Chapter 2

Targeted Gene Silencing into Solid Tumors with Electrically Mediated siRNA Delivery

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Abstract

Short interfering RNAs (siRNAs) represent new potential therapeutic tools, owing to their capacity to induce strong, sequence-specific gene silencing in cells. However, their clinical development requires new, safe, and efficient in vivo siRNA delivery methods. In this study, we report an efficient in vivo approach for targeting gene knockdown in solid tumors by the use of external electric field pulses. We show that gene silencing is efficiently obtained in vivo with chemically synthesized siRNA after targeted electrical delivery in the tumor-bearing mouse. The associated gene silencing was followed on the same animal by fluorescence imaging and confirmed by qPCR. Gene silencing obtained in tumors lasted from 2 to 4 days after a single treatment. Therefore, this method should allow gene function analysis or organ treatment by a localized delivery of siRNAs.

Key words: RNA interference, siRNA, tumor, GFP, fluorescence, in vivo imaging, electroporation, mice, therapy, electropulsation.

1. Introduction

In the last few years, RNAi has become a powerful experimental tool for knocking down the expression of genes of interest *(1)*. Treatment of cells with chemically synthesized small interfering RNAs (siRNAs) is now used as a routine technique for in vitro functional analysis of cellular processes. Furthermore, RNAi-based approaches for clinical applications are currently under intensive development. Among the different limitations encountered in the use of siRNAs for therapy, efficient intracellular

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uptake and safe in vivo uptake remain critical issues. In addition to different administration modes of the siRNA molecules and chemical modification of the siRNA itself, a variety of chemical and physical methods have been developed to improve the final in vivo delivery of these molecules *(2, 3)*. Among the physical methods, electropulsation (electroporation) has proved to be successful for delivery of siRNAs in a large number of organs and tissues in rodents *(4)*. Electropulsation has indeed been known for more than 25 years to strongly increase in vitro intracellular uptake of molecules and drugs.

In vivo, electric pulses have been extensively used for drug and plasmid delivery in a large number of organs and tissues *(5–7)*. A key feature is that the delivery of molecules is restricted to the volume where the electric field is generated. A physical targeting of the effect is therefore possible. In order to assess the contribution of electrical treatment to siRNA-mediated endogenous gene silencing in solid tumors, we used as a reference experimental model B16-F10 mouse melanoma cell lines stably expressing an enhanced green fluorescent protein (EGFP). The extent of EGFP gene suppression, a model of constitutive gene silencing, in subcutaneous B16-EGFP tumors was subsequently monitored over time in the living animal by whole-body fluorescence imaging.

2. Materials

2.1. Cell Culture

1. B16-F10 mouse melanoma cells and their EGFP-expressing derivatives were obtained after retroviral vector and in vitro transduction (see *(8)* for further details).
2. Cells were cultured in Eagle Minimum Essential Medium (EMEM) (Gibco LifeTechnologies, France) supplemented with 10% fetal calf serum (Gibco), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (0.58 mg/ml).
3. Cells were grown on Petri dishes in a 5% CO_2 humidified incubator at 37°C.
4. Dulbecco's PBS buffer (Eurobio, Les Ulies, France) was used to rinse and/or resuspend the cells.
5. Trypsin/EDTA (Eurobio) was used to detach the cells from the Petri dishes.

2.2. siRNAs and DNA Oligonucleotides

1. siRNA Suspension Buffer: 100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4.
2. 10 mM Tris-HCl, pH 8.0 (T8.0): autoclaved and stored at 4°C.
3. RNAse inhibitor RNAsin^{R} (Promega, Madison, WI).

4. Upon receipt, lyophilized, preannealed double-stranded siRNAs (Qiagen) are resuspended at a concentration of 100 μM in siRNA Suspension Buffer, heated to 90°C for 1 min, and incubated at 37°C for 60 min. Resolubilized siRNAs are stored at –80°C.
5. 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 GFP mRNA, and was designed according to *(9)* (*see* **Note 1**).
6. 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 P76 mRNA and shows no significant homology to mouse transcripts. It is used as a control for specificity of the siRNA construct.
7. Single-stranded DNA oligonucleotides (Sigma) used as primers for quantitative PCR are resuspended in T8.0 buffer at a concentration of 100 μM and stored at –20°C.
8. The primers used for EGFP amplification are EGFP214f (5′-GCA GTG CTT CAG CCG CTA C-3′) and EGFP309r (5′-AAG AAG ATG GTG CGC TCC TG-3′), which were previously described by *(10)*. Primers used for amplification of reference housekeeping genes used for GeNorm calculations are: For β-glucuronidase, primers β-glucF (5′-ACT TTA TTG GCT GGG TGT GG-3′) and β-glucR (5′-AAT GGG CAC TGT TGA TCC TC-3′); for YWHAZ tyrosine 3-monooxygenase, primers mYWHAZ-481F (5′-CGT GGA GGG TCG TCT CAA GT-3′) and mYWHAZ-560R (5′-CTC TCT GTA TTC TCG AGC CAT CTG-3′); for β actin-2, primers mActbeta2-5′ (5′-AGC CAT GTA CGT AGC CAT CCA-3′) and mActbeta2-3′ (5′-TCT CCG GAG TCC ATC ACA ATG-3′).

2.3. In Vivo Experiments

1. Female C57Bl/6 mice were purchased from Rene Janvier (St ISLE, France). The C57Bl/6 mice were 9–10 weeks old at the beginning of the experiments, weighing 20–25 g and were considered as young mice. They were maintained at constant room temperature with 12-h light cycle in a conventional animal colony. Before the experiments, the C57Bl/6 mice were subjected to an adaptation period of at least 10 days.
2. Hair removal cream (Veet, Massy, France).
3. Hamilton syringe with a 26G needle.
4. Isoflurane (Forene, Abbott, Rungis, France).
5. Gas anesthesia system composed of an air compressor (TEM, Lormont, France) and an isoflurane vaporizer (Xenogene, Alameda, CA).

6. Electropulsator PS 10 CNRS (Jouan, St Herblain, France). All pulse parameters were monitored on-line with an oscilloscope (Metrix, Annecy, France). An electronic switch cutting the pulse as soon as its intensity reached 5 amp ensured safety against current surge.
7. Plate parallel electrodes (length 1 cm, width 0.6 cm) (IGEA, Carpi, Italy).
8. Conducting paste (Eko-gel, Egna, Italy).

2.4. In Vivo Visualization of Gene Expression and Gene Silencing

1. A fluorescence stereomicroscope (Leica MZFL III, Germany). The fluorescence excitation was obtained with a Mercury Arc lamp (HBO, Osram, Germany) GFP2 filter (Leica).
2. Cooled CCD Camera Coolsnap fx (Roper Scientific, Evry, France).
3. MetaVue software (Universal, USA) drives the camera from a computer and allowed quantitative analysis of the GFP fluorescence level.

2.5. mRNA Extraction and Analysis

1. RNAlater^{TM} RNA stabilization reagent (Qiagen).
2. RNeasy^{R} RNA isolation kit (Qiagen).
3. FastPrep^{R} oscillating grinding system and lysing matrix D beads (MP Biomedicals, Solon, OH).
4. RNAse-free water: Add diethylpyrocarbonate (DEPC, Sigma) to deionized water to a final concentration of 0.05% (*see* **Note 2**). Incubate overnight at room temperature and then autoclave for 30 min to eliminate residual DEPC. Store at room temperature.
5. TAE electrophoresis buffer (1X): 40 mM Tris-acetate, pH 8.0, 1 mM Na_2EDTA. Prepare as a 50X stock solution, autoclave, and store at room temperature.
6. Ethidium bromide (10 mg/ml solution, Sigma) (*see* **Note 3**).
7. Thermoscript^{TM} RT-PCR system from Invitrogen.
8. β-Mercaptoethanol (molecular biology grade, Sigma).

3. Methods

In this study, electrotransfer of synthetic siRNA against GFP mRNA was used to show the efficiency of in vivo electroadministration after intratumoral injection in solid tumors. We compared treatment groups using egfp22 siRNA, electric field alone, and nonrelevant p76 siRNA. It is important to use the same amount of siRNA among groups as well as the same volumes for injection to obtain reliable results. The first step was to graft the tumor expressing the GFP, and then to determine

whether the injection of egfp22 siRNA affects the GFP fluorescence expression. The expression of the fluorescent reporter gene was determined by in vivo fluorescence stereomicroscopy. Since RNAi-mediated gene knockdown acts through RISC-mediated mRNA degradation, validation of gene knockdown was also done by comparing the steady-state levels of target mRNA molecules in control and siRNA-treated samples by quantitative real-time RT-PCR (qPCR).

3.1. Tumor Cells Injection

1. Two days before the injection, an area of at least 1 cm in diameter is shaved on the back of the mouse with the cream (*see* **Note 4**).
2. B16-F10 cells are passaged when confluent with trypsin/EDTA to provide new culture on a 175 cm^2 flask. After 2 days, they are harvested by digestion with trypsin/EDTA and resuspended in PBS buffer at a final dilution of 10^7 cells/ml.
3. A volume of 100 μl of the cell suspension is injected subcutaneously under the shaved area (*see* **Note 5**).
4. The tumor growth is followed daily by measuring the diameter of the tumor by fluorescence imaging (*see* **Note 6**).

3.2. In Vivo Electropulsation

1. Eight to ten days after the subcutaneous injection, the tumor reaches a mean diameter of 5–7 mm (*see* **Note 7**).
2. Anesthetize mice by isoflurane inhalation (*see* **Note 8**).
3. Immediately prior to injection, dilute siRNAs to 17 μM in 50 μl of autoclaved PBS supplemented with 40 units of the RNAse inhibitor RNAsin^R. For all manipulations of the siRNAs, use DNase- and RNase-free buffers and RNase-free plasticware (microtubes and pipette tips). Always wear gloves.
4. Using a Hamilton syringe with a 26-gauge needle, slowly (over about 15 s) inject the siRNA solution into the tumor. In the control "no siRNA" condition, replace the volume of added siRNA solution with the same volume of siRNA Suspension Buffer to keep the injection conditions similar.
5. Apply conducting paste to insure good contact between the skin and the electrodes (*see* **Note 9**).
6. Approximately 30 s after the injection, fit the parallel-plate electrodes around the tumor (*see* **Note 10**) and deliver a train of four pulses plus four additional pulses in the reverse polarity (electrical conditions: 480 V, 20 ms pulse duration, and 1 Hz pulse frequency) (**Fig. 2.1**). Carefully control the delivery of the pulses on the oscilloscope.

3.3. Whole-Body Imaging

Upon electrically mediated transfer, GFP gene expression in the tumor is detected directly on the anesthetized animal by digitized

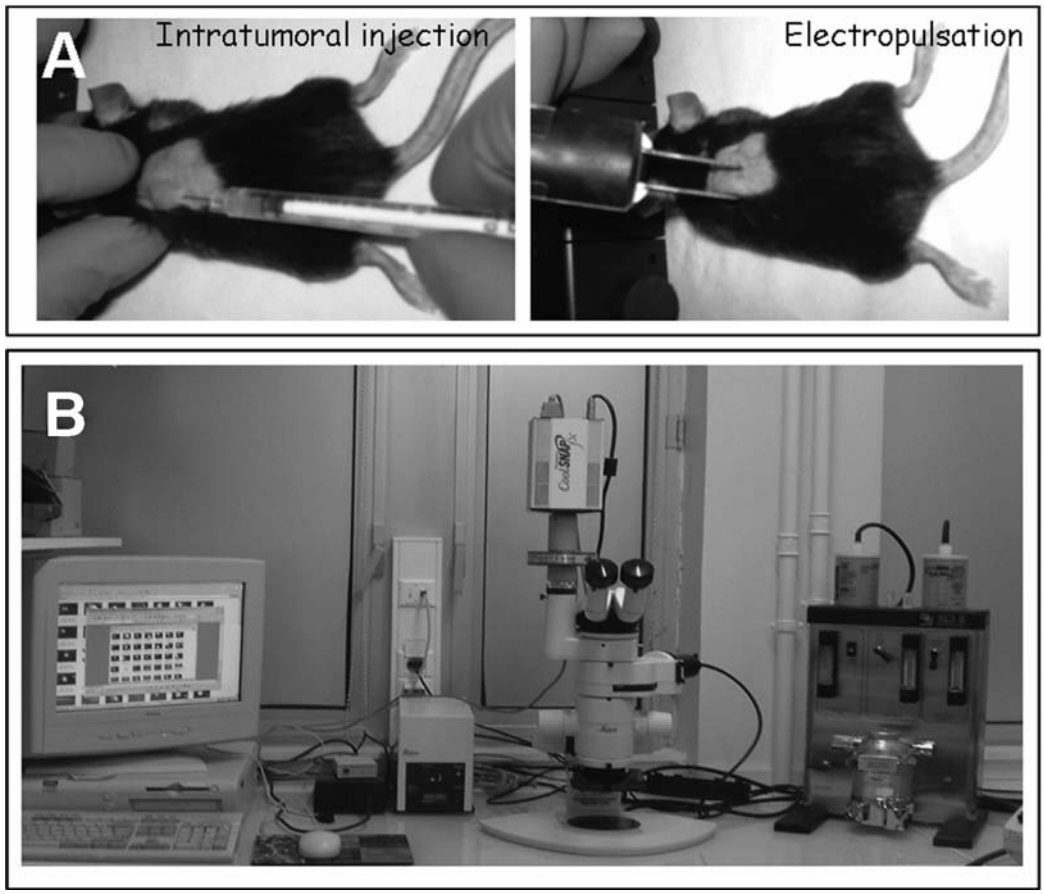

Fig. 2.1. Experimental set-up. **A.** Intratumoral injection of an anesthetized mouse. A volume of 50 μl was injected slowly into the tumor. Electrodes positioned on the tumor allow application of a train of four plus four inverted square wave. **B.** Digitized stereomicroscopy imaging set-up.

stereomicroscopy. The GFP fluorescence from the tumor is quantitatively evaluated at different days (*see* **Fig. 2.2**).

3.3.1. Fluorescence Data Acquisition

1. Anesthetize the mouse.
2. Place the mouse under the stereo-fluorescence microscope, with the tumor on the top of the animal. The whole tumor is observed as a 12-bit, 1.3-M pixel image using a cooled CCD Camera. MetaVue software drives the camera. Take a light picture (*see* **Note 11**).
3. The fluorescence excitation is obtained with a Mercury Arc lamp. The exposure time is set at 1 s with no binning. Acquire by selecting the GFP2 filter (*see* **Note 12**).

3.3.2. Fluorescence Data Analysis

1. The mean fluorescence in the gated area (whole tumor) is quantitatively estimated (measure/region measurement). In our experiments, the background fluorescence is sufficiently

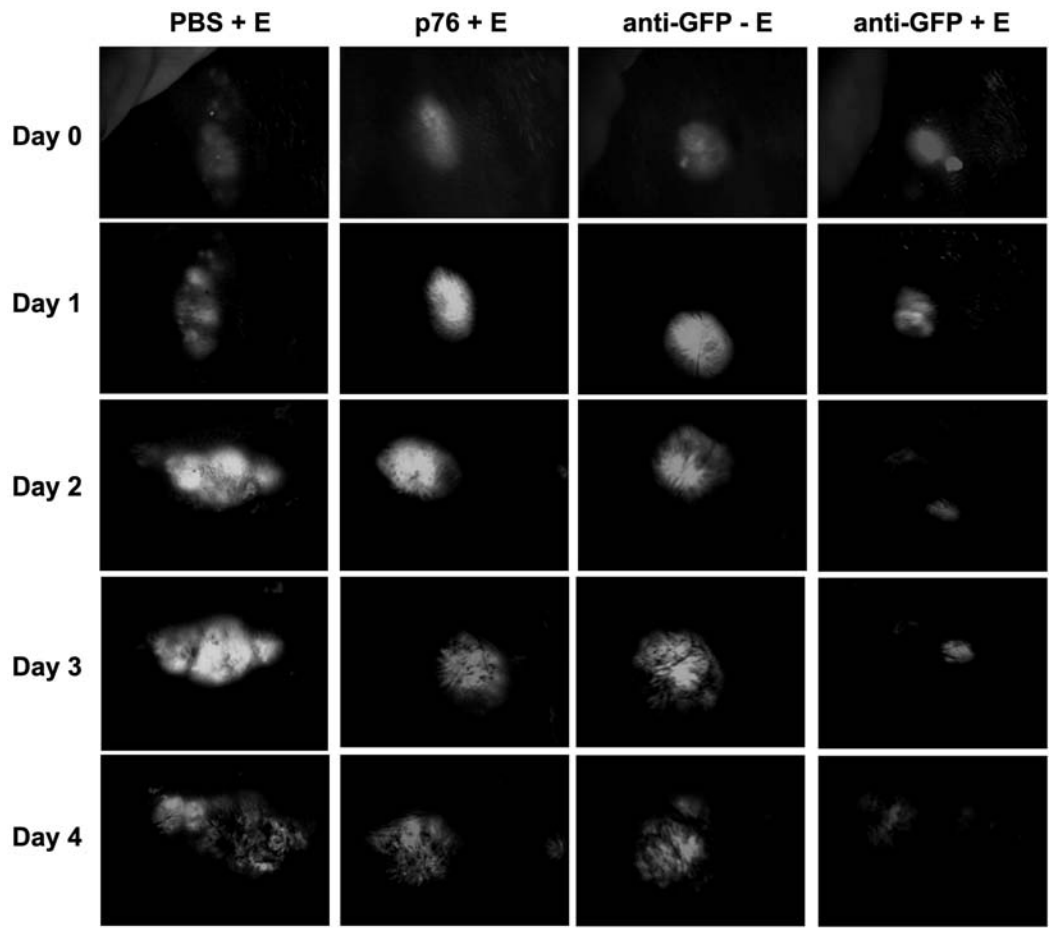

Fig. 2.2. **Representative images of EGFP fluorescence in B16-F10 tumors observed by noninvasive imaging in live animals before (day 0) and after (day 1 to day 4) the different treatments**. Tumors having a mean diameter of 5–7 mm were treated by intratumoral injection of 50-μl saline solution followed by application of electric pulses (PBS + EP); by intratumoral injection of 50 μl of saline solution containing 17 μM of p76 siRNA followed by application of electric pulses (p76 + EP); by intratumoral injection of 50 μl of saline solution containing 17 μM of egfp22 siRNA followed (antiGFP + EP) or not followed (antiGFP – EP) by application of electric pulses. B16-F10 GFP-derived tumors are clearly detected under the animal's skin upon fluorescence excitation and tumor margin can be easily defined. This enables measurement of the tumor area and fluorescence intensity over a period of 4 days after treatment.

low so that it does not interfere with quantitation when GFP emission is present.

2. To quantify the relative time-dependence of knockdown induced by siRNA (**Fig. 2.3**), use the respective intensity of each tumor at day 0 as an animal-specific internal control. The relative fluorescence on day *x* is represented by the ratio of the fluorescence on day *x* to this "internal control."

3.4. Statistical Analysis

Six different animals are treated for each condition. Fluorescence level differences between conditions are statistically compared by an unpaired *t*-test using Prism software (version 4.02, Graphpad).

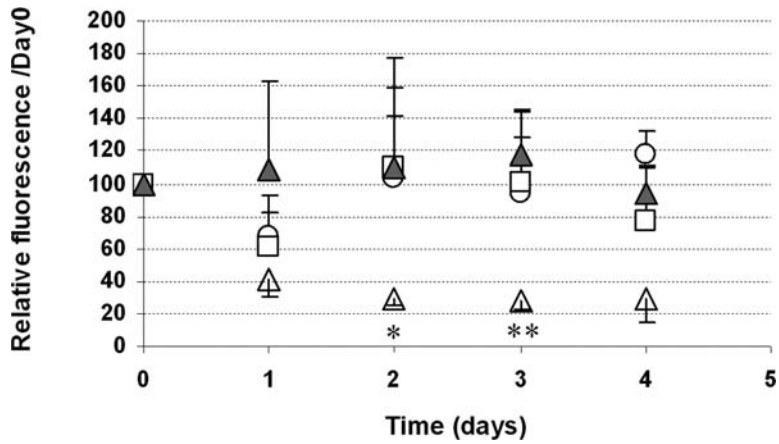

Fig. 2.3. **Time-lapse follow-up of fluorescence levels in B16-F10 GFP tumors after siRNA electrotransfer**. Digital imaging is used to quantify, at different time points, the fluorescence of B16-F10 GFP tumors. On each animal, the mean fluorescence of the tumor is quantitated on a relative scale, using as a reference the fluorescence intensity measured just before treatment (day 0). EGFP fluorescence in tumors following injection of PBS alone with electric field (◯); unrelated p76 siRNA electrotransferred (□); egfp22 siRNA without electroporation (▲); and egfp22 siRNA, electrotransferred (△). Vertical bars represent standard deviation. Differences in fluorescence levels between AntiGFP – EP and AntiGFP + EP conditions were statistically compared using an unpaired two-sided *t*-test using KyPlot software. $^{*}p < 0.05$ and $^{**}p < 0.01$ were plotted when observed.

3.5. Extraction of Total RNA from Tumors for RNA Knockdown Validation

3.5.1. Tumor Preparation and RNA Stabilization of Isolated Tumors

1. Process the tumor immediately after recovery. Work as quickly as possible until tumor tissue reaches the RNA stabilization step (*see* **Note 13**).
2. Briefly dip the recovered tumor in ice-cold PBS and dry by rolling the tumor a few seconds on a sterile paper towel.
3. Quickly weigh the tumor. If required, cut the tumor tissue into pieces of up to 50 mg each using a sterile scalpel blade.
4. Place each tumor sample in a 1.5-ml microtube prefilled with at least 10 volumes (i.e., 10 μl/mg of tissue) of RNAlater™ RNA stabilization reagent (making sure that the sample is fully submerged in the solution). Incubate samples overnight at 4°C prior to RNA extraction. For long-term storage, place the tubes at –20°C after an initial overnight incubation at 4°C.

3.5.2. Total RNA Extraction from Stabilized Tissues

1. In order to avoid possible RNA degradation, the duration time of manipulation of the stabilized tumor should be kept to a minimum. Tumors should therefore be processed one

at a time until the homogenization step in Qiagen RNeasy^R RLT lysis buffer has been completed.

2. Prior to extraction, prefill commercial lysing matrix D tubes with 800 µl of RNeasy^R RLT lysis buffer and 8 µl β-mercaptoethanol. Vortex the tubes for a few seconds.
3. Recover the stabilized tumor (stored at 4°C or –20°C) from the RNAlater solution using forceps, and briefly dry it by rolling on a sterile paper towel.
4. Add tumor to the lysing matrix D tube containing RLT lysis buffer and immediately proceed to sample homogenization for 25 s at a setting of 6.0 using the FastPrep^R grinding device.
5. Cool the lysing matrix D tube for 30 s on ice, and then store tube at room temperature.
6. When all tumors have been homogenized, centrifuge lysing matrix D tubes for 3 min at 12,000×*g* at room temperature.
7. Transfer supernatants in 1.5-ml microtubes by pipetting, and recentrifuge for another 1 min at 12,000×*g*.
8. Transfer supernatants to new 1.5-ml microtubes, avoiding any turbid material (if present).
9. Process to RNA purification according to Qiagen's RNeasy^R Mini Handbook protocol without any modification (*see* **Note 14**).

3.5.3. Determination of RNA Yield and Control of Integrity

1. For handling of purified RNA, use DNase- and RNase-free buffers, RNase-free plasticware, and wear gloves (*see* **Note 15**).
2. Determine RNA yield by measuring absorbance at 260 mm (OD_{260}) on a 100-fold dilution of the sample in RNase-free water using the formula $OD_{260} \times 4 =$ µg RNA per µl.
3. RNA integrity is routinely checked by agarose gel electrophoresis under native conditions (*see* **Note 16**). Prepare autoclaved 1X TAE for the gel and electrophoresis buffer. Prepare a 1% agarose gel in autoclaved 1X TAE electrophoresis buffer containing 0.5 µg/ml ethidium bromide.
4. Load 1–2 µg purified RNA in 1X RNase-free loading buffer. Run the gel at up to 10 V/cm until the bromophenol blue dye reaches the lower third of the gel.
5. Photograph under UV illumination (no destaining is required). Under these conditions, two high-intensity, sharp, major bands corresponding to the 28S and 18S ribosomal RNAs should be detected (**Fig. 2.4**). By visual inspection, no significant smearing (indicating partial degradation) should be visible below the rRNA bands, and no single band at the top of the gel (indicating DNA contamination) should be present. If quantitation of RNA bands is possible with the device used for gel image acquisition, the 28S and 18S

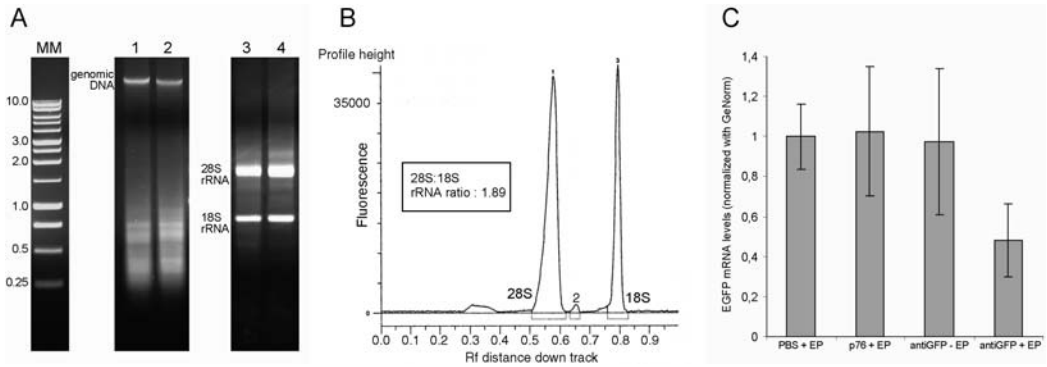

Fig. 2.4. Total RNA extraction from B16-F10 GFP tumors followed by quantitative RT-PCR analysis of EGFP mRNA levels. (**A**) Native gel agarose electrophoresis of total RNA extracted from tumors. Lanes 1 and 2 show examples of RNA initially obtained from tumors by using poorly denaturing RNA extraction buffers and moderately efficient grinding conditions. Degraded RNA appears as a smear in the gel. Note the presence of contaminating genomic DNA at the top of the gel. Lanes 3 and 4 show total RNA extracted from tumors using the described protocol. Two major bands corresponding to 28S and 18S ribosomal RNAs are present. No contaminating genomic DNA is detectable. MM: molecular mass marker. (**B**) Quantification of ethidium bromide fluorescence of sample shown in lane 3. 28S and 18S rRNAs give sharps peaks with a 28S:18S ratio close to 2.0 indicative of intact RNA. (**C**) EGFP reporter gene steady-state transcript levels were determined by qPCR analysis of RNA extracted from tumors 4 days after treatments with: electric field (PBS + EP), unrelated siRNA, electrotransferred (p76 + EP), egfp22 siRNA without electrotransfer (AntiGFP – EP), and egfp22 siRNA followed by electrical treatment (AntiGFP + EP). EGFP transcript levels were determined using a normalization factor calculated from three independent constitutive housekeeping genes using the GeNorm VBA applet. Three independent tumors were analyzed in each case. Histograms represent mean value ± standard deviation.

rRNAs should appear as sharp peaks with 28S:18S quantitative ratios of 1.8–2.0.

3.5.4. qPCR Analysis of Target mRNA Levels

1. Total RNA (5 μg) isolated from the different tumor samples is used to generate cDNAs through reverse transcription by the Thermoscript™ reverse transcriptase (Invitrogen) using random hexamers as primers and subsequent RNase H treatment.
2. qPCR is then performed on 5 μl of a 25-fold dilution of the cDNA synthesis reaction using an ABI Prism 7000 system (Applied Biosystems) and SYBR Green dye fluorescence measurements to quantify amplicon production.
3. Three independent endogenous mouse genes giving the most highly reproducible constitutive expression levels in tumors (*see* **Section 2.2**) are selected over a panel of tested "housekeeping" mouse genes using the *GeNorm* Visual Basic application for Microsoft Excel *(11)*. This application is freely accessible on the Web for noncommercial academic research.
4. The normalization factor calculated by GeNorm from the selected genes is then used to determine target gene mRNA levels.

4. Notes

1. Selection of the optimal siRNA sequences remains a critical issue for RNAi experiments in vivo (for a general discussion *see (12, 13)*). Literature mining may be a valuable source for the identification of "validated" active siRNAs directed against a gene of interest. In parallel, most companies commercializing siRNAs now propose various sets of "predesigned siRNAs" against any mouse or human gene, which are designed by robust, proprietary, algorithms. Similar freely accessible algorithms can also be found on the Internet (e.g., *(13–15)*). A validation and selection of the most active siRNAs in cultured cells should always precede their in vivo use.
2. DEPC is highly toxic and volatile. It must be used only in a laboratory chemical fume hood.
3. Ethidium bromide is a potent mutagen. Always wear gloves and minimize handling. Use specific procedures (such as charcoal filtration) for disposal of ethidium bromide-containing buffers. Use dedicated electrophoresis tanks.
4. The cream should be used 2 days before fluorescence imaging because some components of the cream fluoresce under blue-light excitation. This cream should be used carefully as it may cause some irritations in the mouse skin. Rinse the cream under a flow of water.
5. The subcutaneous injection should be performed under the shaved area to allow direct visualization of the GFP-expressing cells under the skin.
6. GFP expression in the tumor cells is detected directly through the skin on the anesthetized animal by digitized fluorescence stereomicroscopy. This procedure allows observation of GFP expression in the same animal over several days.
7. Hair on the back may re-grow; re-shave the skin above the tumor if necessary.
8. Isoflurane inhalation is safe; mice recover very quickly after the electrical treatment and can be observed daily for in vivo imaging.
9. Conducting paste is very important to insure optimal electrical contact with the skin. One should pay attention that the paste is not continuous between the two electrodes, as the field will pass through the paste and not through the tumor.
10. One person should perform the tumor injection. As the tumor may have different nodules, pay attention when injecting the whole tumor.

11. One person should be responsible for holding the mice to avoid erratic conditions of exposure to the fluorescence excitation beam.
12. Make sure that no saturation of pixels occurs in the area of interest upon image acquisition. Then use identical settings for subsequent acquisitions.
13. In our experiments, extraction of nondegraded RNA from tumors required use of tumor tissue pretreated with an RNA stabilizing agent together with highly denaturing RNA extraction buffers (containing guanidine isothiocyanate), as well as harsh homogenization conditions. We assume that this may be due to the presence of necrotic regions in the tumors, which may release high levels of nucleases in the tissue. Although the reagents and materials used in this protocol may be substituted with others, we recommend using extraction conditions that fulfill the criteria mentioned above.
14. Contamination of the purified RNA sample by genomic DNA sequences may be detrimental to sensitive applications such as qPCR. However, in our hands, additional treatments performed in order to eliminate putative DNA contamination did not modify the qPCR amplification patterns.
15. The electrophoresis tank should have been pretreated with an RNase-removal reagent (e.g., RNaseZap^{R} RNase decontamination solution from Ambion) to avoid any RNA degradation during electrophoresis due to contaminating RNases.
16. Although denaturing conditions provide the greatest resolution for RNA analysis, direct electrophoresis under native conditions was found sufficient to assess the integrity and overall quality of purified RNA. In addition, native RNA staining is much more sensitive than that of denatured DNA.

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