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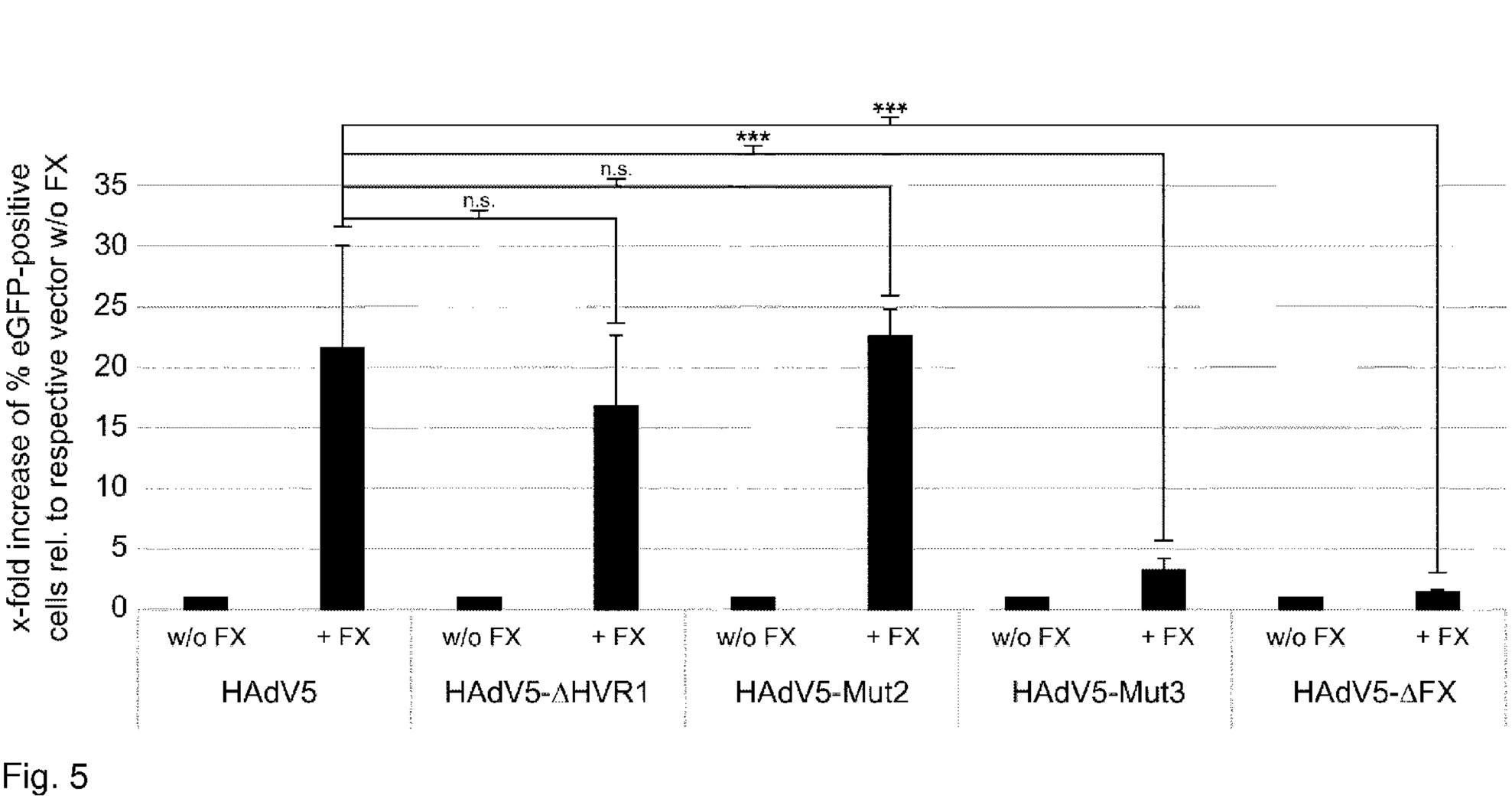


Fig. 5

(57) Abstract: The invention discloses a human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1). The invention further discloses the adenovirus of the disclosure for use in treating or preventing a human disease. The invention further discloses a nucleic acid encoding the modified adenovirus hexon protein. The invention further discloses the use of an adenovirus according to the disclosure for transducing mesenchymal stromal cells (MSCs) or tumor cells. The invention further discloses an in vitro method for transducing MSCs and a transduced MSC obtainable by the method. The invention further discloses the transduced MSC of the disclosure for use in treating a disease.

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Adenovirus comprising a modified adenovirus hexon protein

Field of the invention

The present invention relates to a human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1). The invention further relates to the adenovirus of the invention for use in treating or preventing a human disease. The invention further relates to a nucleic acid encoding the modified adenovirus hexon protein. The invention further relates to the use of an adenovirus according to the invention for transducing mesenchymal stromal cells (MSCs) or tumor cells. The invention further relates to an *in vitro* method for transducing MSCs and a transduced MSC obtainable by the method. The invention further relates to the transduced MSC of the invention for use in treating a disease.

Background of the invention

Adenoviruses are non-enveloped viruses belonging to the virus family Adenoviridae. They carry a linear double-stranded DNA genome with a size of about 36 kilobases (kb). Currently, there are 89 different Human Adenovirus (HAdV) types that are known and that are grouped into seven species, designated species A to G. HAdV species C comprises six types, namely types 1, 2, 5, 6, 57 and 89.

Recombinant adenoviruses can be used for the transfer of nucleic acids, proteins or other molecules into cells of a patient in preventive or therapeutic settings. However, efficient delivery to, e.g., tumors after systemic HAdV administration is still challenging due to several cellular and non-cellular non-target interactions and sequestration mechanisms.

Mesenchymal stromal cells (MSCs) have a natural migration behaviour to tumor tissue and are thus an interesting potential carrier of adenoviruses to tumor tissue. However, while adenoviruses can infect most types of target cells, MSCs, which

do not express the coxsackievirus and adenovirus receptor (CAR), are hardly infected by adenoviruses in vitro.

Therefore, new tools and methods are needed that overcome the current limitations for using adenoviruses in medicine.

Summary of the invention

In a first aspect, the present invention relates to a human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1).

In a second aspect, the present invention relates to the adenovirus of the invention for use in treating or preventing a human disease.

In a third aspect, the present invention relates to a nucleic acid encoding a modified adenovirus hexon protein of a human adenovirus species C, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence of SEQ ID NO.: 1.

In a further aspect, the present invention relates to the use of an adenovirus according to the invention for transducing mesenchymal stromal cells (MSCs) or tumor cells.

In a further aspect, the present invention relates to an *in vitro* method for transducing MSCs, the method comprising the step of:
contacting a plurality of MSCs with an adenovirus according to the invention.

In a further aspect, the present invention relates to a transduced MSC obtainable by the method of the invention.

In a further aspect, the present invention relates to the transduced MSC of the invention for use in treating a disease.

Brief description of the figures

Fig. 1 shows an alignment of the amino acid sequences of hypervariable region 1 of HAdV5 wildtype and HAdV5-Mut3 hexon proteins (Fig. 1A) and of hypervariable regions 1 (HVR1), 5 (HVR5) and 7 (HVR7) of HAdV5 wildtype, HAdV5-ΔHVR1, HAdV5-Mut2, HAdV5-Mut3, HAdV5-Mut4, HAdV5-Mut5, HAdV5-Mut6 hexon proteins (Fig. 1B). Negatively charged amino acids are depicted in bold letters. Inserted lysine residues are depicted in italic letters.

Fig. 2 shows an alignment of the nucleotide sequences encoding hypervariable region 1 of HAdV5 wildtype and HAdV5-Mut3 hexon proteins. Nucleotides encoding for the inserted lysine residues are depicted in capital letters.

Fig. 3 shows that the hexon protein appeared smaller in silver staining for HAdV5-ΔHVR1 and HAdV5-Mut3 than for HAdV5-Mut2 and HAdV5 wildtype (HAdV5). $5x10^9$ virus particles were separated by SDS-PAGE and stained by silver staining.

Fig. 4 shows that the surface charge of HAdV5-Mut3 is significantly higher than for HAdV5 wildtype (HAdV5), HAdV5-Mut2 and HAdV5-ΔHVR1. $2x10^{11}$ virus particles of HAdV5, HAdV5-Mut2, HAdV5-Mut3 and HAdV5-ΔHVR1 were dialyzed overnight, purified with PD MiniTrap G-25 column and measured at ZetaSizer Nano-ZS. Results are given as mean ± standard deviation (n = 5-6). *** $p \leq 0.0005$.

Fig. 5 shows that HAdV5-Mut3 shows significantly less Factor X (FX)-mediated transduction of CAR-negative cells than HAdV5 wildtype (HAdV5), HAdV5-Mut2 and HAdV5-ΔHVR1. $2x10^4$ SKOV-3 cells were treated with FX (+ FX) (8 µg/ml) or without FX (w/o FX) and infected with $2x10^7$ virus particles (pMOI 1000), incubated for 72 h and analyzed for eGFP expression by flow cytometry. Results are given as mean ± standard deviation (n = 14-15). *** $p \leq 0.0005$.

Fig. 6 shows that the uptake of HAdV5-Mut3 by scavenger receptors is significantly reduced compared to HAdV5 wildtype (HAdV5). $1x10^5$ J774.A1 cells were treated with polyinosinic acid (Poly-(I)) (30 µg/ml) for 1 h if indicated, followed by infection with $2x10^8$ virus particles (pMOI 2000), incubated for another 21 h, harvested followed by DNA isolation and qPCR analysis. Results are given as mean ± standard deviation (n = 6). ** $p \leq 0.005$.

Fig. 7 shows that FX binding-ablated HAdV5-Mut3 vector particles escape from neutralization by natural IgMs. HAdV5 wildtype (HAdV5), HAdV5-ΔFX or HAdV5-Mut3 were incubated with PBS or Ad-naïve, hirudinized human or murine plasma samples of different donors or mouse strains for 10 min at 37 °C in a ratio of 2E6 VP/µl. Subsequently, A549 cells were transduced with a pMOI of 1,000. The eGFP expression by the cells was analyzed 24 h post transduction by flow cytometry. Results are given as mean ± standard deviation (n = 7-12). *** $p \leq 0.0005$.

Fig. 8 shows a significantly enhanced secretion of HAdV5-encoded therapeutic protein TSG-6 by MSCs in the presence of Factor X. MSCs were transduced with different pMOIs of the TSG-6-expressing adenoviral vector HAdV5-TSG6 in the absence or presence of Factor X. 72 h post transduction, the TSG-6 concentration in cell culture supernatants was quantified by ELISA (n=1).

Fig. 9 shows that the transduction of several tumor cell lines is improved or preserved when using HAdV5-Mut3 compared to HAdV5 wildtype (HAdV5). HAdV5-Mut3 and HAdV5 were used to transduce UM-SCC-11B, MiaPaCa, Huh7, HepG2 and A549 cells with a pMOI of 300 and 1000. 24 hours post transduction, the percentage of eGFP positive cells was determined by flow cytometry. Results are given as mean ± standard deviation (n = 3). * $p \leq 0.05$.

Fig. 10 shows a significantly enhanced transduction of MSCs by pre-incubation of HAdV5 wildtype (HAdV5) with enhancers or by utilization of HAdV5-Mut3. MSCs were transduced by HAdV5 (either pre-incubated with the respective enhancer or not) or HAdV5-Mut3 with a pMOI of 1000. 72 hours post transduction, eGFP expression was analyzed by flow cytometry. Results are given as mean ± standard deviation (n = 3). * $p \leq 0.05$.

Fig. 11 shows an improved adenoviral replication in MSCs by using enhancer pre-incubated HAdV5wt or by using HAdV5-Mut3wt. MSCs were infected by HAdV5wt (either pre-incubated with the respective enhancer or not) or HAdV5-Mut3wt with a pMOI of 300 and 1000. 24, 48, 72 and 97 hours post infection, infectious adenoviral particles produced by MSCs were quantified. Results are given as mean ± standard deviation of biological duplicates (n = 1).

Fig. 12 shows that a combination of HAdV5-Mut3 with enhancers results in an enhanced eGFP expression. MSCs were transduced by HAdV5-Mut3 (either pre-incubated with the respective enhancer or not) with a pMOI of 100, 300, 600, 900, 1000 or 10,000. The eGFP expression was analyzed 72 hours post transduction by flow cytometry. Results are given as mean value of two donors (biological triplicates each) ± standard deviation (n = 2).

Fig. 13 shows that transduction of MSCs does not inhibit their migration towards UM-SCC-11B cells. Migration of MSCs, not-transduced or transduced using HAdV5 wildtype (HAdV5) in combination with enhancing molecules or using HAdV5-Mut3, was analyzed in a Boyden-Chamber-Assay. 18 hours after starting the migration assay, cells were fixed and stained with DAPI. Migrated cells were quantified by counting DAPI-stained nuclei. Results are given as mean ± standard deviation.

Fig. 14 shows an alignment of the amino acid sequences of HVR1 of HAdV5 wildtype and HAdV5-Mut7, HAdV5-Mut8 and HAdV5-Mut9 hexon proteins. Inserted lysine residues are depicted in bold letters. Further inserted amino acids are depicted in italic letters.

Fig. 15 shows that the transduction of several tumor cell lines is improved or preserved when using HAdV5-Mut3 (HAdV-5-M3) compared to HAdV5 wildtype (HAdV-5) and that the transduction of several tumor cell lines is improved when using HAdV5-ΔCAR-Mut3 (HAdV-5-ΔCAR-M3) compared to HAdV5-ΔCAR (HAdV-5-ΔCAR). The specified adenoviral vectors were used to transduce UM-SCC-11B, MiaPaCa, Huh7, HepG2 and A549 cells with a pMOI of 1000. 24 hours post transduction, the percentage of eGFP positive cells was determined by flow cytometry. Results are given as mean ± standard deviation (n = 9). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Fig. 16 shows a significantly enhanced transduction of MSCs by utilization of HAdV5-Mut3 (HAdV-5-M3) and HAdV5-ΔCAR-Mut3 (HAdV-5-ΔCAR-M3) and no transduction of MSCs by utilization of HAdV5-ΔHVR1 (HAdV-5-ΔHVR1) and HAdV5-Mut2 (HAdV-5-M2). MSCs were transduced by the specified adenoviral vectors with a pMOI of 1000. 24 hours post transduction, eGFP expression was

analyzed by flow cytometry. Results are given as mean ± standard deviation (n = 3). * $p \leq 0.05$, **** $p \leq 0.0001$.

Detailed description of the invention

In a first aspect, the present invention relates to a human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1).

Human adenovirus (HAdV) species C comprises six types, namely types 1, 2, 5, 6, 57 and 89. Complete nucleotide sequences representing all adenovirus species C prototypes are available as GenBank entries: HAdV-C1 (AC_000017.1), HAdV-C2 (AC_000007.1), HAdV-C5 (AC_000008.1), HAdV-C6 (FJ349096.1), HAdV-C57 (HQ003817.1) and HAdV-C89 (MH121097.1).

Adenoviruses have an icosahedral-shaped capsid. The outer shell of the capsid comprises three major types of proteins: hexon, penton base and fiber. These three capsid proteins contribute to the majority of activities required for the early stages of adenovirus infection. The adenovirus hexon protein accounts for the majority of the outer shell of the capsid, forming 240 homo-trimers that encapsidate the majority of the virus, including the viral genome and associated proteins. The fiber protein protrudes from each of the 12 vertices of the icosahedron, while the penton base lies at the base of each fiber protein.

The adenovirus hexon protein has seven hypervariable regions (HVRs), designated HVR1 to HVR7. The modified adenovirus hexon protein of the adenovirus of the invention has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1). In other words, the modified HVR1 region comprises the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1). The amino acid sequence of SEQ ID NO.: 1 is derived from the wildtype amino acid sequence of HVR1 of the HAdV type 5 hexon protein by replacing 13 consecutive amino acid residues of the wildtype sequence, namely the amino acid stretch EEEDDDNEDEVDE (SEQ ID NO.: 6), by four consecutive ly-

sine residues. Due to the modified HVR1 region, the modified adenovirus hexon protein differs from the respective wildtype adenovirus hexon protein.

The modified HVR1 region may comprise additional amino acids located N-terminal or C-terminal of SEQ ID NO.: 1. Typically, the modified HVR1 region has five additional amino acids which are located C-terminal of SEQ ID NO.: 1 and which correspond to the respective wildtype amino acids.

The invention is based on the finding that a modified adenovirus hexon protein that has a modified HVR1 region, wherein the modified HVR1 region has the amino acid sequence of SEQ ID NO.: 1, leads to an adenovirus with improved characteristics. Importantly, the improved characteristics could not be observed for other similar modifications of the HVR1 region.

The inventors set out to produce six different modified (mutant) adenovirus vectors, each having a different type of modified HVR1 region in the hexon protein (Fig. 1B). All mutants were designed with a view to reducing the negative surface charge of the viral particles. The mutant designated HAdV5-Mut3 has a modified HVR1 region that has the sequence of SEQ ID NO.: 1 (Fig. 1A). The complete sequence of the HVR1 region of the modified hexon protein of HAdV5-Mut3 is: DEAATALEINLKKKKQAEQQKTHVF (SEQ ID NO.: 43).

The inventors found that certain modifications to the HVR1 region render the production of the mutant virus vectors unfeasible. Production of the mutant vectors designated HAdV5-Mut4, HAdV5-Mut5, HAdV5-Mut6 was unfeasible.

Production of three further modified (mutant) adenovirus vectors designated HAdV5-Mut7, HAdV5-Mut8 and HAdV5-Mut9, each having a different type of modified HVR1 region in the hexon protein (Fig. 14), was also found to be unfeasible.

The inventors further found that deleting the negatively charged HVR1 loop (mutant HAdV5-ΔHVR1) resulted only in a slight reduction of the negative surface charge of virus particles. The replacement of four aspartic acids by lysines in case of mutant HAdV5-Mut2 further reduced the negative surface charge. However and interestingly, HAdV5-Mut3, which comprises the modified HVR1 region of

SEQ ID NO.: 1, showed a significant reduction of the negative surface charge in comparison to HAdV5 wildtype, HAdV5-ΔHVR1, and HAdV5-Mut2.

Surprisingly, the inventors further found that HAdV5-Mut3 showed a significant reduction of human blood coagulation factor X (FX)-mediated transduction of CAR-negative cells in comparison to HAdV5 wildtype, HAdV5-ΔHVR1, and HAdV5-Mut2. Binding of FX to HAdV5 mediates transduction of hepatocytes and therefore triggers the sequestration of particles, which is one of the major obstacles for efficient HAdV delivery to, e.g., tumors after systemic HAdV administration. The adenovirus of the invention overcomes this obstacle. In contrast, HAdV5-ΔHVR1 and HAdV5-Mut2 showed no reduced FX-binding although their HVR1 region modifications are similar to the one of HAdV5-Mut3.

The significantly reduced FX binding to HAdV5-Mut3 and the thereby reduced hepatocyte transduction of HAdV5-Mut3 is an unexpected finding. According to Alba et al. (Alba et al., 2009), FX interaction with HAdV5 capsid is via binding to HVR5 and HVR7 of the adenovirus hexon protein. FX interaction with HVR3, HVR5 and HVR7 of the adenovirus hexon protein has also been reported. In contrast, a role of HVR1 in FX binding was neither known nor expected. Therefore, it is a surprising finding that a modification in the HVR1 region of the adenovirus hexon protein affects FX binding. Importantly, for FX binding to be affected, the type of modification of the HVR1 region is decisive. This is evidenced by the fact that HAdV5-ΔHVR1 and HAdV5-Mut2 showed no reduced FX-binding although their HVR1 region modifications are similar to the one of HAdV5-Mut3.

More specifically, the findings of the inventors show that just introducing a number of lysine resides such as four or six lysine residues may not be sufficient. In HAdV5-Mut4, five glutamic acid residues and one aspartic acid residue were replaced by lysine residues. HAdV5-Mut4 was not viable. In HAdV5-Mut2, four aspartic acid residues were replaced by lysine residues. However, HAdV5-Mut2 showed no reduced FX binding.

The findings of the inventors show that reduced FX binding is only observed for HAdV5-Mut3, where four lysine residues, which are arranged in a consecutive

manner, have been introduced. Thus, the findings show that it is necessary to introduce four consecutive lysine residues.

Likewise, the findings of the inventors show that just deleting the stretch of 13 amino acids in the HVR1 region as in HAdV5-ΔHVR1 without introducing any lysine residues is not sufficient to affect FX binding.

In addition, the inventors found that the adenovirus of the invention also overcomes another obstacle for efficient HAdV delivery to, e.g., tumors after systemic HAdV administration, namely the neutralization by natural IgM antibodies. This finding was unexpected since HAdV5-ΔFX particles, which also show reduced FX binding, were almost completely neutralized by natural IgMs upon incubation with both human and murine plasma. However and interestingly, the inventors did not observe this effect when HAdV5-Mut3 was incubated with human or murine plasma samples, even though these particles show a reduced FX binding comparable to that of HAdV5-ΔFX. Thus, the inventors showed that the modified HVR1 region of HAdV5-Mut3 allows vector particles devoid of FX-shielding to escape from natural IgMs. This also prevents IgM-mediated binding of the adenovirus of the invention to erythrocytes which is another barrier after systemic, in particular intravenous, administration of HAdV.

Yet another problem of efficient systemic administration of HAdV are liver-residential macrophages called Kupffer cells. Kupffer cells have scavenger receptors on their cell surface, which bind and capture negatively charged molecules, thereby causing HAdV uptake. While the uptake by murine macrophages was significantly reduced compared to HAdV5 wildtype for all three mutant vectors HAdV5-ΔHVR1, HAdV5-Mut2 and HAdV5-Mut3, the effect was most pronounced for HAdV5-Mut3.

Taken together, the adenovirus of the invention overcomes multiple barriers of HAdV systemic administration faced to date. Accordingly, the adenovirus of the invention is particularly useful for systemic administration.

The adenovirus of the invention was further found to have a transduction efficiency of tumor cells that is either improved or similar compared to the respective

HAdV wildtype. Therefore, the adenovirus of the invention is also particularly useful for utilization as an oncolytic virus.

The term "transduction of cells" as used herein refers to the process of transferring one or more nucleic acids into the cells using an adenovirus. The adenovirus is typically a recombinant adenovirus that has been produced by a suitable packaging cell. An important application of the transduction of cells by recombinant adenoviruses is the virus-mediated delivery of one or more genes into target cells, for example into target cells of a patient in gene therapy.

The term “transduction efficiency” as used herein refers to the proportion or percentage of target cells that are successfully transduced by adenovirus after transduction of the cells.

The term "target cell” as used herein refers to a cell that is to be transduced (infected) by an adenovirus.

Even though HAdV5 was used in the studies underlying the present invention, the present invention is not limited to HAdV5. The findings of the inventors likewise apply to all HAdV species C types comprising the modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region having the sequence of SEQ ID NO.: 1.

In a preferred embodiment, the adenovirus is adenovirus type 5. Adenovirus type 5 is also referred to as HAdV type 5, HAdV-C5, HAdV-5 or HAdV5 herein. Viral vectors based on HAdV type 5 belong to the most commonly used vectors in gene therapy research. They have been extensively studied over many years both preclinically and clinically, in particular as oncolytic adenoviruses for cancer therapy. The amino acid sequence of SEQ ID NO.: 1 is derived from a portion of the wildtype amino acid sequence of the HVR1 region of the HAdV type 5 hexon protein.

In another embodiment, the adenovirus is adenovirus type 1, type 2, type 6, type 57 or type 89.

In a preferred embodiment, the modified adenovirus hexon protein has an unmodified (wildtype) HVR5 and/or HVR7 region.

In a preferred embodiment, the adenovirus is an adenovirus vector or an oncolytic adenovirus.

The term "adenovirus vector" as used herein refers to a replication-deficient adenovirus vector. There are different replication-deficient vector types based on adenovirus. Adenoviral (Ad) vectors usually have at least deletions of the E1A and E1B genes and are therefore replication-deficient in human cells. Production takes place in human complementing cell lines, which express the E1A and E1B proteins and in which the E1A and E1B genes are chromosomally integrated. For example, the DELTA E1 Ad vector (also called E1-deleted Ad vector or first-generation Ad vector) is widely used as laboratory tool, in pre-clinical research and development, in clinical studies and in product development in the context of gene therapy or genetic vaccination. This vector type is made replication-defective by removal of the E1 region encoding the E1A and E1B proteins. In addition, this vector type may also contain deletions in the E3 region. Second generation Ad vectors comprise, in addition to deletions of the E1 genes (and optionally deletions in the E3 region), deletions of the E2 genes and/or the E4 genes. In high-capacity Ad (HC-Ad) vectors (also called helper-dependent Ad vectors), all viral coding sequences are replaced by the transgene(s) of interest.

Adenovirus vectors normally carry expression cassettes, in which, under control of a promoter sequence, RNAs are expressed that are either coding for proteins or that are non-coding for protein, for example rather coding for non-coding RNAs such as small-hairpin RNAs (shRNAs) or micro RNAs (miRNAs).

The term "oncolytic adenovirus" as used herein refers to a replication-competent adenovirus. Replication-competent adenoviruses are in clinical development mainly for the treatment of cancers, in particular solid cancers. These viruses multiply in neoplastic tumor cells resulting in their destruction. Regarding safe clinical use, tumor-selective replication has been considered to reduce or prevent damage to non-neoplastic, healthy tissue. Therefore, they also have been named as conditionally-replication competent adenovirus. Many different strategies have been pursued to achieve tumor-selective activity. A common strategy relates to the deletion of the Rb-binding site in E1A (referred to as Delta-24), resulting in transcrip-

tion factor E2F-dependent replication of adenovirus in tumor cells but not in non-neoplastic cells. Other strategies rely on the use of tumor- or tissue-specific regulatory promoter elements to control expression of an essential adenovirus gene (for example E1A) to specific cell types. Overall, oncolytic adenoviruses are promising tools for cancer therapy.

As mentioned above, the adenovirus of the invention was found to have a transduction efficiency of tumor cells that is either improved or similar compared to the respective HAdV wildtype. Therefore, the adenovirus of the invention is particularly useful for utilization as an oncolytic virus.

In a preferred embodiment, the adenovirus comprises a transgene. The term "transgene" as used herein refers to a gene or genetic material that is non-native to the adenovirus and that is delivered to a target cell by means of transducing the target cell with the adenovirus comprising the transgene. In a preferred embodiment, the adenovirus comprises one or several transgenes, preferably two or three transgenes.

In a preferred embodiment, the capsid has at least one additional capsid modification. The additional capsid modification can be present, for example, in the modified adenovirus hexon protein and/or in a different adenovirus capsid protein such as the adenovirus fiber protein, the penton base protein or the minor capsid protein IX. The introduction of several modifications in these proteins in order to obtain adenoviruses with altered characteristics is known.

In a preferred embodiment, the additional capsid modification is a modified adenovirus fiber protein. In a preferred embodiment, the modified adenovirus fiber protein lacks coxsackievirus and adenovirus receptor (CAR) binding due to ablation of the CAR-binding site of the fiber protein by genetic modification of the viral gene coding for the fiber protein. Ablated CAR binding prevents binding of HAdV to erythrocytes and thus prevents sequestration of viral particles by erythrocytes, especially upon intraveneous administration. Accordingly, by using both the modified adenovirus hexon protein with the modified HVR1 region having the sequence of SEQ ID NO.: 1 and a modified adenovirus fiber protein that lacks CAR binding, an adenovirus that is free from non-target interactions with hepatocytes, from non-

target interactions with natural IgM antibodies, and from IgM- or CAR-mediated non-target interactions with erythrocytes is obtained. Such an adenovirus is particularly useful for systemic administration.

In a second aspect, the present invention relates to the adenovirus of the invention for use in treating or preventing a human disease. As discussed above, the adenovirus of the invention is particularly useful for systemic administration. Accordingly, the adenovirus of the invention is particularly suited for use in treating or preventing a human disease.

In a preferred embodiment, the disease is treated or prevented by gene therapy. The term "gene therapy" as used herein refers to the therapeutic delivery of a nucleic acid into cells of a patient to treat or prevent a disease. The nucleic acid encodes a therapeutic molecule such as, for example, a therapeutic protein, that is expressed by the transduced cells.

In a preferred embodiment, the disease is treated or prevented by genetic vaccination. The term "genetic vaccination" as used herein refers to the delivery of a nucleic acid into cells of a subject to produce a protective or therapeutic immunological response in order to protect the subject against a disease or to treat an existing disease. The nucleic acid encodes an immunogen or an antigen that is expressed by the transduced cells and that induces the immunological response.

In a preferred embodiment, the disease is cancer. As mentioned above, oncolytic adenoviruses are promising tools for treating cancer and the adenovirus of the invention is particularly useful for utilization as an oncolytic virus.

In a third aspect, the present invention relates to a nucleic acid encoding a modified adenovirus hexon protein of a human adenovirus species C, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence of SEQ ID NO.: 1.

In a preferred embodiment, the nucleic acid has the sequence of SEQ ID NO.: 2.

In a further aspect, the present invention relates to the use of an adenovirus according to the invention for transducing mesenchymal stromal cells (MSCs) or tumor cells.

Mesenchymal stromal cells (MSCs), also termed mesenchymal stem cells, show a natural ability to migrate to tumors. They are thus interesting potential carrier cells to carry adenoviruses to tumor tissue. Transduced MSCs hide the viral particles from sequestration mechanisms, increase the viral load due to intracellular virus replication, and transport them to the tumor site where newly produced particles are set free. However, MSCs are hardly transduced by commonly used HAdV type 5 wildtype vectors. Surprisingly, the inventors found that when using the adenovirus according to the invention, the transduction of MSCs with HAdV vectors was greatly enhanced. In addition, the inventors found that transduction of MSCs with the adenovirus according to the invention leads to an improved adenoviral replication in the MSCs compared to transduction with wildtype HAdV. The inventors further confirmed that the migration behaviour of MSCs is not affected by transduction of MSCs with the adenovirus according to the invention. Therefore, the adenovirus of the invention is particularly useful for transducing MSCs.

In a preferred embodiment, the MSCs are human MSCs.

As mentioned above, the adenovirus of the invention was found to have a transduction efficiency of tumor cells that is either improved or similar compared to the respective HAdV wildtype. Therefore, the adenovirus of the invention is also useful for transducing tumor cells.

In a preferred embodiment, the tumor cells are human tumor cells.

In a preferred embodiment, the adenovirus is used in combination with a transduction enhancer for transducing MSCs. The inventors found that the transduction efficiency of MSCs with the adenovirus according to the invention can be further increased by using a transduction enhancer.

In a preferred embodiment, the transduction enhancer is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin.

In a preferred embodiment, the transduction enhancer is coagulation factor X, spermidine, spermine or hexadimethrine bromide.

In a further preferred embodiment, the transduction enhancer is coagulation factor X.

In a further aspect, the present invention relates to an *in vitro* method for transducing MSCs, the method comprising the step of:

contacting a plurality of MSCs with an adenovirus according to the invention.

As discussed above, the adenovirus of the invention is particularly useful for transducing MSCs. This paves the way for using MSCs as carrier of adenoviruses to tumor tissue.

In a preferred embodiment, the plurality of MSCs is further contacted with a transduction enhancer, wherein the transduction enhancer preferably is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin.

In a further aspect, the present invention relates to a transduced MSC obtainable by the method of the invention.

In a further aspect, the present invention relates to the transduced MSC of the invention for use in treating a disease.

In a preferred embodiment, the disease is a human disease.

In a preferred embodiment, the disease is treated by gene therapy.

In a preferred embodiment, the disease is treated by cell therapy. The term "cell therapy" as used herein refers to the therapeutic delivery of cellular material into a patient to treat a disease. The cellular material are generally intact, living cells such as transduced MSCs which are obtainable by the method of the invention.

Further disclosed is an *in vivo* method for transducing tumor cells, the method comprising the step of:

contacting a plurality of tumor cells with an adenovirus according to the invention.

Further disclosed is an *in vitro* method for transducing tumor cells, the method comprising the step of:

contacting a plurality of tumor cells with an adenovirus according to the invention.

As discussed above, the adenovirus of the invention is particularly useful for transducing tumor cells.

In a preferred embodiment, the plurality of tumor cells is further contacted with a transduction enhancer, wherein the transduction enhancer preferably is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin. The use of a transduction enhancer further increases the transduction efficiency of tumor cells with the adenovirus of the invention.

Further disclosed is a transduced tumor cell obtainable by the *in vitro* method for transducing tumor cells according to the disclosure.

Further disclosed is a human adenovirus having a capsid which comprises a modified adenovirus hexon protein of SEQ ID NO.: 3.

The amino acid sequence of the modified adenovirus hexon protein of SEQ ID NO.: 3 is derived from the wildtype amino acid sequence of the HAdV type 5 hexon protein by replacing 13 consecutive amino acid residues in the HVR1 region of the wildtype sequence by four consecutive lysine residues. The modified adenovirus hexon protein of SEQ ID NO.: 3 has a modified HVR1 region having the sequence of SEQ ID NO.: 1. Accordingly, the presence of the modified adenovirus hexon protein of SEQ ID NO.: 3 confers improved characteristics to the adenovirus. More specifically, as discussed above, such an adenovirus is particularly useful for systemic administration.

In a preferred embodiment, the adenovirus is an adenovirus species C, preferably adenovirus type 5.

Further disclosed is a human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region comprises three to eight consecutive lysine or arginine residues.

The modified HVR1 region having the sequence of SEQ ID NO.: 1 comprises four consecutive lysine residues. It can be reasonably expected that the effects observed for adenoviruses having a respectively modified hexon protein will also be

obtained in case the four consecutive lysine residues will be inserted at a position in the HVR1 region that is different from their position in SEQ ID NO.: 1. It can further be expected that the effects will likewise be obtained in case the modified HVR1 region comprises three to eight consecutive lysine residues. Since arginine residues have chemically similar properties as lysine residues, in particular a positive charge, it can further be expected that the effects will likewise be obtained in case the modified HVR1 region comprises three to eight consecutive arginine residues.

In a preferred embodiment, the three to eight consecutive lysine or arginine residues are directly adjacent to one or several amino acid residues that are not negatively charged. The directly adjacent amino acid residues are amino acid residues that are located N-terminal and/or C-terminal of the three to eight consecutive lysine or arginine residues.

In a preferred embodiment, the modified HVR1 region comprises four to eight consecutive lysine or arginine residues.

In a preferred embodiment, the modified HVR1 region comprises three to eight, preferably four to eight, consecutive lysine residues.

In a further preferred embodiment, the modified HVR1 region comprises four consecutive lysine residues.

In a further aspect, the present disclosure relates to an *in vitro* method for transducing MSCs, the method comprising the step of:
contacting a plurality of MSCs with an adenovirus and with a transduction enhancer, wherein the transduction enhancer is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin.

MSCs, which do not express coxsackievirus and adenovirus receptor (CAR), are hardly infected by adenoviruses *in vitro*. The inventors found that by using a transduction enhancer, the transduction of MSCs with adenoviruses can be significantly enhanced. This facilitates the use of MSCs as carrier of adenoviruses to tumor tissue. The inventors further found that the use of transduction enhancers

leads to an improved adenoviral replication in the MSCs, which will also benefit the medical use of MSCs as carrier of adenoviruses. The inventors further confirmed that the migration behaviour of MSCs is not affected by the use of transduction enhancers.

In a preferred embodiment, the adenovirus is a human adenovirus species C, wherein the adenovirus preferably is adenovirus type 5.

In a preferred embodiment, the transduction enhancer is coagulation factor X, spermidine, spermine or hexadimethrine bromide.

In a further preferred embodiment, the transduction enhancer is spermidine or spermine. The inventors found that the enhanced transduction of MSCs with adenoviruses was most pronounced when spermidine or spermine was used as transduction enhancer.

In another further preferred embodiment, the transduction enhancer is coagulation factor X. The inventors found that the expression and subsequent secretion of a therapeutic protein by MSCs transduced with a respective recombinant adenovirus was significantly enhanced in the presence of coagulation factor X as transduction enhancer.

In a preferred embodiment, the adenovirus comprises a transgene.

Sequences

The HAdV5 hexon amino acid sequence is given according to GenBank AY339865.1, position 18,842 – 21,700. The hyper variable region 1 (HVR1) of the hexon protein is depicted in bold letters, respectively, according to Khare et al. 2012.

Amino acid sequence of HAdV5 wildtype hexon:

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTV
APTHDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTY
FDIRGVLDRGPTFKPYSGTAYNALAPKGAPNPCEW**DEAATALEINLEEED**
DDNEDEVDEQAEQQKTHVFGQAPYSGINITKEGIQIGVEGQTPKYADKTF
QPEPQIGESQWYETEINHAAGRVLKKTTPMKPCYGSYAKPTNENGGQGIL
VKQQNGKLESQVEMQFFSTTEAAAGNGDNLTPKVVLYSEDVDIETPDTHI
SYMPTIKEGNSRELMGQQSMPNRPNYIAFRDNFIGLMYYNSTGNMGVLAG
QASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRI
IENHGTEDELPNYCFPLGGVINTETLTKVKPKTGQENGWEKDATEFSDKN
EIRVGNNFAMEINLNANLWRNFLYSNIALYLPDKLKYSPSNVKISDNPNT
YDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRNAGLRYRSMLL
GNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQSSLGND
LRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAA
NMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYT
YSGSIPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVD
GEGYNVAQCNMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQP
MSRQVVDDTKYKDYQQVGILHQHNNSGFVGYLAPTMREGQAYPANFPYPL
IGKTAVDSITQKKFLCDRTLWRIPFSSNFMSMGALTDLGQNLLYANSAHA
LDMTFEVDPMDEPTLLYVLFEVFDVVRVHQPHRGVIETVYLRTPFSAGNA
TT

(SEQ ID NO.: 5)

Amino acid sequence of HVR1 of HAdV5 wildtype hexon:

DEAATALEINLEEEDDDNEDEVDEQAEQQKTHVF

(SEQ ID NO.: 42)

Amino acid sequence of a modified adenovirus hexon protein of an adenovirus of the invention, namely amino acid sequence of HAdV5-Mut3 hexon:

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTV
APTHDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTY
FDIRGVLDRGPTFKPYSGTAYNALAPKGAPNPCEW**DEAATALEINLKKKK**
QAEQQKTHVFGQAPYSGINITKEGIQIGVEGQTPKYADKTFQPEPQIGES
QWYETEINHAAGRVLKKTTPMKPCYGSYAKPTNENGGQGILVKQQNGKLE
SQVEMQFFSTTEAAAGNGDNLTPKVVLYSEDVDIETPDTHISYMPTIKEG
NSRELMGQQSMPNRPNYIAFRDNFIGLMYYNSTGNMGVLAGQASQLNAVV
DLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRIIENHGTEDE
LPNYCFPLGGVINTETLTKVKPKTGQENGWEKDATEFSDKNEIRVGNNFA
MEINLNANLWRNFLYSNIALYLPDKLKYSPSNVKISDNPNTYDYMNKRVV
APGLVDCYINLGARWSLDYMDNVNPFNHHRNAGLRYRSMLLGNGRYVPFH
IQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQSSLGNDLRVDGASIK
FDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAANMLYPIPAN
ATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSGSIPYLD
GTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQC
NMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDT
KYKDYQQVGILHQHNNSGFVGYLAPTMREGQAYPANFPYPLIGKTAVDSI
TQKKFLCDRTLWRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDP
MDEPTLLYVLFEVFDVVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 3)

Amino acid sequence of HVR1 of HAdV5-Mut3 hexon:
DEAATALEINLKKKKQAEQQKTHVF
(SEQ ID NO.: 43)

The HAdV5 hexon nucleotide sequence is given according to GenBank AY339865.1, position 18,842 – 21,700. The HVR1 of the hexon is depicted in bold letters, respectively, according to Khare et al. 2012. Nucleotides encoding for the inserted lysine residues are depicted in capital letters.

Nucleotide sequence of HAdV5 wildtype hexon:

18842 atggctacc ccttcgatga tgccgcagtg gtcttacatg cacatctcgg gccaggacgc
18901 ctcggagtac ctgagccccg ggctggtgca gtttgcccgc gccaccgaga cgtacttcag
18961 cctgaataac aagtttagaa accccacggt ggcgcctacg cacgacgtga ccacagaccg
19021 gtcccagcgt ttgacgctgc ggttcatccc tgtggaccgt gaggatactg cgtactcgta
19081 caaggcgcgg ttcaccctag ctgtgggtga taaccgtgtg ctggacatgg cttccacgta
19141 ctttgacatc cgcggcgtgc tggacagggg ccctactttt aagccctact ctggcactgc
19201 ctacaacgcc ctggctccca agggtgcccc aaatccttgc gaatgg**gatg aagctgctac**
19261 **tgctcttgaa ataaacctag aagaagagga cgatgacaac gaagacgaag tagacgagca**
19321 **agctgagcag caaaaaactc acgtatttgg** gcaggcgcct tattctggta taaatattac
19381 aaaggagggt attcaaatag gtgtcgaagg tcaaacacct aaatatgccg ataaaacatt
19441 tcaacctgaa cctcaaatag gagaatctca gtggtacgaa acagaaatta atcatgcagc
19501 tgggagagtc ctaaaaaaga ctacccaat gaaaccatgt tacggttcat atgcaaaacc
19561 cacaaatgaa aatggagggc aaggcattct tgtaaagcaa caaaatggaa agctagaaag
19621 tcaagtggaa atgcaatttt tctcaactac tgaggcagcc gcaggcaatg gtgataactt
19681 gactcctaaa gtggtattgt acagtgaaga tgtagatata gaaaccccag acactcatat
19741 ttcttacatg cccactatta aggaaggtaa ctcacgagaa ctaatgggcc aacaatctat
19801 gcccaacagg cctaattaca ttgcttttag ggacaatttt attggtctaa tgtattacaa
19861 cagcacgggt aatatgggtg ttctggcggg ccaagcatcg cagttgaatg ctgttgtaga
19921 tttgcaagac agaaaacacag agctttcata ccagcttttg cttgattcca ttggtgatag
19981 aaccaggtac ttttctatgt ggaatcaggc tgttgacagc tatgatccag atgttagaat
20041 tattgaaaat catggaactg aagatgaact tccaaattac tgctttccac tgggaggtgt
20101 gattaataca gagactctta ccaaggtaaa acctaaaaca ggtcaggaaa atggatggga
20161 aaaagatgct acagaatttt cagataaaaa tgaaataaga gttggaaata attttgccat
20221 ggaaatcaat ctaaatgcca acctgtggag aaatttcctg tactccaaca tagcgctgta
20281 tttgcccgac aagctaaagt acagtccttc caacgtaaaa atttctgata acccaaacac
20341 ctacgactac atgaacaagc gagtggtggc tcccgggcta gtggactgct acattaacct
20401 tggagcacgc tggtcccttg actatatgga caacgtcaac ccatttaacc accaccgcaa
20461 tgctggcctg cgctaccgct caatgttgct gggcaatggt cgctatgtgc ccttccacat
20521 ccaggtgcct cagaagttct ttgccattaa aaacctcctt ctcctgccgg gctcatacac
20581 ctacgagtgg aacttcagga aggatgttaa catggttctg cagagctccc taggaaatga
20641 cctaagggtt gacggagcca gcattaagtt tgatagcatt tgcctttacg ccaccttctt
20701 ccccatggcc cacaacaccg cctccacgct tgaggccatg cttagaaacg acaccaacga
20761 ccagtccttt aacgactatc tctccgccgc caacatgctc taccctatac ccgccaacgc
20821 taccaacgtg cccatatcca tcccctcccg caactgggcg gctttccgcg gctgggcctt
20881 cacgcgcctt aagactaagg aaaccccatc actgggctcg ggctacgacc cttattacac
20941 ctactctggc tctataccct acctagatgg aacctttac ctcaaccaca cctttaagaa
21001 ggtggccatt acctttgact cttctgtcag ctggcctggc aatgaccgcc tgcttacccc
21061 caacgagttt gaaattaagc gctcagttga cggggagggt tacaacgttg cccagtgtaa
21121 catgaccaaa gactggttcc tggtacaaat gctagctaac tataacattg gctaccaggg
21181 cttctatatc ccagagagct acaaggaccg catgtactcc ttctttagaa acttccagcc
21241 catgagccgt caggtggtgg atgatactaa atacaaggac taccaacagg tgggcatcct
21301 acaccaacac aacaactctg gatttgttgg ctaccttgcc cccaccatgc gcgaaggaca
21361 ggcctaccct gctaacttcc cctatccgct tataggcaag accgcagttg acagcattac
21421 ccagaaaaag tttctttgcg atcgcaccct ttggcgcatc ccattctcca gtaactttat
21481 gtccatgggc gcactcacag acctgggcca aaaccttctc tacgccaact ccgcccacgc
21541 gctagacatg acttttgagg tggatcccat ggacgagccc acccttcttt atgttttgtt
21601 tgaagtcttt gacgtggtcc gtgtgcacca gccgcaccgc ggcgtcatcg aaaccgtgta
21661 cctgcgcacg cccttctcgg ccggcaacgc cacaacataa

(SEQ ID NO.: 4)

Nucleotide sequence of HAdV5-Mut3 hexon:

18842 atggctacc ccttcgatga tgccgcagtg gtcttacatg cacatctcgg gccaggacgc
18901 ctcggagtac ctgagccccg ggctggtgca gtttgcccgc gccaccgaga cgtacttcag
18961 cctgaataac aagtttagaa accccacggt ggcgcctacg cacgacgtga ccacagaccg
19021 gtcccagcgt ttgacgctgc ggttcatccc tgtggaccgt gaggatactg cgtactcgta
19081 caaggcgcgg ttcaccctag ctgtgggtga taaccgtgtg ctggacatgg cttccacgta
19141 ctttgacatc cgcggcgtgc tggacagggg ccctactttt aagccctact ctggcactgc
19201 ctacaacgcc ctggctccca agggtgcccc aaatccttgc gaatgg**gatg aagctgctac**
19261 **tgctcttgaa ataaacctaA AAAAGAAAAA Gcaagctgag cagcaaaaaa ctcacgtatt**
19321 **t**gggcaggcg ccttattctg gtataaatat tacaaaggag ggtattcaaa taggtgtcga
19381 aggtcaaaca cctaaatatg ccgataaaac atttcaacct gaacctcaaa taggagaatc
19441 tcagtggtac gaaacagaaa ttaatcatgc agctgggaga gtcctaaaaa agactacccc
19501 aatgaaacca tgttacggtt catatgcaaa acccacaaat gaaaatggag ggcaaggcat
19561 tcttgtaaag caacaaaatg gaaagctaga aagtcaagtg gaaatgcaat ttttctcaac
19621 tactgaggca gccgcaggca atggtgataa cttgactcct aaagtggtat tgtacagtga
19681 agatgtagat atagaaaccc cagacactca tatttcttac atgcccacta ttaaggaagg
19741 taactcacga gaactaatgg gccaacaatc tatgcccaac aggcctaatt acattgcttt
19801 tagggacaat tttattggtc taatgtatta caacagcacg ggtaatatgg gtgttctggc
19861 gggccaagca tcgcagttga atgctgttgt agatttgcaa gacagaaaca cagagctttc
19921 ataccagctt ttgcttgatt ccattggtga tagaaccagg tacttttcta tgtggaatca
19981 ggctgttgac agctatgatc cagatgttag aattattgaa aatcatggaa ctgaagatga
20041 acttccaaat tactgctttc cactgggagg tgtgattaat acagagactc ttaccaaggt
20101 aaaacctaaa acaggtcagg aaaatggatg ggaaaaagat gctacagaat tttcagataa
20161 aaatgaaata agagttggaa ataattttgc catggaaatc aatctaaatg ccaacctgtg
20221 gagaaatttc ctgtactcca acatagcgct gtatttgccc gacaagctaa agtacagtcc
20281 ttccaacgta aaaatttctg ataacccaaa cacctacgac tacatgaaca agcgagtggt
20341 ggctcccggg ctagtggact gctacattaa ccttggagca cgctggtccc ttgactatat
20401 ggacaacgtc aacccattta accaccaccg caatgctggc ctgcgctacc gctcaatgtt
20461 gctgggcaat ggtcgctatg tgcccttcca catccaggtg cctcagaagt tctttgccat
20521 taaaaacctc cttctcctgc cgggctcata cacctacgag tggaacttca ggaaggatgt
20581 taacatggtt ctgcagagct ccctaggaaa tgacctaagg gttgacggag ccagcattaa
20641 gtttgatagc atttgccttt acgccacctt cttccccatg gcccacaaca ccgcctccac
20701 gcttgaggcc atgcttagaa acgacaccaa cgaccagtcc tttaacgact atctctccgc
20761 cgccaacatg ctctacccta tacccgccaa cgctaccaac gtgcccatat ccatccccta
20821 ccgcaactgg gcggctttcc gcggctgggc cttcacgcgc cttaagacta aggaaacccc
20881 atcactgggc tcgggctacg acccttatta cacctactct ggctctatac cctacctaga
20941 tggaaccttt tacctcaacc acacctttaa gaaggtggcc attacctttg actcttctgt
21001 cagctggcct ggcaatgacc gcctgcttac ccccaacgag tttgaaatta agcgctcagt
21061 tgacggggag ggttacaacg ttgcccagtg taacatgacc aaagactggt tcctggtaca
21121 aatgctagct aactataaca ttggctacca gggcttctat atcccagaga gctacaagga
21181 ccgcatgtac tccttcttta gaaacttcca gcccatgagc cgtcaggtgg tggatgatac
21241 taaatacaag gactaccaac aggtgggcat cctacaccaa cacaacaact ctggatttgt
21301 tggctacctt gcccccacca tgcgcgaagg acaggcctac cctgctaact tccccctatcc
21361 gcttataggc aagaccgcag ttgacagcat tacccagaaa aagtttcttt gcgatcgcac
21421 cctttggcgc atcccattct ccagtaactt tatgtccatg ggcgcactca cagacctggg
21481 ccaaaacctt ctctacgcca actccgccca cgcgctagac atgacttttg aggtggatcc
21541 catggacgag cccaccttc tttatgtttt gtttgaagtc tttgacgtgg tccgtgtgca
21601 ccagccgcac cgcggcgtca tcgaaaccgt gtacctgcgc acgccttct cggccggcaa
21661 cgccacaaca taa

(SEQ ID NO.: 2)

Nucleotide sequence of HVR1 of HAdV5 wildtype hexon:

gatgaagctgctactgctcttgaaataaacctagaagaagaggacgatgacaacgaagac-

gaagtagacgagcaagctgagcagcaaaaaactcacgtattt

(SEQ ID NO.: 44)

Nucleotide sequence of HVR1 of HAdV5-Mut3 hexon:

gatgaagctgctactgctcttgaaataaacctaAAAAAGAAAAAGcaagctgagcag-

caaaaaactcacgtattt

(SEQ ID NO.: 45)

Amino acid sequence of HVR1 of HAdV5-ΔHVR1 hexon:

DEAATALEINLQAEQQKTHVF

(SEQ ID NO.: 46)

Amino acid sequence of HVR1 of HAdV5-Mut2 hexon:

DEAATALEINLEEEKKKNEKEVDEQAEQQKTHVF

(SEQ ID NO.: 47)

Amino acid sequence of HVR1 of HAdV5-Mut4 hexon:

DEAATALKINLKKNKVKQAKQQKTHVF

(SEQ ID NO.: 48)

Amino acid sequence of HVR1 of HAdV5-Mut5 hexon:

DEAATALEINLKKKKQAEQQKTHVF

(SEQ ID NO.: 43)

Amino acid sequence of HVR1 of HAdV5-Mut6 hexon:

DEAATALKINLKKNKVKQAKQQKTHVF

(SEQ ID NO.: 48)

Amino acid sequence of HVR1 of HAdV5-Mut7 hexon:

DEAATALEINLKKKKKKQAEQQKTHVF

(SEQ ID NO.: 51)

Amino acid sequence of HVR1 of HAdV5-Mut8 hexon:

DEAATALEINLKKKKKKKKQAEQQKTHVF

(SEQ ID NO.: 52)

Amino acid sequence of HVR1 of HAdV5-Mut9 hexon:

DEAATALEINLGGSGGGSGKKKKKKKKGSGGGSGGQAEQQKTHVF

(SEQ ID NO.: 53)

The portions of the HVR1 regions of human adenovirus type 1 (HAdV1), type 2 (HAdV2), type 6 (HAdV6) and type 57 (HAdV57) hexon proteins, which have been replaced by the amino acid sequence of SEQ ID NO.: 1, are as follows (aa, amino acids):

HAdV1: aa 136-173 of HAdV1 Hexon (GenBank: BAG48778.1):

EQEEPTQEMAEELEDEEEAEEEEAEEEAEAPQADQKVK

(SEQ ID NO.: 7)

HAdV2: aa 136-171 of HAdV2 Hexon (GenBank: CAC67477.1):

EQTEDSGRAVAEDEEEEDEDEEEEEEEQNARDQATK

(SEQ ID NO.: 8)

HAdV6: aa 136-167 of HAdV6 Hexon (GenBank: BAU36782.1):

EQNETAQVDAQELDEEENEANEAQAREQEQAK

(SEQ ID NO.: 9)

HAdV57: aa 136-166 of HAdV57 Hexon (GenBank: BBF89158.1):

DEDDTQVQVAAEDDQDDDEEEEQLPQQRNGK

(SEQ ID NO.: 10)

HAdV89: aa 136-172 of HAdV89 Hexon (GenBank: AZR67181):

EQTEDSGRAVAEDEEEEEDEDEEEEEEEQNARDQATK

(SEQ ID NO.: 49)

The resulting amino acid sequences of the modified adenovirus hexon proteins are given below.

Amino acid sequence of a modified adenovirus hexon protein of an adenovirus of the invention, wherein the adenovirus is adenovirus type 1 (amino acid sequence of HAdV1-Mut3 hexon):

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPT
HDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLD
RGPTFKPYSGTAYNALAPKGAPNSCEWDEAATALEINLKKKKQAEQQKTHVYAQ
APLAGEKITANGLQIVSDTQTEGNPVFADPTYQPEPQVGESQWNEAEATASGG
RVLKKTTPMKPCYGSYARPTNKNGGQGILVANNQGALESKVEMQFFAPSGTAM
NERNAVQPSIVLYSEDVNMETPDTHISYKPSKTDENSKAMLGQQAMPNRPNYIA
FRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTR
YFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGVTDTYQGIKSNGNGN
PQNWTKNDDFAARNEIGVGNNFALEINLNANLWRNFLYSNIALYLPDKLKYTPTN
VEISPNPNSYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRNAGLR
YRSMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQSSLG
NDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAANML
YPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSGSIPYL
DGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQCNMT
KDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKYKDYQ
QVGILHQHNNSGFVGYLAPTMREGQAYPANFPYPLIGKTAVDSITQKKFLCDRTL
WRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLFEVFD
VVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 11)

Amino acid sequence of a modified adenovirus hexon protein of an adenovirus of the invention, wherein the adenovirus is adenovirus type 2 (amino acid sequence of HAdV2-Mut3 hexon):

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPT
HDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLD
RGPTFKPYSGTAYNALAPKGAPNSCEWDEAATALEINLKKKKQAEQQKTHVYAQ

APLSGETITKSGLQIGSDNAETQTKPVYADPSYQPEPQIGESQWNEADANAAGG
RVLKKTTPMKPCYGSYARPTNPFGGQSVLVPDEKGVPLPKVDLQFFSNTTSLND
RQGNATKPKVVLYSEDVNMETPDTHLSYKPGKGDENSKAMLGQQSMPNRPNYI
AFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRT
RYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGVTDTYQAIKANGNG
SGDNGDTTWTKDETFATRNEIGVGNNFAMEINLNANLWRNFLYSNIALYLPDKLK
YNPTNVEISDNPNTYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHR
NAGLRYRSMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVL
QSSLGNDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLS
AANMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSG
SIPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQ
CNMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKY
KEYQQVGILHQHNNSGFVGYLAPTMREGQAYPANVPYPLIGKTAVDSITQKKFLC
DRTLWRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLF
EVFDVVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 12)

Amino acid sequence of a modified adenovirus hexon protein of an adenovirus of the invention, wherein the adenovirus is adenovirus type 6 (amino acid sequence of HAdV6-Mut3 hexon):

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPT
HDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLD
RGPTFKPYSGTAYNALAPKGAPNSCEWDEAATALEINLKKKKQAEQQKTHVYAQ
APLSGIKITKEGLQIGTADATVAGAGKEIFADKTFQPEPQVGESQWNEADATAAG
GRVLKKTTPMKPCYGSYARPTNSNGGQGVMVEQNGKLESQVEMQFFSTSTNA
TNEVNNIQPTVVLYSEDVNMETPDTHLSYKPKMGDKNAKVMLGQQAMPNRPNY
IAFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRT
RYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGITDTFQAVKTTAANG
DQGNTTWQKDSTFAERNEIGVGNNFAMEINLNANLWRNFLYSNIALYLPDKLKY
NPTNVEISDNPNTYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRN

AGLRYRSMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQ
SSLGNDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSA
ANMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSGS
IPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQC
NMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKYK
DYQQVGIIHQHNNSGFVGYLAPTMREGQAYPANVPYPLIGKTAVDSITQKKFLCD
RTLWRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLFE
VFDVVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 13)

Amino acid sequence of a modified adenovirus hexon protein of an adenovirus of the invention, wherein the adenovirus is adenovirus type 57 (amino acid sequence of HAdV57-Mut3 hexon):

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPT
HDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLD
RGPTFKPYSGTAYNALAPKGAPNSCEWDEAATALEINLKKKKQAEQQKTHVYAQ
APFAGEAINKNGLQIGTNGAATEGNKEIYADKTYQPEPQIGESQWNEAESSVAG
GRVLKKTTPMKPCYGSYARPTNSNGGQGVMVEQNGKLESQVEMQFFSTSVNA
MNEANAIQPKLVLYSEDVNMETPDTHLSYKPGKSDDNSKAMLGQQSMPNRPNYI
AFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRT
RYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGVTDTYQAIKATNGN
GGATTWAQDNTFAERNEIGVGNNFAMEINLNANLWRNFLYSNIALYLPDKLKYN
PTNVEISDNPNTYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRNA
GLRYRSMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQS
SLGNDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAA
NMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSGSI
PYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQC
NMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKYK
DYQQVGILHQHNNSGFVGYLAPTMREGQAYPANFPYPLIGKTAVDSITQKKFLC
DRTLWRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLF

EVFDVVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 14)

Amino acid sequence of the modified adenovirus hexon protein of the adenovirus of the invention, wherein the adenovirus is adenovirus type 89 (amino acid sequence of HAdV89-Mut3 hexon):

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPT
HDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLD
RGPTFKPYSGTAYNALAPKGAPNSCEWDEAATALEINLKKKKQAEQQKTHVYAQ
APLSGETITKSGLQIGSDNAETQAKPVYADPSYQPEPQIGESQWNEADANAAGG
RVLKKTTPMKPCYGSYARPTNPFGGQSVLVPDEKGVPLPKVDLQFFSNTTSLND
RQGNATKPKVVLYSEDVNLETPDTHLSYKPGKGDENSKAMLGQQSMPNRPNYI
AFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRT
RYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGIGVTDTYQAIKANGNG
AGDNGNTTWTKDETFATRNEIGVGNNFAMEINLNANLWRNFLYSNIALYLPDKLK
YNPTNVEISDNPNTYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHR
NAGLRYRSMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVL
QSSLGNDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLS
AANMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGSGYDPYYTYSG
SIPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQ
CNMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKY
KDYQQVGIIHQHNNSGFVGYLAPTMREGQAYPANVPYPLIGKTAVDSITQKKFLC
DRTLWRIPFSSNFMSMGALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLF
EVFDVVRVHQPHRGVIETVYLRTPFSAGNATT
(SEQ ID NO.: 50)

Further aspects of the invention will be apparent to the person skilled in the art by the enclosed description of the examples, in particular the scientific results.

Examples

Materials and methods

Cells

All cells were cultivated at 90% humidity, 5% CO2 and 37 °C and passaged twice a week to indicated split rates. Cells were detached with 0.05% trypsin-EDTA, except for J774A.1 cells, which were detached by scrapping.

A549 cells (ATCC # CCL-185; split rate: 1:8) were propagated in Minimum Essential Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin/glutamine.

J774A.1 cells (ATCC #TIB-67; split rate: 1:10) were propagated in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin/glutamine.

SKOV-3 cells (ATCC # HTB-77; split rate: 1:3) were propagated in Roswell Park Memorial Institute 1640 Medium (Gibco) supplemented with 5% FBS and 1% penicillin/streptomycin/glutamine.

UM-SCC-11B cells (carcinoma cells derived from a human head-and-neck squamous cell carcinoma, kindly provided by Prof. Dr. Brunner, University Clinic; split rate: 1:10) were propagated in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin/glutamine.

MiaPaCa cells (ATCC #CRL-1420, split rate 1:7 – 1:15) were propagated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco) with 10 % FBS 1x GlutaMAX™ and 1x penicillin/streptomycin.

Huh7 cells (JCRB0403, split rate 1:10) were propagated in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin/glutamine.

HepG2 cells (ATCC #HB-8065, split rate 1:4 – 1:7) were propagated in Minimum Essential Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin/glutamine.

Human mesenchymal stromal cells (MSCs) (kindly provided by Institute for Clinical Transfusion Medicine and Immunogenetics, German Red Cross Ulm, Germany, and prepared as described in Fekete, Rojewski et al., 2012) were propagated in BioWhittaker® Alpha Minimum Essential Medium (Lonza) supplemented with 8 % irradiated pooled human platelet lysate (PL) (prepared as described in Fekete, Gadelorge et al., 2012 and provided by the Institute for Clinical Transfusion Medicine and Immunogenetics, German Red Cross Ulm, Germany) and 500 Units of Heparin (Ratiopharm).

Viruses and vectors

Replication-incompetent human adenovirus type 5 (HAdV5) vector particles had a deleted E1 region (GenBank ID: AY339865.1, sequence from nt 1 to 440 and from nt 3523 to 35935), and carried a CMV promoter-driven enhanced GFP (eGFP) expression cassette, subcloned from a pEGFP-N1 plasmid (Clontech 6085-1) that was inserted in reverse orientation in the deleted E1 region.

HAdV5-ΔCAR vector particles carried additionally a point mutation in the fiber knob (Y→477A) that significantly reduces CAR-binding (Kirby et al., 2000).

HAdV5-ΔFX vector particles carried additionally a point mutation in the hyper variable region (HVR) 7 of the hexon protein (E→451Q) that significantly reduces but not abolishes binding of blood coagulation factor X to the vector capsid (Krutzke et al., 2016).

HAdV5-ΔHVR1 particles carried a 13 amino acid deletion within a negatively charged region of HVR1 (ΔEEEDDDNEDEVDE (SEQ ID NO.: 6); nt 19280-19318) of the hexon protein to reduce the negative surface charge of HAdV5 particles (Alemany et al., 2000).

In case of HAdV5-Mut3 particles, these 13 amino acids within HVR1 were replaced by four lysine residues (EEEDDDNEDEVDE (SEQ ID NO.: 6) → KKKK (SEQ ID NO.: 15); nt 19280-19318).

For HAdV5-Mut2 some of the aspartic acids in this 13 amino acid stretch were replaced with lysine residues (EEEDDDNEDEVDE (SEQ ID NO.: 6) → EEEKKKNEKEVDE (SEQ ID NO.: 16); nt 19289-19306).

For HAdV5-Mut4 some negatively charged amino acids within HVR1 were replaced with lysine residues (EINLEEEDDDNEDEVDEQAE (SEQ ID NO.: 17) → KINLKKNKVKQAK (SEQ ID NO.: 18)).

For HAdV5-Mut5 some amino acids within HVR1 (EEEDDDNEDEVDE (SEQ ID NO.: 6) → KKKK (SEQ ID NO.: 15)), within HVR5 (STTEAAAGNGDNLTPK (SEQ ID NO.: 19) → STTKAAAGNGKNLTPK (SEQ ID NO.: 20)), and within HVR7 (GGVINTETLTKVKPKTGQENGWEKDATEFSDKNEIRVGNNF (SEQ ID NO.: 21) → GGVINTETLTKVKPKTGQKNGWKKKATEFSDKNEIRVGNNF (SEQ ID NO.: 22)) were replaced with lysine residues.

For HAdV5-Mut6 some negatively charged amino acids within HVR1 (EINLEEEDDDNEDEVDEQAE (SEQ ID NO.: 17) → KINLKKNKVKQAK (SEQ ID NO.: 18)), within HVR5 (STTEAAAGNGDNLTPK (SEQ ID NO.: 19) → STTKAAAGNGKNLTPK (SEQ ID NO.: 20)), and within HVR7 (GGVINTETLTKVKPKTGQENGWEKDATEFSDKNEIRVGNNF (SEQ ID NO.: 21) → GGVINTETLTKVKPKTGQKNGWKKKATEFSDKNEIRVGNNF (SEQ ID NO.: 22)) were replaced with lysine residues.

For HAdV5-Mut7, the 13 amino acids of SEQ ID NO.: 6 within HVR1 were replaced by six lysine residues.

For HAdV5-Mut8, the 13 amino acids of SEQ ID NO.: 6 within HVR1 were replaced by eight lysine residues.

For HAdV5-Mut9, the 13 amino acids of SEQ ID NO.: 6 within HVR1 were replaced by 24 amino acids, namely by a stretch of eight lysine residues that is flanked by a flexible GS-linker of eight amino acids selected from glycine and serine on each side.

For HAdV5-ΔCAR-Mut3 vector particles, the 13 amino acids of SEQ ID NO.: 6 within HVR1 were replaced by four lysine residues and the vector particles carried

additionally a point mutation in the fiber knob (Y→477A) that significantly reduces CAR-binding (Kirby et al., 2000).

HAdV5-TSG6 vector particles carried, instead of an eGFP expression cassette, in the E1 region a cDNA coding for the human Tumor Necrosis Factor (TNF) stimulated Gene 6 (TSG-6) protein (nucleotide accession GenBank: AJ419936.1) under control of the human CMV promoter. TSG-6 is a protein with anti-inflammatory activity and thus can be used as anti-inflammatory therapeutic.

Replication-competent wildtype adenovirus particles (HAdV5wt) had no deletions, but carried the eGFP expression cassette that was inserted in forward orientation in a non-coding region between E1A and E1B (position 1648/1649).

Vector and virus rescue and production

Replication-incompetent vectors were produced in E1-transcomplementing N52.E6 cells, replication-competent viruses were produced in A549 cells.

Vector and virus DNAs, respectively, were excised from circular bacmid DNA by SwaI restriction enzyme digestion, with subsequent purification using standard procedures. Producer cells (N52.E6 or A549 cells) were transfected with the linear DNAs, prepared as described above, using the transfection agent polyethylenimine (PEI). Vector and virus particles, respectively, were amplified by consecutive harvest and infection cycles with increasing cell numbers. After infection of $2x10^8$ – $4x10^8$ cells, cells were harvested 48 h post infection, resuspended in 50 mM HEPES, 150 mM NaCl, pH 7.4, and lysed by three consecutive freeze/thawing cycles. Particles were purified by one CsCl step gradient (density bottom: 1.41 g/ml; density top: 1.27 g/ml; 2 h at 176,000 x g, 4 °C) and one consecutive continuous CsCl gradient (density: 1.34 g/ml; 20 h at 176,000 x g and 4 °C). Subsequently, particles were desalted using PD-10 size exclusion columns (GE Healthcare) and stored in buffer (50 mM Hepes, 150 mM NaCl, pH 7.4) with 10% glycerol at -80 °C. Physical titers were determined by optical density measurement at OD260nm of isolated virus/vector DNA.

Homologous recombination

Modification of the HVR1, HVR5 and HVR7 region in HAdV5-ΔHVR1, HAdV5-Mut2, HAdV5-Mut3, HAdV5-Mut4, HAdV5-Mut5, and HAdV5-Mut6 vectors, as applicable, were done by homologous recombination according to bacmid kit "Counter-Selection BAC Modification Kit Red/ET Recombination". Therefore, a bacmid carrying the HAdV5 sequence was transformed into streptomycin-resistant *Escherichia coli* (*E.coli*) strain ElectroMAX™ DH10B™ by electroporation. Bacteria were streaked on lysogeny broth medium (LB) agar plates supplemented with chloramphenicol (20 µg/ml) and cultured overnight at 37 °C. After streptomycin (50 µg/ml) and chloramphenicol (20 µg/ml) resistance was verified, *E. coli* was transformed by electroporation with pRed/ET plasmid provided by kit. Bacteria were streaked on LB agar plates containing tetracycline (3 µg/ml) and chloramphenicol (20 µg/ml). Plates were incubated for 20 h at 30 °C. The next day single colonies were picked, cultured overnight at 30 °C and the expression of the recombination proteins Redα and Redβ from the Red/ET plasmid was induced by L-arabinose and a temperature shift to 37 °C. Subsequently, bacteria were transformed with polymerase chain reaction (PCR) product carrying the *rpsL-neo* cassette with homologous arms for the gene region of interest. Bacteria suspension was streaked on LB agar containing tetracycline (3 µg/ml), chloramphenicol (20 µg/ml) and kanamycin (15 µg/ml) and cultured for more than 24 h at 30 °C. Next, clones were picked selected by streptomycin sensitivity and deoxyribonucleic acid (DNA) integrity confirmed by restriction enzyme analysis. After confirmation of introduced *rpsL-neo* cassette, expression of Red/ET genes was again induced by L-arabinose and a temperature shift to 37 °C. Bacteria were transformed with a second PCR product carrying the mutation for either HVR1, HVR5 or HVR7, respectively. These second PCR products also carried homologous arms for the gene region of interest and successfully recombined clones were selected on LB agar containing chloramphenicol (20 µg/ml) and streptomycin (50 µg/ml) overnight at 37 °C. Single colonies were analyzed by DNA restriction analysis for the expected mutations. After large scale bacmid preparations, positive clones were further verified by sequencing.

Polymerase chain reaction (PCR) for the generation of PCR products for homologous recombination

For the generation of HAdV5-ΔHVR1, HAdV5-Mut2, HAdV5-Mut3, HAdV5-Mut4, HAdV5-Mut5, and HAdV5-Mut6, the PCR product encoding the respective *rpsL-neo* cassette, carried flanking homology arms that were complementary to the gene region of interest. The used primers consisted of 74 base pairs (bp) (5´-50 bp complementary to the gene region of interest plus 24 bp complementary to *rpsL-neo*-3´). For PCR reaction 5 µl Pfx amplification buffer (10x), 1 µl $MgSO_4$ (50 mM), 2 µl dNTP´s (10 mM), 1 µl of each primer 10 µM

(for HVR1:
rpsL-neo fw:
caaatccttgcgaatgggatgaagctgctactgctcttgaaataaacctaggcctggtgatgatggcgggatcg
(SEQ ID NO.: 23),
rpsL-neo rev:
ccagaataaggcgcctgcccaaatacgtgagtttttgctgctcagcttgtcagaagaactcgtcaagaaggcg
(SEQ ID NO.: 24);

for HVR5:
rpsL-neo fw:
ggagggcaaggcattcttgtaaagcaacaaaatggaaagcta-
gaaagtcaagGGCCTGGTGATGATGGCGGGATCG
(SEQ ID NO.: 25),
rpsL-neo rev:
gtctggggtttctatatctacatcttcactgtacaataccactttaggagtcTCAGAA-
GAACTCGTCAAGAAGGCG
(SEQ ID NO.: 26);

for HVR7:
rpsL-neo fw:
catggaactgaagatgaacttccaaattactgctttccactgg-
gaggtgtgGGCCTGGTGATGATGGCGGGATCG
(SEQ ID NO.: 27),
rpsL-neo rev:

ctccacaggttggcatttagattgatttccatggcaaaattatttccaactcTCAGAA-
GAACTCGTCAAGAAGGCG
(SEQ ID NO.: 28);

all primer sequences indicated in 5' → 3' direction),

0.5 µl *rpsL-neo* cassette template and 0.5 µl Platinum® Pfx DNA polymerase (2.5 U/µl) were mixed in a final volume of 50 µl. PCR cycles were performed as follows: 1 cycle: 95°C for 5 min (initial denaturation); 27 cycles: 95°C for 45 s (denaturation), 60°C for 45 s (annealing), 68°C for 2 min (elongation); 1 cycle: 68°C for 10 min (final elongation).

The second PCR-products to replace the previously inserted *rpsL-neo* cassette were produced using synthesized templates from Invitrogen GeneArt that carried the desired mutations for either HAdV5-ΔHVR1, HAdV5-Mut2, HAdV5-Mut3, HAdV5-Mut4, HAdV5-Mut5, or HAdV5-Mut6. For PCR reaction 5 µl Pfx amplification buffer (10x), 1 µl $MgSO_4$ (50 mM), 2 µl dNTP´s (10 mM), 1 µl of each primer 10 µM

(HVR1 fw: ttttaagccctactctggcactgc (SEQ ID NO.: 29),
HVR1 rev: CCTTCGACACCTATTTGAATACCC (SEQ ID NO.: 30);
HVR5 fw: cacaaatgaaaatggagggcaagg, (SEQ ID NO.: 31),
HVR5 rev: gtgggcatgtaagaaatatgagtg (SEQ ID NO.: 32);
HVR7 fw: gacagctatgatccagatgttagaa (SEQ ID NO.: 33),
HVR7 rev: ctccacaggttggcatttagattg (SEQ ID NO.: 34);
all primer sequences indicated in 5' → 3' direction),

100 ng template plasmid DNA and 0.5 µl Platinum® Pfx DNA polymerase (2.5 U/µl) were mixed in a final volume of 50 µl. PCR cycles were performed as follows: 1 cycle: 95°C for 5 min (initial denaturation); 27 cycles: 95°C for 45 s (denaturation), 55°C for 45 s (annealing), 68°C for 2 min (elongation); 1 cycle: 68°C for 10 min (final elongation).

PCR products were separated by agarose gel electrophoresis on tris-acetic acid-ethylenediaminetetraacetic acid (TAE) gels. The desired band was cut out and purified by phenol-chloroform extraction followed by ethanol precipitation.

The same procedure was applied for the generation of HAdV5-Mut7, HAdV5-Mut8, and HAdV5-Mut9.

Silver staining

$5x10^9$ vector particles dissolved in 20 µl PBS including 1x SDS-loading buffer with ß-mercaptoethanol, were denatured for 5 min at 70 °C. Subsequently, viral proteins were separated by SDS-PAGE (5% stacking gel, 8% separation/running gel). For visualization of viral proteins silver staining was performed. Proteins were fixed for 30 min in fixation buffer (50% methanol, 12% acetic acid, 0.05% formaldehyde 37%), washed 15 min with wash buffer (50% ethanol) and then pretreated with equilibration buffer (0.8 mM sodium thiosulfate) for 1 min. Next, the gel was washed three times with dH_2O followed by an incubation for 20 min in impregnation buffer (11.78 mM silver nitrate, 0.05% formaldehyde 37%). Staining of proteins occurred by incubation of gel in development buffer (0.57 M sodium carbonate, 0.05% formaldehyde 37%, 15.8 µM sodium thiosulfate) for a reasonable time. Development was stopped with stop buffer (50% methanol, 12% acetic acid), when the protein bands were very clear but the bands of marker were hardly seen.

Zeta potential measurement

The measurement of the zeta potential was performed with ZetaSizer Nano-ZS (Malvern, UK). $2x10^{11}$ vector particles was dialyzed three times (2 h followed by overnight and 6 h) with 50 mM HEPES (pH 7.4) in a Slide-A-Lyzer Dialysis cassettes (3.5K MWCO 0.5 ml, Thermo Fisher Scientific) under easy stirring at 4°C. Subsequently, a size exclusion chromatography using PD MiniTrap G-25 column (GE Healthcare Life Sciences) was performed according to the manufacturer without filter to remove glycerol. Because the needed volume for clear disposable zeta cell cuvettes (Malvern, UK) was 1 ml, purified vector particles were filled up to 1 ml with 50 mM HEPES (pH 7.4 sterile filtered) (Krutzke et al., 2016). The follow-

ing settings were applied for measurement and analysis in DTSNano 5.10 software: Measurement type zeta potential; sample material Protein RI 1.450, Absor 0.00; sample dispersant water temp 25°C, viscosity 0.8872CP, RI 1.330, dielectric constant 78.5; sample general options Smoluchowski; sample temperature 25°C, at start of measurement 2 min equilibration time; sample cell DTS 1060C clear disposable zeta cell; measurements automatic minimum 10, maximum 15; measurements number of measurements 1.

FX-mediated transduction of SKOV-3 cells

SKOV-3 cells were seeded at a density of $2x10^4$ cells/well in 200 µl serum-containing Roswell Park Memorial Institute 1640 medium. Next day, cells were washed and 100 µl serum-free Roswell Park Memorial Institute 1640 medium containing 8 µg/ml FX or not (as control) was provided. Cells were transduced with a pMOI 1000 ($2x10^7$ VP). After an incubation for 3 h at 37 °C cells were washed and 200 µl serum-containing Roswell Park Memorial Institute 1640 medium was added. 72 h post transduction, cells were harvested and the eGFP expression analyzed by flow cytometry.

Scavenger receptor-mediated uptake

J774A.1 cells were seeded in a density of 1 x 10^5 cells/well in a 24 well plate in 1 ml serum-containing Dulbecco´s Modified Eagle Medium and cultured overnight at 37 °C. The next day, cells were washed and 500 µl serum-free Dulbecco´s Modified Eagle Medium was provided. If indicated, polyinosinic acid (Poly-(I)) (30 µg/ml) dissolved in Dulbecco´s phosphate buffered saline was added to cells and cells were incubated at 37 °C for 1 h. Finally, cells were transduced with a pMOI of 2000 ($1x10^8$ VP). After 3 h of incubation at 37 °C, cells were washed twice and subsequently incubated in 1 ml serum-containing Dulbecco´s Modified Eagle Medium. Cells were harvested 24 h post transduction by adding 200 µl Dulbecco´s phosphate buffered saline, 20 µl proteinase K and 20 µl RNase per well. After incubation for 2 min at room temperature, additionally 200 µl AL buffer ("QIAmp DNA Mini Kit") was added and cells incubated at 56 °C for 10 min. Subsequently, DNA of lysed cells was isolated according to the protocol of "QIAmp DNA Mini Kit" (Krutzke et al., 2016).

To quantify the adenoviral content of isolated total DNA samples, quantitative PCR (qPCR) for the adenoviral E4 gene was performed. Murine β-actin copy numbers were used for normalization to exclude dissent cell harvest efficiencies. The qPCR reaction contained 10 µl Kapa SYBRE FAST qPCR master mix, 0.4 µl of each primer (10 µM)

(for murine β-actin:

fw: caaggagtgcaagaacacag (SEQ ID NO.: 35);

rev: gccttggagtgtgtattgag (SEQ ID NO.: 36);

for Ad5 E4:

fw: tagacgatccctactgtacg (SEQ ID NO.: 37);

rev: ccggacgtagtcatatttcc (SEQ ID NO.: 38);

all primer sequences indicated in 5' → 3' direction)

and 2 µl isolated total DNA in a final volume of 20 µl.

PCR cycles were performed as follows: 1 cycle 95°C for 10 min (initial denaturation); 40 cycles 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 20 s (elongation); 1 cycle 95°C for 1 min (denaturation), 55°C for 30 s (annealing); 1 cycle 95°C 30 s (final denaturation).

<u>IgM-mediated neutralization</u>

A549 cells were seeded in 200 µl serum-containing minimum essential medium (Gibco) in 96-well plates. The next day, indicated viral vector particles were incubated for 10 min at 37 °C with PBS or plasma samples in a ratio of 2E6 VP/µl. Ad-naïve human and murine plasma samples were prepared from whole blood by centrifugation at 800 x g for 10 min. To preserve complement activity, blood samples were anti-coagulated with 100 µg/ml hirudin (Celgene). Cells were washed and transduced with pre-incubated viral vectors with a pMOI of 1000 in 100 µl serum-free medium and incubated for 3 h at 37 °C. Subsequently, cells were washed and supplemented with 200 µl serum-containing medium. After 24 h incubation at 37 °C, cells were detached and the eGFP expression analyzed by flow cytometry.

Transduction of MSCs with HAdV5-TSG6 and Coagulation Factor X (FX) as transduction enhancer

2-3x10^5 MSCs were seeded on a 6 cm dish in 6 ml PL containing alpha-Minimum Essential Medium (Lonza) and cultured overnight at 37°C. Next day, FX (2 µg/ml) was pre-incubated in alpha-Minimum Essential Medium with corresponding amount of TSG-6-expressing vector particles HAdV5-TSG6 in a final volume of 290 µl for 20-30 min at 37°C. During pre-incubation of FX with vector particles, cells were washed, 2.7 ml alpha-Minimum Essential Medium without any supplement was provided per 6 cm dish. Finally, the pre-incubated vector particle solution was added to cells for an incubation for 3 h at 37°C followed by three times washing with alpha-Minimum Essential Medium. The cells were cultured for further 72 h in PL containing alpha-Minimum Essential Medium at 37°C. Cell culture supernatant was harvested and the concentration of TSG-6 in cell culture supernatants was analyzed by a sandwich TSG-6 ELISA.

To this end, a 96-well MaxiSorb Nunc plate was incubated overnight at 4°C with 100 µl/well of coating solution (1 µg/ml TSG-6 monoclonal mouse IgG Santa Cruz SC-65886 in 0.1 M sodium carbonate/bicarbonate buffer pH 9.6). Next day, plate was washed with an ELISA plate washer “Well Wash Versa” three times with 300 µl/well of 0.05% Tween-PBS followed by an incubation for 1 h with 300 µl/well SuperBlock (Thermo Fisher) on a shaker at room temperature. After another round of washing, 100 µl/well of sample in corresponding dilutions or TSG-6 protein standard (R&D 2104-TS-050) was added to wells and incubated for 1 h at room temperature on a shaker. Again, plate was washed and 100 µl/well of detection solution (0.25 µg/ml anti-TSG-6 goat polyclonal IgG R&D BAF2104 in PBS) was incubated for 1 h at room temperature on a shaker. Before adding 100 µl/well of streptavidin-horseradish peroxidase conjugate (Dako P0397 c = 0.020 µg/ml) followed by an incubation for 1 h at room temperature on a shaker, plate was washed again. Finally, after another washing step 100 µl/well of detection substrate (1-StepUltra TMB ELISA Substrate Thermo Fisher 34028) was given per well to start the enzymatic reaction in dark. After 15 min reaction was stopped by

addition of 100 µl/well sulfuric acid (2 M) followed by a measurement at 450 nm in an ELISA reader.

Transduction of Tumor Cell Lines with HAdV5 and HAdV5-Mut3 and with HAdV5-ΔCAR-Mut3 and HAdV5-ΔCAR

Different tumor cell lines (UM-SCC-11B, MiaPaCa, Huh7, HepG2, A549) were seeded in 200 µl of the respective medium in 96-well plates. The next day, cells were washed and transduced with a pMOI of 300 or 1000 by either HAdV5 or HAdV5-Mut3 in 100 µL serum-free medium. After incubation at 37 °C for 2 hours, cells were washed and 200 µL of serious medium was added. 24 hours post transduction, cells were harvested and the eGFP expression was analyzed by flow cytometry.

The same procedure was applied for transduction of the different tumor cell lines with HAdV5-ΔCAR-Mut3 and HAdV5-ΔCAR with a pMOI of 1000.

Transduction of MSCs by HAdV5 or HAdV5-Mut3, with or without enhancing molecules

MSCs were seeded in 1 mL PL-containing BioWhittaker® Alpha minimum essential medium (Lonza) in 24-well plates. The next day, the indicated viral vector particles were pre-incubated with or without enhancing molecules for 30 minutes at 37 °C in a total volume of 300 µL in PL-free medium. As enhancing molecules, Factor X (250 – 2000 ng/mL), Spermidine (1 – 625 ng/µL), Spermine (1 – 1000 ng/µL), Polybrene (0.08 – 81 µg/µL), Poly-L-Lysine (1-25 % v/v) and Lactoferrin (1 – 1000 µg/mL) were used. Subsequently, MSCs were washed and 500 µL PL-free medium was provided. Cells were transduced by adding 300 µL of the pre-incubated viral particles (resulting in the indicated pMOI) and incubated for 2 hours at 37 °C. Afterwards, cells were washed, and 1 mL of PL-containing medium was added. 72 hours after transduction, MSCs were detached and eGFP expression was analyzed by flow cytometry.

Amount of enhancers used for pre-incubation of HAdV5:

Enhancing molecule	Amount
Factor X	4 fg / viral particle
Spermidine	500 fg / viral particle
Spermine	1250 fg / viral particle
Polybrene	18 fg / viral particle
Poly-L-Lysine	4% v/v
Lactoferrin	1250 fg / viral particle

Adenoviral Replication in MSCs after infection by HAdV5wt or HAdV5-Mut3wt, with or without enhancing molecules

MSCs were seeded in 1 mL PL-containing BioWhittaker® Alpha minimum essential medium (Lonza) in 24-well plates. The next day, the indicated viral vector particles were pre-incubated with or without enhancing molecules for 30 minutes at 37 °C in a total volume of 300 µL in PL-free medium. As enhancing molecules, Factor X (4 fg / viral particle), Spermidine (500 fg / viral particle), Polybrene (18 fg / viral particle) were used in combination with HAdV5wt. HAdV5-Mut3wt was used without enhancer. Subsequently, MSCs were washed and 500 µL PL-free medium was provided. Cells were infected by adding 300 µL of the pre-incubated viral particles (resulting in a pMOI of 300 or 1000) and incubated for 2 hours at 37 °C. Afterwards, cells were washed, and 1 mL of PL-containing medium was added. 24, 48, 72 and 96 hours post infection, cells and supernatant was harvested and lysed. MSC lysates were used to re-infect A549, previously seeded into 24-well plates 24 hours earlier. 4 hours post re-infection, A549 DNA was isolated using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The obtained A549 DNA samples were used to quantify the infectious adenovirus produced by MSCs using real-time quantitative PCR (qPCR). For qPCR analysis, a part of the adenoviral E4 transcription unit was amplified

(forward primer: TAGACGATCCCTACTGTACG (SEQ ID NO.: 37);
reverse primer: GGAAATATGACTACGTCCGG (SEQ ID NO.: 39);
all primer sequences indicated in 5' → 3' direction).

For normalization β-actin was analyzed, too

(forward primer: GCTCCTCCTGAGCGCAAG (SEQ ID NO.: 40);
reverse primer: CATCTGCTGGAAGGTGGACA (SEQ ID NO.: 41);
all primer sequences indicated in 5' → 3' direction).

The Kapa SYBR FAST qPCR Universal Master Mix (PEQLAB Biotechnologie) was used according to the manufacturer's protocol.

MSC Migration Assays of not-transduced and transduced MSCs

To assess MSC migration (not-transduced or transduced with HAdV5 or HAdV5-Mut3), boyden chamber assays were performed. To do so, MSCs were seeded in 3 mL PL-containing BioWhittaker® Alpha minimum essential medium (Lonza) in 6-well plates. The next day, the indicated viral vector particles were pre-incubated with or without enhancing molecules for 30 minutes at 37 °C in a total volume of 300 µL in PL-free medium. As enhancing molecules, Factor X, Spermidine, Spermine, Polybrene, Poly-L-Lysine and Lactoferrin were used. Subsequently, MSCs were washed and 3000 µL PL-free medium was provided. Cells were transduced by adding 300 µL of the pre-incubated viral particles (resulting in the indicated pMOI) and incubated for 2 hours at 37 °C. Afterwards, cells were washed, and 1 mL of PL-containing medium was added. 4 hours after transduction, MSCs were detached and seeded into transwell inserts (8 µM pore size, $1x10^4$ cells per transwell). The transwells were placed on 24-well plates in which $1x10^5$ UM-SCC-11B cells were seeded the day before. As a control, migration of MSCs towards UM-SCC-11B cultivation medium (DMEM with 3% FBS) was analyzed. 18 hours after seeding MSCs into the transwells, the transwells were washed with PBS and cells were fixed using ice-cold methanol. Subsequently, MSCs were stained with 10 µg/mL 4′,6-Diamidin-2-phenylindol (DAPI) and 1% Triton-X diluted in PBS. Migrated cells were quantified by counting DAPI-stained nuclei.

Transduction of MSCs by HAdV5, HAdV5-Mut3, HAdV5-ΔCAR-Mut3, HAdV5-ΔHVR1 or HAdV5-Mut2

Transduction of MSCs with a pMOI of 1000 was performed as described in section "Transduction of MSCs by HAdV5 or HAdV5-Mut3, with or without enhancing molecules", except that MSCs were detached and eGFP expression was analyzed by flow cytometry 24 hours after transduction. No enhancing molecules were used.

Statistical analysis

Results are given as mean ± standard deviation. Statistical analysis was performed using unpaired two-sample (Welch) student's t-test or Wilcox test. Calculations were done with RStudio-software Version 2.15.0 or GraphPad Prism Version 6.07. P-values ≤ 0.05 were considered statistically significant.

Results

Production and characterization of mutant vectors

Fig. 1 shows an alignment of the amino acid sequences of HVR1 of HAdV5 wildtype and HAdV5-Mut3 hexon proteins (Fig. 1A) and of HVR1, HVR5 and HVR7 of HAdV5 wildtype, HAdV5-ΔHVR1, HAdV5-Mut2, HAdV5-Mut3, HAdV5-Mut4, HAdV5-Mut5, HAdV5-Mut6 hexon proteins (Fig. 1B). Negatively charged amino acids are depicted in bold letters. Inserted lysine residues are depicted in italic letters.

Of note, production of the mutant vectors HAdV5-Mut4, HAdV5-Mut5, HAdV5-Mut6 was unfeasible, since there was no virus rescue after transfection of cleaved bacmid DNA in N52.E6 cells, indicating that this vector is not viable.

Fig. 14 shows an alignment of the amino acid sequences of HVR1 of HAdV5 wildtype and HAdV5-Mut7, HAdV5-Mut8 and HAdV5-Mut9 hexon proteins. Inserted lysine residues are depicted in bold letters. Further inserted amino acids are depicted in italic letters.

Production of the mutant vectors HAdV5-Mut7, HAdV5-Mut8, HAdV5-Mut9 was unfeasible, since there was no virus rescue after transfection of cleaved bacmid DNA in N52.E6 cells, indicating that this vector is not viable and that no functional virus particles can be formed, respectively.

Fig. 2 shows an alignment of the nucleotide sequences encoding hypervariable region 1 of HAdV5 wildtype and HAdV5-Mut3 hexon proteins. Nucleotides encoding for the inserted lysine residues are depicted in capital letters.

The integrity of viral proteins was confirmed by silver staining (Fig. 3). As a control, unmodified vector particles (HAdV5) were used and revealed a molecular weight of 108 kDa for the hexon protein. Silver staining verified the deletion of the negative loop of HVR1 in HAdV5-ΔHVR1 as well as the mutation of the HVR1 region in HAdV5-Mut3. Both of which alterations resulted in a respective, visible reduction of the molecular weight of the hexon protein. In contrast, the hexon protein with inserted lysines instead of aspartic acids (HAdV5-Mut2) showed the same molecular weight like unmodified hexon protein as it was expected.

Zeta potential measurements for determining the surface charge of the vector particles clearly revealed a negative surface charge of -22.8 mV for the control vector HAdV5, which is in accordance with the literature (Fig. 4). Interestingly, the deletion of the negatively charged HVR1 loop (HAdV5-ΔHVR1) resulted only in a not statistically significant, slight reduction of the negative surface charge of particles (-19.4 mV). The replacement of aspartic acids by lysines in case of HAdV5-Mut2 further reduced the negative surface charge (-17.33 mV). However and interestingly, HAdV5-Mut3 showed a statistically significant reduction of the negative surface charge in comparison to HAdV5, HAdV5-ΔHVR1, and HAdV5-Mut2 (-8.1 mV; compared to HAdV5: $p<4.303\times10^{-5}$; compared to HAdV5-ΔHVR1: $p< 2.738\times10^{-4}$; compared to HAdV5-Mut2: $p<1.139\times10^{-7}$).

<u>HAdV5-Mut3 shows significantly less FX-mediated transduction of CAR-negative SKOV-3 cells than HAdV5 wildtype (HAdV5), HAdV5-Mut2 and HAdV5-ΔHVR1</u>

Binding of human blood coagulation factor X (FX) to HAdV5 mediates transduction of hepatocytes and therefore, triggers the sequestration of particles. The relevant binding residues for FX are located within HVR5 and HVR7 of hexon protein (Alba et al., 2009). To exclude CAR-mediated cell transduction, the inventors used CAR-negative SKOV-3 cells (Fig. 5) and analyzed if FX enhances cell transduction of mutant vectors. HAdV5-ΔFX vector particles are known to exhibit significantly reduced FX binding due to a point mutation within HVR7 (Krutzke et al., 2016) and were used as a control. FX-mediated cell transduction by HAdV5-ΔFX was significantly reduced compared to that of HAdV5 in the presence of FX ($p<3.686\times10^{-5}$). Surprisingly, similar effects were observed with HAdV5-Mut3, which showed statistical significantly reduced cell transduction in the presence of FX compared to HAdV5 ($p<1.135\times10^{-5}$). Interestingly, FX-mediated transduction efficiency of HAdV5-ΔHVR1 was not significantly reduced compared to HAdV5. The introduction of lysine residues instead of aspartic acid residues in HVR1 (HAdV5-Mut2) showed no reduction of FX-mediated uptake. Taken together, the results indicate that HAdV5-ΔHVR1 and HAdV5-Mut2 showed no reduced FX binding, while HAdV5-Mut3 showed significantly reduced FX binding that was comparable to that of HAdV5-ΔFX.

The uptake of HAdV5-Mut3 by scavenger receptors is significantly reduced compared to HAdV5 wildtype (HAdV5)

The major sink of systemically administered HAdV5 are liver-residential macrophages called Kupffer cells (Alemany et al., 2000; Khare et al., 2012). These cells are known to possess scavenger receptors on their cell surface, which bind and capture negatively charged molecules. HAdV5 exhibits an overall negative surface charge that is mainly attributed to a stretch of negatively charged amino acids within HVR1 (Khare et al., 2012). To reduce the negative surface charge of particles, the inventors either deleted the negatively charged HVR1 loop (HAdV5-ΔHVR1), inserted lysine instead of aspartic acid in the stretch (HAdV5-Mut2) or replaced the entire stretch by four lysine residues (HAdV5-Mut3) (Fig. 4). Results clearly showed that the uptake of all three mutant vectors HAdV5-ΔHVR1, HAdV5-Mut2 and HAdV5-Mut3 by murine macrophages was significantly reduced compared to control vector HAdV5 (HAdV5-ΔHVR1 $p<0.001462$; HAdV5-Mut2 $p<0.001219$; HAdV5-Mut3 $p<0.001241$) (Fig. 6). This effect was even more pronounced with HAdV5-Mut3. The uptake of HAdV5 particles by macrophages was significantly inhibited in the presence of polyinosinic acid (Poly-(I)). Poly-(I) binds to and saturates scavenger receptors, thus confirmed a scavenger receptor-mediated virus uptake mechanism. Pre-incubation of cells with Poly-(I) had no effect on the uptake of HAdV5-ΔHVR1 and HAdV5-Mut2 by macrophages. Interestingly, pre-incubation of cells with Poly-(I) increased the uptake of HAdV5-Mut3 particles by macrophages.

FX binding-ablated HAdV5-Mut3 vector particles escape from neutralization by natural IgMs

Others and the inventors have shown that FX shields adenovirus type 5 vector particles from neutralization by natural IgM antibodies (Krutzke et al., 2016). Since the inventors showed that HAdV5-Mut3 shows significantly decreased FX binding (Fig. 5), they analyzed if these particles become susceptible for the neutralization by human and murine IgMs. Therefore, the inventors incubated vector particles with verifiably Ad-naïve human and murine plasma samples of different donors and mouse strains and subsequently analyzed A549 cell transduction (Fig. 7).

Control HAdV5 vector particles, which bind FX normally (Fig. 5), transduced A549 cells in presence of human or murine plasma with the same efficiency as when incubated in PBS. In contrast, pre-incubation of HAdV5-ΔFX particles, which show reduced FX binding (Fig. 5), were almost completely neutralized by natural IgMs upon incubation with both human and murine plasma. However and interestingly, the inventors did not observe this effect when HAdV5-Mut3 was incubated with human or murine plasma samples (p<5x10-11 compared to respective pre-incubations of HAdV5-ΔFX), even though these particles show a reduced FX binding comparable to that of HAdV5-ΔFX (Fig. 5). Thus, the inventors show that the introduced Mut3 mutation allows vector particles devoid of FX-shielding to escape from natural IgMs.

Significantly enhanced secretion of HAdV5-encoded therapeutic protein TSG-6 by MSCs transduced with HAdV5-TSG6 in the presence of Factor X as transduction enhancer

It was found that the expression and subsequent secretion of TSG-6, as a representative therapeutic protein, by MSCs was significantly enhanced in the presence of Factor X (FX) (Fig. 8). TSG-6 concentrations in supernatants of cells transduced with pMOI 900 in the presence of FX were about 3-fold higher compared to TSG-6 concentrations in supernatants of cells transduced with pMOI 20,000 in the absence of FX.

Transduction of several tumor cell lines is improved or preserved when using HAdV5-Mut3 compared to HAdV5 wildtype (HAdV5)

It was found that the transduction efficiencies of several tumor cell lines are either improved or preserved when comparing HAdV5-Mut3 to HAdV5, suggesting benefits for utilization of HAdV5-Mut3 as an oncolytic virus (Fig. 9). For example, the proportion of eGFP positive UM-SCC-11B cells can be increased from about 20% to about 75% when HAdV5-Mut3 is used compared to HAdV5 (pMOI 300).

Significantly enhanced transduction of MSCs by pre-incubation of HAdV5 wildtype (HAdV5) with enhancers or by utilization of HAdV5-Mut3

It was found that although nearly no eGFP expression is detected when MSCs are transduced by solely HAdV5, all enhancing molecules (enhancers) as well as the mutant HAdV5-Mut3 viral vector improved the mean fluorescence intensities (MFI) statistically significantly up to over 800-fold (Fig. 10). Strikingly, transduction by HAdV5-Mut3 results in high eGFP expression (over 600-fold increased MFI compared to HAdV5 transduction) without the use of enhancing molecules.

Combination of HAdV5-Mut3 with the enhancing molecules Spermidine (500 fg / viral particle), Spermine (1250 fg / viral particle) and Factor X (4 fg / viral particle) was also investigated (Fig. 12). It was found that the enhancing molecules slightly further enhanced transduction of cells by HAdV5-Mut3 (3-fold).

Improved adenoviral replication in MSCs by using enhancer-pre-incubated HAdV5wt or by using HAdV5-Mut3wt

It was found that enhancing molecules as well as HAdV5-Mut3wt improve adenoviral replication compared to infection with HAdV5wt alone. Strikingly, HAdV5-Mut3wt shows a maximum of about $5x10^3$ infectious particles / MSC after 48 hours, independent of the used pMOI. Only combination of Spermidine with a pMOI of 1000 of HAdV5wt results in a similarly high virus yield (~ $4.7x10^3$ viral particles / MSC). All other combinations of HAdV5wt with enhancers results in accumulation of infectious adenoviral particle over time (probably caused by constant reinfection of initially uninfected MSCs) without a detectable peak.

Transduction of MSCs does not inhibit their migration towards UM-SCC-11B cells

It was found that MSCs show a significantly increased migration towards UM-SCC-11B cells compared to the sole cultivation medium (Fig. 13). Strikingly, migration towards UM-SCC-11B cells was not inhibited by transduction of cells with HAdV5 or HAdV5-Mut3. None of the tested enhancing molecules showed negative effects.

Transduction of several tumor cell lines is improved or preserved when using HAdV5-Mut3 (HAdV-5-M3) compared to HAdV5 wildtype (HAdV-5) and is improved when using HAdV5-ΔCAR-Mut3 (HAdV-5-ΔCAR-M3) compared to HAdV5-ΔCAR (HAdV-5-ΔCAR)

It was found that the transduction efficiencies of several tumor cell lines are either improved or preserved when comparing HAdV5-Mut3 to HAdV5 (Fig. 15, see also Fig. 9).

In addition, it was found that the transduction efficiencies of several tumor cell lines are significantly improved when using HAdV5-ΔCAR-Mut3 compared to HAdV5-ΔCAR (Fig. 15). This shows that CAR-independent transduction of tumor cells is enabled by HAdV5-ΔCAR-Mut3.

Significantly enhanced transduction of MSCs by utilization of HAdV5-Mut3 (HAdV-5-M3) and HAdV5-ΔCAR-Mut3 (HAdV-5-ΔCAR-M3) and no transduction of MSCs by utilization of HAdV5-ΔHVR1 (HAdV-5-ΔHVR1) and HAdV5-Mut2 (HAdV-5-M2)

It was found that MSCs are efficiently transduced by HAdV5-Mut3 and HAdV5-ΔCAR-Mut3 vectors while nearly no eGFP expression is detected when MSCs are transduced by HAdV5 wildtype (HAdV-5) (Fig. 16, for HAdV5-Mut3 and HAdV5 see also Fig. 10).

It was further found that MSCs are not transduced by HAdV5-ΔHVR1 and HAdV5-Mut2 (Fig. 16).

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Claims

1. A human adenovirus species C having a capsid which comprises a modified adenovirus hexon protein, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence DEAATALEINLKKKKQAEQQ (SEQ ID NO.: 1).

2. The adenovirus of claim 1, wherein the adenovirus is adenovirus type 5.

3. The adenovirus of claim 1 or 2, wherein the adenovirus is an adenovirus vector or an oncolytic adenovirus.

4. The adenovirus of any one of claims 1 to 3, wherein the adenovirus comprises a transgene.

5. The adenovirus of any one of claims 1 to 4, wherein the capsid has at least one additional capsid modification, wherein the additional capsid modification preferably is a modified adenovirus fiber protein.

6. The adenovirus of any one of claims 1 to 5 for use in treating or preventing a human disease.

7. A nucleic acid encoding a modified adenovirus hexon protein of a human adenovirus species C, wherein the modified adenovirus hexon protein has a modified HVR1 region, wherein the modified HVR1 region has the sequence of SEQ ID NO.: 1.

8. The nucleic acid of claim 7, wherein the nucleic acid has the sequence of SEQ ID NO.: 2.

9. Use of an adenovirus according to any one of claims 1 to 5 for transducing mesenchymal stromal cells (MSCs) or tumor cells.

10. The use according to claim 9, wherein the adenovirus is used in combination with a transduction enhancer for transducing MSCs.

11. The use according to claim 10, wherein the transduction enhancer is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin.

12. An *in vitro* method for transducing MSCs, the method comprising the step of:
contacting a plurality of MSCs with an adenovirus according to any one of claims 1 to 5.

13. The method according to claim 12, wherein the plurality of MSCs is further contacted with a transduction enhancer, wherein the transduction enhancer preferably is selected from the group consisting of coagulation factor X, spermidine, spermine, hexadimethrine bromide, poly-L-lysine and lactoferrin.

14. A transduced MSC obtainable by the method of claim 12 or 13.

15. Transduced MSC of claim 14 for use in treating a disease.

A

Hypervariable region 1 of hexon

HAdV5 wildtype	**DE**AATAL**E**INL**EEEDDD**N**EDE**V**DE**QA**E**QQKTHVF
HAdV5 Mut3	**DE**AATAL**E**INL----*KKKK*-----QA**E**QQKTHVF

B

	HVR1		HVR5		HVR7
HAdV5 wildtype	**DE**AATAL**E**INL**EEEDDD**N**EDE**V**DE**QA**E**QQKTHVF	(...)	STT**E**AAAGNG**D**NLTPK	(...)	GGVINT**E**TLTKVKPKTGQ**E**NGW**E**K**D**AT**E**FS**D**KN**E**IRVGNNF
HAdV5-ΔHVR1	**DE**AATAL**E**INL----------QA**E**QQKTHVF	(...)		(...)	
HAdV5 Mut2	**DE**AATAL**E**INL**EEE***KKK*N**E**K**E**V**DE**QA**E**QQKTHVF	(...)		(...)	
HAdV5 Mut3	**DE**AATAL**E**INL----*KKKK*-----QA**E**QQKTHVF	(...)		(...)	
HAdV5 Mut4	**DE**AATAL*K*INL*KK*----N*K*--V*K*-QA*K*QQKTHVF	(...)		(...)	
HAdV5 Mut5	**DE**AATAL**E**INL----*KKKK*-----QA**E**QQKTHVF	(...)	STT*K*AAAGNG*K*NLTPK	(...)	GGVINT**E**TLTKVKPKTGQ*K*NGW*KK**K*AT**E**FS**D**KN**E**IRVGNNF
HAdV5 Mut6	**DE**AATAL*K*INL*KK*----N*K*--V*K*-QA*K*QQKTHVF	(...)	STT*K*AAAGNG*K*NLTPK	(...)	GGVINT**E**TLTKVKPKTGQ*K*NGW*KK**K*AT**E**FS**D**KN**E**IRVGNNF

Fig. 1

Hypervariable region 1 of hexon

HAdV5 wildtype	gatgaagctgctactgctcttgaaataaacctagaagaagaggacgatgac
HAdV5 Mut3	gatgaagctgctactgctcttgaaataaaccta----------AAAAAG
HAdV5 wildtype	aacgaagacgaagtagacgagcaagctgagcagcaaaaaactcacgtattt
HAdV5 Mut3	AAAAAG----------caagctgagcagcaaaaaactcacgtattt

Fig. 2

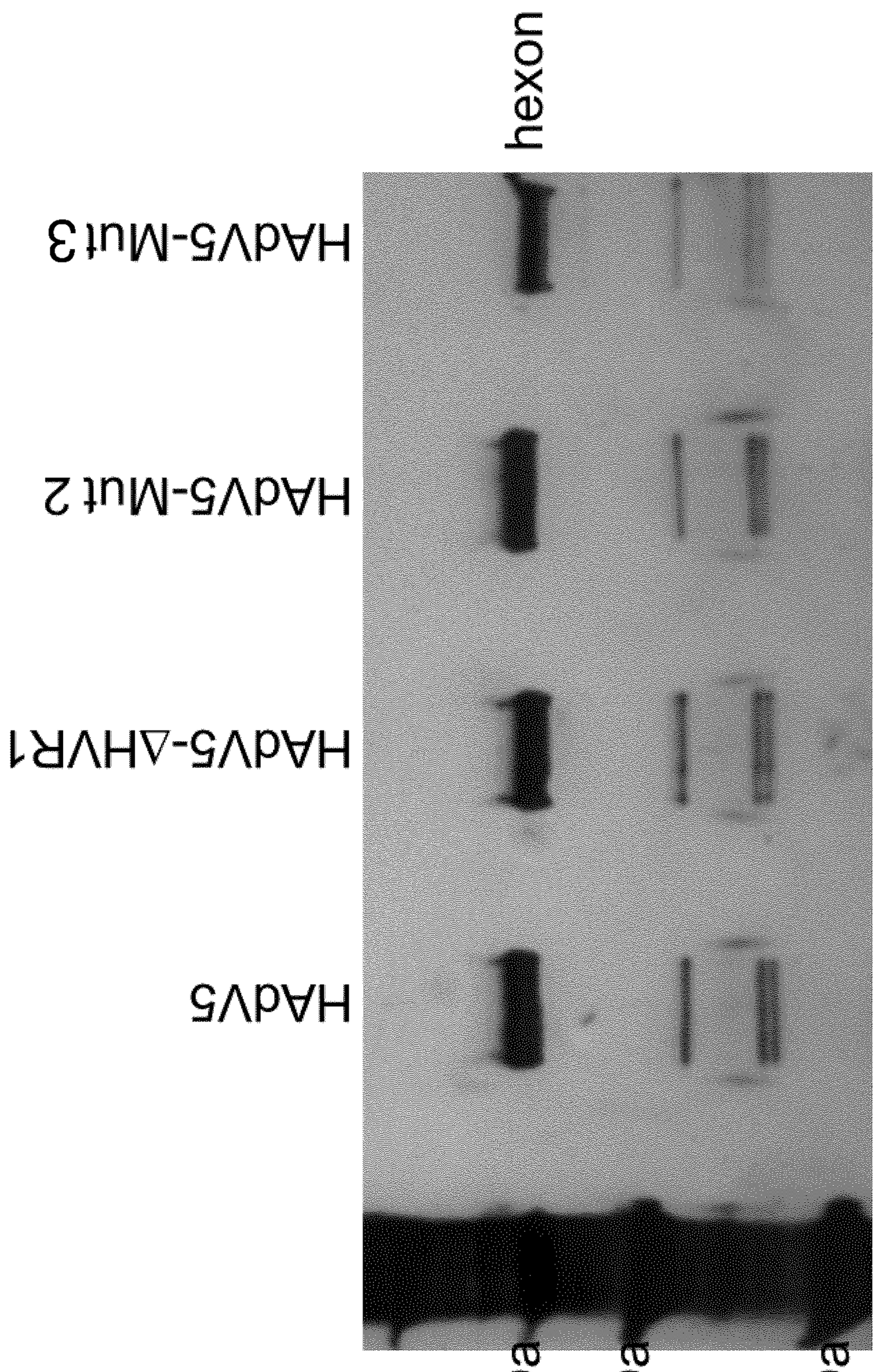

Hexon
HAdV5-ΔHVR1
HAdV5-Mut 2
HAdV5-Mut 3
HAdV5
100 kD
75 kD
50 kD

Fig. 3

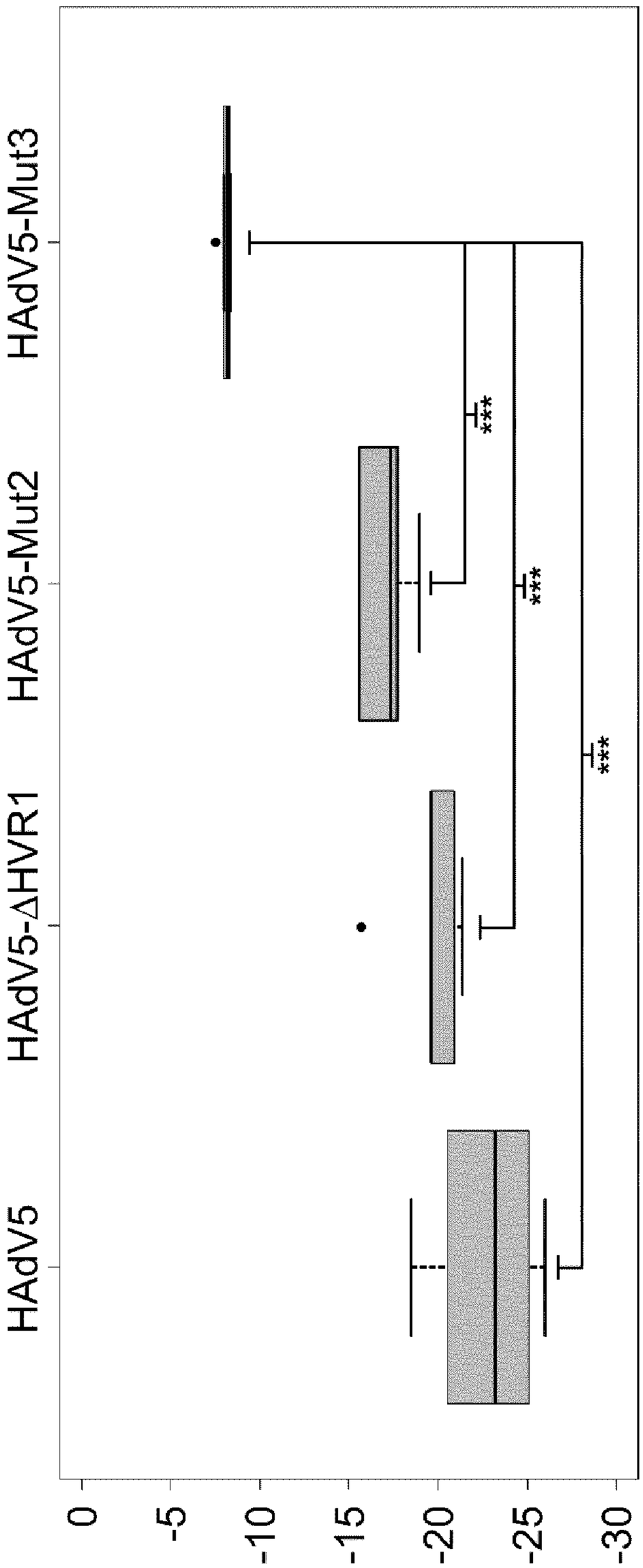

HAdV5
HAdV5-ΔHVR1
HAdV5-Mut2
HAdV5-Mut3
***
***
***
*
0
-5
-10
-15
-20
-25
-30
Zeta (ζ) potential [mV]

Fig. 4

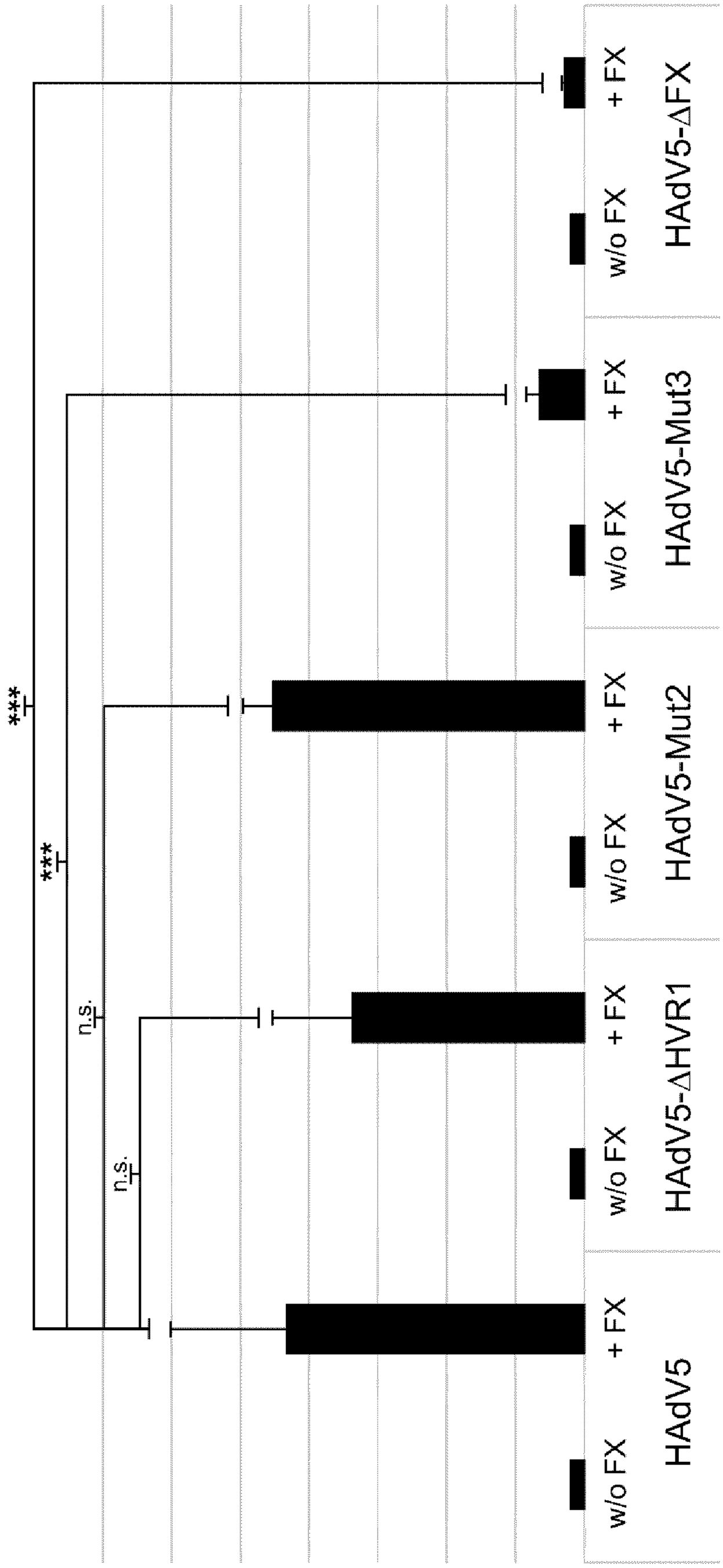

x-fold increase of % eGFP-positive cells rel. to respective vector w/o FX
0
5
10
15
20
25
30
35
w/o FX
+ FX
HAdV5
w/o FX
+ FX
HAdV5-ΔHVR1
n.s.
n.s.
w/o FX
+ FX
HAdV5-Mut2
***
***
w/o FX
+ FX
HAdV5-Mut3
w/o FX
+ FX
HAdV5-ΔFX

Fig. 5

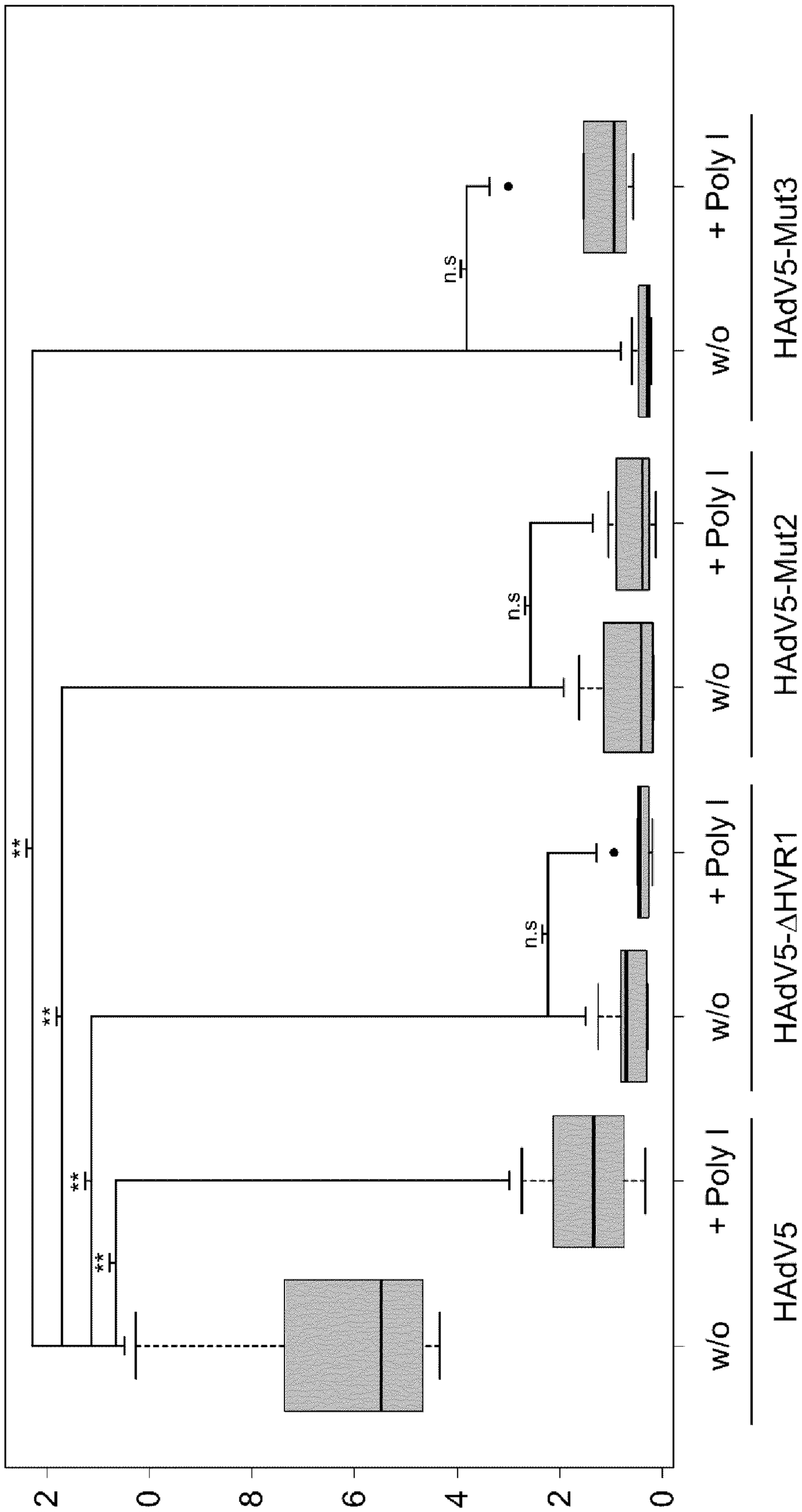

vector copy number per cell (x10³)
0
2
4
6
8
10
12
**
**
**
**
n.s
n.s
n.s
w/o
+ Poly I
HAdV5
w/o
+ Poly I
HAdV5-ΔHVR1
w/o
+ Poly I
HAdV5-Mut2
w/o
+ Poly I
HAdV5-Mut3

Fig. 6

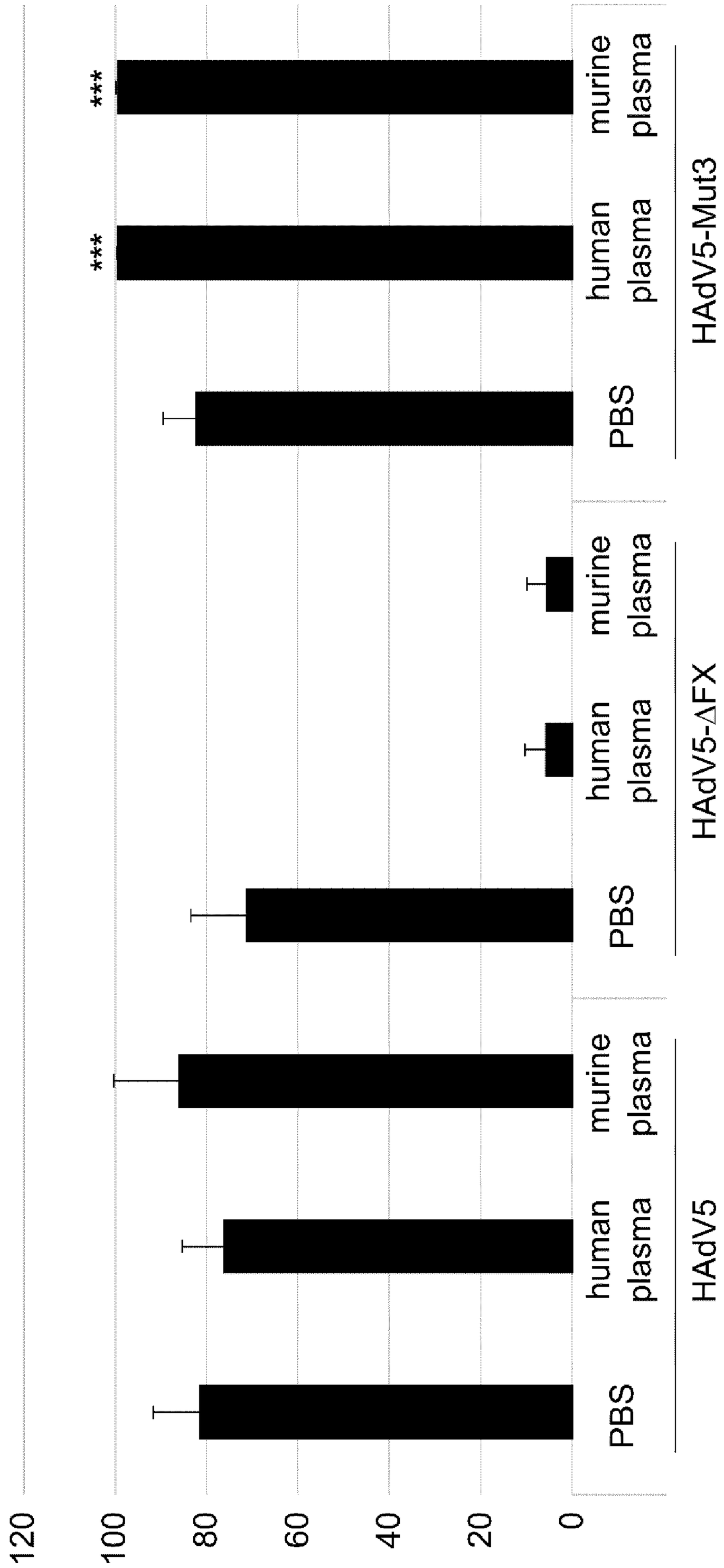

% eGFP-positive cells
120
100
80
60
40
20
0
HAdV5-Mut3
HAdV5-ΔFX
PBS
human plasma
murine plasma
***
***

Fig. 7

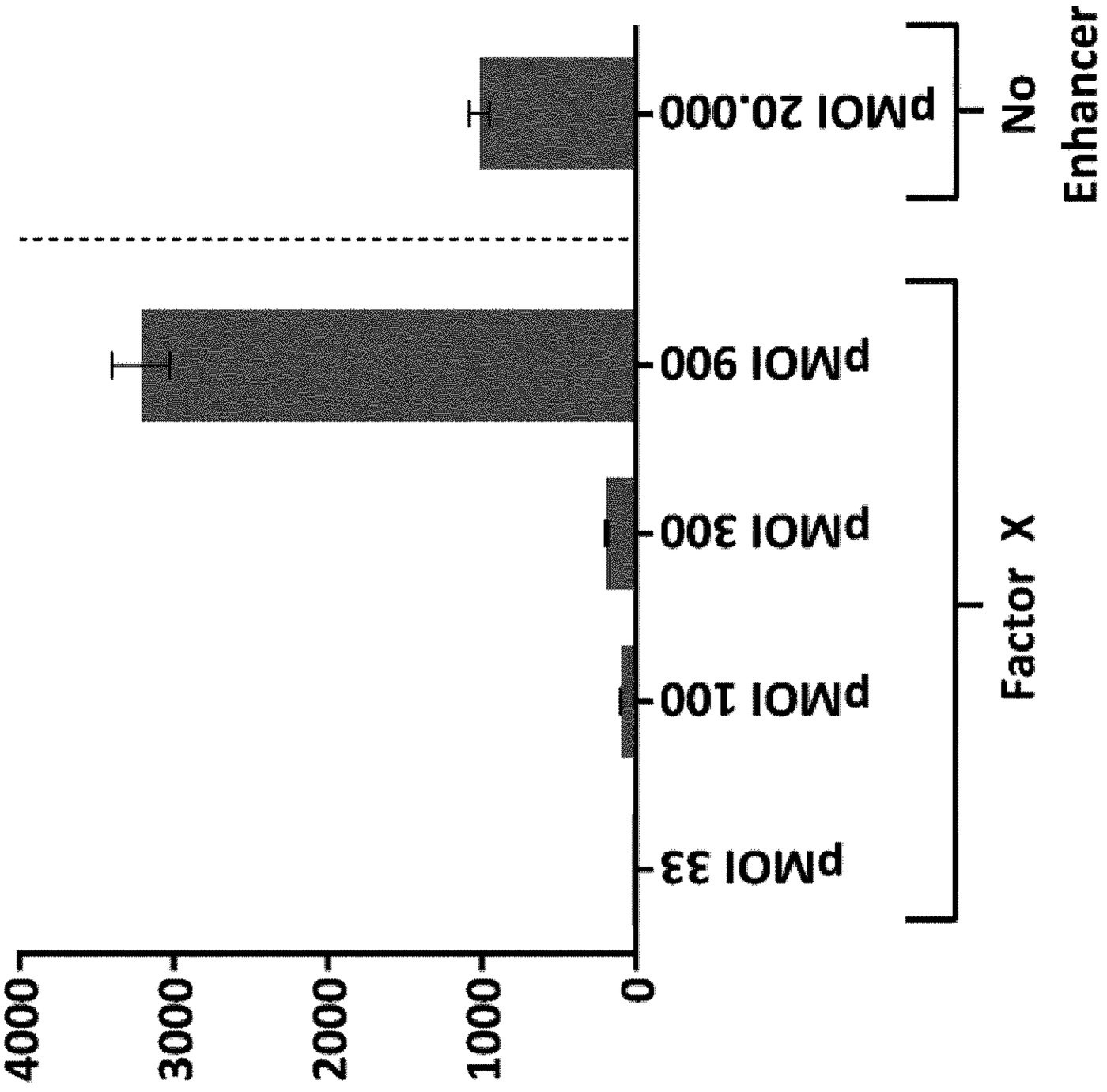

No Enhancer
pMOI 20.000
Factor X
pMOI 900
pMOI 300
pMOI 100
pMOI 33
0
1000
2000
3000
4000
TSG-6 concentration
[ng/mL]

Fig. 8

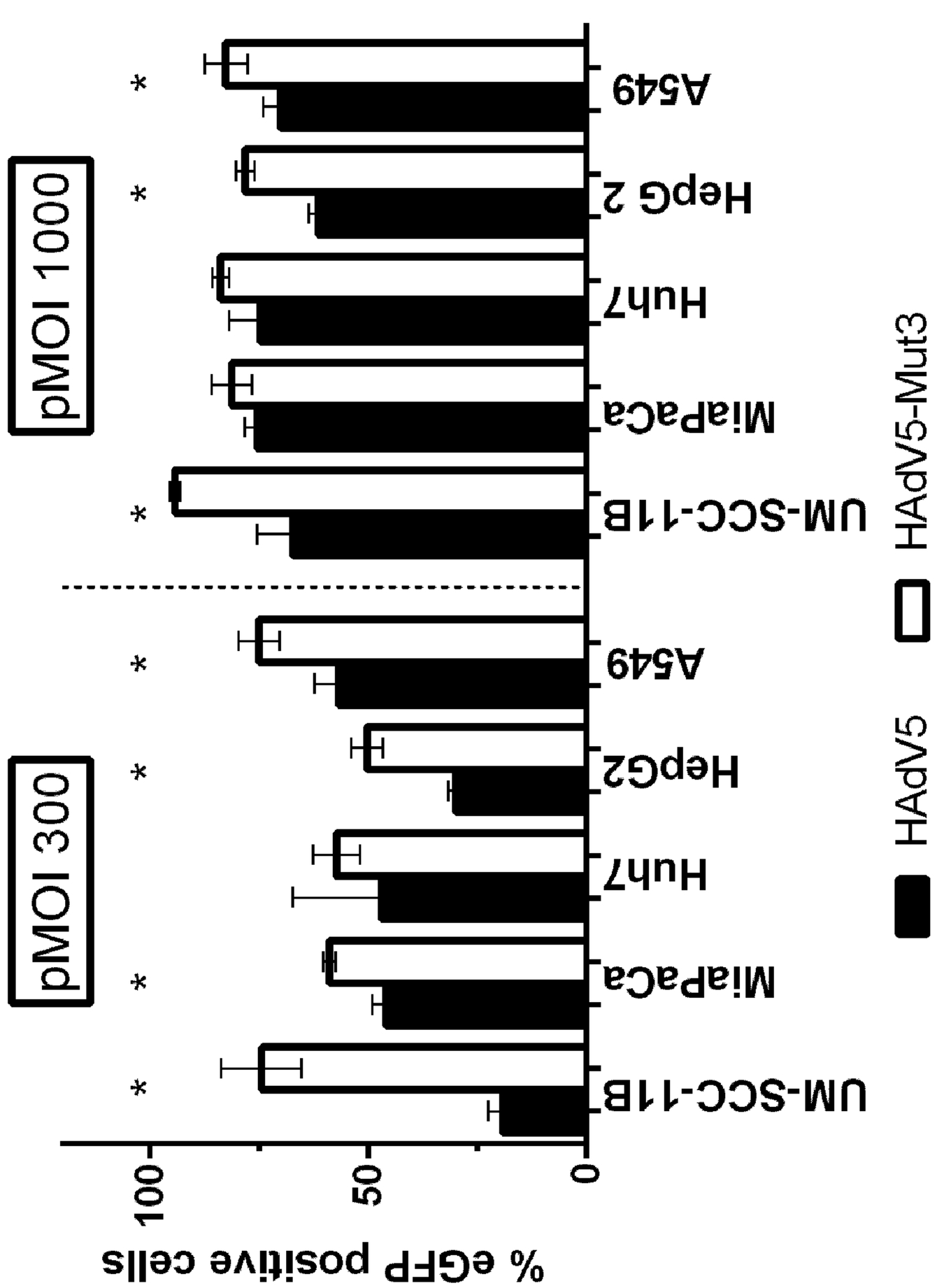

% eGFP positive cells
100
50
0
pMOI 300
pMOI 1000
UM-SCC-11B
MiaPaCa
Huh7
HepG2
A549
HAdV5
HAdV5-Mut3
*

Fig. 9

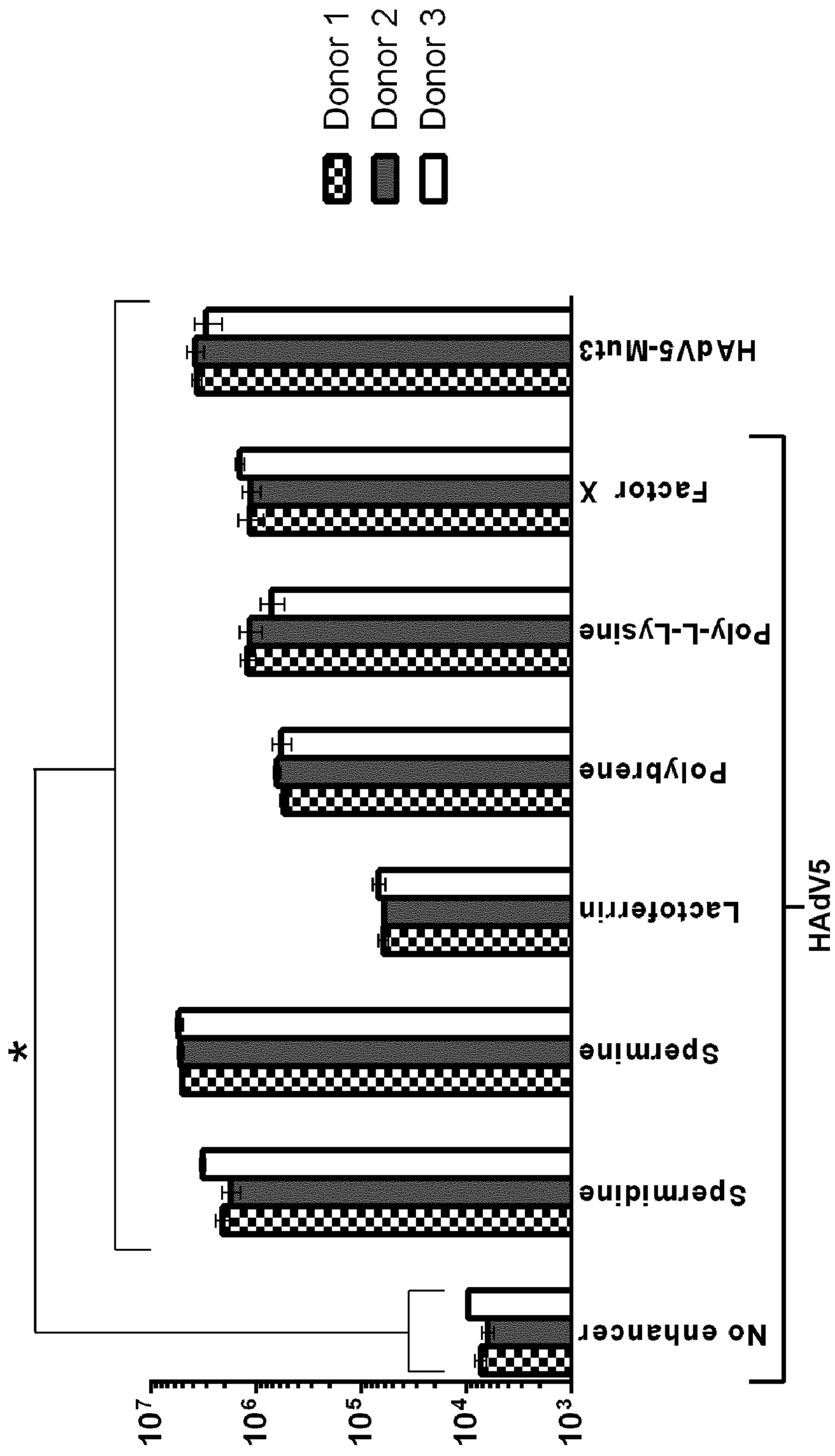

Donor 1
Donor 2
Donor 3
HAdV5-Mut3
Factor X
Poly-L-Lysine
Polybrene
Lactoferrin
Spermine
Spermidine
No enhancer
HAdV5
*
10^7
10^6
10^5
10^4
10^3
Mean fluorescence intensity
(FITC)

Fig. 10

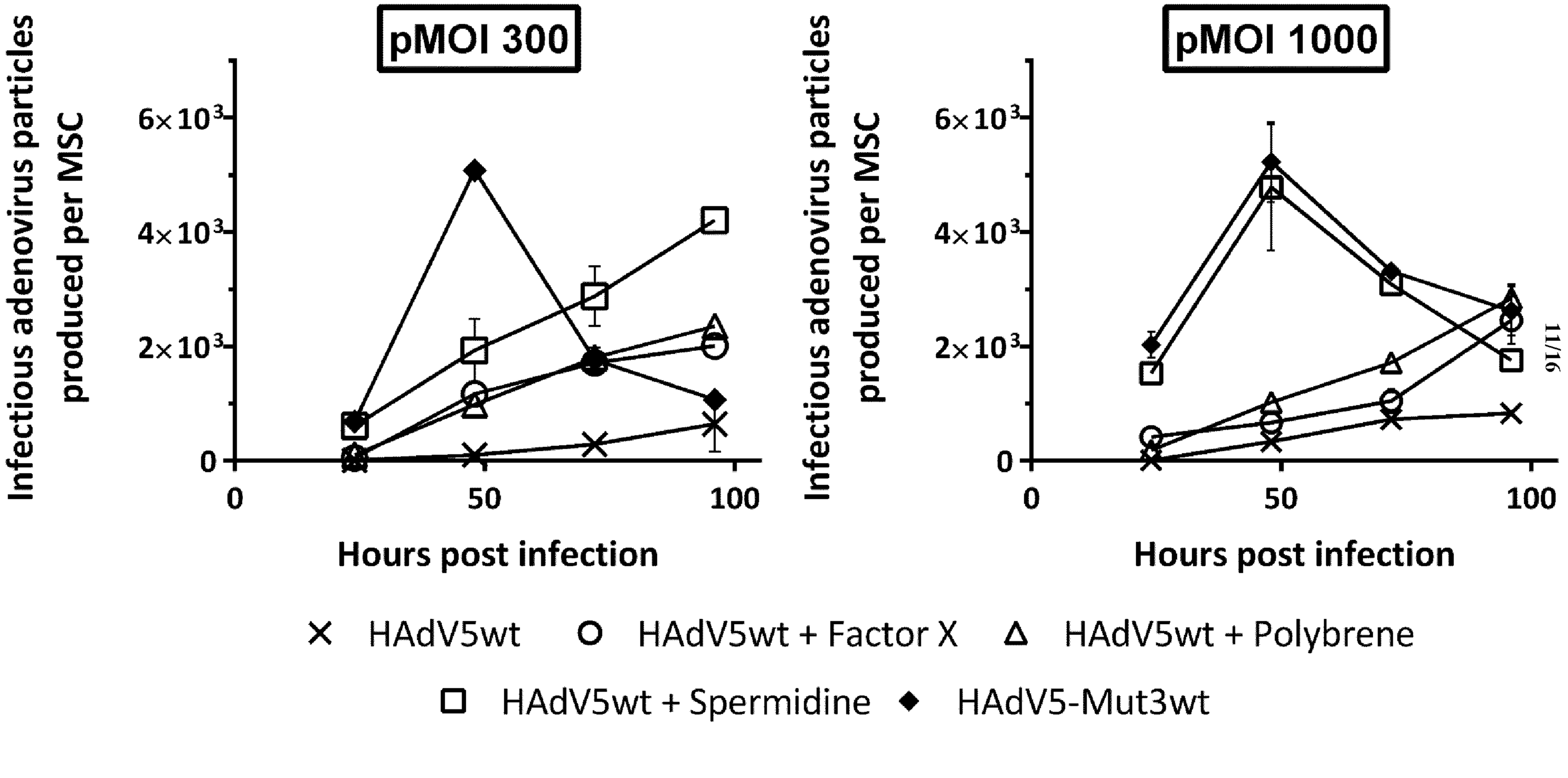

pMOI 300
pMOI 1000
Infectious adenovirus particles produced per MSC
6×10³
4×10³
2×10³
0
0
50
100
Hours post infection
HAdV5wt
HAdV5wt + Factor X
HAdV5wt + Polybrene
HAdV5wt + Spermidine
HAdV5-Mut3wt

Fig. 11

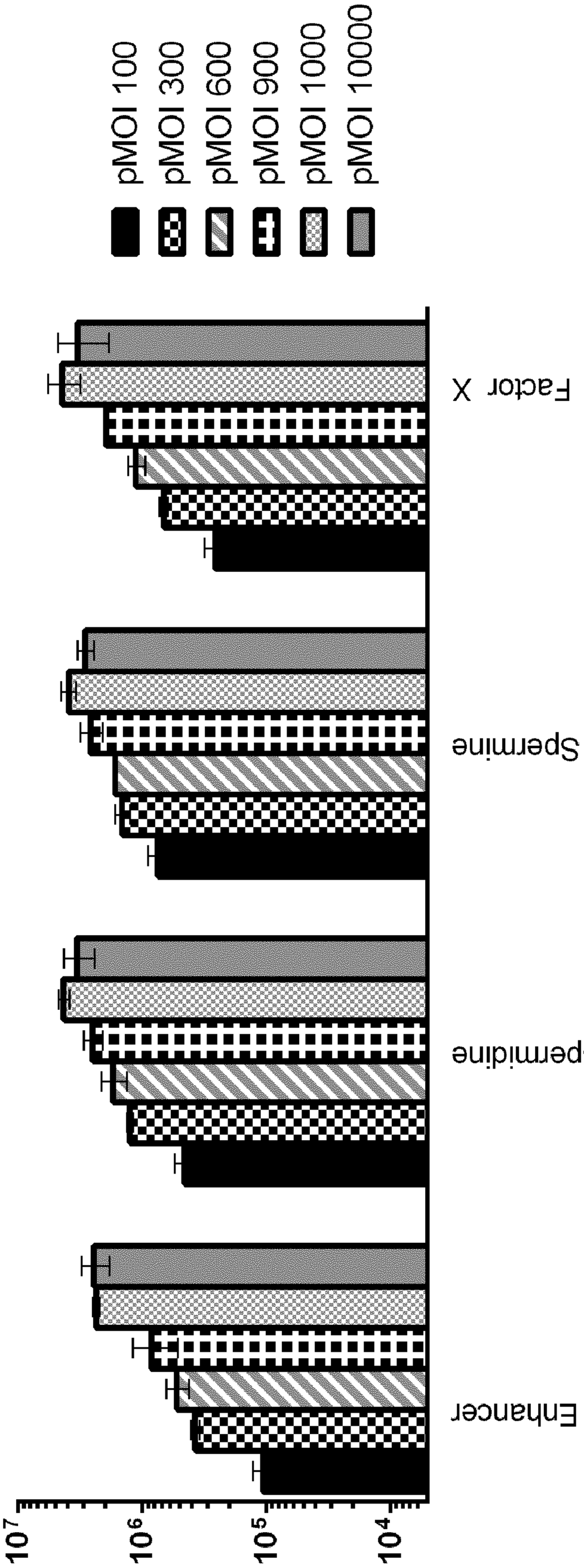

pMOI 100
pMOI 300
pMOI 600
pMOI 900
pMOI 1000
pMOI 10000
Factor X
Spermine
Spermidine
No Enhancer
10^7
10^6
10^5
10^4
Mean fluorescence intensity
(FITC)

Fig. 12

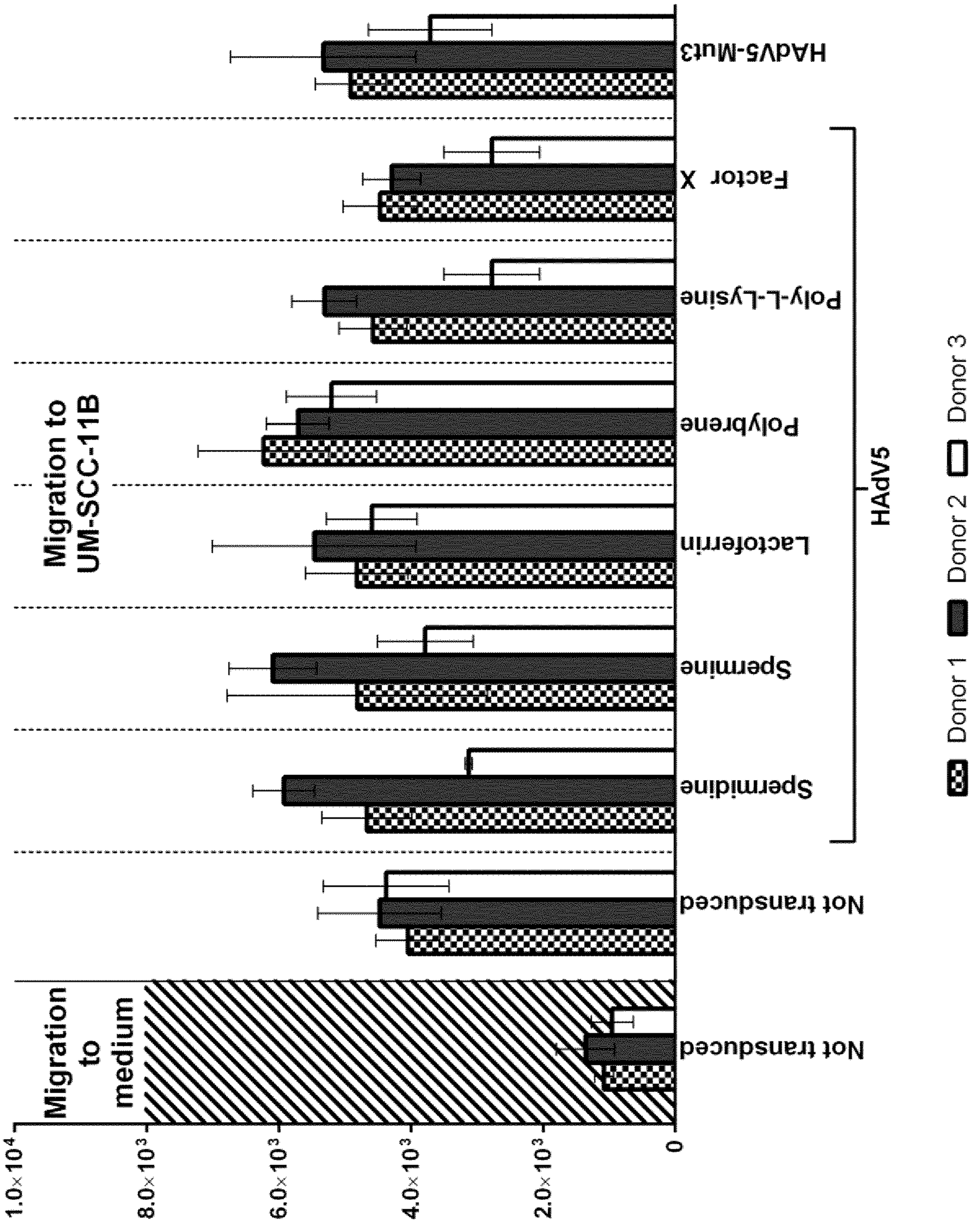

Number of migrated cells
(/Transwell)
1.0×10⁴
8.0×10³
6.0×10³
4.0×10³
2.0×10³
0
Migration to medium
Not transduced
Not transduced
Migration to UM-SCC-11B
HAdV5
HAdV5-Mut3
Spermidine
Spermine
Factor X
Poly-L-Lysine
Polybrene
Lactoferrin
Donor 1
Donor 2
Donor 3

Fig. 13

Hypervariable region 1 of hexon

HAdV5 wildtype	DEAATALEINLEEEDDDNEDEVDEQAEQQKTHVF
HAdV5 Mut7	DEAATALEINL**KKKKKK** - - - - - - - QAEQQKTHVF
HAdV5 Mut8	DEAATALEINL**KKKKKKKK** - - - - - QAEQQKTHVF
HAdV5 Mut9	DEAATALEINL*GGSGGGSG***KKKKKKKK***GSGGGSGG*QAEQQKTHVF

Fig. 14

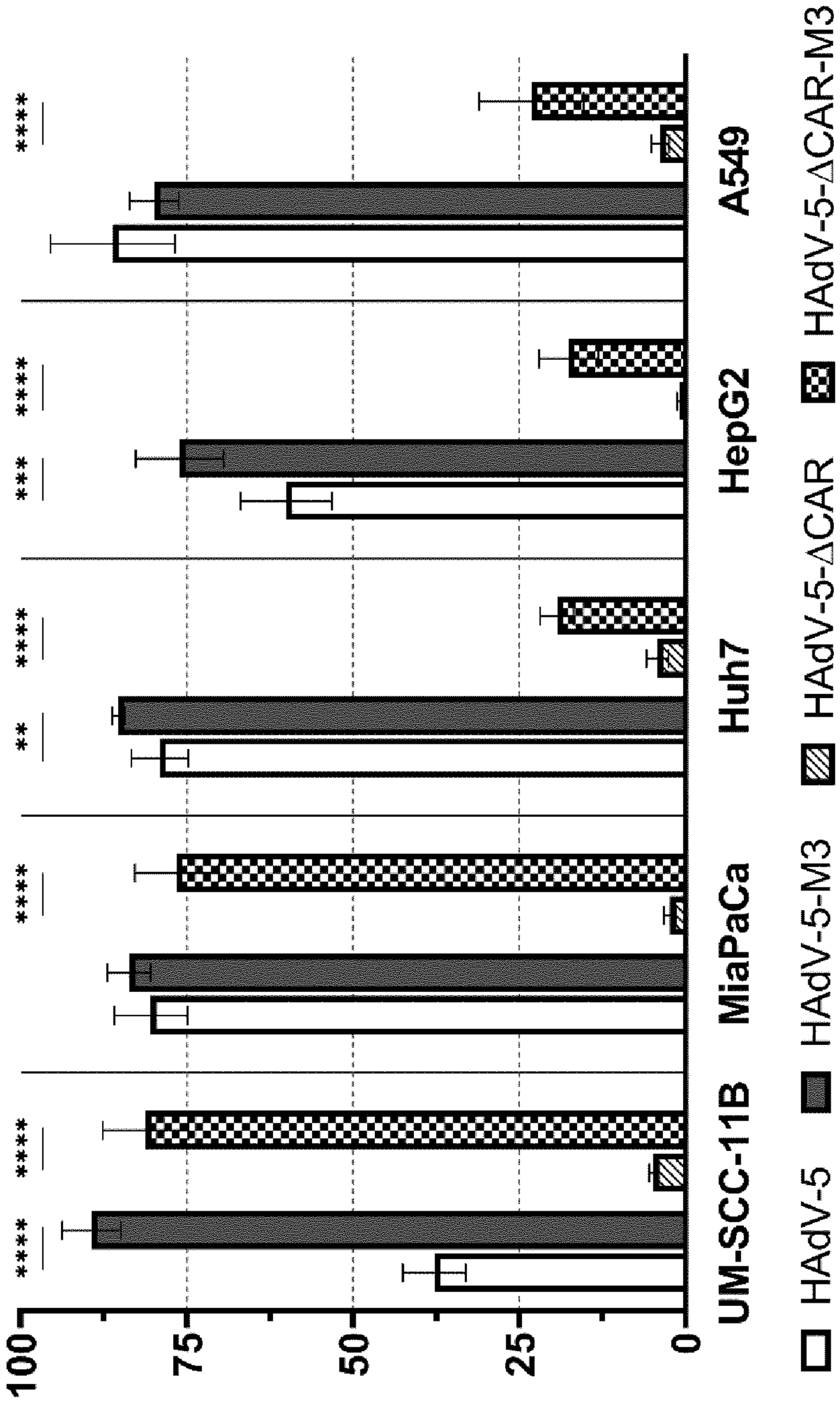

% eGFP positive cells
100
75
50
25
0
HAdV-5
HAdV-5-ΔCAR
HAdV-5-M3
HAdV-5-ΔCAR-M3
UM-SCC-11B
MiaPaCa
Huh7
HepG2
A549
**
****
***
****
****
****
****
****

Fig. 15

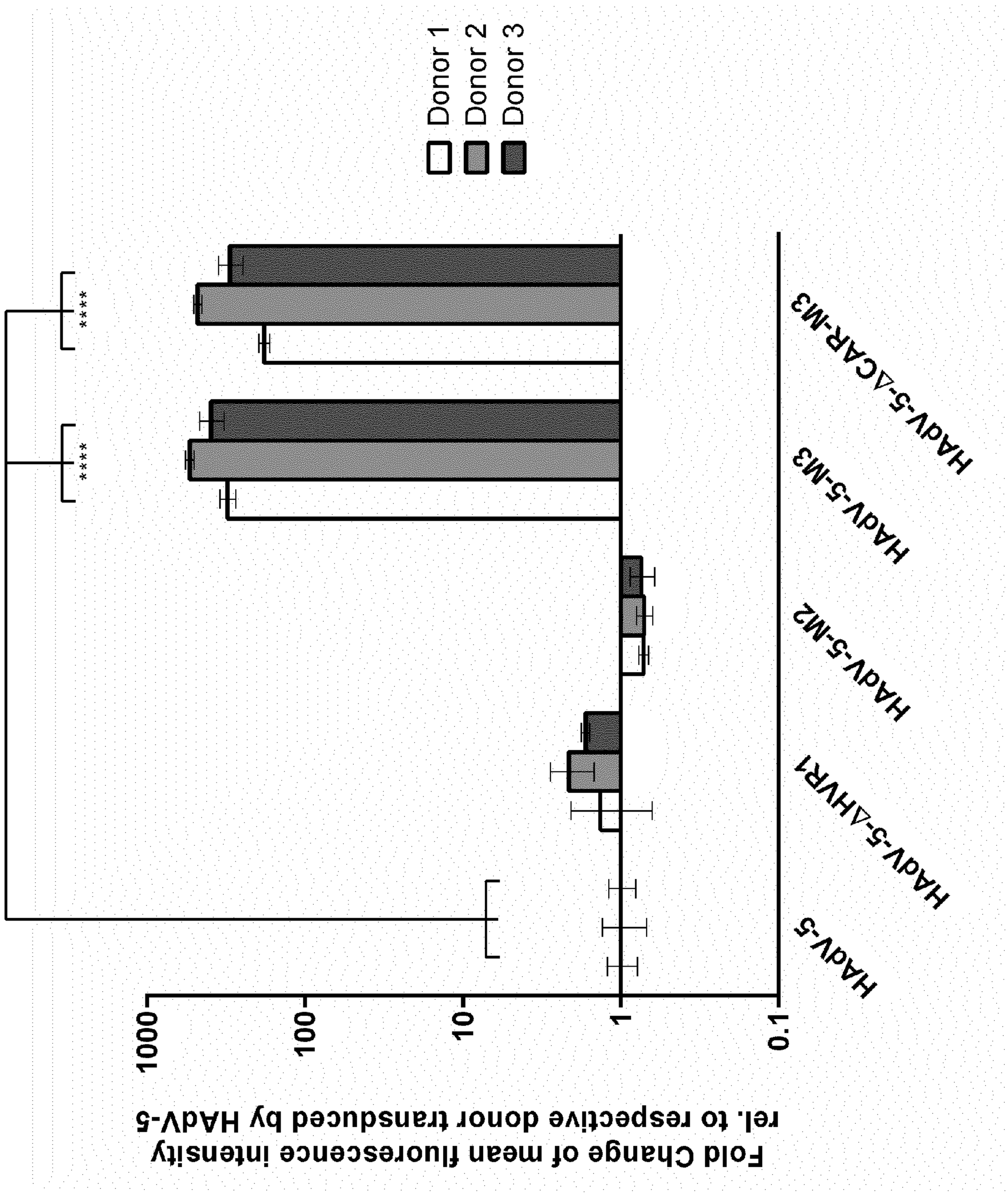

Donor 1
Donor 2
Donor 3
****
****
HAdV-5
HAdV-5-AHVR1
HAdV-5-M2
HAdV-5-M3
HAdV-5-ACAR-M3
1000
100
10
1
0.1
Fold Change of mean fluorescence intensity
rel. to respective donor transduced by HAdV-5

Fig. 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/079494

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/86 A61K35/761 C12N15/34 C12N5/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/302313 A1 (YU DI [SE] ET AL) 14 November 2013 (2013-11-14) see the whole document, in particular the cited passages; paragraphs [0028], [0030], [0064]; figures 2,9,11; sequence 1 -----	1-15
A	WO 2016/118433 A1 (SHAYAKHMETOV DMITRY M [US]; DI PAOLO NELSON C [US]) 28 July 2016 (2016-07-28) see mutant "Ad-3M"; paragraphs [0076] - [0077], [0154] - [0155]; figure 22 -----	1-15

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 2021	25/01/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brenz Verca, Stefano

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/079494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	KRUTZKE L ET AL: "Substitution of blood coagulation factor X-binding to Ad5 by position-specific PEGylation: Preventing vector clearance and preserving infectivity", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 235, 11 June 2016 (2016-06-11), pages 379-392, XP029633357, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2016.06.022 cited in the application the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/079494

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		JP 2018504141 A	15-02-2018
		US 2018099015 A1	12-04-2018
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