Chapter 3

Liposomal siRNA for Ovarian Cancer

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Abstract

Discovery of RNA interference (RNAi) has been one of the most important findings in the last ten years. In recent years, small interfering RNA (siRNA)-mediated gene silencing is beginning to show substantial promise as a new treatment modality in preclinical studies because of its robust gene selective silencing. However, until recently, delivery of siRNA in vivo was a major impediment to its use as a therapeutic modality. We have used a neutral liposome, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), for highly efficient in vivo siRNA delivery. Using siRNA tagged with Alexa-555, incorporated in DOPC liposomes, we have demonstrated efficient intra-tumoral delivery following either intraperitoneal or intravenous injection. Furthermore, EphA2-targeted siRNA in DOPC liposomes showed significant target modulation and anti-tumor efficacy.

Key words: RNA interference, siRNA delivery, neutral liposome, DOPC, ovarian carcinoma, EphA2.

1. Introduction

RNA interference (RNAi) is thought to have evolved as a form of innate immunity against viruses and has become a powerful tool for highly specific gene silencing and drug development. RNAi is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid (dsRNA) inhibits the expression of genes with complementary nucleotide sequences. Long dsRNA is cleaved by Dicer, which is an RNAse III family ribonuclease. This process yields small interfering RNAs (siRNAs) about 21 nucleotides long. These siRNAs are incorporated into a multi-protein RNA-induced silencing complex (RISC) that

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is guided to target mRNA, which is then cleaved *(1–4)*. In mammalian cells, the related microRNAs (miRNAs) are found, which are short, ~22 nucleotides long, RNA fragments. These miRNAs are generated after Dicer-mediated cleavage of longer (~70 nucleotide) precursors with imperfect hairpin RNA structures. The miRNA is incorporated into a miRNA–protein complex (miRNP), which leads to translational repression of target mRNA.

siRNA-mediated gene silencing offers options for targeting genes that cannot be targeted with other approaches such as small-molecule inhibitors or monoclonal antibodies. However, for clinical development, two major barriers must be overcome, nuclease digestion and in vivo delivery. Several approaches including plasmids and viral vectors have been used *(5)* with some successful in vitro applications, but limited potential for in vivo use *(6)*. Liposomes are self-assembled, closed structures composed of lipid layers formed spontaneously upon the addition of water to a dried lipid film. We have shown that nano-liposomes can serve as an effective vehicle for the delivery of siRNA. Liposomal formulations have been used to incorporate and deliver a wide variety of therapeutic and diagnostic agents. The circulating half-life of liposomes can be prolonged by the addition of neutral, hydrophilic polymers such as poly(ethylene glycol) (PEG) to the outer surface *(7)*. An extended circulation half-life allows for sustained availability in order to take advantage of the enhanced permeability of tumor vasculature, resulting in increased delivery to target sites *(8–11)*. Moreover, it offers opportunities to further enhance the specificity and efficiency of siRNA delivery to tumor tissue, by appropriately "flagging" the liposomal surface with molecular tags, to minimize the non-specific toxic effects.

Our goal was the development of a safe, biodegradable, and biocompatible carrier system for siRNA that will protect siRNA from nuclease digestion and increase delivery into tumors. Liposomes, in general, have been shown to be safe in a number of clinical trials using a wide variety of anticancer and antimicrobial drugs. The liposomes' charge, size, and lipid composition will impact their safety, distribution, and uptake by cells and tissues. For example, negatively charged liposomes will be avidly taken up by phagocytic cells and may not result in optimal loading efficiency due to the negative charge of the siRNA *(12)*. Cationic liposomes have low delivery efficiency because of their electrostatic interactions with other cells and biomolecules along the delivery path. Therefore, we have focused on the use of a neutral liposome, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), for in vivo delivery of siRNA.

We have recently demonstrated that siRNA incorporated into nano-liposomes, composed of DOPC, are able to penetrate deep into tumors in vivo while avoiding phagocytic uptake (we will use

the term DOPC to mean liposomes composed of DOPC). For these initial delivery studies, we used Alexa-555-tagged siRNA. Our initial proof-of-concept studies focused on the use of DOPC for targeting a frequently overexpressed tyrosine kinase, EphA2, in ovarian cancer *(13–15)*. EphA2 is a tyrosine kinase receptor belonging to the ephrin family that plays a key role in neuronal development. In adults, it is expressed to a low degree, primarily in epithelial cells *(14)*. This differential expression in tumor cells makes EphA2 an attractive therapeutic target. We demonstrated that EphA2 siRNA-DOPC given either i.v. or i.p. resulted in EphA2 silencing in orthotopic experimental ovarian tumors, leading to reduced tumor growth as a single agent or in combination with a taxane *(14, 16)*.

2. Materials

2.1. Cell Culture

1. Roswell Park Memorial Institute-1640 (RPMI) Medium supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamycin.
2. Trypsin solution (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM).
3. Hank's Balanced Salt Solution (Serum free) with calcium and magnesium.
4. BCA assay kit.

2.2. Transfection of Cancer Cells with siRNA

1. A non-silencing fluorescent siRNA sequence tagged with Alexa-555, 5′-AATTCTCCGA-ACGTGTCACGT-3′, control siRNA (same sequence without Alexa-555), and EphA2-targeted sequence 5′-AATGACATGCCGATCTACATG-3′ were synthesized and purified.
2. siRNA suspension buffer (100 mM potassium acetate, 30 mM HEPES-potassium hydroxide, 2 mM magnesium acetate, pH 7.4).
3. RNAiFect transfection reagent.
4. 1, 2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC).
5. Molecular weight limit filters.

2.3. Western Blotting

1. Modified radioimmunoprecipitation assay (RIPA) lysis buffer: 50 mM Tris, 150 mM NaCl, 1% Triton, 0.5% deoxycholate plus protease inhibitors (25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mM EDTA, 1 mM sodium orthovanadate).
2. Running buffer: Prepare 10X stock with 1.92 M glycine, 250 mM Tris, 1% SDS. Dilute 100 mL with 900 mL distilled water (1X) for use.

3. Transfer buffer: Prepare 10X stock of glycine and Tris as said above. Add 20% methanol plus 0.05% (w/v) SDS to 100 mL of stock and make up to 1 L with distilled water.
4. Tris-buffered saline with Tween-20 (TBST): Prepare 10X stock with 100 mM NaCl, 50 mM Tris-base. Adjust pH to 7.5 with concentrated HCl and make up to 1 L with distilled water. Add 0.1% Tween-20 to 100 mL stock and make up to 1 L (1X) before use.
5. Blocking buffer: 5% (w/v) nonfat dry milk in TBST.
6. Stacking gel: 0.5 M Tris-HCl buffer with pH 6.8.
7. Resolving gel: 1.5 M Tris-HCl buffer with pH 8.8.
8. Ready gel blotting sandwich containing 0.45 μm nitrocellulose membrane with filter paper (7 × 8.5 cm).
9. Primary anti-EphA2 antibody in 1% nonfat dry milk in TBST.
10. Anti-beta-actin antibody.
11. Secondary horseradish peroxidase (HRP)-conjugated anti-mouse IgG in 1% nonfat dry milk in TBST.
12. Enhanced chemiluminescent (ECL) detection reagents.

2.4. Immunohistochemistry

2.4.1. Determination of Uptake of Alexa-555 Fluorescent siRNA by Tumor Tissues

1. Normal horse and goat serum.
2. Hoechst 33342 trihydrochloride, trihydrate.
3. Rat anti-mouse CD31 primary antibody.
4. Rat anti-mouse f4/80 primary antibody.
5. Goat anti-rat Alexa-488 secondary antibody.
6. Fluorescent mounting medium: Glycerol-propyl galate in PBS.
7. Fluorescent Zeiss Axioplan 2 microscope.

2.4.2. Immunohistochemical Staining for EphA2 in Tumors

1. Mouse IgG Fragment (Fc) blocker.
2. Primary EphA2 antibody (Anti-EphA2 clone EA5, a kind gift from Dr. Michael Kinch).
3. Biotinylated horse anti-mouse antibody.
4. Streptavidin-HRP.
5. 3, 3-Diaminobenzidine substrate.
6. Gill No. 3 hematoxylin.

3. Methods

3.1. Cell Culture

1. Ovarian cancer HeyA8 cells were maintained in log phase of cell growth by culturing in RPMI-1640 medium supplemented with 15% FBS and 0.1% gentamicin sulfate at 37°C in 5% CO_2/95% air.

2. All in vitro experiments were carried out in triplicates using 70–80% confluent cells.

3.2. Preparation of Cells for In Vitro Transient siRNA Transfection

1. The purified siRNAs were dissolved in suspension buffer to a final concentration of 20 μM, heated to 90°C for 60 s, and incubated at 37°C for 60 min prior to use to disrupt any higher aggregates formed during synthesis.
2. To check the downregulation of targeted protein EphA2 in vitro using a target siRNA sequence, in vitro transient transfection was carried out using HeyA8 cells.
3. HeyA8 cells (70–75% confluent) were washed with PBS twice and detached using trypsin-EDTA solution. Trypsin activity was neutralized by RPMI-1640 serum containing media. Cells were counted using trypan blue and 3.5×10^5 cells were seeded on six-well tissue culture dishes. The cells should be plated 24 h prior to transfection and avoid stressing the cells without medium during washing steps (*see* **Note 1**). After 24 h, transfection was carried out using RNAiFect transfection reagent. SiRNA (5 μg) was mixed with 65 μL RPMI-1640 media with serum and 30 μL RNAiFect transfection reagent (*see* **Note 2**). SiRNA and transfection reagent mixture was incubated for 15–20 min to allow for the formation of RNA–lipid complex at room temperature and then mixture was added on to cells. Cells were collected after 48 h to check the downregulation of EphA2 using Western blotting.

3.3. Preparation of Liposomal siRNA for In Vivo Delivery

1. For in vivo delivery studies, siRNA was incorporated into DOPC.
2. siRNA and DOPC were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA/DOPC. The mixture along with Tween-20 was vortexed, frozen in an acetone/dry ice bath, and lyophilized.
3. To estimate the amount of siRNA not taken up by liposomes (unbound siRNA), free siRNA was separated from liposomes by centrifuging the liposomal-siRNA suspension using 30,000 nominal molecular weight limit filters at 5,000 × *g* for 40 min at room temperature. Fractions were collected and the material trapped in the filter was reconstituted with 0.9% NaCl. siRNA in the collected fraction and elute was measured by spectrophotometer.
4. Before in vivo administration, to achieve the desired dose in 200 μL per injection per mouse, lyophilized liposomal siRNA complex was suspended in 0.9% NaCl.

3.4. Orthotopic In Vivo Model of Advanced Ovarian Cancer

1. Female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD).

2. Animals were housed in specific pathogen-free conditions and taken care according to the guidelines of the American Association for Accreditation of Laboratory Animal Care and the USPHS "Policy on Human Care and Use of Laboratory Animals".
3. All studies were approved and supervised by the University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee.

3.5. Preparation of Cells for In Vivo Injections

Tumors were established by i.p. injection of cells prepared as below. For in vivo injection, 60–80% confluent HeyA8 cells were trypsinized and centrifuged at 1,000 rpm for 7 min at 4°C (*see* **Note 3**). Then cells were washed twice with PBS, and reconstituted in serum-free HBSS at a concentration of 1.25×10^5 cells/mL to inject 200 μL i.p. injections per animal. Tumors established in the animals by using this method reflect the i.p. growth pattern of advanced ovarian cancer *(17, 18)*. For treatments, siRNA–DOPC complex was mixed gently with PBS or 0.9% NaCl with pipetting up and down before injection (*see* **Note 4**). Do not freeze the liposomal-siRNA complex after suspending with PBS or normal saline (*see* **Note 5**).

3.6. Determination of Uptake and Distribution of Fluorescent siRNA in Ovarian Tumors

1. A non-silencing fluorescent siRNA sequence tagged with Alexa-555 (5′-AATTCTCCGAACGTGTCACGT-3′), which did not share any sequence homology with any known human mRNA sequences, was used to determine uptake and distribution in various tissues when given in vivo. Same sequence without an Alexa-555 tag was used as control siRNA for tissue background.
2. Determination of uptake of a single dose of fluorescent siRNA in tumor and different organs was initiated once i.p. tumors reached a size of 0.5–1.0 cm^3 as assessed by palpation (~17 days after tumor cells injection).
3. Non-silencing Alexa-555 siRNA–DOPC or control siRNA–DOPC (5 μg) in 100 μL of normal saline was given through i.v. bolus into the tail vein under normal pressure.
4. Tumor and other tissues were harvested at various time points after injection (1 h, 6 h, 48 h, 4 days, 7 days, or 10 days).
5. Tissue specimens were placed in OCT and frozen rapidly in liquid nitrogen for frozen slide preparation.

3.7. Determination of EphA2 Downregulation in Ovarian Tumors

1. To characterize the efficacy of targeted delivery of siRNA packaged into neutral nano-liposomes, HeyA8 ovarian cancer cells were used. A target siRNA sequence, 5′-ATGACATGCCGATCTACATG-3′, was used to downregulate EphA2 in vitro and in vivo.

2. To determine the optimal dose and frequency of dosing, a single injection of EphA2 siRNA–DOPC was given i.v. once tumor size reached 0.5–1.0 cm^3, as assessed by palpation (~17 days after tumor cells injection).
3. Targeted EphA2 siRNA–DOPC or control siRNA–DOPC (5 μg in 100 μL normal saline) was given through i.v. bolus into the tail vein under normal pressure.
4. Tumors were harvested at several time points following siRNA injection. Tissue specimens were either snap frozen for protein assessment, fixed in formalin for paraffin embedding, or frozen in OCT for immunofluorescence.
5. To assess tumor growth for long-term experiments, therapy was started one week after tumor cell injection. Mice were divided into five groups ($n = 10$ per group): (a) empty liposomes; (b) non-targeting control siRNA-DOPC; (c) EphA2 siRNA-DOPC; (d) control siRNA-DOPC + paclitaxel; and (e) EphA2 siRNA-DOPC + paclitaxel. Paclitaxel (100 μg/mouse) was injected i.p. once weekly and siRNAs (non-specific or EphA2-targeted, 150 μg/kg) injected i.v. twice weekly.
6. Mice were monitored for adverse effects. When mice in any group began to appear moribund (approximately after 3–4 weeks of therapy), all animals were sacrificed and tumors were harvested.
7. Mouse weight, tumor weight, and distribution of tumor were recorded. Tumor tissue specimens were collected for Western blotting and immunohistochemistry studies as described above. Results are shown in **Fig. 3.1**.

3.8. Western Blotting

1. HeyA8 cells were grown to 80% confluence, washed two times with PBS, and then lysed in modified RIPA buffer plus protease inhibitors on ice for 20 min.
2. Cell lysates were scraped off, kept on ice for 20 min, and centrifuged at 12,000 rpm for 20 min at 4°C.
3. The protein content of the supernatants was measured by the BCA assay kit.
4. The cell lysates were adjusted for equal amount of protein, mixed with 3X sample buffer, and boiled for 5 min.
5. Thirty micrograms of cell lysate protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and electrophoretically transferred on to a nitrocellulose membrane.
6. The membrane was blocked overnight in 5% (w/v) nonfat dry milk in TBS containing 0.1% Tween-20.
7. The membrane was probed with an EphA2 antibody at 1:1,000 dilution overnight at 4°C and washed with TBST for 1 h at room temperature by changing the TBST every 10 min.

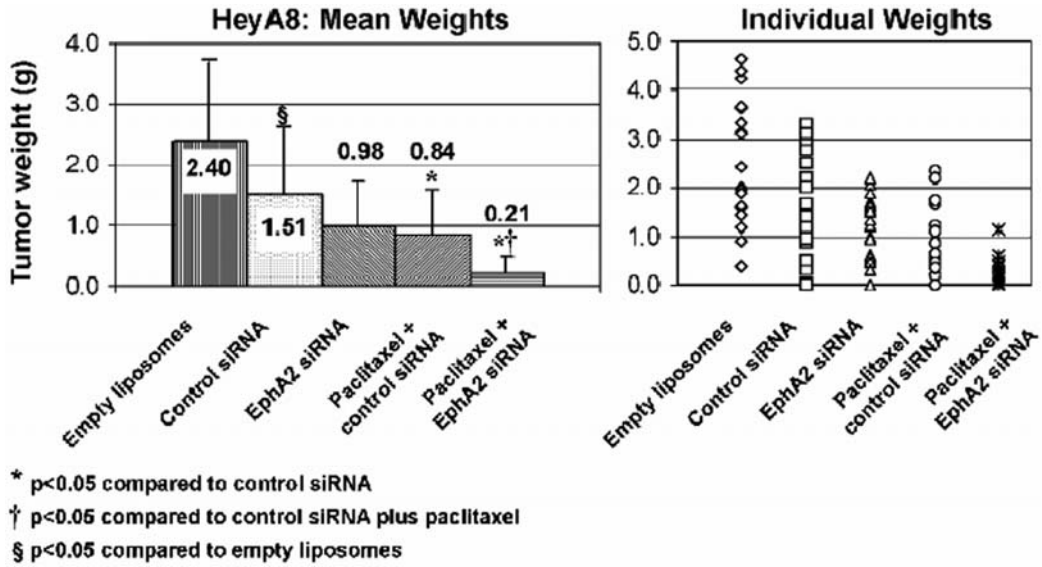

Fig. 3.1. Therapeutic effects of siRNA-mediated EphA2 downregulation on HeyA8 ovarian tumors. A total of 2.5×10^5 HeyA8 cells were injected i.p. into nude mice. Mice were randomly divided into five groups: (**A**) empty liposomes, (**B**) control siRNA–DOPC, (**C**) EphA2 siRNA–DOPC, (**D**) control siRNA–DOPC + paclitaxel, and (**E**) EphA2 siRNA–DOPC + paclitaxel. Therapy was started on the seventh day by injecting liposomal siRNA twice weekly (150 μg/kg) and paclitaxel (100 μg/mouse) once weekly. Animals were killed when control mice became moribund (3–5 weeks after cell injection); mouse weight, tumor weight, and tumor location were recorded. On left, mean tumor weight, and on right, individual tumor values were shown. (Reproduced from Ref. *(16)* with permission from American Association for Cancer Research).

8. The membrane was reprobed with secondary anti-mouse IgG HRP at 1:2,000 dilution.
9. Secondary antibody was removed and membrane was washed for 1 h at room temperature by changing the TBST every 10 min.
10. The enhanced chemiluminescence system was used to detect the reaction between antigen and antibody. After final wash with PBS, remaining steps were done in a dark room. Aliquots (1 mL) of each portion of the ECL reagent was mixed and added onto the blot and then rotated by hand for 1 min to ensure even coverage of the ECL reagent. The blot was removed from the ECL reagent and blotted.
11. The membrane was placed in X-ray film cassette with film for a suitable exposure time, typically a few minutes.
12. The membrane was stripped and reprobed with an anti-beta-actin antibody at a dilution of 1:2,000 to ensure even loading of proteins in the different lanes (**Fig. 3.2A**).

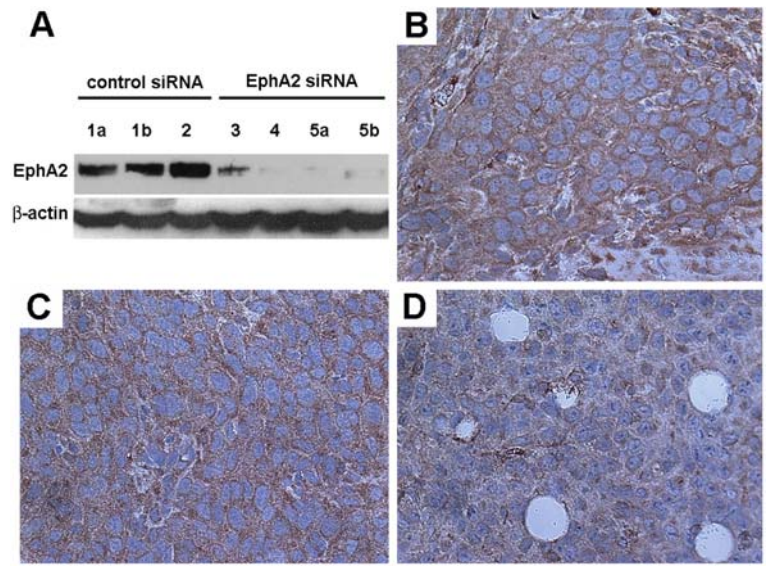

Fig. 3.2. In vivo downregulation of EphA2 using target siRNA. (**A**) Western blot of lysate from tumors collected after 48 h of single administration of control siRNA–DOPC (lanes 1 and 2) or EphA2-targeting siRNA–DOPC (lanes 3–5). Lanes 1a and 1b are separate preparations from same tumor treated with control siRNA–DOPC. Similarly 5a and 5b are from same tumor treated with EphA2-targeted siRNA–DOPC with different preparations. Lanes 2–4 are from separate mice treated with either control or EphA2 siRNA with DOPC. (**B**) Immunohistochemical staining for EphA2 in tumor treated with control siRNA–DOPC. (**C**) Immunohistochemistry 48 h after a single injection of EphA2-targeted siRNA without transfection agent (naked siRNA) is shown and had no detectable effect on EphA2 expression. (**D**) Treatment with EphA2-targeted siRNA in DOPC showed effective downregulation of EphA2 48 h after single injection. B–D, original magnification ×400. (Reproduced from Ref. *(16)* with permission from American Association for Cancer Research).

3.9. Immunohistochemistry

3.9.1. Immunohistochemical Staining for EphA2

1. Sections of formalin-fixed, paraffin-embedded tumor samples (8-μm thick) were heated to 60°C.
2. Sections were dehydrated in xylene, 100% ethanol, 95% ethanol, and 80% ethanol.
3. Then, tissues were rehydrated with PBS.
4. Antigen retrieval was done with 0.2 mol/L Tris/HCl (at pH 9.0) for 20 min in a steam cooker.
5. Slides were allowed to cool for 20 min at room temperature, followed by repeated rinsing with PBS for three times for 5 min.
6. Individual slides were removed, and dried around the tissue. A circle was drawn around the tissue with a Pap pen and kept on the slide holder. A drop of PBS was added to tissue and

placed in a humidified chamber. This step was performed for all immunohistochemistry and confocal microscopic studies.
7. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 5 min and washed three times with PBS for 5 min.
8. Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with non-immune mouse IgG in place of primary antibody (**Fig. 3.2B–D**).

3.9.2. Determination of Uptake of Alexa-555 Fluorescent siRNA by Tumor Tissues

1. Tissues were collected from sacrificed mice, immediately placed in OCT, and frozen rapidly in liquid nitrogen (**Section 3.6**).
2. Frozen sections were cut at 8-μm thickness and fixed with fresh cold acetone for 10 min.
3. Tissues were washed three times with PBS for 5 min and slides were placed on the slide holder as mentioned above in **Section 3.9A**. The humidified chamber was covered with an aluminum foil to protect from light.
4. Samples were exposed to 1.0 μg/mL Hoechst in PBS (at 1:10,000 dilution) for 10 min to stain the nucleus.
5. After three washes for 5 min with PBS, tissues were covered with mounting medium glycerol-propyl-gallate in PBS, coverslipped, and examined with a fluorescent Zeiss Axioplan 2 microscope, Hamamatsu ORCA-ER Digital camera, and ImagePRO Software using red and blue filters for Alexa-555 siRNA and nuclei, respectively (**Fig. 3.3A**). Since the fluorescent signal becomes weaker over time, pictures were taken within 4 days (*see* **Note 6**).

3.9.3. Immunofluorescence Detection of Macrophages

1. OCT frozen sections of 8 μm thickness were fixed with fresh cold acetone for 10 min and washed three times with PBS for 5 min.
2. Slides were placed in a humidified chamber as mentioned above and blocked with protein block for 20 min at room temperature.
3. Extra protein block was drained off and tissues were incubated with 10 μg/mL anti-f4/80 primary macrophage antibody at 4°C overnight.
4. Tissues were washed with PBS three times for 5 min each and then re-incubated with 4 μg/mL secondary goat anti-rat Alexa-488 for 1 h at room temperature.
5. Tissues were washed three times with PBS and counterstained with Hoechst, which was diluted 1:10,000 in PBS.
6. Tissues were washed with PBS for three times, mounted using mounting media glycerol-propyl-gallate in PBS, and then coverslipped.
7. Samples were visualized using conventional fluorescence microscope using appropriate filters such as red filter for

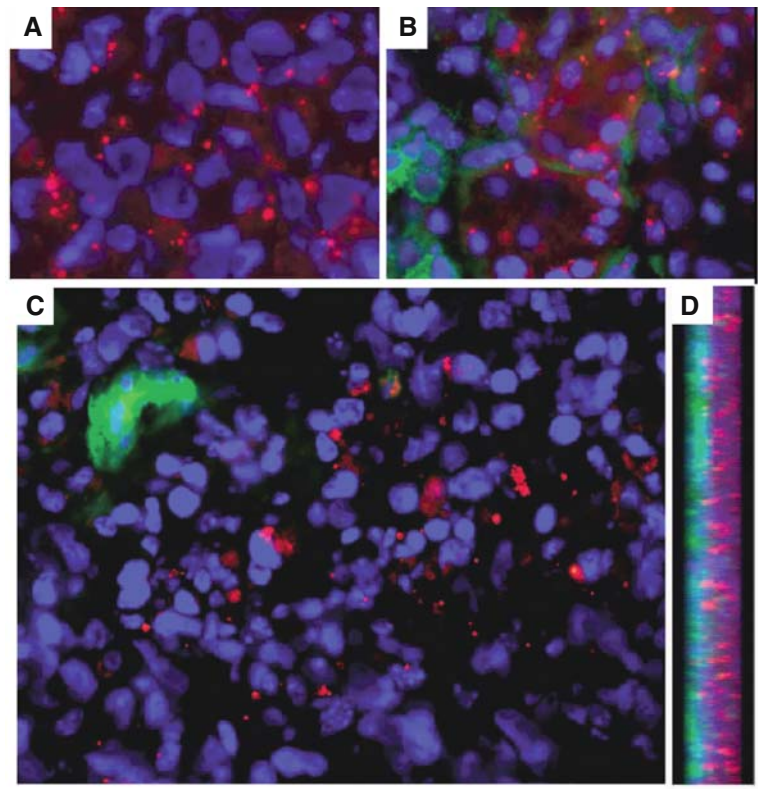

Fig. 3.3. In vivo siRNA distribution in HeyA8 tumor (i.p.) after a single siRNA dose. Tumors were harvested at different time points, frozen in OCT medium, fixed in acetone, and exposed to Hoechst to stain nuclei *blue*. (**A**) Fluorescent siRNA distribution in tumor tissue (original magnification $\times$400). (**B** and **C**) To detect scavenger macrophages and endothelial cells, tumor tissues were processed as mentioned above, additionally exposed to anti-f4/80 and anti-CD31 antibody and then to Alexa-488-tagged secondary antibody (*green*). (**D**) Sections of 30 μm thickness were examined with confocal microscopy. Photographs taken every 1 μm were stacked and examined from the lateral view. Nuclei were labeled blue and fluorescent siRNA (*red*) was seen throughout the section. Fluorescent Alexa-488-tagged secondary antibody (*green*) is trapped on the surface since it is too large to penetrate the tissue. (Reproduced from Ref. *(16)* with permission from American Association for Cancer Research).

fluorescent siRNA, green for macrophage, and blue for nucleus (**Fig. 3.3B**).

3.9.4. Evaluation of the Tumor Vasculature

1. OCT frozen sections were cut at 8 μm thickness.
2. Tissues were fixed with fresh cold acetone for 10 min and then washed with PBS three times for 5 min.
3. Tissues were placed in humidity chamber on the slide holder as mentioned above in **Section 3.9A**.
4. Tissues were incubated with protein block (5% normal horse serum plus 1% normal goat serum in PBS) for 20 min.

5. Excess of protein block was removed and tissues were incubated with primary rat anti-mouse CD31 antibody, which was diluted in protein block at dilution of 1:800 at 4°C overnight.
6. Slides were washed three times with PBS for 5 min each and incubated with secondary goat anti-rat Alexa-488 (1:400 dilution) for 1 h at room temperature. The humidity chamber was covered with aluminum foil to protect from light.
7. Tissues were washed three times with PBS for 5 min and counterstained with Hoechst (1:10,000 dilution) for 10 min for the detection of nucleus.
8. Tissues were mounted using propyl-gallate and coverslipped.
9. Samples were visualized for CD31-positive blood vessels by fluorescent Zeiss Axioplan 2 microscope, Hamamatsu ORCA-ER Digital camera, and ImagePRO Software using red, blue, and green filters for Alexa-555 siRNA, nuclei, and blood vessels, respectively (**Fig. 3.3C**).

3.10. Confocal Microscopy

1. For confocal microscopy, tissues were collected from sacrificed mice, immediately placed in OCT, and frozen rapidly in liquid nitrogen.
2. Frozen sections were cut at 30-μm thickness, fixed with fresh cold acetone for 10 min, and washed three times with PBS for 5 min.
3. Sections were exposed with 10 nmol/L Sytox green in PBS for 10 min to stain the nucleus.
4. After three washes for 5 min with PBS, tissues were covered with glycerol-propyl-gallate, coverslipped, and examined with Zeiss LSM 510 confocal microscope and LSM 510 Image Browser software (**Fig. 3.3D**).

4. Notes

1. Avoid stressing cells with temperature shifts and long periods without medium during washing steps. It is very important that the cell density is not too low at transfection. Ideally, the cells should be plated 24 h prior to transfection.
2. For in vitro siRNA transfection of ovarian cancer cells, we optimized the ratio between siRNA and RNAiFect as 1:6. If there is toxicity seen for other cell types with this ratio, we recommend decreasing the amount of RNAiFect reagent.
3. For in vivo injections, grow cells to 60–80% confluence. Do not use over-confluent cells and cells that have propagated more than 10 passages (since the last thaw), as they will have lower take rates.

4. Mix siRNA–DOPC with PBS (without calcium or magnesium) or normal saline before injection of the complex into mice. Do not vortex the siRNA–liposome complex (degradation of liposomes may occur with vortexing). Mix gently with pipetting up and down.
5. Once liposomal siRNA is mixed with PBS or saline, do not freeze the solution because the freezing process could fracture or rupture the liposomes leading to a change in size distribution and loss of internal contents.
6. When Alexa-555 fluorescently labeled siRNA is used, we recommend taking pictures within 3–4 days after staining because the fluorescent signal may become weaker over time.

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