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Plasmid Transgene Expression *in vivo*: Promoter and Tissue Variables

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1. Introduction

Ensuring an appropriate level and duration of expression is essential in achieving an efficient and safe gene therapy. While the length of time a gene must be expressed for efficacy depends on both the therapeutic strategy and the disease, many gene therapy approaches prove ineffective as the therapeutic is expressed for a limited duration (Frank et al. 2004). 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; Greenland et al. 2007). Vector related elements or activity also contribute to duration of gene expression post administration. Adenovirus is known to stimulate severe innate and adaptive immune responses, and can induce cellular and humoral responses to the transgene product and its capsid proteins resulting in failure to provide long-term gene expression (Jooss et al. 1998; Yuasa et al. 2002; Louboutin et al. 2005; Wang et al. 2005).

Plasmid electroporation, on the other hand, has been shown not to elicit such transgene gene silencing immune responses (Jooss et al. 1998; Mir et al. 1999), 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 plasmid based systems offer certain advantages, they do, however, have drawbacks. The magnitude of transgene expression is generally lower with plasmid vectors than that with viruses. In addition, most plasmids are not passed on to daughter cells following cell division leading to eventual loss of expression in rapidly dividing tissues. This can result in sub-therapeutic effects, a significant problem

with gene therapy. Efforts have been made to ensure that therapeutic protein production is active for an appropriate length of time to address some of these failings. To counteract the effects of episomal DNA loss, the use of integrating DNA in the form of retroviruses or transposon containing plasmids has been examined and shown some efficacy (Sandrin et al. 2003; Ohlfest et al. 2005). Delivery in this fashion would lead to long lasting, possibly indefinite gene expression. Although this addresses one failing of plasmid delivery, the potential of indefinite and uncontrollable protein production to cause unexpected side effects is an issue. Unlike the current situation, where therapy related complications results in withdrawal of the medication, the “offending gene” cannot easily be removed, and may continue to cause significant side effects. In addition, integration of foreign DNA is not ideal as it can lead to mutagenesis, with subsequent alteration in the patient’s protein expression profile and potentially carcinogenesis. With this in mind, methods of prolonging and/or controlling episomal gene expression are preferred, provided this expression is of sufficient magnitude.

Plasmid loss alone may not fully account for the temporal loss of expression seen with these vectors. Epigenetic modification of the therapeutic has also been implicated in gene silencing, but the exact mechanisms by which this occurs have not yet been fully elucidated. It has been demonstrated that duration of transgene expression may be increased by use of ‘native’ promoters of mammalian origin rather than viral promoters (Gazdhar et al. 2006). The postulated mechanism behind this difference of expression relates to the presence and subsequent methylation of CpG sequences on promoters. This methylation is a naturally occurring phenomenon and reports have correlated methylation of CpG-rich sequences with silencing of gene expression (Gazdhar, Bilici et al. 2006). Native mammalian promoters possess fewer CpG sequences than their viral counterparts and are theoretically less prone to silencing. By employing mammalian promoters, the duration of gene expression may be extended, allowing for sustained therapeutic production. Anecdotal evidence suggests that the degree of viral promoter silencing varies between tissue types, and that the duration of gene expression in tumour tissue in particular may be short-lived (Jaenisch et al. 1985; Momparler & Bovenzi 2000; Bartoli et al. 2003). This may, in part, be due to abnormal cell turnover in tumour tissue, but the disorganised methylation pattern in tumour tissue could also play a role.

In this chapter, we assess the influence of promoter type on electroporated plasmid transgene expression in murine models. Expression is examined by utilising the reporter gene luciferase. The activity of luciferase can then be measured *in vivo*, allowing for repeated assessment of gene expression in the same test subjects over time. The pattern of expression is also examined in different tissue types as is the role of epigenetic modification in gene silencing.

2. Materials and methods

2.1. DNA constructs

pGL3-Control and pCMV-luc were purchased from Stratagene (Techno-Path, Limerick, Ireland) and Promega (Medical Supply Co., Dublin, Ireland) respectively. pDRIVE03-UbiquitinB(h) v02 was purchased from Invivogen (Cayla SAS, Toulouse, France). A version of this

plasmid, designated pUb-luc, containing the firefly *luciferase* gene transcriptionally controlled from the human Ubiquitin-B promoter was constructed, by excising the firefly *luciferase* gene from pGL3-Control using restriction enzymes Nco1 and Xba1 (New England Biolabs, USA) and cloning it in the Nhe1 (site 2) and Nco1 sites of pDRIVE03-UbiquitinB(h) downstream of the ubiquitin promoter. Plasmid copy number was calculated using the formula number DNA copies = weight/(Plasmid size \times 1.096×10^{-21}) with pUb-luc = 4.3×10^3 , pCMV-luc = 5.9×10^3 and pGL3 = 5.2×10^3 bp respectively. Endotoxin-free plasmid DNA was isolated from TOP10F *E.coli* (Invitrogen) using the MegaPrep kit (Qiagen, West Sussex, England).

2.2. Animals and tumour induction

Murine JBS fibrosarcoma tumour cells were maintained in culture in Dulbecco's Modified Essential Medium (DMEM) (GIBCO, Invitrogen Corp., Paisley, Scotland) as previously described (Collins, C. G. et al. 2006; Collins, S. A. et al. 2010). Female Balb/C and MF1nu/nu mice of 6–8 weeks of age were obtained from Harlan Laboratories (Oxfordshire, England). For routine tumour induction, 2×10^6 JBS cells suspended in 200 μ l serum free DMEM were injected subcutaneously into the flank.

2.3. *In-vivo* gene delivery

For tumour experiments, mice were treated at a tumour volume of approximately 100 mm³ in volume (5-7 mm major diameter). Mice were anaesthetized during all treatments by intraperitoneal (i.p.) administration of 200 μ g xylazine and 2 mg ketamine. For liver transfection, a 1 cm subcostal incision was made over the liver and the peritoneum opened. The right lobe of the exposed liver was administered plasmid by electroporation as described below (Casey et al. 2010; Collins, S. A. et al. 2011). The wound was closed in two layers, peritoneal and skin, using 4/0 prolene sutures (Promed, Killorglin, Ireland). For plasmid delivery by electroporation, a custom-designed applicator with 2 needles 4 mm apart was used, with both needles placed through the skin central to the tissue. Tissue was injected between electrode needles with 8×10^{11} copies of plasmid DNA in sterile injectable saline in an injection volume of 50 μ l. After 80 seconds, square-wave pulses (1200 V/cm 100 μ sec \times 1 and 120 V/cm 20 msec, 8 pulses) were administered in sequence using a custom designed pulse generator (Cliniporator (IGEA, Carpi, Italy)).

2.4. Inhibition of DNA acetylation *in vivo*

Individual animals were weighed and dosed by i.p. injection of trichostatin A (TSA) (Sigma) at 10 mg/kg in 60 μ l 10% (v/v) dimethyl sulfoxide in filtered peanut oil, daily for the duration of the experiment. *In vivo* luciferase activity was assessed 4 hours after administration of TSA.

2.5. Whole body 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 i.p.

and intratumourally where appropriate. 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, Caliper Life Sciences, England). Exposure conditions were maintained at identical levels so that all measurements would be comparable. All data analysis was carried out on Living Image 2.5 software (Xenogen). Luminescence levels were calculated using standardised regions of interest (ROI) 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 plasmids, luminescence was represented as p/sec/cm²/sr/plasmid copy.

2.6. Assessment of plasmid DNA in liver tissue using PCR analysis

To determine the presence of plasmid DNA in liver tissue, pCMV-Luc was delivered to the livers of 9 mice using electroporation as previously described. Luciferase expression was assessed by IVIS imaging at the time of sampling, 24 hr, 3 days and 10 days post treatment. Livers from three mice were excised at each time-point and snap frozen in liquid nitrogen. Livers were homogenized in TRIZOL Reagent (Invitrogen) using an Ultra Turrax T25 homogeniser (IKA Werke GmbH & Co. KG, Staufen, Germany) and total DNA was extracted as per the manufacturer's protocol. The presence of the plasmid DNA in the total DNA was determined by PCR using *luciferase* specific primers (For- 5'-AATCCATCTTGCTCCAA-CAC-3' Rev- 5'ATCTCTTTTCCGTCATCGTC-3'). PCR conditions were: Initial denaturation at 95 °C for 15mins followed by 35 cycles (95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min) and a final extension of 10 m at 72 °C. The resulting PCR products were analyzed on a 1 % agarose gel.

2.7. 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

Plasmid DNA encoding the luciferase gene transcribed from either the CMV (pCMV-luc) or Ubiquitin-B (pUb-luc) promoter was delivered to murine liver or quadriceps muscle by *in vivo* electroporation. Live whole body imaging (IVIS) was performed at various times over 370 days to determine luciferase expression. Expression mediated by the CMV promoter in liver, while initially high, reduced rapidly to background level by day 7 (figure 1a). When

the Ub promoter plasmid was examined in livers (figure 1b), luminescence was initially low but increased during the first week post transfection, before decreasing slowly, and remained higher than the CMV levels up to day 25. To examine other viral promoter activity in liver, pGL3 (SV40 promoter) was assessed (figure 1c). Like pCMV-luc, pGL3 displayed significantly faster reduction in expression than pUb-luc. A different temporal pattern of expression was observed in muscle for both the CMV and Ub promoters. Although promoter activity fluctuated over the period examined, a gross reduction in expression over time was not observed in this tissue with either CMV or Ub promoters (figure 1).

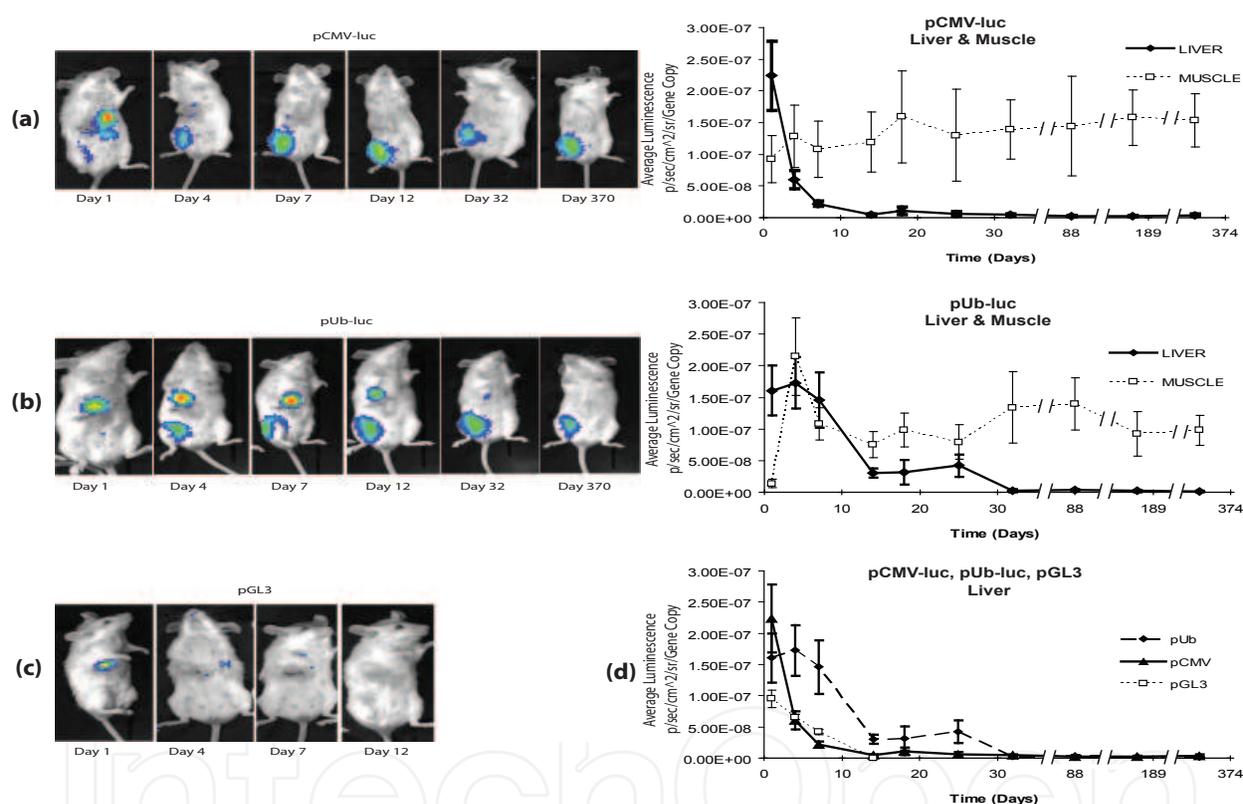


Figure 1. Duration of CMV and Ub promoter activity *in vivo* in liver and muscle pCMV-luc, pUb-luc or pGL3 was delivered to liver and quadriceps muscle (n=8) by electroporation and luminescence analysed *in vivo* over time using IVIS imaging. **(a)** CMV activity in liver was initially high, reducing to background levels by day 7. **(b)** Initial Ub activity levels were lower than those detected for the CMV promoter but increased and remained higher than that detected for CMV at later time points up to day 25. A gross reduction in expression over time was not apparent in muscle with either CMV or Ub promoters. **(c)** SV40 expression in liver decreased to background levels by Day 7. **(d)** Expression levels from both viral promoters (CMV and SV40) decline significantly faster than Ub in liver.

The kinetics of CMV, Ub and SV40 promoter activity were also analysed in tumour bearing mice. pCMV-luc, pUb-luc or pGL3 DNA was electroporated to subcutaneous (s.c.) JBS fibrosarcoma tumours upon reaching 80 mm³ in volume. IVIS imaging over 18 days (the lim-

it of tumour monitoring before animals required culling) demonstrated that the initially high expression driven by the CMV promoter was rapidly reduced to background level by day 4-post transfection (figure 2). Reduction was also observed with SV40 promoter, albeit with a heterologous temporal expression pattern to CMV, with pGL3 expression peaking at day 4 before rapidly reducing to background levels. Ub promoter activity was still evident at the final time point. pCMV-luc and pGL3 displayed statistically similar ($p = 0.98$) maximum to minimum rates of silencing (2.9×10^{-7} p/sec/cm²/sr/gene copy per day), higher than that of pUb-luc (6.8×10^{-8} p/sec/cm²/sr/gene copy per day). pCMV-luc expression was also found to rapidly reduce in s.c. human MCF7 breast carcinoma tumours growing in athymic mice (data not shown). Ubiquitin-B promoter transcriptional activity may be related to the normal functions of ubiquitin in cells, which is expressed constitutively for removing abnormal proteins and for modification of histones leading to gene activation, and so may not be subject to the down-regulation observed with many viral promoters (Ciechanover et al. 2000; Yew et al. 2001). Ubiquitin is also induced in response to cell stress, and expression might be up-regulated in response to cellular necrosis and apoptosis, which is especially relevant in growing tumours. Given that pUb-luc expression is evident long after viral promoter activity diminishes (up to day 25 for pUb-luc as opposed to day 7 for pCMV-luc and pGL3; figure 2), it is plausible that viral promoter plasmids remain present in liver post cessation of expression.

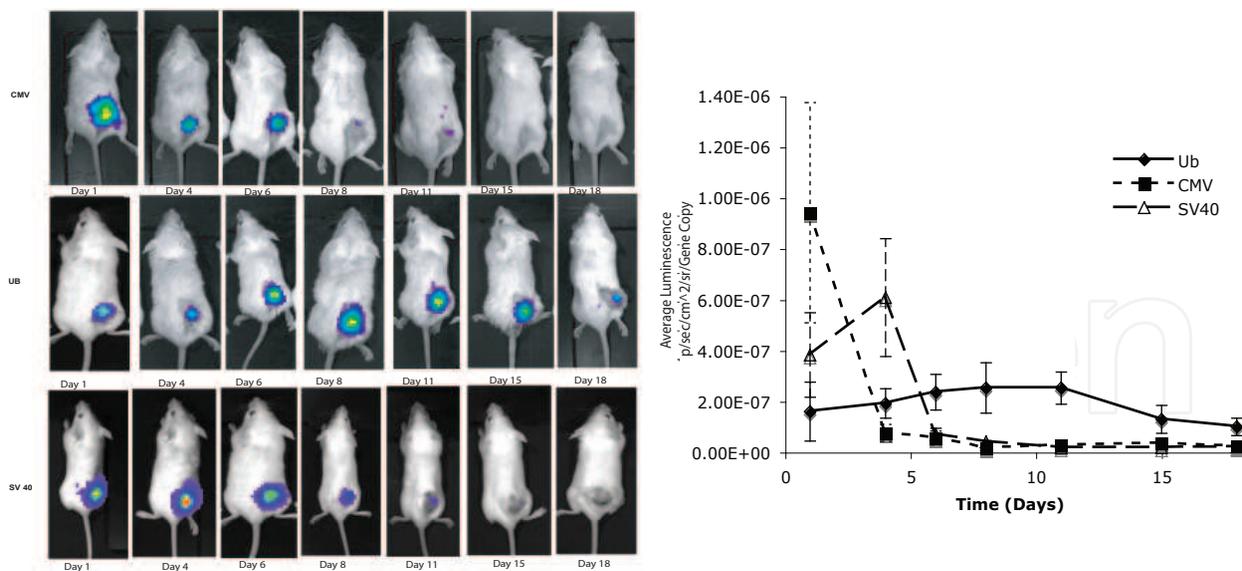


Figure 2. Duration of viral and native promoter activity in tumour pCMV-luc, pGL3 or pUb-luc was delivered *in vivo* to growing tumours ($n=6$) by electroporation and luminescence analysed *in vivo* over time using IVIS imaging. Expression from both viral promoters (CMV and SV40) rapidly diminished, whereas Ub promoter activity was still evident at the final time point (day 18) when mice required culling due to tumour size. Ub mediated expression levels were at 39.4 % of maximal level on final time-point, compared with 2.4 % and 3.5 % for CMV and SV40 respectively.

To test for the presence of plasmid, DNA was extracted from murine livers at various times post transfection with pCMV-luc and PCR analysis performed. DNA PCR results from days 1, 3 and 10 confirmed the presence of *luciferase* DNA in tissue after cessation of expression, suggesting that inhibition of transgene expression occurred at the level of or post transcription (figure 3). Our findings indicate that both viral promoters examined provided short-lived expression in tumours and liver, whereas use of Ub promoter significantly prolonged transgene expression. Importantly, we also found that viral promoter activity was dependent on target tissue, since no reduction in expression was observed in plasmid electroporated muscle with both viral and mammalian promoters.

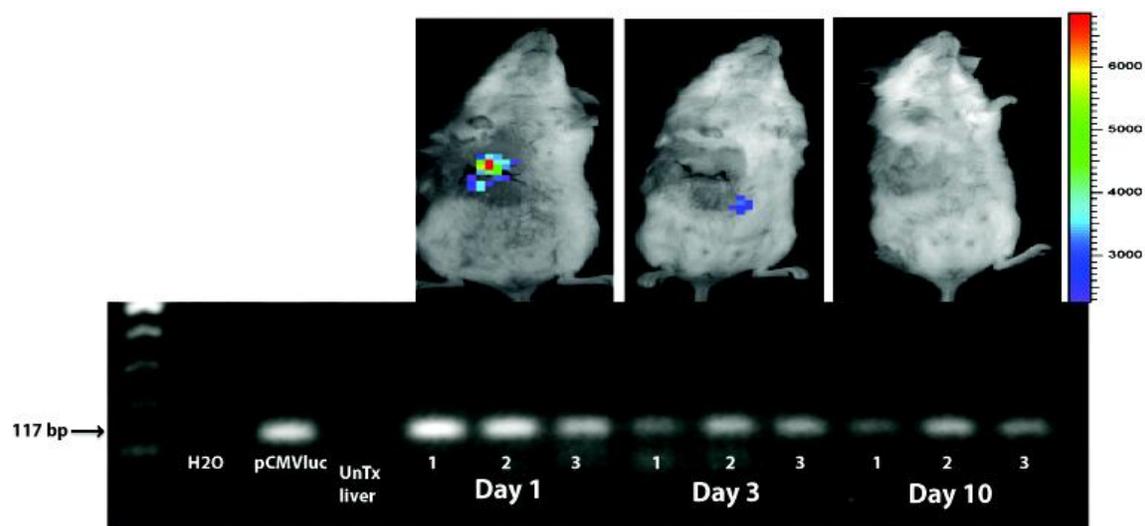


Figure 3. Plasmid DNA persists in liver after cessation of expression PCR analysis of DNA extracted from murine livers (n=3) on days 1, 3 or 10-post electroporation with pCMV-luc. A representative mouse from which DNA was extracted at each time-point is shown. PCR using primers specific for the *luciferase* gene indicates presence of plasmid. Untransfected liver samples did not yield PCR product.

In order to examine any effects of T-cell mediated immune activity on viral promoter construct expression, pCMV-luc expression in livers of athymic mice was examined. No difference in the magnitude or duration of expression was observed between immune competent Balb/C and T-cell deficient mice, suggesting that cellular immune responses were not involved in the observed reduction in hepatic expression of pCMV-luc (figure 4a). Other studies have indicated that luciferase protein has low immunogenicity, and immune-mediated destruction of luciferase-producing cells does not occur in mice (Davis et al. 1997), while the persistence of expression in muscle here also makes this unlikely as a cause for silencing in other tissues. The observation of indefinite expression in plasmid electroporated muscle is in direct contrast to Ad expression in quadriceps muscle, which has been shown to be eliminated through T cell and antibody immune activities and/or CMV promoter methylation (Jooss, Yang et al. 1998; Brooks et al. 2004).

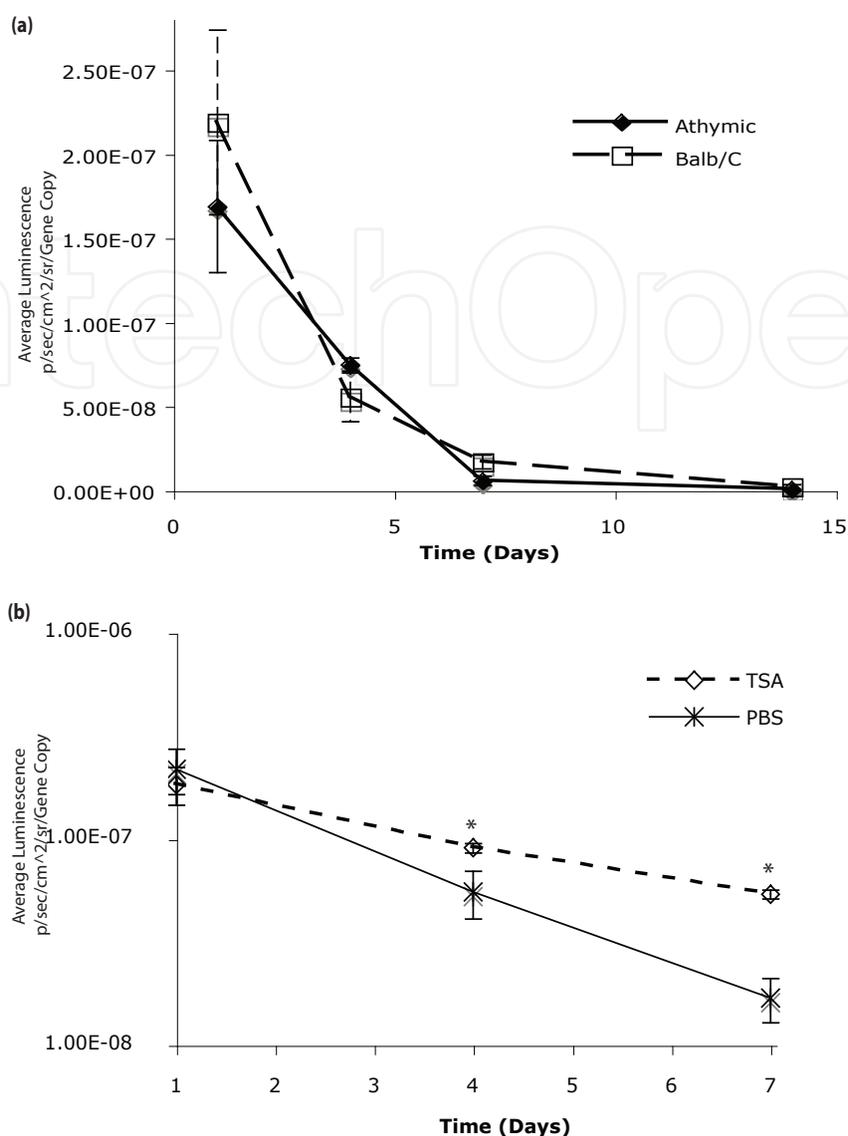


Figure 4. (a) pCMV-luc is silenced in livers in absence of T cells pCMV-luc was electroporated *in vivo* to livers of athymic mice and IVIS imaged (n=4). No difference was observed in plasmid expression at any time point when compared with expression in immunocompetent Balb/C mice ($p > 0.25$). **(b) Effect of deacetylation agent on pCMV-luc expression *in vivo*** pCMV-luc was delivered to livers by electroporation (n=4). TSA or PBS was i.p. administered daily. Gene expression was analysed using the IVIS imaging system. The magnitude and duration of gene expression in animals treated with TSA was significantly increased. * denotes statistically significant difference between groups, $p < 0.05$.

4. Discussion

We did not determine the reasons for the observed tissue-specific nature of viral promoter silencing, and it remains unclear as to why liver and tumour, but not muscle, affected plasmid expression. Plasmids function predominantly in an episomal fashion and copy number per cell is reduced proportional to cell replication. As such, genes would be expected to be

diluted rapidly in tissues with a high mitotic index. Liver hepatocytes and skeletal myocytes are fully differentiated and have a low turnover, unlike tumour cells. (Ayers & Jeffery 1988) It may be hypothesised that the static nature of cell turnover in muscle compared with tumour is relevant in this context. However, this cannot fully account for the observed loss of expression, since in our study, the rate of reduction of expression for plasmids with promoters of mammalian and viral origin was different. Also, previous studies have shown no alteration in longevity of transgene expression when cell turnover was inhibited (Herweijer et al. 2001). Furthermore, we demonstrated by PCR that pCMV-luc persisted in liver cells after expression ceased. We think it is unlikely that the reduction in pCMV-luc expression was due to a parallel reduction in the plasmid DNA as this was not seen for the Ub promoter where similar plasmid copy numbers would be expected.

It has previously been demonstrated that plasmid transgene expression can be modulated with chromatin remodelling agents (Bartoli, Fettucciari et al. 2003). To this end, murine livers were electroporated with pCMV-luc and mice systemically administered the histone deacetylase inhibitor trichostatin-A (TSA) daily for the duration of experiment. TSA is a specific inhibitor for histone deacetylase (HDAC) and is known to enhance gene expression in viral and plasmid-transfected cells *in vitro* and *in vivo* (Vanniasinkam et al. 2006). It has been shown that HDAC binds to the CMV promoter, and TSA may act to overcome such transcriptional repression (Tang & Maul 2003). In our experiments, TSA administration significantly increased levels of expression at later time points, compared with control ($p < 0.002$ on day 7; figure 4b). Interestingly, a further increase was noted when 5' azacytidine (aza-C), a non-specific methylation inhibitor, and trichostatin were used in combination, while aza-C in isolation had no effect (data not shown).

While this study did not generate data to correlate RNA levels with luminescence, differences in transcription appears to be the key element in observed expression levels. Firefly luciferase protein is known to have a short half-life *in vivo*, in the region of 1 - 4 hours (Baggett et al. 2004; Tangney & Francis 2012), and any luminescence detected in our experiments was due to recently transcribed gene. Furthermore, given that pUb-luc expression is evident long after viral promoter activity diminishes (up to day 25 for pUb-luc as opposed to day 7 for pCMV-luc and pGL3; figure 1), it is likely that viral promoter plasmids remain present in liver post cessation of expression, and we demonstrated by PCR that pCMV-luc DNA was present in liver 10 days post transfection. There exist numerous reports linking viral promoter DNA methylation with transcriptional silencing in gene therapy settings *in vitro* and *in vivo* (Di Ianni et al. 1999; Brooks, Harkins et al. 2004; Al-Dosari et al. 2006).

Our findings are consistent with previous studies in lung tissue where the levels and duration of transgene expression *in vivo* were compared using plasmid vectors coding for the CMV or Ubiquitin promoters (Gill et al. 2001; Yew, Przybylska et al. 2001; Gazdhar, Bilici et al. 2006). Further specific methylation assays may elucidate the precise mechanism of viral promoter silencing here. Given that many tumour types have been shown to have abnormal methylation, this phenomenon may represent a serious hindrance to cancer gene therapy which use of native promoters may abrogate as demonstrated here (Kanai 2008). Furthermore, the finding of indefinite high-level expression in plasmid electroporated muscle irre-

spective of the promoter type has important therapeutic implications. Skeletal muscle is a large and accessible tissue, within which a plasmid-based gene therapy might be a safe and efficient method for systemic protein production, particularly when combined with either endogenous or exogenous regulatable systems. We have previously demonstrated the application of an inducible plasmid based system in *in vivo* murine tissue (Morrissey et al. 2012). In addition to providing an “off switch” to safeguard against side effects, this also allows optimal temporal delivery of therapeutic, tailored to when it can most efficiently achieve a biological response.

5. Conclusion

In summary these results highlight the importance of promoter, tissue and vector variables in achieving appropriate transgene expression for DNA therapeutic strategies.

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