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A Human Papillomavirus (HPV) - based pseudoviral gene delivery system for the non-viral, Episomally Replicating Vector pEPI-1

Research Article

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Abbreviations: Bovine Papillomavirus, (BPV); Episomally Replicating Vector, (pEPI-1); Epstein-Barr Virus, (EBV); Human Papillomavirus, (HPV); pEPI-pseudoviruses, (pEPI-PV); Pseudoviral particles harbouring pEPI-1, (pEPI-PV); scaffold/matrix attachment region, (S/MAR); Simian Virus 40, (SV40)

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Summary

Non-viral episomal vector systems might allow a safe and reproducible genetic modification of eukaryotic cells and organisms. However, they share with all non-viral systems the problem of low efficient delivery to the target cells. To overcome this limitation we have utilized a Human Papillomavirus type 16 (HPV16)-based pseudoviral gene delivery system for the non-viral episomally replicating plasmid vector pEPI-1. Pseudoviral particles harbouring pEPI-1 (pEPI-PV) were prepared by co-transfection of the pEPI-1 plasmid DNA and a plasmid expressing the capsid proteins L1 and L2 of HPV16. Here we demonstrate that the resulting pEPI-PV were able to transduce target CHO cells with high efficiency under maintenance of the episomal character of pEPI-1. The combination of HPV-based pseudoviral gene delivery and non-viral, episomally replicating plasmids will provide a powerful new tool for biotechnical and possibly *in vivo* biomedical applications.

I. Introduction

In principle, non-viral, episomally replicating vectors could be considered most useful for the safe and reproducible genetic modification of mammalian cells and organisms and for *in vivo* gene therapy. These constructs are free from the problems related with currently used viral vector systems which include insertional mutagenesis, stimulation of the host immune system, or only transient expression of the transgene (Lipps et al, 2003; Glover et al, 2005). The prototype of such an episomal vector was recently constructed in our lab. This vector, pEPI-1 (**Figure 1A**), is 6700 bp in size, replicates at a copy number of around 5-10 molecules per stably transfected cell, does not integrate into the host cell genome, but remains episomally stable even in the absence of selection (Piechaczek et al, 1999; Baiker et al, 2000; Jenke et al, 2002). Recent work demonstrated that an upstream transcription unit directed into the scaffold/matrix attachment region (S/MAR) is necessary for its function (Stehle et al, 2003). These unique characteristics result in a very homogeneous protein expression pattern in transfected cells (Jenke et al, 2004a) making it a valuable tool for the reproducible analysis of candidate genes eliminating the need for screening a vast number of clones. Due to its extrachromosomal replication mechanism pEPI-1 derived vectors do not cause insertional mutagenesis as known from retroviral and lentiviral vector systems (Marshall, 2000; Check, 2002). Furthermore pEPI-1-based vectors do not require any viral trans-activating proteins for their function, such as the

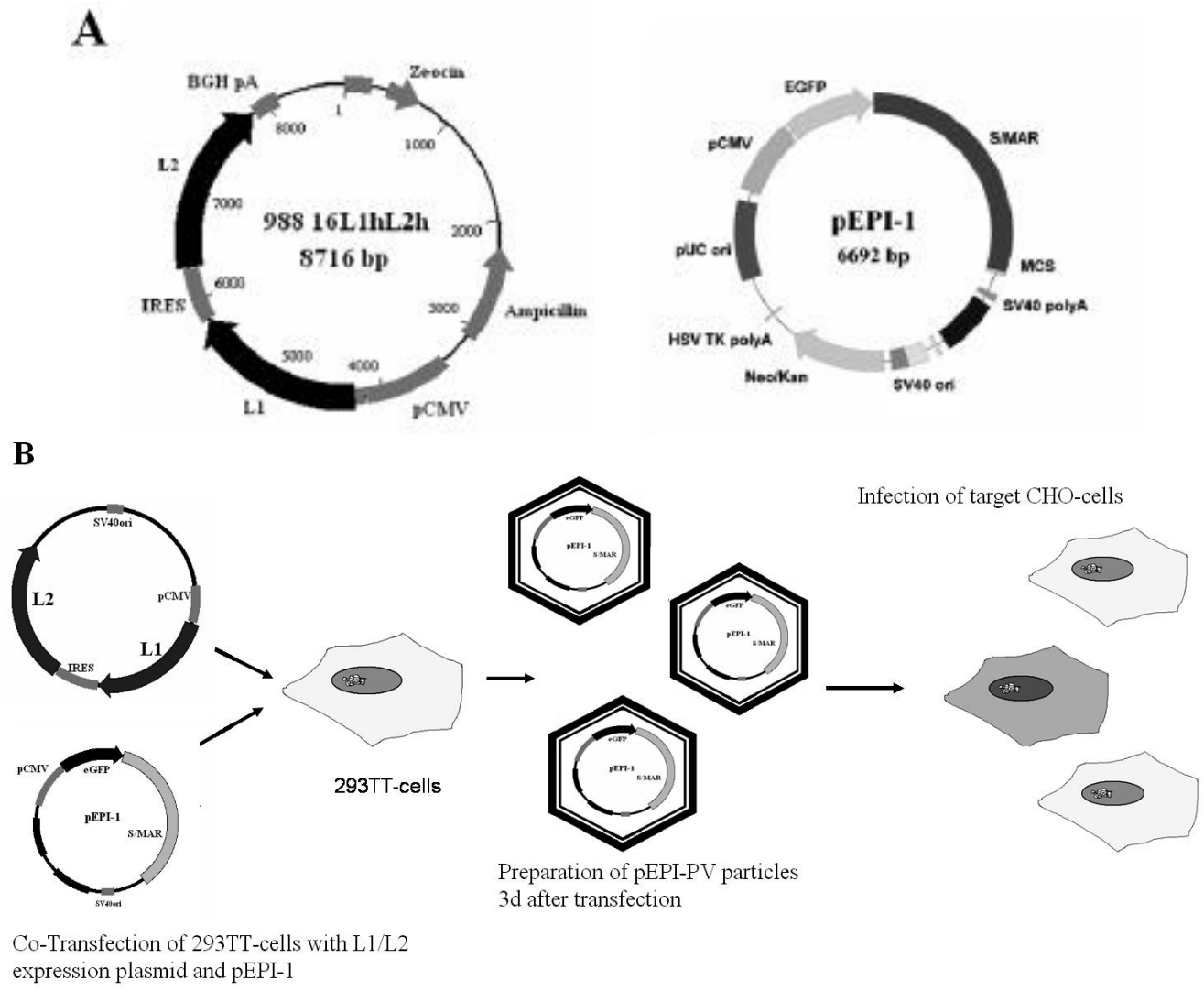

Figure 1. Strategy of pEPI-PV production. (**A**) Vectors used for the construction of pEPI-PV: the episomal replicating vector pEPI-1 and the L1/L2 capsid protein expressing vector 988 16L1hL2h. pCMV: CMV immediate early promotor, EGFP: enhanced green fluorescent protein, S/MAR: scaffold/matrix attached region sequence, MCS: multiple cloning site, SV40 ori: SV40 promotor and origin of replication, neo/kan: neomycin (Geneticin) resistence gene, L1,L2: humanized form of the HPV16 L1,L2 capsid protein, IRES: internal ribosomal entry site, BGH pA: Bovine Growth Hormone polyadenylation signal, HSV TK polyA: Herpes Simplex Virus thymidine kinase polyadenylation signal. (**B**) Overview of the pEPI-PV production.

large T-Antigen of Simian Virus 40 (SV40) or the EBNA-1 protein of Epstein-Barr Virus (EBV) that are known to cause cell transformation (Fanning and Knippers, 1992; Marechal et al, 1997) and to trigger the immune system (Chen et al, 1999). However, this vector shares with all non-viral systems the problem of low efficient delivery to the target cells *in vivo*. Some cell lines might be transfected fairly easily by electroporation, liposomes or chemical reagents *in vitro*, but these methods are not very efficient for the genetic manipulation of more sensitive primary cells, stem cells or tissues *in vivo* (Lipps and Bode, 2001; Lipps et al, 2003). One solution to overcome this problem could be the construction of pseudoviruses as a shuttle system for the *in vivo* delivery of pEPI-1. Papillomaviruses are small double-stranded, circular DNA viruses that replicate episomally and comprise a genome of around 8000 bp. The construction principle of Papillomavirus-based pseudoviruses relies on the ability of the viral capsid proteins L1 and L2 to bind and package small DNA molecules efficiently and to self-assemble into pseudoviral particles that resemble wildtype viruses. The size of the packaged plasmid DNA is limited to 10,2 kb and it has been described that one out of 10^4 pseudoviral particles carries a packaged plasmid molecule (Zhao et al, 1998, 2000; Patterson et al, 2005). Pseudoviral particles are able to transduce target cells efficiently by introducing the packaged DNA into the nucleus of target cells. Zhao et colleagues, 1998 demonstrated that plasmids containing the SV40 origin of replication could be packaged as Bovine Papillomavirus (BPV) – based pseudoviruses and that the resulting pseudoviral particles were able to transduce target COS cells and to deliver the respective plasmid even under maintenance of its episomal character into these large T-Antigen expressing cells. In this report, combining two established technologies we show that the non-viral, episomally replicating vector pEPI-1 can be

delivered to target cells via a Human Papillomavirus type 16 (HPV16)-based pseudovirus. The pEPI-pseudoviruses (pEPI-PV) can be used to efficiently transduce CHO target cells. The pEPI-1 plasmid DNA is delivered into the nucleus of the cells and is able to establish an episomal status. The combination of HPV-based pseudoviral gene delivery and non-viral, episomally replicating plasmids enables new strategies for the safe and reproducible genetic manipulation of mammalian cells *in vitro* and possibly also *in vivo*.

II. Materials and methods

A. Cells and vectors

CHO cells were grown as described previously (Piechaczek et al, 1999). 293TT cells, that express two copies of the SV40 Large T-Antigen, were cultured in Dulbecco's modified Eagle medium (DMEM, Biochrom AG), supplemented with 10% fetal calf serum and 4.5 g/l D-glucose, and selected with Hygromycin B (200µg/ml). Schematic maps of the vectors pEPI-1 and 988 16L1hL2h are displayed in **Figure 1A.**

B. Production of the pEPI-PV and infection of target CHO cells

$7x10^6$ 293TT cells were co-transfected with 7.5µg of pEPI-1 plasmid DNA and 7.5µg of 988 16L1hL2h plasmid DNA in a 10cm dish using METAFECTENE™ (Biontex, Munich). Three days after transfection the 293TT cells were harvested and resuspended in PBS. Pseudoviral particles were released after incubation of the cells in lysis buffer (PBS + 0.5% Brij58 + 1U/µl Benzonase) for 45 min at 37°C. Cell lysates were incubated on ice for 15 min and NaCl was added to a final concentration of 850 mM. To remove cellular debris, the cell lysate was centrifuged at 3000 g for 10 min at 4°C. The pEPI-PV containing supernatant was dialyzed against PBS, aliquoted and stored at -80°C. Several dilutions of the aliquots were used to transduce CHO target cells. Transduction efficiency was monitored by GFP expression of the infected target CHO cells by FACS analyses.

C. DNA isolation and Southern analysis

For Southern analysis total cellular DNA was isolated, digested with *Bgl* II, a restriction enzyme that linearizes the 6.7 kb pEPI-1 vector DNA, separated on 1% agarose gels, blotted onto nylon membranes and hybridised with a ^{32}P-labeled pEPI-1 probe.

III. Results

For the production of pEPI-PV we used the method described by Buck and colleagues (Buck et al, 2004). Briefly, 293TT cells that express two copies of the SV40 Large T-Antigen, were co-transfected with pEPI-1 DNA and the plasmid 988 16L1hL2h (**Figure 1A**), that expresses codon-modified (humanized) forms of the L1 and L2 capsid proteins of HPV 16 (Leder et al, 2001). Three days post transfection the 293TT cells were harvested and pEPI-PV particles were released by lysing the cells. In order to prevent plasmid DNA contaminations the obtained cell lysates were treated with Benzonase. **Figure 1B** gives an overview about pEPI-PV production.

To assess the titer of the resulting pEPI-PV particles, 4 x 10^5 CHO cells were transduced with various dilutions of the obtained cell lysates in 3ml of medium. At 3 days post transduction, the CHO target cells were harvested and subjected to FACS analysis to detect the GFP signal resulting from cells transduced with pEPI-1. The results of these analyses are summarized in **Figure 2A**. Using a dilution of 1:5000 of the 293TT cell lysate 4.3% of the cells expressed GFP. A 1:1000 dilution resulted in 16,5% GFP expressing cells, a 1:500 dilution of 30.2% and a 1:200 dilution of 37.5% GFP expressing cells. The titers of the transducing pseudoviral particles were calculated according to the following formula:

$$\text{Transducing particles/ml} = \frac{(\%\ \text{GFP(+) cells}/100) \times (4 \times 10^5\ \text{target cells})}{(3\ \text{ml culture volume}) \times (\text{dilution factor})}$$

Typical pEPI-PV titers obtained by this procedure ranged from 1-2 x 10^7 transducing particles per ml. Other experiments to increase the transduction efficieny of the pseudoviral gene delivery, such as purification and concentration, or the use of positively charged polymers (e.g. Polybrene) to facilitate the cellular uptake of pseudoviral particles have not been performed.

The transduction efficiency of the pEPI-PV particles could be reduced to background levels by preincubation of the cell lysates with the neutralizing polyclonal rabbit antiserum #4543 directed against the HPV16 capsid protein L1 (Leder et al, 2001), demonstrating the specificity of the pseudoviral gene delivery (**Figure 2B**).

In further experiments we focused on the analysis of stably selected clones derived from CHO cells infected with the pEPI-PV. For this purpose, the pseudovirally transduced CHO cells were selected with Geneticin (G418) at a concentration of 500µg/ml and then grown in the presence or absence of the antibiotic for further two months. Interestingly in only 2-5% of the transduced cells the vector DNA was stably established. This phenomenon is not related to the pseudoviral gene delivery, since other transfection methods, such as electroporation, calcium phosphate transfection (Batard and Wurm, 2001), or cationic liposomes (Tseng et al, 1997) have been studied in parallel with the same results (Piechaczek et al, 1999). The FuGENE 6 reagent (Roche Diagnostics, Mannheim, Germany), for example, resulted in pEPI-1 plasmid transfection efficiencies of some 36% in CHO cells as measured by GFP expression. But also here, only in 2-5% of the transfected cells the vector DNA was stably established after G418 selection. It seems that the establishment of the stable episomal status of the plasmid pEPI-1 within cells is a rare event and distinct from transient effects. Similar effects were observed with other episomal constructs, for example the OriP/EBNA-1 replicon (Leight and Sugden, 2001) and it is assumed that the establishment of episomal replicons is dependent on infrequent epigenetic events not yet understood.

To analyse the status of the vector in stable clones, cellular DNA from 20 clones was isolated, digested with *Bgl* II, which cuts once in the pEPI-1 plasmid, and subjected to Southern blot analyses using ^{32}P labelled pEPI-1 DNA as a probe. 18 out of 20 (90%) stably with pEPI-PV transduced CHO cell clones maintained the pEPI-1 plasmid DNA as an episome. The hybridisation pattern of 10 clones is shown in **Figure 3**. In one out of 10 clones the vector was integrated into the host genome

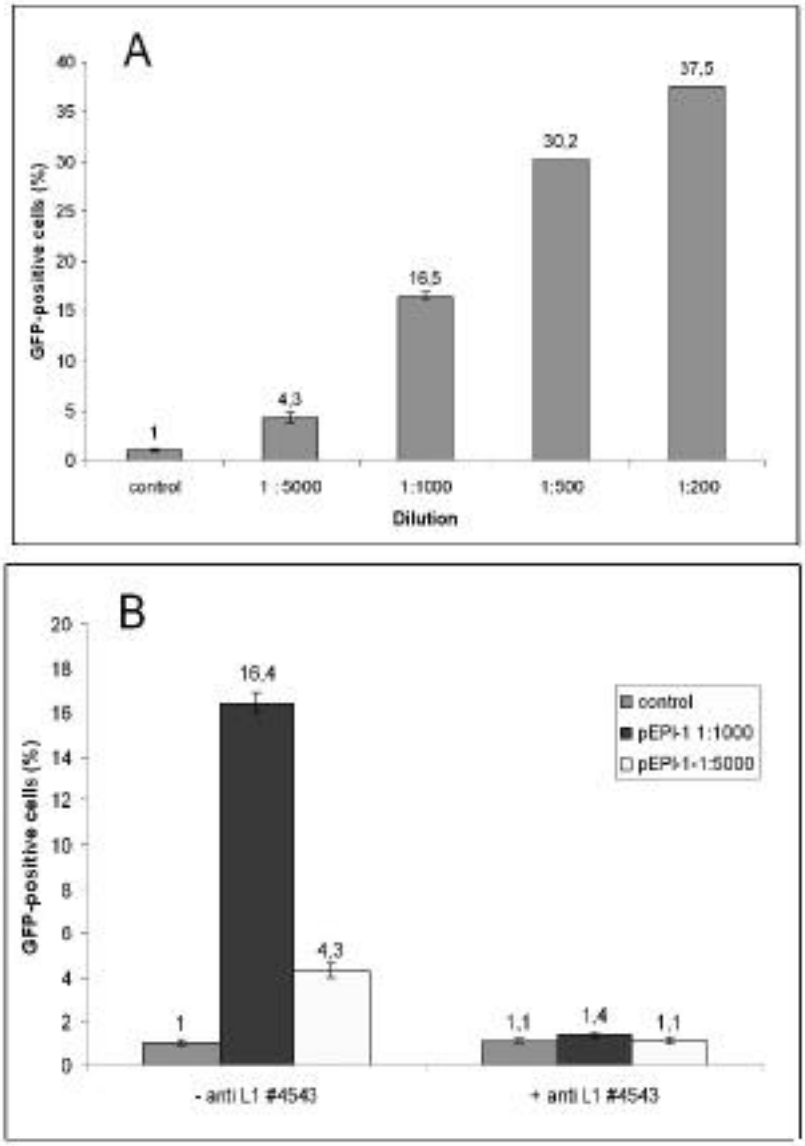

Figure 2. Transduction of CHO cells with pEPI-PV. (**A**) GFP expression rates of target CHO cells transduced with serial dilutions of the pEPI-PV containing 293TT cell lysates. These numbers were used to determine the pseudoviral titers. (**B**) 1:1000 and 1:5000 dilutions of the pEPI-1 and 988 16L1hL2h co-transfected 293TT cell lysates were used to transduce target CHO cells. Incubation of the target CHO cells with the pEPI-PV was done either in the presence of an unspecific rabbit antiserum (left panel) or presence of the neutralizing rabbit antiserum #4543, directed against epitopes of the L1 capsid protein (right panel). In the presence of the neutralizing antiserum #4543 the transduction efficiency of the pEPI-PV particles was significantly decreased to background level (1,4% and 1,1% respectively). Experiments were performed in quadruplicate to obtain standard error bars.

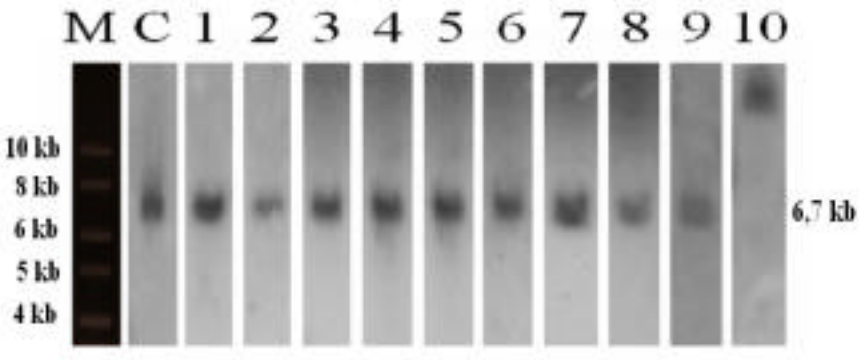

Figure 3. Southern blot analyses of CHO cells stably transduced with pEPI-1 by pseudoviral gene delivery. Total DNA was digested with *Bgl* II, separated on a 1% agarose gel and probed with ^{32}P- labelled pEPI-1 DNA. Lane M: marker (SMART ladder, Eurogentec), Lane C: control (10ng pEPI-1 plasmid DNA), Lane 1-10: 5µg of total DNA isolated from pEPI-PV transfected CHO clone 1-10 respectively. Clones 1-9 show the same restriction pattern as the control demonstrating the episomal character of the pEPI-1 plasmid DNA. Clone 10 shows a different restriction pattern typical for integrated vectors

(lane 10) while the restriction pattern of nine clones (lanes 1-9) was identical to that of the original plasmid pEPI-1 (lane C). The integration of a low percentage of vector may be explained by shearing of the DNA molecule during transient transfection of the 293TT cells, a phenomenon described before (Jenke et al, 2004b), or by rare breaking events of the transduced plasmid within the target CHO cells. Southern blot analyses of cells grown for 2 months in the absence of selection showed the same hybridisation signals demonstrating that pEPI-1 delivered as a pseudovirus is stably maintained as reported earlier for other transfection methods (Piechaczek et al, 1999).

IV. Discussion

The result of this work shows, that the episomally replicating plasmid pEPI-1 can be packaged efficiently as a pseudoviral particle in 293TT cells by co-transfection of pEPI-1 plasmid DNA and a plasmid that expresses the L1 and L2 capsid proteins of HPV 16. The pEPI-PV particles were present in the cellular extract of the transiently co-transfected 293TT cells and could be used to transduce target CHO cells with high efficiency. The pEPI-1 plasmid DNA is transported to the nucleus of the transduced target cell, as shown by the transient expression of the GFP reporter protein. Around 2-5% of the originally transduced cells could be stably selected to form cell lines. In 90% of the examined pEPI-PV transduced CHO cell lines, the pEPI-1 DNA could be detected as an episome. HPV based pseudoviral particles have been successfully used to transduce a variety of mammalian cell lines in vitro. Among them kidney cells, keratinocytes, fibroblasts and epithelial cells (Patterson et al, 2005). The major advantage of the pseudoviral gene delivery over standard non-viral transfection procedures such as electroporation, calcium phosphate transfection and liposomal transfection lies in the fact, that pseudoviral particles are able to transduce target cells *in vivo* (Nardelli-Haefelinger et al, 2005; Sasagawa et al, 2005). Another advantage of HPV based pseudoviral vectors worth mentioning is the fact that there are around 100 serotypes of Human Papillomaviruses that do not cross-react with each other (Combita et al, 2002; Giroglou et al, 2001), enabling strategies for repeated gene therapeutic treatments without immunological complications. A low establishment efficiency of 2-5% of the episomes in the transduced target cells should be sufficient for many gene therapeutic approaches in vivo if a controlled expression of the transgene is desirable. The current low transduction efficiency with stable inheritance of our pEPI-PV has to be weighed up carefully against high transduction efficiencies with potentially dangerous integration events of retroviral vector systems. Taken together these data suggest, that the pseudoviral gene transfer in combination with small, non-viral, episomally replicating vectors enable novel perspectives for biotechnological and biomedical applications *in vivo*.

Acknowledgements

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Lower row (from left to the right): Gero Weyer, Stefan Juranek, Katharina Heyse, Matthias Moellenbeck, Jan Postberg, and Kathrin Peschke.