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In vivo inhibition of microRNA to decrease tumor growth in mice

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Corresponding Author:	Julia Ramirez-Moya Instituto de Investigaciones Biomedicas Alberto Sols Madrid, Madrid SPAIN
Corresponding Author's Institution:	Instituto de Investigaciones Biomedicas Alberto Sols
Corresponding Author E-Mail:	jramirez@iib.uam.es
Order of Authors:	Julia Ramirez-Moya
	León Wert-Lamas
	Adrián Acuña-Ruiz
	Miguel A Zaballos
	Pilar Santisteban
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TITLE:

In vivo Inhibition of MicroRNA to Decrease Tumor Growth in Mice

AUTHORS AND AFFILIATIONS:

Julia Ramírez-Moya[1,2], León Wert-Lamas[1], Adrián Acuña-Ruiz[1,2], Miguel A. Zaballos[1,2] and Pilar Santisteban[1,2]

(1) Instituto de Investigaciones Biomédicas, CSIC-UAM, Madrid, Spain
(2) Ciberonc, Instituto de Salud Carlos III, Madrid, Spain

Julia Ramírez-Moya: jramirez@iib.uam.es
León Wert-Lamas: Leon_WertLamas@DFCI.HARVARD.EDU
Adrián Acuña-Ruiz: aacuna@iib.uam.es
Miguel A. Zaballos: mazaballos@iib.uam.es
Pilar Santisteban: psantisteban@iib.uam.es

KEYWORDS:

miRNA inhibitor, thyroid, tumor growth, mouse models, miRNA-based treatment, miR-146b, thyroid cancer

SUMMARY:

This protocol describes xenograft and orthotopic mouse models of human thyroid tumorigenesis as a platform to test microRNA-based inhibitor treatments. This approach is ideal to study the function of non-coding RNAs and their potential as new therapeutic targets.

ABSTRACT:

MicroRNAs (miRNAs) are important regulators of gene expression through their ability to destabilize mRNA and inhibit translation of target mRNAs. An ever-increasing number of studies have identified miRNAs as potential biomarkers for cancer diagnosis and prognosis, and also as therapeutic targets, adding an extra dimension to cancer evaluation and treatment. In the context of thyroid cancer, tumorigenesis results not only from mutations in important genes, but also from the overexpression of many miRNAs. Accordingly, the role of miRNAs in the control of thyroid gene expression is evolving as an important mechanism in cancer. Herein, we present a protocol to examine the effects of miRNA-inhibitor delivery as a therapeutic modality in thyroid cancer using human tumor xenograft and orthotopic mouse models. After engineering stable thyroid tumoral cells expressing GFP and luciferase, cells are injected into nude mice to develop tumors, which can be followed by bioluminescence. The in vivo inhibition of a miRNA can reduce tumor growth and upregulate miRNA gene targets. This method can be used to assess the importance of a determined miRNA in vivo, in addition to identifying new therapeutic targets.

INTRODUCTION:

Thyroid cancer is an endocrine malignancy with an increasing incidence, although in general terms it has a good outcome[1]. Nevertheless, some patients develop aggressive forms of the disease that are untreatable and the molecular bases are poorly understood[2].

miRNAs are 22-nucleotide-long non-coding RNAs that regulate gene expression in many tissues, typically by base-pair binding to the 3' untranslational region (3'UTR) of target messenger RNAs (mRNAs), triggering mRNA degradation or translational repression[3,4]. There is increasing evidence demonstrating that the deregulation of microRNA expression is a hallmark of cancer, as these molecules modulate proliferative signaling, migration, invasion and metastasis, and can provide resistance to apoptosis[5,6]. In recent years, many studies have identified miRNAs as potential biomarkers for cancer diagnosis and prognosis as well as therapeutic targets[7], providing a new dimension to cancer evaluation and treatment.

miRNAs have taken center stage in human molecular oncology as key drivers of human thyroid neoplasms[8-12]. Among the miRNAs up-regulated, miR-146b is highly overexpressed in Papillary Thyroid Carcinoma (PTC) tumors and was shown to significantly increase cell proliferation, and to be associated with aggressiveness and dismal prognosis[6,12-15]. Furthermore, miR-146b regulates several thyroid genes involved in differentiation[12], and also important tumor suppressor genes such as PTEN[16] and DICER1[17]. Despite their importance in cancer biology, miRNA-based cancer therapy is still in its early stages, and very few studies have addressed thyroid cancer – the most frequent of the endocrine tumors[18]. Here we describe a protocol using two different mouse models with human-derived tumors, in which the administration of a synthetic miRNA-inhibitor (antagomiR) that specifically inhibits a cellular miRNA can block tumor growth. We first used a common xenograft model, and the local intratumor administration of an antagomiR decreased tumor growth measured as a reduction in tumor bioluminescence[16]. Because the establishment of robust mouse models mimicking human tumor progression is essential to develop unique therapeutic approaches, orthotopic implantation of primary human tumors is a more valuable platform for clinical validation of new drugs than subcutaneous implantation models. Thus, in order to better assess the therapeutic potential of the antagomiR, we used an orthotopic mouse model with systemic delivery in the blood stream, obtaining the same results.

PROTOCOL:

Animal experimentation was performed in compliance with the European Community Law (86/609/EEC) and the Spanish law (R.D. 1201/2005), with approval of the Ethics Committee of the Consejo Superior de Investigaciones Científicas (CSIC, Spain).

1. Flank inoculation of cells and intratumoral antagomiR treatment

1.1. Cell preparation

1.1.1. Engineer a Cal62 human thyroid cancer cell line ($KRAS^{G12R}$ and $p53^{A161D}$ mutations) to overexpress a transgenic construct that constitutively expresses GFP and luciferase (CMV-Firefly Luc-IRES-eGFP). Select the transgenic cells by antibiotic resistance or by sorting the GFP-positive cells with flow cytometry.

1.1.2. Grow the cells in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with penicillin, streptomycin and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2.

1.1.3. Suspend 1 x 10^6 cells in 50 µL of phosphate buffered saline (PBS) at 4 °C.

1.2. Cell inoculation into the flanks of mice

1.2.1. Mix the cells with the same amount of basement membrane matrix. For example, add 1 x 10^6 cells in 50 µL of PBS to 50 µL of basement membrane matrix (see **Table of Materials**) and mix gently.

NOTE: The membrane matrix polymerizes to produce a reconstituted, biologically-active stable matrix, which is effective for the attachment of the transformed cells to enhance their tumorigenicity. It is extremely important that the basement membrane matrix and all the material coming in contact with it is pre-chilled/ice-cold, since the matrix will start to gel above 10°C. Handle under sterile conditions using pre-cooled pipet tips.

1.2.2. Inject 100 µL of the sample (cells in PBS + basement membrane matrix) subcutaneously into the left flank of 6-week-old immunodeficient BALB/c *nu*/*nu* mice using a 1 mL insulin syringe with a 27G 1/2'' (0.4x13 mm) needle.

1.3. Intratumoral antagomiR treatment.

NOTE: Perform this step 2 weeks after the cell injection, when tumor formation is apparent.

1.3.1. Suspend the antagomiR (see **Table of Materials**) or the negative control in 500 µL of RNase-free distilled water.

1.3.2. Prior to the injection, prepare 2 nmol of the antagomiR or the control together with an in vivo delivery reagent (see **Table of Materials**) for each injection.

1.3.2.1. First mix the antagomiR solution (see **Table of Materials**) and the complexation buffer (see **Table of Materials**) in a 1:1 ratio. For example, add 80 µL of miRNA-inhibitor solution (16 nmol) to 80 µL of complexation buffer (included in the in vivo delivery reagent kit, see **Table of Materials**).

1.3.2.2. Bring the in vivo delivery reagent to room temperature. Add 160 µL to a 1.5-mL tube and immediately add 160 µL of diluted antagomiR solution. Return remaining reagent to -20 °C. If necessary, store the reagent at 4 °C for up to one week after thawing.

1.3.2.3. Vortex immediately (10 s) to ensure complexation of the in vivo delivery reagent-antagomiR.

1.3.2.4. Incubate the in vivo delivery reagent-antagomiR mixture for 30 min at 50 °C.

Centrifuge the tube briefly to recover the sample.

1.3.2.5. Dilute the complex 6-fold by adding 1360 µL of PBS pH 7.4 and mix well.

1.3.2.6. Proceed with in vivo delivery of the reagent-antagomiR complex (8 mice/condition), or store the complex at 4 °C for up to one week prior to injection. Inject 200 µL intratumorally into each tumor (2 nmol of antagomiR).

NOTE: The volume and quantity of the antagomiR is independent of the tumor volume.

1.3.3. Perform the treatment 3 times each week (Monday, Wednesday and Friday) for 2 weeks.

1.4. Analysis of tumor growth

NOTE: Determine the tumor bioluminescent signal twice weekly to calculate tumor growth.

1.4.1. Inject 50 µL of a 40 mg/mL solution of D-luciferin substrate (see **Table of Materials**) subcutaneously at each time point with a 1 mL syringe with a 27G 1/2'' (0.4 mm x 13 mm) needle.

1.4.1.1. At 8 min post-injection, anesthetize the mice using 3% isoflurane mixed with oxygen. Assess the level of anesthesia by pedal reflex (firm toe pinch) and adjust anesthetic delivery as appropriate to maintain surgical plane.

1.4.1.2. Apply ophthalmic ointment to both eyes to prevent desiccation.

1.4.2. Image the bioluminescent signal with in vivo imaging software (see **Table of Materials**).

NOTE: Calipers can also be used to measure the tumor volume and the tumor growth.

1.4.3. Once the bioluminescence signals are obtained, analyze the tumoral growth comparing both treatments and determine the significance by using a t-test. To analyze the within-group variance use the SEM.

1.5. Tumor excision

1.5.1. Seven days after the end of the antagomiR treatment, sacrifice the mice, excise the tumor and extract protein and/or RNA for future analysis. Alternatively, section the tumors and fix them for immunohistochemistry.

2. Thyroid orthotopic inoculation of cells and systemic antagomiR treatment

2.1. Cell preparation

2.1.1. Engineer a Cal62 human thyroid cancer cell line to overexpress a transgenic construct that

constitutively expresses GFP and luciferase. Select the transgenic cells by antibiotic resistance or by sorting the GFP-positive cells with flow cytometry.

2.1.2. Grow these cells in DMEM supplemented with antibiotics (penicillin and streptomycin) and 10% FBS at 37 °C and 5% CO_2.

2.1.3. Suspend 1 x 10^5 cells in 5 µL of PBS.

2.2. Cell inoculation into the thyroid gland of mice

2.2.1. Inject 100 µL of analgesic (buprenorphine) and 100 µL of antibiotic (cephalosporin) subcutaneously into 7-week-old BALB/c *nu*/*nu* mice.

2.2.2. Inject 5 µL of the cell solution into the thyroid gland of the mice.

2.2.2.1. Anesthetize the mouse using 3% isoflurane mixed with oxygen and place it under a stereomicroscope in a sterile flow cabinet. Assess the level of anesthesia by pedal reflex (firm toe pinch) and adjust anesthetic delivery as appropriate to maintain surgical plane.

2.2.2.2. Apply ophthalmic ointment to both eyes to prevent desiccation.

2.2.2.3. For each mouse, disinfect the neck with iodopovidone and make an incision of approximate 2 cm in the skin with scissors. Once open, expose the neck by displacing the salivary glands.

2.2.2.4. Dissect the strap muscles using dissection forceps and/or scissors to expose the trachea and the thyroid gland. Using a 10 µL microliter syringe inject 5 µL of the cell solution into the right thyroid lobule, located at the side of the cricoid cartilage.

2.2.2.5. Reposition the salivary glands and suture the incision with silk using braided, coated, non-absorbable sutures.

2.2.2.6. Add iodopovidone to the wound area and place the mouse on a thermic blanket while it recovers from the anesthesia.

2.2.3. The following day post-surgery, inject subcutaneously analgesic (buprenorphine) and add 3 mL of liquid ibuprofen (40 mg/mL) into 250 mL of the drinking water for 1 week.

2.3. Systemic antagomiR treatment

NOTE: Perform this step 2-3 weeks after the cell injection, when the bioluminescent signal is detectable.

2.3.1. Suspend the antagomiR or the negative control in distilled RNase-free water.

2.3.2. Prior to the injection, prepare 7 nmol of the antagomiR or the control together with the in vivo delivery reagent for each injection.

2.3.2.1. For the antagomiR and the in vivo delivery reagent solution preparation see step 1.3 but add 7 nmol of the miRNA-inhibitor per mouse.

2.3.3. Administer the solution intravenously by retro-orbital injection of the venous sinus of the mouse. Use isoflurane to induce anesthesia.

NOTE: Assess the level of anesthesia by pedal reflex (firm toe pinch).

2.3.4. Treat the mice 3 times a week (Monday, Wednesday and Friday) for 2 weeks.

2.4. Analysis of tumor growth

2.4.1. Determine the tumor bioluminescent signal twice weekly to calculate tumor growth.

2.4.1.1. Inject 50 µL of a 40 mg/mL solution of D-luciferin substrate at each time point via subcutaneous injection.

2.4.1.2. At 8 min post-injection, anesthetize the mice using 3% isoflurane mixed with oxygen and image the bioluminescent signal with in vivo imaging software.

2.4.2. Once the bioluminescence signals are obtained, analyze the tumoral growth comparing both treatments and determine the significance by using a t-test. To analyze the within-group variance use the SEM.

2.5. Tumor excision

2.5.1. Seven days after the end of the antagomiR treatment period, sacrifice the mice, excise the tumor and extract protein and/or RNA for future analysis. Alternatively, section the tumors and fix them for immunohistochemistry.

REPRESENTATIVE RESULTS:

We used two different mice models to determine whether the neutralization of a miRNA could suppress tumor growth. Accordingly, human tumor thyroid Cal62-luc cells were subcutaneously injected into the flanks of nude mice to generate a xenograph model. After two weeks, tumors were established and could be measured with calipers. At that time point, mice were injected intratumorally with the miR-146b-inhibitor, or an appropriate control, and tumor volume was followed for a further two weeks (**Figure 1A**). As shown in **Figure 1B**, the growth of tumors intratumorally injected with the miR-146b-inhibitor (anti-146b) (n=8) was significantly suppressed with respect to the negative control group (n=4) or the saline control group (not shown). miRNAs are an important feature of gene regulation, as they regulate several target

mRNAs. Accordingly, oncomiRs are important regulators of tumor suppressor genes. This protocol allows for the analysis of intratumoral expression levels of these genes, which can be tested after treatment with the miRNA-inhibitor. In addition, the levels of some proliferation markers can be also studied, illustrated by the lower expression of proliferating cell nuclear antigen (PCNA) in antagomiR-treated tumors than in control-treated tumors (**Figure 2**). Also the recovery of the miRNA-targets can be studied through the analysis of tumor-extracted RNA or protein, as illustrated by the higher expression of PTEN in the antagomiR(anti-146b) treated tumors (**Figure 2**). Collectively, these data reveal that the in vivo inhibition of a miRNA is effective and may be exploited therapeutically for thyroid cancer treatment.

To better assess the therapeutic potential of a miRNA-inhibitor and improve the mouse model, we generated an orthotopic model, in which human tumor thyroid Cal62-luc cells were directly seeded into the right thyroid lobe of nude mice. After 3 weeks, the thyroid tumors were established, as demonstrated by bioluminescence signals in the neck of the mice and by gross tissue analysis and immunohistochemistry, with hematoxylin and eosin and GFP staining of cells, respectiviely. As shown in **Figure 3**, the epithelial cells surrounding the colloid demonstrate the thyroid follicle architecture from the mouse. GFP-positive cells interspersed with the follicles, unequivocally demonstrating that human Cal62 cells have been injected correctly and proliferate within the mouse thyroid. Once the tumors were formed, we injected 13 mice intravenously with the miR-146b-inhibitor (n=8) or an appropriate control (n=5), and tumor volume was followed for a further two weeks (**Figure 4A**). We found that the tumor growth was significantly decreased in the systemic antagomiR-treated group (**Figure 4B**). Notably, the expression of the newly described miR-146b-target DICER1 in the primary tumor was increased after the anti-miR-146b treatment (**Figure 5**). These data demonstrate that the inhibition of endogenous miRNA expression and therefore the restoration of its target genes could be used as a therapy in thyroid cancer[16,17].

FIGURE AND TABLE LEGENDS:

Figure 1. **The miR-146b-inhibitor impairs established human thyroid tumor growth.** Cal62-luciferase expressing cells (Cal62-luc) were injected subcutaneously. After xenografts were established, a synthetic miR-inhibitor (anti-146b) (n=8) or a negative control (n=4) was administered intratumorally. (**A**) Timeline. (**B**) Left: The image shows the endpoint bioluminescent signal of the treated tumors imaged with in vivo imaging software. Right: The graph shows tumor radiance increase at the indicated times in xenografts from treatment onset with the miRNA-inhibitor (dark grey) or the negative control (grey). (**C**) Representation of the endpoint radiance increase of the treated tumors. Values represent mean ± SEM. *$p < 0.05$; **$p < 0.01$. Figure adapted from Ramírez-Moya et al.[16].

Figure 2. **PCNA protein expression decreases and PTEN protein expression increases in miR-146b-inhibitor-treated tumors.** Protein from the tumors treated with control or miR-146b inhibitor was obtained for western blotting with antibodies to PCNA or PTEN. Figure adapted from Ramírez-Moya et al.[16].

Figure 3. Intratumoral mouse thyroid follicles in the orthotopic model. Staining with hematoxylin and eosin (upper panel) and or an antibody to GFP (bottom panel) of the orthotopic mice tumors 28 days after inoculation with Cal62-luc GFP-positive cells.

Figure 4. The miR-146b-inhibitor impairs established human thyroid tumor growth. Cal62-luciferase expressing cells (Cal62-luc) were injected in the thyroid gland of the mice. After the tumors were established (3 weeks), a synthetic miRNA-inhibitor (anti-146b) (n=8) or a negative control (n=5) was administered systemically. (**A**) Timeline. (**B**) Left: The image shows the endpoint bioluminescent signal of the treated tumors imaged with in vivo imaging software. Right: The graph shows tumor radiance increase at the indicated times from treatment onset with the miRNA-inhibitor (blue) or the negative control (green). (**C**) Representation of the endpoint radiance increase of the treated tumors. Values represent mean ± SEM. *$p < 0.05$. Figure adapted from Ramírez-Moya et al.[17].

Figure 5. DICER1 protein expression increases in miR-146b-inhibitor-treated tumors. Immunohistochemistry with a DICER1 antibody in the orthotopic tumors after treatment with miR-146b-inhbitor or control.

DISCUSSION:

This paper describes a method for studying the in vivo function of a miRNA in order to better understand its role in tumor initiation and progression, and its potential as a therapeutic target in thyroid cancer. The tumor xenograft models here described are based on the use of cells that can be tracked by their bioluminescence signal, permitting the measurement of tumor growth in vivo under the influence of a treatment. In addition, we describe the use of a miRNA-based treatment for thyroid cancer, which is currently in the early stages.

We first tested the feasibility of a miRNA as a therapeutic target using a subcutaneous xenograft model, in which thyroid tumor cells were injected into the flanks of immunodeficient mice. We then intratumorally injected the antagomiR. This model can be used for several cancer types to test the significance of specific miRNAs or oncomiRs in vivo in human cancer cells. Advantages of this technique are that it is relatively simple to perform and also quick, with minimal animal suffering. A major disadvantage of the technique, however, is that it does not closely mimic the human condition because the cells are injected subcutaneously and not into the organ-of-origin. To overcome this limitation, we used a more robust model by performing surgical orthotopic implantation of bioluminescent thyroid tumor cells into the thyroid of the mice. Although this technique is more complicated and time consuming, it allows the study of tumor growth in its "native" environment, the thyroid. A strength of this method is that it permits the study of tumoral cell dissemination as metastasis, usually in the lungs, which can be evaluated with in vivo imaging software. Another strength is that the systemic delivery of the antagomiR mimics a potential human treatment and allows for the analysis of the thyroid response. Furthermore, the systemic injection of the antagomiR did not cause adverse effects on the animals, as parameters such as serum glucose levels or liver morphology were not altered[17].

There are a few critical steps. In the orthotopic mouse model, the injection of the tumoral cells

should be precisely performed in the thyroid gland and not in neighboring tissue. Thus, it is necessary to demonstrate with immunohistochemistry that the thyroid architecture is maintained and that the cells injected have infiltrated the thyroid and are not present out with the gland. In addition, for both xenograft and orthotopic models, the size of the tumors in the different groups of animals should be similar at the beginning of the treatment in order to follow a similar growth pattern in all cases. Finally, it is important to demonstrate that the expression of the targets downstream of the microRNA (in our case miR-146b) are altered after antagomiR treatment

As a possible future application of this model, patient cells could be injected into mouse models in order to analyze how the inhibition of a miRNA could affect the tumor growth of a specific patient, a useful approach for precision and personalized medicine based on miRNAs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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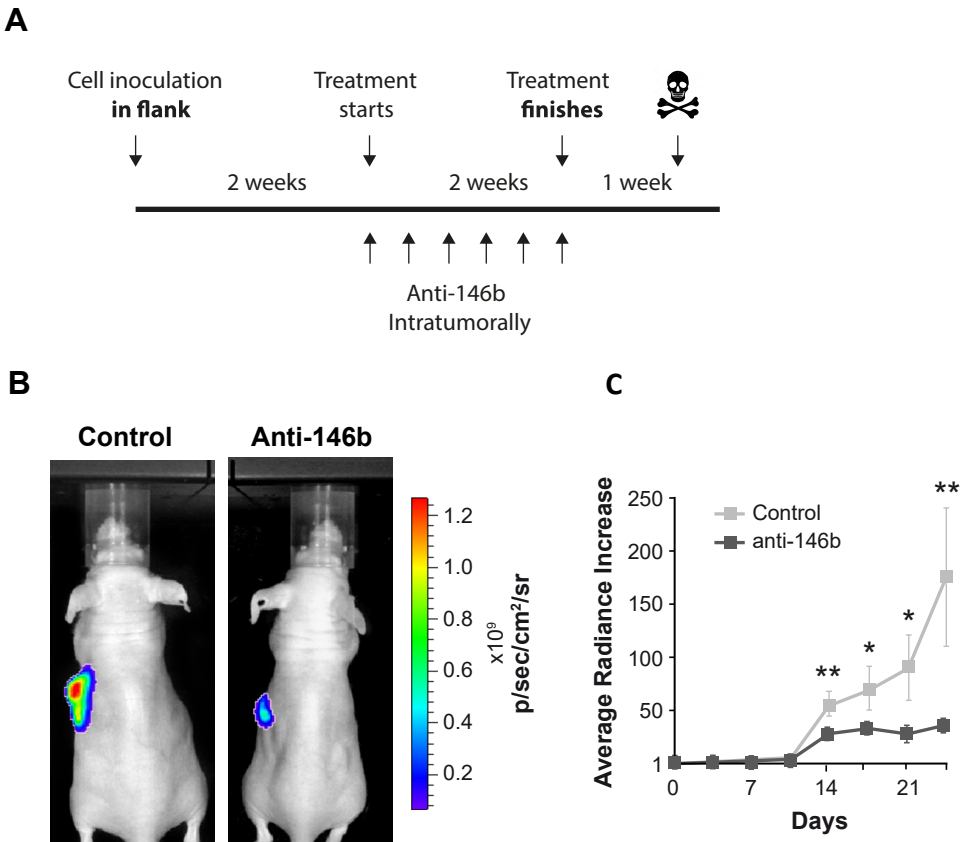

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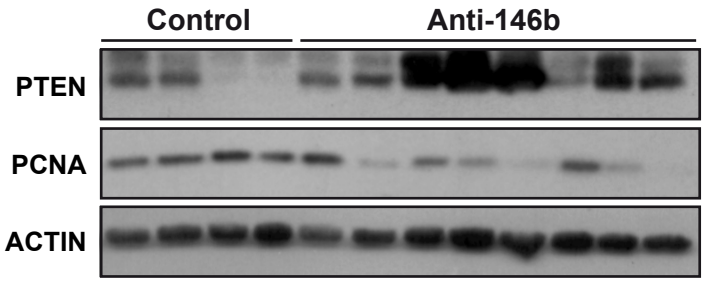

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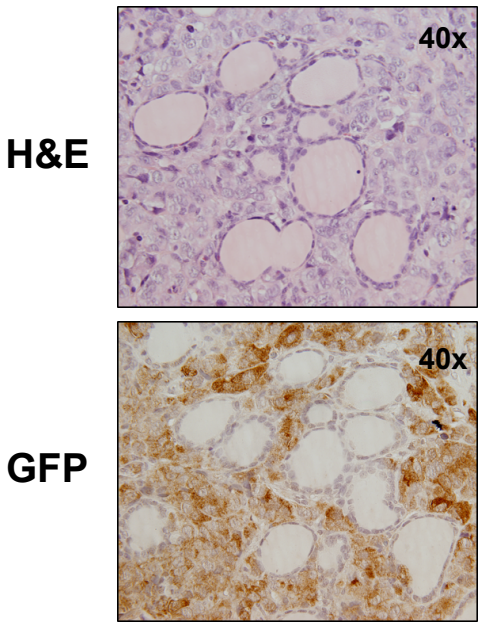

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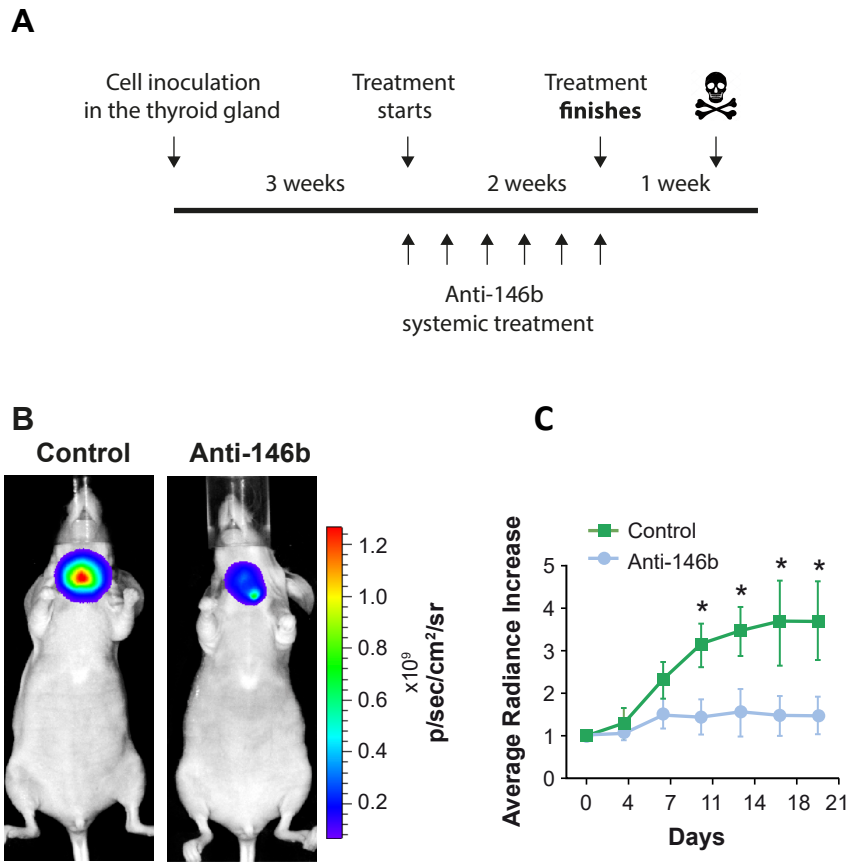

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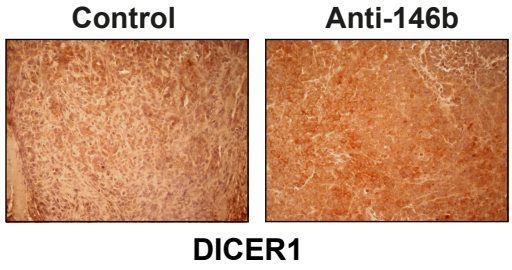

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Name	Company	Catalog Number	Comments
AntagomiR: mirVana miRNA inhibitor	Thermo Fisher	4464088	In Vivo Ready
Basement Membrane Matrix: Matrigel Basement Membrane Matrix High Concentration	Corning	#354248	
DICER antibody	Abcam	ab14601	IHQ: 1/100
In vivo delivery reagent: Invivofectamine 3.0 Reagent	Thermo Fisher	IVF3005	
In vivo imaging software: IVIS-Lumina II Imaging System	Caliper Life Sciences		
Negative control: mirVana miRNA Inhibitor, Negative Control #1	Thermo Fisher	4464077	In Vivo Ready
PCNA antibody	Abcam	ab92552	WB: 1/2,000
PTEN antibody	Santa Cruz	sc-7974	WB: 1/1,000
XenoLight D-Luciferin - K+ Salt Bioluminescent Substrate	PerkinElmer	122799	Diluted in PBS

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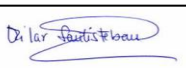
CORRESPONDING AUTHOR

Name:	Pilar Santisteban
Department:	Department of Cancer Biology
Institution:	Instituto de Investigaciones Biomédicas Alberto Sols
Title:	Professor
Signature:	
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Madrid, March-27-2019

Dear Editor:

Thank you for give us the opportunity to resubmit the manuscript JOVE_59322_R0_203110. Also we appreciate the two extension in the deadline.

Please enclosed find the answer to Editor comments and to Reviewers.

Yours sincerely

Pilar Santisteban

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Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Done

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3. Please revise lines 43-46, 183-185, and 199-202 to avoid previously published text.

We have revised these lines

4. Please provide an email address for each author.

Done
In addition please note that due to requirements of new experiments, there are two new authors

5. Keywords: Please provide at least 6 keywords or phrases.

Done

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We have changed as follow:
Matrigel : basement membrane matrix
Invivofectamine: in vivo delivery reagent
IVIS-Lumina : In vivo imaging software
Hamilton, microliter syringe
Betadine: Iodopovidone

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the **imperative tense** in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be

written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Done

8**. Please add more details to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

9. 1.3.1: Please reference the Table of Materials for the antagomiR /miR inhibitor used in this step. What volume of distilled water is used?

Done

10. 1.3.2.1: Please provide the composition of the complexation buffer.
This is a commercial buffer from the Invivofectamine 3.0 kit, from ThermoFisher.

11. 1.3.2.6: Please add more details about the in vivo delivery of the Invivofectamine 3.0-antagomiR complex.

Done

12. 1.3.3: Please specify on which days of the week the treatment is performed.

Done

13. 1.4.1.2, 2.2.2.1: Please specify the concentration of isofluorane used and mention how proper anesthetization is confirmed.

Done

14. 2.2.2.2: How large is the incision? Please specify all surgical instruments used.

Done

15. Please include single-line spaces between all paragraphs, headings, steps, etc.
Done

16. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Done

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done

19. Discussion: Please discuss critical steps within the protocol.

We have added a few lines in the discussion regarding the critical steps.

20. Table of Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The protocol presented by Ramirez-Moya describes the delivery of miR inhibitors/antagomiRs in xenograft and in orthotopic model of thyroid cancer using human thyroid cancer cell line.

It allows to study the effect of miR and antagomiR in vivo and eventually discover therapeutic effects of miR.

Major Concerns:

Our main problem relates to the orthotopic model protocol. To be called orthotopic, authors should demonstrate that the injection of Cal62 cells in the thyroid effectively occurred in the thyroid.
- H/E staining showing the typical thyroid architecture combined with GFP activity to reveal the presence of the Cal62 cells is required.

We believe that we did indeed generate orthotopic tumors in mice. As can be seen in Figure 3 in the H/E staining of the tumor appears the typical thyroid architecture.

In addition, we have performed GFP staining, to visualize the tumoral thyroid cells injected in the gland. As shown in Figure 3, the cells injected (positive for GFP) are interspersed between the thyroid cells of the mouse. We believe that we have generated a real orthotopic thyroid tumor model.

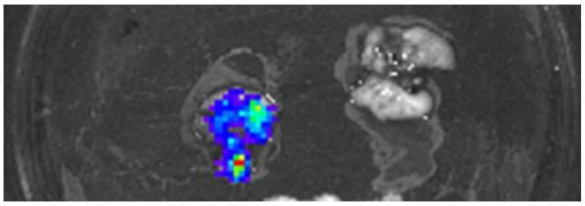
- Effect on tumor growth should also be shown.

The systemic treatment of anti-miR-146b in the orthotopic mouse generated blunts tumor growth, as shown in the new B of Panel of Figure 4 and explained in the text.

- dissemination of tumoral cells to the lungs?

We observed GFP-positive cells in the lung of orthotopic thyroid mouse (see the figure included below for the reviewer).

Lungs: 25 days after cell inoculation in the thyroid gland



Luciferase Positive lungs | Luciferase Negative lungs

Minor Concerns:

- line 49 : sentence is too general for a single reference. Either give more references or be specific by mentioning miR146. It would also be nice to have one or two background information sentences on miR146.

Done

- line 60 : computer-assisted mistake : orthotopic instead of orthotropic.

Done

- line 72 : mention the mutation(s) found in this cell line, the details of the GFP and Luc construct.

Done

- line 77 : suspend cell in cold, RT or warm PBS ?

Done

- line 85 : Matrigel instead of Matirgel

Done

- line 86 : ...using (a) pre-cooled pipet tips ....and not pipet.

Done

- line 87: mention the type of syringe and needle used

Done

- line 88: inject 200 ul.... state that the volume injected is independent (or dependent) of the tumor volume.

The volume and quantity of miR-inhibitor is independent of the tumor volume. Nevertheless, as explained in the critical steps (Discussion) we always try to have tumors of similar sizes

- line 109: final concentration: 2 nmol. This is not a concentration but a quantity

Done

- line 110: specify the days of the treatments (3 times each week) : monday, wednesday and friday ?

Done

- line 113 : remove 1.4.1 and place the sentence after the title : Analysis of tumor growth.

Done

- line 114 : mention the type of syringe and needle used for the injection.

Done

- line 116 : details on isoflurane dose, flux could be given

Done

- line 142 : mention if your work under a sterile flow and with a stereomicroscope ....

Done

- line 145 : mention the type of Hamilton syringe you are using ...

Done

- could 2.4. Analysis of tumor growth comes before 2.3. Systemic antagomiR/miR inhibitor treatment ?

The process 2.3 and 2.4 are simultaneous at the beginning and since the final aim is to measure the size of the tumor, we believe that the analysis of the tumor growth is the final step before 2.5 ”tumor excision”

- line 159 : inhibitor instead go imhibitor

Done

- line 161 : retro-orbital vein injection on anesthetized animals ?

Done

- line 168 : 8 or 9 minutes ?

Done

- line 172 : 7 or Seven ....

Done

- line 172 : sacrifice the mice before excising the tumors ?

Done

- A time line showing the 3 retro-orbital injection and the 2 bioluminescence recording per week could be illustrated in the figures.

We made a time line (Figs 1 and 4 panels A) representing different steps (cell inoculation, treatment starting and finishing). We think that adding also bioluminescence recording will make the figure overly busy.

- line 181 : mirVana ... I would mention antagomiR or miR inhibitor and not the brand. When describing transfection, the brand was not mentioned....

Done

- lines 212 and 216 : SEM and SD : choose and adapt.

The correct is SEM

- graphs in figures do not contain any unit

The graphs and Figures contain the units

Reviewer #2:

Manuscript Summary:

In this work, Moya and colleagues presents a protocol in which after thyroid cancer cells injection in xenograft and orthotopic mouse models, they monitored the effects of AntagomiR as a therapeutic approach in thyroid cancer. The authors showed that, the inhibition of microRNA is able to reduce tumor growth in mice. Overall the work is interesting however there are several points that need to be addressed.

Major Concerns:

1. Is the CAL62 cell line authenticated?

Yes, we authenticate in our laboratory the cells every 6 month

2. What is the exact sample size (number) of xenograft and orthotopic mice used for each experimental group? Please add a detailed description.

Done

3. Are statistical tests justified as appropriate? The authors should report the statistical methods used in Protocol paragraph.

Done

4. Is there an estimate of variation within each group of data? This would be important as well for comparison.

We used the SEM to analyze the within-group variance as described in the figure legend and in steps: 1.4.3 and 2.4.2.

5. In literature it is reported that miR-146b promotes thyroid cancer progression by targeting PTEN, such as reported in Reference 10 of your manuscript. Consequently, it is very important know the level of PTEN protein in the miR-inhibitor treated tumor.

We have added these data (see Figure 2)

6. Please provide a more detailed Material and Methods section.

Done