

Comparison of DNA Delivery and Expression Using Frequently Used Delivery Methods

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1. Introduction

Ensuring an appropriate level and duration of expression is essential in achieving an efficient and safe gene therapy. The delivery of the therapeutic gene to target cells has to be sufficiently high to elicit a response, and minimum therapeutic thresholds may vary dramatically between therapeutic strategies. Delivery modalities can be broadly grouped into biological, chemical or physical methods. Biological modalities used in our laboratory include the viral vectors Adeno-Associated Virus and replication incompetent Adenovirus; physical modalities currently being studied include electroporation and sonoporation, while commercially available lipofection reagents are also widely used. Non-viral methods delivering plasmid DNA are argued to present a relatively safe alternative to viral vectors. They are less immunogenic, toxicity is generally very low, plasmid DNA has greater potential for delivery of larger genetic units and large-scale production is relatively easy. Physical methods such as electroporation have been utilised effectively for *in vivo* plasmid delivery to accessible tissues (Collins, C. G. et al. 2006; Mir 2008). Like electroporation, low intensity ultrasound, or sonoporation, can induce transient permeabilisation of the cell membrane and facilitates intracellular delivery of plasmid DNA (Larkin et al. 2008; Rome et al. 2008).

However, all the described methods have associated problems; the transfer of naked DNA is typically an inefficient process, with cell & tissue damage caused by administration of physical and chemical modalities, while adenoviral-mediated gene transfer is complicated by a host immune response to both the vector and transduced target cells (Jooss et al. 1998; Heller et al. 2008). In addition, only transient transgene expression is typically achieved by these approaches. Proposed causes of transient expression include loss of DNA due to cell turnover, immune responses against transfected cells and/or expressed proteins, and inhibition of transcription through host cell methylation of microbial DNA sequences (Prosch et al. 1996; Scheule 2000). AAV vectors have been shown not to elicit strong immune responses in general (Jooss, Ertl et al. 1998) (although immune responses against AAV have been reported following liver administration (Manno et al. 2006)) and the levels of transgene

expression following AAV mediated delivery have been shown to increase post delivery in heart, brain and muscle tissues *in vivo* (Lo et al. 1999; Vassalli et al. 2003; Collins, S. A. et al. 2008; Collins, S. A. et al. 2010). However, transduction is receptor dependant and rAAV has a size limit for DNA carrying capacity. Also, for therapeutic purposes, immediate gene expression is generally desirable. The optimal length of time for gene expression varies between therapeutic strategy and disease. For cancer gene therapy for example, short-lived transgene expression for cytokine production may be sufficient for immune sensitisation and containment of tumours (Collins, C. G., Tangney et al. 2006), while sustained secretion of therapeutic molecules is preferable with anti-angiogenic therapies (Malecki et al. 2005; Buhles et al. 2009).

The optimal gene delivery method for a given therapy will be dependant on tissue location, and type, as well as therapeutic strategy. While certain studies have been reported comparing the efficiencies from different vectors (Wang, A. Y. et al. 2004; Kealy et al. 2009), given the paucity of information regards direct comparisons between various delivery techniques, especially in the cancer setting, this study assesses the level and duration of reporter gene expression within target murine tissues when delivered by a range of commonly used gene delivery techniques; electroporation, sonoporation, lipofection, Adenovirus and Adeno-Associated Virus.

2 Materials and methods

2.1 DNA constructs

pCMV-luc plasmid, which expresses firefly luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter, was purchased from Promega (Wisconsin, USA). pCMV-LacZ plasmid, which expresses β -Galactosidase under the transcriptional control of the CMV promoter, was purchased from Plasmid Factory (Bielefeld, Germany). Plasmid concentration was determined using the Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Labtech Int, East Sussex, UK). Replication incompetent recombinant Adenovirus 5 particles encoding the luciferase gene under the transcriptional control of the CMV promoter were a kind gift from Prof. Andrew Baker, University of Glasgow, they were generated and titrated as described previously (Waddington et al. 2008). Replication incompetent Adenovirus 5 particles encoding the β -galactosidase gene under the transcriptional control of the CMV promoter were a kind gift from the Regenerative Medicine Institute, NUI Galway, they were generated and titrated as described previously (Sharif et al. 2006). An AAV plasmid expressing firefly luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter was constructed by first excising the firefly *luciferase* gene from pGL3 (Promega Medical Supply Company, Dublin, Ireland) using NcoI and XbaI, and cloning the Klenow enzyme treated fragment into the EcoRI and XbaI sites of pAAV-MCS plasmid (Stratagene, Agilent, Dublin) downstream of the CMV promoter. Inserts were confirmed by sequencing (MWG Biotech) and restriction enzyme analysis. This plasmid and the commercially available pAAVLacZ (Stratagene) were used to generate rAAV using the AAV Helper-Free System (Stratagene, Agilent, Dublin). The rAAV were purified using the Virakit AAV Purification Kit (Virapur, San Diego, USA) per manufacturer's instructions. Purified recombinant AAV-2 preparations were titrated using real time PCR. The samples were first pre-treated with DNase. For DNase digestion, 1 μ l of the viral sample was incubated with 350 Unit of DNase in a final volume of 10 μ l at 37 °C for 30 min followed by inactivation at 65 °C for 10 min. 1 μ l of Proteinase K (10mg/ml) was

added to each sample and it was incubated at 50 °C for 60 min followed by inactivation at 95 °C for 20 min. PCR was performed using the Lightcycler FastStart DNA Master Sybr Green system (Roche Molecular Biochemicals, Mannheim, Germany). PCR was carried out in a final volume of 20 µl using 0.5 µl of each primer (0.25 µM), 3 mM MgCl₂ and 2 µl of the template. The PCR was performed in a lightcycler (Roche) with a 10 min pre-incubation at 95 °C followed by 40 cycles of 15 s at 95 °C, 4 s at 56 °C, 4 s at 72 °C. PCR products were subjected to melting curve analysis using the light cycler system to exclude the amplification of unspecific products. The PCR products were analysed by conventional agarose gel electrophoresis. Primers were synthesized by MWG Biotech, Germany. The following primers were used to detect the CMV promoter sequence, forward: 5' aaatgggaggtaggcgtgta 3', reverse: 5' gatcgggtcccgtgtctct 3'. A fragment of length 124 bp is expected using the primers.

2.2 Cell lines and tissue culture

Murine JBS fibrosarcoma tumour cells (Collins, C. G., Tangney et al. 2006) and murine CT26 colonic adenocarcinoma cells (obtained from ATCC) were maintained in culture at 37 °C in a humidified atmosphere of 5 % CO₂, in Dulbecco's Modified Essential Medium (GIBCO, Invitrogen Corp., Paisley, Scotland) supplemented with 10 % iron-supplemented donor calf serum (Sigma Aldrich Ireland, Ireland), 300 µg/ml L-glutamine, and 10 mM HEPES (1-Piperazineethane sulfonic acid, 4-(2-hydroxyethyl) monosodium salt), (Sigma Aldrich Ireland, Ireland) pH 7.4. The murine MGC8 gastric carcinoma cell line was kindly provided by Dr. Robert Kammerer, Ludwig-Maximilians-University, Germany (Nockel et al. 2006), and was maintained in RPMI (Roswell Park Memorial Institute- Gibco) medium supplemented with 10 % iron-supplemented donor calf serum (Sigma Aldrich Ireland, Ireland) and 1mM sodium pyruvate (Sigma Aldrich Ireland, Ireland). Cell densities were determined by visual count using a haemocytometer. Cells were at 80 % confluency on the day of transduction *in vitro*. Cell viability was confirmed by Trypan Blue Dye Exclusion (Sigma Aldrich Ireland, Ireland) to be > 95 % for tumour inoculation.

2.3 *In vitro* gene delivery

The efficacies of the different delivery methods *in vitro* were determined using the JBS, CT26 and MGC8 cell lines. Transductions were carried out accordingly to manufacturers' protocols or under optimal conditions where appropriate. Electroporation: 10 µg pCMV-Luc or pCMV-LacZ DNA was added to 1 X 10⁶ cells in 100 µl ZAP buffer (250 mM sucrose, 10 mM K₂HPO₄ 1 mM MgCl₂ ph 7.4). Electroporations were carried out in a 1.0 mm cuvette and the conditions used were 8 X 0.1 ms pulses of 140 V with a 0.1 sec pulse interval. Cells were incubated at room temperature for 15 min. Following the addition of growth medium the treated cells were seeded into a tissue culture dish and incubated for 24 h at 37 °C, 5 % CO₂. Ultrasound: 1 X 10⁶ cells were seeded in a 6-well tissue culture dish 3 h prior to treatment. Prior to US application, the medium was replaced with serum free medium and 10 µg of the pCMV-Luc or pCMV-LacZ plasmid was added. The US conditions used were 1.0 W/cm², 20 % duty cycle, 2 min. Cells were incubated for 15 min before replacing serum free medium with supplemented medium and cells incubated for 24 h at 37 °C 5 % CO₂. Lipofectamine2000: Cells were seeded in a 12-well plate in complete medium 24 h before transfection. On the day of transfection cells were 80 % confluent. Prior to transfection, the cells were rinsed and incubated in serum free medium. 1.6 µg pCMV-Luc or pCMV-LacZ DNA was complexed to Lipofectamine2000 (Invitrogen, Biosciences Ltd, Dublin, Ireland),

according to manufacturer's instructions and incubated with cells for 4 h. Serum free medium was then replaced with complete medium and cells incubated for 24 h 37 °C, 5 % CO₂. AAV: Growth medium was removed and replaced with 0.5 ml AAV permissive growth medium (DMEM, 0.5 mM Tyrphostin (Calbiochem, Merck, UK; (Mah et al. 1998)) per well. The plates were incubated for 2 h at 37 °C, 5 % CO₂. Permissive medium was subsequently removed and cells washed twice with 0.5 ml/well DMEM. 5 X 10⁸ genome copies (GC) of AAVCMVLuc or 3.5 X 10¹⁰ GC of AAVCMVLacZ in a 0.5 ml volume of transduction medium (DMEM, 2 % FBS) was added to individual wells containing the permissive cells. The plates were incubated for 2 h at 37 °C, 5 % CO₂ with gentle rocking at 30 min intervals during the incubation. 0.5 ml post infection medium (DMEM, 18 % FBS) was added to each well and incubated at 37 °C, 5 % CO₂ for a further 24 h. Ad: 1.5 X 10⁹ viral particles (VP) of AdCMVLuc or 4.5 X 10⁸ VP of AdCMVLacZ in 0.5 ml transduction medium (DMEM, 2 % FBS) was added to individual wells of 6-well plates containing the cells. Plates were incubated for 2 h at 37 °C, 5 % CO₂ with gentle rocking at 30 min. 0.5 ml post infection medium (DMEM, 18 % FBS) was added to each well and incubated at 37 °C, 5 % CO₂ for a further 24 h.

2.4 *In vitro* luciferase assay

Treated cells were analysed for luciferase activity using the Luciferase Assay System (Promega MSC, Dublin) 24 hr post transfection. Treated cells were counted and resuspended to 10⁴ cells in 50 µl DMEM medium. 50 µl 1X lysis buffer was added to each of the samples and incubated for 5 min at room temperature. 100 µl Luciferase assay reagent was then added to each sample and the luminescence was measured with a Junior LB 9509 luminometer (Berthold Technology, Promega MSC, Dublin).

2.5 *In vitro* β-galactosidase assay

Treated cells were analysed for β-Galactosidase activity using the Roche β-Gal Staining set (Roche Diagnostics GmbH, Penzberg, Germany) as per the manufacturer's protocol. Briefly, cells were washed with PBS, and incubated in fixative (2% formaldehyde, 0.2 % gluteraldehyde in PBS) for 15 min. Cells were incubated in staining solution o/n at 37 °C. Cells were analysed in PBS under a light microscope and transfection efficiency (% stained cells) was calculated from 10 random viewing fields per well.

2.6 DNA/RNA extraction

Transfections were carried out as previously outlined with the CMVLacZ constructs. At 24 hr post transfection, cells were harvested for simultaneous DNA/RNA extraction using the Qiagen Allprep DNA/RNA Kit (Qiagen Crawley, West Sussex). Briefly, treated cells were counted and resuspended in 350 µl of Buffer RLT containing β-mercaptoethanol and vortexed. The DNA and RNA extraction was carried out as per the manufacturer's protocol. 5 µg RNA was treated with DNase 1 (DNAfree, Ambion) to remove contaminating genomic DNA. cDNA synthesis was carried out using 500 ng of the DNase treated RNA with the Qiagen Omniscript RT kit, per manufacturer's instructions. The resulting cDNA was brought to a 50 µl volume using nuclease free water.

2.7 Quantitative real-time PCR

PCR was performed using the Lightcycler FastStart DNA Master Sybr Green system (Roche). PCR was carried out in a final volume of 20 µl using 0.5 µl of each primer (0.25 µM),

3 mM MgCl₂. PCR was performed in a lightcycler (Roche) with a 15 min pre-incubation at 95 °C followed by 40 cycles of 15 s at 95 °C, 5 s at 60 °C, 5 s at 72 °C. PCR products were subjected to melting curve analysis using the light cycler system to exclude the amplification of unspecific products. PCR products were analysed by conventional agarose gel electrophoresis. Primers were synthesized by MWG Biotech. The following primers were used to detect the *LacZ* sequence, forward: 5' GCGTGGATGAAGACCAGC 3' and reverse: 5' CGAAGCCGCCCTGTAAAC 3'. A standard curve was generated using the pCMV-LacZ plasmid DNA, ranging from 5 X 10³ to 5 X 10⁷ plasmid copies. 50 ng of DNA or 5 µl of cDNA reaction from each delivery method was used for the real time PCR detection of LacZ.

2.8 Animals and tumour induction

All murine experimentation was approved by the University College Cork Animal Ethics Committee. Mice were obtained from Harlan Laboratories (Oxfordshire, England). They were kept at a constant room temperature (22 °C) with a natural day/night light cycle in a conventional animal colony. Standard laboratory food and water were provided *ad libitum*. Before experiments, the mice were afforded an adaptation period of at least 14 days. Female Balb/C mice in good condition, without fungal or other infections, weighing 16–22 g and of 6–8 weeks of age, were included in experiments. For routine tumour induction, 2 × 10⁶ JBS cells suspended in 200 µl of serum free DMEM were injected subcutaneously into the flank.

2.9 *In vivo* gene delivery

Mice were randomly divided into experimental groups and subjected to specific experimental protocols. For tumour experiments, mice were treated at a tumour volume of approximately 100 mm³ in volume (5–7 mm major diameter). For liver transfection, a 1 cm subcostal incision was made over the liver and the peritoneum opened. The proximal portion of the liver was exposed and DNA administered as described below. The wound was closed in two layers, peritoneal and skin, using 4/0 prolene sutures (Promed, Killorglin, Ireland). For muscle experiments, a single intramuscular injection was carried out into the right or left quadriceps muscle of the animal. Mice were anaesthetized during all treatments by intraperitoneal (IP) administration of 200 µg xylazine and 2 mg ketamine.

For plasmid delivery by electroporation, a custom-designed applicator with 2 needles 4 mm apart was used, with both needles placed central to the tissue. Tissue was injected between electrode needles with plasmid DNA in sterile injectable saline in an injection volume of 50 µl. Concentration of plasmid was adjusted to administer 4 × 10¹² gene copy numbers. After 80 seconds, square-wave pulses (1200 V/cm 100 µsec × 1 and 120 V/cm 20 msec, 8 pulses) were administered in sequence using a custom designed pulse generator (Cliniporator (IGEA, Carpi, Italy).

For plasmid delivery by ultrasound, tissue was injected with plasmid DNA as above. The ultrasound probe was then applied to the tissue and ultrasonic waves delivered at 1.0 W/cm², 20 % duty cycle for 5 min (Sonoporator, Sonidel, Dublin, Ireland).

For plasmid delivery using Lipofection, tissue was injected with plasmid DNA/Lipofectamine 2000 complex in an injection volume of 100 µl. Concentration of plasmid was adjusted to administer 4 × 10¹² gene copies.

Viral vector particles were administered by direct intratumoural, intramuscular or intra-hepatic injection in a volume of 50 µl. 2 × 10⁸ – 2 × 10⁹ GC of replication incompetent recombinant AAV2 particles, or 1 × 10⁹ VP of replication incompetent recombinant Adenovirus 5 particles were used per administration.

2.10 Whole body luciferase imaging

In vivo luciferase activity from tissues was analysed at set time points post-transfection as follows: 80 μ l of 30 mg/ml firefly luciferin (Biosynth, Basil, Switzerland) was injected IP (for muscle and liver experiments) and/or intratumourally. Mice were anaesthetised as before. Ten minutes post-luciferin injection, live anaesthetised mice were imaged for 3 min at high sensitivity using an intensified CCD camera (IVIS Imaging System, Xenogen, UK). The exposure conditions were maintained at identical levels so that all measurements would be comparable. Data analysis was carried out on the Living Image 2.5 software package (Xenogen, UK). Luminescence levels were calculated using standardised regions of interest (ROIs) for all three anatomical areas. Actual levels were obtained by subtracting the corresponding ROI of an untransfected mouse to account for background luminescence. For comparison between vectors, luminescence was represented as p/sec/cm²/sr/gene copy.

2.11 Statistical analysis

The primary outcome variable of the statistical analyses was luminescence per cell per gene copy administered in each cell line or luminescence per gene copy administered in each organ measured at each time point. The principal explanatory variables were the delivery modalities used. *In vivo* luminescence was analysed as continuous. At specified time points, a two-sampled t-test was used to compare mean luminescence per gene copy administered for each delivery modality. Microsoft Excel 11.0 (Microsoft) and GraphPad Prism Version 4.0 (GraphPad Prism Software Inc, San Diego, CA, USA) were used to manage and analyze data. Statistical significance was defined at the standard 5 % level.

3. Results

3.1 Comparison of transgene expression levels *in vitro*

Reporter gene expression was analysed following *in vitro* delivery of CMV firefly luciferase gene cassette by the various delivery systems to JBS fibrosarcoma cells. The optimal protocol for each delivery method was utilised in all *in vitro* experiments. Data were standardised by reporting results for each system as expression/cell at time of administration/gene copy administered. The results displayed in Figure 1a correspond to expression from viable cells at time of assay (24 h post-delivery) and cell cytotoxicity relating to transfection is not taken into account. Cell death resulted in 99.8 % and 89 % cells exposed to US and EP respectively, while no significant cell death was observed in the course of Ad, AAV or Lipofectamine transfections (data not shown). Reproducible levels of luciferase expression per cell per gene copy resulted from the various methods with expression from AAV achieving the highest levels (AAV > Ad > Lipo > EP > US).

In order to assess consistency across cell lines, delivery to a range of tumour cell models was examined. Efficiencies arising from Lipofectamine, Ad and AAV delivery to JBS was compared with those from CT26 and MGC8 (Nockel, van den Engel et al. 2006). Cancer cell type-specificity was clearly observed for each vector (Figure 1b). AAV also achieved the highest levels of expression in CT26 cells as with JBS cells. However, AAV failed to transduce MGC8 cells. There were also considerable relative differences observed in Lipofectamine transfected cells ($p < 0.05$), while Ad delivery resulted in expression in all cell lines, albeit with statistically significant variation between each ($p < 0.05$).

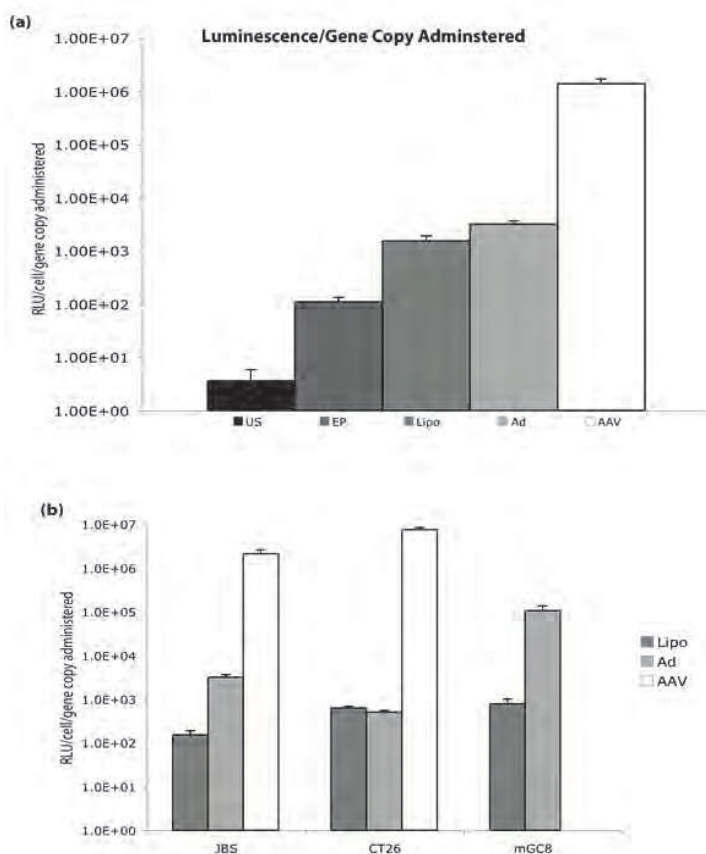


Fig. 1. *In vitro* transfection/transduction (a) Comparison of transgene expression in JBS cells *in vitro* pCMV-Luc was delivered to JBS cells *in vitro* using US, EP or Lipofection, while the corresponding CMVLuc cassette was delivered via Ad (AdCMVLuc) or AAV2 particles (AAVCMVLuc). Luciferase expression was assayed 24 h post-delivery and expressed as relative light units (RLU) per cell per gene copy. Data represent the mean \pm S.E. of triplicate values. Intra-modality differences were statistically different in all cases ($p \leq 0.02$). (b) Cell line specificity in transfection/transduction Average luminescence arising from Lipo, Ad and AAV delivery to JBS, CT26 and mGC8 are shown. There was significant variation between cell line efficiencies for each vector (Ad $p < 0.05$; Lipo $p < 0.05$; AAV $p < 0.0001$). AAV failed to transduce MGC8 cells. Data represent the mean \pm S.E. of triplicate values.

3.2 Analysis of DNA delivery and transcription efficiencies *in vitro*

The CMV-*LacZ* gene cassette was delivered to JBS cells *in vitro* using each of the delivery methods. To determine percentages of cells transfected/transduced by each method, treated cells were fixed 24 h post delivery and stained for β -Galactosidase activity. The number of positive cells was expressed as a percentage of the total number of cells (Figure 2a). Viral

and chemical methods of delivery resulted in significantly higher proportions of reporter gene expressing cells in comparison with the physical methods (ultrasound and electroporation) ($p < 0.02$), possibly reflecting the high cell death rates associated with these systems. Again, AAV resulted in the highest number of transduced cells in comparison with other methods.

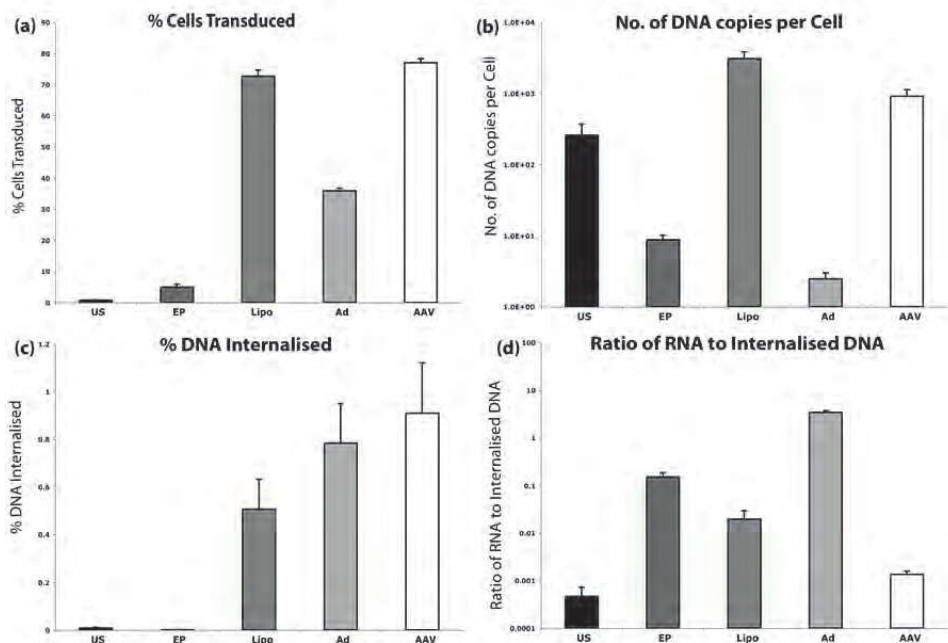


Fig. 2. *In vitro* DNA delivery and transcription efficiencies (a) Percentage of transgene-expressing cells pCMV-LacZ was delivered to JBS cells *in vitro* using US, EP or Lipo, while the corresponding CMVLacZ cassette was delivered via Ad (AdCMVLacZ) or AAV2 particles (AAVCMVLacZ). β -Galactosidase expression was assayed 24 h post-delivery and expressed as % cells transfected/transduced. (b) Number of transgene copies per cell The number of transgene copies in total DNA from a known number of cells was quantified using real time PCR 24 h post-delivery and expressed as the number of transgene copies per cell. (c) Efficiency of DNA delivery to cells The number of transgene copies in total DNA was quantified using real time PCR and was expressed as a percentage of the total number of copies administered for each delivery method. (d) Transcription Efficiency Quantitative real time PCR was used to determine the number of copies of transgene mRNA 24 h post-delivery. This was expressed as a percentage of the number of internalised DNA copies. For (a-d) data represent the mean \pm S.E. of triplicate values.

In order to assess and compare DNA entry efficiency and subsequent transcription efficiencies for each delivery method, LacZ reporter gene DNA and mRNA was quantified by PCR. Prior to DNA/RNA extraction at 24 h post delivery, the number of cells was determined using trypan blue exclusion. The total number of LacZ DNA copies was

expressed per cell at 24 h (Figure 2b). The highest number of DNA copies per cell was observed with Lipo, followed by AAV, which were both significantly higher than US, EP and Ad methods ($p < 0.02$). Lipofection delivery does not involve physical generation of pores required by the other plasmid methods for delivery, nor is it dependent on the presence of cell surface receptors (e.g. CAR), which may be poorly expressed in certain cancers, which may in turn explain the poor Ad uptake by JBS cells observed here. The efficiency of DNA entry to cells was also calculated by comparing the number of transgene copies in extracted DNA and expressing it as a percentage of the number of transgene copies initially administered (Figure 2c). Results correlated with the above % cell transfection data, with viral and chemical methods displaying the highest efficiencies of gene delivery to cells in comparison with the physical modalities ($p < 0.02$). There was no significant difference between AAV, Ad and Lipo methods ($p > 0.05$).

Transcription efficiency was determined using qPCR analyses on LacZ DNA and mRNA (Figure 2d). The number of copies of transgene mRNA 24 h post-delivery was expressed as a percentage of the number of internalised DNA copies. Ad resulted in significantly higher ratio of mRNA:DNA compared with all other delivery methods ($p < 0.01$). There was no significant difference in transcription efficiencies between the remaining delivery methods. With US and Lipo, while these methods may efficiently mediate delivery of plasmid DNA to the cytoplasm, subsequent trafficking to the nucleus and transgene transcription is not ensured. For AAV, the low level observed can be attributed to the rate limiting step associated with AAV mediated expression, involving synthesis of double stranded DNA from the single stranded genome prior to transcription (Ferrari et al. 1996). EP transcription efficiency was also significantly higher than US, AAV and Lipo ($p < 0.02$). The combination of high and low voltage pulses used for electroporation here is believed to create transient pores in both the cell and nuclear membranes enhancing DNA entry and subsequent nuclear localisation (Gothelf & Gehl ; Chang 1992).

3.3 Duration of transgene expression in tumour, liver and skeletal muscle *in vivo*

In vivo luciferase expression was examined from murine liver, quadriceps muscles and subcutaneous JBS tumour (following consistent results with the JBS cell line *in vitro*) using IVIS whole body imaging at various time-points up to 250 days post delivery with Ad, AAV or plasmid. Vector related luminescence expressed as a percentage of maximum expression observed for that vector is graphed in Figure 3. Background thresholds for each vector varied as a result of expressing per gene copy or as a percentage of maximum, and while imaging continued for the full duration of experiments, values below background are not shown. All plasmid methods (Lipo, US and EP) displayed similar time-points of maximal luminescence within the first month (data not shown), and only EP is shown as representative for plasmid.

Luminescence from plasmid and Ad reduced dramatically within 48 h post delivery to tumour and liver (Figure 3), and both plasmid and Ad reduced to background levels from day 7 in tumour, and day 14 (EP) or day 21 (Ad) in liver. Day 5 was the earliest practical time point for imaging of AAV for reasons including safety guidelines for animal experimentation with this vector. AAV-related expression also decreased in tumour, to background levels by day 16-post administration. However, a different pattern of transgene expression was observed for AAV in liver and quadriceps muscle, with an overall increase in luminescence over time. When muscle related expression was examined with plasmid,

prolonged sustained luminescence was observed, with equivalent expression seen at day 370 and day 18 post electroporation (data not shown). However, unlike plasmid, complete loss of Ad mediated luciferase activity was observed when muscle was examined, with Ad expression increasing up to day 7, before reducing to background levels by day 21.

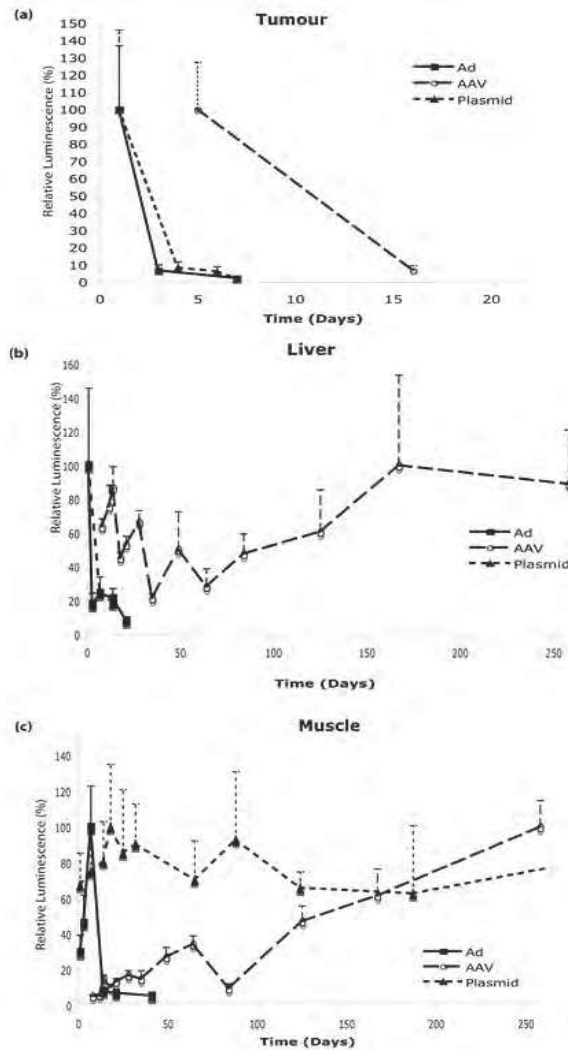


Fig. 3. Vector specific kinetics of transgene expression in tumour, liver and muscle *In vivo* luciferase expression from (a) tumour, (b) liver and (c) muscle was assessed using live whole body imaging (IVIS) at various time-points up to 250 days post delivery ($n \geq 3$). Relative average luminescence as a percentage of maximum expression for each vector is shown. Values below appropriate background for each vector are not shown. Data represent the mean \pm S.E.

3.4 Comparisons between transgene expression levels in tumour, liver and skeletal muscle *in vivo*

To directly compare *in vivo* expression levels between vectors, the time-point of maximum luminescence in a given tissue within one month post gene delivery was used. Luminescence/gene copy in JBS tumour, liver and quadriceps muscles from each vector at the appropriate time-point is displayed in Figure 4.

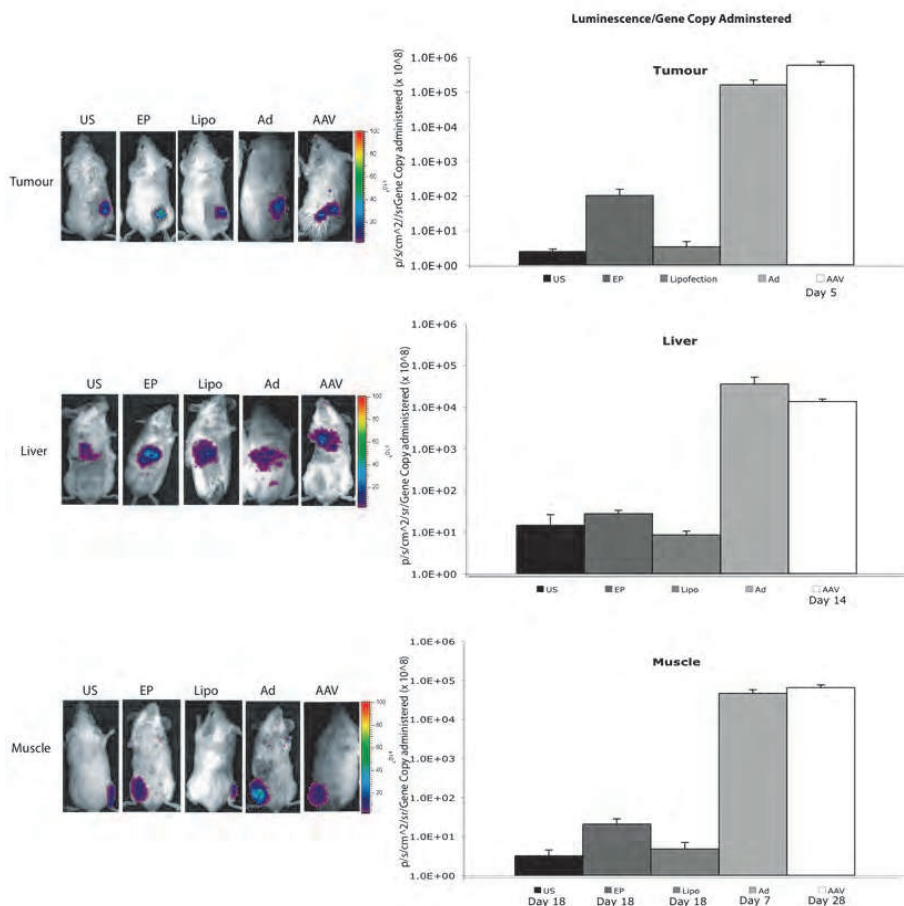


Fig. 4. *In vivo* transfection efficiency Vector constructs were examined *in vivo* in murine subcutaneous JBS tumour, liver and quadriceps muscle ($n \geq 3$). Luciferase expression *in vivo* was assessed using IVIS whole body imaging, and maximum gene expression level achieved within 1 month post administration is reported in all cases, corresponding to 24 h unless otherwise stated. Resulting average luminescence per gene copy is shown. Data represent the mean \pm S.E.

It can be seen that the intra-modality pattern of expression differed from that observed *in vitro*, with AAV and Ad resulting in the highest expression in all tissues *in vivo*, while EP

provided the highest non-viral mediated expression. Significant intra-tissue variation in luciferase activity was observed for all vectors (Figure 5). Tumour produced the highest luminescence for EP and both viral vectors, while liver produced highest expression from US and Lipo. The least consistent results (largest standard error of the mean) were observed with the mechanical methods US and EP. It should be noted that firefly luciferase associated luminescence is dependent on the availability of luciferin substrate and oxygen to cells, which may vary between tissues. However, excess substrate was administered in all experiments in an attempt to achieve saturation levels, and the finding that each vector displayed unique intra-tissue patterns of expression supports the validity of the findings.

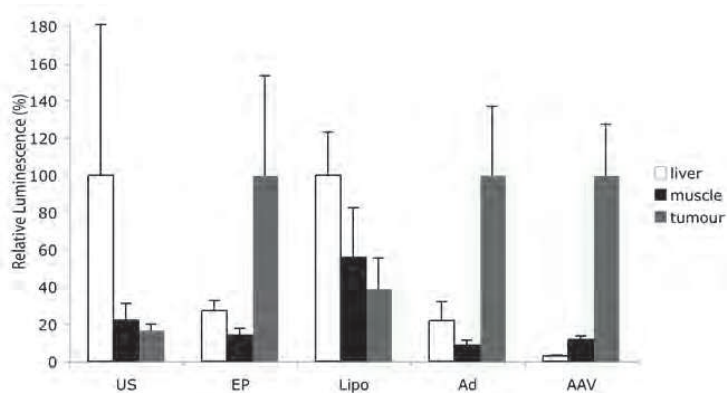


Fig. 5. Intra-tissue variations in reporter gene expression *In vivo* luminescence from liver, muscle or JBS tumour is represented according to the method of gene delivery. Relative average luminescence as a percentage of maximum expression (time-points as per figure 3) for each vector is shown. Data represent the mean \pm S.E (n \geq 3).

4. Discussion

The method employed to deliver genes of interest is the primary parameter related to expression in a target tissue, and consequently has important therapeutic implications. Our findings delineate the relative efficiencies of five well-described delivery modalities, and highlight target organ/tissue specific variations in transfection capability. Furthermore the kinetics of gene expression arising from each modality were compared.

In order for data to be generated *in vitro*, it was necessary to employ different parameters for various delivery methods. Different quantities of DNA and cell numbers were used, and typhostin was added to medium to aid in *in vitro* AAV transduction. Attempts to standardise these experimental parameters produced no reportable data, as a result of total cell killing and/or lack of transfection. Numerical results were instead standardised post experimentation, and all *in vitro* results were reported as expression/administered cell/gene copy administered. We standardized the quantity of vector employed in each case by utilising total vector copy numbers for all vectors, as per Materials and Methods. As such, both viable and non-viable gene constructs are factored in results, and take into account non-infectious viral particles and naked plasmid DNA, which does not successfully enter cells (e.g. due to degradation by extracellular nucleases). Determination and use of

productive viral vector particle numbers increases resulting efficiency rates of DNA cell entry (data not shown).

Given the mechanism of AAV single-stranded DNA virus transduction of cells, it is likely that at the time of measurement of AAV-transduced cells *in vitro* (24 h), second strand synthesis was not completed and protein production had yet to reach maximum levels. The majority of cells were killed by the physical methods *in vitro*, most likely due to heat and physical stresses. Given the distance from clinical reality of the *in vivo* tissue situation, and the known cytotoxic effects of the non-viral modalities examined here, *in vitro* experiments do not accurately represent the more cell protective environment of tissue, since three-dimensional structural architecture, blood flow etc. are not represented. Furthermore, *in vivo* targets are not homogenous populations, particularly in the case of tumours, and gene expression observed from tumours most likely does not represent expression solely from tumour cells, as various stromal and phagocytic cells may also take up DNA.

Uniform conditions were used for all tissues in this study. In the absence of titrations to determine the precise optimal parameters for each method for each target cell or tissue, we cannot rule out that the relative differences reported between vectors *in vitro* may change following targeted optimisation for each vector.

Considerable variation in transgene expression was observed between modalities and cell types. Lipofectamine consistently transfected all cell types examined *in vitro*. It does not have the same cell killing effects associated with EP and US nor is it dependent on cell surface receptors for internalisation like the viral methods. Ad transduced all cell types examined with varying degrees of efficiency, correlating with the ubiquitous expression of the CAR receptor at different levels in cells (Lyons et al. 2006). The finding that AAV2 was unable to transduce all cancer cell types examined, is consistent with a lack of expression of its primary receptor, heparin sulfate proteoglycans (HSPG), which may be absent or deleted on certain tumour types (Summerford & Samulski 1998), and/or CD9, which can mediate AAV-2 infection in certain cancer cells in the absence of HSPG (Kurzeder et al., 2007). The HSPG and CD9 status of the tumour cell lines used in this study is unknown. Several serotypes of AAV exist and corresponding tissue tropism varies considerably (Wu et al. 2006). The AAV2 serotype examined here is known to transduce a wide range of tissue types, including liver and muscle, albeit at a lower efficiency than other serotypes more specific for individual cell types. Therefore, use of an AAV serotype with a high tropism for the target tissue would be expected to produce efficiencies higher than reported here for AAV, in addition to providing a level of selectivity in terms of vector safety. "Cross-packaging" strategies to generate pseudotyped AAV vectors, where AAV2 vector genome is packaged together with capsid proteins of a different serotype, have been shown to improve target specificity and efficiency (Harding et al. 2006; Nathwani et al. 2008).

<i>In vitro</i> analyses at 24 h	% Cell Transfection	DNA Delivery	Copy Number	Transcription Efficiency	Net Expression
AAV	High	High	High	Low *	High
US	Low	Low	Medium	Low	Low
EP	Low	Low	Low	Medium	Medium
Lipo	High	Medium	High	Low/Medium	Medium
Ad	Medium	High	Low	High	Medium/High

* As measured at 24 h

Table 1. Relative efficiencies of DNA delivery and subsequent expression *in vitro*.

Efficient reporter gene expression requires cell and nuclear DNA molecule entry, followed by transcription, translation and enzymatic activity on substrate. We analysed various steps for each delivery modality *in vitro*. Table 1 highlights the combined findings from *in vitro* analyses of DNA delivery, transcription and expression efficiencies. It is evident from these studies that while cell entry is an obvious prerequisite, the efficiency of nuclear localisation and transcription may be the major rate limiting step for all methods, as exemplified by the high expression observed with viruses examined here, both capable of mediating their entry to the nucleus. With plasmids, poor nuclear uptake through the restrictive nuclear membrane pore limits expression even when high copy numbers are delivered to the cytosol (e.g. lipofection). The inclusion of nuclear localisation sequences to plasmid DNA has been shown to improve plasmid transgene expression (Manam et al. 2000). Regional variation in cell mitotic rates may also affect plasmid nuclear uptake and subsequent gene expression *in vivo*, particularly in tumours. Reporter gene associated luminescence was utilised *in vivo* as a measure of transfection efficiency of these vectors. Since multiple factors influence transgene expression *in vivo*, differences in luminescence observed between tissues may reflect variations in cell turnover, CMV promoter activity or local immune activity, in addition to the above described *in vitro* factors.

There were significant inter-vector and inter-tissue variations in the times at which highest luminescence values were observed *in vivo*. Since maximum luciferase expression levels afforded by AAV were not observed for many months post transduction of muscle and liver, reporter gene expression levels observed for each modality at times of maximal expression within the first month post administration were used to provide indicative expression levels for comparison purposes here. Firefly luciferase protein has previously been shown to have a short half-life *in vivo*, in the region of 1 - 4 hours, and any luminescence detected in our experiments was due to recently transcribed gene (Baggett et al. 2004). Both Ad and plasmid-based gene delivery systems have previously been shown to provide short-term gene expression in several tissues (Jooss, Ertl et al. 1998; Wang, L. et al. 2005). Increasing expression profiles for AAV2 have previously been reported (Lo, Qu et al. 1999; Vassalli, Bueler et al. 2003), and sustained AAV expression is proposed to be mediated by episomal persistence of the vector (Flotte et al. 1994). Since all the vector systems examined in this study are non-integrating, loss of transgene with cell division may be the major reason for reductions in expression loss in transfected/transduced cells, especially in rapidly growing tumours. Inhibition of transcription through host cell methylation of viral promoter DNA sequences can also lead to transient expression (Di Ianni et al. 1999; Al-Dosari et al. 2006). Given that luciferase transcription from all constructs examined in this study relied upon the CMV promoter, such activity is likely to have been involved with all vectors examined in tumour and liver. While Ad delivered transgene expression levels in skeletal muscle have been demonstrated to reduce as a result of immune clearance of transfected cells (Jooss, Ertl et al. 1998), muscle related expression has been shown to increase over time with both AAV and plasmid vectors, albeit in a gene product dependent manner, with certain proteins eliciting silencing immune responses (Mir et al. 1999; Yuasa et al. 2002). Luciferase protein has low immunogenicity, and immune responses are believed not to occur in mice (Davis et al. 1997).

While the patterns of luciferase expression from Ad and plasmid were similar in tumour and liver, this was not the case for muscle. It has previously been demonstrated with Ad gene delivery to muscle, that associated Ad transduction of Dendritic Cells resulted in presentation of transgene as antigen and subsequent T cell elimination of transgene

expressing muscle cells (Jooss, Ertl et al. 1998). When we examined Ad mediated expression in quadriceps of athymic mice, no reduction was evident for up to 2 months, unlike in immunocompetent Balb/C mice where luminescence was absent from day 21-post muscle transduction (figure 3d). This suggests the involvement of T-cell inactivation of adenoviral-transduced cells in immunocompetent mice. Plasmid electroporation, on the other hand, has been shown not to elicit such transgene silencing immune responses (Vicat et al. 2000), and presents an attractive option in achieving long-term gene expression, especially in light of recent improvements in plasmid vectors (Gill et al. 2009).

Although Ad provided the highest immediate gene expression in all tissues, the potential for high level expression from AAV is highlighted in this study, with AAV2 providing expression in the same order of magnitude as Ad within the first month post administration, increasing to higher levels over time. Sustained transgene expression is desirable for many therapies and there is also potential to overcome transient expression from plasmids by inclusion of integrating transposon or S/MAR elements (Gill, Pringle et al. 2009). EP yielded the highest transfection among the non-viral techniques, in all tissues, unlike *in vitro* where lipofection displayed optimum efficiency of plasmid delivery, validating the common use of lipofection for *in vitro* laboratory transfections. Identical EP conditions were used for all experiments in this study. However, EP parameters can be optimised for specific tissue/cell types, and higher efficiencies than reported here might be achieved by use of cell-specific parameters (Mir 2008). This may also be true for sonoporation. Sonoporation is the least characterised of these plasmid delivery systems, and the finding that gene expression resulted in all situations examined, albeit it lower than the other non-viral methods, indicates potential for this strategy, especially given the attractive possibility of focusing ultrasound beams externally on internal organs. However, the lack of consistent levels of transfection observed (figure 4) highlight the need for technological improvement. Use of US in combination with 'micro-bubble' technology may increase the efficacy of transfection and enable localisation of systemically administered DNA complex through focussed US mediated sonoporation (Shimamura et al. 2004).

Overall, the data generated here clearly define the relative efficiencies of the various delivery systems in a wide range of tissues *in vivo*, and a range of tumour cell lines *in vitro*, providing the researcher with valuable information to aid in design of experimentation and clinically applicable strategies for gene therapies. Vectors achieve gene delivery with different efficiencies depending on the target cells. As such, the optimal conditions for delivery to one target may differ completely to another target. This study aims at comparing the maximum protein produced by the vectors, but this does not mean that with further optimization, it is not possible to achieve a higher level of expression.

5. Conclusion

The results clearly define the relative efficiencies of these delivery systems in a range of situations, providing researchers with valuable information to support vector choice in therapeutic strategy design.

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