siRNA. Figure 2 showed that the 0.5% atelocollagen/DharmaFECT1/siRNA complex rather than the 0.05% atelocollagen /DharmaFECT1/siRNA complex reduced luciferase activity and that the DharmaFECT1 free complex did not reduce it. To date, a collaborator usually uses DharmaFECT1 in the atelocollagen-mediated systemic gene delivery by i.v. administration, because this reagent improves it (Takeshita F, unpublished observation, 2010).

In peritoneal metastasis model mice, the i.p. administration of *NEDD1* siRNA was able to inhibit tumor growth and prolong survival even without any side effects (Figs 5,7). If targets such as *NEDD1* function in the cell cycle regulation, the slow gene release arising from protection from nucleases by atelocollagen may provide an advantage for long acting and for reducing the number of administrations. As shown in Figure 7, we administered the *NEDD1* siRNA complex five times every 3 days for 15 days in this study; however, that number may possibly be reduced. In another report for intraperitoneal administration of siRNA targeting nuclear factor-κB with only DharmaFECT1, the siRNA prolonged the survival of mice only by the administration of paclitaxel, whereas the siRNA/DharmaFECT1 complex alone could not succeed.[25] Taken together, the atelocollagen/DharmaFECT1/siRNA complex is very useful for gene delivery to the peritoneal cavity.

In the mouse model used, the number of tumor cells (1×10^5 cells) implanted into the mouse peritoneal cavity was estimated to be still very large compared with the number of tumor cells in the peritoneal cavity in human GC patients with cytology positive, who often showed peritoneal metastasis within 2 years. Therefore, the present i.p. delivery system of siRNA has a great potential for treatment of such GC patients.

Although *in vitro* cell growth inhibition was observed by the double siRNA treatment of *ELK1* and *MSX2* (Fig. 3b), no significant difference on *in vivo* tumor growth and mouse survival was found (Fig. 3c and data not shown). Investigation of other hedgehog components (SMO, GLIs, ISL1, BMP4, FOXM1, and FOXA2) and EMT-regulators (SIP1/ZEB2, TWIST2) remains for a future study, because cross-talk between hedgehog and EMT signals is specific to diffuse-type GC.[18]

Atelocollagen has also been reported to efficiently deliver microRNA.[26] Recently, genome-wide microRNA expression profiles of 353 GC samples have shown that some microRNAs including let-7b, miR-214, and miR-433 are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of diffuse-type GC.[27] Thus, these microRNAs may be candidates for SGC therapy. In addition, transforming growth factor-β (TGF-β) has been reported to induce apoptosis of a subset of diffuse-type GCs whose receptor is not inactivated.[28,29] Therefore, adenovirus-mediated TGF-β or the downstream targets such as Gasdermin/GasderminA delivery also have great potential for SGC therapy.

In conclusion, we developed a novel i.p. delivery system of siRNA to disseminated tumor cells in the peritoneal cavity that successfully prolongs the survival of model mice. The present mouse model is for an adjuvant therapy after surgical resection. The ability of atelocollagen/DharmaFECT complex is keeping siRNA from nucleases, leading slow gene release and reducing the amount of administration that results in effective eradication of residual tumor cells in the peritoneal cavity.

Thus, considering other potential targets of the diffuse-type GCs, this system is a highly flexible therapeutic platform for the treatment of peritoneal dissemination.

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Disclosure Statement

The authors have no conflict of interest.

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Chapter 17

In Vivo Imaging of Oligonucleotide Delivery

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Abstract

RNA interference (RNAi) has rapidly become a powerful tool for drug-target discovery and therapeutics. Cancer is an important application for RNAi therapeutics, since abnormal gene regulation is thought to contribute to the pathogenesis and maintenance of the metastatic phenotype of cancer. Many oncogenic genes present enticing therapeutic target possibilities for RNAi. Small interfering RNA (siRNA) and microRNA (miRNA) are potent and specific examples of RNAi are able to silence tumor-related genes and multiple oncogenic pathways and appear to be a rational approach to inhibit tumor growth. In subsequent in vivo studies, an appropriate animal model must be developed for a better evaluation of gene-silencing effects on tumors. How to evaluate the effect of siRNA and miRNA in an in vivo therapeutic model is also important. Bioluminescence imaging is an optical imaging method that can evaluate RNAi in vivo.

Key words: siRNA, MicroRNA, Cancer, Delivery, Imaging, Luciferase, Oligonucleotides

1. Introduction

RNAi can effect posttranscriptional gene silencing. The introduction into an organism of double-stranded RNA (dsRNA) corresponding to a transcribed sequence results in degradation of the corresponding mRNA (1–6). With RNAi, dsRNA blocks gene expression in a sequence-specific manner. When introduced into cells, dsRNA is processed by the RNase III family nuclease Dicer into siRNA, 21-basepair dsRNA with two overhanging bases at each 3′ terminus. The double-stranded siRNA is passed to the RNA-induced silencing complex (RISC), an RNA–nuclease complex, which is activated as it unwinds the duplex and incorporates one of the antisense strands. The RISC then selectively degrades

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RNA containing the sequence complementary to the incorporated antisense strand.

Antisense oligonucleotide drugs were used prior to the discovery of RNAi and several antisense molecules are currently in late-stage preclinical or clinical development (7). Although researchers continue to explore and develop antisense reagents for therapeutic use by morpholino oligomers, a fourth class of oligonucleotide-based compounds, consisting of siRNAs, has recently become widely used for gene knockdown in vitro and in vivo.

Another group of catalytically-active RNA molecules (ribozymes) has also been considered for therapeutic use. However, only a few ribozymes have turned out to be efficient compounds in clinical trials. RNAi is effective because siRNA is highly specific for the target gene, and the single-strand RNA molecule incorporated into the RISC is used to recognize multiple copies of the target RNA. Therefore, an extremely small amount of siRNA can generate reliable gene suppression, making toxicity less of a concern. Furthermore, effective antisense oligonucleotide sequences are determined empirically, resulting in uncertain efficacies.

MicroRNA (miRNA), an endogenously-expressed form of siRNA, approximately 22 nucleotides in length, also works for gene silencing. It is estimated that there are over 1,000 miRNAs in humans. It is believed that a single miRNA can regulate several hundred genes. Current understanding of the molecular mechanism of any disease, including cancer (8, 9), would be incomplete without factoring in the functional significance of miRNA. Misexpression of miRNAs has been observed in various types of cancers and is also associated with the clinical outcome of cancer patients. Consistently, miRNAs have been implicated in the regulation of various cellular processes that are often deregulated during tumor development and progression (10, 11), suggesting that these miRNAs might be targets for cancer therapy.

The most direct way for molecules to correct expression of altered genes and miRNAs is treatment by RNA oligonucleotides. For this purpose, an in vivo delivery system is a key issue. Here, we describe an in vivo imaging method of delivery of oligonucleotides such as siRNA and miRNA.

2. Materials

2.1. Cell Lines and Medium

1. PC-3M-luc cells (Xenogen Corp., Alameda, CA).
2. Cell culture medium: RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Kerrville, TX) and 0.2 mg/ml zeocin (Invitrogen Corp.).

2.2. Oligonucleotide Delivery Mixture

1. Oligonucleotide delivery system: atelocollagen (12–15) for local use AteloGene™ #1390 and systemic use AteloGene™ #1391 (Koken, Tokyo).
2. Oligonucleotides: 5–10 and 20–40 μM oligonucleotide solutions for local and systemic delivery in vivo.

2.3. In Vivo Imaging

1. For in vivo imaging with Renilla luciferase: ViviRen (5 mg/kg, Promega).
2. For in vivo imaging with firefly luciferase: D-luciferin (150 mg/kg, Xenogen).
3. Data analysis: LivingImage software (version 2.50, Xenogen) (16).

3. Methods

Recent progress in the optical imaging of cancers in animal models presents many potential advantages for recreating the disease process, disease detection, screening, diagnosis, drug development, and treatment evaluation. Fluorescence-based imaging (17–21) and bioluminescence-based imaging (12, 13, 22–29) are well developed and allow specific, highly-sensitive, and quantitative measurements of a wide range of tumor-related parameters in mice.

A major advantage of GFP-labeling is that imaging requires no preparative procedures and hence allows for direct visualization in living tissue. In contrast, luciferase imaging requires exogenous injection of luciferin substrate which can stress the animals. In addition, the intensity of the luciferase signal may sometimes be variable and unstable. Furthermore, RFP imaging is about 1,000 times stronger than that of luciferase in vivo. Therefore, for monitoring the tumor metastasis process at the single-cell level, fluorescence imaging may be the more practical method. In fact, fluorescence-based orthotopic metastatic models have been used to study mechanisms and drug discovery. Here, we have used the bioluminescence signal from the luciferase reporter gene in our metastasis model. Luciferase genes in our tumor cells can function stably over significant periods of time in tumors and in their metastases.

3.1. Preparation of Dual Luciferase Expressing Cells

1. For construction of 3′-UTR-Renilla luciferase plasmid and reporter assays, amplify the segment of 3′-UTR of the Bcl2 gene by PCR using genomic DNA from normal human prostate epithelial cells (PrEC, CT-2555, Lonza Walkersville, Inc., Walkersville, MD).
2. Insert the PCR product into a pGL4.75 [HRuc/CMV] vector (Promega, Madison, WI), using the XbaI site immediately

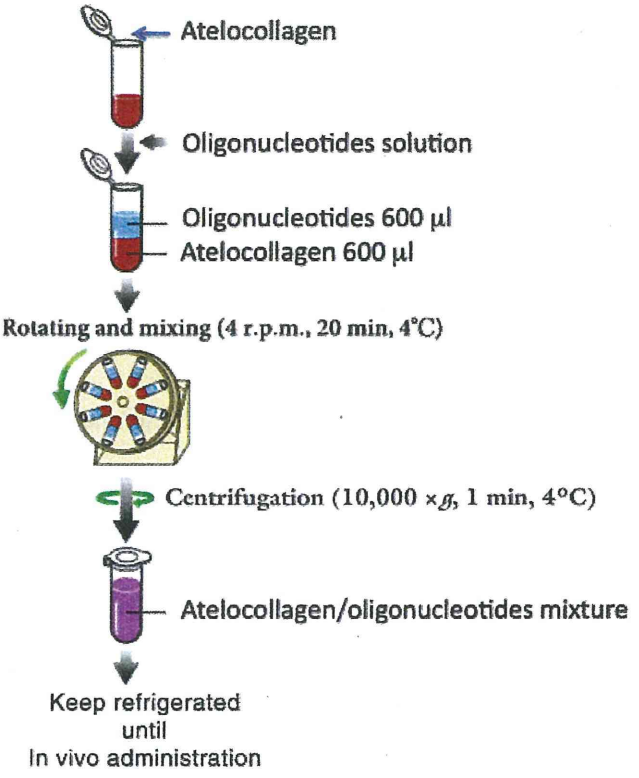

Fig. 1. Preparation of oligonucleotides delivery mixture. Gently add 600 µl of the oligonucleotide solution to 600 µl of the atelocollagen solution. Rotate the mixture solution for 20 min. Set the rotating speed at about 4 rpm when a 20-cm diameter holder is used. After mixing, centrifuge the tube for 1 min at 10,000 x *g* to deform the mixed solution.

downstream from the stop codon of Renilla luciferase (pGL4.75[HRuc/CMV]-Bcl2 3′UTR).

3. For reporter assays, transfect 2 µg pGL4.75[HRuc/CMV]-Bcl2 3′UTR using LipofectAMINE™ 2000 (Invitrogen Corp.) into PC-3M-luc cells.
4. Select stable transfectants in hygromycin (0.2 mg/ml; Invitrogen Corp.) using the Dual-luciferase assay-system (Promega). The intensity of Renilla luciferase is normalized by firefly luciferase. Clones expressing both luciferase genes are named PC-3M-luc/Rluc-Bcl2 3′UTR.

3.2. Oligonucleotides Delivery

1. Gently add 600 µl of the oligonucleotide solution to 600 µl atelocollagen solution.
2. Rotate the mixture solution for 20 min. Set the rotating speed at approximately 4 rpm when using a 20-cm diameter holder (see Note 1).
3. After mixing, centrifuge the tube for 1 min at 10,000×*g* to deform the mixed solution (see Note 2). The procedure is shown in Fig. 1.

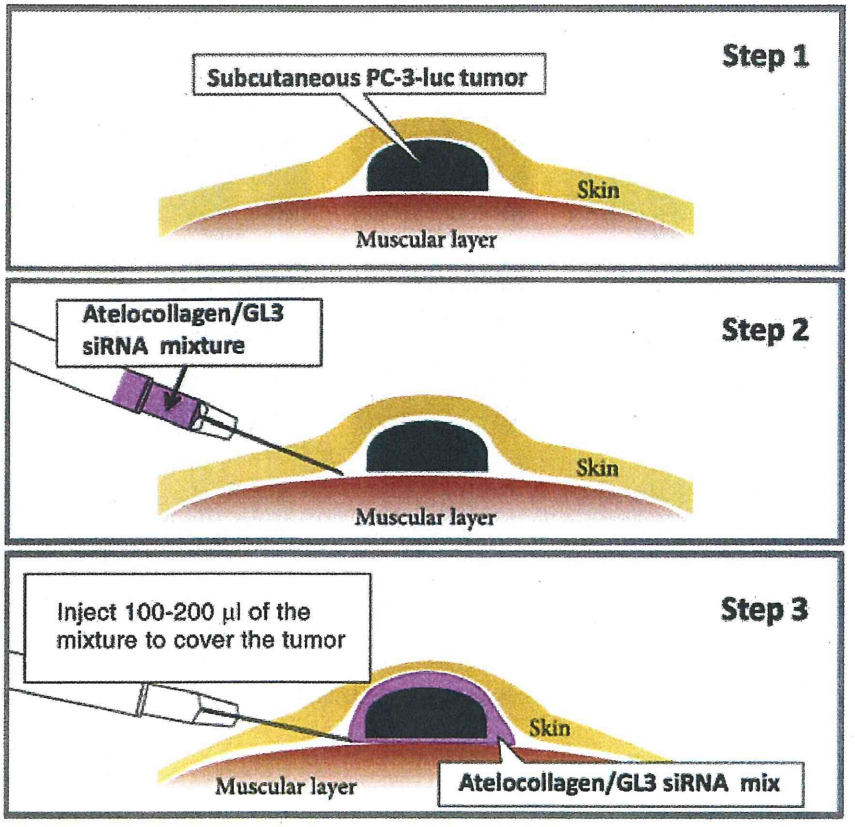

Fig. 2. Administration of the mixture to subcutaneous tumors. Step 1: PC-3-luc subcutaneous tumor; Step 2: insert a 26 G needle subcutaneously approximately 5 mm to the side of the tumor; Step 3: lay the needle parallel to the skin and insert it for 2–3 mm in the direction of the tumor, and then inject the mixture for 20–30 s. It is effective when the mixture is administered so as to cover the whole target site.

3.3. Imaging of Local Delivery of Oligonucleotides

1. Prepare 7- to 10-week-old male athymic nude mice (CLEA Japan, Shizuoka, Japan).
2. To generate a subcutaneous tumor model, the animals are injected with 1×10^6 PC-3-luc cells suspended in 100 ml sterile DPBS.
3. When a tumor develops to 5×5 mm a mixture of GL3 siRNA (specifically knock down firefly luciferase) and atelocollagen are prepared according to the described method.
4. Set the 18-G needle in the disposable syringe and slowly draw the atelocollagen/oligonucleotide mixture (see Note 3).
5. Replace the needle of the syringe with a 26-G injection needle and keep the syringe refrigerated until administration.
6. Insert the injection needle from approximately 5 mm to the side of the subcutaneous tumor with the cut face of the needle turned upward.
7. Lay the needle parallel to the skin and insert it 2–3 mm in the direction of the tumor, and then gently inject 200 µl of the mixture. The procedure is shown in Fig. 2 (see Note 4).

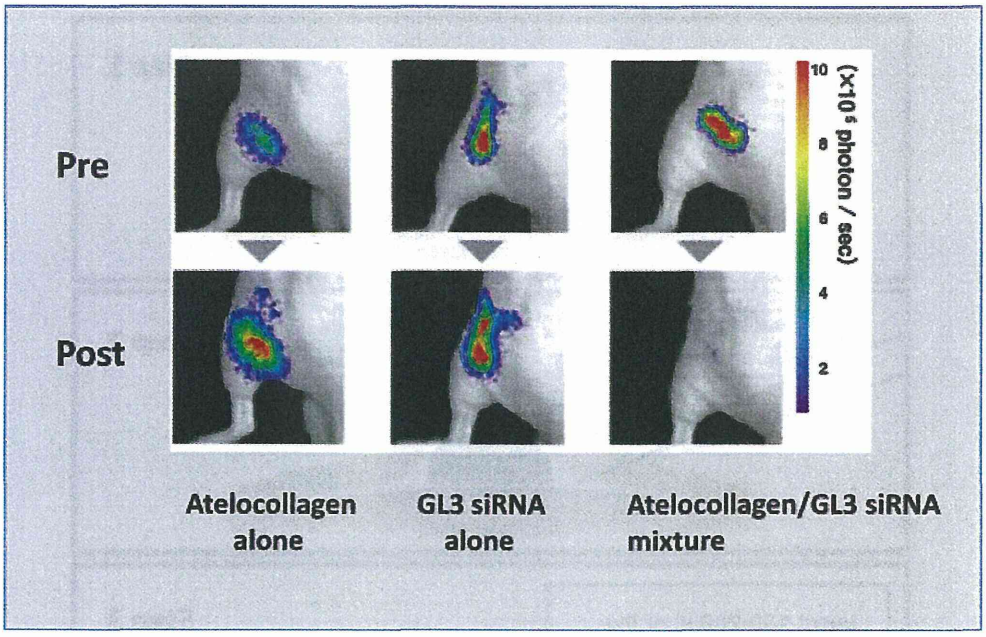

Fig. 3. In vivo imaging of local delivery of oligonucleotides. Firefly luciferase-expressing cells formed subcutaneous tumors. Atelocollagen/GL3 siRNA significantly inhibited the photon count of luciferase as compared to atelocollagen alone and GL3 siRNA alone.

8. 24–48 h after injection (see Note 5), the animals are subjected to bioimaging analysis. An example result is shown in Fig. 3.

3.4. Imaging of Systemic Delivery of Oligonucleotides

1. To generate an experimental metastasis model, the anesthetized animals are injected with 2×10^6 PC-3M-luc cells suspended in 100 ml sterile DPBS into the left heart ventricle (see Note 6).
2. When metastasis develops, a mixture of GL3 siRNA and atelocollagen is prepared according to the above method.
3. Anesthetize the animals, if necessary.
4. Set the 18-G needle in a disposable syringe and slowly draw the atelocollagen/oligonucleotide mixture. In a systemic injection, 100–200 μl of the mixture is used.
5. Replace the syringe needle with the 26-G injection needle and keep the syringe refrigerated until administration.
6. Disinfect the tail of the animal with ethanol.
7. Insert the needle into the vein at a position 1/4 from the tail end.
8. Confirm that the injection needle has entered the vessel and then slowly inject 100–200 μl of the mixture (see Note 7).
9. 24–48 h after injection, the animals are subjected to bioimaging analysis. An example result is shown in Fig. 4.

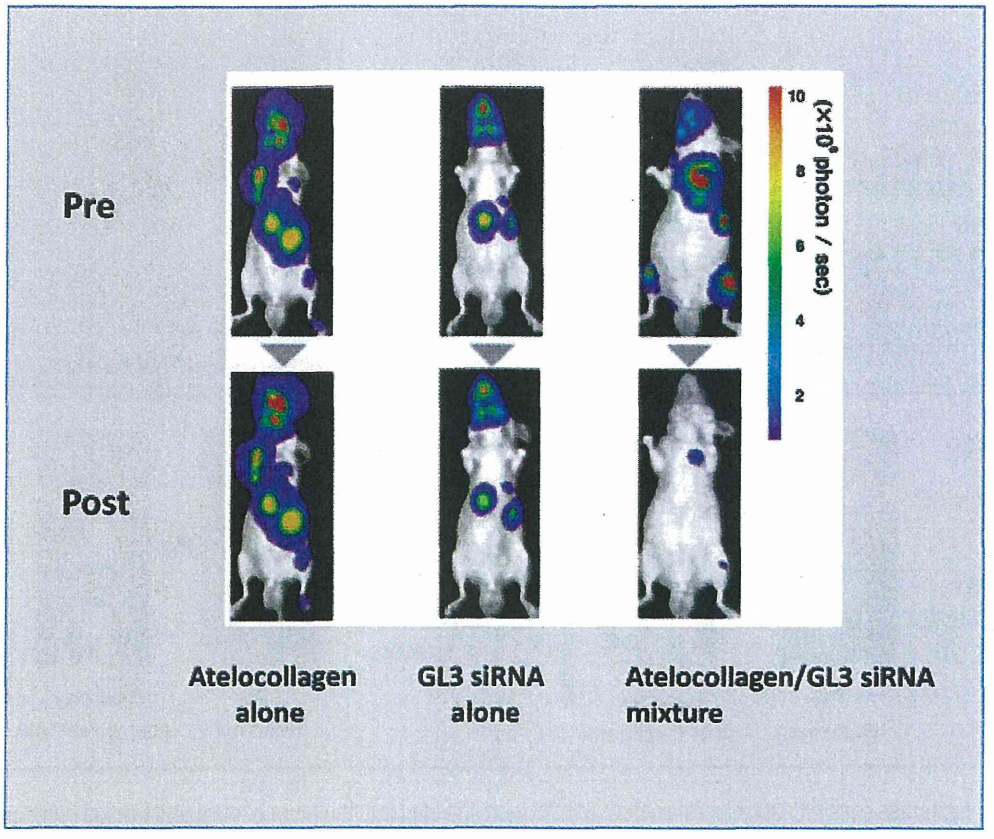

Fig. 4. Firefly-luciferase-expressing cells with formed bone metastasis in mice. Intra-cardiac administration of atelocollagen/GL3 siRNA significantly inhibited the photon count of luciferase as compared to atelocollagen alone and GL3 siRNA alone.

3.5. Dual Luciferase Imaging System for Delivery of Oligonucleotides

1. Seven- to ten-week-old male athymic nude mice (CLEA Japan, Shizuoka, Japan) are anesthetized by exposure to 3% isoflurane on day 0 and subsequent days.
2. On day 0 of the experiments, to generate an experimental metastasis model, the anesthetized animals are injected with 2×10^6 PC-3M-luc/Rluc-Bcl2 3′UTR cells, suspended in 100 ml sterile DPBS, into the left heart ventricle.
3. When metastasis develops, a mixture of miRNA16 and atelocollagen is prepared according to the described method.
4. For systemic injection of the atelocollagen/miRNA mixture, repeat steps 4–8 in Subheading 3.2.
5. For in vivo imaging, the mice are injected with ViviRen (5 mg/kg, Promega) by intravenous tail vein injection and imaged immediately to count the photons from the animal body.
6. After the bioluminescence from Renilla luciferase disappears, the mice are administered D-luciferin (150 mg/kg, Xenogen) by intraperitoneal injection.
7. Ten minutes later, photons from firefly luciferase are counted. An example result is shown in Fig. 5.

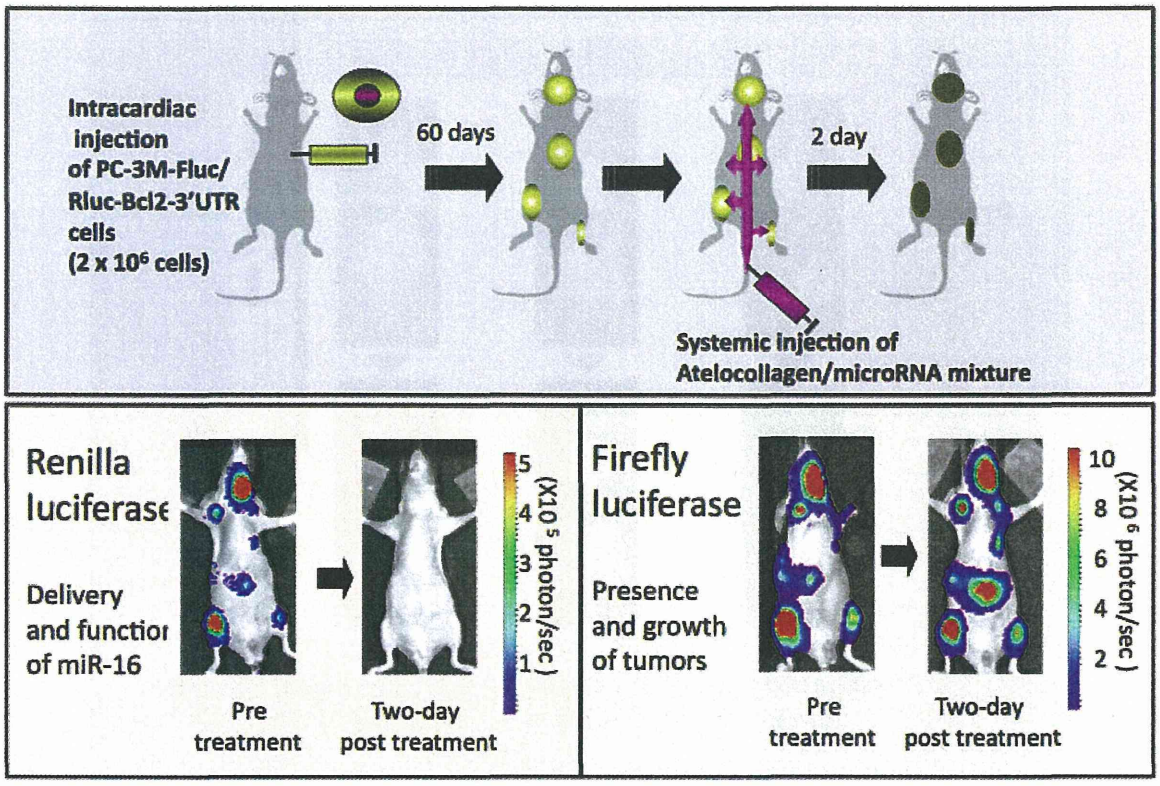

Fig. 5. Dual luciferase cells (PC-3M-Fluc/Rluc-Bcl2-3'UTR) were injected into the heart of mice and formed bone metastases. When the expression of Renilla luciferase from tumor cells could be detected, 50 μg miR-16/atelocollagen was injected intravenously. As can be seen at the bottom of the figure (left side), the photons from the Renilla luciferase were dramatically suppressed one day post-treatment. This result indicates that atelocollagen has the potential for delivering miR-16 throughout the whole body, including bone metastatic sites. Firefly luciferase was not affected, because large-sized tumors like these were not inhibited by a single treatment of miR-16 (29).

4. Notes

1. Avoid mixing by vortexing; otherwise, large aggregates may generate and cause a less efficient delivery of the oligonucleotides.
2. After mixing, ensure there are no visible aggregates.
3. Draw the mixture slowly to avoid incorporation of bubbles.
4. Intratumoral injection is also possible for delivery of atelocollagen/siRNA.
5. The effect of siRNA, such as miRNA delivery by atelocollagen, differs depending on the oligonucleotide sequence, expression level of the target oligonucleotides, the difference in target tumor cells, tissues, site of the tumors, etc. Investigating the