Gene therapy strategies to target post-interventional vascular remodeling

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Prolonged *in vivo* gene silencing by electroporation mediated plasmid delivery of siRNA

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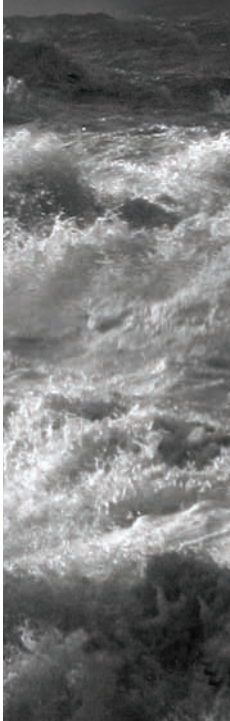

Abstract

For the successful application of RNA interference (RNAi) *in vivo*, it is desired to achieve (local) delivery of siRNAs and long-term gene silencing. Non-viral electrodelivery is very suitable to obtain local and a prolonged expression of transgenes. By intramuscular electrodelivery of a plasmid in which two opposing human polymerase III promoters (H1 and U6) drive the expression of siRNA-constructs that form functional double stranded small interfering RNAs (siRNA), in combination with *in vivo* bioluminescence imaging, we were able to knock-down exogenous delivered luciferase for at least 100 days in murine calf muscles. This effect was sequence specific, since scrambled siRNA had no effect. Moreover, we were able to demonstrate *in vivo* reduction of endogenous Toll-like receptor (TLR)-4 expression for at least one week, using a similar vector expressing a siRNA for TLR4 in the muscle. In this study, we demonstrate that long-term *in vivo* suppression of both endogenous (for at least one week) and introduced genes (>100 days) is feasible via plasmid-driven siRNA expression after electroporation-mediated intramuscular gene transfer. With this approach the short-term effect of oligonucleotides and the drawbacks of viral gene delivery, like immunological responses, could be circumvented. Therefore, this application of RNA interference is a very useful tool to investigate gene function and might be promising as a therapeutic tool for locally acting diseases like restenosis or tumors.

Introduction

The power of RNA interference (RNAi) to evaluate gene function as well as the therapeutic potentials of small interfering RNA (siRNA) have been demonstrated by many studies now, mainly using double-stranded oligo nucleotides and *in vitro* cultures[1-5]. For efficient *in vivo* use of RNAi technology it is essential to deliver siRNA to the proper target tissue and to inhibit gene function for a sufficient period[4,6]. For this purpose, efficient delivery, stability and prolonged effects of siRNAs are prerequisites. General approaches to achieve this are either based on local delivery to the target tissue of synthetic double-stranded oligonucleotides or vector driven local expression of double-stranded RNA constructs[7].

Using synthetic double-stranded oligonucleotides to knock down genes has several advantages, including the availability of validated sequences and controlled quantities of oligos supplied to the tissue. Since many pre-designed and evaluated sequences are offered commercially and highly purified available, this has become easy. Moreover, no misfolding of the RNA structures occurs, because no transcription is needed. Important drawbacks are the difficulty to deliver these oligonucleotides to the target tissue or cells *in vivo*, their single use and instability. Therefore, the effect of gene silencing usually is relatively short.

The use of vectors, viral or non-viral, to express double-stranded RNA constructs has its own benefits: delivery of siRNA to the target cell is feasible and because of the constitutive expression of siRNA, a prolonged inhibition of genes is possible.

Viral delivery is reported to be efficient for delivering siRNA[8]. Nevertheless, inflammatory and immunological responses and low transduction efficiency when delivered locally, particularly adenoviral transduction in musculature, are major drawbacks of this approach. However, some viral vectors, like adeno-associated viruses, maybe useful to transduct skeletal muscle. Especially when several serotypes are used in combination with repeated administrations, expression levels may increase. Nevertheless, efficient myofiber transduction following rAAV2-mediated gene transfer has been disappointing in some case, due to the immune reaction and insufficient level of gene expression[9,10].

As an alternative, non-viral vectors are useful as a way to deliver siRNAs. A requirement is that these plasmids must be suitable to drive the expression of short siRNA [21-25 nucleotides). Recently, an efficient, plasmid based system for expressing siRNAs, was reported [11]. This system, based on the pHippy-vector (H1 inverted U6 promoter plasmid), is remarkable because of

its simplicity. It contains two opposing human polymerase III promoters to drive the expression of both strands of a template DNA cloned in between the promoters. This pHippy-vector proved to be effective in expressing functional siRNAs in human cells *in vitro* that knock down the expression of both ectopic reporters and endogenous genes[11].

In general, the transfection efficiency using plasmid vectors *in vivo* is rather low and the duration of transgene expression is relatively short. Although long-term expression in muscular tissue has been demonstrating by many authors, starting with Wolff and colleagues in 1992[12], electroporation mediated gene delivery has shown to improve the transfection efficiency of plasmid DNA and siRNA oligos *in vitro* and *in vivo*[13,14]. Nowadays, this method has been applied in many different types of tissue (e.g. skeletal muscle, liver, lung and vasculature)[15,16]. Electroporation not only improves the transfection efficiency but also gives prolonged transgene expression in time, especially in muscular tissue. Especially, Luciferase expression can be monitored in the individual animals in time using the new bioluminescence imaging technology.

In this study, we combine the advantages of long-term local gene expression after electroporation mediated *in vivo* gene transfer and *in vivo* bioluminescence imaging, with the advantages of pHippy driven siRNA expression, in order to silence genes *in vivo* for a prolonged period.

To study this, we first demonstrate that pHippy with its two opposing human polymerase III H1 and U6 promoters, is functional in murine cells. Furthermore, we show the efficacy of pHippy mediated endogenous and exogenous gene silencing *in vivo* in mice after intramuscular gene transfer by means of electroporation. In particular, the long-term silencing effect underscores the potential of this method.

Materials and methods

Vectors and siRNA design

A pcDNA3.1 vector (Invitrogen) encoding the *Photinus pyralis* (firefly) luciferase driven by a CMV promoter (pcDNA3.1-Luc) was constructed by subcloning the firefly luciferase reporter gene from the pGL3control vector (Promega) into the pcDNA3.1(+) expression cassette (Invitrogen). The pHippy-siLuciferase plasmid (pHippy-siLuc) was previously described[11]. The oligo sequences used to express siRNA against luciferase are: 5'AAaaggctcctcagaaacagctc3' (sense) and 5'AAaagagctgtttctgaggagcc3' (anti-sense). To knock-down the endogenous gene Toll-like receptor (TLR)-4, the

following oligo sequences were used: 5'AAaaggtgtgaaattgaaacaat 3' (sense) and 5'AAaaattgtttcaatttcacacc 3' (antisense). As a negative control pHippy-empty was constructed by blunting of the cloning sites. Subsequently, the vector was recircularized. Also an irrelevant siRNA (pHippy-siScrambled) was constructed using the following oligo sequences: 5'-AAaatcgactagcatggtcagtaca-'3 (sense) and 5'-Aaaatgtactgaccatgctagtcga-3' (antisense). Finally, the identity of all constructs was verified by DNA sequence analysis. The graphic map of the pHippy siRNA expression vector (pHippy-siTLR4) is depicted in figure 7.1. All plasmid DNA was prepared using DH5α *E.coli* (Invitrogen) and QIAfilter Plasmid Maxi Kits (Qiagen). Plasmid DNA was dissolved in Endofree Tris-EDTA buffer (Qiagen) at a final concentration of 3.5 mg/ml.

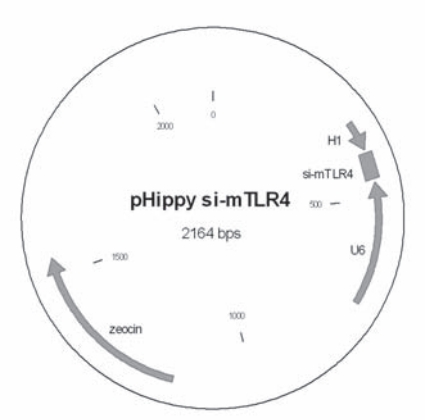

Figure 7.1. Map of the pHippy-vector.
Graphic representation of the pHippy-siRNA expression vector, pHippy-siTLR4.

Cell culture and transfection *in vitro*

Murine and human cell lines were used to test the siRNA vectors *in vitro* (NIH 3T3, obtained from the American Type Culture Collection and HER 911, kindly provided by Dr. F. Fallaux[17], respectively). Cells were grown in DMEM (Gibco) supplemented with 10% FBS (Gibco), penicillin (100U/ml) and streptomycin (100mg/ml, BioWhitakker and Cambrex Bio Science) at 37°C, 5% CO_2. Transfection of cell cultures with plasmid vectors were performed in 6 well plates with Lipofectamin 2000 (Invitrogen) on cell cultures that were approximately 90-95% confluent, according to manufacturer's protocol (4μg DNA per well). Unless indicated otherwise, cell lysates were prepared to determine luciferase activity, 24 hours after transfection.

Animal experiments and electroporation

Mice

All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO). For all experiments, 8 week old male FVB mice (Harlan), with a mean weight of 25.4±0.6 grams, were used. All mice received water and food (chow diet) ad libitum. Before electroporation and bioluminescence imaging, mice were anaesthetized by a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen-Cilag).

Intramuscular electroporation

To increase transfection efficiency, both legs of the mice were shaved and the calf muscles were injected with 30µl of hyaluronidase (in total 13.5U, Sigma-Aldrich), one hour before electroporation as reported by McMahon *et al.*[18] Subsequently, plasmid DNA (in total 50 µg per leg), dissolved in 30 µl TE buffer, 140 mM NaCl, was injected in each of the calf muscles followed by eight 10 millisecond electrical pulses at 200V/cm with a frequency of one Hertz. The pulses were generated with a Square Wave Electroporator ECM 830 and administered using Caliper Electrodes (BTX, Harvard Apparatus).

Luciferase imaging and reporter assay

In vitro experiments

Luciferase activity after transfection of cells was measured with a reporter assay and/or with bioluminescence imaging. Luciferase assays were performed according to the Luciferase Assay Protocol (Promega) using a luminometer (Luminat LB9507, Berthold Technologies). To quantify luciferase activity with *in vitro* imaging, luciferase was detected with a cooled charged-coupled device (CCCD) bioluminescence camera (The Night-OWL LB 981 UltraSens Frontlit, Berthold Technologies) as described previously[19]. Briefly, luciferin (95 µg/ml culture medium, Synchem OHG) was added to each 10 cm^2 well with transfected cells one minute before imaging. Next, cells were placed in the dark chamber and a gray-scale image of cell plate was recorded. Hereafter, photon emission was integrated over a period of 30 seconds and recorded as pseudo-color images (pixel binning 7 x 7). For co-localization of the bioluminescent photon emission on the cells, gray-scale and pseudo-color images were combined by using WinLight software (Berthold Technologies). Localization and measurement of luminescence emitted from the cells was performed by using the overlay of the real image and the luminescence scan.

Data were expressed as photon flux (counts/s). All light measurements were performed under the same conditions.

In vivo imaging

For all *in vivo* luciferase activity measurements, mice were anesthetized and injected i.p. with luciferin (90 mg/kg body weight, Synchem). Five minutes after injection, luciferase activity was measured with the same Night-OWL bioluminescence camera as mentioned above. Gray-scale images and the photon emission pseudo-color images were combined as described before[20,21].

RNA isolation and Real-Time quantitative RT-PCR procedure

To assess the effect of siRNA directed against endogenous TLR4, pHippy-siTLR4 or pHippy-empty as a control was electroporated in the calf muscles (right and left leg, respectively; n=5 per group). Mice were sacrificed and calf muscles were collected one week after administering the siRNAs. To stimulate TLR4 mRNA expression, 100 µg/kg Lipopolysaccharide (LPS from *Escherichia coli* 055:B5, Fluka, Sigma-Aldrich) was injected i.v. 6 hours before sacrifice, in both groups. Directly after harvesting, total calf muscles were snap-frozen in liquid nitrogen and grinded with pestle and mortar. RNA isolation was performed using RNeasy Fibrous Tissue Midi Kit (Qiagen) according to manufacturer's protocol. cDNA was synthesized by means of Ready-To-Go Beads (Amersham Biosciences).

For TLR4 and HPRT, intron-spanning primer-probe sets were designed (Table 7.1) using Primer Express™ 1.5 software (Applied Biosystems). HPRT was used as a housekeeping gene. The PCR reaction was performed using qPCR Mastermix (Eurogentec). Analysis of mRNA expression by real time-PCR was performed on an ABI Prism™ 7700 sequence detection system (Perkin Elmer Biosystems).

Table 7.1. Taqman Probes and Primers for Real-Time quantitative RT-PCR

TLR4	Sense:5' CATGGAACACATGGCTGCTAA 3' Antisense: 5' GTAATTCATACCCCTGGAAAGGAA 3' Probe: 5' CCTTCTGCCCGGTAAGGTCCATGCTATAG 3'
HPRT	Sense: 5' GGCTATAAGTTCTTTGCTGACCTG 3' Antisense: 5' AACTTTTATGTCCCCCGTTGA 3' Probe: 5' CTGTAGATTTTATCAGACTGAAGAGCTACTGTAATGACCA 3'

To analyze the results, the average cycle threshold per time point was subtracted from the average cycle threshold of the housekeeping gene HPRT (ΔCt). The difference between ΔCt-values of the TLR4 electroporated legs and the control group was used to determine ΔΔCt. Data are presented as mean fold induction compared to the normalized pHippy-empty treated legs, calculated as $2^{-\Delta\Delta Ct}$.

Multi-Plex cytokine array analysis

An additional set of experiments was performed to analyze the potential inflammatory responses after electroporation with either the pHippy-vector or the siRNA by itself. A Multi-Plex cytokine array analysis, which utilizes Luminex-based technology[22] was used to analyze plasma of four groups of mice (n=3 per group). For the current experiment, a Mouse 23-Plex Panel was used according to manufacturer's protocol (Bio-Rad). The first group consisted of untreated mice (controls). Group 2 was electroporated without addition of any plasmids to assess the effect of electroporation by itself. Group 3 was electroporated with the pHippy-siScrambled vector and finally group 4 with pcDNA3.1-Empty. Plasma samples were collected before electroporation, 24 hours and 72 hours after electroporation.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was calculated in SPSS 11.5 for Windows. Groups were compared to their controls and significant differences were determined using the Student's T-Tests. A p-value of less than 0.05 was considered statistically significant.

Results

pHippy-siLuciferase induced silencing of luciferase expression *in vitro*

HER 911 cells were transfected with a mixture of pcDNA3.1-Luc and pHippy-siLuc or with a combination of pcDNA3.1-Luc and pHippy-empty in different ratios (3:1, 1:1, 1:3 and 1:9), with a constant amount of plasmid DNA (4 µg) by varying the quantity of luciferase plasmid and pHippy-vectors, to test the efficiency of the pHippy-siLuc vector in human cells. All ratios showed a considerable reduction of luciferase expression; however, complete silencing of luciferase expression in HER 911 cells, as quantified with the bioluminescence camera, was achieved at a ratio of 1:9 (Figure 7.2A and 7.2B).

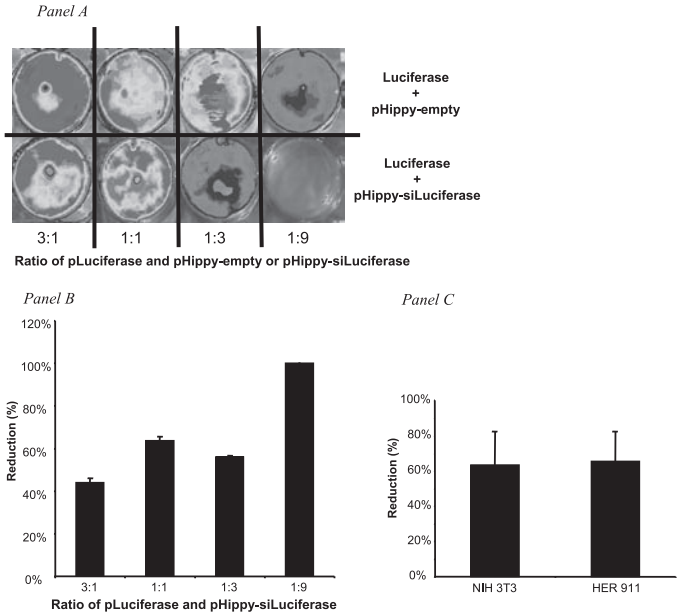

Figure 7.2. Effect of pHippy-siLuc on luciferase expression *in vitro*.

Panel A shows the luciferase expression in HER 911 cells, detected with a bioluminescence camera, one day after transfection with pcDNA3.1-Luc and pHippy-siLuc (bottom wells) or pHippy-empty as a control (upper wells) in different ratios. Panel B represents the relative reduction of luciferase expression in HER 911 cells. Sufficient luciferase expression was obtained with all ratios and the ratio 1:9 showed a complete silencing of luciferase expression. Panel C: Relative reduction of luciferase expression in NIH 3T3 and HER 911 cells after transfection with pcDNA3.1-Luc and pHippy-siLuc or pHippy-empty (ratio 1:1). In both human and murine cell types an obvious reduction of luciferase expression was monitored.

The efficiency of the human polymerase III dependent promoters, H1 and U6, in murine cells, was tested using cultured human HER 911 and murine 3T3 cells. These cells were transfected with a combination of pcDNA3.1-Luc and pHippy-siLuc or with a mixture of pcDNA3.1-Luc and pHippy-empty as a negative control (ratio 1:1). 24 hours after transfection, cell lysates were prepared and luciferase activity was determined. In both human and murine cell types, a marked reduction of luciferase activity could be observed after transfection with pcDNA3.1-Luc and pHippy-siLuc (65.4±17.0% in human and 63.2±19.1% in murine cells, respectively), demonstrating the efficacy of the human H1 and U6 promoters in murine cells (Figure 7.2C).

Long-term luciferase expression *in vivo*

In a first group of 3 mice, 50 µg pcDNA3.1-Luc was injected in the calf muscle and electroporated as described. Luciferase activity was non-invasively monitored and was shown detectable up to 140 days after electroporation. High levels of luciferase expression were detected in the first two weeks after intramuscular electroporation. Expression levels slowly decreased in time, but remained detectable up to 140 days. This indicates that expression up to half a year is feasible (Figure 7.3). In spite of some interanimal variations, the expression profiles in all animals or even all individual muscles show a common trend.

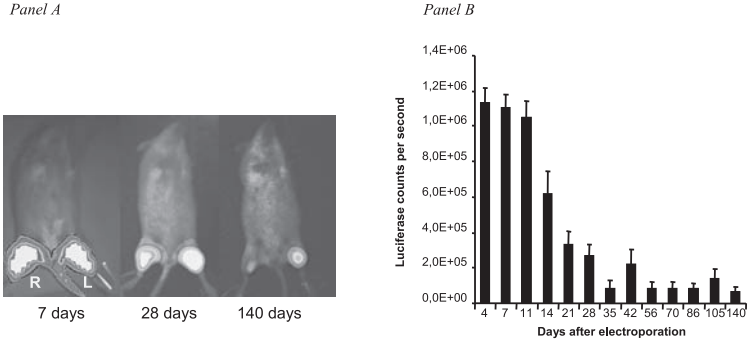

Figure 7.3. Expression of pcDNA3.1-Luc in time after electroporation of the calf muscle.

Panel A: Representative bioluminescence images of intramuscular luciferase expression at t=7, 28 and 140 days. Panel B: Quantitative reproduction of luciferase expression of all time points (n=3). Luciferase expression was detectable at least up to 140 days, but the intensity slowly declined.

pHippy-siLuciferase mediated silencing of luciferase expression *in vivo*

To investigate the effect of pHippy-siLuc *in vivo*, calf muscles of male FVB mice (n=6) were injected and electroporated with a mixture of pcDNA3.1-Luc and pHippy-siLuc (right leg) and a mixture of pcDNA3.1-Luc and pHippy-empty (left leg). The ratio of the injected plasmids in this experiment was 1:9, as stipulated to be the optimal ratio in the *in vitro* experiments. Luciferase activity was non-invasively monitored in time from day one to day 35 after electroporation (Figure 7.4A). In this period, the mean reduction of luciferase activity in the pHippy-siLuc treated group compared to the control was 54.8±6.9%. Even 35 days after electroporation, the effect of pHippy-siLuc was still present; an 84.3±15.7% reduction as compared to the contralateral control was observed. The absolute luciferase activity decreased slightly in time (Figure 7.4B).

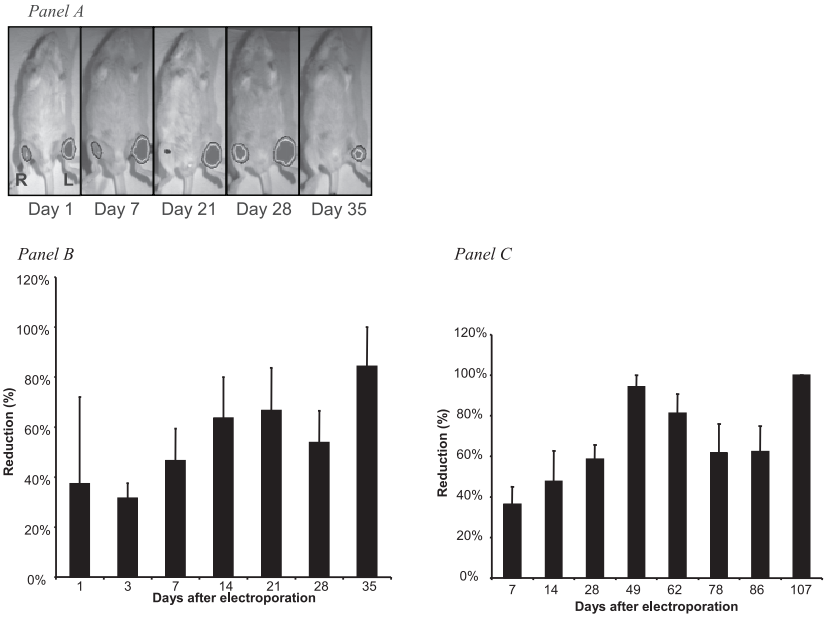

Figure 7.4. Inhibition of luciferase expression *in vivo* after treatment with pHippy-siLuc.

Panel A: Representative bioluminescence images of luciferase expression at several time points. Right legs are treated with pcDNA3.1-Luc and pHippy-siLuc, whereas left legs are electroporated with pcDNA3.1-Luc and pHippy-empty. Panel B represents the calculated silencing effect of pHippy-siLuc (n=6). Panel C: Prolonged silencing effect of pHippy-siLuc. Reduction of luciferase expression could be measured up to 107 days after electrotransfer of pHippy-siLuc (n=3).

After these experiments, three additional mice were electroporated with the same mixture of pcDNA3.1-Luc and pHippy-siLuc or pHippy-Empty (ratio 1:9), to assess the long-term effect of pHippy-siLuc. With the same threshold for detecting by the bioluminescence camera as used in the previous experiment, luciferase activity was measurable up to 107 days after electroporation. Mean reduction of luciferase activity caused by pHippy-siLuc in the whole experiment was 67.8±7.9% (Figure 7.4C). The differences in relative reduction between the two experiments might be due to the difference in expression efficiency of the individual luciferase and siLuc vectors in time, affecting the ratio of inhibition. After 107 days, in the control leg luciferase was detectable and in the treated leg no luciferase activity was seen, suggesting a 100% inhibition. However, the expression levels were so low, that the experiment was stopped at this point. These data indicate that pHippy-siLuc is able to silence luciferase expression *in vivo*. Moreover, long-term RNA silencing is possible with electroporation mediated plasmid-based siRNA delivery.

pHippy-siLuciferase mediated silencing *in vivo* is sequence specific without inducing an inflammatory response

Many reports have published the last couple of years that so called off-target or non-siRNA target specific effects may be responsible for inhibition of mRNA expression. To corroborate that above described results are a pHippy-siLuc specific effect and not a coincidental non siRNA-target side effect (after all, the used control vector is an empty vector not expressing any dsRNA) an extra time course experiment with a scrambled siRNA was performed (Figure 7.5). In this experiment the right leg was co-transfected with pcDNA3.1-Luc and pHippy-siLuc and the left limb with pcDNA3.1-Luc and pHippy-siScrambled (ratio 1:9). Luciferase activity was monitored for one month. Mean reduction of luciferase activity in the pHippy-siLuciferase treated group as compared to the siScrambled group was 80.4±6.1% (n=3) in this month. This experiment proves that the reduction of Luciferase expression after electroporation with pHippy-siLuc is really a siLuciferase sequence specific effect and not non-target sequence specific effect.

To assess potential inflammatory reaction or toxic effect of the pHippy-vectors, an additional experiment was performed. Protein levels of inflammatory cytokines and interferons were analyzed in plasma with a Multi-Plex cytokine array analysis, after electroporation with pHippy-vectors and relevant controls. No significant differences in plasma levels of the several inflammatory markers studied between all groups at all time points were

found, indicating that neither electroporation nor the pHippy-vectors do have an inflammatory effect in our model (data not shown).

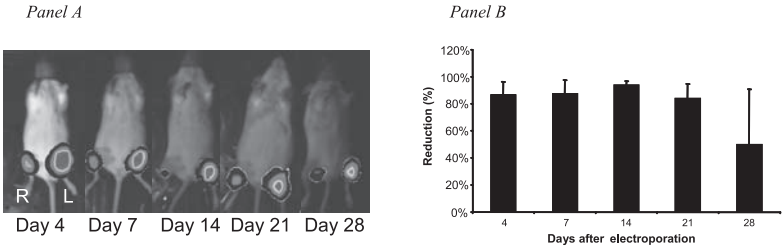

Figure 7.5. Inhibition of luciferase expression *in vivo* after electroporation with pcDNA3.1-Luc and pHippy-siLuc (right legs) and pcDNA3.1-Luc and pHippy-siScrambled (left).

Panel A: Representative bioluminescence images of luciferase expression during 1 month. Panel B represents the calculated silencing effect of pHippy-siLuc (n=3).

Silencing of endogenous TLR4 mRNA expression *in vivo*

The endogenous gene Toll-like receptor (TLR)-4, an important signal transducer that mediates innate immune and inflammatory responses to pathogens, was selected to verify if the pHippy-vector is also suitable to silence endogenous genes. TLR4 is, among other tissues, expressed by skeletal muscular tissue[23]. Seven days after intramuscular electroporation with pHippy-siTLR4 and pHippy-empty as a control, the expression of TLR4 mRNA in the total calf muscle was monitored and was reduced by 27.2±5.4% in the pHippy-siTLR4 treated legs as compared to the controls (p=0.01, Figure 7.6). Although this silencing effect at first glance seems somewhat disappointing, it should be realized that when targeting an endogenous gene by plasmid driven siRNA

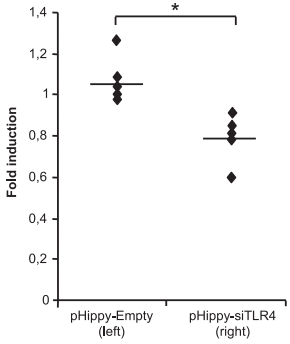

Figure 7.6. Effect of pHippy-siTLR4 on endogenous mRNA TLR4 expression in skeletal muscles.

Relative mRNA expression of TLR4 is determined in total murine calf muscles, seven days after electroporation with pHippy-siTLR4 and pHippy-empty as a control (right and left leg respectively, n=5). A reduction of 27.2±5.4% could be observed in the pHippy-siTLR4 treated legs as compared to the normalized controls (* represents P< .05).

constructs, not all the cells within the tissue are transfected by the siRNA expression vector. A 50% transfection efficiency for the total muscle would be really high. However, total RNA is isolated from the total tissue, including the non-transfected cells. Therefore, this silencing effect on TLR4 might be less strong as compared to reduction of the transfected exogenous luciferase expression as monitored by bioluminescence imaging.

Discussion

Currently, the possibilities of the application of RNA interference are increasing very rapidly. Both in cell cultures as well as in mammals, the function of specific genes and, arising from this, the biologic effect and therapeutic potentials of silencing genes are being explored with the help of RNAi. Because up till now, the effect of RNAi is not long-lasting, it is very complicated to study the long-term effects of silencing genes. An alternative is the use of knock-out cells or mammals. However, these knock-out cells and mammals are difficult to produce and the development of knock-out organisms is time consuming. In this study we demonstrate that long-term silencing of exogenous and endogenous genes *in vivo*, by means of electroporation mediated delivery of siRNA expressing plasmids, is feasible.

Electroporation mediated transfer of short interfering RNA in murine muscle was previously described by two other groups[13,14]. However, using synthetic oligonucleotides siRNAs, they found the maximum duration of inhibition of gene expression to be 7 and 11 days, respectively. Matsuda and Cepko reported plasmid-based delivery of siRNAs in rodent retina by electroporation. Although they did prove that organ specific delivery of siRNA encoding vectors via electroporation is feasible, no long-term effects were examined[24].

In the present study, we show that electroporation mediated plasmid delivery in the murine calf muscle, results in a luciferase expression of at least 20 weeks. Depending of the target tissue, other groups reported similar results: at least nine months expression in muscular tissue[25,26] seven days in vessels[27] and up to 50 days in the retina[24]. In addition, electroporation has several other advantages above other delivery methods. For example, local, organ specific and repeated delivery of plasmids is possible, the vectors are easy and relatively cheap to produce and most important: electroporation is safe because no immune response directed against the vehicle is provoked[15,28-30].

The *in vivo* imaging techniques using the bioluminescence camera enables real-time, non-invasive, analysis of luciferase expression *in vivo* in individual animals and selected organs[31,32]. Therefore, the exogenous gene luciferase is very appropriate to assess the silencing effect of pHippy in time. Besides, because the same mice were followed in a time-course, the total number of mice used could be reduced significantly. Especially this bioluminescence analysis method is versatile and extremely potent for these kinds of studies.

Where several vectors are available to drive expression of short siRNAs, most vectors contain a single polymerase III promoter, needing a hairpin loop to produce double-stranded RNA, so called short-hairpin RNAs (shRNA).

Because the pHippy-vector contains two opposing polymerase III promoters, no hairpin is needed. However, because of the presence of two human promoters in this vector, the question arises whether pHippy also works in murine cells. Consequently, the efficiency of pHippy in murine cells was tested. The reduction of luciferase activity in 3T3 fibroblasts was comparable to the reducing effect in HER 911 cells, indicating the functionality of the human U6 and H1 promoter in murine cells.

The combination of the electroporation mediated gene transfer to the murine muscle *in vivo* and the elegance and simplicity of the pHippy-vector as siRNA expression vector resulted in a potent and prolonged *in vivo* reduction of luciferase expression in mice. Additionally, by using an irrelevant scrambled siRNA as an extra control, unrelated siRNA side effects, like off-target and non target sequence specific effects after dsRNA expression could be excluded, since reduction of Luciferase expression after electrotransfer of pHippy-siLuc as compared to the scrambled siRNA sequence was obvious as well (Figure 7.5). Besides, since no significant differences in plasma levels of the several inflammatory markers studied between all groups were found, this indicates that neither electroporation nor the pHippy-vectors do have an inflammatory effect in our model.

In this study, we found an evident knock-down effect of the pHippy-siLuc vector over a time period of at least 3 months. This period is most probably sufficient to study the effect of knocking-out genes in several disease models.

Hydrodynamic vein tail injection (or volume overload) of siRNAs is an alternative and also effective method to silence (endogenous) genes, especially in the liver[33,34]. Wooddell and colleagues reported up to 20 weeks of silencing effect of two different genes after co-transfecting with the reporter gene and its siRNA, which is comparable with our results[35]. However, disadvantages of this method are that particularly the liver is targeted and effects on gene expression in other organ are difficult to achieve.

To explore the role of gene functions *in vivo*, long-term silencing of endogenous genes will be necessary. The expression of endogenous TLR4 mRNA in the whole calf muscle was reduced with approximately 27%, one week after electroporation with pHippy-siTLR4. With the described intramuscular electroporation method, transfection efficiency levels up to 50% could be reached[18]. In addition, TLR4 mRNA expression was determined in the whole calf muscle. Therefore, the effect of pHippy-siTLR4 is less powerful as compared to the silencing effect of luciferase after a co-transfection with plasmids encoding for luciferase and siLuc in the same muscle fibers. So, however this method is promising to study the functional effects of endogenous genes in skeletal muscle tissue, it is necessary to further optimize electrotransfer of siRNAs into skeletal muscle to increase the silencing effect.

In conclusion, the present study demonstrates, that electroporation mediated pHippy plasmid-based delivery of siRNA results in a long-term knock-down of luciferase and TLR4 expression for at least one week in mice. With this technique, delivery problems of siRNA *in vivo* could be overcome. Because of the prolonged silencing effect after a single intramuscular electroporation, this gene delivery method is very suitable to investigate the function of (endogenous) genes. Moreover, this application of RNA interference might be promising as a locally therapeutic molecular targeting instrument for gene related diseases like post-interventional vascular remodeling (restenosis) or tumors.

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