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Effects of APC De-Targeting and GAr Modification on the Duration of Luciferase Expression from Plasmid DNA Delivered to Skeletal Muscle

Maria C. Subang[1], Rewas Fatah[1], Ying Wu[1], Drew Hannaman[2], Jason Rice[3], Claire F. Evans[2], Yuti Chernajovsky[1] and David Gould[1,*]

[1]*Queen Mary University of London, Bone & Joint Research Unit, London, UK;* [2]*Ichor Medical Systems, San Diego, USA;* [3]*Cancer Research UK Centre, Southampton General Hospital, Southampton, UK*

Abstract: Immune responses to expressed foreign transgenes continue to hamper progress of gene therapy development. Translated foreign proteins with intracellular location are generally less accessible to the immune system, nevertheless they can be presented to the immune system through both MHC Class I and Class II pathways. When the foreign protein luciferase was expressed following intramuscular delivery of plasmid DNA in outbred mice, expression rapidly declined over 4 weeks. Through modifications to the expression plasmid and the luciferase transgene we examined the effect of de-targeting expression away from antigen-presenting cells (APCs), targeting expression to skeletal muscle and fusion with glycine-alanine repeats (GAr) that block MHC-Class I presentation on the duration of luciferase expression. De-targeting expression from APCs with miR142-3p target sequences incorporated into the luciferase 3'UTR reduced the humoral immune response to both native and luciferase modified with a short GAr sequence but did not prolong the duration of expression. When a skeletal muscle specific promoter was combined with the miR target sequences the humoral immune response was dampened and luciferase expression persisted at higher levels for longer. Interestingly, fusion of luciferase with a longer GAr sequence promoted the decline in luciferase expression and increased the humoral immune response to luciferase. These studies demonstrate that expression elements and transgene modifications can alter the duration of transgene expression but other factors will need to overcome before foreign transgenes expressed in skeletal muscle are immunologically silent.

Keywords: Gene therapy, luciferase, microRNA, plasmid DNA, skeletal muscle, tissue-specific promoter, transgene immunogenicity.

INTRODUCTION

Gene therapy has the potential to revolutionise the way that diseases are treated. Genetic diseases can be reversed by delivery of the correct gene copy or a correction can be made to an existing gene. In other diseases, gene therapy could be utilised for the long-term delivery of secreted or intracellular therapeutic molecules. Despite the huge potential, progress with gene therapy has been slow. Initial clinical success was achieved in patients who had a compromised immune system (e.g. X-SCID) and more recently gene therapy has been performed in immune privileged sites (e.g. Leber congenital amaurosis [1]). Gene therapy approaches that involve the expression of 'foreign' or any immunogenic protein, for which the recipient immune system has not been tolerised, will likely be hampered in an immunocompetent patient. The most pertinent example is expression of a correct gene in a genetic disease where clearly there is no tolerance to the correct protein. In other situations, such as pharmacologically regulated gene therapy [2, 3], expression of an immunogenic transactivator is often necessary [4, 5]. Therefore, an important hurdle for the wider application and success of gene therapy is to develop ways to avoid rejection of 'foreign' transgenes.

Plasmid DNA has advantages as a gene therapy vector in terms of immunology because it is devoid of protein components and does not typically elicit anti-vector immune responses, but for gene delivery it is relatively poor because it does not efficiently enter cells. Transfection is observed following plasmid injection in skeletal muscle [6] which can result in long-term gene expression in experimental models, but in clinical trials it has been less efficient with low level, transient (less than 2 weeks) expression observed [7]. The efficiency of transfection of skeletal muscle in experimental models can be enhanced by physical methods such as electroporation (EP) [8] or hydrodynamic delivery through the vasculature [9]. In these studies, long-term expression of proteins occurs because transfected myocytes are terminally differentiated, non-dividing cells so episomal plasmid DNA is not rapidly lost.

Immune responses to foreign transgenes (antigen: Ag) expressed in muscle develop because the Ag in myoblasts activated with IFN can be presented on expressed MHC Class I and II [10, 11] and are the target for Ag-specific cytotoxic CD8+cells which are observed within 2 weeks of DNA delivery [12]. Transfected resident APCs [13, 14] will present intracellular transgenes *via* their MHC class I leading

*Address correspondence to this author at the Bone & Joint Research Unit, Queen Mary University of London, William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ, UK; Tel: 44-(0)-20-7882-3992; Fax: 44-(0)-20-7882-6121; E-mail: d.j.gould@qmul.ac.uk

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to activation of cytotoxic CD8+ T cells. Extracellular Ag (released during cell death) can be taken up by recruited macrophages {McMahon, 1998 #729} and APCs by pinocytosis and presented by MHC Class II to activate CD4+ T helper cells which play a direct role in the clearance of transfected cells through MHC Class II interaction and the Fas/FasL signalling pathway [15, 16]. When there are high concentrations of extracellular Ag 'cross-presentation' through MHC Class I to CD8 cells by a route dependent on mannose receptor mediated endocytosis also occurs [17].

Strategies to avoid immunogenicity have been aimed at preventing APCs from processing and presenting Ag via MHC Class I in order to inhibit CD8 priming. In one approach termed 'de-targeting', transgene expression in APCs is averted through the use of a tissue specific promoter in combination with micro RNA (miR) target sequences in the 3'UTR. When EGFP was expressed from a liver specific albumin promoter and miR 142-3p target sequences were included in the 3'UTR (to prevent transgene expression in APCs), EGFP immunogenicity was avoided [18]. A subsequent study has shown that this de-targeting strategy results in the formation of Ag-specific regulatory T cells which promote immunological tolerance [19]. There are also viral proteins that are able to evade immune detection such as EBNA-1, an Epstein Barr Virus nuclear protein, which has been shown to persist in infected B cells without formation of cytotoxic CD8 cells. Full length EBNA-1 is 641 amino acid (aa) long and contains a glycine-alanine repeat (GAr) sequence composed of over 230 aa. This GAr sequence has several proposed effects which contribute to evading the immune system, these include inhibition of proteasomal processing [20] which could be through destabilising the interaction of ubiquinated substrate with the proteasome [21]. Other attributes include ribosomal effects resulting in self inhibition of synthesis [22] potentially due to the purine rich mRNA sequence [23], as well as delayed assembly of the initiation complex on its own mRNA [24]. The consequence of these properties is that EBNA-1 is expressed at low levels and is not efficiently processed for MHC Class I presentation. Importantly, some of these effects of the GAr sequence have also been replicated with shorter GAr sequences (as short as 7 aa) [25]. There has been some success in utilising either the full length GAr [26] or a shorter GAr sequence (24 aa, [27]) to prolong foreign transgene expression *in vivo*.

In this study we have utilised the reporter gene luciferase as a prototype foreign intracellular transgene. Through the use of bioluminescent imaging we are able to monitor persistence of luciferase expression. The decline in luciferase expression in outbred mice is associated with development of a T cell response and antibodies [28]. Here we examine alterations to the vector so that luciferase expression is either targeted to myocytes with a specific promoter, or away from APCs through the use of miR 142-3p target sequences in the luciferase 3'UTR and the influence of fused GAr elements on the duration of luciferase expression and humoral immune response.

MATERIALS & METHODS

Cells, Bacteria and Chemicals

Plasmid DNA was propagated in DH5-α Escherichia coli and was purified using a standard Plasmid Mega Kit (Qiagen Ltd., Crawley, West Sussex, UK) when DNA was prepared for *in vivo* application, the EndoFree™ Plasmid Mega Kit (Qiagen Ltd.) was used. The mouse myoblast cell line C2C12 (ECACC no. 91031101), human embryonic kidney epithelial cell line 293T, and human monocytic cell line U937 (ECACC no. 85011440) were grown at 10% CO_2 in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium) supplemented with 10% foetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA), glutamine (2 mM) (Cambrex Bio Science Verviers S.p.r.l.), penicillin (100 U/ml) (Cambrex Bio Science), and streptomycin (100 μg/ml) (Cambrex Bio Science). Unless stated otherwise, chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and DNA-modifying enzymes from New England BiolabsUk Ltd (Hitchin, Herts, UK).

DNA Constructs

The vector pcLuc+ that expresses cytoplasmic luciferase from the CMV promoter has been described previously [29]. To ensure that only luciferase was expressed from this vector, expression of the neomycin resistance gene (present in the original pcDNA 3 vector), was prevented by removing part of the promoter and start codon of NeoR by digestion with the restriction enzymes SexAI and Tth III generating the plasmid pcLuc+/-NeoR. Two repeats of the hsa-miR-142-3p target with exact complimentary sequence (in bold) were incorporated into the luciferase 3'UTR using the following oligonucleotides: 5'-CTAG**TCCATAAAGTAGGAAACACTACA**CGAT**TCCATAAAGTAGGAAACACTACA**TCTAGAATTCGGGCC-3' and 5'-CGAATTCTAGA**TGTAGTGTTTCCTACTTTATGGA**ATCG**TGTAGTGTTTCCTACTTTATGGA**-3' which were annealed overnight and inserted into pcLuc+/-NeoR linearised with XbaI and ApaI. The resulting plasmid pcLuc+/miR2T/-NeoR was digested with XbaI and ApaI and additional annealed oligonucleotides (miR 2T) were inserted to generate the pcLuc+/miR4T/-NeoR.

The synthetic muscle specific promoter c5-12 [30] (gift from ADViSYS, Inc., Texas, USA) was cloned into firefly luciferase containing vectors pcLuc+/-NeoR and pcLuc+miR4T/-NeoR by digesting them with NruI and BamHI to remove the CMV promoter. The pAV0243 plasmid (containing the c5-12 promoter) was digested with SacI, blunted with Klenow and digested with BamHI to isolate the c5-12 muscle specific promoter which was cloned into the prepared vectors. The resulting plasmids were called c5-12Luc+/-NeoR and c5-12Luc+miR4T/-NeoR.

To insert glycine-alanine repeats in the C-terminal end of luciferase protein, luciferase containing constructs had to be modified to generate plasmids with an Nhe1 restriction site just after the start codon. Both Luc+/-NeoR and Luc+miR4T/-NeoR were amplified by polymerase chain reaction using the following primers: Luc+ 5' EcoRI-NheI: 5'-ccgGAATTC accatgGCTAGCgaagacgccaaaaacata-3' and pcDNA3 Apa1: 5'-gacactatagaataGGGCCC-3'. The PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen Ltd, Paisley, UK). The pCR-Luc+/NheI and pCR-Luc+/miR4T/NheI vectors were then digested with EcoR1 and ApaI, purified and inserted into the pcDNA3/-NeoR vector that was previously cut with EcoR1 and ApaI. GAr30 contain-

ing constructs were generated by annealing the following oligonucleotides before ligation in to these vectors digested with EcoRI and NheI: Forward primer (1) 5' AATTC ACC ATG TCT AGA GCT GGA GCA GGC GGT GGA GCA GGT GCT GGA GGT GCA G and Reverse primer (1) 5' TCC ACC TGC ACC TCC AGC ACC TGC TCC ACC GCC TGC TCC AGC TCT AGA CAT GGT G; Forward primer (2) 5' GT GGA GCA GGC GGT GCA GGA GCA GGT GGT GCA GGT GCT GGA GGT GGA GCA GGT A; Reverse primer (2) 5' C TAGT ACC TGC TCC ACC TCC AGC ACC TGC ACC ACC TGC TCC TGC ACC GCC TGC.

A fragment from EBNA-1 consisting of the first 1260 nucleotides, including the sequence encoding the full length GAr (located between aa 90 and 328) was amplified by PCR from pCEP-4 (Invitrogen Ltd.): Forward primer 5' TCG GAA TTC ACC ATG TCT AGA GAG GGG CCA GGT ACA and Reverse primer 5' GG ACT AGT GCC ACC TTC TTG GTG GTA. PCR products were cloned into the TA cloning vector pCR2.1. The newly generated pCR-E1260 vector was then digested with EcoRI and SpeI to excise the first 1260 bp of EBNA-1, purified and subcloned into the pcLuc+/-NeoR and pcLuc+/miR4T/-NeoR vectors linearised by EcoRI and NheI digestions producing the vectors pcLuc+/GAr/-NeoR and pcLuc+/GAr/miR4T/-NeoR, respectively.

The plasmid pcLuc+/GAr30/miR4T/-NeoR was generated by digesting pcLuc+/miR4T/-NeoR plasmid with MluI and XcmI and discarding the 1534 bp fragment and replacing it with the CMV-Luc+GAr30/MluI/XcmI fragment excised from the pcLuc+/GAr30/-NeoR plasmid.

To modify the luciferase gene by adding GAr30 in the c5-12 plasmids, the c5-12Luc+/-NeoR and c5-12Luc+miR4T/-NeoR plasmids were digested with BamHI and XcmI removing an 885 bp fragment. The fragment Luc+GAr30/BamHI/XcmI excised from pcLuc+GAr30/-NeoR was then subcloned into the linearised vectors producing the plasmids c5-12Luc+/GAr30/-NeoR and c5-12Luc+/GAr30/mir4T/-NeoR. To generate the plasmids c5-12Luc+/GAr/-NeoR and c5-12Luc+/GAr/miR4T/-NeoR a Luc+/GAr/BamHI/NarI fragment was excised from pcLuc+/GAr/-NeoR and was subcloned into the c5-12Luc+/-NeoR and c5-12Luc+miR4T/-NeoR plasmids linearised with BamHI and NarI.

All vectors constructed in this study were validated by DNA sequencing and are depicted schematically in Fig. (**1a**).

Cell Transfection

Plasmids were transfected into 293T cells by calcium phosphate precipitation or introduced into human monocytic U937 cells and mouse myoblastic C2C12 cells using the AMAXA Nucleofector® device (Lonza Group Ltd., Basel, Switzerland with cell line Nucleofector® Kit C and the recommended protocol for the cells) and Fugene® 6 (Roche Diagnostics Ltd, Burgess Hill, UK), respectively. The plasmid pRL-CMV (Promega Corporation, Madison, WI, USA), that encodes the renilla luciferase gene, was used as a control for transfection efficiency and was introduced to all cells. Cells were lysed 24 or 48 hours after transfection and lysates were used for dual luciferase assays. Luciferase activity was measured with an MLX Microtiter® Plate Luminometer (Dynex Technologies, Chantilly, VA, USA).

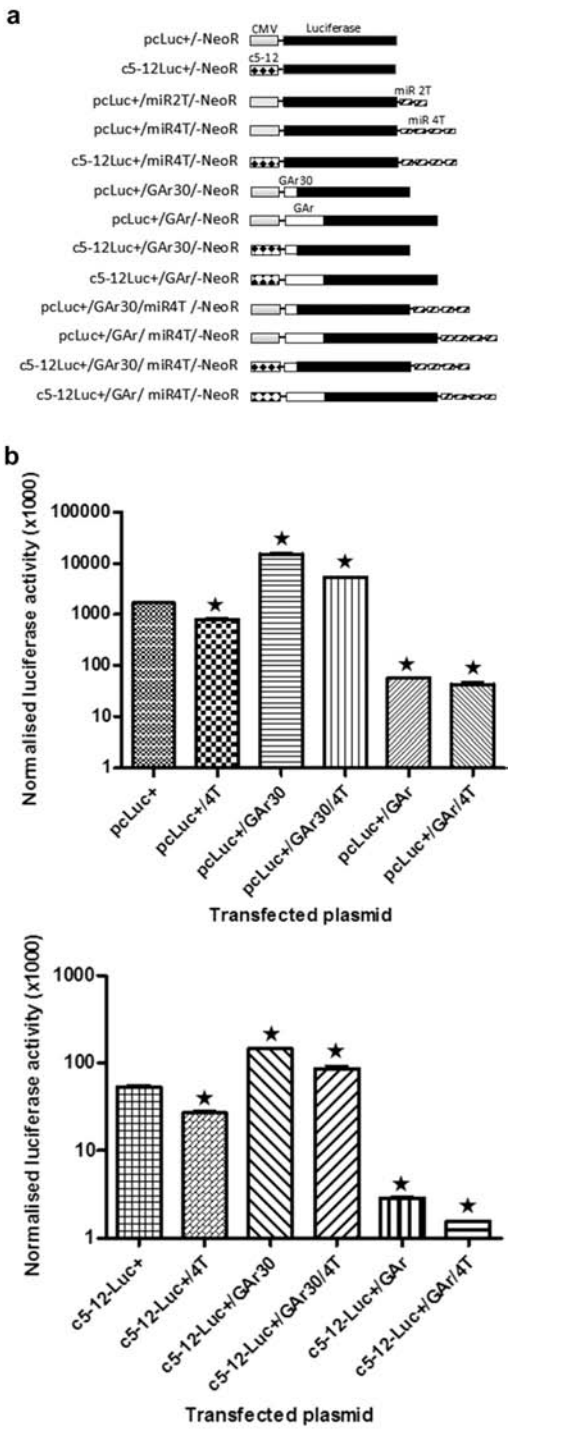

Fig. (1). Illustration of expression cassettes and luciferase expression in transfected cells. (**a**) In the constructs either the ubiquitous CMV (grey) or skeletal muscle specific c5-12 (check) promoters were used to drive expression of luciferase (black) either unmodified or with a GAr (white) fusion at the amino terminus. In some constructs 142-3p miR target sequences (striped) were incorporated in the 3'UTR. (**b**) Luciferase expression was assessed by transient co-transfection of C2C12 cells ($1x10^6$) with the construct of interest (2 ng) and pRL-CMV (0.2 ng) by nucleofection using the Amaxa Nucleofector® device, with luciferase expression assessed after 24 hours. Values are the mean of triplicate readings with vertical bars representing SEM and ★representing a significant ($p<0.05$) difference from pcLuc+ or c5-12-Luc+ in the respective histograms.

Western Blot

Plasmid DNA (20 μg) was transfected into 293T cells through calcium phosphate precipitation. Seventy-two hours after the transfection cells were washed with cold PBS and lysed in RIPA buffer containing a protease inhibitor cocktail and sodium orthovanadate. Protein concentration of the lysates were determined using the Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc.) and equal amounts of protein were diluted in Laemmli sample buffer and run on SDS-PAGE electrophoresis gels [31] and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham International Plc, UK) by electroblotting. Immunoreactive mouse sera were used to detect luciferase protein and a secondary antibody for detection of bands was HRP conjugated F(ab')2 goat anti-mouse IgG (Santa Cruz Inc, CA, USA).

PCR Amplification of miRs

To determine the presence of miRNA 142-3p in different cell lines, including U937, C2C12 and HEK 293T cells, the small RNA enriched fraction was isolated using the mirVana miRNA isolation kit (Applied Biosystems, Warrington, UK). Reverse-transcription and polymerase chain reaction (PCR) were performed to detect miRNA 142-3p and miRNA 16 using SBI QuantiMir RT kit.

In vivo Gene Delivery

Mice were treated according to approved Home Office and institutional guidelines. Outbred 8 to 10 week old male NIH Swiss mice (Harlan UK Ltd; Bicester, Oxon, UK) and Beige SCID immunodeficient mice, strain 250, from Charles River were anaesthetised with isofluorane (isoflurane, Baxter Healthcare Ltd., Norfolk, UK) using Boyle's apparatus (Linde AG, Wiesbaden, Germany). The fur covering the left anterior tibialis was shaved and the exposed skin was sprayed with disinfectant. Endotoxin-free plasmid for injection was prepared in a solution of 0.9% NaCl at a concentration of 1166 μg/ml. DNA (30 μl) was administered by intramuscular (i.m.) injection at a single site using a TriGrid needle array (Ichor Medical Systems Inc. San Diego, CA, USA). The muscle was then electroporated (four pulses at 220 V/cm, pulse duration 10 ms, frequency 6 Hz) using a BTX Electro Square Porator ECM 830 (Harvard Apparatus) in groups of 5 mice. This all-in-one injection and electroporation array has the added advantage that the injection site is the same muscle depth in all mice [32]. Plasmid delivery to lungs was performed by complexing DNA with jetPEI™ (Polyplex Transfection, Cedex, France) at a N/P ratio of 10 and a final glucose concentration of 5%. DNA lipid complex formed during 20 minutes incubation at room temperature then female Balb/c mice (Harlan UK Ltd; Bicester, Oxon, UK) were injected i.v. with 100 μl of complex containing 20 μg DNA.

Whole Body Bioluminescent Imaging

In vivo expression of luciferase was monitored by non-invasive imaging. Mice were given an i.p. injection of 200 μl of luciferin K+ salt (30 mg/ml; Promega Corp). Mice were then anaesthetised with isofluorane. Anaesthetised mice were then photographed (0.2-second exposure) and imaged for light emission between 1 second and five minutes on high sensitivity with the IVIS® 100 series (Caliper Life Sciences Inc, Hopkinton, MA, USA). Luciferase images were overlaid on the photograph, and emission of light was quantified as photons per steradian per square centimetre using Living Image® software version 3 (Caliper Life Sciences Inc.) from a defined region of interest around the anterior tibialis muscle and from control areas of the same size on the abdomen of the same mouse.

Anti-Luciferase Antibody Measurement

Levels of anti-luciferase activity in sera were measured by Elisa. Microtitre plates were coated overnight at 4°C with 100 μl of recombinant luciferase (2 μg/ml Roche Diagnostics Ltd) prepared in bicarbonate buffer (pH 9.5). Plates were washed with PBS and then blocked with 2% marvel solution in PBS for 1 hour at room temperature. Plates were again washed with PBS/Tween (0.05%) and then day 28 serum (diluted from 1:10 up to $1:1x10^8$ with PBS Tween) was incubated for 3 hours at room temperature. After washing with PBS/Tween, bound antibody was detected using HRP conjugated sheep anti-mouse IgG diluted 1:1000 (OBT 1508P; AbDSerotec, Kidlington, UK). After 1 hour the signal was detected using the TMB microwell substrate system (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) and the reaction stopped by addition of 4M sulphuric acid (100 μl). Absorbance measurements were performed at 450 nm using a Tecan-GENios microplate reader (Tecan Group Ltd; Männedorf, Switzerland) with Magellan 4 software. Absorbance readings were compared against a standard generated with a rabbit anti-luciferase IgG fraction of sera which was detected with a secondary HRP conjugated sheep anti-rabbit IgG diluted 1:1000 (Star 54; AbDSerotec).

Splenocyte Stimulation

Spleens were collected from mice at the end of the experiment and cells were dispersed using a nylon cell strainer (70 μm; Becton Dickinson Labware, FranklinLakes, NJ, USA). Cell suspensions were centrifuged and contaminating erythrocytes removed by treatment with red blood cell lysing buffer. Cells were then resuspended in complete DMEM and seeded at $3x10^6$/well in 96-well microtitre plates and were stimulated with luciferase (2 μg/ml) or ConA (3 μg/ml) for 48 hours, after which supernatants were collected and stored at –80°C until measurement of IL-2 by ELISA. IL-2 levels from Con A and luciferase stimulated cells were adjusted for levels produced by non-stimulated cells.

Statistical Methods

Significant differences were calculated using the Student's t test (Microsoft Excel 97) and area under the curve was calculated using GraphPad Prism software (GraphPad Software Inc. version 4).

RESULTS

Luciferase Expression Vectors

The luciferase expression plasmids constructed in this study were co-transfected into C2C12 cells by nucleofection and were all shown to express luciferase (Fig. **1b**). From the

results it can be seen that higher levels of expression were observed with the CMV promoter compared to the c5-12 promoter, reflecting the weak activity of the c5-12 promoter in the undifferentiated C2C12 cells compared to the CMV promoter [30]. The results also show that the GAr modifications had a significant effect on levels of luciferase expression. The GAr30 domain increased expression levels of luciferase from both promoters whilst the full length GAr caused a dramatic reduction in expression levels. By contrast, addition of the miR 142-3p target sequence only caused a small decrease in luciferase expression. The relative expression of the constructs *in vivo* is displayed in Table **1** as area under the curve (AUC) and absolute values, interestingly the different elements have a similar effect on expression *in vivo* as they do *in vitro*.

Longevity of Luciferase Expression in the Absence of an Adaptive Immune Response

When plasmid encoding luciferase was delivered by IM injection in combination with EP to Beige SCID mice, there was a significant decrease in luciferase expression within the first two weeks of plasmid delivery before stable expression was maintained at approximately 50% of the day 7 maximum (Figs. **2a** and **b**). Due to the absence of an adaptive immune system in SCID mice this decrease can be attributed to the innate immune response (with reduced NK cell activity), tissue remodelling following electroporation and promoter silencing. It provides an indication of the gene expression kinetics which could be achieved if the adaptive immune response can be evaded.

Outbred Mice Provide a Stringent Model to Monitor Duration of Luciferase Expression

As shown in Fig. (**3a**), luciferase expression in immunocompetent mice that received pcLuc+/-NeoR was dramatically reduced within 28 days of delivery. In these mice there is a rapid reduction in luciferase expression which after 4 weeks is reduced by 93% compared to the day 7 peak, and is associated with production of antibodies (Fig. **3b**) and reactive T cells (Supplementary Fig. **6bc**). Table **1** provides summary data on the production of anti-luciferase antibodies in these mice.

Skeletal Muscle Targeted Transgene Expression

The synthetic muscle-specific promoter c5-12 has previously been shown to selectively target expression to skeletal muscle *in vivo* and to have equivalent strength to the CMV promoter [30]. The tissue specific characteristic of c5-12 was confirmed in this study by *in vivo* transfection methods for skeletal muscle and mouse lung. Luciferase expression levels from the c5-12 promoter ($6.65\pm0.63x10^5$ photons/steradians/cm^2) were approximately half that seen for the CMV promoter ($1.49\pm0.34x10^6$ photons/steradians/cm^2) in skeletal muscle on day 7 (Fig. **4a**), but in lung transfected by Jet-PEI™ delivery of plasmid DNA (20 μg), expression of luciferase was only observed with pcLuc+ (Fig. **4b**). When luciferase expression was examined in outbred NIH Swiss mice the kinetics profile was similar for the c5-12 (AUC=6.83) and CMV (AUC=5.99) promoters (Figs. **4c** and **d**). However, targeting expression to skeletal muscle significantly ($p\leq0.05$) reduced the level of anti-luciferase antibody production at day 28 (Table **1**, Fig. **9b**).

Table 1. Summarised data for the different treatment groups.

Vector	Protein Expressed	Group Size[a]	Day 7[b] Luciferase	AUC/s[c] ($x10^6$)	Anti-Luc-Ab[d] ($x10^6$)	Anti-Luc Ab ÷ AUC/s[e]
pcLuc+/-NeoR	Luc	5 (9)	885,980	5.99	52	10.18
pcLuc+/4T/-NeoR	Luc	5 (8)	508,410	3.07	2.2	0.81
pcLuc+/GAr30/-NeoR	GAr30-Luc	5	2,134,044	10.7	56.6	5.22
pcLuc+/GAr30/4T/-NeoR	GAr30-Luc	5	776,300	6.96	9.7	1.59
pcLuc+/GAr/-NeoR	GAr-Luc	5	654.7	0.00341	7.4	2869.86
pcLuc+/GAr/4T/-NeoR	GAr-Luc	5	102,824	0.499	9.1	20.09
c5-12Luc+/-NeoR	Luc	4 (7)	675,214	6.83	18.3	3.35
c5-12Luc+/4T/-NeoR	Luc	5 (9)	743,600	6.62	13.1	1.96
c5-12Luc+/GAr30/-NeoR	GAr30-Luc	5	1,759,869	10.8	42.9	5.02
c5-12Luc+/GAr30/4T/-NeoR	GAr30-Luc	5	655,866	7.25	18.2	4.54
c5-12Luc+/GAr/-NeoR	GAr-Luc	5	357,090	1.70	16.6	10.98
c5-12Luc+/GAr/4T/-NeoR	GAr-Luc	5	302,918	1.04	10.6	16.87

[a] Group size is number of mice with parallel expression and antibody measurements, Values in brackets are total number of mice used for expression data.
[b] Represents the actual luciferase activity at day 7 in steradians/cm^2/sec
[c] Area under the curve (AUC) calculated from actual luciferase activity values up to day 28. Readings are adjusted to values per second of imaging.
[d] Anti-Luc Ab (IgG) in day 28 sera determined by Elisa and is a mean value for the group expressed as pg/ml
[e] Anti-Luc Ab/AUC/s: This value is calculated by dividing the luciferase antibody level by the AUC value, which gives a ratio of antibody production relative to the luciferase activity.

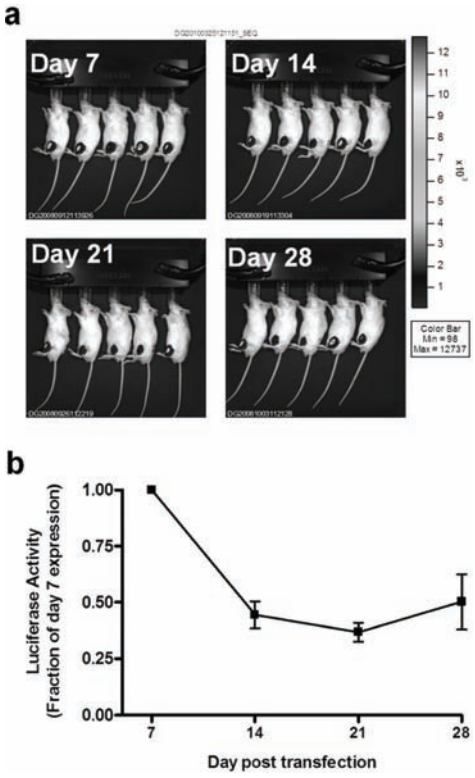

Fig. (2). Luciferase expression in SCID mice. The constitutively encoding luciferase plasmid pcLuc+/-NeoR (35 μg) was delivered by i.m. injection in combination with EP and expression of luciferase was monitored by bioluminescent imaging at weekly intervals until day 28. Bioluminescent images for day 7 to 28 are depicted in (**a**). Luciferase activity in these images was quantified using living-image® software. The reduction in luciferase expression from the day 7 peak is illustrated in (**b**) as the fraction of remaining expression, with mean values plotted +/- SEM.

APC De-Targeted Transgene Expression

The target sequence for miR-142-3p should prevent expression of luciferase in haematopoietic cells, including APCs. We cloned two and four copies of the target sequence for miR-142-3p into the 3'UTR of pcLuc+. Endogenous miRNA can be detected in cells of haematopoietic lineage - monocytic U937 cells and Ramos B cell line, but not in myoblastic C2C12 cells nor in human embryonic kidney 293T cells (Fig. **5a**). The specificity of expression of the different constructs generated was initially tested *in vitro*. C2C12 and 293T cells express luciferase after being transfected with luciferase containing plasmids with or without the 142-3p miR target sequences (Fig. **5b**). This expression of luciferase was abrogated in cells transfected with pcLuc+/miR2T or miR4T by co-delivery of miR-142-3p mimics (data not shown). In monocytic U937 cells, however, luciferase expression was only observed after being transfected with the luciferase encoding plasmid devoid of the miR 142-3p target sequences confirming APC de-targeted expression with these miR target sequences (Fig. **5b**). When assessed *in vivo* in NIH Swiss mice, luciferase expression from pcLuc+miR4T/-NeoR again declined quickly with kinetics similar to the unmodified pcLuc+ plasmid (Figs. **5c**). De-targeting luciferase expression from APCs significantly reduced anti-luciferase IgG levels ($2.2x10^6$pg/ml) compared to the control group ($52 x10^6$pg/ml) ($p \leq 0.05$) (Table **1**, Fig. **9b**).

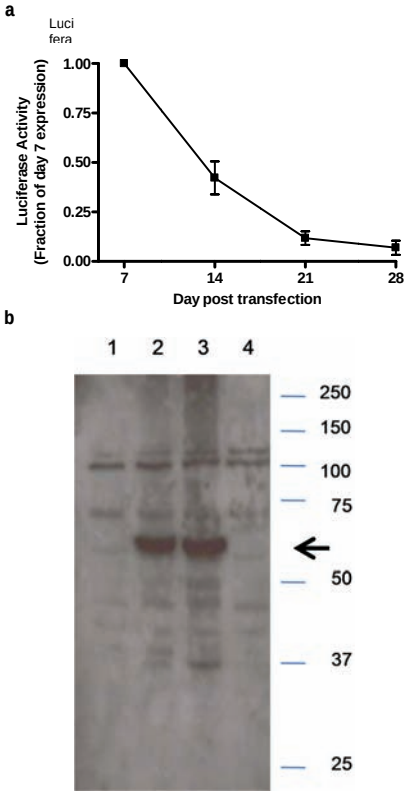

Fig. (3). Luciferase immunogenicity in NIH Swiss mice. The plasmid pcLuc+/-NeoR (35 μg) encoding luciferase was delivered in Swiss mice by IM injection with EP and expression was visualised by bioluminescent imaging between 7 and 28 days after delivery (n=9). Images are shown in (Fig. **S1a**) with the decline in luciferase activity shown in (**a**) as the fraction of remaining activity compared to the day 7 peak. At day 28 antibodies reactive to luciferase protein were detected by western blot (**b**). The samples are lysates of 293T cells transfected with 1-control plasmid pcDNA3; 2 and 3 pcLuc+; and 4 a control transfection and probed with pooled sera (diluted 1:100) collected on day 28 from treated mice.

The Effect of GAr Stabilisation on Duration of Transgene Expression

We have modified the synthetic tetracycline transactivator (rtTA2^S-M2) [33] with GAr domains and demonstrated expression, retained activity and an increase in t1/2 by pulse chase experiments indicating at effect on protein stabilisation by the same GAr30 domain that was used to modify luciferase (Fig. **S2**). In a previous study luciferase was modified with the full length GAr domain from EBNA-1 with some loss of enzyme activity observed [26]. We cloned the GAr30 sequence and the full length EBNA-1 GAr into the luciferase reporter construct. When GAr modified versions of luciferase were expressed *in vivo* from the CMV promoter luciferase activity for the GAr30 molecule was expressed at a slightly higher level than the unmodified luciferase molecule, whilst expression of the full length GAr molecule was significantly ($p \leq 0.005$) lower (Table **1**, Fig. **9a**). The duration of expression of GAr modified versions of luciferase in

mice was significantly reduced compared to the unmodified luciferase (Figs. **6a** & **b**). The low level expression of GAr-luciferase also resulted in a reduction in the level of anti-luciferase antibodies in the sera (Table **1**, Fig. **9b**).

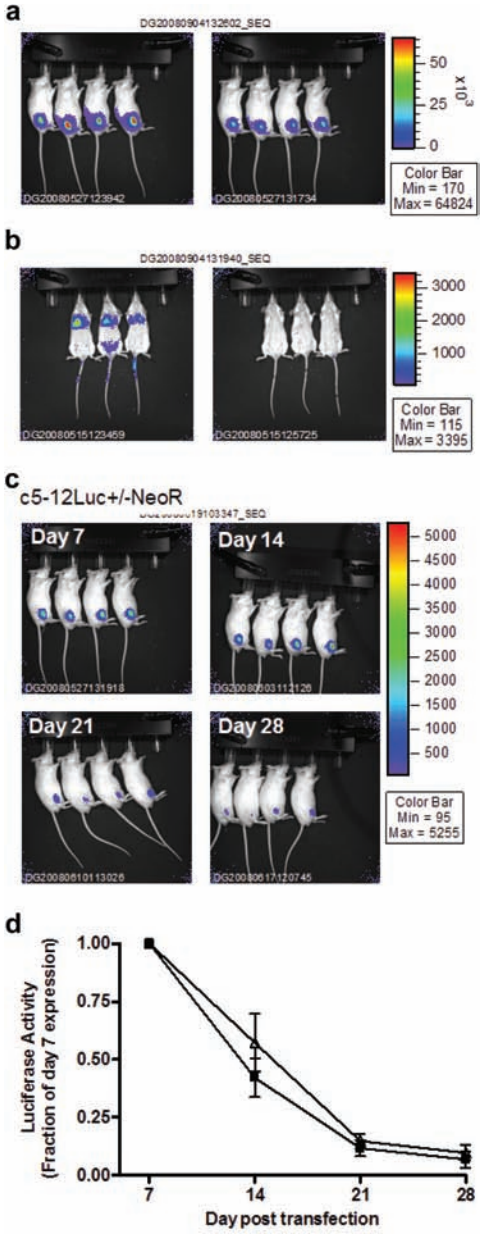

Fig. (4). Effect of targeting luciferase expression to skeletal muscle. Plasmids pcLuc+/-NeoR and pc5-12Luc+/-NeoR were delivered to skeletal muscle by IM injection with EP (**a**) or to lung with JetPEI (**b**). Expression in the muscle of NIH Swiss mice was determined 7 days after DNA (35 μg) delivery and images are captured for 10 seconds at high resolution. For lung expression DNA (20 μg) complexed with JetPEI was delivered by i.v. injection in female Balb/c mice and luciferase expression was determined 24 hours later with images captured for 5 minutes at high resolution. The muscle specific expression plasmid pc5-12-Luc+/-NeoR (35 μg; n=7) was delivered to NIH Swiss mice and luciferase expression monitored by bioluminescent imaging. Weekly images are depicted in (**c**), whilst the decline in luciferase expression from the day 7 peak is shown in (**d**) as mean values +/- SEM (open triangles) and are plotted against the pcLuc+ controls (black squares).

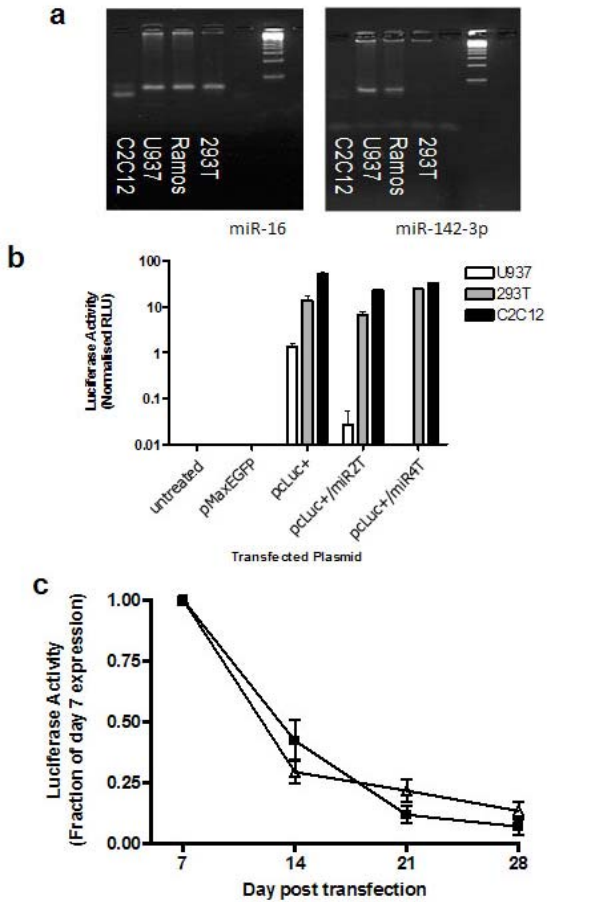

Fig. (5). The effect of de-targeting luciferase expression away from haematopoetic cells. Detection of the ubiquitous miR-16 and the haematopoetic selective miR-142-3p in cells lines by PCR amplification from the purified small RNA fraction (**a**). Expression of luciferase from pcLuc+, and versions with 2 and 4 target sequences for miR 142-3p in U937, C2C12 and 293T cells (**b**). Detection of luciferase expression by bioluminescent imaging on days 7 and 28 after delivery of plasmid DNA pcLuc+/-NeoR or pcLuc+/4T/-NeoR with 4 miR target sequences for 142-3p (Fig. **S1b**). Images were captured for 10 seconds at high resolution. The decay of luciferase expression from the day 7 peak is illustrated for pcLuc+ (black squares) and pcLuc+/4T (open triangles; n=8) (**c**). Values in diagrams are the mean of at least triplicate readings and vertical bars are +/- SEM.

Combined Muscle Specific Promoter and De-Targeted Expression from APC

We then examined the combination of myotube expression and haematopoietic cell de-targeting using the c5-12 promoter with miR 142-3p target sequence in the vector c5-12-Luc+miR4T/-NeoR. We observed a significant increase in maintenance of luciferase expression at all time-points compared to the pcLuc+/-NeoR plasmid (Fig. **7**). The kinetics of luciferase expression decline are interesting because levels are maintained at near steady state up to day 14 (93% of day 7 peak), after which there is a decline, which at day 28 is still 20% of the day 7 peak. This expression context also led to significantly ($p \leq 0.05$) reduced anti-luciferase IgG levels compared to the unmodified expression vector group (Table **1**, Fig. **9b**).

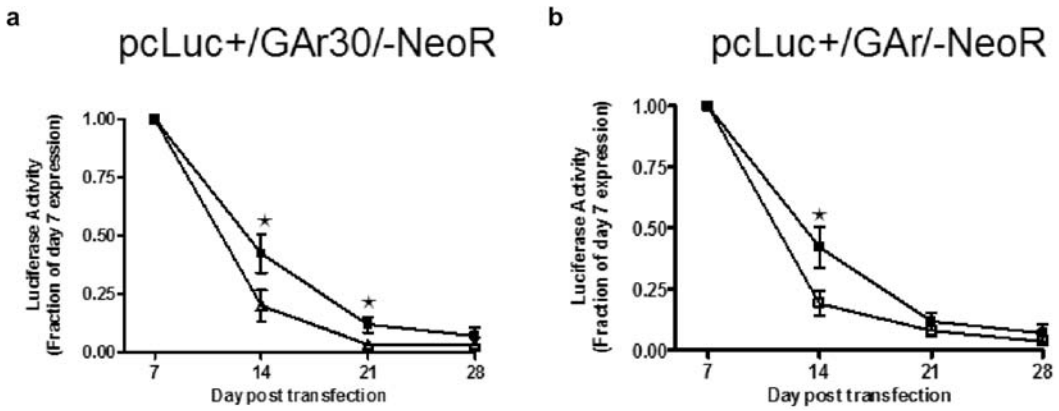

Fig. (6). The effect of GAr modification on the duration of luciferase expression. Bioluminescent imaging of mice that received plasmid pcLuc+/GAr30/-NeoR (captured for 1 second; n=5) and pcLuc+/GAr/-NeoR (captured for 10 minutes; n=5) are shown in Fig. **S3a** whilst the relative decay in the luciferase signal in these groups is compared to the day 7 peak illustrated in **a** and **b**, respectively. Values are the mean +/- SEM and significant differences between the pcLuc+ group (black squares) and the GAr modified groups (white symbols) of $p \leq 0.05$ are indicated by ★.

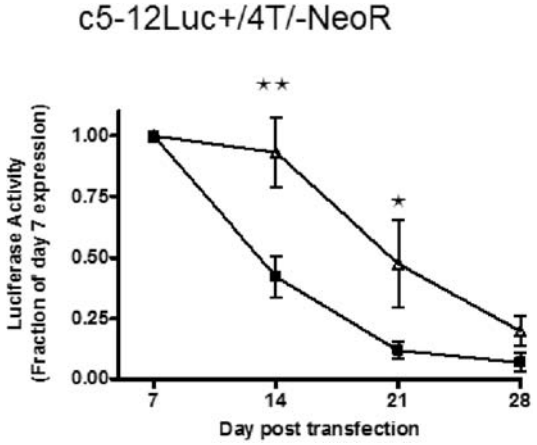

Fig. (7). Combined muscle targeting and APC de-targeting on duration of luciferase expression. Mice were delivered the plasmid c5-12Luc+/-NeoR/4T (n=9) and luciferase activity was determined by bioluminescent imaging at time points between day 7 to day 28. Bioluminescent images are depicted in Fig. **S3b**. The averaged data for the group (open triangles) shows the decay in luciferase activity compared to the day 7 peak and is plotted along with the pcLuc+ group (black squares). Significant differences between the pcLuc+/-NeoR group and the c5-12Luc+/4T/-NeoR group of $p \leq 0.005$ and $p \leq 0.05$ are indicated by ★★ and ★, respectively.

A Combination of miR Sequences and Promoter Targeting of GAr Modified Luciferase

When expression of GAr30 luciferase was de-targeted from haematopoietic cells with the miR 142-3p target sequences, the initial decline in expression was as rapid as unmodified luciferase, the rate of decline then slowed significantly by day 21 ($p \leq 0.005$ and was almost statistically significant at day 28 $p=0.056$) (Fig. **8a**) reflecting the significantly ($p \leq 0.05$) reduced anti-luciferase IgG levels in these mice (Table **1**, Fig. **9b**). When expression of GAr30 luciferase was targeted to myocytes with the c5-12 promoter, the kinetics of the decline in luciferase activity was comparable to that seen with unmodified luciferase (Fig. **8b**). Interestingly, combining the muscle specific promoter and APC de-targeting, GAr30 luciferase expression was significantly enhanced at day 14 compared to expression from pcLuc+/-NeoR (Fig. **8c**). When the same targeting and de-targeting strategies were employed with luciferase containing the full length GAr there were increased levels of luciferase activity and reduced levels of anti-luciferase IgG compared to expression from the CMV promoter (Table **1**), but luciferase expression levels were still rapidly reduced (Fig. **S5**).

Relationship Between Luciferase Expression Profile and the Immune Response

Real time imaging provides a read out of luciferase expression at several time-points in the same mouse which means that the expression profile over the course of the experiment for each mouse can then be related to the anti-luciferase IgG measured in day 28 serum. In Fig. (**9a**) we can see that the expression profile of luciferase expressed as AUC is significantly reduced from all vectors where the full length GAr is fused to the luciferase protein. The only other group where the kinetics of luciferase expression are significantly reduced are those treated with the vector pcLuc+/-NeoR/4T which should prevent expression of luciferase in APCs (Fig. **9a**). When anti-luciferase IgG levels are measured they are significantly reduced in all groups compared to the pcLuc+/-NeoR control with the exception of groups where GAr30 modified luciferase is expressed without miR de-targeting from APCs (Fig. **9b**). Because expression level influences the immune response we can normalise antibody levels with expression levels by simple division as illustrated in Fig. **9c**. The data clearly show that despite the lower expression of full length GAr modified luciferase from the constitutive CMV promoter the relative immune response that develops is greater than observed with the control vector pcLuc+/-NeoR (Fig. **9c**). Similarly, T cell stimulation studies (Fig. **S6**) suggest that IL-2 production in response to luciferase protein stimulation is also elevated (Fig. **S6C**) relative to the level of luciferase expression. By contrast, most vectors in which luciferase expression was de-targeted from APCs with miR 142-3p target sequences displayed a reduced humoral immune response (Fig. **9c**) confirming that this strategy can reduce immunogenicity of foreign transgenes expressed in the correct context.

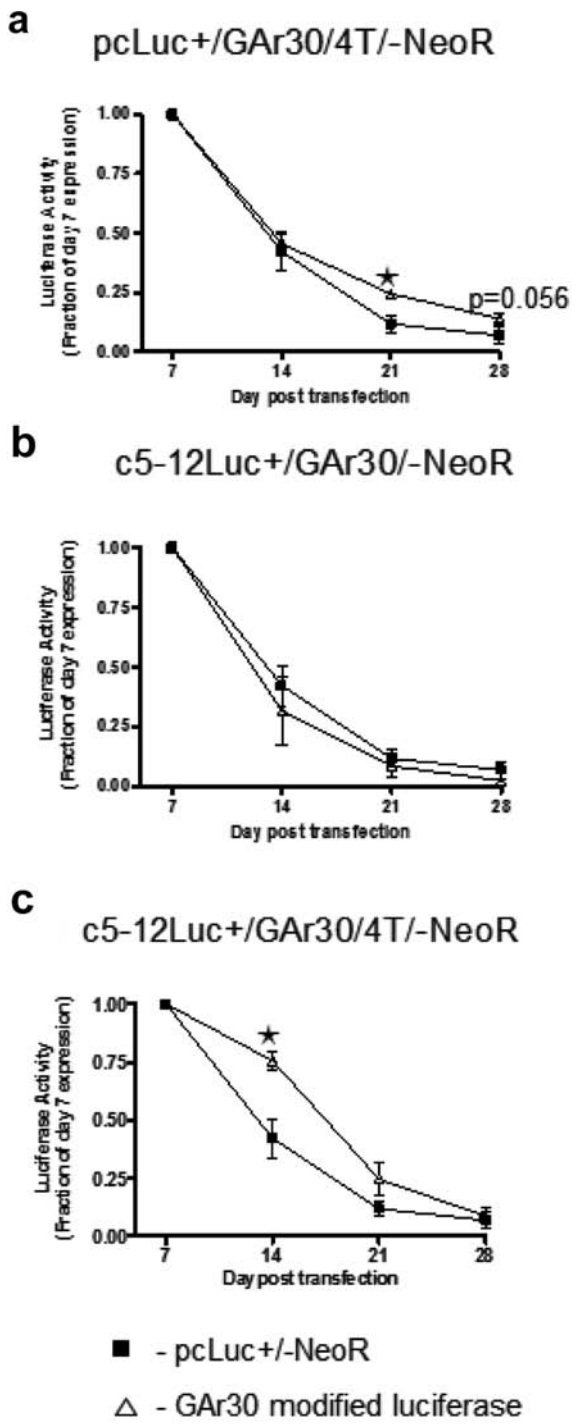

Fig. (8). The effect of APC de-targeting and muscle targeting on duration of GAr30-luciferase expression. In the plasmid pcLuc+/GAr30/4T/-NeoR, GAr30 modification and miR 142-3p de-targeting are combined. Bioluminescent images of NIH Swiss mice are shown in Fig. (**S4**) whilst graphs of averaged data for the decrease in expression from the day 7 peak for pcLuc+/GAr30/4T/-NeoR is shown (**a**). When expression of the same GAr 30 modified luciferase was targeted to skeletal muscle with c5-12 promoter from the plasmid c5-12Luc+/GAr30/-NeoR the expression data is illustrated (**b**). When the muscle promoter and miR de-targeting are combined averaged data is shown in (**c**). Significant differences from the decline in luciferase expression from pcLuc+/-NeoR (black squares) of $p \leq 0.005$ are indicated by ★ (n=5 for each group).

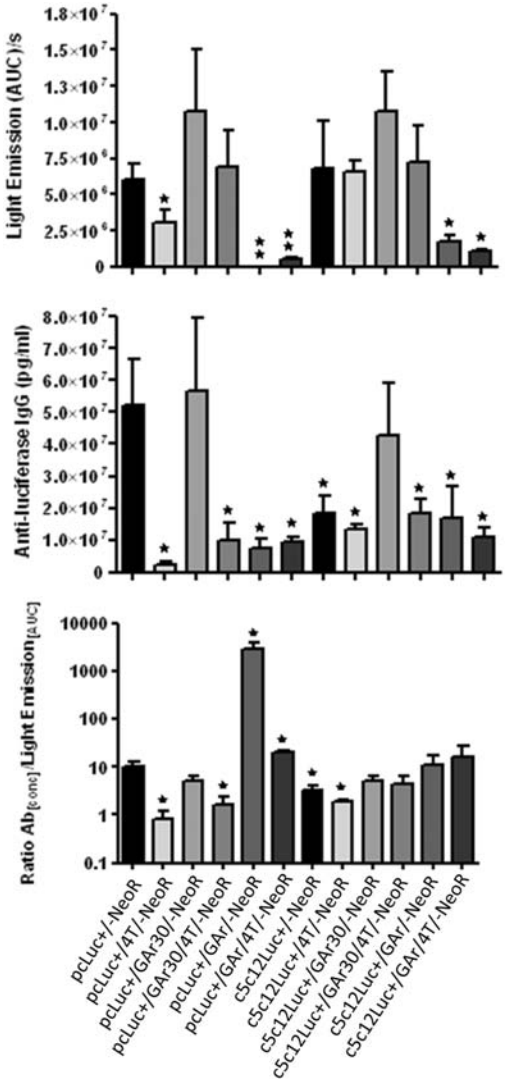

Fig. (9). Anti-luciferase IgG levels relative to luciferase expression levels. Total luciferase expression during the 28 day experiment was calculated as AUC (**a**) and anti-luciferase IgG in sera collected at day 28 were determined by Elisa (**b**) levels of anti-luciferase IgG relative to luciferase expression levels are shown in (**c**). Significant differences of $p<0.05$ and $p<0.005$ from the pcLuc+/-NeoR group are indicated by ★ and ★★, respectively.

DISCUSSION AND CONCLUSION

The method of gene delivery is an important factor affecting immune responses to expressed transgenes. Plasmid DNA contains CpG motifs that are immunostimulatory, acting through TLR9, and cytoplasmic dsDNA also triggers IFN production through intracellular sensors such as DAI (DNA-dependent activator of IFN-regulatory factors) [34] and AIM2 (absent in melanoma 2) [35]. In addition, delivery methods for plasmid DNA typically cause some tissue damage so there is also interaction with the immune system via clearance of cell debris [36]. These attributes of plasmid gene expression has led to the development of DNA vaccination, which is also enhanced by EP [37] and is being actively pursued for clinical application [38, 39]. Firefly luciferase is a foreign protein in mice yet it has been expressed long-term in some inbred strains without sign of immune rejection [6].

Wolff *et al.*, [28] reported that luciferase is immunogenic in outbred ICR mice. We also observed luciferase immunogenicity in outbred ICR mice (data not shown) and in outbred NIH Swiss mice following plasmid delivery to skeletal muscle by injection and EP with the formation of reactive antibodies and T cell responsiveness *in vitro* to exogenous luciferase. Through the use of bioluminescent imaging we were able to observe the decline in luciferase activity in real-time and over a period of 28 days we consistently saw that activity decreased by approximately 95% from the peak level at day 7. This imaging technology gives us a unique approach to follow the reduction in luciferase expression and to readily examine strategies to prolong the duration of luciferase expression. Clearly, both the innate and adaptive immune systems have the potential to affect luciferase expression along with tissue remodeling and promoter silencing. Experiments in SCID mice give an insight of the contribution of all these components to the reduction of luciferase expression with the exception of the adaptive immune system. In these mice we see that luciferase activity is rapidly reduced between day 7 and 14 to approximately 50% where it then stabilises until day 28. In this study we were interested in counteracting the adaptive immune response that accounts for the additional elimination of the foreign transgene we observed in outbred mice.

The strategies we have employed target different aspects of the immune response. Directing expression to myocytes through the use of the c5-12 promoter did not alter the duration of luciferase expression compared with using the ubiquitous CMV promoter. The amount of expressed foreign protein is an important factor in the induction of immune responses, and we confirm here that the synthetic c5-12 promoter is of similar strength to the CMV promoter. Use of a weaker promoter and hence lower levels of expressed foreign protein contributes to evasion of the immune system. This is illustrated with luciferase immunogenicity in ICR mice following hydrodynamic plasmid delivery to muscle which showed that expression persisted at 50% of maximum after 2 months when expressed with the MCK promoter which was 35 times weaker than the CMV promoter after 7 days in this study [28].

By de-targeting luciferase expression from APCs with miR 142-3p, CD8 priming from endogenous antigen via MHC Class I should be prevented and B cell activation via MHC Class II should also be reduced. Indeed we observed reduced antibody production when 142-3p sequences were used in most constructs, however, this strategy alone did not alter the duration of luciferase expression in Swiss mice. This is not too surprising as the main inducer of the immune response is thought to be Ag expressed in non-lymphoid tissues that is transferred to APCs [40]. But combined de-targeting with myotube specific expression did reduce the anti-luciferase IgG levels and significantly prolonged the duration of luciferase expression, with luciferase activity falling by 80% after 28 days. When Wolff *et al.* [28] de-targeted plasmid expression of luciferase in skeletal muscle of ICR mice with the same miR sequences and the MCK promoter they did not see any change in the luciferase rejection compared with the MCK promoter alone, partly because the weaker MCK promoter significantly reduced immunogenicity on its own. In both studies luciferase remained immunogenic (both decline in expression and antibody formation) suggesting that foreign transgene expressed from plasmid DNA in skeletal muscle cannot be completely prevented by this de-targeting strategy in contrast to observations with lentivirus expression of EGFP in the liver [18]. Further research is necessary to establish the exact reason for this discrepancy which may be related to the mouse strain (outbred *vs.* Balb/c), target tissue (skeletal muscle *vs.* liver), delivery vector (plasmid *vs.* lentivirus) or foreign transgene (luciferase *vs.* EGFP). Indeed the complexity of the issue was highlighted in a recent study where immunogenicity of AAV1 expressed human sacroglycan protein could be prevented in C57Bl/6 mice using 142-3p sequences alone, but rejection was only delayed in sacroglycan-deficient dystrophic mice [41]. However, the inflammatory state of the muscle at the time of gene delivery will also differ between these mice.

The full length GAr domain from EBNA-1 should prevent processing for MHC Class I presentation of expressed transgene which has been previously demonstrated in *in vitro* studies [26, 42]. This effect should prevent the MHC Class I presentation route in transfected APCs and also prevent the MHC Class I presentation in myocytes which targets them for destruction by cytotoxic T cells. The long GAr domain will not, however, prevent CD8 priming following cross-presentation [26].

When we used the full length GAr domain from EBNA-1, we observed reduced levels of luciferase expression and a rapid decline in luciferase expression compared with expression of unmodified luciferase. Furthermore, the duration of expression of the full length GAr modified luciferase was unaltered by combinations of targeting and/or de-targeting. These observations contrast with use of the full length GAr domain for maintenance of LacZ transgene delivered with adenovirus in Balb/c skeletal muscle where expression persisted for 19 days, but was only detected in 1 of 4 mice delivered the unmodified LacZ [26]. An important difference is the more stringent immune response observed in the outbred mice that we have utilised in our study.

Fusing the small GAr30 sequence (which we show increased the t1/2 of rtTA-2^{S}M2) to luciferase increased the level of luciferase expression *in vitro* and *in vivo* suggesting a similar stabilisation, but still did not affect the elimination rate of luciferase expression. Although, de-targeting expression from APCs with miR 142-3p target sequences did significantly slow the reduction in luciferase activity which may be due to reduced MHC class I presentation so evading clearance of transfected cells by CTLs. When GAr30 modified luciferase was also targeted to myoblasts there was a further improvement in the duration of luciferase activity, but not better than when the same strategy was employed with unmodified luciferase.

Whilst this study has not unlocked a strategy to achieve persistent foreign transgene expression the de-targeting approach was shown to prolong luciferase expression and GAr 30 modification did display some improvement when expression was de-targeted from APCs. Plasmid DNA is not itself immunogenic, but it does trigger several intra-cellular sensors (TLR9, DAI, AIM-2) and physical delivery techniques combine to provide a strong 'danger' signal to the

immune system. Delivery methods that do not stimulate the immune system could potentially dampen this signal and permit more subtle gene delivery. This study has also shown that luciferase has advantages as a foreign transgene for monitoring duration of expression *in vivo* because of the ability to repeatedly perform real-time measurements. Greater analysis of the immune response could be achieved if immunodominant epitopes of luciferase were characterised, reactive T cell clones were available, and there was a better understanding of the relative contributions of direct and cross-priming to the immune response.

LIST OF ABBREVIATIONS

Ag = Antigen

APC = Antigen presenting cell

CMV = Cytomegalovirus

CTL = Cytotoxic T lymphocyte

EBNA = Epstein-barr nuclear antigen

EGFP = Enhanced green fluorescent protein

EP = Electroporation

GAr = Glycine-alanine repeats

HRP = Horseradish peroxidase

MHC = Major histocompatibility complex

miR = microRNA

NK = Natural killer

PBS = Phosphate buffered saline

UTR = Untranslated region

CONFLICT OF INTEREST

D. Hannaman and C.F. Evans are full-time employees of Ichor Medical Systems.

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PATIENT CONSENT

Declared none.

SUPPLEMENTARY MATERIALS

Supplementary material is available on the publishers web site along with the published article.

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