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ORIGINAL ARTICLE

Development of inducible EIAV-based lentiviral vector packaging and producer cell lines

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Large-scale production of gene therapeutics comprising equine infectious anaemia virus (EIAV) -based lentiviral vectors (LVs) would benefit from the development of producer cell lines enabling the generation of larger quantities of vector than achievable by transient systems. Such cell lines would contain three vector components (Gag/Pol, VSV-G envelope and genome expression constructs). As the vesicular stomatitis virus (VSV-G) envelope protein is cytotoxic, its expression must be regulated. It is also desirable to regulate Gag/Pol expression to minimise metabolic burden on the cell. The Tet repressor (TetR) system was selected to regulate expression of VSV-G and Gag/Pol, necessitating the introduction of a fourth construct, encoding TetR, into the cell line. We have generated an inducible packaging cell line that shows tight control of the packaging components, and high-titre vector production on transient transfection of the EIAV genome. The cell line is stable for at least 7 weeks in the absence of selective pressure. To verify that this packaging cell line can support the generation of producer cell lines it was transfected stably with an EIAV genome cassette encoding ProSavin; a gene therapeutic for Parkinson's disease. Producer cell lines were generated, which on induction, yielded ProSavin with titres comparable to the transient system.
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Keywords: *lentiviral vectors; EIAV; packaging cell line; producer cell line; Tet repressor; inducible expression*

Introduction

The use of lentiviral vectors (LVs) for gene therapy is attractive because of their ability to integrate relatively large therapeutic cassettes into the chromosomes of slowly and non-dividing cells. LVs derived from non-primate lentiviruses, not known to be infectious or pathogenic for humans, such as equine infectious anaemia virus (EIAV), have been developed and their ability to transduce non-dividing cells established.[1–5] EIAV based LVs are being developed for a number of different gene therapy applications for a variety of diseases.[6–13] Recent focus has been on the development of ProSavin, which is an EIAV-based LV for the treatment of Parkinson's disease encoding three enzymes required for the dopamine synthesis.[6] Currently, ProSavin is in phase I/II clinical trials that require relatively small amounts of vector material. This was generated by transient co-transfection of human embryonic kidney 293T (HEK293T) cells with three plasmids that encode the necessary components for vector production (Vector genome, EIAV Gag/Pol and a heterologous envelope (VSV-G)).[2] However, this method is labour intensive and not easily scalable. Development of a stable producer cell line for phase III manufacture and product commercialisation is desirable as this should enable the generation of large quantities of vector and reduce variation between batches.

Stable packaging and producer cell lines have been developed for the production of human immunodeficiency virus 1-based LVs[14–25] but, to date, none have been described for EIAV-based LVs. The glycoprotein of the vesicular stomatitis virus (VSV-G) is often used for pseudotyping LVs because of its high stability, broad tropism and generation of high-titre viral stocks.[26,27] A disadvantage of using VSV-G is that VSV-G pseudotyped LVs are inactivated by human serum complement.[28] This is not likely to be problematic for the ProSavin gene therapeutic as the route of administration is by intrastriatal injection and exposure to human serum complement is minimal. However, a complicating factor is that VSV-G is cytotoxic[26,29] and therefore its expression must be regulated. For many of the human immunodeficiency virus 1 based packaging cell lines VSV-G expression is controlled using tetracycline-inducible systems,[15,16,18,20,21,24] enabling VSV-G expression to be switched on at the time of vector production. In these systems, gene expression is controlled by expression of the tetracycline transactivator fusion protein comprising the tetracycline repressor (TetR) and the transcription activation domain of VP16.[30] However, it has been suggested that tetracycline transactivator is toxic, which is attributed to the VP16 component squelching general cellular transcription.[30,31] As it has been established that if the tetracycline operators ($TetO_2$) are placed in a position such that the first nucleotide is 10 bp from the 3′ end of the last nucleotide of the TATA box of the human cytomegalovirus immediate early enhancer/

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promoter (hCMVp), then TetR alone is capable of acting as a repressor.[32] This system therefore avoids the potentially toxic effect of the VP16 component. This is known as the tetracycline-regulated expression system (T-REx) system and is commercially available from Invitrogen. In this system, expression of the gene of interest is controlled by an hCMV promoter into which two copies of the $TetO_2$ sequence have been inserted in tandem. TetR homodimers in the absence of an inducing agent (tetracycline or its analogue doxycycline (dox)) bind to the $TetO_2$ sequences and physically block transcription from the upstream hCMV promoter.[33] When present, the inducing agent binds to the TetR homodimers, causing allosteric changes such that it can no longer bind to the $TetO_2$ sequences, resulting in gene expression. We have codon optimised the TetR open reading frame (coTetR) and found that this resulted in improved control of $TetO_2$-regulated gene expression (data not shown).

Here, we describe the generation of an HEK293T based cell line that constitutively expresses coTetR. This cell line (HEK293T-coTetR) was used for the generation of an inducible EIAV packaging cell line. In addition to regulating VSV-G it was decided to regulate EIAV Gag/Pol expression to prevent possible selection against cells expressing high levels of Gag/Pol because of the additional metabolic burden this may cause. Inducible expression cassettes encoding VSV-G and Gag/Pol were introduced into the HEK293T-coTetR cell line and a clonal inducible EIAV packaging cell line (PC48.2) was established. Following transient transfection of EIAV genome and induction with dox this cell line was capable of generating high titre vector. Furthermore, very little vector was detected in the absence of dox. To generate a producer cell line, PC48.2 was stably transfected with an EIAV genome cassette encoding ProSavin. On induction of selected ProSavin producer cell lines, vector was produced with titres that were comparable to those obtained by the transient transfection process.

Results

Construction of a coTetR expressing cell line

HEK293T cells were transfected with linearised plasmid encoding the coTetR expression cassette and a puromycin selectable marker (pPuro. coTetR, Figure 1). Following puromycin selection clones (twenty in total) were isolated and the clone (HEK293T-coTetR) that showed the highest level of coTetR protein expression by western blot analysis (data not shown) was chosen for further study. TetR-mediated regulation in this clone was investigated by examining repression and activation of gene expression in the absence and presence of dox, respectively. This was achieved by transient transfection of plasmids encoding either Gag/Pol (pGagPol, Figure 1) or VSV-G (pVSV-G, Figure 1) under the control of an hCMVp containing two $TetO_2$ sequences. Expression of Gag/Pol and VSV-G after culturing the cells with or without dox was then examined. Additional pPuro.coTetR was also transiently co-transfected to see if repression could be improved by increasing the levels of coTetR present. Forty-eight hours post-transfection (and addition of dox, if appropriate) cells were harvested and lysates analysed by western blot for evaluation of either VSV-G (Figure 2a) or Gag/Pol (Figure 2b) expression. The western blot analyses show that expression from the transfected plasmids is repressed in the absence of dox, but is switched on in its presence.

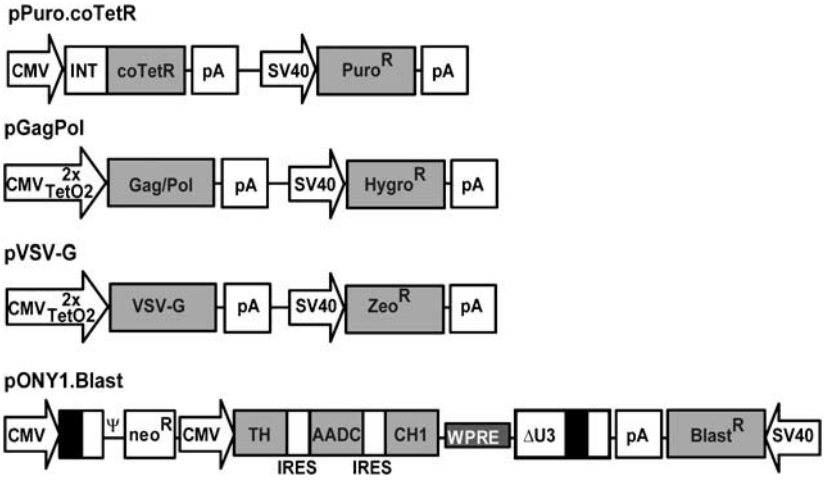

Figure 1 Schematic representation of constructs used to generate the packaging and producer cell lines. The human cytomegalovirus immediate-early enhancer/promoter (CMV), chimeric intron (INT), codon optimised TetR (coTetR), Simian virus 40 promoter (SV40), puromycin resistance gene ($Puro^R$), polyadenylation signal (pA), hybrid promoter consisting of the human CMV promoter and two tetracycline operator two sites (CMV $2 \times TetO_2$), Gag/Pol, hygromycin resistance gene ($Hygro^R$), glycoprotein of vesicular stomatitis virus (VSV-G) and zeocin resistance gene (Zeo^R) are indicated were present in the various constructs. The ProSavin vector genome used for generating the producer cell lines is known as pONY1.Blast. The full-length vector genome transcript is expressed from a CMV/EIAV long-terminal repeat (LTR) RU5 chimeric promoter. The vector contains a deletion in the U3 region (ΔU3) of the 3′-LTR resulting in self-inactivation (SIN) of the vector on transduction. The EIAV packaging signal (Ψ), neomycin resistance gene (neo^R) and Woodchuck post-transcriptional regulatory Element (WPRE) are indicated were present. The ProSavin transgenes: tyrosine hydroxylase (TH), aromatic L-amino acid dopa decarboxylase (AADC) and cyclohydrolase 1 (CH1) are expressed downstream of an internal CMV promoter. For selection purposes the blasticidin resistance gene ($Blast^R$), under the control of the SV40 promoter, was placed downstream of the EIAV vector genome (in the opposite orientation).

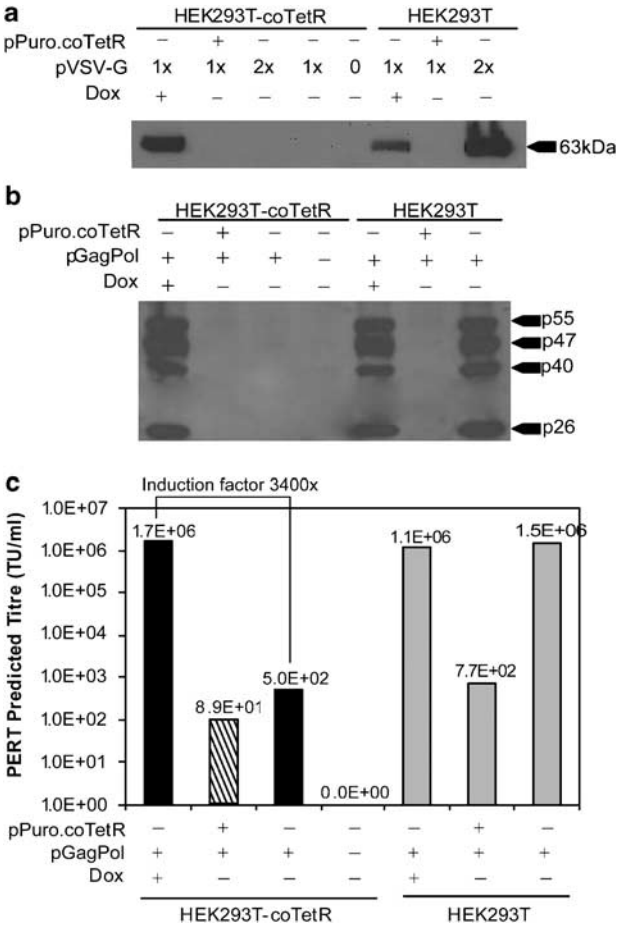

Figure 2 Repression/induction of gene expression in HEK293T-coTetR cells. Cell lysates were prepared from HEK293T-coTetR and HEK293T cells transiently transfected with pPuro-coTetR and/or $TetO_2$ containing plasmid pVSV-G or pGagPol and cultured in the presence or absence of dox as indicated. Western blot analysis was performed to examine expression of (**a**) VSV-G (VSV-G protein is indicated by an arrow) and (**b**) Gag/Pol (full-length Gag precursor p55, the intermediates p47 (MA-CA-NC) and p40 (MA-CA) and the mature capsid protein p26 are indicated by arrows). Cell culture supernatants were examined for the reverse transcriptase activity, measured by PERT analysis (**c**). The induction factor was also calculated. Hatching indicates a predicted titre below the accurate quantitation limits of the assay.

Expression of VSV-G was not detected even when twice the normal amount of VSV-G plasmid DNA was transfected into the HEK293T-coTetR cell line (Figure 2a). As expected, expression of VSV-G and Gag/Pol was detected in control HEK293T cells and not dependent on the addition of dox, unless the coTetR plasmid was co-transfected.

The cell culture supernatants from the Gag/Pol transfected HEK293T-coTetR and HEK293T cells were harvested and analysed for reverse transcriptase (RT) activity by the product-enhanced reverse transcriptase (PERT) assay (Figure 2c). An induction factor of 3400-fold was calculated based on the difference in PERT predicted titres for HEK293T-coTetR cells cultured with and without dox. When additional pPuro.coTetR was transfected into the HEK293T-coTetR cell line, levels of RT activity, in the absence of dox, were below the accurate quantitation limits of the assay.

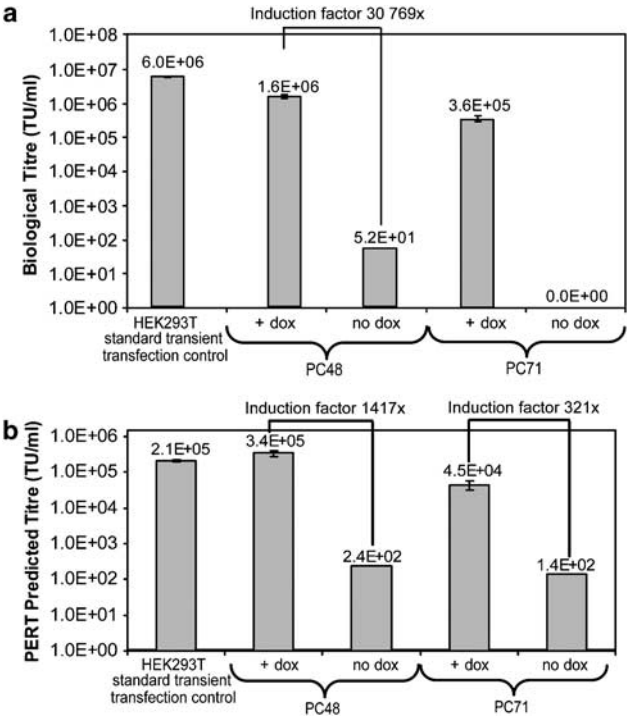

Figure 3 Vector production from EIAV packaging clones with and without dox induction. Two EIAV packaging clones (PC48 and PC71) were transiently transfected with an EIAV genome encoding β-galactosidase (pONY8.4GCZ), and vector production was analysed with (+dox) and without (no dox) induction and compared with an HEK293T standard transient transfection control. Vector production was assessed by, (**a**) biological titre assay and (**b**) PERT analysis. Data shown for the HEK293T standard transient transfection control and +dox samples are the mean values ± s.d. ($N = 2$).

These data show that the HEK293T-coTetR cell line is capable of repressing $TetO_2$ containing expression cassettes, which can be modestly enhanced by increasing the level of coTetR.

Development of inducible packaging cell lines

To develop an EIAV packaging cell line the HEK293T-coTetR cell line was co-transfected with the packaging constructs pVSV-G and pGag/Pol (Figure 1). Following selection of antibiotic resistant colonies and cloning by limiting dilution, fifty-four clones were isolated and screened for vector production by transfection with a genome encoding a reporter gene (see Materials and methods). The two clones that yielded the highest vector titres (PC48 and PC71) were analysed further by scale-up of vector production, and the induction factors also evaluated. Cell culture supernatants were screened by biological titre assay (Figure 3a), which measures functional vector by scoring lacZ colony forming units on transduced cells, and by PERT assay to measure RT activity, which is a measure of particle production (Figure 3b). Results from the biological titre assay (Figure 3a) show production of high titre vector from both clones when cultured in the presence of dox. Titres are comparable to that of vector produced from an HEK293T standard transient transfection control. In the absence of dox very little vector is produced, showing tight regulation of Gag/Pol and VSV-G by coTetR. The induction factor from the biological titre assay is

calculated to be greater than 30 000 for both clones. These results are in agreement with data from the PERT assay (Figure 3b), which show high-predicted titres, comparable to the transient control, when clones had been cultured in the presence of dox, and very low-predicted titres in its absence. From the PERT data, an induction factor of 1417 was observed for PC48 and 321 for PC71. The discrepancy between the induction factors from the biological and PERT assays is most likely because of the PERT assay measuring only Gag/Pol induction, whereas the biological titre assay measures functional vector particles, which also requires the induction of VSV-G.

Overall PC48 showed the highest functional and PERT predicted titres on induction when compared with those obtained for PC71, thus PC48 was selected as the candidate EIAV packaging cell line.

Vector production is not limited by packaging component expression

To establish whether expression of either of the packaging components is limiting vector production in PC48, additional VSV-G (pHG) and/or Gag/Pol (pESGPK) was transiently co-transfected with an EIAV genome encoding β-galactosidase (pONY8.4GCZ). To analyse vector production and expression of the EIAV packaging components biological titre (Figure 4a) and PERT analyses (Figure 4c) were performed on the resulting vector harvests, and VSV-G western blot analysis (Figure 4b) on the producer cell lysates.

Co-transfection of additional VSV-G and Gag/Pol into PC48 did not increase vector production, as measured by functional vector titres, indicating that levels of Gag/Pol and VSV-G are not limiting (Figure 4a). The results from the VSV-G western blot analysis (Figure 4b) showed high-level VSV-G expression, with no obvious increase when extra VSV-G was transfected. Results from the PERT assay (Figure 4c) showed that when additional Gag/Pol was transfected into PC48 the PERT predicted titre did not increase. Overall, the introduction of additional Gag/Pol and/or VSV-G did not increase levels of vector production, VSV-G protein expression or RT activity. Therefore, it appears that the packaging components in PC48 are expressed at maximal levels.

Packaging cell line 48.2 is stable in the absence of selective pressure

PC48 was sub-cloned to ensure isolation of a true clonal population. A stability study was conducted on the selected sub-clone (PC48.2) to ensure that this cell line was capable of producing high titre vector following extensive passaging in the absence of antibiotic selection. PC48.2 was passaged regularly for 49 days (23 passages) in the presence or absence of antibiotics (zeocin, hygromycin and puromycin). At regular intervals, the cell line was tested for its ability to produce vector (+/− dox) on transient transfection of pONY8.4GCZ (Figure 5). TetR-mediated regulation was maintained whether cultured with or without antibiotic selection as very little vector was produced in the absence of dox confirming stable coTetR expression for the duration of the experiment (49 days). In the presence of dox comparable titres were observed at all time points, whether with or without antibiotic selection, throughout the study.

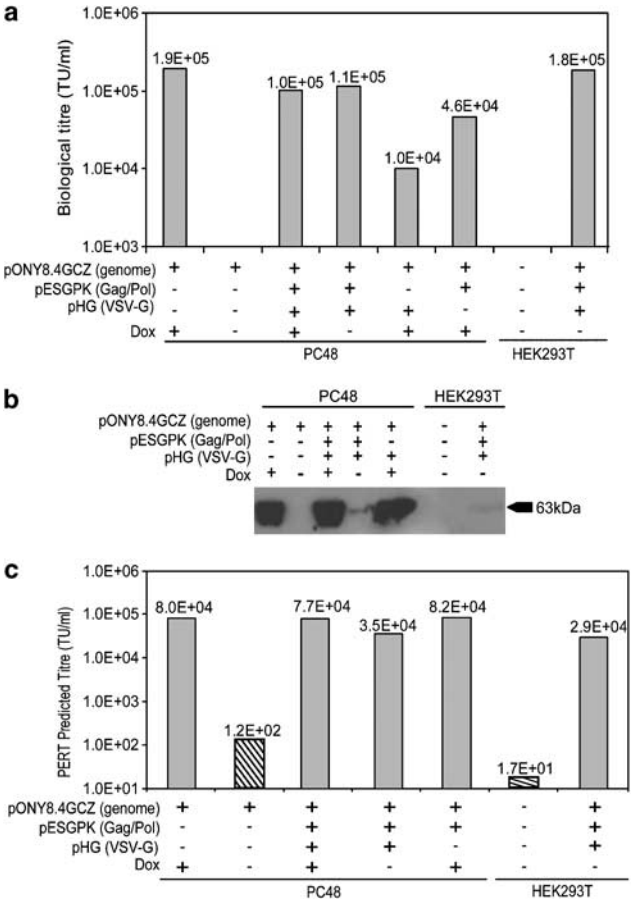

Figure 4 Vector production from packaging cell line PC48 is not limited by either of the packaging components. PC48 cells were transiently transfected with each of the packaging components (pESGPK and pHG) either separately or in combination as indicated. The pONY8.4GCZ EIAV genome was simultaneously co-transfected and cells cultured in the presence or absence of dox as indicated. As a positive control HEK293T control cells were co-transfected with all three plasmids, and for the negative control untransfected HEK293T were included. Vector production was assessed by, (**a**) biological titre assay, (**b**) VSV-G western blot of cell lysates and (**c**) PERT analysis. Hatching indicates a titre below the accurate quantitation limits of the assay.

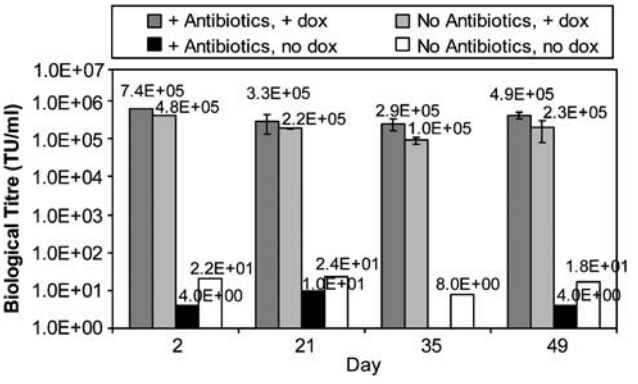

Figure 5 Stability study of packaging cell line PC48.2. The stability of PC48.2 was assessed for 49 days in culture with and without antibiotic selection pressure. The production of EIAV vector was assessed at days 2, 21, 35 and 49 following transient transfection of pONY8.4GCZ genome, +/− dox. Vector titres were established by biological titre assay. Data shown are the mean values ± s.d.

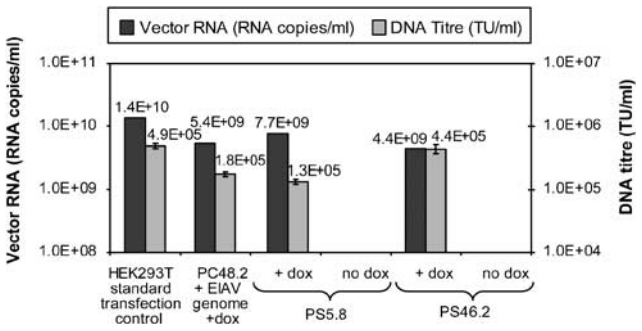

Figure 6 Vector production from two ProSavin producer cell lines. Vector production from ProSavin producer cell lines PS5.8 and PS46.2 was assessed with (+dox) and without (no dox) induction. For comparison ProSavin vector was produced by transfection of the packaging cell line (PC48.2) with the ProSavin genome plasmid (pONYK1-ORT) and by standard transient transfection of HEK293T cells (with pONYK1-ORT, pESGPK and pHG). Vector production was assessed by vector RNA and DNA integration assays. Data shown are the mean values ± s.d.

These data indicate that PC48.2 is stable in the absence of selective pressure and that vector production capacity is maintained during prolonged culture periods.

Generation of inducible producer cell lines

Packaging cell line 48.2 was transfected with linearised plasmid encoding a ProSavin genome expression cassette and the resistance marker for blasticidin (pONY1.Blast, Figure 1). Following selection in blasticidin S containing media and cloning by limiting dilution, seventy-eight clones were isolated and screened for EIAV vector production by measuring RNA genome copies. Those showing high RNA vector genome copies were further screened by DNA integration assay (see Materials and methods) and sub-cloned by limiting dilution. Two sub-clones (PS5.8 and PS46.2) from different parental clones that on screening showed the highest vector RNA levels (as predicted by RNA copies per ml), were selected as candidates for further analysis. The vector production from these two sub-clones was scaled up and analysed by both viral RNA and DNA integration assays (Figure 6).

These data show that the titres of vector from both sub-clones, PS5.8 and PS46.2, are within fourfold of the HEK293T standard transient transfection control. For sub-clone PS46.2 the DNA integration titre (4.4×10^5 TU per ml) is equivalent to that of the HEK293T standard transient transfection control (4.9×10^5 TU per ml).

In the absence of dox the levels of vector could not be quantified by vector RNA or DNA integration assays for either of the producer clones, as they are below the lower level of quantification for both assays. The limit of quantification for the vector RNA assay was 2.0E+06 RNA copies per ml and therefore the induction factors for PS5.8 and PS46.2 are at least 3700 and 2100, respectively. The DNA integration assay is less sensitive and the background relatively high at approximately 1.4E+03 TU per ml, therefore the induction factors for PS5.8 and PS46.2 are calculated to be at least 92 and 314, respectively. Thus, two-tightly regulated producer cell lines have been developed that, when induced, produce ProSavin at high titre. These data show that the packaging cell line can support generation of producer cell lines that are capable of producing vector at comparable titres to those obtained by the transient transfection process.

Discussion

Development of stable producer cell lines is desirable, as it would facilitate the establishment of a robust manufacturing process for the reproducible production of large amounts of vector. To date, there have been no reports describing the generation of producer or packaging cell lines for EIAV based lentivectors. We have developed an HEK293T based inducible EIAV packaging cell line that, on transient transfection with EIAV vector genome, is capable of high titre vector production when induced. Furthermore, this cell line is stable in the absence of antibiotic selection for at least 49 days. This packaging cell line can support generation of producer cell lines, as verified by the stable transfection of a ProSavin vector genome expression cassette and resultant producer cell lines (PS5.8 and PS46.2) capable of high titre production of ProSavin.

On co-transfection of the packaging cell line with genome and additional non-regulated packaging components (either individually or simultaneously), neither vector production, VSV-G protein expression nor RT activity increased. To ensure that the packaging components had been transfected efficiently in the PC48 cells, cells were also co-transfected with genome and the packaging components in the absence of dox. Resultant vector was of equivalent biological titre (within twofold) to that obtained when PC48 was transfected with genome in the presence of dox, thereby confirming that the PC48 could be efficiently transfected with the packaging components. Overall, these data suggest that the packaging components are expressed at sufficient levels to achieve vector production at maximum efficiency. Transfection of the packaging components individually seemed to reduce vector production; this was seen to a greater extent with VSV-G. One hypothesis for this is that optimal VSV-G and Gag/Pol levels are being produced and more of either one of the components adversely affects stoichiometry, which has been observed earlier when examining the effects of stoichiometry of retroviral components on virus production.[34,35] This theory is further supported by observations from experiments to optimise the transient transfection system: the ratio of components is critical for obtaining maximal titres, in particular vector titres are adversely affected when relatively large amounts of VSV-G compared with Gag/Pol plasmid are transfected (data not shown). From western blot analysis it appears that the packaging cell line expresses VSV-G at higher levels than HEK293T cells in the standard transient transfection system. As titres are comparable to those obtained from the transient transfection process, it does not appear that high-level VSV-G expression is adversely affecting vector production in this cell line, perhaps because Gag/Pol expression is sufficiently high to maintain correct stoichiometry. Furthermore, high expression of cellular VSV-G has been shown to directly correlate with efficient pseudotyping of vector particles, which is an important parameter in improving the transduction efficiency of certain target cells.[36]

A stability study was performed on the sub-cloned packaging cell line PC48.2 showing that this cell line is capable of producing high titre vector on induction for the duration of the study (49 days), with and without antibiotic selection pressure. This indicates that the cell line is stable and, within this time frame, loss or silencing of the packaging components and/or coTetR does not occur. An earlier published study using a human immunodeficiency virus 1 producer cell line, reported that with extended culture (2–3 months) expression of the integrated components and vector production declined over time. However, this was not because of gene loss as the copy number per cell remained constant, therefore it was most likely an indication of gene silencing.[21] Therefore, it is of importance that vector production from PC48.2 or derivative producer cell lines is monitored closely particularly beyond the time frame of this stability study.

The ability of packaging cell line PC48.2 to support generation of stable producer cell lines was examined. As the requirement for a ProSavin producer cell line for large-scale manufacture, producer cell lines were developed by stable transfection of PC48.2 with a ProSavin genome expression cassette. Two sub-clones were selected (PS5.8 and PS46.2) from a total of 78 that yielded ProSavin titres within fourfold of the HEK293T transient transfection control. It is possible that screening a greater number of clones could lead to the isolation of a cell line that produces even higher yields of ProSavin than shown by PS5.8 and PS46.2 and by the standard transient transfection process. However, when PS5.8 and PS46.2 were transiently transfected with additional ProSavin genome vector production did not increase (data not shown). This suggests that ProSavin genome levels are not limiting in these ProSavin producer cell lines. Therefore, it is likely that these clones have reached maximal ProSavin production and that screening more clones would not lead to the isolation of a cell line that could produce ProSavin at higher titres.

In the absence of induction the levels of vector in the cell culture supernatant from both PS5.8 and PS46.2 could not be quantified by either the viral RNA or DNA integration assays, corroborating the stringent control of VSV-G and Gag/Pol expression mediated by the coTetR-system. This has a number of advantages, first it is likely that the cells can be grown for extended periods before vector production with no adverse effects on cell growth/viability because of constitutive or leaky VSV-G expression, second in the absence of vector shedding the producer cells should not be auto-transduced (which would result in modification of the genotype of the cells over time as a result of accumulating integration events). A study to examine cell growth/viability and integrated vector copy numbers following extended culture in the absence of dox is currently ongoing to confirm this. The tight regulation of vector production is likely because of codon-optimisation of the TetR open reading frame, allowing high expression of the regulator, and also the fact that expression of two elements essential for functional vector production (Gag/Pol and VSV-G) are simultaneously repressed limiting the availability of both. The level of regulation provided by coTetR, and the rate that vector is produced post-induction seems to be superior to other inducible systems, particularly the Tet-Off system, in which leaky expression was seen in the off-state.[16,21] Furthermore, the Tet-Off system relies on withdrawal of dox/tetracycline to relieve repression which takes a minimum of 4 days to induce vector production,[16,18–20,25] and up to 13 days to reach maximal levels.[20] In this study, vector production is observed within 24 h of induction. The timing of dox addition and harvest is being further investigated with a view to optimising vector yields and collection of multiple harvests.

Sodium butyrate is used to increase EIAV vector yields from the transient transfection process (by approximately fivefold). However, whether it is required to enhance expression and subsequent vector production from stable producer cell lines is unclear. In this study, sodium butyrate was used during vector production from both the packaging and producer cell lines for direct comparison with the transient transfection process. It would be advantageous to omit the sodium butyrate induction step as it is expensive and is toxic to cells at the concentrations used. Furthermore, removal of sodium butyrate would reduce the number of manipulations required during the manufacturing process and also eliminate the requirement for testing of the final product for this chemical. Therefore, a study to examine the effect of sodium butyrate on vector yields from the ProSavin producer cell lines is in progress.

Stability testing of the producer cell lines is being performed to confirm that high titre vector production capacity can be maintained following prolonged culture in the absence of antibiotic selection pressure. This stability study will be performed for a period of time sufficient to encompass generation of master cell and working cell banks and production runs at large scale. The packaging and producer cell lines are also being further characterised to examine the copy number and integrity of the various integrated elements. An *in vivo* study is ongoing to examine gene transfer and immunogenicity of ProSavin generated by producer cell lines compared with that produced by the standard transient transfection process. This is of particular importance given the high level of VSV-G expression observed in the producer cell lines as VSV-G is known to be immunogenic.[26,29] These experiments are beyond the scope of the current publication and will be reported separately. Data from these studies will enable a decision to be made as to which, if either, ProSavin producer cell line is most suitable for the large-scale production and manufacture of clinical grade ProSavin.

Materials and methods

Cell lines

An HEK293T master cell bank was produced using cells derived from the Stanford University HEK293T cell stock deposited with the ATCC (SD-3515; Lot# 2634366) and tested in accordance with International Conference on Harmonisation (ICH) and Good Manufacturing Practice (GMP) guidelines. The HEK293T parental cells used to generate the coTetR, packaging and producer cell lines were derived from this master cell bank. HEK293T cells used for transient transfection controls were obtained from M Calos (Stanford University). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma, Poole, UK, cat. no. D5671) containing 10%

(v/v) foetal calf serum (FCS) supplemented with 2 mM L-Glutamine (Sigma, cat. no. G7513) and 1% non-essential amino acids (Sigma, M7145) as were D17 (canine osteosarcoma) cells. Gamma-irradiated tetracycline approved free FCS of Australian origin (PAA, A15-527) was used to maintain the packaging and producer cell lines; for all other cell culture FCS was obtained from Moregate. Induction was performed with dox (Sigma, cat. no. D9891) at a concentration of 1 μg ml^{-1}. Dox has a half-life of 48 h and as induction occurred less than 48 h before harvest of vector repeat administration of dox to cultures was not necessary. During selection of the TetR, packaging and producer cell lines, cells were maintained in conditioned media. Conditioned media consisted of 50% filtered (0.45 μM) Dulbecco's modified Eagle's medium 10% FCS media taken from HEK293T cells that had been cultured for a period of time, and 50% fresh Dulbecco's modified Eagle's medium 10% FCS media.

Plasmids

Plasmids used for transient transfection. The minimal EIAV genome plasmids pONY8.4GCZ[37] and pONYK1-ORT[36] are SIN vectors that contain the *lacZ* gene and a tricistronic cassette (encoding three enzymes required for dopamine synthesis), respectively, under the control of an internal hCMVp. The VSV-G envelope plasmid, pHG, has been described earlier.[36] pESGPK contains codon optimised EIAV Gag/Pol and is a derivative of pESYNGP,[38] in which the ampicillin resistance gene has been replaced by kanamycin.

Plasmids used for stable transfections. To generate pPuro.coTetR the TetR gene was codon optimised (Eurofins MWG Operon, Ebersberg, Germany) and cloned downstream of the CMV promoter in pIRESpuro (Clontech, Saint-Germain-en-Laye, France, cat. no. 6031-1) digested with *Hin*dIII replacing the MCS, intron and IRES regions. Simultaneously, an SV40 enhancer/early promoter sequence was cloned upstream of the puromycin resistance gene. pVSV-G was derived from pcDNA4 (Invitrogen, Paisley, UK, V1020-20) digested with *Xho*I in which a *Xho*I–*Xho*I fragment containing the VSV-G coding sequence from pHG was inserted. pGagPol was derived from pcDNA5 (Invitrogen V1033-20) digested with *Afl*II/*Not*I, in which the *Afl*II/*Not*I fragment containing the Gag/Pol coding sequence from pESGPK was inserted. The EIAV ProSavin genome plasmid pONY1.Blast was constructed by amplifying the blasticidin resistance gene (*bsr*) by PCR, using pBlasthEndo (InVivogen, Toulouse, France, cat. pblahendo) as a template. The PCR was performed using a forward primer incorporating a *Bgl*II site (5′-CGATA GATCTGTCTGACGCTCAGTGGAACG-3′) and a reverse primer incorporating a *Pci*I site (5′-CGATACATGT GAGCGATCGCAGATCCTTCG-3′). The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, cat. no. K2800-20). This plasmid was digested with *Bgl*II/*Pci*I and the *bsr* gene isolated and inserted into pONYK1[39] that had been digested with *Bsp*HI/*Pci*I thereby replacing the kanamycin resistance gene with the *bsr* gene.

Stable cell lines and drug selection

HEK293T-coTetR cell lines were generated by transfecting a 10 cm dish containing 3×10^6 HEK293T cells with 10 μg of *Ahd*I linearised pPuro-coTetR using Lipofectamine 2000 CD (Invitrogen, cat. no. 12566-101) according to the manufacturer's instructions. Media was replaced with conditioned media containing puromycin (0.6 μg ml^{-1}) 48 h post-transfection to select transfectants. Two weeks post selection individual clones were isolated from the mixed population by limiting dilution in conditioned media.

EIAV-VSV-G packaging cell lines were generated by transfecting 3×10^6 HEK293T-coTetR cells with 8 μg of *Ahd*I linearised pGagPol and 1 μg *Ahd*I linearised pVSV-G using Lipofectamine 2000 CD. At 48 h post-transfection cells were passaged into 6-well plates at a density of 1.5×10^5 cells per well. Cells were grown in conditioned media containing puromycin (0.6 μg ml^{-1}), hygromycin (300 μg ml^{-1}) or zeocin (400 μg ml^{-1}) for 4 days each. Cloning by limiting dilution in conditioned media was performed to isolate individual clones.

ProSavin producer cell lines were generated by transfecting 3×10^6 PC48.2 cells with 20 μg of *Bgl*II linearised pONY1.Blast using Lipofectamine 2000 CD. The media was replaced with conditioned media containing blasticidin S (20 μg ml^{-1}) 48 h post-transfection. One week after addition of blasticidin S the other selection antibiotics (hygromycin, zeocin and puromycin) were added to the culture medium. Two weeks post-selection individual clones were isolated by limiting dilution cloning in conditioned media.

Examining coTetR mediated regulation of gene expression in HEK293T-coTetR cells

To examine the ability of the HEK293T-coTetR cell line to regulate gene expression cells were transiently transfected with either pVSV-G or pGagPol. HEK293T-coTetR cells were seeded at 1.0×10^5 in 24-well plates and 24 h later transfected with either pVSV-G or pGagPol using Lipofectamine 2000 CD (Invitrogen, cat. no. 12566-101) according to the manufacturer's instructions. Briefly, 0.14 μg of plasmid was added to 10.4 μl OptiPRO (Gibco Invitrogen, Paisley, UK, cat. no. 12309-019) and this DNA mix was then added to a mix containing 0.9 μl Lipofectamine CD 2000 and 10.9 μl OptiPRO. At 14–18 h after transfection, sodium butyrate (Sigma, cat. no. B3887) was added to a final concentration of 10 mM and the media was changed 6–8 h later. At 21–23 h later the cells were harvested for VSV-G and Gag/Pol western blot analysis. Cell culture supernatants from pGagPol transfected cells were harvested and filtered through a 0.45 μm syringe filter for PERT analysis. When induction with dox was required it was added at the time of sodium butyrate addition, and at the 6–8 h media change. Further to these transfection experiments additional pPuro.coTetR plasmid was transfected at a ratio of 1:4 for pVSV-G (pPuro.TetR:pVSV-G) and 1:3 for pGag-Pol (pPuro.coTetR:pVSV-G). In addition, twice the amount (0.28 μg) of pVSV-G was transfected into HEK293T-coTetR cell line. As a control these transfection experiments were also performed in HEK293T cells.

Vector production

Transient transfection of HEK293T cells. Transient vector production was carried out by three plasmid co-transfections of HEK293T cells (seeded at 3.5×10^6 in 10 cm dishes 24 h earlier), using Lipofectamine 2000 CD

according to the manufacturer's instructions. The following quantities of plasmid were added to 340 μl OptiPRO: 4 μg genome plasmid (pONY8.4GCZ or pONYK1-ORT), 2 μg pESGPK and 0.08 μg pHG. This DNA mix was then added to a mix containing 25 μl Lipofectamine CD 2000 and 315 μl OptiPRO. At 14–18 h after transfection, sodium butyrate was added to a final concentration of 10 mM. Media was changed 6–8 h after sodium butyrate induction, and 21–23 h later vector was harvested and filtered through a 0.45 μm syringe filter. For vector production in 12-well plates quantities of cells, DNA plasmids and reagents were scaled according to surface area (a factor of 14.5).

Vector production from packaging cell lines. Vector production from packaging cells was performed as described above but only genome plasmid was transfected. When induction with dox was required it was added as described above.

Vector production from ProSavin producer cell lines. Cells were seeded at a density of up to 1×10^7 cells per 10 cm dish. The following morning cells were induced with sodium butyrate (10 mM) and dox (1 μg ml^{-1}) (when required), and 6–8 h later the media was replaced with media containing dox (1 μg ml^{-1}). Twenty-two hours later vector was harvested and filtered (0.45 μm).

Western blot analysis

Cells were lysed in fractionation buffer (0.1 M Tris.Cl, pH 7.3, 0.2% (v/v) Nonidet P40 (BDH, cat. no. 56009)) and 10 μg of total protein was loaded onto 4–20% polyacrylamide denaturing gels. Western blotting was performed by using a peroxidase conjugated anti-VSV-G antibody (Sigma, cat. no. A5977), or anti-Gag/Pol antibody (VMRD, Pullman, WA, U.S.A, cat. no. alpha-E1A) followed by a peroxidase conjugated anti-mouse secondary antibody (Dako, Ely, UK, cat. no. PO447). Visualisation was performed with ECL Advance Western Blotting Detection Kit (GE Healthcare UK Ltd, Little Chalfont, UK).

Quantification assays

Product enhanced reverse transcriptase assay. Product enhanced reverse transcriptase assay assay measures the amount of RT activity within vector preparations.[38] The RT associated with vector particles was released by treatment with a mild detergent (particle disruption buffer[40]) and used to synthesise cDNA using MS2 bacteriophage RNA (Roche, Burgess Hill, UK, cat. no. 165948) as template. The amount of cDNA resulting was proportional to the amount of RT released from the particles and was quantified using an ABI TaqMan 7900 quantitative PCR (qPCR) machine. The PCR mix contained TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Warrington, UK, cat. no. 4324018), 500 nm PERT forward (5′-TCCTGCTCAACTT CCTGTCGA) and reverse (5′-CACAGGTCAAACCTCC TAGGAATG) primers and 500 nm probe (5′-FAM-CGA GACGCTACCATGGCTATCGCTGTAG-TAMRA). The number of molecules of cDNA generated by the RT activity represents the relative number of vector particles present. An EIAV vector reference standard of known titre was included in each PERT assay and comparison of results from the unknown test vector samples to the vector standard allowed estimation to be made of the actual particle number, and reported as PERT predicted titre in transducing units per millilitre (TU per ml).

Biological titre assay. Biological titration of vector encoding the *lacZ* marker gene was performed by limiting dilution and transduction of canine osteosarcoma (D17) cells in the presence of polybrene (8 μg ml^{-1}; Sigma, cat. no. H9268). Cells were cultured for 2–3 days at 37 °C and then incubated in X-gal solution. Single transduction events, represented by a small colony of blue cells, were scored and the titre in TU per ml was calculated by multiplying the number of blue colonies by the dilution factor.

RNA genome assay. RNA genome assay quantifies the number of genomic RNA molecules within a given vector preparation by using qPCR technology and primers specific to the EIAV packaging signal sequence (Ψ).[38] It is important to appreciate that the number of genomic RNA molecules in a vector preparation does not necessarily equate to the number of functional vector particles. The RNA in the vector preparations was isolated (Qiagen, Crawley, UK, cat. no. 52906) and treated with DNaseI (Ambion, Austin, TX, U.S.A, cat. no. 1906) to remove contaminating DNA. Negative and positive (reference vector) controls were processed in parallel. A dilution series of an RNA standard of known copy number containing the EIAV Ψ was used to prepare a standard curve. The PCR mix consisted of one-step RT-PCR master mix containing RT and RNaseI inhibitor (Applied Biosystems, cat. no. 4309169), 500 nm EIAV Ψ forward (5′-ATTGGGAGACCCTTTGACATTG) and reverse (5′-ACCAGTAGTTAATTTCTGAGACCCTTGTA) primers and 500 nm EIAV Ψ probe (5′-FAM-CACCTT CTCTAACTTCTTGAGCGCCTTGCT-TAMRA). Parallel control reactions were set-up in which the RT was omitted to assess levels of DNA contamination. Samples were transferred to a 96-well optical plate and the RNA genome copy number (RNA copies per ml) was quantified using an ABI TaqMan 7900 qPCR machine.[38]

Integration (DNA) titre assay. Integration (DNA) titre assay provides a functional titre by measuring the number of integration-competent vector particles (transducing units) within a vector preparation. This assay uses a qPCR approach to quantify the EIAV Ψ that is present in transduced cells.[11] Analysis of ProSavin vector samples by DNA integration titre assay was performed by transduction of HEK293T cells in the presence of 10 μg ml^{-1} polybrene. A reference standard of known titre is included with each assay. Transduced cells were passaged three times and at day 10 the cells were harvested. Total DNA was extracted (Qiagen, cat. no. 51104) and the DNA concentration ascertained using a calibrated spectrophotometer, 150 ng of DNA sample was amplified in duplicate using an ABI TaqMan 7900 qPCR machine.[38] The PCR mix consisted of 1 × TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems, cat. no. 4304437) and EIAV Ψ primers and probe as described above. A stock of plasmid DNA was used to prepare standards of known copy number to prepare a standard curve. DNA prepared from HEK293T

cells containing a single EIAV vector copy was used as an internal reference standard. The predicted number of TU per ml of each sample was calculated from the standard curve. Titres were adjusted to account for the number of cells at the time of transduction and the dilution and volume of the vector applied.

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