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Optimising ultrasound-mediated gene transfer (sonoporation) *in vitro* and prolonged expression of a transgene *in vivo:* Potential applications for gene therapy of cancer

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ABSTRACT

Therapeutic approaches using gene-based medicines promise alternatives or adjuncts to conventional cancer treatment. Because of its non-invasive nature, ultrasound, as a membrane-permeabilising stimulus has the potential to be highly competitive with viral gene delivery and existing non-viral alternatives. In optimising ultrasound-mediated, microbubble-assisted (MB101) gene tranfection *in vitro,* we demonstrate efficiencies of up to 18% using ultrasound at 1 MHz at a duty cycle of 25% at intensities ranging from 1 to 4 W cm^{-2}. Using ultrasound-mediated transfection together with an episomal plasmid-based gene expression system, we demonstrate prolonged functional gene expression of luciferase in mouse hind leg muscle and in tumours *in vivo*.

1. Introduction

Delivery of nucleic acid to a target tissue together with a means of facilitating efficient entry of that nucleic acid into cell populations in that tissue remain amongst the principle challenges to effective gene therapy in the treatment of a variety of disorders including cancer, cardiovascular disease and inherited immune deficiencies. In the past viral vectors have been employed in attempts to circumvent these challenges and indeed such approaches have provided much evidence supporting the vast potential of gene therapy in medicine. Nevertheless, it has become increasingly clear from clinical trials data that approaches based on the use of viral vectors carry some considerable risk to the patient [1,2]. In addition approaches using viral vectors usually result in the elicitation of immune responses to the vector, so that therapies necessitating repeat dosing may be precluded [3]. Alternatives to viral vectors that have been employed in the past to facilitate entry of therapeutic nucleic acid into cells have been based on methods that induce transient cell membrane permeabilisation and to date, electroporation or electric field-mediated cell membrane permeabilisation, has received most attention [4].

More recently however, ultrasound as a means of stimulating cell membrane permeabilisation (sonoporation) for the purposes of transferring nucleic acids into cells has been increasingly reported in the literature and offers advantage over its competing technologies, primarily as a result of its relatively non-invasive nature [5,6]. Extracorporeal stimulation using ultrasound provides the advantage of accessing sites deep within the body that may be less accessible to electroporative methodologies. Approaches using ultrasound for stimulating entry of nucleic acids such as plasmids encoding therapeutic phenotypes into cells, have included the use of ultrasound alone or in combination with microbubble preparations more commonly employed as diagnostic contrast agents [7,8]. Developments in this area have been complicated by the lack of

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consistency throughout the field, particularly in terms of ultrasound exposure configurations. Ultrasound exposure configurations for studying ultrasound-mediated gene transfer *in vitro* have consisted of exposing cells to ultrasound in tissue culture wells and the effect of exposure configuration on the results obtained have been clearly demonstrated by Dunshani-Eschet & Machluf [9]. In this study it was found that if the ultrasound was transmitted up through the bottom of the well containing the target cells, viability following exposure was much lower than viability when the ultrasound was transmitted into the medium and absorbed following passage through the bottom of the tissue culture well. This decrease in cell viability encountered in the configuration where ultrasound was transmitted up through the tissue culture well was attributed to either reflection from the air–liquid interface resulting in exposure of the cells to higher energy densities and/or the establishment of standing waves in the system which would lead to the generation of excessive heating [9]. In addition to the above, many other ultrasound exposure configurations have been described and in many cases, the ultrasound exposure conditions employed for *in vitro* transfection do not necessarily translate directly to *in vivo* ultrasound transfection systems. Indeed in many cases, the ultrasound energy densities/power densities employed for *in vitro* ultrasound-mediated transfection have been much lower than those employed for *in vivo* stimulation of gene transfer [10–12]. In a relatively recent report, a commercially-available cell culture system that was ideally suited to studies involving exposure of cell-based targets to non-invasive transfection stimuli such as ultrasound was described [13]. Although the authors reported relatively low transfection efficiencies ($\leqslant$4%), the exposure configuration did seem attractive since it suggested that conditions optimised in such a system would be more applicable to an *in vivo* scenario. Therefore in the studies presented here we describe the use of this system to optimise ultrasound and microbubble exposure conditions in order to deduce conditions that may be directly applicable *in vivo*.

In developing an alternative to viral gene transfer or indeed to further enhance the advantages associated with the use of ultrasound to facilitate gene transfer for the purposes of achieving gene expression as a mediator of cancer therapy, expression of the therapeutic gene must be prolonged. In the past, conventional plasmid-based systems have been used as the genetic element in many ultrasound-mediated gene transfection studies [10–13]. In such cases, vectors are only transiently expressed and this has negative repercussions for prolonged gene expression and retention of any introduced therapeutic effect. Such approaches therefore would necessitate repeat treatments and in many cases this would not be desirable particularly where transfection of solid tumours would be required. In 1999 Piechaczek et al., described a plasmid vector system, pEPI-1, that contained the SV40 origin of replication in association with the scaffold/matrix attached region (S/MAR) from the 5′-region of the human interferon β-gene [14]. These sequences are often found in association with chromosomal origins of bidirectional replication. The authors demonstrated that the vector containing this sequence replicated at low copy number in mammalian cells and was stably maintained without selection in tissue culture for 100 generations. Therefore, in order to further enhance the benefits of using non-viral vector systems and in particular, to enhance the use of ultrasound-mediated gene transfection for the purposes of gene therapy, the episomal plasmid system offered advantage in terms of prolonging transgene expression.

In this study we describe the use of an ultrasound exposure configuration to ascertain the appropriate ultrasound conditions to facilitate enhanced gene transfection *in vitro*. In addition we describe the use of the optimised ultrasound conditions together with the episomal plasmid, pEPI-1-Luc, to examine expression of the luciferase transgene in the hind leg of mice. We further describe prolonged transgene expression in mouse syngenic tumours and suggest a possible use of this ultrasound-based approach in the application of gene therapy to the treatment of cancer.

2. Materials and methods

2.1. Plasmids, cell culture and establishing tumours

Plasmid pEGFP-C1 was supplied by BEX (Tokyo, Japan). The episomal plasmid pEPI-1-Luc was a gift from Plasmid Factory GmbH and Co, Germany and contains the scaffold/matrix attached region (S/MAR) from the 5′-region of the human interferon β-gene [14]. This vector also contains the luciferase gene under the control of a CMV promoter. HeLa cells were employed as an *in vitro* transfection target in these studies and was maintained in DMEM tissue culture medium (with glutamine and pyruvate) (GibcoBRL, UK), supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) non-essential amino acids, in a 5% CO_2 humidified atmosphere at 37 °C. When required, single cell suspensions were prepared by treating with a 0.05% (w/v) solution of trypsin containing 0.02% (w/v) EDTA in phosphate buffered saline. RIF-1 cells were maintained in RPMI 1640 medium supplemented with glutamine (GibcoBRL, UK) and 10% (v/v) foetal bovine serum. Cells were grown to confluence and harvested by centrifugation after treatment with trypsin–EDTA solution. Tumours were established in C3H/HeN (8-week-old) mice essentially as described previously [15]. All animals were treated humanely and all manipulations were in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act 1986.

2.2. Ultrasound-mediated transfection in vitro

In these studies a Sonidel SP 100 sonoporation device (Sonidel Ltd., Ireland) emitting ultrasound at a frequency of 1 MHz was employed for all treatments. The transducer had an effective radiating area of 0.8 cm^2 and the ultrasound intensity/power density setting on the instrument was expressed in W cm^{-2} (SATP, spatial average, temporal peak). In some specific cases a modification to the conventional Sonidel SP100 platform was employed and this incorporated an over-riding pulse frequency of 40 kHz as described previously [16]. HeLa cells represented the tar-

get in all *in vitro* studies and these were inoculated into 10 ml culture units (Sonidel Ltd., Ireland) and grown to 90% confluence. In these studies two microbubble preparations were employed as sonoporation aids and these consisted of either the Sonidel MB101® microbubble (Sonidel Ltd, Ireland) or SonoVue® (Bracco, Switzerland). The former consists of a perfluorocarbon gas core encapsulated in a stabilised lipid monolayer. The average particle size was 2.7 μm in diameter and was supplied at a concentration of 8.5×10^8 particles per ml. The latter consists of a sulphur hexafluoride core in a lipid shell and had an average particle size of 2.5 μm. Both microbubble preparations were employed at a final concentration of 4×10^7 particles per ml. Prior to treatment with ultrasound, the cell culture vessel was washed twice with Opti-MEM, filled with 9 ml of the latter together with DNA to yield a final concentration of 12 μg/ml and the unit was then placed at 37 °C for 30 min. After this time the required quantity of microbubbles was diluted in PBS for the Sonidel MB101® microbubble and in the provided saline for the SonoVue® preparation, respectively, to yield a total volume of 1 ml and this was added to the cell culture vessel to yield a total volume of 10 ml. The cell culture units were then placed in a water bath with an ultrasound-absorbing pad placed beneath the unit to minimise echogenic effects and the ultrasound probe was placed in contact with the unit. Ultrasound treatment was then performed at the required intensity, duty cycle and for the indicated time. Following treatment, the cell container was placed in an incubator at 37 °C for a period of 1 h. After this period 1 ml of the Opti-MEM was removed from the unit, replaced with 1 ml of foetal bovine serum and the cell-containing unit was retained at 37 °C for a period of 20–24 h. Cells were subsequently examined for expression of green fluorescent protein (GFP) using fluorescence microscopy and transfection efficiency was expressed as the number of cells expressing GFP as a % of the total number of cells in any given field examined as determined by viewing under white light microscopy. Essentially, following treatment, areas of the cell-containing unit that had received exposure to ultrasound were clearly marked. Initially, at least 10 points within each area were chosen for direct observation and photomicrographs were taken under white light and fluorescence. Photomicrographs were then employed to determine the number of cells per observation area and the number of cells exhibiting green fluorescence in those areas. Using this methodology the number of cells per observation areas under white light was in the region of 500–1000 cells per area. Statistical analysis of significance was conducted using ANOVA and data groups were compared with the Tukey Multiple Comparison Test (MCT) using GraphPad Prism version 4.

2.3. Ultrasound-mediated gene transfer in vivo

In these studies the episomal plasmid pEPI-1-Luc, encoding luciferase under the control of a CMV promoter was employed to monitor transfection. DNA was used at a concentration of 1 mg/ml and for either intramuscular or intratumoural injections, 20 μl of plasmid was mixed with a 20 μl volume of the Sonidel MB101® microbubble to achieve a final concentration of 4×10^8 microbubbles per ml in each injection. Forty microlitre quantities of microbubble-plasmid mixtures were injected into the hind leg muscle of C3H/HeN (8-week-old) mice. Where tumours were used, RIF-1 tumours were grown on the back of the animals until they reached a volume of 150–200 mm^3. The microbubble-plasmid mixture was administered to tumours by intratumoural injection. Target areas were then treated with ultrasound by direct contact with the ultrasound-emitting head and contact was mediated using an ultrasound contact gel. Treatments were carried out at 1.9 W cm^{-2} for 6 and 3 min, respectively, at a duty cycle of 25% in the presence or absence of a superimposed high frequency pulse regime of 40 kHz. Following ultrasound-mediated transfection, *in vivo* imaging was performed to assess functional expression of luciferase. Following induction of anaesthesia (Hypnorm:Hypnoval, IP), luciferin (200 μl of 20 mg/ml) was administered by intraperitoneal injection. Photonic imaging to detect the presence of luminescence was performed using a Xenogen IVIS® Lumina imaging system supported by Living Image® software version 2.60. In all *in vivo* experiments, four animals were employed per treatment group and data were collected from three individual experiments. Once again, statistical analysis of significance was conducted using ANOVA and data groups were compared with the Tukey MCT using GraphPad Prism version 4.

3. Results and discussion

3.1. Optimisation of ultrasound-mediated transfection ***in vitro***

Although a number of physical configurations reported for ultrasound treatment for the purposes of gene transfer *in vitro* have been described, many of those describe optimised ultrasound parameters that do not necessarily translate to facilitating optimal gene transfer *in vivo* [10–12,17]. In many of those cases this results from beam distortion issues relating to the use of rigid plastics commonly employed for tissue culture or from the existence of an air-medium interface resulting in adverse echogenic effects [9,13]. The cell culture device employed in our studies involved culturing cells on a gas permeable and ultrasound transparent membrane and, in addition to obviating the negative impact of those issues referred to above, this also served to reduce contamination risk during exposure to ultrasound. In addition, since a lack of consensus appeared to exist in the literature relating to the type of ultrasound generator employed in many studies, we also employed a precision device that was specifically supplied to the research community and designed solely for the purposes of sonoporation.

Therefore in order to examine the effect of ultrasound intensity/energy density on the efficiency of transfection, the target cell population was exposed to a variety of treatment parameters that would have previously been shown to induce cell permeabilisation [9,16]. In these experiments adhered cells were exposed to ultrasound intensities ranging from 0.5 to 4 W cm^{-2} and these intensities related to supplying total energy densities of 135–1080 Jcm^{-2}. The latter power density conversions were calculated on the basis of 1 W = 1 J s^{-1}. A duty cycle of 60% was employed and this represented a pulse repetition rate of 100 Hz. In addition, SonoVue® was employed as the microbubble preparation. Twenty-four hours after treatment, areas of the cell culture unit that had been exposed to ultrasound were examined using fluorescence microscopy to detect expression of GFP. Cell viability was also determined using trypan blue dye exclusion. The data obtained are shown in Fig. 1 and they demonstrate that a maximum transfection efficiency of 13.5% was obtained at an ultrasound intensity of 4 W cm^{-2} (1080 J cm^{-2}). When analysed for statistical significance (ANOVA and Tukey MCT) it was found that no significant difference in transfection efficiency existed between treatments at 0.5 and 1 W cm^{-2}. However, when compared with treatments at 0.5 W cm^{-2}, treatments at 2 and 4 W cm^{-2} yielded transfection efficien-

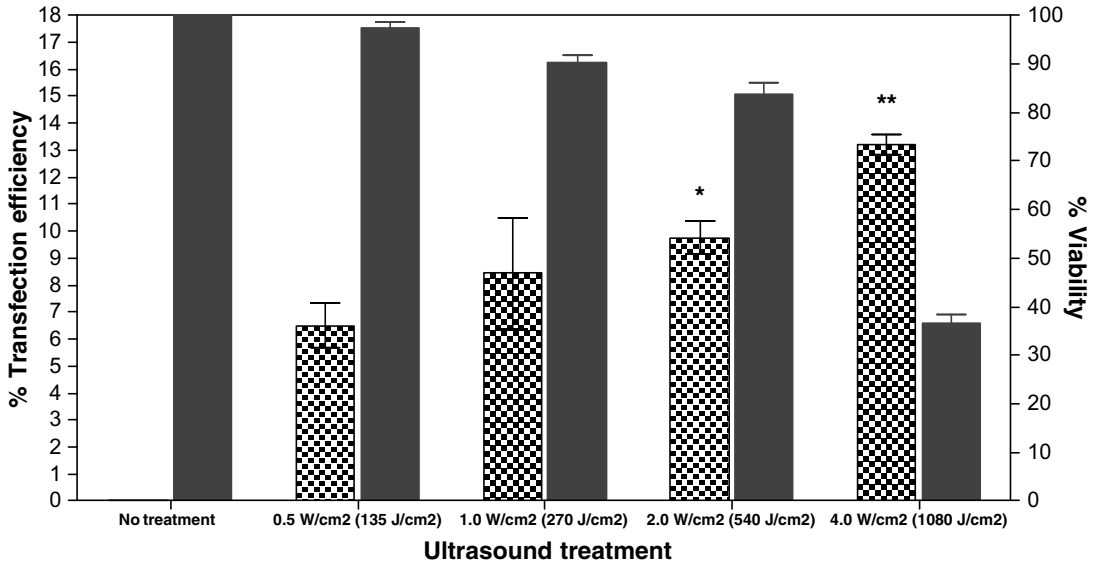

Fig. 1. Ultrasound-mediated transfection of HeLa cells using the plasmid pEGFP-C1. SonoVue® was employed as the microbubble and ultrasound was delivered over a period of 7.5 min using a duty cycle of 60%. Black bars represent cell viabilities and relate to the right *Y*-axis and chequered bars represent transfection efficiencies. The asterisks indicate statistically significant increases in transfection efficiencies when compared with the transfection efficiency observed at 0.5 W cm^{-2} as determined using ANOVA and Tukey MCT where *represents $p < 0.01$ and **represents $p < 0.001$. Error bars represent ±SEM where n (number of experiments) = 3.

cies that were significantly higher ($p < 0.01$ and 0.001, respectively). However, at 4 W cm^{-2}, cell viability was severely compromised with only 37% of cells remaining viable and this reduction in viability was found to be independent of the presence of plasmid in transfection mixtures, i.e. similar reductions in cell viability were observed by exposing cells to ultrasound in the presence of microbubbles and in the absence of plasmid. In overall terms these data compare very favourably with those previously reported using a similar ultrasound exposure configuration and in that case the efficiency of transfection was reported to be in the region of 4% [13]. These authors obtained optimal transfection at acoustic pressures of 0.25 MPa. Here our calculated acoustic pressures range from 0.09 to 0.24 MPa with optimal transfection observed at a calculated acoustic pressure of 0.24 MPa. Cell viabilities at higher ultrasound intensities (4 W cm^{-2}) were however, less favourable than those reported by Duvshani-Eschet & Machluf [9] despite employing similar energy densities. In the latter case however, energy densities of 1080 J cm^{-2} were delivered to the target at an acoustic pressure of 0.17 MPa over a 30 min period using a 30% duty cycle whereas in our case we delivered the energy over a much shorter time frame (7.5 min.) by increasing the intensity (calculated acoustic pressure = 0.24 MPa) and increasing the duty cycle to 60%.

Since we had previously demonstrated that superimposition of a 40 kHz pulse regime on the above ultrasound conditions (1 MHz at duty cycle of 60%) led to enhanced membrane permeabilisation to lower molecular weight agents such as fluorogenic markers and a chemotherapeutic drug [16] it was of interest to determine whether or not such an approach would provide advantage in terms of enhancing gene transfer *in vitro*. To this end cells were treated in a manner similar to that described above, except that the ultrasound intensity was set at 1 W cm^{-2}, with a duty cycle of 60% and with a superimposed pulse repetition rate of 40 kHz as described previously [16]. This was performed in the presence and absence of SonoVue® as the sonoporation aid. Expression of GFP was employed as a measure of transfection efficiency as described above and the results shown in Fig. 2 were obtained. The data demonstrated that low, but detectable levels of transfection were observed in the absence of microbubbles with the introduction of the 40 kHz pulse regime yielding an increased degree of transfection although using ANOVA and the Tukey MCT for group comparisons this difference was not found to be statistically significant. When microbubbles were added to transfection mixtures, a very significant increase ($p < 0.001$) in transfection efficiency was observed when compared with those obtained in the absence of microbubbles. In addition, it was found that introduction of the 40 kHz pulse regime increased the transfection efficiency from 10 to 15% and this was again found to be statistically significant ($p < 0.05$). In these experiments, cell viabilities remained above 85% during all treatments. In previously reported work by others, most notably Rahim et al. [13], increasing the pulse repetition rate above 1 kHz had no significant influence on transfection efficiency. However, the upper limit of the range examined in that study was 2.5 kHz. Here we confirm our earlier findings that enhanced membrane permeabilisation occurs in the presence of the high frequency pulse regime and we demonstrate that such an approach facilitates enhanced ultrasound-mediated transfection *in vitro*.

In the above studies, SonoVue® was employed as the sonoporation aid and it was of interest to determine whether or not an alternative microbubble, MB101® specifically designed for the purposes of sonoporation, would further enhance transfection efficiency. In addition, in order to reduce ultrasound treatment times, it was decided to examine the effect of increasing the intensity (acoustic pressure) above that employed in the work that generated data for Fig. 2 in combination with reducing the low frequency duty cycle aspect. Therefore in these studies, the ultrasound intensity was increased from 1 to 1.9 W cm^{-2} with the latter having a calculated acoustic pressure of 0.167 MPa. Also in the latter case exposure of the target to ultrasound was adjusted so that the overall energy density received by each sample was 85.5 J cm^{-2}. The data obtained are shown in Fig. 3 and they demonstrate a number of interesting points. In the absence of the high frequency pulse regime, sonoporation efficiencies were statistically similar in the presence of both microbubble preparations although there appeared to be an upward trend in sonoporation efficiency using the MB101® microbubble. When the high frequency pulse regime was engaged it was found that the transfection efficiency with SonoVue® tended to increase although this was not found to be statistically significant. However, when the MB101® microbubble was employed, the transfection efficiency increased to 18% and this was found to be statistically significant when compared with transfection efficiencies obtained in the absence of the 40 kHz pulse regime ($p < 0.01$) and when compared with transfection efficiencies obtained using SonoVue® in the presence of the 40 kHz pulse regime ($p < 0.01$). Although both microbubbles are relatively similar in size, they differ in the type of gas incorporated with SonoVue® containing sulphur hexafluoride and MB 101® a perfluorocarbon gas. A recent study examined the influence of microbubble gas on the ability of microbubbles to generate cell membrane pores and this study indicated little difference between the size of pores generated by either perfluorocarbon and SF_6-containing micro-

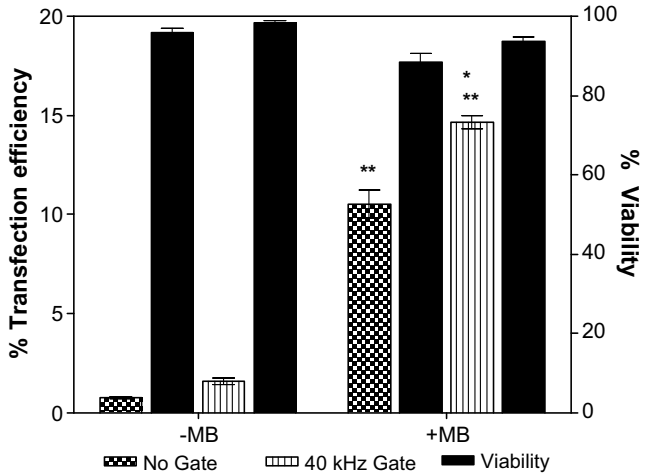

Fig. 2. Ultrasound-mediated transfection of HeLa cells using the plasmid pEGFP-C1 in the presence and absence of a 40 kHz pulse regime. SonoVue® was employed as the microbubble and ultrasound was delivered at an intensity of 1 W cm^{-2} at a duty cycle of 60% (100 Hz) and when the high frequency pulse regime was engaged, this was superimposed over this low frequency pulse regime. Black bars represent cell viabilities and relate to the right-hand *Y*-axis. Statistical analyses were carried out using ANOVA together with the Tukey MCT for inter-group comparisons. * represents a statistically significant increase ($p < 0.05$) in transfection efficiency when compared with that obtained in the presence of microbubbles and in the absence of the 40 kHz pulse regime. ** represents a statistically significant increase ($p < 0.001$) in transfection efficiency when compared with the relevant transfection in the absence of microbubbles. Error bars represent ±SEM where *n* (number of experiments) = 5.

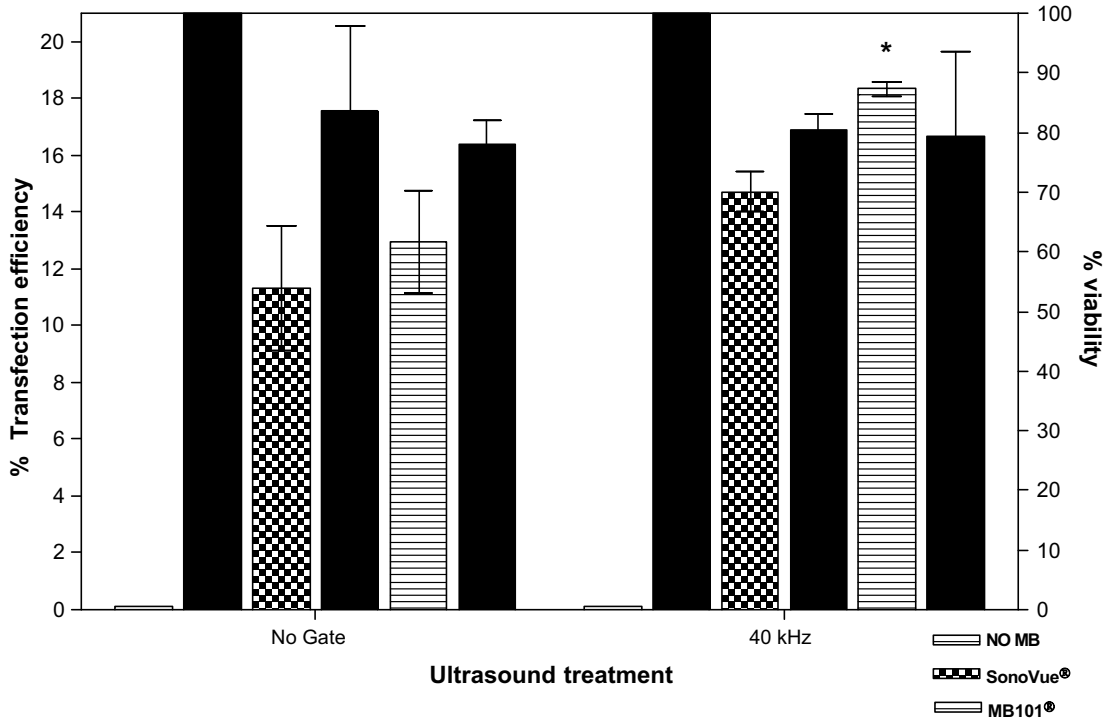

Fig. 3. Comparing the effect of SonoVue® and MB101® in assisting ultrasound transfection of HeLa cells with the plasmid pEGFP-C1 in the presence and absence of a 40 kHz pulse regime. Ultrasound was delivered at an intensity of 1.9 W cm^{-2} at duty cycle of 25% (100 Hz) and in cases where the 40 kHz high frequency pulse regime was engaged this was superimposed over the low frequency pulse regime. Black bars represent cell viabilities and relate to the right-hand *Y*-axis. Statistical analyses were carried out using ANOVA together with the Tukey MCT for inter-group comparisons. * represents a significant ($p < 0.01$) increase in transfection efficiency when comparing the indicated group with either transfection efficiencies obtained in the absence of the 40 kHz pulse regime with either microbubble or in the presence of the SonoVue® in the presence of the 40 kHz pulse regime. Error bars represent ±SEM where *n* (number of experiments) = 3.

bubbles [17]. The results obtained here again confirm that incorporation of the high frequency pulse regime enhanced gene transfection, particularly when the MB 101® microbubble was employed with these ultrasound conditions. In addition it was found that in all cases the cell viabilities remained at or above 80% using these particular ultrasound conditions. It is interesting to note that here, in the absence of the high frequency pulse regime, we subjected cells to energy densities of 85 J cm^{-2} and obtaining transfection efficiencies of 11–13% whereas in our earlier studies shown in Figs. 1 and 2, we employed much higher energy densities to achieve the same results. Indeed as shown in Fig. 1, the results obtained here are much higher than those obtained even at the lowest energy density of 135 J cm^{-2} in those previous studies. These results suggest that, in treating samples for the purposes of achieving gene transfection *in vitro*, it is the ultrasound intensity (SATP)/acoustic pressure and not necessarily the overall ultrasound energy density deposited into the target sample that plays the major role in facilitating enhanced gene transfection.

3.2. Ultrasound-mediated gene transfer into mouse hind leg muscle in vivo

Since the ultrasound conditions employed together with the microbubble MB101® yielded the highest transfection efficiencies as shown in Fig. 3, it was decided to employ these conditions for *in vivo* transfection studies. It was also decided to employ these conditions because treatment times were shorter and this was accomplished as mentioned above by increasing the ultrasound intensity (acoustic pressure). The latter would also provide advantage in terms of tissue penetration for *in vivo* applications and reduce stimulus (ultrasound) dissipation as a result of tissue attenuation. In these studies it was decided to employ bioluminescence as a marker for transfection and gene expression. In addition it was further decided to employ the plasmid pEPI-1-Luc in order to determine whether or not such an approach would facilitate prolonged gene expression following ultrasound-mediated gene transfection. To the above ends plasmid was mixed together with the MB101® microbubble preparation and this was injected into the hind leg muscle of recipient animals. Immediately following injection, ultrasound was applied at 1.9 W cm^{-2} (85.5 J cm^{-2}) at a duty cycle of 25% in the presence or absence of the 40 kHz pulse regime. In addition, animals were also injected with DNA in the absence of microbubbles and treated with ultrasound. Animals were analysed for luciferase expression using photonic imaging at the indicated times following transfection. The data obtained are shown in Fig. 4 and they clearly demonstrate luciferase expression in the animals. The photograph in Fig. 4A illustrates the quality of image obtained following photonic imaging from which the data in Fig. 4 were derived. The signal on the left leg indicates the signal obtained when ultrasound was applied in the presence of the 40 kHz pulse regime and that on the right was obtained following treatment in the absence of the 40 kHz pulse regime. From the data shown in Fig. 4 and the image data shown in Fig. 4A, it is clear that higher luminescent signals are detected when transfection was performed in the presence of the 40 kHz pulse regime. Choosing data harvested from animals on day 5 when bioluminescence peaked and performing statistical analysis (ANOVA and Tukey MCT) on those data, the increase in transfection efficiency obtained in the absence of microbubbles using the 40 kHz pulse regime is statistically significant ($p < 0.001$) when compared with data obtained at that time point in the absence of the 40 kHz pulse regime and in the absence of microbubbles. Indeed these data support the trend obtained using *in vitro* systems with the plasmid pEGFP-C1 in the presence and absence of the 40 kHz pulse regime and in the absence of microbubbles (Fig. 3). It should be noted however that it is very difficult to quantitatively compare the *in vitro* and *in vivo* systems in terms of gene expression, since the analysis providing data for Fig. 3 measured the number of transfected cells and the analysis providing data for Fig. 4 measured actual gene expression (catalytic activity of the gene product) in the hind leg muscle. Nevertheless it was interesting to note that in the former, inclusion of the high frequency pulse regime increased the proportion of transfected cells by 30% whereas inclusion of the high frequency pulse regime in the *in vivo* experiments increased gene expression in the hind leg muscle by 18%. In overall terms however, and in the presence of microbubbles, it would appear that the positive contribution yielded by inclusion of the 40 kHz pulse regime was not overly dramatic when used *in vivo*.

As shown in Fig. 4, when luciferase expression was monitored for a prolonged period of time, it was found that the signal remained elevated for a period of up to 84 days and this is in dramatic contrast to previously reported results. Aoi et al. reported that luciferase expression vanished or was severely reduced 2 days after microbubble-assisted, ultrasound mediated transfection of mouse tissues (tumours) with a luciferase-expressing plasmid, pGL3-control [18]. Interestingly, in our study, when the reporter plasmid, pCMV-Luc (Plasmid Factory, Germany) was employed using ultrasound-mediated transfection, expression of luciferase activity was still evident at day 30 although the bioluminescent signal had started to deteriorate (data not shown). Allera-Moreau et al. [19] have recently described an expression vector system based on the use of internal ribosome entry sites (IRESs) which facilitates long-term expression of luciferase. When electroporation was exploited to facilitate transfection of this vector *in vivo*, the approach was shown to significantly enhance the longevity of luciferase expression in mouse skeletal muscle, although expression of luciferase could not be detected beyond 45 days [19]. The results obtained here, demonstrating functional expression of luciferase encoded by the pEPI-1-Luc plasmid for periods up to 85 days, suggest that an approach incorporating the use of plasmid vector systems based on S/MAR sequences may contribute positively to overall approaches exploiting ultrasound-mediated transfection for applications such as gene therapy. It is interesting to note that even at day 150 although significant luciferase activity could be detected in the animals transfected using the pEPI-1-Luc plasmid, little or no activity could be detected in control animals transfected with the plasmid, pCMV-Luc (results not shown). These results further suggest that prolonged expression of the ultrasound-transfected luciferase-expressing plasmid, pEPI-1-Luc, resulted at least in part from the nature of the plasmid employed in our study since the transfection conditions were similar in both cases.

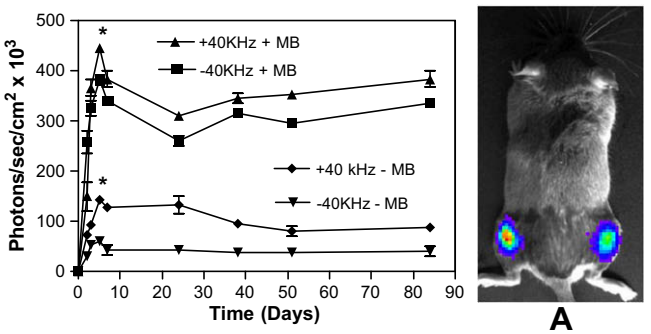

Fig. 4. Expression of the luciferase gene in mouse hind leg following ultrasound-mediated and MB101®-assisted transfection *in vivo* using the plasmid, pEPI-1-Luc. (A) A representative animal in which the left hind leg was treated with ultrasound incorporating the high frequency pulse regime and the right hind leg received ultrasound in the absence of the high frequency pulse regime. Expression was monitored for the indicated times using photonic imaging. Data on day 5 were analysed for statistical significance using ANOVA and the Tukey MCT. *represents a statistically significant increase where $p < 0.001$. Error bars represent ±SEM where n (number of experiments) = 3.

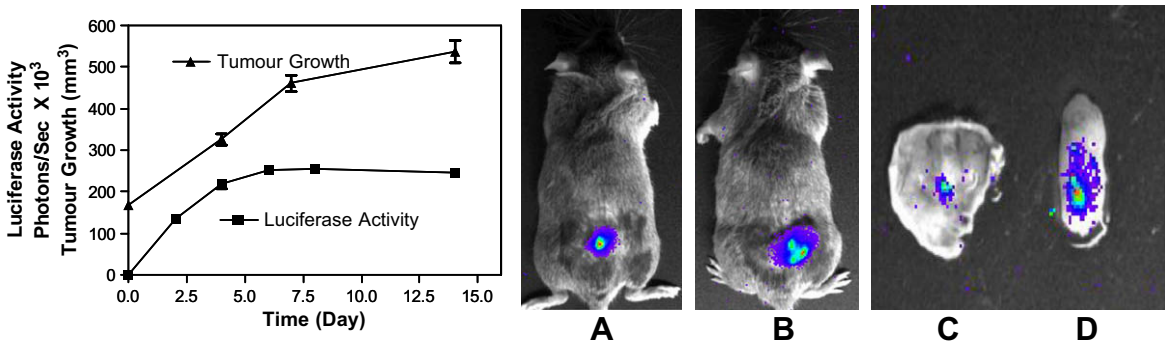

Fig. 5. Expression of the luciferase gene in mouse tumours following ultrasound-mediated and MB101®-assisted transfection *in vivo* using the plasmid pEIP-1-Luc. (A) The signal obtained from an animal at day 4 following transfection and (B) the same animal at day 20 following transfection. (C and D) The luminescent signal generated by the skin overlying the tumour and the excised tumour, respectively.

In previous studies, it has been found that ultrasound, together with microbubbles may contribute to the induction of apoptosis [20] and for general applications in gene therapy, minimising such a phenomenon would be desirable. In terms of the conditions employed in this study, Nie et al. [21] employed similar ultrasound intensities but much higher energy densities delivered over similar treatment periods (compare 85 J cm^{-2} used here with 300 J cm^{-2}) to treat hepatocellular carcinoma-based targets *in vivo* and demonstrated no significant increase in apoptosis indices. This observation in the literature is supported by the animals in this study exhibiting no adverse effects in terms of limb mobility following treatment and recovery from anaesthesia.

3.3. Ultrasound-mediated gene transfer into tumour tissues

Since the above work had demonstrated that the use of the episomal plasmid pEPI-1-Luc together with ultrasound-mediated transfection enabled prolonged expression of the transgene and since this strategy appeared to address the challenge of transient expression, it was felt that such an approach would impact positively on gene therapy-based approaches to treating cancer. In order to test this hypothesis it was decided to examine the possibility of using this approach to transfect the luciferase gene into tumours. To this end, RIF-1 tumours were established in syngenic mice and the optimised ultrasound conditions identified above, together with the MB101® microbubble, were employed to facilitate transfection of those tumours with the plasmid, pEPI-1-Luc. Both growth of tumours and expression of the luciferase gene was monitored using photonic imaging and the data obtained are shown in Fig. 5. It was found that expression of luciferase increased to a maximum by day 6 and the signal remained relatively constant between days 6 and 15. Interestingly, when the tumour was small the area covered by the signal was relatively small. However, as the tumour increased in size, so also did the area covered by the luminescent signal. Indeed as shown in Fig. 5A and B, the area of the signal at day 4 increased almost 2-fold by day 20. Such an observation could suggest that as the tumour increased in size, the plasmid was replicated in daughter cells, leading to an increase in the area covered by the luminescent signal.. It should however be noted that despite the bioluminescent signal remaining relatively constant between days 6 and 15, the tumours did continue to grow and one might have expected the bioluminescent signal to increase accordingly. Reasons for this are currently being examined. In order to confirm that transfection had occurred in the tumour mass and not in the overlying skin tissue it was decided to excise the tumour from one of the animals. The tumour tissue was surgically separated from the overlying skin and both tumour and skin were immersed in the luciferin substrate solution. When both tissues were examined using photonic imaging the majority of the luminescent signal (>90%) resided in the tumour tissue with only a small proportion residing in the overlying skin (Fig. 5C and D). It should also be noted that when tumours were divided, macroscopically, into cross-sections, it was found that bioluminescent signals penetrated to a depth of 1.5 mm from the (skin-facing) surface into the tumour mass. These results demonstrated that ultrasound-mediated transfection of tumours *in vivo* facilitated functional expression of the luciferase transgene throughout the life of the tumour. The results demonstrated that the luminescent signal, indicating gene expression, did not diminish during growth of the tumour and this suggested replication of the plasmid during expansion of the tumour. The results presented here compare very favourably with previously reported results for luciferase expression in tumours *in vivo* using ultrasound as a transfection stimulus. Aoi et al. [18] demonstrated that, following ultrasound-mediated transfection with the luciferase-encoding plasmid, pGL3-control, expression of the luciferase gene was severely reduced 2 days after transfection.

In overall terms the above study demonstrates that conditions employing ultrasound may be combined with judicious choice of an appropriate plasmid to facilitate long-term expression of a transgene *in vivo*. This strategy serves not only to circumvent many of the problems associated with viral gene transfer for applications relating to gene therapy, but also demonstrate a competitive advantage of the somewhat more invasive electroporation-based gene transfection systems. In terms of treating cancer, and since lesions may occur at any point deep in tissues within the body, the above approach employing an ultrasound-based, non-invasive transfection stimulus together with prolonged expression of a therapeutic transgene at a desired locus would have obvious advantages for the application of gene therapy to the treatment of cancer.

Conflict of interest

The authors wish to confirm that they have no conflict of interest relating to the generation for publication of the data contained in this manuscript.

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References

[1] A. Fischer, S. Hacein-Bey-Abina, M. Cavassana-Calvo, Gene therapy of metabolic disease, J. Inherit. Metab. Dis. 29 (2006) 409–412.
[2] W. Qasim, H.B. Gaspar, A.J. Thrasher, Update on clinical gene therapy in childhood, Arch. Dis. Child. 92 (2007) 1028–1031.
[3] A. Zaldumbide, R.C. Hoeben, How not to be seen: immune-evasion strategies in gene therapy, Gene Ther. 15 (2008) 239–246.
[4] L.C. Heller, R. Heller, In vivo electroporation for gene therapy, Human Gene Ther. 17 (2006) 890–897.
[5] G. ter Haar, Therapeutic applications of ultrasound, Prog. Biophys. Mol. Biol. 93 (2007) 111–129.
[6] T. Yamashita, S. Sondo, R. Suzuki, N. Arimura, K. Tachibana, K. Maruyama, T. Sakamoto, A novel bubble liposome and ultrasound-

mediated gene transfer to ocular surface. RC-1 cells i*n vitro* and conjuctiva *in vivo*, Exp. Eye Res. 85 (2007) 741–748.
[7] M.J.K. Blomley, Which US microbubble contrast agent is best for gene therapy?, Radiology 229 (2003) 297–298.
[8] X. Wang, H.D. Lliang, B. Dong, Q.-L. Lu, M.J.K. Blomley, Gene transfer with microbubble ultrasound and plasmid DNA into skeletal muscle of mice: comparison between commercially-available microbubble contrast agents, Radiology 237 (2005) 224–229.
[9] M. Duvshani-Eshet, M. Machluf, Therapeutic ultrasound optimisation for gene delivery: a key factor achieving nuclear DNA localisation, J. Control. Release 108 (2005) 513–528.
[10] T. Li, K. Tachibana, M. Kuroki, M. Kuroki, Gene transfer with echo contrast agents: comparison between Albunex, Optison and Levovist in mice – initial results, Radiology 229 (2003) 423–428.
[11] R. Suzuki, T. Takizawa, Y. Negishi, N. Utoguchi, K. Maruyama, Effective gene delivery with novel liposomal bubbles and ultrasonic destruction technology, Int. J. Pharm. 354 (2008) 47–55.
[12] R. Suzuki, T. Takizawa, Y. Negishi, N. Utoguchi, K. Sawamura, K. Tanaka, E. Namai, Y. Oda, Y. Matsumura, K. Muruyama, Tumour specific ultrasound enhanced gene transfer *in vivo* with novel liposome bubbles, J. Control. Release 125 (2008) 137–144.
[13] A. Rahim, S.L. Taylor, N.L. Bush, G.R. ter Haar, J.C. Bamber, C.D. Porter, Physical parameters affecting ultrasound/microbubble-mediated gene delivery efficiency *in vitro*, Ultrasound Med. Biol. 32 (2006) 1269–1279.
[14] C. Piechaczek, C. Fetzer, A. Baiker, J. Bode, H.L. Lipps, A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells, Nucleic Acid Res. 27 (1999) 426–428.
[15] A.M. Rollan, A. Smyth, P. Hughes, C.N. Reid, A.P. McHale, Electro-sensitisation of mammalian cells and tissues to ultrasound: a novel tumour treatment modality, Cancer Lett. 222 (2005) 49–55.
[16] Y.S. Li, C.N. Reid, A.P. McHale, Enhancing ultrasound-mediated cell membrane permeabilisation (sonoporation) using a high frequency pulse regime and implications for ultrasound-aided cancer chemotherapy, Cancer Lett. 29 (2008) 156–162.
[17] Y.Z. Zhao, Y.K. Luo, C.T. Lu, J.F. Xu, J. Tang, M. Zhang, Y. Zhang, H.D. Liang, Phospholipids-based microbubbles sonoporation pore size and reseal of cell membrane cultured *in vitro*, J. Drug Target. 16 (2008) 18–25.
[18] A. Aoi, Y. Watanabe, S. Mori, M. Takashi, G. Vassaux, T. Kodama, Herpes Simplex virus thymidine kinase-mediated suicide gene therapy using nano/microbubbles and ultrasound, Ultrasound Med. Biol. 34 (2008) 425–434.
[19] C. Allera-Moreau, A. Delluc-Clavieres, C. Constano, L. Van de Berghe, M. Golzio, M. Moreau, J. Teissie, J.-F. Arnal, A.-C. Prats, Long-term expression of bicistronic vector driven by the FGF-1 IRES in mouse muscle, BMC Biotechnol. 7 (2007) 1–12.
[20] L.B. Feril, T. Kondo, Q.-L. Zhao, R. Ogawa, K. Tachibana, N. Judo, S. Fujimoto, S. Nakamura, Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents, Ultrasound Med. Biol. 29 (2003) 331–337.
[21] F. Nie, H-X. Xu, M-D. Lu, Y. Wang, Q. Tang, Anti-angiogenic gene therapy for hepatocellular carcinoma mediated by microbubble-enhanced ultrasound exposure: an *in vivo* experimental study, J. Drug Target. 16 (2008) 389–395.