 vectors in human cells was in agreement with previous reports [22; 23].

Comparative transduction efficiencies of PAd-GFP in murine MT1A2, WEHI, EL4 and AML12 cell lines were comparable to those of HAd-GFP. Whereas, transduction of NIH 3T3 and MS-K68 cell lines with PAd-GFP was approximately 674 and 50% of those with HAd-GFP (Fig. 4B). Similarly, comparative transduction efficiencies of BAd-GFP in MT1A2, WEHI and EL4 cell lines were comparable to those of HAd-GFP. However, transduction of NIH 3T3, MS-K68 and AML12 cell lines with BAd-GFP was approximately 587, 35 and 45% of those with HAd-GFP (Fig. 4B). This extremely high transduction of NIH 3T3 cells, which are known to be low CAR-expressing [1; 17], by both PAd-GFP and BAd-GFP vectors suggested that transduction by these vectors was CAR-independent and that the mouse would serve as a good animal model for both PAd3 and BAd3 vectors.

Both PAd-GFP and BAd-GFP efficiently transduced all cell lines of bovine origin tested except FBK-34, in which BAd-GFP transduction was significantly better than that of PAd-GFP (Fig. 4C). BT-5705 and EBL cell lines that are poorly transduced by HAd-GFP were efficiently transduced by both PAd-GFP and BAd-GFP (Fig. 4C). All three vectors, HAd-GFP, PAd-GFP and BAd-GFP efficiently transduced all cell lines of porcine origin tested except PT-K75, in which BAd-GFP and PAd-GFP transductions were significantly better than those of HAd-GFP (Fig. 4D). The comparative transduction results in porcine and bovine cells indicated that PAd3, BAd3 and HAd5 vectors would be useful as gene delivery systems both in pigs and cattle. Since pigs could serve as an animal model for a number of metabolic diseases including cardiovascular disorders [24] and diabetes [25], our results signify that various genetic intervention strategies could be evaluated in pigs using human and nonhuman adenoviral vectors.

We have previously demonstrated that PAd3-, BAd3-, or HAd5-specific neutralizing antibody response was not cross-neutralizing [11]; therefore, for developing Ad vector-based recombinant vaccines for bovine and porcine while circumventing preexisting species-specific vector immunity, we anticipate that PAd3 and BAd3 vectors will be better suited for bovine and porcine, respectively. Our results of this study further support the use of PAd3 and BAd3 vectors as an alternative or supplement to HAd vectors. Overall, the transduction profile of PAd3 and BAd3 vectors in various cell lines tested in this study suggests that internalization of these vectors is CAR-independent. Since wide tropism of HAd5 vectors has been attributed to their CAR-dependent transduction [14], CAR-independent tropism of these nonhumans Ad vectors may have implications in designing novel vectors with restricted tropism and decreased vector toxicity.

ACKNOWLEDGEMENTS

We thank Mary Woodruff for providing HRT, MS-K68, AML12, BT-5705, EBL and PT-K12 cell lines, Steve Hooser for providing HepG2 cell line, Chang Kim for providing Jurkat and EL4 cell lines, William Muller, Department of Biology, McMaster University, Hamilton, Ontario, Canada for providing MT1A2 cell line, and Jane Kovach for excellent secretarial assistance. This work was supported by NIH, Purdue Research Foundation, and Hatch grants.

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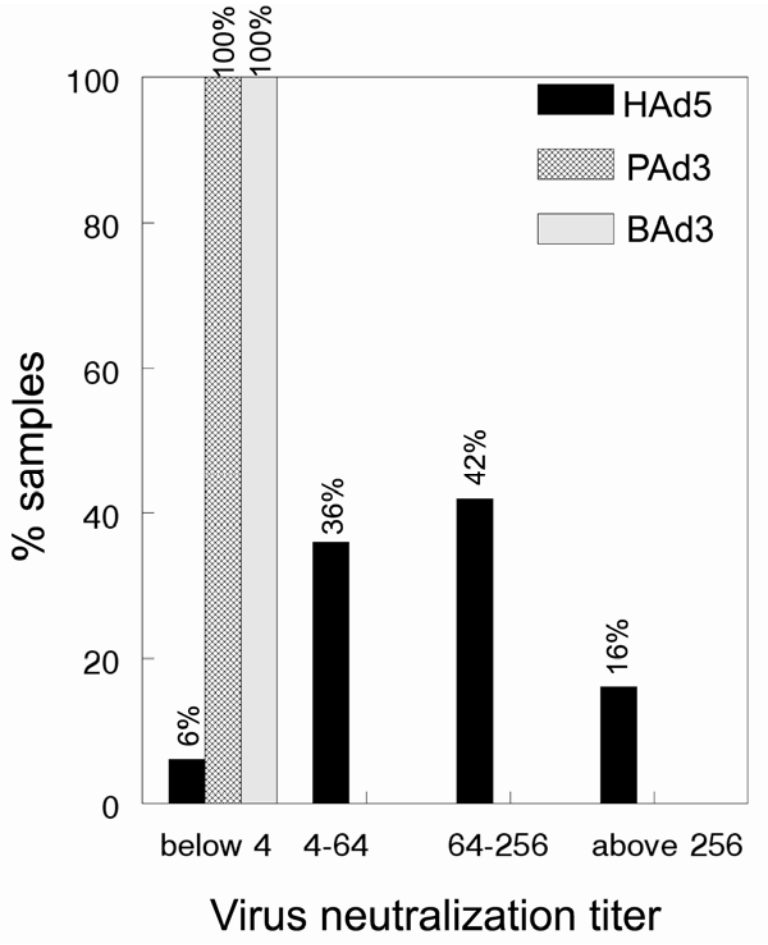

Fig. 1. Detection of preexisting HAd5-, PAd3-, or BAd3-neutralizing antibodies in human serum samples. Fifty serum samples collected from healthy individuals were tested for the presence or absence of HAd5-, PAd3- or BAd3-neutralizing antibodies by virus neutralization assays. The reciprocal of the highest serum dilution, which prevented viral c.p.e., was taken as the neutralization titer. The percent serum samples that display virus-specific neutralization titers of < 4, 4-64, 64-256, and >256 are shown. Data for HAd5 and PAd3 neutralization are obtained from [1]

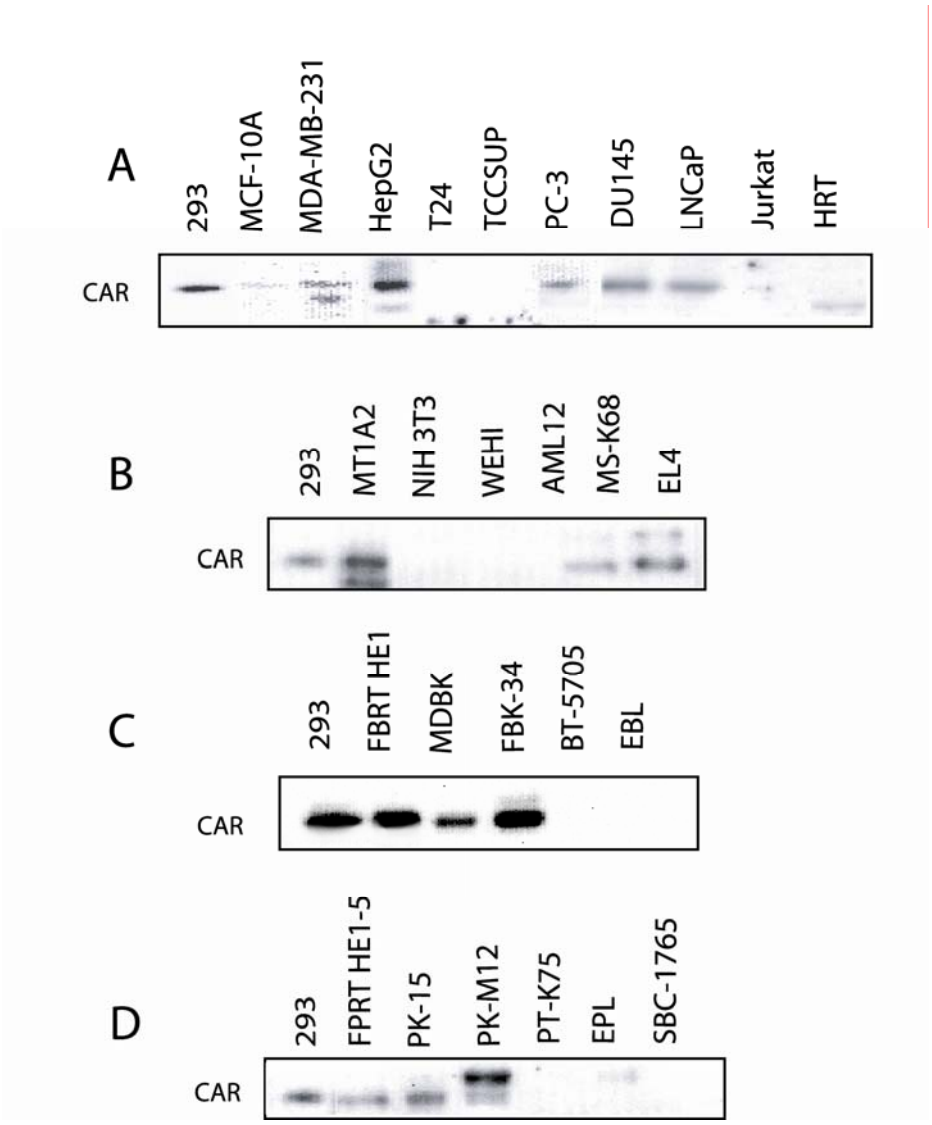

Fig. 2. Level of CAR expression in human, murine, bovine and porcine cell lines. A number of cell lines of **A**) human (293, MCF-10A, MDA-MB-231, HepG2, T24, TCCSUP, PC-3, DU145, LNCaP, Jurkat, and HRT) **B**) murine (MT1A2, NIH 3T3, WEHI, AML12, MS-K68 and EL4), **C**) bovine (FBRT HE1, MDBK, FBK-34, BT-5705 and EBL), and **D**) porcine (FPRT HE1-5, PK-15, PK-M12, PT-K75, EPL and SBC-1765) origin were lysed and cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot using a rabbit polyclonal anti-human CAR antibody and a secondary HRP-conjugated anti-rabbit antibody.

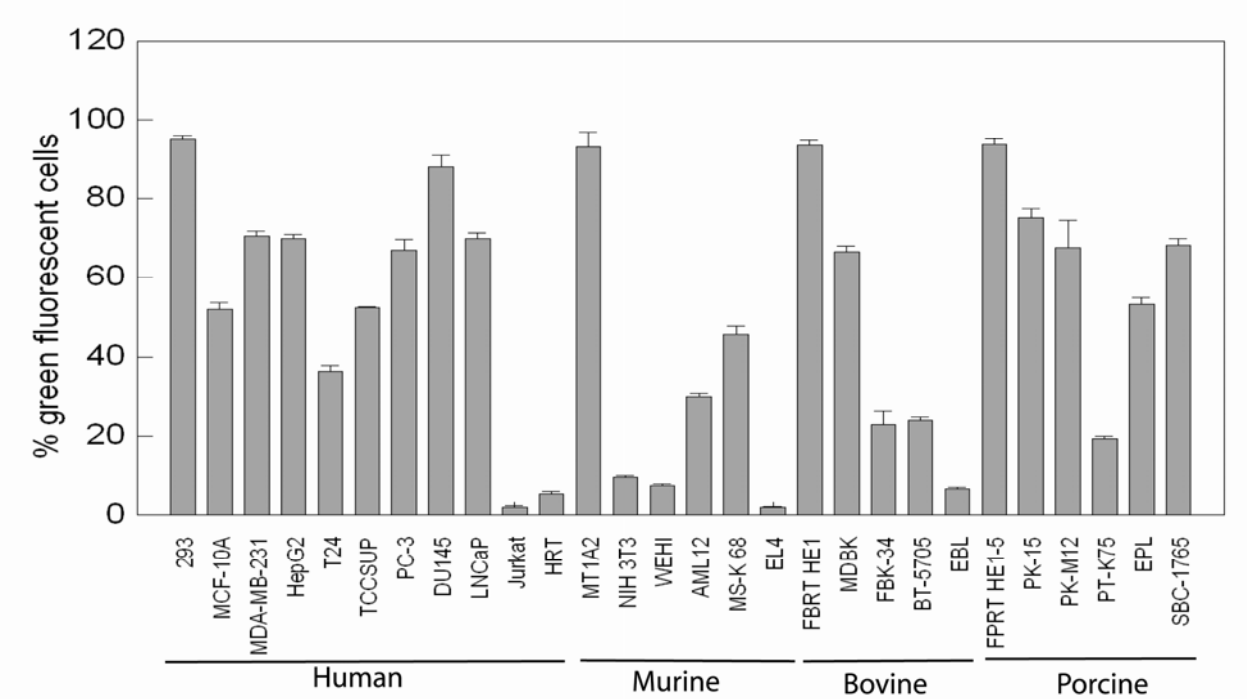

Fig. 3. Transduction efficiencies of HAd-GFP in a number of cell lines of human, murine, bovine, or porcine origin. Various cell lines of human (293, MCF-10A, MDA-MB-231, HepG2, T24, TCCSUP, PC-3, DU145, LNCaP, Jurkat, and HRT), murine (MT1A2, NIH 3T3, WEHI, AML12, MS-K68 and EL4), bovine (FBRT HE1, MDBK, FBK-34, BT-5705 and EBL), and porcine (FPRT HE1-5, PK-15, PK-M12, PT-K75, EPL and SBC-1765) origin grown in 12-well tissue culture plates were infected with HAd-GFP at an m.o.i. of 25 p.f.u. per cell. Thirty-six hours post infection the cells were harvested and sorted by flow cytometry. Mock-infected cells served as negative control. Each data point represents the mean ± standard deviation of three independent observations.

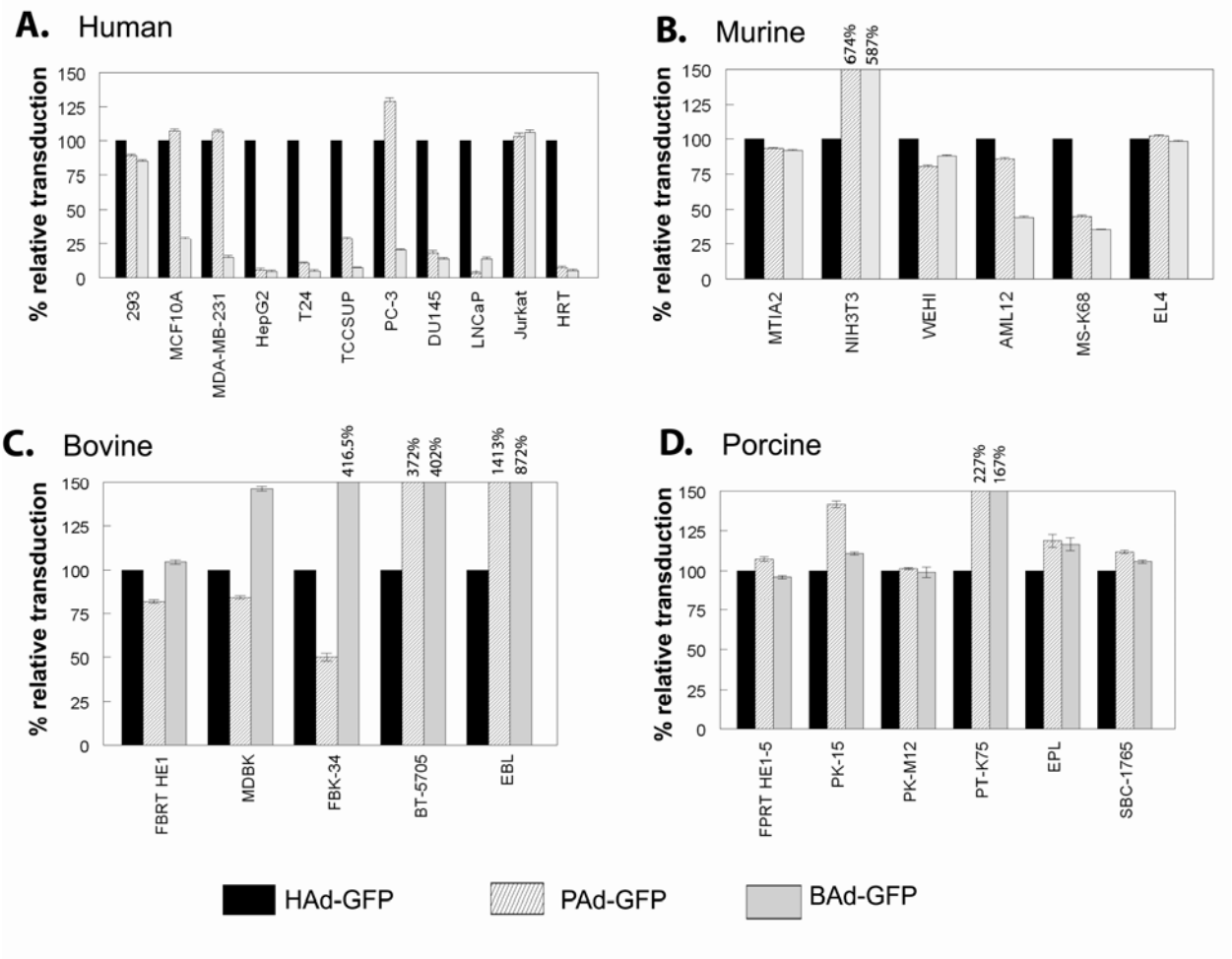

Fig. 4. Comparative transduction of HAd-GFP, PAd-GFP, or BAd-GFP in various cell lines of A) human, B) murine, C) bovine, and D) porcine origin. A number of cell lines grown in 12-well tissue culture plates were infected with HAd-GFP, PAd-GFP, or BAd-GFP at an m.o.i. of 25 p.f.u. per cell. Thirty-six hours post infection the cells were harvested and sorted by flow cytometry for GFP expressing cells. For calculating relative transduction efficiencies of PAd-GFP or BAd-GFP in various cell lines, the transduction efficiency of HAd-GFP for each cell line was considered as 100%. Each data point represents the mean ± standard deviation of three independent observations.

Table 1. Cell lines of human, murine, bovine and porcine origin used in this study

Cell line	Species	Origin	Source (reference)
293	Human	Embryonic kidney	ATCC
MCF-10A	Human	Mammary epithelium	ATCC
MDA-MB-231	Human	Malignant breast cancer cell	ATCC
HepG2	Human	Hepatocarcinoma	ATCC
T24	Human	Transitional cell carcinoma	ATCC
TCCSUP	Human	Transitional cell carcinoma	ATCC
PC-3	Human	Prostate cancer metastases site	ATCC
DU145	Human	Prostate cancer metastases site	ATCC
LNCaP	Human	Prostate cancer metastases site	ATCC
Jurkat	Human	T cell leukemia	ATCC
HRT	Human	Rectal tumor	ATCC
MT1A2	Murine	Mammary tumor transformed with SV40 T antigen	[26]
NIH 3T3	Murine	Mouse fibroblast	ATCC
WEHI	Murine	Fibrosarcoma	ATCC
AML12	Murine	liver	ATCC
MS-K68	Murine	Spleen	ADDL, Purdue University
EL4	Murine	T cell	ATCC
FBRT HE1	Bovine	Fetal retina transformed with HAd5 E1	[12]
MDBK	Bovine	Kidney	ATCC
FBK-34	Bovine	Kidney transformed with BAd3 E1	[12]
BT-5705	Bovine	turbinate	ATCC
EBL	Bovine	Embryonic lung	American BioResearch, TN, USA
FPRT HE1-5	Porcine	Fetal reina transformed with HAd5 E1	[1]
PK-15	Porcine	kidney	ATCC
PK-M12	Porcine	Kidney cell transformed with SV40 T antigen	Produced in our lab
PT-K75	Porcine	turbinate	ADDL, Purdue University
EPL	Porcine	Embryonic lung	Produced in our lab
SBC-1765	Porcine	Derived from buffy coat cells	ADDL, Purdue University