

Research Article

Control and Augmentation of Long-Term Plasmid Transgene Expression *In Vivo* in Murine Muscle Tissue and *Ex Vivo* in Patient Mesenchymal Tissue

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Purpose. *In vivo* gene therapy directed at tissues of mesenchymal origin could potentially augment healing. We aimed to assess the duration and magnitude of transgene expression *in vivo* in mice and *ex vivo* in human tissues. **Methods.** Using bioluminescence imaging, plasmid and adenoviral vector-based transgene expression in murine quadriceps *in vivo* was examined. Temporal control was assessed using a doxycycline-inducible system. An *ex vivo* model was developed and optimised using murine tissue, and applied in *ex vivo* human tissue. **Results.** *In vivo* plasmid-based transgene expression did not silence in murine muscle, unlike in liver. Although maximum luciferase expression was higher in muscle with adenoviral delivery compared with plasmid, expression reduced over time. The inducible promoter cassette successfully regulated gene expression with maximum levels a factor of 11 greater than baseline. Expression was re-induced to a similar level on a temporal basis. Luciferase expression was readily detected *ex vivo* in human muscle and tendon. **Conclusions.** Plasmid constructs resulted in long-term *in vivo* gene expression in skeletal muscle, in a controllable fashion utilising an inducible promoter in combination with oral agents. Successful plasmid gene transfection in human *ex vivo* mesenchymal tissue was demonstrated for the first time.

1. Introduction

Gene therapy is now a realistic prospect for the treatment of a variety of musculoskeletal disorders because of the emerging knowledge concerning proteins that govern the processes of growth and regeneration of tissues [1, 2]. Many orthopaedic tissues do not heal well after injury [3]. Research has identified numerous growth factors and other gene products with the ability to promote regeneration [4]. The most extensively studied are members of the transforming growth factor (TGF) superfamily, collectively known as bone morphogenetic proteins (BMPs). Because gene therapy provides the gene rather than just a degradable protein, this technique may result in higher and more reproducible levels of protein production with the potential for long-term protein delivery. This would allow either local protein production for tissue

healing and so forth or potentiate long-term systemic delivery. Unlike current methods, protein delivery could also be intracellular. Various methods have been employed to deliver genetic material. The most commonly used in the orthopaedic field have been viral vectors and *ex vivo* manipulation with considerable preclinical success [5, 6]. There are concerns regarding viral recombination/infectivity, immunogenicity, and possible carcinogenicity [7–9]. They are expensive to produce and as vectors have biophysical and genetic limitations. Also many gene therapy strategies prove ineffective, suffering from gene silencing [10]. Non-viral therapy has not been extensively studied in orthopaedic research [11, 12]. In theory, non-viral therapy lacks some of the drawbacks associated with viral systems. In this study, the long-term viability of murine skeletal muscle plasmid gene expression *in vivo* was assessed using live whole body

imaging of luciferase expression, along with the potential for expression control using an inducible model. Finally an *ex vivo* model was developed in murine musculoskeletal tissue and conditions replicated to examine plasmid gene expression in human *ex vivo* mesenchymal tissue.

2. Materials and Methods

2.1. Plasmids. The commercially available plasmid pCMV-luc was purchased from Promega (Wisconsin, USA). The plasmid pGTRTL was generously provided by Dr. David Gould (Bone & Joint Research Unit, Barts and The London, Queen Mary's School of Medicine and Dentistry, University of London, London, England).

2.2. Animals. B-alb C 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 mice in good condition, without fungal or other infections, weighing 16–22 g and of 6–8 weeks of age, were included in experiments.

2.3. In Vivo and Ex Vivo Gene Delivery. All *in vivo* and *ex vivo* animal experiments were approved by the ethics committee of University College Cork. Mice were randomly divided into experimental groups and subjected to specific experimental protocols. Mice were anaesthetized during all treatments by i.p. administration of 200 µg 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. The wound was closed in two layers—peritoneal and skin—using 4/0 prolene sutures (Promed). All *ex vivo* human tissue experiments were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Informed consent was obtained from patients scheduled for lower limb amputation and the surgeon's permission obtained. Upon removal of sample, it was immediately immersed in culture medium and brought to the laboratory. Samples were cut to a size approximating the medial thigh muscle of Balb-C mice (1.5 cm³).

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 plasmid DNA in sterile injectable saline in an injection volume of 50 µl. Concentration of plasmid was adjusted to administer 8×10^{11} gene copy numbers. Plasmid concentration was determined using the Nanodrop spectrophotometer (ND-1000 Spectrophotometer, Labtech Int, East Sussex, UK).

The number of plasmid copies was determined using the following calculation:

$$\begin{aligned} &\text{mass of plasmid DNA (g)} \\ &= \text{no. of gene copies (bp)} \quad (1) \\ &\quad \times (\text{plasmid size} \times 1.096 \times 10^{-21}). \end{aligned}$$

Plasmid sizes for pCMV luc and pGTRTL were 6.9 kbp and 7.5 kbp, corresponding to a mass of 6.05×10^{-6} g and 6.576×10^{-6} g, respectively.

After 80 seconds, square-wave pulses (1200 V/cm $100 \mu\text{sec} \times 1$ and 120 V/cm 20 msec, 8 pulses) were administered in sequence using a custom-designed pulse generator (CythorLab, Aditus, Lund, Sweden). In all cases, a drop in impedance measurement was taken as evidence for successful tissue cell poration.

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 [13]. Viral vector particles were administered by direct intramuscular injection in a volume of 50 µl. 1×10^9 VP of replication incompetent recombinant Adenovirus 5 particles was used per administration.

2.4. Ex Vivo Optimisation. The entire medial thigh muscle tissue was harvested from sacrificed B-alb C mice (1.5 cm³). Samples were stored as per the experimental protocol to assess survival in either RPMI (Roswell Park Memorial Institute medium) or DMEM (Dulbecco/Vogt modified Eagle's minimal essential medium) at 37°C for 24 hours. The effect of electroporation on cell survival was assessed by administering the plasmid as described previously and assessing viability at 24 hours.

Cell viability was assessed using the propidium-iodide-based NucleoCounter kit as previously described [14]. Samples were washed and processed through a fine mesh filter (BD Biosciences, Oxford, UK), using a pestle and PBS (phosphate buffered saline) until a suspension was obtained. The suspension was made up to a standard volume, and cell viability was assessed. Viability at time zero was taken as 100% relative survival. Subsequent viabilities were expressed as a percentage relative to this.

2.5. Doxycycline Administration. Solutions were prepared by adding 200 µg/mL of doxycycline (Sigma) to distilled water containing 10% sucrose as previously described. All drinking bottles were wrapped in aluminium foil and were renewed every 2–3 days. Noninduced groups were administered solutions of distilled water containing 10% sucrose. A 20 µg/mL solution was added to wells with pGTRTL *ex vivo* samples as the 200 µg/mL resulted in excessive cell death (data not shown).

2.6. In Vivo Imaging. *In vivo* luciferase activity from tissues was analysed at set time points after transfection as follows: 80 µl of 30 mg/mL firefly luciferin was injected intraperitoneally. Mice were anaesthetised as before. Ten minutes after

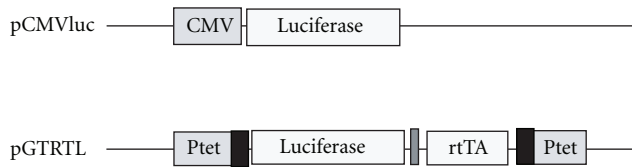


FIGURE 1: Plasmid Constructs. Schematic representations of firefly luciferase coding constructs used in this study. Promoters are outlined in grey.

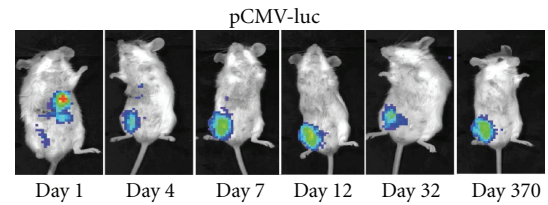
luciferin injection, live anaesthetised mice were imaged for 3 min at high sensitivity using an intensified CCD camera (IVIS Imaging System, Xenogen). The exposure conditions were maintained at identical levels so that all measurements would be comparable. All data analysis was carried out on the Living Image 2.5 software package (IVIS Imaging System, Xenogen). Luminescence levels were calculated using standardised regions of interest (ROIs) for all 3 anatomical areas. Actual levels were obtained by subtracting the corresponding ROI of an untransfected mouse to account for background luminescence. For comparison between plasmids, flux was calculated per gene copy number.

2.7. Statistical Analysis. At each time point, either one-way or a two-sampled *t*-test was used to compare mean luminescence within each experimental group. Microsoft Excel 11.3 (Microsoft) was used to manage and analyze data. Statistical significance was defined at the standard 5% level.

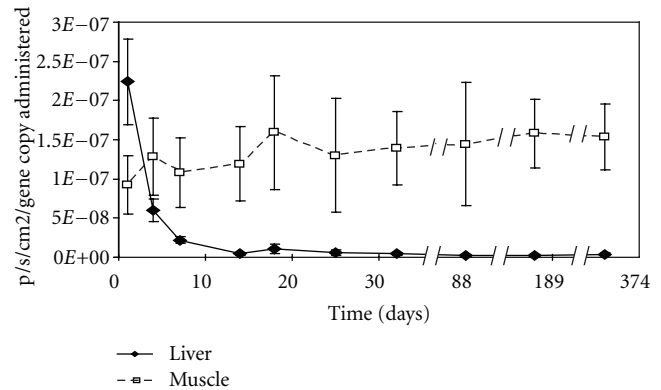
3. Results

3.1. Duration of Gene Expression In Vivo in Muscle and Liver. Luciferase-coding plasmid DNA featuring the CMV (pCMV-luc) promoter (Figure 1) was delivered to liver or thigh muscle by electroporation. IVIS live whole body imaging was used at time points over 370 days to determine luciferase expression (Figure 2). Expression driven by the CMV promoter in liver was initially high but reduced rapidly to background levels by day 14. A different temporal pattern of expression was observed in muscle tissue, with no gross reduction in expression over time observed. Total luminescence for the period was 1.33×10^{-6} p/sec/cm²/sr/plasmid copy. Unlike liver, muscle therefore has the potential to act as a long-term conduit for gene expression.

3.2. Comparison of the Duration and Magnitude of Gene Expression In Vivo in Muscle following Plasmid and Viral Delivery. Luciferase-coding plasmid DNA featuring the CMV (pCMV-luc) promoter or Adenovirus 5 particles encoding the luciferase gene under the transcriptional control of the CMV promoter was delivered to murine thigh muscle as described. IVIS live whole body imaging was used at time points over 18 days to compare luciferase expression between the two constructs. Maximum luciferase expression for both constructs is shown in Figure 3(a). Maximum expression was significantly higher using adenoviral delivery when compared to that of plasmid delivery. Expression



(a)



(b)

FIGURE 2: Duration of plasmid-based CMV promoter activity *in vivo* in liver and muscle. pCMV-luc was delivered to liver and thigh muscle ($n = 10$) by electroporation and luminescence analysed *in vivo* over time using IVIS live whole body imaging. CMV activity in liver is initially high but reduced to background level by day 14. A gross reduction in expression over time was not apparent in muscle.

following adenoviral delivery, however, decreased over the study period to background levels at 18 days (Figure 3(b)).

3.3. Regulation of Gene Expression. Luciferase expression from an inducible “tet-on” promoter was examined in muscle tissue using the pGTRTL plasmid, both in the presence and absence of induction by doxycycline (Figure 4). There was a low initial expression from reporter gene preinduction from the time of delivery. However, following induction with doxycycline at day 8, expression was increased by a factor of 11 ($P = 0.0001$) at day 11. Following withdrawal of doxycycline at day 12, this had returned to baseline by day 16 ($P = 0.08$). Expression was reinduced at day 105 and had increased by a factor of 9.2 by day 108 ($P = 0.001$) and by a factor of 10.7 at day 116. Expression remained constant at a maximal level between days 108 and 116 with no statistically significant change in magnitude observed ($P = 0.77$). Following withdrawal of doxycycline at day 117, this had returned to baseline by day 119 ($P = 0.06$). There was no difference in maximal expression between the two induction periods ($P = 0.28$). pGTRTL gene expression is thus rapidly induced with doxycycline and dissipates on withdrawal. It can also be reinduced on a temporal basis to give reproducible levels of expression.

3.4. Murine Ex Vivo Tissue Survival and Optimisation. Cell viability following incubation in DMEM at 37°C for 1, 4, and

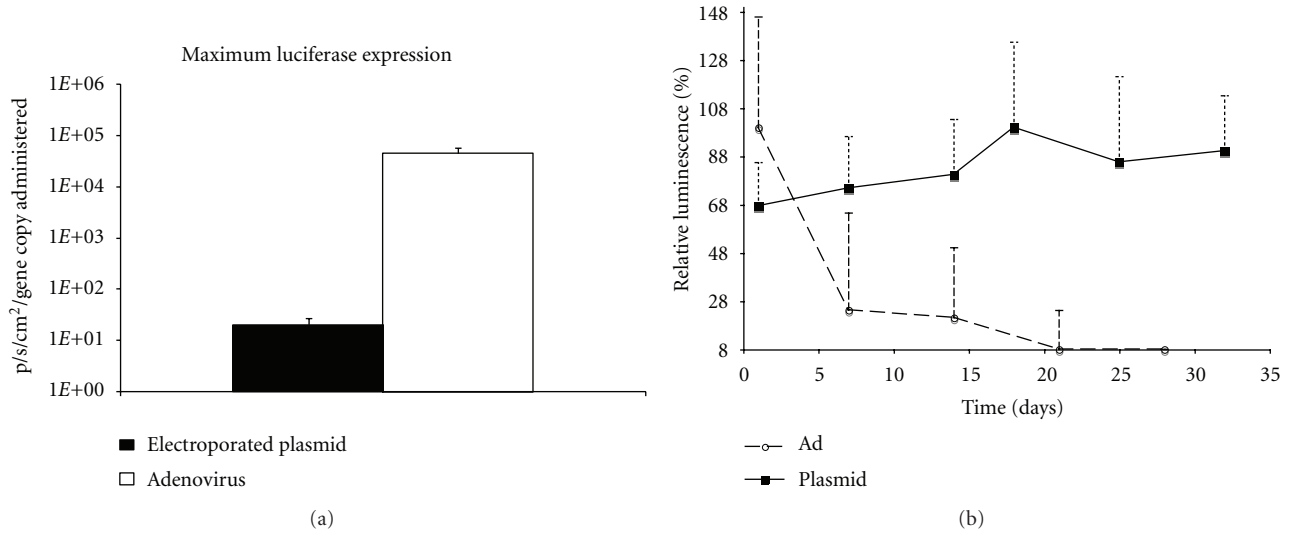


FIGURE 3: Adenoviral vector and electroporated plasmid-mediated transgene expression *in vivo*. Thighs of mice were *in vivo* administered pCMV-luc by electroporation or AdCMV-luc ($n = 5$). Luminescence was analysed *in vivo* over time using IVIS live whole body imaging. (a) Magnitude of electroporated plasmid-mediated luciferase expression compared with Ad-mediated expression (day 18 and day 1, resp.). (b) Plasmid versus Ad transgene expression over time. Although luminescence was higher with Ad delivery, it was subject to rapid silencing to background levels by day 21, unlike electroporated plasmid.

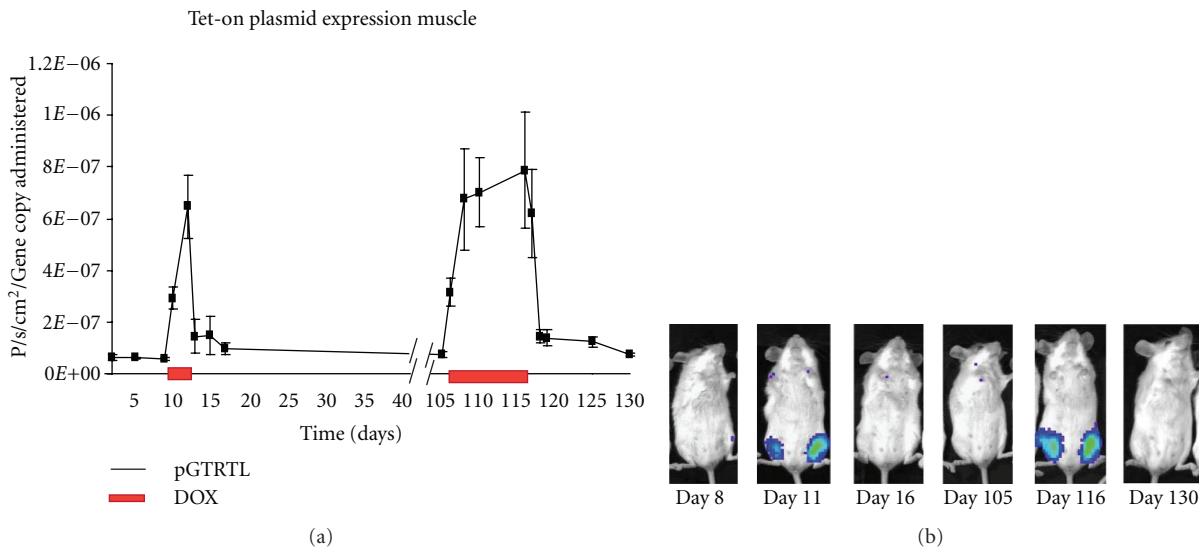


FIGURE 4: Long-term controllable transgene expression using doxycycline-inducible plasmid. pGTRTL was delivered to quadriceps muscle ($n = 22$) by electroporation and luminescence analysed *in vivo* over time using IVIS live whole body imaging. Doxycycline was administered from days 8–11 and days 105–118 inclusively (red bar). Efficient, reproducible, high-magnitude expression was noted on induction with doxycycline.

24 hours was 85%, 82%, and 38%, respectively (Figure 5). This was significantly better than cell survival following incubation in RPMI (22%, $P < 0.05$). There was no difference in cell survival following plasmid electroporation when compared to untreated samples (34% and 38%, resp., $P = 0.5$).

3.5. Ex Vivo Delivery to and Transgene Expression in Patient Mesenchymal Tissues. To assess translation of the murine

findings to a clinical setting, samples of muscle, tendon, and bone with intact periosteal tissue were harvested from a human leg amputated for end-stage peripheral vascular disease. Absolute ischaemic time following amputation before immersion in media was less than 40 minutes. 4 samples were taken of each tissue type and transfected with pCMV-luc or pGTRTL as previously described. These were incubated overnight in conditions described above and luminescence assessed at 24 h (Figure 6). Luciferase

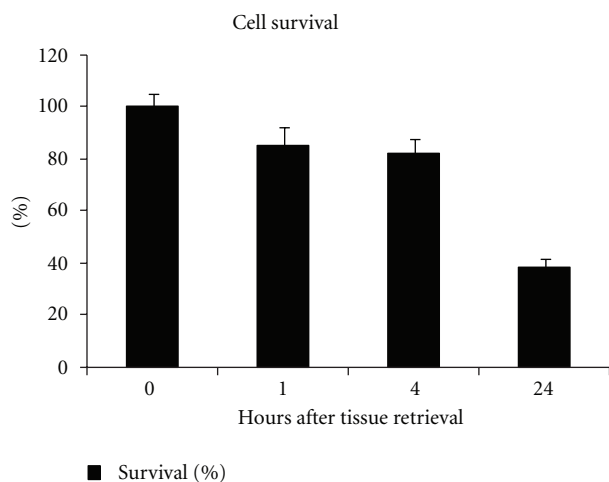


FIGURE 5: Murine *ex vivo* tissue survival in DMEM over time. Murine thigh muscle was harvested immediately after culling, immersed in DMEM, and stored at 37° centigrade. Cell survival was assessed at 0, 1, 4, and 24 hours with 38% viability at 24 hours when compared to 0 hours.

gene expression was observed in all tissues electroporated with plasmid, indicating successful plasmid delivery and subsequent gene expression in this setting.

4. Discussion

Localised gene expression offers certain advantages in the setting of tissue healing. It may provide a more accurate system of protein delivery to the desired tissue, whilst lessening systemic side effects. In addition, it has the potential for sustained protein delivery, both intracellularly and extracellularly without the need for repeated administration. However, inactivation of gene expression in specific cell types has important therapeutic implications, as does the prospect of uncontrolled protein production [10]. Here, we have demonstrated that plasmid-based gene expression in murine muscle does not decrease with time. Long-term plasmid gene expression has been observed in muscle previously, although not using an *in vivo* luminescence system for a period greater than one year [15, 16]. *In vivo* luminescence has previously been shown to be a reliable indicator of transgene expression [17]. It is also an ideal method of assessing an inducible agent as an “on-off” switch to control protein expression, by allowing repeated measurements in the same subjects over a long time period.

Successful bone and tendon healing in animal models have been reported following adenoviral transfer of BMP 2 and BMP 14 [5, 6, 18]. Here, we have compared luciferase expression between a plasmid construct and viral particles. Although maximum expression was significantly greater with adenovirus, this was rapidly silenced to background levels by day 18. The attraction of the plasmid system would therefore lie in its capacity for sustained levels of predictable expression over time. Electroporation-assisted intramuscular delivery of plasmid DNA has been shown to

efficiently transfect cells of myogenic origin with no evidence of transgene expression in infiltrating inflammatory cells [19]. This is broadly in keeping with our observation of long-term expression, with transfection of a nontransient cell population in muscle and the absence of a significant immune response. Silencing has previously been observed with adenovirus, with the mechanism postulated as methylation of the CMV promoter [10]. Why this does not occur when the promoter is delivered using a plasmid construct, however, is unclear.

Uncontrolled long-term gene expression, however, has serious potential side effects. For instance, in the clinical use of recombinant bone morphogenetic protein (rhBMP) for augmentation of fracture healing and joint fusion, heterotopic ossification and other complications have been reported [20]. In this instance, the protein is eventually cleared from the body. Following gene delivery, however, it would be difficult to arrest protein production in the event of unwanted side effects. It is, therefore, desirable to have a plasmid with an “on-off” switch. We have demonstrated that this can be achieved using pGTRTL, a tet-on inducible promoter. After plasmid delivery, gene expression initiated with the commencement of oral doxycycline administration and promptly ceased upon withdrawal. This strategy has also proven to be successful in a preclinical arthritis study [21]. In the clinical setting, should side effects occur, gene expression could be turned off simply by omitting the oral agent.

In addition, growth factors may not be clinically beneficial until days after intervention, while the delivery of rhBMP at the time of surgery has been shown to impair tissue healing by inducing antagonist factors [18, 22]. By using pGTRTL, we have demonstrated that the timing and duration of gene expression can be tightly controlled *in vivo*. This would decrease the chance of antagonist induction, whilst optimising the timescale of protein production. Furthermore, expression was reinduced efficiently and to a reproducible level. This would allow us to repeatedly treat tissue with a known quantity of protein in recalcitrant situations. As the system is orally administered, there would be no need for interference with the physical environment of tissue healing after the initial application. One issue with tetracycline compounds is that they do have an effect on the tissue healing environment and as such may not be the ideal tool for the investigation of therapeutic plasmid use [23]. In terms of principle, however, it demonstrates the potential applicability of an inducible plasmid-based system in a clinical setting.

Finally, we have demonstrated, for the first time, successful plasmid-based gene delivery to human *ex vivo* muscle tissue. Levels of luciferase expression in *ex vivo* tendon and muscle were similar when examined at 24 hours. This was surprising, given the increased cellularity of muscle tissue. Although the exact reasons for the similar levels of expression are unclear, it may be related to a threshold effect or differential tissue survival in the *ex vivo* environment. Expression may differ in the human *in vivo* setting, particularly with regard to increased metabolic activity in muscle tissue. Nonetheless, we believe this to be an important step in the demonstration of the applicability of this technique in

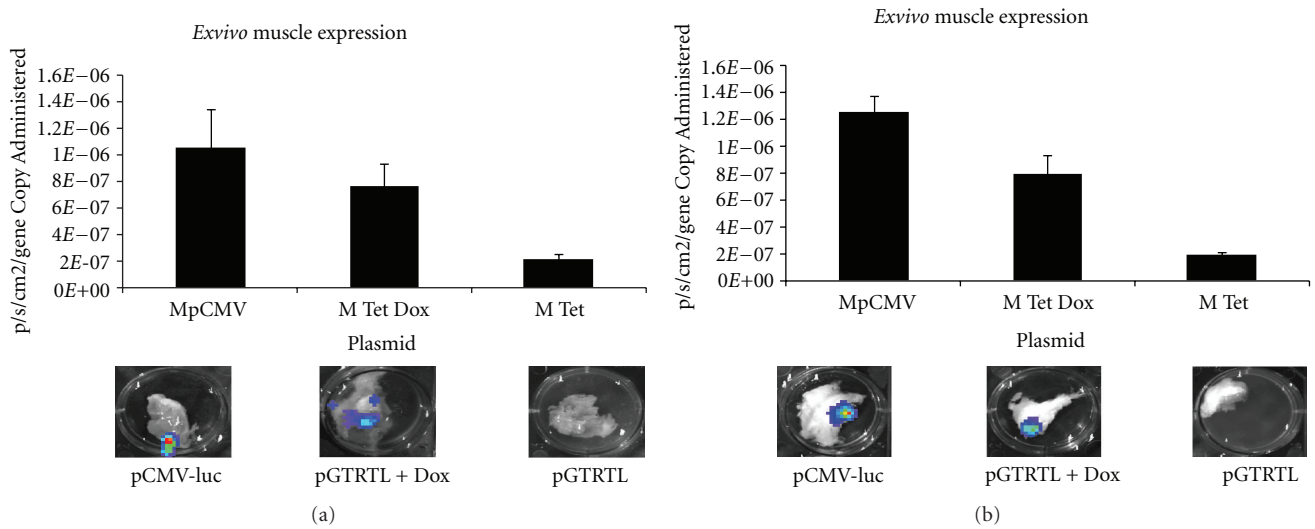


FIGURE 6: Human musculoskeletal tissue electrotransfected *ex vivo* with either pCMV-luc or pGTRTL. Expression was observed in all samples. Levels of expression for the “tet-on” plasmid were significantly increased in the presence of doxycycline.

the *in vivo* setting, as expression capability cannot be definitely extrapolated from the murine model to the human setting. The expression of plasmid-based delivery in human *ex vivo* musculoskeletal tissue is encouraging on two levels; firstly, it proves that the system can function in human tissue, and secondly, it raises the possibility of autograft or allograft transfection, which could lead to the acceleration of graft incorporation [24].

The luminescence imaging strategy utilised here provided a rapid, robust and reliable method for *ex vivo* assessment of transgene expression in patient samples. The method of culturing tissue *ex vivo* has been demonstrated to provide high viability for up to 1 week post resection, while bioluminescence imaging provides a highly sensitive readout for real-time transgene expression in viable cells [25]. Other reporter gene imaging techniques may also be applicable in this context (fluorescence, PET etc.) [26, 27]. In summary, these results indicate, when administered using electroporation, that plasmid constructs result in long-term *in vivo* gene expression and that this expression can be reliably induced with oral agents. Finally, for the first time, successful plasmid gene transfection with electroporation in human *ex vivo* mesenchymal tissue has been demonstrated.

Disclosure

The authors have no declared professional or financial affiliations pertinent to this work.

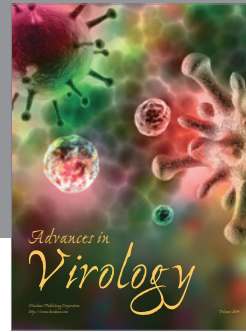
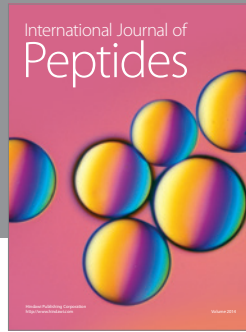
Acknowledgment

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