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► **To cite this version:**

Aurélie Paganin-Gioanni, Jean-Michel Escoffre, Laurent Mazzolini, Marie-Pierre Rols, Justin Teissie, et al.. In vivo imaging of siRNA electrotransfer and silencing in different organs. 11th Mediterranean Conference on Medical and Biological Engineering and Computing 2007, Jun 2007, Ljubljana, Slovenia. hal-04280340

HAL Id: hal-04280340

<https://hal.science/hal-04280340>

Submitted on 10 Nov 2023

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In vivo imaging of siRNA electrotransfer and silencing in different organs

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Abstract—RNA interference (RNAi)-mediated gene silencing approaches appear very promising for therapies based on the targeted inhibition of disease-relevant genes. The major hurdle to the therapeutic development of RNAi strategies remains however the efficient delivery of the RNAi-inducing molecules, the short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs), to the target tissue. In this study we have investigated the contribution of electrically-mediated delivery of siRNA and/or shRNA into muscles or tumors stably expressing a green fluorescent protein (EGFP) target reporter gene. The silencing of EGFP gene expression was quantified over time by fluorescence imaging in the living animal. Our study indicate that electric field can be used as an efficient method for RNAi delivery and associated gene silencing into cells of muscle and solid tumors *in vivo*.

Keywords— Electroporation, RNA interference, imaging, siRNA, ShRNA.

I. INTRODUCTION

Since its discovery¹, RNA interference has been described and extensively characterized in a number of organisms²⁻⁴. The identification of the short interfering RNAs (siRNAs) involved in this process and their use for sequence specific gene silencing has offered a new approach for molecular therapeutics by taking advantages of the progress in genomics^{5,6}.

This development requires, however, new safe and efficient *in vivo* siRNA delivery methods⁷. SiRNAs appear as a very promising new therapeutical agent but besides the problem of delivery, an unanswered problem is to know how long its effect lasts after a single dose delivery⁸. Most recently published *in vivo* results were obtained by « hydrodynamic transfection ». This stringent approach (injection within a few seconds in the tail vein of a volume one tenth the mass of the animal) appears to bring siRNA (and DNA) delivery mainly targeted to the liver⁹⁻¹³. Other methods were described where a systemic or a localized (portal vein injection) delivery was obtained by adding different chemical compounds to the siRNA solution¹⁴⁻¹⁷. SiRNA gene silencing could be obtained *in vivo* on reporter as well as endogenous genes. Delivery remains a critical issue for the development of siRNA as an effective therapeutic⁷. More recently, fluorescently labelled siRNA were

injected IV or IP in mice being complexed with cationic lipid liposomes¹⁶. Rather high amounts (100 µg) were injected in this experiment. Clearly the need for a delivery method suitable for targeting a broad range of tissues remains required. The demonstration in 1998 of drug and plasmid electrotransfer and gene expression in tumours^{18,19} led to the proposal that *in vivo* electropulsation was a promising tool for exogenous agents delivery²⁰. Furthermore it was observed that a very efficient *in vivo* electroloading of large molecules other than plasmids was obtained for proteins¹⁹, dextran²¹, and antisense oligonucleotides²². Electrically mediated gene transfer had been shown to be effective on many tissues: liver²³, skin²⁴, muscle^{21,25} and heart²⁶. Delivery is targeted to the volume where the field pulse is applied, *i.e.* under the control of the electrode localization. This technology allows delivery to almost all tissues, after a small surgery when needed. Impressive results were described in the case of muscles where treatment with non-invasive contact electrodes brought a long lasting expression of therapeutical genes²⁷.

Recent developments in optical imaging provide continuous monitoring of gene delivery and expression in living animals²⁸. Indeed, reporter gene activity can be accurately followed of the same animal as a function of time with no adverse effects either on the reporter gene product or on the animal itself. This increases the statistical relevance of a study, while decreasing the number of animals required. Exogenous gene expression of fluorescent reporter proteins such as GFP can be detected by the associated emission using a highly sensitive CCD camera.

II. MATERIALS AND METHODS

A. siRNAs

All siRNAs were purchased from Qiagen Xeragon (Germantown, MD, USA). The egfp22 siRNA (sense: 5' r(GCA AGC UGA CCC UGA AGU UCA U), antisense: 5' r(GAA CUU CAG GGU CAG CUU GCC G)) is directed against GFPmRNA. The P76 siRNA (sense: 5' r (GCG GAG UGG CCU GCA GGU A)dTT, antisense: 5' r(UAC CUG CAG GCC ACU CCG C)dTT) is directed against an unrelated human mRNA and shows no significant homology to mouse transcripts. It was used as a control for specificity of

the siRNA construct. ShRNA were provided by Cayla InvivoGen.

B. In vivo Electropulsation

Saline solution (*i.e.* PBS containing 40 units of the RNase inhibitor RNasin^R (Promega, Madison, WI) and either egfp22 or p76-siRNA were slowly injected (about 15s) with a Hamilton syringe through a 26G needle (Hamilton, Bonaduz, Switzerland) into the muscle or the tumor, under anesthesia (with or without plasmid pEGFPC1). In control conditions, the volume of siRNA was replaced by the siRNA suspension buffer to keep the injection conditions similar. 30s after injection, an electric pulsation was applied. Plate parallel electrodes (length 1 cm, width 0.6 mm, interelectrode distance 6 mm) (IGE, Carpi, Italy) were fitted around the muscle or the tumor. The skin was previously shaved with a cream (Veet). A good electric contact was obtained between the skin and the electrodes using a conducting paste (Eko-gel, Egna, Italy). The tissue was electropulsed with selected parameters using the PS 10 CNRS Electropulsator (Jouan, St Herblain, France). Square waved pulses were delivered. Voltage, pulse duration and frequency of pulses were all pre-set on the Electropulsator depending on the tissue (see legend of the figures). All parameters were monitored on line with an oscilloscope (Metrix, Annecy, France).

C. Non invasive stereomicroscopy fluorescence imaging in live animals:

GFP expression in the tissue cells was detected directly through the skin on the anaesthetized animal by digitized fluorescence stereomicroscopy. This procedure allowed the observation of GFP expression on the same animal during several days. High magnification images were obtained with an epifluorescence stereomicroscope using the 0.8 magnification (Leica MZFL III, Germany) and a cooled CCD camera (Roper Coolsnap fx). A 15-mm² part of the animal was observed, which was suitable to observe the treated tissue. Camera was driven by the MetaVue software 2.6 (Universal, USA) from a Dell computer. The exposure time was set at 1 s. with no binning. The fluorescence excitation was obtained with a Mercury Arc lamp (HBO, Osram, Germany) and a GFP filter (Leica) for emission. The GFP fluorescence was quantitatively evaluated at different days. A transmission picture and a picture with GFP filter were taken on each tissue.

From the transmission picture, the tissue was located and manually gated to give the region of interest (ROI). On the picture taken with the GFP filter, the mean fluorescence in the gated area was quantitatively estimated. Another

ROI was defined next to the non treated part of the tissue to determine the mean fluorescence background on the skin and normal tissue. Numerical values obtained by Metavue analysis software were transferred to Microsoft Excel and used for data analysis. To quantify the knockdown induced by the delivery of the siRNAs, the fluorescence intensity of the tissue on day 0 (*i.e.* before the treatment) was set as the 100% value for each animal and the "relative fluorescence on day X" values were in reference to this initial value. This allowed to get rid of the observed variations in the initial GFP fluorescence of tissue between the different animals.

III. RESULTS

A. Electrotransfer of siRNA in muscles

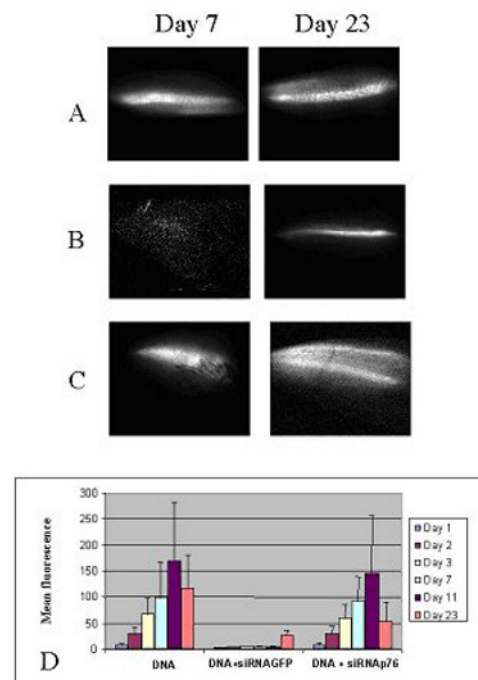


Fig. 2: RNA interference in 9 weeks old C57Bl/6 mouse leg muscle. Representative images of the GFP fluorescence from the mouse leg (Each image is 1 cm wide).

- A- GFP expression resulting from plasmid alone electrotransfer as observed on days 7 and 23 in the same leg.
- B- GFP expression silencing as observed on days 7 and 23 in the same leg when the plasmid was cotransferred with the specific egfp22 siRNA.
- C- GFP expression remained unaffected when an unrelated siRNA (p76) was cotransferred with the plasmid.
- D- Changes in the mean fluorescence emission with time. Sample standard deviations are shown. (n=4)

Plasmid electrotransfer and expression in muscles are known to be very efficient. Up to 70-80 % of the fibers can be transfected after injection of 25 µg of a GFP coding plasmid and by using adequate electrical conditions. Emission of the green protein was high 7 days after the electrical treatment. When the specific siRNA was electro-transferred (DNA +AntiGFP), significant decrease of the GFP expression was observed. Our fluorescence analyses also led us to conclude that inhibition of gene expression lasts more than 11 days. SiRNA delivery therefore occurs in almost all fibers.

B. Electrotransfer of shRNA in muscles

Inhibition of GFP expression was observed by the decreased of fluorescence emission in the muscle after co-transfection of a plasmid encoding GFP and the plasmid

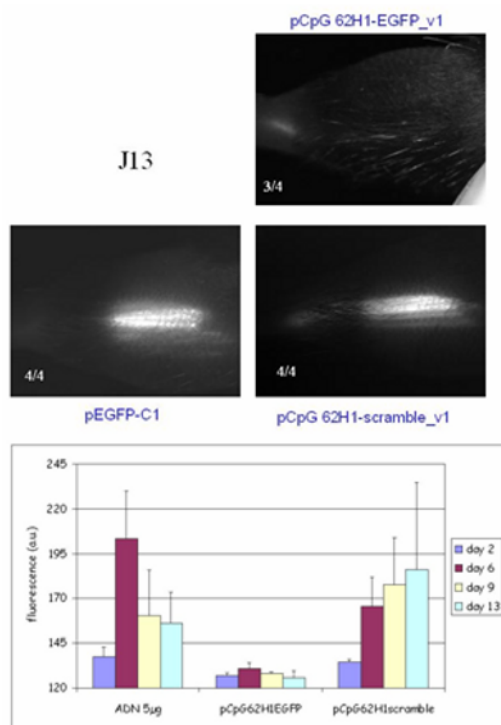


Fig. 3: RNA interference in 9 weeks old C57Bl/6 mouse leg muscle. Representative images of the GFP fluorescence from the mouse leg (Each image is 1 cm wide).

- A- GFP expression resulting from plasmid alone electrotransfer as observed on days 13. GFP expression silencing was observed when the plasmid pEGFP-C1 was co-transferred with the specific pCpG 62H1 EGFP_v1 shRNA (ratio 1/10, respectively). GFP expression remained unaffected when an unrelated pCpG 62H1 EGFP_v1 was cotransferred with the plasmid.
- B- Changes in the mean fluorescence emission with time. Sample standard deviations are shown. (n=4)

encoding shRNA. The use of shRNA leads to a stronger inhibition of the GFP expression (more than 50 days) than synthetic siRNA.

C. SiRNA transfer in tumors

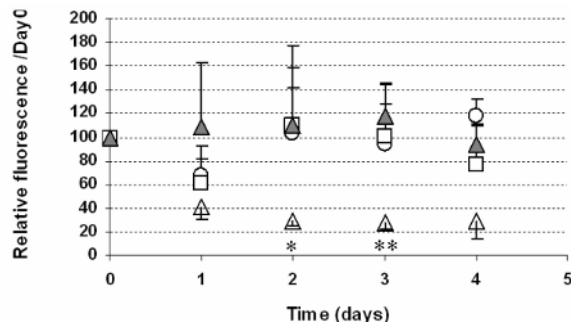


Fig. 4: Time dependence of mean fluorescence intensity of the B16F10 GFP tumors

Time dependence of mean fluorescence intensity of B16F10 GFP tumors in control untreated tumors and in tumors treated with; electric field : PBS + EP (○), unrelated siRNA : p76 + EP (□), 12 µg of egfp22 siRNA alone : AntiGFP - EP (▲) and egfp22 siRNA electro-transferred : AntiGFP + EP (△). On each animal, the mean fluorescence of the tumor was evaluated on a relative scale using the observation just before the treatment as a reference. (N= number of mice per group; for days 1 and 2: 7<N<9, days 3 and 4: 4<N<5).

Differences in fluorescence levels between AntiGFP - EP and AntiGFP + EP conditions were statistically compared by used an unpaired t-test two-sided using the KyPlot software.

* P<0.05 and ** P<0.01 were plotted when observed.

In vivo imaging was used to follow fluorescent tumors and fluorescence intensity as a function of time. GFP fluorescence emission was quantified by digital imaging. Tumor growth was not affected by the treatment (neither siRNA intra-tumoral injection nor electric pulses i.e. EP). When the specific siRNA was electro-transferred (AntiGFP + EP), significant decrease of the GFP expression was observed within 2 days following the treatment. As shown in the pictures of the figures 1 and 2, the fluorescence associated to the tumor disappeared in the treated group (AntiGFP + EP) whereas the fluorescence remained the same in the different control groups.

IV. CONCLUSIONS

In this study, we defined an experimental system allowing visualizing and quantifying the down regulation of a expressed EGFP reporter gene in muscles and subcutaneous B16-F10 mouse melanoma tumors using non invasive *in vivo* fluorescence imaging. This allowed us to follow *in situ* the kinetics of siRNA-mediated inhibition of gene expression, the topology of the effect and to test the impact

of electrical treatment on the establishment of gene knock-down.

ACKNOWLEDGMENT

This work was supported by grants from the CNRS ("Imagerie du petit animal" program), the EU cliniporator project, the Region Midi-Pyrénées, the AFM and the ARC (Association pour la Recherche sur le Cancer).

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