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## Rational and random approaches to adenoviral vector engineering

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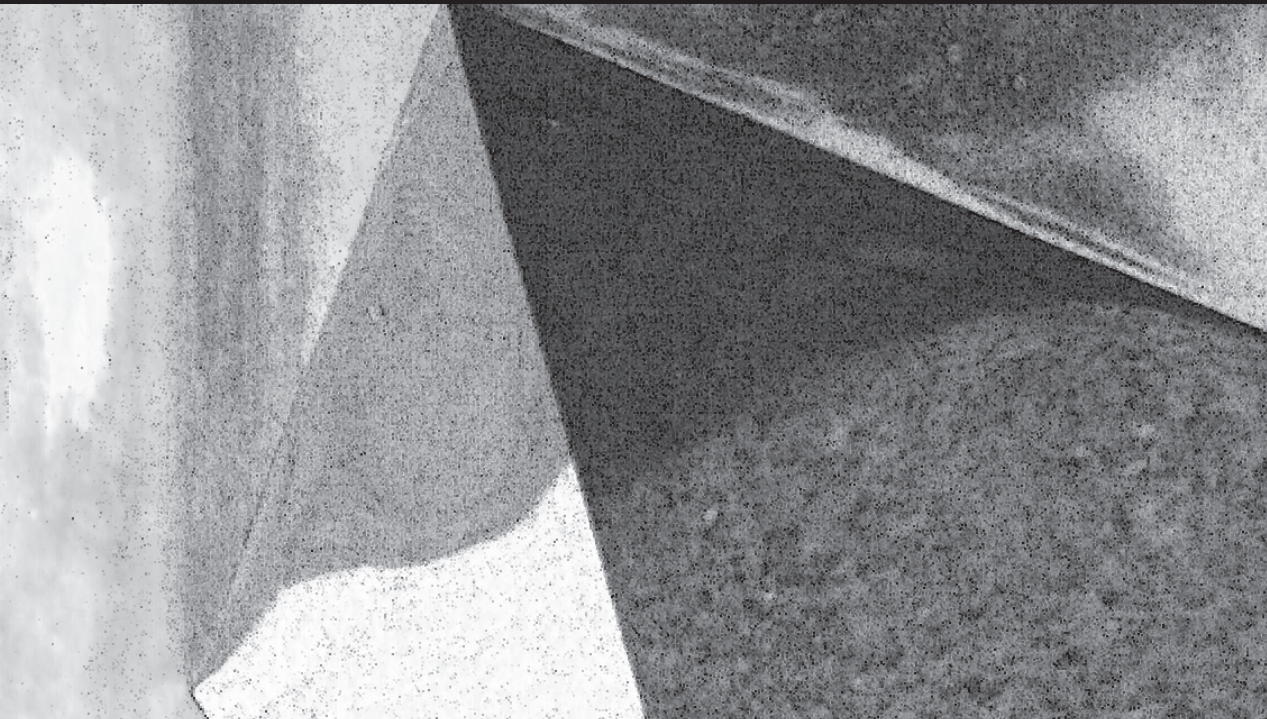
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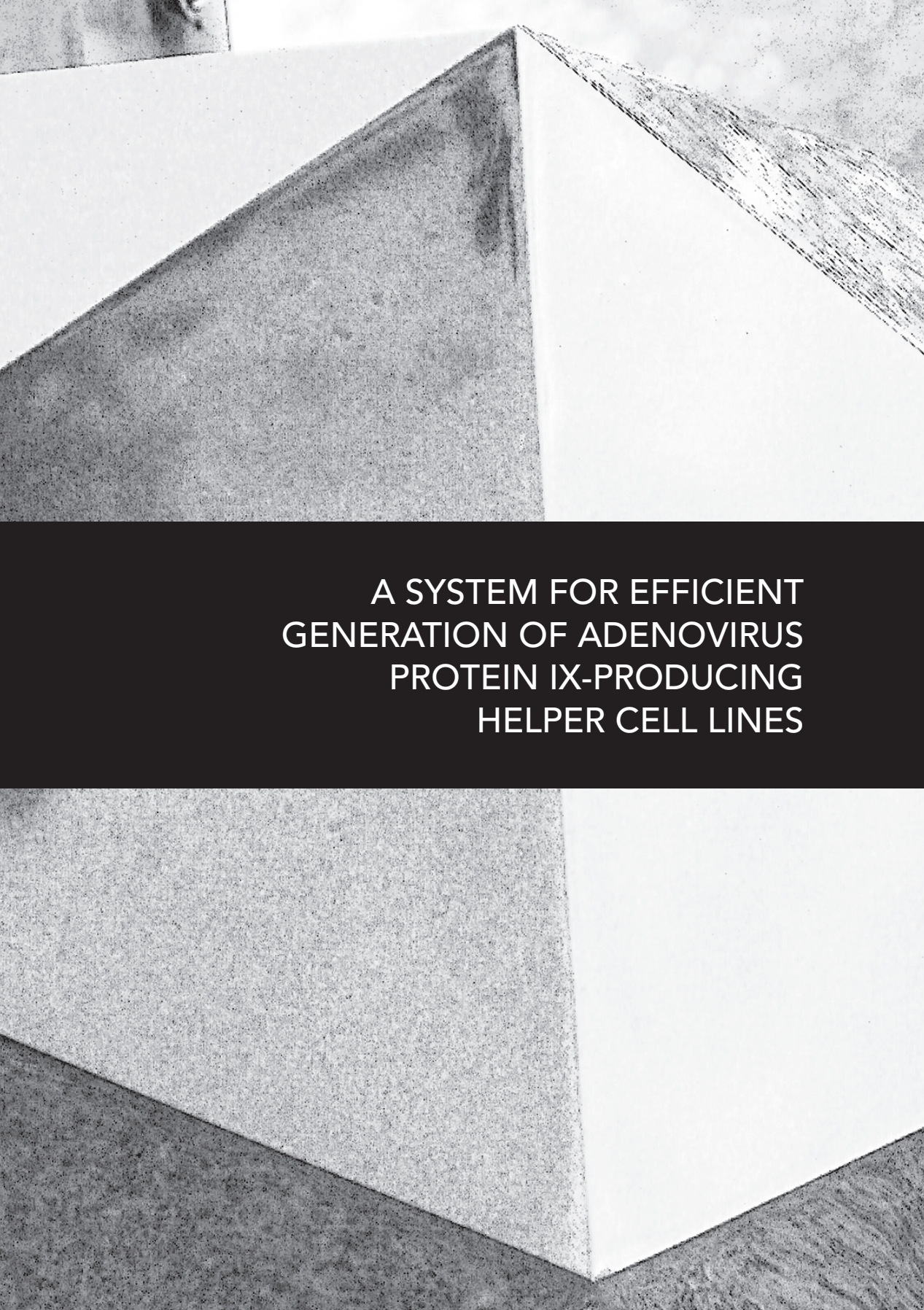
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A SYSTEM FOR EFFICIENT  
GENERATION OF ADENOVIRUS  
PROTEIN IX-PRODUCING  
HELPER CELL LINES

## ABSTRACT

**Background:** The adenovirus 14.3 kDa hexon-associated protein IX (pIX) functions in the viral capsid as 'cement' and assembles the hexons in stable groups-of-nine (GONs). Although viruses lacking pIX do not form GONs, and are less heat-stable than wild-type (wt) viruses, they can be propagated with the same kinetics and yields as the wt viruses. To facilitate 'pseudotyping' of adenoviral vectors we have set up an efficient system for the generation of pIX-producing helper cell lines.

**Methods:** With a lentiviral pIX-expression cassette, monoclonal and polyclonal helper cell lines were generated, which express wt or modified pIX genes at levels equivalent to wt HAdV-5 infected cells. The incorporation efficiency into pIX gene deleted viruses was examined by Western analysis, immuno-affinity electron microscopy, and heat-stability assays.

**Results:** Immuno-affinity electron microscopy on viruses lacking the pIX gene demonstrated that more than 96% of the particles contain pIX protein in their capsids after propagation on the pIX-expressing helper cell lines. In addition, the pIX level in the helper cells was sufficient to generate heat-stable particles. Finally, the ratio between pIX and fiber was equivalent to that found in wt particles. The pIX-producing cell lines are very stable, demonstrating that pIX is not toxic to cells.

**Conclusion:** These data demonstrate that lentivirus vectors can be used for the establishment of pIX-complementing helper cell lines.

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## INTRODUCTION

The human adenoviruses (HAdVs) have been studied extensively as gene transfer vehicles for experimental gene therapy. The modification of the vector tropism to enhance the efficiency and cell-type specificity of gene transfer is an important topic in the adenovirus (Ad)-derived vector design. Successful transgene delivery with HAdV vectors is dependent on efficient transduction of target cells, with minimal transduction of non-target cells (1). One approach to enhance the efficiency and specificity relies on the coupling of targeting devices to the viruses after their production and purification. Such targeting devices could be single-chain antibodies, synthetic ligands, synthetic soluble receptors, etc. (2). Alternatively, the tropism of HAdV receptors can be changed by genetic modification of capsid components. Modification of the major capsid proteins II (hexon) (3–5), III (penton base) (6), and IV (fiber) (7–9) has been exploited for insertion of targeting ligands. More recently, the use of the minor capsid protein IX (pIX) of human and bovine Ad as an anchor for linking targeting ligands has been evaluated (10–13). Protein IX is a 14.3 kDa protein, of which 240 copies are present in the capsid. It arranges the hexons in stable assemblies consisting of groups-of-nine (GONs) (14,15). HAdVs deficient for pIX can be propagated with yields similar to wild-type (wt) Ads; however, such particles are heat-labile and rapidly inactivated at 48 °C (16). Image analyses have positioned pIX at a buried position, approximately 65 Ångstroms lower than the tops of the hexons (17). *In vitro* transduction data and immuno-affinity electron microscopy techniques have demonstrated that the ligand accessibility is augmented by the insertion of an alpha-helical spacer between the carboxyl terminus of pIX and the targeting ligand. The lengths of the inserted spacers positively correlate with the accessibility of the ligands at the outer surface of the viral capsid (18). These data make pIX a promising candidate for the addition of foreign ligands (2).

The modified pIX gene can be inserted into the vector backbone. Alternatively, helper cell lines can be used to provide the pIX *in trans*. The latter approach would lead to particles that do not carry the genetic information for the tropism-modifying capsid modification. Previous data showed that expression of the pIX gene had an adverse effect on cell viability (19). Nevertheless, monoclonal pIX-producing cell lines could be isolated that could restore the pIX deficiency (19–21).

Here we describe a new efficient and robust technique for the generation of stable pIX-producing helper cell lines without the need for clonal selection. The resulting cell populations could effectively complement the pIX deficiency and, as a result, the heat stability of the pIX-gene deleted viruses was fully restored. The availability of a technique for the efficient generation of pIX trans-complementing helper cells will facilitate the use of 'pseudotyping', as opposed to genetic retargeting, as a strategy for modifying the tropism of replication-deficient HAdV vectors.

## MATERIALS AND METHODS

### Cells

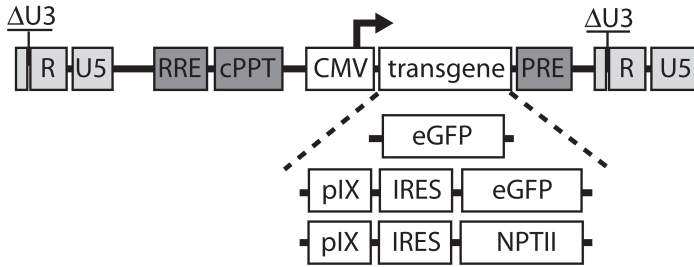
The HAdV-5 E1-transformed cell lines 293 (22), PER.C6 (23), and 911 (23,24) were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS; Gibco-BRL) and 0.3% glucose (J.T. Baker, Deventer, The Netherlands). For PER.C6 cells this medium was supplemented with 10 mM MgCl<sub>2</sub>. The 911 cells were used to propagate and titer adenovirus vectors. Infections of the cells with HAdVs were carried out in infection medium containing 2% FBS. 293T cells were transfected with the calcium phosphate co-precipitation technique (25). 911 cells were transfected with the calcium phosphate co-precipitation technique using pEGFPc1 (Clontech, BD Biosciences, The Netherlands).

### Production of recombinant lentiviruses

The lentiviral vectors used in this study are so-called SIN vectors (26), and contain the Rev-responsive element sequence (27), the central polypurine tract (cPPT) (28–30), and the human hepatitis B virus-derived post-transcriptional regulatory element. All lentivirus vectors were derived from the plasmid pRRL-cPPT-CMV-eGFP- PREsense-SIN (31), here named pLV-CMV-eGFP. Plasmids pLV-CMV-IRES-eGFP, pLV-CMV-pIX-IRES-eGFP, pLV-CMV-pIX-IRES-NPTII, pLV-CMV-pIX.MYC-IRES-NPTII, and pLV-CMV-pIX.flag.75.MYC-IRES-NPTII were constructed by standard cloning procedures. The encephalomyocarditis virus internal ribosomal entry site (IRES) was obtained from pTM3 (32) and the NPTII-coding region was isolated from pEGFP-N2 (Clontech, BD Biosciences, The Netherlands). The genes for pIX, pIX.MYC, and pIX.flag.75.MYC were obtained from the pCDNA3.1-based constructs pAd5pIX, pAd5pIX.MYC, and pAd5pIX.MYC.flag.75.MYC, respectively (18). A schematic outline of these vectors is provided in Figure 1.

The lentiviral vectors were produced as described previously (33). Briefly, the three 'helper' plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope were co-transfected together with the pLV construct into 293T cells. After overnight incubation, the cells were washed and cultured in fresh medium. The medium was harvested 48 and 72 h post-transfection, passed through a 0.45- $\mu$ m filter, and stored at -80 °C until use. The viruses were quantified by antigen-capture enzyme-linked immunosorbent assay (ELISA) measuring HIV p24 amounts (ZeptoMetrix Corp., New York, NY, USA) as described previously (34). Approximately 1 ng p24 is equal to  $2 \times 10^3$  transducing units (30).





**Figure 1. Schematic representation of the lentiviral vectors.** The vectors are derived from a self-inactivating, third-generation HIV-1-derived vector. Upon integration, the vector loses its capacity to produce RNA other than the mRNA from the transgene-expression cassette. The positions of the Rev-responsive element (RRE), the central polypurine tract (cPPT), and the posttranscriptional regulatory element (PRE) are indicated. The pIX transgenes were inserted upstream of the internal ribosome entry site (IRES) and the neomycin phosphotransferase (NPTII) selection marker or the enhanced green fluorescent protein (eGFP).

## Lentiviral transduction

For transduction the lentiviral supernatant was added to fresh medium supplemented with 8  $\mu\text{g/ml}$  Polybrene (Sigma Aldrich, Zwijndrecht, The Netherlands). After overnight incubation the medium was replaced with fresh medium. Cells transduced with lentiviral vectors containing the neomycin selection gene were cultured in medium supplemented with 200  $\mu\text{g/ml}$  G418 (Invitrogen, Breda, The Netherlands).

## Adenovirus vectors

The pTrackCMV-GFP/LUC is an E1-deleted HAdV-5-based shuttle plasmid that carries a green fluorescent protein (GFP) and a firefly luciferase (LUC) transgene, each under the control of the human cytomegalovirus (CMV) immediate-early promoter (35). The  $\Delta\text{pIX}$  pTrack CMV.GFP/LUC plasmid was constructed as described previously for  $\Delta\text{pIX}$  pTrackCMV-GFP (18). Replication-incompetent HAdV-5 vectors HAdV-5CMV.GFP/LUC and HAdV-5 $\Delta\text{pIX}$ .CMV.GFP/LUC were generated with the pAdEasy-1 system as described elsewhere (35). Wild-type (wt) HAdV-5 and HAdV-5-*d1313* were obtained from the virus collection of the Department of Molecular Cell Biology of the Leiden University Medical Center. The HAdV-5-*d1313* virus lacks 2307 bp of the E1 region including the 5' portion of the pIX gene. This mutant does not express detectable amounts of pIX (16). The infectious titer of the adenovirus vectors was determined by plaque assay (24). For heat-stability assays, 150- $\mu\text{l}$  aliquots were incubated at 45  $^{\circ}\text{C}$  for 0, 4, 6, 8, and 10 min and then quickly cooled on ice for 5 min. U2OS cells were infected with 100  $\mu\text{l}$  virus suspension from each aliquot. After 24 h viral titers were determined by measuring the luciferase activity, as described previously (36).

## Western analysis

Cell lysates were made in RIPA lysis buffer (50 mM Tris.Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC and 1% NP40). Protein concentrations were measured with the BCA protein assay (Pierce, Perbio Science B.V., Etten-Leur, The Netherlands). Virus lysates were prepared by adding  $5 \times 10^9$  virus particles [measured by a standard OD260 protocol (37)] directly to Western sample buffer. The Western blotting and detection procedures have been previously described (18). For detection of fiber the 4D2 antibody was used (1 : 2000; Abcam, Cambridge, UK).

## FACS analysis

For fluorescence-activated cell sorting (FACS) analyses the cells were trypsinized and suspended in phosphate-buffered saline supplemented with 1% FBS, and subsequently kept on ice. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson), as described (18). Data analysis was performed using CellQuest 3.1 software (Becton Dickinson). For each sample, 10 000 events were collected.

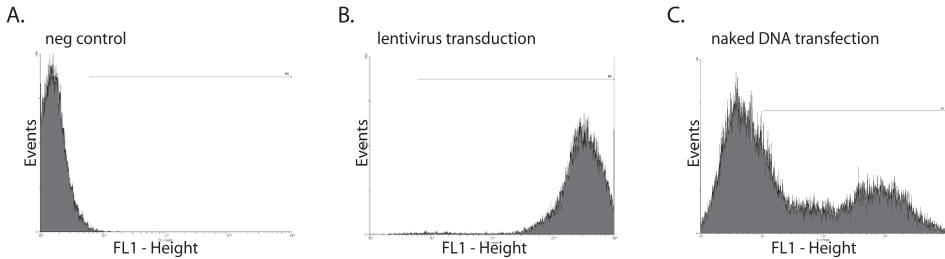
## Immuno-affinity electron microscopy

The presence of the modified pIX molecules in the viral capsids was visualized with antisera and gold-labeled protein-A, as described (18). Subsequently, these samples were fixed in 1.5% glutaraldehyde in cacodylate buffer and negatively stained with 1% uranyl acetate for 15 min. The viruses were examined with a Philips EM 410-LS transmission electron microscope at 80 kV.

## RESULTS

It has been shown previously that pIX is an attractive candidate for use as anchor for polypeptide ligands (10–12,18,38–40). Since pIX is dispensable in the capsid, generation of helper cell lines that synthesize pIX may allow 'pseudotyping' of HAdV vectors. Therefore, we designed and evaluated a lentivirus-based system for the generation of new pIX-complementing cell lines. To test the efficiency of a lentivirus-based system for generating E1- and pIX-complementing helper cell lines, 911 cells were transduced with LV-CMV-eGFP at 40 ng p24 per  $10^5$  cells. After 48 h the transduced cells were harvested and the number of eGFP-positive cells was determined by FACS analysis (Figure 2). The 911 cell line was efficiently transduced. Similar results were obtained when using the E1-complementing helper cell lines 293 and PER.C6 (data not shown). Note that the expression of the transgene is generally higher and more homogenous than in cells transfected with the calcium phosphate co-precipitation technique (Figure 2).



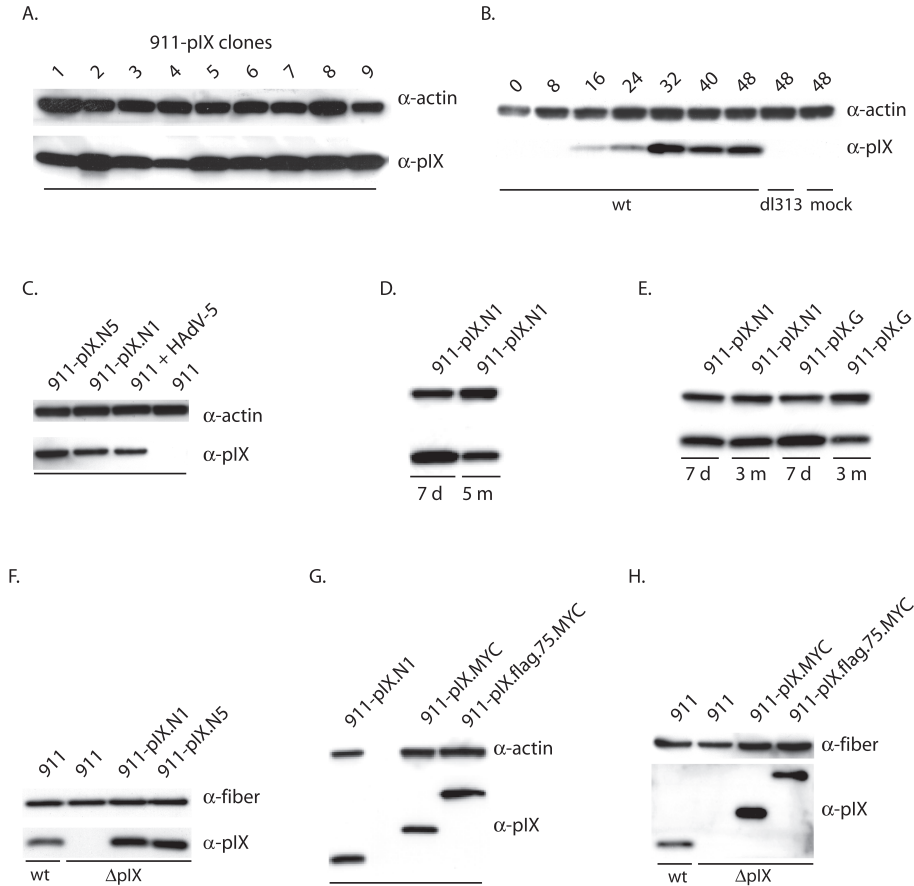


**Figure 2. Transduction-efficiency assay.** GFP-positive cells were visualized by FACS analysis. (A) Negative control. (B) The helper cell line 911 transduced with LV-CMV-eGFP at 40 ng p24 per  $10^5$  cells. (C) 911 cells transduced with the calcium phosphate co-precipitation technique using 10  $\mu$ g pEGFP-C1 per semi-confluent 9-cm dish.

To examine the pIX production after lentivirus-mediated pIX-gene transfer, 911 cells were exposed to LV-CMV-pIX-IRES-NPTII at 40 ng p24 per  $10^5$  cells, and nine monoclonal G418-resistant cell lines were isolated after limiting dilution. Similar pIX amounts are detected in all 911-pIX cell lines (Figure 3A), which is consistent with the homogenous pIX signals observed by immunohistochemistry (data not shown). Therefore, in all subsequent experiments, polyclonal cultures of G418-resistant lentivirus-transduced cells were used.

Next, pIX amounts were assessed during wt HAdV-5 replication in 911 cells. For this, 911 cells were infected with wt HAdV-5 at multiplicity of infection (MOI) of 5. HAdV-5-*dI313*, which lacks a functional pIX gene, was used as a control. At various time points the cells were collected in lysis buffer and the pIX amounts were determined by Western analysis (Figure 3B). The pIX amount peaked at 32 h post-infection. The peak amounts of pIX after HAdV-5 infection were compared with the amounts of pIX in LV-CMV-pIX-IRES-NPTII-transduced polyclonal cell populations established after transduction of 911 cells with LV-CMV-pIX-IRES-NPTII at 40 ng (N1) and 200 ng (N5) p24 per  $10^5$  cells. After 7 days in medium containing 200  $\mu$ g/ml G418, cells were harvested and protein samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Equivalent amounts of pIX were detected in the LV-CMV-pIX-IRES-NPTII-transduced cells and in HAdV-5-infected cells at 32 h post-infection. In the 911-pIX.N5 cells more pIX was present than in 911-pIX.N1 cells (Figure 3C). This probably reflects a higher copy number of integrated pIX vector in the 911-pIX.N5 cells. We noted such a strict copy-number dependency of reporter gene expression previously in differentiated adipocytes and 293T cells (33).

To test the stability of these cell lines, the cells were passaged for 5 months on G418 selection. Only a moderate reduction in pIX amounts occurred over the course of this period, demonstrating that these cell lines are reasonably



**Figure 3. Western blot analyses.** (A) Comparison of pIX amounts of the monoclonal 911 cell lines after lentivirus-mediated transfer of the pIX gene. The cell lines were generated after transduction of 911 cells with LV-CMV-pIX-IRES-NPTII. (B) Protein IX amounts during wt HAdV-5 and HAdV-5-*dl313* infection. 911 cells were infected with HAdV-5 (MOI = 5). Cell lysates were prepared at 0, 8, 16, 24, 32, 40 and 48 h post-infection and a Western analysis was performed using anti-pIX serum. (C) pIX amounts of the 911-pIX.N1 and 911-pIX.N5 cell lines. Equal amounts of lysates of the 911-pIX.N1, 911-pIX.N5, and as control 911 cells infected with HAdV-5 at 32 h post-infection, were assayed by Western analysis. (D) pIX amounts of the 911-pIX.N1 cell line 7 days (7 d) and 5 months (5 m) post-transduction. (E) pIX amounts of the 911-pIX.G cells and 911-pIX.N1 cells 7 days (7 d) and 3 months (3 m) post-transduction. (F) Complementation efficiency of the 911-pIX.N1 and 911-pIX.N5 helper cell lines. To test the incorporation efficiency of pIX produced by the 911-pIX.N1 and 911-pIX.N5 cells, HAdV-5ΔpIX.CMV.GFP/LUC was propagated on the cell lines and purified by CsCl centrifugation, after which protein lysates of the purified viruses samples were made for Western analysis. The amounts of pIX in HAdV-5ΔpIX.CMV.GFP/LUC propagated on 911-pIX.N1 and 911-pIX.N5 cell lines were compared with wt HAdV-5, with anti-pIX serum and, as a virus-particle loading control, the 4D2 antibody directed against the fiber protein. (G) pIX.MYC and pIX.flag.75.MYC amounts in the complementing cell lines 911-pIX.MYC and 911-pIX.flag.75.MYC were compared with the pIX amounts in the 911-pIX.N1 cell line. Equal amounts of cell lysate were assayed as described above. (H) The incorporation efficiency of the pIX.MYC and pIX.flag.75.MYC variants produced by the helper. The HAdV-5ΔpIX.CMV.GFP/LUC vector was propagated and purified as described above and pIX amounts were compared with the amount of pIX in wt HAdV-5 particles.

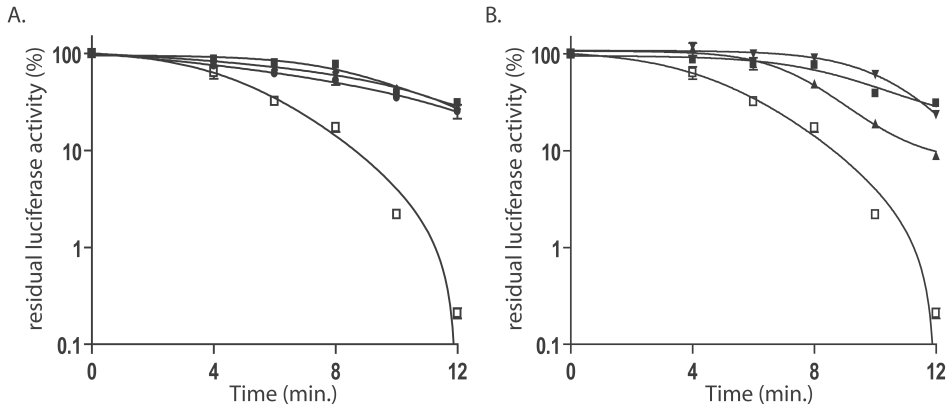


stable (Figure 3D). To test the stability of pIX-producing cell lines without selection, 911 cells were transduced with LV-CMV-pIX-IRES-eGFP at 40 ng p24 per  $10^5$  cells (911-pIX.G). The pIX amounts in the unselected 911-pIX.G cells were compared with that in 911-pIX.N1 cells grown in parallel in medium containing G418 (Figure 3E). Only a slight reduction in pIX amounts was detected after 3 months without selection in the 911-pIX.G cells, while there was no decrease in pIX amounts in the 911-pIX.N1 cells. This demonstrates that pIX is not toxic for 911 cells. For further experiments we used cells transduced with the lentiviral vectors harboring the NPTII selection gene.

Next, we examined the incorporation of pIX into the capsid of the HAdV-5 vector HAdV-5 $\Delta$ pIX.CMV.GFP/LUC. This vector lacks a functional pIX gene and carries the eGFP and the firefly luciferase reporter genes under control of two separate CMV promoters. HAdV-5 $\Delta$ pIX.CMV.GFP/LUC viruses was propagated on the 911-pIX.N1 and 911-pIX.N5 cell lines, and harvested and purified via the conventional CsCl purification method. During purification, the particle-associated pIX is separated from the non-associated pIX since variants of pIX that cannot be incorporated into the capsid do not co-purify with the particles of CsCl gradients (18). To evaluate the amount of pIX in the particles,  $5 \times 10^9$  CsCl-gradient purified particles from each virus preparation were analyzed by Western blot analysis (Figure 3F). The amounts of pIX in the pIX-loaded HAdV-5 $\Delta$ pIX.CMV.GFP/LUC particles is similar to, or maybe even slightly higher than, the amounts in wt HAdV-5 particles, while the fiber contents are identical. The yield of the HAdV-5 $\Delta$ pIX.CMV.GFP/LUC vector in the pIX-complementing cells was similar to the yield routinely obtained on 911 cells (data not shown).

To further characterize the pIX loading in HAdV-5 $\Delta$ pIX.CMV.GFP/LUC particles that were propagated on the pIX-complementing cells 911-pIX.N1 and 911-pIX.N5, we tested these viruses for their heat-stability. The HAdV-5 $\Delta$ pIX.CMV.GFP/LUC rapidly decreased in titer in response to incubation at 45 °C, whereas the vector containing a wt pIX gene (HAdV-5.CMV.GFP/LUC) was thermostable (Figure 4A) (16). The HAdV-5 $\Delta$ pIX.CMV.GFP/LUC particles that were loaded with pIX via propagation on complementing cell lines 911-pIX.N1 and 911-pIX.N5 were as thermostable as HAdV-5.CMV.GFP/LUC.

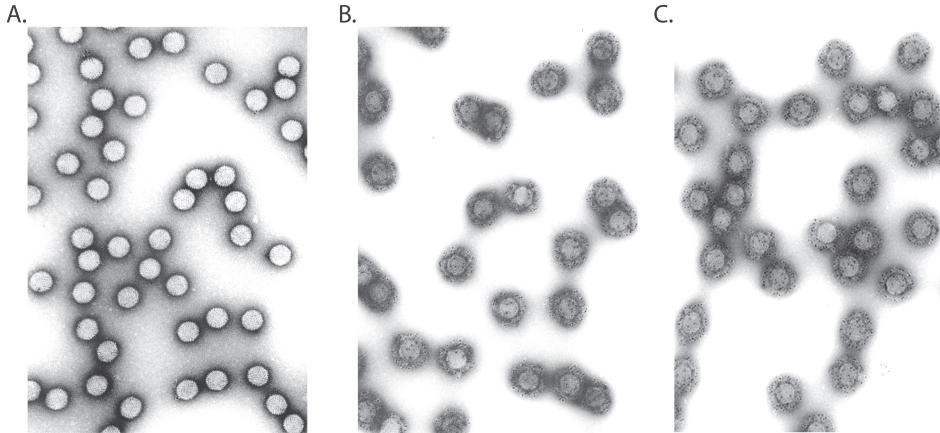
To verify that the majority of particles carried pIX upon propagation on the pIX-complementing cell lines, we used the two MYC-tagged pIX variants pIX.MYC and pIX.flag.75.MYC (18). New 911 cell lines were generated via transduction with LV-CMV-pIX.MYC-IRES-NPTII and LV-CMV-pIX.flag.75.MYC-IRES-NPTII, resulting in the 911-pIX.MYC and 911-pIX.flag.75.MYC helper cell lines, respectively. To test the expression level in the newly produced cell lines, samples were taken and used for Western analysis (Figure 3G). The amounts of pIX.MYC and pIX.flag.75.MYC are similar to the pIX amount in the 911-pIX.N1 cell line. After propagation of HAdV-5 $\Delta$ pIX.CMV.GFP/LUC



**Figure 4. Virus-stability assay.** Thermostability of pIX gene-deleted HAdV vectors carrying pIX molecules produced by the trans-complementing helper cells and, as control, pIX gene-containing HAdV vectors. Infectivity of HAdV-5ΔpIX.CMV.GFP/LUC particles produced on pIX trans-complementing helper cells, and on 911 cells, as determined by their capacity to transfer the luciferase reporter gene to indicator cells. The results are presented as the percentage residual luciferase activity. Each bar represents the mean  $\pm$  standard deviation (SD) of triplicate analyses. **(A)** Comparison of HAdV-5ΔpIX.CMV.GFP/LUC viruses propagated on the 911-pIX.N1 (●) and 911-pIX.N5 (◆) with HAdV-5.CMV.GFP/LUC (■) and HAdV-5ΔpIX.CMV.GFP/LUC (□) produced on 91 cells. **(B)** HAdV-5\_pIX.CMV.GFP/LUC viruses produced on 911-pIX.MYC (▼) and 911-pIX.flag.75.MYC (▲) cells, compared with HAdV-5.CMV.GFP/LUC (■) and HAdV-5ΔpIX.CMV.GFP/LUC (□) produced on 91 cells. The data from **(A)** and **(B)** are derived from the same experiment.

on the 911-pIX.MYC and 911-pIX.flag.75.MYC cell lines, the viruses were harvested and purified by CsCl banding. The MYC-tagged proteins efficiently incorporated (Figure 3H). The presence of the MYC-tagged pIX in the HAdV-5ΔpIX.CMV.GFP/LUC particles was visualized by immuno-affinity electron microscopy, with gold-labeled anti-MYC antibodies, as described previously (18). Of over 1000 particles counted, more than 96% of the particles bound the gold-labeled antibodies (Figure 5). There was no gold label present on HAdV-5ΔpIX.CMV.GFP/LUC lacking the MYC tag.

Subsequently, we examined the heat stability of the adenoviruses containing the MYC-tagged pIX molecules (Figure 4B). The HAdV-5ΔpIX.CMV.GFP/LUC loaded with pIX.MYC was as stable as HAdV-5.CMV.GFP/LUC. Whereas the virus loaded with pIX.flag.75.MYC was significantly stabilized compared to the pIX-deficient HAdV-5ΔpIX.CMV.GFP/LUC particles, it was less stable than the particles loaded with either pIX or pIX-MYC.



**Figure 5. Immuno-affinity electron microscopic analysis of HA $\Delta$ V-5 $\Delta$ pIX.CMV.GFP/LUC particles loaded with pIX.MYC and pIX.flag.75.MYC.** The fraction of particles loaded with the MYC-tagged pIX was assessed by detection of the MYC tag on the virions visualized by the presence of gold particles. There is no MYC tag detected on the HA $\Delta$ V-5 $\Delta$ pIX.CMV.GFP/LUC particles (A), whereas at least 96% of the virions are loaded with pIX.MYC (B) and pIX.flag.75.MYC (C).

## DISCUSSION

Here we describe the use of lentiviral vectors for the generation of pIX-complementing helper cell lines. Lentiviruses are a versatile and efficient tool to transfer transgenes (33,41–44). We have used a third-generation nonreplicating HIV-1-derived vector. The VSV-G-pseudotyped lentiviral vectors can transduce a wide variety of cell types of different species (45). Also the conventional helper cells for adenovirus production can be efficiently transduced. The transduction of cells with lentiviral vectors results in modest changes in gene expression and cellular transcription (46). The propagation of HA $\Delta$ V-5 vectors on the transduced helper cells 911-pIX.N1 and 911-pIX.N5 was not impaired.

Previously, we evaluated the use of modification of pIX for retargeting of HA $\Delta$ V vectors (18). This system was based on transient expression of modified pIX genes in helper cells. The use of lentiviral vectors circumvents the need to sub-clone the stably modified clones after transduction. After lentiviral-vector-mediated pIX gene transfer, the cells in a polyclonal cell population have homogenous pIX amounts as evident from immunohistochemistry and Western analyses.

HA $\Delta$ V-5 lacking the pIX gene can be efficiently propagated on the pIX-producing helper cell lines 911-pIX.N1 and 911-pIX.N5. Intriguingly, the amounts of pIX in the particles seem even slightly higher than in wt HA $\Delta$ V-5 particles. Contamination of the purified virus with non-virus-associated

pIX protein can be excluded: a variant-pIX protein with a deletion in the N-terminus is not present in the purified virus preparation (18). There are two possible alternative explanations. Firstly, if the pIX-gene-deleted virions have lost their pentons (47), we could have loaded more particles, without increasing the fiber signal on the blots. Alternatively, the pIX-gene expression may be deregulated if a wt HAdV-5 virus is grown on helper cells. This could be the result of deregulation of the transcription of the E1B gene of the virus in the helper cells, and E1B transcription affects pIX-gene expression (48). However, the heat-inactivation data, the electron microscopy data, and our particle quantization data do not support these explanations.

By propagation on the transduced cell line populations the heat-stable phenotype could be fully restored. Previous studies have demonstrated that pIX can be incorporated into the HAdV capsid upon heterologous expression in helper cell lines, after transient expression (18), and after generating stable cell lines using EBV replicons and plasmid vectors (20). In the latter studies selected stable cell lines could restore the heat-stable phenotype. Moreover, pIX-deficient HAdV vectors with a genome size larger than wt HAdV-5 could be efficiently packaged on the pIX-producing cell lines, but not on the parental, non-modified helper cells (20). However, the selection and characterization of monoclonal cell lines is a laborious and time-consuming process, making the efficient lentivirus-based system described here a valuable extension of this technology.

To further exploit the possibility to examine pIX-fusion proteins via this system we tested two MYC-tagged pIX variants, i.e. pIX.MYC and pIX.flag.75. MYC, which can be efficiently incorporated into the virus capsid (18). Here we showed that the two MYC-tagged pIX variants incorporate with efficiencies equivalent to that of wt HAdV-5. However, the variant with the flag.75.MYC extension exhibited moderately reduced heat stability. This suggests that the flag.75.MYC extension has an effect on the capsid-stabilizing function of pIX. This is unlikely to be due to inhibition of the pIX-trimer formation, since recent data demonstrated that trimer formation is required neither for pIX capsid incorporation, nor for the heat-stable phenotype (49). The addition of relatively large ligands has previously been shown to influence the capsid stability (38), suggesting that steric hindrance may hinder bona fide capsid stabilization.

In conclusion, the use of lentiviral vectors for the generation of pIX-producing helper cell lines may allow development of systems for 'pseudotyping', rather than genetic retargeting, of HAdV-vectors. By pseudotyping, HAdV vectors are retargeted to a specific cell type or tissue by propagating them on the appropriate pIX-complementing helper cell line. So far few studies have been focused on HAdV pseudotyping. Stable cell lines expressing the fiber gene have been described but it has proven difficult to achieve expression levels sufficiently high to fully load all particles (50,51). The availability of a robust



system for generating pIX-producing cell lines, together with the observation that trimer formation of pIX is not required for its incorporation into the capsid, makes modification of pIX a feasible approach for developing a system for development and generation of batches of 'pseudotyped' HAdV vectors.

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