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Protease-deleted adenovirus as an alternative for replication-competent adenovirus vector

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ABSTRACT

For cancer therapy and vaccination an amplified expression of the therapeutic gene is desired. Previously, we have developed a single-cycle adenovirus vector (SC-AdV) by deleting the adenovirus protease (PS) gene. In order to keep the E1 region intact within the PS-deleted adenoviruses, we examined the insertion of two transgenes under the control of a constitutive or inducible promoters. These were inserted between E4 and the right inverted terminal repeat in a wide variety of backbones with various combinations of PS, E3 and E4 deletion. Our data showed that PS-deleted adenoviruses, expressed transgenes as strongly as replication-competent AdVs in HEK293A and a variant of HeLa cells. In a head-to-head comparison in four human cell lines, we demonstrated that SC-AdV, was comparable for transgene expression efficacy with its replication-competent counterpart. However, the SC-AdV expresses its transgene 10 to 16,000 times higher than its replication-defective counterpart.

1. Introduction

For gene and cancer therapy and vaccination applications, an amplified expression of the therapeutic gene is desired (For review see: Abudoureyimu et al., 2019; Goradel et al., 2018, Kiyokawa and Wakimoto, 2019, Nattress and Halldén, 2018, Stepanenko and Chekhonin, 2018). Furthermore, since the beginning of the COVID-19 pandemic, adenovirus vector (AdV) has been the most common type of viral vectors for the production of COVID-19 vaccines (WHO web page). The benefit of increased transgene expression through AdV replication in permissive species has been well illustrated in several articles (Eloit and Adam, 1995; Massie et al., 1998a; Zhang et al., 1996). This could be attributed mainly to the enhanced transgene expression following replication. However, in spite of their higher efficiency, fully replication AdVs raise biosafety issues related to viral shedding. To prevent virus dissemination, only a few types of single-cycle adenoviruses (SC-AdV) have been generated so far. For example, Crosby and his colleagues generated SC-AdVs with deletion or selective repression of different late genes by preserving DNA replication, but blocking production of infectious progeny viruses. The SC-AdV with deletion of the capsid cement protein IIIa was the most promising. They showed SC-AdV produced 30 to 300-fold more antigen than replication-defective, E1-deleted AdVs (RD-AdV) (Crosby and Barry, 2014, 2017; Crosby et al., 2015).

Several years ago, we developed an original AdV system based on the deletion of one of the essential late viral genes, the protease (PS) (Oualikene et al., 2000). Viral proteins cleaved by the PS include the pTP, pVI, pVII, pVIII, pIIIa, L1-52 K, and 11 K DNA-binding protein. The PS is therefore essential for the maturation and assembly of viral proteins, and for the release of virions from infected cells (Chen et al., 1993, Weber, 1995). Furthermore, viral entry into host cells requires that the PS be packaged within the mature virion (Cotten and Weber, 1995; Greber et al., 1996). Consequently, deleting the PS gene is a simple approach to obtain a SC-AdV with a block in virus assembly and release (Oualikene et al., 2000). The ability of protease-deleted adenovirus (AdPS-) to undergo a single round of replication within the host cell, leads to higher expression level of the therapeutic gene compared to RD-AdV (Bourbeau et al., 2007; Haq et al., 2019; Young et al., 2018). Since no infectious viral particles are assembled in the absence of PS, there is no risk of viral shedding. Thus, AdPS- satisfies both efficiency and safety concerns.

Transgenes can be inserted in the adenovirus genome as autonomous expression cassettes under control of an exogenous promoter or directly inserted into adenoviral transcription units. Farrera-Sal et al. (2020a)

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Abbreviations

AdPS	protease-deleted Adenovirus
AdV	adenovirus vector
dpi	day post-infection
FI	Fluorescence Index
GFP	Green Florescent Protein
GOI	gene of interest
LG	Renilla Luciferase fused with GFP gene
hpi	hours post infection
IRES	internal ribosome entry site
LITR & RITR	left and right inverted terminal repeats
MOI	multiplicity of infection
MLP	major late promoter
ORF	open reading frame
PS	protease
RC-AdV	replication-competent adenovirus vector
RD-AdV	replication-defective adenovirus vector
RLU	Relative Light Units
RLuc	Renilla Luciferase
SC-AdV	single-cycle adenovirus vector

reviewed the effect of transgene location at different possible sites within oncolytic AdVs. For the purpose of the current study, we are focusing only on sites for which the transgene is under the control of exogenous promoters. These sites are (a) the early region 1 (E1), (b) early region 3 (E3) and (c) between the early region 4 (E4) and right inverted terminal repeat (RITR). Transgenes can be inserted in each site in either forward or backward orientations. The E1 and E3 regions are the most commonly used sites for transgene insertion. As E1 is essential for viral replication, E1-deleted AdV becomes replication-defective (RD-AdV) and they need to be grown in complementary cell lines such as HEK293A (Graham et al., 1977). Usually in RD-AdV, the E3 region is also deleted to increase the cargo capacity of the vector up to 8 kb. Importantly, in contrast to E1 deletion, the deletion of E3 does not prevent AdV growth in cell culture. Recombinant AdVs with insertion in E3 are therefore replication-competent adenovirus vectors (RC-AdV). Another region for AdV transgene insertion is between the E4 and RITR. The E4 region of adenovirus is located at the right end of the genome and transcription proceeds from right to left. Seven different open reading frames (ORFs) have been identified in the E4 region. Since adenoviruses that express only ORF 6 can grow to near wild type levels (Bridge and Ketner, 1989; Huang and Hearing, 1989), deletion of the other E4 ORFs that are not required for virus growth in cell culture allows a 1.9 kb increase in the cloning capacity of AdV.

To regulate both the level and the duration of expression for genes whose constitutive expression might not be tolerated by the cells, we have generated an inducible expression system known as the “cumate gene switch” (Mullick et al., 2006). In the activator configuration, a chimaeric transactivator “cTA” binds to several copies of the cumate operator placed upstream of a minimal CMV promoter and activates transcription in the absence of cumate. Upon cumate addition, the transactivator can no longer bind to the DNA and therefore is no longer able to activate transcription from the basal promoter. For the transgene expression, the cTA can be provided by another AdV or by a cell line previously engineered to produce it in a stable manner. In the repressor configuration, regulation is mediated by the binding of the repressor (CymR) to the operator site (CuO), placed downstream of a strong constitutive promoter. The resulting promoter is named CMV5-Cuo. The addition of cumate relieves the repression and the gene is expressed.

Because replication deficient Adenovirus has been developed as a gene delivery vector for several decades already, a good number of studies have been published on the vectorology of this vector (Gao et al., 2019; Danthinne and Imperiale, 2000; Farrera-Sal et al. 2020a,b; Suzuki et al., 2015; Thomas et al., 2013; Word et al., 2013). However, no major work is available on the vectorology of protease deleted AdV. For generating AdPS-, as well as for other types of SC-AdV, the E1 activity, or at least E1A activity, should be conserved (Bourbeau et al., 2007; Haq et al., 2019). Within the genome of a SC-AdV, there are three possible locations for transgene insertion: (a) the E3 region, (b) between E4 and RITR and (c) in the E1 region, if the E1A gene is co-expressed with the gene of interest (GOI). Insertion in the deleted E3 region gives variable results with respect to transgene expression and/or viral growth depending on the configuration and the orientation of the expression cassette (Bramson et al., 1996; Ilan et al., 1997; Mittal et al., 1993; Schneider et al., 1989; Suzuki et al., 2015; Yarosh et al., 1996). We have already generated several AdPS- for which the transgene and E1A are in a bicistronic configuration through the use of an internal ribosome entry site (IRES), and the expression cassette was inserted in the deleted E1 region of AdV (Bourbeau et al., 2007; Haq et al., 2019). The problem with this strategy is that both genes (GOI and E1A) are under the control of the same promoter and it has been shown that the expression level of the gene is lower when it is downstream of IRES instead of upstream (Al-Allaf et al., 2018). In the current study, we examined the third possibility, that is, insertion between the E4 region and the RITR, in the context of SC-AdV and RC-AdV. This was done by comparing undeleted AdV with AdV having different combinations of deletions in the PS gene, E3 and E4 (except for E4 ORF6). We tested two different transgenes, green fluorescent protein (GFP) and a Renilla Luciferase (RLuc) fused with GFP (LG) with either the strong constitutive CMV5 promoter (Massie et al., 1998b) or the cumate-inducible CR5 promoter (Mullick et al., 2006). The RC-AdV were generated by insertion of the PS gene back in its original position (inserted into adenoviral transcription units). We compared the level of transgene expression and virus titer of AdPS- with AdV having the PS gene in its original position. Our data showed that insertion of the expression cassette between E4 and RITR in a PS-deleted backbone resulted in transgene expression as efficient as in RC-AdV.

2. Materials and methods

2.1. Generation of adenovirus transfer vectors

The construction of two transfer vectors with RLuc fused with GFP gene under the control of a strong constitutive CMV5 promoter for which the E4 region is either intact, or deleted (except for ORF6 and E4 promoter), has been described previously (Gilbert et al., 2014; Nazemi-Moghaddam, 2006) and is presented in more detail in the supplementary material section. To construct the transfer vector with GFP regulated by the cumate inducible system, the fragment of GFP gene under the control of the CR5-inducible promoter was generated by PCR using pAdCR5-GFP as template (Mullick et al., 2006) and inserted in the *Sna*BI site of pAdE4ext (Nazemi-Moghaddam, 2006).

2.2. Cells and viruses

Eight different cell lines were used in the current study. Five cell lines were purchased from ATCC: HEK293A, a human embryonic kidney cell line expressing the E1A gene of adenovirus, (Graham et al., 1977, ATCC, CRL-1573), [The letter ″A″ in HEK293A cells refers to the Adherent form of HEK293 to differentiate it from HEK293S or HEK293SF that are adapted to suspension culture, in the presence or in the absence of FBS.]; A549, a human epithelial lung carcinoma (ATCC, CCL-185); MRC-5, a diploid cell line from human lung fibroblasts (ATCC, CCL-171); Hep G2, a human hepatocyte carcinoma cell line (ATCC, HB-8065) and finally HT-29, a human colorectal adenocarcinoma (ATCC- HTB-38). Three remaining cell lines were developed in our laboratory; The HEK293A-PS-CB6 (Castagner, 2002), and HEK293A-CymR-PS (Elahi et al., 2023) are derived from HEK293A expressing the Adenovirus type

2 PS gene under the control of a heat shock and a cumate-inducible promoter (CMV5-Cuo) respectively. The HeLa-cTA (Nazemi-Moghaddam, 2006) is a clone derived from HeLa cells that stably expresses the cumate transactivator of cTA. The HEK293A, HEK293A-PS-CB6, HEK293A-CymR-PS and A549 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 5% of fetal bovine serum and 1% glutamine. The MRC-5 and Hep G2 were grown in Eagle's minimum essential medium (EMEM, Gibco) and HT-29 was grown in RPMI medium supplemented with 10% of fetal bovine serum and 1% glutamine.

The AdPTG3602 (Oualikene et al., 2000), which is a wild-type adenovirus type-5 without any deletion, AdPS+/E1-/CR5-GFP (named as AdCR5-GFP in the original publication, Mullick et al., 2006) which is a RD-AdV and all the RC-AdV were amplified in HEK293A as previously described (Acsadi et al., 1994; Massie et al., 1995). The AdPS- (wt) (a wild type AdV deleted of the protease gene) (Oualikene et al., 2000) and all the PS deleted AdVs (SC-AdV) were generated in HEK293A-PS-CB6 cells using the same protocol as for HEK293A. The HEK293A-CymR-PS stable cell line was use for comparing the virus growth and TCID50 assay of AdPS- viruses.

2.3. Construction of recombinant adenoviruses

All the recombinant AdVs were generated by homologous recombination in mammalian cells. The AdPS- were generated by co-transfection of *Eco*RI digested viral DNA and *Fse*I digested transfer vectors in HEK293A-PS-CB6 cell line (Acsadi et al., 1994). There is a possibility of homologous recombination between the adenovirus DNA and the transfer vector after the E3 deletion region of the transfer vectors. If this happens, the recombinant virus conserves its E3 region. If the recombinant viruses turned out to be E3-deleted, the HEK293A-PS-CB6 cells were co-transfected with the E3-viral DNA and a 5.6 kb *Hin*dIII fragment of wild type adenovirus that contains the E3 region. The AdPS- was used as backbone for insertion of PS gene in its original position by the infection/transfection method (Elahi et al., 2002). Briefly, HEK293A cells in 60 mm dish were infected at a Multiplicity of infection (MOI) of 0.1 with each AdPS-. Five hours later, to insert the PS gene in its original location, the cells were transfected with 1 μg of a 5.2 kb *Xho*I/*Nde*I fragment or with an 8 kb *Hin*dIII fragment of wild type adenovirus DNA which contains the PS gene in addition to 4 μg of salmon sperm DNA. The virus without transgene was generated by removing the transgene through a recombination event between DNA of a recombinant AdV with deletion of E3 and E4 region (except for ORF6) and an empty AdV transfer plasmid. The deletion positions corresponding to the full-length sequence of Adenovirus type 5 are as follows; E3 region: nucleotides 28, 122 to 30,465; E4 region: nucleotides 32,816 to 35,574; PS gene: nucleotides 21,733 to 22,347. Viruses were isolated after at least three rounds of plaque purification and amplified in HEK293A-PS-CB6 or HEK293A cell lines as described elsewhere (Jani et al., 1997). They were then analyzed by PCR and/or digested by restriction enzymes to determine the presence of transgene in the E4 region.

2.4. Expression of transgene by AdVs

Six-well plates of HEK293A were infected in duplicate with each unpurified seed stock of AdV expressing LG or GFP with MOI of 50, and the cells were harvested at 24, 48 and 72 h post infection (hpi). The luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega). The GFP expression of AdVs with CR5 promoter, was analyzed in HeLa-cTA and HEK293A cells by infecting these cells with an MOI of 50 of each AdVs in duplicate. To shut down the transgene expression in HeLa-cTA, cumate was added to the medium at a concentration of 50 μg per mL. In HEK293A cells, for ON condition the cTA transactivator was supplied by co-infecting the cells with an adenovirus expressing cTA (Mullick et al., 2006) at MOI 10. For OFF condition, mock infected cells (no Ad-cTA) were used. Both conditions (ON and OFF) were without cumate addition. The cells were harvested 24 and 48 h later and analyzed by flow cytometry using a Coulter EPICSTM XL-MCL flow Cytometer (Beckman-Coulter) as described previously (Belkhiri et al., 2002).

2.5. Virus progeny of AdVs

The HEK293A or HEK293-CymR-PS cells were each infected with seed stock of AdVs at a MOI of 5. In the case of HEK293-CymR-PS cells, cumate at concentration 50 μg/mL was added at 5 hpi to initiate the expression of PS by the cells. At 48 hpi the infected cells were harvested and virus titers were measured with $TCID_{50}$ assay using HEK293A or HEK293A-CymR-PS cells (in the presence of 50 μg cumate/mL) in triplicate as described previously (Elahi et al., 2021). Data was analyzed using GraphPad Prism (GraphPad® Software, San Diego, CA). Statistical significance of the difference between groups was calculated by 1-way analysis of variance (ANOVA) with Tukey's multiple comparison test or unpaired *t*-test. Significance is considered as not significant (ns) for $p > 0.05$, and significant as * for p values < 0.05.

2.6. Head-to-head comparison of SC-AdV with RC-AdV and RD-AdV

In a head-to-head comparison, the expression level of GFP by AdPS-/GFP-R (a SC-AdV) was compared with AdPS+/GFP-R (a RC-AdV) and AdPS+/E1-/GFP-L (a RD-AdV) in four human cell lines; A549, MRC-5, Hep G2 and HT-29. The 12 well-plates were seeded at a concentration of 150,000 cells per well. The day after, the wells were infected with MOI of 5 and 50 of each virus in duplicate. The cells were harvested at 24, 48, 72 h and 1 week p.i., and were analyzed by flow cytometry using a BD FACS LSRFortessa™ cell analyzer (BD Biosciences). The fluorescence index ([percentage of cells expressing GFP] × [the mean fluorescence intensity of the GFP positive population]) was calculated to compare the level expression of GFP between each virus in different cell lines during the period of infection.

3. Results

3.1. Generation of adenovirus transfer vectors and recombinants AdVs

Fig. 1 shows the map of genomic organization and nomenclature of AdVs used in the current study. To facilitate the understanding of the genomic organization of AdVs, they were divided into different subtypes. A schematic representation of the expression cassettes at E4 region in different backbones of AdV is presented in Fig. S1 (Supplementary Materials). To generate some of the AdVs, the two transfer vector backbones were used for insertion of two GOI between the E4 region and the RITR of AdV. In one of these transfer vectors, the E4 region was left intact. In the other one, the E4 region was first deleted and ORF6 under control of E4 promoter was subsequently reinserted. A fusion of GFP gene with RLuc gene under the control of a constitutive CMV5 promoter was inserted in orientation toward the RITR in both vectors. In other constructions, the GFP gene was inserted in vectors with intact E4 region under the control of a cumate-inducible promoter "CR5" in orientation toward the right ITR. The method used to generate the of adenovirus transfer vectors is described in supplementary materials.

All the recombinant viruses were generated by *in vivo* homologous recombination between overlapping sequences of linearized transfer vectors or fragments of adenovirus DNA (containing E3 region or PS gene) and full-length digested adenovirus backbone DNA, or following infection with AdV. In the case of AdPS- viruses, depending on the site of recombination, position #1 (before the E3 region) or position #2 (after E3 region), the resulting AdVs are either deleted or positive for E3 (Fig. S2, in supplementary materials).

The AdVs with the PS gene in their natural position were generated by infecting HEK293A cells with different parental AdVs and

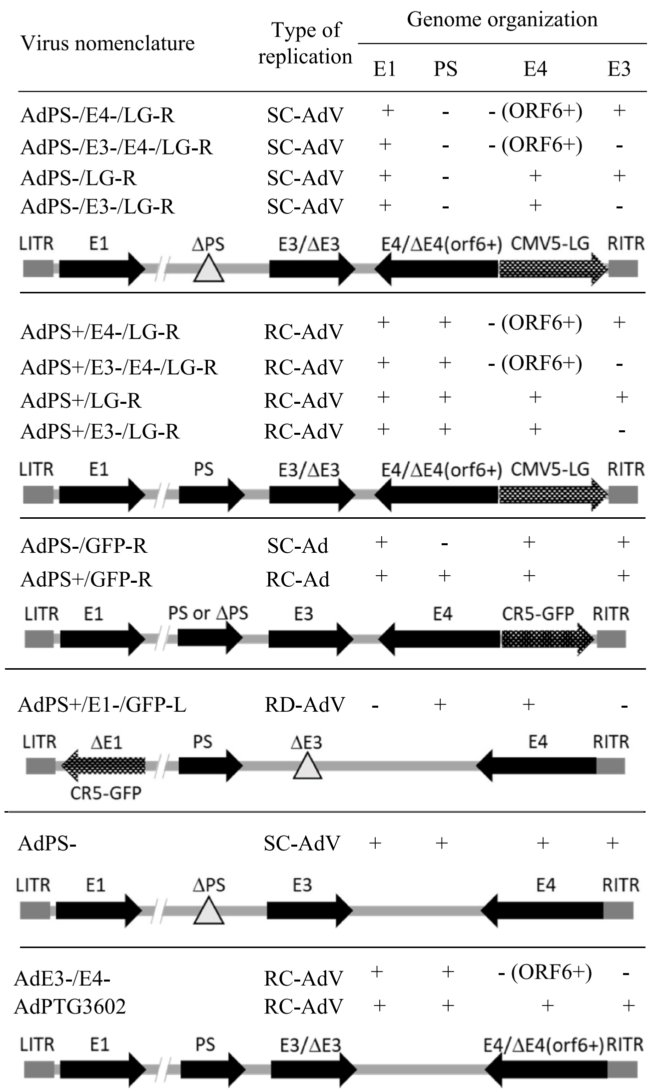

Fig. 1. Genomic organization and nomenclature of AdVs. The presence or absence of E1, E3 and E4 regions, the type of transgene and its orientation are shown for each AdV with a diagram for each group. The AdVs were named on the basis of the presence or absence of PS as follows: AdPS-, PS gene is deleted; AdPS+, PS gene in natural position. AdVs with E3 deletion are shown as E3-and those with E4 deletion, except for ORF6, as E4-. Deletions of E1, E3 and E4 regions are indicated in the AdV nomenclature, otherwise these regions are intact. The abbreviations for transgenes are GFP for Green Fluorescence Protein and LG for the fusion of Renilla Luciferase and GFP. The insert orientation is shown as R (toward the LITR) or L (toward the LITR). The promoters for transgene expression are abbreviated as CMV5 for a modified cytomegalovirus immediate-early promoter or CR5, a cumate inducible promoter. AdPS+/E1-/GFP-L is a RD-AdV in which GFP under control of the CR5 promoter, was inserted in E1 region of adenovirus. AdTG3602 and AdPS- (wt) are the wild type Ad5 with and without PS gene respectively. AdTG3602, AdPS- (wt) and AdPS+/E3-/E4-do not express any GOI.

transfecting the infected cells with a fragment of adenovirus DNA containing the PS gene. Only AdVs which contain PS gene would generate plaques in HEK293A cells after one cycle of freezing and thawing. The reason for producing this set of viruses was to compare the level of expression of the GOI and the virus growth of SC-AdVs with their corresponding RC-AdVs. In summary, thirteen different AdV variants were generated for the current study.

3.2. Analysis of luciferase activity of AdVs with constitutive promoter

The luciferase activity was measured at 24, 48 and 72 hpi in HEK293A infected cells to compare the expression of the GOI between SC-AdV and RC-AdV (Fig. 2). The luciferase expression of SC-AdVs level at 24 hpi was significantly lower compared to the corresponding RC-AdVs. However, in all the SC-AdVs tested, the expression of luciferase reached its maximum level at 48 hpi with no significant difference compared to the luciferase expression in the counterpart RC-AdVs. Insertion of the expression cassettes between RITR and E4 resulted in similar transgene expressions between SC-AdVs and RC-AdVs. Despite the apparent difference in the luciferase expression at 48 hpi between AdPS-/LG-R and AdPS-/E3-/LG-R, the difference is not significant due to the high standard error of the mean between two samples of AdPS-/E3-/LG-R. However, expression of luciferase at 48 hpi was significantly higher for all E4 deleted (except ORF 6) versions than viruses with the intact E4 region. The luciferase expression at 72 hpi in all the samples was reduced due to apparition of CPE and cell death. In an independent experiment, we compared the CPE caused by SC-AdV with RC-AdV. HEK293A cells were infected with MOI of 5 or 50. Cells infected with SC-AdV show the same level of CPE as cells infected with RC-AdV (Fig. S3).

3.3. Analysis of GFP expression of AdVs with inducible promoter

When the GOI is toxic or when it interferes with the replication of AdVs, it is beneficial to utilize an inducible promoter to control the level and the duration of expression of the GOI. Therefore, we evaluated the regulatory capability of the cumate inducible promoter, CR5, in the context of RC-AdV and SC-AdV. HeLa-cTA cells were infected with AdPS-/GFP-R or AdPS+/GFP-R in the absence (ON condition) or presence (OFF condition) of cumate. The mean GFP intensity measured at 24 and 48 hpi by flow cytometry (Fig. 3) was similar between SC-AdV (AdPS-/GFP-R) and its counterpart RC-AdV (AdPS+/GFP-R). Deletion

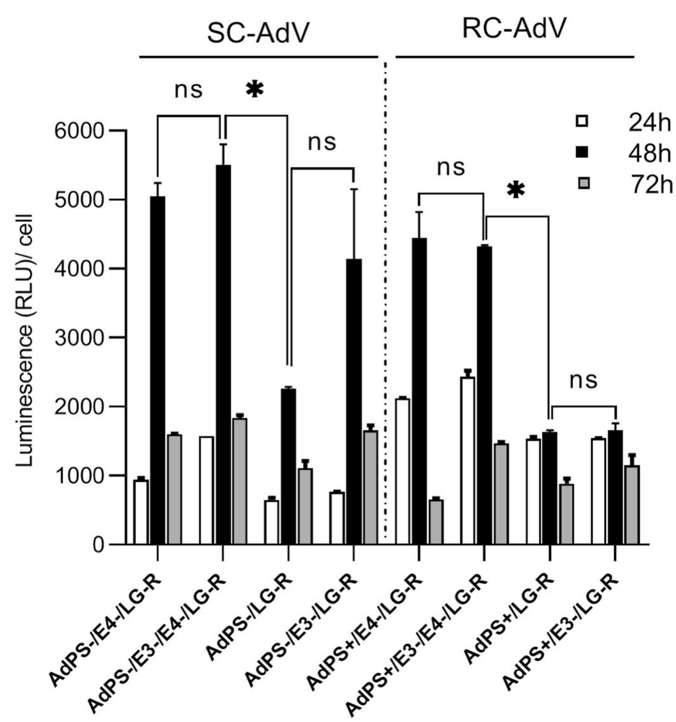

Fig. 2. Luciferase expression of AdVs in infected HEK293A at 24, 48 and 72 hpi. The luciferase activity was measured in duplicate. The data are presented as the mean of Relative Light Units (RLU)/cell ± SEM. Ns: not significant; *: $p < 0.05$.

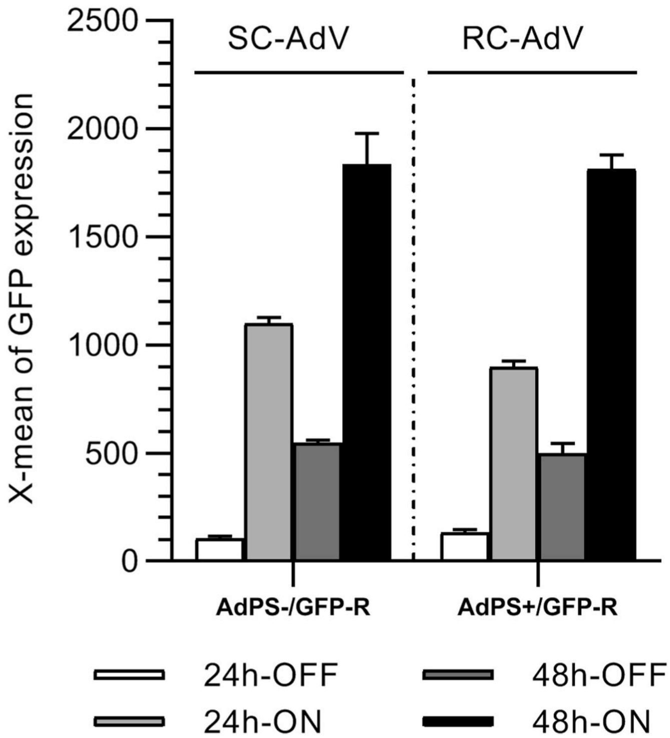

Fig. 3. GFP expression of AdVs regulated by the cumate inducible promoter in HeLa-cTA cells in the presence (OFF) or absence (ON) of cumate. Data shown is the average intensity of GFP in the cell population (X mean) ± SEM. Difference between ON and OFF condition for both viruses at 24 and 48 hpi are significance but difference between the AdPS-/GFP-R and AdPS+/GFP-R at each condition are not significant.

of the PS gene has no impact on the level of GFP expression. We also observed the same results when we evaluated the expression level of GFP in HEK293A cells. In this experiment, for the ON condition, the transactivator (cTA) was supplied by co-infecting HEK293A cells with a recombinant adenovirus expressing cTA (for more details please see Fig. S4; in supplementary material). We also observed that the background level of GFP expression in HeLa-cTA cells, in comparison with cTA-infected HEK293A cells (which do not express the cTA naturally), was higher for both the SC-AdV and RC-AdV viruses. This is because in HeLa-cTA cells, the cTA transactivator is present in both ON and OFF conditions, and adding the cumate (in OFF condition) did not completely prevent the binding of the cTA to CR5 promoter.

3.4. Virus progeny of AdVs in HEK293A and HEK293A-CymR-PS cell lines

The amount of AdVs produced was measured on infected HEK293A or HEK293A-CymR-PS cells for AdPS+ and AdPS- respectively at 48 hpi (Fig. 4). If we compare viruses with the same backbone, the titers of SC-AdVs and RC-AdV were not significantly affected by the insertion of the cassette near the RITR. Hence the difference in titer between AdPS-/LG-R and AdPS- (wt) and between AdPS+/E3-/E4-/LG-R and AdPS+/E3-/E4-were not significant. Among the RC-AdV, the deletion of E3 reduced the titer up to six folds, but only in the E4+ backbone. The viral titer produced by AdPS- (wt) is 4.7 times lower than AdPTG3602 (wild type Ad5 with intact PS gene). The recombinant AdPS-, except for AdPS-/E3-/LG-R, have lower titer (2.8–4 times) compared to their counterpart RC-AdVs. All the recombinant AdPS- produce the same amount of infectious virus particles per cells regardless of their backbone.

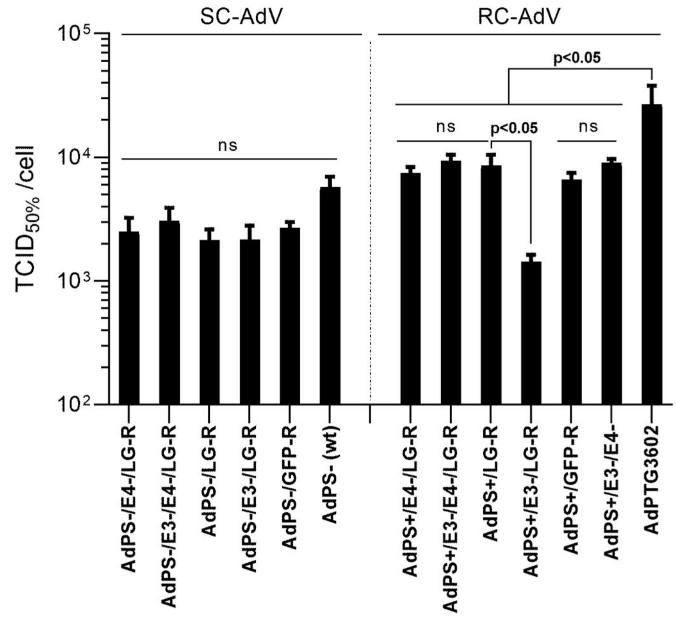

Fig. 4. Amount of AdV produced in HEK293A cells line (for RC-AdV) or HEK293A-CymR-PS cell line (for SC-AdV) at 48 hpi. The titration was performed in triplicate by the TCID$_{50}$ assay using the same cells used for production. Data represents titers obtained and are expressed as mean ± SEM. Ns: not significant; *: $p < 0.05$.

3.5. Head-to-head comparison of SC-AdV with RC-AdV and RD-AdV

In a head-to-head comparison, the expression of GFP by a SC-AdV was compared with its RC-AdV and RD-AdV counterparts in four human cell lines isolated from a normal tissue and three carcinomas. The Fluorescence Index (FI) was measured at 24, 48, 72 hpi and 7-day post-infection (dpi) in A549, MRC-5, Hep G2 and HT-29 cells lines infected with AdPS-/GFP-R, AdPS+/GFP-R and AdPS+/E1-/GFP-L. The FI for AdPS-/GFP-R and AdPS+/GFP-R increased significantly from 24 hpi to 48 hpi in all the cell lines and reached its maximum at 72 hpi except for A549 in which the maximum of FI was reached at 48 hpi in some conditions (Fig. 5). The FI at 7 dpi was reduced significantly in all the cell lines except for HT-29 cells when infected with MOI of 5 (Fig. 5D). At 7 dpi, the FI of HT-29 cells infected with SC-AdV or RC-AdV was significantly higher compared to the three other cell lines infected with the same viruses.

The MRC-5 cell line (a diploid lung fibroblasts cell line derive from a human fetus) was less susceptible to adenovirus infection than A549 cell line (a human epithelial lung carcinoma). At 72 hpi, only 25–35% of MRC-5 cells that were infected with MOI of 50 of AdPS-/GFP-R or AdPS+/GFP-R were GFP positives, compared with 96% and 97% of A549 respectively. Overall, in four cell lines infected with 2 different MOI, only in A549 at MOI of 5, and MRC-5 and HepG2 infected with MOI of 50, the FI obtained with AdPS-/GFP-R was significantly lower than AdPS+/GFP-R. The FI in these three conditions was only 63%, 34% and 17% respectively less than AdPS-/GFP-R. However, when we compared the expression level of GFP between SC-AdV and RD-AdV, an extremely large difference was observed. In the MRC-5, Hep G2 and HT-29 cell lines infected with RD-AdV expression of GFP was barely detectable in both MOI (Fig. 5B–D). Only when A549 cells were infected with an MOI of 50 with RD-AdV, we detected significant amount of GFP compared to the other cell lines (Fig. 5A). The difference in FI between each SC-AdV and its counterpart RD-AdV is indicated in Fig. 5. For the cells infected with MOI of 5, the difference was between 275 and 16,132 times; but it was reduced to 10.6 to 402 times in cells infected with MOI of 50.

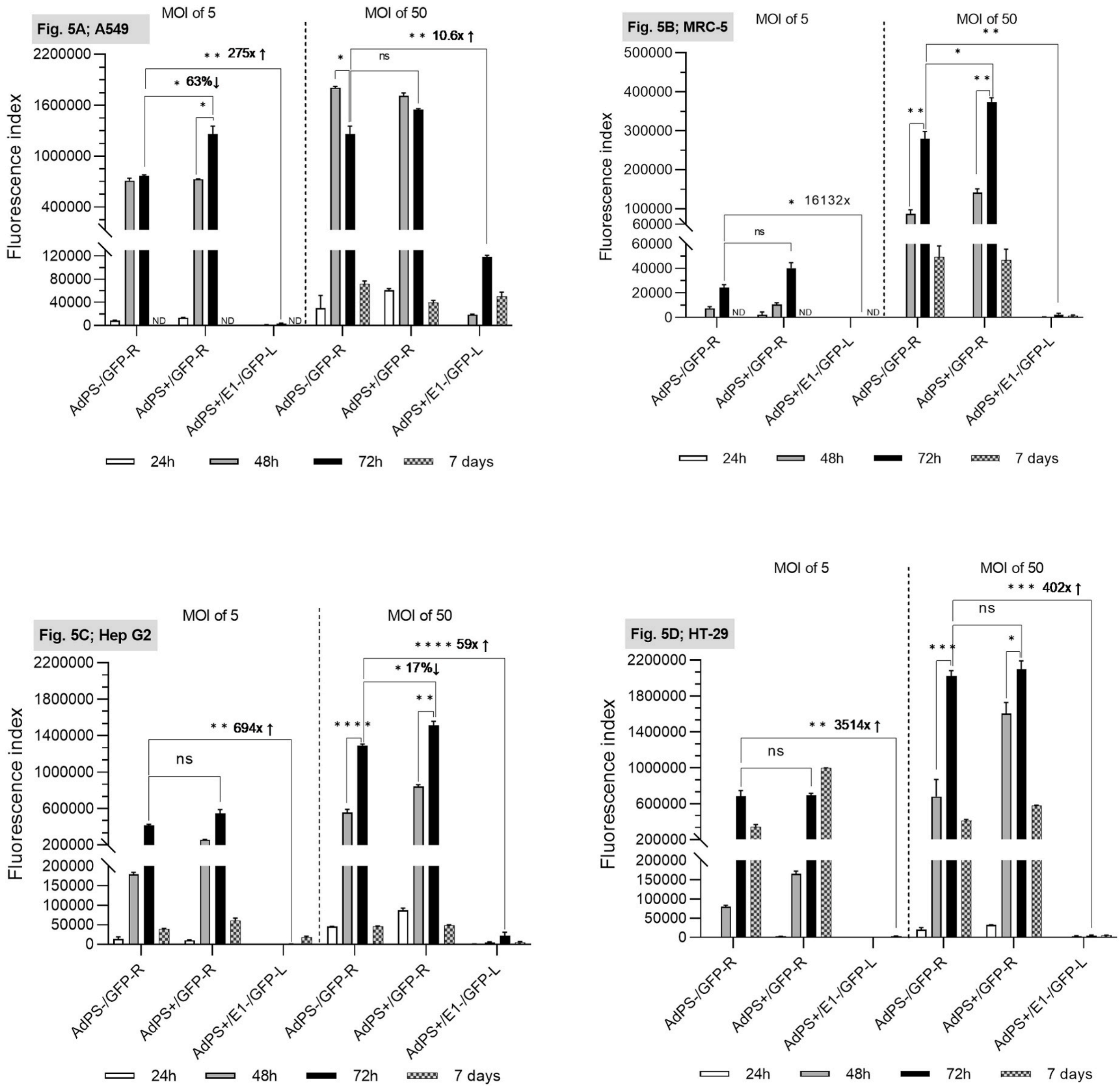

Fig. 5. Head-to-head comparison of SC-AdV with RC-AdV and RD-AdV. In a head-to-head comparison, the expression level of GFP by AdPS-/GFP-R was compared with AdPS+/GFP and AdPS+/E1-/GFP-L in four stable cell lines; A549 (Fig. 5A), MRC-5 (Fig. 5B), Hep G2 (Fig. 5C) and HT-29 (Fig. 5D) up to 1 week after infecting each cell line (n = 2) with MOI of 5 and 50. In the case of A549 and MRC-5 cell lines infected with MOI of 5, the cytometry analysis was not done (ND) at 7 dpi. Fluorescence Index was calculated as the percentage of cells expressing GFP multiplied by the mean of fluorescence value. Data are expressed as mean ± SEM. If the difference of FI between AdPS-/GFP-R and AdPS+/GFP-R is statistically significant, the percentage of reduction is written above the bars. Also, the fold increase of FI between AdPS-/GFP-R and RD-AdV (AdPS+/E1-/GFP-L) is written above the bars. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ and NS -not significant.

4. Discussion

It is well documented that RC-AdV express transgene *in vivo* better and induce a more potent immunity (higher cellular and humoral immune response) than RD-AdV (Peng et al., 2005; Robert-Guroff, 2007). Our collaborators and us have also confirmed this finding when different transgenes were expressed by AdPS- that are replicating but not disseminating AdV (SC-AdV) *in vitro* and *in vivo* (Bourbeau et al., 2007; Girard et al., 2013; Girard et al., 2014; Robert-Guroff, 2007; Roques et al., 2013).

Expression of GOI was evaluated in HEK293A and HeLa-cTA where the GOI expression is controlled by the inducible promoter. We chose the HeLa-cTA because it expresses the cTA transactivator and not the E1 region of adenovirus. The SC-AdVs and RC-AdVs both replicate in these cell lines but only RC-AdV can disseminate. To compare the insertion site and its orientation, several researchers used also one (Suzuki et al., 2015; Thomas et al., 2013) or two (Mizuguchi et al., 2003) cell lines. All the viruses with E4 deleted (except ORF 6) showed higher luciferase expression than viruses with the intact E4 region. The reason for this difference is not clear to us, but it has already been shown that reducing the distance between the adenovirus major late promoter (MLP) and the expression cassette increases the level of expression (Farrera-Sal et al., 2020b). Higher expression of GOI in E4 deleted (except ORF 6) backbones can also be attributed to absence of E4 ORF 3 which has repressive effect on transcription from viral genomes through interaction between E4 ORF 3 and E1A (Soriano et al., 2019).

The absence of PS expression in SC-AdVs, and consequently the lack of virus assembly, did not affect the transgene expression. We have shown previously by Western blot analysis that when infecting A549 cell lines (lacking the E1 gene) at low MOI, AdPS- carrying the ErbB2 gene expression cassette produced the ErB2 protein 27 times more than a first generation AdV (Haq et al., 2019). However, increasing the MOI reduces the difference in GOI expression between SC-AdV and RD-AdV (Haq et al., 2019). It has already been shown that the lack of replication of

RD-AdV can be overcome in cultured cells at high MOI (Armentano et al., 1997). We did not see a difference between SC-AdV and RC-AdV since both of their DNA replicate in A549 contrary to RD-AdV whose virus replication is aborted at early phase.

We also generated two other RC-AdV constructs (data not shown) with GFP expression cassette. In HEK293A cells, one of the constructs which has E3 and E4 deleted (except for ORF6) and with the orientation of GFP toward RITR, GFP background expression level (GFP intensity under the "OFF condition") increases about 3 times compared to AdPS+/GFP-R (the construct with intact E3 and E4 deletion). We hypothesized that reducing the distance between the adenovirus MLP and the expression cassette is responsible for this difference. In another construct where the GFP is orientated toward LITR, the ON/OFF ratio is about 7 time higher than AdPS+/GFP-R (data was no shown). Most likely the opposite orientation of the GFP expression cassette relative to the adenovirus MLP was the reason for this high ON/OFF ratio and lower background expression level. The lack of a SC-AdV version for the virus with GFP orientation toward LITR, prevented us from confirming the gene orientation effect in SC-AdVs. However, as for all the viruses in this study, we did not see a significant difference in transgene expression at 48 hpi between SC-AdV and RC-AdV. We expect to have the same result in the case of SC-AdV with insert orientation toward LITR.

We observed six folds reduction in AdPS+/E3-/LG-R titer compare with his E3+ counterpart. However, no significant different was observed in AdPS- versions. We speculate that the deletion of E4 compensated the lack of E3. It has already been shown in the literature that RD-AdV E3+ vector possesses a growth advantage over its E3- counterpart (Youil et al., 2003).

The lower titer of SC-AdV compare with RC-AdV may have been caused by inadequate expression (amount and/or timing) of PS by the complementary cell line. Our preliminary data showed that premature overexpression of PS is deleterious to viral growth (data not shown). All the recombinant AdPS- produce the same amount of infectious virus particles per cells regardless of their backbone. However, their titers are comparable to many RD-AdV that we routinely produce in our laboratory (data not shown). As the suboptimal expression of E1 gene in HEK293A cells also caused a reduction of 2–3 times of titer of RD-AdV, which are comparable to SC-AdV. Barry (2018) has also observed that SC-AdVs (deleted in capsid pIIIa gene) propagate slower than RC-AdVs in 293-IIIa complementing cell lines.

In the current study, we demonstrated that insertion of a transgene between the E4 region and the RITR is a suitable location for generating SC-AdV. Because the E1, or at least the E1a region, should be present in RC-AdV and SC-AdV to allow replication, the cargo capacity of these viruses is reduced considerably. Additional deletion is needed to accommodate bigger inserts. Deletion of E3 and E4 except for ORF6 increases the cargo capacity in addition of increasing the GOI expression level compared to AdV version with intact E3/E4 regions. However, additional deletion of the AdV might reduce its ability to replicate *in vivo*. For example, Armentano et al. (1995) have shown that a type 2 AdV, which has been modified in the E4 region to contain only ORF6, replicates in cotton rat with a magnitude 3 log lower than wild type adenovirus.

In the case of RC-AdV, we showed that when expression under the control of an inducible promoter is desirable, it is better to have the insert orientation for transcription from the RITR toward the LITR to reduce leaky gene expression of the GOI under the OFF condition. Based on the similarity in transgene expression level that we found between RC-AdV and SC-AdV in all the viruses tested in the current study, we conclude the orientation toward left ITR is the best choice for expression of gene in SC-AdV.

We used HEK293A and HeLa cells for comparing SC-AdV and RC-AdV. However, these cells were transformed by the expression of E1 protein and the Human Papilloma Virus proteins respectively, which could have affected the behavior of the adenovirus. For that reason, in a different set of experiments, we compared the efficacy of SC-AdV and RC-AdV in four different human cell lines. Also, at the same time we took the opportunity to show the superiority of RC-AdV over RD-AdV. We chose AdPS-/GFP-R and AdPS+/GFP-R as the model pair of viruses because a RD-AdV with the same expression cassette inserted into the E1 region (AdPS+/E1-/GFP-L) was available. Two human lung cell lines, one with a normal diploid karyotype (MRC-5) and a cancerous one (A549) were utilized in this study as *in vitro* model. We also included the Hep G2 (a human hepatocyte carcinoma) and HT-29 (a human colorectal adenocarcinoma) cell lines in our study. Between the two lung cell lines, RC-AdV infected better and expressed higher levels of GFP in A549 than MRC-5 cells. In two other cell lines (both carcinoma), GOI was expressed roughly at the same level as A549.

In all the conditions tested, the SC-AdV expressed GFP as the same level or slightly lower (not more than 63%) than RC-AdV. We could not compare long term expression of the GOI because SC-AdV and RC-AdV viruses produced CPE in infected cells. At 7 dpi, the viability of the cells dropped below 44% in all the cell lines infected with SC-AdV and RC-AdV.

The difference between SC-AdV and RD-AdV was remarkable. In cells infected with low MOI (5) the FI of SC-AdV was 275–16,132 times higher than RD-AdV. By increasing the MOI, the difference was reduced to 10.6 to 402 times due to higher copy number of RD-AdV that infected each cell. To refute the hypothesis that lower expression of GFP by AdPS+/E1/GFP-L was due to an unknown mutation in the backbone, AdPS+/E1/GFP-L was compared with AdPS-/GFP-R in HEK293A cell line. Both viruses can replicate their DNA in this cell line. The FI of RD-AdV infected cells was only 35% less than RC-AdV infected ones. This finding exactly aligns with the lower replication rate of RD-AdV in HEK293A compared to RC-AdV (Crosby et al., 2017 and unpublished data) and confirms that the low-level expression of RD-AdV in the four cell lines that do not complement the E1 gene is due to the lack of virus DNA replication in these cell lines in contrast to SC-AdV and RC-AdV which can replicate.

5. Conclusion

Vectorology is a central pillar of cancer and gene therapy as well as for the development of vaccines. The shortage of reagents (media and consumables) for production of AdV especially since the beginning of the COVID-19 pandemic, accentuates this view. Any improvement on viral titer or on the increase of the GOI expression level resulting from vector optimization, permits us to use viral vectors at lower doses and consequently has a significant impact on the viral vector being a successful candidate for gene or cancer therapy or vaccination. The current study is the first to assess vectorology of recombinant AdPS- using different backbones. Contrary to previous publications where only one recombinant SC-AdV was compared with the RD-AdV and/or RC-AdV counterparts in cell culture and *in vivo* (Bourbeau et al., 2007; Girard et al., 2013, 2014; Haq et al., 2019; Young et al., 2018), we have focused our effort on mostly one cell line (HEK293A) and have compared five different backbones of SC-AdV with their RC-AdV counterparts. This strategy allowed us to draw some general conclusions (due to the high number of viruses that we have tested) regarding the best backbone, promoter and orientation for GOI expression. Our data showed that the SC-AdV are as good as their RC-AdV counterparts for expression of GOI in HEK293A and HeLa cell lines. A backbone with E3/E4 deletions (except for ORF 6) with a GOI oriented toward LITR is the best for optimum expression of GOI. To draw a better picture of efficacy of SC-AdV in normal diploid and cancerous cell lines we also chose one SC-AdV and one RC-AdV which express GFP under control of the same promoter and compared them with their RD-AdV counterpart in four different human cell lines. The SC-AdV expresses the GOI to a comparable level as for RC-AdV. However, when we compared SC-AdV with RD-AdV, the SC-AdV expressed GOI remarkably higher than RD-AdV. Additional studies, that are beyond the scope of the current work, will need eventually to be performed, to confirm our results *in vivo*. If the efficacy of

AdPS- with insertion of the GOI in E4 region is confirmed *in vivo*, this type of vector could be employed as substitute for its RC-AdV counterpart. The superiority of SC-AdV over RD-AdV is already undisputable. The SC-AdV could be used at a much lower MOI compared to RD-AdV to obtain the same GOI expression level or at the same MOI for increased expression level. Since no infectious viral particles are assembled in the absence of PS, there is no risk of viral shedding. Thus, AdPS- might contribute to further increase the efficiency and safety of adenovirus making it an excellent candidate for the development of vaccines or for gene and cancer therapy.

Credit author statement

Conceptualization and experimental design: SM Elahi; Performed the experiments: SM Elahi, N Nazemi-Moghadam; Data analysis: SM Elahi, R Gilbert; Writing: SM Elahi, R Gilbert. All authors have read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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